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# Changes in bioactive compounds, phenolic compounds, and element contents of sour orange seeds following sonication in NaOH, lye, and water

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## Abstract

In the present work, the differences in bioactive compounds, phenolic constituents, and mineral quantities of sour orange seeds, which are by-products of citrus industries, were investigated by sonicating them in NaOH, lye, and water,. The total phenolic and flavonoid contents of sour orange seeds sonicated in NaOH, lye, and water were between 43.70 mg GAE/100 g (lye) and 94.85 mg GAE/100 g (control), and between 50.43 mg/100 g (NaOH) and 144.943 mg/100 g (control), respectively. Antioxidant activities of the control (untreated) and treated sour orange seeds were between 3.39 mmol/kg (control) and 5.95 mmol/kg (water). Sonication agents had significant effects on the total phenolic compounds of sour orange seeds. Kaempferol and quercetin amounts of the sour orange seeds were between 20.96 mg/100 g (water) and 30.18 mg/100 g (NaOH), and between 2.81 mg/100 g (control) and 7.67 mg/100 g (NaOH), respectively. P and K amounts of the control and treated sour orange seeds were between 885 mg/kg (water) and 4,235 mg/kg (control), and between 372 mg/kg (water) and 6,012 mg/kg (control), respectively. The highest microelements detected in the samples were Fe, Zn, and B, in decreasing order.

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## Introduction

Recently, studies have begun to be conducted on citrus seeds, which have attracted increasing attention due to their nutritional and economic values (Mahato *et al.*, 2019; Mahawar *et al.*, 2020). Valorising citrus industry by-products into other beneficial products can mitigate environmental pollution issues (Zema *et al.*, 2018).

Citrus plants are cultivated in many countries with tropical or subtropical climates, and belong to the Rutaceae family (Matthaus and Özcan, 2012; Liu *et al.*, 2022; Suri *et al.*, 2022). The portion of citrus fruits consumed by humans does not exceed 45 - 60%, and the remaining inedible part is thrown into landfills or used as animal feed (Zayed *et al.*, 2021). Most citrus fruits contain 20 - 25% seeds, and this seed contains more than 40% oil. Citrus seeds have been found to contain more oil than most seeds and fruits such as cotton, soybean, and olive (Anwar *et al.*, 2008; Al Juhaimi *et al.*, 2018; Suri *et al.*, 2022).

Citrus seed oil has been found to contain bioactive substances such as carotenoids, phenolic compounds, phytosterols, and saturated and unsaturated fatty acids (Waheed *et al.*, 2009; Al Juhaimi *et al.*, 2018; Atolani *et al.*, 2020; Liu *et al.*, 2022). The most basic fatty acids of citrus oils are linoleic, oleic, and palmitic acids (Al Juhaimi *et al.*, 2018; Liu *et al.*, 2022). Parts of citrus fruits are rich in polyunsaturated fatty acids, and are a dietary source (Matthaus and Özcan, 2012; Sicari *et al.*, 2017; Liu *et al.*, 2022). In previous studies, the chemical compositions of various fruit wastes such as core, peel, pulp, stem, leaf, and seed were determined and a significant amount of bioactive components were identified (Monagas *et al.*, 2003; Xu *et al.*, 2016). Since fruit waste creates a great economic impact on businesses from handling to unloading, the evaluation of fruit waste reduces the waste load in storage areas, thus it is important to encourage producers to establish a sustainable economy (Campos *et al.*, 2020).

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Ultrasonic-assisted extraction has great importance in the food and herbal product industry due to its practicality, cost-effectiveness, time and energy saving, low temperature extraction, and preservation of the quality of the extract. It has been reported that ultrasonic extraction system increases the extraction efficiency of bioactive compounds higher than the traditional extraction method (Barba *et al.,* 2016). Ultrasonic-assisted extraction method can also increase the oil yield extracted from seeds. Chemical effects produced during acoustic cavitation can be used advantageously to increase the functionality of certain food compounds. It is thought that citrus seeds obtained as a result of the intensive consumption of citrus fruits and their processing in the food industry, such as fruit juice, can be a good source of oil due to the high oil content in order to add to the economy and protect the environment.

Minimising waste in food processing is one of the most fundamental issues for the modern food industry. Since the jam, marmalade, and fruit juice industry produces a significant amount of waste every year, the disposal of these wastes leads to significant economic burden and environmental pollution. Therefore, the evaluation of wastes from the food industry as a source of new bioactive compounds will provide significant added value to the agricultural industrial field. The purpose of the present work was to define the changes in bioactive compounds, phenolic constituents, and mineral quantities of sour orange seeds, which are by-products of citrus industries, by sonicating them in NaOH, lye, and water.

## Materials and methods

### *Materials*

The sour orange fruits (25 kg) obtained from Gülnar (Büyükeceli) district of Mersin were cut in half, and the seeds were cleaned with water after removal from the pulp. After seeds were dried in the open air, they were powdered using a grinder. Then, the ground seeds were kept at low temperature (+4°C) until subsequent analysis.

### *Methods*

#### *Pre-treatments*

Ground seeds (2 g) were separately mixed with 20 mL of NaOH (2%), ash (20%), and pure water. The samples were shaken in a water bath for 2 h, filtered, added with 25 mL of the same solvents, and subjected to an ultrasonic water bath for 25 min. The filtered samples were washed five times with pure water, laid on filter paper, and dried for 1 h. Samples were used in analyses after pre-treatments.

#### *Moisture content*

The KERN & SOHN GmbH infrared moisture analyser was used for measuring the moisture of sour orange seeds (AOAC, 2005).

#### *Protein content*

The protein content of the sour orange seeds was determined using AOAC (2005) method (Eq. 1):

Protein (%): Nitrogen × 6.25 (Eq. 1)

#### *Oil content*

After 10 g of cleaned, dried, and ground sour orange seeds were weighed into the Soxhlet cartridge, they were put in a balloon-mounted extractor containing petroleum ether. They were extracted with solvent in the Soxhlet for 6 h. Then, the petroleum ether was evaporated from the oil solvent-mixed micella by evaporator, and the oil content of the seeds was determined (AOAC, 2005).

#### *Extraction procedure*

The extraction process for determining the bioactive components and antioxidant activities of the seeds was performed according to Garcia-Salas *et al.* (2013). Pretreated samples (2 g) were added to 10 mL of methanol. After the solution was sonicated in a sonication system at a frequency of 35 Hz for 30 min, the solution was centrifuged at 6,000 rpm for 10 min. The volume of the extract concentrated at 37°C in an evaporator was brought to 10 mL with methanol, and filtered.

#### *Total phenolic content*

The Folin-Ciocalteu reagent was used to determine the total phenolic content of extracts according to Yoo *et al*. (2004).

#### *Total flavonoid content*

The total flavonoid content of the samples were determined using the $NaNO_2$ and $AlCl_3$ colorimetric method according to Hogan *et al*. (2009).

#### *Antioxidant activity (DPPH assay)*

The antioxidant activity of extracts was determined using 1,1-diphenyl-2-picrylhydrazyl according to Lee *et al*. (1998). After pre-processing, the absorbance was recorded at 517 nm.

*Identification of phenolic constituents*

HPLC (Shimadzu) equipped with a PDA detector and an Inertsil ODS-3 (5 µm; 4.6 × 250 mm) column was applied for chromatographic separation of phenolic compounds of citrus seeds sonicated in different solvents (Figure 1). The mobile phase was a mixture of 0.05% acetic acid in water (A) and acetonitrile (B) at a flow rate of 1 mL/min at 30°C. The injection volume was 20 µL. The peaks were recorded at 280 nm using a PDA detector. The elution programme was employed: 0 - 0.10 min 8% B; 0.10 - 2 min 10% B; 2 - 27 min 30% B; 27 - 37 min 56% B; 37 - 37.10 min 8% B; and 37.10 - 45 min 8% B. The total running time per sample was 60 min.

*Mineral determination*

After 0.5 g powdered sour orange seeds were dried in an oven at 70°C till constant weight, it was incinerated in 5 mL of 65% $HNO_3$ and 2 mL of 35% $H_2O_2$ in a microwave. After making up the resulting solution volume (20 mL) with distilled water, the mineral amounts of the solution were detected by the ICP-OES.

*Statistical analyses*

All analyses were carried out in three replicates, and the results were expressed as mean ± standard deviation. Means of the main sources of variation showing statistically significant differences were examined using the Tukey multiple comparison test, and the level of statistical significance was set at $p < 0.05$. Analysis of variance (ANOVA) was performed using JMP version 9.0.

## Results and discussion

*Moisture contents and bioactive properties of sour orange seeds sonicated with different sonication solvents*

Moisture contents and bioactive properties of sour orange seeds extracted with NaOH, lye, and water are presented in Table 1.

Sonication had significant effect on sour orange seeds ($p < 0.05$). The moisture contents of sour orange seeds treated by NaOH, lye, and water were between 5.46% (control) and 77.89% (water). The moisture content of the seeds sonicated with NaOH and lye was lower than that of the seeds sonicated with water. Total phenolic and flavonoid contents of the sour orange seeds sonicated by NaOH, lye, and water were between 43.70 mg GAE/100 g (lye) and 94.85 mg GAE/100 g (control), and between 50.43 mg/100 g (NaOH) and 144.943 mg/100 g (control), respectively. Antioxidant capacities of the control (untreated) and treated sour orange seeds were between 3.39 mmol/kg (control) and 5.95 mmol/kg (water). The amount of bioactive compounds and antioxidant activities of samples sonicated in water were higher than those sonicated in NaOH and lye solutions. In addition, flavonoid compounds were found to be more soluble in water, while the solubility of phenolic compounds was slightly lower. This could have been due to the fact that a significant portion of the phenolic compounds are in bound form. While the moisture content in the seed was found to be quite low (5.46%), the moisture content of sour orange seeds treated with sonication solutions increased significantly, and varied between 72.87% (lye) and 77.89% (water).

Sonication of sour orange seeds was effective on total phenolics, total flavonoids, and antioxidant activities depending on the solvent used. The total flavonoid content of untreated sour orange seed (94.85 mg/100 g) was lower than the total flavonoid content of lemon seed oil (11.6 mg/g) reported by Ordoudi *et al*. (2018). Phenolic constituents have been found to be significantly present in citrus seeds (Zayed *et al*., 2021). İnan *et al*. (2018) pointed out that the total phenolic contents of 19 citrus seed oils varied between 46.6 and 92.8 mg GAE/kg. Nayak *et al*. (2015) determined the antioxidant capacity (DPPH assay) of *C. sinensis* peel with microwave and ultrasound-assisted extraction at 337.16 and 433.09 mL/L, respectively. Chen *et al*. (2017) determined the antioxidant activities of dried *C. reticulata* exocarp and pericarp extracts at 0.52 to 0.68 mg/mL (DPPH assay). In another study, the antioxidant capacity of aqueous or methanolic extracts of lemon pomace was found to be 0.17 and 0.13 mg TE/g sample (DPPH assay), respectively (Papoutsis *et al*., 2016). Some differences were observed when the total phenolic contents, total flavonoid contents, and antioxidant activities of sour orange seeds were compared with the results of previous studies (Papoutsis *et al*., 2016; Chen *et al*., 2017; İnan *et al*., 2018; Zayed *et al*., 2021). These differences could have been due to the cultivar, species, harvest time, maturity status, sonication process parameters, and some other analytical conditions.

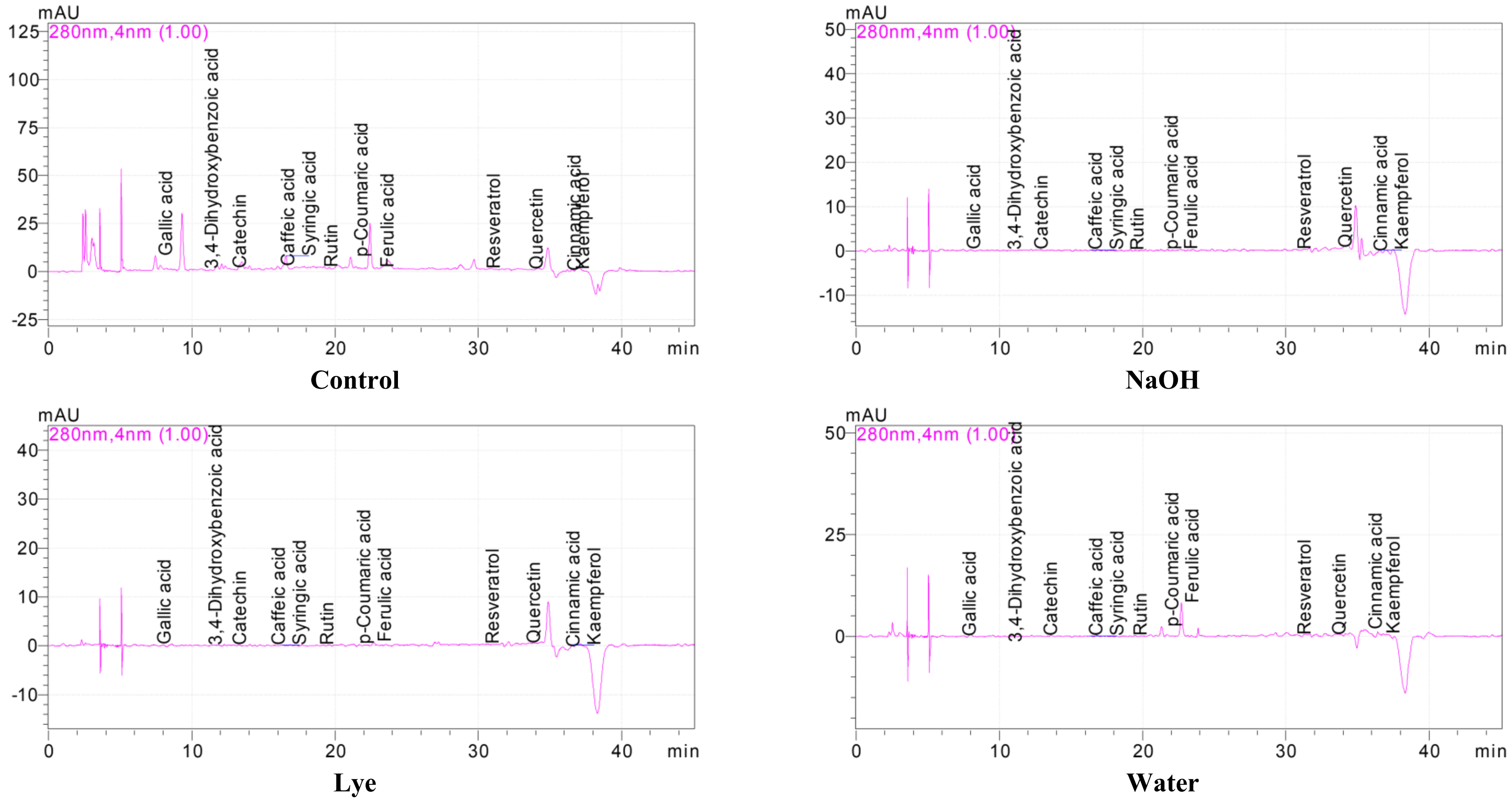

**Figure 1.** Phenolic chromatograms of sour orange seeds following sonication by different solvents, and control.

**Table 1.** Bioactive properties of sour orange seeds following sonication by different solvents, and control.

| Pretreatment | Moisture content (%) | Total phenolic content (mg GAE/100 g) | Total flavonoid content (mg/100 g) | Antioxidant activity (mmol/kg) |
|---|---|---|---|---|
| Control | 5.46 ± 0.22$^{d}$ | 94.85 ± 4.07$^{a}$ | 144.93 ± 6.48$^{a}$ | 3.39 ± 0.04$^{d}$ |
| NaOH | 74.74 ± 1.81$^{b}$ | 44.52 ± 2.25$^{c}$ | 50.43 ± 2.97$^{d}$ | 4.34 ± 0.00$^{b}$ |
| Lye | 72.87 ± 1.38$^{c}$ | 43.70 ± 1.60$^{cd}$ | 70.63 ± 4.19$^{c}$ | 4.08 ± 0.01$^{bc}$ |
| Water | 77.89 ± 0.88$^{a}$ | 69.32 ± 1.81$^{b}$ | 81.30 ± 1.80$^{b}$ | 5.95 ± 0.03$^{a}$ |

Values are mean ± SD. Means with different lowercase superscripts in similar column are significantly different ($p < 0.05$).

### *Phenolic compounds of sour orange seeds sonicated with different sonication solvents*

The phenolic compounds of sour orange seeds sonicated with different sonication agents (NaOH, lye, and water) were characterised by HPLC, and their quantitative values are depicted in Table 2.

Sonication agents had significant effects on the phenolic compounds of sour orange seeds. Kaempferol, quercetin, gallic acid, catechin, and rutin were the main phenolic constituents of control and sonicated sour orange seeds. Kaempferol and quercetin quantities of the sour orange seeds were between 20.96 mg/100 g (water) and 30.18 mg/100 g (NaOH), and 2.81 mg/100 g (control) and 7.67 mg/100 g (NaOH), respectively. In addition, gallic acid and catechin quantities were 0.39 mg/100 g (control). Rutin amounts of control and sonicated sour orange seeds were between 0.58 mg/100 g (lye) and 2.66 mg/100 g (control). In general, sonication decreased the phenolic compounds in the samples. The highest decrease in phenolic compounds of seeds was established in NaOH and lye treatments.

Partial differences were monitored in the amounts of phenolic compounds in sonication with lye and water. This could have been due to the slower penetration of NaOH into the seeds. However, it can be said that sonication with lye and water was more effective than sonication with NaOH. The key phenolic constituents characterised in *Citrus amblycarpa* seeds were 1,2-dihydroxybenzene, catechin, kaempferol, and isorhamnetin (Yang *et al.*, 2020). Citrus seed varieties contained 13.00 - 15.25 mg/kg catechin, 52.59 - 80.01 rutin, 234.28 - 321.03 naringin, 890.01 - 909.67 hesperidin, 6.00 - 10.01 kaempferol, 28.01 - 42.43 gallic acid, 5.01 - 7.13 syringic, 222.97 - 363.30 ferulic acid, 58.08 - 78.01 rosmarinic acid, 41.65 - 48.01 *p*-2-hydroxycinnamic acid, 4.01 - 7.35 lutein, and 4.01 - 7.64 mg/kg total carotenoid. The main basic components of hydroxycinnamic acids found in the cell wall of citrus fruits were chlorogenic, ferulic, *p*-coumaric, caffeic, and sinapic acids (especially lemon) (Nayak *et al.*, 2015; Papoutsis *et al.*, 2018). The dominant phenolic compounds of different citrus fruits were gallic, protocatechuic, *p*-hydroxybenzoic, and vinylic acids. Citrus seed varieties contained 13.00 - 15.25 catechin, 52.59 - 80.01 rutin, 234.28 - 321.03 naringin, 890.01 - 909.67 hesperidin, 6.00 - 10.01 kaempferol, 28.01 - 42.43 gallic acid, 5.01 - 7.13 syringic, 222.97 - 363.30 ferulic acid, 58.08 - 78.01 rosmarinic acid, 41.65 - 48.01 *p*-2-hydroxycinnamic acid, and 4.01-7.35 mg/kg lutein. While the types of phenolic compounds in our findings were similar to the literature data, differences were observed in their amounts. These differences could have been due to the type of solution applied, sonication, plant variety, plant growing conditions, and genetic structure.

### *Elemental contents of sour orange seeds sonicated with different sonication solvents*

The elemental contents of sour orange seeds sonicated with NaOH, lye, and water are given in Table 3. Compared to the control, the effect of solvents on the elemental contents was limited and not very effective. However, among the solvents, the elemental contents of the seeds in the extraction with lye were slightly higher than the extraction with NaOH and water. It was observed that sour orange seeds were rich in macro-elements such as P, K, Ca, and Mg. P and K amounts of the control and treated sour orange seeds were between 885 mg/kg (water) and 4,235 mg/kg (control), and 372 mg/kg (water) and 6,012 mg/kg (control), respectively. Furthermore, Ca amounts of the sour orange seeds were between 763 mg/kg (control) and 4,747 mg/kg (control), while Mg contents were between 164 mg/kg (NaOH) and 1,294 mg/kg (control). Fe and Zn amounts of the control and treated sour orange seeds

**Table 2.** Phenolic compounds of sour orange seeds following sonication by different solvents, and control.

| Phenolic compound (mg/100 g) | Control | NaOH | Lye | Water |
|---|---|---|---|---|
| Gallic acid | 3.21 ± 0.27$^{b}$ | 0.39 ± 0.04$^{d}$ | 2.29 ± 0.50$^{c}$ | 5.71 ± 1.33$^{a}$ |
| 3,4-Dihydroxybenzoic acid | 0.95 ± 0.28$^{a}$ | 0.38 ± 0.01$^{c}$ | 0.29 ± 0.02$^{d}$ | 0.52 ± 0.07$^{b}$ |
| Catechin | 3.08 ± 1.13$^{a}$ | 1.24 ± 0.04$^{d}$ | 1.36 ± 0.09$^{c}$ | 2.01 ± 0.20$^{b}$ |
| Caffeic acid | 0.80 ± 0.15$^{a}$ | 0.25 ± 0.02$^{bc}$ | 0.26 ± 0.02$^{b}$ | 0.26 ± 0.02$^{b}$ |
| Syringic acid | 3.18 ± 0.21$^{a}$ | 0.30 ± 0.05$^{d}$ | 0.45 ± 0.08$^{c}$ | 0.50 ± 0.01$^{b}$ |
| Rutin | 2.66 ± 0.41$^{a}$ | 0.77 ± 0.03$^{c}$ | 0.58 ± 0.03$^{d}$ | 1.14 ± 0.06$^{b}$ |
| *p*-Coumaric acid | 0.93 ± 0.07$^{a}$ | 0.19 ± 0.01$^{cd}$ | 0.20 ± 0.01$^{c}$ | 0.85 ± 0.08$^{b}$ |
| Ferulic acid | 1.56 ± 1.59$^{b}$ | 0.39 ± 0.01$^{c}$ | 0.36 ± 0.02$^{cd}$ | 6.25 ± 0.02$^{a}$ |
| Resveratrol | 0.16 ± 0.02$^{c}$ | 0.16 ± 0.01$^{c}$ | 0.43 ± 0.05$^{b}$ | 0.48 ± 0.04$^{a}$ |
| Quercetin | 2.81 ± 0.77$^{d}$ | 7.67 ± 0.65$^{a}$ | 3.18 ± 0.66$^{c}$ | 4.49 ± 0.92$^{b}$ |
| Cinnamic acid | 0.63 ± 0.23$^{d}$ | 2.02 ± 0.22$^{c}$ | 2.39 ± 0.23$^{b}$ | 4.10 ± 0.34$^{a}$ |
| Kaempferol | 29.50 ± 7.11$^{b}$ | 30.18 ± 1.01$^{a}$ | 22.63 ± 0.29$^{c}$ | 20.96 ± 0.65$^{d}$ |

Values are mean ± SD. Means with different lowercase superscripts in similar row are significantly different ($p < 0.05$).

**Table 3.** Protein (%) and mineral contents (mg/kg) of sour orange seeds following sonication by different solvents, and control.

| Solvent | Protein | P | K | Ca | Mg |
|---|---|---|---|---|---|
| Control | 12.03 ± 0.18$^{A}$ | 4235 ± 21.65$^{A}$ | 6012 ± 61$^{A}$ | 4747 ± 83.50$^{A}$ | 1294 ± 5.42$^{A}$ |
| NaOH | 1.22 ± 0.025$^{C}$ | 1141 ± 88.31$^{C}$ | 491 ± 34$^{C}$ | 1154 ± 25.10$^{B}$ | 164 ± 4.29$^{B}$ |
| Lye | 1.24 ± 0.046$^{C}$ | 1471 ± 46.00$^{B}$ | 3257 ± 206$^{B}$ | 1202 ± 71.01$^{B}$ | 183 ± 15.68$^{B}$ |
| Water | 2.02 ± 0.15$^{B}$ | 885 ± 37.41$^{C}$ | 372 ± 9$^{C}$ | 763 ± 36.09$^{C}$ | 165 ± 2.12$^{B}$ |
| **Solvent** | **Fe** | **Zn** | **Cu** | **Mn** | **B** |
| Control | 13.81 ± 0.59$^{A}$ | 11.51 ± 0.32$^{B}$ | 6.34 ± 0.05$^{A}$ | 5.47 ± 0.02$^{A}$ | 20.70 ± 0.26$^{A}$ |
| NaOH | 3.39 ± 0.03$^{B}$ | 2.46 ± 0.21$^{B}$ | 0.87 ± 0.01$^{B}$ | 0.87 ± 0.01$^{B}$ | 1.84 ± 0.11$^{C}$ |
| Lye | 4.59 ± 0.49$^{B}$ | 2.80 ± 0.11$^{B}$ | 0.93 ± 0.03$^{B}$ | 0.93 ± 0.30$^{B}$ | 2.80 ± 0.09$^{B}$ |
| Water | 2.90 ± 0.19$^{B}$ | 2.81 ± 0.13$^{A}$ | 0.58 ± 0.04$^{C}$ | 0.62 ± 0.02$^{C}$ | 2.50 ± 0.06$^{BC}$ |

Values are mean ± SD. Means with different uppercase superscripts in similar column are significantly different ($p < 0.05$).

were between 2.90 mg/kg (water) and 13.81 mg/kg (control), and 2.46 mg/kg (NaOH) and 11.51 mg/kg (control), respectively. The fact that the macro- and micro-elemental contents in the seeds were not very soluble in the applied solvents could have been due to the fact that alkaline solutions not able to penetrate the seeds well. These results indicated that the relatively high solubility of phenolic compounds, along with the limited dissolution of mineral components in the extract, may improve the seeds' suitability for use in dietary supplements. Consequently, the seeds can be regarded as a promising source of bioactive compounds with potential nutritional and functional benefits.

## Conclusion

The amount of bioactive compounds and antioxidant activities transferred to water following sonication were higher than those treated with NaOH and lye solutions. In addition, flavonoid compounds were found to be more soluble with water, while the solubility of total phenolic contents slightly decreased. Sonication agents had significant effects on the total phenolic compounds of sour orange seeds. In general, the sonication treatments applied to sour orange seeds caused a decrease in phenolic compounds in raw seeds. Among the solvents, the elemental contents of the seeds in the extraction with lye were slightly higher than the extraction with NaOH and water. The fact that the macro- and micro-element contents in the seeds were not very soluble in the applied solvents might have been due to alkaline solutions not being able to penetrate the seeds very much. These findings suggested that the relatively high solubility of phenolic compounds, coupled with the limited release of mineral constituents into the extract, may enhance the suitability of the seeds as candidates for dietary supplement applications. Therefore, the seeds can be considered a promising source of bioactive compounds with potential nutritional and functional value.

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