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Research Article

Extraction of Nutraceutical Bioactive Compounds from Native Algae Using Solvents with a Deep Natural Eutectic Point and Ultrasonic-Assisted Extraction

Seyedeh Bahar Hashemi[1], Alireza Rahimi[2*], Mehdi Arjmand[3]

[1] *Seneca College, Ontario, Canada*
[2] *Department of Civil, Environmental and Sustainable Engineering, Biodesign Institute, Arizona State University, Tempe, AZ, USA*
[3] *Department of Chemical Engineering, Islamic Azad University, South Tehran Branch, Tehran, Iran*

Keywords

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Abstract

Food is the source of energy and growth through the breakdown of its vital components and plays a vital role in human health and nutrition. Many natural compounds found in plant and animal materials play a special role in biological systems and the origin of many such compounds can be *algae*. *Algae* are an enormous source of polysaccharides and have gained much interest in human flourishing. In this study, *algae* biomass extractions were conducted using natural deep eutectic-based solvents (NADES) and Ultrasound-assisted extraction (UAE). The aim of this research is to extract bioactive compounds including total carotenoid, antioxidant activity and polyphenolic contents. For that purpose, the influence of three important extraction parameters, namely biomass-to-solvent ratio, temperature, and time was studied regarding their impact on the recovery of carotenoids and phenolics and on the extracts' antioxidant activity. An experimental design was implemented, and the Response Surface Methodology (RSM) was employed for the process optimization. The influence of the independent parameters on each dependent one was determined through Analysis of Variance (ANOVA). The results showed that UAE for 50 min proved to be the best extraction condition, and proline:lactic acid (1:1) and choline chloride:urea (1:2) extracts showed the highest total phenolic contents (50.00 ± 0.70 mgGAE/gdw) and antioxidant activity [60.00 ± 1.70 mgTE/gdw and 70.00 ± 0.90 mgTE/gdw in 2.2-diphenyl-1-picrylhydrazyl (DPPH) and 2.2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) methods, respectively]. The results confirmed that the combination of UAE and NADES provide an excellent alternative to conventional solvents namely methanol (MeOH) and water for sustainable and green extraction, and have huge potential for use in industrial applications involving the extraction of bioactive compounds from *algae*. This study is the first attempt to optimize effects of ultrasonic-assisted extraction, ultrasonic devices, deep natural eutectic point and their application in extraction of total carotenoids, antioxidant activity, and polyphenolic contents from *algae*. The future perspective of ultrasound technology is also discussed, which will help to better understand the complex mechanism of ultrasonic-assisted extraction and further guide its application in *algae*.

* Corresponding Author: Alireza Rahimi
E-mail address: alireza_rahimi@ymail.com, ORCID: https://orcid.org/0000-0001-5431-8717

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Abbreviation	Full Form
NADES	Natural Deep Eutectic-based Solvents
UAE	Ultrasound-Assisted Extraction
DPPH	2,2-diphenyl-1-picrylhydrazyl
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
RSM	Response Surface Methodology
ANOVA	Analysis of Variance
HBD	Hydrogen Bond Donor
HBA	Hydrogen Bond Acceptor
FRAP	Ferric Reducing Antioxidant Power
ORAC	Oxygen Radical Absorbance Capacity
HPLC	High Performance Liquid Chromatography

1. Introduction

Seaweeds, one of the largest biomass producers of marine environment and there are about 10,000 seaweed species globally [30]. Seaweeds are reported to be rich repository of bioactive compounds such as sulphated polysaccharides, carotenoids, phytosterols, phycobiliproteins, phloroglucinol, fatty acids, flavonoids, proteins, tocopherols and phytosterols, a relatively higher amount of unsaturated fatty acids, potentially promoting protection against neurodegenerative disorders with potential application in food, cosmetic and pharmacological industries, being notably distinct from terrestrial plants [68] that are classified into three classes: Brown (Phaeophycean), Red (Rhodophyceae) and green (Chlorophyceae) based on their pigmentation [8]. Due to these biocompounds, *Algae* present a wide variety of bioactive such as antioxidant, anti-viral, anti-fungal, antibacterial, antiproliferative, anti-inflammatory, neuroprotective, adipogenesis and antidiabetic [32]. At the same time, high fibers content due to the presence of non-digestible polysaccharides in their cell wall, such as fucoidan and laminarin, have been shown to have anti-viral effects, anti-tumor, anti-inflammatory, hypoglycemic and also antioxidant activity [18]. Historically, seaweed compounds have been used as gelling, thickening, and emulsifying agents in various foodstuffs and are now considered a source of health-promoting compounds that depend on growing conditions such as water temperature, salinity, nutrients and light [38].

As a result of the wide variability of classes of target biocompounds, and their physical and chemical properties, it is essential to find the most efficient methods for extracting these bioactive compounds subsequently, to optimize the extraction protocol. Furthermore, yields and extract composition are influenced by the extraction conditions. It has been reported that factors such as the applied solvent, the solid:liquid ratio, the extraction time and temperature impact these parameters [59]. To this end, a search for the optimization of this technique culminated in developing several more efficient, fast, and environmentally friendly techniques for extracting bioactive compounds from natural sources [59].

Since 2014, NADES have been developing as a special group of DES. These emerging systems are made by the combination of natural molecules, allowing further improvement in the impact on the environmental and toxicity risk compared with other deep eutectic solvents. NADES have been studied since 2011 and a rising number of papers highlighted their applications in several fields of chemistry. Among the reported studies, since 2014, the focus on NADES' applications, physiochemical properties, and combination with innovative extraction techniques was particularly detailed for their applications in foods analysis [10]. NADES as an extracting solvent can increase the solubility and bioactivity of these compounds compared to that of conventional solvents. The use of NADES can also increase the stability and shelf life of compounds in extracts. In addition, the bioavailability of compounds dissolved in NADES was also reported to increase [12,55, 91].

NADES are a new derivative of DES. NADES are considered "natural" because the constituent components of the eutectic mixture are primary metabolite groups (which are naturally used by the plant itself for survival), such as sugars, organic acids and bases, and amino acids. NADES can be grouped into: (1) ionic liquid NADES (made from an acid and a base), (2) neutral NADES (made from sugars only or sugars and polyalcohol), (3) neutral NADES with acids (made from sugar/polyalcohol and organic acids), (4) neutral NADES with bases (made from sugar/polyalcohol and organic bases), and (5) amino acids-based NADES (made from amino acids and organic acids/sugars). The NADES are considered renewable and biodegradable, biocompatible, stable with high solubilizing power and depending on their composition, that exhibit a wide range of polarity and described for the extraction of phenolics, anthocyanins, flavonoids, phlorotannin, ascorbic acid and fucoxanthin [31,52,56,82] and are useful for the extraction of different active compounds from natural sources.

Generally, the subgroup of NADESs uses hydrogen bond donor (HBD) and acceptors (HBA) made by sugars, alcohols, organic acids, amino acids, or amines naturally occurring in plant metabolism or eucaryotic cellular systems. NADESs have been indicated as possible solvents present in living cells, thus explaining the presence of compounds at much higher concentrations than what is soluble in aqueous solutions. Furthermore, NADESs reduce physicochemical constraints of metabolite transport and cellular processes through the formation of liquid microenvironments. The mostly used HBA are chlorine chloride (ChCl) and betaine for their low cost, non-toxicity, and biodegradability but also proline, glycine, alanine, histidine, lidocaine, acetylcholine chloride, and nicotinic acid are currently used as eco-friendly and biocompatible molecules. Natural

carboxylic acids (gallic acid, benzoic acid), hydroxycinnamic acid derivatives (coumaric acid and caffeic acid) different sugars (xylitol, glucose, fructose), organic acids (oxalic acid, malic acid), and fatty acids (stearic acid, oleic and linoleic acid) are the used as natural HBD combined with HBA. [6,24,44,63].

The advantage of these solvents is related to chemical properties, such as low melting points, low volatility, nonflammability, low vapor pressure, polarity, chemical and thermal stability, and miscibility solubility. Additionally, a convenient atom economy and a low environmental impact are related to their low costs and high yields of production. Their assembly through intermolecular hydrogen bond interactions does not involve chemical reaction hence there is no production of secondary compounds reducing the need for further purification steps, and no waste is usually produced. The current review is focused on the topic of NADES combined with different extraction techniques to design processes that reduce energy consumption, ensure the use of safe alternative solvents, and optimize high extraction efficiency of target molecules occurring in several fields of foods analysis [26,11,72,86,89,92].

2. Ultrasound-Assisted Extraction (UAE) Fundamentals

Ultrasound is an intensification technique which is widely used for the extraction of bioactive compounds from natural products with applications in industries such as food and pharmaceuticals [4]. The intensification process is based on the acoustic cavitation phenomena, which consists of the formation of stable or transient gas bubbles by the compression and expansion cycles caused by the passage of ultrasonic waves through the liquid and the subsequent rupture, which causes the release of bioactive compounds, and this rupture depends on the extraction conditions [16,77]. Increased solubilization of the mixture formed in the extraction is also one of the effects of cavitation since the ultrasonic waves allow movement of the liquid from shear forces and turbulence caused by bubbles imploding [79,53]. In general, UAE can be explored in more sustainable processes due to the high efficiency associated with its use, allowing lower consumption of solvents and energy. UAE also provides faster extractions, with high reproducibility, rapid return on investment, simplification of manipulation and processing, and higher purity of the final product when compared to conventional extraction methods [60,14]. UAE can be used in many types of matrices, such as fruits, teas, seeds, vegetables, or flowers, which are sources of many bioactive compounds of different classes [69,75]. However, the conditions and the combinations of the variables of the process must be cautiously defined according to the type of sample and compound to be extracted, including factors such as the frequency used and usually between 20 kHz and 100 MHz, ultrasound power, time, temperature, quantity and preparation of the sample, and selection, volume, and concentration of solvent [65,22].

3. Parameters Influencing UAE Processes

The extraction of natural products is a complex process, where each variable, individually or combined with others, can affect the results. It is of the utmost importance to evaluate each component of the process and their interactions, such as solvent, sample, power, frequency, and intensity, temperature, time, and make of the equipment. UAE has been demonstrated to enhance the extraction efficiency of phenolics, utilizing acoustic cavitation to destruct the cell-wall structure. Moreover, the use of NADES instead of traditional organic solvents in this process was reported to further increase the extraction efficiency of phenolics from bioactive compounds. However, research focusing on phenolic extraction from bioactive using NADES-UAE remains scarce. [62,84]. In this study, NADES and UAE were investigated for capacity to extract bioactive compounds from *algae* and this is the first study investigating the antioxidant properties of *algae* using green solvents and also the first report evaluating capacity of NADES-based plant extracts. The phenolic in the extracts were identified and quantified by high performance liquid chromatography-high resolution mass spectrometry (HPLC-HRMS), the antioxidant activity evaluated using different methods. In the available literature, we have not found information regarding the ability of common NADES for several trace elements co-extraction from *algae*. The aim of this study was to investigate the ability of common NADES for several trace elements co-extraction from *algae* by different extraction techniques.

4. Materials and Methods

4.1. Pre-Extraction Sample Preparation

Sample preparation is the first step that usually comprises washing, drying, and grinding of the sample to optimize the extraction efficiency and the safety of the final extract. Fresh seaweed samples, usually collected from coastal areas or beaches, are usually subject to cleaning steps before extraction procedures. First, seaweeds are washed with filtered seawater and then with distilled water to remove residual sediments, impurities, and salts [66]. After the cleaning step, algal materials are then dried using different techniques: surface-dried with a paper towel, chopped into small fragments and freeze dried at a temperature of −110 ◦C to −80 ◦C for 72 h [81,70] oven-dried (70 ◦C, 72 h) [1], dehydrated in a food dehydrator (60 ◦C), 41 ◦C or even sun (40 ◦C) or shade (38 ◦C) dried for 24 h [73]. Posteriorly, for extraction purposes, seaweed samples are finely ground using a mechanical blender to ensure uniformly distributed mass and a higher surface-to-volume ratio [38,70]. The dried and ground material is then subjected to different extraction techniques to prepare extracts rich in bioactive compounds. The most common extraction method is solid-liquid extraction (SLE), but more environmentally friendly advanced extraction techniques, namely, NADES, UAE are also used. The following chapters will discuss a literature review on applying these techniques.

4.2. Extraction Solvent

The choice of solvent is the primary variable in any extraction method. The extraction solvent should be chosen based mainly on the solubility and intensity of the interactions with the matrix [57]. Consideration should also be given to the extraction parameters and their suitability for the solvent, the intermediate and final products to be used, and how the solvent can react with the target compounds under extraction conditions. An important factor is the biochemical and physicochemical properties of the solvents because they define the nature of the medium in addition to interacting with the treated material and extracted compounds [57]. A solvent with low vapor pressure, at the adjusted temperature, facilitates cavitation increasing the effects of ultrasound in the process. The extraction performance can also be affected by the solvent pH by altering the ionic strength, which affects the solubility of the compounds and their interactions with the sample matrix. On pH above 7,0, lower extraction yields were recorded. For the ultrasound-assisted extraction of bioactive compounds from the Citrus reticulata bark, slightly acidic electrolyzed water (pH 6.20) produced the best results for the extraction of total phenolic compounds [57].

4.3. Sampling Procedure

Depending on the target compounds, the sample may be fresh or dry (*algae*), and the structure, moisture, plasticity, and composition of the material will entail the recovery of compounds from the sample matrix. Thus, the preparation of the sample matrix before extraction is of paramount importance, especially because some compounds are sensitive to the processes of preparation, such as drying, homogenization, and sifting. Such high ratios may be adequate when considering the production of a concentrated extract. Still, when the objective of the extraction is sample preparation for quantitative analysis of flavonoids, a higher solvent amount (1:50, 1:100, or even higher) may be required to ensure that target compounds were removed entirely from the sample matrix. The ranges of the solid / solvent ratio are reported in the literature and it is often not indicated whether it is on a dry or wet basis. It is understood that it usually refers to fresh and not dry material. In the case of material that has been dried, it is necessary to hydrate the matrix to allow the solubilization of the compounds of interest and consequently it may be necessary to increase the ratio. Higher efficiency can be achieved using sequential extraction processes as in each extraction, as the fresh solvent will be available and will improve solubility. Still, additional steps will be required between extractions, such as centrifugation or filtration. Particle size is also a factor that influences the efficiency of the UAE. It should be evaluated according to the matrix, and as a function of the compounds to be extracted.

4.4. Ultrasound Power, Frequency, and Intensity

Ultrasound power directly affects the cavitation and shear forces in the extraction medium. As ultrasound power increases, so does the cavitation and its mechanical effects as well as mass transfer of compounds from the sample matrix to the solvent. However, excessive power can negatively affect the extraction process due to the degradation of target compounds, reducing yields. Another critical parameter in UAE is frequency. The frequency used for the extraction of bioactive compounds from natural products usually ranges between 20 and 120 kHz. Still, higher antioxidant capacity was observed in the extracts obtained with higher frequency (80 kHz), indicating that frequency can affect the composition of the extract. It is also essential to consider ultrasound intensity, which is the energy emitted per second per area of the emitting surface, being directly connected with the amplitude of the transducer and the sound wave.

4.5. Temperature and Extraction Time

The temperature of the medium should be closely related to the properties of the solvent since the temperature increase causes a decrease of the viscosity and surface tension of the solvent but increases the vapor pressure. Increased vapor pressure decreases the effectiveness of the cavitation process and leads to lower extraction efficiency. The vapor pressure of the liquid influences the cavitation process, and lower vapor pressure solvents are more advisable in UAE extractions because they induce a more significant collapse between cavitation bubbles.

4.6. NADES Preparation

The preparation of NADES was based on the heating and stirring method [9]. The mixtures, with a known amount of distilled water to facilitate dissolution, were heated at 50-80 ◦C (this time was adjusted to generate a homogenous transparent liquid) in a constant temperature heating magnetic stirrer.

The different types of mixtures prepared and used in the extraction experiments, their abbreviated designations, the molar ratios of their components and visual appearance are shown in Table 1.

4.7. Extraction Procedure

The plant material was extracted in 0.5 mL Erlenmeyer flasks at 50 ◦C using two distinct techniques-maceration (M) and UAE and different NADES combinations (Table 1) with a plant/solvent proportion of 0.5:10 (w/v) with the aim to compare extraction efficiency of NADES and conventional solvents, water, methanol (MeOH) were also tested as extractant solvents. For maceration, the extraction was performed in a SW22 Shaking Water Bath (Julabo, Seelbach, Germany) at 100 rpm for 50 min. Regarding UAE, an Elmasonic S 100 H (220-240 V, 550 W) ultrasound bath (Elma Hans Schmidbauer GmbH and Co. KG, Singen, Germany) with 8 L of water was used at a frequency of 37 kHz (in sweep-function) at different extraction periods (15,30 and 50 min). Since flask

positioning in the ultrasound bath has been shown to affect the extraction efficiency, during extraction procedure all Erlenmeyer flasks were kept in the same position and the water was kept above the level of the solvent in the flasks. All extracts were filtered through Whatman n. ◦1 filter paper (Whatman Int.Ltd., Maidstone, England) and the filtrates were stored at -20 ◦C until use.

Table 1. Composition of Natural Deep Eutectic Solvents (NADES) used in this study and detail concerning the synthesis thereof.

Abbreviation	Component 1 (HBA)	Component 2 (HBD)	Molar Ratio	Appearance
Gly:U	Glycerol	Urea	1:1	Transparent colorless liquid
CC:U	Choline Chloride	Urea	1:2	Transparent colorless liquid
CC:X	Choline Chloride	Xylitol	2:1	Transparent colorless liquid
CC:MA	Choline Chloride	Malic Acid	1:1	Transparent colorless liquid
CC:LA	Choline Chloride	Lactic Acid	1:2	Transparent colorless liquid
Pro:LA	Proline	Lactic Acid	1:1	Transparent colorless liquid

4.8. Determination of Phenolic Compounds from the Extracts

4.8.1. Total Phenolics Contents (TPC) by Folin-Ciocalteu (F-C) Method

The total phenolic contents were determined by a spectrophotometric method which used Folin-Ciocalteu (F-C) reagent as described [3]. F-C reagent 10% (v/v) (100 µL) was mixed with each extract diluted in phosphate buffer (75 mM, pH 7.0) (100 µL) and Na2CO3 (700 mM) (700 µL). After an incubation period of 2 h, at room temperature in the dark, the absorbance of the reaction mixture was measured at 475 nm. Gallic acid was used as standard and the results were expressed as milligrams of gallic acid equivalents per gram of dry weight (mgGAE/gdw), determined using a gallic acid standard curve (0.004–0.5 mM).

4.8.2. Phenolic Profile Analysis by HPLC-HRMS

The diluted plant extracts (1:4) were analyzed using a Dionex Ultimate 3000 HPLC system comprising of a HPLC pump and an autosampler operating at 4 ◦C (Thermo Scientific, San Jose, CA, USA). The injection volume was 5 µL, and the reverse phase separations were carried out using a 150 × 4.6 mm i.d. 5 µm 100 A C18 Kinetex column (Phenomenex, UK) maintained at 40 ◦C and eluted at a flow rate of 1.0 mL/min. The chromatographic conditions were carried out following those used with slight modifications [34]. The solvents used as the mobile phase were water (A) and acetonitrile (B), both with 0.1% formic acid. The gradient flow was as follows: 0-5 min—5-9% B; 5-15 min—9% B; 15-22 min—9-11% B; 22-38 min—11-18% B; 38-43 min—18-23% B; 43-44 min—23-90% B and 44-45 min—80-90% B. The column eluate was split, and 0.5 mL/min directed to an Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, CA, USA) fitted with a heated electrospray ionization probe (HESI) operating in negative ionization mode, scanning the ions in the m/z range from 100 to 1000. Full scans were recorded with a resolution of 50,000 and with a fully automatic gain control (AGC) target of 1,000,000 charges, using 2 microscans. The analyses were also based on in-source collision-induced dissociation scans at 25 eV. The capillary temperature was 320 ◦C, the heater temperature was 150 ◦C, the sheath gas and the auxiliary gas flow rate were 25 and 5 units, respectively, and the spray voltage was 4.00 kV. Data acquisition and processing were carried out using Xcalibur software (Thermo Fisher Scientific, San José, CA, USA). The Exactive Orbitrap was externally calibrated weekly using ready-to-use calibration mixtures (Pierce ESI Negative Ion Calibration Solution and Pierce LQT ESI Positive Ion Calibration Solution, both available from Thermo Fisher Scientific, San José, CA, USA). A quality control (QC) sample was applied to assess and ensure that the analytical process was performed appropriately. The QC sample, composed of identical aliquots of a representative pool of the samples (plant extracts), was injected regularly throughout the run. This QC sample represented both the sample matrix and metabolite composition of the samples and was used to monitor drifts and to determine the variance of a metabolite feature (below 20%).

4.9. Antioxidant Capacity

DPPH Free Radical Scavenging Assay Antioxidant activity was measured using the DPPH radical assay, as described by Soler-Rivas et al. [30] with slight modifications. DPPH methodology consists of the scavenger of the free radical DPPH• by the action of an antioxidant. Thirty microliters of plant extract were added to 300 µL of 90 µM DPPH methanolic solution. The mixture was diluted with 500 µL of methanol 80% and after an incubation period of 30 min, the absorbance was read at 475 nm. Trolox (0.025–0.3 mM) was used as standard and the results were expressed as milligrams of Trolox equivalents per gram of dry weight (mgTE/gdw).

4.10. Statistical Analysis

All tests were carried out in triplicates and data represent mean ± standard error for the total number of experimental results. Data were analyzed by one-way ANOVA, and Duncan's new multiple range test ($p < 0.05$), and correlations were calculated using Pearson's test. Statistical analyses were carried out using IBM SPSS Statistics for Windows, Version 26.0. Armonk, NY: IBM Corp. Data were auto-scaled and a principal component analysis (PCA) was performed using the statistical

software SOLO v. 8.6 (Eigenvector Research Inc., Manson, WA, USA).

5. Results and Discussion

5.1. Optimization of Extraction Conditions

Overall, UAE 50 was the most adequate to extract phenolic compounds from *Algae* and was further used to evaluate the effect of extraction solvent on phenolic profile and bioactivity of the extracts. In order to compare the efficiency of UAE to maceration, the extractions were performed with control of temperature and using the same solvents (conventional and NADES) for 50 min, since some authors reached the maximum recoveries of phenolics with this extraction period. The desirable extraction temperature ranges from 25 ◦C to about 50 ◦C. In this study 50 ◦C was used as extraction temperature. The TPC of the different extracts, determined by F-C method, were compared to assess the best extraction conditions. UAE was more efficient (TPC from 50.00 ± 0.70 mgGAEgdw) for all solvents, excepting proline:lactic acid (1:1) and choline chloride:urea (1:2) in which maceration extracted a larger amount of phenolics and Similar results were obtained by other authors [2,7,51,71].

5.2. NADES as Extraction Solvent

Many studies report that NADES can extract both hydrophilic and hydrophobic phytonutrient compounds, depending on the constituent components. Interestingly, hydrophilic NADES, such as the aqueous NADES family, can dissolve some lipophilic compounds, unlike conventional solvents such as water. Eutectic mixtures with organic acid components generally have the highest polarity, followed by those based on amino acids. On the other hand, a mixture of NADES with polyalcohol and sugar components has a lower polarity than that of the two previous components [20,21,43,44]. Due to the superiority of their "natural" constituent components, NADES support green technology and can be applied in the food, pharmaceutical, and cosmetic industries. These solvents were proven to efficiently produce extracts of plant metabolites with higher yields than those of conventional organic solvents. Because of their green nature, these solvents have increased in demand in a shorter time compared to that of conventional organic solvents. Moreover, the high stabilization and solubilization of NADES can make them excellent candidate to replace conventional organic solvents. However, the main weakness of NADES are the viscosity and variations in the types of constituent components used to synthesize their mixtures. NADES were widely used for extraction from the matrix of natural substances (to obtain a bioactive compound target), as catalysts in enzymatic or chemical processes, and as carriers for insoluble (hydrophobic) compounds for pharmaceutical applications. As extracting solvents, NADES have two mechanisms of action, namely, (1) direct action (interacting with target compounds, usually through hydrogen bonding) and (2) indirect action (damaging the cell wall, releasing the target compound from the plant matrix). In this mechanism, NADES act as a pretreatment solvent [15,39,78].

5.3. Solvents Effect on Phenolic Compounds (F-C method and HPLC-HRMS)

Ten NADES were prepared by heating and stirring methods using four groups of HBAs—glycerol, glucose, choline chloride and proline—in combination with three groups of HBDs—three organic acids, Luteolin-7-O-glucoside and Luteolin, Malic Acid, Lactic Acid and ferulic Acid and urea (Table 1). The NADES components selected to be tested in this work were approved as safe by FDA, as can be attested by their CRFs (Code of Federal Regulations) [9,28].

5.4. Total and Individual Phenolic Contents

A total of phenolic compounds was identified and quantifiable amount (Table 2). Sulfonic Acid was detected in all the extracts but below the limit of detection. The identification of these compounds led to their distribution into five structurally related classes/groups, i.e., hydroxycinnamic and phenolic acids, flavones and flavanones (flavonoids), and a coumarin derivative (Table 2).

The most abundant compounds in all the extracts were the hydroxycinnamic acids, in agreement with the literature available for other species [5,19,87]. Although no significant differences were observed among the conventional solvents MeOH and EtOH 80 and the choline chloride (CC) and lactic acid (LA)-based NADES in the extraction of Lactic acid and Malic acid, the green solvents, namely CC:U and CC:LA, were more efficient to extract ferulic acid than MeOH. Similar results were obtained being CC:U the best solvent for extracting ferulic acid ($p < 0.05$) compared to other NADES and conventional solvents [85]. Overall, no significant differences were observed among the conventional solvents MeOH and water and NADES containing LA or CC in their formulation, for the Sulfonic Acid (Table 2), showing Pro:LA and CC:LA significant better results than MeOH. Our results are in agreement with other researches. NADES, including the CC as MA or LA as Pro:LA, showed a comparable potential to extract (total) phenolic acids as MeOH (Table 2). Regarding flavonoids, just overcome by water, the Pro:LA, CC:LA, Glu:LA and CC:X NADES and the convectional solvents MeOH were the second-best extractants. Moreover, as for phenolic acids, LA-based NADES showed a good efficiency to extract flavonoids, indicating that the polarity of these green solvents and their hydrogen bonding interactions with these compounds appears to be very important. Overall, HPLC data suggests that NADES, including CC or LA in their composition, with special emphasis for Pro:LA and CC:U proved to be equally efficient (or better in some cases) to extract phenolic compounds when compared to conventional solvents (Table 2). In this sense, the methodology proposed by means of NADES for the extraction of phenolics from *Algae* can be considered as a

greener alternative in comparison with organic solvents used until now for the same purpose such as n-hexane, dichloromethane, ethyl acetate and methanol and methanol/water (50:50 v/v) [45].

5.5. Antioxidant Activity

Algae extracts are multicomponent mixtures exceedingly complex and for this reason it is important to evaluate the antioxidant capacity by more than one assay. In this work it was evaluated by using four different chemical assays with two distinct mechanisms, one single electron transfer-based method–FRAP, one atom hydrogen transfer-based method–ORAC, and two mixed methods using hydrogen-atom transfer and single-electron transfer–DPPH and ABTS. In all the assays the conventional solvents MeOH and water displayed better results. In ORAC and FRAP, MeOH proved to be a better extractant than water ($p < 0.05$), whereas in DPPH, no significant differences were obtained between those solvents. Conversely, in ABTS, water showed higher antioxidant activity values than hydroalcoholic solution (60.00 ± 1.70 mgTE/gdw and 70.00 ± 0.90 mgTE/gdw).

5.6. Pearson's Correlation Between the Different Parameters (Phenolics, Antioxidant Capacities)

In this study, a strong correlation was observed between antioxidant results and total phenolic contents by F-C method ($p < 0.01$). The phenolic contents by HPLC were strongly correlated with antioxidant results observed for FRAP ($p < 0.01$) and moderately correlated for DPPH, ABTS and ORAC ($p < 0.05$) (Table 3). The high correlations found between F-C assay results and the antioxidant results obtained by spectrophotometric methods, in relation to the analysis performed by HPLC, can be explained because the F-C method presents some limitations (e.g., poor specificity). This assay can be influenced by any other substances that can be oxidized by the Folin reagent producing an overestimation of the results.

Table 2. Qualitative and quantitative (µg/g of extract, mean) analysis by high performance liquid chromatography-high resolution mass spectrometry (HPLC-HRMS) of phenolic profile from *Algae* extracts obtained by ultrasound-assisted extraction for 50 min.

Compound	Conventional Solvents		NADES					
	MeOH	Water	Gly:U	CC:U	CC:X	CC:MA	CC:LA	Pro:LA
Lactic Acid	<LOD	<LOD	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Malic Acid	<LOD	<LOD	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ferulic Acid	1846 ^{e}	3657 ^{ab}	2868 ^{cd}	3774 ^{a}	2557 ^{d}	2979 ^{c}	3417 ^{b}	3417 ^{b}
Sulfonic Acid	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Total Phenolic Acid	10646 ^{ab}	10646 ^{ab}	5649 ^{cd}	11473 ^{ab}	11473 ^{ab}	9307 ^{abc}	10934 ^{ab}	11967 ^{ab}
Luteolin-7-O-glucoside	58.11 ^{cde}	<LOD	<LOD	45.98 ^{de}	44.07 ^{e}	52.84 ^{cde}	91.15 ^{a}	83.54 ^{ab}
Luteolin	154.1 ^{c}	<LOD	<LOD	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Total Phenolic Compounds	11283 ^{a}	9623 ^{ab}	3940 ^{c}	11968 ^{a}	11094 ^{a}	9759 ^{ab}	11625 ^{a}	12709 ^{a}

Notes: n.d.: not detected; LOD: Limit Of Detection; LOQ: Limit Of Quantification. The results were analyzed using one-way ANOVA followed by Duncan's new multiple range test. Different letters (a-h) in each row and for each phenolic compound mean significant differences ($p< 0.05$) among extracts.

Table 3. Pearson's correlation coefficients between antioxidant activity measured by the different assays (DPPH, FRAP, ABTS, and ORAC) and total phenolic contents measured by F-C and HPLC

Individual Phenolic Compounds	Antioxidant Activity			
	DPPH	FRAP	ABTS	ORAC
Lactic Acid	0.799 **	0.561 **	0.688 **	0.605 **
Ferulic acid	0.846 **	0.708 **	0.846 **	0.717 **
Luteolin-7-O-glucoside	−0.142	0.432	−0.483 *	0.211
Total phenolic contents HPLC	0.429 *	0.731 **	0.463 *	0.463 *

DPPH: 2.2-diphenyl-1-picrylhydrazyl; FRAP: ferric reducing antioxidant power; ABTS: 2.2 -azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); ORAC: oxygen radical absorbance capacity; HPLC: high performance liquid chromatography. ** Correlation is significant ($p < 0.01$). * Correlation is significant ($p < 0.05$).

Lactic acid is an acid ester found in a variety of Algaes family that has shown several interesting biological activities, including antioxidant activity. Although it is possible to observe a strong correlation between TPC and antioxidant activity and besides, lactic acid is the most abundant compound in almost all extracts, in our study this compound did not show a strong correlation with antioxidant activity results, unlike ferulic acid, which revealed a correlation higher than 0.846 ($p < 0.01$) in all antioxidant tests (Table 3). Therefore, it is possible that ferulic acid may be one of the greatest contributors to the antioxidant potential of Algaes extracts observed in this work. [33,40,42,90]. Although Sulfonic acid and Luteolin-7-O-glucoside are present in low amounts in the extracts in comparison to the major compounds, there is a strong correlation ($p < 0.01$) between these compounds and most of the antioxidant results, suggesting that these compounds

might also contribute to the radical scavenging capacity displayed by the extracts.

5.7. Extraction Solvent

The characteristics of the solvent to be observed are, among others, polarity, pH, viscosity, surface tension, vapor pressure, melting point, boiling point, density, specific gravity, as well as the effect on purity, and activity of the extracted compound [50]. These factors should be thoroughly thought out, mainly because they decrease the cavitation threshold, disfavoring the removal of the compounds from the matrix [50]. The possible changes that can occur in the solvents during the extraction process can have significant effects on the stability of the flavonoids and the efficiency of the treatments [23]. In contrast, viscous solutions, such as oils, increases the amplitude of the waves, hindering the propagation of ultrasound, and mechanical effects on the sample caused by the cavitation [67,29]. Several studies have evaluated the optimum pH to extract flavonoids from plant matrices. A recent report [48] investigated the influence of the pH of the solvent on the recovery of Euonymus alatus flavonoids and suggested that recoveries increased in acidic pH (2.5–3.5) and decreased at higher pH. Another report, which in this case evaluated polyphenols, indicated that its extraction from pomegranate peel is affected by the solvent's pH, with the best results being observed in acidic medium [54]. Still, higher yields of flavonoids were reported with acid electrolyzed water pH 3.24 [74]. Another example of the influence of the pH was reported for the ultrasound-assisted extraction of polyphenols from Satsuma mandarin leaves, where a higher yield of total flavonoids was observed at pH 2 in water. The highest amounts of total phenolic compounds and total flavonoids were achieved in acidic media [17]. The reports available in the literature suggest that higher flavonoids yields are usually produced in acidic medium. For polyphenols, this trend can be explained by the fact that an acidic pH supports the cleavage of phenolics bound to proteins and carbohydrate polymers [37]. With a low pH value, phenols are protonated that take the hydrophobic nature to molecules that interact more strongly with the hydrophobic micellar surfactant and, therefore, readily penetrate the micelles [25]. At a higher pH, phenols are deprotonated, and their ionic characteristics increase, leading to a decrease in the solubility of hydrophobic phenolic compounds in micelles due to the higher activity of protons. Thus, the number of phenols extracted increases with the reduction of the pH [36,25]. Therefore, based on earlier findings, the pH in this investigation has been set at 2.

5.8. The Importance of Sample Preparation

In addition to preserving the matrix compounds, the sample preparation also ensures the extraction efficiency as it can eliminate interferences, increase the concentration of the analyte in the mixture and provide the optimum particle size [64]. The ratios between sample quantity and solvent, as well as particle size, are also factors that should be taken into consideration to maximize extraction yield because they influence the cavitation phenomena and final concentration of the extracts [79]. It has been suggested that a proportion between 1:5 and 1:10 sample/solvent (solid vegetal material) for ultrasonic bath extraction is suitable for the recovery of bioactive compounds from plants [80]. In general, small particles remain on the solvent surface and are not affected by cavitation bubbles, while large particles decrease the permeability or diffusion of solvent in the sample [41]. A study developed for Arecanut polyphenol extraction tested particle sizes between 841, 425, 250, and 180µm, and the highest recoveries were obtained with a particle size of 250µm [13]. Another study using UAE for orange peel flavone extraction tested particle sizes between 0.5, 1.0, 1.5, 2.0, and 2.5 cm^2, with the best yielding particle size being 2 cm^2.

5.9. Ultrasound Power, Frequency, and Intensity

To fully explore UAE as an intensification technique, it is required to adjust power considering sample moisture, the temperature of the medium, and solvent used [83]. There are some reports that frequency can modulate the removal of different compounds from the sample matrix [46]. In the case of phenolics from grapes lower frequency of 40 kHz provided higher yields than 120 kHz [35]. On another recent report, a higher yield of phenolics from pomegranate peels was also achieved with lower frequency (37 kHz). High frequencies do not allow the process of cavitation to happen fully, because they decrease the time of expansion of the bubbles, thus reducing the size and impact of these in the sample [50]. On the other hand, in low frequencies, the bubbles are in smaller quantities, but with larger diameters, which assists the physical effects generated in the sample, such as the transfer of masses between the sample and the solvent [27]. Thus, the higher the ultrasonic intensity, the greater the amplitude, and the better the extraction efficiency. Higher amplitudes are associated with the more significant collision between the bubbles originating from the cavitation and the sample. However, very large amplitudes can also lead to the rapid deterioration of the ultrasonic transducer, leading to liquid agitation and not to the cavitation phenomenon. Thus, attention should be paid to the amplitude, especially considering the solvent, where high amplitudes are suitable in more viscous solvents such as the oils [76,46].

5.10. Temperature and Extraction Time

Solvents with high vapor pressure and, consequently, high boiling temperatures, do not fully explore the potential of UAE due to reduced cavitation [29]. High temperature, however, also facilitates the increase of cavitation bubbles, increasing the contact area and diffusion between solid and solvent. Thus, for better effects of ultrasound associated with cavitation, medium to low temperatures are indicated (between 20 and 70◦C), depending on the sample, and especially for thermosensitive ones [60,61]. Maran et al. studied the effect of extraction temperature, among other parameters, on the recovery of anthocyanin, flavonoids, and total phenolics of Nephelium lappaceum bark extracts obtained through the UAE. It was observed that the yield was increased due to the increase in porosity of the material, more significant solvation, and mass transfer when the extraction temperature increased. On the other hand, the required extraction time of the process will depend on

several factors. The overall extraction kinetic curve can be divided into three stages: constant extraction rate (CER), where compound is more easily extracted from the sample matrix; falling extraction rate (FER), where compounds being extracted present some interactions with the matrix, hindering their removal; and diffusional controlled (DC), where compounds depend on diffusion to be removed [60]. When considering the production of extracts, the extraction time is usually determined by the conditions that are most favorable to the mass transfer from the matrix to the medium, generally between CER and FER part of the extraction curve. However, the exact extraction time will depend on several economic factors and manufacturing costs, but especially the raw material cost [60]. In contrast, for analytical purposes, it is necessary to ensure that target compounds where completely removed from the sample, which is usually done by overextending the extraction time. Applications for obtaining concentrated extracts of flavonoids, which usually exploit the first stages of the extraction process (CER and FER), can also explore the benefits of the use of ultrasound. There is a more significant potential of this technique in the preparation of samples for quantitative analysis since ultrasound has a substantial effect on diffusion and can accelerate the final phase of the diffusion-controlled extraction process (CD). There are other important aspects associated with extraction time, including the amount and type of solvent used, the amount and characteristics of the sample (protein content for example), temperature, flow rate (in dynamic extractions), ultrasound intensity and frequency, potential degradation of target compounds, among others, making this one of the most challenging techniques to be optimized. Usually, extraction time is determined experimentally on a case-by-case basis [57].

5.11. Applications of UAE to Flavonoids from Natural Products

During the last decade, several UAE methods have been developed, and a significant number of applications for the recovery of flavonoids can be found in the literature. Some of these applications are shown in Table 2. The use of ultrasound has grown in recent years. In most cases, the produced results indicate that it accelerates the extraction process and decreases the number of solvents used [88,58]. The most used solvents for the extraction of flavonoids are ethanol, mixtures with water at different proportions, and NADES, which are based on their ability to solubilize moderately polar flavonoids with a relatively low cost and environmental impact. Regarding ultrasound, the most used type of equipment is the ultrasonic bath operating at 40 kHz with fixed power. Specific conditions, such as temperature and extraction time, vary significantly due to the particularity of the composition of each raw material. Recent studies have shown that green extraction methods offer excellent alternatives to traditional methods. Many studies are still being carried out in this field, to improve these new green extraction techniques further, with the intention always to reduce the cost of extraction, the time consumed, the quality of the extract, the environmental safety, and health. Also, the combination of extraction methods usually presents advantages to overcome the limitations of a particular approach. Ironically, the massive volume of information available regarding the extraction of flavonoids from the most diverse types of samples makes it difficult to draw overall conclusions. However, the variability of the results reported in the literature for similar compounds is intrinsically related to the sample characteristics, which play a critical role in releasing them to the extraction solvent [57].

6. Conclusion

This study focused on the extraction of bioactive compounds, including total carotenoids, antioxidant activity, and polyphenolic contents, from algae using ultrasound-assisted extraction (UAE) and natural deep eutectic-based solvents (NADES). The aim was to optimize the extraction conditions and evaluate the efficiency of NADES as an alternative to conventional solvents. The results showed that UAE for 50 minutes was the most effective extraction condition, and NADES, particularly proline:lactic acid (1:1) and choline chloride:urea (1:2) extracts, exhibited high total phenolic contents and antioxidant activity. The use of NADES as extraction solvents offers several advantages, including their ability to extract both hydrophilic and hydrophobic compounds, their green nature, and their potential for use in various industries. The NADES used in this study, which consisted of combinations of organic acids, amino acids, polyalcohols, and sugars, demonstrated good extraction efficiency for phenolic compounds. In particular, CC:U and Pro:LA NADES were found to be equally or even more efficient than conventional solvents, such as methanol (MeOH) and water. Furthermore, the analysis of the phenolic profile of the extracts revealed the presence of various phenolic compounds, including hydroxycinnamic acids, flavones, flavanones, and a coumarin derivative. The NADES-based extracts showed comparable or better extraction efficiency for these compounds compared to conventional solvents. The HPLC-HRMS analysis provided valuable insights into the composition of the extracts. Moreover, a strong correlation was observed between antioxidant results and total phenolic contents, as determined by the Folin-Ciocalteu (F-C) method ($p < 0.01$). The phenolic contents analyzed by HPLC were strongly correlated with antioxidant results obtained through spectrophotometric methods such as FRAP ($p < 0.01$) and moderately correlated for DPPH, ABTS, and ORAC ($p < 0.05$) (Table 3). It is important to note that the F-C method has certain limitations, such as poor specificity, which can lead to overestimation of the results as it can be influenced by other substances that can be oxidized by the Folin reagent. Furthermore, although sulfonic acid and luteolin-7-O-glucoside were present in relatively low amounts in the extracts compared to the major compounds, there was a strong correlation ($p < 0.01$) between these compounds and most of the antioxidant results, indicating their potential contribution to the radical scavenging capacity exhibited by the extracts.

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