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

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

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

1. Introduction

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

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

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

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

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

2. Materials and Methods

2.1. Chemicals

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

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

2.2. Plant Material

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

2.3. Extraction Procedures

2.3.1. Accelerated Solvent Extraction

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

2.3.2. Ultrasound Assisted Extraction

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

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

2.4. HPLC Analysis

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

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

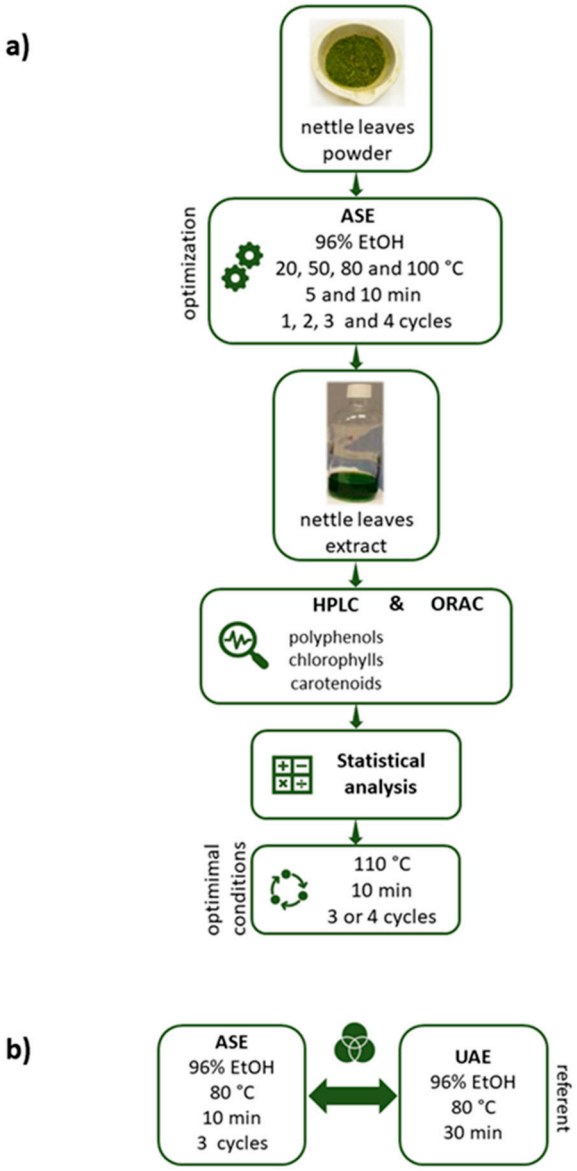

Figure 1. Experimental setup: (**a**) optimization of ASE conditions, (**b**) comparison of ASE and UAE efficiency. (ASE = accelerated solvent extraction, UAE = ultrasound assisted extraction).

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

2.5. Antioxidant Capacity

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

2.6. Statistical Analysis

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

3. Results and Discussion

3.1. Influence of Accelerated Solvent Extraction on Polyphenols Recovery

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.3. Influence of Accelerated Solvent Extraction on Antioxidant Capacity

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

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

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

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

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

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

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

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

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

4. Conclusions

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

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

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