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Research Article

Enhancing the extraction of pumpkin seed (*Cucurbita pepo* L) for increasing oil yield and its phytosterol content

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Abstract

Pumpkin seeds (*Cucurbita pepo*) were harvested in 2018 containing 41.23±1.23% oil; 33.18±1.61% protein; and 1575.97 ±67.79 mg phytosterol in 100g seeds (dried weight). Solvent (hexane) extraction (SE), Ultrasound assisted solvent (hexane) extraction (UAE), Enzyme *Alcalase* assisted extraction (EAE) and Enzyme *Alcalase*_Ultrasound assisted extraction (E_UAE) were studied. The oil's yields and phytosterol in oil obtained were 90.07±0.17% and 1657.6±82.9 mg/100ml oil; 95.46±0.06% and 2017.5±100.1 mg/100ml oil; 89.65±0.11% and 1992.7±99.5 mg/100ml oil; 91.87± 0.03% and 2327.7 ±110.4 mg/100ml oil, respectively. The highest oil yield of 95.56% was obtained when UAE method was applied, while it was found that the highest phytosterol content of 881.7 mg/100g seeds can be obtained in the case of using E_UAE.

Keywords: extraction, phytosterol, pumpkin seed's oil

Abbreviations:

SE - Solvent (hexane) extraction

EAE - Enzyme (*Alcalase*) assisted extraction

UAE - Ultrasound assisted solvent (hexane) extraction

E_UAE - Enzyme (*Alcalase*)_Ultrasonic assisted extraction

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Introduction

In 2013 the yield of pumpkin in worldwide was 1797.19 thousand hectares, 137.32 quintal/ hectare and 24.679 M tones (FAOSTAT 2015). According to the USDA National Agricultural Statistics Service, nearly 65,900 acres of pumpkins were harvested in the US in 2018, producing more than 1.5 billion pounds of usable pumpkins with more than 2 billion produced (Ag Marketing Resource Center 2021). The countries with the highest volumes of pumpkin consumption in 2018 were China (7.9M tones), India (5.9M tones) and Russia (1.3M tones), together accounting for 53% of global consumption, and the countries with the highest levels of pumpkin per capita consumption in 2018 were Ukraine (15.778 kg per 1000 persons), Iran (13.096 kg per 1000 persons) and Russia (8.784 kg per 1000 persons) (Index box 2021). Pumpkin seeds are used mainly in the rough form such as roasting and drying, however many researches showed pumpkin seeds contain valuable phytosterol which has roles in cholesterol reduction (Lee et al. 2003; Chen et al. 2014), anti-inflammatory (Rasmussen et al. 2008), antioxidant (Lee et al. 2003), prevention of cancer (Woyengo et al. 2011). In Vietnam, statistically calculated, 1 hectare of pumpkin produces 2000-2500 kg of pumpkin seeds, equivalent to 72-90 kg of pumpkin seed oil or 110-140 kg of dried pumpkin powder or 44.6 - 55.7 kg phytosterol (Tran Huong Nga 2019). Our previous study showed that the oil yield of 78.1% has reached with 1.25 mg *Bromelain*, 1mg *Cellulase*, and 1mg *Amylase* in 100g pumpkin seed, at 45°C, pH = 7 for 5 hours (Tran Huong Nga 2019). Hrabovski et al. (2012) reported that the oil yields obtained by extraction from *C. pepo* with hexane, petroleum ether and the supercritical CO₂ were 43.37; 44.65; 36.17 (%) respectively, the total phytosterol content in supercritical CO₂ extract (294mg/100g oil) was about 30% and 20% higher than that of hexane and petroleum ether extract (Hrabovski et al. 2014). Additionally, a number of methods for extracting oils from seeds have been investigated, including mechanical compression (Karaj and Müller 2011), ultrasonic extraction (Ozkan et al. 2007), microwave extraction (Kumaran and Karunakaran 2007), and supercritical fluid extraction (Louli et al. 2004). Compared with traditional Soxhlet extraction, ultrasonic extraction provides higher

selectivity, is less time-consuming, has lower energy consumption and reduced emissions (Appelqvist 1971; Ward et al. 1985), and produces higher-quality oil (Ofori-Boateng et al. 2012). The aim of this study was to investigate the effect of enzyme *Alcalase* and ultrasound on oil extracted enriching of phytosterol from *C. pepo* seeds harvested in Vietnam.

Materials and Methods

Pumpkin seeds (*C. pepo*) were collected in 2018 from Hung Yen province in Vietnam. Pumpkin seeds were dried, stored, sealed with a polyethylene bag at 4°C until oil analysis and extraction. Enzyme *Alcalase* 2.4L with the enzyme activity of 3.9 U/ml and β -sitosterol 95% were purchased from Novozyme (Denmark) and Shanghai yuanye (Biotechnology Co. Ltd), respectively.

Preliminary methods. Moisture content, ash content, oil content and starch content were determined by Vietnam National methods (TCVN 10788:2015; TCVN 10761:2015; TCVN 10730:2015). Starch and protein content of raw material were analyzed by Graxianop and Kjeldahl method.

Oil yield in case of employing different extraction methods. *Solvent (hexane) extraction (SE):* Pumpkin seeds were cleaned, grounded then subjected to oil extraction using n-hexane and diethyl ether with the ratios of raw material to solvent: 1/2; 1/4, 1/6, 1/8 and 1/10; extraction temperature: 50, 55, 60, 65 and 68°C; extraction time: 3 hours, 4 hours, 5 hours, 6 hours and 7 hours; 2 extraction repetition.

Ultrasound assisted solvent (hexane) extraction (UAE): at the same extraction condition of SE with ultrasonic frequency of 28; 35 and 40 kHz. *Enzyme Alcalase assisted extraction (EAE):* *Alcalase* pretreatment of pumpkin seed was performed under the optimal temperature (55°C) and pH = 6.96 of *Alcalase* and at the following conditions: enzyme to substrate (v/w%) was 1.61, 2.15, 2.69, 3.23, 3.77 and 4.31; hydrolysis time (hrs) was 2, 3, 4, 5, 6 and 7 hours; stirring speed (rpm) was 0, 30, 60, 90, 120, and 150.

U_EAE: The combination of EAE and EAE at the same condition.

Oil yield (H, %) from extraction was calculated by the formula:

$$H, \% = \frac{M_1}{M} \cdot 100 \quad (1)$$

where:

M₁ - mass of oil obtained from extraction, g

M - oil content in pumpkin seeds to be extract with hexane by Soxhlet extractor, g

Determining the total phytosterol content. Total phytosterol content was analyzed by UV-VIS spectrophotometry (Araújo et al. 2013). The procedure was based on the quantification at 625 nm followed the Liebermann-Burchard reaction; constructed with the β-sitosterol standard solution at five concentration levels (0.02-0.10 mg/ml). Samples are diluted at appropriate concentration, then 1ml of each was added 2ml LB (acetic anhydride and sulfuric acid; 10:1) after 5 min the absorbances were measured in the UV-VIS spectrophotometer, using chloroform to the blank. Measurement was carried out in triplicate.

Total phytosterol was calculated by following formula:

$$\text{Total phytosterol content} = C_s \cdot \frac{A_a}{A_s} \cdot F \quad (2)$$

where:

C_s - standard concentration

A_a - absorbance of the sample

A_s - absorbance of the standard

F - dilution

Statistical analysis. Results were reported as means ± SD. SPSS 11.5 for Windows were employed to interpret the results.

Results and Discussion

Pumpkin seed's composition. The water content; kernel seeds/seeds; total ash; starch and protein content of the sample were 8.10±0.05%; 71.57±1.26%; 2.80±0.02%; 7.04±0.03% and 33.18±1.61% respectively.

Oil yielded in 100g dried pumpkin seeds were 41.23±1.23% and 1575.97±67.79 mg total phytosterol content in 100g dry seeds (UV-VIS spectrophotometry).

The result in this study was similarly to previously reported (Rodríguez-Miranda et al. 2014) the oil contents were 37% for *C. pepo* from Italy, 43% for *C. moschata* from Egypt (Al-Khalifa 1996) and 43.69% for *C. maxima* from Saudi Arabia (Alfawaz 2004).

Solvent extraction of pumpkin seed's oil (SE).

Table 1 showed with the solvent ratio of 1/6 (Efthymiopoulos et al. 2019), 3hrs extraction (Efthymiopoulos et al. 2019; Li et al. 2016) at a temple boiling temperature of hexane and diethyl ether (68°C and 34°C), the yield of oil extraction was of 86.71% and 73.82% respectively. Hexane was chosen for the pumpkin oil extraction.

In hexane extraction, Table 1 showed that, at 60°C; 3hrs of extraction, the oil yield at the ratio of pumpkin seeds to hexane of 1/6 reached 85.09% higher than that at the ratio of 1/2 and 1/4. While the ratio of 1/8 and 1/10 did not give the significant difference in the oil yield.

Extraction temperature higher than 60°C and extraction time longer than 3hrs did not affect on the oil yield (Table 1).

Duplicate extraction was carried out. The first extraction at the ratio of 1/6 (raw material to hexane), 60°C; 3hrs, gave the oil's yield of 87.59±0.07%. (Table 1) The 2nd extraction was at the same condition, the yields obtained were 1.57%±0.03%; 2.48%±0.1% and 2.35±0.66%, for 1hr, 1.5hrs and 2hrs, respectively. The total oil

yield of 7.5hrs was 90.07±0.17% and the phytosterol content was 1657.6 mg in 100ml oil (UV-VIS spectrophotometry); this was 4.07% higher than reported by (Rodríguez-Miranda et al. 2014) previously studied of 5hrs extraction of *C. pepo* at 60°C, and with very high ratio of the raw material to hexane (1/20).

Table 1. Factors effected on the extraction of pumpkin seed oil by SE

Factors	Survey parameters	The yield of oil (%)
Solvent	Hexane	86.71±0.39
	Diethyl ether	73.82±0.16
Ratio of pumpkin seed and hexane	1/2	76.54±0.86
	1/4	84.1±0.08
	1/6	85.09±0.2
	1/8	85.39±0.04
	1/10	85.95±0.01
Extraction temperature (°C)	50	70.08±0.17
	55	76.48±0.09
	60	85.85±0.1
	65	86.28±0.21
	68	86.71±0.04
Extraction time (hrs)	3	85.59±0.02
	4	86.21±0.04
	5	86.47±0.25
	6	87.59±0.07
	7	87.69±0.07
	8	87.59±0.07
The total time of twice extraction (hrs)	6	89.16±0.05
	7	89.16±0.05
	7.5	90.07±0.17
	8	89.94±0.06
*Results are significantly influenced (p ≤0.05)		

Ultrasound assisted extraction (UAE).

Ultrasound assisting reduced the extraction time from 6 hours to 3 hours (Table 2) at the condition investigated (pumpkin seeds to hexane ratio of 1/6 and extraction temperature of 60°C, 40kHz), it gave the oil yield of 93.17 ± 0.1% for the 1st extraction. At the same condition, second extraction for 1.5h, gave 2.29±0.02% the oil yield. This means the yiled of 2 replications of extraction was 95.46% and in 100 ml obtained oil there is 2017.5 mg phytosterol extraction. At the same condition, second extraction for 1.5h, gave 2.29±0.02% the oil yield. This means the yiled of 2 replications of extraction was 95.46% and in 100 ml obtained oil there is 2017.5 mg phytosterol.

Table 2. Factors effected on the extraction of pumpkin seed oil by UAE

Factors	Survey parameters	The yield of oil (%)
Ultrasonic frequency (kHz)	25	72.79±0.18
	38	76.39±1.73
	40	84.81±0.09
Ratio of pumpkin seed and hexane	1/4	76.75±0.94
	1/6	84.81±0.09
	1/8	85.57±0.03
	1/10	85.57±0.44
Extraction time (hrs)	2.5	74.41±0.02
	3	84.81±0.09
	3.5	84.82±0.01
	4	84.43±0.1
	4.5	84.68±0.02
Extraction temperature (°C)	40	75.18±0.01
	45	79.52±0.04
	50	84.81±0.09
	55	88.72±0.01
	60	93.17±0.1
The total time of twice extraction (hrs)	3	93.17±0.1
	4	94.97±0.97
	4.5	95.46±0.06
	5	92.17±0.04
*Results are significantly influenced (p ≤0.05)		

The oil yield of UAE was 5.39% higher than that of the solvent extraction. Solvent extraction yielded only 1657.6 mg phytosterol/100ml oil, while UAE this figure of phytosterol in oil showed up to 2017.5 mg phytosterol/100ml oil (UV-VIS spectrophotometry).

Table 3. Factors effected on the hydrolysis of pumpkin seeds by *Alcalase*

Factors	Survey parameters	Mass of oil (g/100g seeds)	Conditions to hydrolyze pumpkin seeds
Stirring speed (rpm)	0	10.16±0.01	E/S= 2.15%; pH = 6.96; 3 hrs; 55 °C
	30	17.69±0.01	
	60	17.82±0.01	
	90	17.63±0.05	
	120	17.62±0.05	
Ratio <i>Alcalase</i> to substrate	1.61	17.24±0.01	60rpm; pH = 6.96; 3 hrs; 55 °C
	2.15	17.82±0.01	
	2.69	17.92±0.01	
	3.23	18.41±0.19	
	3.77	19.55±0.01	
	4.31	19.54±0.01	
Hydrolysis time (hrs)	2	1.02±0.01	60rpm; pH=6.96; 55 °C; E/S= 3.77%
	3	19.54±0.01	
	4	20.06±0.02	
	5	22.97±0.01	
	6	28.62±0.01	
	7	28.63±0.01	
*Results are significantly influenced (p ≤0.05)			

Enzyme assisted extraction (EAE). *Alcalase* was studied for pumpkin seed treatment prior extraction in term of stirring speed, ratio *Alcalase* (3.9 U/ml) to the substrate and hydrolysis time. Extraction of pumpkin seeds after being hydrolyzed using *Alcalase* 3.77% (v enzyme/w substrate) at pH = 6.96, 55°C was performed for 4, 5 and 6 hours with with 60 revolutions per minute of stirring speed. (Table 4.).

Table 4. Factors effected on the extraction of pumpkin seed oil by EAE

Factors	Survey parameters	The yield of oil (%)
Ratio of pumpkin seed and hexane	1/6	63.88±0.01
	1/8	66.21±0.3
	1/10	73.49±0.02
Extraction temperature (°C)	50	73.49±0.02
	55	77.49±0.12
	60	81.32±0.01
Extraction time (hrs)	3	81.32±0.01
	4	83.21±0.02
	5	83.15±0.04
	6	83.29±0.03
The total time of twice extraction (hrs)	4	83.21±0.02
	5	88.52±0.09
	6	89.65±0.11
	7	89.6±0.04
	8	89.52±0.03
*Results are significantly influenced (p ≤0.05)		

Table 4 showed that longer extraction time than 4hrs did not give remarkable increasing yield. Result of *Alcalase* assisted extraction is presented in Table 4. After *Alcalase* hydrolysis, pumpkin seed was extracting twice. The total oil yield was 89.65±0.11% and there was 1992.7 mg phytosterol in 100ml oil (UV-VIS spectrophotometry).

Enzyme and Ultrasound assisted extraction (E_UAE). *Alcalase* assisted extraction was improved by ultrasound. Pumpkin seeds were hydrolyzing using *Alcalase* and extracted at 40 kHz of ultrasound, the oil yields was 85.08±0.01% after 3hrs (Table 5). Two extraction by E_UAE gave oil yield of 91.87 ± 0.03% and the phytosterol content was 2327.7 mg in 100ml oil (UV-VIS spectrophotometry) (Table 5).

Table 5. Factors effected on the extraction of pumpkin seed oil by U_EAE

Factors	Survey parameters	The yield of oil (%)
Ratio of pumpkin seed and hexane	1/4	74.54±0.9
	1/6	76.34±0.01
	1/8	76.67±0.14
	1/10	76.82±0.01
Extraction temperature (°C)	50	76.34±0.01
	55	80.3±0.03
	60	85.08±0.01
Extraction time (hrs)	2	72.82±0.02
	3	85.08±0.01
	4	84.04±0.03
	5	85.86±0.04
The total time of twice extraction (hrs)	3	85.08±0.01
	4	86.77±0.2
	5	91.87±0.03
	6	91.93±0.07
*Results are significantly influenced (p ≤0.05)		

Table 6. SE, EAE, UAE, E_UAE for *C. pepo* seed oil enriched in phytosterol

Extraction	Extraction time (hrs)	The total oil yield of twice extraction (%)	Phytosterol (mg/ 100ml oil)- (UV-VIS spectrophotometry)	Phytosterol (mg/100g seeds) -(UV-VIS spectrophotometry)
		(% 1 st extraction)		
SE	7.5	90.07± 0.17 (87.59±0.51)	1657.6± 82.9	642.2± 32.1
EAE	6	89.65± 0.11 (83.21±0.10)	1992.7± 99.5	736.56± 36.8
UAE	4.5	95.46± 0.06 (93.17±0.01)	2017.5± 100.1	794.1± 39.4
E_UAE	5	91.87± 0.03 (85.08±0.40)	2327.7± 110.4	881.69± 41.8

SE, EAE, UAE and E_UAE. Extraction was performing combined enzyme and ultrasound. The comparison of SE, EAE, UAE, E_UAE is presented in Table 6. Table 6 showed that oil extraction is efficient with UEA while oil enriched phytosterol extraction is efficient with E_UEA. Other researches (Nakić et al. 2006; Hrabovski et al. 2012) on *C. pepo* by solvent extraction (hexane) illustrated the phytosterol obtained was 317.2mg phytosterol/

100g oil, was about 5 times lower than that of SE in the study.

Conclusion

The oil yield and phytosterol content in the oil obtained from the UAE are 95.46 ± 1.00% and 2017.5mg phytosterol/100ml oil, which were higher than those of the Hexane extraction roughly 5.39% and 359.9 mg phytosterol/100 ml oil. Phytosterol content in oil extracted from the E_UAE was the highest (2327.7 mg phytosterol/100 ml oil) compared to three other extraction processes. For *C. pepo* seed, the oil extraction was efficient with the UEA while the phytosterol extraction was efficient with the E_UEA.

In conclusion, E_UAE was recommended to extract pumpkin seed oil enriching phytosterol for the industrial practice.

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