# DEVELOPMENT AND VALIDATION OF SPECTROFLUOROMETRIC METHOD FOR THE DETERMINATION OF ASCORBIC ACID IN SEVERAL DOSAGE FORMS BY USING METHYLENE BLUE

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## ABSTRACT

Ascorbic acid or vitamin C is one of the most widely consumed supplement and available at the market in several preparations such as tablet, sweetlet, effervescent tablet, and ready to drink product. Spectrofluorometric method has been developed in this study for quantitative determination of ascorbic acid in various forms of products based on the reaction between ascorbic acid and methylene blue. Methylene blue concentration decreased along with the addition of ascorbic acid due to a redox reaction which caused the formation of colorless leuco-methylene blue and dehydroascorbic acid. The fluorescence intensity of methylene blue was measured at excitation and emission wavelengths of 664 nm and 686 nm respectively. The proposed method was found to have a good selectivity, linearity, accuracy, and precision. Validation studies demonstrated a good linearity for the method with a correlation coefficient > 0.999 and Vx0 < 2%. The accuracy test also met the requirements with the recoveries were not less than 90.0% and not more than 110.0%. Precision test gave a relative standard deviation (% RSD) not more than 2%. The ascorbic acid content in several marketed products was evaluated by this new spectrofluorometric method and the result was in good agreement for effervescent tablet, isotonic drink and lozenges with the label claim and specification. Due to its simplicity, the method can be used to analyze vitamin C in different product forms.

**Keywords:** ascorbic acid, methylene blue, spectrofluorometric, redox

## PENGEMBANGAN DAN VALIDASI METODE SPEKTROFLUOROMETRI UNTUK PENENTUAN ASAM ASKORBAT DALAM BEBERAPA BENTUK SEDIAAN DENGAN MENGGUNAKAN METILEN BIRU

## ABSTRAK

Asam askorbat atau vitamin C merupakan salah satu suplemen yang paling banyak dikonsumsi dan tersedia di pasaran dalam berbagai bentuk sediaan seperti tablet, *sweetlet*, tablet *effervescent*, dan produk siap minum. Metode spektrofluorometri telah dikembangkan dalam penelitian ini untuk penentuan kuantitatif asam askorbat dalam berbagai bentuk produk berdasarkan reaksi antara asam askorbat dan metilen biru. Konsentrasi metilen biru menurun seiring dengan penambahan asam askorbat akibat reaksi redoks yang menyebabkan terbentuknya leuco-metilen biru dan asam dehidroaskorbat yang tidak berwarna. Intensitas fluoresensi metilen biru diukur pada panjang gelombang eksitasi dan emisi masing-masing 664 nm dan 686 nm. Metode yang dikembangkan memiliki selektivitas, linieritas, akurasi, dan presisi yang baik. Studi validasi menunjukkan linearitas yang baik untuk metode ini dengan koefisien korelasi > 0,999 dan Vx0 < 2%. Uji akurasi juga memenuhi persyaratan dengan perolehan tidak kurang dari 80,0% dan tidak lebih dari 110,0%. Uji presisi memberikan standar deviasi relatif (% RSD) tidak lebih dari 2%. Kadar asam askorbat pada beberapa produk komersial dievaluasi dengan metode spektrofluorometri ini dan hasilnya menunjukkan kesesuaian kandungan untuk tablet *effervescent*, minuman isotonik dan tablet hisap dengan label klaim dan spesifikasi. Karena kesederhanaannya, metode ini dapat digunakan untuk menganalisis vitamin C pada berbagai bentuk produk yang berbeda.

**Kata kunci**: asam askorbat; biru metilen; spektrofluorometri; redoks

## INTRODUCTION

Ascorbic acid has a molecular formula of $C_6H_8O_6$ and a molecular weight of 176.13 g/mol, official in pharmacopoeia (Ditjen POM Kemenkes RI 2014). It is an antioxidant that involve in many body functions such as formation of collagen, fight bacterial infections, wound healing, maintenance of teeth, bones, skin, capillaries and also play an important role in a number of metabolic functions including the activation of the B vitamin, folic acid, the conversion of cholesterol to bile acids and the conversion of the amino acid, tryptophan, to the neurotransmitter, serotonin (Dave and Patil 2017). Ascorbic acid can be found in fruits and vegetables. This substance cannot be produced or stored by humans and must therefore be obtained from food (Martí *et al*. 2009). Ascorbic acid, which is also famous for its trade name vitamin C, is one of the most popular supplements and is consumed in various forms of products, from pharmaceutical preparations such as tablets, lozenges, effervescent tablets, injections, to beverage products.

Methylene blue is a synthetic substance and occurs in the form of several different hydrates, but not as trihydrate (Rager *et al*. 2012). It has a molecular formula of $C_{16}H_{18}ClN_3S$ and molecular weight (anhydrous form) of 319.85 g/mol. Methylene blue is an oxidation-reduction agent and is widely used as a redox indicator in analytical chemistry. Methylene blue is also used in human and veterinary medicine for several therapeutic and diagnostic procedures, including as a stain in bacteriology, as a redox colorimetric agent, as a targeting agent for melanoma, as an antihaemoglobinaemic, as an antidote, and as an antiseptic and disinfectant (National Toxicology Program 2008, O'Neil *et al*. 2006)

Several analytical methods, including iodimetry titration, spectrophotometric (Gómez Ruiz *et al*. 2016), electrochemically methods such as amperometry (Scremin *et al*. 2018), voltammetry (Phong *et al*. 2018), potentiometry (Elbehery *et al*. 2019), and chromatography such as high performance liquid chromatography (Cefali *et al*. 2018), gas chromatography (Silva 2005), and ultra-performance liquid chromatography (Cotruț and Bădulescu 2016, Turak *et al*. 2017) have already been reported for ascorbic acid analysis. One of methods that still be able to be improved for the analysis of ascorbic acid is the fluorimetry method by using a spectrofluorometer. Fluorometry analysis is well known for its sensitivity, accuracy, and speed of the measurement (Bose *et al*. 2018, Nahata 2011). However ascorbic acid cannot be determined directly by this method due to the structure of ascorbic acid does not have a fluorophore. To overcome this limitation several methods are applied. Suitable chromophore or fluorophore reagents are used in the derivatization process to enhance the sensitivity and selectivity of detection for ascorbic acid by spectrofluorometric method. Gradual decrease in fluorescence intensity of some compounds due to the presence of ascorbic acid also be used for indirectly assay of ascorbic acid in the sample (Abd Ali *et al*. 2019, Duan *et al*. 2017, Haskovic *et al*. 2011, Klepo *et al*. 2016)

The purpose of the present work was to develop a spectrofluorimetric assay for ascorbic acid indirectly that can be applied for several preparations utilizing a decrease in the intensity of fluorescence from methylene blue which is reduced due to the redox reaction with ascorbic acid. Different samples such as lozenges, effervescent tablet, and ready to drink product were used for the validation of the method to see the reliability of this developed method on different sample matrices.

## MATERIALS AND METHODS

### Apparatus

Fluorescence measurements were carried out on a FP-6300 spectro-fluorimeter (Jasco, Japan) equipped with a 150 W xenon lamp and 1 cm quartz cells. Consort P400® digital pH-meter was used for pH adjustment.

### Materials and reagents

All solvents and reagents used throughout the work were of analytical grade and double distilled water was used.

### General procedures

### Preparation of stock standard solutions

A stock standard solution of ascorbic acid 500 ppm was prepared by weighing accurately 50 mg of standard ascorbic acid and dissolving in 100 ml distilled water. This stock standard solution of ascorbic acid shown to be stable within 3 hours. A stock standard solution of methylene blue in distilled water was prepared with concentration of 25 ppm.

### Fluorescence stability study of methylene blue

Fluorescence intensity of methylene blue 1 ppm was carried out at the maximum excitation wavelength for 20 minutes.

### Specificity

The specificity was determined by comparing the emission spectra of three samples (lozenge, effervescent tablet, and isotonic drink) were checked and compared to the emission spectra of methylene blue to ensure accuracy and specificity

of the analyte of interest in the presence of other components in the matrix.

**Linearity and range**

Solutions of ascorbic acid standard were prepared with different concentrations in the range between 1 to 8 ppm. To each standard solution 1 ppm of methylene blue solution was added. The fluorescence intensity at the optimum condition against the concentration of ascorbic acid then was plotted to obtain the linear range of ascorbic acid. A correlation coefficient (r) and coefficient of variation (Vx0) from the linear regression were determined to check the linearity.

**Limits of detection and quantification**

Limit of detection (LOD) and limit of quantification (LOQ) of the method were determined from the linear regression curve obtained from the linearity of ascorbic acid. The slope and standard deviation (Sy/x) of the responses from the linear regression curve were used. The LOD was calculated using the formula 3.3* Sy/x/slope, and the LOQ was calculated using the formula 10* Sy/x/slope.

**Precision and accuracy**

Precision and accuracy of the developed method were estimated by standard addition method whereby the ascorbic acid was spiked directly to the analyzed samples with three different spiked level, 80%, 100%, and 120% of the actual amount of ascorbic acid in the sample. The precision of the assay was estimated by calculating the relative standard deviation (RSD) for the analysis of each spiked sample in three replicates. The accuracy was calculated based on the given formula; (mean concentration found/ concentration taken) × 100%.

## RESULTS AND DISCUSSION

The reaction that occurs between methylene blue and ascorbic acid is a redox reaction. Methylene blue reacts with ascorbic acid to produce a reduced form of methylene blue, namely leuco-methylene blue and an oxidized form of ascorbic acid, dehydroascorbic acid. Based on this reaction, ascorbic acid which has no fluorophore can be determined with spectrofluorometric indirectly by measuring the decrease of fluorescence intensity of fluorescent methylene blue. The reaction between ascorbic acid and methylene blue occurs in a span of a few seconds to several minutes. Reaction rate is influenced by ascorbic acid concentration while the light has no observable effect. The study began with determining the maximum excitation wavelength of methylene blue. From this experiment it was found that the maximum excitation wavelength of methylene blue was at a wavelength of 664 nm. After the maximum excitation wavelength of methylene blue had been obtained, the emission spectrum of methylene blue at a wavelength of 600-800 nm was measured. From this measurement the maximum intensity was observed at a wavelength of 686 nm. The spectrum of excitation and emission from methylene blue are shown in figure 1.

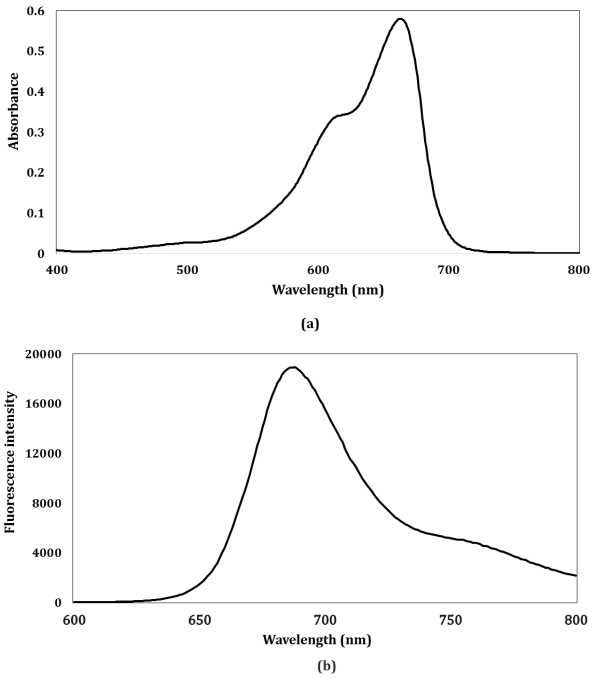


**Figure 1**. Excitation spectrum (**a**) and emission spectrum of methylene blue (**b**).

Stability of the fluorescence intensity of methylene blue after reacted with ascorbic acid under the optimum reagent concentrations then was tested against time. Fluorescence intensity measurements of methylene blue solution with and without ascorbic acid were performed every minute for approximately 20 minutes. The results showed stability was achieved on minutes 15 to 20 as can be seen in figure 2. The stability of fluorescence intensity of methylene blue after ascorbic acid addition was obtained after a while. This was due to the reaction between ascorbic acid and methylene blue which had not been complete. The reaction between ascorbic acid and methylene blue usually occurs in a span of a few seconds to several minutes.

Method validation in this study was performed by examining numerous assessments designed to verify that the spectrofluorometric method is capable to provide beneficial and legitimate analytical data in compliance with International Conference on Harmonization (ICH) guidelines and criteria (ICH 2005). A validation examine includes testing several validation parameters like

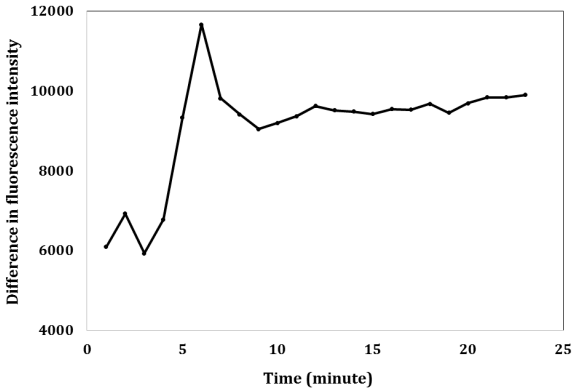


**Figure 2.** The stability of fluorescence intensity of methylene blue after ascorbic acid was added over time.

specificity, precision, accuracy, linearity, limit of detection (LOD) and limit of quantitation (LOQ) (Harmita 2004). Specificity of an analytical method was conducted to test its ability to measure accurately methylene blue in the presence of interferences that may be expected to be present in the sample. At this stage, the emission spectra of three samples (lozenge, effervescent tablet, and isotonic drink) were checked and compared to the emission spectra of methylene blue. Three emission spectra of the samples were superimposed with the methylene blue spectra. From overlap of the spectra, peak of methylene blue was not interfered by the peaks of samples at its maximum emission wavelength that indicate the spectrofluorometric method has good specificity as shown in figure 3.

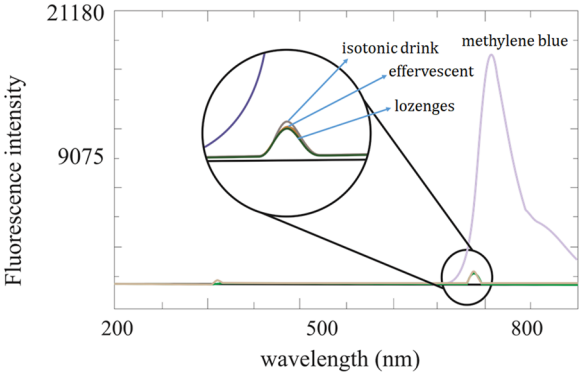


**Figure 3.** Emission spectrum from methylene blue, lozenges, effervescent tablet, and isotonic drink.

The study was continued by testing the linearity of the method. For this purpose, a series of solutions was made consisting of nine concentration of ascorbic acid solutions; 0, 1, 2, 3, 4, 5, 6, 7, and 8 ppm to each of which was added 1 ppm of methylene blue. The emission spectrum of each concentration was then measured at a wavelength of 686 nm. A decrease in fluorescence intensity of methylene blue occurs with increasing concentration of ascorbic acid in solution wherein the relationship was proportional. A calibration curve was made by calculating the difference in fluorescence intensity of methylene blue before and after reacted with ascorbic acids which was plotted on the y-axis, while ascorbic acid concentrations on the x-axis. The method was specific and showed good linearity (r = 0.9996, Vx0 = 1.33%) at concentration between 1 and 8 ppm of ascorbic acid (**Figure 4**).

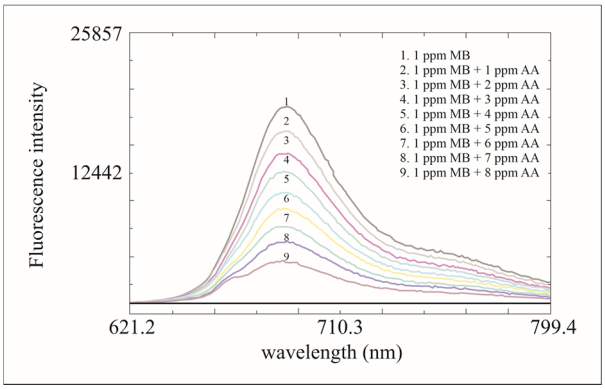


**Figure 4**. Emission spectrum of a series of solutions for calibration curve.

Limit of detection (LOD) and limit of quantitation (LOQ) of this method were obtained from the statistical calculation of the calibration curve that had been made. From the calculation, LOD and LOQ were 0.198 ppm and 0.601 ppm, respectively. Accuracy was measured by spiking the sample of interest (lozenge, effervescent tablet, and isotonic drink) with a known amount of ascorbic acid standard and analyzing the sample using the spectrofluorometric method after it was reacted

**Table 1.** Results of Accuracy and Precision Test (n=3)

| Sample | Spiked level (%) | Average of recovery (%) | RSD (%) | Pooled RSD (%) |
|---|---|---|---|---|
| Lozenges | 80 | 99.1 | 0.9 | 0.9 |
| | 100 | 99.0 | 0.9 | |
| | 120 | 97.6 | 0.8 | |
| Effervescent tablet | 80 | 99.8 | 1.2 | 0.8 |
| | 100 | 98.8 | 0.8 | |
| | 120 | 100.3 | 1.0 | |
| Isotonic drink | 80 | 90.0 | 1.3 | 0.7 |
| | 100 | 89.6 | 1.3 | |
| | 120 | 90.8 | 0.9 | |

**Table 2.** Content of Ascorbic Acid in Three Marketed Products Determined by the Proposed Method (n=3)

| Sample | Calculated ascorbic acid content as percentage of label claim | RSD (%) |
|---|---|---|
| Lozenges | 98.6 | 1.7 |
| Effervescent tablet | 102.5 | 1.8 |
| Isotonic drink | 107.1 | 4.9 |

with methylene blue. From the accuracy data obtained, it was concluded that the method met the criteria. The percentage of recovery that is allowed for solutions with a concentration range of 1-10 ppm are not less than 80.0% and not more than 110.0% of the proper concentration.

To measure degree of repeatability among individual test results when the method is applied repeatedly to multiple samplings, a precision test was carried out. Precision is usually expressed as a relative standard deviation (%RSD) of statistically significant sample measurements. Measurements were made three times for each spiked sample on the same day to determine precision. RSD values of precision were less than 2% which proved the suggested spectrofluorometric method was precise for estimation of ascorbic acid (**Table 1**).

After the spectrofluorometric was validated, it was applied to the commercial samples. The results of determining ascorbic acid levels in three samples are shown in table 2. The ascorbic acid content in several marketed products was evaluated by this new spectrofluorometric method. The result was in good agreement for effervescent tablet with the label claim and the Pharmacopeia's specifications. The result for isotonic drink and lozenges also met the label claim and specification for ascorbic acid content in isotonic drink products.

## CONCLUSIONS

The developed spectrofluorimetric method is simple, accurate, precise, and selective for the analysis of ascorbic acid in lozenges, isotonic drink, and effervescent tablet. The high percentage recoveries obtained for various amounts of ascorbic acid in formulated sample suggested that there is no interference to the analysis. The proposed method is simple and precise and do not suffer from any interference coming from the excipients.

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