

**ISOLATION AND MOLECULAR AND NUTRITIONAL  
PROPERTIES OF PROTEINS FROM *ERUCA SATIVA* LEAVES  
AND SEEDS**

**By**

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**Suggested Short Title:**

**PROTEIN PROPERTIES OF *ERUCA SATIVA* PLANT**

## ABSTRACT

Arugula (*Eruca sativa*) protein isolates from seeds and leaves were extracted by NaOH and precipitated by isoelectric, ethanol and cryoprecipitation techniques. Molecular and nutritional properties of protein isolates were examined by electrophoresis, differential scanning calorimetry (DSC), fourier transform infrared (FTIR) spectroscopy, degree of hydrolysis (DH), and antioxidant activity techniques. SDS-PAGE of protein isolates showed 6 major bands for seed isolates and 5 major bands for whole plant isolates ranged between 108-17 kDa and 131-17 kDa, respectively. Native-PAGE revealed 1 intense band for each protein isolate in seeds (536 kDa) and leaves (600 kDa) except for isolates that were precipitated by ethanol and cryoprecipitation that did not show any bands. DH by trypsin ranged from 34-51 % for seed and 44-65 % for leaf protein isolates with continuous increase. Through trypsin-chymotrypsin mixture, there was increase until 90 minutes and after that a decrease was observed. Final results were 57-66 % for seed and 50-61 % for leaf protein isolates. Protein hydrolysates had higher antioxidant activity than protein isolates for all assays that they were subjected to; (1,1-diphenyl-2-picrylhydrazyl) DPPH and metal chelating assays. Antioxidant activity ranged between 5-15 % for protein isolates and 12.5-27.5 % for protein hydrolysates. DSC results demonstrated high thermal stability for both seed and leaf protein isolates where highest temperature of denaturation ( $T_d$ ) was for arugula whole plant control (123.9 °C) and lowest  $T_d$  referred to arugula whole plant protein isolate-cryoprecipitation (105.8 °C). FTIR results were mostly  $\beta$  structures in amide I and amide II regions for all protein isolates.  $\beta$ -sheet structures are confirmation of protein denaturation compared to raw seed powder that showed only  $\alpha$ -helix and random coil secondary structure.

## Résumé

Des isolats de protéines de roquette (*Eruca sativa*) furent extraits à partir de graines et de feuilles par NaOH et précipités par techniques isoélectriques, éthanol et cryoprécipitation. Les propriétés moléculaires et nutritives des isolats de protéines ont été examinés par des techniques d'électrophorèse, de calorimétrie différentielle à balayage (DSC), de spectroscopie infrarouge à transformée de Fourier (FTIR), de degré d'hydrolyse (DH), et d'activité antioxidante. L'analyse des isolats de protéines par électrophorèse SDS-PAGE a démontré 6 bandes majeures pour les isolats provenant des graines et 5 bandes majeures pour ceux provenant de plants complets, entre 108-17 kDa et 131-17 kDa, respectivement. L'électrophorèse sous conditions non-dénaturantes révéla une bande intense pour chaque isolat de protéines provenant des graines (536 kDa) et des feuilles (600 kDa), sauf pour les isolats qui ont été précipités par éthanol et cryoprécipitation où aucune bande n'a été détectée. L'analyse DH par trypsine des isolats de protéines résulta entre 34-51% pour les graines et 44-65% pour les feuilles avec une augmentation continue de l'hydrolyse. Avec un mélange de trypsine et de chymotrypsine, la DH augmenta jusqu'à 90 minutes suivi par une baisse. Les résultats finaux ont été 57-66% pour les isolats de protéines provenant des graines et 50-61% pour ceux provenant des feuilles. Les hydrolysats de protéines avaient une plus grande activité antioxidante que les isolats de protéines pour toutes les analyses réalisées; analyse par (1,1-diphényl-2 picrylhydrazyl) DPPH et par chélateur de métaux. L'activité antioxidante a varié entre 5-15% pour les isolats de protéines et 12.5-27.5% pour les hydrolysats de protéines. Les résultats obtenus par analyse DSC ont démontré une grande stabilité thermique pour les isolats de protéines provenant des graines ainsi que ceux provenant des feuilles, où la plus haute température de dénaturation ( $T_d$ ) a été enregistrée pour le contrôle de plante de roquette complète (123.9°C) alors que la plus basse  $T_d$  a été obtenue avec l'isolat de

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## ABBREVIATIONS

NaOH	Sodium hydroxide
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Native-PAGE	Native polyacrylamide gel electrophoresis
DPPH	1,1-diphenyl-2-picrylhydrazyl
DSC	Differential scanning calorimetry
FTIR	Fourier transform infrared
H	Hydrolysates
ES	Emulsion stability
FS	Foam stability
WAC	Water absorption capacity
TCA	Trichloroacetic acid
DIGE	Difference gel electrophoresis
$\Delta H$	Enthalpy
$T_d$	Temperature of denaturation
IEP	Isoelectric precipitation isolates
AWPI	Arugula whole protein isolates
ASPI	Arugula seed protein isolates
CP	Cryoprecipitation

APS	Ammonium per sulphate
TEMED	Tetramethylenediamine
OPA	O-phthadialdehyde
CuSO <sub>4</sub>	Copper sulfate
RCS	Refrigerated Cooling System
T <sub>m</sub>	Onset temperature
ATR	Attenuated total reflectance
ANOVA	Analysis of variance
ASC	Arugula seeds control
AWC	Arugula whole plant control
EP	Ethanol precipitation
STD	Standard
ASS	Arugula seed supernatant
AWS	Arugula whole plant supernatant
DH	Degree of hydrolysis
BSA	Bovine serum albumin
T <sub>1/2</sub>	Peak width at half height

# CHAPTER 1

## INTRODUCTION

*Eruca sativa* Mill. is known as rocket leaves as well as "Euzomon" in Greek which means good broth because of its good flavoring. In ancient Hebrew it is called "Gargir" from which Arabic language derived the same name for this vegetable. Other countries also call it arugula and rocca. Scientists confirm that it is mentioned in the Bible as "Oroth". Based on previous studies, Islamic sources and Jewish considered arugula leaves as a garden crop and spice (Yaniv et al., 1998). Countries that had traditionally grown arugula were Italy, Portugal, Egypt, and Turkey. It has also been discovered as a recent crop for Indiana, Central Europe and US Midwest, where it can be cultivated in two ways either in open fields and protectorates. It was presented on the market in Central Europe as "forth generation" vegetable. Before 1990, arugula plant was gathered from the wild because it was not grown on a large scale. At the end of 1990s, a big project involved in preserving and utilizing rocket genetic material to extent genetic diversity for future development of this crop (Doležalová et al., 2013).

Arugula plant is usually eaten raw in salads. In Italy it is used in pizza toppings, pasta and added as seasoning to meat and fish. Latin America use arugula in mozzarella cheese and sundried tomatoes. In the Middle East, arugula vegetable is eaten regularly in the diet as a salad. It is also well known in Brazil where they also add it raw to salads. Ischia Island in Gulf of Naples makes an alcohol called rucolino from arugula plant. In India, where sometimes there is scarcity of rain, arugula seeds are squeezed and pressed to get the oil which is used in pickling, cooking and salad dressing (Doležalová et al., 2013). It is used to make arugula sauce where the leaves are mixed with sugar or honey, vinegar and toasted bread (Nuez and Bermejo, 1994).

Improving the genetic diversity of arugula plants is accomplished by having many institutes and research centers for this purpose. It has been found that the biggest collections of arugula germplasm are at the Institute of Germplasm in Italy and India. There are other smaller collections in Afghanistan, Canada, Germany, Hungary, Pakistan, Poland, Sweden and Sudan (Nuez and Bermejo, 1994).

## **Objectives**

The overall objectives of this research were to isolate *Eruca sativa* proteins from leaves and seeds and to study the molecular weight characteristics and nutritional properties of proteins from arugula leaves and seeds. The specific objectives were:

- 1) To isolate proteins from arugula leaves and seeds by sodium hydroxide extraction/ isoelectric, ethanol and cryo-precipitation techniques.
- 2) To study molecular properties of proteins in arugula leaves and seeds by electrophoresis techniques sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), native polyacrylamide gel electrophoresis (Native-PAGE).
- 3) To investigate the digestibility of proteins in arugula leaves and seeds by trypsin and chymotrypsin enzymes.
- 4) To study the antioxidant activity of arugula protein isolates and protein hydrolysates in leaves and seeds by DPPH (1,1-diphenyl-2-picrylhydrazyl) assay and metal ion chelation assays.
- 5) To study thermal properties of arugula protein extracts in leaves and seeds by differential scanning calorimetry (DSC).

6) To examine the secondary structure of arugula protein extracts in leaves and seeds by fourier transform infrared (FTIR).

## **CHAPTER 2**

### **LITERATURE REVIEW**

#### **2.1 Botanical Aspects**

*Eruca sativa* Mill. Known as arugula belongs to Brassicaceae (Cruciferae) family and *Eruca* and *Diplotaxis* genera (Barillari et al., 2005). Brassicaceae family is also called Mustard family (Franzke et al., 2011). This family is classified as the second worldwide in production and consumption through all other vegetables (Bennett et al., 2007). Arugula plant is widely spread in the world started from Roman ages for its significant health promoting benefits in addition to its pungent peppery flavor (Jin et al., 2009). Nowadays, more interest and greater attention is directed to those plants from Middle East and European countries. Leaves were the main part that is used post-harvest, but now people are also using the plants' flowers, sprouts and seeds as well (Bennett et al., 2007).

#### **2.2 *Eruca sativa* Characteristics**

Arugula is an annual plant which has lance-shaped leaves and four-pelated cream colored flowers (Blazevic and Mastelic, 2008). Plant leaves are found all over the year, while flowers just from May to August. It grows up to 80 cm length with erect stem. Lower leaves are lyrate-pinnatisect. Seeds color varies from yellow green to brown and also vary in length from 1.7-3 mm (Padulosi, 1994). It is well known for its property of bearing drought. As a result, it is produced in barren regions around the world (Tariq et al., 2011).

#### **2.3 Applications**

Arugula is used in food as a salad. It can be steamed also and placed in soups as well. Plants' flowers are also used in salad as a garnishment.



In medicine, leaves are antiscorbutic, aphrodisiac, diuretic, stimulant and they help in digestion. Seeds are rube facial and tonic (Rani et al., 2010). Also, leaves have other characteristics such as: antiphlogistic, astringent, emollient, antacid, anti-inflammatory for colitis (Bennett et al., 2006). It is also advisable to include arugula in the daily diet for those who suffer from vitamin A deficiency and macular degeneration (Znidarcic et al., 2011). People usually use the seed meal for animal feed because of its high protein content (Kim and Ishii, 2006).

Industries use arugula seed oil in soap production, as lubricating substance and in producing biodiesel fuel (Tariq et al., 2011).

### **2.3.1 Food Benefits of *Eruca sativa***

Arugula leaves benefits can be divided into two categories: food benefits and non-food benefits. Health benefits are a sub-section of food benefits. Health advantages like having positive effects on kidneys malfunction and eye problems such as contagion. Combination of B-carotene and vitamin C that are found in arugula with high amounts can reduce the risk of eye problems such as age-related macular degeneration significantly (Johnson, 2002). Glucosinolates are commonly found in green leafy vegetables. Regular consumption of glucosinolates is associated with lowering risk of cancer and cardiovascular disease. Glucoraphanin is one of the most common glucosinolates found in arugula leaves. It is the end product from sulforaphane hydrolysis. This product helps the liver to produce enzymes that play a role in detoxifying chemicals that cause cancer (Cataldi et al., 2007).

### **2.3.2 Non-Food Benefits of *Eruca sativa***

Non-food benefits include using it in cosmetics. Grinding arugula seeds and putting them as cream on the face can cure teen's pimples. They can be used as deodorant as well. When eating

arugula leaves on an empty stomach, it removes sweat's bad odors. It has been used in early ages to produce an extract that protects from dogs bites. As being type of spices, arugula seeds are grinded and used the mash in meat flavoring (Yaniv et al., 1998). Arugula seeds has antibacterial and antifungal effects, but to a different extent. Those factors are considered as non-food benefits. It is best used as antibacterial agents and less efficient for fungal growth. In early studies, this plant has shown its antimicrobial effect on *Escherichia coli*, *Salmonella typhi*, and *Bacillus subtilis* only. Later studies showed that using crude water extract from the plant, methanolic extract and aqueous extract from seeds are the ways to inhibit the growth of those microorganisms. Microorganisms that are inhibited in the presence of mentioned extracts are: *Spadicoides stoveri*, *Paecilomyces variotii*, *Penicillium funiculosum*, *Penicillium lilacinum*, *Enterobacter agglomerans*, and *Hafnia alvei* (Rani et al., 2010).

#### **2.4 Chemical Composition of Leaves and Seeds in *Eruca sativa***

The main useful parts of arugula are leaves and seeds. There are higher percentages of crude proteins, total protein and crude fats in seeds than in leaves. Crude fiber, total carbohydrates and total minerals are higher in leaves than those in seeds (Bukhsh et al., 2007). Table 2.1 shows the chemical composition of seeds and leaves from arugula plant.

**Table 2.1** Chemical composition of seeds and leaves of arugula plant.

Chemical Composition	Arugula	
	Seeds (%)	Leaves (%)
Crude Protein	26.26	16.6
Total Protein	18.2	12.1
Crude Fat	25.6	6.6
Crude Fiber	2.5	14
Total Carbohydrate	15.6	16.9
Total Minerals (Ash)	8.5	9.5
Trace Elements	Seeds (ug/g)	Leaves (ug/g)
Ca	1900	700
Cd	0.0135	0.00532
Cr	10.663	4.72
Cu	32	21
Fe	60.62	37.5
K	720	313
Mg	695	709
Mn	19	10.6
Mo	0.19	nr
Na	1100	440
Ni	0.0423	0.139
P	3183	348
Zn	56.1	1.12

(Bukhsh et al., 2007)

nr= Not reported

## **2.4.1 Proteins**

Nowadays proteins' demand has increased due to increase population in the world and increase protein malnutrition as well because of unbalanced diet. Proteins are used as food, for feeding animals and in food industry. As a result, people started to depend on plant protein since some of them have good protein quality (Chayen et al., 1961). In the past, there were two main reasons behind not consuming plants' proteins which were: Presence of anti-nutritional and toxic compounds such as high concentrations of nitrate and oxalate and presence of indigestible fiber (Gupta and Wagle, 1988).

### **2.4.1.1 Amino Acid Composition of Total Proteins**

Proteins are composed of amino acids. Some of those amino acids are synthesized by our body and others are derived from food. Those amino acids are called essential amino acids. Plants contain proteins which are below par than those from animal origin because they lack some essential amino acids. People can overcome this problem and get all their daily requirements from protein by having complementary proteins which is combining more than one plant protein source in the meal. Plants have two main types of proteins, leaves protein and seed protein (Chayen et al., 1961).

### **2.4.1.2 Albumin**

Albumin is named for any protein that has a property of being soluble in water. Especially in plants' seeds, there are albumins which are known as storage proteins. 2S albumin is the sediment coefficient of those storage proteins in seeds (Mylne et al., 2014). 2S albumins are small storage proteins that are rich in sulphur found in plants (Boutilier et al., 1999). In arugula seeds, there is high level of albumin fractions within total protein. Those proteins shattered

during seed germination to provide enough amounts of sulfur and nitrogen which are important for newly formed seedlings. In the time of seed maturation those proteins go through modifications before they are accumulated in specified vacuoles (Mylne et al., 2014). Albumin comprises four fractions that are found in the supernatant through gel electrophoresis. These bounds are weak and can be eluted easily through DEAE-cellulose ion-exchange chromatography (Kaushal et al., 1982).

### **2.4.1.3 Globulin**

Globulins are storage proteins that are important for seeds germination and young seedlings nutrition. They have higher molecular weight than albumins. Most common structures of plant seeds are 11S and 7S globulins. Globulins represent 40% of total extracted proteins in arugula seeds. They can be separated into three fractions through gel electrophoresis and eluted in salt solution through DEAE-cellulose ion-exchange chromatography. They are the precipitated part in gel filtration technique. The ratio of globulins to albumins is 80:20 through the same technique. *Brassica napus* which is from the same family of arugula has isolated 12S globulin that contains close values of amino acids in globulins of arugula seeds (Kaushal et al., 1982). Mustard (*Brassica alba*) which is also from the same family of arugula has structure of seed storage globulins as 35.6 and 11.7 for alpha helix and beta sheet fractions respectively (Marccone et al., 1998). A summary of the major amino acids from arugula seeds and mustard leaves is presented in Table 2.2.

**Table 2.2** Major amino acids from arugula seeds and mustard leaves.

<b>Amino acids</b>	<b>Arugula Seeds g/100g</b>	<b>Mustard Leaves g/16g of N</b>
Alanine	5.0	6.407
Aspartic acid	8.0	13.59
Arginine	7.5	nr
Glycine	6.1	8.079
Glutamic acid	17.0	6.272
Histidine	3.2	0.338
Isoleucine	4.5	16.603
Lysine	7.2	3.219
Methionine	1.6	nr
Cystine	3.9	nr
Leucine	8.3	0.626
Serine	2.3	5.980
Threonine	2.7	6.381
Phenylalanine	4.7	0.116
Valine	6.1	17.921
Tyrosine	2.0	nr
Tryptophan	Nr	nr
Proline	6.9	8.301

(Kaushal et al., 1982) and (Gupta and Wagle, 1988)

nr= Not reported

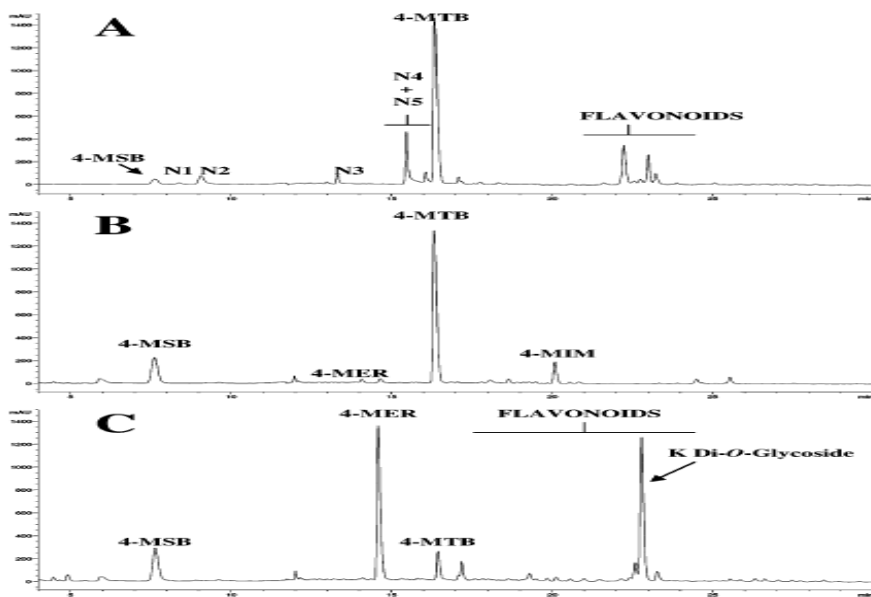
## **2.5 Antioxidant properties of *Eruca sativa***

Free radicals are produced from pollution in the air, normal respiration, UV radiation and many other causes (Sarmadi and Ismail, 2010). Those reactive oxygen species and reactive nitrogen species are responsible of renal failure (Alam et al., 2007), cancer development (Barillari et al., 2005) atherosclerosis, diabetes mellitus and Alzheimer's disease (Singh and Singh, 2008). Antioxidant is any substance that has the capacity to postpone or inhibit an

oxidation process (Aparadh et al., 2012) or interfere with any one of three major steps of oxidative process (initiation, propagation, and termination) (Singh and Singh, 2008).

Erucin (1-isothiocyanato-4-(methylthio) butane) is the major isothiocyanate in arugula leaves. It constitutes 60% of the total essential oils. Fresh weight seeds contain around 40-60 mg/g of erucin compound (Bennett et al., 2006). Glucoerucin is the precursor of erucin which represents 95% of total glucosinolates in arugula seeds (Barillari et al., 2005). Figure 2.1 shows the amount of erucin (4-MTB) in seeds (A), roots (B) and young leaves (C) (Bennett et al., 2006). Erucin is commonly found in cabbage, kale, cauliflower and arugula leaves. This compound has critical functions for human health. It is an active antioxidant, inhibits the metastasis of cancer cells especially lung, breast, liver and blood cancer (Azarenko et al., 2014). Arugula seeds contain high levels of carotenoids, flavonoids (Barillari et al., 2005) as well as gallic acid equivalent which was about 227.48 mg/g in a study conducted by Alam et al., 2007. That reveals a high antioxidant capacity in this plant.

**Figure 2.1** Amount of glucosinolates and N-heterocycles in arugula seeds, roots and leaves.

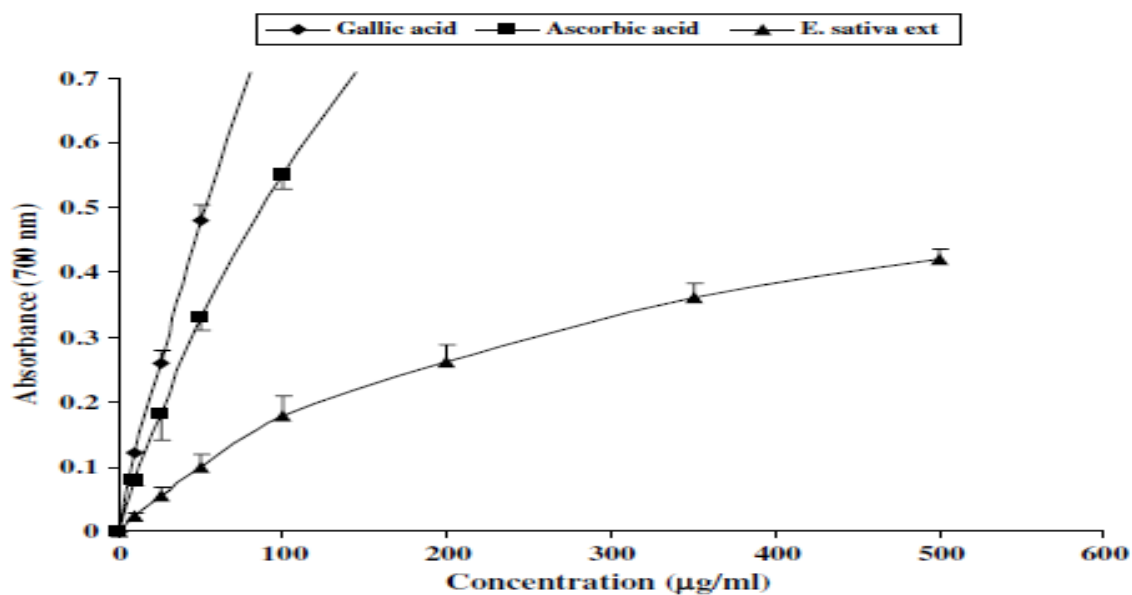


(Bennett et al., 2006)  
 4-MTB = Erucin, 4-MSB = Glucoerucin, 4-MER = 4-mercaptobuty and N = N-heterocycles  
 Seeds (A), Roots (B)

## 2.5.1 Antioxidant Assays

Antioxidants are divided into two groups, indigenous like enzyme antioxidants and exogenous like scavenging and chain breaking antioxidants. They can also be classified according to their function to primary and secondary antioxidants. Primary antioxidants donate hydrogen atom in order to create new stabilized radical. Secondary antioxidants retard radical initiators in order to eliminate the initiation process (Singh and Singh, 2008). There are many assays that can be used depending on the goal of the experiment. For example, using  $\beta$ -carotene method is to measure the antioxidant activity of bioactive compounds, reducing power method to assess proteins and peptides capacity to donate electrons, DPPH radical scavenging activity method to evaluate the scavenging activity in natural complexes (Yust et al., 2012) and metal ion chelating activity assay especially ferrous ions to operate as lipid oxidation pro-oxidant (Gülcin, 2006). A study done by Alam et al., 2007 assessed the antioxidant activity of arugula seeds through many methods such as reducing power, DPPH assay and metal chelating activity. The results showed that samples' extract scavenged 100%, 64.4 % and 39.9% of DPPH with 150  $\mu\text{g/ml}$ , 50  $\mu\text{g/ml}$  and 100  $\mu\text{g/ml}$  respectively. Figure 2.2 shows the amount of reducing power of the same extract.





**Figure 2.2** Reducing power of alcoholic extract of arugula seeds in comparison to that of gallic acid and ascorbic acid (Alam et al., 2007).

## 2.6 Functional Properties of Proteins

Functional property is defined as any physiochemical property that affects the protein's demeanor in food that can be identified and shown in the final product. Some of the functional properties are: viscosity, emulsion stability, foam stability, solubility, water absorption capacity, fiber formation, curdling, flavor binding, and thermal stability. Having good functionality will ease protein choice during food production. Examples of some functional properties that should be examined for few types of food are color control and viscoelastic properties for bread, curdling property in cheese production, and gelation property in marshmallows. There are many factors that affect functionality such as protein type, isolation mechanisms and drying techniques in addition to some environmental factors such as acidity and alkalinity levels, temperature and the strength of ions. There are broad divisions of functional properties in proteins and each of them has some specific terms that can be studied in order to cover and comprehend all functionality aspects from that broad class. For example, hydration is a general property and to

study this property other particular terms should be considered such as solubility, rheology, water holding capacity and viscosity (Kinsella and Melachouris, 1976).

### **2.6.1 Functional Properties of *Eruca sativa* Seed**

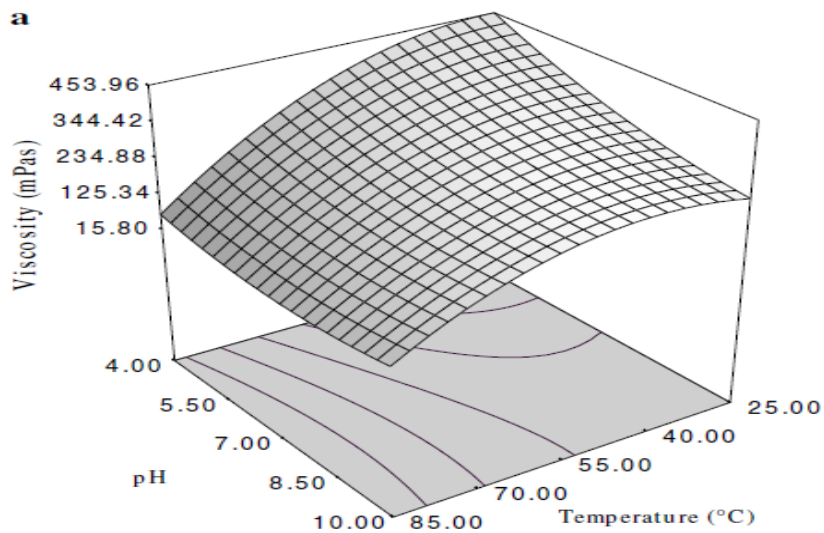
Arugula seeds are known as “Taramira”. Seeds contain between 20-25 % protein and around 30-35% oil. That’s why the seeds are called oilseed crop because of its high oil content. In order to study the proteins in seeds, they should be isolated or extracted then examined. Usually food proteins are separate proteins and each one has its own characteristics. As a result, when studying a particular type of protein it is not necessary that it will affect whole proteins in the sample (Kinsella and Melachouris, 1976).

The results of some analyzed properties were 357 mPas for viscosity, 87 % for emulsion stability (ES), 10.3% for extraction yield, 28.5% for solubility, 9.3 g/g for water absorption capacity (WAC), and 87.5 % for foam stability (FS). Many factors and mainly pH and temperature affect results of mentioned functional properties. The ways of influence will be discussed in following sections (Koocheki et al., 2012).

### **2.6.2 Viscosity**

Proteins swell when they absorb water. As a result, viscosity increases which can be shown in thickening of the final product. So, after doing some enhancement in a product, rheological properties can be examined. Checking viscosity is one of the markers to show the changes in protein structure. Globulins and albumins which are the major proteins in arugula leaves have low viscosity because they are very soluble and they do not swell (Kinsella and Melachouris, 1976). Regarding arugula seeds, viscosity can be increased in acidic media. The highest viscosity is measured when pH is around 5 and the temperature is 33.5 degrees celsius. So, to improve

extraction of arugula seeds, the best way is to reduce pH and temperature in order to get satisfactory results as shown in Figure 2 (Koocheki et al., 2012).



**Figure 2.3** The effect of temperature and pH on the viscosity of arugula seed mucilage

(Koocheki et al., 2012)

### 2.6.3 Solubility

Usually vegetable proteins' solubility is low or in another way, they are not so homogeneous. Ammonia absorption through high pH value can improve the solubility as well as other functional protein properties. This can be obtained through the preparation of NaOH in alkaline solution before drying (Kinsella and Melachouris, 1976). Regarding arugula, temperature and pH values are the most important factors that affect the solubility. With increasing temperature, solubility increases. Also, the ratio of water to seed and different pH values has a large effect on solubility. Increasing water to seed ratio decreases solubility. Optimum solubility (88%) for arugula seed mucilage is obtained with 10 pH values, 22:1 water to seed ratio and 84.5 °C (Koocheki et al., 2012). For other vegetable leaves, fluted pumpkin, best solubility is obtained in acidic and alkaline medias when pH= 1 and pH= 11. Least solubility is clearly shown through 4-5 pH values (Fasuyi, 2006).

## 2.7 Protein Extraction in Plants

There are different techniques of extractions depending on the type of protein. Some proteins need specific methods that cannot be applied to another protein. For example, in specific plants, the cellulose is rigid and needs to be trimmed in order to allow all the contents to be released before extraction. Another example, is presenting phenolic compounds that may degrade the protein which affect its activity. As a result, it is important to manipulate such conditions before doing the extraction. Studying proteins solubility is important as well before extraction (Shewry and Fido, 1996). There are various factors in plant cell that affect extraction and separation of proteins negatively such as tannins, waxes, polyphenols, proteases and pigments. They reduce protein diversity by 25%. Doing a step by step separation with using multiple solvents reduces the intricacy of proteins and improves discovering scarce proteins. To have good protein solubilization, buffer is used that has similar constitution of intracellular medium in order to dissolve proteins.

Trichloroacetic acid (TCA) and phenol extraction through using different buffer extractors are two ways to get total plant protein extract. TCA is one of the most efficient methods for protein extraction. The reasons behind that are: the ability to release membrane proteins, deactivate proteases and precipitate proteins. That happens when the sample is homogenized in 10% of TCA and dissolved in acetone. Phenol extraction, the second method, it is solubilizing the protein in phenol followed by precipitating step using methanol and ammonium acetate. This method is common for hydrophobic proteins. Table 2.12 shows different extraction methods for different plants (Malcevski and Marmioli, 2012). Alkaline solutions are efficient and practical methods to extract proteins from plant sources. Proteins nature in plant cells is described as hydrophobic and they have disulphide bonding between their molecules. As a result, high

alkaline solutions help to break the hydrogen bonds and detach hydrogen from carboxylic and sulphate groups. This leads to better solubility in water (Shen et al., 2008). A study have been conducted by Guo et al., 2005 shown that 55% protein extraction from rice was achieved with 0.1 M NaOH solution.

**Table 2.3** Most common extraction methods of proteins from different plant tissues/organs.

Tissue/Organ	Extraction Methods
<b>Suspension culture</b>	TCA/Acetone
<b>cereal seeds</b>	TCA/Acetone
<b>Xylem and Phloem sap</b>	TCA/Acetone
<b>Wood and other recalcitrant plant tissues</b>	Phenol
<b>chloroplasts</b>	Sorbitol/Percoll
<b>mitochondria</b>	Mannitol/Percoll
<b>nucleus</b>	Glycerol/Ficoll
<b>cell wall</b>	LiCl
<b>pollen</b>	TCA/Acetone
<b>plasma membrane</b>	Glycerol/Dextran/PEG

(Malcevski and Marmiroli, 2012)

### 2.7.1 Conditions that Influence Protein Extraction Efficiency

There are many conditions for extractions that are considered as a part to prepare protein isolates. Temperature, pH value, solid/solution ratio, time and extracting solution are examples of extraction conditions that should be kept in consideration prior this process. Solubility is an important factor for separation process. Its efficiency depends on the previous mentioned conditions. To obtain extraction, two steps have to be done which are precipitation and solubilization. Those techniques depend on solubility. Globulins and albumins are two main proteins in oilseed meals. They are soluble in saline solutions and water soluble respectively. Globulins have best extraction at 7 pH value, while albumins are best extracted at 12 pH values. When doing some enzymatic changes, therefore getting desired properties can be achieved such

as for charges, breaking and producing peptide bonds. Those properties can improve the solubility and exclude any undesirable components such as anti-nutritional compounds (Rodrigues et al., 2012).

## **2.7.2 Protein Identification and Quantitation**

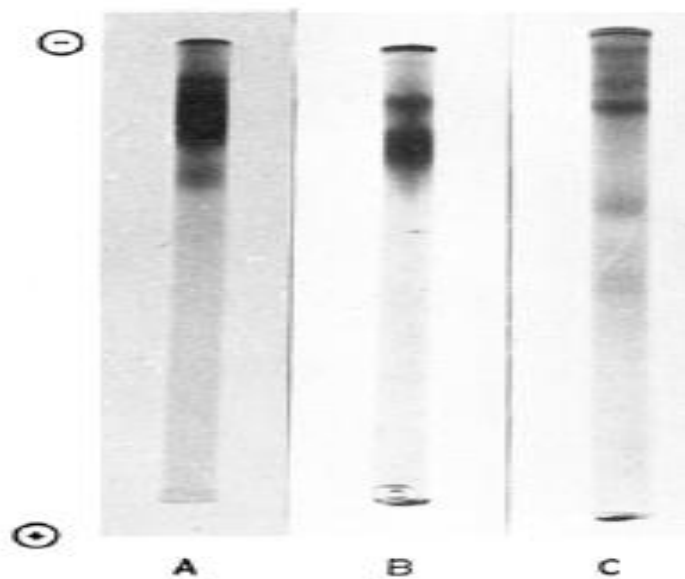
Food has a complex of proteins which can be named as proteomics. As a result, it is hard to identify and quantify those proteins. Technological insufficiency is another reason for inability to identify large amounts of proteins in plant cells that can reach up to  $10^6$  as protein concentrations and up to  $10^7$  for storage proteins. Because of different functional roles of proteins in plants such as energy producing proteins and others for transcription, it has led to heterogeneous proteins community. There are two techniques to identify proteins and both of them depend on mass spectrometry. The first one uses peptide mass fingerprinting which is matrix-assisted laser desorption ionization. The second one is specified for peptide fragment which is electrospray. This method shows peptides structures that can facilitate protein identity (Malcevski and Marmioli, 2012).

By moving to the other point which is protein quantification, it is logically the step that comes after identification. The amount of proteins is known as quantification. Difference gel electrophoresis (DIGE) is one of the methods to quantify proteins. Their abundance can be affected by UV light, aluminum stress, freezing and much more (Malcevski and Marmioli, 2012).

### **2.7.2.1 Polyacrylamide Gel Electrophoresis**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique is an easy method that is used for grouping the germplasm and discovering the polygenetic correlation.

Results can be obtained in a short period of time. Through studying 102 Taramira (arugula seeds) sets by SDS-PAGE, it shown that there were a total of 17 protein polypeptide bands. Six of them were monomorphic and 11 were polymorphic. By checking the size of protein bands, the range was between 15 to 220 kDa. Four bands from the total number of bands (17) were found in all sets (Shinwari et al., 2013). Another study done by Kaushal et al., 1982 studied arugula seeds as well by SDS-PAGE. It showed the total proteins as intensive scattering band and a heterogeneous band. Globulins were separated into three bands with one main broad band whereas albumins were separated into four bands as shown in figure 2.3. Globulin fraction subunits molecular weights were 60000, 35000, 26600, and 18000 Dalton. Another study done by Damiani et al., 2013 showed that the molecular weight for arugula proteins were 17.6, 67.5, 83.7, 127.8 kDa. Those were clearly four bands for raw (fresh) samples extract. Two bands with molecular weight of 59.7 and 73.9 kDa for cooked arugula extract.



**Figure 2.4** Polyacrylamide gel electrophoretic pattern of arugula seed proteins. (A) Total proteins, (B) globulins, (C) albumins.

(Kaushal et al., 1982)

## **2.8 Nutritional Quality Assessment Techniques of Proteins**

Protein quality is the nutritive value of food that relies on the amount and type of amino acids in this particular protein and on the physiological uses of those amino acids after digestion, absorption and oxidation rates. The higher the ratio of essential and vital amino acids, the better quality is obtained. On the other hand, proteins that are lacking one or more amino acids are poor in quality. Amino acids have an important role which is providing essential nitrogen for protein synthesis. There are many techniques that measure protein quality such as percentage of amino acid availability, digestibility, net protein utilization, nitrogen balance method, protein efficiency ratio, plasma amino acid ration, reference protein, amino acid score and relative nutritive value (Friedman, 1996).

### **2.8.1 Protein Quality Comparison from Different Protein Sources**

Measuring protein quality for different types of food depends on amino acids composition and their digestibility. It is important to know the quality of protein because many food proteins have high quality before processing, but after processing the quality decreases sharply. Usually food labels provide people with the amount of protein in food product not the quality of protein, although quality affects our body more than quantity .So, it is more important to declare protein quality. The following table illustrates different protein quality markers for some common types of food. Rapeseed its scientific name is brassica napus which is from the same family (Brassica) of arugula (Friedman, 1996).



**Table 2.4** Ranking of Diets by Five Different Protein Quality Indices.

Diet	PER	NPR	NU	RPER	RNPR
<b>Rapeseed Protein Concentrate</b>	3.29	4.59	4.90	81	87
<b>Soy Protein</b>	1.60	2.74	2.79	39	51
<b>Whole Wheat Flour</b>	0.95	2.35	2.29	23	44
<b>egg white solids</b>	3.71	5.08	5.43	91	95

Protein Efficiency Ratio (PER), Net Protein Ratio (NPR), Relative Protein Efficiency Ratio (RPER), and Relative Net Protein Ratio (RNPR).

(Friedman, 1996)

### **2.8.2 Digestibility of Plant Proteins**

Digestibility is the ability of peptide bonds to hydrolyze and it is different than availability which is maintaining the unity of amino acids by resisting the factors that try to break them such as heat, pH, enzymes, oxidation and others. Both of them (digestibility and availability) are required to measure the nutritive value of proteins. Apparent digestibility differs than true digestibility. Crude protein apparent digestibility varies depending on the concentration of protein in the diet while true digestibility does not rely on concentration. True digestibility for some amino acids such as methionine, cysteine and tryptophan are around 27% lower than those for crude protein, whereas true digestibility of other amino acids as lysine in wheat and some other grains are 14% lower than those in crude protein (Friedman, 1996). Presence of amino acids in lower digestible parts and protease inhibitors such as amylase inhibitors, lectins, phytates, and tannins may be the reason for lower digestibility (Savelkoul et al., 1992). Those inhibitors can be reduced by soaking the sample containing proteins in water and as a result will enhance the digestibility (Kataria et al., 1992). Alkaline medium causes racemization of amino acids and lysinoalanine formation in food proteins. Lysinoalanine deteriorates nutritional quality of foods by reducing the amount of essential L-amino acids, decreasing digestibility and

bioavailability of proteins. It can affect the safety of food as well by forming toxic products (Friedman, 1996). A study was conducted by Negi et al., 2001 on moth beans showed that processing and cooking those beans enhanced their protein digestibility by 14-16% than unprocessed ones. Another study proved that increasing the processing time and soaking period improved protein digestibility of mung bean and black gram (Kataria et al., 1992).

### **2.8.3 Comparison between Cooked and Raw *Eruca sativa* Ingestion**

Allergic reaction happens because of the presence of proteins in food. Allergic test was done previously by in vivo study (Damiani et al., 2013) to show IgE antibodies reaction when ingesting raw (fresh) and cooked arugula through analyzing proteins profile. Specific IgE binding was detected when proteins were transferred and incubated with patient's blood for one night. The Immunoblotting analysis showed reactivity of IgE only for raw arugula extract with bands that have molecular weight 60-70 kDa, but did not show any reactivity to proteins in cooked extract. Same test was done for other brassica family plants to confirm the result. Same result obtained to raw plants only and confirmation of inactivation for some proteins during cooking. The reason behind this result was because of some thermolabile proteins. There was a reduction in protein bands for cooked extract compared to raw plant in protein profile analysis. There were four bands for raw arugula whereas only two bands were found for cooked one (Damiani et al., 2013). Allergic reactions could be reduced by inhibiting IgE antibodies to bind with  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin through using some hydrolysates produced by pancreatic enzymes (Asselin et al., 1989).

## **2.8.4 Protein Hydrolysates**

Protein hydrolysates are peptides and amino acids resulted from enzymes that hydrolysed those proteins (Sarmadi and Ismail, 2010). Protein hydrolysates have many advantages such as having better digestibility due to unfolding of native protein structure through hydrolysis (Van Der Plancken et al., 2003), increasing functional properties than the entire intact protein (Barac et al., 2006), higher absorbance efficiency than large peptide molecules (Wu et al., 2008) and higher antioxidant activity due to having higher hydrophobicity (Li et al., 2008). High antioxidant rate may be referred to certain structure (Sarmadi and Ismail, 2010), sequence of amino acids (Yust et al., 2012) and configuration of peptides (Sarmadi and Ismail, 2010). Example of certain structure that can increase the anti-oxidative rate is having cysteine with SH group which will react directly with radicals (Qian et al., 2008). The study conducted by Barac et al., 2012 revealed that extensive hydrolysis can have adverse effects on some functional properties such as foaming and solubility. Protein hydrolysates are divided into three categories; first one can be used to improve functional properties when the degree of hydrolysis is low; second category can be used to enhance flavors which have moderate degree of hydrolysis and last group can be used as nutritional supplements when there is a high rate of degree of hydrolysis (Vioque et al., 2000).

## **2.9 Thermal Properties of Food Proteins**

Thermal denaturation of proteins involves changes from the native structure and breaking down chemical forces that maintain the structural unity of the protein molecules. Treatments with heat can lead to protein aggregation. Protein functional properties such as gelation, emulsification and foaming are affected by denaturation as well (MA and Harwalkar, 1988). There are other factors that can affect protein folding such as pH and salt. Low salt concentration

causes water molecule protection around the protein resulting in protein hydration, while high salt concentration leads to protein precipitation because of low amount of water and as a result the protein will have higher thermal stability. Every protein has different pH level that it is stable through that range which is the isoelectric point. If pH levels are higher or lower than the isoelectric point, denaturation will occur because of repulsion forces. Also, glycosylated parts of proteins increases thermal stability due to increase solubility of proteins. As a result, sugars are considered as protective molecules against protein denaturation (Meng and Ma, 2001).

### **2.9.1 Differential Scanning Calorimetry**

Differential scanning calorimetry (DSC) is a thermal method of analysis for observing changes in physical and chemical features of materials such as proteins through temperature by detecting heat changes accompanied with these processes (Biliaderis, 1983). Enthalpy ( $\Delta H$ ) and denaturation temperature ( $T_d$ ) are the most two parameters that are used in analyzing protein denaturation through DSC. Changes that happen to protein are either endothermic such as breaking hydrogen bonds or exothermic such as rupturing hydrophobic forces and protein aggregation. Enthalpy is the area under the endothermic peak which indicates native, undenatured, protein (Meng and Ma, 2001). Decrease in enthalpy shows protein denaturation and increase in denaturation temperature means having high thermal stability (MA and Harwalkar, 1988). As previously mentioned protein is denatured in acidic or alkaline pH and that means having low enthalpy and low denaturation temperature (Meng and Ma, 2001). According to a study that was done by Sessa, 1992 on 7S and 11S storage proteins of soybeans, a decrease in 11S  $\Delta H$  was observed in cracked bean and defatted soy flour when moisture increased above 9%. When moisture was above 28%, a decrease in 7S  $\Delta H$  was observed. Results of cracked beans and soy flour showed that  $\Delta H$  decreased for both 7S and 11S with

increase moisture. Regarding denaturation temperatures, pure 7S and 11S increased with decrease in moisture.

## **2.9.2 Advantages of Food Proteins' Thermal Properties in Food Industry**

Foods are exposed to thermal treatments every day during preparation and processing through different procedures such as blanching, cooking and roasting. Thermal treatments can reduce protein quality such as lowering the digestibility and bioavailability of this particular protein. As a result, using DSC is helpful to assess protein quality of food samples. Some of the functional properties such as gelation are affected by thermal stability of proteins. Also 7S globulin of soybean is affected by heating temperatures and DSC properties. Some undesirable functional properties of oilseeds proteins are caused by high heat that affected their structural stability. So, it is important to use DSC in order to maintain protein quality of foods (Meng and Ma, 2001). Regarding DSC analysis of arugula leaves, there is no information in the literature. That made it interesting to analyze protein isolates of leaves and seeds to observe thermal transition and melting peaks of each different technique (isoelectric, ethanol and cryo-precipitations) and compare them together. After that, there will be a clear vision on whether different treatments affected the quality of proteins or not.

## **2.10 Secondary Structure of Proteins**

Most important stages in protein synthesis are first and second one where primary and secondary structures are formed. In the primary structure, linear sequence of amino acids is arranged in polypeptide chain whereas the secondary structure refers to the folding of a polypeptide chain. Secondary structures are observed as two types in proteins. The first one is alpha helix structure that is presented as a coiled spring. The second type is beta pleated sheet

which appears as folded or pleated. All structures are held or secured by hydrogen bonds which in turn are the stabilizing forces of secondary structure in proteins. The whole structure of the protein determines its function in food samples. As a result, studying the secondary structure to look for any changes in it is important for food systems. There are many instruments that can show the secondary structure of the protein such as X-ray crystallography, nuclear magnetic resonance, circular dichroism and infrared spectroscopy. X-ray crystallography has a limitation that the protein sample should be in a crystalline state. That is impossible for some samples which are in solutions or other states where their functions are best when they are in solutions. Nuclear magnetic resonance does not show accurate results. In addition, it is only used only for small proteins. Circular dichroism is mostly used for quantifying the content of alpha helix. Infrared spectroscopy methods proved to be the best one for determining the secondary structure no matter its state either liquid, solid or gel (Kumosinski and Farrell, 1993).

### **2.10.1 Fourier Transform Infrared Spectroscopy**

Fourier transform infrared spectroscopy (FTIR) is a spectroscopic technique that depends on molecules' vibrations. Whenever the molecules interact with infrared light and absorb energy, they vibrate faster. Fourier transformation, which is part of the method's name, is a mathematical technique that is accomplished by the computer to convert or decode the interferograms to a frequency spectrum (Kumosinski and Farrell, 1993). This method is used to determine any secondary structure changes in the protein at any physical state.

Studying alpha-helical, beta-pleated sheet, aperiodic conformations and turns can be determined by the amide I region which is composed of C=O stretching vibrations in 1700–1600  $\text{cm}^{-1}$  region (Ellepola et al., 2005) and amide II region which is formed by deformation of the N-H bonds (Kumosinski and Farrell, 1993). A, B, and I–VII are nine modes formed for the

amide band of proteins and peptides. Their order is upon decreasing frequency (Carbonaro and Nucara, 2010). Table 2.5 shows all amide bands and their absorption frequencies.

**Table 2.5** Infrared absorption bands from proteins and peptides.

Band	Frequency (cm <sup>-1</sup> )	Description
Amide A	3,300	NH stretching
Amide B	3,100	NH stretching
Amide I	1,600–1,690	C=O stretching
Amide II	1,480–1,575	CN stretching; NH bending
Amide III	1,229–1,301	CN stretching; NH bending
Amide IV	625–767	OCN bending
Amide V	640–800	Out-of-plane NH bending
Amide VI	537–606	Out-of-plane C=O bending
Amide VII	200	Skeletal torsion

(Carbonaro and Nucara, 2010)

Table 2.6 shows amide I frequencies of secondary structures that have been collected by referring to a spectrum of homo polypeptides and proteins with mainly  $\alpha$ -helical or  $\beta$ -sheet structures, normal mode analysis and theoretical calculations.

**Table 2.6** Amide I frequencies assigned to protein secondary structure.

Secondary structure	Range	Average
A-helix	1,648–1,660	1,654
$\alpha$ -helix turns <sup>a</sup>	1,630	1,630
$\beta$ -sheet	1,612–1,641	1,625
	1,626–1,640	1,633
	1,670–1,694	1,682
Turns	1,662–1,684	1,673
Random coil	1,640–1,650	1,645

<sup>a</sup> According to Murayama and Tomida (2004)  
(Carbonaro and Nucara, 2010)

### 2.10.2 Applications in Food Industry

Fourier transform infrared (FTIR) spectroscopy can be used to check whether the food has been exposed to radiations or not. A study was done by Dogan et al. 2007 on hazelnuts showed alterations in the structure of irradiated hazelnut proteins by finding aggregation of protein molecules. Quality of food can be investigated as well through checking protein changes in

heated food samples. A study done by Wu et al., 2007 revealed that there were some gain of random structures and aggregated beta sheets after a fast heating of pork meat.

Nutritional aspects can be evaluated by FTIR also. A study was done on lentils to monitor the changes of secondary structure after heating the samples. It was specifically to check 7S protein. By looking at amide I region, the content of beta sheet decreased from 47% to 13% and there was an increase of aggregates with the appearance of random coil structures. Getting high amounts of aggregates after heating food has harmful effects on digestibility of storage proteins (Carbonaro et al., 2008). By using FTIR characteristics, arugula leaf proteins can also be evaluated especially because there is no literature on that plant with FTIR.



## **CHAPTER 3**

### **MATERIALS AND METHODS**

#### **3.1 Materials**

Arugula leaves (*Eruca sativa*) were cultivated in one of the farms of Al-Wafra, in the state of Kuwait. They were randomly harvested after 1 month. After harvesting, arugula plants were washed without their roots with water, bunched, and transferred to the laboratory within 2 hours. Fresh green arugula leaves with stems were submerged in 85°C water for 3 minutes as a blanching step. They were immediately cooled by adding ice-water, and the excess water was drained. After that the samples were freeze dried and stored in plastic containers at 4 °C. While arugula seeds were bought from agricultural stores. They were packed tightly and never opened before use. Before usage, seeds were grinded with seeds grinder.

#### **3.2 Sodium Hydroxide Extraction**

Protein extraction was done using 0.02% NaOH. Adding 50 ml of the prepared solution to 5g of the sample (arugula seeds, arugula whole plant) and stirred the mixture for 1 hour. The next step was centrifuging (BECKMAN, Model J2-21 Centrifuge) the mixture for 15 minutes with 10000 rpm. Filtering the supernatant after the centrifugation was completed by using cotton wool. The extract is now called NaOH extract.

#### **3.3 Isoelectric Precipitation**

By adjusting the pH to 4.5 by diluted HCl (6M), proteins precipitate. The centrifugation for 15 minutes at 10000 rpm was done for separating the proteins. The separated proteins are known as isoelectric precipitation isolates (NaOH-IEP). Arugula leaves and seeds were called as arugula whole protein isolates (AWPI-IEP) and arugula seed protein isolates (ASPI-IEP) respectively.

### **3.4 Ethanol Precipitation**

Precipitating rocket proteins by ethanol was done through adding ethanol (drop wise) to NaOH protein extracts. When precipitation obtained, then centrifugation was done for 15 minutes at 10000 rpm. This technique represents EtOH isolates. Sample at this stage with ethanol precipitation is called arugula whole protein isolates (AWPI-EP) and arugula seed protein isolates (ASPI-EP) respectively.

### **3.5 Cryoprecipitation**

The extracts were refrigerated overnight (16-18 hours) at 4°C. The precipitated proteins were then centrifuged for 15 minutes at 10000 rpm. This technique presented proteins that are known as cryoprecipitation (CP) isolates. Samples at this level are called arugula whole protein isolates (AWPI-CP) and arugula seed protein isolates (ASPI-CP) respectively.

### **3.6 Electrophoresis**

Electrophoresis was done by using Mini- PROTEAN II Electrophoresis Cell unit. This unit is connected to electrophoresis power supply (Bio-Rad, Hercules, CA).

#### **3.6.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

##### **3.6.1.1 Protein Extraction**

Sample was powdered and 0.1 gram of the powder was added into a micro-tube. After that, 400µl of protein extract was mixed with buffer (sample buffer). The buffer consisted of Tris-HCl 0.5M (pH 6.8), SDS 10%, distilled water, 2- mercaptoethanol, glycerol and 0.5% Bromophenol blue. Samples were mixed by centrifugation at 15,000rpm for 10-12 minutes at room temperature, and kept at - 4 °C (Shinwari et al., 2013).

### **3.6.1.2 Gel Preparation**

Electrophoresis was done through 20% polyacrylamide slab gels in a non-continuous buffer system. The separating (resolving) gel composed of 20% acrylamide and 0.135% N.N-methylene-acrylamide in 0.5M Tris-HCl buffer (pH 8.8) with 0.27% SDS, 10% Ammonium per sulphate (APS) and 15 microliters Tetramethylethylenediamine (TEMED). The stacking gel solution consisted of 30% acrylamide and 0.8% N.N-methylene-bis-acrylamide in 0.25M Tris-HCL buffer (pH 6.8) with 0.2% SDS. Electrode buffer was a mixture of Tris-glycine (9.0g Tris- HCl and 43.2g glycine per 3 liters buffer solution at pH 8.9) and SDS 3.0g (Shinwari et al., 2013).

### **3.6.1.3 Electrophoresis Running and Staining and Destaining**

Protein sample (10-12 microliters) was loaded into the wells. Electrophoresis ran at 90 volt for 1-1.5 hours until blue marker reached the bottom of the gel. The molecular weights of separated protein bands were compared with standard protein bands ranging from 10 to 220 KDa (Shinwari et al., 2013). After the process was done, gels were stained with commassie blue solution from 40-60 minutes. Then, they were destained for more than 2 hours with destaining solution that consists of 5% acetic acid, 20% methanol and distilled water with ratio of 5:20:75 (v/v) (Shinwari et al., 2013).

## **3.6.2 Native Polyacrylamide Gel Electrophoresis (Native-PAGE)**

### **3.6.2.1 Sample Preparation**

Sample was powdered and 0.1 gram of the powder was added into a micro-tube. After that, 400 $\mu$ l of protein extract were diluted in Native sample buffer (glycerol, 0.5% bromophenol Blue, 0.5 M Tris-HCL, pH 6.8 and distilled water). Samples were heated at 95 $^{\circ}$ C for 5 minutes before loading 20 $\mu$ l into sample wells. Protein standard for native electrophoresis (GE Healthcare Ltd. 17-0445-01, UK) mixed with 100  $\mu$ l native sample buffer and loaded into the sample wells. Protein standard

ladder for native electrophoresis contains thyroglobulin (669 kDa), Ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and bovine serum albumin (66 kDa).

### **3.6.2.2 Gel Preparation**

Polyacrylamide gradient separation gel (10%) was prepared with other reagents which were: distilled water, 30% acrylamide, 1.5 M Tris-HCL pH 8.8, 10% ammonium persulfate (APS) and tetra methylethylene-diamine (TEMED). 4% acrylamide in stacking gel made up with the same reagents except using Tris-HCL pH 6.8 instead of Tris-HCL pH 8.8. 10% separating gel was poured into the gel sandwich and allowed it to polymerize within 10 min, followed by 4% stacking gel until it fills the gel sandwich. A comb like plastic piece inserted into the sandwich in order to form sample wells. 20  $\mu$ l of sample extract was loaded by Hamilton syringe (Reno, Nevada, USA) in the wells, while 10  $\mu$ l of protein standard for native electrophoresis was loaded (Davis, 1964).

### **3.6.2.3 Electrophoresis Running and Staining and Destaining**

The gels ran in Tris-glycine buffer pH 8.3 for around 1.5-2 hours at a voltage of 120 using a mini protein III Electrophoresis cell unit (Bio-Rad, Hercules, CA). The gels were stained for 18 hours in Coomassie Brilliant Blue R-250 reagent. The gels were then destained by a fixing solution that is composed of 70% distilled water, 20% methanol and 10% acetic acid solution and finally scanned (Davis, 1964).

## **3.7 Preparation of *Eruca sativa* Leaves and Seeds Proteins and Protein Extract Hydrolysates**

### **3.7.1 Enzymatic Hydrolysis of *Eruca sativa* Protein Extract (Digestibility)**

Arugula whole protein isolates and arugula seed protein isolates were digested by trypsin and a mixture of two enzymes; trypsin and chymotrypsin. Sodium phosphate buffer were used to adjust arugula leaves extract to pH 8.0 and then incubated at 37 °C. The ratio of enzyme to substrate was

1:20. 0.5 ml of incubated mixture was withdrawn at zero time and after every 30 minutes of digestion at time intervals between 0 to 120 minutes. Stopping the enzymatic reaction was by heating for 10 minutes at 95 °C followed by centrifugation by mini Spin plus, eppendorf and then filtration. Lysozyme was used as standard.

### **3.7.2 Determination of Degree of Hydrolysis**

Digestibility of arugula leaves proteins extract was measured by Adebisi et al., 2009 method. About 50 µl of hydrolysed extract was mixed with 2 ml of o-phthalaldehyde (OPA) reagent. OPA was prepared by 0.1 M sodium tetraborate, 20% SDS, β- mercaptoethanol. 40 mg of OPA was dissolved in 1 ml methanol and then the mixture was kept for 2 minutes at room temperature. After that, spectrophotometer (Ultrospec 2100 pro UV/Visible Spectrophotometer) was used to measure the absorbance at 340 nm. Finally, digestibility was calculated by equation (1).

$$\text{Degree of Hydrolysis (\%)} = (\text{MW } \Delta \text{ 340 nm}) / (\text{d. e. p}) \times 100 \rightarrow (1)$$

Where:

MW = Average molecular weight of amino acids (120)

$\Delta$  340 nm = Absorbance at 340 nm

d = Dilution factor

e = Average molar absorption of amino acids (6000 M<sup>-1</sup> cm<sup>-1</sup>)

p = protein concentration\*

\*Protein concentration of arugula protein extract was determined by Bradford technique.

## **3.8 Determination of Antioxidants Activity**

### **3.8.1 DPPH Scavenging Activity Assay**

Antioxidant activity of arugula protein isolates and protein hydrolysates was measured by using DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay according to Li et al. 2008. 1 ml of each sample solution at different concentration (0.5-1 mg/ml) was added to 1 ml 0.1 mM DPPH

dissolved in 95% ethanol. The mixture left at room temperature for 30 minutes in a dark place. The absorbance of the mixture was measured at 517 nm by spectrophotometer (Ultrospec 2100 pro UV/Visible Spectrophotometer). The blank contained ethanol solution (95%) instead of the sample. Experiment was done in triplicates including blank and the average was taken for results. The scavenging activity assay was calculated by using the following equation:

$$\text{DPPH scavenging activity (\%)} = [(\text{Blank absorbance} - \text{Sample absorbance}) / \text{Blank absorbance}] \times 100$$

### 3.8.2 Metal Ion Chelating Activity Assay

Arugula protein isolates and protein hydrolysates ability to chelate  $\text{Cu}^{+2}$  and  $\text{Fe}^{+2}$  was determined according to Zhang et al., 2011. For  $\text{Cu}^{+2}$  chelation assay, preparation of aqueous solution was done by mixing one milliliter of 2mM copper sulfate ( $\text{CuSO}_4$ ) with one milliliter of 10% pyridine and 20 $\mu\text{l}$  of 0.1% pyrocatechol violet. One milliliter of samples solution at concentration 1mg/ml was added to the mixture. The absorbance was measured at 632 nm by spectrophotometer (Ultrospec 2100 pro UV/Visible Spectrophotometer). In  $\text{Fe}^{+2}$  chelation assay, aqueous solution was prepared by adding one milliliter of 20 $\mu\text{M}$  iron (II) chloride to one milliliter of 0.5mM ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid sodium salt). The absorbance was measured at 562 nm. Distilled water was used in the blank solution instead of the sample. Experiment was done in triplicates including blank and the average was taken for results.  $\text{Cu}^{+2}$  and  $\text{Fe}^{+2}$  chelating activity of the protein isolates and their hydrolysates was calculated by using the following equation:

$$\text{Metal ion chelating activity (\%)} = [(\text{Blank absorbance} - \text{Sample absorbance}) / \text{Blank absorbance}] \times 100$$

### **3.9 Differential Scanning Calorimetry (DSC)**

#### **3.9.1 Sample Preparation**

Protein isolates were mixed with micro pipetting weighed amounts of distilled water. Sample to water ratio were 1:3 to increase the moisture content in the samples' powder. 11 to 13 mg of hydrated sample was weighed into tared DSC aluminum hermetic pans and then covered and sealed by Tzero Sample Encapsulating press. Covered samples were kept 1 hour prior to analysis in order to achieve higher percent of moisture.

#### **3.9.2 Thermal Properties of *Eruca sativa* Protein Extracts in Leaves and Seeds**

Differential scanning calorimetry (DSC) (TA Q2000, TA Instruments, New Castle, DE, USA) instrument was employed to observe the thermal denaturation of protein isolates. A sealed empty pan was used as a reference. Refrigerated Cooling System (RCS) 90 was used for cooling within an operating range of -90°C to 550°C. In the beginning of the experiment the cell was preheated followed by an equilibration at an initial temperature using indium and sapphire for temperature and heat capacity calibration. Then, holding isothermal for five minutes, heating at constant rate to a final temperature and holding isothermal for 5 minutes was performed. Pans containing samples were run at a 10 °C/min heating/cooling ramp in heating–cooling cycles in a nitrogen atmosphere (flow rate 50 mL/min) from -10° to 140°C. Onset temperature ( $T_m$ ), denaturation temperature ( $T_d$ ) and enthalpy of denaturation ( $\Delta H$ ) were computed from the thermograms by Universal Analysis 2000 (version 4.5A, TA Instruments, New Castle, DE, USA). All analysis was performed in triplicate.

### **3.10 Fourier Transform Infrared (FTIR) Spectroscopy**

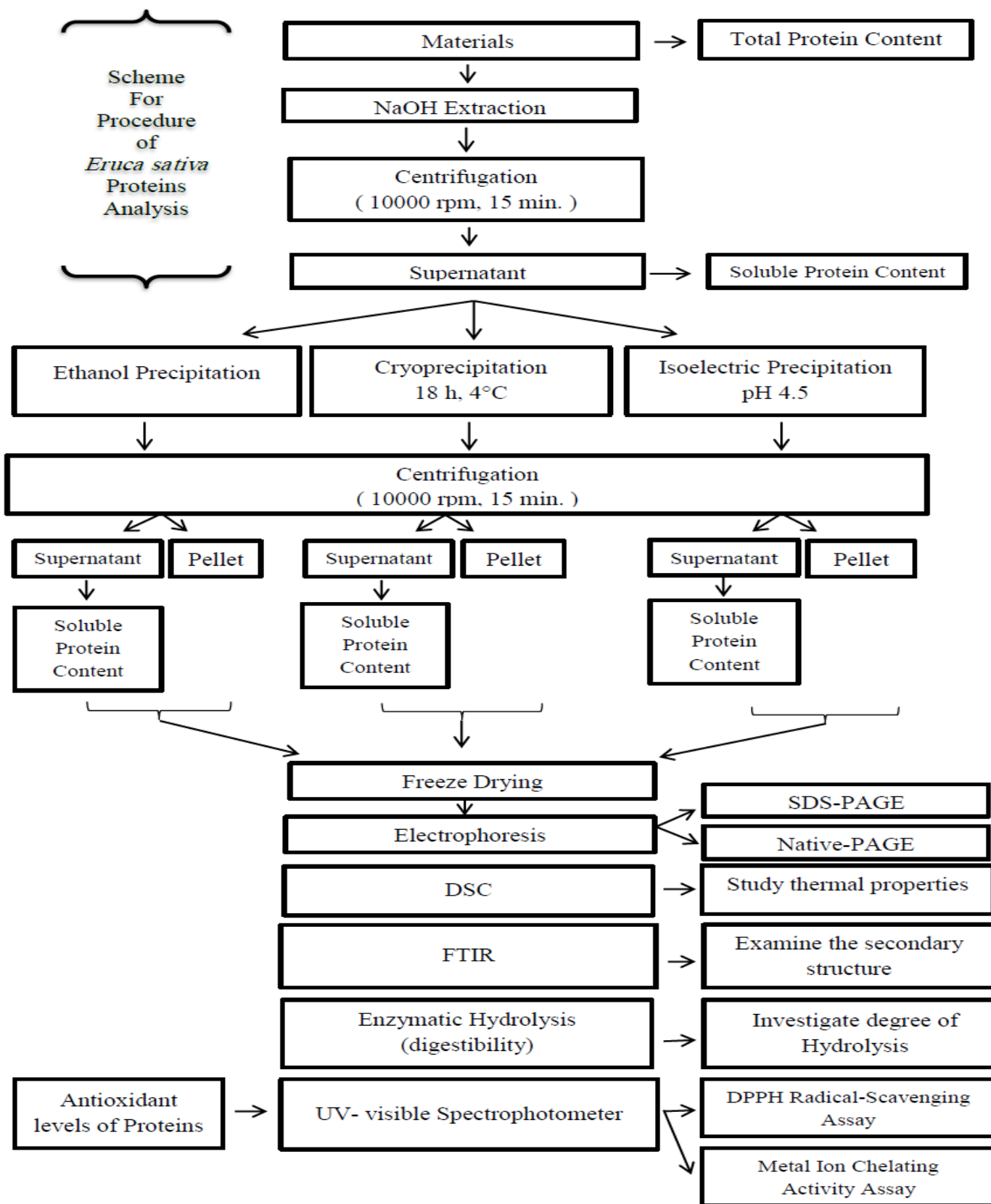
#### **3.10.1 Secondary Structure by FTIR**

The FTIR spectra of raw arugula leaves and seeds and protein isolates from arugula plant and seeds were obtained by using a Nicolet iS5 FT-IR Spectrometer (Thermo Scientific, Madison, WI, USA) over a wavelength range of 400–4000  $\text{cm}^{-1}$  equipped with an OMNIC operating system software (Version 9.0, Thermo Scientific, Madison, WI, USA) to obtain FTIR spectra. Powder samples were covered on the surface in contact with attenuated total reflectance (ATR) on a multi-bounce plate of Zn-Se crystal at 25 °C. All spectra were background corrected using an air spectrum, which was renewed after each scan. Each spectrum was collected from an average of 32 scans with a resolution of 4  $\text{cm}^{-1}$  and the results were reported as mean values. Figure 3.1 shows a scheme for procedure of arugula protein analysis.

#### **3.11 Statistical Analysis**

Data collected from this study was analysed statistically using analysis of variance (ANOVA) in Excel. A p value < 0.05 was considered statistically significant. Data presented as means  $\pm$ SD.





**Figure 3.1** Scheme for arugula protein analysis procedure.

## CHAPTER 4 RESULTS AND DISCUSSION

### 4.1 Electrophoresis

#### 4.1.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

##### 4.1.1.1 SDS-PAGE of Protein Isolates

Molecular weights of subunits of arugula protein isolates are reported in Table 4.1. Molecular weights of fractions ranged between 131- 17 kDa for seeds and leaves. Soluble protein content before obtaining precipitation process also included in the table as arugula control (AC). AC in turn contained the highest subunits in which different precipitation techniques could not obtain the same subunits. Those subunits were in seeds control (ASC) lane with molecular weights 131.8, 78.7, 43.3, 31.6 and 28 kDa. 130 kDa reported as  $\beta$ -Galactosidase (Ullmann et al., 1968), 76.5 documented as transferrin (Folta-Stogniew and Williams, 1999), 43 kDa known as ovalbumin (Castellino et al., 1968), 31.6 kDa could be DNase I (Neville, 1971) and 27.6 kDa referred to subtilisin (Smith et al., 1966). Regarding seeds, Cryo-precipitation (ASPI-CP) contained the highest amount of subunits (9) and most intense ones. They were 108 kDa, 82 kDa, 69.8 kDa, 47 kDa might be fumarase as reported in Kanarek et al., 1964, 37 kDa which is D-amino acid oxidase (Henn et al., 1969), 34 kDa is aspartate transearbamylase (Wiley et al., 1968), 24.9 kDa might be chymotrypsinogen which has 25.7 kDa molecular weight, 23.9 kDa could be trypsin with 23.3 kDa and finally 17.4 kDa which is mostly myoglobin with molecular weight 17.2 kDa (Weber and Osborn, 1969). Myoglobin was found in all lanes of seeds and leaves including control of whole plant (AWC), but the concentration was low that did not show the band clearly. Plus cryo-precipitation (ASPI-CP) showed the largest molecular weights of subunits between 108.1-69.8 kDa which most of them were absent in ethanol (ASPI-EP) and isoelectric precipitation (ASPI-IEP). There were four major subunits that were identical in all techniques of precipitation. Those subunits are with 47, 34.2, 24.9 and 17.4 kDa. Isoelectric

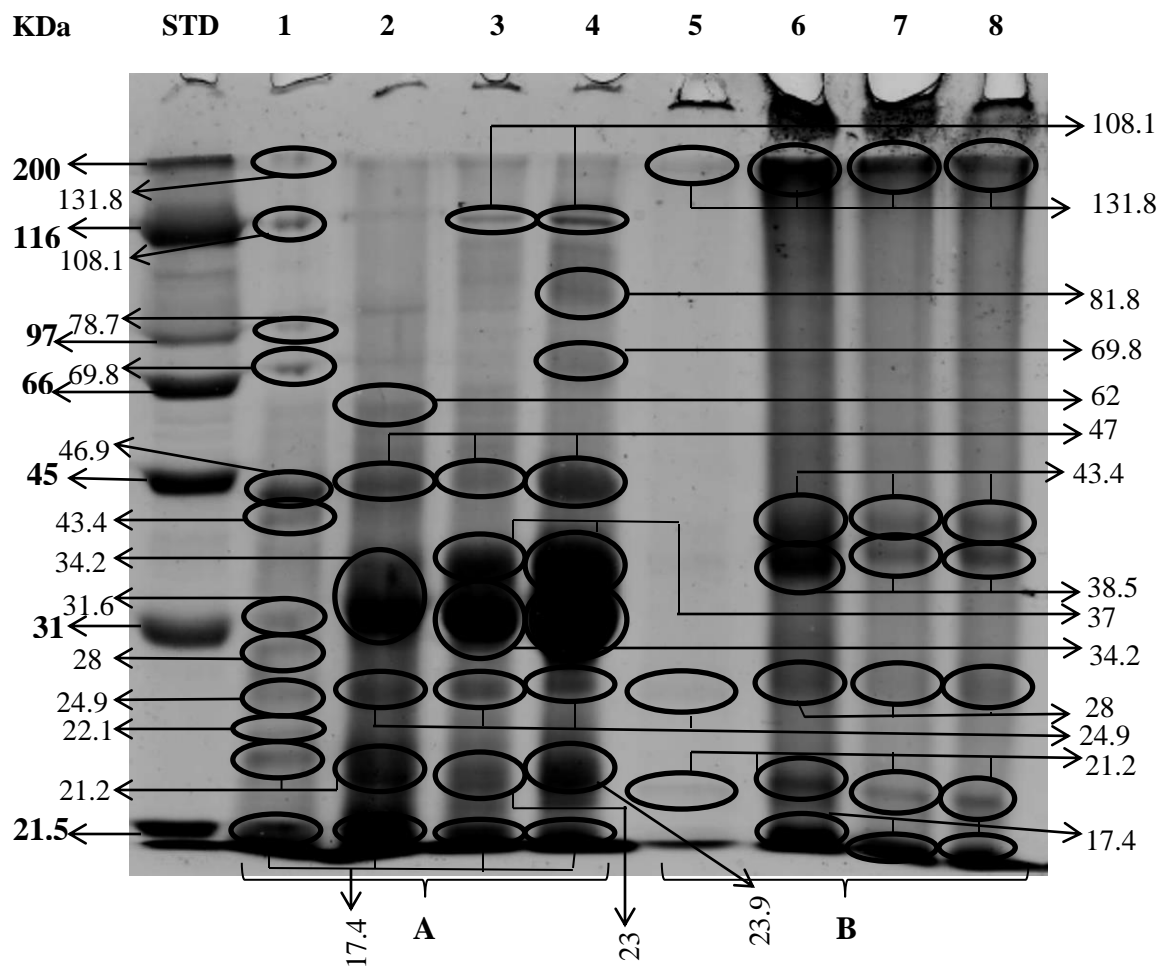
precipitation (ASPI-IEP) revealed two subunits with 62 and 21.2 kDa which were not shown with the other precipitation techniques in seeds. It might be referred to L-amino acid oxidase which has a molecular weight of 63 kDa (Singer et al., 1950) and trypsin inhibitor which has molecular weight of 20.5 kDa (Folta-Stogniew and Williams, 1999) respectively. Ethanol lane also got a subunit that was not found in other lanes with 23 kDa and can be known as papain (Weber and Osborn, 1969).

A study done by Kaushal et al., 1982 about arugula seeds reported 4 subunits by SDS PAGE with molecular weights 60, 35, 26.6 and 18 kDa. All of them were found in the present work and they are the same major subunits except for 60 kDa that only could be found with isoelectric precipitation (ASPI-IEP) technique. All those 4 subunits were major fractions of globulin which is a typical storage glycoprotein (Kaushal et al., 1982). A recent study conducted by Shinwari et al., 2013 on arugula seeds, found 17 protein bands of seed storage proteins and their molecular weights ranged from 15 to 220 kDa. Canola seeds which are from the same family of arugula (Brassica) had 4 major polypeptides with molecular weights of 16, 18, 30 and 53 kDa (Aluko et al., 2001).

On the other hand arugula leaves, the other half of table 4.1, had much less subunits in the control and the three precipitation techniques. Control lane (AWC) contained only three subunits. 24.9 kDa was not found in any precipitation techniques just presented in the control lane. The same thing happened for four of the major subunits that were presented identically in all the precipitation techniques and absent in the control lane. Molecular weights with 131.8 and 21.2 kDa (trypsin inhibitor) were matching with the control and all the different precipitation techniques lanes. A study conducted by Sadia et al., 2009 proved the presence of the band with 20 kDa molecular weight in the leaves by SDS-PAGE. A subunit in a recent study with 32 kDa

has been identified by SDS-PAGE done by Gupta et al., 2013 on arugula leaves. It showed that this subunit is chitinase enzyme, fungal resistance, produced only in pathological situations and during the process of natural senescence of attached or detached leaves (Lers et al., 1998). In the recent work there were two subunits in leaves with 28 and 43 kDa found in all precipitation techniques. It might be chitinase enzyme which could be referred to the senescence process of arugula leaves. The same molecular weights (28 and 42 kDa) were referred to chitinase enzyme by Lers et al., 1998.

In general, seeds had more subunits and some of the high molecular weights were absent in leaves. There was variation between control lanes and different precipitation techniques.



**Figure 4.1** SDS-PAGE of arugula leaf and seed protein isolates. STD= standard proteins; column: 1= ASC (arugula seed control); 2= ASPI- IEP (arugula seed protein isolate-isoelectric precipitation) ; 3= ASPI-EP (arugula seed protein isolate-ethanol precipitation) ; 4= ASPI-CP (arugula seed protein isolate-cryoprecipitation); 5= AWC(arugula whole plant control); 6= AWPI-IEP (arugula whole plant protein isolate-isoelectric precipitation); 7= AWPI-EP (arugula whole plant protein isolate-ethanol precipitation) ; 8=AWPI-CP (arugula whole plant protein isolate-cryoprecipitation). (A): Arugula seeds and (B): Arugula whole plant.

**Table 4.1** Molecular weights of arugula seed and leaf protein isolates.

ASC	ASPI-IEP	ASPI-EP	ASPI-CP	Reference (1)	AWC	AWPI-IEP	AWPI-EP	AWPI-CP
131.8	-	-	-	-	131.8	131.8	131.8	131.8
108.1	-	108.1	108.1	-	-	-	-	-
78.7	-	-	81.8	-	-	-	-	-
69.8	-	-	69.8	-	-	-	-	-
	62	-	-	60	-	-	-	-
46.9	47	47	47	-	-	-	-	-
43.4	-	-	-	-	-	43.4, 38.5	43.4, 38.5	43.4, 38.5
31.6	34.2	37, 34.2	37, 34.1	35	-	-	-	-
28	-	-	-	-	-	28	28	28
24.9	24.9, 23.9	24.9	24.9, 23.9	26.6	24.9	-	-	-
22.1	-	23	-	-	-	-	-	-
21.2	21.2	-	-	-	21.2	21.2	21.2	21.2
17.4	17.4	17.4	17.4	18	-	17.4	17.4	17.4

ASC (arugula seed control); ASPI- IEP (arugula seed protein isolate-isoelectric precipitation) ; ASPI-EP (arugula seed protein isolate-ethanol precipitation) ; ASPI-CP (arugula seed protein isolate-cryoprecipitation); AWC(arugula whole plant control); AWPI-IEP (arugula whole plant protein isolate-isoelectric precipitation); AWPI-EP (arugula whole plant protein isolate-ethanol precipitation); AWPI-CP (arugula whole plant protein isolate-cryoprecipitation).

**Reference 1:** Kaushal G P., Sital J S. and Bhatia I S. (1982). Studies on Taramira Seed (*Eruca sativa* Lam.) Proteins, *J. Agric. Food chem.* **30**: 431-435.

#### 4.1.1.2 SDS-PAGE of Protein Isolates' Supernatant

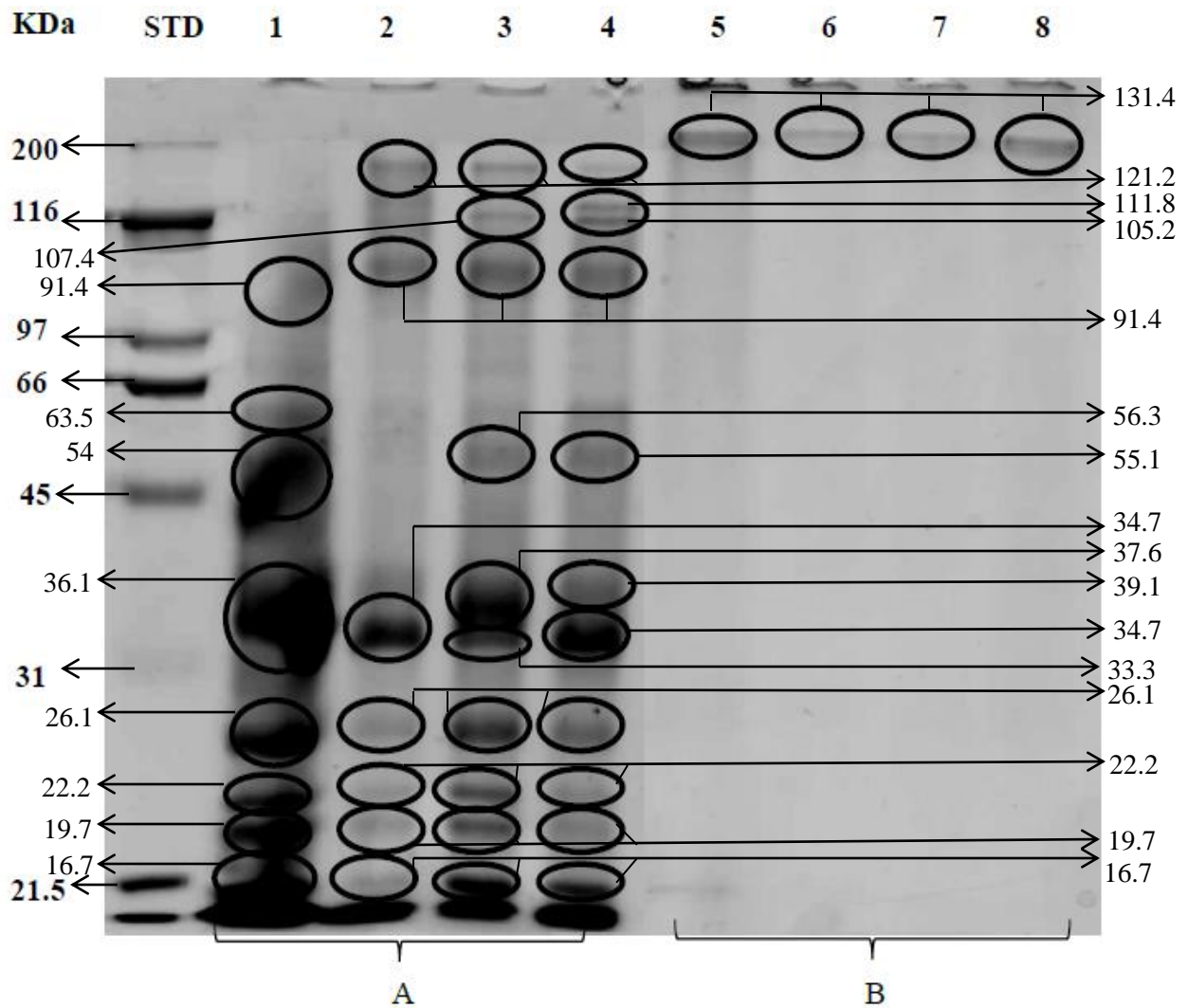
Protein isolates supernatant was obtained after precipitation processes. Table 4.2 shows molecular weights of protein isolates in seeds and leaves. Regarding seeds, molecular weights ranged between 121.2 to 16.7 kDa. Control lane has four absent subunits 121.2, 105.2, 111.8 and 107.4 kDa which were presented in other precipitation techniques. The fraction with 91.4 kDa may be referred to (phosphorylase a) with a molecular weight 94 kDa (Seery et al., 1967). The same lane matches all subunits in other precipitation techniques except the one with 63.5 kDa was found only in the control lane (ASC). 54 kDa could be glutamate dehydrogenase (Ersenberg and Tiomkins, 1968) that was found in control (ASC), ethanol (ASPI-EP) lane with 56 kDa and cryo-precipitation (ASPI-CP) lane with 55 kDa. All seed lanes had fractions between 33 and 36 kDa that can be referred to pepsin with 35 kDa (Bovey and Yanari, 1960). The smallest subunit that was found in all seeds lanes was with 16.7 kDa. Generally all the subunits are found less intense than the ones in the extract. This deduces that those subunits were mainly removed from the extract during different precipitation techniques and minor amounts were left in the supernatant.

In leaf isolates, there was only one subunit in the control lane (AWC) and the three precipitation techniques lanes. It is the same subunit with 131.4 kDa, but also less intense than isolates in figure 4.1.

To improve precipitation process, study was done by Fic et al., 2010 compared four protein precipitating reagents which were chloroform, TCA, methanol and acetone. It was concluded that TCA precipitation was one of the best methods and acetone precipitation yielded the highest protein recovery when compared with the supernatant. Another study conducted to enhance the quality of proteins showed that mixing TCA/acetone and methanol rinsing with phenol extraction resulted in better protein profiles when compared with supernatant for a variety of leaves such as

pine, olive, grape, banana, apple and lemon. This method is effective for samples high sugar content, high polyphenols rates, high pigment content, low protein content, and high acidity (Wang et al., 2006). TCA in acetone was used as well in protein extraction from seeds of soybeans (Xu et al., 2007).



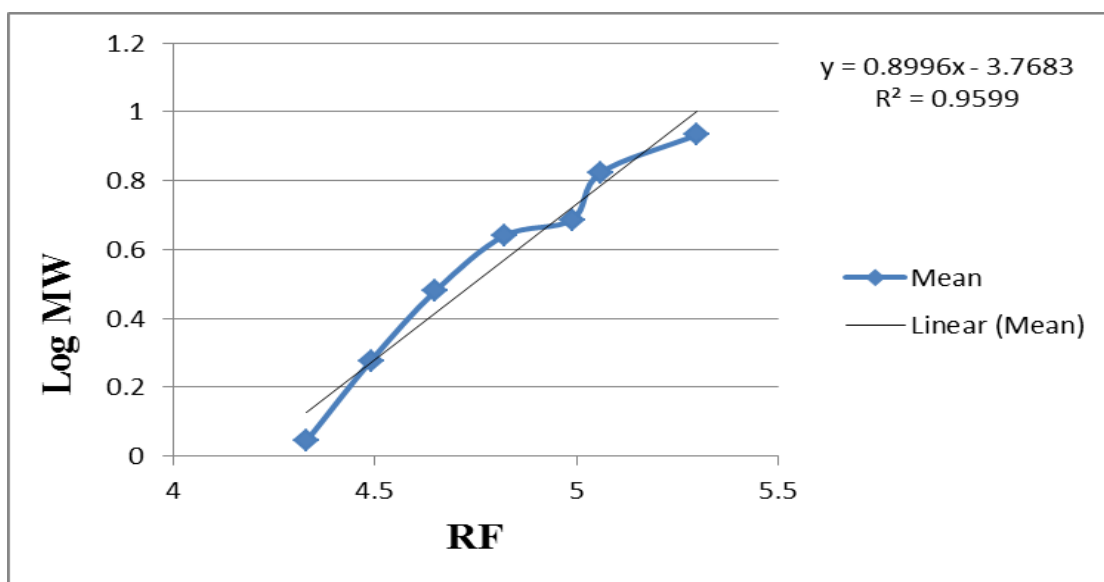


**Figure 4.2** SDS-PAGE of arugula protein supernatant. STD= standard proteins; column: 1= ASC (arugula seed control); 2= ASS-IEP (arugula seed supernatant-isoelectric precipitation); 3= ASS-EP (arugula seed supernatant-ethanol precipitation); 4= ASS-CP (arugula seed supernatant-cryoprecipitation); 5= AWC (arugula whole plant control); 6= AWS-IEP (arugula whole plant supernatant-isoelectric precipitation); 7= AWS-EP (arugula whole plant supernatant-ethanol precipitation); 8=AWS-CP (arugula whole plant supernatant-cryoprecipitation). (A): Arugula seeds and (B): Arugula whole plant.

**Table 4.2** Molecular weights of arugula seed and leaf protein supernatants.

ASC	ASS-IEP	ASS-EP	ASS-CP	AWC	AWS-IEP	AWS-EP	AWS-CP
-	121.2	121.2	121.2	131.8	131.8	131.8	131.8
-	-	107.4	111.8, 105.2	-	-	-	-
91.4	91.4	91.4	91.4	-	-	-	-
63.5	-	-	-	-	-	-	-
54	-	56.3	55.1	-	-	-	-
36.1	34.7	37.6, 33.3	39.1, 34.7	-	-	-	-
26.1	26.1	26.1	26.1	-	-	-	-
22.2	22.2	22.2	22.2	-	-	-	-
19.7	19.7	19.7	19.7	-	-	-	-
16.7	16.7	16.7	16.7	-	-	-	-

ASC (arugula seed control); ASS-IEP (arugula seed supernatant-isoelectric precipitation); ASS-EP (arugula seed supernatant-ethanol precipitation); ASS-CP (arugula seed supernatant cryoprecipitation); AWC (arugula whole plant control); AWS-IEP (arugula whole plant supernatant-isoelectric precipitation); AWS-EP (arugula whole plant supernatant-ethanol precipitation); AWS-CP (arugula whole plant supernatant-cryoprecipitation).



**Figure 4.3** Standard curve used for SDS-PAGE generated by plotting the log of the molecular weight of protein standards vs. the relative mobility.

## **4.1.2 Native Polyacrylamide Gel Electrophoresis (Native-PAGE)**

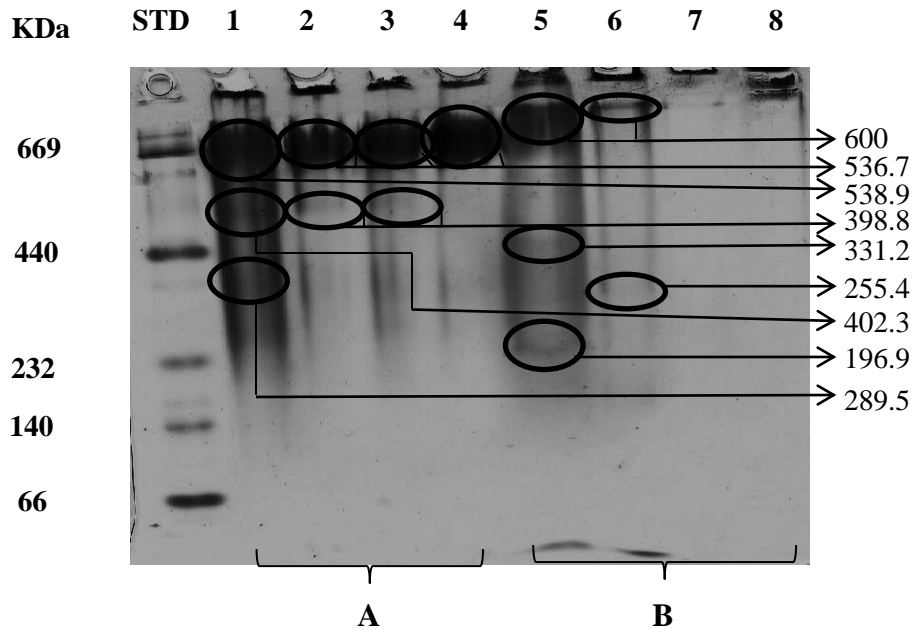
### **4.1.2.1 Native-PAGE of Protein Isolates and Supernatant**

Molecular weights of fractions in protein isolates are documented in table 4.3. Control lanes of seeds and leaves had three fractions, but with different molecular weights. Control lanes and lanes of precipitation techniques had approximately similar major component in the protein fraction with different minor bands in each lane. Ethanol precipitation (AWPI-EP) and cryo-precipitation (AWPI-CP) in leaf lanes did not show any major or minor fractions. In isoelectric precipitation (AWPI-IEP) there was a protein with 255.4 kDa which most probably catalase with molecular weight 247.5 kDa (Sumner and Gralen, 1938). On contrary, seeds had mostly similar fractions in control and multiple precipitation lanes.

Regarding protein supernatant reported in table 4.4, results were totally different. None of the fractions which were found in protein isolates matches the ones in the supernatant exactly, but some of them are near to other molecular weights. Ornstein, 1964 reported that the molecular weight 400 kDa referred to fibrinogen protein. All lanes of seeds in protein isolates and supernatant contained similar molecular weight between 395-410 kDa that might be same protein (fibrinogen). Present work had this molecular weight in protein isolates and supernatant. In control lane of leaves (AWC) in protein supernatant, there was a protein with 459.5 kDa that might be apoferritin with molecular weight 466.9 kDa (Hofmann and Harrison, 1963). In addition, AWPI-EP and AWPI-CP lanes in the supernatant contained three and four fractions respectively. One of those bands with 216 kDa is myosin (Gershman and Dreizen, 1970). Seeds in protein supernatant and control lane in protein isolates, had four bands between 292-276 kDa that could be referred to ERD4 (early responsive to dehydration plant protein) which has exactly molecular weight of 300 kDa (Kjell et al., 2004). Both whole plant in protein supernatant and

seeds in protein isolates had proteins with molecular weights between 536.7-544.6 kDa which could be Rubisco protein (550 kDa) (Sanchez de Jiménez et al., 1995). Whole plant in protein isolates and supernatant contained 3 bands between 331-339 kDa and 4 bands between 190-197 kDa which might be considered as gallic acid decarboxylase (330 kDa) reported by Zeida et al., 1998 and vicilin-like proteins (190 kDa) respectively documented by González-Pérez and Arellano, 2009. Some of the largest molecular weights in seed protein supernatants and whole plant protein isolates were 576.4 kDa and 600 kDa respectively. Those proteins might be plasma membrane proton ATPase-like that has molecular weight between 550-600 kDa (Kjell et al., 2004).

In general, bands found in protein supernatant were intense especially minor ones, and their molecular weights vary from the ones in protein isolates. Major and minor bands approximately match each other in leaves and seeds. Differences in bands types and molecular weights between protein supernatant and protein isolates might be due to the inappropriate precipitation techniques that were used in sample preparation. If more efficient techniques were used like TCA/acetone which was mentioned in part 4.1.1.2, results of protein profiles might have been better.

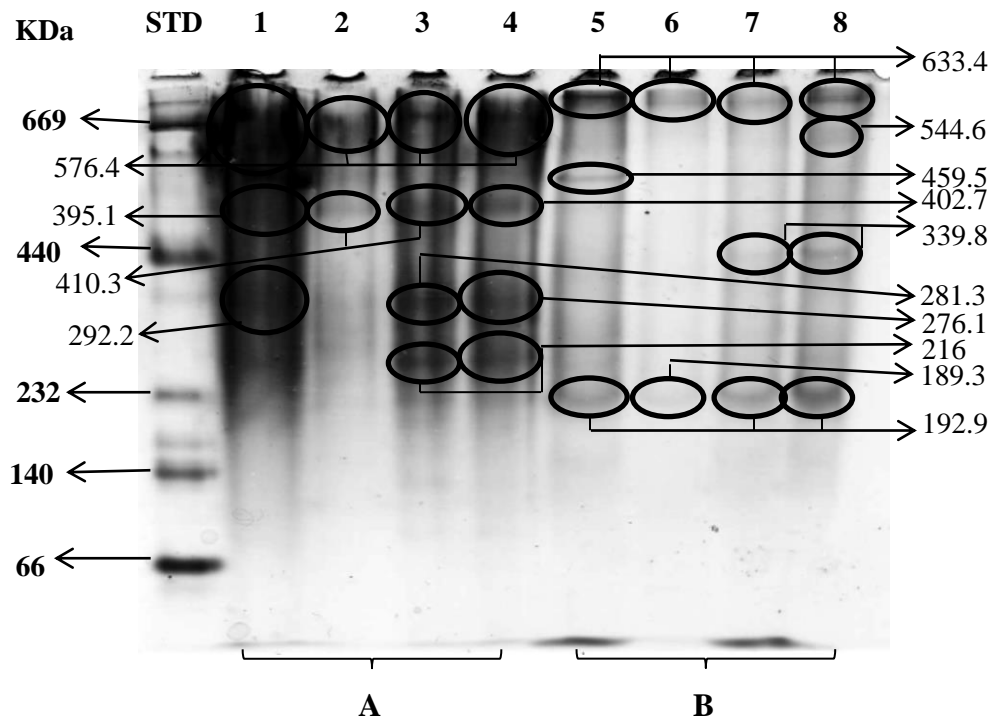


**Figure 4.4** Native-PAGE of protein isolates in arugula leaves and seeds. STD= standard proteins; column: 1= ASC (arugula seed control); 2= ASPI-IEP (arugula seed protein isolate-isoelectric precipitation); 3= ASPI-EP(arugula seed protein isolate-ethanol precipitation); 4= ASPI-CP (arugula seed protein isolate-cryoprecipitation); 5= AWC (arugula whole plant control); 6= AWPI-IEP (arugula whole plant protein isolate-isoelectric precipitation); 7= AWPI-EP (arugula whole plant protein isolate-ethanol precipitation); 8= AWPI-CP (arugula whole plant protein isolate-cryoprecipitation). (A): arugula seeds and (B): arugula whole plant.

**Table 4.3** Molecular weights of native protein isolates from arugula seeds and leaves.

ASC	ASPI-IEP	ASPI-EP	ASPI-CP	AWC	AWPI-IEP	AWPI-EP	AWPI-CP
-	-	-	-	600	600	-	-
<b>538.9</b>	536.7	536.7	536.7	-	-	-	-
<b>402.3</b>	398.8	398.8	-	-	-	-	-
-	-	-	-	331.2	-	-	-
<b>289.5</b>	-	-	-	-	-	-	-
-	-	-	-	-	255.4	-	-
-	-	-	-	196.9	-	-	-

ASC (arugula seed control); ASPI-IEP (arugula seed protein isolate-isoelectric precipitation); ASPI-EP(arugula seed protein isolate-ethanol precipitation); ASPI-CP (arugula seed protein isolate-cryoprecipitation); AWC (arugula whole plant control); AWPI-IEP (arugula whole plant protein isolate-isoelectric precipitation); AWPI-EP (arugula whole plant protein isolate-ethanol precipitation); AWPI-CP (arugula whole plant protein isolate-cryoprecipitation).

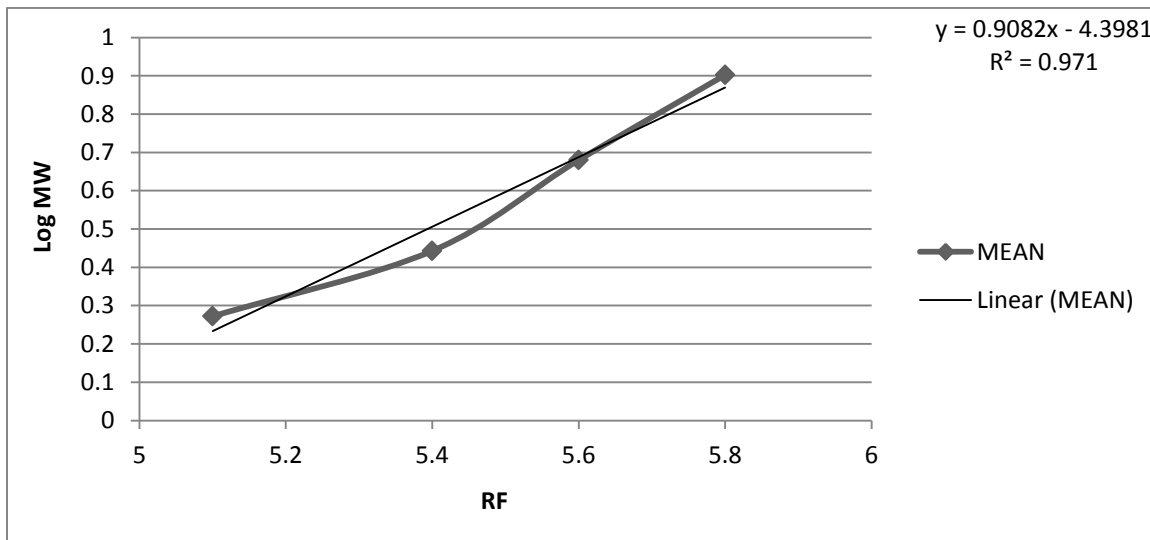


**Figure 4.5** Native-PAGE of protein supernatant in arugula leaves and seeds. STD= standard proteins; column: 1= ASC (arugula seed control); 2= ASS-IIEP (arugula seed supernatant-isoelectric precipitation); 3= ASS-EP (arugula seed supernatant-ethanol-precipitation); 4= ASS-CP (arugula seed supernatant-cryoprecipitation); 5= AWC (arugula whole plant control); 6= AWS-IIEP (arugula whole plant supernatant-isoelectric precipitation); 7= AWS-EP (arugula whole plant supernatant-ethanol precipitation); 8= AWS-CP (arugula whole plant supernatant-cryoprecipitation). (A): arugula seeds and (B): arugula whole plant.

**Table 4.4** Molecular weights of protein supernatant in arugula seeds and leaves.

ASC	ASS-IEP	ASS-EP	ASS-CP	AWC	AWS-IEP	AWS-EP	AWS-CP
-	-	-	-	633.4	633.4	633.4	633.4
<b>576.4</b>	576.4	576.4	576.4	-	-	-	-
-	-	-	-	-	-	-	544.6
-	-	-	-	459.5	-	-	-
<b>395.1</b>	410.3	410.3	402.7	-	-	-	-
-	-	-	-	-	-	339.8	339.8
<b>292.2</b>	-	281.3	276.1	-	-	-	-
-	-	216	216	-	-	-	-
-	-	-	-	192.9	189.3	192.9	192.9

ASC (arugula seed control); ASS-IEP (arugula seed supernatant-isoelectric precipitation); ASS-EP (arugula seed supernatant-ethanol-precipitation); ASS-CP (arugula seed supernatant-cryoprecipitation); AWC (arugula whole plant control); AWS-IEP (arugula whole plant supernatant-isoelectric precipitation); AWS-EP (arugula whole plant supernatant-ethanol precipitation); AWS-CP (arugula whole plant supernatant-cryoprecipitation).



**Figure 4.6** Standard curve used for Native-PAGE generated by plotting the log of the molecular weight of protein standards vs. the relative mobility.



## 4.2 Digestibility of *Eruca sativa* Protein Derivatives *in vitro*

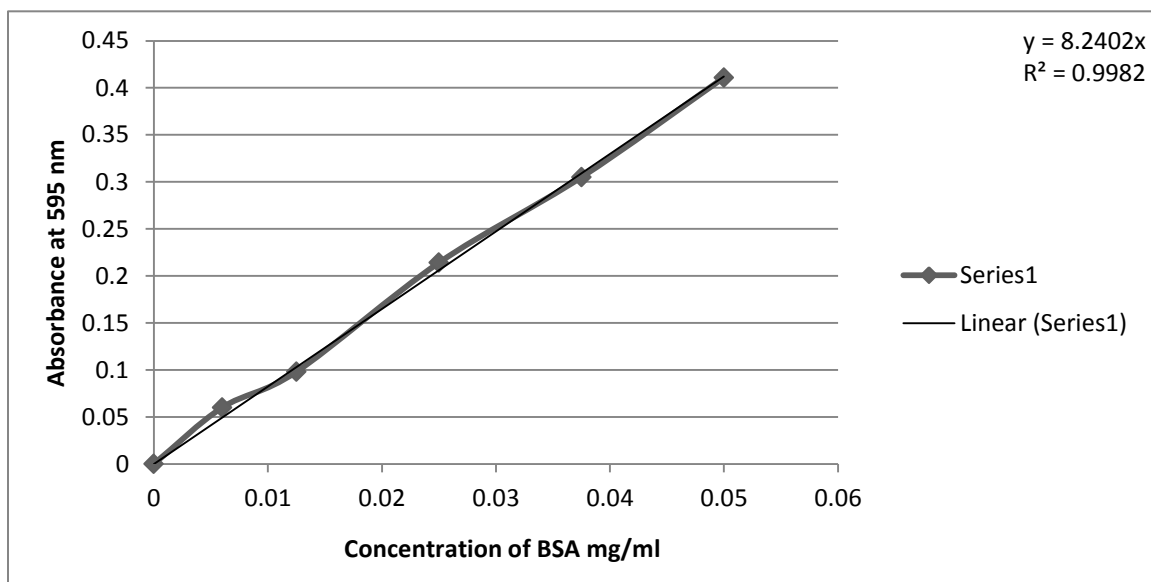
### 4.2.1 Enzymatic Hydrolysis of *Eruca sativa* Protein Derivatives by Trypsin

Trypsin is a serine protease that hydrolyses proteins specifically the peptide bond on carboxyl side of lysine and arginine (Damodaran, 1997) to produce bioactive peptides and enhance functional properties of original proteins. Table 4.6 and figures 4.11 and 4.12 show the results of degree of hydrolysis (DH) during 120 minutes for protein extracts from seeds and leaves. Lysozyme results were presented as standard. Degree of hydrolysis of protein extracts ranged from 34 % in seeds with CP to 65% in leaves with isoelectric precipitation (ASPI-IEP). The standard reached 89 % at 120 minutes.

Degree of hydrolysis usually increased with time especially for trypsin because of converting the insoluble compounds to soluble ones (Yin et al., 2008). *In vivo* study done on pigs by Jansman et al., 1994 showed that trypsin activity reached 70%. Glucosinolates known that they inhibit the digestive proteases (Pedroche et al., 2004) and a study done by Barillari et al., 2005 proved that glucuerucin, major glucosinolate in arugula plant, found in seeds with higher amounts than leaves. So, this might be the reason for having degree of hydrolysis percentages in seeds less than leaves. Trypsin hydrolyses storage proteins (7S and 11S) in seeds very well as reported by Vaintraub et al., 1976. This study showed that 90% and 82% of 11S and 7S of *vicia sativa* were hydrolysed respectively. Seed results could have been better if there were no tannins in them because tannins are known to reduce digestibility (Feeny, 1969) by at least 24% because they bind with digestive enzymes and inhibit their activity (Bravo, 1998). Although seeds do not contain a lot of tannins only 4% (Gulfraz et al., 2011), but it might increase DH around 10% with zero tannins. Also isoelectric precipitation gave one of the best results for trypsin hydrolysis (Vaintraub et al., 1976) which matches the present work where isoelectric precipitation for leaves had the highest DH among other types of

precipitation techniques. Isoelectric precipitation (ASPI-IEP) of seeds had higher DH% than cryo-precipitation (ASPI-CP) one.

Other studies done on different kinds of foods and plants and hydrolysed by trypsin gave varieties of results. Hemp had DH of (2.3-6.7%) (Yin et al., 2008), soybean protein and chickpea protein had DH of 11.5 % and 12.3% respectively (Singh, 2011). DH of flaxseed protein fractions ranged from 9.4-24.5% (Ayad, 2010). Rapeseed had 7.7% DH when using Alcalase and Flavourzyme (Vioque et al., 2000). Whey protein isolates reached around 36% DH when using Alcalase for 5 hours (Ayad, 2010).

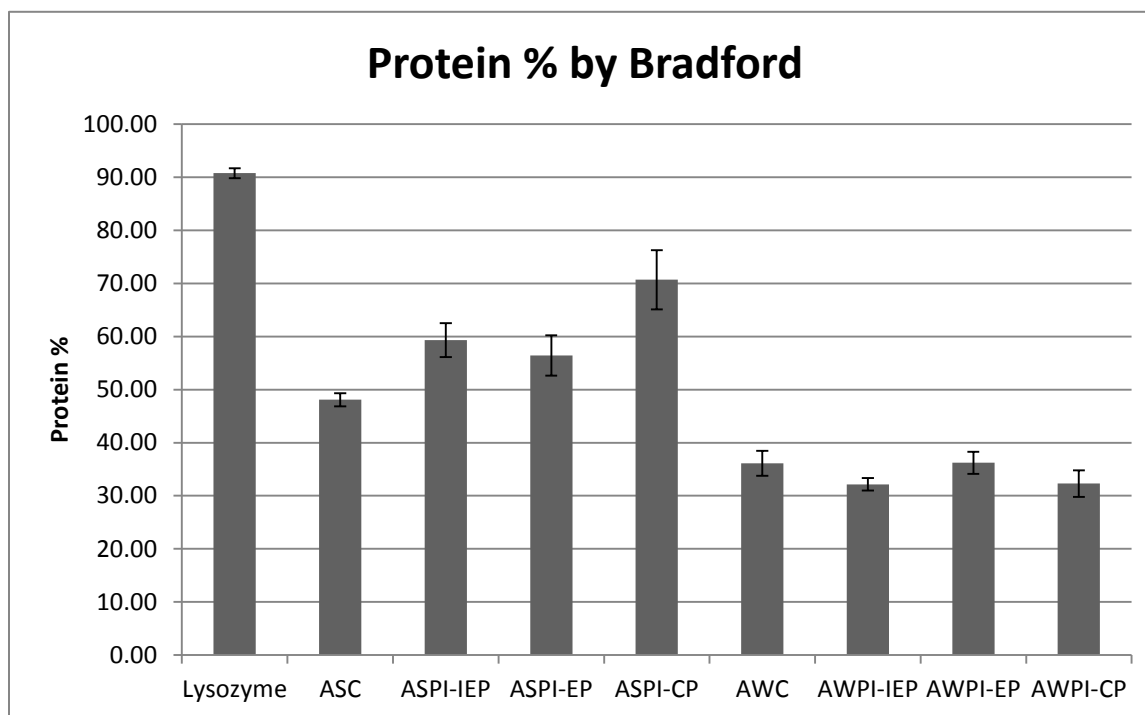


**Figure 4.7** Standard curve for bovine serum albumin (BSA) which was used to calculate protein concentration.

**Table 4.5** Protein content of samples by Bradford method using spectrophotometer.

SAMPLE	Average of triplicate (SD)
Lysozyme	90.73 (0.96)
ASC	48.08 (1.22)
ASPI-IEP	59.31 (3.18)
ASPI-EP	56.40 (3.79)
ASPI-CP	70.67 (5.59)
AWC	36.09 (2.35)
AWPI-IEP	32.15 (1.18)
AWPI-EP	36.21 (2.10)
AWPI-CP	32.31 (2.50)

ASC (arugula seed control); ASPI-IEP (arugula seed protein isolate-isoelectric precipitation); ASPI-EP (arugula seed protein isolate-ethanol-precipitation); ASPI-CP (arugula seed protein isolate-cryoprecipitation); AWC (arugula whole plant control); AWPI-IEP (arugula whole plant protein isolate-isoelectric precipitation); AWPI-EP (arugula whole plant protein isolate-ethanol precipitation); AWPI-CP (arugula whole plant protein isolate-cryoprecipitation).



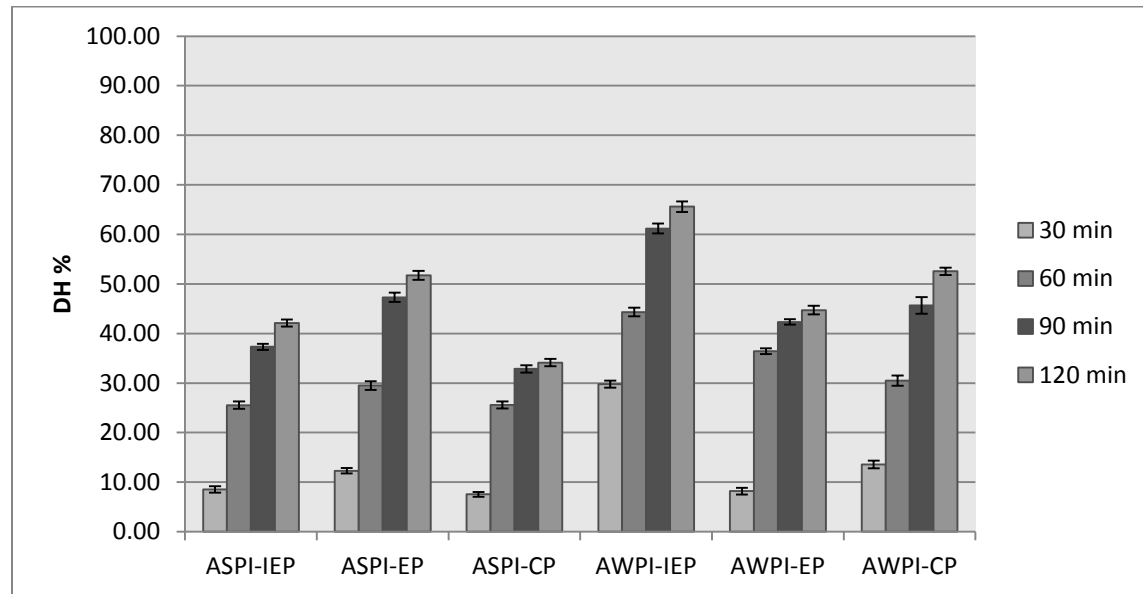
**Figure 4.8** Protein content of arugula seed protein isolate and arugula whole plant protein isolate). ASC (arugula seed control); ASPI-IEP (arugula seed protein isolate-isoelectric precipitation); ASPI-EP (arugula seed protein isolate-ethanol-precipitation); ASPI-CP (arugula seed protein isolate-cryoprecipitation); AWC (arugula whole plant control); AWPI-IEP (arugula whole plant protein isolate-isoelectric precipitation); AWPI-EP (arugula whole plant protein isolate-ethanol precipitation); AWPI-CP (arugula whole plant protein isolate-cryoprecipitation).

**Table 4.6** Degree of hydrolysis of arugula leaf and seed protein isolates by trypsin.

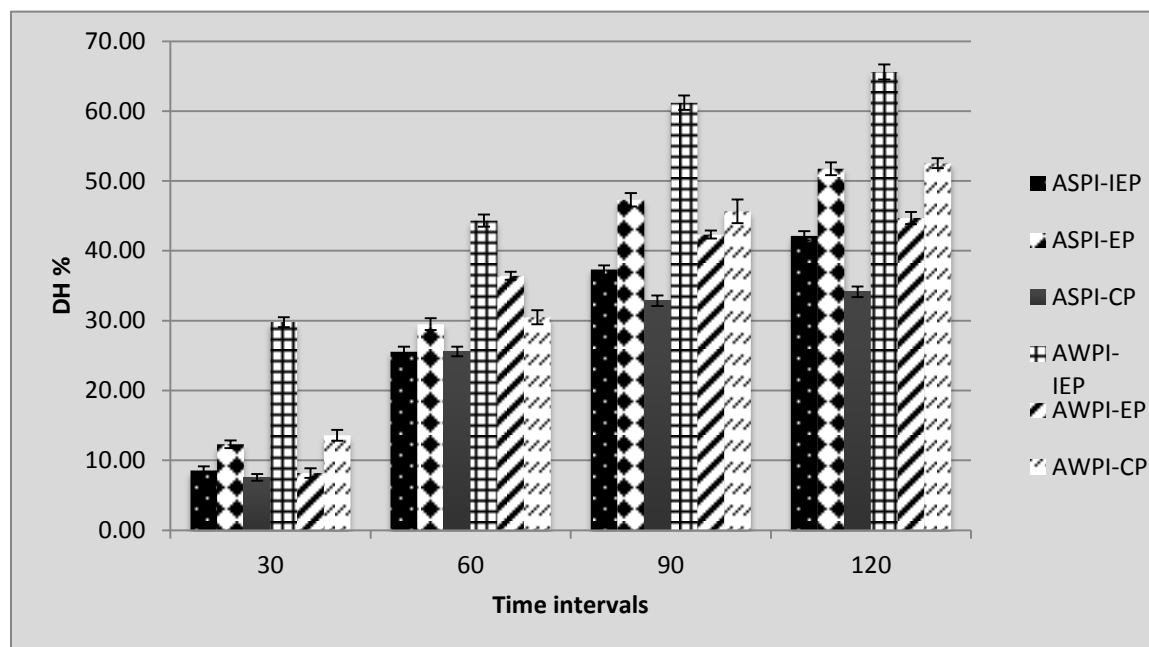
Time	Lysozyme (SD)	ASPI-IEP (SD)	ASPI-EP (SD)	ASPI-CP (SD)	AWPI-IEP (SD)	AWPI-EP (SD)	AWPI-CP (SD)
0	0.14 (0.12) <sup>ab</sup>	5.64 (0.35) <sup>a</sup>	4.44 (0.48) <sup>a</sup>	2.51 (0.79) <sup>ab</sup>	11.49 (0.54) <sup>b</sup>	1.21 (0.5) <sup>a</sup>	1.40 (0.52) <sup>a</sup>
30	7.42 (0.34) <sup>ab</sup>	8.53 (0.62) <sup>a</sup>	12.31 (0.55) <sup>a</sup>	7.56 (0.48) <sup>ab</sup>	29.80 (0.71) <sup>b</sup>	8.19 (0.69) <sup>a</sup>	13.59 (0.78) <sup>a</sup>
60	55.35 (0.66) <sup>ab</sup>	25.54 (0.75) <sup>a</sup>	29.51 (0.87) <sup>a</sup>	25.58 (0.69) <sup>ab</sup>	44.34 (0.88) <sup>b</sup>	36.42 (0.58) <sup>a</sup>	30.50 (1.02) <sup>a</sup>
90	75.41 (1.06) <sup>ab</sup>	37.31 (0.62) <sup>a</sup>	47.30 (0.95) <sup>a</sup>	32.86 (0.74) <sup>ab</sup>	61.19 (1.02) <sup>b</sup>	42.33 (0.56) <sup>a</sup>	45.66 (1.69) <sup>a</sup>
120	90.32 (0.72) <sup>ab</sup>	42.11 (0.69) <sup>a</sup>	51.74 (0.91) <sup>a</sup>	34.12 (0.75) <sup>ab</sup>	65.60 (1.06) <sup>b</sup>	44.71 (0.87) <sup>a</sup>	52.54 (0.73) <sup>a</sup>

ASPI-IEP (arugula seed protein isolate-isoelectric precipitation); ASPI-EP (arugula seed protein isolate-ethanol-precipitation); ASPI-CP (arugula seed protein isolate-cryoprecipitation); AWPI-IEP (arugula whole plant protein isolate-isoelectric precipitation); AWPI-EP (arugula whole plant protein isolate-ethanol precipitation); AWPI-CP (arugula whole plant protein isolate-cryoprecipitation).

Columns with the same letters (a-b) are not significant ( $p \leq 0.05$ ).



**Figure 4.9** Degree of hydrolysis of protein isolates by trypsin. ASPI-IEP (arugula seed protein isolate-isoelectric precipitation); ASPI-EP (arugula seed protein isolate-ethanol-precipitation); ASPI-CP (arugula seed protein isolate-cryoprecipitation); AWPI-IEP (arugula whole plant protein isolate-isoelectric precipitation); AWPI-EP (arugula whole plant protein isolate-ethanol precipitation); AWPI-CP (arugula whole plant protein isolate-cryoprecipitation).



**Figure 4.10** Degree of hydrolysis of protein isolates by trypsin grouped by time intervals. ASPI-IEP (arugula seed protein isolate-isoelectric precipitation); ASPI-EP (arugula seed protein isolate-ethanol-precipitation); ASPI-CP (arugula seed protein isolate-cryoprecipitation); AWPI-IEP (arugula whole plant protein isolate-isoelectric precipitation); AWPI-EP (arugula whole plant protein isolate-ethanol precipitation); AWPI-CP (arugula whole plant protein isolate-cryoprecipitation).

#### **4.2.2 Enzymatic Hydrolysis of *Eruca sativa* Protein Derivatives by Trypsin-Chymotrypsin Mixture**

Mixture of trypsin-chymotrypsin was used in order to get more powerful hydrolysis. Table 4.7 and figures 4.13 and 4.14 show results of enzymatic hydrolysis during 120 minutes for protein extract in leaves and seeds. Lysozyme was used as standard. Chymotrypsin specificity is peptide bonds that have phenylalanine, tyrosine and tryptophan (Damodaran, 1997). DH ranged from 86-61% during first 90 minutes and then decreased to 50-66% in the last 30 minutes interval which was in 120 minutes.

It was mentioned that trypsin-chymotrypsin mixture has capacity to break 25 bonds per mole of substrate (Hill and Schmidt, 1962) and they also documented that complete hydrolysis can be obtained by using only proteolytic enzymes. Having higher percentage of enzymatic hydrolysis, increases the protein solubility due to releasing small units of polypeptide from protein. Unfolding native protein results in enhancing digestibility (Kamara et al., 2010). Study done by Vaintraub et al., 1976 reported that hydrolysis became faster only after denaturation. That might be an explanation of having high percentage of DH from the beginning of hydrolysis because the combined trypsin-chymotrypsin was so powerful that affected the protein and denatured it immediately. They also reported that chymotrypsin did not invade the non-hydrolyzable part of trypsin digest and both of them had equal effects regarding storage proteins. That could be another reason that DH% did not increase very much during 120 minutes only slight increase from 30 minutes until 90 minutes. It was not high as compared to lysozyme.

There are some factors that can reduce digestibility or inhibit hydrolysis process such as protein aggregation because of covering the catalytic location by aggregation (Yin et al., 2008). This can be considered as clarification of decrease in DH at the last 30 minutes. Another reason

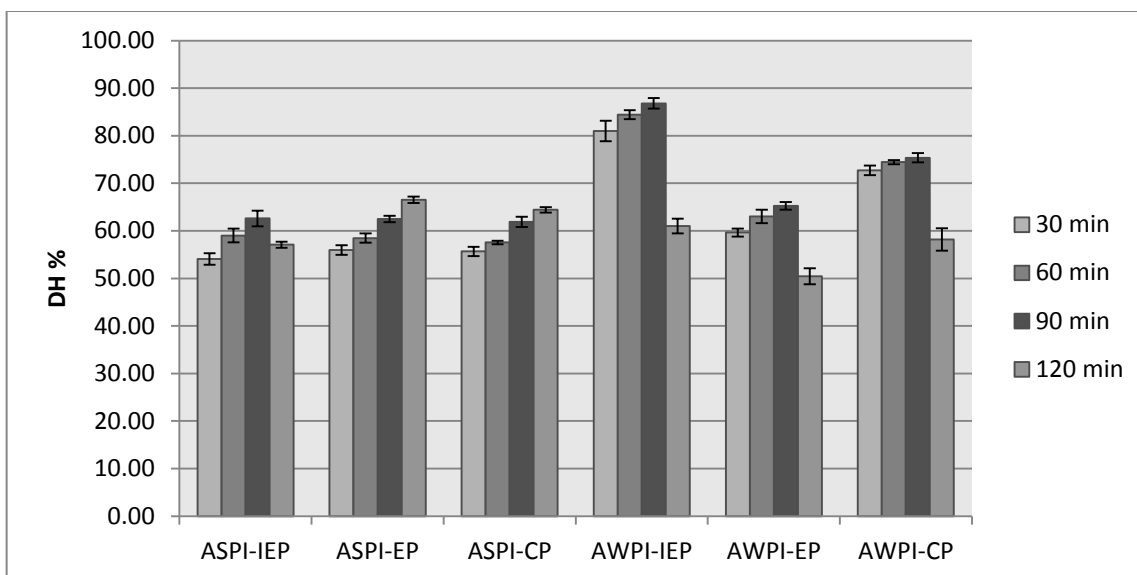
was discovered by Jansman et al., 1994. They justified their research results that were similar to the present work in which the mixture activity decreases after first hours by referring to the dilution of enzymes by the digesta flow. Another study done by Schneeman, 1978 found that fibres could reduce digestive enzymes activities because of the adsorption of those enzymes to the fibre matrix. This might also accounted as a reason because the protein extract not pure, it could be accompanied with some fibres especially in leaves where fibres are higher than seeds. This could be shown clearly by the present results in table 4.7 where seeds had higher DH% than leaves. Low, 1982 reported that there are many factors that affect enzymes activities such as enzymes denaturation, auto-digestion of enzymes and physiochemical conditions of digested food. Hassan, 2012 got similar results as in present work regarding decline of hydrolysis after 120 minutes. He proved that phenolic compounds and flavonoids affect enzymatic hydrolysis negatively when increasing flavonoid concentration. This might had affected present work because flavonoids and phenols in arugula seeds are 24.4% and 26.9% respectively (Gulfraz et al., 2011).



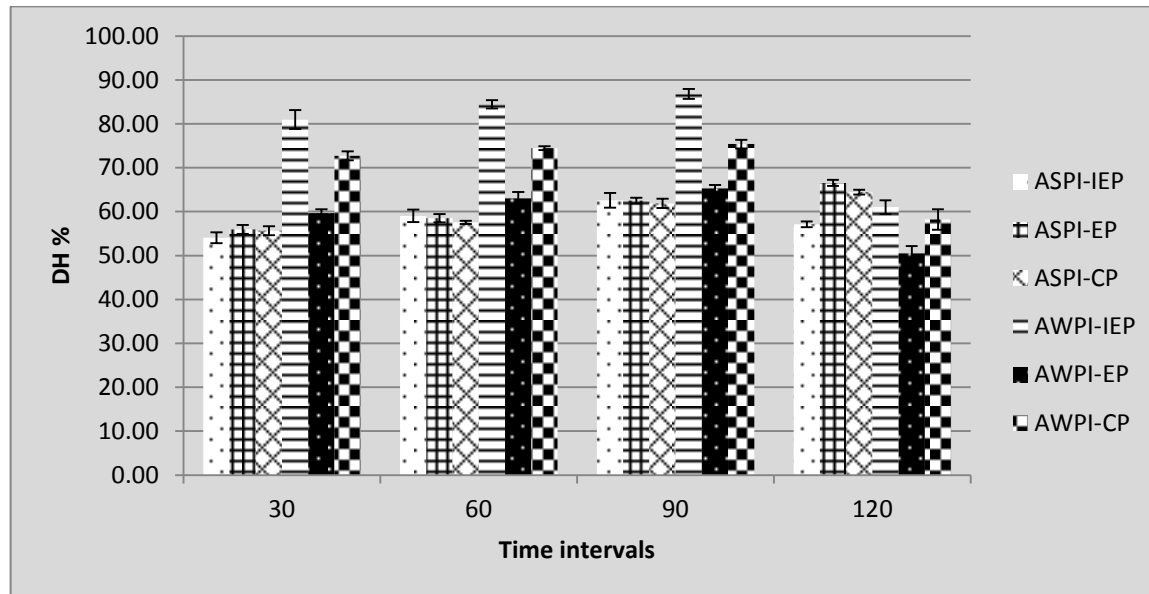
**Table 4.7** Degree of hydrolysis of arugula leaf and seed protein isolates by mixture of trypsin chymotrypsin.

<b>Time</b>	<b>Lysozyme (SD)</b>	<b>ASPI- IEP (SD)</b>	<b>ASPI- EP (SD)</b>	<b>ASPI- CP (SD)</b>	<b>AWPI- IEP (SD)</b>	<b>AWPI- EP (SD)</b>	<b>AWPI- CP (SD)</b>
<b>0</b>	63.41 (3.11) <sup>ad</sup>	49.29 (2.47) <sup>bc</sup>	51.88 (1.48) <sup>bd</sup>	50.83 (1.61) <sup>cde</sup>	77.42 (3.52) <sup>a</sup>	54.50 (1.73) <sup>be</sup>	70.40 (1.85) <sup>d</sup>
<b>30</b>	72.80 (1.35) <sup>ad</sup>	54.07 (1.23) <sup>bc</sup>	55.94 (1.02) <sup>bd</sup>	55.66 (1.0) <sup>cde</sup>	81 (2.15) <sup>a</sup>	59.65 (0.85) <sup>be</sup>	72.71 (1.02) <sup>d</sup>
<b>60</b>	79.75 (1.13) <sup>ad</sup>	59.02 (1.42) <sup>bc</sup>	58.48 (0.96) <sup>bd</sup>	57.56 (0.38) <sup>cde</sup>	84.43 (0.94) <sup>a</sup>	63.02 (1.43) <sup>be</sup>	74.47 (0.44) <sup>d</sup>
<b>90</b>	85.83 (2.90) <sup>ad</sup>	62.60 (1.68) <sup>bc</sup>	62.49 (0.66) <sup>bd</sup>	61.88 (1.05) <sup>cde</sup>	86.81 (1.14) <sup>a</sup>	65.26 (0.80) <sup>be</sup>	75.37 (0.98) <sup>d</sup>
<b>120</b>	95.96 (1.60) <sup>ad</sup>	57.08 (0.66) <sup>bc</sup>	66.54 (0.67) <sup>bd</sup>	64.43 (0.56) <sup>cde</sup>	61.01 (1.55) <sup>a</sup>	50.45 (1.70) <sup>be</sup>	58.21 (2.35) <sup>d</sup>

ASPI-IEP (arugula seed protein isolate-isoelectric precipitation); ASPI-EP (arugula seed protein isolate-ethanol-precipitation); ASPI-CP (arugula seed protein isolate-cryoprecipitation); AWPI-IEP (arugula whole plant protein isolate-isoelectric precipitation); AWPI-EP (arugula whole plant protein isolate-ethanol precipitation); AWPI-CP (arugula whole plant protein isolate-cryoprecipitation).  
Columns with the same letters (a-e) are not significant ( $p \leq 0.05$ ).



**Figure 4.11** Degree of hydrolysis of protein isolates by trypsin-chymotrypsin mixture. ASPI-IEP (arugula seed protein isolate-isoelectric precipitation); ASPI-EP (arugula seed protein isolate-ethanol-precipitation); ASPI-CP (arugula seed protein isolate-cryoprecipitation); AWPI-IEP (arugula whole plant protein isolate-isoelectric precipitation); AWPI-EP (arugula whole plant protein isolate-ethanol precipitation); AWPI-CP (arugula whole plant protein isolate-cryoprecipitation).



**Figure 4.12** Degree of hydrolysis of protein isolates by trypsin-chymotrypsin mixture grouped by time intervals. ASPI-IEP (arugula seed protein isolate-isoelectric precipitation); ASPI-EP (arugula seed protein isolate-ethanol-precipitation); ASPI-CP (arugula seed protein isolate-cryoprecipitation); AWPI-IEP (arugula whole plant protein isolate-isoelectric precipitation); AWPI-EP (arugula whole plant protein isolate-ethanol precipitation); AWPI-CP (arugula whole plant protein isolate-cryoprecipitation).

### **4.3 Antioxidants Activity of *Eruca sativa* Protein Isolates and Hydrolysates**

#### **4.3.1 DPPH Scavenging Activity Assay and Metal Ion Chelating Activity Assay**

Table 4.7 and figures 4.15, 4.16 and 4.17 illustrate results of antioxidant activity assays of 1mg/ml of hydrolysed (trypsin-chymotrypsin mixture) and non-hydrolysed arugula protein isolates from seeds and leaves. DPPH scavenging activity ranged from 12.5-27% for hydrolysates and from 4.2-10.6% for non-hydrolysates. Same trend for metal chelating assay, the range for copper chelation activity was from 17.7-21.4% for digested proteins and 6.6-12.4% for undigested ones. Regarding iron chelation assay, 27.4-23.3% was the range for hydrolysed proteins and 5.4-15.2% for non-hydrolysed proteins. Generally, in all the antioxidant activity assays hydrolysed protein had higher percentage than non-hydrolysed.

Properties of antioxidants in proteins depend mostly on structure, sequence and hydrophobicity of amino acids (Chen et al., 1998). For example, the sequence Proline-Histidine-Histidine revealed the highest antioxidant activity among other sequences (Sarmadi and Ismail, 2010). Methionine, Tryptophan and tyrosine have the highest antioxidant activity (Zhang et al., 2008). Histidine is the second major antioxidant containing peptides. It has the ability to donate hydrogen and metal ion chelation capacity (Rajapakse et al., 2005). In the present work, hydrolysates might have contained histidine which produced higher antioxidative results.

Hydrophobicity plays role in solubility. The more hydrophobic amino acids, the more they are soluble in lipids. As a result, it will be easier to react with free radicals to stop chain reaction (Kim et al., 2007). Hydrophobicity is a result of hydrolysis process because proteolysis enzymes unfold protein chain (Sarmadi and Ismail, 2010). So, hydrolysates might be more hydrophobic than protein isolates because they have higher DPPH scavenging activity. Li et al., 2008 documented that there is a positive correlation between DPPH scavenging activity and metal chelating activity (Pownall et al., 2010) and hydrophobicity. Phenylalanine, leucine, valine and tryptophan are

considered as highly hydrophobic amino acids (Pownall et al., 2010). Leucine is one of the highest amino acids in arugula seeds as reported in table 2.2 and leaves (Nurzynska-Wierdak, 2015).

Aromatic amino acids such as tyrosine, histidine, tryptophan and phenylalanine have the capacity to donate protons, so they will convert radicals into stable molecules. As a result, they have good radical scavenging properties with maintaining their own stability through resonance structure (Rajapakse et al., 2005). Usually radical scavenging happens through hydrogen donation of the hydroxyl group in the aromatic amino acids (Cumby et al., 2008). Metal chelating activity has a positive correlation with aromatic amino acids (Pownall et al., 2010).

Hydrolysates have higher antioxidant activity because through hydrolysis more active amino acid R groups will be exposed (Sarmadi and Ismail, 2010). Enzymatic hydrolysis produces smaller peptides which will enhance antioxidant activity. It has been reported in Li et al., 2008 that peptides with molecular weight range 500-1500 Da have more powerful antioxidant activity than higher than 1500 Da or less than 500 Da (Sarmadi and Ismail, 2010). Flavourzyme produces peptides with low molecular weights (Hamada, 2000) and as a result its hydrolysates will have higher antioxidant activity.

Qian et al., 2008 reported DPPH scavenging% of chymotrypsin and trypsin hydrolysates were 25.6 and 35.8 respectively. Seeds extract of arugula had 39.9% of DPPH scavenging activity in 100 µg/ml (Alam et al., 2007). Study was done by Kim and Ishii, 2006 proved that leaves powder of arugula has higher antioxidative properties than roots powder. Barillari et al., 2005 reported that glucoerucin in seeds which contained 95% of total glucosinolates has direct antioxidant activity due to its capacity to break up hydroperoxides and hydrogen peroxides. Some other proteins antioxidant activities are 45-90% DPPH radical scavenging for hydrolysed flaxseed and soybean and 31-81% for non-hydrolysed ones (Ayad, 2010), 16.8-22% DPPH radicals% for canola

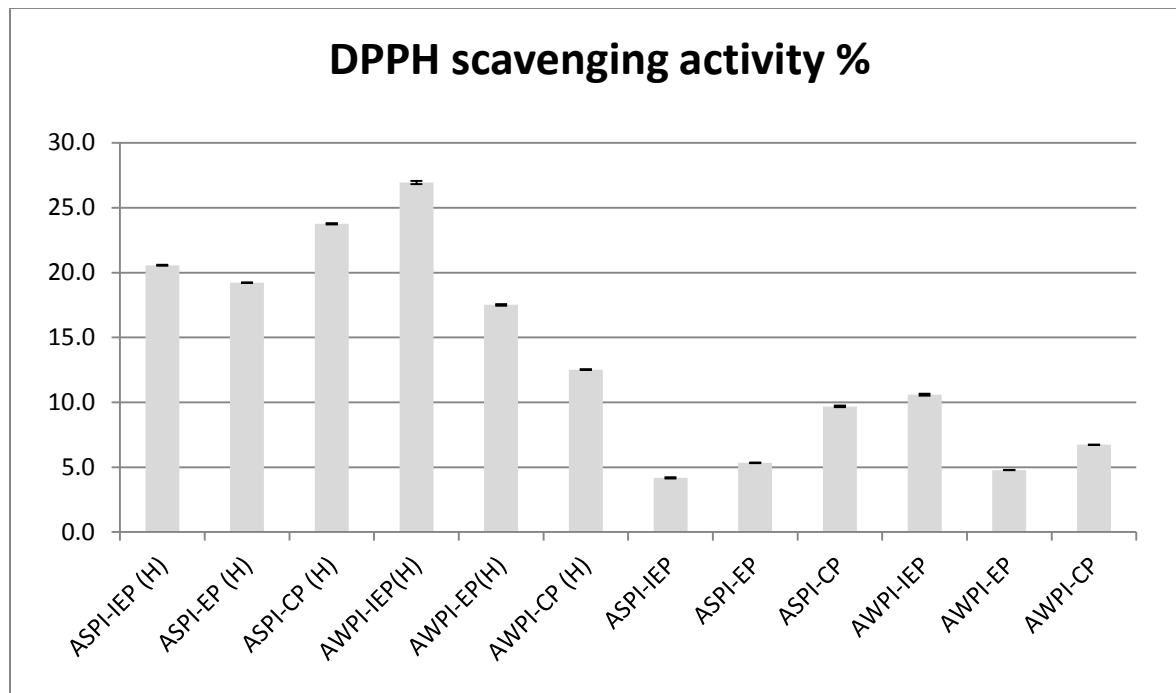
hydrolysates (Cumby et al., 2008), 76.9% and 63.1% for  $\text{Cu}^{2+}$  and  $\text{Fe}^{2+}$  chelation capabilities, respectively (Zhang et al., 2011), around 62% of metal ion chelation of African yam bean seed protein hydrolysates (Ajibola et al., 2011) and 90% of scavenging effect on DPPH radical by crude rapeseed peptides (Zhang et al., 2008).

**Table 4.8** Scavenging activity of protein isolates and protein hydrolysates.

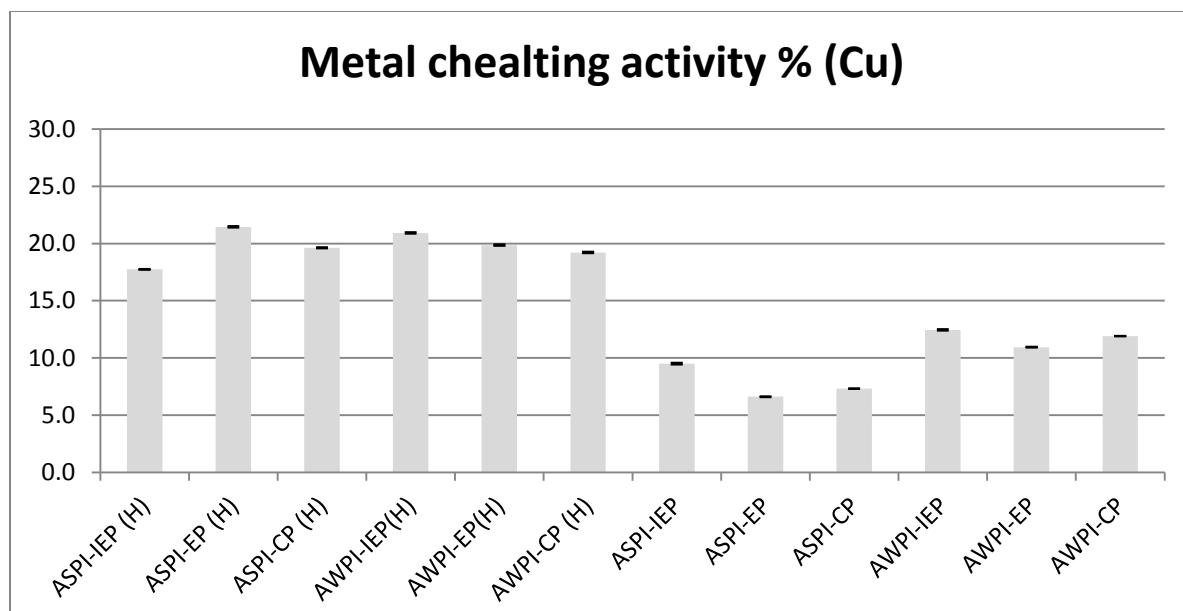
Assays	ASPI-IEP (H) <sub>1</sub>	ASPI-EP (H)	ASPI-CP (H)	AWPI-IEP (H)	AWPI-EP (H)	AWPI-CP (H)	ASPI-IEP	ASPI-EP	ASPI-CP	AWPI-IEP	AWPI-EP	AWPI-CP
<b>DPPH scavenging activity % (SD)</b>	20.6 (0.038) <sup>ac</sup>	19.2 (0.029) <sup>a</sup>	23.8 (0.044) <sup>ad</sup>	27.0 (0.121) <sup>ad</sup>	17.5 (0.061) <sup>ac</sup>	12.5 (0.008) <sup>ae</sup>	4.2 (0.053) <sup>b</sup>	5.3 (0.025) <sup>b</sup>	9.7 (0.071) <sup>be</sup>	10.6 (0.080) <sup>ce</sup>	4.8 (0.010) <sup>bd</sup>	6.7 (0.005) <sup>bcd</sup>
<b>Metal chelating activity (Cu)% (SD)</b>	17.7 (0.036) <sup>ac</sup>	21.4 (0.067) <sup>a</sup>	19.6 (0.061) <sup>ad</sup>	20.9 (0.064) <sup>ad</sup>	19.9 (0.072) <sup>ac</sup>	19.2 (0.070) <sup>ae</sup>	9.5 (0.074) <sup>b</sup>	6.6 (0.043) <sup>b</sup>	7.3 (0.038) <sup>be</sup>	12.4 (0.069) <sup>ce</sup>	10.9 (0.041) <sup>bd</sup>	11.9 (0.025) <sup>bcd</sup>
<b>Metal chelating activity (Fe)% (SD)</b>	27.4 (0.013) <sup>ac</sup>	23.8 (0.011) <sup>a</sup>	24.5 (0.012) <sup>ad</sup>	23.3 (0.008) <sup>ad</sup>	24.5 (0.016) <sup>ac</sup>	25.9 (0.015) <sup>ae</sup>	14.9 (0.012) <sup>b</sup>	5.4 (0.025) <sup>b</sup>	10.5 (0.022) <sup>be</sup>	9.7 (0.001) <sup>ce</sup>	15.2 (0.018) <sup>bd</sup>	13.2 (0.016) <sup>bcd</sup>

ASPI-IEP (arugula seed protein isolate-isoelectric precipitation); ASPI-EP (arugula seed protein isolate-ethanol-precipitation); ASPI-CP (arugula seed protein isolate-cryoprecipitation); AWPI-IEP (arugula whole plant protein isolate-isoelectric precipitation); AWPI-EP (arugula whole plant protein isolate-ethanol precipitation); AWPI-CP (arugula whole plant protein isolate-cryoprecipitation). (H)<sub>1</sub>: hydrolysates.

Columns with the same letters (a-e) are not significant (p≤0.05).

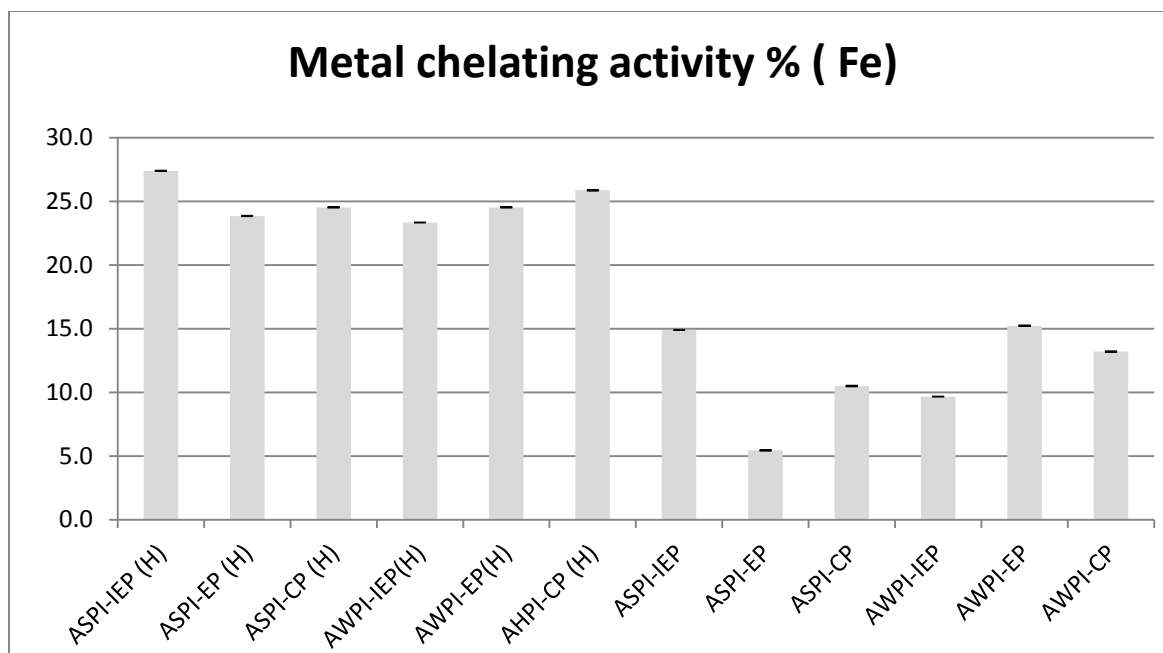


**Figure 4.13** DPPH scavenging activity of protein isolates and protein hydrolysates. ASPI-IEP (arugula seed protein isolate-isoelectric precipitation); ASPI-EP (arugula seed protein isolate-ethanol-precipitation); ASPI-CP (arugula seed protein isolate-cryoprecipitation); AWPI-IEP (arugula whole plant protein isolate-isoelectric precipitation); AWPI-EP (arugula whole plant protein isolate-ethanol precipitation); AWPI-CP (arugula whole plant protein isolate-cryoprecipitation). (H): hydrolysates.



**Figure 4.14**  $\text{Cu}^{+2}$  chelating activity of protein isolates and protein hydrolysates. ASPI-IEP (arugula seed protein isolate-isoelectric precipitation); ASPI-EP (arugula seed protein isolate-ethanol-precipitation); ASPI-CP (arugula seed protein isolate-cryoprecipitation); AWPI-IEP (arugula whole plant protein isolate-isoelectric precipitation); AWPI-EP (arugula whole plant protein isolate-ethanol precipitation); AWPI-CP (arugula whole plant protein isolate-cryoprecipitation). (H): hydrolysates.





**Figure 4.15** Fe<sup>+2</sup> chelating activity of protein isolates and protein hydrolysates. ASPI-IEP (arugula seed protein isolate-isoelectric precipitation); ASPI-EP (arugula seed protein isolate-ethanol-precipitation); ASPI-CP (arugula seed protein isolate-cryoprecipitation; AWPI-IEP (arugula whole plant protein isolate-isoelectric precipitation); AWPI-EP (arugula whole plant protein isolate-ethanol precipitation); AWPI-CP (arugula whole plant protein isolate-cryoprecipitation). (H): hydrolysates.

#### 4.4 Differential Scanning Calorimetry (DSC)

Thermodynamic properties such as enthalpy of denaturation ( $\Delta H$ ), temperature of denaturation ( $T_d$ ), peak width at half height ( $T_{1/2}$ ) and melting temperature ( $T_m$ ) obtained by differential scanning calorimetry (DSC). Results are illustrated in figures 4.9, 4.10 and 4.11 and table 4.5. In all results, peaks were from endotherms which resulted from disruption of hydrogen bonds (Tang and Wang, 2010).  $T_d$  was high for almost all protein isolates and soluble protein content in controls. In general whole plant protein isolates (AWPI) had  $T_d$  and  $T_m$  higher than seed ones. Seed protein isolates (ASPI) had higher  $\Delta H$  than whole plant protein isolates. Results varied regarding  $T_{1/2}$ , where the highest was in seeds with ethanol precipitation (17 °C) and the lowest in control (soluble protein content) seeds (3.97 °C).

The peak of temperature of denaturation ( $T_d$ ) measures protein's thermal stability (Meng and Ma, 2001). High  $T_d$  indicates higher compact and tight protein molecules to the network structure (Ma and Harwalkar, 1988) and low  $T_d$  indicates lower thermal stability due to protein denaturation (Meng and Ma, 2001). From present work results, control of seeds (ASC) and leaves (AWC) had the highest thermal stability because they are still in their native state, while cryo-precipitation in seeds (ASPI-CP) contained the highest denatured protein because of its low  $T_d$ . There are many factors that affect  $T_d$  such as isolation method, degree of protein purity, minimal amounts of salts and other reagents used in isolation methods (Sessa, 1992) and different pHs (Meng and Ma, 2001). High  $T_d$  in controls might be due to glycosylation because sugars are protein protective that enhance thermal stability (Harwalkar and Ma, 1992). Also Morrissey et al., 1987 documented that proteins were more stable in isoelectric point. That's why arugula whole plant protein isolates with isoelectric precipitation (AWPI-IEP) had the highest  $T_d$  among all protein isolates from whole plant except for AWC and arugula seed protein isolate

with isoelectric precipitation (ASPI-IEP) had higher temperature of denaturation than arugula seed protein isolate with cryo-precipitation (ASPI-CP) and close to  $T_d$  in arugula seed protein isolates with ethanol precipitation (ASPI-EP). Water content also affects  $T_d$  by increasing it and decreasing  $\Delta H$  at the same time when moisture content is lower than the significant water levels (Colombo et al., 2010). Another study done by Rouilly et al., 2003 proved that the same phenomenon happened during seeds extraction of sunflower and concluded that those results obtained due to increased pressure in sample pans. They clarified that proteins should be stable in dry environment, so presence of water caused previous results. That might be the explanation of results obtained in ASC and AWC and AWPI-IEP. Ma and Harwalkar, 1988 reported that decrease in  $T_d$  and increase in  $\Delta H$  especially in soluble fraction of heated protein can be due to separation of globulin oligomers into smaller subunits which are less stable than oligomers. As a result, thermal stability will decrease and so  $T_d$  will. Same results were obtained in recent work for seed protein isolates with all types of precipitation, but it was without using any thermal procedure within extraction or precipitation. It could be referred to heating during DSC run that broke protein into smaller subunits.

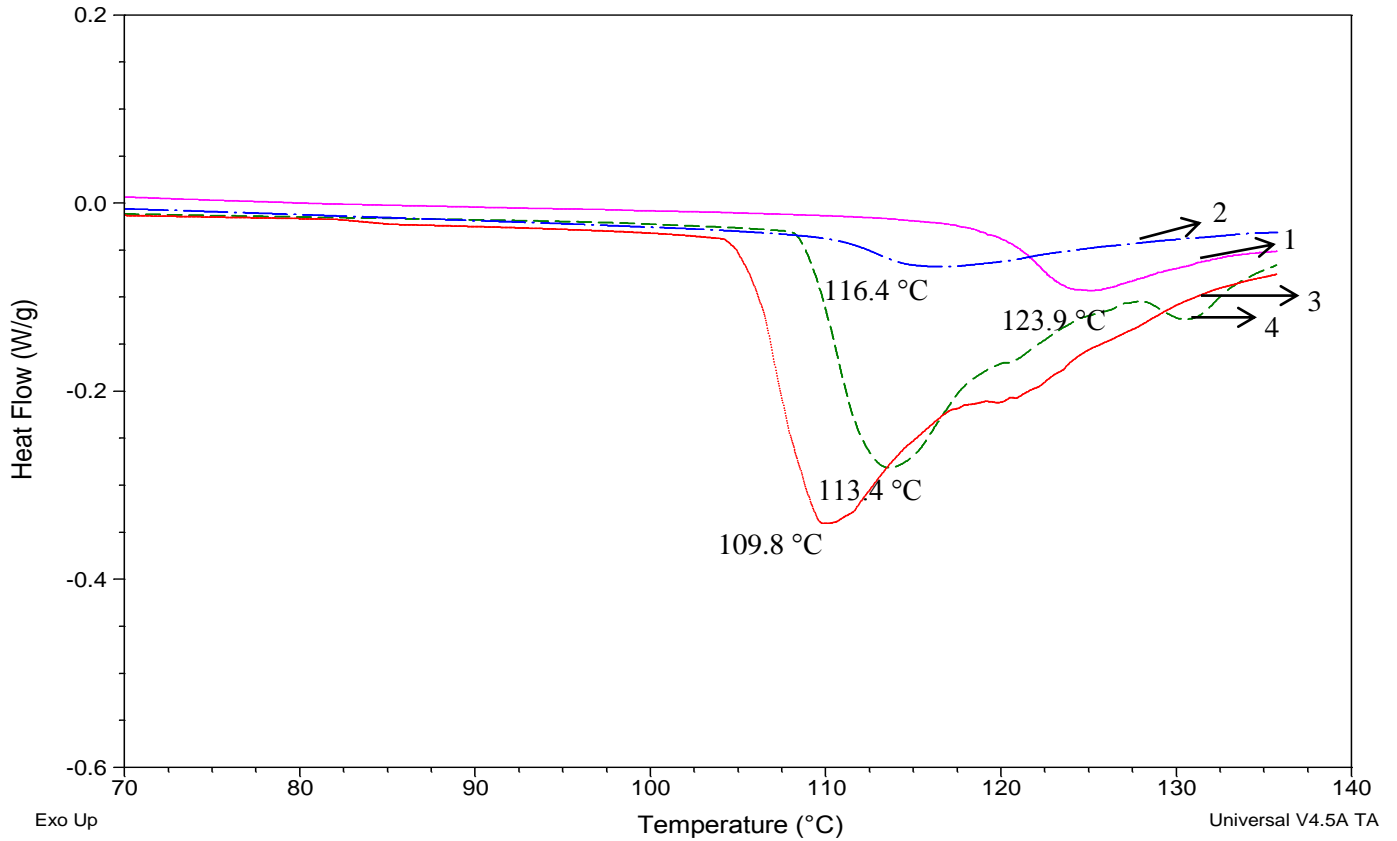
Enthalpy of denaturation ( $\Delta H$ ) shows the degree of un-denatured structure of protein (Arntfield and Murray, 1981). The lower  $\Delta H$ , the more denatured is the protein. It means less thermal energy is needed to complete protein denaturation (Ma and Harwalkar, 1988). There are multiple factors that affect  $\Delta H$  value. One of those factors is the amount of polyphenols presented in the sample. The higher polyphenols, the lower the  $\Delta H$  value is (Rawel et al., 2002). This might be a clear reason of the low  $\Delta H$  in almost all protein isolates of whole plant except arugula whole plant protein isolates with cryo-precipitation (AWPI-CP) because arugula leaves have the highest amount of flavonoids as mentioned in figure 2.1. Another factor is pH where the

highest value of  $\Delta H$  can be obtained in alkaline pH at 7 (Meng and Ma, 2001). This elucidates  $\Delta H$  result obtained in cryo-precipitation from seed protein isolates which was 86.4 °C. The reason is that cryo-precipitation only included protein extraction with NaOH which gave an alkaline pH around 8-9 and then precipitated by keeping samples in the fridge for 18 hours. Third factor is time of storage. The longer freeze-dried proteins are stored in middle to high humidity place, the higher polymerization degree will happen. So, low heat energy ( $\Delta H$ ) will be needed to denature proteins (Sessa, 1992). This may be another reason of low enthalpy of denaturation for whole plant protein isolates although they were stored all the time in the freezer. The same study related the low  $\Delta H$  in storage proteins to the interactions between protein and non-protein components. This may be a better reason with phenol content for both controls in seeds and leaves because they were not pure protein extract, but more as mixture of other components like sugars.

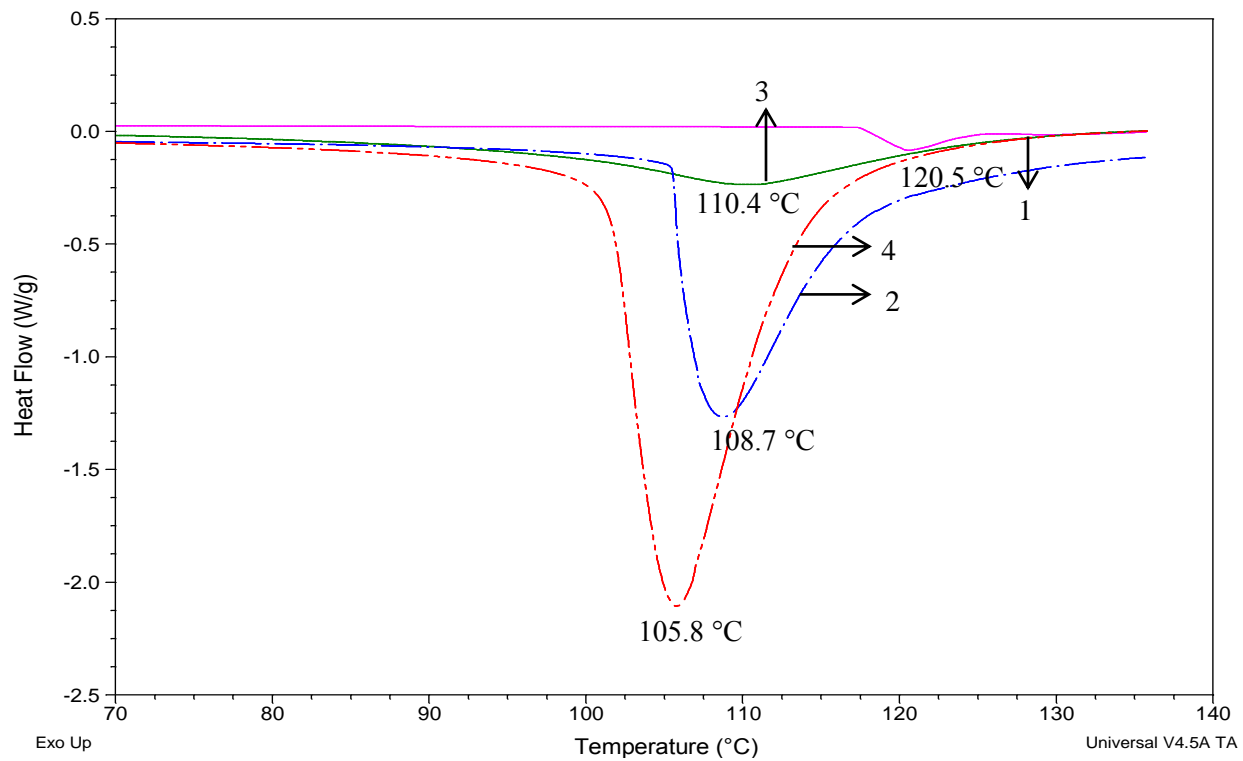
Melting temperature or onset temperature ( $T_m$ ) is the temperature in the beginning of denaturation or when the protein is partially (50%) denatured. It has a positive relation with  $T_d$  (Ma and Harwalkar, 1988). It measures thermal stability as well; through high thermal stability it will be high and vice-versa. As a result,  $T_m$  had the same trend of  $T_d$  in recent results where the highest  $T_m$  was for AWC and the lowest for ASPI-CP.

Peak width at half height ( $\Delta T_{1/2}$ ) measures cooperativity of protein denaturation (Colombo et al., 2010). Low  $\Delta T_{1/2}$  means there are many bonds such as hydrogen and disulfide bonds are broken at the same time (Colombo et al., 2010). If the denaturation is highly cooperative and endothermic peaks are sharp,  $\Delta T_{1/2}$  decreases with increasing in  $T_m$  at the same time (Ma and Harwalkar, 1988). This matches our recent work by having opposite relation between  $T_m$  and  $\Delta T_{1/2}$ . Table 4.5 shows the highest  $\Delta T_{1/2}$  (17 °C) for ASPI-EP and  $T_m$  had the lowest value (94.4

°C) for the same sample. On the other hand, lowest  $\Delta T_{1/2}$  (3.97 °C) was for ASC had second highest  $T_m$  (117.4 °C).



**Figure 4.16** DSC curves for arugula whole plant protein isolates. 1= AWC (arugula whole plant control); 2= AWPI-IEP (arugula whole plant protein isolate-isoelectric precipitation); 3= AWPI-EP (arugula whole plant protein isolate-ethanol precipitation) and 4= AWPI-CP (arugula whole plant protein isolate-cryoprecipitation).



**Figure 4.17** DSC curves for arugula seed protein isolates. 1= ASC (arugula seed control); 2= ASPI-IEP (arugula seed protein isolate-isoelectric precipitation); 3= ASPI-EP (arugula seed protein isolate-ethanol precipitation); and 4= ASPI-CP (arugula seed protein isolate-cryoprecipitation).

**Table 4.9** Thermal transition characteristics of protein isolates from arugula seeds and leaves.

Samples	T <sub>m</sub> (°C)	ΔH (J/g)	T <sub>d</sub> (°C)	T <sub>1/2</sub> (°C)
ASC	117.4	3.2	120.5	3.97
ASPI-IEP	105.4	47.8	108.7	7.0
ASPI- EP	94.4	24.1	110.4	17
ASPI- CP	101.3	86.4	105.8	6.7
AWC	119.6	3.0	123.9	7.9
AWPI- IEP	111.0	2.7	116.4	11.9
AWPI- EP	105.7	23.3	109.8	13.2
AWPI- CP	108.0	6.5	113.4	6.15

ASC (arugula seed control); ASPI-IEP (arugula seed protein isolate-isoelectric precipitation); ASPI-EP (arugula seed protein isolate-ethanol-precipitation); ASPI-CP (arugula seed protein isolate-cryoprecipitation; AWC (arugula whole plant control) AWPI-IEP (arugula whole plant protein isolate-isoelectric precipitation); AWPI-EP (arugula whole plant protein isolate-ethanol precipitation); AWPI-CP (arugula whole plant protein isolate-cryoprecipitation).

T<sub>m</sub>: melting point temperature; ΔH: enthalpy of denaturation; T<sub>d</sub>: temperature of denaturation; T<sub>1/2</sub>: peak width at half height.



### 4.3 Fourier Transform Infrared (FTIR) Spectroscopy

Secondary structure of *Eruca sativa* protein fractions in leaves and seeds was investigated by studying FTIR spectrum. Recent study focused on amide I and II which are the most important regions in studying the secondary structure. Figures 4.12 and 4.13 show the general shape of amide I and II for arugula protein isolates from leaves and seeds and raw seeds and leaves spectrum. Figures 4.14, 4.15, 4.16 and 4.17 elucidate more the peaks in each region of all protein isolates and raw samples. Tables 4.6 and 4.7 clarify the peaks of amide I and II respectively. Table 4.6 refers each peak frequency to a specific peak assignment.

Regarding amide I region, results of arugula seed protein isolates with cryo-precipitation (ASPI-CP) protein isolates and arugula seed control (ASC) showed that  $\beta$ -sheet was the major structure, while for arugula seed protein isolates with isoelectric precipitation (ASPI-IEP) and arugula seed protein isolates with ethanol precipitation (ASPI-EP) protein isolates had  $\alpha$ -helix and  $\beta$ -sheet content with equal area amounts for each of them. Raw seeds powder had  $\alpha$ -helix as the main secondary structures. Marcone et al., 1998 documented that seed globulins from plants have higher levels of  $\beta$ -sheet than  $\alpha$ -helix. In general, bands for raw seeds powder were shifted to lower frequencies with other protein isolation techniques. Zhao et al., 2008 reported that solubilization can change the secondary structure by shifting the bands to lower frequencies. The same study clarified the reason behind lower frequencies that is caused by increasing the effect of hydrogen-bonding. NaOH extraction in recent results might have done the same effect that was reported by the study. All other bands had similar frequencies except for ASC had a unique band ( $1614.7\text{ cm}^{-1}$ ) that was only found in that sample. Jackson and Mantsch, 1992 attributed the finding of that band to the formation of intermolecular  $\beta$ -sheet networks that

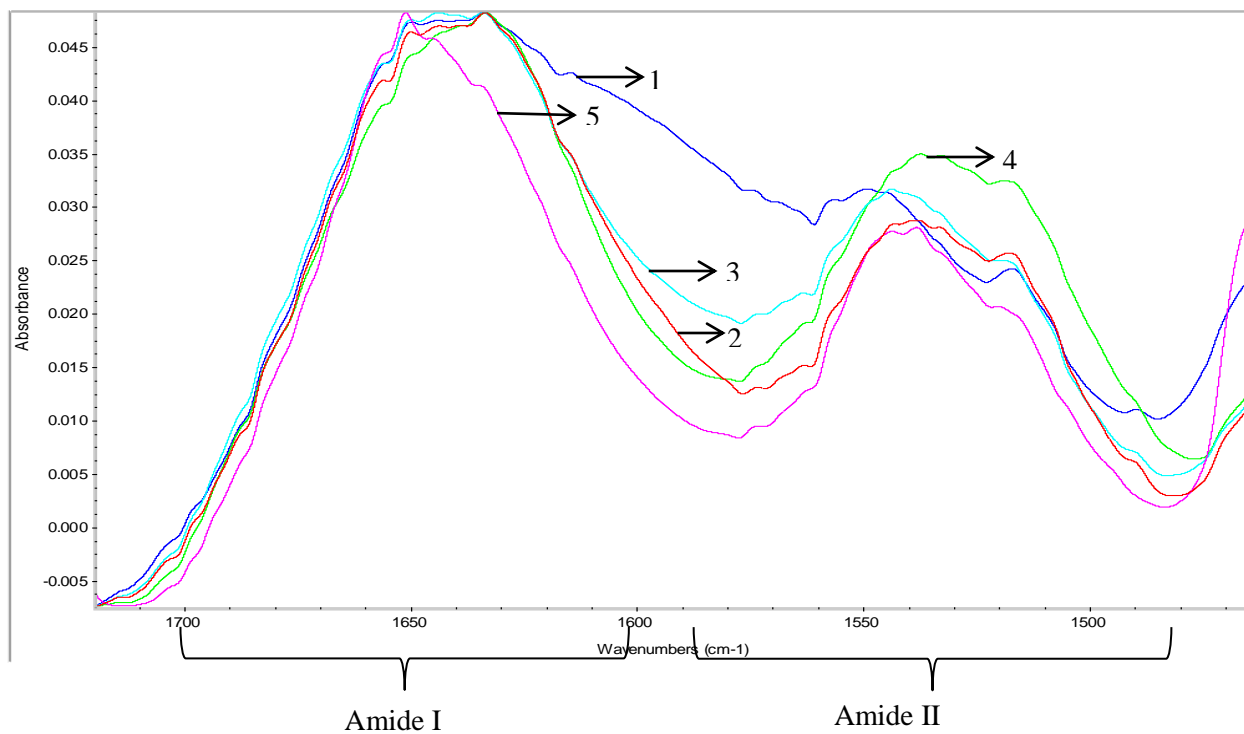
happens through aggregation. Bands at 1630-1650  $\text{cm}^{-1}$  in raw seed powder had lower absorbance intensities than all other protein isolates from seeds. Ellepola et al., 2005 reported that alkaline environment reveals an obvious change in spectral properties such as decrease in frequency and increase in absorbance intensity. That shows a large protein denaturation which maybe a clarification of recent results between raw seeds powder and ASC.

On the other hand, leaves had much less bands in amide I region. The major structure in almost all protein isolates from leaves was  $\beta$ -sheet. Only arugula whole plant protein isolates with isoelectric precipitation (AWPI-IEP) had a band in  $\alpha$ -helix region. There was only one band (1602  $\text{cm}^{-1}$ ) which was not detectable, but it could be referred to side chain vibrations because they are the lowest frequencies in amide I region (1611-1612  $\text{cm}^{-1}$ ) (Ellepola et al., 2005). Generally, the antiparallel  $\beta$ -sheet that resulted from aggregation was higher in leaves than seeds samples. The band at 1625.4  $\text{cm}^{-1}$  in raw leaves had less intensity than in arugula whole plant control (AWC) which was also shifted to lower frequency (1620  $\text{cm}^{-1}$ ). That also shows the extensive protein denaturation due to using alkaline pH through extraction (Ellepola et al., 2005).

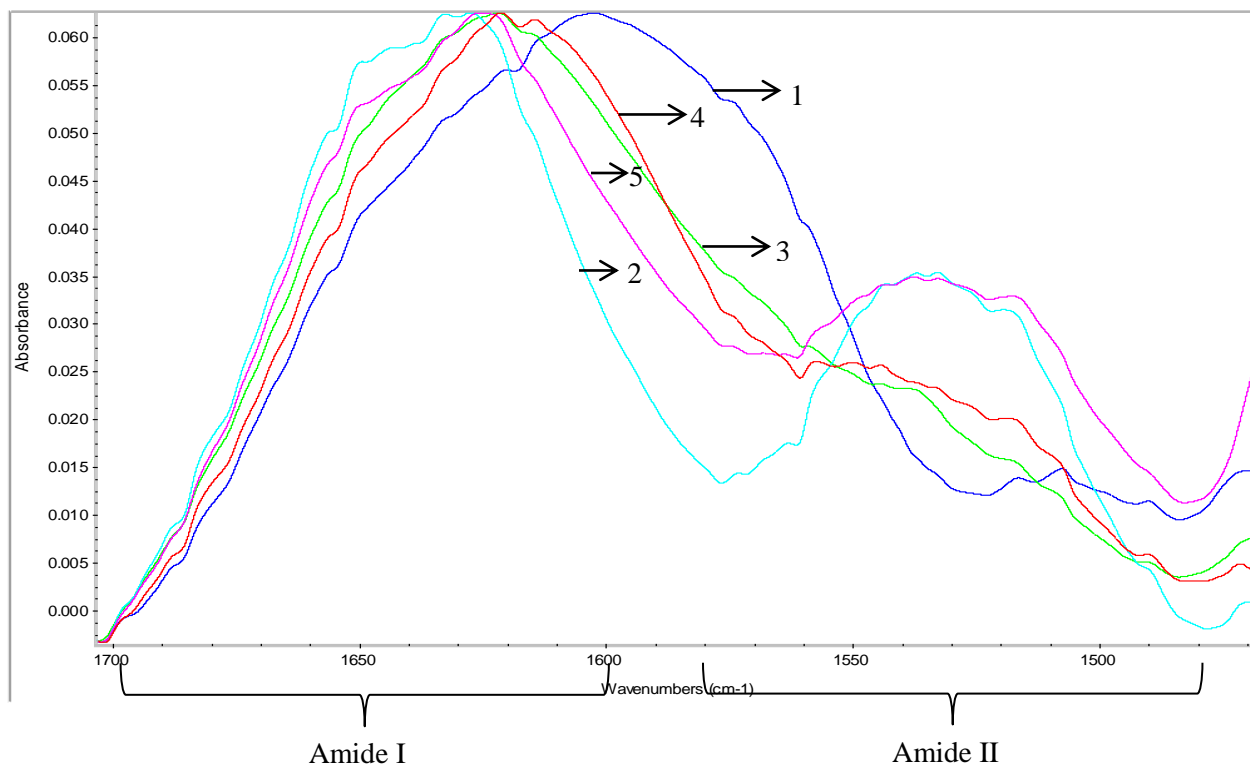
Regarding amide II region, it is from band 1480-1575  $\text{cm}^{-1}$  (Chen et al., 2013). Bands at 1550-1530  $\text{cm}^{-1}$  are assigned to parallel  $\beta$ -sheet, antiparallel  $\beta$ -sheet at 1530-1510  $\text{cm}^{-1}$  (Pelton and Mclean, 2000), 1556-1575  $\text{cm}^{-1}$  are assigned to  $\beta$ -turns (Kumosinski and Farrell, 1993) and  $\alpha$ -helix is for the band at 1537  $\text{cm}^{-1}$  (Nevskaya and Chirgadze, 1976). In recent results, amide II region was mainly  $\beta$ -sheet and  $\beta$ -turns structures. Few samples contained  $\alpha$ -helix structure.

There were lots of shiftings from higher to lower frequencies and vice-versa from raw seeds and whole plant powder to other NaOH extractions with different precipitation techniques. ASPI-CP had the highest absorbance intensity between 1500-1550  $\text{cm}^{-1}$  and the raw seeds powder had the lowest intensity within same frequency range. On the other hand, AWPI-IEP and raw powder of whole plant showed the highest intensities between 1550 to 1520  $\text{cm}^{-1}$ , while AWC revealed the lowest absorbance intensity at 1520-1545  $\text{cm}^{-1}$ .

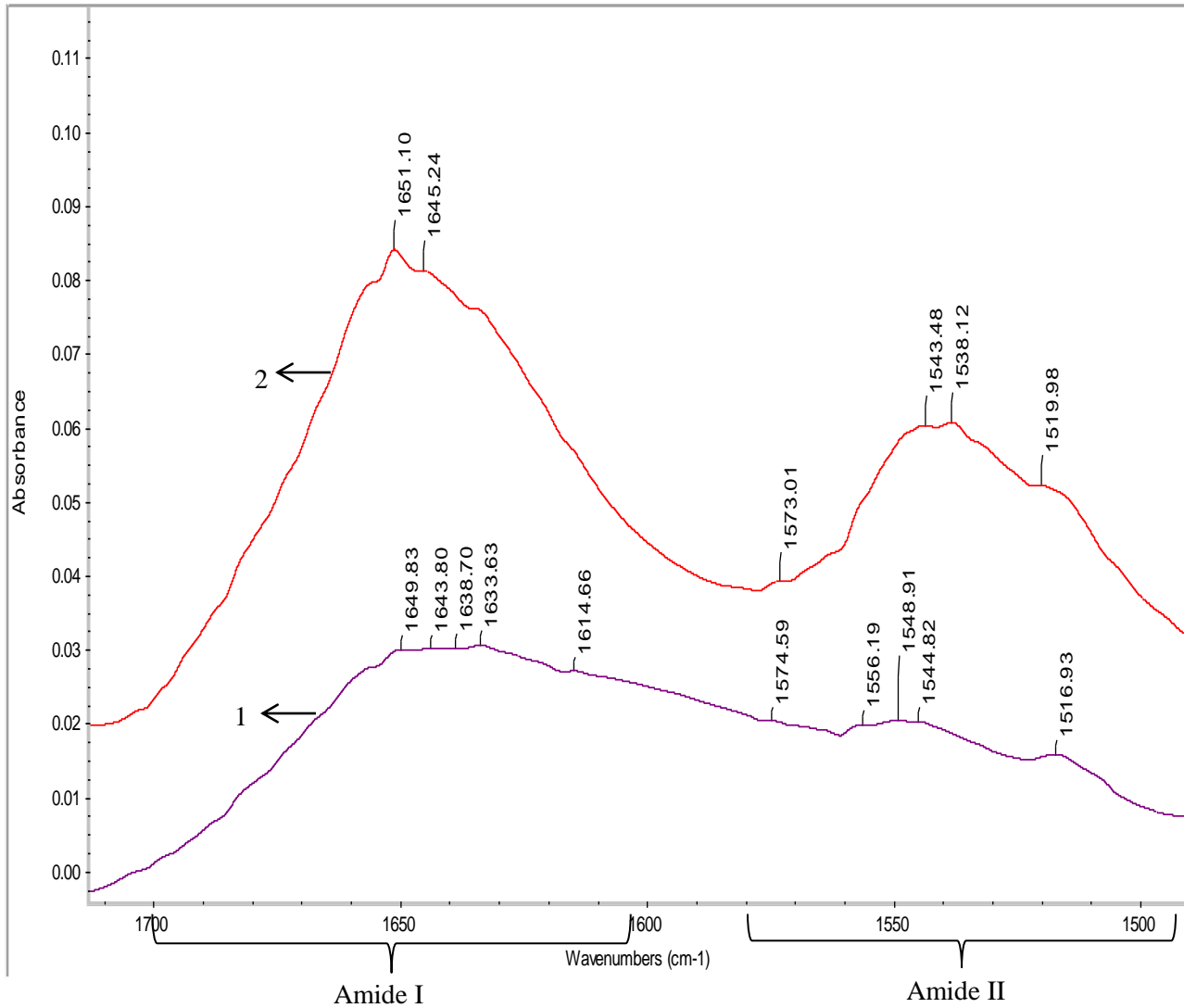
A study done by Carbonaro et al., 2008 on lentil flour found that its major secondary structure was antiparallel  $\beta$ -sheet structures. Another study done on buckwheat seeds showed that the main secondary structure composition was  $\beta$ -strand with 36.8% (Tang and Wang, 2010). Rice globulin seeds contained over 50%  $\beta$ -sheet and turn structures which were the main composition with around 30%  $\alpha$ -helix (Ellepola et al., 2005).



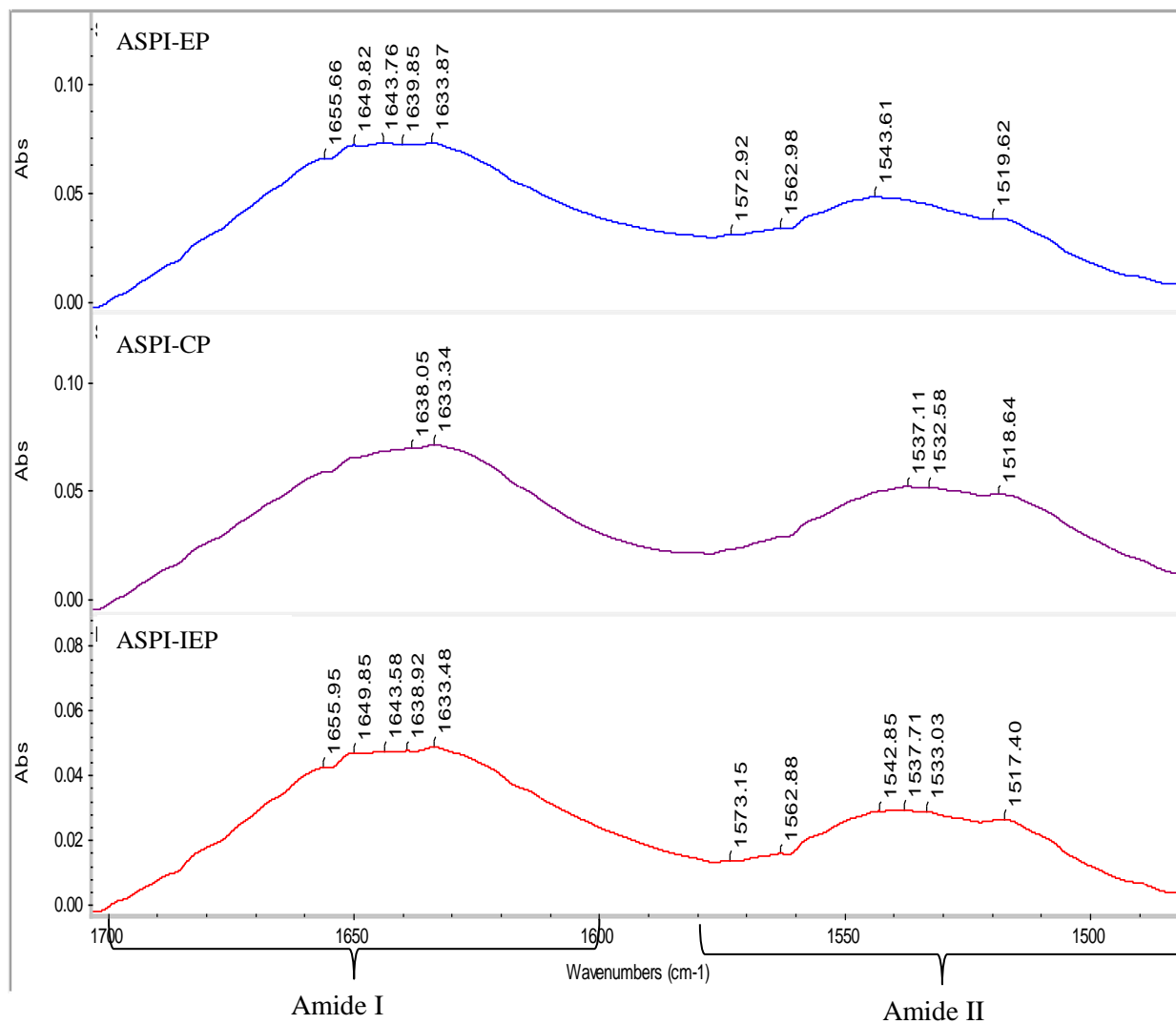
**Figure 4.18** FTIR of arugula seed protein isolates for amide I and amide II bands. 1= ASC (arugula seed control); 2=ASPI-IEP (arugula seed protein isolate-isoelectric precipitation); 3= ASPI-EP (arugula seed protein isolate-ethanol precipitation) 4= ASPI-CP (arugula seed protein isolate-cryoprecipitation); and 5= raw arugula seed powder.



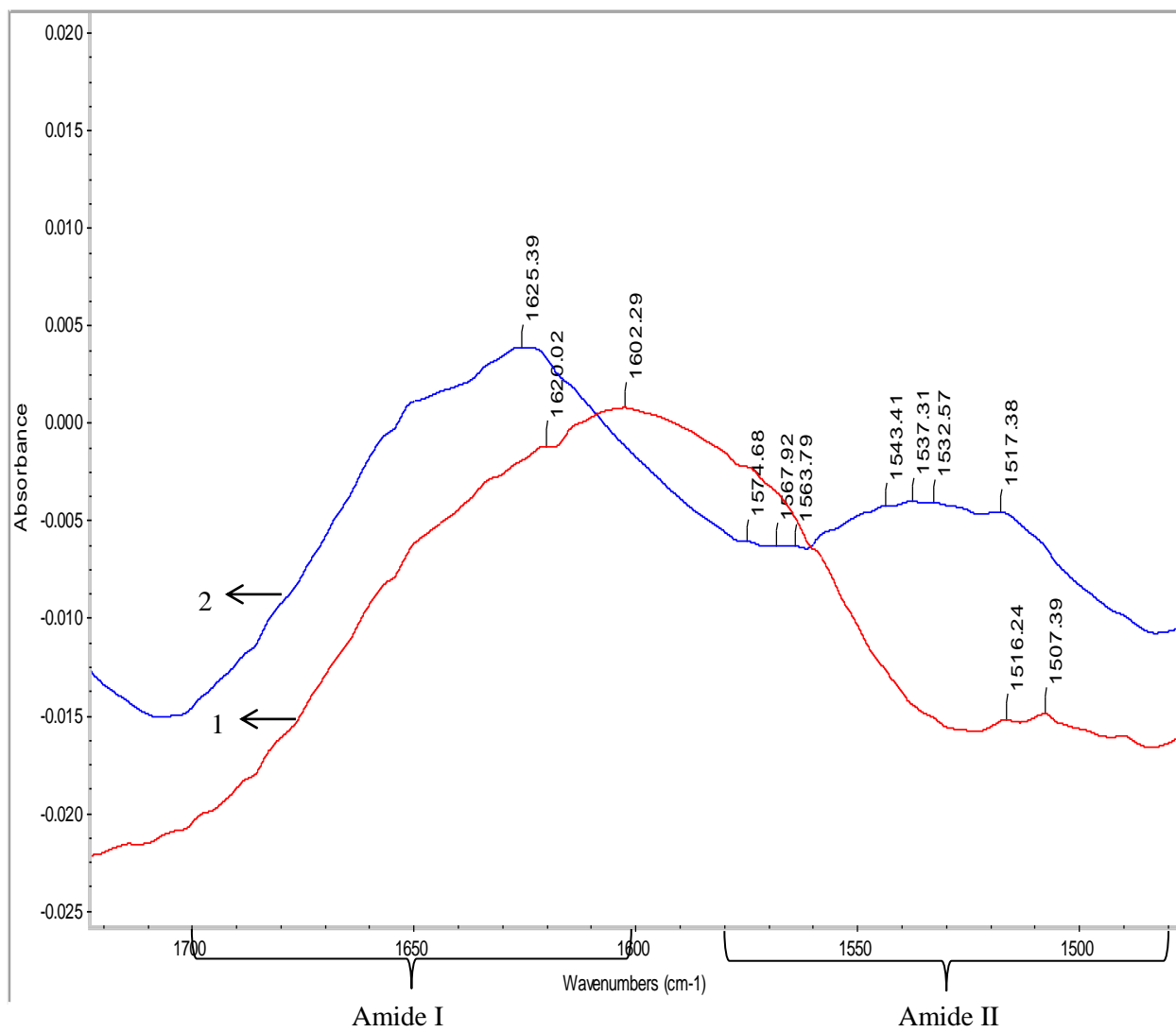
**Figure 4.19** FTIR of whole arugula protein isolates for amide I and amide II bands. 1= AWC (arugula whole plant control); 2= AWPI-IEP (arugula whole plant protein isolate-isoelectric precipitation); 3= AWPI-EP (arugula whole plant protein isolate-ethanol precipitation); 4= AWPI-CP (arugula whole plant protein isolate-cryoprecipitation); and 5= raw arugula whole plant powder.



**Figure 4.20** FTIR of seeds for amide I and II bands. 1= ASC (arugula seed control) and 2= raw seed powder.

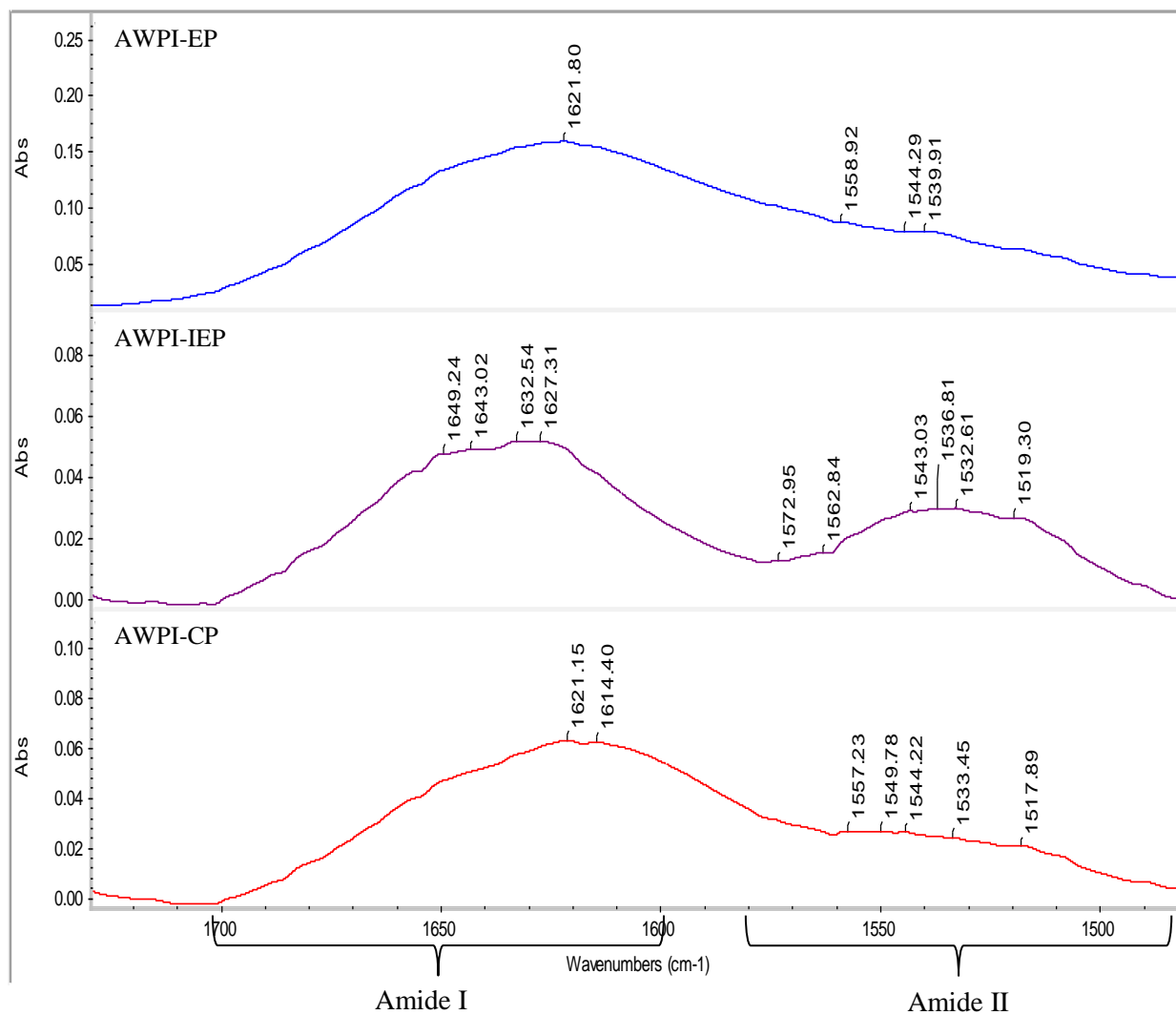


**Figure 4.21** FTIR of arugula seed protein isolates for amide I and II bands. ASPI-IEP (arugula seed protein isolate-isoelectric precipitation); ASPI-EP (arugula seed protein isolate-ethanol precipitation); and ASPI-CP (arugula seed protein isolate-cryoprecipitation).



**Figure 4.22** FTIR of whole plant for amide I and II bands. 1= AWC (arugula whole plant control) and 2= raw whole plant powder.





**Figure 4.23** FTIR of arugula whole plant protein isolates for amide I and II bands. AWPI-IEP (arugula whole plant protein isolate-isoelectric precipitation); AWPI-EP (arugula whole plant protein isolate-ethanol precipitation); and AWPI-CP (arugula whole plant protein isolate-cryoprecipitation).

**Table 4.10** Major band assignments of amide I spectral region of protein isolates and controls in arugula seed and whole plant.

Sample	Amide I				
	$\alpha$ -helix	$\beta$ -strand	Antiparallel $\beta$ -sheet	$\beta$ -sheet	Random Coil
<b>ASC</b>	1650	1633.5	1614.7	1638.7	1643.8
<b>ASPI-IEP</b>	1650 / 1655.9	1633.5	-	1638.7	1643.6
<b>ASPI-EP</b>	1650 / 1655.7	1633.9	-	1639.9	1643.8
<b>ASPI-CP</b>	-	1633	-	1638.1	-
<b>Raw Seed Powder</b>	1651.1	-	-	-	1645.2
<b>AWC</b>	-	-	1620	-	-
<b>AWPI-IEP</b>	1649.2	1632	-	1627.3	-
<b>AWPI-EP</b>	-	-	1621.8	-	-
<b>AWPI-CP</b>	-	-	1621.2 / 1614.4	-	-
<b>Raw Whole Plant</b>	-	-	-	1625.4	-

ASC (arugula seed control); ASPI-IEP (arugula seed protein isolate-isoelectric precipitation); ASPI-EP (arugula seed protein isolate-ethanol precipitation); ASPI-CP (arugula seed protein isolate-cryoprecipitation); AWC (arugula whole plant control); AWPI-IEP (arugula whole plant protein isolate-isoelectric precipitation); AWPI-EP (arugula whole plant protein isolate-ethanol precipitation); and AWPI-CP (arugula whole plant protein isolate-cryoprecipitation).

(Ellepola et al., 2005) and (Carbonaro and Nucara, 2010)

**Table 4.11** Major band assignments of amide II spectral region of protein isolates and controls in arugula seed and whole plant.

Sample	Amide II			
	$\alpha$ -helix	Antiparallel $\beta$ -sheet	$\beta$ -sheet	$\beta$ -turns
<b>ASC</b>	-	1516.9	1548.9 / 1544.8	1574.6 / 1556.2
<b>ASPI-IEP</b>	1537.7	1517.4	1533 / 1542.9	1562.9 / 1573.2
<b>ASPI-EP</b>	-	1519.6	1543.6	1562.9 / 1572.9
<b>ASPI-CP</b>	1537.1	1518.6	1532.6	-
<b>Raw Seed Powder</b>	-	1519	1538.1 / 1543.5	1573
<b>AWC</b>	-	1516	-	-
<b>AWPI-IEP</b>	1536.8	1514.3	1532.6 / 1543	1562.8 / 1572.9
<b>AWPI-EP</b>	-	-	1539.9 / 1544.3	1558.9
<b>AWPI-CP</b>	-	1517.9	1533.5 / 1544.2 / 1544.8	1557.2
<b>Raw Whole Plant</b>	1537.3	1517.4	1532.6 / 1543.4	1563.8 / 1567.9 / 1574.7

ASC (arugula seed control); ASPI-IEP (arugula seed protein isolate-isoelectric precipitation); ASPI-EP (arugula seed protein isolate-ethanol precipitation); ASPI-CP (arugula seed protein isolate-cryoprecipitation); AWC (arugula whole plant control); AWPI-IEP (arugula whole plant protein isolate-isoelectric precipitation); AWPI-EP (arugula whole plant protein isolate-ethanol precipitation); and AWPI-CP (arugula whole plant protein isolate-cryoprecipitation).

(Nevskaya and Chirgadze, 1976), (Kumosinski and Farrell, 1993) and (Pelton and Mclean, 2000)

## CHAPTER 5

### GENERAL CONCLUSION

In this research, properties of extracted protein from arugula seeds and leaves were analysed. SDS-PAGE electrophoresis revealed molecular weights of fractions ranged between 131- 17 kDa for seeds and leaves. Cryo-precipitation (CP) contained highest amount of subunits and most intense ones in arugula seeds isolates. On the other hand arugula leaves, had much less subunits. Subunits in SDS supernatant were found less intense than the ones in the extract.

For Native PAGE electrophoresis, none of the fractions which were found in protein isolates matches the ones in the supernatant. In general bands found in protein supernatant were intense especially minor ones and their molecular weights vary from the ones in protein isolates. Differences between protein supernatant and protein isolates might be due to the inappropriate precipitation techniques.

Degree of hydrolysis (DH) for protein isolates in arugula seeds and leaves were in continuous increase with trypsin. DH ranged from 34-51 % for seeds and 44-65 % for leaves. Through trypsin-chymotrypsin mixture, results were different. There was an increase until 90 minutes then decrease in the last 30 minutes. Final results were 57-66 % for seeds and 50-61 % for leaves. Results referred to fibers in leaves that reduced the digestibility and flavonoid content that affected enzymes activity negatively.

Metal chelating assays and DPPH scavenging activity assay showed higher antioxidant activity percentages in protein hydrolysates than protein isolates. The highest antioxidant activity

found in metal chelating assay ( $\text{Fe}^{+2}$ ). Results ranged from 23-27 % for protein hydrolysates and 5-15% for protein isolates.

Differential scanning calorimetry (DSC) results demonstrated high thermal stability for both seed and leaf protein isolates. Whole protein isolates had higher thermal stability than seed protein isolates which was referred to protein content for each sample. Enthalpy of denaturation ( $\Delta H$ ) confirmed results by having low  $\Delta H$  for high denatured isolates and high  $\Delta H$  for less denatured isolates.

Secondary structure of protein isolates was investigated by fourier transform infrared (FTIR) spectroscopy. Results of amide I range showed that raw seed powder had  $\alpha$ -helix and random coil secondary structure whereas seed isolates had  $\beta$ -sheet and  $\beta$ -strand in addition to  $\alpha$ -helix structures. Whole plant isolates had mostly antiparallel  $\beta$ -sheet secondary structures. Beta structures referred to aggregation of proteins and a result of denaturation.

Overall results of this study demonstrated potential nutritional role of seed and leaf protein isolates in terms of having good digestibility and antioxidant activity for protein hydrolysates. In addition to their high thermal stability which maintains their quality during processing. HPLC is suggested in further studies in order to identify fractions that were found by SDS-PAGE electrophoresis.

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