# การเปรียบเทียบฤทธิ์ต้านอนุมูลอิสระในอาสาอาสมัครสุขภาพดีที่ได้รับสารสกัดขมิ้นชันในขนาดการใช้ที่ต่างกันด้วยวิธี ออกซิเจนแรดดิคอลแอบซอร์แบนซ์คาพาซิตี้

นายคณิต ปึงเจริญกุล



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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# COMPARISON OF THE *IN VIVO* TOTAL ANTIOXIDANT CAPACITY IN HEALTHY VOLUNTEERS AT THE DIFFERENT CURCUMINOIDS DOSES BY OXYGEN RADICAL ABSORBANCE CAPACITY ASSAY

Mr. Kanit Pungcharoenkul



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| By | Mr. Kanit Pungcharoenkul |
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| Thesis Advisor | Associate Professor Phensri Thongnopnua, Ph.D. |

Accepted by the Faculty of Pharmaceutical Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

..... Dean of the Faculty of Pharmaceutical Sciences

(Associate Professor Pintip Pongpech, Ph.D.)

THESIS COMMITTEE

..... Chairman

(Associate Professor Darawan Tanyavutti)

..... Thesis Advisor

(Associate Professor Phensri Thongnopnua, Ph.D.)

..... External Examiner

(Associate Professor M.L. Sumarn Saraya, Ph.D.)

..... Examiner

(Assistant Professor Kulwara meksawan, Ph.D.)

คณิต ปึงเจริญกุล : การเปรียบเทียบฤทธิ์ต้านอนุมูลอิสระในอาสาอาสมัครสุขภาพดีที่ได้รับสารสกัดขมิ้นชันในขนาดการใช้ที่ต่างกันด้วยวิธี ออกซิเจนแรดดิคอลแอบซอร์แบนซ์คาพาซิตี้. (COMPARISON OF THE *IN VIVO* TOTALANTIOXIDANT CAPACITY IN HEALTHY VOLUNTEERS AT THE DIFFERENT CURCUMINOIDS DOSES BY OXYGEN RADICAL ABSORBANCE CAPACITY ASSAY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. เพ็ญศรี ทองนพเนื้อ, 127 หน้า.

การศึกษาฤทธิ์ต้านอนุมูลอิสระในอาสาสมัครสุขภาพดีที่ได้รับสารสกัดขมิ้นชัน ในขนาดต่างกันด้วยวิธีออกซิเจนแรดดิคอลแอบซอร์แบนซ์คาพาซิตี้ ใช้อาสาสมัครสุขภาพดีทั้งชายและหญิง จำนวน 24 คน แบ่งออกเป็น 3 กลุ่ม กลุ่ม A และ B ได้รับสารสกัดขมิ้นชันในขนาด 500 มิลลิกรัมต่อวัน และ ขนาด 6 กรัมต่อวัน ตามลำดับ ส่วนกลุ่ม C ได้รับผลิตภัณฑ์เสริมวิตามินอีในขนาด 200 IU ต่อวัน โดยอาสาสมัครทุกคนได้รับสารสกัดขมิ้นชันหรือ ผลิตภัณฑ์เสริมวิตามินอี เป็นระยะเวลานาน 7วัน ตัวอย่างเลือดที่เก็บจากอาสาสมัครก่อนการทดลอง และในวันที่ 1 กับวันที่ 7 หลังการรับประทานเป็นเวลา 12 ช.ม. ถูกทำการปั่นแยกทันทีเพื่อแยกพลาสมาเก็บไว้ที่ -48 องศาเซลเซียส และนำไปวิเคราะห์ต่อไป การศึกษานี้ไม่พบอาการไม่พึงประสงค์จากการใช้สารสกัดขมิ้นชัน และผลิตภัณฑ์เสริมวิตามินอี ตลอดระยะเวลาที่ทำการศึกษาและหลังทำการศึกษา 7วัน ก่อนเริ่มการศึกษา ความเข้มข้นของระดับแอลฟาโทโคเฟอรอลในพลาสมาอยู่ในช่วงค่าปกติของคนสุขภาพดี (7.58 – 19.52 µg/ml) ค่าฤทธิ์ต้านอนุมูลอิสระเปรียบเทียบก่อนและหลังรับประทานสารสกัดขมิ้นชัน ในอาสาสมัครกลุ่ม A วัดได้ 5,797-15,689 และ 8,269-20,186 µmol TE/L ตามลำดับ ส่วนของกลุ่ม B วัดได้ 9,022-24,834 และ 3,530-20,595 µmol TE/L ตามลำดับ ไม่พบการเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ ของค่าฤทธิ์ต้านอนุมูลอิสระ ภายหลังรับประทานสารสกัดขมิ้นชันทั้งในกลุ่ม A และกลุ่ม B ( $p$= 0.270 และ 0.33 ตามลำดับ) สำหรับกลุ่ม C ค่าฤทธิ์ต้านอนุมูลอิสระก่อนและหลังรับประทานผลิตภัณฑ์เสริมวิตามินอี วัดได้ 8,422-13,922 และ 9,216-23,574 µmol TE/L ตามลำดับ สำหรับการวิเคราะห์ความเข้มข้นของแอลฟาโทโคเฟอรอลในพลาสมา พบว่ามีการเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติของระดับแอลฟาโทโคเฟอรอลในพลาสมา โดยเพิ่มขึ้นจาก 9.16 $\pm$ 1.21 เป็น 14.38 $\pm$ 2.85 µg/ml ($p$ = 0.007) ซึ่งการเพิ่มขึ้นของระดับแอลฟาโทโคเฟอรอลมีความสัมพันธ์กันกับค่าฤทธิ์ต้านอนุมูลอิสระของวิตามินอี อย่างไรก็ตาม แต่ก็ไม่พบการเพิ่มขึ้นอย่างมีนัยสำคัญของค่าฤทธิ์ต้านอนุมูลอิสระ ($p$ =0.585) ดังนั้น จึงได้ข้อแนะนำว่า การรับประทานสารต้านอนุมูลอิสระใดๆ อาจไม่มีความจำเป็นสำหรับคนปกติที่มีสุขภาพดี อีกทั้งร่างกายอาจจะมีกลไกสมดุลบางอย่างในการควบคุมฤทธิ์ต้านอนุมูลอิสระในร่างกายไม่ให้มีมากจนเกินไป



ภาควิชา.....อาหารและเภสัชเคมี..... ลายมือชื่อนิสิต....คณิต ปึงเจริญกุล.....

สาขาวิชา.....เภสัชเคมี..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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KEYWORDS : *IN VIVO* TOTAL ANTIOXIDANT CAPACITY/ ORAC/ CURCUMINOIDS/ CURCUMIN/ ANTIOXIDANT

KANIT PUNGCHAROENKUL: COMPARISON OF THE *IN VIVO* TOTAL ANTIOXIDANT CAPACITY IN HEALTHY VOLUNTEERS AT THE DIFFERENT CURCUMINOIDS DOSES BY OXYGEN RADICAL ABSORBANCE CAPACITY ASSAY. THESIS ADVISOR: ASSO. PROF. PHENSRI THONGNOPNUA, Ph.D., 127 pp.

The total antioxidant activity in healthy volunteers obtaining the different dosages of curcuminoids extract was determined using the oxygen radical absorbance capacity assay (ORAC). Twenty four male and female Thai healthy volunteers were divided into three groups. Group A and B were administrated with curcuminoids extract at the dose of 500 mg/day and 6g/day, respectively. Group C received vitamin E supplement at the dose of 200 IU/day. Every subject was treated for seven days. Blood samples were collected before experimentation and on the first and seventh days up to 12 hrs. after administration, were immediately centrifuged to separate plasma and kept at the -48°C for subsequent analysis. No any adverse reaction was observed in any subject during and after 7 days of curcuminoids extract and vitamin E supplementations. Before experimentation, the concentrations of α-tocopherol of all subjects were within the normal range of healthy people (7.58 – 19.52 μg/ml). The ORAC values comparing before and after curcuminoids extracts administration of subjects in group A were ranged from 5,797-15,689 and 8,269-20,186 μmol TE/L, respectively, and those of subjects in group B were ranged 9,022-24,834 and 3,530-20,595 μmol TE/L, respectively. No significant increase in the ORAC values were observed either in group A subjects or group B subjects ($p$=0.270 and 0.33, respectively). For the subjects in group C, the ORAC values before and after vitamin E supplementations were ranged from 8,422-13,922 and 9,216-23,574 μmol TE/L, respectively. From the analysis of plasma endogenous α-tocopherol, there was statistically significant increased in α- tocopherol concentration from $9.16 \pm 1.21$ to $14.38 \pm 2.85$ μg/ml ($p$ =0.007). The increasing of α-tocopherol concentration was related to antioxidant activity of α-tocopherol. However, there were no statistically significant increased in the ORAC values ($p$ =0.585). Therefore, it is suggested that the supplementation of any antioxidants may not be essential for any healthy people. In addition, there are possibly balance functions to regulate the maximum antioxidant activity in our body.

Department : Food and Pharmaceutical Chemistry Student's Signature Kanit Pungcharoenkul
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# CONTENTS

# LIST OF TABLES

**Table No.** **Page**

# LIST OF FIGURES

**Figure No.** **Page**

**Figure No.** **Page**

# LIST OF ABBREVIATIONS

| | |
|---|---|
| % | percent |
| ˚C | degree Celsius |
| µg | microgram |
| µl | microlitter |
| AUC | area under the curve |
| $C_{max}$ | maximum observed concentration |
| Conc. | concentration |
| ESR | electron spin resonance technique |
| FRAP | Ferric Reducing Ability of Plasma |
| g | gram |
| HPLC | high performance liquid chromatography |
| hr | hour |
| IS | Internal standard |
| kg | kilogram |
| L | litter |
| LLOQ | lower limit of quantification |
| mg | milligram |
| min | minute |
| ml | milliliter |
| nM | nanomolar |
| No. | number |
| NS | non significance |
| ORAC | oxygen radical absorbance capacity assay |
| PA | peak area |
| PAR | peak area ratio |
| PH | peak height |
| PHR | peak height ratio |
| $R^2$ | coefficient of determination |
| ROS | reactive oxygen species |
| RSD | relative standard deviation |
| S | significance |

| | |
|---|---|
| SD | standard deviation |
| SE | standard error |
| TE | trolox equivalent |
| TEAC | trolox equivalent antioxidant capacity |
| $T_{max}$ | time to reach maximum observed concentration |
| TRAP | total radical-trapping antioxidant potential assay |
| w/w | weight by weight |

# CHAPTER I

# INTRODUCTION

Curcumin or diferuloylmethane, (1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3, 5- dione) is the major constituent of curcuminoids compound in the rhizome of turmeric (*Curcuma Longa* L.) (Itokawa et al., 2008). Curcumin is the low molecular weight polyphenol which was firstly characterized in the year of 1815 by Vogel and Pellatier (Vogel and Pelletier, 1815). It is known as a potent antioxidant comparable to α-tocopherol. The antioxidant activity of curcumin has been presented since 1975 (Sharma, 1976). The *in vitro* and the *in vivo* studies of curcumin and its derivatives emphasized in their antioxidant activities have been extensively reported.

## The *in vitro* antioxidant activity of curcumin

In the *in vitro* study, curcumin is proven as a scavenger of oxygen free radical. It exhibits strong antioxidant activity comparable to vitamin C and E (Toda, et al., 1985). Curcumin can significantly inhibit the generation of reactive oxygen species (ROS) (Elizabeth and Rao, 1990; Tcnnesen and Greenhill, 1992; Reddy and Lokesh, 1992; Sreejayan and Rao, 1994; Kumuda and Chandan, 2002; Kim et al., 2002; Tuba and Ilhami, 2008) as superoxide anion, hydrogen peroxide ($H_2O_2$) and nitrite radical generation. These free radicals are the important factors for the initiation of lipid peroxidation. The occurrence of lipid peroxidation within our bodies is reported to be related to the major causes of inflammation, heart disease and cancer. These aforementioned *in vitro* studies utilizing curcumin in the wide concentration range of

0.25-270 μM. However, the antioxidant activities of curcumin were all confirmed in these studies (Elizabeth and Rao, 1990; Tcnnesen and Greenhill, 1992; Reddy and Lokesh, 1992; Sreejayan and Rao, 1994; Selvam et al., 1995; Chatterjee, Desai and Thomas, 1999; Kumuda and Chandan, 2002; Kim et al., 2002; Jayaprakasha, Jaganmohan and Sakariah, 2006; Deng et al., 2006; Somparn et al., 2007; Banerjee et al., 2008; Tuba and Ilhami, 2008).

Generally, the scavenging capacity of samples (eg. pure compounds and turmeric extract) can be determined from their ability in inhibiting free radicals which are generated from the generating free radical- reagent in test tube. The high performance liquid chromatographic (HPLC) method with ultraviolet and visible (UV-VIS) detector is usually utilized for quantification comparing to the standard.

The antioxidant activity of curcumin in samples are expressed in several terms such as the percentage of the inhibition of lipid peroxidation by sample (%inhibition), the capacity of sample to scavenge the free radical (scavenging activity of sample) etc. ( Elizabeth and Rao, 1990; Tcnnesen and Greenhill, 1992; Reddy and Lokesh, 1992; Sreejayan and Rao, 1994; Selvam et al., 1995; Chatterjee, Desai and Thomas, 1999; Kumuda and Chandan, 2002; Kim et al., 2002; Jayaprakasha, Jaganmohan and Sakariah, 2006; Deng et al., 2006; Somparn et al., 2007; Banerjee et al., 2008; Tuba and Ilhami, 2008)

Table 1 is the examples of the *in vitro* antioxidant activity studies of curcumin. Due to the different expression of the antioxidant activities, the results are hardly comparable. However, according to these reports, it is confirmed that curcumin and/or its derivatives exert the antioxidant activities *in vitro*.

**Table 1.** Examples of the *in vitro* antioxidant activity studies of curcumin

| No. | Sample | Curcumin in PBS [1] | Curcumin concentration[2] (µg/ml) | Free radical | Results | Ref. |
|---|---|---|---|---|---|---|
| 1 | curcumin pure compound | 0.1µM | 0.0368 | hydroxyl radical | % inhibition of the reduction of Fe(III) to Fe(II) = 50% at 0.1 µM | Tcnnesen and Greenhill, 1992 |
| 2 | | 0.61- 27.00 µM | 0.22- 9.94 | hydroxyl radical | % hydroxyl radical scavenging activity (mean± SE) = 33.3 ±3.3 to 62.5+ 5.6 | Elizabeth and Rao, 1990 |
| 3 | | 1.35 to 54.00 µM | 0.50-19.87 | hydroxyl radical | % hydroxyl radical scavenging activity (mean± SE) = 1.3 ± 0.1 to 33.3 ± 1.3 | Elizabeth and Rao, 1990 |
| 4 | | 1.35 to 270.00 µM | 0.50-99.36 | superoxide radicals | % superoxide radical scavenging activity (mean± SE) = 6.3 ± 0.4 to 76.8 ± 1.4 | Elizabeth and Rao, 1990 |
| 5 | | 15µg/ml | 15 | superoxide radicals | %superoxide radical scavenging activity (mean± SE) = 42.7 ± 8.1 | Tuba and Gulcin, 2008 |
| 6 | | 4µM | 1.472 | peroxynitrite | $IC_{50}$ [3] for scavenging peroxynitrite radical = 4.0 ± 0.04 µM | Kim et al., 2002 |
| 7 | | 1 to 15µM | 0.368 - 5.52 | singlet oxygen radical | $IC_{50}$ [3] of TEMPO adduct formation = 2.75µM | Kumuda and Chandan, 2002 |
| 8 | | 0.25-20µM | 0.092 - 7.36 | lipid peroxidation | TBARS value [5] (mean± SE) = 3.2 ± 1.0 to 100.0 | Sreejayan and Rao, 1994 |
| 9 | | 5-50 µM | 1.84 - 18.4 | lipid peroxidation | $IC_{50}$ [3] of lipid peroxidation = 25 µM | Reddy and Lokesh, 1992 |
| 10 | | 5-50 µM | 1.84 - 18.4 | lipid peroxidation | $IC_{50}$ [3] of lipid peroxidation = 23.2 ± 2.5 µM | Banerjee et al., 2008 |
| 11 | | 0 to 15 µM | 0 - 5.52 | antioxidant activity | the inhibition time of the RBCs = 132 ± 4 to 229 ± 6 min | Deng et al., 2006 |
| 12 | | 15-45 µg/ml | 15 - 45 | antioxidant activity | $EC_{50}$ [4] = 34.86µg/ml | Tuba and Ilhami, 2008 |
| 13 | | 100 ug/ml | 100 | antioxidant activity | antioxidant activity values = 3099 ±66 µmol/g of ascorbic acid equivalent | Jayaprakasha, Jaganmohan and Sakariah, 2006 |
| 14 | | 100 ug/ml | 100 | antioxidant activity | % inhibition of linoleic peroxidation = 81.98 % at 120 h. | Jayaprakasha, Jaganmohan and Sakariah, 2006 |
| 15 | curcumin, demethoxy-curcumin, bis demethoxy-curcumin pure compound | 1 to 12µM | 0.3-4.4 | antioxidant activity | $IC_{50}$ [3] of lipid peroxidation = 46.2, 20.3, 10.0 min/µM for curcumin, demethoxycurcumin, bis demethoxycurcumin respectively | Somparn et al., 2007 |
| 16 | | 12 to 200µM | 4.4-73.6 | antioxidant activity | $IC_{50}$ [3] of lipid peroxidation = 35.1, 53.4 and 200 µM for curcumin, demethoxycurcumin, bis demethoxycurcumin respectively | |
| 17 | turmeric extract (aqueous) | 0.02% | | lipid peroxidation | $IC_{50}$ [3] of lipid peroxidation =50 µg/ml | Selvam et al., 1995 |
| 18 | turmeric extract ( aqueous) | 0.10% | | lipid peroxidation | TBARs value[5] = 99.83 ± 0.27 % | Chatterjee, Desai and Thomas, 1999 |

[1] The studied concentration

[2] Calculated concentration to µg/ml

[3] IC50 = Half maximum inhibitory concentration

[4] EC50 = Half maximum effective concentration

[5] TBARs value or thiobarbituric acid related substance values = the % inhibition of lipid peroxidation by thiobarbituric acid related substances method (TBARs method); TBARs method is the method for determining of malondialdehyde level which is the marker of oxidative stress.

## The *in vivo* antioxidant activity of curcumin

Not only the *in vitro* antioxidant activity of curcumin was extensively studies, the *in vivo* studies using laboratory animals were also performed but only in limited reports. (Reddy and Lokesh, 1994; Kalpana and Menon, 2004; Pari and Murugan, 2004; Eylb, Kotyzova and Bludovska, 2004; Suryanarayana et al., 2007; Cekmen et al., 2009) Rats and mice are the animal's models utilized by oral feeding them with aqueous turmeric extract or the solutions of curcumin with or without its metabolites in phosphate buffer saline (PBS). The concentrations of curcumin used were ranged between 20-200 mg/kg and the duration was from 1 day to 22 weeks. (Reddy and Lokesh, 1994; Kalpana and Menon, 2004; Pari and Murugan, 2004; Eylb et al., 2004; Suryanarayana et al., 2007; Cekmen et al., 2009)

The principle of the *in vivo* antioxidant activity determination is similar to the *in vitro* study, only that the samples used, are blood, plasma, serum, urine or tissue organs which obtained from the treated animals. The reaction response was usually quantified via HPLC technique with UV-VIS detector or mass spectrometer detector (LC-MS) having α-tocopherol or ascorbic acid as standard antioxidant calibration. The studies are mostly comparable to the control group that treated with the placebo compound.

According to these *in vivo* antioxidant activity studies, it was indicated that curcumin or tetrahydrocurcumin (THC) can decrease the level of lipid peroxidation and increase the activities of enzymatic antioxidant as illustrated in Table 2.

**Table 2** Examples of the *in vivo* antioxidant activities study of curcumin, tetrahydrocurcumin (THC) or turmeric powders

| No. | Substance | Animal | Route | Dose | Duration | Sample | Effect | Ref. |
|---|---|---|---|---|---|---|---|---|
| 1 | **turmeric powder** | rats | p.o.[1] | diet supplement with 1%w/w turmeric powder | 10 weeks | serum and liver tissue | - decrease level of lipid peroxidation by TBARS method<br>- increase enzyme antioxidant activity levels | Reddy and Lokes, 1994 |
| 2 | **curcumin** | rats | p.o.[1] | 80mg/kg curcumin in PBS | 22 weeks | liver, lung and kidney tissue | - decrease level of lipid peroxidation in tissue<br>- increase the enzyme antioxidant activity levels in tissue | Kalpana and Menon, 2004 |
| 3 | **curcumin** | rats, mice | p.o.[1] | 50 mg/kg curcumin in PBS | 3 days | liver tissue | - decrease the level of cadmium-induced lipid peroxidation<br>- increase the glutathione levels | Eylb, Kotyzova and Bludovska, 2004 |
| 4 | **curcumin** | rats | p.o.[1] | 0.002% - 0.01% of curcumin in PBS | 8 weeks | whole blood | - decrease the level of TBARs and protein carbonyl in rats' tissue. | Suryanarayana et al, 2007 |
| 5 | **curcumin** | rats | p.o.[1] | curcumin in aqueous solution dose 200 mg/kg | single admini-stration | serum and kidney tissue | - decrease the level of TBARs of acetaminophen induced lipid peroxidation | Cekmen et al, 2009 |
| 6 | **THC** | rats | p.o.[1] | THC 20, 40, 80 mg/kg in aqueous solution | 15 days | serum | - decreased TBARs values in serum from 0.36 (in control) to 0.17 mmol/dl (in curcumin gr.)<br>- increased the activity of AST, ALT and ALP enzyme | Pari and Murugan, 2004 |

[1]p.o. = Oral administration

There are several studies in patients related to antioxidant activity of curcumin. By consuming curcumin capsule in the dose of 500 mg/day for 12 weeks to children with hemoglobin H disease, the level of lipid peroxidation which related to antioxidant activity could be improved. (Bunchongsilp, 2004).

In addition, curcumin has also been proven as the effective compound for the treatment of disease originated from oxidative stress in patients, for instance, cardiovascular disease (Wongcharoen and Phrommintikul, 2009), rheumatoid arthritis, neurodegradation disease: alzheimer's disease and parkinson's disease (Diplock et al., 1998; Aggarwal et al., 2009), cancer (Kuttan, et al., 1985; Cheng et al., 2001) and ageing (Oldham, 1998; Cornelli, 2009). In these studies, curcumin was used in the regimen ranged 20-16000 mg/day and the durations of the experiments were froms for 5 days to 3 months. (Deohar, Sethi and Srimal, 1980; Satoskar, Shah and Sheroy, 1986; Soni and Kuttan, 1992; Venkatesan, 1998; Ramirez et al., 2000; Lim et al., 2001; Wu et al., 2006; Goel et al., 2008)

Apart from the disease states, curcumin is well known as an effective supplement among Thai people. As a supplement, curcumin is taken once or twice a day in the amount of 250-500 mg. No any reports or documents in country mentioned the benefit or risk of these curcumin administrations. About the effectiveness of antioxidant compound, there are still the controversies of increasing antioxidant status by inducing the antioxidant compound into our body. Some reports recommend the improvement of antioxidant status by the administration of antioxidant compounds. Meanwhile there are also the warning of continually administration of antioxidant compounds that related to the indication of oxidative stress in our body (Jacob et al, 2002; Voronin et al, 2003; Koren et al, 2008).

Therefore, it is interesting to directly determine the total antioxidant activity in our body following administration of curcumin. The different amount of curcumin were used in previous studies, the high dosing regimen up to 8 g/day (Cheng et al., 2001) for studying in patients with various diseases especially cancer and the supplement regimen in the dose 250-500mg/day. To determine whether is there any distinction in antioxidant activity of different curcumin dosage in subjects, this research study was then set up.

## Objectives of the study

1. To determine total antioxidant activity of curcumin at different doses in Thai healthy volunteers.

2. To compare the total antioxidant activity of curcumin administration with vitamin E administration.

3. To determine the correlation of plasma concentrations of curcumin and its metabolites and the plasma total antioxidant activity following administered curcumin extract.

4. To determine the correlation of plasma endogenous α-tocopherol concentration and the total antioxidant activity in plasma following administered of vitamin E supplements.

## Significance of this study

1. To obtain the information of total antioxidant activity of curcumin extract in Thais.

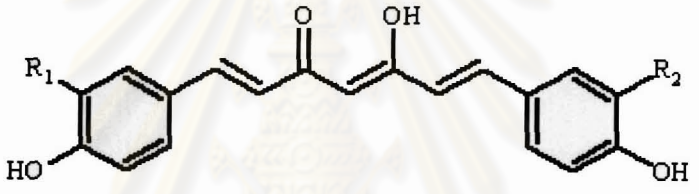
2. To clarify the significance of curcumin dose on total antioxidant activity in human.

3. To obtain the model study for other herbal compounds in the future.

# Theoretical Background

## Turmeric

Turmeric (*Curcuma Longa L.*) belongs to the Zingiberacea family. It's extensively used as a spice, coloring agent and traditional medicine for the treatment of inflammation in India, China and South East Asia. (Chatlopadyhyay et al., 2004) The major chemicals in turmeric are essential oils and curcuminoids. Three major compositions of curcuminoids are curcumin, demethoxycurcumin and bis-demethoxycurcumin




| | $R_1$ | $R_2$ |
|---|---|---|
| **Curcumin** | $OCH_3$ | $OCH_3$ |
| **Demethoxycurcumin** | $OCH_3$ | H |
| **Bisdemethoxycurcumin** | H | H |

**Figure 1** The chemical structures of curcumin, demethoxycurcumin and bisdemethoxycurcumin

The chemical structure of curcumin was firstly proposed by Vogel and Pellatier, as the bis - α, β - unsaturated alkyl biphenyl structures. The bis-keto form is the predominate form in acidic and neutral aqueous solutions and in the cell membrane. Curcumin is also light sensitive. Therefore, it is recommended that samples containing curcumin should be light protection. (Wang et al., 1997)

At pH 3-7, curcumin acts as an extraordinary potent H-atom donor. This is due to the keto form of curcumin; the heptadienone linkage between the two methoxyphenol rings contains a highly activated carbon atom. The C-H bonds on this carbon are very weak due to the delocalization of the unpaired electron on the adjacent oxygen. In contrast, above pH 8, the enolate from of the heptadienone chain predominates and curcumin acts as electron donor (Figure 2), a mechanism more typical for the scavenging activity of the phenolic antioxidants. Curcumin is insoluble in water but it is soluble in acetone, dimethyl sulphoxide and methanol (Sharma, Gescher and Steward, 2005)

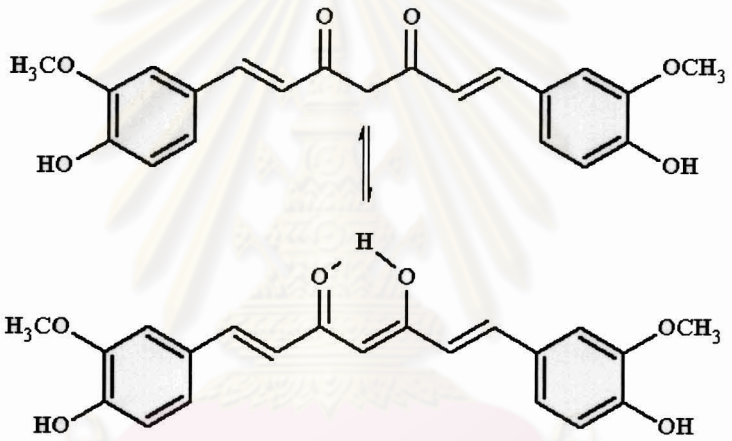



**Figure 2** Tautomerism of curcumin under physiological conditions.

**Pharmacokinetics and biotransformation of curcuminoids**

By intra-peritoneal administration of curcumin in the dose of 0.1g/kg to mouse, curcumin was metabolized to tetrahydrocurcumin (THC) in which it was subsequently converted to mono and di- glucuronide conjugates (Min-Hsiung, Tsang-Miao and Jen-Kun, 1998). The study in rats by feeding curcumin with the dose of 500 mg/kg, the very low concentration of curcumin in plasma could be observed along with the higher levels of curcumin glucuronide and curcumin sulfate in plasma. The

hexahydrocurcumin (HHC) and octahydrocurcumin (OHC) were also detected at same sampling tubes. (Ireson et al., 2001)

In human, the pharmacokinetics of curcumin in Thai healthy volunteers was reported. (Thongnopnua et al., 2007) Administrated of curcuminoids capsules in the dose of 6 g to 12 male subjects, only curcumin could be detected in plasma samples with the maximum concentration (Cmax) 35 – 550 nM at 1.5 - 4.0 hrs. Demethoxycurcumin and bis-demethoxycurcumin in plasma could only be detected at the very low concentrations in some subjects. THC, HHC and OHC were all three detected metabolites of curcumin in human plasma. Glucuronide conjugation of curcumin, demethoxycurcumin and bis-demethoxycurcumin as well as THC and HHC were also observed. (Thongnopnua et al, 2007)

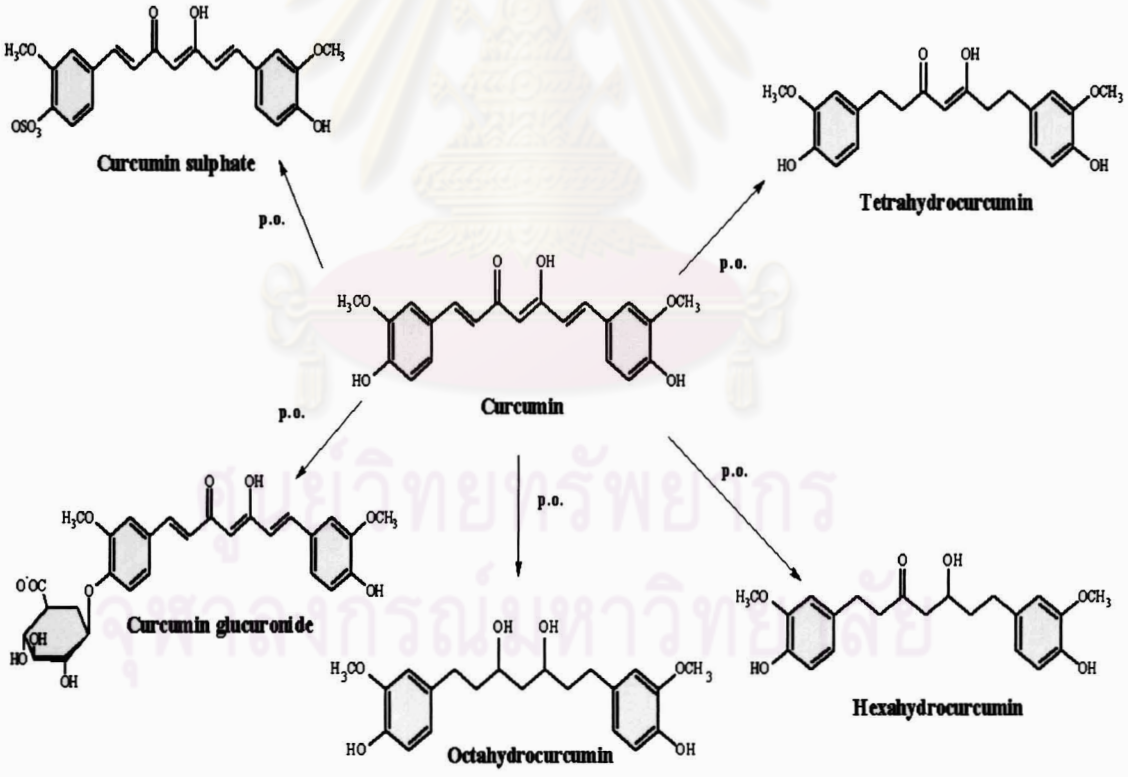



**Figure 3** Pathway of biotransformation of curcumin and its metabolites in rat and human following curcumin administration.

**Biological activities of curcumin**

Curcumin and its metabolites were reported to exert various bio-activities such as anti-inflammatory (Chatopadhyay et al., 2004; Sharma et al., 2005; Itokawa et al., 2008;), antibacterial (Jayaprakasha et al., 2006), anticancer (Satoskar, Shah and Sheroy, 1986; Ajay, Ajaikumar and Bharat, 2007), and antioxidant activities (Cikrkci, Mozioglu and Yilmaz, 2008).

The antioxidant activity of curcumin is prominent according to various *in vitro* and *in vivo* studies (Miquel et al., 2002; Jai et al., 2004; Murugan and Pari, 2006; Somparn et al., 2007; Tuba and Ilhami, 2008). It was shown that curcumin could reduce the propagation of various diseases related to the high level of free radical in human body such as cardio-vascular disease, alzheimer's disease, diabetes, parkinson's disease, and cancer (Kuttan et al., 1985; Cheng et al., 2001).

**Biomedical analysis of curcumin**

The curcuminoids solution exhibit strong light absorption at the wavelength between 420-430 nm. Commercial curcuminoids/turmeric products contain mixture of curcumin, demethoxycurcumin and bisdemethoxycurcumin. It is not possible to quantify the individual curcuminoids with spectrophotometric method. The gas chromatography (GC) method is also not appropriate for quantifying curcuminoids due to the low volatility and thermally labile nature of the curcuminoids (Jayaprakasha et al, 2006). Spectroscopic methods including infra-red (IR), nuclear magnetic resonance (NMR) and mass spectrometry (MS) are widely used for the identification and characterization of the curcuminoids (Asai and Miyazawa, 2000; Anasari et al., 2005; Anchang et al., 2006). Liquid chromatography-mass

spectrometry (LC-MS) and gas chromatography- mass spectrometry (GC-MS) are meaningful techniques for both qualitative and quantitative determination of curcuminoids (Hiserodt et al., 1996). In 1998, He et al., reported the methods for analyzing curcuminoids in a fresh turmeric extract by using the on-line-HPLC-UV diode array and electro-spray mass spectrometer. The curcuminoids are identified at column temperature set at 48°C using gradient elution with ammonium acetate-acetic acid and acetonitrile. But the organic salts in the mobile phase is contaminated the mass spectrometer ion source (He et al., 1998)

Very few reports on curcumin analysis in plasma were evident. Only HPLC technique was presented with UV or MS detection (Christopher et al., 2001; Cheng et al., 2001; Pak et al., 2003; Heath et al., 2003; Thongnopnua et al., 2007). Thongnopnua et al. reported the HPLC technique with UV detection for curcumin, demethoxycurcumin, bisdemethoxycurcumin and their metabolites in plasma, having ethyl acetate and isopropanol as the extracting solution. In this study the method of Thongnopnua et al. was used with no modification.

## Free radicals, oxidative stress, antioxidants

Free radical can be defined as atoms or molecules or molecular fragments containing one or more unpaired electrons. (Halliwell and Gutteridge, 1999) These radicals could be deriving from oxygen generated in living systems to be reactive oxygen species (ROS). The production of ROS from both endogenous and exogenous substances could be observed by various methods such as the ultraviolet-visible spectrophotometric method (UV-Vis spectrophotometric), the electron spin resonance (ESR) and HPLC. The potential endogenous sources of ROS include mitochondria, cytochrome P450 metabolism, and inflammatory cell activation. (Valko et al., 2006)

ROS is well recognized in playing a dual role as both deterious and beneficial species. When the excessive quantities of ROS overwhelm host antioxidant defenses, there are in the occurrence of cell damages and oxidative stress. (Wood, Gibson and Garg, 2006) The oxidative stress is claimed to be related to various disorder symptoms, for instance, nephropathy, type II diabetes, rheumatoid arthritis, neurodegradation, ischemia-reperfusion injury (Abuja and Albertini, 2001.)

The defense mechanism of our body to oxidative stress is taking role by antioxidants. (Valko et al., 2006) The antioxidants in our body can be divided into three groups:



### 1. Enzymatic antioxidants

Enzymatic antioxidant removes ROS that can initiate oxidation. The hydrogen atom transfer is the main mechanism that these enzymes used to scavenge the free radicals. The three major enzymatic antioxidants in our body are:

**1.1 Catalase (CAT)**

Catalase is an enzyme that presents in the cells of plants, animals and aerobic bacteria. Catalase is located in the peroxisome organelle which has the ferri protoporphyrin as the major components. Catalase acts as the catalyst in converting hydrogen peroxide to water and oxygen:

$$2H_2O_2 + 2H^+ \xrightarrow{CAT} 2\,H_2O + O_2 \quad .....(1)$$

**1.2 Superoxide dismutase (SOD)**

Superoxide dismutase exists in several isoforms, differing in the nature of active metal center and the place where its works. In humans there are three forms of SOD based on the metal ion in their active sites: manganese-superoxide dismutase (Mn SOD; SOD2) and copper-zinc superoxide dismutase (CuZnSOD; SOD1) are in the mitochondria and the extra-cellular superoxide dismutase (EC-SOD; SOD3) is in the extracellular fluid. All SODs are the catalyst for the dismutation of superoxide radical to hydrogen peroxide and to the less-reactive species hydrogen peroxide:

$$2H^+ + 2O_2^{-\bullet} \xrightarrow{SOD} O_2 + H_2O_2 \quad .....(2)$$

**1.3 Glutathione peroxidase (GSH-Px)**

Glutathione peroxidase is the seleno-protein in human tissue. GSH-Px eliminates hydroperoxide compounds such as lipid peroxide (LOOH) and hydrogen peroxide. GSH-Px acts in conjunction with the glutathione (GSH) by decomposing peroxides to water and glutathione disulfide (GSSG).

$$H_2O_2 + 2GSH \xrightarrow{GPx} GSSG + H_2O \quad .....(3)$$

$$ROOH + 2GSH \xrightarrow{GPx} ROH + GSSG + H_2O \quad .....(4)$$

**2. Biological metal chelators**

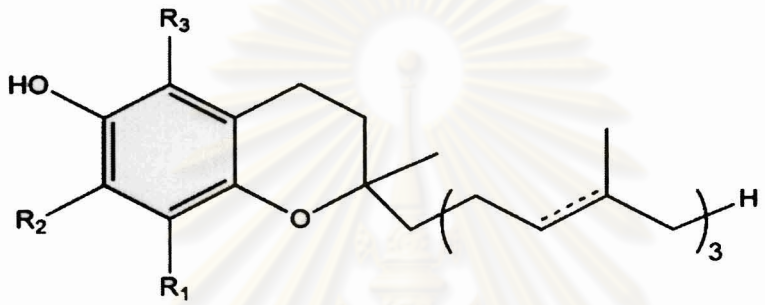
Biological metal chelators is the protein which preventing the participation of transient metal ions in formation of hydroxyl radical and other reactive oxidants by trapping metals ion and from complex compounds. The biological metal chelators in human are transferrin, ferritin and albumin.

**3. Non-enzymatic antioxidants**

Non-enzymatic antioxidants are the antioxidants in blood circulation and lymph circulation. This antioxidant can receive electron from the free radical to form the stable products. Most of non-enzymatic antioxidants in human are obtained from drugs and food administrations. The common non enzymatic antioxidants in our body are vitamin C, glutathione (GSH) and vitamin E.

## Vitamin E (α-tocopherol)

Vitamin E (α-tocopherol) is the fat–soluble vitamin that exists in eight different forms (Figure 4). α-tocopherol is the most active from of vitamin E in human. It is the powerful biological antioxidant which is considered to be the major membrane-bound antioxidant employed by the cell. (Valko et al., 2006) Vitamin E could not be synthesized in animal and human, it must be supplied from the diet.




α-tocopherol, $R_1 = R_2 = R_3 = CH_3$
α-tocotrienol, $R_1 = R_2 = R_3 = CH_3$

β-tocopherol, $R_1 = R_3 = CH_3$; $R_2 = H$
β-tocotrienol, $R_1 = R_3 = CH_3$; $R_2 = H$

γ-tocopherol, $R_1 = R_2 = CH_3$ $R_3 = H$
γ-tocotrienol, $R_1 = R_2 = CH_3$ $R_3 = H$

δ-tocopherol, $R_1 = R_2 = R_3 = H$
δ-tocotrienol, $R_1 = R_2 = R_3 = H$

**Figure 4** The chemical structure of vitamin E

The biological activities among the different commercial vitamin E froms are different. In general, the biopotency of vitamin E in pharmaceuticals, dietaries and cosmetics is labeled in terms of international unit (IU). The international unit (IU) defines as the biological activity of vitamin E from rat fetal resorption test. One IU is defined as the activity of 1 mg of all-rac-α-tocopherol acetate. In addition, the National Research Council defines the biological activity of vitamin E in term of RRR-α-tocopherol equivalence (Duthie, 2000). The biological activities of commercial vitamin E are shown in Table 3.

**Table 3** Biological activities of commercial vitamin E (Duthie, 2000)

| Vitamin E | Biologic activities | |
|---|---|---|
| | IU/mg | Compared to RRR- α-tocopherol (%) |
| RRR- α-tocopherol | 1.49 | 100 |
| All-rac- α-tocopherol | 1.10 | 74 |
| RRR- α-tocopherol acetate | 1.36 | 91 |
| All-rac- α-tocopherol acetate | 1.00 | 67 |

**Absorption and distribution of vitamin E in human**

Vitamin E could be absorbed by intestinal mucosa following oral administration either from food or supplements. By the assistance of bile salts, the absorbed vitamin E could form micelle called chylomicron that consists of triglyceride, free and esterified cholesterol, phospholipids and apolipoproteins before secrete into lymph and blood circulation. Vitamin E in plasma is reported to compose approximately 88% of α-tocopherol, and only 2% and 10 % of β- and γ-tocopherol, respectively. No tocotrienols is observed in plasma. (Traber and Serbinova, 1999; Nelis, D'Haese and Vermis, 2000)

Most of α-tocopherol is transported to liver by using plasma lipoproteins like high density lipoproteins (HDL) and low density lipoprotein (LDL) as the carrier for the distribution. The half life of vitamin E in plasma is approximately 48 hrs.

α-tocopherol could be maintained in human body by equilibrating plasma α-tocopherol with the tissues. Erythrocytes, liver and spleen are tissues that could be rapidly equilibrated with α-tocopherol. Therefore, the change of α-tocopherol level in plasma can higher reflect its change in erythrocytes, liver and spleen than other organs.

**Function of α-tocopherol in human**

α-tocopherol functions *in vivo* as a chain breaking antioxidant that prevents propagations of free radical damage. When lipid hydroperoxide is oxidized to peroxyl radicals ($ROO^{\bullet}$), it reacts 1,000 times faster with α-tocopherol (α-T-OH) than with poly-unsaturated fatty acids (PUFAs or RH). Thus, the presence of α-tocopherol could protect PUFAs in such the way that the phenolic hydroxyl groups of α-tocopherol ( α-T ) react with an organic radical ($ROO^{\bullet}$) to form organic hydroperoxide (ROOH) and the α-tocopheroxyl radical ($\alpha\text{-T-O}^{\bullet}$)(equation 5). In the absence of α-tocopherol, peroxyl radical can react with the other PUFAs, the oxidation chain reaction is then occurred and damaging the tissues. The α-tocopheroxyl radical ($\alpha\text{-T-O}^{\bullet}$) can be recoverd to α-tocopherol by reacting with vitamin C (equation 6) (Traber and Serbinova, 1999).

$$ROO^{\bullet} + \alpha\text{-T-OH} \longrightarrow ROOH + \alpha\text{-T-O}^{\bullet} \quad \text{.....(5)}$$

The α-tocopheroxyl radical ($\alpha\text{-T-O}^{\bullet}$) reacts with vitamin C ($Asc^{\bullet}H$)

$$\alpha\text{-T-O}^{\bullet} + Asc^{\bullet}H \longrightarrow \alpha\text{-T-OH} + Asc^{-\bullet} \quad \text{.....(6)}$$

**The analysis of α-tocopherol in human plasma**

The varieties of techniques have been reported for the determination of plasma α-tocopherol such as GC-MS (Kock et al., 1997; Melchert and Pabel, 2000), HPLC (Chou et al., 1985; MacCrehan and Schonberger, 1987; Seta, Nakamura and

Okayama, 1990; Bortolotti et al., 1993; Gonzalez-Corbella et al., 1994; Teissier et al., 1996; Cooper, Thadwal and Cooper, 1997) and capillary electrochromatography (CEC) (Fanali et al., 2002). Even though GC-MS and CEC methods are usable for determining different froms of tocopherols in plasma, both method seem to be impractical and technically over-complex for routine analysis of only α-tocopherol. GC-MS methods need two steps of sample preparation followed by chemical derivatization. These methods are tedious and time-consuming, while the CEC method requires too large of sample volume (2.0 ml). Therefore, HPLC methods are widely used for α-tocopherol analysis.

Without chemical derivatization, however, most of the HPLC methods also cannot avoid the time-consuming two-step sample preparation (Cooper et al., 1997; Julianto, Yuen and Noor, 1999). Recently, with the deproteinization technique which reported by Nirungsan (Nirungsan and Thongnopnua, 2005), the determination of α-tocopherol in plasma by HPLC technique was more practically used than GC , CEC or other sample preparation techniques for the HPLC system. Therefore this analytical method was carried on to this research study without any modification.

## The determination of antioxidant activity

The determination of antioxidant activity is the study of the capability of antioxidant to be the free radical scavenging. The antioxidant activities are expressed in term of the amount of free radical that is scavenged by an antioxidant. The antioxidant activity can be determined from either the *in vitro* and *in vivo* study.

All of the methods for the *in vitro* antioxidant activity studies are to determine the inhibition of oxidation reaction by antioxidants. Meanwhile the *in vivo* studies are to study the activity of antioxidant in biological sample obtained from animal or human following antioxidant administration.

For the *in vivo* antioxidant activity determination, they are divided into four methods dependent upon the direct marker and the indirect marker for determining the antioxidant activity. In the direct marker method, the antioxidant levels of samples would be increased as the antioxidant activity was increased. In the contrary to the indirect marker, the antioxidant supplement on markers of oxidative stress would be decreased if the antioxidant activity was increased. The antioxidant activity can be determined from the antioxidant concentrations or total antioxidant activity. In order to indirectly determine antioxidant activity, the oxidative stress marker and the measurement of reactive oxygen species are measured (Wood, Gibson and Grag, 2006).

The methods that used to determine the *in vivo* antioxidant activity are:

### 1. The determination of antioxidant concentrations

The direct method for determining the concentrations of non enzymatic antioxidants in biological samples. The analyzed non-enzymatic antioxidant can be

either ascorbic acid or glutathione or α-tocopherol by using the HPLC techniques or spectrophotometric techniques. (Wood et al., 2006)

**2. Measurement of oxidative stress marker**

The marker of oxidative stress can be indirectly determined the antioxidant activity. When there is oxidative stress in our body the peroxidation products are produced from the reaction between free radicals and some biological components, including lipids, protein and DNA. This peroxidation products, malondialdehyde or proteins carbonyl, are then used as the marker of oxidative stress. The spectrophotometric method is usually used for determining in sample. The low antioxidant activity is expressed if the level of marker is elevated. (Abuja and Albertini, 2001)

**3. Measurement of reactive oxygen species (ROS)**

The measurement of ROS is proposed to be an indirect method for determining the *in vivo* antioxidant activity. The ROS or free radical has the paramagnetism property that could be detected by the electron spin resonance technique (ESR). The ESR measures the absorption of energy as a result of the interaction of various free radicals with an applied external magnetic field. The strength of the signal produced by the sample is proportional to the concentration of the free radical present. Spin trapping agents are required for this technique. These agents react with transient free radicals to form spin adducts, which are stable enough for the measurement free radical by ESR. (Wood et al., 2006).

## 4. Measurement of total antioxidant capacity (TAC)

The measurement the total antioxidant capacity is the one of the direct method for determine the antioxidant activity. There are a number of assays designed to measure overall antioxidant activity, as an indication of total antioxidant capacity. The methods for the determination of total antioxidant capacity are divided into two groups according to the mechanism of antioxidants in scavenging free radicals. They are the single electron transfer (SET) and hydrogen atom transfer (HAT) mechanisms. The methods that based on the SET mechanism are trolox equivalent antioxidant capacity (TEAC) and ferric reducing ability of plasma (FRAP). The methods that based on the HAT mechanism are total radical-trapping antioxidant potential assay (TRAP) and oxygen radical absorbance capacity assay (ORAC).

The methods of total antioxidant capacity based on SET mechanism are:

### 4.1 Trolox Equivalent Antioxidant Capacity (TEAC)

The TEAC assay is one of the widely used assays for the determination of total antioxidant activity. In 1993, Miller et al. developed this method to monitor the antioxidant status in premature neonates. The method measured the absorbance of the 2, 2'-azonobis-(3-ethylbenzothiazoline-6- sulphonic acid radical cation ($ABTS^{\bullet+}$) at the maxima absorbance wavelength of 734 nm. $ABTS^{\bullet+}$ is the product from the reaction between 2,2'-azonobis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), hydrogen peroxide and metmyoglobin. In the presence of antioxidant, the absorbance of $ABTS^{\bullet+}$ is quenched related to the antioxidant capacity (Figure 5). Trolox, which is

a water soluble vitamin E analogue (Figure 6), was used as a standard (Miller et al., 1993).

**The limitations of TEAC method**

The ABTS radical that used in TEAC method, is not the usual radicals found in mammalian biology. Thus this method does not represent the scavenging activity of antioxidant to the real free radical which found in mammalian (Phipps et al., 2007).

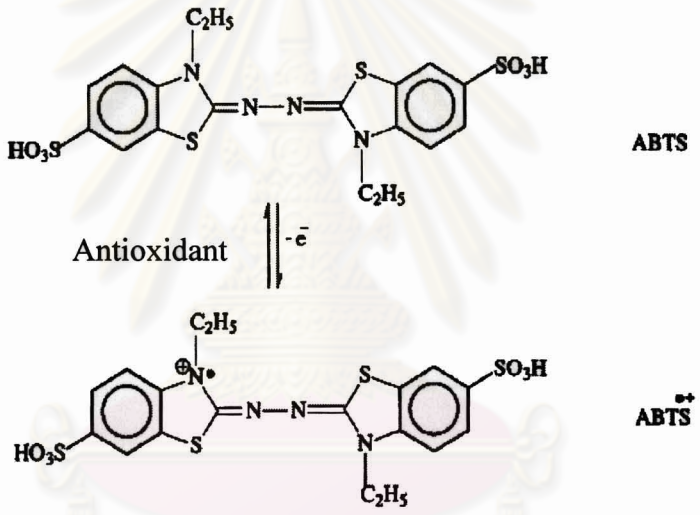
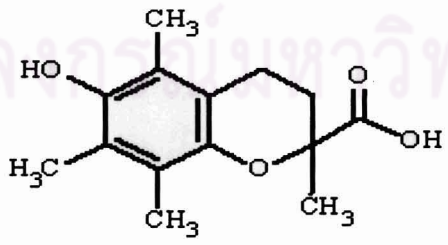



**Figure 5** The principle of TEAC assay (Huang et al., 2005)



Trolox

(6-Hydroxy-2,5,7,8-tetra methy chroman-2-carboxylic acid)

**Figure 6** Trolox structure

### 4.2 Ferric Reducing Ability of Plasma (FRAP)

The FRAP is a measurement of the reducing ability of antioxidants without using any radical generator. Therefore, this method is distinguished from many other test systems, only the reducing ability of antioxidants is measured.

This assay was first described in 1996 by Benzie and Strain using an automatic analyzer to perform the assay (Benzie and Strain, 1996). The sample was reacted with the FRAP reagent, which compose of ferric chloride and 2, 4, 6-Tripyridyl-s-triazine (TPTZ). Antioxidant samples were reduce the Fe (III) in reagent to Fe(II). The absorbance of Fe(II)-TPTZ complex was measured at 593 nm after 8.0 min of reaction between sample and the ferric-TPTZ complex. The reducing ability of Fe (III)-TPTZ complex is calculated using the difference in absorbance (Δ Abs.) values between sample and blank compared with Fe (II) standard solution. (Phipps et al., 2007)

**The limitations of FRAP method**

The FRAP method is unable to measure the SH group-containing antioxidants, such as glutathione or protein. Thus, it induces a serious underestimation of antioxidant activity in serum. Furthermore the FRAP method measures only the reducing ability based on ferric ion, under the condition of pH .3.6 for soluble ferric. This specified condition is not relevant to our normal physiological pH (pH 7.6).

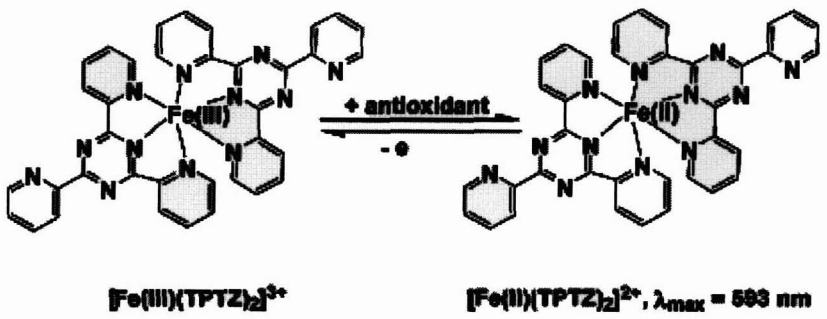



**Figure 7** The principle of FRAP assay (Huang et al., 2005)

The methods of total antioxidant capacity based on HAT mechanism are:

### 4.3 Total Radical-Trapping Antioxidant Potential Assay (TRAP)

The TRAP assay is based on the measuring of antioxidant capability to inhibit the oxidation reaction between the fluorescent molecule (R-phycoerythrin) and the free radical generators such as azo-initiator compounds. A 2, 2'–azobis-2-methyl-propanimidamide, dihydrochloride (AAPH) as an azo-initiator that could release the peroxyl radical by the thermal decomposition. The reaction of peroxyl radicals with fluorescent molecule resulting in the loss of fluorescent property of fluorescent molecule. The antioxidant activity is determined as the time to consume all of the antioxidant, by extension of the lag time for appearance of the fluorescent probe when antioxidants are present. The antioxidant activity was expressed as a lag time of the sample compared to the lag time of standard trolox. (Bartosz, 2003; Phipps et al., 2007)

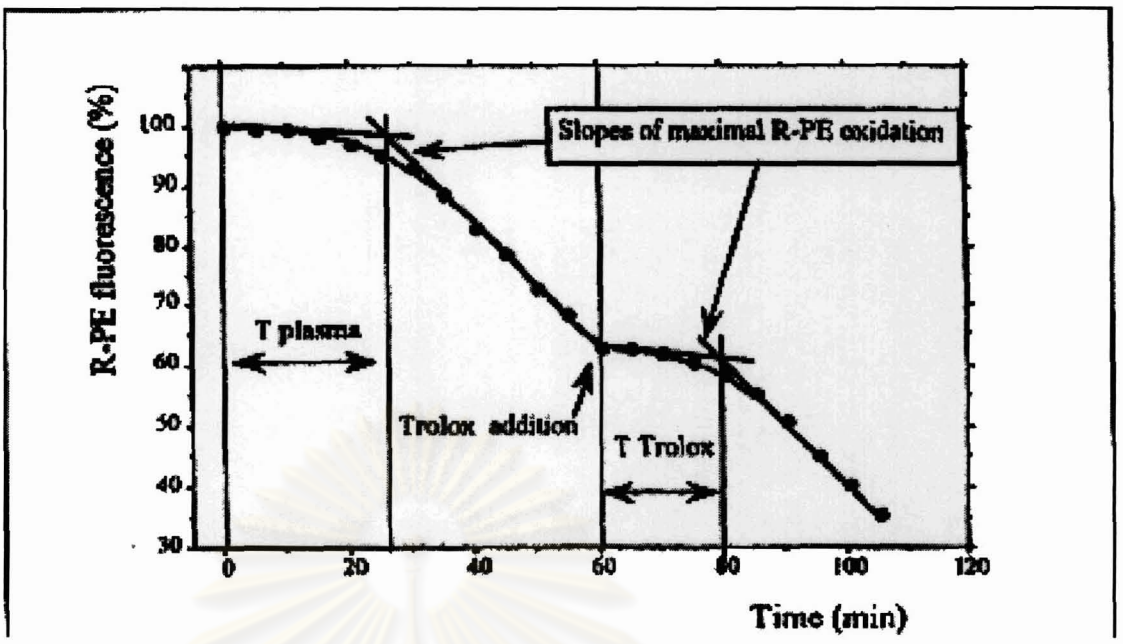



**Figure 8** The kinetics of R-PE oxidation initiated by 5 mM AAPH in the presence of plasma (8μl) before and after added standard trolox. The antioxidant activity of each plasma sample is calculated by comparing the two lag phase obtained in the presence and the absence of trolox. (Bartosz, 2003)

**The limitation of TRAP method**

By TRAP method, many different endpoints as time at the first change to the maximum slope of the fluorescence-intensity curve have been used. Therefore the comparisons of the results among laboratories seem difficult. (Phipps et al., 2007)

### 4.4 Oxygen Radical Absorbance Capacity Assay (ORAC)

The assay measures the oxidative degradation of the fluorescent molecule (either beta-phycoerythrin or fluorescein) after being mixed with free radical generators such as azo-initiator compounds. Azo-initiators are considered to produce the peroxyl radical by heat. This peroxyl free radical could react with the fluorescent molecule, resulting in the loss of fluorescent property. Antioxidant is able to protect

the fluorescent molecule from this oxidative degeneration. The degree of protection will be quantified using a fluoro-spectometer. Fluorescein is commonly used as a fluorescent probe.

The ORAC values were calculated from the area under the intensity curve (AUC) of the fluorescence intensity and time and expressed as the trolox equivalent (TE) in the unit of μmole/L of sample. The net AUC is the AUC of sample or standard which subtract with the AUC of blank that used in the assay. The AUC and net AUC were calculated according to equation 7 and equation 8, respectively.

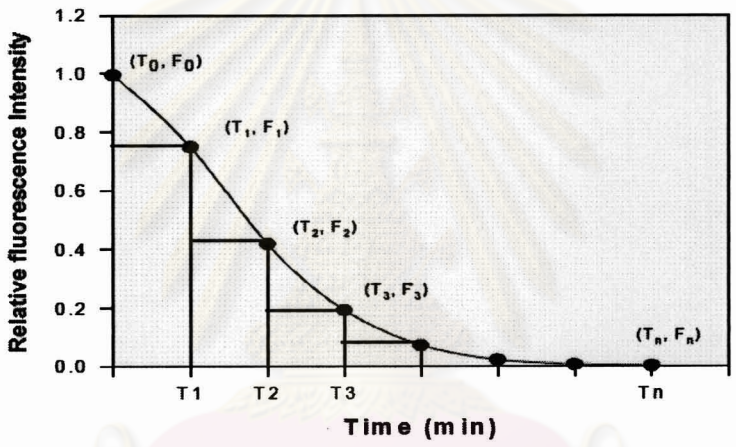



**Figure 9** Calculation of the area under the curve

$$AUC = [0.5 + \left(\frac{F1}{F0}\right) + \left(\frac{F2}{F0}\right) + \left(\frac{F3}{F0}\right) + \cdots + \left(\frac{Fn}{F0}\right)] \quad \text{..... (7)}$$

$F_0$ = The fluorescence intensity at the initiation of reaction

$F_1, F_2 \ldots$ = The fluorescence intensity at the 2,3,... minute measurement

$F_n$ = The fluorescence intensity at the last measurement

$$\text{Net AUC} = \text{AUC}_{\text{sample or standard}} - \text{AUC}_{\text{blank}} \quad \text{..... (8)}$$

The calibration curve was obtained by plotting the net AUC against their trolox concentrations. The ORAC values of samples were calculated by replaced the net AUC of sample into the regression equation obtain from the calibration curve.

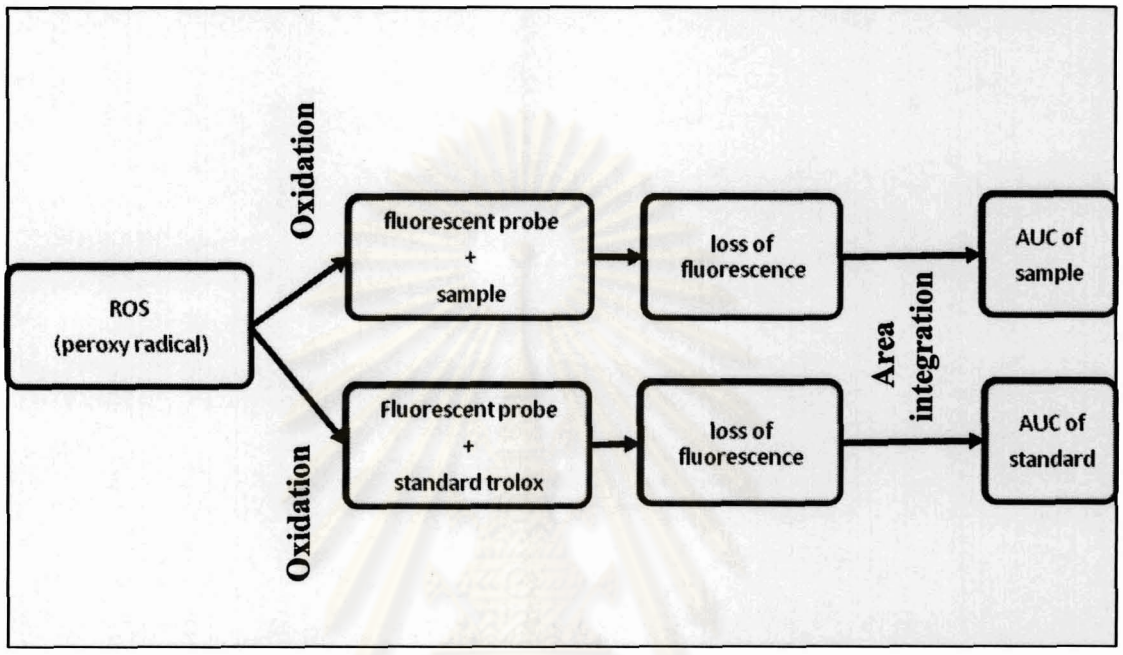



**Figure 10** The principle of ORAC assay (Huang et al., 2005)

**Table 4** Comparison methods for the assessing total antioxidant activity

| antioxidant assay | free radical | mechanism | endpoint | quantitation |
|---|---|---|---|---|
| TEAC | $ABTS^{\bullet+}$ | SET | time | Δ absorbance at fixed time |
| FRAP | -[1] | SET | time, varies | Δ absorbance at fixed time |
| TRAP | $ROO^{\bullet}$ | HAT | lag phase | $IC_{50}$ lag time |
| ORAC | $ROO^{\bullet}$ | HAT | fixed time | AUC |

[1] - = no radicals used in the assay

In these total antioxidant activity assays mentioned, this study select the ORAC method with the following reasons:

1. ORAC methods measure antioxidant inhibition of peroxyl radical induced oxidation and thus reflects a classical chain-breaking antioxidant capacity by hydrogen atom transfer (HAT) (Huang et al., 2005; Phipps et al., 2007). Curcumin, its metabolites (Jovanovic et al., 1999) and vitamin E (α-tocopherol) (Traber and Serbinova, 1999; Leopoldini et al., 2004) have been proposed to have the mechanism of chain-breaking antioxidant capacity by HAT mechanism for their antioxidant activity used to scavenge the free radicals.

2. Comparing with the TRAP that used the same HAT mechanism for scavenging the free radicals, the ORAC can determine the % inhibition and inhibition time in the same assay by plot the curve between time and the fluorescent intensity of the sample then calculate the area under curve (AUC). From the time –fluorescent intensity curve, the length of time that the antioxidant used to inhibit the reaction between the fluorescent probe and the peroxyl free radical could be measured. Furthermore, the area under curve that calculated from these curve are represented to the capacity of antioxidant to inhibit the reaction of free radical and fluorescence probes. While, the TRAP method determine only the length of time that antioxidant used to inhibit the reaction of free radical and fluorescent probe. (Huang et al., 2005)

# CHAPTER II

# MATERIALS AND METHODS

## Materials

### Chemical compounds and reagents

Reference standards in the study were as following:

- α-tocopherol, Lot. No. 32K1243, purity 99%; Sigma, St. Louis, USA.
- α-tocopherol acetate, Lot. No. 30K1172, purity 97.9%; Sigma, St.Louis, USA.
- Turmeric extracted powder, contains curcuminoids in which having 89.41% of curcumin, the 8.14 % of demethoxycurcumin and the 2.45 % of bisdemethoxycurcumin; Lot.No.420724,Thai-China Flavours &Fragranced Industry co. Ltd. (TCFF), Thailand.
- Mefenamic acid, purity 100.35%, Ningbo Smart Chemical & Pharmaceutical, China.
- Trolox, purity 99.9% ,Sigma, St. Louis, USA.
- Standard of Tetrahydrocurcumin, Hexahydrocurcumin and Octahydrocurcumin were generously supplied from the Faculty of Sciences, Ramkhamhaeng University, Thailand.

Supplements for the volunteer's administration:

- GPO-curmin® (Each capsule consists of turmeric extract equivalent to curcumin 250 mg), Lot No. K510061, Mfg. date 02/07/08, Exp. date 02/07/10, GPO, Thailand.
- Vitamin E (D-alpha Tocopherol) 200 IU, Lot No. 135553, Mfg. date 11/2007, Exp. date 11/2012, R.P.Scherer GmbH. Eberbach/Bader,W. Germany.

The other chemical compounds and reagents were of analytical grade and used as received. The reagents for HPLC analysis were all HPLC grade. All of the chemical used were as following:

- AAPH (2,2-azobis (2-amidinopropane) hydrochloride); Wako Chemical , USA.
- Acetic acid glacial; Lab Scan Analytical Science, Thailand.
- Acetonitrile, HPLC Grade; Burdick& Jackson, USA.
- Ethyl acetate; Fisher Scientific, UK.
- Fluorescein sodium salt; Sigma, St. Louis, USA.
- Hydrochloric acid; Lab Scan Analytical Science, Thailand.
- Isopropanol; Riedel-deHaen, Germany.
- Methanol, HPLC Grade; Burdick& Jackson, USA.
- Methanol; Burdick& Jackson, USA.
- Potassium dihydrogen phosphate; Sigma, St. Louis, USA.
- Potassium hydroxide; Sigma, St. Louis, USA.
- Sodium hydrogen phosphate; Sigma, St. Louis, USA.

**Apparatus**

- 96-wel polypropylene plate , black , flat bottom, Costar®, USA.
- Analytical balance; Mettle-Toledo AG 245 balances, Switzerland.
- Centrifuge, Mikro 22R Zentrifugen, Hettich, Germany.
- Freezer –20◦C, Sanyo SF-C95, Sanyo Universal Electric Co.Ltd., Thailand.
- Freezer –48◦C, HLLF-240, Heto-Hatten A/S, Denmark.
- Micropipette; Socorex®, USA.
- Microplate reader Wallac model 1420 with fluorescence filter, Perkin Elmer, USA.
- pH meter, 744 pH meter, Metrohm ion analysis, Switzerland.
- Sonicator bath, RK 103H, Bandelin sonorex, Germany.
- UV-Visible Spectrophotometer, Shimadzu corporation, Japan.
- Vortex mixer, Vortex-Genie; Scientific, Germany.
- High Performance Liquid Chromatography (HPLC), Waters Associates Pty, LTD., Massachusetts. USA. Waters 2695 separations module composes of quaternary solvent gradient pump, four-channel inline vacuum degasser, column heater , sample heater/cooler and 120-vial capacity sample management- auto sampler, Waters 2487 dual λ absorbance detector and Empower® 2002 software.
- HPLC columns and guard column were purchased from Waters Associates Pty, Ltd., Massachusetts, USA.

  For curcuminoids and its metabolites analysis:

  - Symmetry Shield ®, RP18, (150 X 3.0 mm, i.d.) 5 μm.

- Guard column, (20 x 2.0 mm, i.d.) Symmetry Shield® RP18, 5 μm, Par No. 186000107.

For α-tocopherol analysis:

- μBondapak® C18, (300x 3.9 mm, i.d.) 10 μm.
- Guard column, (20 x 2.0 mm, i.d.) packed with Corosil® C18, 37-50 μm.

**Human plasma**

In the bioanalytical method validation, human plasma was purchased from the plasma division, Thai Red Cross Society, Thailand.

**Laboratory Condition**

The analysis of plasma for either curcuminoids or α-tocopherol was performed in the room that illustrated with yellow light for preventing the degradation of curcuminoids and α-tocopherol.

## Methods

The study was divided into three parts:

## Part I Analytical methods used in this study

Three analytical methods were involved in this study. They were for the determination of antioxidant activity in plasma, the analytical methods for HPLC

analysis of plasma curcumin and its metabolites (THC, HHC and OHC) and the analytical method for the determination of endogenous α-tocopherol in plasma by HPLC. The bio-analytical method validation was performed for each analytical method before experimentation.

## Part II Contents and total antioxidant capacity of curcumin and α-tocopherol in supplement preparations

The content of curcumin and α-tocopherol supplements of each individual batch study was determined by HPLC method. For the total antioxidant activity of each curcumin and α-tocopherol supplements, the ORAC method was used.

## Part III Experimentation on volunteer subjects

In this part of study, plasma samples from curcumin administration groups were used for determining the total antioxidant capacity by ORAC method, the concentration of curcumin and its metabolites by the validated HPLC method in Part I, and the plasma concentration of α-tocopherol also from the validated method for α-tocopherol in Part I.

The plasma samples for α-tocopherol administration group were used for determining the total antioxidant capacity by ORAC method and plasma concentration of α-tocopherol by the validated HPLC method in Part I .

## Part I Analytical methods used in this study

### 1. The analytical method for the determination of total antioxidant capacity

The determination of plasma total antioxidant capacity by ORAC assay in this study was performed by utilizing ORAC method established in the year of 2001 by Ou, Woodill and Prior (Ou, Woodill and Prior, 2001). The suitable condition of the method was determined having trolox as the standard antioxidant before performing the analytical method validation. The advantage of ORAC method in biological sample analysis was that no sample preparation is required. The sample that used in the assay must be diluted for decreasing the viscosity or for reducing the concentration of antioxidant in biological samples. The calibration curve was performed using standard trolox in the concentration ranged of 6.25 to 100.00 μM.

**Procedure for the ORAC method:**

Trolox solutions in the concentrations of 6.25, 12.50, 25.00, 50.00, 75.00 and 100.00μM were used as the standard antioxidant. In each well of microplate, a 25 μl of standard trolox or diluted plasma sample (as sample) or 0.075 M phosphate buffer pH 7.0 (as blank) were mixed with 150 μl of fluorescein solution (4.0 nM) and incubated at 37 °C in the incubator for 30 minutes. Then, a 25 μl of warmed of AAPH solution (153 mM) was added before shaking at the maximum intensity of the machine for 10 seconds. The fluorescence of each well was then measured every one minute up to 100 minute. at the excitation wavelength of 485 nm and the emission wavelength of 535 nm. The observed fluorescein intensity was plotted against time

period of AAPH-fluorescein reaction. The ORAC values were calculated from the area under the intensity curve (AUC) of the fluorescence intensity and time and expressed as the trolox equivalent (TE) in the unit of μmole/L of sample.

### 1.1 Bioanalytical method validation of the ORAC assay

The analytical method for determining total antioxidant activity in plasma was validated to ensure the linearity, accuracy and precision of the method according to the criteria of analytical method validation that proposed by Ou et al. (Ou et al., 2001)

#### Linearity

The linear relationship between standard trolox concentrations and net AUC of standard trolox were determined by analyzing a series of standard trolox concentration according to the described method. The reproducibility of linear relationship was confirmed by analyzing three replications of the series of standard trolox. The % RSD of the slope and $R^2$ calculated should be less than 15%.



#### Accuracy and precision

##### The intra-day accuracy and precision

Six replications of standard trolox concentrations of 10.0, 40.0 and 80.0 μM were analyzed according to the described method along with the series of standard trolox concentrations.

The accuracy of analytical method was presented in the term of the percentage (%) bias (equation 9) in which it should be within $\pm$ 15 %.

%bias = [(analyzed conc. – added conc.)/ added conc.] x 100 .....(9)

The precision of analytical method was determined in the term of the percentage of relative standard deviation (%RSD) of trolox concentration. For the acceptable precision, the %RSD should not more than 15% for all trolox concentrations studied.

**The inter-day accuracy and precision**

The aforementioned procedure for intra-day analysis was followed but only one replication of standard trolox was analyzed on six separate days. The same criteria for the intra-day accuracy and precision were followed.

**2. The analysis of curcumin, THC, HHC and OHC in plasma by HPLC**

The analytical method for determining curcumin, THC, HHC and OHC in human plasma by HPLC reported by Thongnopnua et al (Thongnopnua et al., 2007) was utilized.

**Procedure for the curcumin, THC, HHC and OHC analysis:**

A series of calibration solutions and quality samples were analyzed along with plasma samples for every assay. A 375µl of plasma sample was pipetted into eppendorf containing 150 µl of purified water and 15 µl of 6N hydrochloric acid

solution, the mixture was vortex-mixed and extracted by ethyl acetate and isopropanol for 5 minutes. The mixture was centrifuged at 15,300 xg for 30 minutes. The extracted layer was separated and evaporated to dryness using nitrogen gas. It was then reconstituted with 100 µl of mefenamic acid solution as internal standard (0.048µg/ml) and directly injected to the HPLC system.

The series concentration of standard curcumin, THC, HHC and OHC were prepared in the range of 0.04-1.60 µg/ml for OHC and THC. For HHC and curcumin, the concentration ranges were 0.08-3.20 and 0.012-1.00 µg/ml, respectively.

HPLC conditions

| | |
|---|---|
| analytical column: | Symmetry Shield® C18, (150 x 3.0 mm. i.d) 5 µm |
| guard column: | Symmetry Shield®RP18, (20 x 2.0 mm, i.d.) 5 µm, |
| mobile phase: | acetonitrile: 0.15% aqueous glacial acetic acid solution; the proportional gradient of 20 to 50 % of acetonitrile over 50 minutes |
| flow rate: | 0.75 to 0.55 ml/min within 50 minutes |
| auto sampler: | 4 ◦C |
| column temperature: | 30 ◦C |
| detection wavelength: | dual wavelength detector at 420 and 282 nm. |
| injected volume: | 50 µl |

**2.1 The bioanalytical method validation**

The analytical method for curcumin, THC, HHC and OHC in plasma was validated to ensure the linearity, accuracy, precision, sensitivity and specificity of

the method according to the criteria of bioanalytical method validation. (US.FDA, 2001)

**Linearity**

The linear relationship between curcumin, THC, HHC and OHC concentrations with their peak area ratios (PAR) were determined by analyzing a series of standard plasma curcumin, THC, HHC and OHC concentrations according to the described method. The reproducibility of linear relationship was confirmed by analyzing three replications of the series of standard plasma. The % RSD of the slope and $R^2$ calculated should be less than 15%.

**Accuracy and precision**

**The intra-day accuracy and precision**

The standard plasma was spiked with curcumin, THC, HHC and OHC at three different concentrations. The concentrations for curcumin were 0.1, 0.2 and 0.8 μg/ml; for OHC and THC, they were 0.15, 0.3 and 0.9 μg/ml and for HHC, the concentrations were 0.3, 0.6 and 1.8μg/ml. The six replications of standard plasma curcumin, THC, HHC and OHC were analyzed according to the described method along with the series of standard curcumin, THC, HHC and OHC for the calibration curve.

The accuracy of analytical method was presented in the term of the % bias (equation 9) in which it should be within $\pm$ 15 %. The precision of analytical method was determined in the term of the %RSD of curcumin, THC, HHC

and OHC concentration. For the acceptable precision, the %RSD should not more than 15% for all curcumin, THC, HHC and OHC concentrations studied.

**The inter-day accuracy and precision**

The aforementioned procedure for intra-day analysis was followed but only one replication of standard plasma spiked curcumin was analyzed on six separated days. For the acceptable accuracy, the % bias should be within $\pm$ 15 %, The %RSD of not more than 15% for the acceptable precision was followed for all curcumin, THC, HHC and OHC concentrations studied.

**Sensitivity**

The sensitivity of an analytical method can be described in term of the lowest limit of quantitation (LLOQ). LLOQ was the lowest concentration of the calibration curve that can be measured with acceptable accuracy and precision.

To confirm the sensitivity of the method that described by Thongnopnua et al. (Thongnopnua et al, 2007), six replications of spiked standard plasma in the concentration of curcumin 0.012 μg/ml, OHC 0.04 μg/ml, HHC 0.08 μg/ml and THC 0.04 μg/ml were analyzed according to the described method along with the series of calibration curve. The variation of analysis in term of % bias should be within $\pm$ 20 % and % RSD should not more than 20 %.

**Specificity**

The detector that used in the study was dual wavelength in separated file. To determine the specificity of the analytical method, the retention time of

curcumin, THC, HHC, OHC and internal standard (IS) obtained from the analysis of standard solution and spiked standard plasma sample should be identical. In addition, no endogenous peak should be observed at the retention time of each compound.

### 3. The analysis of α-tocopherol in plasma by HPLC

The analytical method for determining α-tocopherol in human plasma reported by Nirungsan and Thongnopnua was utilized in this study (Nirungsan and Thongnopnua, 2005).

**Procedure for the α-tocopherol analysis:**

A 100 µl of plasma was pipetted into eppendorf containing a 10 µl standard solution of α-tocopherol acetate (as internal standard) (0.6mg/ml).The deproteinizing agent (acetonitrile and isopropanol in the proportion of 7:3 ) was added and vortex-mixed for 1 minute before being centrifuged at 17,000 xg for 10 minutes. The separated 20 µl supernatant was used for HPLC analysis.

The series of standard plasma α-tocopherol concentration were 0, 0.50, 1.0, 5.0, 10.0, 15.0, 20.0 and 30.0µg/ml.



HPLC conditions

| | |
|---|---|
| analytical column: | µBondapak® C18, (300 x 3.9 mm., i.d.) 10 µm |
| guard column : | Corosil ® C18, (20x2.0 mm, i.d.) 37-50µm |
| mobile phase: | 100 % methanol |
| flow rate: | 1.0 ml/min |
| auto sampler: | 4 ◦C |

column temperature: 30 ◦C

detection wavelength : 292 nm.

injected volume: 20 µl

### 3.1 Bioanalytical method validation

The analytical method for α-tocopherol in plasma was validated to ensure the linearity, accuracy, precision, sensitivity and specificity of the method according to the criteria of bioanalytical method validation. (US.FDA, 2001)

#### Linearity

The linear relationship between α-tocopherol concentrations with PHR were determined by analyzing a series of standard plasma α-tocopherol concentrations according to the described method. The reproducibility of linear relationship was confirmed by analyzing three replications of the series of standard plasma. The % RSD of the slope and $R^2$ calculated should be less than 15%.

#### Accuracy and precision

**The intra-day accuracy and precision**

Six replications of spiked α-tocopherol standard plasma in the concentrations of 5.0, 12.0 and 24.0 µg/ml were analyzed according to the described procedure along with the series of standard α-tocopherol in plasma for the calibration curve. For the acceptable accuracy, the % bias should be within $\pm$ 15 %. The acceptable precision was confirmed from the % RSD to be less than 15% for all α-tocopherol concentrations studied.

**The inter-day accuracy and precision**

The aforementioned procedure for intra-day analysis was followed but only one replication of standard plasma spiked α-tocopherol was analyzed on six separated days. The same criteria for the intra-day accuracy and precision were followed with the % bias within ± 15 % and the % RSD of not more than 15 % for all α-tocopherol concentrations studied.

**Sensitivity**

The sensitivity of an analytical method can be described in term of the lowest limit of quantitation (LLOQ). LLOQ was the lowest concentration of the standard curve that can be measured with the acceptable accuracy and precision.

To confirm the sensitivity of the method in determining endogenous α-tocopherol, six replications of endogenous α-tocopherol in blank plasma were analyzed along with the standard plasma for preparing the calibration curve. The concentration of endogenous α-tocopherol was determined from the calibration curve by the standard addition method (Meier and Zund, 2000).

The endogenous α-tocopherol in the prepared spiked plasma was diluted with deproteinizing agent before injecting to HPLC. The variation of analysis in term of % bias should be within ± 20 % and not more than 20 % for % RSD.

**Specificity**

To determine the specificity of the analytical method, the retention time of α-tocopherol and internal standard obtained from the analysis of standard

solution and spiked internal standard plasma sample should be identical. In addition, endogenous peak shouldn't interfere with α-tocopherol and internal standard.

**Part II Contents and total antioxidant capacity of curcumin and α-tocopherol in supplement preparations**

Supplement preparations used in this study were curcuminoids extract in the form of capsules and vitamin E capsules. Before experimentation, the antioxidant amounts of curcuminoids and α-tocopherol have to be determined in each individual preparation. Only the amount that was within the percentage labeled claimed could be used. Meanwhile, the total antioxidant capacity of both compound have also been determined.

**2.1 To determine the content of curcumin in supplement preparation**

The dosage form of the curcuminoids extract that use in this study was hard capsule. Follow the USP 30, the uniformity of dosage unit likes hard gelatin capsule which containing the active ingredient more than 25 mg, can be demonstrated by the weight variation.

Ten capsules were individually accurately weighed. The content of each capsule was removed by a suitable means. The emptied shells were accurately weighed individually. The net weight of its contents was calculated for each capsule by subtracting the weight of the shell from the respective gross weight. Then, the content within capsules were mixed and brought to the assay.

For the assay, the content within capsule was accurately weighed equivalently to 50 mg of curcumin, dissolved and made up to 100 ml with methanol

( n=3). The aliquot curcumin solution of 2.0 μg/ml was used for injecting into the HPLC system comparing to curcuminoids standard solution.

The content of curcumin was expressed as % of label claim according to equation (10) and the result from the assay was used to calculate the acceptance value.

$$\% \text{ label claim} = 100(C_u/C_s)(PA_s/PA_u) \quad \text{..... (10)}$$

$C_u$ = concentration of active compound in sample preparation based on the label claim

$C_s$ = concentration of active compound in standard preparation

$PA_s$, $PA_u$ = PA from standard preparation and the sample preparation

The acceptance criteria for the content uniformity of dosage unit was that the acceptance value of 10 of dosage units was less than or equal to 15 %. Calculate the acceptance value (AV) according equation (11).

$$\text{Acceptance value (AV)} = |M - \bar{X}| + ks. \quad \text{..... (10)}$$

M = reference value

$\bar{X}$ = mean of individual contents expressed as a % of label claim

k = acceptability constant; if number of sample =10, k=2.4

s = sample standard deviation

**2.2 To determine the content of α-tocopherol in vitamin E supplement**

The dosage forms of vitamin E supplement that use in this study were soft gelatin capsule. Follow the USP 30, the uniformity of dosage unit likes soft gelatin capsule which containing the solution of active ingredient more than 25 mg, can be demonstrated by the weight variation.

Ten capsules were individually accurately weighed. The content of each capsule was removed by a suitable means. The emptied shells were accurately weighed individually. The net weight of its contents was calculated for each capsule by subtracting the weight of the shell from the respective gross weight. Then, the content within capsules were mixed and brought to the assay.

For the assay, ten vitamin E supplement soft gelatin capsules were individually weighed before and after removed the shell of capsule. The α-tocopherol liquids were accurately weighed equivalently to 20 mg of α-tocopherol, dissolved and made up to 100 ml with methanol (n=3). The aliquot α-tocopherol solution of 0.2 mg/ml was used for injecting into HPLC system comparing to α-tocopherol standard solution.

The content of α-tocopherol was expressed as % of label claim according equation (10) and the result from the assay was used to calculate the acceptance value. The acceptance criteria for the content uniformity of dosage unit was that the acceptance value of 10 of dosage units was less than or equal to 15 %. Calculate the acceptance value (AV) according equation (11).

**2.3 To determine the total antioxidant capacity of curcuminoids and α-tocopherol in supplement preparations**

A 2.0 μg/ml methanol of curcuminoids from supplement preparation was prepared as well as 0.2 mg/ml of α-tocopherol supplement. These samples were used to determine the total antioxidant activity of the preparation according to the described method in part I. and expressed in term of trolox equivalent.

**Part III Experimentation on volunteer subjects**

This part of the study had been approven by the Ethic Committee at Faculty of Pharmaceutical Sciences, Chulalongkorn University. (No. 019/2551)

### 3.1 Inclusion of volunteer subjects

Twenty five to thirty volunteers, both male and female, in the age ranged of 20-40 years with the body mass index (BMI) that not more than 30 $kg/m^2$ were included. They have to pass the physical and biochemical examinations. They were included as the volunteer subjects for the study only after they were willing to sign the informed consent form voluntarily. Also, one month before the day of experiment, they should not be on therapy with any antioxidant supplement especially with curcuminoids extract and vitamin E.

As the volunteer subjects, they were asked to fill up the questionnaire associate to their past meal's habit. They have also to record the details of each meal during experimentation.



### 3.2 Experimentation

All subjects were blind randomized separated into three groups: A, B and C. Each group was assigned for different treatments. Each subject in group A have to administer curcuminoids extract in the dose of 500 mg/day up to 7 days. For group B, the dose of curcuminoids extract 6 g/day was administrated to each subject for 7 days. The subjects in group C were administrated vitamin E supplement in the dose of 200 IU/day for 7 days. At the seventh days of experimentation, blood sample of each

subjects were withdrawn from the forearm vein before supplements administration, separated into portions for the ORAC analysis, the curcumin analysis, the α-tocopherol analysis and also for determine the level of cholesterol, triglyceride, AST enzyme and ALT enzyme.

The curcuminoids extract in the dosage of 6 g/day was the reported amount used the clinical study of cancer patients (Cheng et al, 2001). For curcuminoids extract in the dose of 500 mg/day was the recommended amount for supplementation. The use of 200 IU/day vitamin E supplement was the dose that had been proven to increase the level of endogenous α-tocopherol in Thai people. (Thongnopnua, 2006)

The time schedule for administering any supplement during experimentation was the time before breakfast. The compliance of each subject was followed and any adverse side effect was recorded. For the first and the seventh day of supplement administration, every subject has to follow the requirements according to blood sample collection.

**Blood sample collections**

A 5 ml of blood from forearm vein was withdrawn from each subject at each sampling time of each group.

For subjects in group A and B that administrated curcuminoids supplement, blood sample was collected from the forearm vein before supplementation and at 1, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0 hours post administrations on the first and the seventh day of experimentation.

The subjects participated in group C with the administration of vitamin E supplement was scheduled for blood sampling prior dosing and post dose at the seventh day morning.

Blood sample was immediately centrifuged at 1500 xg for 15 min. The plasma was separated into three portions for determining the total antioxidant capacity, analyzing curcumin, THC, HHC and OHC by HPLC method and the last portion for plasma α-tocopherol analysis by HPLC techniques. All plasma samples were stored light protection at -48 ˚C for subsequent analysis within 2 weeks.

### 3.3 Analysis of plasma samples

Plasma samples were thawed at room temperature before analyzing along with the quality control samples (QC samples) and the series of calibration solutions for each day of analysis.

- **The determination of total antioxidant capacity of samples by ORAC method**

The validated method (Part I section 1) for total antioxidant capacity determination by ORAC method was used to determine the total antioxidant capacity in plasma samples. The total antioxidant capacity was expressed in the term of ORAC values or trolox equivalents.

- **The determination of curcuminoids in plasma samples**

The validated method (Part I section 2) for curcumin, THC, HHC and OHC determination in plasma was used to analyzed the plasma sample from volunteer subjects. The concentration of curcumin and its metabolites in plasma samples were calculated through the regression equations of each calibration curve.

- **The determination of endogenous α-tocopherol in plasma**

The validated method (Part I section 3) for plasma α-tocopherol analysis was used for determination of plasma α-tocopherol in the plasma samples. The concentration of plasma α-tocopherol was calculated through before and after administration.

### 3.4 Data interpretation

- The ORAC values of plasma samples in each sampling time of each subject were determined. The ORAC values were compared between before experimentation and after seven days administration in each individual group. The student paired t' test at the significant level of 0.05 (α=0.05) was monitored.

Compare the ORAC values after seventh day administration in subjects administered curcumin extract at the different dosages (group A and B).and compared the ORAC values after administrated the supplements between group A, B and C by using ANOVA at the significant level of 0.05 (α=0.05).

- The concentration of curcumin, HHC, THC and OHC in the first day and the seventh day of experiment of each individual group A and B. The patterns of the concentration-time profile were observed relationship between group A and B.

- The endogenous α-tocopherol in plasma of each individual group were determine. Comparing the concentrations of α-tocopherol before and after administration in group A, B and C.

# CHAPTER III

# RESULTS AND DISCUSSION

## Part I Analytical methods used in this study

### 1. The analytical method for the determination of total antioxidant capacity

#### 1.1 Bioanalytical method validation of the ORAC assay

**Linearity**

The net AUC was confirmed to relate with trolox concentration in the range of 6.25-100 μM. The linear regression of the calibration curve was determined with the representative calibration curve as shown in Figure 10. The representative regression equation was net AUC = 0.33 Conc. + 2.95. The variation of slope and the $R^2$ of the curve was 6.48 and 0.14 %RSD, respectively (Table 5). The average $R^2$ was 0.9951 that could be acceptable according to Ou et al reported ($R^2$ was $\geq$ 0.99) (Ou et al., 2005).

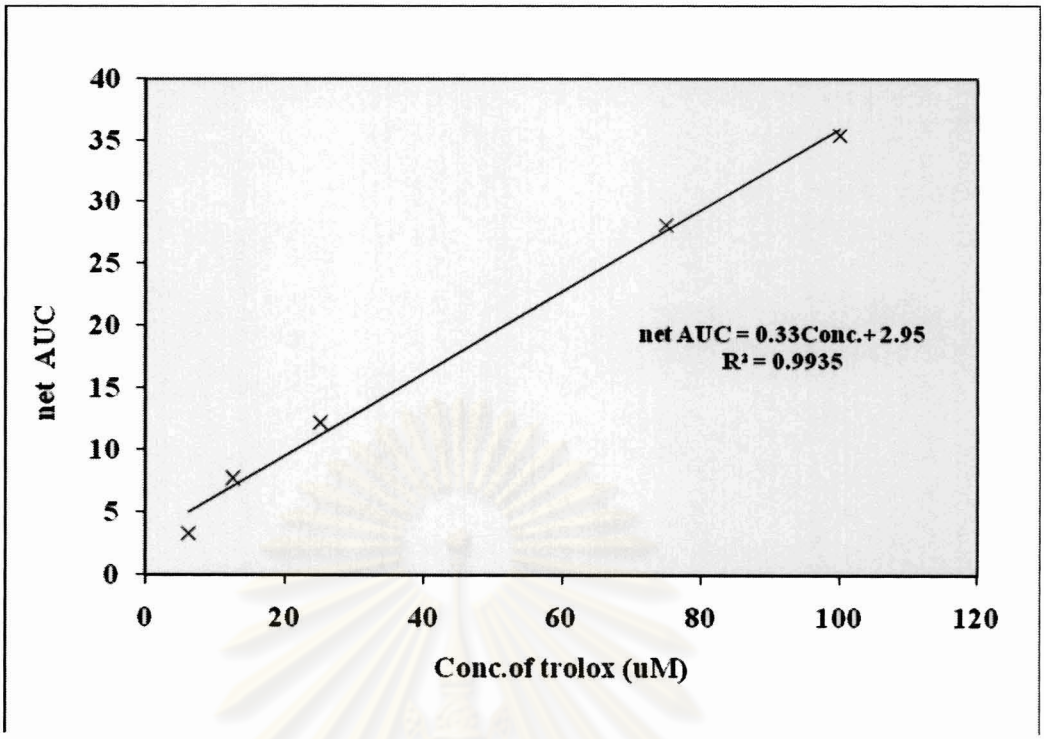



**Figure 11** The representative calibration curve for trolox as the standard in antioxidant activity determination

**Table 5** Parameters of calibration curve for standard trolox (6.25 -100 μM)

| N | Parameter | | |
|---|---|---|---|
| | **Slope** | **Intercept** | **$R^2$** |
| 1 | 0.29 | 4.59 | 0.9959 |
| 2 | 0.30 | 3.73 | 0.9960 |
| 3 | 0.33 | 2.95 | 0.9935 |
| **Mean** | 0.31 | 3.75 | 0.9951 |
| **SD** | 0.02 | 0.82 | 0.0014 |
| **%RSD** | 6.48 | | 0.14 |

**Accuracy and precision**

The percentage of bias of standard trolox in the intra-day and the inter-day accuracy were ranged from – 4.68 to 3.13% and -1.72 to 2.45%, respectively (Table 6).

The percentages of RSD were 7.70 - 10.84% and 3.40 - 6.87% for the intra-day and the inter-day precision, respectively (Table 6).

Both expressions of accuracy and precision for the intra-day and inter-day analysis were within the acceptable range of ± 15% bias and not more than 15% RSD. Therefore, the method was accepted to be accurate and precise enough for determining total antioxidant activity by ORAC method.

**Table 6** The intra-day and inter-day accuracy and precision of standard trolox

| Conc. of trolox (uM) | Intra-day study (n=6) | | | Inter-day study (n=6) | | |
|---|---|---|---|---|---|---|
| | Mean ± SD | %RSD | %Bias | Mean ± SD | %RSD | %Bias |
| 80.0 | 82.50 ± 7.03 | 8.53 | 3.13 | 79.83 ± 4.16 | 5.22 | -0.22 |
| 40.0 | 39.89 ± 3.06 | 7.70 | -0.28 | 40.98 ± 2.82 | 6.87 | 2.45 |
| 10.0 | 9.53 ± 1.03 | 10.84 | -4.68 | 9.83 ± 0.33 | 3.40 | -1.72 |

## 2. The analysis of curcumin, THC, HHC and OHC in plasma by HPLC

### 2.1 The bioanalytical method validation

#### Linearity

The three replications of calibration curves for curcumin, THC, HHC and OHC showed that the % RSD values for the slope and $R^2$ less than 15 % (Table 7). These results confirmed the usable of these calibration curves. The representative regression equation of each compound is expressed in Table 8. Figure 11 shows the representative calibration curve for curcumin.

**Table 7** Parameters of the calibration curve

| | Parameter ( n=3) | | | | |
|---|---|---|---|---|---|
| | Slope | | Intercept | $R^2$ | |
| Sample | Mean ± SD | %RSD | Mean ± SD | Mean ± SD | %RSD |
| curcumin | 57.89 ± 8.23 | 14.21 | 1.8240 ± 0.70 | 0.9949 ± 0.0010 | 0.10 |
| THC | 1.38 ± 0.14 | 10.05 | -0.0077 ± 0.04 | 0.9937 ± 0.0020 | 0.20 |
| HHC | 2.27 ± 0.27 | 11.81 | -0.1904 ± 0.12 | 0.9970 ± 0.0017 | 0.17 |
| OHC | 1.4192 ± 0.20 | 13.76 | -0.0358 ± 0.03 | 0.9953 ± 0.0040 | 0.40 |

**Table 8** The representative regression equations of THC, HHC, OHC and curcumin

| Sample | Regression equation | $R^2$ |
|---|---|---|
| **curcumin** | PAR = 57.03 Conc. – 2.62 | 0.9958 |
| **THC** | PAR = 1.26 Conc. – 0.02 | 0.9960 |
| **HHC** | PAR = 2.26 Conc. – 0.07 | 0.9989 |
| **OHC** | PAR = 1.43 Conc. – 0.02 | 0.9977 |

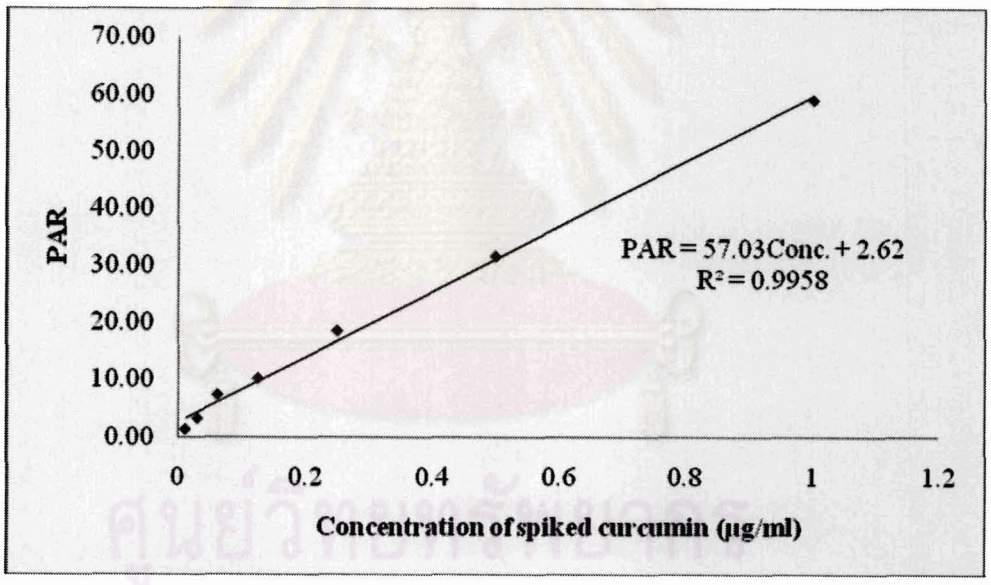



**Figure 12** The representative calibration curve of curcumin

**Accuracy and precision**

**Intra-day and inter-day accuracy**

The intra-day and inter-day accuracy of spiked curcumin, THC, HHC and OHC were all less than ± 15 % bias as shown in Table 9. The percentage of bias in the intra-day and intra-day assay of the spiked curcumin, THC, HHC and OHC in plasma were ranged from – 9.66 to 7.54% and -8.84 to 1.28 % bias respectively. The % bias of the inter-day and intra-day of curcumin, THC, HHC and OHC were within the range ± 15 %. Therefore, it was confirmed that the method was accurate for the determination of curcumin, THC, HHC and OHC in plasma.

**Intra-day and inter-day precision**

The intra-day and inter-day precision of spiked curcumin, THC, HHC and OHC were all less than 15 % RSD as shown in Table 9. The percentage of RSD of the intra-day and inter-day assay of spiked curcumin, THC, HHC and OHC were ranged from 4.26 to 11.20 and 3.65 to 12.06, respectively. Both of the % RSD of the intra-day and inter-day precision assay of all curcumin, THC, HHC and OHC were less than 15 %, so this method that used to determine the concentration of curcumin, THC, HHC and OHC in plasma precised enough.

Both accuracy and precision of intra-day and inter-day were within the range (± 15% bias and not more than 15% RSD), so this method was acceptable for the criteria. It could be confirmed that this method was accurate and precise for analysis curcumin and its metabolites in plasma.

**Table 9** The intra-day and inter-day accuracy of curcumin, THC, HHC and OHC in spiked plasma

| Sample | Spiked conc. (μg/ml) | Analyzed concentration (μg/ml) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Intra-day study (n=6) | | | Inter-day study (n=6) | | |
| | | Mean ± SD | %RSD | %Bias | Mean ± SD | %RSD | %Bias |
| curcumin | 0.10 | 0.10 ± 0.01 | 10.12 | -3.72 | 0.10 ± 0.01 | 5.05 | -3.42 |
| | 0.20 | 0.18 ±0.01 | 7.15 | -7.52 | 0.19 ±0.01 | 3.84 | -6.32 |
| | 0.80 | 0.86 ±0.05 | 5.57 | 7.54 | 0.81 ±0.05 | 6.21 | 1.28 |
| THC | 0.14 | 0.14 ±0.01 | 4.26 | -2.83 | 0.14 ±0.01 | 5.00 | -6.02 |
| | 0.29 | 0.27 ±0.02 | 8.55 | -7.35 | 0.27 ±0.02 | 6.47 | -7.43 |
| | 0.96 | 0.90 ±0.08 | 8.99 | -6.62 | 0.92 ±0.11 | 12.06 | -4.44 |
| HHC | 0.29 | 0.28 ±0.03 | 9.76 | -2.59 | 0.29 ±0.03 | 9.37 | -0.15 |
| | 0.58 | 0.61 ±0.04 | 6.04 | 5.33 | 0.57 ±0.02 | 3.65 | -1.23 |
| | 1.92 | 1.95 ±0.17 | 8.52 | 1.56 | 1.75 ±0.09 | 5.22 | -8.84 |
| OHC | 0.14 | 0.13 ±0.02 | 11.20 | -7.89 | 0.14 ±0.01 | 7.07 | -2.82 |
| | 0.29 | 0.28 ±0.02 | 6.43 | -2.20 | 0.27 ±0.01 | 4.76 | -6.73 |
| | 0.96 | 0.87 ±0.06 | 6.74 | -9.66 | 0.95 ±0.06 | 5.95 | -1.56 |

**Sensitivity**

The lowest concentration of curcumin, THC, HHC and OHC, which could be analyzed with acceptable % bias and % RSD were 0.012, 0.04, 0.08 and 0.04 μg/ml respectively (Table 10). This LLOQ concentration would be used as the first concentration in each individual calibration curve.

**Table 10** The lower concentrations of curcumin, THC, HHC and OHC in spiked plasma

| Sample | Concentration (μg/ml) | Analyzed Conc. (n=6) | | |
|---|---|---|---|---|
| | | Mean ±SD | %RSD | %Bias |
| **Curcumin** | 0.012 | 0.013 ±0.001 | 9.11 | -4.33 to 17.38 |
| **THC** | 0.04 | 0.046 ±0.001 | 2.26 | 13.49 to 19.03 |
| **HHC** | 0.08 | 0.094 ±0.002 | 2.59 | 10.89 to 19.32 |
| **OHC** | 0.04 | 0.044 ±0.001 | 2.41 | 5.98 to 12.85 |

**Specificity**

The specificity of the analytical methods for curcumin, THC, HHC and OHC is displayed in Figure 13 for 420 nm detection wavelength and Figure 14 for 282 nm. The retention time of curcumin, demethoxycurcumin, bisdemethoxycurcumin in standard solution were 40.91 min, 42.94 min. and 45.44 min., respectively. The retention time of THC, HHC and OHC in standard solution were, 33.50 min., 23.22 min. and 19.15 min., respectively. For the spiked standard plasma, the retention time of curcumin, demethoxycurcumin, bisdemethoxycurcumin were 40.91 min., 42.92 min. and 45.42 min., respectively. The retention time of THC, HHC and OHC in spiked solution were 33.49 min., 23.22 min. and 19.17 min., respectively. While the retention time of mefenamic acid (IS) in standard solution, in blank plasma spiked with IS and in spiked standard plasma were 49.495 min., 49.487 min. and 49.490 min., respectively. The chromatograms indicated that curcumin, its metabolites and internal standard could be resolved completely without any interference.

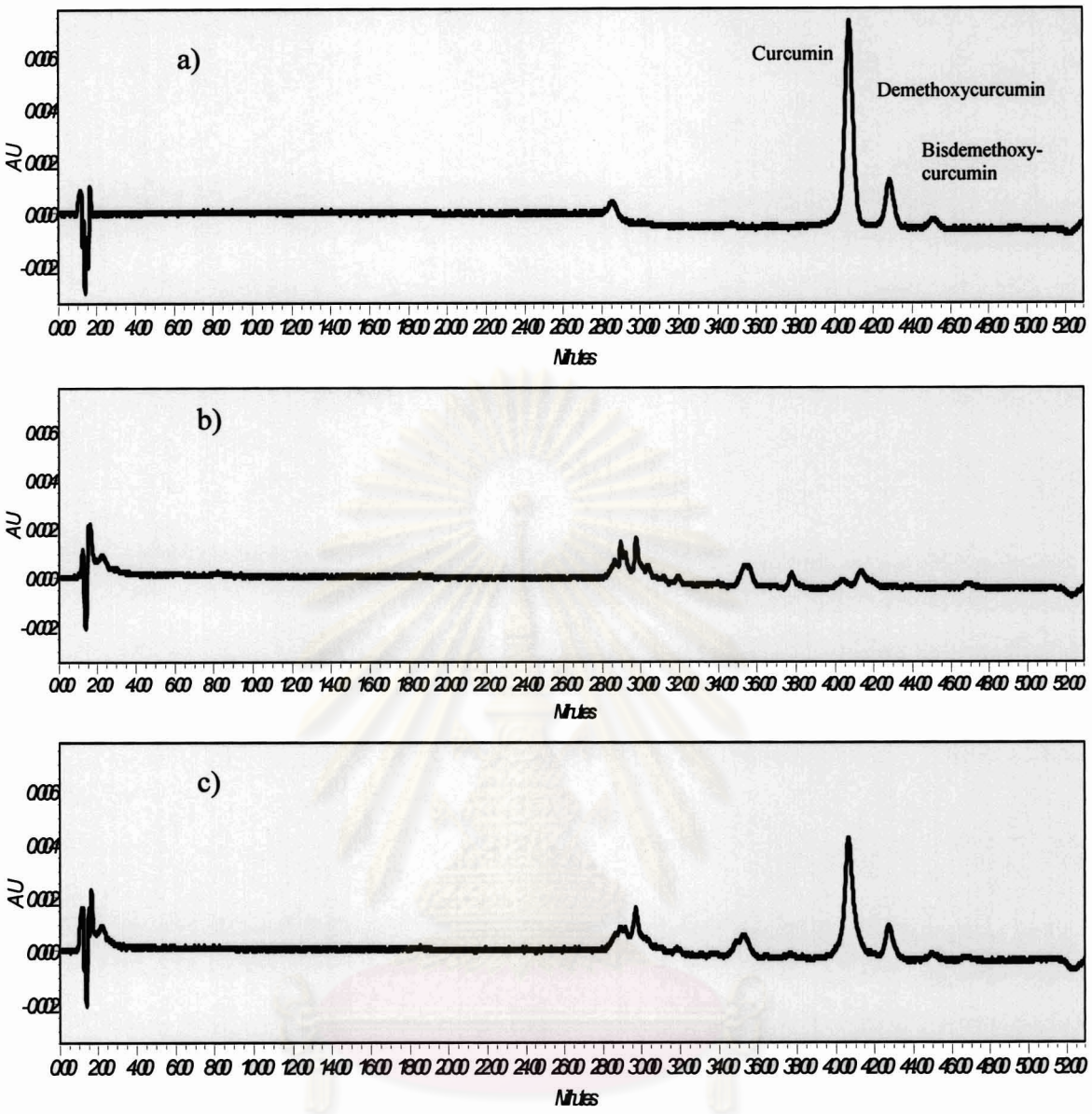



**Figure 13** Chromatograms of curcumin, THC, HHC, OHC and internal standard (IS) at wavelength 420 nm.

a) Standard solution of curcumin 0.25 μg/ml, THC 0.4 μg/ml, HHC 0.8 μg/ml, OHC 0.4 μg/ml and mefenamic acid as Internal standard (IS) 0.048 μg/ml

b) Blank plasma with IS concentration 0.048 μg/ml

c) Spiked plasma sample (at the same concentration as (a) ) with IS concentration 0.048 μg/ml

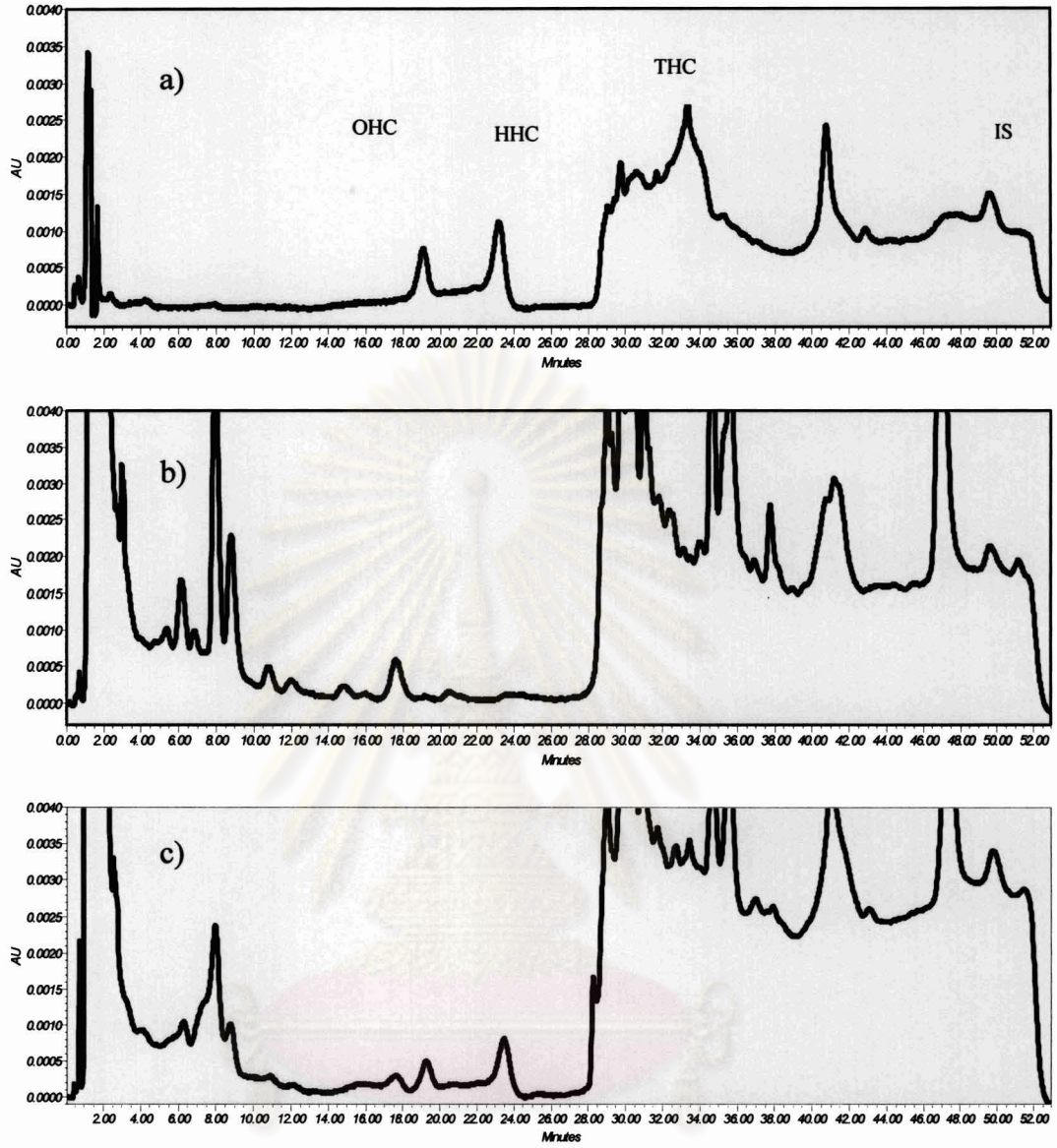



**Figure 14** Chromatograms of curcumin, THC, HHC, OHC and internal standard (IS) at wavelength 282 nm.

a) Standard solution of curcumin 0.25 μg/ml, THC 0.4 μg/ml, HHC 0.8 μg/ml, OHC 0.4 μg/ml and mefenamic acid as Internal standard (IS) 0.048 μg/ml

b) Blank plasma with IS concentration 0.048 μg/ml

c) Spiked plasma sample (at the same concentration as ( a) ) with IS concentration 0.048 μg/ml

## 3. The analysis of endogenous α-tocopherol in plasma by HPLC

### 3.1 The bioanalytical method validation

#### Linearity

The peak height ratio (PHR) was confirmed to relate with α-tocopherol concentration in the range of 0-30 μg/ml. The linear regression of the calibration curve was determined with the representative calibration curve as shown in Figure 14. The representative regression equation was PHR = 0.10(Conc.) + 0.82. The variation of slope and the $R^2$ of the curve were determined to be 0.78 and 0.14%RSD, respectively (Table 11). They could be acceptable according to the criteria of the bioanalytical method validation (US.FDA, 2001).

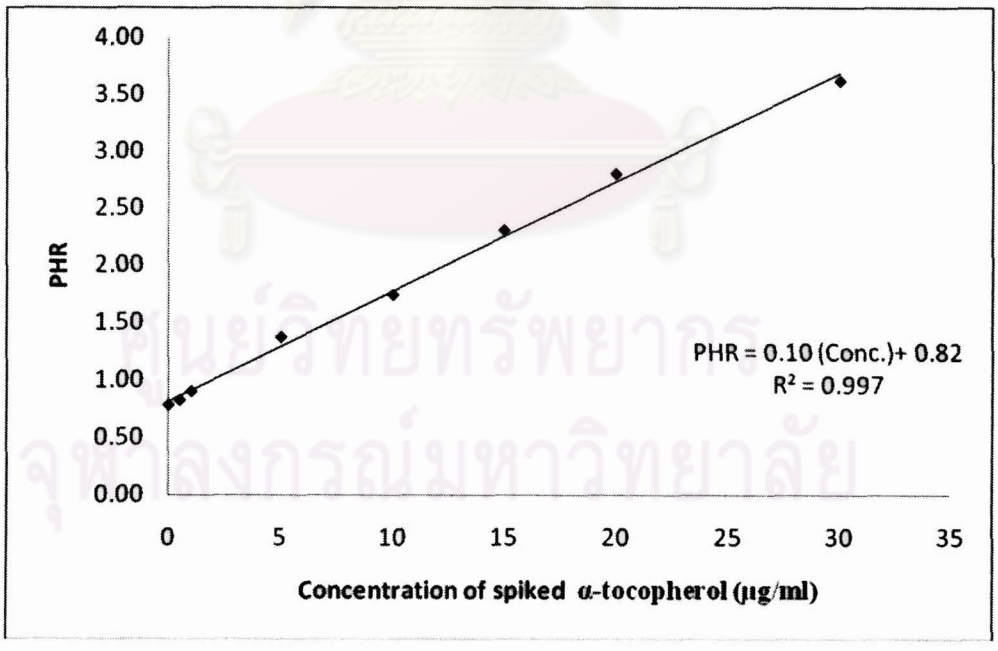



**Figure 15** The representative calibration curve of α-tocopherol

**Table 11** Parameters of calibration curve (0-30 μg/ml)

| | Parameter by PHR (n=3) | |
|---|---|---|
| | **Mean ± SD** | **%RSD** |
| **Slope** | 0.10 ± 0.00 | 0.78 |
| **Intercept** | 0.79 ± 0.02 | 2.51 |
| **$R^2$** | 0.9955 ± 0.00 | 0.14 |

**Accuracy and precision**

**Intra-day and inter-day accuracy**

The intra-day and inter-day accuracy of spiked α-tocopherol in plasma in the term of % bias were all less than ± 15 % as shown in Table 12. The percentage of bias in the intra-day and intra-day assay of the spiked α-tocopherol in plasma were ranged from - 4.58 to - 1.65% and – 1.89 to 0.67 % bias respectively. The method was then considered to be accurate enough for analyzing α-tocopherol in plasma samples.

**Intra-day and inter-day precision**

The intra-day and inter-day precision of spiked α-tocopherol were all less than 15 % RSD as shown in Table 12. The percentages of RSD of the intra-day and inter-day assay of spiked α-tocopherol were ranged from 5.50 to 9.01% and

6.07 to 7.10, respectively. The method was then precise enough determination of α-tocopherol in plasma samples.

Both accuracy and precision of intra-day and inter-day were within the range ( ± 15% for bias and 15% RSD ). It was therefore confirmed that the method was accurate and precise for analysis α-tocopherol in plasma.

**Table 12** The intra-day and inter-day accuracy of α-tocopherol in spiked plasma

| Spiked concentration (μg/ml) | Intra-day (n=6) | | | Inter-day (n=6) | | |
|---|---|---|---|---|---|---|
| | Mean ±SD | %RSD | %Bias | Mean ±SD | %RSD | %Bias |
| 6.0 | 5.72 ±0.52 | 9.01 | -4.58 | 5.89 ±0.42 | 7.10 | -1.89 |
| 12.0 | 11.80 ±0.65 | 5.5 | -1.65 | 12.08 ±0.76 | 6.27 | 0.67 |
| 24.0 | 23.17 ±1.63 | 7.03 | -3.45 | 24.11 ±1.46 | 6.07 | 0.47 |

**Sensitivity**

The lowest concentration of α-tocopherol in plasma that could be quantified was 0.41 μg/ml. Comparing to the lower concentration of α-tocopherol among difference reported by Nirungsan and Thongnopnua was 0.72 μg/ml (Nirungsan and Thongnopnua, 2005), the LLOQ in this study was superior. This discrimination would possibly be due to the difference in UV detector used. For accurate preparation of this concentration, the lowest concentration of α-tocopherol in the calibration curve was prepared to be 0.5 μg/ml plasma.

**Table 13** The lower limit of quantitation (LLOQ) of α-tocopherol in spiked plasma

| Conc. of α-tocopherol (μg/ml) | N | Analyzed Conc. of α-tocopherol (μg/ml) | % Bias |
|---|---|---|---|
| 0.41 | 1 | 0.48 | +17.07 |
| | 2 | 0.49 | +19.51 |
| | 3 | 0.46 | +12.20 |
| | 4 | 0.46 | +12.20 |
| | 5 | 0.48 | +17.07 |
| | 6 | 0.45 | +9.76 |
| **Mean** | | 0.47 | |
| **SD** | | 0.02 | |
| **%RSD** | | 3.30 | |

**Specificity**

As shown in Figure 16, the specificity of the analytical method was presented in which α-tocopherol in standard solution and endogenous α-tocopherol in blank plasma had the retention time at 6.57 and 6.57 minute respectively while the retention time of α-tocopherol acetate (IS) in standard solution and in blank plasma spiked with IS was also at 7.63 and 7.63 minute, respectively. The chromatogram indicated that both α-tocopherol and α-tocopherol acetate could be resolved completely without any interference.

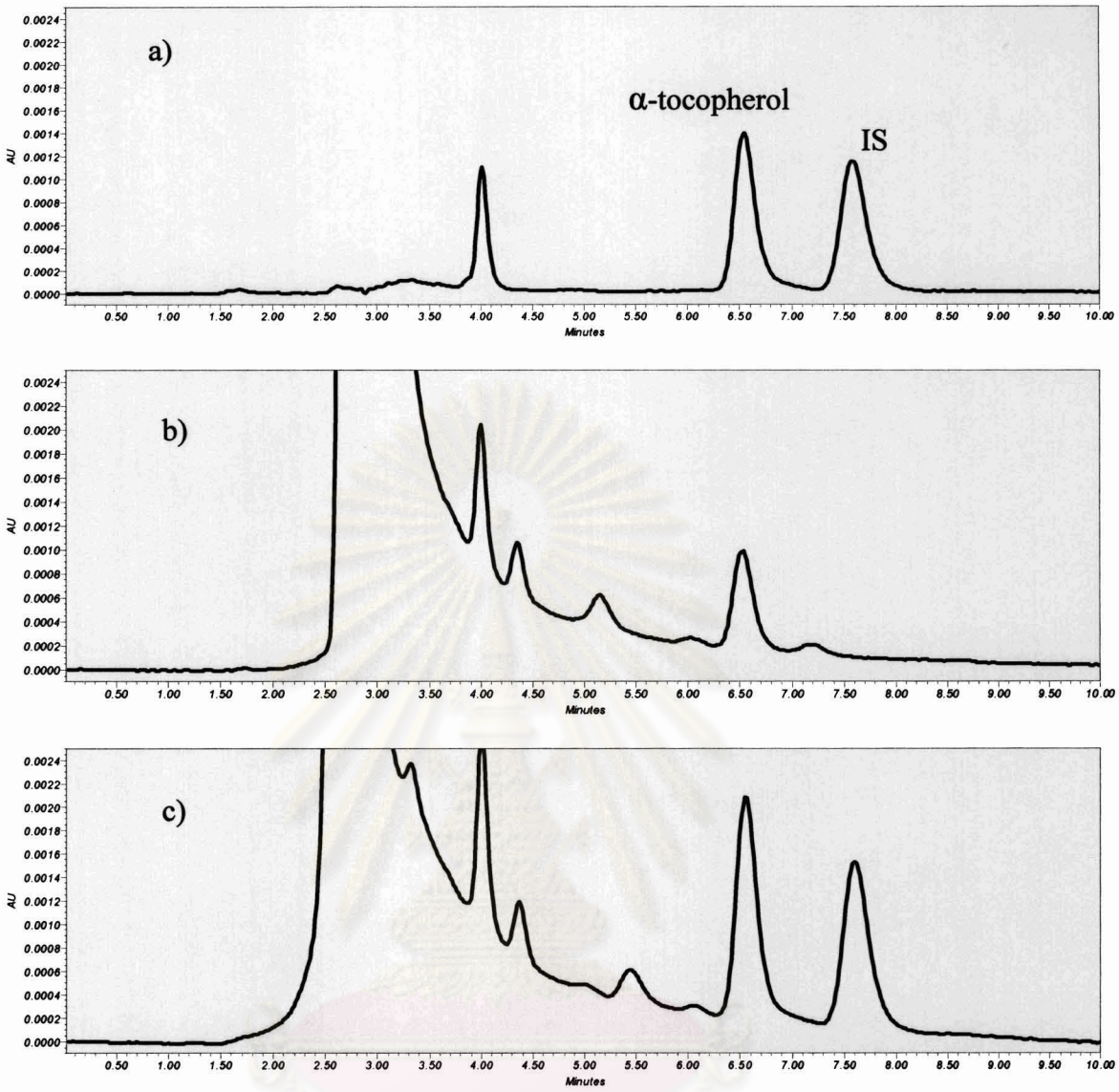



**Figure 16** Chromatograms of α-tocopherol and internal standard (IS) at 292 nm.

a) Standard solution of α-tocopherol (10μg/ml) and α-tocopherol acetate as internal standard (IS) (0.6 mg/ml)

b) Blank plasma with endogenous α-tocopherol

c) Spiked plasma sample with IS (0.6 mg/ml) ( at the same concentration of a )

## Part II Contents and total antioxidant capacity of curcumin and α-tocopherol in supplement preparations

### 2.1 To determine the content of curcumin in supplement preparation

As shown in Table 15, the mean % labeled amount of curcuminoids extract from the assay was 94.57 % of label claim (%LA) calculated as curcumin. Because the average of %LA =94.57 ,according to the criteria of USP 30, if average of the individual contents were less than 98.5%, then M = 98.5, $\bar{X}$ = 94.57, k = 2.4 and s =1.585. The acceptance value of ten capsules was 7.734. According to the content uniformity of dosage units in USP 30, the acceptance value of the hard gelatin capsule, which contained active ingredients more than 25 mg was less than or equal to 15%. Therefore, this curcuminoids extract supplements pass the acceptance criteria and could be used in the study with safety.

**Table 14** Content uniformity of curcuminoids extract

| No. of capsules | Weight of (g) | | |
|---|---|---|---|
| | capsule shell and curcuminoids extract | Capsule shell | Curcuminoids extract |
| 1 | 0.4300 | 0.0997 | 0.3303 |
| 2 | 0.4276 | 0.0954 | 0.3322 |
| 3 | 0.4305 | 0.0941 | 0.3364 |
| 4 | 0.4313 | 0.0982 | 0.3331 |
| 5 | 0.4462 | 0.1009 | 0.3453 |
| 6 | 0.4394 | 0.0992 | 0.3402 |
| 7 | 0.4220 | 0.0957 | 0.3263 |
| 8 | 0.4277 | 0.0990 | 0.3287 |
| 9 | 0.4374 | 0.1007 | 0.3367 |
| 10 | 0.4340 | 0.0996 | 0.3344 |
| Average | 0.4326 | 0.0982 | 0.3343 |
| SD | 0.0069 | 0.0023 | 0.0056 |
| %RSD | 1.60 | 2.41 | 1.68 |

**Table 15** The %LA of curcuminoids extract of each capsules

| No. of capsules (n=10) | %LA |
|---|---|
| 1 | 93.42 |
| 2 | 93.96 |
| 3 | 95.14 |
| 4 | 94.21 |
| 5 | 97.66 |
| 6 | 96.22 |
| 7 | 92.29 |
| 8 | 92.97 |
| 9 | 95.23 |
| 10 | 94.58 |
| Mean | 94.57 |
| SD | 1.585 |
| %RSD | 1.68 |

$$\%LA = \frac{\%\text{ LA average from assay} \times (\text{wt. of curcuminoids extract in each capsule})}{\text{Average wt. of 10 capsules of curcuminoids extract}}$$

**2.2 The content of α-tocopherol in vitamin E supplements**

According to Table 17, the mean % labeled amount (%LA) of vitamin E supplements 200IU/caps was determined to be 98.42 % label claimed. Because the average of %LA from the assay = 98.42% , according to the criteria of USP 30, if average of the individual contents were less than 98.5%, then M = 98.5, $\bar{X}$ = 98.42, k = 2.4 and s =0.80. The acceptance value of ten capsules was 2.0. According to the content uniformity of dosage units in USP 30, the acceptance value of the hard gelatin capsule, which contained active ingredients more than 25 mg was less than or equal to

15%. So, this vitamin E supplements passed the acceptance criteria and could be used safely.

**Table 16** Content uniformity of vitamin E supplement

| No. of capsules | Weight of (g) | | | Calculated %LA (%) |
|---|---|---|---|---|
| | capsule shell and α-tocopherol | Capsule shell | α-tocopherol | |
| 1 | 0.3630 | 0.1355 | 0.2275 | 99.23 |
| 2 | 0.3620 | 0.1370 | 0.2250 | 98.13 |
| 3 | 0.3629 | 0.1386 | 0.2243 | 97.83 |
| 4 | 0.3635 | 0.1390 | 0.2245 | 97.92 |
| 5 | 0.3630 | 0.1340 | 0.2290 | 99.88 |
| 6 | 0.3625 | 0.1383 | 0.2242 | 97.79 |
| 7 | 0.3632 | 0.1400 | 0.2232 | 97.35 |
| 8 | 0.3630 | 0.1381 | 0.2249 | 98.09 |
| 9 | 0.3629 | 0.1360 | 0.2269 | 98.96 |
| 10 | 0.3630 | 0.1360 | 0.2270 | 99.01 |
| Average | 0.3629 | 0.1373 | 0.2257 | 98.42 |
| SD | 0.0004 | 0.0019 | 0.0018 | 0.80 |
| %RSD | 0.11 | 1.35 | 0.81 | 0.81 |

**Table 17** The %LA of vitamin E supplements of each capsules

| No. of capsules (n=10) | %LA |
|---|---|
| 1 | 99.23 |
| 2 | 98.13 |
| 3 | 97.83 |
| 4 | 97.92 |
| 5 | 99.88 |
| 6 | 97.79 |
| 7 | 97.35 |
| 8 | 98.09 |
| 9 | 98.96 |
| 10 | 99.01 |
| **Mean** | 98.42 |
| **SD** | 0.80 |
| **%RSD** | 0.81 |

$$\%LA = \frac{\%\text{ LA average from assay} \times (\text{wt. of curcuminoids extract in each capsule})}{\text{Average wt. of 10 capsules of curcuminoids extract}}$$

**2.3 To determine the total antioxidant capacity of curcuminoids extract and α-tocopherol in supplement preparations.**

From the ORAC method, the ORAC values of curcumin in 0.2 μg/ml methanol of curcuminoids were 1392.88 ± 5.33 μmol TE / g of curcuminoids extract and the ORAC values of 0.2 mg/ml of α-tocopherol in vitamin E supplements were 0.92 ± 0.03 μmol TE /g of vitamin E supplements. As the label claimed that curcuminoids extract 1 capsule had 250 mg of curcumin equivalent, thus, the ORAC value of curcumin in 1 capsule was 348.22 ± 5.33 μmol TE/g. According to the dose

used in the experiments, the ORAC values for curcuminoids extract at the dose of 500 mg and 6 g were approximately 696.44 and 3857.28 μmol TE/g, respectively.

For the vitamin E supplement, the label claimed that each capsule had d-alpha tocopherol 200 IU (approximately 134.2282 mg of α-tocopherol), so the ORAC values of α-tocopherol in one capsule was $0.12 \pm 0.03$ μmol TE / g.

## Part III Experimentation on volunteer subjects

### 3.1 Volunteer Characteristics

The overall thirty volunteers were recruited into this study according to their blood chemistry examination (as shown in appendix B). Nevertheless, only twenty-four subjects were experimented because one subject did not present on the day of experimentation, and the other five subjects did not meet the inclusion criteria. The twenty-four volunteers in this study were sixteen males and eight females. Their characteristics were shown in Table 18.

During the weeks of supplementation and a week afterward, no any severe adverse effects were observed among the subjects. However, there were the occurrences of flatulent effect and rather dark yellow stools in the subjects who were on 6 g/day curcuminoids extract supplementation.

**Table 18** Physical characteristics of all 24 volunteers

| | **Male** | **Female** |
|---|---|---|
| **No. of subjects** | 16 | 8 |
| **Age ( Mean ± SD) year** | 29.25 ± 5.93 | 29.38 ± 4.34 |
| **Weight( Mean ± SD) kg** | 69.63 ± 12.09 | 51.13 ± 3.48 |
| **BMI( Mean ± SD) kg/m$^2$** | 23.99 ± 3.30 | 20.25 ± 1.17 |

**The level of cholesterol and the level of liver enzymes (AST and ALT)**

According to the blood biochemistry of subjects before and after curcuminoids extract administration, the cholesterol levels of group A and group B subjects were statistically significant decreased comparing to the first day of the experimentation ($p$=0.003 for the group A subjects and $p$=0.039 for group B subjects, respect) ( Table19). This result was similar to the report of Soni and Kuttan (1992).

After the curcuminoids extract administration at dose 6g/day, no statistically significant differences in the level of AST and ALT enzyme were observed ($p$=0.204 and 0.667, respectively) (Table 20). This confirmed the safety used of curcuminoids extract at this high dose.

**Table 19** The level of cholesterol in the subjects' plasma before and after seven days of curcuminoids extract administration in the dose 500 mg/day (group A) and the dose of 6 g/day (group B)

| | Cholesterol level in the plasma (mg/dl) | | | | |
|---|---|---|---|---|---|
| **Code** | **Gr. A before** | **Gr. A after** | **Code** | **Gr. B before** | **Gr. B after** |
| **A01** | 221 | 184 | **B01** | 238 | 232 |
| **A02** | 190 | 166 | **B02** | 157 | 160 |
| **A03** | 199 | 134 | **B03** | 196 | 186 |
| **A04** | 242 | 176 | **B04** | 155 | 154 |
| **A05** | 203 | 202 | **B05** | 188 | 171 |
| **A06** | 219 | 162 | **B06** | 232 | 224 |
| **A07** | 231 | 183 | **B07** | 200 | 204 |
| **A08** | 172 | 167 | **B08** | 270 | 249 |
| **A09** | 191 | 137 | **B09** | 194 | 161 |
| **A10** | 180 | 188 | | | |
| **Mean ±SD** | 204± 22 | 169 ± 21 | | 203 ± 37 | 193 ± 35 |
| **paired student t-test (α = 0.05)** | *S ($p$=0.003) | | | *S ($p$=0.039) | |

**Table 20** The level of AST and ALT enzyme in the serum of subjects before and seven days after curcuminoids extract administration at the dose 6 g/day (group B)

| | Level of AST enzyme (U/L) | | Level of ALT enzyme (U/L) | |
|---|---|---|---|---|
| **Code** | **before** | **after** | **before** | **after** |
| **B01** | 29 | 19 | 16 | 6 |
| **B02** | 55 | 71 | 82 | 102 |
| **B03** | 58 | 20 | 7 | 6 |
| **B04** | 50 | 16 | 9 | 7 |
| **B05** | 27 | 20 | 7 | 12 |
| **B06** | 32 | 21 | 38 | 29 |
| **B07** | 16 | 29 | 22 | 18 |
| **B08** | 29 | 29 | 20 | 17 |
| **B09** | 32 | 27 | 28 | 20 |
| **Mean ± SD** | 36± 14 | 28 ± 17 | 25± 24 | 24 ± 30 |
| **paired student t-test (α = 0.05)** | *NS ($p$=0.204) | | *NS ($p$=0.667) | |

## 3.2Before experimentation

### 3.2.1 Endogenous α-tocopherol concentration in plasma before experimentation

The concentrations of endogenous α-tocopherol in volunteers' plasma prior to administration were ranged from 7.58 – 19.52 μg/ml. No statistically significant difference in the plasma level of endogenous α-tocopherol among three experimental groups were observed ($p$ = 0.099) (Table 21). Therefore, all subjects were implied to contained normal antioxidant level. An endogenous α-tocopherol in Thais' plasma has been recorded to be 7.44 – 17.58 μg/ml (Thongnopnua et al, 2006).

**Table 21** The concentrations of endogenous α-tocopherol in healthy volunteers before administration

| | code | Concentration of endogenous α-tocopherol in plasma (μg/ml) | code | Concentration of endogenous α-tocopherol in plasma (μg/ml) | code | Concentration of endogenous α-tocopherol in plasma (μg/ml) |
|---|---|---|---|---|---|---|
| | **A01** | 14.49 | **B01** | 14.67 | **C01** | 8.49 |
| | **A02** | 8.05 | **B02** | 11.30 | **C02** | 8.59 |
| | **A03** | 13.26 | **B03** | 10.73 | **C03** | 8.80 |
| | **A04** | 15.97 | **B04** | 7.58 | **C04** | 11.32 |
| | **A05** | 19.52 | **B05** | 10.13 | **C05** | 8.63 |
| | **A06** | 10.98 | **B06** | 13.71 | | |
| | **A07** | 12.38 | **B07** | 8.42 | | |
| | **A08** | 9.78 | **B08** | 14.02 | | |
| | **A09** | 9.35 | **B09** | 9.61 | | |
| | **A10** | 12.21 | | | | |
| **N** | | 10 | | 9 | | 5 |
| **Average ± SD** | | 12.60±3.42 | | 11.13 ±2.52 | | 9.16±1.21 |
| **one-way ANOVA (α = 0.05)** | NS ( $p$= 0.099 ) | | | | | |

NS = Non statistically significance

### 3.2.2 The ORAC values in plasma before experimentation

The ORAC values in volunteers' plasma prior experimentation were determined to be 7,272-24,834 μmol TE./L of plasma. Statistically significant difference in the ORAC values in plasma among the three experimental groups were observed ($p$ = 0.018) (Table 22).These observed ORAC values of Thai healthy volunteers were in the ranged of the other previous report, utilizing the same ORAC methods (Ou, Hampsch-Woodill and Prior, 2001). The ORAC values of normal sera of American reported by Ou, Hampsch-Woodill and Prior (2001) to be 7,310-8,250 μmol TE./L of sample (n=3) (Ou, Hampsch-Woodill and Prior, 2001) and from Spanish subjects to be 14,366 to 21,668 μmol TE./L of sample (n=8) (Fernandez-Pachon et al, 2005). The ORAC values were also different between plasma and serum sample used (Bartoze, 2003).

The ORAC values are defined to measure the total antioxidant activity in plasma (Cao, Alessio and Cutler, 1993). From the results of endogenous α-tocopherol in plasma (Table 21) and the ORAC values (Table22), it is impressed that the overall antioxidant status for anyone would not possibly be related to only the concentration of plasma endogenous α-tocopherol.

**Table 22** The ORAC values of plasma samples before experimentation

| Code | ORAC value of Gr. A | Code | ORAC value of Gr. B | Code | ORAC value of Gr. C |
|---|---|---|---|---|---|
| **A01** | 12028 | **B01** | 10060 | **C01** | 8422 |
| **A02** | 9945 | **B02** | 24834 | **C02** | 7272 |
| **A03** | 11243 | **B03** | 11270 | **C03** | 10185 |
| **A04** | 12984 | **B04** | 9022 | **C04** | 13922 |
| **A05** | 5797 | **B05** | 16455 | **C05** | 13683 |
| **A06** | 11639 | **B06** | 16078 | | |
| **A07** | 13062 | **B07** | 21339 | | |
| **A08** | 15689 | **B08** | 22786 | | |
| **A09** | 8949 | **B09** | 16959 | | |
| **A10** | 12082 | | | | |
| **Average ± SD** | 11342± 2668 | | 16534± 5673 | | 10697± 3020 |
| **one-way ANOVA ( α = 0.05)** | S ( $p$=0.018) | | | | |

S = Significant difference

## 3.3 Experimentations

### 3.3.1 Group A (Curcuminoids extract administration of dose 500 mg/day)

**The total antioxidant activity (ORAC values) in plasma samples before and after experimentation**

The mean total antioxidant capacity in term of ORAC values (n=10) before curcuminoids extract administration was in the ranged of 5,797 to 15,689 μmol TE. /L of plasma. After experimentation, the mean ORAC values were within the range of 8,269 to 20,186 μmol TE. /L of plasma as showed in Table 23. There was no statistically significant difference in ORAC values before and after supplementation ($p$= 0.270). Although no statistically significant increased in the

ORAC values after supplementation was observed, the increase in the mean ORAC values after experimentation was notified.

**Table 23** The ORAC values in plasma samples before and after supplementation with curcuminoids extract at the dose of 500 mg/day

| | **ORAC values** (μmol TE. /L of sample) | |
|---|---|---|
| **Code** | **before** | **after** |
| **A01** | 12028 | 15220 |
| **A02** | 9945 | 10645 |
| **A03** | 11243 | 12484 |
| **A04** | 12984 | 20186 |
| **A05** | 5797 | 12874 |
| **A06** | 11639 | 10863 |
| **A07** | 13062 | 8269 |
| **A08** | 15689 | 10242 |
| **A09** | 8949 | 13578 |
| **A10** | 12082 | 15352 |
| **Average ± SD** | 11342± 2668 | 12971± 3379 |
| **Paired student t-test (α = 0.05)** | NS ($p$=0.270) | |

NS = non-significance difference

**The total antioxidant activity during the first and the seventh days of experimentation**

By examining the ORAC values profiles during 12 hrs of curcuminoids administration (Figure 17), only a minor deviation of the ORAC values during 12 hrs. after the first and the seventh dose. It was implied that the curcuminoids extract amount of 500 mg/day may be unable to significant elevated the total antioxidant activity in plasma.

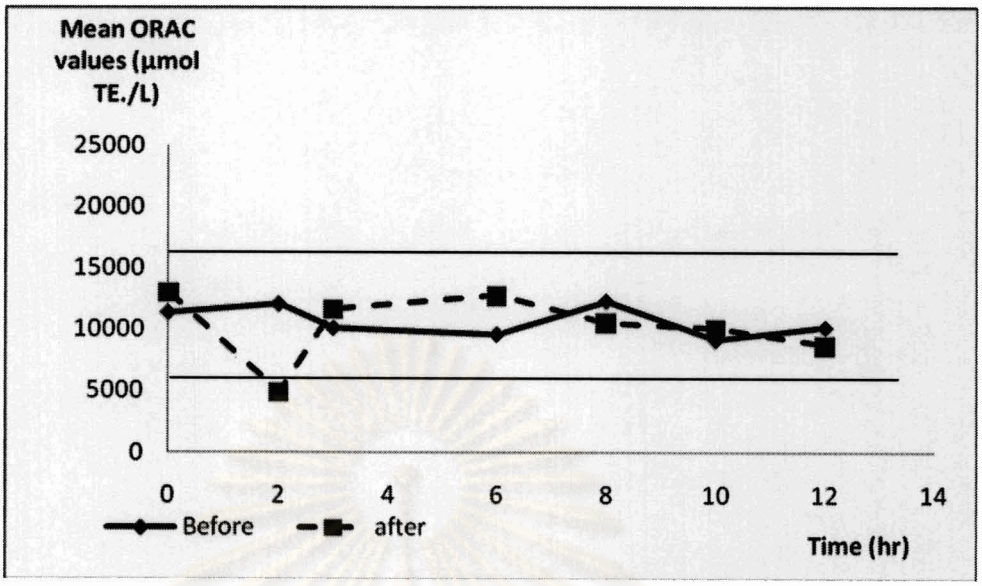



**Figure 17** Mean ORAC values –time profiles of subjects following the first and the seventh day of administration of curcuminoids extract in dose 500 mg/day

**The determination of curcumin, THC, HHC and OHC concentrations in plasma samples**

The plasma curcumin concentration in every subject was undetectable at any sampling time-points. The undetectable of curcumin and its metabolites (THC, HHC and OHC) were similarly to the previous pharmacokinetic study in the patients with high-risk or pre-malignant lesions at the curcuminoids extract dose of 500-2,000 mg, in which the concentration of curcumin in serum were also barely detectable. Also, no curcumin could be detected in the urine (Cheng et al, 2001).

Concentration of curcumin could not detectable due to its very low concentration in plasma sample. Asia and Miyazawa (2000) used the LC-MS for analyzing curcumin in plasma of the rat that administered curcumin at the dose of 100

mg/kg. They had to transform glucuronide and glucuronide/ sulfates metabolites of curcumin for increasing the concentration of curcumin for the possible detected curcumin concentration (Asia and Miyazawa, 2000).

In this study, the plasma samples of the subjects were treated with enzyme β-glucuronidase to recover any glucuronide conjugates into the parents compounds. It was showed that the curcumin and its metabolites were also not detectable. This should be due to the sensitivity of HPLC detector used. This study used UV detector that is less sensitive than the MS detector used by Ireson et al. (Ireson et al, 2001).

**The determination of endogenous α-tocopherol in plasma samples**

The concentration of endogenous α-tocopherol in plasma samples of subjects supplemented with curcuminoids extract were determined before and after experimentation to be in the range of 8.05 to 19.52 and 8.00 to 15.58 ug/ml, respectively. No statistically significant differences in plasma α-tocopherol concentrations were observed. This result indicates that the administration of curcuminoids extract do not induce the endogenous α-tocopherol in our body (Figure 18).

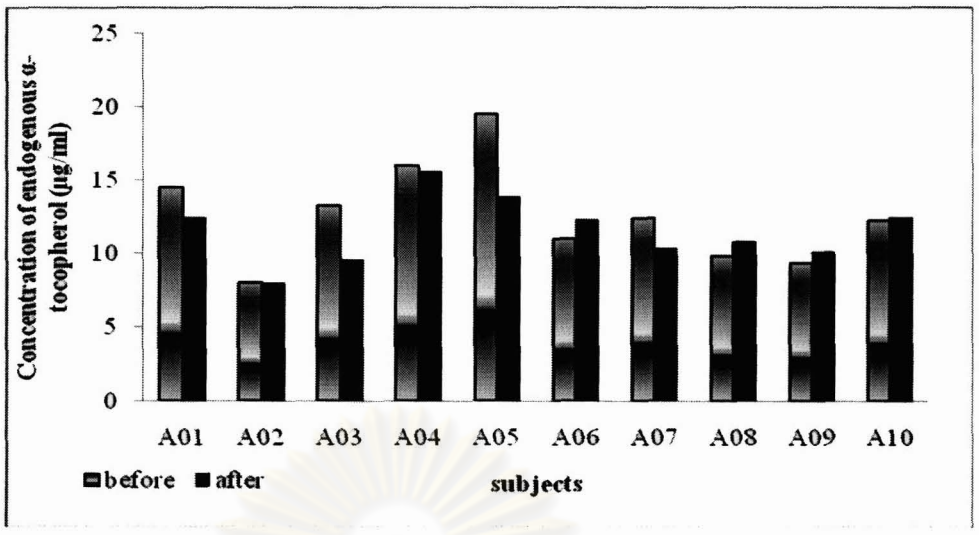



**Figure 18** The endogenous α-tocopherol in subjects group A before and after curcuminoids extract administration (500 mg/day).

It was also found that during the first and the seventh day of curcuminoids administration, the level of α-tocopherol during 12 hrs. after curcuminoids extract administration was not affected by plasma curcumin concentrations (Figure 19).

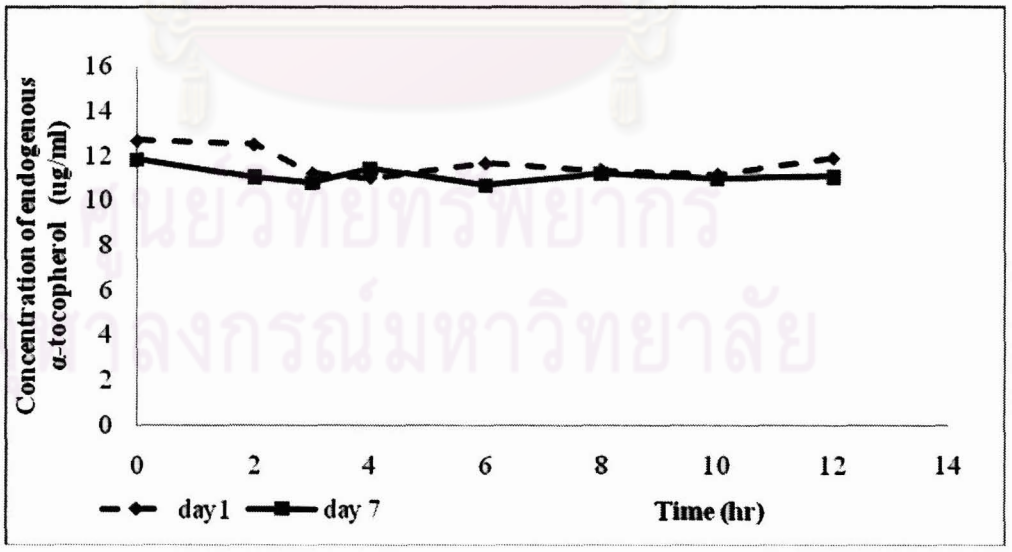



**Figure 19** The mean concentration of endogenous α- tocopherol- time profiles on the first and the seventh day of curcuminoids extract administration (500mg/day).

### 3.3.2 Group B (Curcuminoids extract administration of dose 6g/day)

**The total antioxidant activity (ORAC values) in plasma samples**

The ORAC values for subjects in group B (n=9) comparing between before and after administration of curcuminoids extract in the dose of 6 g/day were in the range of 9,022 to 24,834 μmol TE./L and 3,530 to 20,595 μmol TE. /L, respectively (Table 24). No statistically significant difference in the ORAC values was observed ($p$=0.330).

**Table 24** The ORAC values of plasma samples obtaining before and after curcuminoids extract administration in the amount of 6 g/day.

| | **ORAC values** (μmol TE. /L of sample) | |
|---|---|---|
| **Code** | **Before** | **After** |
| B01 | 10060 | 14050 |
| B02 | 24834 | 13165 |
| B03 | 11270 | 13782 |
| B04 | 9022 | 3530 |
| B05 | 16455 | 18203 |
| B06 | 16078 | 13738 |
| B07 | 21339 | 16633 |
| B08 | 22786 | 18829 |
| B09 | 16959 | 20595 |
| Average ±SD | 16534± 5673 | 14725±4963 |
| Paired Student t-test ($\alpha$ = 0.05) | NS ($p$=0.330) | |

NS= non-significant difference

According to the ORAC values of curcuminoids extract supplements that determined in the part II, the ORAC values of curcuminoids extract at the dose 6 g/day were approximately 3857.28 μmol TE/g. It was supposed that after curcuminoids extract administration, the ORAC values in plasma samples should be induced due to the antioxidant activity of curcumin. However, the result obtained seems to be opposed.

The possibility of this occurrence would be explained in term of some balancing mechanism in our body. As notified from the ORAC values profiles within 12 hrs. after curcuminoids extract administration on the first and the seventh day (Figure 20), there were ORAC values differences at each sampling time. The overall profile for the seventh day exhibited the systematically decreased for the whole sampling time period. It may be possible that the first day of curcuminoids extract administration could stimulate the antioxidant activity due to its high dose. After six days of curcuminoids extract administration, the balancing mechanism in our body try to manage the antioxidant activity into the normal range. Therefore, the mean ORAC values on the seventh day were reduced to the normal range and then lower than the mean ORAC values on the first day. The ORAC values profiles on the seventh day of high curcuminoids extract administration was within the normal ORAC values.

It was noticeable that at two hrs after curcuminoids extract administration the ORAC values from each subject was dropped resulting in the mean ORAC values that less than the other sampling time points. This pattern was also observed from group A subjects at the same sampling time. This may be associated with curcumin is the polar compound that the absorption or metabolism could be raised by fed state rather than fast state (Thongnopnua et al, 2007). At this time point

was the sampling time that subjects were two hrs. fasted after curcuminoids extract administration. It is therefore suggested that the two hrs. fasted after administration may not appropriate.

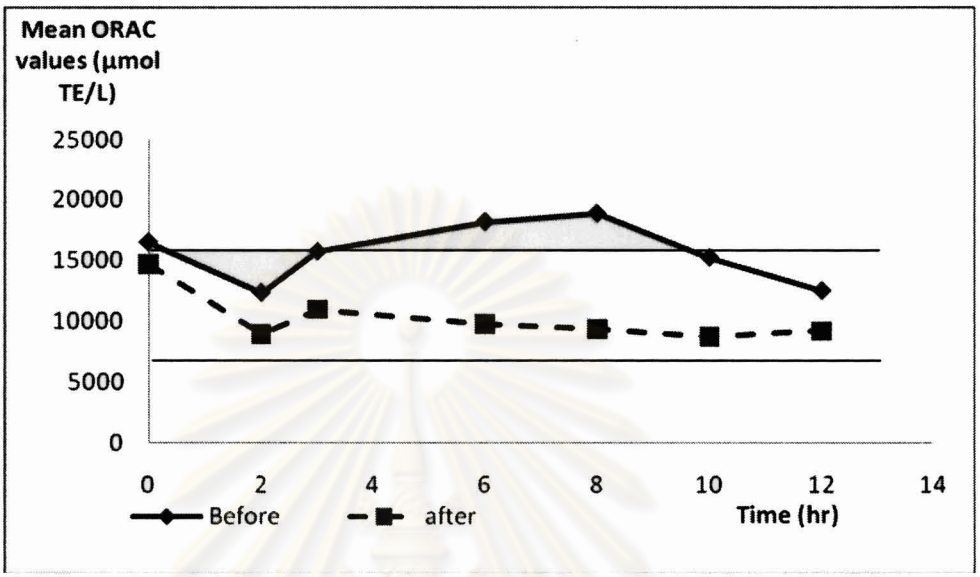



**Figure 20** Mean ORAC values –time profiles of subjects following the first and the seventh day of administration of curcuminoids extract in the dose of 6 g/day

**The determination of curcumin, THC, HHC and OHC in plasma samples of group B subjects**

Following curcuminoids extract administration, it was found that curcumin concentration in plasma could be determined in very low concentration with maximum concentration ($C_{max}$) of 0.9 to 17.6 nM at time ($T_{max}$) 3-5 hr. THC which is the major active metabolites of curcumin could be observed in almost every subjects at the higher concentration than curcumin with the $C_{max}$ ranged 26.9 to 457.0 nM at $T_{max}$ 5-6 hr. HHC compound could also be detected in some subjects (A-06 and A-10). For the OHC compound, which is the more polar metabolite than HHC and THC,

the level was found to be very high in some subjects (A-04 and A-06). Since curcumin could be metabolized to many metabolites, the levels of metabolites in plasma imply the different metabolites activities of each subject.

Not only being metabolized to THC, HHC, and OHC, curcumin concentrations were also recovered after treating plasma sample with enzyme β-glucuronidase. These findings correspond with the pharmacokinetic study of curcumin in Thai healthy volunteers (Thongnopnua et al, 2007).

For subjects that no any curcumin concentrations could be detected within 12-15hrs. following curcuminoids extract administration, the plasma samples were treated with enzyme β-glucuronidase to recover any glucuronide conjugates in samples. The result clearly showed that curcumin, demethoxycurcumin and bisdemethoxycurcumin were found in the sample plasma after treated with enzyme β-glucuronidase (Figure 21 to 24). Therefore, the undetectable curcumin could be due to its biotransformation to glucuronide conjugate (Ireson et al, 2001).

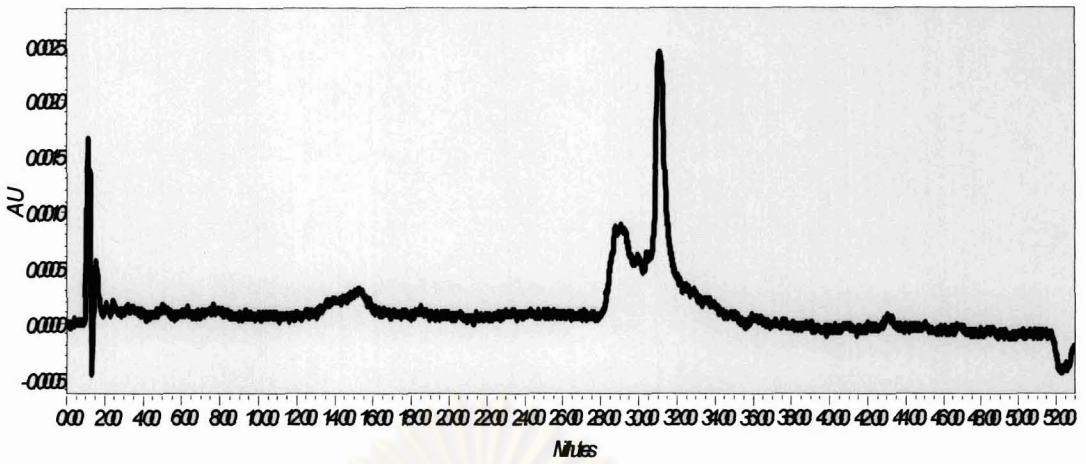



**Figure 21** The chromatogram of non-treat plasma sample obtained from subject B08 at 2 hrs after administration of curcuminoids extract on the first day. The UV detection wavelength was set to 420 nm for curcuminoids detection. (non-treat β-glucuronidase)

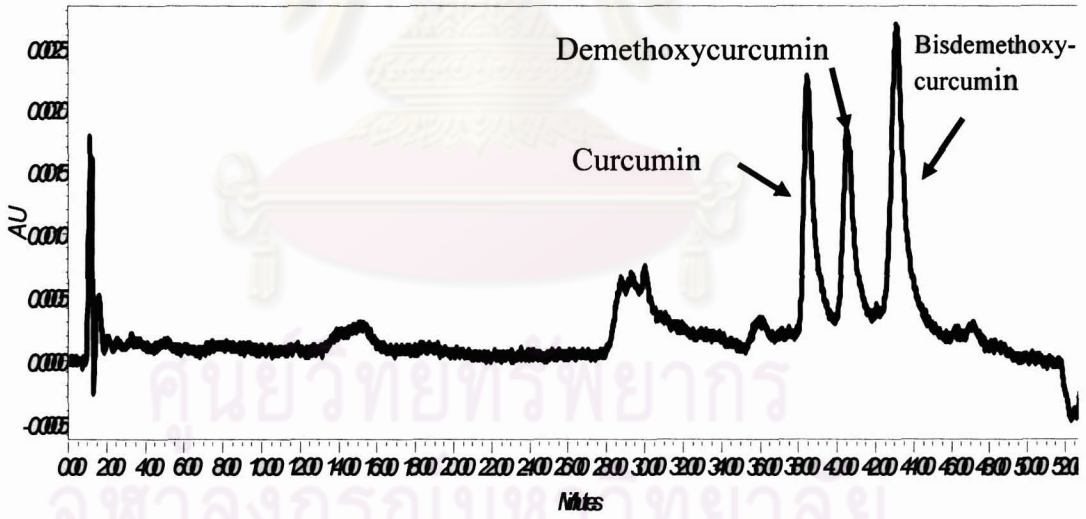



**Figure 22** The chromatogram of non-treat plasma sample obtained from subject B08 at 2 hrs after administration of curcuminoids extract on the first day. The UV detection wavelength was set to 420 nm for curcuminoids detection. (treat β-glucuronidase)

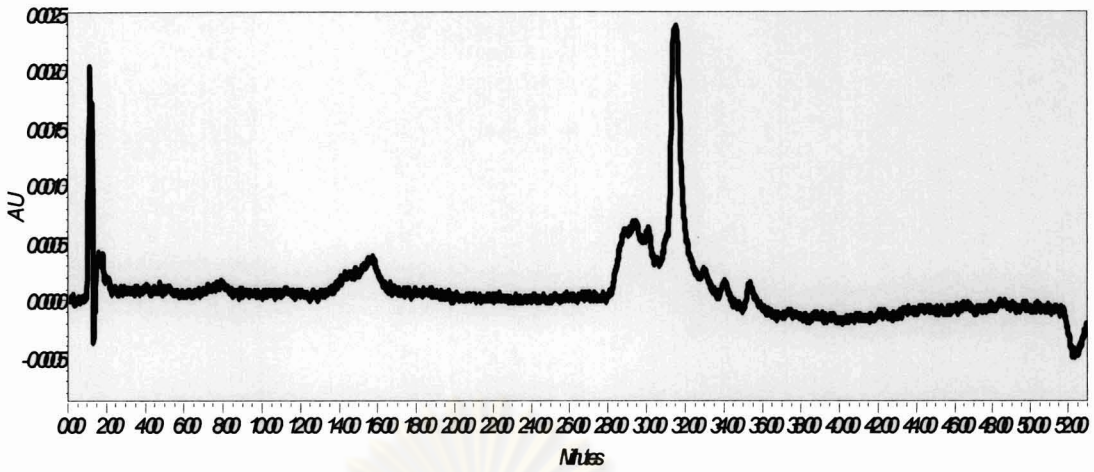



**Figure 23** The chromatogram of non-treat plasma sample obtained from subject B08 at 2 hrs after administration of curcuminoids extract on the seventh day. The UV detection wavelength was set to 420 nm for curcuminoids detection. (non-treat β-glucuronidase)

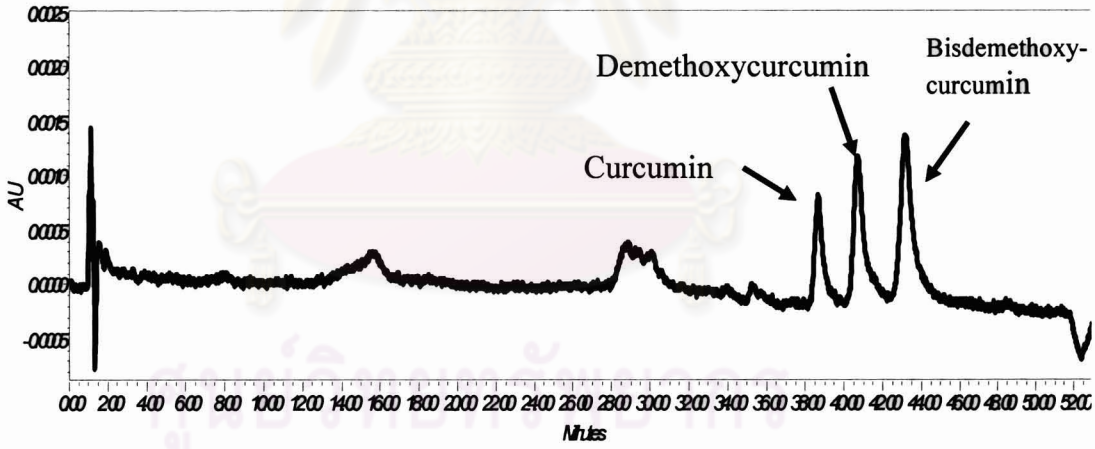



**Figure 24** The chromatogram of non-treat plasma sample obtained from subject B08 at 2 hrs after administration of curcuminoids extract on the seventh day. The UV detection wavelength was set to 420 nm for curcuminoids detection. (treat β-glucuronidase)

**The determination of endogenous α-tocopherol in plasma samples**

The concentration of endogenous α-tocopherol in plasma samples of subjects administration curcuminoids extract were determined to be in the range of 7.58 to 14.67 and 7.32 to 14.00 ug/ml before and after experimentation, respectively. No statistically significant difference in plasma α-tocopherol concentration was observed. This result indicated that the administration of curcuminoids extract did not induce the α-tocopherol in our body (Figure 25).

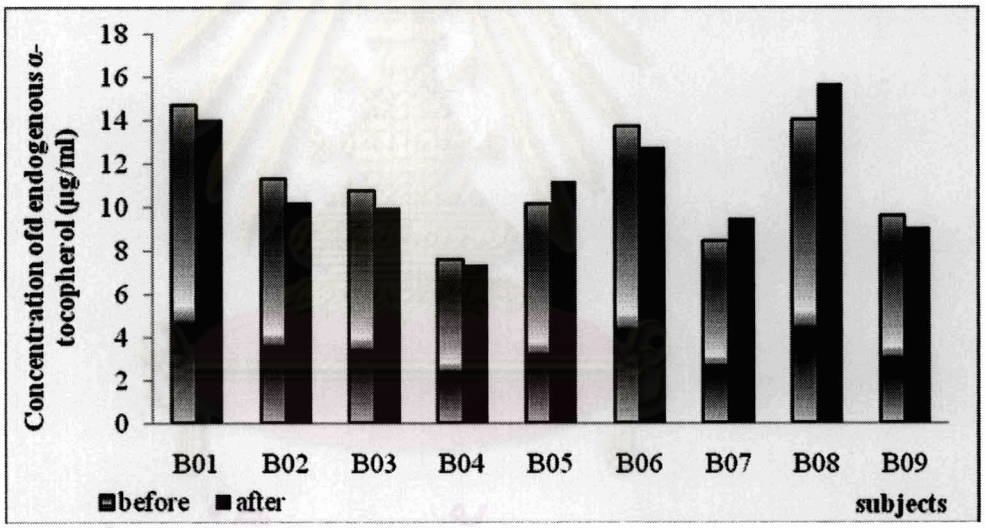



**Figure 25** The endogenous α-tocopherol in subjects group B before and after curcuminoids extract administration (6 g/day).

It was also found that during the first and the seventh day of curcuminoids administration, the level of α-tocopherol following 12 hrs. of administration was not affected by curcumin concentration in plasma (Figure 26).

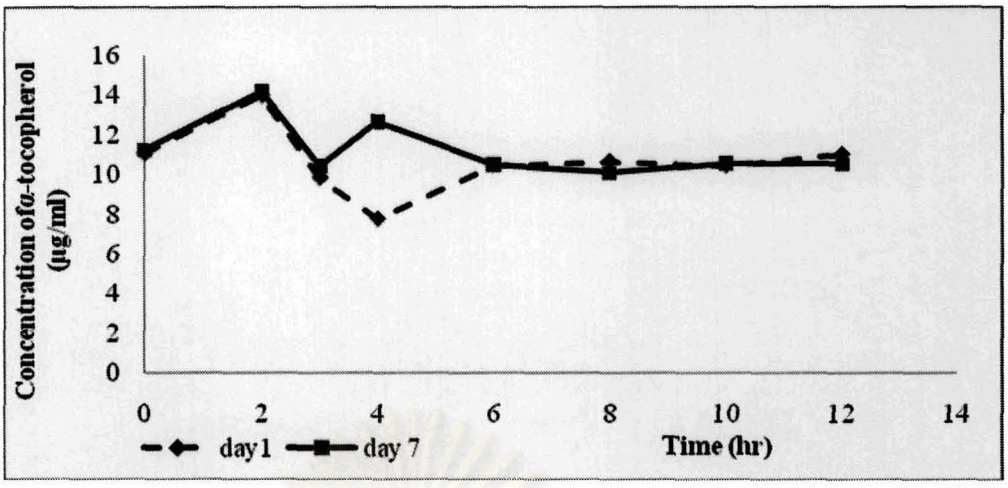



**Figure 26** The mean concentration of endogenous α- tocopherol- time profiles during curcuminoids extract administration (6 g/day) at the first day and the seventh day

### 3.3.3 Group C

**The total antioxidant activity (ORAC values) in plasma samples**

The ORAC values in plasma samples of subjects in group C (n=5) , before and after administration of vitamin E supplement at the dose of 200 IU/day were ranged from 7,272 to 13,922 µmol TE./L and 9,216 to 23,574 µmol TE. /L respectively. The ORAC values after vitamin E supplementation were significantly increased ($p$=0.045), confirming the antioxidant property of vitamin E.

**Table 25** The ORAC values of plasma samples following vitamin E supplements (200IU/day) before and after administration.

| Code | ORAC values (μmol TE. /L of sample) | |
|---|---|---|
| | before | after |
| C01 | 8422 | 9216 |
| C02 | 7272 | 10661 |
| C03 | 10185 | 12605 |
| C04 | 13922 | 23574 |
| C05 | 13683 | 20788 |
| Average ±SD | 10697±3020 | 15369±6409 |
| Paired Student t-test (α = 0.05) | NS (p=0.045) | |

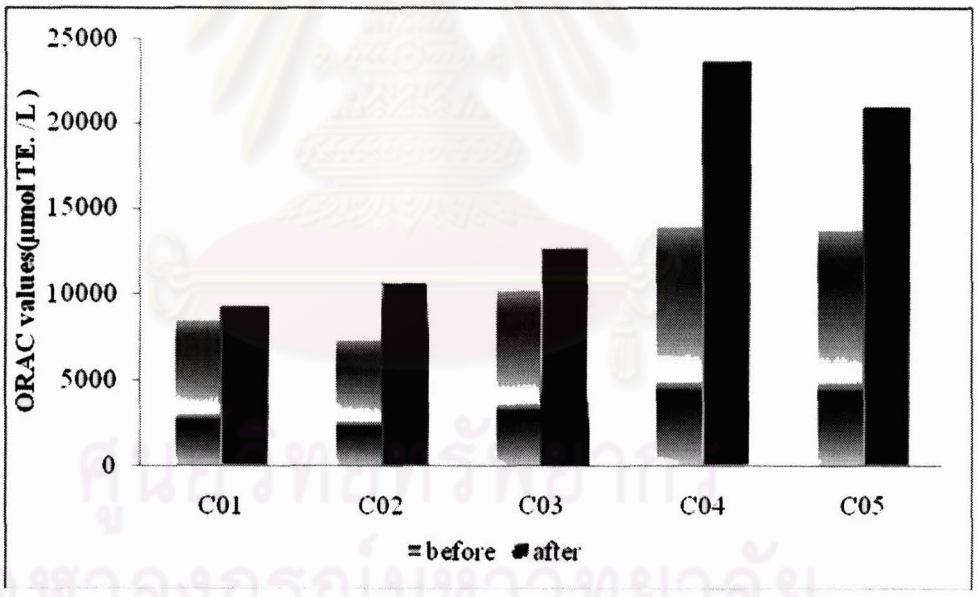



**Figure 27** Mean ORAC values of each subject following before and after administration of vitamin E supplements in dose 200IU/day

**The determination of endogenous α-tocopherol in plasma samples of subjects in group C**

The concentrations of endogenous α-tocopherol in plasma samples of each subject are shown in Figure 28. There was statistical significant increase in the endogenous α-tocopherol concentration in plasma after vitamin E supplementation (200 IU/day) for 7 days (from $9.16 \pm 1.21$ to $14.38 \pm 2.85$ μg/ml) ($p$=0.007). The concentration of endogenous α-tocopherol in plasma after administrated vitamin E supplement in this study were lower than that previously reported in plasma of the Thai people after administrated vitamin E supplements in dose 200 IU/day (11.1-26.5 ug/ml) (Thongnopnua et al, 2006).

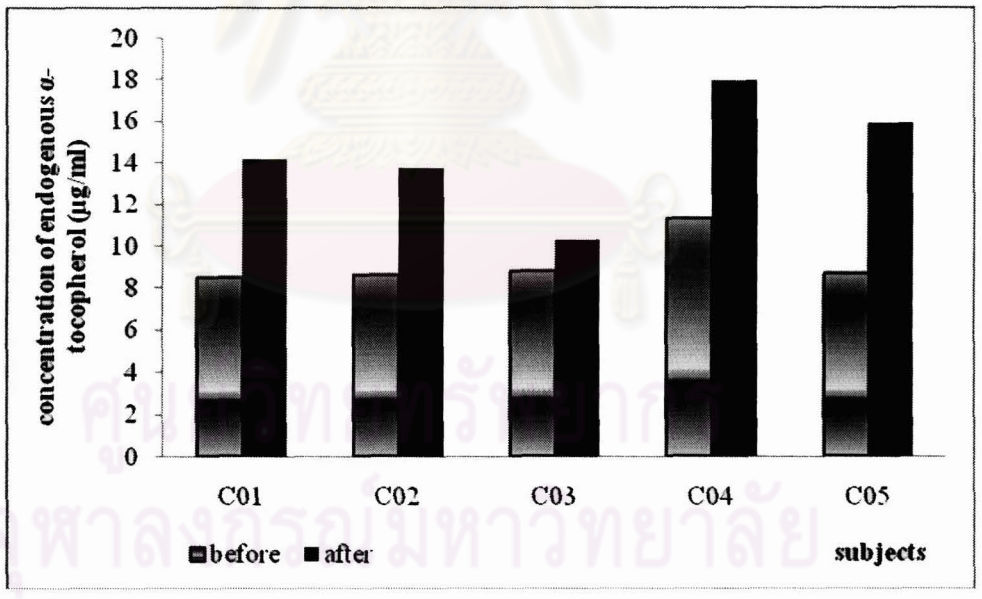



**Figure 28** The endogenous α-tocopherol in subjects group C before and after administration of vitamin E supplements in dose 200IU/day.

## 3.4 The comparison of antioxidant activity in plasma between group A and group B

### 3.4.1 The ORAC values before and after curcuminoids extract administration.

The antioxidant activities in plasma samples of group A and group B subjects after the administration of curcuminoids extract were shown in Figure 29 and 30, respectively. Although there were no statistically significant difference in the ORAC values between before and after administration of curcuminoids extract in the dose of 500 mg/day ($p$=0.270) (Table 23), seven subjects in this group had the increased ORAC values after administration and the other three subjects showed the decreased ORAC values. In addition, there were also no statistically significant difference in the ORAC values ($p$=0.330) (Table 24) between before and after curcuminoids extract administration among the subjects in group B. However, five subjects showed the decreased ORAC values, while four subjects showed the increased ORAC values.

Under the acceptable criteria that the determinations of total antioxidant activity in plasma or serum samples aim to reflect our antioxidant status, the results from this study could be explained in many viewpoints. The inter-subject variations were significantly observed in either group A or group B subjects. These variations could be reduced by increasing the number of subject studied in the future. Nevertheless, the ORAC values in group B subjects tended to be decreasing more than to be increasing. This observation supported the study of Jacob and Venor. Jacob et al determined the effect of antioxidant supplements (consists of 723 mg of vitamin

C, 31 mg of all-rac-α-tocopherol and 400 μg of folic acid) in taken every day for 90 days to the healthy men that having in taken low fruit and vegetable habits. No any effect on the biomarker of oxidative damage after 90 days of the administration(Jacob et al, 2003). Veronin et al. determined the total antioxidant capacity of blood plasma from fifteen healthy volunteers which receiving vitamin and mineral complex tablets at the dose of 2 tablets /day for 4 weeks also shown that the level of the total antioxidant capacity by TEAC method does not change after 4 weeks of the administration (Voronin et al, 2004).

Furthermore, there are also have many research that show the ORAC method can determine the antioxidant activity and results were contrast to our results. Cao et al. determined the antioxidant activity of human plasma after consumption of the controlled diet high in the fruit and vegetable for 16 days and found that the ORAC values were increased after the consumption (Cao et al, 1998). Nazza et al. determine the antioxidant activity in serum of healthy volunteers after supplementation with blueberry supplement in the dose of 100 g/day (contained 1.2g of total anthocyanin) were statistically significant increased after 4 hrs of the supplementation (Mazza et al,2002). Futher more the other studied shown that the ORAC values were increased in the elderly women and the patients with psychiatric and renal disease. Cao et al. determine the ORAC values of the serum in elderly women after 14 hrs of the consumption of strawberries (240 g) or spinach (294 g) or red wine (300 ml) or vitamin C (1250 mg). The ORAC values from serum were increased after 14 hrs of the consumption ( Cao, et al, 1998). Sofic et al. determine the ORAC values of serum from the patients with neurological disease, psychiatric, renal disease and cardiomyopathy. The results shown that the antioxidant activity from these patients were statistically significant decreased in the patients with

neurological disease and cardiomyopathy and found statistically significant increased in the patients with psychiatric and renal disease. The patient with psychiatric and renal disease were have the high oxidative stress that make the increasing of the high level of antioxidant compound in their body so the ORAC values were increased from the normal people ( Sofic et al, 2002).

These studied could be also explained in the concept of Cornelli ( Cornelli , 2009). He proposed the nutritional paradigm leading to the sound reasonable of antioxidant activity in healthy and patient subjects. He explained that the antioxidant compound would be usable only for those who contain the lower antioxidant level. For example in elder person, patients with chronic disease etc. Whenever the antioxidant level meet the daily allowance, the extra antioxidant in taken may become the pro-oxidant resulting in toxic state. However, this concept needs to be more explore with more study in various antioxidant compound and various subjects status.

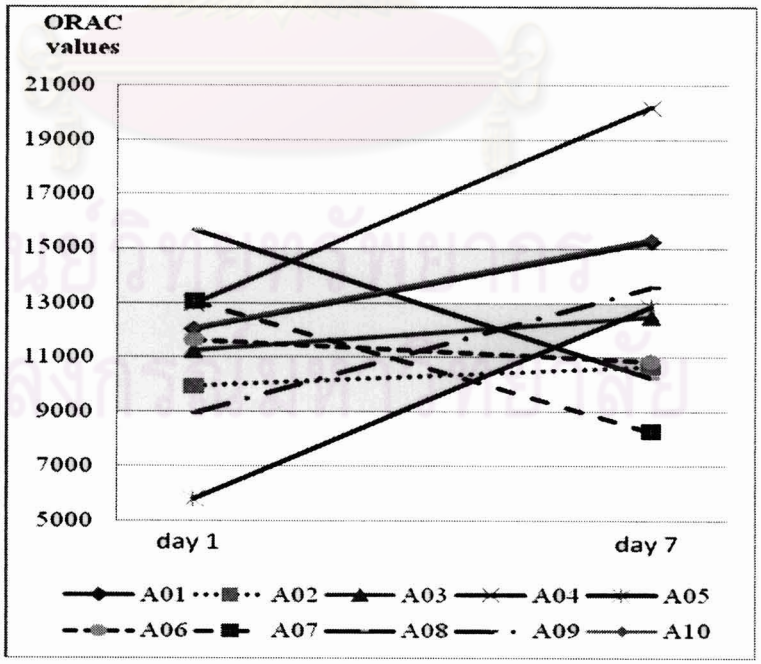



**Figure 29** The ORAC values of each subject before and after curcuminoids extract administration at the dose of 500 mg/day

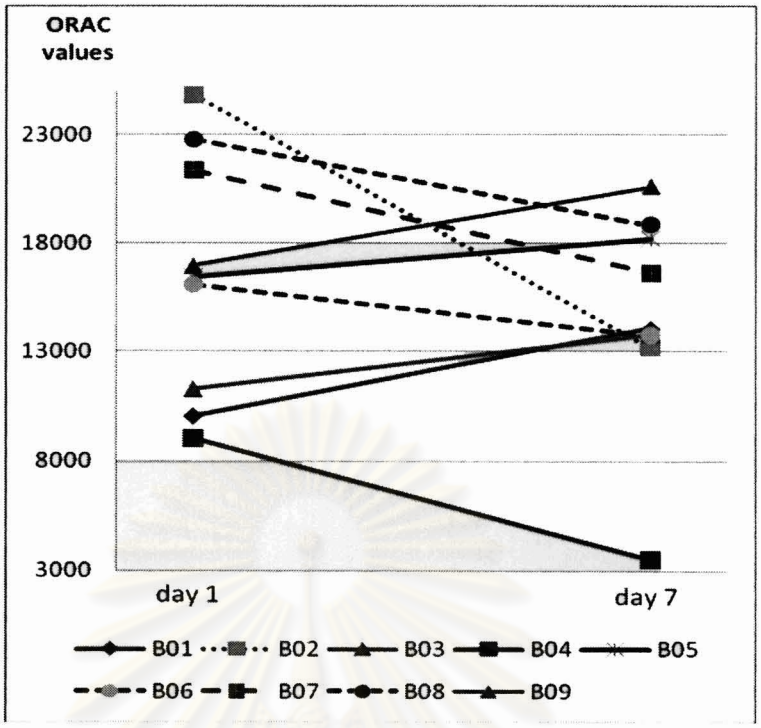



**Figure 30**The ORAC values of each subject before and after curcuminoids extract administration at the dose of 6 g/day

### 3.4.2 The total antioxidant activity after curcuminoids extract administration and after the vitamin E supplementations.

Total antioxidant activity in plasma of each subject after seven days treatment with curcuminoids extract is illustrated in Table 26. It was clearly shown that the ten times of curcuminoids extract dose in group B subjects could not significantly induce the antioxidant activity. The mean ORAC values after curcuminoids extract supplementation in group A and group B subjects were ranged from 8,269 to 20,186 μmol TE./L and from 3,530 to 20,595μmol TE./L , respectively.

**Table 26** The ORAC values plasma sample from group A and group B subjects before drug administration in the seventh day of administration.

| Code | Gr. A day7 | Code | Gr. B day 7 |
|---|---|---|---|
| **A01** | 15220 | **B01** | 14050 |
| **A02** | 10645 | **B02** | 13165 |
| **A03** | 12484 | **B03** | 13782 |
| **A04** | 20186 | **B04** | 3530 |
| **A05** | 12874 | **B05** | 18203 |
| **A06** | 10863 | **B06** | 13738 |
| **A07** | 8269 | **B07** | 16633 |
| **A08** | 10242 | **B08** | 18829 |
| **A09** | 13578 | **B09** | 20595 |
| **A10** | 15352 | | |
| **Mean ± SD** | 12971± 3379.88 | | 14725± 4963.89 |
| **one-way ANOVA ( α = 0.05)** | *NS ($p$=0.376) | | |

NS = non statistical significant

From the results that show in Table 26, there were no statistically significant differences of the ORAC values between group A and B after the curcuminoids extract administrations. As both group A and group B subjects in this study was healthy subject, the need for the additional supplement might not be potent. The ORAC values from both treatment groups were then not quite different. Furthermore, the number of subjects in the group A and B may be too small. In the other points of views, the increasing in the number of subjects studied is strongly recommended.

**Table 27** The ORAC values plasma sample from each volunteers before drug administration in the seventh day of administration.

| Code | Gr. A day7 | Code | Gr. B day 7 | Code | Gr. C day 7 |
|---|---|---|---|---|---|
| **A01** | 15220 | **B01** | 14050 | **C01** | 9216 |
| **A02** | 10645 | **B02** | 13165 | **C02** | 10661 |
| **A03** | 12484 | **B03** | 13782 | **C03** | 12605 |
| **A04** | 20186 | **B04** | 3530 | **C04** | 23574 |
| **A05** | 12874 | **B05** | 18203 | **C05** | 20788 |
| **A06** | 10863 | **B06** | 13738 | | |
| **A07** | 8269 | **B07** | 16633 | | |
| **A08** | 10242 | **B08** | 18829 | | |
| **A09** | 13578 | **B09** | 20595 | | |
| **A10** | 15352 | | | | |
| **Mean ± SD** | 12971± 3379.88 | | 14725± 4963.89 | | 15369± 6409.89 |
| **one-way ANOVA ( α = 0.05)** | *NS ($p$=0.585) | | | | |

NS = non statistical significant

When comparing the ORAC values after supplementation in group A and group B subjects to subjects in group C that administer vitamin E supplement, no statistically significant difference in the ORAC values were observed with the $p$-value of 0.585 (Table 27). By examining the mean ORAC values after vitamin E administration, it was increased higher than the mean ORAC values from group B and group A, respectively.

There were one or two reasons for the results that shown no statistically significance difference in the ORAC values after administration of antioxidant supplements. Hence, it would probably that there is an auto-balance of the antioxidant in our body in order to control and eliminate the excessive supplement unless the subjects contain oxidative stress. Despite these considerations, the daily dietary intake of each subject would also affect the final ORAC values obtained.

The other reasons in observing minor changed of the antioxidant activity after curcuminoids extract administration at supplement and treatment dose would be the method for antioxidant activity determination. The principle of the ORAC method seems to be reliable for determining the antioxidant activity in plasma. The method is based on the hydrogen atom transfer which is the mechanism for scavenging free radicals (Huang et al., 2005; Phipps et al., 2007). This is the antioxidant mechanism of curcumin (Jovanovic et al., 1999) and α-tocopherol (Traber and Sabinova, 1999; Leopoldini et al., 2004). The ORAC is the method that determined the overall antioxidant activity in our body. The result could also be affected by any antioxidant activity within our body. The increase in antioxidant activity by curcumin extracts or α-tocopherol may be too small when compared with the other antioxidant in our body.

### 3.4.3 The ORAC values and curcumin concentration profiles.

The concentration of curcumin and THC could detectable only in group B subjects that intaken curcuminoids extract in the dose of 6 g/day. Contrast to the ORAC values which could be detectable in both group A and group B subjects. Therefore, the antioxidant activity in the term of ORAC values could not be related to the curcumin and metabolites concentrations.

As shown in Figure 31, the mean ORAC values at the first day curcuminoids extract administration of the group B were higher than group A. It may be due to the high dose of curcuminoids extract administration. But for the seventh day administration (Figure 32), the ORAC values of group B were reduced comparing to the first day.

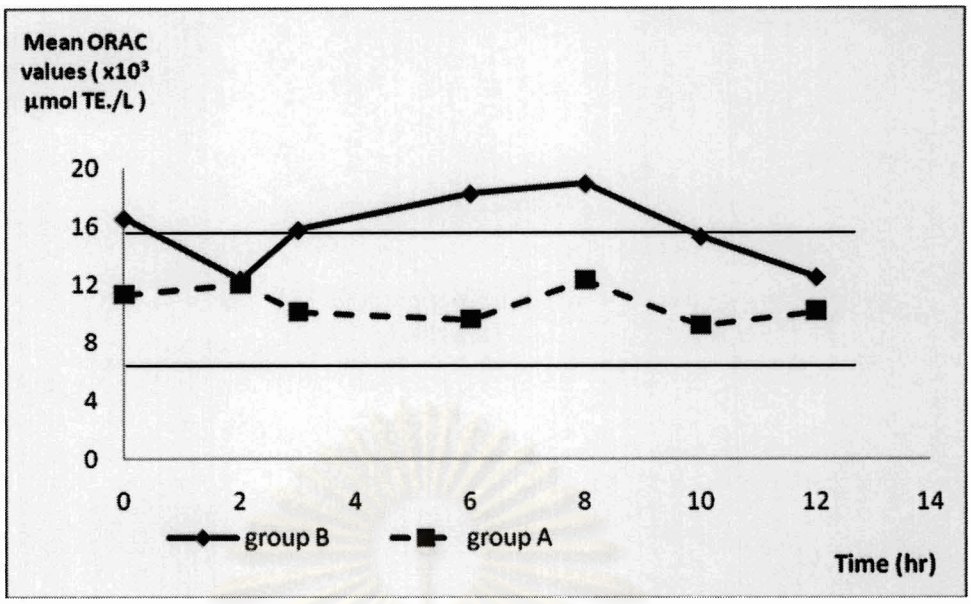



**Figure31** Mean ORAC values –time profiles of subjects following the first day of administration group A ( dose 500 mg/day ); group B( dose 6 g/day ).

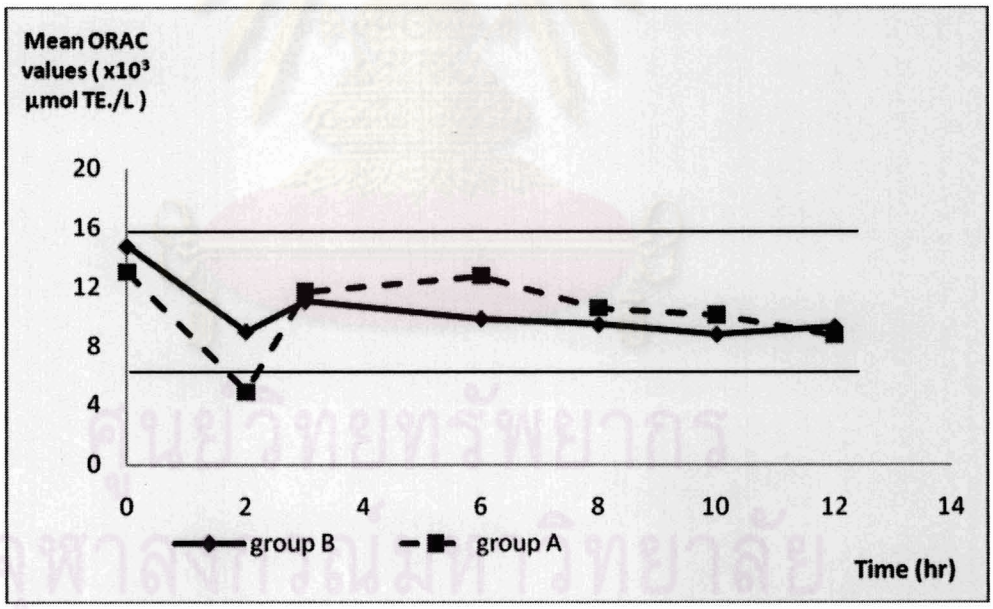



**Figure32** Mean ORAC values –time profiles of subjects following the seventh day of administration group A (dose 500 mg/day ); in group B(dose 6 g /day )

### 3.4.4 The ORAC values and subjects' habit of fruit and vegetable consumptions

As shown in Table 28, the ORAC values of all subjects (16 M, 8 F) can divided in to two groups based on the habit of fruit and vegetable consumptions (High veg. group; consumption fruit and vegetable more than 10 times among the seven days of experimentation, Low veg. group; consumption fruit and vegetable less than 5 times among the seven days of experimentation). Found no statistically significant difference of the ORAC values of both group (before and after administration) ($p$=0.290 for high veg. and fruit group and $p$=0.800 for low veg. and fruit group). There were the pattern of vegetable and fruit consumption were not change in the individual subject during the period of the experimentation, this result implied that the vegetable and fruit consumptions may not be the main effect did not effect to the ORAC values.

**Table 28** The mean ORAC values comparing the high fruit and vegetable consumption and low fruit and vegetable consumption groups

| Group | High Veg. & fruit (n=9) | Low Veg. & fruit (n=15) |
|---|---|---|
| ORAC values before curcuminoids extract adm. (Mean ± SD μmol TE./L of sample) | 12646 ± 3707 | 14002 ± 6336 |
| ORAC values after curcuminoids extract adm. (Mean ± SD μmol TE./L of sample) | 13871 ± 3715 | 14558 ± 6049 |
| Paired Student t-test (α = 0.05) | *NS ($p$=0.290) | *NS ($p$=0.800) |

NS = non statistical significant

Furthermore, the other study that shown the effect of the 16 days after consumption of diets which contained the high fruit and vegetables can increased the level of plasma antioxidant capacity (Cao et al, 1998). Contrast to this study that used periods of the study were only the seven days, if there were more period times of the study the information about the effect of food consumption would be clearly.

This study took only seven days experimentations. With this short experimentation period, the effect of fruit and vegetable consumption may not be sufficiently evaluated.

# CHAPTER IV

# CONCLUSION

To complete this study, the feasibility of the analytical methods used have to be proven from their validation reports.

The ORAC method was reliable for antioxidant activity determination. The HPLC method for plasma determination of curcumin, THC, HHC and OHC in samples was specific and sensitive enough for all sample analysis. Also, the HPLC method for plasma α- tocopherol was successfully used for endogenous α- tocopherol analysis.

Subject experimentations can be concluded as following:

1) No any adversed or undesirable effects were observed from any subject either curcuminoids extracts or vitamin E supplements administration. In addition, the difference in gender didn't affect the result.

2) Before experimentation, the endogenous α- tocopherol concentration of all subjects were determined to be within the normal range of healthy people (7.58 – 19.52 μg/ml)

Also, the ORAC values from all subjects were determined to be 7,272-24,834 μmol TE. /L of sample which has been reported for healthy human.

3) During experimentation, the ORAC profiles during the 1st and 7th day of curcuminoids administration showed only a little variation, The ORAC values with 12 hr. of group A subjects on the 1st day and on the 7th day were 5,797 to 15,689 and 8,269 to 20,186 μmol TE. /L, respectively. The ORAC values with 12 hr. for group B subjects were 9,022 to 24,834 μmol TE. /L and 3,530 to 20,595 μmol TE. /L

for the 1st day and the 7th day of treatment, respectively. This would then be followed to the nutritional paradigm.

4) After administration of curcuminoids extracts, no significance increased in the ORAC values were observed either in group A subjects or group B subjects (p=0.270 for group A and p=0.330 for group B , respectively) were observed. This would also explain within the concept of the nutritional paradigm.

Therefore, it is supported that, no supplementation is essential for any healthy people. The mega-dose of supplementation may lead to the pro-oxidant effect in the long-term usage. There are still no definite conclusion on the curcuminoids supplementation and the resulting oxidative stress

Suggestion for further study:

As many methods are reported for antioxidant activity determination and each method is uncomparable. It is therefore recommended that more than one method should be used for any antioxidant determination in the future.

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# APPENDICES

# Appendix A

Physical characteristics of 24 volunteers

| Code of subject | Sex | Age (years) | Weight ( kg ) | Height (cm.) | BMI ($kg/m^2$) |
|---|---|---|---|---|---|
| A-01 | male | 23 | 93 | 177 | 29.68 |
| A-02 | male | 34 | 73 | 168 | 25.86 |
| A-03 | male | 33 | 58 | 158 | 23.23 |
| A-04 | male | 39 | 65 | 168 | 23.03 |
| A-05 | female | 35 | 56 | 165 | 20.57 |
| A-06 | female | 30 | 51 | 159 | 20.17 |
| A-07 | male | 26 | 58 | 172.5 | 19.49 |
| A-08 | male | 22 | 63 | 172 | 21.30 |
| A-09 | female | 25 | 50 | 158 | 20.03 |
| A-10 | male | 24 | 63 | 173 | 21.05 |
| B-01 | male | 29 | 71 | 171 | 24.28 |
| B-03 | female | 36 | 57 | 160 | 22.27 |
| B-04 | female | 26 | 50 | 153 | 21.36 |
| B-05 | female | 31 | 49 | 161 | 18.90 |
| B-06 | male | 28 | 61 | 168 | 21.61 |
| B-07 | male | 23 | 93 | 177 | 29.68 |
| B-08 | male | 34 | 73 | 168 | 25.86 |
| B-09 | male | 39 | 65 | 168 | 23.03 |
| B-10 | male | 33 | 58 | 158 | 23.23 |
| C-01 | male | 34 | 90 | 174 | 29.73 |
| C-02 | male | 22 | 63 | 172 | 21.30 |
| C-03 | female | 26 | 48 | 155 | 19.98 |
| C-04 | male | 25 | 67 | 177 | 21.39 |
| C-05 | female | 26 | 48 | 160 | 18.75 |
| **Mean $\pm$ SD** | | 29.29 $\pm$5.35 | 63.46 $\pm$ 13.36 | 166.35$\pm$ 7.41 | 22.74 $\pm$ 3.28 |

# Appendix B

Biochemistry parameters of 24 volunteers

| Content | Range | Normal Range |
|---|---|---|
| Hemoglobin (g/dl) | 11.20 -14.69 | (Male) 14.00-18.00<br>(Female) 12.00-16.00 |
| Hematocrit (%) | 34.00 - 43.46 | (Male) 42.00-52.00<br>(Female) 37.00-47.00 |
| Platelet (x10^3/ul) | 115.00 - 196.63 | 150.00-400.00 |
| BUN (mg/dl) | 6.00 -11.83 | 6.00-20.00 |
| Creatinine ( mg/dl) | 0.70 - 1.03 | 0.50-1.50 |
| Uric acid (ug/dl) | 2.50 - 4.63 | 2.50-7.00 |
| Cholesterol (mg/dl) | 155.00 - 202.33 | 120.00-220.00 |
| Triglyceride ( mg/dl) | 43.00 - 97.21 | 60.00-150.00 |
| AST (SGOT) (U/L) | 15.00 - 28.92 | Up-40.00 |
| ALT (SGPT) (U/L) | 7.00 - 24.54 | Up-40.00 |
| ALK. Phosphatase ( U/L) | 32.00 - 55.75 | 26.00-117.00 |
| HDL ( mg/dl) | 35.00 - 54.63 | 55.00-100.00 |
| LDL (mg/dl) | 73.00 - 128.25 | <150.00 |

# Appendix C

## แบบบันทึกอาสาสมัคร

วันที่บันทึกข้อมูล วันที่ ..... เดือน..... พ.ศ. .....

ผู้บันทึก ชื่อ .....นามสกุล.....

**1. ข้อมูลพื้นฐาน**

ชื่อ ..... นามสกุล.....

อายุ..... ปี อาชีพ.....

อาศัยอยู่บ้านเลขที่ .....หมู่ที่ .....ซอย.....ถนน.....

แขวง.....เขต.....จังหวัด.....รหัสไปรษณีย์.....

โทรศัพท์ที่ติดต่อได้สะดวก.....

**2. ข้อมูลสุขภาพ**

น้ำหนัก.....กิโลกรัม ส่วนสูง.....เซนติเมตร ความดันโลหิต.....มิลลิเมตรปรอท

2.1 ท่านมีปัญหาสุขภาพในเรื่องต่อไปนี้หรือไม่

| | มีปัญหา | ไม่มีปัญหา |
|---|---|---|
| โรคหัวใจและหลอดเลือด | ( ) | ( ) |
| โรคเบาหวาน | ( ) | ( ) |
| โรคตับ/เกี่ยวกับตับ | ( ) | ( ) |
| ภาวะไขมันในเลือดสูง | ( ) | ( ) |
| ภาวะไขมันในเลือดต่ำ | ( ) | ( ) |
| โรคทางเดินปัสสาวะ / ไต | ( ) | ( ) |
| โรคระบบทางเดินอาหาร | ( ) | ( ) |

ปัญหาสุขภาพอื่นๆ (โปรดระบุ) .....

2.2 ท่านมีการรับประทาน วิตามิน เกลือแร่ หรืออาหารเสริม ภายในระยะเวลา 3เดือน ก่อนเข้าร่วมโครงการหรือไม่ ( ) รับประทาน ( ) ไม่ได้รับประทาน

หากรับประทานโปรดระบุรายละเอียด ชนิด ปริมาณ และระยะเวลาที่ใช้.....

.....

.....

**3.ข้อมูลด้านโภชนาการ**

ในชีวิตประจำวัน ระบุความถี่ในการรับประทานอาหารแต่ละประเภท ดังต่อไปนี้

**3.1 อาหารประเภททอดหรือผัดที่ปรุงจากน้ำมันพืช**

( ) มากกว่า 10 ครั้งต่อสัปดาห์

( ) 5 - 10 ครั้งต่อสัปดาห์

( ) น้อยกว่า 5 ครั้งต่อสัปดาห์

อาหารปรุงเอง ( ) อาหารสำเร็จรูป ( )

ระบุชนิดของน้ำมันพืชที่ใช้ ( หากทราบ ) .....

3.2 **ผักใบเขียว** ผักสด ( ) ผักต้ม ( ) ผักปรุง ( )

( ) มากกว่า 10 ครั้งต่อสัปดาห์

( ) 5 - 10 ครั้งต่อสัปดาห์

( ) น้อยกว่า 5 ครั้งต่อสัปดาห์

ผักที่รับประทาน ได้แก่ ( ) คะน้า ( ) ผักบุ้ง

(ระบุจำนวนครั้งในวงเล็บ) ( ) ผักกระเฉด ( ) ผักกวางตุ้ง

( ) แครอท

อื่นๆ โปรดระบุชนิด.....

3.3 **ผลไม้** สด ( ) หมักดอง ( ) คั้นน้ำ ( )

( ) มากกว่า 10 ครั้งต่อสัปดาห์

( ) 5 - 10 ครั้งต่อสัปดาห์

( ) น้อยกว่า 5 ครั้งต่อสัปดาห์

ระบุชนิดผลไม้ที่รับประทานบ่อยที่สุด 3 ชนิด

.....

.....

รับรองข้อมูลถูกต้อง

..... อาสาสมัคร

วันที่..... เดือน..... พ.ศ. .....

# Appendix D

Inclusion and exclusion criteria for volunteer subject.

Criteria for volunteers

Inclusion criteria

Thai volunteers in the age range of 20 – 40 years, body mass index (BMI) less than 30 $kg/m^2$ , and pass the physical examination were include in the study. All volunteers don't have systemic disease such as cardiovascular disease, diabetes and cancer. They have to sign the informed consent and follow the following recommendation:

- No taking of particular drug or curcumin supplements or antioxidant supplements especially antioxidant vitamin such as vitamin A, C and E.
- No alcohol or caffeine uptake on the day before experiment.
- Foods which consist of turmeric powder such as curry, yellow curry must be avoids along the experiment periods.
- Foods has to obtained at least 8-10 hours before drug administration (water was allowed).

Exclusion criteria

- Volunteers would be dropped out if fail to pass the physical examination.
- Not willing to continue the experiment.

Volunteers who passed the including criteria were selected into the study and would be explain about the detail of experiment and they also have to fill in the questionnaires about their lifestyle.

# VITA

Mr. Kanit Pungcharoenkul was born on September 14, 1981 in Ubonratchathani, Thailand. He had received his Bachelor of Science in Pharmacy in 2004 from the Faculty of Pharmaceutical Sciences, Chulalongkorn, Thailand. After his graduation, he has work in Quality Assurance Department, The Government Pharmaceutical Organization, Bangkok, Thailand.