Final Report

Project Title

Development of High Throughput Quantitative Assays for Determining Antioxidant Capacities and interactions in Oil-in-Water and Water-in-Oil Emulsions

By ATIKORN PANYA

May / 2559

Contract No. TRG5780061

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This project granted by the Thailand Research Fund

Abstract

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Project Title: Development of High Throughput Quantitative Assays for Determining Antioxidant Capacities and interactions in Oil-in-Water and Water-in-Oil Emulsions

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Project Period: June 2014 to June 2016

Abstract:

Four versions of CAT based assays (CAT, ApoCAT, Fenton-CAT and Fenton-ApoCAT assays) have been developed in this research to elucidate the complexity of antioxidant behaviors of the individual and combined antioxidants in O/W emulsion and food model systems. Protocatechuic and gallic acid and their alkyl esters were selected as antioxidants with different polarities in order to compare in the developed models. Results suggested that the ApoCAT and Fenton-ApoCAT assays were able to predict precisely the performances of antioxidants with different polarities in the O/W emulsion, and roasted peanut systems except for the bulk oil system. It could be due to the structure of bulk oil system may differ from other model systems. However, in terms of the antioxidant interactions, the Fenton-CAT, ApoCAT and Fenton-ApoCAT assays did not show the similar relationship with the actual lipid oxidation in O/W emulsions. Only the antioxidant interactions observed in the CAT assay were similar to those observed in the oxidation in O/W emulsions. In summary, oxidation dynamics (oxidation hierarchy and free radical distribution) should be one of the important keys to understand lipid oxidation mechanism in particular food systems. In addition, together with antioxidant dynamics (antioxidant distribution and exchange) in the systems, one could be able to design a better antioxidant assay that could predict precisely both antioxidant performances and interactions.

Keywords: 3-5 words antioxidant interaction, synergistic, CAT, ApoCAT, Fenton

บทคัดย่อ

รหัสโครงการ: TRG5780061

หัวข้อวิจัย: การพัฒนาวิธีการวิเคราะห์ความสามารถและการปฏิสัมพันธ์ของสารต้านอนุมูลอิสระในระบบอิมัลชั่นชนิดน้ำในน้ำมันและน้ำมันในน้ำ

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บทคัดย่อ

ในโครงการวิจัยนี้ วิธีการ CAT ถูกพัฒนาขึ้นมาใน 4 รูปแบบ เช่น CAT ApoCAT Fenton-CAT และ Fenton-ApoCAT เพื่อใช้ในการศึกษาความซับซ้อนของพฤติกรรมของสารต้านอนุมูลอิสระชนิดเดียว หรือสองชนิดที่ทำงานร่วมกัน ในระบบอิมัลชันชนิดไขมันในน้ำ และระบบอาหารอื่นๆ กรดโปรโตแคทชูอิค และ กรดแกลลิต และอนุพันธ์แบบอัลคิล ถูกนำมาใช้เป็นสารต้านอนุมูลอิสระ อันเนื่องมาจากสารเหล่านี้มีความมีขั้วแตกต่างกัน ผลการทดลองพบว่าวิธี ApoCAT และ Fenton-ApoCAT สามารถทำนายการความสามารถในการป้องกันออกซิเดชันในระบบ อิมัลชันชนิดไขมันในน้ำ และระบบถั่วคั่วบดได้ดี แต่ไม่สามารถทำนายในระบบน้ำมันได้ ทั้งนี้อาจเป็นเนื่องมาจากระบบน้ำมันมีโครงสร้างของอาหารที่แตกต่างจากระบบอิมัลชันของระบบ CAT และอาหารชนิดอื่นๆ อย่างไรก็ตาม ทางด้านอันตรกิริยาของสารต้านอนุมูลอิสระ พบว่า วิธี CAT ให้ค่าดัชนีอันตรกิริยาของสารต้านอนุมูลอิสระ คล้ายคลึงกับที่พบในระบบอาหารจำลองอิมัลชันชนิดไขมันในน้ำ จากการทดลองสรุปได้ว่า การกระจายตัวหรือการแลกเปลี่ยนของอนุมูลอิสระเป็นปัจจัยสำคัญในการเข้าใจการเกิดออกซิเดชันในระบบอาหาร นอกจากนี้ การกระจายตัวหรือการแลกเปลี่ยนของสารต้านอนุมูลอิสระ ก็มีส่วนสำคัญ ต่อสามารถของสารต้านอนุมูลอิสระ จากองค์ความรู้เหล่านี้ เราสามารถออกแบบระบบหรือวิธีการวัดให้มีแม่นยำสูงขึ้น ทั้งด่านการทำนายความสามารถในการต้านออกซิเดชัน และการทำนายอัตรกิริยา

คำสืบค้น อันตรกิริยาของสารต้านอนุมูลอิสระ การทำงานแบบเสริมฤทธิ์ แคท อะโปแคท เฟนทอน

Executive summary

Four versions of CAT based assays (CAT, ApoCAT, Fenton-CAT and Fenton-ApoCAT assays) have been developed in this research to elucidate the complexity of antioxidant behaviors of the individual and combined antioxidants in O/W emulsion and food model systems. Protocatechuic and gallic acid and their alkyl esters were selected as antioxidants with different polarities in order to compare in the developed models. According to the current research, the ApoCAT and Fenton-ApoCAT assays were able to predict precisely the performances of antioxidants with different polarities in the O/W emulsion, and roasted peanut systems except for the bulk oil system. It could be due to the structure of bulk oil system may differ from other model systems.

However, in terms of the antioxidant interactions, the Fenton-CAT, ApoCAT and Fenton-ApoCAT assays did not show the similar relationship with the actual lipid oxidation in O/W emulsions. Only the antioxidant interactions observed in the CAT assay were similar to those observed in the oxidation in O/W emulsions.

In summary, oxidation dynamics (oxidation hierarchy and free radical distribution) should be one of the important keys to understand lipid oxidation mechanism in particular food systems. In addition, together with antioxidant dynamics (antioxidant distribution and exchange) in the systems, one could be able to design a better antioxidant assay that could predict precisely both antioxidant performances and interactions.

Objectives

1. To develop high throughput quantitative assays for analyzing synergistic, additive and antagonistic antioxidant interactions in O/W emulsions and food models such as bulk oil and roasted peanut systems
2. To understand how locations and/or types of free radical initiators affecting antioxidant capacities and interactions

Research methodology

Chemicals reagents

Trichloroacetic acid (TCA), Butylated hydroxyl toluene, thiobarbituric acid, cumene hydroperoxide and 1,1,3,3,- tetrahydroxypropane were purchased from Merck (USA). Ethylenediaminetetraacetic acid (EDTA), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), silicic acid, folin-Ciocalteau's reagent, sodium carbonate, ferrous sulfate, ferric chloride, ferrozine, Tung oil, Brij 35, ascorbic acid, Polyoxyethylene sorbitan monolaurate (TWEEN® 20), barium chloride and ammonium thiocyanate, ferrous sulfate, cobalt fluoride were purchased from Sigma-Aldrich (St. Louis, USA). Absolute ethanol was purchased from RCI Labscan (Thailand). 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), dimethyl 2,2'-azobis(2-methylpropionate) or V601 and activated charcoal were purchased from Wako Chemical (Japan). Medium chain triglyceride (MCT) mixture (Miglyol®812N) was purchased from Sasol Germany Gmbh (Witten, Germany). Protocatechuic acid, ethyl protocatechuate, gallic acid, methyl gallate, propyl gallate, butyl gallate, octyl gallate and laury gallate were purchased from Sigma-Aldrich (St. Louis, USA). Cold pressed perilla oil and refined soybean oil were purchased from a local market in Thailand. Deionized water was used for the preparation of all solutions. All organic solvents used in this study were analytical grade.

Methodology

1. Selection of antioxidants and synthesis of rosmarinate esters and chlorogenate esters

To understand the influence of location and/or hydrophobicity of antioxidants on their antioxidative performance in model studies, ester forms of antioxidants, so called phenolipids, will be used as a tool in this research. As illustrated in Figure 1, protocatechuic acid and gallic acid were selected to be esterified with several alkyl chain lengths. Antioxidant capacities of protocatechuic acid and its esters and gallic acid and its esters were evaluated in various models such as the CAT, the ApoCAT, Fenton-CAT, and Fenton-ApoCAT assays. In addition, gallic acid and some of its esters were selected to use for further studying on their performances in food models and antioxidant interaction of combinations. As shown in table 1 and 2, Some antioxidants were purchased from Sigma-Aldrich Co. LLC. However, some antioxidants from chemical synthesis were supplied from a laboratory at CIRAD agricultural research for development, Montpellier, France. The chemo-esterification of these antioxidants was performed as the method described by Grajeda-Iglesias, et al., 2015.

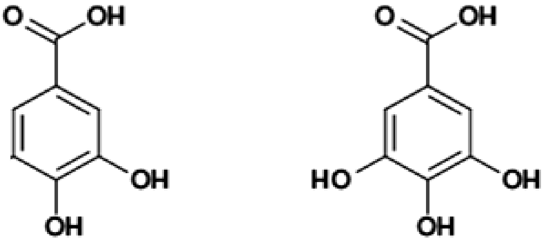

Figure 1 Phenolipids used in this research; Protocatechuic (A) and gallic acid (B)

Table 1 Gallic acid and it esters used in this research

Name	Alkyl chain length (carbon)	Molecular weight	Source
Gallic acid	0	170.12	Sigma Aldrich
Methyl gallate	1	184.15	Sigma Aldrich
Propyl gallate	3	212.20	Sigma Aldrich
Butyl gallate	4	226.23	Sigma Aldrich
Octyl gallate	8	282.33	Sigma Aldrich
Dodecyl gallate	12	338.44	Sigma Aldrich
Tetradecyl gallate	14	366.54	Synthesis
Hexadecyl gallate	16	394.60	Synthesis
Octadecyl gallate	18	422.66	Synthesis

Table 2 Protocatechuic acid and it esters used in this research

Name	Alkyl chain length (carbon)	Molecular weight	Source
Protocatechuic acid	0	154.12	Sigma Aldrich
Methyl protocatechuate	1	168.15	Synthesis
Ethyl protocatechuate	2	182.18	Sigma Aldrich
Butyl protocatechuate	4	210.24	Synthesis
Hexyl protocatechuate	6	238.30	Synthesis
Octyl protocatechuate	8	266.36	Synthesis
Decyl protocatechuate	10	294.42	Synthesis
Dodecyl protocatechuate	12	322.48	Synthesis
Tetradecyl protocatechuate	14	350.54	Synthesis
Hexadecyl protocatechuate	16	378.60	Synthesis
Octadecyl protocatechuate	18	406.66	Synthesis

2. Comparison of antioxidant capacities of phenolipids in various models

To investigate the effect of types and locations of free radical initiators on determination of antioxidant capacities, three new versions of the CAT assay were developed. First, apolar radical-initiated conjugated autoxidizable triene (ApoCAT) assay was developed. Second, conjugated autoxidizable triene via Fenton reaction (Fenton-CAT) assay was developed using a water-soluble free radical initiator (H_2O_2 and Fe^{2+}). Thirst, apolar radical-initiated conjugated autoxidizable triene via Fenton reaction (Fenton-ApoCAT) assay was developed using a lipid-soluble free radical initiator (cummene hydroperoxide and Fe^{2+}).

In addition, gallic acid and its esters (up to 12 carbons) were further selected to investigate their antioxidant performances in three food models including oil-in-water emulsion, bulk oil, and dried moisture food systems.

2.1. Antioxidant behaviors of phenolipids performed in conjugated autoxidizable triene (CAT) assay: using a water-soluble free radical initiator

The conjugated autoxidizable triene (CAT) assay method was performed with some modifications (Panya et al., 2015). Conjugated triene triacylglycerols from Tung oil were used as the oxidizable UV probe for an oxidation reaction. All of the reagents and samples were dissolved and diluted in phosphate buffer (50 mM, pH7.2). The CAT assay protocol was composed of 2 steps described as follow.

1. Emulsion preparation

Phosphate buffer (PB) solution (50 mM, pH 7.2) containing 34 µM Brij 35 was added to 6±1 mg of stripped Tung oil. The mixture was then homogenized (Ultra-TurraxT25 basic, IKA® Werke, Germany) at 6,500 rpm for 90 s. The emulsion was centrifuged at 10,000 g, 10 °C for 5 min to remove the unstable part.

2. Reaction mixture

A 50-µl volume of 50 mM PB containing samples (desirable concentration) was transferred to a UV-Star 96-well plate followed by 125 µl of emulsion. The 96-well plate containing antioxidants and emulsions was mixed at 1,200 rpm, 37 °C for 5 min by a microplate thermomixer (Thermomixer comfort, eppendorf, Germany). After mixing, 25 µl of 8mM AAPH solution in PB solution was added into the mixtures. The final mixture volume was 200 µl with 17 µM Brij 35, 1 mM AAPH and various concentrations of antioxidants. Changes in the absorbance at 273 nm of mixtures were then recorded using a UV-domain microplate reader in kinetic mode for 3 h. The CAT values were expressed as trolox equivalent by calculating the relationship between net protection areas under the curve (AUC) and concentrations of antioxidants compared to that of trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

Tung oil was striped prior using to remove the minor polar components. Briefly, 100 g of silicic acid was washed with distilled water for three times with a total volume of 3 L, then the washed silicic acid was filtered with Whatman filter paper and dried at 110 °C for 20 h. The glass chromatographic column (3.0 cm internal diameter × 35 cm height) was packed sequentially with 22.5 g of washed silicic acid followed by 5.63 g of activated charcoal and 22.5 g of washed silicic acid. The washed silicic acid and activated charcoal were suspended in 100 ml and 70 ml of n-hexane, respectively, before packing into a glass column. Thirty grams of Tung oil were dissolved in 30 ml of n-hexane, and passed through the column by eluting with 270 ml of n-hexane. The collected triacylglycerols were kept in an ice bath and covered with an aluminum foil in order to minimize lipid oxidation during stripping process. To remove the n-hexane from the collected stripped Tung oil after the elution completed, the rotary evaporator (BÜCHI Rotavapor R-114, Switzerland) was performed at 35 °C under vacuum. The nitrogen flushing was adapted to remove traces of hexane remaining in the oil. After flushing for 10 min, 3 ml of stripped oil was transferred into vials and flushed with nitrogen again. The aliquots of stripped Tung oil were kept at −80 °C before use.

2.2. Antioxidant behaviors of phenolipids performed in apolar radical-initiated conjugated autoxidizable triene (ApoCAT) assay: using a lipid-soluble free radical initiator

In this research, the ApoCAT assay was newly developed and published as shown in Appendix B. Based on the original CAT assay, all procedures of the ApoCAT assay were performed in the same manner as the original CAT assay except for the free radical initiator. Instead of using AAPH, dimethyl 2,2'-azobis(2-methylpropionate) or V-601, an lipid-soluble free radical initiator was used in this assay. To deliver the lipid radical initiator into emulsion mixtures, Medium chain triglycerides (MCT) emulsions were prepared.

Briefly, 10 mM PB solution (pH 7.2) containing 34 µM Brij 35 was added to 6±1 mg of MCT oil. The mixture was then homogenized at 6,500 rpm for 90 s. The V601 radical initiator was prepared by dissolving in the MCT emulsions to obtain a final concentration of 8 mM. To remove oil residues and other non-stable parts, the emulsion was centrifuged at 10,000 g, 10 °C for 5 min. The MCT emulsion containing 8 mM V-601 was collected from the supernatant. To begin the oxidation of the ApoCAT assay, 25 µl of the MCT emulsion with V601 was added into the prepared Tung oil emulsions with antioxidants as described in the CAT assay.

2.3. Antioxidant behaviors of phenolipids performed in conjugated autoxidizable triene via Fenton reaction (Fenton-CAT) assay: using a water-soluble free radical initiator

Based on the original CAT assay, all procedures of the Fenton-CAT assay were performed in the same manner as the original CAT assay except for the free radical initiator. Instead of using AAPH, hydrogen peroxide (H_2O_2) and metal ions (Fe^{2+} or Co^{2+}) were used in this assay.

Briefly, H_2O_2 (7 mM), $FeSO_4$ (14 μM) and CoF_2 (14 μM) solutions were prepared. To initiate the oxidation, 25 μl of H_2O_2 solution was added into the antioxidant/tung oil emulsion mixture. Immediately, 25 μl of $FeSO_4$ or CoF_2 solution was added to start the oxidation. Changes in the absorbance at 280 nm of mixtures were then recorded using a UV-domain microplate reader in kinetic mode for 6 h.

2.4. Antioxidant behaviors of phenolipids performed in apolar radical initiated conjugated autoxidizable triene via Fenton reaction (Fenton-ApoCAT) assay: using a lipid-soluble free radical initiator

Based on the ApoCAT assay, all procedures of the Fenton-ApoCAT assay were performed in the same manner as the original ApoCAT assay except for the free radical initiator. Instead of using V601, cummene hydroperoxide and metal ions (Fe^{2+} or Co^{2+}) were used in this assay.

Briefly, cummene hydroperoxide (7 mM) in the MCT emulsion, $FeSO_4$ (115 μM) and CoF_2 (115 μM) solutions were prepared. To initiate the oxidation, 25 μl of cummene hydroperoxide in MCT solution was added into the antioxidant/tung oil emulsion mixture. Immediately, 25 μl of $FeSO_4$ or CoF_2 solution was added to start the oxidation. Changes in the absorbance at 280 nm of mixtures were then recorded using a UV-domain microplate reader in kinetic mode for 6 h.

2.5. Antioxidant behaviors of phenolipids in various in food models: oil-in-water emulsions, bulk soybean oils and roasted peanuts (dried moisture food)

From previous experiments, the antioxidant capacities of selected antioxidants were performed in the original CAT assay and the newly developed CAT assays. Moreover, conventional assays for antioxidant activities including TPC, ABTS, FRAP, ORAC assays were performed in comparison with the CAT assays as shown in Appendix A. The differences in the antioxidant activity patterns because of difference in the polarity of antioxidants were analyzed and further compared with food models. It was noted that water-in-oil emulsion was excluded from this study. Two food models (bulk soybean oils and roasted peanuts) were selected due to that they are more relevant to actual food applications.

2.5.1. Oil-in-water (O/W) emulsion preparation

In this study, oil-in-water (O/W) emulsions were used to represent a heterogeneous food model for evaluating the antioxidative performance of gallic acid and its alkyl esters. The perilla oil was stripped prior to use in the same manner as Tung oil as desceibed in the CAT assay. The O/W emulsion was prepared with slightly modifications. Shortly, 1% of stripped perilla oil was mixed with tween 20 at the emulsifier/oil ratio of 1:10 in PB (10 mM, pH 7.0) by homogenizing at 9,500 rpm for 2 min (Ultra-Turrax T25 basic, IKA® Werke, Germany). Then, the coarse emulsion mixture was homogenized with ultrasonic treatments using a 20 kHz 130 W ultrasonic processor (Sonic, Vibra-cell™ VCX 130, USA) equipped with a 13 mm diameter tip (with 100% amplitude). Total pulse duration was 2 min (30 s each pulse, with 10 s interval).

2.5.2. Oxidative stability of oil-in-water (O/W) emulsions

The perilla O/W emulsions was added with gallic acid and its alkyl esters to obtain final concentration of 50 µM. The O/W emulsions containing tested antioxidants were transferred into 96-deep well plates. The foiled cover sheets were used to minimize the evaporation of emulsions in each well. The plates were then incubated at 30 °C. Samples were taken periodically to determine the oxidation products. Formation of lipid hydroperoxide values (PV) and thiobarbituric acid reactive substances (TBARs) were evaluated as the indicators of primary and secondary lipid oxidation products, respectively.

2.5.3. Measurements of lipid hydroperoxides

Formation of lipid hydroperoxide was determined with some modifications (Panya et a., 2012). Fifty microliter of emulsions were first mixed with 450 isooctane: isopropanol (3:1) then centrifuged (3,200 g) for 3 min to break emulsions. Then, 50 µl of the upper layer of organic solvent phase was collected and 200 µl of methanol: butanol (2:1) was added. The reaction begins with the addition of 20 µl of ammonium thiocyanate reagent. The ammonium thiocyanate reagent was prepared by mixing $FeSO_4$ (0.144 M) with BaCl2 (0.132 M) at the ratio 1:1. After that, the mixture was centrifuge at 3,200 rpm for 2 min. The upper clear phase of centrifuged mixture was mixed 3.94 M of ammonium thiocyanate solution at the ratio of 1:1. The reaction mixture of was left at room temperature for 20 min, and the absorbance was recorded at 510 nm using a microplate reader. Cummene hydroperoxide was used as a standard lipid hydroperoxide.

2.5.4. Thiobarbituric acid reactance (TBAR) assay

Thiobarbituric acid reactance (TBAR) assay was performed with slightly modifications (Panya et al., 2012). Briefly, TBA reagent was prepared by mixing 20% of TCA, 0.5% of TBA and 0.2% of EDTA in 30mM HCl. Immediately before analysis, 30 ml of TBA reagent was mixed with 450 µl of 3% BHT in absolute ethanol solution. Emulsion (150 µl) was reacted with the TBA reagent (300 µl) in 96-deep well plates. The plates containing mixture were covered and heated at 90 °C for 20 min. Then, 96-deep well plates were transferred into an ice bath to stop the reaction for 10 min. Then, the plates were centrifuged at 3,200 g for 5 min. The supernatant was collected to measure the absorbance at 532 nm. A calibration curve was prepared with 1,1,3,3-tetrahydroxypropane as a standard for quantification.

2.5.5. The expression of oxidative stability of oil-in-water (O/W) emulsions

The oxidative stability of emulsions was expressed as the induction periods (IP) or oxidation lag time obtained from the formation of hydroperoxide and TBARs. The induction periods were defined as the first data point that statistically increased from the previous data points during the oxidation studies. Comparisons of the means were performed using Duncan’s multiple range tests.

2.5.6. Oxidative stability of bulk soybean oils and roasted peanuts

The antioxidative performances of gallic acid and its alkyl esters were investigated in different food matrices including bulk soybean oils and roasted ground peanuts using Rancimat test. Commercial soybean oil (TVO public company limited, Thailand and freshly prepared roasted ground peanuts were used in this experiment. The soybean oil and roasted ground peanuts were mixed with the ethanolic solution of gallic acid and its alkyl esters to obtain the final concentration of 2 mmoles per kilogram. For soybean oil samples, ethanol was evaporated out using a rotary evaporator operating at 35 °C under vacuum for 10 min. For roasted grounded peanut, samples were hand shaken to obtain homogeneous mixture for 3 min after the addition of antioxidants.

After that, the roasted grounded peanut samples were dried in a hot air oven 35 °C for 30 min to remove the remaining ethanol. After soybean oil and roasted grounded peanut were completed mixed with gallic acid and its alkyl esters, the commercial instrument called “Rancimat” machine (Rancimat 743, Metrohm CH-9101, Herisau, Switzerland) was adapted to determine the oxidative stability of foods. The instrument was set at 110 °C and air flow rate of 20L/h[15]. Roasted peanut were further ground before tested. The sample size of bulk soybean oils and roasted ground peanuts were three grams and one gram, respectively. Samples were weighed into the reaction tube and placed in the Rancimat instrument. The conductivity of deionized water was recorded kinetically. The induction times (IT) of oxidation were calculated using 743 Rancimat software (Metrohm CH-9101, Herisau, Switzerland).

2.5.7. Data analysis

All analyses were performed on triplicate samples. The relationship between antioxidant evaluation assays were analyzed by principle component analysis (PCA) using SPSS 17 (http://www.spss.com; SPSS Inc., Chicago, IL).

3. Comparison of antioxidant interactions in various models

Besides evaluating individual antioxidants, another perspective of using antioxidant assays was that these assays should be relevant to food emulsions in terms of combinations of antioxidants or interactions. It was hypothesized that different locations of free radical initiators might have different impacts on antioxidant interactions, resulting in variation in synergistic, additive and antagonistic effects.

3.1 Study on antioxidant interaction in various CAT assays

In this experiment, interaction between selected antioxidants were investigated. Two types of the combination were designed. First, the combination between a water-soluble antioxidant (gallic acid, G0) and a lipid-soluble antioxidant (tetradecyl gallate, G14) was selected. Second, the combination between two lipid-soluble antioxidants (dodecyl gallate, G12 and tetradecyl gallate, G14) was used.

The combination ratios of combined antioxidants were prepared at 5:1, 3:1, 1:1, 1:3 and 1:5 (mole to mole) ratios. Antioxidant capacities of individual and combined antioxidants were compared in terms of interaction index as illustrated in the following equation (Eq.4)

$$\text{Interaction Index} = \frac{\text{(Observed slope of the combination)}}{\text{(Expected slope of the combination)}} \qquad (1)$$

Interaction indexes were expressed as synergistic (> 1), additive (≈ 1) and antagonistic (< 1) antioxidant effects.

3.2 Study on antioxidant interaction in food emulsion models

In this experiment, two types of the combination were performed in oil-in-water emulsions. First, the combination between a water-soluble antioxidant (gallic acid, G0) and a lipid-soluble antioxidant (tetradecyl gallate, G14) was selected. Second, the combination between two lipid-soluble antioxidants (dodecyl gallate, G12 and tetradecyl gallate, G14) was used. The combination ratios of combined antioxidants were prepared at 3:1, 1:1 and 1:3 (mole to mole) ratios. Lipid hydroperoxide and TBARs formation in emulsion solutions will be determined according to the method described by Panya and coworkers (6) with some modifications.

The induction periods of the emulsions with antioxidants will be investigated by analyzing lipid hydroperoxide and TBARs formation. Interaction indexes of various antioxidants were calculated based on the oxidation lag times of lipid hydroperoxides and hexanal formation as described by Panya et al. (2015). Lag times were determined as the first data point that was statistically ($p<0.05$) greater than time zero.

Briefly, the oxidation lag times of individual antioxidants were used to estimate the expected oxidation lag times of its combination. Interaction indexes were calculated from the ratio between the obtained oxidation lag times of the combination and the expected oxidation lag time of the combination with the following equation (Eq. 5):

$$\text{Interaction Index} = \frac{(\text{Observed lag time of the combination})}{(\text{Expected lag time of the combination})} \qquad (5)$$

Interaction indexes were expressed as synergistic (> 1), additive (≈ 1) and antagonistic (< 1) antioxidant effects

Results

1. Antioxidant behaviors of phenolipids performed in conjugated autoxidizable triene (CAT) assay: using a water-soluble free radical initiator

In this study, gallic acid and protocatechuic acids and their esters were used in order to investigate the impact of changes in the polarities of antioxidants on their antioxidant capacities in the CAT assay. As previously hypothesized, using AAPH as a water-soluble radical initiator in the CAT assay could influence on the oxidation behaviors of oil-in-water emulsions that might be irrelevant to the oxidation dynamics of oil-in-water emulsions.

As shown in Figure 2 and 3, antioxidant capacities of gallic and protocatechuic acids and their esters were performed in the CAT assay. Even though gallic acid and its esters have one more hydroxyl group (-OH) in comparison with protocatechuic acid and its esters, interestingly, gallic acid and its esters exhibited higher antioxidant capacities than protocatechuic acid and its esters. In comparison with their alkyl esters, butyl gallate had the highest antioxidant capacities, while ethyl protocatechuate exhibited the highest antioxidant capacities.

It was noticed that two cut-off effects were observed in Figure 2 and 3. In general, one cut-off effect was expected when the polarity of antioxidants was decreased according to the polar paradox hypothesis. For the gallate series, two peaks of antioxidant capacities of the gallate were observed at ethyl gallate (2 carbons) and hexadecyl gallate (16 carbons). In the case of the protocatechuate series, first peak of the antioxidant capacity was observed at ethyl protocatechuate, and the second peak of the antioxidant capacity was in addition observed at dodecyl- and tetradecyl gallate (12 and 14 carbons).

From these phenomena, it was hypothesized that it could be due to the distribution of free radicals in the system. Since the CAT assay used AAPH to accelerate the oxidation, at the begin of the oxidation, radicals should be located/mainly distributed at aqueous phase. As the oxidation progressed, radicals in the lipid phases could be then initiated. Thus, there are radicals distributed in both aqueous and lipid phases. As a result, water-soluble antioxidants especially butyl gallate and ethyl protocatechuate had higher antioxidant capacities than expected, because polar antioxidants, mainly distributed in aqueous phase could inactivate the water soluble radicals in the aqueous phase before they could further initiate lipid soluble radicals in the lipid phases.

The red arrows in both figures were used to indicate the possible antioxidant capacities of the antioxidants what if there were no the impacts of water soluble radicals.

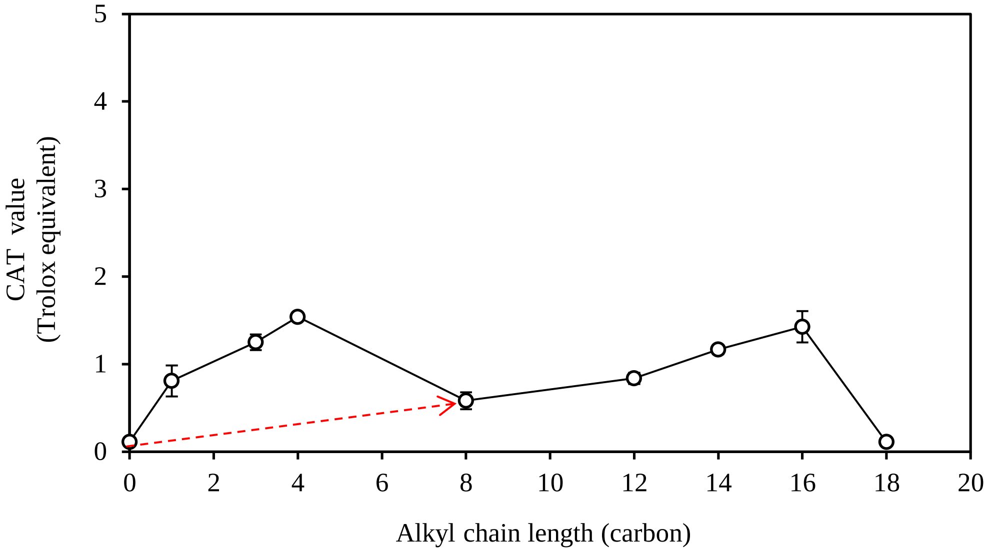

Figure 2 Antioxidant capacities of gallic acid and its esters using the CAT assay

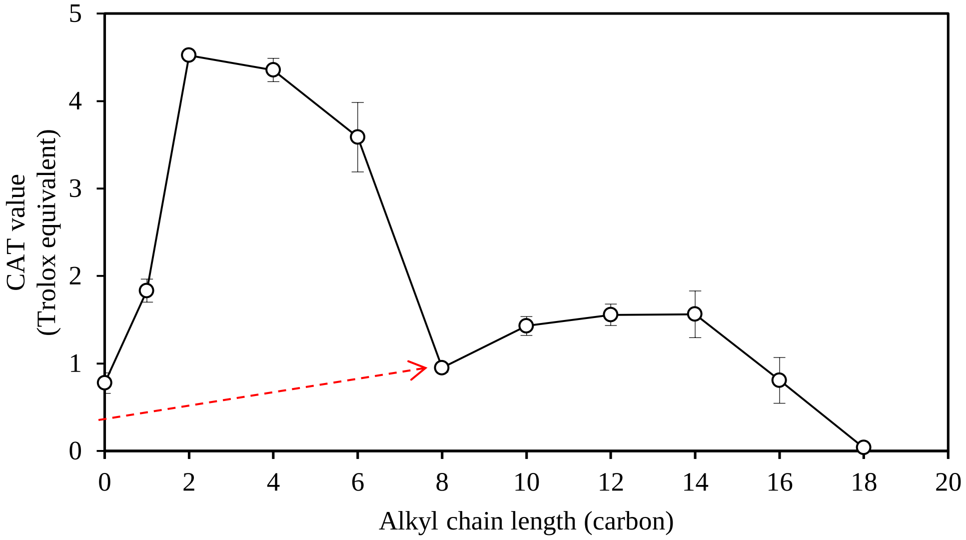

Figure 3 Antioxidant capacities of protocatechuic acid and its esters using the CAT assay

3. Antioxidant behaviors of phenolipids performed in apolar radical-initiated conjugated autoxidizable triene (ApoCAT) assay: using a lipid-soluble free radical initiator

As shown in the Appendix A, the ApoCAT had been developed and published in Journal of Agricultural and Food Chemistry. In this study, gallic acid and protocatechuic acids and their esters were used in order to investigate the impact of changes in the polarities of antioxidants on their antioxidant capacities in the ApoCAT assay. The MCT emulsion was used in order to deliver a lipid soluble azo radical initiator, V601 or AIBME. By relying on dynamic exchanges of components between tung oil and MCT oil emulsions, both antioxidants and lipid soluble radicals could be transferred.

Results showed that the performances of polar antioxidants were dramatically decreased in comparison with those of polar antioxidants in the CAT assay. In contrast to observed in the CAT assay, it was noticed that gallic acid and its esters exhibited higher the ApoCAT value than protocatechuic acid and its esters. Only one cut-off effect was observed in the gallate series at which the highest antioxidant capacity was found at tetradecyl and hexadecyl gallates (14 and 16 carbons). A linear relationship between changes in the polarities and antioxidant capacities was observed up to 16 carbons. However, for protocatechuate series, two cut-off effects were still observed at hexyl and dodecyl protocatechuates (6 and 12 carbons).

From this observation, even though the distribution of aqueous phase radicals was compromised by using AIBME in MCT emulsion, the second cut-off effect was observed in the case of protocatechuic acid and its esters. This suggested that lipid-soluble radicals in tung oil and/or MCT emulsions might somehow behave like aqueous phase radicals in the system.

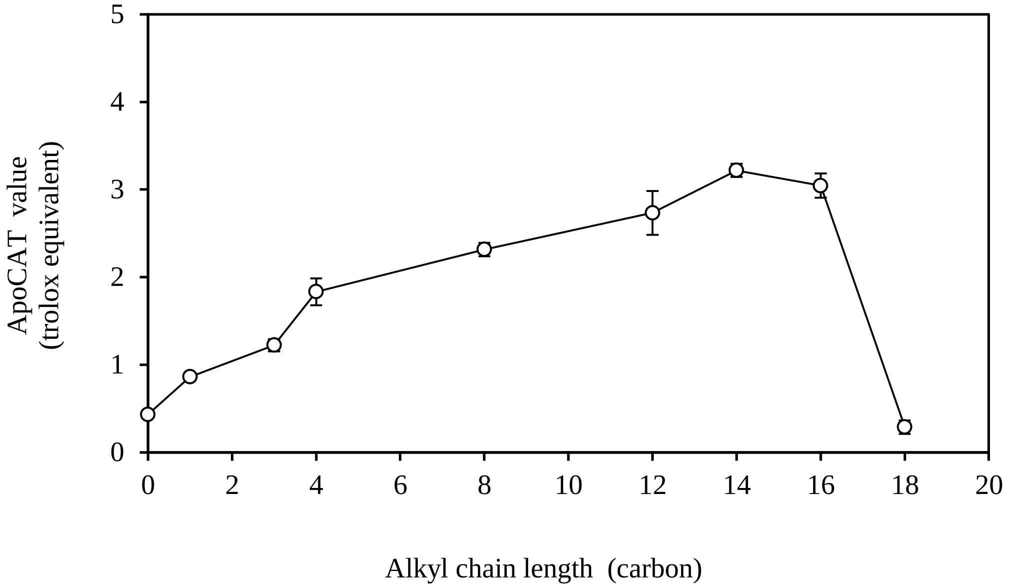

Figure 4 Antioxidant capacities of gallic acid and its esters using the ApoCAT assay

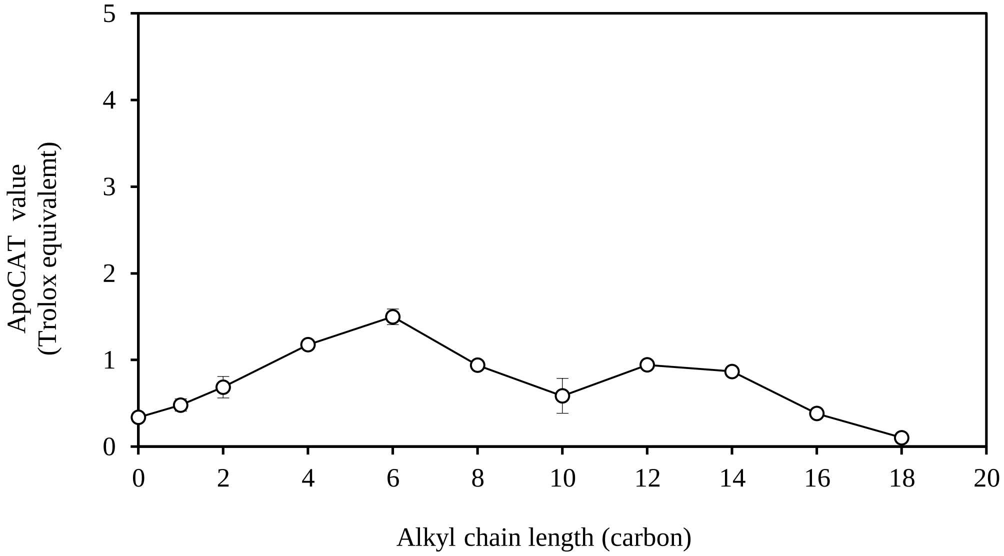

Figure 5 Antioxidant capacities of protocatechuic acid and its esters using the ApoCAT assay

4. Development of conjugated autoxidizable triene via Fenton reaction (Fenton-CAT) assay and apolar radical initiated conjugated autoxidizable triene via Fenton reaction (Fenton-ApoCAT) assay

At the beginning, there were two versions of the Fenton-CAT assays. They differed in metal ions in Fenton's reaction. The first model used the combination between hydrogen peroxide (H_2O_2) and $FeSO_4$ (Fe^{2+}), and the second model used the combination between hydrogen peroxide (H_2O_2) and CoF_2 (Co^{2+}).

As illustrated in Figure 6 and 7, antioxidant capacities of gallic acid and its esters exhibited only one cut-off effect in both assays. For the Fenton-CAT with Fe^{2+}, antioxidant capacities were increased as the increase in alkyl chain lengths up to 3 carbons. However, increasing the alkyl chain lengths beyond this points until 16 carbons, there were no significant differences in antioxidant capacities from G3 to G16. The cut-off effect was observed when the alkyl chain length was higher than 16 carbons.

For the Fenton-CAT with Co^{2+}, antioxidant capacities were increased as the increase in alkyl chain lengths up to 8 carbons, however, there were no significant differences in antioxidant capacities from G8 to G16. The cut-off effect was observed when the alkyl chain length was higher than 16 carbons. Surprisingly, the Fenton CAT value obtained from the Fenton-CAT with Co^{2+} exhibited very high Fenton-CAT value than expected. Compared to trolox, gallic acid and its esters showed the Fenton-CAT value ranging from 2 to 9 trolox equivalence at which the value may not represent the true performance of the antioxidants Thus, the Fenton-CAT assay with Fe^{2+} was preferred for a further study with protocatechuic acid and its esters.

The same was true for the study in the Fenton-ApoCAT assays with Fe^{2+} and Co^{2+}. As shown in Figure 8, the Fenton-ApoCAT assay with cummene hydroperoxide and Fe^{2+} exhibited a linear relationship between increases in alkyl chain length and antioxidant capacities of gallic acid and its esters. The cut-off effect was observed beyond G16. For the Fenton-ApoCAT assay with Co^{2+}, two cut-off effects were observed at G1 and G16. The Fenton-ApoCAT assay with Fe^{2+} was selected for a further study with protocatechuic acid and its esters.

As shown in Figure 10 and 11, one cut-off effect was observed in both Fenton-CAT and Fenton-ApoCAT assays. Butyl protocatechuate (PC4) exhibited the highest Fenton-CAT value, while hexyl protocatechuate (PC6) had the highest Fenton-ApoCAT value.

Interestingly, it seems like that not only could antioxidant polarities and radical exchange dynamics in terms of free radical distributions, but also the dynamic exchanges of antioxidants among phases in the system influence on overall antioxidant behaviors. It was hypothesized that protocatechuic acid and its esters might be exchanged among phases faster than gallic acid and its esters due to smaller molecules.

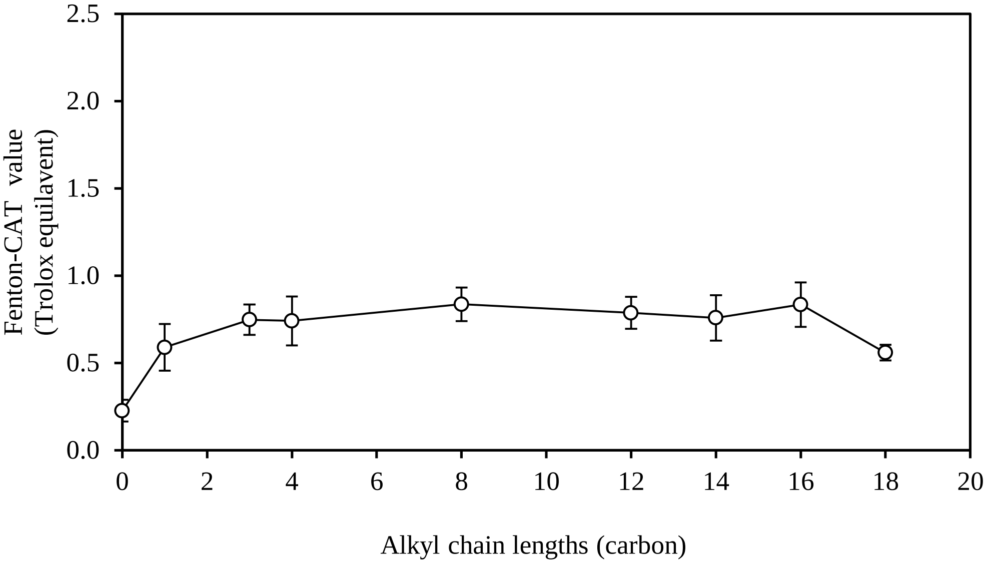

Figure 6 Antioxidant capacities of gallic acid and its esters in Fenton-CAT assay using H_2O_2 (7 mM) and $FeSO_4$ (14 µM)

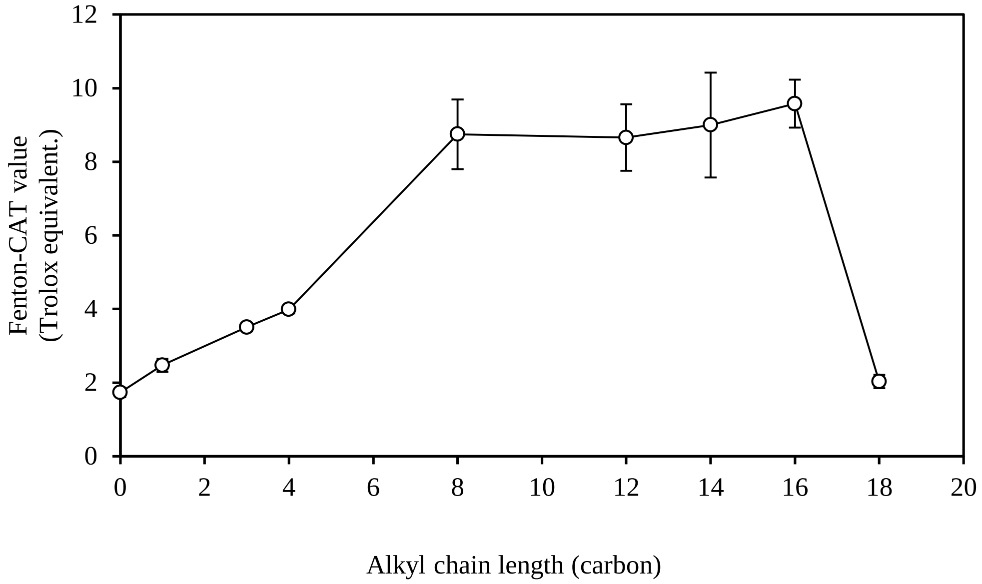

Figure 7 Antioxidant capacities of gallic acid and its esters in Fenton-CAT assay using H_2O_2 (7 mM) and CoF_2 (14 µM)

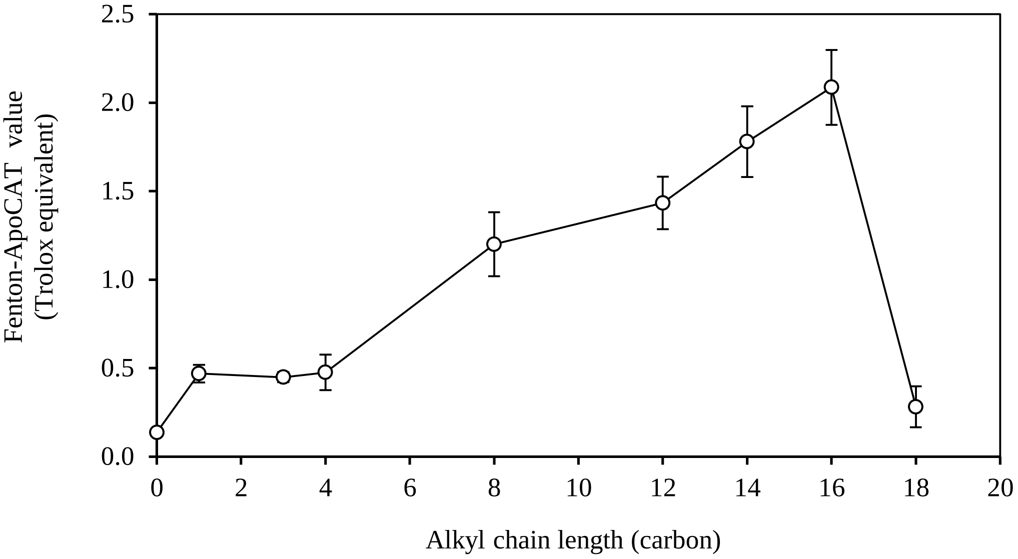

Figure 8 Antioxidant capacities of gallic acid and its esters in Fenton-CAT assay using cummene hydroperoxide (7 mM) and $FeSO_4$ (115 μM)

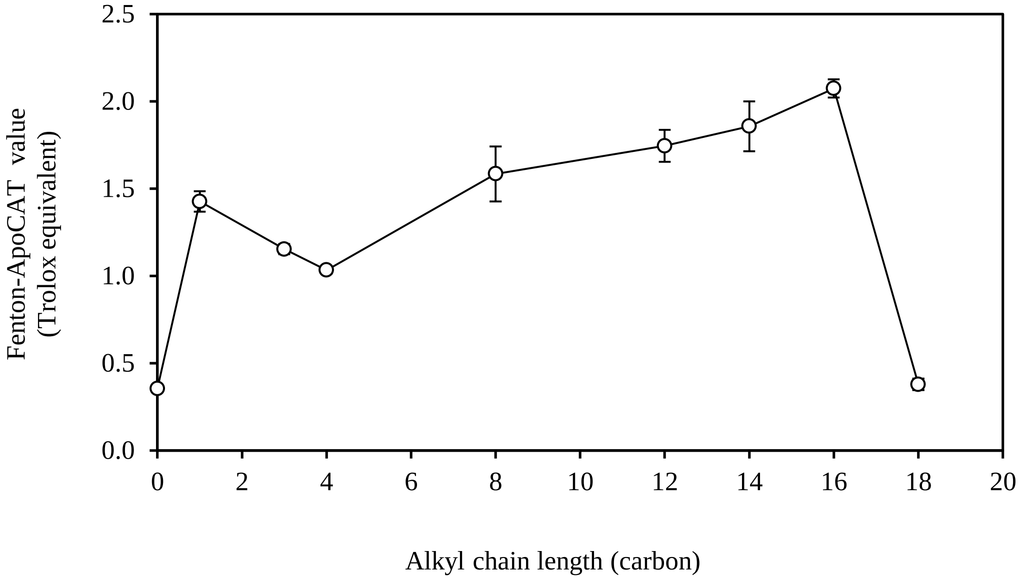

Figure 9 Antioxidant capacities of gallic acid and its esters in Fenton-CAT assay using cummene hydroperoxide (7 mM) and CoF_2 (115 μM)

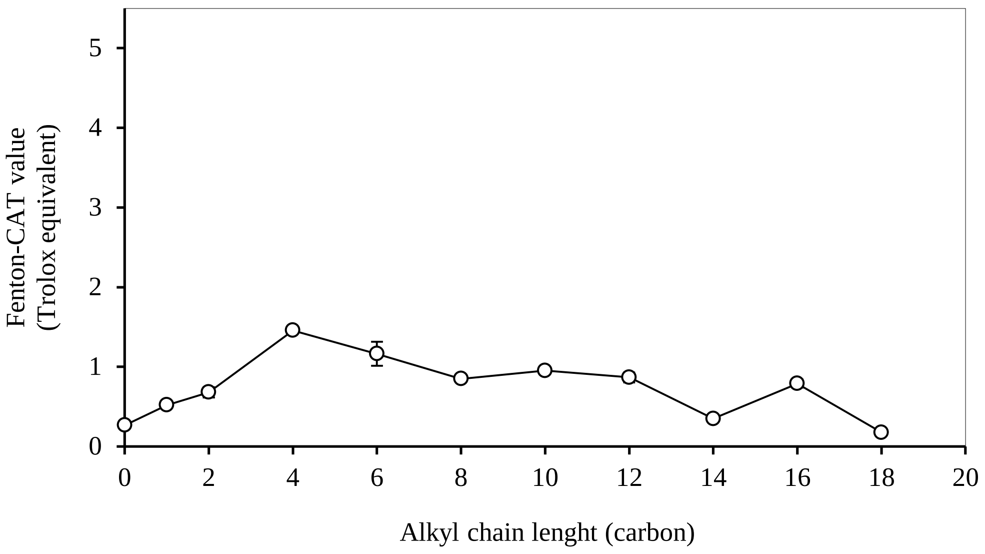

Figure 10 Antioxidant capacities of protocatechuic acid and its esters in Fenton-CAT assay using H_2O_2 (7 mM) and $FeSO_4$ (14 μM)

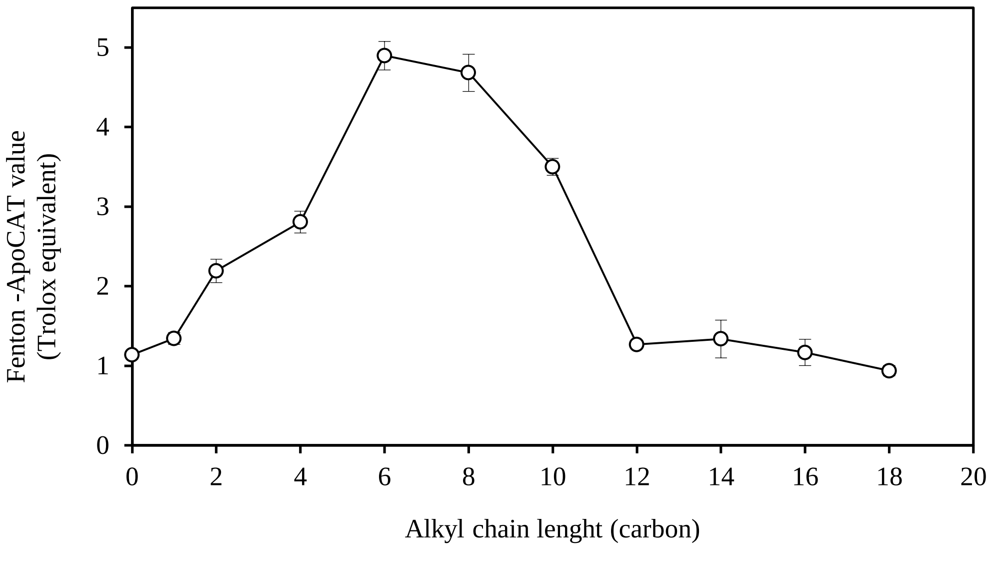

Figure 11 Antioxidant capacities of protocatechuic acid and its esters in Fenton-CAT assay using cummene hydroperoxide (7 mM) and $FeSO_4$ (115 μM)

6. Performances of phenolipids in several food models

6.1. Antioxidant activities of gallic acid and its alkyl esters in homogeneous antioxidant assays

According to the homogeneity of mediums, the TPC, ABTS, FRAP and ORAC assays representing examples of homogeneous antioxidant assays have been commonly used in many research areas to evaluate the ability of compounds to donate hydrogen atoms and/or electrons. As shown in Table 3, antioxidants performing in the non-competitive assays (the TPC, ABTS and FRAP assays) exhibited a similar behavioral pattern that increasing alkyl chain lengths of a free acid form of gallic acid (G0) to methyl gallate (G1) dramatically decreased the antioxidant activity. Further decreases in the antioxidant activities were observed when alkyl chain lengths were increased. Gallic acid had the highest TPC, ABTS and FRAP values following by methyl gallate (G1), propyl gallate (G3), butyl gallate (G4), octyl gallate (G8) and lauryl gallate (G12), respectively.

However, in the ORAC assay, increases in alkyl chain lengths from G0 to G3 improved the antioxidant activity as indicated by the ORAC value. Propyl gallate (G3) exhibited significantly higher ORAC value than gallic acid (G0). This result was consistent with the previous study by Alamed and co-workers that propyl gallate exhibited higher ORAC value than gallic acid. It should be noted that further increases in alkyl chain lengths beyond G3, antioxidant activities decreased as shown in Table 3.

Since the ABTS and FRAP assays were performed in aqueous solutions the antioxidant values of gallate esters decreased according to their alkyl chain length. This phenomenon could be explained that the polarities of gallate alkyl esters were decreased resulting in their lower solubility in the aqueous system. Thus, the reduction of solubility of gallate alkyl ester could influence their ability to scavenge free radical as compare to gallic acid. Moreover, the gallate alkyl esters could be self-aggregated by the hydrophobic effect as explained by self-assembly of the nonpolar substances in aqueous or polar solvents. The aggregation of gallate alkyl esters might occur as micelles, lamellar structures and other association colloids.

With the increasing of alkyl chain lengths, G1, G3 and G4 exhibited higher ORAC values than G0. This result might be explained by the esterification reaction increasing the radical scavenging activity of substances, which was consistent to the study of Lu and co-workers (Lu et al., 2006). However, the ORAC value decreased at G8 and observed lowest at G12, it might be due to the aggregation of lipophilic molecules in water phase, which may decrease the solubility of antioxidants resulting in lower antioxidant activities.

Table 3 Antioxidant activities of gallic acid and its alkyl esters using homogeneous antioxidant evaluation assays

Antioxidants	TPC (Gallic acid equivalent)	ABTS (Ascorbic acid equivalent)	FRAP (Ascorbic acid equivalent)	ORAC (Trolox equivalent)
Gallic acid (G0)	1^{*d}	2.91 ± 0.14^{d}	2.25 ± 0.03^{d}	1.53 ± 0.17^{c}
Methyl gallate (G1)	0.091 ± 0.00^{c}	1.08 ± 0.04^{c}	0.73 ± 0.01^{c}	1.76 ± 0.20^{cd}
Propyl gallate (G3)	0.087 ± 0.01^{c}	1.09 ± 0.01^{c}	0.77 ± 0.04^{c}	1.88 ± 0.17^{d}
Butyl gallate (G4)	0.088 ± 0.00^{c}	0.98 ± 0.02^{c}	0.73 ± 0.03^{c}	1.78 ± 0.13^{cd}
Octyl gallate (G8)	0.060 ± 0.01^{b}	0.65 ± 0.93^{b}	0.56 ± 0.03^{b}	1.23 ± 0.11^{b}
Dodecyl gallate (G12)	0.055 ± 0.00^{a}	0.09 ± 0.00^{a}	0.30 ± 0.00^{a}	0.32 ± 0.04^{a}

$^{a\text{-}d}$ Means within a column with different letters are significantly different ($p<0.05$).

The * indicates the value of approximately 1 due to the antioxidant was used as the standard.

6.2. Antioxidant activity of gallic acid and its alkyl esters in heterogeneous antioxidant assays

The conjugated autoxidizable triene (CAT) assay has been developed to determine the antioxidant activity in oil-in-water emulsions. Since the CAT assay is conducted in oil-in-water emulsion, the locations of antioxidant in the system have to be accounted for their antioxidative performances. According to the polar paradox hypothesis, the non-polar antioxidant is more active in emulsified systems. Results showed that butyl gallate (G4) exhibited the highest value of the CAT assay followed by propyl gallate (G3), methyl gallate (G1), lauryl gallate (G12), octyl gallate (G8) and gallic acid (G0), respectively (Table 4). According to this result, it was inconsistent with the polar paradox hypothesis. The non-linear trend was observed after increased the alkyl chain length to G4. Interestingly, the antioxidant activity of alkyl gallate esters was sharply dropped at G8 before increasing again at G12. According to the AAPH (water soluble radical) used in the CAT assay, it was hypothesized that an overestimation of the antioxidant capacity of water soluble antioxidants, and on the contrary, an underestimation of the antioxidant performance of a lipid soluble antioxidant may be observed. Therefore, the ApoCAT assay, a modified version of the CAT assay, has been developed. Instead of using AAPH, a lipid soluble radical initiator called V601or AIBME was used to initiate oxidation reaction. As illustrated in Table 4, results indicated that lauryl gallate (G12) exhibited the highest ApoCAT and Fenton-ApoCAT values followed by octyl gallate (G8), butyl gallate (G4), propyl gallate (G3), methyl gallate (G1) and gallic acid (G0). Unlike the CAT assay, the linear trends between antioxidant values and alkyl chain lengths were observed in the ApoCAT and Fenton-ApoCAT assays.

Table 4 Antioxidant activities of gallic acid and its alkyl esters using heterogeneous antioxidant evaluation assays

Antioxidants	CAT (Trolox equivalent)	ApoCAT (Trolox equivalent)	Fenton-CAT (Trolox equivalent)	Fenton-ApoCAT (Trolox equivalent)
Gallic acid (G0)	0.64±0.08^{a}	0.28±0.03^{a}	0.23±0.06^{a}	0.14±0.01^{a}
Methyl gallate (G1)	1.65±0.15^{c}	0.76±0.09^{b}	0.59±0.13^{b}	0.47±0.05^{b}
Propyl gallate (G3)	1.84±0.10d^{e}	1.16±0.25^{c}	0.75±0.09^{c}	0.45±0.03^{c}
Butyl gallate (G4)	1.98±0.06^{e}	1.62±0.17^{d}	0.74±0.14^{d}	0.48±0.10^{d}
Octyl gallate (G8)	1.26±0.11^{b}	2.4±0.14^{e}	0.84±0.10^{e}	1.20±0.18^{e}
Dodecyl gallate (G12)	1.69±0.15^{cd}	3.26±0.17^{f}	0.79±0.09^{f}	1.43±0.15^{f}

$^{a-f}$ Means within a column with different letters are significantly different ($p<0.05$).

6.3. Antioxidative performances of gallic acid and its alkyl esters in oil-in-water (O/W) emulsions

The performances of gallic acid and its alkyl esters to prevent lipid oxidation in oil-in-water (O/W) emulsions were evaluated using the induction periods obtained from the formation of primary lipid oxidation products (peroxide value or PV value) and secondary lipid oxidation products (thiobarbituric acid reactance or TBAR value). As shown in Table 5, the induction periods were estimated from the end of lag periods at which the PV and TBAR values were statistically increased from the previous values. The induction periods calculated based on the PV and TBAR values of O/W emulsion treated with 50 µM of gallic acid (G0), methyl gallate (G1), propyl gallate (G3), butyl gallate (G4), octyl gallate (G8) and lauryl gallate (G12). The linear trend of antioxidative performance in O/W emulsions was observed according to the alkyl chain lengths. This observation could be explained by the polar paradox hypothesis that the non-polar antioxidants are more active than their polar homologues in O/W emulsion systems.

Table 5 Oxidative stabilities of oil-in-water emulsions (O/W) treated with the addition of gallic acid and its alkyl esters at 50 µM. Results were expressed as the induction period (IP) of peroxide (PV) and thiobarbituric acid reactance (TBAR) values

Antioxidants	Induction period (day)*	
	PV	TBAR
Gallic acid (G0)	3	3
Methyl gallate (G1)	4	4
Propyl gallate (G3)	5	5
Butyl gallate (G4)	6	6
Octyl gallate (G8)	8	8
Dodecyl gallate (G12)	9	9

* Data obtained from 3 individual replicate samples

6.4. Antioxidative performances of gallic acid and its alkyl esters in bulk soybean oils

Bulk soybean oil was used as the bulk oil model to evaluate the antioxidant performances of gallic acid and its alkyl esters. In bulk oil, lipid oxidation mechanisms are different from emulsified system due to many factors[1]. It was hypothesized that polar antioxidants are more effective in bulk oil than their non-polar homologues.

The antioxidative performances in bulk soybean oil of gallic acid (G0), methyl gallate (G1), propyl gallate (G3), butyl gallate (G4), octyl gallate (G8) and lauryl gallate (G12) were evaluated using Rancimat test. The increasing of conductivity by trapping lipid oxidation products in water was monitored kinetically. Result showed that G0 exhibited the highest induction time followed by G4, G8, G12, G3 and G1, respectively (Figure 12).

The result was consistent to the polar paradox hypothesis that gallic acid (polar homologue) was the most effective antioxidant in bulk soybean oil compared to its non-polar homologues (gallate esters). However, increasing alkyl chain length to G3, the performances of gallate esters in bulk soybean oil were increased. As illustrated in Figure 12, the result indicated that antioxidant performances of gallic acid and its alkyl esters in bulk soybean oil were depended regardless of the polarities. This result was in agreement with the study on chlorogenic acid and its alkyl esters which hydrophobicity did not exert a strong influence on antioxidant capacity. It is worth mentioned that there might be some other minor components and water remained in soybean oil. Thus, the association colloids could form in bulk soybean oil resulting in the high susceptibility of bulk soybean oil to oxidation reaction. This result could give a good provide that not only the intrinsic factors such as hydrophobicity of antioxidant influences on the antioxidant capacity but also the extrinsic factors of environmental system could exert the high influencing of the antioxidant capacity.

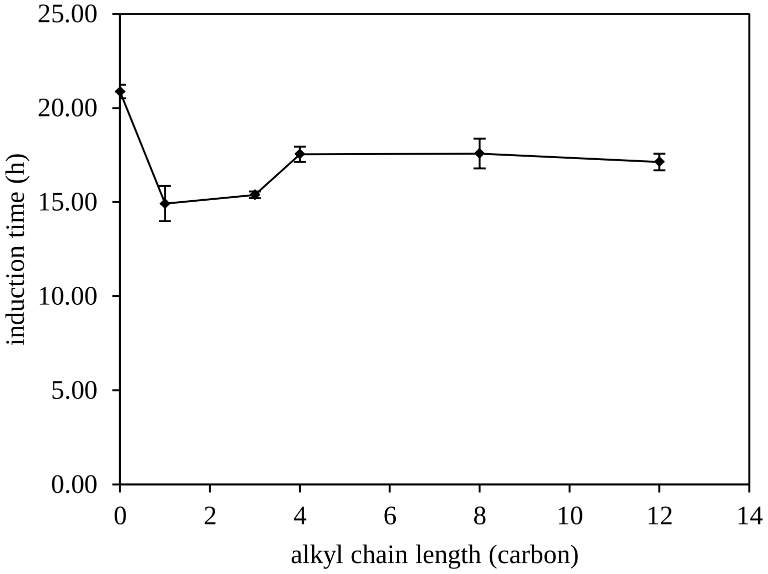

Figure 12 Oxidative induction times of bulk soybean oils treated with gallic acid and its alkyl esters at 2 mmoles per kilogram of sample using the Rancimat test at 110 °C with airflow rate of 20 L/h

6.5. Antioxidative performances of gallic acid and its alkyl esters in roasted peanuts

According to the studies of antioxidative performances of gallic acid and its alkyl esters in oil-in-water emulsions and bulk soybean oil, the results showed that the antioxidant ability of substance to protect lipid oxidation was not depended on the only intrinsic factors of antioxidant. However, the extrinsic factors such as the nature of food system also play the key role on the antioxidative performances. To better understand the antioxidative performances of substances in various food models, roasted peanut was chosen as a low moisture food containing lipid model.

The antioxidative performances of gallic acid and its alkyl esters were determined in roasted peanut using Rancimat test. Roasted peanuts treated with lauryl gallate (G12) exhibited the highest induction time followed by octyl gallate (G8), butyl gallate (G4), methyl gallate (G1), propyl gallate (G3) and gallic acid (G0), respectively (Figure 13).

In recent, the information on the antioxidant effectiveness in low moisture food is limited (Barden 2014). This result suggested that the antioxidant effectiveness in roasted peanuts increased with increasing the hydrophobicity of antioxidants. Results were in agreement with the study of rosmarinic acid and its alkyl esters in cracker that similar to gallic acid, rosmarinic acid exhibited the lowest antioxidant efficacy to inhibit lipid oxidation in low moisture foods. The reason for these results might be due to the exclusion of polar antioxidants from the lipid phase resulting in the separation of antioxidants from lipid oxidation reaction site[24]. On the contrary, increasing the hydrophobicity of antioxidant would make antioxidants more capable to migrate to lipid oxidation site thus the more effectiveness to prevent lipid oxidation were observed in low moisture food.

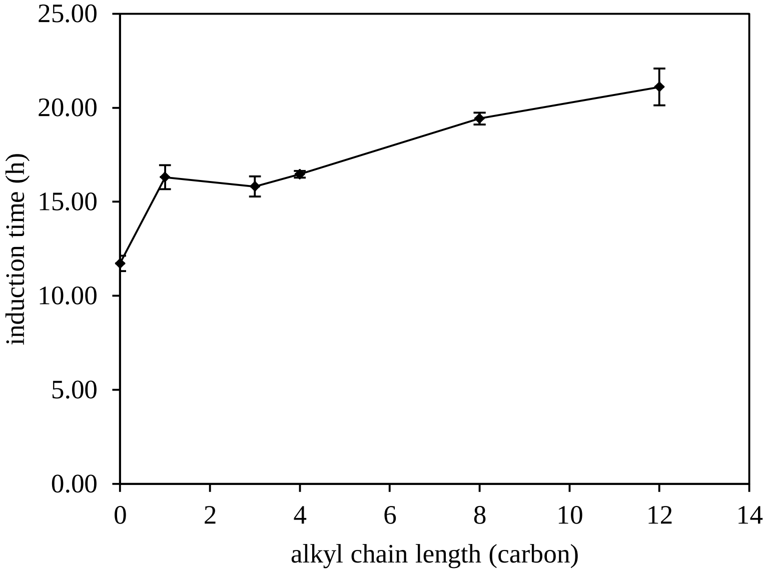

Figure 13 Oxidative induction times of roasted peanut treated with gallic acid and its alkyl esters at 2 mmoles per kilogram of sample using the Rancimat test at 110 °C with airflow rate of 20 L/h

6.6. Relationship between antioxidant activities in various antioxidant assays and antioxidative performances in food models of gallic acid and its alky esters

The Relationship between antioxidant activities in various antioxidant assays and antioxidant performances in different food matrices of gallic acid and its alky esters were investigated using principle component analysis (PCA). As illustrated in Figure 14A, from ten parameters, they could be reduced into two components (PC1 and PC2) explaining 95.40 % of total variance. Each component was responsible for 52.38% and 43.02%, respectively. The PC1 represented the Fenton-ApoCAT value, ApoCAT value, induction periods from PV and TBAR values of oil-in-water emulsions and the oxidative stability of roasted peanuts (IT_RP), while the PC2 explained the TPC, ABTS and FRAP values, and the oxidative stability of bulk soybean oils (IT_SBO). The PCA score plot of gallic acid and it alkyl esters as shown in Figure 14B suggested that lauryl gallate (G12) and octyl gallate (G8) were strongly explained by the assay in PC1. In contrast, gallic acid was highly correlated with the assays categorizing in PC2. However, methyl gallate (G1), propyl gallate (G3) and butyl gallate (G4) were intermediate correlate with the assays in both PC1 and PC2.

The results were consistent with previous studies that the ORAC assay was not related to the TPC, ABTS and FRAP assays. However, some studies had shown the good correlation between the ORAC assay and others assays (the TPC, DPPH and FRAP assays). Although, the good relationship of the ORAC assay and others were found in some experiments, many researchers suggested that when antioxidants were different kinds, the correlation between the ORAC and other methods would be low because of the different kinetics and reaction mechanisms of the various antioxidants present.

Furthermore, the results were in agreement with the study on relationship between radical scavenging activity and antioxidant activity in foods which reported that the ORAC assay was not able to predict the antioxidant activity of compounds in oil-in-water emulsions and cook ground beef. The lack of relationship between antioxidant activities obtained from homogeneous assay such as TPC, ABTS, FRAP and ORAC and antioxidant activities of compound in food might be due to the complexity of food. Thus, food matrices are the important part providing the multitude factors influencing on the ability of compound to inhibit lipid oxidation in food system.

Recently, the CAT assay has been developed to evaluate the antioxidant activity of substances in a heterogeneous system (O/W emulsions). However, our results showed that the CAT assay was not able to predict antioxidant performance in all three types of food systems (O/W emulsions, bulk soybean oils and roasted peanuts). The CAT and Fenton-CAT assays could not predict the actual antioxidant activity in foods due to the unrealistic oxidation reaction occurring in the tested systems. The CAT assay used AAPH which is water soluble radical initiator to initiate lipid oxidation thus making the radical locating in water phase of emulsion. From this reason, it is unlike lipid oxidation in complex food systems in which lipid oxidation occurs at the oil/water interface regions of emulsion droplets[1]. To solve this problem, the ApoCAT assay has been developed to use the lipid soluble radical initiator instead of AAPH (a water soluble radical initiator). According to the lipid soluble radical initiator in the Fenton-ApoCAT and ApoCAT systems, the lipid oxidation could occur at the interphase of water-oil droplets, and thus the phenomenon might relate to lipid oxidation in actual food systems.

Interestingly, as illustrated in Figure 14A, our results exhibited a close relationship among the Fenton-ApoCAT/ApoCAT assays with the induction periods of PV and TBAR formation in oil-in-water emulsions and the oxidative stability of roasted peanuts (IT_RP). However, the Fenton-ApoCAT and ApoCAT assays had a poor correlation with the oxidative stability of bulk soybean oils (IT_SBO). This result could be explained that lipid oxidation mechanisms in bulk oil systems might be different from emulsion systems.

From the PCA score plot (Figure 14B), results suggested that high polar antioxidant (G0) exhibited high antioxidant values in non-competitive homogeneous assays while non-polar antioxidant (G8 and G12) exhibited high antioxidant value in heterogeneous assays. In addition, the correlations among all tested methods were calculated and showed that the Fenton-ApoCAT and ApoCAT assays were significantly correlated with the oxidative induction periods of oil-in-water (O/W) emulsions (PV and TBAR) and the oxidative induction times of roasted peanuts (IT_RP) (data not shown).

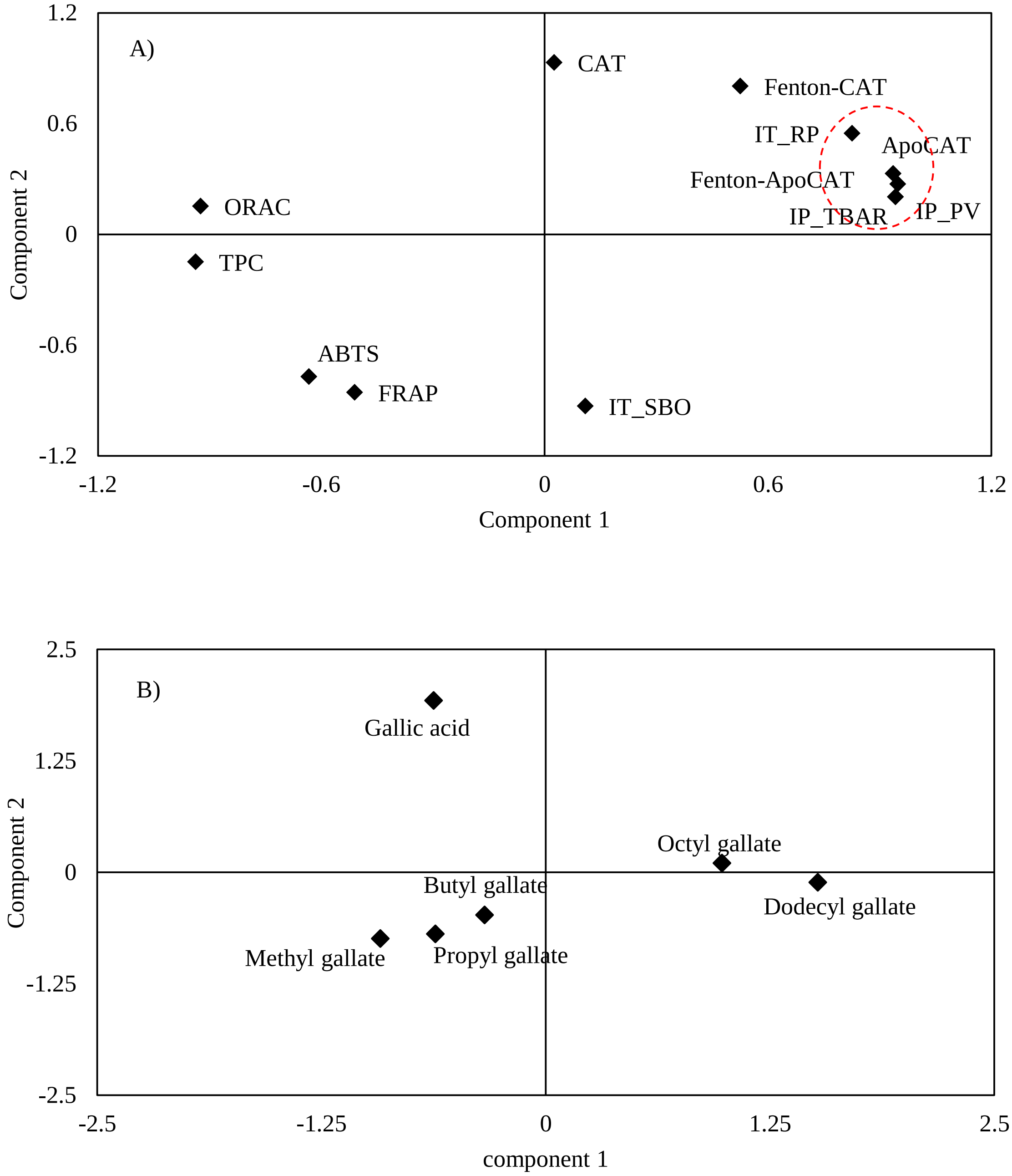

Figure 14 PCA loading (a.) and score (b.) plots obtained from total phenolic content (TPC assay), antioxidant activities (ABTS, FRAP, ORAC, CAT, Fenton-CAT, ApoCAT, Fenton-ApoCAT assays), the oxidative induction periods of PV and TBAR formation in oil-in-water emulsions, and the oxidative induction time of bulk soybean oils (IT_SBO) and roasted peanuts (IT_RP)

7. Comparison of antioxidant interactions in various models

7.1. Study on the antioxidant interactions in the CAT assays

In this study, two types of antioxidant combinations were used to investigate the antioxidant interactions influenced by different distribution of radicals in the CAT assay. Antioxidant capacities and interactions between gallic acid (G0) and tetradecyl gallate (G14) at different combination ratios were shown in Figure 15 and 16.

As indicated by the interaction index, the combination at higher ratios of the water-soluble antioxidant (G0) to the lipid-soluble antioxidant (G14) exhibited strong the synergistic effect. Decreases in the ratio of gallic acid in the mixture resulted in the reduction of synergistic effect to additive effect.

It was hypothesized that the distribution of radicals and antioxidants in the CAT assay could be an important factor impacting on antioxidant interactions. In the CAT assay, water-soluble radicals were firstly distributed in aqueous phase. Then, these radicals initiated lipid oxidation in O/W emulsions resulting the formation of lipids soluble radicals. Thus, the major radicals could be in the aqueous phase, and the minor radicals might be in the lipid phase.

Results demonstrated that the water-soluble antioxidant (G0) could inactivate the water-soluble radicals in the aqueous phase. As the result, the oxidation rate of O/W emulsions could be decreased indirectly without the inhibition of lipid oxidation by inactivating lipid-soluble radicals in the emulsion droplets. On the other hands, lipid-soluble antioxidants (G14) could neutralize the radicals mainly in lipid phase.

In the case of antioxidants with the similar polarity (G12/G14), as shown in Figure 17 and 18, additive effects were observed. This phenomenon could be explained by the similarity of mechanisms in order to inactivate radicals in the systems.

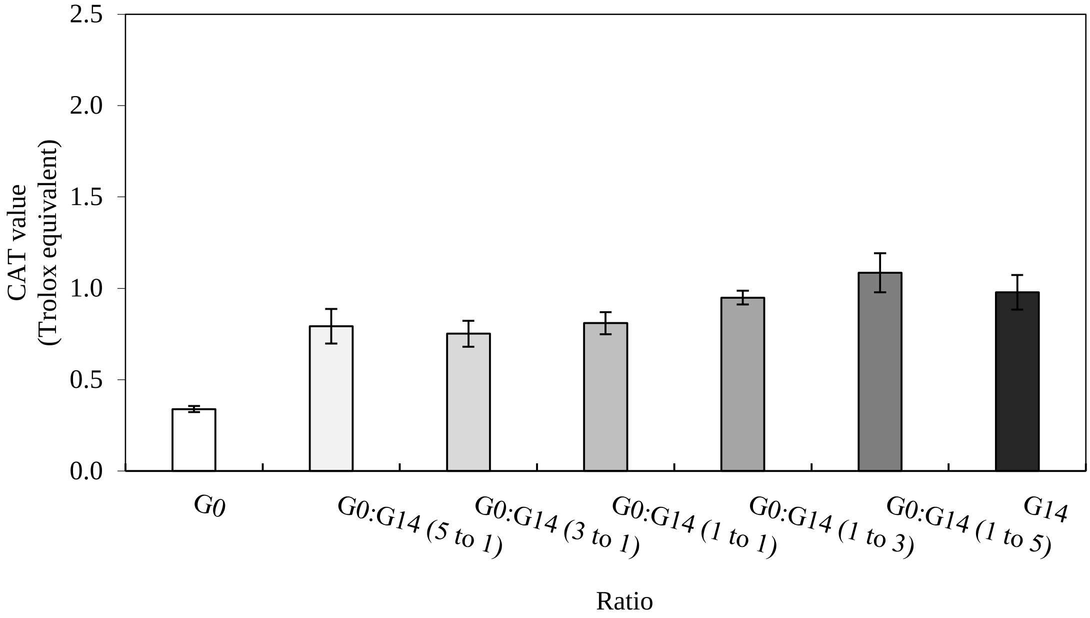

Figure 15 Antioxidant capacities between gallic acid (G0) and tetradecyl gallate (G14) in the CAT assay at different combination ratios (mole/mole)

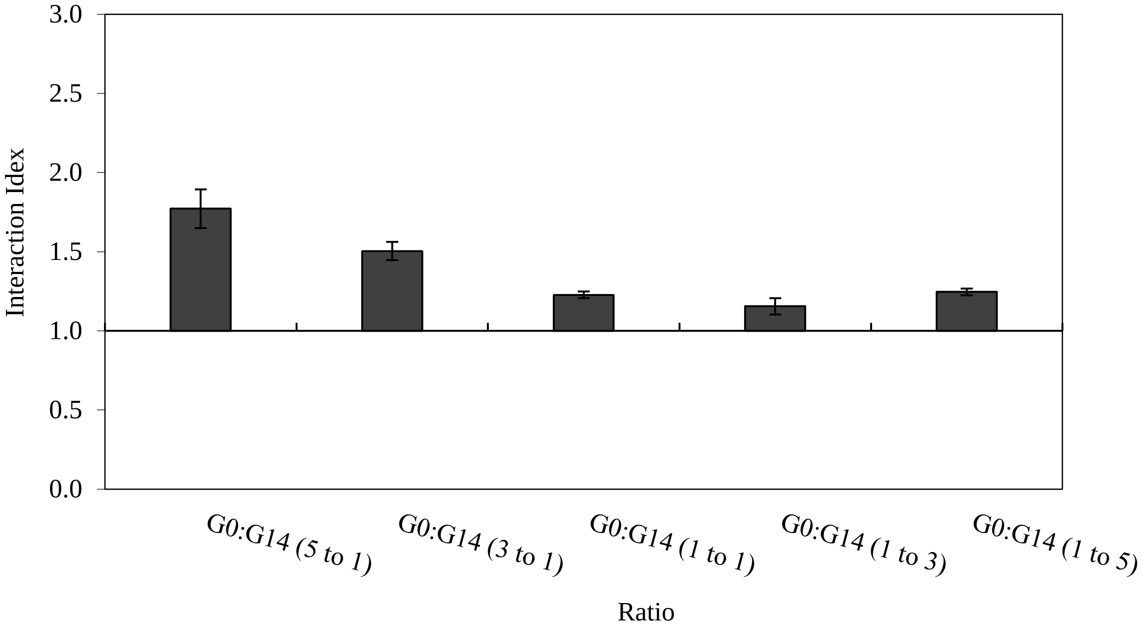

Figure 16 Antioxidant interactions between gallic acid (G0) and tetradecyl gallate (G14) in the CAT assay at different combination ratios (mole/mole)

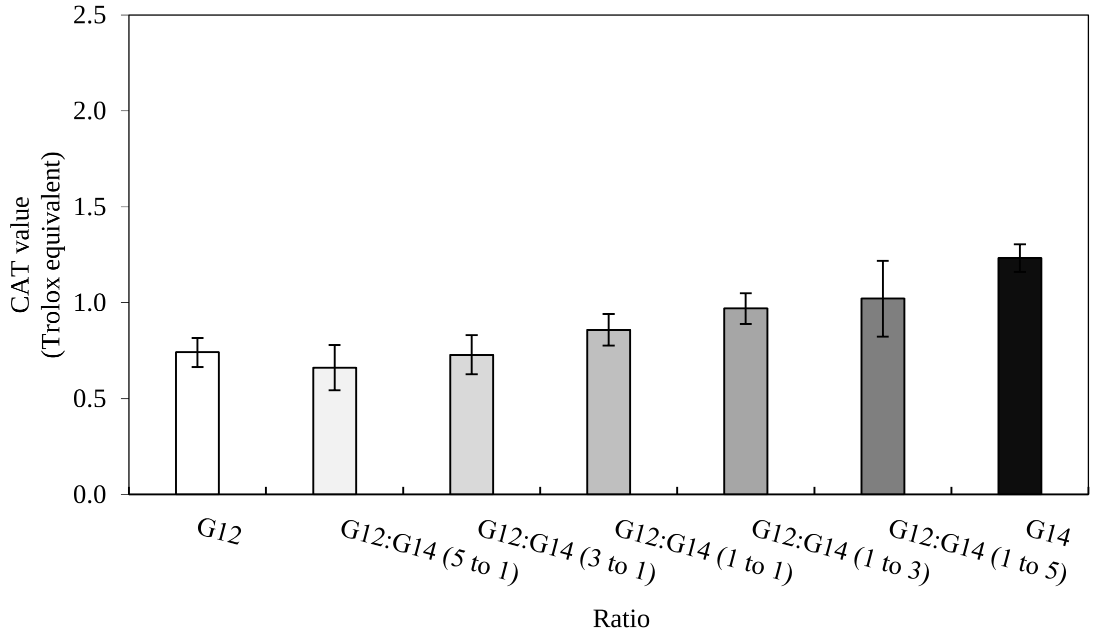

Figure 17 Antioxidant capacities between dodecyl gallate (G12) and tetradecyl gallate (G14) in the CAT assay at different combination ratios (mole/mole)

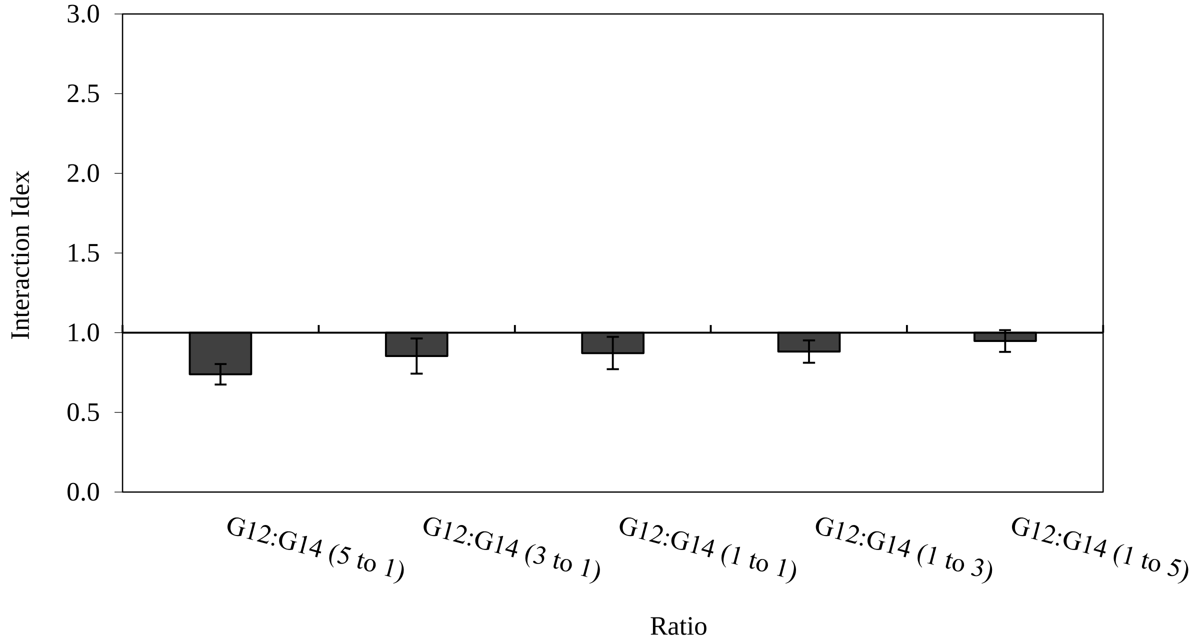

Figure 18 Antioxidant interactions between dodecyl gallate (G12) and tetradecyl gallate (G14) in the CAT assay at different combination ratios (mole/mole)

7.2. Study on the antioxidant interactions in the ApoCAT assays

In this study, two types of antioxidant combinations were used to investigate the antioxidant interactions influenced by different distribution of radicals in the ApoCAT assay. Antioxidant capacities and interactions between gallic acid (G0) and tetradecyl gallate (G14) at different combination ratios were shown in Figure 19 and 20.

As indicated by the interaction index, increases in the ratios of the water-soluble antioxidant (G0) to the lipid-soluble antioxidant (G14) did not exhibit a strong impact on antioxidant interactions resulting approximately additive effects.

It was hypothesized that the distribution of radicals and antioxidants in the ApoCAT assay could be an important factor impacting on antioxidant interactions. In the ApoCAT assay, lipid-soluble radicals could be mainly distributed in lipid phases of both tung oil and MCT emulsions. Thus, the distribution of water phase radicals could be compromised.

In the case of antioxidants with the similar polarity (G12/G14), as shown in Figure 21 and 22, interestingly, increases in the ratio of G14 in the mixtures resulted in the increasing of the synergistic effects. However, it was unclear why increasing the ratio of G14 in the mixture could synergistically improve the antioxidant capacities of the mixtures.

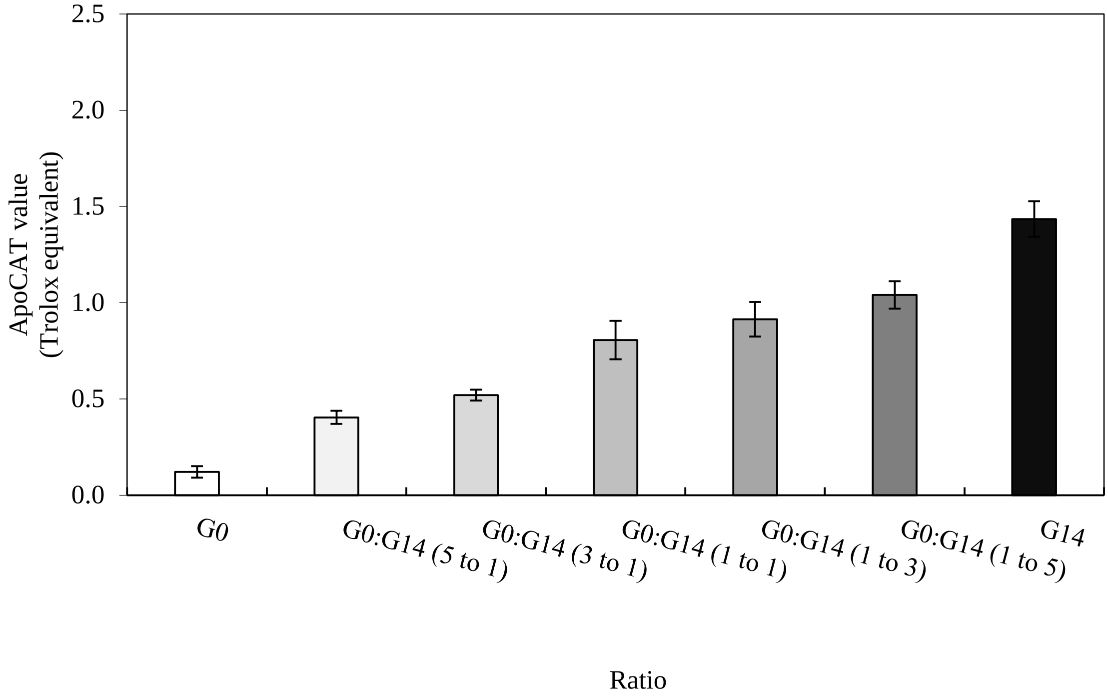

Figure 19 Antioxidant capacities between gallic acid (G0) and tetradecyl gallate (G14) in the ApoCAT assay at different combination ratios (mole/mole)

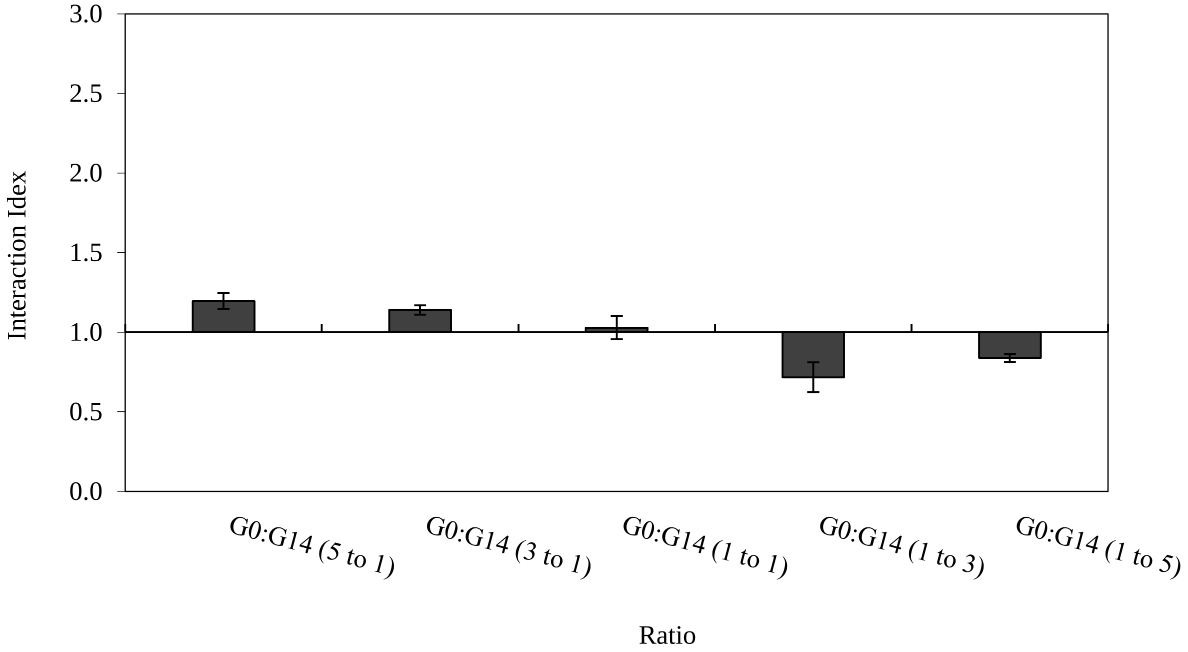

Figure 20 Antioxidant interactions between gallic acid (G0) and tetradecyl gallate (G14) in the ApoCAT assay at different combination ratios (mole/mole)

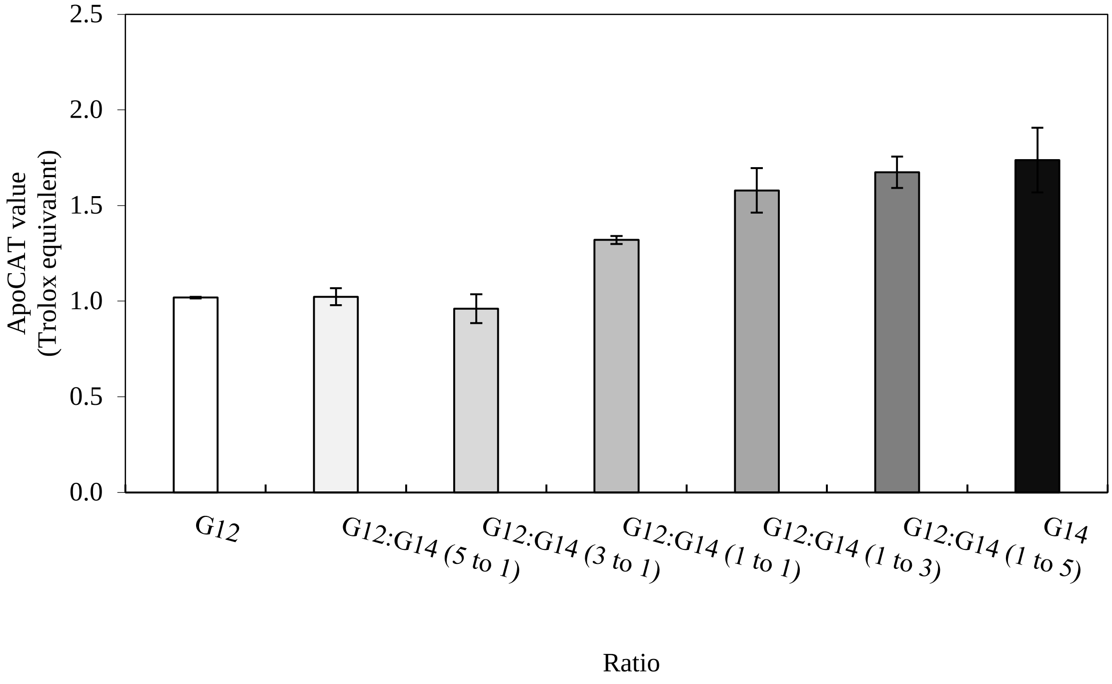

Figure 21 Antioxidant capacities between dodecyl gallate (G12) and tetradecyl gallate (G14) in the ApoCAT assay at different combination ratios (mole/mole)

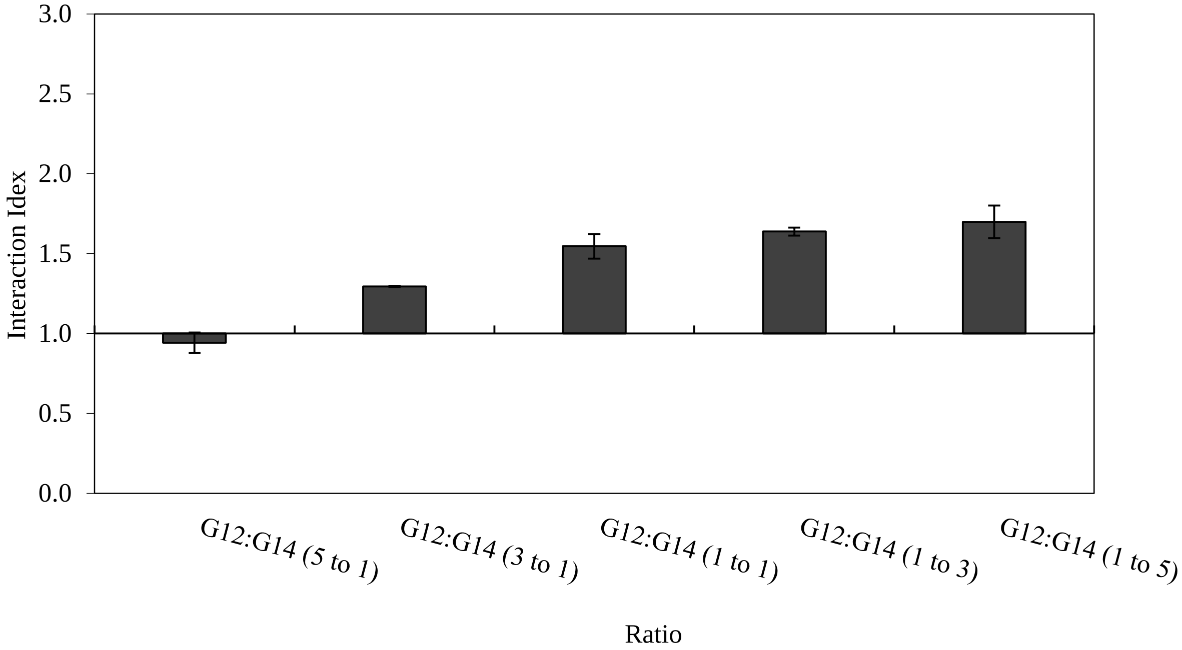

Figure 22 Antioxidant interactions between dodecyl gallate (G12) and tetradecyl gallate (G14) in the ApoCAT assay at different combination ratios (mole/mole)

7.3. Study on the antioxidant interactions in the Fenton-CAT assays

In this study, two types of antioxidant combinations were used to investigate the antioxidant interactions influenced by different distribution of radicals in the Fenton-CAT assay. Antioxidant capacities and interactions between gallic acid (G0) and tetradecyl gallate (G14) at different combination ratios were shown in Figure 23 and 24.

Similar to the results from the CAT assay, as indicated by the interaction index, the combination at higher ratios of the water-soluble antioxidant (G0) to the lipid-soluble antioxidant (G14) exhibited strong the synergistic effect. Decreases in the ratio of gallic acid in the mixture resulted in the reduction of synergistic effect to additive effects.

It was hypothesized that the distribution of radicals and antioxidants in the Fenton-CAT assay could be an important factor impacting on antioxidant interactions. In the Fenton-CAT assay, water-soluble radicals (hydroxyl radicals) were firstly distributed in aqueous phase. Then, these radicals initiated lipid oxidation in O/W emulsions resulting the formation of lipids soluble radicals (lipid hydroxyl radicals). Thus, the major radicals could be in the aqueous phase, and the minor radicals might be in the lipid phase.

Results demonstrated that the water-soluble antioxidant (G0) could inactivate the water-soluble radicals in the aqueous phase. As the result, the oxidation rate of O/W emulsions could be decreased without the inhibition of lipid oxidation by inactivating lipid-soluble radicals located in the emulsion droplets. On the other hands, lipid-soluble antioxidants (G14) could neutralize the radicals mainly in lipid phase.

In the case of antioxidants with the similar polarity (G12/G14), as shown in Figure 25 and 26, additive effects were observed similar to what found in the CAT assay. This phenomenon could be explained by the similarity of mechanisms in order to inactivate radicals in the systems.

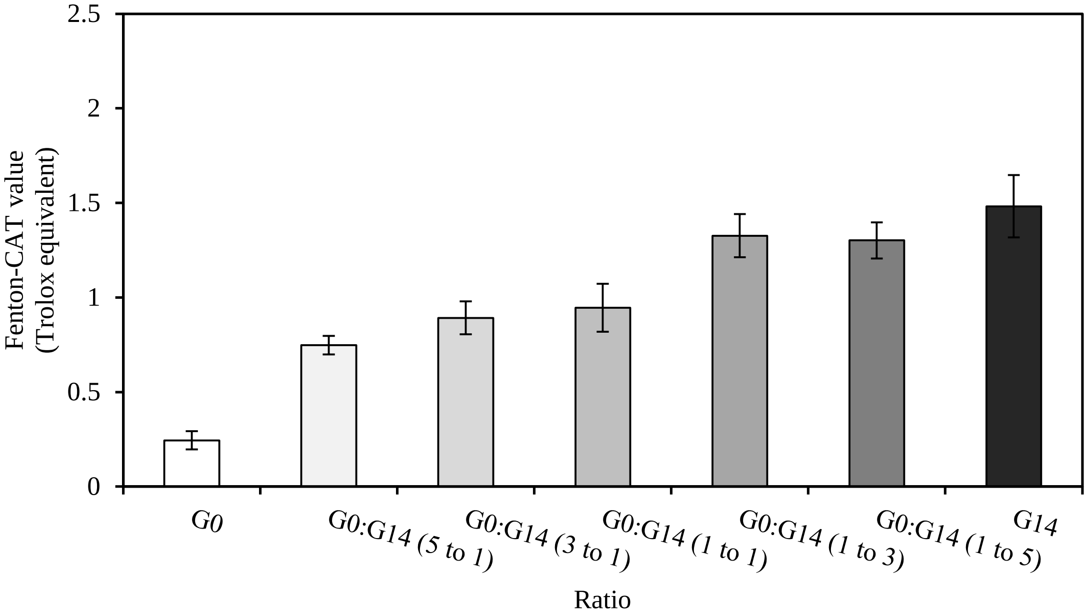

Figure 23 Antioxidant capacities between gallic acid (G0) and tetradecyl gallate (G14) in the Fenton-CAT assay at different combination ratios (mole/mole)

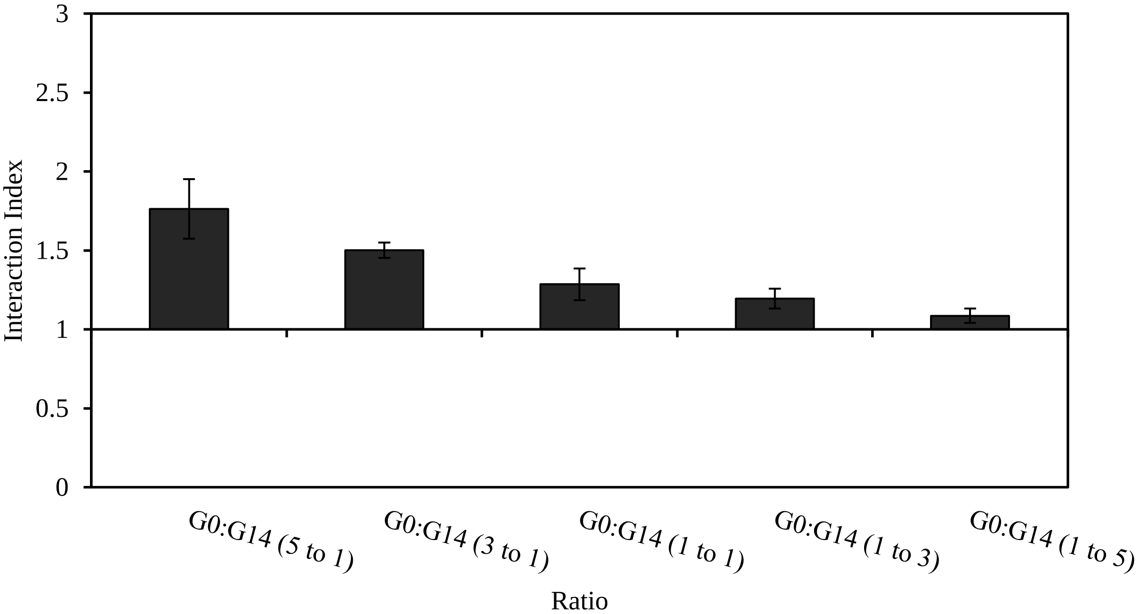

Figure 24 Antioxidant interactions between gallic acid (G0) and tetradecyl gallate (G14) in the Fenton-CAT assay at different combination ratios (mole/mole)

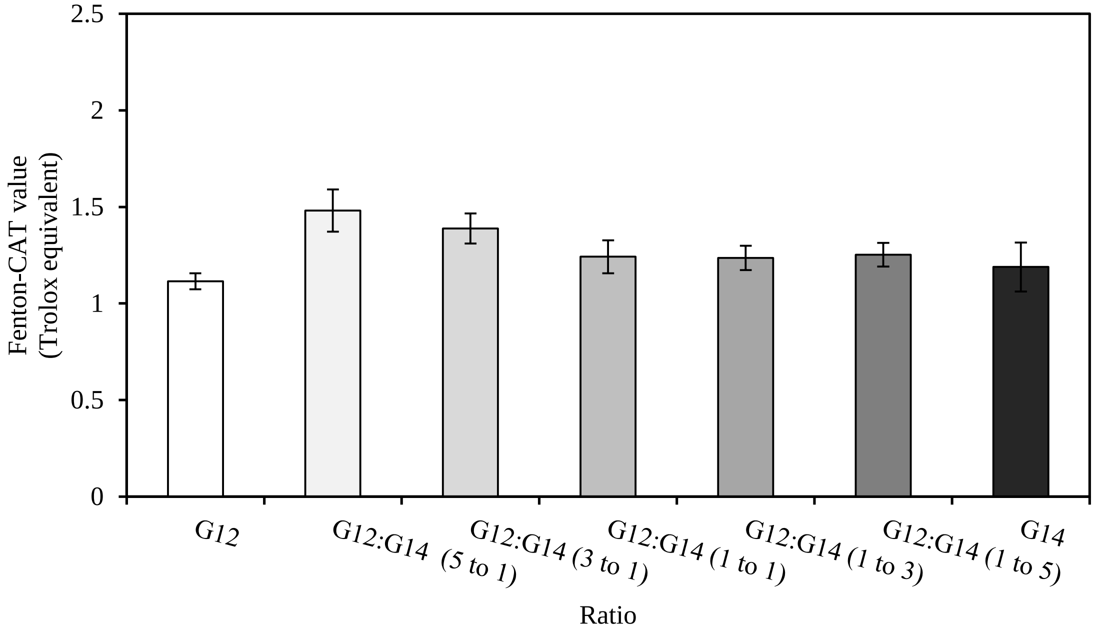

Figure 25 Antioxidant capacities between dodecyl gallate (G12) and tetradecyl gallate (G14) in the Fenton-CAT assay at different combination ratios (mole/mole)

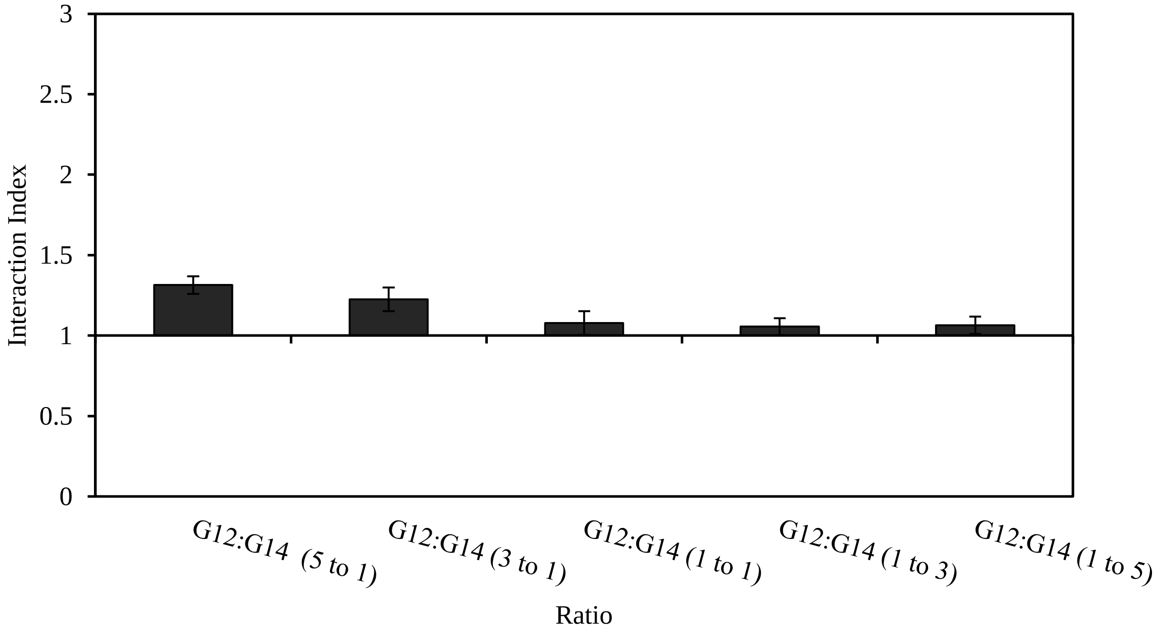

Figure 26 Antioxidant interactions between dodecyl gallate (G12) and tetradecyl gallate (G14) in the Fenton-CAT assay at different combination ratios (mole/mole)

7.4. Study on the antioxidant interactions in the Fenton-ApoCAT assays

In this study, two types of antioxidant combinations were used to investigate the antioxidant interactions influenced by different distribution of radicals in the Fenton-ApoCAT assay. Antioxidant capacities and interactions between gallic acid (G0) and tetradecyl gallate (G14) at different combination ratios were shown in Figure 27 and 28.

As indicated by the interaction index, increases in the ratios of the water-soluble antioxidant (G0) to the lipid-soluble antioxidant (G14) exhibited a strong antagonistic effect (5 to 1 ratio of G0 and G14). Further decreases in G0 in the mixture, additive effects were observed.

In this case, it was hypothesized that G0 might exhibit a prooxidative effect such as the reduction of Fe^{2+} to Fe^{3+}, which is the well-known prooxidative mechanism in the presence of metal ions such as Fe, Cu and Co ions.

In the case of antioxidants with the similar polarity (G12/G14), their interaction indexes were additive effects as shown in Figure 28 and 29.

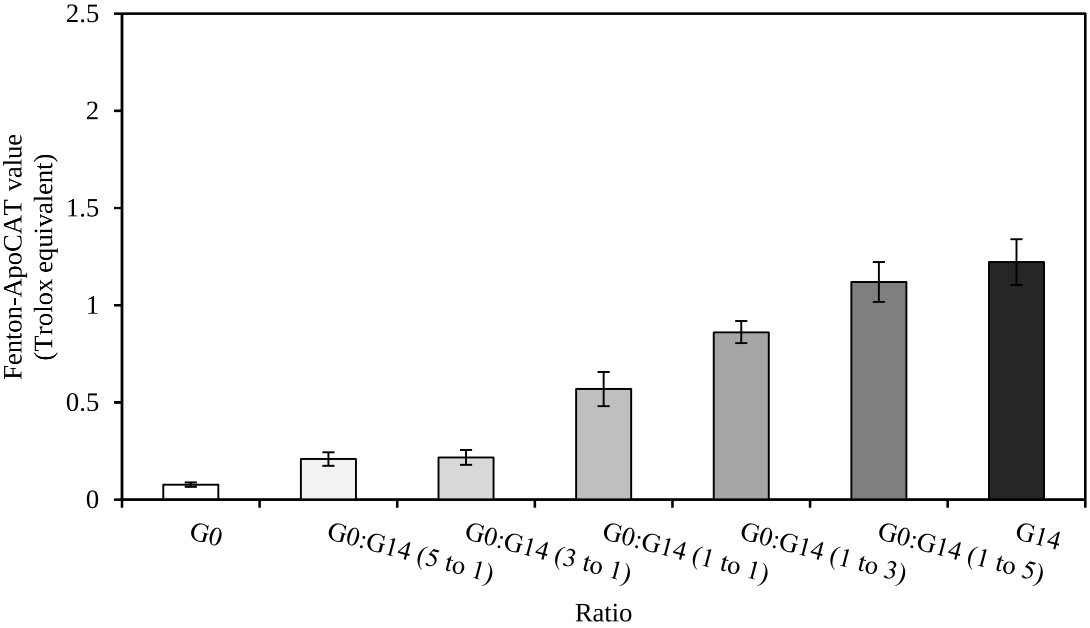

Figure 27 Antioxidant capacities between gallic acid (G0) and tetradecyl gallate (G14) in the Fenton-ApoCAT assay at different combination ratios (mole/mole)

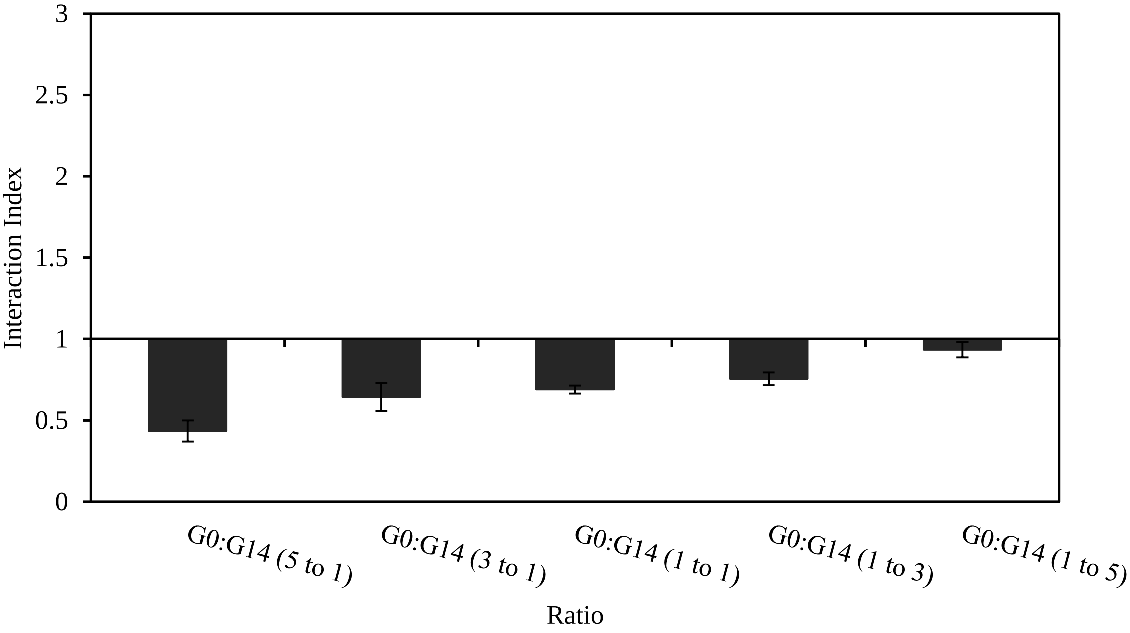

Figure 28 Antioxidant interactions between gallic acid (G0) and tetradecyl gallate (G14) in the Fenton-ApoCAT assay at different combination ratios (mole/mole)

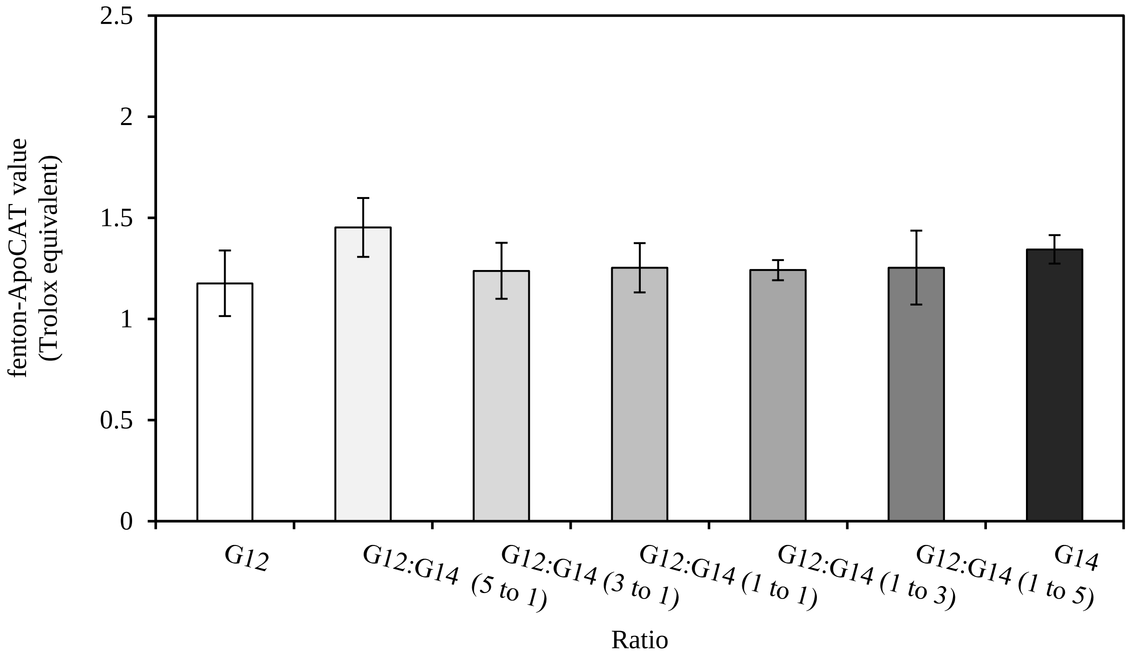

Figure 28 Antioxidant capacities between dodecyl gallate (G12) and tetradecyl gallate (G14) in the Fenton-ApoCAT assay at different combination ratios (mole/mole)

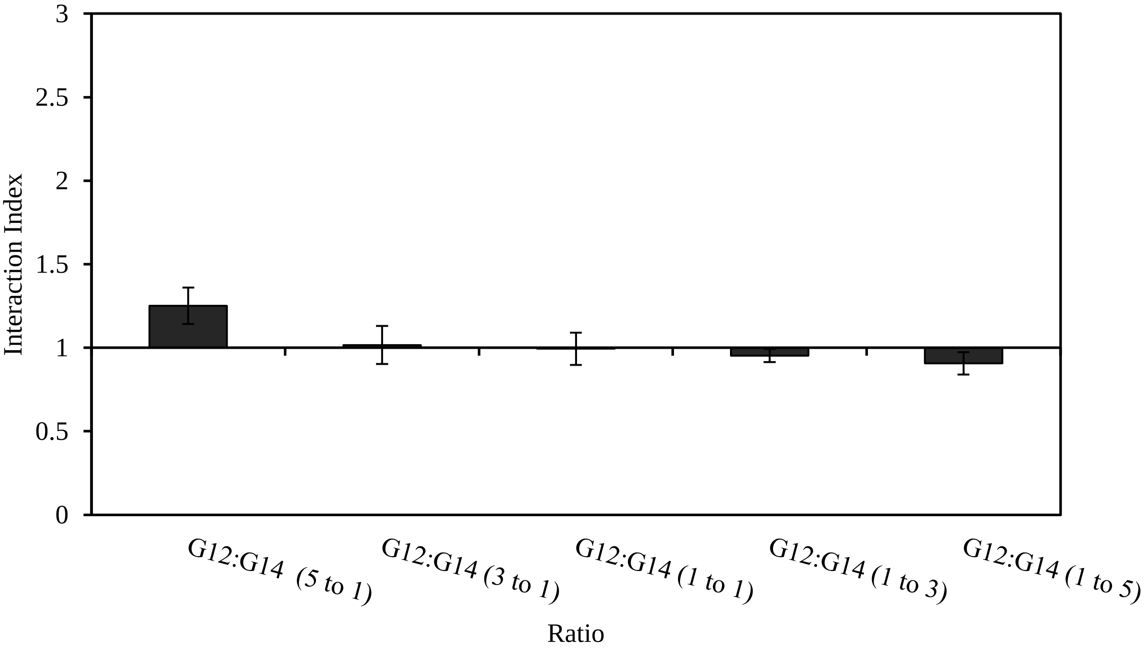

Figure 29 Antioxidant interactions between dodecyl gallate (G12) and tetradecyl gallate (G14) in the Fenton-ApoCAT assay at different combination ratios (mole/mole)

7.2 Study on antioxidant interaction in food emulsion models

Without adding radical initiators, lipid oxidation of O/W emulsions were performed in order to compare with the results obtained from the CAT and modified CAT assays. As illustrated in Figure 30A, the combination of G0/G14 samples at different ratios exhibited synergistic effects. It was noted that this phenomenon was similar to the CAT and Fenton-CAT assays. In the case of the combination of G12/G14, slight antagonistic effects were observed as shown in Figure 30B. Interestingly, the results were similar to the CAT assay.

Overall, when it came to the antioxidant interaction, however, the CAT assay exhibited a similar antioxidant interaction as lipid oxidation in O/W emulsions (autoxidation). Although the CAT assay showed some bias in terms of the activity of water-soluble antioxidant compared to the ApoCAT and Fenton-ApoCAT assays.

Results suggested that lipid oxidation in O/W emulsions might be more complex than our expectation. In addition, the results emphasized the localization and dynamic exchanges of lipid soluble radicals and/or other prooxidants between aqueous and lipid phases.

From the results, we hypothesized that lipid soluble radicals in the lipid phases could be also distributed in aqueous phase due to their incorporation into surfactant micelles and/or small emulsion particles. There small particle loaded with lipid soluble radicals might behave similar to water-soluble radicals in the aqueous phase.

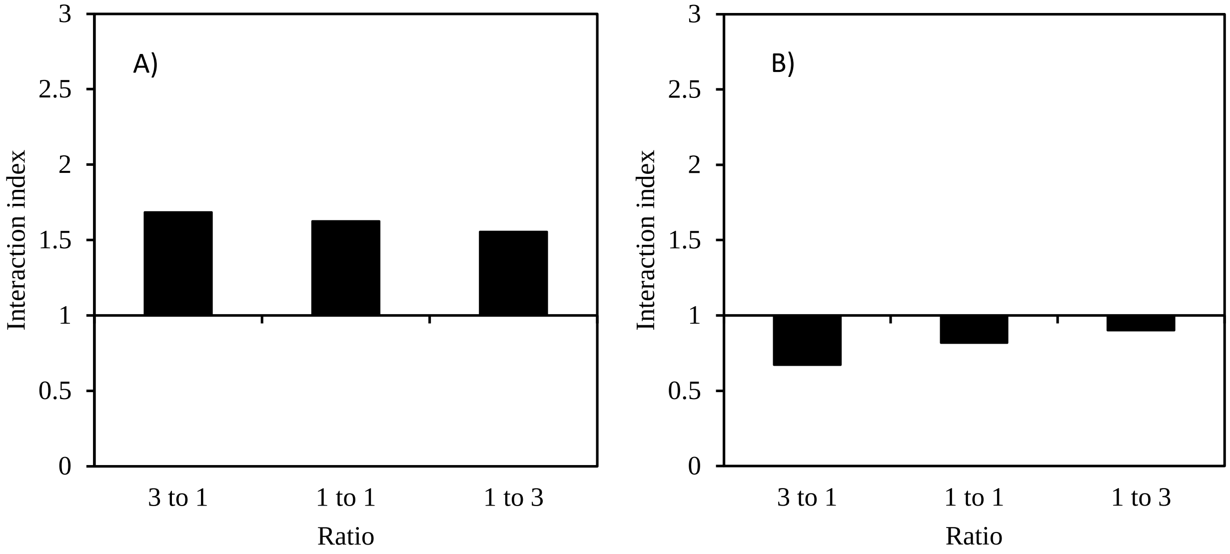

Figure 30 Antioxidant interactions of the gallic acid and tetradecyl gallate (G0/G14: A), and the dodecyl gallate and tetradecyl gallate (G12/G14; B) in autoxidation of oil-in-water emulsions at different combination ratios (mole/mole)

Conclusion and Discussion

Several CAT based assays have been developed in this research to elucidate the complexity of antioxidant behaviors of the individual and combined antioxidants in O/W emulsion and food model systems. The ApoCAT and Fenton-ApoCAT assays were developed in order to address the oxidation site concerns in the CAT assay that might be not relevant to a natural lipid oxidation in O/W emulsions. Results suggested that the ApoCAT and Fenton-ApoCAT assays exhibited a strong linear relationship with changes in their polarities, and in addition, one cut-off effect in comparisons with other CAT assays. In addition, the ApoCAT and Fenton-ApoCAT assays were able to predict precisely the performances of antioxidants with different polarities in the O/W emulsion, and roasted peanut systems except for the bulk oil system. It could be due to the structure of bulk oil system may differ from other model systems.

It should be noted that the water-in-oil (W/O) emulsion system was intentionally excluded from this research by the author due to that the structure of W/O emulsions may totally differ from the most food emulsions. In addition, only few W/O emulsions in foods were known for example butter. Thus, in this research, the author decided to perform other food model systems that should be more relevant to most food applications such as O/W emulsions, bulk oils, and dried moisture foods.

However, in terms of the antioxidant interactions, the Fenton-CAT, ApoCAT and Fenton-ApoCAT assays did not show the similar relationship with the actual lipid oxidation in O/W emulsions. Only the antioxidant interactions observed in the CAT assay were similar to those observed in the oxidation in O/W emulsions.

In conclusion, oxidation dynamics (oxidation hierarchy and free radical distribution) should be one of the important keys to understand lipid oxidation mechanism in particular food systems. In addition, together with antioxidant dynamics (antioxidant distribution and exchange) in the systems, one could be able to design a better antioxidant assay that could predict precisely both antioxidant performances and interactions.

Appendix A

1. Total phenolic content (TPC) Assay

Total phenolic content (TPC) of gallic acid and its alkyl esters, and plants extracted was determined using Folin-Ciocalteu method with some modifications (Deetae et al., 2012). The reaction was done in 96-well microplates. Samples (30 µl) were mixed with 150 µl of Folin-Ciocalteau's reagent (10-time dilution) and 120 µl of sodium carbonate (7.5% w/v). The mixtures were mixed using a microplate mixer (Thermomixer comfort; eppendorf, Germany) at 600 rpm for 1 min, and kept in the dark for 30 min. The absorbance of mixture was read at 765 nm using a microplate reader (Spectra MAX 190, Molecular Devices, USA). Gallic acid was used as a standard phenolic compound. Results were expressed as gallic acid equivalent.

2. ABTS radical decolorization (ABTS) assay

The free radical scavenging activity of plants extracts was performed using the ABTS assay (Deetae et al., 2012). ABTS cation radicals ($\text{ABTS}^{\bullet}$+) were generated by reacting 7 mM ABTS stock solution (10 ml) with 140 mM potassium persulfate (179µl). Then, the mixture was incubated at room temperature for 16 h under the dark. The $\text{ABTS}^{\bullet}$+ solution was diluted to obtain OD of 0.700±0.050 at wavelength 734 nm before use. Twenty microliters of samples were mixed with 280 µl of the $\text{ABTS}^{\bullet}$+ solution and then incubated in the dark at room temperature for 6 min. The absorbance of mixture was then read at 734 nm using a microplate reader. The amount of the absorbance decrease was expressed against the standard curve of various concentrations of ascorbic acid. Results were expressed as ascorbic acid equivalent.

3. Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) assay was performed using the modified method as described by Deetae et al. 2012. The FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl and 20 mM FeCl3.6H2O at a ratio of 10:1:1. The reactions were started by mixing 30 µl of samples with 270 µl of FRAP reagent. The reaction was performed in 96-well microplates. The total volume of reaction was 300 µl. The microplates containing reactive mixture were kept in the dark at 37 °C for 8 min. The increase in absorbance of mixtures was read at 595 nm using a microplate reader. Using ascorbic acid as a standard curve, results were expressed as ascorbic acid equivalent.

4. The oxygen radical absorbance capacity (ORAC) assay

The oxygen radical absorbance capacity (ORAC) assay method was performed with some modifications (Nikhili et al., 2011). Fluorescein was used as the fluorescent probe, and AAPH was used as the oxidizable substrate. The final reaction mixture consisted of fluorescein (160 µl, 78 nM), sample (20 µl) and AAPH (20 µl, 12 mM). All reagents were prepared in 75 mM phosphate buffer solution, pH 7.4. The ORAC assay was done in black 96-well plates (Eppendorf Microplate 96/U-PP). Fluorescent intensity of the mixture was recorded each minute with 5 s agitation using a fluorescent microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 520 nm in kinetic mode for 3 h

Appendix B

(International publication)

Apolar Radical Initiated Conjugated Autoxidizable Triene (ApoCAT) Assay: Effects of Oxidant Locations on Antioxidant Capacities and Interactions

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Running Title: Effects of hydrophilic and lipophilic radical initiators on antioxidative performances in the CAT assay and its modification

ABSTRACT

Development of an antioxidant assay explaining antioxidant behaviors in complex food systems has been a challenging topic for food scientists. This research aimed to investigate antioxidant capacities and interactions of selected synthetic antioxidants and commercial natural antioxidant extracts using the CAT assay and a newly developed ApoCAT assay, which used water- and lipid-soluble azo radical initiators, respectively. Results suggested that the higher hydrophobicity of an antioxidant, the higher antioxidant capacity of an antioxidant was observed in the ApoCAT assay. Relationship between two different assays was explained by the ratio between the ApoCAT and the CAT values. Interestingly, all lipophilic derivatives of the antioxidants exhibited higher ApoCAT/CAT ratios than their hydrophilic derivatives. In the case of the commercial food-grade antioxidants, green tea extract and mixed tocopherols showed a higher antioxidant capacity in the ApoCAT assay than in the CAT assay, while grape seed and rosemary extracts didn't show significant different changes in behaviors in both assays. The study on antioxidant interactions revealed that additive, synergistic and antagonistic effects between hydrophilic antioxidants and natural extracts, and mixed tocopherols could be observed in both the CAT and the ApoCAT assays, depending on the combined ratios. In most cases, at a particular ratio, synergistic effect reached the maximum level before suddenly dropping to additive and antagonistic effects in both assays.

Keywords: Lipid oxidation; Natural antioxidant extracts; Antioxidant capacity; The CAT assay; modified CAT assay

INTRODUCTION

Under atmospheric oxygen, foods and living organisms are susceptible to lipid oxidation, causing deterioration of food quality and health complications-related to oxidative stress. Several strategies to prevent lipid oxidation have been proposed, for example, the use of antioxidants. Free radical scavenging activity is one of the most important antioxidative mechanisms of antioxidants, which is essential for foods and living organisms to retard oxidation processes.

To evaluate antioxidant capacity of substances, several antioxidant assays have been developed, for example, 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid or ABTS cation radical scavenging assay, DPPH radical scavenging assay, ferrous reducing ability power (FRAP) assay, oxygen radical absorbance capacity (ORAC) and etc. Generally, they have been used in many studies on antioxidants derived from plants and food components (*1*-*8*). Especially, the ORAC database for various plant extracts was developed, since a water soluble peroxyl radical generated from 2, 2'-azobis-2-methyl-propanimidamide, dihydrochloride (AAPH) was thought to be more relevant to peroxyl radicals observed in biological systems.

However, some drawbacks of the ORAC assay have been discussed (*9*-*11*). The kinetic reaction between an antioxidant and a substrate was unrealistic because the concentration of the substrate (fluorescein) was much lower than the tested antioxidant (*9*). Most importantly, the ORAC assay was performed under a homogeneous system (phosphate buffer solution) which is very different than foods. For example, the ability of antioxidants to partition at the interfaces of emulsions (oil-in-water or water-in-oil) and/or biological membranes has been found to be more important in the activity of an antioxidant compared to it electron donating ability (*12*-*15*).

To solve these problems, conjugated autoxidizable triene (CAT) assay, a novel spectrophotometric method was developed by Laguerre et al. (2008). Instead of using fluorophore, the oxidation process in the CAT assay was monitored by determination of the degradation of conjugated triene fatty acids (octadecatrienoic acid) found in tung oil using UV spectrophotometer at 273 nm. The CAT assay has several advantages over the ORAC assay.

First, the CAT assay was performed under a heterogeneous system (oil-in-water emulsions). Thus, effect of locations of antioxidants in O/W emulsions (antioxidant partitioning) on their antioxidative performance has been incorporated into the model. Second, the amount of antioxidants used in the CAT assay was approximately 100 times less than the substrate. Under this condition, the reaction kinetic was realistic trend in terms of natural antioxidant phenomena (*9*).

Recently, the CAT assay has been used in many researches to explore an optimum degree of lipophilization of phenolic compounds (*16*) using AAPH (a water soluble peroxyl radical initiator) to accelerate lipid oxidation in oil-in-water emulsions. However, due to the complexity of lipid oxidation in emulsion systems, locations of free radicals in the system could be differently partitioned not only into water phase, but also the oil/lipid interfacial region and/or lipid core at which lipid oxidation was hypothesized to occur in emulsions. Thus, it is worth to investigate the influence of using a lipid-soluble free radical initiator on antioxidant behaviors in the CAT-based assay as an additional complementary method to the original CAT assay using different ways to initiate lipid oxidation.

In this research, apolar radical-initiated conjugated autoxidizable triene (ApoCAT) assay, a modified CAT assay, was proposed by using a lipid soluble free radical azo initiator (dimethyl 2,2'-azobis(2-methylpropionate) so called V601or AIBME) instead of AAPH. According to chemical information, AIBME is a non-nitrile azo initiator which is very insoluble in water. It was hypothesized that the ApoCAT assay may be more relevant to autoxidation of oil-in-water emulsions; since it was believed that lipid oxidation in O/W emulsions occurs at the interface of oil droplets (*17, 18*) instead of acceleration from aqueous phase.

In addition, these two assays may be suitable for studying combinations of antioxidants. It was reported that locations of antioxidants in O/W emulsions also impacted overall antioxidative performances of combined antioxidants, resulting in different interaction behaviors such as synergistic, additive and antagonistic effects (*19*).

This research aimed to investigate the effects of hydrophilic and lipophilic peroxyl radical initiators on antioxidative performances of some synthetic antioxidants and natural extracts, to study the relationship between CAT and ApoCAT assay, and to explore antioxidant interactions using these assays.

MATERIALS AND METHODS

Chemicals and Materials

Ethylenediaminetetraacetic acid (EDTA), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ). silicic acid, Folin-Ciocalteau’s reagent, sodium carbonate, ferric chloride, tung oil, Brij 35, ascorbic acid, ascorbyl palmitate, gallic acid, lauryl gallate and mixed tocopherols were purchased from Sigma-Aldrich (St. Louis, USA). Hydrochloric acid was purchased from Fisher Scientific (Loughborough, UK). Absolute ethanol was purchased from RCI Labscan (Thailand). 2, 2'-azobis-2-methyl-propanimidamide, dihydrochloride (AAPH),dimethyl 2,2'-azobis(2-methylpropionate) (AIBME) or V601 and activated charcoal were purchased from Wako Chemical (Japan). Miglyol®812N, medium chain triglyceride (MCT) mixture was purchased from Sasol Germany Gmbh (Witten,Germany). Commercial grape seed (95%

oligomeric proanthocyanidins; OPC) and rosemary (60% carnosic acid) extracts were purchased from Changsha Nutramax Inc. (Changsha, China). Commercial green tea extract was purchased from Specialty Natural Products Co., Ltd. (Chonburi, Thailand). All commercial extracts were processed without any additive and carriers. Double-distilled and deionized water was used for the preparation of all solutions. All organic solvents used were analytical grade.

Total Phenolic Content (TPC) Assay

Total phenolic contents of antioxidants and plant extracts were determined by the method of Deetae et al. (2012) (*8*) with some modifications. The reaction was performed in a 96-well microplate. Briefly, thirty microliter of sample was mixed with 150 µl of Folin-Ciocalteau's reagent (10-times dilution). Then, 7.5% w/v of sodium carbonate (120 µl) was added. The mixtures were kept in the dark for 30 min. The absorbance of mixture was recorded at 765 nm using a microplate reader (SpectraMax 190, Molecular Devices, USA). Gallic acid was used as a standard phenolic compound. In the case of synthetic antioxidants, result is expressed as millimole of gallic acid per one mole of antioxidant (mmole GAE/mole antioxidant). In the case of natural extracts, result is expressed as mg of gallic acid per one gram of antioxidants (mg GAE/g extract).

ABTS Radical Scavenging Activity Assay

Free radical scavenging activity of antioxidants and plant extracts was performed according to the ABTS assay described by Deetae et al. with some modification (*8*). ABTS cation radical ($ABTS^{\bullet+}$) was generated by reacting 10 ml of 7 mM ABTS stock solution with

179 µl of 140 mM potassium persulfate. Then, the mixture was incubated at room temperature for 16 h. $ABTS^{\bullet+}$ solution was diluted to obtain an absorbance of 0.7 at a wavelength of 734 nm before use. Twenty microliters of samples were added into 280 µl of $ABTS^{\bullet+}$ solution in a microplate. The mixtures were mixed using the microplate mixer, and then incubated in the dark at room temperature for 6 min. The absorbance of mixture was then read at 734 nm by a microplate reader (Spectra Max 190, Molecular Devices, USA). Ascorbic acid was used as a standard phenolic compound. In the case of synthetic antioxidants, result is expressed as millimole of ascorbic acid per one mole of antioxidant (mmole ascorbic acid/mole antioxidant). In the case of natural extracts, result is expressed as mg of ascorbic acid per one gram of antioxidants (mg ascorbic acid/g extract).

Ferrous Reducing Antioxidant Power (FRAP) Assay

Ferric reducing antioxidant power (FRAP) assay was performed using a method described by Deetae et al. with some modification (*8*). Shortly, FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ at the ratio of 10:1:1. Sample (30 µl) was added into 270 µl of FRAP reagent in a 96-well microplate. The microplate was kept in the dark at 37 °C for 8 min. The absorbance of the mixture was measured at 595 nm using a microplate reader (SpectraMax 190, Molecular Devices, USA). Ascorbic acid was used as a standard phenolic compound. In the case of synthetic antioxidants, result is expressed as millimole of ascorbic acid per one mole of antioxidant (mmole ascorbic acid/mole antioxidant). In the case of natural extracts, result is expressed as mg of ascorbic acid per one gram of antioxidants (mg GAE/g extract).

Oxygen Radical Absorbance Capacity (ORAC) Assay

Oxygen radical absorbance capacity (ORAC) assay was performed according to Nkhili et al. (*10*). Briefly, a series of varying concentrations of antioxidant was prepared. Twenty microliters of samples were added into a 96-well black microplate (Eppendorf). Then, 160 µl of 78 nM fluorescein in PBS solution (pH 7.2) was added. The mixture was incubated at 37℃ for 5 min in the dark. The reaction started when 12 mM AAPH (20 µl) was added. Fluorescent intensity of the mixture was recorded using a fluorescent microplate reader (SpectraMax M5, Molecular Devices, USA) with an excitation wavelength of 485 nm and an emission wavelength of 520 nm at 2 min intervals for 3 hours in kinetic mode.

Conjugated Autoxidizable Triene (CAT) Assay

Conjugated autoxidizable triene (CAT) assay was assessed as described by Laguerre and coworkers (*20*) with some modification. Conjugated triene triacylglycerols in tung oil was used as a substrate and UV probe for monitoring the progress of the oxidation reaction. Polar compounds of tung oil were stripped by using a method explained by Boon et al. (*21*). Briefly, silicic acid (100 g) was washed three times with a total of 3 L of distilled water followed by drying at 110℃ for 20 h.

A chromatographic column (3.0 cm internal diameter35 cm height) was then packed sequentially with 22.5 g of silicic acid followed by 5.625 g of activated charcoal and then another layer of silicic acid (22.5 g). Tung oil (30 g) was dissolved in 30 ml of n-hexane and passed through the column. Tung oil was eluted with addition of n-hexane (270 ml). The collected triacylglycerols were held in an ice bath, and covered with aluminum foil to inhibit lipid oxidation. The solvent in stripped tung oil was removed using a vacuum rotary evaporator (BÜCHI Rotavapor R-114, Switzerland) at 37℃, and traces of the remaining solvent were removed by flushing with nitrogen. The stripped tung oil was transferred into small vials, and stored at -80℃ until performing an experiment.

The CAT assay was performed with two main steps as the following details. First, for emulsion preparation, 25 ml of PBS solution (pH 7.2) containing 34 µM Brij 35 was added into a beaker containing 6(± 0.5) mg of stripped tung oil. Oil-in-water emulsions were made by homogenizing the mixture at 6,500 rpm for 90 s (Ultra-Turrax T25 basic, IKA® Werke, Germany). The emulsion was then centrifuged using a centrifuge (Model 5810 R, Eppendorf, Germany) at 10,000 g, 10 °C for 5 min in order to remove the unstable part (large oil droplets), providing more homogeneous size distribution of O/W emulsions.

Second, a series of different concentrations of samples in PBS solution (50 µl) was transferred to a UV-Star 96-well microplate (Costar, Corning, USA), followed by addition of the prepared emulsion (125 µl). The samples were mixed at 1,200 rpm, 37℃ for 5 min using a microplate mixer (Thermomixer R, Eppendorf, Germany). After mixing, the samples were immediately mixed with 25 µl of 4 mM AAPH solution in PBS solution and recorded at 273 nm at 1-min intervals for 3 hours using a microplate reader (SpectraMax 190, Molecular Devices, USA). Trolox was used as a standard antioxidant to compare against other treatments.

The CAT value, expressed as mg of trolox per one gram (or mole) of an antioxidant, was calculated from a relationship between net protection areas under the curve (AUC) and concentrations of antioxidants compared to trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

Apolar Radical-Initiated Conjugated Autoxidizable Triene (ApoCAT) Assay

The ApoCAT assay was adapted from the original CAT assay described by Laguerre et al. (*20*). Instead of using AAPH, the lipid-soluble free radical initiator, dimethyl 2, 2'-azobis (2-methylpropionate), was used. All procedures were performed as described in the CAT assay except for the preparation of free radical initiator.

In brief, MCT oil-in-water emulsion was prepared exactly the same way as described in tung oil-in-water emulsion. MCT emulsion solutions was mixed directly with the lipid-soluble free radical initiator to obtain a final concentration of 8 mM dimethyl 2, 2'-azobis (2-methylpropionate) in MCT oil-in-water emulsion. Twenty milliliters of V601 loaded MCT oil-in-water emulsion were mixed with 120 ml of tung oil-in-water emulsions to start the oxidation reaction.

Calculation of Antioxidant Interaction Indexes in the CAT and ApoCAT Assays

Interaction indexes of combined effects between α-tocopherol (lipid soluble) and water soluble antioxidants were investigated using the CAT and the ApoCAT assays. Different ratios of water to lipid soluble antioxidants (1:3, 1:1, 3:1, 5:1 and 7:1) were prepared in ethanolic solution based on molar mass basis of antioxidants. To estimate the interaction indexes, individual and combined antioxidants were performed in the same 96-well microplate. Antioxidant capacities of individual antioxidant could be observed directly. For the expected antioxidant capacities of the combination, it could be estimated as described in the following equation.

$$\text{Interaction Index} = \frac{\text{Observed CAT value of the combination}}{\text{Expected CAT value of the combination}}$$

$$= \frac{the\ CAT\ value\ of\ combined\ (A+B)}{the\ CAT\ value\ of\ A\ x\ F1 + the\ CAT\ value\ of\ B\ x\ F2}$$

Where, A and B represent α-tocopherol and another antioxidant, respectively. Individual CAT or ApoCAT values of A and B were used to calculate the expected CAT or ApoCAT of the combined A and B. F1and F2 represent fractions of the combination. For example, a ratio between antioxidants was 3 to 1. The F1 and F2 are 3/4 and 1/4, respectively. Interaction indexes were expressed as synergistic (> 1), additive (≈ 1) and antagonistic (< 1) antioxidant effects.

Statistical Analysis

All analyses were performed on triplicate samples. In all cases, comparisons of the means were performed using Duncan's multiple-range tests. A significance level of $p < 0.05$ was defined as being statistically different. All calculations were performed using SPSS18.0 (http://www.spss.com; SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Effect of hydrophilic and lipophilic azo radical initiators on antioxidant capacities in the CAT assay

Water soluble (AAPH) and lipid soluble (AIBME) azo radical initiators were used to investigate influences of different oxidant locations in the CAT assay on antioxidant capacities of a variety of antioxidants. Instead of using water-soluble AAPH, the ApoCAT assay has been developed by using lipid-soluble AIBME in the original CAT assay. One of the problems in the development of ApoCAT assay was finding a suitable delivery system for the incorporation of AIBME into the tung oil emulsions. To avoid large impacts on antioxidant distributions in the emulsion system from using solvents and surfactants, AIBME incorporated MCT oil-in-water emulsions were prepared using the same protocol as the tung oil emulsions. It was observed that using ethanol as a solvent to dissolve AIBME impacted on the emulsion stability of tung oil emulsions (data not shown). Hence, using excess surfactant concentrations could change the distribution of antioxidants in the systems as previously reported by Panya et al. (2012) that the antioxidant activity of medium-chain of alkyl ester (4-12 carbons) of antioxidants (rosmarinic acid) was decreased, while the activity of long-chain of alkyl ester (20 carbons) of rosmarinic acid was improved by the addition of Tween 20 (*15*).

Figure 1 demonstrates proposed mechanisms of oxidants and antioxidants in the CAT and ApoCAT assays. In the CAT assay, water soluble antioxidants are supposed to be mainly distributed in water phase, so they have high possibility to react with radicals generated in water phase. Since, the limitation of lipid soluble antioxidants to dissolve in water, lipid soluble antioxidants could only react with radicals generated near the oil/water interfacial regions.

However, in the case of the ApoCAT assay, lipid-soluble radicals and non-polar antioxidants could be partitioned into several hydrophobic regions such as lipid cores of tung and MCT oil droplets, micelles, and the oil/water interfaces. As a result, lipid-soluble antioxidants could mainly inactivate free radicals generated in the lipid phase, while water-soluble antioxidants could only interact with lipid-soluble radicals at the interfacial regions. It should be noted that dynamic exchanges of lipid soluble radicals and antioxidants between the tung oil and MCT oil emulsion droplets could be also facilitated by surfactants due to the formation of micelles.

As illustrated in Figure 2, without addition of Trolox, oxidation rates of tung oil-in-water emulsions in the ApoCAT assay was slightly slower than that of the CAT assay. At the same concentration of Trolox, oxidation rates of tung oil emulsions of the ApoCAT assay were also slower than what observed in the CAT assay. As a result, the net protection areas against various concentrations of Trolox observed in the ApoCAT assay was higher than observed in the CAT assay. It could be due to 10 hours half-life decomposition temperature of AIBME is 66℃, while that of AAPH is 44℃. Thus, in general, the radical generation rate of AIBME is slower than that of AAPH at the same experiment condition.

From a kinetic point of view, Trolox exhibited different behaviors in both assays. Trolox could suppress oxidation more effective in the CAT assay than in the ApoCAT assay as indicated by larger oxidation lag phase and/or slower initial oxidation rates. Laguerre and co-workers (*9*) have also reported that Trolox exhibited as retarder behavior in the CAT assay. However, it was noted that in this research, Trolox showed a pure retarder behavior (with an insignificant oxidation lag phase) in the ApoCAT assay. Results implied that a water soluble antioxidant could exhibit higher antioxidant activity in the CAT assay than that observed in the ApoCAT assay.

The relationships between the CAT and ApoCAT assays were investigated using several water-and lipid-soluble antioxidants. Kinetics of absorbance bleaching in the presence of water soluble (gallic acid and ascorbic acid) and lipid soluble (lauryl gallate and ascorbyl palmitate) antioxidants performed in the CAT and ApoCAT assays are shown in Figure 3 and 4, respectively. Results suggested that there was a strong influence of oxidant locations on the oxidation behaviors of water-and lipid-soluble antioxidants as indicated by changes in decay kinetics of tung oil emulsions with several antioxidants.

In the ApoCAT assay, gallic acid exhibited as a strong retarder as a result of faster decay rate of the oxidation in comparison with what observed in the CAT assay. The same was true in the case of lauryl gallate. In the CAT assay, lauryl gallate showed a significant lag phase, while in the ApoCAT assay, lauryl gallate didn’t exhibit any lag phase. As illustrated in Figure 4, similar results were observed for ascorbic acid and ascorbyl palmitate. Ascorbic acid exhibited higher antioxidant activity in the CAT assay (Figure 4A) than in the ApoCAT assay (Figure 4B). However, ascorbyl palmitate showed higher antioxidant activity in the ApoCAT assay (Figure 4D) than in the CAT assay (Figure 4C). It should be noted that ascorbyl palmitate exhibited as a strong chain-breaker due to a significant lag phase.

From these results, it should be noted that kinetic behaviors of antioxidants as indicated by lag times and net protective areas under the curve (AUC) were changed according to how the systems were accelerated. It could be due to that antioxidant partitioning was different in the CAT and ApoCAT systems. It was postulated that water soluble antioxidants, mostly distributed in aqueous phase, could be able to mainly react with radicals generated in aqueous phase and near the oil-water interface of tung oil emulsions in the case of the CAT assay, and both tung oil and MCT oil emulsions in the case of ApoCAT. In comparison with the CAT assay, aqueous antioxidants in the ApoCAT model may have less chance to react with aqueous phase radicals, so decreases in antioxidant performance of water soluble antioxidants were observed, resulting in shorter or insignificant oxidation lag phases.

In the case of lipid soluble lauryl gallate (12 carbons), its oxidation lag time was clearly observed in the CAT assay, while it was not found in the ApoCAT assay. These results were different in comparison with ascorbyl palmitate (16 carbons) with an oxidation lag time being longer in the ApoCAT assay than in the CAT assay. It was hypothesized that it could be due to that the polarity of ascorbyl palmitate was lower than that of lauryl gallate, so the distribution of ascorbyl palmitate should be mainly associated with the emulsion droplets. However, antioxidant performance of lauryl gallate had dramatically changed in both assays. Results suggested that the majority of lauryl gallate molecules may be distributed in water phase instead of oil droplets

Relationships between net AUC of selected antioxidants in the CAT and the ApoCAT assays are shown in Figure 5 (A and B), respectively. Results suggested that the AUC of the ApoCAT assay was higher than that of the CAT assay. It could be due to the slower oxidation rate of the AIBME in comparison to AAPH because the 10 hour half-life decomposition temperature of AIBME (66℃) was higher than that of AAPH (44℃). In addition, the same concentrations of AAPH and AIBME were used in both assays.

Results suggested that the ApoCAT assay showed a strong impact on antioxidant activities of non-polar antioxidants (ascorbyl palmitate and lauryl gallate) in the same manner. For instance, it was noticed that antioxidant activities of ascorbic acid and ascorbyl palmitate were strongly impacted. Ascorbic acid exhibited a strong antioxidant activity in the

CAT assay, while it showed a poor antioxidant activity in the ApoCAT assay. On the contrary, ascorbyl palmitate exhibited higher antioxidant activity in the ApoCAT assay than in the CAT assay. Overall, it could be concluded that non-polar antioxidants performed better in the ApoCAT assay than in the CAT assay, and vice versa.

Comparisons of the performance of the CAT and ApoCAT assays to conventional antioxidant activity assays

As illustrated in Table 1, antioxidant capacities of ascorbic acid, gallic acid and their esters were investigated in TPC, ABTS, FRAP, ORAC assays in comparisons with the CAT and the ApoCAT assays. It should be noted that TPC, ABTS, FRAP and ORAC assays were considered as homogeneous systems, while the CAT and the ApoCAT assays were concerned as heterogeneous systems. Results showed that ascorbic acid and gallic acid exhibited higher antioxidant capacities than their ester forms in TPC, ABTS, FRAP, and ORAC assays. Hence, the solubility of antioxidants in the aqueous systems should be one of the important factors affecting on the overall antioxidant activities.

However, in the CAT assay, ascorbyl palmitate exhibited lower antioxidant capacity than ascorbic acid, while lauryl gallate showed higher antioxidant capacity than gallic acid, based on trolox equivalents. Results indicated that the cut-off effect could be observed in ascorbyl palmitate. Laguerre and co-workers (*9*, *22*) explained the cut-off effect in several heterogeneous systems that too low polarity of an antioxidant may impact on locations of the antioxidant, distributing into lipid core inside and/or self-aggregation outside emulsion droplets. However, in the case of the ApoCAT assay, it was noted that a different antioxidant behavior was observed. Ascorbyl palmitate exhibited higher antioxidant capacity than ascorbic acid, based on trolox equivalents.

In addition, these models were performed on various commercial antioxidant extracts as shown in Table 2. Based on homogeneous models, TPC, ABTS and FRAP assays showed a similar antioxidant behavior that green tea extract had the lowest antioxidant capacity followed by rosemary extract, mixed tocopherol and/or grape seed extract. On the contrary, the obtained results from the ORAC assay differed from other assays. In this model, mixed tocopherol had the lowest antioxidant capacity followed by green tea, grape seed, and rosemary extracts, respectively.

The CAT and the ApoCAT assays showed a similar antioxidant pattern that green tea had the lowest antioxidant capacities, while rosemary extract had the highest antioxidant capacity. To understand the effect of oxidant locations, the ApoCAT/CAT ratios of tested antioxidants were calculated as illustrated in Figure 6. It should be noted that the higher the ApoCAT/CAT ratio, the better antioxidative performance of an antioxidant was observed in lipid phase radical-initiating system. As shown in Figure 6A, all ester forms had higher ApoCAT/CAT ratios than their acid forms. For example, ascorbyl palmitate showed an improved performance in the ApoCAT assay, while ascorbic acid showed the opposite trend. In addition, the same was true for gallic acid and its ester.

Overall, the ApoCAT/CAT ratio could provide more information on characteristics of antioxidants preferring towards free radicals in water- and/or lipid-phases. This concept was also applied for characterizing some commercial antioxidants and natural plant extracts as shown in Figure 6B. As expected, mixed tocopherols performed better in the ApoCAT assay since they are lipid soluble antioxidants. Interestingly, the performances of green tea and rosemary extracts have been also increased, while grape seed extract showed no significant difference in both assays. Results suggested that mixed tocopherols, green tea extract and rosemary extract could be good antioxidant candidates for preventing oxidation in oil-in-water emulsion systems.

Investigation of antioxidant interactions using the CAT and the ApoCAT assays

Combined effects of selected antioxidants and were investigated using the CAT and ApoCAT assays at various ratios. Figure 7 shows linear relationships between observed individual and combined antioxidants, and expected combined antioxidants of various ratios of ascorbic acid/α-tocopherol mixtures based on mass basis. Results indicated that increasing the ratio of ascorbic acid to α-tocopherol from 1:1 to 5:1 changed antioxidant interactions from synergism to antagonism. In the case of synergistic effect, the observed slope of the antioxidant activity was higher than the expected slope of the antioxidant activity, and vice versa in antagonistic effect. Using this calculation, the relationships between ratios of polar and non-polar antioxidants, and antioxidant interactions in O/W emulsions could be investigated.

As shown in Figures 8 and 9, increasing the ratios of various polar antioxidants to mixed tocopherol (non-polar antioxidant) resulted in altered antioxidant interactions and overall antioxidant capacities. Depending on antioxidants and evaluation methods, optimal combination ratios to obtain the highest antioxidant synergy and capacities could be different. As illustrated in Figure 8, combining ascorbic acid or green tea with α-tocopherol exhibited the highest interaction index at 3:1 ratio. It should be noted that increasing the ratio further than this level resulted in antagonistic effect (at 5:1ratio).

Similar behaviors of the combinations were observed in the combined effects of grape seed and rosemary extracts. For the combinations with grape seed extract, the optimal ratios for synergy were observed around 1:1 to 3:1 ratios, and however, for rosemary extract, the highest synergy was observed at 1:3 ratios. It was noted that the combination with ascorbic acid (Figure 8A) and rosemary (Figure 9A) extracts exhibited similar combination behaviors in such a way that the CAT assay provided a significant higher interaction index in comparison with the ApoCAT assay. However, green tea (Figure 8C) and grape seed (Figure 9C) extracts exhibited a weak synergy in the ApoCAT assay higher than in the CAT assay.

Overall, results demonstrated that performance of antioxidants could be improved by combined strategy between water-and lipid- soluble antioxidants. It was hypothesized that water-soluble antioxidants could provide active prevention against radicals generated from AAPH in the water phase in addition to passive prevention of the radicals generated at the oil-in-water interface of the emulsion droplets. This could explain why the strong synergy was strongly observed in the CAT assay rather than the ApoCAT assay.

However, due to the complexity of antioxidant mechanisms, it is impossible to explain antioxidant interactions using a simple explanation. Several reports showed that antioxidant regeneration was one of the important mechanisms to obtain a strong antioxidant synergy. By principle, the primary antioxidant is regenerated by the secondary antioxidant based on differences in their reduction potentials. For instance, ascorbic acid is able to regenerate α-tocopherol, which is more active in the system (*23*). In addition, several compounds were reported to exhibit some antioxidant interactions with α-tocopherol such as β-carotene, amino acids, peptides, and phospholipids (*24-30*).

ACKNOWLEDGMENTS

We would like to express our thanks to Young Scientist and Technologist Program (YSTP), NSTDA (Grant No. SP-56-BT05) and Thailand Research Fund (Grant No. TRG5780061) for supporting this research project.

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Figure list

Figure 1 Proposed mechanisms of oxidants and antioxidants in the CAT and ApoCAT assays

Figure 2 Kinetic decay of conjugated triene in the CAT (left) and the ApoCAT (right) assays treated with trolox (A and B)

Figure 3 Kinetic decay of conjugated triene in the CAT (left) and the ApoCAT (right) assays treated with gallic acid (A and B) and lauryl gallate (C and D)

Figure 4 Kinetic decay of conjugated triene in the CAT (left) and the ApoCAT (right) assays treated with ascorbic acid (A and B) and ascorbyl palmitate (C and D)

Figure 5 The area under the curve (AUC) vs. concentrations of antioxidants in the CAT (A) and ApoCAT (B) assays

Figure 6 Ratio of antioxidant capacity of pure chemical antioxidants between the CAT and the ApoCAT assays. Synthetic antioxidants are ascorbic acid (Asc), ascorbyl palmitate(AP), gallic acid(G) and lauryl gallate(LG). Natural crude antioxidants are mixed tocopherol (mToco), grape seed extract (GSE), green tea extract (GTE) and rosemary extract (RME). Values are the means of three replicates. The different letters (a, b, c and d) of interaction index obtained from different ratios of the same assay indicate significant differences ($p < 0.05$).

Figure 7 Demonstration of estimating interaction index of ascorbic acid and α-tocopherol at the ratio of 1 to 1 and 5 to 1 in the CAT assay

Figure 8 Antioxidant interactions (left) and capacities (right) between selected antioxidants and mixed tocopherols in both CAT and ApoCAT assays. Combination of ascorbic acid and mixed tocopherols (A and B), and combination of green tea extract and mixed tocopherols (C and D)

Values are the means of three replicates. The different letters (a and b) of interaction index from the CAT and the ApoCAT assays at the same ratio indicate significant differences ($p < 0.05$). The different letters (A, B, C and D) of interaction index obtained from different ratios of the same assay indicate significant differences ($p < 0.05$).

Figure 9 Antioxidant interactions (left) and capacities (right) between selected antioxidants and mixed tocopherol in both CAT and ApoCAT assays. Combination of grape seed extract and mixed tocopherol (A and B), and combination of rosemary extract and mixed tocopherol (C and D) Values are the means of three replicates. The different letters (a and b) of interaction index from the CAT and the ApoCAT assays at the same ratio indicated significant differences ($p < 0.05$). The different letters (A, B, C and D) of interaction index obtained from different ratios of the same assay indicates significant differences ($p < 0.05$).

Table list

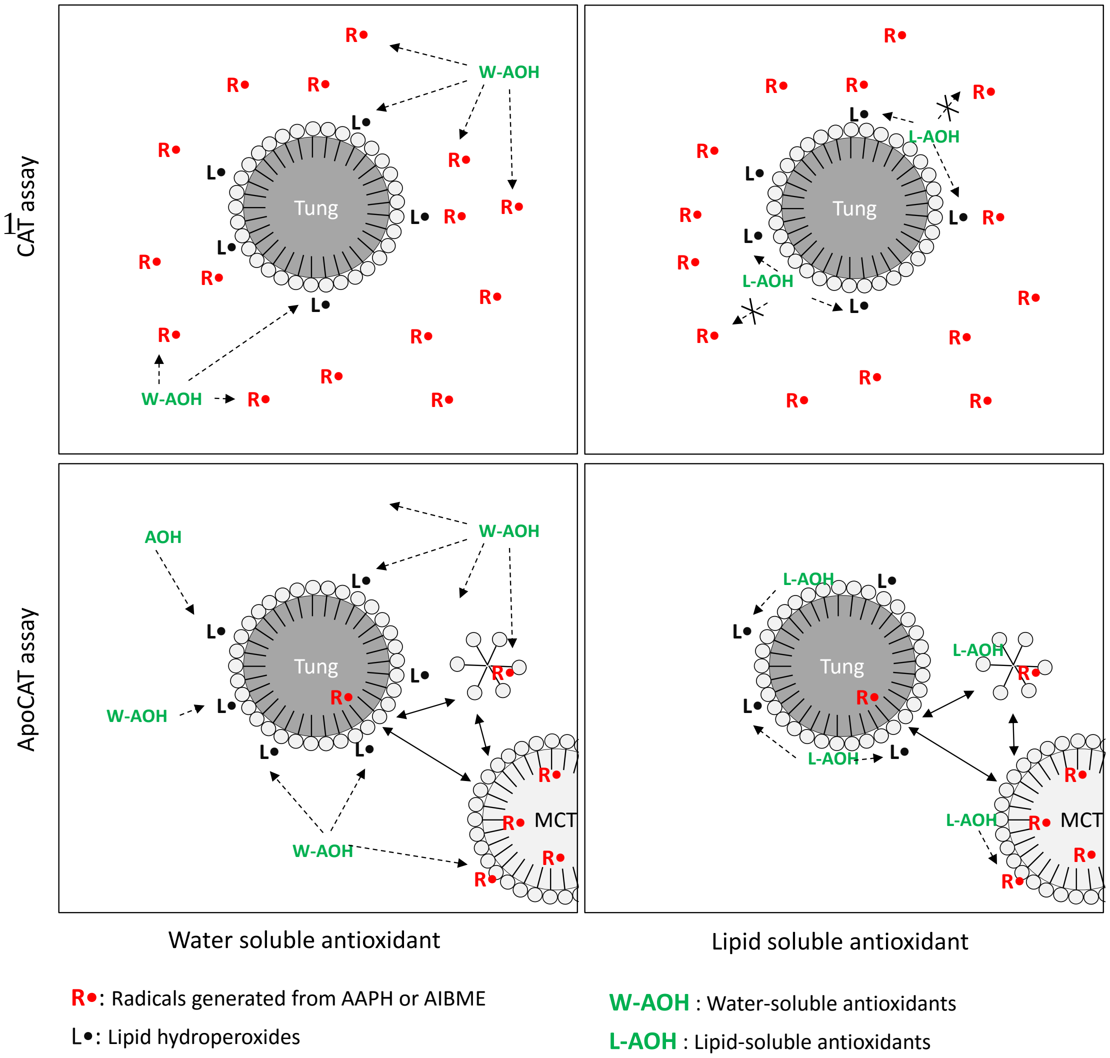
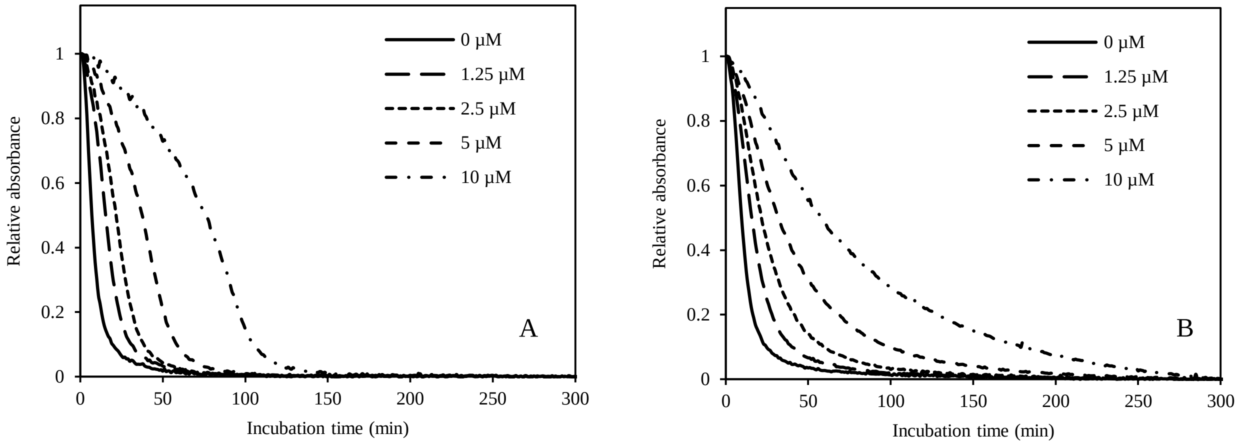

CAT assay
ApoCAT assay
R•
L•
W-AOH
L-AOH
AOH
Tung
MCT
Water soluble antioxidant
Lipid soluble antioxidant
R•: Radicals generated from AAPH or AIBME
L•: Lipid hydroperoxides
W-AOH : Water-soluble antioxidants
L-AOH : Lipid-soluble antioxidants

Figure 1


Relative absorbance
Incubation time (min)
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1.25 µM
2.5 µM
5 µM
10 µM
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B
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0.2
0.4
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0.8
1
0
50
100
150
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250
300

Figure 2

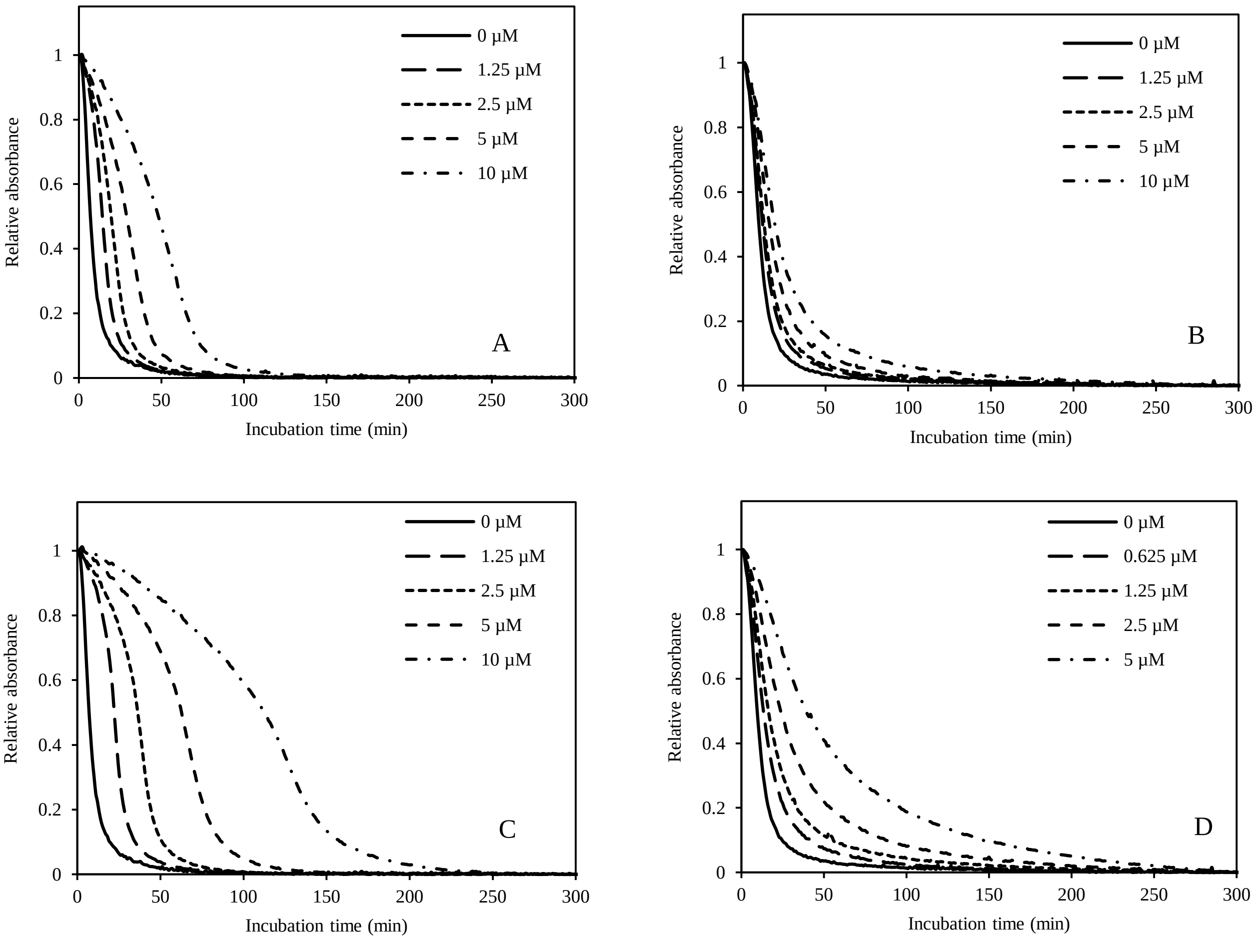


Figure 3 Gallic acid and Gallate (ApocAT is on the right)

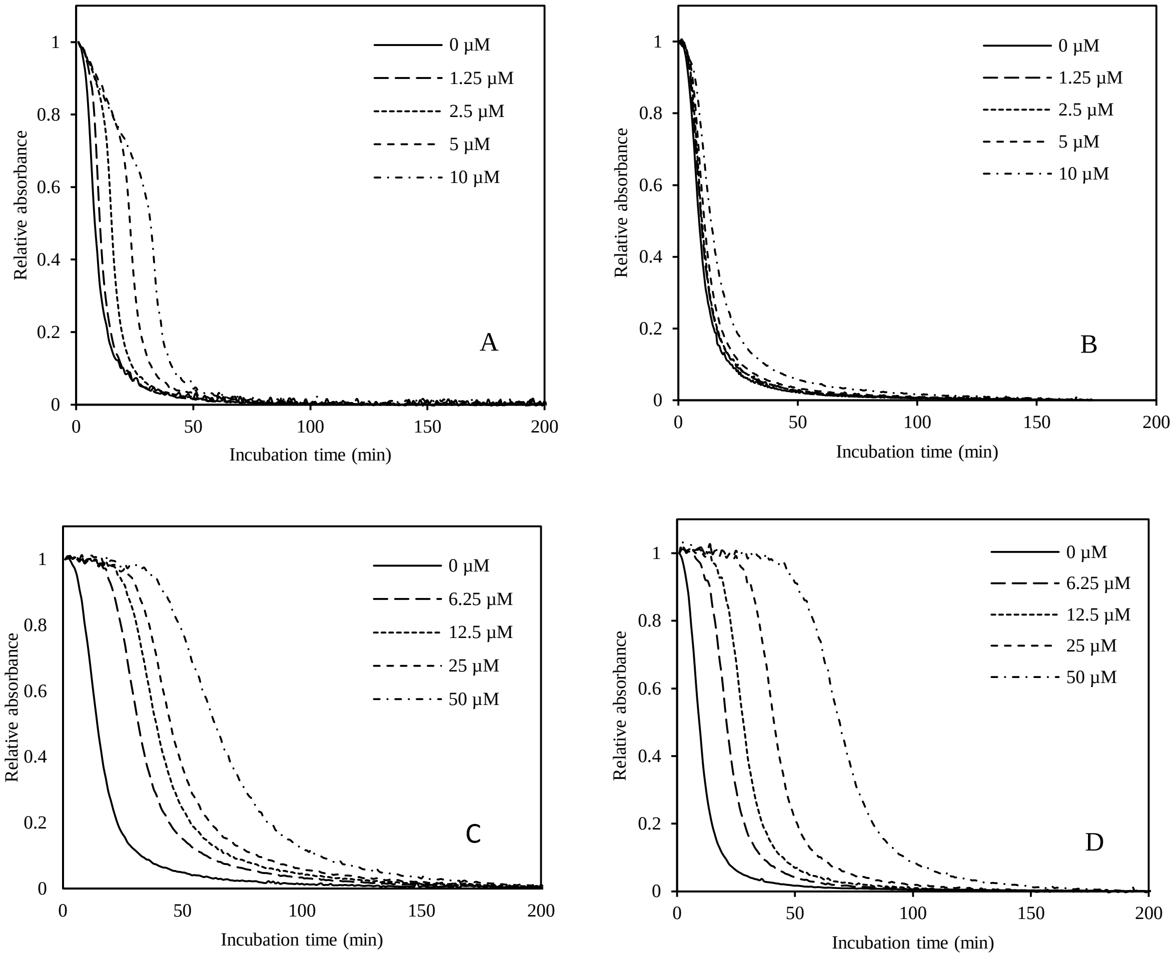
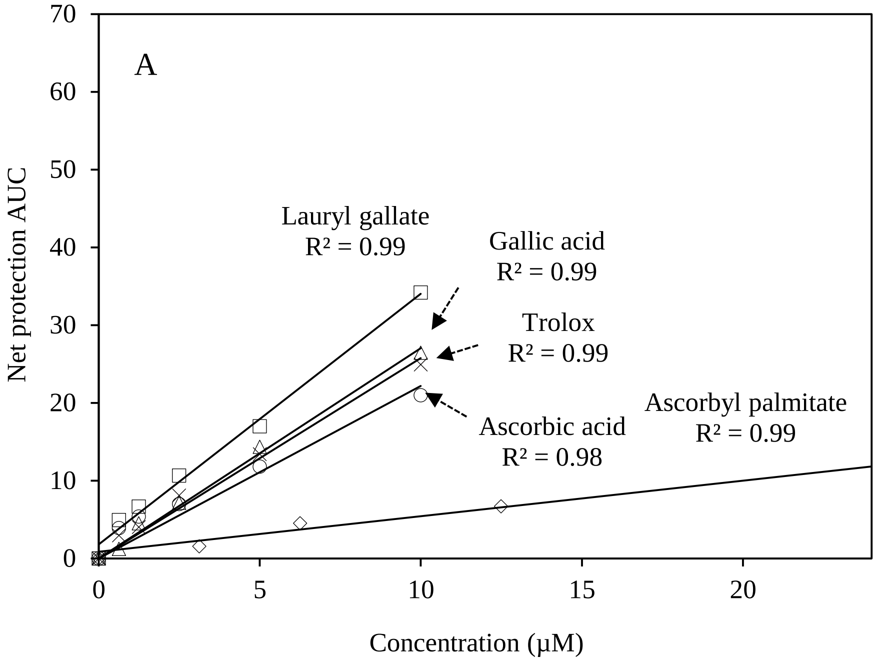


Figure 4 A and AP (ApoCAT is on the right)


A
Net protection AUC
70
60
50
40
30
20
10
0
0
5
10
15
20
Concentration (µM)
Lauryl gallate
R² = 0.99
Gallic acid
R² = 0.99
Trolox
R² = 0.99
Ascorbic acid
R² = 0.98
Ascorbyl palmitate
R² = 0.99

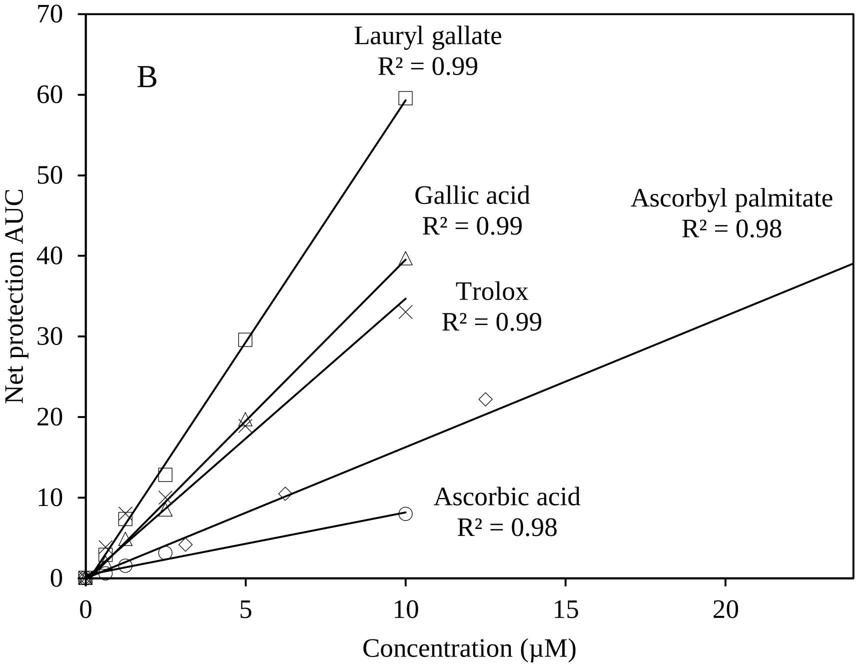

B
Net protection AUC
70
60
50
40
30
20
10
0
0
5
10
15
20
Concentration (µM)
Lauryl gallate
R² = 0.99
Gallic acid
R² = 0.99
Trolox
R² = 0.99
Ascorbyl palmitate
R² = 0.98
Ascorbic acid
R² = 0.98

Figure 5

Table 1 Comparisons of antioxidant capacities of synthesized antioxidants.

Antioxidants	TPC Gallic acid equivalents	ABTS Ascorbic acid equivalents	FRAP Ascorbic acid equivalents	ORAC Trolox equivalents	CAT Trolox equivalents	ApoCAT Trolox equivalents
Ascorbic acid	0.59 ± 0.00^{a}	1.00*	1.00*	0.95 ± 0.02^{b}	0.56 ± 0.04^{c}	0.19 ± 0.01^{d}
Ascorbyl palmitate	0.39 ± 0.01^{c}	0.74 ± 0.02^{c}	1.23 ± 0.01^{b}	0.96 ± 0.05^{b}	0.17 ± 0.00^{d}	0.56 ± 0.02^{b}
Gallic acid	1.00*	3.39 ± 0.09^{a}	2.99 ± 0.06^{a}	1.46 ± 0.00^{c}	0.67 ± 0.08^{b}	0.31 ± 0.05^{c}
Lauryl gallate	0.56 ± 0.01^{b}	1.22 ± 0.05^{b}	0.90 ± 0.03^{c}	0.23 ± 0.05^{a}	1.75 ± 0.12^{a}	3.27 ± 0.12^{a}

$^{a\text{-}d}$ Means within a column with different letters are significantly different($p<0.05$).

The * indicates the value of approximately 1 due to the antioxidant was used as the standard.

Table 2 Comparisons of antioxidant capacities of natural antioxidant extracts.

Antioxidants	TPC (mg gallic acid/g sample)	ABTS (mg ascorbic acid/g sample)	FRAP (mg ascorbic acid/g sample)	ORAC (mg trolox/g sample)	CAT (mg trolox/g sample)	ApoCAT (mg trolox/g sample)
Mixed tocopherols	268.5 ± 5.3^{b}	344.2 ± 6.9^{a}	360.6 ± 2.6^{b}	60.0 ± 14.1^{a}	503.9 ± 31.5^{c}	723.9 ± 41.5^{ab}
Green tea extract	40.0 ± 0.9^{d}	50.9 ± 0.0^{c}	58.9 ± 0.9^{d}	536.2 ± 29.3^{b}	195.9 ± 13.3^{d}	314.7 ± 25.5^{c}
Grape seed extract	365.0 ± 24.8^{a}	253.4 ± 1.2^{b}	582.8 ± 7.8^{a}	2101.7 ± 97.7^{c}	670.2 ± 34.0^{b}	640.9 ± 57.3^{b}
Rosemary extract	178.9 ± 0.0^{c}	45.8 ± 0.10^{c}	174.7 ± 6.5^{c}	2402.1 ± 79.4^{d}	736.2 ± 45.7^{a}	782.4 ± 57.7^{a}

$^{a\text{-}d}$ Means within a column with different letters are significantly different($p<0.05$).

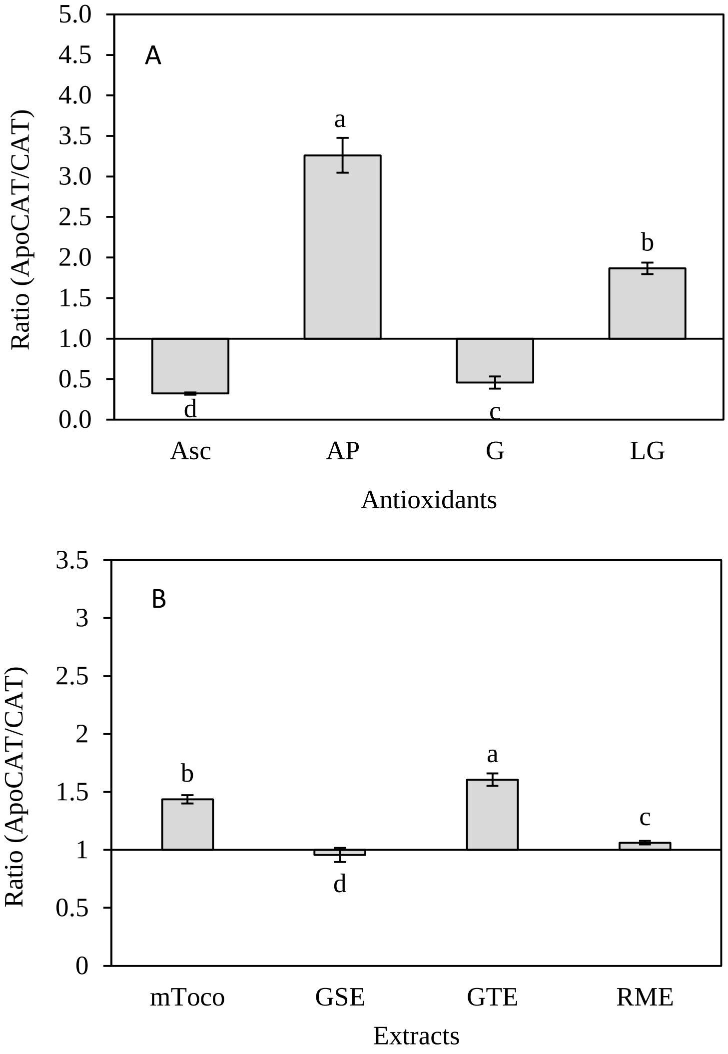

A
Ratio (ApoCAT/CAT)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
d
c
Asc
AP
G
LG
Antioxidants
B
Ratio (ApoCAT/CAT)
3.5
3
2.5
2
1.5
1
0.5
0
b
a
c
d
mToco
GSE
GTE
RME
Extracts

Figure 6

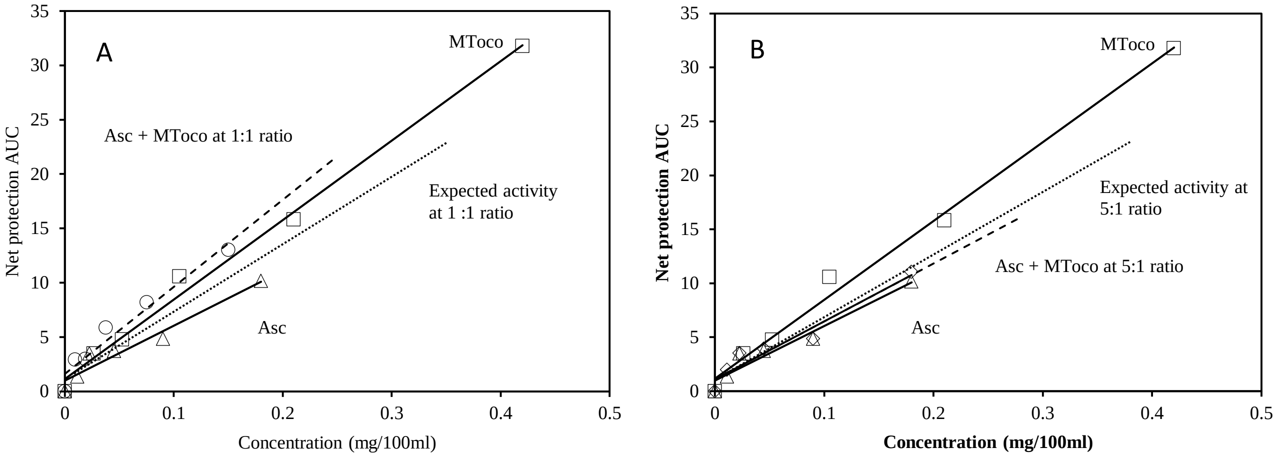

A
MToco
Asc + MToco at 1:1 ratio
Expected activity at 1 :1 ratio
Asc
Net protection AUC
Concentration (mg/100ml)
0
5
10
15
20
25
30
35
0
0.1
0.2
0.3
0.4
0.5
B
MToco
Expected activity at 5:1 ratio
Asc + MToco at 5:1 ratio
Asc
Net protection AUC
Concentration (mg/100ml)

Figure 7

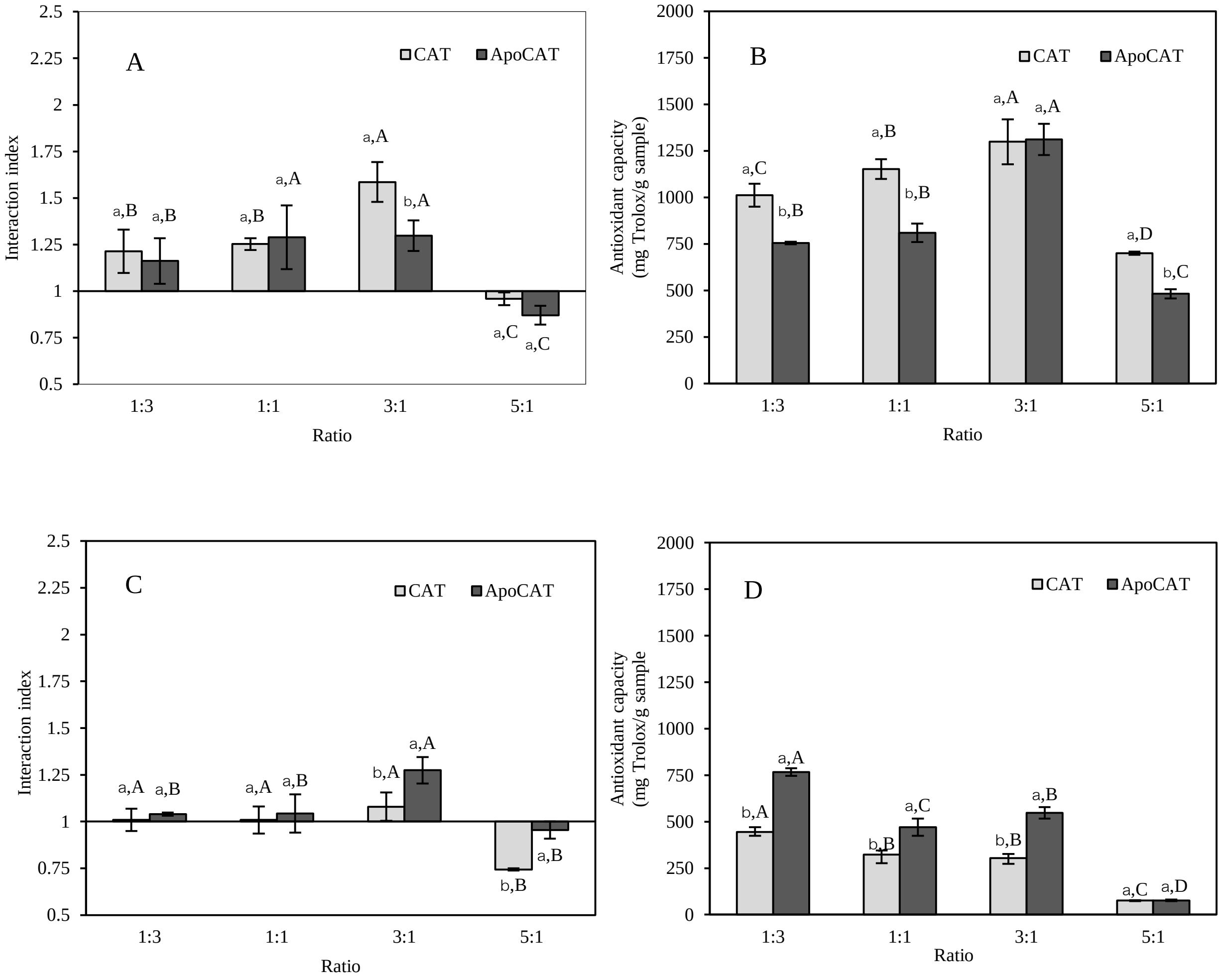

A
B
C
D
CAT
ApoCAT
Interaction index
Antioxidant capacity (mg Trolox/g sample)
Ratio
1:3
1:1
3:1
5:1
0.5
0.75
1
1.25
1.5
1.75
2
2.25
2.5
0
250
500
750
1000
1250
1500
1750
2000
a,B
a,B
a,B
a,A
a,A
b,A
a,C
a,C
a,C
b,B
a,B
b,B
a,A
a,A
a,D
b,C
a,A
a,B
a,A
a,B
b,A
a,A
b,B
a,B
b,A
a,A
b,B
a,C
b,B
a,B
a,C
a,D

Figure 8

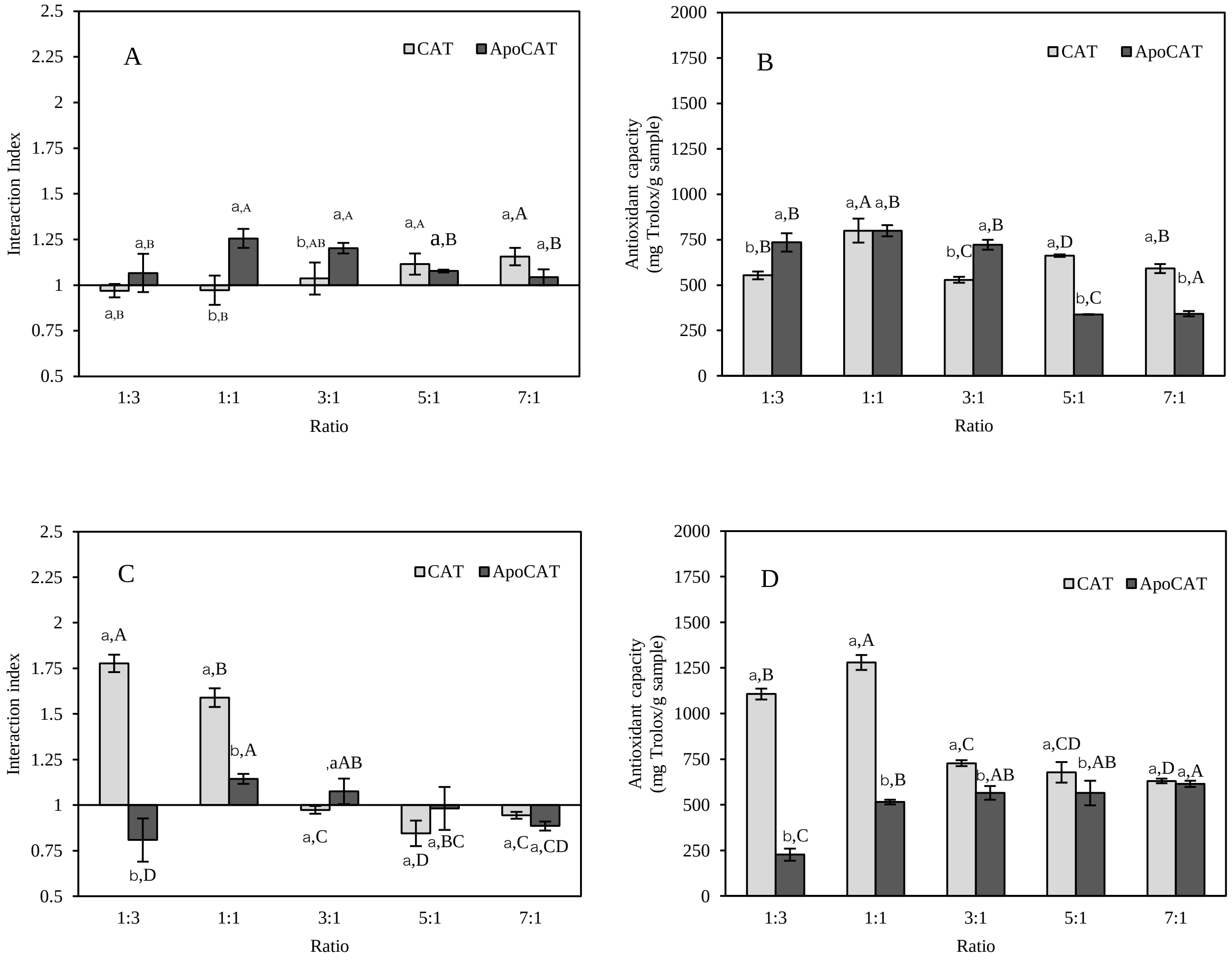

A
□CAT ■ApoCAT
Interaction Index
2.5
2.25
2
1.75
1.5
1.25
1
0.75
0.5
a,B
a,B
a,A
b,B
b,AB
a,A
a,A
a,B
a,A
a,B
1:3
1:1
3:1
5:1
7:1
Ratio
B
□CAT ■ApoCAT
Antioxidant capacity (mg Trolox/g sample)
2000
1750
1500
1250
1000
750
500
250
0
b,B
a,B
a,A
a,B
b,C
a,B
a,D
b,C
a,B
b,A
1:3
1:1
3:1
5:1
7:1
Ratio
C
□CAT ■ApoCAT
Interaction index
2.5
2.25
2
1.75
1.5
1.25
1
0.75
0.5
a,A
b,D
a,B
b,A
a,C
,aAB
a,D
a,BC
a,C
a,CD
1:3
1:1
3:1
5:1
7:1
Ratio
D
□CAT ■ApoCAT
Antioxidant capacity (mg Trolox/g sample)
2000
1750
1500
1250
1000
750
500
250
0
a,B
b,C
a,A
b,B
a,C
b,AB
a,CD
b,AB
a,D
a,A
1:3
1:1
3:1
5:1
7:1
Ratio

Figure 9

Appendix C

(Submitting Manuscript)

Comparison of Antioxidant Evaluation Assays for Investigating Antioxidative Activity of Gallic Acid and Its Alkyl Esters in Different Food Matrices

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A. Decker and Atikorn Panya[1*],

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Running title: Relationship between antioxidant evaluation assays and food matrices

ABSTRACT

The addition of antioxidants into food is one of the strategies to inhibit lipid oxidation, a major cause of food deterioration, which leads to rancidity development and nutritional losses. However, several studies have been reported that conventional antioxidant assays could not predict antioxidant performance in several foods. In this study, antioxidants with different polarities (gallic acid and its alkyl esters) were used to evaluate their antioxidant activities using both conventional antioxidant assays (TPC, ABTS, FRAP and ORAC) and two new assays (CAT and ApoCAT). The results indicated that the polarities of the antioxidants have a strong impact on antioxidant activities. Moreover, results from among the CAT and ApoCAT assays suggested that oxidant locations (free radicals) and antioxidant polarities could impact on the overall antioxidant performances in particular tested systems. The antioxidative performance of gallic acid and its alkyl ester was investigated in oil in water (O/W) emulsions, bulk soybean oil, and roasted peanut as the lipid food models. The result showed that the ApoCAT assay was able to predict the antioxidative performances in O/W emulsions regardless of the antioxidant polarities. This study demonstrated that the relevance of antioxidant assays to food models is strongly dependent on the similarity between the tested assays and the food structure matrices.

Keywords: Lipid oxidation; Antioxidant; Antioxidative performance; Food matrices

INTRODUCTION

Lipid oxidation is the important reaction in either biological and food systems. In food containing lipid, lipid oxidation has been considered as the deteriorative reaction [1]. To solve the lipid oxidation problem, numerous strategies have been incorporated into food products which one of the most effective strategies is the addition of antioxidant [2]. Antioxidants are substances which prevent and/or delay the oxidation reaction of others molecules. Antioxidants can prevent lipid oxidation by various mechanisms including scavenging free radical, chelating metal ions, decomposing lipid peroxides, quenching reactive oxygen species, preventing formation of peroxides, breaking the antioxidative chain reaction, and reducing localized O_2 concentrations [3].

Recently, there is no standardized and/or single method to determine the antioxidant capacity of substances[4]. Nowadays, various methods have been used to determine antioxidant activities based on both non-competitive and competitive reaction schemes. The non-competitive assays for example; ABTS and FRAP assays, measure the antioxidative ability of substance to directly reduce an oxidant. On the contrary, the competitive assays are the methods which measured the ability of antioxidant competing with the oxidizable substrate for an oxidant. For example, the ORAC assay is one of the competitive antioxidant evaluation assays which measured the ability of antioxidant competing with fluorescein as the substrate for peroxyl radicals generated through the thermally decomposition of azo compound [5]. In the past, researchers have been tried to set the standard antioxidant evaluation assay. In 2007, the USDA released the database for the ORAC values of 277 food items. In addition, the ORAC values of 49 food items were added into the database in 2010 for a total of 326 food items [6]. However, the ORAC database was removed from the USDA's Nutrient Data Laboratory (NDL) database due to various evidences supporting that the ORAC assay have no relevance to the antioxidative abilities of bioactive compounds, including polyphenols on human health [7].

Moreover, the relationships between the antioxidant abilities of compounds in various assays and their antioxidative performances in actual food models have been studied. Due to the complexity of food matrices, the evidences supported that antioxidant values obtained from homogeneous assays such as DPPH, FRAP and ORAC were poorly to predict the antioxidant effectiveness of compounds in food systems [2, 8]. In foods, lipids are generally consisted with others components as dispersion. Thus, the partitioning of antioxidant in food plays a crucial role on the effectiveness of preventing lipid oxidation as rationalized in polar paradox hypothesis.

Recently, the heterogeneous based antioxidant evaluation assay called the conjugated autoxidizable triene (CAT) assay has been created [9]. The CAT assay measures antioxidant ability of substances in oil-in-water (O/W) emulsions. Thus, the partitioning effect of antioxidants in the system has been incorporated in this assay. The apolar radical-initiated conjugated autoxidizable triene (ApoCAT) assay has been created base on the CAT assay to eliminate the free radical bias from the water soluble free radical initiator by using a lipid soluble free radical initiator (V601)[10]. In the recent, there are no studies supporting the relationship between homogeneous and heterogeneous antioxidant evaluation assays, and their ability to predict the antioxidant activity in the actual food systems. Therefore, the aims of this study were to investigate the antioxidant activities of gallic acid and its alkyl esters using various antioxidative evaluation assays (TPC, ABTS, FRAP, ORAC, CAT and ApoCAT) and the relationship between antioxidant evaluation assays and their ability to predict the antioxidant effectiveness in various food models.

MATERIALS AND METHODS

Chemicals reagents

Trichloroacetic acid (TCA), Butylated hydroxyl toluene, thiobarbituric acid, cumene hydroperoxide and 1,1,3,3,- tetrahydroxypropane were purchased from Merck (USA). Ethylenediaminetetraacetic acid (EDTA), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), silicic acid, folin-Ciocalteau's reagent, sodium carbonate, ferrous sulfate, ferric chloride, ferrozine, Tung oil, Brij 35, ascorbic acid, Polyoxyethylene sorbitan monolaurate (TWEEN® 20), barium chloride and ammonium thiocyanate were purchased from Sigma-Aldrich (St. Louis, USA). Absolute ethanol was purchased from RCI Labscan (Thailand). 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), dimethyl 2,2'-azobis(2-methylpropionate) or V601 and activated charcoal were purchased from Wako Chemical (Japan). Medium chain triglyceride (MCT) mixture (Miglyol®812N) was purchased from Sasol Germany Gmbh (Witten, Germany). Gallic acid, methyl gallate, propyl gallate, butyl gallate, octyl gallate and laury gallate were purchased from Sigma-Aldrich (St. Louis, USA). Cold pressed perilla oil was purchased from a local market in Thailand. Deionized water was used for the preparation of all solutions. All organic solvents used in this study were analytical grade.

Total phenolic content (TPC) Assay

Total phenolic content (TPC) of gallic acid and its alkyl esters, and plants extracted was determined using Folin-Ciocalteu method with some modifications [11]. The reaction was done in 96-well microplates. Samples (30 µl) were mixed with 150 µl of Folin-Ciocalteau's reagent (10-time dilution) and 120 µl of sodium carbonate (7.5% w/v). The mixtures were mixed using a microplate mixer (Thermomixer comfort; eppendorf, Germany) at 600 rpm for 1 min, and kept in the dark for 30 min. The absorbance of mixture was read at 765 nm using a microplate reader (Spectra MAX 190, Molecular Devices, USA). Gallic acid was used as a standard phenolic compound. Results were expressed as gallic acid equivalent.

ABTS radical decolorization (ABTS) assay

The free radical scavenging activity of plants extracts was performed using the ABTS assay [11]. ABTS cation radicals (ABTS●+) were generated by reacting 7 mM ABTS stock solution (10 ml) with 140 mM potassium persulfate (179µl). Then, the mixture was incubated at room temperature for 16 h under the dark. The ABTS●+ solution was diluted to obtain OD of 0.700±0.050 at wavelength 734 nm before use. Twenty microliters of samples were mixed with 280 µl of the ABTS●+ solution and then incubated in the dark at room temperature for 6 min. The absorbance of mixture was then read at 734 nm using a microplate reader. The amount of the absorbance decrease was expressed against the standard curve of various concentrations of ascorbic acid. Results were expressed as ascorbic acid equivalent.

Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) assay was performed using the modified method as described by [11]. The FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl and 20 mM FeCl3.6H2O at a ratio of 10:1:1. The reactions were started by mixing 30 µl of samples with 270 µl of FRAP reagent. The reaction was performed in 96-well microplates. The total volume of reaction was 300 µl. The microplates containing reactive mixture were kept in the dark at 37 °C for 8 min. The increase in absorbance of mixtures was read at 595 nm using a microplate reader. Using ascorbic acid as a standard curve, results were expressed as ascorbic acid equivalent.

The oxygen radical absorbance capacity (ORAC) assay

The oxygen radical absorbance capacity (ORAC) assay method was performed with some modifications [12]. Fluorescein was used as the fluorescent probe, and AAPH was used as the oxidizable substrate. The final reaction mixture consisted of fluorescein (160 µl, 78 nM), sample (20 µl) and AAPH (20 µl, 12 mM). All reagents were prepared in 75 mM phosphate buffer solution, pH 7.4. The ORAC assay was done in black 96-well plates (Eppendorf Microplate 96/U-PP). Fluorescent intensity of the mixture was recorded each minute with 5 s agitation using a fluorescent microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 520nm in kinetic mode for 3 h

The conjugated autoxodizable triene (CAT) assay

The conjugated autoxidizable triene (CAT) assay method was performed with some modifications [9]. Conjugated triene triacylglycerols from Tung oil were used as the oxidizable UV probe for an oxidation reaction. All of the reagents and samples were dissolved and diluted in phosphate buffer (50 mM, pH7.2). The CAT assay protocol was composed of 2 steps described as follow.

1. Emulsion preparation

Phosphate buffer (PB) solution (50 mM, pH 7.2) containing 34 µM Brij 35 was added to 6±1 mg of stripped Tung oil. The mixture was then homogenized (Ultra-TurraxT25 basic, IKA® Werke, Germany) at 6,500 rpm for 90 s. The emulsion was centrifuged at 10,000 g, 10 °C for 5 min to remove the unstable part.

2. Reaction mixture

A 50-µl volume of 50 mM PB containing samples (desirable concentration) was transferred to a UV-Star 96-well plate followed by 125 µl of emulsion. The 96-well plate containing antioxidants and emulsions was mixed at 1,200 rpm, 37 °C for 5 min by a microplate thermomixer (Thermomixer comfort, eppendorf, Germany). After mixing, 25 µl of 8mM AAPH solution in PB solution was added into the mixtures. The final mixture volume was 200 µl with 17 µM Brij 35, 1 mM AAPH and various concentrations of antioxidants. Changes in the absorbance at 273 nm in of mixtures were then recorded using a UV-domain microplate reader in kinetic mode for 3 h. The CAT values were expressed as trolox equivalent by calculating the relationship between net protection areas under the curve (AUC) and concentrations of antioxidants compared to that of trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was .

Tung oil was striped prior using to remove the minor polar components. Briefly, 100 g of silicic acid was washed with distilled water for three times with a total volume of 3 L, then the washed silicic acid was filtered with Whatman filter paper and dried at 110 °C for 20 h. The glass chromatographic column (3.0 cm internal diameter×35 cm height) was packed sequentially with 22.5 g of washed silicic acid followed by 5.625 g of activated charcoal and 22.5 g of washed silicic acid. The washed silicic acid and activated charcoal were suspended in 100 ml and 70 ml of n-hexane, respectively, before packing into a glass column. Thirty grams of Tung oil were dissolved in 30 ml of n-hexane, and passed through the column by eluting with 270 ml of n-hexane. The collected triacylglycerols were kept in an ice bath and covered with an aluminum foil in order to minimize lipid oxidation during stripping process. To remove the n-hexane from the collected stripped Tung oil after the elution completed, the rotary evaporator (BÜCHI Rotavapor R-114, Switzerland) was performed at 35 °C under vacuum. The nitrogen flushing was adapted to remove traces of hexane remaining in the oil. After flushing for 10 min, 3 ml of stripped oil was transferred into vials and flushed with nitrogen again. The aliquots of stripped Tung oil were kept at −80 °C before use [13].

The apolar radical-initiated conjugated autoxidizable triene (ApoCAT) Assay

The ApoCAT assay was performed with slightly modifications [10]. Based on the original CAT assay, all procedures of the ApoCAT assay were performed in the same manner as the original CAT assay except for the free radical initiator. Instead of using AAPH, dimethyl 2,2'-azobis(2-methylpropionate) or V-601, an lipid-soluble free radical initiator was used in this assay. To deliver the lipid radical initiator into emulsion mixtures, Medium chain triglycerides (MCT) emulsions were prepared. Briefly, 10 mM PB solution (pH 7.2) containing 34 µM Brij 35 was added to 6±1 mg of MCT oil. The mixture was then homogenized at 6,500 rpm for 90 s. The V601 radical initiator was prepared by dissolving in the MCT emulsions to obtain a final concentration of 8 mM. To remove oil residues and other non-stable parts, the emulsion was centrifuged at 10,000 g, 10 °C for 5 min. The MCT emulsion containing 8 mM V-601 was collected from the supernatant. To begin the oxidation of the ApoCAT assay, 25 µl of the MCT emulsion with V601 was added into the prepared Tung oil emulsions with antioxidants as described in the CAT assay.

Oil-in-water (O/W) emulsion preparation

In this study, oil-in-water (O/W) emulsions were used to represent a heterogeneous food model for evaluating the antioxidative performance of gallic acid and its alkyl esters. The perilla oil was stripped prior to use in the same manner as Tung oil as desceibed in the CAT assay. The O/W emulsion was prepared with slightly modifications. Shortly, 1% of stripped perilla oil was mixed with tween 20 at the emulsifier/oil ratio of 1:10 in PB (10 mM, pH 7.0) by homogenizing at 9,500 rpm for 2 min (Ultra-Turrax T25 basic, IKA® Werke, Germany). Then, the coarse emulsion mixture was homogenized with ultrasonic treatments using a 20 kHz 130 W ultrasonic processor (Sonic, Vibra-cell™ VCX 130, USA) equipped with a 13 mm diameter tip (with 100% amplitude). Total pulse duration was 2 min (30 s each pulse, with 10 s interval) [13].

Oxidative stability of oil-in-water (O/W) emulsions

The perilla O/W emulsions was added with gallic acid and its alkyl esters to obtain final concentration of 50 µM. The O/W emulsions containing tested antioxidants were transferred into 96-deep well plates. The foiled cover sheets were used to minimize the evaporation of emulsions in each well. The plates were then incubated at 30 °C. Samples were taken periodically to determine the oxidation products. Formation of lipid hydroperoxide values (PV) and thiobarbituric acid reactive substances (TBARs) were evaluated as the indicators of primary and secondary lipid oxidation products, respectively.

Measurements of lipid hydroperoxides

Formation of lipid hydroperoxide was determined with some modifications[14]. Fifty microliter of emulsions were first mixed with 450 isooctane: isopropanol (3:1) then centrifuged (3,200 g) for 3 min to break emulsions. Then, 50 µl of the upper layer of organic solvent phase was collected and 200 µl of methanol: butanol (2:1) was added. The reaction begins with the addition of 20 µl of ammonium thiocyanate reagent. The ammonium thiocyanate reagent was prepared by mixing $FeSO_4$ (0.144 M) with BaCl2 (0.132 M) at the ratio 1:1. After that, the mixture was centrifuge at 3,200 rpm for 2 min. The upper clear phase of centrifuged mixture was mixed 3.94 M of ammonium thiocyanate solution at the ratio of 1:1. The reaction mixture of was left at room temperature for 20 min, and the absorbance was recorded at 510 nm using a microplate reader. Cummene hydroperoxide was used as a standard lipid hydroperoxide.

Thiobarbituric acid reactance (TBAR) assay

Thiobarbituric acid reactance (TBAR) assay was performed with slightly modifications[2]. Briefly, TBA reagent was prepared by mixing 20% of TCA, 0.5% of TBA and 0.2% of EDTA in 30mM HCl. Immediately before analysis, 30 ml of TBA reagent was mixed with 450 µl of 3% BHT in absolute ethanol solution. Emulsion (150 µl) was reacted with the TBA reagent (300 µl) in 96-deep well plates. The plates containing mixture were covered and heated at 90 °C for 20 min. Then, 96-deep well plates were transferred into an ice bath to stop the reaction for 10 min. Then, the plates were centrifuged at 3,200 g for 5 min. The supernatant was collected to measure the absorbance at 532 nm. A calibration curve was prepared with 1,1,3,3-tetrahydroxypropane as a standard for quantification.

The expression of oxidative stability of oil-in-water (O/W) emulsions

The oxidative stability of emulsions was expressed as the induction periods (IP) or oxidation lag time obtained from the formation of hydroperoxide and TBARs. The induction periods were defined as the first data point that statistically increased from the previous data points during the oxidation studies. Comparisons of the means were performed using Duncan’s multiple range tests.

Oxidative stability of bulk soybean oils and roasted peanuts

The antioxidative performances of gallic acid and its alkyl esters were investigated in different food matrices including bulk soybean oils and roasted ground peanuts using Rancimat test. Commercial soybean oil (TVO public company limited, Thailand and freshly prepared roasted ground peanuts were used in this experiment. The soybean oil and roasted ground peanuts were mixed with the ethanolic solution of gallic acid and its alkyl esters to obtain the final concentration of 2 mmoles per kilogram. For soybean oil samples, ethanol was evaporated out using a rotary evaporator operating at 35 °C under vacuum for 10 min. For roasted grounded peanut, samples were hand shaken to obtain homogeneous mixture for 3 min after the addition of antioxidants. After that, the roasted grounded peanut samples were dried in a hot air oven 35 °C for 30 min to remove the remaining ethanol. After soybean oil and roasted grounded peanut were completed mixed with gallic acid and its alkyl esters, the commercial instrument called “Rancimat” machine (Rancimat 743, Metrohm CH-9101, Herisau, Switzerland) was adapted to determine the oxidative stability of foods. The instrument was set at 110 °C and air flow rate of 20L/h[15]. Roasted peanut were further ground before tested. The sample size of bulk soybean oils and roasted ground peanuts were three grams and one gram, respectively. Samples were weighed into the reaction tube and placed in the Rancimat instrument. The conductivity of deionized water was recorded kinetically. The induction times (IT) of oxidation were calculated using 743 Rancimat software (Metrohm CH-9101, Herisau, Switzerland).

Statistical analysis

All analyses were performed on triplicate samples. The relationship between antioxidant evaluation assays were analyzed by principle component analysis (PCA) using SPSS 17 (http://www.spss.com; SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Antioxidant activities of gallic acid and its alkyl esters in homogeneous antioxidant assays

According to the homogeneity of mediums, the TPC, ABTS, FRAP and ORAC assays representing examples of homogeneous antioxidant assays have been commonly used in many research areas to evaluate the ability of compounds to donate hydrogen atoms and/or electrons. Similar to ABTS and FRAP assay, the total phenolic content (TPC) assay also evaluates the electron donating ability of compounds by evaluating reduction of molybdate in

Folin-Ciocalteu (F-C) reagent via oxidation-reduction reactions in comparison to gallic acid as a phenolic standard[16]. Dependent upon the nature of the reaction, these assays can be categorized into competitive (the ORAC assay) and non-competitive systems (the TPC, ABTS and FRAP assays). In the ORAC assay, antioxidants have to compete with oxidants (AAPH) in order to prevent the oxidation of substrates (fluorescence), while in the TPC, ABTS and FRAP assays; antioxidants are able to react directly to oxidants. Thus, antioxidants performing well in the ORAC assay should have not only the ability or capacity to give electrons, but also high reactivity to radicals.

As shown in Table 1, antioxidants performing in the non-competitive assays (the TPC, ABTS and FRAP assays) exhibited a similar behavioral pattern that increasing alkyl chain lengths of a free acid form of gallic acid (G0) to methyl gallate (G1) dramatically decreased the antioxidant activity. Further decreases in the antioxidant activities were observed when alkyl chain lengths were increased. Gallic acid had the highest TPC, ABTS and FRAP values following by methyl gallate (G1), propyl gallate (G3), butyl gallate (G4), octyl gallate (G8) and lauryl gallate (G12), respectively.

However, in the ORAC assay, increases in alkyl chain lengths from G0 to G3 improved the antioxidant activity as indicated by the ORAC value. Propyl gallate (G3) exhibited significantly higher ORAC value than gallic acid (G0). This result was consistent with the previous study by Alamed and co-workers that propyl gallate exhibited higher ORAC value than gallic acid[2]. It should be noted that further increases in alkyl chain lengths beyond G3, antioxidant activities decreased as shown in Table 1.

In terms of the kinetic point of views, decreasing patterns of the absorbance of fluorescein during the ORAC assay indicated that all antioxidants exhibited clear oxidation lag times. Results indicated that chain-breaking behaviors were observed regardless of alkyl chain lengths (data not shown). This result suggested that peroxyl radicals generated from

AAPH could react with gallic acid in the same manner as other alkyl gallate esters. However, the higher concentrations, the longer lag times of antioxidants were observed.

Since the ABTS and FRAP assays were performed in aqueous solutions the antioxidant values of gallate esters decreased according to their alkyl chain length. This phenomenon could be explained that the polarities of gallate alkyl esters were decreased resulting in their lower solubility in the aqueous system. Thus, the reduction of solubility of gallate alkyl ester could influence their ability to scavenge free radical as compare to gallic acid. Moreover, the gallate alkyl esters could be self-aggregated by the hydrophobic effect as explained by self-assembly of the nonpolar substances in aqueous or polar solvents[17]. The aggregation of gallate alkyl esters might occur as micelles, lamellar structures and other association colloids[18].

With the increasing of alkyl chain lengths, G1, G3 and G4 exhibited higher ORAC values than G0. This result might be explained by the esterification reaction increasing the radical scavenging activity of substances, which was consistent to the study of Lu and co-workers (Lu et al., 2006). However, the ORAC value decreased at G8 and observed lowest at G12, it might be due to the aggregation of lipophilic molecules in water phase, which may decrease the solubility of antioxidants resulting in lower antioxidant activities.

Antioxidant activity of gallic acid and its alkyl esters in heterogeneous antioxidant assays

The conjugated autoxidizable triene (CAT) assay has been developed to determine the antioxidant activity in oil-in-water emulsions[9]. Since the CAT assay is conducted in oil-in-water emulsion, the locations of antioxidant in the system have to be accounted for their antioxidative performances. According to the polar paradox hypothesis, the non-polar antioxidant is more active in emulsified systems[19].vResults showed that butyl gallate (G4) exhibited the highest value of the CAT assay followed by propyl gallate (G3), methyl gallate (G1), lauryl gallate (G12), octyl gallate (G8) and gallic acid (G0), respectively (Table 2). According to this result, it was inconsistent with the polar paradox hypothesis. The non-linear trend was observed after increased the alkyl chain length to G4. Interestingly, the antioxidant activity of alkyl gallate esters was sharply dropped at G8 before increasing again at G12. According to the AAPH (water soluble radical) used in the CAT assay, it was hypothesized that an overestimation of the antioxidant capacity of water soluble antioxidants, and on the contrary, an underestimation of the antioxidant performance of a lipid soluble antioxidant may be observed. Therefore, the ApoCAT assay, a modified version of the CAT assay, has been developed[10]. Instead of using AAPH, a lipid soluble radical initiator called V601or AIBME was used to initiate oxidation reaction.

As illustrated in Table 2, results indicated that lauryl gallate (G12) exhibited the highest ApoCAT value followed by octyl gallate (G8), butyl gallate (G4), propyl gallate (G3), methyl gallate (G1) and gallic acid (G0). Unlike the CAT assay, the linear trend between antioxidant values and alkyl chain lengths was observed in the ApoCAT assay.

There were some other experiments observing the nonlinear trend of antioxidant behaviors in emulsion systems. For example, the antioxidant activity performing in the CAT assay of chlorogenic acid and its alkyl ester were collapsed after dodecyl chain[20]. Furthermore, other studies also observed the nonlinear trend against the polar paradox hypothesis using various antioxidants with different polarities in emulsion systems[14, 21]. According to the nonlinear trend of antioxidant behaviors, the cut-off hypothesis has been introduced to explain these phenomena[18]. The maximum alkyl chain length at which an antioxidant exhibited the highest antioxidative activity in emulsions was regarded as critical chain length or CCL. The cut-off effect of antioxidants in oil in water emulsion has been explained by three putative mechanisms of action including the reduced mobility, the internalization and the self-aggregation hypotheses. First, the reduced mobility, when the alkyl chain of antioxidant was lengthened, their mobility to move toward the oxidation sites was reduced due to the strongly bounding to the molecular environment by hydrophobic interactions[18]. Another mechanism of action explaining the cut-off hypothesis is internalization. The internalization hypothesis explained that increasing the hydrocarbon chain from medium to long chain resulting in the deeply burying of antioxidants into the lipid droplet core. Consequently, the poor antioxidative performance is observed due to the antioxidant compounds located far away from the oil/water interface where the oxidation occurs[18]. For the last hypothesis, self-aggregation, beyond the CCL, the antioxidant activity is decreased according to aggregation of antioxidants with each other in order to form micelles in the water phase of emulsions. When the self-aggregation occurs, the concentration of antioxidants at the oxidation sites may be decreased, resulting in the lower ability of antioxidant to prevent lipid oxidation in emulsified systems[18].

In contrast, the cut off effect was not found in the ApoCAT assay. Due to the lipid soluble free radical initiator used in the ApoCAT assay, free radical would locate at oil-water interphase. Thus, the scavenging reaction between the antioxidant and free radical in water phase was not occurred and the overestimation of water soluble antioxidant may not observe.

Moreover, the ratio between ApoCAT and CAT value was generated. The ApoCAT/CAT ratios of gallic acid (G0), methyl gallate (G1), propyl gallate (G3), butyl gallate (G4), octyl gallate (G8) and lauryl gallate (G12) were calculated as illustrated in Figure 1. The ratio of ApoCAT/CAT provided more information on characteristics of antioxidants preferring towards free radical in water- and/or lipid-phases. As expected, the lipophilic antioxidants (G8 and G12) performed better in ApoCAT system as their calculated values were above 1. In contrast, it might be summarized that G0, G1, G3 and G4 exhibited the preferential activity toward water soluble free radical. This result was consistent to the study on the effect of oxidant location on antioxidant capacities using ApoCAT and CAT ratio from Panya and co-workers which demonstrated that the esters forms of antioxidants had higher ratio than their acid forms[10]. It is worth mentioned that this ApoCAT/CAT ratio could be applied for characterizing the unknown antioxidant or natural crude extracts.

From the kinetic point of views of the CAT assay as shown in Figure 2, lag phase was observed in only methyl gallate (G1) and octyl gallate (G8) while no clearly lag phases were observerd in gallic acid (G0), propyl gallate (G3), butyl gallate (G4) and lauryl gallate (G12). Results indicated that only G1 and G8 showed the antioxidant behaviors as chain breaker while others showed retarder behaviors in the CAT assay. However, in the ApoCAT assay, no lag phases were observed for all tested compounds (Figure 3). Results indicated that gallic acid and its alkyl esters exhibited the antioxidant behaviors as retarders in the ApoCAT assay. According to the CAT assay, the result was consistent to previous study of Laguerre and co-workers who also found the retarder antioxidant behavior of gallic acid[9].

Antioxidative performances of gallic acid and its alkyl esters in oil-in-water (O/W) emulsions

The performances of gallic acid and its alkyl esters to prevent lipid oxidation in oil-in-water (O/W) emulsions were evaluated using the induction periods obtained from the formation of primary lipid oxidation products (peroxide value or PV value) and secondary lipid oxidation products (thiobarbituric acid reactance or TBAR value). As shown in Table 3, the induction periods were estimated from the end of lag periods at which the PV and TBAR values were statistically increased from the previous values. The induction periods calculated based on the PV and TBAR values of O/W emulsion treated with 50 µM of gallic acid (G0), methyl gallate (G1), propyl gallate (G3), butyl gallate (G4), octyl gallate (G8) and lauryl gallate (G12). The linear trend of antioxidative performance in O/W emulsions was observed according to the alkyl chain lengths. This observation could be explained by the polar paradox hypothesis that the non-polar antioxidants are more active than their polar homologues in O/W emulsion systems.

Antioxidative performances of gallic acid and its alkyl esters in bulk soybean oils

Bulk soybean oil was used as the bulk oil model to evaluate the antioxidant performances of gallic acid and its alkyl esters. In bulk oil, lipid oxidation mechanisms are different from emulsified system due to many factors[1]. It was hypothesized that polar antioxidants are more effective in bulk oil than their non-polar homologues[22].

The antioxidative performances in bulk soybean oil of gallic acid (G0), methyl gallate (G1), propyl gallate (G3), butyl gallate (G4), octyl gallate (G8) and lauryl gallate (G12) were evaluated using Rancimat test. The increasing of conductivity by trapping lipid oxidation products in water was monitored kinetically. Result showed that G0 exhibited the highest induction time followed by G4, G8, G12, G3 and G1, respectively (Figure 4).

The result was consistent to the polar paradox hypothesis that gallic acid (polar homologue) was the most effective antioxidant in bulk soybean oil compared to its non-polar homologues (gallate esters). However, increasing alkyl chain length to G3, the performances of gallate esters in bulk soybean oil were increased. As illustrated in Figure 4, the result indicated that antioxidant performances of gallic acid and its alkyl esters in bulk soybean oil were depended regardless of the polarities. This result was in agreement with the study on chlorogenic acid and its alkyl esters which hydrophobicity did not exert a strong influence on antioxidant capacity [23]. It is worth mentioned that there might be some other minor components and water remained in soybean oil. Thus, the association colloids could form in bulk soybean oil resulting in the high susceptibility of bulk soybean oil to oxidation reaction [1]. This result could give a good provide that not only the intrinsic factors such as hydrophobicity of antioxidant influences on the antioxidant capacity but also the extrinsic factors of environmental system could exert the high influencing of the antioxidant capacity.

Antioxidative performances of gallic acid and its alkyl esters in roasted peanuts

According to the studies of antioxidative performances of gallic acid and its alkyl esters in oil-in-water emulsions and bulk soybean oil, the results showed that the antioxidant ability of substance to protect lipid oxidation was not depended on the only intrinsic factors of antioxidant. However, the extrinsic factors such as the nature of food system also play the key role on the antioxidative performances. To better understand the antioxidative performances of substances in various food models, roasted peanut was chose as a low moisture food containing lipid model.

The antioxidative performances of gallic acid and its alkyl esters were determined in roasted peanut using Rancimat test. Roasted peanuts treated with lauryl gallate (G12) exhibited the highest induction time followed by octyl gallate (G8), butyl gallate (G4), methyl gallate (G1), propyl gallate (G3) and gallic acid (G0), respectively (Figure 5).

Morover, the kinetic conductivity curves of roasted peanuts were different from soybean oil (data not shown). At the beginning period, the conductivities of distilled water obtained from roasted peanut oxidation were rapidly increased. After that, the conductivities were stable and increased again after the rate of oxidation significantly increased. According to the homogeneity, roasted peanuts are commonly composted of other organic components, for example carbohydrates and proteins. Thus, the oxidation reaction scheme in roasted peanuts might occur differently from bulk soybean oil. In roasted peanut, some oil component might not mix with the antioxidant, resulting in the oxidation reaction at the beginning period of the Rancimat test.

In recent, the information on the antioxidant effectiveness in low moisture food is limited (Barden 2014). This result suggested that the antioxidant effectiveness in roasted peanuts increased with increasing the hydrophobicity of antioxidants. Results were in agreement with the study of rosmarinic acid and its alkyl esters in cracker that similar to gallic acid, rosmarinic acid exhibited the lowest antioxidant efficacy to inhibit lipid oxidation in low moisture foods. The reason for these results might be due to the exclusion of polar antioxidants from the lipid phase resulting in the separation of antioxidants from lipid oxidation reaction site[24]. On the contrary, increasing the hydrophobicity of antioxidant would make antioxidants more capable to migrate to lipid oxidation site thus the more effectiveness to prevent lipid oxidation were observed in low moisture food.

Relationship between antioxidant activities in various antioxidant assays and antioxidative performances in food models of gallic acid and its alky esters

The Relationship between antioxidant activities in various antioxidant assays and antioxidant performances in different food matrices of gallic acid and its alky esters were investigated using principle component analysis (PCA). As illustrated in Figure 6a, from ten parameters, they could be reduced into two components (PC1 and PC2) explaining 95.40 % of total variance. Each component was responsible for 52.38% and 43.02%, respectively. The PC1 represented the ApoCAT value, induction periods from PV and TBAR values of oil-in-water emulsions and the oxidative stability of roasted peanuts (IT_RP), while the PC2 explained the TPC, ABTS and FRAP values, and the oxidative stability of bulk soybean oils (IT_SBO). The PCA score plot of gallic acid and it alkyl esters as shown in Figure 6b suggested that lauryl gallate (G12) and octyl gallate (G8) were strongly explained by the assay in PC1. In contrast, gallic acid was highly correlated with the assays categorizing in

PC2. However, methyl gallate (G1), propyl gallate (G3) and butyl gallate (G4) were intermediate correlate with the assays in both PC1 and PC2.

The results were consistent with previous studies that the ORAC assay was not related to the TPC, ABTS and FRAP assays[25]. However, some studies had shown the good correlation between the ORAC assay and others assays (the TPC, DPPH and FRAP assays)[26]. Although, the good relationship of the ORAC assay and others were found in some experiments, many researchers suggested that when antioxidants were different kinds, the correlation between the ORAC and other methods would be low because of the different kinetics and reaction mechanisms of the various antioxidants present [27].

Furthermore, the results were in agreement with the study on relationship between radical scavenging activity and antioxidant activity in foods which reported that the ORAC assay was not able to predict the antioxidant activity of compounds in oil-in-water emulsions and cook ground beef [2]. The lack of relationship between antioxidant activities obtained from homogeneous assay such as TPC, ABTS, FRAP and ORAC and antioxidant activities of compound in food might be due to the complexity of food. Thus, food matrices are the important part providing the multitude factors influencing on the ability of compound to inhibit lipid oxidation in food system.

Recently, the CAT assay has been developed to evaluate the antioxidant activity of substances in a heterogeneous system (O/W emulsions). However, our results showed that the CAT assay was not able to predict antioxidant performance in all three types of food systems (O/W emulsions, bulk soybean oils and roasted peanuts). The CAT assay could not predict the actual antioxidant activity in foods due to the unrealistic oxidation reaction occurring in the tested systems. The CAT assay used AAPH which is water soluble radical initiator to initiate lipid oxidation thus making the radical locating in water phase of emulsion. From this reason, it is unlike lipid oxidation in complex food systems in which lipid oxidation occurs at the oil/water interface regions of emulsion droplets[1]. To solve this problem, the ApoCAT assay has been developed to use the lipid soluble radical initiator instead of AAPH (a water soluble radical initiator). According to the lipid soluble radical initiator in ApoCAT test system, the lipid oxidation could occur at the interphase of water-oil droplets, and thus the phenomenon might relate to lipid oxidation in actual food systems.

Interestingly, as illustrated in Figure 6a, our results exhibited a close relationship between the ApoCAT assay with the induction periods of PV and TBAR formation in oil-in-water emulsions and the oxidative stability of roasted peanuts (IT_RP). However, the ApoCAT had a poor correlation with the oxidative stability of bulk soybean oils (IT_SBO). This result could be explained that lipid oxidation mechanisms in bulk oil systems might be different from emulsion systems.

From the PCA score plot (Figure 6b), results suggested that high polar antioxidant (G0) exhibited high antioxidant values in non-competitive homogeneous assays while non-polar antioxidant (G8 and G12) exhibited high antioxidant value in heterogeneous assays. In addition, the correlations among all tested methods were calculated and showed that the ApoCAT assay was significantly correlated with the oxidative induction periods of oil-in-water (O/W) emulsions (PV and TBAR) and the oxidative induction times of roasted peanuts (IT_RP) (data not shown).

CONCLUSION

In conclusion, to be able to accurately predict antioxidant performances in complex food systems using the rapid determination of antioxidants, new knowledge on influences of food matrices on oxidation and antioxidant mechanisms should be taken into consideration. In this study, we demonstrated that the ApoCAT assay was able to predict only the activities of antioxidant in oil-in-water emulsions, suggesting that the heterogeneous antioxidant evaluation assays could predict the antioxidant activities in food models having similar matrix to the tested system not only in terms of food structures, but also oxidant locations.

Table list

Figure list

Figure 1 The ratio between the ApoCAT and CAT values of gallic acid (G0), methyl gallate (G1), propyl gallate (G3), butyl gallate (G4), octyl gallate (G8) and lauryl gallate (G12)

Figure 2 Kinetic decay of conjugated triene in the CAT assay treated with gallic acid (a.), methyl gallate (b.), propyl gallate (c.), butyl gallate (d.), octyl gallate (e.), lauryl gallate (f.)

Figure 3 Kinetic decay of conjugated triene in the ApoCAT assay treated with gallic acid (a.), methyl gallate (b.), propyl gallate (c.), butyl gallate (d.), octyl gallate (e.), lauryl gallate (f.)

Figure 4 Oxidative induction times of bulk soybean oils treated with gallic acid and its alkyl esters at 2 mmoles per kilogram of sample using the Rancimat test at 110 °C with airflow rate of 20 L/h

Figure 5 Oxidative induction times of roasted peanut treated with gallic acid and its alkyl esters at 2 mmoles per kilogram of sample using the Rancimat test at 110 °C with airflow rate of 20 L/h

Figure 6 PCA loading (a.) and score (b.) plots obtained from total phenolic content (TPC assay), antioxidant activities (ABTS, FRAP, ORAC, CAT and ApoCAT assays), the oxidative induction periods of PV and TBAR formation in oil-in-water emulsions, and the oxidative induction time of bulk soybean oils (IT_SBO) and roasted peanuts (IT_RP)

Table 1 Antioxidant activities of gallic acid and its alkyl esters using homogeneous antioxidant evaluation assays

Antioxidants	TPC (Gallic acid equivalent)	ABTS (Ascorbic acid equivalent)	FRAP (Ascorbic acid equivalent)	ORAC (Trolox equivalent)
Gallic acid (G0)	1*^{d}	2.91±0.14^{d}	2.25±0.03^{d}	1.53±0.17^{c}
Methyl gallate (G1)	0.091±0.00^{c}	1.08±0.04^{c}	0.73±0.01^{c}	1.76±0.20^{cd}
Propyl gallate (G3)	0.087±0.01^{c}	1.09±0.01^{c}	0.77±0.04^{c}	1.88±0.17^{d}
Butyl gallate (G4)	0.088±0.00^{c}	0.98±0.02^{c}	0.73±0.03^{c}	1.78±0.13^{cd}
Octyl gallate (G8)	0.060±0.01^{b}	0.65±0.93^{b}	0.56±0.03^{b}	1.23±0.11^{b}
Dodecyl gallate (G12)	0.055±0.00^{a}	0.09±0.00^{a}	0.30±0.00^{a}	0.32±0.04^{a}

$^{a-d}$ Means within a column with different letters are significantly different ($p<0.05$).

The * indicates the value of approximately 1 due to the antioxidant was used as the standard.

Table 2 Antioxidant activities of gallic acid and its alkyl esters using heterogeneous antioxidant evaluation assays

Antioxidants	CAT (Trolox equivalent)	ApoCAT (Trolox equivalent)
Gallic acid (G0)	0.64±0.08^{a}	0.28±0.03^{a}
Methyl gallate (G1)	1.65±0.15^{c}	0.76±0.09^{b}
Propyl gallate (G3)	1.84±0.10d^{e}	1.16±0.25^{c}
Butyl gallate (G4)	1.98±0.06^{e}	1.62±0.17^{d}
Octyl gallate (G8)	1.26±0.11^{b}	2.4±0.14^{e}
Dodecyl gallate (G12)	1.69±0.15^{cd}	3.26±0.17^{f}

$^{a-f}$ Means within a column with different letters are significantly different ($p<0.05$).

Table 3 Oxidative stabilities of oil-in-water emulsions (O/W) treated with the addition of gallic acid and its alkyl esters at 50 µM. Results were expressed as the induction period (IP) of peroxide (PV) and thiobarbituric acid reactance (TBAR) values

Antioxidants	Induction period (day)*	
	PV	TBAR
Gallic acid (G0)	3	3
Methyl gallate (G1)	4	4
Propyl gallate (G3)	5	5
Butyl gallate (G4)	6	6
Octyl gallate (G8)	8	8
Dodecyl gallate (G12)	9	9

* Data obtained from 3 individual replicate samples

Table 4 Correlations among total phenolic content (TPC) antioxidant values (ABTS, FRAP, ORAC, CAT, and ApoCAT values), the oxidative induction period of oil-in-water emulsions (the PV and TBAR values), the oxidative induction times of bulk soybean oils (IT_SBO) and the oxidative induction times of roasted peanuts (IT_RP)

	TPC	ABTS	FRAP	ORAC	CAT	ApoCAT	PV	TBAR	IT_SBO	IT_RP
TPC	1	.931**	.976**	0.131	-.856*	-0.611	-0.630	-0.630	.830*	-0.793
ABTS		1	.988**	0.483	-0.733	-.844*	-.844*	-.844*	0.655	-.952**
FRAP			1	0.344	-0.796	-0.754	-0.761	-0.761	0.749	-.901*
ORAC				1	0.085	-0.81	-0.760	-0.760	-0.176	-0.689
CAT					1	0.369	0.359	0.359	-0.786	0.501
ApoCAT						1	.993**	.993**	-0.166	.943**
PV							1	1.000**	-0.166	.943**
TBAR								1	-0.166	.943**
IT_SBO									1	-0.442
IT_RP										1

** Correlation is significant at the 0.01 level (2-tailed)

* Correlation is significant at the 0.05 level (2-tailed)

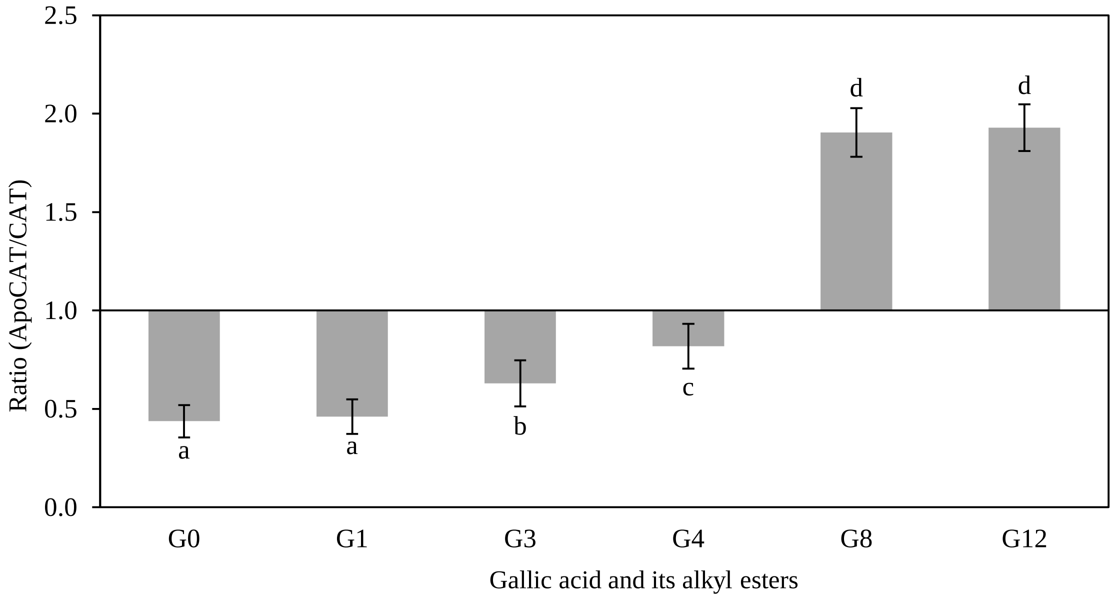

Figure 1 The ratio between the ApoCAT and CAT values of gallic acid (G0), methyl gallate (G1), propyl gallate (G3), butyl gallate (G4), octyl gallate (G8) and lauryl gallate (G12)

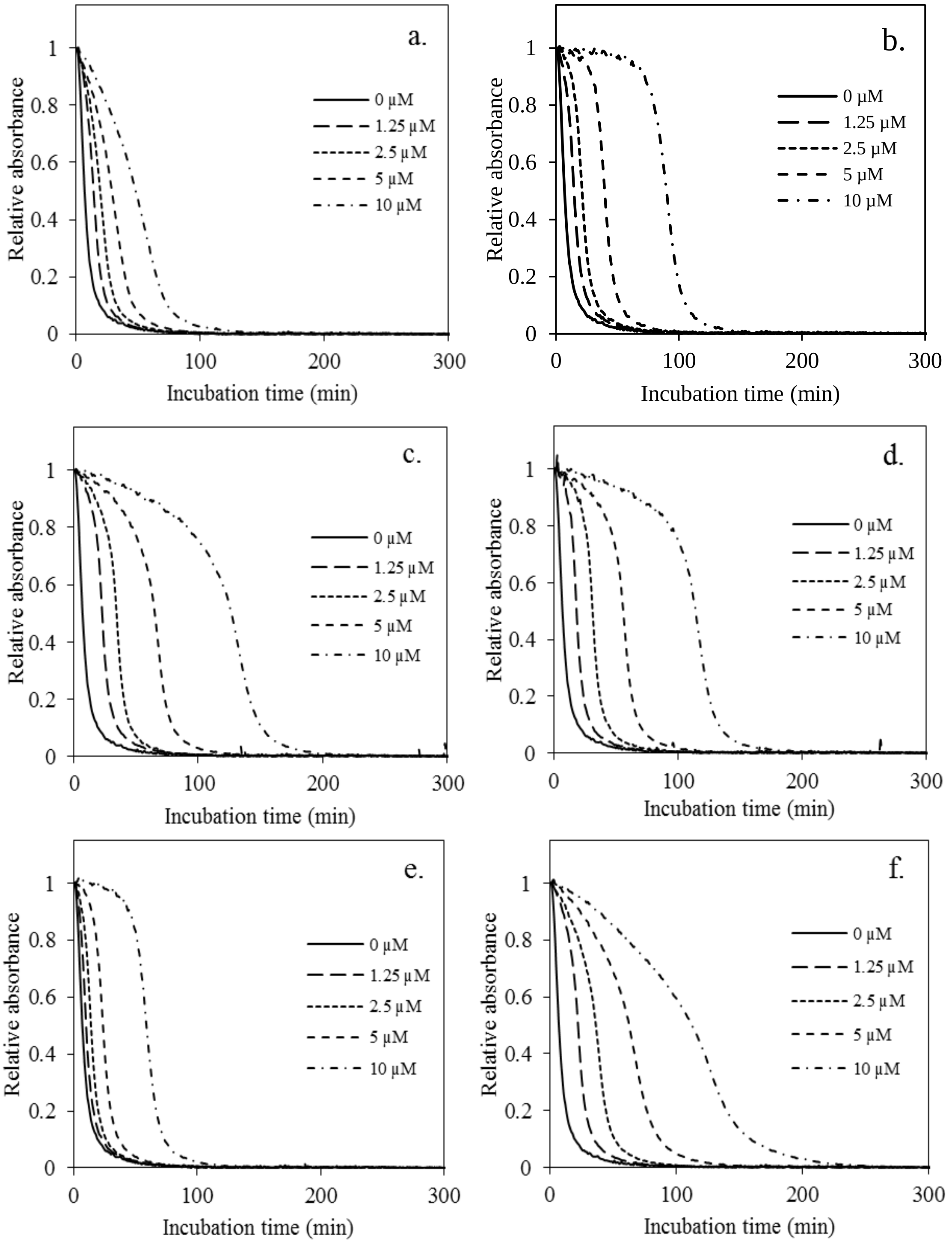

Figure 2 Kinetic decay of conjugated triene in the CAT assay treated with gallic acid (a.), methyl gallate (b.), propyl gallate (c.), butyl gallate (d.), octyl gallate (e.), lauryl gallate (f.)

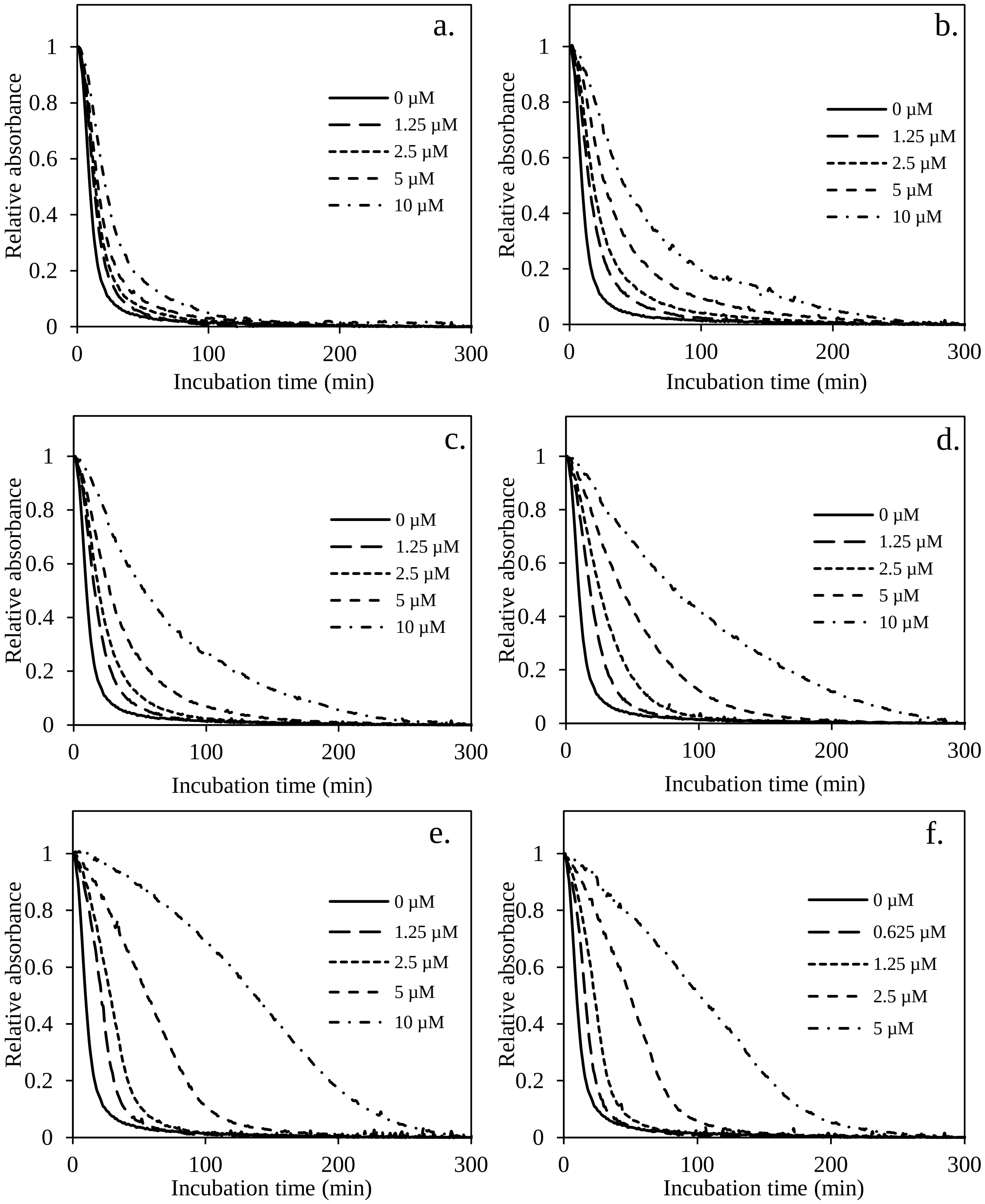

Figure 3 Kinetic decay of conjugated triene in the ApoCAT assay treated with gallic acid (a.), methyl gallate (b.), propyl gallate (c.), butyl gallate (d.), octyl gallate (e.), lauryl gallate (f.)

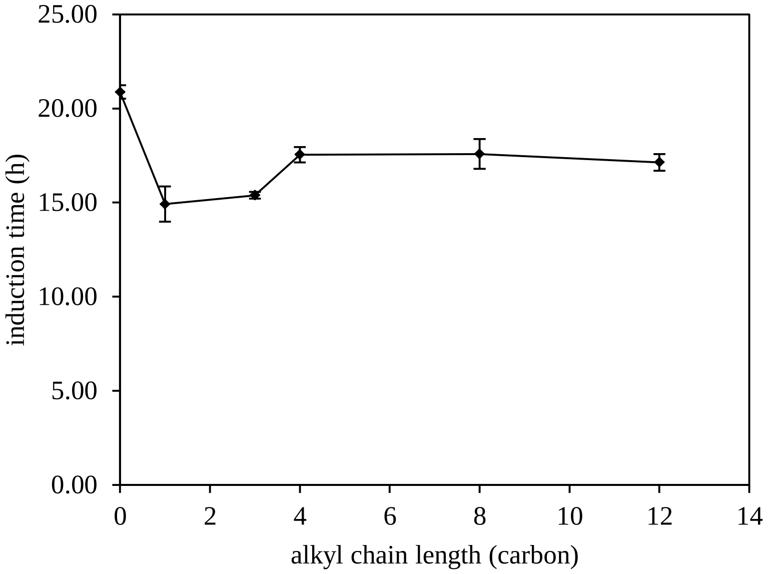

Figure 4 Oxidative induction times of bulk soybean oils treated with gallic acid and its alkyl esters at 2 mmoles per kilogram of sample using the Rancimat test at 110 °C with airflow rate of 20 L/h

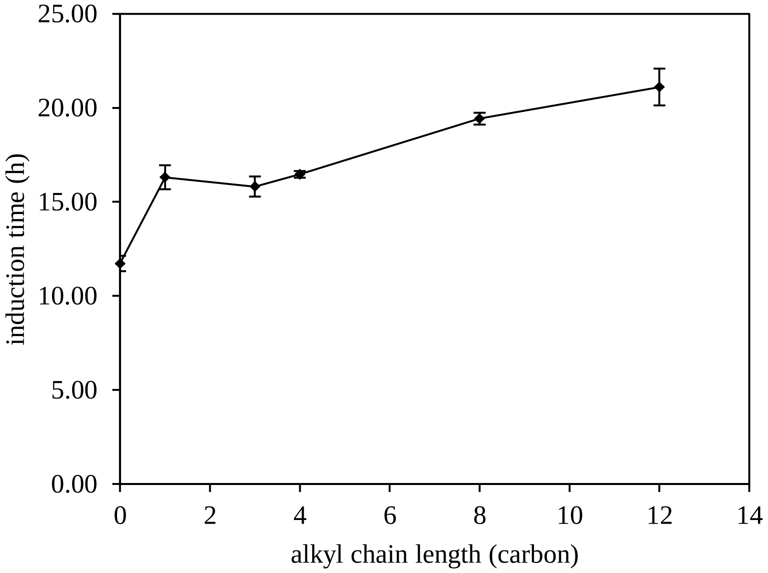

Figure 5 Oxidative induction times of roasted peanut treated with gallic acid and its alkyl esters at 2 mmoles per kilogram of sample using the Rancimat test at 110 °C with airflow rate of 20 L/h

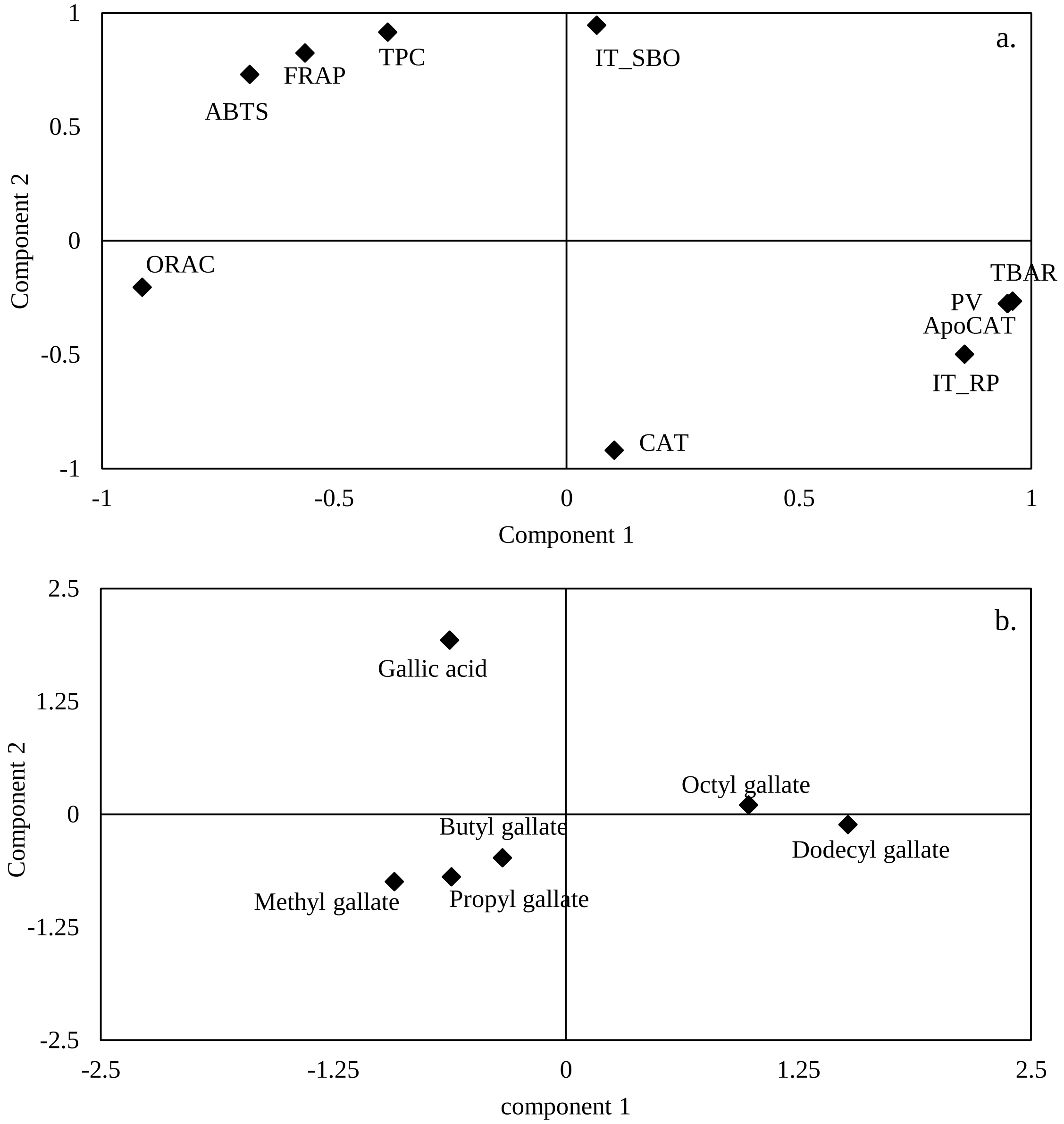

Figure 6 PCA loading (a.) and score (b.) plots obtained from total phenolic content (TPC assay), antioxidant activities (ABTS, FRAP, ORAC, CAT and ApoCAT assays), the oxidative induction periods of PV and TBAR formation in oil-in-water emulsions, and the oxidative induction time of bulk soybean oils (IT_SBO) and roasted peanuts (IT_RP)

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Output (Acknowledge the Thailand Research Fund)

International Journal Publication

1. Panya, A., Temthawee, W., Phonsatta, N., Charoensuk, D., Deetae, P., Visessanguan, W and Decker, E.A. (2015) Apolar Radical Initiated Conjugated Autoxidizable Triene (ApoCAT) Assay: Effects of Oxidant Locations on Antioxidant Capacities and Interactions. Journal of Agricultural and Food chemistry. 63(34):7546-55. DOI:10.1021/acs.jafc.5b02493
2. Phonsatta, N., Deetae, J., Luangpituksa, P., Visessanguan, W Decker, E.A. and Panya, A. Comparison of Antioxidant Evaluation Assays for Investigating Antioxidative Activity of Gallic Acid and Its Alkyl Esters in Different Food Matrices. (Submitting to Journal of Agricultural and Food chemistry) the manuscript as shown in Appendix C.

National conference

รางวัล สกว. เสนอผลงานวิจัยดีเยี่ยมแบบโปสเตอร์

ดร. อติกร ปัญญา นักวิจัย หน่วยวิจัยเทคโนโลยีชีวภาพอาหาร ได้รับรางวัลนำเสนอผลงานวิจัยดีเยี่ยมแบบโปสเตอร์ ได้นำเสนอผลงานแบบโปสเตอร์ เรื่อง Apolar Radical Initiated Conjugated Autoxidizable Triene (ApoCAT) Assay: Effects of oxidant locations on antioxidant capacities and interactions ในการประชุมนักวิจัยรุ่นใหม่ พบเมธีวิจัยอาวุโส สกว. ครั้งที่ 15 ในวันที่ 6-8 มกราคม 2559 ณ โรงแรมเดอะรีเจ้นท์ ชะอำ บีช รีสอร์ท จังหวัดเพชรบุรี

International conference

To be a invited speaker at Food Science, Singapore on 16-17 November 2016

http://www.sepscience.com/Conferences-and-Workshops/Food-Science-2016/Speakers