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Contributed Paper

Prediction of Antioxidant Capacity of Thai Vegetable Extracts by Infrared Spectroscopy

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ABSTRACT

The antioxidant capacity of extracts from 33 local Thai vegetables was determined by ABTS, DPPH, FRAP and FCRC assays. The extracts had a wide range of antioxidant activities and could be categorized into three groups: high, moderate and low antioxidant activities. The antioxidant activities of the four different assays were highly correlated ($r > 0.98$). Fourier transform infrared (FTIR) spectra and partial least square (PLS) regressions were used to predict the measured antioxidant capacities. The adjusted R^2 was higher than 0.97 for prediction models and higher than 0.89 for cross-validation models, which could be applied on liquid extracts or freeze-dried extract. FTIR spectroscopy in combination with PLS regression successfully predicted the antioxidant capacity of several Thai vegetables.

Keywords: Thai vegetables, antioxidant, infrared spectroscopy, partial least square regression

1. INTRODUCTION

Consumption of vegetables is associated with a lower risk of chronic diseases, e.g., hypertension, cardiovascular disease and stroke, and may also lower risk of cancer, type 2 diabetes mellitus, dementia and osteoporosis [3]. These health-promoting effects were ascribed in part to the antioxidant characteristics of polyphenols found in the vegetables [25].

Many *in vitro* methods have been developed to determine antioxidant capacities. However, a single antioxidant assay cannot represent the total antioxidant capacity of samples. Therefore, it has been recommended that different antioxidant assays should be carried out to analyze how they correlated [11] but to perform all these assays is time-consuming and requires many chemical reagents. To address this, infrared spectroscopy with multivariate analysis has been applied to rapidly quantify the antioxidant capacities of food samples.

However, most applications of Fourier transform infrared spectroscopy (FTIR) in combination with partial least square (PLS) regression were focused on the same plant samples, e.g., red wine [29], onion and shallot [12], while studies on different plant species were focused on fruit samples [24].

The objective of this study was to investigate the potential of FTIR and PLS regressions as a rapid substitute for chemical-based antioxidant capacity evaluation methods.

2. MATERIALS AND METHODS

2.1 Materials

Local vegetables were purchased from a market or obtained directly from farms in Chiang Mai, Thailand during August-September 2012 (Table 1). Moisture content of the vegetables was determined by the AOAC method [1].

Analytical grade of ethanol was obtained from J.T. Baker (Coopersburg, PA, USA). Folin-Ciocalteu phenol reagent, 1,1-diphenyl 2-picrylhydrazyl (DPPH),2,22-azinobis (3-ethylbenzothiazo-line-6-sulfonic acid) diammonium salt (ABTS), 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ), gallic acid and (±)-6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid (Trolox) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Table 1. Antioxidant capacities of vegetable extracts.

No.	Name	Part	Antioxidant capacities[a]			
			FCRC[b]	DPPH[c]	ABTS[c]	FRAP[c]
1	*Mangifera indica* L. cv. Talapnak	Leaves	17.77	250.79	247.83	240.77
2	*Mangifera indica* L. cv. Chokanan	Leaves	16.28	220.36	221.97	219.23
3	*Mangifera indica* L. cv. Namdokmai	Leaves	19.47	279.16	247.77	265.82
4	*Ficus lacor* Buch.	Leaves	19.07	258.09	201.79	260.12
5	*Spondias pinnata* Kurz.	Leaves	19.95	310.15	245.78	306.13
6	*Cratoxylum cochinchinense* (Lour.) Blume	Leaves	6.40	76.69	59.68	48.26
7	*Melissa officinalis* L.	Leaves	6.86	92.36	81.51	98.19
8	*Ocimum basilicum* L.	Leaves	5.39	71.81	62.69	71.05
9	*Neptunia oleracea* Lour.	Leaves	5.99	94.62	76.39	69.79
10	*Gymnema inodorum* Decne.	Leaves	2.23	14.10	14.93	12.97
11	*Apium graveolens* L.	Leaves	1.67	18.10	18.02	17.21
12	*Leucaena glauca* Benth.	Leaves	9.46	111.24	97.78	122.30
13	*Acanthopanax trifoliatum* Merr.	Leaves	4.20	56.88	48.57	50.28
14	*Polygonum odoratum* Lour.	Leaves	4.75	68.61	60.68	58.25
15	*Ocimum gratissimum* L.	Leaves	5.49	60.29	85.29	90.67
16	*Acacia pennata* (L.) Willd	Leaves	2.35	4.45	6.57	4.41
17	*Tiliacora triandra* Diels	Leaves	1.43	16.56	13.26	14.81
18	*Ocimum sanctum* L.	Leaves	1.72	23.78	20.35	25.45
19	*Sphenoclea zeylanica* Gaertn.	Leaves	1.60	15.66	12.66	14.44
20	*Ocimum×citriodorum*	Leaves	1.46	17.22	16.12	18.85
21	*Basella alba* L.	Flowers	2.05	1.04	8.03	5.86
22	*Garcinia cowa* Roxb.	Leaves	1.32	8.27	6.62	13.36

Table 1. Continued.

No.	Name	Part	Antioxidant capacities[a]			
			FCRC[b]	DPPH[c]	ABTS[c]	FRAP[c]
23	*Eryngium foetidum* L.	Leaves	0.98	7.48	10.51	10.00
24	*Moringa oleifera* Lam.	Leaves	1.41	13.46	10.83	11.76
25	*Diplazium esculentum* (Retz.) Swartz	Leaves	1.46	4.39	9.45	4.61
26	*Colubrina asiatica* (L.) Brongn.	Leaves	1.13	9.35	8.03	7.73
27	*Spilanthes acmella* Murr.	Leaves	0.95	7.90	9.07	9.16
28	*Amaranthus lividus* L.	Leaves	0.99	4.08	9.16	8.46
29	*Basella alba* L.	Flowers	1.87	0.79	8.37	7.09
30	*Piper sarmentosum* Roxb. Ex Hunter	Leaves	0.81	2.26	6.10	4.74
31	*Telosma minor* Craib	Flowers	1.49	6.63	12.91	7.82
32	*Morinda citrifolia* L.	Leaves	0.90	6.14	5.64	5.01
33	*Sesbania javanica* Miq.	Flowers	0.87	8.91	7.40	5.78

[a] Means of triplicate analysis.
[b] Unit of FCRC was g GAE equivalent/100 g dry weight
[c] Unit of DPPH, ABTS, FRAP was mmol Trolox equivalent/100 g dry weight

2.2 Preparation of Vegetable Extracts

Edible parts of 33 vegetables, without bruise or visual defect, were selected and washed with tap water for about 1 min. Each vegetable (10 g) was extracted with 60% ethanol (50 ml) using a blender (Model MR 4050 CA, Braun, Spain) for 30 sec. The solution was filtered through double layer of muslin cloth and then centrifuged at 2,500 g for 20 min. The samples were divided into two parts. The first part was subjected to antioxidant capacity assay while the other part was evaporated, freeze-dried and ground into powder. The dry powder was re-dissolved in 50% ethanol and then analyzed for antioxidant capacity. Three different extractions were performed for each vegetable.

2.3 Antioxidant Capacity Assays

Folin-Ciocalteu reducing capacity (FCRC) was analyzed by the modified method of Dudonnei *et al.* [6]. In brief, 20 mL of sample was mixed with 100 mL of 10% Folin-Ciocalteu reagent and 80 mL of 7.5% sodium carbonate. The mixture was allowed to stand for 1 h and then the absorbance was measured at 755 nm by Synergy HT microtitre plate reader (Biotek Instruments, Winooski, VT, USA). The result was expressed as gram gallic acid equivalent (GAE)/100 g dry plant, or gram GAE/100 g extract.

ABTS radical scavenging capacity (RSC) was analyzed according to the method of Re *et al.* [21]. The stock solution of 7 mM ABTS and 2.45 mM potassium persulfate were mixed at the ratio of 1:1 and left to stand in the dark at room temperature for 12-16 h. Then, 20 mL of plant extract was mixed with 280 mL of $ABTS^{\bullet+}$ solution and the absorbance was read at 730 nm. The result was expressed as mmol Trolox equivalent (TE) /100 g dry plant, or mmol TE/100 g extract.

DPPH radical scavenging capacity (RSC) assay was determined according to the modified method of Payet *et al.* [19]. In brief,

20 mL of sample was mixed with 280 mL of 1 mM DPPH solution and incubated for 1 h in dark condition. The absorbance was recorded at 520 nm. The result was expressed as mmol TE/100 g dry plant, or mmol TE/100 g extract.

Ferric reducing antioxidant power (FRAP) was determined according to the method of Benzie and Strain [2]. FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2, 4, 6-tris (2-pyridyl)-s-triazine (TPTZ) solution (in 40 mM HCl) and 20 mM $FeCl_3$ solution in the proportions of 10:1:1 (v/v) and warmed to 37 °C in a water-bath prior to use. Twenty mL of sample was mixed with 280 mL of FRAP reagent and incubated for 4 min before measuring the absorbance at 593 nm. The result was expressed as mmol TE/100 g dry plant, or mmol TE/100 g extract.

TE value was calculated using the following equation:

$$\text{mmol TE/100 g DW of vegetable} = \frac{A \times V \times D}{10 \times W \times (100 - M)}$$

$$\text{mmol TE/100 g freeze-dried extract} = \frac{A \times V \times D}{10 \times W}$$

where

A = TE calculated from standard curve (mM)

V = volume of solvent used for vegetable extraction or extract solubilization (ml)

D = dilution factor

W = fresh weight of vegetable or weight of freeze-dried extract (g)

M = moisture content of vegetable (%)

2.4 FTIR Spectroscopy

Vegetable extract powders (~1 mg) were mixed with KBr (10 mg) and compressed into pellets. Fourier transform infrared (FTIR) spectra were measured by Tensor 27 (Bruker Optics, Ettlingen, Germany) at wavelengths varying from 4,400 to 400 cm^{-1} with a resolution of 2 cm^{-1}.

2.5 Statistical Analysis

Statistical analysis was performed by R version 2.15.2 (http://cran.r-project.org/). K-means clustering was analysed by 'cluster' package. Partial least square (PLS) regression was performed by 'pls' package. Data scaling was performed prior to statistical analysis. FTIR spectra were used as explanatory variables while antioxidant capacities were used as reference values. Cross-validation was performed by the leave-one-out method. The optimum number of PLS components was selected based on the minimum value of the root mean square error (RMSE) of validation to avoid over-fitting of the model [24].

3. RESULTS AND DISCUSSION

3.1 Antioxidant Capacities of Vegetable Extracts

The samples, based on all measured antioxidant values (Table 1), could be categorized into three groups based on their antioxidant values: high (Group 1), middle (Group 2) and low (Group 3) (Figure 1). The first two components explained 99.68% of the point variability. Loadings of the first component were similar between all variables (0.499 to 0.500), indicating that all antioxidant assays were equally important for the model. Samples 1 to 5 exhibited high antioxidant values, and were classified together into Group 1. Samples 6 to 9 and 12 to 15 exhibited moderate antioxidant values, and were classified together into Group 2. The remaining 20 samples, with low antioxidant values, were combined into Group 3.

Folin-Ciocalteu reagent contains phosphomolybdic/phosphotungstic acid

complex which can be reduced by phenolic compounds and other reducing species to form blue complex [13]. This method has been widely used to measure total phenolic content (TPC) of food and agricultural products. However, the reagent is not specific to phenolic compounds only. It can also be reduced by other non-phenolic species, e.g., proteins, thiols, many vitamin derivatives, Fe^{2+}, Mn^{2+}, I^- and SO_3^{2-} [7]. Therefore, the name of this assay has been proposed as Folin-Ciocalteu reducing capacity (FCRC), and has been cited in other works [10; 14].

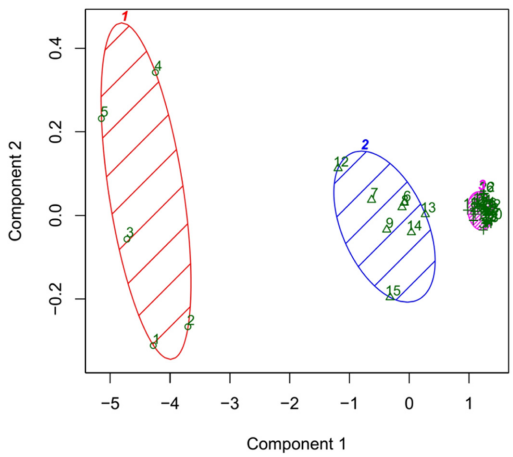

Figure 1. K-means clustering of samples based on ABTS, DPPH, FRAP and FCRC values.

FCRC values of the plants used in this study varied from 0.81 to 19.95 g GAE/100 g dry weight (DW). The highest value was found in *Spondias pinnata* Kurz. leaves. Average FCRC values for the extracts in Groups 1, 2 and 3 were 18.51, 6.07 and 1.43 g GAE eq./100 g DW, respectively. The FCRC values were in the range of the methanolic extracts of 133 Indian medicinal plants (0.06 to 41.47 g GAE/100 g DW) [28] and 112 Chinese medicinal plants (0.19 to 50.20 g GAE/100 g DW) for the aqueous extract and 0.22 to 50.30 g GAE/100 g DW for the methanolic extracts [4]. The results were also similar to those of nine culinary herbs and spices (1.85 to 14.7 g GAE/100 g DW) [9]. Another study of 26 Thai indigenous plants reported FCRC values in the range of 0.53 to 17.8 g GAE/100 g DW [15] which was similar to this study. However, the FCRC values were much higher than those reported in 32 herbs, with values up to 15.15 mg GAE/100 g DW [30].

DPPH, ABTS and FRAP assays are widely used in antioxidant assays based on electron transfer from antioxidants to the stable non-biological radicals [10]. DPPH, ABTS and FRAP values of Thai vegetable extracts ranged from 0.79 to 310.15, 5.64 to 247.83 and 4.41 to 306.13 mmol TE/100 g DW, respectively. *Spondias pinnata* Kurz had the highest antioxidant values in DPPH and FRAP assays while *Mangifera indica* L. cv. Talapnak had the highest ABTS radical scavenging capacity. The TE value obtained in this study was similar to the range reported in previous studies. ABTS and FRAP values of Indian medicinal plants ranged from 0.16 to 500.70 and n.d. to 679.69 mmol Trolox eq./100 g DW, respectively [28].

In this study, the TE values obtained from different assays were not significantly different. The mean values were 64.90, 59.14 and 63.95 mmol Trolox eq./100 g DW for DPPH, ABTS and FRAP, respectively. This result differs from other studies. In onion and shallot, the value was similar between FRAP and DPPH assays, but higher in ABTS assay [12]. For Indian medicinal plants, the values were similar for ABTS and DPPH but higher in FRAP (means of 27.07, 28.05 and 6.56 mmol Trolox/g DW, respectively) [28]. For herbs from Poland, the values were different among all assays, with means of 34.58, 234.14 and 167.21 mmol Trolox/100 g DW for ABTS, DPPH and FRAP,

respectively [30].

Significant correlations were found between ABTS, DPPH, FRAP and FCRC values ($r > 0.98$, $P < 0.05$) (Figure 2) which has also been reported extensively in the literature [28; 30]. The correlation of antioxidant values was due to the same electron transfer antioxidant mechanism of these assays [4]. These assays used oxidant or free radical with color ($DPPH^{\bullet}$, $ABTS^{\bullet+}$, $[Fe(III)(TPTZ)_2]^{3+}$ in FRAP and Mo(VI) in FCRC) as a probe. When the probe obtained electrons from antioxidants, it turned into reduced form and the color changed [10].

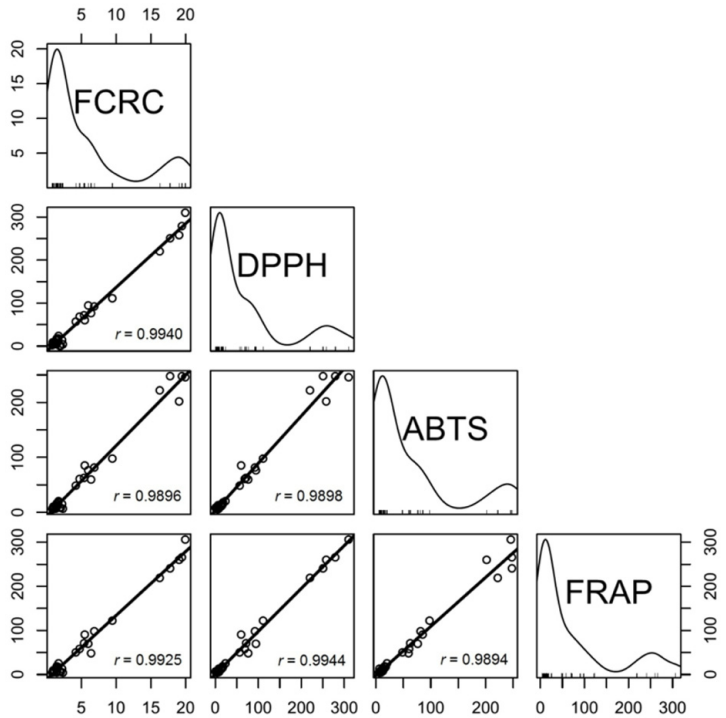

Figure 2. Correlation between FCRC, DPPH, ABTS and FRAP assays with Pearson's correlation coefficient (r).

3.2 FTIR-PLS Regression of Antioxidant Capacity

FTIR spectra of the freeze-dried vegetable extracts are shown in Figure 3. These spectra were used for PLS regression in combination with antioxidant capacities of the liquid extracts or the re-dissolved freeze-dried extract. The adjusted R^2 of the calibration model was higher than 0.97 for all response variables while the value of the cross-validation model was lower, ranging between 0.89 and 0.95 (Table 2). The calibration error was 6 to 11% for the freeze-dried sample and 12 to 15% for the liquid extract. The cross-validation error (CVE) was 15 to 38%. These values were similar to PLS regression of anthocyanins in wine, which showed 25 to 48% CVE [24]. CVE values were low for FCRC and FRAP compared to DPPH and ABTS models in both extracts.

The correlation between the infrared spectra and antioxidant capacities in the PLS regression model was presented as the coefficient value. Regression coefficients of all regression models had similar patterns for both the liquid extract (data not shown) and the freeze-dried extract (Figure 4). For a

transmittance scale of infrared spectrum, the sample with more functional groups exhibited the lower value in the infrared spectrum. Therefore, a negative regression coefficient indicated the presence of a functional group with electron-donating effect and was responsible for the antioxidant activities of the sample, while the positive coefficient value should represent a functional group with electron-withdrawing effect that reduced the antioxidant activities of the samples.

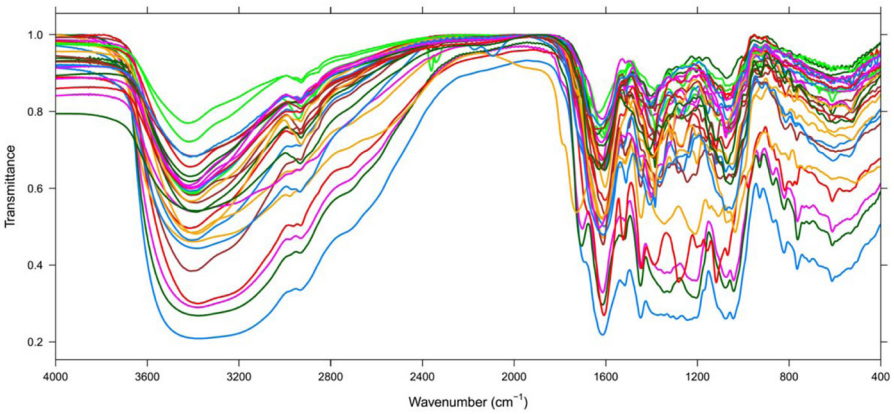

Figure 3. FTIR spectra of vegetable extracts.

Table 2. Parameters and results of PLS regression.

Antioxidant capacities	Data[a]		No. of samples	PLS components	Calibration			Cross-Validation		
	Mean	Range			Adj.-R^2	RMSE	% CE	Adj.-R^2	RMSE	% CE
Liquid extract										
FCRC	4.87	1.05-15.91	27	5	0.9723	0.61	12.54	0.9123	1.08	22.11
DPPH	26.36	0.12-102.23	29	7	0.9773	3.93	14.92	0.8999	8.18	31.04
ABTS	25.43	0.88-99.88	31	10	0.9876	3.36	13.23	0.8944	9.70	38.15
FRAP	30.22	2.18-127.78	27	7	0.9853	3.68	12.19	0.9336	7.71	25.53
Freeze-dried extract										
FCRC	22.12	4.87-51.78	29	9	0.9881	1.49	6.75	0.9331	3.51	15.87
DPPH	125.76	2.67-405.52	30	11	0.9879	13.87	11.02	0.8968	40.04	31.84
ABTS	98.72	6.22-358.18	30	11	0.9904	9.69	9.81	0.9061	29.87	30.26
FRAP	103.16	9.32-313.40	28	9	0.9879	10.17	9.86	0.9512	20.29	19.67

[a] Unit of FCRC was g GAE equivalent/100 g dry weight; unit of DPPH, ABTS, FRAP was mmol Trolox equivalent/100 g dry weight

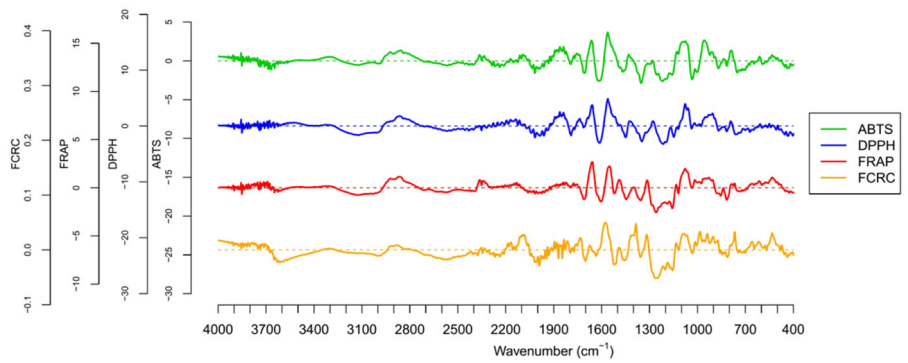

Figure 4. Regression coefficients for the prediction of antioxidant capacity of freeze-dried vegetable extracts.

Many negative coefficient peaks can be assigned to the vibration of the aromatic ring. This aromatic ring is a structural composition of phenolic compounds, e.g., flavonoid, anthocyanidin and hydroxycinnamic acid - antioxidants commonly found in fruits and vegetables [18]. Vibrations of other functional groups of flavonoid ring are shown in Table 3.

The vibration of the hydroxyl group of alcohol, phenol, carboxylic acid and flavonoids can be assigned from negative coefficient peaks. Radical scavenging activity (ABTS and DPPH methods) of phenolic compounds is influenced by the number and position of hydroxyl groups. For example, antioxidant activity of gallic acid (trihydroxybenzoic acid) was higher than those of protocatechuic acid (dihydroxybenzoic acid) and salicylic acid (monohydroxybenzoic acid) [23]. Hydroxylation at 3, 3' and 4' positions of flavonoid ring greatly enhanced peroxynitrite scavenging activity [8].

Table 3. Negative peaks of regression coefficient and their designated functional groups.

Negative peaks of coefficient (wavenumber, cm^{-1})				Reference range	Functional group vibration mode	References
FCRC	DPPH	ABTS	FRAP			
	3852		3852	3843	C-H stretch of flavonoids	[17]
3618		3669	3647	3600-3700	O-H stretch of alcohol or phenol	[27]
	3121	3119	3125	3033-3452	O-H stretch of gallic acid	[16]
3001	3013	2993	3028	3010-3040	Vinyl C-H stretch	[5]
2563	2565	2565	2567	2500-3000	O-H stretch of carboxylic acid	[39]
1991, 1846, 1701	1962, 1792	1792, 1707	1713	1650-2000	Several bands of substituted benzene rings	[27]
1636, 1522, 1508	1614	1614	1605, 1522	1580-1615, 1450-1510, 1657, 1552	Aromatic C=C-C stretch, C-O stretch of flavonoids, Rings A and B deformation of flavonoids	[5; 17]

Table 3. Continued.

Negative peaks of coefficient (wavenumber, cm^{-1})				Reference range	Functional group vibration mode	References
FCRC	DPPH	ABTS	FRAP			
	1449	1466	1447	1430-1470, 1439	Methyl C-H asymmetrical bend, C-C-O asymmetric stretch of flavonoids	[5; 17]
	1348	1352		1330-1430, 1310-1390, 1384	In-plane O-H bend of alcohol, C-O-H deformation of phenol, C-O-H in-plane bend of flavonoids	[22; 27]
1261		1238	1260	1200-1300	C-O stretch of aromatic carboxylic acid	[27]
1105, 1032	1215, 1186	1219, 1186, 1034	1157, 1144	950-1225, 970-1250	Aromatic C-H in-plane bend; Alcohol or phenol C-O stretch	[5; 22]
814	868, 818	870, 818		735-900	Disubstituted benzene	[5]
700	762	750	729	590-770	O-H out-of-plane bend of alcohol or phenol	[5]
438	434		426	406	O-H out-of-plane bend of flavonoid	[17]

The vibration of C=C was found on both positive (ν 1660 cm^{-1}) and negative (ν 1610 cm^{-1}) coefficient peaks. Reduction of the 2,3 double bond in the C ring of quercetin into taxifolin (dihydroquercetin) greatly reduced the antioxidant activity. However, reduction of the 2, 3 double bond in the C ring of kaempferol into dihydroxykaempferol had no significant effect on antioxidant activity [23]. In contrast, substitution of a double bond with the hydroxyl group, especially in the B-ring, improved antioxidant activity of flavonoid [26].

Vibration of the methyl group (ν 2860 cm^{-1}) correlated with the reduction of antioxidant activity. Methylation and glycosylation had a steric effect on the hydroxyl group at B ring and suppressed the antioxidant activity of flavonoids [23]. Quercetin aglycone had higher antioxidant activity than glucuronide and rutinoside of quercetin. Kaempferol glycosides had much lower antioxidant activity than the corresponding glycosides of quercetin, but were not different from kaempferol aglycone [20].

In addition, based on our unpublished data using online HPLC-ABTS and HPLC-MS data, the major antioxidants in the samples were hydroxycinnamic acids and flavonoids. For example, rosmarinic acid, chicoric acid, caftaric acid and dihydroxy-dimethoxyflavone were major antioxidants in Lamiaceae plants. Caffeoylquinic acids were found in *Ficus lacor, Acanthopanax trifoliatum*, *Amaranthus lividus* and *Moringa oleifera*. Epigallocatechin gallate and myricetin rhamnoside were major antioxidants in *Leucaena glauca*. These results confirmed the correlation between antioxidant activities and functional group data obtained from infrared spectroscopy-PLS regression.

FTIR spectroscopy and PLS regression exhibited the potential to predict four types of antioxidant activities of vegetable extracts from different species. A linear relation between measured and predicted antioxidant values was found in all assays, with $R^2 > 0.97$ (Figure 5). However, the difference between RMSE in the calibration and validation models was quite high. Therefore, a larger number of samples was required to better establish the PLS regression model. Samples from a broader range of species, different parts of plants, a variety of geographical conditions or different harvesting times should be included in further analysis.

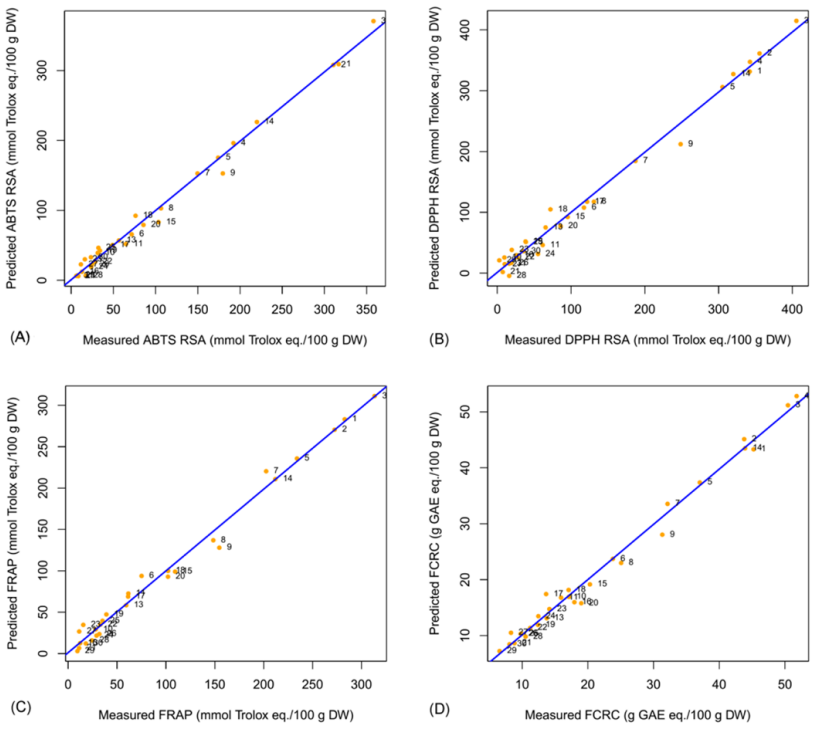

Figure 5. Measured and predicted antioxidant activities of freeze-dried extracts.

4. CONCLUSIONS

Thai vegetable extracts exhibited a wide range of antioxidant activities which could be categorized into three groups. The antioxidant activities of the four different assays were highly correlated. FTIR spectroscopy in combination with PLS regression was a simple and rapid method for predicting antioxidant capacities based on electron transfer mechanism, including FCRC, FRAP, DPPH and ABTS radical scavenging capacity of liquid and freeze-dried vegetable extracts. Further investigation should be conducted on antioxidant capacity based on hydrogen atom transfer mechanism.

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