

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

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University of Zagreb

Faculty of Food Technology and Biotechnology

Ena Cegledi

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DOCTORAL DISSERTATION

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Supervisor:

Verica Dragović-Uzelac, Ph.D., Full professor

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.



Ena Cegledi

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

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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).*





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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

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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, pigmenta 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

**Rad sadrži:** 139 stranica, 4 slike, 4 tablice, 174 reference

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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

## Prošireni sažetak

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

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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:**

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### Publication No.3

**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 (Otlés 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.

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# 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](http://www.shutterstock.com) accessed 23<sup>rd</sup> 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 (Otlés 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)

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

It also contains a great amount of vitamins, especially vitamins A, B complex (B<sub>1</sub>, B<sub>2</sub>, B<sub>3</sub>, B<sub>6</sub>), 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- <i>O</i> -rutoside, kaempferol-3- <i>O</i> -glucoside, isorhamnetin-3- <i>O</i> -glucoside, quercetin-3- <i>O</i> -glucoside, quercetin-3- <i>O</i> -rutoside, 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, <i>p</i> -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               | $\beta$ -carotene, lutein, 13- <i>cis</i> -lutein, 9- <i>cis</i> -lutein, neoxhantin, violaxanthin, zeaxanthin   | Guil-Guerro et al., (2003); Rafajlovska et al., (2002)                          |
|                    | chlorophylls              | chlorophyll <i>a</i> , chlorophyll <i>b</i>  | 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, $\alpha$ -linoleic acid, palmitoleic acid, oleic acid   | Tarasevičienė 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, <i>p</i> -coumaric acid, ferulic acid, quinic acid, caffeic acid   | Otles and Yalcin, (2012); Kregiel et al., (2018)                                |
|                    | chlorophylls              | chlorophyll <i>a</i> , chlorophyll <i>b</i>  | Hojnik et al., (2007)   |
| ROOTS              | phytosterols              | $\beta$ -sitosterol, stigmasterol, campesterol, campestanol, $\Delta^5$ -avenasterol, $\Delta^5$ -stigmasterol, cycloartenol, 24-methylene cycloartenol, obtusifoliol, citrostadienol  | Obranović et al. (2022); Kovacheva et al. (1990)                                |
|                    | pentacyclic triterpenoids | $\alpha$ -amyrin, $\beta$ -amyrin, $\beta$ -amyrin acetate   | Obranović et al., (2022)  |
|                    | phenolic acids            | <i>p</i> -hydroxybenzoic acid, protocatechuic acid, quinic acid, <i>p</i> -coumaric acid, ferulic acid, caffeic acid, 5- <i>O</i> -caffeoylquinic acid   | Francišković et al., (2017)   |
|                    | flavonoids                | myricetin, quercetin, kaempferol, rutin  | Otles and Yalcin, (2012)  |
|                    | coumarins                 | esculetin, scopoletin  | Francišković et al., (2017); Sajfirtova et al., (2004)                          |
|                    | fatty acids               | behenic acid, stearic acid   | Tarasevičienė et al., (2023)  |
|                    | lignans                   | neo-olivil, secoisolariciresinol, dehydrodiconiferyl alcohol, isolariciresinol, pinosresinol, 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 (Braidly 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 ( $\alpha$ -carotene,  $\beta$ -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  $\alpha$ -amyrin and  $\beta$ -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**

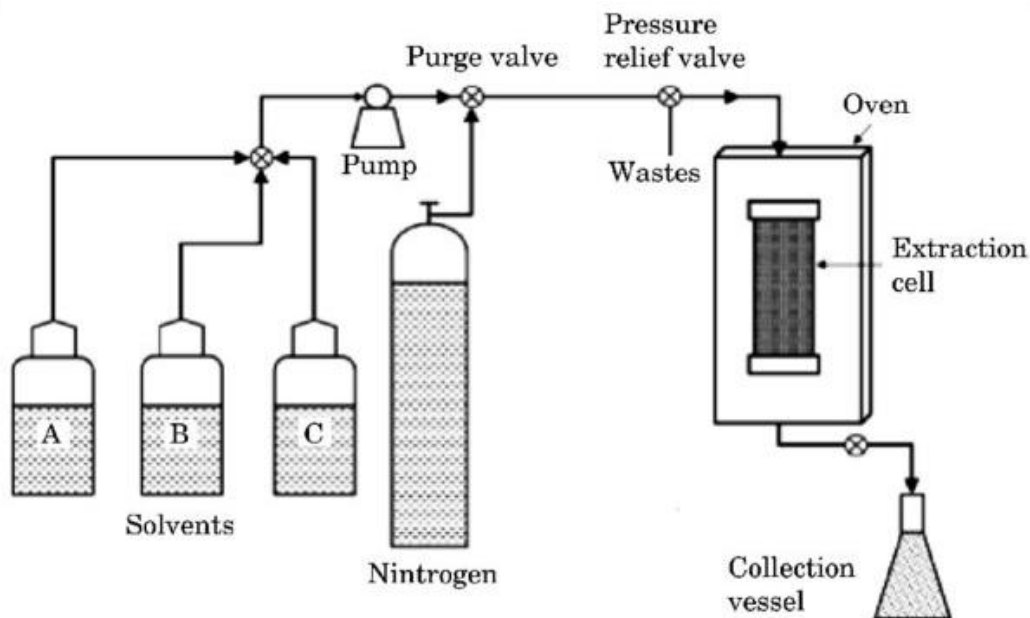
In order to isolate bioactive molecules from plant material, various extraction techniques are used. Extraction is a process for selective isolation or separation of desired substance or component from a mixture using a suitable solvent or extraction technique. In this process, the target substance is 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 Oniszczyk, 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 Dobrosłavić 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 (Mottaleb 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 molecules, 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**

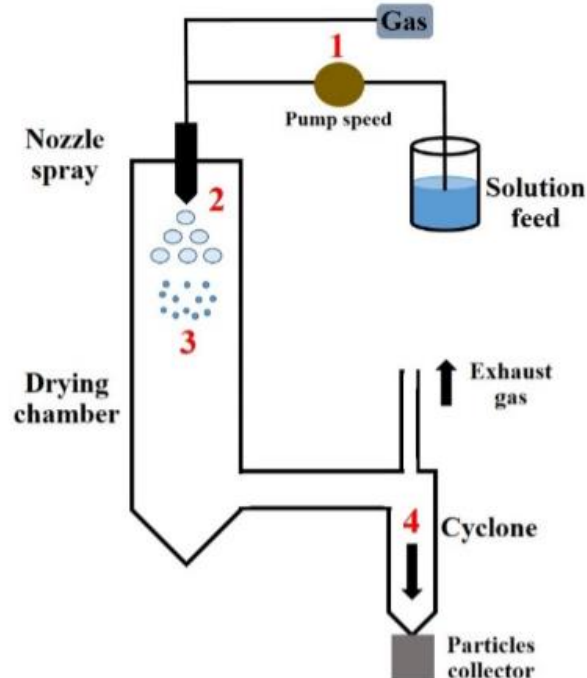
Although liquid plant extracts are most commonly used by consumers, the stability of bioactive molecules in such extract depends on numerous factors such as light, oxygen, heat and moisture (Shishir and Chen, 2017), which affect the reduction or complete loss of their biological activity over time. The 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 techniques, 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  $\mu\text{m}$  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 (Timislina 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  $\alpha$ -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),  $\alpha$ -,  $\beta$ - and  $\gamma$ -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  $\beta$ -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  $\beta$ -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 content 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 forms 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

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# 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

Repajić, M., **Cegledi, E.**, Kruk, V., Pedisić, S., Çınar, F., Bursać Kovačević, D., Žutić, I., Dragović-Uzelac, V. (2020) Accelerated Solvent Extraction as a Green Tool for the Recovery of Polyphenols and Pigments from Wild Nettle Leaves. *Processes*, 8(7), 803.

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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 ( $\geq 95\%$ ), *p*-coumaric acid ( $\geq 98\%$ ), ferulic acid ( $\geq 99\%$ ), quercetin-3-glucoside ( $\geq 99\%$ ), (–)- $\beta$ -carotene,  $\alpha$ -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  $103 \pm 2$  °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

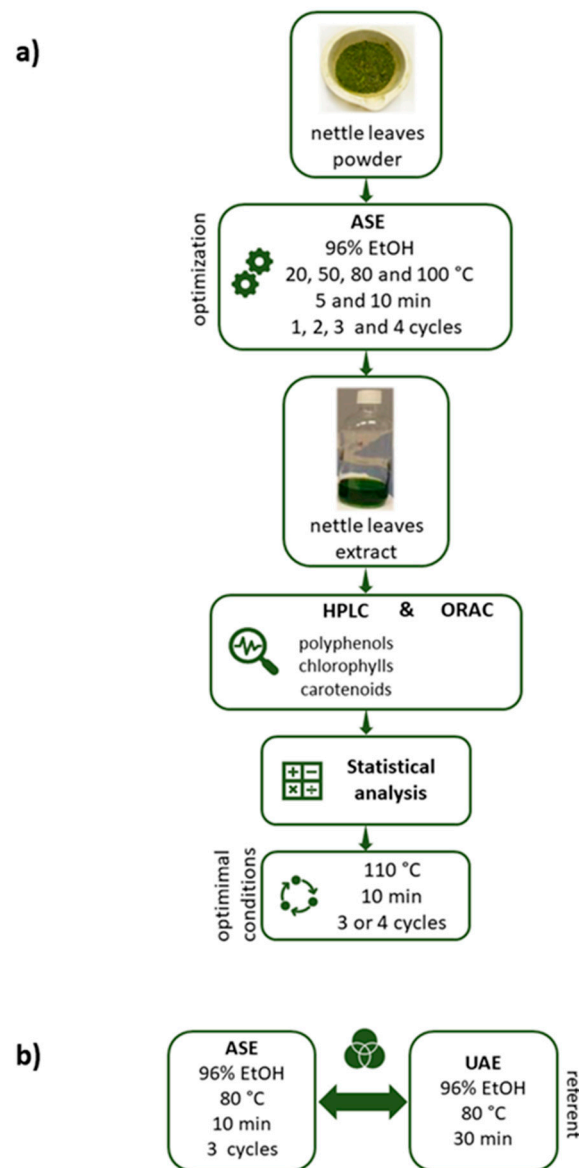
In order to compare ASE efficiency, an ultrasound assisted extraction (UAE) of NL polyphenols and pigments was simultaneously conducted at previously optimized conditions. Briefly, sample (0.5 g) was put into a sealed test tube (50 mL) and 25 mL of ethanol (96%) was added and homogenized on the Vortex 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<sup>-1</sup>. 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-Aqueous C 30, 3  $\mu\text{m}$ , (250  $\times$  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  $\text{min}^{-1}$  and the injection volume 10  $\mu\text{L}$ . 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 ( $\alpha$ - and  $\beta$ -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  $\beta$ -carotene,  $\alpha$ -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  $\beta$ -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  $\text{g}^{-1}$  of dry matter, as mean values  $\pm$  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  $\mu\text{M}$  phosphate buffer (pH 7.4). Briefly, 25 mL of appropriate diluted samples were added in a 96-well black plate containing 150  $\mu\text{L}$  of fluorescein solution (70.3 nM). The plate was incubated for 30 min at 37  $^{\circ}\text{C}$  and after the first three cycles (representing the baseline signal), AAPH (240 mM) was injected into each well to initiate the peroxy radical generation. On each plate, different dilutions of Trolox (3.37–107.88  $\mu\text{M}$ ) 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  $\text{g}^{-1}$  of dry matter, as mean values  $\pm$  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  $\pm$  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<sup>-1</sup> 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<sup>-1</sup> dm, TF (from 3.67 to 80.16 mg 100 g<sup>-1</sup> dm) and TP (from 20.54 to 335.67 mg 100 g<sup>-1</sup> 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<sup>-1</sup> dm, TF = 50.47 mg 100 g<sup>-1</sup> dm, TP = 214.78 mg 100 g<sup>-1</sup> 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<sup>-1</sup> dm) and ORAC values (mmol TE 100 g<sup>-1</sup> dm) in extracts obtained at ASE different conditions.

| Temperature (°C) | Static Time (min) | Cycle Number | ChA            | <i>p</i> -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<sup>-1</sup> dm), pigments (mg 100 g<sup>-1</sup> dm) and ORAC values (mmol TE 100 g<sup>-1</sup> dm).

| Source of Variation      | THCA              | TF                | TP                | TCH               | TCAR              | TPG               | ORAC              |
|--------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| <b>Temperature (°C)</b>  | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 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     |
| <b>Static time (min)</b> | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 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     |
| <b>Cycle number</b>      | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 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     |
| <b>Grand mean</b>        | 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<sup>-1</sup>) 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<sup>-1</sup> dm), pigments (mg 100 g<sup>-1</sup> dm) and ORAC values (mmol TE 100 g<sup>-1</sup> dm) in UAE extracts obtained at optimal extraction conditions (80 °C/30 min).

| Compounds                   |                      | Concentration  |
|-----------------------------|----------------------|----------------|
| Polyphenols                 | ChA                  | 76.84 ± 3.32   |
|                             | <i>p</i> -CA         | 53.23 ± 1.07   |
|                             | Q-3-G                | 19.02 ± 2.77   |
|                             | K-3-R                | 5.18 ± 0.45    |
| Chlorophylls                | CHL <i>b</i> der 1   | 8.42 ± 0.06    |
|                             | CHL <i>a</i> der 1   | 36.65 ± 2.10   |
|                             | CHL <i>a</i> der 2   | 38.62 ± 2.44   |
|                             | CHL <i>b</i>         | 230.46 ± 15.17 |
|                             | CHL <i>a</i>         | 589.04 ± 22.36 |
| Carotenoids                 | CHL <i>a</i> der 5   | 5.07 ± 0.02    |
|                             | VIOLAX der           | 1.17 ± 0.10    |
|                             | NEOX                 | 2.38 ± 0.15    |
|                             | VIOLAX               | 1.10 ± 0.08    |
|                             | 13'- <i>cis</i> -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'- <i>cis</i> -LUT  | 1.78 ± 0.11    |
| α-CAR                       | 6.12 ± 0.41          |                |
| β-CAR                       | 14.14 ± 1.03         |                |
| LYC der 10                  | 0.32 ± 0.01          |                |
| <b>Antioxidant capacity</b> |                      |                |
| 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<sup>-1</sup> 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<sup>-1</sup> 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),  $\alpha$ -carotene ( $\alpha$ -CAR),  $\beta$ -carotene ( $\beta$ -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<sup>-1</sup> dm with the highest yield achieved at 110 °C/10 min/2 cycles.  $\beta$ -CAR was the following carotenoid with a maximum value of 19.02 mg 100 g<sup>-1</sup> dm, while the least present carotenoid was LYC der 10 (0.32–0.85 mg 100 g<sup>-1</sup> 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,  $\beta$ -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<sub>2</sub> extraction were used to characterize NL chlorophylls and carotenoids, it was revealed that chlorophylls content was lower (CHL *a* 73 mg 100 g<sup>-1</sup> dm, CHL *b* 100 100 g<sup>-1</sup> dm), while LUT and  $\beta$ -CAR contents were higher (LUT 39 mg 100 g<sup>-1</sup> dm,  $\beta$ -CAR 24 mg 100 g<sup>-1</sup> 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<sup>-1</sup> dm) in extracts obtained at ASE different conditions.

| Temperature (°C) | Static Time (min) | Cycle Number | CHL <i>b</i> der 1 | CHL <i>a</i> der 1 | CHL <i>a</i> der 2 | CHL <i>b</i>   | CHL <i>a</i> der 3 | CHL <i>a</i> der 4 | CHL <i>a</i>   | CHL <i>a</i> 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<sup>-1</sup> 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 ± 0.18 | 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, NEOF = neoxanthin, VIOLAX = violaxanthin, 13'-cis-LUT = 13'-cis-lutein, LUT 5,6-ep = lutein 5,6-epoxide, NEOF 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<sup>-1</sup> dm, TCAR 31.48 vs. 65.81 mg 100 g<sup>-1</sup> 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<sup>-1</sup> dm), at the static time of 10 min (992.77 mg 100 g<sup>-1</sup> dm) and at the fourth cycle of extraction (928.82 mg 100 g<sup>-1</sup> 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  $\beta$ -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  $\beta$ -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  $\alpha$ - and  $\beta$ -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<sub>2</sub> extraction), the levels of CHL *a* + *b* and  $\beta$ -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<sup>-1</sup> 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<sup>-1</sup> dm) compared to the value at the initial temperature (5.52 mmol TE 100 g<sup>-1</sup> 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<sup>-1</sup> dm), pigments (mg 100 g<sup>-1</sup> dm) and ORAC values (mmol TE 100 g<sup>-1</sup> 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 compared to UAE extracts (22.07 vs. 13.26 mmol TE 100 g<sup>-1</sup> 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  $\beta$ -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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## References

1. Otles, S.; Yalcin, B. Phenolic compounds analysis of root, stalk, and leaves of nettle. *Sci. World J.* **2012**, *2012*, 1–12. [[CrossRef](#)] [[PubMed](#)]
2. Đurović, S.; Pavlič, B.; Šorgić, S.; Popov, S.; Savić, S.; Petronijević, M.; Radojković, M.; Cvetanović, A.; Zeković, Z. Chemical composition of stinging nettle leaves obtained by different analytical approaches. *J. Funct. Foods* **2017**, *32*, 18–26. [[CrossRef](#)]
3. Grauso, L.; de Falco, B.; Lanzotti, V.; Motti, R. Stinging nettle, *Urtica dioica* L.: Botanical, phytochemical and pharmacological overview. *Phytochem. Rev.* **2020**. [[CrossRef](#)]
4. Đurović, S.; Vujanović, M.; Radojković, M.; Filipović, J.; Filipović, V.; Gašić, U.; Tešić, Ž.; Mašković, P.; Zeković, Z. The functional food production: Application of stinging nettle leaves and its extracts in the baking of a bread. *Food Chem.* **2020**, *312*. [[CrossRef](#)] [[PubMed](#)]
5. Tomczyk, M.; Zaguła, G.; Dżugan, M. A simple method of enrichment of honey powder with phytochemicals and its potential application in isotonic drink industry. *Lwt* **2020**, *125*. [[CrossRef](#)]
6. Hayward, L.; Wedel, A.; McSweeney, M.B. Acceptability of beer produced with dandelion, nettle, and sage. *Int. J. Gastron. Food Sci.* **2019**, *18*. [[CrossRef](#)]
7. Marchetti, N.; Bonetti, G.; Brandolini, V.; Cavazzini, A.; Maietti, A.; Meca, G.; Mañes, J. Stinging nettle (*Urtica dioica* L.) as a functional food additive in egg pasta: Enrichment and bioaccessibility of Lutein and  $\beta$ -carotene. *J. Funct. Foods* **2018**, *47*, 547–553. [[CrossRef](#)]
8. Moldovan, L.; Gaspar, A.; Toma, L.I.A.N.A.; Craciunescu, O.A.N.A.; Saviuc, C.R.I.N.A. Comparison of polyphenolic content and antioxidant capacity of five Romanian traditional medicinal plants. *Revista de Chimie -Buchar.* **2011**, *62*, 299–303.
9. Orčić, D.; Francišković, M.; Bekvalac, K.; Svirčev, E.; Beara, I.; Lesjak, M.; Mimica-Dukić, N. Quantitative determination of plant phenolics in *Urtica dioica* extracts by high-performance liquid chromatography coupled with tandem mass spectrometric detection. *Food Chem.* **2014**, *143*, 48–53. [[CrossRef](#)]
10. Carvalho, A.R.; Costa, G.; Figueirinha, A.; Liberal, J.; Prior, J.A.V.; Lopes, M.C.; Cruz, M.T.; Batista, M.T. *Urtica* spp.: Phenolic composition, safety, antioxidant and anti-inflammatory activities. *Food Res. Int.* **2017**, *99*, 485–494. [[CrossRef](#)]
11. Guil-Guerrero, J.L.; Rebolloso-Fuentes, M.M.; Torija Isasa, M.E. Fatty acids and carotenoids from Stinging Nettle (*Urtica dioica* L.). *J. Food Compos. Anal.* **2003**, *16*, 111–119. [[CrossRef](#)]
12. Hojnik, M.; Škerget, M.; Knez, Ž. Isolation of chlorophylls from stinging nettle (*Urtica dioica* L.). *Sep. Purif. Technol.* **2007**, *57*, 37–46. [[CrossRef](#)]
13. Ibrahim, M.; Rehman, K.; Razzaq, A.; Hussain, I.; Farooq, T.; Hussain, A.; Akash, M.S.H. Investigations of phytochemical constituents and their pharmacological properties isolated from the genus *urtica*: Critical review and analysis. *Crit. Rev. Eukaryotic Gene Expr.* **2018**, *28*, 25–66. [[CrossRef](#)]
14. Martínez-Aledo, N.; Navas-Carrillo, D.; Orenes-Piñero, E. Medicinal plants: Active compounds, properties and antiproliferative effects in colorectal cancer. *Phytochem. Rev.* **2020**, *19*, 123–137. [[CrossRef](#)]
15. Ziaei, R.; Foshati, S.; Hadi, A.; Kermani, M.A.H.; Ghavami, A.; Clark, C.C.T.; Tarrahi, M.J. The effect of nettle (*Urtica dioica*) supplementation on the glycemic control of patients with type 2 diabetes mellitus: A systematic review and meta-analysis. *Phytother. Res.* **2019**, *34*, 282–294. [[CrossRef](#)]

16. Jacquet, A.; Girodet, P.-O.; Pariente, A.; Forest, K.; Mallet, L.; Moore, N. Phytalgic®, a food supplement, vs placebo in patients with osteoarthritis of the knee or hip: A randomised double-blind placebo-controlled clinical trial. *Arthritis Res. Ther.* **2009**, *11*. [[CrossRef](#)]
17. Cencic, A.; Chingwaru, W. The role of functional foods, nutraceuticals, and food supplements in intestinal health. *Nutrients* **2010**, *2*, 611–625. [[CrossRef](#)]
18. Granato, D.; Barba, F.J.; Bursac Kovačević, D.; Lorenzo, J.M.; Cruz, A.G.; Putnik, P. Functional foods: Product development, technological trends, efficacy testing, and safety. *Annu. Rev. Food Sci. Technol.* **2020**, *11*, 93–118. [[CrossRef](#)]
19. Stanojević, L.P.; Stanković, M.Z.; Cvetković, D.J.; Cakić, M.D.; Ilić, D.P.; Nikolić, V.D.; Stanojević, J.S. The effect of extraction techniques on yield, extraction kinetics, and antioxidant activity of aqueous-methanolic extracts from nettle (*Urtica dioica* L.) leaves. *Sep. Sci. Technol.* **2016**, *51*, 1817–1829. [[CrossRef](#)]
20. Chemat, F.; Vian, M.A.; Cravotto, G. Green extraction of natural products: Concept and principles. *Int. J. Mol. Sci.* **2012**, *13*, 8615–8627. [[CrossRef](#)]
21. Mottaleb, M.A.; Sarker, S.D. Accelerated Solvent Extraction for Natural Products Isolation. In *Natural Products Isolation*, 3rd ed.; Sarker, S.D., Nahar, L., Eds.; Springer: New York, NY, USA, 2012; pp. 75–88. [[CrossRef](#)]
22. Putnik, P.; Barba, F.J.; Španić, I.; Zorić, Z.; Dragović-Uzelac, V.; Bursac Kovačević, D. Green extraction approach for the recovery of polyphenols from Croatian olive leaves (*Olea europaea*). *Food Bioprod. Process* **2017**, *106*, 19–28. [[CrossRef](#)]
23. Bursac Kovačević, D.; Barba, F.J.; Granato, D.; Galanakis, C.M.; Herceg, Z.; Dragović-Uzelac, V.; Putnik, P. Pressurized Hot Water Extraction (PHWE) for the green recovery of bioactive compounds and steviol glycosides from *Stevia rebaudiana* Bertoni Leaves. *Food Chem.* **2018**. [[CrossRef](#)]
24. Ameer, K.; Shahbaz, H.M.; Kwon, J.-H. Green extraction methods for polyphenols from plant matrices and their byproducts: A Review. *Compr. Rev. Food Sci. F* **2017**, *16*, 295–315. [[CrossRef](#)]
25. Jentzer, J.-B.; Alignan, M.; Vaca-Garcia, C.; Rigal, L.; Vilarem, G. Response surface methodology to optimise accelerated solvent extraction of steviol glycosides from *Stevia rebaudiana* Bertoni leaves. *Food Chem.* **2015**, *166*, 561–567. [[CrossRef](#)] [[PubMed](#)]
26. Pena-Pereira, F.; Lavilla, I.; Bendicho, C. Miniaturized preconcentration methods based on liquid–liquid extraction and their application in inorganic ultratrace analysis and speciation: A review. *Spectrochim. Acta Part B* **2009**, *64*, 1–15. [[CrossRef](#)]
27. Alexovič, M.; Dotsikas, Y.; Bober, P.; Sabo, J. Achievements in robotic automation of solvent extraction and related approaches for bioanalysis of pharmaceuticals. *J. Chromatogr. B* **2018**, *1092*, 402–421. [[CrossRef](#)] [[PubMed](#)]
28. Johnson, T.A.; Sohn, J.; Inman, W.D.; Bjeldanes, L.F.; Rayburn, K. Lipophilic stinging nettle extracts possess potent anti-inflammatory activity, are not cytotoxic and may be superior to traditional tinctures for treating inflammatory disorders. *Phytomedicine* **2013**, *20*, 143–147. [[CrossRef](#)]
29. Johnson, T.A.; Morgan, M.V.C.; Aratow, N.A.; Estee, S.A.; Sashidhara, K.V.; Loveridge, S.T.; Segraves, N.L.; Crews, P. Assessing Pressurized Liquid Extraction for the high-throughput extraction of marine-sponge-derived natural products. *J. Nat. Prod.* **2010**, *73*, 359–364. [[CrossRef](#)]
30. AOAC. *Official Methods of Analysis: Changes in Official Methods of Analysis Made at the Annual Meeting*, 15th ed.; Association of Official Analytical Chemists: Rockville, Maryland, 1990; Volume I, pp. 40–64.
31. Dent, M.; Dragović-Uzelac, V.; Penić, M.; Brnčić, M.; Bosiljkov, T.; Levaj, B. The effect of extraction solvents, temperature and time on the composition and mass fraction of polyphenols in dalmatian wild sage (*Salvia officinalis* L.) extracts. *Food Technol. Biotech.* **2013**, *51*, 84–91.
32. Akbay, P.; Basaran, A.A.; Undeger, U.; Basaran, N. *In vitro* immunomodulatory activity of flavonoid glycosides from *Urtica dioica* L. *Phytother. Res.* **2003**, *17*, 34–37. [[CrossRef](#)] [[PubMed](#)]
33. Farag, M.A.; Weigend, M.; Luebert, F.; Brokamp, G.; Wessjohann, L.A. Phytochemical, phylogenetic, and anti-inflammatory evaluation of 43 *Urtica* accessions (stinging nettle) based on UPLC–Q–TOF–MS metabolomic profiles. *Phytochemistry* **2013**, *96*, 170–183. [[CrossRef](#)] [[PubMed](#)]
34. Pinelli, P.; Ieri, F.; Vignolini, P.; Bacci, L.; Baronti, S.; Romani, A. Extraction and HPLC analysis of phenolic compounds in leaves, stalks, and textile fibers of *Urtica dioica* L. *J. Agric. Food Chem.* **2008**, *56*, 9127–9132. [[CrossRef](#)]

35. Castro-Puyana, M.; Pérez-Sánchez, A.; Valdés, A.; Ibrahim, O.H.M.; Suarez-Álvarez, S.; Ferragut, J.A.; Micol, V.; Cifuentes, A.; Ibáñez, E.; García-Cañas, V. Pressurized liquid extraction of *Neochloris oleoabundans* for the recovery of bioactive carotenoids with anti-proliferative activity against human colon cancer cells. *Food Res. Int.* **2017**, *99*, 1048–1055. [[CrossRef](#)]
36. Gupta, P.; Sreelakshmi, Y.; Sharma, R. A rapid and sensitive method for determination of carotenoids in plant tissues by high performance liquid chromatography. *Plant. Methods* **2015**, *11*, s13007–s13015. [[CrossRef](#)] [[PubMed](#)]
37. Sözgen Başkan, K.; Tütem, E.; Özer, N.; Apak, R. Spectrophotometric and chromatographic assessment of contributions of carotenoids and chlorophylls to the total antioxidant capacities of plant foods. *J. Agric. Food Chem.* **2013**, *61*, 11371–11381. [[CrossRef](#)] [[PubMed](#)]
38. Prior, R.L.; Wu, X.L.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290–4302. [[CrossRef](#)]
39. Bender, C.; Graziano, S.; Zimmerman, B.F.; Weidlich, H.H. Antioxidant potential of aqueous plant extracts assessed by the cellular antioxidant activity assay. *Am. J. Biol. Life Sci.* **2014**, *2*, 72–79.
40. Rodríguez-Rojo, S.; Visentin, A.; Maestri, D.; Cocero, M.J. Assisted extraction of rosemary antioxidants with green solvents. *J. Food Eng.* **2012**, *109*, 98–103. [[CrossRef](#)]
41. Carabias-Martínez, R.; Rodríguez-Gonzalo, E.; Revilla-Ruiz, P.; Hernández-Méndez, J. Pressurized liquid extraction in the analysis of food and biological samples. *J. Chromatogr. A* **2005**, *1089*, 1–17. [[CrossRef](#)]
42. Vajić, U.-J.; Grujić-Milanović, J.; Živković, J.; Šavikin, K.; Gođevac, D.; Miloradović, Z.; Bugarski, B.; Mihailović-Stanojević, N. Optimization of extraction of stinging nettle leaf phenolic compounds using response surface methodology. *Ind. Crops Prod.* **2015**, *74*, 912–917. [[CrossRef](#)]
43. Moreira, S.A.; Silva, S.; Costa, E.M.; Saraiva, J.A.; Pintado, M. Effect of high hydrostatic pressure extraction on biological activities of stinging nettle extracts. *Food Funct.* **2020**, *11*, 921–931. [[CrossRef](#)] [[PubMed](#)]
44. Zeipina, S.; Alsiņa, I.; Lepse, L. Stinging nettle—the source of biologically active compounds as sustainable daily diet supplement. *Res. Rural. Dev.* **2014**, *20*, 34–38.
45. Ciulu, M.; Quirantes-Piné, R.; Spano, N.; Sanna, G.; Borrás-Linares, I.; Segura-Carretero, A. Evaluation of new extraction approaches to obtain phenolic compound-rich extracts from *Stevia rebaudiana* Bertoni leaves. *Ind. Crops Prod.* **2017**, *108*, 106–112. [[CrossRef](#)]
46. Zgórka, G. Pressurized liquid extraction versus other extraction techniques in micropreparative isolation of pharmacologically active isoflavones from *Trifolium* L. species. *Talanta* **2009**, *79*, 46–53. [[CrossRef](#)] [[PubMed](#)]
47. Hossain, M.B.; Barry-Ryan, C.; Martin-Diana, A.B.; Brunton, N.P. Optimisation of accelerated solvent extraction of antioxidant compounds from rosemary (*Rosmarinus officinalis* L.), marjoram (*Origanum majorana* L.) and oregano (*Origanum vulgare* L.) using response surface methodology. *Food Chem.* **2011**, *126*, 339–346. [[CrossRef](#)]
48. Erdoğan, S.; Erdemoğlu, S. Evaluation of polyphenol contents in differently processed apricots using accelerated solvent extraction followed by high-performance liquid chromatography–diode array detector. *Int. J. Food Sci. Nutr.* **2011**, *62*, 729–739. [[CrossRef](#)]
49. Gomes, S.V.F.; Portugal, L.A.; dos Anjos, J.P.; de Jesus, O.N.; de Oliveira, E.J.; David, J.P.; David, J.M. Accelerated solvent extraction of phenolic compounds exploiting a Box-Behnken design and quantification of five flavonoids by HPLC-DAD in *Passiflora* species. *Microchem. J.* **2017**, *132*, 28–35. [[CrossRef](#)]
50. Esclapez, M.D.; García-Pérez, J.V.; Mulet, A.; Cárcel, J.A. Ultrasound-Assisted Extraction of natural products. *Food Eng. Rev.* **2011**, *3*, 108–120. [[CrossRef](#)]
51. Zengin, G.; Cvetanović, A.; Gašić, U.; Stupar, A.; Bulut, G.; Şenkardes, I.; Dogan, A.; Ibrahime Sinan, K.; Uysal, S.; Aumeeruddy-Elalfi, Z.; et al. Modern and traditional extraction techniques affect chemical composition and bioactivity of *Tanacetum parthenium* (L.) Sch.Bip. *Ind. Crops Prod.* **2020**, *146*. [[CrossRef](#)]
52. Barros, F.; Dykes, L.; Awika, J.M.; Rooney, L.W. Accelerated solvent extraction of phenolic compounds from sorghum brans. *J. Cereal Sci.* **2013**, *58*, 305–312. [[CrossRef](#)]
53. Alkema, J.; Seager, S.L. The chemical pigments of plants. *J. Chem. Educ.* **1982**, *59*. [[CrossRef](#)]
54. Ferruzzi, M.G.; Bohm, V.; Courtney, P.D.; Schwartz, S.J. Antioxidant and antimutagenic activity of dietary chlorophyll derivatives determined by radical scavenging and bacterial reverse mutagenesis assays. *J. Food Sci.* **2002**, *67*, 2589–2595. [[CrossRef](#)]

55. Jaime, L.; Rodríguez-Meizoso, I.; Cifuentes, A.; Santoyo, S.; Suarez, S.; Ibáñez, E.; Señorans, F.J. Pressurized liquids as an alternative process to antioxidant carotenoids' extraction from *Haematococcus pluvialis* microalgae. *Lwt Food Sci. Technol.* **2010**, *43*, 105–112. [[CrossRef](#)]
56. Mustafa, A.; Trevino, L.M.; Turner, C. Pressurized hot ethanol extraction of carotenoids from carrot by-products. *Molecules* **2012**, *17*, 1809–1818. [[CrossRef](#)]
57. Sovová, H.; Sajfrtová, M.; Bártlová, M.; Opletal, L. Near-critical extraction of pigments and oleoresin from stinging nettle leaves. *J. Supercrit. Fluids* **2004**, *30*, 213–224. [[CrossRef](#)]
58. Saha, S.; Walia, S.; Kundu, A.; Sharma, K.; Paul, R.K. Optimal extraction and fingerprinting of carotenoids by accelerated solvent extraction and liquid chromatography with tandem mass spectrometry. *Food Chem.* **2015**, *177*, 369–375. [[CrossRef](#)]
59. Cha, K.H.; Lee, H.J.; Koo, S.Y.; Song, D.-G.; Lee, D.-U.; Pan, C.-H. Optimization of Pressurized Liquid Extraction of carotenoids and chlorophylls from *Chlorella vulgaris*. *J. Agric. Food Chem.* **2010**, *58*, 793–797. [[CrossRef](#)]
60. Kim, J.-S.; An, C.G.; Park, J.-S.; Lim, Y.P.; Kim, S. Carotenoid profiling from 27 types of paprika (*Capsicum annuum* L.) with different colors, shapes, and cultivation methods. *Food Chem.* **2016**, *201*, 64–71. [[CrossRef](#)]
61. Rafajlovska, V.; Djarmati, Z.; Najdenova, V.; Cvetkov, L. Extraction of stinging nettle (*Urtica dioica* L.) with supercritical carbon dioxide. *Balikesir Üniversitesi Fen Bilimleri Enstitüsü Dergisi* **2016**, *4*, 49–52.
62. Plaza, M.; Santoyo, S.; Jaime, L.; Avalo, B.; Cifuentes, A.; Reglero, G.; García-Blairsy Reina, G.; Señorans, F.J.; Ibáñez, E. Comprehensive characterization of the functional activities of pressurized liquid and ultrasound-assisted extracts from *Chlorella vulgaris*. *Lwt-Food Sci. Technol.* **2012**, *46*, 245–253. [[CrossRef](#)]
63. Koo, S.Y.; Cha, K.H.; Song, D.-G.; Chung, D.; Pan, C.-H. Optimization of pressurized liquid extraction of zeaxanthin from *Chlorella ellipsoidea*. *J. Appl. Phycol.* **2011**, *24*, 725–730. [[CrossRef](#)]
64. Cheng, S.H.; Khoo, H.E.; Kong, K.W.; Prasad, K.N.; Galanakis, C.M. Extraction of carotenoids and applications. In *Carotenoids: Properties, Processing and Applications*; Galanakis, C.M., Ed.; Academic Press: New York, NY, USA, 2020; pp. 259–288. [[CrossRef](#)]
65. Skapska, S.; Marszałek, K.; Woźniak, L.; Zawada, K.; Wawer, I. Aronia dietary drinks fortified with selected herbal extracts preserved by thermal pasteurization and high pressure carbon dioxide. *Lwt-Food Sci. Technol.* **2017**, *85*, 423–426. [[CrossRef](#)]
66. Tian, Y.; Puganen, A.; Alakomi, H.-L.; Uusitupa, A.; Saarela, M.; Yang, B. Antioxidative and antibacterial activities of aqueous ethanol extracts of berries, leaves, and branches of berry plants. *Food Res. Int.* **2018**, *106*, 291–303. [[CrossRef](#)]
67. Howard, L.; Pandjaitan, N. Pressurized Liquid Extraction of flavonoids from spinach. *J. Food Sci.* **2008**, *73*, C151–C157. [[CrossRef](#)]
68. Benchikh, Y.; Louailèche, H. Effects of extraction conditions on the recovery of phenolic compounds and in vitro antioxidant activity of carob (*Ceratonia siliqua* L.) pulp. *Acta Botanica Gallica* **2014**, *161*, 175–181. [[CrossRef](#)]
69. Četković, G.; Čanadanović-Brunet, J.; Djilas, S.; Tumbas, V.; Markov, S.; Cvetković, D. Antioxidant potential, lipid peroxidation inhibition and antimicrobial activities of *Satureja montana* L. subsp. *kitaibelii* extracts. *Int. J. Mol. Sci.* **2007**, *8*, 1013–1027. [[CrossRef](#)]
70. Miguel, M.G. Antioxidant activity of medicinal and aromatic plants. A review. *Flavour Fragr. J.* **2010**, *25*, 291–312. [[CrossRef](#)]
71. Zenão, S.; Aires, A.; Dias, C.; Saavedra, M.J.; Fernandes, C. Antibacterial potential of *Urtica dioica* and *Lavandula angustifolia* extracts against methicillin resistant *Staphylococcus aureus* isolated from diabetic foot ulcers. *J. Herbal Med.* **2017**, *10*, 53–58. [[CrossRef](#)]
72. Böhm, V.; Puspitasari-Nienaber, N.L.; Ferruzzi, M.G.; Schwartz, S.J. Trolox equivalent antioxidant capacity of different geometrical isomers of  $\alpha$ -carotene,  $\beta$ -carotene, lycopene, and zeaxanthin. *J. Agric. Food Chem.* **2002**, *50*, 221–226. [[CrossRef](#)]
73. Ben-dor, A.; Steiner, M.; Gheber, L.; Danilenko, M.; Dubi, N.; Linnewiel, K.; Zick, A.; Sharoni, Y.; Levy, J. Carotenoids activate the antioxidant response element transcription system. Carotenoids activate the antioxidant response element transcription system. *Mol. Cancer Ther.* **2005**, *4*, 177–186. [[PubMed](#)]

74. Dewick, P.M. *Medicinal Natural Products: A Biosynthetic Approach*; John Wiley & Sons Ltd.: Chichester, UK, 2009.
75. Yunbo, L. Carotenoids. In *Antioxidants in Biology and Medicine: Essentials, Advances, and Clinical Applications*; Tsisana Shartava, M.D., Tbilisi, G., Eds.; Nova Science Publishers Inc.: New York, NY, USA, 2011.



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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*

*Foods*





Publication No.2

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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 stable 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,  $\alpha$ -carotene,  $\beta$ -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  $-18\text{ }^{\circ}\text{C}$ , 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  $103 \pm 2\text{ }^{\circ}\text{C}$  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/<br>Latitude/Longitude         | Weather<br>Parameters | Phenological Stage    |       |                        |       |                        |         |
|--------------------|---|---|-----------------------|-----------------------|-------|------------------------|-------|------------------------|---------|
|                    |   |   |                       | I<br>Before Flowering |       | II<br>During Flowering |       | III<br>After Flowering |         |
|                    |   |   |                       | April                 | May   | June                   | July  | September              | October |
| Continental        | Sela Žakanjska                          | 244 m<br>45°36′27.80″N<br>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<br>45°34′14.88″N<br>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<br>45°36′34.38″N<br>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<br>45°51′31.10″N<br>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<br>45°49′8.69″N<br>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<br>45°48′47.23″N<br>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/<br>Latitude/Longitude | Weather<br>Parameters | Phenological Stage    |       |                        |       |                        |         |
|---------------|---------------|---------------------------------|-----------------------|-----------------------|-------|------------------------|-------|------------------------|---------|
|               |               |                                 |                       | I<br>Before Flowering |       | II<br>During Flowering |       | III<br>After Flowering |         |
|               |               |                                 |                       | April                 | May   | June                   | July  | September              | October |
| Mountain      | Ogulin        | 320 m                           | a.d. T (°C)           | 10.8                  | 12.4  | 21.6                   | 21.4  | 15.6                   | 13.0    |
|               |               | 45°15'47.84"N                   | T min (°C)            | 0.5                   | 0.5   | 11.8                   | 8.3   | 2.8                    | 3.0     |
|               |               | 15°13'42.36"E                   | 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                           | a.d. T (°C)           | 9.4                   | 11.1  | 20.1                   | 19.7  | 14.0                   | 10.6    |
|               |               | 44°49'44.07"N                   | T min (°C)            | −2.1                  | −1.3  | 7.6                    | 5.5   | −2.0                   | 1.2     |
| 15°17'57.29"E |               | 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         | a.d. T (°C)                     | 9.4                   | 11.1                  | 20.1  | 19.7                   | 14.0  | 10.6                   |         |
|               | 44°49'50.05"N | T min (°C)                      | −2.1                  | −1.3                  | 7.6   | 5.5                    | −2.0  | 1.2                    |         |
|               | 15°17'57.18"E | 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                          | a.d. T (°C)           | 13.0                  | 14.5  | 24.3                   | 24.9  | 19.4                   | 15.7    |
|               |               | 45°13'37.03"N                   | T min (°C)            | 3.9                   | 6.0   | 13.2                   | 13.4  | 7.3                    | 6.3     |
|               |               | 13°35'39.64"E                   | 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                            | a.d. T (°C)           | 13.0                  | 14.5  | 24.3                   | 24.9  | 19.4                   | 15.7    |
|               |               | 45°7'56.45"N                    | T min (°C)            | 3.9                   | 6.0   | 13.2                   | 13.4  | 7.3                    | 6.3     |
|               |               | 13°39'13.78"E                   | 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                           | a.d. T (°C)           | 13.4                  | 14.4  | 23.9                   | 24.5  | 19.8                   | 15.5    |
|               |               | 45°2'25.93"N                    | T min (°C)            | 4.9                   | 4.5   | 13.8                   | 13.6  | 7.5                    | 5.8     |
|               |               | 13°47'8.88"E                    | 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         | a.d. T (°C)                     | 13.4                  | 14.4                  | 23.9  | 24.5                   | 19.8  | 15.5                   |         |
|               | 44°57'28.79"N | T min (°C)                      | 4.9                   | 4.5                   | 13.8  | 13.6                   | 7.5   | 5.8                    |         |
|               | 13°51'6.10"E  | 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         | a.d. T (°C)                     | 11.1                  | 12.5                  | 22.2  | 22.3                   | 16.4  | 13.1                   |         |
|               | 45°14'30.84"N | T min (°C)                      | 0.5                   | 1.1                   | 11.2  | 9.5                    | 2.3   | 2.5                    |         |
|               | 13°48'38.44"E | 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<sup>-1</sup>), capillary voltage, +4 –3.5 kV<sup>-1</sup> 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<sup>-1</sup> 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<sup>-1</sup>. 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 ( $\alpha$ - and  $\beta$ -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  $\alpha$ -carotene,  $\beta$ -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  $\beta$ -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<sup>-1</sup> 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 peroxy 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)  $100\text{ g}^{-1}$  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 protocatechuic (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 loss 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 Francišковиć 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 isorhamnetin 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        |          |                  |                      |                 |                         |                         |   |
| <b>2</b>             | 0.828    | 105              | 9                    | -               | 153                     | 109                     | Protocatechuic acid (3,4-dihydroxybenzoic acid) |
| <b>14</b>            | 0.992    | 100              | 9                    | -               | 153                     | 109                     | Gentisic acid (2,5-dihydroxybenzoic acid)       |
| <b>31</b>            | 8.837    | 90               | 7                    | -               | 197                     | 182                     | Syringic acid                                   |
| <b>34</b>            | 11.358   | 80               | 10                   | -               | 137                     | 93                      | <i>p</i> -hydroxybenzoic acid                   |
| <b>35</b>            | 11.375   | 100              | 10                   | -               | 169                     | 125                     | Gallic acid *                                   |
| Cinnamic acids       |          |                  |                      |                 |                         |                         |   |
| <b>12</b>            | 0.975    | 80               | 10                   | -               | 179                     | 135                     | Caffeic acid *                                  |
| <b>15</b>            | 1.254    | 80               | 10                   | -               | 353                     | 191                     | Chlorogenic acid *                              |
| <b>19</b>            | 3.332    | 80               | 10                   | -               | 163                     | 119                     | <i>p</i> -coumaric acid *                       |
| <b>21</b>            | 4.490    | 100              | 5                    | -               | 147                     | 103                     | Cinnamic acid                                   |
| <b>25</b>            | 6.158    | 80               | 5                    | -               | 193                     | 178                     | Ferulic acid *                                  |
| <b>32</b>            | 11.012   | 100              | 17                   | -               | 223                     | 193                     | Sinapic acid *                                  |
| Other phenolic acids |          |                  |                      |                 |                         |                         |   |
| <b>16</b>            | 1.620    | 150              | 20                   | -               | 191                     | 85                      | Quinic acid *                                   |
| Flavonols            |          |                  |                      |                 |                         |                         |   |
| <b>3</b>             | 0.842    | 120              | 15                   | +               | 625                     | 317                     | Isorhamnetin rutinoside                         |
| <b>4</b>             | 0.856    | 120              | 15                   | +               | 595                     | 287                     | Kaempferol-3-rutinoside *                       |
| <b>5</b>             | 0.880    | 100              | 5                    | +               | 449                     | 303                     | Quercetin rhamnoside                            |
| <b>6</b>             | 0.880    | 30               | 5                    | +               | 449                     | 287                     | Kaempferol hexoside                             |
| <b>8</b>             | 0.907    | 140              | 25                   | +               | 319                     | 273                     | Myricetin *                                     |
| <b>10</b>            | 0.938    | 130              | 15                   | -               | 301                     | 151                     | Quercetin                                       |
| <b>17</b>            | 1.855    | 100              | 5                    | +               | 465                     | 303                     | Quercetin-3-glucoside *                         |
| <b>18</b>            | 2.461    | 120              | 5                    | +               | 611                     | 303                     | Quercetin-3-rutinoside *                        |
| <b>24</b>            | 5.963    | 160              | 21                   | -               | 315                     | 300                     | Isorhamnetin                                    |
| <b>27</b>            | 7.106    | 100              | 5                    | +               | 419                     | 287                     | Kaempferol pentoside                            |
| <b>28</b>            | 7.256    | 100              | 5                    | +               | 435                     | 303                     | Quercetin pentoside                             |
| <b>29</b>            | 7.930    | 100              | 5                    | +               | 433                     | 287                     | Kaempferol rhamnoside                           |
| <b>30</b>            | 8.242    | 100              | 10                   | +               | 507                     | 303                     | Quercetin acetylhexoside                        |
| <b>33</b>            | 11.232   | 100              | 15                   | +               | 653                     | 303                     | Quercetin acetylrutinoside                      |
| <b>36</b>            | 11.391   | 100              | 15                   | +               | 597                     | 303                     | Quercetin pentosylhexoside                      |
| <b>39</b>            | 11.758   | 120              | 15                   | +               | 581                     | 287                     | Kaempferol pentosylhexoside                     |
| <b>41</b>            | 11.822   | 130              | 0                    | -               | 285                     | 285                     | Kaempferol                                      |
| Flavan-3-ols         |          |                  |                      |                 |                         |                         |   |
| <b>23</b>            | 4.728    | 100              | 5, 15                | +               | 459                     | 289, 139                | Epigallocatechin gallate *                      |
| <b>37</b>            | 11.615   | 100              | 10                   | +               | 291                     | 139                     | Epicatechin *                                   |
| <b>38</b>            | 11.621   | 100              | 5                    | +               | 291                     | 165                     | Catechin *                                      |
| <b>40</b>            | 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<br>(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<sup>-1</sup> dm, among which cinnamic acids were the most abundant group (GM 179.22 mg 100 g<sup>-1</sup> dm), followed by flavonols (GM 134.60 mg 100 g<sup>-1</sup> dm), flavones (GM 24.56 mg 100 g<sup>-1</sup> dm), flavan-3-ols (GM 20.70 mg 100 g<sup>-1</sup> dm) and benzoic acids (GM 10.20 mg 100 g<sup>-1</sup> dm). Coumarins, isoflavones and other acids were present in lower concentrations: GM values 5.31, 3.09 and 2.88 mg 100 g<sup>-1</sup> dm, respectively, while the least represented group of polyphenols were flavanones (GM 0.34 mg 100 g<sup>-1</sup> 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<sup>-1</sup> fw, wild 2.58 mg g<sup>-1</sup> fw) as opposed to nettle stalks (cultivated 3.670 mg g<sup>-1</sup> fw, wild 0.750 mg g<sup>-1</sup> 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 Kószegi 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<sup>-1</sup> 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 |
|---------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| <b>Plant part</b>         | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 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    |
| <b>Phenological stage</b> | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 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    |
| <b>Region/Habitat</b>     | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * |
| Sela                      | 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    |
| Žakanjska                 |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |
| Sopčič 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    |
| Koretici                  | 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    |
| <b>M</b>                  |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |
| 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    |
| <b>S</b>                  |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |
| 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    |
| <b>Grand mean</b>         | <b>10.20</b>      | <b>179.22</b>     | <b>2.88</b>       | <b>134.60</b>     | <b>20.70</b>      | <b>24.56</b>      | <b>3.09</b>       | <b>0.34</b>       | <b>5.31</b>       | <b>380.90</b>     |

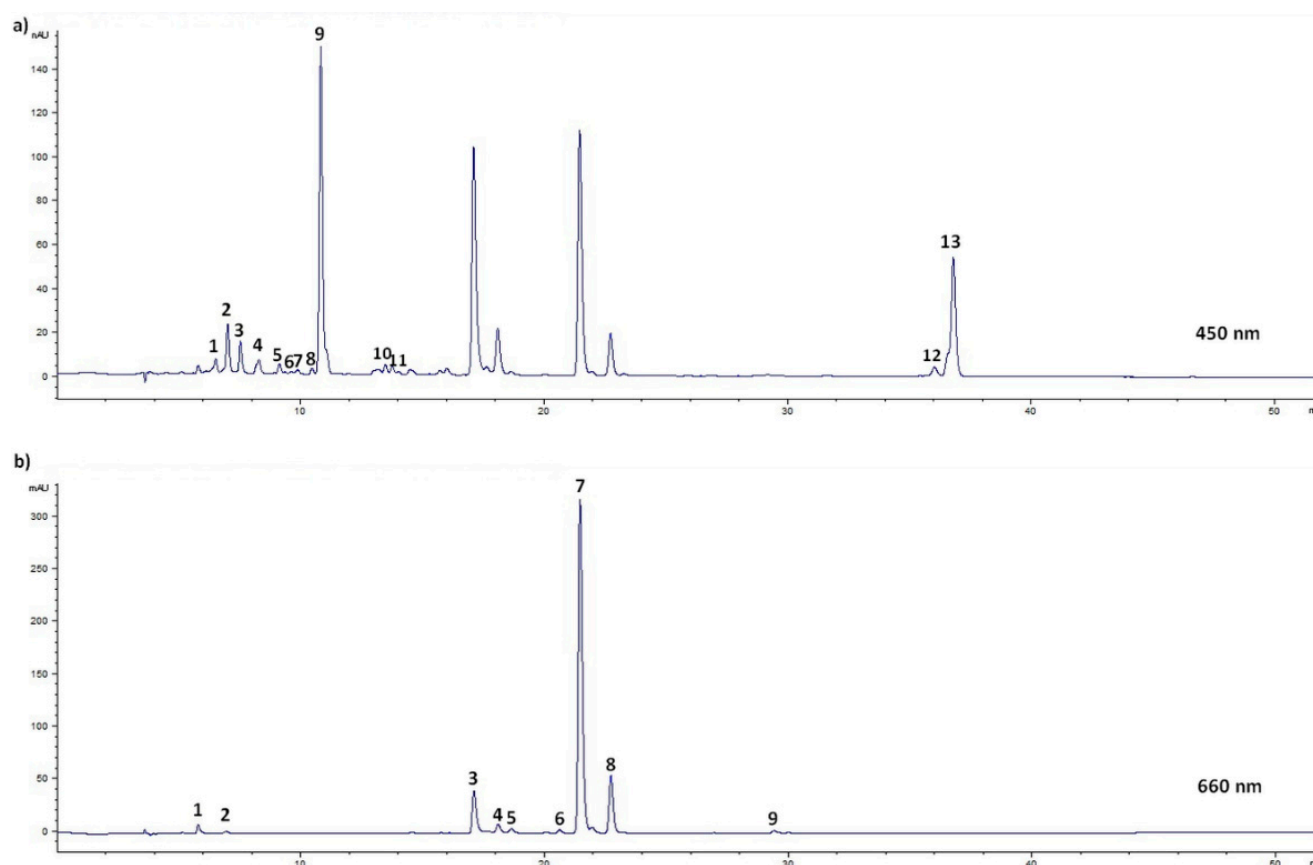
C = continental, M = mountain, S = seaside. \* Statistically significant variable at  $p \leq 0.05$ . Results are expressed as mean ± SE (N = 4). Values with different letters within column are statistically different at  $p \leq 0.05$ .

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 ( $513.12 \pm 1.03 \text{ mg } 100 \text{ g}^{-1} \text{ dm}$ ), benzoic ( $19.39 \pm 0.10 \text{ mg } 100 \text{ g}^{-1} \text{ dm}$ ) and cinnamic acids ( $227.10 \pm 0.70 \text{ mg } 100 \text{ g}^{-1} \text{ dm}$ ), flavan-3-ols ( $32.13 \pm 0.11 \text{ mg } 100 \text{ g}^{-1} \text{ dm}$ ), flavones ( $42.44 \pm 0.12 \text{ mg } 100 \text{ g}^{-1} \text{ dm}$ ) and isoflavones ( $5.29 \pm 0.05 \text{ mg } 100 \text{ g}^{-1} \text{ dm}$ ). Contrarily, Ogulin, situated in mountain areas, was characterized with the highest amounts of other acids ( $4.62 \pm 0.07 \text{ mg } 100 \text{ g}^{-1} \text{ dm}$ ), flavonols ( $182.65 \pm 0.38 \text{ mg } 100 \text{ g}^{-1} \text{ dm}$ ), flavanones ( $0.56 \pm 0.02 \text{ mg } 100 \text{ g}^{-1} \text{ dm}$ ) and coumarins ( $6.77 \pm 0.04 \text{ mg } 100 \text{ g}^{-1} \text{ dm}$ ). 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,  $\alpha$ -carotene,  $\beta$ -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  $644.22 \text{ mg } 100 \text{ g}^{-1} \text{ dm}$ , most of which were chlorophylls (GM  $611.19 \text{ mg } 100 \text{ g}^{-1} \text{ dm}$ ), while carotenoids were less present (GM  $33.03 \text{ mg } 100 \text{ g}^{-1} \text{ dm}$ ). 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.  $16 \text{ mg } \text{g}^{-1} \text{ extract}$ ). 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 =  $\alpha$ -carotene, 13 =  $\beta$ -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 ( $691.46 \text{ mg } 100 \text{ g}^{-1} \text{ dm}$ ) when compared to the amount of carotenoids ( $36.97 \text{ mg } 100 \text{ g}^{-1} \text{ dm}$ ). 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  $\beta$ -carotene concentrations in nettle leaves occurred during the flowering stage ( $184$  and  $6.7 \mu\text{g g}^{-1} \text{ dm}$ , 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<sup>-1</sup> dm) and ORAC values (mmol TE 100 g<sup>-1</sup> dm) in wild nettle (*Urtica dioica* L.) upon plant part, phenological stage and habitat.

| Source of Variation       |                | Carotenoids       | Chlorophylls      | Total Pigments    | ORAC              |
|---------------------------|----------------|-------------------|-------------------|-------------------|-------------------|
| <b>Plant Part</b>         |                | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 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      |
| <b>Phenological Stage</b> |                | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 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      |
| <b>Region/Habitat</b>     |                | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * | <i>p</i> < 0.01 * |
| C                         | Sela Žakanjska | 37.29 ± 0.21h     | 701.89 ± 1.76g    | 739.18 ± 1.87h    | 11.76 ± 0.06i     |
|                           | Sopčič 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      |
| <b>Grand mean</b>         |                | <b>33.03</b>      | <b>611.19</b>     | <b>644.22</b>     | <b>9.67</b>       |

C = continental, M = mountain, S = seaside. \* Statistically significant variable at *p* ≤ 0.05. Results are expressed as mean ± SE (N = 4). Values with different letters within column are statistically different at *p* ≤ 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<sup>-1</sup> 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<sup>-1</sup> dm vs. 7.37 mmol TE 100 g<sup>-1</sup> dm). Similar ORAC values in nettle leaves were recorded in study of Repajić et al. (2020) [11], while Česlova 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, Kirca 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<sup>-1</sup> dm) and ORAC values (mmol TE 100 g<sup>-1</sup> 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 *     |

\*  $p \leq 0.05$ .

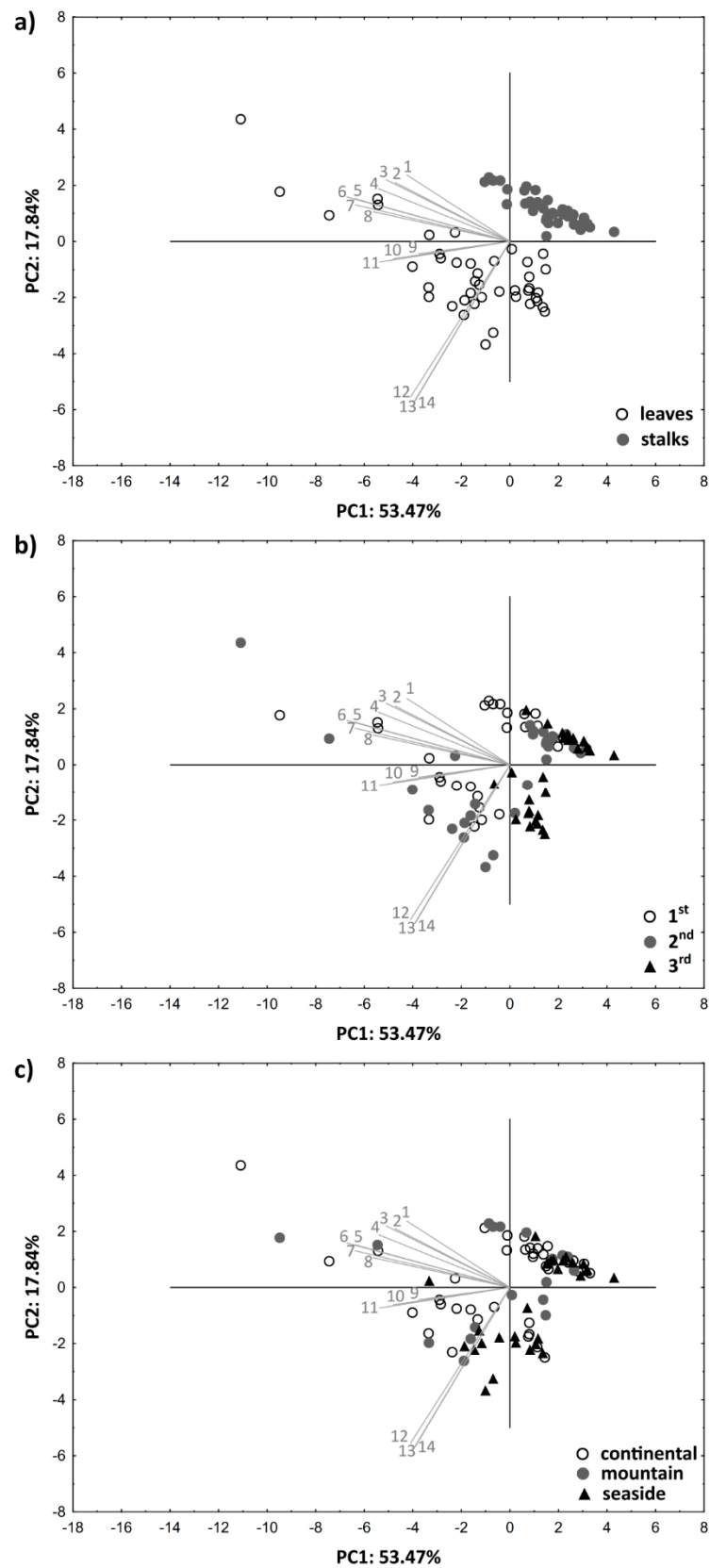
Obtained results clearly demonstrated the importance of the appropriate plant part selection as well as its phenological stage with the presence of the highest bioactive compounds accumulation in order to obtain the maximally enriched product, which will be beneficial for consumers.

### 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<sup>-1</sup> dm and 57.44–383.25 mg 100 g<sup>-1</sup> dm, respectively), while chlorophylls were the most abundant natural pigments (4.26–1934.38 mg 100 g<sup>-1</sup> dm). Moreover, the ORAC values of obtained nettle extracts ranged from 3.05 to 19.83 mmol TE 100 g<sup>-1</sup> 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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#### References

1. Di Virgilio, N.; Papazoglou, E.G.; Jankauskiene, Z.; Di Lonardo, S.; Praczyk, M.; Wielgusz, K. The potential of stinging nettle (*Urtica dioica* L.) as a crop with multiple uses. *Ind. Crop. Prod.* **2015**, *68*, 42–49. [[CrossRef](#)]
2. Kregiel, D.; Pawlikowska, E.; Antolak, H. *Urtica* spp.: Ordinary plants with extraordinary properties. *Molecules* **2018**, *23*, 1664. [[CrossRef](#)] [[PubMed](#)]
3. Upton, R. Stinging nettles leaf (*Urtica dioica* L.): Extraordinary vegetable medicine. *J. Herb. Med.* **2013**, *3*, 9–38. [[CrossRef](#)]

4. Guil-Guerrero, J.L.; Reboloso-Fuentes, M.M.; Isasa, M.E.T. Fatty acids and carotenoids from Stinging Nettle (*Urtica dioica* L.). *J. Food Compos. Anal.* **2003**, *16*, 111–119. [[CrossRef](#)]
5. Sajfrtová, M.; Sovová, H.; Opletal, L.; Bártlová, M. Near-critical extraction of  $\beta$ -sitosterol and scopoletin from stinging nettle roots. *J. Supercrit. Fluids* **2005**, *35*, 111–118. [[CrossRef](#)]
6. Pinelli, P.; Ieri, F.; Vignolini, P.; Bacci, L.; Baronti, S.; Romani, A. Extraction and HPLC analysis of phenolic compounds in leaves, stalks, and textile fibers of *Urtica dioica* L. *J. Agric. Food Chem.* **2008**, *56*, 9127–9132. [[CrossRef](#)]
7. Otlés, S.; Yalcin, B. Phenolic compounds analysis of root, stalk, and leaves of nettle. *Sci. World J.* **2012**, *2012*. [[CrossRef](#)]
8. Carvalho, A.R.; Costa, G.; Figueirinha, A.; Liberal, J.; Prior, J.A.V.; Lopes, M.C.; Cruz, M.T.; Batista, M.T. *Urtica* spp.: Phenolic composition, safety, antioxidant and anti-inflammatory activities. *Food Res. Int.* **2017**, *99*, 485–494. [[CrossRef](#)]
9. Đurović, S.; Pavlič, B.; Šorgić, S.; Popov, S.; Savić, S.; Pertoničević, M.; Radojković, M.; Cvetanović, A.; Zeković, Z. Chemical composition of stinging nettle leaves obtained by different analytical approaches. *J. Funct. Foods* **2017**, *32*, 18–26. [[CrossRef](#)]
10. Marchetti, N.; Bonetti, G.; Brandolini, V.; Cavazzini, A.; Maietti, A.; Meca, G.; Mañes, J. Stinging nettle (*Urtica dioica* L.) as a functional food additive in egg pasta: Enrichment and bioaccessibility of Lutein and  $\beta$ -carotene. *J. Funct. Foods* **2018**, *47*, 547–553. [[CrossRef](#)]
11. Repajić, M.; Cegledi, E.; Kruk, V.; Pedisić, S.; Çınar, F.; Bursać Kovačević, D.; Žutić, I.; Dragović-Uzelac, V. Accelerated Solvent Extraction as a Green Tool for the Recovery of Polyphenols and Pigments from Wild Nettle Leaves. *Processes* **2020**, *8*, 803. [[CrossRef](#)]
12. Dhoubi, R.; Affes, H.; Salem, M.B.; Hammami, S.; Sahnoun, Z.; Zeghal, K.M.; Ksouda, K. Screening of pharmacological uses of *Urtica dioica* and others benefits. *Prog. Biophys. Mol. Biol.* **2020**, *150*, 67–77. [[CrossRef](#)] [[PubMed](#)]
13. Wagner, H.; Willer, F.; Samtleben, R.; Boos, G. Search for the antiprostatic principle of stinging nettle (*Urtica dioica*) roots. *Phytomedicine* **1994**, *1*, 213–224. [[CrossRef](#)]
14. Orcic, D.; Franciškovic, M.; Bekvalac, K.; Svircev, E.; Beara, I.; Lesjak, M.; Mimica-Dukic, N. Quantitative determination of plant phenolics in *Urtica dioica* extracts by high-performance liquid chromatography coupled with tandem mass spectrometric detection. *Food Chem.* **2014**, *143*, 48–53. [[CrossRef](#)] [[PubMed](#)]
15. Chrubasik, J.E.; Roufogalis, B.D.; Wagner, H.; Chrubasik, S. A comprehensive review on the stinging nettle effect and efficacy profiles. Part II: *Urticae radix*. *Phytomedicine* **2007**, *14*, 568–579. [[CrossRef](#)] [[PubMed](#)]
16. Brown, D. Encyclopedia of herbs and their uses. In *Encyclopedia of Common Natural Ingredients Used in Food, Drugs, and Cosmetics*; Leung, Y.A., Foster, S., Eds.; Wiley: Hoboken, NJ, USA, 1995.
17. Mottaleb, M.A.; Sarker, S.D. Accelerated solvent extraction for natural products isolation. In *Natural Products Isolation*; Springer: Berlin/Heidelberg, Germany, 2012; pp. 75–87.
18. Hojnik, M.; Škerget, M.; Knez, Ž. Isolation of chlorophylls from stinging nettle (*Urtica dioica* L.). *Sep. Purif. Technol.* **2007**, *57*, 37–46. [[CrossRef](#)]
19. Kőszegi, K.; Békássy-Molnár, E.; Koczka, N.; Kerner, T.; Stefanovits-Bányai, É. Changes in Total Polyphenol Content and Antioxidant Capacity of Stinging Nettle (*Urtica dioica* L.) from Spring to Autumn. *Period. Polytech. Chem. Eng.* **2020**, *64*, 548–554. [[CrossRef](#)]
20. Ncube, B.; Van Staden, J. Tilting plant metabolism for improved metabolite biosynthesis and enhanced human benefit. *Molecules* **2015**, *20*, 12698–12731. [[CrossRef](#)]
21. Moore, M. *Medicinal Plants of the Mountain West*; Museum of New Mexico Press: Santa Fe, NM, USA, 2003; ISBN 0890134545.
22. Tolić, M.T.; Krbavčić, I.P.; Vujević, P.; Milinović, B.; Jurčević, I.L.; Vahčić, N. Effects of weather conditions on phenolic content and antioxidant capacity in juice of chokeberries (*Aronia melanocarpa* L.). *Pol. J. Food Nutr. Sci.* **2017**, *67*, 67–74. [[CrossRef](#)]
23. González-Talice, J.; Yuri, J.A.; del Pozo, A. Relations among pigments, color and phenolic concentrations in the peel of two Gala apple strains according to canopy position and light environment. *Sci. Hortic.* **2013**, *151*, 83–89. [[CrossRef](#)]
24. Saini, R.K.; Nile, S.H.; Park, S.W. Carotenoids from fruits and vegetables: Chemistry, analysis, occurrence, bioavailability and biological activities. *Food Res. Int.* **2015**, *76*, 735–750. [[CrossRef](#)] [[PubMed](#)]
25. AOAC. *Official Methods of Analysis: Changes in Official Methods of Analysis Made at the Annual Meeting. Supplement*; Association of Official Analytical Chemists: Rockville, MD, USA, 1990; Volume 15.
26. Elez Garofulić, I.; Zorić, Z.; Pedisić, S.; Brnčić, M.; Dragović-Uzelac, V. UPLC-MS2 Profiling of Blackthorn Flower Polyphenols Isolated by Ultrasound-Assisted Extraction. *J. Food Sci.* **2018**, *83*, 2782–2789. [[CrossRef](#)] [[PubMed](#)]
27. Castro-Puyana, M.; Pérez-Sánchez, A.; Valdés, A.; Ibrahim, O.H.M.; Suarez-Álvarez, S.; Ferragut, J.A.; Micol, V.; Cifuentes, A.; Ibáñez, E.; García-Cañas, V. Pressurized liquid extraction of Neochloris oleoabundans for the recovery of bioactive carotenoids with anti-proliferative activity against human colon cancer cells. *Food Res. Int.* **2017**, *99*, 1048–1055. [[CrossRef](#)] [[PubMed](#)]
28. Sozgen Baskan, K.; Tutem, E.; Ozer, N.; Apak, R. Spectrophotometric and chromatographic assessment of contributions of carotenoids and chlorophylls to the total antioxidant capacities of plant foods. *J. Agric. Food Chem.* **2013**, *61*, 11371–11381. [[CrossRef](#)]
29. Gupta, P.; Sreelakshmi, Y.; Sharma, R. A rapid and sensitive method for determination of carotenoids in plant tissues by high performance liquid chromatography. *Plant Methods* **2015**, *11*, 5. [[CrossRef](#)]
30. Prior, R.L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290–4302. [[CrossRef](#)]

31. Bender, C.; Graziano, S.; Zimmerman, B.F.; Weidlich, H.H. Antioxidant potential of aqueous plant extracts assessed by the cellular antioxidant activity assay. *Am. J. Biol. Life Sci.* **2014**, *2*, 72–79.
32. Gruz, J.; Novák, O.; Strnad, M. Rapid analysis of phenolic acids in beverages by UPLC-MS/MS. *Food Chem.* **2008**, *111*, 789–794. [[CrossRef](#)]
33. Parejo, I.; Jauregui, O.; Sánchez-Rabateda, F.; Viladomat, F.; Bastida, J.; Codina, C. Separation and characterization of phenolic compounds in fennel (*Foeniculum vulgare*) using liquid chromatography-negative electrospray ionization tandem mass spectrometry. *J. Agric. Food Chem.* **2004**, *52*, 3679–3687. [[CrossRef](#)]
34. Francišковиć, M.; Gonzalez-Pérez, R.; Orčić, D.; de Medina, F.S.; Martínez-Augustin, O.; Svirčev, E.; Simin, N.; Mimica-Dukić, N. Chemical Composition and Immuno-Modulatory Effects of *Urtica dioica* L. (Stinging Nettle) Extracts. *Phyther. Res.* **2017**, *31*, 1183–1191. [[CrossRef](#)]
35. Zeković, Z.; Cvetanović, A.; Švarc-Gajić, J.; Gorjanović, S.; Sužnjević, D.; Mašković, P.; Savić, S.; Radojković, M.; Đurović, S. Chemical and biological screening of stinging nettle leaves extracts obtained by modern extraction techniques. *Ind. Crop. Prod.* **2017**, *108*, 423–430. [[CrossRef](#)]
36. Chen, Y.; Yu, H.; Wu, H.; Pan, Y.; Wang, K.; Jin, Y.; Zhang, C. Characterization and Quantification by LC-MS/MS of the Chemical Components of the Heating Products of the Flavonoids Extract in Pollen Typhae for Transformation Rule Exploration. *Molecules* **2015**, *20*, 18352–18366. [[CrossRef](#)] [[PubMed](#)]
37. Bucar, F.; Britzmann, B.; Streit, B.; Weigend, M. LC-PDA-MS-profiles of phenolic compounds in extracts of aerial parts of *Urtica* species. *Planta Med.* **2006**, *72*. [[CrossRef](#)]
38. Pinheiro, P.F.; Justino, G.C. Structural Analysis of Flavonoids and Related Compounds—A Review of Spectroscopic Applications. In *Phytochemicals—A Global Perspective of Their Role in Nutrition and Health*; Rao, V., Ed.; InTech: Rijeka, Croatia, 2012; pp. 33–56.
39. Farag, M.A.; Weigend, M.; Luebert, F.; Brokamp, G.; Wessjohann, L.A. Phytochemical, phylogenetic, and anti-inflammatory evaluation of 43 *Urtica* accessions (stinging nettle) based on UPLC-Q-TOF-MS metabolomic profiles. *Phytochemistry* **2013**, *96*, 170–183. [[CrossRef](#)] [[PubMed](#)]
40. Proestos, C.; Boziaris, I.S.; Nychas, G.-J.; Komaitis, M. Analysis of flavonoids and phenolic acids in Greek aromatic plants: Investigation of their antioxidant capacity and antimicrobial activity. *Food Chem.* **2006**, *95*, 664–671. [[CrossRef](#)]
41. Nencu, I.; Vlase, L.; Istudor, V.; Duțu, L.E.; Gird, C.E. Preliminary research regarding the therapeutic uses of *Urtica dioica* L. Note I. The polyphenols evaluation. *Farmacia* **2012**, *60*, 493–500.
42. Wang, F.; Jiang, K.; Li, Z. Purification and Identification of Genistein in Ginkgo biloba Leaf Extract. *Chin. J. Chromatogr.* **2007**, *25*, 509–513. [[CrossRef](#)]
43. Mazimba, O. Umbelliferone: Sources, chemistry and bioactivities review. *Bull. Fac. Pharm. Cairo Univ.* **2017**, *55*, 223–232. [[CrossRef](#)]
44. Ioana, N.; Viorica, I.; Diana-Carolina, I.; Valeria, R. Preliminary research regarding the therapeutic uses of *Urtica dioica* l note ii. The dynamics of accumulation of total phenolic compounds and ascorbic acid. *Farmacia* **2013**, *61*, 276–283.
45. Roslon, W.; Weglarz, Z. Polyphenolic acids of female and male forms of *Urtica dioica*. In Proceedings of the International Conference on Medicinal and Aromatic Plants (Part II), Budapest, Hungary, 8–11 July 2001; pp. 101–104.
46. Biesiada, A.; Kucharska, A.; Sokół-Łętowska, A.; Kuś, A. Effect of the Age of Plantation and Harvest Term on Chemical Composition and Antioxidant Activity of Stinging Nettle (*Urtica dioica* L.). *Ecol. Chem. Eng. A* **2010**, *17*, 1061–1068.
47. Biesiada, A.; Wołoszczak, E.; Sokół-Łętowska, A.; Kucharska, A.Z.; Nawirska-Olszańska, A. The effect of nitrogen form and dose on yield, chemical composition and antioxidant activity of stinging nettle (*Urtica dioica* L.). *Herba Pol.* **2009**, *55*, 84–93.
48. Naser Aldeen, M.G.; Mansoor, R.; AlJoubbeh, M. Fluctuations of phenols and flavonoids in infusion of lemon verbena (*Lippia citriodora*) dried leaves during growth stages. *Nutr. Food Sci.* **2015**, *45*, 766–773. [[CrossRef](#)]
49. Zeipiņa, S.; Alsiņa, I.; Lepse, L. Stinging nettle—The source of biologically active compounds as sustainable daily diet supplement. *Res. Rural Dev.* **2014**, *20*, 34–38.
50. Kong, D.X.; Li, Y.Q.; Wang, M.L.; Bai, M.; Zou, R.; Tang, H.; Wu, H. Effects of light intensity on leaf photosynthetic characteristics, chloroplast structure, and alkaloid content of Mahonia bodinieri (Gagnep.) Laferr. *Acta Physiol. Plant.* **2016**, *38*, 120. [[CrossRef](#)]
51. Pajević, S.; Krstić, B.; Katić, S.; Nikolić, N.; Mihailović, V. Some photosynthetic parameters of alfalfa (*Medicago sativa* L.) leaves at different phenological stages and in different cuttings. *Matica Srp. Proc. Nat. Sci.* **1999**, *35*. Available online: [http://www.maticasrpska.org.rs/stariSajt/casopisi/prirodne\\_nauke\\_097.pdf#page=38](http://www.maticasrpska.org.rs/stariSajt/casopisi/prirodne_nauke_097.pdf#page=38) (accessed on 18 January 2021).
52. Cândido, T.L.N.; Silva, M.R.; Agostini-Costa, T.S. Bioactive compounds and antioxidant capacity of buriti (*Mauritia flexuosa* Lf) from the Cerrado and Amazon biomes. *Food Chem.* **2015**, *177*, 313–319. [[CrossRef](#)]
53. Česlová, L.; Šilarová, P.; Fischer, J. Effect of harvesting and processing of stinging nettle on the antioxidant capacity of its infusions. *Sci. Pap. Univ. Pardubice Ser. A Fac. Chem. Technol.* **2016**, *22*, 23–33.
54. Kırca, A.; Arslan, E. Antioxidant capacity and total phenolic content of selected plants from Turkey. *Int. J. Food Sci. Technol.* **2008**, *43*, 2038–2046. [[CrossRef](#)]

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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

Ena Cegledi, \*  Maja Repajić,  Sandra Balbino,  Matea Peričić and Verica Dragović-Uzelac 

## 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<sup>-1</sup>, with nine sterols and three pentacyclic triterpenoids identified and quantified within a total range of 42.81–76.57 mg 100 g<sup>-1</sup> and 0.69–1.68 mg 100 g<sup>-1</sup> dried root, respectively. The most abundant sterol and pentacyclic triterpenoid were  $\beta$ -sitosterol and  $\beta$ -amyrin acetate, with mean values of 50.21 mg 100 g<sup>-1</sup> and 0.56 mg 100 g<sup>-1</sup> 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. © 2022 Society of Chemical Industry.

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

## INTRODUCTION

Nettle (*Urtica dioica* L.) is a medicinal plant from the *Urticaceae* family, distributed worldwide from temperate to tropical areas.<sup>1,2</sup>

Because of the presence of various bioactive molecules, nettle possesses anti-inflammatory, anti-cancer, antioxidant, cardiovascular and diuretic effect.<sup>3–5</sup> Previous studies have shown that nettle leaves are rich in vitamins, minerals, phenolic compounds and pigments.<sup>6</sup> Considering their nutritional properties, they are used in soups, sauces, teas and juices.<sup>7–9</sup> On the other hand, nettle root is rich in protein lectin, sterols, polysaccharides, lignans, coumarins and phenols.<sup>10–12</sup> 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),<sup>13</sup> antioxidant and anti-inflammatory effects.<sup>12,14</sup> 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 Chrusasik *et al.*,<sup>13</sup> 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 Rübbergen<sup>15</sup> 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<sup>16</sup> 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.<sup>17,18</sup> 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  $\beta$ -sitosterol, campesterol and stigmasterol.<sup>19,20</sup> As structural components of cell membranes, sterols possess protective, structural, transport and signal function in plants.<sup>21</sup> 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.<sup>22</sup> Furthermore, sterols act as antioxidants, reduce the risk of cardiovascular diseases and cancer, and are used in anti-aging preparations,<sup>23,24</sup> and they also play a significant role in the treatment of BPH attributed to nettle root.<sup>25</sup> 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.<sup>24,26,27</sup> 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  $\alpha$ - and  $\beta$ -amyrin. Their biological effects include anti-inflammatory, antimicrobial, anticancer, antidiabetic and antioxidant properties; thus, they also are used as dietary supplements.<sup>17,28-31</sup>

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.<sup>32,33</sup> 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).<sup>34,35</sup> 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.<sup>36,37</sup> Furthermore, PLE has a number of other advantages over conventional methods: shorter extraction time, lower solvent consumption, controlled temperature and pressure, and automation.<sup>38-40</sup>

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<sub>2</sub>O<sub>3</sub> 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  $\alpha$ -cholestanol,  $\alpha$ -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; Sprzet 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<sup>41</sup> 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<sup>2-4</sup> 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<sup>®</sup> instrument (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the method previously described by Repajić *et al.*<sup>2</sup> and Balbino *et al.*,<sup>42</sup> 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.<sup>43</sup> Nettle root lipid fraction (0.25 g) was refluxed for 15 min with 5 mL of ethanolic KOH solution (0.5 mol L<sup>-1</sup>) after adding 1 mL of  $\alpha$ -cholestanol (1 mg mL<sup>-1</sup>) 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<sup>-1</sup> root; mean  $\pm$  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 $\pm$ 0.05               | 42.81 $\pm$ 0.04 | 0.69 $\pm$ 0.02                 |
| 2      | 110              | 5                 | 4                | 0.83  | 66.36 $\pm$ 0.14               | 65.14 $\pm$ 0.14 | 1.22 $\pm$ 0.00                 |
| 3      | 110              | 15                | 2                | 0.93  | 72.12 $\pm$ 0.01               | 70.59 $\pm$ 0.11 | 1.53 $\pm$ 0.10                 |
| 4      | 110              | 10                | 3                | 1.00  | 65.55 $\pm$ 0.19               | 64.43 $\pm$ 0.18 | 1.12 $\pm$ 0.01                 |
| 5      | 150              | 10                | 4                | 1.35  | 78.26 $\pm$ 0.35               | 76.57 $\pm$ 0.07 | 1.68 $\pm$ 0.42                 |
| 6      | 150              | 10                | 2                | 1.63  | 72.98 $\pm$ 0.33               | 71.53 $\pm$ 0.33 | 1.45 $\pm$ 0.00                 |
| 7      | 110              | 10                | 3                | 0.87  | 71.25 $\pm$ 0.14               | 69.87 $\pm$ 0.13 | 1.38 $\pm$ 0.01                 |
| 8      | 70               | 15                | 3                | 0.53  | 61.12 $\pm$ 0.02               | 59.99 $\pm$ 0.07 | 1.13 $\pm$ 0.05                 |
| 9      | 110              | 10                | 3                | 0.92  | 65.36 $\pm$ 0.10               | 64.06 $\pm$ 0.09 | 1.30 $\pm$ 0.00                 |
| 10     | 70               | 10                | 4                | 0.55  | 62.61 $\pm$ 0.19               | 61.37 $\pm$ 0.02 | 1.24 $\pm$ 0.17                 |
| 11     | 110              | 15                | 4                | 0.88  | 64.42 $\pm$ 0.18               | 63.04 $\pm$ 0.04 | 1.38 $\pm$ 0.14                 |
| 12     | 150              | 5                 | 3                | 1.57  | 70.11 $\pm$ 0.17               | 68.65 $\pm$ 0.16 | 1.46 $\pm$ 0.01                 |
| 13     | 110              | 5                 | 2                | 0.68  | 53.43 $\pm$ 0.11               | 52.39 $\pm$ 0.00 | 1.03 $\pm$ 0.11                 |
| 14     | 110              | 10                | 3                | 0.79  | 62.92 $\pm$ 0.06               | 61.57 $\pm$ 0.05 | 1.35 $\pm$ 0.01                 |
| 15     | 110              | 10                | 3                | 1.02  | 66.31 $\pm$ 0.13               | 64.80 $\pm$ 0.11 | 1.51 $\pm$ 0.02                 |
| 16     | 150              | 15                | 3                | 0.87  | 49.50 $\pm$ 0.21               | 48.38 $\pm$ 0.12 | 1.11 $\pm$ 0.10                 |
| 17     | 70               | 10                | 2                | 0.53  | 57.59 $\pm$ 0.04               | 56.15 $\pm$ 0.03 | 1.44 $\pm$ 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  $\mu$ L per 1 mg of unsaponifiable matter. The tube was closed and heated at 105 °C for 15 min, cooled and centrifuged at 4711  $\times$   $g$  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  $\times$  0.32 mm, 0.25  $\mu$ m (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<sup>-1</sup> 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<sup>-1</sup> and the split ratio was 13.3:1. The injected sample volume was 1.0  $\mu$ L. 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,  $\alpha$ -amyrin,  $\beta$ -amyrin and  $\beta$ -amyrin acetate were also identified. The content and composition of sterols and

pentacyclic triterpenoids were expressed in mg 100 g<sup>-1</sup> dried nettle root as the mean  $\pm$  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<sup>-1</sup> dried root) and the  $\beta$ -sitosterol content (mg 100 g<sup>-1</sup> 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:<sup>44</sup>

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

where  $Y$  is predicted response;  $\beta_0$  is fixed response at central point;  $\beta_i$ ,  $\beta_{ii}$  and  $\beta_{ij}$  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 analysis 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  $\pm$  SD ( $n = 2$  for extraction yield and  $n = 4$  for content of total triterpenoid derivatives and  $\beta$ -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.*,<sup>8</sup> the content of saponifiable matter in nettle root was 0.1 g 100 g<sup>-1</sup> sample, which is lower than the results of this study. Sajfrtová *et al.*<sup>11</sup> extracted the lipid fraction from nettle root using supercritical and liquid CO<sub>2</sub> and the highest yield of lipid extract was 9 mg g<sup>-1</sup> 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<sup>-1</sup> 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<sup>-1</sup> dried root with a mean of 62.43 mg 100 g<sup>-1</sup> and total pentacyclic triterpenoids were present in a range from 0.69 to 1.68 mg 100 g<sup>-1</sup> dried root with mean of 1.30 mg 100 g<sup>-1</sup> (Table 1). Kovacheva *et al.*<sup>45</sup> 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 the present study in which content of sterols, converted to the lipid extract obtained, was in the range

4.39–11.25%. Jafari *et al.*,<sup>46</sup> who analyzed nettle seeds, determined a total plant sterols content of 93.1 mg 100 g<sup>-1</sup> oil, which is even lower compared to the results of the present study. In addition, Balbino *et al.*<sup>42</sup> examined sterols in various Apiaceae seeds and total content of sterols were in the range 468.8–903.9 mg 100 g<sup>-1</sup> oil, whereas Ham *et al.*<sup>47</sup> determined the average sterols content in oily extract from saw palmetto berries to be 186.4 mg 100 g<sup>-1</sup> 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.*,<sup>48</sup> the content of sterols (expressed as oil content) in different vegetable oils was investigated, and the content ranged from 27.23 mg 100 g<sup>-1</sup> in coconut oil to 616.97 mg 100 g<sup>-1</sup> in evening primrose oil. Also, in other edible oils, the mean value of the different varieties was 511.82 mg 100 g<sup>-1</sup> for sesame seed oil,<sup>49</sup> and variation of sterols was in the range 170–190 mg 100 g<sup>-1</sup> for tomato seed oil<sup>50</sup>. 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<sup>-1</sup> 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  $\Delta^5$ -sterols,  $\Delta^7$ -sterols, stanols, sterol precursors and

**Table 2.** Content of sterols (mg 100 g<sup>-1</sup> root; mean  $\pm$  SD,  $n = 2$ ) in nettle root lipid fraction obtained under different PLE conditions

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

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

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

When observing individual sterols, the most dominant sterol in all samples was  $\beta$ -sitosterol, with a mean of 50.21 mg 100 g<sup>-1</sup> dried root (i.e. 80.42% of total sterols). It was followed by campesterol (mean 6.57 mg 100 g<sup>-1</sup> dried root), stigmasterol (mean 1.47 mg 100 g<sup>-1</sup> dried root), citrostadienol (mean 1.27 mg 100 g<sup>-1</sup> dried root) and cycloartenol (mean 1.15 mg 100 g<sup>-1</sup> dried root). The results obtained are in accordance with Ostlund *et al.*,<sup>53</sup> who reported that  $\beta$ -sitosterol, campesterol and stigmasterol are the most abundant sterols in plant species in general. Means of other determined sterols were < 1 mg 100 g<sup>-1</sup> dried root, where the least represented was obtusifoliol (mean 0.16 mg 100 g<sup>-1</sup> dried root). The results obtained are reasonably consistent with the data in the literature. Horník *et al.*<sup>54</sup> documented that the predominant sterol in the nettle root extract was  $\beta$ -sitosterol with a content of 81%, whereas Sajfrtová *et al.*<sup>11</sup> extracted  $\beta$ -sitosterol and scopoletin from nettle root and the maximum yield of  $\beta$ -sitosterol was 0.63 mg g<sup>-1</sup> dry matter. Moreover, Petkova *et al.*<sup>55</sup> reported that the most dominant sterol in nettle seed oil was  $\beta$ -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.*<sup>56</sup> 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 ( $\beta$ -sitosterol) increased by 35.72%. Yang *et al.*<sup>57</sup> studied the composition of sterols from different vegetable oils. The most significant sterol in all examined oils was  $\beta$ -sitosterol, accounting for approximately 50% of total sterols. Besides the main representatives of sterols ( $\beta$ -sitosterol, campesterol and stigmasterol), other sterols were also recorded: the content of  $\Delta^5$ -avenasterol ranged from 3.64 mg 100 g<sup>-1</sup> in peony oil to 157.41 mg 100 g<sup>-1</sup> in rice bran oil, cycloartenol ranged from 4.71 mg 100 g<sup>-1</sup> in soybean oil to 156.25 mg 100 g<sup>-1</sup> in rice bran oil, whereas the content of 24-methylene-cycloartanol varied from 12.33 mg 100 g<sup>-1</sup> in camellia oil to 222.88 mg 100 g<sup>-1</sup> in rice bran oil. The sterol content determined in the present study was similar to that of the mentioned vegetable oils, except for  $\beta$ -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  $\alpha$ -amyrin,  $\beta$ -amyrin and  $\beta$ -amyrin acetate. Their individual contents are given in Table 3, where it can be seen that the most abundant triterpenoid compound was  $\beta$ -amyrin acetate, with mean of 0.56 mg 100 g<sup>-1</sup> dried root. It was followed by  $\beta$ -amyrin (mean 0.50 mg 100 g<sup>-1</sup> dried root), whereas  $\alpha$ -amyrin was determined in the lowest content (mean 0.24 mg 100 g<sup>-1</sup> dried root). When combining the GC-MS with nuclear magnetic resonance spectroscopy, Grauso *et al.*<sup>58</sup> identified  $\alpha$ - and  $\beta$ -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.*<sup>42</sup> identified two pentacyclic triterpenoids,  $\alpha$ - and  $\beta$ -amyrin, in Apiaceae seed lipid extracts obtained by PLE.  $\beta$ -amyrin was present in higher content than  $\alpha$ -amyrin, which is consistent with the results of current study. Klein *et al.*<sup>59</sup> compared  $\alpha$ - and  $\beta$ -amyrin concentrations in extracts from uvaia (*Eugenia pyriformis* Cambess.) leaves obtained by SFE and UAE under different conditions. In both extracts, the concentration of  $\beta$ -amyrin was also higher compared to  $\alpha$ -amyrin. According to Zhang *et al.*,<sup>60</sup> who studied the composition of various phytosterols in 31 vegetable oils,  $\alpha$ - and  $\beta$ -amyrin were also detected.  $\alpha$ -amyrin was detected only in 10 vegetable oils, whereas  $\beta$ -amyrin was detected in 15 vegetable oils. The content of  $\alpha$ -amyrin ranged from 2.53 mg 100 g<sup>-1</sup> oil in samara oil to 1721 mg 100 g<sup>-1</sup> oil in shea butter oil, and the content of  $\beta$ -amyrin ranged from 3.47 mg 100 g<sup>-1</sup> oil in poppy seed oil to 271 mg 100 g<sup>-1</sup> oil in shea butter oil. Compared with the results of the present study, the content of  $\alpha$ - and  $\beta$ -amyryns was higher in nettle lipid fraction, except for shea nut butter, which had higher levels of the mentioned amyryns. On the other hand, Czaplicki *et al.*<sup>61</sup> studied the composition of nine unconventional vegetable oils and no amyryns 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

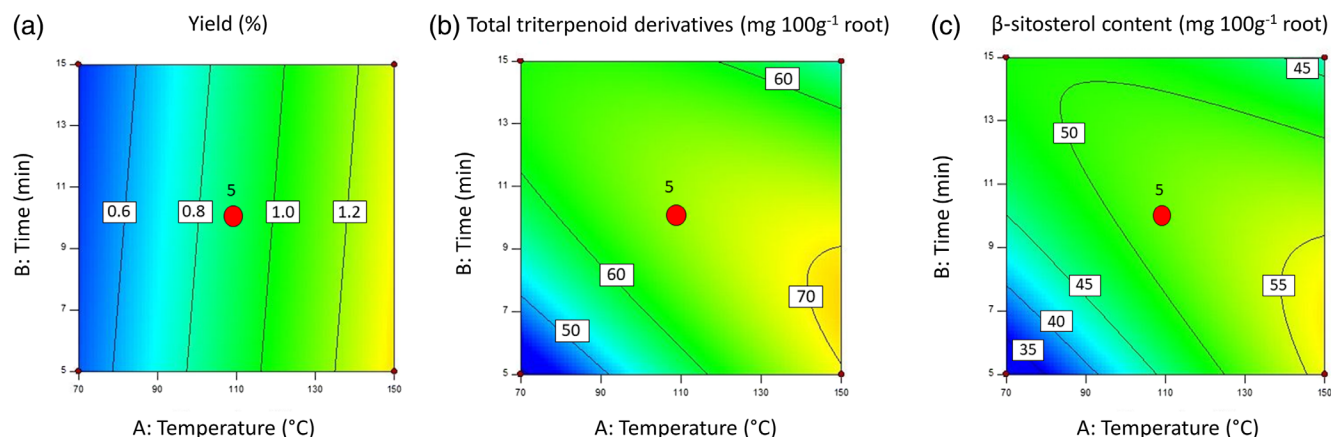
Lipid fraction yield, content of total triterpenoid derivatives and  $\beta$ -sitosterol, as dominant bioactive compounds showing a positive effect on health,<sup>62-64</sup> were included in further data analyses (i.e. RSM) as the most important variables for testing the influence

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

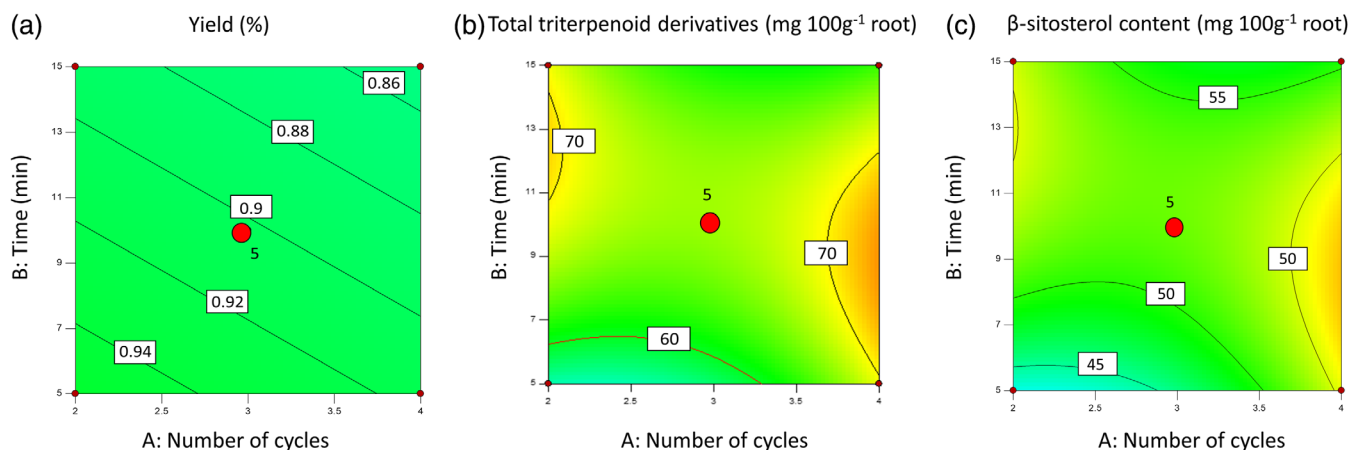
As presented in Table 1, the lowest yield was obtained at 70 °C during 5 min and three cycles and the highest yield was obtained at 150 °C, during 10 min and two extraction cycles. The results of ANOVA show that temperature had a statistically significant effect ( $P \leq 0.05$ ) on the yield of the 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.<sup>34,36</sup> Lohani *et al.*<sup>65</sup> 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.*<sup>42</sup> 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.<sup>34</sup> The purpose of the extraction cycle is to introduce fresh solvent during extraction and maintain a favorable extraction balance.<sup>66</sup> It is useful for samples with high analyte concentrations and those where it is difficult to penetrate the matrix with the solvent.<sup>34</sup> 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<sup>-1</sup> root) and (c) content of β-sitosterol (mg 100 g<sup>-1</sup> 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<sup>-1</sup> root) and (c) content of β-sitosterol (mg 100 g<sup>-1</sup> 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<sup>-1</sup> root) and  $\beta$ -sitosterol (mg 100 g<sup>-1</sup> 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 <sup>-1</sup> 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*  |
| $X_1X_3$   | $9.43 \times 10^{-4}$   | 0.976   |
| $X_2X_3$   | 6.13  | 0.043*  |
| Lack of fit  | 3.00  | 0.158   |
| $R^2$  | 0.778   |         |
| Model  | $Y = -50.9182 + 1.0597X_1 + 14.2962X_2 - 16.7376X_3 - 0.0478X_1X_2 + 0.0016X_1X_3 - 1.0317X_2X_3 - 0.0020X_1^2 - 0.2800X_2^2 + 4.8029X_3^2$ |         |
| $\beta$ -sitosterol (mg 100 g <sup>-1</sup> 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.*<sup>67</sup> 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.*,<sup>68</sup> higher concentrations of amyriins 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.*<sup>69</sup> 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  $\beta$ -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  $\beta$ -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  $\beta$ -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  $\beta$ -sitosterol content, with their combination showing the same effect on  $\beta$ -sitosterol content as for total triterpenoid derivatives (Figs 1c and 2c). Shen & Shao<sup>70</sup> 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  $\beta$ -sitosterol content accompanied with temperature increase. de Oliveira et al.<sup>71</sup> studied the effect of the PLE parameters (solvent and static time) on the quantification of  $\beta$ -sitosterol from Babassu almond oil and concluded that a lower extraction time (about 3 min) was sufficient to obtain the maximum  $\beta$ -sitosterol content.

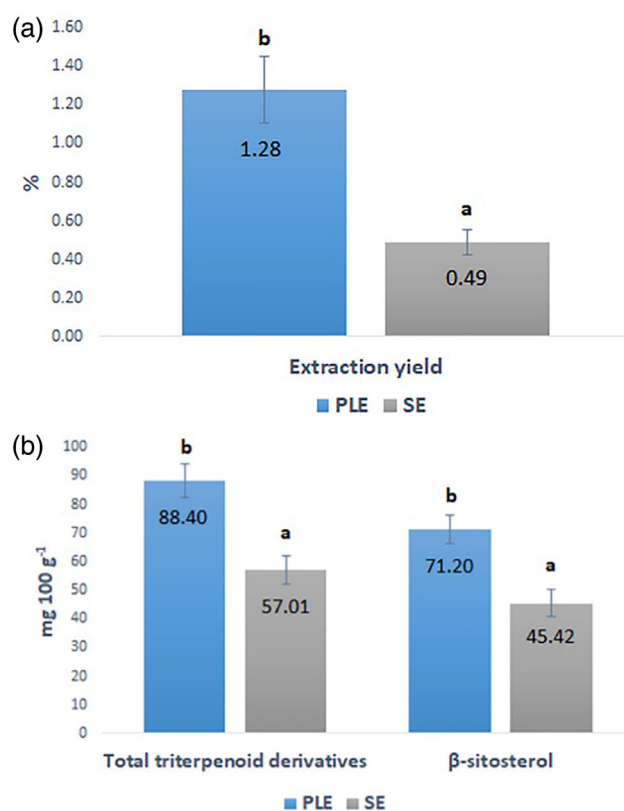
To predict values of lipid fraction yield, contents of total triterpenoid derivatives and  $\beta$ -sitosterol were calculated for each value of temperature, static time and number of cycles in linear, quadratic and interaction coefficients combined. The equations of the regression model are presented 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.<sup>72</sup> 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.<sup>73</sup>

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  $\beta$ -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<sup>-1</sup> dried root of total triterpenoid derivatives and 66.31 mg 100 g<sup>-1</sup> dried root of  $\beta$ -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<sup>-1</sup> dried root for total triterpenoid derivatives and 71.20 mg 100 g<sup>-1</sup> dried root for  $\beta$ -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  $\beta$ -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  $\beta$ -sitosterol, was compared and results obtained are given in Fig. 3.

When comparing the results obtained with both methods, PLE was found 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  $\beta$ -sitosterol and total triterpenoid derivatives. Results are presented as the mean  $\pm$  SD ( $n = 2$  for extraction yield and  $n = 4$  for content of total triterpenoid derivatives and  $\beta$ -sitosterol). Different lowercase letters indicate a statistically significant difference at  $P \leq 0.05$ .

triterpenoid derivatives as well as  $\beta$ -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<sup>-1</sup>,  $\beta$ -sitosterol 71.2 versus 45.42 mg 100 g<sup>-1</sup>) (Fig. 3b). In the study by Pères et al.,<sup>74</sup> 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  $\beta$ -sitosterol content. Additionally, Hirondart et al.<sup>75</sup> 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.<sup>76</sup> 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<sup>-1</sup> 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  $\beta$ -sitosterol, whereas  $\beta$ -amyirin 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  $\beta$ -sitosterol and triterpenoid derivatives were significantly affected by all three parameters examined. Moreover, the contents of  $\beta$ -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  $\beta$ -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.

## REFERENCES

- di Virgilio N, Papazoglou EG, Jankauskiene Z, di Lonardo S, Praczyk M and Wielgusz K, The potential of stinging nettle (*Urtica dioica* L.) as a crop with multiple uses. *Ind Crops Prod* **68**:42–49 (2015).
- Repajić M, Cegledi E, Zorić Z, Pedišić S, Garofulić IE, Radman S *et al.*, Bioactive compounds in wild nettle (*Urtica dioica* L.) leaves and stalks: polyphenols and pigments upon seasonal and habitat variations. *Foods* **10**:190 (2021).
- Alisi C, Emejulu A, Alisi PN, Nwaogu L and Onyema O, Decreased cardiovascular risk and resistance to hyperlipemia-induced hepatic damage in rats by aqueous extract of *Urtica dioica*. *Afr J Biochem Res* **2**:102–106 (2008).
- Hajhashemi V and Klooshani V, Antinociceptive and anti-inflammatory effects of *Urtica dioica* leaf extract in animal models. *Avicenna J Phytomed* **3**:194 (2012).
- Extraordinary vegetable medicine, Upton R. stinging nettles leaf (*Urtica dioica* L.). *J Herb Med* **3**:9–38 (2013).
- Paulauskienė A, Tarasevičienė Ž and Laukagalis V, Influence of harvesting time on the chemical composition of wild stinging nettle (*Urtica dioica* L.). *Plan Theory* **10**:686 (2021).
- Đurović S, Pavlić B, Šorgić S, Popov S, Savić S, Pertonijević M *et al.*, Chemical composition of stinging nettle leaves obtained by different analytical approaches. *J Funct Foods* **32**:18–26 (2017).
- Guil-Guerrero JL, Reboloso-Fuentes MM and Torija Isasa ME, Fatty acids and carotenoids from stinging nettle (*Urtica dioica* L.). *J Food Compos Anal* **16**:111–119 (2003).
- Orčić D, Frančišković M, Bekvalac K, Svircev E, Beara I, Lesjak M *et al.*, Quantitative determination of plant phenolics in *Urtica dioica* extracts by high-performance liquid chromatography coupled with tandem mass spectrometric detection. *Food Chem* **143**:48–53 (2014).
- Kamyab S, Zamani A, Mahasti P and Zojaji M, Evaluation of physico-chemical properties of nettle leaf oil. *J Food Biosci Technol* **5**:77–86 (2015).
- Sajfrtová M, Sovová H, Opletal L and Bártlová M, Near-critical extraction of  $\beta$ -sitosterol and scopoletin from stinging nettle roots. *J Supercrit Fluids* **35**:111–118 (2005).
- Semalty M, Adhikari L, Semwal D, Chauhan A, Mishra A, Kotiyal R *et al.*, A comprehensive review on phytochemistry and pharmacological effects of stinging nettle (*Urtica dioica*). *Curr Tradit Med* **3**:156–167 (2017).
- Chrubasik JE, Roufogalis BD, Wagner H and Chrubasik S, A comprehensive review on the stinging nettle effect and efficacy profiles. Part II: *Urticae radix*. *Phytomedicine* **14**:568–579 (2007).
- Liao JC, Wei ZX, Ma ZP, Zhao C and Cai DZ, Evaluation of a root extract gel from *Urtica dioica* (Urticaceae) as analgesic and anti-inflammatory therapy in rheumatoid arthritis in mice. *Trop J Pharm Res* **15**:781–785 (2016).
- Schneider T and Rübber H, Brennesseltrockenextrakt (Bazoton®-uno) in der Langzeittherapie des benignen Prostata-syndroms (BPS). *Der Urologe A* **43**:302–306 (2004).
- Lichius J and Muth C, The inhibition effects of *Urtica dioica* root extracts on experimentally induced prostatic hyperplasia in the mouse. *Planta Med* **63**:307–310 (1997).
- Xu F, Huang X, Wu H and Wang X, Beneficial health effects of lupenone triterpene: a review. *Biomed Pharmacother* **103**:198–203 (2018).
- Kushiro T and Ezibuka Y, Triterpenes, In: *Comprehensive Natural Products II: Chemistry and Biology*, Vol. 1, Elsevier, London, pp. 673–708 (2010).
- Ferrer A, Altabella T, Arró M and Boronat A, Emerging roles for conjugated sterols in plants. *Prog Lipid Res* **67**:27–37 (2017).
- Rogowska A and Szakiel A, The role of sterols in plant response to abiotic stress. *Phytochem Rev* **19**:1525–1538 (2020).
- Valitova JN, Sulkarnayeva AG and Minibayeva FV, Plant sterols: diversity, biosynthesis, and physiological functions. *Biochemistry* **81**:819–834 (2016).
- Gil-Ramírez A, Caz V, Martín-Hernandez R, Marín FR, Largo C, Rodríguez-Casado A *et al.*, Modulation of cholesterol-related gene expression by ergosterol and ergosterol-enriched extracts obtained from *Agaricus bisporus*. *Eur J Nutr* **55**:1041–1057 (2016).
- Jones PJH and Abumweis SS, Phytosterols as functional food ingredients: linkages to cardiovascular disease and cancer. *Curr Opin Clin Nutr Metab Care* **12**:147–151 (2009).
- Kopylov AT, Malsagova KA, Stepanov AA and Kaysheva AL, Diversity of plant sterols metabolism: the impact on human health, sport, and accumulation of contaminating sterols. *Nutrients* **13**:1623 (2021).
- Madersbacher S, Sampson N and Culig Z, Pathophysiology of benign prostatic hyperplasia and benign prostatic enlargement: a mini-review. *Gerontology* **65**:458–464 (2019).
- Jain T and Bathla S, Plant sterols for human health—a review. *J Appl Nat Sci* **7**:1081–1087 (2015).
- Li F, Wang H and Mei X, Preparation and characterization of phytosterol-loaded microcapsules based on the complex coacervation. *J Food Eng* **311**:110728 (2021).
- Jäger S, Trojan H, Kopp T, Laszczyk MN and Scheffler A, Pentacyclic triterpene distribution in various plants - rich sources for a new group of multi-potent plant extracts. *Molecules* **14**:2016–2031 (2009).
- Ludwiczuk A, Skalicka-Woźniak K and Georgiev MI, Terpenoids, in *Pharmacognosy: Fundamentals, Applications and Strategy*, Elsevier, London, pp. 233–266 (2017).
- Morales D, Oak trees (*Quercus* spp.) as a source of extracts with biological activities: a narrative review. *Trends Food Sci Technol* **109**:116–125 (2021).
- Xu J, Cao J, Yue J, Zhang X and Zhao Y, New triterpenoids from acorns of *Quercus liaotungensis* and their inhibitory activity against  $\alpha$ -glucosidase,  $\alpha$ -amylase and protein-tyrosine phosphatase 1B. *J Funct Foods* **41**:232–239 (2018).
- Handa SS, *Extraction Technologies for Medicinal and Aromatic Plants*. Earth, Environmental and Marine Sciences and Technologies, Trieste, Italy (2008).
- López-Bascon MA and Luque de Castro MD, Soxhlet extraction, in *Liquid-Phase Extraction*, Elsevier, London, pp. 327–354 (2019).
- Mottaleb MA and Sarker SD, Accelerated solvent extraction for natural products isolation. *Methods Mol Biol* **864**:75–87 (2012).

- 35 Zengin G, Cvetanović A, Gašić U, Stupar A, Bulut G, Senkardes I et al., Chemical composition and bio-functional perspectives of *Erica arborea* L. extracts obtained by different extraction techniques: innovative insights. *Ind Crops Prod* **142**:111843 (2019).
- 36 Hossain MB, Barry-Ryan C, Martin-Diana AB and Brunton NP, Optimisation of accelerated solvent extraction of antioxidant compounds from rosemary (*Rosmarinus officinalis* L.), marjoram (*Origanum majorana* L.) and oregano (*Origanum vulgare* L.) using response surface methodology. *Food Chem* **126**:339–346 (2011).
- 37 Wang P, Zhang Q, Wang Y, Wang T, Li X, Ding L et al., Evaluation of Soxhlet extraction, accelerated solvent extraction and microwave-assisted extraction for the determination of polychlorinated biphenyls and polybrominated diphenyl ethers in soil and fish samples. *Anal Chim Acta* **663**:43–48 (2010).
- 38 Azwanida N, A review on the extraction methods use in medicinal plants, principle, strength and limitation. *Med Aromat Plants* **4**: 2167–0412 (2015).
- 39 de Oliveira NA, Cornelio-Santiago HP, Fukumasu H and de Oliveira AL, Green coffee extracts rich in diterpenes – process optimization of pressurized liquid extraction using ethanol as solvent. *J Food Eng* **224**:148–155 (2018).
- 40 Saini RK and Keum YS, Carotenoid extraction methods: a review of recent developments. *Food Chem* **240**:90–103 (2018).
- 41 AOAC, Official methods of analysis, in *Changes in Official Methods of Analysis Made at the Annual Meeting*. Association of Official Analytical Chemists, Rockville, MD (1990).
- 42 Balbino S, Repajić M, Obranović M, Medved AM, Tonković P and Dragović-Uzelac V, Characterization of lipid fraction of Apiaceae family seed spices: impact of species and extraction method. *J Appl Res Med Aromat Plants* **25**:100326 (2021).
- 43 ISO method 12228. ISO 12228-1, *Determination of Individual and Total Sterols Contents — Gas Chromatographic Method — Part 1: Animal and Vegetable Fats and Oils*. International Standard Organization, Geneva (2014).
- 44 Khuri AI and Mukhopadhyay S, Response surface methodology. *Wiley Interdiscip Rev Comput Stat* **2**:128–149 (2010).
- 45 Kovacheva E, Ganchev G, Neicheva A, Ivanova I, Konoushlieva M and Andreev V, Gas chromatographic determination of sterols in fat-soluble concentrates obtained from plant materials. *J Chromatogr* **509**:79–84 (1990).
- 46 Jafari Z, Samani SA and Jafari M, Insights into the bioactive compounds and physico-chemical characteristics of the extracted oils from *Urtica dioica* and *Urtica pilulifera*. *SN Appl Sci* **2**:1–8 (2020).
- 47 Ham B, Jolly S, Triche G, Williams P and Wallace F, A study of the physical and chemical properties of saw palmetto berry extract. *Science Direct Working Paper* **4**:S1574–S0331 (2002).
- 48 da Silva SA, Sampaio GR and da EAF ST, Phytosterols content in vegetable oils of Brazil: coconut, safflower, linseed and evening primrose. *Braz Arch Biol Technol* **63**:1–8 (2020).
- 49 Anastasi U, Sortino O, Tuttobene R, Gresta F, Giuffrè AM and Santonoceto C, Agronomic performance and grain quality of sesame (*Sesamum indicum* L.) landraces and improved varieties grown in a Mediterranean environment. *Genet Resour Crop Evol* **64**:127–137 (2017).
- 50 Giuffrè AM and Capocasale M, Sterol composition of tomato (*Solanum lycopersicum* L.) seed oil: the effect of cultivar. *Int Food Res J* **23**:116–122 (2016).
- 51 Flores-Sánchez IJ, Ortega-López J, Del M, Montes-Horcasitas C and Ramos-Valdivia AC, Biosynthesis of sterols and Triterpenes in cell suspension cultures of *Uncaria tomentosa*. *Plant Cell Physiol* **43**: 1502–1509 (2002).
- 52 Schaller H, The role of sterols in plant growth and development. Progress in lipid research. *Planta* **42**:163–175 (2003).
- 53 Ostlund RE, Phytosterols in human nutrition. *Annu Rev Nutr* **22**:533–549 (2002).
- 54 Horník Š, Sajfrtová M, Karban J, Sýkora J, Březinová A and Wimmer Z, LC-NMR technique in the analysis of phytosterols in natural extracts. *J Anal Methods Chem* **2013**:1–7 (2013).
- 55 Petkova ZY, Antova GA and Angelova-Romova MY, Biologically active components and health benefits of nettle seed oil. *Grasas Aceites* **71**:347 (2020).
- 56 Fedorovska M, Yarema I, Polovko N and Ivanchuk I, Substantiation of technological parameters of the oil extract obtained from *Urtica dioica* roots based on the yield determination of phytosterols using capillary gas chromatography method. *ScienceRise Pharm Sci* **6**:27–36 (2021).
- 57 Yang R, Xue L, Zhang L, Wang X, Qi X, Jiang J et al., Phytosterol contents of edible oils and their contributions to estimated phytosterol intake in the Chinese diet. *Foods* **8**:334–346 (2019).
- 58 Grauso L, Emrick S, Bonanomi G and Lanzotti V, Metabolomics of the alimurgic plants *Taraxacum officinale*, *Papaver rhoeas* and *Urtica dioica* by combined NMR and GC–MS analysis. *Phytochem Anal* **30**: 535–546 (2019).
- 59 Klein EJ, Santos KA, Palú F, Vieira MGA and da Silva EA, Use of supercritical CO<sub>2</sub> and ultrasound-assisted extractions to obtain A/B-amyriinrich extracts from uvaia leaves (*Eugenia pyriformis* Cambess.). *J Supercrit Fluids* **137**:1–8 (2018).
- 60 Zhang T, Xie L, Liu R, Chang M, Zhang H, Jin Q et al., Revisiting the 4,4-dimethylsterols profile from different kinds of vegetable oils by using GC-MS. *LWT* **124**:109163 (2020).
- 61 Czaplicki S, Ogrodowska D, Derewiaka D, Tańska M and Zadernowski R, Bioactive compounds in unsaponifiable fraction of oils from unconventional sources. *Eur J Lipid Sci Technol* **113**:1456–1464 (2011).
- 62 Saeidnia S, The story of Beta-sitosterol- a review. *European J Med Plants* **4**:590–609 (2014).
- 63 Baskar AA, Ignacimuthu S, Paulraj GM and Al Numair KS, Chemopreventive potential of  $\beta$ -sitosterol in experimental colon cancer model-an In vitro and In vivo study. *BMC Complement Altern Med* **10**:1–10 (2010).
- 64 Bin SM, Karim S, Sharmin T and Morshed M, Critical analysis on characterization, systemic effect, and therapeutic potential of Beta-sitosterol: a plant-derived orphan phytosterol. *Medicines* **3**:29 (2016).
- 65 Lohani UC, Fallahi P and Muthukumarappan K, Comparison of ethyl acetate with hexane for oil extraction from various oilseeds. *J Am Oil Chem Soc* **92**:743–754 (2015).
- 66 Mustafa A and Turner C, Pressurized liquid extraction as a green approach in food and herbal plants extraction: a review. *Anal Chim Acta* **703**:8–18 (2011).
- 67 Gil-Ramírez A, Aldars-García L, Palanisamy M, Jiverdeanu RM, Ruiz-Rodríguez A, Marín FR et al., Sterol enriched fractions obtained from *Agaricus bisporus* fruiting bodies and by-products by compressed fluid technologies (PLE and SFE). *Innov Food Sci Emerg Technol* **18**: 101–107 (2013).
- 68 de ARC S, Stefanov S, Bombardelli MCM, Corazza ML and Stateva RP, Assessment of composition and biological activity of *Arctium lappa* leaves extracts obtained with pressurized liquid and supercritical CO<sub>2</sub> extraction. *J Supercrit Fluids* **152**:104573 (2019).
- 69 Repajić M, Cegledi E, Kruk V, Pedisić S, Činar F, Kovačević DB et al., Accelerated solvent extraction as a green tool for the recovery of polyphenols and pigments from wild nettle leaves. *Processes* **8**:803 (2020).
- 70 Shen J and Shao X, A comparison of accelerated solvent extraction, Soxhlet extraction, and ultrasonic-assisted extraction for analysis of terpenoids and sterols in tobacco. *Anal Bioanal Chem* **383**:1003–1008 (2005).
- 71 de Oliveira NA, dos Santos Garcia AB, Mazalli MR, Fukumasu H and de Oliveira AL, Babassu almonds oil extracted with alternative pressurized green solvents, its triacylglycerol prediction and  $\beta$ -sitosterol composition. *J Food Process Eng* **42**:13139 (2019).
- 72 Li F, Zhao H, Xu R, Zhang X, Zhang W, Du M et al., Simultaneous optimization of the acidified water extraction for total anthocyanin content, total phenolic content, and antioxidant activity of blue honeysuckle berries (*Lonicera caerulea* L.) using response surface methodology. *Food Sci Nutr* **7**:2968–2976 (2019).
- 73 Mendes LC, de Menezes HC, Aparecida M and da Silva AP, Optimization of the roasting of robusta coffee (*C. canephora* conillon) using acceptability tests and RSM. *Food Qual Prefer* **12**:153–162 (2001).
- 74 Péres VF, Saffi J, Melecchi MIS, Abad FC, de Assis Jacques R, Martinez MM et al., Comparison of soxhlet, ultrasound-assisted and pressurized liquid extraction of terpenes, fatty acids and vitamin E from *Piper gaudichaudianum* Kunth. *J Chromatogr A* **1105**:115–118 (2006).
- 75 Hironart M, Rombaut N, Fabiano-Tixier AS, Bily A and Chemat F, Comparison between pressurized liquid extraction and conventional Soxhlet extraction for rosemary antioxidants, yield, composition, and environmental footprint. *Foods* **9**:584 (2020).
- 76 Hu J, Guo Z, Glasius M, Kristensen K, Xiao L and Xu X, Pressurized liquid extraction of ginger (*Zingiber officinale* roscoe) with bioethanol: an efficient and sustainable approach. *J Chromatogr A* **1218**:5765–5773 (2011).

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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<sup>-1</sup>. 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  $\beta$ -cyclodextrin ( $\beta$ -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,  $\beta$ -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  $\beta$ -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, synapic 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 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  $\beta$ -CD, where MD and  $\beta$ -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 <sup>-1</sup> ) | 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                   | 120              | 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                   | 120              | 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                   | 120              | 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                   | 160              | 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                   | 160              | 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 <sup>-1</sup> ) | 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                   | 200              | 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                   | 200              | 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, Kirchleugern, 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 ( $\text{g } 100 \text{ g}^{-1}$ ) according to the following equation:

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

where  $m_7$  is the mass (g) of 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  $\mu$ L 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<sub>3</sub> 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<sub>3</sub>. 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. Subsequently, 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  $\mu$ L of distilled water, 80  $\mu$ L of sample and 2080  $\mu$ L 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  $\mu$ M).

##### 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  $\mu\text{M}$ ).

#### 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  $2000\times$  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  $0.45$   $\mu\text{m}$  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  $\text{h}^{-1}$ ), capillary voltage  $+4 -3.5$   $\text{kV}^{-1}$  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  $100 \times 2.1$  mm with a particle size of  $1.8$   $\mu\text{m}$  (Agilent, Santa Clara, CA, USA). The column temperature was set at  $35$  °C, and the injection volume was  $2.5$   $\mu\text{L}$ . 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 ( $R_t$ ) 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  $\text{mg } 100 \text{ g}^{-1}$  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),  $\beta$ -CD,  $\beta$ -CD:GA (1:1) and  $\beta$ -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

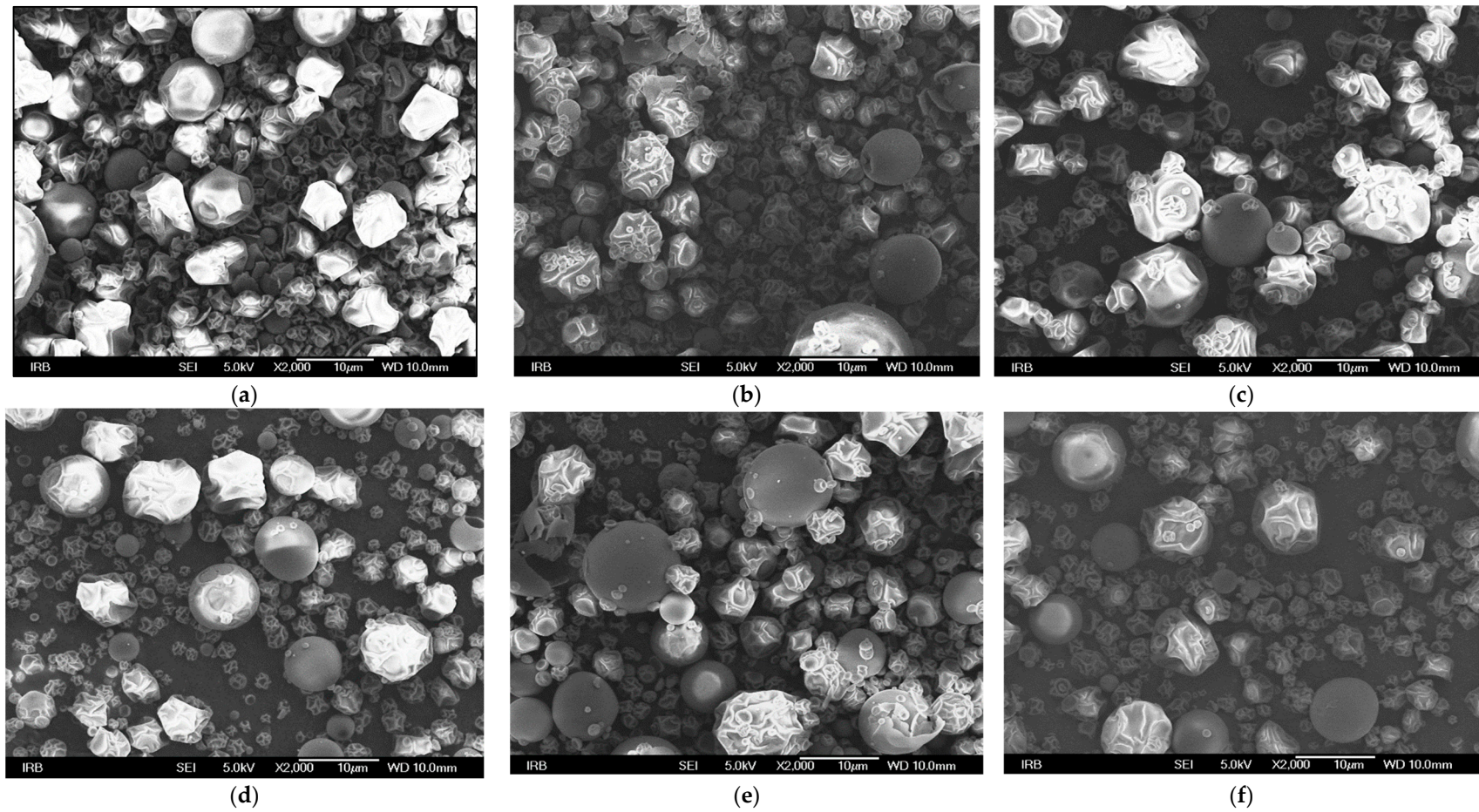
In order to obtain an encapsulated nettle extract with the best physical and chemical properties and with the highest retention and stability of polyphenols, the spray drying encapsulation process needs to be optimized. In addition to the inlet temperature, the type and proportion of the carrier also 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 <sup>-1</sup> ) | Encapsulation Capacity (%) | Loading Capacity (%)       |
|-----------------------------|----|----------------------------|---------------------------|----------------------------|---|----------------------------|----------------------------|
| <b>Temperature (°C)</b>     |    | $p = 0.23$                 | $p < 0.01$                | $p = 0.12$                 | $p = 0.26$                              | $p < 0.01$                 | $p = 0.36$                 |
| 120                         | 36 | 75.32 ± 0.51 <sup>a</sup>  | 4.78 ± 0.19 <sup>c</sup>  | 85.46 ± 0.84 <sup>a</sup>  | 20.70 ± 0.89 <sup>a</sup>               | 97.62 ± 0.13 <sup>c</sup>  | 11.75 ± 0.58 <sup>a</sup>  |
| 160                         | 36 | 76.77 ± 0.91 <sup>a</sup>  | 3.98 ± 0.22 <sup>b</sup>  | 84.00 ± 1.57 <sup>a</sup>  | 22.88 ± 0.97 <sup>a</sup>               | 97.38 ± 0.11 <sup>b</sup>  | 12.74 ± 0.58 <sup>a</sup>  |
| 200                         | 36 | 75.55 ± 0.78 <sup>a</sup>  | 3.29 ± 0.15 <sup>a</sup>  | 85.37 ± 1.54 <sup>a</sup>  | 21.29 ± 0.76 <sup>a</sup>               | 96.61 ± 0.11 <sup>a</sup>  | 11.53 ± 0.49 <sup>a</sup>  |
| <b>Carrier</b>              |    | $p < 0.01$                 | $p = 0.28$                | $p < 0.01$                 | $p < 0.01$                              | $p < 0.01$                 | $p < 0.01$                 |
| MD                          | 18 | 78.94 ± 0.94 <sup>b</sup>  | 3.69 ± 0.21 <sup>a</sup>  | 86.55 ± 0.73 <sup>ab</sup> | 18.08 ± 1.16 <sup>a</sup>               | 97.51 ± 0.19 <sup>b</sup>  | 10.93 ± 0.93 <sup>ab</sup> |
| MD:GA (1:1)                 | 18 | 76.90 ± 0.96 <sup>ab</sup> | 4.52 ± 0.37 <sup>a</sup>  | 88.66 ± 0.74 <sup>c</sup>  | 21.05 ± 1.35 <sup>ab</sup>              | 97.51 ± 0.23 <sup>b</sup>  | 10.37 ± 0.62 <sup>a</sup>  |
| MD:GA (3:1)                 | 18 | 77.82 ± 0.83 <sup>b</sup>  | 4.38 ± 0.27 <sup>a</sup>  | 87.87 ± 0.86 <sup>b</sup>  | 24.22 ± 1.05 <sup>b</sup>               | 97.41 ± 0.09 <sup>b</sup>  | 11.55 ± 0.80 <sup>ab</sup> |
| $\beta$ -CD                 | 18 | 73.11 ± 0.83 <sup>a</sup>  | 3.59 ± 0.30 <sup>a</sup>  | 74.43 ± 3.04 <sup>a</sup>  | 18.86 ± 1.05 <sup>a</sup>               | 96.43 ± 0.21 <sup>a</sup>  | 13.07 ± 0.53 <sup>ab</sup> |
| $\beta$ -CD:GA (1:1)        | 18 | 75.27 ± 1.24 <sup>ab</sup> | 4.22 ± 0.33 <sup>a</sup>  | 89.74 ± 0.28 <sup>c</sup>  | 24.90 ± 1.07 <sup>b</sup>               | 97.35 ± 0.11 <sup>b</sup>  | 12.08 ± 0.68 <sup>ab</sup> |
| $\beta$ -CD:GA (3:1)        | 18 | 73.24 ± 0.86 <sup>a</sup>  | 3.72 ± 0.29 <sup>a</sup>  | 82.41 ± 1.45 <sup>ab</sup> | 22.62 ± 1.00 <sup>ab</sup>              | 97.01 ± 0.13 <sup>ab</sup> | 13.54 ± 0.86 <sup>b</sup>  |
| <b>Ratio sample:carrier</b> |    | $p < 0.01$                 | $p < 0.01$                | $p < 0.01$                 | $p < 0.01$                              | $p = 0.16$                 | $p < 0.01$                 |
| 1:1                         | 36 | 72.39 ± 0.57 <sup>a</sup>  | 4.53 ± 0.13 <sup>b</sup>  | 82.08 ± 1.66 <sup>a</sup>  | 27.32 ± 0.57 <sup>b</sup>               | 97.02 ± 0.10 <sup>a</sup>  | 15.47 ± 0.31 <sup>c</sup>  |
| 1:2                         | 36 | 75.80 ± 0.46 <sup>b</sup>  | 3.97 ± 0.24 <sup>ab</sup> | 83.70 ± 1.35 <sup>a</sup>  | 19.65 ± 0.61 <sup>a</sup>               | 97.32 ± 0.12 <sup>a</sup>  | 10.76 ± 0.18 <sup>b</sup>  |
| 1:3                         | 36 | 79.45 ± 0.70 <sup>c</sup>  | 3.56 ± 0.24 <sup>a</sup>  | 89.05 ± 0.46 <sup>b</sup>  | 17.89 ± 0.50 <sup>a</sup>               | 97.27 ± 0.17 <sup>a</sup>  | 9.80 ± 0.54 <sup>a</sup>   |

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



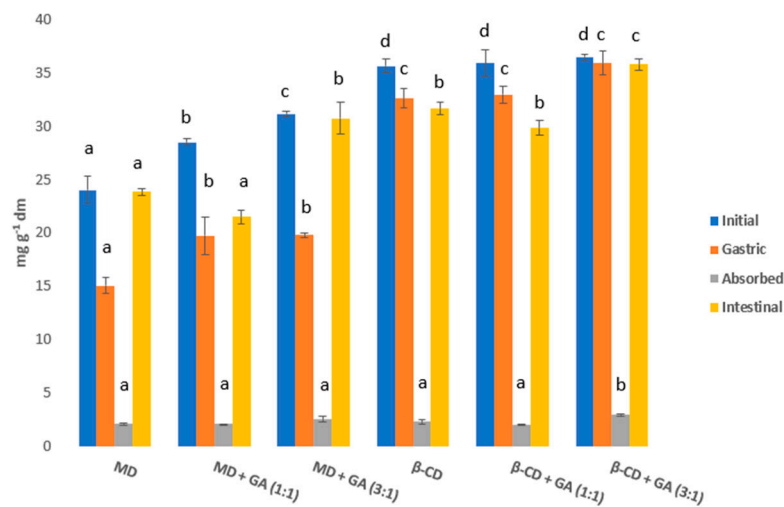
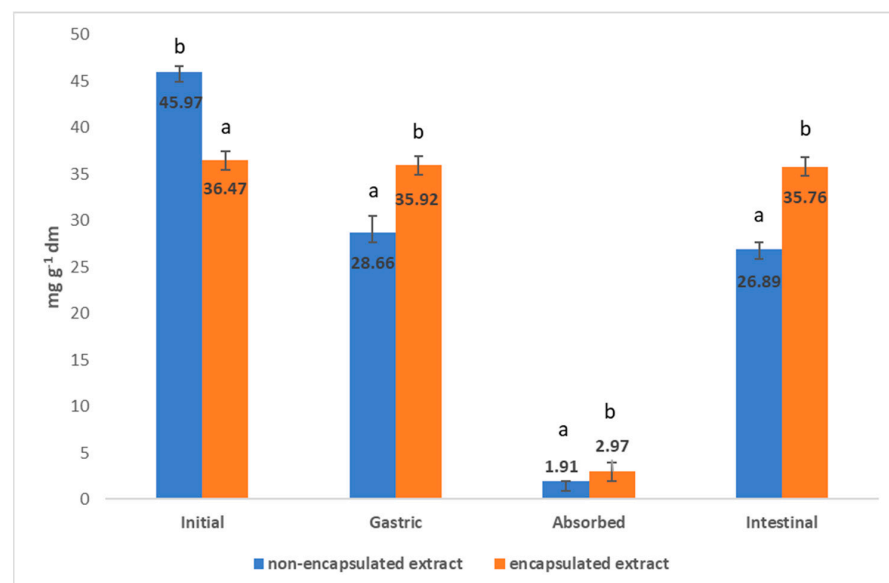


**Figure 1.** SEM images of the microcapsules of encapsulated nettle leaves extract with different carriers: (a) MD (b) MD:GA (1:1) (c) MD:GA (3:1) (d)  $\beta$ -CD (e)  $\beta$ -CD:GA (1:1) (f)  $\beta$ -CD:GA (3:1) at 160 °C and sample:carrier ratio 1:3.

**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 <sup>-1</sup> dm) | DPPH (mmol TE 100g <sup>-1</sup> dm) |
|--------|---------------|-----------------------|------------------|--------------------------------------|--------------------------------------|
| 31     | MD            | 1:3                   | 160              | 6.13 ± 0.18 <sup>a</sup>             | 9.10 ± 0.08 <sup>a</sup>             |
| 32     | MD:GA (1:1)   |                       |                  | 8.57 ± 0.40 <sup>b</sup>             | 12.42 ± 0.16 <sup>b</sup>            |
| 33     | MD:GA (3:1)   |                       |                  | 8.30 ± 0.62 <sup>b</sup>             | 12.11 ± 0.03 <sup>b</sup>            |
| 34     | β-CD          |                       |                  | 9.59 ± 0.16 <sup>bc</sup>            | 12.74 ± 0.12 <sup>b</sup>            |
| 35     | β-CD:GA (1:1) |                       |                  | 10.55 ± 0.20 <sup>c</sup>            | 12.13 ± 0.34 <sup>b</sup>            |
| 36     | β-CD:GA (3:1) |                       |                  | 11.04 ± 0.27 <sup>cd</sup>           | 15.57 ± 0.01 <sup>c</sup>            |

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.

| Compound                     | Rt (min) | Mass Spectrometric Data |                    | Concentration (mg 100 g <sup>-1</sup> ) |
|------------------------------|----------|-------------------------|--------------------|---|
|                              |          | 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                             |
| Isorhamnetin                 | 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.

|                               |        |     |     |               |
|-------------------------------|--------|-----|-----|---------------|
| <i>p</i> -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 |
| <i>p</i> -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  $\beta$ -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  $\beta$ -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  $\beta$ -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  $\beta$ -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  $\beta$ -CD was used as a carrier. This is because of  $\beta$ -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  $\beta$ -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<sup>-1</sup> (Table 1) with mean of 21.62 g 100 g<sup>-1</sup>. 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  $\beta$ -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  $\beta$ -CD with GA (1:1) proved to be most effective in achieving the best encapsulation capacity, while the lowest capacity was achieved when  $\beta$ -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  $\beta$ -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  $\mu\text{m}$ . 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  $\beta$ -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  $\beta$ -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  $\beta$ -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  $\text{mg g}^{-1}$  dm of extract. The polyphenol concentrations in the gastric phase were slightly lower (15.09–35.92  $\text{mg g}^{-1}$  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  $\text{mg g}^{-1}$  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  $\beta$ -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  $\beta$ -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  $45.97 \text{ mg g}^{-1} \text{ dm}$  of extract, and in an *in vitro* digestion test,  $1.91 \text{ mg g}^{-1} \text{ dm}$  of extract was absorbed, corresponding to 4.16%. On the other hand, the concentration of initial encapsulated extract was  $36.47 \text{ mg g}^{-1} \text{ dm}$  of extract and was absorbed by  $2.97 \text{ mg g}^{-1} \text{ dm}$  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  $100\text{g}^{-1} \text{ dm}$  of the extract and for DPPH ranged from 9.10 to 15.57 mmol TE  $100\text{g}^{-1} \text{ dm}$  of the extract. In both tests, the lowest value was obtained when MD was used as the carrier and the highest value when  $\beta$ -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  $\beta$ -CD and concluded that the powder obtained with  $\beta$ -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  $\beta$ -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<sup>-1</sup>), followed by caffeic acid (194.82 mg 100 g<sup>-1</sup>). 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 represented 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<sup>-1</sup>). 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  $\beta$ -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,  $\beta$ -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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## References

1. Veiga, M.; Costa, E.M.; Silva, S.; Pintado, M. Impact of Plant Extracts upon Human Health: A Review. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 873–886. [[CrossRef](#)] [[PubMed](#)]
2. Bornkessel, S.; Bröring, S.; Omta, S.W.F.; van Trijp, H. What Determines Ingredient Awareness of Consumers? A Study on Ten Functional Food Ingredients. *Food Qual. Prefer.* **2014**, *32*, 330–339. [[CrossRef](#)]
3. Grauso, L.; Emrick, S.; Bonanomi, G.; Lanzotti, V. Metabolomics of the Alimurgic Plants *Taraxacum Officinale*, *Papaver Rhoëas* and *Urtica dioica* by Combined NMR and GC–MS Analysis. *Phytochem. Anal.* **2019**, *30*, 535–546. [[CrossRef](#)]
4. Di Virgilio, N.; Papazoglou, E.G.; Jankauskiene, Z.; di Lonardo, S.; Praczyk, M.; Wielgusz, K. The Potential of Stinging Nettle (*Urtica dioica* L.) as a Crop with Multiple Uses. *Ind. Crops Prod.* **2015**, *68*, 42–49. [[CrossRef](#)]
5. Kukrić, Z.Z.; Topalić-Trivunović, L.N.; Kukavica, B.M.; Matoš, S.B.; Pavičić, S.S.; Boroja, M.M.; Savić, A.V. Characterization of Antioxidant and Antimicrobial Activities of Nettle Leaves (*Urtica dioica* L.). *Acta Per. Technol.* **2012**, *43*, 257–272. [[CrossRef](#)]
6. Repajić, M.; Cegledi, E.; Zorić, Z.; Pedisić, S.; Garofulić, I.E.; Radman, S.; Palčić, I.; Dragović-Uzelac, V. Bioactive Compounds in Wild Nettle (*Urtica dioica* L.) Leaves and Stalks: Polyphenols and Pigments upon Seasonal and Habitat Variations. *Foods* **2021**, *10*, 190. [[CrossRef](#)] [[PubMed](#)]
7. Otlés, S.; Yalcin, B. Phenolic Compounds Analysis of Root, Stalk, and Leaves of Nettle. *Sci. World J.* **2012**, *2012*, 564367. [[CrossRef](#)] [[PubMed](#)]
8. Upton, R. Stinging Nettles Leaf (*Urtica dioica* L.): Extraordinary Vegetable Medicine. *J. Herb. Med.* **2013**, *3*, 9–38. [[CrossRef](#)]
9. Dai, J.; Mumper, R.J. Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties. *Molecules* **2010**, *15*, 7313–7352. [[CrossRef](#)]
10. Tonon, R.V.; Brabet, C.; Hubinger, M.D. Influence of Process Conditions on the Physicochemical Properties of Açai (*Euterpe oleraceae* Mart.) Powder Produced by Spray Drying. *J. Food Eng.* **2008**, *88*, 411–418. [[CrossRef](#)]
11. Buljeta, I.; Pichler, A.; Šimunović, J.; Kopjar, M. Polysaccharides as Carriers of Polyphenols: Comparison of Freeze-Drying and Spray-Drying as Encapsulation Techniques. *Molecules* **2022**, *27*, 5069. [[CrossRef](#)] [[PubMed](#)]
12. Igual, M.; García-Herrera, P.; Cámara, R.M.; Martínez-Monzó, J.; García-Segovia, P.; Cámara, M. Bioactive Compounds in Rosehip (*Rosa Canina*) Powder with Encapsulating Agents. *Molecules* **2022**, *27*, 4737. [[CrossRef](#)] [[PubMed](#)]
13. Ferrari, C.C.; Germer, S.P.M.; de Aguirre, J.M. Effects of Spray-Drying Conditions on the Physicochemical Properties of Blackberry Powder. *Dry Technol.* **2012**, *30*, 154–163. [[CrossRef](#)]
14. Murugesan, R.; Orsat, V. Spray Drying for the Production of Nutraceutical Ingredients—A Review. *Food Bioproc. Technol.* **2012**, *5*, 3–14. [[CrossRef](#)]
15. Kurozawa, L.E.; Morassi, A.G.; Vanzo, A.A.; Park, K.J.; Hubinger, M.D. Influence of Spray Drying Conditions on Physicochemical Properties of Chicken Meat Powder. *Dry Technol.* **2009**, *27*, 1248–1257. [[CrossRef](#)]
16. Peng, Z.; Li, J.; Guan, Y.; Zhao, G. Effect of Carriers on Physicochemical Properties, Antioxidant Activities and Biological Components of Spray-Dried Purple Sweet Potato Flours. *Food Sci. Technol.* **2013**, *51*, 348–355. [[CrossRef](#)]
17. Grgić, J.; Šelo, G.; Planinić, M.; Tišma, M.; Bucić-Kojić, A. Role of the Encapsulation in Bioavailability of Phenolic Compounds. *Antioxidants* **2020**, *9*, 923. [[CrossRef](#)]
18. Vulić, J.; Šeregelj, V.; Kalušević, A.; Lević, S.; Nedović, V.; Šaponjac, V.T.; Čanadanović-Brunet, J.; Četković, G. Bioavailability and Bioactivity of Encapsulated Phenolics and Carotenoids Isolated from Red Pepper Waste. *Molecules* **2019**, *24*, 2837. [[CrossRef](#)]
19. Kalajahi, M.S.E.; Ghandiha, S. Optimization of Spray Drying Parameters for Encapsulation of Nettle (*Urtica dioica* L.) Extract. *LWT* **2022**, *158*, 113149. [[CrossRef](#)]
20. Elez Garofulić, I.; Malin, V.; Repajić, M.; Zorić, Z.; Pedisić, S.; Sterniša, M.; Možina, S.S.; Dragović-Uzelac, V. Phenolic Profile, Antioxidant Capacity and Antimicrobial Activity of Nettle Leaves Extracts Obtained by Advanced Extraction Techniques. *Molecules* **2021**, *26*, 6153. [[CrossRef](#)]
21. Zhang, L.; Zeng, X.; Fu, N.; Tang, X.; Sun, Y.; Lin, L. Maltodextrin: A Consummate Carrier for Spray-Drying of Xylooligosaccharides. *Food Res. Inter.* **2018**, *106*, 383–393. [[CrossRef](#)]
22. Anderson, R.A.; Anderson, R.A.; Conway, H.F.; Pfiefer, V.F.; Griffin, E.L. Roll and Extrusion-Cooking of Grain Sorghum Grits. *Cereal Sci. Today* **1969**, *14*, 373–381.
23. Robert, P.; Gorená, T.; Romero, N.; Sepulveda, E.; Chavez, J.; Saenz, C. Encapsulation of Polyphenols and Anthocyanins from Pomegranate (*Punica granatum*) by Spray Drying. *Int. J. Food Sci. Technol.* **2010**, *45*, 1386–1394. [[CrossRef](#)]
24. Hu, L.; Zhang, J.; Hu, Q.; Gao, N.; Wang, S.; Sun, Y.; Yang, X. Microencapsulation of Brucea Javanica Oil: Characterization, Stability and Optimization of Spray Drying Conditions. *J. Drug Deliv. Sci. Technol.* **2016**, *36*, 46–54. [[CrossRef](#)]
25. Saénz, C.; Tapia, S.; Chávez, J.; Robert, P. Microencapsulation by Spray Drying of Bioactive Compounds from Cactus Pear (*Opuntia ficus-indica*). *Food Chem.* **2009**, *114*, 616–622. [[CrossRef](#)]

26. Mcdougall, G.; Dobson, P.; Shpiro, F.; Smith, P.; Stewart, D.; Fyffe, S. Assessing Bioavailability of Soft Fruit Polyphenols In Vitro. *Int. Symp. Hum. Health Eff. Fruits Veg.* **2005**, *744*, 135–148. [[CrossRef](#)]
27. Gunathilake, K.D.P.P.; Ranaweera, K.K.D.S.; Rupasinghe, H.P.V. Change of Phenolics, Carotenoids, and Antioxidant Capacity Following Simulated Gastrointestinal Digestion and Dialysis of Selected Edible Green Leaves. *Food Chem.* **2018**, *245*, 371–379. [[CrossRef](#)]
28. Dobroslavić, E.; Elez Garofulić, I.; Šeparović, J.; Zorić, Z.; Pedisić, S.; Dragović-Uzelac, V. Pressurized Liquid Extraction as a Novel Technique for the Isolation of *Laurus nobilis* L. Leaf Polyphenols. *Molecules* **2022**, *27*, 5099. [[CrossRef](#)]
29. Elez Garofulić, I.; Zorić, Z.; Pedisić, S.; Brnčić, M.; Dragović-Uzelac, V. UPLC-MS2 Profiling of Blackthorn Flower Polyphenols Isolated by Ultrasound-Assisted Extraction. *J. Food Sci.* **2018**, *83*, 2782–2789. [[CrossRef](#)]
30. Bhandari, B.R.; Datta, N.; Howes, T. Problems Associated with Spray Drying of Sugar-Rich Foods. *Dry Technol.* **1997**, *15*, 671–684. [[CrossRef](#)]
31. Fang, Z.; Bhandari, B. Effect of Spray Drying and Storage on the Stability of Bayberry Polyphenols. *Food Chem.* **2011**, *129*, 1139–1147. [[CrossRef](#)]
32. Vidović, S.S.; Vladić, J.Z.; Vaštag, Ž.G.; Zeković, Z.P.; Popović, L.M. Maltodextrin as a Carrier of Health Benefit Compounds in Satureja Montana Dry Powder Extract Obtained by Spray Drying Technique. *Powder Technol.* **2014**, *258*, 209–215. [[CrossRef](#)]
33. Şahin-Nadeem, H.; Dinçer, C.; Torun, M.; Topuz, A.; Özdemir, F. Influence of Inlet Air Temperature and Carrier Material on the Production of Instant Soluble Sage (*Salvia fruticosa* Miller) by Spray Drying. *Food Sci. Technol.* **2013**, *52*, 31–38. [[CrossRef](#)]
34. Navarro-Flores, M.J.; Ventura-Canseco, L.M.C.; Meza-Gordillo, R.; Ayora-Talavera, T.D.R.; Abud-Archila, M. Spray Drying Encapsulation of a Native Plant Extract Rich in Phenolic Compounds with Combinations of Maltodextrin and Non-Conventional Wall Materials. *J. Food Sci. Technol.* **2020**, *57*, 4111–4122. [[CrossRef](#)] [[PubMed](#)]
35. Şahin-Nadeem, H.; Torun, M.; Özdemir, F. Spray Drying of the Mountain Tea (*Sideritis stricta*) Water Extract by Using Different Hydrocolloid Carriers. *Food Sci. Technol.* **2011**, *44*, 1626–1635. [[CrossRef](#)]
36. Daza, L.D.; Fujita, A.; Fávoro-Trindade, C.S.; Rodrigues-Ract, J.N.; Granato, D.; Genovese, M.I. Effect of Spray Drying Conditions on the Physical Properties of Cagaíta (*Eugenia dysenterica* DC.) Fruit Extracts. *Food Bioprod. Process.* **2016**, *97*, 20–29. [[CrossRef](#)]
37. Sablania, V.; Bosco, S.J.D. Optimization of Spray Drying Parameters for *Murraya koenigii* (Linn) Leaves Extract Using Response Surface Methodology. *Powder Technol.* **2018**, *335*, 35–41. [[CrossRef](#)]
38. Tran, T.T.A.; Nguyen, H.V.H. Effects of Spray-Drying Temperatures and Carriers on Physical and Antioxidant Properties of Lemongrass Leaf Extract Powder. *Beverages* **2018**, *4*, 84. [[CrossRef](#)]
39. Fazaeli, M.; Emam-Djomeh, Z.; Kalbasi Ashtari, A.; Omid, M. Effect of Spray Drying Conditions and Feed Composition on the Physical Properties of Black Mulberry Juice Powder. *Food Bioprod. Process.* **2012**, *90*, 667–675. [[CrossRef](#)]
40. Bastías-Montes, J.M.; Choque-Chávez, M.C.; Alarcón-Enos, J.; Quevedo-León, R.; Muñoz-Fariña, O.; Vidal-San-martín, C. Effect of Spray Drying at 150, 160, and 170 °C on the Physical and Chemical Properties of Maqui Extract (*Aristotelia Chilensis* (Molina) Stuntz). *Chil. J. Agric. Res.* **2019**, *79*, 144–152. [[CrossRef](#)]
41. Quek, S.Y.; Chok, N.K.; Swedlund, P. The Physicochemical Properties of Spray-Dried Watermelon Powders. *Chem. Eng. Process.* **2007**, *46*, 386–392. [[CrossRef](#)]
42. Gunjal, S.D.; Shirolkar, S.V. An Overview of Process Parameters and Spray Drying Agents Involved in Spray Drying of Herbal Extracts. *Paed. J.* **2020**, *13*, 102–118.
43. Giovagnoli-Vicuña, C.; Briones-Labarca, V.; Romero, M.S.; Giordano, A.; Pizarro, S. Effect of Extraction Methods and In Vitro Bio-Accessibility of Microencapsulated Lemon Extract. *Molecules* **2022**, *27*, 4166. [[CrossRef](#)] [[PubMed](#)]
44. Susantikarn, P.; Donlao, N. Optimization of Green Tea Extracts Spray Drying as Affected by Temperature and Maltodextrin Content. *Int. Food Res. J.* **2016**, *23*, 1327–1331.
45. Phisut, N. Spray Drying Technique of Fruit Juice Powder: Some Factors Influencing the Properties of Product. *Int. Food Res. J.* **2012**, *19*, 1297–1306.
46. Fernandes, R.V.D.B.; Borges, S.V.; Botrel, D.A. Gum Arabic/Starch/Maltodextrin/Inulin as Wall Materials on the Microencapsulation of Rosemary Essential Oil. *Carbohydr. Polym.* **2014**, *101*, 524–532. [[CrossRef](#)]
47. Pudziuvelyte, L.; Marksa, M.; Jakstas, V.; Ivanauskas, L.; Kopustinskiene, D.M.; Bernatoniene, J. Microencapsulation of Elsholtzia Ciliata Herb Ethanolic Extract by Spray-Drying: Impact of Resistant-Maltodextrin Complemented with Sodium Caseinate, Skim Milk, and Beta-Cyclodextrin on the Quality of Spray-Dried Powders. *Molecules* **2019**, *24*, 1461. [[CrossRef](#)]
48. Zokti, J.A.; Baharin, B.S.; Mohammed, A.S.; Abas, F. Green Tea Leaves Extract: Microencapsulation, Physicochemical and Storage Stability Study. *Molecules* **2016**, *21*, 940. [[CrossRef](#)]
49. Ali, B.H.; Ziada, A.; Blunden, G. Biological Effects of Gum Arabic: A Review of Some Recent Research. *Food Chem. Toxicol.* **2009**, *47*, 1–8. [[CrossRef](#)]
50. Rodríguez-Hernández, G.R.; González-García, R.; Grajales-Lagunes, A.; Ruiz-Cabrera, M.A.; Abud-Archila, M. Spray-Drying of Cactus Pear Juice (*Opuntia streptacantha*): Effect on the Physicochemical Properties of Powder and Reconstituted Product. *Dry Technol.* **2005**, *23*, 955–973. [[CrossRef](#)]
51. Tonon, R.V.; Freitas, S.S.; Hubinger, M.D. Spray Drying of Açai (*Euterpe oleraceae* Mart.) Juice: Effect of Inlet Air Temperature and Type of Carrier Agent. *J. Food Process. Preserv.* **2011**, *35*, 691–700. [[CrossRef](#)]

52. Mishra, P.; Mishra, S.; Mahanta, C.L. Effect of Maltodextrin Concentration and Inlet Temperature during Spray Drying on Physicochemical and Antioxidant Properties of Amla (*Emblica officinalis*) Juice Powder. *Food Bioprod. Process.* **2014**, *92*, 252–258. [[CrossRef](#)]
53. Zorić, Z.; Pelaić, Z.; Pedisić, S.; Elez Garofulić, I.; Bursać Kovačević, D.; Dragović-Uzelac, V. Effect of Storage Conditions on Phenolic Content and Antioxidant Capacity of Spray Dried Sour Cherry Powder. *Food Sci. Technol.* **2017**, *79*, 251–259. [[CrossRef](#)]
54. Watson, M.A.; Lea, J.M.; Bett-Garber, K.L. Spray Drying of Pomegranate Juice Using Maltodextrin/Cyclodextrin Blends as the Wall Material. *Food Sci. Nutr.* **2017**, *5*, 820–826. [[CrossRef](#)]
55. Chong, P.H.; Yusof, Y.A.; Aziz, M.G.; Nazli, N. Mohd.; Chin, N.L.; Muhammad, S.K.S. Effects of Spray Drying Conditions of Microencapsulation of *Amaranthus Gangeticus* Extract on Drying Behaviour. *Agric. Agric. Sci. Proc.* **2014**, *2*, 33–42. [[CrossRef](#)]
56. Shahidi, F.; Peng, H. Bioaccessibility and Bioavailability of Phenolic Compounds. *J. Food Bioact.* **2018**, *4*, 11–68. [[CrossRef](#)]
57. Ydjedd, S.; Bouriche, S.; López-Nicolás, R.; Sánchez-Moya, T.; Frontela-Saseta, C.; Ros-Berruazo, G.; Rezgui, F.; Louaileche, H.; Kati, D.E. Effect of in Vitro Gastrointestinal Digestion on Encapsulated and Nonencapsulated Phenolic Compounds of Carob (*Ceratonia siliqua* L.) Pulp Extracts and Their Antioxidant Capacity. *J. Agric. Food Chem.* **2017**, *65*, 827–835. [[CrossRef](#)]
58. Tuan, M.P.; Hoang, T.V.A.; Le, T.H.C.; Vu, N.Q.C.; Dang, T.B.O.; Dang, B.K.; Pham, D.T.M.; Mai, H.T.; Nguyen, T.T.S.; Dam, S.M.; et al. Extraction and Encapsulation of Polyphenols of Guava Leaves. *Annals. Food Sci. Technol.* **2016**, *17*, 34–40.
59. Jovanović, M.; Drinić, Z.; Bigović, D.; Zdunić, G.; Mudrić, J.; Šavikin, K. Effect of Carrier Type on the Spray-Dried Willowherb (*Epilobium angustifolium* L.) Leaves Extract, Powder Properties and Bioactive Compounds Encapsulation. *Lek. Sirovine* **2021**, *41*, 41–45. [[CrossRef](#)]
60. Bonetti, G.; Tedeschi, P.; Meca, G.; Bertelli, D.; Mañes, J.; Brandolini, V.; Maietti, A. In Vitro Bioaccessibility, Transepithelial Transport and Antioxidant Activity of *Urtica dioica* L. Phenolic Compounds in Nettle Based Food Products. *Food Funct.* **2016**, *7*, 4222–4230. [[CrossRef](#)]
61. Bhusari, S.N.; Kumar, P. Antioxidant activities of spray dried tamarind pulp powder as affected by carrier type and their addition rate. In Proceedings of the International Conference on Food, Biological and Medical Sciences (FBMS-2014), Bangkok, Thailand, 28–29 January 2014. [[CrossRef](#)]
62. Fernandes, M.R.V.; Kabeya, L.M.; Souza, C.R.F.; Massarioli, A.P.; Alencar, S.M.; Oliveira, W.P. Antioxidant Activity of Spray-Dried Extracts of *Psidium Guajava* Leaves. *J. Food Res.* **2018**, *7*, 141. [[CrossRef](#)]
63. Sharayei, P.; Azarpazhooh, E.; Ramaswamy, H.S. Effect of Microencapsulation on Antioxidant and Antifungal Properties of Aqueous Extract of Pomegranate Peel. *J. Food Sci. Technol.* **2020**, *57*, 723–733. [[CrossRef](#)]
64. Pinho, E.; Grootveld, M.; Soares, G.; Henriques, M. Cyclodextrins as Encapsulation Agents for Plant Bioactive Compounds. *Carbohydr. Polym.* **2014**, *101*, 121–135. [[CrossRef](#)] [[PubMed](#)]
65. Carvalho, A.R.; Costa, G.; Figueirinha, A.; Liberal, J.; Prior, J.A.V.; Lopes, M.C.; Cruz, M.T.; Batista, M.T. *Urtica* Spp.: Phenolic Composition, Safety, Antioxidant and Anti-Inflammatory Activities. *Food Res. Int.* **2017**, *99*, 485–494. [[CrossRef](#)] [[PubMed](#)]
66. Pinelli, P.; Ieri, F.; Vignolini, P.; Bacci, L.; Baronti, S.; Romani, A. Extraction and HPLC Analysis of Phenolic Compounds in Leaves, Stalks, and Textile Fibers of *Urtica dioica* L. *J. Agric. Food Chem.* **2008**, *56*, 9127–9132. [[CrossRef](#)]



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

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

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.

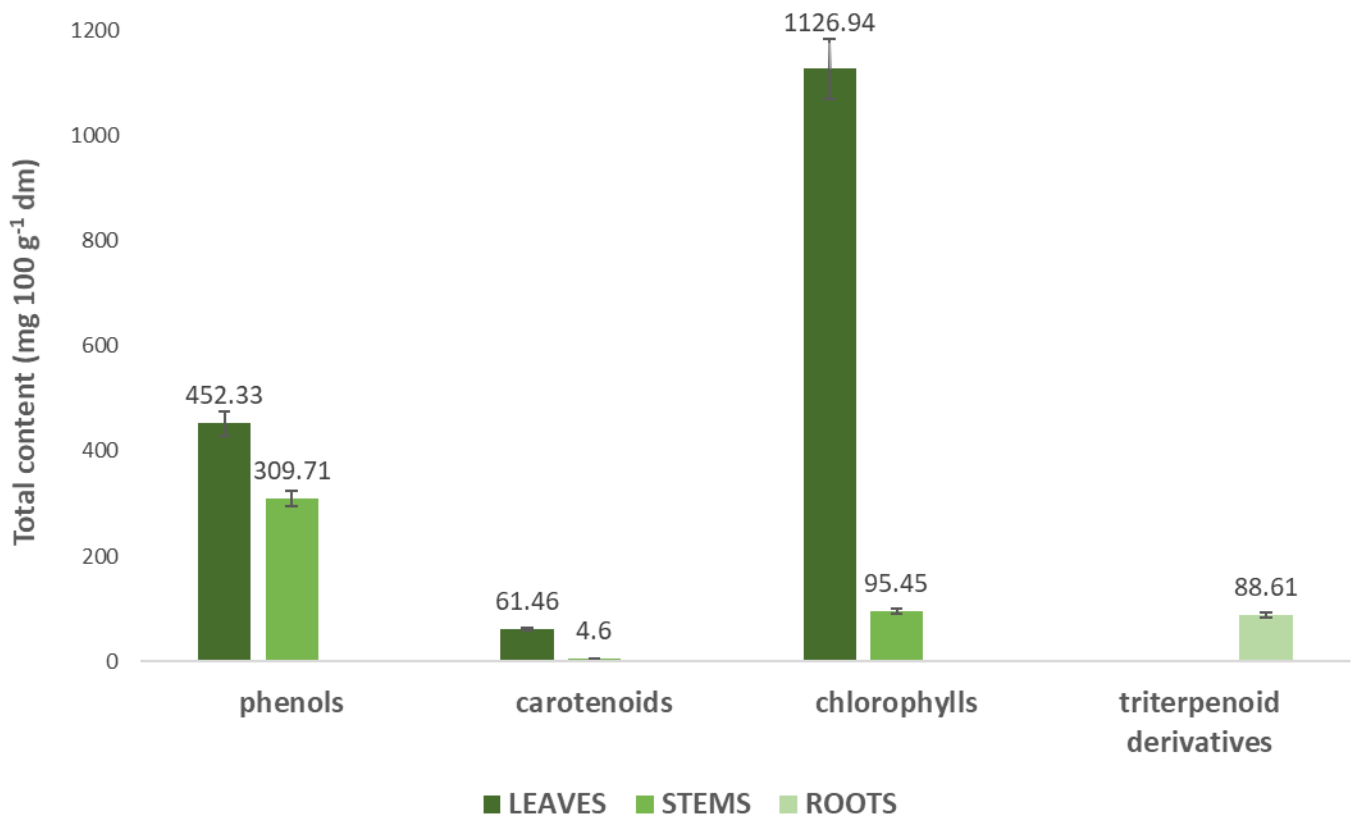
In the *Publication No. 3*, the optimal ASE conditions for the efficient isolation of the oil fraction from nettle root were compared with Soxhlet extraction, the conventional, most commonly used technique for extracting the oil fraction. The main focus was on the comparison of extraction yield, total triterpenoid derivatives and  $\beta$ -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  $\beta$ -sitosterol was also significantly higher in the extracts obtained using ASE (88.40 mg 100 g<sup>-1</sup> dm and 71.20 mg 100 g<sup>-1</sup> dm) in comparison with the extracts obtained by Soxhlet (57.01 mg 100 g<sup>-1</sup> dm and 45.42 mg 100 g<sup>-1</sup> 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<sup>-1</sup> dm, while in the stem it was 309.71 mg 100 g<sup>-1</sup> 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<sup>-1</sup> 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<sup>-1</sup> dm), followed by

flavonols (grand mean 134.60 mg 100 g<sup>-1</sup> 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<sup>-1</sup> dm) and stems (4.6 mg 100 g<sup>-1</sup> dm). Among the analyzed groups of pigments, chlorophylls are the most represented. Their approximate amount was 1126.94 mg 100 g<sup>-1</sup> dm in the nettle leaves, while it was much lower in the stems (95.45 mg 100 g<sup>-1</sup> 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<sup>-1</sup> dm), and among chlorophylls it was chlorophyll *a* (594.67 mg 100 g<sup>-1</sup> 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<sup>-1</sup> dm, which is slightly higher than the amounts reported by Obranović et al. (2023). Phytosterols and pentacyclic 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,  $\beta$ -sitosterol dominated with a mean value of 50.21 mg 100 g<sup>-1</sup> dm (80.42% of the total phytosterols), while among the pentacyclic

triterpenoids,  $\beta$ -amyryn acetate dominated with a mean value of  $0.56 \text{ mg } 100 \text{ g}^{-1} \text{ dm}$ . 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 ( $513.12 \text{ mg } 100 \text{ g}^{-1} \text{ dm}$ ).

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 ( $799.30 \text{ mg } 100 \text{ g}^{-1} \text{ dm}$ ). 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,  $\beta$ -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  $\beta$ -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.



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# 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  $\beta$ -sitosterol and  $\beta$ -amyirin 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,  $\beta$ -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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# References





- Abdel-Aal, E. S. M., Akhtar, H., Rabalski, I., Bryan, M. (2014) Accelerated, microwave-assisted, and conventional solvent extraction methods affect anthocyanin composition from colored grains. *Journal of Food Science*, **79**(2), C138-C146.
- Akbarbaglu, Z., Peighambardoust, S. H., Sarabandi, K., Jafari, S. M. (2021) Spray drying encapsulation of bioactive compounds within protein-based carriers; different options and applications. *Food Chemistry*, **359**, 129965.
- Alhallaf, W., Bishop, K., Perkins, L. B. (2022) Optimization of accelerated solvent extraction of phenolic compounds from chaga using response surface methodology. *Food Analytical Methods*, **15**(10), 2777-2790.
- Aliakbarian, B., Painsi, M., Casazza, A. A., Perego, P. (2015) Effect of encapsulating agent on physical-chemical characteristics of olive pomace polyphenols-rich extracts. *Chemical Engineering Transactions*, **43**, 97-102.
- Alvarez-Rivera, G., Bueno, M., Ballesteros-Vivas, D., Mendiola, J. A., Ibanez, E. (2020) Pressurized liquid extraction. In *Liquid-phase extraction*, Elsevier. 375-398
- Ameer, K., Shahbaz, H. M., Kwon, J. H. (2017) Green extraction methods for polyphenols from plant matrices and their byproducts: A review. *Comprehensive Reviews in Food Science and Food Safety*, **16**(2), 295-315.
- Antony, A., Farid, M. (2022) Effect of temperatures on polyphenols during extraction. *Applied Sciences*, **12**(4), 2107.
- Astley, S., Finglas, P. (2016) Nutrition and health. In: Reference Module in Food Science, Elsevier, Amsterdam <https://doi.org/10.1016/B978-0-08-100596-5.03425-9>
- Balbino, S., Repajić, M., Obranović, M., Medved, A. M., Tonković, P., Dragović-Uzelac, V. (2021) Characterization of lipid fraction of Apiaceae family seed spices: Impact of species and extraction method. *Journal of Applied Research on Medicinal and Aromatic Plants*, **25**, 100326.
- Belščak-Cvitanović, A., Komes, D., Benković, M., Karlović, S., Hečimović, I., Ježek, D., Bauman, I. (2012) Innovative formulations of chocolates enriched with plant polyphenols from *Rubus idaeus* L. leaves and characterization of their physical, bioactive and sensory properties. *Food Research International*, **48**(2), 820-830.
- Bhusal, K. K., Magar, S. K., Thapa, R., Lamsal, A., Bhandari, S., Maharjan, R., Shrestha, S., Shrestha, J. (2022). Nutritional and pharmacological importance of stinging nettle (*Urtica dioica* L.): A review. *Heliyon*, **8**, e09717.
- Bitwell, C., Sen, I. S., Luke, C., Kakoma, M. K. (2023) A review of modern and conventional extraction techniques and their applications for extracting phytochemicals from plants. *Scientific African*, **19**, e01585.
- Blicharski, T., Oniszczyk, A. (2017) Extraction methods for the isolation of isoflavonoids from plant material. *Open Chemistry*, **15**(1), 34-45.

- Bohn, T. (2014) Dietary factors affecting polyphenol bioavailability. *Nutrition Reviews*, **72**, 429–452.
- Braidy, N., Behzad, S., Habtemariam, S., Ahmed, T., Daglia, M., Nabavi, S. M., Sobarzo-Sanchez, E., Nabavi, S. F. (2016) Neuroprotective effects of citrus fruit-derived flavonoids, nobiletin and tangeretin in Alzheimer's and Parkinson's disease. *CNS & Neurological Disorders - Drug Targets*, **15**(6), 667-677. doi: 10.2174/1871527315666160131234938
- Bratovic, A., Suljagic J. (2019) Micro-and nano-encapsulation in food industry. *Croatian journal of food science and technology*, **11**(1), 113-121.
- Buljeta, I., Pichler, A., Šimunović, J., Kopjar, M. (2022) Polysaccharides as carriers of polyphenols: Comparison of freeze-drying and spray-drying as encapsulation techniques. *Molecules*, **27**(16), 5069.
- Bursać Kovačević, D., Maras, M., Barba, F. J., Granato, D., Roohinejad, S., Mallikarjunan, K., Montesano, D., Lorenzo, J. M., Putnik, P. (2018) Innovative technologies for the recovery of phytochemicals from *Stevia rebaudiana* Bertoni leaves: A review. *Food Chemistry*, **268**, 513-521. doi:10.1016/j.foodchem.2018.06.091
- Caleja, C., Barros, L., Barreira, J. C., Ciric, A., Sokovic, M., Calhelha, R. C., Beatriz, M., Oliveira, P.P., Ferreira, I. C. (2018) Suitability of lemon balm (*Melissa officinalis* L.) extract rich in rosmarinic acid as a potential enhancer of functional properties in cupcakes. *Food Chemistry*, **250**, 67-74.
- Caliskan, G., Dirim, S. N. (2013) The effects of the different drying conditions and the amounts of maltodextrin addition during spray drying of sumac extract. *Food and Bioprocess Processing*, **91**(4), 539-548.
- Chaurasia, N., Wichtl, M. (1987) Sterols and sterol glycosides from *Urtica dioica*. *Journal of Natural Products*, **50**(5), 881-885.
- Cho, S. K., Abd El-Aty, A. M., Choi, J. H., Kim, M. R., Shim, J. H. (2007) Optimized conditions for the extraction of secondary volatile metabolites in *Angelica* roots by accelerated solvent extraction. *Journal of Pharmaceutical and Biomedical Analysis*, **44**(5), 1154-1158.
- Corona, G., Vauzour, D., Amini, A., Spencer, J.P.E. (2014) Chapter 44—The impact of gastrointestinal modifications, blood-brain barrier transport, and intracellular metabolism on polyphenol bioavailability: An Overview. In: *Polyphenols in Human Health and Disease*; Watson, R.R., Preedy, V.R., Zibadi, S., Eds.; Academic Press: San Diego, CA, USA, pp. 591–604.
- Corrêa-Filho, L. C., Lourenço, M. M., Moldão-Martins, M., Alves, V. D. (2019) Microencapsulation of  $\beta$ -carotene by spray drying: effect of wall material concentration and drying inlet temperature. *International journal of food science*, **2019**, 1-12.
- Dai, J., and Mumper, R. J. (2010) Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules*, **15**(10), 7313-7352.

- de Barros Fernandes, R. V., Borges, S. V., Botrel, D. A. (2014) Gum arabic/starch/maltodextrin/inulin as wall materials on the microencapsulation of rosemary essential oil. *Carbohydrate Polymers*, **101**, 524-532.
- Devkota, H.P., Paudel, K.R., Khanal, S., Baral, A., Panth, N., Adhikari-Devkota, A., Jha, N.K., Das, N., Singh, S.K., Chellappan, D.K., Dua, K., Hansbro P.M. (2022) Stinging Nettle (*Urtica dioica* L.): Nutritional Composition, Bioactive Compounds, and Food Functional Properties. *Molecules*, **27**, 5219. <https://doi.org/10.3390/molecules27165219>
- Di Virgilio, N., Papazoglou, E.G., Jankauskiene, Z., Di Lonardo, S., Praczyk, M., Wielgusz, K. (2015) The potential of stinging nettle (*Urtica dioica* L.) as a crop with multiple uses. *Industrial Crops and Products*, **68**, 42–49.
- Dias, D. R., Botrel, D. A., Fernandes, R. V. D. B., Borges, S. V. (2017) Encapsulation as a tool for bioprocessing of functional foods. *Current Opinion in Food Science*, **13**, 31-37.
- Dimitrijević, V. D., Krstić, N. S., Stanković, M. N., Arsić, I., Nikolić, R. S. (2016) Biometal and heavy metal content in the soil-nettle (*Urtica dioica* L.): System from different localities in Serbia. *Advanced Technologies*, **5**(1), 17-22.
- Dobrosłavić, E., Elez Garofulić, I., Šeparović, J., Zorić, Z., Pedisić, S., Dragović-Uzelac, V. (2022) Pressurized Liquid Extraction as a Novel Technique for the Isolation of *Laurus nobilis* L. Leaf Polyphenols. *Molecules*, **27**(16), 5099.
- Dobrosłavić, E., Elez Garofulić, I., Zorić, Z., Pedisić, S., Roje, M., Dragović-Uzelac, V. (2023) Physicochemical Properties, Antioxidant Capacity, and Bioavailability of *Laurus nobilis* L. Leaf Polyphenolic Extracts Microencapsulated by Spray Drying. *Foods*, **12**(9), 1923.
- Dobson, C. C., Mottawea, W., Rodrigue, A., Pereira, B. L. B., Hammami, R., Power, K. A., Bordenave, N. (2019) Impact of molecular interactions with phenolic compounds on food polysaccharides functionality. *Advances in food and nutrition research*, **90**, 135-181.
- Durmaz, Y., Kilicli, M., Toker, O. S., Konar, N., Palabiyik, I., Tamtürk, F. (2020) Using spray-dried microalgae in ice cream formulation as a natural colorant: Effect on physicochemical and functional properties. *Algal Research*, **47**, 101811.
- Đurović, S., Pavlić, B., Šorgić, S., Popov, S., Savić, S., Petronijević, M., Radojković, M., Cvetanović, A., Zeković, Z. (2017) Chemical composition of stinging nettle leaves obtained by different analytical approaches. *Journal of Functional Foods*, **32**, 18–26.
- Efthymiopoulos, I., Hellier, P., Ladommatos, N., Russo-Profili, A., Eveleigh, A., Aliev, A., Kay, A., Mills-Lamphey, B. (2018). Influence of solvent selection and extraction temperature on yield and composition of lipids extracted from spent coffee grounds. *Industrial Crops and Products*, **119**, 49-56.
- Eldamaty, H. (2018) Effect of adding nettle leaves (*Urtica dioica* L.) powder on basal diet to lower diabetes in rats. *Egyptian Journal of Food Science*, **46**(1), 141-151.

- Elez Garofulić, I., Malin, V., Repajić, M., Zorić, Z., Pedisić, S., Sterniša, M., Smole Možina, S., Dragović-Uzelac, V. (2021) Phenolic profile, antioxidant capacity and antimicrobial activity of nettle leaves extracts obtained by advanced extraction techniques. *Molecules*, **26**(20), 6153.
- Elez Garofulić, I., Zorić, Z., Pedisić, S., Dragović-Uzelac, V. (2016) Optimization of sour cherry juice spray drying as affected by carrier material and temperature. *Food Technology and Biotechnology*, **54**(4), 441.
- Elshafie, H. S., Camele, I., Mohamed, A. A. (2023) A Comprehensive review on the biological, agricultural and pharmaceutical properties of secondary metabolites based-plant origin. *International Journal of Molecular Sciences*, **24**(4), 3266.
- Erb, M., Kliebenstein, D. J. (2020) Plant secondary metabolites as defenses, regulators, and primary metabolites: the blurred functional trichotomy. *Plant physiology*, **184**(1), 39-52.
- Fang, Z., Bhandari, B. (2011) Effect of spray drying and storage on the stability of bay berry polyphenols. *Food Chemistry*, **129**, 1139-1147.
- Fatima, T., Naseer, B., Hussain, S. Z. (2018) Stinging Nettle: A herb with tremendous pharmacological potential. *International Journal of Unani and Integrative Medicine*, **2**(2), 24-28.
- Fernandez-Panchon, M. S., Villano, D., Troncoso, A. M., Garcia-Parrilla, M. C. (2008) Antioxidant activity of phenolic compounds: from in vitro results to in vivo evidence. *Critical reviews in food science and nutrition*, **48**(7), 649-671.
- Ferrari, C. C., Germer, S. P. M., de Aguirre, J. M. (2012) Effects of spray-drying conditions on the physicochemical properties of blackberry powder. *Drying Technology*, **30**, 154-163.
- Ferruzzi, M. G., Böhm, V., Courtney, P. D., Schwartz, S. J. (2002) Antioxidant and antimutagenic activity of dietary chlorophyll derivatives determined by radical scavenging and bacterial reverse mutagenesis assays. *Journal of food Science*, **67**(7), 2589-2595.
- Fikselová, M., Šilhár, S., Mareček, J., Frančáková, H. (2008) Extraction of carrot (*Daucus carota* L.) carotenes under different conditions. *Czech Journal of Food Sciences*, **26**(4), 268-274.
- Flórez, M., Cazón, P., Vázquez, M. (2022) Antioxidant extracts of nettle (*Urtica dioica*) leaves: evaluation of extraction techniques and solvents. *Molecules*, **27**(18), 6015.
- Francišković, M., Gonzalez-Pérez, R., Orčić, D., Sanchez de Medina, F., Martínez-Augustin, O., Svirčev, E., Simin, N., Mimica-Dukić, N. (2017) Chemical Composition and Immuno-Modulatory Effects of *Urtica dioica* L. (Stinging Nettle) Extracts. *Phytotherapy research*, **31**(8), 1183-1191.
- Garmendia, A., Raigón, M. D., Marques, O., Ferriol, M., Royo, J., Merle, H. (2018) Effects of nettle slurry (*Urtica dioica* L.) used as foliar fertilizer on potato (*Solanum tuberosum* L.) yield and plant growth. *PeerJ*, **6**, e4729.
- Ghosh, S., Sarkar, T., Das, A., Chakraborty, R. (2021) Micro and nanoencapsulation of natural colors: A holistic view. *Applied Biochemistry and Biotechnology*, **193**, 3787-3811.

- Giergielewicz-Możajska, H., Dąbrowski, Ł., Namieśnik, J. (2001) Accelerated solvent extraction (ASE) in the analysis of environmental solid samples—some aspects of theory and practice. *Critical Reviews in Analytical Chemistry*, **31**(3), 149-165.
- Goh, Y. S., Karunakaran, T., Murugaiyah, V., Santhanam, R., Abu Bakar, M. H., Ramanathan, S. (2021) Accelerated solvent extractions (ASE) of *Mitragyna speciosa* Korth.(Kratom) leaves: Evaluation of its cytotoxicity and antinociceptive activity. *Molecules*, **26**(12), 3704.
- González-Burgos, E., Gómez-Serranillos, M. P. (2021) Effect of phenolic compounds on human health. *Nutrients*, **13**(11), 3922. <https://doi.org/10.3390/nu13113922>
- González-Talice, J., Yuri, J.A., del Pozo, A. (2013) Relations among pigments, color and phenolic concentrations in the peel of two Gala apple strains according to canopy position and light environment. *Scientia Horticulturae*, **151**, 83–89.
- Grauso, L., de Falco, B., Lanzotti, V., Motti, R. (2020). Stinging nettle, *Urtica dioica* L.: Botanical, phytochemical and pharmacological overview. *Phytochemistry Reviews*, **19**, 1341-1377.
- Grgić, J., Šelo, G., Planinić, M., Tišma, M., Bucić-Kojić, A. (2020) Role of the encapsulation in bioavailability of phenolic compounds. *Antioxidants*, **9**(10), 923.
- Guil-Guerrero, J. L., Reboloso-Fuentes, M. M., Isasa, M. T. (2003) Fatty acids and carotenoids from Stinging Nettle (*Urtica dioica* L.). *Journal of Food Composition and Analysis*, **16**(2), 111-119.
- Guajardo-Flores, D., Rempel, C., Gutiérrez-Urbe, J. A., Serna-Saldívar, S. O. (2015) Influence of excipients and spray drying on the physical and chemical properties of nutraceutical capsules containing phytochemicals from black bean extract. *Molecules*, **20**, 21626-21635.
- He, J., Balasubramanian, R. (2009) Determination of atmospheric polycyclic aromatic hydrocarbons using accelerated solvent extraction. *Analytical letters*, **42**(11), 1603-1619.
- Hojnik, M., Škerget, M., Knez, Ž. (2007) Isolation of chlorophylls from stinging nettle (*Urtica dioica* L.). *Separation and Purification Technology*, **57**(1), 37-46.
- Hozawa, A., Jacobs, D. R., Steffes, M. W., Gross, M. D., Steffen, L. M. (2007) Circulating carotenoid concentrations and incident hypertension: The Coronary Artery Risk Development in Young Adults (CARDIA) study. *Journal of Hypertension*, **25**(12), 2377-2383. doi: 10.1097/HJH.0b013e3282f0ddc3
- Huang, D., Ou, B., Prior, R.L. (2005) The Chemistry behind Antioxidant Capacity Assays. *Journal of Agricultural and Food Chemistry*, **53**, 1841–1856, doi:10.1021/jf030723c
- Hudec, J., Burdová, M., Kobida, L. U., Komora, L., Macho, V., Kogan, G., Turianica, I., Kochanova, R., Ložek, O., Haban, M., Chlebo, P. (2007) Antioxidant capacity changes and phenolic profile of *Echinacea purpurea*, nettle (*Urtica dioica* L.), and dandelion (*Taraxacum officinale*) after application of polyamine and phenolic biosynthesis regulators. *Journal of agricultural and food chemistry*, **55**(14), 5689-5696.

- Jan, K. N., Zarafshan, K., Singh, S. (2017) Stinging nettle (*Urtica dioica* L.): a reservoir of nutrition and bioactive components with great functional potential. *Journal of food measurement and Characterization*, **11**, 423-433.
- Jeszka-Skowron, M., Zgoła-Grześkowiak, A., Frankowski, R., Grześkowiak, T., Jeszka, A. M. (2022) Variation in the content of bioactive compounds in infusions prepared from different parts of wild polish stinging nettle (*Urtica dioica* L.). *Molecules*, **27**(13), 4242.
- Jha, A. K., Sit, N. (2022) Extraction of bioactive compounds from plant materials using combination of various novel methods: A review. *Trends in Food Science & Technology*, **119**, 579-591.
- Joshi, B. C., Mukhija, M., Kalia, A. N. (2014) Pharmacognostical review of *Urtica dioica* L. *International Journal of Green Pharmacy*, **8**(4), 201-209.
- Juan, C., González, L., Soriano, J. M., Moltó, J. C., Mañes, J. (2005) Accelerated solvent extraction of ochratoxin A from rice samples. *Journal of agricultural and food chemistry*, **53**(24), 9348-9351.
- Kalajahi, S. E. M., Amjadi, S., Ghandiha, S. (2022) Development of the spray-dried nettle (*Urtica dioica* L.) extract-loaded nanoliposome powder for application as a natural additive in cake. *Journal of Food Processing and Preservation*, **46**(12), e17229.
- Kandasamy, S., Naveen, R. (2022) A review on the encapsulation of bioactive components using spray-drying and freeze-drying techniques. *Journal of Food Process Engineering*, **45**(8), e14059.
- Kavalali, G. M. (2003) *Urtica*: therapeutic and nutritional aspects of stinging nettles, Taylor & Francis, London
- Khan, N., Mukhtar, H. (2013) Cancer and metastasis: prevention and treatment by green tea polyphenols. *Cancer and Metastasis Reviews*, **32**(1-2), 25-49. doi: 10.1007/s10555-012-9411-8
- Kovacheva, E., Ganchev, G., Neicheva, A., Ivanova, I., Konoushlieva, M., Andreev, V. (1990) Gas chromatographic determination of sterols in fat-soluble concentrates obtained from plant materials. *Journal of Chromatography A*, **509**(1), 79-84.
- Kregiel, D., Pawlikowska, E., Antolak, H. (2018) *Urtica* spp.: Ordinary plants with extraordinary properties. *Molecules*, **23**(7), 1664.
- Kukrić, Z. Z., Topalić-Trivunović, L. N., Kukavica, B. M., Matoš, S. B., Pavičić, S. S., Boroja, M. M., Savić, A. V. (2012) Characterization of antioxidant and antimicrobial activities of nettle leaves (*Urtica dioica* L.). *Acta periodica technologica*, **43**, 257-272.
- Kumar, S. M., Chandrasekar, M. J. N., Nanjan, M. J., Suresh, B. (2005) Herbal remedies for acne. *Natural Product Radiance*, **4**(4), 328-334.
- Kumar, K., Srivastav, S., Sharanagat, V. S. (2021) Ultrasound assisted extraction (UAE) of bioactive compounds from fruit and vegetable processing by-products: A review. *Ultrasonics sonochemistry*, **70**, 105325.

- Kumara, S. S., Gayathri, D., Hariprasad, P., Venkateswaran, G., Swamy, C. T. (2020). In vivo AFB1 detoxification by *Lactobacillus fermentum* LC5/a with chlorophyll and immunopotentiating activity in albino mice. *Toxicon*, **187**, 214-222.
- Kushiro, T., Ezibuka, Y. (2010) Triterpenes, In: *Comprehensive Natural Products II: Chemistry and Biology*, Vol. 1, Elsevier, London, pp. 673–708
- Langi, P., Kiokias, S., Varzakas, T., Proestos, C. (2018) Carotenoids: From Plants to Food and Feed Industries. In: Barreiro, C., Barredo, J.L. (eds) *Microbial Carotenoids. Methods in Molecular Biology*, vol 1852. Humana Press, New York, NY. [https://doi.org/10.1007/978-1-4939-8742-9\\_3](https://doi.org/10.1007/978-1-4939-8742-9_3)
- Lechanteur, A., Evrard, B. (2020) Influence of composition and spray-drying process parameters on carrier-free DPI properties and behaviors in the lung: A review. *Pharmaceutics*, **12**(1), 55.
- Lin, K. H., Hsu, C. Y., Huang, Y. P., Lai, J. Y., Hsieh, W. B., Huang, M. Y., Yang, C.M., Chao, P. Y. (2013) Chlorophyll-related compounds inhibit cell adhesion and inflammation in human aortic cells. *Journal of medicinal food*, **16**(10), 886-898.
- Lu, W., Yang, X., Shen, J., Li, Z., Tan, S., Liu, W., Cheng, Z. (2021) Choosing the appropriate wall materials for spray-drying microencapsulation of natural bioactive ingredients: Taking phenolic compounds as examples. *Powder Technology*, **394**, 562-574.
- Ludwiczuk, A., Skalicka-Woźniak, K., Georgiev, M. I. (2017) Terpenoids. In *Pharmacognosy*, Academic Press., 233-266
- Luthria, D., Vinjamoori, D., Noel, K., Ezzell, J. (2019) Accelerated solvent extraction. In: *Oil Extraction and Analysis*; AOCS Publishing: Urbana, IL, USA, 25-38.
- Ma, L., Yan, S. F., Huang, Y. M., Lu, X. R., Qian, F., Pang, H. L., Xu, X.R., Zou, Z.J., Dong, P.C., Wang, X., Sun, T.T., Dou, H.L., Xiao, X. (2012). Effect of lutein and zeaxanthin on macular pigment and visual function in patients with early age-related macular degeneration. *Ophthalmology*, **119**(11), 2290-2297. doi: 10.1016/j.ophtha.2012.06.014
- Mahlangeni, N. T., Moodley, R., Jonnalagadda, S. B. (2016) The distribution of macronutrients, anti-nutrients and essential elements in nettles, *Laportea peduncularis* susp. *peduncularis* (River nettle) and *Urtica dioica* (Stinging nettle). *Journal of Environmental Science and Health, Part B*, **51**(3), 160-169.
- Manousi, N., Sarakatsianos, I., Samanidou, V. (2019) Extraction techniques of phenolic compounds and other bioactive compounds from medicinal and aromatic plants. In: *Engineering tools in the beverage industry* (pp. 283-314). Woodhead Publishing.
- Maoka, T. (2020) Carotenoids as natural functional pigments. *Journal of natural medicines*, **74**(1), 1-16.
- Marchetti, N., Bonetti, G., Brandolini, V., Cavazzini, A., Maietti, A., Meca, G., Mañes, J. (2018) Stinging nettle (*Urtica dioica* L.) as a functional food additive in egg pasta: Enrichment and bioaccessibility of Lutein and  $\beta$ -carotene. *Journal of Functional Foods*, **47**, 547-553.



- Martins, N., Barros, L., Ferreira, I. C. (2016) In vivo antioxidant activity of phenolic compounds: Facts and gaps. *Trends in Food Science & Technology*, **48**, 1-12.
- Michalska, M., Gluba, A., Mikhailidis, D. P., Nowak, P., Bielecka-Dabrowa, A., Rysz, J., Banach, M. (2010) The role of polyphenols in cardiovascular disease. *Medical Science Monitor*, **16**(5), RA110-9.
- Mitrović, J., Nikolić, N., Karabegović, I., Lazić, M., Nikolić, L., Savić, S., Pešić, M., Šimurina, O., Stojanović-Krasić, M. (2021) The effect of thermal processing on the content and antioxidant capacity of free and bound phenolics of cookies enriched by nettle (*Urtica dioica* L.) seed flour and extract. *Food Science and Technology*, **42**, 62420.
- Mohammad, S., Mohd, Y., Faqeer, M. (2015) Plant phenolics: A Review on Modern extraction techniques. In book: *Recent Progress in Medicinal Plants: Vol. 41- Analytical and Processing Techniques* Publisher: Studium Press LLC, USA
- Morais, R. M. S. C., Morais, A. M., Dammak, I., Bonilla, J., Sobral, P. J. D. A., Laguerre, J. C., Afonso, M.J., Ramalhosa, E. C. (2018) Functional dehydrated foods for health preservation. *Journal of Food Quality*, **2018**, 1-29.
- More, P. R., Jambrak, A. R., Arya, S. S. (2022) Green, environment-friendly and sustainable techniques for extraction of food bioactive compounds and waste valorization. *Trends in Food Science & Technology*, **128**, 296-315. <https://doi.org/10.1016/j.tifs.2022.08.016>
- Mottaleb, M. A., Sarker, S. D. (2012) Accelerated solvent extraction for natural products isolation. In: *Natural product isolation*. 3.ed., (Sarker, S. D., Nahar, L., edit.) Humana Press, New York, 75-87.
- Nabi, B. G., Mukhtar, K., Ahmed, W., Manzoor, M. F., Ranjha, M. M. A. N., Kieliszek, M., Bhat, Z.F., Aadil, R. M. (2023) Natural pigments: Anthocyanins, carotenoids, chlorophylls, and betalains as food colorants in food products. *Food Bioscience*, **52**, 102403.
- Ncube, B., Van Staden, J. (2015) Tilting plant metabolism for improved metabolite biosynthesis and enhanced human benefit. *Molecules*, **20**, 12698–12731.
- Nedović, V., Kalušević, A., Manojlović, V., Petrović, T., Bugarski, B. (2013) Encapsulation systems in the food industry. In: *Advances in food process engineering research and applications*, Springer, Berlin, 229-253.
- Obranović, M., Balbino, S., Repajić, M., Robić, K., Ritoša, E., Dragović-Uzelac, V. (2023). Wild nettle (*Urtica dioica* L.) root: Composition of phytosterols and pentacyclic triterpenes upon habitat diversity. *Food Chemistry Advances*, **2**, 100262.
- Oktay, M., Gülçin, İ., Küfrevioğlu, Ö. İ. (2003) Determination of in vitro antioxidant activity of fennel (*Foeniculum vulgare*) seed extracts. *LWT*, **36**, 263-271. doi:10.1016/s0023-6438(02)00226-8

- Olech, M., Łyko, L., Nowak, R. (2020) Influence of accelerated solvent extraction conditions on the LC-ESI-MS/MS polyphenolic profile, triterpenoid content, and antioxidant and anti-lipoxygenase activity of rhododendron luteum sweet leaves. *Antioxidants*, **9**(9), 822.
- Opačić, N., Radman, S., Fabek Uher, S., Benko, B., Voća, S., Šic Žlabur, J. (2022) Nettle Cultivation Practices—From Open Field to Modern Hydroponics: A Case Study of Specialized Metabolites. *Plants*, **11**(4), 483.
- Orčić, D., Francišković, M., Bekvalac, K., Svirčev, E., Beara, I., Lesjak, M., Mimica-Dukić, N. (2014) Quantitative determination of plant phenolics in *Urtica dioica* extracts by high-performance liquid chromatography coupled with tandem mass spectrometric detection. *Food chemistry*, **143**, 48-53.
- Ostlund Jr., R. E. (2002) Phytosterols in human nutrition. *Annual Review of Nutrition*, **22**(1), 533-549. doi: 10.1146/annurev.nutr.22.010402.102807
- Otles, S., Yalcin, B. (2012) Phenolic compounds analysis of root, stalk, and leaves of nettle. *The Scientific World Journal*, **2012**, 1-12. <https://doi.org/10.1100/2012/564367>
- Paini, M., Aliakbarian, B., Casazza, A. A., Lagazzo, A., Botter, R., Perego, P. (2015) Microencapsulation of phenolic compounds from olive pomace using spray drying: A study of operative parameters. *LWT-Food Science and Technology*, **62**(1), 177-186.
- Palabiyik, I., Durmaz, Y., Öner, B., Toker, O. S., Coksari, G., Konar, N., Tamtürk, F. (2018) Using spray-dried microalgae as a natural coloring agent in chewing gum: effects on color, sensory, and textural properties. *Journal of applied phycology*, **30**, 1031-1039.
- Palma, M., Piñeiro, Z., Barroso, C.G. (2001) Stability of phenolic compounds during extraction with superheated solvents. *Journal of Chromatography A*, **921**, 169–174.
- Pasrija, D., Ezhilarasi, P. N., Indrani, D., Anandharamakrishnan, C. (2015) Microencapsulation of green tea polyphenols and its effect on incorporated bread quality. *LWT-Food Science and Technology*, **64**(1), 289-296.
- Paulauskienė, A., Tarasevičienė, Ž., Laukagalis, V. (2021) Influence of Harvesting Time on the Chemical Composition of Wild Stinging Nettle (*Urtica dioica* L.). *Plants*, **10**(4), 686. <https://doi.org/10.3390/plants10040686>
- Phisut, N. (2012) Spray drying technique of fruit juice powder: some factors influencing the properties of product. *International Food Research Journal*, **19**, 1297-1306.
- Pimpley, V. A., Maity, S., Murthy, P. S. (2022) Green coffee polyphenols in formulations of functional yoghurt and their quality attributes. *International Journal of Dairy Technology*, **75**(1), 159-170.
- Piñeiro, Z., Palma, M., Barroso, C. G. (2004) Determination of catechins by means of extraction with pressurized liquids. *Journal of Chromatography A*, **1026**(1-2), 19-23.

- Pinelli, P., Ieri, F., Vignolini, P., Bacci, L., Baronti, S., Romani, A. (2008) Extraction and HPLC analysis of phenolic compounds in leaves, stalks, and textile fibers of *Urtica dioica* L. *Journal of agricultural and food chemistry*, **56**(19), 9127-9132.
- Polat, G.D., Durmaz, Y., Konar, N., Toker, O. S., Palabiyik, I., Tasan, M. (2020) Using encapsulated *Nannochloropsis oculata* in white chocolate as coloring agent. *Journal of Applied Phycology*, **32**, 3077-3088.
- Popescu, C., Pruteanu, A., Voicea, I., Ivancu, B., Gageanu, G., Popa, L., Vladut, V. (2014) Study regarding biochemical characterization and some preparations from nettle and wormwood in order to capitalize them as bioinsecticide/biofertilizers in organic agriculture. *Annals of the University of Craiova-Agriculture, Montanology, Cadastre Series*, **44**(2), 175-185.
- Precedence Research (2023) Functional Food Market <https://www.precedenceresearch.com/functional-food-market>. Accessed July 22, 2023
- Putnik, P., Barba, F. J., Lucini, L., Rocchetti, G., Montesano, D. (2019) Conventional, nonconventional extraction techniques and new strategies for the recovery of bioactive compounds from plant material for human nutrition. *Food Research International*, **123**, 516-517. doi:10.1016/j.foodres.2019.05.010
- Rafajlovska, V., Djarmati, Z., Najdenova, V., Cvetkov, L. (2002) Extraction of stinging nettle (*Urtica dioica* L.) with supercritical carbon dioxide. *Balkesir Üniversitesi Fen Bilimleri Enstitüsü Dergisi*, **4**(2), 49-52.
- Randall, C., Randall, H., Dobbs, F., Hutton, C., Sanders, H. (2013) Randomized controlled trial of nettle sting for treatment of base-of-thumb pain. *The Journal of the Royal Society of Medicine*, **106**(4), 143-149. doi:10.1177/0141076813476043
- Ras, R. T., Geleijnse, J. M., Trautwein, E. A. (2014) LDL-cholesterol-lowering effect of plant sterols and stanols across different dose ranges: A meta-analysis of randomized controlled studies. *British Journal of Nutrition*, **112**(2), 214-219. doi: 10.1017/S0007114514000705
- Rasul, M. G. (2018) Conventional extraction methods use in medicinal plants, their advantages and disadvantages. *International Journal of Basic Sciences and Applied Computing*, **2**, 10-14.
- Repajić, M., Ekić, S., Kruk, V., Dragović-Uzelac, V. (2020) Effect of accelerated solvent extraction conditions on the isolation of bioactive compounds from fennel (*Foeniculum vulgare* Mill.) seeds. *Croatian Journal of Food Technology, Biotechnology and Nutrition*, **15**(3-4), 102-106.
- Roschek Jr, B., Fink, R. C., McMichael, M., Alberte, R. S. (2009) Nettle extract (*Urtica dioica*) affects key receptors and enzymes associated with allergic rhinitis. *Phytotherapy Research*, **23**(7), 920-926.
- Ross, C.F., Hoye, C., Fernandez-Plotka, V.C. (2011) Influence of Heating on the Polyphenolic Content and Antioxidant Activity of Grape Seed Flour, *Journal of Food Science*, **76**, 884–890.

- Rutto, L. K., Xu, Y., Ramirez, E., Brandt, M. (2013) Mineral properties and dietary value of raw and processed stinging nettle (*Urtica dioica* L.). *International journal of food science*, **2013**, 1-9.
- Saavedra-Leos, Z., Leyva-Porras, C., Araujo-Díaz, S.B., Toxqui-Terán, A., Borrás-Enríquez, A.J., (2015) Technological Application of Maltodextrins According to the Degree of Polymerization. *Molecules*, **20**(12), 21067-81. doi: 10.3390/molecules201219746.
- Safarinejad, M. R. (2005) *Urtica dioica* for treatment of benign prostatic hyperplasia: a prospective, randomized, double-blind, placebo-controlled, crossover study. *Journal of Herbal Pharmacotherapy*, **5**(4), 1-11. doi:10.1300/J157v05n04\_01
- Saha, S., Walia, S., Kundu, A., Sharma, K., Paul, R. K. (2015) Optimal extraction and fingerprinting of carotenoids by accelerated solvent extraction and liquid chromatography with tandem mass spectrometry. *Food chemistry*, **177**, 369-375.
- Şahin-Nadeem, H., Dinçer, C., Torun, M., Topuz, A., Özdemir, F. (2013) Influence of inlet air temperature and carrier material on the production of instant soluble sage (*Salvia fruticosa* Miller) by spray drying. *LWT-Food Science and Technology*, **52**(1), 31-38.
- Said, A. A. H., Otmani, I. S. E., Derfoufi, S., Benmoussa, A. (2015) Highlights on nutritional and therapeutic value of stinging nettle (*Urtica dioica*). *International Journal of Pharmacy and Pharmaceutical Sciences*, **7**(10), 8-14.
- Saini, R.K., Nile, S.H., Park, S.W. (2015) Carotenoids from fruits and vegetables: Chemistry, analysis, occurrence, bioavailability and biological activities. *Food Research International*, **76**, 735–750.
- Sajfrtová, M., Sovová, H., Opletal, L., Bártlová, M. (2005). Near-critical extraction of  $\beta$ -sitosterol and scopoletin from stinging nettle roots. *The Journal of supercritical fluids*, **35**(2), 111-118.
- Samanta, K. K., Roy, A. N., Baite, H., Debnath, S., Ammayappan, L., Nayak, L. K., Singha, A., Kundu, T. K. (2021) Applications of Nettle Fibre in Textile: A Brief Review. *International Journal of Bioresource Science*, **8**(1), 39-45.
- Santos, D., Maurício, A. C., Sencadas, V., Santos, J. D., Fernandes, M. H., Gomes, P. S. (2018) Spray drying: an overview. In: *Biomaterials-Physics and Chemistry-New Edition*, IntechOpen, London, 9-35.
- Scalbert, A., Johnson, I. T., Saltmarsh, M. (2005) Polyphenols: antioxidants and beyond. *The American Journal of Clinical Nutrition*, **81**(1), 215S-217S. doi: 10.1093/ajcn/81.1.215S
- Schöttner, M., Ganßer, D., Spiteller, G. (1997) Lignans from the roots of *Urtica dioica* and their metabolites bind to human sex hormone binding globulin (SHBG). *Planta medica*, **63**(06), 529-532.
- Serafini, M., Peluso, I., Raguzzini, A. (2010) Flavonoids as anti-inflammatory agents. *Proceedings of the Nutrition Society*, **69**(3), 273-278. doi: 10.1017/S002966511000162X

- Semalty, M., Adhikari, L., Semwal, D., Chauhan, A., Mishra, A., Kotiyal, R., Semalty, A. (2017) A comprehensive review on phytochemistry and pharmacological effects of stinging nettle (*Urtica dioica*). *Current Traditional Medicine*, **3**(3), 156-167.
- Sesso, H. D., Buring, J. E., Norkus, E. P., Gaziano, J. M. (2004) Plasma lycopene, other carotenoids, and retinol and the risk of cardiovascular disease in men. *American Journal of Clinical Nutrition*, **79**(1), 47-53.
- Shahid, M. O. H. A. M. M. A. D., Yusuf, M. O. H. D., Mohammad, F. (2016) Plant phenolics: A Review on Modern extraction techniques. *Recent Progress in Medicinal Plants*, **41**, 265-287.
- Shams, K. A., Abdel-Azim, N. S., Saleh, I. A., Hegazy, M. E. F., El-Missiry, M. M., Hammouda, F. M., Tahrir, E. (2015). Green technology: Economically and environmentally innovative methods for extraction of medicinal & aromatic plants (MAP) in Egypt. *Journal of Chemical and Pharmaceutical Research*, **7**(5), 1050-1074.
- Shin, H. Y., Shim, S. H., Ryu, Y. J., Yang, J. H., Lim, S. M., Lee, C. G. (2018) Lipid extraction from *Tetraselmis* sp. microalgae for biodiesel production using hexane-based solvent mixtures. *Biotechnology and bioprocess engineering*, **23**, 16-22.
- Shishir, M. R. I., Chen, W. (2017) Trends of spray drying: A critical review on drying of fruit and vegetable juices. *Trends in food science & technology*, **65**, 49-67.
- Shonte, T. T., Duodu, K. G., de Kock, H. L. (2020) Effect of drying methods on chemical composition and antioxidant activity of underutilized stinging nettle leaves. *Heliyon*, **6**(5), e03938.
- Singh, S., Dixit, D. (2014) A review on spray drying: emerging technology in food industry. *International Journal of Applied Engineering and Technology*, **4**(1), 1-8.
- Sun, H., Ge, X., Lv, Y., Wang, A. (2012) Application of accelerated solvent extraction in the analysis of organic contaminants, bioactive and nutritional compounds in food and feed. *Journal of Chromatography A*, **1237**, 1-23.
- Tarasevičienė, Ž., Vitkauskaitė, M., Paulauskienė, A., Černiauskienė, J. (2023). Wild stinging nettle (*Urtica dioica* L.) leaves and roots chemical composition and phenols extraction. *Plants*, **12**(2), 309.
- Thi Anh Dao, D., Van Thanh, H., Viet Ha, D., Duc Nguyen, V. (2021) Optimization of spray-drying process to manufacture green tea powder and its characters. *Food Science & Nutrition*, **9**(12), 6566-6574.
- Timilsena, Y. P., Haque, M. A., Adhikari, B. (2020) Encapsulation in the food industry: A brief historical overview to recent developments. *Food and Nutrition Sciences*, **11**(6), 481-508.
- Tolić, M.T., Krbavčić, I.P., Vujević, P., Milinović, B., Jurčević, I.L., Vahčić, N. (2017) Effects of weather conditions on phenolic content and antioxidant capacity in juice of chokeberries (*Aronia melanocarpa* L.). *Polish Journal of Food and Nutrition Sciences*, **67**, 67–74.

- Tolve, R., Condelli, N., Caruso, M. C., Barletta, D., Favati, F., Galgano, F. (2018) Fortification of dark chocolate with microencapsulated phytosterols: Chemical and sensory evaluation. *Food & function*, **9**(2), 1265-1273.
- Tonon, R. V., Brabet, C., Hubinger, M. D. (2008) Influence of process conditions on the physicochemical properties of açai (*Euterpe oleraceae* Mart.) powder produced by spray drying. *Journal of Food Engineering*, **88**, 411–418.
- Tran, T. T., Nguyen, H. V. (2018) Effects of spray-drying temperatures and carriers on physical and antioxidant properties of lemongrass leaf extract powder. *Beverages*, **4**(4), 84.
- Truong, D. H., Nguyen, D. H., Ta, N. T. A., Bui, A. V., Do, T. H., Nguyen, H. C. (2019) Evaluation of the use of different solvents for phytochemical constituents, antioxidants, and in vitro anti-inflammatory activities of *Severinia buxifolia*. *Journal of food quality*, **2019**, 1-9. <https://doi.org/10.1155/2019/8178294>
- Tungmunnithum, D., Thongboonyou, A., Pholboon, A., Yangsabai, A. (2018) Flavonoids and Other Phenolic Compounds from Medicinal Plants for Pharmaceutical and Medical Aspects: An Overview. *Medicines*, **5**(3), 93. <https://doi.org/10.3390/medicines5030093>
- Upton, R. (2013) Stinging nettles leaf (*Urtica dioica* L.): Extraordinary vegetable medicine. *Journal of Herbal Medicine*, **3**, 9–38.
- Velderrain-Rodríguez, G. R., Palafox-Carlos, H., Wall-Medrano, A., Ayala-Zavala, J. F., Chen, C. O., Robles-Sánchez, M., Astiazaran-García, H., Alvarez-Parrilla, E., González-Aguilar, G. A. (2014). Phenolic compounds: their journey after intake. *Food & function*, **5**(2), 189-197.
- Viotti, C., Albrecht, K., Amaducci, S., Bardos, P., Bertheau, C., Blaudez, D., Bothe, L., Cazaux, D., Ferrarini, A., Govilas, J., Gusovius, H-J., Jeannin, T., Lühr, C., Müssig, J., Pilla, M., Placet, V., Puschenreiter, M., Tognacchini, A., Yung, L., Chalot, M. (2022) Nettle, a Long-Known Fiber Plant with New Perspectives, *Materials*, **15**(12), 4288. <https://doi.org/10.3390/ma15124288>
- Wang, D., Miao, X., Li, Q. X. (2008) Analysis of organochlorine pesticides in coral (*Porites evermanni*) samples using accelerated solvent extraction and gas chromatography/ion trap mass spectrometry. *Archives of environmental contamination and toxicology*, **54**, 211-218.
- Wunderlich, A. L. M., Azevedo, S. C. S. F., Yamada, L. A., Bataglini, C., Previante, C., Campanholi, K. S. S., Pereira, P.C.S., Caetano, W., Kaplum, V., Nakamura, C.V., Nakanishi, A.B.S., Comar, J.F., Pedrosa, M.M.D., Godoi, V. A. F. (2019). Chlorophyll treatment combined with photostimulation increases glycolysis and decreases oxidative stress in the liver of type 1 diabetic rats. *Brazilian Journal of Medical and Biological Research*, **53**. <https://doi.org/10.1590/1414-431X20198389>
- Xu, F., Huang, X., Wu, H., Wang, X. (2018) Beneficial health effects of lupenone triterpene: a review. *Biomedicine and Pharmacotherapy*, **103**,198–203

- Yin, G., Tang, D., Dai, J., Liu, M., Wu, M. (2018) Recent advances in pentacyclic triterpenoids and their analogues as anticancer agents. *Anti-Cancer Agents in Medicinal Chemistry*, **18**(6), 806-816. doi: 10.2174/1871520618666171017161114
- Yoshida, Y., Niki, E. (2003) Antioxidant effects of phytosterol and its components. *Journal of nutritional science and vitaminology*, **49**(4), 277-280.
- Yunus, M. A. C., Hasan, M., Othman, N., Mohd-Setapar, S. H., Salleh, L. M., Ahmad-Zaini, M. A., Idham, Z., Zhari, S. (2013) Effect of particle size on the oil yield and catechin compound using accelerated solvent extraction. *Jurnal Teknologi*, **60**(1), 21-25.
- Zengin, G., Cvetanović, A., Gašić, U., Stupar, A., Bulut, G., Senkardes, I., Dogan, A., Seebaluck-Sandoram, R., Rengasamy, K.R.R., Sinan, K.I., Mahomoodally, M. F. (2019) Chemical composition and bio-functional perspectives of *Erica arborea* L. extracts obtained by different extraction techniques: Innovative insights. *Industrial Crops and Products*, **142**, 111843.
- Zhang, Q. W., Lin, L. G., Ye, W. C. (2018) Techniques for extraction and isolation of natural products: A comprehensive review. *Chinese medicine*, **13**, 1-26.
- Zhiqiang, Z. H. A. O., Hao, J. I. A. O. (2022) Effect of chlorophyll derivatives on wound healing, inflammatory edema and expression of tissue angiogenic factors after hemorrhoid surgery. *Chinese journal of integrative medicine*, **30**(2), 132-137.
- Zhong, S., Bird, A., Kopec, R. E. (2021) The Metabolism and Potential Bioactivity of Chlorophyll and Metallo-chlorophyll Derivatives in the Gastrointestinal Tract. *Molecular Nutrition & Food Research*, **65**(7), 2000761.
- Zorić, Z., Pelaić, Z., Pedisić, S., Garofulić, I. E., Kovačević, D. B., Dragović-Uzelac, V. (2017) Effect of storage conditions on phenolic content and antioxidant capacity of spray dried sour cherry powder. *LWT-Food Science and Technology*, **79**, 251-259.
- Zulfiqar, S., Sharif, S., Saeed, M., Tahir, A. (2021). Role of Carotenoids in Photosynthesis. In: Zia-Ul-Haq, M., Dewanjee, S., Riaz, M. (eds) *Carotenoids: Structure and Function in the Human Body*. Springer, Cham. [https://doi.org/10.1007/978-3-030-46459-2\\_5](https://doi.org/10.1007/978-3-030-46459-2_5)

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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.

## List of authors publications

### Original scientific papers indexed in Web of Science (Current Contents Connect)

- Marčac, N., Balbino, S., Tonković, P., Medved, A.M., **Cegledi, E.**, Dragović, S., Dragović-Uzelac, V., Repajić, M. (2023) Hydrodistillation and Steam Distillation of Fennel Seeds Essential Oil: Parameter Optimization and Application of Cryomilling Pretreatment. *Processes*, **11**, 2354. <https://doi.org/10.3390/pr11082354> (Q2)
- Jović, T., Elez Garofulić, I., Čulina, P., Pedisić, S., Dobrosravić, E., **Cegledi, E.**, Dragović-Uzelac, V., Zorić, Z. (2023) The Effect of Spray-Drying Conditions on the Characteristics of Powdered *Pistacia lentiscus* Leaf Extract. *Processes*, **11(4)**, 1229. <https://doi.org/10.3390/pr11041229> (Q2)
- **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> (Q1)
- **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> (Q1)
- 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> (Q1)
- Repajić, M., **Cegledi, E.**, Kruk, V., Pedisić, S., Çınar, F., Bursać Kovačević, D., Žutić, I., Dragović-Uzelac, V. (2020) Accelerated Solvent Extraction as a Green Tool for the Recovery of Polyphenols and Pigments from Wild Nettle Leaves. *Processes*, **8(7)**, 803. <https://doi.org/10.3390/pr8070803> (Q3)

### **Abstracts in Book of abstracts**

- Dobrinčić, A., Dobrosravić, E., **Cegledi, E.**, Elez Garofulić, I., Dragović-Uzelac, V. (2022) Influence of microwave-assisted extraction parameters on brown algae *Dictyota dichotoma* polyphenols. In: *Book of Abstracts of the 4th International Congress on "Green Extraction of Natural Products" (GENP2022)*, Poreč, Croatia, 49-49.
- Dobrinčić, A., Dobrosravić, E., Piasecka, I., **Cegledi, E.**, Zorić, Z., Pedisić, S., Dragović-Uzelac, V. (2022) Polyphenol profiles and antioxidant capacity of different berry fruit pomace and seeds. In: *Book of Abstracts of the 10th International Congress of Food Technologists, Biotechnologists and Nutritionists*, Zagreb, Croatia, 199-200.
- **Cegledi, E.**, Repajić, M., Marčac, N., Elez Garofulić, I., Cegledi, K., Dobrosravić, E., Dragović-Uzelac, V. (2022) Influence of temperature and carrier ratio on fennel essential oil powder obtained by spray drying. In: *Book of Abstracts of the 10th International Congress of Food Technologists, Biotechnologists and Nutritionists*, Zagreb, Croatia, 11-12.
- Repajić, M., Elez Garofulić, I., Pufek, P., **Cegledi, E.**, Levaj, B., Dragović-Uzelac, V. (2022) Application of pressurized liquid extraction for the isolation of phenols from sea buckthorn leaves. In: *Book of Abstracts of the 10th International Congress of Food Technologists, Biotechnologists and Nutritionists*, Zagreb, Croatia, 39-40.
- Dobrosravić, E., **Cegledi, E.**, Dobrinčić, A., Elez Garofulić, I., Dragović-Uzelac, V. (2022) Isolation of *Thymus Vulgaris* L. Leaf polyphenols by microwave-assisted extraction. In: *Book of Abstracts of the 4th International Congress on "Green Extraction of Natural Products" (GENP2022)*, Poreč, Croatia, 44-44.
- **Cegledi, E.**, Dobrinčić, A., Dobrosravić, E., Repajić, M., Dragović-Uzelac, V. (2022) Influence of solvent and material proportion during microwave-assisted extraction of *Dictoyota dichotoma* and *Thymus vulgaris* pigments. In: *Book of Abstracts of the 2nd International Conference on Advanced Production and Processing*, Novi Sad, Serbia, 84-84.
- **Cegledi, E.**, Repajić, M., Elez Garofulić, I., Zorić, Z., Radman, S., Dragović-Uzelac, V. (2022) The influence of extraction and enviromental parameters on the isolation of bioactive compounds from nettle (*Urtica dioica* L.). In: *Book of Abstracts of the 4th*

*International scientific and expert conference "Natural resources, green technology and sustainable development/4-GREEN2022"*, Zagreb, Croatia, 104-104.

- Dobrinčić, A., Brunović, B., Repajić, M., **Cegledi, E.**, Dobroslavić, E., Dragović-Uzelac, V., Levaj, B. (2022) Stability of minimally processed apple treated with laurel (*Laurus nobilis* L.) essential oil. In: *Book of Abstracts of the 2nd International Conference on Advanced Production and Processing*, Novi Sad, Serbia, 96-96.
- Dobroslavić, E., **Cegledi, E.**, Dobrinčić, A., Elez Garofulić, I., Zorić, Z., Pedisić, S., Dragović-Uzelac, V. (2022) Phenolic content and antioxidant activity of carob (*Ceratonia siliqua* L.) leaf extracts obtained by green extraction techniques. In: *Book of Abstracts of the 2nd International Conference on Advanced Production and Processing*, Novi Sad, Serbia, 83-83.
- Levaj, B., Dobrinčić, A., **Cegledi, E.**, Dobroslavić, E., Čošić, Z., Dragović-Uzelac, V., Repajić, M. (2022) Shelf-life of fresh-cut potatoes treated with laurel essential oil during refrigerated storage. In: *CEFood Congress Book "Food, technology and nutrition for healthy people in a healthy environment"*, Čatež ob Savi, Slovenia, 192-192.
- Dobrinčić, A., **Cegledi, E.**, Dobroslavić, E., Cvitković, D., Mrvčić, J., Dragović-Uzelac, V. (2022) Green synthesis of silver nanoparticles using seaweed *Fucus virosides* and *Cystoseira barbata* extracts. In: *Book of Abstracts of the 4th International scientific and expert conference "Natural resources, green technology and sustainable development/4-GREEN2022"*, Zagreb, Croatia, 128-128.
- **Cegledi, E.**, Repajić, M., Marčac, N., Elez Garofulić, I., Balun, M., Dobroslavić, E., Dragović-Uzelac, V. (2022) Inkapsulacija eteričnog ulja komorača primjenom sušenja raspršivanjem. In: *International conference 19. Ružička days "TODAY SCIENCE-TOMORROW INDUSTRY"*, Vukovar, Croatia, 94-94.
- Elez Garofulić, I., Repajić, M., **Cegledi, E.**, Pedisić, S., Dragović-Uzelac, V. (2022) Spray-drying encapsulation of nettle and fennel bioactive molecules. In: *Book of Abstracts of the 4th International scientific and expert conference "Natural resources, green technology and sustainable development/4-GREEN2022"*, Zagreb, Croatia, 105-105.
- Repajić, M., **Cegledi, E.**, Marčac, N., Balbino, S., Elez Garofulić, I., Levaj, B., Dragović-Uzelac, V. (2022) Isolation of fennel essential oil by conventional and advanced extraction techniques. In: *Book of Abstracts of the 4th International scientific and expert conference*

“Natural resources, green technology and sustainable development/4-GREEN2022“, Zagreb, Croatia, 103-103.

- **Cegledi, E.**, Dobroslavić, E., Dobrinčić, A., Cvitković, D., Mrvčić, J., Dragović-Uzelac, V. (2022) Green synthesis of silver nanoparticles using nettle (*Urtica dioica* L.), myrtle (*Myrtus communis* L.) and bay laurel (*Laurus nobilis* L.) leaf extracts. In: *Book of Abstracts of the 4th International scientific and expert conference “Natural resources, green technology and sustainable development/4-GREEN2022*, Zagreb, Croatia, 133-133.
- Balbino, S., Repajić, M., Dobroslavić, E., Cvitković, D., **Cegledi, E.**, Dobrinčić, A., Dragović-Uzelac, V. (2022) Brown seaweed alginate as carrier for *Myrtus communis* essential oil stabilization by electrostatic extrusion. In: *Book of Abstracts of the World Aquaculture 2021*, Mérida, Mexico, 23-23.
- Repajić, M., Balbino, S., Dobroslavić, E., Cvitković, D., **Cegledi, E.**, Levaj, B., Dragović-Uzelac, V. (2022) Alginate-based beads: encapsulation of sage essential oil by electrostatic extrusion. In: *Book of Abstracts of the World Aquaculture 2021*, Mérida, Mexico, 205-205.
- Dobroslavić, E., **Cegledi, E.**, Zorić, Z., Elez Garofulić, I., Dragović-Uzelac, V. (2022) Bioavailability of *L. nobilis* leaf polyphenols encapsulated by spray-drying. In: *Book of Abstracts of the 7th Croatian Congress of Microbiology with International Participation*, , Sveti Martin na Muri, Croatia, 52-52.
- Dragović-Uzelac, V., Repajić, M., Elez Garofulić, I., Dent, M., Pedisić, S., Zorić, Z., Levaj, B., **Cegledi, E.**, Lisica, P. (2021) The influence of microwave assisted extraction on the recovery of phenolic acids and flavone glycosides from sage (*Salvia officinalis* L.). In: *Book of Abstracts of the 35th EFFoST International Conference 2021 Healthy Individuals, Resilient Communities, and Global Food Security*, Lausanne, Switzerland, 2305-2305.
- **Cegledi, E.**, Repajić, M., Balbino, S., Obranović, M., Peričić, M., Lončarić, S., Dragović-Uzelac, V. (2021) Comparison of conventional Soxhlet and advanced accelerated solvent extraction in isolation of phytosterols from nettle (*Urtica dioica* L) roots. In: *Book of Abstracts of the 35th EFFoST International Conference 2021 Healthy Individuals, Resilient Communities, and Global Food Security*, Lausanne, Switzerland, 2306-2306.
- **Cegledi, E.**, Mikulić, A., Elez Garofulić, I., Dragović-Uzelac, V. (2021) Inkapsulacija fenolnih spojeva ekstrakta lista koprive primjenom sušenja raspršivanjem. In: *Book of*

*Abstracts of the 13th International Scientific and Professional Conference WITH FOOD TO HEALTH*, Osijek, Croatia, 61-61.

- Levaj, B., Dobrinčić, A., **Cegledi, E.**, Dobroslavić, E., Cvitković, D., Dragović-Uzelac, V., Repajić, M. (2021) Fresh-cut potatoes treated with fennel essential oil: shelf-life during refrigerated storage. In: *Book of Abstracts of the 13th International Scientific and Professional Conference WITH FOOD TO HEALTH*, Osijek, Croatia, 152-152.
- Repajić, M., **Cegledi, E.**, Palčić, I., Marčac, N., Dragović-Uzelac, V. (2021) Essential oils in wild Istrian fennel (*Foeniculum vulgare* Mill.): variability in the content and composition. In: *Book of Abstracts of the 13th International Scientific and Professional Conference WITH FOOD TO HEALTH*, Osijek, Croatia, 134-134.