Production of Proanthocyanidins From Grape Seeds

By

Evren ALTIOK

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We approve the thesis of **Evren ALTIOK**

Date of Signature

08.09.2003

Prof. Semra ÜLKÜ

Supervisor

Department of Chemical Engineering

08.09.2003

Prof. Şebnem HARSA

Co-Supervisor

Department of Biotechnology and Bioengineering

08.09.2003

Asst. Prof. Oğuz BAYRAKTAR

Co-Supervisor

Department of Chemical Engineering

08.09.2003

Prof. Devrim BALKÖSE

Department of Chemical Engineering

08.09.2003

Asst. Prof. Durmuş ÖZDEMİR

Department of Chemistry

08.09.2003

Prof. Şebnem HARSA

Head of Interdisciplinary

Biotechnology and Bioengineering Program

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ABSTRACT

Proanthocyanidin is a potent antioxidant that is why it directly affects many important diseases such as coronary heart disease, cholesterol etc. Thus, proanthocyanidin is one of the important dietary supplements, which is mainly found in grape seeds. In this study the extraction of this valuable antioxidant from grape seeds was studied. For the extraction of proanthocyanidins, different solvents such as ethanol, methanol, acetone and their aqueous forms were used. The maximum extraction yield was achieved after 24 h at room temperature. It was observed that 70% aqueous acetone was the best solvent with the highest yield of 17.5%. Yield of proanthocyanidin extracted with 70% aqueous acetone was 3 times greater than proanthocyanidins extracted with other solvents.

The extract was freeze dried to obtain the product as powdered form. To compare the product with commercial ones HPLC chromatograms and FTIR spectra were taken and similar spectra were obtained. With HPLC, monomers of proanthocyanidins; (+)-catechin and (-)- epicatechin were determined. (+)-catechin was observed at 17.2 min as 3.06%w/w. Also, (-)-epicatechin was observed at 42.8 min as 1.99%w/w.

Most of the drying methods are performed at high temperatures which may result negative effects on proanthocyanidin`s antioxidant capacity. Even the applications at low temperatures in case of freeze-drying have some disadvantages e.g. the process takes a long time, it is expensive and has some difficulties. Therefore as an alternative to other concentration and drying techniques, adsorption process was thought to be a promising technique. Although adsorption of proanthocyanidins on zeolites were not obtained, removal of excess water from the extracts can be achieved by using zeolites. This would possess as a promising technique for the concentration of the end product.

The antioxidant capacities of proanthocyanidins from the grape seed extracts were also determined. Proanthocyanidin has an antioxidant capacity with 57 TEAC value, which is more than synthetic antioxidants; BHT, BHA, TBHQ and PG, 6.66, 0.593, 1.168 and 12.62 TEAC respectively.

ÖZ

Potansiyel bir antioksidan olan proantosiyanidin, koroner kalp rahatsızlığı, kolesterol gibi birçok hastalığı önler. Bu sebeple, proantosiyanidin önemli diyet katkı maddelerinden olup başlıca üzüm çekirdeğinde bulunur. Bu çalışmada bu değerli antioksidanın üzüm çekirdeğinden ekstraksiyonu üzerine çalışılmıştır. Proantosiyanidin ekstraksiyonunda etanol, methanol, ve aseton gibi değişik çözgenler ve bunların sulu çözeltileri kullanıldı. Maksimum ekstraksiyon verimine oda sıcaklığında 24 saatte ulaşıldı. 70% sulu aseton sağladığı 17.5% verimle en iyi çözgen olduğu belirlendi. 70% sulu asetonla ekstrakt edilen proantosiyanidin verimi diğer çözgenlerle ekstrakt edilen proantosiyanidin veriminden 3 kat fazla olduğu görüldü.

Ürünü toz halde elde etmek için ekstrakt dondurularak kurutuldu. Ürünü diğer ticari ürünlerle karşılaştırmak için HPLC kromatogramları ve FTIR spektrumları alındı ve benzer spektrumlar elde edildi. HPLC ile proantosiyanidin monomerleri olan (+)-kateşin ve (-)-epikateşin belirlendi. (+)-kateşin 17.2. dakikada ve 3.06% ağırlık olarak, (-)-epikateşin ise 42.8. dakikada ve 1.99% ağırlık olarak gözlendi.

Kurutma metotlarının çoğu, yüksek sıcaklıklarda uygulanır. Yüksek sıcaklık proantosiyanidinlerin antioksidan kapasitelerini olumsuz etkiler. Düşük sıcaklıklarda uygulanmasına rağmen, dondurarak kurutma işleminin uzun zaman alması, pahalı olması ve uygulamasındaki bazı zorluklar bu işlemin bazı dezavantajlarıdır. Bu nedenle diğer konsantrasyon ve kurutma tekniklerine alternative olarak adsorpsiyon işlemi gerçekleştirildi. Proantosiyanidinlerin zeolite iyi adsorpsiyonunun sağlanamamış olmasına rağmen, ekstrakttaki fazla su zeolitle uzaklaştırılmıştır. Bu da son ürünün konsantrasyonunu sağlayan etkili bir işlem olduğu düşünülmektedir.

Üzüm çekirdeği ekstraktından elde edilen proantosiyanidinlerin antioksidan kapasiteleri belirlendi. Proantosiyanidinin antioksidan kapasitesi 57 TEAC değeri olup sentetik antioksidanlarınkinden daha yüksektir. Sentetik antioksidanlardan BHT, BHA, TBHQ ve PG'nin antioksidan kapasiteleri sırasıyla 6.66, 0.593, 1.168 ve 12.62 TEAC olarak belirlenmiştir.

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CHAPTER-1

INTRODUCTION

In last decades, dietary supplements have been become to find it's way in application to fight against various diseases such as coronary heart disease, stroke, Alzheimer and Parkinson's disease, high cholesterol level, inflammation, blood vessel plugging, cancer etc. which are the biggest problem of new century. For this purpose, dietary supplements have been developed; their effects on human body, limits and toxicity tests have been carried out. Within dietary supplements, natural resources have great importance because of their effectiveness and safety properties against to treatment of these diseases.

Grape seed extract is one of the important dietary supplements with its high antioxidant capacity, safety, and radical scavenging ability. Everyday, free radicals are metabolized in human body because of the daily exposures of stress, toxic substances-smokes, industrial and environmental wastes-, high cholesterol diet etc. These free radicals attack to human body cells and cause lipid peroxidation that result in the cell death. To scavenge these free radicals dietary supplement, especially grape seed extract is widely used in all over the world.

Grape seed extract has a complex structure, which has polymeric forms of flavonoids. Flavonoids are important plant origin substances that have high antioxidant activity capacity and radical scavenging ability. Grape seed extract is also known as proanthocyanidins, OPC`s, anthocyanidins. Proanthocyanidins are condensed tannins that are of the flavonoid class substances.

In the world, there are various trademarks of grape seed extract, which are produced with different techniques under different patents. Because of the land, weather, soil properties, and production techniques, grape seed extracts have different characteristics. For example, the kind of soil affects the antioxidant capacity of proanthocyanidin.

In Turkey, the production of grape is important as raw material in winery, raisin, and pekmez production. Within these industries as by-products, grape seeds, stems, skins are separated as waste. In Turkey, there is not any production of grape seed extract despite of these large resources. Moreover, it is imported from foreign countries with

very high prices. By economical meaning, it is very important to produce this high value added product in our country.

For this purpose, the extraction, characterization and determination of antioxidant activity of proanthocyanidins from grape seeds were investigated in this study.

Efforts have been done to substitute adsorption technique using zeolite against the solvent extraction methods. However, proanthocyanidin did not show any specific affinity towards zeolites. Therefore, natural zeolites were used to discard the excess water from the prepared extracts, and thought to be used as a concentration step.

The characterization of proanthocyanidin is important for the determination of grape seed extract's purity. High performance liquid chromatography (HPLC) is the best technique in that grape seed extract is both qualitatively and quantitatively determined. However, there are difficulties in the exact determination of all fractions of proanthocyanidins by HPLC. Within this study, the suitable method for HPLC has been tried to build up. Beside the HPLC, the other method FTIR spectra, which let us fingerprint comparison of lyophilized product with the other commercial products, are investigated.

The objectives of this study are extraction of the grape seed proanthocyanidins, the comparison of them with the commercial products, determination of their antioxidant activity capacity and investigation of their behaviors on the natural zeolite.

CHAPTER 2

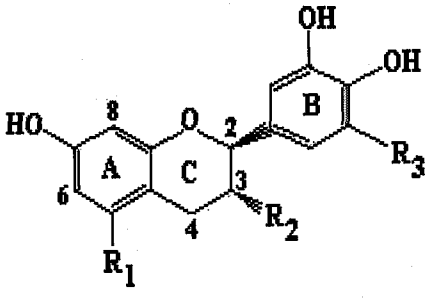
PRODUCTION AND PROPERTIES OF PROANTHOCYANIDINS

2.1. Flavonoids

Foods of plant origin are rich sources of polyphenolic compounds, many of which are potent antioxidants. Flavonoids are the most abundant subgroup and appear to be the most biologically active overall. They are also the most abundantly available antioxidant compounds in the human diet. Further, flavonoids possess many properties in addition to their antioxidant capacities, which have potentially beneficial effects on cardiovascular health. Flavonoids, which are biologically active in humans, are often referred to as bioflavonoids.

2.1.1. Flavonoids in the Plant Kingdom

Flavonoids are ubiquitous among vascular plants. More than 5000 have been identified so far within the parts of plants normally consumed by humans. It is also likely that there is many times more in nature that has yet to be discovered, since only a small percentage of plant species have been systematically examined for flavonoid constituents. Every flavonoid shares a basic polyphenolic C_6-C_3-C_6 3-ring structure. Figure 2.1. shows this structure and the standard numbering system. These compounds are formed from the aromatic amino acids, phenylalanine and tyrosine. The various flavonoids differ primarily by degree of saturation and number of hydroxyl groups. Many contain additional conjugates as well (Buelga, Scalbert, 2000).



R1	R3	Class
OH	H	Proanthocyanidin
OH	OH	Prodelphinidin
H	H	Profisetinidin
H	OH	Prorobinetinidin

Figure 2.1. The basic repeating unit in condensed tannin.

Flavonoid compounds are crucial to the survival of plants. Evolution within the plant kingdom corresponds with the creation of new chemical components that serve to react to and meet various environmental challenges. The flavonoids exhibit a wide variety of biochemical, physiological and ecological activities. They provide plants with means of protection from a wide range of threats such as facilitating reproduction. Within plants, flavonoids act as potent antioxidants and as metal chelators, sequestering dangerous metal ions such as iron and copper (Buelga, Scalbert, 2000; Bruyne et al., 1999). They interfere in various ways with viral, bacterial and fungal reproduction, growth and development (Bruyne et al., 1999). They also play a role in inhibiting animal and insect feeding through the production of toxic substances. The destructive behavior of ultraviolet light is another universal threat to plants. The conjugated aromatic structure of flavonoids enables them to act as potent screens against UV light (Buelga, Scalbert, 2000).

The flavonoids tend to be highly concentrated in the most colorful parts of plants, the flowers, fruit and leaves. They are responsible for many of the colorful pigments, which attract creatures and promote the reproductive process (Bruyne et al., 1999). It has also been found that flavonoids, along with other phenolic acids, contribute to the structural stability of plants. It is interesting to note that, although flavonoids in general are ubiquitous in the plant kingdom, their distribution varies by plant type and species, which is evidence for the great variation in their activities. The varieties of flavonoids, which evolved within a particular plant, would likely depend on the kinds of environmental challenges faced.

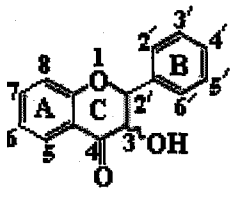
Beside the importance of flavonoid in plant, they have important health effects. Although it was back in 1936 when Szent-Gyorgyi first isolated two flavonoids from a food source, hesperidins and eriocitrin in lemon, and found they could improve capillary resistance, it has only been in the past two decades that flavonoids have been investigated seriously for their ability to help prevent and slow the progress of cardiovascular disease. It is obvious that, the possibility that just as plants have evolved to create numerous types of flavonoids as protection, our physiologies perhaps have also evolved to make use of these compounds.

2.1.2. Flavonoids in Food

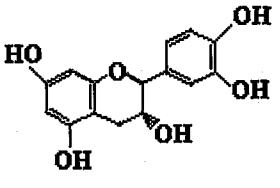
Since flavonoids are omnipresent in plant matter, they are obviously also so in foods of plant origin. Fruits, vegetables and herbs are generally high in flavonoid content. Beverages such as teas and red wine, made from plant foods, have particularly high concentrations of certain flavonoids. Flavonoids also occur in nuts, seeds and legumes. Cocoa has also been found to contain substantial quantities of flavonoids.

The bioflavonoids are classified into four main subgroups based on structure: (1) flavonols, (2) catechins or flavan-3-ols, (3) flavanones, (4) flavones (Buelga, Scalbert, 2000). All flavonoids have hydroxyl groups at the 4, 5 and 7 positions and an oxygen-containing pyran ring at the center of the skeleton. Chalcones, the metabolic precursors to flavonols, which do not contain a complete pyran ring, sometimes are considered to be a sixth subgroup.

Figure 2.2. illustrates the structures of the four subgroups. Flavonols are the most unsaturated with a 2-3 double bond and keto group at carbon 4 as well as an additional hydroxyl group at carbon 3. Catechins are at the lowest oxidation state due to their saturated C ring. The basic structure also has a hydroxyl group at carbon 3, which is often esterified by gallic acid. Flavanones have a saturated pyran ring with a keto group at carbon 4. Flavones also have a keto group at carbon four but an unsaturated pyran ring devoid of conjugates. The type and color of foods are usually indicative of the variety of flavonoids that are present in highest concentration.



(A)



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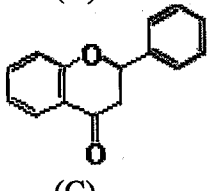

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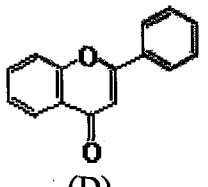


(D)

Figure 2.2. Structures of flavonoid subgroups. (A) Flavonols (B) Catechins (C) Flavanones (D) Flavones.

The most common flavonoids and common foods that are known to contain them in high concentration are shown in Table 2.1. Dimers, trimers and polymers of these compounds are also found in these foods.

Table 2.1. Common flavonoids and common foods that contain them.

Flavonoid		Food
Flavonoids	Quercetin	onion, red apple, lettuce, broccoli, cranberry, berries, olive oil, tea, red wine
	Myricetin	cranberry, grapes, red wine
	Kaempferol	endive, leek, broccoli, radish, grapefruit, tea
Catechins	Catechin	green and black tea, red wine
	Epicatechin	green and black tea, red wine
	Epigallocatechin	green and black tea, red wine
	Epicatechin-gallate	green and black tea, red wine
Anthocyanidins	Cyanidin	cherry, raspberry, strawberry, grapes
	Proanthocyanidin	red wine, red grapes
Flavanones	Hesperidin	citrus fruits
	Naringin	citrus fruits
Flavones	Apigenin	apple skins, celery, parsley
	Chrysin	fruit skins, berries

Estimates of the average amount of flavonoids consumed by an adult per day range from 23 mg to 1 g. There is a lack of comprehensive data on the flavonoid content of foods. There does not exist a single, robust analytical system that can measure all of the most prominent flavonoids in foods at once. The structural diversity of flavonoids makes creating such a system very difficult. It must also be kept in mind that there are

still new flavonoids continuously being discovered and that the study of flavonoids in the context of the human diet is very new compared to vitamins and minerals.

The flavonol, quercetin, is the most common and best-described flavonoid, often reported as responsible for a high percentage of flavonoid intake. It is found in many fruits and vegetables, including broccoli and leafy greens but is particularly abundant in red apples and onions. Onions have been reported to contain about 300 mg quercetin /kg black tea and red wine appear to contribute a large percentage of flavonoid consumption in most of the populations studied so far. The various catechins are the primary flavonoids in tea and the anthocyanins are most abundant in red wine. Interestingly, apples, grapes and onions, which have all been found to be excellent sources of flavonoids with high quality antioxidant capacities, are unusually low in the antioxidant vitamins.

Flavonoids are often found in high concentrations in the skins and peels of fruits and vegetables. Climate, soil conditions and genetic variability likely affect the flavonoid content of individual plants. A study that evaluated the flavonoid content of several different wines, for instance, found considerable variability, which appeared to depend on the variety of grape, processing techniques, climate, location and other environmental factors. In general, however, red wines have a very high concentration of flavonoids as well as other phenolic acids when compared to other foods.

2.2. Tannins

Tannins are flavonoids, which are found in vascular plant tissues such as leaves, needles, bark and seeds. They are found in many plants in particular, in fruits, legumes, different beverages (wine, green tea etc.), and in cacao. They are water-soluble and having a molecular weight between 500-3000 units. Tannins have ability to interact with alkaloids, gelatin and other proteins, permitting their use in the production of leather from hide. It is precisely this capacity to precipitate proteins (Naczk et al., 2001), in particular the salivary proteins (Manchado et al., 1999) in the oral cavity, which is believed to give them an astringent character easily recognized in tannin-rich food (Cheynier, 2000; Ricardo-Da-Silva et al., 1990; Buelga, Scalbert, 2000; Peleg et al., 1999).

Tannins can be divided into two groups;

- hydrolysable tannins and
- condensed tannins.

2.2.1. Hydrolysable Tannins

The hydrolysable group is the esters of phenolic acids and a polyol, usually glucose. Gallotannins, that are the polyesters of gallic acid, and ellogitannins, derived from the hexahydroxydiphenic acid, can be given as examples to this group.

2.2.2. Condensed Tannins (Proanthocyanidins)

The condensed tannins, which are commonly referred to as proanthocyanidins or more broadly as polyflavanoids, are widely distributed in the plant kingdom and represent a ubiquitous group of plant phenolics. They are most common in our diet, in that edible fruits are one of the richest sources of proanthocyanidins, and we consume these compounds every day from fresh fruits, wines and the processed foods made from fruits. They consist of oligomers and polymers of flavonoid compounds.

Structurally, tannins possess 12-16 phenolic groups and 5-7 aromatic rings per 1000 units of relative molecular mass. This feature, together with their high molecular weight, clearly makes the tannins and similar phenolic polymers found in processed products such as red wine or black tea different both in structure and properties from the low-molecular-weight phenolic acids and monomeric flavonoids. The phenolic polymers are formed by enzymatic and/or chemical transformation of simple flavonols (Buelga, Scalbert, 2000).

2.2.2.1. History of Proanthocyanidins

Oligomeric proanthocyanidins (OPCs) gained prominence in the 1990's. They were first extracted from pine bark in 1951 by a French investigator who found that they shared many biochemical and physiologic effects with Vitamin C (Kemper, 1999).

Scientists first became interested in grape seed extract in the latter 20 th century due to an outgrow of research about the "French Paradox". Atherosclerosis and coronary heart diseases (CHD) have been linked to excessive consumption of dietary

saturated fat and cholesterol. However, examination of World Health Organization epidemiological data showed marked differences in CHD mortality among various countries and regions. An intriguing anomaly existed in a certain French region, in spite of high saturated fat consumption, comparable plasma cholesterol and similar risk factors showed considerably lower incidence of death from CHD than Americans did. This apparent discrepancy popularly known as the French Paradox triggered a scientific scrutiny for this advantageous anomaly. Table 2.2. shows CHD mortality against plasma cholesterol levels for major world countries.

Table 2.2. Coronary heart diseases mortality against plasma cholesterol level (MegaNatural Gold).

MORTALITY FROM CORONARY HEART I
(AGE STANDARDIZED MALE, 35-64 YEARS)

	Mortality per 10^4	**Plasma Cholesterol (mg/dl)**
Japan	33	-
Toulouse (Fr.)	78	224
France (Gen)	102	216
Stanford (USA)	182	209
United Kingdom	380	240

Multivariate analysis showed that consumption of wine was the only dietary factor that showed a negative correlation with CHD, suggesting that intake of wine counteracts the effects of saturated fat and reduces the incidence of CHD mortality.

The epidemiological data also suggested that alcohol consumption, especially red wine, showed superior protection compared to other beverages such as beer, thus, providing evidence that the beneficial effects of red wine (Carando et al., 1999) are, at least in part, contributed by components other than alcohol. There was another study, which showed that CHD mortality was significantly reduced in populations that consume wine.

Most of the research on OPC's has been done in Europe, primarily in France, where grape seeds are abundant. The French use OPC's to improve capillary stability, decrease venous stasis and bruising, enhance lymphatic drainage and reduce

lymphedema. Based on their ability to improve night vision and recovery from glare, OPC`s are also used to treat macular degeneration and diabetic retinopathy.

2.2.2.2. The Chemical and Physiological Properties of Proanthocyanidins

Proanthocyanidins are polymeric flavan-3-ols whose elementary units are linked by C-C and occasionally C-O-C bonds. The flavan-3-ol units have the typical C_6-C_3-C_6 flavonoid skeleton. The three rings are distinguished by the letters A, B and C (Fig 1.1.). They differ structurally according to the number of hydroxyl groups on both aromatic rings and the stereochemistry of the asymmetric carbons of the heterocycle. The most common proanthocyanidins in food are procyanidins with a 3',4'-dihydroxy substitution on the B-ring and prodelphinidins with a 3',4',5'-trihydroxy substitution. Procyanidins or mixed procyanidins / prodelphinidins are most common in food. Propelargonidins with 4'-hydroxy B-rings are relatively rare in food sources. So far, a unique propelargonidin dimer has been isolated as a minor compound in oolong tea and propelargonidins were found by acid depolymerisation to account for 3% of the total proanthocyanidins in tea. The three carbons C2, C3 and C4 of the flavanol heterocycle are asymmetric and may occur in different configurations. With some very rare exceptions, the configuration of C2 is R. Flavan-3-ol units with the 2S configuration are distinguished by the prefix enantio (ent-). The stereochemistry of the C2-C3 linkage may be either trans (2R, 3S) or cis (2R, 3R) as in (+)-(gallo)catechin and (-)-epi(gallo)catechin polymers respectively. The interflavan bond at C4 is always trans with respect to the hydroxy group at C3.

Proanthocyanidins are oligomeric and polymeric flavan-3-ol units, which are (+)-catechin and (-)-epicatechin and their gallate forms (Figure-2.3.).

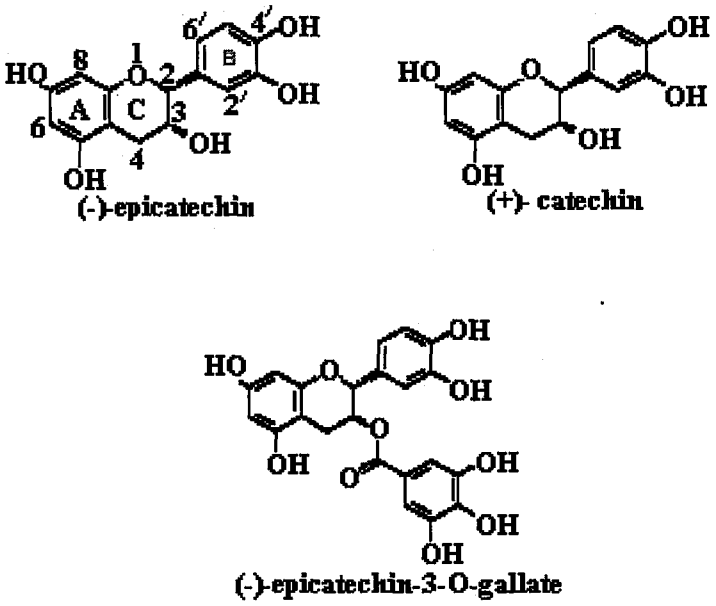


Figure 2.3. Flavan-3-ol units; (+)- catechin, (-)-epicatechin and its gallate ester.

They easily react with the atmospheric oxygen and are condensed to oligomers and polymers. Their interflavanol linkages are carbon- carbon bonds between C4- C6 and C4-C8 (Gabetta et al., 2000) as can be seen in Figure 2.4.

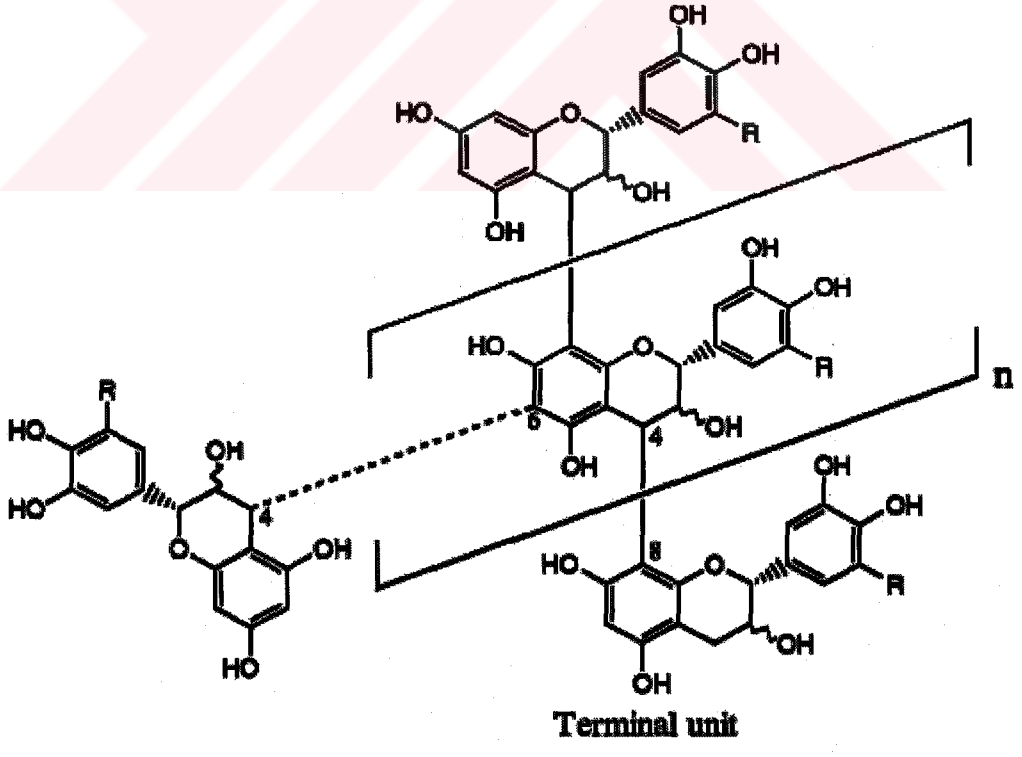


Figure-2.4. Flavon-3-ol units are linked each other via generally a C4-C6 and sometimes C6-C8 bonds in that these result in different structure and properties.

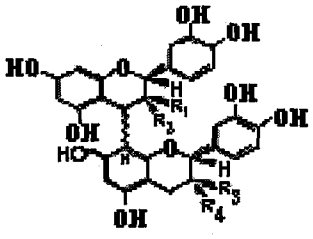
Proanthocyanidins comprise a great diversity of structures, which may show different properties and reactivities, due to the occurrence of:

• numerous constitutive units, which differ by the hydroxylation pattern on the general flavanol skeleton (e.g. catechin, gallocatechin), 2,3 stereochemistry (e.g. catechin, epicatechin) and the presence of substituents (e.g. epicatechin 3-gallate);

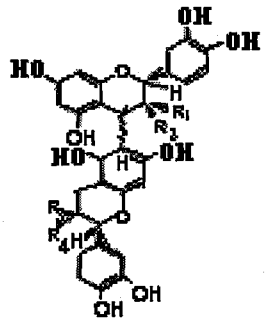
• different linkage positions (C4-C6 or C4-C8 bonds in the case of B-type proanthocyanidins, additional ether linkages for A-type ones);

• variable number of units in the molecule.

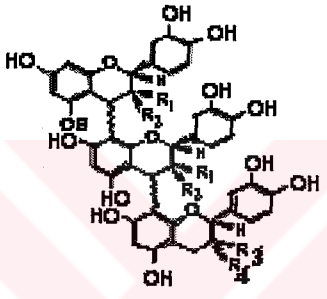
Initially, oligomeric proanthocyanidins were named by an alphanumeric system, with a letter A, B or C to describe the type of interflavanol linkage; a number was added to the letter as they were detected. A new nomenclature was later introduced to name an increasing number of new structures. It is based on that utilized for the polysaccharides. In this nomenclature, the elementary units of the oligomers are designated with the name of the corresponding flavan-3-ol monomers. The interflavanol linkage and its direction are indicated in parentheses with an arrow (4→) and its configuration at C4 is described as α or β. In type-A doubly linked proanthocyanidins, both linkages are indicated within the parentheses. It is unnecessary to indicate the oxygen in the additional ether bond since it is obvious from the substitution pattern of catechin lower units. For instance, according to this nomenclature, procyanidin dimer B1 becomes epicatechin-(4β→8)-catechin and dimer A2 becomes epicatechin-(2β→7, 4β→8)-epicatechin (Fig 2.5.).



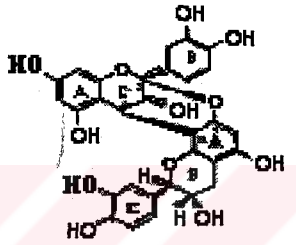
B_1: R_1= OH; R_2= H; R_3= H;R_4= OH
B_2: R_1= OH; R_2= H; R_3=OH;R_4=H
B_3: R_1= H ; R_2= OH; R_3= H;R_4= OH
B_4: R_1= H ; R_2= OH; R_3=OH;R_4=H



B_5: R_1= OH; R_2= H; R_3= H;R_4= OH
B_6: R_1= H ; R_2= OH; R_3=OH;R_4=H
B_7: R_1= OH; R_2= H; R_3=OH;R_4=H
B_8: R_1= H ; R_2= OH; R_3= H;R_4= OH



C1: R_1= OH; R_2=H
C2: R_1=H; R_2= OH



A2:R_1= OH; R_2= H; R_3=OH;R_4=H

Figure 2.5. Structures of some proanthocyanidin dimers and trimmers of the A, B and C types.

The distribution of these B types (B1 to B7) and C types in grape seeds, wines and apple are illustrated in table 2.3.

Table 2.3. Contents of proanthocyanidin oligomers in some foodstuffs (mg per 100g) and beverages (mgL^{-1}) (Buelga, Scalbert, 2000)

	Proanthocyanidins							
	B1	B2	B3	B4	B7	C1	EEC	Others
Grape	9-26	4-19	3-18	3-15	tr	3-15	5-18	<7 (galloyled dimers)
Red Wine	1-218	tr-73	tr-66	tr-36	nd-18	tr-24	tr-49	<10(galloyle d dimers)
White Wine	nd-2	nd-2	nd-2					
Sherry Wine	12-21	7-13	10-15	3-9				
Apple	tr-1	Tr-5				Tr-4		<1(B5)
Apple Juice	nd-28	nd-139	nd-3	nd-2		nd-120		14-36 (unknown)

C: (+)-catechin E: (-)-epicatechin
B1, E-(4,8)-C; B2, E-(4,8)-E; B3, C-(4,8)-C; B4, C-(4,8)-E; B5, E-(4,6)-E; B7, E-(4,6)-C; C1, E-(4,8)-E-(4,8)-E; C2, C-(4,8)-C-(4,8)-C; EEC, E-(4,8)-E-(4,8)-C;

Since the elucidation in the 1960s of the PA basic structure, more than 200 PA oligomers with polymerization degree as high as 5 have been identified and fully characterized. However, the bulk of PA polymers in plants usually have a higher degree of polymerization (DP). Such polymers are then characterized by chemical degradation in the presence of a nucleophile, usually phloroglucinol or benzylmercaptan and NMR spectroscopy. Such methodologies allow characterization of the nature of terminal and inner flavanol units and the average length of the chain. More recently, the introduction of electrospray mass spectrometry techniques coupled to liquid chromatography led to a more detailed characterization of PA polymers and to the non-ambiguous identification of a PA molecule with a DP of 17 in an extract of cider apple.

The polymerization degree was found to vary widely with the species, tissues or methods of extraction. For example, the average DP of extracts prepared from barks originating from different tree species varied between 3 and 8. In cider apple extracts it varied between 4 and 11 according to the tissue zones in the fruit. In these same apple samples, PAs with the highest DPs were better extracted by aqueous acetone than aqueous methanol. Some PAs resist extraction, either because of a poor solubility or because of secondary chemical reactions with the insoluble matrix. High molecular weight PAs are usually more strongly absorbed on a polar matrix such as silica than PAs of lower molecular weight.

2.2.2.3. Health Effects of Proanthocyanidins

Plant proanthocyanidins are known as the functional food factors that possess a variety of physiological activities such as antioxidant, antimicrobial, anti-allergy, hair-growth promotion, anti-caries, chemopreventive effects (Joshi et al., 2000), anti-hypertensive and inhibition against the activities of some physiological enzymes (Bruyne et al., 1999) and receptors. The greater part of these activities of proanthocyanidins largely depends on their structures and particularly their degree of polymerization. To elucidate the physiological mechanisms of proanthocyanidins and to provide these oligomers for a subsequent in vivo study, it is required to establish an effective separation and fractionation method of proanthocyanidin oligomers according to their degree of polymerization. This is consistent with that monomeric and oligomeric fractions are absorbed by the body and polymers are unabsorbed.

There are prospective epidemiological studies suggesting that high intakes of vitamin E, vitamin C and flavonoids are associated with reduced coronary heart disease (CHD) risk (Kumpulainen, Salonen, 1996, Yamakoshi et al., 1999). Several flavonoids including proanthocyanidins have been reported to inhibit either enzymatic or non-enzymatic lipid peroxidation (Kumpulainen, Salonen, 1996), which is an oxidative process implicated in several pathologic conditions, including atherosclerosis, hepatoxicity and inflammation. Phenolic compounds extracted from red wine are inhibitors of LDL oxidation which is a process involved CHD. Furthermore, they might trap reactive oxygen species in aqueous series such as plasma and interstitial fluid of the arterial wall, thereby inhibiting oxidation of LDL and showing an antiatherosclerotic activity (Yamakoshi et al., 1999).

2.2.2.4. Antioxidant Properties of Proanthocyanidins

Food products undergo a chain of change in the natural matrix due to ripening, harvesting, primary processing, and storage. These changes are caused by several factors including browning reactions, microbial spoilage, and autoxidation of lipids. Of the various factors, autoxidation of lipids contributes significantly to the deterioration and reduction in the self-life of many products. Lipid oxidation is a free- radical chain reaction that causes a total change in the sensory properties and nutritive value of food products. Changes in color, texture, odor and flavor; loss of vitamins; and damage to

proteins are some of the effects of lipid oxidation. The onset of lipid oxidation can be delayed by the addition of antioxidants. The use of antioxidants are extended to a wide variety of products including high-fat foods, cereals and even products containing very low levels of lipids. In recent years, naturally occurring antioxidants have come to be preferred by both consumers and food manufacturers mainly because of concern raised over the safety of synthetic antioxidants (Madhavi et al., 1996). Based on their function, food antioxidants are classified as primary or chain breaking antioxidants, synergistic and secondary antioxidants.

Primary antioxidants terminate the free- radical chain reaction by donating hydrogen or electrons to free radicals and converting them to more stable products. They may also function by addition in reactions with the lipid radicals, forming lipid-antioxidant complexes. Both hindered phenolics (e.g. synthetic antioxidants including, BHA- Butylated hydroxyanisole, BHT- Butylated hydroxytoluene, TBHQ- tert-butyl hydroquinone and tocopherols) and polyhydroxyphenolic (e.g., gallates) antioxidants belong to this group. Many of the naturally occurring phenolic compounds like flavonoids, eugenol, and vanillin and rosemary antioxidant also have chain- breaking properties. Other group, synergistic antioxidants can be broadly classified as oxygen scavengers and chelators. They may act as hydrogen donors to the phenoxy radical, thereby regenerating the primary antioxidant. Hence, phenolic antioxidants can be used at lower levels if a synergist is added simultaneously to the food products. Secondary antioxidants such as thiodipropionic acids function by decomposing the lipid peroxides into stable end products. Compounds listed under miscellaneous antioxidants, such as flavonoids and related compounds and amino acids function as both primary antioxidants and synergists (Madhavi et al., 1996).

In recent years, consumers and food manufacturers have been opting for products with "all natural" labels. The volume of such products increase day by day. Consequently, a lot of emphasis is given to the identification and incorporation of novel, natural antioxidants in food products. The area of natural antioxidants developed enormously in the past decade mainly because of the increasing limitations on the use of synthetic antioxidants and enhanced public awareness of health issues. In general, natural antioxidants are preferred by consumers because they are considered safe. Some of the natural antioxidants listed in Table 2.4. The tocopherols and vitamin C are used widely in food products. They have also been synthesized on a commercial scale.

Table 2.4. Some naturally occurring antioxidants

Vitamin E, Vitamin C	Uric Acids
Amino acids	Sterols
Lecithin	Protein hydrolysates
Proteins	Saponins
Flavonoids	Tartaric Acid
Lignans	Vanillin
Phenolic acids	Carotenoids

Numerous reports have been published on the identification of novel, naturally occurring antioxidants from plants, animals, microbial sources, and processed food products. Recent reports in this area discuss young green barley leaves, a medicinal herb, pea bean, wild rice and grape seeds (Madhavi et al., 1996).

Antioxidants are also required for the purpose of some health evidence. Because of its radical scavenging activity the dose of antioxidants recommended daily taken as dietary supplements. Occupational exposure to chemically and structurally diverse environmental pollutants including pesticides, toxic chemical wastes, direct and second hand cigarette smoke, gasoline exhaust, urban air pollutants ozone and radiation, and physical stress, produce similar toxic effects on human health (Bagchi, 1999). These environmental pollutants have been demonstrated to produce enormous amounts of free radicals, resulting in oxidative deterioration of lipids, proteins and DNA, activation of procarcinogens, inhibition of cellular and antioxidant defense systems, depletion of sulfhydryls, altered calcium homeostasis, changes in gene expression and induction of abnormal proteins, and contribute significantly to human disease pathophysiology (Bagchi, 1999; Bouhamidi et al., 1998). Antioxidants/free radical scavengers function as inhibitors at both initiation and promotion/ propagation/ transformation stages of tumor promotion/ carcinogenesis and protect cells against oxidative damage. The potential role of the antioxidant vitamins such as vitamin C and E, β-carotene and proanthocyanidins, antioxidant minerals such as zinc and selenium, and antioxidant enzymes such as glutathione, superoxide dismutase and catalase, has been extensively studied in the prevention of numerous degenerative diseases including tumor growth and carcinogenesis (Shrikhande, 2000). Proanthocyanidins are antioxidants because of their chemical nature: hydroxyl groups attached to the phenyl ring (Hollman, 2001).

These free radicals are; HO_2 –hydroperoxy radicals, $O_2^{\bullet-}$ -superoxide anion radical, $^1O_2$ - singlet oxygen, $HO^\bullet$ - hydroxyl radical, H_2O_2 – hydrogen peroxide. They threat human body and cause some important diseases such as coronary heart disease, low-density lipoprotein (LDL) oxidation and cholesterol, vision problem, carcinogenesis and tumor promotion, Alzheimer and Parkinson disease (Bagchi, 1999) and lots of the others. Humans possess a wide array of antioxidant physiological defenses, which scavenge radicals, chelate metals involved in their formation and repair damage. The consumption with the diet of polyphenols together with other natural antioxidants such as vitamins C and E and carotenoids also contributes to these defenses.

Eventhough plant phenols are not always treated as real antioxidants in the literature, many in vitro studies have demonstrated the antioxidant potential of phenols as direct aqueous phase radical scavengers.

Catechin metabolites were significantly more effective than intact catechin in inhibiting monocyte adhesion and infiltration in blood vessels. A study investigating the antioxidant activity of epicatechin metabolites in rat plasma found that most epicatechin had been metabolized to a conjugated form, epicatechin-glucuronide being the most common (Da Silva, 1991).

Proanthocyanidins, owing to the lack of easily available substrates, have received much less attention than their monomeric counterparts. They were shown to scavenge $O_2^{\bullet-}$, $HO^\bullet$ and the synthetic radical cation $ABTS^+$ in aqueous solutions, often as efficiently as quercetin or butylated hydroxytoluene. Galloylation increased the scavenging capacity of both proanthocyanidins (Bors et al., 2001; Hagerman et al., 1998) and theaflavins (Buelga, Scalbert, 2000). The doubly linked A-type dimers were less effective than their B-type counterparts. The influence of the polymerization degree was not as clear. In some experiments, no difference was observed between monomers, dimers and trimers. In other experiments, for a given per weight concentration, the scavenging capacity increased up to the trimers then decreased for higher molecular weights, or was the same for catechin monomers and dimers and then decreased for larger polymerization degree (Buelga, Scalbert, 2000).

Procyanidins B1 and B3 were evaluated as antioxidants for linoleic acid in aqueous systems. They showed stronger anti-oxidant activity than ascorbic acid and α-tocopherol (Bagchi, 1999). From a study of their radical scavenging properties

towards radicals induced from an aqueous dispersion of methyl linoleate, it was seen that the dimeric procyanidins could trap eight peroxyl radicals (cf. ascorbic acid traps one radical; α-tocopherol traps two radicals). The higher the degrees of polymerization, the more radicals are scavenged per molecule (Bruyne et al., 1999).

Radical scavenging action of galloylated condensed tannins for 1,1-diphenyl-2-picrylhydrazyl (DPPH)-radical, as well as for superoxyde anions and for $^{\bullet}OH$ and $^{\bullet}OOH$. All mentioned radicals were scavenged dose-dependently. The authors also decided that the ability for radical scavenging was proportional to the degree of polymerization, which was contradicted by Ricardo Da Silva et al. (1991), since with higher oligomerisation, also the number of galloyl groups increased in the investigated compounds (Bruyne et al., 1999).

Ricardo Da Silva et al. (1991) tested various procyanidins for their scavenging activities for superoxyde radical $O2^{-\cdot}$ and hydroxyl radical •OH in aqueous models. Galloylation, increases the scavenging ability for both O_2^- and OH O_2-scavenging was more prominent for (4 ⟶ 8)-coupled dimers than for (4⟶ 6)-linked procyanidins; a difference not seen for $OH^{\bullet}$ scavenging activity.

All the major polyphenolic constituents of food show greater efficacy as antioxidants on a molar basis than the antioxidant nutrients vitamin C, vitamin E, and β-carotene (Buelga Scalbert, 2000). Grape seed proanthocyanidin is important plant phenolic having a high antioxidant capacity. It is nearly 20 times and 50 times more than vitamin C and vitamin E respectively. Proanthocyanidin contains all monomeric, oligomeric and polymeric forms and it has wide applications in pharmaceuticals.

2.2.2.5. Proanthocyanidins as Dietary Supplement

Researched extensively in Europe, proanthocyanidin has been used as a nutritional supplement throughout the world since 1969. Proanthocyanidins has been studied and its benefits documented in many nutritional and medical journals. Proanthocyanidins is safe and non-toxic and has been tested in mutagenic and carcinogenic studies around the world. Proanthocyanidins, as a dietary supplement, is patented in the US, and in many countries around the world. The patented proanthocyanidins varieties sold in one country under a given name may be sold in another country under a different name. These products are now being sold to the rapidly growing dietary supplement industry.

Table 2.5. shows the number of grape phenolic products that have proliferated in the market in US.

Table 2.5. Number of grape products of health relevance in the US (Shrikhande, 2000)

Grape Seeds	22
Grape Skins	5
Red wine powder	7
Anthocyanin extract	5
Other (grape extract)	4

The grape skin extracts are also being marketed due to their more complex structure consisting of anthocyanins from skins and procyanidins from seeds. Such products are normally prepared from aqueous steeping of grape pommace in the presence of sulphur dioxide. The aqueous phenol extracts are clarified and concentrated as thick liquids or spray dried to powders. These products are already in the trade as natural colors and approved by the FDA for coloring of beverages.

2.2.2.6. Major Source of Proanthocyanidins

Proanthocyanidins are widely distributed in plant kingdom, for example in cacao (Subagio et al., 2001), coffee (Colmenares et al., 1998) in apple (Yanagida et al., 2000), in unripe almond (Terasa et al., 1998), in various types of berry fruits, such as strawberry, raspberry, blueberry, bilberry etc. On the other hand, bark of the oak tree and the pine tree and grape seeds are the powerhouses of proanthocyanidins. OPC`s are also found in green tea (Camellia sinensis) and account for some of its antioxidant effects.

Table-2.6. Proanthocyanidin content in different foodstuffs and beverages. (Buelga, Scalbert, 2000)

Type of Foodstuff and beverages	Content[a]	Proanthocyanidin Type[b]
Cereals and pulses (mg. Per 100 g.)		
Lentil	316-1040(dry weight)	PC, PD
Faba Bean	nd-740	PC
Sorghum	nd-3900	PC
Barley	64-126	PC, PD
Fruits and berries (mg. Per 100 g.)		
Apple	17-50 (dry weight)	PC
Pear	07-12	PC
Grape	1-160	PC, PD, gall
Sweet cherry	10-23	PC, PD
Blueberry	1-7	PC, PD
Red raspberry	2-48	PC, PD
Strawberry	2-50	PC, PD, gall
Blackberry	9-11	PC
Juices and Drinks (mgl^{-1})		
Apple Juice	nd- 298	PC
Pear Juice	11-74	PC
Grape Juice	3.5-46	PC
Cider	2290-3710	PC
Red Wine	nd-500	PC, PD
White Wine	Tr-7	PC
Wine Vinegar	4-414	PC
Beer	3.5-19.5	PC, PD
Others		
Cacao Bean	260-1200	PC

a Expressed as catechin equivalent. HPLC values only include oligomers (DP=2±3).
b PC, procyanidins; PD, prodelphinidins; gall, galloylated derivatives.

Grape seed contains high amounts of these polyphenols (Kennedy et al., 2000; Bouhamidi et al., 1998). The major identified polyphenols include the C, EC and ECG flavan-3-ol monomers, as well as the oligomeric and the polymeric procyanidins (Bouhamidi et al., 1998). After the grinding of grapes in the red wine production, must are fermented with grape seed pommace, including stems, seeds, skins etc. Then, a significant proportion of these polyphenols is extracted from seeds during red wine production (Sun et al., 1999), and wine production practices such as maceration time, pressing, maturation and fining probably affect their eventual concentrations in red wine. In red wine, the polyphenolic flavan-3-ol monomers and proanthocyanidins are relatively important components, because these compounds provide red wine its bitter and astringent properties (Cheynier , 2000; Kennedy et al., 2000).

Grape seed is the major source of proanthocyanidin. The important information about the grape seed and its extract can be given as;

- Botany

Family: Vitis vinifera is the family of grape and its seeds are generally used for producing grape seed proanthocyanidins.

- Common names:

The naming of these compounds is complex and is made more confusing by the fact that one of the generic terms, pycnogenols, is also patented as a trade name for one particular product, Pycnogenol. Other names include condensed tannins, grape seed extract, leucoanthocyanidins, nonhydrolyzable tannins, oligomeric proanthocyanidins, polyphenolic oligomers, and pycnogenols. Pycnogenolis a brand name for a patented OPC made from maritime pine bark.

- Sources

Large amount of the grape seeds are obtained in winery industry, which are discarded from the wine processing.

Grape seeds containing pommace can be obtained from the production of pekmez.

- Quality of extract

There exists a great deal of diversity between commercial seed extracts varying in total phenol concentration, monomers, oligomers and polymers (Ricardo-Da-Silva et al., 1990). Such diversity is based upon seed selection, the processing method employed and perhaps application. Total phenol ranges from the mid 40s to mid 90s, which clearly indicates that higher numbers are the result of greater purification of phenols from other seed substances, possibly, requiring multiple solvent extractions and precipitations. Such processes are expensive, less flexible and provide lower yields. The quality of seed extracts, in general, is considered better if they contain higher than 90% total phenols, greater than 10% monomers, greater than 65% oligomers (2±7 units of catechins) and usually less than 15% polymers. To achieve these quality standards, varietals seed selection becomes very important with higher monomeric and oligomeric fractions (Shrikhande, 2000).

To determine the quality of extracts various methods have been developed. Most reliable methods are spectrophotometric methods such as Fourier Transfer Infrared Spectroscopy (FTIR), UV- Visible spectrophotometer, Nuclear Magnatic Resonance Spectroscopy (NMR), mass spectroscopy (Schofield et al., 2001); chromatographic

techniques such as High Performance Liquid Chromatography (HPLC) (Labarbe et al., 1999), Gel Permeation Chromatography, Thin Layer Chromatography (TLC) and some analytical methods such as Porter assay (Makkar et al., 1999), thiolysis etc. (Schofield et al., 2001). However, analyzing the condensed tannins is complicated by the diversity of structures (Schofield et al., 2001).

Condensed tannins can be quantified using HPLC. Both normal phase and reversed phase columns have been applied (Lazarus et al, 1999; Schofield et al., 2001; Peng et al., 2001). Reversed phase HPLC has been used for separation of lower molecular weight condensed tannins (Schofield et al., 2001).

2.2.2.7.Extraction and Production of Grape Seed Proanthocyanidin

There are various methods to extract the polyphenols from the grape seeds. Principally, there are three principle techniques that may be used: (1) solvent extraction, (2) solid-phase extraction, (3) supercritical extraction.

Supercritical extraction is reliable and safest method to extract desired material from the solid matrices. However, the cost of this system is so high that, in industry, it is not chosen preferably.

The basic system to extract the phenolic substances is the solvent extraction. This is a process designed to separate soluble phenolic compounds by diffusion from a solid matrix (plant tissue) using a liquid matrix (solvent). The process can be divided into two stages;

1. Initial Stage. Swelling of the particles is observed due to sorption of the solvent in the solid phase. This sorption is caused by osmotic forces, by capillarity and by solvation of the ions in the cell. In this stage, the soluble components are dissolved.

2. Diffusion Stage. Diffusion takes place in two steps; an internal step within the solid phase and another external step through the outer layers that surround the particles.

Factors that influence the efficiency of solvent extraction are type of solvent, temperature, number of steps and volume and composition of solvent and particle size in the sample. The most widely used solvent for extracting phenolic substances is acetone and acetone- water mixtures (Buelga, Scalbert, 2000). Other solvents such as methanol, ethyl acetate and solvent mixtures have also been utilized, but they usually provide lower yields. The efficiency of the extraction increases along with the number

of extraction steps. In this sense, it is more efficient to carry out extractions with 50 mL of solvent (Pekic et al., 1998). Particle size, grinding of solid and homogenization favor the extraction process and it provides to contact solvent with the solid matrix. However, grinding of grape seeds increase the contaminants and decrease the purity of extract (Pekic et al., 1998).

It is advisable to complete the extraction using dry, frozen and lyophilized samples since some phenolics are unstable or can be degraded by enzyme action in undried plant material (Palmer et al., 2000). Avoiding the thermal degradation, air-drying can be applicable steps to the grape seeds.

2.2.2.8. Use of Zeolite in Proanthocyanidin Extraction

Zeolites are commercially valuable because of their unique ion-exchange, molecular sieving, and catalytic properties. The potential uses of zeolites in industry are given in Table 2.7.

Table.2.7. The Present and Potential Uses of Natural Zeolites

Ion exchange
Adsorption and molecular sieving
Hydration and dehydration
Radioactive waste removal
Air separation
Animal nutrition
Natural gas purification
Phosphate substitutes in detergents
Hydrogen sulfide removal from hydrocarbon gas streams

Zeolites are hydrated aluminosilicates of the alkaline and alkaline earth metals. The primary building block of zeolite is a tetrahedron of four oxygen atoms surrounding a central silicon atom $(SiO_4)_4^-$.

When Al^{3+} is substituted for Si^{4+} in the zeolite framework, a net negative charge on the molecule results. This is compensated for by a 'nonframework' cation (e.g., Na^+), which is 'held' in the pores of the structure. Because this cation is not a part of the

crystalline lattice, it is relatively mobile and easily exchangeable for other cations (Whitelaw, 1994). Because of the exchangeable cations, zeolites are polar adsorbents. Molecules such as water or ammonia (high dipole), CO_2, N_2 (quadrupolar) or aromatic hydrocarbons (π layer interaction) therefore adsorb more strongly than nonpolar compounds of similar molecular weight. This affinity generally increases with increasing charge on the exchangeable cation and decreasing cation radius, but its effect may be masked by water, which, because it is strongly bound to a zeolite, will reduce the zeolite's affinity for other, less polar molecules.

Natural zeolite can be beneficially used to adsorb proanthocyanidins from the extraction solvent. In addition, the use of zeolite in dehydration step prior to freeze drying of proanthocyanidin will ease the production of grape seed proanthocyanidin

CHAPTER-3

EXPERIMENTAL

3.1. Materials

The experiments were carried out by using the seeds of grapes grown at Muğla harvested in 2001-2002. These seeds were the by-products of Pekmez production. They were separated from the pommace immediately after grape pressing, and air-dried in a thin- layer.

Chemicals, reagents and equipments, which were used in all experiments presented in this study, are given in Appendix A.

3.2. Methods

3.2.1. Pretreatment of Grape Seeds

Grape seeds were dried at 40 ^{0}C in Memmert-800 oven for 24 h. After that the moisture content of grape seeds were determined by heating grape seeds at 105 ^{0}C (Pekic et al., 1997) and weighing them using a moisture analyzer Sartorius- MA 100 until the constant value were obtained.

3.2.2. Extraction of Grape Seed Proanthocyanidins

Proanthocyanidins from grape seeds were extracted according to the method described by Pekic et al. (1997). Whole grape seeds without grinding were used for the extraction. Although, grinding of grape seeds could shorten the extraction time, it did not increase the yield of Proanthocyanidins. Moreover, the grinding causes a significant increase in the extraction of undesired contaminant components, which hindered obtaining of a preparation with an increased content of proanthocyanidins (Dumon, 1990, Pekic et al.. 1997). For these reasons, all experiments presented in this study were performed using whole grape seeds. Experimental layouts can be given as in Figure 3.1.

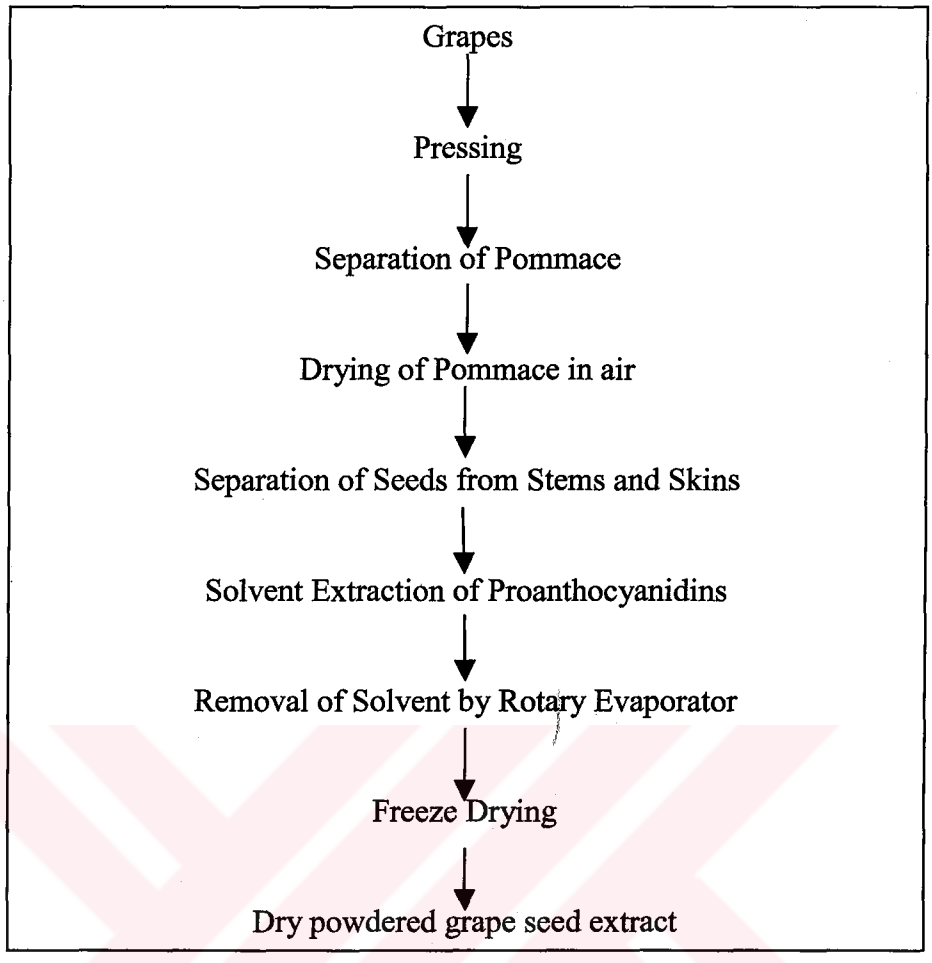


Figure 3.1. Flow diagram of experimental layout for production of grape seed proanthocyanidins

10g. of grape seeds were weighed and 50 ml. of solvent- water mixtures were added. Extraction was carried out one day in Gerhard Thermo Shaker at 30 ^{0}C and 180 rpm for 24 hours. After the extraction, the extract was centrifuged using Nüve centrifuge for 5 min. at 5000 rpm. Samples were removed in time intervals from the supernatant part and subjected to subsequent analysis.

Different solvents and their mixtures such as acetone, methanol, water, ethanol, and their aqueous forms were used to extract the grape seed proanthocyanidins. In order to investigate the most effective solvent on the extraction procedure; 20%, 40%, 50%, 60%, 70%, 80%, 90%, 100 % (v/v) water-solvent ratios were used.

3.2.3. Lyophylisation

Before lyophylisation, grape seed extract was distilled by rotary evaporator to remove organic solvent at 40 ^{0}C for 20 minutes with a 120 rpm rotation under vacuum. Then, solvent free grape seed extract was freeze dried to obtain a dried extract powder using a Labconco Freeze Drier System at –45 ^{0}C and 65 10^{-3} MPa.

3.2.4. Analyses

3.2.4.1. Porter Assay

The butanol-HCl-iron method is widely used for measurement of extractable condensed tannins (proanthocyanidins) in foods and feeds. This colorimetric reaction uses an acid-catalyzed oxidative depolymerization of condensed tannins to yield red anthocyanidins. It is diagnostic for polyflavan structure. In other words, this method is not applicable to determine flavan-3-ols, which are monomers of proanthocyanidins; (+)- catechin and (-)- epicatechin (Bagchi, 1999). Proanthocyanidin structure is depolymerized in acidic environment with butanol and ferric reagent at 100 ^{0}C. Under these conditions, fraction of depolymerized proanthocyanidins give pink-reddish coloured anthocyanidins (Figure 3.2.).

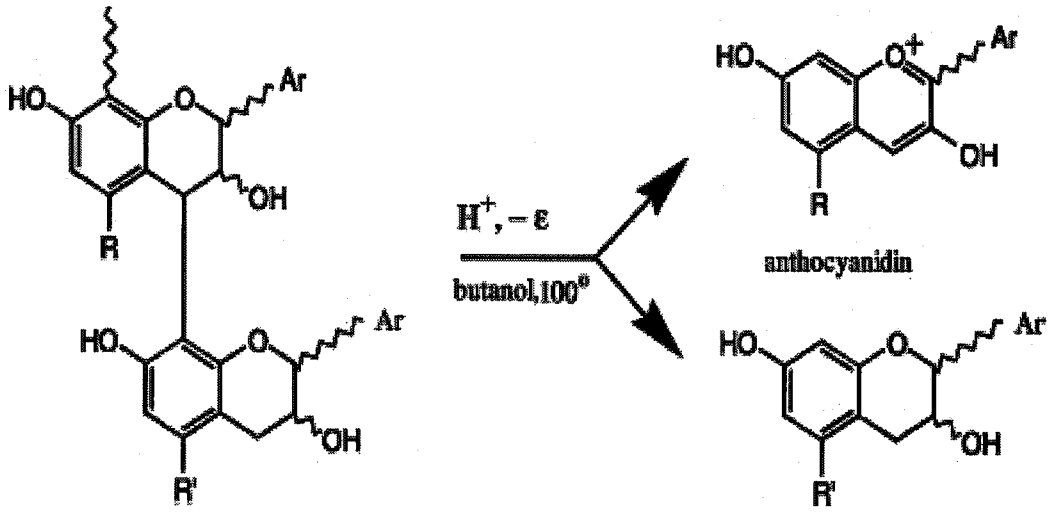


Figure 3.2. Chemistry of acid- butanol reaction. The reaction involves oxidation and that the terminal unit does not give a colored anthocyanidin product structure.

Shimadzu UV-visible spectrophotometer was used for the qualitative and quantitative determination of proanthocyanidins in Porter assay. Peaks were seen at around the 550 nm. wavelength as shown in Figure 3.3.

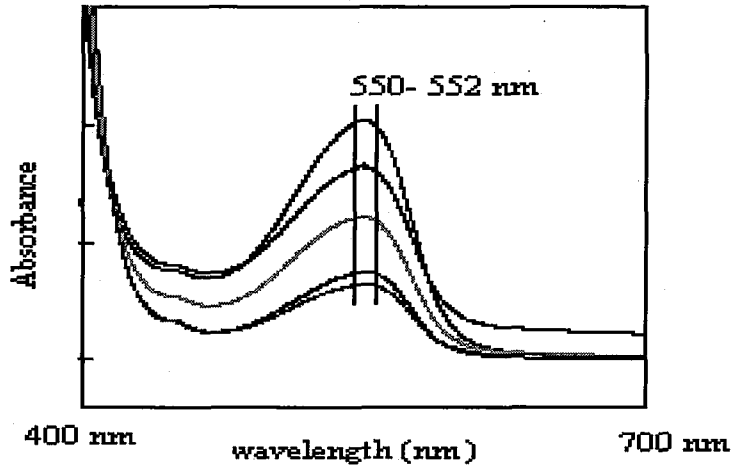


Figure 3.3. Visible spectrum screening within the range of 400nm- 700 nm for Porter assay.

By using the reference products of proanthocyanidins, standard curve of proanthocyanidin concentration vs. absorbance was obtained with R^2 =0.99 for Meganatural Gold ^{TM} (Appendix-B.2.) at 552 nm.

The Porter procedure was explained in detailed in Appendix B.1.

3.2.4.2. FTIR Analysis of Proanthocyanidins

Fourier Transform Spectroscopy of proanthocyanidins in different commercial preparates and sample extracts obtained in this study were carried out. FTIR spectra were obtained by using FTIR-1600, Shimadzu, Co. All samples were prepared by pressing the ground samples with dried crystalline KBr. The measurements were performed from 500 to 4500cm^{-1}.

3.2.4.3. HPLC Characterization of Monomers of Proanthocyanidins

Monomers of proanthocyanidin; (+)- catechins and (-)- epicatechin were determined by using HPLC. The HPLC system is composed of Perkin Elmer Series 200 pump, photo diode array detector, Series 900 interface and a computer. The system is controlled by a software, Turbochrom Navigator.

The HPLC analyses were done under gradient conditions using a Reversed Phase C-18 column (BTR separation and Metachem). The column temperature was maintained at 30 ^{0}C with a MetaTherm column oven. The column was eluted with Mobile phase A: %5 acetic acid (CH_3COOH) in deionized water; Mobile phase B: Methanol (MeOH) in A (20:80) at a flow rate of 0.8 ml min^{-1}. The linear gradient condition was applied in HPLC analysis at the wavelength of 280nm. The operating and analysis conditions of the HPLC system are given in Appendix C.1. In addition, the calibration curves for catechin and epicatechin can be seen in Appendix C.2.

3.2.4.4. Determination of Antioxidant Activity of Proanthocyanidin

Antioxidant activity determination is important for indicating and comparing the antioxidant capacity of proanthocyanidin with the other antioxidants. As indicated in Chapter 2, antioxidants are used as dietary supplement against some diseases. The use of natural antioxidants instead of synthetic ones is preferable for human health. The antioxidant capacity of proanthocyanidin differs according to the environment, land property, weather, extraction techniques, and process steps. For this reason, by determining the antioxidant activity, we can evaluate our product quality. In this study, the commercial products and our product were compared.

A number of assays have been introduced for the measurement of the total antioxidant activity of body fluids, food extracts, and pure compounds. Each method relates to the generation of a different radical, acting through a variety of mechanisms and the measurement of a range of end points at a fixed time point or over a range. Two types of approach have been taken, namely, the inhibition assays in that the extent of the scavenging by hydrogen- or electron-donation of a pre-formed free radical is the marker of antioxidant activity, as well as assays involving the presence of antioxidant system during the generation of the radical.

Generation of the ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation forms the basis of one of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activity of solutions of pure substances, aqueous mixtures and beverages. The improved technique for the generation of $ABTS^+$ involves the direct production of the blue/green $ABTS^+$ chromophore through the reaction between ABTS and potassium persulfate. This has absorption maxima at wavelengths 415 nm, 645 nm, 734 nm and 815 nm, as reported previously (Re et al.,

1999; Kumpulainen, Salonen, 1996). Addition of antioxidants to the pre-formed radical cation reduces it ABTS, to an extent and on a time-scale depending on the antioxidant activity, the concentration of the antioxidant and the duration of the reaction. Thus, the extent of decolorization as percentage inhibition of the $ABTS^{+}$ radical cation is determined as a function of concentration and time and calculated relative to the reactivity of Trolox, which is a water-soluble Vitamin E derivative, as a standard, under the same conditions. The procedure was described in Appendix D.1. Also, the calibration curve for the determination of Trolox Equivalent Antioxidant Capacity (TEAC) value by using Trolox is given in Appendix D.2. The method is applicable to the study of both water-soluble and lipid-soluble antioxidants, pure compounds, and food extracts such as proanthocyanidins (Buelga, Scalbert, 2000; Kumpulainen, Salonen, 1996).

The protocol described by Re R. (1999), was modified for this study. Methanol (MeOH) and ethanol (EtOH) were used as solvents for the extraction of proanthocyanidins compared to the protocol of Re R., in which ethanol was used as a solvent for food extracts.

3.2.5. Adsorption properties of proanthocyanidins on clinoptilolite

The adsorption behavior of grape seed proanthocyanidins on clinoptilolite was investigated.

Zeolite (particle diameter of 106-150μm) was weighed, washed to remove water-soluble impurities and dried at 300 ^{0}C for 24 hours in an oven. 2g. and 4g. of pretreated zeolites were contacted with crude grape seed extracts and standard proanthocyanidins dissolved in aqueous solvent mixtures. The solid/liquid ratio used in these experiments were 0.2 and 0.4 g zeolite/ mL extract. Adsorption of proanthocyanidins from the aqueous solution on zeolite was carried out using an orbital thermo shaker at 180 rpm at room temperature.

Samples were taken within two hours, centrifuged for 5 min at 8000 rpm in a microcentrifuge and proanthocyanidin contents of each samples were determined by Porter assay. Also, the amount of (+)- catechin and (-)- epicatechin were determined by HPLC to investigate the concentration increments in the extract.

CHAPTER-4

RESULTS AND DISCUSSION

4.1. Extraction of Proanthocyanidins from Grape Seeds

4.1.1. Extraction Kinetics

Extraction of proanthocyanidins from grape seeds were carried out in the aqueous solutions of acetone, methanol and ethanol with 70 %, 10 % and 70 % concentrations, respectively. The proanthocyanidin concentrations were determined according to Porter assay. Extraction kinetics of proanthocyanidins can be seen in Fig. 4.1.

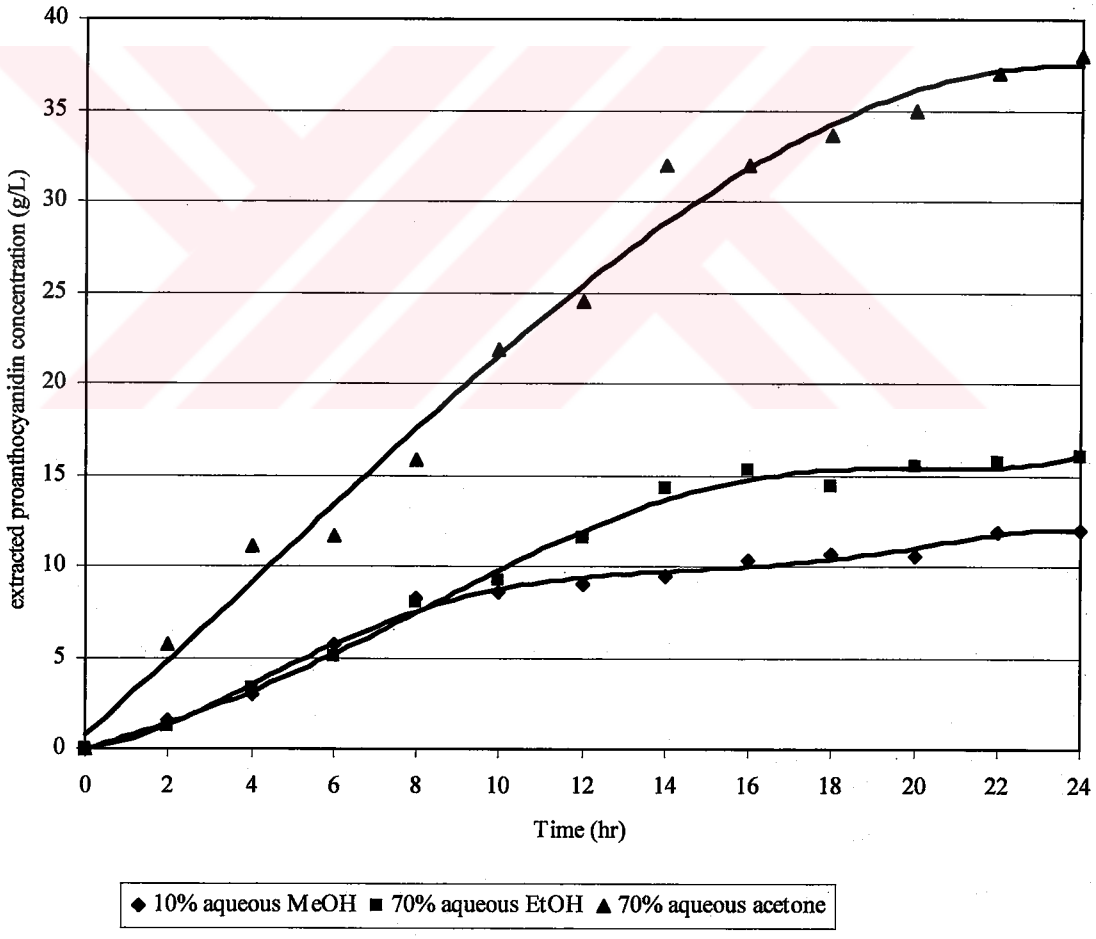


Figure 4.1. Extraction kinetics of proanthocyanidins

As seen in Figure 4.1., 10 g/L; 15 g/L and 32 g/L proanthocyanidins from grape seeds were extracted at 16 hours with 10% MeOH, 70% EtOH and 70% acetone respectively. The equilibrium was reached within 16 hours when MeOH and EtOH were used as extraction solvents. In case of acetone extraction of proanthocyanidins, the equilibrium has not been attained with this period. Longer extraction time was needed to extract 37 g/L proanthocyanidin with acetone at 24 hours, which is also the equilibrium time for proanthociyanidin/acetone extraction system.

Diffusion coefficients (D) were determined for the extractions of proanthocyanidin from grape seeds as in Appendix F.3. From the figure in Appendix F.3, diffusion coefficients were close to each other as found as 0.0160 mm^2/h, 0.0163 mm^2/h and 0.0146 mm^2/h for 70% aqueous ethanol, 10 % aqueous methanol and 70% aqueous acetone respectively.

4.1.2. Effect of Solvent Type on Extraction

Grape seeds were extracted with acetone, methanol, ethanol and their water mixtures. For this purpose, pure solvents, 20%, 40%, 50%, 60%, 70%, 80%, 90% aqueous solvents and 100% water; were used to investigate the effect of solvent type and water content.

Extraction yield can be defined as the percentage of g extracted proanthocyanidin per g of seeds. Figure 4.2 shows the effect of solvents type and solvent/water ratio on the extraction yield of proanthocyanidin for 24 hours.

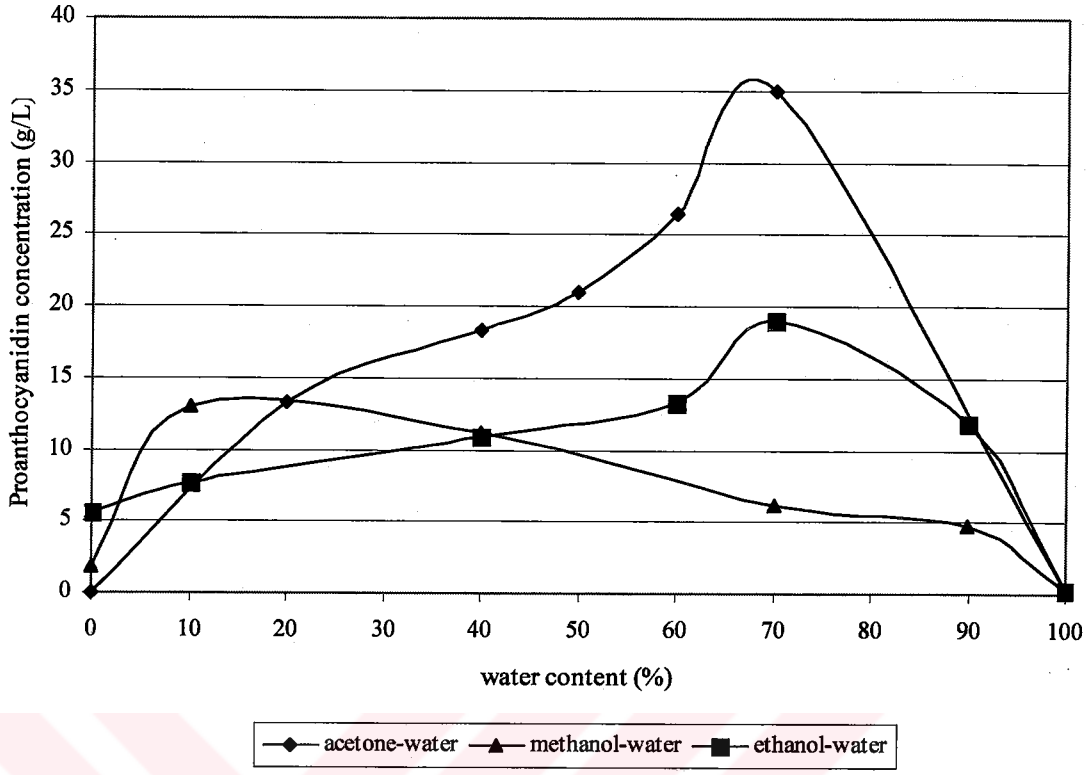


Figure 4.2. Effect of solvents and water contents of them on extraction for 24 h.

The comparison of the extraction yields for different solvents after 24 hrs. are shown in Figure 4.3.

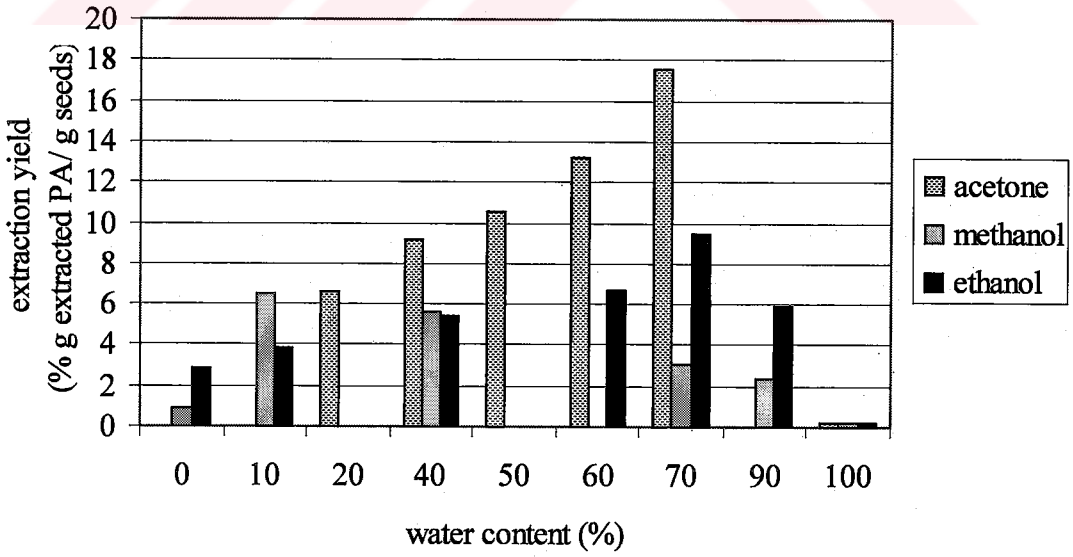


Figure 4.3. Comparison of extractable proanthociyanidins using different solvents

Extraction procedure was completed within 24 hours period and the values at this time are given in order to allow complete extraction. The highest yield of proanthocyanidin extraction was obtained as 17.5% with 70% aqueous acetone. On the other hand, 70% aqueous ethanol and 10% aqueous methanol gave the yield values of 9.5% and 6.5%, respectively. Since the extraction yields are very low in case of EtOH and MeOH when compared with acetone (70%); it can be said that acetone is the best solvent- water mixture to obtain highest yield of proanthocyanidin.

Water was important in the case that, yield of 70% aqueous acetone was around 700 fold increase than proanthocyanidins extracted without water. It was obvious that the presence of water increased permeability of grape seeds and thus enabled a better mass transport by molecular diffusion. However, pure water could not be able to extract proanthocyanidins with high yield. 0.33 g/L proanthocyanidins were extracted with water, which was 100 times less than extractable proanthocyanidins.

Since proanthocyanidins from grape seeds were extracted with low yields and also the limitation of using methanol in food industry; 70% aqueous acetone seems to have promising alternative for the proanthocyanidin extraction from grape seeds.

4.2. FTIR Spectra of Proanthocyanidins

FTIR spectra of (+)-catechin, commercial proanthocyanidin extracts and crude extract obtained in this study are shown in Figure 4.4.

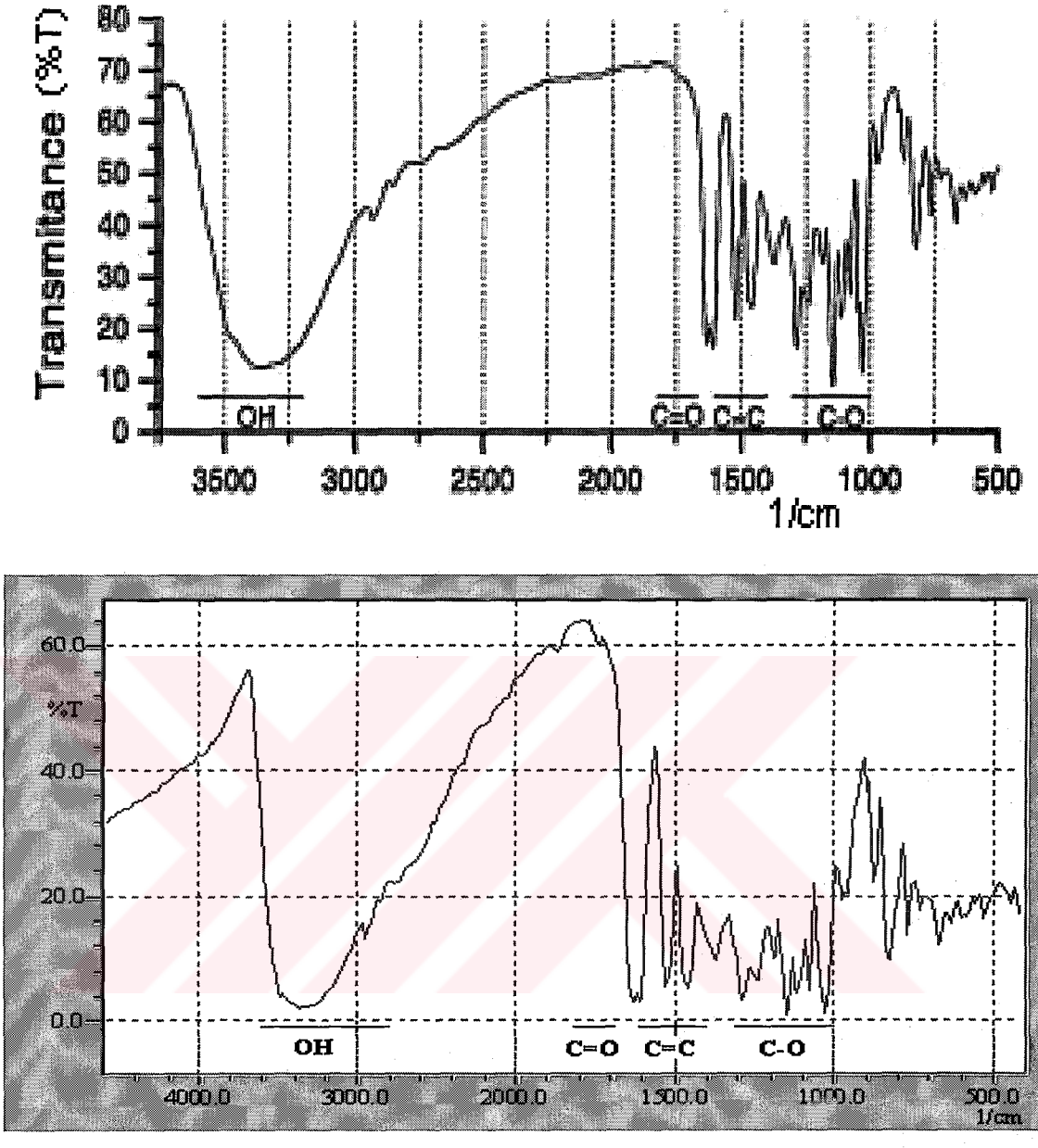


Figure 4.4. FTIR spectra (%Transmitance vs. 1/cm) of A: literature spectrum of (+)- catechin (Tejada et al., 2002); B: FTIR spectrum of (+)-catechin

Similar FTIR spectra were obtained for Meganatural Gold™, Leucoselect`s Leucocianidine™, and lyophilized product (Figure 4.5.).

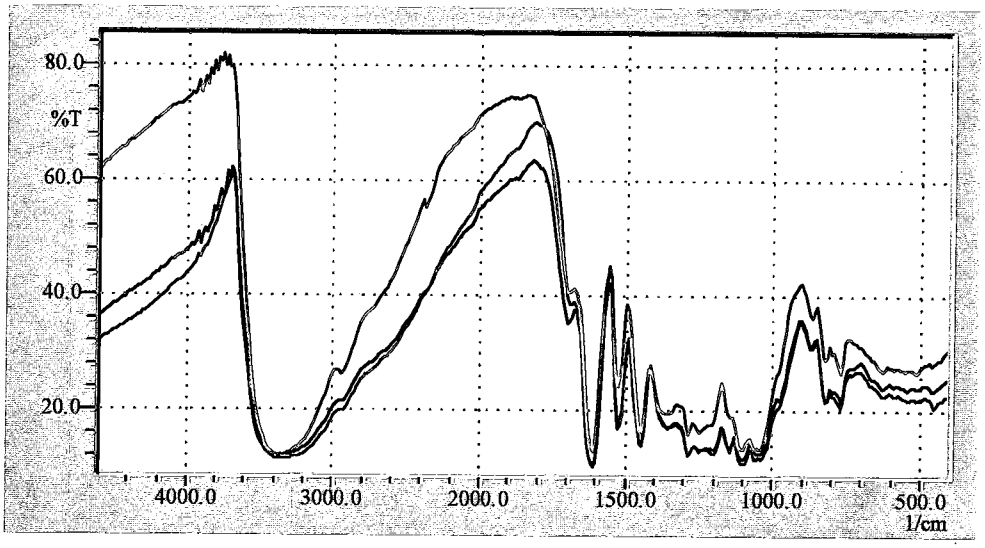


Figure 4.5. Spectrum of Meganatural Gold ™ (green), Leucoselect`s Leucocianidine ™ (blue), and lyophilized product (red).

There were 6 characteristic peaks of different intensity between 2931 and 455 wavenumber (cm^{-1}) and a broad OH group peak between 3400 and 3100 cm^{-1}. The exact values of the peak wavenumbers and their intensities for (+)- catechin, Meganatural Gold ™, Leucoselect`s Leucocianidine ™, and lyophilized product in KBr disc were collected in Table 4.1. while in Appendix E were shown characteristic vawenumber regions for different groups, which might be present in the tested sample.

Table 4.1. FTIR wavenumbers characteristic for the (+)- catechin, commercial samples and lyophilized product.

Wavenumber (cm^{-1})	Transmittance (%)	Wavenumber(cm^{-1})	Transmittance (%)
(+)- catechin peaks			
412.7	17.24	1458.1	5.24
821.6	9.71	1519.8	5.40
867.9	22.26	1604.7	2.95
975.9	19.24	2499.6	31.73
1029.9	1.32	3355.9	2.02
1076.2	4.97		
Leucoselect`s Leucocianidine ™ peaks			
455.2	23.53	1519.8	17.00
1037.6	12.71	1608.5	10.68
1446.5	14.15	2931.6	21.66
Meganatural Gold ™ peaks			
447.5	20.77	1519.8	17.96
1037.6	11.85	1608.5	10.47
1442.7	14.04	2931.6	20.01
Lyophilized product peaks			
455.2	27.97	1608.5	13.27
1041.5	13.53	2931.6	26.37
1446.5	16.27	3363.6	12.28
1519.8	24.58		

Analyzing the spectra at Figure 4.5. , only intensity differences were found between the commercial samples and our product. FTIR spectra indicated that O-H groups had a broad spectrum that could because of not only water but also O-H groups found in proanthocyanidins structure. The close peak intensities were obtained between the regions of 1400- 1700, which indicated that, the amount of C=C bonds in extracts were same with the commercial products. There were slight differences between the regions of 1400-800, especially around 1200 cm^{-1}, where instead of two peaks in the commercial samples, one sharp peak appeared. These peaks belong to C-O groups. In FTIR spectrum of (+)-catechin was different at that region, that was probably because of the conversion of catechin molecules to the other forms; dimers, oligomers or to the polymers. In general, FTIR spectra of commercial products and our lyophilized product show similar trend with a good comparison.

4.3. HPLC Results

High performance liquid chromatography lets the reliable qualitative and quantitative analyses. However, the complex structure of grape seed proanthocyanidin, which contains monomers, dimers, trimers, oligomers, polymers and their gallate forms, makes difficult to build a suitable method. By the reversed phase HPLC, it was possible to determine monomers of proanthocyanidin; (+)- catechin, (-)- epicatechin. The quantitation of (+)-catechin and (-)-epicatechin were achieved by an external standard procedure, using multiple-point calibration (Appendix-C.1. and C.2.). Results were expressed as (+)-catechin and (-)- epicatechin equivalents. Under these conditions retention times for (+)-catechin and (-)-epicatechin were 17.2 minute and 42.8 min. respectively.

The determination of monomers is important because, these monomers, which are not biologically active in human body, are considered as impurities of grape seed extract. It was possible to compare grape seed extract with the other trademark products. There are many other trademark extracts with different names and properties. To classify our extract, HPLC was used and comparison of it with GNC ^{TM}, Leucoselect`s Leucocianidine ^{TM}, and Meganatural Gold ^{TM} have been carried out. The chromatograms of these trademark products, grape seed extract after extraction and lyophilized product were investigated (Figure 4.6.). And also the amounts of (+)-catechin and (-)- epicatechin were detected in the extract and compared with different trademark products. Based on the values tabulated in Table 4.2; the purity of proanthocyanidin extract can be estimated. Meganatural Gold ^{TM} , GNC ^{TM}, Leucoselect`s Leucocianidine ^{TM}, extract and lyophilized product has 93.36 %, 94.1%, 95.84%, 93.98%, 94.95% pure proanthocyanidin respectively.

Table 4.2. The amount of (+)-catechin and (-)- epicatechin

Product	(+)-catechin (%)	(-)- epicatechin (%)
Commercial Products		
Meganatural Gold ™	4.22	2.42
GNC ™	3.18	2.61
Leucoselect`s Leucocianidine ™	2.68	1.48
Our product and extract		
Extract	3.26	2.76
Lyophilized product	3.06	1.99

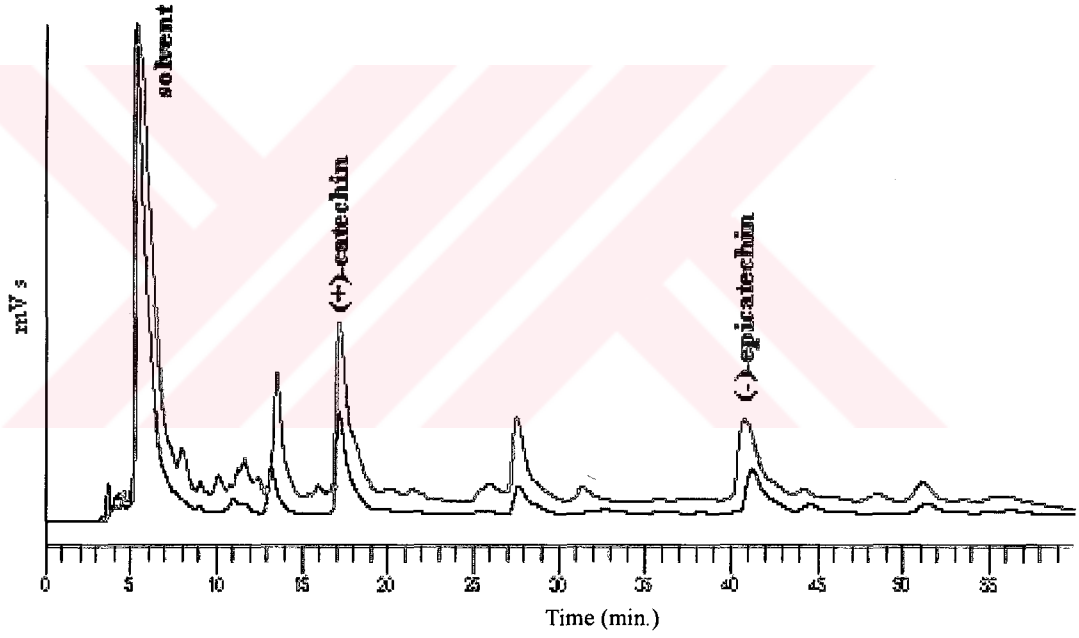


Figure 4.6. Chromatogram of blue: extract and red: Leucoselect`s Leucocianidine ™

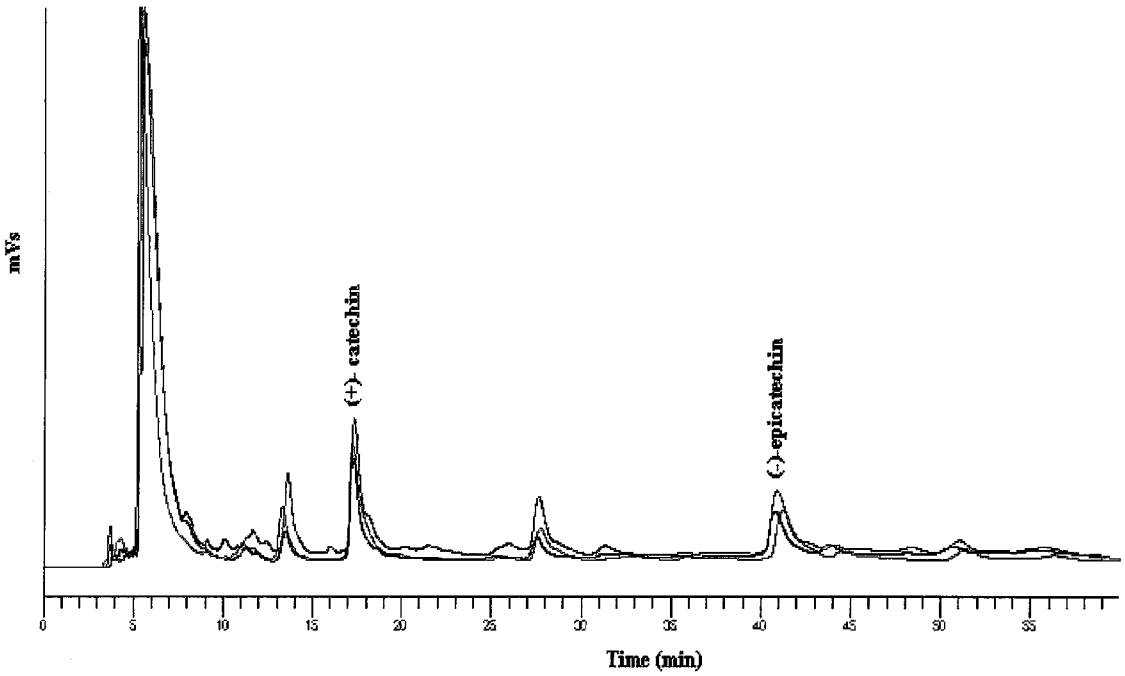


Figure 4.7. Comparison of the chromatograms of blue: Meganatural Gold ™ product , red: extract with 70% aqueous acetone, black: freeze dried extract

As can be seen from the Figure 4.7. there was not great diversity between these products and our extract. The peaks except monomers can be dimers or trimers of proanthocyanidins. However, it cannot be possible to exactly determine them namely with this method. For this reason, preparative HPLC will be beneficial to detect the other peaks by collecting the fractions separately.

4.4. Adsorption of Proanthocyanidin on Natural Zeolite, Clinoptilolite

After the extraction of grape seed proanthocyanidins, further purification methods and dehydration of extract are required. For this reason, the possibility of proanthocyanidin adsorption on clinoptilolite was investigated. 2g and 4g of Gördes clinoptilolite were contacted with the grape seed proanthocyanidin extract and with the extract prepared from the GNC ™ capsule and Meganatural Gold ™ proanthocyanidins. These commercial products were prepared both with the pure acetone and with the 70% aqueous acetone. Table 4.3 indicates that proanthocyanidin adsorption was observed from the solution of extract, which was pure acetone. Although the highest extraction yield was observed by 70% aqueous acetone in extraction step, the proanthocyanidin

adsorption on clinoptilolite was not achieved. As it is known that, clinoptilolite has hydrophilic structure and it shows affinity to water molecules in the extract. As a result, clinoptilolite cannot be used to separate proanthocyanidins specifically from the extract solution.

Table 4.3. Adsorption of proanthocyanidin on natural zeolite.

Extract with-	Amount of Clinoptilolite	Time (min.)	Initial Conc. (g/L)	Final conc. (g/L)	Adsorbed proanthocyanidins (g/g zeolite)*
70% aqueous acetone	2g	15	9	8.08	0.0092
	4g	15	9	8.44	0.0028
Extract prepared with Meganatural Gold ™ in-					
Pure acetone	2g	15	7.35	1.05	0.063
10% aqueous acetone	2g	15	13.5	10.5	0.03
20% aqueous acetone	2g	15	26.5	24.5	0.02
30% aqueous acetone	2g	15	12.5	11.8	0.007
Lyophilized extract solved in-					
Pure acetone	2g	15	9.3	0.25	0.086
Extract prepared with GNC ™ in-					
70% aqueous acetone	2g	15	8.35	7.67	0.0068
70% aqueous acetone	4g	15	8.25	7.49	0.0038

* The adsorbed proanthocyanidins can be determined as in Appendix F.1.

With pure acetone, the adsorption of proanthocyanidins was observed (Table 4.3.). Thus, the adsorption phenomenon was investigated by controlling the adsorption time (Figure 4.8.). For this purpose, separate samples were treated with the zeolite for 2 hours. The proanthocyanidin content of samples were determined by Porter assay. The adsorption was occurred within 5 minutes.

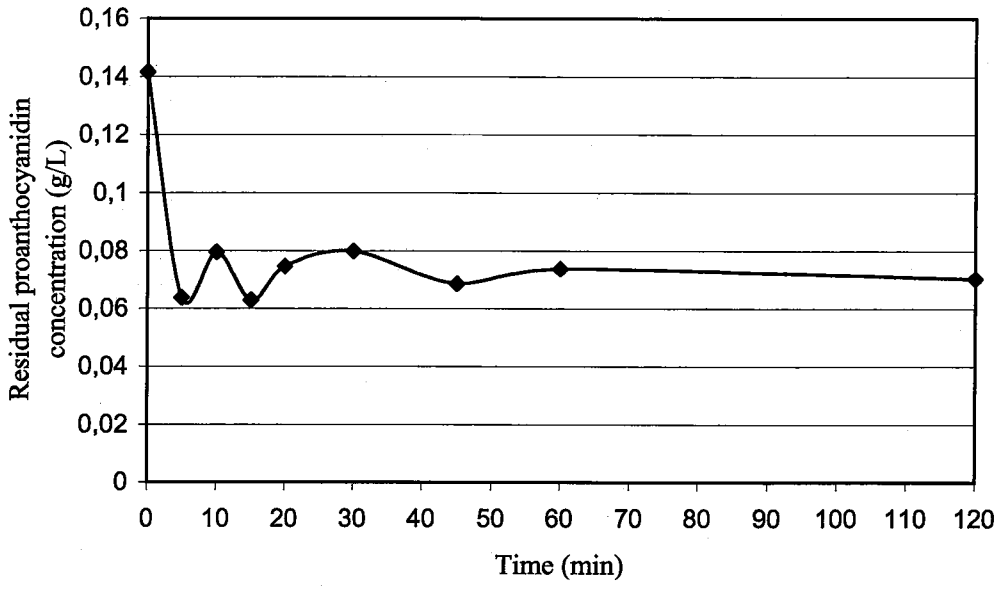


Figure 4.8. Adsorption of Proanthocyanidins against time for Meganatural Gold™ capsule prepared in pure acetone.

To investigate the effect of water on adsorption, the solutions with different water content were prepared and reacted with clinoptilolite for 15 minutes (Table 4.3). In Figure 4.9., water was the main factor, which affects directly the adsorption of proanthocyanidins.

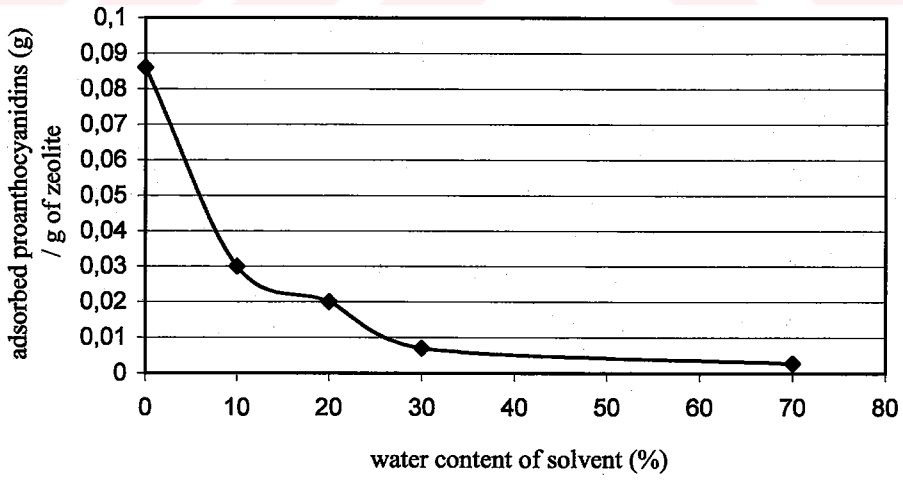


Figure 4.9. : Water content of solvent (%) vs. adsorbed proanthocyanidin content (g) per g of natural zeolite.

After the extraction with 70 % aqueous acetone, proanthocyanidin powders were obtained by freeze drying of the extract. Freeze drying is an expensive and long process. Because of the water affinity of zeolite, dehydration process can be applied to the extract. Thus, the amount of water in extract can be reduced and the process becomes easier. For this reason, the water adsorption capacity of clinoptilolite was investigated. The solid/liquid ratio used in dehydration experiments were 0.4 g zeolite/ mL extract (Tıhmınlıoğlu, 1993).

In this study; based on finding of Tıhmınlıoğlu (1993) zeolite was prepared as 106-150 μm particle size, washed with water and held at 300 ^{0}C for 24 hours. The concentration of (+)- catechin was determined against time and results are given in Figure 4.10.

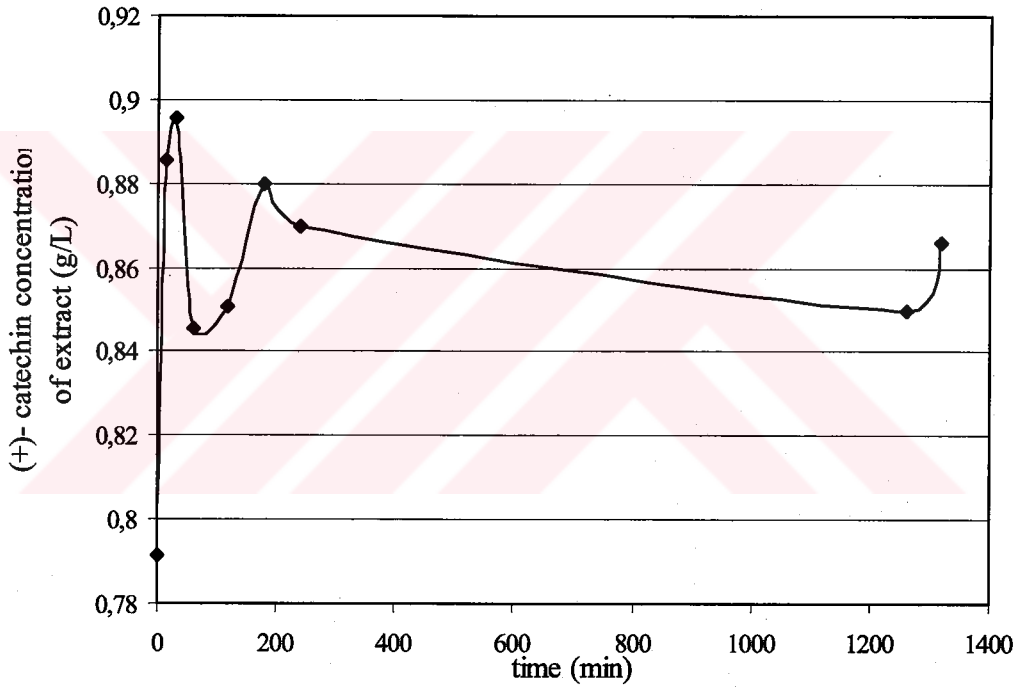


Figure 4.10. The concentration changes of (+)-catechin in solution vs. time determined by HPLC.

The adsorption of water on zeolite was detected by analyzing the (+)-catechin concentration in solution by HPLC. There was an increase in (+)-catechin concentration in the solution that could be explained by the adsorption of water on zeolite structure (Figure 4.10.). Amount of adsorbed water on g zeolite was calculated as in Appendix F.2. Figure 4.11. shows the amount of water adsorbed on g zeolite.

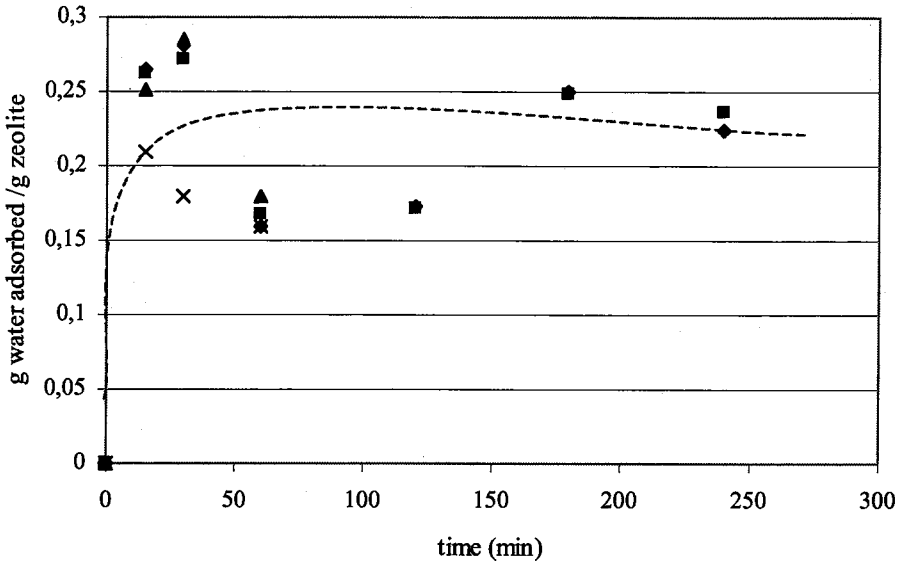


Figure 4.11. Water adsorption kinetics for proanthocyanidin/zeolite system.

Nearly 0.2 g water was adsorbed on g of zeolite. This will help to decrease water content of extract and will ease the freeze-drying process, which takes 200 minutes. However, temperature rise was recorded during the process, especially initial contact of natural zeolite with the extract, which was because of the heat of adsorption. During the process, temperature increased from 29 ^{0}C to 39 ^{0}C within 5 seconds. Instant increase in temperature above the 40 ^{0}C degraded the proanthocyanidin structure and inhibited its antioxidant property. For this reason, adsorption could have been performed at controlled temperature. Moreover, the controlled addition of zeolite may prevent sharp temperature rise.

Figure 4.12 shows the comparison of HPLC chromatograms of proanthocyanidin extracts before and after the treatment with zeolite. It was observed that the amounts of monomers did not change significantly, this supports our findings from the adsorption experiments that proanthocyanidin monomers does not show affinity to zeolite structure.

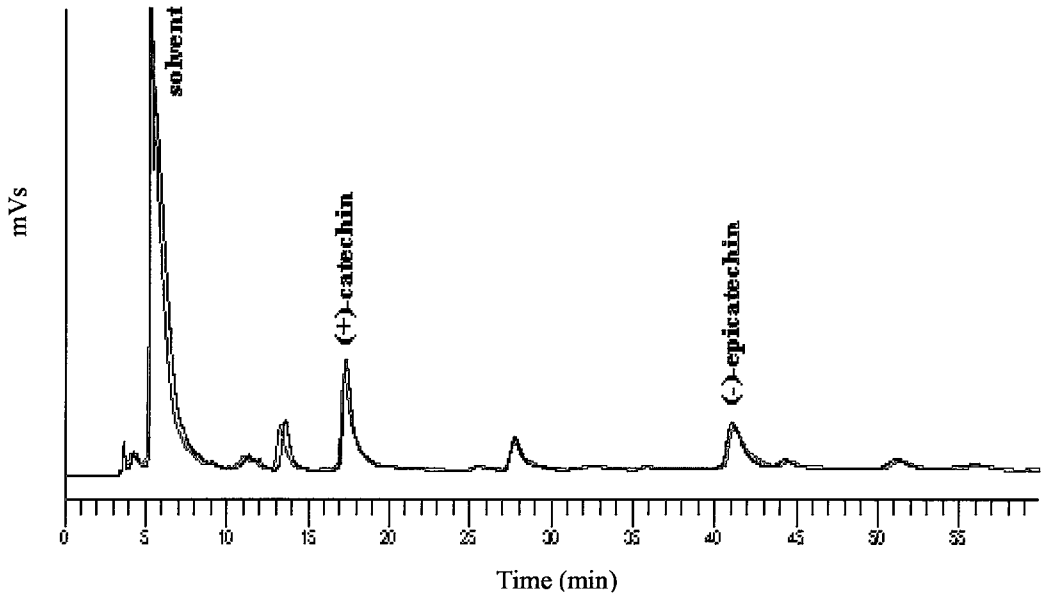


Figure 4.12. The proanthocyanidin extract, which was extracted with %70 aqueous acetone (red chromatogram) and 20 g zeolite treated extract (blue chromatogram)

4.5. Antioxidant Activity of Grape Seed Proanthocyanidin Extracts

The standard Trolox curve was used for the determination of Trolox Equivalent Antioxidant Capacity (TEAC) value. For this purpose, the concentration-absorbance curve for stock standards of Trolox and it's time dependency were obtained and they were illustrated in Fig. 4.13.

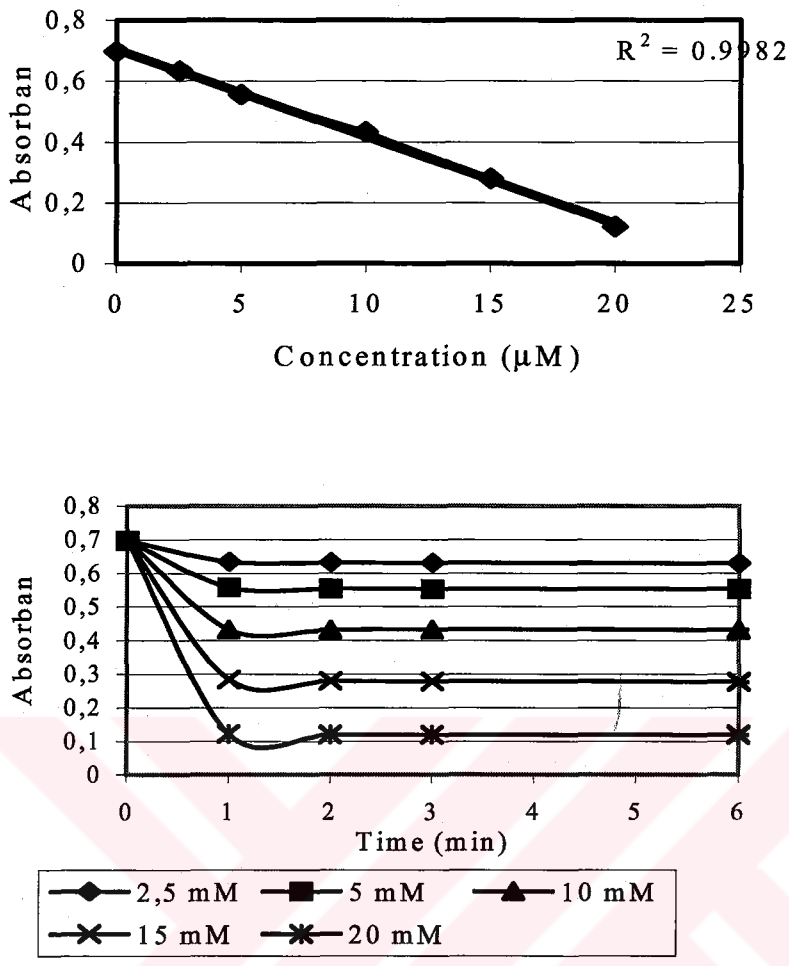


Figure 4.13 Absorbance vs. concentration curves of sequentially prepared Trolox stock solutions and it's time dependency.

To obtain the standard Trolox curve, % inhibition versus concentration curve of Trolox was plotted as in Appendix D.2., which was used for the determination of TEAC values.

Proanthocyanidin is a potent radical scavenger. Since it is an antioxidant; the antioxidant capacities were determined and compared for the proanthocyanidin tablet from GNC ™ prepared both in MeOH and EtOH. TEAC values were determined using Trolox standard curve.

The % inhibition vs. concentration and absorbance vs. time curves were illustrated in Figure 4.14.

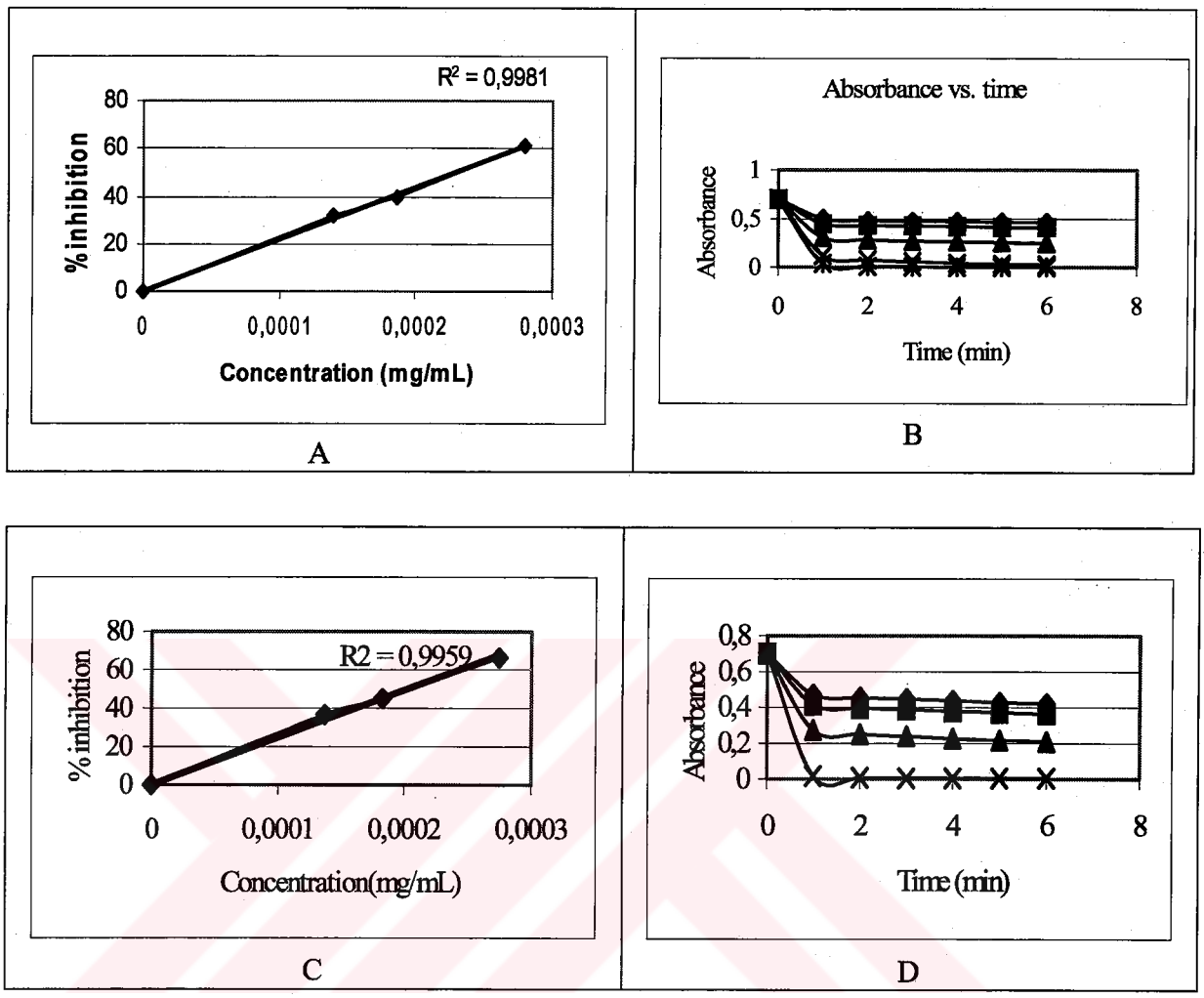


Figure 4.14. The effects of concentration of the proanthocyanidin on the inhibition of the $ABTS^{+}$, (A) GNC™ tablet in the solvent of MeOH; (B) it`s absorbance time dependency within different concentrations; (C) GNC™ tablet in the solvent of EtOH; and (D) it's absorbance- time dependency.

From Figure 4.14., the % inhibition values were calculated for different proanthocyanidin concentrations. By using the standard curve in Appendix D.2.., the responses for the % inhibition values were obtained. These responses were converted to the TEAC values by the multiplications with the dilution factors.

The TEAC values of extracts were calculated as the concentration of Trolox (mM) that showed the same antioxidant activity as 1 g. of the analyzed substance. The TEAC values were found as 56 mM trolox/ g extract and 60.7 mM trolox/ g extract in

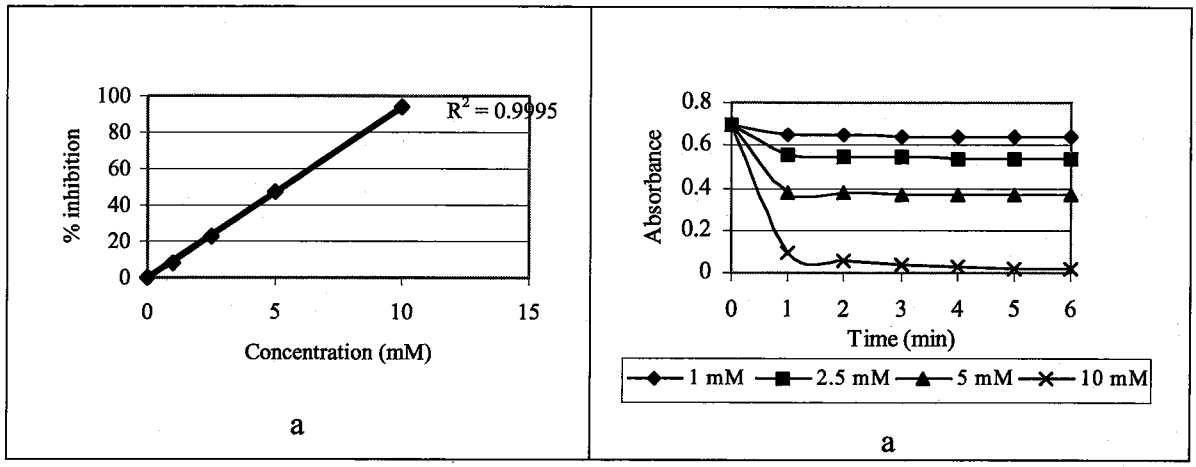
methanol and ethanol respectively. In this method, the TEAC values of proanthocyanidins prepared in both MeOH and EtOH are close to each other. As a conclusion, this method can be adaptable by using MeOH as a solvent.

Proanthocyanidin has higher antioxidant capacity compared with other synthetic antioxidants such as BHT, TBHQ and natural antioxidants as ascorbic acid and tocopherol. The comparison of the antioxidant activity of proanthocyanidin with synthetic antioxidants; Butylated Hydroxy Anisole (BHA), Butylated Hydroxy Toluene (BHT), Tert- Butylated Hydro kinone (TBHQ) and Propyl gallate (PG) were investigated. The % inhibition and concentration curves and time dependency of them are illustrated in Appendix D.3.

The concentration of antioxidant giving the same percentage inhibition of absorbance of the radical cation at 734 nm as 1 mM Trolox was calculated in terms of the Trolox Equivalent antioxidant activity (TEAC). This value was given as mM Trolox for mM antioxidant. This calculation was performed by the gradient of the plot of the percentage inhibition of absorbance vs. concentration plot for the antioxidant in question is divided by the gradient of the plot for Trolox. From Appendix D.3., the TEAC values of BHA, BHT, TBHQ and PG were calculated as 1.21 TEAC, 0.117 TEAC, 1.085 TEAC and 2.08 TEAC respectively. Generally the reaction was occurred regularly within 1 minute except BHT. This means the reduction of $ABTS^{+}$ was completed within 1 min.

On the other hand, the TEAC values were also calculated as the concentration of Trolox in mM exhibiting the same antioxidant activity with the 1 g of questioned antioxidant. This calculation is useful for the extractable substances such as cherries, hibiscus-fruits, elderberries, grape seeds etc. Thus, the TEAC values of synthetic antioxidant were 6.66, 0.593, 1.168 and 12.62 for BHA, BHT, TBHQ and PG respectively. The TEAC value of grape extract from GNC ^{TM} was calculated as 55.98 ($\pm$ 5.5) using Figure 4.14.

The TEAC values of monomer of Proanthocyanidins; (+)- catechin and ascorbic acid (Vitamin-C) were investigated (Figure 4.15).



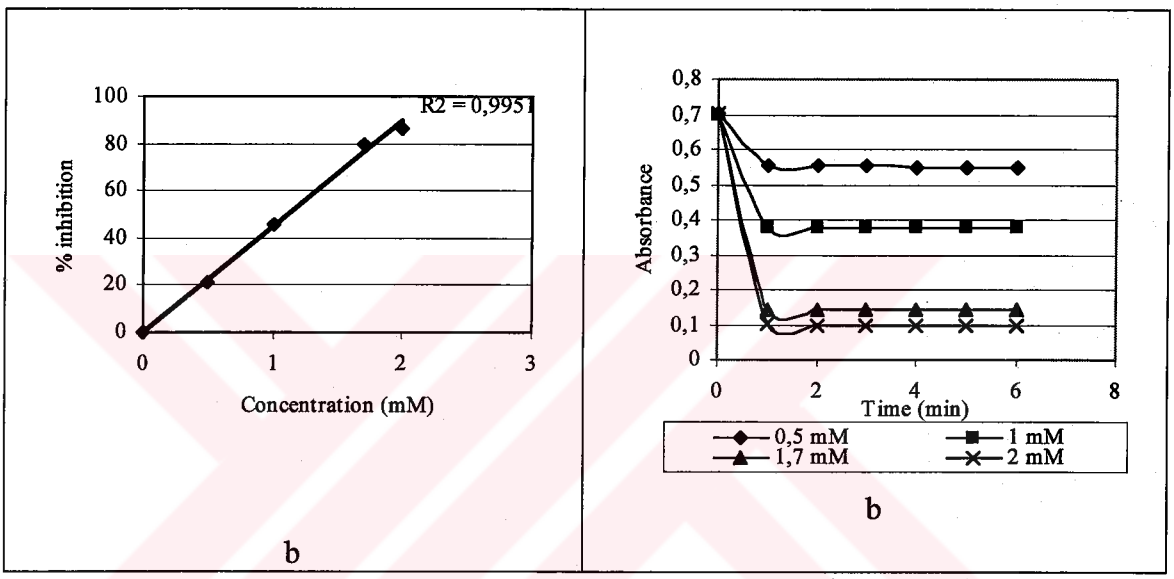


Figure 4.15. % inhibition- concentration of (+)- catechin (a), ascorbic acid (b) and their time dependencies.

For the purpose of controlling the reliability of method, the TEAC values were determined both as mM trolox/ mM. antioxidant and mM Trolox/ g of antioxidant. For (+)- catechin and ascorbic acid, TEAC values were determined as 2.32 TEAC and 8.01 TEAC and 1.107 TEAC and 6.45 TEAC as stated above respectively.

From the Table 4.4, the TEAC value of (+)-catechin was found around from 2.47 to 3.42 TEAC (mM Trolox/mM. antioxidant), which is close to our result.

Table 4.4. TEAC values of different structures of flavonoids.

Compounds		TEAC values	References
Flavon-3-ols	Quercetin	3.4±0.05	Re et al., 1999
	Kaempferol	1.45±0.08	Ishige et al., 1999
	(+)-catechin	3.11±0.48	Ishige et al.,
	(-)-epicatechin	3.16- 4.8	Ishige et al., Spencer et al.
α-tocopherol		0.97 ±0.06	Re et al., 1999
Ascorbic acid		1.05±0.02	Re et al., 1999
Trolox		1	Re et al., 1999 ; Ishige et al.
Catechin Gallate		5.12±0.55	Ishige et al.
Rasveratrol		2.88±0.15	Ishige et al.
(+)-catechin		2.32	PRESENT STUDY
Ascorbic acid		1.107	PRESENT STUDY

The TEAC value of proanthocyanidin was determined around 55-60, which is very noticeable. The higher $ABTS^{+}$ scavenging capacity of proanthocyanidin can be explained, structurally, by the high number of conjugated structures between the catechol groups in the B-rings and the 3-OH free groups of the polymeric polyphenolic skeleton (Castillo et al., 2000).

Lyophilized Proanthocyanidins were used to determine its antioxidant activity (Figure 4.16.) to compare our freeze-dried product with the other commercial products (Appendix D.4.).

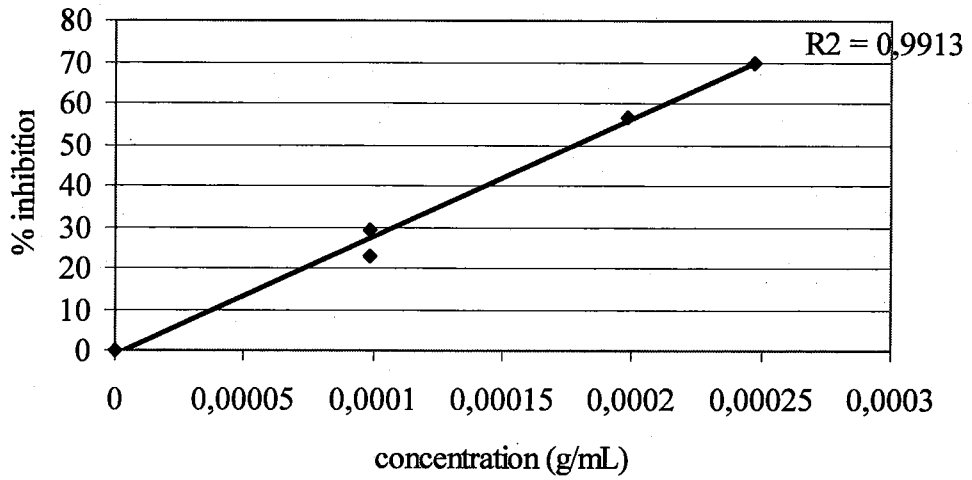


Figure 4.16. Lyophilized Proanthocyanidins extracted with %70-aqueous acetone

By using the standard curve of trolox the antioxidant activity of lyophilized extract can be found as 57.28 TEAC (mM. Trolox/ g. extract), which is less from other commercial mark grape seed extracts which were 59 TEAC for GNC™ tablets, 88 TEAC for Meganatural Gold™ and 100 TEAC for Leucoselect Leucocyanidine™ (Table 4.5). These capacities were changed according to the extraction techniques, type of grapes, soil properties, climate etc. The antioxidant capacity of our products, commercial ones and its monomer (+)- catechin and ascorbic acid were given in Table 4.5.

Table 4.5. TEAC values of different products, (+)- catechin and ascorbic acid

Commercial Products	GNC™ tablets	59 TEAC*
	Meganatural Gold™	100 TEAC
	Leucoselect Leucocyanidine™	88 TEAC
Our Lyophilized product		57.28 TEAC
(+) catechin		8.01 TEAC
Ascorbic acid		6.45 TEAC

* TEAC value was determined as mM trolox/ g antioxidant

CHAPTER-5

CONCLUSION

Grape seeds are the main source of proanthocyanidin, which has high antioxidant capacity. Proanthocyanidin is used as dietary supplement in all over the world. Turkey is the main producer of grape, which is used for the production of wine, pekmez etc. Grape seeds are the waste material of these industries which contains valuable products such as proanthocyanidins. To extract the proanthocyanidins from grape seeds, various solvents were investigated in this study and it was observed that, 70 % aqueous acetone was the best solvent with its 17.5% yield for 24 hour. Water was important in that, it increased the permeability and provided better mass transport.

In industry, lyophilization was used to dry the extract. As in industry, our extract was freeze dried and it was analyzed by various techniques such as FTIR, HPLC, their antioxidant capacities to compare our product with the commercial ones. FTIR gave the good comparison and our lyophilized product was similar to the commercial ones. HPLC was used to determine its monomers; (+)- catechin and (-)- epicatechin at 17.2 min as 3.06%w/w and at 42.8 min as 1.99%w/w respectively. Monomers of proanthocyanidins are not biologically active so, they are accepted as impurities in grape seed extracts. For this reason, the determination of monomers is important to determine the extract's purity. It was observed that, our product included 3.06 % (+)- catechin and 1.99 % (-)- epicatechin which were 4.22 % and 2.42 % for Meganatural Gold ^{TM} , 3.18 % and 2.61 % for GNC ^{TM}, and 2.68 % and 1.48 % for Leucoselect`s Leucocianidine ^{TM}. It should be noted that there was not significant difference between our products and commercial ones. Beside the determination of monomers, other fractions were tried to be determined by HPLC. There were other peaks on chromatogram but it was not possible to characterize them. For this reason, fractionation was required and further techniques should be developed. Preparative HPLC will be useful to determine the other fractions by collecting the fractions separately.

Lyophilization processes are expensive, slow and difficult to carry out; another concentration technique has been investigated as an alternative in this study. Since adsorption processes can be used for concentration purposes; proanthocyanidin

adsorption on clinoptilolite were performed. It was observed that there was not any proanthocyanidin adsorption on zeolite because extraction solution contain 70 % water, which was hindered the process. In pure acetone, the adsorption on zeolite was observed as around 0.08 g proanthocyanidin per gram of zeolite. Increase in water content of solvent decreased the proanthocyanidin adsorption on zeolite. For this purpose, the affinity of zeolite on water was observed. Then, to decrease the water content of extract immediately after the extraction, the water adsorption capacity of zeolite from the extract solution was investigated. It was calculated that, around 0.2 g water was absorbed by g of zeolite. This will help to decrease water content of extract and will ease the freeze-drying. Beside the natural zeolite, other adsorbent materials can be examined such as hydroxyapatite, chitin, chitosan, synthetic polymers etc.

Antioxidant property of proanthocyanidin was also investigated and compared with the commercial products. Trolox equivalent antioxidant capacity (TEAC) determination was applied to determine the antioxidant capacity and the TEAC values were determined as mM trolox per gram of questioned substances. This was why the molecular weight of proanthocyanidin was not determined exactly because of the complexity of proanthocyanidin structure. By comparing the grape seed extract with its monomer (+)- catechin and ascorbic acid (Vitamin C) and also by the synthetic antioxidants such as BHT, BHA, TBHQ and PG, it was observed that proanthocyanidin had high antioxidant capacity as 59 TEAC values, where (+)- catechin had 8.01 TEAC, ascorbic acid had 6.45 TEAC, BHT had 0.593 TEAC, BHA had 6.66 TEAC, TBHQ had 1.168 TEAC, and PG had 12.62 TEAC. This was clearly seen that proanthocyanidin had 10-30 times more antioxidant capacity than the synthetic and natural antioxidants.

The comparison of antioxidant activities of our lyophilized product with the commercial ones were carried out and it was observed that, commercial trademarks' TEAC values were between the 55- 100 which was very noticeable. It can be indicated that the TEAC values can vary according to land properties, soil types, weather conditions, type of grapes, and type of extraction process.

The most important point of this study is that of, in Turkey it was the first time to initiate using the natural resources and organic wastes beneficially, Hence, these products are to be produced as valuable natural antioxidants and consumed as dietary supplement with its high benefits against some important diseases.

This study is the preliminary step to discover our countries' valuable natural resources to produce antioxidant property substances. Also, it can be considered as

being the starting point for further studies of its kind, using natural resources and organic wastes beneficially in Turkey. Grape seed proanthocyanidin should be investigated by the means of toxicology, its effects on cell system and its dosage to use in human body.

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APPENDICES

APPENDIX A. CHEMICALS, REAGENTS AND EQUIPMENTS

For High Performance Liquid Chromatography (HPLC); HPLC grade acetone, methanol (MeOH), n-Butanol, ethanol (EtOH) are supplied by Merck ; (+)-catechin, (-)-epicatechin, (-)- epicatechin gallate are from Sigma, Steinheim, Germany; Columns which were used in experiments were C 18 column from Metachem and BTR-Seperation C18 column.

For Porter analysis; Ferric Ammonium Sulfate, Hydrochloric acid, n-Butanol are supplied by Merck, Darmstadt, Germany.

For antioxidant determination; ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation from Sigma, Steinheim, Germany; Trolox ((±)-6-Hydroxy-2,5,7,8-tetra-methylchromane-2-carboxylic acid), potassium persulfate ($K_2O_8H_8$), Butylated Hydroxy Anisole (BHA), Butylated Hydroxy Toluene (BHT), Tert- Butylated Hydro kinone (TBHQ) and PG from Fluka, Steinheim, Germany; Ascorbic Acid from Riedel.

Trademark products of Proanthocyanidins of GNC™ , Meganatural Gold ™ , and Leucoselect`s Leucocianidine ™ were used for comparison and standard curves.

Distilled or deionized water was used in all preparations.

Natural zeolite, clinoptilolite from the region of Gördes was used to determine the adsorption behavior of proanthocyanidin on it.

Perkin- Elmer Series of HPLC with photodiode array detector, Shimadzu UV-Visible Spectrophotometer, Gerhard Thermo Shaker, Sartorius- MA 100 Moisture analyzer, Buchi R-3000 Rotary Evaporator, Elma Transsonic 780/H Ultrasonic water bath, Labconco Freeze Drier System.

APPENDIX B. PORTER ASSAY

B.1. Procedure

Reagents

* Butanol-HCl reagent (butanol-HCI 95:5 v/v): Mix 950 ml n-butanol with 50 ml concentrated HCl (37%).

* Ferric reagent (2% ferric ammonium sulfate in 2N HCl): Make 16.6 ml of concentrated HCl up to 100 ml with distilled water to make 2N HCl. Dissolve 2.0 g ferric ammonium sulfate in this volume of 2N HCl. This reagent should be stored in a dark bottle.

In a 100 mm x 12 mm glass test tube, 0.50 ml of the grape seed extract diluted with acetone was pipetted. The quantity of acetone should be large enough to prevent the absorbance (552 nm) in the assay from exceeding 0.6. That was why the correlation coefficient decreased significantly. It will depend on the quantity of condensed tannin expected in the sample, and occasionally will need to be determined by trial and error. To the tubes 3.0 ml of the butanol-HCl reagent and 0.1 ml of the ferric reagent were added. After to vortex the tubes, the mouth of each tube was covered with a glass marble and the tubes were put in a heating block adjusted at 97 to 100°C (or in a boiling water bath) for 60 min. Then, the tubes were cooled and absorbance was recorded at 552 nm. Subtract a suitable blank, which was the absorbance of the unheated mixture.

B.2. Calibration Curve of Porter Assay

Calibration curve for Porter Assay was obtained according to Meganatural Gold™.

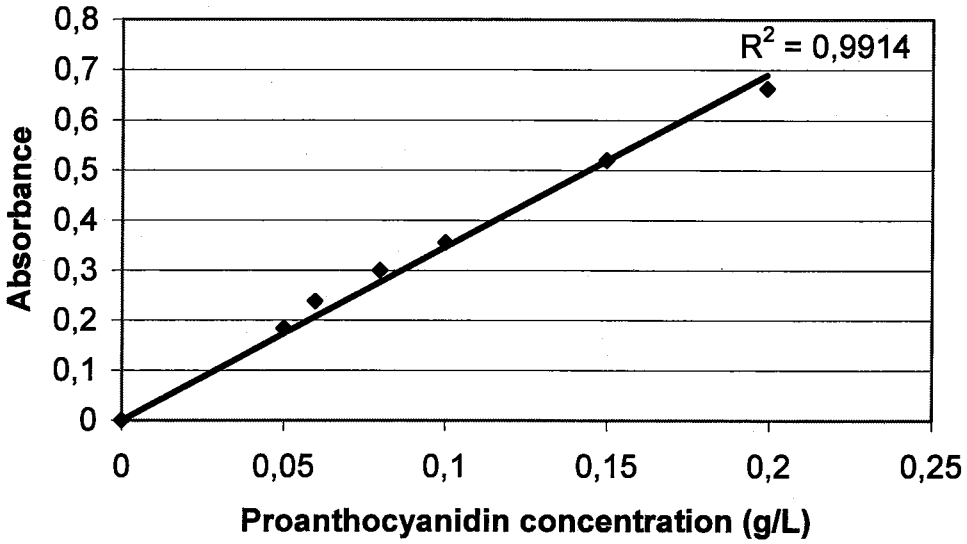

Figure B.2. Calibration curve of Porter Assay

APPENDIX C. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

C.1. Operating Conditions of HPLC System

Properties of High Performance Liquid Chromatography (HPLC) are given as below;

Property	Values or Attributes
Column	BTR-seperation RP-C18 column, Metachem C18 column
Column length	150 mm
Column diameter	5 mm
Particle size	4.8μm
Column cleaning solvent	%5 acetic acid
Mobile phase	Mobile phase A: %5 acetic acid in deionized water; Mobile phase B: MeOH in A (20:80)
Flow rate	0.8 ml min^{-1}
Temperature	30 °C
Detector	Photo Diode Array Detector

Gradient condition is;

Time (min)	Mobile Phase A	Mobile Phase B
0	100	-
50	70	30
10	100	-

C.2. HPLC Calibration Curves

C.2.1. HPLC Calibration Curve for (+)- catechin

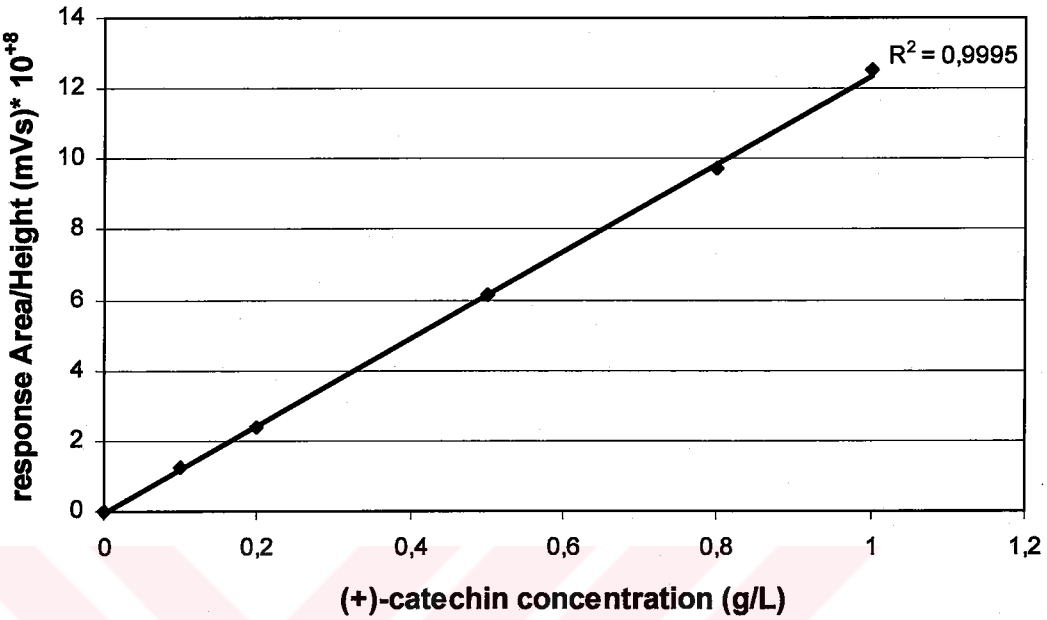

Figure C.2.1. Calibration curve of (+)- catechin

Multiple- point standard calibration curve of (+)- catechin with 0.9995 R^2.

C.2.2. HPLC Calibration Curve for (-)- epicatechin

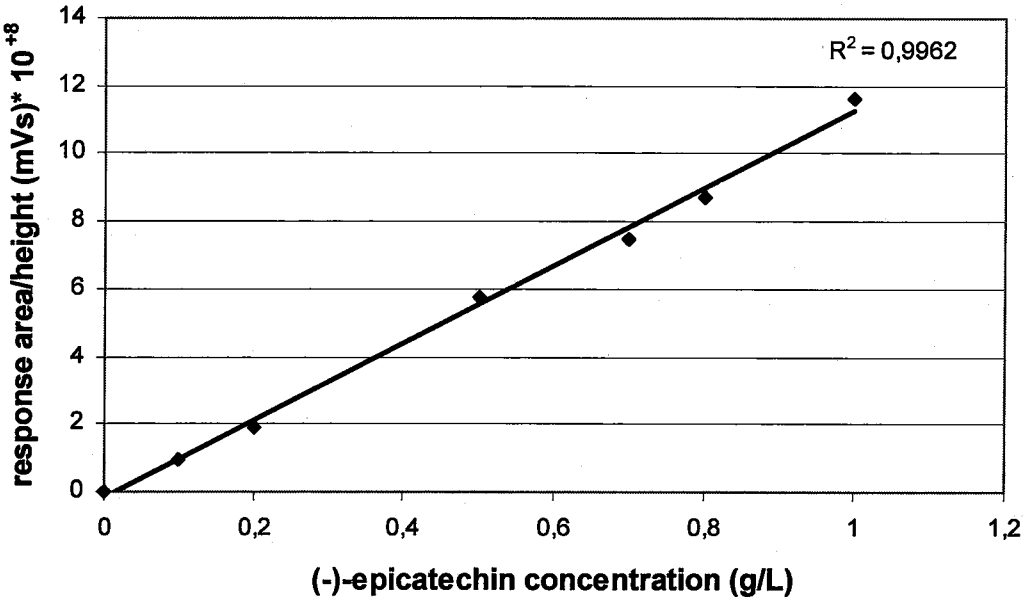

Figure C.2.2. Calibration curve of (-)- epicatechin

Multiple- point standard calibration curve of (-)- epicatechin with 0.9962 R^2.

APPENDIX D. DETERMINATION OF ANTIOXIDANT ACTIVITY

D.1. Procedure

In the method, ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. Because ABTS and potassium persulfate react stoichiometrically at a ratio of 1:0.5, this will result in incomplete oxidation of the ABTS. Oxidation of the ABTS commenced immediately, but the absorbance was not maximal and stable until more than 6 h had elapsed (Re et al., 1999). The radical was stable in this form for more than two days when stored in the dark at room temperature (Re et al., 1999 and Koleva et al., 2001). For the study of phenolic compounds and food extracts, the $ABTS^{\bullet+}$ solution was diluted with EtOH and MeOH to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30°C. Stock solutions of phenolics in EtOH and MeOH were prepared as they produced between 20%–80% inhibition of the blank absorbance. After addition of 2.0 ml of diluted $ABTS^{\bullet+}$ solution (A_{734nm}= 0.700±0.020) to 20 µl of antioxidant compounds or Trolox standards (final concentration 0–2 mM) in EtOH or MeOH, the absorbance reading was taken at 30°C exactly 1 min after initial mixing and up to 6 min. Appropriate solvent blanks were run in each assay. All determinations were carried out at least three times, and in triplicate, on each occasion and at each separate concentration of the standard and samples. The percentage inhibition of absorbance at 734 nm is calculated and plotted as a function of concentration of antioxidants and of Trolox for the standard reference data.

D.2. Calibration Curve of Antioxidant Activity Prepared by Trolox

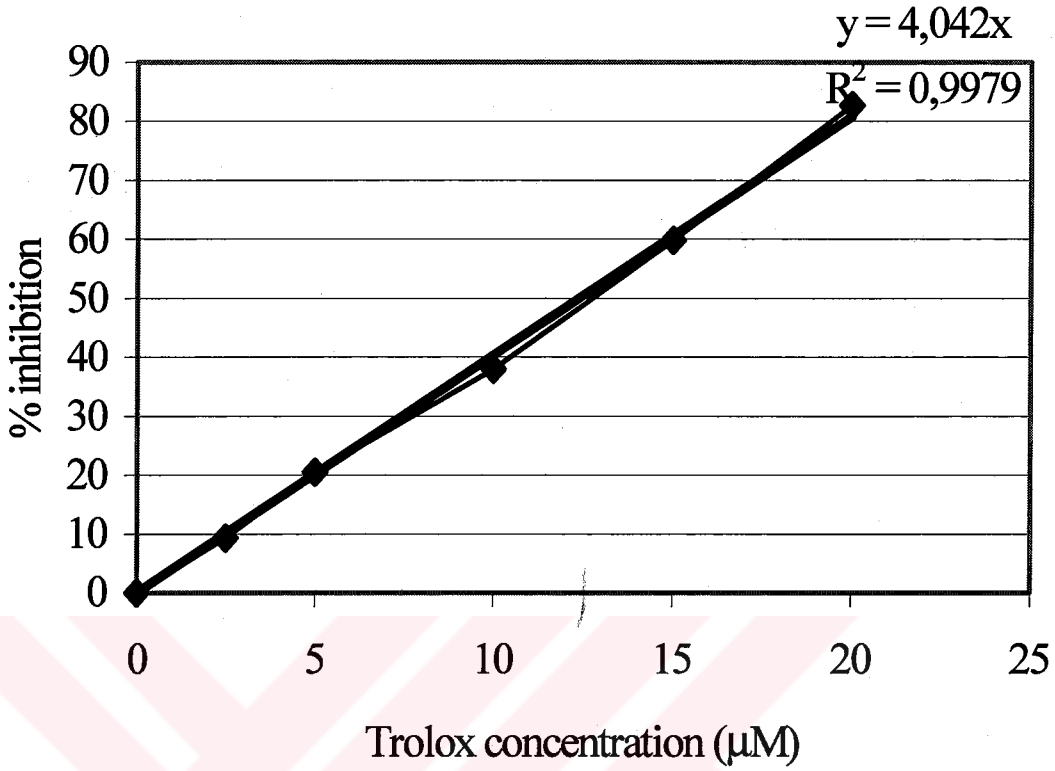

Figure D.2. Calibration curve for antioxidant activity determination prepared by Trolox

% inhibition vs. concentration curve for the absorbance at 734 nm. for $ABTS^{+}$ as a function of concentration of standard Trolox solution from Figure 5.13 was used as a calibration curve for TEAC determination.

D.3. Determination of Teac Values of Synthetic Antioxidants

D.3.1. % Inhibition vs. Concentration Curve and Time Dependency Curve of BHA

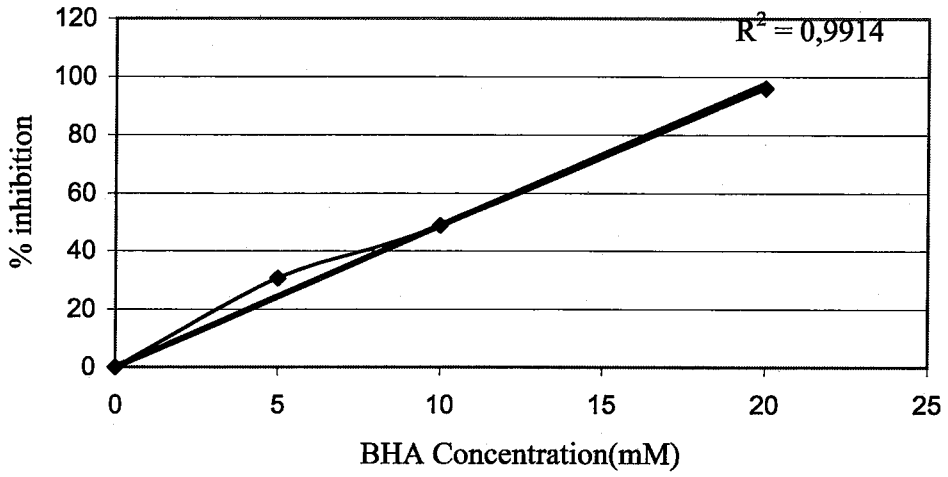

Figure D.3.1.1. % inhibition vs. concentration curve of BHA.

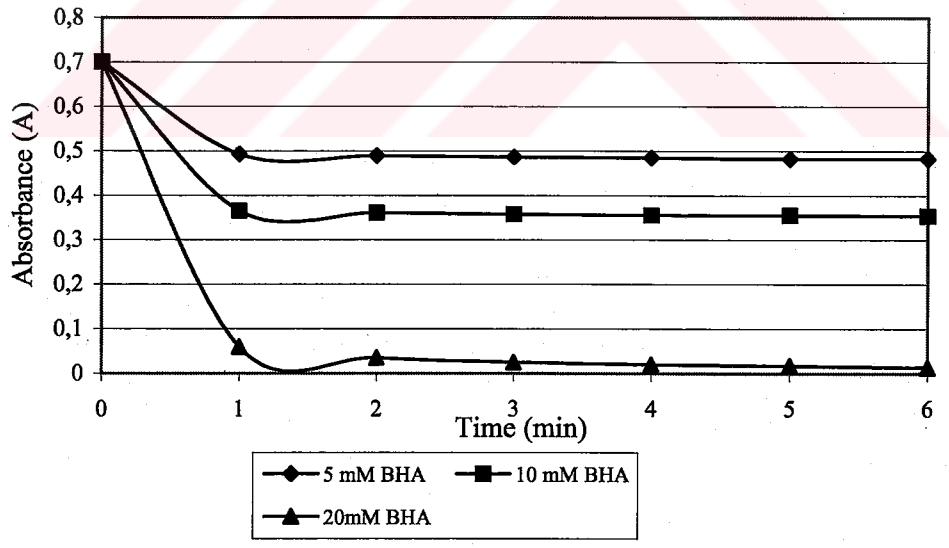

Figure D.3.1.2 Time dependency of BHA.

D.3.2. % Inhibition vs. Concentration Curve and Time Dependency Curve of BHT

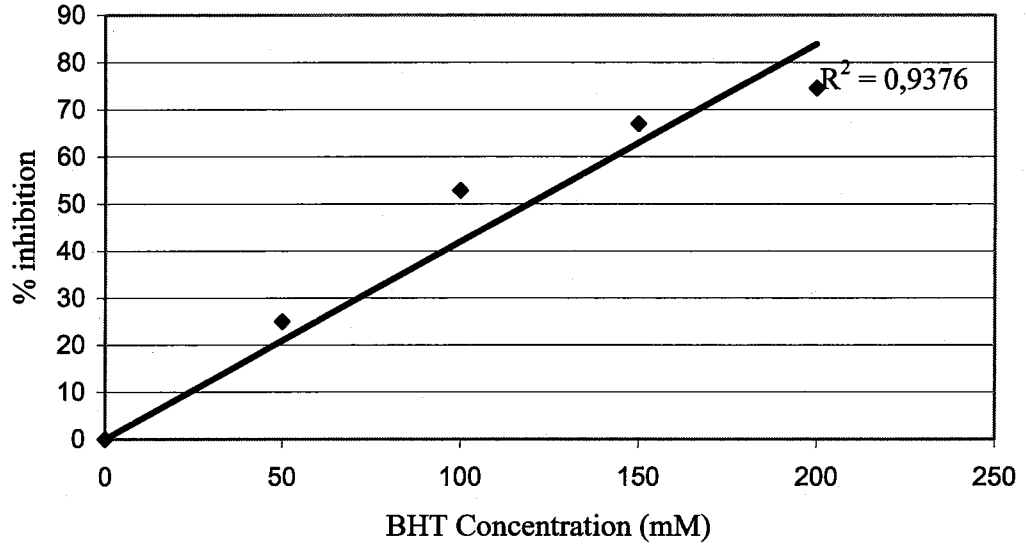

Figure D.3.2.1. % inhibition vs. concentration curve of BHT.

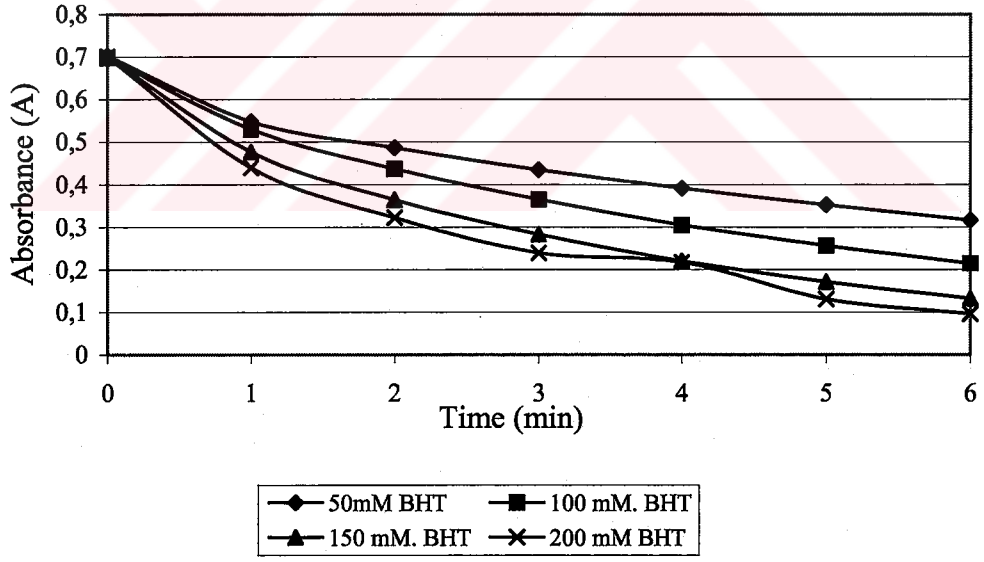

Figure D.3.2.2 Time dependency of BHT.

D.3.3. % Inhibition vs. Concentration Curve and Time Dependency Curve of TBHQ

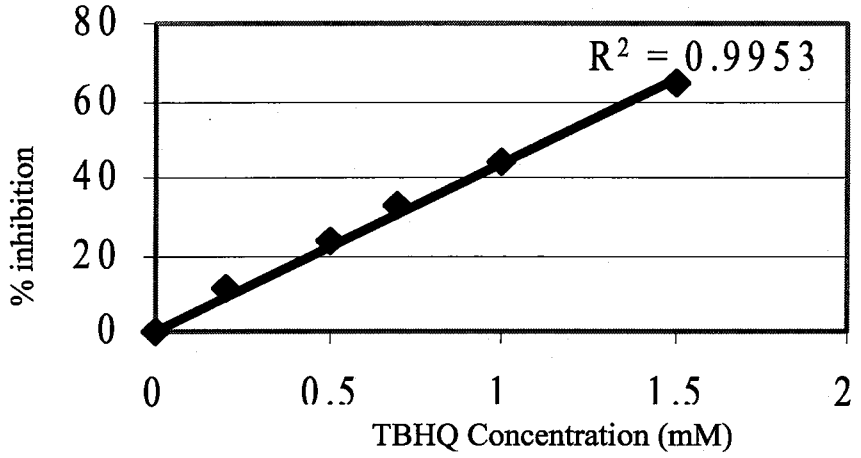

Figure D.3.3.1. % inhibition vs. concentration curve of TBHQ.

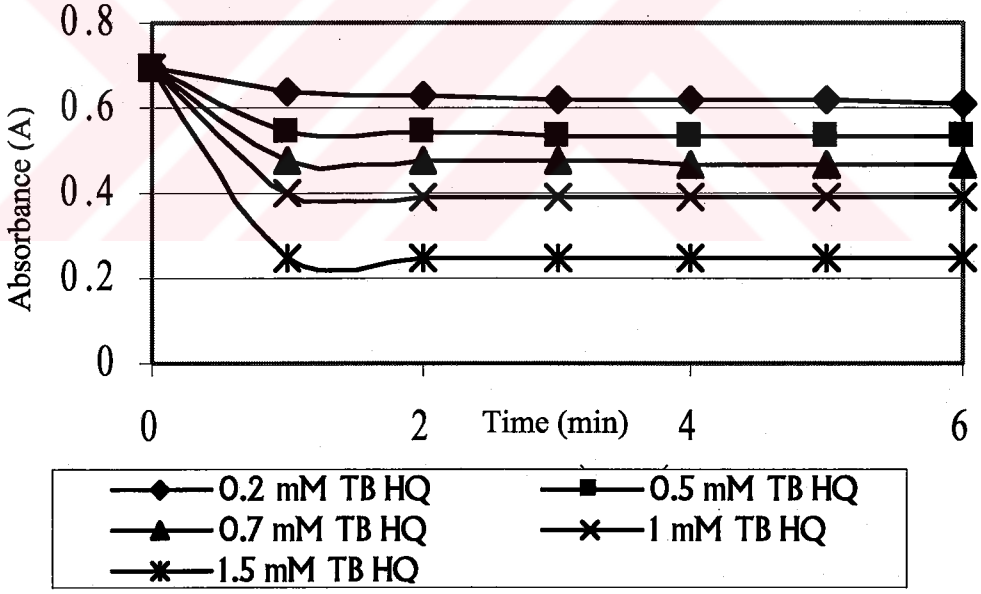

Figure D.3.3.2 Time dependency of TBHQ.

D.3.4. % Inhibition vs. Concentration Curve and Time Dependency Curve of PG

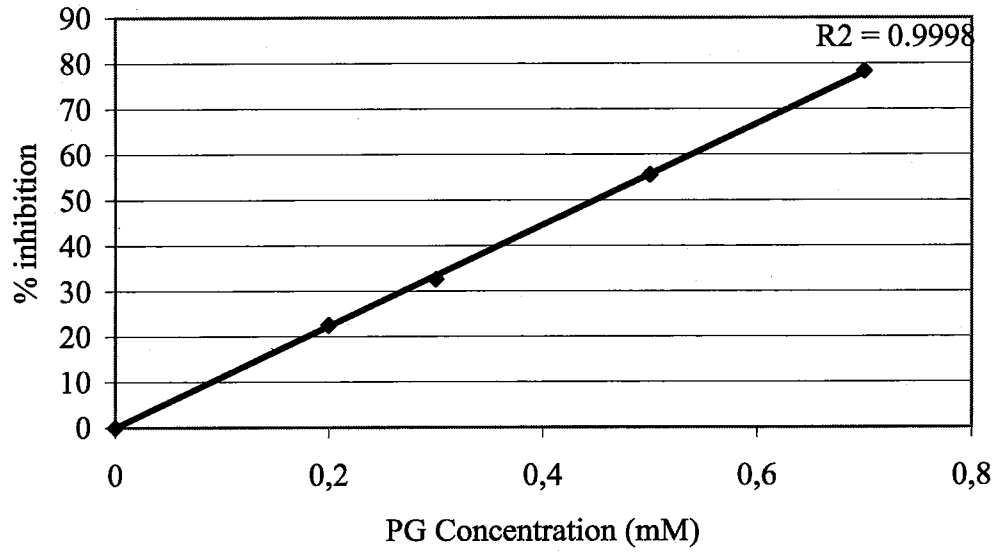

Figure D.3.4.1. % inhibition vs. concentration curve of PG.

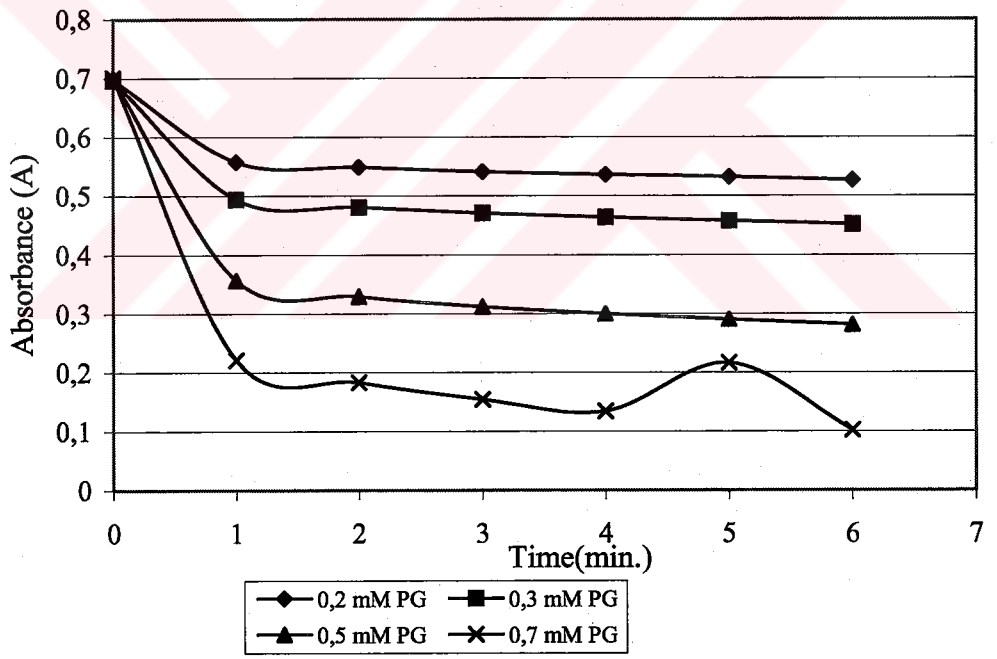

Figure D.3.4.2 Time dependency of PG.

D.4. Determination of TEAC Values of Commercial Proanthocyanidin Products

D.4.1. % Inhibition vs. Concentration Curve and Time Dependency Curve of Meganatural Gold™.

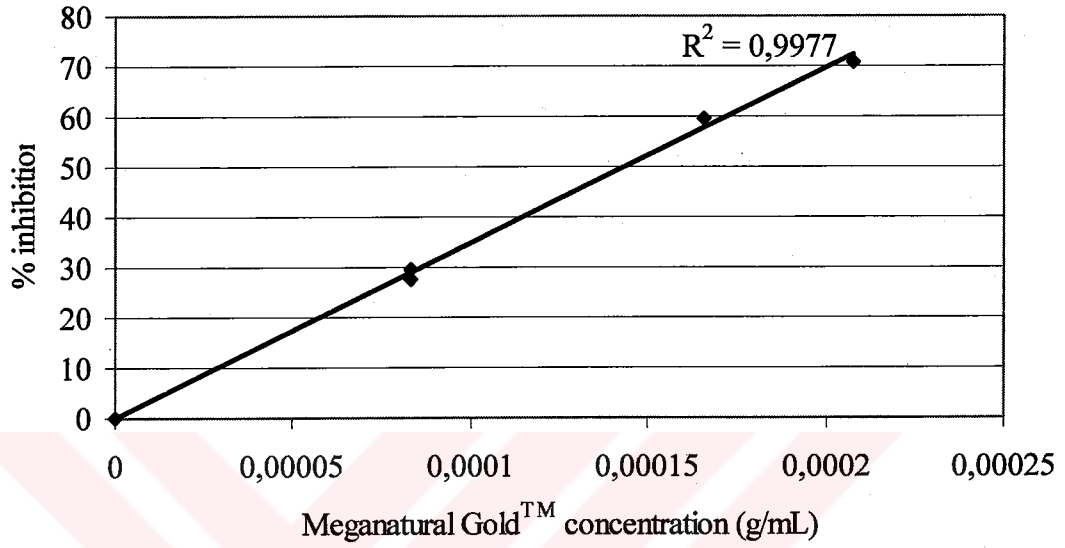

Figure D.4.1.1. % inhibition vs. concentration curve of Meganatural Gold™.

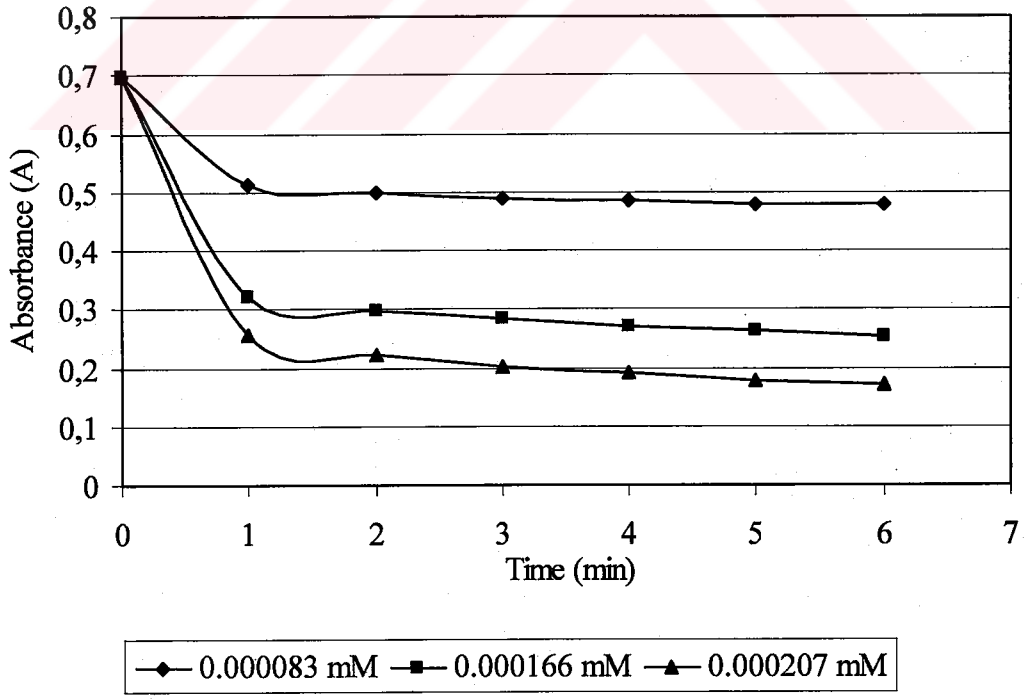

Figure D.4.1.2. Time dependency of Meganatural Gold™.

D.4.2. % Inhibition vs. Concentration Curve and Time Dependency Curve of Leucoselect Leucocianidine ™.

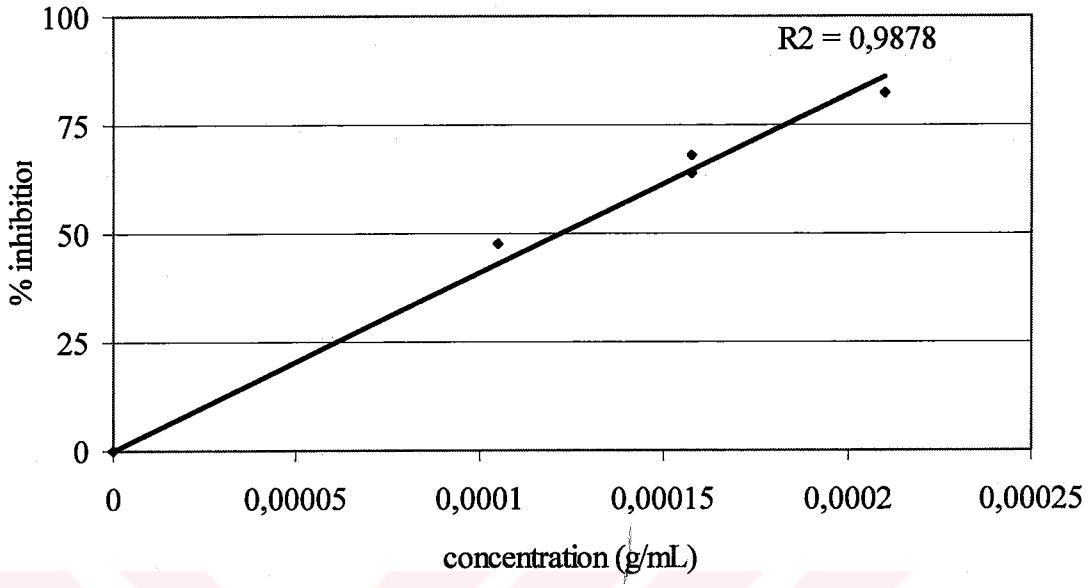

Figure D.4.2.1. % inhibition vs. concentration curve of Leucoselect Leucocianidine ™.

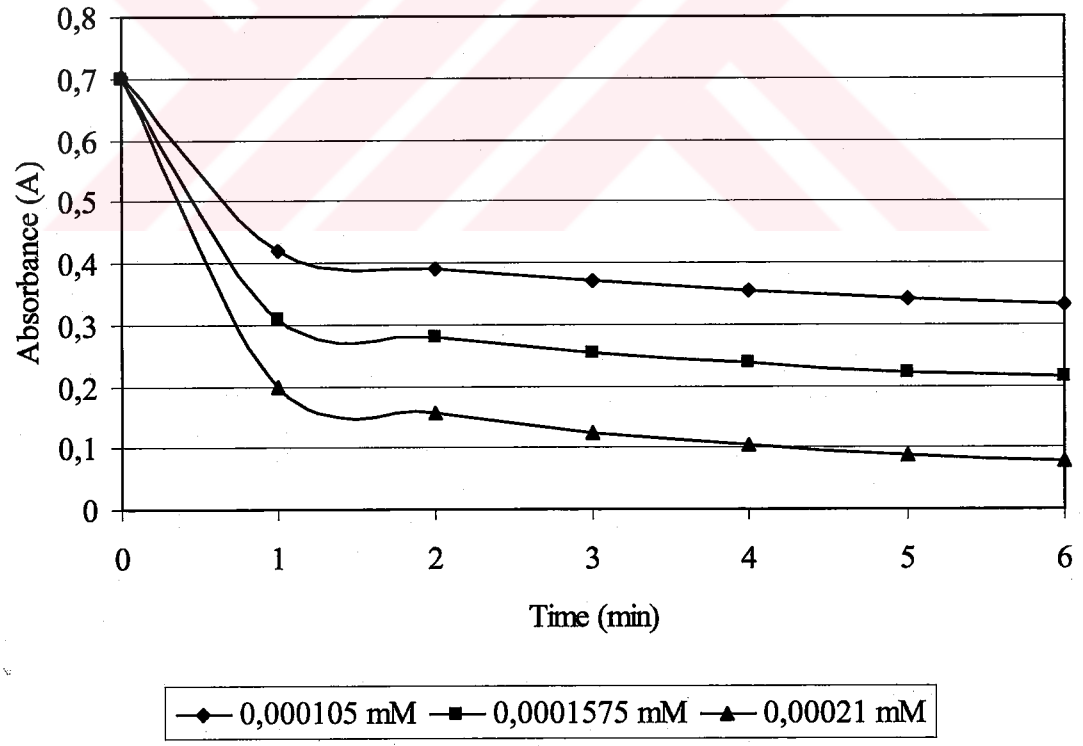

Figure D.4.2.2. Time dependency of Leucoselect Leucocianidine ™.

APPENDIX E. CHARACTERISTIC PEAK BONDS ON FTIR SPECTRA FOR DIFFERENT GROUPS

Group	Wave number region (cm^{-1})	Characteristic
O-H	3600–3200	H-bonded broad and strong
C-H	3100–3000	aromatic medium
C-H	3000–2850	alkane medium, sharp (stretch)
C=C	1600–1400	aromatic medium-weak, series of sharp bands
C=O	1820–1670	ester and carbonyl generally strong, conjugated lower
-C-H	1480–1350	alkane variable (bending)
C-O	1300–1000	alcohol and ether strong, ester two bands or more
1,2-disubstituted	1200–900	benzene ring, three peaks, two medium, one strong
1,3-disubstituted	1100–700	Benzene ring, four peaks, two medium, two strong

APPENDIX F. CALCULATIONS

F.1. Determination of the Amount of Adsorbed Proanthocyanidin on Zeolite by Porter Assay

Adsorbed proanthocyanidin was determined with this formula;

(C_i-C_t) x Vi / M_{zeo} = g adsorbed proanthocyanidin/ g zeolite

where,

C_i = Initial concentration of extract (g/L)
C_t = Concentration of extract after Zeolite treatment (g/L)
V_i = Volume of extract, which was treated with the zeolite (L)
M_{zeo} = Amount of zeolite (g)

Sample calculation;

20 mL of lyophilized product with a 9.3 g/L initial proanthocyanidin concentration was treated with the 2 g of clinoptilolite. The adsorbed proanthocyanidin per gram of zeolite was calculated as;

(9.3- 0.7) x 0.02 / 2 = 0.086 g praonthocyanidin/ g zeolite

F.2. Determination of the Amount of Adsorbed Water on Zeolite by the determination of the concentration of (+)- catechin

By the increase in concentration of (+)-catechin, final volume of extract can be determined by (1).

$C_i \times V_i = C_t \times V_t$

$Vt = C_i/C_t \times V_i$ (1)

where,

C_i = Initial concentration of (+)- catechin (g/L)
C_t = Concentration of (+)- catechin after Zeolite treatment (g/L)
V_i = Volume of extract, which was treated with the zeolite (L)
V_t = Final volume of extract (g/L)

The difference between the final volume and initial volume, which is because of the adsorption of water on zeolite, is observed as in (2).

mL of water loss= $C_i - C_t$

$= Ci- (C_i/C_t \times V_i)$ (2)

The g amount of water adsorbed on zeolite can be determined by multiplying the volume of water loss with the density of water (3).

g of water loss = $\rho_{water} \times Ci- (C_i/C_t \times V_i)$ (3)

Finally, g adsorbed water on g zeolite can be determined as

g adsorbed water on g zeolite = g water loss / M_{zeo}

$= \rho_{water} \times Ci- (C_i/C_t \times V_i) / M_{zeo}$ (4)

where,

M_{zeo} = Amount of zeolite (g)

ρ_{water} = Density of water (g/mL)

F.3. Determination of the Diffusion Coefficients

Diffusion coefficients were calculated with the formula of;

$$\frac{Mt}{M\infty} = \frac{6}{r} \times \sqrt{\frac{D}{\pi}} \times t^{1/2} \quad \text{where,}$$

Mt = concentration of extracted proanthocyanidins (g/L) at time =t

M_∞= maximum concentration of extracted proanthocyanidin (g/L)

r = Average radius of grape seeds (mm)

D= Diffusion coefficient (mm^2/h)

t = Time (h)

Grape seeds were accepted as in spherical shape. Thus, the average diameter of grape seeds were determined as 4.18 mm.

The curve of M_t/M_∞ vs $t^{1/2}$ was plotted as seen in Figure F.3.1 and slope of this figure was used in determination of diffusion coefficient (D).

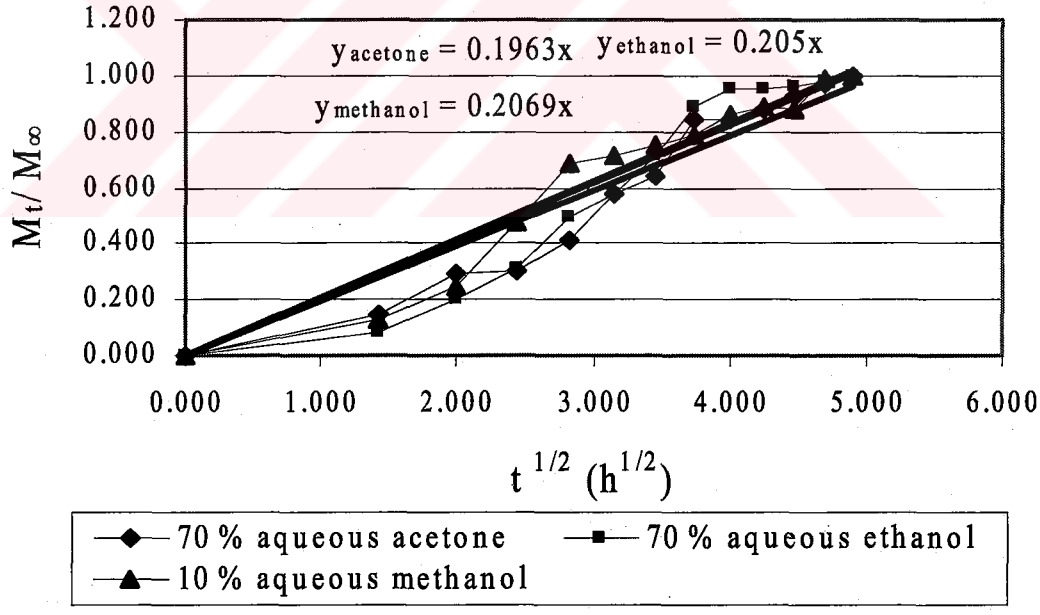

Figure F.3.1. M_t/M_∞ vs $t^{1/2}$ was plotted for 70 % aqueous acetone, 70% aqueous ethanol and 10% aqueous methanol.