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Effect of ethanol solvent concentrations in pepino melon fruit (*Solanum muricatum* Aiton) extraction on total flavonoid, phenolic, and β-carotene content

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ABSTRACT

The quality of an extract is determined by the type and level of compounds contained therein. The solvent is one of the elements that influences the extract's quality. Therefore, extraction must be carried out using a solvent that can extract bioactive substances. This study was aimed at obtaining the optimal concentration of ethanol in extracting flavonoids, phenolics, and β-carotene from pepino melon fruit. In this study, dried pepino melon fruit was extracted using ethanol of 50%, 70%, and 96%. The extracts were analyzed qualitatively for phenolic and flavonoid using thin layer chromatography (TLC), and quantitatively using a spectrophotometry. Meanwhile, β-carotene levels were determined using high pressure liquid chromatography (HPLC). The data of level flavonoid, phenolic, and β-carotene were statistically analyzed using multivariate analysis of variance. The results showed that 50%, 70%, and 96% ethanol solvent produced extracts with a yield value of 51.8%; 87.4%; 54.6%; total flavonoid content of 0.298 ± 0.04 mgQE/g; 0.559 ± 0.03 mgQE/g; 0.289 ± 0.01 mgQE/g; total phenolic content of 4.763 ± 0.08 mgGAE/g; 3.631 ± 0.12 mgGAE/g; 3.317 ± 0.10 mgGAE/g; β-carotene level of 0.157 ± 0.02 mg/g; 0.910 ± 0.16 mg/g; 1.054 ± 0.13 mg/g, respectively. The statistical analysis showed that there was a significant difference in different ethanol concentrations in extraction with the content of total flavonoids, total phenolics, and β-carotene ($p<0.05$). The optimal ethanol solvent for extracting flavonoids, phenolic compounds, and β-carotene from pepino melon fruit was 70% ethanol.

Keywords: β-carotene, flavonoid, pepino melon, phenolic

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INTRODUCTION

Pepino melon fruit (*Solanum muricatum* Aiton) can thrive and develop well in highlands such as in West Java, Dieng-Central Java, and the city of Batu Malang (Ide, 2010). Pepino melon fruit has many health benefits including anti-inflammatory, diabetes, stroke, high blood pressure, digestive disorders, anti-tumor, cancer, kidney, constipation, and hemorrhoids (Ahmad et al., 2014; Hsu et al., 2011; Shathish & Guruvayoorappan, 2014; Sudha et al., 2012). It is known that pepino melon fruit contains a large amount of vitamin C, as well as carotenoids, which give the flesh a yellow color (Hsu et al., 2011). Research by Scala et al. (2011) shows that pepino melon fruit has a much higher phenolic content than its vitamin C content.

Pepino melon fruit is a plant that has the potential to contain flavonoids, phenolic compounds, and β-carotene. Pepino melon fruit has a total phenolic level of 20.43 mg GAE/g and total flavonoid level of 53.85 mgQE/g (Sudha et al., 2012). While the level of β-carotene in Pepino melon fruit is 27 mg/100 g (Maheshwari et al., 2014). However, information about which solvent is the most optimal for extracting the active compounds in pepino melon fruit is limited.

Based on the concept of like dissolves like, a solvent tends to dissolve a compound that has the same polarity as the solvent (Zhuang et al., 2021). Differences in ethanol concentration affect the level of polarity of the ethanol so that it will affect the extracted bioactive compounds and the bioactivity of the extract (Pradal et al., 2016). Therefore, it is necessary to consider the choice of solvent concentration. The research was aimed to determine the total flavonoid, phenolic, and β-carotene levels of pepino melon fruit extracted with various ethanol concentrations of 50%, 70%, and 96%. This study is expected to reveal the optimal concentration of the ethanol used to extract pepino melon fruit.

MATERIALS AND METHOD

Materials

Pepino melon fruit (*Solanum muricatum* Aiton) was obtained from Melody Orchard in Wonolelo Village, Sawangan District, Magelang Regency on March 30, 2022. The authentication of the pepino melon fruit identity had been carried out by the Herbal Laboratory of Materia Medica Batu, Malang, East Java (Reference number: 074/581/102.20-A/2022). Aquadest, technical ethanol, ethanol p.a (Merck), methanol p.a (Merck), chloroform p.a (Merck), acetone p.a (Merck), acetic acid (Merck), ammonia, $FeCl_3$, $AlCl_3$ (Merck), CH_3COONa (Merck), *Folin-Ciocalteu* (Merck), gallic acid (Aldrich), quercetin (Aldrich), β-carotene (Sigma), acetonitrile for HPLC (Merck), dichloromethane for HPLC (Merck), methanol for HPLC (Merck).

Methods

Sample extraction

Ethanol solvent was used to macerate up to 150 grams of pepino melon fruit powder (1:10). The concentration of each ethanol solvent was 50%, 70%, and 96% which was used for soaking for 24 hours then maceration was carried out twice at room temperature. In addition, a vacuum rotary evaporator at 50°C is used to filter it and evaporate it. The liquid extract was re-evaporated over the water bath until a thick extract was once produced. For future use, the extract was stored at 4°C (Sudha et al., 2012 with slight modification).

Thin layer chromatography

Silica gel 60 F_{254} stationary phase or TLC plate was prepared and then activated in an oven at 100°C for 10 minutes. Before being spotted onto the stationary phase, 10 mg of the extract and standard were dissolved in 1 mL of methanol. To identify flavonoid compounds use the mobile phase water: methanol: chloroform (0.4: 2.6: 7) was applied. Rutin and quercetin were used for standard. The spots were observed using UV 254 and 366 nm and visually using ammonia vapor. The mobile phase used was chloroform: acetone: acetic acid (2: 3: 1) to identify the phenolic compound. Gallic acid was

used for standard. The spots were observed using UV light and visually sprayed using $FeCl_3$ solution (Pratama et al., 2018; Moldovan et al., 2020 with slight modification).

Quantification of total flavonoid levels

This determination involved measuring the total flavonoid content of a sample using a colorimetric method. A series of standard concentrations of quercetin was made at 25, 50, 75, and 100 ppm. The extracted sample was weighed as much as 80 mg dissolved in 10 mL of ethanol p.a. 0.5 mL sample and the standard solutions were added 1.5 mL of ethanol p.a, 0.1 mL of 10% $AlCl_3$, 0.1 mL of CH_3COONa 1 M and 2.8 mL of aquadest. Then, it was left to incubate for 25 minutes. UV-Vis spectrophotometry at a maximum wavelength of 437 nm was used to measure the absorbance. Measurements were replicated 3 times. The total flavonoid content obtained was expressed in mgQE/g (Kemenkes RI, 2017).

Quantification of total phenolic levels

Folin-Ciocalteu testing is used to measure total phenolic levels. A series of gallic acid concentrations were made at 25, 50, 75, and 100 ppm. The extracted sample was weighed as much as 80 mg in 10 mL of methanol p.a. The sample solution and standard solution were pipetted as much as 1 mL, and 5 mL of the 7.5% Folin-Ciocalteu solution were added. After 8 minutes of standing, 4 mL of 1% NaOH were added, and the mixture was incubated for an hour. At a maximum wavelength of 730 nm, the absorbance was measured. Measurements were replicated 3 times. The measured total phenol content was expressed as mgGAE/g (Kemenkes RI, 2017).

Quantification of β-carotene levels

Quantification of β-carotene levels was carried out by High-Performance Liquid Chromatography (HPLC) using mobile phase acetonitrile: methanol: dichloromethane (37: 10: 53 v/v/v). At a flow rate of 1 mL/min, a Pepino fruit ethanol extract sample of up to 20 µL was injected into the HPLC and read at 450 nm. The β-carotene level was quantified based on the results of the area value in each extracted sample.

The parameters of specificity, linearity, precision, accuracy, LOD, and LOQ are included in the validation of the analytical method. Linearity by making β-carotene at concentrations of 5, 10, 20, 30, and 35 ppm to make a linear equation of the relationship between concentration and area. The CV values of six replicate samples at a level of 20 ppm β-carotene were used to calculate precision. Accuracy was determined by the value of data recovery in 3 concentration variations (5, 20, and 35 ppm) and repeated 3 times. LOD & LOQ were obtained by injecting standard β-carotene at concentrations of 0.05; 0.1; 0.2; 0.3; 0.4; 0.6 ppm to obtain the standard curve equation for LOD & LOQ (Sugihartini et al., 2019).

Data Analysis

Data were analyzed using SPSS with Multivariate Analysis of Variance (MANOVA) which includes a homogeneity test using the Levene Test, then the Tests of Between-Subjects Effects test and the MANOVA Post Hoc test are performed. The value $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Extraction

In this work, we investigated the concentration of ethanol solvent that is effective in dissolving the active chemicals found in pepino melon fruit. Extract yield, total flavonoid, total phenolic, and beta-carotene content were among the parameters measured.

Table 1 shows the yield of pepino melon fruit extract. The yield of 70% ethanol extraction is higher than that of 96% and 50% ethanol extraction. A 70% ethanol solvent may dissolve the bulk of the components found in pepino melon fruit, resulting in a high extract yield. Because of its optimal water content of 30%, 70% ethanol can successfully extract secondary metabolites (Rahardhian et al., 2019).

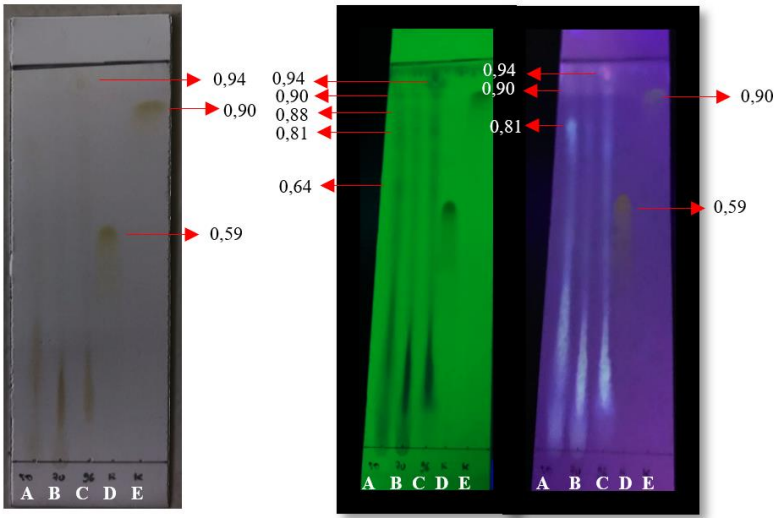
These findings are consistent with prior research that found that 70% ethanol provided an optimal extract yield (Suhendra et al., 2019; Rahardhian et al., 2019).

Table 1. The yield of pepino melon fruit extract

Extraction Solvent	Weight of dry fruit (g)	Weight extract (g)	Yield (%)
50% Ethanol	150	77.7	51.8
70% Ethanol	150	131.1	87.4
96% Ethanol	150	81.9	54.6

Thin layer chromatography (TLC)

Based on Figure 1, the Rf values obtained in the 50%, 70%, and 96% ethanol extract samples were the same as the quercetin comparison at Rf 0.90, it can be said that the three samples contained flavonoid compounds, namely quercetin. The results are by the research of Hsu et al. (2011) which stated that four flavonoids (myricetin, naringenin, quercetin, and rutin) were detected using HPLC separation in pepino melon fruit. On 50% ethanol extract, the spot at Rf 0.81 was light blue fluorescence after being given ammonia vapor under UV 366 nm. The increase in the intensity of the lighter blue fluorescence after being vaporized with ammonia indicated that the isoflavone group of flavonoid compounds did not contain free 5-OH (Markham, 1988). The spots that were suspected to be of the flavonoid group were spots with Rf of 0.81, 0.90, and 0.94.



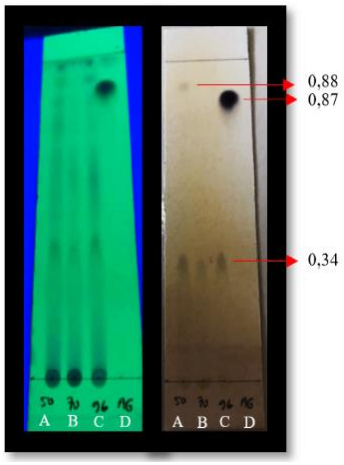
(1) (2)

Figure 1. TLC profile of flavonoid detection. (1) visually after being vaporized by ammonia (2) UV 254 nm and 366 nm after being vaporized by ammonia

Description: A: 50% ethanol extract; B: 70% ethanol extract; C: 96% ethanol extract; D: rutin; E: quercetin

Figure 2 shows the results of the TLC profile for phenolic testing. The TLC profile visually shows a blue color after being sprayed with $FeCl_3$. It means that the sample contains phenolic compounds. The reaction between phenol and $FeCl_3$ produces a Fe^{3+} complex so that the color of the spots becomes darker (black) (Nurmalasari et al., 2019). Extract with 50% ethanol has an Rf of 0.88 which is close to gallic acid which is 0.87 so the extract with 50% ethanol contains phenolic compounds with similar

polarity to gallic acid. The three samples extract with 50%, 70%, and 96% ethanol also contained other phenolic compounds with lower polarity at Rf 0.34.



(1) (2)

Figure 2. TLC Profile of Phenolic Test (1) UV 254 nm (2) Visually after being sprayed with $FeCl_3$. A = 50% ethanol extract; B = 70% ethanol extract; C = 96% ethanol extract; D = Gallic acid

Quantification of total flavonoid levels

Colorimetric reactions revealed the total flavonoid content. The formation of complexes between $AlCl_3$ and the group of ketone on the C-4 atom, as well as the OH- group on the adjacent C-3 or C-5 atom of flavones and flavonols, is the fundamental principle of colorimetric reactions (Lindawati & Ma'ruf, 2020). Figure 3 depicts the reaction that results in the formation of the flavonoid-$AlCl_3$ complex.

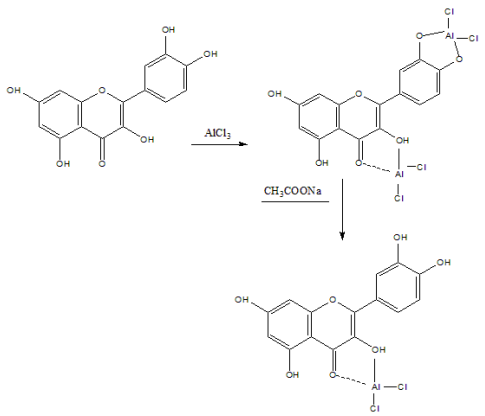


Figure 3. Reaction for the formation of complex flavonoid-$AlCl_3$ (Lindawati & Ma'ruf, 2020)

The quercetin regression equation was obtained y=0.0088x-0.005. Linearity obtained correlation coefficient value of 0.9979. The results of total flavonoid content can be sorted as follows: 70% > 50% > 96% (Table 2). The extract with 70% ethanol had the highest total flavonoid value (0.559 ± 0.03 mgQE/g extract). The same results are from the previous research which gave the highest total flavonoids in 70% ethanol (Suhendra et al., 2019; Rahardhian et al., 2019; Dong et al., 2022). Sucipto et al. (2022) reported that optimization results of ethanol concentration showed that 70% ethanol was optimal for extracting flavonoids. The total flavonoids level are smaller than the previous study from

Sudha et al. (2012) which obtained a result of 53.85 mgRE/g because they used ethyl acetate solvent which has semi-polar properties.

Flavonoid compounds break down into several types, every kind of flavonoid has a specific polarity relying on the position and number of the OH- group so that the solubility of flavonoids is affected by the solvent (Hikmawanti et al., 2021). The 70% ethanol gives the highest total flavonoids. It explains that the characteristics of the flavonoid compound in that extract have the same polarity as the solvent 70% ethanol. These results are the same as the yield obtained, namely the highest yield at 70% ethanol. So that the pepino melon fruit extract with 70% ethanol produces the highest content of flavonoid compounds according to the principle "like dissolves like". Which means that the solvent will only extract active substances that have similar polarities Suhendra et al., 2019; Hijazi et al., 2015).

Table 2. Total flavonoid (TFC), total phenolic (TPC), and β-carotene content of pepino fruit extract at different ethanol concentrations

Extraction Solvent	TFC (mgQE/g)	TPC (mgGAE/mg)	β-Carotene (mg/g)
50% Ethanol	0.298 ± 0.04	4.763 ± 0.08^{a}	0.157 ± 0.02^{c}
70% Ethanol	0.559 ± 0.03*	3.631 ± 0.12^{b}	0.910 ± 0.16^{b}
96% Ethanol	0.289 ± 0.01	3.317 ± 0.10^{c}	1.054 ± 0.13^{a}

Data in the table are represented as a mean value of replication ± SD (n = 3). *Significant to 50% and 96% Ethanol. The differences between the letters are significantly different ($p < 0.05$)

Quantification of total phenolic levels

The Follin-Ciocalteu technique was used for the test. The ability of a phenolic group to convert the Follin-Ciocalteu reagent's phosphomolybdate-phosphotungstate complex into a blue molybdenum-tungsten (Mo-W) complex that can be identified by spectrophotometry UV-Vis is the basis of the method (Haeria et al., 2014) (Figure 4).

The equation for the gallic acid calibration curve is y=0.0081x+0.1276. Measurement of the calibration curve resulted in an R-value of 0.996. This value (R) is shut to 1, so the gallic acid calibration curve equation is linear. The greatest total phenolic level obtained was: extract with ethanol of 50% > 70% > 96%. The extract with 50% ethanol produced the best total phenolic level, namely 4.763 ± 0.08 mgGAE/g extract. When compared to 70% and 96% ethanol, the comparatively high water content in 50% ethanol solvent has created more polar conditions, which can facilitate the extraction of polyphenolic chemicals (Jovanović et al., 2017). According to Ballesteros et al. (2014), ethanol concentrations ranging from 20% to 60% can extract phenolic chemicals more effectively. The same study showed that 50% ethanol gave the highest total phenolic content (Hikmawanti et al., 2021). Another study showed the highest total phenolic content (4.50 ± 0.06 mgGAE/g) in 50% ethanol compared to 60%, 70%, and 80% ethanol (Jokić et al., 2009).

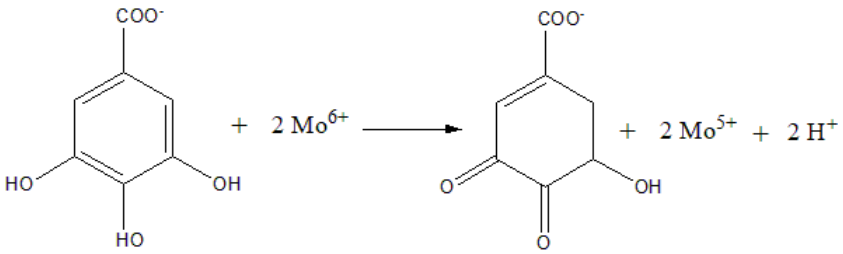


Figure 4. The reaction of gallic acid and Folin-Ciocalteu reagent (Nunes et al., 2011)

The greater the composition of water in the ethanol solvent, the higher the phenolic compounds (flavonoid glycosides) such as gallic acid (3,4,5-trihydroxy benzoate) which are easy to dissolve in water and often bind to sugar as glycosides and usually in cell vacuoles. In addition, it has the ability to also extract a class of compounds with a wider polarity (Lohvina et al., 2022). It has been mentioned that the total phenolic level obtained in the use of mixed solvent ethanol-water is greater than water alone (Maulana et al., 2019).

Quantification of β-carotene content

The outcomes of the validation of the analytical method are presented in Table 3. The validation of the analytical method aims to prove that the assay method used can provide precise and accurate results (Purwanto et al., 2021). All test parameters have met the requirements so that the technique can be used for the quantification of β-carotene content. The level of β-carotene must be greater than the LOD of 0.131 ppm and the LOQ of 0.437 ppm.

Table 3. The results of the validation analysis method of β-carotene

Parameters	Results	Requirement
Specificity	Rs = 3.563	Rs ≥ 1.5
Linearity	R = 0.999	R ≥ 0,999
Precision	The actual level of β-carotene is 20 and the level obtained is 20.677 with a CV of 1.32%	CV < 2%
Accuracy	101.2%; 102.3%; 101.3% at levels of 5, 20, and 35 ppm	Percent Recovery = 98 – 102%
LOD (Limit of Detection)	0.131 ppm	
LOQ (Limit of Quantification)	0.437 ppm	

Table 2 shows that the highest β-carotene content (1.054 ± 0.13 mg/g) was in the 96% ethanol extract. The obtained beta carotene levels can be sorted as follows: extract with ethanol of 96% > 70% > 50%. The chromatograms of the three samples can be seen in Figure 5. The β-carotene content of this research was greater than the previous research which contained β-carotene content ranging from 57-68 µg/g in the ripe pepino melon samples (Kola, 2010). Similarly, the β-carotene content of pepino melon fruit was reported as 27 mg/100 g (Maheshwari et al., 2014).

β-carotene molecule is nonpolar and has a lower solubility in water compared to water-ethanol. Increasing the percentage of ethanol concentration will reduce the dielectric constant, thereby increasing the solubility of β-carotene (Mohammadi et al., 2020). So, the β-carotene compound dissolves well in 96% ethanol compared to 50% and 70% ethanol.

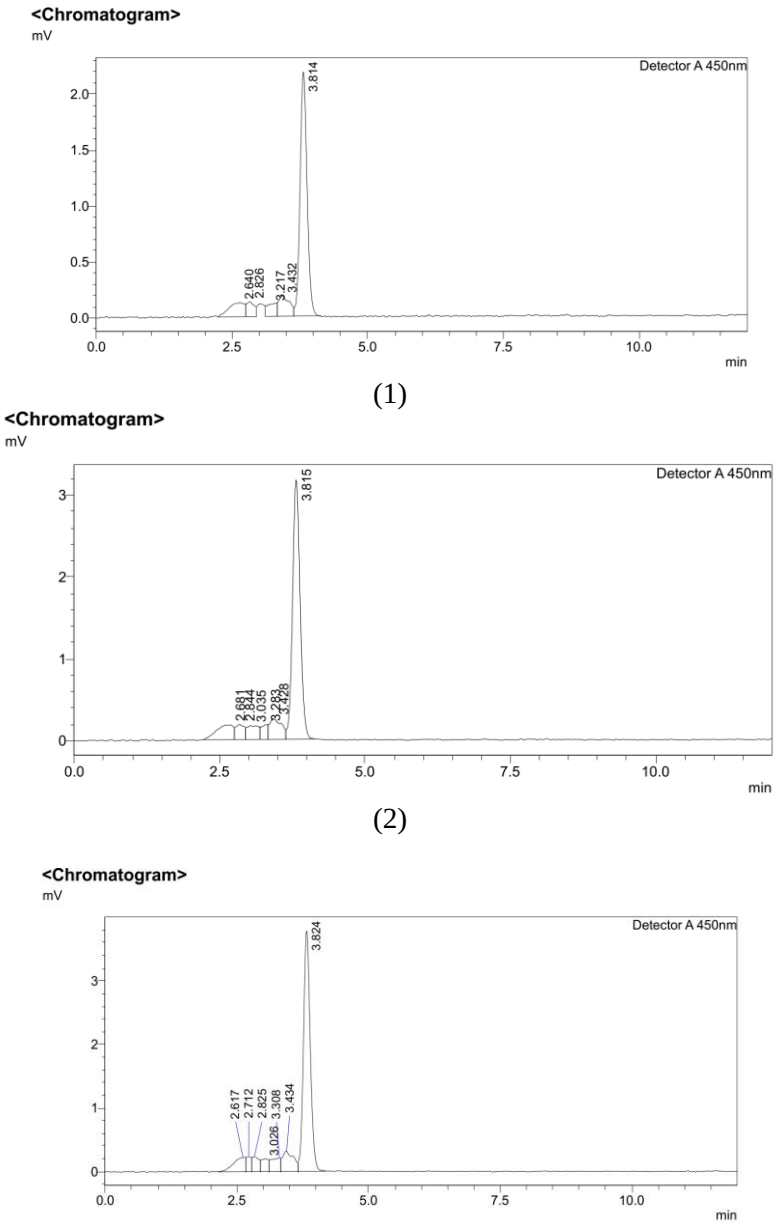


Figure 5. The β-carotene chromatogram of pepino melon fruit extract. (1) 50% ethanol (2) 70% ethanol (3) 96% ethanol

CONCLUSION

Variations in ethanol concentration affected the extract yield, total flavonoid, phenolic, and β-carotene content of pepino melon fruit. The 70% ethanol was the optimal solvent concentration for extracting pepino fruit.

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