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Investigation of total phenolic contents, antioxidant activities and analyses of active compounds in some sweet peppers (*Capsicum annuum* L.)

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ABSTRACT

This work focused on the investigation of total phenolic contents, antioxidant activities and analyses of active compounds in red, yellow and green sweet peppers (*Capsicum annuum* L.). All crude samples were extracted from dried samples preparing from two different methods; an oven-dried technique (at 70°C) and a freeze-dried technique. Antioxidant activities of all crude extracts were investigated using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method, while total phenolic compounds were determined *via* the Folin-Ciocalteu Colorimetric method. It was found that antioxidant activities and total phenolic contents of all crude extracts from freeze-dried samples were better or higher than those from oven baked samples. Yellow sweet pepper crude extracts showed the best antioxidant activity (IC_{50} = 304.44 ppm) and highest contents of total phenolic compounds (74.58 mg/g of crude extract). Analyses of active compounds such as phenolic and flavonoid compounds in all crude extracts were carried out *via* a high performance liquid chromatography (HPLC) technique. The results showed that five active compounds including caffeic acid, *p*-coumaric acid, feruilc acid, luteolin and quercetin were present in all crude extracts preparing from freeze dried samples. In the case of crude extracts preparing from oven baked samples, four active compounds including *p*-coumaric acid, feruic acid, luteolin and quercetin were detected in yellow sweet pepper crude extracts, while quercetin was not detected in red and green sweet pepper crude extracts.

Keywords: phenolic content, antioxidant activity, sweet pepper, Folin-Ciocalteu Colorimetric method

Introduction

Nowadays, much attention has been paid on sources of free radicals because they are the main cause of human illness. Free radicals arise 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, B. *et. al.*, 2009].

Even though free radicals can be eliminated by enzyme [Velioglu, Y. S.L. *et.al.*, 2009; Chanwitheesuk, A. *et.al.*, 2005], too many free radicals produced in the body to be eliminated may cause damages to cells and issues, which will essentially affect health. Thus, it is crucial to have essential sources containing additional antioxidant. Antioxidant can reduce risks of many illnesses, such as cancer, diabetes, heart disease, brain-related illness, e.g. alzheimer. In addition, it has been reported that antioxidant compounds can also slow down some aging progresses [Nooman A. K. *et.al.*, 2008; Mathew S. *et.al.*, 2006].

We can take antioxidant into the body by eating food containing antioxidant. The essential sources of antioxidant include fruits and vegetables, which contain many crucial substances that can work against free radicals. Among the variety of phenolic compounds, phenolic acids have attracted considerable interest in the past few years due to their many potential health benefits. Phenolic acids are efficient antioxidants and have been reported to show antibacterial, antiviral, anticarcinogenic, anti-inflammatory and vasodilatory activities [Breinholt V., 1999; Duthie G.G. *et.al*., 2000]. At present, many works have conducted researches for study the amounts of overall phenolic compounds in vegetables and fruits, as well as its properties as an oxidant in a distilled. Both vegetables and fruits have high amounts of phenolic compound, and possess different degree of antioxidants [Nooman A. K. *et.al.*, 2008; Caia Y. *et.al.*, 2004]. Therefore, the aim of this research was to investigate total amounts of some phenolic compounds and their antioxidant activities in crude extracts of red, yellow and green sweet peppers (*Capsicum annuum* L.). These sweet peppers are accessible and popularly consumed in Thailand. They are used as an ingredient or to decorate a variety of dishes. They give beautiful coloring and contain many properties such as stimulating the stomach's metabolism, better appetite, increase perspiration, reduce gases in stomach, reduce the phlegm, cure the symptoms of vomiting, cure scabies and tinea corporis. It also reduces blood pressure as it 'softens' blood vessels and allows good blood circulation. In the current work, crude extracts of sweet peppers (*Capsicum annuum* L.) were prepared *via* two different methods; an oven-dried method (70 °C) and a freeze-dried method. Furthermore, quantitative and qualitative analyses of various active compounds in the crude extracts were also investigated.

According to previous researches, there have already been some studies on antioxidant properties and the total phenolic compounds from the crude extracts of some vegetables and fruits. For example, Marja has reported antioxidant activities using a DPPH free radical scavenging method and total phenolic contents *via* the Folin-Ciocalteu colorimetric method in some vegetables [Marja P., 1999]. The samples were dried at 70-80^{o}C before the analyses. It was found that all crude extracts showed either moderate or good antioxidant properties. Mattila has studied total phenolic compounds in the crude extracts of potatoes and some vegetables [Mattila P., 2007]. It was discovered that crude extracts of some vegetables such as spinach, cabbages and red cabbages contained high amounts of phenolic compounds.

In addition, some researches have been focused on the studies in antioxidant activities and total phenolic compounds in crude extracts of sweet peppers (*Capsicum annuum* L.). For example, Kaur has studied antioxidant activities of

some red sweet peppers using a DPPH free radical scavenging method [Kaur C., 2006]. In this report, the sample was dried *via* a freeze-drying technique. It appeared that the crude extract of Bomby red sweet peppers showed good antioxidant activities. Another work from Kaur has been focused on the investigation of antioxidant activities and total phenolic compounds in many genotypes of the red sweet pepper crude extracts [Kaur C., 2007]. They discovered that within each genotype, amounts of phenolic compounds and antioxidant activities in each samples were different. In addition, Manonk, et. al. have presented antioxidant activities of crude extracts from 18 types of herbs [Manonk C., 2012]. The samples were dried at 70-80 ^{o}C. The results showed that the crude extracts of cashew, great morinda, broken bones tree, roselle and capsicum showed good antioxidant activities. The totals of phenolic compound determined *via* the Folin-Ciocalteu Colorimetric method, were following; cashew (747.667 ppm), great modinda (580.000 ppm), broken bones tree (84.333 ppm), roselle (74.000 ppm) and sweet pepper (45.333 ppm).

However, the recent research data on comparisons of antioxidant activity, total phenolic content and types of active compounds in sweet pepper (*Capsicum annuum* L.) crude extracts is quite limited. Therefore, the main objective of this research was to investigate total phenolic contents, antioxidant activities and types of some active compounds in crude extracts of red, yellow and green sweet peppers. Two different sample preparation methods were carried out; an oven-dried method (70^{o}C) and a freeze-dried method. The DPPH; 2,2-diphenyl-1-picrylhydrazyl free radical scavenging agent was used to investigate antioxidant activities and the Folin-Ciocalteu colorimetric method was employed to determine total amounts of phenolic compound. In addition, quantitative analyses of active compounds in sweet peppers crude extracts were performed *via* a high performance liquid chromatography (HPLC) technique.

Experimental

Materials and Apparatus

Plant Materials: All sweet peppers (*Capsicum annuum* L.) were from the Royal Project Foundation, Chiang Mai, Thailand. They were kept at -4 °C until used.

Chemicals and reagents: The standards of phenolic acids were obtained from two manufacturers. Caffeic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, ferulic acid, quercetin and kaempferol were purchased from Sigma Chemical Co., while catechin, ellagic acid, epicatechin, gallic acid, luteolin and cinnamic acid were obtained from Fluka. 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-2-picrylhydrazyl (DPPH) and BHT were obtained from Fluka, and the Folin-Ciocalteu's reagent was obtained from Merck.

Apparatus: HPLC analyses were performed on Agilent 1100 Series HPLC with a diode array detector operating at wavelengths between 200 and 600 nm. The column was VertiSep UPS C18 HPLC column, 4.6 x 250 mm. Spectrophotometric

determinations were performed on a PerkinElmer 554 UV–VIS spectrophotometer with 1-cm path length cuvettes.

Preparation of dried sweet pepper samples

Three sweet peppers (*Capsicum annuum* L.), including red, yellow and green sweet peppers, were chopped into small pieces and then divided into two equal proportions. The first part was dried at 70 °C. The second part was freeze dried for 2-3 days. All dried samples were mashed up and kept at -20 ^{o}C until used.

Sample extraction and hydrolysis

Extraction was carried out as previously described by Mattila [Mattila P., 2007]. Briefly, phenolic compounds were extracted from 0.5 g of dried samples in 7 mL of a mixture of methanol, containing 2 g/L of butylatedhydroxyanisole (BHA) and 10% acetic acid (85:15). The extract was stirred for 1 hour at room temperature and made up to a volume of 10 mL with distilled water. Afterwards, the extract was added with 12 mL of distilled water containing 1% ascorbic acid and 0.415% ethylenediaminetetraacetic acid tetrasodium salt dehydrate (EDTA). Then, 5 ml of 10% w/v NaOH as alkaline hydrolysis was added to the mixture to cleave phenolic acids bound. The reaction was allowed for 16 hour. After the alkaline hydrolysis, the extracts were acidified to pH 2 with conc.HCl, followed by extraction with diethyl ether (DE) and ethyl acetate (EA, 1:1 v/v). The organic layer was separated and stored as the first portion. The residue was added with 2.5 ml of conc.HCl as acid hydrolysis and incubated in a water bath at 60 ^{o}C for 1 hour. After the acid hydrolysis, the extracts were again extracted similarly to above procedure. The second portion of the organic layer was combined with the first part, followed by evaporation to dryness. The extracts were then dissolved into 2 mL of methanol and the mixture was filtered and analyzed for total phenolic acids *via* HPLC.

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

1.00 g of dried red, yellow and green sweet peppers (*Capsicum annuum* L.) was extracted with 10 ml of 1% of acetic acid in ethanol at room temperature for 1 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 repeated twice. Once completed, the organic layers from the 3 trials were combined and concentrated under vacuum using a rotary evaporator at 70^{o}C. The extracts were stored at 4^{o}C for further use.

Investigation of antioxidant activities using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method

The antioxidant activity of the crude extracts was studied by using 1,1-diphenyl-2-picrylhydrazyl (DPPH)-free radical scavenging method [Nooman A. K. *et.al*, 2008; Marxen K. *et.al.*, 2007]. The stock solution of crude extracts (3 mg/ml) was prepared by dissolving in absolute ethanol. The sample solutions (0,100,200,300,400,500,600 and 700 ppm) of the crude extracts were prepared from the stock solutions with suitable dilutions. Briefly, 1.5 mL of sample solutions and standard solutions were pipetted into a test tube. Then, 1.5 mL of DPPH solutions in ethanol at the concentration of 3×10^{-4} M was added. The solution was shaken and

placed in dark at room temperature for 30 min. Afterwards, the solution's absorbance (Abs) was measured at 517 nm using Spectrophotometer and ethanol was used as a blank. The result was used in the calculation to determine the percentage of inhibition in comparison with the absorbance of DPPH solutions using the equation below:

$$\% \text{ Inhibition} = 100 - \%\text{Activity}$$
$$\% \text{ Activity} = (\text{Abs of the sample solution}) / (\text{Abs of DPPH}) \times 100$$

Investigation of total phenolic contents using the Folin-Ciocalteu colorimetric method

Total phenolic content was estimated using the Folin-Ciocalteu colorimetric method described previously [Tarola A.M. *et.al.,* 2013; Serea C. *et.al.,* 2011]. Briefly, 0.5 ml of each crude extract in methanol (1 mg/ml) was mixed with 1.5 ml of the Folin–Ciocalteu reagent and then 2.0 ml of 7.5% (w/v) sodium carbonate solution was added. The samples were kept in dark at room temperature for 30 min. Percent absorbance was measured by means of a spectrophotometric method at 765-nm wavelength. A calibration curve was established from a gallic acid standard solution (10, 20, 30, 40, 50, 60, 70 ppm) with methanol as a blank.

Quantitative analyses of active compounds using an HPLC technique

All active compounds were analyzed on an Agilent 1100 Series HPLC with a diode array detector and detected at 280, 320 and 360 nm. The spectra of all compounds were recorded between 200 and 600 nm. The column was VertiSep UPS C18 HPLC column, 4.6 x 250 mm, 5 µm operated at 25ºC. The injection volume was 20 µL and the flow rate was set at 1 mL/min. The elution solvents were acetonitrile (A) and 0.2% acetic acid in water (B). The samples were eluted according to a linear gradient: 0-25 min, 15-25%A; 25-30 min, 25-40%A; 30-42 min, 40%A; 42-45 min, 40-15%A and 45-50 min, 15%A isocratic and then washing and reconditioning of the column. Identification and quantification of all active compounds in the samples were obtained according to the chromatographic retention times and areas of standard compounds. The calibration curves of phenolic acid standards were established using known concentrations of the standard compounds.

Results and discussion

Investigation of antioxidant activities of sweet pepper (Capsicum annuum L.*) crude extracts from oven baked dried samples and freeze-dried samples*

In this study, antioxidant activities of the all sweet pepper crude extracts from oven baked samples and freeze-dried samples were determined using a DPPH free radical scavenging method. The DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to decolorize in the presence of antioxidant. When DPPH reacts with an antioxidant compound, which can donate hydrogen, the DPPH is decolorized. The changing of colour can be quantitatively measured from absorbance (Abs) at 517-nm wavelength. The use of DPPH provides an easy and

rapid way to evaluate antioxidants [Nooman A. K. *et.al,* 2008; Marxen K. *et.al.*, 2007]. Antioxidant activities of three types of sweet pepper crude extracts were studied and the 50% inhibition (IC_{50}) values for all samples were determined and shown in Table 1.

Table 1. IC_{50} values of sweet pepper (*Capsicum annuum* L.) crude extracts from oven-dried samples and freeze-dried samples

crude extracts	IC_{50} (ppm)	
	oven baked dried samples	freeze-dried samples
red sweet pepper	337.60	311.70
yellow sweet pepper	304.77	304.44
green sweet pepper	520.57	502.45

From Table 1, the IC_{50} values of yellow sweet pepper crude extract were lower than those of red and green sweet pepper crude extracts, indicating that yellow sweet pepper crude extracts had the strongest antioxidant activities and showed a highest percentage inhibition (IC_{50}), while the lowest percentage inhibition was observed in the case of green sweet pepper crude extracts. This was attributed to the presence of flavonol, a flavonoid group that gives the yellow colour, in yellow sweet peppers. Yellow sweet pepper showed good antioxidant properties and thus contains high amounts of vitamin E and vitamin C. In addition, it was also found that sweet pepper crude extracts from freeze-dried samples displayed better antioxidant activity when compare to those from oven baked samples. This was attributed to the decomposition of some active compounds when heating them at high temperature (70^{o}C) during sample preparation procedure.

Investigation of total phenolic contents in sweet pepper (capsicum annuum L.*) crude extracts from oven baked dried samples and freeze- dried samples*

The Folin-Ciocalteu colorimetric method was selected to investigate the total phenolic contents.

The Folin-Ciocalteu reagent is formed from a mixture of phosphotungstic acid, $H_3PW_{12}O_{40}$, and phosphomolybdic acid, $H_3PMo_{12}O_{40}$, which, after oxidation of the phenols, is reduced to a mixture of blue oxides of tungsten, W_8O_{23}, and molybdenum, Mo_8O_{23}. The blue coloration produced has a maximum absorption at 750 nm. This method is simple, sensitive, and precise [Serea C. *et.al*., 2011; Prior R. L. *et. al.*, 2005]. Total phenolic contents were expressed as gallic acid equivalents (GAE) in mg/g crude extract, using a standard curve of gallic acid, with concentrations varying between 0 and 70 µg/ml complying with the standardized method as shown in Fig.1. The choice of gallic acid as standard is based on the availability of the stable and pure substance. In addition, the response of gallic acid has been shown to be equivalent to most other phenolic in vegetables on a mass basis [Prior R. L. *et. al.*, 2005].

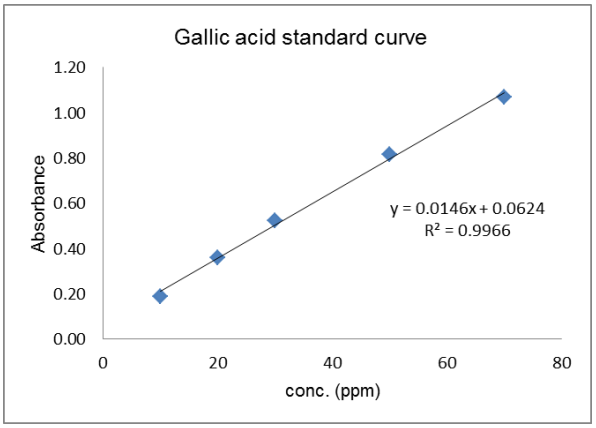


Figure 1. The standard curve of gallic acid, with concentrations varying between 0 and 70 μg/ml

All sweet pepper crude extracts from freeze-dried sample showed the total phenolic content higher than those from oven baked dried samples as shown in Table 2. This was attributed to the decomposition of unstable phenolic compounds at high temperature (70°C), resulting the loss of some phenolic compounds. In addition, the comparison of total phenolic contents in each capsicum crude extract indicated that the yellow sweet pepper crude extract has the highest amounts of phenolic compounds, followed by red and the green capsicum crude extracts respectively. This may be because yellow sweet pepper has high content of flavonol compounds, a kind of phenolic compound, which provides the yellow color. In addition, it was also found that sweet pepper crude extracts from freeze-dried samples displayed better result in antioxidant activity when compare to those from oven baked dried samples.

Table 2. Total phenolic contents found in red, yellow and green sweet peppers

Crude extracts	Total phenolic contents (mg gallic acid/ g crude extract)	
	Oven baked dried sample	Freeze- dried samples
red sweet pepper	32.06	71.56
yellow sweet pepper	35.36	74.58
green sweet pepper	25.70	64.28

Analysis of active compounds in sweet pepper (capsicum annuum L.*) crude extracts via HPLC technique*

Standard phenolic acid compounds used in this work included gallic acid, *p*-hydroxybenzoic acid, ellagic acid, caffeic acid, *p*-coumaric acid, ferulic acid, and cinnamic acid, while standard flavonoid compounds were catechin, epicatechin, luteolin, quercetin, and kaempferol. The chromatographic conditions have been optimized using a standard mix of the actives compounds listed above, with the aim of ensuring that all the compounds were well resolved. The HPLC conditions used in this work can clearly differentiate all standard active compounds as shown in Fig.2. The optimized time of the chromatographic run was 50 min; it can separate 12 standard compounds with kaempferol (peak#12) being the last compound eluted at approximately 41 min. Three wavelengths have been selected for the quantification of phenolic compounds in this study: 280 nm for gallic acid cinnamic acids, catechin, *p*-hydroxybenzoic acid, ellagic acid and epicatechin; 320 nm for caffeic acid, ferulic acid and p-coumaric acids; 360 nm for luteolin, quercetin and kaempferol as shown in Table 3.

To establish the calibration curves, five different concentrations of all standard active compounds of the phenolic acid groups and flavonoids were performed. The standard compound concentrations (in milligrams per liter) (x) were plotted against peak areas (y), and correlation coefficient (R^2) was then analyzed. It was discovered that the correlation coefficient: R^2 of all standards ranged between 0.9960 and 0.9980. The minimum concentration reliably detectable for the method (LOD) and the minimum concentration reliably measurable (LOQ) were determined and shown in Table 3.

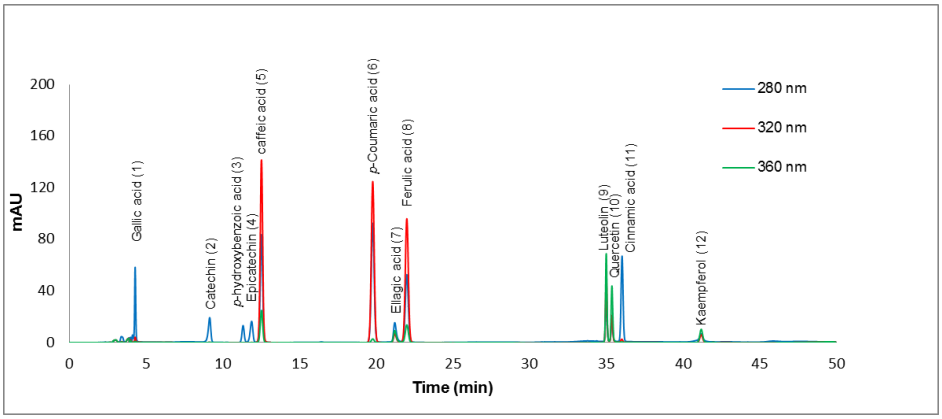
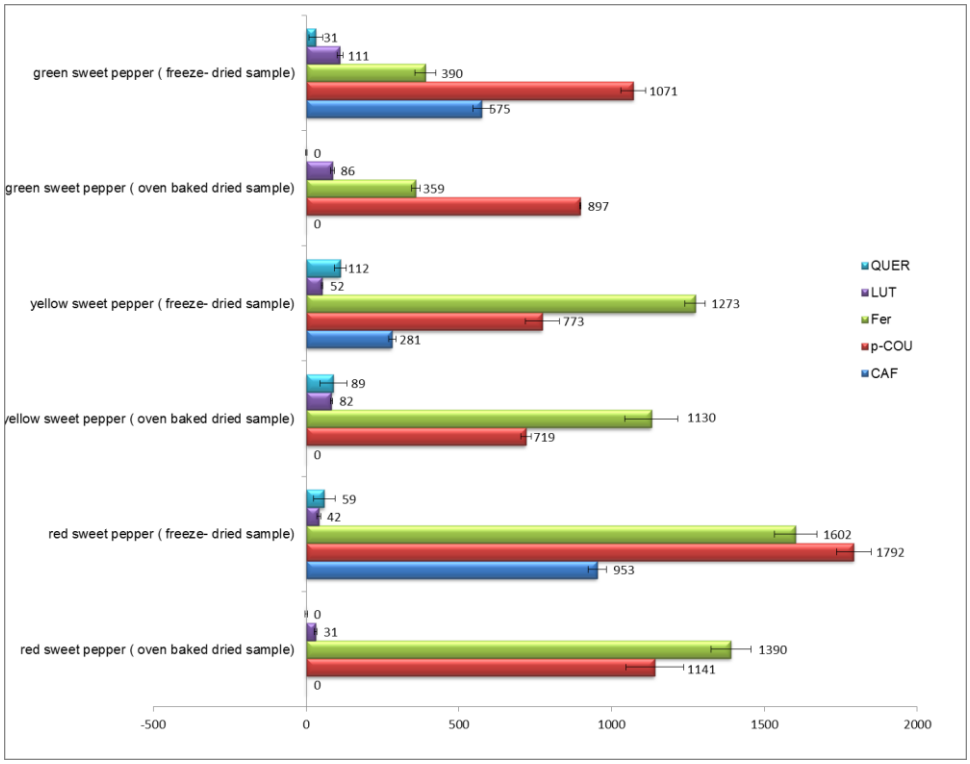


Figure 2. HPLC chromatograms of the separation of twelve active compound standards in methanol detected at 280 , 320 and 360-nm wavelengths

Table 3. Retention time (t_R), quantification absorbance (λ), correlation coefficient (R^2) of the calibration curves, LOD, and LOQ of all active compounds

Active compounds	t_R (min)	λ (nm)	LOD(mg/L)	LOQ (mg/L)	R^2
gallic acid	4.27	280	0.06	0.20	0.9995
p-hydroxybenzoic acid	11.31	280	0.09	0.30	0.9999
ellagic acid	21.20	280	0.25	0.80	0.9972
caffeic acid	12.51	320	0.03	0.10	0.9974
p-coumaric acid	19.77	320	0.03	0.10	0.9971
ferulic acid	21.98	320	0.06	0.20	0.9979
cinnamic acid	36.03	280	0.03	0.10	0.9984
catechin	9.14	280	0.25	0.80	0.9997
epicatechin	11.87	280	0.25	0.80	0.9986
luteolin	34.99	360	0.03	0.10	0.9977
quercetin	35.37	360	0.03	0.10	0.9961
kaempferol	41.19	360	0.03	0.10	0.9969

Fig. 3 shows the types and active compounds contents in the unit of µg of active compound/100 g of fresh sample. *p*-Coumaric acid and feruic acid were the predominant phenolic compounds found in all sweet pepper crude extracts. It was also found that five active compounds including caffeic acid, *p*-coumaric acid, feruic acid, luteolin and quercetin were found in all sweet pepper crude extracts from freeze-dried samples. As expected, the amounts of active compounds in sweet pepper crude extracts from oven baked dried samples, were less than those from freeze-dried samples. Four active compounds including *p*-coumaric acid, feruic acid, luteolin and quercetin were found in the yellow sweet pepper crude extracts, while quercetin was not detected in red and green sweet pepper crude extracts. It is worth to note that the preparation of the samples via an oven-drying method (70^{o}C) may affect to the types of active compounds found due to the decomposition of some active compounds. As a result, the numbers of active compound found in all capsicum crude extracts were lower than those from freeze-dried samples.



*CAF=Caffeic acid, *p*-COU=*p*-Coumaric acid, Fer=Feruic acid, LUT=Luteolin, QUER=Quercetin

Figure 3. Quantitative and qualitative analyses of all active compounds in the unit of µg /100 g of fresh sample

CONCLUSION

Investigations of total phenolic contents of green, red and yellow sweet pepper (*capsicum annuum* L.) crude extracts from oven-dried and freeze-dried samples were presented in this work. All sweet pepper crude extracts from freeze-dried samples showed better antioxidant activity and contained higher amounts of phenolic compounds than those from oven baked dried samples. The yellow sweet pepper crude extracts showed high antioxidant activity and total phenolic content, followed by red and green sweet pepper crude extracts, respectively. A HPLC method was employed for identification and quantification of the active compounds in sweet pepper crude extracts. The results showed that sweet pepper crude extracts from freeze-dried samples had higher amounts of phenolic acids and flavonoids than those from the other method. In addition, it was also found that five active compounds, including caffeic acid, *p*-coumaric acid, feruilc acid, luteolin and quercetin, were present in all crude extracts preparing from freeze dried samples. In the case of crude extracts preparing from oven baked samples, four active

compounds including *p*-coumaric acid, feruic acid, luteolin and quercetin were detected in yellow sweet pepper crude extracts, while quercetin was not detected in red and green sweet pepper crude extracts.

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