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Extraction and identification of proanthocyanidins from grape seed (*Vitis Vinifera*) using supercritical carbon dioxide

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ABSTRACT

In this study, supercritical carbon dioxide extraction of proantocyanidins (PRCs) was performed and the effect of different pressure, temperature and ethanol percentage was investigated. High performance liquid chromatography was used for the analysis of the compounds and it was found that the most effective parameter on the extraction was the amount of the ethanol percentage. Each compound was extracted from grape seeds at their maximum level when different parameters were used which was probably because of their different polarities. Gallic acid (GA), epigallocatechin (EGC) and epigallocatechingallate (EGCG) were extracted at their maximum level when the 300 bar 50 °C and 20% of ethanol was used. The maximum amount of catechin (CT) and epicatechin (ECT) were obtained when 300 bar 30 °C and 20% of ethanol was used for extraction, and 250 bar, 30 °C and 15% of ethanol was needed to extract highest amount of epicatechingallate (ECG).

1. Introduction

Grape seed is a residue of wine and grape juice industry and is generally used as an animal feed. The investigation on grape seed has been increasing since its positive effects was shown on human health. Grape seed contains 8–15% oil and this oil contains high amounts of unsaturated fatty acids and antioxidant-rich compounds [1]. These antioxidants are mostly phenolic compounds like cathecins and procyanidins [2–4]. These phenols may act against the in vitro oxidation of low density lipoproteins [5], and also have anticarcinogenic, antiviral and antimutagenic [6] activities, besides these they prevent the thrombosis, reduces cholesterol and regulates the autonomic nerves [7]. The applications of the grape seed oil have been increasing in pharmaceuticals, medical, cosmetic and food industry due its properties.

The extraction of seed oil includes many different stages like cleanup of the biomass, drying, crushing and pressing. By the application of pressing most of the oil can be extracted but nevertheless a considerable amount of oil remains in the cake, and this remaining oil can be extracted by hexane; which should be evaporated after the process [8]. The main drawback of this process is the usage of n-hexane at the last stages [1].

The methods for the extraction of phenolic compounds from grape seed include usage of methanol [3,4,9], ethanol [6,10,11] and acetone [12]. Different extraction temperatures are being used with different times that range from minutes to several hours.

Due to the complexity of the procedure to extract oil and phenolic compounds, newer methods are in consideration. Supercritical fluid extraction (SFE) is one of these methods, extensively studied nowadays because of its unique properties. SFE has many advantages, like absence of light and air, over the conventional extraction procedures. Air and light can cause degradation of both the phenolic compounds and unsaturated fatty acids (UFAs). Among many supercritical fluids carbon dioxide is the one that is mostly used owing to its non-toxicity, cheap and non-flammable properties [1]. Besides these properties CO_2 has moderate critical pressure (7.28 MPa) and temperature (304.1 K) which in turn prevents the thermal degradation of phenolic compounds. Supercritical carbon dioxide extraction of grape seed oil has been extensively applied [1,7,8,13], however; for the extraction of phenolic compounds a co-solvent like methanol or ethanol should be used, because carbon dioxide is a non-polar substance and polar substances like phenolic compounds cannot be extracted from grape seeds.

In this study, the extraction of phenolic compounds from grape seed using supercritical carbon dioxide with ethanol as a co-solvent was performed. The effect of different temperature, 30 and 50 °C, pressure, 250, 275 and 300 bar, and co-solvent percentages, 5, 10, 15 and 20 wt%, to the extraction amount has been investigated. The amounts of the phenolic compounds extracted were analyzed using RP-HPLC with a gradient program.

2. Material and methods

2.1. Material

The carignan type of grape seeds used in the study was kindly given by Kavaklidere Winery. The marc given was dried

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Table 1
The amount of the PRCs (ppm) extracted from the grape seeds at 30 °C, 250, 275 and 300 bar.

mg of component extracted/kg of grape seed													
		P = 250 bar				P = 275 bar				P = 300 bar			
Rt	Compound	5%	10%	15%	20%	5%	10%	15%	20%	5%	10%	15%	20%
8.05	GA	2.6 ± 0.0^{a}	5.9 ± 0.0^{a}	19.6 ± 7.1^{b}	13.6 ± 5.0^{ab}	4.5 ± 0.3^{a}	10.4 ± 1.6^{ab}	13.6 ± 2.4^{b}	16.7 ± 4.2^{b}	4.9 ± 0.3^{a}	9.5 ± 0.3^{ab}	15.1 ± 1.1^{b}	29.3 ± 7.0^{c}
19.03	EGC	9.3 ± 0.3^{a}	19.0 ± 0.1^{ab}	83.9 ± 32.6^{b}	51.9 ± 32.6^{ab}	9.6 ± 0.1^{a}	33.2 ± 4.5^{ab}	45.0 ± 12.5^{bc}	66.6 ± 19.4^{c}	12.7 ± 0.6^{a}	32.8 ± 2.6^{a}	59.6 ± 17.7^{a}	173.1 ± 55.8^{b}
20.57	CT	ND	5.8 ± 0.1^{a}	44.1 ± 18.8^{b}	25.1 ± 16.5^{ab}	ND	12.6 ± 2.8^{a}	21.5 ± 7.2^{a}	48.6 ± 17.0^{b}	1.3 ± 0.2^{a}	12.0 ± 0.7^{a}	22.8 ± 7.3^{a}	90.3 ± 28.5^{b}
27.76	ECT	ND	4.8 ± 0.0^{a}	20.9 ± 7.1^{b}	13.3 ± 6.0^{ab}	ND	9.0 ± 1.0^{a}	13.1 ± 3.6^{ab}	23.5 ± 6.9^{b}	5.2 ± 0.2^{a}	9.9 ± 0.8^{a}	14.6 ± 2.0^{a}	43.1 ± 11.8^{b}
28.44	EGCG	11.3 ± 0.0^{a}	13.5 ± 0.6^{a}	24.0 ± 6.2^{b}	15.3 ± 2.6^{ab}	12.2 ± 0.1^{a}	26.3 ± 4.2^{b}	23.3 ± 3.3^{b}	10.3 ± 0.6^{a}	13.0 ± 0.3^{a}	25.2 ± 3.4^{b}	25.9 ± 0.2^{b}	27.3 ± 3.1^{b}
33.08	ECG	1.1 ± 0.0^{a}	7.2 ± 0.1^{b}	8.5 ± 0.3^{c}	6.8 ± 0.0^{b}	ND	ND	ND	2.5 ± 0.0^{a}	0.9 ± 0.2^{a}	1.1 ± 0.2^{a}	1.5 ± 0.3^{ab}	1.9 ± 0.4^{b}

$SC\text{-}CO_2$ was modified with ethanol as a co-solvent and the percentages were 5, 10, 15 and 20% and the temperature was 30 °C. The extraction with SCF was performed twice. Extraction of PRCs from the oil extracted with SFE was performed in 2 parallels. Rt = retention time of the components in HPLC (min), ND = not detected. a–c: Means within the same pressure value and same row with different superscript letters are different ($p < 0.05$).

at room temperature and the grape seeds were separated from their peels, after removing the peels the seeds were kept at −18 °C until they were used. The standards used for HPLC analysis were (+)-catechin hydrate (96%), (−)-epicatechin (>97%), (−)-epigallocatechin (from green tea > 95%), (−)-epicatechin gallate (from green tea, 98%), (−)-epigallocatechin gallate (97%) and were obtained from Sigma–Aldrich (Steinheim, Germany). Ethanol which was used as a co-solvent and methanol, used for extraction of PRCs from the grape seed oil, were obtained from Riedel de-Haen (Germany). HPLC grade acetic acid (100%) was from Sigma–Aldrich (Germany) and acetonitrile was from Merck (Darmstadt, Germany).

2.2. Methods

2.2.1. Sample preparations

Seeds, which were kept at −18 °C were equilibrated to room temperature and then were crushed with a mixer. The seeds were crushed for 2 min, after every 30 s. The samples were waited for 30 s to prevent seeds from warming up. Crashed seeds were then sieved and the ones that were between 425 and 230 μm. 30 g of the sieved seeds were weighed and were put into the extraction vessel.

2.2.2. Supercritical fluid extraction of grape seeds

The extraction of antioxidant substances from the seed was performed using and analytical supercritical fluid extractor (SFE-100-2-FMC10, Thar Instruments, PA, USA). The instrument was equipped with automated back pressure regulator, 100 ml extraction vessel, 500 ml collection vessel, six zone temperature controller, high pressure P-50 series pump, cooling systems filled with glycol, and a series III pump for co-solvent (cannot operate over 400 bar). The co-solvent pump was purged before each extraction in order to ensure that co-solvent entered the system (Thar Instruments, Series III Pump, Manuel).

The carbon dioxide flow rate was fixed to 5 g/min and was same for all the extraction parameters. Low flow rate was selected in order to ensure that the residence time was longer in the supercritical fluid extraction vessel. Due to the fact that PRCs could not be extracted with CO_2, because of its non-polar nature, the CO_2 was modified with ethanol at the level of 5, 10, 15 and 20% (wt%). Ethanol was selected because it is a polar solvent is permitted in food industry. Other than the ethanol percentages 3 different pressures (250, 275 and 300 bar) and 2 different temperatures, 30 and 50 °C were used during the extractions. The length of the extraction was for 1 h. After the extraction the collection vessel was washed with 25 ml of ethanol to clean the surfaces of the vessel, and to minimize the losses that can occur. The grape seed oil, PRCs was collected in a 250 ml volumetric flask and the ethanol was removed with rotary evaporator (Büchi, B465, Switzerland) at 45 °C. After removal of the ethanol the oil containing the PRCs were analyzed with HPLC (Agilent 1100). The extractions with $SC\text{-}CO_2$ were performed in two replications.

2.2.3. HPLC analysis of the extracts

The samples obtained from the SFE process were analyzed according to the methods of [14,15]. According to those methods 1 ml of the oil in the volumetric flask was transferred to a centrifuge tube and 1 ml of methanol was added and was vortexed for 2 min. The vortexed samples were then centrifuged 10 min at 3000 rpm (Nüve, NF 1215, Istanbul, Turkey) and the supernatant was taken to a test tube. The extraction was repeated twice and the supernatants were mixed and were analyzed with Agilent 1100 RP-HPLC. The experiments were performed in 2 parallels. The analysis in HPLC were performed with a nucleosil C18 HPLC column (250*4.6 mm, Supelco Inc., Bellefonte, PA, USA), at 280 nm and 35 °C using a DAD detector. The flow rate was 1 ml/min and the injection volume was 10 μl. Gradient flow was used with 2 mobile phases which are 2%