PRESSURIZED LIQUID EXTRACTION AND ULTRASOUND-ASSISTED EXTRACTION OF FUNCTIONAL INGREDIENTS FROM C*HLORELLA VULGARIS*. CHEMICAL CHARACTERIZATION USING HPLC-DAD AND GC-MS.

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ABSTRACT

Functional foods have been incorporated to the human nutrition in the last few years for their proven benefits to the health, since they contain substances capable to prevent diseases. Most of these substances have a natural origin and are found in plants and/or algaes. Among them, the unicellular green algae *Chlorella vulgaris* contains many bioactive substances with medical properties such as antitumor, hepato-protective, antioxidant, and antibacterial, etc.

The objective of the present work was to study the ability of two different extraction techniques, pressurized liquid extraction (PLE) and ultrasound-assisted extraction (UAE), to obtain functional ingredients from *Chlorella vulgaris* and to evaluate their effect in the final chemical composition of the extracts. PLE is based on the extraction at temperature and pressure high enough to maintain the solvent in the liquid state during the whole extraction procedure. As a result, this extraction technique provides shorter extraction times, is automated and usually does not require toxic organic solvents compared to other traditional extraction processes; furthermore, PLE involves extracting the sample in an oxygen and light-free environment. Ultrasound-assisted extraction consists in ultrasound transmission into the alga material through the phenomenon of cavitation produced in the solvent by the ultrasonic wave. Ultrasound also exerts a mechanical effect, allowing greater penetration of solvent into the alga.

Several PLE conditions have been used; different combinations solvent-temperatures (water, ethanol, acetone at 50ºC, 100ºC, 150ºC and 200ºC) have been tested to obtain bioactive ingredients from *C. vulgaris.* For UAE, several solvents have been also tested (acetone, ethanol and hexane) with and amplitude wave of 20 %. Yields obtained by PLE were considerably higher than yields of UAE.

INTRODUCTION

Pressurized liquid extraction (PLE) is a newer technique which has been successfully applied to extract a variety of organic compounds from complex solid samples.

Traditional solvent techniques use large quantities of toxic organic solvents, are labour-intense, need long extraction times, posses low selectivity, and/or low extraction yields and can expose the extracts to excessive heat, light, and oxygen. Opposite to that, PLE uses less solvent in a shorter period of time, is automated, and involves retaining the sample in an oxygen and light-free environmental conditions. Such techniques, like supercritical fluid extraction (SFE), are frequently used to obtain functional compounds from natural sources, however, PLE has not been widely applied as a routine tool in natural product extraction but recent studies have demonstrated the advantage of PLE for the extraction of natural compounds from different matrices [**1**].

PLE provides higher selectivity, shorter extraction times and frequently does not require large amounts of toxic organic solvents; furthermore, water can be used as medium polarity solvent when working at high temperatures and moderate pressures, that is, at subcritical conditions. A drawback of PLE is its requirement of especial instrumentation in order to get relative high

pressures together with high temperatures. Moreover, there are not data available on solubility of natural compounds in the solvent at the pressures and temperatures employed in PLE.

Ultrasound-assisted extraction (UAE) has been proven to significantly decreased extraction time and increase extraction yields in many vegetables materials **[2].** The application of ultrasound in the food industry can be divided into two distinct categories of low-intensity high frequency (f >100 kHz) and high-intensity low frequency (20 kHz $\leq f \leq$ 100 kHz) ultrasound. Low-intensity ultrasound does not alter the physical or chemical properties of the material through which the ultrasonic wave propagates. High-intensity shock wave generates intense pressures, shear and temperature gradient due to the bubble of cavitation inducing the majority of ultrasonic effects within a material, which can produce physical, chemical and mechanical effects. The effect of high-intensity ultrasound on process and products which cause physical disruption or promote certain chemical reaction (e.g. oxidation) depends on many variables including reaction medium characteristics, ultrasonic parameters, ultrasonic generator performance, size and geometry of treatment tank.

Chlorella spp. is a genus of single-celled green algae, belonging to the phytum Chlorophyta. *Chlorella spp.* contains the green photosynthetic pigments chlorophyll a and b in its chloroplast. The unicellular algae *Chlorella vulgaris* contains many bioactive substances with medical properties. Experimental studies have demonstrated the medical properties of *Chlorella spp*. antitumor effect, hepato-protective properties, antioxidant properties, and antibacterial effects, etc **[3]**.

Chlorella contains many dietary antioxidants such as lutein, α-carotene, β-carotene, ascorbic acid and α-tocopherol. These bioactive compounds have the capacity to scavenge the free radicals **[3]**.

MATERIALS AND METHODS

Samples

Microalgae samples (*Chlorella vulgaris*) consisted of air-dried microalgae, from Las Palmas de Gran Canarias University (Canarias, Spain), stored under dry and dark conditions.

Chemicals

Acetone, hexane and methanol, HPLC grade, provided from Lab Scan (Dublin, Ireland), ethanol, was purchased from VWR BDH Prolabo (Madrid, Spain), and chloroform was obtained from Merck (Darmstadt, Germany). Methanolic base, provided from Supelco (Bellefante, USA), and Sodium sulphate was purchased from Fluka Chemie AG (Buchs, Switzerland). Ammonium acetate was purchased from Panreac (Barcelona, Spain). Carotenoid standards isolated from phytoplankton used in LC-DAD (β-cryptoxanthin, canthaxanthin, echinenone, fucoxanthin, lutein, neoxanthin, violaxanthin and zeaxanthin) and chlorophylls (a y b) were purchased from DHI Water and Environment (Hoersholm, Denmark) and β-carotene was obtained from Sigma-Aldrich (Madrid, Spain). The water used was Milli-Q Water (Millipore, Billerica, MA, USA).

Mixture from n-undecane to n-octacosane used in GC-MS, was from Aldrich (Sigma-Aldrich Chemie, Steinhein, Germany).

Pressurized liquid extraction (PLE). PLEs of *C. vulgaris* were performed using an accelerated solvent extractor (ASE 200, Dionex Corp., Sunnyvale, CA) equipped with a solvent controller. Three different solvents (i.e., acetone, ethanol, and water) were used to obtain extracts with different compositions. Extractions were performed at four different extraction temperatures (50, 100, 150, and 200 ºC), extraction time was 20 min. An extraction cell heat-up was carried out for a given time prior to any extraction, the warming-up time changed depending on the extraction temperature (i.e., 5 min when the extraction temperature was 50 and 100 ºC, 7 min if the extraction temperature was 150 ºC, and 9 min if the extraction temperature was 200 ºC). All

extractions were done using 11 mL extraction cells, containing 1.5 g of sample in ethanol extracts, 1 g in water extracts and 0.8 g in acetone extracts. When water was used for the extraction, the extraction cell was filled with sand between the sample (6.0 and 2.0 g of sand at the bottom and top, respectively) to prevent the clogging of the system.

The extraction procedure was as follows (Fig.1): (i) sample was loaded into the cell; (ii) cell was filled with solvent to a pressure of 1500 psi; (iii) initial heat-up time was applied; (iv) a static extraction with all system valves closed was performed; (v) the cell was rinsed (with 60 % cell volume using extraction solvent); (vi) solvent was purged from the cell with N2 gas; and (vii) depressurization took place. Between extractions, a rinse of the complete system was made to avoid carryover. For solvent evaporation, a Rotavapor R-210 (from Büchi Labortechnik AG, Flawil, Switzerland) was used for the extracts obtained using organic solvents. For water extracts, a freze-dryer (Labconco Corporation, Missouri, USA) was employed.

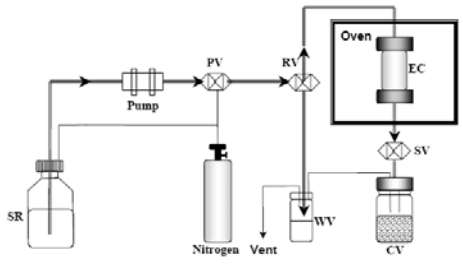

Figure 1. Schematic diagram of the PLE apparatus. SR, solvent reservoir; PV, purge valve; RV, Pressure relief valve; EC, extraction cell; SV, Static valve; CV, collector vial; WV, waste vial.

Ultrasound-assisted extraction (UAE). Extraction of *Chlorella vulgaris* were performed using an ultrasound-assisted extractor (BRANSON Digital sonifier). Three different solvents (acetone, hexane, and ethanol) were used to achieve extracts with different composition from a natural matrix. The sample (0.5 g) was placed into a falcon tube, and this tube was set into ice bath. 15 ml of solvent were added to each falcon tube and the samples were sonicated, at a controlled temperature (< 25 ºC), for times periods between 25-30 min with and amplitude wave of 20 % (Table 1). After sonication, the samples were removed from the ice bath, centrifuged and the supernatant removed. The samples were evaporated with a Rotavapor R-210 (from Büchi Labortechnik AG, Flawil, Switzerland).

Table 1. UAE conditions (solvent, extraction temperature, amplitude wave and extraction time)

Solvent	V (mL.)	T (ºC)	A (%)
Ethanol (t: 25 min)	15	25	20
Acetone (t: 30 min)	15	25	20
Hexane (t: 25 min)	15	25	20

V: solvent volume, T: temperature, A: amplitude wave, t : extraction time

HPLC analysis. Dry extracts were dissolved before injection into the HPLC. The ethanol extracts were dissolved in ethanol to a concentration 10 mg/ml, hexane and acetone extracts were dissolved in acetone, and water extracts were dissolved in ethanol.

The pigment composition of the extracts was analyzed by HPLC using a Hewlett-Packard HP 1090 Liquid Chromatograph equipped with a DAD. The separation was carried out in a Novapack C18 column 150 mm x 3.9 mm, 4 μm particle sizes from Waters. The mobile phase was a mixture of solvent A (methanol:ammonium acetate 0.1 N; 7:3) and solvent B (methanol) at 0.9 mL/min according to a step gradient, lasting 35 min, starting from 25% B, changing to 50% in one minute, rising up to 100% B at minute 10, then the mobile phase composition was kept constant until the end of the analysis. Injection volume was 20 μL. The identification of the peaks was performed, when possible, using standards. When no standards were available, tentative identification was based on UV-vis spectral characteristics and comparison with data appearing in the literature.

GC analysis.

Gas chromarography-mass spectometry (GC-MS) analysis of the volatile fraction. The volatile fraction of the PLE and UAE extracts was analyzed with an Agilent-6890N GC system with a programmed split/splitless injector coupled to an Agilent-5973N quadrupole mass spectrometer (Agilent, Palo Alto, Calif.). The system was controlled by means of Agilent MSD Chemstation software. The column used in the GC was a 30 m by 0.25-mm internal diameter fused silica capillary column coated with a 0.25-μm layer of SE-54 (HP-5MS, Agilent). The injector was heated to 250 ºC in split mode (ratio 1:20). The volume of sample injected was 1 μl. The extracts were injected at a concentration of 10 mg/ml. Helium was the carrier gas (7 psi). The oven temperature was programmed as follows: 40 ºC as the initial temperature (maintained for 2 min) to 150 ºC in 24 min at 5 ºC/min, and from 150 ºC to a final temperature of 300 ºC at 15 ºC/min.

Compounds were tentatively identified by MS in SCAN mode, using a mass interval ranging from 35 to 450. Their spectra were compared with those in an MS library (Wiley Registry of Mass Spectral Data), with data found in the literature and with standards when available. Additionally, to identify compounds more precisely, their linear retention indices (RIs) were used when possible. Mixture fom *n*-undecane to *n*-octacosane (Aldrich, Sigma-Aldrich Chemie, Steinheim, Germany) dissolved in *n*-hexane were employed for linear RI calculations.

Gas chromarography-mass spectometry (GC-MS) analysis of the fatty acid fraction

Derivatization procedure. To prepare methyl esters of free and esterified fatty acids, samples were mixed with chloroform and methanol, (2:1, v/v) at a known concentration (2 mg/ml) and methylated by adittion of 1 ml of a solution methanolic base (0.5 N). This mixture was allowed to react at 65 ºC for 45 min. After addition of 500 μl of water, the resulting mixture was extracted with two volumes of 1 ml of *n*-hexane, and the final extract was then dried with sodium sulfate.

Analysis was carried out by GC-MS using an Agilent-6890N GC system with a programmed split/splitless injector coupled to an Agilent-5973N quadrupole mass spectrometer (Agilent, Palo Alto, Calif.). The system was controlled by means of Agilent MSD Chemstation software. A Carbowax column (Polyethyleneglycol) from Quadrex (Woodbridge, Conn.) was used (30 m by 0.25-mm internal diameter and 0.5- μm film thickness). The injection was carried out at 250 ºC in split mode (ratio of 1:2). The volume of sample injected was 3 μl. Helium was the carrier gas (7 psi) The oven was programmed so that the initial temperature was 100 ºC and reached 220 ºC at a rate of 20 ºC/min, maintaining this temperature for 25 min before reaching 270 ºC at 15 ºC/min. The final temperature was held for 10 min.

RESULTS

PLE and UAE extracts from *Chlorella vulgaris.*

PLE. The three solvents were selected in order to cover a wide range of dielectric constants (see Table 2) allowing the evaluation of the influence of the solvent polarity on the extraction of functional compounds from the microalga. Additionally, different extraction temperatures were also tested, because this is the primary variable involved in the PLE process, as has been previously shown with different microalgae **[4]**

UAE. The *Chlorella* samples were extracted in sonicator for different periods of time and with different solvents (i.e., acetone, ethanol, hexane), in order to determine the contact time required to achieve the maximum yield. The results suggest that the ultrasonic extraction period for achieving maximum yield is about 25 min in ethanol and hexane extracts, and 30 min in acetone extract.

The extraction conditions employed as well as the extraction yields obtained for the different solvents are shown in Table 2. With PLE, the yields were higher than UAE. As can be observed, the extraction yield (percentage dry weight of starting material) was maximum when extractions

were carried out with ethanol. On the other hand, extracts obtained using hexane provided lower extraction yields, whereas intermediate and similar values were observed when using water and acetone. From Table 2 it can also be deduced that the extraction yield increased with the extraction temperature, and that this trend was independent of the solvent used. This can be explained by an increase of mass transfer from the sample to the pressurized solvent with temperature. From the data it could be suggested that *Chlorella* contained mainly medium-low-polarity compounds (i.e., higher yields were obtained with medium-low-polarity solvents).

Table 2. PLE and UAE conditions (solvent, extraction temperature and extraction time) of *Chlorella vulgaris* extractions and yield produced (% dry weigh).

Solvent (dielectric constant)	Extraction Temp (ºC)	Extraction time	Yield
Acetone (PLE) (20.7)	50	20	4.27
	100	20	33.29
Ethanol (PLE) (24.3)	50	20	8.63
	100	20	32.57
Water (PLE) (78.5)	50	20	6.80
	100	20	39.31
Acetone (UAE) (20.7)	25	30	3.06
Ethanol (UAE) (24.3)	25	25	5.05
Hexane (UAE) (1.9)	25	25	0.70

Extracts characterization

HPLC-DAD analysis

PLE and UAE extracts were analyzed by HPLC-DAD to obtain a chromatographic profile of the compounds that are present in the whole extracts (Fig. 3 and 4). The carotenoid content and profile was very similar in both extraction systems. The most representative carotenoid in *C. vulgaris* was lutein, and the most representative chlorophyll was chlorophyll a. Lutein is not only an important natural food colorant and additive, but is also effective in delaying chronic disease, stimulating immune response and hampering the development of cataracts and atherosclerosis **[5].** β-Carotene was found in minor quantities. This carotenoid in converted into retinol in the body and also possesses provitamin A activity. Due to its antioxidant activity, β-carotene may play important roles in preventing degenerative diseases.

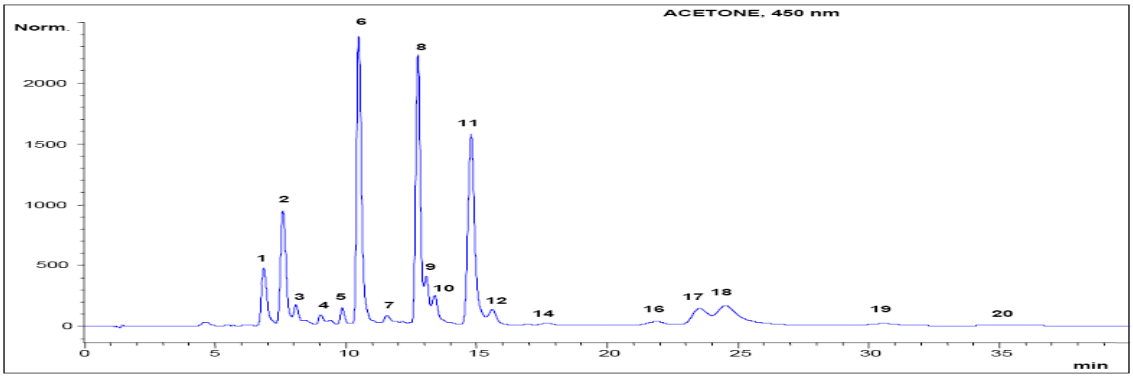

Figure 3. UAE-HPLC chromatograms (450 nm) of an acetone extract. Peak assignment: 1, fucoxanthin; 2, neoxanthin; 3, violaxanthin; 6, lutein; 8, chlorophyll b; 9, chlorophyll b´; 11, chlorophyll a; 12, chlorophyll a´; 17, β-carotene isomers; 18, β-carotene; 4, 5, 7, 10, 13, 14, 15, 20, carotenoid not identified; and 16, 19, chlorophyll not identified.

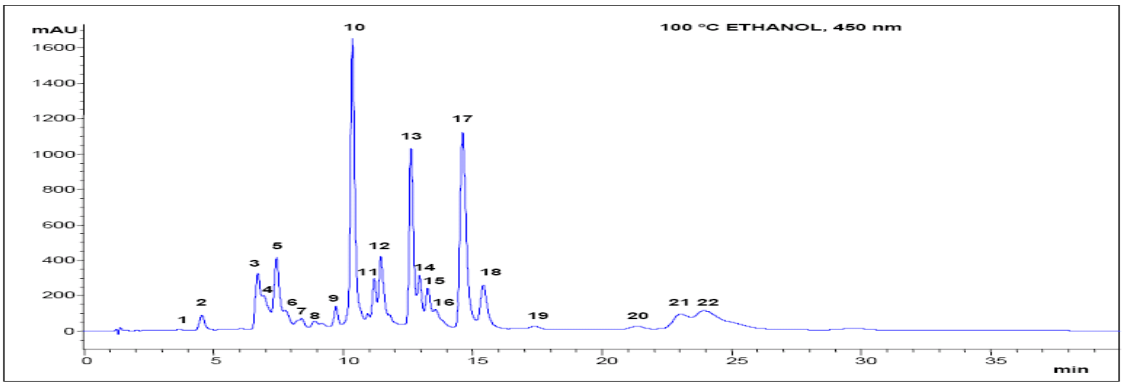

Figure 4. PLE-HPLC chromatograms (450 nm) of an ethanol extract obtained at 100 ºC. Peak assignment: 3, fucoxanthin; 5, neoxanthin; 10, lutein; 13, chlorophyll b; 14, chlorophyll b´; 16, chlorophyll a derivative; 17, chlorophyll a; 18, chlorophyll a´;21, α-carotene; 22, β-carotene; 4, 8, 9, 11, 12, 15, 19, carotenoid not identified; and 1, 2, 6, 7, 20, chlorophyll not identified.

GC-MS analysis of the volatile fraction

By GC-MS analysis, several volatile compounds with different functional activity, such as, antimicrobial (phytol, neophytadiene, hexadecanoic acid) antioxidant (vitamin E), positive effect against cardiovascular diseases (vitamin E, phytosterols) and anticarcinogenic activity (vitamin E) were detected. Composition was quite similar using the different solvents and different extraction systems. Total areas were higher with ethanol.

GC-MS analysis of the fatty acid fraction

The consumption of certain fatty acids can modulate different pathological situations in individual (i.e., positive effect against cardiovascular diseases, anticarcinogenic, anti-inflammatory and antimicrobial activity …) **[6]**

Hexadecanoic acid (palmitic acid); 7,10,13-Hexadecatrienoic acid; 9,12-Octadecadienoic acid (linoleic acid), 9-Octadecanoic acid (oleic acid), and 9,12,15-Octadecatrienoic acid (α-linolenic acid) were determined to be the main compounds, representing approximately 67% of the total chromatogram area. Other compound that also was present in high percentage was phytol (2-hexadiene-1-ol,3,7,11,15-tetramethyl). The fatty acids determined in the mentioned PLE extracts were the same that in the UAE extracts and their percentage was very similar. In PLE and UAE extracts, total areas were higher with acetone (200 ºC working with PLE).

CONCLUSION

This work showed the ability of an environmentally friendly extraction technique such as Pressurized Liquid Extraction (PLE) and a traditional extraction technique such as Ultrasound-Assisted Extraction (UAE), to obtain functional compounds from natural sources such as the microalga *Chlorella vulgaris.* Several compounds that could be responsible of these functional activities were characterized by HPLC-DAD and GC-MS. The chemical characterization of the extracts proved the complex relationships between composition and functional activities. The results showed the presence of important families of compounds such as carotenoids (lutein, fucoxanthin, neoxanthin, β-carotene …), chlorophylls, sterols, phytols, and fatty acids (among others) with demonstrated biological activity.

Ethanol was considered the more appropriate solvent to extract compounds with biological activities from *Chlorella* not only for the higher yields and chemical composition obtained but also for the GRAS consideration. PLE extracts had higher yield than UAE extracts. In this work, it has been demonstrated that the use of the combined protocol PLE-HPLC-DAD-GC-MS allows in a fast and simple way the extraction, purification, and preliminary characterization of several functional compounds from *Chlorella vulgaris* microalga

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