ADSORPTION OF OLIVE LEAF ANTIOXIDANTS ON SILK FIBROIN

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by
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ABSTRACT

ADSORPTION OF OLIVE LEAF ANTIOXIDANTS ON SILK FIBROIN

This study focused on producing protein based functional food with antioxidative and antimicrobial properties by adsorption of olive leaf antioxidants on silk fibroin. In accordance with this aim, extraction and adsorption of two most abundant polyphenols in olive leaves, oleuropein and rutin were studied. Furthermore, desorption studies were performed to increase the purity of polyphenols.

In this study, effect of various parameters on extraction and adsorption were investigated by HPLC and antioxidant analyses. The adsorption isotherms of oleuropein and rutin were plotted at different temperatures, pH values and solid-liquid ratios. After adsorption, the adsorbed polyphenols on silk fibroin was subjected to SEM, FTIR, color, antioxidant and antimicrobial analyses to observe the changes in the silk fibroin.

The experimental data of adsorption isotherms were well-fitted to Langmuir model. The maximum adsorption capacity was determined as 108 mg oleuropein/g silk fibroin and 21 mg rutin/g silk fibroin. In desorption process, 81% of rutin and 85 % of oleuropein were removed from the adsorbent surface in 70 % aqueous ethanol solution.

After adsorption of oleuropein and rutin, the antioxidant capacity of silk fibroin increased from 1.93 mmol TEAC/g to 3.61 mmol TEAC/g. Silk fibroin also gained antimicrobial activity against *Staphylococcus aureus* and *Klebsiella pneumoniae* after adsorption of olive leaf antioxidants. Desorbed polyphenols exhibited higher antioxidant capacity than the same amount of olive leaf extract due to the increase in the purity of oleuropein and rutin after desorption.

Consequently, silk fibroin is a promising protein for the production of functional food or dietary supplements and for the purification of oleuropein and rutin.

ÖZET

ZEYTİN YAPRAĞI ANTİOKSİDANLARININ İPEK PROTEİNİ ÜZERİNE ADSORPSİYONU

Bu çalışmada, zeytin yaprağı antioksidanlarının ipek fibroin üzerine adsorpsiyonu ile protein bazlı, antioksidatif ve antimikrobiyel özelliğe sahip olan fonksiyonel gıda üretimi amaçlanmıştır. Zeytin yaprağında en çok bulunan oleuropein ve rutinin ekstraksiyon, adsorpsiyon ve desorpsiyon çalışmaları yapılarak, saflık dereceleri arttırılmaya çalışılmıştır.

Birçok parametrenin zeytin yaprağı antioksidanlarının ekstraksiyonu ve adsorpsiyonu üzerindeki etkileri HPLC ve antioksidan analizleri ile değerlendirilmiştir. Bu çalışmalar sonucunda, oleuropein ve rutinin adsorpsiyon izotermleri değişik sıcaklık, pH ve katı-sıvı oranlarında çizilmiştir. Adsorpsiyondan sonra, ipek fibroin üzerinde tutunan polifenollerin etkisini görmek amacıyla SEM, FTIR, renk, antioksidan ve antimikrobiyel analizleri yapılmıştır.

Deneysel adsorpsiyon verileri Langmuir modeline uyum göstermiştir. İpek fibroinin maksimum adsorpsiyon kapasitesi oleuropein için 108 mg/g, rutin için ise 21 mg/g olarak belirlenmiştir. Desorpsiyon işleminde ise % 70'lik etanol çözeltisi içinde adsorplanan rutinin %81'i, oleuropeinin ise % 85'i ipek proteini üzerinden ayrılmıştır.

Adsorpsiyon işleminden sonra, ipek fibroinin antioksidan kapasitesi 1.93 mmol TEAC/g'dan 3.61 mmol TEAC/g'a artmıştır. Bunun yanında, ipek proteini *Staphylococcus aureus* ve *Klebsiella pneumoniae* bakterilerine karşı antimikrobiyel özellik kazanmıştır. Desorpsiyon edilen polifenoller ise aynı miktardaki zeytin yaprağı özütüne göre daha yüksek antioksidan etki göstermiştir. Oleuropein ve rutinin saflık derecesinin artması desorpsiyon olmuş polifenollerin antioksidan aktivitesini arttırmıştır.

Sonuç olarak, ipek fibroini hem adsorpsiyon işleminden sonra fonksiyonel gıda yada sağlık destekleyici madde olarak kullanılabilecek hem de oleuropein ve rutinin saflaştırılmasında kullanılabilecek potansiyel bir proteindir.

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ABTS	2,2'-azinobis 3-ethylbenzothiazoline-6-sulphonic acid
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
C_{eq}	Equilibrium concentration mg/ml
C_i	Initial concentration mg/ml
E.coli	*Esherichia coli*
FT-IR	Fourier transform infrared
HPLC	High performance liquid chromatography
K	Langmuir constant, mg/ml
K. pneumoniae	*Klebsiella pneumoniae*
MA	Methacrylate
OLE	Olive leaf extract
PVA	Polyvinyl alcohol
PVPP	Polyvinylpolypyrrolidone
P. aeruginosa	*Pseudomonas aeruginosa*
q	Adsorbed amount on adsorbent, mg/g
q_0	Maximum adsorption capacity, mg/g
S. aureus	*Staphylococcus aureus*
SDVB	Styrene-Divinylbenzene Resin
SEM	Scanning Electron Microscopy
SF	Silk fibroin
S/L	Solid-liquid ratio
TEAC	Trolox equivalent antioxidant capacity
θ_i	Surface coverage

CHAPTER 1

INTRODUCTION

Phytochemicals are known as the lifesavers in the last decades since they play a major role in the prevention and therapeutics of the mortal chronic diseases. Clinical studies prove that the health benefits of plant polyphenols is mostly due to their bioactivities such as antioxidative, antimicrobial, antiviral, anti-inflammatory, and anticarcinogenic effects. Because of these properties, plant polyphenols are preferred in the pharmaceutical, food, cosmetics and chemical industry instead of the synthetic compounds. These natural phenolic structures are used as dietary supplements, functional foods, food additives, and natural colorants in the industry.

Plant polyphenols are strong antioxidants and they are capable of quenching and stabilizing the free radicals, which are the life threatening atoms or molecules, formed during the oxidative metabolism in the body and foodstuff. Antioxidants prevent the oxidative damage of free radicals to biomolecules such as DNA, lipids and proteins that cause chronic diseases such as cancer and cardiovascular diseases. Furthermore, plant polyphenols can be used as antimicrobial agents because of their ability to complex with extracellular and soluble proteins and bacterial cell walls to inhibit the bacterial growth.

Olive leaf is one of the potent plant polyphenols having antioxidative, antimicrobial, antiinflammatory and antiviral properties due to its components. The mostly abundant and bioactive components of olive leaf are oleuropein and rutin. In order to use these components effectively in the industry, they should be recovered from the olive leaf.

Solvent extraction is a favorable process to recover the biological substances from solid. It is used for many of the plants, like grape seed, gingko biloba and olive leaf in order to obtain the crude extract of these plants. The further purification of these crude extracts is necessary in order to get rid of undesired structures and to recover the certain high bioactive compounds. For this reason, solid phase extraction, membrane filtration, adsorption and many other separation techniques are used after solvent extraction process.

Adsorption is the most preferred technique among the others because of its low-

cost, applicability to large and small scales and its simplicity. Many scientists have been studying on the adsorption of plant polyphenols on synthetic or natural adsorbents (Xu et al. 2001, Ribeiro et al. 2002, Liao et al. 2003, Scordino et al. 2004). Unfortunately, the use of synthetic adsorbents brings the necessity of desorption process after adsorption which increases the cost of the whole process. If natural and edible adsorbents can be used instead of synthetic adsorbents, the need for desorption will be eliminated and adsorption of two natural compounds will highly benefit to humankind.

Silk fibroin is an edible protein, which has functional aminoacids in its structure, and it is preferred in many biotechnological applications such as drug delivery and tissue engineering. Because of its hydrophobic and bounding characters, it has been also used as adsorbent in adsorption studies. However, to our knowledge, silk fibroin has not been used as an adsorbent for the removal of any polyphenolic structures.

The aim of this study was to achieve the adsorption of olive leaf antioxidants, especially oleuropein and rutin on edible biopolymer, silk fibroin in order to produce protein based functional food with antimicrobial and antioxidative properties. In accordance with this aim, the effects of parameters on adsorption were investigated and the antioxidative and antimicrobial properties of olive leaf antioxidants adsorbed on silk fibroin were determined.

CHAPTER 2

LITERATURE REVIEW

2.1. Free Radicals

Oxidative metabolism is essential for the survival of cells in the body and in foodstuffs whereas the side effect of this dependence is the production of free radicals (Antolovich et al. 2002). A free radical can be defined as a high energy (caused from light, smog, tobacco, radiation, alcohol, polyunsaturated fats, etc.), unstable atom with its extra electron as shown in Figure 2.1.

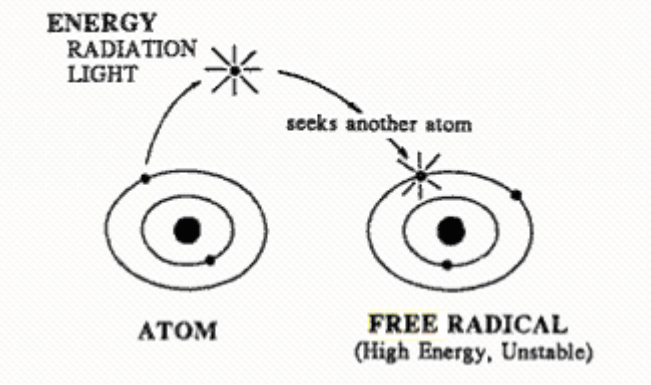


Figure 2.1. Free radical formation (Source: Simone 1992).

Unstable free radicals try to become stable by transferring their high energy to nearby substances. In the body, this high energy is transferred to the body tissues and by this way, excess amount of free radicals, in the body, can cause destructive and lethal cellular effects by oxidizing membrane lipids, cellular proteins, DNA and enzymes, thus shutting down the cellular respiration, schematically shown in Figure 2.2 (Antolovich et al. 2002, Simone 1992).

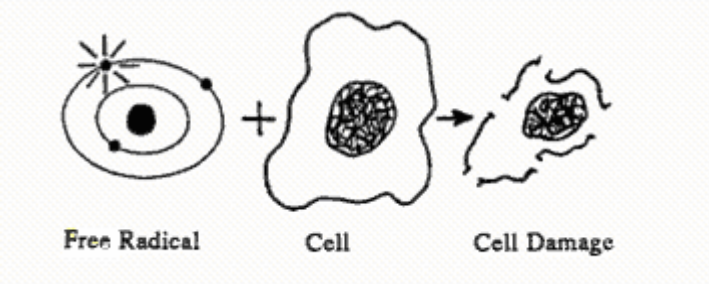


Figure 2.2. Cell damage by free radicals (Source: Simone 1992).

For instance, the interaction of free radicals with molecules of a lipidic nature produces new radicals such as hydroperoxides and different peroxides. This group of radicals (superoxide, hydroxyl and lipoid peroxides) may interact with biological systems in a cytotoxic manner (Garcia et al. 2000).

One of the ways of lipid oxidation is non-enzymatic free-radical mediated chain reaction, which consists of initiation, propagation, branching and termination steps. The process may be initiated by external agents such as heat, light, ionizing radiation or by chemical initiation involving metal ions or metalloproteins.

Initiation:

$$LH + R^{\bullet} \rightarrow L^{\bullet} + RH \tag{2.1}$$

where LH represents the substrate molecule in other words a lipid with $R^{\bullet}$ as the initiating oxidizing radical. The oxidation of the lipid generates a highly reactive allyl radical ($L^{\bullet}$) that can rapidly react with oxygen to form a lipid peroxyl radical ($LOO^{\bullet}$).

Propagation:

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \tag{2.2}$$

$$LOO^{\bullet} + LH \rightarrow L^{\bullet} + LOOH \tag{2.3}$$

The peroxyl radicals are the chain carriers of the reaction that can further oxidize the lipid, producing lipid hydroperoxides (LOOH), which in turn break down to a wide range of compounds, including alcohols, aldehydes, alkyl formates, ketones and hydrocarbons and radicals including the alkoxyl radical ($LO^{\bullet}$).

Branching:

$$LOOH \rightarrow LO^{\bullet} + HO^{\bullet} \quad (2.4)$$

$$2LOOH \rightarrow LOO^{\bullet} + LO^{\bullet} + H_2O \quad (2.5)$$

The breakdown of the hydroperoxides involves transition metal ion catalysis, in reactions analogous to that with hydrogen peroxide. The reaction yields lipid peroxyl and lipid alkoxyl radicals.

$$LOOH + M^{n+} + H^{+} \rightarrow LO^{\bullet} + M^{(n+1)+} + H_2O \quad (2.6)$$

$$LOOH + M^{(n+1)+} + OH^{-} \rightarrow LOO^{\bullet} + M^{n+} + H_2O \quad (2.7)$$

Termination reaction occurs in the case of the combination of radicals forming non-radical products.

Termination:

$$LO^{\bullet} + LO^{\bullet} \rightarrow \quad (2.8)$$

$$LOO^{\bullet} + LOO^{\bullet} \rightarrow non - radical - products \quad (2.9)$$

$$LO^{\bullet} + LOO^{\bullet} \rightarrow \quad (2.10)$$

There are differences between the reactions occuring in vivo and in foods because of the temperature, storage or processing conditions (Antolovich et al. 2002).

All these groups of radicals are the primary cause of certain human diseases involving many organs. For example, when a malignant change occurs in certain segments of DNA, the genetic code can be altered leading to cancer. The following are medical conditions associated with free radical formation:

- Heart and cardiovascular disease
- Cancers: all types
- Lung disease
- Alcohol-related diseases
- Immune system related diseases
- Eye diseases

- Central nervous system diseases
- Radiation injury
- Kidney diseases
- Gastrointestinal diseases
- Skin diseases
- Aging

(Simone 1992).

Moreover, oxidation is the major cause of chemical spoilage in foods. Oxidation results in deterioration of the nutritional quality, color, flavour, texture and safety of foods.

These negative effects of free radicals in the body and in foodstuffs can be defenced by the action of the various antioxidants (Antolovich et al. 2002). Antioxidants are the protective substances, which prevent the harmful effects of oxygen on tissue via free radical formation (Simone 1992). In addition to vitamins and provitamins, many of the plant polyphenols have been found to demonstrate strong antioxidant property (Chu et al. 2002). For example, several plant polyphenols have been reported to inhibit either enzymatic or non-enzymatic lipid peroxidation of free radicals (Kumpulainen and Salonen 1996). For this reason, consumption of fruits and vegetables, especially Mediterranean diet has been associated with the reduced risk of diseases and aging in the last years.

2.2. Plant Polyphenols

"An apple a day keeps the doctor away."

Traditional American rhyme (Cowan 1999)

Traditional rhymes prove that finding healing powers in plants is an ancient idea. Recent epidemiological studies indicate that increased intake of fruits and vegetables reduce the risk of many chronic diseases. Since prevention of these diseases is more effective than the treatment, nutritional factors such as foods and plants are essential for human health (Nicoli et al. 1999, Sun et al. 2002).

The health promoting capacity of plants is due to their phytochemical

content. Phytochemicals especially polyphenols in plants are the major bioactive compounds because of their antioxidative, antimicrobial, antiproliferative, antiviral and antiinflammatory properties (Sun et al. 2002, Chu et al. 2002, Cowan 1999). Polyphenols are secondary plant metabolites and serve as plant defense mechanisms. Plant polyphenols (tannins) are traditionally classified into three groups. These are:

- *Condensed Tannins:* They are essentially oligomeric derivatives of flavon-ols (flavon-3-ols and flavan-3,4-diols or a mixture of two), such as catechin and epicatechin which are a group of flavonoids. The structure of flavonoids is characterized by the diphenylpropane ($C_6C_3C_6$) skeleton. The family includes monomeric flavonoids, flavanones, anthocyanidins, flavones, and flavonols, and these compounds are found in almost every plant and they accumulate in epidermal cells of plant organs such as flowers, leaves, stems, roots, seeds and fruits, being found in glycosidic forms (glycosides) and non-glycosidic forms (aglycones). Consumption of plants and plant products that are rich in flavonoids, such as cocoa, wine, tea, olive leaves, grapes and berries, has been related with protective effects against cardiovascular disease and certain forms of cancer (Papadopoulou et al. 2005, Sakihama et al. 2002, Tang et al. 2003).

- *Hydrolysable Tannins:* They are esters of gallic acid and its derivatives.

- *Complex Tannins:* They have structural characteristics of both of the condensed tannins and hydrolysable tannins (Liao et al. 2003, Tang et al 2003).

Phenolics in plants can be found in soluble free form or bound forms. Bound phenolics are generally in the form of β-glycosides. In the human gastrointestinal system, food can be digested in stomach (acid environment with enzymes), small intestine (mild base environment with enzymes), and colon (neutral pH environment with intestinal microflora). Bound phytochemicals can not be digested by human enzymes and also they can survive from small intestine and reach the colon intact, where they are released to exhibit their bioactivity with health benefits (Sun et al. 2002).

Because of their antioxidative, antimicrobial, anticarcinogenic and antiviral properties, certain plant ploypheno1s are suggested as dietary supplements. For instance, flavonoids, phenols and oleuropeosides all have antioxidant activity towards the free radicals, which is mainly based on the redox properties of their phenolic hydroxyl groups and their chemical structure (Garcia et al. 2000).

2.3. Antioxidative Activity of Plant Polyphenols

Antioxidative properties of polyphenols protect the tissues against free radicals and lipid oxidation involved in several pathological conditions (Hollman and Katan 1999). Antioxidants may be defined as any substance that when present at low concentration compared to those of oxidizible substrates (lipids, proteins, DNA or carbohydrates) significantly delays or prevents oxidation of these substrates (Aehle et al. 2004).

The primary antioxidants, 'AH', when present in trace amounts, may delay or inhibit the initiation step by reacting with a lipid radical or inhibit the propagation step by reacting with peroxyl or alkoxyl radicals.

$$L^{\bullet} + AH \rightarrow LH + A^{\bullet} \quad (2.11)$$

$$LOO^{\bullet} + AH \rightarrow A^{\bullet} + LOOH \quad (2.12)$$

$$LO^{\bullet} + AH \rightarrow A^{\bullet} + LOH \quad (2.13)$$

The antioxidant free radical may interfere with chain-propagation reactions and form peroxy antioxidant compounds.

$$LOO^{\bullet} + A^{\bullet} \rightarrow LOOA \quad (2.14)$$

$$LO^{\bullet} + A^{\bullet} \rightarrow LOA \quad (2.15)$$

The activation energy of the reactions increases with increasing A-H and L-H bond dissociation energy. Thus, the efficiency of the antioxidant increases with decreasing A-H bond strength (Antolovich et al. 2002).

By destroying free radicals and reducing cellular damage, antioxidants can:

- Promote eye health and prevent macular degeneration, cataracts, and other degenerative eye diseases.
- Keep the immune system in good shape, or boost the immune system when it has been compromised.
- Prevent age-related neurodegeneration (decline of the brain and nervous system).
- Prevent DNA damage and therefore have anticarcinogenic effects.
- Have antiatherogenic effects (that are, promote cardiovascular health and help in prevention of artherosclerosis, heart attacks, strokes, and other cardiovascular diseases).
- Antioxidants can inhibit LDL (low-density lipoprotein) oxidation and exhibit remarkable cholesterol-lowering properties (Visioli et al. 2005).

These health benefits of antioxidants make them essential for lives of human and many other mammals. Ascorbic acid (vitamin C), vitamin A, vitamin E, selenium, resveratrol, kaempferol, proanthocyanidins, rutin, quarcetin, luteolin, apigenin, oleuropein, naringenin, catechin, epicatechin are well-known nutritional antioxidants. Furthermore, many antioxidants such as ascorbic acid, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), citric acid, acetic acid, pectin and rosmarinic acid are well-known food preservatives used in the food industry.

Due to the side effects of synthetic antioxidants, plant polyphenols having antioxidative activity are studied to be isolated in recent years. Moreover, clinical studies prove that these plant polyphenols exhibit antimicrobial activity besides their antioxidative activity.

2.4. Antimicrobial Activity of Plant Polyphenols

Although various chemicals have been used for the treatment of infectious diseases since the 17^{th} century, the rapid development in the antimicrobial therapy began in 1935, with the discovery of the sulfonamides by Domagk. The discovery,

development and clinical use of antibiotics during the 20th century have decreased the mortality from bacterial infections. However, since 1980, there has been a decline in the new antimicrobial agents for clinical use and there is an alarming increase in bacterial resistance to existing agents because of the short life span of antibiotics (Brooks et al. 1991). This resistance problem and many other problems made the clinical microbiologists to become interested in the topic of antimicrobial plant extracts.

Finding healing powers in plants is an ancient idea and previous studies on phytochemicals seem to lead the scientists into the arsenal of antimicrobial drugs. There are many driving factors for the renewed interest in plant antimicrobials other than the ineffectiveness of traditional antibiotics. For instance, people are increasingly becoming aware of problems with the overprescription and injudicious use of traditional antibiotics and they are interested in self-medication of the plant compounds (Cowan 1999). Moreover, there is a growing concern about the quality and safety of foods especially, meat which has led to numerous developments in meat preservation. The usage of synthetic preservatives in meat has limited due to their side effects. For this reason, the use of bioactive phytochemicals as natural preservatives to control pathogenic growth has become preferred by customers and industry in order to eliminate microbial contamination in meat (Ahn et al. 2004, Sallam et al. 2004).

An ideal antimicrobial agent exhibits selective toxicity towards to the parasite. The antimicrobial mechanism is not completely understood but it can be placed under four headings:

- Inhibition of cell wall synthesis
- Inhibition of cell membrane function
- Inhibition of protein synthesis
- Inhibition of nucleic acid synthesis (Brooks et al. 1991)

Plant phytochemicals demonstrate different antimicrobial mechanism and activity due to their structure and properties. For instance, flavonoids are known to be synthesized by plants in response to microbial infection so their effect to a wide array of microorganisms in vitro is not surprising.

The antimicrobial activity of flavonoids is probably due to their ability to complex with extracellular and soluble proteins and bacterial cell walls. More lipophilic

flavonoids may also disrupt microbial membranes. Another potent antimicrobial agent in plants is quinone. It is known to complex irreversibly with nucleophilic amino acids in proteins that causes the inactivation of the protein and loss of its function. It generally targets surface-exposed adhesins, cell-wall polypeptides, and membrane bound enzymes in microbial cell (Cowan 1999).

Effective antimicrobial compounds might act on more than one target site on the bacterial membrane, resulting in leakage or autolysis and inhibition of growth or even death of the cell. Because of the absence of an outer membrane in the cell-membrane of gram-positive bacteria, they are more sensitive to antimicrobial agents whereas the lipopolysacharides in the cell membrane of gram negative bacteria provides a barrier to many antimicrobial agents, including some of the polyphenols (Abu-Shanab et al. 2004, Ahn et al. 2004).

Polyphenols of cranberry, onion, grape and rosemary are all known to exhibit antimicrobial activity (Cowan 1999). Olive leaf is one of the potent plants having many flavonoids and oleuropeosides in its structure and there exist many studies due to its antimicrobial and antioxidative properties.

2.5. Olive Leaf and Its Antimicrobial and Antioxidative Properties

In recent years, there is a growing interest obtaining the biologically active compounds from natural sources. The potential danger of some synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been documented and this has stimulated the substitution of synthetic antioxidants by natural ones (Aehle et al. 2006, Bonilla et al. 2006). The olive tree is one of the potential natural antioxidant sources because of its phenolic content (Bonilla et al. 2006, All-Azzawie et al. 2006, Garcia et al. 2000).

Olive leaf has known as a symbol of Mediterranean Region and peace since ancient times (Bonilla et al. 2006, All-Azzawie et al. 2006, Garcia et al. 2000), and it is the first botanical mentioned in the Bible (Lee-Huang et al. 2003) among the thirty botanicals. Historically, olive leaf has been used as a folk remedy for several diseases such as fevers and malaria. Moreover, it has been used to lower the blood pressure in animals, to increase the blood flow in coronary arteries, to relieve arrhythmia, and to prevent intestinal muscle spasms (Garcia et al. 2000). In recent times, clinical research

and experience have shown that olive leaf extract has a wide range of successes against viral, bacterial, fungal, and protozoan infections, HIV-AIDS, flu, cold, meningitis, Epstein-Barr virus, encephalitis, shingles, chronic fatigue, hepatitis B, pneumonia, tuberculosis, gonorrhea, severe diarrhea, blood poisining, ear, dental, urinary tract and surgical infections (Page 2002).

These health benefits of olive leaf are probably due to its rich polyphenolic content. Olive leaf extract from *Olea europea* leaves mainly contain five groups of compounds. These are oleuropeosides (oleuropein and verbascoside); flavones (luteolin-7-glucoside, apigenin-7-glucoside, diosmetin-7-glucoside, luteolin and diosmetin); flavonols (rutin); flavan-3-ols (catechin) and substituted phenols (tyrosol, hydroxytyrosol, vanillin, vanillic acid and caffeic acid) (Garcia et al. 2000). The general structures of some of these compounds are given in Figure 2.3.

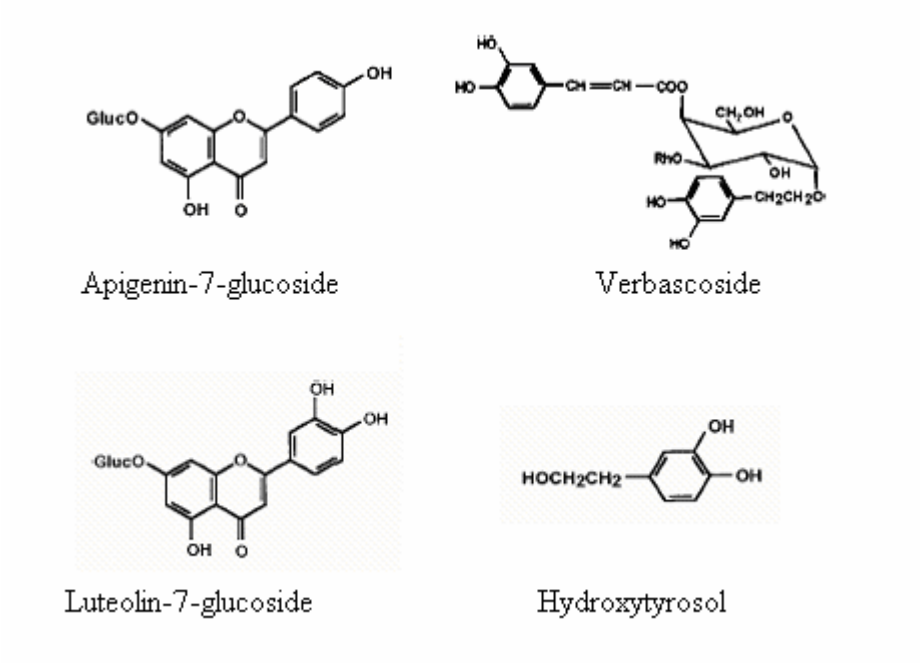


Figure 2.3. Phenolic compounds of olive leaf extract and their chemical structure.

There are main structural groups determining the antioxidative capacity of the flavonoids. These are;

• The *o*-dihydroxy (catechol) structure in the B-ring, which confers greater stability to aroxyl radicals

- The 2,3-double bond conjugated with a 4-oxo function, responsible for electron delocation from the B-ring
- 3- and 5-hydroxyl groups for maximal radical-scavenging capacity.

The main structure, which confers the antioxidant properties to the polyphenols of the olive leaf extract, is the *o*-dihydroxy (catechol) structure in the moities (Garcia et al. 2000).

Garcia et al (2000) reported the sequence of the antioxidant capacity of the flavanoids in olive leaf extract as rutin > catechin ≈ luteolin > OL ≈ hydroxytyrosol > diosmetin > caffeic acid > verbascoside > oleuropein > luteolin -7- glucoside ≈ vanillic acid ≈ diosmetin-7-glucoside > apigenin -7- glucoside > tyrosol > vanillin.

The high antioxidant capacity of rutin, catechin and luteolin show the importance of the flavonoid B-ring catechol structure (rutin, catechin, luteolin); the 3-hydroxyl free or glycosylated group (catechin and rutin); and the 2,3-double bond conjugated with a 4-oxo function (rutin and luteolin). Although the 2,3-double bond conjugated with a 4-oxo function is absent in the catechin, its antioxidant activity is nearly the same with luteolin which confirms the importance of the flavonoid B-ring catechol and free 3-hydroxyl group. The antioxidant activity of oleuropein is mainly due to the hydroxytyrosol moiety in its structure but because of its high molecular weight, its antioxidant capacity is lower than the hydroxytyrosol (Garcia et al. 2000).

Besides its antioxidant capacity, olive leaf extract has shown antimicrobial activities. It is effective against many microorganisms such as *E. coli, Pseudomonas aeruginosa, S. aureus, K. pneumoniae, Trichophyton mentagrophytes, Microsporum canis, T. rubrum* and *Candida albicans* (Markin et al. 2003). Furthermore, olive leaf extract exhibited antiviral activity against viral haemorrhagic septicaemia rhabdovirus (VHSV) (Micol et al. 2005).

Consequently, olive leaf is a potent antioxidant source having antimicrobial, antiviral, antifungal properties and it is reported that its significant effect comes from its major component, oleuropein (Page 2002). The HPLC chromatogram (shown in Figure 2.4) of olive leaf shows that the most abundant flavonoid in olive leaf is oleuropein (Garcia et al. 2000, Savournin et al. 2001, Lee-Huang et al. 2003).

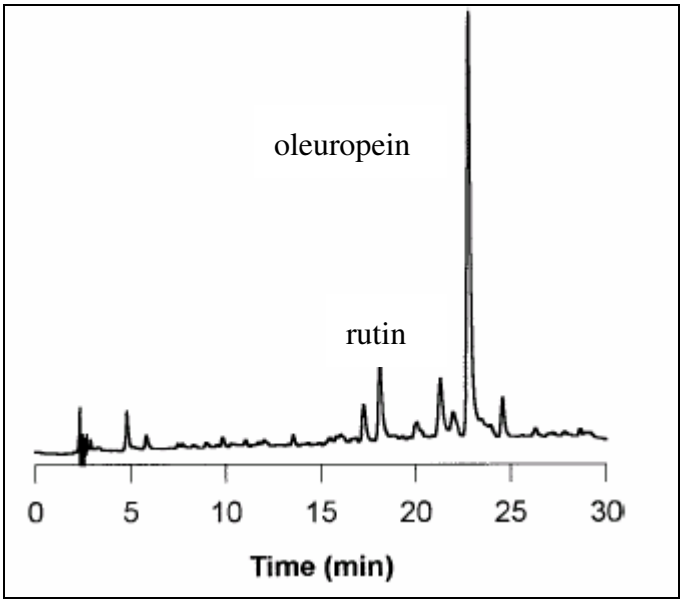


Figure 2.4. HPLC chromatogram of olive leaf extract (Source: Garcia et al. 2000).

As it is definite in Figure 2.4, rutin follows the oleuropein as being the second most abundant component in olive leaf. The percentages of other components present in olive leaf extract are lower compared with these two compounds.

2.5.1. Oleuropein

The principal active component of olive leaf extract, oleuropein, has been intensively studied for its promising effects on human health and its medical potential. Oleuropein is a natural product of the secoiridoid group, shown in Figure 2.5. Oleuropein can produce other bioactive substances, elenolic acid and 3, 4-dihydroxy-phenylethanol (hydroxytyrosol) upon hydrolysis (Al-Azzawie et al. 2006, Bonilla et al. 2006).

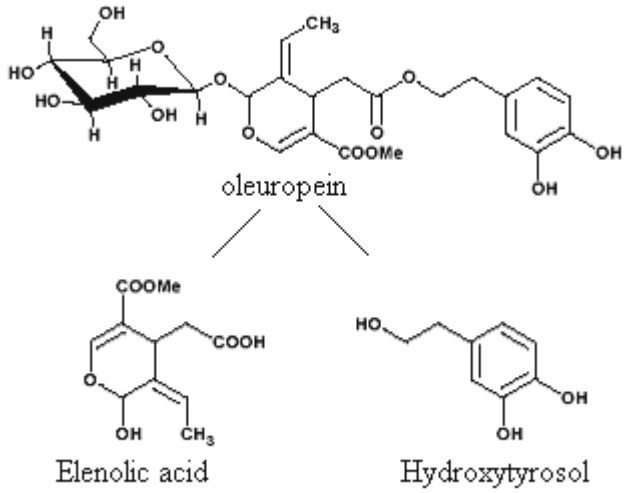


Figure 2.5. Structure of oleuropein and its metabolites elenolic acid and hydroxytyrosol.

As a result of its antioxidative properties, oleuropein has a wide range of health promoting properties, including antiarrhythmic, antiatherogenic, spasmolytic, immune-stimulant, cardioprotective (inhibition of low-density lipoprotein oxidation and platelet aggregation), hypotensive, hypoglycemic, antihypertensive (vasodilatator), antiviral (even against the HIV virus) and anti-inflammatory (by inhibiting the 5-lipoxygenase enzyme) effects, cytostatic (against the McCoy cells), molluscicidal, endocrinal, and an enzyme modulator (Ranalli et al. 2006, Al-Azzawie et al. 2006). Furthermore, oleuropein is converted into elenoic acid in the body, which may prevent viruses and bacteria from replicating.

Oleuropein acts as an antioxidant at both prevention and intervention levels. Oleuropein prevents the free radicals formation by chelating metal ions, such as copper and iron, which catalyze the free radical generation. Another prevention mechanism of oleuropein is due to its inhibitory effect on several inflammatory enzymes like lipoxygenases. In the intervention level, when the free radicals are present, oleuropein directly neutralizes and quenches free radicals by providing hydroxyl groups.

Oleuropein and its metabolite hydroxytyrosol both possess the structural requirement (a catechol group) needed for optimum antioxidant and/or scavenging activity. Both oleuropein and hydroxytyrosol have been shown to be scavengers of superoxide anions, and inhibitors of the respiratory burst of neutrophils and hypochlorous acid-derived radicals. Hydroxytyrosol and oleuropein are also reported to

be effective scavengers of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (Al-Azzawie et al. 2006).

Furthermore, Micol et al. (2005) reported the antiviral activity of oleuropein against viral haemorrhagic septicaemia rhabdovirus (VHSV) which infects continental and sea farmed fish and a wide range of marine species in Europe, Japan and North America. The antiviral effect of oleuropein was possibly due to its interaction with the surface of phospholipid bilayers. Because of its effect on lipid/protein components, it might induce changes on the VHSV envelope, which could interfere the interaction of glycoprotein G with anionic phospholipid domains of membranes, and by this way, it inhibited the viral fusion (Micol et al. 2005).

Consequently, oleuropein has a wild range of bioactivities known and unknown. In order to get benefits from these activities, it should be isolated from the other constituents in olive leaf extract. Since synergistic effect between the polyphenols can occur in a negative way, and olive leaf extract contains many substances other than polyphenols, it is essential to obtain pure oleuropein.

2.5.2. Rutin

Rutin (quercetin-3-O-rutinose) is one of the most bioactive flavonoids and the second abundant component found in olive leaves. This flavonoid is also known as vitamin P and was thought to be an activating factor for vitamin C. It is 3',4',5,7-tetrahydroxyflavone-3β-$_D$-rutinoside and the corresponding chemical structure is given in Figure 2.6.

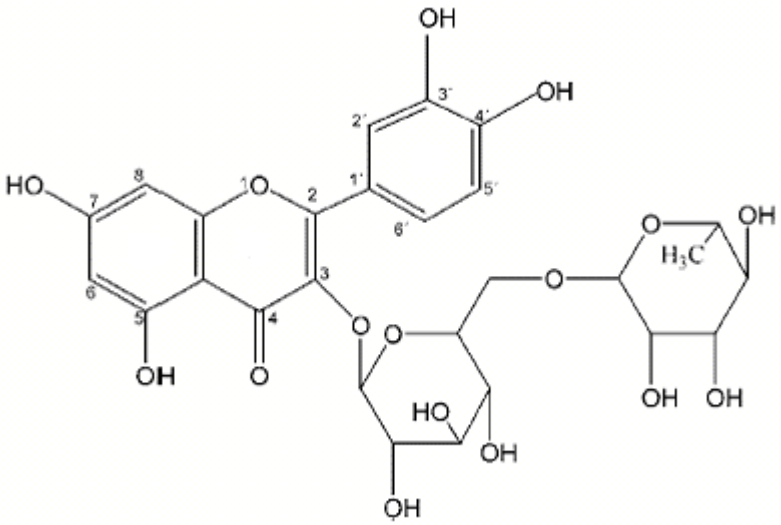


Figure 2.6. Structure of rutin.

Rutin has a broad range of physiological activities such as anti-inflammatory, antitumor, antibacterial properties (Ghica and Brett 2004), antioxidative, antihypertensive, and antihemorrhagic activity, strengthening of the capillaries of blood vessels and the regulation of the capillary permeability. These properties are potentially beneficial in preventing diseases and protecting the stability of the genome (Krusiwa et al. 2003). There exist many studies on the health effects and mechanisms of rutin. For instance, Sheu et al. (2004) investigated the mechanism of rutin in inhibition of platelet aggregation and found that rutin may be an effective agent in treating thromboembolic-related disorders. It can be used as antiplatelet agents such as aspirin and ticlopidine in intravascular thrombosis.

Rutin has found in many of the plants. For example, it was reported that the upper youngest asparagus shoot tissues contained the highest amount of rutin in the shoot and it was 0.03-0.06% of tissue fresh weight (Wang et al. 2003). On average buckwheat leaves, stems, and flowers were reported to contain, about 300, 1,000, and 46,000 ppm of rutin respectively (Kreft et al. 1999).

The absorption of rutin in vivo was also investigated but there are conflicting statements in the literature. It was reported that dietary rutin was recovered in a substantial concentration in rat plasma as two conjugated metabolites. Rutin could be hydrolyzed by the intestinal microflora with R-rhamnosidase and β-glucosidase to isoquercitrin (quercetin3-glucoside) and quercetin. Then appreciable amounts of

quercetin was absorbed, and the absorbed quercetin was excreted into the bile and urine as glucuronide and sulfate conjugates within 48 h. Besides, it was reported that quercetin was further degraded as phenolic acids such as 3-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid by intestinal bacteria and isoquercitrin is hydrolyzed to quercetin during transport across the intestinal membrane (Shimoi et al. 2003).

On the other hand, Song and Wang (2001) reported that rutin was absorbed into the bloodstream in the upper part of the small intestine. Excessive amounts were excreted in the urine. These two conflicting statements demonstrate the sorption of rutin is certain in vivo but its absorption mechanism has not been enlightened yet.

Rutin is another bioactive polyphenol having different properties. Scientists have tried to isolate it since 1940's (Naghski et al. 1947, Krewson and Couch 1947).

Extraction is the main process used to remove polyphenols from plants since it is a low-cost and simple technique. The purity of oleuropein and rutin can also be increased with solvent extraction process.

2.6. Extraction

Many biological, inorganic and organic substances occur in a mixture of different components in a solid. Soluble biological materials are generally found in the cells. In order to seperate these solute constituents or remove an undesirable solute component from the solid phase, the solid is contacted with a liquid phase, which provides the diffusion of the solutes from the solid to the liquid phase. This separation method is called liquid-solid leaching in other words solid-liquid extraction or solvent extraction (Geankoplis 1993).

In the food and biological industries, many products are separated from their natural structure by solid-liquid extraction (Geankoplis 1993). Since ancient times, extraction has been used in its simplest form. According to Chinese legend, emperor Shen-Nung first brewed tea in 2737 B.C. when a few leaves accidentally fall into boiling water. Coffee has been brewed in Arab countries for centuries. The earliest commercial application of extraction was the sugar-beet factory, which was founded in Germany in 1803.

Extraction is less energy-intensive compared with the distillation and evaporation, depending on the choice of solvent and recovery method. Extraction is particularly applicable to food, pharmacological and biochemical products because of its possibility of recovering heat-sensitive materials at low temperatures (Segado 1995).

Solvents used in extraction of the food and biological materials should be;

- Nontoxic
- Have high capacity
- High distribution coefficient
- High selectivity for the solutes
- Low miscibility with the feed
- Easily recoverable
- Have favorable physical properties
- Stable and inert
- Nonflammable
- Nonexplosive
- Environmentally safe
- Inexpensive

Solvent extraction involves at least three components and two or more phases. The extent of the extraction is determined by equilibrium between phases. Equilibrium information is generally reached by phase diagrams and distribution curves (Segado 1995).

2.6.1. Extraction of the Polyphenolic Compounds

The extraction of polyphenols from plants is important for the manufacturing of polyphenol-rich products, which are highly used in the pharmacological, food and cosmetic industry.

Equilibrium state and the mass transfer rate are the two aspects that control the extraction process (Shi et al. 2005). In the solvent extraction of the soluble phenolic compounds from the solid matrix (plant tissue), two stages take place. The dissolution of each polyphenolic compound at the cellular level in the plant material matrix and their diffusion in the external solvent medium (Shi et al. 2005).

Initial Stage: Firstly, the sorption of the solvent in the solid matrix is provided with osmotic forces, capillarity and the solvation of the ions in the cells. In this stage, a certain percentage of the polyphenols damaged in grinding are extracted directly by washing and the soluble components are dissolved.

Diffusion Stage: Diffusion takes place in the solid phase and through the outer layers that surround the particles. The color change of the solvent is observed in this stage (WEB_1, 2004).

In order to save time and energy, the efficiency of the extraction should be improved. Nature of the solvent, pH of the extraction medium, temperature, number of extraction steps, solvent to solid ratio, concentration gradient, particle size, shape and area, diffusion coefficient, viscosity are all the criteria which affect the extraction efficiency (Cacae and Mazza 2002, WEB_1, 2004).

Nature of the solvent: The solvent should remain stable throughout the process and it should not react with the polyphenols. Choosing the right solvent affects the amount and rate of the polyphenols that are extracted. The most widely used solvent is ethanol-water mixtures. Ethanol is a safe solvent since it is the active component of the alcoholic beverages and even it is found in the final extract, it is also safe for human consumption. Another common solvent in the extraction process is hot water. However, hot water can cause denaturation of the polyphenols for this reason, the temperature of the medium and the period of the exposure should be carefully established (Shi et al. 2005, Shrikande et al. 2002).

pH of the extraction medium: The degree of the solubility of compounds change due to the pH of the medium for this reason suitable pH should be determined (WEB_1, 2004).

Viscosity: Lower solvent viscosities increase the rate of extraction since they enhance the diffusion coefficient.

Solvent to solid ratio: As the solvent to solid ratio increases until a threshold value, concentration of polyphenols are diluted and a higher concentration gradient is formed between the inside and surface of the seeds (Shi et al. 2005).

Number of extraction steps: The efficiency of the extraction increases with staged (successive) extractions. This method is important when the first extraction does not provide a suitable amount of desired product. Quantitative yields are obtained when 3-5 sequential extraction are performed (Shi et al. 2005, WEB_1, 2004).

Particle size and shape: Homogenization and grinding enhance the extraction kinetics. Grinding shortens the path that the solvent has to travel and by this way, it enhances the diffusion and reduces the extraction time (Shi et al. 2005, WEB_1, 2004).

Temperature: Increasing the temperature increases the extraction rates since the heat increases the permeability of the cell walls; solubility and diffusion coefficient of the compounds extracted and decreases the viscosity of the solvent. Since too high temperatures denature the polyphenols and make them useless for human consumption, temperatures higher than 25°C are uncommon in the extraction process (Shi et al. 2005, WEB_1, 2004, Gadow et al. 1997).

2.6.2. Why Extracting Polyphenols from Plants?

Oxidative stress has been associated with a variety of pathologic conditions in humans such as cardiovascular dysfunctions, atherosclerosis, inflammation, carcinogenesis, drug toxicity, reperfusion injury and neurodegenerative diseases (Toyokuni et al. 2003). Due to the special health promoting and disease preventing effects of polyphenols, the efficient methods for extracting polyphenols from different plants have being developed (Shi et al, 2003). In the last decades, there is a trend to substitute the currently used synthetic antioxidants with the naturally occurring antioxidants because of their negative health effects. Many plant species have been investigated for their novel antioxidants and some natural antioxidants such as rosemary and sage are already exploited commercially either as antioxidant additives or as

nutritional supplements (Koleva et al. 2002).

The need for natural antioxidants has leaded the researchers to study on the extraction of plant polyphenols. In order to increase the antioxidative, antimicrobial activity of plant extracts, the scientists try to increase the extraction efficiency by changing temperature, pH, solid/liquid ratio, and particle size. Many potent plant extracts' antioxidative, antimicrobial property and polyphenolic content have been investigated for production of the dietary supplements.

For instance, garlic has been used as an herbal medicine for thousands of years because of its health benefits. There are many studies in the literature on the extraction of garlic to find out its antioxidative capacity. Arnault et al. (2003) established an analytical method in order to quantify the compounds in the garlic powder extracts. Nuitila et al. (2003) compared the antioxidant activities of parts of garlic and onion and they concluded with the higher radical scavenging effect of red onion. Especially the skin extracts of onion had the highest activities. Moreover, Benkeblia (2004) suggested the essential oil extracts of garlic and onions to be used as natural antimicrobial additives for incorporating in various food products because of their inhibition effects towards bacteria, *Staphylococcus aureus* and *Salmomella enteritidis*.

Grape seeds and skins are other good sources of phytochemicals since they contain gallic acid, catechin, and epicatechin, which are suitable raw materials for the production of antioxidative dietary supplements. Yılmaz and Toledo (2004) established the HPLC chromatogram and antioxidant capacity of grape seed extracts and they found out the most radical scavenging activity of phenolics present in grape seeds as resveratrol.

Another potent antioxidant, olive leaf extract, was investigated by Garcia et al. (2000) and its constituents were quantified, their antioxidative properties were found. Also olive leaf extracts' anti-viral property was investigated by Micol et al. (2005) and they demonstrated that olive leaf extract and its major compound, oleuropein inhibited the in vitro infectivity of the viral haemorrhagic septicaemia virus (VHSV), a salmonid rhabdovirus. Incubation of virus with olive leaf extract before infection reduced the viral infectivity to 10 %.

There exist many other studies in which the antioxidative property of extracts were studied such as wheat cereals (Baublis et al., 2000), virgin olive oil (Gimeno et al. 2002, Beltran et al. 2005), spinach (Aehle et al., 2004), green tea (Valcic et al. 1999),

ginkgo biloba (Xu et al. 2001, Xu et al. 2000), etc…

Great number of studies on plant extracts was established and still there is an increasing trend towards different kind of plant extracts and their health benefits on the human and animal health. Today, many of these plant extracts such as garlic, grape, olive leaf and ginkgo biloba are commercially used as dietary supplements, shown in Figure 2.7.

Figure 2.7. Commercially used plant extracts (a) Ginkgo biloba extract, (b) Olive leaf extract (c) Grape seed extract. (Source: WEB_2, 2003, WEB_3, 1999, WEB_4, 2005).

Unfortunately, in most of the plant extractions, many other compounds other than polyphenols exist in the plant extract. Generally, a significant amount of carbohydrate ends up in the crude extract. In order to remove this and increase the purity of the extract, many methods are used. One of these methods is the solid phase extraction, which is achieved by packing a column with poly-acrylic resins or non-ionic polystyrene, or other materials such as polyamide or silica gel. As the polyphenol containing solution elutes through this system, the polyphenols adsorb on the system whereas the sugars are eluted. Then the column is washed with a solvent to gather the retained extract in column. The solvent used for washing is evaporated in order to obtain the flavonoids. Then the fractionation is done using the polyamide or cellulose as the stationary phases and a suitable solvent as mobile phase. Then Sephadex LH 20,

which is based on molecular size, and H - bonding interactions, is used to achieve the final purification. However, these techniques are very expensive compared to, for example, paper chromatography. Paper chromatography is a low cost, convenient technique but only milligram quantities can be obtained by running several one-dimensional paper chromatograms (Swinny and Markham 2003).

Consequently, it is meaningless to use high cost separation techniques such as solid phase extraction or supercritical fluid extraction and other chromatographic methods since obtained products from these methods are low-volume.

Membrane filtration systems can be alternative methods for further purification of flavonoids but blocking of the pores decrease the efficiency of the membranes and cleaning of them takes huge time.

Among these separation techniques, adsorption is the best method because of its low-cost and applicability to large or small scales. Furthermore, high quantities of product can be obtained by this system. As a result, adsorption is a promising technique for further purification of polyphenols from crude plant extract.

2.7. Adsorption

Adsorption is a physical and/or chemical process in which one or more components of gas or liquid mixtures or solutions are accumulated at the interface of solid – liquid or solid-gas mixtures. This accumulation provides seperation of the components from one phase to another. The substance which is being removed from the liquid phase to the interface is called as adsorbate and the solid phase is called as adsorbent (Montgomery 1985, Geankoplis, 1993). On the other hand, desorption denotes the converse process of adsorption in which the amount adsorbed decreases (Sing et al. 1985).

There are many application areas of gas and liquid adsorption in industry. Applications of liquid phase adsorption include removal of organic compounds from water or organic solutions, coloured impurities from organics, and various fermentation products from fermentor effluents. Applications of gas-phase adsorption include removal of water from hydrocarbon gasses, sulfur compounds from natural gas, solvents from air and other gasses, and odors from air (Geankoplis, 1993).

In the adsorption from liquid solution, generally two components take place, solvent and solute. The presence of the solvent makes the adsorption from liquid solution more complicated than that from the gas phase. Although the adsorption of the solute is of interest, all the interactions between solute-surface, solvent-surface as well as solute-solvent are involved in the liquid-phase adsorption (Yang 2003). Adsorbate and solvent molecules may compete to adsorb on the surface of the adsorbent as it is schematically illustrated in Figure 2.8. For this reason, suitable solvent should be used in order to accumulate the target adsorbate on the adsorbent.

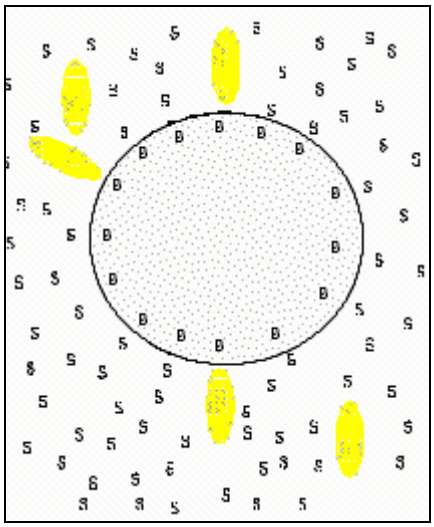


Figure 2.8. Adsorption from solution (B-binding site; S-solvent molecule, yellow elipse-adsorbate molecule).

2.7.1. Mechanisms of Adsorption

Adsorption from solution takes place because of the attractive forces between the adsorbate and adsorbent. The adsorption of the adsorbate on the solid surface can be thermodynamically described. The adsorbate is driven onto the surface to the lower energy state (where it has a lower free energy than in solution). By this way, it keeps with the second law of thermodynamics during equilibration (Montgomery 1985, Dehoff 1993).

Adsorption can be categorized as either physical adsorption (physisorption) or chemical adsorption (chemisorption) depending on the nature of the bonding between adsorbate and adsorbent.

2.7.1.1. Physical Adsorption

Physical adsorption is relatively non-specific, the bonds are much weaker, and hence the process is reversible. Bonds formed in physical adsorption are held by columbic (or electrostatic) forces or hydrophobic bonding. The columbic forces originate in the ionic atoms and polar groups on the surface. The physical interactions among molecules, based on electrostatic forces, include dipole-dipole interactions, dispersion interactions and hydrogen bonding. When there is a net separation of positive and negative charges within a molecule, it is said to have a dipole moment. When two dipoles are near each other, the attraction between these molecules is called *dipole-dipole interaction*. Hydrogen bonding is a special case of dipole-dipole interaction in which the hydrogen atom in a molecule has a partial positive charge and attracts an atom on another molecule, which has a partial negative charge. When two neutral molecules, which have no permanent dipoles, approach each other, a weak polarization is induced because of quantum mechanical interactions between their distributions of charge. This weak attraction is known as the dispersion interaction or the London-van der Waals force.

Nonpolar compounds are adsorbed more strongly to nonpolar adsorbents. This is known as hydrophobic bonding. Longer hydrocarbon chain is more nonpolar, so, degree of this type of adsorption increases with increasing molecular length (Montgomery 1985, Yang 1999).

2.7.1.2. Chemical Adsorption

The attraction between adsorbent and adsorbate is more powerful in chemisorption. Covalent or electrostatic chemical bonds with shorter bond length and higher bond energy occur between atoms. Chemisorption involves electron transfer and is essentially two-dimensional chemical reaction. The bond between adsorbate and surface is specific to particular sites or functional groups on the surface of the adsorbent (Montgomery 1985, Yang 1999).

2.7.2. Adsorption Equilibrium

Practically, maximum capacity of adsorbent cannot be fully utilized because of mass transfer effects involved in the adsorption systems. In order to find out adsorption capacity of adsorbent, it is essential to have information on adsorption equilibrium. When an adsorbent and a certain composition of adsorbate is in contact, adsorption takes place and after a sufficiently long time, the adsorbent and the adsorbate reach equilibrium. The relation between amount adsorbed q, and concentration of the adsorbate C, at constant temperature T is called adsorption isotherm (Suzuki, 1990). Adsorption isotherms are described in many mathematical forms and graphics depending on the interactions.

In the case of adsorption from solution, the isotherms fall into two main types.

Type L: Isotherm is concave to the concentration axis, having a long well-defined plateau which is associated with monolayer adsorption of the solute and minimal competition from the solvent.

Type S: Isotherm is first convex and then concave to the concentration axis. This isotherm is explained by the existence of a different balance between the adsorbate-adsorbent and adsorbate-adsorbate interactions (Rouquerol et al. 1999).

2.7.2.1. Adsorption Isotherms

Langmuir Adsorption Isotherm

The Langmuir adsorption isotherm has found wide applicability in adsorption from solution because of its simplicity and its ability to fit a broad range of experimental data (Suzuki 1990, Montgomery, 1985). Due to the Langmuir Isotherm;

- Energy of adsorption is independent of degree of coverage.
- Bonding is reversible
- Monolayer adsorption takes place (Montgomery, 1985).

The curved shape of Langmuir isotherm as it is shown in Figure 2.9 is because of the competition between the adsorbate molecules for adsorption sites (Janson and Ryden, 1989). The equilibrium concentration in this figure is represented by C_e, the adsorbate amount per unit weight of adsorbent is represented by q.

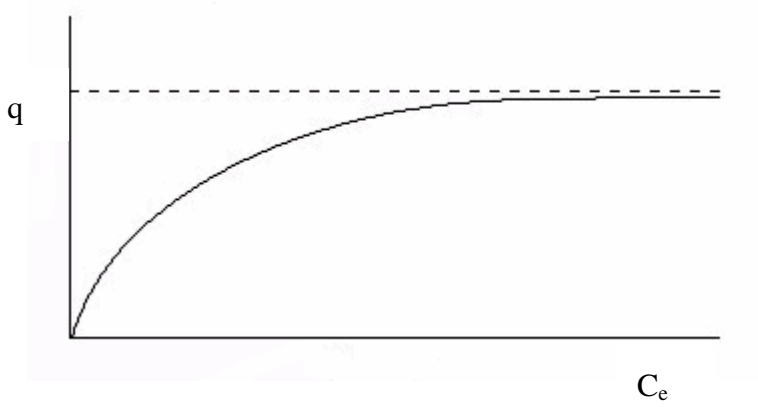


Figure 2.9. Langmuir isotherm.

When surface coverage or fractional filling of the micropore is

$$\theta = \frac{q}{q_0} \tag{2.16}$$

and the concentration in the fluid phase is C, then the equilibrium relation of the adsorption and desorption rates is defined as follows.

$$q = \frac{q_0 c}{K + c} \tag{2.17}$$

Where,

q_0 = Maximum adsorbed amount (adsorbate amount per unit weight of adsorbent)
K= Langmuir Constant (adsorbate amount per unit volume of fluid)

This equation was derived due to the assumptions of Langmuir Isotherm. Mass adsorbed, q is assumed to approach a saturating value, q_0. A plot of 1/q versus 1/c

approximates a line with slope of K/q_0 and intercept of $1/q_0$ (Geankoplis 1993).

Due to the Langmuir model, energy of adsorption is the same for all surface sites and not dependent on degree of coverage. However, in reality, the energy of adsorption varies because real surfaces are heteregenous (Montgomery 1985).

Freundlich Adsorption Isotherm

The Freundlich Adsorption Isotherm accounts the variety of the energy of adsorption on real surfaces. Due to the Freundlich isotherm;

- The frequency of sites associated with a free energy of adsorption decreases exponentially with increasing energy (Montgomery 1985).
- Isotherm equation is empirical (Geankoplis 1993).

The Freundlich Isotherm equation approximates data for many physical adsorption systems and useful for liquids. Figure 2.10 shows the general shape of the Freundlich isotherm. The equilibrium concentration in this figure is represented by C_e, the adsorbate amount per unit weight of adsorbent is represented by q.

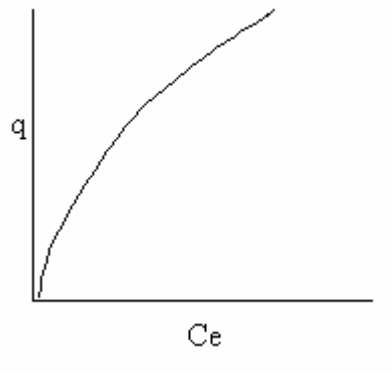


Figure 2.10. Freundlich isotherm.

The Freundlich Isotherm equation is;

$$q = Kc^n \tag{2.18}$$

Where;
K and n are constants. If a log-log plot of q versus c is made, the slope is dimensionless exponent n and the intercept is logK. The units of K depend on the value of n (Geankoplis 1993).

Effect of Parameters on Adsorption Isotherms

Many parameters affect the adsorption and adsorption isotherms like pH, temperature, nature of the solvent, nature of support, ionic strength, particle size, presence of extraneous ions, and solid/liquid ratio. Among these solid/liquid ratio, temperature and pH are the most important factors affecting the adsorption from solution.

pH effect: The pH of the medium strongly affects the adsorption in aqueous solutions. It directly affects the surface charge of the adsorbent. At point of zero charge (PZC), the negatively and positively charged molecular species are present in equal concentration. Zero net charge in proteins is called isoelectric point (pI). Due to their isoelectric point or point of zero charge, molecules behave variously at different pH values. If the medium of the pH is lower than pI, surface of the adsorbent is positively charged and electrostatic interactions begin to occur between the adsorbent and adsorbate. When the medium of the pH is higher than pI, surface of the adsorbent is negatively charged and again the electrostatic interactions take place in the adsorption medium (Agrawal et al. 2004).

Temperature: Temperature can decrease or increase the adsorption capacity of adsorbents. If the adsorbed amount increases with the increasing temperature, then the process is endothermic. On the other hand, if the adsorption decreases with the increasing temperature, then the process is said to be exothermic and the heat of adsorption is negative.

Solid/Liquid Ratio: The adsorption efficiency can increase or decrease with increasing solid/liquid ratio. This is due to the availability of the surface area of the adsorbent during the adsorption (Agrawal et al. 2004).

2.7.3. Adsorption of Polyphenolic Compounds

Polyphenol extracts from the plants are used as food additive, nutritional supplement or herbal medicine because of their therapeutic activities. Although the organic solvents provide the extraction of the metabolites from plants, further purification can be essential in order to obtain concentrated specific components selectively since many other compounds such as sugars, protein or metals may exist in the plant extracts (Yoon et al. 1997, Aehle et al. 2003). These compounds do not contribute to the antioxidant activity and may even act as prooxidants (Aehle et al. 2003). For the selective recovery of target plant metabolites from the crude solvent extracts, adsorption is preferred by many of the researchers, since it is a low-cost separation technique (Yoon et al, 1997). For the further purification of the polyphenols, adsorption and desorption processes can take place respectively. Adsorption process is also used for the removal of undesired compounds from the plant extracts. Moreover, because of the worldwide tendency toward the natural pigments to replace synthetic colorants, anthocyanins, which are responsible for red, blue and purple colors, have received growing importance as European food colorants and again adsorption is the preferred technique to recover these flavonoids especially from citrus processing (Scordino et al. 2004).

There are many studies related with the selective adsorption of polyphenols from different plant extracts by different synthetic and natural adsorbents. These studies also enlighten the interactions between various adsorbent materials and polyphenols. Some of which are illustrated in Table 2.1 and also they are briefly discussed in the following section.

Table 2.1. Some of the adsorption studies of flavonoids and their attributes in the literature.

REFERENCE	ADSORPTION SYSTEMS	ANALYTICAL TECHNIQUE	ATTRIBUTES
Mauro et al. 1999	Hesperidin/Styrene-Divinylbenzene Resin (SDVB)	HPLC	Adsorption and desorption studies
Xu et al. 2000	Ginkgo biloba leaves/Methacrylate (MA)-Divinylbenzene (DVB) beads	HPLC	High adsorption selectivity
Xu et al. 2001	Ginkgo flavonol glycosides and terpene lactones/ Gelatin-Polyvinyl alcohol (PVA)	HPLC	Hydrogen bonding of flavonol glycosides
Ribeiro et al. 2002	Limonin and Naringin/XAD-4, XAD-7, XAD-16	HPLC	Hydrophobic Interactions/Langmuir and Freundlich Isotherm
Liao et al. 2003	Polyphenols/Collagen Fiber	HPLC	Hydrogen bonding and Hydrophobic Interactions
Tang et al. 2003	Polyphenols/Collagen and Cellulose	TLC	Hydrophobic Interactions, Flexibility of gallolyl groups, molecular size
Aehle et al. 2004	Spinach/Four resins and charcoal adsorbents	HPLC DPPH antioxidant analysis	Over 95 % adsorption efficiency of flavonoids
Kim et al. 2004	Narirutin/Amberlite XAD-7	HPLC	Freundlich Isotherm
Scordino et al. 2004	Cyanidin 3-glucoside /13 commercial resins	UV-visible spectrophotometry	Langmuir and Freundlich Isotherms
Takagai et al. 2005	*trans*-Resveratrol/ Cellulose Cotton	V-570 double beam spectrophotometer	Hydrogen bonds/Salting, temperature effect

Yoon et al. 1997 developed a simple purification method in which flavonoid compounds of *Ginkgo Biloba Leaves* from its methanol extract were selectively adsorbed onto a polycarboxyl ester resin (XAD-7). The adsorption process was primarily controlled by hydrophobic interactions by adjusting the solution polarity of methanol extract according to the water content. Desorption process followed the adsorption for further purification of flavonoids (Yoon et al. 1997).

Ribeiro et al. 2002 studied on the removal of limonin and naringin from citrus juices since these flavonone glycosides cause the bitterness of orange juices. Activated diatomaceous earths, granulated activated carbon and synthetic neutral resins (Amberlite XAD-4, XAD-7 and XAD-16) were used as adsorbents. They observed a favorable adsorption of limonin and naringin onto neutral resins (XAD-4 and XAD-7) compared to the other components such as carotenoids and sugars in citrus juices because hydrophobic interactions played a great role for adsorption of limonin and naringin (apolar solutes) from aqueous solution onto neutral adsorbents (Ribeiro et al. 2002).

Furthermore, many biopolymers have been used as adsorbents for the recovery of polyphenols and the interaction between the biopolymers and antioxidative polyphenols have been widely investigated. Tang et al. 2003 studied polyphenol interactions with both cellulose and collagen by using TLC with twenty-four different polyphenolic compounds. For the polyphenol interactions with cellulose and collagen, they concluded with the following results:

- The galloyl group of polyphenols is the functional group
- The strength of interactions increase with the increasing molecular size, the number of galloyl groups and the hydrophobicity of polyphenols
- The hydrophobic interactions are significantly important
- The interactions are strongly dependent on the flexibility of galloyl groups (Tang et al. 2003).

Liao et al. (2003) concluded with the same result as Tang et al. (2003) about their study on collagen fibers. They used the collagen fibers to remove the vegetable hydrolysable tannins since they are useless and harmful components. Removal of hydrolysable tannins from plant extracts is important since they are easily degraded in

the biological system by nonspecific esterases and the hydrolyzed products could lead to liver or kidney toxicity once the level in blood is beyond the detoxification capability of these organs. The collagen fiber selectively adsorbed the hydrolysable tannins that are rich in gallolyl groups (Liao et al. 2003).

Cellulose and collagen are nearly the most common biopolymer used as an adsorbent for purification of plant extracts. For instance, Takagai et al. 2005 achieved to recover 93.8 % of the *trans*-Resveratrol on cellulose cotton by increasing the salt concentration and decreasing the temperature of the solution. *Trans*-Resveratrol is one of the important natural polyphenols found in the grapes and related products. Its importance is due to its anticarcinogenic, antioxidization and antiinflammation effect (Takagai et al. 2005).

Some studies in the literature enlighten the parameters that affect the adsorption of flavonoids and show the fitness of the experimental data to various adsorption isotherms.

Adsorption of cyanidin-3-glucoside on 13 commercial resins with different properties was studied in order to see their adsorption capacities by Scordino et al. (2004). The Food and Drug Administration approved these resins were suitable for food contact use. They had different surface areas, pore radii and different hydrophobicity. The styrene-divinylbenzene EXA-118 resin, having the highest surface area proved to be the most efficient adsorbent through the other tested ones for removing cyanidin 3-glucoside. The acrylic resins showed low efficiency because of their partially hydrophilic nature. The equilibrium experimental data were well fitted to Langmuir and Freundlich isotherms. Furthermore, Scordino et al. (2004) demonstrated that adsorption was not influenced by pH variations of the solution within the range of 1.0-4.5 (Scordino et al. 2004). Xu et al (2001) also investigated the pH effect on the adsorption from aqueous solution. They worked on the separation of ginkgo flavonol glycosides and terpene lactones by using gelatin-PVA adsorbent. They saw different effects of pH on the adsorption of ginkgo flavonol glycosides and terpene lactones. The adsorption affinity for flavanol glycosides became higher whereas it became lower for terpene lactones as the pH increased (Xu et al. 2001).

Adsorption studies also take place in the recovery of flavonoids from the waste materials. In citrus juice processing, an enormous amount of peels is discharged as waste material. Thus, many researchers attempt to find a use for these citrus peels. Kim

et al. (2005) established the recovery process of narirutin from waste water during the washing step of citrus peels in the pectin production and their equilibrium data were well fitted to Freundlich isotherm (Kim et al. 2005).

There are also patents about the isolation of polyphenols by low-cost adsorption techniques. This invention was on the adsorption of the cocoa extract on polyvinylpolypyrrolidone (PVPP). PVPP selectively adsorbed catechin and epicatechin from the cocoa extract. The adsorption was more efficient at a temperature below 30°C whereas desorption was more successful at a temperature above 30°C (Hoving et al. 2003).

All these studies and others in the literature prove that adsorption is a powerful technique in order to remove or recover the polyphenols. Because of its low cost and regeneration possibility, it is also a suitable technique for industrial applications. All these studies in the literature enlighten the main adsorption mechanism of polyphenolic structures. Because of their hydrophobic character, the adsorption efficiency is higher when non-polar adsorbents are used. Hydrophobic force is one of the occurred attractive forces in the physisorption. Due to the estimation of Ribeiro et al. (2002), selective adsorption of limonin and naringin was a physisorption process because the free energy of adsorption was lower than -13.3 kJ/molK (Ribeiro et al. 2002).

The experimental data of these studies generally well fitted to Langmuir, Freundlich Isotherms since adsorption takes place in solution, and these isotherms find wide applicability in adsorption from solution studies.

Unfortunately, these studies do not give any clear information about the pH, temperature and solid/liquid ratio effect on polyphenol adsorption. In all these studies, desorption behavior of the compounds were studied after adsorption since it is aimed to increase the purity of the polyphenols.

Generally, commercial adsorbents were tried on the removal of flavonoids from citrus peels or juices compared to the natural ones. There is an increasing trend to purify many other polyphenols from plants, which have antioxidative properties. One of these plants is olive leaf, which have antimicrobial, antioxidative and anti-inflammatory effects as discussed in this study. Because of their promising health effects, oleuropein and rutin are the two essential components of the olive leaf. They can be removed from crude olive leaf extract by adsorption techniques. If an edible and medicinal adsorbent such as silk fibroin can be used instead of these commercial resins in the adsorption of

oleuropein and rutin, then there will be no need for desorption step and by this way silk fibroin will gain antioxidative, antimicrobial and anti-inflammatory properties for further use in industry, as functional food or dietary supplement.

2.8. Silk Fibroin

Silkworm silk produced by *Bombyx mori* has been used as textile-grade fibers for at least 2000 years and recently extensive research related to silk has been performed. Silk usually consists of a fibrous protein, fibroin, and a coating protein, sericin (Saitoh et al. 2004). Silk fibroin is a protein polymer (Lee et al. 2003) which consists of highly repetitive regions. These can be represented by the repetitions of six amino acid residues, Gly-Ala-Gly-Ala-Gly-Ser (Saitoh et al. 2004). The highly repetitive primary sequence in silk fibroin leads to significant homogenity in secondary structure, β-sheets, shown in Figure 2.11. The strong hydrogen bonding and the hydrophobic nature of the silk fibers provides its environmental stability and its insolubility in most of the solvents (Altman et al. 2003). Only very concentrated aqueous salt solutions such as lithium bromide (LiBr), lithium thiocyanate LiSCN, sodium thiocyanate (NaSCN), calcium thiocyanate ($Ca(SCN)_2$), calcium chloride ($CaCl_2$) and some copper salts can dissolve the silk fibers (Tsukada et al. 1994, Xu et al. 2005).

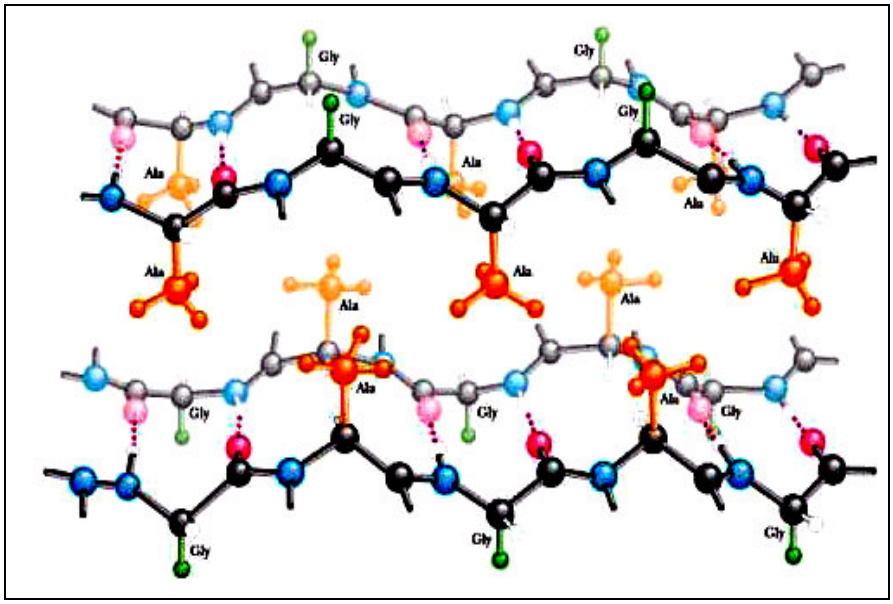


Figure 2.11. Structure of silk fibroin (WEB_5, 2006).

These fibrous proteins exhibit important mechanical properties and have better environmental stability compared with the globular proteins. For this reason, silks are suggested to be used in the fields of controlled drug release, biomaterials, scaffolds (Altman et al. 2003), enzyme immobilization matrix as being a good biosensor, wound dressing artificial skin (Park et al. 1999) and catalyst applications. Furthermore, silk has been commercially used in cosmetics and also it is suggested as a pharmacological agent and as a food additive because of its amino acid content and health benefits. These aminoacids have different functions to the body. For instance, glycine reduces the cholesterol levels, cures high blood pressure and is important for a having strong immune system. Alanine is important for muscle tissue, the brain and central nerve system, strengthens the immune system by producing antibodies and protects the liver. Serine reduces the cholesterol levels as glycine and it strengthens the immune system by producing the antibodies (Luo et al. 2003).

2.8.1. Silk Fibroin as an Adsorbent

Silk fibroin is an insoluble protein and has a well-defined composition, for this reason; it is preferred in many adsorption studies since 1940's. The scientists tried to enlighten the bounding mechanisms of silk fibroin and its adsorption capacity towards various compounds. For example, Coulombre and Moore (1949) investigated adsorption of sulfanilamide on silk fibroin in unbuffered solutions. They observed a decrease in the adsorption capacity of silk fibroin when pH was changed from 6 to 5 and also they investigated the adsorption behavior at various temperatures in order to calculate heat of adsorption. These experiments proved that the bounding mechanism of sulfanilamide on silk fibroin was due to the probable bounding of the basic side chains of the protein with the sulfanilamide anion and the large van der Waals forces between the benzene ring of tyrosine and that of sulfanilamide (Coulombre and Moore, 1949).

In 1945, Pauling (1945) investigated the adsorption of water on many proteins, including silk. The results showed that there was an encouraging agreement between the number of molecules of water initially adsorbed by the proteins and the number of polar groups that exist in the proteins. The agreement was especially satisfactory for silk since its amino-acid composition was reliable. Silk had the minimum adsorption capacity for water among the other proteins since it possesses the minimum number of polar groups

(moles/10^5 gr) compared with the other proteins such as gelatin, collagen, serum albumin, zein, overalbumin, wool, salmin and β-lactoglobulin (Pauling 1945).

In another study, the thermodynamics of adsorption of laccaic acid on silk was investigated and it was noted that the adsorption was an exothermic process. As the temperature increased, the adsorption capacity of silk yarn was decreased and the heat of adsorption was calculated as -13.2 kcal/mol. The experimental data of this study were well fitted to Langmuir Isotherm (Kongkachuichay et al. 2002).

On the other hand, there are many other researches in which the silk fibroin was used as an adsorbate because of its hydrophobic character, coating technology is an example for this (Wang et al., 2005).

Consequently, silk fibroin has been used as adsorbent or adsorbate in many of the studies because of its bounding mechanisms such as its hydrophobic character, insolubility in water, polar groups and basic side chains. Because of its promising health effects and bounding mechanisms, silk fibroin can be considered as a potent adsorbent for the isolation of antioxidants from the crude extracts. Silk fibroin is a more suitable adsorbent for the recovery of hydrophobic polyphenols compared to the other biopolymers since it has less polar groups and more hydrophobic than others. Furthermore, because of its edible and medicinal properties, it is a beneficial material for human health.

2.9. Functional Food

Functional foods are food products that provide health benefits by their nutritional quality. The general types of functional foods are:

Fortified products: The simplest types of functional foods are the fortified products. The nutrient contents of these products are increased with additional nutrients such as addition of Vitamin C to various fruit juices.

Enriched products: In this type of functional foods, new nutrients or components are added in a particular food. For instance, addition of prebiotics to food, which are typically fermentable dietary fibres that provide a gastrointestinal environment in which beneficial bacteria can thrive.

Altered products: These kinds of products replace existing components with beneficial components. A good example of this is the use of high fiber fat replacers produced from grain products.

Enhanced commodities: An exciting class of functional foods is enhanced commodities. Plant breeders can develop amazing varieties of products that have potentially important benefits. Examples include high lysine corn, fruits and vegetables with enhanced content of vitamins, and overproduction of phytonutrients in a variety of fruits and vegetables such as carotenoid containing potatoes (Spence, 2006).

Functional foods provide tremendous health benefits such as the dietary supplements. Fruits and vegetables are good dietary sources of natural antioxidants for dietary prevention of degenerative diseases. The main contribution to the antioxidant capacity of a fruit or vegetable is likely to come from their phytochemicals such as phenolics, thiols, carotenoids and tocopherols, which may protect us against chronic diseases. Therefore, increased consumption of fruits and vegetables has been recommended. An increasingly growing market for nutraceuticals and functional foods has triggered the study on natural sources of antioxidants and their potential for nutraceutical and functional foods (Shui and Leong, 2006).

The high amino acid content and the health benefits of silk fibroin are wildly known due to the previous studies. Therefore, silk fibroin can be suggested as a functional food or dietary supplement after adsorption of olive leaf antioxidants on its structure. Because of its high amino acid content, it may become an enriched product after addition of antioxidants to its structure since its nutritional quality increases with the antioxidative, antimicrobial and the antiviral properties of olive leaf constituents.

CHAPTER 3

OBJECTIVES

The aim of this study was producing protein based functional food with antioxidative, antimicrobial and possibly anticarcinogenic properties by the adsorption of olive leaf antioxidants on silk fibroin. In accordance with this aim, the adsorption of two most abundant antioxidants in olive leaf, oleuropein and rutin on silk fibroin was studied.

The goals of this study can be summarized as follows:

- To study the effects of solvent and temperature on extraction of the olive leaf antioxidants
- To qualify and quantify oleuropein and rutin in olive leaves
- To investigate the adsorption of oleuropein and rutin on silk fibroin
  - To understand the biopolymer-polyphenol interactions by changing the solvent of adsorption medium
  - To study the adsorption kinetics and adsorption isotherms
  - To study effects of temperature, pH, and solid-liquid ratio on adsorption capacity
  - To characterize silk fibroin before and after adsorption
- To get insight into the antioxidative and antimicrobial properties of silk fibroin, olive leaf and adsorbed polyphenols on silk fibroin
- To study desorption of olive leaf polyphenols from silk fibroin.

CHAPTER 4

EXPERIMENTAL STUDY

4.1. Materials

Olive leaves used in all experiments were collected from the olive trees grown in the campus of Izmir Institute of Technology in Izmir in Turkey. Oleuropein and coumarin (internal standard) were obtained from Extrasynthese (Genay, France) whereas rutin (98.5 + %) was obtained from Merck Co. (Darmstadt, Germany). High performance liquid chromatography (HPLC) grade ethanol (for extraction) from Riedel-de Haën (Seelze, Germany), HPLC grade acetonitrile (mobile phase for HPLC) from Sigma-Aldrich Chemie (Steinheim, Germany) and HPLC grade acetic acid (mobile phase for HPLC) from Merck Co. (Dermstadt, Germany) were used in this study. Citric acid monohydrate (for phosphate buffer) was supplied from Sigma-Aldrich Chemie (Steinheim, Germany) and di-sodium hydrogen phosphate (99 + %) (for phosphate buffer) was supplied from Merck Co. (Darmstadt, Germany).

ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) from Sigma (Steinheim, Germany), Trolox (6-hydroxy-2,5,7,8,-tetramethylchroman-2-carboxylic acid) and potassium persulfate ($K_2O_8H_8$) from Fluka (Steinheim, Germany) were used for antioxidant analysis.

Hydrophobic silk fibroin from Silk Biochemical Co., LTD. (China) was used as adsorbent to determine the adsorption behavior of olive leaf antioxidants on it.

4.2. Methods

The methods included in this study can be summarized in three parts. The first part is the pretreatment and extraction of olive leaf antioxidants. This part includes the identification of olive leaf phenolic compounds. Moreover, solvent and temperature effects on the performance of extraction process were investigated by using chromatographic and antioxidant analyses. The second part is the adsorption of olive

leaf antioxidants on silk fibroin. In this part, the characterization of olive leaf antioxidants and the characterization of silk fibroin before and after adsorption were carried out. Moreover, antimicrobial and antioxidative properties of the samples were determined. The last part is desorption of antioxidants from silk fibroin. The followed experimental procedure in this study is schematically represented in Figure 4.1.

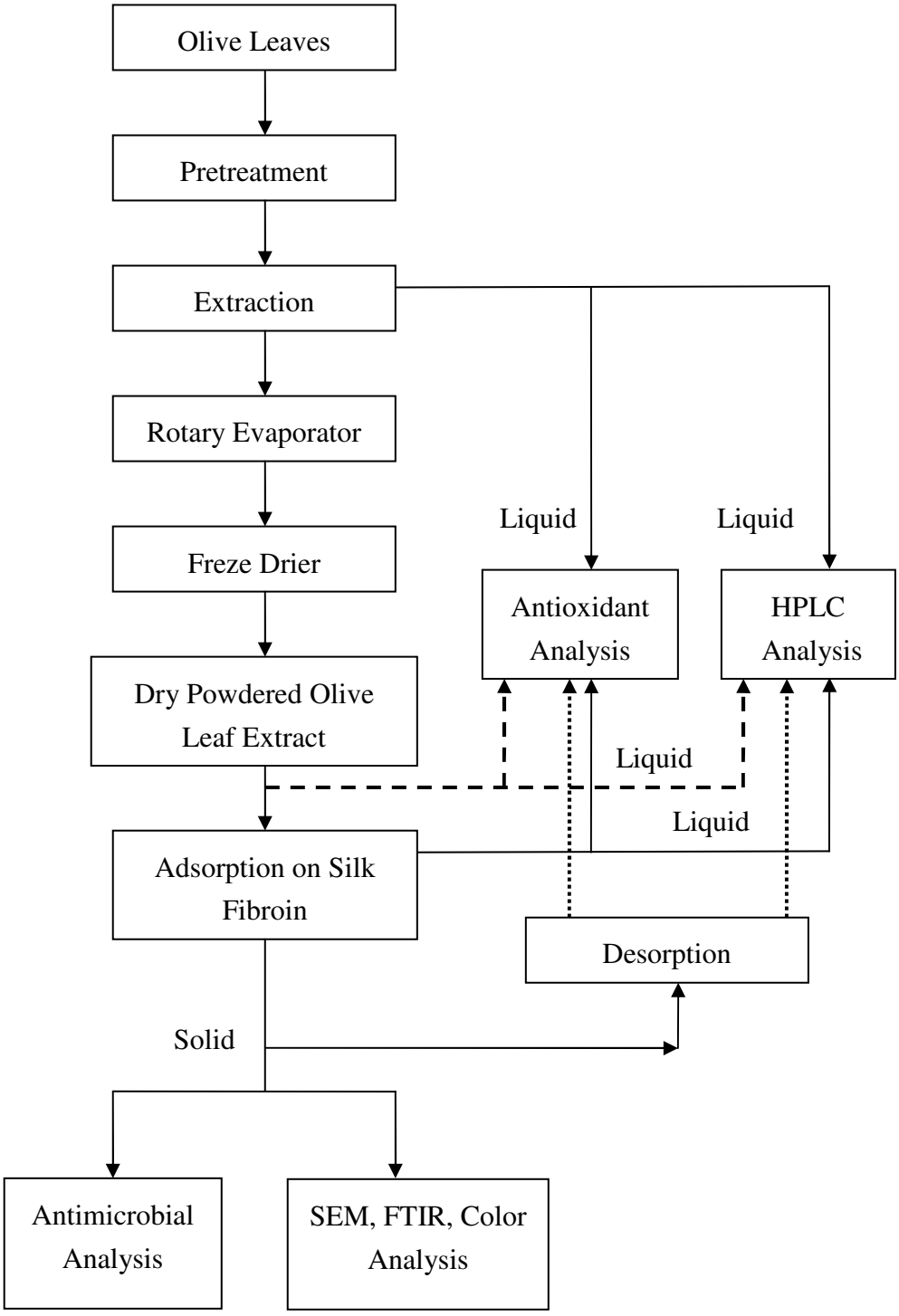


Figure 4.1. Schematic representation of experimental procedure.

4.2.1. Pretreatment and Extraction of Olive Leaves

4.2.1.1. Pretreatment of Olive Leaves

Collected olive leaves were washed with deionized water and then they were dried at 37 °C in Memmert-800 oven for three consecutive days. The drying temperature was chosen as 37 °C since there is a degradation risk of antioxidants over 40 °C. The moisture content of the leaves each day was controlled by a moisture analyzer Sartorius – MA 100 until a constant value was obtained. Then the dried leaves were pulverized in a blender in order to decrease the particle size of the leaves. The size of the used leaves was approximately 90 – 150 µm. During this process, the frictional heating of the leaf powder was prevented by stopping the blender at each 5 minute. Then the olive leaf powder was stored in light protected glass bottles for further use.

4.2.1.2. Extraction of Olive Leaf Polyphenols

Olive leaves were extracted after the pretreatment process. Due to their nontoxic nature and low-cost, water and aqueous ethanol solution were preferred as extracting solvent. Extraction was carried out in an orbital shaking incubator (Comecta Thermoshaker) at 250 rpm for 2 hours since 2 hours is adequate for this system to reach at equilibrium. The solid/liquid ratio was selected as 1/20 for all trials due to the previous studies.

The procedure was investigated at five different conditions in order to find out the most suitable one for the extraction of olive leaf antioxidants. 70 %, 50 % and 20 % ethanol-water mixtures at 25 °C and water at 40 °C and 70 °C were used as the extracting solvent. Then the mixture was centrifuged in Beckman Coulter Centrifuge at 5,000 rpm for 15 minutes in order to separate the solid particles from the extraction medium.

After the optimum extraction condition was decided by the HPLC analysis and antioxidant analysis, this extraction condition was used during this study. In order to obtain dried olive leaf extract at this optimum condition, the solvent of the extracted medium was removed by Heidolph rotary evaporator at 38 °C with a 120 rpm rotation under vacuum. The temperature was carefully adjusted to 38 °C since the antioxidative

property of olive leaf polyphenols decrease above 40 °C. Then, the solvent free olive leaf extract was dried using a Telstar Cryodos freeze drier system at -52 °C and below 0.2 mbar. After these steps the HPLC, antioxidant and antimicrobial analyses of olive leaf extract were made and then it was stored in light protected glasses until further use in adsorption studies.

4.2.1.3. HPLC Analysis and Identification of Phenolic Compounds

In order to quantify and qualify the phenolics in the olive leaf extract, high performance liquid chromatography (HPLC) analysis of the samples was done at each step. Firstly, the samples were filtered through 0.45 µm membrane before injection to HPLC (1100 Agilent Series). The operating conditions and properties of HPLC are given in Table 4.1.

Table 4.1. Operating conditions and properties of High Performance Liquid Chromatography

Property	Values or Attributes
Column	C18 LiChrospher 100 analytical column
Column Length	250 mm
Column Diameter	4 mm
Particle Size	5 µm
Mobile Phase	Mobile Phase A: 2.5 % acetic acid in deionized water Mobile Phase B: 100 % acetonitrile
Flow Rate	1 ml/min
Temperature	30 °C
Detector	Diode Array Detector
Absorbance	280 nm.

A linear gradient elution system was adopted with the mobile phases, given in the Table 4.2.

Table 4.2. HPLC elution program .

Time (min)	Mobile Phase A (2.5% acetic acid)	Mobile Phase B (100 % Acetonitrile)
0	95 %	5 %
20	75 %	25 %
40	50 %	50 %
50	20 %	80 %
60	5 %	95 %

The two most abundant phenolic compounds in olive leaf extract, oleuropein and rutin were identified by comparing their retention times with the corresponding standards. Also calibration curves were drawn for these two compounds by using internal standard and external standard methods. Coumarin was used in internal standard method. The calibration curves of these two compounds provided the quantification of these materials at each experiment.

4.2.1.4. Antioxidant Analysis

A number of assays have been introduced for the measurement of the total antioxidant activity of body fluids, food extracts, and pure compounds (Re et al. 1999). In this study, the used method was based on the ability of olive leaf antioxidants to scavenge the $ABTS^{\cdot+}$ (ABTS radical cation) compared with a standard antioxidant (Trolox) in a dose-response curve.

7 mM aqueous ABTS solution was mixed with 2.45 mM potassium persulfate solution to form $ABTS^{\cdot+}$. The mixture standed in the dark at ambient temperature for 12-16 hours in order to complete the reaction. While studying phenolic compounds, the $ABTS^{\cdot+}$ solution was diluted with ethanol to an absorbance of 0.7 (±0.02) at 734 nm and equilibrated at 30 °C. On the other hand, stock solutions of phenolics in ethanol were prepared as they produced inhibition between 20 % - 80 %.

20 µl of phenolic compounds was added to 2 ml of diluted $ABTS^{\cdot+}$ solution and the absorbance was taken at each 1 minute during 6 minutes at UV-Visible Spectrophotometer (Perkin Elmer-Lambda 25). All samples were analyzed at least three

times at different concentrations. The percentage inhibition of absorbance at 734 nm was calculated and the trolox equivalent antioxidant capacity (TEAC) value was determined (Re et al. 1999).

4.2.2. Adsorption of Olive Leaf Antioxidants

Adsorption procedure followed the extraction procedure of olive leaf antioxidants. After the crude olive leaf extract was obtained at the optimum condition, it was dissolved in proper solvent and mixed with silk fibroin in a batch system for the adsorption process. Adsorption process was performed in the thermoshaker at 250 rpm during 30 hours. Several parameters were investigated in the adsorption procedure.

Firstly, the solvent effect on adsorption was investigated by using 70 % ethanol-water solution and water in order to understand the adsorption mechanism of the system.

Secondly, different forms of silk fibroin were tried in adsorption. Silk fibroin in the structure of foam, fiber and powder were used to demonstrate which has the highest adsorption capacity.

Then the adsorption kinetics of this system was followed during 30 hours at 25°C when the solid/liquid ratio was 1/20. After gaining knowledge about its kinetics, adsorption behavior of the system at different concentrations was investigated to plot the adsorption isotherms.

The pH effect on the system was investigated at four different pH. The pH of the solution was adjusted by using citric acid monohydrate buffer. It was adjusted to 2.4, 4, and 7.6. Also water at pH 6 was used to see its effect on adsorption isotherms. At the optimum pH, the experiment was run at three different temperatures 25 °C, 30 °C and 35 °C to show the effect of temperature on the adsorption isotherms. Then at the optimum pH and temperature, solid liquid ratio was changed from 0.025 to 0.1 to demonstrate its effect on the adsorption.

After adsorption, the samples were centrifuged in Hettich Rotina Centrifuge at 8,000 rpm for 15 minutes. Then the liquid and solid were separated. The supernatant of the samples before and after adsorption were analyzed at HPLC after centrifugation. The results of HPLC provided to plot the adsorption isotherms of oleuropein and rutin at different initial concentrations and at different conditions.

4.2.2.1. Silk Fibroin Characterization Before and After Adsorption

In order to see the difference between before and after adsorption, silk fibroin was characterized by instrumental analyses techniques.

Scanning Electron Microscopy (SEM) (Philips XL 30S FEG) analyses were performed to show the morphological changes on silk fibroin before and after adsorption.

Fourier Transform Infrared Spectroscopy (FTIR) analyses was carried out in the region of 500-7000 cm^{-1} using an FTIR spectrophotometer (Digilab FTS 3000 Mx).

The color of the silk fibroin changed from white to dark yellow and to brown during the adsorption. As the initial concentration increased, the adsorbed quantity was increased and the color of the silk fibroin became darker. After adsorption, the silk fibroin's color at each concentration was measured by Color Measurement Spectrophotometer (Avantes Avamouse) in order to see the change in its color and effect of adsorption on the color of silk fibroin.

4.2.2.2. Antioxidant Analysis of Adsorbed Polyphenols on Silk Fibroin

It was a question mark whether or not the antioxidative property of flavonoids on silk fibroin was effective after adsorption. For this reason, the solid phase of the system, silk fibroin and the adsorbed olive leaf antioxidants were dissolved in Ajisawa's reagent ($CaCl_2$/ethanol/water, 111/92/144 in weight) since it is impossible to dissolve silk fibroin in other solvents. Then the antioxidant capacity of this adsorbent – adsorbate mixture was measured by UV-Visible Spectrophotometer. Also the antioxidant capacity of silk fibroin, Ajisawa solution and olive leaf extract were measured just for control.

4.2.2.3. Antimicrobial Tests

Antimicrobial tests were done in order to see the effect of olive leaf extract against the growth of certain bacteria. Four samples in the form of pellets with 14 mm diameter were pressed in Hydraulic Press Machine. These four pellets are demonstrated in Figure 4.2.

(a) (b) (c) (d)

Figure 4.2. The image of the pellets.

(a) 0.15 gr olive leaf extract

(b) 0.15 gr physically mixed silk fibroin and olive leaf extract in the ratio of 2 to 1

(c) 0.15 gr silk fibroin

(d) 0.15 gr the adsorbed polyphenols on silk fibroin

The antibacterial action of these four samples against *Esherichia coli, Staphylococcus aureus, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were determined by disk diffusion method. Bacterial cultures were grown on Mueller–Hinton agar medium at 37 °C for overnight. One of the colonies was picked up and dissolved in 1ml of phosphate buffer solution (PBS); the turbidity was adjusted to McFarland no. 1 (10^9 CFU). After placing a sterile cotton swab in the bacterial suspension, the swab was streaked in three directions over the surface of the Mueller–Hinton agar to obtain uniform growth. Four different pellets were placed into the plates of each bacterium. The plates were incubated at 37 °C for one day. Besides this, three commercial antibiotics, Penicillin, Vancomycin and Gentamicin pellets in 6 mm diameter were placed in each plates of the bacterium for positive control. Then the width of inhibition zone of each sample and commercial antibiotics in the plates was measured.

4.2.3. Desorption of Olive Leaf Antioxidants

The purification of polyphenolic compounds is an important downstream process, which has high costs. After adsorption, the dried remaining solid phase, silk fibroin and olive leaf constituents were mixed with 70 % ethanol to perform the desorption. The desorption process was performed in the thermoshaker at 250 rpm and

25 °C during 4 days. Then the mixture was centrifuged to separate the liquid phase from the solid phase. The supernatant was analyzed in HPLC to determine the desorption and purification efficiency of this system. Furthermore, the antioxidant capacity of this sample was measured to see whether or not the olive leaf flavonoids still had the antioxidant activity after adsorption.

CHAPTER 5

RESULTS AND DISCUSSION

5.1. Pretreatment and Extraction of Olive Leaves

In order to obtain a highly bioactive plant extract, extraction of olive leaves were carried out at different conditions. Aqueous ethanol solutions and water were used as the extracting media due to their favorable properties such as nontoxic nature and safety. 70 %, 50 % and 20 % ethanol-water mixtures at 25 °C and water at 40 °C and 70 °C were used as the extracting media. The High Performance Liquid Chromatography (HPLC) and antioxidant analyses were performed to find out the suitable extracting medium to obtain a highly bioactive olive leaf extract.

5.1.1. HPLC Analysis and Identification of Phenolic Compounds

Oleuropein and rutin are the two major compounds of olive leaf extract and the efficiency of the extraction is highly dependent on the amount of these two polyphenols since they increase the bioactivity of olive leaf extract. For this reason, oleuropein and rutin in the olive leaf extract were firstly qualified by comparing their retention times with the corresponding standards and then they were quantified by external and internal calibration methods. These calibration methods are explained in Appendix A and the calibration curves for oleuropein (Figures B1 and B2) and rutin (Figures B3 and B4) are given in Appendix B.

The HPLC chromatogram (Figure 5.1) of olive leaf demonstrates that the amount of other polyphenols is not as much as the oleuropein and rutin.

Furthermore, this chromatogram shows that the retention time of oleuropein and rutin is 17.296 and 22.403 min., respectively. Elution time of polyphenols is directly proportional to their polarity in reversed phase columns. The column used in this study was nonpolar and the mobile phase was polar so the high polarity solutes eluted first, which means rutin is more polar than oleuropein.

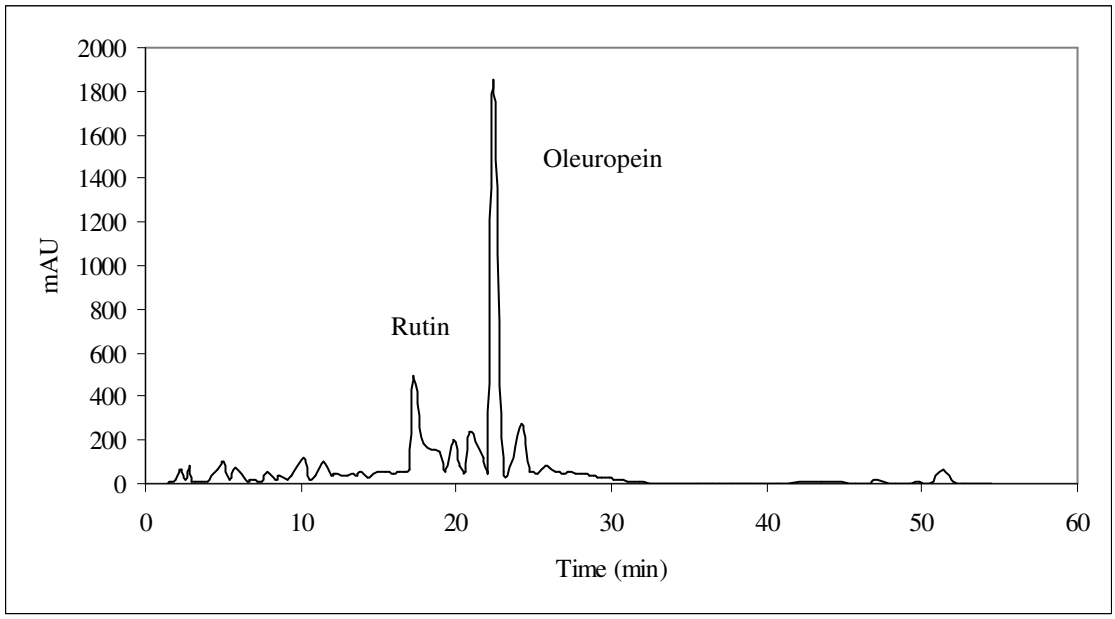


Figure 5.1. HPLC chromatogram of olive leaf.

After extracting the polyphenols in different conditions, each sample was injected to HPLC and the amount of oleuropein and rutin were calculated using the calibration curves. One sample calculation for this is given in Appendix B.

Figure 5.2 shows the effect of solvent type and solvent/water ratio on the extraction yield of oleuropein and rutin which is defined as mg extracted oleuropein or rutin per gram of olive leaf in this study.

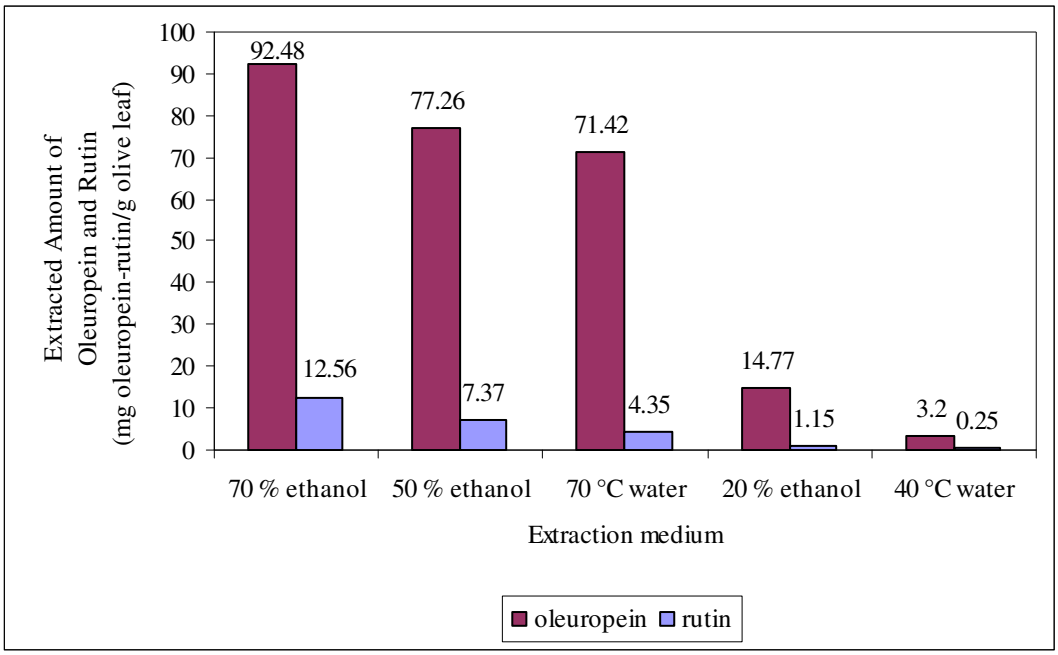


Figure 5.2. Effect of solvent on extracted amount of oleuropein and rutin per gram of olive leaf.

The highest yield of oleuropein and rutin extraction was obtained in 70 % aqueous ethanol solution. On the other hand, the extracted oleuropein amount was closer to each other in 50 % aqueous ethanol solution and 70 °C water whereas the extraction efficiency was lower in 20 % aqueous ethanol solution and 40 °C water. Increasing the temperature of water highly affected the extraction yield because the selectivity of water for the solutes increased and its extracting capacity was nearly reached to 50 % aqueous ethanol solution.

Besides this, decrease in the ethanol amount decreased the efficiency of the extraction. Due to the previous studies, also increase in ethanol amount over 70 % also decreased the yield of the extraction. As a result, presence of water played an important role for the mass transfer of the soluble biological compounds.

Although the highest yield was obtained in 70 % aqueous ethanol solution, the extraction efficiency of 50 % aqueous ethanol solution and water at 70 °C were also favorable. Each of these three extraction media had different advantages due to their properties. For example, water is cheaper than ethanol.

The solvents used in the extraction of the food and biological materials should have some properties. For instance, they should be easily recoverable because high

volumes of solvents are required in the industry. Using a mixture of solvent, even it is aqueous ethanol solution, brings the necessity of another procedure for the production of crude plant extracts. For instance, if 70 % aqueous ethanol solution was chosen as the extracting medium, firstly ethanol and then water should be separated to obtain the crude olive leaf extract but if 70 °C water was chosen as the extracting medium, only water should be separated. All these reasons made 70 °C water favorable for choosing as the extracting medium. However, high temperatures can cause the decomposition of oleuropein and rutin. For this reason, before choosing the extraction medium, the antioxidant capacities of samples in these three favorable conditions were analyzed by free radical scavenging method.

5.1.2. Antioxidant Analysis

One of the major bioactivities of plants is the antioxidant capacity. For this reason, the extraction efficiency is also dependent on antioxidant capacity of olive leaves. In order to choose the optimum extracting medium throughout 70 % and 50 % aqueous ethanol solution and 70 °C water, their antioxidant capacities were determined in terms of Trolox Equivalent Antioxidant Capacity (TEAC).

Olive leaf antioxidants are able to scavenge or inhibit the ABTS radical cations. This inhibition can be observed as a decrease in the absorbance values at 734 nm in UV-Visible Spectrophotometer. The percentage inhibitions of absorbance at different concentrations were calculated to be represented in graphs (Figures C2, C3 and C4). These graphs and inhibition percentage calculations are given in Appendix C. The TEAC values of olive leaves were calculated by comparing the slope of these curves with the slope of Trolox dose-response curve given in Appendix C, Figure C1. Figure 5.3 shows the TEAC values of extracted olive leaves in different media.

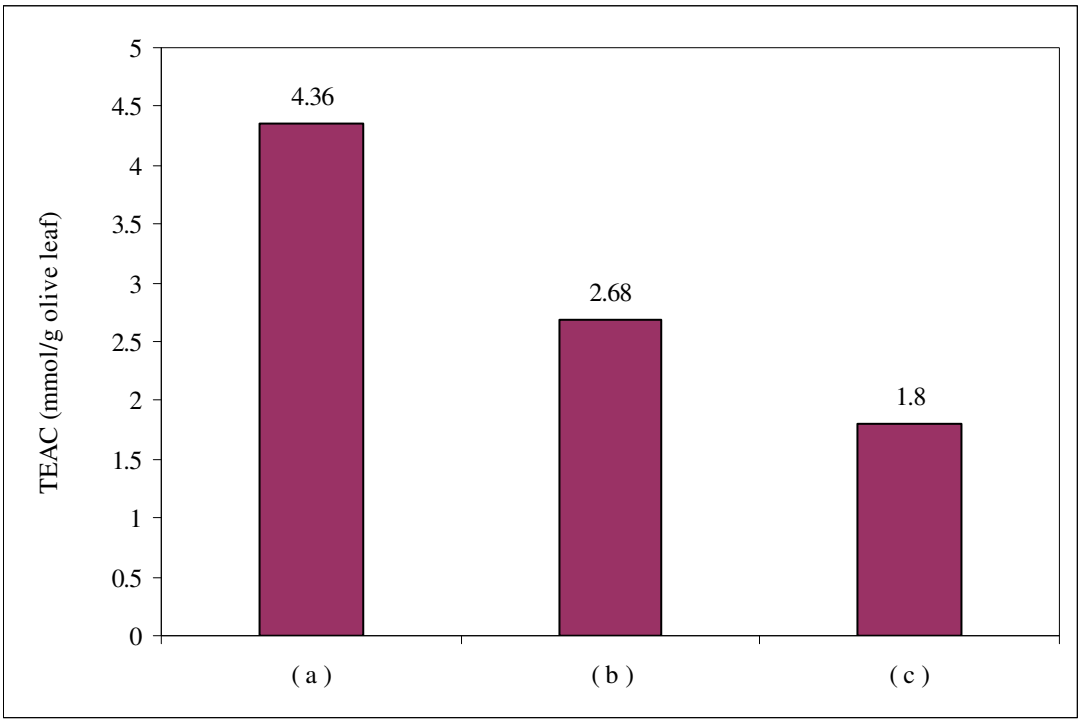


Figure 5.3. Antioxidant capacities of olive leaf extract. (a) Olive leaf in 70 % aqueous ethanol solution (b) Olive leaf in 50 % aqueous ethanol solution (c) Olive leaf in 70 °C water.

The highest antioxidant capacity was observed in 70 % aqueous ethanol solution. The antioxidant capacity of extracting media decreased due to decreasing oleuropein and rutin contents, which prove that oleuropein and rutin have significant effect in olive leaf bioactivity. Although the oleuropein content of 50 % aqueous ethanol solution and 70 °C water were closer to each other, their antioxidant capacity differed. Increase in temperature decreased the antioxidant capacity of olive leaves.

The HPLC and antioxidant analyses showed that 70 % aqueous ethanol solution was a promising medium to obtain a highly bioactive olive leaf extract.

5.1.3. Properties of Olive Leaf Extract

Studies continued with extracting the olive leaves in 70 % aqueous ethanol solution to obtain crude olive leaf extract. The efficiency of the extraction was increased with four successive extractions. The yield of extraction from olive leaf after four successive extractions was calculated as 52 % whereas the yield of first extraction was

calculated as 46 %. As a result, first extraction provided a suitable amount of product and there was no need to use extra solvents for successive extractions.

The HPLC and antioxidant analyses of olive leaf extract were performed and they were compared with the literature. The oleuropein and rutin content of one-gram olive leaf extract was found as 134.4 mg and 18.25 mg, respectively so the percentages of oleuropein and rutin in olive leaf extract were 13.4 % and 0.18 %, respectively. Savournin et al. (2001) investigated the oleuropein and rutin content of 14 cultivated olive tree leaves and they reported that the oleuropein content changed from 9.27 % to 13.43 % whereas rutin content changed from 0.07 % to 0.35 % depending on the type of the trees.

The antioxidant analysis of olive leaf extract was also performed and its antioxidant capacity was determined as 7.52 mmol of TEAC /gr olive leaf extract. The antioxidant capacity of olive leaf extracts was calculated in many of the studies but they were not reported in terms of mmol of TEAC /gr olive leaf extract, for this reason, it was difficult to compare this result with the literature. However, Laporta et al. (2006) reported that the antioxidant capacity of olive leaf extract and green tea extract were nearly the same and Arts et al. (2002) reported the antioxidant capacity of green tea extract as 7.3 mmol of TEAC/g of freeze dried green tea. These statements proved that our result was consistent with the literature.

5.2. Adsorption of Olive Leaf Antioxidants

In order to obtain concentrated specific polyphenols, further purification was necessary after the extraction procedure. Insoluble, hydrophobic silk fibroin was used for the removal of oleuropein and rutin from the crude olive leaf extract. The olive leaf extract was analyzed in HPLC before and after adsorption in order to determine the concentration of oleuropein and rutin in the liquid phase and the adsorbed amounts on silk fibroin were calculated from the following equation. One sample calculation is given in Appendix B in sample calculation part.

$$q = (C_i - C).\frac{V}{m} \tag{5.1}$$

Where,

q = Equilibrium solute phase concentration (mass solute/mass adsorbent)

C_i = Initial liquid phase concentration (mass solute/volume solution)

C = Equilibrium solute concentration in the aqueous phase (mass solute/volume solution)

V = Volume of liquid phase

m = Mass of the adsorbent

The adsorption was performed in two different solutions on three different forms of silk fibroin.

5.2.1. Solvent Effect and Effect of Adsorbent Form on Adsorption

The competition between the solute-adsorbent and solute-solvent makes the liquid adsorption more complicated compared with the gas adsorption. For this reason, choosing the solvent in liquid adsorption is the most important step to obtain a high yield of adsorption. The surface and form of the adsorbent are other important criteria, which affect the efficiency of adsorption system.

In order to decide the solvent type and form of the adsorbent for the removal of olive leaf antioxidants, adsorption was performed in 70 % aqueous ethanol solution and deionized water on fiber, foam and powder form of home-made silk fibroin and on approximately 90 µm. commercial silk fibroin powder.

After adsorption, there were visible changes in the color and structure of the silk fibroin. The color of the silk fibroin turned to light green from white when the adsorption was in 70 % aqueous ethanol solution and it turned to dark yellow when the adsorption was in water. Besides this, silk fibroin in the form of fibers got lump when adsorption was performed in water whereas there wasn't any change in 70 % ethanol solution. The oleuropein and rutin content of liquid phase was determined before and after adsorption by using HPLC. Figure 5.4 represents the adsorbed quantities of oleuropein and rutin per gram of adsorbent in these different conditions.

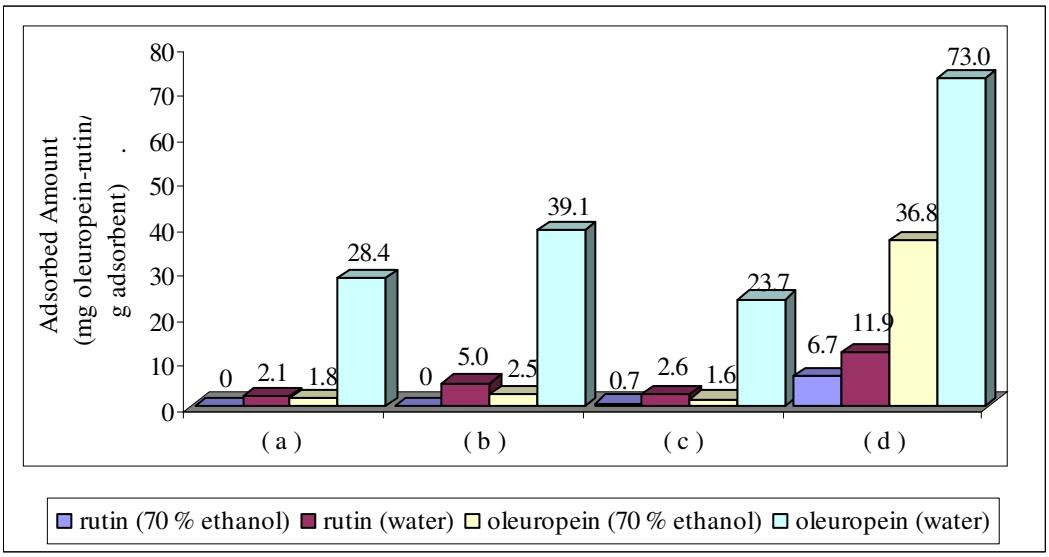


Figure 5.4. Adsorbed amount of rutin and oleuropein on silk fibroin in 70 % aqueous ethanol solution and water (a) Home-made foam silk fibroin (b) Home-made fiber silk fibroin (c) Home-made powder silk fibroin (d) Commercial silk fibroin powder.

Figure 5.4 directly gives the answer of two important questions. Firstly, the highest quantities of oleuropein and rutin were adsorbed in water so the adsorption was more efficiently performed in water compared with 70 % aqueous ethanol solution. The folding of fibers and higher adsorption efficiency in water showed that probably hydrophobic interactions take place between the olive leaf antioxidants and silk fibroin. The interactions between the biopolymers and polyphenols are mainly hydrophobic interactions and hydrogen bonding. In hydrophilic solvents, hydrophobic interactions are favored whereas in hydrophobic solvents, hydrogen bonding is favored.

Tang et al. (2003) performed a similar study in order to understand the main interactions between cellulose and polyphenols. They used precoated cellulose thin layer chromatography (TLC) to find out the retardation factors of twenty-four tannins in two different solvents. While one of the solvents was hydrophilic, similar to water, the other solvent was more hydrophobic. After comparing the retardation factors in these two solvent systems, they concluded with the existence of strong hydrophobic interaction between polyphenols and cellulose.

In our system, 70 % aqueous ethanol solution is more hydrophobic than water. If the hydrogen bonding were the main interaction between olive leaf antioxidants and silk

fibroin, then the adsorption would perform efficiently in ethanol solution. The olive leaf antioxidants stayed in the solution instead of accumulating on silk fibroin. Their preference staying in 70 % ethanol solution and preference accumulating on silk fibroin in water proved that the adsorption of olive leaf antioxidants on silk fibroin strongly depends on the hydrophobic interactions.

Secondly, Figure 5.4 represents that the commercial silk fibroin powder had the highest adsorption capacity among the others. This was probably due to the contact area between the polyphenols and adsorbents. As the contact area increases in the adsorption systems, mass transfer increases as increasing the adsorption on adsorbents. The average size of the used commercial silk fibroin powder was 90 µm providing sufficient contact area for efficient adsorption.

In order to understand whether or not the silk fibroin selectively adsorbed the rutin and oleuropein, the two HPLC chromatograms before adsorption and after adsorption were overlapped in a graph.

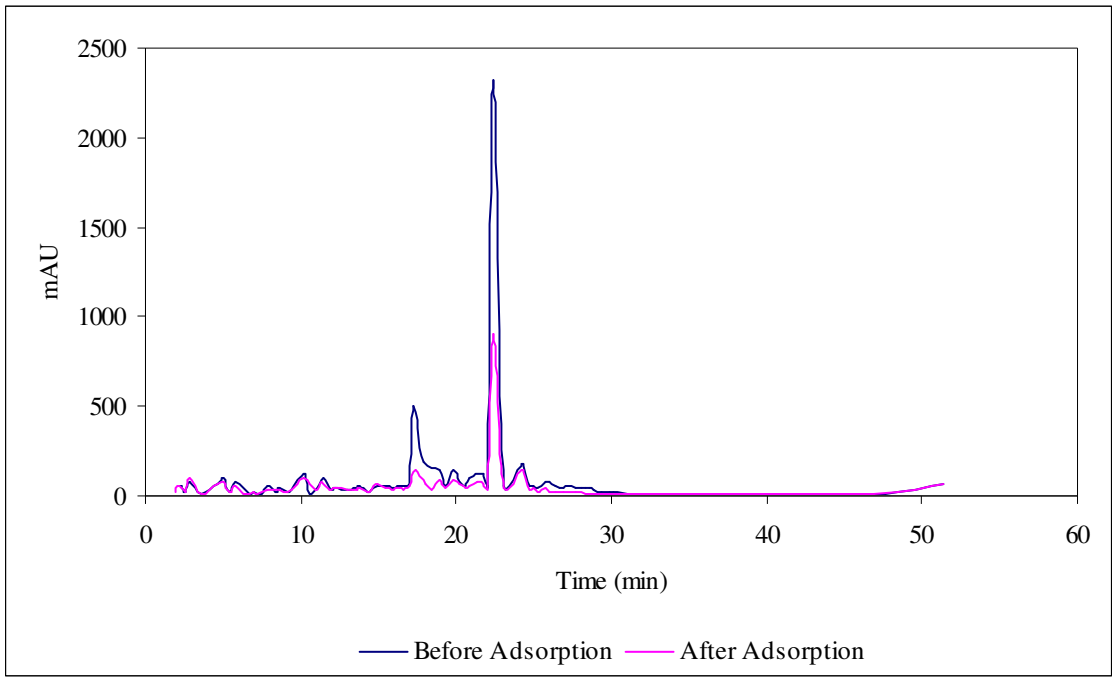


Figure 5.5. The HPLC chromatograms of olive leaf extract before and after adsorption.

Silk fibroin selectively adsorbed rutin and oleuropein compared with the other polyphenols as seen in Figure 5.5. In addition, the other polyphenols such as verbascoside, apigenin-7-glucoside, diosmetin-7-glucoside which have closer retention

times to retention time of rutin and oleuropein, were adsorbed on silk fibroin. As it was indicated before, HPLC separates the polyphenols due to their polarity. Figure 5.5 also represents that relatively nonpolar polyphenols were removed from the olive leaf extract solution. The accumulation of the nonpolar compounds on the hydrophobic silk fibroin can be explained by the presence of strong hydrophobic interactions between silk fibroin and polyphenols during the adsorption process.

5.2.2. Adsorption Equilibrium

When a certain composition of adsorbate is in contact with adsorbent, adsorption begins to take place and after a sufficiently long time, adsorbent saturates and the adsorption reaches at equilibrium. The adsorption behavior of oleuropein and rutin was followed during 30 hours. Figure 5.6 and Figure 5.7 both show the adsorbed amount of oleuropein and rutin depending on time.

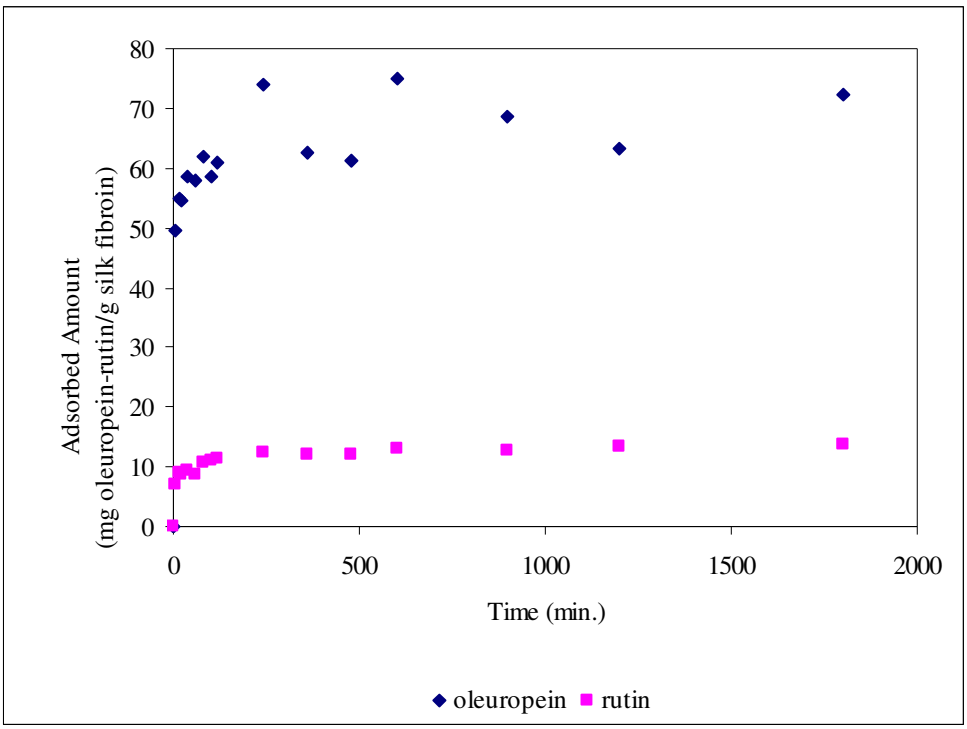


Figure 5.6. Adsorption behavior of silk fibroin during 30 hours.

Figure 5.6 shows that the system reaches equilibrium before 30 hours. The adsorbed quantities did not much differ after two hours time. For this reason, the change during two hours is represented in Figure 5.7.

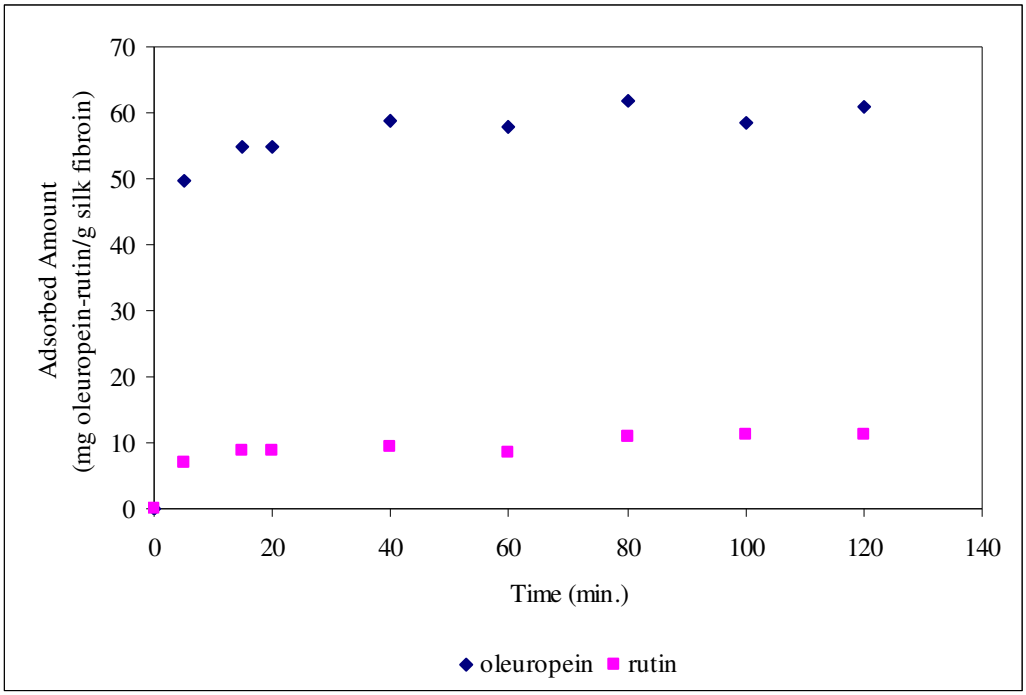


Figure 5.7. Adsorption behavior of silk fibroin during 2 hours.

The adsorption significantly occurred in the first five minutes and then the adsorbed quantity slightly increased in 40 minutes. After 40 minutes, the adsorption reached equilibrium. Consequently, 40 minute was sufficient for the removal of olive leaf antioxidants and after 40-minute contact time, the steady-state assumption was valid for this system. The studies in the literature established that approximately 2 or 3 hour-adsorption time was generally adequate for the recovery of polyphenols (Ribeiro et al. 2002, Kim et al. 2005, Liao et al. 2003, Scordino et al. 2004). The equilibrium time has great importance to find out the maximum adsorption capacity of the system and to form adsorption isotherms.

5.2.3. Adsorption Isotherms

The relation between the adsorbed amount and concentration of the adsorbate at equilibrium gives the adsorption isotherm of that system. Adsorption isotherms are the best systems that describe the interactions and the maximum capacity of the adsorbents. For this reason, adsorption isotherms of oleuropein and rutin at different conditions were established, successively.

5.2.3.1. Adsorption Isotherms of Oleuropein

Oleuropein is the most abundant compound in the olive leaf. Because of this and its nonpolar character, a high amount of oleuropein was adsorbed on silk fibroin. In order to find out the maximum capacity of silk fibroin for the adsorption of oleuropein, the adsorption was accomplished at different initial concentrations. Figure 5.8 shows the effect of initial concentration on the adsorption of oleuropein and the adsorption isotherm of oleuropein at 25 °C is given in this figure.

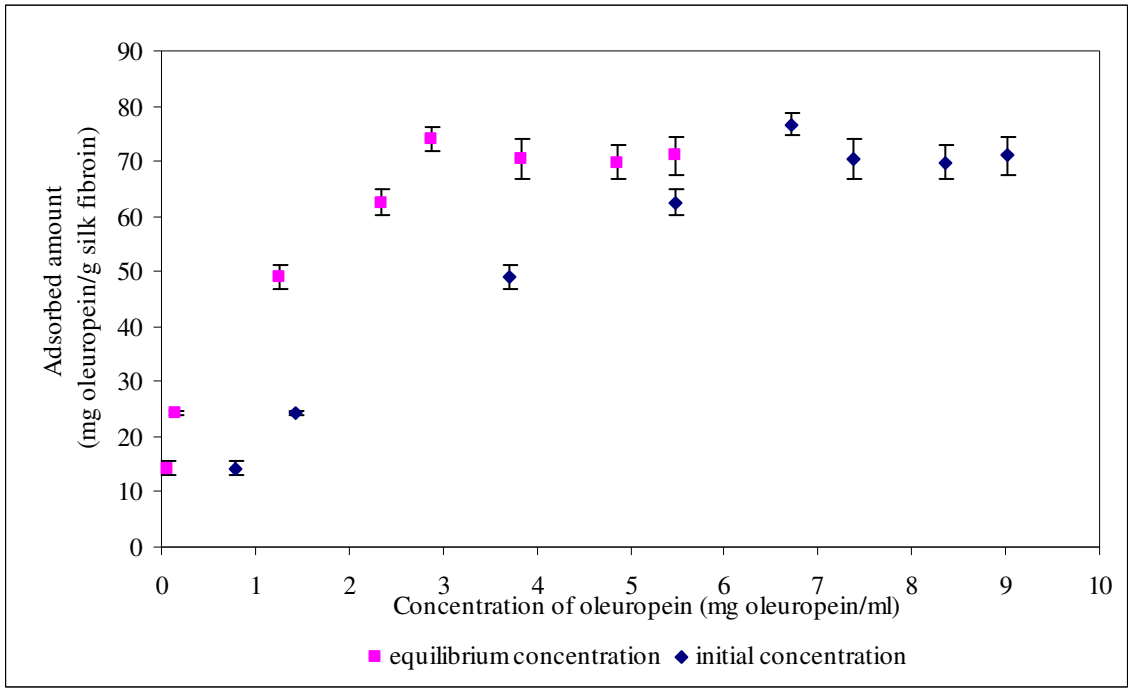


Figure 5.8. Adsorption behavior of oleuropein at different initial concentrations.

Increase in the amount of olive leaf extract increased the concentration of oleuropein in the solution. The initial concentration of oleuropein was changed from 0.79 mg/ml to 9.03 mg/ml. A significant increase in the adsorption of oleuropein was observed until the initial concentration of oleuropein was set to 6.72 mg/ml. and concentrations over 6.72 mg/ml did not affect the adsorption of oleuropein.

The adsorption isotherm was plotted by using the equilibrated oleuropein amount in the solution and on the adsorbent. As it is seen in Figure 5.8, this isotherm is concave to the concentration axis and it has a long plateau, which shows that this isotherm is a *Type L* isotherm. *Type L* isotherms are associated with the monolayer coverage of the solutes and minimal competition from the solvent. Since the adsorption took place in water, water did not compete with the adsorbent causing the equilibrated oleuropein quantities to form *Type L* isotherm.

Langmuir and Freundlich models were applied to equilibrium data of the adsorption isotherm. Langmuir model demonstrated considerable superiority to Freundlich model since there is a limit in the adsorption capacity of silk fibroin. The Langmuir parameters of the adsorption isotherm were calculated by using the Langmuir equation.

$$q = \frac{q_0 c}{K + c} \tag{5.2}$$

Where,

q_0 = Maximum adsorbed amount (mg oleuropein/gr silk)

K = Langmuir Constant (mg oleuropein/ml water)

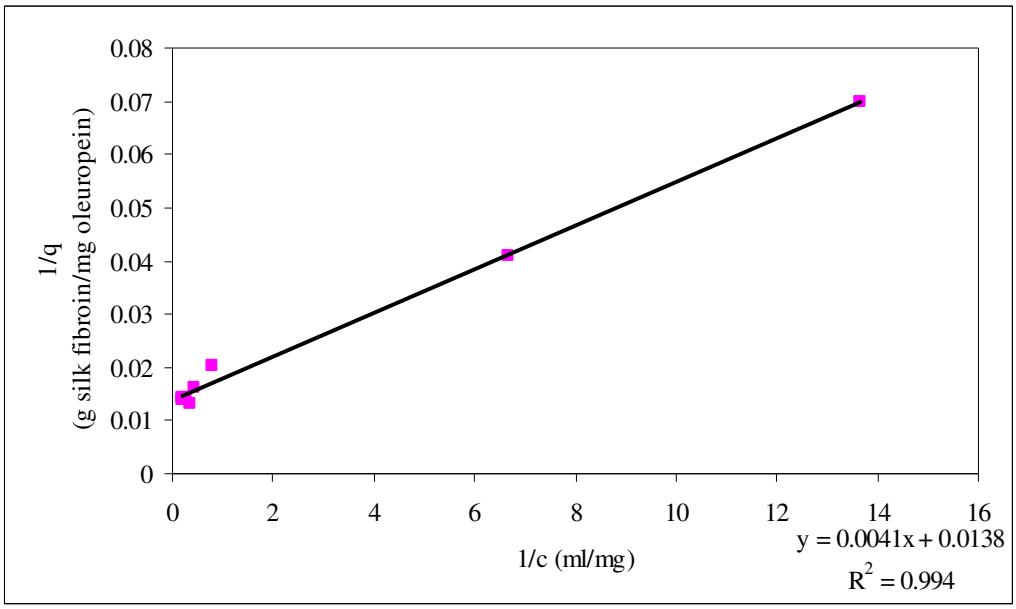


Figure 5.9. Langmuir Model Curve.

The Langmuir parameters were calculated by using the equation of the Langmuir model curve. Intercept of this curve gives $1/q_0$ whereas the slope of the curve is equal to K/q_0.

$1/q_0$ = 0.0138

q_0 = 72.46 mg oleuropein/g silk

K/q_0 = 0.0041

K = 0.297 mg/ml

The maximum adsorption capacity of silk fibroin for oleuropein was calculated as 72.46 mg oleuropein/g silk by using the Langmuir equation and assuming the monolayer coverage was valid. From the plateau of the adsorption isotherm given in Figure 5.8, the maximum adsorption capacity was determined as 71.02 mg oleuropein/g silk. The proximity of these values with the model and experimental data proved the validity of the monolayer coverage.

Temperature, pH and solid-liquid ratio are the other parameters, which affect the adsorption capacity of adsorbents. Because of this, their effects were investigated. Firstly, the effect of temperature was investigated.

Effect of Temperature on Adsorption of Oleuropein

Due to their low-energy nature of adsorptive separation processes, adsorption is more preferred in the large-scale operations. For this reason, it is meaningless to accomplish an adsorption procedure at very low and very high temperatures since both of them require energy and money. Takagai et al. (2005) observed the increase in the adsorption of resveratrol on cellulose cotton when they decreased the temperature from 18 °C to 5 °C. However, 5 °C is not a practical temperature for industrial scales.

Furthermore, at high temperatures, higher than 40 °C, there is always a risk of degradation of both polyphenols and proteins. Because of these reasons, adsorption was established at three different temperatures 25, 30 and 35 °C. All these experiments were run in water and the solid-liquid ratio was always 1/20 to see only the effect of temperature on adsorption.

The adsorption isotherms obtained at these three temperatures are demonstrated in Figure 5.10.

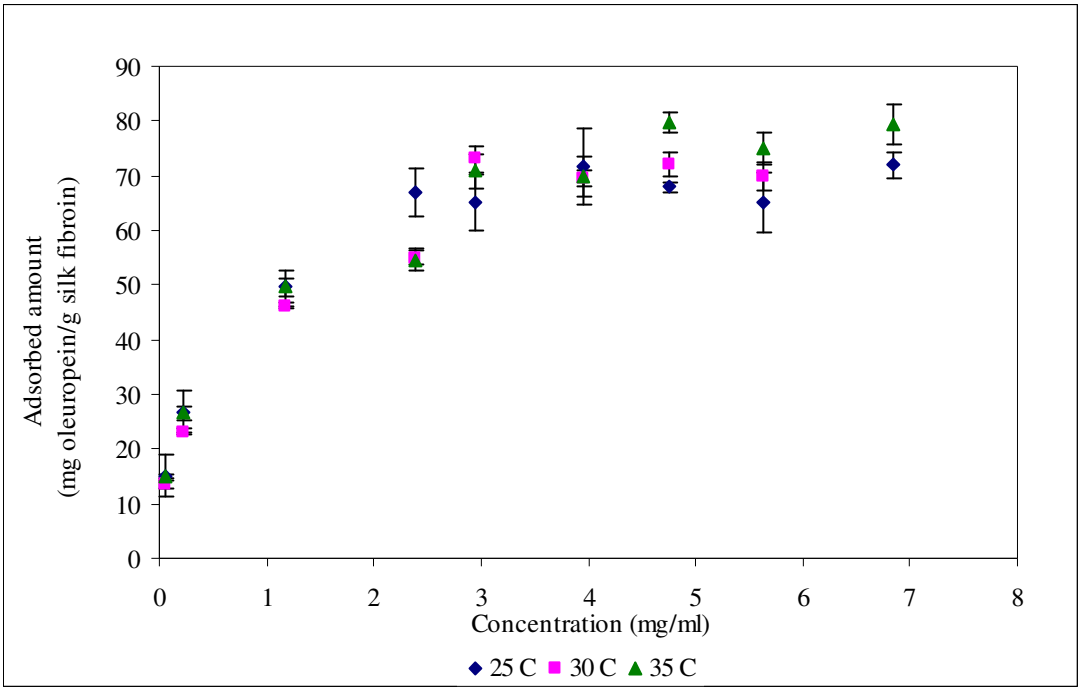


Figure 5.10 Effect of temperature on adsorption of oleuropein.

A clear difference could not be detected between the adsorption capacities of

silk fibroin at these three temperatures within the range of error margins. This was probably due to the proximity of temperatures but as it is indicated, it is not meaningful to perform the adsorption lower than 25 °C and higher than 35 °C. The Langmuir model curves of these isotherms are given in Appendix D, in Figures D1, D2 and D3. The calculated Langmuir constants are tabulated in Table 5.1.

Table 5.1. Langmuir Constants at different temperatures.

Temperature	q_0	K	1/K	R^2
25 °C	72.46	0.297	3.37	0.994
30 °C	68.49	0.260	3.84	0.992
35 °C	72.46	0.254	3.93	0.990

Increase in the values of q_0 with the increase in temperature indicates the endothermic nature of the process whereas decrease in q_0 with the increase in temperature indicates the exothermic nature of the process. However, the maximum adsorption capacity values of this process did not increase or decrease in an order. For this reason, q_0 values could not give any opinion about the nature of the process.

On the other hand, 1/K values are directly related to the energy of adsorption. Increasing values of 1/K with the increase of temperature were accompanied by high-energy requirement for the oleuropein adsorption. Thus, under optimized conditions, the equilibrium adsorption capacity of oleuropein was found to be 72.46 mg oleuropein/g silk at 25 °C.

For this reason, following experiments were run at 25 °C.

Effect of pH on Adsorption of Oleuropein

The adsorption behavior of proteins differs due to the pH of the medium. At the pH values lower than pI, surface of the adsorbent is positively charged and at pH values higher than pI, the surface is negatively charged. The isoelectric point of silk fibroin is approximately 4, that's why the experiments were run at pH 2.4, 4, 6 and 7.6. The pH of the system was adjusted to 2.4, 4, and 7.6 by phosphate buffer. The other experiment was run in deionized water, which was at pH of 6. Figure 5.11 demonstrates the adsorption behaviour of silk fibroin at these 4 different pH values.

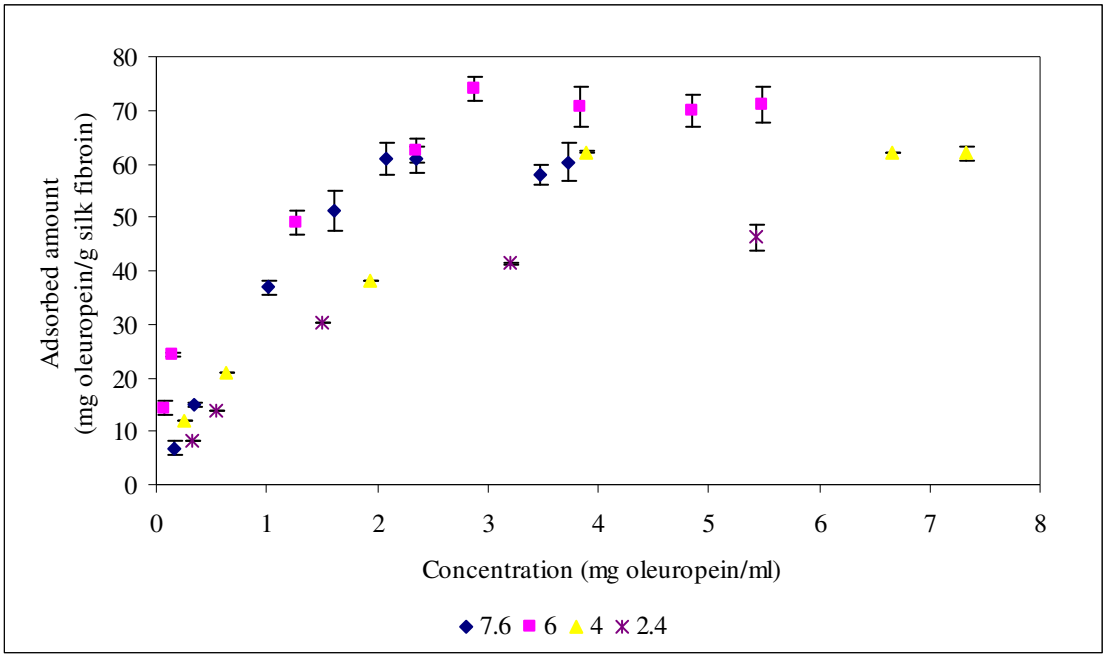


Figure 5.11. Effect of pH on adsorption of oleuropein.

The adsorptive properties of silk fibroin were closer to each other at all of the pH values, except at pH value of 2.4. The adsorbed amount of oleuropein was significantly less at pH 2.4 showing that the electrostatic interactions between the adsorbent and adsorbate were not as strong as the hydrophobic interactions. From the plateau of the adsorption isotherms, the maximum adsorbed amount was found as 61, 71.02, 62, and 45 for the pH values of 7.6, 6, 4 and 2.4, respectively. The adsorbed amount of oleuropein was nearly the same at pH 4 and 7.6 whereas maximum adsorption was achieved at pH 6. Similar results were concluded for the adsorption of polyphenols. Xu et al. (2001) observed that the adsorption affinity of gelatin-polyvinyl alcohol for flavonol glycosides was higher between pH 5 and 6.5. In the phenol adsorption, typically the amount adsorbed decrease at both low and high pH values and the maximum adsorption values for the irreversible systems are generally obtained in unbuffered systems (Yang 2003).

In order to find out the maximum adsorption, corresponding to the saturated monolayer of adsorbate molecules on adsorbent surface, Langmuir model curves at different pH values were plotted and they are given in Figures D4, D5 and D6 in Appendix D. The Langmuir constants are tabulated in the following table.

Table 5.2. Langmuir constants at different pH values.

pH	q_0	K	R^2
7.6	70.42	0.751	0.992
6	72.46	0.297	0.994
4	69.93	1.293	0.991
2.4	60.61	2.078	0.995

The data well fitted to the Langmuir equation as shown by the regression coefficients in Table 5.2. The maximum adsorption capacities in the case of monolayer coverage are higher than the maximum adsorbed quantities at pH 7.6, 4 and 2.4. Thus, monolayer coverage was not achieved in these conditions and addition of buffer solution affected coverage of the monolayer in this system. Due to the Langmuir model, the maximum adsorption capacity of silk fibroin did not change significantly between pH 4 and pH 7.6, showing the hydrophobic interactions are the main interactions in this system. The experimental data and the model values all represented that pH 6 was the optimum condition that's why the following experiments were run in water.

Effect of Solid – Liquid Ratio on Adsorption of Oleuropein

Solid-liquid ratio is another important criterion in the adsorption from solution, which affects the economy of the system. If total removal of the solutes can be achieved at low amounts of adsorbent then there is no need to increase the concentration of adsorbent in the solution.

The adsorption behavior of oleuropein was observed in three different solid-liquid ratios at 25 °C in water. Solid-liquid ratio was adjusted to 0.025, 0.05 and 0.1 by changing only the adsorbent amount.

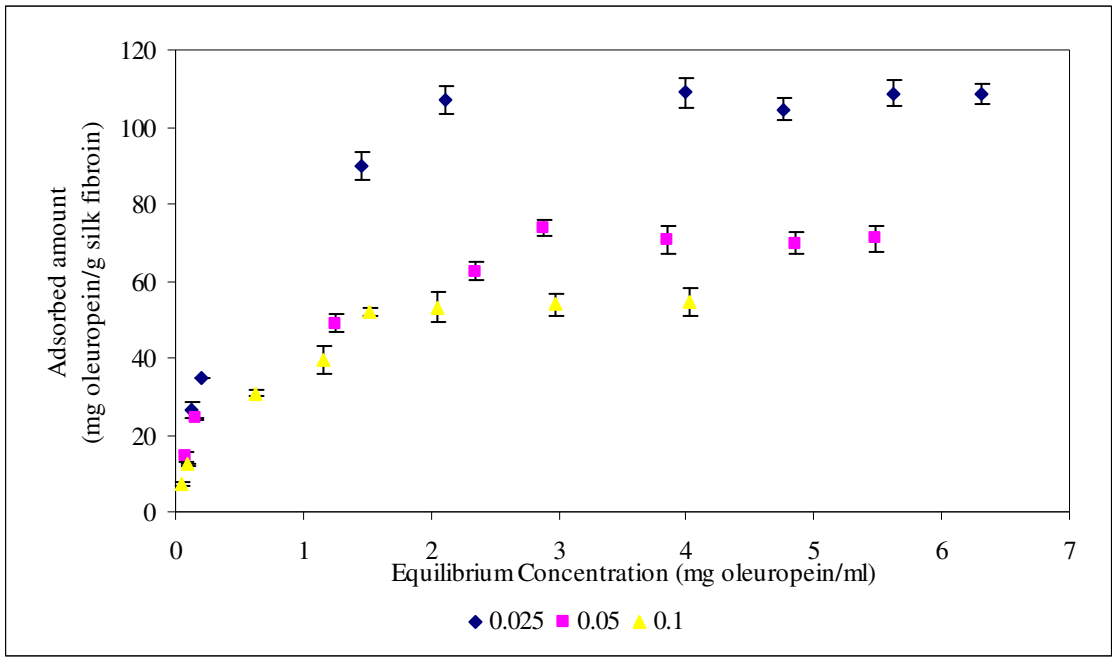


Figure 5.12. Effect of solid-liquid ratio on adsorption of oleuropein.

The adsorption efficiency of oleuropein was increased with the decreasing adsorbent amount at the constant initial concentrations of oleuropein. This might be due to the total removal of the solute even with low adsorbent concentration. Since the adsorbent was saturated when the solid liquid ratio was 0.025, the adsorbed amount per g of adsorbent was lower when the solid liquid ratio increased from 0.025 to 0.1.

The Langmuir model curves were plotted (Figures D7 and D8) to calculate the Langmuir constants, represented in Table 5.3.

Table 5.3. Langmuir constants at different solid-liquid ratio values.

S/L	q_0	K	R^2
0.025	117.65	0.447	0.9963
0.05	72.46	0.297	0.994
0.1	55.55	0.338	0.9959

Due to the model, the maximum adsorption was 117.65 mg oleuropein/g silk fibroin whereas the plateau of the adsorption isotherm indicated it as 108 mg oleuropein/g silk fibroin, showing that monolayer was not fully covered in this system.

Table 5.3 obviously represents the importance of solid-liquid ratio in the adsorption systems. The adsorbed amount at 0.025 solid-liquid ratio was 1.5 times of the adsorbed amount at 0.05 solid-liquid ratio and it was nearly 2 times of the adsorbed amount at 0.1 solid-liquid ratio. Because of this, the decrease with the mass of the adsorbent can be represented by a power equation as given in Figure 5.13.

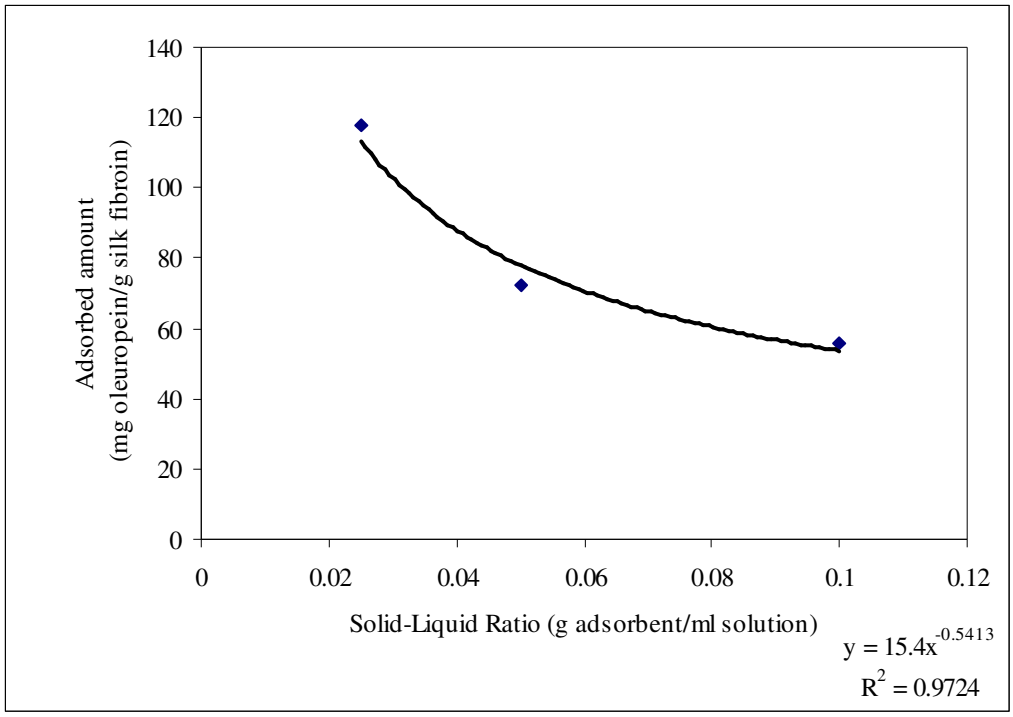


Figure 5.13. Adsorption efficiency of oleuropein at different solid-liquid ratios.

The adsorption of limonin and naringin on various natural and synthetic adsorbents also increased by a power equation as the solid-liquid ratio was decreased (Ribeiro et al. 2002).

Consequently, maximum adsorption capacity of silk fibroin for oleuropein at 25 °C, in water was found as 108 mg oleuropein/g silk fibroin when the solid-liquid ratio was 0.025. To our knowledge, there are not any studies about the adsorption of polyphenols on silk fibroin or adsorption of olive leaf antioxidants on any adsorbents because of this, it is difficult to compare this value with other studies.

5.2.3.2. Adsorption Isotherms of Rutin

Although rutin is the second most abundant compound in olive leaf extract, rutin content of olive leaves is very low compared with the oleuropein content. For this reason, the adsorbed rutin amount on silk fibroin was lower compared to adsorbed amount of oleuropein. The adsorbed amount of rutin was determined by using the results of the HPLC analysis, too. Firstly, the effect of initial concentration on the adsorption of rutin was observed and its adsorption isotherm at 25 °C was plotted as it is shown in Figure 5.14.

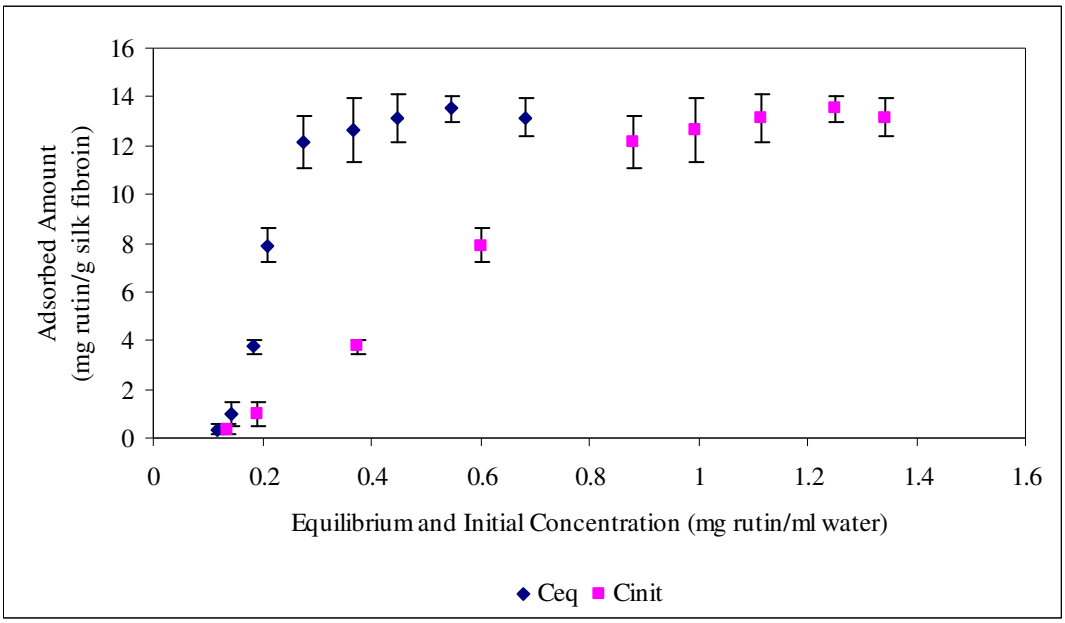


Figure 5.14. Adsorption behavior of rutin at different initial concentrations and adsorption isotherm of rutin.

As the concentration of olive leaf extract was increased, the concentration of rutin increased, too. The initial concentration of rutin was changed from 0.13 mg/ml to 1.34 mg/ml. In this range, its adsorption on silk fibroin was increased until its initial concentration was set to 0.88 mg rutin/ml and the adsorbed amount was nearly the same over this concentration.

The adsorption isotherm of rutin was plotted within this range of initial concentration by using the equilibrium values of rutin in the solution and on the

adsorbent. The same results were concluded as the oleuropein adsorption on silk fibroin. Since the isotherm was concave to the concentration axis and had a long plateau, it was a *Type L* isotherm and the experimental data well-fitted to Langmuir model compared with the Freundlich model. However, because of the low concentration values, 1/q and 1/c values of linear part of the adsorption isotherm were used in the modeling instead of using all the experimental data.

The Langmuir model curve of rutin adsorption is given in Figure D9. The maximum adsorption capacity of silk fibroin for rutin was calculated as 16.47 mg rutin/g silk from this curve whereas the maximum adsorption capacity was determined as 13.5 mg rutin/g silk from the plateau of the adsorption isotherm. These values demonstrate that the monolayer coverage was not achieved 100 %.

The parameters such as temperature, pH and solid-liquid ratio also affected the adsorption of rutin on silk fibroin. Firstly, the effect of temperature was investigated.

Effect of Temperature on Adsorption of Rutin

The adsorption behavior of silk fibroin for rutin was investigated at the temperatures of 25°C, 30°C and 35°C, which are demonstrated in Figure 5.15. The experimental data of these adsorption isotherms were fitted to Langmuir model and these Langmuir model curves are represented in Figures D9, D10 and D11, respectively. Their Langmuir constants are given in Table 5.4.

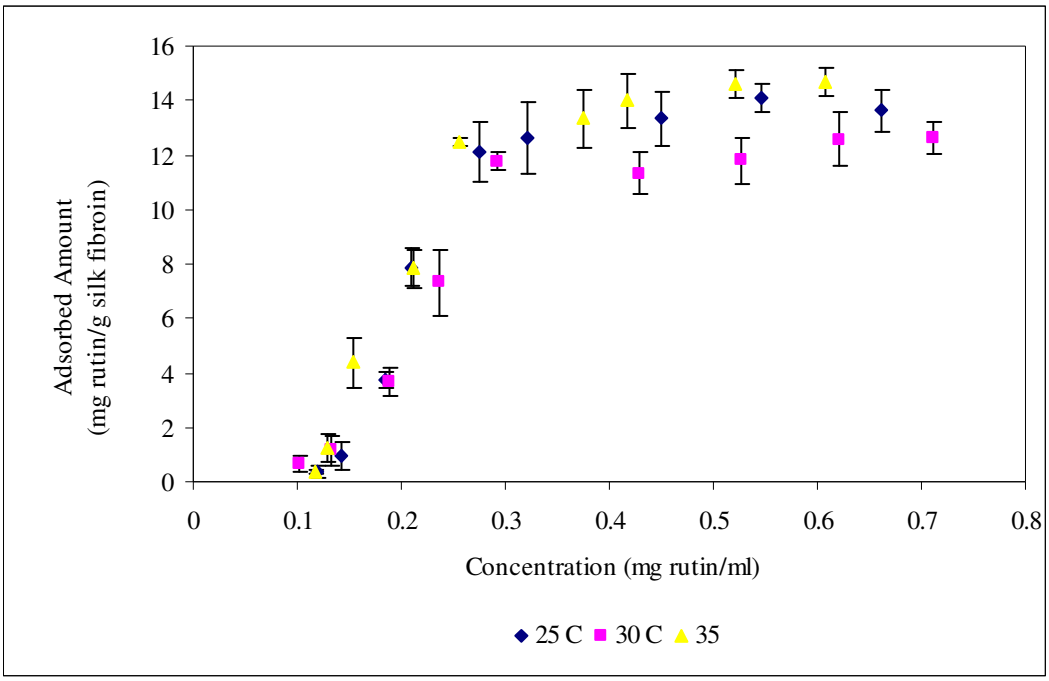


Figure 5.15. Effect of temperature on adsorption of rutin.

The proximity of temperatures again caused the same problem in rutin adsorption as the oleuropein adsorption. A clear difference could not be detected between the adsorption capacities of silk fibroin at these three temperatures within the range of error margins. The maximum adsorption capacities obtained from the model are given in Table 5.4.

Table 5.4. Langmuir Constants at different temperatures.

Temperature	q_0	K	1/K	R^2
25	16.47	0.099	10.1	0.983
30	15.45	0.159	6.29	0.988
35	17.83	0.127	7.8	0.991

The maximum adsorption capacity and 1/K values of this process did not increase or decrease significantly. For this reason, the nature of the process can not be understood but 25°C seems to be favorable condition for the adsorption of rutin.

Effect of pH on Adsorption of Rutin

The adsorption behavior of silk fibroin for rutin at basic and acidic pH values was investigated. Figure 5.16 demonstrates the adsorption isotherms at pH 7.6, 6, 4 and 2.4.

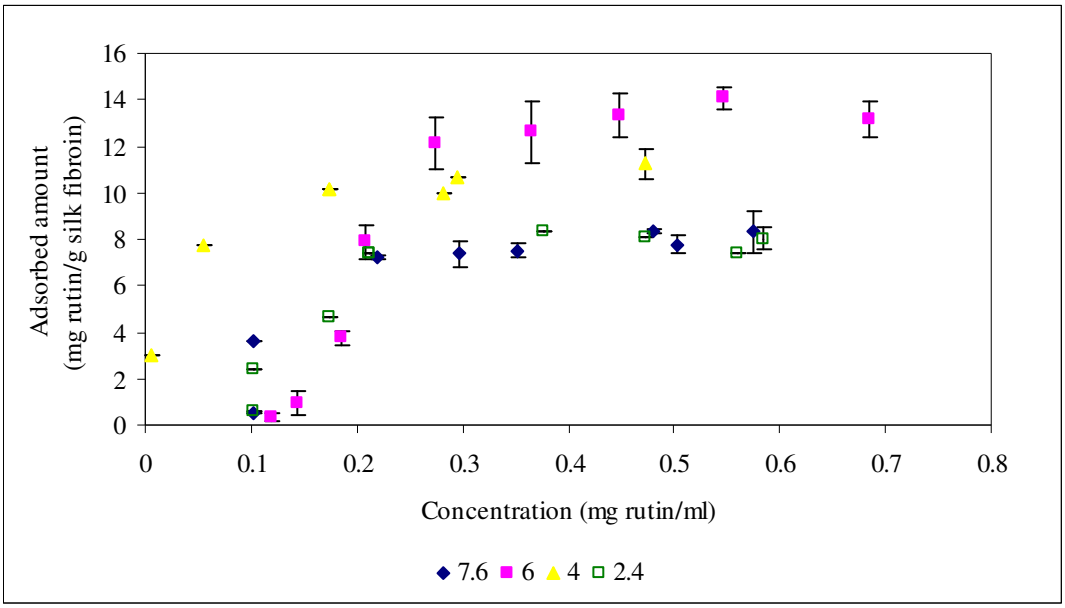


Figure 5.16. Effect of pH on adsorption of rutin.

The adsorbed amount of rutin was significantly less at pH 2.4 and 7.6 showing that the electrostatic interactions were not the main interactions between the silk fibroin and rutin. From the plateau of the adsorption isotherms, the maximum adsorbed amount was found as 8.3, 13.5, 10.8 and 7.5 for the pH values of 7.6, 6, 4 and 2.4, respectively. The maximum adsorption was again accomplished at pH 6 as in the case of oleuropein adsorption. In order to find out the maximum adsorption in case of monolayer coverage, Langmuir model curves at different pH values were plotted and they are given in Figures D12, D13 and D14 in Appendix D. The Langmuir constants are tabulated in Table 5.5.

The linear portion of the adsorption isotherms well fitted to the Langmuir equation as shown by the regression coefficients in Table 5.5.

Table 5.5. Langmuir constants at different pH values.

pH	q_0	K	R^2
7.6	8.47	0.05	0.989
6	16.47	0.099	0.983
4	10.76	0.014	0.9951
2.4	10.26	0.14	0.963

The maximum adsorption values calculated from the equation are higher than the maximum adsorbed values found from the plateau of the adsorption isotherms, showing that the monolayer coverage was not accomplished. The addition of buffer solution also affected the adsorption capacity of silk for rutin as it is seen in Table 5.5. For this reason, the effect of solid liquid ratio on the system was investigated in water at pH 6.

Effect of Solid-Liquid Ratio on Adsorption of Rutin

The solid-liquid ratio had inversely affected the adsorption of oleuropein on silk fibroin. Therefore, the rutin adsorption was also expected to decrease as the solid-liquid ratio was increased. The adsorbed amount of rutin was found when the solid-liquid ratio was 0.025, 0.05 and 0.1 at different initial concentrations.

As it is obvious in Figure 5.17, maximum adsorption efficiency was obtained when the solid liquid ratio was 0.025. This was probably due to the total removal of rutin with low amount of silk fibroin.

The maximum adsorbed amounts were determined as 21, 13.5, and 8.5 mg rutin/g silk fibroin for solid-liquid ratio of 0.025, 0.05 and 0.1, respectively.

The Langmuir model curves of these adsorption isotherms are given in Figures D15 and D16 and the calculated Langmuir constants are tabulated in Table 5.6.

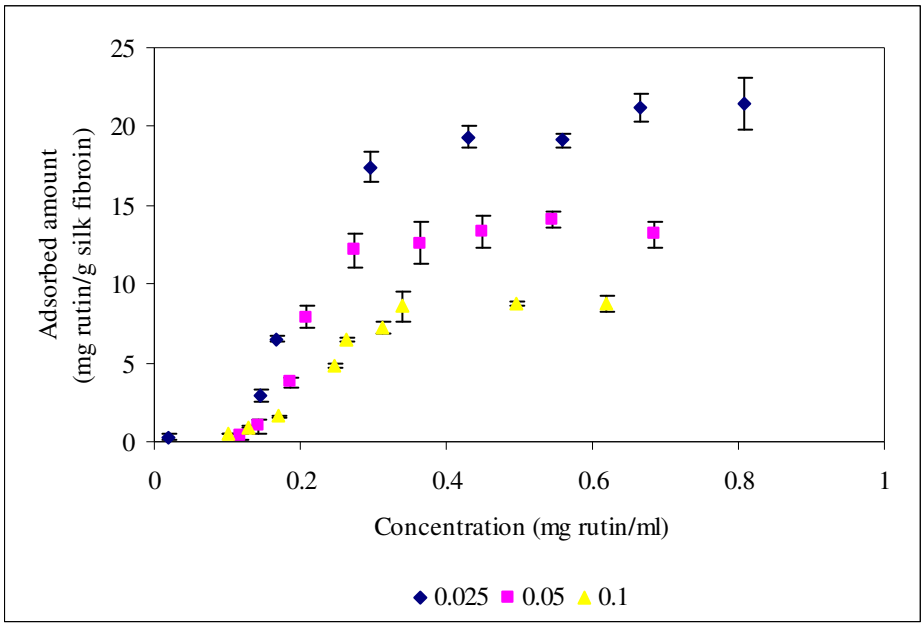


Figure 5.17. Effect of solid-liquid ratio on adsorption of rutin.

There are slightly differences in the maximum adsorption capacity of model and experimental data, showing that monolayer was not fully covered in this system.

Table 5.6. Langmuir constants at different solid-liquid ratio values.

S/L	q_0	K	R^2
0.025	25.06	0.13	0.994
0.05	16.47	0.099	0.983
0.1	12.53	0.236	0.963

The solid-liquid ratio significantly affected the adsorption of rutin. Figure 5.18 represents the decrease in the adsorption efficiency with the mass of the adsorbent by a power equation.

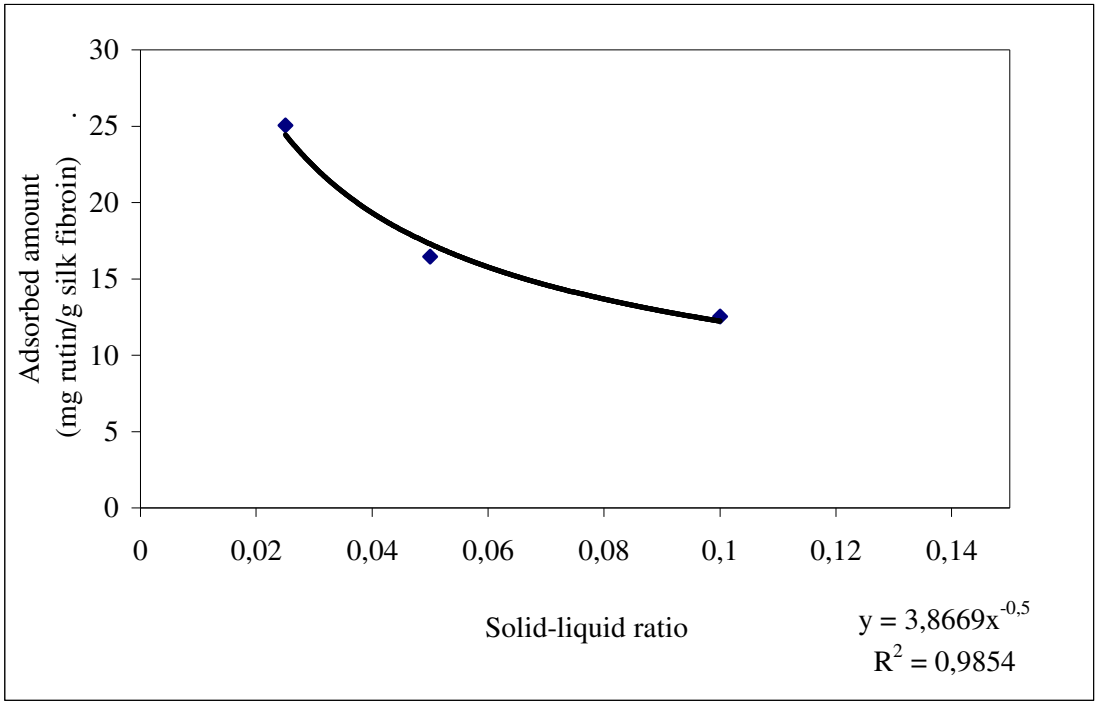


Figure 5.18. Adsorption efficiency of rutin at different solid-liquid ratios.

As a result, maximum adsorption capacity of silk fibroin for rutin at 25 °C, in water was found as 21 mg rutin/g silk fibroin when the solid-liquid ratio was 0.025.

Since both rutin and oleuropein were removed in this system, this system can be defined as multicomponent adsorption.

5.2.3.3. Multicomponent Adsorption of Oleuropein and Rutin

The objective of adsorption in most real systems is to remove several adsorbates instead of one. This complicates the theoretical picture of equilibrium among adsorbates and adsorbent. The Langmuir model may be generalized from single to multicomponent adsorption (Montgomery, 1985). The Langmuir equation for multicomponent systems is given in the following.

$$\theta_i = \frac{(1/K)C_i}{1+\sum_{i=1}^{n}(1/K)C_i} \tag{5.3}$$

Where,

θ_i = Surface coverage

Using this equation, one can estimate the fraction of the surface covered by oleuropein, rutin or other polyphenols. However, this model is successful under the following conditions:

- The competing components must obey the assumptions of the Langmuir-like equation for single solutes
- The capacity of the surface for each component must be the same
- Adsorbates must be competing for the same binding sites.

Due to the equation 5.3, the surface coverage of both oleuropein and rutin were calculated as 0.29 and 0.66. The calculation procedure is given in Appendix D. Since other polyphenols also adsorbed on silk fibroin as demonstrated in Figure 5.5, they covered the left 0.05 portion of available silk fibroin. The surface coverage of silk fibroin was also found by using the experimental data of rutin and oleuropein by assuming no other polyphenols were adsorbed on silk fibroin. In this case, the surface coverage of rutin and oleuropein was found as 0.16 and 0.84.

The difference between the surface coverage values of oleuropein and rutin are probably due to the differences in molecular sizes or differences in orientations of them on the surface of silk fibroin. Alkhamis and Wurster (2002) reported that if adsorption occurs on sites that are not equally accessible to all of the competing adsorbates, the Langmuir model is not expected to yield accurate results.

The adsorption affinity factors in most of the studies are defined as the equilibrium ratio in the initial linear region of the adsorption isotherms since the number of occupied sites is small as compared to the total active sites, which can be taken as constant and a measure of the affinity for the solute (Ribeiro et al. 2002).

The affinity factors for oleuropein and rutin were found from Figure 5.12 and 5.17, respectively when the adsorption was performed at 25 ºC and the solid-liquid ratio was adjusted to 0.025 in water. The slopes of the initial linear region of these

curves were determined as 60.06 for rutin and 50.41 for oleuropein showing the fact that adsorption affinity for rutin was higher than oleuropein. Although oleuropein content was higher than rutin, adsorption affinity for rutin was higher. This was probably due to the difference of the molecular weights between rutin and oleuropein. The molecular weight and molecular formula of rutin are 610.53 and $C_{27}H_{30}O_{16,}$ respectively. Molecular weight and molecular formula of oleuropein are 540.53 and $C_{25}H_{32}O_{13}$, respectively (Walter et al. 1973). Increase in the molecular weight increases the interactions between biopolymers and polyphenols. Since molecular weight of the rutin is higher than molecular weight of the oleuropein, the adsorption affinity for rutin was higher than oleuropein.

Consequently, the recovery of oleuropein and rutin were increased from 54 % and 65 % to 80 % and 88 % by changing the adsorption conditions. Solid-liquid ratio was the most effective parameter in the adsorption of olive leaf antioxidants. As a result of this study, the purity of oleuropein and rutin were increased and polyphenol rich silk fibroin was obtained. In order to understand whether or not these polyphenols exhibit their properties on silk fibroin, their antioxidant analyses were performed. Since here, the liquid phase of the system was investigated. The solid phase of the system, polyphenol rich silk fibroin is investigated in the following parts.

5.2.4. Silk Fibroin Characterization Before and After Adsorption

5.2.4.1. Morphological Examination of Silk Fibroin

Scanning Electron Microscopy (SEM) analyses of silk fibroin were performed in order to examine its morphology and morphological changes before and after the adsorption process. Figure 5.19 shows the SEM photomicrographs of silk fibroin.

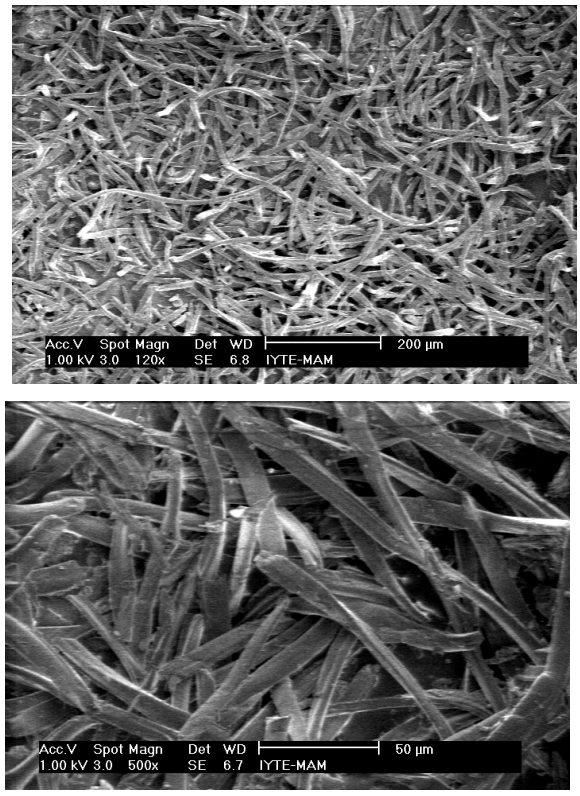


Figure 5.19. SEM micrographs of silk fibroin.

The taken SEM micrographs at different magnification (120x, 500x) show that the commercial silk fibroin used in this study was formed from approximately 90 µm. fibers in length and 12 µm. in width. These fibers were not regular in shape and in order. There wasn't any change between the SEM micrographs taken before and after adsorption so adsorption did not influence the morphology of silk fibroin.

5.2.4.2. FT-IR Analysis

Fourier Transform Infrared Spectroscopy (FTIR) analyses were performed for olive leaf extract, oleuropein, rutin and silk fibroin. The main goal of this analysis was to investigate the change in the molecular conformation and orientation of silk fibroin before and after adsorption of polyphenols and examine whether the characteristic peaks of oleuropein can be seen on silk fibroin after adsorption.

Firstly, IR spectrum of silk fibroin was examined. The secondary structure of *Bombyx mori* silk fibroin mainly consists of two major conformations, random coils, α-form (silk I) and β-sheet (silk II). Silk I is the water-soluble structure of silk fibroin whereas silk II is the water insoluble part of the silk fibroin. Since the used silk fibroin was highly hydrophobic, it was expected to observe amide bonds mainly attributed to β-sheet conformation.

The random coils show strong absorption bands in the region of 1655-1665 cm^{-1} (amide I), 1540-1542 cm^{-1} (amide II) and 1230-1235 cm^{-1} (amide III). On the other hand, the characteristic bands of β-sheet conformations appear in the region of 1628-1630 cm^{-1} (amide I), 1516-1533 cm^{-1} (amide II), 1265 cm^{-1} (amide III) and 700 cm^{-1} (amide V) (Um et al. 2003, Ayutsede et al. 2005, Nam and Park, 2000, Tsuboi et al. 2001, Li et al. 2003). Amide I is the vibrational transition bands of C=O stretching, whereas amide II and III are the bands of N-H de-formation and C-N stretching, respectively (Bayraktar et al. 2005).

As it is seen in Figure 5.20, silk fibroin showed strong absorption bands at 1629 cm^{-1} (amide I) and 1516 cm^{-1} (amide II) which were attributed to the silk II conformation. Moreover, the existence of absorption bands at 1265 cm^{-1} (amide III) and 700 cm^{-1} (amide V) also represented the β-sheet conformation of silk fibroin. Only the absorption band at 1228 cm^{-1} was attributed to the random coil conformation of silk fibroin.

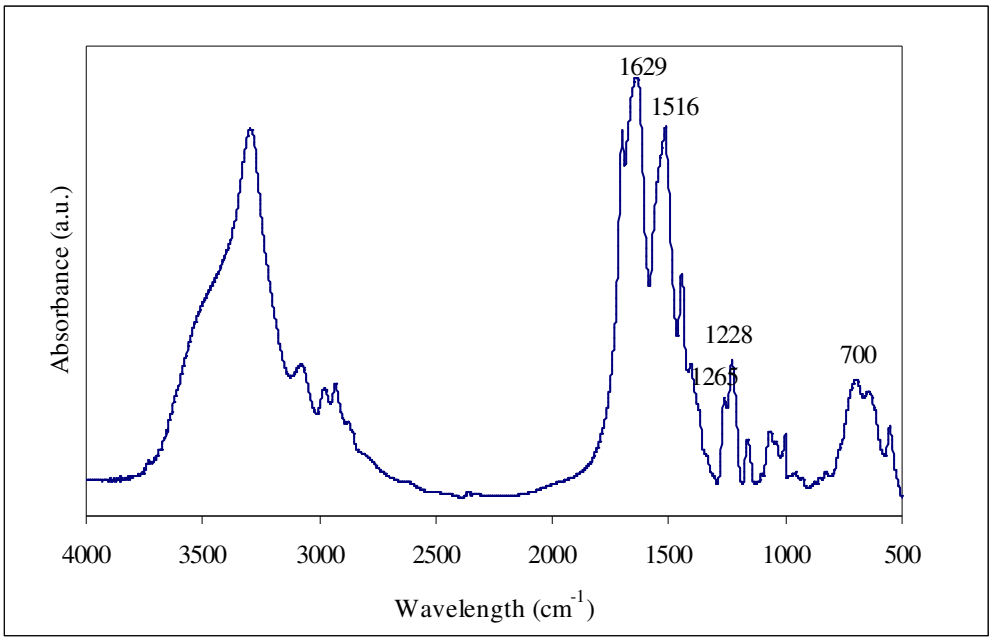


Figure 5.20. FTIR spectrum of silk fibroin.

In summary, the absorption bands attributed to β-sheet conformation were dominant compared to the random coil conformation, proving the hydrophobic character of silk fibroin.

Up to now, the liquid phase of the system was analyzed and there was not any proof that the adsorbed oleuropein and rutin really existed on the adsorbent. FTIR spectra of samples would be the proof of the adsorption process that showed the oleuropein and rutin adsorption on the solid phase. For this reason, silk fibroin was analyzed after adsorption. The IR spectrum of silk fibroin before adsorption was subtracted from the IR spectrum of silk fibroin after adsorption to see if any bands were left. In other words, IR spectrum of silk fibroin was taken as background to understand which peaks were belonged to the adsorbed polyphenols. In addition to this, analysis of olive leaf extract and oleuropein were performed in order to see which absorption bands were attributed to oleuropein in olive leaf extract. Figure 5.21 gives the FTIR spectra of olive leaf extract, oleuropein and adsorbed polyphenols.

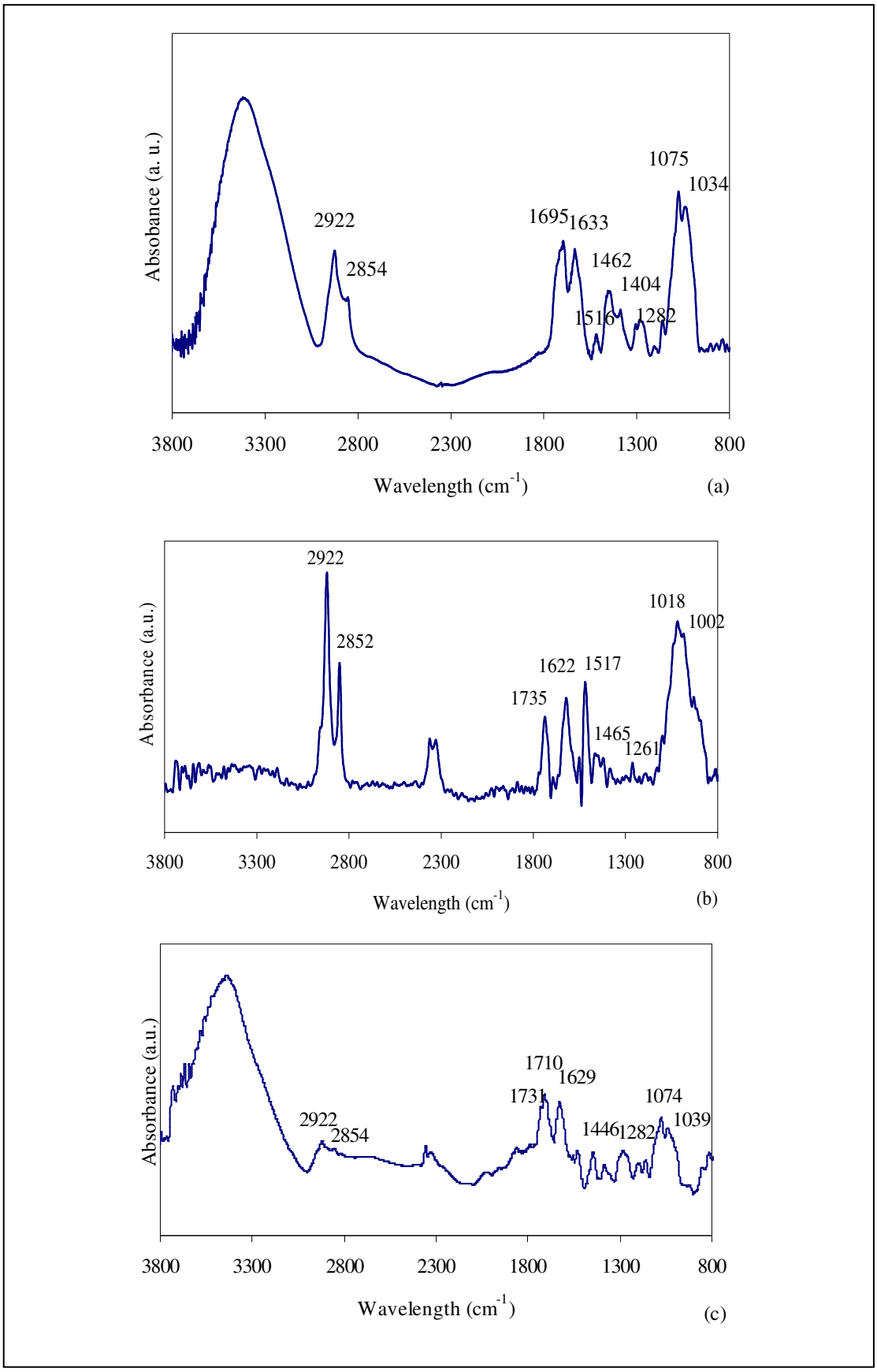


Figure 5.21. FTIR spectra of (a) olive leaf extract, (b) adsorbed polyphenols and (c) oleuropein.

The existence of absorption bands in the FTIR spectrum of adsorbed polyphenols proved the accumulation of oleuropein on the silk fibroin since silk fibroin was taken as background. The presence of peaks at certain adsorption bands in oleuropein, olive leaf extract and adsorbed polyphenols represented that the adsorption of oleuropein on silk fibroin was accomplished.

The appearance of C-H stretching bands in the region of 2852-2854 cm^{-1} and at 2922 cm^{-1} in the IR spectra of olive leaf extract and oleuropein shows that they are the characteristical bands of oleuropein. The observed peaks at 2922 and 2852 cm^{-1} in the spectrum of adsorbed polyphenols prove the adsorption of oleuropein on silk fibroin. The peaks at 1516 cm^{-1} and 1517 cm^{-1} in the spectrum of olive leaf extract and adsorbed olive leaf polyphenols represented the existence of aromatic rings.

C-O stretching in phenols produces an absorption band in the 1300-1100 cm^{-1} region. This band was appeared at 1282 cm^{-1} in both of the oleuropein and olive leaf extract whereas it was appeared at 1261 cm^{-1} in the adsorbed polyphenols. Furthermore, the C=O stretching was observed at 1710 cm^{-1}, 1731 cm^{-1} in the spectrum of oleuropein, at 1735 cm^{-1} in the spectrum of adsorbed polyphenols and at 1695 cm^{-1} in the spectrum of olive leaf extract. The broad strong peak between 3600-3200 cm^{-1} regions shows the O-H stretching. In the spectra, peaks at 1075, 1039 and 1002 cm^{-1} were responsible for C-O stretching. As a result, the observed principal bands in the spectra of oleuropein and olive leaf extract were also observed in the spectrum of adsorbed polyphenols, proving the oleuropein and rutin adsorption on silk fibroin (Stuart, 2004).

5.2.4.3. Color Analysis of Silk Fibroin

Plant polyphenols have colors and because of this property, they are used as natural colorants in the industry. In this study, the coloring property of olive polyphenols was also observed. Due to the initial concentration and adsorbed amount on silk fibroin, the color of the silk fibroin turned from white to dark yellow and light brown. The color change of the silk fibroin was measured by Avantes Avamouse and it was represented in a coordinate plane.

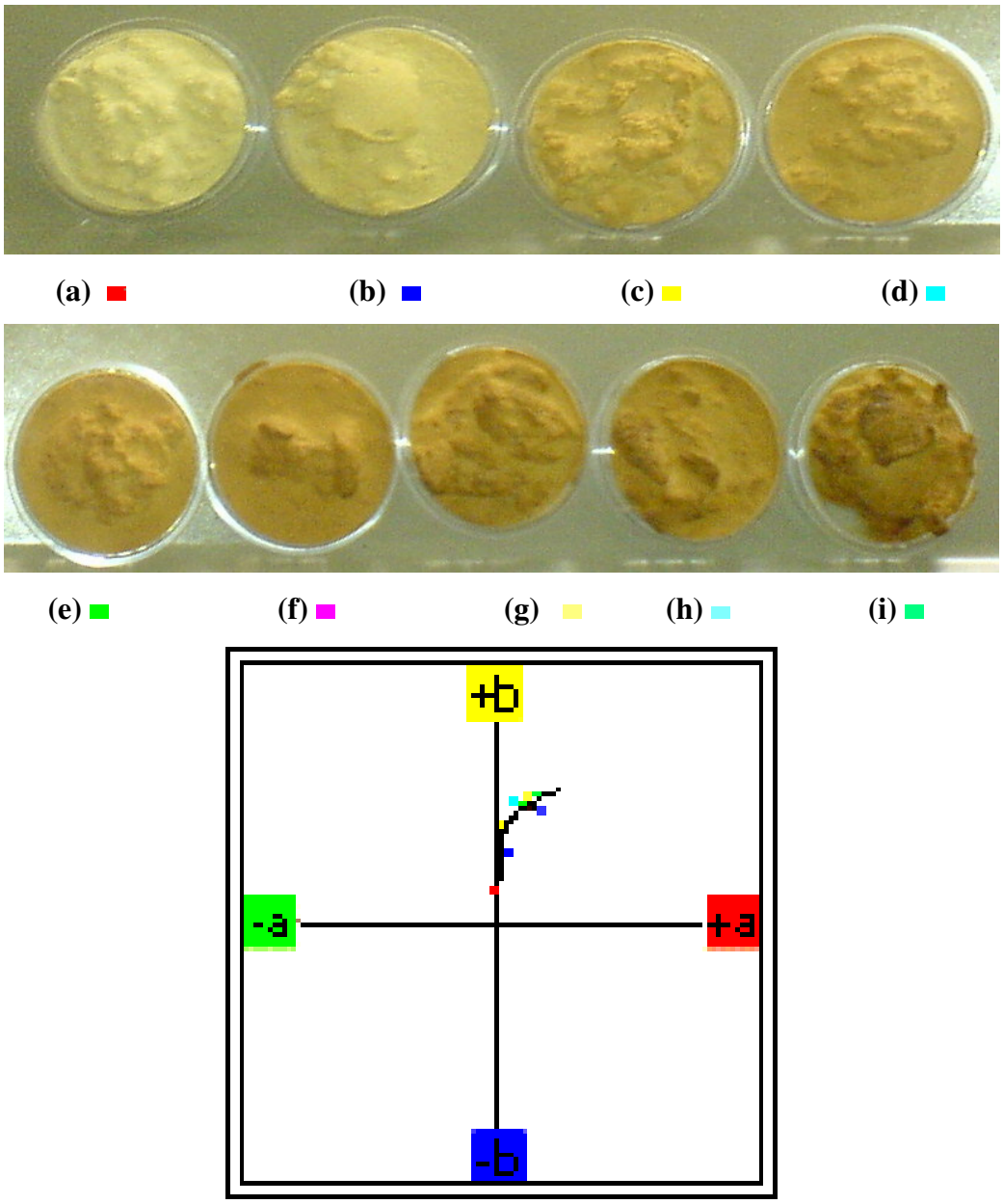


Figure 5.22.The color change of silk fibroin in (a) 5 mg/ml olive leaf extract (b) 10 mg/ml olive leaf extract (c)25 mg/ml olive leaf extract (d) 37.5 mg/ml olive leaf extract (e) 50 mg/ml olive leaf extract (f) 62.5 mg/ml olive leaf extract (g)75 mg/ml olive leaf extract (h) 87.5 mg/ml olive leaf extract (i) 100 mg/ml olive leaf extract.

As it is seen in Figure 5.22, the colors of the samples were designated by points and these points were connected by a line, showing the change in the color of silk fibroin. The color of the silk fibroin firstly increased sharply to dark yellow and then it

slightly changed to light brown and at the end, it reached a plateau. The shape of this line resembles the shape of the adsorption isotherms especially Langmuir Isotherm, determined for oleuropein and rutin. Thus, as the silk fibroin saturated with the adsorbates, its color also reached plateau.

The color change of the silk fibroin was also an indication of the adsorption. As a result, after adsorption process silk fibroin can be used as a natural colorant in the food, textile and leather industry.

5.2.5. Antioxidant Analysis of Adsorbed Polyphenols on Silk Fibroin

The aim of this study was to produce a protein based functional food having antioxidative and antimicrobial properties. In accordance with this aim, it should be known whether or not olive leaf polyphenols show their functionality after adsorption. Since it was impossible to find out the antioxidant capacity of solid phase including silk fibroin and olive polyphenols, it was dissolved in Ajisawa's reagent to determine the antioxidant activity.

Four samples were analyzed in UV-Visible Spectrophotometer. The first sample was Ajisawa solution, second was the olive leaf extract solution containing the same amount of adsorbed polyphenols, third was the adsorbed polyphenols and silk fibroin and the fourth was the silk fibroin solution. Ajisawa reagent did not exhibit any antioxidative activity and it did not induce the antioxidant activity of the samples. The percentage inhibition versus concentration curves of these samples are given in Appendix, in Figures C6, C7 and C8. The antioxidant activities of these samples were determined in terms of TEAC (mmol/g) and they are represented in Figure 5.23.

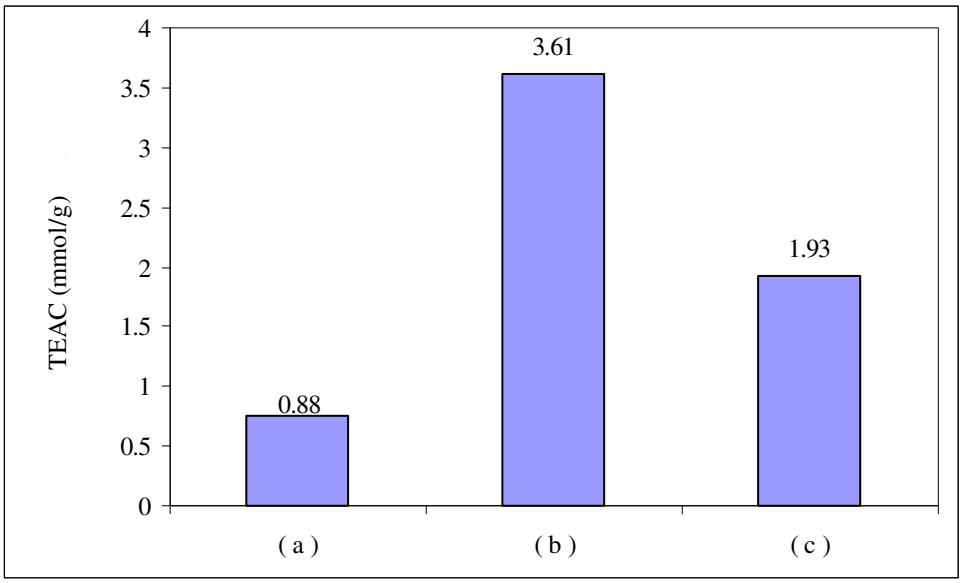


Figure 5.23. Antioxidant capacities of (a) Olive Leaf Extract in Ajisawa (b) Adsorbed Polyphenols and Silk Fibroin in Ajisawa (c)Silk Fibroin in Ajisawa.

Silk fibroin also exhibited antioxidant activity towards ABTS radical cation and its antioxidant capacity was increased from 1.93 TEAC mmol/g to 3.61 TEAC mmol/g after adsorption. On the other hand, the same amount of olive leaf extract as the adsorbed polyphenols exhibited 0.88 TEAC mmol/g, lower antioxidant activity compared to the adsorbed polyphenols on silk fibroin. Olive leaf phenolics exhibit inhibition in their radical scavenging capacity when they are together as in the case of olive leaf extract (Garcia et al. 2000). The purity of oleuropein and rutin in adsorbed polyphenols is higher compared with the olive leaf extract and they exhibited higher antioxidant activity.

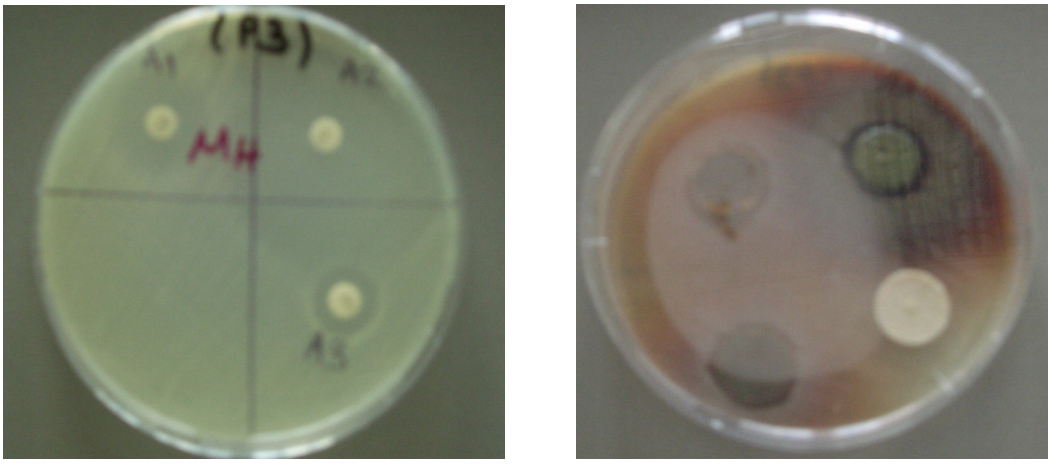
In the literature, the antioxidant capacities of proanthocyanidin complexes with polysaccharides and proteins were investigated and it was concluded that the antioxidant activity of the system in this kind of complex decreases because of the proanthocyanidin structure.

In this study, adsorption of olive leaf polyphenols increased the antioxidant activity of silk fibroin. Their effects on several pathogen bacteria were determined by antimicrobial tests.

5.2.6. Antimicrobial Tests of Adsorbed Polyphenols on Silk Fibroin

The antimicrobial tests of silk fibroin (SF), olive leaf extract (OLE), physical mixture of olive leaf extract with silk fibroin and adsorbed polyphenols on silk fibroin were investigated towards four different bacteria, *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae*. Furthermore, the effects of three different commercial antibiotics, gentamicin, penicillin, vancomycin towards these bacteria were studied as positive control.

The samples and antibiotics were all in pellet form with 14 mm and 6 mm in diameter, respectively. After one day, inhibition zones around the samples and antibiotics were observed. However, a color change was also observed around the olive leaf extract containing samples, which showed the diffusion of the polyphenolic structures. This is demonstrated in the Figure 5.24.



(a) (b)

Figure 5.24. Antimicrobial effect of (a) antibiotics and (b) samples towards *S. aureus.*

The inhibition zones of samples and antibiotics are given in Table 5.7. All the samples except silk fibroin demonstrated antimicrobial activity. Mainly samples were resistant towards *S. aureus*. Only the adsorbed polyphenols on silk fibroin inhibited the growth of *K. pneumoniae* among the samples.

Table 5.7. Antimicrobial Analysis.

	Inhibiton zone for Different Bacteria			
	E. coli	*K. pneumoniae*	*S. aureus*	*P. aeruginosa*
Olive Leaf Extract	-	-	19 mm	-
Physical mixture of OLE and SF	-	-	20 mm	-
Silk Fibroin	-	-	-	-
Adsorbed OLE on SF	-	16 mm	20 mm	-
Gentamicin	24 mm	8 mm	20 mm	21 mm
Penicillin	-	-	44 mm	-
Vancomycin	-	-	14 mm	-

The measured inhibition zone of adsorbed OLE on SF was the same with the physical mixture of OLE and SF. Thus, adsorption did not have any inverse effect on the antimicrobial activity of polyphenols. The inhibition effect of adsorbed OLE on SF towards *K. pneumoniae* probably was due to the selective adsorption of oleuropein. It was reported that oleuropein was the significant compound in the antimicrobial activity of olive leaves (Page 2002). Since the amount of oleuropein on SF was higher after adsorption, this sample hindered the growth of *K. pneumoniae.*

S. aureus is a gram-positive bacterium whereas other bacteria used in this study are gram-negative. The gram-positive bacteria are more sensitive to antimicrobial agents since they do not have outer membrane for their cell membrane. Since the effect of olive leaf polyphenols against gram-positive bacteria is higher compared to the gram-negative bacteria, they are probably causing inhibition of cell membrane function.

5.3. Desorption of Olive Leaf Antioxidants

The isolation and purification of antioxidants is a desired process for the food, cosmetics and pharmacological industry. New and low-cost commercial techniques have been searched especially for their use in dietary supplements. In this part of the study, the adsorption of oleuropein and rutin was accomplished in high percentages and they continued to exhibit their antimicrobial and antioxidative properties still on the silk

fibroin. If desorption of olive leaf antioxidants can be successfully achieved, then they will be recovered in large amounts from crude olive leaf extract.

Desorption of oleuropein and rutin could not be managed in water at 25°C. In order to provide it, temperature should be increased. Since there is a risk of denaturation at high temperatures, the temperature was not increased. For this reason, desorption was performed in 70 % aqueous ethanol solution. The desorption behavior of oleuropein ad rutin was followed during 4 days. The system reached equilibrium in 20 hours.

The maximum adsorption amounts of rutin and oleuropein (when the solid-liquid ratio was 0.025) were 21 mg rutin/g silk fibroin and 108 mg oleuropein/g silk fibroin. In desorption process, 17 mg rutin/g silk fibroin and 91.8 mg oleuropein/g silk fibroin were removed from the adsorbent. Thus, 81% of rutin and 85 % of oleuropein were removed from the adsorbent surface. As a result of this study, 1 g silk fibroin can provide the removal of 69 % of oleuropein and 93 % of rutin from 1 g olive leaf extract.

After desorption, the color of the silk fibroin again turned to light yellow as shown in Figure 5.25. This also represents that the total removal of antioxidants was not accomplished by desorption. Maybe other solvents could be used to achieve 100 % desorption but ethanol was the safest solvent.

Figure 5.25. The color of the silk fibroin after desorption.

The antioxidant capacity of desorbed polyphenols was analyzed to control whether or not they lost their antioxidant property during desorption process. It was compared with the antioxidant capacity of the olive leaf extract containing the same amount of desorbed polyphenols.

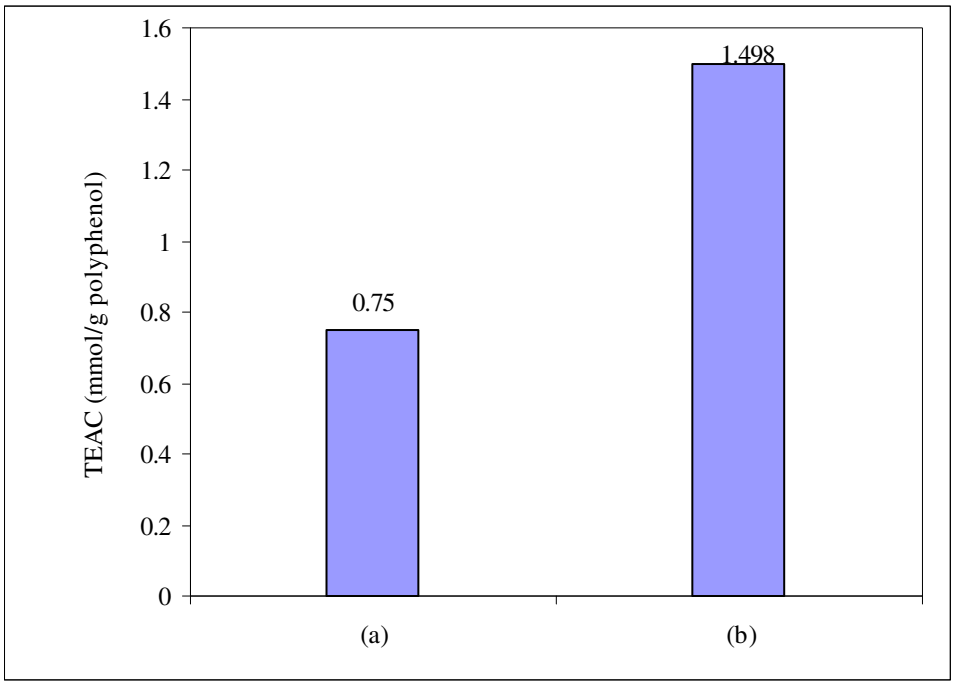


Figure 5.26. Antioxidant Capacities of (a) Desorbed amount of polyphenols in OLE (b) Desorbed polyphenols.

The antioxidant activity of desorbed polyphenols was nearly two times fold of the olive leaf extract (containing the same amount of desorbed polyphenols). Similar result was obtained in the investigation of the antioxidant capacity of adsorbed polyphenols. Consequently, purification of oleuropein and rutin increased their antioxidant capacity. Because of inhibition effect, olive leaf extract could not exhibit as much antioxidant capacity as the partially purified polyphenols.

Furthermore, desorption and adsorption processes did not affect the antioxidant capacity of polyphenols since they were done under control and at low temperatures. If Figure 5.23 and 5.26 are investigated together, the synergistic effect between the olive leaf antioxidants and silk fibroin can be understood. The antioxidant capacity of desorbed olive leaf antioxidants was 1.5 TEAC mmol/g. Antioxidant capacity of silk fibroin was increased from 1.93 TEAC mmol/g to 3.61 TEAC mmol/g after adsorption. Consequently, the antioxidant capacity of the system was increased after adsorption.

CHAPTER 6

CONCLUSION

Olive leaf is a potent antioxidant having antimicrobial, anti-inflammatory and antiviral properties due to its major polyphenols. This study focused on the two major polyphenols of olive leaf, oleuropein and rutin. Extraction, adsorption and desorption of olive leaf antioxidants were performed successively to increase the purity of oleuropein and rutin.

Firstly, the effects of solvent and temperature on extraction were determined by HPLC and antioxidant analyses. Both of these analyses demonstrated that the yield of extraction was maximum in 70 % aqueous ethanol solution at 25 °C. The extracted one gram of olive leaf at these conditions contained 134.4 mg oleuropein and 18.25 mg rutin and its antioxidant capacity was determined as 7.52 mmol of TEAC/g olive leaf extract.

The adsorption process was performed in both 70 % aqueous ethanol solution and water to understand the biopolymer-polyphenol interactions. The yield of adsorption was higher in water than the yield in ethanol solution. This demonstrated that the accumulation of nonpolar compounds on the hydrophobic silk fibroin in water was probably due to the strong hydrophobic interactions between silk fibroin and olive leaf polyphenols.

The effects of temperature, pH and solid-liquid ratio on adsorption were investigated. The adsorption behavior of oleuropein and rutin were observed at temperatures between 25°C and 35°C within the pH range of 2.4 and 7.6. Furthermore, the solid-liquid ratio was changed from 0.025 to 0.1. The adsorption isotherms of oleuropein and rutin were plotted at these conditions by using the HPLC analysis. The experimental data of these isotherms were well fitted to Langmuir model. The maximum adsorbed amount of oleuropein and rutin was obtained at 25°C in deionized water at pH of 6 when the solid-liquid ratio was 0.025. The maximum adsorption capacities at these conditions were calculated as 108 mg oleuropein/g silk fibroin and 21 mg rutin/g silk fibroin.

The adsorption of olive leaf antioxidants on silk fibroin were confirmed by FTIR, color, antimicrobial and antioxidant analyses. The principal bands observed in

the IR spectra of oleuropein and olive leaf extract were also observed in the adsorbed polyphenols on silk fibroin. This proved the accumulation of certain polyphenolic compounds on silk fibroin.

The color of the silk fibroin was changed from white to yellow to light brown due to the adsorbed amount of oleuropein and rutin during the adsorption process. This color change was investigated by Avantes Spectrocam and represented in a coordinate plane by points. These points demonstrated a sharp increase in the beginnings and it reached a plateau at the end similar to Langmuir isotherm.

Silk fibroin alone showed antioxidative activity whereas it did not exhibit antimicrobial activity against any bacteria. After adsorption, the antioxidative property of silk fibroin increased from 1.93 mmol TEAC/g to 3.61 mmol TEAC/g. Moreover, silk fibroin gained antimicrobial activity against *Staphylococcus aureus* and *Klebsiella pneumoniae* after adsorption of olive leaf antioxidants.

Desorption studies were performed in 70 % aqueous ethanol solution at 25°C in order to increase the purity of oleuropein and rutin. 81 % of rutin and 85 % of oleuropein were removed from the adsorbent surface. Their antioxidant analyses was performed after desorption. Desorbed polyphenols exhibited higher antioxidant capacity than the same amount of olive leaf extract. Increase in the purity increased the antioxidant capacity of olive leaf extract since it eliminated the inhibition effect in crude olive leaf extract.

In conclusion, silk fibroin was determined as a promising protein in the production of antioxidative and antimicrobial functional food or dietary supplement after adsorption of olive leaf antioxidants. Furthermore, it can be used in the further purification of olive leaf antioxidants from the crude olive leaf extracts.

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APPENDIX A

CALIBRATION METHODS

Calibration was required to calculate the amount of oleuropein and rutin in olive leaf extract. External calibration and internal calibration methods were applied in this study.

In external calibration, a stock solution of rutin and oleuropein were prepared. Oleuropein was dissolved in 50 % acetonitrile-water solution at 25 °C whereas rutin was dissolved in 70 % ethanol-water solution at 35 °C. Different concentrations of oleuropein and rutin were prepared by diluting the stock solution. Each concentration was injected to HPLC. The responses of HPLC, in terms of area were recorded due to the changing concentrations. The concentration versus area curves for both of the polyphenols were plotted to form the calibration curves.

Coumarin was used as an internal standard. The reasons for choosing coumarin as internal standard are:

- Coumarin is not found in olive leaves.
- It does not react with any phenolics in olive leaves.
- Its retention time in HPLC is closer to the retention times of oleuropein and rutin
- Its structure is similar to the structure of polyphenols in olive leaves.

In internal calibration method, stock solutions for oleuropein, rutin and coumarin were prepared. Coumarin was dissolved in 50 % acetonitrile-water solution. Different concentrations of oleuropein and rutin were prepared. For the oleuropein calibration, a solution containing 60 % oleuropein and 40 % coumarin was prepared at each injection. The same procedure was also done for rutin calibration. Then the responses of HPLC at different concentrations were recorded. The ratio of the area of oleuropein to area of coumarin versus concentration of oleuropein was plotted for oleuropein calibration whereas the ratio of the area of rutin to area of coumarin versus rutin concentration was plotted for rutin calibration curve.

The calibration curves of these samples are given in Appendix B. Each data point in these curves were repeated three times and their standard deviations are shown on the points.

APPENDIX B

CALIBRATION CURVES

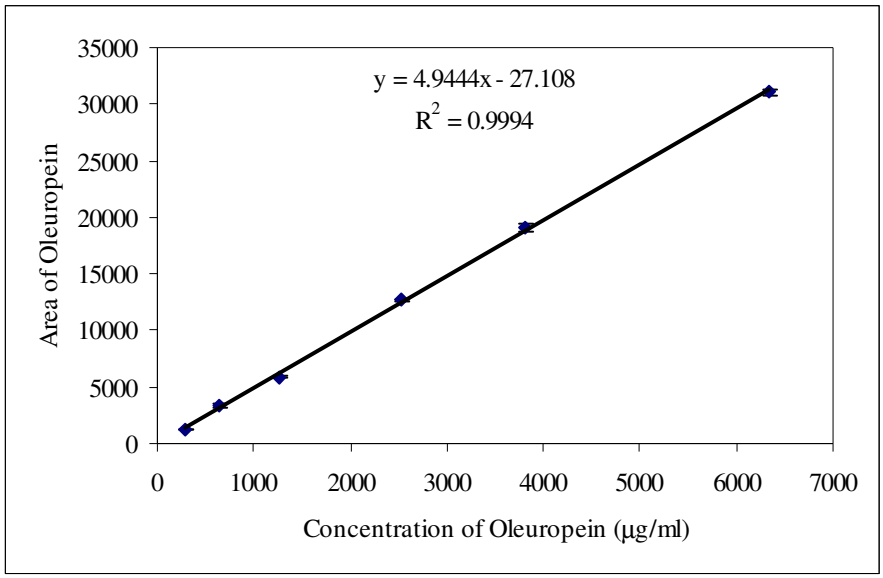

Figure B1. Oleuropein External Calibration Curve

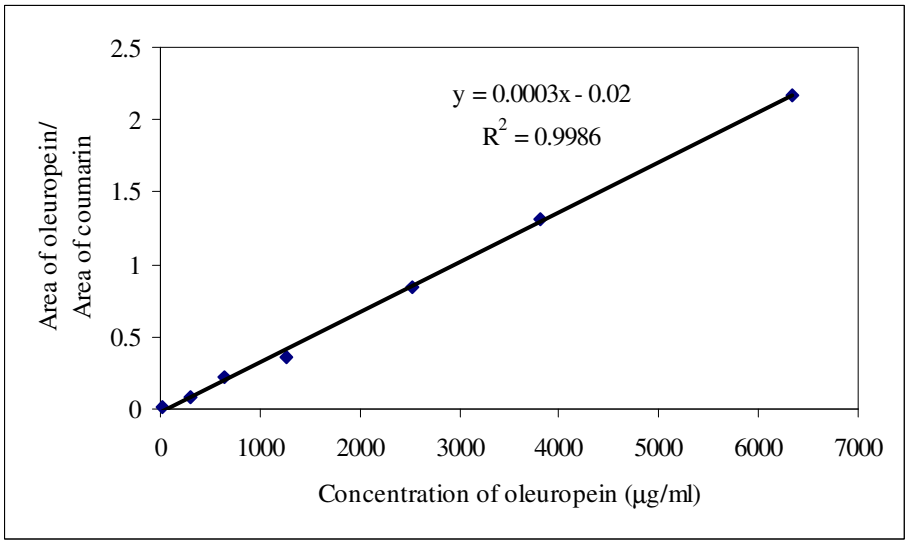

Figure B2. Oleuropein Internal Calibration Curve

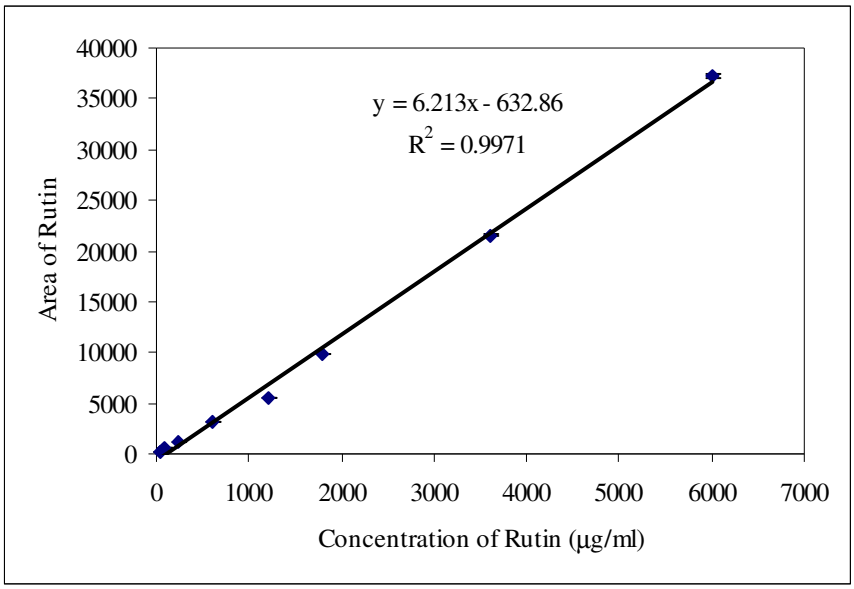

Figure B3. Rutin External Calibration Curve.

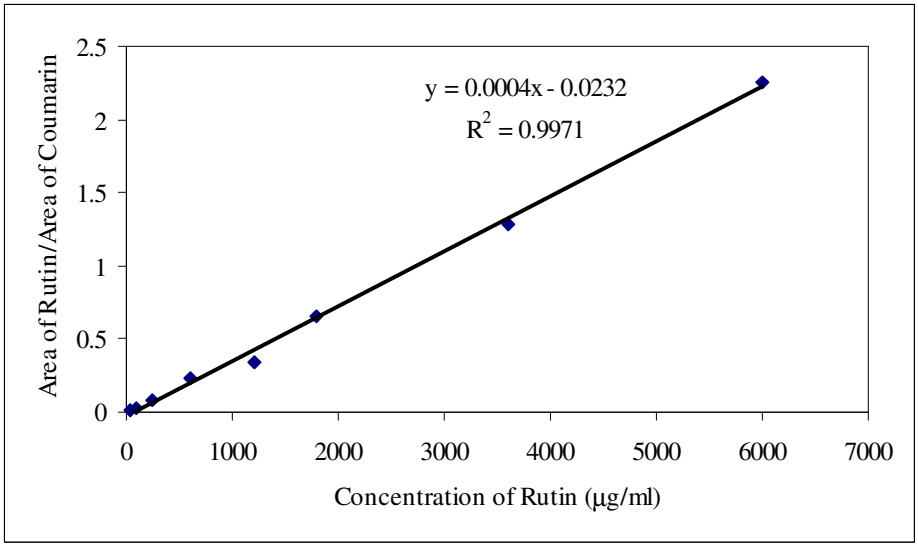

Figure B4. Rutin Internal Calibration Curve.

Sample Calculation:

In order to find out the amount of oleuropein and rutin in olive leaf extract, the responses of HPLC in terms of areas were recorded. By using these areas and calibration curves, their concentrations were calculated. For instance, when olive leaf prepared in the solid-liquid ratio of 1/20 was injected to HPLC, the HPLC response for

oleuropein was 22815. The equation of external calibration curve for oleuropein is:

y = 4.94 x-27.108

where,

y = area

x = concentration

22815 = 4.94 x -27.108

x = 4624 µg/ml = 4.624 mg/ml.

The solid-liquid ratio was 1/20 so one gram of olive leaf contains 92.5 mg oleuropein.

The oleuropein and rutin content of olive leaf extract were analyzed before and after adsorption in HPLC. In order to find out the adsorbed amounts, the responses in terms of areas were converted to concentrations by using the calibration curves. Then the following equation was used.

$$q = (C_i - C) \times \frac{V}{m} \quad \text{(A.1)}$$

Where,

q = Equilibrium solute phase concentration, mg polyphenol/g silk fibroin

C_i = Initial liquid phase concentration, mg polyphenol/ml olive leaf extract

C = Equilibrium solute concentration in the aqueous phase, mg polyphenols/ml olive leaf extract

V = Volume of liquid phase, ml

m = Mass of the adsorbent, g

When adsorption was performed at 25°C, in water and the solid-liquid ratio was set to 0.05, the initial concentration and equilibrium concentration were determined as 6.7 mg/ml and 3.65 mg/ml, respectively by using calibration curves. Then the adsorbed amount can be calculated as follows.

$$q = (6.7 - 3.65) \times \frac{1}{0.05}$$

q = 61 mg oleuropein/g silk

The same calculations were done for all of the parameters, initial concentration, solid-liquid ratio, temperature and pH.

APPENDIX C

ANTIOXIDANT CAPACITY CURVES

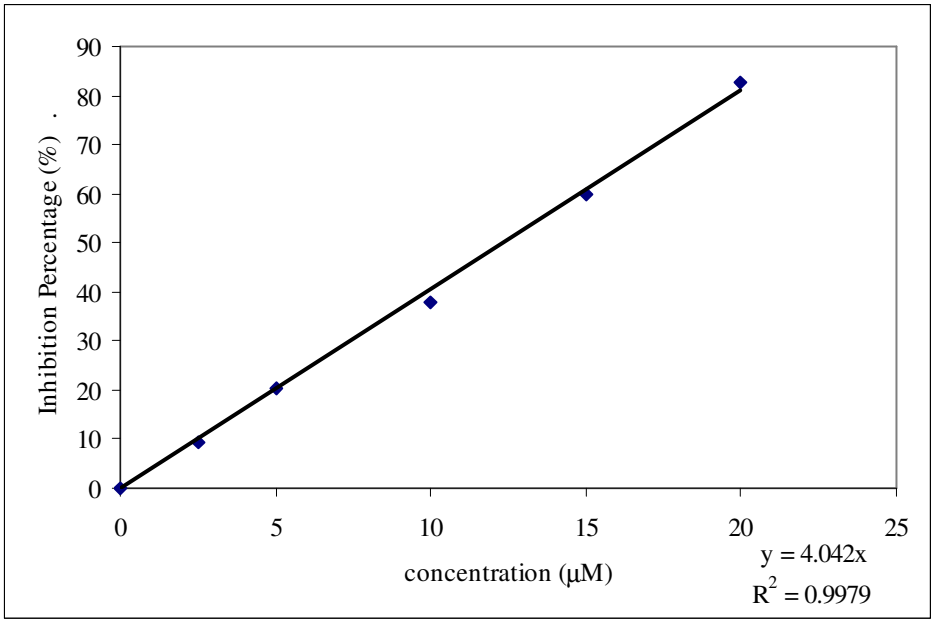

Figure C1. Calibration curve for trolox.

Sample Calculation for percentage inhibition:

The olive leaf polyphenols scavenge the ABTS radical cations and they exhibit inhibition. This inhibition can be observed as a decrease in the absorbance values at 734 nm in UV-Visible Spectrophotometer. Firstly, the absorbance of ABTS radical cation solutions was measured. It was adjusted to absorbance of 0.7 ± 0.02. Then olive leaf extract solution was added to ABTS radical cation solution and the absorbance values were taken at each 1 minute during 6 minutes. Average of these 6 data was taken and its decrease from the absorbance value of ABTS radical cation solution was calculated in order to find out percentage inhibition.

In one of the samples, absorbance value of ABTS radical cation was 0.6694. The six measured absorbance values after adding the olive leaf extract solution were, 0.4154, 0.4055, 0.3993, 0.3921, 0.387 and 0.3819. The average of these values were

calculated as 0.396. Then the inhibition percentage is,

$$\frac{(0.6694-0.396)}{0.6694\times100}=40.71\,\%$$

The inhibition percentage of samples were measured at least three different concentrations and at three replicates.

Antioxidant Capacity Curves

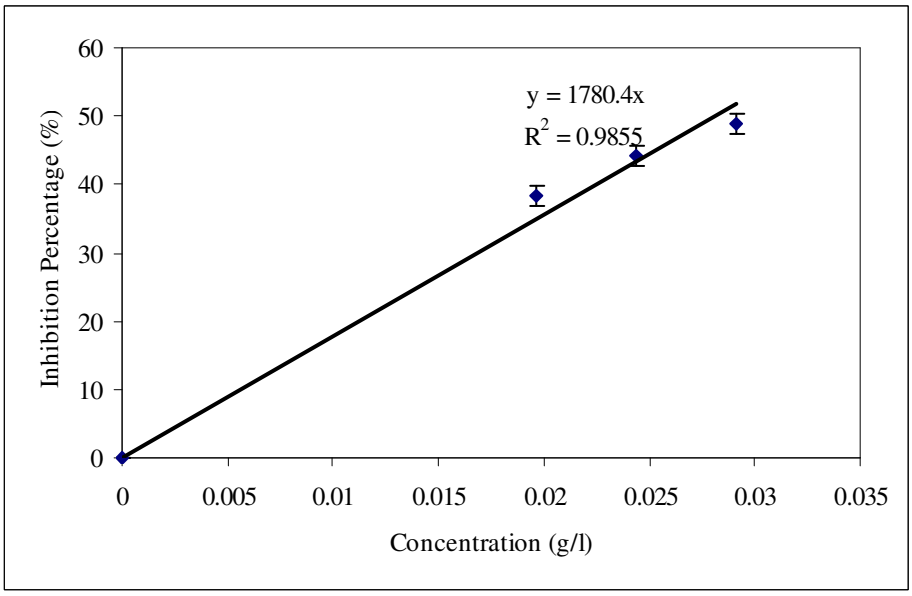

Figure C2. Inhibition percentage of olive leaf in 70 % ethanol-water.

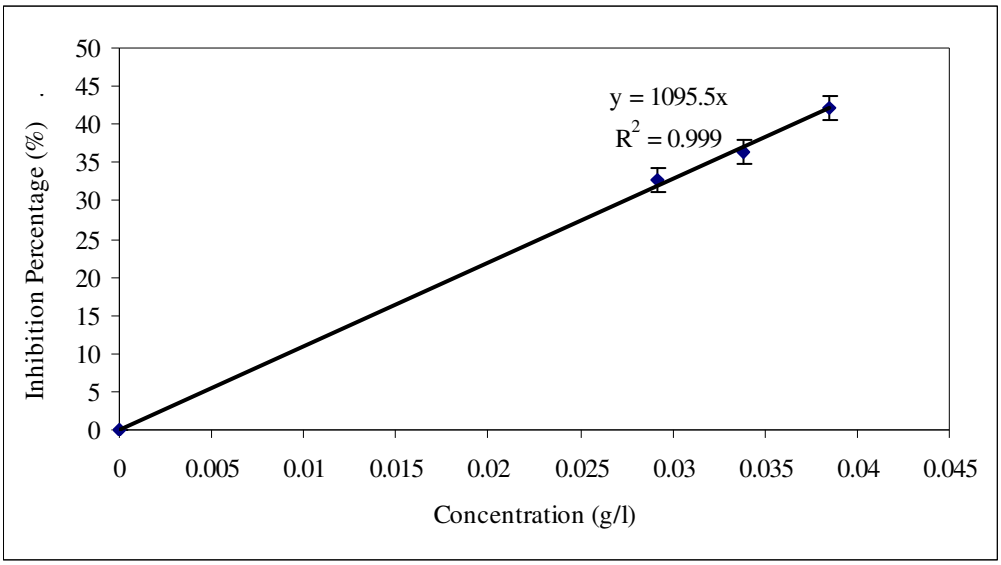

Figure C3. Inhibition percentage of olive leaf in 50 % ethanol-water.

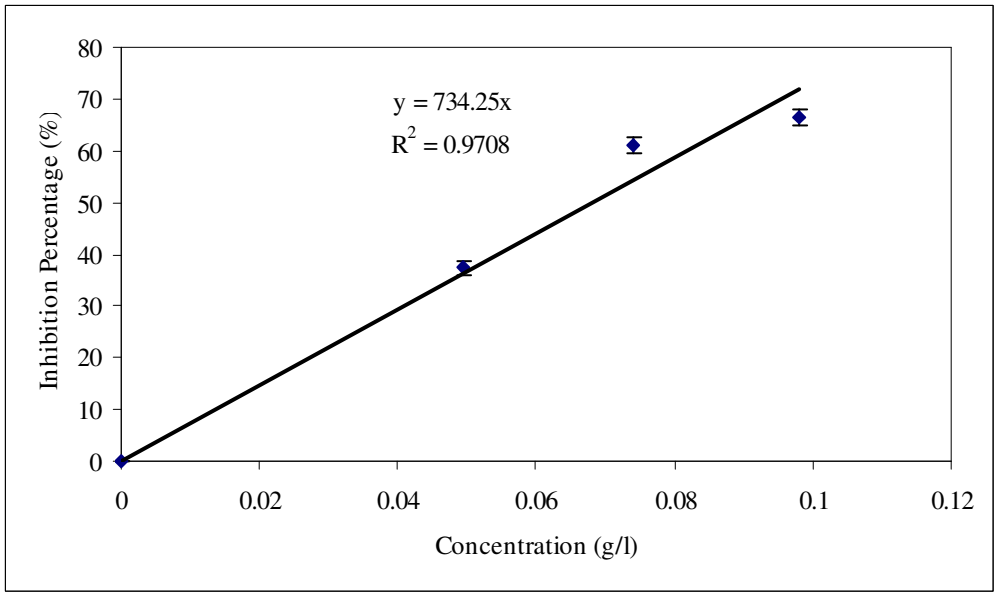

Figure C4. Inhibition percentage of olive leaf in 70 °C water.

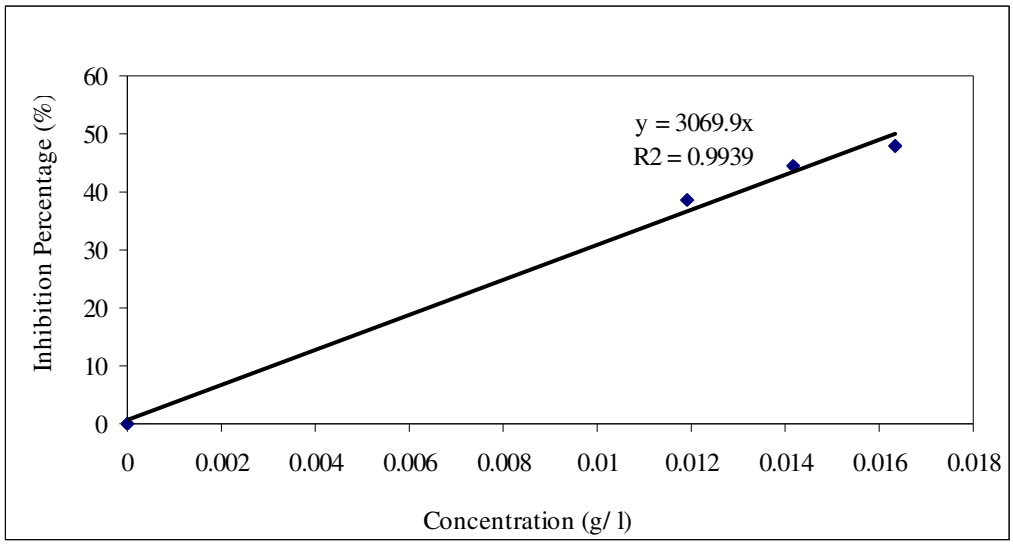

Figure C5. Inhibition percentage of olive leaf extract in Ajisawa.

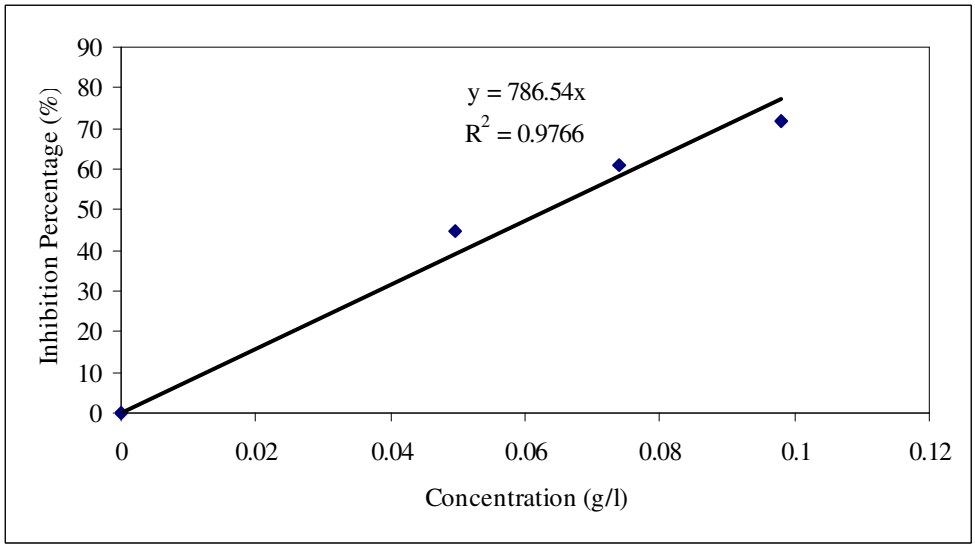

Figure C6. Inhibition percentage of silk fibroin in Ajisawa.

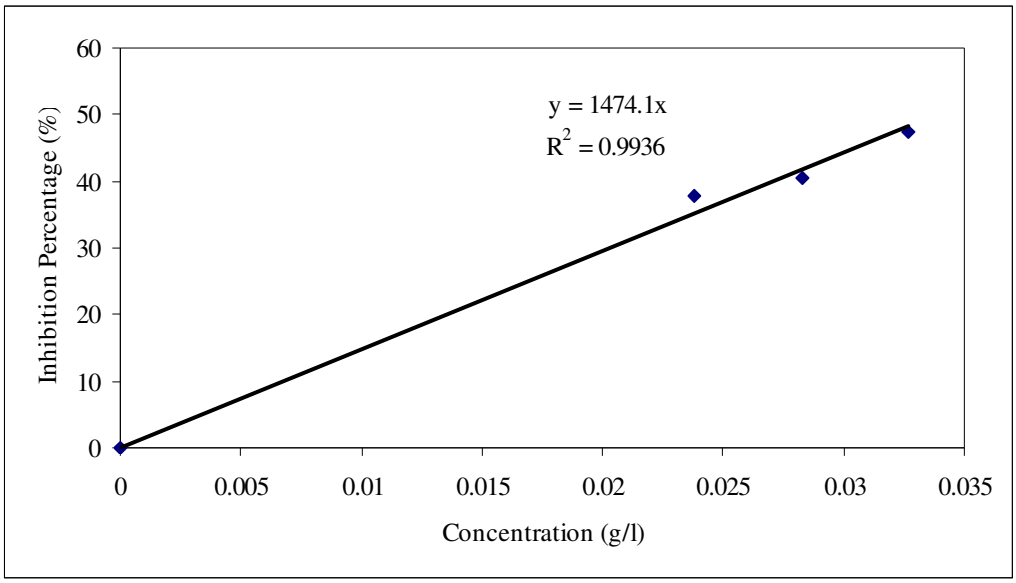

Figure C7. Inhibition percentage of adsorbed polyphenols and silk fibroin in Ajisawa.

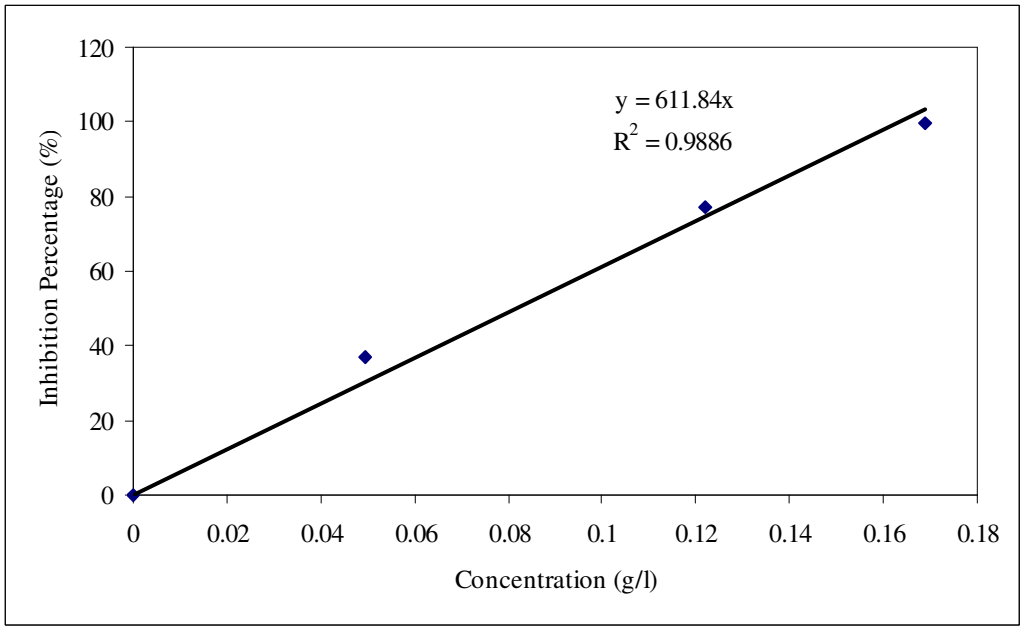

Figure C8. Inhibition percentage of desorbed polyphenols in 70 % ethanol-water.

APPENDIX D

LANGMUIR MODEL CURVES

Langmuir Model Curves of Oleuropein

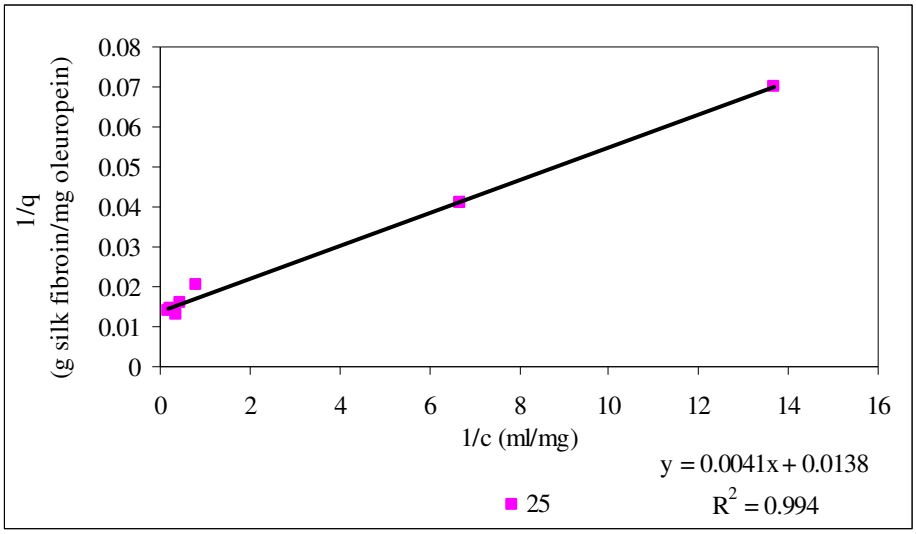

Figure D1.Langmuir model curve of oleuropein when temperature is at 25 °C, S/L=0.05 and pH = 6

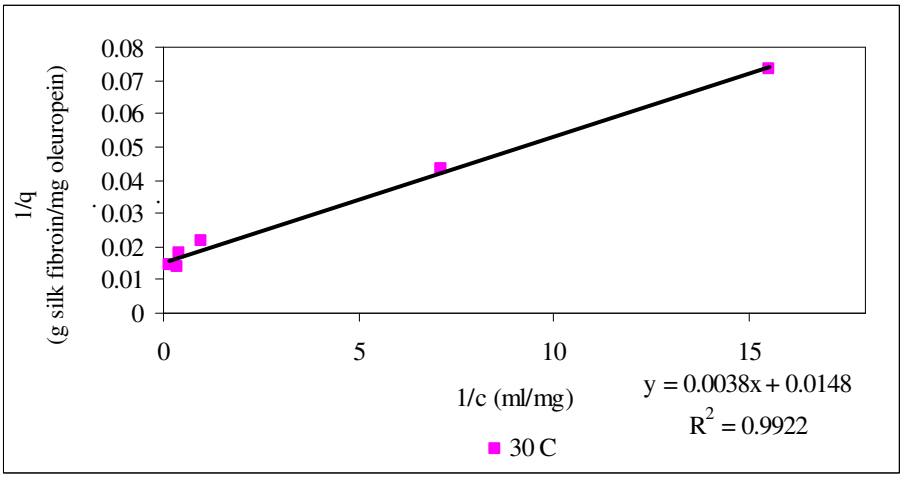

Figure D2. Langmuir model curve of oleuropein when temperature is at 30 °C, S/L=0.05 and pH = 6

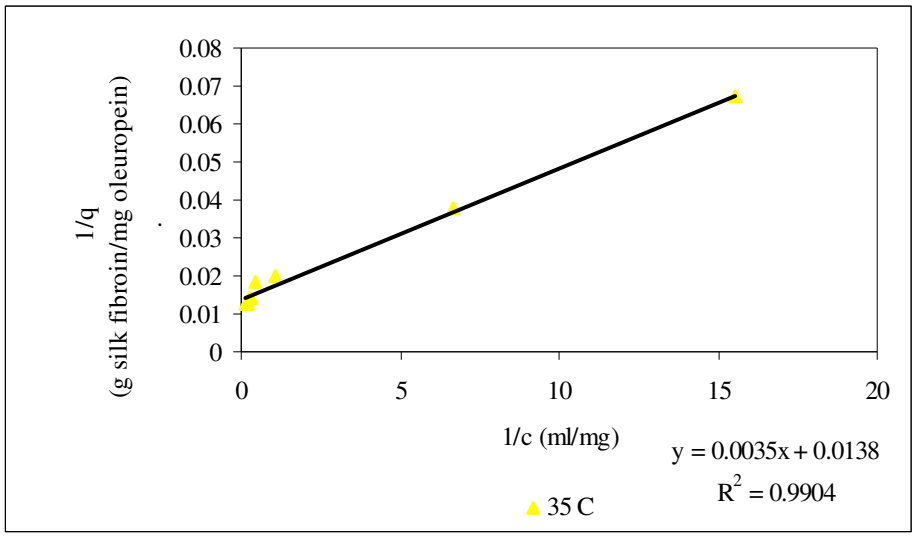

Figure D3. Langmuir model curve of oleuropein when temperature is at 35 °C, S/L= 0.05 and pH = 6

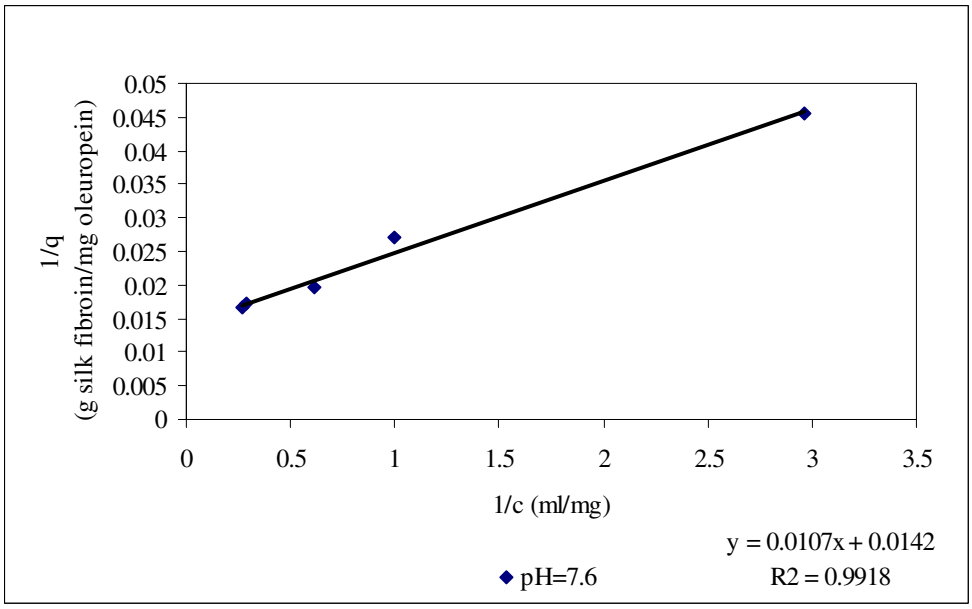

Figure D4. Langmuir model curve of oleuropein when temperature is at 25 °C, S/L=0.05 and pH = 7.6.

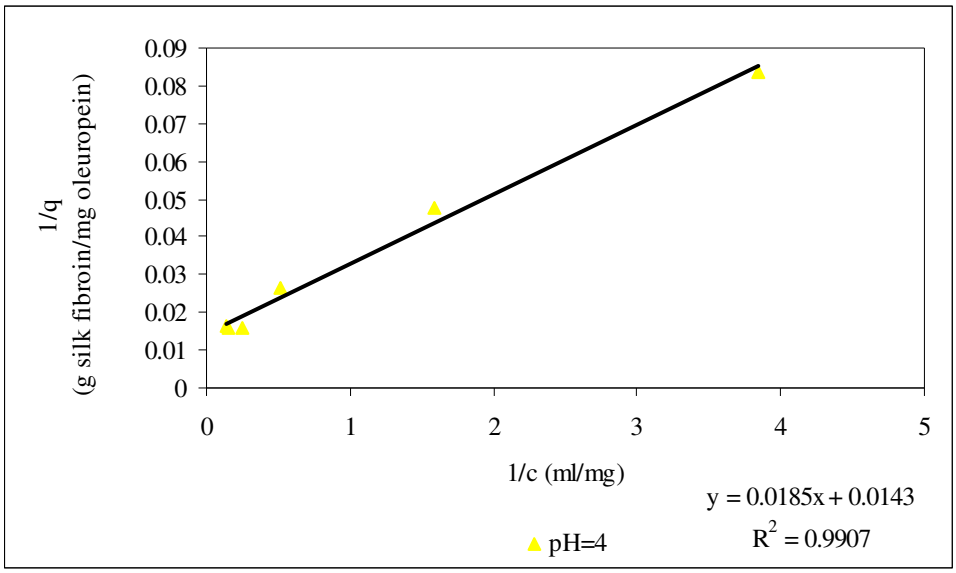

Figure D5. Langmuir model curve of oleuropein when temperature is at 25 °C, S/L= 0.05 and pH = 4

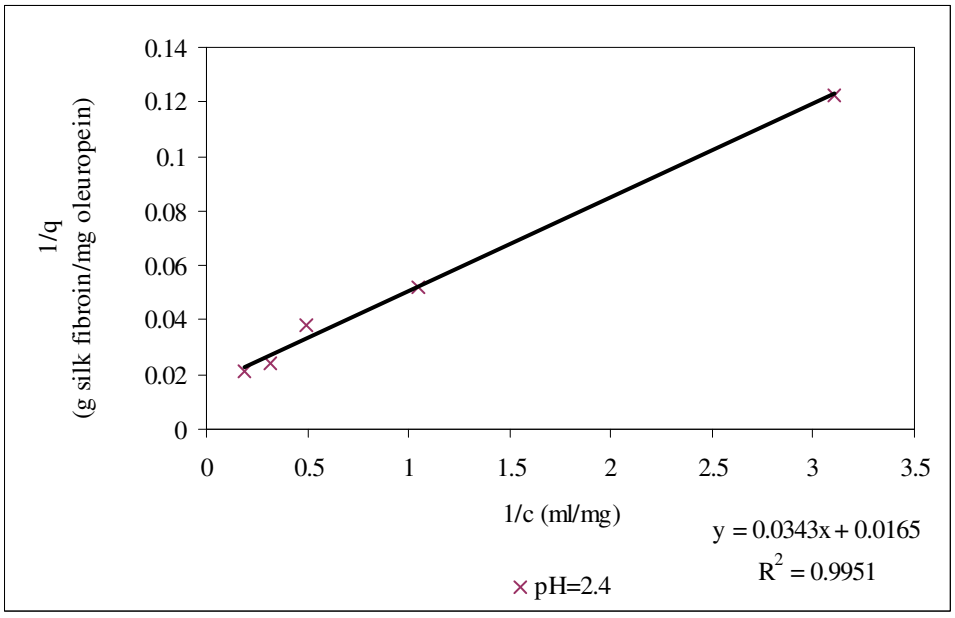

Figure D6. Langmuir model curve of oleuropein when temperature is at 25 °C, S/L= 0.05 and pH = 2.4

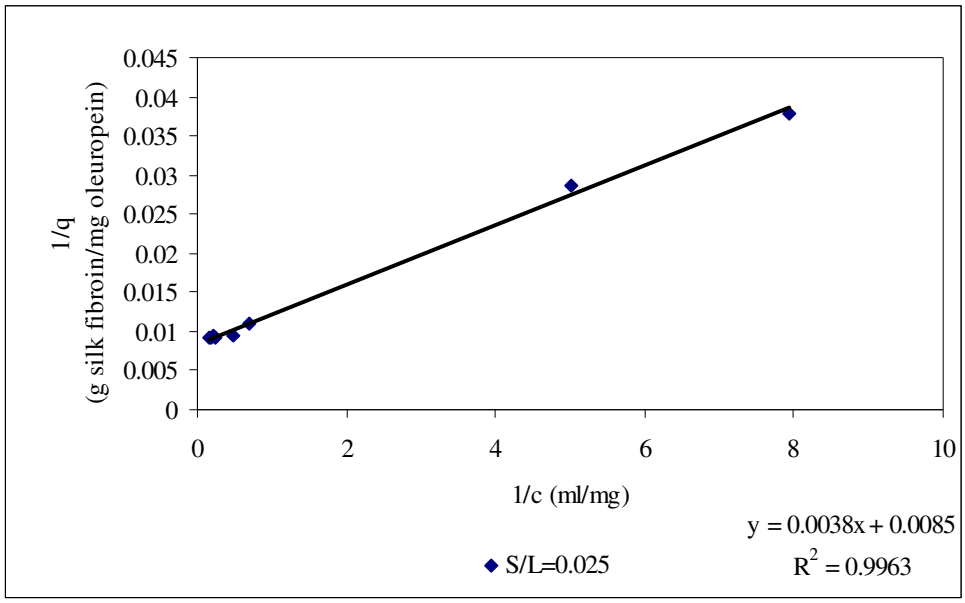

Figure D7. Langmuir model curve of oleuropein when temperature is at 25 °C, S/L= 0.025 and pH = 6

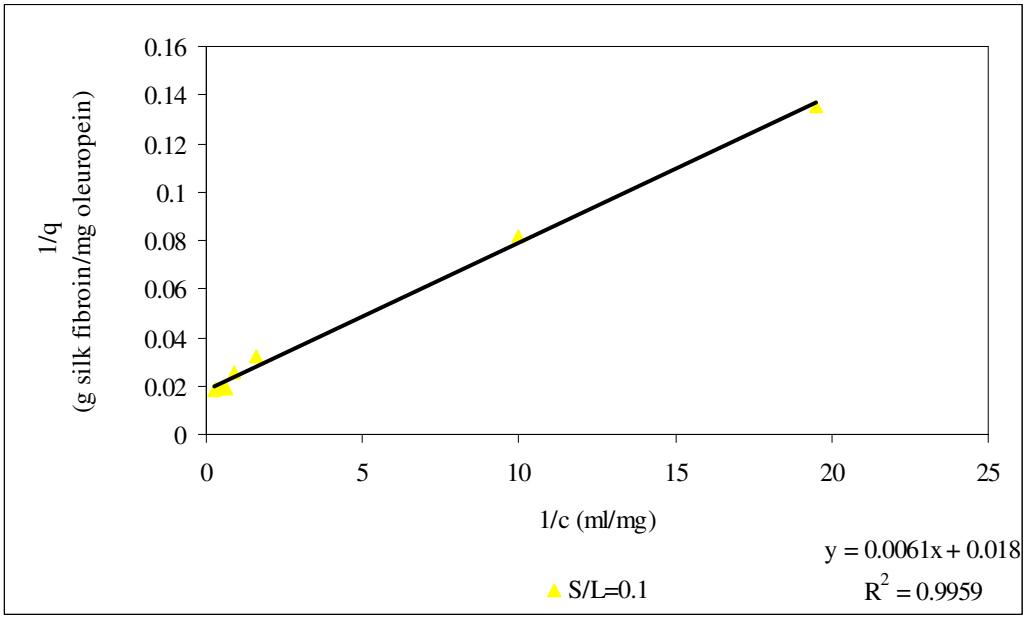

Figure D8. Langmuir model curve of oleuropein when temperature is at 25 °C, S/L= 0.1 and pH = 6

Langmuir Model Curves of Rutin

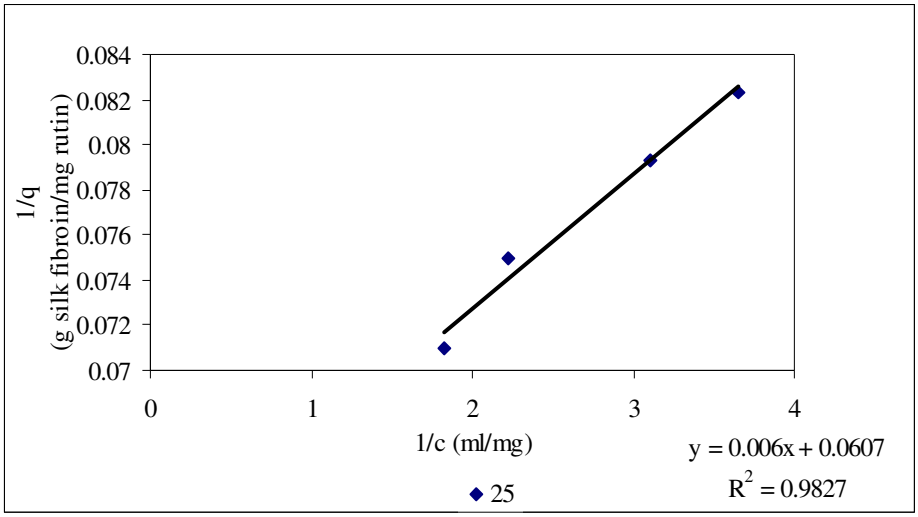

Figure D9. Langmuir model curve of rutin when temperature is at 25 °C, S/L= 0.05 and pH = 6

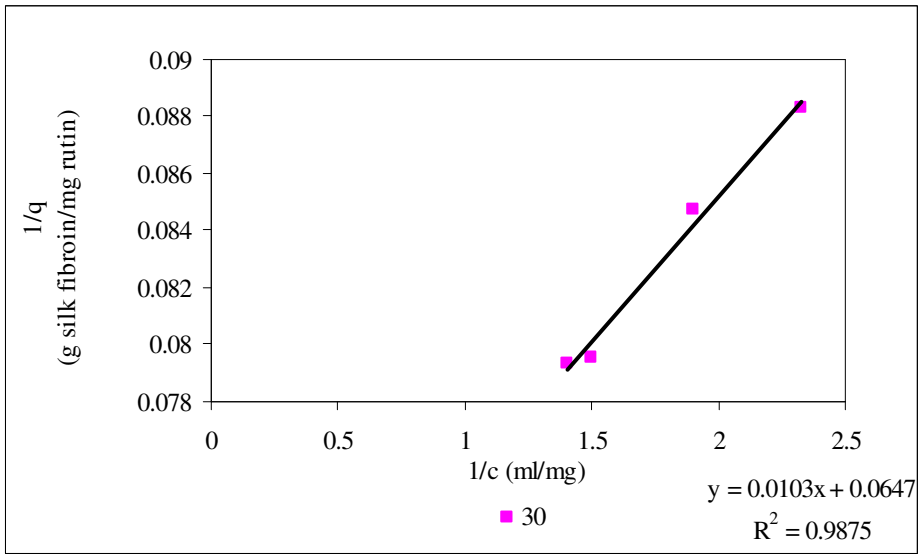

Figure D10. Langmuir model curve of rutin when temperature is at 30 °C, S/L= 0.05 and pH = 6

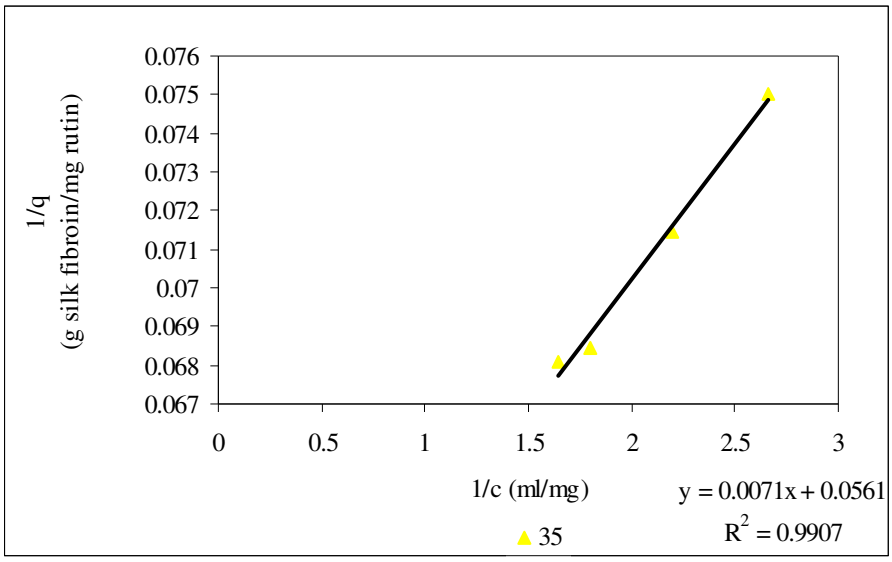

Figure D11. Langmuir model curve of rutin when temperature is at 35 °C, S/L= 0.05 and pH = 6

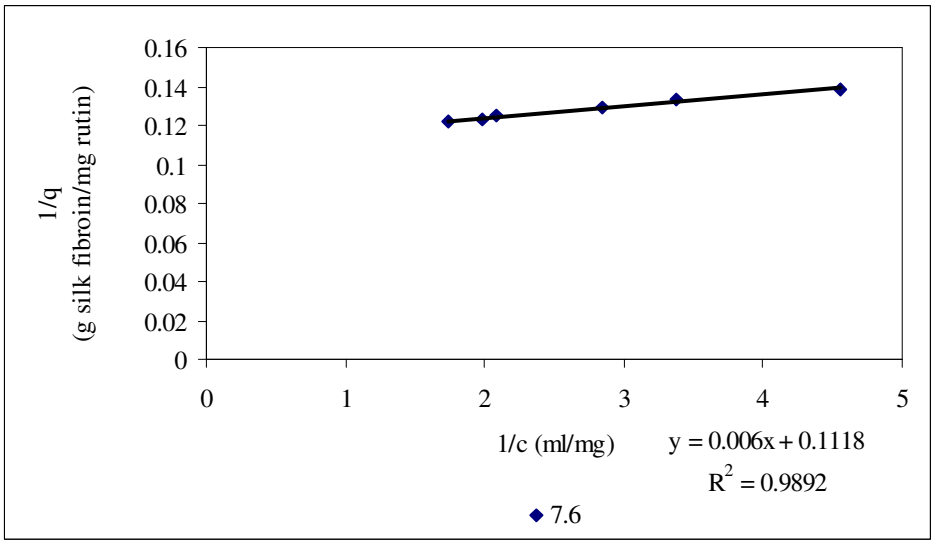

Figure D12. Langmuir model curve of rutin when temperature is at 25 °C, S/L= 0.05 and pH = 7.6

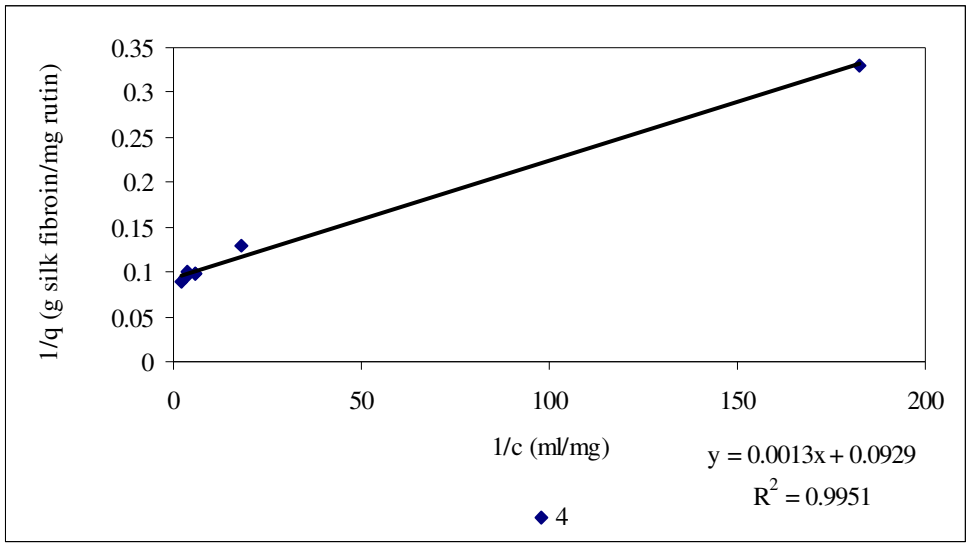

Figure D13. Langmuir model curve of rutin when temperature is at 25 °C, S/L= 0.05 and pH = 4

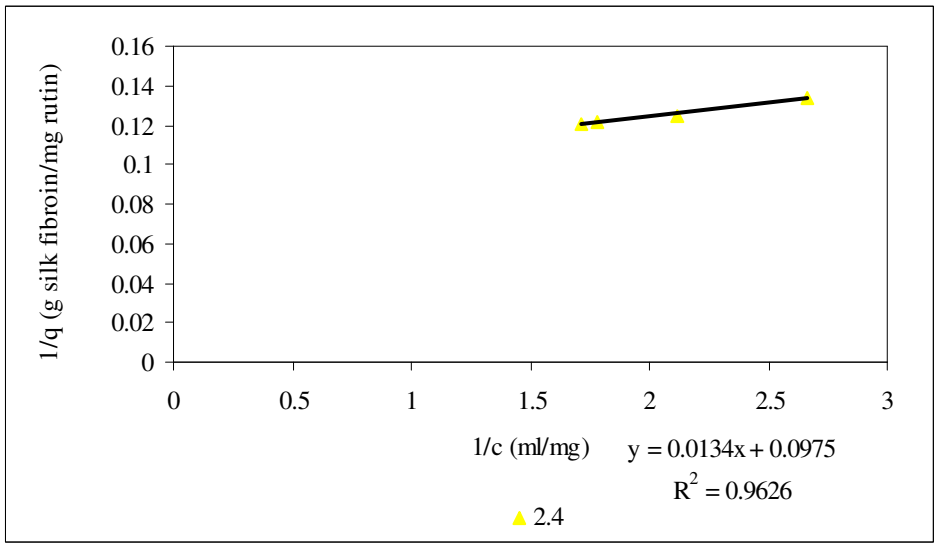

Figure D14. Langmuir model curve of rutin when temperature is at 25 °C, S/L= 0.05 and pH = 2.4

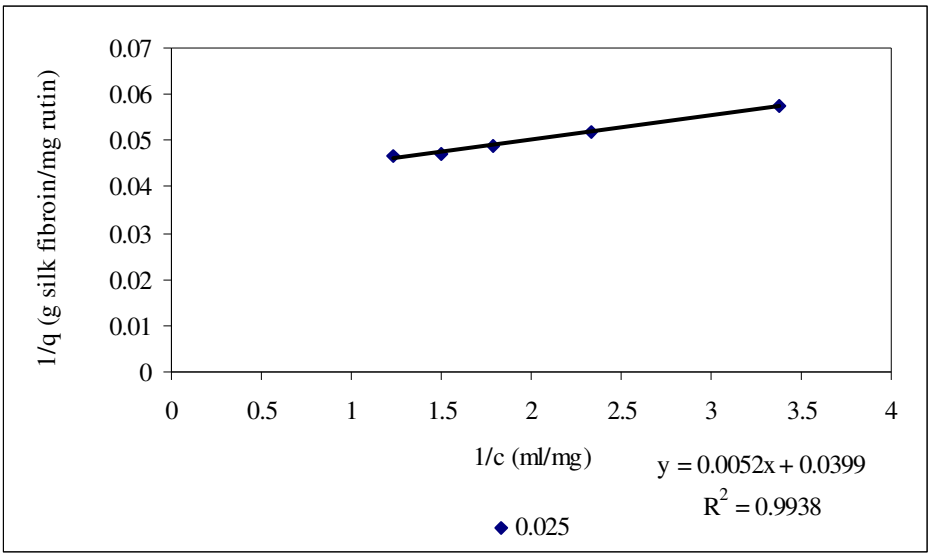

Figure D15. Langmuir model curve of rutin when temperature is at 25 °C, S/L= 0.025 and pH = 6

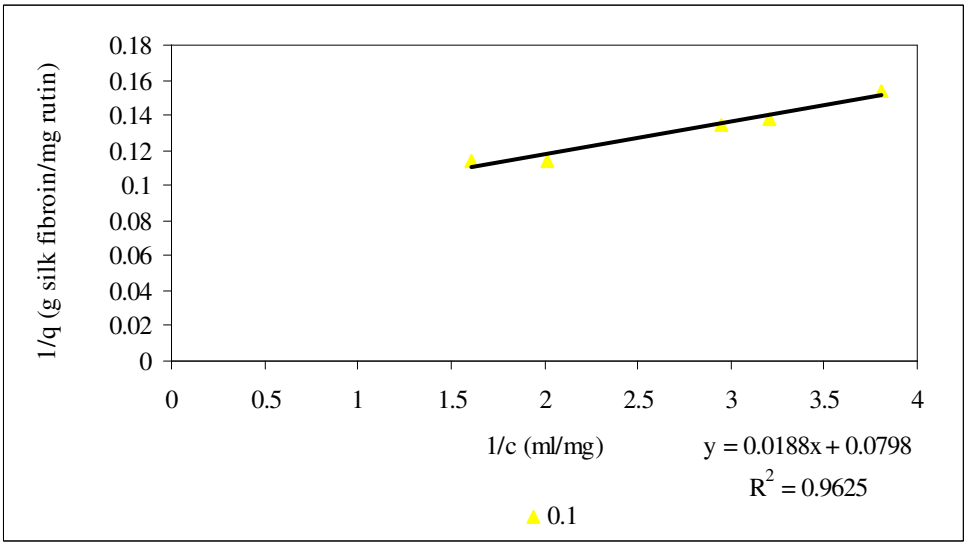

Figure D16. Langmuir model curve of rutin when temperature is at 25 °C, S/L= 0.1 and pH = 6

Surface Coverage Calculation

Maximum adsorption was occurred in water at 25°C when the solid/liquid ratio was 0.025. At these conditions, 1/K values were 7.7 and 2.24 for rutin and oleuropein, respectively. The concentrations were 0.8 and 6.3 for rutin and oleuropein, respectively. The surface coverage formula is:

$$\theta_i = \frac{(1/K)C_i}{1+\sum_{i=1}^{n}(1/K)C_i} \tag{D.1}$$

Surface coverage for rutin:

$$\theta_i = \frac{7.7\times 0.8}{1+(7.7\times 0.8+2.24\times 6.3)} = 0.29$$

Surface coverage for oleuropein:

$$\theta_i = \frac{2.24\times 6.3}{1+(2.24\times 6.3+7.7\times 0.8)} = 0.66$$