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Short Communication

Effect of Glucose and Ascorbic Acid on Total Phenolic Content Estimation of Green Tea and Commercial Fruit Juices by Using Folin Ciocalteu and Fast Blue BB Assays

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ABSTRACT

Folin Ciocalteu (FC) assay had been widely used in the estimation of total phenolics content (TPC) in foods. However, the main disadvantage of this assay is that the reagent reacts with reducing substances and measures the total reducing capacity of a sample, not just phenolic compounds. The Fast Blue BB (FBBB) assay is another assay that can be used to estimate the total phenolic content in foods instead. The aim of the study was to estimate and compare the total phenolic content of green tea and commercial fruit juices using FC and FBBB assays, and also the effects of glucose and ascorbic acid on both assays. Green tea had the highest ascorbic acid content and TPC value among the samples. TPC estimated using FC assay were significantly higher than FBBB assay in all the samples. FC and FBBB assays were not affected by glucose at different concentrations. However, FC assay was significantly affected by the presence of ascorbic acid compared to FBBB assay. In conclusion, FC assay overestimated the TPC value in the sample extracts and was significantly affected by the presence of ascorbic acid. The FBBB assay can be considered as an alternative for estimation of total phenolic content in ascorbic acid-rich foods.

Keywords: Ascorbic acid, Fast Blue BB assay, Folin Ciocalteu assay, green tea, total phenolic content

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INTRODUCTION

Folin Ciocalteu (FC) assay has been widely used in the estimation of total phenolics content in foods. The FC reagent, a mixture of phosphomolybdate and phosphotungstate, is used for the colorimetric *in vitro* assay of phenolic and polyphenolic antioxidants (Simoni et al., 2002). The assay was originally developed as an improvement to the Folin Denis assay, which was used to determine total protein concentration by measuring tyrosine and tryptophan contents in the sample (Folin & Denis, 1912; Sánchez-Rangel et al., 2013). Eventually, the FC assay was adapted further for determining total phenolic content in food samples. The assay was applied to a wide variety of food samples, from plants (Baba & Malik, 2015), and also beverages such as wine (Hosu et al., 2014; Singleton & Rossi, 1965) and tea (Alarcon et al., 2008).

However, a major disadvantage of the FC assay is that the reagent reacts with any reducing substance and measures the total reducing capacity of a sample, not just phenolic compounds. The reagent has been shown to be reactive towards thiols, vitamins, nucleotide bases (adenine, guanine, cytosine, uracil, and thymine), organic acids such as uric acid and oleic acid, and proteins (Prior et al., 2005). Also, some inorganic substances such as hydrazine, hydroxyammonium chloride, iron ammonium sulfate, iron sulfate, manganese sulfate, potassium nitrite, sodium cyanide, sodium metabisulfite, sodium phosphate, sodium sulfite, and tin chloride may also react with the FC reagent to give elevated apparent phenolic concentrations (Prior et al., 2005). Another disadvantage of the assay is that it is quite general, in a sense that it only measures the total phenolic content, without determining the specific phenolic compounds present in the sample. Hence, overestimation of the total phenolic content in food samples are more likely to occur when using FC assay due to the interference by the compounds stated earlier. As a result, food with low phenolic content may be falsely accepted as good source of phenolic compound due to overestimation.

The Fast Blue BB (FBBB) assay is another assay used in estimating the total phenolic content in food. It was developed by Medina (2011a) and utilizes FBBB diazonium salt where the diazonium group only reacts to reactive phenolic hydroxyl groups under alkaline conditions, forming stable azo complexes which can be measured at 420 nm (Lester et al., 2012). The advantage of this assay is that the diazonium salt only reacts with phenolic hydroxyl groups and leaving the non-phenolic compounds behind, thus providing a more accurate quantification of total phenolic content in the sample (Medina, 2011b). However, this assay is also quite general, where it can only quantify the total phenolic content of the samples, instead of determining every phenolic compounds present and their exact amount in the samples.

Tea is made from the leaves of the *Camellia sinensis* plant and is considered as one of the most commonly consumed beverage in the world (Hilal & Engelhardt, 2007). Green tea is made by inactivating the enzymes in the fresh leaves by drying or steaming for a short time, which prevents

the enzymatic oxidation of catechins (Wang et al., 2000). Green tea also contains considerable amount of antioxidant and ascorbic acid, albeit a low sugar content. Similarly, commercial fruit juices also contain considerable amount of ascorbic acid and sugars, similar to their fresh fruit counterparts. Since both ascorbic acid and sugars are commonly present inside fruits and vegetables, finding an assay that will not be affected by these compounds can be very useful in estimating the total phenolic content of plant samples accurately.

In this study, the effects of different concentrations of glucose and ascorbic acid on the total phenolic content of green tea and selected fruit juices estimated using FC assay and FBBB assay were determined. In addition, the ascorbic acid and total phenolic content of each samples estimated using both assays was also compared.

MATERIALS AND METHODS

Chemicals and Reagents

Absolute methanol (CH_3OH, 99.9%) was purchased from J. Kollin Chemicals (UK). Folin Ciocalteu (FC) reagent, metaphosporic acid (HPO_3) and sodium carbonate (Na_2CO_3) pellets were obtained from Merck (Darmstadt, Germany). Gallic acid, 2, 6-dichlorophenol indophenol (DCPIP) sodium salt, ethylenediaminetetraacetic acid (EDTA), and 4-benzoylamino-2, 5-dimethoxybenzene diazonium chloride hemi (-zinc chloride) salt (FBBB salt) were obtained from Sigma Aldrich (Missouri, USA). Glucose standard was obtained from Scharlau Chemicals (Barcelona, Spain).

Preparation of Sample

The tea leaves were harvested and processed into green tea at Kakuda Seicha Tea Plantation in Yame-shi (Fukuoka prefecture, Japan), before delivered to Kyushu Institute of Technology (KIT) (Fukuoka Prefecture, Japan). The green tea was placed in sealed plastic before shipped to Universiti Putra Malaysia. The leaves were stored at -20°C until used. Prior to the extraction process for estimation of total phenolic content, the leaves were ground into powder using a commercial blender from Waring Commercial (Connecticut, USA) and passed through a 0.025mm sieve.

Commercial orange & guava juices of a particular brand were purchased from several supermarkets around Serdang district (Selangor, Malaysia).The fruit juices were stored at 6°C until used. Prior to the extraction process for estimation of total phenolic content, the juices were freeze dried for 3 days and then stored at -20°C before being re-dissolved in 70% methanol.

Determination of Ascorbic Acid Content

Ascorbic acid content was determined using indophenol titration method (Official Method of Analysis [OMA], 2012). 6% and 3% HPO_3 – EDTA solutions were prepared by mixing 6% HPO_3 with 0.005M EDTA solution, and mixing 3% HPO_3 with 0.0025M EDTA solution, respectively. The sample (100g) was mixed with 100 mL of 6% HPO_3 – EDTA solution and blended. The homogenous mixture (10mL) was taken and diluted to 100mL with 3% HPO_3 – EDTA solution and then filtered. The

filtrate (10 mL) was taken and titrated with 0.025% DCPIP solution to a faint pink end point which lasted for 15 sec. Ascorbic acid equivalent value was determined by taking 5 mL of ascorbic acid standard (100 mg ascorbic acid + 400 mL of 3% HPO_3 – EDTA solution) and diluting it with 5 mL of 3% HPO_3 – EDTA solution before titration with DCPIP solution. The ascorbic acid content, in mg per 100 g of sample, was calculated using formulas (1) and (2):

$$\text{Ascorbic acid content (mg per 100 g)} = \frac{\text{Vsample (mL)} \times 100}{\text{Vstandard (mL)} \times \text{Ws (g)}} \quad (1)$$

Vsample = amount of dye used during titration of sample
Vstandard = Amount of dye used during titration of standard
Ws = Weight of sample in aliquot of filtrate of diluted sample used for titration

$$\text{Ws} = \text{Va(mL)} \times \frac{\text{W1 (g)}}{\text{Vb(mL)}} \times \frac{\text{Vc(mL)}}{100} \quad (2)$$

Va = Volume of aliquot of mixture diluted with 3% HPO_3
Vb = Final volume of mixture (sample + 6% HPO_3)
Vc = Volume of filtrate used for titration
W1 = Weight of sample used

Sample Extraction for Total Phenolic Content Determination

The method of extraction was adapted based on the method for analysis of phenolic compounds in grains and beverages described by Medina (2011a). One gram of dry, powdered sample was extracted with 20 mL of 70% methanol. The mixture was centrifuged at 2683 × g for 30 min and the filtered using a syringe filter. The collected supernatant was stored at 6°C before analysis.

Folin Ciocalteu (FC) Assay

FC assay was done according to Medina (2011a) with some modification (higher dilution factor). The pre-diluted (50 times dilution factor) sample extract (0.1 mL) was transferred into borosilicate tubes, followed by the addition of 1.7 mL of distilled water and 0.1 mL of 1N FC reagent. Then, 0.1 mL of saturated 20% Na_2CO_3 was added and mixed, and the solution was allowed to react for 90 min. The absorbance of the mixture was measured at 750 nm using UV-1800 UV spectrophotometer from Shimadzu Corporation (Japan). Gallic acid standard at varying concentrations (0.001M, 0.005M, 0.01M, 0.015M, 0.02M, and 0.025M) were prepared.

Fast Blue BB (FBBB) Assay

FBBB assay was done according to Medina (2011a) with some modification (higher dilution factor). The pre-diluted (50 times dilution factor) sample extract (0.1 mL) was transferred into borosilicate tubes followed by the addition of 1.7 mL of distilled water.

Next, 0.1 mL of sonicated 0.1% FBBB salt was added. The solution was mixed for 30 sec before 0.1 mL of 20% Na_2CO_3 was added and mixed for another 30 sec. The resulting solution was incubated in the dark for 90 min at room temperature. The absorbance was measured at 420 nm using UV-1800 UV spectrophotometer from Shimadzu Corporation (Japan). Gallic acid standard at varying concentrations (0.001M, 0.005M, 0.01M, 0.015M, 0.02M, and 0.025M) were prepared.

Effect of Glucose and Ascorbic Acid on Total Phenolic Content

Different concentrations of glucose (0.01M, 0.02M, 0.03M, 0.04M, and 0.05M) and ascorbic acid (0.001M, 0.002M, 0.003M, 0.004M, and 0.005M) were prepared in distilled water. Next, a mixture containing 50 µL of pre-diluted sample (50 times dilution factor) and 50 µL of the glucose or ascorbic acid solution was prepared and assayed according to the FC and FBBB assays stated earlier. The resulting total phenolic content (TPC) value was calculated. The assays were repeated using different concentrations of glucose and ascorbic acid.

Statistical Analysis

The results were analyzed using SPSS version 21. The experiments were done in triplicates and the average value was obtained. The total phenolic content obtained was expressed as gallic acid equivalent (GAE) per 100 g dry weight with standard deviation (SD). ANOVA (p-value set at $p<0.05$) was used to determine whether there were significant differences for ascorbic acid content and total phenolic content of each samples obtained using both assays. Independent sample t-test (p value set at $p<0.05$) was used to determine any significant difference in the TPC content obtained using the two assays in the presence of glucose and ascorbic acid.

RESULTS AND DISCUSSION

Sugar Composition and Ascorbic Acid Content

Total sugar content of green tea, orange juice and guava juice had been previously reported by several studies. Among the samples, orange juice showed the highest amount of sugar, followed by guava juice and green tea. Abel and Aidoo (2016) reported that the total sugar content of commercial orange juice was 10.72 g per 100 mL of sample, with the highest amount represented by sucrose (4.21 g per 100 mL), fructose (3.39g per 100mL), and lastly glucose (3.11 g per 100 mL). Sanz et al. (2004) reported that fructose is the main sugar (2.74 g per 100 mL) found inside fresh guava juice, followed by glucose (0.95g per 100mL) and sucrose (0.57 g per 100 mL). For green tea, the main sugar found in it was fructose (0.72 g per 100 g), glucose (0.68 g per 100 g), and sucrose (0.71 g per 100 g) according to Shanmugavelan et al. (2013).

Ascorbic acid content in the samples determined using indophenol titration method is tabulated in Table 1. Overall, significant difference ($p<0.05$) was observed between all the samples. The highest amount of ascorbic acid obtained was from green tea

(295 ± 9 mg per 100 g of sample), followed by commercial guava juice (68 ± 4 mg per 100 g of sample), and commercial orange juice (38 ± 5 mg per 100 g of sample). The value obtained by the samples in this study was almost similar to the values reported by previous studies. According to Somanchi et al. (2017), the amount of ascorbic acid available in green tea ranges between 250 to 280 mg per 100 g of dried leaves. Green tea usually have higher ascorbic acid content than processed tea such as black and oolong tea, possibly due to the lack of manufacturing and fermentation process during its production (Cabrera et al., 2006; Lee & Kader, 2000).

Table 1
Ascorbic acid content of the samples

Samples	Ascorbic acid content (mg/100 g) ± SD
Green Tea	295 ± 9 [a]
Commercial Guava Juice	68 ± 4 [a]
Commercial Orange Juice	38 ± 5 [a]

Note. The values were expressed as mean of mg per 100 g sample ± SD (n = 3).Same superscript shows significant difference ($p<0.05$) in ascorbic acid content between each samples

As for commercial orange juice, the value obtained in this study was slightly lower than the value reported by Bungau et al. (2011) which was 56.4 mg per 100 g of sample. Similarly, Dauda and Sadisu (2013) reported that the ascorbic acid content of guava fruit was 180 mg per 100 g of sample, which was higher than the value obtained from the commercial guava juice in this study. However, it should be noted that the value reported by both of these studies was obtained from fresh fruits, not manufactured juice. The reason for commercialised fruit juices having lower ascorbic acid content than fresh fruit is probably due to the packaging process during manufacture. Kaleem et al. (2015) stated that processes such as clarification, filtration and pasteurization could easily destroy vitamin C. In addition, temperature and oxygen also play a major role in the loss of vitamin-C during processing of the fruit juices (Kaleem et al., 2015).

Total Phenolic Content

The total phenolic contents (TPC) of green tea, commercial guava juice, and commercial orange juice extracts using Folin Ciocalteu (FC) and Fast Blue BB (FBBB) assays are tabulated in Table 2. The TPC of the green tea extract estimated using FC assay was 2744 ± 99 mg GAE per 100 g DW, and FBBB assay was 1504 ± 37 mg GAE per 100 g DW. In the guava juice extract, TPC estimated using FC assay was 2403 ± 74 mg GAE per 100 g DW, and FBBB assay was 1408 ± 55 mg GAE per 100 g DW. The orange juice extract's TPC estimated by FC and FBBB assays were 2428 ± 67 mg GAE per 100 g DW, and 1346 ± 51 mg GAE per 100 g DW, respectively. Statistical analysis showed significant difference ($p<0.05$) between the total phenolic content estimated using both assays in all of the samples.

In the FC assay, green tea extract had the highest TPC value, followed by orange juice and guava juice extracts. However, there were no significant differences ($p>0.05$) in

Table 2
Total phenolic content of the samples

Extract	Assay	Total phenolic content (mg GAE/100 g DW) ± S.D
Green tea	Folin Ciocalteu	2744 ± 99 [a]
	Fast Blue BB	1504 ± 37 [a]
Guava juice	Folin Ciocalteu	2403 ± 74 [b]
	Fast Blue BB	1408 ± 55 [b]
Orange juice	Folin Ciocalteu	2428 ± 67 [c]
	Fast Blue BB	1346 ± 51 [c]

Note. The values were expressed as mean of mg GAE per 100 g DW ± SD (n = 3).Same superscript shows significant difference ($p<0.05$) in TPC between assays in each sample extract

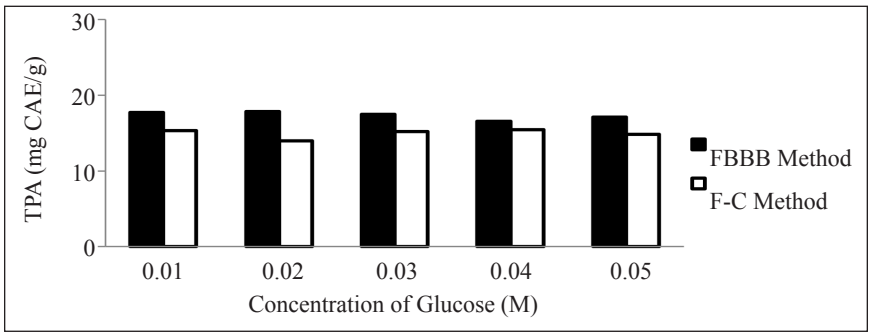
the TPC values between all three extracts for FC assay. In the FBBB assay, green tea extract also had the highest TPC value, followed by guava juice and orange juice extracts. Significant difference ($p<0.05$) was found between the green tea and orange juice extracts.

In general, FC assay estimated a higher level of TPC in all the sample extracts compared to FBBB assay. The differences in the estimated value of TPC using both assays can be attributed to several factors. The same sample extract can give out different values when analysed using different assays. As shown in the present study, FC assay showed a higher TPC in all the samples compared to FBBB assay. However, the TPC obtained using FC assay was probably affected by the presence of ascorbic acid in the samples. As stated earlier, the amount of ascorbic acid in the samples ranged from 38 mg to 295 mg per 100 g of sample. The presence of ascorbic acid in the samples could affect the TPC obtained using FC assay, resulting in a significantly higher TPC value than FBBB assay.

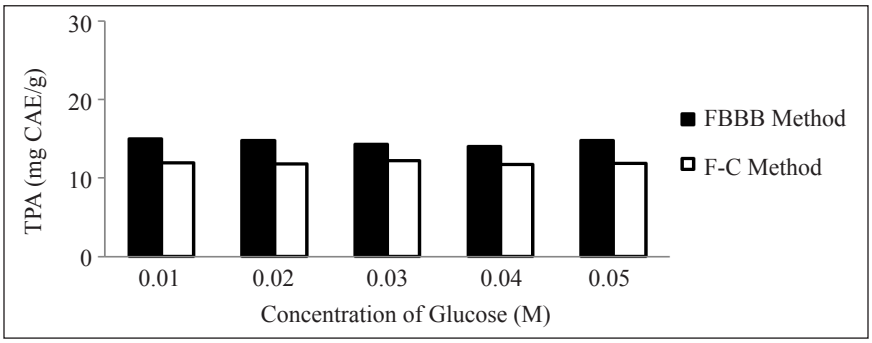
The TPC of a sample can also be influenced by the method of sample preparation, type of solvent used and its concentration, and the extraction temperature (Jahangiri et al., 2011). A study by Jahangiri et al. (2011) showed that higher incubation temperature might provide higher yield of total phenol recovery. A reason for this is that incubation in hot water may break down some pectic polysaccharides from the cell wall (Sun et al., 2002), and weaken the cell wall's integrity, allowing the solvent to get into contact with the phenolic compounds.

Effect of Glucose and Ascorbic Acid Concentration on TPC value

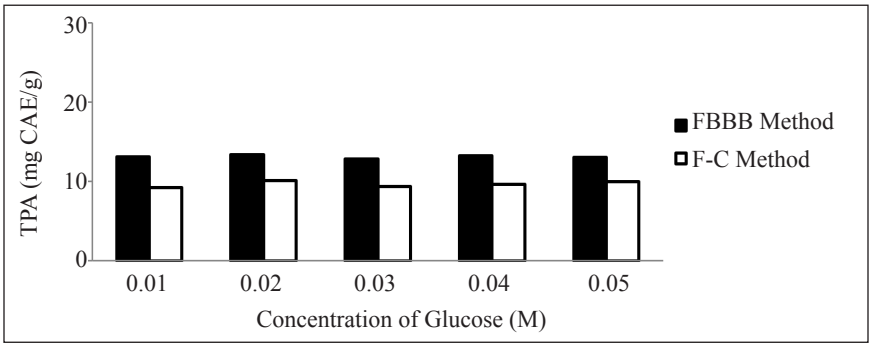
Figure 1 shows the effect of different glucose concentrations on the TPC value (mg GAE/g) obtained by FC and FBBB assays. Based on the results, FBBB assay showed higher TPC values at every glucose concentration compared to FC assay for all the samples. Statistical analysis showed significant difference ($p<0.05$) between the TPC values obtained by both assays. However, the TPC value did not increase linearly with the glucose concentration for both assays. It shows that glucose, or possibly other sugars, will not affect the absorbance value of both assays significantly,



(a)



(b)



(c)

Figure 1. Effect of different glucose concentrations on the TPC value of (a) Green tea, (b) Commercial guava juice and (c) Commercial orange juice using FC and FBBB assays. P-value was set at ($p<0.05$)

regardless of its concentration in the sample. It corresponds with a previous study done by (Lester et al., 2012) where neither FC nor FBBB gave a response to sugar (fructose, glucose and sucrose) standards; as sugars were reported to interfere with the FC assay only when heated (Slinkard & Singleton, 1977). As such, both assays can be used to estimate the total phenolic content of sugar-rich food samples without interference as long as it is done at room temperature.

Figure 2 shows the effect of different concentrations of ascorbic acid on the TPC value (mg GAE/g) obtained by both assays. In FC assay for all the samples, there was a significant difference ($p<0.05$) in the

TPC value as it increased linearly with the ascorbic acid concentration. On the other hand, the FBBB assay did not show any significant difference in the TPC value as the ascorbic acid concentration increased. This indicated that TPC value obtained by FC assay can be influenced by ascorbic acid amount in the samples and thus providing an overestimation of the sample's phenolic content. Hence, the total phenolic content of food samples with high ascorbic acid content, such as fruits, will be much higher than its actual value when assayed using FC assay. The result corresponded with

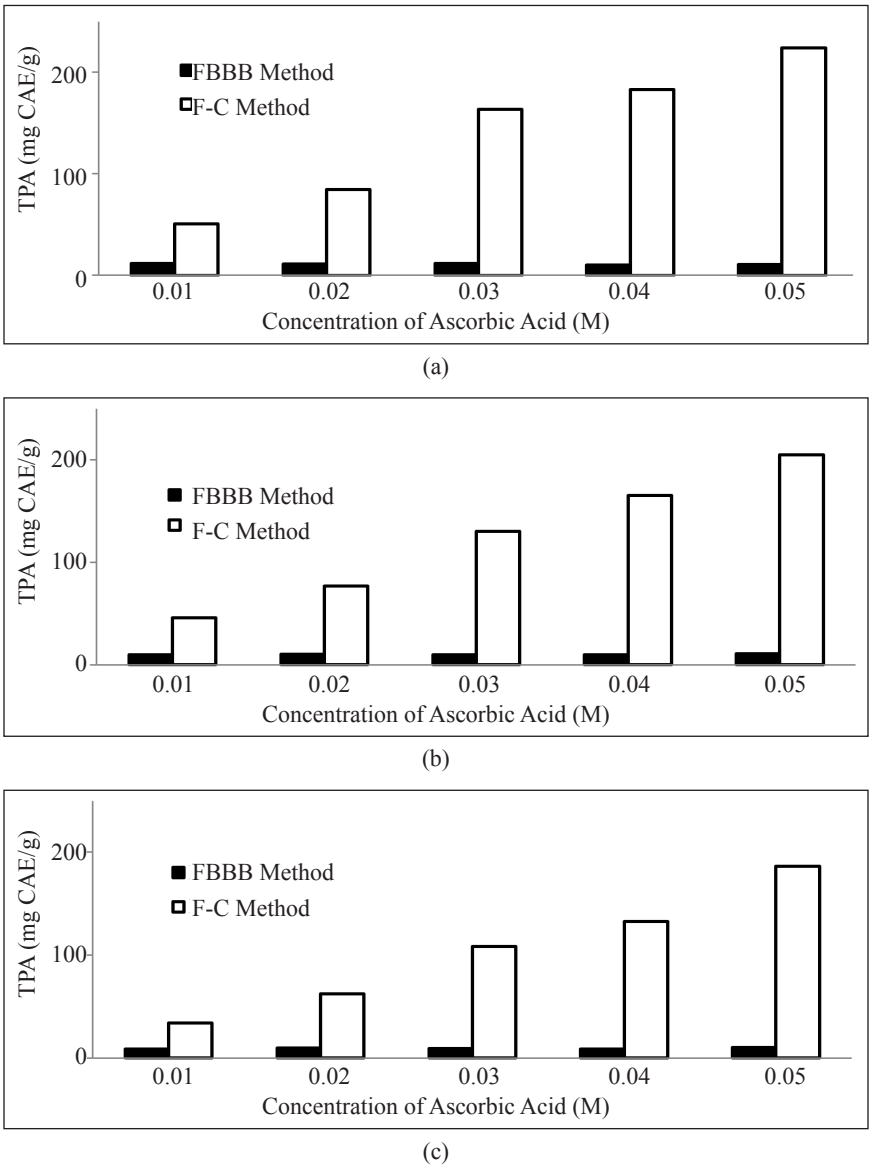


Figure 2. Effect of different ascorbic acid concentrations on the TPC value of (a) Green tea, (b) Commercial guava juice and (c) Commercial orange juice using FC and FBBB assays. P-value was set at ($p<0.05$)

previous studies that stated that the FC assay suffered from a number of interfering substance including ascorbic acid (Musa & Abdullah, 2009; Prior et al., 2005). The presence of ascorbic acid, most notably in fruit and its juices, provides a strong non-phenolic FC signal that can easily exceed the magnitude of that from actual phenolics (Singleton et al., 1999).

FC reagent is a yellow coloured solution consisting of a mixture of the heteropoly acids, phosphomolybdic and phosphotungstic acids in which the molybdenum and the tungsten are in the positively-charged oxidation state (Agbor et al., 2014). Under alkaline conditions, the phenolic compound dissociates a proton, leading to the formation of phenolate ion which reduces the FC reagent and forming the molybdenum blue and the tungsten blue which gives the mixture its blue colour (Agbor et al., 2014). The presence of ascorbic acid in the sample can interfere with the previous reaction by rapidly reacting with the FC reagent without requiring an alkaline condition, giving a blue colour right after mixing the plant extract and the reagent (Sánchez-Rangel et al., 2013).

On the contrary, FBBB assay uses the coupling property of a diazonium salt with phenolic compounds to form azo compounds in its reaction. The aromatic diazonium ion is coupled to electron-rich substrates such as phenols (Medina, 2011b). The aromatic diazonium ions ($-^{+}N{=}N-$) specifically couples with reactive phenolic hydroxyl (–OH) groups by releasing a proton (Medina, 2011a), resulting in an electrophilic aromatic substitution (Medina, 2011b). Under alkaline conditions, the coupling process results in azo compounds with wavelengths that can be read using spectrophotometer (Medina, 2011b). As a result, FBBB assay showed very minimal reaction towards ascorbic acid even at a higher concentration based on the absorbance value which is in correspondence with the results reported by Lester et al. (2012). It indicates that the absorbance value obtained by FBBB assay was not significantly influenced by the ascorbic acid in the sample. Therefore, FBBB assay is more suitable for estimating the total phenolic content of food samples with high ascorbic acid content as it will provide a reliable estimation compared to FC assay.

CONCLUSION

Based on the present study, green tea had the highest ascorbic acid content and TPC among the samples. TPC value obtained using FC assay was significantly higher than TPC value obtained using FBBB assay for all samples. Significant increase in the TPC value estimated using FC assay as the ascorbic acid concentration increased indicated that FC assay is severely affected by the presence of ascorbic acid. On the contrary, FBBB assay was not affected by the presence of glucose and ascorbic acid. Hence, Fast Blue BB assay can be considered as an alternative assay in estimating the total phenolic content in food samples with high ascorbic acid content more accurately. The present study only investigated the effects of two compounds on the estimation of TPC

using FC and FBBB assays. Future studies should include other types of compounds that might have the possibility to affect the TPC determination assays so that estimation of phenolic content of plant samples can be conducted more accurately.

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