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Effect of ultrasounds and high pressure homogenization on the extraction of antioxidant polyphenols from lettuce waste

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ABSTRACT

The possibility to exploit ultrasound (US) and high pressure homogenization (HPH) to obtain ethanolic antioxidant extracts from lettuce waste was studied. The application of US (400 W, 24 kHz) for 120 s led to polyphenol extraction yield (81 μg/mL) and antioxidant activity (101 μg TE/mL) significantly higher than those obtained by traditional solid-liquid extraction at 50 °C for 15 min. Despite the intense cell rupture effect, the application of HPH pre-treatments resulted in 25% lower phenolic yields as compared to US solely, possibly due to the 40% activation of polyphenoloxidase (PPO) upon HPH treatment.

Industrial relevance: The waste generated by fresh-cut processing of lettuce poses environmental and economic issues to companies, leading to the need for alternative strategies for its management. US can be successfully exploited as time-saving extraction procedure for obtaining antioxidant extracts from lettuce waste.

1. Introduction

Lettuce can be commercialized as whole lettuce heads or as fresh-cut product. In this regard, lettuce is the most important fresh-cut vegetable, representing 50% of the entire fresh-cut market in Europe and US (Cook, 2015; Rabobank International, 2010), with an estimated production of about 100 million kg a year in Italy (Casati & Baldi, 2012). The production of fresh-cut lettuce from whole-heads requires the preliminary removal of external leaves and core, leading to waste amounts up to 50% of the initial lettuce head weight (Plazzotta, Manzocco, & Nicoli, 2017). Lettuce waste is currently composted to obtain fertilizers or anaerobically digested to produce biogas. However, these strategies pose different issues, including the risk of pathogen development during composting and the low biogas production potential due to the poor carbohydrate content of lettuce waste (Zheng, Phoungthong, Lü, Shao, & He, 2013). For these reasons, this waste must be co-composted or co-digested in centralized plants, with high management costs.

Based on these considerations, alternative valorisation strategies are required to exploit lettuce waste. Previous studies have demonstrated that lettuce might be an interesting and cheap source of health-promoting antioxidant polyphenols (Llorach, Martínez-Sánchez, Tomás-Barberán, Gil, & Ferreres, 2008; Llorach, Tomás-Barberán, & Ferreres, 2004). To this regard, discarded external leaves of lettuce have been reported to show higher phenol content than the edible portions, due to the intense secondary metabolism activated by external stresses (Viacava, Gonzalez-Aguilar, & Roura, 2014). Fresh-cut processing further promotes polyphenol production in lettuce tissue, as a response to cell injury such as leaf cutting or shredding (Cantos, Espín, & Tomás-Barberán, 2001).

Literature attention has been mainly focused on the extraction of lettuce polyphenols by traditional solid-liquid extraction with organic solvents at concentrations of about 50–80% (DuPont, Mondin, Williamson, & Price, 2000; Llorach et al., 2008; Viacava et al., 2014; Viacava, Roura, & Agüero, 2015). In these works, methanol and acetone came up as suitable solvents to reach good phenolic extraction yields. Procedures reported in the literature usually require the homogenization of lettuce with the extractive solution, followed by the maintenance at temperature usually in the range 4–50 °C for increasing time up to 48 h. Besides being time consuming, these procedures do not allow obtaining food-grade polyphenol extracts (Gil-Chávez et al., 2013). To solve these issues, along with the use of non-toxic and GRAS (generally recognized as safe) extraction solvents such as ethanol, alternative "green" extraction technologies might be exploited. Based on the definition reported by Chemat, Rombaut, Meullemiestre, et al. (2017), Green Food Processing "*is based on the discovery and design of technical processes which allow a reduction of energy and water consumption as well*

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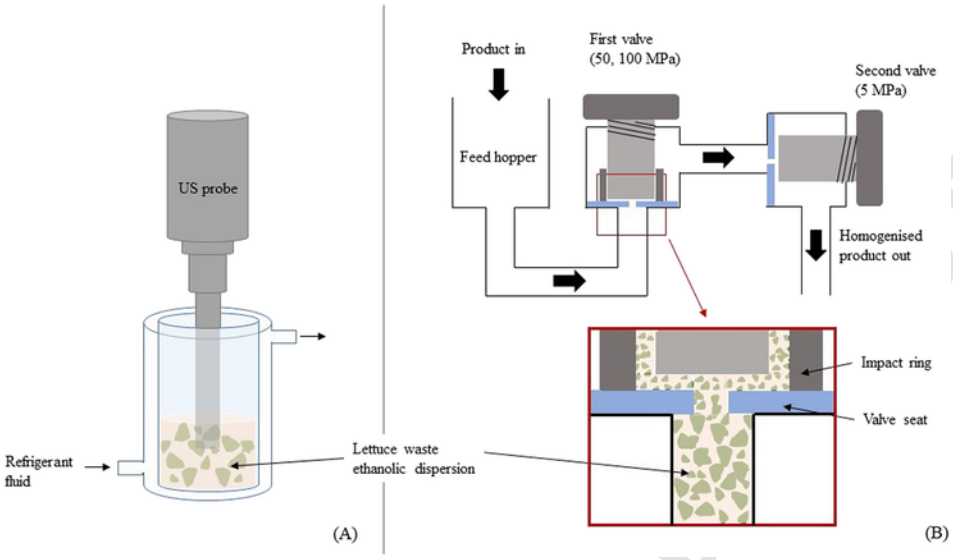


Fig. 1. Schematic diagram of the ultrasonication device (A) and of the continuous lab-scale high-pressure homogenizer supplied with two homogenization valves (B) used for the treatment of lettuce waste ethanolic dispersions.

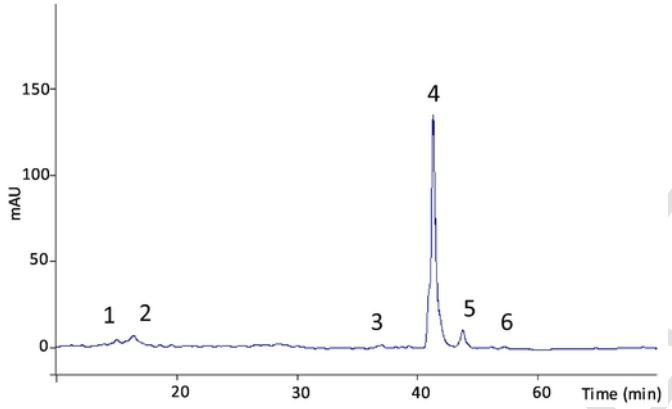


Fig. 2. HPLC profile of lettuce waste extract obtained by traditional solid-liquid extraction with 750 mL/L ethanol aqueous solution for 60 min. Peak identification: (1) 3-O-caffeoylquinic acid; (2) caffeoyltartaric acid; (3) isochlorogenic acid; (4) chicoric acid; (5) luteolin-7-O-glucuronide; (6) quercetin 3-O-glucuronide. AU = arbitrary units.

as recycle of by-products while guaranteeing safety and quality of the final product". In this regard, ultrasound assisted extraction has been attracting large attention in the last years, due to its promising effects, including enhanced yields, shorter times, simplicity of use, reduced operating and maintenance costs (Chemat, Rombaut, Meullemiestre, et al., 2017).

High energy ultrasounds exploit low frequency sound waves (usually 24–50 kHz) to enhance extraction efficacy due to cavitation phenomena. The latter refer to the formation and subsequent collapse of cavitation bubbles produced during the propagation of sound waves into the extractive solutions. Cavitation bubble implosion generates microjets and solvent flows, which favour cell rupture and mass transfer, leading to an enhanced release of target bioactive compounds (Dranca & Oroian, 2016; Espada-Bellido et al., 2017). US have been demonstrated to be an efficacious technology for obtaining phenolic antioxidant extracts from different vegetable by-products, including apple pomace, orange peel and spent coffee grounds (Al-Dhabi, Ponmurugan, & Maran Jeganathan, 2017; Khan, Abert-Vian, Fabiano-Tixier, Dangles, & Chemat, 2010; Pingret, Fabiano-Tixier, Le Bourvellec, Renard, & Chemat, 2012). In the case of lettuce, US have been mainly indirectly delivered to sample through a sonication bath (Parente, Lima, Moreira, Barros, & Guido, 2013; Viacava et al., 2014). However, times longer than 30 min have been reported for this indirect US procedure, due to energy attenuation through bath medium and sample container walls. The application of direct US procedures, by using a probe, is expected to reduce extraction time, being US energy fully delivered to the sample (Chemat, Rombaut, Sicaire, et al., 2017).

Although representing an additional processing step, possibly enhancing global processing time and energy, preliminary cell disruption has been reported to largely improve extraction efficacy (Meullemiestre, Breil, Abert-vian, & Chemat, 2016). In this regard, high pressure homogenization (HPH) has been reported as pre-treatment for the enhancement of the extraction of different target compounds such as proteins and lipids (Cho et al., 2012; Dong et al., 2011; Safi et al., 2014; Samarasinghe, Fernando, Lacey, & Faulkner, 2012). During HPH, the fluid is forced to pass through a narrow gap in the homogenization valve, where it is submitted to a rapid acceleration. The resulting pressure drop simultaneously generates intense mechanical forces, elongation stresses, cavitation and turbulence in the medium, leading to cellular disruption (Balasubramaniam, Martínez-Monteagudo, & Gupta, 2015; Bot et al., 2017). Despite these evidences, no indications are available about HPH pre-treatments in combination with US for the extraction of polyphenols.

Based on these considerations, the aim of this work was to evaluate the potentialities of US and HPH in the extraction of polyphenols from fresh-cut lettuce waste, to be used as dietary supplements or food antioxidants. To this purpose, lettuce waste was submitted to extraction with ethanolic food-grade solutions by: (i) traditional solid-liquid extraction at 50 °C for increasing time up to 60 min; (ii) US treatments for increasing time up to 120 s; (iii) HPH at increasing pressure up to 100 MPa as pre-treatment to sonication. Extraction yields were assessed by analysing total phenols, phenol profile and antioxidant capacity. Results were discussed in relation to the effect of the treatments on cell structure and polyphenoloxidase activity.

2. Materials and methods

2.1. Crude extract preparation

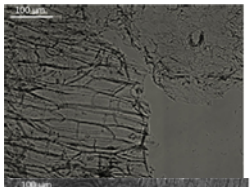
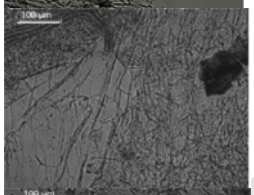
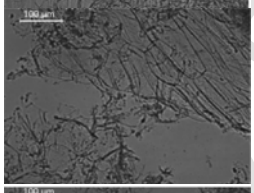
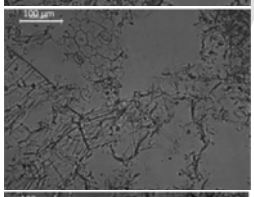
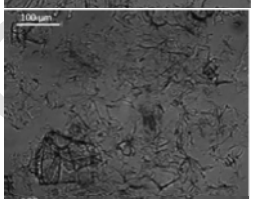
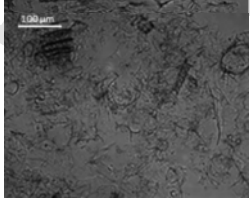
A 10-kg batch of iceberg lettuce (*Lactuca sativa* var. *capitata*) was purchased at the local market and stored overnight at 4 °C. Outer leaves were manually removed from lettuce heads, simulating operations that are industrially carried out during fresh-cut lettuce processing. Lettuce waste amounted to 299 ± 15 g/kg of the entire processed

Table 1
Individual and total (TPC) polyphenol content, and antioxidant activity of lettuce waste extracts obtained by the application of traditional, ultrasound (US) and high pressure homogenization (HPH) treatments.

Extraction	Time (min)	HPH (MPa)	US (s)	3-O-caffeoylquinic acid (μg/mL)	Caffeoyltartaric acid (μg/mL)	Isochlorogenic acid (μg/mL)	Chicoric acid (μg/mL)	Luteolin 7-O-glucuronide (μg/mL)	Quercetin-3-O-glucuronide (μg/mL)	TPC (μg/mL)	Antioxidant activity (μg TE/mL)
Traditional	2	–	–	2.67 ± 0.01 d	7.05 ± 0.37 f	1.39 ± 0.19 ab	35.26 ± 0.10 de	2.37 ± 0.01 d	0.68 ± 0.01 bc	49.42 ± 0.69 cd	74.60 ± 1.30 bc
	15 (control)	–	–	3.45 ± 0.01 f	8.06 ± 0.07 g	1.38 ± 0.34 ab	38.09 ± 0.17 fg	3.00 ± 0.09 e	0.70 ± 0.02 bc	54.68 ± 0.70 f	88.42 ± 1.77 ef
	30	–	–	3.46 ± 0.03 f	7.84 ± 0.04 g	1.43 ± 0.03 ab	34.42 ± 0.29 de	4.24 ± 0.01 g	0.60 ± 0.03 ab	51.09 ± 0.45 de	87.70 ± 1.90 ef
	45	–	–	3.84 ± 0.05 g	6.47 ± 0.05 de	2.25 ± 0.1 b	36.13 ± 0.49 ef	1.57 ± 0.02 c	0.94 ± 0.06 ef	51.20 ± 0.80 de	77.11 ± 2.73 cd
	60	–	–	5.10 ± 0.17 i	6.33 ± 0.24 de	1.19 ± 0.01 a	34.62 ± 0.19 de	0.47 ± 0.01 a	0.84 ± 0.05 cd	48.55 ± 0.28 cd	64.11 ± 2.27 a
US	–	–	20	3.04 ± 0.04 e	6.65 ± 0.02 ef	2.12 ± 0.14 b	39.20 ± 0.49 g	0.51 ± 0.01 a	0.59 ± 0.01 a	52.11 ± 0.70 ef	92.71 ± 3.26 fg
	–	–	70	8.72 ± 0.0 j	9.61 ± 0.01 i	3.80 ± 0.24 c	44.52 ± 0.13 h	1.69 ± 0.01 c	0.88 ± 0.01 cd	69.72 ± 0.49 h	99.00 ± 1.90 gh
	–	–	120	9.11 ± 0.17 k	12.37 ± 0.01 j	4.93 ± 0.43 d	49.02 ± 0.01 i	4.05 ± 0.08 g	1.14 ± 0.19 fg	80.62 ± 0.89 i	101.31 ± 2.45 h
HPH	–	50	–	2.54 ± 0.01 d	6.10 ± 0.14 d	1.92 ± 0.34 ab	25.32 ± 0.17 ab	0.47 ± 0.09 a	0.95 ± 0.02 ef	37.30 ± 0.77 a	81.88 ± 0.27 de
	–	100	–	2.12 ± 0.02 c	5.97 ± 0.06 cd	1.69 ± 0.11 ab	29.09 ± 0.29 c	1.03 ± 0.01 b	1.30 ± 0.04 g	41.40 ± 0.31 b	88.18 ± 2.01 ef
HPH-US	–	50	20	2.14 ± 0.0.02 c	7.12 ± 0.01 f	1.90 ± 0.05 ab	24.45 ± 0.30 a	3.45 ± 0.15 f	0.69 ± 0.01 bc	39.84 ± 0.52 ab	71.69 ± 0.82 ab
	–	50	70	1.57 ± 0.03 a	7.17 ± 0.03 f	1.99 ± 0.24 ab	25.13 ± 0.81 ab	5.70 ± 0.03 h	0.70 ± 0.01 bc	42.27 ± 1.07 b	70.67 ± 1.09 ab
	–	50	120	1.65 ± 0.06 a	5.39 ± 0.13 b	2.09 ± 0.43 ab	25.05 ± 1.38 ab	8.41 ± 0.16 i	0.63 ± 0.05 ab	43.22 ± 1.84 b	89.00 ± 2.46 ef
	–	100	20	1.73 ± 0.01 ab	4.81 ± 0.20 a	4.37 ± 0.05 cd	29.38 ± 0.48 c	5.87 ± 0.02 h	1.58 ± 0.09 h	47.74 ± 0.11 c	84.96 ± 1.79 df
	–	100	70	2.00 ± 0.10 bc	5.47 ± 0.02 bc	4.41 ± 0.18 cd	26.91 ± 0.71 b	8.72 ± 0.01 j	1.29 ± 0.02 g	48.80 ± 1.04 cd	90.54 ± 4.08 fg
	–	100	120	4.23 ± 0.19 h	8.61 ± 0.01 h	3.57 ± 0.03 c	32.92 ± 1.20 d	9.55 ± 0.04 k	1.29 ± 0.03 g	60.77 ± 1.50 g	92.65 ± 2.67 fg

a–k: in the same column, mean values indicated by different letters are significantly different ($p < 0.05$).

Table 2
Microscopic images and polyphnoloxidase (PPO) activity of control lettuce waste extracts and of extracts obtained by the application of ultrasounds (US) for 20, 70 and 120 s, and high pressure homogenization (HPH) at 50 and 100 MPa. The energy density (E_V) of HPH and US treatments is also reported.

Extraction	HPH (MPa)	US (s)	E_V (J/mL)	Microscopic image	PPO activity (U/mg)
Traditional (15 min, control)	–	–	–		3.49 ± 0.03 a
US	–	20	23.4		3.43 ± 0.05 a
	–	70	81.9		3.45 ± 0.07 a
	–	120	140.4		3.37 ± 0.05 a
HPH	50	–	50.0		4.82 ± 0.07 b
	100	–	100.0		4.63 ± 0.26 b

a–b: in the same column, mean values indicated by different letters are significantly different ($p < 0.05$).

Table 3
Investment cost, energy consumption and carbon emissions of traditional solid-liquid extraction at industrial scale and of ultrasound-assisted extraction at both laboratory and industrial scale.

Extraction technique	Scale	Operative conditions	Maximum capacity (L)	Cost (€)	Energy consumption (kWh/kg of extracted polyphenols)	Carbon emission (kg CO_2/kg of extracted polyphenols)
Traditional solid-liquid	Industrial (medium)	15 min, 50 °C	6.0	6000	2286.0	964.7
Ultrasound assisted	Laboratory	2 min, 50 °C	0.1	3000	2067.3	872.4
	Industrial (small)	2 min, 50 °C	2.0	10,000	248.1	104.7
	Industrial (medium)	2 min, 50 °C	6.0	25,000	206.7	87.2

lettuce. Lettuce waste was washed with flowing water (18 ± 1 °C) and sanitized 20 min in a chlorinated bath containing 200 mg/L of NaClO with a 0.1 g/mL lettuce/water ratio. Waste was then rinsed with flowing water and centrifuged in a manual kitchen centrifuge (mod. ACX01, Moulinex, France) for 1 min (Plazzotta, Calligaris, & Manzocco, 2018). To reduce sample variability without affecting phenol composition, lettuce waste was submitted to freeze-drying. In particular, waste was frozen in single layers at −80 °C for 24 h and freeze dried for 72 h

at 4053 Pa by using the pilot plant model Mini Fast 1700 (Edwards Alto Vuoto, Milan, Italy). Waste was then finely ground using a ball mill (MM2, Retsch, Hann, Germania) for 5 min. Ground lettuce waste was then dispersed in hydro-alcoholic solutions at 500 and 750 mL/L ethanol concentration (Carlo Erba, Milan, Italy) and with a lettuce/solvent ratio of 0.02 g/mL. Lettuce waste dispersions were then immediately submitted to extraction protocols, filtered using 0.45 μm membrane filters (GVS, Meckenheim, Germany) and stored at 4 °C until use.

2.2. Traditional solid-liquid extraction

Lettuce waste dispersions were blended using a high-speed homogenizer (Polytron, PT 3000, Cinematica, Littau, Swiss) at 4000 rpm for 30 s. Then, 5 identical 100 mL samples, each in separate glass vessels (250 mL capacity, 110 mm height, 60 mm internal diameter) were maintained at 50 °C under gentle mixing. At defined time interval (2, 15, 30, 45 and 60 min), one of the samples was randomly selected and analysed.

2.3. Ultrasound assisted extraction

An ultrasonic processor (Hielscher Ultrasonics GmbH, mod. UP400S, Teltow, Germany) with a titanium horn tip diameter of 22 mm was used. The instrument operated at constant ultrasound amplitude, power and frequency of 100 μm, 400 W and 24 kHz, respectively. As schematically shown in Fig. 1, aliquots of 100 mL of lettuce dispersion were introduced into 250 mL capacity (110 mm height, 60 mm internal diameter) glass vessels. The tip of the sonicator horn was placed in the centre of the solution, with an immersion depth in the fluid of 50 mm. The ultrasound treatments were performed for 20, 70 and 120 s. The temperature was controlled using a cryostatic cooling system set at 4 °C to dissipate the heat generated during the treatment, allowing sample temperature to be maintained at values lower than 50 °C.

2.4. High pressure homogenization

A continuous lab-scale high-pressure homogenizer (Panda Plus 2000, GEA Niro Soavi, Parma, Italy) supplied with two Re + type tungsten carbide homogenization valves, as schematically shown in Fig. 1, with a flow rate of 10 L/h, was used to treat 150 mL of lettuce dispersion. The first valve was the actual homogenization stage and was set at increasing pressure of 50 and 100 MPa. The second valve was set at the constant value of 5 MPa.

2.5. Temperature measurement

Temperature was measured with a copper-constantan thermocouple probe (Ellab, Hillerød, Denmark) connected to a portable data logger (mod. 502A1, Tersid, Milan, Italy).

2.6. Optical microscopy

Two droplets of each sample were placed on a glass slide, covered, and observed at room temperature using a Leica DM 2000 optical microscope (Leica Microsystems, Heerbrugg, Switzerland). The images were taken at 200× magnification using a Leica EC3 digital camera and elaborated with the Leica Suite Las EZ software (Leica Microsystems, Heerbrugg, Switzerland).

2.7. HPLC analysis

Extracts were analysed using a HPLC system equipped with a Prostar 230 pump (Varian, Walnut Creek, USA) and a Prostar 330 diode array detector (Varian, Walnut Creek, California, USA). To this aim, 20 μL extract was injected in a C18 column (Alltima, 5 μm, 250 × 4.6 mm, Grace, Lokeren, Belgium). The mobile phase was water with 50 mL/L formic acid (Fluka, St. Louis, Missouri, USA) (solvent A) and HPLC grade methanol (Chromasol ≥ 99.9%, Sigma-Aldrich St. Louis, Missouri, USA) (solvent B) at a flow rate of 1 mL/min. The linear gradient started with 10% B in A to reach 20% B at 25 min, 50% B at 40 min, 50% B at 45 min and 90% B at 60 min (Llorach et al., 2004). Chromatograms were recorded at 335 nm. Data elaboration was performed by Polyview program (v. 5.3). Phenolic compounds identification was based on their UV spectra and retention times (DuPont et al., 2000; Llorach et al., 2004; Mai & Glomb, 2013; Tomás-Barberán, Loaiza-Velarde, Bonfanti, & Saltveit, 1997). Chicoric acid was quantified (Lee & Scagel, 2013) using an external standard while other compounds were quantified as 3-O-caffeoylquinic acid by comparison with external standard (Sigma-Aldrich, St. Louis, Missouri, USA). To this aim, seven-point external standard calibration curves (concentration 0.4–10 μg/mL) were produced, whose linearity was acceptable (0.994 and 0.990 for 3-O-caffeoylquinic acid and chicoric acid, respectively).

The total content of polyphenols in the extracts was determined as the sum of the amount of the individually quantified compounds and expressed as μg per mL of extract.

2.8. Polyphenoloxidase activity (PPO)

The polyphenoloxidase (PPO) activity was assayed spectrophotometrically (Shimadzu UV-2501PC, UV–Vis recording spectrophotometer, Shimadzu Corporation, Kyoto, Japan) at 25 °C according to the methodology of Kahn (1985). The reaction was started by the addition of 500 mL of extract to 2 mL of 0.1 mol/L potassium phosphate buffer pH 7 and $1.5 \cdot 10^{-3}$ mol/L L-Dopa (Carlo Erba, Milan, Italy). The absorbance at 420 nm was monitored each minute for 10 min. The changes in absorbance per min were calculated by linear regression, applying the pseudo zero order kinetic model. The eventual final stationary phase was excluded from regression data. The slope of the very first linear part of the reaction curve was used to determine PPO specific activity. The latter was defined as the amount of enzyme that converted 1 μmol of substrate per min (U), expressed per mg of protein (U/mg). The latter was determined based on *Iceberg* lettuce protein content of official databases (USDA, 2018).

2.9. Antioxidant activity (DPPH• assay)

The chain-breaking activity was measured following the bleaching rate of a stable free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH•) in the presence of the sample (Manzocco, Mastrocola, & Nicoli, 1998). A volume of 150 μL of 6.1×10^{-5} mol/L DPPH• (Sigma-Aldrich, St. Louis, Missouri, USA) methanol solution was used. The reaction was started by the addition of 10 μL of lettuce waste extract. DPPH• bleaching was followed at 515 nm (Sunrise™, Tecan, Männedorf, Switzerland) at 25 °C for 10 min. DPPH• bleaching rate was proportional to sample concentration. Trolox (Sigma-Aldrich, St. Louis, Missouri, USA) was used as the standard for the calibration curve in the assay (5–150 μg/mL, $R^2 = 0.999$), and the antioxidant capacity was expressed as μg of Trolox equivalents (TE) per mL of extract.

2.10. Energy density

The energy density (E_v, J/mL) transferred from the ultrasound probe to the sample was determined calorimetrically by recording the temperature (T, K) increase during the homogenization process (Raso, Mañas, Pagán, & Sala, 1999). The following Eq. (1) was used:

$$E_V = \frac{mc_p\,(\partial t/\partial T)}{V} \times t \quad (1)$$

where m is the sample mass (g), c_p is the solvent specific heat (2.89 $Jg^{-1}\,°C^{-1}$), V is the sample volume (mL), $\delta T/\delta t$ (°C/s) is the heating rate during the treatment, and t (s) is treatment time.

The energy density transferred from the homogenization valve to the sample was determined as described by Stang, Schschmann, and Schubert (2001), according to Eq. (2):

$$E_V = \Delta P \quad (2)$$

where ΔP is the pressure difference operating at the nozzles (MPa).

2.11. Economic, environmental and scaling-up investigation

To get a first insight into the possible industrialization of the best-performing extraction procedure, a multi-objective approach was adopted (Simeoni, Nardin, & Ciotti, 2018). To this aim, a survey was conducted on companies producing industrial traditional extractors as well as US plants, the latter both at laboratory and industrial scale. This information was used to identify the possible investment cost (€), the energetic demand (kWh/kg of extracted polyphenols) and the environmental impact (kg CO_2/kg of extracted polyphenols) of the proposed procedures. In particular, the environmental impact was measured as carbon dioxide emission, calculated through the proper emission conversion factors of electricity (0.422) for the Italian electricity production system (Simeoni et al., 2018).

2.12. Statistical analysis

For each set of processing conditions, the experiments were performed in duplicate and each collected sample was analysed in triplicate. The mean values and standard deviations of experimental data were calculated. Statistical analysis was performed by using R v. 2.15.0 (The R foundation for Statistical Computing). Bartlett's test was used to check the homogeneity of variance, one-way ANOVA was carried out and Tukey test was used to determine statistically significant differences among means ($p < 0.05$).

3. Results and discussion

3.1. Traditional solid-liquid extraction

Lettuce waste was maintained in contact with hydro-alcoholic solutions at 500 and 750 mL/L ethanol concentration at 50 °C for up to 60 min. In agreement with literature data, these preliminary trials showed that the highest extraction yields were obtained at 750 mL/L ethanol concentration, which was thus selected for further experimentation (DuPont et al., 2000; Llorach et al., 2004; Mai & Glomb, 2013; Viacava et al., 2014, 2015). The chromatographic profile of 750 mL/L hydro-alcoholic extracts obtained by 60 min traditional solid-liquid extraction is reported in Fig. 2. The identified phenolic compounds were mainly represented by caffeoylquinic and caffeoyltartaric acid derivatives and, among them, the main derivative identified was dicaffeoyltartaric acid (chicoric acid) (peak 4), followed by caffeoyl tartaric acid (peak 2), in agreement with literature data (Llorach et al., 2008, 2004; Mai & Glomb, 2013). In addition, an isomer of chlorogenic acid (3-O-caffeoylquinic acid, peak 1) and isochlorogenic acid (peak 3) as well as flavonoid compounds (luteolin and quercetin derivatives, peak 5 and 6) were identified. These compounds have been previously reported in *Iceberg* lettuce (Llorach et al., 2008, 2004; Tomás-Barberán et al., 1997).

Independently on extraction time up to 60 min, all samples submitted to traditional solid-liquid extraction showed similar phenolic profiles (data not shown). However, extraction time differently affected the amount of the individual phenolic compounds (Table 1). 3-O-caffeoylquinic acid was progressively extracted up to 60 min while the concentration of caffeoyltartaric acid, chicoric acid and luteolin was maximized after 15–30 min extraction. Similarly, the concentration of isochlorogenic acid and quercetin increased up to 45 min extraction while a decrease in their concentration was observed by prolonging extraction time (Table 1). These results can be explained by the possible degradation of phenolic compounds with the increase in extraction time. This agrees with literature studies reporting a maximum increase in extraction efficacy in the first stage of solid-liquid extraction, followed by a decrease, due to the counterbalancing effect of bioactive extraction and degradation (Amendola, De Faveri, & Spigno, 2010; Silva, Rogez, & Larondelle, 2007). As a result, a maximum total phenolic amount of about 50 μg/mL (corresponding to 12.5 mg/100 g fresh weight) was obtained after 15 min of extraction, which was thus selected as control extraction procedure. Despite the inherent vegetable variability and the application of different extraction parameters and quantification methods, the obtained extraction yield is consistent with literature data relevant to traditional solid-liquid extraction of phenols from green-leaf lettuce. For example, Llorach et al. (2008) obtained a total identified phenolic content of 18.2 mg/100 g fresh weight upon extraction with 50% methanol of *Iceberg* lettuce; similarly, Llorach et al. (2004) obtained a total identified phenolic content of 24 and 14 mg/100 g fresh weight upon thermal extraction (100 °C) of *Iceberg* lettuce waste in water and methanol, respectively. By extracting butterhead lettuce in 70% ethanol solutions, Viacava et al. (2015) obtained a total phenolic content of 44 mg/100 g fresh weight, quantified with Folin-Ciocalteu method. Although this is one of the most used methods for determining TPC in vegetable matrices, it is not specific for phenolic compounds. Rather, it measures the ability of both phenolic and non-phenolic compounds in alkaline medium to reduce the phosphomolybdic/phosphotungstic acid reagent, possibly leading to an overestimation of TPC value (Singleton, Orthofer, & Lamuela-Raventos, 1999). The antioxidant activity of the extracts obtained by the application of traditional solid-liquid extraction was determined using DPPH• method. Consistently with literature data, a positive correlation ($R^2 = 0.81$) was found between the phenolic content and the antioxidant activity of the extracts. It is well known that the antioxidant activity of plant extracts containing polyphenol components is due to their capacity to donate hydrogen atoms or electrons and scavenge free radicals (Altemimi, Choudhary, Watson, & Lightfoot, 2015; Chen et al., 2018; Llorach et al., 2004; Vijayalaxmi, Jayalakshmi, & Sreeramulu, 2015). A maximum antioxidant activity of 88 μg TE/mL (corresponding to 17.7 μmol TE/g and 4.4 mg TE/g of lettuce dry weight) was exerted by the extract obtained upon 15 min extraction (Control). Such value is consistent with values commonly found in green-leaf lettuces. In this regard, extracts obtained from external leaves of butterhead lettuce by Viacava et al. (2014) resulted of about 12 μmol TE/g dry weight. Similarly, Llorach et al. (2004) found a DPPH• scavenging activity around 20 mg TE/g dry weight in *Iceberg* lettuce waste extracts obtained by thermal extraction up to 60 min.

3.2. Ultrasound assisted extraction

Data showing the effect of US treatments at increasing time (20, 70, 120 s) on phenolic concentration of lettuce waste extracts are reported in Table 1. The 20 s US treatment resulted in a total polyphenol amount not significantly different from that of the control extract obtained by traditional extraction carried out for 15 min. However, the application of US treatments for 70 and 120 s resulted in a 28 and 47% increase in phenolic extraction (Table 1). Low frequency US, usually in the range 20–50 kHz, have been widely reported as efficient strategy for phenol extraction from different vegetable waste materials (Khan et al., 2010; Pingret et al., 2012). Microscopic images of lettuce tissue submitted to traditional control extraction and to US treatments for increasing time are reported in Table 2. Compared to control sample,

which showed well-maintained cell structures, a progressive loss of cell integrity was observed with the increase in US treatment. A similar progressive destruction of cellular organization upon US application was also observed on other vegetable materials such as tomato, microalgal biomass and onion (Anese, Mirolo, Beraldo, & Lippe, 2013; Halim, Rupasinghe, Tull, & Webley, 2013; Rajewska & Mierzwa, 2017). Since the main accumulation sites of soluble phenols in lettuce are represented by vacuoles (Goupy, Varoquaux, Nicolas, & Macheix, 1990), it can be desumed that US promoted the permeabilization of these cellular structures, leading to phenol extraction. In this regard, recent studies conducted on rosemary leaves and eggplant peels proved that the effects of US are not limited to vegetable surface but involve several subsequent mechanisms, leading to the gradual physical damage of vegetable tissue and thus intra-cellular organelles (Ferarsa et al., 2018; Khadhraoui et al., 2018). Such chain of mechanisms promoted by US cavitation starts with the erosion of surface leaf structures (e.g. waxy cuticles, intact and broken trichomes, stomata) and proceeds with the deformation of remaining surface structures by US shear forces, generating micro-fractures. The latter increase in size and number (sonoporation), finally leading to tissue fragmentation. The resulting increase in surface area exposed to the extraction solvent favours its capillary penetration into inner structures, enhancing phenol extraction. Upon further US treatment, complete tissue destructuration is obtained (Khadhraoui et al., 2018). Based on microscopic analysis (Table 2), the application of US treatments up to 120 s was not able to cause complete lettuce tissue destructuration, since many intact cells were still present. It can be thus hypothesized that a further increase in US time would lead to more intense destructuration and thus to higher phenolic concentrations. However, literature data indicate that the increase in US treatment time is usually associated to an initial fast extraction phase and a subsequent decrease in extraction rate until reaching a *plateau* value, possibly followed by a the decrease of the target compound concentration (Chan, See, Yusoff, Ngoh, & Kow, 2017; Chan, Yusoff, & Ngoh, 2013; Pan, Qu, Ma, Atungulu, & McHugh, 2012). This has been attributed to phenol degradation during US, due to extreme pressure and temperature conditions reached in the hot-spots generated by cavitation bubble collapse as well as to the loss of metal by the sonication probe surface, which can induce radical formation (Meullemiestre et al., 2016). In our experimental conditions, a progressively increasing phenolic concentration of the extracts, despite the increase in treatment time, was observed. This can be attributed to the mild conditions applied in the present work (times shorter than 120 s and temperature values lower than 50 °C), possibly accounting for reduced phenol degradation phenomena. This hypothesis is further supported by the absence of changes in the phenolic profile (data not shown) as compared to that of the sample obtained by traditional extraction (Fig. 2).

It can be inferred that treatments more energetic than those here applied would be required to reach the extraction *plateau*. In this regard, Chan et al. (2017) reported energy density (E_V) (Eq. (1)) higher than 250 J/mL to be required for reaching the maximum extraction of Java tea bioactive compounds by using a 70% ethanolic solution. In our conditions, the E_V transferred to samples by 20, 70 and 120 s US treatments resulted lower than 150 J/mL (Table 2), suggesting that further increase in treatment energy could lead to higher extraction performances.

Consistently with polyphenolic extraction data, the antioxidant activity of samples obtained by US treatments progressively increased with sonication time (Table 1). Extracts obtained by the application of 20 s showed an antioxidant activity similar to that of the control sample ($p \geq 0.05$). Upon 70 and 120 s US treatment, extracts showed an increase in antioxidant activity of 12 and 15%, respectively. Also in this case, a strong positive correlation ($R^2 = 0.98$) was found between TPC and antioxidant activity of sonicated lettuce waste extracts.

3.3. Effect of high pressure homogenization pre-treatment

HPH has been widely reported to increase the extractability of plant bioactive compounds by disrupting vegetable tissues (Guan et al., 2016; Velázquez-Estrada, Hernández-Herrero, Rüfer, Guamis-López, & Roig-Sagués, 2013). For this reason, it has been suggested as a pre-treatment for increasing the yield of target compounds during a subsequent extraction process (Cho et al., 2012; Safi et al., 2014; Spiden et al., 2013). Table 2 reports the light microscope images of lettuce waste extracts obtained upon 50 and 100 MPa HPH treatment. The disruption efficacy of HPH increased with the applied pressure. Samples treated at 50 MPa still presented a number of intact cells, although the broken cell material was the most abundant. By contrast, no intact cells were observed in the samples submitted to 100 MPa, in which the broken cell material was quite uniformly distributed. The increase of HPH pressure results in an increase of energy density (E_V) transferred to the sample (Eq. (2), Table 2), due to the intensification of forces experienced by vegetable particles. In this regard, several mechanisms underlie HPH efficiency in cell breakage, favouring the release of target compounds. A pressure increase resulted in a higher pumping speed of the lettuce ethanolic dispersion and thus in a more intense collision of suspended particles with valve seat (Fig. 1). During the passage through the valve, sample particles underwent fluid dynamic stresses, initially leading to their deformation and, beyond a certain pressure value, to their breakage. The sudden pressure drop at the valve exit induced rapid sample acceleration and cavitation, leading to high kinetic energy, which is responsible for intensive collisions among particles and between particles and instrument walls (especially the so-defined impact ring, Fig. 1) (Coccaro, Ferrari, & Donsì, 2018). Since in this experimentation a two-stage homogenizer was used (Fig. 1), also the back pressure (5 MPa) applied to the second valve allowed increasing cavitation intensity and breaking aggregates, possibly formed by inter-particle collisions (Freudig, Tesch, & Schubert, 2003; Sharabi, Okun, & Shpigelman, 2018). Differently from US, thus, HPH-induced tissue disruption is not gradual, but involves concomitant events, so that energy density lower than that applied by US was required to obtain even higher tissue destructuration levels (Table 2).

Although tissue disruption induced by HPH resulted much more intense than that promoted by US (Table 2), HPH carried out as a pre-treatment to US extraction resulted in lower phenolic yields as compared to US solely (Table 1). For example, the 50 and 100 MPa HPH pre-treatments associated to 120 s resulted in a TPC value about 46 and 25% lower than that obtained by the application of 120 s US treatment alone. These results suggest that cellular disruption solely cannot account for a higher efficacy of US assisted extraction.

The inefficacy of HPH treatments can be possibly explained by the decompartmentalising effect of this treatment on the oxidative enzymes entrapped in the plant matrix. It can be inferred that, upon cell disruption induced by HPH (Table 2), polyphenoloxidase (PPO), which has been reported to be highly active in *Iceberg* lettuce, was no longer separated from its phenolic substrates, which were thus oxidised (Butz, Koller, Tauscher, & Wolf, 1994; Guan et al., 2016; Mai & Glomb, 2013). In addition, cell wounding could promote the release of proteases, responsible for the activation of latent PPO which, differently from the free soluble one, is bounded to the cellular membrane. In particular, latent PPO forms have been reported to account for about 50% of total PPO in *Iceberg* lettuce (Cantos et al., 2001). To confirm this hypothesis, PPO activity upon extraction treatments was determined. The average PPO activity in control extract was found to be about 3.5 U/mg of proteins (Table 2). This value resulted much lower than that found by Mai and Glomb (2013) in the external leaves of *Iceberg* lettuce (about 90 U/mg of proteins), probably due to the procedure used for preparing the extract. The latter, in fact, were obtained from freeze-

dried lettuce and presented a 750 mL/L ethanol liquid phase. Both frozen storage and ethanol presence have been reported to hinder PPO activity (Cantos et al., 2001; Lerici & Manzocco, 2000). US treatments did not modify this activity value ($p \geq 0.05$), suggesting that applied US treatments did not cause changes in PPO conformation. In this regard, PPO inactivation induced by US has been attributed to the modification of secondary and tertiary protein structure upon localized increase of pressure and temperature, and strong shear stress associated to acoustic cavitation. Obtained results confirm literature evidences, in which times longer than 5 min in the same US conditions applied in this study (400 W, 24 kHz, temperature lower than 50 °C) were required to promote a slight decrease in PPO activity (Bot et al., 2018).

By contrast, HPH treatments at 50 and 100 MPa promoted the activation of PPO, which resulted 40 and 34% higher than that of control extract, respectively (Table 2). This activation can be attributed not only to the disruption of cellular structures that physically separate phenols from PPO but also to the possible effect of HPH on PPO conformation and activity. In this regard, a progressive PPO activation was observed also in Chinese pear and mushroom submitted to high pressure microfluidization at pressures in the range from 80 to 200 MPa (Liu, Liu, Liu, et al., 2009; Liu, Liu, Xie et al., 2009), while inactivation has been mostly reported only upon multiple passes in the homogenization valve, due to the combined effect of pressure and thermal effect of these treatments (Bot et al., 2018). The effect of HPH on the specific phenolic compounds (Table 1) could represent a further evidence of the implication of PPO in phenol extraction yield. HPH pre-treatment reduced the extraction of caffeoyl phenolic compounds (3-O-caffeoylquinic acid, caffeoyltartaric acid, isochlorogenic acid, chicoric acid) to a higher extent than the flavonoids luteolin-7-O-glucuronide and quercetin-3-O-glucuronide. This can be attributed to the higher affinity of PPO towards caffeoyl derivatives, which are much better substrates for PPO than flavonoids (Goupy et al., 1990). As expected, the loss of phenolic compounds upon HPH pre-treatments negatively affected the antioxidant activity of the extracts, leading to values 10–30% lower than those obtained without applying HPH before sonication (Table 1).

3.4. Economic, environmental and scaling-up investigation

Although the promising results obtained by the application of US in producing antioxidant extracts from lettuce waste, the lack of knowledge keeps industry from implementing this technology in their processes (Chemat, Rombaut, Meullemiestre, et al., 2017). The availability of data relevant to economic and environmental impact as well as on scaling-up could represent a chance to increase industry confidence in this technology. To get a first insight in these aspects, the investment cost, energy consumption and carbon emission of the best performing US extraction procedure (120 s, 50 °C) on a laboratory and low/medium industrial scale were compared to those of the control traditional solid-liquid extraction (15 min, 50 °C) on a medium industrial scale (Table 3). Obtained data revealed that the scaling-up of ultrasound extractors would allow reducing by about one order of magnitude both the energy consumed and the carbon emitted for extracting 1 kg of polyphenols from lettuce waste. Although the considerably higher investment cost, the energy demand and the carbon emission associated to industrial ultrasound plants would be significantly lower than those associated to traditional solid-liquid extractors. This is mainly due to the long processing time and lower yield of traditional extraction as compared to ultrasound assisted extraction (Meullemiestre et al., 2016).

Although obtained data are to be considered as preliminary and should be validated on real industry context, they can represent a useful starting point to develop a support decision system for industries, to be used as a guidance for investment strategies.

4. Conclusions

Lettuce waste can be considered a cheap and always available source of antioxidant polyphenols, to be potentially used in the food and pharmaceutical sectors. Although further optimisation of processing parameters (e.g. solvent/sample ratio, temperature, time) is needed, ultrasounds can be efficaciously exploited to rapidly prepare food-grade polyphenol extracts from lettuce waste. Preliminary investigations on ultrasound energy demand and environmental impact represent a promising starting point for the scaling-up of this waste valorisation strategy.

Despite the complete disruption of cellular organization, the application of high pressure homogenization pre-treatments can be detrimental for ultrasound extraction yields, probably due to the activation of oxidative enzymes.

The application of alternative pre-treatments able to enhance polyphenolic yield by disrupting cell integrity while inactivating polyphnoloxidase should be considered to further enhance ultrasound extraction efficacy. In this regard, the effect of blanching, pulsed electric fields and high hydrostatic pressures could be investigated.

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