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Different Effects of Microwave and Ultrasound on the Stability of (*all-E*)-Astaxanthin

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Both microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) have been widely applied in the extraction of a variety of biologically active compounds including carotenoids due to their lower pollution to environment, high extraction efficiency, lower cost, and shorter extraction time as compared to conventional extraction techniques. However, there are few reports on their effects on the stability of these compounds. In the present study, the stability of (*all-E*)-astaxanthin, one of the carotenoids, was examined under the action of both ultrasound and microwave. Results showed that microwave induced the isomerization of (*all-E*)-astaxanthin to its *Z* analogues, preferentially to (13*Z*)-astaxanthin as analyzed by HPLC coupled with diode array detection and LC-MS; and the percentage of the isomerization increased with increasing both treatment time and microwave power. In contrast to the microwave, the ultrasound degraded (*all-E*)-astaxanthin to unidentified colorless compound(s) as suggested by HPLC analysis and UV/vis measurements, and the degradation likewise increased as both treatment time and ultrasonic power increased. The results presented here emphasized that both MAE and UAE techniques should be carefully used in the extraction of unstable compounds such as (*all-E*)-astaxanthin.

KEYWORDS: Astaxanthin; ultrasound; microwave; isomerization; degradation; carotenoids

INTRODUCTION

Carotenoids are naturally occurring pigments that provide the yellow, orange, and red colors of fruit, vegetables, plants, and marine animals. These colors are a result of the presence of conjugated bonds that are responsible for their light absorption as well scavenging free radicals activity (*1*). According to epidemiological studies, carotenoids play an important role in the prevention of cancer, cataracts, and aging diseases such as heart disease. There also exists an inverse relationship between the consumption of foods containing carotenoids and the risk of lung, intestinal, skin, and bladder cancer (*2*, *3*). In contrast, recent studies showed that supplements containing β-carotene were harmful to cigarette smokers, resulting in an increase in the incidence of lung cancer and in overall mortality (*4*, *5*). However, the use of natural food colorants including carotenoids in food industry and pharmaceuticals are receiving increasing attention.

Astaxanthin (3,3′-dihydroxy-β,β′-carotene-4,4′-dione) (**Figure 1**) is a commonly encountered keto-carotenoid, which occurs in certain marine animals and plants including salmon, trout, shrimp, lobster, and algae (*6*, *7*). Due to its attractive pink color, it has been used as a colorant in aquaculture. Increasing numbers of studies on food, cosmetic, and medical application of astaxanthin have been undertaken because of its high antioxidant activity (*8*, *9*) and other biological functions (*10*−*12*). Previous studies showed that astaxanthin has about 100−500 times higher antioxidative activity than α-tocopherol (*7*, *13*, *14*).

Extraction is widely used for the separation of these biologically functional carotenoids from various plant, bacteria, or animal sources. The processes of conventional liquid extraction such as stirring extraction and Soxhlet extraction for solid and semisolid materials are generally time-consuming and laborious. Moreover, large volumes of organic solvent are required, which can lead to sample contamination, losses due to volatilization during concentration steps, and environmental pollution from solvent waste. Recently, many new green extraction methods have been used for the separation of carotenoids. One of these methods was matrix solid-phase dispersion (MSPD) which is a gentle and expeditious extraction technique for a solid and viscous sample, but it was primarily applied to a small quantity of samples as the pretreatment method for analyzing samples (*15*). Microwave-assisted extraction (MAE) is known as another popular extraction technology. It carries the advantage of being a simple device and has a wide area of application, high extraction efficiency, good reproducibility, and low consumption of organic solvents and time as well as low environmental pollution. In recent years, this technique has been widely used

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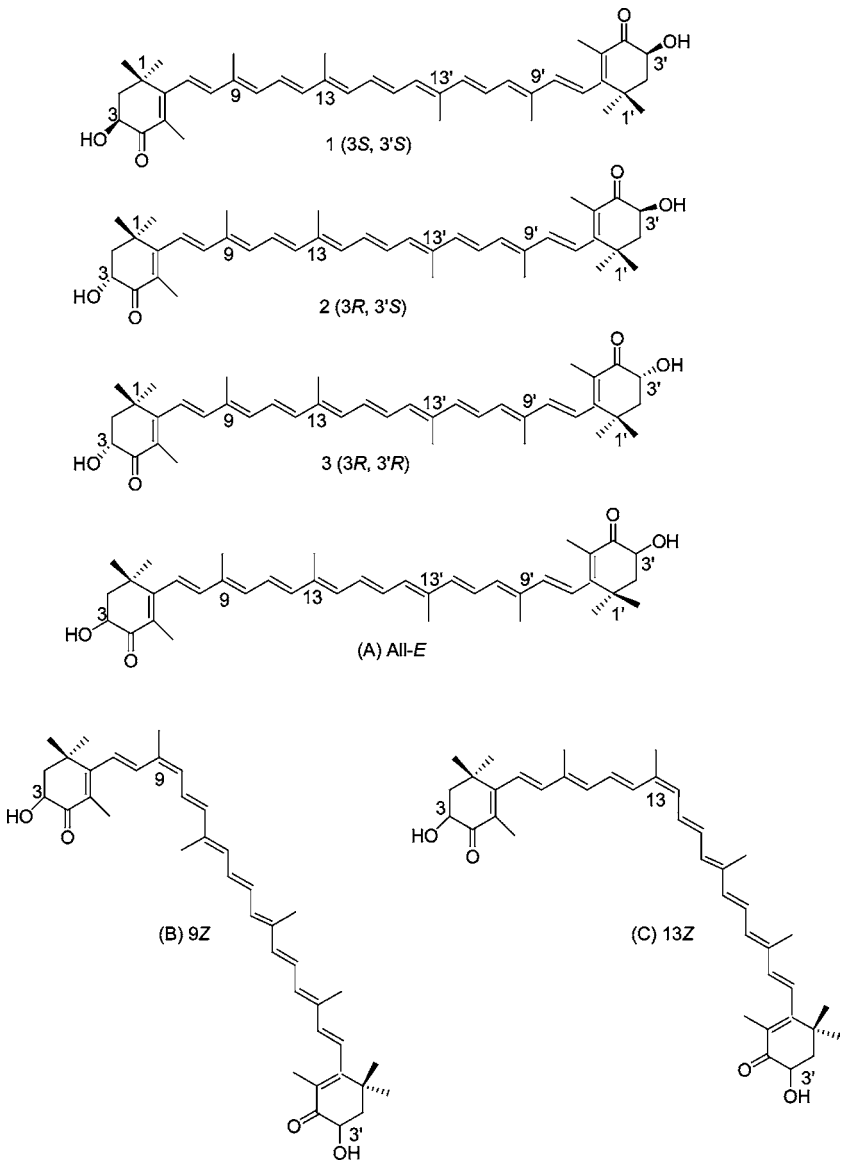

Figure 1. Structures of astaxanthin optical isomers (*all-E*)-(3*S*,3′*S*)- (**1**), (*all-E*)-(3*R*,3′*S*;*meso*)- (**2**), and (*all-E*)-(3*R*,3′*R*)-astaxanthin (**3**) and geometrical isomers (*all-E*)- (**1A**, **2A**, **3A**), (9*Z*)-(**1B**, **2B**, **3B**), and (13*Z*)- astaxanthin (**1C**, **2C**, **3C**).

for the extraction of natural products (*16*, *17*) including carotenoids (*18*, *19*) as well as for the extraction of effective constituents in medicines (*20*). Ultrasound-assisted extraction (UAE) is also an environmentally friendly technique with increased productivity. It uses high-frequency (typically higher than 16 kHz) sound to disrupt the target compound from cells (*21*). To date, there have been several reports on the application of UAE in the separation of various biologically active compounds such as the anti-cancer drug camptothecin from *Nothapodytes foetida* (*16*), isoflavones from freeze-dried ground soybean (*22*), phenolic compounds from alperujo (*23*), and astaxanthin from microorganisms (*24*, *25*).

Recently, our research has focused on the application of the foregoing green extraction techniques such as MAE and UAE in the isolation of the biologically active products such as astaxanthin from food materials. It is important to elucidate the effect of microwave and ultrasound on astaxanthin prior to extraction with these two techniques. However, to date, there have been few reports available about this. In the present study, (*all-E*)-astaxanthin was used as a model compound, and the influence of the microwave and ultrasound on its stability was evaluated.

MATERIALS AND METHODS

Chemicals. HPLC-grade solvents, including methanol, acetonitrile, ethanol, dichloromethane, and chloroform, were purchased from Kangkede Chemical Co. (Tianjin, China). (*all-E*)-Astaxanthin was purchased from Sigma Chemical Co. (St. Louis, MO) and is a mixture of optical isomers (3*R*,3′*R*)-, (3*S*,3′*S*)-, and (3*R*,3′*S*)-astaxanthins with a molar ratio of 1:1:2. This mixture was dissolved in chloroform as a stock solution of which its concentration was 635.3 μM. The stock solution was kept in a brown vial protected with N_2.

Ultrasonic Treatment. Ultrasonic treatment was performed with a high-intensity ultrasonic probe system of 650 W and 25 kHz (JY-92-II, Ningbo Scientz Biotechnology Co., Ningbo, China) equipped with a 6 mm microtip. The stock solution of (*all-E*)-astaxanthin was diluted to 17.2 μM with ethanol. A series of 5 mL aliquots were added to numbered small beakers immersed in an ice bath in darkness, which were treated 1, 2, 3, 4, 5, and 6 min at 100, 200, 300, 400, 500, and 600 W, respectively, through dipping the microtip in the solution 1 cm below the surface. After sonication, all the treated solutions were stored at −18 °C for the concentration determination of (*all-E*)-astaxanthin by HPLC.

Microwave Treatment. All microwave treatments were carried out in a microwave oven of a frequency of 2455 MHz with maximum power of 700 W (NJL07-3, Jiequan microwave instrument Co., Nanjing,

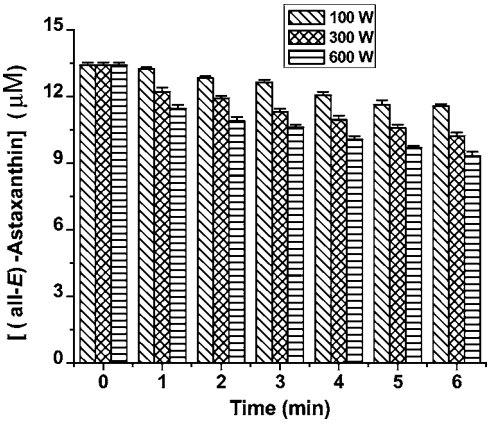

Figure 2. Changes in concentration of (*all-E*)-astaxanthin with time after treatment with microwave at different powers. Each value was expressed as mean ± S.D. ($n = 3$).

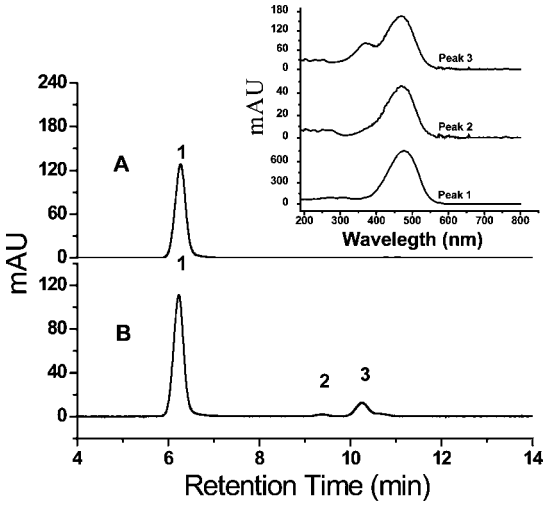

Figure 3. Chromatograms of untreated (*all-E*)-astaxanthin (spectrum A) and (*all-E*)-astaxanthin treated with microwave (spectrum B) for 2 min at 600 W. Inset: UV/vis spectra of peaks 1, 2, and 3 obtained by a diode array detector.

China), which was equipped with a condensor on its top. The stock solution of (*all-E*)-astaxanthin was diluted to 13.5 μM with ethanol. A series of 5 mL aliquots were placed into numbered flasks connected with the condenser at red light. These solutions were treated 1, 2, 3, 4, 5, and 6 min at 100, 200, 300, 400, 500, and 600 W, respectively. All the treated samples were stored at −18 °C in darkness for the measurement of (*all-E*)-astaxanthin concentration.

High-Performance Liquid Chromatography (HPLC). A solution of (*all-E*)-astaxanthin in chlorform (635.3 μM) was prepared, and the solution was diluted to 1.59, 3.18, 6.35, 12.7, 19.1, 25.4, and 31.8 μM with ethanol. These solutions were quantitatively analyzed by a LC-10AT HPLC (Shimadzu, Kyoto, Japan) equipped with a RID-10A photodiode array detector (Shimadzu, Kyoto, Japan) at 480 nm to produce a standard curve of (*all-E*)-astaxanthin concentration versus peak area. Concentrations of (*all-E*)-astaxanthin between 1.59 and 31.8 μM exhibit an excellent linear correlation to the peak area ($R^2 = 0.9999$). The detection limit is 0.0168 μM. On the basis of the standard curve, the content of (*all-E*)-astaxanthin of the samples treated by both ultrasound and microwave were measured by HPLC at the same conditions as the standard samples. The chromatography conditions were as follows: Kromasil C18 column (250 × 4.6 mm), a mixture of dichloromethane, methanol, acetonitrile, and water (5:85:5:5) as the mobile phase, flow rate was 1 mL/min, peaks were detected at 480 nm, the three-dimensional chromatogram was recorded from 200 to 800 nm, the column temperature was 25 °C.

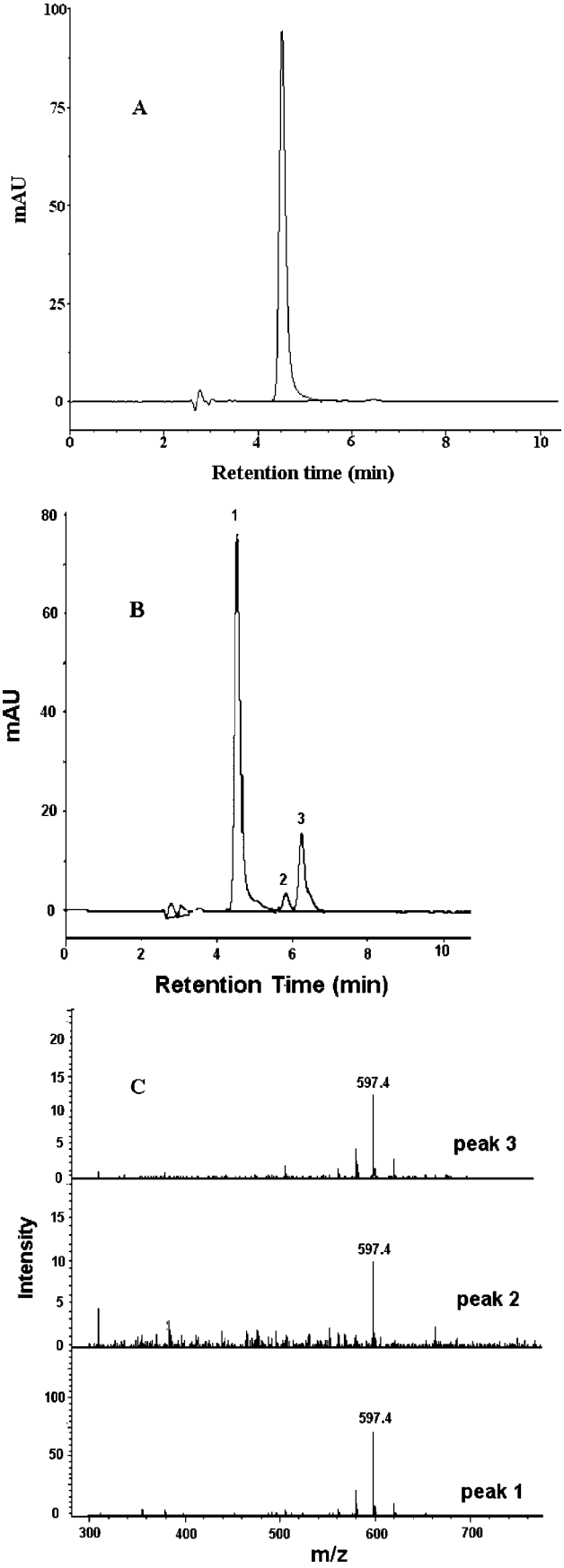

Figure 4. HPLC-APCI-MS analysis of (*all-E*)-astaxanthin treated 6 min by microwave at 600 W. (**A**) the chromatogram of untreated (*all-E*)-astaxanthin. (**B**) the chromatogram of (*all-E*)-astaxanthin treated 6 min by microwave at 600 W. (**C**) Positive ion APCI mass spectra; spectrum A for peak 1, spectrum B for peak 2, and spectrum C for peak 3.

The concentrations of (9*Z*)- and (13*Z*)-astaxanthin were calculated by their peak areas at 480 nm with their respective molar extinction coefficients of 247 660 and 190 512 M^{-1} cm^{-1} (*24*).

HPLC-APCI-MS Analysis. To identify the products produced from the isomerization of (*all-E*)-astaxanthin, a sample treated for 6 min by microwave at 600 W in darkness was analyzed by HPLC-APCI-MS (1100LC/MSD-Trap, Agilent, USA). The chromatography conditions were as follows: Kromasil C18 column (250 × 4.6 mm), a mixture of methanol and acetonitrile (85:15) as mobile phase, flow rate was 1 mL/min, peaks were detected at 480 nm, the column temperature was 25 °C. Mass spectra were monitored in the *m*/*z* range 300−1000. The APCI vaporizer temperature was held at 450 °C, the corona discharge voltage was optimized to 4 kV. The spectrometer had been tuned to optimize the signal of *m*/*z* 597.4 ($[M + H]^+$ of astaxanthin). Detection was performed in the positive mode with an ion injection time of 5 ms and the accumulation of 20 ms for each scan with a maximum ion time of 200 ms for each mass spectrum. All of the above experiments were undertaken in darkness or red room light.

Statistical Analysis. Each ultrasonic and microwave treatment was run in triplicate. Each sample was injected to HPLC three times, and the mean values were obtained. All the data were subjected to analysis of variance and Duncan's multiple range tests, using a SPSS statistical system.

RESULTS AND DISCUSSION

Treatment of *trans*-Astaxanthin with Microwave. Figure 2 exhibited that the concentration of (*all-E*)-astaxanthin varied with treatment time by microwave over the power range of 100−600 W. With increasing the treatment time from 1 to 6 min, the concentration of (*all-E*)-astaxanthin decreased at all three examined microwave powers. Likewise, the concentration of (*all-E*)-astaxanthin also decreased as the microwave power was increased from 100 to 600 W. For example, the concentration of (*all-E*)-astaxanthin decreased by 1.25% (100 W), 7.62% (300 W), and 14.0% (600 W) after 1 min treatment whereas (*all-E*)-astaxanthin decreased by 13.9% (100 W), 23.8% (300 W), and 30.5% (600 W), respectively, after 6 min treatment. Thus the concentration of (*all-E*)-astaxanthin is a function of both treatment time and microwave power. All these results suggest that microwave has a marked influence on the stability of (*all-E*)-astaxanthin, especially at longer treatment times and a higher power.

HPLC and HPLC-APCI-MS Analysis of *trans*-Astaxanthin after Treatment with Microwave. Previous studies showed that organic solvents (*27*, *28*), iodine (*26*, *29*), and Cu-(II) ion (*30*) as well as environmental factors such as heat, light, and oxygen (*31*, *32*) induced (*all-E*)-astaxanthin and its diacetate to convert to their *Z* analogues, especially (13*Z*)- and (9*Z*)-astaxanthin. To examine whether the decrease of (*all-E*)-astaxanthin concentration stems from its (*E*/*Z*)-isomerization induced by microwave, (*all-E*)-astaxanthin was analyzed by HPLC after a 2 min treatment at 600 W. Chromatograms of both untreated and treated (*all-E*)-astaxanthin are shown in **Figure 3**. As expected, only one peak appears at ∼6.3 min in control sample, which corresponds to (*all-E*)-astaxanthin (**Figure 3**, spectrum A), which is actually a mixture of three optical isomers including (3*R*,3′*R*)-, (3*S*,3′*S*)-, and (3*R*,3′*S*)-astaxanthins with a molar ratio of 1:1:2. This assignment was confirmed by its UV spectrum (**Figure 3**, inset) exhibiting a maximum absorbance at ∼480 nm (*27*, *28*, *30*). In contrast, two new peaks appeared at ∼9.4 min (peak 2) and ∼10.5 min (peak 3) after microwave treatment. Moreover, with increasing the treatment time or the microwave power, the area of these two new peaks increased while the peak area corresponding to (*all-E*)-astaxanthin decreased (data not shown), a finding indicating that (*all-E*)-astaxanthin converts to two new species. Isomerization of carotenoids from *E* isomer to *Z* isomer would cause a hypsochromic shift in the main absorption band (*33*). To identify the two species, their UV/vis spectra was obtained by a diode array detector and shown in the inset of **Figure 3**. The UV/vis spectrum of peak 2 exhibited a maximum absorbance at ∼470 nm, which is assigned to (9*Z*)-astaxanthin (**Figure 3**, inset) (*27*, *28*, *30*, *34*). Since (*all-E*)-astaxanthin is a chemical mixture (1:1:2) of (3*R*,3′*R*)-, (3*S*,3′*S*)-, and (3*R*,3′*S*)-astaxanthins, peak 2 represents the mixture of the following four carotenoids: (9*Z*,3*R*,3′*R*)-astaxanthin, (9*Z*,3*S*,3′*S*)-astaxanthin, (9*Z*,3*R*,3′*S*)-astaxanthin, and (9′*Z*, 3*R*, 3′*S*)-astaxanthin. In contrast to UV/vis spectra of peaks 1 and 2, the spectrum of peak 3 exhibited two maximal absorbances appearing at ∼470 nm and ∼373 nm, which is identified as (13*Z*)-astaxanthin (**Figure 3**, inset) (*27*, *28*, *30*, *34*). Similar to peak 2, peak 3 also represents a mixture of four optical isomers containing (13*Z*,3*R*,3′*R*)-astaxanthin, (13*Z*,3*S*,3′*S*)-astaxanthin, (13*Z*,3*R*,3′*S*)-astaxanthin, and (13′*Z*,3*R*,3′*S*)-astaxanthin. In addition, it is worth noting that peak 3 is larger than peak 2, indicating that (*all-E*)-astaxanthin was isomerized to (13*Z*)-astaxanthin more than to (9*Z*)-astaxanthin, being in accord with recent measurements showing that all tested organic solvents (*27*) or Cu^{2+} (*30*) induced (*all-E*)-astaxanthin to preferentially convert to (13*Z*)-astaxanthin rather than its (9*Z*)-form.

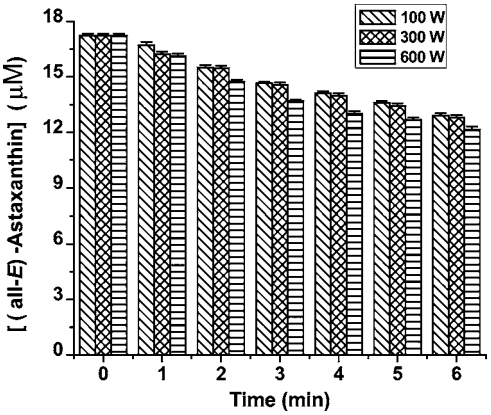

Figure 5. Changes of (*all-E*)-astaxanthin concentration with different treatment times by ultrasonic disintegrator at different powers. Each value was expressed as mean ± S.D. ($n = 3$).

To confirm the assignment of peaks 2 and 3 based on UV/vis spectra, the sample of (*all-E*)-astaxanthin treated for 6 min by microwave at 600 W was further analyzed by HPLC-APCI-MS. As shown in **Figure 3**, spectrum B, there are also three peaks appearing in the chromatogram (**Figure 4B**) although they appear at slightly different retention times from those in **Figure 3** because of the different mobile phase used. However, there is only one peak in the chromatogram of untreated (*all-E*)-astaxanthin (**Figure 4A**). The expected protonated molecular ion, $[M + H]^+$, of *trans*-astaxanthin correspounding to peak 1 appeared at *m*/*z* 597.4 (**Figure 4C**) (*35*). The two other species corresponding to peaks 2 and 3 have the same protonated molecular ion at *m*/*z* (597.4) as (*all-E*)-astaxanthin while their mass spectra are significantly distinct from that of (*all-E*)-astaxanthin, a result demonstrating that the two species are isomers of (*all-E*)-astaxanthin. By comparison with reported spectra (*34*, *35*−*37*), the two species were identified as (9*Z*)-astaxanthin (peak 2) and (13*Z*)-astaxanthin (peak 3), consistent with the above results obtained from UV/vis spectra.

Treatment of (*all-E*)-Astaxanthin with Ultrasound. When (*all-E*)-astaxanthin was treated with ultrasound at 100, 300, and 600 W, the concentration of (*all-E*)-astaxanthin generally decreased as treatment time increased from 1 to 6 min (**Figure**

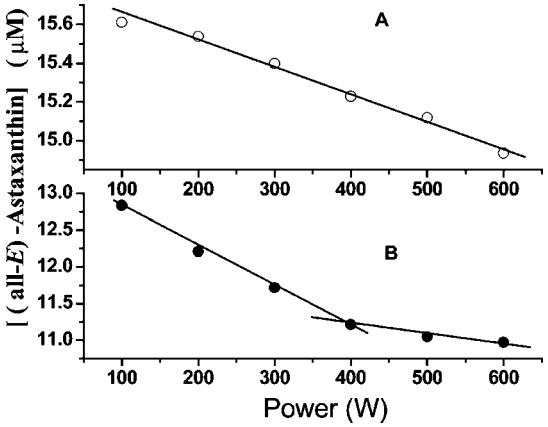

Figure 6. Changes in concentration of (*all-E*)-astaxanthin with power after treated for 2 min with either ultrasound (**A**) or microwave (**B**).

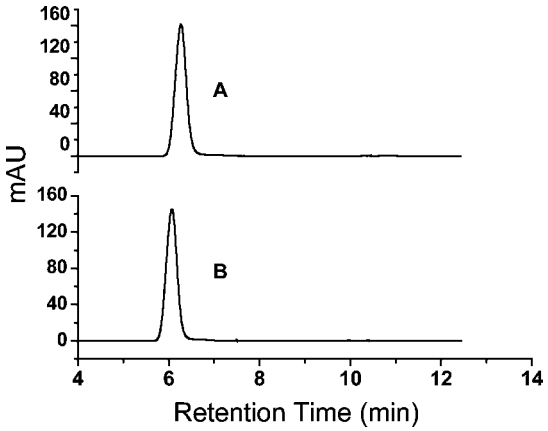

Figure 7. Chromatograms of untreated (*all-E*)-astaxanthin (spectrum A) and (*all-E*)-astaxanthin treated with ultrasound (spectrum B) for 2 min at 600 W.

5). The content of (*all-E*)-astaxanthin decreased by 3.11% (100 W), 5.74% (300 W), and 6.42% (600 W) after 1 min treatment with ultrasound. As expected, 6 min ultrasonic treatment resulted in a larger decrease in the concentration of (*all-E*)-astaxanthin by 25.1% (100 W), 25.5% (300 W), and 29.4% (600 W), respectively. Thus both treatment time and ultrasound power likewise have significant effects on the stability of the (*all-E*)-astaxanthin concentration. However, the decrease of the concentration of (*all-E*)-astaxanthin exhibited a linear dependence ($r^2 = 0.9885$) on the ultrasound power upon 2 min treatment with ultrasound at 100−600 W (**Figure 6A**) while there is a complex relationship (**Figure 6B**) between the decrease of the concentration of (*all-E*)-astaxanthin and the microwave power, indicating that the influences of both ultrasound and microwave on the stability of the (*all-E*)-astaxanthin are distinct.

Consistent with this conclusion, neither (9*Z*)-astaxanthin nor (13*Z*)-astaxanthin appeared in the HPLC chromatogram after (*all-E*)-astaxanthin was treated for 2 min at 600 W by ultrasound (**Figure 7**, spectrum B), suggesting that (*all-E*)-astaxanthin may convert to colorless compound(s). Further support for this view comes from UV/vis measurements of (*all-E*)-astaxanthin from 260 to 700 nm after treatment with ultrasound and microwave, respectively. As compared to the spectrum of control sample (**Figure 8**, spectrum A), ultrasonic treatment resulted in the appearance of two new absorbances at 294 and 324 nm in the UV/vis spectrum of (*all-E*)-astaxanthin (**Figure 8**, spectrum C) while its absorbance at ~480 nm, which is characteristic of (*all-E*)-astaxanthin, decreased. This result suggests that (*all-E*)-astaxanthin may be degraded to colorless compound(s) by

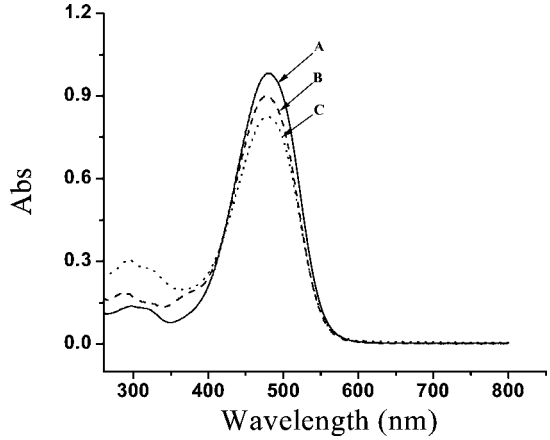

Figure 8. UV/vis spectra of (*all-E*)-astaxanthin (spectrum A), and (*all-E*)-astaxanthin treated for 6 min by mirowave at 600 W (spectrum B) or by ultrasound at 600 W (spectrum C).

ultrasound. In contrast, no significant absorbance was observed with (*all-E*)-astaxanthin sample treated by microwave at both 294 and 324 nm, but an absorbance at ~ 373 nm appeared, which is characteristic of (13*Z*)-astaxanthin (**Figure 8**, spectrum B), again indicating that ultrasound and microwave have different effects on the stability of (*all-E*)-astaxanthin. In addition, it is observed that the 480 nm absorbance decreased more upon treatment with ultrasound (**Figure 8**, spectrum C) than with microwave (**Figure 8**, spectrum B), indicating that the ultrasound has a greater effect on the stability of (*all-E*)-astaxanthin than microwave under the same condition.

It is known that increased extraction efficiency of organic compounds by ultrasound is ascribed to a phenomenon known as cavitation produced in the solvent by the propagation of an ultrasonic wave (*21*). The cavitation involves the formation, growth, and rapid collapse of microscopic bubbles. The chemical effects produced by cavitation generate local high temperatures and mechanical action between solid and liquid interfaces (*38*, *39*), which possibly lead to the degradation of (*all-E*)-astaxanthin into unidentified colorless molecule(s). As compared to ultrasound, microwave is relatively milder, and may cause a lower increase in solution temperature through interaction with the polar molecules, so it only induces the isomerization of (*all-E*)-astaxanthin into its *Z* analogues. The different mechanism of (*all-E*)-astaxanthin destabilized by ultrasound and microwave is currently under study.

Conclusions. This study demonstrates that both ultrasound and microwave have significant effects on the stability of (*all-E*)-astaxanthin. However, their effects are pronouncedly different. Microwave only induces the conversion of (*all-E*)-astaxanthin to its *Z* forms, preferentially to (13*Z*)-astaxanthin, while ultrasound probably degrades this pigment into colorless compound(s). All the results emphasize the careful application of both ultrasound- and microwave-assisted extraction techniques in the extraction of carotenoids or other unstable biological molecules.

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