V DISCUSSION

5.1 General analysis of broccoli seeds

The procedures for determining the moisture content, the ash value and percentage of fat were carried out relatively straight forward and simple, no extra precaution is necessary. Only the 'Green Queen' cultivar demonstrated the unusual mean of 3.96% moisture while the 'Rod Fai' cultivar was 2.41%. The 'Green Queen' also had the highest ash value of 3.05% while the rest were essentially contained approximately the same amount of moisture about 1.8% with an exception 'Top Green #067' of 1.5%. These results, similarly, indicated that no significant difference exist among the moisture and ash percentages in each cultivar. Results also showed the significant difference in ash percentage of 'Green Queen' cultivar among the others, although there was no significant difference in fat percentage among cultivar. In the 'Green Queen' and 'Packman' cultivars were no significant difference in the percentage of moisture and ash, as well as in 'Pak Ging' and 'Top Green #067' cultivars.

5.2 Glucosinolates analysis

5.2.1 Evaluation of the method used

5.2.1.1 Preparation and cleanup of glucosinolates extracts

As it is well known, glucosinolates coexist with myrosinase in the plant, and any process such as cutting or grinding of fresh tissue will initiate a rapid hydrolysis of these compounds. Consequently, the importance of carefully selected conditions for reduction of sample size and extraction of glucosinolates cannot be overemphasized. Thus, broccoli seeds samples were completely dry before disruption of the cellular integrity. Meanwhile, the use of aqueous methanol (70%) for extraction, in combination with high temperatures, inhibited myrosinase activity. The anionic nature of glucosinolates facilitates cleanup procedures based on initial adsorption onto ion-exchange media. Desulfation performed on small ion-exchange columns, which also serve for the broccoli seeds extracts cleanup step, taken at 12 h at room temperature. Thereafter, this procedure with glucosinolates being eluted intact or after treatment with enzyme sulfatase to liberate the neutral desulfoglucosinolates.

5.2.1.2 Optimization of the procedures

Most early techniques relied on thin-layer or paper chromatography of the glucosinolates and/or of their presumptive hydrolysis products. Numerous methods have been utilized for the isolation, identification and quantification of glucosinolates, including steam distillation and titration of volatile isothiocyanates,

UV spectroscopy of oxazolidinethiones, gas chromatography (GC) of volatile isothiocyanates, GC/UV spectroscopy, UV spectroscopy of thiourea derivatives of isothiocyanates and GC of trimethylsilyl derivatives of glucosinolates. With much efforts, G.R. Fenwick and his colleagues have developed the reversed-phase HPLC method for quantitative analysis of desulfoglucosinolates and most widely used today. This method utilizes an on-column enzymatic desulfation treatment of plant extracts followed by HPLC detection of the resultant desulfoglucosinolates.[136] Adaptation of the sulfohydrolase desulfation method as an HPLC method, is still subjected to difficulties in interpretation because of the effects of enzyme activity time and pH on the desulfation products.[94] Typically, this method uses the response factors determined with a purified desulfosinigrin and uses desulfobenzyl glucosinolate as an internal standard. The corresponding of glucosinolates retention times, and by comparing to standardized rapeseed extracts are typically used to validate the chromatographic profiles.

Since the HPLC methods described in the literature for the quantification of glucosinolates compounds in broccoli seeds were not completely validated. This RP-PIC method with photodiode array detection was developed and validated same as an AOCS official method[137] for identification of the individual glucosinolates in various broccoli seed cultivars available in Thailand.

The chromatographic conditions were optimized with the aim of obtaining chromatograms with a good resolution of adjacent peaks within a short analysis time. Several mobile phases of various concentrations were used for the isolation and separation of glucosinolates in this study. Generally, separation of individual

glucosinolates is difficult because these molecules are highly charged and water soluble, resolutions must depend on the properties of the less polar side chains. A structural feature common to all glucosinolates is the presence of strongly acidic sulfate groups. Ion-pair formation between ammonium ions and sulfate groups is extremely favorable. Thus, when counter ions such as tetraoctylammonium are paired with sulfates, the negative charge of the sulfate group is effectively masked and the ion pair behaves like a hydrophobic molecule which can be separated by reverse-phase chromatography. In order to control pH and prevent any degradation of glucosinolates, the solvent condition of 50% acetonitrile containing 5 mM tetraoctylammonium bromide was achieved. Gradient elution was carried out so as to ensure that each run of analysis was completed within a short time. To optimize the mobile phase for a binary gradient profile, different compositions of acetonitrile (B) in acetonitrile (50%) plus de-ionized water (50%) containing 5 mM tetraoctylammonium bromide (A) were used. Under these gradient conditions (initial, 0% B; 0-20 min, 100% B; 20-23 min, 0%B; 23-30 min) peaks were well separated in a short time. A typical glucosinolates chromatogram from broccoli seed is shown in Figure 2.1 (page 66). Flow rates between 0.2 and 1.5 mL/min were studied. The mobile phase flow rate of 0.20 mL/min gave an optimum signal to noise ratio with a reasonable separation time of 30 min. Column performance results for all intact glucosinolates are presented in Table 4.16, page 80. Therefore, HPLC of desulfoglucosinolates was to be preferred that this approach offered better sepatation, constant retention times and longer colum life.

5.2.2 Glucosinolates profiles in broccoli seeds

The accurate assessment of glucosinolate levels in plant foods and feeedstuffs has been prompted by the possible physiological consequences to humans of a high dietary intake of glucosinolates. As a consequence, a considerable amount of data on levels of total and individual glucosinolates in now available. Generally, a plant species contains more than one glucosinolate and often their are both quantitative differences between the roots, leaves and seeds of a plant. The same glucosinolates occur in a particular subspecies regardless of genetic origin and in most species only between one and four glucosinolates are found in relatively high concentrations. Broccoli has recently attracted intense research interest because of their cancer chemoprotective glucosinolates attributes.

Broccoli grows well in most types of soil, but rather prefer those crumbling or friable soils with pH acidity around 6.0-6.5, high soil humidity, and sunlight-preference. It develops best during cool season of the year. The optimal temperatures are between 18-27° C, but preferably at the average temperature of 20° C.[138] In Thailand, the most suitable of period for broccoli cultivation is between November and December. There are various types of F1 hybrid broccoli cultivars in Thailand. As far as commercial seed production is concerned F1 hybrid cultivars are the result of crossing two inbred lines which have been maintained under restricted control, or under the supervision of commercial plant breeders which are known to produce a desirable hybrid. The advantages of F1 hybrid broccoli cultivars include uniformity, increased vigor, earliness, higher yield and

resistance to specific pests and pathogens. This research focused specifically on the seed part, for the content of glucosinolates in various Thai broccoli seed cultivars.

In this subsection, the most and important chemical consituent which was the prime concern in this research investigation is 4-methylsulfinylbutyl glucosinolate (glucoraphanin). The 'Top Green #067' was reported the highest content of glucoraphanin and 'Rod Fai' cultivar was the lowest. The 'Top Green #067' was three times higher than the 'Rod Fai'.

For discussion purposes, assuming that 'Rod Fai' cultivar is on baseline and has value equal to one (1), the approximate normalized for other cultivars would be, 'Pak Ging' cultivar = 1.2, 'Green Queen' = 2.5, 'Packman' = 2.9 and 'Top Green #067' = 3.2 times more in the glucoraphanin content as shown in Table 4.2, page 67. In Figure 4.1, the normal chromatogram of broccoli seed extract demonstrated the clear picture of separation of all interested compounds, the second peak (4-methylsulfinylbutyl glucosinolate) was the outstanding peak and the highest. All the responses either prefer to report as peak height value or peak area value can be assertained by the advent of equipment computer programs and the results from the machine are reliable and accurate. The ChemStation software version 6.03 was specifically installed to the HP model 1090 and all the errors had been debuged.

'Top Green #067' also showed (Table 4.2) the highest value of 4-methylthiobutyl glucosinolate (11.0 μmol/g DW). This compound also be known as glucoerucin. The combination of glucoraphanin and glucoerucin made 'Top Green #067' on the top of the chart in active principle account. In term of total glucosinolates content, 'Top Green #067' is the obvious highest, however,

'Packman' run in second, followed by 'Green Queen', but 'Pak Ging' and 'Rod Fai' were in the third group.

There was a wide range of glucoraphanin concentration among broccoli seeds genotypes. Carlson, *et al.* (1987) reported differences in glucoraphanin concentration in seeds of four broccoli genotypes from USA (31.5–72.2 μmol/g defatted meal). Accordingly, Rangkadilok, *et al.*[139] also identified the concentration of glucoraphanin among 13 Australian broccoli seeds genotypes varied between 44.2–275.1 μmol/g DW. Further, low concentration of glucoraphanin was found in seeds of cabbage (40.5–49.5 μmol/g DW), Brussels sprouts (15.3–49.2 μmol/g DW) and Chinese broccoli (1.2–59.0 μmol/g DW). These results were similar to previous results reported by Carlson, *et al.* (1987), who indicated that glucoraphanin concentration in seeds of cauliflower, Brussels sprouts and Chinese broccoli was lower than in broccoli. However, glucoraphanin concentration in five broccoli seeds from Thailand were small (11.4–48.9 μmol/g DW) compared to Australian broccoli genotypes. It has realized that in the breeding program for Thailand should be improved or developed new elite lines contains higher levels of anticancer glucosinolates for a better health benefits.

5.3. Antioxidant assay

5.3.1 Total antioxidant capacity

5.3.1.1 ABTS radical cation decolourization assay

This spectrophotometric assay is relatively expensive method due to the reagent price. The method is a straight forward simple, no extra precaution. It is a measurement absorbancy of sample against the blank. The results are compared with a known compounds such as ascorbic acid, quercetin or a water soluble vitamin E analogue, Trolox. The results obtained for this assay, in general are considered as secondary in nature and these known compounds were considered at a gold standards. The results presented in Figure 4.11-4.13 showed that both methanol and water could give the broccoli seeds extracts a higher ABTS radical scavenging activity. Ethyl acetate and chloroform extracts were considerably less effective radical scavenger compared to methanolic extracts.

However, many new and innovated methods are discovered everyday, the selected ABTS assay still at lease be used as reference. The R^2 of all three plots were > 0.99 and without intercept value. The methanolic extract represented a better ABTS radical scaventing activity. As the polarity of the extracting solvent was decreasing the scavenging activity became less and less, which fit very well to the theory that less polar solvent will have less extracting power on water soluble compounds.

5.3.1.2 Ferric reducing antioxidant power (FRAP) assay

The FRAP also a spectrophotometric assay by using a different chromophore reagent and the method is as fast as ABTS assay. The solvent power in extraction was explained the same way as ABTS method. The 'Packman' and 'Top Geen#067' cultivars are no significant different in antioxidant activity while 'Green Queen' and 'Pak Ging' cultivars reacted the same ways and closetied to each other and left 'Rod Fai' far behide as the least ferrous ion chelating ability.

5.3.2 Content of phenolics, flavonoids and flavonols

It was reasonable to determine the total amount of phenolics in the selective seed extracts. However, the major constituents consist of phenolic groups which acting as free radical terminators and/or primary consideration as antioxidants. Flavonoids comprise the most widespread and diverse group of polyphenolics plant secondary metabolites. These compounds play an important role in biological and chemical activities including free radical scavenging properties. Such properties are especially distinct for flavonols.

Based on such concept, the content of phenolic compounds in all sample extracts were also evaluated from the regression equation and expressed in gallic acid equivalent unit. The calibration curve itself has intercept and not go to zero but R^2 = 0.99 which indicated that some problems at the lower end of the assay method has detection limit. It is also noteworthy that the highest amounts of phenolics was found in 'Top Green # 067' cultivar, whereas in 'Packman' and 'Pak

Ging' cultivars were no significant differences among the other cultivars. It can be explained that the content of phenolics in the extracts correlates with their antiradical activity in the data from section 5.3.1. The correlation coefficient (R) between data of total phenolic compounds and the scavenging of radical cation 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), (ABTS) assay is 0.65, and in ferric reducing antioxidant power (FRAP) assay, R is 0.87, confirming that phenolic compounds are likely to contribute both free radical-scavenging and metal chelation activity of these seed extracts.

The flavonoids and flavonols expressed in rutin equivalent values. The highest amounts of flavonoids was found in the extracts of 'Top Green # 067' cultivar, while 'Green Queen', 'Packman' and 'Pak Ging' cultivars contained remarkably no significant amounts of these compounds. Relatively low amounts of flavonoids was also detected in 'Rod Fai' cultivar which contained the lowest amount of phenolics. It can be reported that the amount of flavonoids in the analysed seed extracts showed high correlation with the total amount of phenolics (R=0.95). It is known that only flavonoids of a certain structure and particularly hydroxyl position in the molecule demonstrate antioxidant properties, in general these properties depend on the ability to donate hydrogen or electron to a free radical.

Total flavonols were significant differences among the cultivars. Indeed, some correlation between total phenolics and flavonols can be observed, as well as the highest amounts was found in 'Top Green # 067'. Flavonols are known as important compounds in terms of free radical scavenging properties. In our study flavonols contents had higher correlation with flavonoids (R=0.84).

Significant differences in total antioxidant concentrations were also observed within each broccoli seeds cultivars grown in Thailand. The mean total antioxidant concentration was the highest in 'Top Green #067' (4.871 mg/100mg DW) cultivar, whereas the lowest was 'Rod Fai' cultivar (3.292 mg/100mg DW). Total phenolic compounds concentration was higher in 'Top Green #067' cultivar than in 'Green Queen', 'Packman', 'Pak Ging' and 'Rod Fai' cultivars, respectively. While in 'Green Queen', 'Packman' and 'Pak Ging' cultivars tested in this study for the flavonoids concentrations do not differ significantly.

The intercept of the calibration curve for flavonoids and flavonols got even higher value of 0.30. Accordingly, The content of these compounds are expressed in mg/100 g DW or 10 μg/g DW and/or 0.01 μg/mg DW in term of unit of expression which the interested compound is quite small and subject to analytical error for spectrophotometric assay, especially using a chromophore forming reagent. However, this method still considers to be a strong tool. 'Top Green # 067' cultivar showed in flavor in all three test as a top tier as presented in Table 4.21, page 88. More detailed examination of phenolics, flavonoids and flavonols composition in seed extracts is required for the comprehensive assessment of individual compounds exhibiting antioxidant activity.

5.3.2 Individual antioxidant activity

5.3.2.1 Phenolic compounds analysis

Even though the analytical method for detection of catechin, epicaterchin, epigallocatechin gallate, gallic acid, quercetin and rutin are fully developed and validated. There is no detectable level of such compounds show in any of the broccoli samples plants elsewhere. This validation assay procedure, therefore, can be applied to some other.

5.3.2.2 Content of ascorbic acid, β-carotene and tocopherols

Ascorbic acid, β-carotene and tocopherols contents in broccoli seed were extrapolated and extracted the total antioxidant activity as explained in section 4.3. Results also show no significant differences in concentrations of ascorbic acid among 'Green Queen' and 'Pak Ging' cultivars tested. At this point, β-carotene and tocopherols were almost no correlated with ABTS assay (R = 0.28 and 0.58, respectively). There is no surprise because β-carotene and tocopherols are associated with the lipid phase in biological systems, whereas ascorbic acid is found in the aqueous phase.

The LOD (from Table 4.23) of ascorbic acid is 0.05 μg/ml or approximately 0.05 μg/g and the linear detecting range of 0.2-100 μg/ml which is well below the ascorbic present in the broccoli samples i.e. 'Rod Fai' cultivar 0.021 mg/100 g DW or 2.1 μg/g. This analysis can be explained in the same manner for β-carotene and tocopherols assay. Therefore, all data here are proved to be valid.

All the raw data are demonstrated in appendices G1 through G11. Even though the real data were there, by simply use ranking technique, a true picture can be assertained below:

Amount of antioxidants ranking by names of cultivar

	Compounds		
Rank	Ascorbic acid	β-carotene	Tocopherols
1	Top Green #067	Top Green #067	Top Green #067
2	Packman	Pak Ging	Green Queen
3	Green Queen	Packman	Pak Ging
4	Pak Ging	Green Queen	Packman
5	Rod Fai	Rod Fai	Rod Fai

5.4 Method validation

Validation of an analytical method is a process by which it is established that the performance characteristic of the method meet the requirements for the method is intended application. All the relevance performance characteristics have been incorporated in the method validation which are: specificity, linearity, range, precision (repeatability, intermediate precision and reproducibility), accuracy, detection limit and quantitation limit. Peak purity tests were performed through out the development phase, that the analyte chromatographic peak is not attributable to more than one component. Luckily, the diode array detection can be especially useful in this respect, while mass spectrometric detection can provided still greater discriminatory ability (equipment is not available). However, the ICH guidances

were followed. The emphasis of the precision of all the analytical methods can be expressed in the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the precribed condition.

The detection limit was assigned based on signal-to-noise ratio because the analytical procedures exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2: 1 is generally considered acceptable for estimating the detection limit. And this principle applied here.

For further informations please refer to:

1. ICH Harmonized Tripartite Guideline, Q2A, Text on validation of analytical procedures, sterp 4, October 27, 1994. (http://www.IFPMA.org/ICH5Q.html#analytical)
2. ICH Harmonized Tripartite Guideline, Q2B, Validation of analytical procedures: Methodology. (http://www.IFPMA.org/ICH5Q.html#analytical)