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Research Article

Optimization and Validation of Visible-Spectrophotometry Method For Determination Ascorbic Acid in Jeruk Bali *(Citrus maxima)* Fruit From Indonesia

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ABSTRACT

The method validation is prelimnary step in a research when researcher has not familier yet with the instruments, procedures and different sample matrices. The aim is obtaining suitable analytical method. Spectrophotometry is an instrumental method which is simpler, faster and cheaper than other instrumental methods. This study optimized and validated Spectrophotometry method for the determination of ascorbic acid in Jeruk Bali Fruit *(Citrus maxima)*. Ascorbic acid in acidic condition gives blue color when was reacted with ammonium molybdate, then the absorbance was observed in visible wavelength area. Optimization to determine ascorbic acid (250.0 µg) required the addition of 4.0 ml of 5% sulphuric acid, 1.5 ml of 3% metaphosphate acid, 1.5 ml of 5% ammonium molybdate and the resulting color was stabil within 20-30 minutes. Furthermore, optimized condition was applied to validate Spectrophotometry method for the determination of ascorbic acid in Jeruk Bali Fruit. Selectivity test obtained 701 nm as wavelength of choice, linierity test resulted Y=0.0019x+0.071 and r=0.9998 (p=0.000; p<0.01), Coeffisien of Variation (C.V)=0.3512%, recovery was (%) = 100.2128±1.7039. Using optimized and validated condition, the determination of ascorbic acid in Jeruk Bali Fruit from Indonesia =0.0208±0.0129 (%; w/w).

Keywords: Optimization, Validation method, Ascorbic acid, Visible-Spectrophotometry method, Jeruk Bali *(Citrus maxima)* fruit from Indonesia.

INTRODUCTION

Validation of analytical methods depending on the purpose of the methods to be used. Validation activities should be organized so that the experiments are planned to complement their preferred method. The purpose is to ensure the method validation and to confirm that they are appropriate analysis method. Validation of methods is usually to newly created analytical methods and development of methods. Validation of methods is an important step to determine the reliability and reproducibility of the method. Data reliability depends on the reliability analysis tools, methods are valid and well-trained analyst. Methods that require the validation phase is non-standard methods, development of methods, modification methods, application methods at other laboratories and comparison with the standard method[1].

According to USP XXXI (2008)[2], the characteristics of the analysis method is expressed as a parameter analysis, namely selectivity/specificity, linearity, limit of detection, limit of quantitation, precision, accuracy, ruggedness and robustness. Validation methods performed to ensure the reproducibility of the results. Spectrophotometry method is often done because it has several advantages, which have a high sensitivity, the way it is simple, fast and relatively low cost[3].

Ascorbic acid is a vitamin group that also included a group of water-soluble antioxidant[3]. Ascorbic acid is contained in various kinds of fruits, among others in the flesh grapefruit (*Citrus maxima*). Spectrophotometry is a quick and simple method for the determination of ascorbic acid ascorbic acid levels in various sample matrices, but can not be used on a sample matrix is complex has an the absorbance in the ultra violet. To eliminate the influence of the sample matrix, then created derivatives by adding acid molybdate reagent into a colored compound that can be observed no visible area. Ascorbic acid in acidic conditions would provide a blue color when reacted with ammonium molybdate. Blue compound formed, will provide maximum absorption in the visible region. Ascorbic acid with methylene blue (MB), color change can be observed at a wavelength of 665 nm[4].

Determination of ascorbic acid can be determined by a variety of methods such as: HPLC[5-8], Iodometri[9-12], Titimetri[13-16], UV-Spectrophotometry[17-21] and Visible-Spetrophotometry[10,22-25].

This research is the development of methods Visible-Spectrophotometry in the determination of ascorbic acid is applied to the flesh grapefruit which implies complex, so that the necessary implementation phase of optimization and validation of the method of

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determination of ascorbic acid levels in the flesh of grapefruit beforehand.

MATERIALS AND METHODS

Instrument

UV-Vis Spectrophotometer Lambda EZ-201 (Perkim Elmer), Analytical Balance (O-Haus Adventurer), a tool glasses commonly used in pharmaceutical analysis laboratory.

Chemicals

Ascorbic acid (p.a; Sigma), metaphosphate acid (p.a; Merck), ammonium molybdate (p.a; Merck) and sulfuric acid (p.a;Merck), grapefruit *(Citrus maxima)*, distilled water.

Optimization

The addition of metaphosphate acid, ammonium molybdate, , sulfuric acid and color stability of the ascorbic acid 250.0 µg.

Validation Methods

Selectivity

Conducted on a solution of ascorbic acid, grapefruit juice and orange juice were added ascorbic acid. The 3% metaphosphate acid solution is then added, 5% sulfuric acid and 5% ammonium molybdate selected. Observed spectra at a wavelength of 400-800 nm

Linearity

Ascorbic acid with various concentrations increase, added a solution of 5% sulfuric acid, 3% acid ammonium metaphosphate and 5% ammonium molybdat selected, then the observed of the absorbance after the color formed stable.

Precision

A solution of ascorbic acid at a concentration of 25.5 µg/ml was added 5% sulfuric acid, 3% acid metaphosphate and 5% ammonium molybdate selected. Furthermore, the solution was observed of the absorbance after the color is stable as much as ten times.

Accuracy

The sample matrix grapefruit artificial meat that does not contain ascorbic acid can not be made, then the accuracy of the technique is done with the addition. Grapefruit juice made with carefully weighing approximately 50.0 g flesh grapefruit, then transferred to the flask 50.0ml and add distilled water until the limit mark. Ascorbic acid solution with a certain concentration, added numerous volumes of grapefruit juice. Add 5% sulfuric acid, 3% metaphosphate acid and 5% ammonium molybdate with the selected volume. Observed of the absorbance at the wavelength and color stability when elected.

Determination of ascorbic acid levels in the flesh of grapefruit, weighed carefully flesh grapefruit approximately 100.0 g were then taken the juice and insert it into the flask 50.0ml. Add distilled water to the extent of 50.0 ml mark. Grapefruit juice solution of 0.5 ml volume are added various concentrations of ascorbic acid solution given, then added 5% sulfuric acid, 3% acid metaphosphate and 5% ammonium molybdate with the selected volume. Observed of the absorbance at the wavelength and color stability when elected.

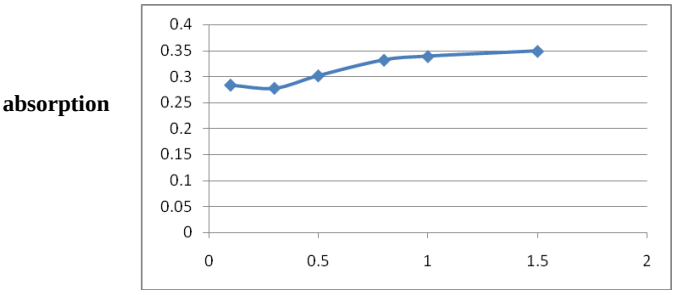

Figure 1: Optimization of the addition 3% metaphosphate acid at 250.0 µg ascorbic acid.

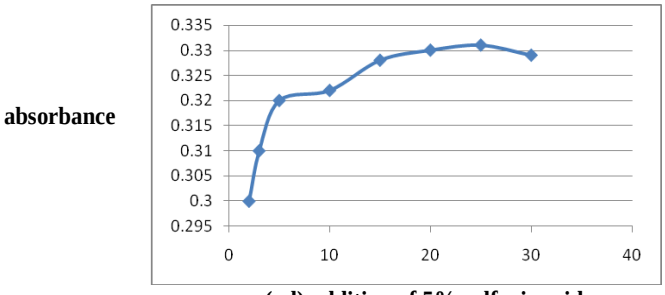

Figure 2: Optimization of the addition of 5% sulfuric acid.

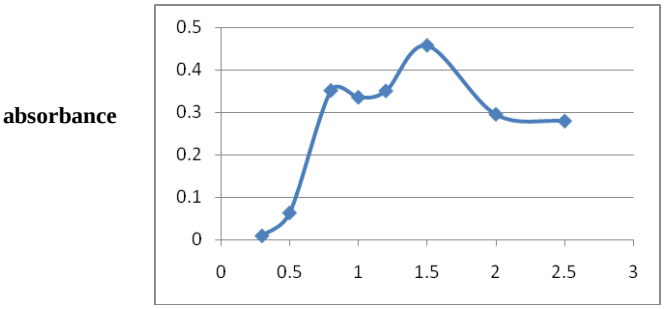

Figure 3: Optimization of the addition of 5% ammonium molybdate at 250.0 µg ascorbic acid.

RESULTS AND DISCUSSION

Optimization

Ascorbic acid in the solution is unstable easily oxidized to form dehydroascorbic acid[26]. So, we need a shift in wavelength of the ascorbic acid from the ultra-violet to visible areas by reacting with ammonium molybdate in addition to the necessary addition of sulfuric acid and a reducing acid metaphosphate[11].

The addition of 3% metaphosphate acid

From the results of the optimization 3% metaphosphate acid addition at 250.0 µg ascorbic acid to obtain optimal results in 1.5 ml (Fig. 1). So for the next determination been the addition 1.5 ml of 3% acid metaphosphate.

On the other investigators[5], addition of metaphosphate acid in the form of 0.5 ml metaphosphate acid-acetic acid and not described ratio metaphosphate acid or a percentage, whereas in this study 3% metaphosphate acid was prepared in glacial acetic acid. The addition of metaphosphate acid required to dissolve ascorbic acid is

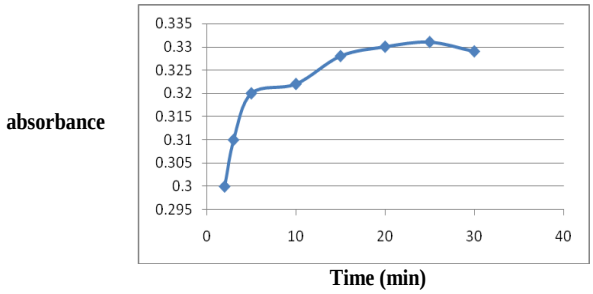

Figure 4: Optimization of color stability

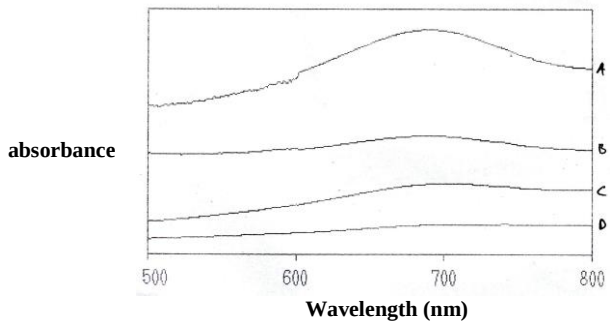

Figure 5: Spectra: A. grapefruit juice + 50.0 µg ascorbic acid. B. grapefruit juice. C. 50µg Ascorbic acid and D. 25.0 µg Ascorbic acid

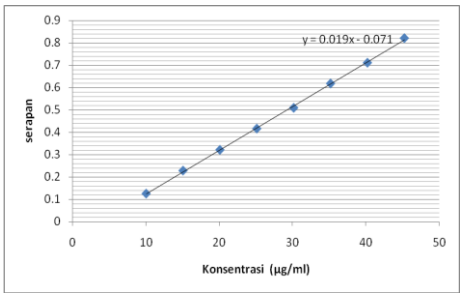

Figure 6 : The linearity of ascorbic acid

Table 1: Precision.

Observation	Absorption
1	0.480
2	0.483
3	0.483
4	0.484
5	0.485
6	0.484
7	0.486
8	0.485
9	0.485
10	0.485
mean	0.484
S.D.	$1.6997.10^{-3}$
C.V.	0.3512%

in foodstuffs and to reduce the oxidation of ascorbic acid or an antioxidant[20].

Optimization of the addition of 5% sulfuric acid

Optimization of the addition of 5% sulfuric acid at 250.0 µg ascorbic acid to obtain optimal results between 3.0-5.0 ml (Fig. 2). No significant differences occurred the addition of 5% sulfuric acid at 3.0 ml, 4.0 ml and 5.0 ml. *at 250.0 µg ascorbic acid*

So in this study have the addition of 5% sulfuric acid as much as 4.0 ml, it is the same with the addition of sulfuric acid other researchers[8]. The addition of acid and reducing agent as necessary as other researchers reported[11].

Optimization of the addition of 5% ammonium molybdate

The result of the addition of 5% ammonium molybdate optimal at 250.0 µg ascorbic acid was at 1.5 ml (Fig.3). So that the subsequent determination of 5% ammonium molybdate used additional of 1.5 ml.

The addition of ammonium molybdate to form colored compounds, which can be observed at visible wavelengths. Total addition of 5% ammonium molybdate depending on the amount of ascorbic acid, resulting in differences in the addition of 5% ammonium molybdate. On the other researchers addition of color reagent are not reported for a number of ascorbic acid[4].

Optimization of color stability

By looking at the color stability optimization results that occurred, there was no significant difference at 20 minutes, 25 minutes and 30 minutes. It was explained that, colors formed stable in the range of 20-30 minutes (Fig.4). So that the subsequent determination, uptake was observed in the range of 20-30 minutes after every addition of reagents. Because the number of researchers in the process using 250.0 µg of ascorbic acid and the process is longer than 30 minutes, then the color stability observations to 30 minutes. And in this study showed a color stable than 20-30 minutes. The time stability of color formed not reported other researchers[4,11,25,27]. Other researchers using dinitrophenylhydrazine reagent color, and color is formed for three hours[26].

Method Validation

Validation of the determination of ascorbic acid levels in the flesh of grapefruit, including an analytical method to calculate the quantitation of the major components, the category one[1]. Parameter validation category of one: selectivity, linearity, precision and accuracy.

Selectivity

Based on the spectra of the above, it can be concluded that the wavelength of 701 nm can be used as the wavelength selected (Fig.5). Reported by other researchers, that the determination of the wavelength of ascorbic acid by adding ammonium molybdate is 880 nm. The different wavelength due to differences in the substance determined that antimony by using reagent ammonium molybdate and orthophosphate[27], and at a wavelength of 665 nm using a reagent methylene blue[4].

Linearity

At the linearity of ascorbic acid, regression equation y = 0.0019x + 0.071; r = 0.9998; (P = 0.000; p <0.01) (Fig.6). Based on these data, it can be concluded that there is a linear relationship between the concentration and absorbance, according to the Beer-Lambert Law.

Other researchers[5], reported that there is a linear relationship between the concentration and absorbance, so that the same according to Lambert-Beer's Law. Linear same result with reagent 2,4-dinitrophenyl hydrazine red

Table 2 : (%) Accuracy of ascorbic acid.

Added of Ascorbic acid (µg)	Ascorbic acid obtained (µg)	(%) Recovery
200.0	203.2475	101.6237
202.0	205.3154	101.6413
226.8	222.7728	98.2243
302.4	300.4700	99.3618
Mean		100.2128
S.D		1.7039
C.V		0.0170

Table 3: (%) Levels of ascorbic acid in the flesh grapefruit from Indonesia.

weighing citrus fruits (g)	weight vitamin c obtained (g)	Levels of ascorbic acid (%;w/w)
100.1116	0.0159	0.0158
100.1235	0.0046	0.0046
100.3300	0.0195	0.0194
100.0620	0.0142	0.0142
100.1225	0.0416	0.0416
100.1200	0.0292	0.0292
	mean	0.0208
	S.D.	0.0129

happened was observed at a wavelength of 521 nm[11]

Precision

Based on the Coefficient of Variation (C.V) = 0.3512% (Table.1), the precision qualify namely C.V.≤2%[28]. Precision (C.V.) on the other investigators are not reported[11,25,26]. Terms C.V maximum is 2%, so that variations in this study meet the requirements.

Accuracy

Based on the accuracy, obtained an average% recovery of (100.2128 ± 1.7039)% with Coefficient of Variation 0.017% (Table.2), so that the accuracy of the results meet the requirements, the accuracy requirements are 98-102%[28].

The accuracy of the measured parameters (%) recovery in this study conducted by techniques addition, because the artificial sample matrices can not be made. Not explained the technique of determining the levels of ascorbic acid in the sample to the other investigators[4]. Yield (%) recovery of ascorbic acid in the flesh grapefruit in this study 100.2128 ± 1.7039%. These results meet the requirement under is 98-102%[28]. While the accuracy of the reported results of other researchers 101.4-108.0%[4].

Determination of ascorbic acid in the meat juices grapefruit from Indonesia

Validation of the results mentioned above, was applied to the determination of ascorbic acid levels in the flesh of grapefruit (Table 3).

Application of optimization and validation conditions on the determination of ascorbic acid levels in the flesh of grapefruit was 0.0208 ± 0.0129 (%; w/w). Variations ascorbic acid levels in the flesh of grapefruit caused each comb/petal flesh grapefruit in one fruit had higher levels of ascorbic acid are different. Other researchers, reported levels of ascorbic acid in fruit pomelo *(Citrus maxima*) is 43 mg/100 g of fruit flesh[16], 43.2-986.6 µg/ml in the various fruit juices in Lagos[21], 984 mg/100 g in the orange pulp[10], 141.34±22.07 mg/100ml in the orange fruit in Ethiopia[12], 25.11 mg/100ml in the sweet orange fruit in Kathmandu, India[24]. Ascorbic acid content difference is due to the method, location, time of collection and a variety of fruits

CONCLUSIONS

Optimization of the determination of ascorbic acid (250 µg), required the addition of reagents: 4.0 ml of 5% sulfuric acid, 1.5 ml of 3% acid metaphosphate, 1.5 ml of 5% ammonium molybdate and stabilized colored compounds which occur in 20-30 minute.

Validation Spectrophotometry method in the determination of ascorbic acid levels in the flesh of grapefruit, qualify include: selectivity at a wavelength of 701 nm, has a linear relationship between the concentration by absorbance (Lambert-Beer's Law), Precision (C.V.) = 0.3512 % (<2%) and accuracy of 100.2128± 1.7039%. Optimization and validation of methods Spectrophotometry can be used for the determination of ascorbic acid levels in the flesh of grapefruit.

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