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EFFECTS OF ENZYMATIC CLARIFICATION TREATMENT ON PHENOLIC COMPOUNDS OF PUMMELO (*CITRUS GRANDIS* L. OSBECK) FRUIT JUICE

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ABSTRACT

Pummelo is known to be the largest of all citrus fruits, with expected ratio of 2:1 (w/v) of producing juice, is an attractive opportunity for Malaysia to expand pummelo's influence and marketability over the international market of juices. However, in order to obtain a bright and clear product, the juice must be clarified and it is expected to affect the phenolic composition. The purpose of this study is to investigate the changes that occur during enzymatic clarification on the phenolic compounds between 2 varieties of pummelo juice: Ledang (PO55) and Tambun (PO52). The changes in the polyphenols composition are monitored through the clarification process using High Performance Liquid Chromatography Diode Array Detection (HPLC-DAD). Clarification of pummelo fruit juice with Pectinase SMASH XXL has statistically no significance influence ($p < 0.05$) on the phenolic compounds of Ledang and Tambun variety. However, the phenolic compounds (naringin) of Tambun variety is statistically high ($p > 0.05$) when compared to Ledang variety and no significant difference was found to naringin's amount after the enzymatic treatment. This study suggests that Pectinase SMASH XXL is to be used as recommended to avoid unfavourable changes to the juice quality while producing a healthy, organic and profitable product.

Keywords: *enzymatic treatment, clarification, pummelo fruit juice, phenolic compounds and antioxidant.*

INTRODUCTION

Citrus fruits are considered as the most important horticultural crops because of their nutritional value and health benefits. Citrus juice has long been believed to have a correlation with good health, due to their antioxidant potency and plasma lipid metabolism [1]. Citrus fruit extracts have also been found to demonstrate anti-cancer, anti-inflammatory, anti-tumour and blood clot inhibition activities [2]. The health benefits of citrus juice are mainly been attributed to the presence of bioactive compounds, such as phenolics, ascorbic acid and carotenoids. Citrus flavonoids have been said to be responsible for the beneficial effects and such compounds are identified as methoxylated flavones, flavonones and flavonone glucosides [3].

Pummelo (*Citrus grandis* (L.) Osbeck), of Rutaceae family, is a citrus fruit native to Southeast Asia and the Indo-China regions. It is also known as pomelo, pommelo, shaddock, limau bali and Chinese grapefruit. The fruit is commonly eaten fresh and the taste varies from mildly sweet and bland to sub acid with a faint touch of bitterness [4]. Popular variations of the fruits are; PO51 (Sha Thing), PO52 (Tambun) and KK2 (Melo Mas). Recently, Department of Agriculture Malaysia has introduced a new hybrid known as Ledang Variety (PO55), where the fruit is sweeter and has less bitter aftertaste pummelo usually known for. In Malaysia, about 1895 hectare of pummelo grown commercially and in 2009, production is estimated at 8830 metric tonnes. The

largest growing state is Johor with 380 hectare and Perak with current 320 hectare of commercially grown trees and the harvest season is usually in January and September [5]. Singapore, Indonesia and Thailand are the biggest importer of pummelo with the amount reaching up to RM5.4million per year.

Naringin, neohesperidin and hesperidin are the major flavonoids formed in *Citrus grandis*, *Citrus paradise*, *Citrus aurantium and Citrus limon* [6]. However, until the moment of writing, identification and/or quantification of flavonoids has been mainly carried out on lemon, orange, and grapefruit species [2, 6, 7, 8, 9, and 10] and very little literature was done on pummelo. Pummelo have been shown to contain coumarins, furocoumarins, flavanones, flavones and flavonols in both free and glycosidic form [7, 8]. The flavonoid profile too, have been shown to vary with the species and cultivars, therefore can be used to distinguish between the different varieties.

In this study, two cultivars of pummelo fruit juice were analysed using high-performance liquid chromatography diode array detection (HPLC-DAD) for the identification and quantification of flavonoids after a specific enzymatic treatment. Enzymatic treatment was done in order to improve clarification of fruit juice before further pasteurization to be done to avoid undesirable turbidity, haze and sediments in the final products. Visual perception of turbidity and haze in fruit juices is the result of light scattering caused by suspended substances. The immediate turbidity in freshly squeezed fruit juices is generally considered to be a result of suspended pectin particles stemming from the plant cell wall and cell materials may also contribute to juice turbidity [11]. During storage, pectinesterase is slowly deactivated because of the decrease in the level of sugar due to its consumption by microorganisms for fermentation. Therefore, cloud loss could be taking place during storage, affecting the quality of pummelo fruit juice [12]. Furthermore, this study could help the food industry to exploit the natural flavonoids in fruit juices to minimize the usage of synthetic antioxidants in bid to have more natural, organic foods.

MATERIALS AND METHODS

Standards and chemicals

Milli-Q water (Millipore, Bedford, MA, USA) was used in all work. HPLC-grade methanol and formic acid (Merck, Darmstadt, Germany) were used after filtration through a 0.45-µm pore size membrane filter and sonication for 30 minutes. Phenolic acids (gallic, caffeic, chlorogenic, *p*-coumaric, ferulic and sinapic acids) and flavanones (naringin, naringenin, narirutin and hesperidin) were purchased from Sigma-Aldrich (Stenheim, Germany).

Preparation of juice sample

Fresh harvests of pummelo fruits, *Citrus Grandis* L. Osbeck, of Ledang variety (PO55) were obtained from Jabatan Pertanian Daerah Segamat, Johor Darul Takzim, Malaysia and Tambun variety (PO52) were obtained from Jabatan Pertanian Daerah Kinta, Ipoh, Perak Darul Ridzuan, Malaysia. Pummelo fruits were kept in a refrigerator at 10°C until they were used for experimental works. Prior to peeling, the pummelos were washed with tap water to eliminate any microbial contaminations to the fruits. The thick fruit skin (flavedo and albedo) was peeled manually after a 1 cm-deep horizontal incision was made using a knife to reveal the juicy segments. Pummelo was then peeled into segments and the inner skin of each segment was peeled and discarded. The white membrane surrounding the juicy segments, including seeds, were removed completely. The juice was extracted using a screw type extractor and nylon-filtered to remove the pulps. This process was repeated three times to optimize the juice extraction. The juice is then kept in a HDPE bottle in a freezer at -5°C until further experimental works.

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Standard chemical analysis

The total titrable acidity were assessed by titration with sodium hydroxide (0.1 N) and expressed as citric acid %. The pH value was measured using a digital Jenway 3505 pH meter (Bibby Scientific Limited, Staffordshire, UK). Total soluble solids were measured as Brix using an Atago refractometer (Atago Co, Tokyo, Japan). Ascorbic acid was determined by visual titration, using 2, 6-dichlorophenolindophenol [10]. Colour of pomelo fruit juice was determined using a Hunter Lab colorimeter (Ultrascan, VA, USA). The clarity of fruit juice was measured directly using a UV-VIS Shimadzu spectrophotometer (Kyoto, Japan) as absorbance at 660 nm with distilled water was used as a reference.

Enzymatic treatment

For each variety, 200 ml pummelo juice was subjected to different enzymatic treatment conditions as shown in Table 1. The optimized range of the variables for enzymatic treatment conditions were based on the preliminary RSM experiments conducted earlier. The independent variables were the incubation time, incubation temperature and concentration of enzyme used. The temperature of the enzymatic treatment was adjusted to the desired level using a constant temperature water bath. The pH of pummelo juice was kept at its natural pH value of 4.0 and was excluded from the RSM experimental design as the pH is considered optimal for *exo*-pectinase [13]. At the end of the enzymatic treatment, the enzyme in the sample was inactivated by chilling the suspension at -2°C for 5 min in a water bath. The treated juices were then centrifuged at 3000g for 10 min and the supernatant was collected. After that, the juice was filtered through a Whatman no. 1 filter paper using vacuum suction at 25 mm Hg. The filtrate was collected for further analysis.

Table 1: Enzymatic treatment

Variety	Enzyme Concentration (%)	Time (minutes)	Temperature (°C)
Ledang (PO55)	0.034	31	57
Tambun (PO52)	0.055	33	23

Determination of total phenolic compounds

Total phenols were determined by the Folin-Ciocalteu procedure [14] as follows; 1mL of 10-fold diluted Folin-Ciocalteu reagent was added to a 0.2mL 1:50 juice sample. After 1 min, 0.8mL of 7.5% (w/v) Na_2CO_3 solution was added and the mixture was shaken. After 2 hours, the absorbance was measured at 765nm using a UV-Vis 1240 Spectrophotometer (Shimadzu, Kyoto, Japan). The phenolic content was expressed as gallic acid equivalents in mg/100 mL.

Liquid chromatographic analysis of phenolic compounds

Samples were filtered through a 0.45-m pore size membrane before injection. An Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) operated by Windows NT based ChemStation software was used. The HPLC equipment was used with a diode array detector (DAD). The system consisted of a binary pump, degasser and auto sampler. The column used was a Thermo Scientific C18 column (Waltham, MA, USA): 250 x 4.6 mm x 5µm. The injection volume of the fruit juice was 20 µL per sample. The mobile phase consisted of two solvents: Solvent A, 0.1% water in formic acid and Solvent B, 100% methanol. Phenolic compounds were eluted under the following conditions [10] with modifications: gradient conditions 0 to 20% solvent B (0 mins), 20 to 30% solvent B (0 to 20 mins), 30 to 50% solvent B (20 to 30 mins), 50 to 90% solvent B (30 to 35 mins), 90 to 20% solvent B (35 to 40 mins), followed by washing and reconditioning of the column. The separations were performed with a flow rate of 1 mL/min, which was directly injected in the ESI source, without any splitting. The column temperature was maintained at 25°C. The analysis time was of 40 minutes.

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The HPLC method was tested on 10 phenolic compounds (coumaric acid, sinapic acid, chlorogenic acid, gallic acid, ferulic acid, caffeic acid, narirutin, hesperidin, naringenin and naringin). The polyphenols standard solutions (10 µg/mL) were prepared in methanol. The ultra-violet-visible-spectra (scanning from 200 to 600 nm) were recorded for all peaks. Triplicate analyses were performed for each sample. The identification of phenolic compounds was obtained by using authentic standards while quantification was performed by external calibration with standards.

Statistical analysis

The data obtained in the study were analyzed using Minitab Release 14 (Minitab Inc., PA, USA). Analysis of variance was performed by ANOVA procedure and significant differences ($p < 0.05$) between means were determined using Tukey's multiple range test. All analyses were done in triplicate.

RESULTS AND DISCUSSION

Standard chemical analysis

The chemical composition of Ledang and Tambun pomelo fruit juice are given in Table 2. It was obvious that the choice of cultivars affected the composition of juice. As can be seen, Tambun juice showed higher concentration of total acidity, brix and ascorbic acid compared to Ledang variety. Vitamin C which derived from ascorbic acid, can be found abundantly in pummelo fruit juice, was found to have a concentration of 509 and 537 mg/L respectively in Ledang and Tambun variety. The Brix/acid ratio was also identified as an important parameter related to the quality of citrus fruit [15]. It was found that the Brix/acid ratio for Ledang and Tambun variety is 1.07 and 1.06 respectively, which suggested that the pummelo is suitable for citrus juice processing. Xu *et al.* [15] also reported that the acidity of citrus plays a great role in terms of bitterness, because under low pH conditions, the A-ring lactone (LARL) can be converted into limonin, a bitter limonoid making juice undesirable. These values are recorded with the purpose of having an initial value of the juice quality prior to enzymatic treatment which could alter the compositions considerably.

Table 2: Chemical composition of Ledang and Tambun pomelo fruit juice

Analysis	Ledang	Tambun
Juice yield (%)	42.2 ± 0.008	38.2 ± 0.004
Total acidity (g/L)*	11.3 ± 0.2	13.43 ± 0.15
pH	3.99 ± 0.02	3.88 ± 0.015
Brix	12.23 ± 0.057	14.17 ± 0.057
Total phenolics (mg/100 mL)	28.67 ± 0.58	46.67 ± 1.53
Clarity	0.49 ± 0.004	0.36 ± 0.006
Colour	75.51 ± 0.29	76.62 ± 0.096
Ascorbic acid (mg/L)	509.13 ± 3.00	537.47 ± 2.89

**As citric acid*

Total phenolic compounds

Total phenolic compounds for Ledang and Tambun varieties including the enzymatically treated are given in Table 3. Phenolics present in fruits and vegetables have received considerable attention because of their potential antioxidant activity. Phenolic compounds undergo a complex redox reaction with the phosphotungstic and phosphor-molybdic acids present in the Folin-Ciocalteu reagent [16]. It was found that Ledang variety had a considerably lower total phenolic contents compared to Tambun variety ($p < 0.05$). This result is in agreement with the liquid chromatography analysis in the next section, with naringin being the major contributor of the phenolic acids. The enzymatically treated juices however, have shown a slight decrease to the total phenolic contents for both varieties. This slight difference might be due to different enzymatic treatment (Table 1) and

could also due to storage conditions after the enzyme treatment. Although non-significant, pre-cautions should be made to avoid further phenolics loss to juice.

Table 3: Total phenolics content (mg/100mL ± standard deviation) of Ledang and Tambun pomelo fruit juice

Ledang (control)	28.67 ± 0.58[a]
Ledang (enzymatically treated)	24.33 ± 1.53[a]
Tambun (control)	46.67 ± 1.53[b]
Tambun (enzymatically treated)	42.67 ± 0.58[b]

**Results are the means of triplicates.*
***For each treatment, the means within the column followed by different letters were significantly different at p <0.05.*

Liquid chromatographic analysis of phenolic compounds

A total of six phenolic compounds were identified and quantified in Ledang and Tambun pummelo fruit juice (Table 4), including hydroxycinnamic acids and flavanones compounds. The total amount of phenolic compounds found though liquid chromatograph analysis was 22.04 and 43.35 mg/100mL in non-enzymatically treated Ledang and Tambun variety respectively, with naringin being the main contributor of the total phenolic compounds. A great difference ($p < 0.05$) of total phenolic contents for the control juice could be affected by the species, growing season, ripening, and environmental factors such as light, temperature and as well as, processing treatment.

Table 4: Phenolics content (mg/100mL ± standard deviation) of Ledang and Tambun pomelo fruit juice

Compounds	Peak no.	Retention Time (min)	LC (mg/100mL)	LE (mg/100mL)	TC (mg/100mL)	TE (mg/100mL)
Hydroxycinnamic acids						
Chlorogenic acid	1	7.85	1.60 ± 0.07	1.58 ± 0.01	2.85 ± 0.61	2.84 ± 0.57
Caffeic acid	2	10.84	0.09 ± 0.01	0.08 ± 0.00	0.11 ± 0.01	0.11 ± 0.01
Coumaric acid	3	16.80	1.44 ± 0.08	1.00 ± 0.01	1.33 ± 0.20	1.22 ± 0.26
Total	-	-	3.13	2.66	4.29	4.17
Flavanones						
Naringin	4	22.75	13.09 ± 0.10	12.88 ± 0.03	26.76 ± 4.28	26.60 ± 4.05
Hesperidin	5	8.36	3.57 ± 0.03	3.70 ± 0.02	5.39 ± 1.10	5.37 ± 1.02
Narirutin	6	28.26	3.00 ± 0.14	2.80 ± 0.14	7.19 ± 1.18	7.21 ± 1.10
Total	-	-	19.66[a]	19.38[a]	39.34[b]	39.18[b]

***** *LC: Ledang-Control, LE: Ledang-Enzymatic treatment: 0.034% enzyme concentration, 57°C, 31 minutes, TC: Tambun-Control and TE: Tambun-Enzymatic treatment: 0.055% enzyme concentration, 23°C, 21 minutes.*
***For each treatment, the means within the row followed by different letters were significantly different at p <0.05.*

Through liquid chromatograph analysis, it was identified that three hydroxycinnamic acids and three flavanones found in both varieties at 280 nm and 320 nm. The summary of both phenolic compounds were shown in Table 4. Two hydroxybenzoic acids that were tested for identification; gallic and protocatechuic acid were not detected in both varieties. Gallic acid, the major contributor to hydroxybenzoic acid, is a naturally abundant plant phenolic compound has been attracting considerable interests for its antioxidant properties [17]. This could be explained by the standard reference that was obsolete at the time of processing.

Three hydroxycinnamic acids identified in the analysis were chlorogenic acid, caffeic acid and coumaric acid. Sinapic and ferulic acid were not in found in either 280 nm or 320 nm chromatogram, with the reason being that the amounts of acids were nominal in comparisons to other acids in the fruit juice for the

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chromatograph to detect it at both wavelength. Chlorogenic acid was the most dominant hydroxycinnamic acid in both varieties, as it accounted for the largest proportion of the total hydroxycinnamic acids contents (Table 4). In both Ledang and Tambun, chlorogenic acid was found with 1.60 - 2.85 mg/100mL, followed by coumaric acid (1.44 – 1.33 mg/100mL) and caffeic acid (0.09 – 0.11 mg/100mL). It was found that the enzymatic treatment gave a slight effect to both varieties, although with no significant different ($p > 0.05$) to the fruit juice, except for coumaric acid that decreased with a significant difference ($p < 0.05$), where in Ledang the decreament was as much as 0.31% as compared to Tambun with decrement of 0.08%. Chlorogenic and coumaric has long been known as the antioxidant that reduced the risk of stomach cancer by reducing the formation of carcinogenic nitrosamines [18].

Flavanone is the major flavonoids found in citrus fruits, especially in pummelo. Three flavanones; naringin, hesperidin and narirutin were identified in both varieties. Table 4 and Figure 1 showed that, the dominant neohesperidosyl flavanones, naringin was the most abundant phenols in all samples. Naringin may be instrumental in inhibiting cancer-causing compounds and thus may have potential chemotherapeutic value. Studies have also shown that naringin interferes with enzymatic activity in the intestines and, thus, with the breakdown of certain drugs, resulting in higher blood levels of the drug [18]. Naringin was found with 13.09 and 26.76 mg/100mL respectively in non-enzymatically treated Ledang and Tambun variety. However beneficial naringin as an antioxidant property, naringin is also known to give a bitter aftertaste to the juice, which may affect the marketability of the juice. The positive observation of Pectinase Smash XXL was the reduced amount of naringin in the enzymatically-treated juice for both varieties. Naringin was reduced to 12.88 (1.6%) and 26.60 (0.59%) respectively. The decrease may not be significant but the potential for the enzyme to be exploited to minimize the bitterness pummelo are known for, is important.

Hesperidin, the second most abundant rutinosyl flavanones found in the Ledang variety, is known to be tasteless and therefore does not contribute to the taste of pummelo juice [19]. Hesperidin was found to increase (3.51%) in Ledang variety after the enzymatic treatment but decreased insignificantly (0.37%) in Tambun variety. The literature reports [16] that hesperidin level in orange juice depends on extraction method, technological treatment and storage. Narirutin was found in contrast to hesperidin level in Ledang and Tambun variety. Narirutin was shown to increase in Tambun (0.28%) and decreased in Ledang by 6.67%. According to an earlier literature [19], narirutin-to-hesperidin ratio has been proposed for quality control of orange juices. On the other hand, narirutin-to-hesperidin ratio can be used to gauge the juice clarity. Since hesperidin is insoluble, it can be found in juices in form of a fine suspension. If the amount of hesperidin is high, narirutin-to-hesperidin ratio will be lower, thus, lucidity of the juice is expected to be lower and a cloudy juice will be produced. In this study, Ledang was recorded to have a ratio of 0.84 and Tambun at 1.33 in comparison to enzymatically-treated which shown the value at 0.76 and 1.34. Which indicate, that Tambun variety produced a cloudy juice and not affected by the enzyme treatment done prior to the liquid chromatograph analysis.

With respect to the phenol levels, it was positive to note that the level of total phenols in no case were decreased significantly ($p > 0.05$) by the alternative treatments. The finding that the measured levels of total phenols and chromatographs were similar in the control and experimentally treated samples (Table 3 and 4), indicated that the influence of the different enzymatic treatment most likely reflected differences in the size and perhaps the shape of the clarity-causing molecules and not in their total content. This seems to be in agreement with the results published by Pinelo *et al.* [11]. Report by Aturki *et al.* [21] have conclusively shown close relationship between phenolic contents and antioxidative activity of fruit juice. Since the chemical composition and structures of active extract components are important factors governing the efficacy of natural antioxidants, the antioxidant activity of an extract could not be explained on the basis of their phenolic contents alone.

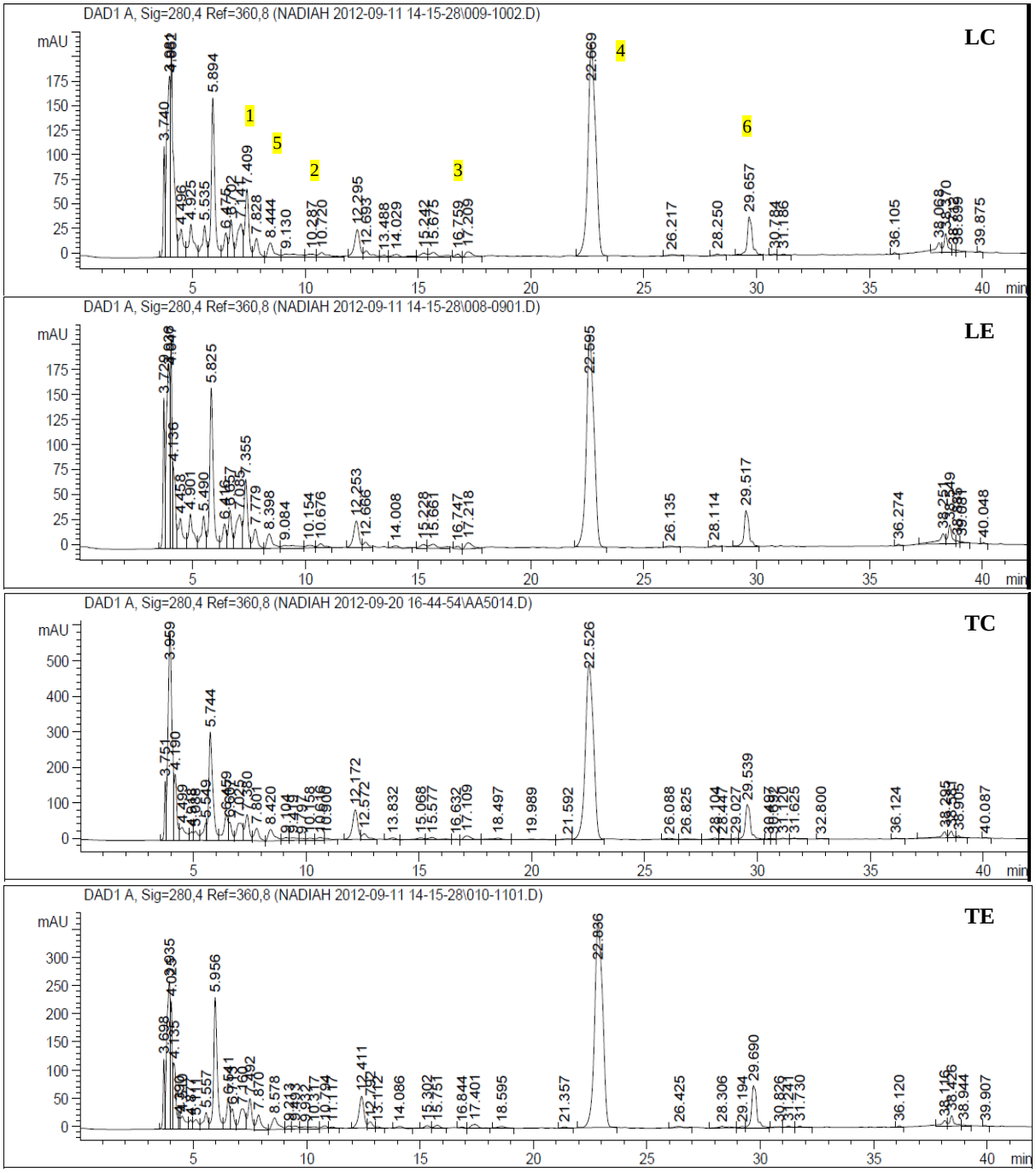

Fig. 1: HPLC-DAD chromatogram at 280 nm of LC (Ledang-Control), LE (Ledang-Enzymatic treatment: 0.034% enzyme concentration, 57°C, 31 minutes), TC (Tambun-Control) and TE (Tambun-Enzymatic treatment: 0.055% enzyme concentration, 23°C, 21 minutes).

CONCLUSION

In the present study, the antioxidant contents from enzymatically treated pummelo fruit juice from two main varieties were evaluated against non-enzymatically treated of the same varieties. Tambun variety was proven to possess high phenolic and flavonoid content, thus higher antioxidant capacity, due to the amount of naringin found in fresh squeezed and enzymatically treated juice. Naringin was the most dominant flavanone and the main contributor of antioxidant property however, decreased twicefold in Ledang eventhough the amount of enzyme concentration was less than that of Tambun. Furthermore, Ledang variety demonstrated to have the least effects (no significant difference, $p > 0.05$) on the juice quality after it was treated with Pectinase Smash XXL to clarify the juice in comparison to Tambun. Although theoretically Tambun would have made a better choice for juice development for the total phenolic content (naringin) it possessed, however, considerations have to be made to suit consumer's taste bud and the amount of enzyme needed to clarify the juice for its marketability.

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