International Food Research Journal 20(1): 331-338 (2013)

Journal homepage: *http://www.ifrj.upm.edu.my*

Ultrasound-assisted extraction of valuable compounds from winter melon (*Benincasa hispida*) seeds

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Article history

Received: 25 May 2012
Received in revised form: 17 June 2012
Accepted:20 June 2012

Keywords

Winter melon *(Benincasa hispida)*
ultrasound-assisted extraction
complete randomised design
crude extraction yield
radical scavenging activity
conventional Soxhlet extraction

Abstract

Ultrasound-assisted extraction (UAE) was applied for the extraction of bioactive valuable compounds from winter melon (*Benincasa hispida*) seeds. Effects of amplitude (25-75%), temperature (40-60°C) and sonication time (20-60 min) on crude extraction yield (CEY) and radical scavenging activities (RSA, % inhibition of DPPH$^{\cdot}$ and ABTS$^{\cdot+}$ free radicals) of extracts were determined using complete randomised design (CRD). The results showed that the CEY and RSA of extracts significantly affected by independent variables. The maximum value of CEY (97.14±0.36 mgg^{-1}), scavenging of DPPH$^{\cdot}$ radicals (32.12 ± 0.38%) and scavenging of ABTS$^{\cdot+}$ radicals (40.52±0.73%) were obtained at the combined treatment conditions of 75%, 55°C and 40 min. The UAE results obtained were compared with those achieved by using conventional Soxhlet extraction (CSE) method. It was found UAE allowed extraction at lower temperature and the extracts obtained posses higher quality compare with CSE. UAE is a promising environment friendly technique for the extraction of bioactive compounds from winter melon (*Benincasa hispida*) seeds.

Introduction

As a result of consumer demand great attention has been focused on replacement of synthetic additives by natural ones. In this regard isolation and characterization of valuable compounds and effective natural antioxidants play a key role. Natural antioxidants could operate as free radical scavengers, reducing agents, complex of pro-oxidants metals and quenchers of the formation of singlet oxygen. These valuable compounds could be consumed in the food industry and their desired biological effects on human body have been proved (Lee *et al*., 2000; Koski *et al*., 2002). Considering to harmful effect of free radical formation in foods and biological systems attention on radical scavenging activity is necessary. Different methods are currently used to determine the antioxidant potency of plant extracts. DPPH$^{\cdot}$ and ABTS$^{\cdot+}$ radical scavenging methods are common spectro-photometric procedures for determining the antioxidant activities of different compounds (Gulcin *et al*., 2004).

Winter melon (*Benicasa hispida*) is widely used as vegetable in tropical countries and belongs to the family of *Cucurbitaceae* (Grover *et al*., 2001). *Benicasa hispida* is called winter melon, white gourd, ash pumpkin, tallow gourd, white pumpkin, ash gourd, wax gourd, gourd melon and Chinese watermelon or Chinese preserving melon in English (Morton 1997; Marr *et al*., 2000; Mohd Zaini *et al*., 2011). Wax gourd is named for the white wax covering on mature fruit which enhaces long-term postharvest life (Grover *et al*., 2001). Mature winter melon is characterised by its thickly deposited hairs with easily removable waxy bloom while the young fruit has fleshy, succulent and hairy attributes (Mohd Zaini *et al*., 2011). Conventional Soxhlet extraction (CSE) is considered as a standard procedure for extraction of valuable compounds from different sources (Wang and Weller, 2006). The CSE application is

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restricted due to the high amount of organic solvents consumption which has harmful effects on human health and environment. Furthermore, degradation of bioactive compounds is unenviable during this method as a result of applying high temperature during long extraction time (Zhang *et al*., 2008). Recently, environmental friendly techniques are being interested to develop the ‘‘Green Chemistry’’ concept (Kamran Khan *et al*., 2010). Therefore, an improved or better extraction technique is necessary and favored. Ultrasound-assisted extraction (UAE) is one of the environmental friendly techniques to isolate different natural valuable compounds from different plant matrices (Ran *et al*., 2009). UAE could be suggested as an alternative to conventional extraction techniques due to its inexpensive, simple and efficient characteristics. Acoustic cavitation phenomenon which generated by passing ultrasound waves through solvent system is caused higher efficiency of this technique. This phenomenon permits better penetration of the solvent into the sample, increasing the release of the solutes from the matrix to the solvent. Temperature could enhance the liberation of solutes from cell matrix through degradation of cell walls (Morelli and Prado, 2012). Considering to reduced operating temperature this technique is advisable for extraction of thermo sensitive compounds. Any type of solvents can be used to separate various types of natural compounds during this process. Scientific documents have described radical scavenging activity (RSA) of different seeds extract (Espin *et al*., 2000; Reische *et al*., 2002; Ramadan *et al*., 2003). So far, no information is currently available on the extraction of valuable compounds from Benincasa hispida seeds using UAE process.

Therefore, this work aimed to outline the potentiality of UAE in the fast preparation of extracts in good yield with strong antioxidant activity from winter melon (*Benincasa hispida*) seeds. Several variables such as amplitude, temperature and sonication time that could potentially affect the crude extraction yield (CEY) and radical scavenging activity (RSA) of seeds extract were investigated. Finally, the UAE results obtained were compared with those achieved by using a conventional Soxhlet extraction (CSE) method.

Material and Methods

Material and reagents

Whole winter melon (*Benincasa hispida*) fruits were purchased from a local market in Serdang, Selangor, Malaysia. Seeds were separated and washed under tap water then dried at 40°C in a ventilated drying oven (1350FX, USA) for 24 h. The seeds were ground in grinder mill (MX-335, Panasonic, Malaysia) for 10 s to produce a powder with an approximate size of 1.5-2.5 mm before extraction.

The following chemicals were used: Ethanol (EtOH, 99.5%, analytical grade) obtained from Scharlau Chemical, European Union. Butylated hydroxytoluene (BHT), 2-2´Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt ($ABTS^{\cdot +}$), 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$) and catechin was purchased from Fisher (Pittsburgh, PA, USA). All other chemicals were either of chromatography or analytical grade.

Ultrasound-assisted extraction

Five grams (5 g) of winter melon (*Benincasa hispida*) seeds sample were extracted in a beaker (100 ml) containing a volume of extraction solvent. The extraction solvent was ethanol (99.5%) as a food grade solvent which is recommended by the US Food and Drug Administration for extraction purposes (Bartnick *et al*., 2006). The extraction was performed by ultrasound equipment (Sonics and Materials Inc., Model VC505, Danbury, CT, USA). Temperature was monitored by immersing the beaker into an automatically temperature control water-bath (Memmert, WNE14. Memmert GmbH Co. KG, Germany) and no significant increase in temperature (below 2ºC) was observed during extraction. Control experiments were carried out without using ultrasound waves. The immersed seeds in extraction solvent were subjected to ultrasonic waves during a period of 20 to 60 min. After extraction, the crude extracts were filtered through the Whatman No. 1. Then, ethanol was removed from the extracts by evaporation under vacuum at 40ºC on a rotary evaporator. Subsequently, the residual solvent was removed by drying in oven at 40ºC for 1 h and by flushing with 99.9% nitrogen. Gravimetric measurement (Eq. 1) was used to obtain the amount of total crude extract weight. The scheme of experimental set-up presents in Figure 1.

$$CEY = \frac{m_e}{m_s} \times 1000 \qquad (\text{Eq. 1})$$

The results of CEY were expressed as mgg^{-1} sample. Where me is the crude extract mass (g) and ms is the extracted sample mass (g). Finally, the winter melon seeds extract were kept for further analysis.

Conventional Soxhlet extraction method

Five gram (5 g) of grounded winter melon (*Benincasa hispida*) seeds was put into extraction thimble and then the thimble was transferred into a Soxhlet apparatus. Approximately, 150 mL of

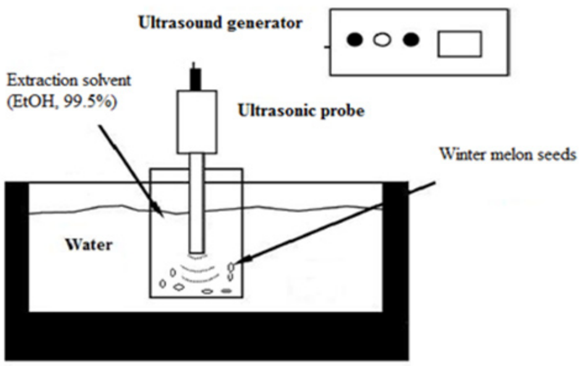

Figure 1. Scheme of experimental set-up for UAE

Table 1. Experimental levels of the variables used in complete randomized design (CRD) full factorial

Independent Variables	Levels		
	1	2	3
Amplitude (%)	25	50	75
Temperature (ºC)	45	55	65
Sonication time (min)	20	40	60

selected solvent (ethanol, 99.5%) were added to each flask which was connected to the extractor. Each extraction was performed in triplicate during 6 hr. The obtained extracts were weighed gravimetrically.

Radical scavenging activity of extracts

The winter melon (*Benincasa hispida*) seed extracts obtained under different conditions of UAE was subjected to antioxidant activity measurement analysis. 2-2´Azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt ($ABTS^{\cdot+}$), 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$) radical scavenging assays were used to determine the radical scavenging activity of extracts.

Determination of $DPPH^{\cdot}$ radical scavenging activity

This assay is based on the color change caused by reduction of the $DPPH^{\cdot}$ radical which was determined by measuring absorbance at 515 nm. The reaction time for the assay was determined 60 min by preliminary experiments. This assay was carried out as described by Zengin *et al*. (2010) with some modifications. Three replications were done for each sample.

Determination of $ABTS^{\cdot+}$ radical scavenging activity

2,2´-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) assay was carried out according to the method of Cai et al. (2004) and Mandana *et al*. (2012). Three replications were done for each sample.

Statistical analysis

The experimental design applied was 3×3×3 factorial design in a frame of Complete Randomized Design (CRD), corresponding to the amplitude, temperature and sonication time. The levels of independent variables were reported in Table 1. The results were statistically analysed by analysis of variance (ANOVA) and mean differences were compared by using Tukey test at 95% of confidence level using the Minitab Release 14.0 (Minitab Inc. State College, PA, USA). The correlation between different RSA and TPC were established according Pearson's linear correlations at 95% of confidence level.

Results and Discussions

Effect of UAE variables on the crude extraction yield

Figure 2 (a-c) presented the effects of different temperature levels at 25, 50 and 75% amplitude during 60 min of sonication time on the crude extraction yield (CEY) of extracts. The CEY ranging 60.40±1.13 to 97.21±0.62 mgg^{-1} under studied conditions. It was clear that by using lower amplitude (25%) the CEY was increased with prolonging sonication time up to 60 min (Figure 2 a). By increasing amplitude from 25 to 50% the extraction was completed during 40 min sonication at the constant temperature of 55 and 65°C (88.73±1.03 and 86.18±0.84 mgg^{-1}, respectively) (Figure 2 b). As shown in Figure 2 (c) applying the highest amplitude (75%) resulted in reaching to constant value of CEY during 40 min of sonication time at the constant temperature of 45, 55 and 65°C (91.67±0.14, 97.14±0.36 and 93.15±0.42 mgg^{-1}, respectively). This finding revealed that ultrasound is more effective in the first 40 min of sonication.

Several studies have shown that acoustic cavitation and mechanical effects can improve the efficiency of UAE process (Rostagno *et al*., 2003; Chemat *et al*., 2004; Li *et al*., 2004). In this regard, penetration of solvent into the plant matrix is facilitated by disrupting cell walls through the acoustic cavitation. Ultrasound waves lead to the greater contact surface area between the solvent and solutes. Therefore, utilization of UAE resulted in reduction of extraction time and solvent consumption (Wu *et al*., 2001). Furthermore, the bioactive and valuable compounds which are thermo sensitive are protected due to the fact that UAE can perform at lower temperatures (Hromadkova *et al*., 1999; Zhang *et al*., 2008). As shown in Figure 2 (c) at the constant amplitude of 75% with increasing

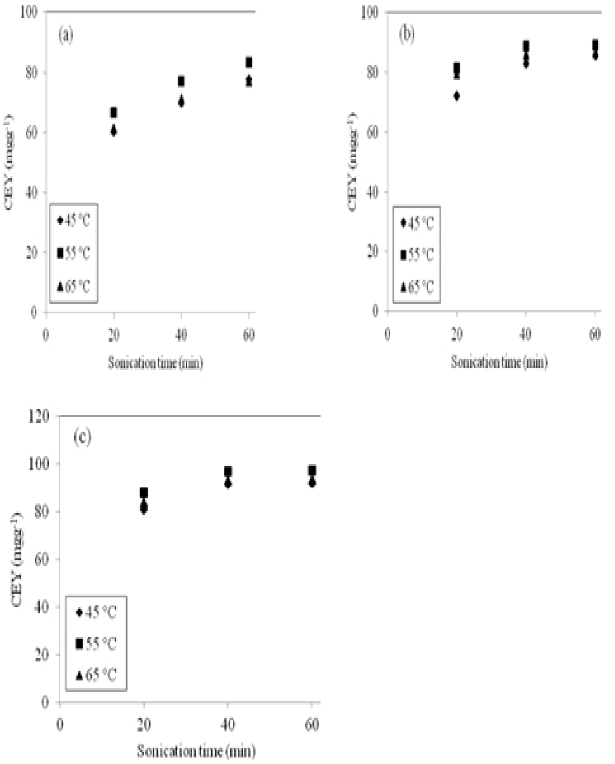

Figure 2. Effect of temperature at (a) 25% amplitude; (b) at 50% amplitude and (c) 75% amplitude on crude extraction yield of extracts during 60 min sonication time (standard deviation bars are smaller than the symbol size)

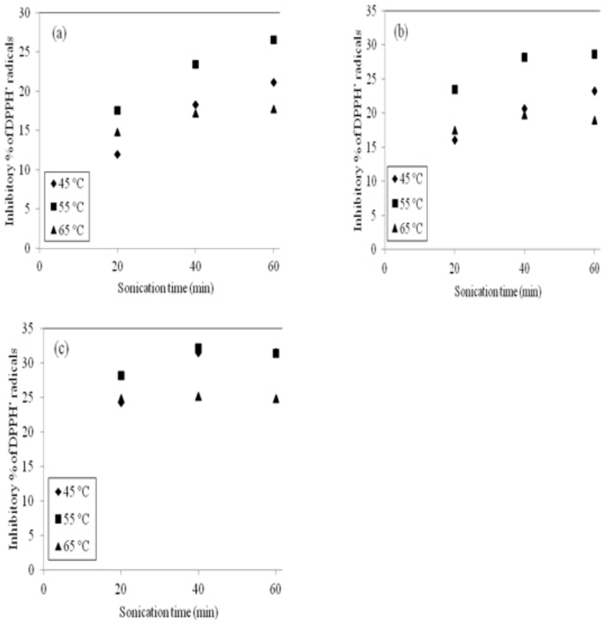

Figure 3. Effect of temperature at (a) 25% amplitude; (b) at 50% amplitude and (c) 75% amplitude on DPPH· scavenging activity of extracts during 60 min sonication time (standard deviation bars are smaller than the symbol size)

temperature from 55 to 65ºC there was an unexpected reduction in the CEY from 97.14±0.36 to 93.15±0.42 mgg^{-1}, respectively during 40 min sonication. This finding is associated with increase of temperature which caused more vapor pressure and strongly affected the generation and intensity of cavitation phenomenon. At lower temperature few bubbles with lower vapor pressure were generated and collapse with high intensity due to higher pressure difference between inside and outside of bubbles and vice versa (Hromadkova *et al*., 1999). The other reason is the lower surface tension at higher temperature which affects the generation and explosion of bubbles. It was pointed out that the mass transfer rate decreased at higher temperature due to the fact that the bubbles might easily collapsed (Zhang *et al*., 2008). Zhang *et al*. (2008) revealed that the extraction yield of flaxseed oil was reduced by increase of temperature. The yield was about 83% at 30°C which was reduced around 6 to 77% at 50°C.

In another study, Lou *et al*., (2010) investigated the influence of ultrasonic waves and extraction time on the chickpea oil separation. They stated that the yield increased rapidly during the first 20 min of extraction. After that, it was increased gradually with the extraction time. However, the oil yield did not increase after 30 min. In current study as shown in Figure 2 (c) the extraction time was shortened to 40 min sonication time by using 75% amplitude. By considering obtaining results, the maximum recovery of CEY (97.14±0.36 mgg^{-1}) was obtained using 75 % amplitude and 55 ºC temperature during 40 min sonication time.

Effect of UAE variables on the radical scavenging activity

Radical scavenging activity (RSA) of winter melon (*Benincasa hispida*) seed extracts was expressed as percentage of DPPH· and ABTS$^{\cdot+}$ free radicals scavenged (%DPPH$_{sc}$ and %ABTS$_{sc}$). The higher inhibition, the stronger RSA of extract. The effects of extraction variables on the %DPPH$_{sc}$ and %ABTSsc were analyzed through the analysis of variance (ANOVA). Based on the probability value (p-value), all the three independent variables had significant effect on the %DPPH$_{sc}$ and %ABTS$_{sc}$ of *Benincasa hispida* seeds extracts.

In Figures 3 (a-c) and 4 (a-c), the effect of different temperature levels at 25, 50 and 75% amplitude during 60 min sonication time on the %DPPH$_{sc}$ and %ABTS$_{sc}$ were presented. It was clear that by using lower amplitude (25%) the %DPPH$_{sc}$ of *Benincasa hispida* seeds extracts increased with the extension of sonication time up to 60 min at temperature of 45 and 55°C which reached to value of 21.12±0.70 and 26.51±0.45% inhibition, respectively (Figure 3 a). Same behavior was observed for ABTS$^{\cdot+}$ free radicals scavenging at the same condition as it reached to value of 30.25±0.56 and 37.04±1.02%

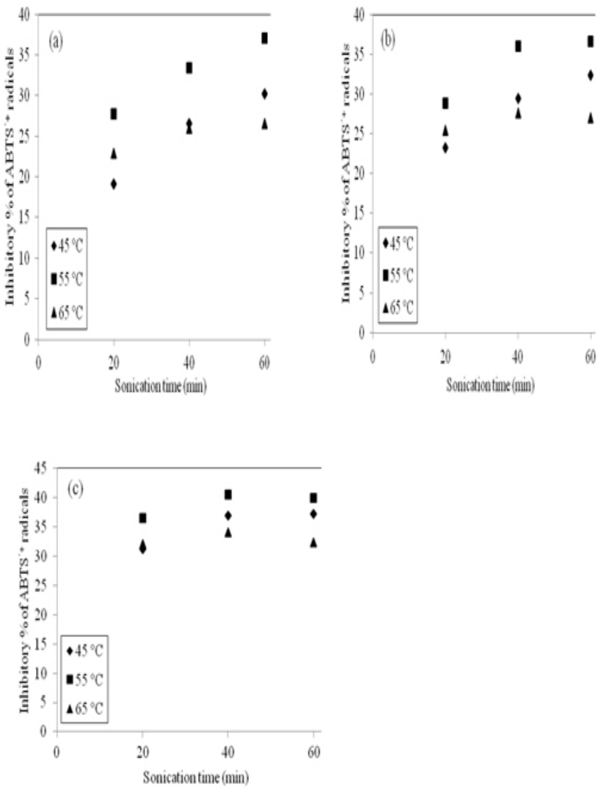

Figure 4. Effect of temperature at (a) 25% amplitude; (b) at 50% amplitude and (c) 75% amplitude on $ABTS^{\cdot +}$ scavenging activity of extracts during 60 min sonication time (standard deviation bars are smaller than the symbol size)

inhibition, respectively (Figure 4 a). It was clear that 40 min sonication time was adequate at the amplitude of 25% by using the highest temperature (65°C) as the RSA of extracts reached to plateau (17.20±0.81 and 29.08±0.85% inhibition of $DPPH^{\cdot}$ and $ABTS^{\cdot +}$ radicals, respectively). Yang *et al*. (2008) observed that by prolonging the sonication time at low ultrasonic power the reducing ability was increased. These findings are consistent with research conducted by Boonkird *et al*. (2008) on extraction of capsaicinoids from *Capsicum frutescens* using UAE. The high rate of capsaicinoids extraction was found at the first stages of process due to the concentration gradient difference between the solvent and plant source. Afterwards, the extraction rate was decelerating significantly due to the lower concentration gradient and location of capsaicinoids in the inner part of plant matrix. Furthermore, this result is linked to dissolving of solutes near the particle surface (rinsing) and diffusion of solutes from the solid particles to the liquid solvent bulk (slow extraction) (Vinatoru 2001).

In current study, it was found that at the constant amplitude (for example 50%), the RSA of extracts at 65°C during 60 min sonication time (19.00±0.82% inhibition of $DPPH^{\cdot}$ and 27.00±0.70% inhibition of $ABTS^{\cdot +}$, respectively) was lower than those obtained at 55°C (28.60±0.67% inhibition of $DPPH^{\cdot}$ and 36.68±0.70% inhibition of $ABTS^{\cdot +}$, respectively). It is documented that less efficiency in cavitation phenomenon was happen at higher temperature (65ºC) (Boonkird *et al*., 2008). Moreover, thermo degradation of valuable compounds which are contributed to the antioxidant activity of extracts could be happen by applying higher temperatures. Kamran Khan *et al*. (2010) found that the optimum temperature for the highest antioxidant activity (69%) of orange peel was 40°C after which the antioxidant activity was reduced.

Using moderate amplitude (50%) and low temperature (45ºC) the RSA of extracts was increased up to 60 min of sonication time (23.20±0.59 and 32.32±0.73% inhibition, respectively). Using same amplitude at higher temperature resulted in reduce the extraction time to 40 min. This could be due to the positive effect of temperature on recovery of solutes from plant matrix. As stated by Zhang *et al*. (2008), since the temperature increased the solubility and diffusivity increased and caused higher mass transfer rate.

As shown in Figures 3 c and 4 c the RSA of extracts did not change significantly ($p>0.05$) by using 75% amplitude after 40 min sonication time. It was reported that as a result of hard cell walls existence which are not permeable after a certain sonication time no further improvement in solutes recovery could be observed (Liu *et al*., 2010). These findings are consistent with those obtained by Sivakumar *et al*. (2007) for UAE of tannin from myrobalan nut. As shown in Figures 3 c and 4 c the RSA of *Benincasa hispida* seed extracts were reduced after 40 min sonication time by applying 75% amplitude at the temperature of 55 and 65°C. This phenomenon was due to the fact that more violently bubble collapse at high amplitude range of ultrasonic waves (Herrera and Luque de Castro, 2005). Moreover, hot spots production with extremely high local temperature and pressure during explosion of bubbles cause thermo sensitive compounds decomposition (Tuulmets and Salmar, 2001). In another study which conducted by Porto *et al*. (2009) it was found that the concentrations of volatile compounds obtained from samples treated at 100% of ultrasonic power was very close with those obtained by hydrodistillation.

In current study, it was found that using 75% amplitude during 40 min at 55°C to be the best operating condition for UAE process since the maximum value of RSA (32.12±0.36% inhibition of $DPPH^{\cdot}$ and 40.52±0.73% inhibition of $ABTS^{\cdot +}$) can be obtained using this condition. Finally, high correlation coefficient obtained between these

Table 2. Comparison between CSE and UAE data

Extraction Mode	CEY (mgg^{-1})[c]	%DPPH$_{sc}$[c]	%ABTS$_{sc}$[c]
CSE	250.00 ± 1.30	28.70 ± 0.7[a]	27.0 ± 0.9[a]
UAE	97.14 ± 0.36	32.12 ± 0.38	40.52 ± 0.73
BHT[b]	-	93.43 ± 0.6	-
Catechin[b]	-	98.10 ± 0.8	97.54 ± 0.7

[a] Mandana *et al*., 2012.
[b] Synthetic antioxidants
[c] Data are expressed as the mean ± standard deviation

two chemical assays (Pearson correlation of 0.96) confirmed the accuracy of these results. The RSA of extracts from *Benincasa hispida* seeds was lower compared with that of synthetic antioxidants at the same concentration (0.1mg/mL) (Table 2). As reported by Liu *et al*. (2009) the antioxidant activity values for seeds oil from *Opuntia dillenii* Haw ranged from 90 to 17% when the concentrations varied from 4 to 0.1 mg ml^{-1}, accordingly. Therefore, it was expected that using higher concentration of extracts this weak point can be settled which is of great interest and need to be further investigated.

Comparison of UAE and CSE

In association with the further effectiveness in evaluating the UAE method the CEY and RSA results were compared with results obtained using CSE method. Different extraction methods have different extraction yield and efficiencies. Higher concentration of natural bioactive compounds in the extracts is an important factor in the production of natural products while a primary task in the industries is lower economic cost (Grigonis *et al*., 2005). Compared the UAE and CSE procedure the CEY and RSA results obtained at both extraction conditions were shown in Table 2. It was found that the CEY obtained under the best condition of UAE was around 38.86% of those obtained from CSE while the RSA of extracts was significantly ($p<0.05$) higher than CSE extracts. It was evident that extraction time was reduced from 6 hours which used in CSE to 40 min using UAE. Some of the main disadvantages of CSE method include: long extraction time, losses of volatile compounds and degradation of unsaturated compounds, resulting unfavourable off-flavour compounds due to heat (Grigonis *et al*., 2005). UAE has different advantages over CSE method such as low operating temperature, thus no thermal degradation of most of the labile compounds and shorter extraction duration leading to saving energy. UAE also seems to be a cost-effective process at laboratory scale, but a precise economic evaluation will need additional experiments for establishing large-scale units (Virot *et al*., 2010). Therefore, it can be recommended as a suitable extraction method to achieve extracts with strong antioxidant activity and rich in bioactive compounds from *Benincasa hispida* seeds.

Conclusion

The findings of current study, for the first time, revealed the potentiality of ultrasound-assisted technique as a simple and effective extraction method for recovery of valuable and bioactive compounds from winter melon (*Benincasa hispida*) seeds. Different process variables investigated to determine their effects on the CEY and RSA of extracts. Amplitude, temperature and sonication time had significant effect ($p<0.05$) on the CEY and RSA of *B. hispida* seed extracts. Compared with CSE method the application of UAE technique reduced the time of extraction to 40 min (UAE) and resulted in extracts which possessed high antioxidant activity. Therefore, UAE of winter melon seeds by using food grade solvent (ethanol, 99.5%) has strong potentiality for industrial application for the preparation of rich extracts in natural antioxidants aimed at replacing synthetic antioxidants. Moreover, a winter melon (*Benincasa hsipida*) seed is a potential source of valuable compounds which introduced new source of natural compounds in the food industry.

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