Simple Quantitative Analysis of Ascorbic Acid in Vitamin C Related Products using High Performance Liquid Chromatography

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Abstract: Antioxidant has gained interest among consumers regarding its significances in daily life. In this research, quantitative analysis of ascorbic acid in two samples of vitamin C related products by High Performance Liquid Chromatography (HPLC) had been studied. A reversed phase HPLC system with stationary phase of Phenomenex C_{18} column (250 x 4.6 mm) was used for the separation at ambient temperature with isocratic mobile phase of 0.1% phosphoric acid. The system was analyzed at the flow rate of 0.5 ml min^{-1} and detection of dual-wavelengths ultraviolet; 245 nm and 270 nm, respectively. Successful separation of ascorbic acid of both samples had been achieved and the peaks resolved completely at retention time between 8 minutes until 10 minutes. The concentration of ascorbic acid for both samples also similar to the label on the bottles, 20 000 mg l^{-1}. The stability test also had been done by keeping the samples in four different storage conditions. The results showed that concentration of ascorbic acid were stable for both samples after being exposed for six hours. The optimized method was further validated according to The International Conference on the Harmonization of the Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) as the standard guidelines. The method showed good system suitability, linearity ($R^2 > 0.99$), recovery (> 20%), precision (%RSD) and sensitivity (limit of detection (LOD) and limit of quantification (LOQ)), indicating that the proposed method could be used for further quantitative analysis of ascorbic acid in vitamin C related products.

Keywords: Antioxidant, Ascorbic Acid, HPLC, Isocratic, Method Validation

1. Introduction

People from all walks of life have concern about the significance of vitamins in life which has a lot of benefits starting from its physical absorption to the consequences of vitamins intake in life. Vitamins are organic substances our body needs to grow and function on a daily basis. Vitamins can be categorized into two main groups which are water soluble and oil soluble vitamins. Water soluble vitamins are thiamine (vitamin B1), niacinamide (vitamin B3), panthothenic acid (vitamin B5), pyridoxine (vitamin B6) and ascorbic acid (vitamin C). On the other hand, oil soluble vitamins are riboflavin (vitamin B2) and folic acid (vitamin B9). One of the important antioxidant that our body really need is ascorbic acid (AA) which also known as vitamin C.

Ascorbic acid or vitamin C is an essential nutrient required by the body for the development and maintenance of scar tissue, blood vessels, and cartilage. Vitamin C has various pharmacological and physiological functions. It includes functions in collagen synthesis, acts as an important biological antioxidant and intestinal absorption of iron and drug metabolism (Tai and Gohda, 2007). Vitamin C also known as free radical scavengers which means it works to regenerate each other such as reduced glutathione regenerates ascorbic acid which then regenerates α–tocopherol from its radical forms (Wang et al., 2012). Furthermore, the vitamin C is said to have the power to relief fever to increase the organism's resistance against microorganism that participates in antibody formation (Hatambeygi et al., 2011).

Despite the various benefits that the Vitamin C offers, it cannot be produced or stored in our body and will be excreted from our body though urine and active tubular reabsorption (Lamarche et al., 2011). Thus, it is important for us to eat high Vitamin C food and fruits such as oranges, berries and papayas to ensure that we get the Vitamin C that our body need. However, in the fast moving world where everything must be fast and "instant', it is difficult to get the recommended amount of essential vitamins daily. Sometimes people just don't have enough time to eat all the nutritional food and sometimes the food itself has lost a lot of nutrients after undergoing a lot of processes such as packaging, cooking and improper storage. As the solution to the hectic lifestyle, a lot of companies sell vitamin C related products either in form of tablets or syrups that are easy to be taken and contain high amounts of Vitamin C. In addition, there are also a few types of Vitamin C product called serum and ampoules to maintain resilient and youthful skin. A few years back, the usage of these Vitamin C products are only limited to the dermatologist, aesthetician and at the clinics, but now with high technology, these products have been sold openly and many cosmetics products also have their own Vitamin C serum.

Due to the labile nature of the vitamin C and the abundance of Vitamin C related products in the market, it is important to check the quantity of vitamin C in the products. The RDA value that is recommended is from 60-90 mg per day for men and 75-90 mg per day for women (Canavese et al., 2005). It also said that the recommended dietary allowance and estimated average requirement and of ascorbic acid are 120 and 100 mg per day respectively (Mitić et al., 2011). On 2 November 2012, the National Pharmaceutical Control Bureau stated that all products should comply with the updated upper daily limits for vitamins and minerals. For vitamin C, the upper daily limit is 1000 mg per day. Excess intake of vitamin C can acidify urine which can cause diarrhea and gastrointestinal irritation. It is one of the most dangerous gastrointestinal tract disorders that can lead to death due to dehydration, loss of electrolyte and hypernatremia in human and animals (Ahmed et al., 2012). It also lower the urine output and decreases the blood pressure.

Over the last decades, several methods have been employed for the analysis of vitamin C in food, including electrochemical (Calokerinos and Hadjiioannou, 1983), spectrophotometric (Liu et al., 1982), spectrofluorimetric (Sánchez-Mata et al., 2000) and chromatographic methods. However, high-performance liquid chromatography (HPLC) methods have shown some advantages regarding specificity, sensitivity or easy operation (Gökmen and Acar, 1996). In this study, the instrument that has been used was the HPLC with UV-Visible detector.

This study has been conducted to determine the concentration of vitamin C in vitamin C related products. This is to ensure the products available are up to the recommended standard by the authority. Furthermore, this study compared the percentage of vitamin C with the said amount stated on the labelled sheet. Since the stability of vitamin C is unstable and has high polarity together with matrices complexity (Jin et al., 2012), suitable place with correct temperature and humidity is needed. Thus, this study also covered the effect of different storage places for further recommendation to the consumers. The optimized method was further validated according to The International Conference on the Harmonization of the Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) as the standard guidelines.

2. Materials and Methods

2.1 Reagents and standard solutions

Ascorbic acid and phosphoric acids were respectively purchased from R & M and Merck. All preparation of standards and samples were done using type II distilled water. The standards were prepared in range of 1 ppm to 14 ppm and subsequently linearity of calibration curve was obtained according to Beer's law.

2.2 HPLC system

Waters 1515 Isocratic Pump with Waters 2487 Dual λ absorbance detector was used in this study. As suggested by Ferin et al., (2013) and Jin et al,. (2012), 245nm and 270nm were set for Channel 1 and 2 respectively. The separation used C_{18} column with 0.1% (v/v) phosphoric acid as the mobile phase at a flow rate of 0.5 ml/min. All analysis was done at ambient temperature.

2.3 Sample preparation

Two tablet form samples, P and K were purchased from local pharmacy store in Pahang. The tablets were pressed gently to fine powder. Next, 1.0 g of powdered tablet was dissolved in mobile phase, shaken vigorously for 5 minutes. The solution was filtered prior analysis through 0.22 μm PTFE membrane syringe. For the stability test, samples which were in solution form were kept in four different storage conditions and labeled as S1; in a handbag S2; in a car with temperature of approximately 31°C, S3; in ambient temperature and S4; in a refrigerator with temperature of approximately 14°C. The samples were left for 6 hours. On the other hand, the recovery test was done by adding 200 μL of 2 ppm of standard solution into 200μL of sample while the precision analysis were measured by triplicates injections of 2 ppm and 10 ppm of standard solutions for two consecutive days.

3. Results and discussion

3.1 Correlation coefficient of ascorbic acid

The equation for wavelength 245nm was Y= 182.548X + 222.214 where, Y is the absorbance and X is the retention time with value of 0.990681 as the correlation coefficient (R^2). Else, for wavelength 270 nm, the equation was Y=95.000X + 110.470 with correlation coefficient (R^2) of 0.991075. Wavelength 270 nm showed better correlation coefficient (R^2) than wavelength 245 nm. AA was eluted at the retention time 8.5 minute to 9.5 minute.

3.2 Analysis of ascorbic acid in tablet P

Tablet P was manufactured in Selangor on November 2012 and expired on October 2015. The amount of AA stated at the bottle was 1000 mg per tablet. Upon dilution, the concentration of one tablet of AA was supposed to be 20 000 mg L^{-1}. One tablet of sample was equal to 0.9989 g. Hence, expected values were 19 978 mg L^{-1} or 998.9 mg per tablet. Based on Table 1 below, the amount of AA that resolved from the HPLC were 1021.50 mg and 990.00 mg.

Table 1. Results for amount of AA in original tablet P

Wavelength	Retention time (min)	Amount (mg per tablet)
245 nm	8.914	1021.5
270 nm	8.914	990.0

3.2.1 Stability test for tablet P for wavelengths 245 nm and 270 nm

Based on Figure 1, the AA content of tablet P did not show drastic changes from the amount of control sample. The respective labels for the conditions were stated in the sample preparation. Amount of AA had the lowest value for condition 4 which was in refrigerator with temperature of 14°C. It proved that storage above 4°C could not cause degradation to AA. Studies conducted by Jin et al., (2012) and Tai et al., (2007) also achieved good sample stabilities upon storage at 4°C. Previous study done by Gallarate et al., (1999), said that AA could undergo oxidation process when it was in solution and produced dehydro-L-ascorbic acid (DHAA) and other degradation products. Surprisingly, it did not degrade to DHAA within six hours. It proved that degradation process could not be happen within six hours as being found by Jin et al., (2012). Their study obtained %RSD values less than 2% which showed AA was reproducible. Wavelength 270 nm also gave similar result as wavelength 245 nm that showed non-drastic changes in amount compared to the control sample. The bar chart showed parallel readings to each other. A small difference in amount of AA was observed between both wavelengths. It proved that both wavelengths could be used for AA analysis.

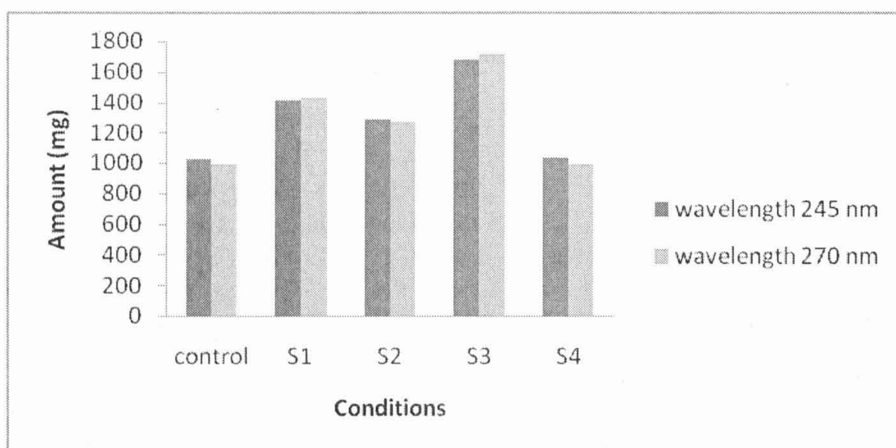

Fig. 1 Amount of AA versus conditions for wavelength 245 nm and 270 nm of tablet P.

3.3 Analysis of ascorbic acid in tablet K

Tablet K was manufactured in Germany on May 2013 and expired on May 2015. It contained vitamin C and zinc and was an effervescent tablet. Effervescent version was already in a form that could be quickly digested upon ingestion. It allowed 100% absorption inside the stomach and easy to swallow. Each bottle contained 10 tablets that weighed approximately 4.000 g per tablet. The label sheet showed that it contained 1000 mg of AA per tablet. The elution of AA could be resolved completely. As for wavelength 270 nm, the concentration of AA also fluctuated just like what happen in wavelength 245 nm.

3.3.1 Correlation coefficient of standard series of Ascorbic Acid

The equation for wavelength 245 nm was Y= 178.096X – 213.479 with value of 0.992645 as the correlation coefficient (R^2). Else, for wavelength 270 nm, the equation was Y=95.001X – 98.218 with correlation coefficient (R^2) of 0.991996. Wavelength 245 nm showed better correlation coefficient (R^2) than wavelength 270 nm. Retention time for AA was in range of 9.8 minute until 10.0 minute for both wavelengths.

3.3.2 Analysis of tablet K

Before conducting the stability test, the concentration of control sample was first analyzed. Each tablet stated containing 1000 mg of AA. Upon dilution, the expected amount of AA in one tablet weighed 3.9444 g was 20 000 mg L^{-1}. Since the amount of sample being analyzed was 1.0008 gram, hence, there was approximately 5075 mg L^{-1} or 253.75 mg of AA per gram of sample.

Table 2. Results for amount of AA in original tablet K

Wavelength	Retention time (min)	Amount (mg per tablet)
245 nm	10	1125.82
270 nm	10	1113.6

The Table 2 shows the concentration of AA for wavelength 245 nm and wavelength 270 nm. After calculation, the amounts resolved from the chromatogram were 1125.82 mg and 1113.60 mg per tablet, respectively. It was slightly higher than the expected amount and showing same pattern of tablet P result previously.

3.3.3 Stability test for tablet K for wavelengths 245 nm and 270 nm

The same procedures and conditions were applied to test the stability of AA in tablet K. Figure 2 shows the trace fluctuation of data by comparing the concentration of AA in different storage places with the control sample. The values of amount of AA were slightly fluctuated. The estimated values should be less than 1000.09 mg. This phenomenon happened might be because of there was AA in the column that not fully flushed out from the previous injection. For HPLC system, this could be considered as one of the limitation for a sensitive instrument. Roughly, we could say that the stability of AA still in stable mode after being exposed to certain conditions that had tendency to make AA degrade to DHAA. The amounts did not decrease drastically proven that it was stable although being exposed for six hours. The value obtained at condition 4 was the lowest compared to the other three conditions. It was probably due to exposure of low temperature in the refrigerator.

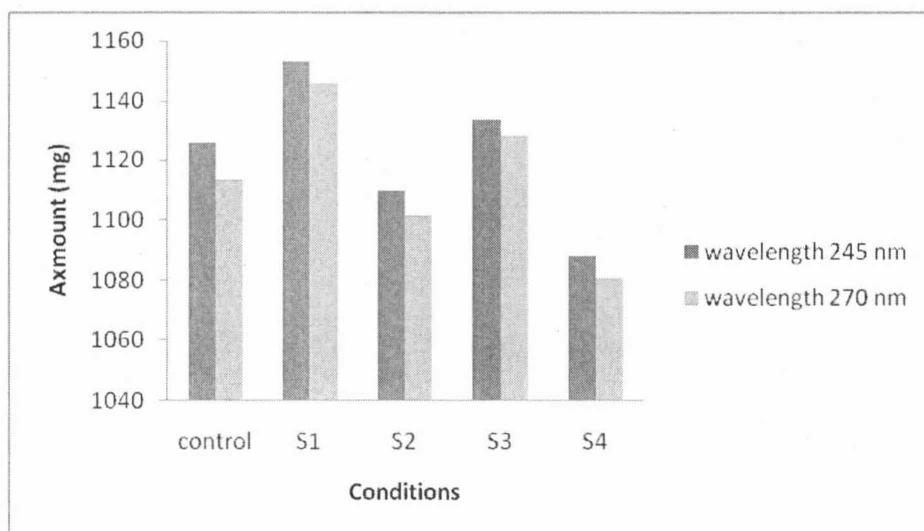

Fig. 2 Amount of AA versus conditions for wavelength 245 and 270 for tablet K.

3.4 Stability of ascorbic acid for both tablets

The results of the present study showed that amount of AA were stable in four different storage conditions hence underlined the possibility to increase the stability of AA in the respective samples. No degradation of AA was observed throughout the analysis after storing them at temperature more than 30 ± 0.1°C. It contrasted with previous study done by Ozkan et al., (2004) reported that degradation of AA into its byproducts happened at temperature of 20, 30 and 40°C. Other than that, by looking at the HPLC chromatograms for stability test, the AA peak was well resolved without any peaks of the byproducts. The trends of values are also higher than the estimated value due to limitation of the analysis. The increased amount was presumed to be due to leftover of AA in column or syringe from the previous injection. Among the limitations was the lacking of guard column and degasser unit. Guard column is important as it increase the life time of analytical column. It positioned ahead of analytical column to remove particulate matter and contaminants from the solvents. The degasser unit functions to sweep out solution by fine bubbles of an inert gas that was insoluble in mobile phase using vacuum pumping system. Due to the limitations, the column was purged with 70% v/v methanol to eliminate contaminants.

3.5 Method validation

In this analysis, the validation of analytical methodology consist of LOD and LOQ, precision and recovery data.

3.5.1 Limit of Detection and Limit of Quantification

LOD and LOQ were obtained from the calculation of the triplicates injection of 2 ppm AA. LOQ were also can be obtained with adequate precision and accuracy from the method setup (Mitic, 2011). The results of triplicate injections of 2 ppm standard solution to calculate the value of LOD and LOQ were tabulated in Table 3.

Table 3. Results for injections of 2 ppm standard solution for wavelength 245 nm and 270 nm.

Wavelength	Injection	Retention time (min)	Concentration (ppm)
245 nm	1	9.996	2.567
	2	10.003	2.465
	3	10.007	2.441
270 nm	1	9.996	2.528
	2	10.003	2.414
	3	10.007	2.402

The average amount of standard solution of 2 ppm quantified was 2.491 ppm with SD of 0.1159 calculated from Equation (1). The calculated values of LOD and LOQ from Equation (2) and (3) were 0.0021 ppm and 0.0065 ppm respectively.

$$SD = \sqrt{\frac{n(\sum x^2) - ((\sum x)^2)}{n-1}} \quad (1)$$

LOD = 3.3 (SD/S) (2)

LOQ = 10 (SD/S) (3)

The values of LOD and LOQ were lower than the average quantified amount that showed high sensitivity of injections done. The average amount of standard solution of 2 ppm quantitated was 2.4480 ppm with SD of 0.1205 calculated from Equation (1). The calculated values of LOD and LOQ from Equation (2) and (3) were 0.0022 ppm and 0.0068 ppm respectively. Wavelength at 270 nm showed higher values of LOD and LOD compared to wavelength 245 nm. Table 4 showed the LOD and LOQ obtained from the previous studies. It showed different values because the dependency and capability of the HPLC system were used in the respective study.

Table 4. Results for LOD and LOQ from previous studies

Previous study	LOD	LOQ
Refin et al.,(2013)	550.8 mg/L	183.6 mg/L
Jin et al.,(2012)	0.137 mg/L	0.410 mg/L
Hatambeygi et al.,(2011)	-	1.50 mg/L
Khan and Iqbal (2011)	$6.0x10^{-5}$ mg/L	$2.0x10^{-4}$ mg/L
Mitic et al.,(2011)	1.95 mg/L	6.5 mg/L
Tai and Gohda (2007)	0.3 mg/L	-
This study (2014)	0.0022 mg/L	0.0068 mg/L

3.5.2 Recovery

Recovery analysis was done to determine the accuracy of analytical method by applying the standard addition method. Table 5 and Table 6 show the results for recovery percentage for wavelength 245 nm and 270 nm, respectively. Since the concentration of samples being altered by addition of standard solution of 2 ppm, the new concentrations of sample S1, S2, S3 and S4 were calculated as 3.926 ppm, 3.816 ppm, 3.876 ppm and 3.761 ppm respectively. Table 3.5 and 3.6 show the percentage of recovery for AA where in range from 43% to 48%.

Table 5. Results for percentage recovery for wavelength 245 nm

Sample	Amount of Non Spiked-sample (ppm)	Amount of Spiked sample (ppm)	Percent recovery (%)
S1	5.851	3.332	43.10%
S2	5.631	3.004	46.70%
S3	5.752	3.087	46.30%
S4	5.521	2.976	46.10%

Table 6. Results for percentage recovery for wavelength 270 nm

Sample	**Amount of Non Spiked-sample (ppm)**	**Amount of Spiked sample (ppm)**	**Percent recovery (%)**
S1	5.851	3.274	44.00%
S2	5.631	2.959	47.50%
S3	5.752	3.053	46.90%
S4	5.521	2.925	47.00%

The recovery of both wavelengths did not achieve 95% to 100% accuracy as compared to other previous studies in Table 7. However, the current method still could be accepted as according to more than 20% as stated in manual for validation of analytical methods and verification of laboratory equipment done by United Nation Office on Drugs and Crime (UNODC). Table 7 shows that higher percentage of recovery was achieved and relative standard deviation (RSD) were less than 2%, indicating high accuracy of the method. Again, the limitation of the HPLC system could give interference to the accuracy of readings.

Table 7. Results for percentage recovery from previous studies

References	**%recovery**	**%RSD**	**CV%**
Ferin et al.,(2013)	94.00	1.40	1.48
Jin et al., (2012)	100.40	0.70	-
Hatambeygi et al.,(2011)	96.10	1.40	-
Khan and Iqbal (2011)	97.61	1.28	-
Mitic et al (2011)	101.39	0.17	-
Tai and Gohda (2007)	92.00	0.30	-
This study (2014)	43-48	0.1205	3.79-8.13

3.5.3 Precision

It consists of two conditions which are intra-assay precision that were done by intra-day multiple injections and inter-assay precision for inter-day multiple injections. The level of repeatability was compared to the value of coefficient of variation (CV). The acceptable value was 20%. The Equation (4) showed the calculation to determine the value of CV.

$$CV = (SD / x) \times 100\% \quad (4)$$

Figure 3 shows simplification of precision data for wavelength 245 and 270 nm for day 1 and day 2. For wavelength 245 nm, average amount of multiple injections of 2 ppm standard solution was 2.214 ppm with SD of 0.18. The value for CV was 8.13 %. Average amount of

multiple injections of 10 ppm standard solution was 9.504 ppm with CV of 3.79%. Both readings showed that it also was precise because it did not exceed the maximum value of 20%. Both of the concentrations showed good repeatability and precise reading for intra-assay precision as it not influenced by the stability of AA that was easily degraded when exposed to sunlight and unsuitable temperature.

After completing the intra-day analysis, inter-day analysis was conducted. Since AA was instable within 12 hours, the inter-day analysis could not be taken into consideration to validate the inter-assay precision. The average amount of AA detected was 0.325 ppm for both concentrations. It was completely showed that AA was degraded into its byproducts where only small amount of AA was detected.

For wavelength 270 nm, average amount of multiple injections of 2 ppm standard solution was 2.169 ppm with SD of 0.18 and CV of 8.12 %. The readings showed that it also was precise similar to like what happened in analysis for wavelength 245 nm, as it did not exceed the maximum value of 20 %. Average amount of multiple injections of 10 ppm standard solution was 9.468 ppm with SD of 0.39 and CV of 4.09%. The readings showed that it also was precise as it did not exceed the maximum value of 20%. The average amount of AA detected was 0.283 ppm for both concentrations. It showed that AA was degraded into its byproducts where only small amount of AA was detected. Table 8 show values of precision that had been obtained from the previous studies compared to this study. All of them showed good precision for both intra-day and inter-day precision.

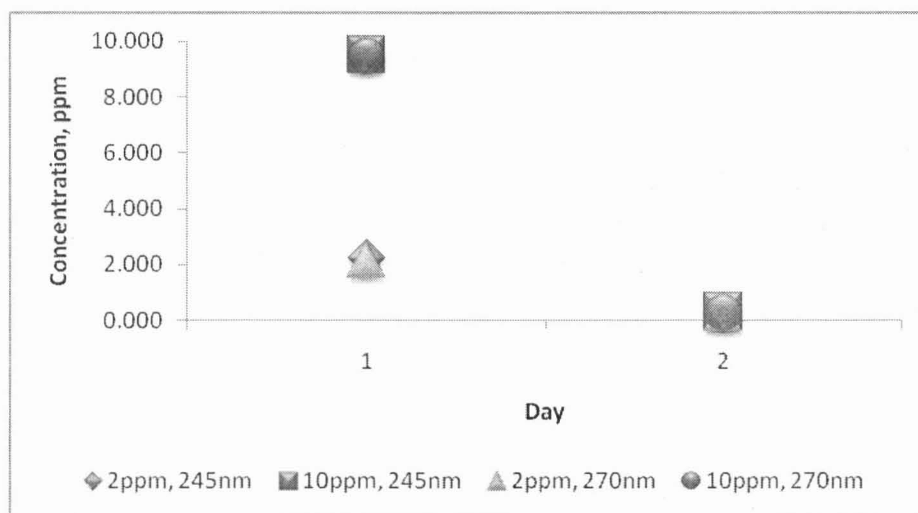

Fig. 3 Concentration of AA versus day for wavelength 245 nm and 270 nm

Table 8. Results of precision from previous studies

References	%RSD (intra-day)	CV%	%RSD (inter-day)	CV%
Ferin et al.,(2013)	1.67	0.16	18.9	10.97
Jin et al.,(2012)	1.75	-	1.92	-
Hatambeygi et al.,(2011)	1.26	-	1.70	-
Khan (2011)	1.7	-	1.83	-
Tai (2007)	1.73	-	1.70	-
This study (2014)	0.18	8.13 (245 nm) 8.12 (270 nm)	-	-

4. Conclusion

In conclusion, the quantitative analysis of ascorbic acid in vitamin C related products was challenging due to their extremely unstable characteristic. In this analysis, the development of simple method with isocratic HPLC system was successful with good sensitivity, linearity, precision and accuracy by analyzing the method validation methods. All samples were well separated from other compounds and degradation products showed that the usage of 0.1% (v/v) phosphoric acid as the diluting solution and mobile phase suitable for the extraction and separation process of ascorbic acid. The concentrations of ascorbic acid present in selected vitamin C related products measured at wavelength 245 nm were to be similar to the amount stated at the label for sample P and sample K which were 1021.50 mg and 1125.82 mg respectively. As for wavelength 270 nm, the amounts were 990.00 mg for sample P and 1113.60 mg for sample K. Both wavelengths of 245 nm and 270 nm were accepted to be used as the wavelength of ascorbic acid analysis. Stability test also showed that the four conditions that consumers used to keep their vitamin C do not affect the quality of AA content. In this study, storage in ambient temperature was recommended for consumer as it away from direct sunlight and extreme temperature as in car or refrigerator. Nevertheless, storage over a long period could degrade the ascorbic acid content in the products.

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