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Original paper

Accelerated Solvent Extraction of Flavan-3-OL Derivatives from Grape Seeds

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Accelerated Solvent Extraction of flavan-3-ols from grape seed was carried out to determine the effect of extraction conditions (solvent type, temperature, extraction time, particle size, and cycle number) at the constant pressure (10.3 MPa). The highest flavan-3-ols amount was obtained with 70% acetone. The recovery of flavan-3-ols increased with extraction temperature and maximized at 120℃. There was an almost two-fold increase in the amount for flavan-3-ols extracted when the average particle size decreased from 0.725 mm to 0.512 mm. The highest extraction yields were achieved at an extraction time of 20 min. Two cycles for ten min each were also found to recover the maximum amount of flavan-3-ols.

Keywords: grape seed, flavan-3-ols, accelerated solvent extraction

Introduction

Grape seeds are one of the major sources of polyphenols, particularly flavan-3-ols and proanthocyanidins, which have been shown to act as strong antioxidants and exert health-promoting effects (Dai and Russell, 2010). Flavan-3-ols are mainly represented by (+)-catechin, (−)-epicatechin and (−)-epicatechin-3-O-gallate. Among the proanthocyanidin, the dimers (dimer B1 and B2) are usually most abundant compounds in seeds (Prieur *et al*., 1994).

Sample preparation is a primary and critical aspect of flavan-3-ol analysis. Conventional solvent extraction is the initial step in the processing of plant extracts rich in phenolic compounds prior to analysis. However, the conventional extraction methods used to obtain these types of products have several drawbacks. They are time-consuming, laborious, and exhibit low selectivity and/or extraction yields; moreover, they usually employ large amounts of organic solvents (Cacace and Mazza, 2007) .

Accelerated Solvent Extraction (ASE) is a solid-liquid extraction process performed at elevated temperatures and high pressures. Extraction is carried out under pressure to maintain the solvent in its liquid state at high temperature. High temperatures and pressures increase the penetration of solvent into the plant material and improve constituent solubilization, enhancing extraction speed and yield (Carabias-Martinez *et al*., 2005).

A number of studies on the extraction of grape phenolics have been performed. Most of them have considered the conventional solvent extraction technique (Spigno *et al*., 2007; Pinelo *et al*., 2005a; Pinelo *et al*., 2005b), and some have explored supercritical fluid extraction (Fioria *et al*., 2009). There are also some studies on the process of using hot water at high temperature and high pressure (PLWE) (Monrad *et al*., 2012). However, water alone also dissolves undesired proteins and polysaccharides, particularly under high pressure and temperature. Although ASE was performed in a few studies for the extraction of proanthocyanidins and anthocyanins from grape pomace or skin, only the effect of temperature and ethanol and acidified organic solvent concentration on the extraction yields of those compounds were studied (Monrad *et al*., 2010; Ju and Howard, 2003).

Therefore, in the present study, the ASE method was applied for the extraction of flavan-3-ols from grape seed to investigate the effects of extraction parameters (solvent type, temperature, average particle size, static extraction time, and number of extractions) on the extraction efficiency of flavan-3-ol derivatives.

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Materials and Methods

Materials Grape seeds generated as waste during the production of red grape juice were kindly provided by Asya Fruit Juice Ind. Inc. (Isparta, Turkey). The moisture content of the seeds was 7.13% (w/w). (+)-Catechin (C), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCG), (−)-epicatechin gallate (ECG), proanthocyanidin B1 and B2 were purchased from Sigma-Aldrich (Taufkirchen, Germany). All other solvents and chemicals used in the extraction and analysis were purchased from E. Merck Co. (Darmstadt, Germany). The dried grape seeds were milled in a coffee grinder for 5 s, and separated by sieving into five particle classes (0.224 − 0.425 mm, 0.425 − 0.600 mm, 0.600 − 0.850 mm, 0.850 − 1.250 mm, and 1.250 − 1.800 mm). Average particle sizes (mm) were calculated as mass mean diameter from the following equation:

$$Dw = \sum X_i D_{pi} \quad \cdots\cdots \text{Eq. 1}$$

where, Dw: mass mean diameter (mm), Xi: mass fraction of given increment, and Dpi: average particle diameter, taken as arithmetic average of smallest and largest particle diameters in increment (McCabe, Smith and Harriott, 2005)

Accelerated solvent extraction (ASE) ASE was performed using a Dionex ASE 100 instrument (Dionex Corp., Sunnyvale, CA, USA). The system consisted of a solvent module, a pump delivering a fixed fluid pressure of 1500 psi, a thermostated extraction cartridge, and an extract collection device. Ten grams of defatted milled grape seed was packed into a 100 mL extraction cartridge. The extraction cartridge was packed with glass beads to fill the void spaces, and a cellulose filter was used at the outlet of the extraction cartridge to remove particles from the extract. The pre-set default conditions were as follows: the solvent flush volume was 60% of the extraction cell; purging was performed for 90 s using pressurized nitrogen (10.3 MPa); and the extract was collected in 60 mL glass vials with Teflon-coated rubber. After the extraction process, the extracts were partitioned with hexane three times to remove oils, and organic solvents were rotary evaporated at 40℃. The remaining aqueous phase was freeze-dried. Extractions were performed in duplicate, and the extraction yields were calculated based on dry weight.

In the first step of the experiment, the effects of extraction solvent type (pure acetone, ethyl acetate, methanol, ethanol, and aqueous solutions containing 50 and 70% methanol, acetone, and ethanol) on the yields of individual flavan-3-ols were tested. Then, the temperature (50℃, 80℃, and 120℃), static extraction time (5, 10, 20, and 30 min), average particle size (0.325 mm, 0.512 mm, 0.725 mm, 1.050 mm, and 1.525 mm), and cycle number (1, 2, 3, 4, and 5) were studied using the solvent determined to be the optimum in the first step of the extraction of phenolic compounds.

Conventional solvent extraction Phenolic compounds were extracted from 5 g of defatted seeds (0.325 mm of average particle) with 50 mL of acetone:water (70:30) at 50℃ for 12 hrs. Aceton from extract was removed by rotary evaporator at 40℃ and remaining aliquot extract was freeze dried.

Monomeric and dimeric flavan-3-ol content by HPLC The equipment used for the HPLC analysis was a Hewlett–Packard HPLC System (Agilent 1100 series, Waldbronn, Germany) consisting of a quaternary pump, diode-array detector (DAD), autosampler, and column oven. For the separation, a 250x4.6 mm i.d., 5-µm, Kromsil C18 (Technocroma, Barcelona, Spain) operating at 30℃ was employed. The flow rate was 1 mL/min. The eluent was composed of (A) water/acetic acid (98:2) and (B) acetonitrile/water/acetic acid (80:19.6:0.4) (Bozan *et al*., 2008). The following linear gradient program was used for the elution: 0 − 10% B for 5 min; 10 − 30% B for 20 min; 30 − 50% B for 10 min; 50 − 60% B for 5 min; 60 − 90% B for 5 min; then, a return to the initial conditions within 5 min and the re-equilibration of the column.

The chromatogram was monitored at 280 nm, and the identification of the phenolics was based on a comparison of the retention times and on-line spectral data to standards. The quantification of phenolic acids was performed using the calibration curves of each standard compound. Two determinations were made for each extract.

Statistical analysis Experimental data were reported as mean ± SD for triplicate determination. Analysis of variance (STATGRAPHICS 3.1) was conducted to identify differences among the means. Multiple comparison of the means was performed by the least significant difference (LSD) test at the $\alpha = 0.05$ level.

Results and Discussion

Effect of extraction solvent The effect of solvent type on the extraction of flavan-3-ols was studied under the following fixed conditions: a temperature of 80℃, an average particle size of 0.512 mm, a seed mass of 10 g, single cycle, and an extraction time of 10 min. As shown in Figures 1, solvent type showed a significant effect ($p < 0.05$) on the purity and extraction yield of the flavan-3-ols. The extracts obtained with the pure organic solvents contained higher flavan-3-ol contents (Figure 1a). The contents of the main monomeric flavan-3-ols, (+)-catechin (C) and (−)-epicatechin (EC), were higher in acetone and ethyl acetate than in the other solvents. However, the extraction yields of those compounds in the pure solvents were very low, especially for ethyl acetate, compared with those in the other solvents (Figure 1b). The highest amount of monomeric and dimeric flavan-3-ols was recovered using 70% acetone. The efficacy of the solvents in terms of their ability to extract individual flavan-3-ols followed the order 70% acetone > 50% acetone > 70% ethanol > 50% ethanol = 70% methanol > 50% methanol > methanol > ethanol > acetone > ethyl acetate.

The experimental results regarding the effects of solvent type on the extraction of phenolic compounds were in accordance with

those of some previous studies, which reported that a binary solvent system was more useful and favorable in the conventional extraction of polyphenols from plant samples than a mono-solvent system (Spigno *et al*., 2007; Yilmaz and Toledo, 2006; Nawaz *et al*., 2006). Different solvents have been used for the extraction for proanthocyanidins and a low degree of flavan-3-ols, principally methanol, acetone, ethanol, ethyl acetate, and their aqueous solutions (Pekic *et al*., 1998). Some studies have reported that methanol and aqueous methanol are the most common solvents for extracting a low degree of flavan-3-ols. Others have indicated that methanol and aqueous acetone are the best solvents for the extraction of catechin and proanthocyanins, respectively (Pineiro *et al*., 2004; Yilmaz and Toledo, 2006). In this study, the aqueous acetone solutions (70% and 50%) were the most effective solvents for the extraction of dimeric proanthocyanidins (proanthocyanidin B1 and B2) as well as catechin and its derivatives. Although ethyl acetate is reported to be a selective solvent for the extraction of a low degree of flavan-3-ols, it is generally used for purification in the solvent partition step of the extraction of these compounds (Spigno *et al*., 2007; Pekic *et al*., 1998). Ethanol and its aqueous solutions are other commonly used solvent systems for the extraction of polyphenols. However, their selectivity for the extraction of flavan-3-ols is found to be lower than that of acetone-water mixtures because they are able to extract non-phenolic

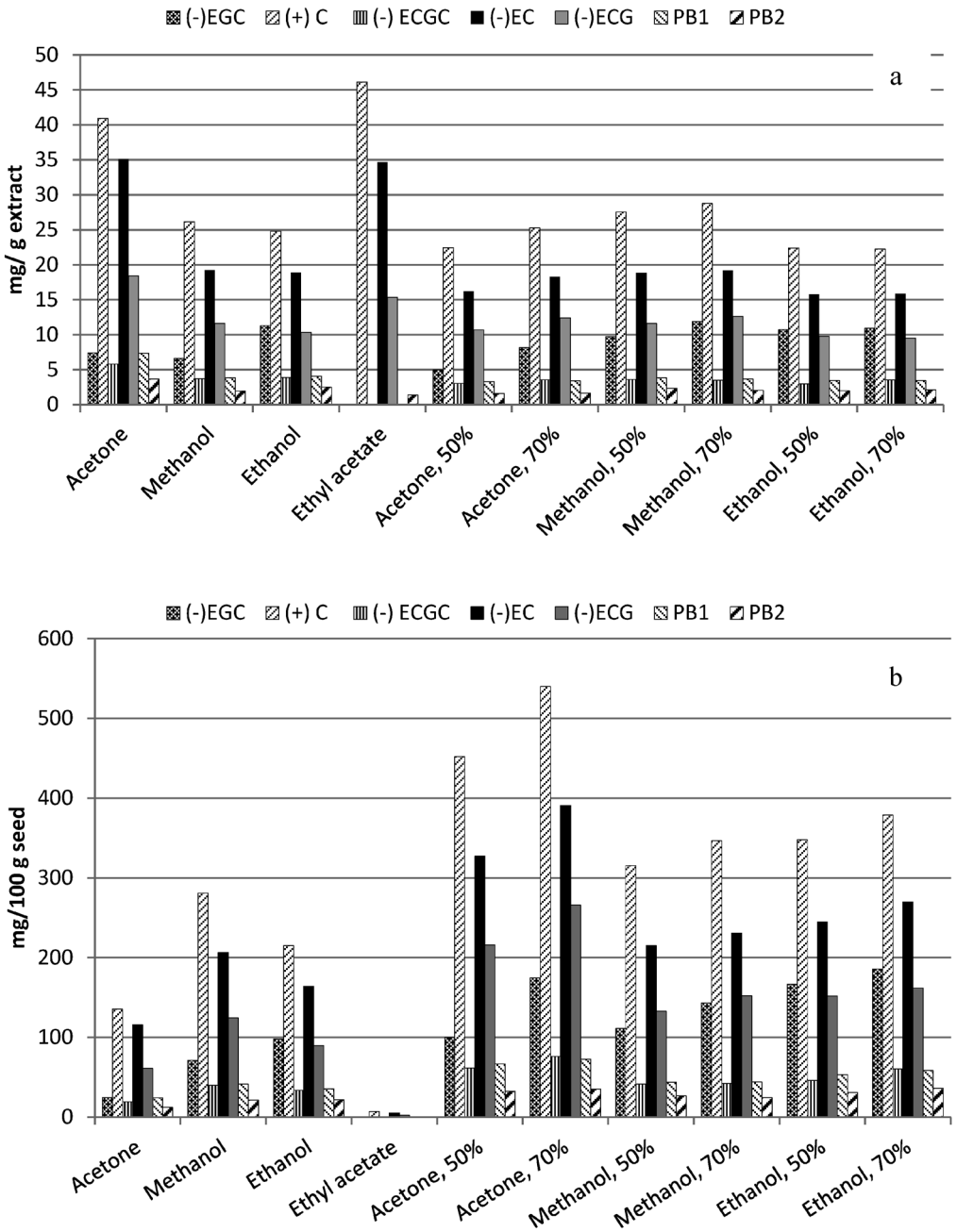

Fig. 1. Effect of extraction solvents on the purity (a) and recovery (b) of individual flavan-3-ols, (n = 3); (80℃, 0.512 mm, 10 min, 1 cycle), C: (+)-catechin, EC: (–)-epicatechin, EGC: (–)-epigallocatechin, EGCG: (–)-epigallocatechin gallate, ECG: (–)-epicatechin gallate, PB1: proanthocyanidin B1 and PB2: proanthocyanidin B2)

compounds such as sugars, pigments, fats, etc. Moreover, acetone-water mixtures are reported to be more effective for the extraction of polyphenols from proteic matrices because they are able to degrade polyphenol-protein complexes (Santos-Buelga *et al*., 2012).

Effect of temperature The pressurized liquid extraction process allows for the use of temperatures well above the normal boiling points of solvents, which is not possible with traditional and other common extraction procedures. In this study, 10 g of grape seeds was extracted at 50, 80, and 120℃ using 70% aqueous acetone and an average particle size of 0.725 mm after 10 min of extraction. Temperature also positively affected both the purity (data not shown) and yields (Figure 2) of flavan-3-ols. As the temperature increased from 50℃ to 80℃, the increments in the yields of (+)-C and (−)-EC were only 15% and 57%, respectively; however, at 120℃, the yields of these compounds increased to almost 2.5 and 1.5 times, respectively, those obtained at 80℃.

Elevated temperature has been reported to improve the efficiency of extraction due to the corresponding increase in the solubility and diffusion rates of compounds to be extracted (Gertenbach, 2001). An increase in extraction yield with temperature was observed during the pressurized water extraction of phenolics from flaxseed (Cacace and Mazza, 2006) and hydroxycinnamates from red grape skin (Ju and Howard, 2003) and the subcritical solvent extraction of proanthocyanidins from grape seeds (Monrad *et al*., 2010; Spigno *et al*., 2007)

Effect of particle size Particle size is another factor that must be considered during extract processing. Using small particles reduces the distance over which solutes diffuse within solids and increases the concentration gradient, which ultimately increases the extraction rate (Gertenbach, 2001). Because the distance over which a solute must diffuse to reach the surface is shorter, the extraction time is reduced. In Figure 3, the flavan-3-ol yields from dry grape seeds with different particle sizes at 80℃ are shown. It can be seen that recovery of flavan-3-ols increased with decreasing particle size as expected. The particle size showed no significant effect on the purity (mg/g extract) of flavan-3-ols (data not shown). Flavan-3-ols yields increased almost two and three fold when the particle size decreased from 0.725 mm to 0.512 mm. No significant differences were found in the yields of flavan-3-ols between average particle sizes of 0.512 mm and 0.325 mm. The effect of particle size on the extraction yields of phenolic compounds was not linear, and similar results were reported by (Devanand and Luthria, 2008). The positive effect of reducing the particle size has been previously reported in other studies on phenolic extraction from grape by-products. However, the positive effects of reducing the particle size are not always obvious because a reduction in the particle size can obstruct the access of the solvent to all of the exposed surface area of the solid and release phenol compounds (Pinelo *et al*., 2005a).

Effect of extraction time and cycle number The extraction time is crucial in minimizing the energy and cost of extraction processes. In accelerated solvent extraction, the extraction time is defined as the period during which the sample interacts with the extraction solvent per cycle in the extraction cell. In this study, the maximum concentration of flavan-3-ols was generally achieved in an extraction time of 20 min. The yields of flavan-3-ols increased with increasing extraction time (Figure 4) up to 20 min. A greater amount of flavan-3-ols was recovered in the extraction performed over two cycles. The amount of flavan-3-ols extracted in the two cycles was not significantly different from the amounts obtained at higher cycle numbers.

Comparison of flavan-3-ols recoveries obtained by ASE and conventional extraction ASE had the highest extraction yield

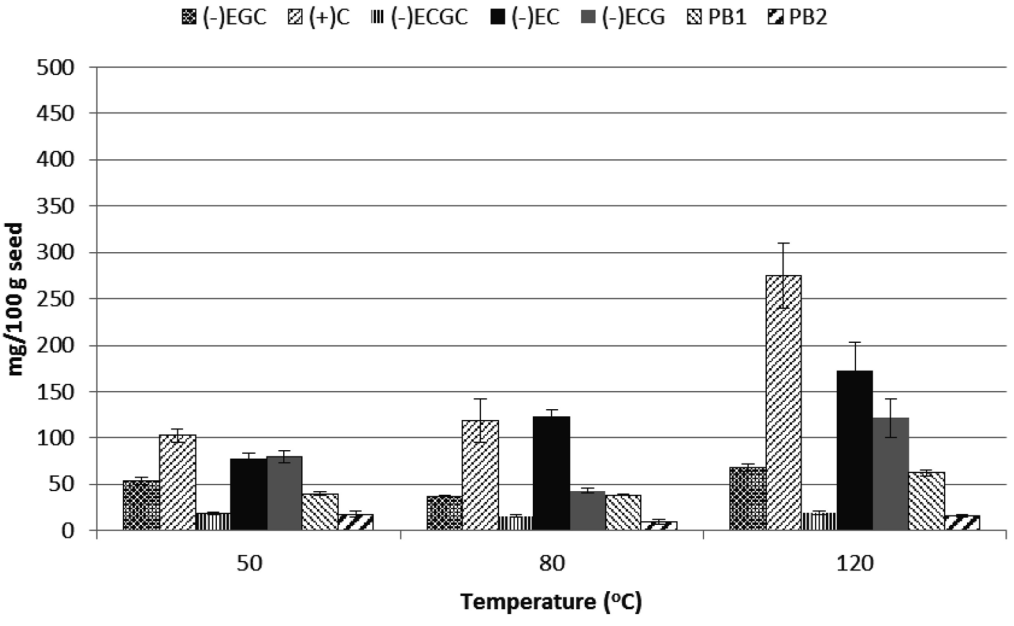

Fig. 2. Recovery of flavan-3-ols at different temperatures (n = 3) (70% acetone, 1.05 mm, 10 min, 1 cycle).

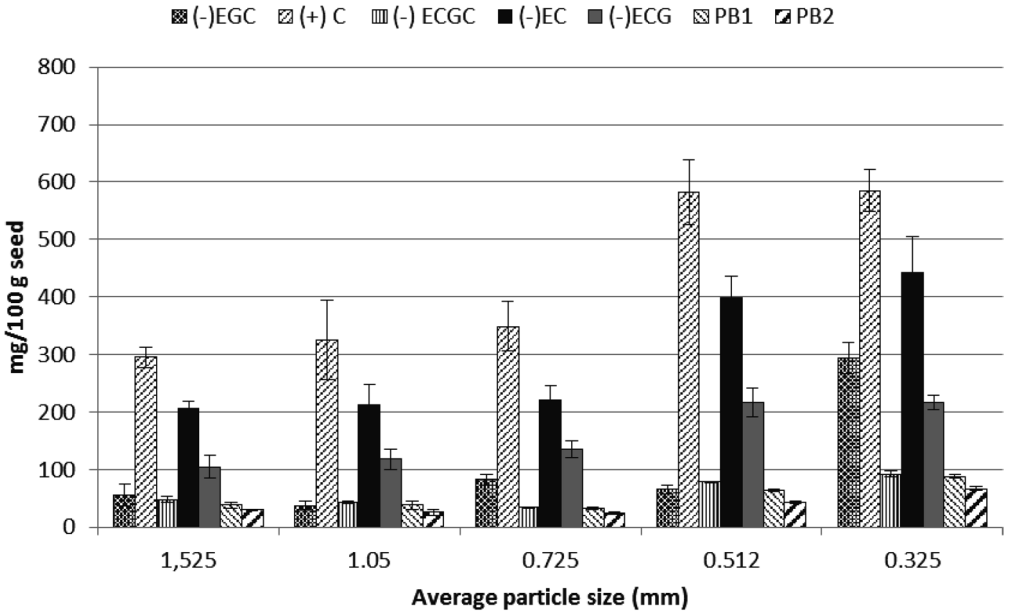

Fig. 3. Effect of particle size on the recovery of flavan-3-ols (n = 3) (70% acetone, 80℃, 10 min, 1 cycle).

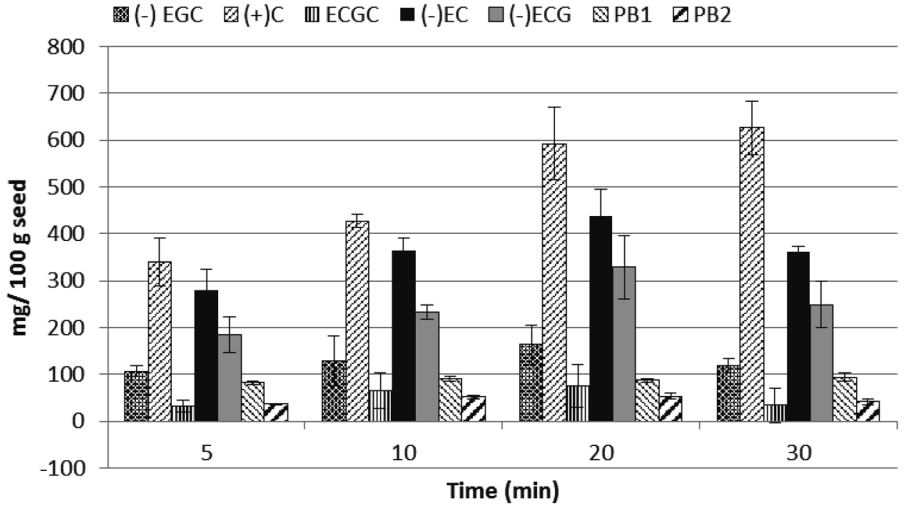

Fig. 4. Recovery of flavan-3-ols at different extraction times (n = 3) (70% acetone, 80℃, 0.512 mm, 1 cycle).

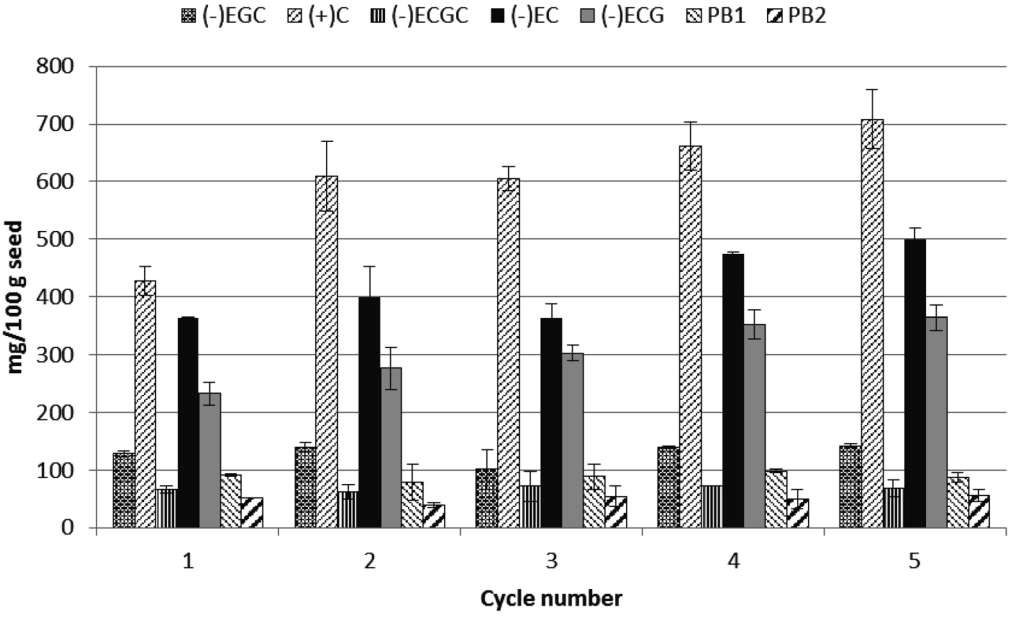

Fig. 5. Recovery of flavan-3-ols at different cycle numbers (n = 3) (70% acetone, 80℃, 0.512 mm, 10 min).

(~25%) at the average particle size of 0.325 mm at 120℃ for 10 min extraction time, whereas the extraction recovery of conventional extraction was 15% at 50℃ for 12 hrs extraction time, due to the improvement in extraction efficiency by the high temperature of ASE. Composition and the amount of flavan-3-ols in the extracts both obtained by ASE and conventional extraction method were similar, since the extraction solvent was the major factor influenced the composition of flavan-3-ols in the extracts.

Conclusion

Among the various solvents studied for the ASE of flavan-3-ol compounds from grape seed, 70% acetone gave the highest extraction yield. The increase in the yield of flavan-3-ols (both monomers and dimers) with the increase in extraction temperature from 80℃ to 120℃ was 90%. The extraction efficiency was almost two or three fold less in the extraction with average particle sizes above 0.512 mm. Twenty min for one cycle or two cycles, 10 min each, were enough for the extraction of monomeric and dimeric flavan-3-ols. The results obtained in this study represent the first step in optimizing the ASE extraction process, and further studies on the optimization of other extraction conditions are required to maximize the extraction efficiency of grape seed flavan-3-ols.

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