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Study the mumefural change and antioxidant properties during lemon essence making

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Lemon is an important crop in Pingtung (Southern Taiwan), and its price is always low due to the supply exceeds the demand in productive periods, so it is necessary to develop diversified products of lemon. There have plenty of commercial plum essence products in the current market, but the development of lemon essence is still unexplored in the laboratory and in the industry. In this study, lemon is processed by the procedure such as squeezing, residue discard and highly concentrated, and obtain the sticky dark black product. Interestingly, the mumefural, a compound generally recognized having the function of enhancing immune system, was formed during the lemon essence processing. The mumefural content will be increased gradually by increasing heating time with the highest mumefural was found at 10 hr heating time, but the content was decreased at 12 hr heating. It is speculated that prolonged over-heating time will lead to the degradation of mumefural. However, the longer the concentration time, lemon essence had the stronger antioxidant activity, especially in 10 and 12 hours. This finding can be further collaborated with antioxidant compound determination.

Keywords: Mumefural, antioxidant, lemon

INTRODUCTION

Elderly people comprise the fastest growing population segment in industrialized society, making the control of circulatory disease an important subject (Ettinger et al., 1994; Tamara et al., 1997). Increasing interest is being paid to the role of diet, particularly functional foods, sometimes called pharmafoods, designer foods, or nutraceuticals in the business press, for the circulatory system function is closely linked to dietary factors. Food material that has relationship with good blood circulation has been studied extensively. Previous study that observe about the effects of traditional Japanese foods on blood fluidity (Kikuchi, 1995) found that the fruit-juice

concentrate of Japanese apricot (scientific name: *Prunus mume* Sieb. et Zucc; Japanese name: Ume), markedly improves the fluidity of human blood. Mumefural is a component of Japanese apricot fruit juice concentrate, which has been used as a traditional non-official medicine in Japan, and was reported to have an activity in improving the blood fluidity (Chuda et al., 1999).

Lemon is an important crop in Pingtung which has annual production of 12,827 tones lemons, planting an area of about 1250 hectares, and often lead to excess because of oversupply and low prices when it's in season. As a result, the development of diversified products is necessary. There are many plum essence products in the market, but the development of lemon essence is still unexplored in the laboratory and in the industry. Therefore, in order to develop the value added of the agroproduct and agricultural export industry, in this study, the

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extracted lemon juice was used, heated by the time interval and mumeifural content and antioxidant ability were observe to show the special function of produced lemon essence, and hopefully it will be able to help lemon farmers increasing their income, and contribute to farmers and the social-economic of the agricultural areas (Sun, 2008).

MATERIALS AND METHODS

Materials

Eureka full-ripen lemon fruit (\pm 85 gram/piece) were collected from Lemon Production and Marketing Cooperation, Jiu Ru Village, Pingtung County, Taiwan. Japanese apricot were obtained from Nakano BC. Co. Ltd, Japan. Chemical reagent including Folin-Ciocalteu's phenol reagent, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 5-hydroxymethyl-2-furaldehyde (HMF), and 22'-azinobis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS) were purchased from Sigma, USA. Other chemical reagents used were in analytical grade.

Methods

Lemon Essence Production

About 50 pieces of fresh lemon were extracted using juice extractor to obtain about 1900 mL lemon juice. Filtered lemon juice were in directly boiled in a pottery pot using small fire, with temperature was about 97-100°C, and stirred constantly by mixing stick to prevent the bottom overheating. Sampling was taken at time of 0, 2, 4, 6, 8, 10, and 12 hours (Nakamura, 1995). After concentrated, lemon essence was freeze dried (PANCHUM FD-5030, Taiwan) and it will be diluted based on concentration for analysis.

Physiological properties determination

During boiling time to obtain lemon essence, several physicochemical properties was determined; including total soluble solids, titratable acidity, water activity (A_w), water content, ascorbic acid content, color determination, and change in viscosity. Total soluble solid was determined by placing small amount of lemon essence into top of prism of Abbe refractometer (Tokyo, Japan), close the daylight plate and brix degree could be seen on the scale. Titratable acidity (organic acid content) was determined by titration of 0.3 grams sample in 100 mL distilled water with 0.1 N NaOH solution with phenolphthalein as color indicator. The titratable acidity value (%) was calculated as following: $0.1N \text{ NaOH (mL)} \times 0.0070055/\text{Sample weight (g)} \times 100$ (Hatanaka and

Kaneda, 1985).

Water activity was measured by loading sample into the water activity meter (AQUALAB CX-2, Washington, USA) and measured at room temperature with 3 times repetition for each sample. Water content was determined using infra-red moisture meter (FD 600, KETT, Japan) by comparing weight before and after heat treatment at 102°C. Color value was determined by Hunter-Lab (TC-1500 DX, DENSOKU, Japan) which L represent lightness, a represent red (+) and green (-) color, and b represent yellow (+) and blue (-) color.

Citric acid, malic acid, HMF and mumeifural contents determination

HMF, mumeifural, citric acid and malic acid contents were determined by HPLC (HITACHI UV detector L-7400, Japan). Sample was prepared by adding 0.3 grams lemon essence with distilled water until 10 mL, shocked with ultrasound for 10-15 minutes and centrifuged at 6000 rpm for 10 minutes. After centrifugation, 0.5 mL supernatant was collected into the SPF column (add 1.5mL distilled water), and added 1.5. mL acetonitrile CH_3CN , then the filtrate were collected and some sample was filtered with 0.2 μm small filter before HPLC analysis (Chuda et al., 1999).

HPLC used in citric acid and malic acid content determination were using YMC-Pack AQ-303 column with S-5 μm , 250 \times 4.6 mm. The flow rate was 0.7 ml min^{-1} . Amount of 10 μL test solution were injected into the system and the spectra was recorded at 280 nm with UV detector. The mobile phase used was 0.1 M of ammonium phosphate monobasic buffer pH 2.0 (Huang et al., 2002).

HPLC analysis for HMF and mumeifural were established using XTerraTM MS C18 column with 3.5 μm 2.1 \times 150 mm (WATERS, USA). The temperature of the column oven was set at 45 °C. The flow rate was 1.2 ml min^{-1} . Amount of 2 μL test solution were injected into the system and the spectra was recorded at 280 nm with UV detector. The mobile phase used was explained in Table 1 (Chuda et al., 1999).

Antioxidant compound determination

Total phenolic compound determination

Total phenolic content were determined by following Chu et al. (2002) method. Filtered lemon essence (0.2 mL) was added with distilled water until 2 mL and 1 mL of Folin-Ciocalteu's Phenol reagent were added. After 3 minutes reaction time, 0.8 mL of 20% Na_2CO_3 was added and reacted again for 30 minutes. Absorbance was measured in 740 nm using spectrophotometer. Gallic acid (225-350 ppm) was used as standard and total phenolic contents were expressed as μg gallic acid equivalent/g

Table 1: The mobile phase of HMF and mumefural determination by HPLC

Time (min)	Acetonitrile (%)	Sodium phosphate buffer pH 2.3 (%)
0	0	100
10	2	98
20	4	96
30	6	94
40	4	96
50	2	98
60	0	100

sample.

Total flavonoid determination

The total flavonoid content of the samples was measured using colorimetric method (Abeyasinghe et al., 2007). Filtered lemon essence (0.5 ml) was added to a test tube containing 1.5 ml of absolute ethanol. After addition of 0.1 mL 1 M $\text{Al}(\text{NO}_3)_3$, 0.1 mL 1 M CH_3COOK , and 2.8 mL of distilled water. Absorbance was measured at 420 nm using spectrophotometer after 30 min reaction time. Quercetin (0-100 ppm) was used as the standard and total flavonoid contents were expressed as μg quercetin equivalents/g sample.

Vitamin C content determination

Vitamin C determination was done by following Gil et al. (2002) with modification. Three grams of sample was added with 0.5% oxalic acid until 100 mL and reacted for 5-10 minutes. Amount of 20 mL reaction mixture were collected into tube, added with half-spoon of activated carbon and mixed well by ultrasonic bath (BRANSON 8210, Japan). After filtered, the liquid layer was transferred into a flask and 3.5 mL of liquid phase were added with oxalic acid until 4 mL. Reaction started with addition of 0.1 and 1 mL of 2,4-dinitrophenylhydrazine (DNP) and incubated in hot water for 10 minutes. After cooling down, 5 mL of 85% H_2SO_4 solution was added and absorbance was measured in O.D 515 nm. Vitamin C contents were calculated from ascorbic acid standard curve.

Antioxidant activity determination

DPPH free radical scavenging activity

DPPH free radical scavenging activity was determined according to Mensor et al. (2001). Freeze dried lemon essence were added with appropriate amount of distilled water for preparation of 250, 125, 62.5, 31.25, 15.63, 9, 6,

3, 1 mg/mL sample concentration. Taking 4 mL of various concentrations of samples and then adding 1 mL of 1 mM DPPH solution. Distilled water was use to replace sample in control. After shaking at room temperature for 30 minutes, absorbance of the sample were measured in 517 nm using HITACHI U-2001 spectrophotometer (Tokyo, Japan) and the DPPH free radical scavenging capacity was expressed in % with formula: $(\text{abs control} - \text{abs sample})/\text{abs control} \times 100$.

TEAC (Trolox Equivalent Antioxidant Activity)

TEAC was determined by comparing ABTS free radical-scavenging activity of each sample according to the method described by Re et al. (1999). The radical cation $\text{ABTS}^{\cdot+}$ was generated by persulfate oxidation of ABTS. A mixture (1:1, v/v) of ABTS (7.0 mM) and potassium persulfate (4.95 mM) was allowed to stand overnight (12-16 hours) at room temperature in the dark to form radical cation $\text{ABTS}^{\cdot+}$. A working solution was diluted with phosphate buffer solution to absorbance values 0.7 ± 0.02 at 734 nm. An aliquot (0.01 ml) of each sample was mixed with the working solution (1 ml), and the decrease of absorbance was measured at 734 nm after 1 minute. Aqueous phosphate buffer solution (1 ml, without $\text{ABTS}^{\cdot+}$ solution) was used as a control. The $\text{ABTS}^{\cdot+}$ scavenging rate was calculated as mmol trolox equivalent antioxidant activity/g sample.

Reducing power

The ability to reduce ferric ions was measured using method described by Yen and Chen (1995). An aliquot (150 μL) of each sample was added with 0.2 M sodium phosphate buffer and 1% $\text{K}_3\text{Fe}(\text{CN})_6$ with the same solution amount and the reaction mixture was incubated at 50°C for 20 min. After cooled down for 3 minutes in ice water, 150 μL 10% TCA, 0.6 mL distilled water and 120 μL 0.1% FeCl_3 were added and reacted for 14 minutes. Absorbance value was measured at 700 nm by spectrophotometer. Higher absorbance show higher reducing power.

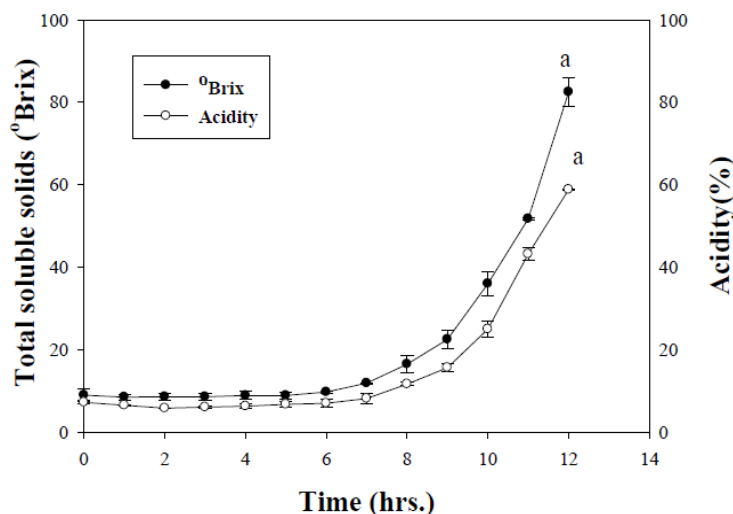


Figure 1: The changes of total soluble solids and titratable acidity in different heating time during lemon essence processing. Data expressed as mean \pm SD (n=3).

Superoxide anion scavenging activity

The effect of scavenging superoxide radical was determined by the nitroblue tetrazolium reduction method (Robak and Gryglewski, 1988). Reagent such as 120 μ M phenazine methosulfate (PMS), 936 μ M nicotinamide adenine-dinucleotide (NADH) and 300 μ M nitroblue tetrazolium (NBT) were prepared by diluting with 0.1 M PBS (pH 7.4). Sample about 0.3 mL were added with 1 mL each of PMS, NADH, and NBT, reacted for 5 minutes and measured in 560 nm spectrophotometer against blank samples, containing all the reagents except the PMS. The lower absorbance value shows the higher scavenging activity. Superoxide radical scavenging activity was expressed in % with formula: $(1 - (\text{abs sample} / \text{abs control})) \times 100$

Statistical analysis

Statistical analyze was done by using Statistical Analysis System (SAS). Statistical analyze that conducted was analysis of variance (ANOVA) using Duncan's multiple range comparison post hoc with $p < 0.05$ considered significant.

RESULTS

Physiological Properties determination

The average soluble solids of fresh lemon juice is about 7.5°Brix. This Brix value is greater than 6.5 of lime CNS (Chinese National Standard). Soluble solids were

increased by the heating time and significant increases were observed at 9-12 hours processing (Figure 1). After 12 hours heating time, Brix value was 82°Brix. The titratable acid also increased during the time heating and obvious changes were found in 9-12 hours. This phenomenon was the similar as the change of total soluble solid.

The moisture content of lemon juice was decreased by the processing time. It was just a small change in the water activity (A_w) value during 0 to 11 hours, but after 11 hour heating time, A_w value was drop from 0.88 until 0.4 (Figure 2). Lower A_w could be applied for food preservation Water content value also decrease as the increase of heating time and obvious decrease was shown in 9-12 hours heating time.

Color value determination results were shown in Table 2. Lightness of lemon juice was decrease as the increase of processing time. After 9 hours processing time, lemon essence change the color become reddish and this color intensity increase as the increase of processing time. Generated reddish color may due to maillard reaction occur in lemon essence processing.

Citric acid, malic acid, HMF and mumefural contents determination

Citric acid, malic acid, and HMF content were determined by comparison with standard curve ($r^2 > 0.995$). The content of citric acid and malic acid, non-volatile acid, on lemon essence (based on the original water content) during the heating processing were fluctuated as shown in Figure 3. These acids content in lemon essence showed tendency to decrease as processing time increase. Both

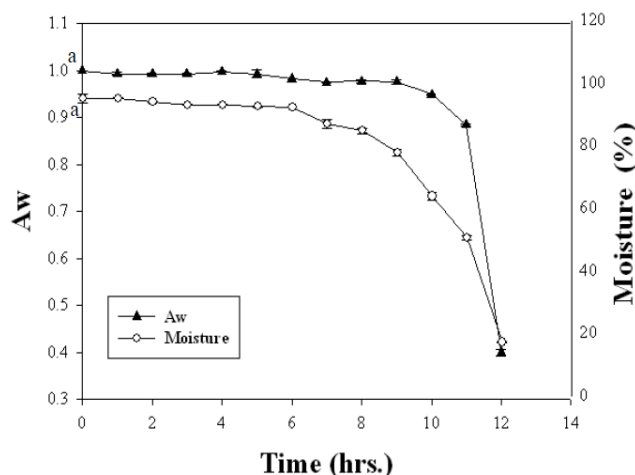


Figure 2: The changes of Aw and moisture content in different heating time during lemon essence processing. Data expressed as mean \pm SD (n=3).

Table 2: Change in color L, a, b value of lemon essence processing

Hour	L	a	b
0	49.425 \pm 0.063 ^a	-3.735 \pm 0.063 ⁱ	9.05 \pm 0.014 ^k
1	47.655 \pm 0.007 ^c	-3.08 \pm 0.014 ^h	12.745 \pm 0.098 ⁱ
2	43.65 \pm 0.035 ^h	-3.21 \pm 0.007 ^h	11.225 \pm 0.007 ^j
3	47.285 \pm 0.021 ^d	-2.415 \pm 0.049 ^g	14.115 \pm 0.035 ^g
4	42.225 \pm 0.042 ^k	-2.415 \pm 0.063 ^g	12.995 \pm 0.021 ^h
5	48.035 \pm 0.007 ^b	-1.45 \pm 0.028 ^e	15.64 \pm 0.014 ^d
6	45.28 \pm 0.028 ^f	-1.66 \pm 0.035 ^f	14.805 \pm 0.007 ^f
7	45.04 \pm 0.042 ^g	-1.29 \pm 0.056 ^e	15.03 \pm 0.021 ^e
8	45.55 \pm 0.014 ^e	-1.085 \pm 0.021 ^d	15.7 \pm 0.0035 ^c
9	42.905 \pm 0.077 ^j	0.145 \pm 0.035 ^e	16.075 \pm 0.007 ^b
10	43.25 \pm 0.028 ⁱ	1.353 \pm 0.410 ^b	17.21 \pm 0.014 ^a
11	16.355 \pm 0.021 ^l	1.67 \pm 0.056 ^b	8.545 \pm 0.021 ^l
12	6.267 \pm 0.056 ^m	7.225 \pm 0.162 ^a	2.273 \pm 0.056 ^m

Each value is expressed as mean \pm SD (n=3). ^{a-l}Values in column with different superscript indicate the significant difference (p<0.05).

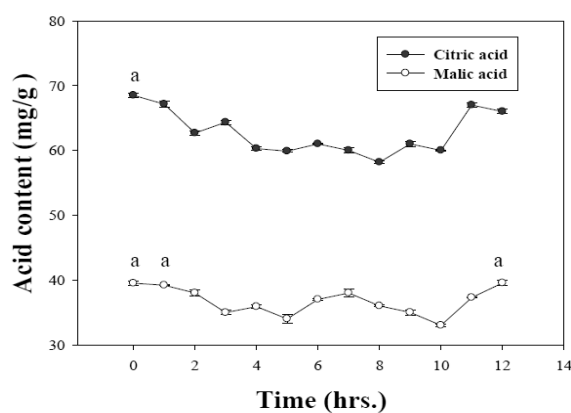


Figure 3: Citric acid and Malic acid contents during in different heating time during lemon essence processing. Data expressed as mean \pm SD (n=3).

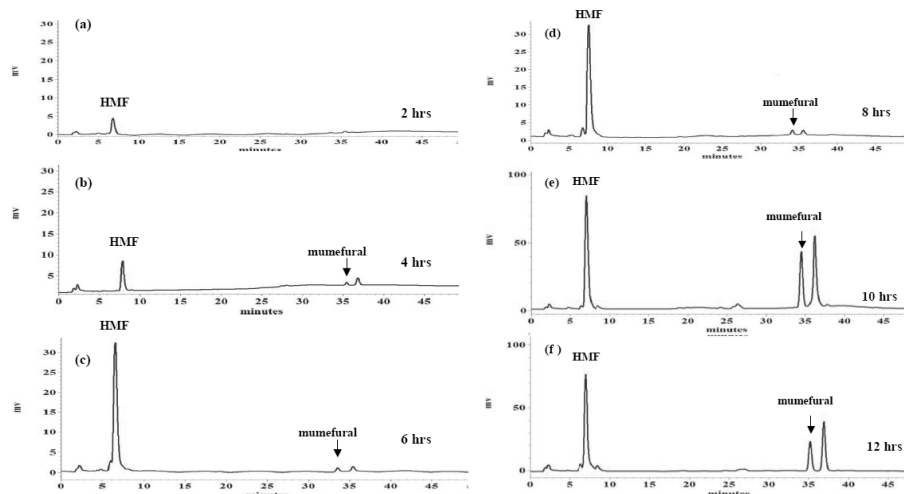


Figure 4: HPLC Chromatograms (280 nm) of HMF and mumeifural contents during the different time processing of lemon essence.

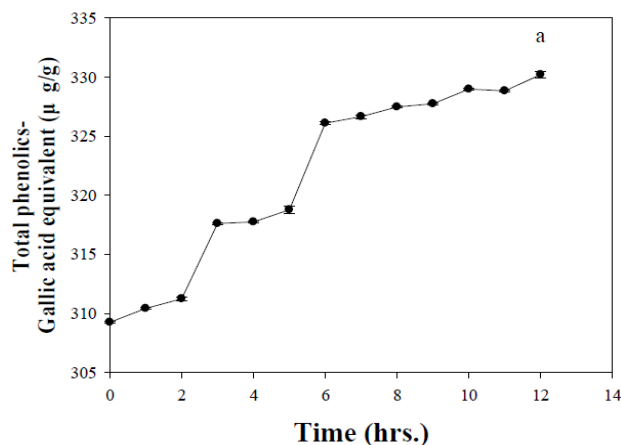


Figure 5: Total phenolic content of lemon essence processing. Data expressed as mean \pm SD (n=3).

of these acids were detected have low contents in 10 hour processing time and become higher again after that time.

HMF was produced after heating for 2 hours. The longer the heating time, the higher HMF content (Figure 4). HMF showed highest content on heating 10 hours (1.3 mg/g) and reduced at 12 hour processing time (1.0 mg/g). There was no mumeifural content in early processing time (2 hours). The mumeifural content increased slowly as heating time increase (4-8 hours). The significant production of mumeifural was exhibit in 10 hours processing time, and its content reduced again at 12 hours processing time.

Antioxidant compound determination

Antioxidant compound that determined were total

phenolic and flavonoid content and vitamin C compound for every hour processing time. Flavonoid is one group compound that included as phenolic compounds. Result shown in Figure 5 and 6 demonstrated that total phenolic and flavonoid contents were increased as the increase of processing time and total flavonoid content increased most at 12 hours heating time. Different with phenolic and flavonoid contents, vitamin C content decrease by the processing time (Figure 7).

Antioxidant activity determination

This result also showed that produced lemon essence have antioxidant activity. Antioxidant activities measured in this study were DPPH free radical scavenging activity, TEAC (Trolox Equivalent Antioxidant Activity), reducing

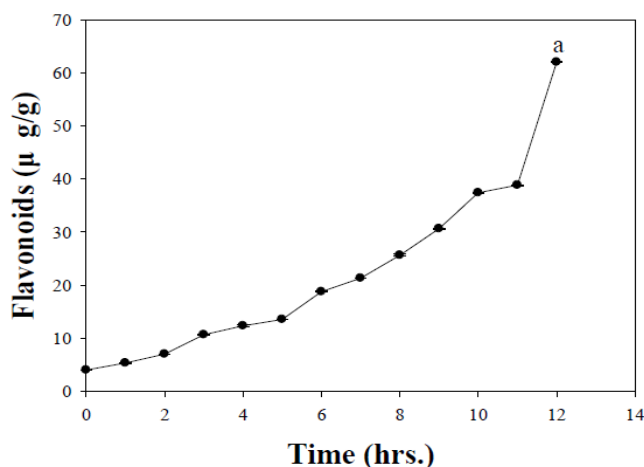


Figure 6: Total flavonoid content of lemon essence processing. Data expressed as mean \pm SD (n=3).

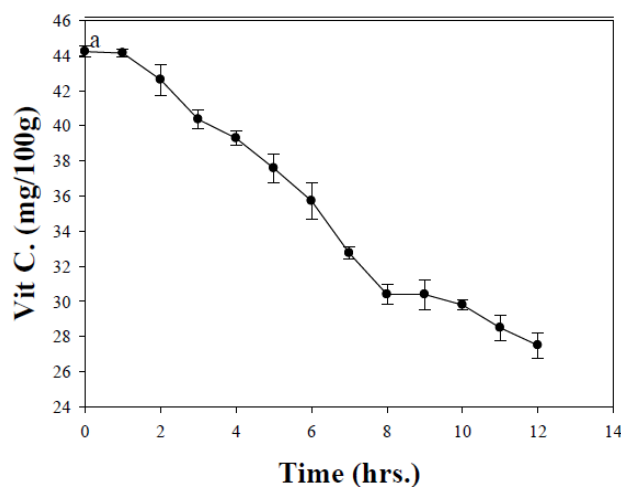


Figure 7: Total vitamin C content of lemon essence processing. Data expressed as mean \pm SD (n=3).

power and superoxide anion scavenging activity. Figure 8 showed that there are no significant difference of DPPH free radical scavenging ability difference between lemon essence and Japanese apricot essence.

All antioxidant activities showed that there are increase in antioxidant properties as increasing processing time and concentration (Figure 9). Superoxide anion scavenging ability showed higher percentage compared to DPPH free radical scavenging ability. In TEAC and reducing power, lemon essence at 10 and 12 hour processing showed significantly higher antioxidant properties compared with other processing time.

DISCUSSION

In this study, lemon is processed by a series of squeezing,

residue removal and highly concentrated, and the sticky dark black product was achieved (Huang, 1986) as shown on its physiological properties. Physiological properties of lemon essence product after 12 hours heating time were higher in Brix value, titratable acidity (organic compounds), reddish color and lower water content, Aw, and lightness value, as compared to lemon juice (before concentrated).

HMF, often used as an index of heat treatment and deteriorative changes in food such as tomato paste, infant milk, honey, and fruit juices, was resulted from Maillard reaction (Hurtado et al., 1998; Vinas et al., 1992). The present study demonstrated HMF formation as heating time increased as the result of Maillard reactions. Mumeferal compound is a compound generally recognized having the function of enhancing immune system. Mumeferal was synthesized by equal molecules of citric acid and HMF (Chuda et al., 1999). Reduced citric

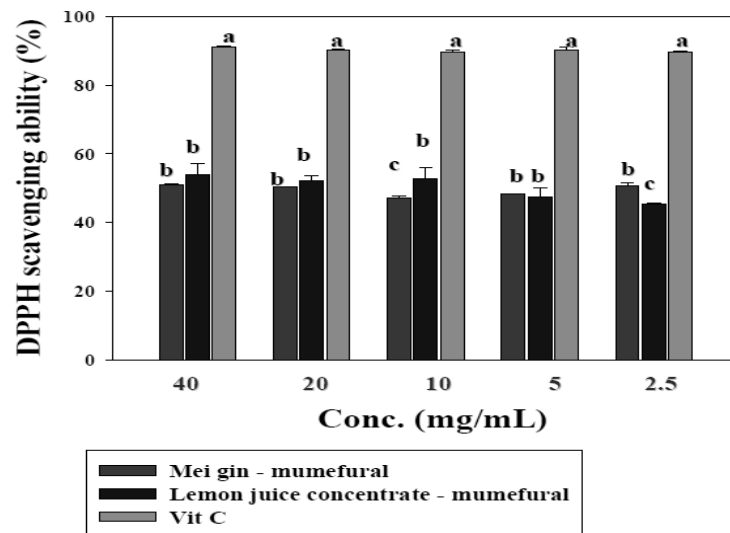


Figure 8: DPPH scavenging activity of lemon essence compared with Japanese apricot essence and vitamin C. Data expressed as mean \pm SD (n=3).
^{a-c}Values with different superscript indicate the significant difference ($p < 0.05$).

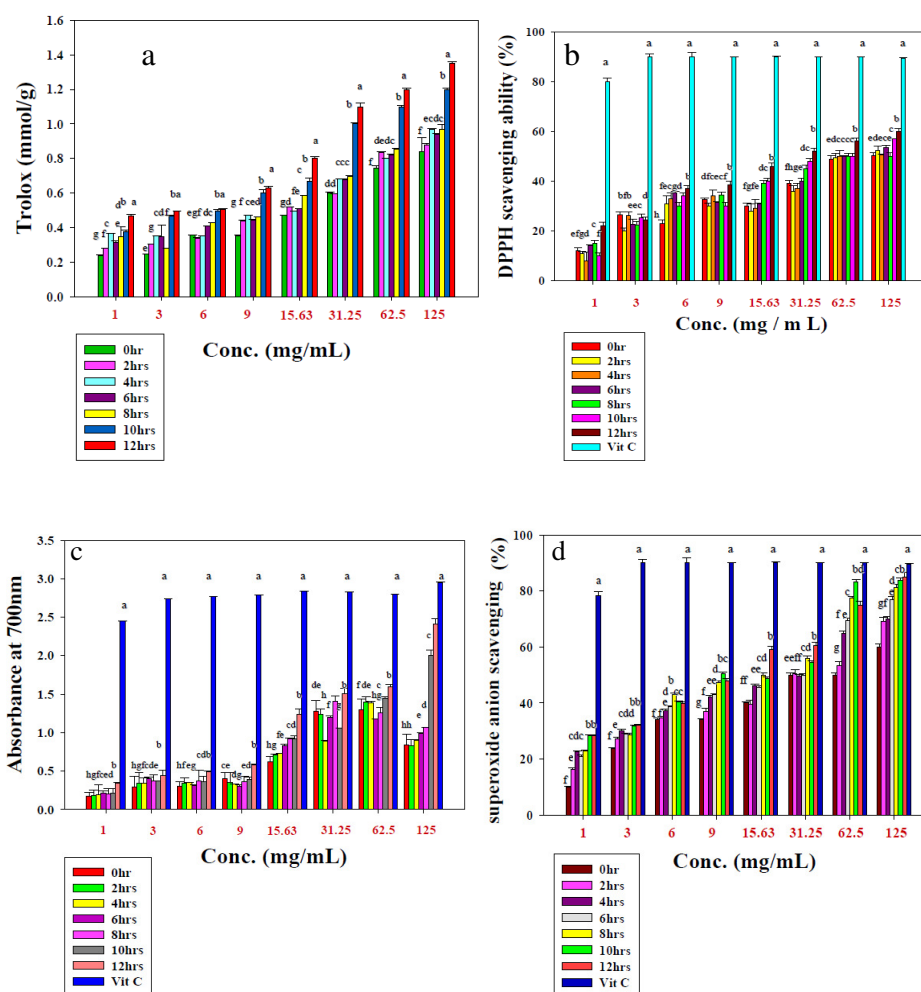


Figure 9: Antioxidant properties of lemon essence (a) DPPH scavenging activity, (b) TEAC, (c) reducing power, and (d) superoxide anion scavenging activity. Data expressed as mean \pm SD (n=3).
^{a-h}Values with different superscript indicate the significant difference ($p < 0.05$).

acid content in 10 hour processing time may due to its usage as substrate for mumefural formation. There is no mumefural presence in the fresh lemon juice. The mumefural content will be increased gradually by increasing heating time with the highest mumefural was found at 10 hr heating time, but the content was decreased at 12 hr heating. It is speculated that prolonged over-heating time will lead to the degradation of mumefural.

Interestingly, the longer the concentration time, lemon essence had the stronger antioxidant activity, especially in 10 and 12 hours. This finding can be further collaborated with antioxidant compound determination. Antioxidant compound that may interfere with antioxidant properties were phenolic, especially flavonoid compound as shown increase as the increase of heating time. According to the report, that antioxidant power of food is not necessarily because of thermal processing could be decreased or loss of power (Nicoli et al. 1999). In some cases, that certain processed food antioxidant capacity could be increased by heating, may be related to the generation of browning products. (Nicoli et al. 1997; Anese et al. 1999). Therefore, HMF and mumefural also suspected as compounds that played important role in antioxidant properties of lemon concentrated product.

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