

Determination of Extractability of Pine Bark Using Supercritical CO₂ Extraction and Different Solvents: Optimization and Prediction

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Bark from *Pinus brutia* was extracted with supercritical fluid extraction (SFE), using CO₂, at various extraction conditions both at laboratory and at pilot scale. Optimized parameters were 200 bar, 60 °C, and 3% ethanol at a solvent/feed ratio of 30. Additionally, the pine bark was sonicated (1 h at 50 °C) by different solvents (*n*-hexane, dichloromethane, ethyl acetate, and ethanol) to investigate the correlation between the different extraction setups and to obtain information on SFE up-scaling possibilities. Analyzed by HPLC, 7.2% of (–)-catechin was extractable at laboratory scale, and 58.4% (800 bar) and 47.8% (200 bar), both with modifiers, at pilot scale. By sonication with ethanol, 46.8% of (–)-catechin and almost 100% of (–)-epicatechin and (–)-catechin gallate were extracted. Ethyl acetate extract revealed high correlations with the laboratory scale SFE ($r = 0.98$) and also pilot scale SFE runs at 200 ($r = 0.99$) and 800 bar ($r = 0.98$) without modifiers.

KEYWORDS: Pine bark; supercritical CO₂ extraction; catechins; polarity; optimization; pilot scale

INTRODUCTION

The use of pine bark dates back to ancient times, and today pine bark extracts and other oligomeric proanthocyanidin complexes are widely consumed as food ingredients or dietary supplements and are attracting more interest in the fields of nutrition, health, and medicine (1). Normally, supercritical CO₂ extraction (SFE) and solvent extraction are used to concentrate substances of interest from valuable plant materials with preference, however, to SFE. The extracts obtained with SFE can be considered to be more sustainable and healthier, conforming with industrial requirements as to healthy food and pharmaceutical products because they usually are solvent-free (if solvents are applied for SFE, they are used as modifiers in small amounts to enhance the extraction). Pine bark extracts, however, contain a great number of phenolic compounds such as (–)-catechin, (–)-epicatechin, taxifolin, and phenolic acids with polar characteristics (2, 3). Due to their high polarity, they are difficult to extract under SFE conditions, and particularly with CO₂, the most widely used supercritical fluid, especially in the food industry because of the safety requirements (4). Although the solvating power of supercritical fluids is adjustable

with pressure, enabling extraction of biomaterials due to the change in their densities and enhanced mass transfer rates during extraction, CO₂ is more suitable for the extraction of nonpolar solutes, which is attributed to there being no permanent dipoles in their structure. However, if a particular modifier with a high dielectricity constant is used, the extractability of polar compounds can be significantly increased (5, 6). Therefore, addition of solvents such as methanol, ethanol, acetone, and water is required for the efficient extraction of compounds with polar properties existing in pine bark. It is a well-known fact that extraction with water and ethanol is the most common industrial method to manufacture pine bark extracts from *Pinus maritima*, which is the most extensively investigated *Pinus* species (7).

The effects of different extraction setups on the quantitative analysis and biological activities of compounds from various pine barks have been reported (8–13). However, there is still a lack of information regarding supercritical CO₂ extraction of *Pinus* species. One study dealt with supercritical CO₂ extraction of ponderosa pine with the aim to evaluate the extraction conditions required to remove resin and fatty acids from wood. The bark was also extracted at 207 bar, as well as 100 and 160 °C in the presence of ethanol. However, no data were available about the composition of the extract, and it was not the objective to extract phenolic compounds (14). Another recent study focused on the fractionated SFE of antioxidants from the bark of *P. maritima*. The authors carried out experiments using CO₂ and CO₂ + ethanol (10%) mixtures in two consecutive steps,

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Table 1. Contents of *P. brutia* Samples (Milligrams per Gram of Extract) Extracted with Different Solvents from Nonpolar to Polar^a

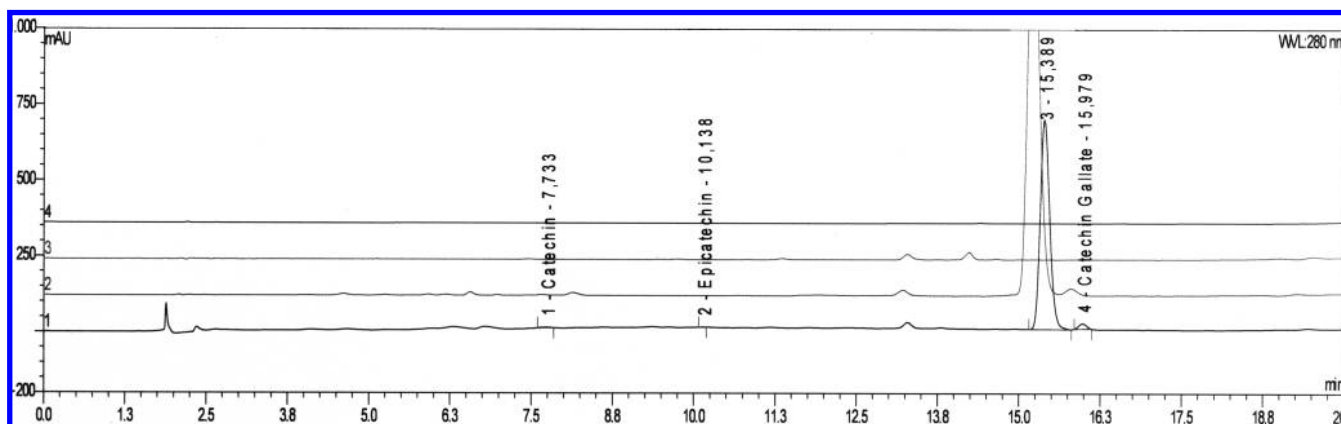
solvent	catechin	epicatechin	catechin gallate	taxifolin	total catechins
<i>n</i> -hexane			0.31 ± 0.01		0.31
dichloromethane	1.63 ± 0.05	0.04 ± 0.02	0.04 ± 0.01		1.71
ethyl acetate	2.40 ± 0.03	0.49 ± 0.05	0.06 ± 0.01	0.102 ± 0.01	2.95
ethanol	2.83 ± 0.27	0.24 ± 0.02	1.73 ± 0.30	0.072 ± 0.01	4.80

^a Data are presented as means ± SEM of duplicate measurements.

Table 2. Effect of Temperature on the Contents of Catechins and Taxifolin (Milligrams per Gram of Extract) Extracted with SFE at 200 bar and a Solvent/Feed Ratio (S/F) of 30^a

SFE parameters	catechin	epicatechin	catechin gallate	taxifolin
27.5 °C, 200 bar, S/F = 30	0.34 ± 0.02	0.83 ± 0.01	0.003 ± 0.00	0.009 ± 0.00
30 °C, 200 bar, S/F = 30	0.24 ± 0.01	0.68 ± 0.31	0.004 ± 0.01	0.004 ± 0.00
40 °C, 200 bar, S/F = 30	0.18 ± 0.01	0.52 ± 0.01	0.003 ± 0.00	0.003 ± 0.00
60 °C, 200 bar, S/F = 30	0.26 ± 0.01	0.78 ± 0.01	0.006 ± 0.00	0.005 ± 0.00
80 °C, 200 bar, S/F = 30	0.10 ± 0.01	0.19 ± 0.02	0.004 ± 0.00	0.003 ± 0.00

^a Data are presented as means ± SEM of duplicate measurements.

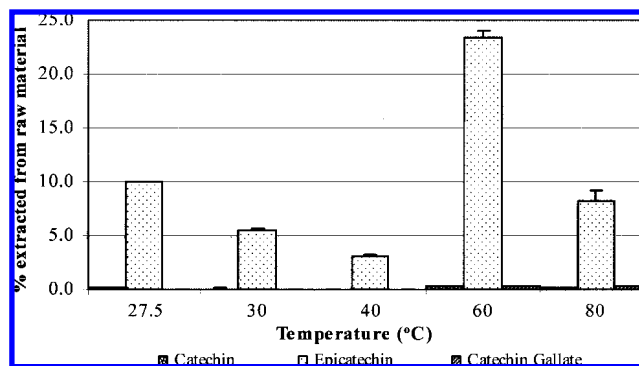
**Figure 1.** HPLC chromatograms of samples extracted with hexane (4), dichloromethane (3), ethyl acetate (2) and ethanol (1).

which made it possible to obtain different extract fractions with diverse antioxidant capacities (15). On the other hand, supercritical CO₂ extraction of green tea was more extensively investigated. The main goal of those studies was to remove caffeine from green tea powder while retaining the catechins (16, 17). In the literature, some specific studies focused on the separation of catechins using supercritical CO₂ extraction from various raw materials or byproduct such as green tea (18), tamarind seed coat (19), and grape seeds and skins (20–22). These studies, however, raised the questions of whether flavan-3-ols and taxifolin, as the main phenolic compounds in pine bark, are extractable with SFE and whether similar extraction efficiencies could be achieved in comparison to solvent extraction.

The primary objective of this research study was to optimize process parameters at SFE and to obtain extracts in both laboratory and pilot scale under optimized conditions, which were then quantified by HPLC. The other was to predict the supercritical CO₂ extractability of the chosen phenolic substances in pine bark by sonication with different solvents, which is not so applicable with SFE. On the basis of these data and solvent extract compositions of the same material, a correlation could be established to compare the efficiencies of both extraction methods.

MATERIALS AND METHODS

Plant Material. *Pinus brutia* bark was collected in Izmir-Deliomer region, Turkey (38° 10' 17.0" N, 27° 03' 46.7" E, altitude = 120 m) in August 2006. The specimen was dried at room temperature, ground by a conventional grinder, and stored at 4 °C.

**Figure 2.** Effect of temperature on the extraction efficiency (percent) of SFE at 200 bar and a solvent/feed ratio of 30. Data are presented as means ± SEM of duplicate measurements.

Extraction of Pine Bark Using Solvents with Different Polarities.

About 5 g of bark was extracted in a sonicator (Bandelin Sonorex Super RK 510H) at 50 °C for an hour using 100 mL each of the following solvents: *n*-hexane (VWR Prolabo), ethyl acetate (Merck), and ethanol (J. T. Baker) (1:20, w/v). Dichloromethane (Carl Roth GmbH) was extracted at 35 °C, which is well below its boiling point (39.8 °C). Subsequently, the extraction mixtures were cooled and filtered, and then the solvents were evaporated in a rotary vacuum evaporator at 50 °C. Additionally, 2 g of bark was extracted with methanol in a sonicator at a ratio of 1:50 (w/v) for 15 min (23), and the contents were analyzed by HPLC to quantify each compound extracted from the raw material and thereby to obtain information on the extraction efficiency.

Supercritical CO₂ Extraction at Laboratory Scale. A Nova Swiss (Nova Werke AG, Effretikon, Switzerland) supercritical fluid extractor

Table 3. Effect of Pressure on the Contents of Catechins and Taxifolin (Milligrams per Gram of Extract) Extracted with SFE at 60 °C and a Solvent/Feed Ratio (S/F) of 30 and Effect of a S/F of 50 at 200 bar and 60 °C^a

SFE parameters	catechin	epicatechin	catechin gallate	taxifolin
60 °C, 100 bar, S/F = 30	0.11 ± 0.00	0.48 ± 0.03	0.006 ± 0.00	0.003 ± 0.00
60 °C, 200 bar, S/F = 30	0.26 ± 0.01	0.78 ± 0.01	0.006 ± 0.01	0.005 ± 0.00
60 °C, 300 bar, S/F = 30	0.18 ± 0.00	0.49 ± 0.01	0.004 ± 0.00	0.002 ± 0.00
60 °C, 200 bar, S/F = 50	0.17 ± 0.01	0.86 ± 0.01	0.004 ± 0.00	0.002 ± 0.00

^aData are presented as means ± SEM of duplicate measurements.

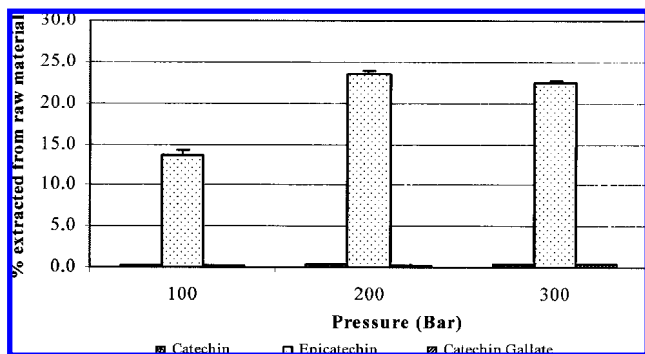


Figure 3. Effect of pressure on the extraction efficiency (percent) of SFE at 60 °C and a solvent/feed ratio of 30. Data are presented as means ± SEM of duplicate measurements.

was used to perform all of the experiments. About 40 g of the sample was placed into a 300 mL stainless steel cartridge. The extraction module was pressurized, and both the module and the separator were thermostated prior to the commencement of extraction. Pure CO₂ and mixtures with 1 and 3% of ethanol as modifier were delivered at a solvent/feed ratio of 30. From experience the chosen amount of modifier has proven to be most effective for the extraction of catechins from raw material (23). Additionally, it is known that solid-phase trapping efficiency is markedly affected by modifier in excess of 2% (24). Different temperatures were selected to investigate the contents of the extracts at room temperature (27.5 °C), subcritical (30 °C), and supercritical conditions (40, 60, and 80 °C) at 200 bar. Then the optimum temperature was identified, and additional trials were conducted at 100, 200, and 300 bar. Subsequently, a solvent/feed ratio of 50 was tested at the optimized pressure and temperature conditions. Extracts were collected from the separator outlet before CO₂ was released into the system.

Supercritical CO₂ Extraction at Pilot Scale. A 6.5 L Natex (Natex Prozesstechnologie GmbH, Ternitz, Austria) supercritical fluid extractor was used to perform the experiments at pilot scale. About 1000 g of sample was placed into the extraction vessel. The extraction vessel was pressurized, and both the vessel and the two separators were thermostated prior to the commencement of extraction. Pure CO₂ and a mixture with 3% of ethanol as modifier were delivered at a solvent/feed ratio of 30 at both 200 and 800 bar at 60 °C.

HPLC Analysis. Sample solutions were prepared by dissolving the extracts in the solvents originally used for extraction except for the ethanol fraction, which was dissolved in methanol (Merck), and the SFE extracts, dissolved in dichloromethane. All extracts were prepared at a concentration of 5.00 mg/mL in a Sonorex sonicator (Bandelin, Berlin, Germany).

The analysis of flavonoids was carried out using an HPLC method modified from that of Perva-Uzunalic et al. (23). A Synergi MAX-RP column (150 × 4.6 mm, 4 μm particle size; Phenomenex) was used. The mobile phase comprised 2% acetic acid (Merck) in water (A) and acetonitrile (Merck) (B). Gradient elution was performed starting with 92 A/8 B, changing the composition to 72 A/28 B in 20 min, followed

by 65 A/35 B in 10 min, and held for 2 min. Each run was finished to permit an equilibration with 92 A/8 B for 5 min. Detection wavelength, flow rate, and column temperature were set to 280 nm, 1 mL/min, and 30 °C, respectively. For all solutions (samples, standards) 10 μL was injected.

Calibration curves were established by dissolving 5.00 mg of (–)-catechin, (–)-catechin gallate (Sigma, Steinheim, Germany), (–)-epicatechin (Roth, Karlsruhe, Germany), and taxifolin (Fluka, Steinheim, Germany) in 5.00 mL of methanol and serially diluting this stock solution with methanol. Within the concentration range injected (1000.0–10 μg/mL) the detector response was linear ($R^2 = 0.9996$), with a detection limit of <0.05 μg/mL (data not shown in detail). Statistical analyses of the data were performed with Student's *t* test. A probability value of $P \leq 0.05$ was considered to denote a statistically significant difference, and $P \leq 0.01$ was also used to show the power of the significance. Data are presented as mean values ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

Extraction of Pine Bark Using Solvents with Different Polarities. *P. brutia* was extracted with four different solvents from nonpolar to polar to investigate if a correlation exists between this approach and supercritical CO₂ extraction. The investigation was based on determination of the amounts of four compounds, (–)-catechin, (–)-catechin gallate, (–)-epicatechin, and taxifolin. **Figure 1** displays the chromatograms of samples extracted with *n*-hexane, dichloromethane, ethyl acetate, and ethanol. (–)-Catechin gallate was the only compound extractable with *n*-hexane, whereas (–)-catechin, (–)-epicatechin, and (–)-catechin gallate could be extracted with dichloromethane and all four compounds were detectable in ethyl acetate and ethanol fractions. Although an increase was noted for all of the compounds from nonpolar to polar, highest amounts were achieved with ethanol. (–)-Catechin was quantified at 1.63 mg/g in the dichloromethane fraction, as well as at 2.40 mg/g in ethyl acetate and at 2.83 mg/g in the ethanol fraction. The contents of (–)-epicatechin (0.49 and 0.24 mg/g) and taxifolin (0.102 and 0.072 mg/g) were similar in ethyl acetate and ethanol. The content of (–)-catechin gallate was 43-fold higher in the ethanol fraction compared to the dichloromethane fraction (**Table 1**).

Starting material was extracted with methanol in a sonicator at a ratio of 1:50 (w/v) and was found to contain 1.19 mg/g of (–)-catechin, 0.05 mg/g of (–)-epicatechin, 0.03 mg/g of (–)-catechin gallate, and 20.03 mg/g of taxifolin. Extraction efficiencies were calculated on the basis of these data. Among solvent-extracted samples, the ethanol fraction displayed the highest extraction efficiencies with 46.8% of (–)-catechin and almost 100% of both (–)-epicatechin and (–)-catechin gallate, but only 0.0004% of taxifolin was extracted from the raw material. Perva-Uzunalic and co-workers (23) focused on the extraction efficiency of major catechins from green tea and reported an extraction efficiency of 77.2% of catechins with ethanol extraction. This value was in the same range of catechins in pine bark with 80.1%. On the other hand, about 6.3% of catechin, 30.3% of epicatechin, 5.7% of catechin gallate, and 0.0001% of taxifolin were extractable with ethyl acetate fraction, whereas only around 1.0% of each flavan-3-ol could be extracted with dichloromethane (**Table 2**).

Supercritical CO₂ Extraction at Laboratory Scale: Optimization. SFE experiments were first carried out at 200 bar with a solvent/feed ratio of 30 (1.2 kg of CO₂) at room temperature (27.5 °C), then at 30 °C to maintain subcritical conditions, and finally at 40, 60, and 80 °C to investigate supercritical conditions. From **Table 2** it can be seen that amounts of catechins were highest at 60 °C and degradation in catechins was observed when treated at 80 °C. In particular,

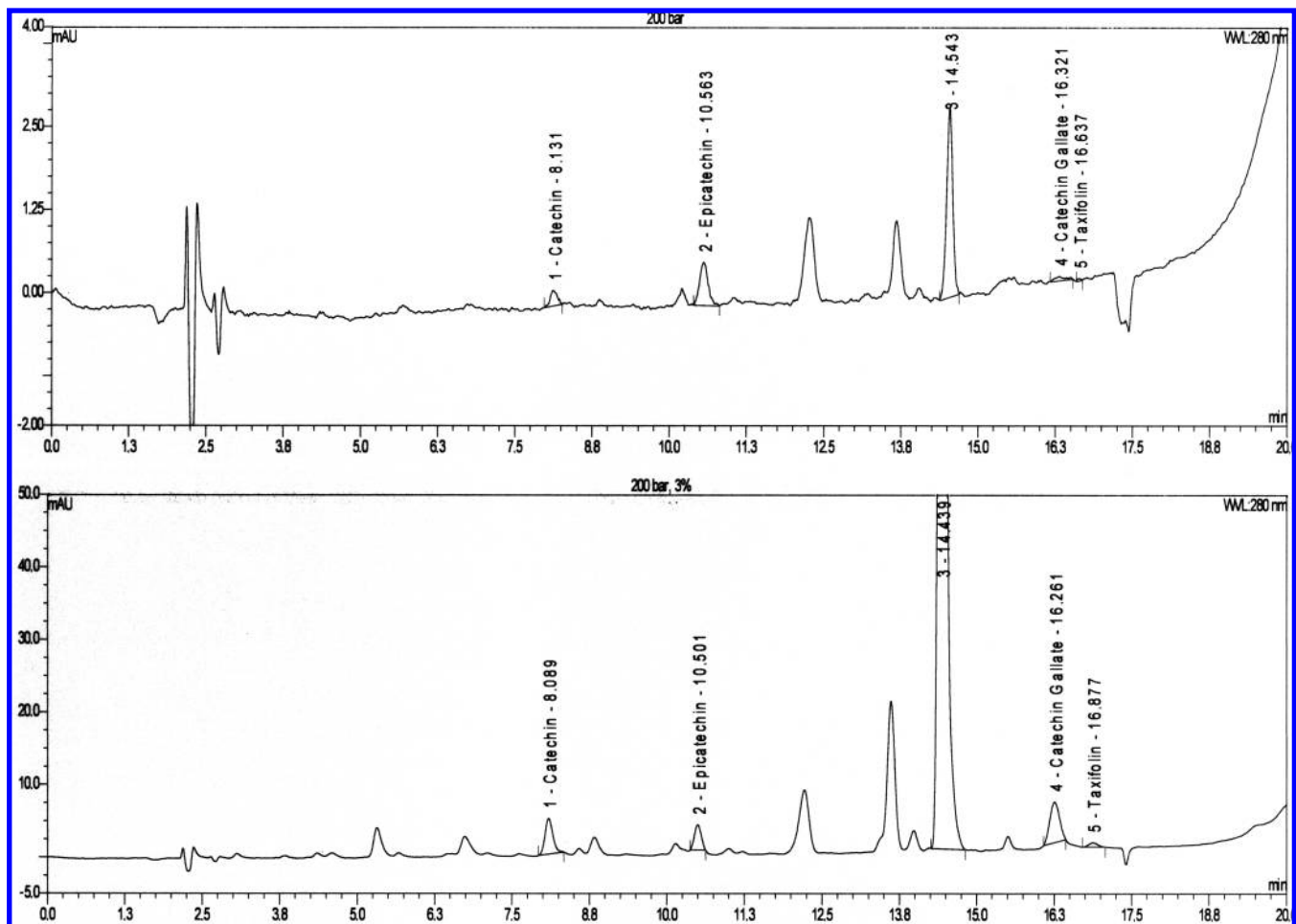


Figure 4. HPLC chromatograms of SFE samples without modifier and 3% of ethanol under the conditions of 200 bar, 60 °C, and a solvent/feed ratio of 30 at laboratory-scale SFE equipment.

Table 4. Effect of Modifier on the Contents of Catechins and Taxifolin (Milligrams per Gram of Extract) Extracted with SFE under the Conditions of 200 bar, 60 °C and a Solvent/Feed Ratio (S/F) of 30^a

SFE parameters	catechin				total catechins
	catechin	epicatechin	hallate	taxifolin	
60 °C, 200 bar, S/F = 30, 1%	0.86 ± 0.01	1.56 ± 0.01	0.09 ± 0.01	0.01 ± 0.00	2.51
60 °C, 200 bar, S/F = 30, 3%	6.12 ± 0.02	3.49 ± 0.05	2.12 ± 0.02	0.27 ± 0.02	11.73

^a Data are presented as means ± SEM of duplicate measurements.

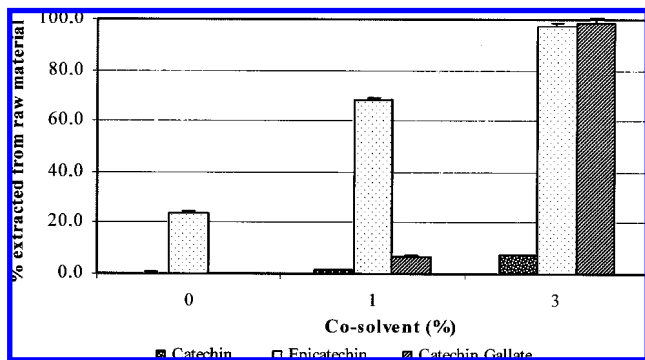


Figure 5. Effect of modifier on the extraction efficiency (percent) of SFE under the conditions of 200 bar, 60 °C, and a solvent/feed ratio of 30. Data are presented as means ± SEM of duplicate measurements.

trace amounts of (–)-catechin gallate and taxifolin were obtained. Therefore, these compounds were not extracted but

rather transferred through the extraction matrix under the experimental conditions tested. **Figure 2** depicts the percentage of each catechin that could be extracted from raw material. (–)-Epicatechin had the highest extractability with 23.5% realized at 60 °C.

The optimum temperature was elicited to be 60 °C, and subsequent SFE experiments were conducted at 100, 200, and 300 bar using a solvent/feed ratio of 30. Amounts of catechins were lowest at 100 bar (**Table 3**). Although the density and dielectric constant and, hence, the solvating power of supercritical carbon dioxide depend on its pressure and temperature and pressure increase enhances the solubility (24), the treatments with 200 and 300 bar did not have a significant effect on the extraction efficiency. About 0.3% of (–)-catechin was extractable at 200 bar and 0.4% at 300 bar. With regard to (–)-epicatechin, 23.5% was extracted at 200 bar and 22.5% at 300 bar and only 0.3% of taxifolin at 200 and 300 bar, respectively (**Figure 3**). This might be due to the fair solubility of catechins in carbon dioxide, which has also been reported in the literature. For instance, Chang and co-workers (18) tried to separate catechins from green tea by SFE under the conditions of 310 bar and 60 °C. The results showed that (–)-epicatechin and epicatechin gallate were not detectable, but epigallocatechin and epigallocatechin gallate were obtained in trace amounts (0.093 and 0.018 mg/g, respectively). Therefore, a solvent/feed ratio of 50 (2 kg of CO₂) was tested in our study to investigate if the solvent/feed ratio parameter would have an effect on the outcome. The results showed that only a slight increase in (–)-

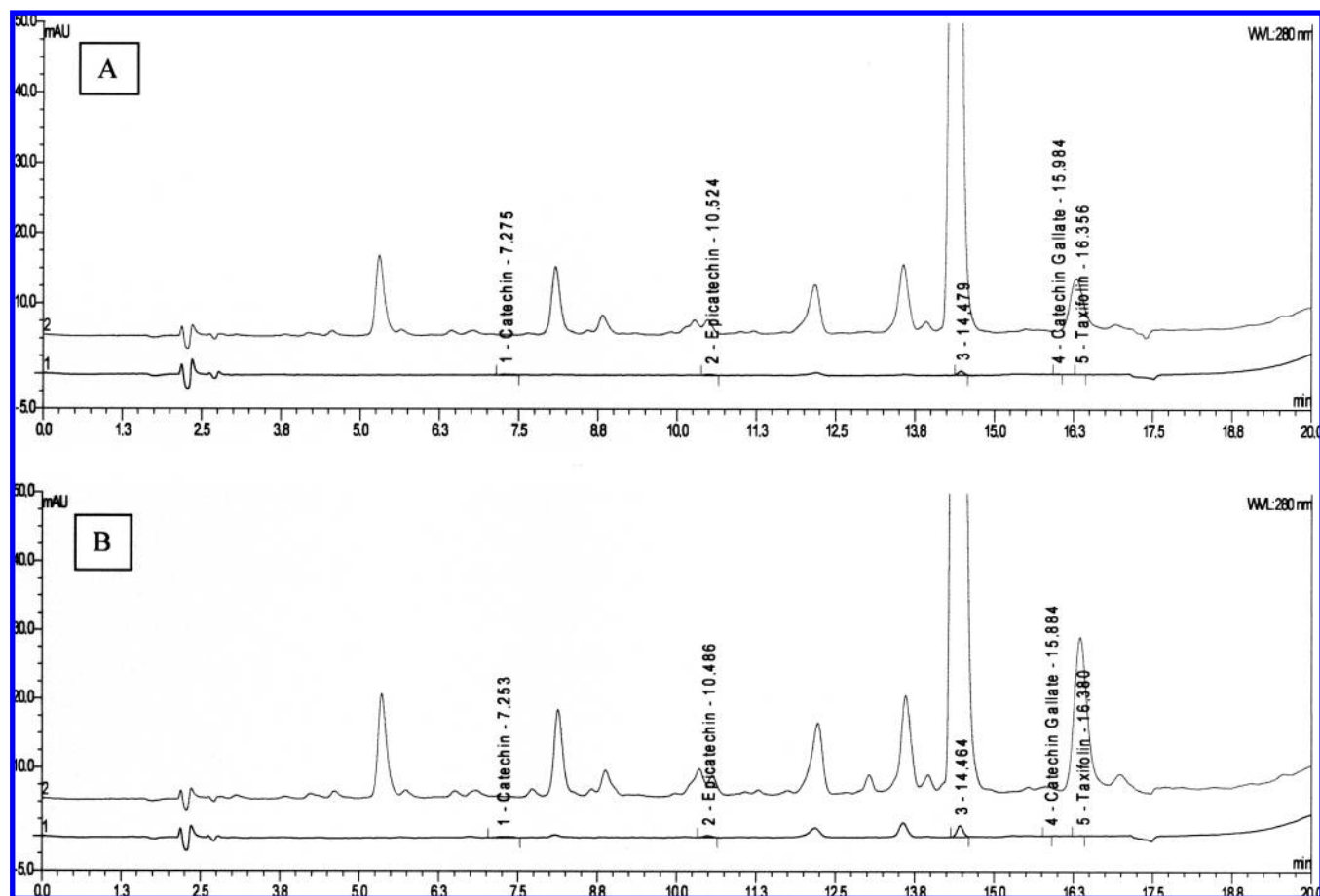


Figure 6. HPLC chromatograms of SFE samples with and without modifier under the conditions of 60 °C and a solvent/feed ratio of 30 at both 200 (A) and 800 bar (B) at pilot-scale SFE equipment.

Table 5. Effect of Pressure and Modifier on the Contents of Catechins and Taxifolin (Milligrams per Gram of Extract) Extracted with 6.5 L Pilot-Scale SFE under the Conditions of 60 °C and a Solvent/Feed Ratio (S/F) of 30^a

SFE parameters at 6.5 L pilot plant	catechin				total catechins
	catechin	epicatechin	gallate	taxifolin	
60 °C, 200 bar, S/F = 30	0.11 ± 0.02	0.09 ± 0.01	0.006 ± 0.00	0.004 ± 0.00	0.21
60 °C, 800 bar, S/F = 30	0.20 ± 0.05	0.25 ± 0.02	0.007 ± 0.00	0.03 ± 0.00	0.46
60 °C, 200 bar, S/F = 30, 3%	13.77 ± 0.03	1.16 ± 0.04	0.400 ± 0.00	4.46 ± 0.05	15.33
60 °C, 800 bar, S/F = 30, 3%	14.47 ± 0.85	1.01 ± 0.02	0.560 ± 0.00	9.87 ± 0.62	16.04

^a Data are presented as means ± SEM of duplicate measurements.

epicatechin yield was achieved but that there were no differences in the amounts of (–)-catechin and (–)-catechin gallate, which indicated that an increased solvent/feed ratio would not enhance the extractability of catechins.

Therefore, for the following experiments, the optimum pressure and solvent/feed ratio were determined to be 200 bar and 30, respectively. The last step at the optimization was to use a polar modifier that is acceptable in food and pharmaceutical applications. The effects of mixtures of carbon dioxide and ethanol at proportions of 1 and 3% were investigated. HPLC chromatograms of SFE samples with and without modifier are shown in **Figure 4**. An overall increase in the amounts of catechins was noted along with increased proportions of the modifier (**Table 4**). An 11.2-fold increase was observed with 3% ethanol and 2.4-fold with 1% as compared to the extraction

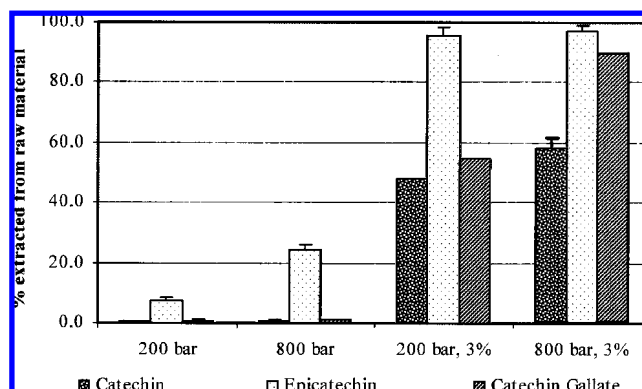


Figure 7. Effects of pressure and modifier on the extraction efficiency (percent) of pilot-scale SFE under the conditions of 60 °C and a solvent/feed ratio of 30. Data are presented as means ± SEM of duplicate measurements.

without modifier. Total catechins in the SFE extract with 3% ethanol were even 2.4-fold higher than in the sonicated extract with ethanol at a ratio of 1:20 (w/v). Ethanol has a critical temperature of 243.4 °C, which is greater than that of carbon dioxide and a critical pressure of 64 bar, slightly lower than that of pure carbon dioxide. The mixture might have been a solvent system with increased solubility due to enhanced hydrogen bonding with polar groups (14). The extraction efficiency increased with increasing proportions of the modifier, and almost 100% of both (–)-epicatechin and (–)-catechin gallate were extracted from the raw material, whereas only 7.2% of the (–)-catechin was extractable when 3% ethanol was used

(Figure 5). Although higher efficiencies could not be achieved for (–)-catechin, 3% of ethanol addition created a 23.5-fold increase in concentration of this compound compared to the application without a modifier.

Supercritical CO₂ Extraction at Pilot Scale. One of the main stimuli of the runs conducted at the 6.5 L SFE pilot plant was to observe the effect of scaling up on the extraction efficiencies, and the other one was the lack of high-pressure SFE application on extractability of catechins. HPLC chromatograms of SFE samples at 200 and 800 bar with and without modifier are shown in Figure 6. Overall increases in the amounts of total catechins were noted with the addition of modifier. The concentration of taxifolin was 4.46 mg/g at 200 bar and 9.87 mg/g at 800 bar, which represents the most significant difference in terms of higher extractability of taxifolin compared to the results obtained from the runs at laboratory-scale SFE (Table 5). The extraction of catechins was tested at 400 bar and 100 °C (20) and at 350 bar and 50 °C (25). Considering that most SFE applications have been carried out in the pressure range between critical pressure of carbon dioxide and 300 bar (26), slightly higher pressures were used than in common applications, and trace amounts of catechins were obtained without a modifier. In our study, although the increase of pressure from 200 to 300 bar did not create any differences in terms of the extractability of catechins at laboratory scale, the increase from 200 to 800 bar doubled the amounts of (–)-catechin and (–)-epicatechin (0.11 and 0.09 mg/g; 0.20 and 0.25 mg/g, respectively), whereas the amounts of (–)-catechin gallate and taxifolin (0.006 and 0.004 mg/g, respectively) remained the same. Nevertheless, an increase in extractability was much notable with the addition of modifier, in particular for taxifolin, reaching values of 4.46 and 9.87 mg at 200 and 800 bar, respectively. In terms of extraction efficiency, 47.8 and 58.4% of (–)-catechin were extracted from the raw material at 200 and 800 bar, whereas 46.8% was extracted by sonication with ethanol. (–)-Epicatechin and (–)-catechin gallate were also totally recovered from the raw material (Figure 7).

Correlation between Solvent and Supercritical Fluid Extraction: Prediction. Extraction efficiencies were considered to investigate if a correlation exists between solvent and supercritical fluid extraction. Given that the time required to set up the optimum experimental conditions, as well as resources and equipment, may often be limited, it is possible to predict the composition of an SFE extract regarding the substances of interest by finding a solvent that in polarity and density resembles supercritical carbon dioxide under optimized extraction conditions. HPLC results of the dichloromethane fraction and laboratory-scale SFE at 200 bar without modifier did not have a correlation ($r = 0.01$). However, the ethyl acetate fraction revealed very good correlations with the same SFE run ($r = 0.98$) and also with pilot-scale SFE runs at both 200 bar ($r = 0.99$) and 800 bar ($r = 0.98$) without modifier. As expected, the ethanol fraction revealed good correlations with SFE runs conducted at both laboratory and pilot scales at 200 bar with modifier ($r = 0.93$ and 0.88 , respectively). It is also worth mentioning that good correlations were noted between SFE runs at laboratory and pilot scale at 200 bar without ($r = 0.99$) and with modifier ($r = 0.78$), which indicates a good reproducibility of results in scaling up.

In conclusion, it has been demonstrated that SFE corresponded to extraction with ethyl acetate, whereas SFE with 3% ethanol corresponded to ethanol with regard to extractability of four monomers from *P. brutia*. Therefore, it was possible to predict the result of a far more elaborate laboratory SFE

extraction simply through extraction with solvent sonication. Furthermore, a scaling-up of SFE to industrial scale can be performed with the obtained data.

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