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Effects of Ultrasonic Treatments on the Polyphenol and Antioxidant Content of Spinach Extracts

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Abstract

The objective was to test ultrasound treatments on spinach leaves during extraction, and conventional extraction was used as a control. The effects of different combinations of the ultrasonic water bath factors tested on phenolic compound yields included frequency (37 and 80 kHz), exposure time (5, 10, 15, 20, 25, and 30 min), temperature (30, 40, and 50 °C), and ultrasonic power (30, 50, and 70 %). The best conditions for extraction yields were ultrasonic frequency of 37 kHz, extraction time of 30 min, reaction temperature of 40 □C, and ultrasonic power of 50 %. The mean yield (mg/ 100g), total phenol (mg gallic acid/ g DW), flavonoids (mg / g DW), % DPPH free-radical scavenging activity, and % ferric reducing antioxidant power were all high (64.88±21.84 , 33.96±11.30 , 27.37±11.85 , 64.18±16.69 and 70.25 ±9.68). Treatments were significantly different. The interaction among the ultrasonic parameters was significant. Temperature and power had significant effects on all other dependent variables.

Keywords: ultrasonic extraction; spinach; DPPH; flavonoids; antioxidant activity

1. Introduction

Consumption of vegetables was associated with reduced risks of many diseases (such as cancer and cardiovascular disease) in epidemiological studies [1]. Numerous studies have attempted to screen commonly eaten vegetables (carrots, potatoes, sweet potatoes, red beets, cabbage, Brussels sprouts, broccoli, lettuce, and spinach) for bioactive compounds and their antioxidant activities using different

assays [2]. Advanced extraction methods have paved the way for rapid extraction of bioactive compounds [3]. Despite assays to show the activity of vegetables' bioactive compounds, little is known about the activity of antioxidant components that can be isolated from these vegetables. Researchers have tended to focus on developing advanced methods to isolate, identify, and measure the activity of natural antioxidant compounds such as flavonoids, phenolic acids, tocopherols, carotenoids, and ascorbic acid [4].

Spinach (*Spinacea olerace L.)* is one of the most popular vegetables in the world [5] .The number of people in the United States who consume spinach increased in the past decades. According to analytical chemists, spinach is a good source of violaxanthin and neoxanthin because these kinds of compounds are not commercially available as supplements [6].Generally, in green vegetables such as spinach, only the green chlorophylls are seen by the consumer because they mask the bright colors of carotenoids. Carotene, lutein, violaxanthin, and neoxanthin are the major carotenoids in raw spinach [7] .The health benefits of spinach are partly due to the photoprotective function of carotenoids. Some of the carotenoids contain provitamins such as carotene which can be converted to vitamin A inside the human body through metabolism. In addition, scientists have confirmed that carotenoids have the ability to protect against certain forms of cancers, eye diseases such as age-related macular degeneration, and cardiovascular diseases [8].

Consumption of spinach is important in both developed and developing countries. Spinach in developed countries is mostly consumed either fresh or blanched, and sometimes after being frozen or canned. Dehydrated spinach is used in many developing countries due to extended shelf life [5,6] .Isolated polyphenols and antioxidants from spinach may be obtained by an extraction and separation process for potential use in functional foods or nutraceuticals. Higashio et al.[9] used methanol to extract and identify phenolic compounds from spinach leaves. Approximately 15 peaks were successfully extracted and separated by by high-pressure liquid chromatography (HPLC), but only quercetin was identified. Other studies reported use of ultrasound to enhance extraction by disrupting cell tissue, such as extracting anthocyanin from grape by-products [10] and phenolics from cranberry products [11] .Recently, Albu et al.[12] used ultrasound to extract phenolic compounds from rosemary. They compared

ultrasonic bath, ultrasonic probe, and shaking water bath extraction methods at diverse temperatures and with different solvents to find the most efficient. In all situations, the operation time was decreased by using an ultrasonic bath or probe system. Similar behavior was reported by Luque-Garcia et al.[13] who used ultrasound due to its positive effects in extraction processes for capsaicinoids of hot peppers. Both mechanical and thermal effects of ultrasound were studied on plant cells and tissues. The thermal effects of ultrasound occurred when ultrasonic waves were converted to heat and absorbed by plant tissue while the mechanical effects of ultrasound caused acoustic cavitation thereby causing a bubble to grow resulting in cell disruption for improved extraction[14,15].

Ultrasonic treatments have not been reported for extraction of antioxidants from spinach, but may prove improve yield over traditional solvent extraction methods. The objectives of this study were to (1) compare phytochemicals extracted from ultrasound and a traditional solvent extraction method; (2) compare ultrasonic treatment at different frequencies, temperatures, power levels, and exposure times on the yield of total phenol, total flavonoids and antioxidant activity; and (3) compare yield of spinach polyphenols between the highest yielding ultrasonic treatment and the traditional extraction method.

2. Materials and methods

2.1 Raw Material

Spinach leaves were provided by Dr. Alan Walters of the Department of Plant, Soil and Agricultural Systems, College of Agricultural Sciences, Southern Illinois University, USA. Raised beds with vermicompost for fertilizer on bare soil were used for organic production. Spinach (cv. ‘Tyee') was planted in double rows (7-10 cm spacing) on the raised beds. Spinach leaves were harvested from several randomly selected plants. Leaves were harvested from several randomly selected plants. The leaves were cleaned, sliced, and crushed in a blender; and then sealed and stored in plastic bags at -18 °C for five days before freeze-drying

2.2 Ultrasonic extraction

An Elmasonic P30 (P30) ultrasonic cleaner (Elma Hans Schmidbauer GMBH, Singen, Germany) with heated bath was used for treatments. User adjustable controls included frequency (37 and 80 kHz),

heated bath temperature, and power level as a percentage of full power (30-100 %). The standard ultrasonic mode was used. Temperature settings used for this study were 40 °C, 50 °C, and 60 °C and power level settings were 30 %, 50 %, and 70%. The manufacturer rated the P30 with an ultrasonic peak power of 480 W and an effective power rating of 120 W. The P30 had a proprietary algorithm to adjust power based on the impedance of the system. For a specific power setting, samples experienced the same degree of cavitation regardless of the load in the tank. For all treatments, the bath of the P30 contained 1.7 L of tap water before treatment containers were added. Ultrasonic power was expressed as W/cm^2, based on the power setting as a percentage of rated power and the volume of the bath solution prior to addition of treatment containers. Ultrasonic peak power for the 30 %, 50 %, and 70% power levels was 85 W/cm^2, 141 W/cm^2, 198 W/cm^2, respectively and effective power was 21 W/cm^2, 35 W/cm^2, 49 W/cm^2 respectively.

2.3 Preparation of crude extracts

The solvent extraction technique of Chang et al [16] was used with slight adjustments. Ten grams of lyophilized spinach were weighed and placed in a 200 mL glass flask. Then 100 mL of methanol was added to the flask. The solution was transferred to a 116 mL polypropylene container with cylindrical shape and screw-on lid before insertion in the P30. For the traditional method, the mixture was placed in the P30 water bath for 30 min at 50 °C without ultrasound to solubilize bioactive compounds from spinach.

For each ultrasonic temperature-power treatment, the Elmasonic P30 was set to the desired temperature and power and the water bath was allowed to reach the set temperature. Then 6 identical samples, each in separate polypropylene containers, were placed in the ultrasonic bath and the ultrasonic treatment was initiated for 30 min. At each 5 min interval (5, 10, 15, 20, 25, and 30 min), one of the samples was randomly selected and removed from the ultrasonic bath. The remaining samples were immediately clustered together at one end of the ultrasonic bath.

All ultrasonic treatments were conducted in a systematic order from lowest to highest temperature (30°C, 40°C and 50°C). Within each temperature setting, power settings were adjusted from low to high

106 (30%, 50% and 70%). Each treatment setting was repeated 3 times before changing to the next setting.
107 The procedure was completed for 37 kHz frequency and duplicated for 80 kHz frequency.
108 After treatment, ultrasound and traditional extraction samples were filtered (Whatman no.1,
109 Whatman International Ltd, Maidstone, United Kingdom). The solids of the lower layer were re-extracted
110 with 100 mL of methanol at room temperature to ensure all soluble bioactive compounds were recovered.
111 The filtered liquids were placed into a rotary evaporator (BUCHI, Labortechnik AG, Flawil, Switzerland)
112 under vacuum at 40 °C to reduce solvent volumes to 10 mL.
113 **2.4 Phytochemical tests**
114 Seven assays were used to identify phytochemical compounds of alkaloids, saponins, glycosides,
115 tannins, phenols, flavonoids, and triterpenoids in each sample according to the methods of Harbone [17]
116 .Three samples of the traditional extract method were analyzed. For the ultrasonic method, one sample of
117 each combination of frequency, temperature, and power level was analyzed.
118 2.4.1 Alkaloids
119 Mayer's reagent was prepared by mixing 13.5 gm of mercuric chloride and 50 gm of potassium
120 iodide with 100 mL distilled water into 100 mL flask. The 50 mg of crude extracts were treated with 1-2
121 mL of hydrochloric acid (2N) and then 1-3 drops of newly prepared Mayer's reagent were added. The
122 appearance of red residue in the test liquid indicated alkaloids in the sample.
123 2.4.2 Saponins
124 Exactly 25 mL of distilled water were added to 2 mL of the spinach samples with manual shaking
125 for 15-20 min. The appearance of a steady foam indicated the presence of saponins.
126 2.4.3 Glycosides
127 Hydrochloric acid, 5 mL of 70 % (v/v) was added to 1 g spinach for hydrolysis in water bath at
128 100 °C. Afterward spinach extracts were treated with chloroform, and then 5 mL of dilute ammonia were
129 added to the supernatant layer. A pink color indicated the existence of glycosides in the samples.
130 2.4.4 Tannins

Drops of distilled water were added to the crude spinach extracts with approximately 0.25 g NaCl. The appearance of tannins was indicated when a blue green color developed after treating samples with 1 mL of ferric chloride (2%).

2.4.5 Phenols

The presence of bioactive compounds with intense green color was observed when 5 mL of 6 % (w/v) of ferric chloride was mixed with 1 mL of samples.

2.4.6 Flavonoids

In the first assay, approximately 3-4 drops of absolute H_2SO_4 and a few drops of 10% (w/v) NaOH were added to the spinach samples. Brown and orange colors were indicative of flavonols and flavones respectively. In a second assay, about 0.5 mL of the spinach extract was added to test tube, then 7-10 drops of 80% (v/v) HCl with a small amount of magnesium ribbon to reach the boiling point after 5-10 min. Either reddish pink or foggy brown color in samples indicated the presence of flavonoids.

2.4.7 Triterpenoids

Approximately 7-10 drops of antimony trichloride were mixed with 2 mg of spinach extract for 10 min. A blue color indicated triterpinoids in the crude samples.

2.5 Ultrasonic treatment performance

Five measures were used to compare ultrasonic extraction methods and to compare the highest yielding ultrasonic method with the traditional extraction method.

2.5.1 Total extraction yield

The total extract yield was measured according to the following equation used by Wang et al.[18].

Total Yield = [dried product (mg) / lyophilized sample (10g)] * 100 (1)

2.5.2 Total phenolics

The Folin–Ciocalteu assay was used to measure total phenolic compound [19]. Sodium carbonate (2 g) was dissolved into 100 mL of distilled water. One g of the crude extracts was dissolved in 46 mL of distilled water with 1 mL of Folin-Ciocalteu solution. The mixture was shaken for 10 min, and 3 mL of

157 the sodium carbonate solution (2 % w/v) was added. The mixture was kept in the dark for two hours with
158 intermittent shaking to homogenize the mixture. The absorbance was measured at 750 nm and compared
159 to a calibration curve prepared with known amounts of Gallic acid (Roth, Karlsruhe, Germany). The
160 results were expressed as mg/g dry matter (DM).
161 2.5.3 Total flavonoids
162 The total flavonoid contents were determined following the method of Taga et al.[20].
163 Approximately 0.2 mL of spinach crude extracts was added to 5 mL of cinnamaldehyde with manual
164 shaking for 30 min at 25 °C. The absorbance was estimated at 640 nm compared to a blank sample
165 without spinach extracts. The standard curve of known amounts of catechin was used for calibration. The
166 calculation of total flavonoids was compared with the standard calibration curve of catechin, and
167 expressed as catechin equivalents.
168 2.5.4 Ferric reducing antioxidant power
169 Antioxidant compounds produce a color complex with potassium ferricyanide, trichloro acetic
170 acid, and ferric chloride, which were measured at 700 nm. The increase in absorbance of the reaction
171 mixture indicates the possibility of using these spinach extracts as antioxidants [21]. Exactly 1 mL of
172 spinach sample was dissolved in 1 mL of distilled water, and 2.5 mL of K3Fe(CN)6 (1% w/v) with 2.5
173 mL of 0.2 M phosphate buffer (pH 6.6) according to method described by Oyaizu et al.[22]. The mixture
174 was incubated for 20 min at a temperature of 50 °C and then 22.5 mL of trichloro acetic acid (10% w/v)
175 was added. An upper layer (2.5 mL) was obtained through centrifugation at 3000 rpm for 10 min. The
176 supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (0.1%, w/v). The absorbance
177 was measured at 700 nm in a spectrophotometer. Ferric reducing antioxidant power was calculated as
178 follows:
179 % ferric reducing antioxidant power= 100-(A/B)*100 (2)
180 Where, A= absorbance of sample ; B=absorbance of control.
181 2.5.5 DPPH-Elisa assay

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution was prepared by dissolving 10 mg of DPPH in 4 mL methanol, and the solution was kept in the dark at 5 °C according to Lee et al.[23]. A stock solution (1000 μg/mL) of spinach crude compounds was prepared in dimethyl sulfoxide (DMSO). Varying concentrations of the stock solution were made (20, 40, 60, 80, 100, 120, 140, and 160 μg/mL). Each concentration was added to a 96-well Elisa plate so that the highest concentration was in the top wells with each decreased concentration following with the lowest concentration in the bottom wells. Later, 5 μL methanolic DPPH solution was added to the each of 96-wells. The Elisa plate was shaken to ensure the DPPH solution was mixed before incubation while covered with aluminum foil. The optical density (OD) of the whole solution was measured at 517 nm after 30 min by using an ELISA Reader. Pure DPPH in a methanolic solution was used as a control sample. The following equation was used to calculate the percentage inhibition of oxidation:

% DPPH free-radical scavenging = {1 - Absorbance (DPPH + sample)/absorbance (control) }* 100 (3)

2.6 Statistical Analysis

The variables of frequency, temperature, power, and exposure time were analyzed as a full factorial ANOVA for each of the five measures of ultrasonic performance. When main effect interactions were significant, simple effects were analyzed for differences. Differences in the simple effect of temperature-power combinations were determined within frequency and with exposure times treated as additional observations. The simple effect of temperature within each frequency at the highest yielding power based on the temperature-power combination analysis was examined for differences. The simple effect of power level within each frequency at the highest yielding temperature based on the temperature-power combination analysis was examined for differences. The simple effect of exposure time within each frequency for the highest yielding temperature-power combination was also determined. SAS 9.2 with $P < 0.05$ was used for statistical analysis. Tukey's HSD (honestly significant difference) test was used for mean separation.

3. Results and Discussion

3.1 Qualitative phytochemicals analysis of spinach

208 The qualitative phytochemicals analysis of ultrasonic extracts resulted in evidence of the presence
209 of flavonoids, phenols, tannins, glycosides, saponins and alkaloids, but triterpenoids were not detected.
210 For the traditional extraction method, phenols, tannins, glycosides, and saponins were detected while
211 flavonoids, alkaloids and triterpenoids were not detected (Table 1). These results were in agreement with
212 Haizhou et al.[24] who mentioned that ultrasonic water bath had the ability to increase the permeability of
213 the plant tissues by inducing cavitation, and thus smoothing to release all compounds compared to the
214 conventional method.

215 **3.2 Effect of ultrasonic frequency, temperature, time, and power on extraction yield (mg/ 100 g**
216 **DW) of spinach.**

217 Results of the full factorial ANOVA analysis indicated significant ($P < 0.0001$) interactions
218 among all combinations of the frequency, temperature, power, and exposure time variables for extraction
219 yield. The simple effect of temperature-power combinations showed a significant difference ($P < 0.0001$)
220 among the treatments for both 37 kHz and 80 KHz frequencies. Within each frequency, the temperature
221 setting of 40 °C and power level of 50 % resulted in a significantly higher extraction yield than the other
222 temperature-power combinations (Table 2). The mean yield of polyphenols from spinach at this
223 combination was 64.88±21.84 and 50.44±12.97 mg/ 100 g for 37 kHz and 80 kHz respectively. For both
224 frequencies, the combinations of 30 °C with power levels of 30 % and 50% were in the grouping of
225 lowest extraction yields. The frequency, temperature, and power of the ultrasound are known to affect the
226 efficiency of extraction; especially the power of ultrasound is affected by the amplitude of the ultrasound
227 waves. The results here indicated that operating ultrasonic equipment with 37 kHz was more effective
228 than 80 kHz in regards to extraction yield. This finding concurred with Zhou et al. [25] who found that
229 increasing ultrasonic frequency had a major effect on extraction yield by decreasing the intensity of
230 cavitation in liquids.

231 At the 50 % power level, there was a significant difference ($P < 0.0001$) among temperatures at
232 both frequencies. The 40 °C temperature had the significantly highest extraction yield at 37 kHz and 80
233 kHz (Table 3). At 37 kHz there was no significant difference between 30 °C and 50 °C temperatures. At

234 80 kHz there was a significant difference in extraction yield among all three temperatures; temperature 40
235 °C gave highest yield followed by 50 °C and 30 °C. Chan et al. [26] reported that the yield of phenolic
236 compounds increased when the temperature increased from 40 °C to 70 °C. Teh and Birch [27] also found
237 that yield was increased when the temperature was raised from 40°C to 50°C. However, at 60°C,
238 extraction yield in flax and canola seed cake extracts decreased, whereas total flavonoids decreased at
239 70°C in hemp seed cake extracts probably due to the destruction of phenolic compounds.
240 Within the 40 °C treatments, there was a significant difference ($P < 0.0001$) among power levels
241 at both frequencies. The 50 % power level had the significantly highest extraction yield within the 40 °C
242 treatments, with no difference between the 30 % and 70 % power levels at both the frequencies (Table 4).
243 This finding was in agreement with Herrera et al. [28] who mentioned that the degradation (nearly100%)
244 of many phenolic compounds from strawberries was caused by ultrasound. However, the yields of sinapic
245 and vanillic acid did not decline significantly with increased extraction time at 40 °C. One of the possible
246 reasons given for this phenomenon was that the stability of these two phenolic compounds at high
247 temperatures was higher. This may be partly ascribed to the differences in their chemical structures.
248 At the temperature-power combination of 40 °C and 50 %, there was a significant difference ($P < 0.0001$)
249 among exposure times for both frequencies with extraction yield significantly increasing for each
250 increased exposure time (Table 5). The exposure time results were in agreement with Sultana et al.[29]
251 who reported a gradual increase from 0 to 60 min in phyllyrin yields, and they ascribed the different
252 availability and class of extractable components were resulting from the varied chemical composition of
253 plants. Probably, it was not just time to rarefaction or compression at high frequency but also time to
254 allow a bubble to grow to a size sufficient to cause disruption and resulting increase in extraction yield.
255 Therefore, the bubbles may need time during rarefaction to collapse through processing. For that reason,
256 the high frequencies will not have the ability to cause enough cavitation in the extracts [30].
257
258

3.3 Effect of ultrasonic frequency, temperature, time, and power on total phenol (mg gallic acid/ g DW) of spinach

Full factorial ANOVA analysis resulted in significant ($P < 0.0001$) interactions among all combinations of the frequency, temperature, power, and time variables on total phenols in spinach extract. According to statistical analyses (Table 2) among temperature-power combinations there was a significant difference ($P < 0.0001$) for both 37 kHz and 80 KHz frequencies. The highest total phenol within each frequency was related to use of 40 °C and power level of 50 % which resulted in a significantly higher total phenol than the other temperature-power combinations. The mean total phenol from spinach at this combination was 33.96±11.30 and 25.52±6.56 for 37 kHz and 80 kHz respectively. The combinations of 50 °C with power levels of 70 % were in the grouping of lowest total phenol for both frequencies. So, this study showed that when the temperature was 50 □C, the total phenols yield decreased with an increase in the percentage of power. The extraction of phenolic compounds was dependent on both the temperature of the ultrasonic water bath and its power percentage. Consequently, single factor analyses might not be effective for optimization of the extraction of a bioactive compound. Hence, this study supported reports that a combination of temperature with power variables was more effective in extracting phenolic compounds than a single factor [31].

For each frequency at the power level of 50 %, there was a significant difference ($P < 0.0001$) among temperatures. According to statistical analyses (Table 3), the results exhibited that the highest total phenol occurred at 40 °C at 37 kHz and 80 KHz. There was no significant difference between 30 °C and 50 °C temperatures for both frequencies (37 KHz and 80 KHz). This is consistent with previous findings of Pinelo et al. [32] who reported that the yields of phenolic compounds from milled berries and grape pomace depended significantly on extraction temperature. However, higher temperatures beyond 50 °C induced the instability of phenolic compounds.

There was a significant difference ($P < 0.0001$) among power levels at both frequencies at 40 °C (Table 4). The results showed that the highest total phenol was at 50 % power level within the 40 °C treatments. Moreover, per statistical analysis there was no difference between the 30 % and 70 % power

levels at both frequencies. The above results agreed with Ma et al. [33] who confirmed the positive effects of increasing the level of power on the yields of phenolic compounds of citrus peel. They observed that by increasing the power from 3.2 to 30 W, the yields of most phenolic compounds were significantly increased and then gradually decreased after 30 W.

Table 5 summarized that for both frequencies, the total phenol significantly ($P < 0.0001$) increased by increasing exposure time at the temperature-power combination of 40 °C and 50 %. Increases in total phenolic yields were observed at each time point from 5 min to 30 min. The findings were in agreement with Marquez et al. [34] who found that the phenolic compounds yield from lyophilized *Laurus nobilis L.* increased when extraction time was increased.

3.4 Effect of ultrasonic frequency, temperature, time, and power on total flavonoids (mg / g DW) of spinach

ANOVA analysis (full factorial) of frequency, temperature, power, and time on total flavonoids resulted in significant ($P < 0.0001$) interactions among all combinations of the independent variables. Table 2 has demonstrated that there was a significant difference ($P < 0.0001$) for both 37 kHz and 80 KHz frequencies among all treatments for temperature-power combinations. The results within each of the frequencies showed that total flavonoids at 40 °C and a power level of 50 % were higher than the other temperature-power combinations. In contrast, a relatively higher temperature of 50 °C with 50 % power reduced flavonoids yield significantly, possibly by the denaturation of cell membranes. This finding concurred with Cacace et al. [35] who discovered that the degradation of some flavonoids might occur when the temperature was raised to 50 □C or more. The mean total flavonoids from spinach at this combination were 27.37 ±11.85 and 15.27±4.88 (mg / g DW) for 37 kHz and 80 kHz respectively. The combinations of 30 °C with power levels of 30 %, and 50 % were in the grouping of lowest total flavonoids at 37 KHz. But, the combinations of 30 °C with power levels of 30 % exhibited lowest total flavonoids at 80 KHz.

According to statistical analyses, there was a significant difference ($P < 0.0001$) among temperatures at both frequencies at the power level of 50 %. The results (Table 3) have shown that the

311 highest total flavonoids were at 40 °C temperature for both 37 kHz and 80 kHz. Also, for both
312 frequencies, there was no significant difference between 30 °C and 50 °C temperatures. This finding was
313 in agreement with Qu et al. [36] who found that a low extraction temperature (below 45°C) and low
314 ultrasonic power were very important to enhance the extractions.
315 At 40 °C, there was a significant difference ($P < 0.0001$ for 37 kHZ and P = 0.0444 for 80 kHz)
316 among power levels at both frequencies (Table 4). The statistical analyses showed that the highest total
317 flavonoid extractions were at 50 % power with the 40 °C treatments at 37 kHz but at both 30 % and 50 %
318 power at 80 kHz. That phenomeneon might be ascribed to the positive effect of ultrasonic power and
319 temperature by enhancing the mass transfer process. Moreover, statistical analyses indicated that that
320 there was no difference between the 30 % and 70 % power levels at 37 KHz. However, at 80 KHz ,there
321 was no difference between the 30 % and 70 % power levels nor between the 30 % and 50 % power levels
322 but each pair differed. The different effect of ultrasonic power on extraction efficiency may have been
323 due to differences in hardness, compactness, solute distribution and eventually cavitation behavior in
324 medium [34]. The results showed here a significant interaction between temperature and power. The
325 extraction of total flavonoids was highly related to both temperature of the ultrasonic water bath and its
326 power percentage.
327 The total flavonoids were significantly ($P < 0.0001$) increased for both frequencies when the
328 exposure time was increased at the temperature-power combination of 40 °C and 50 % (Table 5), with
329 one exception at 80 kHz of 10 min resulting in a higher value than 15 min..
330 **3.5 Effect of ultrasonic frequency, temperature, time, and power on DPPH free-radical scavenging**
331 **activity (%) of spinach**
332 A full factorial ANOVA analysis of frequency, temperature, power, and time on DPPH free-
333 radical scavenging activity showed significant ($P < 0.0001$) interactions among all combinations of the
334 classification variables. The results indicated that for both frequencies, DPPH free-radical scavenging rate
335 in spinach extracts was significantly different ($P < 0.0001$) among temperature-power combinations. The
336 antioxidant activity within each frequency was higher at 40 °C and power levels of 50 % than the other

temperature-power combinations. The mean DPPH free-radical scavenging rate was 64.18±16.69 and 48.72 ±14.68 % for 37 kHz and 80 kHz respectively (Table 2). The combinations of 30 °C with power levels of 30 %, 50 %, and 70 % were among the lowest DPPH free-radical scavenging rates at 37 KHz. However, the combinations of 30 °C with power a level of 30 %, and 50 °C with a power level of 70 % exhibited the lowest DPPH free-radical scavenging rates at 80 KHz. When ultrasonic frequency was increased from 37 kHz to 80 kHz, DPPH free-radical scavenging rate decreased first slowly and then rapidly as temperature and power were increased. These findings were in agreement with Wang et al. [37] who ascribed this phenomenon to the relation between frequency and the number of cavitation bubbles. When the frequency increased, not only did the number of cavitation bubbles increased but also the size of these bubbles became smaller, thereby it may be inferred as reducing and decreasing DPPH free-radical scavenging rates of extracts.

At the power level 50% within each frequency, the statistical analyses showed that there was a significant difference ($P < 0.0001$) among temperatures. Table (3) has shown that the highest DPPH free-radical scavenging rate was at 40 °C temperature for both 37 kHz and 80 kHz. Also, for both frequencies, there was no significant difference between 30 °C and 50 °C temperatures. DPPH free-radical scavenging rate increased as temperature increased from 30 °C to 40 °C with 50 % of power. However, when the temperature was 50 °C, DPPH free-radical scavenging rate decreased because the temperature led to lose some sensitive compounds which might have high antioxidant activity.

The statistical analyses showed that there was a significant difference ($P = 0.0245$) at 40 °C among power levels at 37 kHz, but no difference at 80 kHz (Table 4). The results showed that the highest DPPH free-radical scavenging rate in spinach extracts was at 50 % power within the 40 °C treatments at 37 KHz, but it was not different from the 30 % power among all power levels at 80 KHz. This result confirmed that higher frequency (80 KHz) played a dynamic role, possibly to collapse bubbles. Consequently, high frequency did not allow sufficient time to extract all the target compounds. Moreover, statistical analyses showed that that there was no difference between the 30 % and 50 % power levels at 37 KHz.

For both frequencies, increasing the ultrasonic exposure time significantly ($P < 0.0001$) increased the DPPH free-radical scavenging rate in spinach extracts at the temperature-power combination of 40 °C and 50 % (Table 5). According to statistical analyses, the 30 min extraction time was appropriate for nearly complete leaching for high rates of DPPH free-radical scavenging. The 30 min exposure time was inferred to allow most of the phenolic compounds to be extracted with methanol in the ultrasonic water bath extractions.

3.6 Effect of ultrasonic frequency, temperature, time, and power on ferric reducing antioxidant power of spinach

Full factorial ANOVA analysis showed significant ($P < 0.0001$) interactions among all combinations of the frequency, temperature, power, and time variables on ferric reducing antioxidant power. The ferric reducing antioxidant power for both frequencies increased significantly ($P < 0.0001$) among treatments for temperature-power combinations (Table 2). The ferric reducing antioxidant power at 37 KHz was higher at 40 °C and a power level of 50 % than the other temperature-power combinations. However, there was no difference between the temperature-power combinations of 30 °C & 70 %, 40 °C & 30 %, 40 °C & 50 %, and 40 °C & 70 % at 80 kHz. The positive effects of frequency 37 KHZ may be ascribed to be less degradation in phenolics content with much faster extraction process, causing disruption of plant cell walls that facilitated the release of the cell content into solvent. The mean ferric reducing antioxidant power at 40 °C and power level of 50 % was 70.25±9.68 % and 68.57 ±9.65 % for 37 kHz and 80 kHz respectively. The combinations of 30 °C with power levels of 30 % and 50% were among the lowest ferric reducing antioxidant power percentages for both 37 KHz and 80 KHz.

The ferric reducing antioxidant power at the ultrasonic power level 50% was significantly different ($P < 0.0001$) among temperatures for both frequencies according to the statistical analyses (Table 3). The results showed that the highest ferric reducing antioxidant power was at 40 °C for both 37 kHz and 80 kHz. There was no significant difference between 30 °C and 50 °C temperatures for both frequencies. There was a significant difference ($P = 0.0009$ for 37 kHz and $P = 0.0149$ for 80 kHz) at 40 °C among power levels at both frequencies according to the statistical analyses (Table 4). Therefore, it

was concluded that the highest ferric reducing antioxidant power in spinach extracts was at 50 % power within the 40 °C treatments at 37 KHz, but the statistical analyses showed that that there was no difference between the 30 % and 50 % power levels and the 30 % and 70 % at 80 KHz. The results were in agreement with Jahouach□Rabai et al. [38] who suggested that a higher temperature of ultrasonic extraction with a higher ultrasonic power could destroy some of the phenolic compounds that were disbanded into the extraction medium.

At the temperature-power combination of 40 °C and 50 %, the ferric reducing antioxidant power in spinach extracts for both frequencies increased significantly ($P < 0.0001$) as the ultrasonic exposure time increased from 5-30 min in 5 min increments (Table 5). An ultrasonication time of 30 min changed the yellow color of solution to either green or blue depending on the ferric reducing antioxidant power of spinach samples. Similar results were reported by Teh & Birchin [27]. They found that DPPH and FRAP had the highest antioxidant capacity of seed cake extracts when 30 min of ultrasonication time was used. They ascribed that phenomenon to be providing more time to release bioactive compounds from plants tissue as well as enhancing the diversity of the extracted compounds. According to statistical analyses, the interaction effects of treatment time, temperature, power, and frequency were significant on ferric reducing antioxidant power. The best ultrasonic power conditions was again determined to be lower temperature (40 °C), longer time (30 min) and low frequency (37 KHz).

3.7 Comparison between ultrasonic water bath extraction of spinach polyphenol content and the conventional extraction (control)

In order to compare between ultrasonic water bath technique and the conventional method, the first experiment was conducted to use ultrasonic water bath with temperature 40 □C, power 50 %, 30 min and both 37 KHz and 80 KHz separately while the traditional extraction used 50 □C for 30 min. The results showed that ultra-sonication at 37 KHz significantly increased the yield of total phenolic contents from spinach leaves compared to the conventional extraction ($p < 0.0001$; Fig. 1, 2). For example, the yields were 22.47, 69.32 and 95.76 (g /100 g DW); and total phenolic contents were 11.98, 33.33, and 51 (mg GAE /100 g D.W) for control, ultrasonic (80 KHz), and ultrasonic (37 KHz) respectively. In

addition, total flavonoids of spinach extracted using either ultrasonic frequency of37 KHz or 80 KHz were significantly ($P < 0.0001$) higher than the control (Fig. 3). The antioxidant activity was in agreement with the total phenolic content in the spinach extraction. The results showed that % DPPH free-radical scavenging and ferric reducing antioxidant power activity were significantly ($P< 0.0001$) higher in both ultrasonic frequencies compared to the control (Fig.4, 5). Spinach extracts of control, ultrasonic at 80 KHz), and ultrasonic at 37 KHz exhibited DPPH free-radical scavenging of 20.42 %, 168.91 % and 84.29 % respectively. Furthermore, for control, ultrasonic at 80 KHz and ultrasonic at 37 KHz exhibited ferric reducing antioxidant power of 41.05 %, 80.07 %, and 83.20 % higher than the controls, respectively. The above results were in agreement with Han et al. [39] who confirmed that both ultrasonic power and frequency can play a dynamic role during dispersion of plant materials in the sample.

4. Conclusion

The ultrasound treatment had the capability to increase polyphenol extraction yields from spinach. The results of this study showed that the ultrasonic treatments were reliable and feasible methods for the extraction of phenolic compounds from spinach. According to statistical analyses, the best extraction conditions were at the ultrasound frequency of 37 KHz, ultrasonic power of 50%, treatment time of 30 min and process temperature of 40 □C. In addition, spinach extracts showed strong antioxidant capacity in vitro, and the extracts can be considered as a good source of natural antioxidants. Polyphenol extraction from spinach by ultrasound will be a low cost method because it reduces the amount of solvent used and avoids the need for longer extraction times compared to the conventional extraction method. Ultrasound extraction is strongly recommended as a potential method for extraction of bioactive compounds from diverse plant materials.

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Figure Caption:

Figure 1. Total yield of spinach extract after 30 min at power 50 %, and temperature 40 °C

Figure 2. Total phenol of spinach extract after 30 min at power 50 %, and temperature 40 °C

Figure 3. Total flavonoids of spinach extract after 30 min at power 50 %, and temperature 40 °C

Figure 4. Percent DPPH free-radical scavenging by spinach extract obtained from 30 min at power 50% and temperature 40 °C

Figure 5. Percent ferric reducing antioxidant power of spinach extracts obtained from 30 min at power 50% and temperature 40 °C

Table 1. Qualitative analysis of presence or absence of phytochemicals in spinach resulting from conventional and ultrasonic extraction methods.

Phytochemicals	Extraction Method	
	Conventional (n = 3)	Ultrasonic Bath (n = 18)
Flavonoids	Present	Present
Phenols	Present	Present
Tannins	Present	Present
Glycosides	Present	Present
Saponins	Present	Present
Alkaloids	Absent	Present
Triterpenoids	Absent	Absent

n = number of samples

Table 2. Ultrasonic treatment measures at each temperature-power combination for each frequency.

Temperature-Power Combination	Extraction Yield* (mg/100g DW) (n = 18)	Total phenol*(mg gallic acid/ g DW) (n=18)	Flavonoids* (mg / g DW) (n = 18)	% DPPH free-radical scavenging* (n = 18)	% Ferric reducing antioxidant power* (n = 18)
Frequency = 37 kHZ					
30 °C & 30 %	13.42 ^{d}	13.73^{cd}	6.08^{d}	28.75^{c}	45.54^{c}
30 °C & 50 %	14.16 ^{d}	13.96^{cd}	6.65^{d}	31.07^{c}	51.09^{bc}
30 °C & 70 %	15.57 ^{cd}	14.57^{bcd}	7.93^{cd}	32.13^{c}	54.81^{bc}
40 °C & 30 %	23.42 ^{bc}	14.95^{bcd}	8.99^{cd}	56.89^{ab}	59.97^{b}
40 °C & 50 %	64.88 ^{a}	33.97^{a}	27.37^{a}	64.19^{a}	70.25^{a}
40 °C & 70 %	26.52 ^{b}	18.71^{b}	14.49^{b}	49.79^{b}	59.07^{b}
50 °C & 30 %	25.73 ^{b}	17.37^{bc}	12.17^{bc}	38.34^{c}	55.82^{b}
50 °C & 50 %	24.17 ^{bc}	15.85^{bcd}	10.10^{bcd}	36.46^{c}	52.93^{bc}
50 °C & 70 %	22.15 ^{bcd}	12.62^{d}	9.43^{cd}	33.42^{c}	50.70^{bc}
Frequency = 80 kHZ					
30 °C & 30 %	15.20 ^{f}	14.89^{bc}	7.78^{e}	30.37^{d}	47.13^{d}
30 °C & 50 %	15.92 ^{ef}	15.04^{bc}	9.58^{cde}	31.03^{cd}	55.09^{cd}
30 °C & 70 %	21.62 ^{de}	16.07^{bc}	11.09^{bcd}	32.21^{bcd}	60.58^{abc}
40 °C & 30 %	33.27 ^{bc}	16.76^{bc}	13.34^{ab}	41.40^{ab}	65.56^{ab}
40 °C & 50 %	50.44 ^{a}	25.53^{a}	15.28^{a}	48.73^{a}	68.57^{a}
40 °C & 70 %	37.81 ^{b}	17.56^{b}	11.75^{bc}	40.15^{abc}	59.82^{abc}
50 °C & 30 %	29.12 ^{c}	14.80^{bc}	9.78^{cde}	34.00^{bcd}	58.19^{bc}
50 °C & 50 %	22.59 ^{d}	13.76^{c}	8.57^{cde}	29.61^{d}	56.43^{c}
50 °C & 70 %	20.20 ^{def}	13.48^{c}	8.14^{de}	28.56^{d}	54.97^{cd}

*Means within each column and frequency with the same superscript letter are not significantly different. n = number of samples. DW = dry weight

599 Table 3. Ultrasonic treatment measures by temperature at 50 % power level for each frequency.

Temperature	Extraction Yield* (mg/100 g DW) (n = 18)	Total phenol* (mg gallic acid/ g DW) (n = 18)	Flavonoids* (mg / g DW) (n = 18)	% DPPH free-radical scavenging* (n = 18)	% ferric reducing antioxidant power* (n = 18)
Frequency = 37 kHZ					
30 °C	14.16 [b]	13.96[b]	6.65[b]	31.07[b]	51.09[b]
40 °C	64.88[a]	33.97[a]	27.37[a]	64.19[a]	70.25[a]
50 °C	24.17 [b]	15.85[b]	10.10[b]	36.46[b]	52.93[b]
Frequency = 80 kHZ					
30 °C	15.92 [c]	15.04[b]	9.58[b]	31.03[b]	55.09[b]
40 °C	50.44[a]	25.53[a]	15.28[a]	48.73[a]	68.57[a]
50 °C	22.59 [b]	13.76[b]	8.57[b]	29.62[b]	56.43[b]

600 *Means within each column and frequency with the same superscript letter are not
601 significantly different. n = number of samples. DW = dry weight

602 Table 4. Ultrasonic treatment measures by power level at 40 °C temperature for each frequency.

Power level	Extraction Yield* (mg/100 g DW) (n = 18)	Total phenol* (mg gallic acid/ g DW) (n = 18)	Flavonoids* (mg / g DW) (n = 18)	% DPPH free-radical scavenging* (n = 18)	% ferric reducing antioxidant power* (n = 18)
Frequency = 37 kHZ					
30 %	23.42 [b]	14.95[b]	8.99[b]	56.89[ab]	59.97[b]
50 %	64.88[a]	33.97[a]	27.37[a]	64.19[a]	70.25[a]
70 %	26.52 [b]	18.71[b]	14.49[b]	49.79[b]	59.07[b]
Frequency = 80 kHZ					
30 %	33.27 [b]	16.76[b]	13.34[ab]	41.40[a]	65.56[ab]
50 %	50.44[a]	25.53[a]	15.28[a]	48.73[a]	68.57[a]
70 %	37.81 [b]	17.56[b]	11.75[b]	40.15[a]	59.82[b]

603 *Means within each column and frequency with the same superscript letter are not
604 significantly different. n = number of samples. DW = dry weight

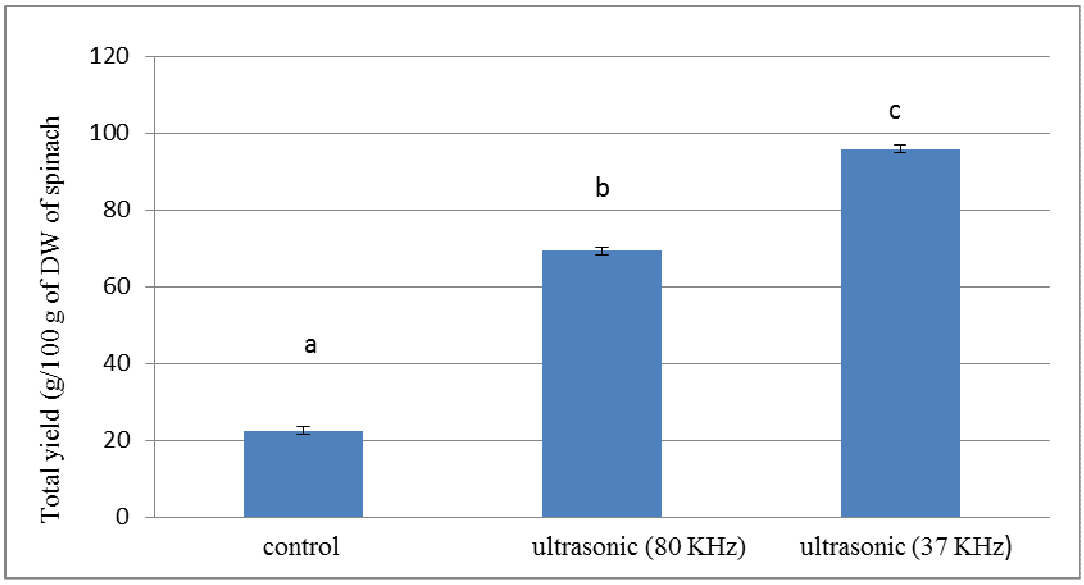
605 Table 5. Ultrasonic treatment performance measures by exposure time at 40 °C temperature
606 and 50 % power level for each frequency.

Exposure Time (min)	Extraction Yield* (mg/100 g DW) (n = 18)	Total phenol* (mg gallic acid/ g DW) (n = 18)	Flavonoids* (mg / g DW) (n = 18)	% DPPH free-radical scavenging* (n = 18)	% ferric reducing antioxidant power* (n = 18)
Frequency = 37 kHZ					
5	35.85 ^{f}	20.11 ^{f}	16.32 ^{e}	38.66 ^{f}	55.87 ^{f}
10	45.09 ^{e}	21.53 ^{e}	15.45 ^{f}	47.87 ^{e}	61.23 ^{e}
15	56.98 ^{d}	31.62 ^{d}	20.31 ^{d}	64.98 ^{d}	69.52 ^{d}
20	70.07 ^{c}	36.98 ^{c}	28.29 ^{c}	70.34 ^{c}	73.34 ^{c}
25	85.55 ^{b}	42.66 ^{b}	36.56 ^{b}	78.98 ^{b}	78.34 ^{b}
30	95.76^{a}	50.90^{a}	47.31^{a}	84.29^{a}	83.20^{a}
Frequency = 80 kHZ					
5	38.22 ^{f}	17.03 ^{f}	10.21 ^{f}	28.34 ^{f}	53.21 ^{f}
10	39.07 ^{e}	17.53 ^{e}	13.53 ^{d}	36.86 ^{e}	60.00 ^{e}
15	43.03 ^{d}	24.62 ^{d}	13.32 ^{e}	41.50 ^{d}	68.91 ^{d}
20	46.74 ^{c}	29.98 ^{c}	13.97 ^{c}	55.21 ^{c}	72.00 ^{c}
25	66.26 ^{b}	30.66 ^{b}	15.32 ^{b}	61.54 ^{b}	77.23 ^{b}
30	69.32^{a}	33.33^{a}	25.32^{a}	68.91^{a}	80.07^{a}

607 *Means within each column and frequency with the same superscript letter are not
608 significantly different. n = number of samples. DW = dry weight

609 Figure 1

610

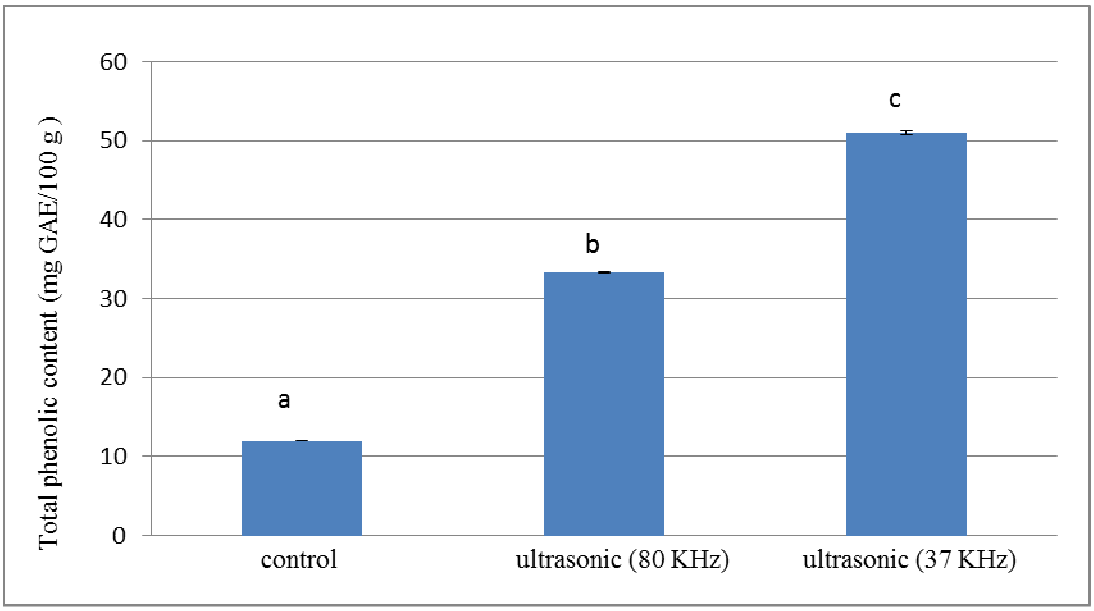


611

612 Figure 1. Total yield of spinach extract after 30 min at power 50 %, and temperature 40 °C

613 Figure 2

614

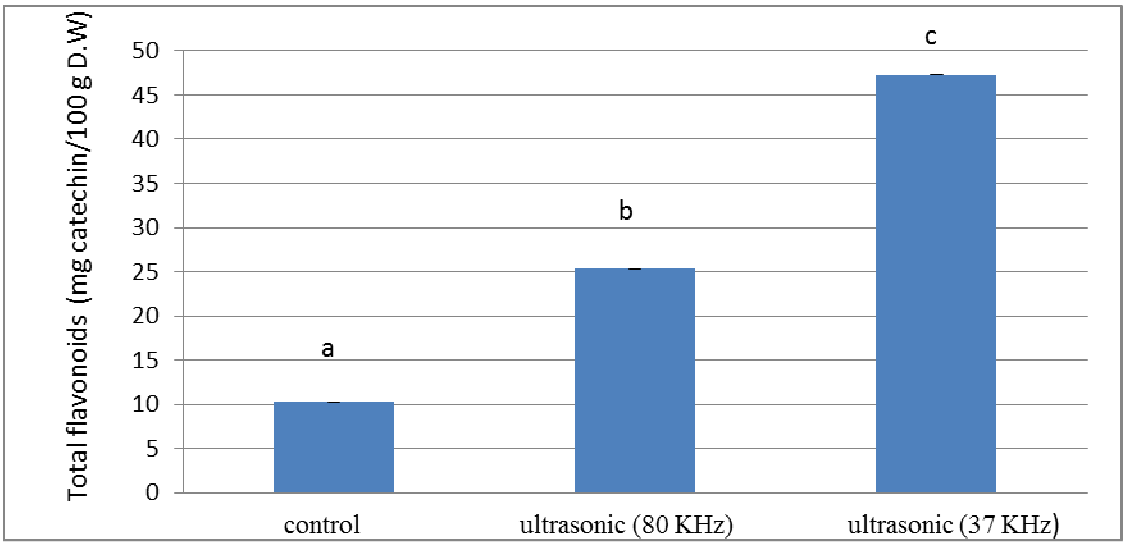


615

616 Figure 2. Total phenol of spinach extract after 30 min at power 50 %, and temperature 40 °C

617 Figure 3

618

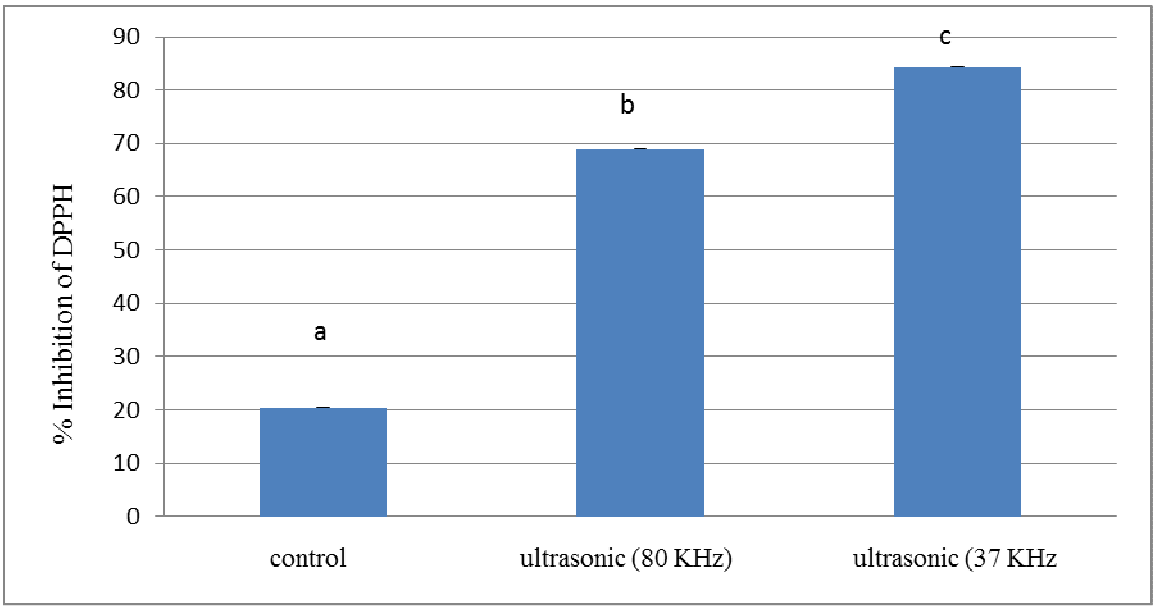


619

620 Figure 3. Total flavonoids of spinach extract after 30 min at power 50 %, and temperature 40 °C

621 Figure 4

622

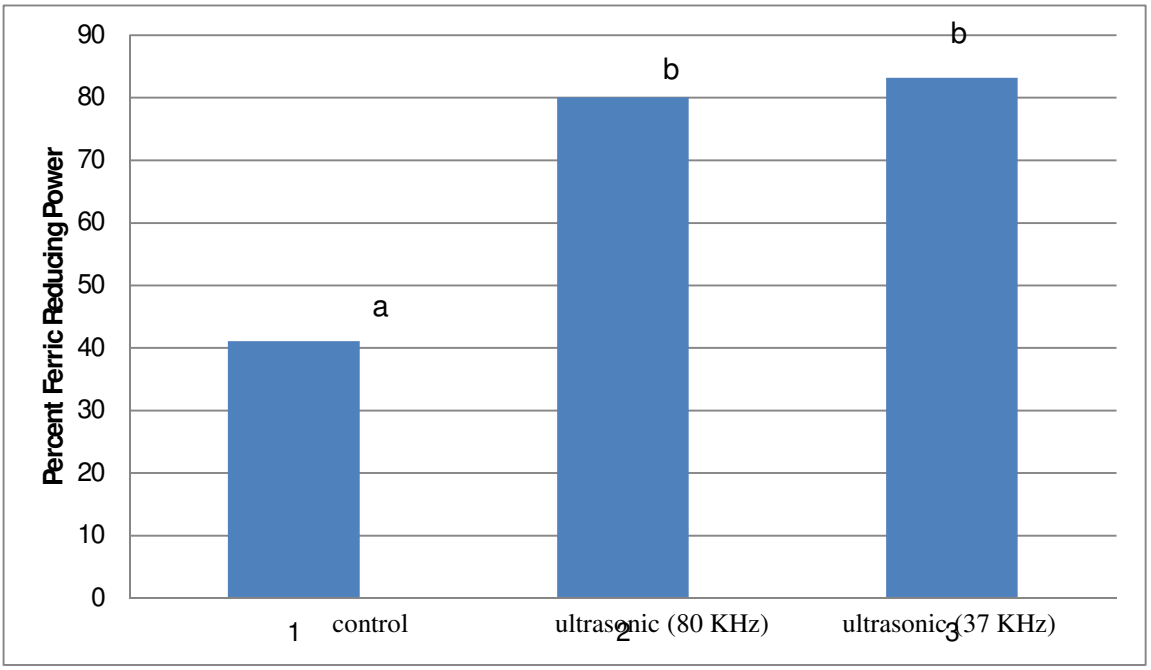


623

624 Figure 4. Percent DPPH free-radical scavenging by spinach extract obtained from 30 min at
625 power 50% and temperature 40 °C

626 Figure 5

627



628

629 Figure 5. Percent ferric reducing antioxidant power of spinach extracts obtained from 30 min at
630 power 50% and temperature 40 °C

631