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Effect of Ultrasonic Treatment on Enzyme Activity and Bioactives of Strawberry Puree

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Abstract

The aim of this study was to evaluate the impact of ultrasound (US) at different frequencies (20, 370, and 583 kHz) and power levels (35 and 48 W) on the residual activity (RA) of peroxidase (POD), polyphenol oxidase (PPO) in strawberry puree. Total anthocyanin content (TAC), total phenolic content (TPC), ferric ion reducing antioxidant power (FRAP) and trolox equivalent antioxidant capacity (TEAC) was also assessed. Results were compared with untreated, thermally treated at 40 ˚C (control), and pasteurised (90 ˚C) strawberry puree. POD and PPO RA was significantly ($p<0.05$) reduced, whilst there was a significant ($p<0.05$) increase in TAC (5 19%) in all US-treated samples in comparison to the untreated samples and the controls. US at 20 kHz (35 W) increased significantly ($p<0.05$) TPC (9%) and FRAP (6%) in strawberry puree, whereas the effect of 583 kHz (48 W) on these parameters was insignificant ($p>0.05$). Pasteurisation inactivated POD and PPO however, decreased dramatically TAC (14%), TPC (17%), and FRAP (9.5%) in strawberry puree. These findings suggest that US is a promising novel non-thermal food technology that can be tailored to improve the quality of strawberry puree by inactivating enzymes responsible for food deterioration whilst maintaining the content of bioactive compounds.

Keywords: ultrasound; peroxidase; polyphenol oxidase; polyphenols; antioxidants; strawberry puree

1. Introduction

Strawberries (*Fragaria* x *ananassa*) are one of the most well-liked fruits globally, while being rich in health-promoting bioactive compounds (Battino *et al.*, 2017). However, strawberries are quite perishable due to flavour, colour, aroma and texture modifications caused by postharvest handling, fungal attack and the activity of the oxidative enzymes polyphenol oxidase (PPO), and peroxidase (POD) (Badawy *et al.*, 2017; Azam *et al.*, 2019). PPO and POD are involved in browning reactions by catalysing the oxidation of phenolic compounds, resulting in loss of the nutritional and market value of food and food products (Cheng *et al.*, 2013; Al-Amrani *et al.*, 2020).

An alternative to conventional thermal technologies for processing and preservation of fruit and vegetable products is ultrasound (US) (Nicolau-Lapeña *et al.*, 2019), i.e. sonic waves above ~16 kHz (Dolatowski *et al.*, 2007); a green food processing technology with a low carbon and water footprint (Chemat *et al.*, 2017). When US propagates into a liquid, produces mechanical, chemical and biochemical effects through the formation and subsequent implosion of cavitation bubbles (Paniwnyk, 2017) resulting in the inactivation of microorganisms and enzymes (Huang *et al.*, 2017). US applications in food technology also include homogenization, mixing, extraction, filtration, crystallization, dehydration, fermentation, degassing, reduction of particle sizes, temporary or permanent modifications of viscosity, modulation of the growth of living cells, cell destruction and dispersion of aggregates, and sterilization of equipment (Gallo *et al.*, 2018).

Sonication at low frequency (20 – 40 kHz) has been reported to extend the shelf-life and inactivate acid-adapted *E. coli* (Yildiz *et al.*, 2019, 2021), increase the content of bioactives namely ascorbic acid, anthocyanins, phenolics and antioxidant activity (Tiwari *et al.*, 2009; Wang *et al.*, 2019), while maintaining colour attributes (Tiwari *et al.*, 2008; Tomadoni *et al.*, 2017) and physicochemical properties (Cheng *et al.*, 2014) in strawberry juice. However, there is no available study examining the impact of higher US frequencies (>100 kHz) on strawberry juice. Earlier studies on model systems of POD (Tsikrika et al., 2018) and PPO (unpublished data) revealed that high frequency US at 378 kHz or 583 kHz was very efficient in the inactivation of these two enzymes. Thus, the current study is focused on the impact of US at a low (20 kHz), and high frequencies (370 kHz, 583 kHz) on POD and PPO activity as well as on the bioactives (TAC, TPC, FRAP and TEAC) in strawberry puree. A control treatment (40 ˚C) and pasteurisation at (90 ˚C) were also carried out for comparison purposes.

2. Materials and methods

2.1. Preparation of strawberry puree

Strawberries (*Fragaria x ananassa* Duch, cultivar Sonata) were acquired from a local grocery store in Dundee, UK. Strawberries were rinsed thoroughly, destemmed, and cut into small pieces prior to blending in a kitchen blender. The puree was stored in polypropylene sterile containers (50 mL) at –25 ˚C. The frozen samples were defrosted (12 h) at 4 °C and then diluted with distilled water (1:1 v/v) before the experiments.

2.2. US and thermal treatments

2.2.1. US at 20 kHz

US at 20 kHz was applied in diluted puree (200 mL; 1:1 v/v dilution) as described in Tsikrika *et al*. (2018) (**S.1**). A 20 mL puree aliquot was removed after 30 and 60 min, instantly dipped in an ice-water bath and then utilised for analysis.

2.2.2. US at 370 and 583 kHz

Sonication at 370 (35 W) and 583 kHz (34 W and 48 W) was conducted as in (Tsikrika *et al.*, 2018) in diluted puree (200 mL, 1:1 v/v dilution) (**S.2**). Sampling and storing of the puree were the same as described in 2.2.1.

The initial temperature for all the US experiments was 20 ± 2 °C and it did not exceed 40 ˚C in all experiments. The temperature profile was recorded every 5 min.

2.2.3. Control treatment (40 °C) and pasteurisation (90 ˚C)

A control treatment at 40 °C (maximum temperature reached during US experiments) was performed for comparison purposes using a thermal bath. Diluted puree (200 mL; 1:1 v/v dilution) was incubated at 40 °C and an aliquot of 20 mL puree was taken after 30 and 60 min of treatment and treated as explained above.

The pasteurisation followed the method of Odriozola-Serrano *et al.* (2008); a conical flask with 200 mL of diluted puree (1:1 v/v dilution) was heated at 90 ˚C, using in a thermostatic bath and 20 mL aliquots were taken out after 30 and 60 s of pasteurisation.

2.3 Enzyme extraction

An adapted method of Sulaiman *et al.* (2015) was employed for the enzyme extraction of the strawberry puree (**S.3**).

2.4. POD activity assay

POD activity (ΔA_{420} min^{-1} g^{-1} FW) assay in strawberry puree was performed according to Kwak *et al.*, (1995) with slight modifications (**S.4**). The residual activity (RA) of POD was calculated by the equation:

$$\text{Residual Activity } (\%) = \frac{A_t}{A_0} \times 100 \quad (1)$$

where A_t is the activity measured after treatment and A_0 is the total initial activity.

2.5 PPO activity assay

PPO activity (ΔA_{420} min^{-1} g^{-1} FW) assay in strawberry puree was conducted following an adapted method of Sulaiman *et al.* (2015) (**S.5**). PPO RA was calculated by Eq (1).

2.6 Antioxidant/Phenolic compounds extraction

The extraction of the bioactive compounds was performed in keeping with the method of Oancea *et al.*(2012) (**S.6**).

2.5 Anthocyanin Analysis (pH shift method)

The total anthocyanin content (TAC) was determined as pelargonidin-3-glucoside (P3g) equivalents (Giusti *et al.*, 1999; Giusti and Wrolstad, 2001; Aaby *et al.*, 2012; Tonutare *et al.*, 2014) following the pH shift method (Ribéreau-Gayon and Stonestreet, 1965) (S.4). TAC was reported as mg anthocyanin per 100 g fresh weight.

2.6 Total Phenolic content (Folin-Ciocalteu method)

The Folin-Ciocalteu method was used for the total phenolic content (TPC) assay (Singleton and Rossi, 1965) (**S.7**). TPC was reported as mg of gallic acid equivalents (GAE) per g fresh weight of strawberry puree.

2.7 Ferric ion reducing antioxidant power (FRAP) assay

FRAP assay was performed in keeping with the method of Benzie and Strain (1996) (**S.8**) and the results were reported as the mean concentration of Fe^{2+} produced (mM).

2.8 *Trolox equivalent antioxidant capacity assay (TEAC)*

A slightly modified method of Pellegrini *et al.* (2003) was used for the TEAC assay (**S.9**). TEAC was reported as mmol of Trolox equivalents per g fresh weight of strawberry puree.

2.9 Statistical analysis

Statistical analysis was performed using a Linear Mixed Model (IBM SPSS Statistics 23). The main effects of time, frequency, and power on every variable as well as possible interaction between them were examined. The probability/significance level was set at a 95%. Experiments were replicated at least twice and analyses were repeated 3 times (n≥6).

3. Results and discussion

3.1 Enzyme activity

Fig. 1 shows POD RA in untreated, US-treated, and control strawberry puree samples. Sonication at 583 kHz and 48 W was the most efficient for POD inactivation, resulting in 29 % and 5 % POD RA after 30 and 60 min of treatment, respectively, while 20 kHz at 35 W led to a 47 % POD RA after 30 min and 24 % POD RA, after 60 min of treatment. POD RA in control samples was 67 % (30 min) and 62 % (60 min) indicating that the low levels of POD RA in the US treated puree were due to an US effect rather than the heat

generated during the process. A 4 % and 2 % POD RA was recorded after pasteurisation for 30 s and 60 s, respectively (data not shown). Treatment duration ($p<0.001$) and US power ($p<0.001$) were the main factors for POD inactivation, whereas the frequency effect was insignificant ($p>0.05$).

These results are in accord with a previous study on a model system of horseradish POD (Tsikrika et al., 2018); US at 583 kHz and 48 W for 60 min led to the total inactivation of the enzyme, whereas 20 kHz and 35 W for 60 min resulted in a 28 % RA. Sonication at 20 kHz and intensities 90, 180, 271, 362, and 452 W/cm^2 for 12 min led to 73 %, 48 %, 38 %, 19 %, and 9 % POD RA in bayberry juice, respectively (Cao *et al.*, 2018). The use of US at 23 kHz and powers of 25 % and 40 % for 150 s caused a 64 % and 16 % POD RA in tomatoes, respectively, whilst a complete inactivation was observed after US at 50 % power for 150 s and 75 % for 90 s (Ercan and Soysal, 2011). A possible mechanism for the inactivation of POD by US has been previously described (Tsikrika et al., 2017); briefly, time resolved fluorescence spectroscopy revealed that US at 378 kHz or 583 kHz led to the subtraction of the haem from the active centre of POD. The presence of a new fluorescent species within the enzyme was also found, which was linked with the synthesis of di-tyrosine

PPO activity in strawberry puree is shown in **Fig. 1**; Interestingly, US at 20 kHz (35 W) was the most efficient treatment, leading to 18 % PPO RA after 30 min and 9 % after 60 min, whereas a 63 % and 37 % PPO RA occurred upon sonication at 583 kHz and 48 W, for 30 and 60 min respectively. The control treatment for 30 and 60 min caused a PPO RA of 75% and 67%, respectively whilst pasteurisation for 30 s and 60 s led to 8 % and 3 % PPO RA. Statistical analysis showed that there was a main effect of treatment duration ($p<0.001$), frequency ($p<0.001$), and power ($p<0.05$) on PPO RA but no significant ($p>0.05$) interactions amongst these factors.

Similarly, Bhat and Goh (2017) reported that US at 25 kHz and 70% power (20 °C) resulted in a significant decrease in PPO activity after 30 and 60 min of treatment. However, there is a wide variation in the literature concerning the influence of US on PPO RA. For instance, Sulaiman et al., (2015) reported that US at 24 kHz and 32.5 W for 10 min caused a 75 % reduction in strawberry PPO RA, whereas Costa et al., (2011) observed an increase in PPO activity in pineapple after the application of US at 19 kHz and intensities from 150 to 300 (W/cm^2) for 2-10 min. On the other hand, PPO activity in mangoes did not change significantly after US treatment at 25 kHz and 55 W/L for 30min (Santos et al. (2015). These contradicting results suggest that PPO inactivation is highly reliant on the source and the sub-type of the enzyme (Cheng et al., 2013).

In general, enzyme inactivation induced by US is attained by a series of phenomena including cavitation, shock waves, and the production of free radicals by water sonolysis (Islam *et al.*, 2014; Cao *et al.*, 2018). As a consequence, alterations may occur on the enzyme structure, resulting in a reduced activity. In parallel, the free radicals produced by US might react with the amino acids present in the enzyme, leading to modifications of the enzyme structure and subsequently of its catalytic action. One or a combination of

the aforementioned mechanisms could also explain the decrease in POD and PPO activity in strawberry puree, as described in this study.

3.3. Total anthocyanin content (TAC)

TAC (P3g equivalents) of untreated, sonicated, and control strawberry puree is presented in **Fig. 2**. Untreated strawberry puree had 15.9 mg P3g eq/ 100 g fresh weight and is in agreement with the literature (Wrolstad *et al.*, 1970; Pilando *et al.*, 1985). All US treatments enhanced TAC in strawberry puree, compared to the untreated puree. The observed increase in TAC can be ascribed to the enhanced extraction of the bound anthocyanins from the suspended pulp caused by US (Tiwari et al., 2008). Control samples had 8 % ($p<0.05$) and 7 % ($p<0.05$) lower TAC after 30 and 60 min of treatment, respectively in comparison to the untreated samples, while TAC in the pasteurised samples was reduced by 15 % ($p<0.05$) and 14 % ($p<0.05$) after 30 and 60 s, respectively. Statistical analysis revealed main effects of frequency ($p<0.05$) and treatment duration ($p<0.05$) but not of power ($p>0.05$) on TAC. The interactions between these factors were not significant ($p>0.05$).

It has been reported that TAC in red raspberry puree was enhanced by 13 % and 7 % after US at 20 kHz and 490 kHz for 10 min, respectively, whereas the effect of 986 kHz was insignificant, which was ascribed to the fact that when the US frequency approximates 1 MHz, the produced cavitation cannot disrupt the cell walls (Golmohamadi *et al.*, 2013). TAC was slightly increased (1 – 2 %) in US-treated strawberry juice at 20 kHz at lower amplitude levels and treatment times, whereas a decrease in TAC followed the use of higher US amplitude levels and a prolonged period (>5 min) (Tiwari et al., 2008), which agrees with the trend in the present study. A slight but significant enhancement in TAC in strawberry juice was also observed upon US at 24 kHz, 0.29 W/mL for 3 min at 55 °C (Yildiz *et al.*, 2021), and at 25 kHz with power set at 70% for 15 and 30 min, at 20 °C (Bhat and Goh, 2017), which was also linked with the rupture of the cell wall in the juice pulp caused by US, leading to the extraction of the bound anthocyanins.

3.4. TPC

Fig. 3 shows the TPC in US treated, control, and untreated strawberry puree samples. TPC in untreated strawberry puree was 1.69 ± 0.07 mg/g fresh weight and it is in agreement with the literature (Cassani et al., 2017; Medina, 2011; Tomadoni et al., 2017; Tulipani et al., 2011). US at 20 kHz and 35 W enhanced the TPC in strawberry puree by 7.2 % ($p<0.05$) and 8.9 % ($p<0.05$), after 30 and 60 min, respectively in comparison with the untreated puree. Higher (poly)phenolic content upon US at frequencies ranging from 20 to 40 kHz has also been reported in strawberry juice (Bhat and Goh, 2017; Tomadoni *et al.*, 2017; Wang *et al.*, 2019; Yildiz *et al.*, 2021). The observed increase in TPC in these juice samples might have occurred by the release of the bound polyphenols following enhanced mass transfer rates and the mechanical disruption of the cell walls due to the formation of microcavities caused by US (Wang *et al.*, 2019). On the other hand, losses in TPC have been reported in red raspberry puree, after the use of US at 490 kHz for 30 min (Golmohamadi *et al*., 2013), and in a phenol model system upon sonication at 358 and 1062 kHz

(Ashokkumar *et al.*, 2008). The latter authors associated the reduction in phenol levels in the sonicated samples with a decrease in $^{\bullet}OH$ radical concentrations, and a concomitant increase in hydroxylated products.

A decreasing trend followed the thermal treatments; TPC in control samples was reduced by 16 % ($p<0.05$) after 30 min of treatment and 18 % ($p<0.05$), after 60 min, in comparison to the untreated samples **(Fig. 3)**, while pasteurisation for 30 s and 60 s resulted in a 12 % ($p<0.05$) and 17 % ($p<0.05$) reduction, respectively (data not shown).

3.5. FRAP

FRAP analysis of strawberry puree is presented in **Fig. 4(a)**. US at 20 kHz and 35 W for 30 and 60 min led to a 6 % ($p<0.05$) and 5 % ($p<0.05$) higher FRAP in strawberry puree respectively in comparison to the untreated puree. Control treatment for 30 and 60 min led to a 9% ($p<0.05$) and 12% ($p<0.05$) decrease in FRAP of strawberry puree, respectively, whilst an 8% ($p<0.05$) and 10% ($p<0.05$) reduction was caused by 30 s and 60 s of pasteurisation, when compared to untreated puree. Statistical analysis showed main effects of frequency ($p<0.001$) and power ($p<0.05$), but not of time ($p>0.05$) for FRAP in US-treated strawberry puree.

A significant enhancement of FRAP has been reported in fresh-cut pineapple samples after sonication at 37 kHz and 25 W or 29 W for 10 – 15 min, and it was correlated to the increase in their total phenolic content (Yeoh and Ali, 2016). A higher reducing power (Fe^{3+}–Fe^{2+} transformation) has also been found in US-treated (40 kHz and 130 W) chokanan mango juice when compared to untreated juice (Santhirasegaram, Razali and Somasundram, 2013) and it was ascribed to the effective removal of occluded oxygen which can also explain the results of the present study.

3.6 TEAC

Fig 4(b) shows the TEAC of untreated, US treated and control strawberry puree. US, and control treatments, as well as pasteurisation, resulted in a slight (<1%) but significant ($p<0.05$) decrease, in the TEAC of strawberry puree, in comparison to untreated puree. Treatment duration had a significant ($p<0.001$) effect on the TEAC of US-treated strawberry puree, whereas that of frequency and power was insignificant ($p>0.05$).

The contradicting results between FRAP and TEAC analysis can be attributed to the different methods they use; FRAP technique measures the ability of antioxidants to reduce ferric iron, whereas the TEAC analysis assesses the scavenging capacity of antioxidants towards long life $ABTS^{\bullet+}$ radicals (Ali *et al.*, 2016). It is known that the antioxidant activity might be due to a combined effect of different compounds, acting either synergistically or antagonistically. For instance, a pro-oxidant activity has been reported for flavonoids (Kurutas, 2016), which may explain the observed decrease in the TEAC of the sonicated strawberry

samples in the current study as opposed to the TAC, and TPC. Ascorbic acid has also been reported to exert pro-oxidant properties (Chakraborthy *et al.*, 2014; Kaźmierczak-Barańska *et al.*, 2020) which might have contributed in the latter findings of the present study.

4. Conclusions

Strawberry puree treated with US at low (20 kHz) and high frequency (370 kHz, and 583 kHz) had significantly lower POD and PPO RA than the control (40 ^{o}C) and the untreated puree. Most US treatments enhanced the TAC, TPC, and FRAP in strawberry puree, compared to the control, pasteurised, and untreated samples. Even though pasteurisation inactivated both enzymes, it had a detrimental impact on the TAC, TPC, FRAP, and TEAC of strawberry puree. These findings suggest that US is a promising alternative to conventional thermal processes of strawberry puree preventing quality losses of the product whilst being effective in inactivating enzymes responsible for food deterioration. Furthermore, considering that high frequency US was more effective than low frequency in decreasing POD activity, future work should focus on this area in relation to other food enzymes.

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Declarations
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Conflicts of Interest The authors declare that they have no conflict of interest.
Data Availability All data and materials are available from the authors upon request.

Ethics Approval Ethics approval was not required for this research.

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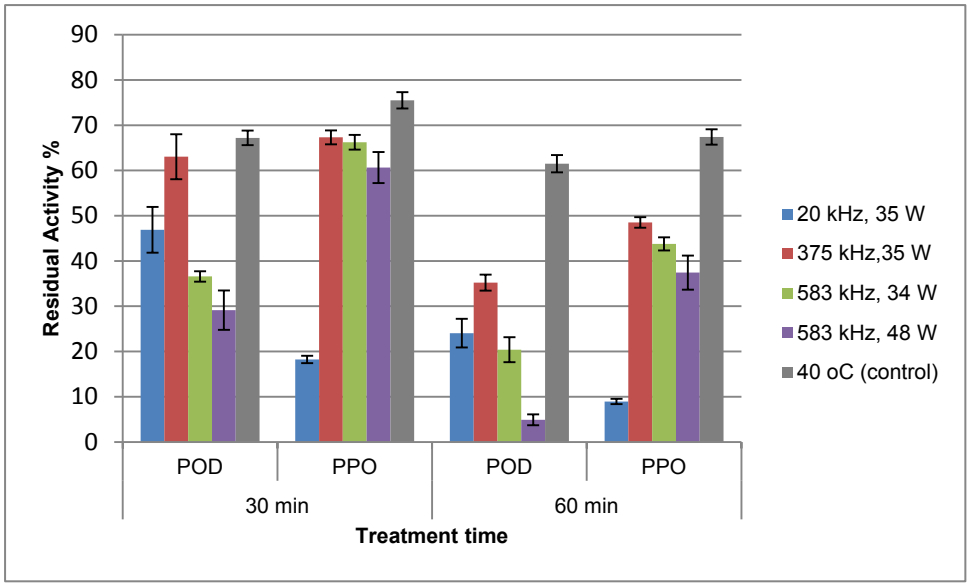

Figure 1 Residual Activity (RA %) of POD and PPO in strawberry puree upon US at low (20 kHz) and high frequency (370 kHz, 583 kHz) and at 34 – 48 W, and control treatment (40 ^{o}C). Values presented are the average (n = 6) ± STDEV.

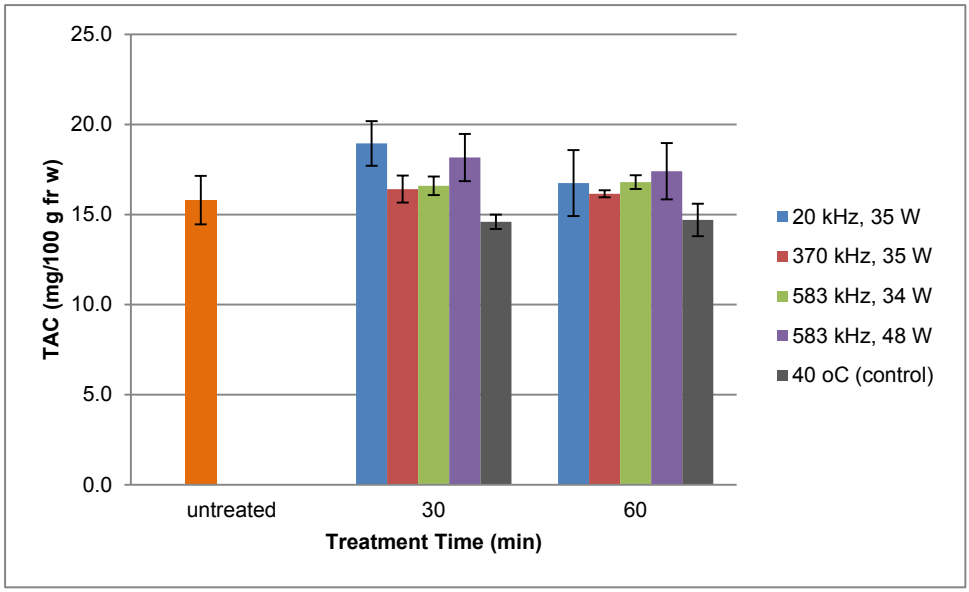

Figure 2. Effect of US and control treatments on total anthocyanin content (TAC) of strawberry puree, compared to untreated samples, and expressed as mg pelargonidin-3-glucoside (P3g) equivalents per 100 g fresh weight. Values presented are the average (n = 9) ± STDEV.

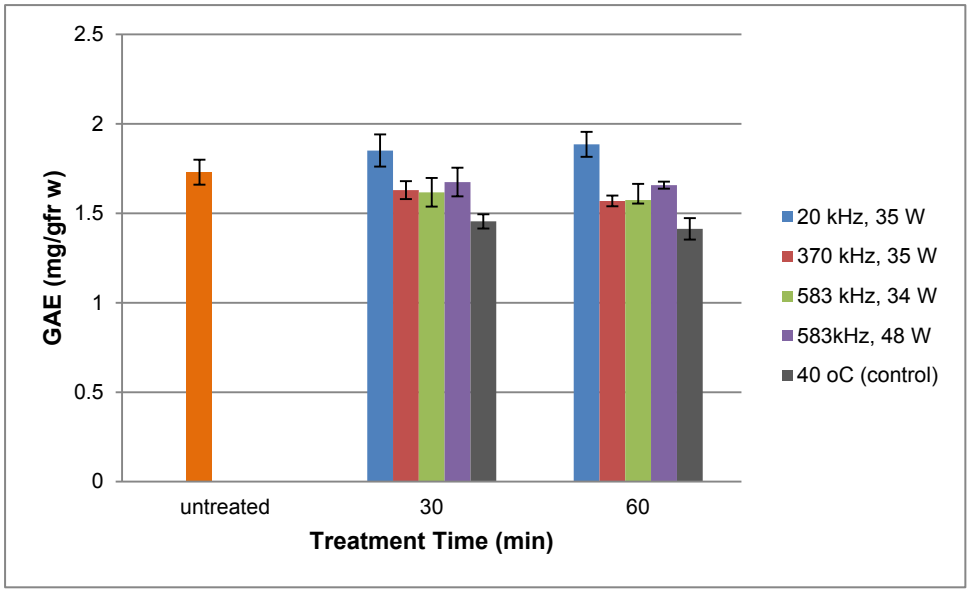

Figure 3. Effect of sonication and control treatment on total phenolic content (TPC) of strawberry puree, compared to untreated puree and reported as gallic acid equivalents (GAE) per g fresh weight. Values presented are the average (n = 9) ± STDEV.

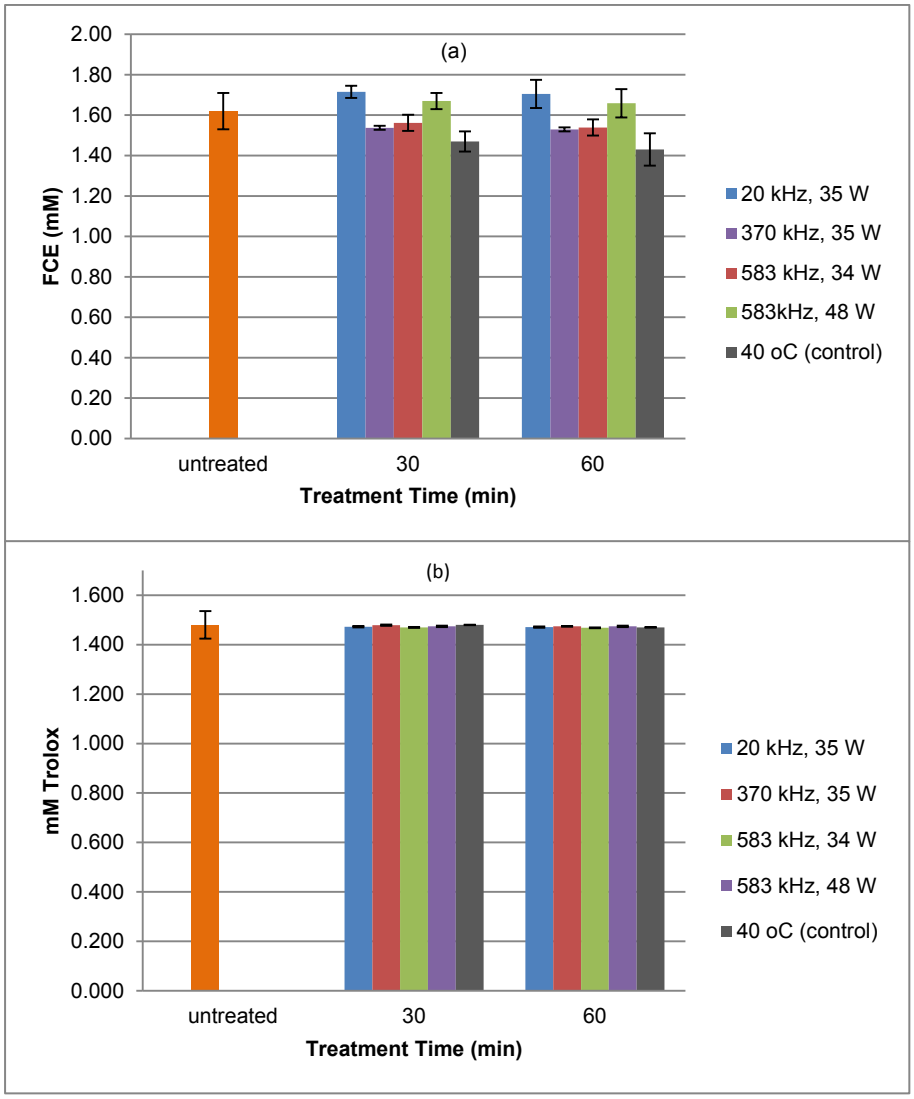

Figure 4 FRAP (mM ferric chloride equivalents (FCE)) (a) and TEAC (mM Trolox equivalents) (b) of untreated, US-treated and control strawberry puree. Values presented are the average (n = 9) ± STDEV.