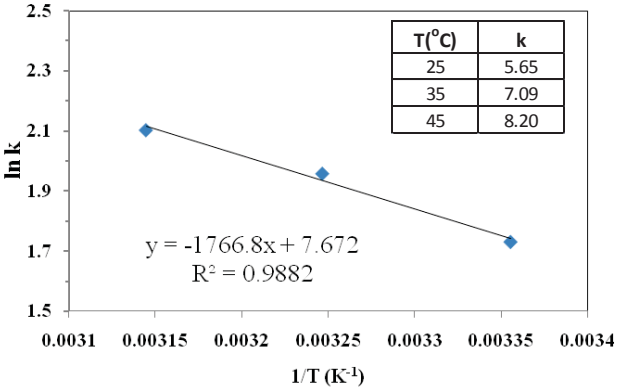

Figure 6. The Arrhenius plot of the rate constant of the reaction on the surface.

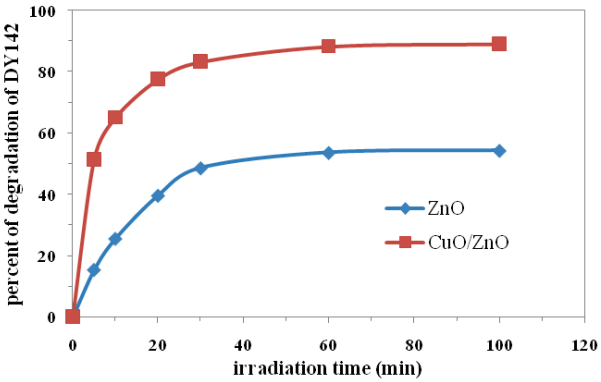

Figure 7. Comparison the photodegradation of DY142 by using ZnO and CuO/ZnO as the catalyst.

Langmuir-Hinshelwood plot for the photodegradation of DY142 can be shown in Fig. 5. The rate constant of the reaction on the surface, k and the adsorption constant, K_{DY142} can be calculated from the slope and y-intercept, respectively. The linear fit with the least-square correlation R^2 of 0.9968 confirms that the kinetics of photodegradation of DY142 with CuO/ZnO as a catalyst under the daylight irradiation can be explained by the Langmuir-Hinshelwood expression with the adsorption equilibrium constant of 0.022 $Lmg^{-1}$and the rate constant of 9.07 $mgL^{-1}min^{-1}$at 35°C.

3.6 Effect of temperature

In this part, we carried out the photodegradation of DY142 at three different temperatures. It was observed as expected that at high temperature the rate of photodegradation was faster than that at low temperature. Within 20 minutes, the percent of degradation of 40.32 mgL^{-1} DY142 at 25°C is 67, while that at 45°C is 81. At each temperature, we performed the experiment again at various initial dye concentrations. The rate constant of the reaction on the catalyst surface at each temperature was obtained by means of the Langmuir-Hinshelwood expression. The temperature dependence of the rate constant is demonstrated in the Arrhenius fashion in figure 6. The activation energy (E_a) can be calculated from the slope of the linear relation between k and 1/T. It results that E_a is 14.7 kJ/mol.

Finally, it is interesting to compare the degradation efficiency when we use CuO/ZnO composite and ZnO as a photocatalyst. The experiments were done with 40.32 mg/L DY142 and a certain amount of catalyst (3.0 g/L). The result is shown in the figure 7. Within 100 minutes the percent of degradation rises up to 90 % if CuO/ZnO composite is used as a catalyst. While the percent of degradation is just 54% if one use ZnO instead.

4. Conclusion

In this study, the photodegradation of DY142 under the daylight irradiation was investigated. It was demonstrated that CuO/ZnO composite can be used as a heterogeneous catalyst with the higher catalytic efficiency than using ZnO by a factor of 1.64. The optimal dosage of CuO/ZnO for the photodegradation of 40.32 mgL^{-1} DY142 was 3.0 g/L. The photo-degradation efficiency did not strongly depend on the pH of the solution. However, performing the reaction in the acidic medium still had the higher efficiency than in the alkaline medium. The kinetics of the photocatalytic degradation of DY142 can be explained by the Langmuir-Hinshelwood model with the adsorption equilibrium constant of 0.022 $Lmg^{-1}$and the rate constant of 9.07 $mgL^{-1}min^{-1}$at 35°C. By using the Arrhenius equation, the activation energy of 14.7 $kJmol^{-1}$ was obtained.

Acknowledgements

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Reference

[1] M.A. Behnajady, N. Modirshahla and R. Hamzavi, *J. Hazard. Mater. B*, **133**(2006), pp. 226-232
[2] H. Anh Le, L. T. Linh, S. Chin and J. Jurng, *Pow. Tech.*, **225**(2012), pp. 167-175
[3] K. Chan-Soo, S. Jung-Woo, A. Sang-Hun, J. Hee-Dong and K. Tae-Oh, *Chem. Eng. J.*, **204-206**(2012), pp.40-47.
[4] X. Wang, H. Fan and P. Ren, *Colloids. Surf. A: Phys. Eng.*, In Press (2012)
[5] X. Liu, Z. Li, Q. Zhang, F. Li and T. Kong, Mat. Lett., **72** (2012) pp. 49-52
[6] R. Saravanan, S. Karthikeyan, V.K. Gupta, G. Sekaran, V. Narayanan and A. Stephen, *Mat. Sci. Eng. C*, **33** (2013), pp. 91-98
[7] J. Sun, Y. Wang, R. Sun and S. Dong, Mat. Chem. Phys., **115**(2009), pp. 303-308
[8] H. Liu, G. Liu and Q. Zhou, *J. Solid Stat. Chem.*, **182**(2009), pp. 3238-3242
[9] P. Sathishkumar, R. Sweena, J.J. Wu and S. Anandan, *Chem. Eng. J.*,**171**(2011), pp. 136-140
[10] Z. Meng, Lei. Zhu, J. Choi, C.Y. Park and W. Oh, *Nano. Res. Lett.*, **6**(2011), pp. 459
[11] Y.Hu, X.H. Zhou, Q. Han, Q.X. Cao and Y.X. Huang, *Mater. Sci. Eng. B*, **99**(2003), pp. 368-376
[12] J. Arana, C.F. Rodrguez, O.G. Diaz, J.A.H. Melian and J.P. Pena, *Catal. Today*, **101**(2005), pp. 261-266
[13] M.R. Sohrabi and M. Ghavami, *J. Harzard. Mat.*, **153**(2008), pp. 1235-1239.

USE OF WASTE TYRE RUBBER COATED WITH CATIONIC POLYMER FOR ARSENATE ADSORPTION

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Abstract: Waste tyre rubber (WTR) powder was coated with cationic polymer, poly(3-acrylamidopropyl) trimethylammonium chloride (p(APTMACl)) which was synthesized via free radical polymerization in aqueous solution. The WTR/p(APTMACl) was utilized as adsorbent for the adsorption of arsenate from aqueous medium at room temperature. The adsorbent was characterized by Fourier transform infrared spectrometry (FT-IR) and the amount of arsenate in solutions was determined by inductively coupled plasma-optical emission spectrometry (ICP-OES). Parameters affecting the adsorption of arsenate including solution pH, concentrations of APTMACl monomer in the preparation step of WTR/p(APTMACl), contact time of adsorption and temperature for drying process of the adsorbent, as well as Langmuir and Freundlich adsorption isotherms were studied. The optimal pH for the adsorption was 8. The adsorption increased gradually with increasing the monomer concentration and contact time. Comparing drying temperatures between 50 and 100 °C, the adsorption amount of arsenate was higher at the lower drying temperature. The adsorption behavior obeyed the Freundlich model with the R^2 of more than 0.98 indicating that the adsorption occurred on a heterogeneous surface. The sorption amount of arsenate increased with increasing the initial arsenate concentrations in the range of 5-30 mg/L.

1. Introduction

Arsenic in drinking water has the greatest impact on the human health. The guideline values for arsenic in drinking water established by the World Health Organization (WHO) and United States Environmental Protection Agency (USEPA) are limited to 10 µg/L [1]. Under reducing conditions, arsenic occurs in trivalent state (arsenious acid) whereas the pentavalent state (arsenic acid) is common in oxidizing conditions in aqueous system [2]. The removal of arsenic is accomplished by adsorption techniques. Waste tyre rubbers (WTR) are an interesting and inexpensive sorbent for the adsorption of toxic metals from aqueous solutions. Common chemicals having been used in the production of tyre rubber are zinc oxide, carbon black and sulfur, one or all of which could be responsible for sorption affinity for metal ions [3]. The carbon black in the rubber is similar to activated carbon, a good adsorbent to remove dissolved organic substances from wastewater. There have been numerous studies on the adsorption of heavy metals from water and wastewater by tyre rubber powder. For instance, Cd(II), Pb(II), Cr(IV), Hg(II) and Cu(II) ions have been removed from aqueous solutions by tyre rubber [4,5]. In this study, WTR was modified by a cationic polymer, poly(3-acrylamidopropyl)trimethylammonium chloride [p(APTMACl)] and used as arsenate adsorbent.

2. Materials and Methods

2.1 Materials and chemicals

WTR was obtained from Union Commercial Development Co. Ltd. (Samut Prakan, Thailand). (3-Acrylamidopropyl)trimethylammonium chloride (APTMACl) (75 wt% solution in water), *N,N"*-methylene bisacrylamide (MBA), *N,N,N',N'*-tetramethyl ethylenediamine (TEMED), and ammonium persulfate (APS) were purchased from Aldrich. The stock solution of 1000 mg As(V)/L was purchased from Sigma-Aldrich.

2.2 Instruments

A Fourier transform infrared spectrometer (FT-IR, model Nicolet 6700, Thermo) was used for the characterization of the prepared materials. An inductively coupled plasma-optical emission spectrometer (ICP-OES, model iCAP 6500, Thermo) was used the determination of As in solutions.

2.3 Preparation of waste tyre rubber powder coated with poly(3-acrylamidopropyl)trimethylammonium chloride

About 0.5 g of WTR was mixed with desired amounts of APTMACl monomer, 10 mM of TEMED and 50 mM of MBA. Then, the mixture was sonicated for 15 min and purged by N_2 for 1 h. About 0.5 mL of 70 mM APS was added and stirred for 1 h and the solid was separated by filtration. After that, it was washed twice with 5 mL of DI water and dried at 50°C for 24 h. The concentration of APTMACl was varied in the range of 250 - 2000 mM and the As(V) adsorption capacities of obtained WTR/p(APTMACl) were investigated. The adsorption efficiency q (mg/g) was calculated using Equation 1.

$$q = \frac{(C_0 - C_f)V}{W} \quad (1)$$

where C_o and C_f are the initial and final As concentration in solution (mg/L), respectively. V is the volume of As solution (L) and W is the weight of adsorbent (g).

2.4 Effect of pH

The effect of pH was studied in the pH range of 3.0-10.0 by using 1% HCl and 1% NaOH solution for pH adjustment. 10 mL of As(V) solution (10 mg/L) and 0.1 g of WTR/p(APTMACl) was shaken in a test tube for 24 h. Then, the solution was filtered and the amount of As(V) was determined by ICP-OES.

2.5 Adsorption isotherm

0.1 g of WTR/p(APTMACl) was stirred with 10 mL of various initial concentrations of As(V) solution in the range of 5 to 30 mg/L using optimal conditions from previous experiments. Langmuir and Freundlich models were studied.

2.6 Effect of adsorption time

The effect of adsorption time (30-360 min) was investigated using 10 mg/L of As(V) solution with the optimal pH value. 10 mL of As(V) solution was added in a test tube containing 0.1 g of WTR/p(APTMACl) and the mixture was stirred at room temperature.

3. Results and Discussion

3.1 FT-IR characterization

FT-IR spectra of WTR/p(APTMACl) and WTR were compared in Figure 1. Figure 1(a) displays the broad peak (3500-3200 cm^{-1}) which was assigned to N-H stretching. The absorption band at around 3000 cm^{-1} was assigned to the stretching of alkyl groups. The strong peaks at 1650 and 1211 were assigned to C=O and C-N stretching of amide groups of p(APTMACl). Therefore, it suggested that WTR/p(APTMACl) was successfully prepared.

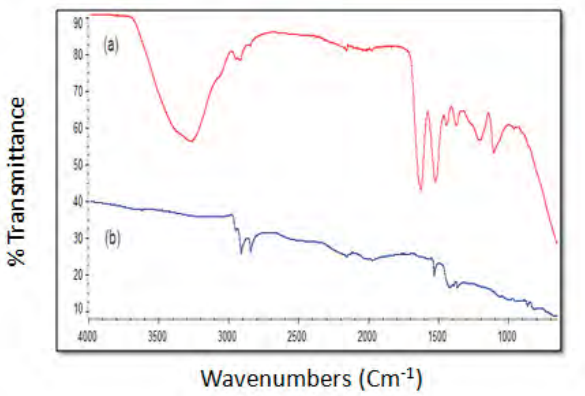

Figure 1. FT-IR spectra of (a) WTR/p(APTMACl) (0.5 g WTR; 10 mM TEMED; 70 mM APS; 500 mM APTMACl and 50 mM MBA) ; (b) WTR..

3.2 Effect of pH

The adsorption amount of WTR/p(APTMACl) was evaluated and compared with WTR by using As(V) solution (10 mg/L) in the pH range of 3-10 with the adsorption time of 24 h. From Figure 2, WTR/p(APTMACl) showed higher efficiency in As(V) adsorption than WTR. As(V) adsorption amount using WTR was almost similar at all initial pH. At pH 6, WTR showed the higher adsorption amount of 0.054 mg/g, while for WTR/p(APTMACl) the higher adsorption amount was observed about 0.35 mg/g at pH 8. This tendency agrees with the deduction that higher pH favors the formation of negative charge of arsenate form [6], leading to higher adsorption of arsenate onto the surface of positively charged WTR/p(APTMACl).

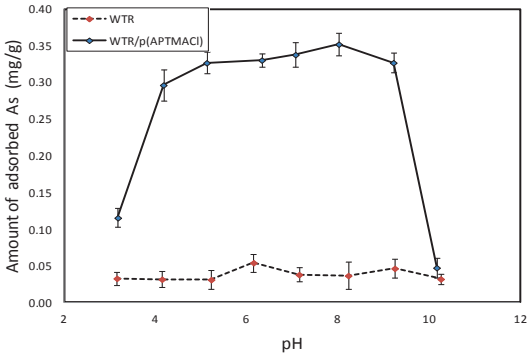

Figure 2. Effect of pH on As(V) adsorption (initial concentration = 10 mg/L (10 mL); adsorption time = 24 h; adsorbent = 0.1 g).

In addition, carbon black, stearic acid and sulfur in the form of thiol groups and polysulfide links in WTR could also behave as an ionic exchanger [7-9]. Furthermore, due to the large available surface areas of p(APTMACl), As(V) would be entrapped onto polymer via electrostatic attraction or van der Waals force [10]. WTR/p(APTMACl) adsorbent was modified with APTMACI as the monomer, where the functional group $-N^{+}(CH_3)_3$ carries a permanent positive charge, which is highly susceptible to ion exchange during the process of adsorbing the anion species [11]. It was expected that the trimethylammonium group would provide the major adsorption sites. The amide group, existing both in the monomer and crosslinker (MBA), is also susceptible to arsenate adsorption. As chloride is initially attached to the $-N^{+}(CH_3)_3$ group for charge balance, in the presence of arsenate species, the exchange between anions may occur during the process of adsorption, in which the mechanism should be ion exchange.

3.3 Effect of ratio of APTMACl to WTR

The increase in a ratio of APTMACl to WTR would be expected to increase the amount of adsorbed As(V). The results presented in Figure 3 indicated that as the ratio of APTMACI to WTR increased, the amount of adsorbed As(V) increased up to 0.48 mg/g. This may be due to an increase of $-N^{+}(CH_3)_3$ groups on the adsorbent. However, the higher adsorbed amount of As(V) with small amount of monomer is preferable. Thus the weight ratio of APTMACl/WTR at 5 (with 0.35 mg As(V)/g sorbent) was selected for further experiments.

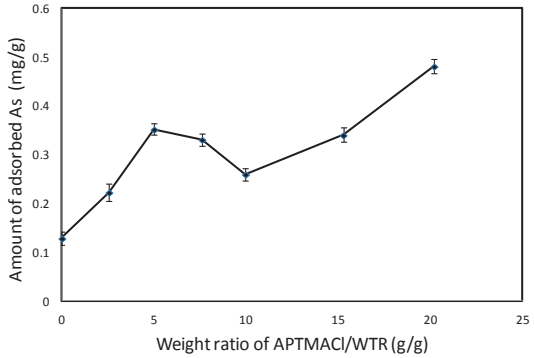

Figure 3. Effect of ratio of APTMACl to WTR (pH 8; drying temperature of adsorbent = 50 °C; adsorbent weight = 0.1 g).

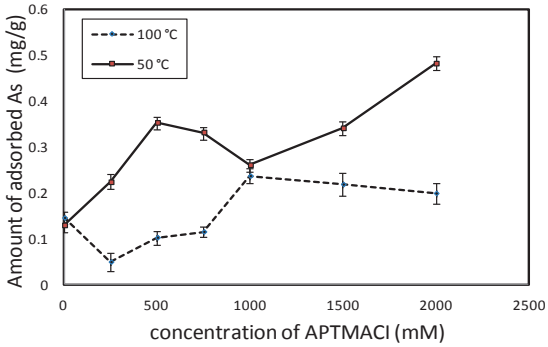

Figure 4. Effect of drying temperatures of WTR/p(APTMACl) at 50 and 100 °C ([As] = 10 mg/L; pH 8; contact time = 24 h).

3.4 Effect of drying temperature of adsorbents

The effect of drying temperature of WTR/p(APTMACl) adsorbents between 50 and 100°C on the adsorption of As(V) was investigated for initial concentration of 10 mg/L As(V) solution, pH 8, contact time of 24 h and in various concentration of APTMACl. From Figure 4, the adsorption amount of arsenate was higher at the lower drying temperature. This suggested that high temperature may cause the degradation of cationic polymer p(APTMACl) on the surface of WTR, resulting in decreasing the efficiency of As(V) adsorption.

3.5 Adsorption isotherm

The adsorption behavior can be investigated by using a fixed amount of WTR/p(APTMACl) at 0.1 g with various concentrations of As(V) solution under the optimum conditions (pH 8 and adsorption time of 24 h) at 25±1°C. Figure 5(a) displays that the amount of As(V) adsorbed onto the adsorbent increased linearly with increasing As(V) concentrations in the range of 5-30 mg/L. Langmuir isotherm explains the monolayer adsorption of adsorbates on homogeneous surface with limited number of active sites and each site can adsorb only one target ion via chemisorptions. Freundlich isotherm model can be applied to multilayer as well as non-ideal sorption on heterogeneous surface with different adsorption energy [12].

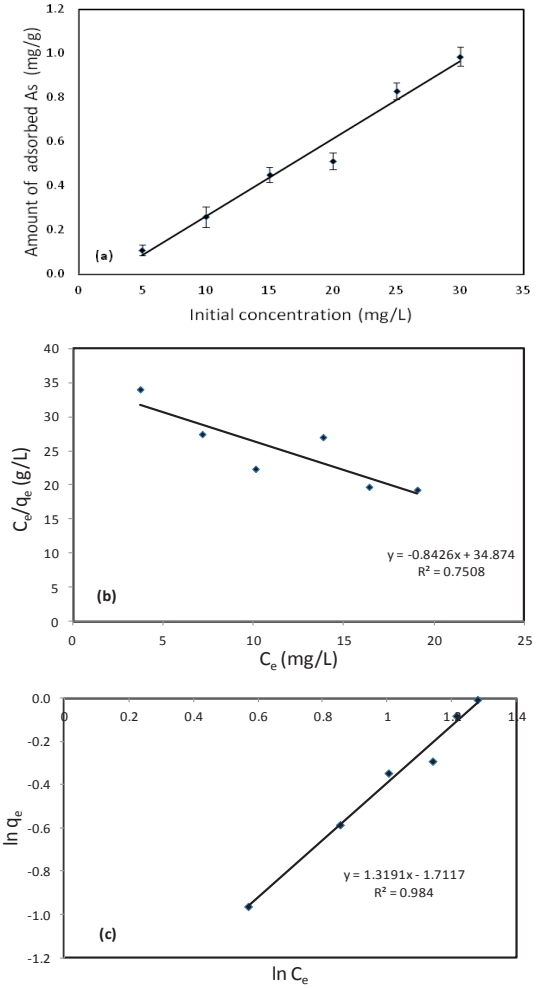

Figure 5. Adsorption efficiency of WTR/p(APTMACl) (pH 8; t = 24 h; 25±1°C; 0.1 g sorbent) (a) adsorption amount; (b) Langmuir isotherm plot. (c) Freundlich isotherm plot.

Linearized equations of Langmuir and Freundlich isotherm model are shown below. The Langmuir equation can be written in linear form in Equation 2.

$$\frac{C_e}{q_e} = \frac{1}{q_m b} + \frac{C_e}{q_m} \qquad (2)$$

where C_e is the equilibrium concentration of As(V) in the bulk solution (mg/L), q_e is the amount of adsorbed As(V) at equilibrium (mg/g), q_m is the maximum adsorption capacity (mg/g) and b is the constant related to the free energy of adsorption (L/mg).

q_m and b can be calculated from the slope and y-axis interception by plotting C_e/q_e versus C_e as shown in Figure 5(b). Furthermore, the linearized Freundlich equation is shown in Equation 3.

$$\ln q_e = \ln K_f + \frac{1}{n} \ln C_e \qquad (3)$$

where K_F is a constant related to adsorption capacity of the adsorbent ($mg^{1-(1/n)}L^{1/n}g^{-1}$) and n is Freundlich constant indicating the adsorption intensity. C_e and q_e

are the same parameters used in Langmuir equation, which are concentration of As(V) (mg/L) and amount of As(V) adsorbed at equilibrium (mg/g), respectively.

The linearized Freundlich isotherm is presented in Figure 5(c) by plotting $ln\ q_e$ versus $ln\ C_e$. From the correlation coefficient (R^2) in Figure 5(b) and 5(c), the experimental data of As(V) adsorption fit to Freundlich model better than Langmuir model. It reveals that As(V) adsorption on the WTR/p(APTMACl) follows Freundlich isotherm suggesting that arsenate ions were adsorbed as multilayer on the surface of WTR/p(APTMACl). This may reflect the irregular energy distribution on the adsorbent surface due to the presence of different groups; (i.e., amide groups and quaternary ammonium) with different activation energies [13]. The adsorption of As(V) was likely to occur on heterogeneous surface as mentioned in Freudlich isotherm assumption. The adsorption capacity (K_F) and adsorption intensity (n) were calculated to be 0.1806 $mg^{1-(1/n)}L^{1/n}g^{-1}$ and 0.7581, respectively. The high n value of Freundlich isotherm is related to the strength of adsorption, which significantly indicates that the interaction between As(V) and the adsorbent is strong.

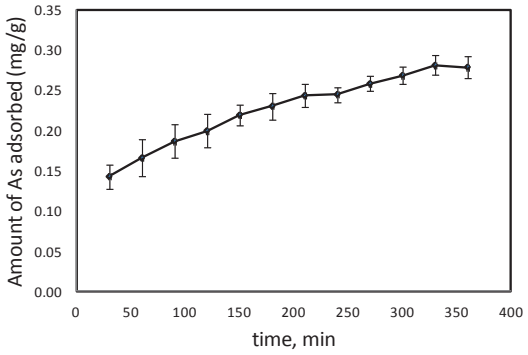

Figure 6. Effect of contact time on the adsorption of As(V) on WTR/p(APTMACl) ([As] = 10 mg/L; pH 8; temperature = 25°C; adsorbent = 0.1g).

3.6 Effect of contact time

In former experiments, the contact time was fixed at 24 h in order to ensure the equilibrium of adsorption according to the literature [4,5]. However, optimal and shorter contact time would be preferred. The effect of contact time was thus studied from 30 to 360 min for the initial concentration of 10 mg/L As(V) solution. It is clear from Figure 6 that the adsorption of As(V) on WTR/p(APTMACl) increased significantly with time and the equilibrium attained at about 330 min.

4. Conclusions

WTR/p(APTMACl) is a suitable adsorbent for the adsorption of arsenate. The adsorption depends on the temperature for drying process of the preparation of adsorbent. FT-IR characterization of adsorbent proved that the sorbent contained amide group which is expected to improve the efficiency of adsorption of arsenate via electrostatic force. A maximum of 0.35 mg As(V)/g sorbent could be achieved at pH 8. The amount of As(V) adsorption increased with increasing concentrations of APTMACl monomer and contact time. However, the adsorption amount of arsenate was higher at the lower drying temperature. The analysis of the equilibrium data of the adsorption process fitted the Freundlich isotherm. The prepared sorbent from waste tyre rubber powder is a promising low cost adsorbent for arsenate removal from wastewater.

Acknowledgements

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References

[1] Y. Tang, T. Wang, N. Gao, *J. Environ. Sci*. **11** (2010) 1689-1694.
[2] P. Lakshmipathiraj, V.R.B. Narasimhan, S. Prabhakar, B.G. Raju, *J. Hazard. Mater*. **136** (2006) 281-287.
[3] P. Danwanichakul, D. Dechojarasrri, S. Meesumrit, S. Swangwareesakul, *J. Hazard. Mater*. **154** (2008) 154, 1-8.
[4] A.S. Asheh, F. Banak, *Adsorpt. Sci. Technol*. **18** (2000) 685-700.
[5] K.N. Hamadi, X.D. Chen, M.M. Farid, Q.G.M. Lu, *Chem. Eng. J*. **84** (2001) 95-105.
[6] J.C. Hsu, J.C. Lin, H.C. Liau, T.S. Chen, *J. Hazard. Mater*. **153** (2008) 817-826.
[7] Z. Yu, S. Peldszus, P. Huck, *Envi. Sci. Tech.* **43** (2009) 1467-1473.
[8] G.A. Rowley, M.F. Husband, B.A. Cunningham, *Water Res.* **18** (1984) 981-984.
[9] L.A. Alamo-Nole, O. Perales-Perez, F.R. Roman-Velazquez, *J. Hazard. Mater*. **185** (2011) 107-111.
[10] A. Denizli, G. Ozkan, M.Y. Arica, *J. Appl. Polym. Sci.* **78** (2008) 81-89.
[11] Q. Tang, X. Sun, Q. Li, J. Lin, J. Wu, *J. Mater. Sci.* **44** (2009) 726-733.
[12] K. Sanuwong, *Preparation of polyethyleneimine-coated carbon nanotubes modified with methyl thioglycolate for extraction of gold(III) ions*, Master's Thesis, Chulalongkorn University, (2011).
[13] M. Constatin, J. Asmarandei, V. Harabagiu, L. Chimici, P. Ascenzi, G. Fundueanu, *Carbohyd. Polym.* **91** (2013) 74-84.

EVALUATION OF INTERNAL DOSE DUE TO THE INGESTION OF ^{210}Po IN SEAFOOD FROM THE GULF OF THAILAND

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Abstract: Environmental assessment of radioactive contamination in the marine organisms requires basic information on radionuclide baseline levels in biotic and abiotic components. A naturally occurring alpha emitter, ^{210}Po accumulates in marine organisms and reflects differences in their diets. The activity concentrations and internal dose of ^{210}Po in crab, fish, shrimp, and squid collected from the Gulf of Thailand were monitor. Samples were freeze dried and radiochemical separation of ^{210}Po was performed. There were differences in the activity concentrations of ^{210}Po in a descending order: shrimp > crab > fish > squid. In addition, it was observed that the ^{210}Po levels in demersal fish were higher than the pelagic fish, probably due to their habitats and feeding habits. The safeness of the environment was analyzed by calculating the internal dose rate. The hazard quotient for the studied seafood was lesser than the global bench mark dose of 1000 μSv.y^{-1}.

1. Introduction

Seafood and their products form one of the major sources of protein for the Thai people and have high great export significance. The demand of global market on seafood has been influenced the country's international trade and the development of fisheries. This is reflected in raising shrimp, squid and pelagic species for export. As a result, Thailand was the second largest global exporter of fishery commodities in terms of value during the period 2001 to 2003[1].

Since a Fukushima incident, the public has become of growing concern on radionuclides contamination in seafood. Marine organisms have the capacity of bioaccumulation radionuclides and other toxic elements from water, and therefore the investigation of radioactivity in marine organisms such as crustaceans, molluscs and fishes is importance in term of public health safety. Consumption of seafood containing radionuclides is the most important route of exposure. Measurement of natural background levels and estimation of dose to public is necessary. Environmental assessment of radioactive contamination in the marine organisms requires basic information on radionuclide baseline levels in biota and abiotic components.

Among the natural radionuclides occurring in the sea, ^{210}Po poses considerable radiation risk even with minimal intake due to its high linear energy transfer (LET). ^{210}Po is a pure α-emitting radionuclide with a half-life of 138.3767 ± 0.0020 days and with a high specific activity of 1.7 x 10^{14} Bq.g^{-1}. The main source of the ^{210}Po is ^{222}Rn emanation which is released from the earth's crust to the atmosphere. ^{210}Po returns to the earth surface by wet and dry deposition. Atmospheric precipitation of ^{210}Po on the surface and coastal waters are the main source of ^{210}Po entering to the marine environment [2].

^{210}Po is the most contributing radionuclide (>90%) in fish and shellfish result in higher radiation dose and toxicity to people whose consuming them [3]. The concentration of ^{210}Po in marine biota can be increased in the marine food chain by bioaccumulation process. According to UNSCEAR report (2000), ^{210}Po deliver about 83% of the annual effective dose to human. In the same report stated that ^{210}Po is the major contributor, with the average dietary fish-product component containing 2 Bq.Kg^{-1} of ^{210}Po [4]. The committed effective dose of ^{210}Po per unit of intake is about 10 to 10 000 times higher than for most other radionuclides. The toxicity of ^{210}Po is connected with a relatively high energy of about 5.3 MeV and that it is concentrated in the soft tissues, such as muscle, liver, kidney, and hemoglobin. ^{210}Po is primarily associated with proteins in living organisms and can also penetrate into the cytoplasm of cells [5].

The purposes of this study were to obtain a baseline data on ^{210}Po activity concentrations in crab, fish, shrimp, and squid collected from the Gulf of Thailand and also determination of the internal dose of ^{210}Po in studied organisms.

2. Materials and Methods

2.1 The study sites

Crab, shrimp, fish, octopus and squid were collected during May-July 2011 from the Gulf of Thailand on the east and the south coast districts (Figure 1).The sampling sites were chosen on the basis of the landform. The marine landform situates along the coast surrounded by many fishing villages in the coast of both locations with fish landing centers and with approximately 200-300 hundred fish workers. The east coast districts were in Chonburi, Rayong and Trad province where the south coast districts were in Prachuap-Kirikhan, Chumphon and Suratthani province. These places are endowed with a rich diversity of marine organisms where on east coast is

primed in squid and bivalves and south coast is in octopus, dermersal and pelagic fishes.

Figure 1 Map showing the study area.

2.2 Sample preparation

The samples were studied in three different groups: fish, crustacean (crab and shrimp) and mollusc (squid and octopus). Fishes were dissected into muscle and remainder (bone and viscera), only muscle was used to analysis. Shells of crab and shrimp were removed and soft tissues were collected. Squid and octopus samples were washed with distilled water, except two exported products (cleaned squids). All samples were freeze dried and then mixed homogeneously. Wet weight and dry weight of each sample was recorded.

2.3 Analytical procedures

Aliquots of the homogenized samples, 5 g dry weight were subjected to radiochemical analysis using ^{209}Po as a yield tracer. The sample was carefully decomposed by wet-ashing with HNO_3, $HCLO_4$ and HCl. Each portion of acids was evaporated to dryness at temperature of about 80^0 C. The residue was then dissolved in 100-150 ml of 0.3M HCl with warming; any insoluble matter was filtered off before proceeding to the polonium spontaneous deposition. Several 10 mg of ascorbic acid were added to the solution until the yellowish color disappeared.

Polonium was spontaneously deposited on a polished silver disc (the backside of which was coated with resin) at room temperature for 16 hr. After completion of the deposition, the silver disc was removed, rinsed with milliQ (18 mΩ) water and dried at room temp. Po was measured by α spectrometer (ORTEC Octete PLUS).

The accuracy and precision of the radiochemical method were evaluated using IAEA reference material (IAEA-414 Fish).

2.4 The annual committed effective dose (CED)

The estimated CED for individuals as a result of radionuclide intake was derived from measured concentrations in sample using the appropriate ingestion dose conversion factors (DCC) for adults recommended by IAEA [6]. The dose was calculated as follows:

$$C_0 = A_i \times D_F \times E_F \times M_F$$

Where C_0 is the CED in μSv.y $^{-1}$, A_i is the activity intake (Bq. kg^{-1} ww), D_F is the DCC (0.43 μSv.y $^{-1}$), E_F is the exposure frequency (y) and M_F is the modifying factor due to decay of ^{210}Po between catch and consumption (0.6) [3].

The Southeast Asia consumption rate used for marine dermersal and pelagic fish, crustacean and mollusc are 16.4, 1.6 and 4.1 kg.y^{-1}, respectively [7].

3. Results and Discussion

3.1 Activity concentrations of ^{210}Po

The activity concentrations range (Bq.kg^{-1}ww) of ^{210}Po measured in the flesh part of crab, shrimp, fish, octopus and squid collected from the east and south coast districts of the Gulf of Thailand are given in Table 1. The results are comparable with the literature data [3, 5, 8, 9].

There were insignificant differences in activity concentrations of ^{210}Po among the collecting sites. For the better comparison of the results obtained, the fishes were classified into two categories based on their habitats as dermersal and pelagic. The samples are classified into three categories as crustacean (crab and shrimp), fish (dermersal and pelagic) and mollusc (octopus and squid).

Table 1. Activity concentrations range (Bq.kg^{-1}ww) of ^{210}Po measured in the flesh part of seafood. In parenthesis is the number of samples analyzed.

Type	^{210}Po level (Bq.kg^{-1}ww)
Crab (3)	23.861 – 32.157
Shrimp (5)	42.316 – 45.908
Dermersal fish (20)	18.692 – 24.246
Pelagic fish (20)	5.822 – 17.251
Octopus (5)	6.023 – 10.728
Squid (30)	3.275 – 12.651
Squid (cleaned) (5)	1.434 – 2.032

The highest value of ^{210}Po activity was found in shrimp and the lowest value was found in squid. The concentration in fish samples varied from 5.822 to 24.246 Bq.kg^{-1}ww. The data showed that the ^{210}Po activity concentration (Bq.kg^{-1}ww) in the groups examined ranged from 23.861 to 45.908 (crustacean), 5.822 to 24.246 (fish), and 3.275 to 12.651 (mollusc). The ranges quoted here suggest some variation ^{210}Po activity concentration among different species according to those feeding habits and bio-mechanisms. Activity levels of ^{210}Po revealed that shrimp > crab >

fish > squid as show in Figure 2. In the case of speceies grouping, the concentration levels indicated crustacean > demersal fish > pelagic fish > mollusc.

Since polonium is absorbed from water and incorporated into the suspended particles, it is suggested that the high concentration in the body tissues might be due to the feeding habits of the marine organisms In this study indicated the relatively low ^{210}Po concentration in mollusc (octopus and squid) when compared with others, may be due to its differential feeding pattern. Similarly, shrimp being a scavenger, accumulated more ^{210}Po compared to other species. The ^{210}Po absorption efficiency for shrimp and fish was reported to approximately 0.33 and 0.5, respectively, and roughly corresponded to the assimilation efficiencies of protein from food [8].

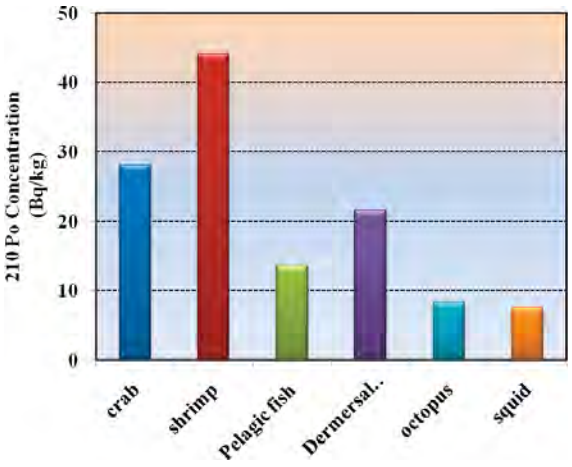

Figure 2 Concentrations of ^{210}Po in crab, shrimp, fish and squid of the Gulf of Thailand environment.

Considered on fish categories, it was found that ^{210}Po in dermersal fish higher than pelagic. Radiation activity measured in these species may be due to the nature of these fishes and their carnivores mode of feeding; these fishes feed on benthic organisms w hich tends to accumulate ^{210}Po from food chain at high rate. Indeed, it is reasonable that the levels in different food may influence the level of ^{210}Po noted amoung tissues of different species of fish because the main source of ^{210}Po accumulated by fish is believed to be the food chain. Since the global data did not take into consideration any classification according to feeding habits, it was difficult to attribute that wide range variation to the difference in feeding habits. The observation results in this study are similar to those given in the literatures [9, 10].

3.2 The annual committed effective dose (CED)

Dietary intake of ^{210}Po plays a major role in the building up of an internal dose to man. The results are showed in Table 2.

Table 2. Annual effective dose range (μSv.y $^{-1}$) of ^{210}Po from intake of seafood. In parenthesis is the number of samples analyzed.

Type	CED (μSv.y $^{-1}$)
Crab (3)	9.850 – 13.274
Shrimp (5)	17.468 – 18.951
Dermersal fish (20)	79.090 –102.590
Pelagic fish (20)	24.634 – 72.992
Octopus (5)	6.371 – 11.348
Squid (30)	3.436 – 13.382
Squid (cleaned) (5)	1.517 – 2.149

It should be noted that the consumption rate in kg per capita of different seafood species reflect the CED in the descending order: fish > shrimp >crab > octopus and squid (Figure 3).

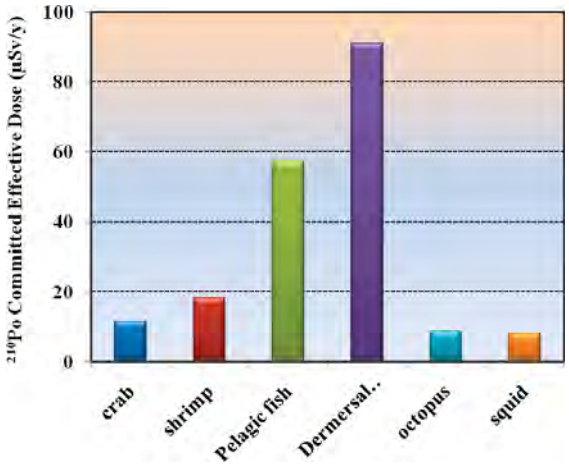

Figure 3. Annual effective dose range (μSv.y $^{-1}$) of ^{210}Po from intake of seafood

It is known that ^{210}Po presents in significant quantities in the marine environment and it contributes a significant portion of the total dose due to the naturally occurring radionuclides in seafood [11]. ^{210}Po ingested in the diet and thus especially with seafood, accounts for a radiation dose higher than in humans consuming no seafood. ICRP [12] has reported a maximum dose of 1000 μSv.y $^{-1}$ from practices in addition to natural radioactivity to public on average of 2400 μSv.y $^{-1}$ [4]. Therefore, human exposure due to intake of seafoods and their product is negligible, because ^{210}Po alone contributes to 5% of the natural radioactivity received on the highest by the coastal public in this study. In general, CED (μSv.y $^{-1}$) of Thai people's due to the consumption of ^{210}Po in seafood were relatively low in comparison with other nations such as Bay of Bengal in India (65.8-2668.9), France (46-125), Italy (50-200),USA (0.4-153.3) and

Kudankulam coast in India (mollusc 32.8-515.6, crustacean 69.3-266.7 and fishes 11.04-100.1).

4. Conclusions

In the present study, the activities of ^{210}Po were measured in seafood collected from the Gulf of Thailand. ^{210}Po activity levels in seafood samples were varied from 1.434 to 45.908 Bq.kg^{-1}. The highest activity of ^{210}Po was observed in crustaceans (crab and shrimp) and fishes followed by mollusc (octopus and squid). ^{210}Po was found to be accumulated more in dermersal fishes than pelagic ones. The variation in accumulation may be due to their habitats and feeding habits. ^{210}Po accumulated in marine organisms probably come from its availability in water and sediment; thus increase via the trophic levels of food chain.

The CED for individuals as a result of ^{210}Po intake varied between 1.517 to 102.590 μSv.y $^{-1}$. The CED values in the descending order were: fish > shrimp. crab > octopus and squid. However, the calculated effective dose was lesser than international guideline was considered to be safe.

This study can be used as a reference and the values compared when the contamination is occurred. It is strongly recommended that further and more complete research is undertaken to study the bioaccumulation of ^{210}Po in the marine environment of Thailand and the safety dose uptake level of seafood, which is a notable source of ^{210}Po in humans.

Acknowledgements

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References

[1] D. Lymer, S. Funge-smith, P. Khemakorn, S. Naruepon and S. Ubolratana, *A review and synthesis of capture fisheries data in Thailand - Large versus small-scale fisheries.* FAO Regional Office for Asia and the Pacific, FAO RAP Publication 2008/17, 51 pp.

[2] K.M. Matthews, C. Kyu Kim, P. Martin, Appl. Radiat. Isot. **65** (2007) 267-279.

[3] M. Feroz Khan and S. Godwin Wesley, http://dx.doi.org/10.1016/j. marpolbul.2012.06.010.

[4] UNSCEAR, 2000. United Nations Scientific Committee on the Effects of Atomic Radiation UNSCEAR 2000 report to the general assembly with scientific annexe, United nations, New York.

[5] M. Strok and B. Smodiš, Chemosphere (2010), doi:10.1016/j.chemospere.2010.10.075.

[6] International Atomic Energy Agency (IAEA), IAEA Tech. Report ser. No. 422 Vienna (2004).

[7] FAO Report of World Review of Fisheries and Aquaculture, FAO Fisheries Circular No.821,(2012), pp. 1-148.

[8] L. Alam and C.A.R. Mohamed, International Food Research Journal **18** (2011) 1-10.

[9] R.K. Hassona, A.K. Sam, O.I. Osman, D.A. Sirelkhatim and J. LaRosa, The Total Environment. **393** (2008) 214-218.

[10] E.M. Catal, A. Ugur, B. Ozden and I. Filizok, Mar. Pollut.Bull(2012)doi:10.1016/j.marpolbul.2012.01.016

[11] A. Ugur, B. Ozden and I. Filizok, Chemosphere **83** (2011) 1102-1107.

[12] ICRP Internationa Commission on Radiological Protection, ICRP Publication no. **60**, Pergamon Press Oxford (1991).

STUDY OF USING BASE-FORM AND SALT-FORM OF POLYANILINE AS SORBENTS FOR POLYCYCLIC AROMATIC HYDROCARBONS FOR PASSIVE SAMPLING TECHNIQUE

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Abstract: Salt-form of polyaniline (PANi-salt) was synthesized via interfacial polymerization and converted to base-form polyaniline (PANi-base) using ammonium hydroxide solution. Both forms were studied for polycyclic aromatic hydrocarbons (PAHs) adsorption by coating PANi on filter paper comparatively. In this work, the selected PAHs: naphthalene, fluorene, fluoranthene, phenanthrene, and pyrene were heated to gaseous phase and the adsorption process was performed in close container by passive sampling technique. The adsorbed PAHs were extracted from PANi-salt and PANi-base coating, with 3.00 mL of cyclohexane and cyclohexane: dichloromethane (90:10 v/v), respectively. Analysis of PAHs were studied by HPLC coupled with UV detector. The separation was performed on Hypersil Green PAH column, using acetonitrile and water mixture as mobile phase at a flow rate of 1.0 mL min^{-1}. The elution were initially operated at fixed ratio of acetonitrile: water (85:15 v/v) for 8 min, then the percentage volume of acetonitrile was increased to 100% within 7 min. Under this condition, the detection limits for the selected PAHs were in the range of 5.0-20.0 ppb. The experimental results can be concluded that filter paper without PANi coating was the best sorbent for naphthalene, fluorene was highly adsorbed by all passive samplers, pyrene and phenanthrene were significantly adsorbed on PANi samplers, while PANi-salt was a good sorbent for fluoranthrene. In addition, the adsorption process for all selected PAHs approached equilibrium after 20 hours.

1. Introduction

Polycyclic aromatic hydrocarbons(PAHs) are environmental pollutions formed from incomplete burning of coal, oil and gas, garbage and other organic substances at high temperature. These compounds are found throughout environment in air, water and soil. They receive a great research interest due to their toxic and carcinogenic effects. The European Union requirement about ambient air quality assessment and monitoring recommended a value of 1 ng m^{-3} as a limit value in ambient air for benzo(a)pyrene and 0.1 ng m^{-3} as a target value. The French National Health Council proposed 0.7 ng m^{-3} as a limit value and 0.1 ng m^{-3} as a target value [1]. Benzo(a)pyrene is considered as marker of all PAHs due to its carcinogenic effect, though it is essentially found in particulate matter. The PAHs containing less than five aromatic rings (naphthalene, phenanthrene, anthracene, fluoranthene and pyrene) are volatile and semi-volatile compounds particularly abundant in gaseous phase and they are known to be less carcinogenic.

Analysis of PAHs in environmental samples has become an important topic in analytical chemistry and the sample collection is the first important step prior to sample preparation and analysis. Passive air samplers are relatively inexpensive and simple monitoring technique for volatile and semi-volatile PAHs in air. Various kinds of material were reported as samplers or sorbents for PAHs. Semipermeable membrane devices (SPMDs) involving a low-density polyethylene (LDPE) membrane enclosing triolein [2], pinus bark [3], LDPE or ethylene vinyl acetate(EVA) film coated on aluminum foil [4] and XAD resins [5], have been employed as passive air samplers. Fiber coated polyaniline(PANi) was employed for solid phase microextraction(SPME) of some PAHs from water samples [6].

Polyaniline is a well-known conducting electro-active polymer and can be easily synthesized either electro- chemically or chemically from acidic aqueous solutions [7]. The redox states of PANi are shown in Figure 1.

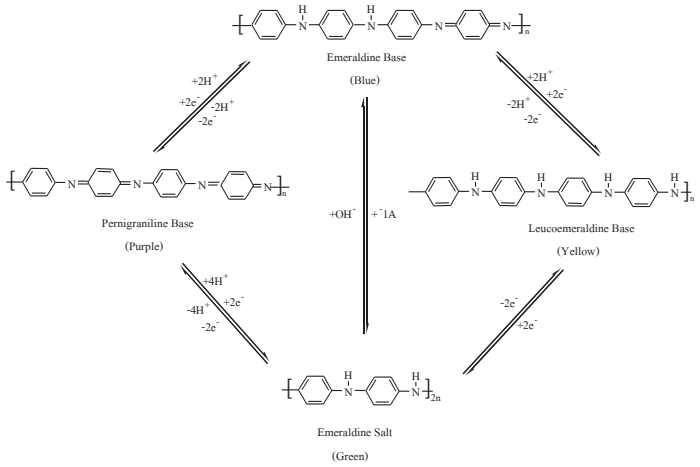


Figure 1. The redox states of PANi [7].

In this work, passive air samplers based on the application of PANi-base(pernigraniline base) and PANi-salt(emeraldine salt) as sorbent for selected PAHs: naphthalene(naph), fluorene(flu), phenanthrene (phen), fluoranthene(fluor) and pyrene(pyr) were studied. The scope of this work included the synthesis of PANi by interfacial polymerization; preparation of PANi passive sampler; experimental studies on the adsorption of the selected PAHs; the optimum

conditions for extraction of PAHs and analysis by HPLC-UV detection.

2. Materials and Methods

2.1 Chemicals and instrument

Naphthalene, fluorene, phenanthrene, fluoranthene, and pyrene were obtained from Supelco, USA (> 99.8 % assay). The stock solution of these compounds was prepared in methanol (HPLC grade, Berdick&Jackson) at concentration of 250.00 mgL^{-1}. The working solutions were prepared by appropriate dilution of the stock solutions to the required concentration. Aniline (99.0% assays, Panreac Quimica, Spain) was used without purification and other chemicals were analytical reagent grade. All solvents used in this study were analytical reagent grade or HPLC grade.

The HPLC analyses were performed on Varian 300 HPLC system with pump model Prostar 230(USA) and 20.0 µL sample loop, equipped with UV detector. Reverse phase analyses were carried out using Hypersil Green PAH column (250x4.6 mm i.d., d_p = 5 µm). The mobile phase at flow rate of 1.0 mL min^{-1} was programmed for separation as follows: initial ratio of acetonitrile to water (85:15 %v/v) was held for 8 min and then changed to 100 %v/v acetonitrile in 7 min. The signals of selected PAHs were detected by UV detector at 254 nm.

2.2 Synthesis and purification of polyaniline [7]

Two solutions were separately prepared as follows: the organic solution contained 3.2 mmol of aniline[$C_6H_5NH_2$] in 10.0 mL chloroform; and the aqueous solution contained 0.8 mmol of ammonium persulfate[($NH_4)_2S_2O_8$], in 10.0 mL of 1.0 M nitric acid. Poured the organic solution carefully into a 20 mL glass vial followed by the aqueous solution and capped the vial. The green PANi occurred at the interface of the two solutions and progressed into the upper aqueous layer. Complete polymerization was observed from the red color of the organic phase. The solution containing PANi(dark green) was drawn into 5.0 M nitric acid to stop the reaction. Transferred the PANi solution into a dialysis tube and immersed in a large volume of deionized water with stirring and the washing step was repeated until deionized water was colorless. Then the PANi solution was filtered, washed the solid with deionized water and acetone, vacuum dried at 25°C and the dark green solid of PANi-salt (emeraldine salt) was obtained. By dissolving PANi-salt in 1.0 M ammonium hydroxide, the PANi-base (purple color) was formed.

2.3 Preparation of PANi membrane

PANi-base solution was prepared by dissolving 0.10 g of PANi -salt in 50.0 mL of 1.0 M NH_4OH, sonicated for 20-30 min, then the blue-purple solution was obtained. PANi-base was coated on filter paper (2.5cm) by dip-coating in the planar direction one time. Dried the filter paper for 2 hours under vacuum at 25°C and weighed before used. The same procedure was applied to the PANi-salt solution that prepared by 0.10g of PANi-salt in 50.0 mL of deionized water.

2.4 Study of the appropriate solvent for extraction

Mixed standard solution (100.0 mgL^{-1}) of selected PAHs was prepared by appropriate dilution of the stock solution. Spotted 50.0 µL of the mixed standard onto PANi coated filter papers (both forms) and air dried for 5 min. The sampler was sonicated in 3.0 mL of solvent (cyclohexane: dichloromethane range from 70:30 to 100:0 % v/v) for 20 min, and removed the membrane. Blank (PANi membrane without PAHs) was extracted simultaneously by each solvent for base line correction. The extract was purged by nitrogen to remove all solvents, then redissolved the extract with 1.00 mL of acetonitrile. The final solution was filtered with 0.45 µm nylon filter and analyzed by HPLC-UV to observe the signal and pattern of chromatogram.

2.5 Calibration curves of PAHs

A series of mixed standard solution were applied on PANi samplers followed by the extraction of PAHs with an appropriate solvent(from 2.4). The final concentrations of the selected PAHs in 1.00 mL of acetonitrile were in range of 10-500 ppb.

2.6 Adsorption study of naphthalene, fluorene, pyrene, phenanthrene, fluoranthene and by PANi membrane

The adsorption experiments were performed in a sealed 16 L dessiccator as shown in Figure 2. The PANi membrane was placed at the back part of the passive holder. Each adsorption batch used 4 PANi samplers (2 PANi-base and 2 PANi-salt) and 2 plain filter papers. Six passive holders were mounted in the perpendicular position. Two hundred microliters of 0.20 mgL^{-1} mixed standard solution inside dessiccator was heated to produce gaseous PAHs. The adsorption time in this work varied from 8,14,20,26 and 32 hours. Extraction of PAHs from PANi membranes and plain filter papers were carried out for each adsorption time and analyzed by HPLC. The experiments were repeated for setting the passive sampler in the horizontal position.

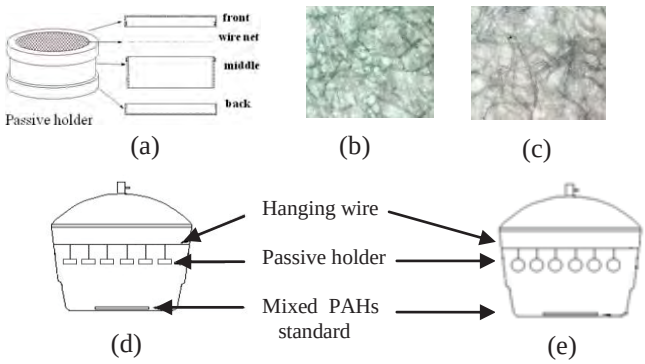


Figure 2. Components for adsorption experiment of PAHs. (a) passive holder, (b) PANi - salt membrane (c) PANi-base membrane (d) horizontal position (e) perpendicular position

3. Results and Discussion

3.1 IR spectrum of polyaniline

Interfacial polymerization of PANi according to the described procedure resulted in an emeraldine salt form (PANi-salt) with average yield of 14.1%. IR spectra of PANi-base and PANi-salt were almost identical (wave number shift in the range of 3-10 cm^{-1}) as shown Figure 3. Table 1 showed the interpretation data corresponded to the functional groups of PANi.

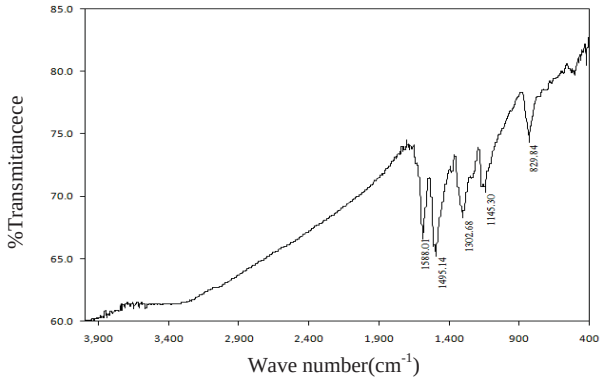


Figure 3. IR spectrum of PANi-base.

Table 1. Interpretation of IR spectrum

Wave number (cm^{-1})	Mode of vibration	Functional group
829.84	C-H bending	aromatic ring
1145.30	C-H bending	aromatic ring
1302.68	C-N sym. stretching	–C-N=, -C-NH-
1495.14	C=C stretching	Benzenoid ring
1588.01	C=C stretching	Quinoid ring

papers (Figure 2b, 2c) were 0.52 ± 0.15 mg. The membranes were stable to all solvents and conditions used in this work (room temperature to 50°C). Observation on chromatograms in term of peak shape, peak area of PAHs and low base line signal, it was found that mixed cyclohexane and dichloromethane (90:10 %v/v) was the appropriate solvent for PAHs extraction from PANi-base, while cyclohexane(100 %) was the best solvent to extract PAHs from PANi-salt. In addition, no peaks were found from blank extract. Figure 4 showed chromatogram of selected PAHs from the extract at optimum condition of separation. The analytical data obtained from calibration graphs and detection limits of selected PAHs were concluded in Table 2.

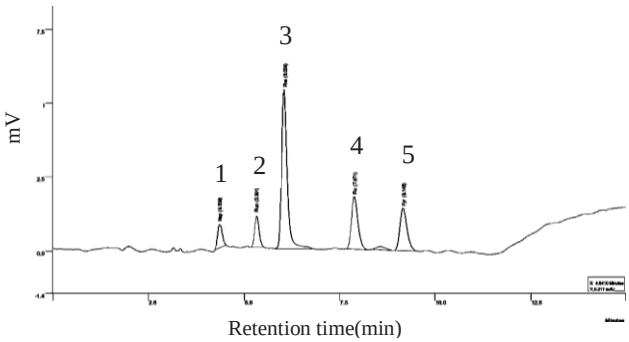


Figure 4. Chromatogram of mixed standard PAHs (500 ppb) extracted from PANi-base membrane. (1) naph, (2) flu, (3) phe, (4) fluor, (5) pyr

3.3 Adsorption study of PAHs by PANi sampler

Adsorption profiles for naphthalene, phenanthrene, fluorene, fluoranthene and pyrene on PANi-base, PANi-salt sampler and on plain filter paper were shown in Figure 5, where the adsorption of all selected PAHs by passive samplers approached

Table 2. Retention time, detection limits and analytical data from calibration graphs

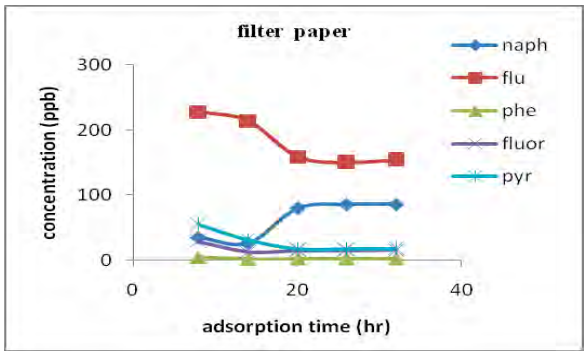
PAHs	Retention time (min)	Detection Limits (ppb)	Adsorption by PANi-base		Adsorption by PANi-salt	
			Calibration equations	R^2	Calibration equations	R^2
Naphthalene	4.39	5	Y = 8.9703x+481.53	0.9974	Y = 7.6955x+618.56	0.9998
Fluorene	5.20	5	Y= 14.372x+180.36	0.9974	Y= 16.384x+170.85	0.9943
Phenanthrene	5.87	5	Y= 110.16x+1070.1	0.9997	Y= 96.314x +18.937	0.9993
Fluoranthene	7.49	10	Y= 28.662x-192.87	0.9985	Y= 19.946x+22.962	0.9971
Pyrene	8.66	20	Y= 24.77x-116.85	0.9978	Y= 14.065x+319.12	0.9965

3.2 Extraction solvents and separation

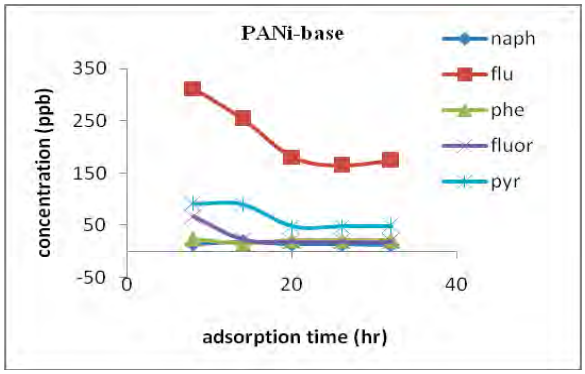
The average weight of PANi-base (pernigraniline base) and PANi-salt (emeraldine salt) coated on filter

equilibrium after 20 hours. From the adsorption profiles at equilibrium, it was found that naphthalene was highly adsorbed by cellulose filter paper but insignicant adsorption was found on PANi samplers.

This may due to the gaseous naphthalene adsorbed and dispersed as a layer on high surface energy of cellulose filter paper, followed by the deposition of new coming naphthalene molecules onto the layer [8]. This mechanism is preferable for naphthalene which is a sublimable compound but not observed on the PANi coated sampler which has low surface energy. Fluorene was almost equally adsorbed by all passive samplers(filter paper and PANi-coated filter paper) with the highest concentration retained. Phenanthrene and pyrene were significantly adsorbed on PANi –base and PANi-salt rather than filter paper, while fluoranthene was adsorbed on PANi-salt more than the PANi-base and filter paper. Their adsorptions on PANi surface may invovled π-π interactions and planarity of PAHs molecules. In addition, the effect of passive sampler mounting positions showed no significant differences in PAHs adsorption at equilibrium as shown in Figure 6.



(a)

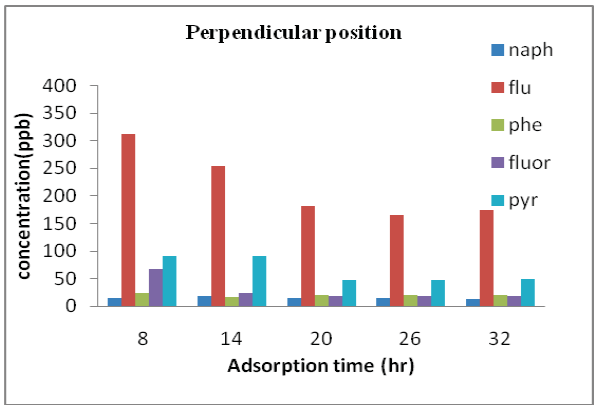


(b)

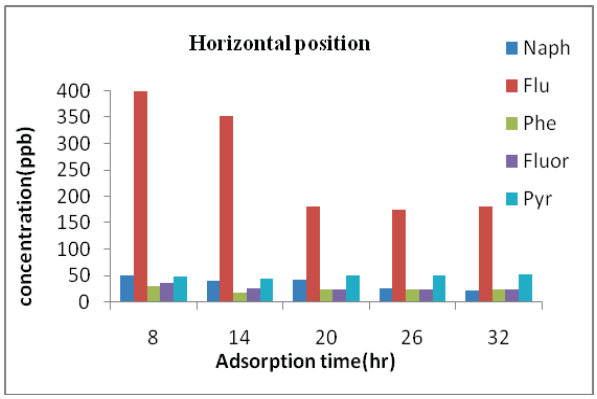
Figure 5. Adsorption profiles of selected PAHs on (a) filter paper (b) PANi-base passive samplers setting in the perpendicular position.

4. Conclusions

According to the experimental results, we could come to the interesting conclusion that PANi-base, PANi-salt and plain filter paper can be used as sorbents for fluorene with relative high adsorption efficiency, comparing to the other selected PAHs. Naphthalene was highly adsorbed on filter paper without PANi coating. Although the adsorption of phenanthrene, fluoranthene and pyrene by PANi was observed, but the concentration retained was much lower than that of fluorene. Adsorption process by passive sampling of all selected PAHs approached equilibrium after 20 hours. However, the effect of position of passive sampler setting (perpendicular and horizontal position) showed insignificant differences in adsorption of PAHs after equilibration time. All experimental results from this work can be used for further study on application of passive sampling of PAHs in ambient air, since the preparation of PANi sampler is simple, inexpensive and less time consuming, and the standard calibration is required.



(a)



(b)

Figure 6. Comparison of the setting positions of PANi-base passive sampler for PAHs adsorption in (a) the perpendicular position and (b) horizontal position.

References

[1] F. Portet-Koltalo, K. Okebdane, L. Robin, F. Dionnet and P.L. Desbene, *Talanta* **71** (2007) 1825-1833.

[2] F.A. Esteve- Turrillas, V. Yusa, A. Pastor and M. Guardia, *Trends Anal. Chem.* **26(7)** (2008) 703-712.

[3] S. Orecchio, A. Ganguzza and L. Culotta, *Environ.Res.* **107** (2008) 371-379.

[4] K.E. Kenedy, D.W. Hawker, J.F. Muller, M.E. Bartkow and R.W. Truss, *Atmos. Environ.* **27** (2007) 5778 -5787.

[5] L. Kusimaki, K. Peltone, P. Mutanen and K. Savela, *Anal. Occupy Hygiene* **47(5)** (2003) 389-398.

[6] H. Bagheri, E. Babanezhad and A. Es-hagi, *J. Chromatogr. A* **1152** (2007) 168-174.

[7] J. Huang, *Pure Appl. Chem.* **78(1)** (2006) 15-27

[8] Y. Tozuka, E. Tashiro, E. Yonemochi, T. Oguchi and K. Yamamoto, *J. Colloid Interface Sci.* **248** (2002) 239-243

BIOACCUMULATION OF ZINC ON SCLERACTINIAN CORALS *ACROPORA FORMOSA* USING Zn-65 AS A RADIOTRACER

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Abstract: Bioaccumulation of zinc on Scleractinian Corals *Acropora formosa* under laboratory controlled conditions was investigated. Zn-65 was used as a radiotracer. The experiment revealed the bioaccumulation of Zn-65 by *A. formosa* whole body and soft tissue followed a simple linear model. The maximum concentration factor (CF_m) at the end of the exposure period (96 hr) was 400 and 47700 for whole coral and soft tissue, respectively. The non linear regression fitting using the statistical package software revealed the uptake rate of 3.90 CF.hr^{-1} or 3.28 Bq.g^{-1}.hr^{-1} with R^2 = 0.93 corresponding to 0.0109 pg.g^{-1}whole coral.hr^{-1}. In soft tissue, the uptake rate was 449.19 CF.hr^{-1}or 377.38 Bq.g^{-1}.hr^{-1} with R^2=0.85 corresponding to 12.56 pg.g^{-1} protein. Loss kinetics of Zn-65 from whole corals and soft tissues followed a two-component exponential model. Using the non-linear regression fitting; short lived components represented a proportion of the total activity 46 and 20%; with the computed half lives ($Tb_{1/2s}$) 1.02 and 1.13 hr, for whole coral and soft tissue, respectively. Long-lived components represented a proportion of the total activity 54 and 80; with the computed half lived ($Tb_{1/2l}$) 198.6 and 58.4 hr, respectively. The experiment revealed *A. formosa* efficiently accumulated Zn-65 in both whole corals and soft tissue and remained in its body with relatively long half-lived.

1. Introduction

Of the micronutrients, some trace metals including Fe, Mn, Zn, Co, Cu, Ni and Mo are essential for the growth and metabolism of all marine organisms. Among these trace elements, Zn is the most important one since it is a cofactor of nearly 300 enzymes [1,2]. In microalgae, carbonic anhydrate, zinc-based metalloenzyme, is involved in the inorganic carbon acquisition from seawater [3, 4]. The activity of this enzyme has been shown to be dependent on the level of CO_2 and on the availability of Zn, thus conferring on Zn a key role in oceanic carbon cycling.

Reef building corals are autotrophic organisms living in symbiosis with dinoflagellate called zooxanthellae. Zn enrichment in the marine environment stimulated the photosynthetic capacity of the corals [5]. However, the concentrations of dissolved Zn in seawater are often very low (27 nmol.L^{-1}).Therefore, the rate of photosynthesis, the transformation and uptake of major nutrients such as carbon of corals might be controlled by the low availability of Zn in parts of the ocean.

While Zn is an important at trace concentrations, it can also be very toxic when in excess, forming dangerous free radicals [6]. Marine organisms have an opportunity to be exposed to high metal concentrations as a result of human activities or natural disasters like Tsunami. Despite these dual relationships, few studies have investigated the rate of trace metal uptake as well as the transfer between tissue and skeleton[7,8]. There is also no clear correlation between metal exposure and metal accumulation [9]. In this study the bioaccumulation in situ of Zn on Scleractinian corals *Acropora formosa* was investigated, for this purpose, the radioactive Zn-65 was used as a radiotracer.

2. Materials and Methods

Acropora formosa colony nubbins were collected from a depth of 5 m around Phuket Island. Samples of 5±1 cm from branch tips and 1 cm wide (~ 4.7±1.1 g) were sectioned to represent growth by cutting terminal portions of branches of parent colonies using bone-cutting pliers[10]. One hundred and forty nubbins were prepared. Coral nubbins were maintained in open-flow aquaria. After approximately 1 month of healing, tissue will cover the break area and the skeleton and coral fragment will be ready for bioaccumulation experiment.

To assess the rate of Zn uptake by coral at in situ concentration, two aquaria containing 30L of aerated seawater were prepared. Seawater in each aquarium was then spiked with micro-litre quantities of the radiotracer Zn-65 (negligible concentration change) to reach the appropriate activity of ~1 Bq.ml^{-1}. Thirty coral nubbins were then randomly chosen and incubated in each tank which contained seawater under controlled conditions: 29-30 ^{o}C temp, 32-34 ppm salinity, 7500-10000 Lux constant luminance, 12:12 h photo period.

The experimental medium was changed every day to maintain Zn-65 activity at a constant level and to avoid depletion of the medium in total Zn due to accumulation by coral. The spiked seawater in each tank was sampling and counted for Zn-65 activity after each seawater change. Accumulation of Zn-65 by corals was followed after 0, 2, 4, 8, 24, 48, 72 and 96 hr by randomly taken three nubbins from each tank, the samples were then processed following the sample processing procedure described below before counting for gamma ray measurement.

To assess the rate of depuration, two aquaria were prepared under the same condition as described above. However, the coral nubbins were incubated in the aquaria for 96 hr without sampling. At the end of the incubation period, the labeled seawater was discharged and the aquaria were flowed through with the unlabeled seawater. The depuration rates were then followed by randomly taken three nubbins from each tank after 0, 2, 4, 8, 24, 48, 72 and 96 hr. The samples were then processed following the sample processing procedure.

Zn-65 gamma activity was measured in the whole colony and tissue after the separation of the two fractions as described below. Zn-65 specific activities measured in whole coral were normalized by weight of each corals while as in soft tissue were normalized by weight of protein in soft tissue extract.

Sample processing

After each sampling time, nubbins were sampled and incubated for 30 min in 50 ml of unlabeled seawater to rinse the coelenteric cavity [10]. Nubbins were then blotted dry on absorbent paper to eliminate any adhering radioactive medium, transferred to counting vials, and counted for gamma emissions for 10 min.

The tissue extraction was done in laminar flow fume cabinet. To a 60 ml test tube, counted coral sample was placed and 25 ml 30% H_2O_2 solution was added. Sample was agitated overnight in ultrasonic bath. H_2O_2 was discarded and sample rinsed with 25 ml Milli-Q water and agitated for 10 min in ultrasonic bath. The rinse is discarded and combined with previously discarded H_2O_2. Zn-65 activity was determined in the tissue portion. Protein concentrations were measured in microplate reader (Tecan : Männedorf, Switzerland) ,using the Bio-Rad Protein assay Kit (Bio-Rad, CA, USA) with bovine serum albumin as the standard. The stable Zn concentration in natural seawater was measured using ICP-MS.

Zn-65 activity was measured via the gamma emission 1115.55 keV using high resolution gamma-spectrometry connected to n-and p- type HpGe detector with relative efficiency of 60% and 40% at 1332 keV, respectively. The detectors were connected to multichannel analyzer and spectral analysis software. The Zn-65 activity was corrected for background and radioactive decay of the radiotracer. Counting efficiency was determined by comparison with standard Zn-65 (IPL-1265-60) of appropriate geometry. Counting time of 10 min was adjusted to give a relative propagated error of <5% at the 1 SD level.

3. Results and Discussion

Experimental conditions

The experimental conditions are summarized in Table 1. Seawater temperature was controlled using a water chiller (Hailer HC 300A). Temperature and light intensity was controlled using HOBO pendant temperature and light. The experimental seawater was sampling and Zn-65 was measured every day. The elemental Zn concentration in natural seawater was measured using ICP-MS and revealed the elemental Zn concentration of < 0.0080 µg/L of seawater.

Table 1 Experimental conditions used for the study bioaccumulation of dissolved Zn-65 in A. Formosa.

Parameter	In situ test
Radiotracer Zn-65 activity ($Bq.ml^{-1}$)	0.8 ± 0.07
pH	7 ± 1
Salinity	30 ± 1
Temperature	29 ± 0.36°C
Light intensity (Lux)	7500 ± 2500
Exposed duration(12hr:12 hr; dark: light period)	96 hr

Uptake Kinetics

Bioaccumulation of radiotracer in marine organisms was described using kinetics model [11,12]. This model are mainly based on concentration factor (CF), which was the ratio of specific activity in the animal ($Bq.g^{-1}$) and the specific activity in seawater ($Bq.ml^{-1}$).

Accumulation kinetic of Zn-65 in *A. formosa* whole body followed a simple linear model and can be displayed as in Figure 1:

$$CF_t = kt$$

Where CF_t was CF of radiotracer at time t; k was the uptake rate constant (hr^{-1}).

Accumulation of Zn-65 on *A. formosa* whole body displayed a maximum concentration factor (CF_m) of 400 at the end of the exposure period (96 hr). The non linear regression fitting (Figure 1a) using the statistical package software [13] revealed the uptake rate of 3.90 $CF.hr^{-1}$ or 3.28 $Bq.g^{-1}.hr^{-1}$ by coral whole body with R^2= 0.93 corresponding to 0.01 pg $Zn.g^{-1}$ whole coral. hr^{-1}. In soft tissue, accumulation of Zn-65 followed the same simple linear model with a maximum concentration factor (CF_m) of 47700 at the end of the exposure period (96 hr). The non linear regression fitting (Figure 1b) revealed the uptake rate of 449.19 $CF.hr^{-1}$ or 377.38 $Bq.g^{-1}protein.hr^{-1}$ with R^2=0.85 corresponding to 12.56 $pg.g^{-1}$ protein.

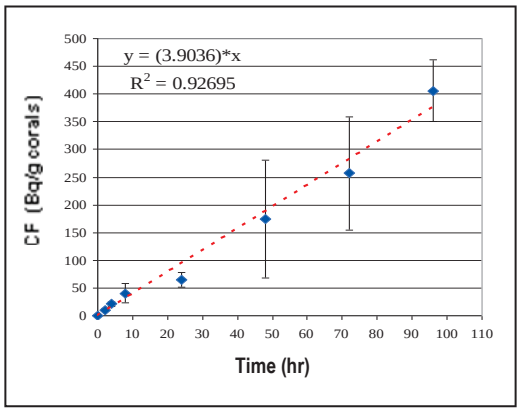

Figure 1a

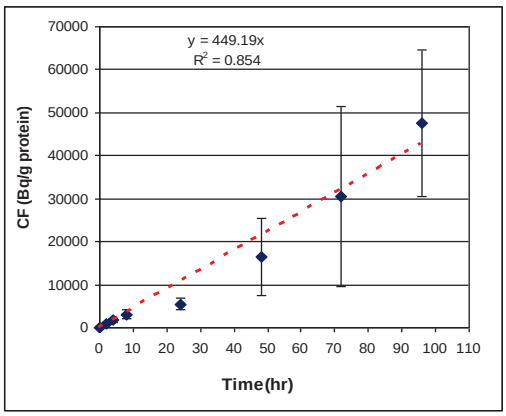

Figure 1b

Figure1. Uptake kinetics [CF; mean±SD, n=6] of Zn-65 on *A. formosa* whole body (Figure 1a) and tissue extract (Figure 1b) exposed to radiotracer in seawater. The parameters of the equation fitting the data were given in Table 2 below.

Table 2 Parameters of the equations describing the whole-body uptake kinetics of Zn-65 on *A. formosa* whole body and tissue extract.

Organ	Model	CF_t	K(SE)	R^2	p-value
Whole body	L	405.45	3.903 (0.171)	0.92695	<0.0005
Tissue extract	L	47633.151	449.192 (13.168)	0.854	<0.0005

L= Linear uptake model, CF_t=kt; CF_t: concentration factors at time t (hr); K= rate constant (hr^{-1}); SE= standard error; R2= determination coefficient; p= probability of the model adjustment

Loss kinetics

Loss of radiotracer was expressed in terms of the percentage of the remaining activity plotted against time.

The results were described by either a single-compartment exponential model:

$$A_t = A_0 e^{-\lambda t}$$

Or by a 2- compartment exponential model:

$$A_t = A_{0s} e^{-\lambda s t} + A_{0l} e^{-\lambda l t}$$

Where A_t, A_0 = remaining radioactivity (%) at time t and 0, λ = biological depuration rate constant (hr^{-1}) , s and l = subscripts for short and long-lived component , respectively.

Loss kinetics of Zn-65 from whole body and soft tissue corals followed over a 96–hr period were described by a two-component exponential model and can be displayed as in Fig 2 below.

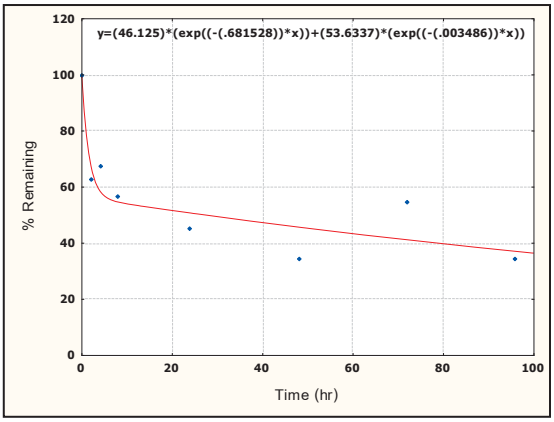

Figure 2a

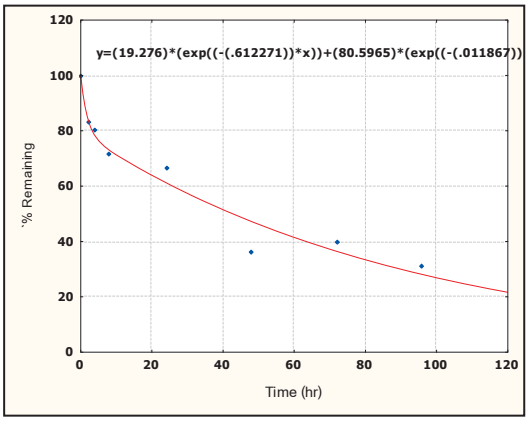

Figure 2b

Figure 2. Loss kinetics [% remaining; mean ± SD, n= 6] in whole body (Figure 2a) and soft tissue (Figure 2b) of *A. formosa* exposed to the radiotracer for 96 hr in seawater. The parameters of the equation fitting the data are given in Table 3.

Table 3 Parameters of the equation describing the whole-body and soft tissue loss kinetics of Zn-65 in *A. formosa* previously exposed to radioisotope for 96 h

Organ	Model	A_{0s},%(SE)	Λ_s(SE)	Tb $_{1/2sh}$	P	A_{0l},%(SE)	Λl(SE)	Tb $_{1/2lh}$	R^2	p
Whole-coral	T	46.137(7.58)	0.68(0.34)	1.017	<0.05	53.63(5.14)	0.0035(0.0019)	198.61	0.821	<0.05
Soft tissue	T	19.28(9.54)	0.61(0.80)	1.132	<0.05	80.60(7.30)	0.012(0.026)	58.39	0.821	<0.05

T= Two component loss model; A_{0s} = short-lived component; A_{0l} = long-lived component; SE= standard error; p= probability and determination coefficient of the model adjustment

Using the non-linear regression fitting; short-lived components represented a proportion of the total activity of 46.14 % with a computed half lives (Tb $_{1/2s}$) of 1.02 hr. Long-lived components represented a proportion of 53.63% with a computed half lives (Tb $_{1/2l}$) of 198.61 hr.

For soft tissue , loss kinetics was also followed a two-component exponential model (Figure 2b), short-lived components represented 19.28 % of a portion of the total activity of with a computed half lives (Tb $_{1/2s}$) of 1.13 hr. Long-lived components represented 80.60% of a portion of the total activity with a computed half lives (Tb $_{1/2l}$) of 58.4 hr.

Concentration of dissolved inorganic zinc measured in natural seawater was <0.0080 µg/L of seawater in consistent with those previously found in oceanic waters, ranging from ca. 0.1 to 100 nM (.0065 to 6.541 µg/L) in surface and deep waters, respectively [14,15].

The uptake kinetics of Zn-65 from seawater by *A. formosa* identified a linear rate of increase of zinc with an incubation time of 96 hr in consistent with those on *Stylophora pistillata* whole colony over an incubation time of 72 hr [5]. The concentration factor of 3.90 for whole coral and 449.19 in soft tissue indicate *A. formosa* accumulate Zn-65 in their soft tissue higher than those in their skeleton. After 96 hr of depuration in clean seawater, 46.14 ± 7.58 % of the total Zn-65 activity of the whole coral was rapidly released. This fraction represented the short-lived loss component characterized by a biological half-life of 1.02 hr. The long-lived component representing the major proportion of Zn-65 accumulated at the end of the exposure period, (i.e. 53.63±5.14%), displayed a depuration rate constant of approximately 198.61 hr. In soft tissue, 19.28±9.54% of the total Zn-65 activity was eliminated in a few hours with a biological half-life of 1.13 hr while 80.60 ±7.30% of the total Zn-65 activity was retained however this amount of Zn was depurated out with a biological half life of 58.39 hr, faster than those from the whole coral. Zn-65 distribution among body compartments at the end of the exposure period indicated that most of the Zn-65 activity was found in tissue (112 times of those in skeleton).

Ferrier-Pages et al [5] found that in a few hours about 30% of the total zinc was eliminated from *S. pistillata* whole colony and suggested that short lived component of Zn was probably those weakly adsorbed to the coral's mucus as it was known to bind metal [16]. The remaining component was therefore metabolized (involving in metabolic process).

Hanna and Muir [9] investigated the bioaccumulation of trace metals on three coral species, *Porites lutea*, *Goniastrea retiformis* and *Pocillopora verrucosa*. The result demonstrated corals had great affinity to accumulate trace metals from the surrounding environment. The metal concentrations were all significantly higher for those corals from polluted areas compared to the unpolluted areas. Pb and Cd were found to be in higher concentrations in skeletal material than in soft tissue. However, soft tissue and detritus of corals were found to have higher concentrations of Zn, Ni, Cu, Mg, and Fe than the skeleton. Zn concentration in soft tissue was even found to be about 150 times of those in the skeleton. Several authors [17,18] have reported similar findings and attributed it to the metabolic, feeding and filtration rate.

Esslemont [19] investigated the potential of *Goniastrea aspera*, *Porites danicornis*, and *Acropora formosa* on accumulation of trace metals, Cd, Pb, Cu, Ni, Cr, and Zn. All three corals species showed affinities to accumulate trace metals both in their soft tissue and skeleton, especially, soft tissue can accumulate trace metals higher than skeleton. Metal concentrations in skeleton were found to be correlated to trace metal concentration in the environment; however, this correlation was not found in soft tissue.

Toxicant uptake and depuration experiments using radiotracer proxies of stable metals (e.g. radioactive Zn-65) offer a unique approach for investigating and understanding the behavior and fate of toxic substances in coastal systems. The resultant transfer factors also referred to as bioaccumulation or concentration factors; indicate the most likely toxicant concentration in organisms relative to its degree of exposure (i.e. time and/or concentration). These studies can be used to better understand the impact of acute exposures to toxicants (as in industrial discharge or natural disaster like tsunami event) by the rate of uptake and loss for predictive modeling

Conclusion

Bioaccumulation of zinc on Scleractinian Corals *Acropora formosa* was investigated using Zn-65 as a radiotracer. The experimental condition was controlled as follows: 29-30^{o}C temp, 32-34 ppm salinity, 7500-10000 Lux constant luminances, 12:12 h photo period. Accumulation of Zn-65 by corals was followed after 0,2,4,8,24,48,72, and 96 hr. The uptake kinetic followed a simple linear model with the maximum concentration factor (CF_m) after an exposure time of 96 hr were 400 and 47700, for whole coral and soft tissue, respectively. The non linear regression fitting revealed the uptake rate of 3.90 $CF.hr^{-1}$ or 3.28 $Bq.g^{-1}.hr^{-1}$ by coral whole body with R^2=0.93 corresponding to 0.01 pg $Zn.g^{-1}$ whole $coral.hr^{-1}$ and 449.19 $CF.hr^{-1}$ or 377.38 $Bq.g^{-1}.hr^{-1}$ with R^2=0.85 corresponding to 12.56 $pg.g^{-1}$ protein in soft tissue, respectively. Loss kinetics followed a two-component exponential model with the short lived components represented a proportion of the total activity 46 and 20%; with the computed half lives ($Tb_{1/2s}$) 1.02 and 1.13 hr, for whole coral and soft tissue, respectively. Long-lived components represented a proportion of the total activity 54 and 80%; with the computed half lived ($Tb_{1/2l}$) 198.6 and 58.4 hr, respectively. *A. formosa* efficiently accumulated Zn-65 in both whole corals and soft tissue and it remained in its body with relatively long half-lived.

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References

[1] MA. Anderson, FMM. Morel, *Limnol Oceanogr*.**239** (1978) 283-295.
[2] FMM. Morel, JR. Reinfelder, SB. Roberts, CP. Chamberlain, JG. Lee, D. Yee, *Nature*. **369** (1994) 740-742.
[3] JT. Cullen, TW. Lane, FMM.Morel, RM. Sherrel, *Nature*. **402** (1999) 165-167.
[4] TW. Lane, MM. Morel. *Plant Physiol*. **123(1)** (2000) 345-352.
[5] C.Ferrier-Pages, F. Houlbreque, E. Wyse, C. Richard, D. Allemand, F. Boisson,*Coral Reefs*. **24** (2005) 636-645.
[6] WG. Sund ,*Biol Oceanogr*. **12** (1991) 411-418
[7] G. Esslemont, VJ.Harriot, DM. McConchie, *Mar. Pollut Bull*. **40(7)** (2000) 637-642
[8] AJ.Reichelt-Brushett , G.McOrist , *Mar Pollut Bull*. **46** (2003). 1573-1582.
[9] RG. Hanna, GL. Muir, *Environmental Monitoring Assessment*. **14** (1990) 211-222.
[10] E.Tambutte,D.Allemand, I. Bourge, JP. Gattuso, J.Jaubert, *Mar. Biol.* **122** (1995)453-459
[11] M. Warnau, JL. Teyssie, SW . Fowler, *Mar.Ecol. prog. Ser.* **14** (1996) 83-94.
[12] JR. Reinfelder, NS. Fisher, WX. Wang, J. Nicholas, SN. Louma, *Sci.Total Environ*. **219** (1998)117-135.
[13] Statsoft.Satistica: The Small BookTulsa,OK.Statsoft Inc (2004)166.
[14] KW. Bruland, *Earth Planet Sci, Lett* **47** (1980) 176-198.
[15] JH. Martin, RM. Gordon, S. Fitzwater, WW. Broenkow VERTEX:. *Deep Sea Res* **36** (1989) 649-680.
[16] R. Howell, *Nematologia* .**28** (1982) 110-114.
[17] AP. Vinogradov, *Sears Found, Mar. Res. Yale Univ.*, Memoir No. **11**(1953),647.
[18] R. Eisler, *TraceMetal Concentration Marine Organism*, Pergamon Press, London, Oxford, UK (1981).
[19] G. Esslemont, *Marine Chemistry*. **71** (2000) 215-231.

CHEMICAL PRETREATMENT OF BAGASSE FOR DYE REMOVAL

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Abstract: In this study, the bagasse, wastes from the sugar industry, was used as a biosorbent for an adsorption of dyes such as methylene blue (MB) and direct Blue 2B (DB). Effects of contact time and initial dye concentration were investigated in batch experiments. The adsorption equilibrium of bagasse was reached at 29 hours and 9 hours for MB and DB respectively. It was seen that the removal of dyes increased with the increasing of initial dye concentration. The amount of dye adsorbed of bagasse increased from 0.30 to 3.66 mg/g for MB and 0.49 to 3.63 mg/g for DB when the initial dye concentration was raised from 10 to 80 mg/L.

Moreover, the bagasse pretreated with 0-3 mol/L alkali (NaOH and KOH) prior to adsorption was used for dye removal. The pretreated bagasse showed greater amount of dye adsorbed than that of unpretreated bagasse at any concentration. At the initial dye concentration of 20 mg/L, the amount of dye adsorbed of unpretreated bagasse was only 0.74 and 0.81 mg/g for MB and DB respectively. However, the amount of dye adsorbed of bagasse pretreatment with 2 mol/L NaOH was increased to 1.87 and 1.99 mg/g for MB and DB respectively. It might be indicated that the chemical pretreatment would be the method for improving the adsorption of both dyes (direct dye and cationic dye) on to the biosorbent.

1. Introduction

Industrial wastewater has become a major and serious problem in recent years. Many industries such as paper, plastics, leather, textile, and paint industries use dyes to color their products. It is found that 10–15% of the dye is lost in the effluent during the dyeing process [1,2]. The presence of dyes in effluents is a major concern for both toxicological and esthetical reasons. The adsorption is effective method for the removal of dyes from wastewaters due to its low cost, simplicity of design, ease of operation and insensitivity to toxic pollutants [3].Various agricultural by-products, which are cheap, renewable, easily available such as rice straw, orange peel, banana pith and corn cob, were used as sorbent in order to remove dyes from aqueous solutions[4-5]. In Thailand especially in Kanchanaburi province has many sugar factories. Thousands of tons of bagasse are discarded daily by these factories, leading to a big environmental problem. One alternative solution for bagasse exploitation might be used as adsorbent for dye removal.

In this present work, the sugarcane bagasse, wastes from sugar industry, was used as adsorbent for dye removal. The amount of dye adsorbed of bagasse for methelene blue and direct blue 2B removal were investigated. In addition, the enhancing of dye removal by sugarcane bagasse pretreated with KOH and NaOH was reported.

2. Materials and Methods

2.1Adsorbent preparation

Unpretreated bagasse

The bagasse used as biosorbent in this experiment was obtained from Thai sugar industry Co.,Ltd in Kanchanaburi province, Thailand. The bagasse was firstly washed with tap water and followed by hot water several times in order to remove retained sugar in bagasse. Then, the bagasse was washed with distillation water and naturally dried under sunlight for three days. After that, it was dried in an oven at 80 ^{o}C for 24 hours. The dried bagasse was crushed in a blender and sieved to obtain a particle size between 0.2-0.5 mm.

Pretreated bagasse

The bagasse was immersed in aqueous alkali solution (KOH, NaOH) in the concentration range of 0-3 mol/L for 2 hours. Then, the bagasse was removed from alkali solution and washed several time with tap water. The bagasse was then neutralized by immersion in 1 mol/L acetate buffer (pH 5.5) for 2 hours. After that, the bagasse was washed again with distillation water until the pH of solution was closed to 7. The bagasse was dried und sunlight for three days. Then, it was further dried in an oven at 80 ^{o}C for 24 hours. The dried bagasse was crushed in a blender and sieved to obtain a particle size between 0.2-0.5 mm.

2.2Adsorbate preparation

Methylene blue(MB) supplied by RFCL limited and direct dye 2B (DB) supplied by Dalian Richon Chem Co.,Ltd. were used as adsorbate. A stock solution of dye (1000 mg/L) was prepared by dissolving a required amount of dye powder in deionized water. In the experiment, the stock solution was diluted with deionized water to obtain the desired concentration ranging from 20 to 80 mg/L.

2.3 Batch sorption studies

Batch sorption experiments were performed by adding an 1 gram of adsorbent into a 250 mL

erlenmeyer flask containing 50 mL of dye solution and then, shaking in orbital shaker at 100 rpm. The adsorbent was separated from solution by filtration and centrifugation prior to analysis in order to minimize the interference of the fine adsorbent particles.

The dye concentrations in solution were determined by absorbance measurement using UV Vis spectrophotometer at 664 nm for MB and 615 nm for DB . Dye concentration was then calculated using the standard calibration curves of MB and DB. The amount of dye, q (mg of dye /g of sorbent) was calculated using the following equation:

$$q = \frac{C_0 - C}{m} V$$

Where C_0 and C represent the concentration of dye (mg/L) in the solution at time, t = 0 and at any time, t. V is the volume of solution (L) and M is the amount of adsorbent used (g).

3. Results and Discussion

Effect of contact time and initial dye concentration

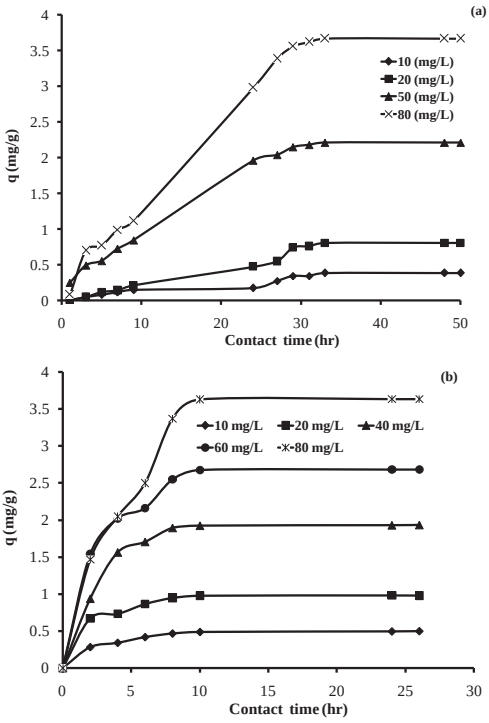

Figure 1. Effect of contact time and initial dye concentration on amount of dyed adsorbed
(a) methylene blue (b) direct blue 2 B

The effect of contact time and initial dye concentration on the amount of dyed adsorbed was investigated using both methylene blue (fig 1a) and direct blue 2B (fig 1b) at various initial dye concentrations (10-80 mg/L). It was observed that the amount of dye adsorbed (q) increases with an increase in initial dye concentration. Moreover, the amount of dye adsorbed gradually increased at the beginning of contact time and then remained constant with the increasing contact time. The equilibrium time for methylene blue and direct blue 2B at any dye concentration were 29 and 9 hours respectively. The data showed that the equilibrium adsorption increases from 0.30 to 3.66 for MB and from 0.49 to 3.63 for DB with the initial dye concentration increased from10 to 80 mg/L. This may be due to the fact that ,by increasing the concentration of dye in solution, the driving force for mass transfer increases and the number of collisions between dye ions and the adsorbent also increase[6], thus enhancing the amount of dye adsorption

Effect of alkali pretreatment

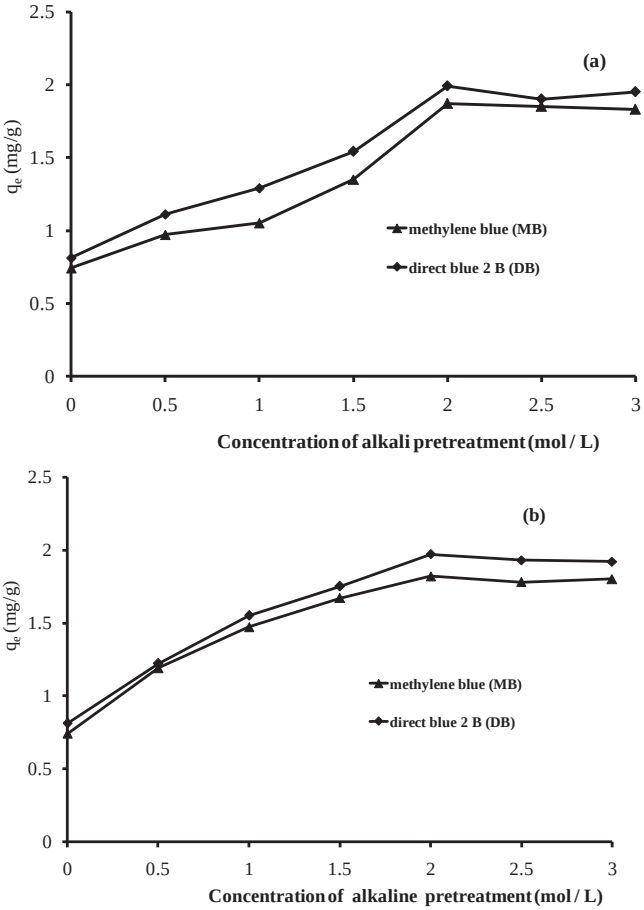

Figure 2. Effect of alkali pretreatment on amount of dyed adsorbed
(a) NaOH pretreatment(b) KOH pretreatment

In this part, the effect of alkali type and alkali concentration on amount of dyed adsorbed at equilibrium (q_e) were studied. This study was done by the pretreatment of the bagasse with alkali prior to perform an adsorption experiment. For the treatment, the concentration of alkali solution was varied from 0 to 3 mol/L. A fixed initial dye concentration of 20 mg/L was used for adsorption experiment. The result of this experiment are shown in fig 2 . The amount of dye adsorbed , for all dyes and for both NaOH and

KOH pretreatment, initially increased up to 2 mol/l, and then remained constant with the increasing alkali concentration. The maximum amount of dye adsorbed of bagasse was obtained at 2 mol/L in both NaOH and KOH pretreatment. The amount of dye adsorbed of untreated bagasse was only 0.74 and 0.81 mg/g. However, the amount of dye adsorbed increase to 1.87 and 1.99 mg/g for MB and DB respectively when pretreatment with 2 mol/L NaOH and increase to 1.82 and 1.97 for MB and DB respectively when pretreatment with 2mol/L KOH

It was known that alkali pretreatment is a method for pretreatment of lignocellulosic biomass. Alkali causes lignin removal from biomass, leading to the decreased degree of polymerization and crystallinity of cellulose in biomass. Thus, the accessibility of chemical substance to fiber and the inner surface area were increased [7]. These might be the reason for enhancing the dye removal of pretreated bagasse as compared to that of untreated bagasse. Moreover, there were no significant differences in amount of dye adsorbed between bagasse pretreated with KOH or with NaOH at any level of alkali concentration.

4. Conclusions

The present work shows that the bagasse, wastes from sugar industry, can be used as an adsorbent for the removal of basic dye such as methylene blue and direct dye such as direct blue 2B from its aqueous solutions. The amount of dye adsorbed was found to vary with both initial dye concentration and contact time. The equilibrium time of MB and DB was reached at 29 hours and 9 hours, respectively. The maximum amount of dye adsorbed of bagasse, obtained at an initial dye concentration of 80 mg/L, was found to be 3.66 mg/g for MB and 3.63 mg/g for DB. In addition, this work presented that the alkali pretreatment can be the process for increasing the amount of dye adsorbed of both basic dye and direct dye. The amount of dye adsorbed was also influenced by alkali type and alkali concentration on the pretreatment step.

Acknowledgements

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References

[1]L. Young and Y.U. Jain, Ligninase-catalyzeddecolorization, *Water Res.***31** (1997) 1187–1193.

[2]L. Lian, L. Guo and C. Guo, Adsorption of Congo red from aqueous solutions onto Ca-bentonite, *J. Hazard. Mater*. **161** (2009) 126–131.

[3] V.K.Garg, R. Kumar and R. Gupta,*Dyes Pigments.***62** (2004), 1–10.

[4] C. Namasivayam, and N. Kanchana, *Chemosphere*.**25** (1992) 1691–1705.

[5] G. Annadurai, R.S. Juang and D.J. Lee, *J. Hazard. Mater*.**B92** (2002) 263–274.

[6] M. Z. B. Mukhlish, M.R. Khan, M.C. Bhoumick and S. Paul, *Water, Air, & Soil Pollutio*. **223**, 4949-4958.

[7] Y. Zheng, Z. Pan and R. Zhang,. Int J Agric & Biol Eng.**2**(2009)51-68.

CAPABILITY OF PLANTS FOR INDOOR NITROGEN DIOXIDE ABSORPTION

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Abstract: Nitrogen dioxide (NO_2) is an important indicator of air pollution. It is considered the most toxic form of nitrogen oxides. Indoor sources of NO_2 combustion appliances and activities such as smoking, gas heating, cooking, etc. Vegetation has also been shown to be the most significant sink area for NO_2, thereby improving air quality. This study aims to test the capability of plants in NO_2 absorption in the testing chamber. A selected ornamental plant, namely dumb cane (*Dieffenbachia seguine*), was put into the sealed chamber and left for 8 hours for the short-term experiment. NO_2 was produced for the experiment by dissolving copper powder in nitric acid in a dessicator. Then, NO_2 was pumped into the chamber along with diluted with air until a concentration of approximately 20 ppm was reached. NO_2 concentration levels were measured using gas analyzer every 30 minutes over 8 hours. A control for the experiment was set by measuring the NO_2 concentration level in the chamber, without plants during the same period of time. The changing of NO_2 concentrations in the chamber was assessed for the plant absorption. It was found that the dumb cane absorbed 4-7 ppm of NO_2 in 8 hours. However, the soil also showed a significant level of impact from NO_2 absorption. It therefore needs to be proven that NO_2 absorption mainly occurs through the plant itself, but also through the soil. If ornamental plants can absorb NO_2 from the environment, it could be developed in a plant-based bio-filtration system in the future.

1. Introduction

Nitrogen dioxide (NO_2) is a major air pollutant that causes the production of photo-oxidants such as ozone by a photochemical reaction with hydroxyl radical produced from volatile organic compounds in the atmosphere [1]. The Organization for Economic Co-operation and Development (OECD) estimates the input of the anthropogenic emissions of NO_x, which includes nitric oxide (NO) and NO_2, at some 50 Tg per year [2]. The main source of NO_2 occurs from human activities involved with the combustion of fossil fuels, such as motor vehicles, while the indoor sources involve combustion appliances and activities, such as smoking, gas heating and cooking. The U.S. Environmental Protection Agency (USEPA) has developed guidelines for acceptable indoor air quality and recommends that the level of NO_2 should not exceed the National Ambient Air Quality Standards (NAAQS) of 0.05 ppm for 1 year period [3]. The World Health Organization has a guideline for indoor NO_2 levels (1 hour and 1 year), which should not exceed 0.1 ppm and 0.004 ppm, respectively [4].

The air quality of the indoor air environment has become a major health consideration in the developed world, since urban-dwellers generally spend 80-90% of their time indoors [5]. Tightly sealed buildings are an additional concern for the health of those who live and work inside. Skolnick (1989) reported that a population living in tight energy efficient buildings contracted upper respiratory diseases at rates 46% to 50% higher than a comparison group who were living in better-ventilated homes [6]. The effects of air pollution on human health are considered very complex. After inhalation, air pollutants affect human health by severely damaging the lungs and respiratory system [7-9]. The United Nations has estimated that over 600 million people worldwide in urban areas are exposed to dangerous from the traffic-generated air pollutants [10]. From many epidemiological studies, it has been shown that this type of pollution does cause a variety of respiratory diseases whether or not it is combined with other forms of air pollution, such as ozone, sulfur oxide, and particulate materials less than 2.5 μm [11]. Plants are also affected by NO_2 exposure, in terms of the reduction of net photosynthesis, their respiration, stomatal conductance, enzyme activities, and growth [12]. High concentrations of NO_2 can cause acute damage to plant leaves, probably through the accumulation of nitrites [13, 14]. Chronic exposure to lower concentrations of NO_2 usually reduces the growth of plants [15-17].

Some plant species have a capacity for NO_2 phytoremediation that occurs from the atmosphere [18, 19] and can assimilate its nitrogen into organic nitrogenous compounds through the primary nitrate assimilation pathway [19, 20]. The capability of plants to take up and assimilate NO_2 depends on the plant species [21] and the NO_2 concentration levels in the atmosphere [22].

This research therefore aims to test the ability of selected plant in absorbing NO_2 in the air. Even though they are many research studies that have been conducted using plants to absorb pollutants, this is the first study that has used dumb cane for NO_2 absorption.

2. Materials and Methods

2.1 The selected plant for NO_2 absorption

Dumb cane (*Dieffenbachia seguine*) is a very common ornamental plant. The leaf of the dumb cane is broad and has white streaks or speckles on a green background. Some leaves are green or cream-colored with a green border. The height is about 0.3 - 1.8 m, while newer hybrids are more compact reaching about 30 - 60 cm [23].

This plant originated in tropical areas and is a popular ornamental plant used in home and office decoration. There are 25-30 species, but the two main species are *Dieffenbachia seguine* and *Dieffenbachia pincta* [24]. Dumb cane is a foliage plant, which can grow indoors. It grows well in bright, indirect sun-light and needs low amounts of water. Based on the properties of the dumb cane mentioned above, it was therefore selected to test its capability in NO_2 absorption from the air.

2.2 Preparation of selected plants

Dumb canes with a height of approximately 20 cm and 6 - 7 leaves, planted in plastic bags were selected from the nursery. They were transferred to the new pots containing soil with a high content of humus and organic matter. They were left indoors with an appropriate amount of indirect sunlight for about 1 week and were watered every 2 days. After that, each plant was put inside the chamber for further experimentation. Each experimental condition was repeated 3 times using new plants.

2.3 Experimental chamber

The schematic diagram of the NO_2 experimental chamber is shown in Figure 1. The chamber was made from acrylic plate (thickness 0.4 cm) in order to minimize NO_2 absorption on the surface. The size of the chamber is 0.5 m (*W*)×0.5 m (*L*)×0.6 m (*H*), which accounts for a total of 0.15 m^3. The chamber was equipped with a NO_2 sensor (Testo 350-XL, Germany), a thermometer, a barometer, a mini-fan and a hygrometer. An NO_2 generator and a pump (flow rate 0.037 m^3/min) were also installed. NO_2 gas was produced by dissolving copper powder in nitric acid in a desicator (generator). Then NO_2 was pumped into the chamber and it was diluted with air until a concentration of approximately 20 ppm and 40 ppm was reached. Concentration levels were measured with a gas analyzer every 30 minutes over 8 hours. The temperature inside the chamber was kept at 27 ± 1^oC, while the relative humidity was recorded at $60\pm2\%$.

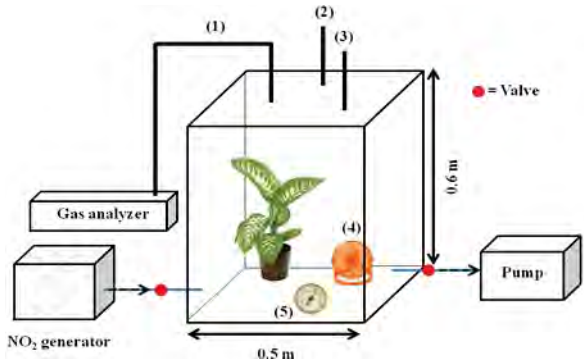


Figure 1. Schematic diagram of the NO_2 experimental chamber (1) NO_2 sensor (2) thermometer (3) barometer (4) minifan (5) hygrometer

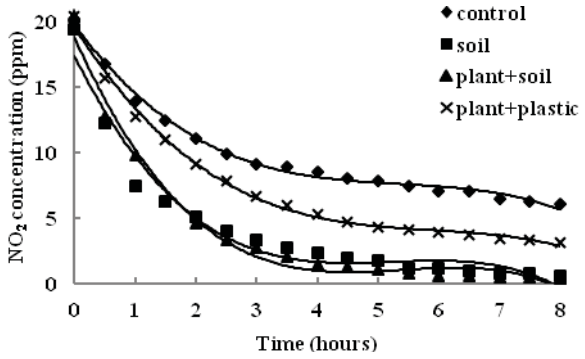
2.4 NO_2 absorption by plants in the closed system

In order to test the capability of dumb cane in NO_2 absorption from the air in the closed system, 4 conditions were set up: 1) a control set; the chamber without a plant, 2) soil; a pot containing soil without a plant, 3) soil+plant; a pot containing soil and a plant and 4) plant; a pot containing soil and a plant with a plastic bag covering the pot to prevent gas absorption through the soil media. The plants were tested in the chamber with NO_2 concentration levels beginning at 20 and 40 ppm. Each condition was repeated 3 times for 8 hours (9.00 am - 5.00 pm). After that, the differentiation of the NO_2 concentration levels in the chamber was assessed for the absorption rate. Moreover, the morphological change of plant was also observed and recorded.

3. Results and Discussion

3.1 NO_2 absorption by dumb cane

(a)



(b)

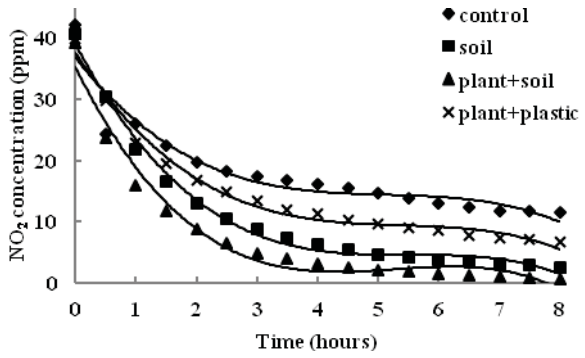


Figure 2. Changing of NO_2 concentrations (ppm) in the chamber over 8 hours with starting concentration levels of (a) 20 ppm and (b) 40 ppm

The average NO_2 concentrations (n=3) from each experimental condition were plotted against time (Figure 2). The graph patterns of the NO_2 concentrations over an 8-hour period for all conditions were almost the same. For the control set, the NO_2 level was quickly reduced within the first 3 hours of the experiment. After that, it slowly decreased and remained quite constant. This is probably due to the presence of some leaks in the chamber and certain amount of loss through the NO_2 sensor. In the case of 20 ppm NO_2, the final concentration after 8 hours was ~ 6 ppm (Figure 2a), while at 40 ppm NO_2, the concentration level was reduced to ~ 11 ppm (Figure 2b). This means that the NO_2 level in the chamber decreased ~ 4 times over 8 hours.

Based on the NO_2 level detected at both concentrations, it was found that the soil and plant showed a capability to absorb NO_2 from air. Based on the Kruskal-Wallis test, there was no significant difference ($\alpha = 0.05$) of NO_2 absorption by the soil alone or by a combination of plant and the soil. They however differed in NO_2 levels remaining in the control set and the set using the plant alone. This could be because NO_2 absorption through the soil was greater than by the plant leaves. However, a combination of plant and soil did provide a higher capacity of absorption, than did soil without plants and/or an individual plant.

(a)

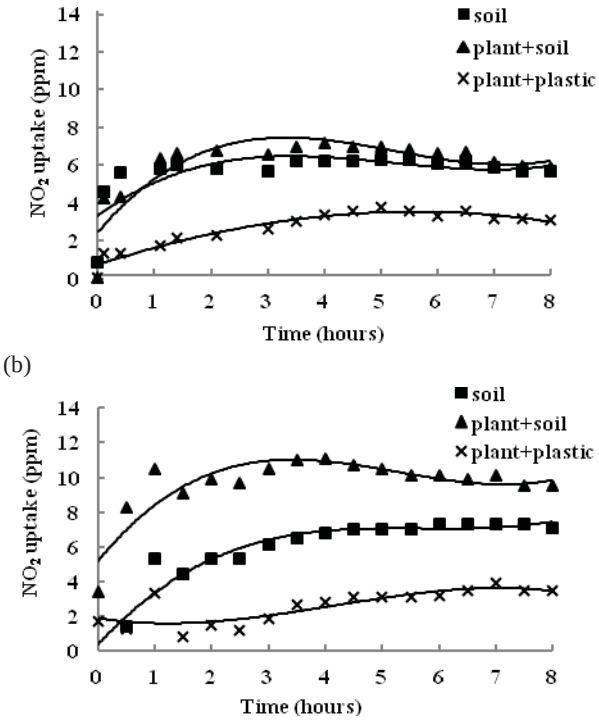


Figure 3. NO_2 uptake (ppm) by dumb came over 8 hours for (a) 20 ppm and (b) 40 ppm of NO_2

The uptake of NO_2 by plants was calculated from the NO_2 concentrations detected under each condition subtracted by those of the control set. It was found that the NO_2 removal by the dumb cane was rapid in the first two hours of exposure. After that the uptake slowed and remained constant (Figure 3). The results showed that the plant and soil could efficiently absorb NO_2 from air in the sealed chamber. It was also found that these 3 conditions were significantly different ($p < 0.05$) in terms of NO_2 uptake from the air. The capability of NO_2 removal of the 3 conditions in descending order were plant+soil > soil > plant. The absorption by the surfaces of the plant and the soil was easily reached in a short time. A dramatic change in the removal concentrations could be due to the uptake by the stomata of the plant and the degradation by microorganisms in the soil media.

Table 1: NO_2 uptake (ppm)

Experimental conditions	NO_2 uptake (ppm)	
	20 ppm (n=3)	40 ppm (n=3)
(1) Soil	4.4-6.1	4.4-7.3
(2) Plant & soil	4.0-7.0^x	8.3-11^y
(3) Plant without soil (covered with plastic)	1.1-3.6	0.8-3.9

$^{x, y}$ represent significant difference at 95% confidence ($\alpha = 0.05$)

The NO_2 uptake by the plant and the soil is shown in Table 1. It was found that the removal concentrations were almost the same at 20 and 40 ppm either by the soil (1) or by the plant (3). Moreover, the soil showed an NO_2 absorption rate at 2-4 times higher than the plant. However, when NO_2 concentration was increased from 20 to 40 ppm, NO_2 removal by the plant & soil (2) also increased from 4-7 ppm to 8-11 ppm. By a comparison of NO_2 uptake between 20 and 40 ppm NO_2 concentrations under each experimental condition, it was found that only the conditions of the plant and soil combination showed a significant difference ($p < 0.05$) of NO_2 uptake according to the Mann-Whitney test. At higher concentrations of NO_2 (40 ppm), the plant and soil showed a higher NO_2 uptake than that of lower concentrations of NO_2 (20 ppm). Similar work has been done in the real environment using trees for ambient NO_2 uptake. It was reported that bullet wood and queen's flower trees could absorb NO_2 from ambient air in highly polluted areas at a range of 6.2-46.1 and 0.4-18.3 ppbv, respectively, whereas, the absorption levels at areas that were classified as being low polluted areas were 3.2-10.3 and 0.7-13.3 ppbv, respectively [25]. It should be noticed that the NO_2 concentration in the real environment is much lower than it was in the experiments carried out in the closed chamber system.

3.2 Effect of NO_2 on dumb cane morphology

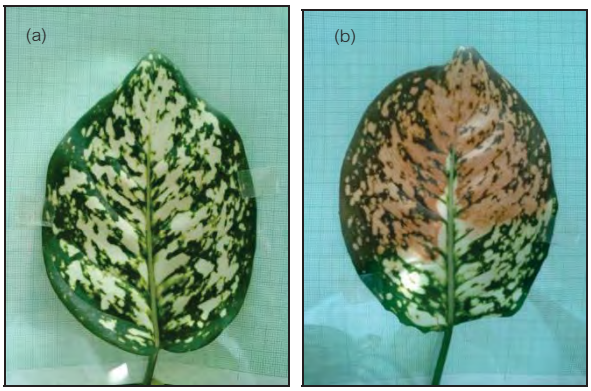


Figure 4. leaf of dumb cane (a) before and (b) after being exposed to 20 and 40 ppm NO_2 for 8 hours

Morphological changes of dumb cane leaves after exposure to high concentrations of NO_2 were observed (Figure 4). It was obvious that NO_2 affected and damaged the leaf of the dumb cane. The external appearance of the healthy leaf (Figure 4a) changed to appear as a burnt leaf (Figure 4b) after exposure to 20 and 40 ppm of NO_2 for 8 hours. About 60% of leaf necrosis was observed after one day of exposure. However not every leaf was affected, about half of the total leaves in one plant were damaged and ultimately died. This is probably due to the short-term exposure. Therefore, plants can still recover and continue to grow after the exposure. The results well agreed with Tingey et al. [26]. They used tobacco, radish, oats and alfalfa plants for NO_2 exposure at a concentration of 8 ppm for 4 hours and found that leaf injuries from NO_2 occurred as marginal and/or as interveinal necrosis on each leaf surface (bifacial) and may result in yield losses for plants grown under field conditions.

4. Conclusion

This study has demonstrated that the dumb cane can absorb NO_2 from the air. Its capability for NO_2 absorption was found to have increased with the NO_2 concentrations in the air. The knowledge of absorption of air pollutants by plants is important not only for determining the dose-response of pollutants, but also to gain an understanding of the removal of pollutants from the atmosphere.

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References

[1] Wellburn, A.R., Wellburn, A.R. *Air Pollution and Climate Change: the BiologicalImpact, second ed. Longman Scientific & Technical,England*. (1994) 57–82.

[2] Organization for Economic Co-operation and Development. *OECD Environmental Data Compendium*. (2002) 14–22.

[3] American Society of Heating, Refrigerating and Air-Conditioning Engineers (ASHRAE). *Ventilation for Acceptable Indoor Air Quality*. (2004).

[4] World Health Organization. *WHO Environmental health* criteria *190: Xylene. Geneva: World health Organization*. (1977)

[5] Brunekreef, B., Holgate, S.T. *Air pollution and health. Lancet* **360** (2002) 1233–1242.

[6] Skolnick, A. *Journal of the American Medical Association*. **262** (1989) 3102-3103.

[7] Sather, M.E., Slonecker, M., Daughtery, J. and Williams, D.D. *Environmental Monitoring and Assessment* **124** (2007) 211-221.

[8] Caciola, R. R., Sarva, M., and Pasola, R., *Atmospheric Pollution Research*. **6** (2002) 57–281.

[9] Kato, T., Tachibana S. and Inden, T. *Environmental Control in Biology*. **12** (1974) l03-107.

[10] Matsumaru, T., Youneyama, T., Tsumugu, T. and Yoshihiro, M. *Soil Science and Plant Nutrition*. **27** (1981) 255-261.

[11] Wellburn, A.R. *Air Pollution and Climate Change: the Biological Impact, second ed. Longman Scientific & Technical*. (1994) 57–82.

[12] Pandey, J. S., Kumar, R., and Devotta, S. Health risks of NO_2, SPM and SO_2 in Delhi (India). *Atmospheric Environment*. **39** (2005), 6868–6874.

[13] Rogers, H.H., Campbell, J.C., Volk, R.J. *Science* **206** (1979) 333–335.

[14] Yoneyama, T., Sasakawa, H. *Plant Cell Physiol*. **20** (1979) 263–266.

[15] Morikawa, H., Higaki, A., Nohno, M., Takahashi, M., Kamada, M., Nakata, M., Toyohara, G., Okamura, Y., Matsui, K., Kitani, S., Fujita, K., Irifune, K., Goshima, N. *Plant Cell Environ*. **21** (1998) 180–190.

[16] Pandey, J.S., Kumar, R., and Devotta, S. Health risks of NO_2, SPM and SO_2 in Delhi (India). *Atmospheric Environment*. **39** (2005), 6868–6874.

[17] Gilbert, L., Goldberg, M.S., Brook, J.R. and Jarret, M. *Atmospheric Environment* **41**(2007) 2670-2673.

[18] Taylor, O.C. and Eaton, F.M. *Plant Physiology*. **41** (1966) 132-135.

[19] Thomson, C.R., Hensel, E.G., Kate, G and Taylor, O.C. *Atmospheric Environment*. **4** (1970) 349-355.

[20] Ashenden, T.W. and Mansgield, T.A. *Nature* **273** (1987) 142-143.

[21] Lucas M.N., Douglas W.D., James H.W., John D.S., Frank E.S. and Benjamin G.F. *American Journal of Epidemiology*. **134** (1991) 204-219.

[22] Bootdee, S. Master.s Thesi, Chiang Mai Unversity, (2009)

[23] http://www.guide-to-houseplants.com/dumb-cane.html (Retrieved December 13, 2012)

[24] McGovern T. *briefs: dumb cane (Lodd.) Schott. Cutis*. **66** (2000) 333-4.

[25] Farrelly, W. 2011. M.S. Thesis, Faculty of Science, Chiang Mai University.

[26] Tingey, D.T., Reinert, R.A., Dunning, J.A. and Heck, W.W. *Phytopathology*. **61** (1971) 1506-1511.

Free radicals/ Antioxidants

TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF SWEET POTATO (*IPOMOEA BATATAS* L.) FLOURS FROM DIFFERENT VARIETIES GROWN IN THAILAND

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Abstract: Sweet potato (*Ipomoea batatas* L.) can be grown in most part of Thailand. Each variety is distinct in its flesh color depended on pigment compound which could contribute to an antioxidant activity to its flour. Four sweet potato varieties different in their flesh color were used in this study, namely 0106-1(white), Phichit 265-1 (deep-yellow), T101 (orange), and Phichit 65-3(deep-purple). Potato (Atlantic variety) was used for comparison. The flours of the sample were prepared by hot-air drying, milling, and sieving. The flours were analyzed their chemical compositions, total phenolic content and DPPH radical scavenging activity. The proximate analysis showed that the flours were significantly ($p \leq 0.05$) different in their chemical compositions. They contained 7.70-12.08% (wet basis; wb) in moisture, 2.72-10.75% (dry basis; db) in protein, 0.79-6.72% (db) in fat, 1.85-5.30% (db) in crude fiber, 2.54-5.10% (db) in ash and 79.92-87.44% (db) in carbohydrate. Sweet potato flours were significantly ($p \leq 0.05$) higher in crude fiber than potato flour. The flours were also different in their total phenolic content which was in the range of 168.27-448.93 mg gallic acid equivalent (GAE)/100g sample and DPPH radical scavenging activity which was in the range of 1.167-9.843 mg flour/ml (as expressed in half maximal effective concentration; EC_{50}). Sweet potato flours were higher in total phenolic content and EC_{50} than potato flour. Among sweet potato, flour from Phichit 65-3 variety had the highest in phenolic content and EC_{50} following by T101, Phichit 265-1, and 0101-1 varieties. This study suggested that sweet potato flours are a good source of fiber and high in antioxidant activity especially, Phichit 65-3 variety.

Keywords: sweet potato; potato; antioxidant; phenolic; DPPH

1. Introduction

Sweet potato (*Ipomoea batatas* L.) is a tuber plant that endures to tropical weather resulting in high growth rate and production yield. It can be grown in most part of the country especially, in the central region of Thailand. Its average annual productivity is 184,353 tons or 1,336.559 million THB [1]. Almost all parts of sweet potato (buds, leaves, tubers, and roots) can be eaten; however, the tuber is mainly consumed due to its high amount of carbohydrate. Sweet potato has many varieties different in flesh color, such as white, brown, yellow, orange, and purple.

Compared with other flours, sweet potato flour is considerably rich in proteins, dietary fibers, vitamins, minerals, and phenolic compounds such as anthocyanins, flavanoids, and carotenoids [2-3]. These chemical compositions vary among sweet potato varieties [4]; especially, with different in flesh color. Purple sweet potato which is high in anthocyanins content, exhibited significantly higher in antioxidant activities than white sweet potato [5]. Due to its acid tolerance, Fan et al. [6] use anthocyanins extracted from purple sweet potato (ZiA0 variety) as a natural colorant in high acid foods. Beta carotene was also found at 83.3 µg/g in orange sweet potato (Tainong 66 variety) [7].

There are many varieties of colored sweet potatoes grown in Thailand, however their chemical compositions and functional properties have not been thoroughly investigated resulting in a limit use in food industry. This study was aimed to investigate the chemical compositions, total phenolic content, and DPPH radical scavenging activity of four main varieties of colored sweet potato grown in Thailand. Potato which is also a tuber plant and has been widely used in the food industry has been also investigated for comparison.

2. Materials and Methods

2.1 Materials

Potato (Atlantic variety) and four sweet potato cultivars varying in flesh colors; namely, 0106-1 (white), Phichit265-1 (deep-yellow), T101 (orange), and Phichit65-3 (deep-purple) were obtained from Department of Agriculture, Thailand. All samples were in the same age and grown in the same environment.

2.2 Preparation of flours

The flour preparation was modified from the method of Yadav [8]. The tubers were cleaned, peeled, and sliced into small cubes (approximately 1x1x1 cm^3). Then, the sample cubes were hot-air dried at 40 ^oC for 10 h. The dried samples were ground and sieved through a 100 mesh. The raw flour was then packed in an aluminum foil bag, and stored at room temperature until used.

2.3 Proximate compositions of flours

The flour was analyzed its moisture, fat, protein, ash, fiber, and carbohydrate using the AOAC Official Methods [9].

2.4 Preparation of flour extracts

A gram of flour was shaken in a water bath (ABQUIP, Thailand) with 25 ml acidified methanol (7% acetic acid in 80% methanol) at 30 ºC for 2 hours and the mixture was filtered using a BÜchner funnel (Eyela, Aspirator A-35, Japan). The residue was then extracted again with an additional 10 ml of acidified methanol as described above. The combined supernatant was concentrated by the rotatory evaporator (Buchi RII, Thailand) and diluted to 25 ml with acidified methanol. The extract solution was stored at 4 ºC in an amber bottle before analysis.This method was modified according to Teow et al. [5].

2.5 Total phenolic assay

Total phenolic content of the flour extract was determined according to the Folin- Ciocalteu method [10]. Flour extract solution or a standard gallic acid solution varying in concentrations (0.1 ml) was pipetted into a 10-ml volumetric flask. Then, distilled water (7 ml) and Folin-Ciocalteau reagent (0.5 ml) were added. The reaction was allowed to take place at room temperature for 1-8 min. After that, saturated sodium carbonate solution (1.5 ml) was added and made up to 10 ml by distilled water. The mixture was shaken and incubated at room temperature for 2 h. The mixture was measured its absorbance at 765 nm by spectrophotometer (Spectronic, Genesys 10UV, USA) with distilled water as a blank. The content of phenol was calculated on the basis of the calibration curves of gallic acid, and was expressed as mg gallic acid per 100 g dry matter.

2.6 Assay of DPPH radical scavenging activity

The scavenging activity of flour extract on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was determined using the modified method of Huang [4]. The flour extracts solution varied in volumes (0.02-2 ml) was mixed with varied amount of acidified methanol up to the final volume of 2 ml in a test tube. Then, freshly prepared 0.1 mM DPPH solution in methanol (3 ml) was added into the flour extract solution. The mixture was shaken vigorously and left to stand for 30 min in the dark. The absorbance of the mixture was then measured at 515 nm by spectrophotometer (Spectronic, Genesys 10UV, USA). The percentage of radical scavenging activity was calculated as follows [1- (A_{sample} / $A_{control}$)] x 100. EC_{50} value (mg/ml) is the effective concentration of sample at which DPPH radical were scavenged by 50% and was obtained by extrapolation from the graph plotted between the percentage of radical scavenging activity and the concentration of the flour extract solution (mg/ml). BHA was also used for comparison.

2.7 Statistical analysis

Proximate compositions analyses were performed in duplicate; while other analyses were performed in triplicate. The data were subjected to analysis of variance (ANOVA) with Duncan's multiple range test ($p \leq 0.05$) using SPSS program versions 16 for windows (SPSS Inc., Chicago, IL).

3. Results and Discussion

3.1 Proximate compositions of flours

Sweet potato and potato flours were significantly ($p \leq 0.05$) different in their chemical compositions (Table1). Carbohydrate was the major components found in both flours. However, sweet potato flours contained higher carbohydrate than potato flour, especially, 0106-1 variety. Yadav [3] also found that flour from red-purple sweet potato grown in India contained carbohydrate as high as 75% (db). This suggested that sweet potato was an appropriate source for flour production. Besides carbohydrate, sweet potato flours were higher in fat and fiber than potato flour, especially T101 and Phichit 65-3 varieties. However, they contained lower amount of protein and ash than potato flour. Reported by Ahmed [11], flours from sweet potato cultivated in South Korea also contained considerably high in fiber (5.26%) but low in protein (3.48%) and ash (3.45%). These high-fiber and low-protein characteristics were also reported by Yadav [3] who studied the flour from red-purple sweet potato grown in India (17.5% in fiber and 6.6% in protein). Although flours of sweet potato from different varieties and growing areas shared the same chemical compositions characteristics, they were still different in detail of each composition.

3.2 Total phenolic contents

Phenolic content of the flour extracts from all sweet potato varieties were higher than that of the flour extract from potato (Table 2).

Table1: Proximate compositions of sweet potato and potato flour.

Flour	Variety	Flesh color	Protein (%dry basis)	Fat (%dry basis)	Fiber (%dry basis)	Ash (%dry basis)	Total Carbohydrate (%dry basis)
Sweet potato	0106-1	white	$2.72^{d} \pm 0.01$	$3.95^{c} \pm 0.11$	$3.35^{c} \pm 0.04$	$2.54^{e} \pm 0.03$	$87.44^{a} \pm 0.03$
	Phichit 265-1	deep-yellow	$4.28^{c} \pm 0.07$	$2.55^{d} \pm 0.08$	$4.06^{b} \pm 0.20$	$2.99^{d} \pm 0.00$	$86.10^{b} \pm 0.18$
	T101	orange	$4.09^{c} \pm 0.03$	$6.72^{a} \pm 0.02$	$5.30^{a} \pm 0.10$	$3.97^{b} \pm 0.04$	$79.92^{d} \pm 0.11$
	Phichit 65-3	deep-purple	$5.23^{b} \pm 0.14$	$4.49^{b} \pm 0.01$	$5.22^{a} \pm 0.28$	$3.27^{c} \pm 0.07$	$81.77^{c} \pm 0.22$
Potato	Atlantic	cream	$10.75^{a} \pm 0.20$	$0.79^{e} \pm 0.08$	$1.85^{d} \pm 0.01$	$5.10^{a} \pm 0.15$	$81.50^{c} \pm 0.44$

The values are the mean ± SD of two independent observations. The values with same superscripts in a column did not differ significantly ($p \leq 0.05$).

Carbohydrate content is calculated from 100 – (moisture -lipid - protein – ash).

Furthermore, all four sweet potato varieties were significantly ($p \leq 0.05$) different in the phenolic content of their flour extracts. The flour extract from deep-purple sweet potato (Phichit 65-3) exhibited the highest phenolic content following by those from orange (T101), deep-yellow (Phichit 265-1), and white (0106-1) sweet potatoes. Rumbaoa [12] also found this positive correlation between flesh color and phenolic content of Phillippine sweet potatoes. Dakol variety, a purple sweet potato, gave the highest in phenolic content (1,159.0 mg GAE/100 g), while Emelda variety, a white sweet potato, had the lowest in phenolic content (192.7 mg GAE/100 g).

Type of pigment in sweet potato flesh probably influences the phenolic content of the flour extract. Teow [5] found that purple sweet potato contained considerably high amount of anthocyanins (0.030 - 0.531 mg/g), while orange and yellow sweet potatoes highly contained carotenoids (1.50-226.00 µg/g). Both pigments are polyphenol compounds; however anthocyanins had more phenolic rings than carotenoids [13]. As a result, Flour with anthocyanin content has higher total phenolic than flour with carotenoid content. Therefore, the flour extract of purple sweet potato showed higher amount of total phenolic content than those of orange, yellow, and white sweet potatoes, respectively. However, the total phenolic content from the same sample could be also varied depended on the extraction time, temperature, and solvent type [14].

Table2: Total phenolic content of sweet potato and potato flour extracts.

Flour	Variety	Flesh color	Total Phenolic (mg GAE/100 g sample)
Sweet potato	0106-1	white	$181.67^{d} \pm 9.44$
	Phichit 265-1	deep-yellow	$234.60^{c} \pm 4.58$
	T101	orange	$394.27^{b} \pm 5.13$
	Phichit 65-3	deep-purple	$448.93^{a} \pm 22.19$
Potato	Atlantic	cream	$168.27^{d} \pm 8.62$

The values are the mean ± SD of three independent observations. The values with same superscripts in a column did not differ significantly ($p \leq 0.05$).

3.3 DPPH radical scavenging activity

Scavenging activities on DPPH, a stable nitrogen radical, of the flour extracts and BHA were presented in Table 3. The activity was determined as EC_{50} value which is the effective concentration of the testing antioxidant solution at which DPPH radicals were scavenged by 50%. Therefore, the lower the EC_{50}, the higher the efficiency of the testing antioxidant is. All flour extracts had the EC_{50} in the range of 1.167-9.843 mg flour/ml, while the EC_{50} of BHA was 0.003 mg/ml. Based on their EC_{50} values, flour extracts could be classified into three groups: the high antioxidant efficiency which were the purple (Phichit 65-3) and orange (T101) sweet potato flours, the medium antioxidant efficiency which was the yellow (Phichit 265-1) sweet potato flour, and the low antioxidant efficiency which were the white (0106-1) sweet potato and potato (Atlantic) flours. These findings were in accordance to the high radical scavenging activity of the flour extract of three Philippine purple sweet potato varieties over those of yellow and white sweet potato varieties [12]. This high antioxidant activity of purple sweet potato flour extract could be due to its high anthocyanins content. Teow [5] revealed the positive correlation between the anthocyanins content and antioxidant activity of the flour extract from purple sweet potato varieties. The antioxidant activity was depended on the type of anthocyanins. The major types of anthocyanins found in sweet potato were peonidin and cyanidin.

Table3: DPPH radical scavenging activity of sweet potato and potato flour extracts.

Flour	Variety	Flesh color	EC_{50} (mg/ml)
BHA	-	-	$0.003^{e} \pm 0.01$
Sweet potato	0106-1	white	$3.638^{b} \pm 0.10$
	Phichit 265-1	deep-yellow	$2.115^{c} \pm 0.02$
	T101	orange	$1.183^{d} \pm 0.05$
	Phichit 65-3	deep-purple	$1.167^{d} \pm 0.04$
Potato	Atlantic	cream	$9.843^{a} \pm 0.37$

The values are the mean ± SD of three independent observations. The values with same superscripts in a column did not differ significantly ($p \leq 0.05$).

According to the total phenolic content and the DPPH radical scavenging activity of flour extract, there was a correlation between these two characteristics. Flour extracts with high amount of phenolic compounds also exhibited high efficiency in its antioxidant activity. This correlation could be implied that the phenolic compounds such as anthocyanins or carotenoids in sweet potato exhibited antioxidant activity. Teow [5] also reported that total phenolic content and antioxidant activities (DPPH and ORAC assay) were highly correlated ($R^2 = 0.870$ and $R^2 = 0.937$, respectively).

4. Conclusions

Four sweet potato varieties grown in Thailand (0106-1, Phichit 265-1, T101, and Phichit 65-3) were appropriate for flour production due to their high in carbohydrate content as potato. Compared to potato flour, sweet potato flours were uniquely high in fiber and fat but low in protein, especially the flour from orange sweet potato (T101). Total phenolic content and DPPH radical scavenging activity of flour extracts were highly related to the flesh color of sweet potato. Flour extract of purple sweet potato (Phichit 65-3) exhibited the highest in total phenolic and DPPH radical scavenging activity, while that of white sweet potato (0106-1) showed the lowest in both values. Sweet potato cultivated in Thailand, especially the colored-flesh varieties, had a high potential for using as a source of flour in healthy starch-based food products.

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References

[1] Office of Agricultural Economics. *Agricultural statistics yearbook of 2007*. Information center of Agricultural, Thailand (2007).
[2] C. Kaur and H. C. Kapoor, *International Journal of Food Science and Technology* **37** (2002) 153-161.
[3] A. R. Yadav, G. Manisha, R. N. Tharanathan and R.S. Ramteke, *LWT*. **39** (2006) 20-26.
[4] Y.-C. Huang, Y.-H. Chang and Y.-Y. Shao, *Food Chem*. **98** (2006) 529-538.
[5] C. C. Teow, V.-D. Truong, R. F. McFeeters, R. L. Thompson, K. V. Pecota and G. C. Yencho, *Food Chem*. **103** (2007) 829-838.
[6] G. Fan, Y. Han, Z. Gu and F. Gu, *LWT*. **41** (2008) 1412-1416.
[7] M.-C. Shih, C.-C. Kuo and W. Chiang, *Food Chem.* **117** (2009) 114-121.
[8] A. R. Yadav, S. Mahadevamma, R. N. Tharanathan and R. S. Ramteke, *Food Chem*. **103** (2007) 1119-1126.
[9] AOAC, *Official Methods of Analysis*, **17th ed**. Association of Official Analysis Chemists. Washington D. C. (2005).
[10] A. L. Waterhouse, in: R. E. Wrolstad, T. E. Acree, E. A. Decker, M. H. Penner, D. S. Reid, S. J. Schwartz, C. F. Shoemaker, D. Smith and P. Sporns (Eds.), *Handbook of Food Analytical Chemistry: Pigment, Colorrants, Flavors, Texture and Bioactive Food Components*, New Jersey: John Wiley & Sons (2005), pp. 463-470.
[11] M. Ahmed, M. S. Akter and J. -B. Eun, *Food Chem*. **121** (2010) 112-118.
[12] R. G. O. Rumbaoa, D. F. Cornago and I. M. Geronimo, *Food Chem*. **113** (2005) 1133-1138.
[13] O. Vajragupta, in: P. Boonchoong, C. Boonyarut and M. Utsintong (Eds.), *Radical Scavenging Agents*, P. S. Prints, Thailand, (2006), pp. 123-127.
[14] W. Tewaruth, *Antioxidant activity of black glutinous rice Oryza sativa L. extracts*, Master's Thesis, Chulalongkorn University, (2007).

EVALUATION OF ANTIOXIDANT AND ANTI – TYROSINASE OF *BRUNFELSIA HOPEANA* BENTH

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Abstract: The methanolic extracts obtained from flowers, leaves and stems of *Brunfelsia hopeana* Benth were examined for their antioxidant properties (AOP) and tyrosinase inhibition. Total phenolic contents (TPC), total flavonoid contents, 2,2′-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azino-bis-(3-ethylbenzothiazotine–6-sulfonic acid) (ABTS) free radical scavenging activities (RSA) were used to evaluate the AOP. Modified 3,4-dihydroxy-L-phenylamine (L-DOPA) method was used to determine tyrosinase inhibitory activity. The results showed that TPC of flowers, leaves and stems were 45.34, 25.19 and 0.045 mg-gallic acid equivalent/g(dry), respectively, and total flavonoid contents of flowers, leaves, and stems with aluminium chloride colorimetric method were 1.72, 5.0 and 0.53 x 10^{-2} mg-quercetin equivalent/g (dry), respectively. The free radical scavenging assay have showed the highest methanolic extracts from the flowers of *Brunfelsia hopeana* Benth. For the investigation of tyrosinase inhibitory activity, it was found that the methanolic extracts yield from flowers exhibited the highest tyrosinase inhibitory activity. The results indicate that this plant is a potential source for the chemicals used as an antityrosinase and an antioxidant.

1. Introduction

Reactive oxygen species (ROS) in the forms of super oxide anion radical ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$), hydrogen peroxide (H_2O_2) can cause oxidative damage to human cells, resulting in diseases such as cancer, cardiovascular disease, osteoporosis, and degenerative diseases [1]. Antioxidants can delay or inhibit the initiation or propagation of oxidative chain reaction and thus prevent or repair oxidative damage done to the body's cells by oxygen [2]. Thus synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxyl toluene (BHT), and tertiary butylhydroquinone (TBHQ) have been widely used in food industry. Synthetic antioxidants, however, are known to induce carcinogenesis by mutagenicity and toxicity against human enzymes and lipids [3,4]. For this reason, studies of natural antioxidants are attractive to researchers for uses in foods or medicinal materials to replace synthetic antioxidants.

Tyrosinase (monophenol monooxygenase, E:C:1.14.18.1), also known as polyphenol oxidase (PPO) and a copper containing enzyme, is involved in the first two steps of melanin biosynthesis [5]. This enzyme is responsible for enzymatic browning in plants, producing undesirable changes in color, flavor and nutritive values of plant-derived foods and beverages [6]. Tyrosinase inhibitors are chemical agents capable of reducing enzymatic reactions, such as food browning and melanisation of human skin. Therefore, tyrosinase inhibitors have become increasingly important in cosmetic and medical products in relation to hyperpigmentation [7].

Brunfelsia has two centers of species diversity. One is in South America, especially south-central and eastern Brazil, the other is in the Antilles, distributed in Cuba, Jamaica, Hispaniola, and Puerto Rico [8]. Species of Brunfelsia, whose flowers change color progressively from violet to white, are popularly known as yesterday-today-tomorrow. *Brunfelsia hopeana*, known in Thai as 'Phut sam sri' or 'Sam rasri. The decrease in anthocyanin concentration in these flowers is extremely rapid and occurs at a specific and well-defined stage [9]. *Brunfelsia* is used in folk medicine, mainly as anesthetic and diuretic. Based on Ethnobotanica surveys, it has been investigated chemically and pharmacologically for expanding the knowledge on the native medical flora and for searching bioactive compounds [10].

There was a little available information on antioxidant properties of *Brunfelsia hopeana*, as well as tyrosinase inhibitory activity. Therefore, the objective of this study was to evaluate total phenolic contents (TPC), total flavonoid contents, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activities (RSA), and 2,2′-azino-bis-(3-ethylbenzothiazotine-6-sulfonic acid) (ABTS) radical scavenging activity of methanolic extracts from flowers, leaves and stems of *Brunfelsia hopeana* . In addition, tyrosinase inhibitory activity of methanolic extracts was also evaluated for the benefit of cosmetic industry as a new source of whitening agents.

2. Materials and Methods

2.1 Sample and preparation

The flowers, leaves and stems of *B. hopeana* were collected from the Department of Chemistry, Faculty of Science, King Mongkut's University of Technology Thonburi, Thongkru, Bangkok, in June - October 2011. The flowers, leaves and stems were freeze-dried at room temperature to dry and keep the weight constant. The dried flowers, leaves and stems were grounded separately to powder. Three grams of the powder were extracted separately with 50 mL methanol for 5 hrs on the shaker. The extracts were filtered via vacuum filtration and the solvent was dried

off using a rotary evaporator at 45°C. The crude extracts were re-dissolved in methanol to make 1 mg/l stock solution and stored at 4°C for further use [11].

2.2 Chemicals and reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis-(3-ethylbenzothiazotine-6-sulfonic acid) (ABTS), butylated hydroxytoluene (BHT), gallic acid, quercetin, Trolox, aluminium chloride, L-3,4-dihydroxyphenylalanine and mushroom tyrosinase were purchased from Sigma Chemical Co. (Thailand). Folin-Ciocalteu's reagent, Dimethyl sulfoxide (DMSO), methanol and sodium carbonate were purchased from Merck Chemical Supplies (Thailand).

2.3 Determination of total phenolic contents

Total phenolic content in the extracts was determined using modified Folin-Ciocalteu's method [12].

An aliquot of the extract (0.25 ml) was mixed with 0.5 ml of the freshly prepared Folin-Ciocalteu's reagent and a further 6.0 ml of distilled water. The mixture was shaken vigorously and 2.0 ml of sodium carbonate (15% w/v) was added and the mixture was again shaken vigorously for 2 min. The final volume was made up to 10.0 ml with distilled water. After the mixture was left to stand for 2 h at room temperature, the absorbance of mixture was then measured at 750 nm using the Hewlett Packard UV-VS spectrophotometer. Total phenolic content was expressed as mg gallic acid equivalent / g sample (dry mass) using the following equation based on the calibration curve: $y = 0.002x+0.014$, $r^2 = 0.9920$, where x was the absorbance and y was the gallic acid equivalent (mg/g (dry mass)).

2.4 Determination of total flavonoid contents

Estimation of the total flavonoid in the crude extract was carried out using the method of Ordon Ez et al [13]. To 5.0 ml of the extract stock solution of a known concentration, 5.0 ml of 2% $AlCl_3$ methanolic solution was added. After one hour at room temperature, the absorbance of the mixture was measured at 420 nm using the Hewlett Packard UV-VS spectrophotometer. A yellow color of the solution indicated the presence of flavonoid compounds. Total flavonoid content was calculated as quercetin (mg/g) using the following equation based on the calibration curve: $y = 0.0310x + 0.023$, $r^2= 0.9990$, where x was the absorbance and y was the quercetin equivalent (mg/g(dry mass)).

2.5 Determination of antioxidant activities

2.5.1 DPPH radical scavenging activity

The stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the crude extracts [14]. Different concentrations (10, 20, 30, 40 and 50 mg/l) of each crude extract were added at an equal volume to 1.0×10^{-4} M methanolic solution of DPPH. After incubation for 30 min at room temperature, the absorbance of the mixture was measured at 517 nm. The experiment was repeated for three times. BHT was used as a standard reference. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following equation:

$$\% \text{ radical scavenging activity} = (A_{control} - A_{sample} / A_{control}) \times 100$$

where $A_{control}$ is the absorbance of the control without extract, and A_{sample} is the absorbance of reaction mixture. The $DPPH^{\cdot}$ radical scavenging activity (IC_{50}) was calculated using a plot of percent radical-scavenging activity against concentration (mg/l) to determine the concentration of extract necessary to reduce $DPPH^{\cdot}$ by 50%. Samples with a lower IC_{50} had a stronger antioxidant activity. All tests and analyses were carried out in triplicate and averaged.

2.5.2 ABTS radical scavenging activity

The ABTS radical cation scavenging assay followed the method of Re et al. [15]. To oxidize the colorless ABTS to the blue-green $ABTS^{\cdot+}$ radical cation, the stock solutions were prepared including 7 mM ABTS and 2.4 mM potassium persulfate . The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was diluted in methanol to an absorbance of 0.70 (±0.02) at 734 nm before used. Then 1.00 ml of sample extract was added to 10.00 ml of $ABTS^{\cdot+}$ solution, stirred for 30 s and allowed to stand for 15 min at room temperature. The absorbance was then determined at 734 nm. A calibration curve was made by absorbance reduction with different concentrations of Trolox. A control consisted of 1.00 ml of methanol and 10.00 ml of $ABTS^{\cdot+}$ solution. The antioxidant solution reduces the radical cation to ABTS which reduces the color. The $ABTS^{\cdot+}$ scavenging capacity of the extract was calculated as ABTS radical scavenging activity (%) = $[(Abs_{control} - Abs_{sample})]/[Abs_{control})]$ x 100 where $Abs_{control}$ is the absorbance of ABTS radical + methanol; Abs_{sample} is the absorbance of ABTS radical + sample extract/standard. The $ABTS^{\cdot}$ activity was calculated using a plot of percent radical-scavenging activity against concentration, as described in IC_{50} for DPPH system.

2.6 Tyrosinase inhibitory activity

Tyrosinase inhibitory activity was measured using the method described by Vanni, Gastaldi, and Giunata [16]. A 5 mg aliquot of the extract was weighed and dissolved in 2 ml of 50% DMSO. Each extract (50 µl) was diluted with 150 µL of 0.02 M sodium phosphate buffer (pH 6.8) in a 96-well plate and then 50 µl of 0.34 mM L-DOPA solution and 50 µl of mushroom tyrosinase (3666 unit/ml) were added into a 96-well plate. The test mixture (300 µl) was mixed well and incubated at 37°C for 15 min. Each sample was accompanied by a blank that contains all

components except L-DOPA. Absorbance was measured at 492 nm. The increase in absorbance at 492 nm due to the formation of DOPAchrom was monitored as a function of time, using miroplates reader spectrophotometer. The extract of *Brunfelsia hopeana* was used as the positive control. Results were compared with a control and a blank containing 50% DMSO in place of the sample solution. BHT and gallic acid were used as the positive contrals.The percent inhibition of tyrosinase by the active compounds was calculated as the following :

inhibition (%)= $[(Abs_{control}–Abs_{sample})]/[Abs_{control})]$x 100

.

The inhibitory effect (%) of the compound was expressed as the inhibitor concentration causing 50% loss of enzyme activity (IC_{50}). The experiment was repeated for three times.

2.7 Statistical analysis

Each of the measurement described above was carried out at least three replicate experiments, and the results are reported as the mean and standard deviation.

3. Results and Discussion

3.1 Total phenolic and flavonoid contents

Phenolic and flavonoid compounds are well known as effective free radical scavenging and antioxidant agents. This activity is believed to be mainly due to their redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. [17]. Results obtained in the present study revealed that the level of the phenolic and flavonoid contents in the methanolic extracts of *Brunfelsia hopeana* were considerable (Table 1).

Total phenolic content was determined with linear gallic acid standard curve (y=0.002x+0.014; r^2=0.9990) and total flavonoid content was determined with linear quercetin standard curve (y=0.031+0.023x; r^2=0.9996). The highest phenolic and flavonoid contents were found in flowers, which were 45.36 ± 0.46 and 1.72±0.04 mg/g (DW), respectively. Earlier studies showed that the fresh leaf and stem extracts of some plants had lower phenolic and flavonoid contents than those of fresh flowers [18].

Table 1: Total phenolic and flavonoid contents of the methanolic extracts of leaves, stems and flowers of *Brunfelsia hopeana*

Brunfelsia hopeana	**Total phenolic * (mg/g (DW)**	**Total Flavonoid****
Flowers	45.35 ±0.46	1.72±0.04
leaves	25.19 ±0.14	5.00 ±0.05
Stems	0.045 ±0.01	$0.53x10^{-2}$ ± $0.06x10^{-3}$

*Expressed as mg gallic acid/g of dry plant extracts

**Expressed as mg quercetin/g of dry plant extracts

3.2 DPPH and ABTS radical scavenging activity

DPPH is a stable free radical compound that has been widely used to evaluate the free radical scavenging activity (RSA) of various plants and pure compounds. Proton radical scavenging is an important attitude of antioxidants. ABTS, a protonated radical, has characteristic absorbance maxima at 734 nm which decreases with the scavenging of the proton radicals [19].

Table 2 shows the dose-response of DPPH RSA and ABTS RSA of the methanolic extracts of the flowers, leaves and stems of *B. hopeana*, compared with BHT.

It was observed that methanolic extracts of the flowers with IC_{50} (22.20±0.20 (DPPH), 20.37±0.15 (ABTS)) showed the highest DPPH RSA and ABTS RSA, but the activities of the extracts were less than IC_{50} (12.50 (DPPH), 11.60 (ABTS)) of standard BHT. The present study showed that the extracts had the proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants.

3.3 Tyrosinase inhibitory activity

Tyrosinase inhibitors are chemical agents capable of reducing enzymatic reactions, such as food browning and melanisation of human skin. Therefore, these agents have good commercial potential in both food processing and cosmetic industries.

Tyrosinase inhibitory activity of *B. hopeana*, gallic acid and BHT are shown in Table 3. Like antioxidant activities, the values of tyrosinase inhibitory activity of *B. hopeana* increased as the concentration increased from 1 to 1000 mg/l. IC_{50} values of *B. hopeana* , Gallic acid and BHT were shown in Table 3.

Table 2: DPPH and ABTS radical scavenging activity of methanolic extracts from *Brunfelsia hopeana*

	DPPH RSA (%) of *B. hopeana*				**ABTS RSA (%) of *B. hopeana***			
Conc. (mg/l(DW))	**Flowers**	**Leaves**	**Stems**	**BHT**	**Flowers**	**Leaves**	**Stems**	**BHT**
10	21.42±0.51	11.27±0.27	10.01±0.36	42.61±0.51	13.45±0.26	13.19±0.42	11.56±1.26	45.96±0.26
20	44.65±0.40	23.17±0.39	29.30±0.72	66.41±0.39	49.55±0.41	25.14±0.44	31.16±1.45	70.21±0.24
30	68.25±0.28	34.24±0.12	36.25±0.63	75.32±0.01	74.74±0.58	64.73±0.62	49.49±1.18	81.03±0.16
40	90.52±1.22	50.33±1.04	45.73±0.41	84.41±0.33	84.03±0.42	71.11±0.49	70.93±1.40	89.60±0.33
50	91.89±0.66	59.79±0.51	57.18±0.24	86.36±0.63	90.26±0.37	75.85±0.44	75.33±0.60	93.94±0.24
IC_{50}	22.20±0.20	39.60±0.87	43.50±0.30	12.50±0.00	20.37±0.15	25.90±0.20	29.67±0.37	11.60±0.00

IC_{50} (mg/l) , concentration for scavenging 50% of DPPH radicals and ABTS radicals concentration

Table 3: Tyrosinase inhibitory activity of the methanol extracts of the *B. hopeana*, compared with BHT and gallic acid.

Conc.(mg/l)	Flowers	Leaves	Stems	Gallic acid	BHT
1	4.40±0.57	5.50±0.78	4.10±0.90	47.14±1.32	41.66±1.37
50	16.89±0.36	12.01±1.03	9.52±0.24	57.23±0.93	52.92±0.81
100	35.46±0.58	30.24±0.63	29.11±0.44	70.29±0.93	65.29±0.72
500	47.64±0.37	48.42±1.53	44.4±0.25	78.29±0.40	69.98±0.37
1000	57.44±0.85	52.55±1.83	53.60±0.32	84.06±1.68	73.91±1.12
IC_{50}	560.00±0.01	610.00±0.06	760.00±0.01	10.00±0.001	40.00±0.001

From Table 3, the tyrosinase inhibitory activity of the extracts varies depending on the concentration. However, the tyrosinase inhibitory activity of the extracts was much less than those of gallic acid and BHT. This study is the first attempt to exhibit the tyrosinase inhibitory activity of the methanolic extracts from flowers, leaves and stems of *B. hopeana*. Further studies are needed to determine tyrosinase inhibitory activity in extracts of various solvents.

4. Conclusions

The present result showed that methanolic extracts from flowers, leaves and stems of *Brunfelsia hopeana* flowers contain abundant phenolic and flavonoid contents and antioxidant activity. This study also found that there was a good correlation between total phenolic and flavonoid contents and antioxidant activity of the extracts. Methanolic extracts from flowers, leaves and stems of *Brunfelsia hopeana* had low tyrosinase inhibitory activity compared to gallic acid and BHT. These results demonstrated that the flowers of *Brunfelsia hopeana* can be considered as a source of natural antioxidants in food industry and suggested that the flowers of *B. hopeana* has the potential as an ingredient in cosmetic products.

Acknowledgements

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References

[1] B. Halliwell and J.M.C. Gutteridge, *Biochem. J.* **218** (1984) 1-4.
[2] Y.S. Veliogu, G. Mazza, L. Gao, B.D. Oomah, *J. Agric. Food Chem.* **46** (1998) 4113-4117.
[3] S. Boxiang and M. Fukuhara, J. *Toxicol*, **122**(1997) 61-72.
[4] M. Hirose, Y. Takesada, H. Tanaka, S. Tamano, T.Kato and T. Shirai, *Carcinogenesis*. **19** (1998) 207-212.
[5] A. Kaioti, A. Protopappa and H. Skalta, *Bioorg. Med. Chem.* **15** (2007) 2708-2714.
[6] M. Friedman, *J. Agric. Food Chem*. **44** (1996) 631-653.
[7] Y. M. Kim, J. Yun, C.K. Lee, H, H. Lee, K.R. Min and Y. Kim, *J. Biol. Chem.* **277** (2002)16340-16344.
[8] N. Filipowicz and S.S. Renner, Mol. Phylogenet. Evol. **64**(2012) 1-11.
[9] H. Vaknin, A. Bar-Akiva, R. Ovadia, A. Nissim-Levi, I. Forer, D. Weiss and M. Oren-shamin, *Planta*. **222**(2005) 19-26.
[10] M.R. Duarte and S. Wolf, Lat. Am. J. Pharm. 29(2010) 1259-1264.
[11] R.S.L. Taylor, F. Edel, N.P. Manandhar and G.H.N. Towers, *J. Ethnopharmacol*. **50** (1996) 97-102.
[12] K. Wolfe, X. Wu and R. H. Liu, *J. Agric. Food Chem*. **51**(2003) 609-614.
[13] A.A.L. Ordon Ez, J.D. Gomez, M.A. Vattuone and M.J. Isla, *Food Chem.* **97** (2006) 452-458.
[14] T.A. Koleva, J. Linssen and L.N Evastatieva, *Phytochem. Anal*. **13**(2002) 8-17.
[15] R. Re, N. Pellegrini, A. Proreggent, A. Pannala, M. Yang and C. Rice-evans, *Free. Radical. Bio. Med.* **26**(1999) 1231-1237.
[16] A. Vanni, D. Gastaldi, G. giunata, *Ann Chim*. **80**(1990) 35-60.
[17] W. Zheng and S.Y. Wang, *J. Agric. Food Chem*. **49**(2001) 5165-5170.
[18] M.A.Soobratte, T. Bahoran, V.S. Neergheen, K. Googoolye and O. Aruoma, *Toxicol. In Vitro.* **22**(2008) 45-56.
[19] S. Mathew and E.T. Abraham. *Food Chem. Toxico.* **44**(2004) 198-206.

RADICAL SCAVENGING ACTIVITY AND TOTAL PHENOLIC CONTENT OF COCONUT OIL WITH EXTRACTED GINGER BLENDED SUNFLOWER OIL

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Abstract: The ginger had phenolic compounds which are powerful antioxidant for stabilization of coconut oil and prevent deleterious effect of free radicals. Coconut oil enriched with phenolic compounds has high oxidative stability which can be used for healthy food. Therefore, the incorporation of coconut oil to sunflower oil increases the stability of the blends. The objective of this work was to prepare sunflower oil blend with coconut oil with extracted ginger to improve oxidative stability of sunflower oil during storage. Coconut oil with extracted of ginger (COEG) blends with sunflower oil (SFO) in ratio of 20%+80%, 50%+50% and 80%+20% (20%COEG + 80%SFO, 50%COEG + 50%SFO and 80%COEG + 20%SFO) was prepared. 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH•) scavenging activity, total phenolic contents and peroxide value in these blends oil were-studied. It was found that total phenolic content in coconut oil with extracted ginger, virgin coconut oil (VCO), sunflower oil and coconut oil with extracted ginger blend : 20%COEG + 80%SFO, 50%COEG + 50%SFO and 80%COEG + 20%SFO were 763, 93, 138, 274, 387, 513 mggallic acid equivalent/100g oil, respectively. In addition, high total phenolic content resulted to high radical scavenging activity and low peroxide value. It was concluded that blending of coconut oil with extracted ginger and sunflower oil provides oxidative stability to the blends oil and greater radical scavenging activity.

1. Introduction

Vegetable oil is an important component of the diet. The fatty acid composition of the substrate is essential for the body's important hormones such as prostaglandins hormones and control many factor in the body (i.e. blood pressure, cholesterol levels) [9]. However, vegetable oils (i.e. soybean oil, sunflower oil, rice bran oil, and sesame oil) are rich in monounsaturates fatty acid and polyunsaturates fatty acid. These vegetable oil have problem for oxidative stability especially catalyzed by the heat that occur free radicals and trans fats easily [7]. Free radicals can be penetrated the epithelial cells are the cause of many chronic diseases (i.e. Ischemic heart, Hypertension, Alzheimer, and Diabetes). Trans fats are dangerous to the body. It is difficult to eliminate from the blood vessels and accumulate in the body. These the problem were solved by adding antioxidants [8] and medium chain fatty acid in vegetable oil. Synthetic antioxidants such as Butylated hydroxyanisole (BHA), Butalated hydroxytoluene (BTH), and ter-butyl hydroguinone (TBHQ) were used as food additives. But these synthetic antioxidants may be implicated in many health risks including cancer. Due to these safety concerns, the natural antioxidant such as polyphenol compound in plant was used in food replaced these synthetic antioxidant. Ginger is one of plant which found polyphenol compound with high antioxidant activity[8].

In addition, increasing the stability of vegetable oil by adding the medium-chain triacyl glycerols contains saturates fatty acid, C8:0, C10:0, and C12:0. This fatty acid is easily absorbed into the body and burnt for energys rather than stored in the body [5]. Coconut oil contains major medium-chain triacyl glycerols (60%), especially C12:0 (50%) as called lauric acid which becomes 2-mono-laurin in the gut and dissolves the lipid envelope that protects most pathogenic bacteria and viruses. Moreover, natural coconut oil in the diet leads to a normalization of body lipid, protects against alcohol damage to the liver, and improves the immune system's anti-inflammatory response [1]. It is very stable against oxidation and hence not prone to peroxide formation. Therefore, the oil for health has rich antioxidant and medium chain fatty acid. The objectives of this work were to study SFO blends with COEG and study the radical scavenging activity, total phenolic content and oxidative stability of coconut oil with extracted ginger blend sunflower oil. These blends oil during storage.

2. Materials and Methods

2.1 Materials

Coconut milk was extracted from coconut 10-12 month which obtain from local markets in Thongsong, Nakhon Si Thammarat, Thailand to produce coconut oil.

2.2 Methods

2.2.1 Preparation of Blends

A 100 g of COEG and SFO was blended in 250-ml beakers and stirrer with mechanical at 180 rpm for 15 min. The three blends are 20% COEG + 80% SFO, 50% COEG + 50% SFO, 80% COEG + 20% SFO were perpared. These oil blends were analyzed oxidative stability, total phenolic, and radical scavenging activity.

2.2.2 Oxidative stability measurement

Blends of COEG with SFO in ratio of 20:80, 50:50, and 80:20, respectively, were prepared in 100 g x 2 batches. These oil blends were placed in beakers (50-ml capacity) and incubated at 65 °C in incubator to determine their peroxide value over a period of 4 weeks (28 days) by using AOCS Method No: Cd 8-53, 1997 [2].

2.2.3 Extraction of phenolic compounds

The procedure reported by Kapila (2009) was slightly modified [5]: Five grams of oil sample was weighed, dissolved in 25-ml hexane and transferred to a separatory funnel. Twenty-five millilter of a methanol-water mixture (80:10 v/v) was added and shaked for 2 min. After that the lower methanol-water layer was separated. The extraction was repeated twice and the methanol-water phase was combined. The methanol-water solvent in extraction of phenolic compounds was removed in rotary evaporatory under vaccum at 40 °C. The dry residue was then diluted in 1 ml of methanol.

2.2.4 Determine of total phenolic content

The content of total phenolic compounds in the VCO and COEG and oil blends was determined by Folin-Ciocalteau reagent [6]. The reaction mixture contains 1 ml of methanol-water extract diluted in methanol, 8 ml of freshly Folin-Ciocalteau reagent and 1 ml of sodium carbonate solution. The mixture was kept in the dark at ambient conditions for 30 min to complete reaction. Ather that the absorbance of thin mixture was measured on spectrophotometric 21 at 645 nm. Gallic acid was used as standard. The content of total phenolic compounds are expressed as mg of Gallic acid per kg of oil.

2.2.5 Radical scavenging activity (RSA) toward DPPH radicals

RSA and the presence of hydrogen donors in the prepared oil blends were examined by reduction of DPPH radicals in ethyl acetate. The procedure was reported by Lee (2007)[3] and was slightly modified : 1 g of oil sample was dissolved with ethyl acetate in 10 ml volumetric flask, then 1 ml of this solution was transferred to DPPH radical solution in 10 ml of volumatric. Reaction was shaken for 10 s with vortex apparatus and allowed to stand in the dark for 30 min against a bank of pure ethyl acetate without DPPH radicals, the decrease in the absorption at 515 nm was measured with spectrophotometer (Biochrom S22) in a 1 cm quartz cell at 60 min. %Inhibition was calculated using the following equation (1)

$$\%\text{Inhibition} = [\{A_{control} - A_{sample}\}/A_{control}] \times 100 \quad --(1)$$

Where $A_{control}$ and A_{sample} are Absorbance of control and sample, respectively.

3. Results and Discussion

3.1 Oxidative stability

Oxidative Stability of oil blends was studied on compared with individual oil (virgin coconut oil, coconut oil with extracted ginger, and sunflower oil). Peroxide value of all oil sample was observed after storage at 65°C upto 28 days. A regular increase in peroxide value in a function of storage time was observed for all the oil sample of all intervals exeepted coconut oil with extracted ginger which has low and constant peroxide value. Initially, the difference in peroxide content of all the oil samples was not noticeable. After the 3rd week, these was tremendous rise in peroxide value of sunflower oil and all oil blends. However, peroxide value for all the oil blends increased but this increase was slower than peroxide value of sunflower oil. These data suggest that coconut oil with extracted ginger enriched phenolic antioxidant inhibit lipid peroxidation for all the oil blends (Fig. 1).

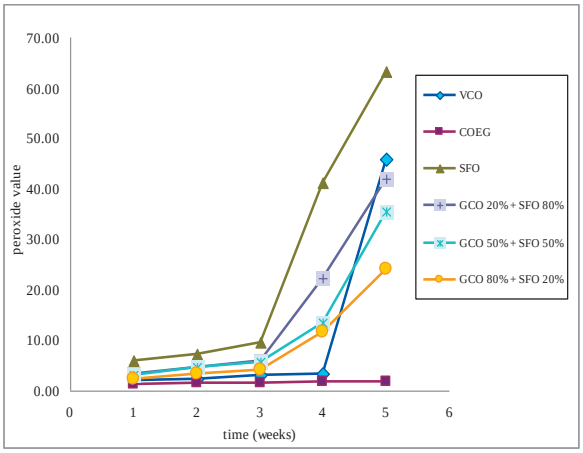

Figure 1 Rates of peroxide formation in the sample oil sample for 4 weeks (28 days)

3.2 Total phenolic content and radical scavenging activity

Table1 Total phenolic content and %inhibition of sample oils at 60 min

Sample	Total phenolic content (mg GAE/100g oil	%Inhibition
α - Tocopherol		95.6±0.0
VCO	93±7.8	2.8±0.0
COEG	763±0.0	92.2±0.0
SFO	138±2.7	46.2±0.0
20% COEG + 80% SFO	274±30.9	74.3±0.0
50% COEG + 50% SFO	387±5.4	74.4±0.0
80% COEG + 20% SFO	513±1.9	78.6±0.0

Total phenolic content was calcuted from linear regression equation at standard curve ($y = 0.5240x$, $R^2 = 0.9902$). Where x and y are concentration of gallic acid as standard solution. Absorbance value of phenolic compound in oil sample was measured at 700 nm. We found that total phenolic content in oil blend samples were lower than coconut oil with extracted ginger but higher than sunflower oil. Total phenolic content in oil blend samples are increase when increase the content of coconut oil with extracted ginger. Therefore, total phenolic content in 80%COEG + 20%SFO (513 mg GAE/100g oil) is higher than in 50%COEG +50%SFO (387 mg GAE/100g oil) and 20%COEG + 80%SFO (274 mg GAE/100g oil) as shown in Table 1. The antioxidant activity of oil sample was determined by DPPH radical scavenging activity assay. The DPPH radicalscavenging activity in shown in Table 1.

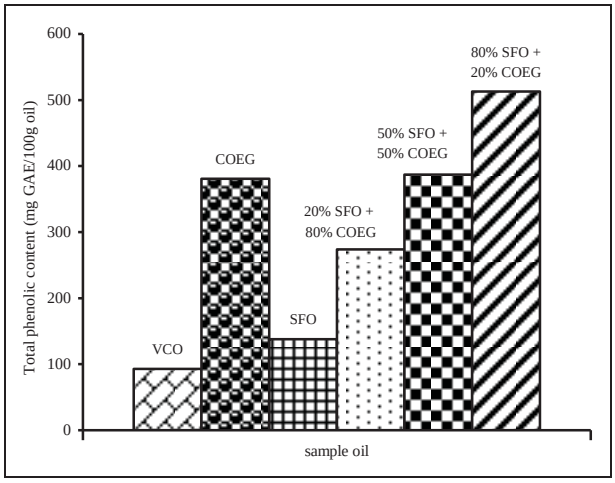

Figure 2 Total phenolic content of sample oils

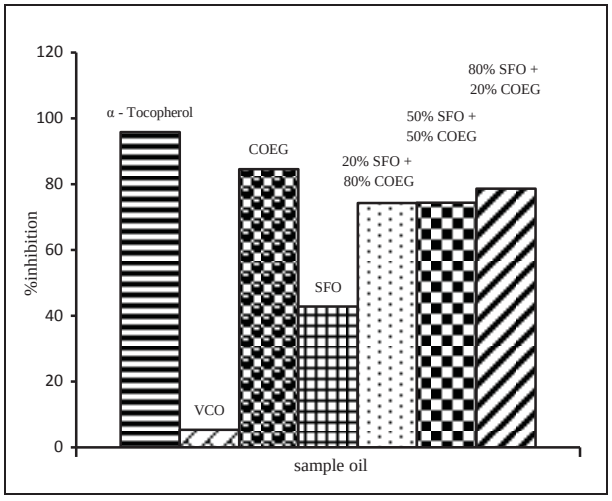

Figure 3 %inhibition of sample oils for 60 min

%Inhibition is a recommended criterion for evaluation of effectiveness of antioxidants and stability of oil sample. %Inhibition were 74.3, 74.4 and 78.6 for 20%COEG + 80%COEG, 50%COEG + 50%SFO and 80%COEG + 20%SFO blends oil, repectively. Which increase the ratio of coconut oil with extracted ginger in blends oil with increasing %inhibition. However, %inhibition of these blends oil were higher than sunflower oil, but lower than coconut oil with extracted ginger. Coconut oil with extracted ginger in blends oil increased the %inhibition at blends oil reveals coconut oil with extracted ginger an increase in stability of blends oil.

4. Conclusions

It was found that the oxidative stability and radical scavenging activity of oil blends depended upon the total phenolic content of the oil blends. The high total phenolic content, would be high oxidative stability and the high the DPPH scavenging activity.

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The Faculty of Science and Technology, Rajamangala University of Technology Srivijaya.

References

[1] Bhatnagar, A. S., Prasanth, P. K., Hemavathy, J. & Gopala Krishna, A. G. (2009). Fatty acid composition, Oxidative stability, and Radical scavenging activity of vegetable oil blends with coconut oil. *J Am Oil Chem Soc 86*:991-999.

[2] Firestone D (ed) (1997) Method no: Cd 8-53. Official Methods and recommended practices of the American Oil Chemists Society, 5th edn, AOCS press, USA

[3] Lee, J.M., Chung, H., Chung, H. and Lee, J.M., Food Chemistry 103(2007) 662-669.

[4] Marina, A. M., Che Man, Y. B., Nazimah, S. A. H. & Amin, I. (2009). Chemical Properties of Virgin Coconut Oil. *J Am Oil Chem Soc 86*:301-307.

[5] Seaton, T. B., Welle, S, L., Warenk, M, K., & Campbell, R. G. (1989). Thermic effect of medium-chain fatty acid and long-chain triglycerides in man. *American Journal of Clinical Nutrition, 44*, 630-634.

[6] Shahid I. & Bhanger, M. I. (2007). Stabilization of sunflower oil by garlic extract during accelerated storage. *Food Chemistry, 100*, 246-254

[7] Shahidi, F. & Wanasundara, U. N. (1992). Stability of canola oil and its stabilization by natural antioxidants: a review note. *In Canola Oil*, 74-188.

[8] Stoilova, I., Krastanov, A., Stoyanova, A., Denev, P. & Gargova, S. (2007). Antioxidant activity of a ginger extract (Zingiber officinale). *Food chemistry, 102*, 764-770.

[9] Walisiewicz-Niekbalska, W., Kosmacinska, B. & Chmielarz, B. (1997). New trends in the processing of vegetable oils and animal fats for technical purposes. *Pollena: Tluszcze,Srodki Piorace Kosmet, 41(5)*, 190-194.

ANTIOXIDANT CAPACITY OF COMMERCIAL CITRUS JUICES FROM SUPERMARKET IN THAILAND

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Abstract:
Citrus juices are the most popular fruits and juices widely consumed around the world. Citrus belongs to Rutaceae family that is cultivated in the tropical zone of Southeast Asia. Our current life style has encountered with free radicals and toxic metabolites in our body. Free radicals have been reported to be responsible for several diseases. Citrus juices contain biologically active compounds which possess antioxidant activity. They are essential components of functional food. Hence, the objective of this study was to determine the total phenolic content, total flavonoid content and the antioxidant activity of six citrus juices obtained from the supermarket in Chon Buri Province, Thailand: lime, leech lime, mandarin, tangerine, pomelo and orange juice concentrate. Antioxidant activity was measured by using DPPH and superoxide radical scavenging methods. Among the juices, lime juice had the highest content of total phenolic and total flavonoid (10.75±0.01mg gallic acid equivalent (GAE)/g sample and 34.18±9.69 mg hesperidin equivalent respectively/g sample). Leech lime juice had the greatest scavenging activity on DPPH radical (IC_{50}= 2.21±0.81% (v/v)) whereas orange juice concentrate had the highest inhibitory effect against superoxide radical (IC_{50}= 0.25±6.41% (v/v)). In addition, a correlation between the total phenolic contents and superoxide radical scavenging activity was observed with the correlation coefficient by 0.9854. The results revealed that phytochemicals from citrus juices might be used as a good source of natural antioxidant compounds.

1. Introduction

In recent years, clinical trials and epidemiological studies have established an inverse correlation between the intake of fruits and vegetables and the occurrence of chronic diseases, the most prevalent causes of death in the world. This protective effect has been attributed to the antioxidant properties, which coordinate and balance the body system to protect tissues and fluids from damage by reactive species or free radicals [1].

Citrus fruits (Rutaceae family) are an important source of antioxidants such as ascorbic acid, carotenoids, flavonoids, and other phenolic compounds, and also some essential minerals for human nutrition. In Asian countries, citrus fruits, such as lime (*Citrus microcarpa* and *Citrus aurantifolia*), lemon (*Citrus limon*), and pomelo (*Citrus grandis*) are widely available and regularly consumed as whole fruits or fruit juices and preserved snacks. Among the phenolic compounds, flavanones are the major group found in citrus [2]. Various studies have shown that intake of flavonones is associated with reduced risk of developing coronary heart disease, degenerative diseases and anti-carcinogenicity because of their anti-lipid peroxidation [3-4].

The objective of the present study was to determine the total phenolic content, total flavonoid content and the antioxidant activity of six citrus juices obtained from the supermarket in Chon Buri, Thailand: lime, leech lime, mandarin, tangerine, pomelo and orange juice concentrate. Such activity was performed using DPPH and superoxide radical scavenging activity.

2. Materials and Methods

2.1 Sample Preparation

Pomelo juice (*Citrus maxima* (Burm.)), tangerine juice (*Citrus reticulata* Blanco), mandarin juice (*Citrus reticulata*) and orange juice concentrate were purchased from supermarkets in Chon Buri province. Lime juice (*Citrus aurantifolia* Swing.) and leech lime juice (*Citrus hystrix DC*) were obtained from local market at Chon Buri province. All juices were filtered through Whatman No.1 (Figure 1). Then, the juices were kept at 4^0C until use.

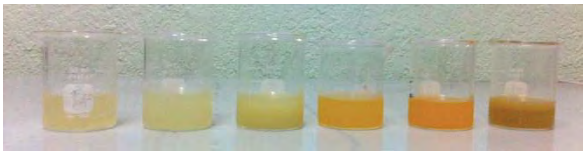

Figure 1. Samples of citrus fruit juice: (from left to right hand) pomelo, lime, leech lime, tangerine, mandarin juices and orange juice concentrate.

2.2 Total Phenolic Content

The phenolic content was determined using Folin-Ciocalteu as reactive reagent. The absorbance of the sample was determined by a spectrophotometer at 765nm after it was incubated for 2h at room temperature. The average of triplicate measurements was used to calculate the phenolic content as mg gallic acid equivalents (GAE)/ml samples. Gallic acid was used for the preparation of standard curve.

2.3 Total Flavonoid Content

The flavonoid content was measured by a modified aluminium chloride colorimetric method. The absorbance was measured at 510nm. The measurement

was compared with a standard curve of hesperidin solutions and expressed as mg hesperidin equivalent; HE per ml sample.

2.4 DPPH Radical Scavenging Assay

The ability of citrus juices to scavenge commercially available and stable free radical DPPH was performed as the protocol mentioned by Aquino et al. [5]. Briefly, 0.2mM 2,2-diphenyl-1-picrylhdrazyl (DPPH) in methanol was prepared and each juice was adjusted to different concentrations. The solution was divided into 3 groups; group A: DPPH in methanol, group B: citrus juice and DPPH, and group C: citrus juice and distilled water. Then, all solutions were incubated at room temperature and avoided from light for 30min. the absorbance was measured at 517 nm using a microplate spectrophotometer (VERSA max Microplate reader, CA). The positive controls were BHT and ascorbic acid. The antioxidant capacity was expressed as %DPPH scavenging activity that calculated by [A-(B-C)]/A x100 where: A = the A_{517} of DPPH in methanol, B = A_{517} of DPPH and sample, C = A_{517} of sample and distilled water.

2.5 Superoxide Scavenging Activity

The superoxide anion scavenging activity was carried out by the method described by Beissenhirtz et al. [6] and it is based on the reduction of cytochrome c. One ml of the extract was mixed with 1 ml of the solution containing 0.07 units per ml of xanthine oxidase, xanthine (c = 100 µmol/l), and cytochrome c (c = 50 µmol/l). After incubation at 20°C for 3 min, the absorbance at 550 nm was determined. All tests were performed in triplicate. The superoxide anion scavenging activity was calculated as follows: Scavenging activity (%) = $[(A_0 - A_1)/A_0] \times 100$ where: A_0 = absorbance of the control (without the sample) and A_1 = absorbance of the mixture containing the sample.

2.6 Statistical Analysis

The data were expressed as means ± standard deviations (SD) of three replicate determinations and then analyzed by SPSS 11.5 for window. One way analysis of variance (ANOVA) and the Turkey's test were used to determine the differences among means. *P* values < 0.05 were regarded to be significant.

3. Results and Discussion

Table 1 shows total phenolic and total flavonoid contents. Lime juice, leech lime juice and orange juice concentrate contained higher contents of phenolic compounds than pomelo, tangerine and mandarin juices. A wide variation in the levels of polyphenols and flavonoids was observed in the analysis. The lowest level was observed in the mandarin juice (5.71±0.01mg GAE/ml) and the highest in the lime juice (10.75±0.01 mg GAE/ml) for total phenolic content and the lowest level was observed in the pomelo juice (0.84±0.13 mg HE/ml) and the highest in the lime juice (34.18±9.69 mg HE/ml) for total flavonoid content. Although citrus juices are recognized as providing an important source of vitamin C and phenolic compounds for human nutrition, there are other parts of the fruit which also contain these bioactive ingredients. However, these other parts of the fruit are not recognized in nutrition because they are generally the inedible components. Xu et al. [7] found that phenolic compounds dominated total antioxidant capacity of citrus fruits. Most phenolic compounds contained in citrus juices were flavonoid glycosides including hesperidin, narirutin, naringin, neohesperidin and phenolic acids such as caffeic acid, *p*-coumaric acid, ferulic acid, sinapic acid, protocatechuic acid, *p*-hydroxybenzoic acid, and vanillic acid.

Table 1. Total phenolic and flavonoid contents of different citrus fruit juices.

Sample	TPC mg GAE/ml	TFC mg HE/ml
Leech lime juice	10.63 ± 0.00	29.27 ± 7.31
Lime juice	10.75 ± 0.01	34.18 ± 9.69
Pomelo juice	7.71 ± 0.00	0.84 ± .0 13
Mandarin juice	5.71 ± 0.01	1.16 ± 0.43
Tangerine juice	7.17 ±0.00	1.43 ±0.0 5
Orange juice concentrate	10.57 ± 0.17	9.09 ± 6.54

TPC = Total phenolic content, TFC = Total flavonoid content, GAE = Gallic acid equivalent, HE = Hesperidin equivalent

The antioxidant capacity of citrus fruits is an important indicator of their *in vitro* potential as health promoters. Several methods have been developed to evaluate the antioxidant capacity of these fruits; for instance, phenolics in fruits have been monitored spectrophotometrically using the Folin–Ciocalteau reagent. The antioxidant capacity by DPPH and superoxide anion scavenging assays was selected to determine the total antioxidant capacities of citrus fruit juices. The results are shown in Tables 2-5.

Among the individual DPPH scavenging activity detected in the citrus juices, Leech lime, lime and pomelo juices exerted the most effective to scavenge the free radical (IC_{50} = 2.21 ± 0.81, 5.63 ± 1.47, 5.90 ± 0.29 %v/v, respectively) (Table 4). This may be due to its high contents of bioactive components. Flavanones in citrus fruits were previously used as markers to differentiate citrus varieties. The main flavanone glycosides in oranges and mandarin species are hesperidin and narirutin [8] that are able to act for this inhibitory phenomenon.

Table 2. % DPPH scavenging activity of different citrus fruit juices.

Concentration (% v/v)	%DPPH scavenging activity					
	Leech lime juice	Lime juice	Pomelo juice	Orange juice concentrate	Mandarin juice	Tangerine juice
0.3	18.10±6.03	12.96±2.99	13.05±2.96	23.07±6.12	-	-
0.6	29.74±13.60	16.06±3.64	13.27±0.74	24.51±9.39	-	-
1.25	41.17±12.90	22.02±5.43	20.60±0.82	25.97±8.80	-	-
2.5	54.25±1.66	32.91±9.64	28.77±2.04	37.58±7.68	-	-
5	87.77±5.97	49.25±18.85	45.35±0.50	61.65±8.72	-	-
10	93.68±0.69	78.52±10.90	76.04±2.97	72.40±4.41	35.07±11.85	39.84±3.75
20	-	-	-	-	56.55±12.43	51.72±5.42
40	-	-	-	-	72.97±16.98	64.80±8.90
60	-	-	-	-	83.76±9.47	75.99±4.42
80	-	-	-	-	95.13±8.97	84.34±9.78
100	-	-	-	-	96.28±10.89	87.21±10.28

Table 3. % Superoxide scavenging activity of different citrus fruit juices.

Concentration % v/v	% Superoxide scavenging activity					
	Leech lime juice	Lime juice	Orange juice concentrate	Pomelo juice	Mandarin juice	Tangerine juice
0.3	26.47±12.82	ND	56.85±14.36	-	-	-
0.6	37.47±9.99	8.70±11.94	59.12±14.26	-	-	-
1.25	47.93±7.76	16.81±12.15	67.47±13.96	-	-	-
2.5	65.01±4.28	35.88±11.64	76.18±21.38	-	-	-
5	79.33±1.52	62.42±8.99	80.29±20.87	-	-	-
10	97.23±0.14	83.92±4.46	99.70±24.04	23.60±13.34	ND	ND
20	-	-	-	39.23±9.39	ND	14.30±4.18
40	-	-	-	49.50±18.91	21.74±2.02	41.93±3.73
60	-	-	-	68.05±13.10	36.43±0.68	55.18±4.50
80	-	-	-	73.61±12.12	50.43±3.53	67.21±5.25
100	-	-	-	88.53±6.72	62.20±4.22	80.16±3.95

ND = Not determined

Table 4. IC_{50} of DPPH scavenging effect of different citrus fruit juices.

Sample	IC_{50} (%; v/v)
Leech lime juice	2.21 ± 0.81^{a}
Lime juice	5.63 ± 1.47^{a}
Pomelo juice	5.90 ± 0.29^{a}
Mandarin juice	16.71 ± 9.60^{b}
Tangerine juice	17.50 ± 5.00^{b}
Orange juice concentrate	4.95 ± 0.61^{a}

$^{a, b}$ significantly difference at $P < 0.05$

Table 5. IC_{50} of superoxide scavenging effect of different citrus fruit juices.

Sample	IC_{50} (%; v/v)
Leech lime juice	$1.96±1.74^{a}$
Lime juice	$4.94±1.04^{a}$
Pomelo juice	$40.27±12.51^{b}$
Mandarin juice	$80.79±8.34^{b}$
Tangerine juice	$57.76±4.77^{b}$
Orange juice concentrate	$0.25±6.41^{a}$

$^{a, b}$ significantly difference at $P < 0.05$

Because superoxide is quite toxic, the alleviation the toxicity by any compounds from edible plants is very interesting challenge. The highest superoxide radical scavenging activity of studied citrus juices was observed in orange juice concentrate with IC_{50} = 0.25±6.41 %v/v following by leech lime and lime juices (IC_{50} = 1.96±1.74 and 4.94±1.04, respectively) (Tables 3 and 5) as they were the most enriched in polyphenolic compounds. Interestingly, the correlations between antioxidant capacities and bioactive compounds of commercial citrus juices were observed (Figures 2A and 2B).

4. Conclusions

In conclusion, the antioxidant capacity of commercial citrus juices from local supermarket correlates both to phenolic and flavonoid constituents. There is increasing interest in the potential health benefits of natural antioxidants contained in this commercial drinks but little is known about their mechanism of actions. In this work, we have evaluated the bioactive ingredients and their functional assays to provide information on the antioxidant properties. We show that experimental approaches could be complementary for the screening of commercial citrus juices that are good candidates for disease prevention.

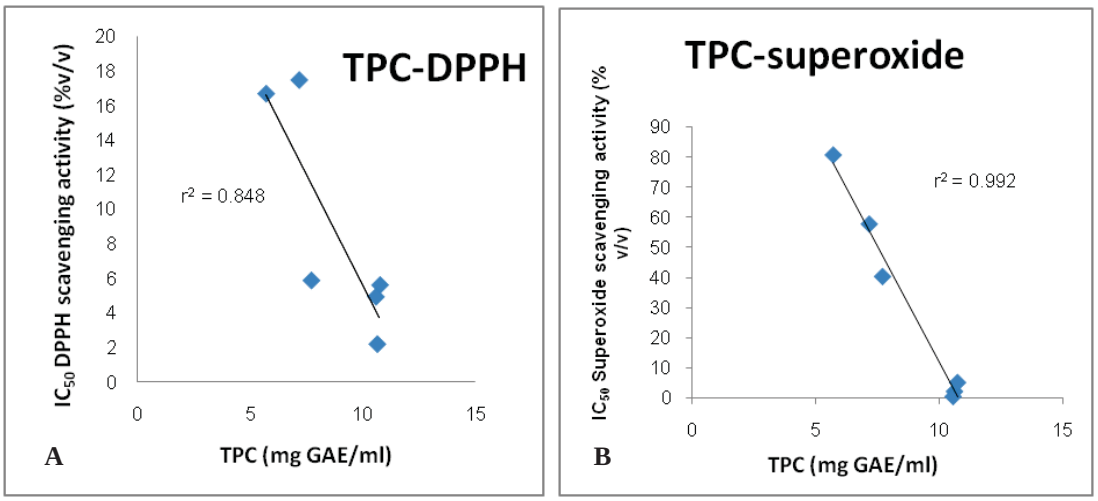

Figure 2. Correlation between total phenolic contents and IC_{50} of DPPH (A) and superoxide scavenging activities (B).

Acknowledgements

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References

[1] H.R.M. Barros, T.A.P.C. Ferreira, M.I. Genovese, *Food Chem.* **134** (2012) 1892-1898.
[2] A.A. Franke, R.V. Cooney, S.M. Henning, L.J. Custer, *J. Agric. Food Chem.* **53(13)** (2005) 5170-5178.
[3] N. Martí, P. Mena, J.A. Cánovas, V. Micol, D. Saura, *Nat. Prod. Commun.* **4(5)** (2009) 677-700.
[4] H.L. Tsai, S.K. Chang, S.J. Chang, *J. Agric. Food Chem.* **55(8)** (2007) Apr 18 2867-72.
[5] R. Aquino, I.S. Morell, M.R. Lauro, S. Abdo, A. Saija, A.Tomaino, *J. Nat. Prod.* **64** (2000) 1019–1023.
[6] M.K. Beissenhirtz, R.C. Kwan, K.M. Ko, R. Renneberg, F.W. Schiller, F. Liskat, *Phytother. Res.* **18** (2004) 149–153.
[7] G. Xu, D. Liu, J. Chen, X. Ye, Y. Ma, J. Shi, *Food Chem.* **106** (2008) 545–551.
[8] M.V. Albertini, E. Carcouet, O. Pailly, C. Gambotti, F.O. Luro, L. Berti, *J. Agric. Food Chem.* **54** (2006) 8335–8339.

THE EFFECT OF *PLUCHEA INDICA* LESS. TEA ON LIPID PEROXIDATION OCCURRED DURING STORAGE

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Abstract

Lipid oxidation and auto-oxidation are major causes of deterioration in the quality of the foods in which they are contained and the reduction in their nutritional value is one of the major factors in deterioration during the food storage that involves a lot of degenerative diseases. The oxidation of polyunsaturated fatty acids leads to serious damage such as coronary, atherosclerosis, emphysemas, and cancers. In this study, to elucidate the storage effect at room temperature in darkness of *Pluchea indica* Less. (Indian marsh fleabane) tea powder on the lipid peroxidation, the thiobarbituric acid reactive substance (TBARS), conjugated diene (CD), peroxide value (PV) assays were investigated and the total phenolic and total flavonoid contents were also determined. The result showed that the storage effect had influenced to markedly elevate the oxidation of lipid. However, TBARS and CD values could be strongly attenuated by *Pluchea indica* Less. tea in dose-dependent manner. IC_{50} against TBARS of extract during 2 months was observed with the significant value by 0.0427 mg/ml for initial time and 0.0111 mg/ml after 2 months, respectively whereas CD during 2 months could be moderately reduced from 60.31% to 46.38% and PV values at 48h could be effectively diminished from 97.65% to 83.22% at the highest concentration, respectively. In addition, total phenolic content of tea extract after 2 months of storage time significantly decreased to 544.28±0.01 mg gallic acid equivalent/g sample and the increase of total flavonoid content of tea extract during 2 months were observed (482.76±0.001 mg quercetin equivalent/g sample). Therefore, the oxidation of lipid significantly elevated in dose-dependent manner for a long period of the storage. Nevertheless, *Pluchea indica* Less. tea could efficiently inhibit the lipid peroxidation. Eventually, it may be used to consume as a healthy food.

1. Introduction

The auto-oxidation of fats is a big problem because of the deterioration in the quality of the foods in which they are contained and the reduction in their nutritional value. The oxidation of polyunsaturated fatty acids leads to serious damage such as coronary atherosclerosis, emphysemas, cancer and cirrhosis [1]. Safeguarding fats against oxidation is normally done by restricting the access of oxygen or adding antioxidants. The most commonly applied antioxidants are synthetic phenols such as butylated hydroxytoluene and butylated hydroxyanisole (BHA). However, the demand for natural antioxidants has recently increased because of the toxicity and carcinogenicity of synthetic antioxidants [2]. Thus, there is an increasing interest in finding natural herbal plants that show high antioxidant activity. Several studies have documented the effectiveness of antioxidative components in herbal plants such as flavonoids, phenolic or polyphenolic and related compounds for the prevention of lipid oxidation [1].

Pluchea indica Less. (Indian March Fleabane) is commonly known in Thai as "Khlu" is taxonomically classified in the Compositae (Asteraceae) family has been used as a therapeutic agent such as anti-hypertensive, anti-diabetic, anti-cardiovascular, diuretic, anti-inflammatory, and anti-hemorroidal activities [3]. In this study, we are interested in this herbal tea on the storage effect at room temperature in darkness of lipid peroxidation and the total flavonoid and total phenolic determination.

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2. Materials and Methods

2.1 Preparation of plant extract

Pluchea indica Less. (Indian March Fleabane) leaves tea imported from Thason community enterprises, Chanthaburi Province, Thailand. Then it was cut to a smaller size particle using a grinder (Moulinex, Type MCU 1A, France). The *P. indica* tea (dried leaves) will be ground into a fine powder. Powder (100 g) was mixed with boiling distilled water (1L) and further heated (90-95 $^{\circ}$C) for 30 min. [4]. In addition, in order to investigate the inhibitory effect of *Pluchea indica* Less. Tea on the lipid peroxidation during storage, it was ground into a fine powder and storage at room temperature in the darkness for 2 months. After that the tea was weighed and kept at -20 ^{0}C in an airtight container until use.

2.2 Oil emulsion preparation

Oil emulsions were prepared by mixing 0.285 g of oil, 0.289 g of Tween20 as emulsifier and 50 ml phosphate buffer (pH7.2). The mixture was homogenized for 5 min according to Stoilova's method [5]. The *P. indica* tea was added at the final

concentrations of 0, 0.0313, 0.0078, 0.0156, 0.0313 and 0.0625 mg/ml of dry extract. The mixture was incubated in an oven at 60^0C for 0, 24, 48 and 72h, respectively. The course of oxidation was monitored by measuring conjugated dienes (CD) formation, peroxide value (PV) and thiobarbituric acid reactive substances (TBARS).

2.3 Determination of total phenolic content

The total phenolic content was determined using Folin–Ciocalteu reagents with analytical grade gallic acid as standard. One ml of extract or standard solutions (0–2 mg/ml) was added to 10 ml deionized water and 0.125 ml of Folin–Ciocalteu phenol reagents. After 6 min, 1.25 ml of 7% sodium carbonate was added to the mixture. After 1.30h in darkness, the absorbance at 700 nm was measured. The concentration of total phenols was expressed as gallic acid equivalent/g sample [6].

2.4 Determination of total flavonoids content

Total flavonoids were measured by a colorimetric assay with some modification. 0.5 mL of sample or standard solutions of quercetin (0 – 0.04 mg L-1, Sigma-Aldrich, St Louis, MO) was added to 2 mL H_2O in a 10 mL volumetric flask. At zero time, 0.15 mL of 5% $NaNO_2$ was added to the flask. After 5 min, 0.15 mL $AlCl_3$ (10%) was added to the mixture and immediately diluted with 2.2 mL of H_2O. Absorbance of the mixture was read at 510 nm with methanol used for the blank. Total flavonoids were expressed as mg quercetin equivalents/g of dry matter [6].

2.5 Determination of conjugated dienes formation (CD)

Aliquots 0.02 ml of the emulsion in 2.2 was taken at different intervals during incubation. After incubation, 2 ml of methanol in deionized water (60%) was added, and the absorbance of the mixture was measured at 233 nm. The conjugated dienes concentration was expressed in mg/ml in each sample. The results were calculated as % inhibition on CD = B x v/wt x 100; where B is the absorbance reading, v denotes the volume (ml) of the sample and wt is the mass (mg) of emulsion measured [7].

2.6 Determination of peroxide value (PV)

Peroxide value was determined according to AOAC method 965.33 (Association of Official Analytical Chemists, 1995) and expressed as mg /g emulsion of sample [8].

2.7 Determination of thiobarbituric acid reactive substances (TBARS) [9].

Thiobarbituric acid reactive substances (TBARS) method was used to measure the antioxidant activity of *Pluchea indica* Less. in terms of inhibition on the lipid peroxidation. 0.2 ml of sample from oil emulsion and the following reagents were sequentially added: 400 µl TBA/trichloroacetic acid (TCA) solution (20 mM TBA in 1.5 g/L TCA) and 6 µl BHT (50 mM BHT in 70% ethanol). The mixture was heated in a 95^0C water bath for 15 min and cooled in ice; the mixture was then mixed and centrifuged at 950 rpm for 10 min. The supernatant was separated and the absorbance of the supernatant was measured at 535 nm in UV-visible spectrophotometer. Malondialdehyde (MDA) standard curve was prepared with different concentrations and TBARS were expressed as mg MDA/g dry matter.

3. Results and Discussion

It is known that plant phenolic compounds constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators. Flavonoids are phenolic compounds mainly present in plant and conserved in plant-derived foods. Flavonoids and phenolic compounds can act as antioxidant by a number of potential pathways. The most important is likely to be by free radical scavenging in which the polyphenol can break the free radical chain reaction. The *P. indica* tea extract had the total phenolic content during storage for 2 months were also evaluated by 781.5 ± 0.57, from the initial time to 544.28±0.01 mg gallic acid equivalent/g sample and total flavonoid content during storage for 2 months were also evaluated by 188.75 ± 0.002, from the initial time to 482.76±0.001 mg quercetin equivalent/g sample, respectively**.** From the test, it was found that the total phenolic and total flavonoid content was significantly influenced by different concentrations of tea extract. Therefore, the total phenolic content decreased with storage time and total flavonoid content significantly elevated in dose-dependent manner for a long period of the storage time. [10].

To complete the study of the storage effect at room temperature in darkness on the antioxidant behavior, it is necessary to evaluate the inhibition of lipid peroxidation in a model similar to those found in foods. Then, several concentrations of *Pluchea indica* Less. tea stored for 1-2 months were tested in oil emulsion and lipid oxidation with different indicators (conjugated dienes formation, peroxide value and TBARS formation). The concentration of conjugated diene increased during storage time (0-72h). Interestingly, the inhibition of the CD after the addition of the tea extract was observed. The percentage of inhibition on the CD formation during the storage time were 54.59%, 60.31% extract concentration 0.0625 mg/ml of storage for 0 month at initial time and after 72h of incubation, respectively and 27.93%, 46.38% after 2 months of storage time, when incubated at 0 and 72h, respectively (Fig. 1). Therefore, *P. indica* tea extract does not affect to the inhibition of CD formation during 2 months of storage time. However, *P. indica* tea extract can inhibit of CD during storage time.

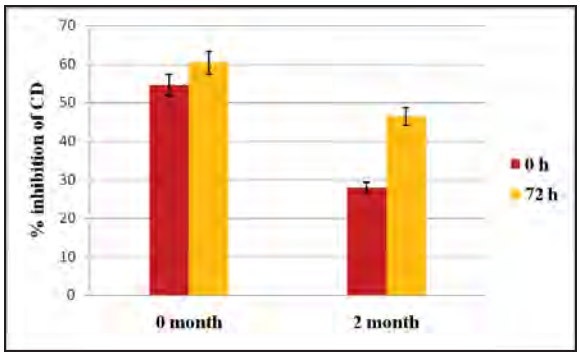


Figure 1. Percent of inhibition on the lipid oxidation measured as CD of *Pluchea indica* Less. tea extract according to the storage time (0, 2 months), when incubated at 0 and 72h.

In case of the peroxide development in the emulsion system, this value was strongly influenced by the addition of the *P. indica* tea extract during the entire test. Fig. 2 showed the percents of inhibition to the lipid oxidation affected by the storage time. The PV was measured by 91.11%, 97.65% at initial time and 72h of incubation time, respectively. After 2 months of storage time, these values appeared by 92.88%, 83.22% when incubated at 0 and 72h, respectively. The result showed that percents of inhibition on the peroxide values could be strongly attenuated by *P. indica* Less. tea in concentration dependent manner during 2 months of storage.

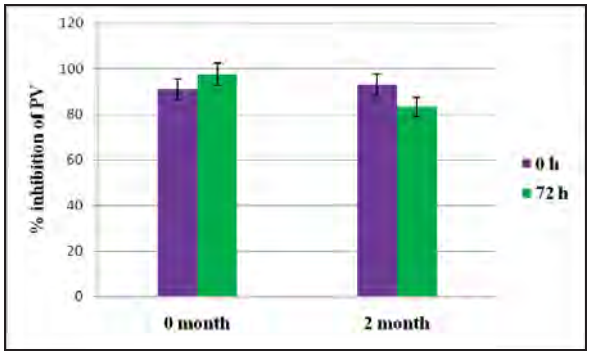
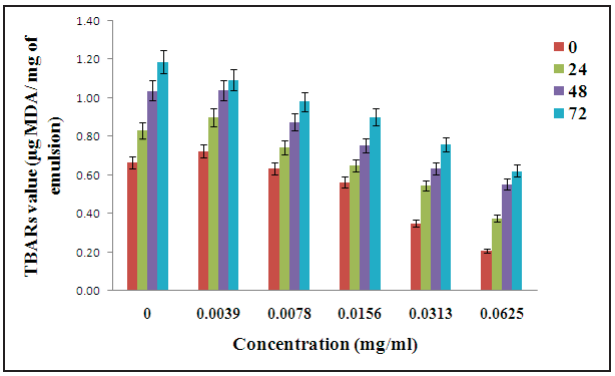
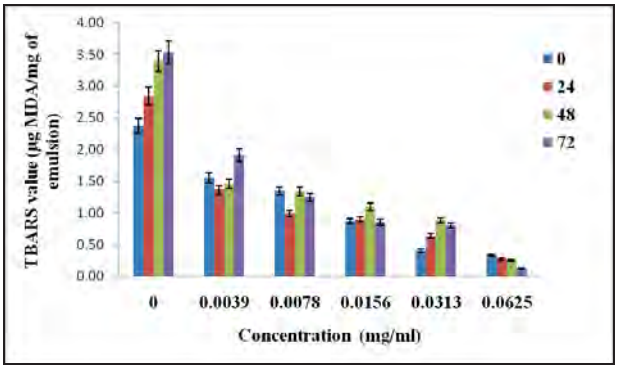


Fig. 2 Percent of inhibition on the peroxide value of *Pluchea indica* Less. tea extract according to the storage time (0, 2 months) when incubated at 0 and 48h.

TBARs analysis expresses the formation of secondary products of lipid oxidation, mainly malondialdehyde, which may contribute off-flavour to oxidized oil. The analysis of variance for the TBARs data indicates that the TBARs values were relatively affected between the control and samples that treated with the various antioxidants throughout storage (Fig. 3). The result showed that the storage effect had influenced to markedly elevate the oxidation of lipid. However, TBARS could be markedly attenuated by *Pluchea indica* Less. tea in concentration-dependent manner. IC_{50} against TBARS of extract during 2 months was observed with the significant value by 0.0427, 0.0111 mg/ml for 0 and 2 months, when incubated at 0, 24, 48, and 72 h, respectively.



[A]



[B]

Fig. 3 Effect of *Pluchea indica* Less. tea extract at different amounts during storage at 60^0C against TBARs value according to the storage time, [A] effect of the amount of TBARS tea extract storage for 0 month, [B] effect of the amount of TBARS tea extract storage for 2 months.

4. Conclusions

In this study, It showed antioxidant activities on the inhibition of lipid peroxidation of *Pluchea indica* Less. tea to support the use *P. indica* Less. herbal tea for healthy benefits particularly in order to protect the lipid peroxidation-related disorders. The oxidation of lipid significantly elevated in dose-dependent manner for a long period of the storage of *P. indica* Less. tea . Nevertheless, *P. indica* Less. tea could efficiently inhibit the lipid peroxidation. Eventually, it may be considered to consume as a healthy food.

References

[1] J. Fernandez, J.A. Perez-Alvarez, J.A. Fernandez-Lopez, *Food Chem*. **59(3)** (1997) 345–353.
[2] A.L. Branen. *J. Am. Oil Chem. Soc*. **52(1)** (1975) 59–63.
[3] A. Traithip. *Phytochemistry and antioxidant activity of Pluchea indica. Thesis (M.Sc.) Pharmacy*

(Pharmacognosy), Faculty of Graduate Studies, Mahidol University. (2005)
[4] J. Ahn, I.U. Grun, L.N. Fernando. *J. Food Sci*. **67** (2002) 1364–1369.
[5] I. Stoilova. *Food Chem.* **102** (2007) 764–770.
[6] D. Kim, S. Jeond, C. Lee. *Food Chem*. **81** (2003) 321–326.
[7] M. Zainol, A. Abd-Hamid, S. Yusof., R. Muse. *Food Chem*. **81** (2003) 575–581.
[8] Association of Official Analytical Chemists. Official methods of analysis (16th ed.). Washington DC: AOAC. (1995).
[9] E. Niki, *Experimental Protocols for Reactive Oxygen and Nitrogen Species*, Oxford, UK. (2000) 156-157.
[10] H. Normala, M.I. Suhaimi. *Pertanika J. Sci. Technol.* **19 (1)** (2011) 19 – 24.

ANTIOXIDATIVE CAPACITY AND ANTI-INFLAMMATORY ACTIVITY OF SOLVENT EXTRACTS FROM CLERODENDRUM DISPARIFOLIUM BLUME STEM, BRANCH AND LEAF ASSESSED BY VARIOUS METHODS

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Abstract: The genus Clerodendrum is ubiquitous throughout Thailand and traditionally used as medicinal plants for inflammatory related diseases. This study focused on the antioxidative capacity and anti-inflammatory activity of both polar (ethanol) and non-polar (hexane) solvent extracts from stem, branch and leaf of *C. disparifolium* Blume. The extracts were assessed for their antioxidant using different in vitro assays i.e. Folin-Ciocalteu assay (phenolic content), aluminium chloride colorimetric assay (flavonoid content), ferric reducing antioxidant power assay (FRAP) and lipid peroxidation assay using ferric thiocyanate (FTC) and thiobarbituric (TBA) methods and for their anti-inflammatory activity against lipid peroxidation catalyzed by soybean lipoxygenase. Both ethanol and hexane extracts from stem, branch, leaf of *C. disparifolium* provided the phenolic content, flavonoid content more than 990 mg GAE/g of dry extract and 10 mg QE/g of dry extract, respectively. FRAP of different extracts increased with the concentration of the extract, which correlated with the amount of antioxidants. In FTC method, the inhibition of the hexane stem extract showed the maximum inhibition of 73.58% at 2 µg/mL, whereas the ethanol branch extract provided maximum inhibition of 99.37% at 40 µg/mL by TBA method. In addition, hexane stem extract possessed the greatest anti-inflammatory activity with IC_{50} value at 35.36 µg/mL. The results obtained in the present study indicate that *C. disparifolium* can be a potential source of antioxidant and anti-inflammatory agents.

1. Introduction

Oxidative stress and inflammation underlie the pathogenesis of illnesses. Physiological and biochemical processes in the human body may produce free radicals as byproducts, which can cause oxidative damage to biomolecules leading to various diseases [1]. A number of species in the genus Clerodendrum (family Lamiaceae) were widely documented in indigenous systems of medicine throughout the world especially Asian and African continents [2]. The presence of antioxidant and anti-inflammatory molecules in these plant species was considerably evident their beneficial actions that led researchers to investigate plant extracts targeting their activity. Antioxidant activity could be characterized with in vitro assays *i.e.* Folin-Ciocalteu assay (phenolic content), aluminium chloride colorimetric assay (flavonoid content), ferric reducing antioxidant power assay (FRAP) and total antioxidant activity using ferric thiocyanate (FTC) and thiobarbituric (TBA) methods [3]. Whereas anti-inflammatory activity was tested against lipoxygenase, which is classified as the lipid-peroxidizing enzymes being involved in the progression of inflammation. It catalyzes the oxygenation of polyunsaturated fatty acids to the corresponding hydroperoxy derivatives and this reaction involves the formation of radical intermediates [4-5].

As mentioned previously, *Clerodendrum disparifolium* Blume was selected to evaluate the antioxidant and anti-inflammatory activities using polar and non-polar extracts from stem, branch and leaf. The extracts were supposed to demonstrate molecules that could scavenge radicals and inhibit lipoxygenase that may partly explain the health attribute.

2. Materials and Methods

2.1 Chemicals

Ammonium thiocyanate, butylated hydroxytoluene (BHT),Folin-Ciocalteu's reagent, sodium tetraboratedecahydrate, quercetin dehydrate, indomethacin, 2-thiobarbituric acid were all purchased form Sigma-Aldrich (St. Louis, MO, USA). Methanol, ethanol and hexane were purchased from J.T. Baker (NJ, USA). Iron (II) chloride ($FeCl_2$) tetrahydrate, linoleic acid and hydrochloric acid were purchased from Fluka (Buchs, Switzerland). Potassium dihydrogen orthophosphate, orthophosphoric acid, sodium hydroxide, potassium ferricyanide, sodium carbonate anhydrous and aluminium chloride ($AlCl_3$) were purchased from Ajax Finechem (NSW, Australia). Gallic acid, calcium acetate monohydrate,trichloroacetic acid, iron(III)chloride ($FeCl_3$) hexahydrate, 5-lipoxygenase and boric acid were purchased from Ascros organics (Geel, Belgium), POCH S.A. (Gliwice, Poland), Unilab (General Santos, Philippines), LOBA Chemie (Mumbai, India), TCI (Tokyo, Japan) and RCI Labscan (Bangkok, Thailand) respectively.

2.2 Instrumentation

All spectra and absorbance measurements were made on UV-Vis spectrophotometer Evolution 600

UV (Thermo Scientific, USA) and microplate UV-Vis spectrophotometer Infinite M200 (TECAN®, USA).

2.3 Preparation of plant extract

C. disparifolium was taken from middle part of Thailand.separated part of freshly stem, branch and leaves were washed, cut into small pieces, air-dried and crushed into powder.The sample powder was extracted with ethanol and hexane by maceration at room temperature for 72h. After filtering and evaporating to dryness, the crude extracts were obtained.

2.4 Total phenolic content

Total phenolic content was determined by Folin-Ciocalteu method [6] using gallic acid as a standard. Briefly, 125 uL of 2 mg/mL plant extract was mixed with 2.5 mL of 2.0% sodium carbonate. Then, 125 µL of Folin-Ciocalteu reagent was added and placed at room temperature for 30 min. The absorbance was measured at 680 nm against a reagent blank.

The standard curve of gallic acid was established and total phenolic contents were calculated and expressed in terms of mg gallic acid equivalent (GAE) /g of dry extract.

2.5 Total flavonoid content

Determination of the total flavonoid content in the plant extracts was carried out using aluminium chloride colorimetric method [7].0.5 mL of 2 mg/mL plant extract, 0.5 mL of 1.2% $AlCl_3$ ethanol solution and 0.5 mL of 0.12 M calcium acetate were mixed at room temperature for 30 min. A yellow colour indicated the presence of flavonoids. The absorbance was measured at 430 nm against a reagent blank.

The calibration of quercetin was established and total flavonoid contents were expressed in terms of mg quercetin equivalent (QE) /g of dry extract.

2.6 Total reductive capability

The Fe^{3+}reductive capability of all extracts were determined using Oyaizu method [8]. One mL of *C. disparifolium* extracts (500-1500 ug/mL) were mixed with 2.5 mL of 0.2 M phosphate buffer (pH = 6.6) and 2.5 mL of 30 mM potassium ferricyanide. The mixture was incubated at 50° C for 20 min. After incubation, 2.5 mL of 0.6 M trichloroacetic acid was added, and the mixture was then centrifuged at 3000 rpm for 10 min. 2.5 mL of the supernatant was transferred to mix with 2.5 mL of distilled water and 0.5 mL of 6 mM $FeCl_3$. The absorbance was measured at 700 nm against a blank.

2.7 Total antioxidant activity

2.7.1 Ferric thiocyanate (FTC) method

The antioxidant activities of *C. disparifolium* extracts were determined according to the FTC method [9] with slightly modification. 2 mL of 1mg/mL extracts, 2.05 mL of 80 M linoleic acid in 99.5% ethanol, 4 mL of 50 mM phosphate buffer (pH 7.0), and 1.95 mL of water were transferred to a vial and placed in an oven at 40°C protecting from light until use. 0.1 mL of the reaction mixture was transferred and mixed with 9.7 mL of 75% ethanol and 0.1 mL of 3.9 M ammonium thiocyanate. Then, 0.1 mL of 20 mM ferrous chloride in 3.5% HCl was added to the reaction mixture. Absorbance of the developed red colour solution was measured in 3 min at 500 nm every 24 h until 1 day after the absorbance of the control reached its maximum. The percent inhibition of linoleic acid peroxidation was calculated according to the following equation:

% Inhibition of linoleic acid = $100-[(A_{sample} / A_{control}) x100]$

Where $A_{control}$ is the absorbance of the control (containing all reagents except the tested compound) and A_{sample} is the absorbance of tested compound.

2.7.2 Thiobarbituric acid method

Two mL of 1.2 M trichloroacetic acid and 2 mL of 46 mM TBA were added to 1 mL of extracts prepared according to the FTC method. The solution was boiled for 10 min and cooled before centrifugation at 3000 rpm for 20 min. The absorbance of supernatant was measured at 532 nm. Antioxidant activity was based on the absorbance on the final day of the FTC method. The percent inhibition of linoleic acid peroxidation was calculated using the same equation as the FTC method [9-10].

2.8 Anti-inflammatory activity

Anti-inflammatory assay was studied using 5-lipoxygenase enzyme and linoleic acid as substrate and [12]. Test solution was dissolved in 25 uL of 2M borate buffer pH 9.0 and added 25 uL of lipoxygenase solution (20,000U/mL). The solution was incubated at 25°C for 5 min. After which, 100 uL of linoleic acid solution (0.6 mM) was added, mixed well and absorbance was measured at 234nm. Indomethacin was used as a reference standard. The percent inhibition was calculated from the following equation:

$$\% \text{ inhibition} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

A response curve was plotted to determine the IC_{50} values. IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum anti-inflammatory activity. All determinations were performed in triplicates.

3. Results and Discussion

3.1 Total phenolic and flavonoid content

The quantitation of phytochemical constituent (*i.e.* total phenolic and flavonoid contents) of *C. disparifolium* ethanol and hexane extract is shown in Table 1. Generally, plant phenols and flavonoids are highly effective free radical scavenging and antioxidant. Both ethanol and hexane extracts from selected parts of *C. disparifolium* provided the phenolic content and flavonoid content in the range of 990-1992 mg GAE/g of dry extract and 10-82 mg

QE/g of dry extract, respectively. Among all the extracts, hexane branch extract showed the highest amount of phenolic compounds, whereas ethanol branch extract exhibited the highest amount of flavonoid contents.

3.2 Total reductive capability

Reducing power assay is utilized to measure the reductive ability of antioxidantand evaluated by the transformation of Fe (III) to Fe (II) in the presence of the sample extracts [13]. The reducing power of *C. disparifolium* extracts is shown in Figure 1. The higher absorbance of the reaction mixture indicated the greater reducing power. Reducing abilities of all extracts increased with increasing in the concentration of extracts.

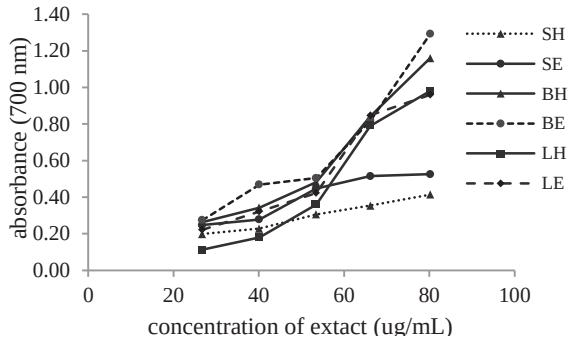

Figure 1 Antioxidant activity of *C. disparifolium* extracts of stem, branch and leaf using reducing power assay.

3.3 Total antioxidant activity

The total antioxidant activities of the *C. disparifolium* extracts were evaluated by the FTC and TBA methods. These methods based on the inhibitory activity against lipid peroxidation, which determined the FTC and TBA reactive substances obtaining in the reaction.

peroxide at the beginning of lipid peroxidation, in which it reacted with $FeCl_2$and subsequently produced ferric ions (Fe^{3+}). Then, ferric ions reacted with ammonium thiocyanate and generated FTC in a red colour. *C. disparifolium*extracts at concentration of 2 µg/mL exhibited mild to moderate activity (9.98%-73.58 %) against linoleic acid oxidation (Table 1) and the hexane stem extract showed the highest inhibition of 73.58%.

3.3.2 Thiobarbituric acid (TBA) method

TBA method measured the total peroxide content, which produced from the final stage of lipid peroxidation. Analyses of *C. disparifolium* extracts at the concentration of 40 µg/mL shows good inhibitory activity against linoleic acid oxidation with 53.72-99.37 %inhibition (Table 1). The ethanol branch extract provided maximum inhibitory activity while the ethanol stem extract offered the minimum inhibition.

In comparison, the antioxidant (%inhibition) of all extracts obtained from TBA method was higher than that of FTC method. This might suggest that the amount of peroxide in the initial stage of lipid per oxidation is less than the amount of peroxide in the final stage. Furthermore, the final product is much more stable for a period of time [14].

3.3.3 Anti-inflammatory activity

All extracts except the hexane leaf extract displayed promising 5-lipoxygenase inhibitory activity with IC_{50} values less than 96 µg/mL (Table 1). Both hexane and ethanol branch extracts generated the greatest inhibition capacity with IC_{50} values of 35.36 and 41.76 µg/mL, which correlated the total phenolic content. Whereas, the hexane leaf extract showed the lowest %inhibition. The results was comparable to the standard indomethacin (IC_{50}=48.65µg/mL) that inhibited the formation of lipoxygenase products.

Table 1 Total phenolic and flavonoid contents, total antioxidant activity and anti-inflammatory activity of *C. disparifolium*extracts

Extract	Total phenolic content (mgGAE/g of dry extract)	Total flavonoid (mg QE/g of dry extract)	FTC method* (%inhibition)	TBA method** (%inhibition)	Anti-inflammatory activity IC_{50} (µg/mL)
SH	1547.81±0.00	14.38±0.01	73.58±0.00	83.06±2.66	75.11±0.96
SE	990.96±0.01	10.30±0.01	30.36±0.00	93.38±3.10	95.59±0.17
BH	1991.56±0.01	23.89±0.00	21.98±0.01	70.47±0.34	35.36±2.39
BE	1217.03±0.01	81.81±0.02	40.37±0.01	99.37±0.90	41.76±2.13
LH	1272.42±0.01	59.91±0.00	13.87±0.00	53.72±1.03	754.28±1.49
LE	1167.21±0.00	13.03±0.01	9.98±0.01	96.17±3.17	53.83±1.01

*Extract: SH = hexane stem extract; SE = ethanol stem extract; BH = hexane branch extract; BE = ethanol branch extract; LH = hexane leaf extract; LE = ethanol leaf extract
**FTC method = Ferric thiocyanate method
***TBA method = Thiobarbituric acid method

4. Conclusions

*C. disparifolium*stem, branch and leaf extracts of different polarity (hexane and ethanol) composed of

varied total phenolic and flavonoid contents. These phytochemical contents determined the antioxidant potential of the extracts. The total antioxidant activities of the *C. disparifolium* extracts were also evaluated by the FTC and TBA methods that based on the inhibitory activity against lipid peroxidation. The hexane stem extract and the ethanol leaf extract showed the highest antioxidant activity. All extracts displayed good reducing power. They were also able to inhibit lipid peroxidation catalyzed by 5-lipoxygenase. The extract with highest amount of phenolics was better inhibit lipoxygenase. The presence of extracts is able to scavenge radicals and inhibits lipoxygenase, which would benefit in health in particular as antioxidant and anti-inflammation.

Acknowledgements

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References

[1] F.Conforti, S. Sosa, M. Marrelli, F. Menichini, G.A. Statti, D. Uzunov, A. Tubaro, F. Menichini and R. D. Loggia, *J .Ethnopharmacol*.**116**(2008) 144-151.
[2] N. Shrivastava and T. Patel, *Medicinal Aromatic Plant Sci. Biotech.***1**(2007) 142-150.
[3] S.P. Wong, L.P. Leong and J.H. William Koh, *Food Chem.***99**(2006) 775-783.
[4] M.N. Xanthopoulou, T. Nomikos, E. Fragopoulou and S. Antonopoulou, *Food Res. Int.***42**(2009) 641-646.
[5] G.E. Anthon and D.M. Barrett, *J. Agric. Food Chem.*, **49**(2000) 32-37.
[6] V.L. Singletonand J.A.Rossi, *Am.J. Enol. Vitic.***16** (1965) 144-158.
[7] R.D. Petry, O.G. González and W.B. *Pharmazie*.**56** (2001) 465–470.
[8] M. Oyaizu,*Jpn J Nutr*.**44** (1986) 307–316.
[9] H. Kikuzaki andN. Nakatani,*J Ethnopharmacol*. **87** (1993) 2007–2010.
[10] A. Ottolenghi,*Arch BiochemBiophys.***79** (1959) 355–363.
[11] J.I. Gray, *J Am Oil Chem Soc*.**55** (1978) 539–546.
[12] U.A. Shinde, K.R. Kulkarni, A.S. Phadke, A.M. Nair, D.V.J. MungantiwarandM.N. Saraf, *Indian. J. Exp. Biol.* **37**(1999) 258-261.
[13] I. Gülçin, M.Oktay, E. KireAci and O.I. Küfrevioglu, *Food Chem*. **83** (2003) 371–382.
[14] M.Zahin, F. Aqiland I.Ahmad, Int.*J. Pharm. Pharm. Sci.***1**(2009) 88-95.

IRRADIATION-INDUCED FREE RADICALS IN THAI KAOLINS

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Abstract: The effects of γ-radiation on EPR spectra from the two most abundant kaolin deposits in Thailand (Ranong in the south and Lampang in the north) are reported and compared with the free radical signals in the natural kaolin samples, where they are assumed to arise from the long-term accumulation of radiation damage centres. The results show that in addition to a dose-response effect of radiation exposure, there are appreciable qualitative differences between the natural and newly-produced free radical centres in the two deposits. Thus such minerals offer the potential to distinguish between recent and ancient radiation exposure, although their sensitivities are strongly influenced by the mineral crystallinity. The potential use of EPR measurements of free radical centres in kaolin minerals is discussed in the context of assessment of recent mineral exposure to γ-radiation as could occur in the event of a nuclear accident.

1. Introduction

Kaolins are a common group of 1:1 layer silicate minerals in which the individual layers are formed by a combination of a sheet of SiO_4 tetrahedra and a sheet of AlO_6 octahedra. The various minerals within this group, namely kaolinite, dickite, nacrite, and halloysite, are distinguished by the stacking order of these layers and whether or not water is incorporated between them. The kaolin group minerals occur on all continents, and are used extensively, especially in the manufacture of paint, paper, and ceramics.

Natural kaolin samples usually contain stable free radical centres that can be detected by electron paramagnetic resonance (EPR) spectroscopy, and these radicals are associated with electronic defects in the oxygen framework of the minerals that are created by reactions with natural γ-radiation. Since the intensity of the EPR signal is proportional to the cumulative radiation exposure to which the mineral has been subjected, the kaolin minerals could act as natural dosimeters to measure environmental radiation exposure over geological time periods. More than 20 years ago, Muller et al. [1] reported that kaolinite produced three clearly-defined paramagnetic defect centres (called A, A', and B) on exposure to γ-radiation, and that these "effectively reflected the cumulative radiation dose". These radiation-induced radicals had different stabilities, and only the A-centre was reported to be stable over geological time periods. On the basis of these results, Muller et al. proposed that the different types of defect centre in kaolinites may "form a record of successive irradiations linked to geochemical conditions prevailing during and after kaolinite formation", since they can be considered as being formed during different time periods. Therefore, kaolinite could represent a sensitive *in situ* dosimeter with the ability to be used for tracing the dynamics of radionuclide transfer in the environment, and it was suggested that the distribution of the paramagnetic defect centres in kaolinites could also be used for assessing the safety of nuclear wastes disposed of in geological settings that contain kaolinites.

The kaolin minerals have diverse compositions and variations in structural order [2], which might be expected to influence both the chemical nature and stability of free radical centres in their structures. Recently, Worasith and Goodman [3] reported major qualitative variations in the EPR spectra from free radical centres in natural kaolins from different parts of the World, a result which appears to confirm that kaolin minerals from different deposits respond differently to radiation exposure. As a consequence, the current work was undertaken to measure the responses of the minerals from the two major kaolin deposits in Thailand to controlled additional radiation dosages. However, instead of simply finding increases in intensity of the free radical signals that are seen in natural kaolin samples, the presence of different signals was also observed. The present paper describes the qualitative characteristics of these free radical spectra determined using EPR spectroscopy, and discusses the abilities of these samples to discriminate between historical and recent damage to the mineral structure. Finally, there is a discussion of the potential of these kaolins to be able to determine the cumulative radiation exposure that a soil could have received as a result of a nuclear accident.

2. Materials and Methods

2.1 Kaolin samples

Ranong kaolin was obtained from the Had Som Pan District, Muang, Ranong, Thailand. It is believed to have been formed by the hydrothermal alteration of granite [4], and contained both kaolinite and halloysite [5]. Lampang kaolin was obtained from the Ban Sa District, Jae Hom, Lampang, and is composed of weathering products of rhyolite in siltstone, sandstone and shale [4] with quartz as an impurity. Both kaolin

samples were washed with distilled water and dried in an oven at 100 ^{o}C for 24 h before use. Additional samples were prepared by (i) extraction with oxalic acid to remove poorly crystalline iron oxides associated with the kaolin minerals, or (ii) with the Lampang sample, grinding to damage the long-range order of the mineral structures. Grinding was performed as described by [5] in a Retsch stainless steel planetary ball mill for 1 hour with a rotation speed of 300 rpm using a weight ratio of balls to kaolin of 30:1.

2.2 Radiation treatment

Irradiation of samples was performed with γ-radiation from a Co-60 source housed in a Gammacell 220 SN 189R (MDS Nordion, Canada) (source activity 375.6 TBq in March 2010). The irradiation dose rate was 2 Gy/sec, and samples were prepared with received dosages of 10, 25, or 100kGy.

2.3 EPR measurements and spectral analysis

EPR spectroscopy was performed at X-band frequencies using a Bruker BioSpin, model A300 spectrometer equipped with a Gunn diode microwave bridge and an ER 4102ST rectangular cavity. Spectra were acquired at ambient temperature as 1st derivatives of the microwave absorption from 50-70 mg of sample (accurately weighed) using the following acquisition parameters: microwave power, 2 mW; centre field, 3520 gauss; sweep width, 200 or 300 gauss; modulation frequency, 100 kHz; modulation amplitude, 3 gauss; conversion time, 81.92 ms; time constant, 81.92 ms; resolution 1024 points, 10 scans; the receiver gain was adjusted as appropriate. Spectral intensities (SI) were calculated by double integration of the 1st derivative spectra, after correcting for any baseline non-linearity, and relative free radical concentrations were calculated as SI/(W x G), where W = sample weight and G = receiver gain

3. Results and Discussion

The paramagnetic A-centre is characterized by an anisotropic EPR spectrum with $g_{//}$ = 2.050 and $g_{\perp}$ = 2.008, and is thought to originate from defects on the oxygen atoms linking the octahedral and tetrahedral sheets [6]. There is also strong evidence that this free radical centre is associated with the boundaries of clusters of trioctahedral cells in the normal dioctahedral structure [7,8]. Usually, the A-centre shows no evidence of hyperfine structure (hfs) (from interaction of the unpaired electron with a nucleus with non-zero spin), although there is one report of several peaks with separations of ~2.3 gauss on the $g_{\perp}$ feature of a sample from Georgia, USA [3]. These peaks probably correspond to hfs from ^{27}Al atoms, which have nuclear spin I = 5/2, and hence produce sextet patterns in the EPR spectra. However, its magnitude is much smaller than for ^{27}Al in the B-centre (see below).

The EPR spectrum of the B-centre contains extensive hfs, although overlap with other paramagnetic components sometimes prevents its resolution. However, in Q-band spectra Clozel et al [9,10] resolved 11 peaks with separations in the range 7.1-8.4 gauss from this resonance; thus it corresponds to two ^{27}Al atoms interacting with the unpaired electron. There is currently no definitive evidence for the location of the B-centre radical, which could in principle be associated with (deprotonated) surface oxygen atoms on the octahedral sheet or oxygen atoms linking the octahedral and tetrahedral sheets.

In addition to the paramagnetic defect centres, the EPR spectra of kaolin group minerals contain components from various transition metal ions, usually Fe(III) along with some V(IV), Mn(II), Mn(IV), or Cu(II) ions. The EPR spectra from the metal ions in the Ranong and Lampang kaolins have been described previously [3,5]; they are not of specific concern in the present work, except for the relationship between the Fe(III) (g=4.3) signal and structural order [11.12].

The Fe(III) signals indicate that the Lampang sample is poorly ordered, whereas the Ranong sample has a much higher degree of structural order. This structural difference could influence the ability of the minerals to stabilize radiation-induced defects, and some differences in the free radical signals are observed Fig. 1(a, c). Although the spectra from both samples can be interpreted primarily in terms of a combination of A- and B-centres, the differences between the spectra in Figs. 1(a, c) suggests that either these kaolin samples have experienced a different radiation history, or they respond differently to exposure to γ-radiation.

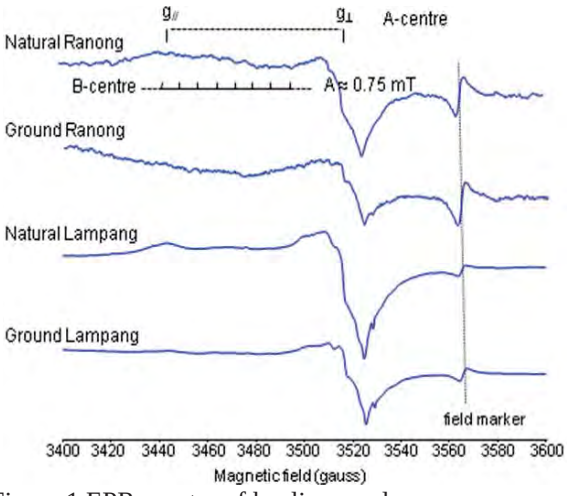

Figure 1 EPR spectra of kaolin samples.

The free radical spectra observed after exposure of these kaolin samples to γ-radiation from Co-60 (100 kGy) are presented in Fig. 2. Major differences are observed in the qualitative composition of these spectra both respect to one another and to the corresponding unirradiated natural samples. With the Ranong sample there was a large increase in the intensity of the signal from the B-centre, 8 peaks of which are clearly resolved with separations of ~7.5 gauss in Fig. 2(a). This result is similar to that reported for kaolinite irradiated with α-particles [13].

In contrast, the Lampang sample showed only minor changes in the A- and B-centres on irradiation, but it produced a completely new signal, which we refer to here as the C-centre. This shows several peaks with separations of ~21 gauss to the low and high field sides of the A-centre, and these probably correspond to an extensive hfs pattern, the central region of which is obscured by resonances from the A- and B-centres. This hfs almost certainly originates from ^{27}Al atoms, since these are likely the only atoms with appropriate nuclear spin that are present in the mineral in significant quantities.

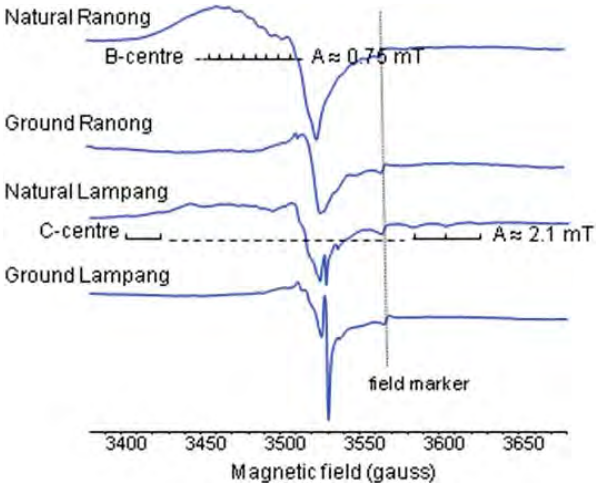

Figure 2 EPR spectra of kaolin samples after 100 kGy radiation dosage.

Close inspection of the spectrum from the natural Ranong sample shows that the high field peaks of the C-centre are also present, but the low field peaks are obscured by the intense B-centre resonance. Identification of the C-centre is difficult to make from these data, but the observed peaks are consistent with an 11-peak hyperfine structure, such as would occur with two equivalent ^{27}Al atoms. This pattern is then similar to that from the B-centre, but the much larger hfs from the C-centre indicates that it has a correspondingly greater unpaired electron density on the Al atoms. Also, its absence from the natural samples indicates that the C-centre is less stable than either the A- or B-centres.

Because of the presence of ^{27}Al hfs in each case, both the B- and C-centre radicals are likely to be located on oxygen atoms that are bound to aluminium atoms in the octahedral sheet. Therefore, it seems plausible that the B- and C-centres correspond to defect centres located on bridging and surface oxygen atoms, respectively. Surface radicals might be expected to be less stable than those associated with oxygen atoms bridging the octahedral and tetrahedral sheets, because of the possibility of direct interaction with O_2 and other paramagnetic species in the environment. However, it is not clear why the relative levels of production of these two centres by γ-irradiation should be so different for these two kaolin mineral samples.

Since the Fe(III) EPR signals indicated that the Ranong kaolin has a much greater degree of order than that from Lampang, the effects of chemical and physical treatments on the subsequent generation of free radicals on irradiation were also investigated. Deferration by extraction with oxalic acid removed some of the background signal from magnetically interacting Fe(III) ions, but had essentially no effect on the free radical signals, either before or after irradiation. This observation is consistent with the deferration treatment simply removing poorly-crystalline iron-rich phases from the sample, and not affecting the kaolin minerals. In contrast, grinding the samples had a major effect on the free radical signals. Before irradiation, the ground samples had much weaker signals from the A- and B-centres than the corresponding natural kaolins (Fig. 1), consistent with a destabilization of the defect centres as a result of grinding-induced breakdown of the mineral structures. Also, the responses of the ground samples to exposure to Co-60 γ-radiation were different from those of the natural mineral samples (Fig. 2), with little if any generation of any of the A-, B-, or C-centres. Although there was a signal in a similar position to that of the $g_{\perp}$ component of the A-centre, this signal is not definitive, because many other free radical centres occur in this region, and it could, therefore, correspond to a minor mineral component in the deposit. Thus the integrity of the kaolin mineral structure is essential for stabilization of the radiation-induced free radical centres in the kaolin minerals. The sharp peak that is seen at ~3525 gauss in the Lampang sample, and which makes a greater contribution after irradiation, probably arises from a defect centre in quartz, which was present as a major impurity in this specimen. The much harder nature of the quartz crystals make them more resistant to grinding, and it has been shown that the presence of quartz increases grinding-induced changes to the kaolin mineral structures [14].

Plots of total free radical signal intensity versus received radiation dosage are presented in Fig. 3.

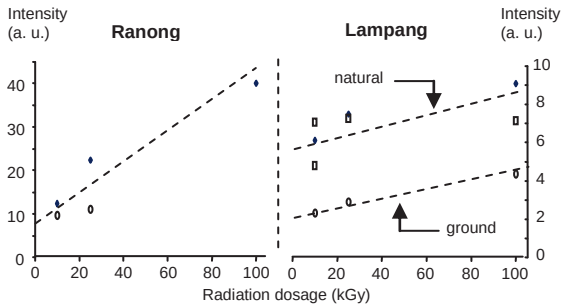

Fig. 3 Variation of EPR free radical spectral intensity with radiation dosage.

These show a linear response of total radical signal intensity to radiation dosage, but the plot for the well crystalline Ranong sample is much steeper than that for the poorly crystalline Lampang sample. Also, the EPR signals from the ground Lampang sample showed a considerably weaker response to γ-radiation

exposure than the original mineral, thus providing further evidence for the importance of mineral crystallinity for stabilizing the free radical defect centres.

It is also significant that the EPR spectra generated in the kaolin minerals by γ-irradiation are appreciably different from those observed in the natural minerals, and also from one another. In the natural minerals the free radical centres are assumed to have accumulated as a result of natural irradiation over geological time periods, and the dominance of the A-centre is the result of its greater stability [1]. However, in the present samples, there appeared to be little generation of the A-centre on irradiation in the laboratory, whereas there was a major production of the B-centre in the Ranong sample and the C-centre in the Lampang sample. Thus the A-centre may represent a secondary radical that is formed by electron hopping from either the B- or C-centres. Furthermore, grinding the samples substantially reduced their abilities to show radiation-induced free radical centres, thus demonstrating that mineral crystallinity has a major effect on the ability of the mineral to stabilize the radiation-induced centres.

With the kaolin minerals investigated in the present work, both qualitative and quantitative changes in the EPR spectra were observed as a result of exposure to γ-radiation in the laboratory. Thus, as proposed by Muller et al. [1], EPR spectroscopy can potentially distinguish between recently-formed free radical centres and those built up over geological periods of time. However, we also observed large qualitative differences between the radiation-induced spectra from specimens from the north and south of Thailand, and substantial quantitative differences in the radiation-induced responses. The quantitative differences in the radiation-induced signals from these specimens may be at least partly the consequence of different sample crystallinities, since grinding the samples also reduced the accumulation of the free radical signal. However, the crystallinity of the samples does not explain the qualitative differences in their responses to exposure to γ-radiation, and this is likely to be the consequence of compositional differences between the minerals in the two kaolin deposits.

4. Conclusions

The EPR spectra observed after exposure to γ-radiation of the Lampang and Ranong kaolins were qualitatively different from those in the natural minerals and from one another. In these samples there was little if any production of the A-centre as a result of irradiation of the sample in the laboratory, and this centre therefore probably corresponds to a secondary radical that is stable over geological time periods. The B-centre was produced in large quantities in the Ranong sample, but not that from Lampang. Since it is also seen in the natural samples, this centre must also be very stable, and its presence cannot be used to indicate a recent radiation event. However, the C-centre has not been reported in natural kaolins, and its presence could, therefore, be taken as evidence for recent irradiation of the mineral. However, there were clear differences in the relative proportions of the B- and C-centres that were produced in the Ranong and Lampang samples as a result of irradiation in the laboratory, so careful calibration of samples is necessary if the C-centre radical intensity is to be used to estimate a received dosage.

Although the kaolin specimens from both deposits showed a linear response of EPR free radical signal intensity to radiation dosage, it was only with the relatively well-crystalline Ranong sample that the slope of the plot is sufficiently steep for this approach to have potential for use in accurately measuring radiation exposure. With the poorly crystalline Lampang sample, the errors in such a measurement are sufficiently high for the approach to have only minor value for calculating the received radiation dosage, although identification of the presence of the C-centre signal can be taken as strong evidence for exposure to a recent radiation event.

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References

[1] J.-P. Muller, B. Clozel, P. Ildefonse and G. Calas. *Appl. Geochem.* **7** (1992) 205–216

[2] B. Velde *Origin and Mineralogy of Clays: Clay and the Environment*. Springer-Verlag, New York (1995).

[3] N. Worasith and B.A. Goodman. *Clay Miner.* **47**, (2012) 539-557.

[4] C. Kuentag and P. Wasuwanich. Clay. Economic Geology Bulletin No. 19, *Economic Geology Division, Department of Mineral Resources*, Thailand (1978).

[5] N. Worasith, B.A. Goodman, N. Jeyachoke and P. Thiravetyan. *Clay Miner.* **46** (2011) 539-559.

[6] K. Lombardi, J.L. Guimarães, A.S. Mangrich, N. Mattoso, M. Abbate, W.H. Schreiner and F. Wypych *J. Braz. Chem. Soc*. **13** (2002) 270-275.

[7] A.H. Cuttler *Clay Miner.* **15** (1980) 429-444.

[8] A.H. Cuttler *Clay Miner.* **16** (1981) 69-80.

[9] B. Clozel, T. Allard and J.-P. Muller. *Clays Clay Miner.* **42** (1994) 657-666.

[10] B. Clozel, J.-M. Gaite and J.-P. Muller. *Phys. Chem. Miner.* **22** (1995) 351-356.

[11] M.M. Mestagh, L. Vielvoye and A.J. Herbillon. *Clay Miner.* **15** (1980) 1-13.

[12] G.W. Brindley, T. Chih-Chun Kao, J.L. Harrison, M. Lipsicas and R. Raythatha. *Clays Clay Miner.* **34** (1986) 239-249.

[13] T. Allard, J.-P. Muller, J.-C. Dran and M.-T. Ménager. *Phys. Chem. Miner.* **21** (1994) 85-96.

[14] É. Makó, R.L. Frost, J. Kristóf and E. Horváth . *J. Coll. Interfac. Sci.* **244**, (2001) 359-364.

ANTIOXIDANTS – FRIEND OR FOE, OR WE DON'T KNOW?

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Abstract: This paper provides a brief summary of the roles played by antioxidant molecules in foods, medicines and nutrition and the methods used to determine their concentrations. Critical assessments are made of the types of molecule that are determined in typical chemical assays of antioxidant activity. These include behaviour as free radical scavengers as well as inhibitors of oxidation processes. However, although the chemistry of some specific molecules is well known, there is still a dearth of information available many others, and specifically on their contributions to the health of consumers.

1. Introduction

Antioxidant molecules, such as polyphenols, are now accepted as important components of healthy diets [1,2], and there is a plethora of chemical assays for measuring "antioxidant status" of various food products and biological systems. As the name implies, the principal function of antioxidant molecules is to provide protection against oxidation by reactive oxygen species (ROS), O_2-related molecules that are more powerful oxidizing agents than $^3O_2$, the stable form of O_2. ROS may be free radicals, such as $O_2^{\cdot-}$ or $^{\cdot}OH$, or precursors of free radicals, such as H_2O_2 or $^1O_2$. It is frequently assumed that antioxidants operate by selectively scavenging ROS, and consequently that they are also free radical scavengers. However, some unstable radicals (e.g. $^{\cdot}OH$) react indiscriminately with organic molecules, so their selective scavenging is impossible in complex systems. Protection against their reaction is thus only achieved by inhibiting their formation. In addition, ROS also play roles in important biological reactions, so their elimination could be fatal for the organism.

According to the *European Food Safety Authority* [3], antioxidants from dietary sources protect cells from free radicals,, protect tissues, lipids, cells and DNA from oxidative damage, and help to strengthen the body's natural defenses against oxidative stress. Some of the most common antioxidant molecules that are consumed on a regular basis are the vitamins, C and E, carotenoids, such as β-carotene, lycopene and lutein, and various types of polyphenols. These are all generally considered to be important or essential micronutrients and are frequently consumed as dietary supplements, in addition to being common food components. However, there is also a wide range of other substances that have antioxidant properties, and many of these are ill-defined and have largely unknown biological functions.

This paper presents a brief overview of our knowledge of antioxidants in food, medicine and nutrition, and raises questions about some of the common views on this important group of molecules. Methodology used for determination of antioxidant contents of food and biological systems is also considered along with some specific reactions which show that molecules which are considered to be antioxidants can also catalyze oxidation reactions.

2. Antioxidants and food quality

Antioxidants are now recognized as important components of foods. They are used to prolong shelf life by inhibiting chemical processes and the development of spoilage organisms that lead to product deterioration. In addition, the consumption of diets rich in antioxidant molecules has been linked to longer life-spans.

The use of antioxidant preservatives in processed foods is now common. These include synthetic molecules, such as butylhydroxyanisole (BHA) and butylhydroxytoluene (BHT), and naturally-occurring molecules, such as ascorbic acid (vitamin C) and α-tocopherol (vitamin E). Developments in food preservation have had a major impact on our eating habits, and although there have been questions raised about the health aspects of consumption of large quantities of synthetic antioxidants, the risks are generally considered to be minimal at typical levels of consumption [4,5]. Furthermore, since unsaturated lipid oxidation is an important aspect of deterioration in many food products, antioxidant preservatives have a major beneficial effect by inhibiting the formation of toxic lipid peroxidation products, such as the 4-hydroxyalkenals [6,7], in addition to delaying development of off-flavors.

In addition to their micro-nutritional properties, many antioxidant molecules, especially polyphenols, exhibit antimicrobial properties [8-12]. Since some spoilage organisms produce potentially dangerous toxins, it could be considered that a major function of antioxidant molecules in foods is the preservation of the integrity of the original product, by inhibiting the formation of potentially dangerous molecules.

Thus there is a dual role for antioxidants in foods; provision of important dietary components and

inhibition of the formation of toxins. Both are probably important, but it should be noted that the epidemiological evidence is not able to distinguish the relative importance of the two types of function.

Finally, although the addition of antioxidant preservatives extends the period of safe consumption for food products, and thus decrease wastage during storage, this extension of "shelf-life" can produce complacency and result in products being sold in sub-optimum conditions as a result of long-term storage.

3. Antioxidants as essential micronutrients

An important function of antioxidants is to provide systemic protection against both degenerative and infectious diseases, and the abundance of plant-derived antioxidants has been suggested as a major factor in the beneficial effects of the Mediterranean diet [13,14]. In addition, antioxidants, such as ascorbic acid, tocopherols and carotenoids, provide protection against degenerative diseases of aging. An adequate intake of these antioxidant molecules can build up reserves to combat oxidative stress events.

The theory of free radicals in aging was originally developed by Harman [16], and has been extensively reviewed (e.g. [17,18]). Essentially, it starts from the generation of superoxide radical anions ($O_2^{\cdot-}$) in the mitochondrial electron transport chain; this is converted to H_2O_2 by the enzyme superoxide dismutase, and subsequently the highly reactive $^{\cdot}OH$ radical. Although catalase can remove H_2O_2, it is not possible to selectively scavenge $^{\cdot}OH$ and damage resulting from its generation is limited by selectively scavenging the (more stable) products of its reaction. It is at this stage in the degenerative aging process that simple antioxidant molecules can be effective. A lifetime of adequate supply of antioxidant molecules to minimize mitochondrila damage combined with a nutritious calorie restricted diet has been suggested to be able to increase average life expectancy at birth by ≥5 years [16]. Also, supplementing a high fat diet with red wine was observed to increase plasma antioxidant capacity, decrease oxidative DNA damage, and normalize endothelial function [19].

In addition to the well-known antioxidant molecules discussed above, many uncharacterized molecules in the average diet have antioxidant properties, e.g. melanoidins in coffee and many cooked foods. At the present time little in known about their specific effects on the human body, and coffee for example has been shown to have both mutagenic and anti-carcinogenic properties (e.g. [20]).

4. Sources of antioxidants in normal diets

For many people the main sources of antioxidants in their diets are the beverages coffee and tea, along with various fruits and vegetables; on the basis of chemical antioxidant assays, coffee has been shown to be the major source of antioxidants in many western diets [21,22]. However, a considerable proportion of the antioxidant activity of coffee originates from the melanoidin fraction [23], the biological properties of which are still not well-known. Polyphenols have attracted more attention, and some of their main natural sources have been reviewed by Dimitrios [24]. In addition to the natural sources of antioxidants, it is important to include the synthetic antioxidants that are added in substantial quantities to processed foods, and dietary supplements, the consumption of which has grown substantially in recent decades.

Thus we need to address the question as to whether instead of addressing deficiency situations, our consumption of antioxidants might be too high. Recommended daily intakes are produced by several governments for individual supplements, but there is little information available on the optimum overall intake of antioxidant molecules. Yet there must be an optimum range, because toxicity situations, though rare, have been reported, and for example, congenital malformations have been associated with both maternal deficiency and over-use of vitamin A [25,26].

5. Medicinal properties of antioxidants

A quick look in magazines, newspapers or the internet shows just how much publicity is given to the value of antioxidant medicines. As was discussed for foods, antioxidant medicines perform two distinctly different functions; protection of the body against natural deteriorative processes (aging and degenerative diseases), and fighting infectious diseases. Systemic protection against aging processes was considered above, but in recent decades there has been a lot of publicity given to antioxidant protection against degenerative diseases, such as cancer, cardiovascular disease, immune-system decline, brain dysfunction, and cataracts [15]. A good example of protection against infectious diseases is that of vitamin C and the common cold, which was strongly advocated by Linus Pauling in the 1970s. However, in a major review of the literature, Hemilä et al [27] concluded that regular ingestion of vitamin C had no effect on common cold incidence, although it did reduce the duration and severity of common cold symptoms. However, for individuals who experience short periods of extreme physical stress (including marathon runners and skiers) vitamin C halved the common cold risk.

Biological systems are extremely complex and it is likely that most antioxidants will exhibit a degree of specificity in their actions. Thus for example, coffee which could be considered to be a traditional medicine, is associated with a decrease in incidences of cancers of the upper digestive tract and the colon / rectum, but an increased risk of stomach cancer [28]

5. Antioxidant Assays

When discussing the contents of antioxidant molecules in complex systems such as foods or live organisms, it is important to consider how these molecules are assessed. Firstly, one must recognise that all antioxidant assays are based on chemical reactions carried out *in vitro*, and thus the values they

produce may not be biologically relevant, since they do not take into account bioavailability, *in vivo* stability, retention by tissues, and reactivity in tissues [29]. Nor in the case of foods or "traditional" medicines, do they take account of reactions with other components.

Antioxidant assays should be rapid, because of the possible occurrence of unwanted oxidation processes during sample analysis, and synergistic effects of antioxidant mixtures in real food samples [30]. Although many assays have been reported for measuring total antioxidant capacity [29,31,32], there are fundamental differences in the chemical processes involved, and thus results correspond to specific chemical properties and do not necessarily relate to biological antioxidant behavior. Measurements made by a single method can be misleading, and assays involving 2 or more different types of chemical reaction are necessary for a realistic assessment of the overall antioxidant properties of a complex sample, such as a food product.

In general, antioxidants are classified as preventive or chain-breaking. Preventive antioxidants, such as the enzymes superoxide dismutase, catalase or peroxidase inhibit formation of ROS, whereas chain-breaking antioxidants, such as vitamin C, vitamin E, uric acid, bilirubin, polyphenols, etc., scavenge ROS and thereby break radical chain reaction sequences.

Methods for the analysis of individual antioxidant molecules are readily available [33] and will not be discussed further here.

Chain breaking antioxidants can function either by (i) hydrogen atom transfer, where an oxygen-derived radical abstracts a hydrogen atom from the antioxidant, resulting in formation of a (relatively) stable antioxidant-derived radical, or (ii) single electron transfer, in which an electron is first transferred to the antioxidant, and then followed by a proton. Both mechanisms occur in parallel at different rates and the net result is the same, although the single electron transfer reaction is solvent dependent because of the need to stabilize the charged species [34].

Antioxidant assays can be classified according to whether they involve competitive or non-competitive reactions [32]. In a competitive reaction, the species being tested competes with the antioxidant for the oxidizing agent. However, one must be aware that the antioxidant capacity is dependent on the relative reaction rates of the test species and the antioxidant with the oxidizing agent, and the concentration ratio of antioxidants and target species. Ideally the target species should react with an oxidant at low concentration, the reaction should be measurable through a drastic spectroscopic change after oxidation, whereas no radical chain reaction after oxidation should occur, and the antioxidant should not react with the target species. In non-competitive assays, antioxidants interact with oxidizing agents without any other competing target molecules present, and only the antioxidant and the reactive species are present in the initial reaction mixture.

For all assays it is important to understand the chemistry that actually occurs [35], and in principle biologically relevant compounds (ROS, RNS) should be utilized. However, this is not the case in most electron transfer reactions, e.g. the quenching of stable radicals such as DPPH or ABTS [36], although these methods are relevant to the scavenging of the relatively stable secondary radicals that are produced after primary reactions by highly reactive species, such as the hydroxyl radical.

6. Antioxidant chemistry

Much is known about the chemical reactions of some specific antioxidant molecules. There are many excellent texts on the subject (e.g. [37-40]), and these should be consulted for more information on antioxidant chemistry. However, it seems to be key property of biological antioxidants that they can redox cycle between (relatively) stable reduced and oxidized forms, and the original reduced forms of antioxidants can be regenerated by reaction of their oxidized forms with natural reducing agents.

It should also be noted that reactions of antioxidant molecules can produce net pro-oxidant effects, especially in complex systems containing transition metal species that can exist in more than one stable oxidation state. An example is the role of antioxidants, such as ascorbic acid, polyphenols, carotenoids in reducing Fe(III) to Fe(II), which can then react with hydrogen peroxide to produce hydroxyl radicals. Such reactions can be important in fighting microorganisms, but they can also be damaging to the host.

7. Conclusions

Although there is strong evidence that diets rich in natural antioxidants are beneficial to health, there is still no definitive evidence for the reason, despite the wide publicity that is given to free radical scavenging. Highly reactive free radicals cannot be selectively scavenged in complex systems, and the only effective approach to prevent their reaction is to inhibit their formation. That means controlling the production and reactivity of precursors, such as H_2O_2.

One important function of antioxidants in foods is to inhibit the formation of potentially toxic compounds, either by preventing the formation of toxic aldehydes or ketones from lipid peroxidation, by inhibiting the development of food spoilage organisms. Thus, it is too simplistic to assume that antioxidants simply provide protection against free radical damage. Indeed, as mentioned in the previous section, antioxidant molecules actually stimulate oxidation reactions.

Understanding the roles played by antioxidants is further complicated by the fact that there is little evidence for a positive effect of antioxidant supplements for healthy individuals consuming a balanced diet, although antioxidant supplementation may be beneficial for people with inadequate diets, or

in stressful situations. As the EFSA has stated in the context of the consumption of dietary polyphenols [3], a cause and effect has not been established between their consumption and the claimed beneficial health effects. Nevertheless, several individual antioxidant molecules clearly have important biological functions, but these are not necessarily determined by the types of chemistry that are measured in assays for antioxidant properties. Thus there are still important questions that must be answered concerning the functions of the wide range of ill-defined molecules that are measured in various types of antioxidant assay.

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References

[1] A. Scalbert and G. Williamson. *J. Nutr.* **130** (2000) 2073S-2085S.
[2] A. Scalbert, I.T. Johnson and M. Saltmarsh. *Am. J. Clin. Nutr*. **81** (2005) 215S-217S.
[3] European Food Safety Authority (EFSA) Panel on Dietetic Products, Nutrition and Allergies (NDA) (2011)
[4] D.V. Parke and D.F.V. Lewis. *Food Addit. Contam.* **9** (1992) 561-577.
[5] R. Kahl and H. Kappus. *Z. Lebensm.Unters. For.* **196** (1993) 329-338.
[6] H. Esterbauer, R.J. Schaur and H. Zollner *Free Radic Biol Med.* **11** (1991) 81-128.
[7] R.J. Mark, M.A. Lovell, W.R. Markesbery, K. Uchida and M.P. Mattson. *J. Neurochem*. 68 (1997) 255–264.
[8] T. Taguri, T. Tanaka, I. Kouno. *Biol. Pharm. Bull.* **27** (2004) 1965-1969.
[9] S. Moreno, T. Scheyer, C.S. Romano and A.A. Vojnov. *Free Radical Res.* **40** (2006) 223-231.
[10] I. Oliveira, A. Sousa, I.C.F.R. Ferreira, A. Bemto, L. Estevinho and J.A. Pereira. *Food Chem. Toxicol.* **46** (2008) 2326-2331.
[11] R. Ksouri, H. Falleh, W. Megdiche, N. Trabelsi, B. Mhamdi, K. Chaieb, A. Bakrouf, C. Magné, C. Abdelly. *Food Chem. Toxicol.* **47** (2009) 2083–2091.
[12] T. Yaltirak, B. Aslim, S. Ozturk and H. Alli *Food Chem. Toxicol.* **47** (2009) 2052-2056.
[13] A. Trichopoulou and E. Vasilopoulou. *Brit. J. of Nutr.* **84** (2000) S205-S209.
[14] A. Trichopoulou, E. Vasilopoulou, A. Lagiou. *Nutr. Rev.* 57 (1999) 253-255.
[15] B.N. Ames, M.K. Shigenaga, and T.M. Hagen. *PNAS* **90** (1993) 7915-7922.
[16] D. Harman. *Mutati. Rese.-DNAging* **275**, (1992) 257–266.
[17] K.B. Beckman and B.N. Ames. *Physiol. Rev.* **78** (1998) 547–581.
[18] E. Cadenas and K.J. Davies. *Free Radical Bio. Med.* **29** (2000) 222-230.
[19] F. Leighton, A. Cuevas, V. Guasch, D.D. Pérez, P. Strobel, A. San Martín, U. Urzua, M.S. Díez, R. Foncea, O. Castillo, C. Mizón, M.A. Espinoza, I. Urquiaga, J. Rozowski, A. Maiz and A. Germain. *Drugs Exp. Clin. Res.* 25 (1999) 133-141.
[20] B.A. Goodman. In: *Coffee Consumption and Health* (Ed. B.A. Goodman), Nova Science Publishers, New York, (2012) pp. 187-219.
[21] A. Svilaas, A. Kaur Sakhi, L. Frost Andersen, T. Svilaas, E.C. Ström, D.R. Jacobs, Jr., L. Ose and R. Blomhoff (2004). *J. Nutr.* **134** (2004) 562-567.
[22] R. Pulido, M. Hernández-García and F. Saura-Calixto *Eur. J. Clin. Nutr.* **57**, (2003) 1275-1282.
[23] S. Opitz, S. Smrke, B.A. Goodman and C. Yeretzian Methodology for the measurement of antioxidant capacity of coffee In: *Processing and Impact on Antioxidants in Beverages* (Ed. V.R. Preedy), Elsevier, in press (2013).
[24] B. Dimitrios *Trends Food Sci. Tech.* **17** (2006) 505-512.
[25] A Bendich and L Langseth. *Am. J. Clin. Nutr.* **49** (1989) 358-371.
[26] K.L Penniston and S.A Tanumihardjo *Am J Clin Nutr* 83 (2006) 191-201
[27] H. Hemilä, E. Chalker and B. Douglas. *Cochrane Database of Systematic Reviews 2007, Issue 3. Art. No.: CD000980.*
[28] B.A. Goodman (ed.). *Coffee Consumption and Health*, Nova, New York (2012).
[29] D. Huang, B. Ou and R.L. Prior. *J. Agric. Food Chem.* **53** (2005) 1841-1856.
[30] A. Blauz, T. Pilaszek, A. Grzelak, A. Dragan, and G. Bartosz. *Food Chem. Toxicol.* **46** (2008) 2365-2368.
[31] R.L. Prior and G.H. Cao. *Free Radical Biol. Med.* **27** (1999) 1173-1181.
[32] L.M. Magalhaes, M.A. Segundo, S. Reis and J.L.F.C. Lima. *Anal. Chim. Acta* **613** (2008) 1-19.
[33] *Official Methods of Analysis of the AOAC*
[34] J.S. Wright, E.R. Johnson and G.A. DiLabio. *J. Am. Chem. Soc.* **123** (2001) 1173-1183.
[35] R.L. Prior, X.L. Wu and K. Schaich. *J. Agric. Food Chem.* **53** (2005) 4290-4302.
[36] E.N. Frankel and A.S. Meyer. *J. Sci. Food Agric.* **80** (2000) 1925-1941.
[37] O.I. Aruoma and B. Halliwell. *Free Radicals and Food Additives*, Taylor & Francis, London (1991)
[38] B. Frei (Editor) *Natural Antioxidants in Human Health and Disease*, Academic Press (1994)
[39] J.M.C. Gutteridge and B. Halliwell. *Antioxidants in Nutrition Health and Disease*. Oxford University Press (1994).
[40] B. Halliwell and J.M.C. Gutteridge. *Free Radicals in Biology and Medicine, 3rd Edn* (1999).

ANTIOXIDANT ACTIVITIES OF THE PHENOLIC EXTRACT FROM THE WOOD VINEGAR OF *GARCINIA MANGOSTANA* FRUIT

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Abstract: The aim of this research was to investigate the antioxidant activities of the phenolic extract from the wood vinegar of *Garcinia mangostana* fruit. The extracts were prepared by three different methods, standing for 3 months at room temperature, evaporating under vacuum and filtering through charcoal. The antioxidant activities of these extracts were analyzed using the DPPH radical scavenging assay (ascorbic acid as a standard) and the ferric reducing antioxidant power assay (FRAP) using ascorbic acid and BHT as a standard. The extract from evaporation exhibited the strongest antioxidant, scavenging the DPPH radical with the IC_{50} value of 5.02 ± 0.18 μg/mL (ascorbic acid, IC_{50} 3.60 ± 0.18 μg/mL). The FRAP result showed that all extracts have higher potential antioxidant than the standard used. The greatest activity was also the phenolic extract from evaporated fraction. These results indicated that the phenolic extract from the wood vinegar of *Garcinia mangostana* fruit might serve as natural antioxidants.

1. Introduction

Wood vinegar is a dark brown condensed liquid from smoke collected during pyrolysis or carbonization of wood in airless and under high temperature conditions. The wood vinegar composed of many chemical components with acetic acid as the major substance [1]. Several reports demonstrated that the wood vinegar contained organic chemicals exhibited the inhibitory activity against bacteria, fungi and termites [1-3].

Garcinia mangostana Linn. (mangosteen), known as the queen of fruits, is a slow-growing tropical tree belonging to the Guttiferae family which widely distributed throughout Southeast Asia including Thailand. The fruit of *G. mangostana* is a dark purple or reddish pericarp (hull) with white, soft and juicy edible pulp. The fruit hulls have been used as a traditional medicine in Thailand for the treatment of diarrhea, skin infections and wounds [4-5]. The fruit hulls of this plant have been composed of a variety of secondary metabolites with xanthones as main bioactive components especially α-, β- and γ-mangostins [6-9]. The activities of xanthones isolated from mangosteen have been shown on several reports such as anticancer [10-11], antiallergy [12], anti-inflammatory [7,13], antimicrobial [14-15], antimalarial [16] and antioxidant [17-18] activities.

Mangosteen fruits are released to markets in excessive volume during harvest season and causes depreciation of value of the produces. It is worthwhile if some principle ingredients in the fruits could be extracted for uses instead of throwing away. Nowadays, there is no report on investigation and evaluation of the wood vinegar from mangosteen-tree. From this reason we are very interested to evaluate the mangosteen fruits concerning antioxidant properties of its wood vinegar.

2. Materials and Methods

2.1 General and chemicals

The absorbance was measured on a UVM 340 micro-plate reader apparatus and GENESYS 20 spectrophotometer. The distillated sample was performed on a Büchi rotary evaporator R-220. Chemicals were purchased from Merck and Sigma-Aldrich. All solvents used were of analytical grade.

2.2 Plant materials

The fruit of *G. mangostana* was collected from Phrom Khiri district, Nakorn Sri Thammarat province in the Southern part of Thailand.

2.3 Preparation of wood vinegar

Wood vinegar was a condensed liquid, which was produced from the smoke of charcoal production. The carbonization process of *G. mangostana* fruit using a 200-liter fuel tank as charcoal brazier and the system was set in air-closed condition. Wood vinegar was cooled by outside air while passing through a chimney and was collected from the vapors under temperatures of 300-400°C. This temperature is indicated by the production of acrid yellowish smoke. To separate tar the raw wood vinegar was differentially prepared by three methods; standing for 3 months at room temperature, evaporating using rotary evaporator under vacuum and filtering through charcoal.

2.4 Preparation of phenolic extract

Each wood vinegar fraction was exhaustingly partitioned with ethyl acetate. The organic layer was concentrated and then extracted with 5% $NaHCO_3$ (w/v) and 5% NaOH (w/v), respectively. The basic portion was acidified by 20% H_2SO_4 (w/v) prior to extract with ethyl acetate several times to obtain phenolic extracts from three different methods; standing at room temperature (SPE), evaporating

under vacuum (EPE) and filtering through charcoal (FPE).

2.5 DPPH radical scavenging assay

The free radical scavenging activity was determined by DPPH radical decolourization assay [19], with slight modifications. Briefly, 190 μL of freshly-prepared 0.002 %w/v DPPH radical solution dissolved in ethanol was mixed with 10 μL of extracts also dissolved in ethanol at the concentration of 200, 100, 50, 20, 10, 5 and 1 μg/mL in a 96-well plate. The absorbance at 517 nm was measured after 10 min. Ethanol, as a solvent blank, was run in each assay. Ascorbic acid was used as a standard for comparison. All measurements were performed in triplicate. The DPPH radical scavenging activity (%) was calculated by the following equation:

$$\text{Scavenging activity (\%)} = (1 - A_{sample}/A_{control}) \times 100$$

where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of tested extract or standard.

2.6 Ferric reducing antioxidant power (FRAP)

The reducing power of extracts was assayed by the method described by Gülçın, Oktay, Kırecçı and Küfrevıoğlu [20]. Briefly, an aliquot of 0.2 mL of extracts at the concentration of 100, 75, 50, 25, 10 and 5 μg/mL was mixed with 0.5 mL of phosphate buffer (0.2 M, pH 6.6) and 0.5 mL of 1%w/v potassium ferricyanide [$K_3Fe(CN)_6$]. After incubation at 50^{o}C for 20 min, 2.5 mL of 10%w/v trichloroacetic acid was added to the reaction mixture, followed by centrifugation at 3000 rpm for 10 min. The 2.5 mL of supernatant was mixed with an equal quantity of distilled water and 0.5 mL of 0.1%w/v $FeCl_3$. The absorbance was measured at 700 nm using spectrophotometer against the blanks that contained all reagents except the extracts. Ascorbic acid and BHT were used as standard for comparison. All measurements were performed in triplicate. The increased absorbance of the reaction mixture indicated increased reducing power.

3. Results and Discussion

3.1 Preparation of phenolic extracts

Wood vinegar from the mangosteen fruit was a dark brown liquid with pH and density ranges 4.08-4.40 and 0.98-1.03 g/mL, respectively. The raw wood vinegar (250 mL each) eliminated tar by standing at room temperature for 3 months, evaporating under vacuum and filtering through charcoal was separately extracted with ethyl acetate yielding organic extracts 0.84, 0.91 and 1.57 g, respectively. After fractionating the phenolic components using 5% NaOH, each method gave the extracted yield of 0.64 g (76.7%), 0.75 g (82.8%) and 1.32 g (84.0%).

3.2 DPPH radical scavenging activity

DPPH radical assay was a suitable method for measuring radical scavenging activity of antioxidants due to the change in color of DPPH solution [21]. The antioxidants could scavenge the radical by hydrogen donation decreased the absorbance of DPPH at 517 nm. Figure 1 exhibited the DPPH radical scavenging activity of phenolic extracts of mangosteen-fruit wood vinegar which was also compared with the commercial antioxidant, ascorbic acid. The results found that the scavenging capacity of all samples tested was dose-dependent. The linear regression equations were y = 6.2707x + 5.7521 (R^2 = 0.988) for SPE, y = 9.0559x + 4.5259 (R^2 = 0.963) for EPE, y = 6.7258x + 0.3432 (R^2 = 0.999) for FPE and y = 8.5612x + 19.223 (R^2 = 0.940) for ascorbic acid.

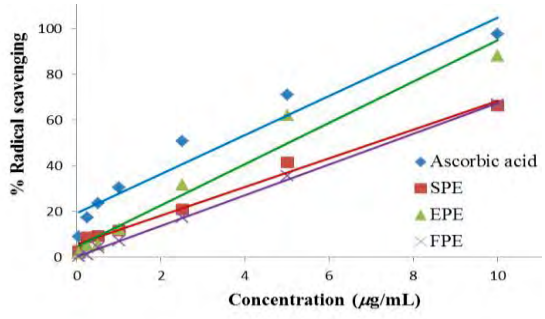

Figure 1. DPPH radical scavenging activity of phenolic extracts and ascorbic acid

The inhibitory concentration of antioxidants required for a 50% decrease in the absorbance of DPPH radical (IC_{50}) of SPE, EPE, FPE and ascorbic acid was 7.06±1.12, 5.02±0.18, 7.38±0.52 and 3.60±0.18 μg/mL, respectively, as shown in Table 1. These results indicated that the evaporating portion (EPE) showed the DPPH radical scavenging activity stronger than SPE and FPE fractions and was nearly those of ascorbic acid.

Table 1: IC_{50} of phenolic extracts and standard compound

Extract/standard	IC_{50} DPPH (μg/mL)
SPE	7.06±1.12
EPE	5.02±0.18
FPE	7.38±0.52
Ascorbic acid	3.60±0.18

3.3 Ferric reducing antioxidant power (FRAP)

Phenolic antioxidants were an electron-donating molecule which used as an indicator for Fe^{3+} reduction [22]. In the FRAP assay, the increased absorbance of samples indicated the increase of reducing power. Figure 2 demonstrated the dose-dependent for the reducing power of phenolic extracts and reference compounds. All phenolic extracts showed the reducing power and also exhibited stronger power than BHT. However, ascorbic acid displayed significant better activity than those of all extracts. The reducing power

decreased in the order of ascorbic acid, EPE, FPE, SPE and BHT in the presence of 100 μg/mL test sample.

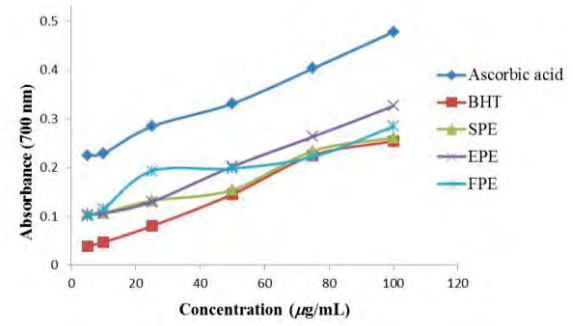

Figure 2. Ferric reducing antioxidant power (FRAP) of phenolic extracts and standard compounds

4. Conclusions

This work is the first report on the study of antioxidant activity of the wood vinegar from *G. mangostana* fruit. Investigation of the antioxidant activities using DPPH radical scavenging and FRAP assay showed that all phenolic extracts exhibited strong activity. The EPE fraction was the most active. These results indicated that the phenolic extracts from the wood vinegar of *G. mangostana* fruit might serve as natural antioxidants.

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References

[1] M. Yatagai, M. Nishimoto, K. Hori, T. Ohira and A. Shibata, *J. Wood Sci.* **48** (2002) 338–342.

[2] J. Radhakrishnan, J.R. Teasdale and C.B. Coffman, *Proceedings of the Northeastern Weed Northeastern Weed Science Society* **56** (2002) 100.

[3] K.I. Seo, K.J. Ha, Y.I. Bae, J.K. Jang and K.H. Shim, *Korean Journal of Postharvest Science Technology* **7** (2000) 337–341.

[4] W. Mahabusarakam, P. Wiriyachitra and W.C. Taylor, *J. Nat. Prod*. 50 (1987) 474–478.

[5] S.C. Pierce, *A Thai Herbal*, Findhorn Press, Scotland, UK (2003).

[6] N. Chairungsrilerd, K.I. Furukawa, T. Ohta, S. Nozoe, and Y. Ohizumi, *Eur. J. Pharmacol*. **314** (1996) 351–356.

[7] N. Chairungsrilerd, K. Takeuchi, Y. Ohizumi, S. Nozoe and T. Ohta, *Phytochemistry* **43** (1996) 1099–1102.

[8] G. Gopalakrishnan, B. Banumathi and G. Suresh, *J. Nat. Prod*. **60** (1997) 519–524.

[9] Y.L. Huang, C.C. Chen, Y.J. Chen, R.L. Huang and B.J. Shith, *J. Nat. Prod*. **64** (2001) 903–906.

[10] K. Mutsumoto, Y. Akoa, E. Kobayashi, K. Ohguchi, T. Ito, M. Iinuma and Y. Nozawa, *J. Nat. Prod*. **66** (2003) 1124–1127.

[11] S. Suksamrarn, O. Komutiban, P. Ratananukul, N. Chimnoi, N. Lartpornmatulee and A. Suksamrarn, *Chem. Pharm. Bull*. **54** (2006) 301–305.

[12] K. Nakatani, M. Atsumi, T. Arakawa, K. Oosawa, S. Shimura, N. Nakahata and Y. Ohizumi, *Biol. Pharm. Bull*. **63** (2002) 1137–1141.

[13] N. Chairungsrilerd, K. Furukawa, T. Ohta, S. Nozoe, and Y. Ohizumi, *Planta med*. **62** (1996) 471–472.

[14] W. Mahabusarakam, P. Wiriyachitra and S. Phongpaichit, *J. Sci. Soc. Thailand*, **12** (1986) 239–242.

[15] S. Suksamrarn, N. Suwannapoch, W. Phakhodee, J. Thanuhiranlert, P. Ratananukul, N. Chimnoi and A. Suksamrarn, *Chem. Pharm. Bull*. **51** (2003) 857–859.

[16] W. Mahabusarakam, K. Kuaha, P. Wilairat and W.C. Taylor, *Planta Med*. **72** (2006) 912–916.

[17] H.A. Jung, B.N. Su, W.J. Keller, R.G. Mehta and A.D. Kinghorn, *J. Agric. Food Chem*. **54** (2006) 2077–2082.

[18] Y.W. Chin, H.A. Jung, H. Chai, W.J. Keller and A.D. Kinghorn, *Phytochemistry* **69** (2008) 754–758.

[19] Q.F. Zhang, Y.X. Guo, X. Shangguan, G. Zheng and W.J. Wang, *Food Chemistry* **133** (2012) 140–145.

[20] İ. Gülçın, M. Oktay, E. Kırecçı and Ö.İ. Küfrevıoğ lu *Food Chemistry* **83** (2003) 371–382.

[21] X. Yuan, M. Gao, H. Xiao, C. Tan and Y. Du, *Food Chemistry*, **133** (2012) 10–14.

[22] Y.N. Sreeraman, V.B. Sashikala and V.M. Pratape, *Food Chemistry*, **133** (2012) 156–162.

Food safety and Food Chemistry

AMYLOPECTIN RECRYSTALLIZATION AND PROPERTIES OF WHITE WHEAT BREAD AS AFFECTED BY SOY FORTIFICATION

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Abstract: The effect of soy fortification on staling and keeping quality of white wheat bread was investigated. Full-fat soy flour (SF) and soymilk residue, or okara (OK), were used to substitute wheat flour in bread recipe at 5-15% by weight. Moisture and freezable water contents of freshly baked soy-containing breads were significantly higher than those of all-wheat control ($p \leq 0.05$). Both moisture and freezable water contents became decreasing with increasing storage time, with a sharper reduction demonstrated in the control. Meanwhile, unfreezable water content was not significantly different among the samples ($p > 0.05$) and remained relatively constant throughout the 7-day storage. Amylopectin recrystallization, as monitored using a differential scanning calorimetry technique, was observed to be most pronounced in the control. The OK bread exhibited slightly lower amylopectin recrystallization as compared to the SF bread at the same substitution level. For instance, at Day 3 of storage, the enthalpy required to melt recrystallized amylopectin in the control, 15% SF and 15% OK samples were equalled to 0.77, 0.35 and 0.23 J/g, respectively. The increasing trend of amylopectin recrystallization was in consistent with the decreasing trend of the water-soluble starch content, implying an increase in the crystalline structure. Crumb hardness was found to be increased at a greater extent in the control in comparison to that of the soy-containing samples. These changes were found to occur rapidly in the first two days of storage. In summary, soy fortification contributed a positive effect on the storage quality of white wheat bread by retarding starch retrogradation.

1. Introduction

A sizable economic loss of bread industry is resulted from bread staling which characterizes by crumb firming, development of crumbliness and flavor changes [1]. Over the years, attempts have been made to elucidate bread staling mechanisms as well as to search for a means to reduce bread staling. The majority of bread staling studies have been focusing on retrogradation of starch, the prime component of the product. Nevertheless, some other studies reported that moisture migration could also play a role in bread staling [2,3].

High protein and high fiber ingredient was reported to help retain moisture in food products and thereby prolong their freshness [4]. Soy flour is a good source of protein and fiber. It contains approximately 40% protein and 5% fiber on a dry basis [5].

Soymilk residue or okara is a by-product of soymilk and tofu manufacture. Even though, a portion of the protein present in soybeans is extracted during soymilk production. A significant amount of protein still remains in the residue. It was reported that okara contains 27% protein and 56% fiber on a dry basis [6,7]. However, it should be noted that okara composition may vary depending on the preparation method used. The protein and fiber, with their excellent water holding capacity and emulsifying property, could be advantageous to the keeping quality of bakery products [8].

This study aimed to investigate the substitution of soy flour or okara to wheat flour in white bread and its effect on staling and keeping quality of the stored bread.

2. Materials and Methods

2.1 Materials

Bread ingredients, including bread flour, instant baker's yeast, salt, sugar and shortening, were commercial products. At a level not exceeding that permitted by the Ministry of Public Health [9], food-grade calcium propionate was used as a mold inhibitor.

Dehulled soybeans for okara preparation was obtained from Thai Cereals World (Bangkok, Thailand) and full-fat soy flour was a product of Doi Kham (Bangkok, Thailand).

2.2 Okara Preparation

Okara was prepared using a hot water-blanching soymilk production method [10] with some modifications. The wet okara obtained after separating soymilk was steamed at 100 ^{o}C for 30 minutes to ensure a complete inactivation of antinutritional factors present in raw soybeans. The steamed okara was then dried at 60 ^{o}C for about 14 hours until obtaining 10% (wb) moisture content.

2.3 Bread Making

Bread samples were prepared using a method adapted from that described earlier [11]. The ingredients, expressed as baker's percentage, for all-wheat control sample included 100% bread flour, 1.2% instant yeast, 4.8% shortening, 4.8% sugar, 1.8% salt, 59.9% water and 0.3% calcium propionate. For the soy-fortified samples, either full-fat soy flour (SF) or okara (OK) was used to substitute bread flour at 5, 10 and 15% by weight.

2.4 Evaluation of Bread Properties

Moisture Content: Moisture content was determined using the AOAC standard method [12].

Freezable and Unfreezable Water Contents: Freezable water was monitored using differential scanning calorimetry (DSC) technique [13]. An endothermic peak transition around 0 ^{o}C is attributed to ice melting [14]. Freezable and unfreezable water contents were calculated using Equations (1) and (2) [13]:

$$\%\text{Freezable water} = h/(\Delta H_{fus} \times M) \quad \ldots(1)$$

Where h is the peak enthalpy (J/g). ΔH_{fus} is the latent heat of fusion of ice (334 J/g) and M is g water/g sample.

$$\%\text{Unfreezable water} = \%\text{moisture} - \%\text{freezable water} \quad \ldots(2)$$

Amylopectin Recrystallization: Amylopectin recrystallization was monitored using DSC technique [13]. An endothermic peak transition around 40-70 ^{o}C is attributed to the heat required to melt amylopectin crystallite [13]. The degree of amylopectin recrystallization was reported in terms of peak enthalpy per g sample.

Water-Soluble Starch Content: Water-soluble starch content was determined according to the method described previously [15].

Crumb Hardness: Texture profile analysis of bread crumb was carried out using a universal materials testing machine (Instron, Norwood, MA). A 2×2×2 cm^3 sample cube was compressed using a 6 cm-diameter cylindrical probe with a cross-head speed of 1.0 mm/s until 70% deformation was achieved. Hardness was obtained from the maximum force of the first penetration.

2.5 Statistical Analysis

Experiments were done in triplicate. A completely randomized design was used. Data were analyzed using Analysis of Variance. A Duncan's new multiple range test was used to determine the difference among sample means at p=0.05.

3. Results and Discussion

3.1 Moisture Content

Moisture content of the bread samples was illustrated in Figure 1. Freshly baked soy-containing breads (Day 0) contained more moisture than the all-wheat control did. Upon storage for a period of 7 days, moisture content of all bread samples decreased in a similar fashion, but the decrease was slightly more pronounced in the control. Moisture reduction of bread during storage has been attributed to moisture migration from the crumb to the crust and also from bread to the surrounding atmosphere [2]. The presence of soy ingredients resulted in an increased water-holding capacity due to their high fiber content [13].

3.2 Freezable and Unfreezable Water Contents

In the current study, it was observed that onset temperature of ice melting in the bread samples was in the range of -14.4 to -12.4 ^{o}C. Freezable and unfreezable water contents of the bread samples are depicted in Figures 2 and 3.

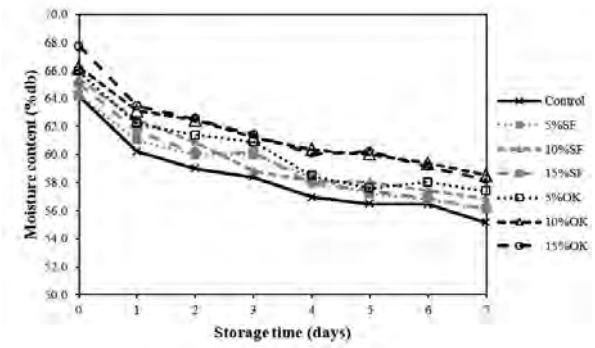

Figure 1 Moisture content of wheat (control), soy flour-substituted (SF) and okara-substituted (OK) breads during 7-day storage

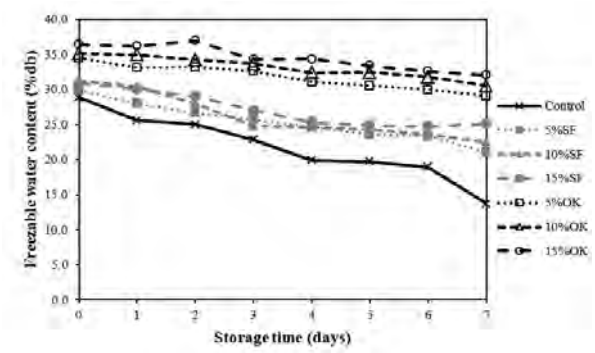

Figure 2 Freezable water content of wheat (control), soy flour-substituted (SF) and okara-substituted (OK) breads during 7-day storage

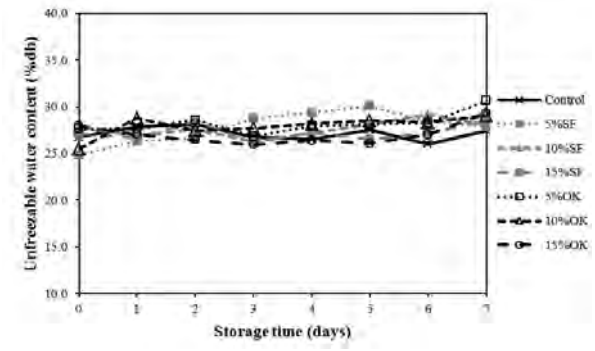

Figure 3 Unfreezable water content of wheat (control), soy flour-substituted (SF) and okara-substituted (OK) breads during 7-day storage

Freshly baked control bread contained 28.9% freezable water on a dry basis (Figure 2). Upon substituting with SF or OK, freezable water content became significantly increased ($p \leq 0.05$), with the OK samples showing higher freezable water content than the SF ones. Freezable water content increased with increasing level of soy substitution. This was in consistent with the higher moisture content of the soy-containing breads.

Freezable water content in all samples became decreasing with increasing storage time. The control exhibited greater reduction in freezable water over time than the soy-containing breads did. This was due to the fact that soy ingredients helped promote homogeneous moisture distribution throughout the bread loaf and the minimal changes in freezable water could be attributed to the low degree of moisture migration in soy-containing bread during storage [16].

In the case of unfreezable water (Figure 3), the content in all freshly baked breads was not significantly different (p>0.05). In addition, the unfreezable water content also remained relatively constant during 7-day storage. This was in good agreement with previous study [13] which suggested that the freezable water, which is more available, was the fraction undergoing detectable changes during storage.

3.3 Amylopectin Recrystallization

Amylopectin crystallite was monitored using a DSC technique. In the current study, an endothermic peak corresponding to the melting of amylopectin crystallite was observed in the temperature range of 65-80 ^{o}C. The energy required to melt amylopectin crystallite was illustrated in Figure 4.

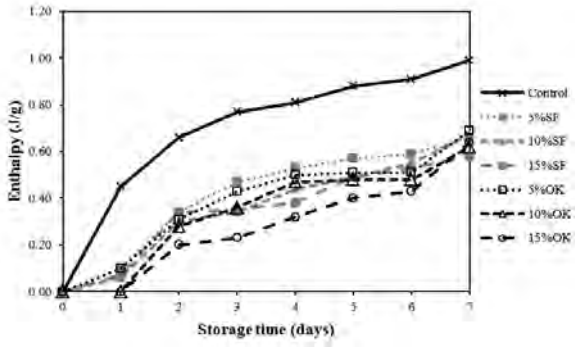

Figure 4 Melting enthalpy of amylopectin crystallite in wheat (control), soy flour-substituted (SF) and okara-substituted (OK) breads during 7-day storage

For all freshly baked samples, no endothermic peak was observed in the amylopectin crystallite melting region. This indicated negligible amount of amylopectin recrystallization in freshly baked breads. With increasing storage time, the melting enthalpy was found to increase, with the most pronounced in the control, followed by the 5%-substituted, 10%-substituted and 15%-substituted samples, respectively. The 15% OK exhibited the lowest amylopectin recrystallization throughout the storage time studied. Generally, OK sample possessed slightly lower degree of amylopectin recrystallization as compared to the SF sample of the same substitution level. Amylopectin recrystallization seemed to rapidly occur during the first two days of storage and the recrystallization rate became leveling off after that.

Previous study [3] suggested that there was a correlation between the degree of amylopectin recrystallization and crust-crumb moisture gradient. Soy ingredients helped promote homogeneous moisture distribution, thus minimizing moisture gradient within the loaf of bread. Besides, the high water-holding capacity of soy components (i.e., soy protein and soy fiber) resulted in less water available for starch component, hence lowering starch recrystallization during storage.

Apart from water-holding and moisture-distributing effect of soy ingredients, it was also proposed that soy components strongly interacted with starch, hence interfering starch-starch interaction and retarding amylopectin recrystallization [17].

3.4 Water-Soluble Starch Content

As starch retrogrades, starch molecules (i.e., amylose and amylopectin) become re-associate via hydrogen bonding with a formation of ordered crystalline structure. The increased crystallinity results in lowered solubility. Water-soluble starch content of the bread samples was shown in Figure 5.

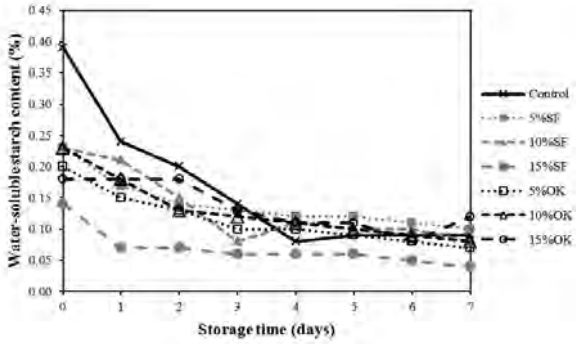

Figure 5 Water-soluble starch content of wheat (control), soy flour-substituted (SF) and okara-substituted (OK) breads during 7-day storage

Freshly baked control bread contained the highest water-soluble starch. Upon substituting with SF or OK, water-soluble starch content was lowered significantly (p≤0.05). All samples exhibited a decrease in water-soluble starch content with increasing storage time but the change was higher in the control. This decrease in water-soluble starch content suggested an increase in the formation of water-insoluble entity or the crystalline structure. This was also in consistent with the increase in amylopectin recrystallization discussed above.

3.5 Crumb Hardness

Changes in crumb hardness during 7-day storage were shown in Figure 6. All samples exhibited increasing crumb hardness over time. As compared to the control, a lesser increase in crumb hardness was observed in the soy-containing samples, implying that incorporation of soy ingredients could help retard bread staling. However, it should be noted that, with soy substitution, particularly at high levels of OK-substitution, freshly baked bread was harder in texture

than the control. This was due to the high fiber content of okara.

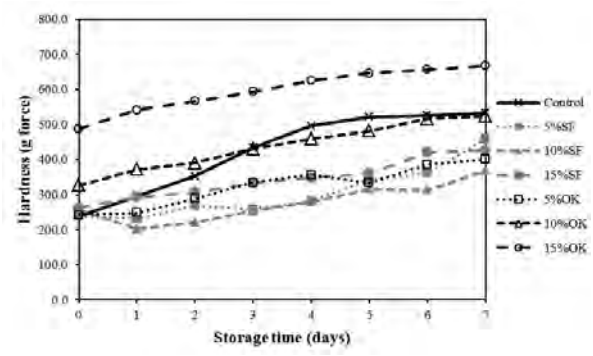

Figure 6 Hardness of wheat (control), soy flour-substituted (SF) and okara-substituted (OK) bread crumbs during 7-day storage

4. Conclusions

The role of soy ingredients, namely soy flour and okara, in retarding starch retrogradation and bread staling was demonstrated. This was due to the moisture-retaining ability of the soy components, protein and fiber, thus promoting homogeneous moisture distribution and minimizing moisture gradient within the loaf of bread. The high water-holding capacity of soy components also resulted in less water available for starch component. In addition, soy components could strongly interact with starch and interfere with starch-starch interaction, resulting in retrogradation-retarding effect.

Acknowledgements

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References

[1] M.E. Bárcenas and C.M. Rosell, *J. Food Eng.* **72** (2006) 92-99.
[2] M.Y. Baik and P. Chinachoti, *Cereal Chem.* **77** (2000) 484-488.
[3] M.Y. Baik and P. Chinachoti, *J. Agric. Food Chem.* **49** (2001) 4031-4038.
[4] W.J. Wolf, *J. Agric. Food Chem.* **18** (1970) 969-976.
[5] J. De Meester, S. Kempener and P. Mollee, *Ind. Proteins* **8** (2000) 5-7.
[6] I. Taruna and V.K. Jindal, *Dry. Technol.* **20** (2002) 1035-1051.
[7] S. Wachiraphansakul and S. Devahastin, *Dry. Technol.* **23** (2005) 1229-1242.
[8] D.K. O'Toole, *J. Agric. Food Chem.* **47** (1999) 363-371.
[9] Ministry of Public Health, *Food Additive Act, Notification of Ministry of Public Health No. 84 (B.E. 2527).*
[10] W.F. Wilkens, L.R. Mattick and D.B. Hand, *Food Technol.* **21** (1967) 1630-1633.
[11] http://www.dianadesserts.com/ (Retrieved May 5, 2010).
[12] AOAC, *Official Methods of Analysis, 17th ed.*, the Association of Analytical Chemists, Washington, D.C. (2000).
[13] E. Vittadini and Y. Vodovotz, *J. Food Sci.* **68** (2003) 2022-2027.
[14] Y. Vodovotz, I. Hallberg and P. Chinachoti, *Cereal Chem.***73** (1996) 264-270.
[15] I.M. Shaikh, S.K. Ghodke and L. Ananthanarayan, *Food Chem.* **101** (2007) 113-119.
[16] A. Lodi and Y. Vodovotz, *Food Chem.* **110** (2008) 554-561.
[17] K.J. Ryan, C.L. Homco-Ryan, J. Jenson, K.L. Robbins, C. Prestat and M.S. Brewer, *J. Food Sci.* **67** (2002) 2385-2390.

EFFECT OF BINDING AGENTS AND TRANSGLUTAMINASE TREATMENT ON PROPERTIES OF FISH BALL PRODUCT

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Abstract: The objective of this study was to examine the effect of different binding agents, namely wheat flour (WF), soy protein isolate (SPI) and the mixture of both, as well as transglutaminase treatment on properties of fish ball product which contained 1.25 or 1.50% by weight of sodium chloride. To study the effect of binding agents, minced fish in the fish ball recipe was substituted with binding agent at 2% by weight. For the effect of transglutaminase treatment, microbial transglutaminase (MTGase) was added at 0.6% level. Among the binding agents studied, SPI was found to significantly improve gel strength, hardness, cohesiveness, springiness and water holding capacity of the product ($p \leq 0.05$). On the other hand, transglutaminase treatment was shown to improve gel strength, hardness, cohesiveness, springiness and water holding capacity of the product. Using SPI and/or WF together with MTGase produced fish ball product with increased gel strength. Neither binding agent nor transglutaminase treatment posed an effect on whiteness of the fish ball. Upon MTGase addition, an increase in protein cross-linking was confirmed by the SDS-PAGE pattern which revealed a decrease in intensity of the bands represented myosin heavy chain (205 kDa) and actin (45 kDa) of the fish protein with a concomitant increase in the band intensity of protein with molecular weight greater than 205 kDa.

1. Introduction

Texture is among the foremost quality attributes of fish ball product. High quality fish balls can be obtained from certain types of fish such as cod and haddock. In Thailand, clown knifefish is considered prime raw material since it gives fish ball with desirable textural quality. Due to high demand, the cost of clown knifefish is relatively high compared to other fish.

Isok barb, or Jullien's golden carp, is a freshwater fish distributed in Mekong, Chao Phraya, Nan, Ta Jeen and Meklong basins of Thailand. At present, the Department of Fisheries supports the aquaculture of Isok barb in a commercial scale. The use of Isok barb for fish ball production is growing in volume because of its incentive price. However, the quality of Isok barb fish ball is still inferior as compared to that made from clown knifefish. Manufacturers have therefore been seeking for techniques for improving texture of fish ball produced from Isok barb, as well as other moderately-priced fish which, by themselves, give a product with merely moderate textural quality.

Tapioca flour is the most common binder used to improve fish ball texture. However, often times, experienced consumers could differentiate between the fish ball made from only minced fish and those added with tapioca flour. Flour-containing fish balls are usually lower in their hardness, cohesiveness and springiness as compared to the all-meat product.

Various proteins are known for their gelation ability. Many protein gels are high in cohesiveness and springiness. Wheat flour contains about 13% protein [1]. Wheat gluten is known for providing strong gel and could therefore help improve gel strength of the fish ball. In addition, the starch could also act as stabilizer hence improving the gel texture. Soy protein isolate, on the other hand, contains mostly protein (≥90% protein). Soy protein gel has been praised for its excellent strength and cohesiveness and could also be used to improve texture of various food products.

Owing to its ability to catalyze the cross-linking between glutamine and lysine residues of a protein, transglutaminase has been successfully used to improve a myriad of properties that are related to the degree of protein-protein interaction. Transglutaminase treatment could therefore be of advantage in improving texture of protein foods, such as fish ball.

This study aimed to investigate the effect of wheat flour and/or soy protein isolate fortification and transglutaminase treatment on properties of fish ball made from Isok barb.

2. Materials and Methods

2.1 Materials

Fresh Isok barb was purchased from a local market (Bangkok, Thailand), ice-packed in an insulated container and immediately transported to the laboratory.

Wheat flour (WF) (United Flour Mill, Bangkok, Thailand) and soy protein isolate (SPI) (Mighty International, Bangkok, Thailand) were commercial products. Microbial transglutaminase (MTGase) (ACTIVA^{TM} TG-AK) was kindly provided by Ajinomoto (Bangkok, Thailand).

2.2 Fish Ball Preparation

Upon arrival at the laboratory, the fish was headed, gutted and washed with chilled water. After that, it was hand-filleted to obtain only white muscle meat and then minced. The minced fish was then processed into fish ball according to the method of the Department of Fisheries [2] with some modifications as detailed in Figure 1.

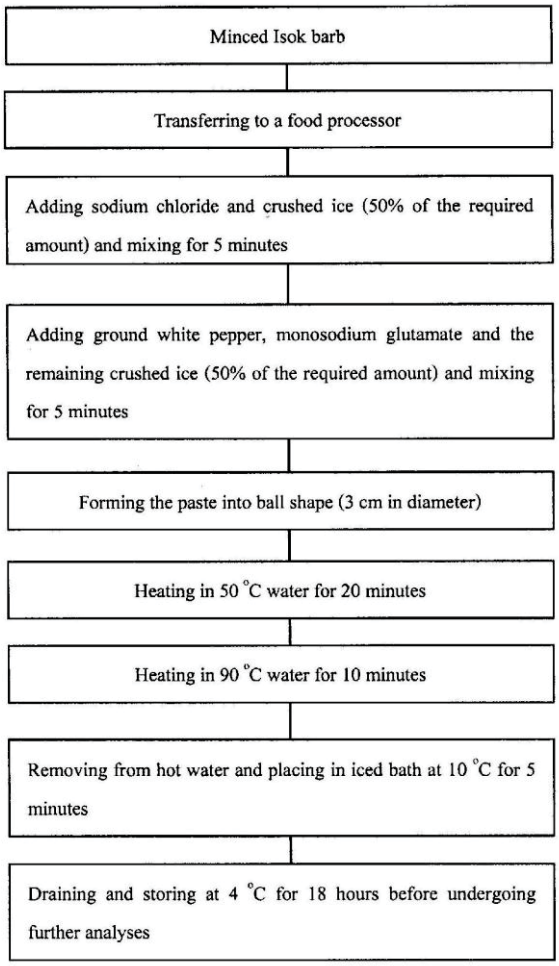

Figure 1 Production flowchart for control fish ball

Sodium chloride was added at either 1.25 or 1.50%. For those samples with WF, SPI and 1:1 WF/SPI mixture, the binder was used to replace the minced fish at 2% by weight. For the effect of transglutaminase treatment, MTGase was added at 0.6% level.

2.3 Evaluation of Fish Ball Properties

Gel Strength: Measurement of the gel strength of fish ball was carried out using a universal materials testing machine (Instron, Norwood, MA). The sample was compressed using a 6 mm-diameter ball probe with a cross-head speed of 1.0 mm/s until 75% deformation was obtained.

Texture Profile Analysis: Texture profile analysis of the fish ball was carried out using a universal materials testing machine (Instron, Norwood, MA). The sample was compressed twice using a 56 mm-diameter cylindrical probe with a cross-head speed of 1.0 mm/s until 50% deformation was obtained. Hardness, cohesiveness and springiness was extracted from the texture profile.

Water Holding Capacity: To determine water holding capacity, the fish ball was cut into a 1×1×2 cm^3 and put into a centrifuge tube with two pieces of 3×3 cm^2 Whatman No.1 filter paper placing over and three pieces placing under the sample piece. The tube was centrifuged at 1500×g for 5 minutes. Water holding capacity was reported in terms of %expressible water, calculated using Equation (1):

$$\%\text{Expressible water} = (W_f\text{-}W_i)100/W_s \quad ...(1)$$

Where W_f is the weight of filter papers after centrifugation; W_i is the weight of filter papers before centrifugation and W_s is the sample weight.

Whiteness: Color was measured using ColorFlex® color meter system (Hunter Associates Laboratory, Reston, VA). The measurement was done in the CIELAB color system using D65 light source. Whiteness was calculated using Equation (2) [3]:

$$\text{Whiteness} = 100\text{-}[(100\text{-}L^*)^2+a^{*2}+b^{*2}]^{1/2} \quad ...(2)$$

Where L^* is the lightness coordinate, a^* it the red/green coordinate and b^* is the yellow/blue coordinate.

2.4 SDS-PAGE

Three grams of fish ball sample were solubilized with 27 mL of solution containing 5% sodium dodecyl sulfate and 0.1% 2-mercaptoethanol. The sample was centrifuged at 17,500×g for 20 minuters to remove undissolved debris. The supernatant was determined for its protein content using a modified Lowry method [4]. The supernatant (0.3 mL) was mixed at 1:3 (v/v) ratio with sample buffer (60 mM Tris-HCl, pH 6.8 containing 2% SDS, 25% glycerol, 14.4 mM 2-mercaptoethanol and 0.1% bromophenol blue) and boiled for 2 minutes. Ten μL of the sample were subjected to SDS-PAGE. The electrophoresis was conducted using a Hoefer mini VE gel electrophoresis unit (Amersham Pharmacia Biotech, Uppsala, Sweden) and stained with Coomassie Brilliant Blue R-250 solution [5].

2.5 Statistical Analysis

Experiments were done in triplicate. A completely randomized design was used. Data were analyzed using Analysis of Variance. A Duncan's new multiple range test was used to determine the difference among sample means at p=0.05.

3. Results and Discussion

3.1 Gel Strength

Gel strength of the fish ball samples was illustrated in Figure 2. It was found that incorporation of a binder (WF, WF/SPI or SPI) helped increase gel strength of the fish ball at both levels of sodium chloride used. Among the binders studied, SPI seemed to be most effective in terms of improving gel strength of the fish ball.

With transglutaminase treatment, gel strength of the fish balls increased to even higher value, as compared to the non-treated sample (Figure 2). This was due to the protein cross-linking ability of MTGase [6, 7, 8]. The sample added with SPI and treated with MTGase exhibited the highest gel strength.

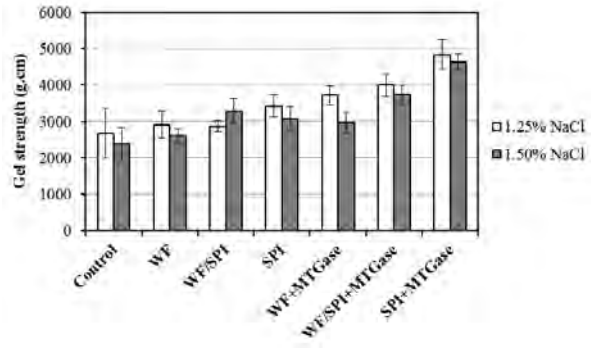

Figure 2 Gel strength of fish balls as affected by binder addition and transglutaminase treatment

3.2 Texture Profile Parameters

Hardness, cohesiveness and springiness as obtained from texture profile analysis of the fish ball samples were shown in Figures 3, 4 and 5.

Hardness was found to be greatly affected by binder addition and transglutaminase treatment (Figure 3). Hardness became increasing with the addition of binder or MTGase, with the sample added with SPI and treated with MTGase exhibited the highest hardness.

Even to a lesser extent as compared to hardness, cohesiveness (Figure 4) and springiness (Figure 5) also increased upon addition of binder or MTGase.

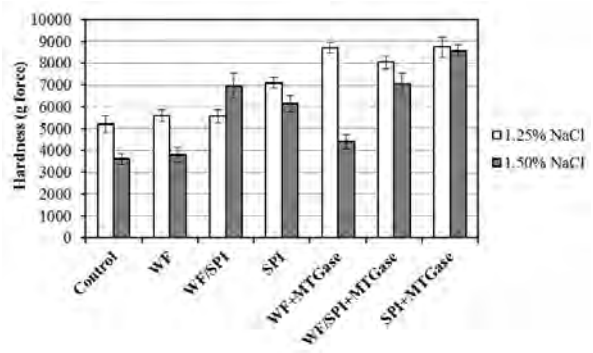

Figure 3 Hardness of fish balls as affected by binder addition and transglutaminase treatment

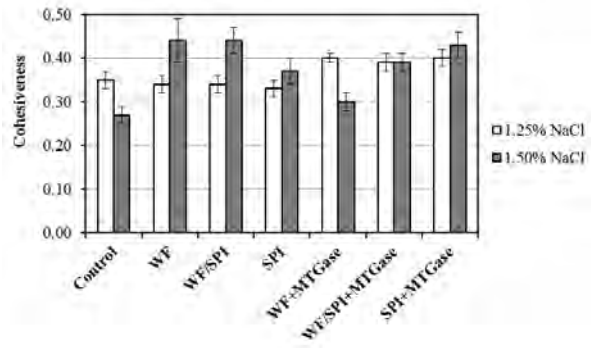

Figure 4 Cohesiveness of fish balls as affected by binder addition and transglutaminase treatment

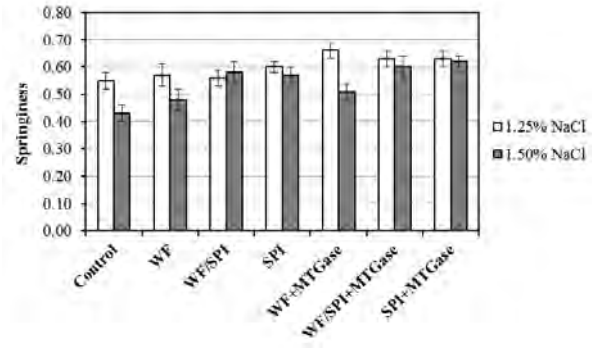

Figure 5 Springiness of fish balls as affected by binder addition and transglutaminase treatment

3.3 Water Holding Capacity

Water holding capacity of the fish balls was reported in terms of expressible water (Figure 6). A sample with high water holding capacity will result in lower amount of expressible water upon centrifugation. It was found that both binder and MTGase helped decrease the amount of expressible water significantly. This was due to the fact that binder and MTGase helped increase the strength of gel network thus increasing it water holding capacity [9].

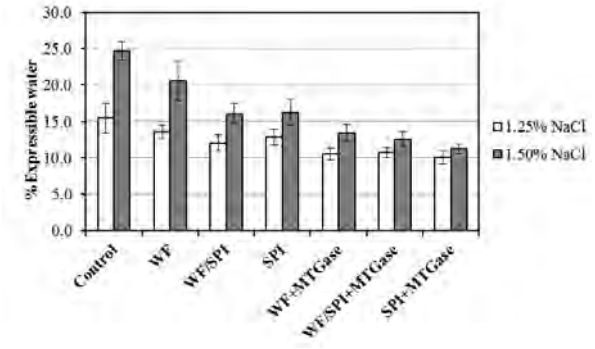

Figure 6 Expressible water of fish balls as affected by binder addition and transglutaminase treatment

3.4 Whiteness

Whiteness of the fish ball samples was shown in Figure 7. Addition of WF, SPI and WF/SPI mixture was found to pose no significant effect on whiteness of the product. This may be due to the low amount of the binders used. A former study [10] reported that use of SPI in the amount greater than 4% resulted in an increase in yellowness of low-fat bologna.

Transglutaminase treatment, on the other hand, did not affect whiteness of the fish ball. The observed effect was similar to previous reports [8, 11].

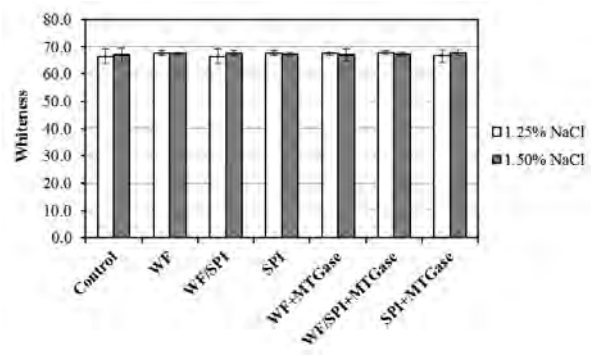

Figure 7 Whiteness of fish balls as affected by binder addition and transglutaminase treatment

3.5 SDS-PAGE

The SDS-PAGE protein pattern of the fish ball samples was shown in Figure 8.

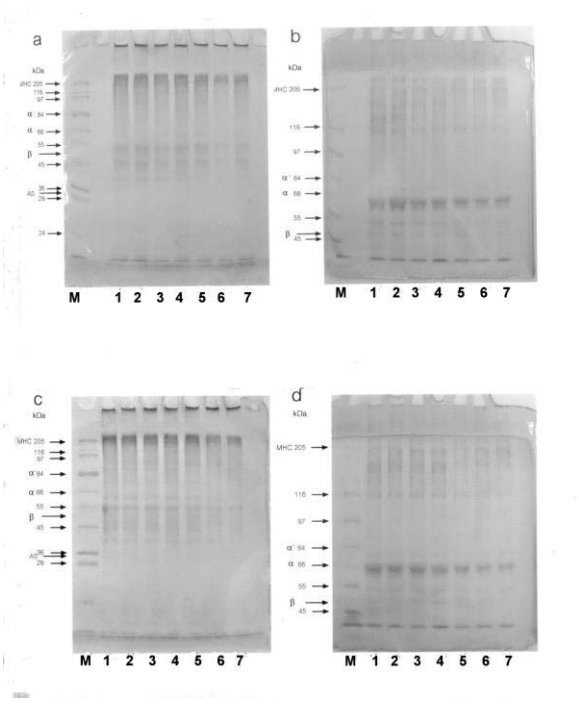

Figure 8 SDS-PAGE protein pattern of (a) 1.25% NaCl fish ball, analyzed using 10% separating gel, (b) 1.25% NaCl fish ball, analyzed using 7.5% separating gel, (c) 1.50% NaCl fish ball, analyzed using 10% separating gel, and (d) 1.50% NaCl fish ball, analyzed using 7.5% separating gel. M represents protein marker; Lane 1 was the control; Lanes 2, 3, 4 were the fish ball samples containing WF, WF/SPI and SPI, respectively; Lanes 5, 6, 7 were the fish ball samples containing WF, WF/SPI and SPI, with added MTGase, respectively. MHC represents myosin heavy chain (205 kDa) of the fish protein. α′, α, and β represent the α′, α, and β subunits of soy β-conglycinin. AS represents the acidic subunit of the soy glycinin.

It was observed that the band intensity of the fish myosin heavy chain (MHC) (205 kDa) (Figure 8a and 8c) as well as that of the fish actin (45 kDa) (Figure 8b and 8d) were not affected by the addition of binders.

In contrast, upon transglutaminase treatment, the bands represent the fish MHC (Figure 8a and 8c) and actin (Figure 8b and 8d) noticeably decreased in intensity. At the same time, there was also an increase in the band intensity of the proteins with molecular weight greater than 205 kDa, shown as darker band on top of the stacking gel in Figures 8a and 8c. These changes indicated the increase in protein cross-linking due to the action of MTGase.

4. Conclusions

Binders, especially soy protein isolate, were proved to be effective in improving gel strength, hardness, cohesiveness, springiness and water holding capacity of the fish ball. Transglutaminase treatment was shown to increase protein cross-linking, thus improving gel strength, hardness, cohesiveness, springiness and water holding capacity of the product. On the other hand, whiteness of the fish ball was not affected by addition of binders or transglutaminase.

Acknowledgements

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References

[1] http://ndb.nal.usda.gov/ (Retrieved June 2, 2012)
[2] http://www.fisheris.go.th/ (Retrieved October 25, 2011)
[3] T.C. Lanier, in: T.C. Lanier, C.M. Lee (Eds.), *Surimi Technology*, Marcel Dekker, New York, (1992), pp. 23-58.
[4] G.L. Peterson, *Meth. Enzymol.* **91** (1983) 95-119.
[5] U.K. Laemmli, *Nature* **227** (1970) 680-685.
[6] I. Cohen, L. Young-Bandala, T. Blankenberg, A.G.E. Siefring and J. Bruner-Lorand, *Arch. Biochem. Biophys.* **192** (1979) 100-111.
[7] D.R. Kahn and I. Cohen, *Biochim. Biophys. Acta* **668** (1981) 490-494.
[8] T.F. Tseng, D.C. Liu and M.T. Chen, *Meat Sci.* **55** (2000) 427-431.
[9] J.Y. Imm, P. Lian and C.M. Lee, *J. Food Sci.* **65** (2000) 200-205.
[10] K.B. Chin, J.T. Keeton, M.T. Longnecker and J.W. Lamkey, *Meat Sci.* **53** (1999) 45-57.
[11] B. Kilic, *Meat Sci.* **63** (2003) 417-421.

EFFECT OF COAGULANTS ON SOLUBILITY AND EMULSIFYING PROPERTIES OF PROTEIN CONCENTRATES FROM MUNG BEAN AND ADZUKI BEAN

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Abstract: This research was aimed at studying the effect of coagulants (hydrochloric acid and citric acid) on solubility and emulsifying properties of protein concentrates from mung bean (*Vigna radiata* (L.) Wilczek) and adzuki bean (*Vigna angularis*). Both mung bean protein concentrate (MPC) and adzuki bean protein concentrate (APC) were prepared using isoelectric precipitation method. The pH of suspension was adjusted to 5.0 and then 4.5, respectively. The obtained protein precipitates were adjusted to pH 7.0 and were dried in a tray dryer at 55 ^{O}C for 8 hours. The minimum solubility of MPC and APC was found at the pH range of 4.0-5.0. The protein solubility increased when the pH was higher or lower than the pH range of 4.0-5.0. However, APC exhibited better solubility than the MPC at all pH studied ($p \leq 0.05$). Protein concentrates from both beans precipitated with hydrochloric acid were more soluble compared to samples treated with citric acid. The highest solubility of MPC and APC treated with hydrochloric acid was found at pH 10 with the value of 80.12% and 91.59%, respectively while that of the MPC and APC treated with citric acid was also found at the same pH with the value of 77.40% and 86.36%, respectively. The water-holding capacity (WHC) of protein concentrates was determined at various pH (3.5, 5.5 and 7.5). It was found that, at the pH studied, MPC and APC treated with hydrochloric acid showed a higher WHC than samples treated with citric acid. The emulsifying property was influenced by type of coagulants. Protein concentrates treated with hydrochloric acid exhibited higher emulsifying activity index but lower emulsifying stability index than those treated with citric acid. The difference in functional properties of MPC and APC prepared using different coagulants may come from the chemical composition of protein concentrates.

Keywords: Protein concentrates; Mung bean; Adzuki bean; Coagulants; Solubility

1. Introduction

Protein concentrates, containing a minimum of 70% protein, are produced from various sources of food such as legumes, cereals and whey. Protein concentrates can be prepared from various processes, for example, acid precipitation, extraction with 20-80% aqueous ethanol and moist heat [1]. One of the most popular techniques is the precipitation with coagulants at isoelectric point of proteins. Legume proteins have the isoelectric point at a pH range of 4.0-5.0 [2]. The commonly used coagulants are acid solutions. They can be either organic acids such as citric acid and acetic acid or inorganic acids, for instance, hydrochloric acid and sulfuric acid [3].

Using different coagulants affect chemical compositions and functional properties of the obtained protein concentrates. It was found that potato protein concentrate precipitated with hydrochloric acid contained higher protein and lipid content than protein concentrate treated with citric acid [4]. In mung bean protein precipitation, using hydrochloric acid gave higher amount of protein than citric acid [5]. Furthermore, coagulants also affect protein functionality. The functional properties of proteins mean those physicochemical properties affecting the behavior of proteins in food systems during preparation, processing, storage and consumption [6]. The main functional properties are the solubility, water holding capacity, oil binding capacity, foaming and emulsifying property. Wongpratheep and Pukruspan [5] reported that mung bean protein concentrate treated with hydrochloric acid exhibited better solubility and emulsifying property than protein concentrate treated with citric acid

Since, proteins from legumes are increasingly interesting as ingredient in food products. Protein concentrate prepared via coagulant precipitation is considered as simple and convenient technique. Thus, this research is aimed at studying the effect of coagulants (hydrochloric acid and citric acid) on functional properties of protein concentrates from mung bean and adzuki bean.

2. Materials and Methods

2.1 Materials

Mung bean from Raitip® brand and adzuki bean from Royal Project Foundation were ground using Soybean grinder (NSB-6, Lita Hatsuyuki, Bangkok, Thailand). Flours from both beans were then passed to 100-mesh sieve and packed in aluminum foil laminated bag.

2.2 Preparation of mung bean and adzuki bean protein concentrates

Each flour (mung bean and adzuki bean) was dispersed in distilled water by 1:10 (w/v) ratio. The slurry was adjusted to pH 9.5 with 1 M NaOH and kept at room temperature (30±2 ^{O}C) for 1 hour. It was then centrifuged at 3,500x*g* for 20 min. The

Table 1 : Chemical composition of mung bean and adzuki bean protein concentrates treated with HCl and citric acid

Chemical composition	MPC-HCl	MPC-CA	APC-HCl	APC-CA
moisture	5.17^{b}±0.31	5.14^{b}±0.29	5.83^{a}± 0.31	5.69^{a}±0.11
lipid	1.10^{c}±0.11	0.84^{c}±0.14	1.61^{a}± 0.27	1.43^{a}±0.48
protein	80.67^{a} ±0.35	77.71^{b}±1.34	73.70^{c}±0.81	71.45^{d}±1.53
ash	4.38^{b}±0.28	4.89^{a}±0.14	4.40^{b}±0.07	4.69^{a}±0.18
carbohydrate†	8.68^{d}±0.45	11.42^{c}±1.12	14.46^{b}±0.94	16.74^{a}±1.88

* (a,b,c,d):Different superscript letters in the same row indicate significant difference ($p \leq 0.05$).
† Carbohydrate content is calculated from 100 – (moisture -lipid - protein – ash)

supernatant was adjusted to pH 5.0, holding about 15 min, and then to pH 4.5 with two different coagulants (6 M HCl and 6 M citric acid). The obtained slurry was then centrifuged at 3,500x*g* for 20 min. The obtained protein precipitate was adjusted to pH 7.0 using 1 M NaOH. The sample was dried in a tray dryer at 55 ^{O}C for 8 hours. Dried protein (moisture content < 6%) was passed to 100-mesh sieve and filled in aluminum foil laminated bag and stored at room temperature (30±2 ^{O}C).

2.3 Chemical composition analysis

Four types of protein concentrates, mung bean protein concentrate treated with either HCl or citric acid (MPC-HCl and MPC-CA) and adzuki bean protein concentrate treated with either HCl or citric acid (APC-HCl and APC-CA), were determined for their moisture, lipid, protein (conversion factor 6.25) and ash content according to the standard methods of analysis [7]. Carbohydrate content was calculated as follows: Carbohydrate content = 100 – (moisture - lipid - protein – ash)

2.4 Functional properties

2.4.1 Protein solubility

The protein solubility was determined according to the method of Adebowale and Lawal [8] with a slight modification. Protein concentrate was dissolved in distilled water to obtain 1% (w/v) solution. The pH of the suspension was adjusted with 1 M HCl or 1 M NaOH to cover the range of 2 to 10. The suspension was stirred for 1 hour at room temperature (30±2 ^{O}C) and then centrifuged at 4,000x*g* for 15 min. The protein content of the supernatant was determined using the Lowry's method [9]. The percentage of protein solubility was calculated as follows: Protein solubility (%) = (amount of protein content in the supernatant)/(amount of protein content in the sample) x 100

2.4.2 Water holding capacity (WHC)

Water holding capacity was determined according to the method of Quinn and Paton [10]. Sample (1 g) was added to 10 ml of distilled water. The pH of the suspension was adjusted with 0.1 M HCl or 0.1 M NaOH. After being stirred for 1 min, the suspension was then centrifuged at 8,200x*g* for 15 min. Three different pH values (3.5, 5.5 and 7.5) were tested. The supernatant was carefully removed and the sample was weighed. The water holding capacity was expressed as gram of water per gram of the sample.

2.4.3 Emulsifying properties

The emulsifying activity index (EAI) and emulsifying stability index (ESI) of the protein concentrates were determined according to the method of Pearce and Kinsella [11]. The emulsion was diluted (1:400 v/v) with 0.1% (w/v) sodium dodecyl sulfate. The absorbance was measured at 500 nm. EAI and ESI were calculated as follows: EAI (m2/g) = (2 x 2.303 x A_0 x dilution factor)/(c x $\emptyset$ x 10000), ESI (min) = (τ x Δt)/$\Delta\tau$ where c is the initial concentration of protein (g/ml), $\emptyset$ is the fraction of oil used to form the emulsion (0.25), A_0 is the absorbance of diluted emulsions, τ is the turbidity at A_0, and $\Delta\tau$ is the change in turbidity after a time interval (60 min), respectively.

2.5 Statistical analysis

All experiments in this study were reported as mean and standard deviation of three replicates. Analysis of variance (ANOVA) was carried out to compare the mean values. Differences in the mean values were determined using Duncan's multiple range tests (SPSS v.11 for Windows®, SPSS Inc., Chicago, IL).

3. Results and Discussion

3.1 Chemical composition

The chemical composition of four protein concentrates is shown in Table 1. The protein concentrates from same type of bean exhibited no significant difference ($p > 0.05$) in moisture and fat contents but the significant difference ($p \leq 0.05$) was found in protein, ash and carbohydrate contents. MPC-HCl and APC-HCl contained considerably higher protein content but lower ash and carbohydrate content than MPC-CA and APC-CA. Using HCl, a strong acid, creates more dissociated intermolecular forces between protein molecules in the protein bodies attached to starch granules than citric acid, a weak acid, does. So protein molecules are better promoted for coagulation and precipitation in the precence of HCl [3,12]. These results corresponded to Wongpratheep and Pukruspan [5] who reported that mung bean protein concentrate treated with HCl

exhibited significantly higher protein content than protein concentrate treated with citric acid ($p \leq 0.05$). Knorr [4] also found that, in potato protein precipitation, using HCl showed higher protein content than citric acid.

Mung bean protein concentrates showed higher protein content but less carbohydrate content than adzuki bean protein concentrates. It was because proteins in adzuki bean are tightly bound to starch structure compared to that in mung bean [13]. This leads to the difficulty in protein extraction.

3.2 Functional properties

3.2.1 Protein solubility

Protein solubility at different pH (2-10) of four protein concentrates is shown in Figure 1. The pH solubility curves obtained for four protein concentrates exhibited minimum value in the range of 4.0 to 5.0. The protein solubility increased when the pH was higher or lower than this pH range. The minimum solubility for most plant proteins is at their isoelectric point at pH 4.0-5.0 [14]. At isoelectric point, net charge on the proteins is close to zero. As a result, there are no repulsive interactions, protein precipitation occurs. At low pH, positive net charge of proteins is induced, while the proteins at high pH are conducted to the negative net charge. Thus, repulsive forces increase, resulting in more solubility of those proteins [15]. This observation is in good agreement with those reported for soy protein concentrate [2], adzuki bean protein isolate [16] showing the minimum solubility at a pH range of 4.0 to 5.0.

MPC-HCl and APC-HCl exhibited better solubility than MPC-CA and APC-CA (Figure 1a and 1b). The highest solubility of MPC-HCl and APC-HCl was found at pH 10 with the value of 80.12% and 91.59%, respectively while that of the MPC-CA and APC-CA was also found at the same pH with the value of 77.40% and 86.36%, respectively. Protein concentrates treated with citric acid had lower solubility. It may be due to the increasing of the hydrophobic amino acid contents on surface of protein molecules [4]. This results in lower protein-water interaction than in protein concentrates treated with HCl.

Protein concentrates from adzuki bean showed higher solubility than that from mung bean at all pH studied ($p \leq 0.05$). It might be possible that protein solubility is affected by the hydrophilic-lipophilic balance of amino acids on the protein molecules [17]. The glutamic and aspartic acids contents in the adzuki bean were reported to be higher in the mung bean [18]. Both amino acids showed negative net charge at a pH higher than pI, thus increasing the protein-water interaction in adzuki bean protein.

3.2.2 Water holding capacity (WHC)

In Table 2, no significant difference ($p > 0.05$) was found in water holding capacity (WHC) of four protein concentrates at pH 3.5 whereas the significant difference ($p \leq 0.05$) was found in WHC of four samples at pH 5.5 and 7.5. All samples showed the highest WHC at pH 7.5; the lowest WHC was at pH 5.5. pH 5.5 was found to be close to isoelectric point of protein concentrates (4.0-5.0, Figure 1). At this pH, it led to a decrease in the formation of hydrogen bonding between water molecules and protein molecules at pH 5.5.

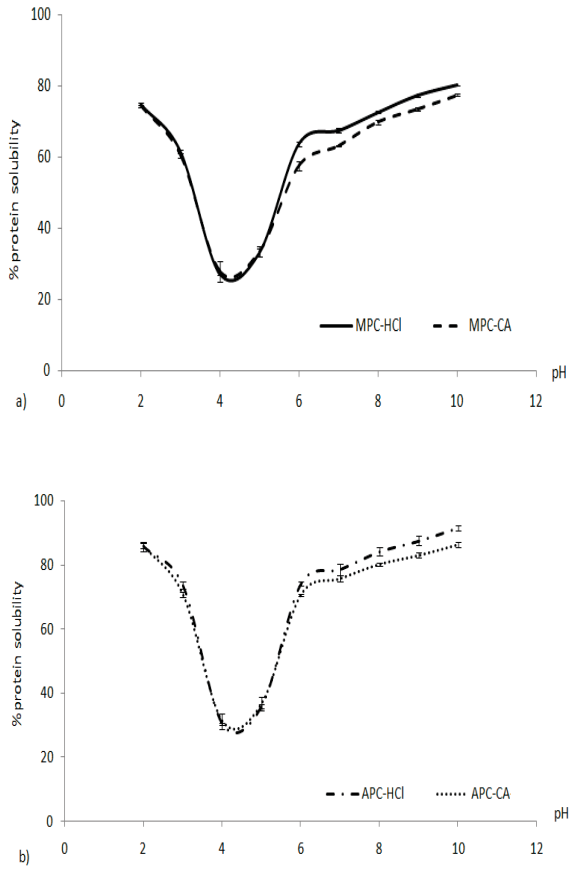

Figure 1. Protein solubility (%) of (a) mung bean protein concentrate and (b) adzuki bean protein concentrate treated with HCl and citric acid at pH-range 2-10

MPC-HCl and APC-HCl showed higher WHC than MPC-CA and APC-CA because the protein content of protein concentrates treated with HCl was higher than protein concentrates treated with citric acid, so they could bind more water. Kinsella [6] reported that water holding capacity increased with increasing protein concentration. These results were agreed well with Wongpratheep and Pukruspan [5] who reported that mung bean protein concentrate treated with HCl exhibited significantly higher WHC than protein concentrate treated with citric acid ($p \leq 0.05$).

Adzuki bean protein concentrates exhibited better WHC than mung bean protein concentrate because protein concentrates from adzuki bean possess higher amount of polar or hydrophilic amino acid compared to mung bean [19]. It resulted in an increase in the

amount of water remaining within the protein molecules by forming hydrogen bonding.

Table 2 : Water holding capacity of mung bean and adzuki bean protein concentrates treated with HCl and citric acid at pH 3.5, 5.5 and 7.5

sample	WHC (g water/g protein)		
	pH 3.5^{ns}	pH 5.5	pH 7.5
MPC-HCl	2.64^{b}±0.12	2.45^{Ab}±0.09	3.13^{Ca}±0.10
MPC-CA	2.53^{b}±0.06	2.23^{Bc}±0.07	2.89^{Da}±0.04
APC-HCl	2.73^{b}±0.14	2.63^{Ab}±0.14	3.67^{Aa}±0.12
APC-CA	2.65^{b}±0.04	2.46^{Ac}±0.06	3.39^{Ba}±0.01

* (A,B,C,D):Different superscript letters in the same column indicate significant difference ($p \leq 0.05$).
* (a,b,c,d):Different superscript letters in the same row indicate significant difference ($p \leq 0.05$).
* ns = no significant differences ($p > 0.05$) in the same column.

3.2.3 Emulsifying properties

Emulsifying properties of all protein concentrates are shown in Table 3. MPC-HCl and APC-HCl exhibited significantly higher emulsifying activity index (EAI) but lower emulsifying stability index (ESI) than MPC-CA and APC-CA ($p \leq 0.05$). The EAI of protein concentrates is related to its solubility [2,16]. The higher value of EAI of mung bean and adzuki bean protein concentrates treated with HCl may be attributed to its higher solubility (Figure 1). The higher solubility of proteins will increase protein migration to oil-water interface which enhances interaction between unfolding proteins and oil at the interface [20]. The lower value of ESI was observed in bean protein concentrates treated with HCl. This was due to the higher amount of protein in HCl-treated bean protein concentrates. High amount of protein may lead to protein-protein interaction instead of protein-oil interaction at oil-water interface [20]. This process promoted flocculation and coalescence, resulting in reducing emulsion stability.

Protein concentrates from adzuki bean exhibited higher EAI but lower ESI than mung bean protein concentrate. The results were attributed to higher solubility of adzuki bean protein concentrates, resulting in improving emulsifying activity. The decreasing in emulsion stability of adzuki bean protein concentrates may be attributed to higher polar amino acid of adzuki bean protein concentrates can reduce protein-oil interaction [19].

Table 3 : Emulsifying properties of mung bean and adzuki bean protein concentrates treated with HCl and citric acid

sample	EAI (m^2/g)	ESI (min)
MPC-HCl	12.36^{b} ± 0.04	135.31^{b} ± 2.50
MPC-CA	10.19^{c} ± 0.09	152.58^{a} ± 0.87
APC-HCl	13.02^{a} ± 0.50	126.00^{c} ± 2.83
APC-CA	12.41^{b} ± 0.15	135.96^{b} ± 1.90

* (a,b,c,d):Different superscript letters in the same column indicate significant difference ($p \leq 0.05$).

4. Conclusions

Various coagulants can be used for protein concentrate preparation. Using different coagulants had a significant effect on chemical composition, protein solubility, water holding capacity and emulsifying properties. The protein precipitated with HCl exhibited higher protein content, solubility, WHC and EAI but lower ESI than those treated using citric acid. Protein concentrates from adzuki bean showed higher solubility, WHC and EAI but lower protein content and ESI than mung bean protein concentrates. This information may be useful for food processors to use these protein concentrates as a food ingredient.

5. Acknowledgements

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References

[1] N. Hettiarachchy, U. Kalapathy and M. Wu In: J.S. David and J.L. Willett, Editors, *Soy protein: a renewable resource for food and nonfood ingredients*, Chapter 13, AOCS publishing, UK (1998).
[2] A. Rao, H.E. Shallo, A.P. Ericson and R.L. Thomas, *J. Food Sci*. **67** (2002) 1412-1418.
[3] S.K. Sathe, S.S. Deshpande and D.K. Salunkhe, *J. Food Sci*. 47 (1982) 491-497.
[4] D. Knorr, *J. Food Sci*. **45** (1980) 1183-1186.
[5] N. Wongpratheep and T. Pukruspan, *Improvement of the production and functional properties of mung bean protein products*. Master's Thesis, Kasertsart University, (2005).
[6] J.E. Kinsella, *J. Am. Chem. Soc*. **56** (1979) 242-258.
[7] AOAC, "*Official Methods of Analysis*". 16th ed. Virginia, USA, (1995)
[8] K.O. Adebowale and O.S. Lawal, *Food Chem*. **83** (2003) 237-246.
[9] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, *J. Biol. Chem*. **193** (1951) 265-275.
[10] J.R. Quinn and D. Paton, *Cereal Chem*. **56** (1979) 38-40.
[11] K. N. Pearce and J.E. Kinsella, *J. Agric. Food. Chem*. **26** (1976) 716-723.
[12] Y. Hrynets, D.A. Omana, Y. Xu and M. Betti, *Poult. Sci*. **90** (2011) 458-466.
[13] H. Tang, K. Watanabe and T. Mitsunaga, *Carbohydr. Polym*. **49** (2002) 13-22.
[14] S.K. Sathe, *Crit. Rev. Biotechnol* . **22** (2002) 175-223.
[15] N. Singh, M. Kaur and K.S. Sandhu, *Dry. Technol*. **23** (2005) 975-988.
[16] S. Sai-Ut, S. Ketnawa, P. Chaiwut and S. Rawdkuen, *As. J. Food Ag-Ind*. **2** (2009) 493–504.
[17] S. Damodaran and A. Paraf, *Food proteins and their applications*. Marcel Dekker, New York (1997).
[18] G.T. Meng and C.Y. Ma, *Food Chem*. **73** (2001) 453-460.
[19] C.H. Tang, X. Sun and S.W. Yin, *Food Hydrocolloids*. **23** (2009) 1771-1778.
[20] O.S. Lawal, *Food Chem*. **86** (2004) 345-355.

STUDY OF THE COCONUT OIL PRODUCTION PROCESS BY USING PROTEASE ENZYME FROM CUCUMBER

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Abstract

Coconut milk is an emulsion which is stabilized by naturally protein occurring in coconut milk. Protein in coconut milk can be decomposed by protease enzyme including erepsin which is commonly found in cucumber. The main objective of this work was comparative study of coconut oil production processes using enzyme from cucumber (37°C, 3h), heating (90°C, 3h), chilling (4°C, 3 hr), fermentation (37°C, 3h) and combination of enzyme from cucumber follow by chilling. The data implied that a combination of enzyme from cucumber follow by chilling provided highest %yield of coconut oil (20.8%) as compared another coconut oil production processes including enzyme from cucumber (18.4%), thermal (13%), chilling (16.0%), fermentation (0.4%). Moreover, Physical and chemical properties including free fatty acid and peroxide value are evaluated for coconut oil. These methods of coconut oil production processes provided coconut oils with low free fatty acid and peroxide value.

1. Introduction

Coconut milk is an oil-water emulsion which is stabilized by the naturally occurring protein such as albumins and globulins and phospholipids such as lecithin and cephalin [1]. This emulsion is destabilized by using extra energy to obtain oil by dry and wet process [2]. In dry process, oil is exposed to high temperature during refining. Disadvantage of oil obtained from dry process is without the natural antioxidant such as vitamin E. Virgin coconut oil (VCO) was obtained from fresh and mature coconuts without thermal treatment and refining which has more beneficial effect than coconut oil obtained from dry process [3]. VCO retains most of the nutritional components, especially antioxidant [4]. VCO is rich medium chain fatty acids and contains 48%-53% lauric acid. The lauric acid in VCO can be used to antibacterial, antifungal and antiviral functions [5]. Moreover, VCO is used in skin care product to prevent wrinkles and sagging [6]. VCO production processes was developed from traditionally method such as fermentation method to new method to obtain high yield and quality of VCO. In fermentation method, the milk is allowed to ferment for a longtime (24-36 h) at ambient temperature. The disadvantages of this method are low oil recovery, fermented odor in the oil and using long time for producing to obtain oil. Alternative of VCO production processes is the use of enzymatic destabilzation of coconut milk emulsion without the need of thermal treament or fermentation. This method is stringent environmental, safety and using short time for producing to obtain high %yield of coconut oil. Protein in coconut milk can be decomposed by protease enzyme including erepsin which is commonly found in cucumber. The main objective of this work was comparative study of coconut oil production processes using enzyme from cucumber, heating, chilling, fermentation and combination of enzyme from cucumber follow by chilling.

2. Materials and Methods

2.1 Materials

Coconut milk was obtained form mature coconuts (10-12 months old). It was procured from the local Thong song market, Nakhon Sri Thammarat. All chemicals as analytical grade were procured from J.T. Baker chemicals, USA.

2.2 Treatment of coconut oil production

2.2.1 Thermal treatment

Freshly extracted coconut milk was heated in a constant temperature (90°C) for 3 h to destabilize the coconut milk emulsion. Finally, clear oil was separated from cream .

2.1.2 Chilling treatment

The coconut milk emulsion was chilled at 4°C for 3h and thawed to ambient condition (37°C). Further, thawed coconut milk emulsion was centrifuged at 6000 rpm for 30 min to obtain oil.

2.1.3 Fermentation

The coconut milk emulsion was thawed at ambient temperature (37°C) for 3 h. Further, thawed coconut milk emulsion was centrifuged at 6000 rpm for 30 min to obtain oil

2.1.4 Enzyme treatment

Fresh cucumber was procured from the local Thong song market. It was washed and spin extractor with pulp mill to obtain solution of crude enzyme. Then solution of crude enzyme was filtered with four layers of cheeseclothes. Crude enzyme was mixed with coconut milk emulsion in ratio 1:1 v/v. This mixture was thawed to ambient condition (37°C) for 3 h and then was centrifuged at 6000 rpm for 30 min to obtain oil.

2.1.5 Combination of crude enzyme from cucumber and chilling treatments

Fresh cucumber was procured from the local Thong song market. It was washed and spins extractor with pulp mill to obtain solution of crude enzyme. Then solution of crude enzyme was filtered with four layers of cheeseclothes. Crude enzyme was mixed with coconut milk emulsion in ratio 1:1 v/v. This mixture was thawed to ambient condition (37°C) for 1 h and following by chilling at 4°C for 3 h. Then it was centrifuged at 6000 rpm for 30 min to obtain oil.

2.3 Chemical properties

Peroxide value and % free fatty acid of coconut oil from different treatments were analyzed according to standard method [7,8]. Free fatty acid and peroxide value were expressed as percentage FFA as lauric acid and meq O_2/kg, respectively.

2.4 Statistical analysis

Oil yield from different treatments was carried out in triplicates for oil from different treatment. Significant differences were determined by t-test using statistical package for social science (SPSS). Significance of differences was defined at $p< 0.05$

3. Results and Discussion

3.1 Oil yield from different treatments

Table 1 Oil yield from different treatments

Treatment	% Oil yield	p-value
Enzyme	18.4 ± 1.40	-
Chilling	16.0 ± 2.00	0.164
Heating	13.0 ± 1.00	0.006
Combined	20.8 ± 0.52	0.099
Ferrmentation	0.4 ± 0.20	0.000

Oil yield obtained from different treatments are significantly different at $p<0.05$

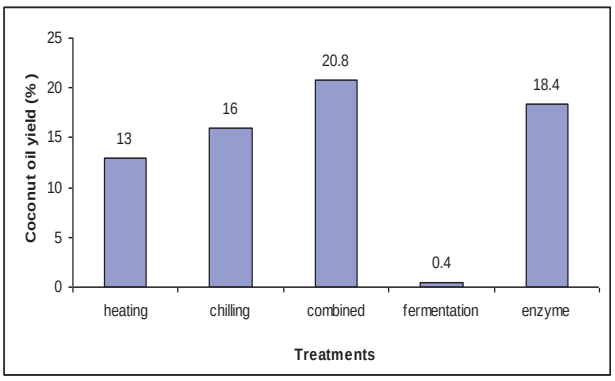

Fig. 1 Coconut oil yield of different treatments

The oil yield from different treatments including enzyme from cucumber, heating, chilling, fermentation and combination of enzyme from cucumber followed by chilling is presented in fig. 1 and table 1. Destabilization of coconut milk emulsion was achieved by employing different treatments such as heating, enzymes from cucumber, chilling and combination of enzyme from cucumber followed by chilling. Oil yield of 18.4% obtained from enzyme from cucumber treatment is slightly difference as compared with combination of using enzyme from cucumber followed by chilling (20.8%) and chilling (16%) treatments but it is significantly different as compared with heating (13%), and fermentation (0.4%) treatments. In enzyme from cucumber treatment, coconut milk emulsion can be destabilized by erepsin as protease enzyme in cucumber which hydrolyzes peptides bonds in the interior of polypeptide chain to obtained shorter fragment of protein/peptides. These shorter fragments of protein/peptides move to the aqueous phase and after that a high oil yield was observed [9]. Moreover, combination of using enzyme from cucumber followed by chilling provide highest yield of coconut oil and was more effective for the destabilization of coconut milk emulsion compared to all other treatments. This result indicated chilling plays an important role in complete destabilization of coconut milk emulsion by enzyme from cucumber treatment. At low temperature in chilling, the solidification of oil take place and during thawing oil globules loose their spherical structure and coalesce to form large droplets, resulting in the high destabilization of the coconut milk emulsion[10].

3.2 Chemical properties

Chemical properties such as peroxide value and free fatty acid of coconut oil obtained from different treatment like enzyme from cucumber, heating, chilling, fermentation and combination of enzyme

from cucumber followed by chilling as shown in Table 2. The free fatty acid content of coconut oil obtained from all treatment was relatively low and within the limits of Asian and Pacific Coconut Community(APCC) standard (<0.5%). It is know that free fatty acid is responsible for an undesirable flavor in oils. Free fatty acid is formed during hydrolytic rancidity, caused by the hydrolysis of and ester by lipase or moisture [11]. Peroxide value was found to be 0.30, 0.33, 2.83, 0.36 and 3.16 meq O_2/kg in oil samples obtained from chilling, heating, combination of enzyme from cucumber followed by chilling, fermentation and enzyme from cucumber treatments, respectively and are within the limits of APCC standard for VCO (<3meq O_2/kg). The peroxide value of oil obtained from all treatment was low indicating their high stability against oxidation. However the stability coconut oil obtained from combination of enzyme from cucumber followed by chilling and enzyme from cucumber treatments is lower than coconut oil obtained from heating, chilling and fermentation treatments.

Table 2 Free fatty acid (% as lauric acid) and peroxide value (meq O_2/kg) of coconut oil

Treatment	Free fatty acid	Peroxide value
chilling	0.30 ± 0.02	0.32 ± 0.02
heating	0.21 ± 0.001	0.33 ± 0.04
combined	0.42 ± 0.01	2.83 ± 0.007
fermentation	0.32 ± 0.00	0.36 ± 0.06
enzyme	0.46 ± 0.00	3.16 ± 0.28

Value are averages $\pm$ SD from tree replication analysis

4. Conclusions

Destabilization of coconut milk emulsion to obtain coconut oil was achieved by enzyme from cucumber, heating chilling, fermentation and combination of enzyme from cucumber followed by chilling. The combination of enzyme from cucumber followed by chilling was found to play a significant role in the complete destabilization of coconut milk emulsion to obtain the highest oil yield (20.08%). Moreover, coconut oil from all treatments was low free fatty acid and peroxide value which are within the limits of APCC standards.

Acknowledgements

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References

[1] Nattapol T, Coupland JN (2005) Effect of heating and homogenization on the stability of coconut milk emulsions. J Food Sci 70(8):466-470

[2] McGlone OC, Canales ALM, Carter JV (1986) Coconut oil extraction by a new enzymatic process. J Food Sci 51 (3): 695-697

[3] Marina AM, Che Man YB, Amin I (2009) Virgin coconut oil: emerging functional food oil. Trends Food Sci Technol 20:481-487

[4] Nevin KG, Rajamohan T (2004) Beneficial effect of Virgin coconut oil on lipid parameters and in virtro LDL oxidation. Clin Biochem 37:830-835

[5] German JB, Dillard CJ (2004) Saturated fats: what dietary intake? Am J Clin Nutr 80:550-559

[6] Rethinam P (2004) Virgin coconut oil applications. Indian Coconut J 4:5-7

[7] AOCS (1998) Offical method and recommentded practices of the American Oil Chemists' Society. In:Firestone D(ed) 5th edn, vol 1. AOCS Press, Champign, USA

[8] AOAC (2000) Offical method method of analysis, 17th end. Association of offical Analytical Chemists, USA

[9] Raghavendra SN, Raghavarao KSMS (2011) Aqueous extraction and enzymatic destabilization of coconut milk emulsions. J Am Oil Chem Soc 88:481-487

[10] Gunetileke KG, Laurentius SF (1974) Conditions for the separation of oil and protein from coconut oil milk emulsion. J Food Sci 39:230-233

[11] Osawa C, Goncalves R (2007) By the official method. Food Comp Anal 20:523-52

DETERMINATION OF CARBON MONOXIDE IN MODIFIED ATMOSPHERE PACKAGED FROZEN MEAT PRODUCTS

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Abstract: Carbon monoxide (CO) has been used in modified atmosphere packaging (MAP) of fresh meat in order to extend the shelf life, maintain their attractive fresh red color appearance and decrease growth of spoilage organisms and pathogenic bacteria. However, CO does not completely inhibit pathogen growth. There is concern that CO-MAP products might look fresh even though bacterial levels are high and the meat products are spoiled. Due to the food safety risk, methods for sample preparation and determination of CO in meat and fish tissues for the trace of CO-MAP were proposed in this research.

The meat drips from tuna, beef and pork tissues were analyzed by the headspace gas chromatography/mass spectrometry (headspace GC/MS) with selected ion monitoring (SIM) mode at m/z 32 (0-5 min) and m/z 28 (5.01-10 min), following the chemical liberation of CO using potassium hexacyanoferrate(III) as a gas-releasing agent. The results had high precision.

Another technique used was the double-wavelength visible absorption spectrophotometry at 420 and 431 nanometers to determine the percentage of carboxymyoglobin in the meat drips from tuna, beef and pork tissues using sodium dithionite solution as a reducing agent. The calculated results showed high matrix interferences and this technique was suitable for tuna meat only.

1. Introduction

The meat industry is continuously developing new technologies to extend the shelf life, maintain their attractive fresh red color appearance and decrease growth of spoilage organisms and pathogenic bacteria. Modified atmosphere packaging (MAP) of perishable food is a well-known and approved method in food industry. The principle of MAP is to replace the normal atmosphere by a single gas or a mixture of gases, such as nitrogen (N_2), carbon dioxide (CO_2), oxygen (O_2), argon (Ar) and carbon monoxide (CO). CO was successfully used in fresh and frozen meat packaging because CO masks the natural surface discoloration of meat by reacting with myoglobin (Mb) in the meat and producing a bright-red color carboxymyoglobin (CO-Mb). [1-3]. CO-Mb is much more resistant to oxidation than oxymyoglobin (oxy-Mb), therefore is not transformed into brown iron(III) metmyoglobin (met-Mb) [4-6]. However, CO does not completely inhibit pathogen growth. CO-treatment of meat product is not allow in the European Community. There is concern that CO-MAP products might look fresh even though bacterial levels are high and the meat products are spoiled. Due to the food safety risk, methods for sample preparation and determination of CO in meat and fish tissues for the trace of CO-MAP were reported, such as gas chromatographic (GC) method with flame ionization (FID) detection [7], gas chromatography/mass spectrometric method (GC/MS) [8] and spectrophotometric method [4,9].

A simple and rapid sample preparation method as well as determination methods for CO in fish and meat products by headspace GC/MS with selected ion monitoring (SIM) mode and double-wavelength visible absorption spectrophotometry were proposed in this research.

2. Materials and Methods

2.1 Chemicals and Reagents

All solutions were prepared with analytical grade reagents and water from a Milli-Q® Water Purification System (Millipore, USA). The 5 M sulfuric acid solution was prepared by appropriate dilution of sulfuric acid (analytical grade, 95-97%, 1.84 g mL^{-1}, Merck, Germany). The 20% (w/v) potassium hexacyanoferrate(III) solution was prepared by dissolving potassium hexacyanoferrate(III) (analytical grade, Merck, Germany) in Milli-Q water. The 20 mg mL^{-1} sodium dithionite solution, used as a reducing agent, was prepared by dissolving sodium dithionite (analytical grade, Sigma-Aldrich, USA) in Milli-Q water. The phosphate buffer solution (PBS, pH 7.0, analytical grade, deoxygenated) was purchased from Merck (Germany).

2.2 Sample preparation

Fresh and frozen samples of tuna, beef and pork were obtained from local markets. Frozen products were thawed in their original packages. The meat drip of sample was collected by a gas-tight syringe, transferred to a vial and closed tightly.

Headspace GC/MS method

A 2.00 mL portion of meat drip was transferred into a 20-mL headspace vial using a gas-tight syringe. The vial was immediately capped and connected to solid phase extraction manifold (Supelco, USA) to pump the air from the top of sample vial for 10-15 minutes. Then, a 3 mL of a gas-releasing agent was added into the vial through a septum with a gas-tight syringe, vortex-mixed and released CO was immediately analysed by headspace GC/MS.

Visible absorption spectrophotometric method

A 200 µL portion of meat drip was transferred into a vial. 10 µL of 20 mg mL^{-1} sodium dithionite solution and 3.00 mL PBS pH 7.0 was added. The mixture was then vortex-mixed, transferred to 1-cm cuvette and analysed by visible absorption spectrophotometry.

2.3 Analysis

Headspace GC/MS method

Headspace GC/MS analysis of CO was performed using an Agilent 6890 N gas chromatograph coupled with an Agilent 5973 mass selective detector (Agilent Technologies, USA). The system was equipped with an Agilent 7694 headspace sampler (Agilent Technologies, USA). Headspace vial equilibration time was set to 15 min, the purging time was 5 min, and headspace temperature was maintained at 70 °C. The GC oven temperature was held constant at 40 °C and injection port temperature was 200 °C. A HP-PLOT MOLSIEVE capillary column, 30 m × 0.322 mm i.d. × 12 µm (J&W Scientific, USA) was used. The carrier gas, helium, was set to a constant flow rate of 1.5 mL min^{-1}. The transfer line was set at 280 °C, and the Agilent 5973 mass selective detector was operated in electron ionization (EI) mode at 70 eV. After 1.00 min solvent delay, the filament was turned on. Selected ion monitoring (SIM) mode at m/z 32 (0-5 min) and m/z 28 (5.01-10 min) was used for O_2 and CO, respectively. A dwell time of 150 ms was chosen.

Visible absorption spectrophotometric method

Visible absorption spectra of the samples were recorded with ultraviolet-visible spectrophotometer (HP 8453, Hewlett Packard, USA). The absorbance was measued at two different wavelengths, 420 and 431 nanometers, corresponding to the absorption maxima of CO-Mb and deoxy-Mb, respectively. The relative amount of CO-Mb (χ_{CO}) in the presence of deoxy-Mb was calculated by Equation (1):

$$\chi_{CO} = \frac{[A_{(420)} \times 0.78] - [A_{(431)} \times 0.67]}{[A_{(420)} \times 0.32] + [A_{(431)} \times 0.55]} \qquad (1)$$

where $A_{(420)}$ and $A_{(431)}$ are the absorbance values at 420 and 431, respectively [9].

3. Results and Discussion

3.1 Headspace GC/MS method

Optimum CO-releasing agent

In this work, headspace GC/MS was used to measured CO released from the meat drips of MAP frozen meat products. Therefore, the results of CO were depended on CO-*releasing agent*. Two types of CO-releasing agent, 5 M sulfuric acid (H_2SO_4) solution [7] and 20% (w/v) potassium hexacyanoferrate(III), $K_3Fe(CN)_6$, solution [8], were compared. As shown in Table 1, the amount of CO liberlated from the meat drips of treated tuna, pork and beef samples via potassium hexacyanoferrate(III) were higher than those obtained with sulfuric acid. Therefore, 20% (w/v) potassium hexacyanoferrate(III) solution was selected for further work.

Table 1: Percentage of CO determined by headspace GC/MS, following the chemical liberation of CO using different gas-releasing agent, sulfuric acid or potassium hexacyanoferrate(III).

Sample	%CO	
	H_2SO_4	$K_3Fe(CN)_6$
Treated tuna	67.45	74.74
Treated pork	63.52	74.31
Treated beef	66.32	76.34

Analysis of frozen treated tuna, pork and beef

Exemplary chromatogram of CO liberated from the meat drip of treated meat sample is presented in Figure 1. Typical retention times were 1.17 min for O_2 peak and 6.43 min for CO peak. The percentage of CO was calculated by the ratio of the area of CO peak and the sum of the area of CO peak and O_2 peak. The exemplary EI mass spectrum of CO liberated from the meat drip of treated tuna are shown in Figure 2. Table 2 shows CO content of gas liberlated from the meat drips of treated tuna, pork and beef as determined by headspace GC/MS, following the chemical liberation of CO using potassium hexacyanoferrate(III) as gas-releasing agent. High percentage of CO detected in these samples indicated that these meat products has been packaged in CO modified atmosphere. Moreover, the results had high precision.

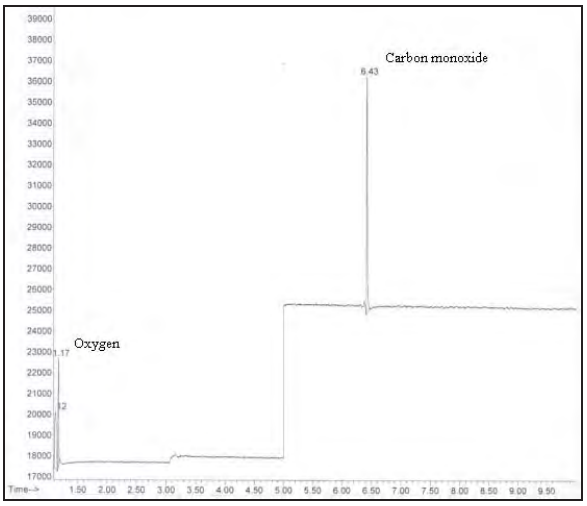

Figure 1. Exemplary chromatogram of gas released from the meat drip of treated tuna sample.

3.2 Visible absorption spectrophotometric method

Optimum amount of reducing agent

To determine the relative amount of CO-Mb (χ_{CO}), the iron(III) met-Mb was reduced to iron(II) deoxy-Mb using 20 mg mL^{-1} sodium dithionite solution. The effect of the amount of reducing agent on χ_{CO} was studied using 10, 20 and 30 µL of 20 mg mL^{-1} sodium dithionite solution. The results in Table 3 showed no significant difference in χ_{CO} when different volumes of sodium dithionite solution were used. Consequently,

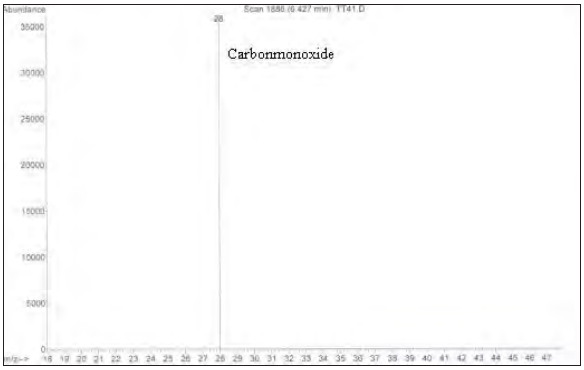

Figure 2. Exemplary EI mass spectrum of CO released from the meat drip of treated tuna sample.

Table 2: CO content from the meat drips as determined by headspace GC/MS, after the chemical liberation of CO using $K_3Fe(CN)_6$ as gas-releasing agent.

Sample	CO (%)	RSD (%)
Treated tuna	74.74 (N = 5)	3.00
Treated pork	74.31 (N = 3)	8.59
Treated beef	76.34 (N = 3)	0.14

Table 3: χ_{CO} determined by visible absorption spectrophotometric method using different volumes of sodium dithionite solution.

Volume of sodium dithionite solution (μL)	Absorbance		χ_{CO} (%)
	420 nm	431 nm	
Treated tuna	0.99800	0.89947	21.59
Treated pork	1.01420	0.91497	21.51
Treated beef	1.32300	1.22240	19.43

10 μL of 20 mg mL^{-1} sodium dithionite solution was used for complete reduction of met-Mb to deoxy-Mb.

Analysis of frozen treated tuna, pork and beef

Table 4 shows χ_{CO} determined by double-wavelength visible absorption spectrophotometric method at 420 and 431 nm. χ_{CO} values from the meat drips of the CO-treated samples are markedly higher than those obtained from the untreated fresh samples. However, χ_{CO} values of both treated and untreated pork and beef are rather too high. This may be caused by high matrix interferences of the meat drips from pork and beef. Then, Equation (1) which previously reported for determination of CO in treated fish tissues [9], was not practical to calculate χ_{CO} values of the meat drips from pork and beef samples. Therefore, this method was suitable for tuna only.

Validation of visible spectrophotometric method

The results of CO determined by visible absorption spectrophotometric method were compared with those

Table 4: χ_{CO} from the meat drips as determined by visible absorption spectrophotometric method using sodium dithionite solution as reducing agent.

Sample	χ_{CO} (%)	%RSD
Fresh tuna	39.97 (N = 3)	5.98
Treated tuna	56.55 (N = 5)	8.89
Fresh pork	62.34	-
Treated pork	137.62	-
Fresh beef	76.52	-
Treated beef	91.05	-

Table 5: Comparison of CO content of the meat drips determined by headspace GC/MS and visible absorption spectrophotometry.

Sample	headspace GC/MS		visible absorption spectrophotometry		
	CO (%)	RSD (%)	χ_{CO} (%)	RSD (%)	RPD*
Treated tuna	74.74	3.00	56.55	8.89	-18.19
Treated pork	74.31	8.59	137.62	-	+63.31
Treated beef	76.34	0.14	91.05	-	+14.71

*RPD = relative percent difference

obtained by high accuracy and precision headspace GC/MS method are shown in Table 5. The relative percent difference (RPD) for analysis of CO in treated tuna by both methods was lower than 20%, which confirmed that the visible absorption spectrophotometric method was acceptable method for determination of CO in treated tuna.

4. Conclusions

A simple and rapid sample preparation method and determination methods for CO in the meat drips from CO-MAP frozen fish and meat products by headspace GC/MS with SIM mode and double-wavelength visible absorption spectrophotometry were developed.

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References

[1] R.A. Mancini and M.C. Hunt, *Meat Science* **71** (2005) 100-121.
[2] L. Martinez, D. Djenane, I. Cilla, J.A. Beltran, and P. Roncales, *Meat Science* **71** (2005) 563-570.

[3] B.H.P. Wilkinson, J.A.M. Janz, P.C.H. Morel, R.W. Purchas, and W.H. Hendriks, *Meat Science* **73** (2006) 605-610.
[4] E. Droghetti, G.L. Bartolucci, C. Focardi, M. Bambagiotti-Alberti, M. Nocentini, and G. Smulevich, *Food Chem.* **128** (2011) 1143-1151.
[5] M. Seyfert, R.A. Mancini, M.C. Hunt, J. Tang, and C. Faustman, C., *Meat Science* **75** (2007) 432-442.
[6] R.A. Wicklund, D.D. Paulson, E.M. Tucker, A.J. Stetzer, F. Desantos, M. Rojas, B.J. Macfarlane, and M.S. Brewer, *Meat Science* **74** (2006) 704-709.
[7] J. Czogala, W. Wardas, and M.L. Goniewicz, *Anal. Chim Acta* **556** (2006) 295-300.
[8] C.R. Anderson and W. Wu, *J. Agric. Food Chem.* **53** (2005) 7019-7023.
[9] G. Smulevich, E. Droghetti, C. Focardi, M. Coletta, C. Ciaccio, and M. Nocentini, *Food Chem.* **101** (2007) 1071-1077.

EFFECTS OF CRUDE EXTRACTS FROM *Tagetes erecta* ON SEED GERMINATION AND SEEDLING GROWTH OF TEST PLANTS

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ABSTRACT: The objective of this study was to investigate the allelopathic effects of *T. erecta* extracts on seed germination and seedling growth of Chinese amaranth (*Amaranthus tricolor* L.) and barnyardgrass (*Echinochloa crus-galli* [L.] Beauv.). The crude methanol extract from the leaves of *T. erecta* was fractionated using four solvents: *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol. The seeds were germinated in petri dishes with varied concentrations (500-4000 ppm) of different fractions; distilled water was used as the control. The results indicated that the *n*-hexane and dichloromethane fractions influenced the seed germination and seedling growth of Chinese amaranth. At 500 ppm, the *n*-hexane and dichloromethane fractions completely inhibited the germination of Chinese amaranth. For the barnyardgrass, the *n*-hexane fraction was observed at 2000-4000 ppm concentrations reduced the seed germination, shoot, and root length while the dichloromethane fraction at 4000 ppm inhibited the shoot and root length. These results indicated that *T. erecta* may contain growth inhibitory substances and possess allelopathic activity. Further studies are required to identify and isolate effective allelochemicals from this plant which can be used as an environmental friendly herbicide for weed control.

1. Introduction

Allelopathy is most commonly defined as the direct or indirect detrimental or beneficial effects of one plant including microorganisms on the germination, growth, or development of other plants through the production of chemicals (allelochemicals) that escape into the environment [1]. Allelopathic interactions between plants have been implicated in the patterning of vegetation and weed growth in an agricultural system and in inhibition of the growth of several crops [2]. There are several natural products or mixtures of natural products, mostly extracts from plant origins, such as essential oils that are commercialized as crop protection products for use in organic agriculture [3]. Allelochemicals are found in all plant parts, including the roots, stems, rhizomes, flowers, inflorescences and leaves [1]. Kamara et al. reported the strong allelopathic activity of *Tetrapleura tetraptera* leaf extracts on cowpea germination and early reduction developments of cowpea dry matter in pot experiments [4]. Organic solvents have always been used for the extraction of bioactive compounds from plant materials with different efficiencies for individual classes of bioactive compounds. Various solvent systems have been used for the extraction of secondary metabolites from plant materials because extraction efficiency depends on the nature of the chemical. *Tagetes erecta* belongs to the family of Asteraceae, a prevalent garden plant and is commonly known as Marigold. It is very popular garden plants and yields a strongly aromatic essential oil (tagetes oil), which is mainly used for the compounding of high-grade perfumes. Different parts of this plant are used in folk medicine to cure various diseases [5]. Phytochemical studies carried out with different species of *Tagetes* have revealed the presence of flavonoids and terpenes displaying pharmacological and insecticidal properties [6,7]. Although much research has been done on *T. erecta* in different aspects, little information is available concerning the allelopathic potential of *T. erecta* on weed. Its was, therefore, of interest to determine the allelopathic potential of *T. erecta* using crude organic extracts of *T. erecta* leaves by evaluating its effect on the seed germination and seedling growth of test plant species, Chinese amaranth (*A. tricolor* L.) and barnyardgrass (*E. crus-galli* [L.] Beauv.), in the laboratory bioassay condition.

2. Materials and Methods

2.1 Plant materials and test plants

The study consisted of 3-factors (i) Test plants: 2 [Chinese amaranth (*A. tricolor*) and barnyardgrass (*E. crus-galli* (L.) Beauv.)], (ii) crude organic extracts in solvents: 4 (hexane, dichloromethane, ethyl acetate and butanol) and (iii) crude organic extracts concentrations: 5 (0, 500, 1000, 2000, 4000 ppm). The seeds of Chinese amaranth were purchased from Thai Seed & Agriculture Co., Ltd., Bangkok, Thailand and the barnyardgrass seeds were collected (August 2011) from paddy fields in the Minburi District, Bangkok, Thailand. The germination of test seeds was > 80%.

Leaves of *T. erecta* were collected from the Faculty of Agricultural Technology at King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand in June of 2011. After collection, the mature and healthy leaves were washed with tap water to remove soil and other debris, then dried in a hot air oven at 45 ^{o}C for 3 days. The dried leaves were kept at room temperature until extraction.

2.2 Extracting of *T. erecta*

The dried leaves (1.2 Kg) of *T. erecta* were ground to powder in an electric blender and macerated in methanol (4 L) at room temperature for 7 days with occasional stirring and shaking. The extract was then filtered first through a fresh cotton plug and finally with Whatman No. 1 filters paper. The filtrate was concentrated to dryness under reduced pressure using a rotary evaporator at 50 ^{o}C. The methanol paste crude extract (216.10 g) was suspended in water (400 mL) and was then further partitioned with *n*-hexane, dichloromethane (DCM), ethyl acetate (EtOAc) and *n*-butanol using a separatory funnel. The collected extracts were evaporated to dryness using a rotary evaporator before left air dried in a fume cupboard. Dried paste extracts were kept in refrigerator at 4 ^{o}C for further bioassay.

2.3 Bioassay

The 4-dried samples concentrated from hexane, dichloromethane, ethyl acetate and *n*-butanol were again dissolved in each solvent to compare their inhibitory effects. Five-hundred µL of each crude extract solutions (5000, 10000, 20000 and 40000 ppm concentrations) were added to petri dishes (9 cm diameter) lined with germination paper. The solutions were allowed to evaporate for 3 h at room temperature. After evaporation, 5 mL of distilled water was added on the germination paper to obtain 500, 1000, 2000 and 4000 ppm concentrations. Then 20 seeds of the test plant were placed on the germination paper as per the treatments. The control received only distilled water. The treatments were replicated 4-times in a completely randomized design. All petri dishes were covered and left at room temperature (32 ^{o}C day and 28 ^{o}C night) under natural light conditions (0600 h – 1800 h). After 7 days, germination (%), shoot and root length were recorded for all treatment. Inhibition (%) relative to control, was calculated as under:

$$\text{Inhibition (\% of control)} = [1-(\text{sample extracts}/\text{control})] \times 100$$

2.4 Statistical analysis

Differences in the percentages of seed germination and root and shoot lengths were assessed by an analysis of variance statistical method. Comparisons between the treatments were made at a 0.05 probability level using Duncan's Multiple Range Test (DMRT).

3. Results and Discussion

3.1 Effects of crude organic extracts of *T. erecta* leaves on seed germination of the test plant species

The inhibitory activity of crude organic extracts of *T. erecta* leaves on the seed germination of Chinese amaranth and barnyardgrass is shown in Table 1. Crude hexane and DCM extracts at concentrations of 1000-4000 ppm completely inhibited the seed germination of Chinese amaranth while crude EtOAc and *n*-butanol had no the inhibitory effect on the seed germination of Chinese amaranth. In barnyardgrass, a crude hexane extract at concentrations of 2000-4000 ppm inhibited the seed germination while the other crude extracts had no inhibitory effect on seed germination.

Table 1: Effect of crude organic extracts of *T. erecta* leaves on germination of the test plant species

Concentrations (ppm)	Chinese amaranth (%inhibition)	Barnyardgrass (%inhibition)
Control (0)	0.00 ± 0.00d	0.00 ± 5.12c
Hexane 500	40.00 ± 8.16b	17.95 ± 0.00bc
Hexane 1000	100.00 ± 0.00a	17.95 ± 0.00bc
Hexane 2000	100.00 ± 0.00a	58.97 ± 5.92ab
Hexane 4000	100.00 ± 0.00a	71.79 ± 6.40a
DCM 500	90.00 ± 0.00a	12.82 ± 5.92bc
DCM 1000	100.00 ± 0.00a	2.56 ± 5.92c
DCM 2000	100.00 ± 0.00a	17.95 ± 0.00c
DCM 4000	100.00 ± 0.00a	33.33 ± 5.92abc
EtOAc 500	0.00 ± 0.00d	5.13 ± 9.82c
EtOAc 1000	2.50 ± 5.00cd	2.55 ± 5.92c
EtOAc 2000	35.00 ± 5.77bcd	0.00 ± 5.12c
EtOAc 4000	42.50 ± 5.00b	5.13 ± 9.82c
n-Butanol 500	0.00 ± 0.00d	20.51 ± 9.82bc
n-Butanol 1000	2.50 ± 5.00cd	15.38 ± 5.12bc
n-Butanol 2000	5.00 ± 5.77bcd	20.51 ± 9.82bc
n-Butanol 4000	15.00 ± 5.77bcd	5.13 ± 5.12c

Mean values in each column followed by the same letter are not significantly different at P=0.05 according to the Duncan's multiple range test.

3.2 Effects of crude organic extracts of *T. erecta* leaves on seedling growth of the test plant species

The effects of the crude extracts of *T. erecta* leaves on the shoot and root length of Chinese amaranth are shown in Table 2. Crude hexane and DCM extracts exhibited marked shoot and root growth inhibition on Chinese amaranth which was also concentration dependent. At 500 ppm concentration, the crude hexane extract reduced root length by 53.46% but it had no inhibitory effect on shoot length while the crude DCM extract reduced both shoot and root length by 79.22% and 84.85%. The results indicate that seedling growth of Chinese amaranth was completely inhibited at 1000-4000 ppm concentrations.

Table 2: Effects of crude organic extracts of *T. erecta* leaves on seedling growth of Chinese amaranth

Concentrations (ppm)	Chinese amaranth (%inhibition)	
	Shoot length	Root length
Control (0)	0.00 ± 5.02cde	0.00 ± 4.57de
Hexane 500	26.94 ± 1.12bcd	53.46 ± 1.19bc
Hexane 1000	100.00 ± 0.00a	100.00 ± 0.00a
Hexane 2000	100.00 ± 0.00a	100.00 ± 0.00a
Hexane 4000	100.00 ± 0.00a	100.00 ± 0.00a
DCM 500	79.22 ± 5.52ab	84.85 ± 1.65ab
DCM 1000	100.00 ± 0.00a	100.00 ± 0.00a
DCM 2000	100.00 ± 0.00a	100.00 ± 0.00a
DCM 4000	100.00 ± 0.00a	100.00 ± 0.00a
EtOAc 500	4.42 ± 4.72cde	-21.48 ± 2.07e
EtOAc 1000	-10.65 ± 4.41de	-7.83 ± 6.38e
EtOAc 2000	-10.91 ± 1.23de	20.82 ± 3.60cde
EtOAc 4000	45.45 ± 3.99bc	54.70 ± 7.96bc
n-Butanol 500	7.79 ± 7.78cde	-21.82 ± 3.07e
n-Butanol 1000	-19.48 ± 6.52de	-13.49 ± 3.63e
n-Butanol 2000	-38.96 ± 5.97e	-14.82 ± 2.37e
n-Butanol 4000	-11.95 ± 2.49de	41.55 ± 5.81cd

Mean values in each column followed by the same letter are not significantly different at *P*=0.05 according to the Duncan's multiple range test.

In barnyardgrass, crude hexane at 2000-4000 ppm concentrations slightly high inhibited shoot and root length while DCM at the highest concentration (4000 ppm) inhibited root length by 71.14% (Table 3). All the applied concentrations of crude EtOAc and *n*-butanol had no inhibitory effect on either shoot or root length of barnyardgrass.

Table 3: Effects of crude organic extracts of *T. erecta* leaves on seedling growth of barnyardgrass

Concentrations (ppm)	Barnyardgrass (%inhibition)	
	Shoot length	Root length
Control (0)	0.00 ± 4.07bc	0.00 ± 2.70cde
Hexane 500	17.49 ± 4.88bc	23.09 ± 1.29cd
Hexane 1000	18.09 ± 2.96bc	32.41 ± 3.81bc
Hexane 2000	66.74 ± 3.34a	71.37 ± 2.83ab
Hexane 4000	68.02 ± 5.11a	83.57 ± 4.37a
DCM 500	-0.97 ± 2.72bc	-1.11 ± 7.09cde
DCM 1000	2.10 ± 7.84bc	4.11 ± 7.18cde
DCM 2000	3.53 ± 1.77bc	32.19 ± 8.28bc
DCM 4000	34.01 ± 3.26ab	71.14 ± 5.23ab
EtOAc 500	2.07 ± 6.78bc	-0.67 ± 2.98cde
EtOAc 1000	1.35 ± 4.69bc	-34.74 ± 4.90e
EtOAc 2000	3.08 ± 1.35bc	15.65 ± 5.21cd
EtOAc 4000	-1.35 ± 4.01c	2.11 ± 0.40cde
n-Butanol 500	0.97 ± 2.78bc	-18.53 ± 2.79de
n-Butanol 1000	-3.68 ± 4.62c	-30.63 ± 0.50e
n-Butanol 2000	-0.60 ± 3.78bc	-19.76 ± 2.09de
n-Butanol 4000	-5.93 ± 4.90c	-6.22 ± 1.12cde

Mean values in each column followed by the same letter are not significantly different at *P*=0.05 according to the Duncan's multiple range test.

The results from this study showed that crude hexane and DCM extracts of *T. erecta* leaves significantly inhibited seed germination and the shoot and root growth of test plants used in this research. This inhibitory activity was proportional to the extract concentrations and higher concentration had the stronger inhibitory effect. Chinese amaranth was more sensitive to the extracts than was barnyardgrass. It has been reported that leaves of *T. erecta* contains essential oils including terpenoids and flavonoids [6,9,10]. Substances broadly recognized for their allelopathic potential, such as essential oils and flavonoids [2] were already reported as components of *T. erecta* leaves. Therefore, the inhibition by the extracts on seed germination and seedling growth of the test plants might be due to the presence of allelopathic substances in *T. erecta* extract. The inhibitory effect might occurr through a variety of mechanisms like reduced mitotic activity in roots and shoots, a reduced rate of ion uptake and inhibition of photosynthesis [8].

4. Conclusions

The current research indicates that the crude hexane and DCM extracts of *T. erecta* leaves have a strong inhibitory effect on Chinese amaranth. The biological activity of extracts increased at higher concentrations. These results suggest that *T. erecta* leaves may contain growth inhibitory substances and possess allelopathic activity. This may be due to the solvents to extract the different constituents having allelopathic activity. Hexane a non-polar solvent was expected to extract the non-polar compounds such as essential oils including terpenoids. DCM a moderately polar solvent was expected to extract the less polar compounds such as flavonoids. Further analysis of possible growth inhibitory substances in *T. erecta* leaves should be done in further studies. The identification of these substances might provide a chemical basis for the development of novel bio-herbicides for environmentally friendly sustainable agricultural systems.

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References

[1] A.R. Putnam, *Weed Technol*, **2** (1998) 510-518.
[2] E.L. Rice, *Allelopath. 2nd Ed*, Academic Press. London, UK (1984).
[3] O. F. Hüter, *Phytochem Rev*. **10** (2011), 185-194.
[4] A.Y. Kamara, I.O. Sangingga and S.C. Jutzi, *J Agron Crop Sci*, **182** (1999) 127-134.
[5] F. Nikon, M. Rowshanul, Z.Alam, M. Rezaul, A. Kumar and S. Zaman, *Int. J. Drug Dev. Res*., **1** (2009), 161-165.
[6] M. Terschuck, M. Riera, G. Castro, and L. Abdala, *J. Ethnopharmacol*, **56** (1997), 227-232.
[7] M. Perich, C. Wells, W. Bertsch, and K. Tredway, *J. Am. Mosq. Control Assoc*., **11** (1995), 307-310.

[8] E.L. Rice, *Allelopathy*, Academic Press, NewYork, USA (1974).
[9] H. Halbwirth, G. Forkman, and K. Stich, *Plant Sci*., **167** 129-135.
[10] I.A. Ogunwande, A. Isiska, and N.O. Olawore, **18** *J. Essen. Oil Res.*, (2006) 366-368.

ANTIOXIDANT ACTIVITIES OF ADZUKI BEAN (*VIGNA ANGULARIS*) PROTEIN HYDROLYSATE

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Abstract: Adzuki bean (*Vigna angularis*) is a major food legume containing various nutrients such as proteins, lipids, vitamins and minerals and some phytochemicals possessing natural antioxidant activities. The objectives of this study were to analyze the chemical compositions of adzuki beans and to investigate antioxidant activities of the enzyme-hydrolyzed adzuki bean protein hydrolysate (ABPH) by ferric reducing antioxidant power (FRAP), the metal ions chelating activity and also by the determination of total phenolic compounds (TPC) by Folin-Ciocalteau method. The hydrolysis was achieved by using Flavourzyme® 500 MG at variable concentrations (1, 3, 5 and 7 % w/w) and hydrolysis time (1, 2, 3 and 4 h). The beans are composed of 25.74 % protein, 1.97 % lipid, 4.30 % ash, 3.61 % crude fiber and 64.38 % carbohydrates (dry basis). Degree of hydrolysis (DH), TPC and metal ions chelating activity of ABPH tended to increase with increasing enzyme concentration and hydrolysis time, when compared with fresh adzuki bean extract but such effects were not remarkably observed for FRAP. ABPH hydrolyzed with 7 % enzyme concentration for 4 h exhibited the highest value of 47.94 % DH and 58.30 % metal ions chelating activity. However, the TPC was the highest at 120.14 µg GAE/g for the sample hydrolyzed by 7 % enzyme concentration for 2 h. Therefore, ABPH reveals the possibility for being applied as an antioxidant in food products.

1. Introduction

Adzuki or small red beans are used as staple foods in many countries [1] and are rich source of nutrients such as proteins, lipids, vitamins and minerals [2] and some phytochemicals such as phenolic compounds possessing natural antioxidant activities [3]. Thus, adzuki bean is one of the important foods for human.

Protein hydrolysates are the chemically or enzymatically hydrolyzed proteins which are broken down into free amino acid and peptides of varying sizes. Some properties of the hydrolyzed food proteins can be altered and are different from those of the native sources [4].

In recent years interest in utilizing natural antioxidants has increased substantially [5]. This has led to new investigations into assessing the antioxidant potential of protein hydrolysates from various food tissues.

This research aimed to analyze the chemical compositions of adzuki beans and to investigate antioxidant activities of the enzyme-hydrolyzed adzuki bean protein hydrolysate. The acquired information will possibly help expand the application of its hydrolysate as antioxidant in food products.

2. Materials and Methods

2.1 Materials

Adzuki beans were purchased from the Royal Project Foundation (Khunphae development center), Chiangmai, Thailand during March 2010.The beans were ground into powder and passed through a 100 mesh sieve. The screened powder was packed in 15 cm × 21cm aluminium-laminated bags (PET/AL/PE) and kept in a desiccator until used.

2.2 Proximate analysis

Chemical compositions including moisture, protein, lipid, ash and crude fiber were analyzed according to the AOAC methods [6]. Carbohydrate content was obtained as difference from 100 %.

2.3 Adzuki bean extraction

The extraction of adzuki bean was achieved with the methods of Xu and Chang [7] and Amarowicz, Estrella, Hernández and Troszyńska [8] with some modifications .Ten grams of sample was mixed with 100 ml of solvent (acidic 70 % acetone, ratio of acetone/water/acetic acid (99.8 %) being 70:29.5:0.5, v/v/v). The mixture was shaken at 100 rpm at 50 °C for 30 min in a shaking water bath. The extraction was repeated twice, supernatants combined and centrifuged at 3,500 rpm for 15 min. The solvent was evaporated in a rotary evaporator. The prepared extract was stored at -20 °C until used.

2.4 Preparation of the enzyme-hydrolyzed adzuki bean protein hydrolysate (ABPH)

Ten grams of sample were mixed with the distilled water at a ratio of 1: 10 (w/v). After that, Flavourzyme® 500 MG were added to the solution at 50 °C and pH 7. Enzyme concentrations were varied at 1, 3, 5 and 7 % (w/w) and hydrolysis times were 1, 2, 3 and 4 h, respectively. These treated samples were inactivated at 70 °C for 10 min, rapidly cooled and centrifuged at 3,500 rpm for 15 min. The supernatants were kept at -20 °C until used. The degree of hydrolysis (DH) was determined by using the *o*-phthaldialdehyde (OPA) method as described by Nielsen, Petersen, and Dhambmann [9].

2.5 Ferric reducing antioxidant power

The FRAP assay was measured according to Benzie and Strain [10] with some modifications. The stock solutions included 300 mM Na acetate buffer, pH 3.6; 10 mM TPTZ (2, 4, 6-tripyridyl-*s*-triazine) solution in 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution was prepared by mixing 25 ml Na acetate buffer, 2.5 ml TPTZ solution and 2.5 ml $FeCl_3 \cdot 6H_2O$ solution and then warmed at 37 °C before use. The sample solution (200 µl) was allowed to react with 2000 µl of the FRAP solution for 30 min in the dark condition. Reading of the colored product (ferrous tripyridyltriazine complex) was taken at an absorbance of 593 nm. The standard curve was linear between 0.05 and 0.3 mM Trolox. Results were expressed in µmol trolox equivalent (TE)/g. Additional dilution was needed if the FRAP value measured was over the linear range of the standard curve.

2.6 Metal ions chelating activity

Metal ions chelating activity was measured according to the modified method described by Xie, Huang, Xu and Jin [11]. One ml of sample solution was premixed with 0.05 ml of iron dichloride ($FeCl_2$) solution (2 mM) and 3.7 ml of distilled water. Afterwards, 0.1 ml of ferrozine solution (5 mM) was added and mixed vigorously and the mixture was left in darkness (20 min) and measured for its absorbance at 562 nm, i.e., distilled water being used as a control. The chelating effect was calculated by using the following equations

$$\text{Chelating effect (\%)} = \left(1 - \frac{\text{A562 sample}}{\text{A562 control}}\right) \times 100$$

2.7 Determination of total phenolic compounds

The TPC was determined by the Folin-Ciocalteau (FC) method, which was adapted from Waterhouse [12]. One ml of sample was added into a 100 ml volumetric flask and added with 70 ml distilled water and 5 ml of 2 N FC reagent. The content was incubated at room temperature (25 °C) for 4 min and added with 15 ml of saturated Na_2CO_3 solution and mixed well. The mixture was made up to volume with distilled water, mixed and incubated at room temperature in the dark for 2 h. The absorbance was measured at 765 nm. The TPC was expressed in µg gallic acid equivalent (GAE)/g.

2.8 Statistical analysis

Determination of proximate compositions was performed and analyzed with triplicates. The data for DH, FRAP, metal ions chelating activity and TPC were analyzed using a symmetric 4×4 factorial in completely randomized design (CRD) with duplicates. Duncan's New Multiple Rang Test (DNMRT) was used to compare the means at $p \leq 0.05$. All the statistical analyses were carried out using SPSS (version 16.0).

3. Results and Discussion

3.1 Proximate Compositions

Adzuki beans contain carbohydrates and proteins as the first two major nutrients whose contents are more than eighty percent (Table 1). These data are similar to those reported by Nwokolo and Smartt [13] who found the chemical compositions of this bean as: moisture 15.0 %, protein 21.1 %, lipid 1.0 %, ash 3.4 %, crude fiber 3.9 % and carbohydrate 59.5 %.Variation of proximate compositions could be due to seed size, cultivar differences and environmental factors during growth [14].

Table 1: Proximate compositions of adzuki bean

Compositions	**% wet basis**
Moisture	10.84 ± 0.28
Protein	22.95 ± 0.90
Lipid	1.76 ± 0.22
Ash	3.83 ± 0.09
Crude fiber	3.22 ± 0.25
Carbohydrate	57.40 ± 0.70

All values are means of triplicate determinations ± SD.

3.2 Degree of hydrolysis

Degrees of hydrolysis of ABPH were ranged from 29.36 % to 47.94 % as shown in Figure 1. It was increased by increasing enzyme concentration and hydrolysis time especially at 2, 3 and 4 h. This was due to cleavage of proteins into free amino acids and peptides of varying sizes. DH affects the functional properties of hydrolysate. Vioque et al. [15] categorized hydrolysates into three main groups based on their DH that determined their application: i) hydrolysates with low DHs were those with improved functional properties features, ii) hydrolysates with various DHs were generally used as flavourings, and iii) hydrolysates with broad DHs were mostly used as nutritional supplements and in special medical diets. Consequently, it was decided that these ABPHs should be investigated for their antioxidant activities which are not only important for food applications but also for health benefits.

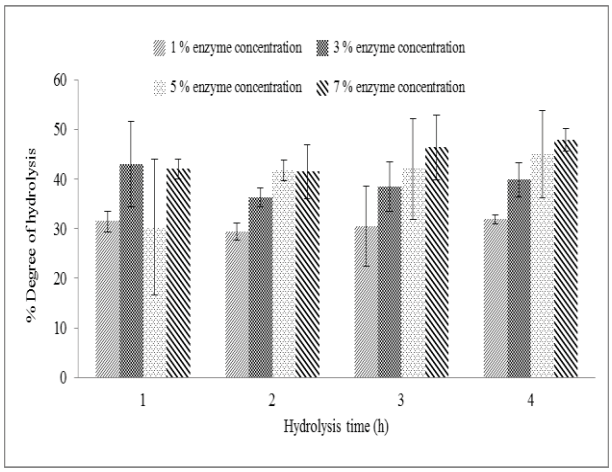

Figure 1. Degree of hydrolysis of ABPH.

3.3 Ferric reducing antioxidant power

FRAP is the method based on electron transfer (ET) used frequently to measure antioxidant activity of peptides and phenolics [16]. In Figure 2, the FRAPs of ABPHs were lower than the control which could possibly be due to fewer amounts of those two groups of compounds especially the phenolics, in the hydrolysates. In addition, there was a tendency that the longer the hydrolysis time the higher the FRAPs, except for those values obtained at 3 h. It might be possible, in the control, that free or unbound phenolic compounds were extracted more and they were solely responsible for the FRAP. However, for the hydrolysates, the effect caused by the resultant peptides seemed to be more pronounced in comparison with that of the phenolics.

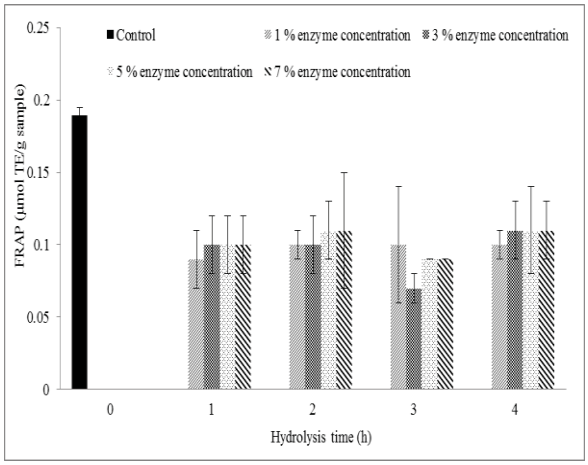

Figure 2. Ferric reducing antioxidant power of ABPH.

3.4 Metal ions chelating activity

The metal ions chelating activities of ABPHs were higher than the control. This could be explained by the higher contents of free amino acids and smaller peptides especially in the samples hydrolyzed with longer hours.

Metal chelation appears to be at least partly responsible for the antioxidant activity that has been found with several free amino acids [17, 18]. Furthermore, antioxidant peptides containing Pro and His have synergistic effect in the presence of other antioxidants such as polyphenols [19]. In addition, chelation of iron by His, Glu, Asp, and Cys results in enhanced iron absorption, and also in reduction of ferric to ferrous ion [20]. Previous study showed that peptides containing acidic and/or basic amino acids might play an important role in Fe^{2+} and Cu^{2+} chelation [21].

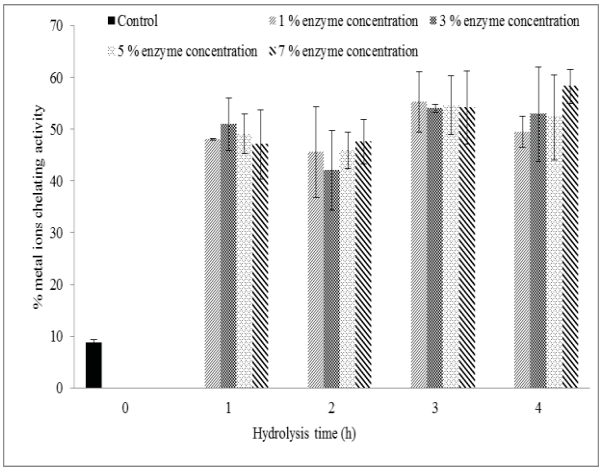

Figure 3. Metal ions chelating activity of ABPH.

3.5 Total phenolic compounds

The total phenolic compounds of ABPHs seemed to be increased with increasing enzyme concentration and hydrolysis time especially at 1 or 2 h. The highest TPC was 120.14 µg GAE/g for the sample hydrolyzed by 7 % enzyme concentration for 2 h and the lowest was 90.70 µg GAE/g for the sample hydrolyzed by 1 % enzyme for 3 h and the value for fresh adzuki bean extract (control) was 108.91 µg GAE/g (Figure 4). Some samples had lower TPC values than the control because some phenolics possibly lost during hydrolysis and the others probably involved in the formation of insoluble complexes with proteins and/or other compounds [22]. Interestingly enough, a half of the hydrolysate samples had higher TPCs than the bean extract although the values were not significantly different. Nevertheless, this might reflect the need of hydrolysis to release more TPCs into the hydrolysates.

Amarowicz et al. [8] reported the dominant phenolics in the adzuki bean extract as catechin, epicatechin glucosides, procyanidin dimers, myricetin, protocatechuic acid and quercetin glucosides, which possess antioxidant activities.

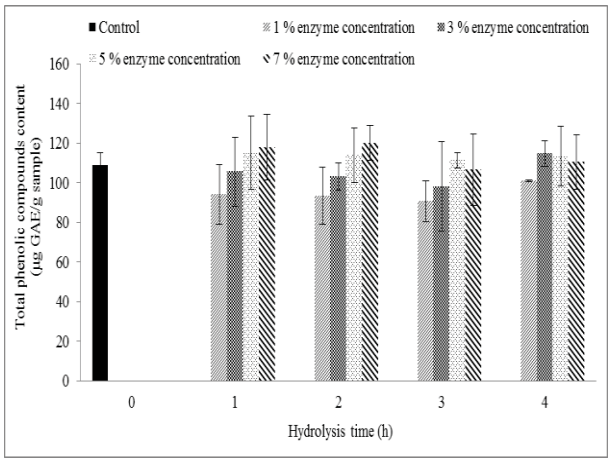

Figure 4. Total phenolic compounds (TPC) of ABPH.

4. Conclusions

Adzuki bean is composed of various nutrients such as carbohydrates, proteins, lipids, ashes and some phytochemicals possessing natural antioxidant activities. The antioxidant activities of ABPHs tended to increase with increasing enzyme concentration and hydrolysis time. Therefore, ABPH reveals the possibility for being applied as an antioxidant in food products.

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References

[1] H. Yoshida, Y. Tomiyama, N. Yoshida, and Y. Mizushina, *Food Chemistry*. **115** (2009) 1224-1429.

[2] S. Sai-Ut, S. Ketnawa, P. Chaiwut, and S. Rawdkuen, *Asian Journal of Food Agro-Industry*. **2(04)** (2009) 493-504.

[3] R. Amarowicz and R.B. Pegg, *European Journal of Lipid Science and Technology*. **110** (2008) 865-878.

[4] H.G. Kristinsson and B.A. Rasco, *Critical Review in Food Science and Nutrition*. **4(1)** (2000) 43-81.

[5] Y. Li, B. Jiang, T. Zhang, W. Mu, and J. Liu, *Food Chemistry*. **106** (2008) 444-450.

[6] AOAC, *Ofiicial Methods of Analysis of AOAC International*, **18th ed**, AOAC International. Gaithersburg. (2006), pp. 1-61.

[7] B.J. Xu and S.K.C. Chang, *Journal of Food Science*. **72(2)** (2007) 159-166.

[8] R. Amarowicz, I. Estrella, T. Hernández, and A. Troszyńska, *Journal of Food Lipids*. **15** (2008) 119-136.

[9] P.M. Nielsen, D. Petersen, and C. Dhambmann, *Journal of Food Science*. **66(5)** (2001) 642-646.

[10] I.F.F. Benzie and J.J. Strain, *Methods in Enzymology*. **299** (1999) 15-27.

[11] Z. Xie, J. Huang, X. Xu, and Z. Jin, *Food Chemistry*. **111** (2008) 370-376.

[12] A.L. Waterhouse In: R.E. Wrolstad, T.E. Acree, E.A. Decker, M.H. Penner, D.S. Reid, S.J. Schwartz, C. F. Shoemaker, D. Smith, and P. Sporn, Editors, *Handbook of Food Analytical Chemistry*, **Vol. 2**, John Wiley and Sons, Hoboken (2008), pp. 463-464.

[13] E. Nwokolo and J. Smartt, Editors, *Food and Feed from Legumes and Oilseeds*, Chapman and Hall, London (1996), pp. 206-207.

[14] T.A. Lumpkin and D.C McClary, Editors, *Azuki Bean: Botany, Production and Uses*, **Chapter 8**, CAB International, Wallingford (1994), pp. 157-172.

[15] J. Vioque, A. Clemente, J. Pedroche, M.M. Yust, and F. Millán, *Grasas Aceites*. **52** (2001) 132-136.

[16] L.K. McDonald-Wicks, L.G. Wood, and M.L. Garg, *Journal of the Science of Food and Agriculture*. **86** (2006) 2046-2056.

[17] W.Y. Huang, K. Majumder, and J. Wu, *Food Chemistry*. **123(3)** (2010) 635-641.

[18] L. Zhu, J. Chen, X. Tang, and Y.L. Xiong, *Journal of Agricultural and Food Chemistry*. **56(8)** (2008) 2714-2721.

[19] D.D. Kitts and K. Weiler, *Current Pharmaceutical Design*. **9** (2003) 1309-1323.

[20] S. Storcksdieck, G. Bonsmann, and R.F. Hurrell, *Journal of Food Science*. **72(1)** (2007) 19-29.

[21] A. Saigo, S. Tanabe, and T. Nishimura, *Journal of Agricultural and Food Chemistry*. **51(12)** (2003) 3661-3667.

[22] F. Shahidi and M. Naczk, Editors, *Food Phenolics: Sources, Chemistry, Effects and Applications*, Technomic Publishing Company, Inc. Lancaster. (1995), pp.13-17.

MECHANICAL AND MOISTURE BARRIER PROPERTIES OF SOY PROTEIN FILM INCORPORATED WITH PHENOLIC-CONTAINING EXTRACTS FROM MULBERRY LEAVES

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Abstract: Phenolic compounds have been shown to possess protein cross-linking ability and, therefore, could be used to improve mechanical properties of a protein film. This study aimed to investigate the effect of different degrees of oxidation of phenolic-containing mulberry leaf extract on mechanical as well as barrier properties of soy protein isolate film. Water extracts of mulberry leaves at three different degrees of oxidation, namely unoxidized (UNOX), mildly oxidized (MILD-OX) and highly oxidized (HIGH-OX), were added to the film-forming solution at three different levels (2, 5 and 10% by weight of protein). UNOX was obtained using water extraction of steamed mulberry leaves to inactivate any oxidizing enzyme endogenous to the leaves, especially polyphenol oxidase. MILD-OX was the obtained from unheated leaves while HIGH-OX was obtained by 60-minute aeration of the unheated-leaf extract. It was found that the film samples containing 10% UNOX, 2, 5, 10% MILD-OX and 2 or 5% HIGH-OX exhibited significantly increased tensile strength ($p \leq 0.05$) as compared to the control. On the other hand, elongation at break was not affected by the extract incorporation. Cross-linking of the soy protein as a result of mulberry phenolics was confirmed by SDS-PAGE. A decrease in water vapor permeability was also demonstrated in the samples containing the extracts. Phenolic-containing extracts from mulberry leaves, especially those with higher degrees of oxidation, could therefore be used to improve the tensile strength and moisture barrier property of soy protein isolate film.

1. Introduction

Due to environmental impact of non-biodegradable waste, biodegradable packaging materials have been growing of interest in recent years. Several biopolymers, including protein from various sources were proved to be a good candidate as raw material for biodegradable film. However, protein film, in general, exhibits poor mechanical properties as compared to plastic films, thereby limiting its uses. Improvement of mechanical properties can be achieved via cross-linking of the protein to produce a strong network structure. Aldehydes, such as formaldehyde, glutaraldehyde and glyoxal, were reported to be effective protein cross-linkers by reacting with amino and sulfhydryl side chains of the polypeptide [1]. In spite of that, safety of aldehyde-added film is still questionable [2].

Many polyphenols naturally occurred in plants are known to react with side chain amino group of peptides, leading to the formation of protein cross-link [3]. Mulberry leaves contain phenolic compounds, with the majority being *p*-coumaric acid, benzoic acid, cathechin, and chlorogenic acid [4] and may therefore be used to improve protein film strength. Additionally, phenolic at different states of oxidation may differ in its ability to cross-link protein. The capability of phenol to be oxidized to a quinone structure was reported to be the important factor underlining its reactivity towards protein [5].

This study therefore aimed to investigate the effect of phenolic-containing mulberry leaf extract at different degrees of oxidation and concentrations on mechanical and moisture barrier properties of soy protein isolate film.

2. Materials and Methods

2.1 Materials

Soy protein isolate (≥ 90% protein) was purchased from Mighty International (Bangkok, Thailand). Glycerol was a product of Ajax Finechem (Taren Point, Australia). Mulberry (*Morus alba* L.) leaves were obtained from Queen Sirikit Sericulture Center (Nan, Thailand).

2.2 Preparation of phenolic-containing mulberry leaf extract

Mulberry leaf extract was prepared using a modified method of that described earlier [6]. For UNOX extract, the leaves were steamed at 90 ^{o}C for 5 min, to inactivate oxidizing enzymes indigenous to the leaves, before extraction. Fresh mulberry leaves were used for MILD-OX and HIGH-OX extraction. To prepare extracts, the leaves were cut and ground with distilled water at room temperature using a leaf-to-water ratio of 1:3. The slurry was then filtered through several layers of cheese cloth and the filtrate was centrifuged at 3000 rpm for 10 min to obtain a MILD-OX extract. For the HIGH-OX extract, the extract obtained using the same method as MILD-OX was further undergone 60-min air bubbling to accelerate the oxidation of phenolic compounds to corresponding quinone. After obtaining the extract, it was freeze-dried, sealed in an aluminum foil-laminated bag together with a satchel of silica gel and oxygen absorber, and kept in a dessicator until needed.

2.3 Determination of total phenolic content

The mulberry leaf extracts were determined for their total phenolic content spectrophotometrically according to the method described earlier [7]. Dry extract powder (0.25 g) was solubilized in 50 mL distilled water. A 0.1 mL aliquot was then mixed with 7 mL of distilled water and oxidized with 0.5 mL of Folin-Ciocalteau's reagent. The reaction was neutralized with 1.5 mL of sodium carbonate solution and incubated for 2 h at room temperature. The absorbance of the resulting blue color was measured at 765 nm using UV/Vis spectrophotometer (model V-530, Jasco, Easton, MD). Total phenolic content was expressed as mg gallic acid equivalents/g of extract powder.

2.4 Film preparation

Film samples were prepared using a modified method of that described previously [8]. Film forming solution was prepared by solubilizing 5 g of soy protein isolate to 75 mL of 0.05 M Tris-HCl buffer (pH 8.0). Glycerol, at a concentration of 55% by weight of soy protein isolate, was used as a plasticizer. The mixture was homogenized using a Ystral homogenizer (Model X10/25, Ballrechten-Dottingen, Germany) at 22,000 rpm for 1 min until a homogeneous solution was obtained. The solution was heated at 70 ^{o}C for 30 min to partly denature the soy protein. The extract powder was added at 2, 5 and 10% by weight of soy protein isolate. To prepare the extract-containing film-forming solution, the extract powder was solubilized in 25 mL of 0.05 M Tris-HCl buffer (pH 8.0) and then added to the plasticizer-containing protein solution. The solution was homogenized again at 22,000 rpm for 2 min. The solution obtained was then undergone ultrasonic degassing technique to remove air bubbles. An aliquot (45 mL) of the film-forming solution was then casted onto 15 cm-square acrylic plate and let dry at 25 ^{o}C for 24 h. The film was then peeled off and stored at 25 ^{o}C in a chamber with 50% relative humidity for a period of 48 h before undergoing further analyses.

2.5 Mechanical properties

Tensile properties (tensile strength and elongation at break) of the film samples were determined using universal materials testing machine (Model 5565, Instron, Norwood, MA) equipped with a 5-kg load cell. A 30 mm×100 mm strip of film sample was measured using a pneumatic side-action grips probe (Instron, Norwood, MA).The film sample was pulled at a constant rate of 5.0 mm/s until failure.

2.6 SDS-PAGE

Protein pattern of the film samples was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using modified method of that described elsewhere [9] with some modifications. Film sample (0.3 g) was dissolved in 4 mL of extracting solution (60 mM Tris-HCl (pH 7.5) containing 2% (w/v) SDS). The mixture was stored at room temperature for 12 h and boiled for 3 min. Supernatant was obtained after centrifugation at 1000 rpm for 15 min. Protein content of the supernatant was determined [10]. The supernatant was then mixed with sample buffer (0.5 M Tris-HCl (pH 6.8) containing 10% (w/v) SDS, glycerol, β-mercaptoethanol and 1% (w/v) bromophenol blue) at a ratio of 7:3 (v/v). Sample (20 µg protein) was loaded onto the polyacrylamide gel made of 4.5% stacking gel and 10% separating gel. The electrophoresis was carried out in Hoefer electrophoresis unit (Model miniVE, Hoefer, Holliston, MA) using a current of 20 mA/gel. After that, the gel was stained with Coomassie blue R-250 in 50% (v/v) ethanol and 10% (v/v) acetic acid and destained with 25% (v/v) ethanol and 10% (v/v) acetic acid. Wide range molecular weight protein markers (Sigma-Aldrich, Munich, Germany) were used to estimate the molecular weight of the proteins.

2.7 Water vapor permeability

Water vapor permeability (WVP) was determined using an ASTM standard method [11]. The film was mounted over a glass permeation cup containing silica gel (0% relative humidity) and sealed with silicone vacuum grease. An O-ring was used to hold the film in place. The cup was then placed in a chamber containing distilled water and stored at 25 ^{o}C. Weight gain of the cup was determined at 2-h interval for a period of 30 h. WVP was calculated using Equation (1):

$$WVP = w\,x/A\,t\,(P_2-P_1) \quad \ldots(1)$$

Where w is the weight gain (mg), x is the film thickness (mm), A is the area of the exposed film (m^2), t is the time (h) and P_2-P_1 is the vapor pressure differential across the film (Pa).

2.8 Statistical analysis

Experiments were done in triplicate. A completely randomized design was used for all experiments. Data were analyzed using Analysis of Variance. A Duncan's new multiple range test was used to determine the difference among sample means at p=0.05.

3. Results and Discussion

3.1 Total phenolic content of extract powder

Total phenolic content of mulberry leaf extract powders are show in Table 1. Total phenolic contents of UNOX, MILD-OX and HIGH-OX, expressed as gallic acid equivalent, were 75.3, 72.4 and 59.2 mg/g extract powder, respectively. The decrease in total phenolic content with increasing degree of oxidation was a result of the conversion of phenolic compounds into their respective quinones.

3.2 Mechanical properties

Tensile strength and elongation at break of the film samples were illustrated in Figures 1 and 2.

Table 1: Total phenolic content of mulberry leaf extract powder at different degrees of oxidation.

Extracts	Total phenolic content*
Unoxidized	75.3 ± 0.336
Mildly-oxidized	72.4 ± 0.336
Highly-oxidized	59.2 ± 0.767

*Expressed as mg gallic acid equivalents/g of extract powder
Values are given as means ± SD (n=3).

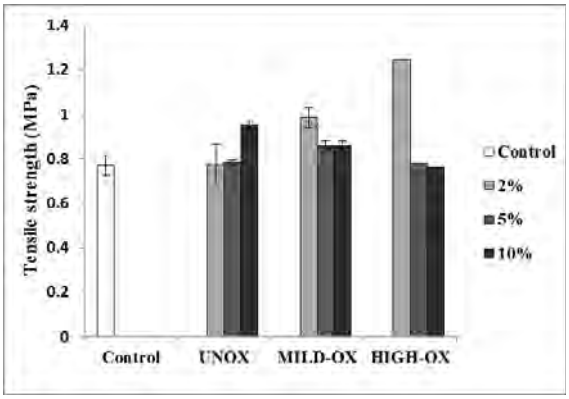

Figure 1. Tensile strength of the film samples containing no extract (control), unoxidized extract (UNOX), mildly oxidized extract (MILD-OX) and highly oxidized extract (HIGH-OX) at 2, 5 and 10% by weight of soy protein isolate.

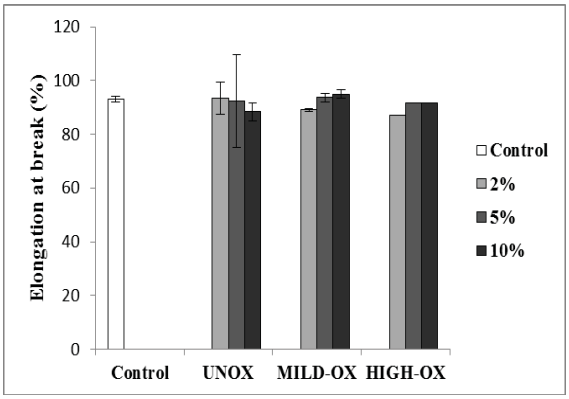

Figure 2. Elongation at break of the film samples containing no extract (control), unoxidized extract (UNOX), mildly oxidized extract (MILD-OX) and highly oxidized extract (HIGH-OX) at 2, 5 and 10% by weight of soy protein isolate.

It was found that the 2 and 5% UNOX films exhibited similar tensile strength to the control soy protein isolate film (Figure 1). However, the 10% UNOX sample possessed significantly higher tensile strength than the control ($p \leq 0.05$). At 2% extract powder fortification, the MILD-OX and HIGH-OX films exhibited an even higher tensile strength, with the 2% HIGH-OX possessed 61.47% increase in tensile strength as compared to the control. The increment in tensile strength could be linked to the formation of a more stable network due to attractive interactions between protein and phenolics. Phenolic compounds could be converted to quinone, a protein cross-linker, in which new covalent cross-link could be formed. Quinones react with amino or sulfhydryl side chains of polypeptides to form covalent C-N or C-S bonds [3]. Oxidized phenolic might contribute to the formation of non-disulfide covalent bond. Thus, the incorporation of oxidized extract effectively improved tensile strength of resulting film [12].

It should be noted here that higher levels (5 and 10%) of MILD-OX and HIGH-OX extract powder addition resulted in inferior tensile strength as compared to 2% addition. The decreased tensile strength might be associated with self-aggregation of phenolic compounds, leading to the loss in capability to cross-link protein. High concentration of phenolic compounds was shown in earlier study to exhibit lower efficiency in interacting with protein [13].

For elongation at break (Figure 2), extract addition seemed to pose less effect on the property, as compared to the tensile strength.

3.3 SDS-PAGE pattern

Protein pattern of the film samples under reducing condition are shown in Figure 3. As compared to the control, the films added with extract powder exhibited lower intensity of the bands representing lower molecular weight proteins. This suggested the cross-linking of proteins induced by phenolic compounds. Incorporation of 10% UNOX, 2% MILD-OX and 2% HIGH-OX resulted in the noticeably decrease in band intensity, implying extensive protein cross-linking in these samples. Non-disulfide covalent bond, disulfide bond as well as other weak bonds were suggested to contribute to the film strengthening effect [14].

3.4 Water vapor permeability

Addition of extract powder into soy protein film significantly lowered its WVP ($p \leq 0.05$) (Figure 4). Increasing amount of cross-links via hydrogen and hydrophobic interaction might produce a protein network with decreased free volume of the polymeric matrix. This, in turn, resulted in the lower WVP of the film [15].

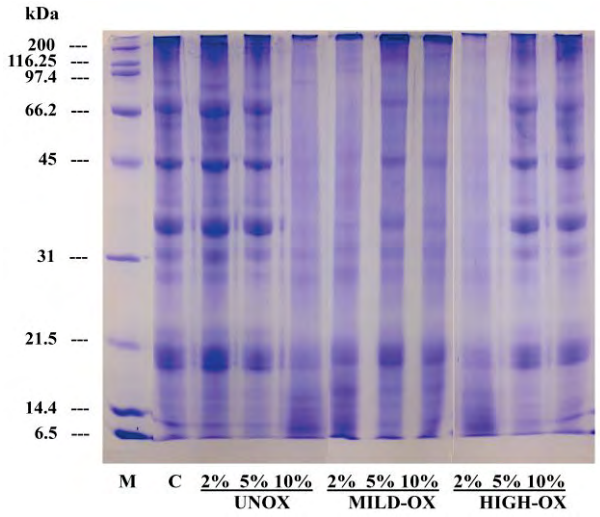

Figure 3. Protein patterns of soy protein films added with mulberry leaf extract at different degrees of oxidation; protein marker (M), control without extract (C), samples with unoxidized extract (UNOX), samples with mildly oxidized extract (MILD-OX) and samples with highly oxidized extract (HIGH-OX).

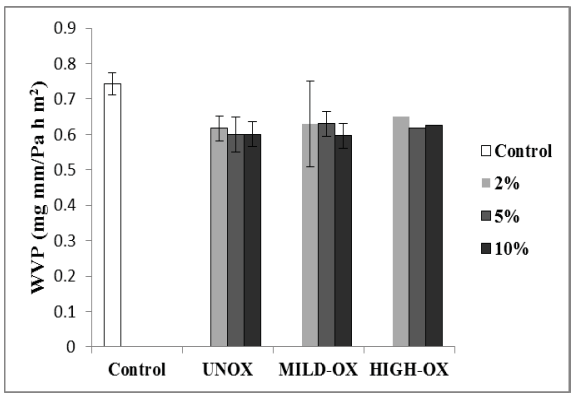

Figure 4. Water vapour permeability of the film samples containing no extract (control), unoxidized extract (UNOX), mildly oxidized extract (MILD-OX) and highly oxidized extract (HIGH-OX) at 2, 5 and 10% by weight of soy protein isolate.

Conclusion

Incorporation of mulberry leaf extracts, especially at higher degrees of oxidation could effectively induce cross-linking of soy protein. This resulted in a film with increased tensile strength and decreased water vapor permeability. However, higher levels of extract addition, especially in the case of MILD-OX and HIGH-OX, may result in inferior tensile strength. Increased protein cross-linking in the sample containing oxidized extract was attributed to the conversion of phenolic compounds to quinones which are effective protein cross-linker.

References

[1] C. Marquié, A.M. Tessier, C. Aymard and S. Guibert, *J. Agric. Food Chem.* **45** (1997) 922–926.
[2] G. Galietta, L. Di Gioia, S. Guilbert and B. Cuq, *J. Dairy Sci.* **81** (1998) 3132–3130.
[3] G. Strauss and S.M. Gibson, *Food Hydrocolloids.* **18** (2004) 81–89.
[4] P. Wanyo, S. Siriamornpun and N. Meeso, *J. Agric. Env. Sci.* **6** (2009) 470–479.
[5] H.M. Rawel, D. Czajka, S. Rohn and J. Kroll, *Int. J. Biol. Macromol.* **30** (2002) 137-150.
[6] M. Naczk, M. Townsend, R. Zadernowski and F. Shahidi, *J. Food Chem.* **128** (2011) 292–298.
[7] A.L. Waterhouse, in: R.E. Wrolstad, T.E. Acree, E.A. Decker, M.H. Penner, D.S. Reid, S.J. Scgwartz, C.F. Shoemaker, D.M. Smith and P. Sporns (Eds.), *Handbook of Food Analytical Chemistry*, Wiley Interscience, London (2005), pp. 461-470.
[8] Y. Jiang, C.H. Tang, Q.B. Wen, L. Li and X.Q. Yang, *Innov. Food Sci. Emerg.* **8** (2007) 218–225.
[9] U.K. Laemmli, *Nature* **227** (1970) 680–685.
[10] G.L. Peterson, *Methods in Enzymology* **91** (1983) 95–119.
[11] ASTM, *Annual Book of ASTM Standards*, the American Society for Testing and Materials, Philadelphia, PA (1999).
[12] S.D. Hoque, S.W. Benjakul and T.N. Prodpran, *Food Hydrocolloids.* **25** (2011) 1085–1097.
[13] V. De Freitas and N. Mateus, *J. Agric. Food Chem.* **49** (2001) 940–945.
[14] T.N. Prodpran, S.W. Benjakul and S.R. Phatcharat, *J. Biol. Macromol.* **51** (2012) 774–782.
[15] A. Gonzalez, M.C. Strumia and C.A. Igarzabal, *J. Food Eng.* **106** (2011) 331–338.

INVESTIGATION OF ACETALDEHYDE IN POLY(ETHYLENE TEREPHTALATE) PET BOTTLES INFLUENCING COLOR CHANGE IN FISH SAUCE

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Abstract: Poly(ethylene terephtalate) PET bottles are commonly used as packaging for Thai fish sauce due to it is less expensive and lighter weight than glass bottles. However, PET bottles still some disadvantages and discoloration in fish sauce is thus far one of the major problems. The color change obviously caused consumer misunderstanding that fish sauce started degrading and becoming unsafe to consume. Previously, several studies reported that reaction between aldehyde from natural source such as glucose degradation with protein in fish sauce was one of the factors leading to discoloration through Millard reaction. Additionally, it was known that PET undergoes 1,5-sigmatropic rearrangement at elevated temperature releasing acetaldehyde trapping inside itself during extrusion blow molding process. To confirm whether acetaldehyde entrapped in PET bottle could contribute to any discolorations in fish sauce, the amount of acetaldehyde trapping inside bottles needed to be evaluated. In this study, Thermal gravimetric analysis mass spectrometer (TGA-MS) and gas chromatography-flame ionization (GC-FID) were employed and it was found that TGA-MS of PET sheet with thickness range from 0.45-0.55 mm (1x1 cm^2) showed a weight loss approximately 0.16% and the acetaldehyde trapping in the PET sheet was not detected under this procedure. To reconfirm the results, a purge and trap technique using GC-FID was further applied. Interestingly, it was found that acetaldehyde was detected in the range of 0.248 to 0.360 (ng mL^{-1}) in PET bottle with SD < 0.039 (n=9). With such a very low concentration of acetaldehyde detected in PET bottle, it was clearly shown that Millard reaction between acetaldehyde trapping in PET bottle and proteins in fish sauce should not affect the drastic color change in fish sauce over periods of 8 months and this might assume that acetaldehyde trapping in PET bottle was not a main factors for discoloration in fish sauce.

1. Introduction

Browning of food is commonly found during processing and storage especially during manufacture of meat, fish, and vegetable products. Browning usually impairs the sensory of products due to associated changes in the color, flavour, and softening besides nutritional properties. [1] Browning effect is originated from both enzymatic and non-enzymatic oxidation of phenolic compounds [2] as well as from Maillard reaction (Figure 1) that occurs when mixtures of amino acids and reducing sugars are heated. [3]

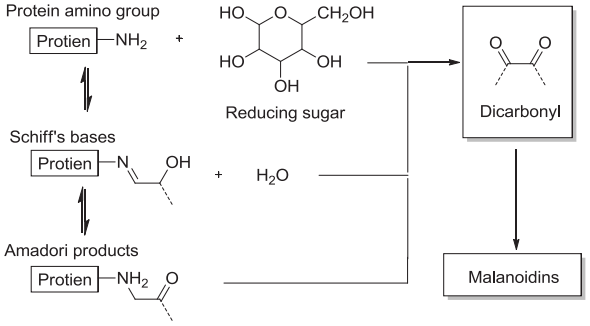


Figure 1 Mechanism of Millard reaction between reducing sugar with amino group of protein.[4]

Fish sauce is a condiment that is the most consumed in Thailand. [5] In the past, fish sauce, the important seasoning in the household and industry, plays an important impact to both local and international economy especially, fishery industry and salt pan. Generally, it has been known that each Thai people regularly consume fish sauce approximately 17-20 ml per day. Additionally, it was discovered that businesses that consume fish sauce at the highest rate were restaurants and eateries and this caused the expansion of fish sauce production. [6] Thus far, the total values of fish sauce were over 1 billion baht annually for both domestic consumption and export. More than 95 percent of production is for local consumption and the rest is exported to the major trade partners such as U.S.A., Japan, Australia, Hong Kong and France. [7] Fish sauce is a condiment that is the most consumed in Thailand. With the largest production and total values over 7,500 million baht annually for domestic consumption and export, Thai's fish sauce industry is perhaps the world's biggest. [6] During this process sucrose can degrade, through the caramelization reaction, leading to non-enzymatic browning and thus colour changes.

Characteristic color of fish sauce is red-brown, browning results from both enzymatic browning reaction and non-enzymatic browning reaction. Browning can occur during processing and product storage. In the case of fish sauce, browning occurs from non-enzymatic causing by three types of reactions; 1) the reaction of carbonyl groups (reducing sugar, aldehydes, ketones, lipid oxidation products) and amino compound (lysine, glysine, peptide, amine,

ammonia proteins), [3] 2) ascorbic acid browning that is the spontaneous thermal decomposition of ascorbic acid under both aerobic and anaerobic conditions either in the presence or absence of amino-compound and 3) lipid browning which is probably oxidative of unsaturated deterioration of glyceride components followed by polymerization which is accelerated by the presence of ammonia, amines or proteins. [8]

In Thailand, fish sauce is generally prepared by adding one part of salt to three parts of small fish such as anchovies or sardines and allowing them to become soluble in closed tanks at room temperature. When most of the fish tissue has been solubilized, the liquid fish sauce is then drained off and filtered to give a clear amber solution or first grade fish sauce. After that, it is usually packed in glass bottles and then transports to consumers via either trucks or ship. The shipment cost is depending on the volume and weigh of fish sauce. Therefore, most of capital cost for fish sauce production is mainly contributed from the weight of glass bottle.

In order to reduce such a high trasportation cost, new container for fish sauce is introduced to the industry. Expectedly, plastic bottle becomes the most attractive choice especially, PET bottles, due to their light weight, durability and cheap. It has been estimated that the PET bottles have been used for packing fish sauce more than 6 million bottles per year. [9]

After employment of PET bottle for a certain period of time, it was found that the shelf-life of the fish sauce is shorter than those in glass bottle using the color change from brown to dark brown as indicator which is easily noticeable with eyes. Interestingly, it was found that the discoloration of fish sauce in plastic bottle occurred within 8 months compared with 24 months in glass bottle leading to an economic loss for both producers and consumers. For producers, it obviously affects consumer's purchasing judgment. For consumers, color of fish sauce changes rapidly once opened and become too dark to consume with the concerns whether it is safe to consume.

Generally, Poly(ethylene terephthalate) (PET) bottles are widely used for beverages especially for mineral water. For this application, high levels on the neutrality of odor and taste of the packaging materials are required. For mineral water in PET bottles, the migration of acetaldehyde is of special interest, because acetaldehyde is detectable in low concentrations in mineral water as a fruity of flavor. Acetaldehyde that found in PET bottles is a degradation product of the PET polymer during pre-form production. After cooling down, acetaldehyde is trapped in the PET bottle wall and migrates into the mineral water after filling and storage. The proposed acetaldehyde formation mechanism is given in Figure 2.

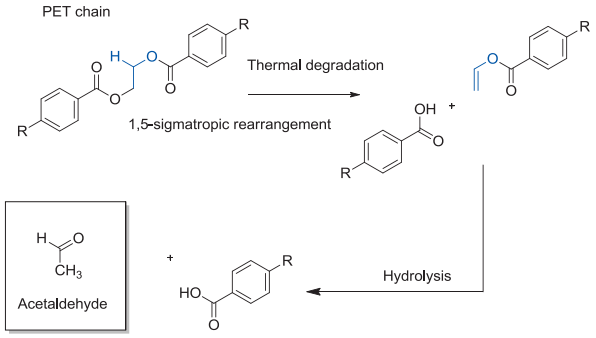


Figure 2 Proposed mechanism of the formation of acetaldehyde from PET polymer during perform production [10]

With such a different in size of PET bottles, employment of raw material and processing procedures should affect to the amount of trapped acetaldehyde in PET bottles that produce in Thailand. In this investigation, the amount of acetaldehyde trapping in PET bottles with size of 750 mL which is a normal size for packing the fish sauce in Thailand were measured in order to observe whether the trapped acetaldehyde in PET bottles could contribute to discoloration in Thai fish sauce.

2. Materials and Methods

2.1 Thermogravimetric analysis-mass spectrometry (TGA-MS)

PET sheet with a size of 1x1 cm^2 was cut from PET bottles size of 750 mL. Thermogravimetric analysis-mass spectrometry (TGA-MS) series mettle-toledo cirrus 2 was conducted at Mettle-Toledo Laboratory with heating rate at 500 ^oC / min from 30-200 ^oC and flow rate of liquid Nitrogen was 50 ml/min.

2.2 Gas chromatography-flame ionization detector (GC-FID) [11]

The sample preparation part is consisted of a laboratory-built heating box. PET bottles was flushed with nitrogen gas for 1 min. and the incubate at 60 ^oC for one hour.[12] The heating process is accelerated the releasing of acetaldehyde. After that, nitrogen gas and residue wwas then transferred to GC-FID (Agilent 6890N series) by vacuum pump. The injector port was modified by integrating a six-port valve (gas sampling valve) that acted as an injector. The DB-1 (15m×0.53 mm× 0.32 μm) capillary column coupled with GC-FID (Agilent 6890N series) was used for analysis. (Figure3)

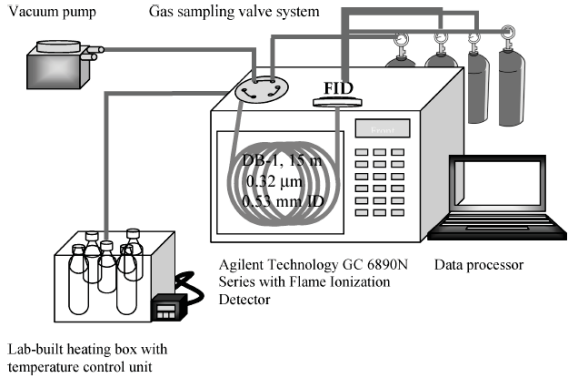


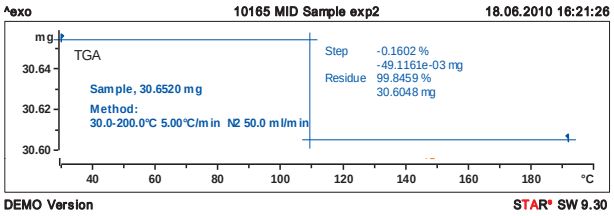
Figure 3 Instrumentation for acetaldehyde analysis using a flame ionization detector [11]

3. Results and Discussion

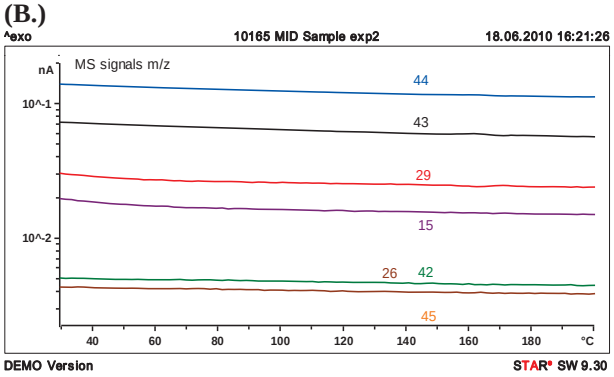
3.1 Determination of acetaldehyde via thermogravi-metricanalysis-mass spectrometry (TGA-MS)

Figure 4 (A.) showed the TGA result of Poly(ethylene terephthalate) (PET). The plot showed the mass as a function of sample temperature for the Poly(ethylene terephthalate) (PET) under a nitrogen purge. Approximately 30.652 mg of sample was heated at a rate of 50 ^{o}C/min. The TGA result displayed that the Poly(ethylene terephthalate) (PET) underwent thermal degradation beginning at 109 ^{o}C and the sample showed a gradual weight loss step from 30 °C to 200 °C. The absolute weight loss of the sample was up to 49 µg corresponding to a percentage of 0.16%. The mass fragments of acetaldehyde were displayed in Figure 4(B.) were monitored in the MID (multi-ion detection) mode with patterns mass signal of acetaldehyde m/z = 29, 44, 43, 42, 26, 15 and 45.

(A.)



(B.)



(C.)

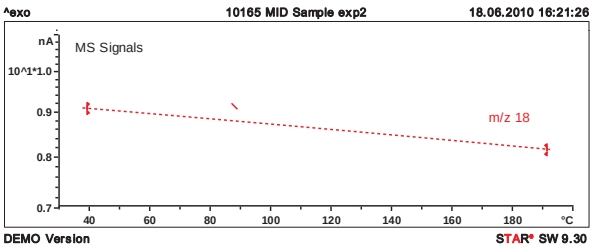


Figure 4 (A)TGA curve of the PET sheet with size of 1x1 cm^2 from PET bottles size 750 mL, (B) Mass fragments of acetaldehyde patterns (C) Mass fragments from the PET sheet

During the experiment, no apparent information was observed which indicates the release of acetaldehyde (Figure 4C) because there is lack of evidence in the MS signals of acetaldehyde. The only mass that was detected is the signal of mass fragment of water (m/z 18). A broad peak was observed within the temperature range corresponding to the weight loss. This indicated that moisture was evolved in the whole weight loss process. The possibility exists that a very small amount of acetaldehyde (below the detection limit of procedure and detection method) was possibly evolved during the weight loss process.

Therefore, the developed rapid and sensitive method by using a lab-built heating system coupled with GC-FID for qualitative and quantitative analysis of acetaldehyde residue in PET bottles with good precision was then further selected for next investigation. The advantages of the method were simple did not require any solvent and had a low detection limit, can be used with industrial applications and is effective for analyzing with a wide range of sizes of bottles.[11]

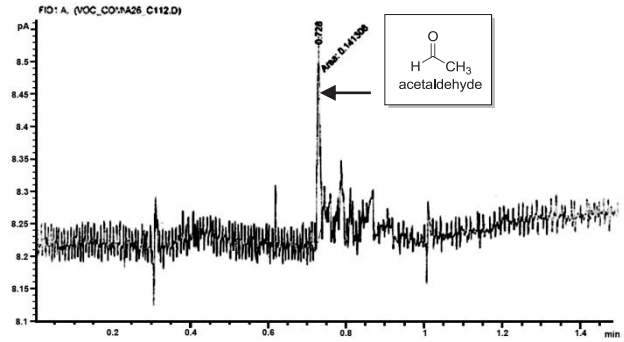


Figure 5 Chromatogram of acetaldehyde in PET bottles.

3.2 Gas chromatography-flame ionization detector (GC-FID) [11]

Heating PET bottles at 60 ^{o}C for one hour to stimulate the movement of PET polymer chain to releasing trapping acetaldehyde, Chromatogram of

acetaldehyde from PET bottles was showed in Figure 5 with a retention time at 0.72 min. In order to calculate the amount of acetaldehyde releasing from PET bottles, it can be determined from the peak height from the equation below [13] and the results was shown in Table 1.

peak height (pA) = 9.82[concentration $_{(ng\ mL^{-1})}$] – 2.37

Table 1 Concentration of Acetaldehyde in PET bottles

Sample	Ret. Time (min)	Area (pA*s)	Height (pA)	Conc. (ng mL^{-1})
1	0.731	0.069	0.155	0.250
2	0.726	0.052	0.133	0.248
3	0.728	0.141	0.322	0.268
4	0.729	0.113	0.265	0.262
5	0.727	0.055	0.154	0.251
6	0.728	0.392	0.828	0.320
7	0.727	0.339	0.730	0.310
8	0.727	0.315	0.740	0.311
9	0.727	0.550	1.223	0.360

From Table 1, it was shown that the average retention of acetaldehyde was approximately 0.728 and the peak height was ranged from 0.133 to 1.223. The concentration of acetaldehyde trapping in PET bottles was 0.248 - 0.360 ng.mL^{-1}. From Millard reaction, trace amount of acetaldehyde that released from PET bottles would eventually react with amino acid in fish sauce producing a trace amount of browning products in a short period of time and leading to slightly change in color of fish sauce. By monitoring the color change in fish sauce by industrial sector, the browning process in fish sauce was however gradually increased over a period of 8 months. Therefore, it can assume that active aldehyde derivatives still continuously generated in the fish sauce and causing discoloration. Clearly, the acetaldehyde trapping in PET bottles should not be associated under the color change incident due to such a low concentration that observed from this investigation.

4. Conclusions

The investigation of acetaldehyde in poly (ethylene terephtalate) PET bottles found that PET undergoes 1,5-sigmatropic rearrangement at elevated temperature releasing acetaldehyde trapping inside PET bottles during extrusion blow molding process. However, concentration of detected acetaldehyde in PET bottles was very low in the range of 0.248 - 0.360 ng.mL^{-1}. It could assume that discoloration of fish sauce in PET bottles over periods of 8 months was not mainly contributed from acetaldehyde trapping in PET bottles.

Acknowledgements

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References

[1] D.Bastos, E.Monaro and M.Sefaro, *Food Industrial Processes-Methodes and Equipment*, **15** (2006) 281-300

[2] J. Agric, *Food Chem.*, **44 (3)** (1996), 631–65

[3] C.G.A.Davies and T.P.Labusa, *Food Industrial Process*, **3** (2000) 1-33

[4] P. Cloos and S.Christgau, *Biogerontology*, **5** (2004) 139-158.

[5] http://www.foodnetworksolution.com/vocab/wordcap//%E0%B8%99%E0%B9%89%E0%B8%B3%E0%B8%9B%E0%B8%A5%E0%B8%B2 (Retrieved December 8, 2012).

[6] http://www.chiantaveecom/index.php?mo=3&art=193588 (Retrieved December 8, 2012).

[7] http://www.awesomethaifood.com/th/fish-sauce-6/ (Retrieved December 8, 2012).

[8] S.Benjamin, *Foodbiochemistry and food processing*, **2nd** (2000)69-75.

[9] S.Kaleemullah, *Food Packaging*,(2001) 5-10

[10] J.Ewender and F.Welle, *Fraunhofer Institute for Process Engineering and Packaging (IVV)*, **35**, 85354

[11] A.Choodum, T.Thavarungkul, P.Kanatharana, *Environmental Science and Health*, **45** (2007) 577-583

[12] Local soft drink company, Airspace *Acetaldehyde Test*,1999.

[13] G.Linssen, H.Reitsma,and G.Cozynsen, *Z. Lebensm. Untersuch. Forsch.* (1995) 201–203.

PRETREATMENT OF COTTON FABRIC WITH SOY PROTEIN TO IMPROVE DYEABILITY OF JACKFRUIT WOOD EXTRACT (MORIN DYE)

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Abstract: The effect of soy protein on dyeability of jackfruit wood extract on cotton fabrics was studied. At first, crude soybean milk as a source of soy protein was treated with 5 g/L NaOH to depolymerize soy protein and then diluted to obtain various solid contents of 5, 10, 15, 20, 25, and 30 g/L. Thus obtained soybean milk was applied onto cotton fabrics. In the mean time, jackfruit wood extract was prepared using various liquor ratios of 1:10, 1:20, 1:30 under the condition as follows: temperature of 100˚C and time for 1 hour. Soy protein treated cotton fabric was then dyed with jackfruit wood extract at temperature of 60˚c for 45 min. The effect of soy protein concentration on dyeability and fastness properties (wash fastness, light fastness and rubbing) of jackfruit wood extract were studied. The result showed that an increase in concentration of soy protein resulted in an increase in colour strength. This indicated that Morin dye flavourably interacted via hydrogen bonding with soy protein presented in the cotton fabric, leading to dyeability enhancement.

1. Introduction

Nowadays, natural dyes are attractive for textile dyeing for the reason of growing health concern associated with synthetic dyes. However, the dyeing of natural dyes still cannot be claimed to be the ecofriendly dyeing process due to the typical usage of toxic mordants such as copper sulphate. Mordants are toxic but important to nature dyes in terms of fastness properties enhancement. Even though, natural dyes exhibit biodegradability and compatibility with the environment but the application of mordants with natural dyes do more harm than good. In search for environmentally friendly mordant, attention has been drawn to metal ion-free mordants such as tannin [1]. Tannin enhances fastness of natural dyes through complexation mechanism. Like tannin, proteins such as silk and wool exhibit higher natural dye uptake than cellulose. Thus, in this work, soy protein was investigated to improve dyeability of jackfruit extract on cotton fabric.

Jackfruit is classified as *Artrocarpus heterophyllus Lam*. Historically, the wood of the jackfruit tree has been used for dyeing silk and cotton fabrics. The main colour component in the jackfruit wood is morin. (Figure 1.) In Thailand, jackfruit wood extract is well-known for yellow colouring of hand-made textiles. However, its usage is not popular due to low fastness property.

In this work, the effect of soy protein on dyeability of jackfruit wood extract on cotton fabrics was studied. Soybean milk as a source of protein was pretreated onto cotton fabric prior to dyeing with jackfruit wood extract. Colour strength as well as wash fastness were evaluated.

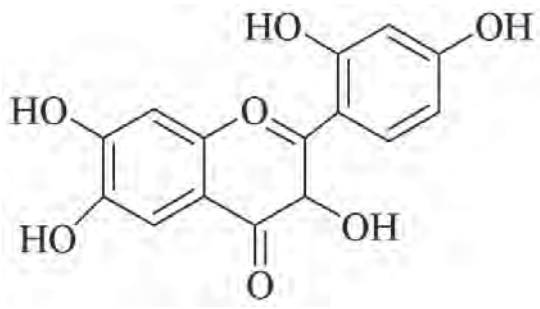


Figure 1. Chemical structure of Morin

2. Materials and Methods

2.1 Materials

Bleached cotton knit fabric was purchased from Boonchuay Industrial Co., Ltd (Thailand). Sodium hydroxide was purchased from Ajax Finechem. Jackfruit wood was obtained from local source. Crude soybean milk was prepared from soybean seed with addition of citric acid to prevent spoiling.

2.2 Pretreatment of cotton fabric

Soybean milk was treated by 5 g/L sodium hydroxide under continuous stirring for half an hour. Then treated soybean milk was diluted to obtain various concentrations of 5, 10, 15, 20, 25, and 30 g/L. The scoured and bleached cotton fabric were immersed into the soybean milk solution and squeezed by the padder roller to obtain about 80 % wet pick-up and then dried at room temperature. The presence of soy protein on fabric surface was analyzed using ATR/FTIR technique.

2.3 Extraction of jackfruit wood and dyeing cotton fabric

Jackfruit wood extract was prepared using various liquor ratios of 1:10, 1:20, 1:30 under the condition as follows: temperature of 100˚C and time for 1 hour. Then treated cotton fabric was dyed with jackfruit wood extract under the condition as follows: temperature of 60˚C and time for 45 min. After dyeing, dyed cotton fabric was washed thruoghly and dried in open air.

2.4 Percent nitrogen content determination on fabric

The percents of nitrogen content on fabric were measured by Elemental Analyzer (PerkinElmer PE2400 seriesII)

2.5 Colour measurement and fastness determination

Colour strength of dyed fabric was evaluated using reflectance spectrophotometer (Machbeth colour-EYE 7000) as expressed by *K/S* and L* a* b*. The colour fastness of dyed fabric was assessed according to the standard method including ISO 105-C06 2010 (A1M) for wash fastness, AATCC16E for light fastness and AATCC Test Method 8-2007 for rubbing.

3. Results and Discussion

3.1 The analysis of soy protein on cotton fabric surface by ATR/FTIR

The soy protein treated fabric was analyzed by ATR/FTIR spectroscopy to confirm the existence of soy protein on the fabric surface. The representative spectra are shown in Figure 2.

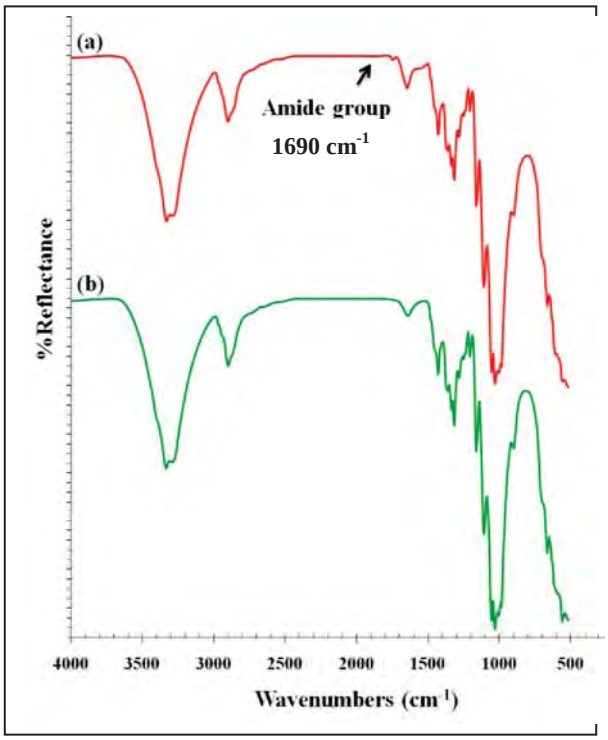


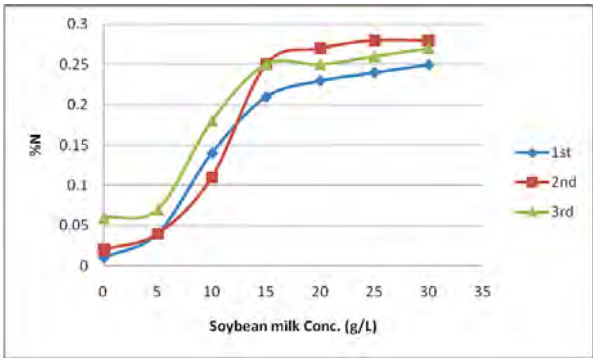
Figure 2. ATR/FTIR spectra of (a) soy protein treated cotton fabric (b) cotton fabric

The spectrum of cotton fabric treated with soy protein exhibits absorption peak at 1690 cm^{-1} corresponding to amide group of protein.

3.2 Application of soy protein onto cotton fabric

Depolymerized soy protein were applied onto cotton fabric. The soy protein content on the fabric surface were estimated via nitrogen determination. The results are showed in Figure 3. From Figure 3, it is found that the nitrogen content slightly increase as the applying concentration of soybean milk increases. These indicated that the nitrogen content increases with an increase in concentration level of soy protein. The average percents of nitrogen content on fabric about 0.25

Figure 3: The nitrogen content on 5.0g cotton fabric



3.3 The effect of soy protein on K/S and colour difference

Table 1 shows the effect of soy protein on K/S and colour difference of dyed fabrics. The results show that fabric treated with soy protein exhibit an increase in colour strength with an increase in percent soy protein add-on. This indicates that protein on the fabric is able to attract morin dye via hydrogen bonding interaction, resulting in an increase in colour strength. The ΔE* is represented to the colour difference calculated from CIE L*a*b* value. The L* value defines the white-dark range; the higher L* the whiter or lighter. The a* defines the red-green range; the higher a* the more reddish. The b* defines the yellow-blue range; the higher b* the more yellowish. CIE L*a*b* value provides information that change in colour shade of morin dyed fabric is derived from the protein-morin dye interaction. Even though the dyed fabric gave the yellow shade, there were not exactly the same. There were a few differences in each yellow shade.

Table 1: Effect of soy protein on dye properties of dyed fabric

Soy bean conc. (g/L)	*K/S* (410 nm)	L*	a*	b*	ΔE*
untreated	1.8	88.4	-5.9	38.5	0.00
5	1.9	88.4	-6.0	39.1	0.6
10	2.0	88.1	-5.9	40.1	1.7
15	2.1	87.7	-5.7	40.5	2.1
20	2.1	87.6	-5.5	40.5	2.2
25	2.2	87.7	-5.5	39.7	2.5
30	2.3	87.0	-5.4	41.7	3.5

3.3 Effect of soy protein on colour fastness

Table 2 shows the effect of soy protein on colour fastness properties (light fastness, wash fastness and rubbing) of dyed fabrics treated with soy protein and untreated fabric. The results show that there is no difference in fastness properties between treated and untreated dyed fabrics. Light fastness rating of morin dye is 3-4. Wash fastness rating is 1-2 which is considered poor. It could be said that even though protein was capable of attracting morin dye but the attractive force mainly hydrogen bonding was not strong enough to withstand washing, resulting poor wash fastness.

Table 2: Fastness properties

Soybean conc. (g/L)	LF	WF		RF	
		Colour change	staining	Dry	Wet
Untreated	3/4	1/2	4/5	4/5	3/4
5	3/4	1/2	4/5	5	3
10	3/4	1/2	4/5	4/5	3
15	3/4	1/2	4/5	4/5	3/4
20	3/4	1/2	4/5	4/5	3/4
25	3/4	1/2	4/5	4/5	3
30	3/4	1/2	4/5	4/5	3

4. Conclusions

In this study, depolymerization of soy protein using sodium hydroxide was carried out and applied onto cotton fabrics. ATR/FTIR analysis provided evidence to confirm that amide group of soy protein presence on the fabric surface. The nitrogen content slightly increased as the applying concentration of soybean milk increased. Effects of soy protein on dyeing properties and fastness were evaluated. It was found that the colour strength increased with an increase in concentration of soy protein. However, there was no difference in light fastness and wash fastness between dyed treated and untreated dyed fabric due to weak bonding interaction of morin dye.

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References

[1] S.M. Burkinshaw and N. Kumar, *Dyes Pigm*, **80** (2009), pp. 53-60.
[2] A.K. Samanta, P. Agarwal and S. Datta, *Indian J Fibre Text Res*, **33** (2008), pp. 171-180.
[3] S. Houshyar and S.H. Amirshahi, *Iran Polym J.*,***11*** (2002), pp 295-301.
[4] C. Suitcharit, F. Awae, W. Sengmama and K. Srikulkit, *Chiang Mai J. Sci*., **38** (2011), pp. 001-12.
[5] H.T. Deo and B.K. Desai, *JSDC*.,**115** (1999), pp. 224-227
[6] S. Kittinaovarat, *J. Sci. Res.Chula. Univ*.,**29** (2004), pp.155-164.
[7] A.K. Samanta, P. Agarwal and S. Datta, *Indian J. Fibre Text Res*, **32** (2007), pp. 466-476.

EVALUATION OF LIPID OXIDATION OCCURRENCE IN SOYBEAN AND SOYMILK PRODUCTS

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Abstract: Lipid Oxidation is one important reaction in food system.The occurrence of this reaction gives the rancidity in food products. The aim of this work was to analyse the malondialdehyde content in soybean and soy milk products that sold in the market by reverse phase high performance liquid chromatography technique. Malondialdehyde is one of an index to indicate the occurrence of lipid oxidation in food. The soymilk products samples were pasteurized soymilk and UHT soymilk which were brought from fresh market and super market in Bangkok. All those soymilk products were analysed as soon as possible after sampling.The results of the analysis showed the malondialdehyde content in soybean approximately $5x10^{-4}$-$6.0x10^{-4}$ ppm and in soymilk samples approximately 0.07-0.16 ppm.This indicated that the lipid oxidation reaction tend to increase after soybean was processed to soymilk products.

1. Introduction

Lipid peroxidation is one of important reaction in food especially food containing fat. This reaction is associated with cell aging and many chronic diseases such as atherosclerosis ,cancer, inflammation, etc. [1–4].The reactive oxygen species (ROS) can react with double bonds of polyunsaturated fatty acids (PUFAs) in food to yield lipid hydroperoxides. The important oxidation products of peroxidized polyunsaturated fatty acids is malondialdehyde (MDA) [5,6], which has been referred to have mutagenic and cytotoxic effects[7]. It may participate in the atherosclerosis [8]. MDA has been used as biomarker to indicate oxidative stress in cell[7,8]. MDA is also widely used in food sciences as an index of lipid oxidation and rancidity in foods and food products too[9]. There have many works that report the MDA content in food systems as cooking oil[10],fish [11],cow milk[12]. However, the analysis of MDA is most frequently determined by spectrophotometrically as thiobarbituric acid reactive substances (TBARS)[8]. There was no report about the content of MDA in soymilk products as drinking milk. The aim of this work was to study the MDA content in soybean and soymilk products using reverse phase high performance liquid chromatography.The soybean was analysed first to compare the MDA in soymilk products. The results from this research may give the detail about the oxidative stress from soybean in processing by heat.Soybean is the one important cereal for vegetarian people,it contain lipid for 19.94 % [13].The soybean was processed to be many products such as snack , soy milk etc. This work attened to prove the lipid peroxidation occurrence in soybean after processing to soymilk samples. However, there are many types of soymilk products that sold in Thailand market such as pasteurized pure soymilk, pasteurized mixed soymilk with sesame or cowmilk ,UHT pure soymilk ,UHT soymilk mixed with other food as .wheat germ ,corn milk, sesame, greentea, chocolate.Thus,all samples are very interested to analysed the lipid oxidation occurrence between processing.

2. Materials and Methods

2.1 *Study on the optimization for analysis by High performance liquid chromatography*

2.1.1 Preparation of Standard solution and Thiobarbituric acid.[14]

The 1,1,3,3-Tetraethoxypropane (TEP)(AR grade from Sigma) 23.0mg was weighed and dissolved in 10 ml of 0.1N HCl.Then, the standard solution was incubated in water bath at 40^{o}C for 40 min. to form standard Malondialdehyde(MDA) 10 mM.The MDA standard was prepared as standard series solution between 0.5 – 10 nM.

The 2-thiobarbituric acid (TBA)(AR grade from Fluka) was prepared to 0.6 %(W/V) in deionised water.

2.1.2 Analysis of Standard MDA solution by High performance liquid chromatography.

Both of standard solution MDA and TBA were mixed together and incubated in water bath at 95 ^{o}C for 60 min. and rapidly cool down to 10 ^{o}C. The standard solution from derivertized products was injected to the reverse phase column chroma-tography(C18, SphereClone 5 µm ODS, 250 x 4.60 mm, Phenomenex) couple with High performance liquid chromatography(Agilent HP1100), detected signal by Diode array UV detector at 532 nm.

2.1.3. Optimization method for analysis.

*2.1.3.1 Study on buffer(KH_2PO_4) ratio to ethanol solution.*The KH_2PO_4 buffer(pH6) for mobile phase system was prepared as 20 mM and mixed with ethanol in the ratio between 50:50 ,55:45,60:40,65:35 and 70:30. Those system was used to separated the MDA-TBA derivertised products in HPLC system in 2.1.2.