Study in Total Phenolic Contents, Antioxidant Activity and Analysis of Glucosinolate Compounds in Cruciferous Vegetables

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Abstract

This research studied in the investigation of total phenolic compounds contents, antioxidant activity and analysis of glucosinolate compounds in cruciferous vegetables including broccoli, kale, choy, cabbage and cauliflower obtained from the Royal Project Foundation, Thailand. Crude extracts were extracted from dried samples prepared via two different drying methods; 1) an oven-drying method (at 70°C) and 2) a freeze-drying method. It was found that all crude extracts from freeze-dried samples showed higher total phenolic contents and better antioxidant activity than those from oven-dried samples. Freeze-dried kale crude extracts showed the highest total phenolic contents and the best antioxidant activity. The analysis of glucosinolate compounds, including sinigrin, progoitrin, glucotropaeolin and glucoraphanin, in all crude extracts was determined. The glucotropaeolin was the major glucosinolate component in all crude extracts. In addition, the types and amounts of glucosinolate compounds in all crude extracts from freeze-dried samples were higher than those from oven-dried samples.

Keywords: Glucosinolates, Antioxidant activities, Phenolic compounds, Cauliflower, Kale

Introduction

Nowadays, people are paying high attention and studying in free radical because it can cause severe oxidative damage to proteins, lipids, enzymes and DNA by covalent binding and lipid peroxidation, with subsequent tissue injury. It arises from many pathways such as metabolism, respiration, excessive exercises, infection, stress and external causes; charred food, preservatives, insecticide, ultra-violet light and much pollution (Halliwell, 1999). Free radicals have been implicated in the development of a number of various diseases such as cancer, diabetes, cardiovascular diseases, autoimmune disorders, neurodegenerative diseases and inflammation, giving rise to the studies in antioxidants for the prevention and treatment of diseases (Babaa & Malik, 2015). In recent years, there has been growing interest in finding natural antioxidants in many plants and food materials because they can prevent oxidative cell damage and anticarcinogenic properties and may also consequently prevent aging and neurodegenerative diseases. Epidemiological studies demonstrated that various vegetables have a lot of natural antioxidants which can prevent and kill many types of cancer cells and one of those vegetables is the cruciferous vegetables. A lot of cruciferous vegetables such as broccoli, brussels sprouts, cabbage, cauliflower, kale, radish and mustards, have received much attention because they have been reported to possess anticancer activity both *in vitro* and *in vivo*. From the observation of the patterns of vegetables consumption among people in Thailand, it was found that Thai people consume more cruciferous vegetables than other vegetable categories (Renuka & Berla, 2010). Epidemiological studies have shown that cruciferous vegetables may contain the important components which have been reported to exhibit a wide range of medicinal properties such as diuretics, expectorants, laxatives, healing leaky gut, spleen tonic,

carminative, antacids, and also have properties of antioxidation (Haina, Shanjing, Yuru, Gongnian & Qi, 2010). Many researchers supported the chemopreventive effect of cruciferous vegetables and their constituents in various animal and clinical experiments. Therefore, many studies revealed that the consumption of cruciferous vegetables can reduce the risk of cancers, such as stomach, lung, prostate, colon and rectum, breast and bladder (Lianga, Yuana, Donga & Liub, 2006). Cruciferous vegetables showed the existence of many antioxidant compounds such as glucosinolates, phenolic acids and flavonoids. Phenolic acids and flavonoids are efficient antioxidants and exhibit a wide range of physiological properties, such as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, anti-thrombotic and vasodilatory effects (Breinholt, 1999). In particular, cruciferous vegetables have been reported as rich sources of glucosinolate (GSL), which is a class of plant secondary metabolite, usually formed as the potassium or sodium salt. They consist of a β-D thioglucose moiety, a sulfate attached through a C=N bond, and a side chain that distinguishes one GSL from another (Warton, John, & Mark, 2001).

Glucosinolates have long been known for their fungicidal, bacteriocidal, nematocidal, allelopathic properties and are fast gaining popularity because several studies have reported their ability to fight certain types diseases of cancers and other chronic and degenerative (Chuanphongpanich, Phanichphant, Bhuddasukh, Suttajit & Sirithunyalug, 2006; Devi & Thangam, 2010). In addition, glucosinolate hydrolysis products, especially the isothiocyanates have been proven to affect human health in the cancer prevention and act as blocking agents against carcinogenesis. Moreover, glucosinolates and their derivatives would prevent carcinogen molecules from reaching the target site or interacting with the reactive carcinogenic molecules or activating the important hepatic enzymes for the protection against several carcinogens (Song, Morrison, Botting & Thornalley, 2005; Totušek et al., 2011). Although many studies have reported in anti-nutritional factors and glucosinolates in several cruciferous vegetables, there are only a few reports focusing on the anti-nutritional factors and types of glucosinolate compounds in cruciferous vegetables cultivated in Thailand. The data form this study might be greatly beneficial as informative base on developing accessions with enhanced health benefits for the people who consume cruciferous vegetables. Therefore, the main aims of this paper were: (i) to determine the diversity of total glucosinolate content including a quantitative and qualitative analysis of the major components in edible parts of some cruciferous vegetables from the Royal Project Foundation, Thailand via a high performance liquid chromatography (HPLC) technique, and (ii) to determine anti-nutritional factors including antioxidant activities using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method and total phenolic acid content via the Folin-Ciocalteu colorimetric method. In addition, the effects of conventional processing-an oven-drying method (70°C) and a freeze-drying method on glucosinolate contents and anti-nutritional factors were also determined.

Methods and Materials

Plant materials and apparatus

Plant materials: Broccoli, calian, cabbage, choy and cauliflower used in this study were obtained from the Royal Project Foundation, Thailand, in November 2015. The plants were kept at -4 °C until used.

Chemicals and reagents: The standards of glucosinolate compounds including snigrin, progoitrin, glucotropaeolin and glucoraphanin were purchased from Fluka. Aryl sulfatase enzyme was obtained from Sigma-Aldrich. Methanol (A.R grade), Abs. ethanol, ethyl acetate (A.R grade),

diethyl ether (A.R grade) and acetonitrile (HPLC grade) were obtained from Labscan. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 3,5-Di-tert-butyl-4-hydroxytoluene (BHT) were obtained from Fluka, and the Folin-Ciocalteu's reagent was obtained from Merck.

Apparatus: HPLC analyses were performed on Agilent LC1200 Series HPLC with a diode array detector operating at wavelengths between 200 and 600 nm. The column was Inertsil ODS-3 (C18), 3.0 x 150 mm. Spectrophotometric determinations were performed on a PerkinElmer 554 UV-VIS spectrophotometer with 1-cm path length cuvettes.

Preparation of dried plant materials

All plant materials were cut into small pieces and then divided into two equal proportions. The first part was dried in an oven at 70 °C and the second part was freeze dried for 2-3 days. All dried samples were ground to a coarse powder using a mechanical blender and stored at -20 °C prior to extraction.

Solvent extraction of dried samples to study in antioxidant activities and total phenolic contents

The powdered dried plant materials were extracted with 10 ml of 1% of acetic acid in methanol at room temperature for an hour. The extracts were shaken for 15 min, centrifuged (3,000 rpm) for 15 min at room temperature and then the organic layer was collected. The extraction process was repeated twice. Then, the extracts from all the three washes were pooled and concentrated under vacuum at 60 °C to obtain a dried crude extract. The crude extracts were stored at 4°C for further use.

Determination of total phenolic contents

Total phenolic content was determined using the Folin-Ciocalteu colorimetric method (Tarola, Velde, Salvagni & Preti 2013). Briefly, 0.5 mL of each crude extract (1 mg/mL) was made up to 1 mL with methanol, mixed thoroughly with 1.5 mL of the Folin-Ciocalteu reagent, followed by an addition of 2 mL of 20% (w/v) sodium carbonate. The mixture was vortexed for 15 sec and allowed to stand for 30 min at room temperature for color development. Absorbance of the solution was measured at 765 nm wavelength. Total phenolic content is expressed as mg/g gallic acid equivalent (GAE) based on the calibration curve of gallic acid standard solutions (10, 20, 30, 40, 50, 60, 70 ppm).

Antioxidant activity testing

The antioxidant activity of the crude extracts was estimated by the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay, according to the method of (Nooman, Ashok, Alo-thman, El-agbar, & Farah, 2008) with slight modification. Briefly, 1.5 mL of various concentrations of extract solutions were mixed with 1.5 mL DPPH solution (3×10^{-4} M) and incubated in dark at room temperature for 1 h. Absorbance of the solution was measured at 517 nm wavelength having ethanol as a blank and BHT as a positive control. The ability of the sample to scavenge DPPH radical (percentage inhibition) was calculated according to the following formula: Percentage inhibition = $(1-A)/A_0) \times 100$ where A_0 is the absorbance of the control reaction (containing all reagents except the extract), and A is the absorbance of the extract.

Glucosinolate analysis

Sample extraction for glucosinolate determination

Glucosinolates were extracted and analyzed according to the procedure of Lee *et. al*. (Lee et al., 2014) Briefly, the powdered dried plant materials (100 mg) and 70% methanol (1.5 mL) were added to centrifuge vials. The vials were quickly sealed, shaken, and stood in a water bath at 70 °C for 5 min. The extracts were cooled down and centrifuged at 12,000 rpm for 10 min and then the supernatant was transferred to another tube and stored as the first portion. The extraction process repeated twice as described above. Once completed, the extracts from all three washes were pooled and concentrated under

vacuum to obtain dried glucosinolate extracts, which were stored at 4°C for further use.

Desulfation of glucosinolate extracts

Glucosinolate extracts were desulfated by passing through an ion exchange column (DEAE Sephadex A-25) in the presence of sulfatase (Helix pomatia, type H1). Briefly, the suspension of DEAE anion exchange resin (Sephadex A-25) in 0.5 M sodium acetate was transferred to a pipette column. The column was then rinsed with 1 ml DI water twice. One milliliter of the glucosinolate extract was added to a prepared column and aryl sulfatase enzyme solution was added to the column (75 μl) and left to react for 16 h at ambient temperature. Then desulfoglucosinolate was eluted with three 1 ml portions of water and the effluent was filtered and analyzed for glucosinolate via HPLC.

Quantitative analyses of glucosinolate compounds using an HPLC technique

The glucosinolates in all samples were analyzed via Agilent LC1200 Series HPLC using a diode array detector. The sample (20 μl) was injected into the column and the signals were monitored at 227 nm. The desulfoglucosinolates were separated using a type C18 column (Inertsil ODS-3 C18 Type Size 3.0 mm × 150 mm, 3 μm) operated at 40°C and elution was carried out using a gradient system consisting of water (A) and acetonitrile (B) at a flow rate of 1.0 mL/min. The total running time was 30 min with a gradient as follows: 100% A and 0% B for 5 min, then in 23 min to 0% A and 100% B and in 7 min back to 100% A and 0% B. Individual glucosinolates were identified in comparison with the retention time of standard compounds and areas of standard compounds. The calibration curves of glucosinolate standards were established using known concentrations of the standard compounds.

Results and Discussion

Determination of total phenolic contents in cruciferous vegetables crude extracts from oven-dried samples and freeze-dried samples

The content of total phenolic was carried out based on the reaction of various crude extract solutions with the Folin-Ciocalteu reagent to yield colored solutions. The absorbance measured at 765 nm wavelength was compared with the standard solutions of gallic equivalents (the standard curve equation: $y = 0.0043x - 0.004$, $r^2 = 0.9989$). The values obtained for the concentration of total phenols are expressed as mg of GAE/g of extract. Data obtained from the total phenolic method agrees well with the key role of phenolic compounds in free radical scavenging. The total phenolic contents in the examined extracts ranged from 18.14 to 40.08 mg GAE/g crude extract at a concentration of 1000 $\mu g.ml^{-1}$. The total phenolic contents in plant extracts of cruciferous vegetables depend on the sample drying methods. It was found that all cruciferous vegetables crude extracts from freeze-dried sample showed the total phenolic content higher than those from oven-dried samples as shown in Figure 1. This was attributed to the decomposition of unstable phenolic compounds at high temperature (70°C) resulting in the loss of some phenolic compounds. According to previous research (Cartea, Francisco, Soengas & Velasco, 2010) factors such as temperature and sample preparation method can influence the remaining phenolic compounds in the samples. When comparing total phenolic contents in all cruciferous vegetable crude extracts, it was found that kale from both oven-dried and freeze-dried methods exhibited the highest amounts of phenolic compounds, followed by broccoli, choy, cauliflower

and cabbage crude extracts, respectively. According to a study by Ismail (Ismail, Marjan, & Foong, 2004), kale exhibited higher phenolic content than other cruciferous vegetables. Moreover, various studies have confirmed that kale was mainly composed of polyphenols such as phenolic acids and flavonoids (Unal, Susanti, & Taher, 2014). These could support that there are abundant of phenolic compounds in kale.

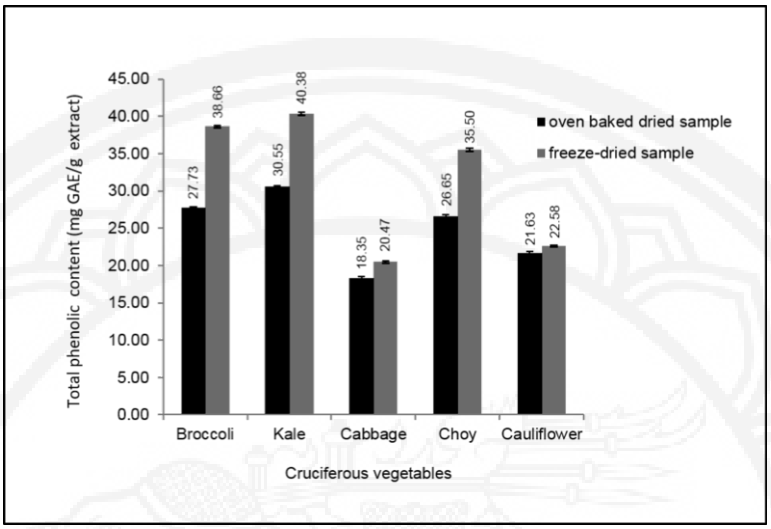

Figure 1 Total phenolic contents of cruciferous vegetables crude extracts determined via the Folin-Ciocalteu assay. The results are the average of triplicates ± SD.

Antioxidant activity testing of cruciferous vegetable crude extracts from oven-dried samples and freeze-dried samples

In this work, antioxidant activities of the all cruciferous vegetable crude extracts were studied using a DPPH free radical scavenging method. This method is widely used in assessing free radical scavenging activity because of the ease and rapid way to evaluate antioxidant activity. In addition, DPPH, a very stable free radical method, has the advantage of being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition. In general, freshly prepared DPPH solution exhibits a deep purple color, which changes to yellow when DPPH reacts with an antioxidant compound. This can provide hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule. The change of colour can be quantitatively measured from absorbance (Abs) at 517 nm wavelength (Milan, 2011). The antioxidant activity of all crude extracts was expressed in terms of IC_{50} values (ppm) as shown in Figure 2. IC_{50} values, which represent the volume of extract required to reduce the absorbance of DPPH radical by half, are calculated and are used to measure antioxidant assay. Lower IC_{50} value indicates higher antioxidant activity in the extract.

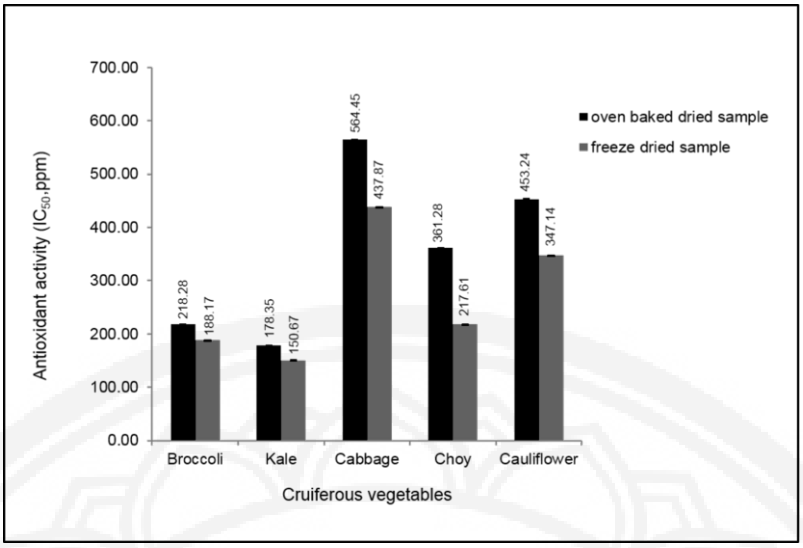

Figure 2 The antioxidant activity of all cruciferous vegetable crude extracts was expressed in terms of IC_{50} values (ppm). The results are the average of triplicates ± SD.

The cruciferous vegetable crude extracts from freeze-dried samples showed a good anti-radical activity in scavenging DPPH radical as compared to those from oven-dried samples (Figure 2). It can be explained that some active compounds were decomposed when heated at high temperature (70°C) during the sample preparation procedure. The comparison of antioxidant activity of all cruciferous vegetables was also investigated. It was found that kale crude extract showed the best result with anti-radical activity in scavenging DPPH radical and with IC_{50} value of 150.56 ppm from oven-dried samples and 178.09 ppm from freeze-dried samples). From another report, it was also demonstrated that kale had higher antioxidant activity than some cruciferous vegetables such as broccoli, choy and cabbage (Kushad et al., 1999). Because of kale contains a lot of antioxidant compounds, e.g. vitamins A, C and K; one has called kale as the healthiest vegetable on earth. In addition, these results were corresponded to the study of total phenolic contents in all crude extracts.

Quantitative analysis of glucosinolate compounds in cruciferous vegetable crude extracts via HPLC technique

The analysis of glucosinolate compounds was carried out via HPLC technique. HPLC method for determining desulfated glucosinolates provides a simple means for obtaining information on the glucosinolate profiles. Four standard glucosinolate compounds used in this work included sinigrin, progoitrin, glucotropaeolin and glucoraphanin as their chemical structures shown in Figure 3. The choice of these compounds as standards is based on the availability of the stable and pure substances. In addition, all of standard compounds were the most abundant and the major glucosinolate in cruciferous vegetables.

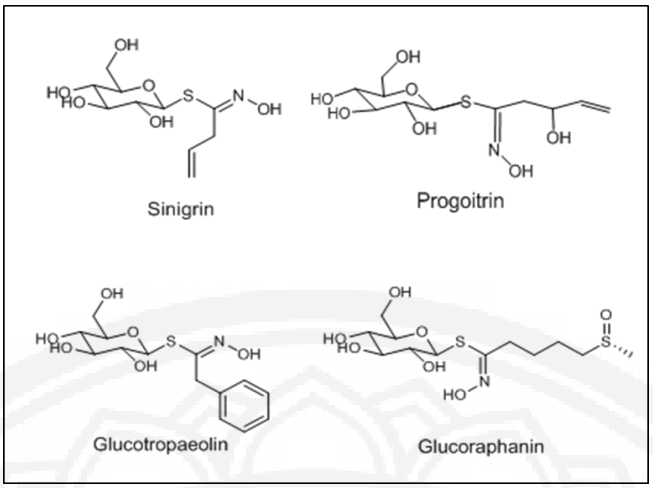

Figure 3 The chemical structures of standard glucosinolate compounds

The chromatographic conditions for the quantification of glucosinolate compounds were optimized by conducting preliminary trials with the standard mix of the glucosinolate compounds, with the aim of ensuring that all the compounds were well resolved. The wavelength at 227 nm has been selected for the analysis of glucosinolate compounds in this study. Form the study, it was found that the optimized HPLC conditions can clearly separate all standard glucosinolate compounds and the optimized time of the chromatographic run was 30 min as shown in Figure 4.

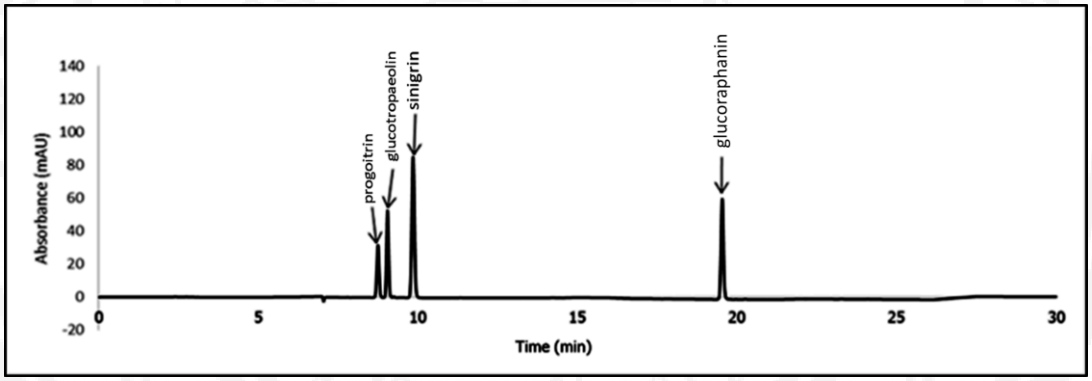

Figure 4 A HPLC chromatogram of standard glucosinolate compounds

The linearity of the standard curve was expressed in terms of the determination correlation coefficient (R^2) from the plots of the integrated peak area and concentration of the standard. Seven different concentrations of all standard active compounds in the range of 10–70 ppm were employed. It was found that the correlation coefficient (R^2) of all standards ranged between 0.9998 and 1.0000. According to the R^2 values of the calibration curves for each glucosinolate, the linearity of all calibration curves was acceptable. The limits of detection (LOD) and limit of quantification (LOQ) under the present chromatographic conditions were determined with the formula (SD)*3; (SD)*10, respectively as shown in Table. 1

Table 1 Retention time (t_R), LOD, and LOQ of glucosinolate standard compounds

Standard compounds	t_R (min)	LOD (μg/ml)	LOQ (μg/ml)	R^2
Progoitrin	8.87	0.04	0.14	1.0000
Glucotropaeolin	9.10	0.04	0.12	1.0000
Sinigrin	9.54	0.02	0.05	0.9998
Glucoraphanin	19.75	0.04	0.13	1.0000

The accuracy was determined in independent samples by means of recovery assays. The recovery of the method was evaluated by the analysis of all crude extract samples, which were spiked with known amounts of standard compounds before extraction with 70% methanol in a water bath at 70°C and the desulfation reaction. After extraction and desulfation, the samples were quantified by HPLC. All crude extracts showed values of recoveries of standard compounds ranged between of 98.50–103.15% and these values were acceptable. Table 2 presents the recovery percentages obtained from these analyses.

Table 2 A summary of the method recovery values of all glucosinolate standard compounds

Standard compounds	%Recovery
Progoitrin	99.09 – 100.32
Glucotropaeolin	98.50 – 99.79
Sinigrin	101.39 – 103.15
Glucoraphanin	100.58 – 101.45

These results indicate that the present method can be used for quantitative analysis of glucosinolate compounds in cruciferous vegetables. The precision values found are in agreement with those obtained in previous related studies (Lee et al., 2014). A content of four glucosinolates in cruciferous vegetables is shown in Figure 5. The data shows the significant differences in the content of individual glucosinolates among the vegetables. The data shows the types of glucosinolates and the contents in the unit of mg of active compound/100 g of fresh sample.

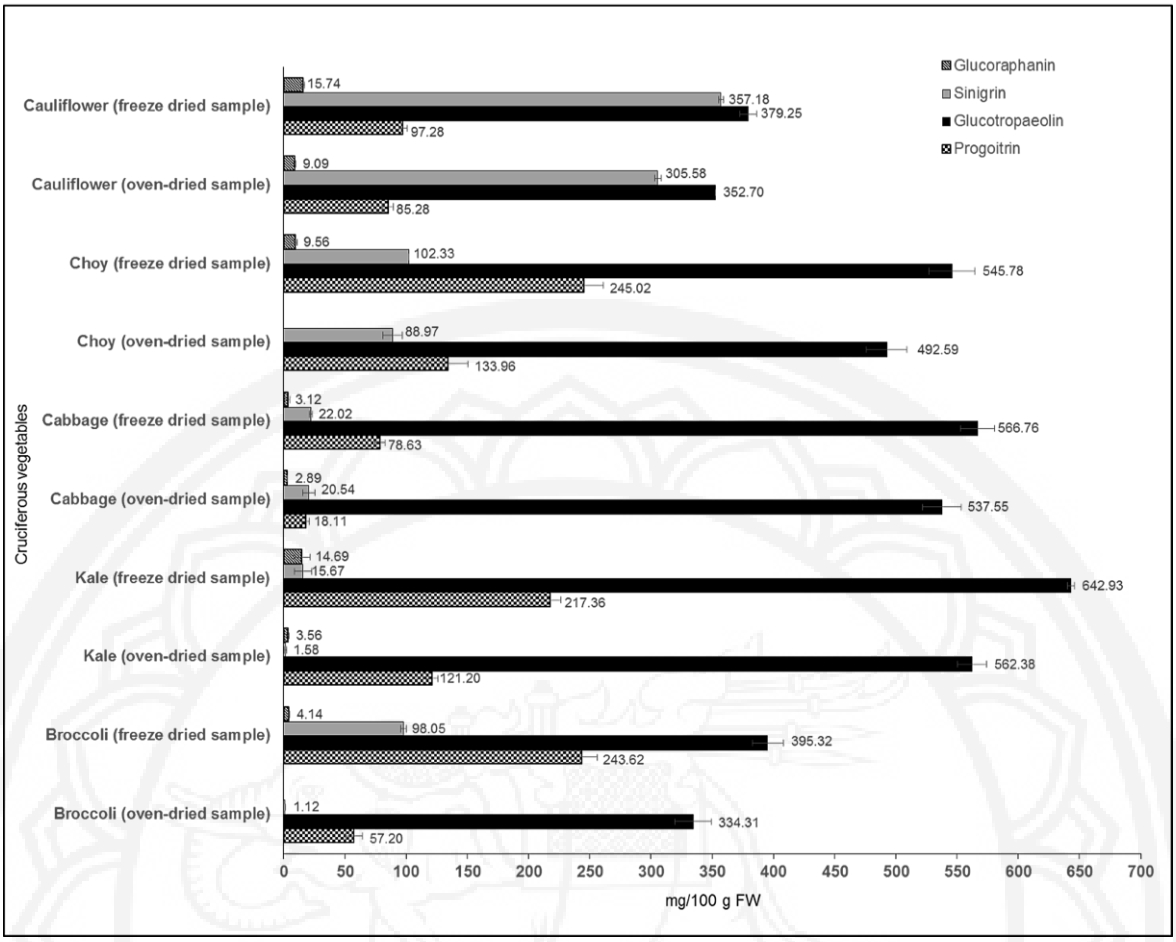

Figure 5 Quantitative analyses of glucosinolate compounds in the unit of mg /100 g of fresh weight

From Figure 5, glucotropaeolin was mainly obtained in cruciferous vegetable crude extracts ranged from 334.31 to 642.93 mg/100 g of fresh weight, while glucoraphanin concentration was the lowest in all crude extracts ranging from 1.12 to 15.74 mg/100 g of fresh weight. Kale from freeze-dried sample contained the highest level of individual glucosinolates, with glucotropaeolin at 642.93 mg/100 g of fresh weight, while choy from freeze-dried sample exhibited the highest level of total glucosinolates. All four glucosinolate compounds were found in all crude extracts from freeze-dried samples, while glucoraphanin was not detected in broccoli and choy crude extracts from oven-dried samples. In addition, the types and amounts of glucosinolate compounds in crude extracts from freeze-dried samples were higher than those from oven-dried samples. Some of the glucosinolates showed the loss after heating. Considering only in the case of glucosinolates, the loss of glucoraphanin and progoitrin was higher than sinigrin and glucotropaeolin. It is worth to note that the preparation of the samples via an oven-drying method may cause significant loss of some glucosinolates due to accessibility of myrosinase to glucosinolates with subsequent enzymatic conversion of glucosinolates to isothiocyanates during heating.

Conclusion

This research focused on the investigation of total phenolic contents, antioxidant activity in cruciferous vegetable crude extracts from oven-dried and freeze-dried samples. The identification and quantification of the glucosinolate compounds in crude extracts was also studied. All cruciferous vegetables crude extracts

from freeze-dried samples showed the total phenolic content, antioxidant activity and amount of glucosinolate compounds higher than those from oven-dried samples. Kale crude extracts from freeze-dried samples exhibited the highest total phenolic contents and the best antioxidant activity. This should be a good choice for human daily consumption. The types and amounts of glucosinolate compounds in cruciferous vegetable crude extracts were then analyzed by using HPLC method. The method was validated and showed acceptable quantitative performance in terms of LOD, LOQ and accuracy. This could be applied to the identification and quantification of glucosinolate compounds in cruciferous vegetables. The result showed that the differences in the individual glucosinolate contents were observed among different vegetables. The major glucosinolate compound in all crude extracts was glucotropaeolin. In addition, it was also found that all glucosinolate compounds were detected in all crude extracts preparing from a freeze-drying method, while an oven-dried method resulted in significant reductions in all types and amounts of glucosinolate compounds.

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