Chemical Profiling and Comparative Evaluation of Bioactive Compounds in Lyophilized and Tray-dried Rocket (*Eruca sativa*)

By

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ABSTRACT

Species of crucifers such as *Eruca sativa* (rocket, roquette) are known to contain a variety of bioactive compounds such as phenolic compounds, flavonols, and isothiocyanates. In this study, two different drying procedures were used to compare the levels of bioactive compounds, antioxidant activity, and chemical profiles of arugula leaves and stems after treatment. The overall comparison of the bioactive compounds and chemical profiles of lyophilized (LR) and tray-dried rocket samples (TDR), conditions of 55^{o} C for 6 hours, was performed using chromogenic assays and chromatographic analysis. Total flavonoid assay showed significantly different concentrations between the LR (3.29 ± 0.15 g/100g) and TDR samples (2.42 ± 0.22 g/100g) measured as quercetin equivalents (QE) although the total phenolic content between the samples showed no difference: 8.67 ± 0.6 g/100g QE and 8.5 ± 0.8 g/100g QE, for LR and TDR respectively. The antioxidant activity in the LR sample as measured using the DPPH• assay indicated a scavenging activity of 28.01% while a result of 27.96 % was obtained for the TDR sample. Moreover, total isothiocyanate contents measured by the cyclocondensation reaction analyzed by GC-MS showed a concentration twofold higher in the TDR sample (6.05 ± 0.83 μg/g) as opposed to the LR sample (3.26 ± 0.59 μg/g). Chemical fingerprinting of the LR and TDR samples using pyrolysis-GC/MS was investigated to identify volatile compounds; this revealed minor differences in compounds observed between the samples. LC-ESI-qTOF-MS analysis was also employed for the identification of possible structural configurations of non-volatile compounds, which indicated the presence of glycosides in the LR sample in contrast to the TDR sample that harbored aglycones instead. Furthermore, infrared spectra obtained using FTIR-ATR for both samples showed minimal variations between those, although TDR displayed a slightly higher number of peaks. Images from Scanning Electron Microscopy (SEM) revealed variations in the average particle diameter in TDR due to thermal processing, and only minor shrinkage in the case of LR particles.

RESUME

Plusieurs espèces de crucifères tel que *Eruca sativa* contiennent une série de substances bioactives, par exemple des composés phénoliques, des flavonols, de même que certains isothiocyanates. La présente étude visait à comparer les effets post-traitement de deux méthodes de déshydratation sur (i) les niveaux de composés bioactifs, (ii) l`activité antioxidative, et (iii) la composition chimique, des feuilles et tiges de laitue roquette (également connue sous le terme arugula). Principalement, des analyses par réactions chromogéniques et par chromatographie ont permis de comparer les composés bioactifs et la composition chimique d`échantillons soit lyophilisés (RL) soit séchés sur plateaux à 55^{o} C durant 6 heures (RSP). La quantification des flavonoïdes totaux a montré des différences significatives entre RL (3.29 ± 0.15 g/100g) et RSP (2.42 ± 0.22 g/100g) tel que mesuré en équivalents quercétine ou EQ; toutefois le contenu en composes phénoliques totaux s`est avéré similaire entre les deux traitements. soit: 8.67 ± 0.6 g/100g EQ et 8.5 ± 0.8 g/100g EQ respectivement. L'activité antiradicalaire mesurée par la méthode de piégeage du DPPH• pour l'échantillon RL a indiqué une activité de 28.01% alors qu'une valeur de 27.96% a été obtenue pour RSP. Par ailleurs, le contenu en isothiocyanates mesuré via cyclocondensation suivie d`injection sur unité GC-MS a révélé une teneur deux fois supérieure dans l`échantillon RSP (6.05 ± 0.83 μg/g vs 3.26 ± 0.59 μg/g). Les empreintes chimiques des deux types d`échantillons ont été obtenues par pyrolyse/GC-MS et la comparaison des composés volatils ainsi identifiés a indiqué une différence mineure entre les deux traitements. Une analyse par LC-ESI-qTOF-MS a également été utilisée afin d`identifier certaines configurations structurales de composés non-volatils; cette étape a permis de confirmer la présence de glycosides dans le produit lyophilisé, contrairement à l`échantillon séché sur plateau qui lui contenait de son côté des aglycones. De plus, l`analyse spectrale par FTIR-ATR des deux types d`échantillons n`a montré qu`une faible variation, malgré un nombre de pics légèrement supérieur pour RSP. Les images par microscopie électronique à balayage (SEM) ont confirmé des variations au niveau du diamètre moyen des particules RSP, conséquentes au traitement thermique et ceci par contraste au phénomène de rétraction observé dans l`échantillon lyophilisé.

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CHAPTER 1

1. INTRODUCTION

1.1. General Introduction

The *Brassicaceae* family is considered a major contributor to vegetable production and consumption. It comprises vegetable crops such as cabbage and broccoli as well as salad species including rocket. Rocket, also known as arugula, is the term used to define different species characterized by their pungent-tasting leaves. The main species used for human consumption are *Eruca sativa* L. (salad rocket), *Diplotaxis tenuifolia* L. (wild rocket), *Diplotaxis erucoides* L. (wall rocket) and *Bunias orientalis* L. (Turkish rocket) (Bennett *et al.*, 2006). Recently, rocket has been receiving great attention as potentially significant commercial and edible species due to their peppery aroma and taste.

Eruca sativa is originally found in the Mediterranean and Middle Eastern countries. Phenolic compounds and flavonoids are the major phytochemicals found in different parts of rocket tissue which contribute to its antioxidant properties. Glucosinolates; sulfur-containing plant secondary metabolites, are responsible for the characteristic bitter taste of rocket salad and were found to have antibacterial, anticarcinogenic, and antioxidant activities (Bell & Wagstaff, 2014; Pasini *et al.*, 2012). Upon disruption of plant tissues, these chemicals are hydrolyzed by an endogenous enzyme myrosinase to produce glucose and unstable intermediate products that rearrange to form degradation products, one of which is isothiocyanates. Isothiocyanates have been potentially considered to have chemopreventive and anti-carcinogenic properties in both cell and animal models (Kim & Ishii, 2007; Lamy *et al.*, 2008; Melchini *et al.*, 2009). In several studies, isothiocyanates showed strong evidence of tumorigensis inhibition by modifying the activity of enzymes involved in biotransformation. *E.sativa* is also a source of vitamins A, C, and K, minerals such as calcium and iron, and phytonutrients including carotenoids (Garg & Sharma, 2014; Lamy *et al.*, 2008).

There is an increased interest in using crucifers as functional foods to deliver high concentrations of health promoting bioactive compounds. To preserve the bioactive compounds in such plants, several drying methods preceding chemical analyses are commonly used. Conventional hot air dryers are generally used to dry food ingredients while freeze-drying or lyophilization is considered a costly alternative to it. However, air-drying or tray-drying is known to decrease product quality due to high drying temperature and long drying time (Potisate

& Phoungchandang, 2010). Lyophilization is a method commonly used to preserve the nutritional and phytochemical profiles of perishable foods, though it is a costly and time-consuming method than other conventional drying methods. In lyophilization, water is removed by dehydration through sublimation of ice in the sample which is preceded by the freezing of the sample. This process assists in the preservation of phenolic compounds and antioxidants (ascorbic acids, tocopherols, carotenoids, etc.) Its wide usage was due to the process's ability of retaining the biological activity, nutrients, color, and flavor of the dried material. During the extended drying time of the lyophilized material due to the equipment's use of low vapor pressure, antioxidants reduction may occur (Shofian *et al.*, 2011). A modification of this method was done in this study to produce a tray-dried powder using less drying time and lower drying temperature to preserve the bioactive components of the leaves.

Rocket leaves of various species are recently acquired as a potential deliverer of flavonoids and antioxidants. They have the potential to be used as functional foods delivering a substantial amount of bioactive compounds especially isothiocyanates and vitamins to humans (Bennett *et al.*, 2006). The evaluation of the stability and degradation patterns of these bioactive compounds is required before they can be used in the development of functional foods (Zainol *et al.*, 2009). The impacts of two postharvest processing methods; lyophilization and tray-drying, on the stability of bioactive compounds were compared using rocket leaves and stems.

1.2. Research Objectives

The overall objective of this research was to compare the bioactive compounds and chemical profiles of lyophilized and tray-dried rocket leaves and stems from one biological variety. The specific objectives were to investigate:

1. Total flavonoids and phenolic compounds, as well as antioxidant activity in lyophilized and tray-dried rocket using UV-VIS spectrophotometric techniques.

2. Total isothiocyanate content and volatile compounds analysis using GC/MS and Py-GC/MS as well as chemical profiling using LC-ESI-qTOF-MS analysis for the nonvolatile compounds of the two processed rocket samples.

3. Functional group profiling using infrared spectroscopy (FTIR-ATR) of the two processed rocket samples.

4. The difference in overall microstructure between lyophilized and tray-dried rocket powder measured by a Scanning Electron Microscope (SEM).

CHAPTER 2

2. LITERATURE REVIEW

2.1. Brassicaceae family

Brassicaceae (Crucifereae) is a large family of flowering plants that include more than 300 genera and nearly 3,500 species, only a few of which are edible (Garg *et al.*, 2014). The word Crucifereae is derived from the cross-shaped petals that distinguish the plants of this family. The limited number of plants fit for human consumption comprise cabbage, cauliflower, broccoli, Brussels sprouts, kale, mustard, and rocket, to name a few. The cruciferous plants can take one, two, or more than two years to complete their biological cycle, also known as annual, biennial, or perennial plants, respectively. They are mainly grown in the northern hemisphere, specifically in the Mediterranean region, and cultivated in various regions of Central Asia, Southwest Asia, Europe, and North America (Dias, 1997; Velasco *et al.*, 2010).

Cruciferous vegetables are recognized as a family of antioxidants; such as vitamins A & C, flavonoids, carotenoids, and glucosinolates. A high intake of cruciferous vegetables was associated with lower cancer risks in humans according to multiple epidemiological studies, due to the presence of phytochemicals and a variety of nutrients that are significant to human health (Khoobchandani *et al.*, 2011; Velasco *et al.*, 2010).

The common feature among crucifers is the presence of glucosinolates, which are a group of water-soluble sulfur-containing organic compounds that exist as secondary plant metabolites. Glucosinolates contribute to the pungent taste in crucifers that is shown to be produced as a defense mechanism against pests and mammalian feeding damage (Brown *et al.*, 2002). Fahey *et al.* (2001) indicated that around 120 individual glucosinolates have been isolated from this particular family in addition to other similar families. One of the plants encompassing a significant amount of glucosinolates is *Eruca sativa* Miller originating from the rocket species. Extensive research has been done on the *Brassicaceae* family concerning these bioactive compounds with minimal research on *E. sativa.*

2.2. Composition of rocket leaves

Rocket is a term used to identify several species within the Brassicaceae family. Rocket is also known as salad rocket, garden rocket, arugula, roquette in France, and rucola in Italy (V. V Bianco, 1995). *Eruca sativa* Mill*., Eruca vesicaria, Diplotaxis tenuifolia* (L.), and *Diplotaxis*

muralis are among the edible rocket species suitable for human consumption. The *E.sativa* plant grows up to be between 10-100 cm tall with a branched stem, compound leaves, a thin taproot, and a characteristic strong smell. The flowers are pale yellow or white with deep violet veins and the seeds are spherical in shape and yellow-brown or reddish in color. (Garg *et al.*, 2014; Pignone, 1996).

2.2.1. Carbohydrates and proteins in rocket leaves

Glucose was found to be the major sugar in leaves of rocket, representing more than 70% of soluble carbohydrates while sucrose, fructose, galactose, arabinose, and mannose were found at lower concentrations. The presence of glucose contributes to the osmotic regulation of rocket, which is known for its capability to support prolonged water deficiency (Villatoro-Pulido *et al.*, 2013).

The crude protein in ground defatted seeds of *E.sativa* cv. Baladi was 31% whereas the main amino acids present were glutamic acid, aspartic acid, leucine and proline. *E.sativa* Lam. seeds, however, contain 20-25% protein (V. V Bianco, 1995).

2.2.2. Aromatic components of rocket

Jirovetz *et al.* (2002) analyzed the aroma compounds of *E.sativa* fresh leaves resulting in 50 components responsible for the characteristic intense green; herbal; horseradish-like aroma of these salad leaves. The constituents are isothiocyanates and derivatives of butane, hexane, octane, and nonane. Also, 4-methylthiobutyl isothiocyanate, *cis*-3-hexen-1-ol, *cis*-3-hexenyl butanoate, 5-methylthiopentyl isothiocyanate, *cis*-3-hexenyl 2-methylbutanoate, and 5-methylthiopentanenitrile were detected and analyzed.

2.2.3. Seed oils of rocket

Hydrodistillation was applied to oils of rocket flowers and leaves with green beans to obtain 4-methylthiobutyl isothiocyanate or erucin, which was the major sulfur/nitrogen component in all oils (9.0-81.7%) depending on the plant material. Jirovetz *et al.* (2002) also found erucin to be the main component making up 14.2% of all volatiles. Other compounds were found such as fatty acids and esters, as the main components, including decanoic acid, hexadecanoic acid, and dodecanoic acid. Moreover, aliphatic alcohols and carbonyls were present in leaves with green beans after autolysis as well as phytols, cyclocitral and (*E*)-β-ionone which were detected only in oils from leaves with green beans (Blaževic & Mastelic, 2008).

2.2.4. Agricultural properties and uses of rocket

Eruca species are adaptable plants, grown on dry lands and can endure the scarcity of water as well as the inhospitable lands. It is usually eaten as raw leaves in salad, cooked as an ingredient in traditional dishes, consumed as a spice and is recently being added to several dishes to add flavor. The sharp taste and odor associated with rocket is dependent on the species, genetic diversity, and environment (V.V. Bianco & Boari, 1996; Garg *et al.*, 2014; Pignone, 1996). In addition to food intake, rocket was traditionally known for its medicinal properties dating back to many years ago. It was used to treat eye infections, act as a deodorant, and a digestive. It was also used as a diuretic, stimulant and a laxative, attributable to the presence of vitamin C and mineral salts. Moreover, rocket seeds were utilized to produce oil similar to rapeseed oil in India, making rocket a multipurpose crop (Bhandari & Chandel, 1996; Doležalová *et al.*, 2013; Yaniv *et al.*, 1998).

2.2.5. Nutrient content of rocket

The cruciferous family is recognized for the antioxidant compounds they possess. As stated by the USDA, raw arugula is calculated to contain 25 kcal per 100g, with a low percentage of carbohydrates, fat, and protein; 3%, 3%, and 5%, respectively. On the other hand, a higher portion of vitamins, minerals, and phytochemicals that help improve health have been shown to be present. The main flavonoids in different rocket species were kaempferol, quercetin, and isorhamnetin (Table 2.1), in addition to the presence of glucosinolates and isothiocyanates (Garg *et al.*, 2014). Vitamin C and folate make up almost 25 wt% and is fairly high in iron and calcium; 16% and 18% RDA (Recommended Daily Allowance), respectively (U.S. Department of Agriculture, 2011) The main fatty acid present in rocket seeds is erucic acid that makes up 41.7 wt%, contributing to a higher oxidative stability (Moser *et al.*, 2010).

Table 2.1. Flavonoid values for the edible portion of raw arugula in milligrams. (U.S. Department of Agriculture, 2011).

Flavonoids	Value per 100g
Isorhamnetin	4.3
Kaempferol	34.9
Quercetin	7.9

2.3. Bioactive components in rocket

2.3.1. Phenolic compounds

Plants have been recognized to contain phenolic compounds and flavonoids that play a significant role in disease prevention. Phenolic compounds are chemical compounds comprised of a hydroxylated aromatic ring that can be classified as polyphenols depending on the number of aromatic rings and hydroxyl groups attached to it (Palacios *et al.*, 2011). They are secondary metabolites ubiquitously present in food of plant origin, and are considered one of the abundant groups of phytochemicals derived from several biological pathways in plants including the pentose phosphate, shikimate, and phenylpropanoid pathways. Phenolics can be divided into three classes including hydroxycinnamic acids, hydroxybenzoic acids and flavonoids, which is the most important phenolic class (Escarpa & González, 2001; Randhir *et al.*, 2004).

Phenolic compounds are essential for plant growth and development besides defense against injury caused by pathogens and predators. They contribute mostly to the organoleptic properties of plant foods due to their abundance in plants, and are responsible for color differentiation as well. Additionally, they provide "anti-allergenic, anti-artherogenic antiinflamatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects" (Balasundram *et al.*, 2006). The physiological benefits displayed by phenolic compounds are due to the antioxidant properties they possess, specifically, free radical scavenging and metal chelating activity as well as electron/hydrogen donation. The antioxidant activity is positively correlated with the degree of hydroxylation of phenolic compounds (Afanes'ev *et al.*, 1989; Balasundram *et al.*, 2006; Dai & Mumper, 2010; Kähkönen *et al.*, 1999).

2.3.2. Flavonoids

Flavonoids, on the other hand, are fifteen carbon compounds with low molecular weight that form the largest group of the many subgroups of phenolic compounds. The basic structure of flavonoids consist of two fused aromatic rings (a phenolic ring and a pyrane ring); A and C, attached to the 1' position of a third ring denoted as ring B with the 2' position of ring C (Figure 2.1) (Balasundram *et al.*, 2006; Rice-Evans *et al.*, 1997).

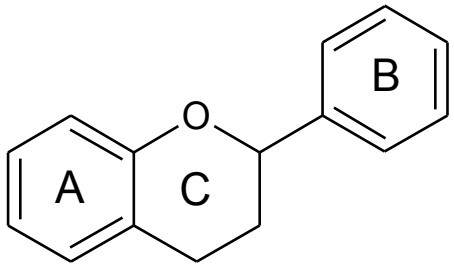


Figure 2.1. Flavonoid basic structure

Along with tannins and phenolic acids, flavonoids are viewed as the major phenolic compounds found in food. They are further divided into groups characterized by structural differences in their phenolic rings and side groups, i.e., flavonols, flavones, flavanones, flavanols, isoflavones, flavanonols, and anthocyanidins (Balasundram *et al.*, 2006; Rice-Evans *et al.*, 1997).

Flavonoids are commonly found in plant stems, barks, flowers, and leaves (Larson, 1988). They provide protection against any kind of damage elicited by pathogens, herbivores, or ultraviolet radiation. Moreover, flavonoids are responsible for color formation especially in berries and vegetables. Close to 4,000 flavonoids were discovered until now; this diversity is due to the substitution of compounds to rings A and B by oxygenation, alkylation, glycosylation, acylation, and sulfation, which will further produce more compounds (Balasundram *et al.*, 2006; Heim *et al.*, 2002).

Flavonoids are characterized by low-molecular weight substances, however, conjugation, glycosylation, or methylation can alter these characteristics affecting hydrophilicity and biological properties as well as increasing the molecular weight of the flavonoid itself (Aherne & O'Brien, 2002). Jungbluth and Ternes (2000) showed the molecular masses of flavonols including quercetin and kaempferol and their oxidation products to be within the range of 286 and 334 g/mol with difference of 16 amu (Table 2.2).

Table 2.2. Molecular weights in (g/mol) of bioactive compounds in *E.sativa*

Compound	Molecular weight	Reference
Kaempferol	286.2	Calderón-Montaño *et al.* (2011)
Quercetin	302.2	Gates and Lopes (2011)
Sulforaphane	177.3	Bolton *et al.,* (2008)
Glucoerucin	421.5	Bolton *et al.,* (2008)
Erucin	161.3	Bolton *et al.,* (2008)

Similar to phenolics, flavonoids exhibit high antioxidant activity, which is determined by the type of substitution on rings B and C. According to van-Acker *et al.* (1996) higher antioxidant activities of flavonoids have been shown when hydroxyl groups were attached to ring B on the 3'-, 4'-, and 5'- positions than those having one hydroxyl group attached. Radical scavenging ability was enhanced when a double bond was formed between C2 and C3 of ring C with a 4-oxo group or a 3 hydroxyl group attached (Balasundram *et al.*, 2006).

As a result of the antioxidant activity that flavonoids possess, they are able to protect the heart from injury produced by LDL oxidation and other factors. Diets rich in flavonoids have shown to decrease mortality rates of older men with coronary heart disease and reduce the risk of occurrence in postmenopausal women. It was also shown that flavonoids were significantly effective in scavenging reactive nitrogen species and their mediators; peroxynitrites, resulting in a lower incidence of coronary heart disease (Haenen *et al.*, 1997; Heim *et al.*, 2002).

Flavonoid compounds are ubiquitous among many types of foods. Flavonols are widely distributed among the Ericaceae fruits; most commonly residing in the skin of the cranberry and blueberry fruits, for example. They constitute several types of compounds mainly isorhamnetin, quercetin, kaempferol, and myricetin. Anthocyanins are the second class of flavonoids; made of up an anthocyanidin molecule that exist with a sugar moiety (Côté *et al.*, 2010). They are responsible for the color found in berries such as bilberries, chokeberries, blackberries, raspberries and strawberries, as well as grapes (Dai *et al.*, 2010). Almost half of the components of green tea compose of flavanols; 26.7% are catechin-gallate components including epigallocatechin gallate, epicatechin gallate, epigallocatechin, epicatechin, and catechin. Flavanones, on the other hand, have the least presence in food. Grapefruit and other citrus fruits

contain the compounds naringin and hesperidin; the main flavonone compounds found in food (Chanet *et al.*, 2012; Rice-Evans *et al.*, 1997).

Flavones are the most commonly occurring flavonoids besides flavonols, because of their ability to form countless structures. Apigenin, luteolin, and rutin found in various vegetables such as celery, green pepper, and parsley belong to the flavone family Soybeans are the main source of isoflavones incorporating the compounds genistein and daidzein, which act as phytoestorgens and show anticarcinogenic activity (Rice-Evans *et al.*, 1997; H.-j. Wang & Murphy, 1994).

2.3.2.1. Kaempferol

The low molecular weight compound, kaempferol, is a member of the flavonoid family and flavonol being the parent compound. The diphenyl propane structure is presented as a simple flavone with a molecular weight of 286.2 g/mol characterized by the presence of a double bond between C2 and C3 and a hydroxyl group situated at C3 on ring C, which plays a role in improving antioxidant activity (Balasundram *et al.*, 2006; Calderón-Montaño *et al.*, 2011). It can be categorized as a sinapic acid and a coumaroyl derivative due to structural differences of kaempferol glycosides. The main characteristics of sinapic acid incorporate anti-inflammatory, anti-oxidant, anti-microbial, and anti-cancer activity (Nićiforovič & Abramovič, 2013).

Flavonoids are rarely found in their aglycone form; instead they are present in their conjugated form in fresh vegetables. However, kaempferol is usually found as *O*-glycosides with conjugation mostly occurring at the third carbon of the C ring. Glucose, rhamnose, galactose, and rutinose are the sugars that commonly form glycosides when attached to kaempferol. The flavonol aglycones that occur in Brassica crops along with kaempferol are quercetin and myricetin (Calderón-Montaño *et al.*, 2011; Cartea *et al.*, 2011; Heim *et al.*, 2002).

The flavonol kaempferol is slightly soluble in water rendering it one of the least polar flavonoids tested. It is mainly found in tea and some vegetables such as endives, leeks, broccoli, cabbage, beans, and tomatoes. In the small intestine where hydrolysis occurs, kaempferols attached to glucosides such as kaempferol-3-glucoside are hydrolyzed by beta-glucosidase activity. (Calderón-Montaño *et al.*, 2011; Heim *et al.*, 2002; Merken & Beecher, 2000b; Rice-Evans *et al.*, 1997).

There are significant beneficial effects of kaempferol on disease risk reduction such as in the case of some types of cancer and cardiovascular disease. A case-controlled study of 558 lung

cancer cases (smokers) that consumed kaempferol-rich foods such as tea, beans, broccoli, spinach, apples, and strawberries of approximately 2 mg of kaempferol per day showed an inverse association with lung cancer risk. A reduced gastric cancer risk was also observed in 354 cases with the consumption of kaempferol-rich foods such as onions, cruciferous vegetables, and green beans. Furthermore, kaempferol consumption was inversely associated with pancreatic cancer risk after a follow-up of a period of 8 years in 183,518 people with an intake of around 3.89 mg/day (Calderón-Montaño *et al.*, 2011). The synergistic action of quercetin and kaempferol was shown to prevent the multiplication of cancer cells in the gut (Cartea *et al.*, 2011). On the other hand, a cohort study of 805 men between the ages of 65 and 84 found a correlation between flavonoid consumption (quercetin, kaempferol, myricetin, apigenin and luteolin) found in black tea, onions and apples, and reduced mortality risk from coronary heart disease and a reduced incidence of myocardial infarction. Another similar cohort study of 361 and 394 men and women, respectively, found that kaempferol consumption was associated with a significant decreased risk of acute myocardial infarction. The flavonol trio, quercetin, kaempferol and isorhamnetin were also advantageous in producing an anti-inflammatory effect in humans (Calderón-Montaño *et al.*, 2011).

Oxidative stress has been found to play an important role in the pathogenesis of many chronic diseases. However, the development of these processes can be prevented by antioxidant agents such as flavonoids. Kaempferol antioxidant activity is due to the structural features of kaempferol that include the presence of the double bond at C2-C3 and the hydroxyl groups at C3, C5, and C4' (van-Acker *et al.*, 1996). Kaempferol was found useful in inhibiting the enzyme xanthine oxidase that is capable of producing ROS In comparison with other flavonoids such as luteolin, kaempferol displayed a greater increase in scavenging activity on 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical, but when compared with other flavonoids such as quercetin, myricetin, and catechin, kaempferol was the least active (IC_{50} 5.93 μM) (Hirano *et al.*, 2001). Kaempferol was also found to chelate iron, prevent lipid peroxidation and is known to scavenge hydroxyl radicals and peroxynitrites at low kaempferol concentrations. (Calderón-Montaño *et al.*, 2011; Ferrali *et al.*, 1997; van-Acker *et al.*, 1996). Also, kaempferol displayed more antioxidant activity against aqueous phase radicals than vitamins E and C (Cos *et al.*, 1998; Rice-Evans *et al.*, 1995; L. Wang *et al.*, 2006).

2.3.2.2. Kaempferol in rocket species

In rocket plants, flavonols are found in substantial amounts. Kaempferol exists in many species of rocket specifically *D.erucoides* and *E.sativa* as kaempferol di-*O*-glycoside; *D.tenuifolia* as kaempferol -3,4'-di-glucoside; *Eruca vesicaria* as kaempferol -3-glucoside, kaempferol-3,4'-di-glucoside, and kaempferol-3-(2-sinapoyl-glucoside)-4'-glucoside (Cartea *et al.*, 2011). Specifically speaking, *E.sativa* is found to contain kaempferol and kaempferol glycosides, in addition to its presence in Turkish rocket, wild rocket, and wall rocket (Calderón-Montaño *et al.*, 2011). Arabbi *et al.* (2004) concluded that *E.sativa* housed the aglycone kaempferol and its glucosides in large concentrations when compared with other dark green vegetables (41 and 104 mg/100 g of FW). Bennett *et al.* (2006) reported that rocket tissues contained significant levels of poly-glycosylated flavonoids, while Michael *et al.* (2011) managed to isolate two novel kaempferols glycosides from *Eruca sativa* leaves; kaempferol 3-*O*-(2"-*O*-malonyl-β-D-glucopyranoside)-4'-*O*-β-D-glucopyranoside and rhamnocitrin 3-*O*-(2"-*O* methylmalonyl-β-D-glucopyranoside)-4'-*O*-β-D-glucopyranoside, along with 7 other identified kaempferols.

2.3.2.3. Quercetin

Quercetin is a ubiquitous flavonol; most prevalent in vegetables as *O*-glycosides. The content of quercetin in foods is negatively affected by cooking, however, a reduction of quercetin levels is due to leaching or heat degradation (Aherne *et al.*, 2002; Garg *et al.*, 2014). In ideal situations, the half-life of quercetin was calculated to be from 20-72 hours, resulting in subsequent plasma quercetin buildup (Heim *et al.*, 2002). Frequent onion consumption, in particular, results in the accumulation of quercetin glycosides in the plasma, since quercetin has been identified in the plasma after 48 hours of the consumption of flavonols, therefore causing an increase in the presence of antioxidants in the blood (Aherne *et al.*, 2002; Hollman & Katan, 1999). As a result, quercetin was able to inhibit LDL oxidation and aid in the production of detoxifying enzymes which assists in the prevention of chronic diseases such as cancer and atherosclerosis along with chronic inflammation (Ackland *et al.*, 2005; Kim & Ishii, 2006; Soengas *et al.*, 2011). Researchers also concluded that humans could absorb significant amounts of quercetin in both aglycone and glycosidic forms. However, quercetin aglycones are found to

be more bioactive than the glycosylated quercetin (Degl'innoocenti *et al.*, 2007; Kähkönen *et al.*, 1999).

Structural orientations are known to affect biological activities of flavonoids such as antioxidant activity. The presence of hydroxyl groups on flavonol molecules gives quercetin and its derivatives a higher activity in comparison with other flavonols. The hydroxyl groups can initiate redox reactions by donating a hydrogen atom or acting as reducing agents and scavenging singlet oxygen species (Bell *et al.*, 2014). The C-3 position is the most equipped followed by the C-7 position. Sugar moieties like D-glucose are commonly attached as well as galactose, arabinose, and lignins. (Aherne *et al.*, 2002) However, the more sugar moieties attach to quercetin, the lower the antioxidant activity. Quercetin decreases oxidative degradation by inhibiting the activity of both xanthine oxidase and nitric-oxide synthase by iron chelation and the prevention of lipid peroxidation. Besides, antigenotixicity characteristics were displayed when quercetin and kaempferol were issued with mutagens (Jin *et al.*, 2009; Nijveldt *et al.*, 2001).

Quercetin prevents the activity of cyclooxygenase and lipoxygenase, decreasing the supply of arachidonic acid production and its inflammatory metabolites. The catechol group attached to ring B of quercetin contributes to its peroxynitrite scavenging activity. Furthermore, in plants, the catechol group in quercetin 3-*O*-glycosides, produced in response to environmental stimuli, contributes to the ability of quercetin to chelate metals and reduce reactive oxygen species when compared with a single hydroxyl group attached to the B ring of a flavonol glycoside. (Haenen *et al.*, 1997; Pollastri & Tattini, 2011).

According to Haenen *et al.* (1997), quercetin is a powerful antioxidant due to its structural configuration and absence of substituents on the hydroxyl groups. The quercetin structure is equipped with 2 hydroxyl groups on its B-ring, an unsaturation and 4-oxo function in its C ring which offers the ability of disabling numerous mediators that may induce cancer. The hydroxyl groups attached to ring B and ring C, specifically at C-3' and C-3, respectively, is linked to an increase in superoxide radical scavenging (Cartea *et al.*, 2011; Cos *et al.*, 1998). A group of flavonols were tested for their antioxidant activity including quercetin, which ranked the highest and most effective for scavenging 50% of the DPPH radical at <3 μm (Hirano *et al.*, 2001). Likewise, quercetin was found to be the strongest among flavonols with an IC_{50} value of 8.05 μm using the DPPH assay. Moreover, quercetin was the most effective, compared with

other flavonoids, when tested for their antioxidant capacity using the radical cation 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) or ABTS, produced from the reaction with hydrogen peroxide activated myoglobin. Quercetin and kaempferol displayed the strongest inhibition of Fenton-mediated hydroxyl radical among flavonols with an estimated IC_{50} of 0.5 μM. In another study, quercetin, catechin, and epicatechin demonstrated strong antioxidant behavior against oxygen radicals, protecting the phospholipid bilayer of the cell membrane. The metal chelating ability of quercetin is beneficial against metal-induced radical reactions including iron and copper, inhibiting LDL oxidation in peroxidation reactions involving copper. The conjugation between A and B rings offers efficacious antioxidant activity for quercetin. However, loss of conjugation in the C ring will contribute to the loss of antioxidant activity (Rice-Evans *et al.*, 1997; L. Wang *et al.*, 2006).

2.3.2.4. Quercetin in rocket species

All parts of the rocket plant including the seeds, leaves, and flowers contain the main flavonoid aglycones kaempferol, quercetin, and isorhamnetin. The aglycones quercetin and kaempferol are usually found attached to a sugar molecule and acylated. This introduces changes in the biological activities of flavonols. The major flavonols in salad rocket was kaempferol di-*O*-glycoside with its isomer, along with quercetin di-*O*-glycoside and a quercetin tri-*O*-glycoside, in addition to an isorhamnetin di-*O*-glycoside. Quercetin 3-*O*-glucoside and quercetin monosinapoyl di-*O*-glycoside were also shown to be present (Bell *et al.*, 2014; Bennett *et al.*, 2006).

Rocket salads, specifically Eruca and Diplotaxis, incorporate an abundance of flavonoid compounds in relation to other salads. Generally, the amount of the flavonol quercetin in rocket species ranges from 0.50 ± 0.37 mg/100g FW and 7.74 ± 2.51 mg/100g FW (Durazzo *et al.*, 2013). *E.sativa* contains 97.8 mg/100 g^{-1} FW of kaempferol-3,4′di-*O*-glucoside according to Bell et al. (2014), calculated as 74% of total phenolic content of that same flavonoid according to Martínez-Sánchez *et al.* (2008). Additionally, a similar finding concluded that kaempferol predominates in *E.sativa* (41 and 104 mg/100 g of FW) and quercetin in minimal amounts (14 mg/100 g of FW) suggesting that rocket was one of the samples in which the largest concentration of total flavonoids was present (Arabbi *et al.*, 2004).

Quercetin was found in other species of rocket such as *D.tenuifolia* in higher amounts. (Martínez-Sánchez *et al.*, 2008) However, in a current study, Weckerle *et al.* (2001) utilized

nuclear magnetic resonance spectroscopy and isolated three new acylated quercetin derivatives, specifically tri-*O*-β-d-glucosides in *E.sativa* leaves. The three novel quercetin triglucosides are quercetin 3,30,40-tri-*O*-*b*-*d*-glucopyranoside, quercetin 30-(6-sinapoyl-*O*-*b*-*d*-glucopyranosyl)-3,40- di-*O*-*b*-*d*-glucopyranoside, and quercetin 3-(2-sinapoyl-*O*-*b*-*d*-glucopyranosyl)-30-(6-sinapoyl-*O*-*b*-*d*-glucopyranosyl)-40-*O*-*b*-*d*-glucopyranoside. A number of quercetin derivatives were also detected in rocket species like quercetin-3-β-glucoside (isoquercetin) and rutin (quercetin-3-*O*-rutinoside). It should be noted that when rocket is present in the diet, it contributes to flavonoid ingestion. (Villatoro-Pulido *et al.*, 2013).

2.3.2.5. Glucosinolates

Glucosinolates are a group of anionic stable secondary plant metabolites. Their structure is composed of thiohydroximates with a *S*-linked *b*-glucopyranoside moiety and an *O*-linked sulfate residue plus an amino acid-derived variable side chain (Figure 2.2). Substituents are attached to the *O, S, N* atoms of the side chain or glucosyl moiety. Glucosinolates are known for their pungent and peppery characteristic flavor. The taste and odor of glucosinolates and its derivatives was mainly produced to protect plants against microbes, herbivores and other predators. They are present at much higher amounts in plants than their metabolic products. The various classes of glucosinolates depend on the amino acid precursor, which divides them into aliphatic glucosinolates derived from methionine, isoleucine, leucine, or valine; aromatic glucosinolates derived from phenylalanine and tyrosine, and heterocyclic (indole) glucosinolates derived from tryptophan. These classes distinguish glucosinolates by the substituents linked to the *O, S, or N* atoms of the side chain of amino acids formed (Agerbirk & Olsen, 2012; Fahey *et al.*, 2001; Velasco *et al.*, 2010).

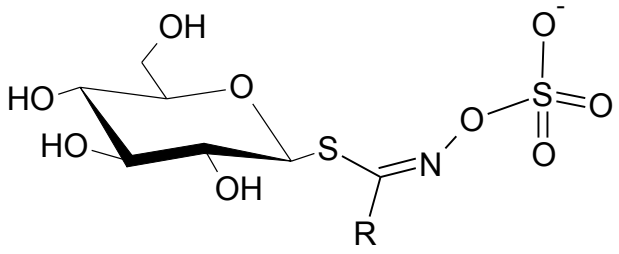


Figure 2.2. Basic structure of glucosinolates.

Glucosinolates and their derivatives, especially isothiocyanates, were known to reside in the Brassicaceae family and have been present throughout the years in the human diet. More than 120 glucosinolates exist in this family (cruciferous vegetables) at 20 μmol/g of dry weight (DW) of vegetable (Clarke *et al.*, 2008; Hayes *et al.*, 2008). In humans, they were shown to have a role in regulating the enzymes involved in phase I drug metabolism and inducing phase II metabolizing enzymes and protein kinases in signaling pathways, as well as acting as chemoprotective agents (Bennett *et al.*, 2006; Fahey *et al.*, 2001).

The glucosinolate's hydrolysis products that are present in crucifers exert protective effects against certain cancers, such as gastrointestinal and breast cancer. However, some glucosinolate products are considered toxic at high concentrations. Even though all the Brassicaceae family contains glucosinolates, the cruciferous vegetables differ by their glucosinolates profile. Each glucosinolate has a different substituent that distinguishes it from the other glucosinolates. Glucobrassicin, glucoraphanin, and gluconasturtin are common glucosinolates present in broccoli, Brussels sprouts, cress, and turnip (Bennett *et al.*, 2006; Bonnesen *et al.*, 2001; Velasco *et al.*, 2010).

The amount of glucosinolate in leaves can be affected by various conditions including climate, cultivation, soil properties, harvest time, storage, physical damage to leaves and food preparation (Bennett *et al.*, 2006; Velasco *et al.*, 2010). When plant tissue is damaged, glucosinolates in cell vacuoles combine with myrosinase enzymes in myrosin cells and form what is referred to as the mustard oil bomb. The myrosinase enzymes are usually stored in separate compartments than glucosinolates in undamaged plants, but upon injury they come into contact allowing the enzymes to hydrolyze the s-glucosyl bond of the glucosinolate to form glucose and an aglycone; thiohydroximate-*O*-sulfonate, that rearranges spontaneously at neutral pH to form isothiocyanates by loss of sulfate (Agerbirk *et al.*, 2012). Besides isothiocyanates, this process yields many hydrolysis products including nitriles, thiocyanates, epithionitriles, indoles, oxazolidine-2-thiones, cyanopithioalkanes, ascorbigens, goitrogens, and epithioalkanes as a defense mechanism against bacteria, fungi, and insects. The formation of these hydrolysis products depend on pH of the reaction, glucosinolate side-chain, and the presence of certain ions and specifier proteins. The hydrolysis products isothiocyanates and nitriles are the two compounds produced in high concentrations. However, stress as well as environmental conditions cause glucosinolates profile and concentrations to fluctuate (Bennett *et al.*, 2006).

Myrosinase activity is also found in the microflora of the intestinal tract where escaped glucosinolates are hydrolyzed, without knowing the efficacy of their action. Nevertheless, plant myrosinase activity, in contrast with intestinal microflora increases the bioavailability of glucosinolate products (Clarke *et al.*, 2008; Zhang, 2004).

2.3.2.6. Glucosinolates in rocket species

One of the main characteristics of rocket species is the presence of high amounts of glucosinolate compounds, ranging from 14.02 – 28.24 μmol/g^{-1} DW (Villatoro-Pulido *et al.*, 2013). The total average glucosinolate content in Eruca accessions ranged from 756 to 2459 mg/kg^{-1} DW (Pasini *et al.*, 2012). A total of thirteen glucosinolates were discovered in rocket leaves; eight aliphatic (glucoerucin, glucoraphanin, gluconapin, glucoiberverin, progoitrin, gluconapoleiferin, glucobrassicanapin and glucosativin), one aromatic (gluconasturtiin) and four indoles (4-hydroxyglucobrassicin, 4-methoxyglucobrassicin, glucobrassicin and neoglucobrassicin.) Glucosativin (4-mercaptobutyl glucosinolate), glucoerucin (4-methylthiobutylglucosinolate), and glucoraphanin (4-methylsulfinylbutyl GSL) are the most dominant, ranging from 3.64 to 12.64 μmol g^{-1} DW for glucoraphanin, 0.14 to 4.03 μmol g^{-1} DW for glucosativin and 8.10 to 11.40 μmol g^{-1} DW for glucoerucin while glucobrassicin was the one of the least dominant compounds. It was lately found that glucosativin was the main glucosinolate in leaves of Eruca and Diplotaxis species. Nevertheless, a minor amount of glucosinolates is required to initiate cell defense against oxidative stress, so increased concentrations do not have an additional effect (Bennett *et al.*, 2006; Villatoro-Pulido *et al.*, 2012).

Indoles have shown programmed cell death in prostate, breast, and bone cancer cell lines. Glucobrassicin was the minor glucosinolate in Italian, European, Spanish, Chinese and North African Eruca accessions while indole-3-carbinol was the major indole in rocket species, which is known to prevent cancer in reproductive organs (Bell *et al.*, 2014; Bennett *et al.*, 2007). Eruca sativa leaf glucosinolate extracts were also shown to prevent oxidative stress, according to Jin *et al.* (2009).

In comparison with other cruciferous vegetables, rocket leaves possess a lower glucosinolate content. Kim *et al.* (2004) present data on the high antioxidant activities using the XYZ-dish method from two desulfo-glucosinolates purified from rocket leaves (Kim *et al.*, 2006). They also concluded that seeds present simpler HPLC profiles and higher glucosinolate

levels when compared with leaves of rocket. The intact leaf powder contains half the glucosinolates than the intact root powder but showed higher activity indicating the presence of other phytochemicals assisting with the antioxidant activities.

2.3.2.7. Isothiocyanates

Isothiocyanates originate from glucosinolate hydrolysis and are recognized by their chemical group R–N=C=S, whose central carbon is highly electrophilic. They are characterized by their pungent taste produced to repel hostile activity which also provides beneficial effects on health. They also possess bioactivity promoting health. Subsequent isothiocyanates can be formed with distinctive side chains dictating certain characteristics including isothiocyanate volatility and hydrophobicity, which in turn affects its activity (Agerbirk *et al.*, 2012; Bell *et al.*, 2014). Sulforaphane is a widely studied isothiocyanate present in crucifers, in addition to allyl isothiocyanate, phenethyl isothiocyanate, and benzyl isothiocyanate. These compounds display chemopreventive effects similar to those of sulforaphane. The exposure of cells to the bioactivity of isothiocyanates helps prevent tumorigenesis and proliferation (Garg *et al.*, 2014; Melchini & Traka, 2010; Velasco *et al.*, 2010).

Cruciferous vegetables are widely known for their ability to protect against cancer. Many aliphatic glucosinolates, specifically methionine-derived isothiocyanates are found to regulate cellular redox status and signaling in mammals, thus help promote the coding of detoxification enzymes namely glutathione *s*-transferase (Grubb & Abel, 2006).

These hydrolysis products exhibit antibacterial and antifungal properties as plant defense. The short exposure time of isothiocyanates in the biological system is able to display beneficial effects against cancer, especially breast cancer in humans. The level of isothiocyanate absorption in the living system is dependent on the consumption of raw, minimally processed food sources. Low concentrations of isothiocyanates (30 mol/L in most cases) are required to exert potential anticarcinogenic benefits while higher concentrations revealed the loss of these benefits (Bell *et al.*, 2014; Velasco *et al.*, 2010; Villatoro-Pulido *et al.*, 2012). Extreme concentrations of isothiocyanates can have toxic effects because of their electrophilic nature and the consequent production of reactive oxygen species. The electrophilic carbon in isothiocyanates reacts with nucelophiles resulting in the activity of isothiocyanates (Lamy *et al.*, 2008; Rose *et al.*, 2003; Zhang, 2004).

Isothiocyanates and indoles displayed chemopreventive effects at different stages. The consumption of isothiocyanates and indoles showed an increase in drug metabolizing enzymes in several organs in rodents and in another study displayed cytotoxicity in cell lines at >12.5 µM concentrations as well as controlling apoptosis (Bell *et al.*, 2014; Velasco *et al.*, 2010). Isothiocyanates alone inhibits the activation of phase I enzymes and induces the detoxification of phase II enzymes involved in the protection against oxidative stress, supported by in vitro and in vivo studies. In mice, isothiocyanates inhibited tumorigenesis in lungs. Thus, the protective effect of cruciferous vegetables is attributable to the hydrolysis products (Bonnesen *et al.*, 2001; N. Hanlon *et al.*, 2009).

At 15 µmol/l concentration, sulforaphane induced apoptosis in human breast cancer cells. Furthermore, sulforaphane was shown to be more potent than erucin in inhibiting the propagation of prostate cancer cells at high concentrations. Sulforaphane was shown to indirectly increase the antioxidant activity of the cell, not as a direct-acting antioxidant. (Melchini *et al.*, 2013; Pledgie-Tracy *et al.*, 2007).

Erucin (4-methylthiobutyl isothiocyanate), on the other hand, is a bioactive chemopreventive isothiocyanate that demonstrate both indirect antioxidant activity by inducing phase II enzymes and direct antioxidant activity by scavenging reactive oxygen species such as hydrogen peroxides and alkyl hydroperoxides and protecting cells against oxidative stress (Blaževic *et al.*, 2008; Garg *et al.*, 2014). Moreover, the induction of apoptosis and prevention of the cell cycle was observed in human cultured cancer cell lines such as lung, liver, breast, and colon cancer and leukemia cells. Erucin also caused an increase in CYP1A1; involved in phase I metabolism, in the lungs of rats at a dose of 3mg/kg/day. CYP1B1 was also increased in liver and lung tissues, which was analogous to the amount of erucin consumed in the human diet. Erucin is mainly characterized by its selectivity in inducing antiproliferative effects on select human cancer cells but not in non-cancerous cells (Azarenko *et al.*, 2014; Melchini *et al.*, 2010).

2.3.2.8. Isothiocyanates in rocket species

Isothiocyanates and their hydrolysis products, including 4-methylthiobutyl isothiocyanate have been found in rocket species. Glucoerucin is the main glucosinolate in rocket, in which the isothiocyanate erucin is produced. Erucin in rocket displayed similar bioactivity of sulforaphane found in broccoli (Azarenko *et al.*, 2014; Bell *et al.*, 2014; Hedges & Lister, 2005).

Rocket plants can provide chemopreventive effects by specific concentrations levels achievable in the average daily consumption of rocket (Bell *et al.*, 2014). A low concentration of isothiocyanates (0.005 g/kg of diet) from the consumption of rocket species in the diet may offer additional benefit to the antioxidant system in mammals. In another study, sulforaphane, iberin, and erucin were detected in rocket leaves, in which glucoraphanin and sulforaphane were not found to be significant (Fahey *et al.*, 2001; Melchini *et al.*, 2009; Villatoro-Pulido *et al.*, 2012). However, it was concluded that sulforaphane is found at higher levels in *E. sativa* Mill and Diplotaxis species than erucin. Sulforaphane levels were measured to be 0.615mg/g DW in E. sativa, while erucin was measured at 8.84 μg/g DW. This suggests variations in enzyme hydrolysis activity within rocket accessions. The synergistic effects of the isothiocyanates in rocket plants are shown to add to the antigenotoxic effects of rocket. Limited human studies were made on the isothiocyanate sulforaphane in rocket species (Bennett *et al.*, 2007; Lamy *et al.*, 2008; Melchini *et al.*, 2009).

E.sativa extracts demonstrated strong antigenotoxicity in human liver cancer cells. At a concentration of 6 μL /mL, Eruca plant extracts showed a decrease in DNA damage by benzo(a)pyrene. Erucin inhibited the progression of human lung cancer cells by increasing the protein expression of p53 and p21 responsible for cell cycle progression, while sulforaphane displayed a stronger potency towards it (Lamy *et al.*, 2008; Melchini *et al.*, 2009). Melchini *et al.* (2013) recently examined the effect of erucin from rocket species in reducing the proliferation of prostate cancer cells. Erysolin, another isothiocyanate extracted from *E.sativa* showed more efficiency than erucin in the inhibition of human liver cancer cells treated with benzo-a-pyrene, using a lower concentration.

Glucosativin (4-mercaptobutylglucosinolate) and sativin isothiocyanates, found in rocket species Eruca and Diplotaxis, are responsible for the sharp flavor and odor they emit in rocket plants. The interaction between glucosinolates, isothiocyanates, vitamins, and antioxidants found in these species are capable of providing bioactive compounds with significant effects on the body, especially antigenotoxicity (Bennett *et al.*, 2007; Bennett *et al.*, 2006; Lamy *et al.*, 2008).

2.4. Phenolic compounds: sample preparation

The extraction of bioactive compounds from fresh, frozen, or dried plants is the first and most important step for the sample to be used as part of functional food ingredients, supplements, and nutraceuticals. Plant samples undergo pre-processing such as lyophilization or

air-drying and subsequently go through a grinding and homogenizing step to form smaller particles with larger surface area. In literature, lyophilization was shown to retain more phenolic compounds than other drying methods such as air-drying. However, other compounds that make up the plant may be affected by the harsh conditions of lyophilization (Dai *et al.*, 2010; Khoddami *et al.*, 2013).

The common extraction technique used for plant samples is solvent extraction because of its feasibility, efficiency, and wide applicability. The type of solvent used with different polarity, time, temperature, and the chemical nature of the sample all affect the extraction yield. As for flavonoids, phenolic compounds do not have a universal extraction procedure to extract all phenolic compounds. Some of these solvents include water, methanol, ethanol, acetone, and ethyl acetate (Khoddami *et al.*, 2013). Methanol is the most widely used solvent as is shown to efficiently extract low molecular weight polyphenols. Moreover, when it is combined with water, water-soluble flavonoids are extracted too. Ethanol, on the other hand, is a safe solvent but is not as efficient as methanol in extracting bioactive compounds. As for anthocyanins, a weak acid is added to the solvent system to assist in denaturing the cell wall and dissolving the anthocyanins (Dai *et al.*, 2010).

Long extraction times and high temperatures are not advised when dealing with phenolic compounds since they are easily hydrolyzed and oxidized. A slight increase in temperature can increase solubility of the sample while the viscosity and surface tension of solvent is decreased allowing better extraction rate. For example, it was shown that a temperature between 20°C and 50°C is suitable for anthocyanin extraction while a higher temperature causes degradation of these compounds. Conventional extraction methods such as soxhlet extraction are not recommended since they are time-consuming, uses large amounts of solvent, and show low efficiency (Dai *et al.*, 2010).

2.5. Phenolic compounds: identification methods

Concerning phenolic compounds quantification, the Folin-Ciocalteu assay is the most widely used assay that is both convenient and reproducible. It depends on the reducing power of the analyte which in turn determines the total polyphenolic content in samples. The basis of the test involves the chemical reduction of the Folin-Ciocalteu reagent, composed of tungsten and molybdenum oxides, by the phenolic compounds under basic conditions. However, the reagent can be reduced by nonphenolic compounds such as vitamin C and Cu(I) (Ho *et al.*, 2012; Huang

et al., 2005). It was shown that the B ring of the flavonoid structure with two hydroxyl groups such as those in catechin and quercetin showed a stronger reduction towards the reagent than other flavonoids used (Ho *et al.*, 2012).

Moreover, the Folin-Ciocalteu was adjusted with a novel micro-method in determining phenolic compounds of plant methanol extracts. It combines time (20 min), temperature (40°C), alkali conditions [5% (w/v) sodium carbonate solution] and alcohol (4%) to measure small concentrations of phenolic compounds in plant extracts. The reaction conditions are optimized to eliminate methanol interferences in the assay (Cicco *et al.*, 2009). Another more specific test introduced the use of polyphenol oxidase to measure total phenolic compounds in tea (Huang *et al.*, 2005). Furthermore, the phosphomolybdate assay was used infrequently to measure total antioxidant activity of extracts. It involves the reduction of molybdenum (VI) to molybdenum (V) forming a green phosphomolybdate complex which is measured spectrophotometrically at 760 nm. (Dai *et al.*, 2010)

2.6. Flavonoid compounds: identification methods

Both separation and non-separation analytical methods were used for the determination of flavonoids. Chromatographic techniques such as HPLC, GC, and capillary electrophoresis coupled with Uv-Vis spectrophotometry or mass spectrometry detection were widely used for the identification and quantification of individual flavonoids. Nevertheless, these methods require sophisticated instruments and numerous standards as well as it being time-consuming. In addition, there are more than 4,000 different flavonoids detected thus it is impractical to determine total flavonoids using separation techniques with the limited availability of authentic standards (Ho *et al.*, 2012; Magalhães *et al.*, 2012; Stalikas, 2007). Other methods including colorimetric methods are widely accepted as they target flavonoids with similar structures. They are more convenient, rapid, and inexpensive and do not require individual analytical standards for flavonoid determination (Chang *et al.*, 2002; Denni & Mammen, 2012). However, there is no chromogenic procedure that detects and quantifies all flavonoids (Chang *et al.*, 2002; Denni *et al.*, 2012; Ho *et al.*, 2012).

The sodium-borohydride/chloranil-based (SBC) assay was recently developed for the quantification of total flavonoids in which flavonoids with a 4-carbonyl group are reduced to flavan-4-ols using sodium borohydride catalyzed with aluminum chloride. Then the flavan-4-ols are oxidized to anthocyanins by chloranil in acetic acid solution and the anthocyanins formed

react with vanillin in concentrated hydrochloric acid forming the vanillin–anthocyanin adducts that are quantified at 490 nm. This procedure is specific but is inconvenient and very time-consuming with many steps as well as the addition of concentrated acids such as acetic and hydrochloric acids, and a heating step at 100 °C for 60 min (Magalhães *et al.*, 2012).

Furthermore, there are several chromogenic analytical methods for the quantification of flavonoids that rely on the type of flavonoids present in the plant species. Several of these methods employ the use of aluminum ions with different reaction conditions to form a color complex with the flavonoid, to quantify the flavonoid content in plant extracts. A procedure involving aluminum chloride, sodium hydroxide and a sodium nitrite solution was shown to best detect flavanols with *o*-dihydroxyl structure as the predominant component of flavonoid at 510 nm, showing less absorbance for flavonols and flavanones and no detection of flavones. The proposed explanation was the preference of the aluminum ion to bind with 3-hydroxyl and 4-keto oxygen or 5-hydroxyl and 4-keto oxygen of the flavonoid molecule rather than the *o*-dihydroxyl groups that is present in flavonols quercetin and rutin, for example, which leads to lower absorbance at 510 nm. However, the stability of the reaction products was found to be poor as the absorbance slowly decreased over time. The reaction occurs in a strong alkaline environment which affects the complex formation between the aluminum and the flavonoid (Ho *et al.*, 2012).

The second one-step procedure uses 2-aminoethyl diphenylborate to detect flavonols especially, measured at 404 nm, with no absorbance shown for products from flavanols and flavones. This test causes a bathochromatic shift of the absorption maximum and is specific to flavonoids with 3-hydroxy-4-keto structures. Also, great stability of the absorption spectra over 60 min was shown (Ho *et al.*, 2012).

Finally, Chang *et al.* (2002) and others developed a chromogenic test that includes aluminum chloride and an aqueous potassium acetate solution in ethanol. The addition of potassium acetate makes this test lean towards flavonols such as quercetin, fisetin, kaempferol, myricetin, and rutin, with weaker chromogenic capability for partial flavones (with 5-hydroxyl structures) measured at 415 nm (Denni *et al.*, 2012). For example, apigenin and chrysin formed a complex but had maximum absorbance at 395 and 385 nm. However, luteolin which included a C-5 hydroxyl group and an *o*-dihydroxyl group showed a strong absorption at 415 nm (Chang *et al.*, 2002). The principle behind this test is the acid-stable complex formation of the aluminum chloride with C-4 keto groups and either C-3 or C-5 hydroxyl groups of flavones and flavonols.

This test was found suitable for samples with flavonols or flavones as the main flavonoid. The reaction products remained stable for quercetin as a standard showing constant intensities over 60 min. The aluminum ion was shown to chelate with *o*-dihydroxyl flavonoid groups to produce bathochromic shifts of absorption bands in the spectrum (Ho *et al.*, 2012).

2.7. Antioxidant activity: determination methods

There are several methods that are frequently used to determining antioxidant capacity in plant extracts among which are the ORAC (oxygen radical absorption capacity), TEAC (trolox equivalent antioxidant capacity), FRAP (ferric reducing antioxidant potential), ABTS (2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonate), DPPH (2,2-diphenyl-1-picrylhydrazyl), and SOD (superoxide dismutase) assay. The antioxidant capacity in plant extracts depends on the method, extraction and the conditions used. DPPH, ABTS, and FRAP assays are commonly used to estimate the antioxidant capacities in plant extracts in which DPPH and ABTS are shown to produce fast and reproducible results. The ORAC requires more time and expensive equipment although it is the only test that allows the free radical to go to completion. This assay, along with the SOD uses a biologically relevant radical source which is a significant difference between the other tests. Furthermore, the DPPH, ABTS, FRAP, ORAC and SOD showed similar results for antioxidant activity for 30 plant extracts (Dudonné *et al.*, 2009).

2.8. Isothiocyanates: determination methods

In the early years, isothiocyanates were quantified using thin layer chromatography (TLC) but with the advancement of technology, GC and HPLC became the analytical tools to identify and quantify isothiocyanates and their metabolites in plant extracts using the cyclocondensation assay. HPLC analysis was coupled with Uv-Vis spectrophotometric techniques to quantify total isothiocyanates based on the cyclocondensation reaction. However, the HPLC-UV photometric method was not as specific and can produce errors for product detection when the absorption bands of the product and the interferents are similar (Choi *et al.*, 2004). Spectrophotometric methods for the analysis of total isothiocyanates that relied on colorimetric dyes were also reported, yet they lack sensitivity and specificity. Moreover, the GC-MS analysis was shown to have reproducible results and is a more reliable and efficient method to determine total isothiocyanates of different samples. It can confirm the identity of the product

formed by taking its mass spectrum and sensitivity will increase if the GC-MS is operated in selected ion monitoring (SIM) mode (Choi *et al.*, 2004).

The central carbon atom in most isothiocyanates is highly electrophilic reacting with oxygen-, sulfur-, and nitrogen-centered nucleophiles to form thiocarbamates, dithiocarbamates, or thiourea derivatives (Marton & Lavric, 2013; Zhang, 2012). Using the cyclocondensation assay, isothiocyanates react quantitatively with 1,2-benzenedithiol with the treatment of myrosinase to release isothiocyanates from the plants, forming the cyclocondensation end product, 1,3-benzodithiole-2-thione, a cyclic thiocarbonyl (Marton *et al.*, 2013). The use of 1,2-benzendithiol is explained by its immediate reaction with almost all isothiocyanates as well as its stability. Also, it is independent of the structure of the side chain of the isothiocyanate and the glucosinolate and provides high sensitivity of detection using GC-MS analysis (Choi *et al.*, 2004).

2.9. Volatile and semi-volatile analysis methods

GC-MS analysis is also used for the analysis of volatile and semi-volatile compounds by the fingerprinting approach. It helps characterize components of a mixture and identifies compounds (Pongsuwan *et al.*, 2008). Pyrolysis is the thermal degradation of macromolecules into volatile and semi-volatile fragments in an inert atmosphere. It is often coupled to a Gas Chromatography – Mass Spectrometry (GC/MS) which is a hyphenated technique used to separate volatile compounds in a test sample and analyze unknown components of plant origin, in our case. It primarily provides qualitative information for the identification of volatile organic compounds. The compounds eluted are detected and compared with the components stored in the NIST library for structural confirmation. The fragments are generated in a reproducible way forming a pyrogram. Moreover, it produces a fingerprint which can be specific to a particular sample with regard to the relative distribution of the fragments. Some of the volatile organic compounds that are detected include aldehydes, alcohols, terpenes, and fatty acids (Keheyan & Guilianelli, 2006; Ravikumar *et al.*, 2012).

2.10. Infrared spectroscopy

Another useful tool for the analysis and fingerprinting of organic compounds is infrared spectroscopy. Compounds in a sample, be it gas, liquid, or solid, usually has an infrared absorption spectrum which begins after the visible region at 700 nm and covers from 2,500 to

50,000 nm (Cheng *et al.*, 2010). It occupies three sub-regions, the far-infrared (400–10 cm^{-1}), mid-infrared (4,000–400 cm^{-1}), and near-infrared (13,000–4,000 cm^{-1}) measured in wavenumber (cm^{-1}). The higher regions are linked to stretching vibrations (S-H, C-H, N-H, and O-H) while the lower regions are associated with bending and carbon skeleton fingerprint vibrations (Baker *et al.*, 2014). The individualistic fingerprint of the compound determines the elucidation of molecular structures depending on molecular vibrations. The principle of infrared spectroscopy is to measure the amount of infrared radiation absorbed by a sample at different regions as a function of the wavelength (Bunaciu *et al.*, 2010). This provides a unique fingerprint of a tested compound. Over time, it has shown great reproducibility and provides high resolution over a wide spectral range. Specifically speaking, FTIR (Fourier Transform Infrared spectroscopy) is used to analyze organic compounds by absorbing infrared photons which leads to the excitement of molecular bonds causing different types of vibrations patterns. The bands produced as used as a fingerprint for the identification of molecular structures (Cheng *et al.*, 2010).

CHAPTER 3

3. MATERIALS AND METHODS

3.1. Materials

The rocket plants (*E. sativa*) were cultivated in Kuwait (Wafra farms), and randomly harvested after 26 days under usual production practices. After harvesting, rocket plants without roots were washed with water, sorted, bunched, and transferred to the laboratory within 2 h. Samples were stored at 4 ˚C, and processed within 18 h (Ahmed *et al.*, 2013).

The DPPH (2,2-diphenyl-1-picrylhydrazyl) reagent, Folin & Ciocalteu's (F-C) phenol reagent (500 mL), sodium carbonate (anhydrous), ~98% (+)-catechin hydrate quercetin, 1,2-benzenedithiol, diphenylamine as an internal standard (I.S.), thioglucosidase (myrosinase, EC 3.2.3.1 from Sinapis alba), buffer solutions HPCE pH 8.5 (20 mM sodium phosphate) and pH 6.5 (20 mM sodium phosphate) was obtained from Sigma Chemicals Co. (St. Louis, MO). Anhydrous sodium sulfate was purchased from Fisher Chemicals Co. (Waltham, MA). All other chemicals were of analytical-reagent grade without further purification.

3.1.1. Preparation of lyophilized rocket powder

Rocket leaves (~10 kg) with stems were soaked in 4 L of water at 85°C for 3 min as a blanching step and cooled to room temperature (25°C) by submerging them into iced water. The rocket leaves with stems were separated and allowed to dry. The rocket leaves and stems were frozen at -30 ˚C in a freezer, and later transferred to the freeze-drier (GAMMA 2-16 LSC; Martin Christ GmbH, Osterode am Harz, Germany) for 38 h at a temperature between -47 ˚C and -50 ˚C, and a pressure of 0.7 Pa. The lyophilized samples were grinded into a powder and stored in air-tight containers at 4°C for further analysis (Ahmed *et al.*, 2013)

3.1.2. Preparation of tray-dried rocket powder

Rocket leaves (~2 kg) were dried uniformly on stainless steel perforated trays in a cabinet dryer at 55°C for 6-7 h. The samples were then grinded and stored in air-tight containers at 4°C for further analysis.

3.2. Total flavonoid analysis

3.2.1. Sample extraction

A total of 500 mg of lyophilized and tray-dried rocket powder (triplicates) was placed in separate glass tubes and 80% aqueous methanol (5 mL) was added to each tube, sonicated for 30 min (Pranson 2510 Sonicator, Branson Ultrasonic Corp., Danbury, CT) and then centrifuged for 10 min. The supernatant was recovered and the residue was extracted with 80% aqueous methanol (5 mL) twice for 30 min and centrifuged for 10 min then filtered using grade 1 Whatman filter paper. The final volume was adjusted to 10 mL with methanol for a final concentration of 5 mg/mL. The blank was reacted and measured and quercetin was used as the standard to produce a calibration curve (250, 100, 50, and 25 μg/mL).

3.2.2. Standard preparation

Quercetin was used as the chemical standard for this assay which was dissolved in methanol, starting with 250 μg/mL and then diluted to 100, 50, and 25 μg/mL. One milliliter of standard solution was measured using the spectrophotometer Evolution 300 BB UV-Vis Spectrophotometer (Thermo Electron Corporation, Cambridge, UK) and the calibration curve was obtained. The analysis was carried out in duplicates and the absorbance was measured at 415 nm. The reaction mixture with methanol in place of the sample was used as the blank control. The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank.

A sample extract of 500 μL was mixed with 1.5 mL of 95% ethanol. The mixture was added to 10% (w/v) aqueous aluminum chloride solution (0.1 mL) and 0.1 mL of 1 M aqueous potassium acetate solution. Distilled water was added to a volume of 5 mL, and the mixture was allowed to stand at room temperature for 30 min. A 1 mL of the mixture was transferred into a quartz cuvette and the absorbance was determined at 415 nm against a prepared reagent blank (Chang *et al.*, 2002).

3.3. DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical assay for antioxidant activity

3.3.1. Reagent preparation

The free radical DPPH• was used to evaluate the free radical scavenging activity by measuring the difference in absorbance at 515 nm after 30 min at room temperature. DPPH• reagent standard solution was prepared by dissolving 0.0039 g of DPPH• was in 100 mL of >99.6% methanol to generate a 0.1 mM DPPH• stock solution which was prepared fresh daily. The DPPH• solution was tightly sealed and kept in the dark to prevent it from air and light.

3.3.2. Sample preparation

Five hundred milligrams of lyophilized and tray-dried rocket powder was measured and placed in separate glass tubes containing 10 mL >99.6% methanol. Samples were mixed for 2-3 min and sonicated for 3 hr, then filtered using grade 1 Whatman filter paper to achieve a clear sample extract which was stored for further analysis.

3.3.3. DPPH• Assay

A Varian Cary 50 Bio Spectrometer system (Agilent, Santa Clara, CA) with VisionPro software was used to obtain all the data in this research. Methanol was used as a background and zeroed before starting. A volume of 980 μL of 0.1 mM DPPH was pipetted into a quartz cuvette, and the absorbance at 515 nm was recorded to determine starting concentration. Twenty microliters of antioxidant solution or lyophilized or tray dried sample was added to achieve a final volume of 1 mL and mixed rapidly. The recording of absorbance was initiated immediately and the rate of absorbance was kept and recorded for 30 min. The rate of absorbance was recorded every 10 s for 30 min to generate reaction curves (Llorach *et al.*, 2004). The antioxidant activity was calculated as:

$$\text{AOA (\%)} = [(\Delta A_{515nm} \text{ of control} - \Delta A_{515nm} \text{ of sample})/\Delta A_{515nm} \text{ of control}] \times 100\%$$

3.4. Folin-Ciocalteu micro-method

3.4.1. Standard preparation

In a 100 mL volumetric flask, 100 mg of quercetin was dissolved with >99.6% methanol. Then 4 mL of the above stock solution were diluted to 10 mL with distilled water to obtain a 40% quercetin concentration in methanol. A 100 mg/L quercetin solution was freshly prepared from the 40% methanol concentration by diluting it with methanol. Then, accurate serial dilutions of quercetin (80, 60, 40, 20 mg/L and 0-blank) were prepared to construct a calibration curve.

3.4.2. Sample preparation

A total of 0.005g of lyophilized and tray-dried powder was extracted with 1 mL 80% methanol separately at 35°C for 3 h. The samples were cooled down and filtered using grade 1 Whatman filter paper.

3.4.3. Analytical procedure for the F-C micro-method

One hundred microliters of sample, calibration solutions or blank were pipetted into separate test tubes and F–C reagent (100 µL) was added to each. The mixture was mixed well and allowed to equilibrate. After exactly 2 min, 800 µL of a 5% (w/v) sodium carbonate solution was added. The mixture was swirled and put in a temperature bath (Thermolyne Dri-bath, Johns Scientific Inc., Dubuque, IA) at 40°C for 20 min. Then, the tubes were rapidly cooled on ice and the color generated was read at its maximum absorption, i.e.745 nm in the case of quercetin. Absorbance was measured in a cuvette by a spectrophotometer Evolution 300 BB UV-Vis Spectrophotometer (Thermo Electron Corporation, Cambridge, UK). One mL of methanol was used as a background and samples were analyzed in triplicates (Cicco *et al.*, 2009).

3.5. Gas Chromatography-Mass Spectrometric (GC-MS) determination of total isothiocyanates using cyclocondensation reaction

3.5.1. Instrumentation

A Varian CP-3800 gas chromatograph coupled to a Saturn 2000 ion trap detector was used for the GC/MS analysis. The separation was performed using a fused silica DB-5MS column (50 m length × 0.2 mm i.d. × 33 μm film thickness).

The GC method used for the analysis of the volatiles was as follows: Helium was used as the carrier gas at flow rate of 1 mL/min. The column temperature was set at 50°C for 3 min increasing to 270°C in increments of 10°C/min and kept at 270°C for 5 min. The injection volume was 1 μL and all the injections were performed in a splitless mode. The samples were detected by using an ion trap mass spectrometer. Electron impact mass spectra were recorded at 70 eV. The injector temperature was set at 250°C and the ions monitored were *m*/*z* 140 and 184 for 1, 3-benzodithiole-2-thione (BDT) and 169 for diphenylamine, respectively. The generated data were analyzed using the AMDIS_32 version 2.69 computer software, and peak identification was done using the NIST version 2.0 mass spectral research program. A Digital Heating Shaking Drybath S08040 (Thermo Scientific™, Marietta, OH) was used in all incubations.

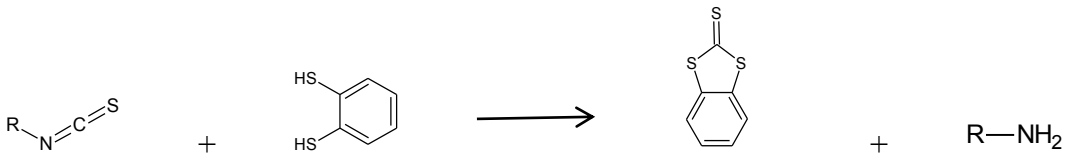
3.5.2. Sample preparation

A total of 0.5g of lyophilized and tray-dried rocket was weighed and placed in a test tube with 10 mL 80% methanol separately. The mixtures were mixed and sonicated separately for 3 h at 35°C and filtered through a grade 1 Whatman filter paper to obtain a clear sample extract solution.

3.5.3. Cyclisation reaction of erucin with 1,2-benzenedithiol

A 0.1 mL aliquot of 15.5μM Erucin in methanol was mixed with 0.1 mL of 10 mM 1,2-benzenedithiol in methanol. A defined amount of 500 μL of 0.07M phosphate buffer solution (0.07M / pH 8.5) was added and mixed thoroughly by vortex (Fisher, Scientific Industries Inc., Bohemia, NY). The mixture was incubated at 65°C in a water-bath shaker for 2 h. Then a 0.5 mL aliquot of $CHCl_3$ was added and the solution was centrifuged for 10 min. The organic layer was pipetted out and passed through anhydrous Na_2SO_4 into a 4 mL vial. The $CHCl_3$ extraction was repeated twice. A 1 μL aliquot of the solution was then injected into the GC-MS under the full

scan mode detection. The cyclocondensation reaction between isothiocyanates and 1,2-benzenedithiol to form 1,3-benzodithiole-2-thione (BDT) is as follows:



ITC 1,2-benzenedithiol BDT

3.5.4. Standard preparation

A standard curve was obtained by dissolving different concentrations of Erucin in $CHCl_3$ starting with 15.5 µM and diluting it to 11.625, 9.3, and 7.75 µM.

3.5.5. GC-MS assay of total isothiocyanates in samples

A 0.125 mL aliquot of sample extract solution was mixed with 0.125 mL of myrosinase solution (10 mg/mL in 20 mM phosphate buffer, pH 6.5) in a capped 2 mL glass tube. The mixture was incubated for 2 h at 37 ◦C in a dry-bath shaker. Then it was cooled to room temperature. Five hundred microliters of 10 mM 1,2-benzenedithiol in methanol and 0.5 mL of 20 mM phosphate buffer (pH 8.5) were added and mixed thoroughly by vortex. The mixture was incubated at 65°C in the dry-bath shaker for 2 h. 0.5 mL of $CHCl_3$ was added and the solution was centrifuged for 10 min. The organic layer was pipetted out and passed through anhydrous Na_2SO_4 into a 4 mL vial. The $CHCl_3$ extraction was repeated twice. The combined organic extract was concentrated under a stream of argon gas. Ten microliters was added to the dried extract and 1µL aliquot of the solution was then injected into the GC-MS (Choi *et al.*, 2004). (see Figure 3.1).

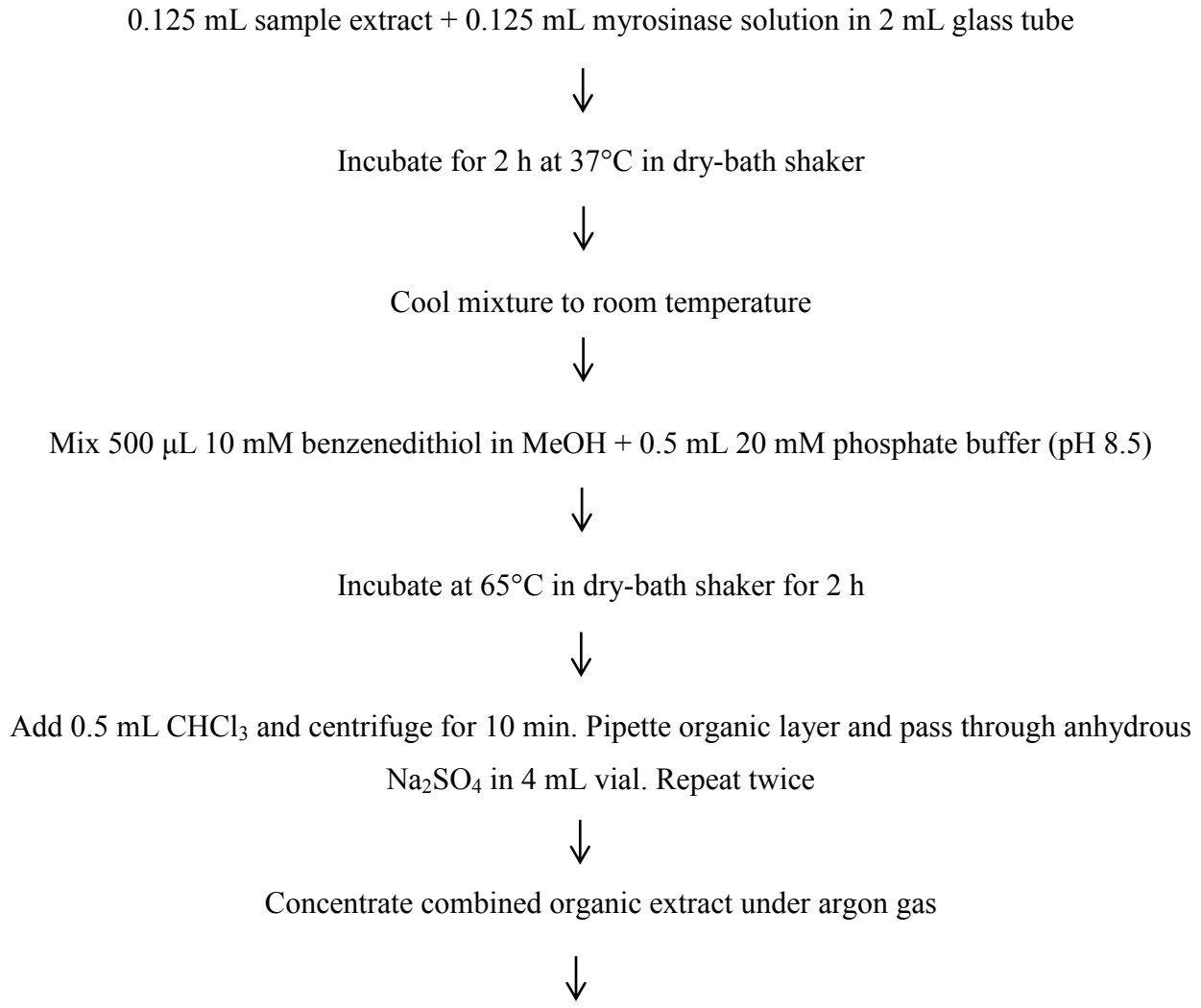


Figure 3.1. Cyclisation reaction of isothiocyanates in lyophilized and tray-dried rocket with 1,2-benzenedithiol.

3.6. Pyrolysis−Gas Chromatography/Mass Spectrometry (Py−GC/MS)

A Varian CP-3800 gas chromatograph coupled to a Saturn 2000 ion-trap detector interfaced to a CDS Pyroprobe 2000 unit through a valved interface (CDS 1500) was used for the desorption of the volatiles from the dried residues. The lyophilized and tray-dried samples were weighed (1.5 mg) into quartz tubes of 0.3 mm thickness sealed at both ends with glass wool (Supelco, Bellefonte, PA, USA) inserted inside the coil probe and pyrolyzed at 250°C for 20 s under helium.

The separation was performed using a fused silica DB-5MS column (50 m length × 0.2 mm i.d. × 33 μm film thickness). The GC method used for the analysis of the volatiles was as follows: GC column flow rate was regulated by an electronic flow controller (EFC) and set at a pressure pulse of 55 psi for first 3 min and then decreased to 32 psi at the rate of 400 psi/min and finally increased to 70 psi at a rate of 1.23 psi/min for the rest of the run. The GC oven temperature was set at −5°C for the first 5 min using CO_2 as the cryogenic cooling source and then increased to 50°C at a rate of 50°C/min. Then, the oven temperature was again increased to 270°C at a rate of 8°C/min and kept at 270°C for 8 min. The samples were detected by using an ion trap mass spectrometer. The MS transfer line temperature was set at 250°C, manifold temperature was set at 50 °C, and ion trap temperature was set at 175°C. The ionization voltage of 70 eV was used, and EMV was set at 1600 V. The data was analyzed using the AMDIS_32 version 2.69 computer software, and peak identification was done using the NIST version 2.0 mass spectral research program.

3.7. Liquid chromatography and electrospray ionization/time-of-flight mass spectrometric (LC-ESI-qTOF-MS) Analysis

The lyophilized and tray-dried rocket samples were dissolved in methanol at a concentration of 10 mg/mL. The sample was then diluted 10-fold in 10% methanol prior to analysis by qTOF/ESI/MS. The LC-electrospray ionization (ESI)-MS analysis consisted of a Dionex Ultimate 3000 RS liquid chromatograph (Dionex, Germering, Germany) coupled to a Bruker Maxis Impact quadruple-time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany) operated in a negative ion mode. A sample of 1 μL was injected into the LC-MS. The electrospray interface settings were the following: nebulizer pressure, 1.0 bar; drying gas, 4 L/min at a temperature of 180°C and capillary voltage of 4500 V. The scan range was from 100 to 1000 m/z. The data was analyzed using Bruker Compass Data Analysis software version 4.1.

3.7.1. Structural Identification

The products were tentatively identified using elemental formulas derived from high resolution ESI/MS data.

3.8. Infrared (FTIR-ATR) spectroscopy

The lyophilized and tray-dried rocket powder was subjected to IR analysis. FTIR spectra were recorded on a Bruker Alpha-P FTIR spectrometer (Bruker Optic GmbH, Ettlingen, Germany) equipped with a deuterated triglycine sulfate (DTGS) detector, a temperature-controlled single-bounce diamond attenuated total reflectance (ATR) crystal, and a pressure application device for solid samples. A total of 32 scans at 4 cm^{-1} resolution were co-added at a scan range of 4000 - 375 cm^{-1}. Processing of the FTIR data was performed using Bruker OPUS software.

3.9. SEM observation for average particle size and tissue structure

The lyophilized and tray-dried rocket particles were examined through a scanning electron microscope (SEM) (JEOL, JSM-5410LV, Tokyo, Japan) for both shape and size. Firstly, each sample was coated with titanium using a sputter coater (Structure Probe, West Chester, PA) before scanning and was photographed at two different magnifications (250× and 4,500×). Then the particle size was measured by the software and the average particle dimensions were calculated. About 32 particles were chosen randomly for the particle size measurement.

3.10. Statistical analysis

All results were presented as means (± standard deviation) of triplicate determinations. Student's t test was used to analyze the data for significance. A value of ($P < 0.05$) was considered as significant. All statistical analyses were done using Excel 2010.

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1. Standard quercetin calibration curve

Figure 4.1 shows the calibration curve for the standard quercetin at 4 different concentrations. The calibration curve was linear over the analyzed concentrations ranges as shown in the equation below:

$$y = 0.0041x + 0.0722$$

$$R^2 = 0.9999$$

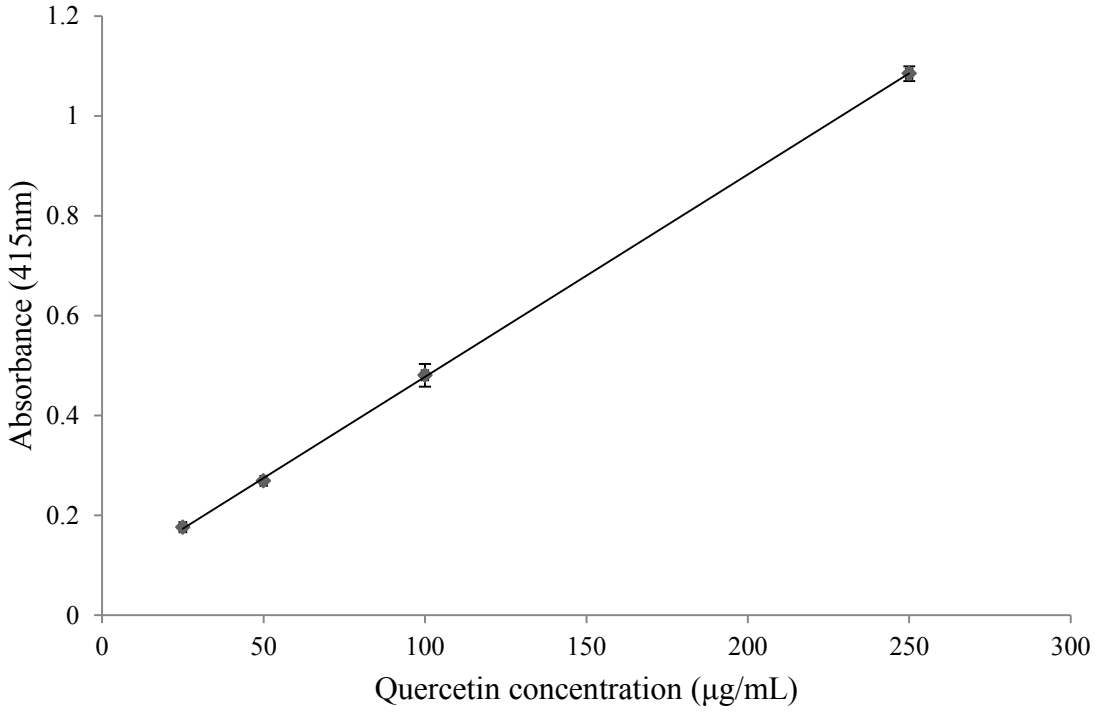


Figure 4.1. Quercetin standard curve using potassium acetate assay.

Table 4.1. Total flavonoid content (TFC), total phenolic content (TPC), total isothiocyanate content (TIC), and antioxidant activity (AOA) of lyophilized and tray-dried *E.sativa* (dry weight)[a]

Drying method	TFC (g QE/100 g)[b]	TPC (g QE/100g)[b]	AOA (%)[c]	TIC (µg /g)
Lyophilized	3.29 ± 0.15a	8.67 ± 0.6a	28.01 ± 0.6a	3.26 ± 0.59a
Tray-dried	2.42 ± 0.22b	8.5 ± 0.8a	27.96 ± 1.0a	6.05 ± 0.83a

[a] Values of TFC, TPC, and AOA of leaves are means ± SD (n=3). For each column values followed by the same letter (a-b) are not statistically different at $P < 0.05$ as measured by student's t test.
[b] Values of TFC and TPC are measured as quercetin equivalence.
[c] Antioxidant activity is measured as average scavenging activity of DPPH.

4.2. Total flavonoid analysis of lyophilized and tray-dried rocket

Total flavonoid results for lyophilized and tray-dried rocket are shown in (Table 4.1). The results show a significantly higher concentration of total flavonoids in lyophilized rocket than in tray-dried rocket. Lyophilized rocket displayed the average total flavonoids of 3.29 ± 0.15 g/100g dry weight (328.6 mg/100g fresh weight) while tray-dried rocket had an average of 2.42 ± 0.22 g/100g dry weight (242.2 mg/100g fresh weight). The difference between the two samples was found to be statistically significant at $p<0.01$. The results of both dried rocket was comparable with the total flavonoids measured in 32 *Eruca* accessions with a difference ranging from 999 to 3139 mg/100g dry weight and an average of 2353 mg/100g dry weight (Pasini *et al.*, 2012). The lyophilized sample was found to be slightly higher than the range while the tray-dried rocket was close to the average total flavonoid content of the 32 *Eruca* accessions. In addition, kaempferol derivatives were the main flavonols detected in the latter study. Moreover, the total flavonoid content for both dried samples was found to be higher than fresh wild rocket which was calculated to be 105 mg/100g fresh weight (Martínez-Sánchez *et al.*, 2005). However, the results range was not in agreement with Arabbi *et al.* (2004) who reported total flavonoids of rocket to be 41-118 mg/100g fresh weight. In fresh young *Eruca sativa* leaves, a lower total flavonoid content was calculated (250-500 µg flavonoid/g fresh weight); kaempferol being the core aglycone (Bennett *et al.*, 2006).

Additionally, rocket was one of the plants to contain the highest concentration of flavonoids among ten other vegetables tested (Arabbi *et al.*, 2004). Variant concentrations of flavonoids in fresh *E.sativa* have been published since flavonoids are affected by light, especially UV light, soil and growing conditions, as well as seasonal variation (Bennett *et al.*, 2006). For example, rocket presented lower amounts of flavonoids (41 mg/100g fresh weight) in the first semester of 2002 than the second semester of 2001 (118 mg/100g fresh weight). In leaves with sharp taste such as chicory and rocket, the flavonol kaempferol was detected in high amounts, 4-11 mg/100 g (fresh weight) and 41 and 104 mg/100 g (fresh weight), respectively. Around 30% of the flavonoid intake in São Paulo, Brazil originated from rocket (Arabbi *et al.*, 2004).

In wild rocket (*D.tenuifolia*), quercetin glycosides were the dominant type of flavonoids present, where the total concentration was around 105 mg/100g (fresh weight). Similar concentrations of phenolic compounds and flavonoids found in wild rocket were found in *E.sativa,* cabbage, and spinach (Martínez-Sánchez *et al.*, 2005).

4.2.1. Effect of drying methods on total flavonoid analysis

Most flavonoids are stable and differ in their thermostability and thus plants can be dried thermally or non-thermally. In a previous study, the destruction of flavonoids varied with different drying treatments where flavonoid loss was lower in freeze dried samples than in tray dried samples. (Cannac *et al.*, 2007; Zainol *et al.*, 2009). Also, lyophilization leads to high extraction efficiency since ice crystals formed in the plant matrix can rupture cell structure causing the release of cellular components. This allows better solvent accessibility and extraction (Chan *et al.*, 2009). So, lyophilization is the best for the preservation of flavonoids regardless of the species-specific differences associated with it (Julkunen-Tiitto *et al.*, 2015).

Furthermore, freezing of onions resulted in lower levels of flavonols while lyophilization increased the amount of flavonols due to the release of phenolic compounds from the matrix from lyophilization (Shofian *et al.*, 2011). However, it was shown that a high concentration of quercetin and myricetin glycosides was achieved at a temperature of 80°C rather than 40°C in *B. pendula* leaves (Julkunen-Tiitto *et al.*, 2015).

4.3. Standard quercetin calibration curve

Figure 4.2 shows the calibration curve for the standard quercetin at 5 different concentrations. The calibration curve was linear over the analyzed concentrations ranges as shown in the equation below:

$$y = 0.0101x + 0.1291$$

$$R^2 = 0.994$$

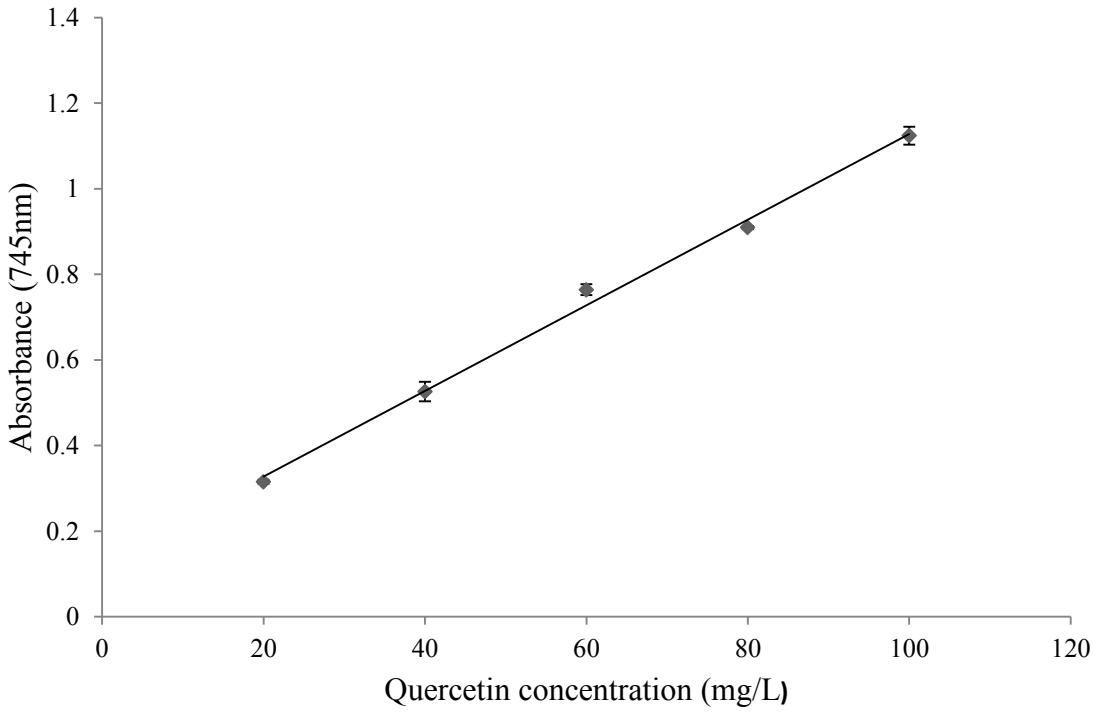


Figure 4.2. Quercetin standard curve using Folin-Ciocalteu micro-method.

4.4. Total phenolics analysis of lyophilized and tray-dried rocket

The total phenolic content results for lyophilized and tray-dried rocket are shown in Table 4.1. The results display comparable concentrations of total phenolic compounds in both lyophilized and tray-dried rocket samples. The lyophilized rocket displayed the average total phenolic content of 8.67 g/100g dry weight (867.0 mg/100g fresh weight) while tray-dried rocket had an average of 8.5 g/100g of dry weight (849.9 mg/100g fresh weight) as quercetin equivalence in which differences were insignificant. Pasini *et al.* (2012) calculated the phenolic contents of various *Eruca* accessions in a dry weight basis which resulted in a variable but moderately lower concentrations of phenolic compounds ranging from 9.99 to 31.39 g/kg DW as rutin equivalents using HPLC-DAD-MS. The total polyphenol content of baby rocket leaves measured in fresh weight basis was also lower; 139.1 ± 11.5 and 132.3 ± 17.1 mg/100g for wild rocket and salad rocket, respectively (Martínez-Sánchez *et al.*, 2008). In contrast, in salad rocket, 208.11 mg gallic acid/100g (fresh weight) and 100.08 mg gallic acid/100g (fresh weight) in wild rocket was detected by Heimler *et al.* (2007), which is almost twice the amount of the salad rocket calculated in the previous study using the same method of analysis. In brief, the phenolic compounds measured in this study using different drying methods and different growing conditions appeared to be higher in comparison to fresh and dried rocket in all the previous studies.

The total phenol assay using the Folin-Ciocalteau method is a simple and reproducible method used routinely by other researchers (Huang *et al.*, 2005). Polyphenolic content, like flavonoids, is affected by genetic and environmental factors as well as the maturity of the leaves and the growing period. The inconsistency in the concentrations may be linked to the different extraction conditions and type of analysis used since this study presents results as dry weight compared to fresh weight of other studies (Heimler *et al.,* 2007). Also, standards of sinapic acid, gallic acid, and rutin were used for quantification in other studies in comparison with the standard (quercetin) used in this experiment (Martínez-Sánchez *et al.*, 2008). Quercetin was used as a standard because according to already published data, the three dominant flavonols that reside in salad rocket are kaempferol, quercetin, and isorhamnetin. Furthermore, quercetin displayed more reproducible and consistent results than kaempferol as a standard. In wild rocket, 94% of the total phenolic content comprise of quercetin, while kaempferol make up 78.8% of the phenolic content of salad rocket (Martínez-Sánchez *et al.*, 2008).

4.4.1. Effect of drying methods on total phenolics content

When comparing fresh and lyophilized fruits, the total polyphenolic content was found to be significantly different. Some authors found that total flavonoid content increased after lyophilization. Flavonols in onions increased when the samples were subjected to lyophilization, explained by the possible release of phenolic compounds during the process of lyophilization (Vuthijumnok *et al.*, 2013). Authors also underline the importance of oven-drying leaves in reducing the enzymatic decomposition that happens when samples are homogenized prior to drying in other methods (Cannac *et al.*, 2007).

There are conflicting reports on the effect of lyophilization on phenolic content which seems to vary according to the plant species. Lyophilized leaves of ginger were shown to have higher values of phenolic compounds than fresh leaves by 26%. Also, minor compounds appeared in the chromatograms of lyophilized leaves than in fresh leaves causing an increase of 32% in total phenolic content than 26% (Chan *et al.*, 2009).

4.5. Antioxidant activity of lyophilized and tray-dried rocket

The antioxidant activity of lyophilized and tray-dried rocket is shown in Table 4.1. Both dried samples exhibited a similar decrease in the DPPH• at a concentration of 25 mg/ml (dry weight). The lyophilized rocket displayed a 28% scavenging activity while the tray-dried rocket showed a 27.96% scavenging activity. The average scavenging activity of both fresh wild rocket and rocket was approximately 20 to 35% at ~7 mg/ml, according to Heimler *et al.* (2007). In other previous work, rocket showed the least antioxidant activity (120 mg/100g FW) when compared with watercress and mizuna leaves using the DPPH assay (Martínez-Sánchez *et al.*, 2008). Furthermore, rocket comprised the least antioxidant activity among ten other freshly consumed salad species. However, Martínez-Sánchez *et al.* (2005) tested the antioxidant activity in fresh wild rocket leaves which resulted in 60.8% scavenging activity.

The inconsistency in the results of a sensitive test such as antioxidant activity depends on different factors among them are plant growth stage and harvesting time that can affect the levels of antioxidant compounds of these plants and their contribution to the diet as well as the post-harvest methods used. Also, there was a poor correlation between the antioxidant activity and phenolics content in rocket, indicating that the mechanisms and the compounds involved in the radical activity depend on the varying plant species (Martínez-Sánchez *et al.*, 2008; Martínez-Sánchez *et al.*, 2005).

Earlier work displayed high concentrations of ascorbic acid in rocket salad (Degl'Innocenti *et al.*, 2007). This is in agreement with Martínez-Sánchez *et al.* (2008) in which rocket was found to contain a high content of vitamin C in comparison with other leaves of *Brassicaceae* species. However, there was an absence of correlation between antioxidant activity and phenolic content explained by the presence of interferences such as ascorbic acid, sugars, and proteins in the food matrix (Apak *et al.*, 2015; Degl'innoocenti *et al.*, 2007; Heimler *et al.*, 2007). Yet, another study suggests that glucosinolates content may also play a role in the antioxidant activity along with the phytochemicals in the plant (Kim *et al.*, 2006).

Furthermore, glucose substitution at 3' or 4' positions of the hydroxyl groups have been shown to reduce the antioxidant power of wild rocket flavonoids according to Martínez-Sánchez *et al.* (2005). Therefore, the antioxidant ability of rocket is not attributed to phenolics and flavonoids alone, but to other non-phenolic compounds that work synergistically to contribute to the antioxidant activity including vitamins, fibers, and other phytonutrients (Llorach *et al.*,

2004). However, the high kaempferol content that is present in great amounts primarily in rocket leaves contributed to the antioxidant power of rocket (Degl'innoocenti *et al.*, 2007). To reduce the DPPH radical by 50%, kaempferol requires 6 min while myricetin requires around 66 min. However, the kaempferol concentration needed to reduce 50% of the DPPH radical (EC_{50}) was higher than most compounds and was not considered significant, yet it showed the highest the antioxidant efficacy of all phenolic compounds tested because of its fast rate of reaction, which is biologically significant (Sharma & Bhat, 2009; Villaño *et al.*, 2007).

On the other hand, it was observed that quercetin in an 80% aqueous methanol solution required 300 milliseconds to react with the DPPH radical, measured using a stopped-flow spectrophotometer. The compounds that reduce the DPPH radical in less than 30 s act by electron transfer (ET) reactions while the compounds that require longer times to reduce the DPPH radical act by hydrogen atom transfer (HAT). Compounds such as ascorbic acid and kaempferol were also shown to exhibit a fast reaction with the DPPH radical indicating an ET reaction, although other flavonols showed differing rates of reaction (Sharma *et al.*, 2009; Villaño *et al.*, 2007).

The main characteristic that distinguishes the antioxidant activity is the quenching rate and steric accessibility. The kinetic parameter which includes the rate at which the antioxidant quenches peroxyl radicals for example, is what determines whether a compound is a good antioxidant or not, not the concentration. Other authors agree with the fact that the initial reaction is the only relevant period for reactivity comparison. Some studies, however, rely on concentration such as EC_{50} (half maximal effective concentration) or the antioxidant concentration required to reduce the DPPH radical by 50%. The EC_{50} is time-dependent and when time is increased, it results in improved compound activity; i.e. lower EC_{50}, thus falsely proving the antioxidant capacity of the compound. This reflects stoichiometry or capacity ignoring the important kinetic information; i.e. how much rather than how fast, thus providing incorrect assessment of antioxidant activity. If an antioxidant requires a long time to quench radicals, then the scavenging activity is irrelevant *in vivo* in cells or in foods. The DPPH radical is stable and remains stable in a solution, while the radicals that are active in foods, being hydroxyl radicals (HO•), superoxide anion (O2 −•), nitric oxide (NO•), lipid peroxyl (LOO•) alkoxyl (LO•) radicals for example, have short lifetimes, measured in milliseconds. So, the time it takes for the reactive group in food to quench the radicals is much more significant than the

number of reactive groups present. The use of concentration (EC_{50}) alone will provide inaccurate information about antioxidant reaction with the DPPH when comparing antioxidants from different structural classes (Foti, 2015; Xie & Schaich, 2014).

The high concentration of total flavonoids measured in the previous test does not seem to contribute to the total antioxidant activity measured by the DPPH assay as seen in Table 4.1. This can be explained by the structure of the flavonoids measured as well as the primary antioxidant mechanism used. If there was a diminishing steric accessibility to the DPPH radical site, it is due to ring adducts and multiple phenolic rings attached to the flavonoids, which is the case of a concentrated plant sample such as lyophilized or tray-dried rocket. At times, when a high concentration of antioxidant is added, it results in crowding around the DPPH radical, which in turn obstructs access to the radical site of DPPH and reduce reactivity. However, small monophenols have easy access to the radical site which allow for the completion of reaction within seconds. (Martínez-Sánchez *et al.*, 2005; Xie *et al.*, 2014) Furthermore, polyphenols are known to exhibit higher antioxidant activity than monophenols but due to steric hindrance, the apparent antioxidant activity is not displayed. Also, the reaction between phenols and DPPH is always less than the total phenolic compounds also due to steric impedance factors that affect the reaction (Brand-Williams *et al.*, 1995; Prior *et al.*, 2005; Schaich & X. Tian, 2015).

4.5.1. Effect of drying methods on antioxidant activity

Different processing methods can improve, decrease, or show no change in antioxidant properties. Most losses in antioxidant activity were reported in vegetables as a result of thermal degradation of phenolic compounds, degradative enzymes, and loss of antioxidant enzyme activities leading to loss of other bioactive properties (Chan *et al.*, 2009). Lyophilization, however was shown to retain ascorbic acid in different fruits depending on the nature of the food. Also, as carotenoids are retained in lipid membranes or in plasma vacuoles, lyophilization may be responsible for the reduction of the concentration of beta carotene in fresh fruits. In two types of fruits, the fresh samples contained relatively higher antioxidant activity using the DPPH assay than lyophilized samples (Shofian *et al.*, 2011).

4.6. Standard erucin calibration curve

Figure 4.2 shows the calibration curve for the standard erucin at 4 different concentrations. The calibration curve was linear over the analyzed concentrations ranges as shown in the equation below:

$$y = 18643x - 54592$$

$$R^2 = 0.9947$$

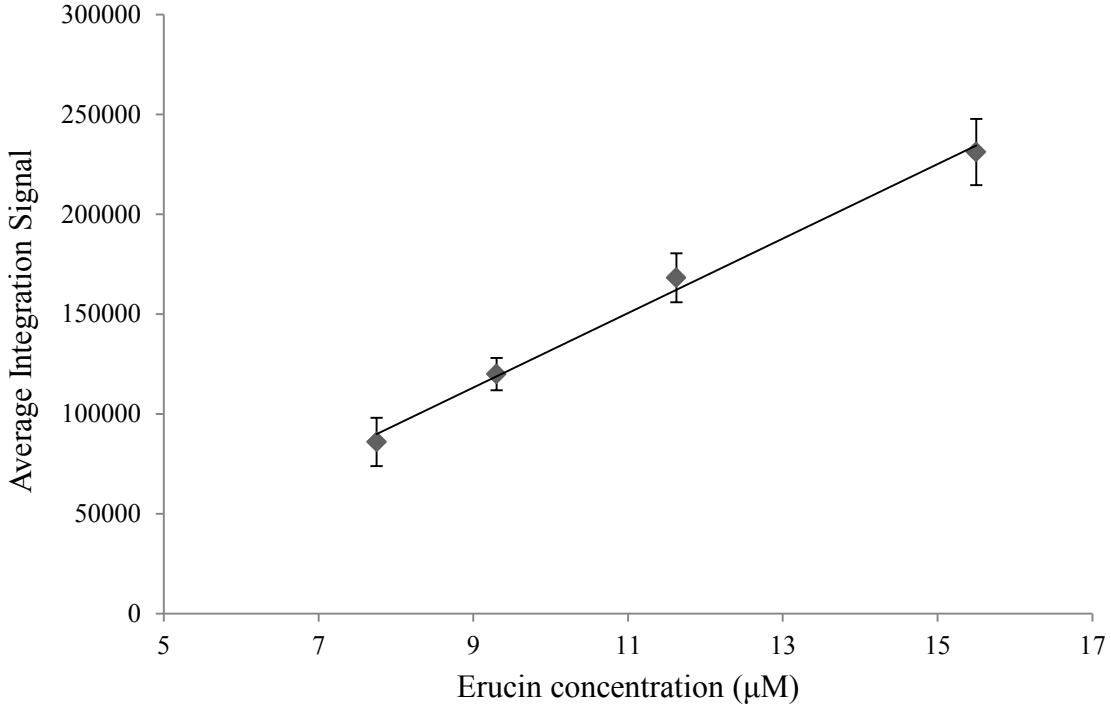


Figure 4.3. Erucin standard curve using cyclocondensation reaction.

4.7. Total isothiocyanate test for lyophilized and tray-dried rocket

The average isothiocyanate concentration of lyophilized and tray-dried rocket is shown in Table 4.1 measured using the cyclocondensation assay. The results show no significant difference between the two samples although the concentration was twofold higher in the heated rocket sample rather than the lyophilized sample, with a concentration of 6.05 ± 0.59 μg/g (dry weight) of Erucin equivalence in oppose to 3.26 ± 0.83 μg/g (dry weight), respectively. However, there was scant information in literature on the total isothiocyanate content in fresh or dried rocket. In one study, *E.sativa* extracts contained sulforaphane at higher levels (0.615 mg/g dry weight) than erucin (8.84 μg/g dry weight) (Melchini *et al.*, 2009). However, in another study, sulforaphane content ranged from 0.14 to 6.30 μmol/g dry weight which was in accordance with the previous study, who reported values of 3.46 μmol of sulforaphane/g dry weight.

Choi *et al.* (2004) measured the total isothiocyanate content of a range of Chinese medicinal herbs one of which was radish seeds (*Raphanus sativus L.*) which contained an average of 20.3 ± 0.73 μmol/g of fresh weight. Mustard seeds were found to contain 670.86 μg/g of total isothiocyanates (Marton *et al.*, 2013). However, in mustard greens, the isothiocyanate content showed a 345-fold difference ranging from 0.4 and 137.9 μmol/100g FW.

4.7.1. Effect of drying methods on total isothiocyanate content

The results obtained from different methods performed in different laboratories indicate the variation in isothiocyanate yield across vegetable type and within each vegetable. This is due to different environmental factors, growing conditions and cultivation practices. Eight raw crucifers were analyzed for their isothiocyanate content measured in wet weight. The average isothiocyanate content was at 16.2 μmol/100g, the lowest being in raw cauliflower (1.5 μmol/100g) and highest in raw mustard greens (61.31 μmol/100g). According to that study, the tray-dried rocket sample contained an average amount of isothiocyanate content when compared with other raw crucifers (Tang *et al.*, 2013).

The conversion of all the isothiocyanates to BDT showed a minimum yield of 90% under experimental conditions, and cyclocondensation products correspond to the total amount of isothiocyanates (expressed as molar concentration). All isothiocyanates except tertiary isothiocyanates such as *tert*-butyl-NCS achieved a complete quantitative cyclocondensation reaction with 1,2-benzenedithiol (Choi *et al.*, 2004). Some studies showed that isothiocyanate-

related compounds such as thiocyanates, cyanates, isocyanates, and cyanide did not interfere with the cyclocondensation reaction (Zhang *et al.*, 1996).

In a study published in 2012, vegetable extracts treated with myrosinase led to a 38-fold increase in isothiocyanate content, proposing that if plant myrosinase is present, it is inadequate for full hydrolysis of glucosinolates. A total of 11 cruciferous vegetables displayed a wide range of total isothiocyanates ranging from 0.8 μmol/g FW in broccoli and 0.05 μmol/g FW in bok choi, where non-cruciferous vegetables were devoid of any detectable isothiocyanates (Zhang, 2012). However in 2013, a study showed conflicting results. It stated that isothiocyanate yield from cruciferous vegetables (broccoli, cauliflower, Brussels sprouts, and cabbage) showed identical figures when measured before and after the sample was treated with exogenous myrosinase. It concluded that the adequate amount of myrosinase present in cruciferous vegetables allow for the complete conversion of glucosinolates to isothiocyanates (Tang *et al.*, 2013).

The different cooking or preparation methods of vegetables, in addition to the cooking temperature and time, can considerably affect the production and release of isothiocyanates from glucosinolates. This is attributable to the inactivation of the enzyme myrosinase and epithiospecifier protein (ESP) as well as the destruction of heat-sensitive isothiocyanates. (Tang *et al.*, 2013). The ESP is a heat-labile, non-catalytic cofactor of myrosinase that has been found in several crucifers. It requires iron for its activity and is responsible for the formation of epithionitriles and nitriles rather than isothiocyanates which has been displayed to be the main isothiocyanate products produced by some crucifers (Matusheski *et al.*, 2004).

The ESP action is favored in vegetables such as broccoli but not in others, as it diverts myrosinase hydrolysis towards nitriles rather than isothiocyanates. It is also specific to certain substrates hydrolyzing aliphatic glucosinolates with a lesser efficiency on aromatic glucosinolates (Tang *et al.*, 2013). However, at 60°C, ESP is destroyed leaving behind myrosinase thus causing a 5 times increase in generation of isothiocyanate (Matusheski *et al.*, 2004; Tang *et al.*, 2013). Isothiocyanates such as sulforaphane was not affected by heat at 60°C while at higher temperatures the content decreased (Totušek *et al.*, 2011). The heated sample in this study was heated at 55°C which may offer an explanation on the higher content of available isothiocyanates measured. Also, air-drying of rocket plants was found to degrade glucosinolates into isothiocyanates (Blaževic *et al.*, 2008).

Mild heating of specific cruciferous plants was identified to be effective in decreasing the formation of nitriles, while favoring the isothiocyanates formation route. At a maximum of 60°C, sulforaphane formation was favored in broccoli, while ESP activity was significantly decreased with heating at 50°C or higher compared to an unheated sample, leading to a decrease in nitrile product formation. Also, this formation of isothiocyanates from the activity of myrosinase is more heat sensitive in broccoli plants than in sprouts (Matusheski *et al.*, 2004).

4.8. Chemical fingerprinting of lyophilized and tray-dried rocket by Pyrolysis-GC/MS

4.8.1. Lyophilized rocket pyrogram analysis

The Py-GC/MS was used to characterize both samples of lyophilized and tray-dried rocket. Figure 4.4 (B) shows the pyrograms and Table 4.3 lists the proposed structures of the peaks observed in the lyophilized rocket sample. The identification of the detected compounds was performed through comparison of their mass spectra with those reported in NIST library. The profile was characterized by the formation of aldehydes; such as 3-methylbutanal, 5-methylfurfural, and cyclopentene and furanone derivatives; such as 2-cyclopenten-1,4-dione, (R)-5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone. Sulfur-containing compounds were also found to be present including dimethyl disulfide and tetrahydrothiophene.

The volatile compound, tetrahydrothiophene, which was previously identified with an allium- and cabbage-like odor, was found in both dried samples and previously detected in the volatiles of *E.sativa* by several authors such as Jirovetz *et al.* (2002), Blaževic *et al.* (2008), and Bell *et al.* (2016). These sulfur compounds along with isothiocyanates may contribute to the characteristics pungent taste of rocket. Pyrrole, a nitrogenous compounds was detected in the lyophilized rocket sample along with ketone derivatives such as 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one, 4-(2,6,6-trimethylcyclohexa-1,3-dienyl)but-3-en-2-one, and 3',5'-dimethoxyacetophenone. Phytol (3,7,11,15-tetramethyl-2-hexadecen-1-ol) which is also found in both dried samples, originates from the breakdown of carotenoids (Blaževic *et al.*, 2008).

4.8.2. Tray-dried pyrogram analysis

A pyrogram obtained from the analysis of tray-dried rocket is shown in Figure 4.4 (A). A total of 12 compounds were identified by comparison to NIST libraries and listed in Table 4.3. The identified compounds comprised several classes including alcohols, alkenes, aldehydes, ketones, nitrogenous and sulfur compounds, and esters; some of which have been reported in other studies (Bell *et al.*, 2016; Blaževic *et al.*, 2008; Jirovetz *et al.*, 2002). Similar to the lyophilized sample, tetrahydrothiophene, 2-cyclopentene-1,4-dione, and 5-methylfurfural were also detected. An aldehyde, furfural, was also present originating from the hydrolysis of plant polysaccharides. Another sulfur compound was present, dimethyl trisulfide, which was previously detected in *E.sativa* plants (Blaževic *et al.*, 2008). Nitrogenous compounds were present such as 1H-pyrrole, 1-ethyl- and 1H-pyrrole, 1-(2-furanylmethyl)- in addition to

pyrazine, 2-ethyl-6-methyl-, the latter being pyrolysis artifacts. Nitrogenous heterocyclic compounds such as pyrrols are mainly produced from the Maillard reaction (Su *et al.*, 2011). Ketones were also part of the volatiles including 4,5-octanedione, 1-(3,4-dimethoxyphenyl)-ethanone. Pyrrolidine-1-dithiocarboxylic acid 2-oxocyclopentyl ester is a sulfur aromatic compound that was formerly detected in rocket volatiles and is present in the tray-dried sample only. Other aliphatic volatile compounds such as dodecanoic acid isooctylester can originate from fatty acid catabolism. Dodecanoic acid was also identified as a major component in the isolated oils from flowers and leaves of *E.sativa*. The compounds with higher alkanes originate from plant waxes which play an important role in protecting the plant from microorganisms as well as protection from plant withering (Blaževic *et al.*, 2008).

The tray-dried sample included several sulfur-containing compounds previously detected in rocket volatile studies in comparison with lyophilized rocket as well as esterified compounds. The rest of the unidentified peaks are column artifacts and minor compounds.

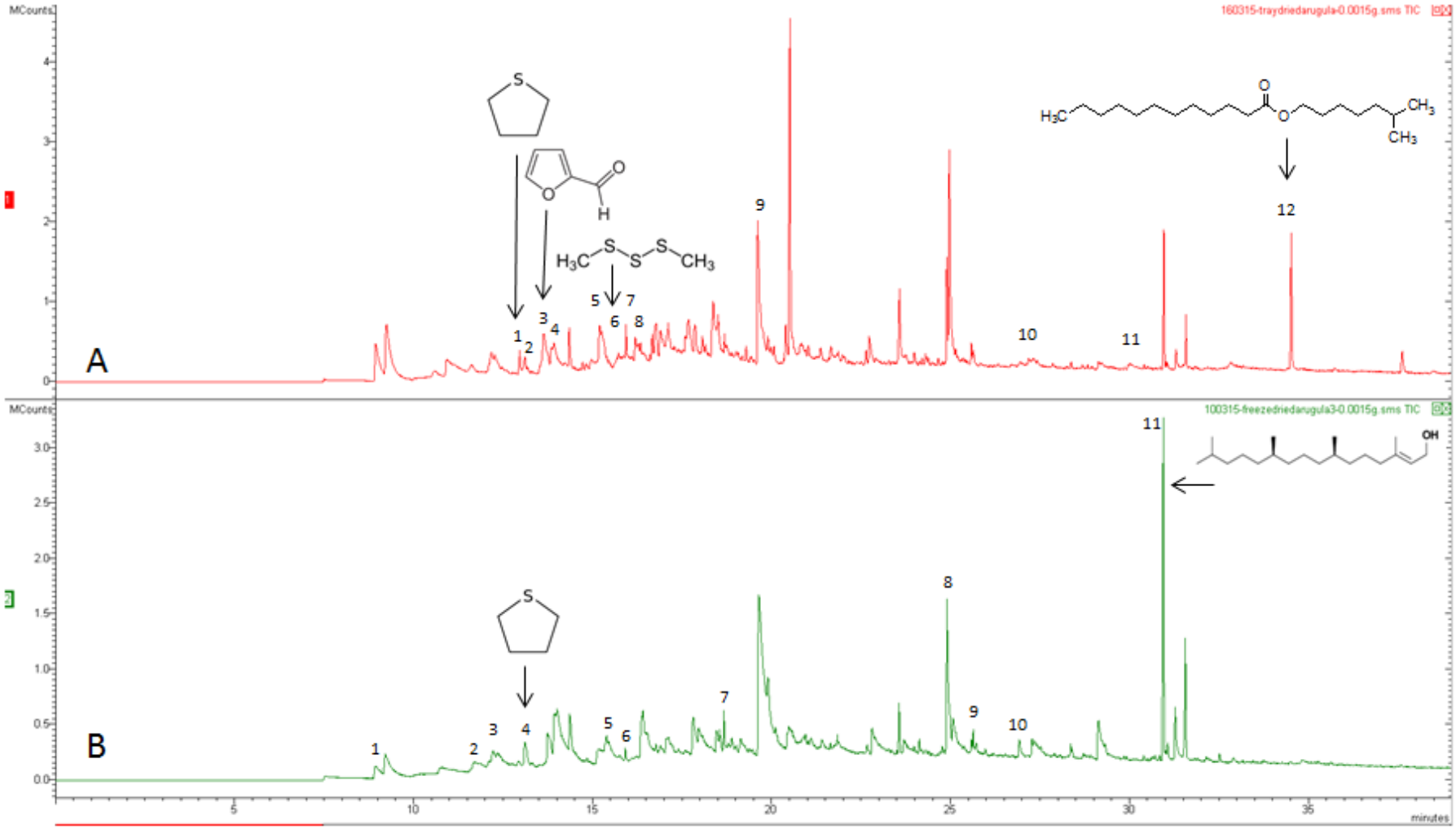


Figure 4.4. Total ion chromatogram obtained from pyrolysis (Py-GC/MS) of tray-dried (A) and lyophilized (B) *E.sativa.*

Table 4.2. Proposed structures of the volatile compounds observed in the lyophilized *E.sativa* pyrogram using GC/MS analysis.

Retention time (min)	Compound	Elemental Composition
8.93	3-Methylbutanal	$C_5H_{10}O$
11.721	Dimethyl disulfide	$C_2H_6S_2$
12.352	Pyrrole	C_4H_5N
13.111	Tetrahydrothiophene	C_4H_8S
15.136	2-Cyclopenten-1,4-dione	C_5H_4O2
16.359	5-Methylfurfural	$C_6H_6O_2$
19.645	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	$C_6H_8O_4$
25.588	4-(2,6,6-Trimethylcyclohexa-1,3-dienyl)but-3-en-2-one	$C_{13}H_{18}O$
26.925	(R)-5,6,7,7a-Tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone	$C_{11}H_{16}O_2$
27.296	3',5'-Dimethoxyacetophenone	$C_{16}H_{12}O_3$
31.282	3,7,11,15-Tetramethyl-2-hexadecen-1-ol (phytol)	$C_{20}H_{40}O$

Table 4.3. Proposed structures of the volatile compounds observed in the tray-dried *E.sativa* pyrogram using GC/MS analysis.

Retention time (min)	Compound	Elemental composition
13.119	Tetrahydrothiophene	C_4H_8S
13.187	1H-Pyrrole, 1-ethyl-	C_6H_9N
13.859	Furfural	$C_5H_4O_2$
14.977	2-Cyclopentene-1,4-dione	$C_5H_4O_2$
16.191	5-Methylfurfural	$C_6H_6O_2$
16.346	Dimethyl trisulfide	$C_2H_6S_3$
16.605	4,5-Octanedione	$C_8H_{14}O_2$
16.671	2-Ethyl-6-methyl-pyrazine[a]	$C_7H_{10}N_2$
20.075	1-(2-Furanylmethyl)-1H-pyrrole	C_9H_9NO
27.196	1-(3,4-Dimethoxyphenyl)-ethanone	$C_{10}H_{12}O_3$
29.134	Pyrrolidine-1-dithiocarboxylic acid 2-oxocyclopentyl ester	$C_{10}H_{15}NOS_2$
34.519	Dodecanoic acid isooctylester	$C_{20}H_{40}O_2$

[a]Pyrolysis artifact

4.9. GC/MS analysis of methanolic extracts of lyophilized and tray-dried rocket

4.9.1. Methanolic extract of tray-dried sample

Figure 4.5 (A) shows the chromatogram of methanolic extract of tray-dried rocket sample. The methanolic extract of the tray-dried rocket sample showed the presence of various functional groups such as aldehydes, alcohols, ketones, and an isothiocyanate that are listed in Table 4.4. Glycerol and phytol are the alcohols retained at the beginning and end of the chromatogram and usually plants use alcohols as a defense mechanism. In other plant species' defensive mechanisms are ruled by the genetic regulation via enzymes, which is expected to be the same for rocket. Alcohols offer a cut-grass aroma that is found in leafy vegetables (Su *et al.*, 2011). Furthermore, 2(5H)-furanone was detected in the tray-dried sample while a similar furanone derivative was found in rocket volatiles; 5-ethyl-2(5H)-furanone (Bell *et al.*, 2016). Other ketones were also detected such as 3,4-hexanedione which is a marker for thermal treatment along with 1-(1H-pyrrol-2-yl)-ethanone, while 2,3-dihydro-3,5-dihydroxy-6 methyl-4H-pyra-4-one was found in both dried samples. Moreover, 5-hydroxymethylfurfural which is derived from the dehydration of certain sugars was present in this sample and not in the lyophilized sample while other furans were detected in fresh samples (Su *et al.*, 2011).

Lastly, 5-methylthiopentanenitrile, which is a sulfur compound, was found in the tray-dried sample but not in the lyophilized sample. It is known that isothiocyanates, nitriles, epithionitriles and thiocyanates are the degradation products that are enzymatically produced in *Brassicaceae* in the glucosinolate-myrosinase reaction (Su *et al.*, 2011). 5-Methylthiopentanenitrile or erucin nitrile is one of the glucoerucin degradation products. Blaževic *et al.* (2008) concluded that air-drying of the rocket plant contributes to the degradation of glucoerucin, therefore producing 5-methylthiopentanenitrile. This compound was identified in oils from dried plant material of flowers and leaves of *E.sativa* along with other isothiocyanates. According to Jirovetz *et al.* (2002), 5-methylthiopentanenitrile was found to make up 5% of the aroma products produced from *E.sativa* salad from Austria. Both 5-methylthiopentanenitrile and tetrahydrothiophene, along with other sulfur compounds which were not detected, contribute to the *Brassicaceae* aroma.

4.9.2. Methanolic extract of lyophilized sample

Figure 4.5 (B) shows the chromatogram of the methanolic extract of lyophilized rocket sample. There were fewer compounds identified in lyophilized rocket methanolic extract as shown in Table 4.5. The two drying processes showed a difference in the nature and the number of volatiles identified. According to Jirovetz *et al.* (2002) 155 mg/kg of volatiles were formed from dried plant leaves in contrast to volatiles formed from fresh plant material 35.0 mg/kg (without autolysis) and 71.7 mg/kg (with autolysis).

In general, aldehydes, alcohols, ketones, indoles, and sulfur compounds were identified in this extract. Glyceraldehyde, 5-methylfurfural, 1,3-dihydroxy-2-propanone and 1,3-dioxolane-4,5-dione were detected in the sample, which as mentioned previously originates form the dehydration of sugars. On the other hand, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one was the only ketone detected in both extracts. A nitrogenous indole was also identified; 5-methoxy-1H-indole. Although, there were no isothiocyanates detected, however, a sulfur-containing heterocyclic compound; thianthrene 5,5-dioxide was detected.

Isothiocyanates are usually present in low amounts relative to other volatiles but the health benefits that they produce in vitro require very low concentrations as mentioned in previous studies (P. R. Hanlon *et al.*, 2007). Bell *et al.* (2016) concluded that at 'day 7' of storing rocket leaves, a decline to less than 1% of these compounds occurred, which indicates that there is a possible drop of nutritional value of rocket leaves as they reach the consumer. Adequate and careful pre-processing of leaves can lead to protection of these volatiles.

In conclusion, isothiocyanate concentrations may vary greatly among rocket plants depending on the intensity of physical damage and wounding that occur during processing of the leaves. Nevertheless, the phytochemical content and volatile organic compounds are also affected by variations in temperature, humidity, and environmental stresses (Bell *et al.*, 2016).

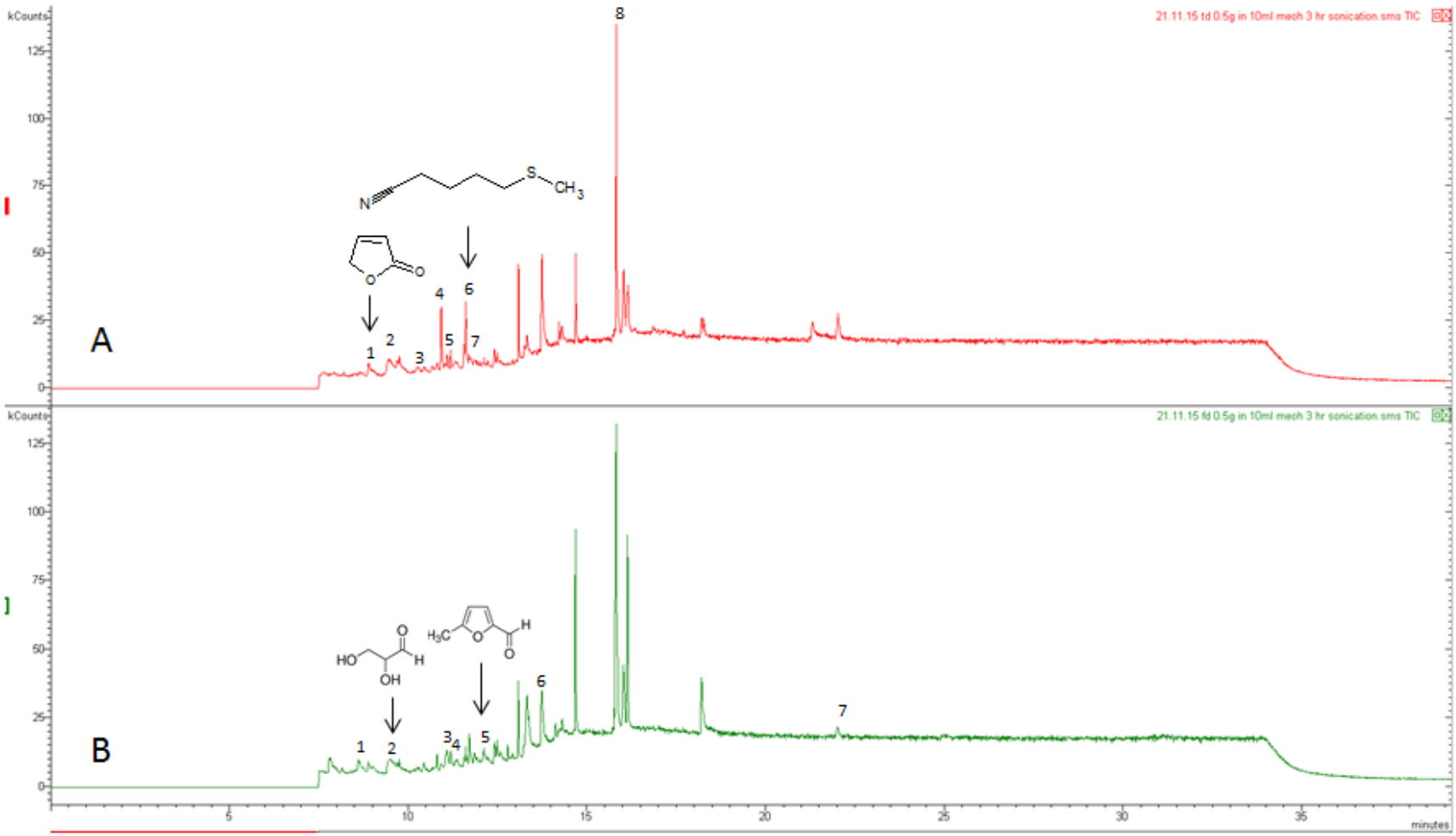


Figure 4.5. Total ion chromatogram of methanolic extracts of tray-dried (A) and lyophilized (B) *E.sativa* using GC/MS.

Table 4.4. Proposed structures of the volatile compounds observed in the tray-dried *E.sativa* methanolic extract using GC/MS analysis.

Retention time (min)	Compound	Elemental composition
8.876	2(5H)-Furanone	$C_4H_4O_2$
9.516	Glycerin	$C_3H_8O_3$
10	3,4-Hexanedione[a]	$C_6H_{10}O_2$
10.404	1-(1H-Pyrrol-2-yl)-ethanone	C_6H_7NO
11.19	2,3-Dihydro-3,5-dihydroxy-6 methyl-4H-pyra-4-one	$C_6H_8O_4$
11.621	5-Methylthiopentanenitrile	$C_6H_{11}NS$
11.715	5-Hydroxymethylfurfural	$C_6H_6O_3$
15.816	3,7,11,15-Tetramethyl-2-hexadecen-1-ol (Phytol)	$C_{20}H_{40}O$

[a]Marker for thermal treatment

Table 4.5. Proposed structures of the volatile compounds observed in the lyophilized *E.sativa* methanolic extract using GC/MS analysis.

Retention time (min)	Compound	Elemental composition
8.62	1,3-Dihydroxy-2-propanone	$C_3H_6O_3$
9.513	Glyceraldehyde	$C_3H_6O_3$
11.031	1,3-Dioxolane-4,5-dione	$C_3H_2O_4$
11.191	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one[a]	$C_6H_8O_4$
11.713	5-Methylfurfural	$C_6H_6O_3$
14.137	5-Methoxy-1H-Indole	C_9H_9NO
22.005	Thianthrene-5,5-dioxide	$C_{12}H_8O_2S_2$

[a]Marker for thermal treatment

4.10. LC-ESI-qTOF-MS analysis of lyophilized and tray-dried rocket

4.10.1. LC-ESI-qTOF-MS analysis of lyophilized rocket

Electrospray ionization mass spectrometry (ESI/MS) is often used to identify flavonoids in plant extracts (Engström *et al.*, 2015). The marker ions specific to quercetin-based flavonoids were m/z 301 and 300 and for kaempferol-based flavonoids were ion at m/z 285 and 284, and for myricetin-based flavonoids were m/z 317 and 316 (Engström *et al.*, 2015). Moreover, glycosylation sites also have an influence on the formation of these ions. For example, previous studies showed that 3-*O*-glycosylated flavonoids prefer the formation of ions at m/z 300, 284, and 316, while 4′- and 7-glycosylated analogs tend to form ions at m/z 301, 285, and 317. This suggests that the diverse flavonol fingerprint that are produced from a plant extract offers insight on the type of glycosylation of flavonols (Engström *et al.*, 2015)

In this study the LC/ESI-MS analysis of the samples in the negative ion mode was found to be more sensitive than the positive ion mode. The chromatogram displayed numerous unidentified negatively charged ionic compounds but two targeted compounds were found to match the masses obtained from the chemical profile of the lyophilized sample. The analysis showed two glucosides of quercetin and kaempferol; quercetin-3-glucoside and kaempferol 3,4'-diglucoside, respectively (see Table 4.6). Previously, Bell *et al.* (2014) identified these flavonol compounds using their parent ions in *E.sativa* by LC/ESI-MS in negative ion mode with numerous other flavonols were undetected in our sample. Kaempferol 3,4'-diglucoside was also present in other species of rocket including *Diplotaxis*. Martínez-Sánchez *et al.* (2008) found that kaempferol 3,4′-di-*O*-glucoside was the main flavonoid in salad rocket leaves, representing 74% of total polyphenol content which is in agreement with Pasini *et al.* (2012) who found that it makes up 71.4 -82.2% of the phenolic compounds in all rocket accessions tested.

4.10.2. LC-ESI-qTOF-MS analysis of tray-dried rocket

In the tray-dried rocket samples LC-ESI-qTOF-MS analysis indicated the detection of smaller flavonol aglycones (see Table 4.6). The largest identified compound and the most significant was glucoerucin, which is hydrolyzed by myrosinase to form erucin. This glucosinolate is one of the three most abundant glucosinolates in rocket found to date (Bell *et al.*, 2014). The two remaining flavonols identified were kaempferol and isorhamnetin, one of which is the hydrolyzed version of the glucoside found in the lyophilized sample. This supports what is proposed by Engström *et al.* (2015) that the characteristic ions for kaempferol-based flavonoids were m/z 285 and 284.

An LC/MS analysis of seeds, flowers, and leaves of three types of rocket showed kaempferol, quercetin, and isorhamnetin to be the core aglycones of the flavonoid glycosides. The major flavonol glycosides found were kaempferol di-*O*-glycoside and a kaempferol di-*O*-glycoside isomer, as well as a quercetin di-*O*glycoside and a quercetin tri-*O*-glycoside, along with an isorhamnetin di-*O*-glycoside. However, other studies argue that kaempferol derivatives of *E.sativa* leaves are predominant with minor amounts of quercetin and isorhamnetin glycosides, while D. tenuifolia accumulates mainly quercetin (Bennett *et al.*, 2006; Martínez-Sánchez *et al.*, 2008). Although isorhamnetin aglycones are mutual to both species, they are present in lower concentrations. The significance of each flavonol depends on their hydroxyl group arrangement and glycosylation since it affects the antioxidant activity by permitting molecules to act as hydrogen/electron donors, act as scavengers or reducing agents. Quercetin derivatives rank the highest when it comes to antioxidant activity followed by kaempferol and isorhamnetin (Bell *et al.*, 2014).

Table 4.6. Proposed elemental composition of targeted masses $[M - H]^-$ (m/z) of nonvolatile compounds identified by LC-ESI-qTOF-MS (negative ion mode) of lyophilized and tray-dried rocket.

Elemental composition	$[M - H]^-$ (m/z)	Chemical name	Error (ppm)
Lyophilized rocket			
$C_{27}H_{29}O_{16}$	609.1382	Kaempferol 3,4'-diglucoside ($C_{27}H_{30}O_{16}$)	12.082
$C_{21}H_{19}O_{12}$	463.0817	Quercetin-3-glucoside ($C_{21}H_{20}O_{12}$)	12.851
Tray-dried rocket			
$C_{15}H_9O_6$	285.0405	Kaempferol ($C_{15}H_{10}O_6$)	2.059
$C_{16}H_{11}O_7$	315.0510	Isorhamnetin ($C_{16}H_{12}O_7$)	-2.0
$C_{12}H_{22}NO_9S_3$	420.0460	Glucoerucin ($C_{12}H_{23}NO_9S_3$)	0.789

4.11. FTIR-ATR analysis of lyophilized and tray-dried rocket

The infrared spectra of lyophilized and tray-dried rocket are shown in Figure 4.6. The analysis of the spectra offered additional information on the substructures and functional groups associated with the sample (Weckerle *et al.*, 2001). The major types of vibrations are characteristic of different molecular functional groups that provide distinctive spectral absorption features which in turn provides fingerprints for many compounds (Luz, 2006).

There was a moderate infrared signal for both dried samples at 3275 cm^{-1} (O–H) indicating an alcohol/phenol functional group. Distinct peaks were shown in the 2900 cm^{-1} and 2850 cm^{-1} region (C–H) representative of alkanes for both samples as well. The minor signals at ~1740 cm^{-1} for the two samples may indicate the presence of conjugated ester moieties in the molecules (Weckerle *et al.*, 2001). A sharp peak was depicted at 1635 cm^{-1} indicating a flavonol-like structure such as quercetin (α,β-unsaturated C=O) for the tray-dried rocket sample. The peak at 1625 cm^{-1} found in the lyophilized rocket sample indicate the presence of a benzene ring (C=C). However, the peak at 1530 cm^{-1} which appeared for the lyophilized sample shows an aliphatic (N-O) nitro compound while the peak at 1350 cm^{-1} which was characteristic to the tray-dried sample, shows and an aromatic (N-O) nitro compound, although the lyophilized sample showed a similar peak at 1373 cm^{-1}. Additional peaks were displayed in the tray-dried sample than in the lyophilized sample such as 1558 cm^{-1} and 1549 cm^{-1} characteristic to (N-O) nitro compounds. At ~1420, (C-H) bending appears for the tray-dried sample but not in the lyophilized sample. In the fingerprint region, a moderately intense peak was seen at ~1240 cm^{-1} which is characteristic to phenols (Schulz & Baranska, 2007) and the band situated near 825 cm^{-1} may be also related to phenolic compounds (Luz, 2006). There was an intense band in the two samples close to 1000 cm^{-1} which is mainly attributable to the presence of alkenes. The peaks positioned at the fingerprint region showed slight differences between the two dried samples, yet overall, variances are shown to be less than 10% between the samples.

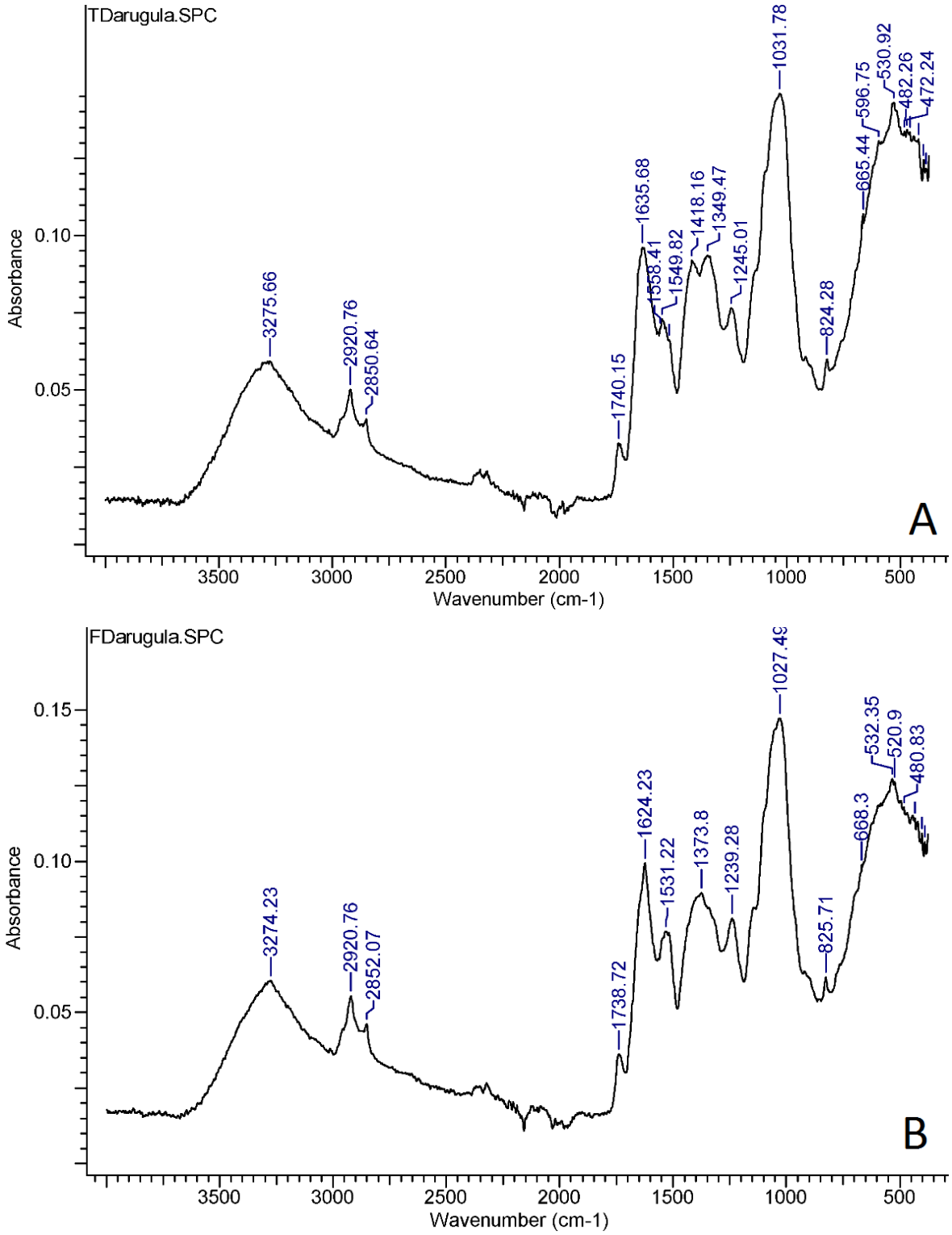
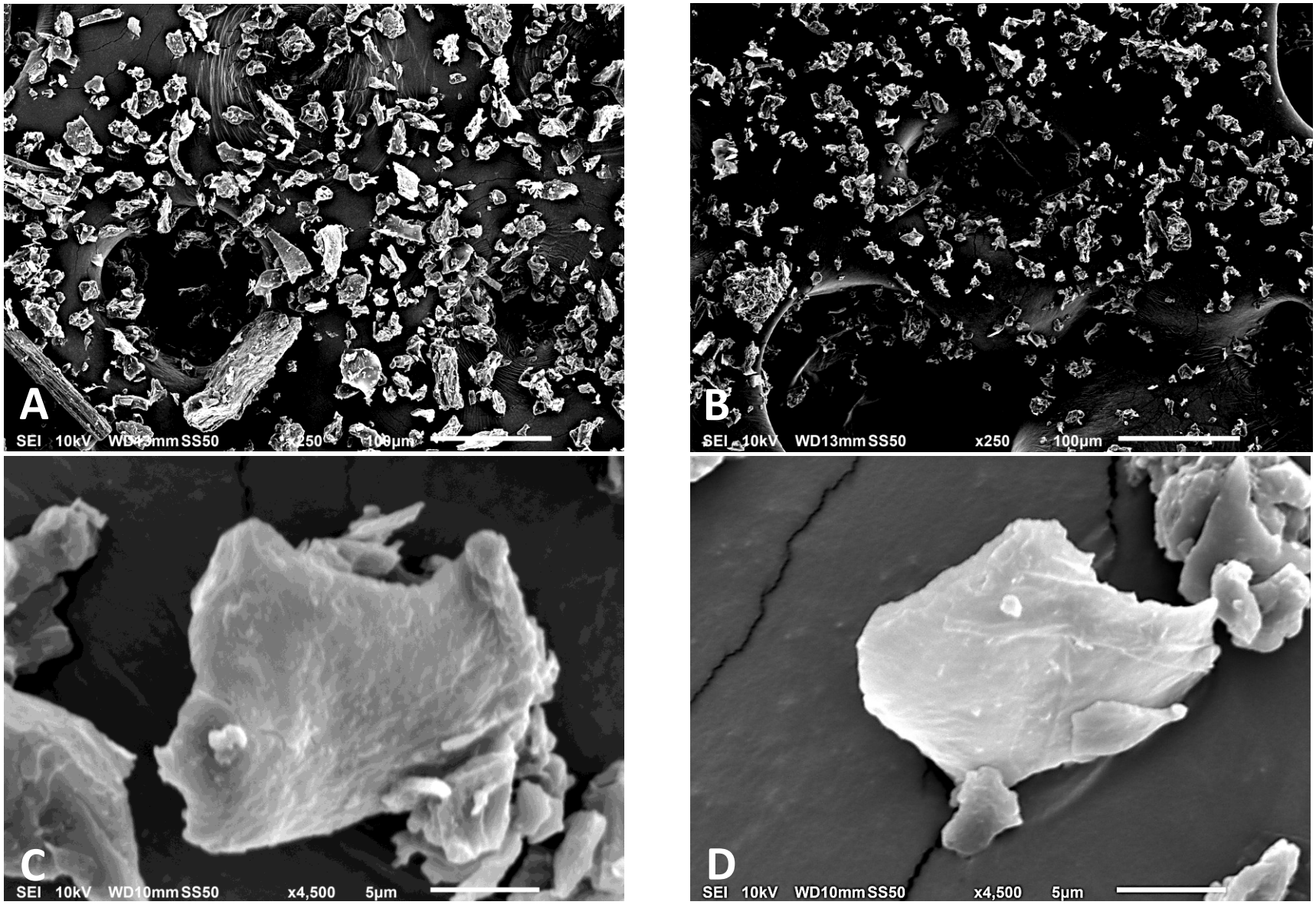


Figure 4.6. FTIR spectra of tray-dried (A) and lyophilized (B) *E.sativa*.

4.12. SEM analysis of lyophilized and tray-dried rocket

Scanning Electron Microscopy (SEM) was used in this study to show the impact of extraction and processing on tissue integrity. Figure 4.7 shows the scanning electron micrographs of rocket samples dried by two methods at different magnifications. In general, the high resolution images obtained by a Scanning Electron Microscope (SEM) assists in the examination of plant structures at high resolution depicting microstructural morphology of the plants (Pathana *et al.*, 2009). The average diameters of the tray-dried sample ranged from 51 to 8 μm and the corresponding values for the lyophilized sample was 117 and 10.5 μm. The mean diameter of the lyophilized rocket particles was calculated to be 31.3 μm. However, the tray-dried rocket particles (15.9 μm) appeared to be almost half the average size of the lyophilized rocket. The lyophilized rocket particles seem to have unevenness in their structure showing droplet-like wider surfaces (Figure 4.7 E and F) while the tray-dried rocket showed more shriveled and dried but flat smaller particles (Figure 4.7 C and D). Both samples exhibited irregular shapes however the difference in particle size distribution is prominent. It is evident that the application of thermal processing leads to major loss of integrity of the rocket leaves, however in lyophilized rocket, the particles showed more intact structures and the cellular integrity have not been completely destructed by the drying procedure.

During lyophilization of berries in general, insignificant shrinkage (5-15%) was reported as opposed to air drying which showed high level of shrinkage (80%). Air drying was accompanied with decrease in volume and subsequent wrinkle formation along with deformation and color change, which was more evident in the tray-dried rocket sample (Ratti, 2001).


A
SEI 10kV WD13mm SS50 x250 100µm
B
SEI 10kV WD13mm SS50 x250 100µm
C
SEI 10kV WD10mm SS50 x4,500 5µm
D
SEI 10kV WD10mm SS50 x4,500 5µm

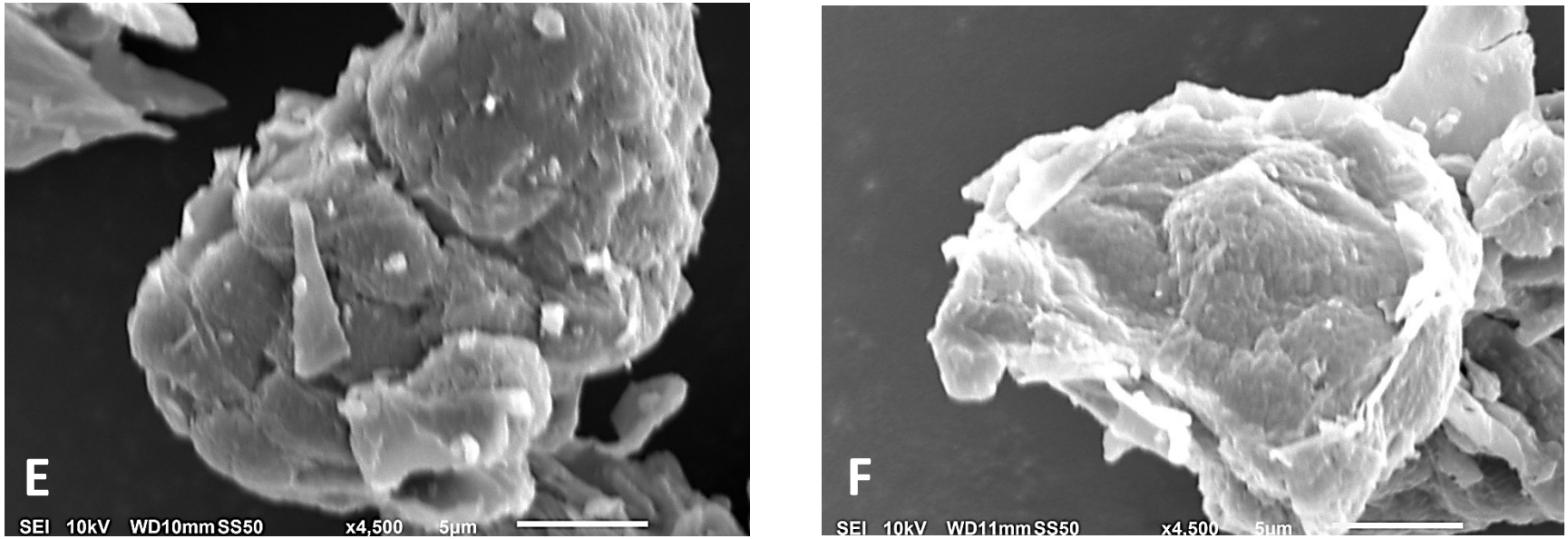


Figure 4.7. Scanning electron micrographs of lyophilized and tray-dried rocket at different magnifications. (A) Lyophilized rocket 250×, (B) Tray-dried rocket 250×, (C) & (D) Tray-dried rocket 4,500×, (E) & (F) Lyophilized rocket 4,500×.

GENERAL CONCLUSION

The objective of this study was to compare the effect of lyophilization and tray-drying on the levels of bioactive compounds and chemical profiles in rocket leaves and stems mainly grown in Kuwait. Rocket is found in almost every household in Kuwait and is consumed regularly since it is grown in abundant amounts. Significant quantities are wasted yearly due to lack of processing and storage facilities. Therefore, different drying techniques were applied to help preserve this plant and its bioactive compounds the best way possible.

Firstly, the total flavonoid test was the only test that showed significant differences in concentration between the lyophilized and tray-dried rocket samples. The total phenolic content and the antioxidant activity displayed similar results between the two dried samples. However, the total isothiocyanate content was higher in the tray-dried sample in comparison with the lyophilized sample although the difference was not significant. The volatile compounds present also showed minor differences between the two drying procedures. Furthermore, the analysis of non-volatile compounds showed the presence of glycosides in the lyophilized sample. However, the corresponding aglycones were observed in the tray-dried sample. The infrared spectra also showed minimal variations between the samples although the tray-dried samples displayed slightly more peaks. The SEM images revealed a variation in the average diameter of the rocket particles indicating loss of integrity of the tray-dried sample due to thermal processing and minor shrinkage of the lyophilized rocket particles.

In general, although similar concentrations of bioactive compounds were detected in both samples, the lyophilized rocket sample is potentially the better choice since it retained the flavonoid glycosides found formerly in fresh rocket while maintaining particle integrity and color of the sample. This investigation can serve as a basis for further studies to compare the composition of bioactive compounds, specifically that of isothiocyanates in fresh rocket samples to that of processed samples.

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