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การวิเคราะห์วิตามินต้านออกซิเดชันในพืชไทย 30 ชนิด โดยวิธีโครมาโตกราฟีเหลวสมรรถนะสูง

Analysis of antioxidant vitamins in 30 Thai vegetables by high-performance liquid chromatographic method

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บทคัดย่อ :

การวิจัยนี้ได้วิเคราะห์หาปริมาณวิตามินต้านออกซิเดชั่นในสมุนไพรไทยที่รับประทานได้ จำนวน 30 ชนิด การหาปริมาณวิตามินซีซึ่งละลายในน้ำในตัวอย่างพืช ใช้วิธีสกัดด้วย 6% กรดออร์โธฟอสฟอริดและฉีดเข้าคอลัมน์ชนิดฟีนิลโดยใช้ isocratic mobile phase สำหรับการหาวิตามินที่ละลายในไขมันในตัวอย่างพืชใช้วิธีสกัดด้วยอะซิโตน และต่อมาทำ saponification แล้ว จึงสกัดด้วยส่วนผสม เฮกเซน-ไดเอทิลอีเธอร์ และฉีดเข้าคอลัมน์ชนิด reverse phase C_{18} ที่มี gradient mobile phase การประเมินวิธีการทดลองสำหรับสารมาตรฐานวิตามิน พบว่าให้ความสัมพันธ์กับความเข้มข้นเป็นเส้นตรง โดยมีค่า r^2 เท่ากับ 0.9997, 0.9987, 0.9998 และ 1.000 สำหรับวิตามินซี, เรตินอล, แอลฟาโทโคเฟอรัล และ เบตาคาโรทีน ตามลำดับ สำหรับการคำนวณหา % Bias และ CVs ของการวิเคราะห์แบบ intra และ inter เท่ากับ 2.54(1.22) - (1.99(2.23) และ -3.99 (0.32) - 1.61 (0.42); 0.35(0.43) - 1.41 (0.59) และ 0.18 (0.17) - 1.19 (1.36) ตามลำดับ

จากพืช 30 ชนิดที่วิเคราะห์สามารถตรวจพบวิตามินซีในพืช 20 ชนิด (มีปริมาณตั้งแต่ 2.04-114.89 มิลลิกรัม/100 กรัม) พืชทุกชนิดมีเบตาแคโรทีน (มีปริมาณ 0.15-6.78 มิลลิกรัม/100 กรัม) พบเรตินอลในพืช 12 ชนิด (มีปริมาณตั้งแต่ 0.0033-0.22 มิลลิกรัม/100 กรัม) ส่วนแอลฟาโทโคเฟอรัล พบในพืชเพียง 5 ชนิด (มีปริมาณตั้งแต่ 0.02-0.45 มิลลิกรัม/100 กรัม) พืชที่มีวิตามินสูง 5 อับดับแรก คือ ผักหวานบ้าน แค มะรุม ถอบแถบเครือ และมะระขี้นก งานวิจัยนี้ให้ข้อมูลที่สนับสนุนประโยชน์ของการบริโภคผักพื้นบ้านที่พบในภาคตะวันออกเฉียงเหนือ

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Abstract

High-performance liquid chromatographic (HPLC) method was applied to determine four antioxidant vitamins in 30 edible indigenous Thai plants. Plant samples for water-soluble vitamin C analysis were extracted with 6% orthophosphoric acid then injected onto a phenyl column using an isocratic mobile phase. Samples for fat-soluble vitamin determination were firstly extracted with acetone and after saponification the samples were subsequently extracted with hexane/diethyl ether. Then a reverse phase C_{18} column with a gradient mobile phase was used. The method validation for standard vitamins revealed linear concentration relationship with the square of correlation coefficient (r^2) of 0.9997, 0.9987, 0.9998 and 1.0000 for ascorbic acid, retinol, α-tocopherol and β-carotene, respectively. The accuracy expressed in terms of % Bias was from 2.54 (1.22) to 1.99 (2.23) and -3.99 (0.32) to 1.61 (0.42) for intra- and inter-day assay, respectively. % Coefficients of variation (CVs) were 0.35 (0.43) to 1.41 (0.59) and 0.18 (0.17) to 1.19 (1.36) for intra- and inter- day assay, respectively.

Among 30 studied plants, ascorbic acid was detected in 20 plants (ranging 2.04-114.89 mg/100g). All plants had β-carotene (ranging 0.15-6.75 mg/100g). Retinol was found in 12 plants (ranging 0.0033-0.22 mg/100g), whereas α-tocopherol was found in only 5 plants (ranging 0.02-0.45 mg/100g). The top five plants having high antioxidant vitamin content are *Sauropus androgynus* Merr., *Sesbania grandiflora* Derv., *Moringa oleifera* Lam., *Connarus semidecandrus* Jack., and *Momordica charantia* Linn. This study supports the claimed beneficial effects for consuming local edible vegetables of the people in the Northeast of Thailand.

Keywords: antioxidant vitamins, ascorbic acid, β-carotene, HPLC, retinol, Thailand, α-tocopherol, vegetables

Introduction

Low antioxidant vitamin levels have been associated with chronic oxidative damages and attributed to several states of diseases including cancers, heart and HIV- diseases (Nomura, Stemmermann, Heibrun, Salkeld & Vuillenmier, 1985; Pace & Leaf, 1995.) A number of epidemiological studies have shown that low dietary intake of antioxidant vitamins is correlated with the increased incidence of mortality from cancers (Basu, Temple & Hodgson, 1988; Zeigler, 1989). The inverse association between plasma or serum antioxidant vitamins and the risk of cancers has been demonstrated in various types of cancer including breast (Kim, Ahn & Lee-Kim, 2001), lung (Willis & Wians, 2003) and gastric (Choi, Kim & Yu, 1999).

Fruits and vegetables are rich sources of antioxidant compounds. Polyphenols, flavonoids and antioxidant vitamins are granted for these beneficial effects. Studies have been performed on the quantification of polyphenols and flavonoids in several plants such as fresh herbs in diets (Justesen& Knuthsen,

2001), olive drupes (Owen et al, 2003), grapefruits (Gorinstein et al, 2004), citrus fruit juices (Kanaze, Kokkalou, Glorgarakis & Niopas, 2003), and beverages including beer (Nardini & Ghiselli, 2004), and teas (Kilmartin &Hsu, 2003). High-performance liquid chromatography has been successfully used for the antioxidant vitamin determination in foods (Pelletier, 1985; Ball, 1992), human tissues and plasma (Talwar, Ha, Cooney, Brownlee & Reilly, 1998; Lunetta et al, 2002; Sripanidkulchai, Vaikrutta, Sriamporn, Vatanasapt, Sripanidkulchai & Sirisangtrakul, 2003).

Several plant leaves are common nutritious vegetables such as *Sauropus androgynus* Merr., *Moringa oleigera* Lam., *Centella asiatica* Linn. (Padmavathi & Rao, 1990; Davadas, Chandrasekhar, Premakumari&Saishree, 1996; Seshadri & Nambiar, 2003). Traditionally, green leaf vegetable consumption is very popular among people in the Northeast of Thailand. At least 30 kinds of plants are sold as side-dish vegetables in the market. It is claimed that eating young leaves of the indigenous plants is a health benefit according to the vitamin and fiber contents. However, the existing vitamin types of these plants have not been reported. It is the aim of this study to investigate the antioxidant vitamin contents of the indigenous plants by the HPLC method.

Materials and Methods

Chemicals

Ascorbic acid (vitamin C), retinol (vitamin A), α-tocopherol (vitamin E), β-carotene, sodium dihydrogen phosphate were abtained from Fluka Biochemika (Swizerland). Acetone, acetonitrile, butylated hydroxy toluene (BHT), methanol, orthophosphoric acid, potassium hydroxide, sodium sulfate and tetrahydrofuran were purchased from Carlo Erba Reagent (Italy). Diethyl ether was obtained from Anala (England) and hexane from JT Baker (USA).

Determination of standard vitamins

The methods described for determination of vitamin C (Lloyd et al, 1988) and fat soluble vitamins (Ball, 1992) were modified in this study.

Standard solution and chromatographic system for vitamin C determination

A stock solution of ascorbic acid of 100 μg/ml was prepared in 6% orthophosphoric acid. The working standard solutions were 4, 8, 12, 16 and 20 μg/ml. The chromatographic system used was a Waters 600 HPLC (USA) with Nova-Pak Phenyl column (3.9 × 150mm.) connected to Nova-Pak C_{18} guard column with an isocratic mobile phase (0.05M sodium dihydrogen phosphate in 85% orthophosphoric acid, pH3), The flow rate was 1ml/min and the temperature was set at 25°C. Photodiode array detection (Waters 996) was recorded at 243 nm.

Standard solutions andchromatographic system for fat-soluble vitamin determination

For analysis of vitamins A and E, stock solutions of retinol and α-tocopherol were prepared at 100 μg/ml concentration in methanol. The working

standard solutions were 1.5, 3, 6, 12, 15, 25, 50 μg/ml and 1.5, 20, 40, 60, 80, 100 μg/ml for retinol and α-tocopherol, respectively. A stock solution of β-carotene was prepared at 100 μg/ml in acetonitrile and the working standard solutions were 0.16, 5, 10, 15, 20 μg/ml. A Nova-Pak reversed phase C_{18} column (4μm, 3.9×150mm) equipped with a Nova-Pak C_{18} guard column was used with gradient mobile phases. Mobile phase A consisting of methanol: acetonitrile (55:45) was programmed at 0-10 min for vitamins A and E detection and mobile phase B consisting of methanol: tetrahydrofuran (95:5) was continuously used at 11-35 min for β-carotene detection. The flow rate was 1ml/min and photodiode array detection was recorded at 291, 324, 450 nm for vitamin E, A and β-carotene, respectively. Millenium 32 software system was used in cell calculation

Method validation

For linearity, the peak area was evaluated with the square of correlation coefficient (r^2) by least-squares linear regression analysis. The accuracy of this method was expressed as % Bias which referred to [(Calculated concentration- added concentration)/added concentration]×100. The intra-day precision was determined from % CV of 4 different concentrations and 6 repeated assay, whereas the inter-day precision was the data of 3 days analysis.

Preparation of plant samples

Thirty fresh edible plants were collected from the local market in Ubonratchathani Province, in the Northeast of Thailand. For vitamin C determination, 3g of clean plant was grounded with 50ml of 6% orthophosphoric acid in a mortar, then vortex mixed for 5 min and filtered. For fat-soluble vitamins determination, 3 g of clean plant was ground with acetone until a clear solution was obtained. The sample was passed through 5g sodium sulfate by vacuum filtration and then dried by rotary evaporation at a temperature lower than 40°C. The dried sample was saponified with 100 ml of 10% KOH containing 0.1% vitamin C as an antioxidant, at room temperature for 24 h under the light protection and continuously stirred. Then partition extraction with 100 ml of hexane: diethyl ether (7:3) containing 0.1% BHT was performed. The solvent layer was collected and dried at a temperature below 40 °C. The dried sample was dissolved in 1 ml acetonitrile and kept in a light protected container and filtered before HPLC injection.

Determination of plant vitamins

The methods for standard vitamin C and fat-soluble vitamins as above described were applied for the plant samples.

Results

As shown in Figure 1, the chromatogram demonstrated retention times of 1.642, 2.060, 6.320 and 24.788 min for vitamin C, A, E and β-carotene standards, respectively. The results on the method validation are summarized in Table1. All vitamins determination revealed linear concentration relationship with r^2 of 0.9987-1.0000. The accuracy as expressed

Figure 1 High- performance liquid chromatogram of standard antioxidant vitamins at 10 µg/ml concentration: ascorbic acid (A); retinol (B); α- tocopherol (C); and β-carotene (D).

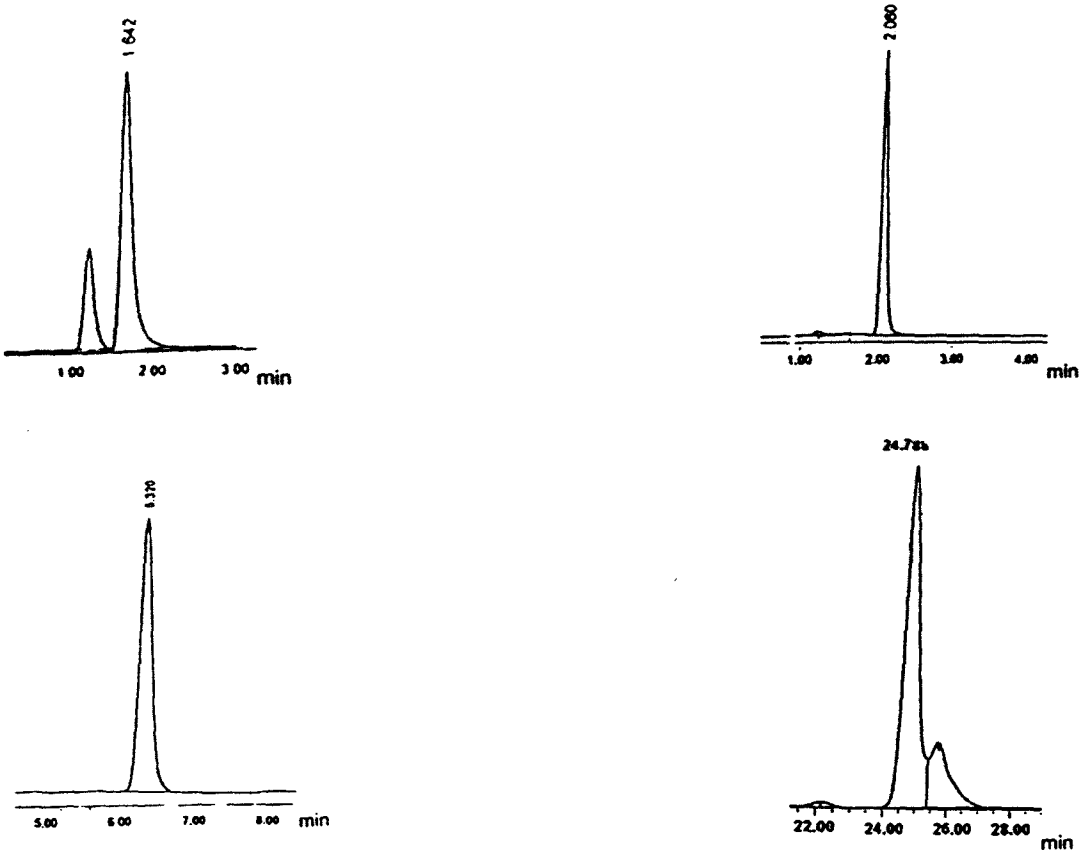

Table 1 Summary of Linearity (r^2), accuracy (% Bias), precision (%CV) and Limit of Quantification (LOQ) for method validation of antioxidant vitamins

vitamin	r^2	% Bias[1] (n=6)		% CV[2] (n=6)		LOQ
		Intra[3]	Inter[3]	Intra[3]	Inter[3]	(µg/ml)
Vitamin C	0.9997	1.19(2.23)	1.61(0.42)	1.41(0.59)	0.54(0.39)	4.0
Vitamin A	0.9987	-1.96(0.34)	-3.99(0.32)	0.35(0.43)	0.31(0.14)	1.5
Vitamin E	0.9998	-1.01(0.5)	-1.45(0.11)	0.51(0.44)	0.18(0.17)	1.5
β-carotene	1.0000	-2.54(1.22)	-1.46(1.22)	1.26(1.53)	1.19(1.36)	0.16

[1] % Bias (=[(calculated concentration-added concentration) / added concentration] x 100) were expressed as mean (SD)

[2] % CV (= SD/meanx100) expressed as mean (SD)

[3] For intra-day assay, 4 different concentrations and 6 repeated samples were performed, whereas inter-day assay was from 3 different days of experiments.

Figure 2 Example of HPLC chromatograms for antioxidant vitamins from plant extracts: ascorbic acid (A) retinol (B), β-carotene (D) peaks from extract of *Sesbania grandiflora* Derv. and α- tocopherol peak (C) from extract of *Connarus semidecandrus* Jack.

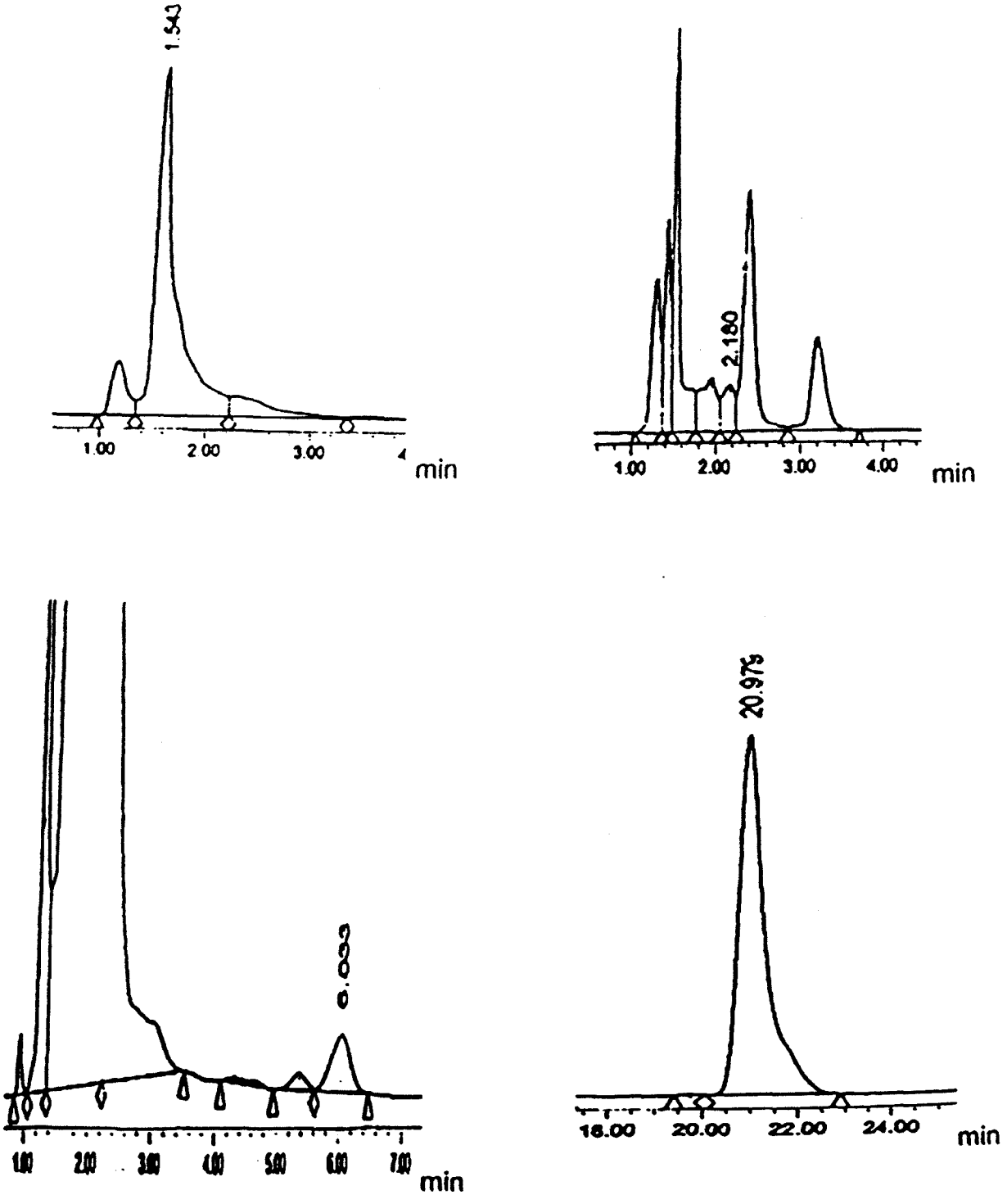

Table 2 Amount of detectable antioxidant vitamins in local edible plants.

Plant (Thai name)	Family	Part used	vitamins			
			C	A	β-carotene	E
1. *Sagittalia guayanensis* H.B.K (Pong)	Alismataceae	leaf	2.04 ± 0.01	-	0.70 ± 0.01	-
2. *Limnocharis flava* (L.) (Ta-la-pat-reu-si)	Alismataceae	Young leaf, flower	2.43 ± 0.01	0.03 ± 0.001	0.89 ± 0.01	-
3. *Aganosma marginata* Roxb. G.Don (Mok-kruea)	Apocynaceae	Young leaf	28.66 ± 0.15	-	0.87 ± 0.01	-
4. *Careya spherica* Roxb. (Kra-don)	Baringtoniaceae	Young leaf	-	0.22 ± 0.001	0.23 ± 0.00	-
5. *Spilanthes acmella* Merr. (Pak-krad-hau-wan)	Compositae	Young leaf	-	0.031 ± 0.001	2.28 ± 0.03	0.05 ± 0.01
6. *Connarus semidecandrus* Jack. (Tob-tab-krau)	Connaraceae	Young leaf	87.64 ± 0.47	-	5.54 ± 0.07	0.45 ± 0.01
7. *Cuscuta chinensis* Linn. (Foy-thong, Sai-mai)	Convolvulaceae	Whole plant	12.93 ± 0.07	-	0.15 ± 0.01	-
8. *Momordica charantia* Linn. (Ma-ra-kee-nok)	Cucurbitaceae	Young leaf	10.49 ± 0.06	0.027 ± 0.001	1.71 ± 0.02	-
9. *Phyllanthus roscus* Beille (Pak-kan-thong)	Euphorbiaceae	Young leaf	-	-	1.38 ± 0.02	-
10. *Sauropus androgynus* Merr. (Pak-wan-ban)	Euphorbiaceae	Young leaf	114.89 ± 0.62	0.009 ± 0.001	4.40 ± 0.05	-
11. *Cratoxylum formosum* (Jack) sup pruniflorum (Kurz. Gagel (Tew)	Guttiferae	Young leaf	-	0.025 ± 0.001	3.97 ± 0.05	-
12. *Garcinia cowa* Roxb. (Cha-muang, Som-mong)	Guttiferae	Young leaf	21.96 ± 0.12	-	2.0 ± 0.02	-
13. *Coleus amboinicus* Lour. (Hu-suea)	Lamiaceae	Young leaf	9.75 ± 0.05	0.03 ± 0.001	2.12 ± 0.03	0.083 ± 0.01
14. *Marsilea crenata* Presl. (Waen)	Marsilcaceae	leaf	-	-	0.41 ± 0.00	-
15. *Cissampelos pareira* L.var. hirsuta (Buch. ex. DC.) (Kruea-ma-noi)	Menispermaceae	Young leaf	20.28 ± 0.11	-	5.87 ± 0.07	-

Plant (Thai name)	Family	Part used	vitamins			
			C	A	β-carotene	E
16. *Tiliacora triandra* Diels. (Ya-nang)	Menispermaceae	leaf	12.80 ± 0.07	0.05 ± 0.001	1.09 ± 0.01	-
17. *Morus alba* Linn. (Mon)	Moraceae	Young leaf	58.72 ± 0.32	-	1.64 ± 0.02	-
18. *Moringa oleifera* Lam. (Ma-rum)	Moringaceae	Young leaf	73.64 ± 0.40	0.08 ± 0.001	6.75 ± 0.08	-
19. *Eugenia grata* Wight.var.Collinsae. Craib. (Mek)	Myrtaceae	Young leaf	-	0.0033 ± 0.001	0.53 ± 0.01	-
20. *Sesbania grandiflora* Derv. (Kae)	Pipillionoideae	Young leaf	104.60 ± 0.56	0.05 ± 0.001	3.23 ± 0.34	-
21. *Ceratopteris thalictroides* Bronga. (Kood)	Parkeriaceae	Young leaf	-	-	1.32 ± 0.02	-
22. *Piper sarmentosum* Roxb. (Cha-plu)	Piperaceae	Young leaf	16.03 ± 0.09	-	5.05 ± 0.06	-
23. *Polygonum oderatum* Louv. (Praew, Pai-nam)	Polygonaceae	Young leaf	22.55 ± 0.12	-	3.76 ± 0.04	-
24. *Fagraea fragrans* Roxb. (Kan-krao)	Gentianaeae	Young leaf	5.07 ± 0.03	-	2.25 ± 0.03	-
25. *Limnophila aromatica* Merr. (Ka – yaeng)	Scrophulariaceae	Whole plant	21.07 ± 0.11	-	0.97 ± 0.01	-
26. *Centella asiatica* Linn. (Bau-bok)	Umbelliferae	leaf	-	-	6.51 ± 0.08	0.05 ± 0.01
27. *Hydrocotyle javanica* Thunb. (Nong, Wan-kaew)	Umbelliferae	leaf	-	-	5.77 ± 0.07	-
28. *Viola betonicifolia* Sm. (Pai-noi)	Violaceae	Young leaf	2.29 ± 0.01	-	2.47 ± 0.03	-
29. *Careya trifolia* (Linn.) Domin. (Tau-kan)	Vitidaceae	Young leaf	-	0.0082 ± 0.001	1.47 ± 0.02	0.02 ± 0.01
30. *Spirogyra* sp. (Tau-nam)	Zygnemataceae	Whole plant	7.36 ± 0.04	-	1.52 ± 0.02	-

Values expressed as average from three sample injections in term of mg/100g of fresh plant

- = not detectable.

in terms of mean (SD), %Bias which was from - 2.54 (1.22) to 1.19(2.23) and - 3.99 (0.32) to 1.61(0.42) for intra- and inter-day analysis, respectively. The precision was clearly shown with %CV as 0.35 (0.43) to 1.41 (0.59) and 0.18 (0.17) to 1.19(1.36) for intra- and inter-day assay, respectively. The limits of quantifications (LOQ) were 4.0, 1.5, 1.5 and 1.16 μg/ml for vitamin C, A, E and β-carotene, respectively. All vitamins showed relative good stability under storage at 4°C in light protected containers for 3 hours.

The HPLC method was successful for the determination of these four antioxidant vitamins in plants as those seen in Figure 2. The amount of each vitamin is summarized in Table 2. The 30 collected edible plants, which are frequently consumed by the local people in the Northeast of Thailand, were in 26 families. Ascorbic acid was detected in 20 plants, ranging from 2.04 ± 0.01 to 114.89 ± 0.62 mg/100g of fresh plant. Retinol was found in 12 plants, ranging from 0.0033 ± 0.001 to 0.22 ± 0.001 mg/100g. β-carotene was found in all 30 plants, ranging from 0.15 ± 0.01 to 6.75 ± 0.08 mg/100g, whereas α-tocopherol was detected in only 5 plants, ranging from 0.02 ± 0.01 to 0.45 ± 0.01 mg/100g.

Discussion

The HPLC method was modified and successfully determined four antioxidant vitamins namely vitamin A, C, E and β-carotene in Thai edible vegetables. The water-soluble vitamin C in the plants was extracted by 6% orthophosphoric acid, whereas the plant fat-soluble vitamins were extracted and saponified to get the free form of the vitamins. The analysis method is very rapid, sensitive and gives good accuracy and precision. The %Bias and %CV of intra- and inter- assay for all four vitamins were very low (<15%) and within limits of acceptance. β-carotene was found to have the lowest detection limit with LOQ of 0.16 μg/ml

The method was useful for determination of plant leaf vitamins. However, it is essential to note that the chromatograms of standard ascorbic acid and β–carotene showed two separated peaks, which may due to the purity of chemicals. In this study the major peaks of these vitamins were used to calculate their amounts in plant extract. Among 30 selected edible vegetables, vitamin C can be detected in 20 plants. The young leaves of top five plants that had relatively high vitamin C content (more than 50mg/ 100g) were *S. androgynus, S. grandiflora, C. semidecandrus, M. oleifera. and M. alba.* β-carotene can be measured in all 30 plants. The plants with high β-carotene content (more than 5mg/100g) were *M. oleifera*, C. asiatica, C. pareira, H. javanica, C. *semidecandrus* and P. *sarmentosum.* The finding of high β-carotene content in M. *odeifera* confirmed previous studies on nutritional values and recommendation on consumption of this plant for vitamin A deficiency (Freiberger et al, 1998; Nambiar& Seshadri, 2001). Moreover, M. oleifera leaves also have high Zn content (Barminas, Charles & Emmanuel, 1998), Therefore, it suggests the health benefit of this plant's leaves in addition to the traditional consumption of the plant drumstick among Thais.

The vitamin A and E contents of these vegetables were relatively low. C. *amboinicus* was the only

plant that all four vitamins were detected. Five plants seemed to be good sources of antioxidant vitamins according to the high detectable amount of vitamin C, A and β-carotene; they were S. *androgynus*, S. *grandiflora*, M. *oleifera*, T. *triandra*, and M. *charantia*. All 30 plants are generally consumed along with the main local dish especially for lunch meals. These vegetables are also reported to have high antioxidant activity detected by the DPPH method (Sripanidkulchai, Sirisangtrakul, Priprem, Wangboonsakul, Tattawasart & Chantaranothai, 2003)

Our findings suggested the potential sources of antioxidant vitamins in vegetables in addition to fruits. Therefore, the consumption of local vegetables should be recommended to protect free radical caused chronic diseases such as heart disease and cancers.

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