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Comparison of vitamin C content in citrus fruits by titration and high performance liquid chromatography (HPLC) methods

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Abstract

Vitamin C is one of the essential vitamins for human and animal. Many methods were developed for the determination of vitamin C such as spectrophotometry, electrophoresis, titration, and high performance liquid chromatography (HPLC). This study aims to compare vitamin C content of citrus fruits (orange, grapefruit, lemon, lime, kaffir lime and musk lime) using indophenol titration and HPLC-PDA methods. In the titration method, orange has the highest vitamin C content (58.30 mg/100g) followed by grapefruit (49.15 mg/100g), lemon (43.96 mg/100g), kaffir lime (37.24 mg/100g), lime (27.78 mg/100g) and musk lime (18.62 mg/100g). While, in the HPLC method orange also leads with the highest vitamin C content (43.61 mg/100g) followed by lemon (31.33 mg/100g), grapefruit (26.40 mg/100g), lime (22.36 mg/100g), kaffir lime (21.58 mg/100g) and musk lime (16.78 mg/100g). Orange is the best source of vitamin C while musk and kaffir lime have lower content. Significant differences were observed in vitamin C of samples by both methods. Both methods are suitable for the determination of vitamin C, however HPLC method is more accurate, precise and specific.

Introduction

Vitamin C, also is known as ascorbic acid is one of the most important vitamins and essential for human and animal life. This water soluble vitamin contributes to many health benefits such as prevention of scurvy and cancer, relief from common cold, stimulate collagen synthesis and play a significant role in wound healing process (Iqbal *et al*., 2004). According to Teucher *et al.* (2004), vitamin C helps to enhance availability and absorption of iron from non-heme sources. Vitamin C also has antioxidant properties since it can easily lose the electron to neutralize and inhibit free radicals from being oxidized in preventing cell damage. It is also commonly used as food additive which acts as antioxidant (Whitney and Rolfes, 2008).

Vitamin C is an organic compound consists of carbon, hydrogen and oxygen (Chinnici *et al*., 2005). The terms vitamin C is not only used for ascorbic acids, but it includes all compounds exhibiting biological activity such as oxidized, ester and synthetic form. The main biological form of vitamin C is L-ascorbic acid, and it can reversibly change to oxidized form called dehydroascorbic acid (Fenolland Martinez, 2010). Many factors can cause oxidation of vitamin C such as pH, light, temperature, presence of oxygen and metal ion (Wantz *et al*., 2005).

Human is unable to synthesize their own vitamin C supply as human cells cannot perform the crucial last step in vitamin C biosynthesis,the conversion of L-gulono-g-lactone into ascorbic acid which is catalyzed by gulonolactone oxidaseenzyme (LinsterandVan Schaftingen, 2007). Therefore, they require vitamin C for maintaining the physiological functions. To meet the requirement, vitamin C must be consumed from diet. The recommended nutrient intake of vitamin C for Malaysian adult is 70 mg per day. An intake of 45 mg/day will ensure measurable amount of ascorbate be present in the plasma of most people and available to supply tissue requirements for metabolism or repair at sites of depletion or damage (MOH, 2005).

Many methods can be used for determination of vitamin C such as spectrophotometry, electrophoresis, titration, and high performance liquid chromatography (HPLC) (Tang and Wu, 2005; Dong *et al*., 2007; Spinola *et al.*, 2012). The most commonly used method is oxidation-reduction titration method where ascorbic acids are oxidized to dehydroascorbic acid and the indophenol dye is reduced to a colorless compound. The end point of the titration can be easily detected when an excess of the unreduced dye give a rose pink color in an acid

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solution (Tee *et al.*, 1996). It is a simple and easy method to determine vitamin C in fruits and fruit juices. However, the method is not suitable for fruits that have reddish-purplish color. The titration method also is time-consuming and lack of specificity due to interference of reducing substances in the food such as ferrous iron, stannous tin, cuprous copper, sulphur dioxide, sulphite orthiosulphate (Eitenmiller *et al.*, 2008).

Several other methods to determine vitamin C content like spectrometric, spectrofluorimetric, and electrophorhesis, still some of them are not practical and need re-evaluation due to insufficient of sensitivity and selectivity (Agar, 1995). Quiroz and Fernandez (2009) stated that the most preferred method of determining vitamin C content in foods is a chromatographic method using HPLC due to the rapid, high accuracy and consistency.

Previously, there were many studies done on citrus fruits mainly focusing on determination of vitamin C using HPLC by modifying the stationary phase, mobile phase, type of detector and sample preparation (Gazdik *et al*., 2008; Spinola *et al*., 2012) but there was not many studies done comparing two or more different methods in determining vitamin C in citrus fruits (Ullah *et al*., 2012; Spinola *et al*., 2013). Moreover, most studies are just focusing on single type of lime and do not focus on another type of limes such as kaffir lime and musk lime.Thus, the purpose of this study was to provide the comparison between titration and HPLC method in determination of vitamin C content in citrus fruits.

Materials and Methods

Sample preparation of titration method

Sample and standard preparation and quantification of vitamin C in titration method was performed using the method described by Tee *et al.* (1996). In brief, fruit samples (200 g-300 g) was blended with 6% metaphosphoric acid (HPO_3) with an equal volume to homogenous slurry and made it up until 500 ml of volume. The homogenous mixture was measured around 10 to 30 ml and diluted into 100 ml volumetric flask with 3% of metaphosphoric acid (HPO_3). The diluted sample was then filtered to remove away suspension using vacuum pump before 10 ml aliquote of the filtrate was pipetted into a small Erlenmayer flask. The filtrate was immediately titrated with a dye solution 2, 6-dichlorophenol-indophenol to a faint pink end point. Triplicate titration was conducted for all samples.

Preparation of standard solution for titration method

Vitamin C standard solution (0.2 mg/ml) was prepared by dissolving 100 mg vitamin C in 3% of metaphosphoric acid (HPO_3) solution and diluted to 500 ml with the same solvent. Then, 5 ml aliquot of the vitamin C standard solution was diluted (containing 1 mg vitamin C) with 5 ml 3% metaphosphoric acid (HPO3). Next the diluted vitamin C standard solution was titrated with dye solution 2, 6-dichlorophenol-indophenol to a faint pink color. Triplicate titration also was conducted for the vitamin C standard.

Quantification of Vitamin C in the extracted solution of titration method

Firstly, weight of the sample in 10 ml filtrate was calculated by the following equation:

$$W = \frac{a}{b} \; x \frac{c}{100ml} \; x \; 10ml$$

Where,

a = Weight of sample will be used

b = Final volume of homogenous slurry [sample + 6% metaphosphoric acids (HPO_3)]

c= Aliquot of homogenous slurry used for dilution to 100 ml with 3% metaphosphoric acid (HPO_3)

Then total vitamin C content (mg per 100 g sample) of each sample was obtained by the following equation:

$$\text{Vitamin C content (mg per 100 g sample)} = \frac{X \; x \; Y \; x \; 100}{W}$$

X = volume of dye used for titration of aliquot of diluted sample (ml)

Y = vitamin C equivalent of dye solution, mg per ml dye solution

W = weight of sample in aliquot of filtrate of diluted sample used for titration (g)

Sample preparation of HPLC method

Sample preparation, chromatography conditions, identificantion and quantification of vitamin C in HPLC method was perfomed using the method described by Czech Agriculture and Food Inspection Security (2005). The homogenous solid sample was measured around 10 to 30g and mixed it with 60 to 80 ml 3% of metaphosphoric acid (HPO_3) for one minute. The obtained extracted was filtered through filtration paper and washed it for few times by using vacuum pump filtration. Next, the filtrate quantitatively transferred into a 100 ml volumetric flask and 3% of metaphosphoric acid (HPO_3) was added up to 100 ml volumetric mark. All the sample

solutions was filtered again through 0.45 µm syringe filter. After that, the samples were run in the HPLC system.

HPLC system or chromatography conditions

The total of vitamin C content in 6 types of fruits were determined by HPLC system. The reversed–phase liquid chromatographic method was used for determination of vitamin C consisted of an isocratic elution procedure with photodiode assay detection at 254 nm. The separations were carried out on Carbon 18 (C_{18}) column of 250mm x 4.6 mm (LiChro CART, Darmstadt, Germany). The mobile phase used was a mixture of methanol - water (5.95, v/v). The flow rate of the mobile phase was 1.0 mL min-1 and 20 µL injection volume of samples and standard were used in quantitative analysis. The temperature of analytical column was kept constant at laboratory temperature which was 25°C. The standard of the vitamin C was an external standard which 1mg/ml natural Vitamin C derivatives. There were different type of concentrations of vitamin C range from 0.5 mg/ml to 200 mg/ml based on a 10 point calibration. If the shape of the plot was deviates from the square waveform during detection of a peak, then the peak was considered as no longer spectrally pure. Standard solutions were filtered through a filter paper and then was filtered again through 0.45 µm syringe filter. To prevent the loss of vitamin C all standards and extracted sample solutions were protected from light by covering it with aluminium foil.

Identification and quantification of vitamin C in extraction solutions

Identification was performed by the comparison of retention time of analyte in the analyzed sample with the retention time of the calibration standard. Quantification was carried out with the external standard method (Vitamin C standards at various concentrations) using the following equation formula to calculate the Vitamin C concentration on samples.

$$CA\ (mg/\ 100g) = \frac{Aa \times D \times RF \times 100}{m}$$

Where,

A_a = Peak area of the analyte

D = Sample factor dilution

RF = Response factor (way to adjust the proportionality of the detector response to the concentration of vitamin C and is calculated the following formula:

$$RF = \frac{Cst}{Ast}$$

Where,

Cst = standard working solution concentration 50 µg/ml

Ast = corresponding peak area

m = weight of samples

Statistical analysis

IBM SPSS version 21.0 software was used for statistical analysis. The total vitamin C concentration in food samples was expressed as mean ± standard deviation. One-way ANOVA was used to determine significance difference of mean value of vitamin C content ($p<0.05$) among the samples for each method. Moreover, independent t-Test was used to determine significance difference of mean value of vitamin C content ($p<0.05$) between the titration and HPLC method. The Pearson correlation test also was used to test the correlation in term of vitamin C content of the samples between oxidation- reduction titration and HPLC method.

Results and Discussion

Weight, diameter and colour of fruit samples

Table 1 shows the weight, diameter and skin colour of citrus fruit samples. Among the six kinds of citrus fruit samples, grapefruit has the highest weight of 243 g followed by orange (151 g), lemon (148 g), kaffir lime (65 g), lime (56 g) and musk lime (3.4 g). Similarly, grapefruit has the highest diameter of 8.0 cm followed by orange (6.5 cm), lemon (5.8 cm), kaffir lime (5.0 cm), lime (4.6 cm) and musk lime (3.0 cm). Table 1 also shows skin colour of the citrus fruits, as the orange fruit is a bright orange while grapefruit is yellow-orange colour. The skin colour of lemon is yellow whereas lime, musk lime and kaffir lime are the greencolour.

Total vitamin C content in fruit samples by titration and HPLC method

Table 2 shows the total vitamin C content in fruit samples by titration method. By comparing the vitamin C concentration of the six citrus fruits, orange has the highest vitamin C content of 58.30 mg/100g followed by grapefruit (49.15 mg/100g), lemon (43.96 mg/100g), kaffir lime (37.24 mg/100g) and lime (27.78 mg/100g). Musk lime has the least vitamin C content at only 18.62 mg/100g. To determine the precision of the data, triplicate samples were analysed, and standard deviation (SD) and coefficient of variation (CV) were calculated. A CV below 5.0% is considered precise whereby an acceptable range for standard deviation is between $-1.00 < SD < 1.00$. Generally, SD of the fruit samples

Table 1. Weight, diameter and colour of fruit samples

Fruit sample	Weight (g)	Diameter (cm)	Skin color
Orange	151	6.5	Bright orange
Grapefruit	243	8.0	Yellow orange
Lemon	148	5.8	Yellow
Lime	56	4.6	Green
Musk lime	3.4	3.0	Green
Kaffir lime	65	5.0	Green

Table 2. Total Vitamin C content (mg/100g) in fruits sample by different method

Fruit Samples	Titration		HPLC	
	Mean (mg/100g)	Coefficient of variation (%)	Mean (mg/100g)	Coefficient of variation (%)
Orange	$58.30 \pm 0.53^{a\#}$	0.91	$43.61 \pm 1.72^{a*}$	3.95
Grapefruit	$49.15 \pm 0.53^{b\#}$	1.08	$26.40 \pm 0.12^{b*}$	0.44
Lemon	$43.96 \pm 0.93^{c\#}$	2.08	$31.33 \pm 0.92^{c*}$	2.93
Lime	$27.78 \pm 0.53^{d\#}$	1.90	$22.36 \pm 0.68^{d*}$	3.04
Musk Lime	$18.62 \pm 0.53^{e\#}$	2.84	$16.78 \pm 0.33^{e*}$	1.96
Kaffir Lime	$37.24 \pm 1.06^{f\#}$	2.84	$21.58 \pm 0.51^{f*}$	2.36

Values in the same column with different superscripts letters are significantly different at $p<0.05$ (ANOVA). Values in same row with different superscript symbol are significantly different at $p<0.05$ (T-test).

(orange, grapefruit, lemon, kaffir lime, lime and musk lime obtained are ± 0.53, ± 0.53, ± 0.92, ± 1.06, ± 0.529 and ± 0.53, respectively) were within the range except for kaffir lime. The CV of fruit samples (orange, grapefruit, lemon, kaffir lime, lime and musk lime obtained were 0.91%, 1.08%, 2.08%, 2.84%, 1.90% and 2.84%, respectively) also were less than 5.0%, thus indicate the precision of the data. Table 2 also shows that the concentration of vitamin C in the fruit samples were significantly different ($p<0.05$).

Table 2 shows the total vitamin C content in fruit samples by high performance liquid chromatography (HPLC) method. By comparing the vitamin C concentration of the six citrus fruit samples, it can be seen that orange remains to contain the highest vitamin C of 43.61 mg/100g followed by lemon (31.33 mg/100g), grapefruit (26.40 mg/100g), lime (22.36 mg/100g), and kaffir lime (21.58 mg/100g). Among the fruit samples, musk lime has the lowest vitamin C content of only 16.78 mg/100g. Similarly, SD of the studied samples was within the acceptable range except for orange while CV of all samples were less than 5.0%, thus indicating the precision of the data.The concentrations of vitamin C in the fruit samples were also significantly different($p< 0.05$).

Comparison of vitamin C content of fruit samples by titration method between a present and previous studies is shown in Table 3. Vitamin C of orange sample in the present study is almost similar to values reported by Cioroi (2006) (56.02 mg/100g), Sanusi *et al.* (2008) (56.00 mg/100g) and Bungau *et al.* (2011) (56.40 mg/100g) but differ in the value reported by Tee *et al*., (1988). Vitamin C of grapefruit in this study is close to the value reported by Cioroi (2006) (48.010 mg/100g) and Sanusi *et al*. (2008) (47.000 mg/100g) but higher than reported by Bungau *et al*. (2011) (39.40 mg/100g). Lemon in the present study contained almost similar amount of vitamin C as published by Tee *et al.* (1988) (46.800 mg/100g), Cioroi (2006) (51.780 mg/100g), Sanusi *et al.* (2008)

Table 3. Comparison of the vitamin C content in citrus fruit sample by titration method between present and previous studies

Samples	Vitamin C content (mg/100g)	Studies
Orange	58.304	Present study
	42.700	Tee *et al.* (1988)
	56.020	Cioroi (2006)
	56.000	Sanusi *et al.* (2008)
	56.400	Bungau *et al.* (2011)
Grapefruit	49.145	Present study
	48.010	Cioroi (2006)
	47.000	Sanusi *et al.* (2008)
	39.400	Bungau *et al.* (2011)
Lemon	43.956	Present study
	46.800	Tee *et al.* (1988)
	51.780	Cioroi (2006)
	41.000	Sanusi *et al.* (2008)
	49.000	Bungau *et al.* (2011)
Lime	27.778	Present study
	41.700	Tee *et al.* (1988)
Musk lime	18.620	Present study
Kaffir lime	37.241	Present study

(41.000 mg/100g) and Bungau *et al.* (2011) (49.000 mg/100g). However, vitamin C in lime sample in this study exhibited considerable difference compared with Tee *et al*. (1988). However, there was no previous study reported the value of vitamin C content in musk lime and kaffir lime.

Table 4 shows comparison of vitamin C content in fruit samples by HPLC method between a present and previous studies. Orange fruit had the almost similar amount of vitamin C with the value reported by Scherer *et al.* (2012) (43.133mg/100g). However, the differences in vitamin C between the studied orange with other studies (52.50 to 64.27 mg/100g) were quite large (Tee *et al.*, 1997; Chebrolu *et al*., 2012). For grapefruit, the vitamin C content of the present study was smaller than reported by Tee *et al.* (1997) (40.000 mg/100g) and Cherbrolu *et al.*, (2012) (30.670 mg/100g).However, the vitamin C content in present study was closeto the value reported by Scherer *et al.* (2012). Previous studies on vitamin C of lemon indicated a large difference of 19.40 to 55.50 mg/100g (Tee *et al*., 1997; Chebrolu *et al*., 2012). In contrast, the vitamin C in lime sample of the present study was lower compared with the report by Tee *et al.* (1997) (36.900 mg/100g) and Cherbrolu *et al.* (2012) (38.960 mg/100g). Similarly, musk lime (41.60 mg/100g) and kaffir lime (37.00 mg/100g) in this study exhibited substantial differences in terms of vitamin C content as compared with value by Tee *et al*. (1997).

Differences of vitamin C content between titration and high performance liquid chromatography (HPLC) methods

Table 2 shows a comparison of mean vitamin C content between titration and high performance liquid chromatography (HPLC) methods. There were significant differences in the content of vitamin C between both methods ($p < 0.05$). Generally, the main advantage of using oxidation-reduction titration method is because of its simplicity using simple equipment and inexpensive chemicals. Furthermore, the reaction of indophenol dye with the ascorbic acid is very fast. However, in some condition, the oxidation-reduction titration may overestimate the vitamin C content of fruit as the end point of titration could be difficult to be detected especially when high colored (e.g.,reddish-purplish)fruit was used (Hernandez *et al.*, 2006). Besides, the presence of reducing substances (ferrous ion, copper ion, sulphur dioxide, sulphite and thiosulphate) in the fruit samples can react with the indophenol dye and cause overestimation of vitamin C in fruit samples (Spinola *et al.*, 2013). When oxidation-reduction titration is not rapid, the exposure of samples to oxygen and light may cause degradation of the

Table 4: Comparison of the vitamin C content in fruit sample by HPLC method between present and previous studies

Samples	Vitamin C content (mg/100g)	Studies
Orange	43.611	Present study
	52.500	Tee *et al*. (1997)
	64.270	Chebrolu*et al*. (2012)
	43.133	Scherer *et al*. (2012)
Grapefruit	26.402	Present study
	40.000	Tee *et al*. (1997)
	30.670	Chebrolu*et al*. (2012)
Lemon	31.329	Present study
	19.400	Tee *et al*., (1997)
	55.500	Chebrolu*et al*. (2012)
	43.167	Scherer *et al*. (2012)
Lime	22.359	Present study
	36.900	Tee *et al*. (1997)
	38.960	Chebrolu*et al*. (2012)
Musk lime	16.779	Present study
	41.600	Tee *et al*. (1997)
Kaffir lime	21.578	Present study
	37.000	Tee *et al*. (1997)

ascorbic acids. Besides, the reduced ascorbic acids (dehydroascorbic acids) also are not quantified in this method. Therefore, the titration method is lack of specificity, do not overcome problem with reducing substances and might cause exposure to the air. High performance liquid chromatography (HPLC) method is a more specific, sensitive and selective technique for determining vitamin C content in fruit samples. Furthermore, HPLC method requires small amount of sample and chemicals, quite rapid and less susceptible to systemic error due to its high specificity (Quiroz and Fernandez, 2009).

Using simple linear correlation,the relationship between titration and HPLC methodsregarding vitamin C content in citrus fruits samples was performed. There was no correlation found between titration and HPLC methods in the studied samples (Table 5). In contrast, Hernandez *et al.* (2006) showed that there was strong significant correlation (r^2=0.980) between titration and HPLC methods. Hernandez *et al.* (2006) also reported no significant differences between the titration and HPLC methods in orange, papaya, mango and pineapple samples. Spinola *et al.* (2013) also showed strong significant correlation (r^2=0.976) between the titration and HPLC methods in samples of lemon, papayas, passion fruits cherimoyas, strawberries and broccoli. Similarly, Spinola *et al*. (2013) also reported no significant differences in vitamin C contents by both methods.

Generally, the variations of vitamin C content in citrus fruit samples could be attributed to many pre-harvest and handling factors includingthe environment. Exposure of fruits to light have strong influence on chemical composition of fruits especially vitamin C. The higher the light intensity exposed to citrus fruit trees, the higher the vitamin C content in the fruits as light is required for photosynthesis to produce energy in the form of glucose to produce more ascorbic acids in the fruits (Stumpf *et al.*, 1988). Besides, Hassan *et al.* (2012) stated that utilization of organic based fertilizer may improve the quality of plants by reduction of nitrate content, increment of vitamin C content, antioxidant activity, nitrogen and calcium contents. Furthermore, ripening process of citrus fruits may reduce the vitamin C content. Igwe (2013) showed that vitamin C concentration of unripe fruits was higher than the matured ripe fruits and both vitamin C levels of ripe and unripe fruits decreased when the temperature and length of exposure of fruits were increased. Storage temperature and handling also are important in maintaining the vitamin C content in fruit samples. In addition, citrus fruits grown in fully irrigated system during flowering, the fruit growing stage and ripening showed increased

Table 5. Pearson's correlations coefficient (r) test between titration and high performance liquid chromatography (HPLC) method in term of total vitamin C content in fruit samples

Total vitamin C in fruit samples by HPLC method	Total vitamin C in fruit samples by titration method (r)					
	Orange	Grapefruit	Lemon	Lime	Musk Lime	Kaffir Lime
	-0.345	-0.907	0.835	0.880	0.237	-0.985
	p = 0.775	p = 0.277	p = 0.371	p = 0.315	p = 0.847	p = 0.110

*p<0.05

concentration of vitamin C compared to control (Aguado *et al*., 2012).

Conclusion

In this study, orange contained the highest vitamin C among the citrus fruits followed by grapefruit, lemon, kaffir lime and lime by titration method. Similarly, orange also has the highest vitamin C by HPLC method followed by lemon, grapefruit, lime andkaffir lime.Musk lime contained the least vitamin C by both methods.Comparison between titration and HPLC method in terms of vitamin C content, showed significant differences in all fruit samples. The vitamin C contents in fruits samples were higher in titration method compared with HPLC method. The significant differences between the two methods could be affected due to many factors such as lack of specificity, presence of the reducing substances, time consuming and exposure to the air. Furthermore, the value of vitamin C content in HPLC method was lower than titration method. This could be due to the high sensitivity, selectivity and specificity of the HPLC method in isolating actual amount of vitamin C in fruit samples without any interference of other substances.

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