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DEVELOPMENT AND VALIDATION OF A RP-HPLC METHOD FOR A SIMULTANEOUS ANALYSIS OF QUERCETIN AND ASCORBIC ACID IN PSIDIUM GUAJAVA FRUIT EXTRACT AT DIFFERENT RIPENING STAGES

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Abstract

A sensitive yet simple but HPLC-UV method is developed to determine quercetin and ascorbic acid in red guava (Psidium guajava Linn.). The flow rate at 1.0 ml/ min, with an injection of 20 µl, was detected using two different wavelengths; 254 nm for ascorbic acid and 370 nm for quercetin. The eluent system consisted of 0.01 mol/ L NH4H2PO4 with pH = 2.67 (mobile phase A) and a 0.1% orthophosphoric acid mixture: methanol 51:49 (mobile phase B). The gradient elution method used is 0-5 minutes: A 100%; 5-45 minutes A 0%; and 45-55 minutes A 100%. The stationary phase is a column C-18, 150 x 4.6 mm id, 5 µm ODS-3 Inertsil GL-Science, and uses a Shimadzu, LC-20AT prominence. The RT for ascorbic acid was 3.5 minutes, and the RT for quercetin was 25.32 minutes, with the overall analysis duration of 55 minutes. It was obtained, from the validation results, that a linear calibration curve with R = 0.999 for quercetin and R = 0.999 for ascorbic acid, respectively. The precision (RSD) is less than 1.35% for quercetin and 1.65% for Ascorbic acid. The limit of detection (LOD) of the two compounds is 0.01 μg / ml. The limit of quantification (LOQ) of the two compounds is 0.02 μg / ml for quercetin and 0.04 μg/ ml for ascorbic acid. The recovery rates for quercetin were 86.77% and 95.75% for ascorbic acid. The specificity value for ascorbic was 2.59 and for quercetin 2.28. Thus, the HPLC analysis method developed has met the analytical requirements and can be used in the analysis of quercetin and ascorbic acid levels of Red Guava. To conclude, it was found that the compound content was in the range 1.11-31.78 μg / ml for quercetin, and 0.87-31.84 μg / ml for ascorbic acid. The content of quercetin compounds is inversely related to the level of ripeness of the guava fruit. Conversely, the levels of ascorbic acid compounds increase with the maturity level of the guava fruit.

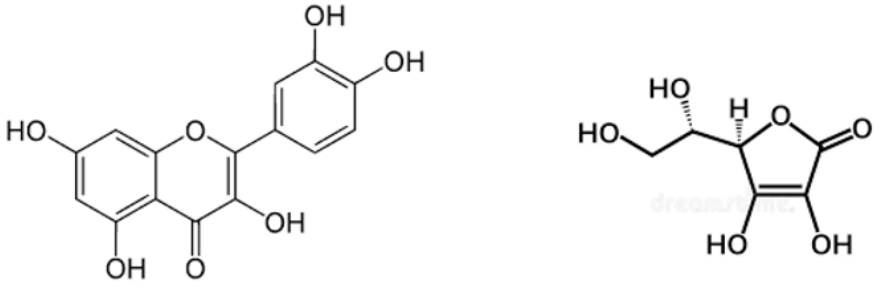
Keywords: Ascorbic acid, Guava, HPLC, Quercetin, Ripening.

1. Introduction

Guava belongs to a small tropical tree growing in the tropics as tall as 35 feet tall and is widely planted for its fruit consumption. Guava is also part of the Myrtaceae family with around 133 genera and over 3,800 species. The leaves and bark of P. guajava tree have been long used in traditional medicine up till now. Psidium guajava (P. guajava) is native to tropical America. It is also called "guayaba" in Spanish speaking countries and "goiaba" in Brazil, and is widely grown in Taiwan, Hawaii, Thailand, Philippines and Malaysia [1]. Guava leaf extract is reported to cure various types of gastrointestinal disorders comprising diarrhea, vomiting, gastroenteritis, peristaltic reflex problem, antimicrobial spasmolytic activity, activity, antioxidants, flatulence, dysentery, and gastric stomach. The important active constituents are essential oils, flavonoids, carotenoids, polyphenol compounds, pentacyclic triterpenoids, esters, and aldehydes [2-6].

However, the positive effects of guava for health are not only caused by these polyphenol compounds, but they are also affected by the role and presence of ascorbic acid. Several previous studies have shown that the content of ascorbic acid in guava is among the highest [7-10]. Therefore, ascorbic acid has a significant contribution to the guava pharmacological activity. Quercetin is one of the polyphenolic compounds whose determination has been widely used, but the method of determining quercetin with ascorbic acid simultaneously is still uncommon. Detailed information on the phytochemicals involved is still focused on the determination of polyphenolic compounds in guava leaves [11-15].

Several studies examining the contents of the fruit have succeeded in determining 13 active phenolic compounds in guava with two different ripeness: green and ripe [16]. However, simultaneous methods for examining polyphenols and ascorbic acid are still intermittent. The structure of quercetin and ascorbic acid is shown in Fig. 1, where it appears that both have many chromophore groups. Reversed phase HPLC with UV / Vis detector (RP-HPLCUV / Vis) is a simple yet reliable method by measuring light absorption by chromophore groups in the range of 200nm to 800nm wavelength [17]. In this study, a simultaneous determination of quercetin and ascorbic acid in guava fruit will be developed and validated. This is important to determine the distribution of the two active compounds in the red guava (Psidium guajava Linn.). Thus, the results of this study can become a basis in choosing the accurate fruit ripeness relevant to the therapeutic or dietary goals of using guava. The developed method is linear, precise, robust and accurate, and has been successfully applied to the determination of ascorbic acid and quercetin in guava fruits extract.



(a) (b)

Fig. 1. Chemical structures of quercetin (a) and ascorbic acid (b).

2. Materials and Methods

2.1. Chemicals and reagents

Reference standards of quercetin hydrate (batch number 337951, purity > 95.0%), and L-Ascorbic acid (batch number A92902, purity > 99.0%), were purchased from Sigma Aldrich, Saint Louis, USA. Methanol (for liquid chromatography) and $NH_4H_2PO_4$ was obtained from Merck, Darmstadt Germany. The Red guava fruits (*Psidium guajava* Linn.) were collected in November 2018 from Cimaung village-Pangalengan, West Java, Indonesia, at 7°4′48.6444S 107°33′41.8068″E and 769 m above sea level. The plant consists of three ripening stages, such as unripe (4 weeks old), medium-ripe (8 weeks old), and ripe (14 weeks old). They were authenticated at the Herbarium Bandungense (No. 4513/I1.CO2.2/PL/2016), The School of Life Science and Technology, Institut Teknologi Bandung, West Java, Indonesia. All other reagents and chemicals were analytical grade.

2.2. Instrumentation and analytical conditions

The analyses in this study were performed on a Shimadzu HPLC system which has been equipped with LC-20AT prominence. The HPLC analysis was performed on an ODS-3 Inertsil GL-Science Shimadzu-C18 column (150mm × 4.6 mm, 5 μm) with a mobile phase A ($NH_4H_2PO_4$ 0.01 mol/L B) and mobile phase B comprising methanol and 0.1% orthophosphoric acid (51: 49, v/v) with a consistent rate of 1.0ml/min. The injection volume was 20μl and the column temperature was set at 25∘C. The wavelength was set at 370 nm for quantitative analysis of quercetin and 254nm for ascorbic acid. The proportion of components in the mobile phase showed in Table 1 which optimized to obtain a well separation of quercetin, and ascorbic acid (IS).

2.3. Preparation of calibration standards and quality control standards

The concentrated stock solutions of quercetin were prepared by dissolving the reference standards ascorbic acid in $NH_4H_2PO_4$ 0.01 mol/L solution [18] and quercetin in methanol to final concentration of 30mg/ml. All solutions were in protection upon light and was in storage at 4°C. There were quality control samples prepared in the same way as the calibration samples which represent three different level concentrations (low, medium, and high) of quercetin and ascorbic acid at 10, 20, and 30 μg/ml, respectively.

2.4. Sample preparation

200mg guava fruit extract was transferred to a 5mL vial to which was added 500 μL methanol then add 5ml with $NH_4H_2PO_4$ 0.01 mol/L for processing. After having been vortex-mixed in 30 seconds, 20 μL of the sample was injected into the column of HPLC to be analysed. Sample preparation method was also applied to determine precision, accuracy, and recovery.

2.5. Method validation

The method carried out in accordance with USP guidelines taking into considerations such factors as specificity, linearity, LOD, LOQ, accuracy, and precision [19].

2.5.1. Specificity

This paper had specificity study aiming at investigating whether endogenous constituents and other substances existing in samples matriks were no interference to the detection of the analytes comparatively analysing blank eluent, corresponding to blank samples spiked with the two analytes, and the samples from guava fruits extract. The acceptance criterion was >2.0 for the percentage of relative standard deviation (% RSD) for retention times for the samples and the peak area.

2.5.2. Linearity, Limit of Detection (LOD) and Limit of Quantification (LOQ)

Linearity was determined by three injections of seven different IS concentrations (0.5, 1, 2, 4, 8, 16, and 32 µg/ml). Linearity is defined as an ability of obtaining test results directly proportional upon the analyte concentration. Peak area in average was plotted against concentrations. The linearity was then evaluated using the calibration curve for calculating coefficient of correlation, slope and intercept. In general, a value of correlation coefficient, which is (R) > 0.998, is considered evident upon an acceptable fit for the data to the regression line (Table 2). The LOD, which was defined as the lowest concentration in the detectable calibration curve, and LOQ, defined as the lowest concentration in the calibration curve with mathematic equation [20].

2.5.3. Precision and accuracy

The precision test was aimed to determine the degree of closeness between individual test results. It was measured through the distribution of individual results that were determined repeatedly from a homogeneous mixture under the same analysis conditions. Accuracy experiments were carried out on at least six replica samples taken from a sample mixture with a homogeneous matrix. It is better if the accuracy is determined for the actual sample in the form of a mixture with a pharmaceutical preparation carrier (placebo) to see the effect of the carrier matrix on this similarity [19]. The total precision of the method was expressed as the relative standard deviation (%RSD). In this paper, the precision was determined by six replicate analyses at a concentration of 40 mg/mL of samples solution using the developed method and % RSD < 2% was accepted.

2.5.4. Analysis of real samples

Three stages of ripening guava fruits extract were analysed with three replicates. All the results were expressed as means ± standard deviation (SD) of three replicates. The profile of their ascorbic acid and quercetin level content as can be seen in Fig 3.

3. Results and Discussion

3.1. Method development

3.1.1. Optimization of system suitability test

The determination of the conditions for the analysis of mixed compounds simultaneously was done initially by optimizing the analytical conditions for each compound. It was aimed to identify the most optimum conditions as the basis for mixed compound analysis. The optimization was done by using gradient elution for each compound. Subsequently, it was followed by adjusting the time and eluent composition

until the optimum conditions were achieved for both compounds. Therefore, the change in gradient time was also used as one of the parameters to be optimized.

In the system suitability test, there are several parameters that must be met. They include the theoretical plate value (*N*), tailing factor (*T*), resolution (r_s) and the relative standard deviation value (% RSD) for the area of 5 injections and the results are summarized in Table 1. Quercetin standards and ascorbic acid were injected into the HPLC system and the data obtained from the injection was processed using software connected to the HPLC system. The data obtained were compared with the requirements of these parameters in the USP. To determine the standard separation of ascorbic acid and quercetin, a standard mixture of 30 mg/L was analysed. From the separation chromatogram of Ascorbic acid and quercetin, it was revealed that the theoretical plate value (*N*) of each standard peak was more than 2000, this indicated that the efficiency of the column used is quite high, the Resolution value of $R \geq 2.0$ indicates that the separation of the two compounds is effective. The %RSD which is lower than 2% for both of ascorbic acid and quercetin indicate that the system appropriate to analyse the two of them simultaneously. Capacity factor for the quercetin is below 2, however, the capacity factor value has no theoretical meaning for the gradient system, so it can be ignored for this condition. Based on the result, it can be concluded that the chromatogram had met the criteria and suitable to identify ascorbic acid and quercetin simultaneously.

Table 1. System suitability test parameters.

Chromatographic parameters	Ascorbic acid (30 µg/ml)	Quercetin (30 µg/ml)	Acceptance criteria
Retention time min, (*n*=5)	3,777±0,036	25,32±0,438	-
%RSD, Retention time (*n*=5)	0,96	1,73	≤ 2,0%
%RSD, Peak area (*n*=5)	1,58	1,444	
Theoretical plates (N)	3.935	12.260	>1000[a]
Capacity factor (*k'*)	5,2	0,63	>2.0
Tailing factor (T)	1,508	1,71	≤2.0
Resolution (r_s)	5,23	13,64	>2.0
Specificity	2.54	2.28	>2.0

Table 2. Linearity, LOD, and LOQ for the analysis of quercetin and ascorbic acid in guava fruit extract solutions.

Compound	Linearity (µg/ml)	Calibration curve	Correlation coefficient (*R*)	LOD (µg/ml)	LOQ (µg/ml)
Ascorbic acid	0.87-31.84	y=56364x-5726.5	0.999	0.01	0.04
Quercetin	1.11-31,78	y=114750x-46495	0.999	0.01	0.02

Table 3. The precision, and accuracy of quercetin and ascorbic acid in guava fruit extract solutions (*n*=6).

Compounds	Spiked conc. (µg/ml)	Found conc (µg/ml)	Precision (RSD%)	Accuracy (%)	Recovery (%)
Ascorbic acid					
80%	3,5	5,63±0,40	7,13	95,75	88,55
100%	4,4	7,19±0,95	13,27		
120%	5,3	8,52±0,55	6,41		
Quercetin					
80%	0,80	1,36±0,03	1,82		
100%	1,00	1,34±0,07	4,86	86,79	97,56
120%	1,20	2,18±0,12	5,42		

3.1.2. Optimization of chromatographic conditions

The initial test was carried out by single injection of each standard Ascorbic acid and quercetin with a concentration of 10.20,30 mg / l. The volume of injection in the HPLC system was 20 µl. The test was carried out using a C18 column, 150x4.6 mm id., 5 µm with a mixed mobile phase composition. Compositions of mobile phase systems (acetonitrile-water, methanol-water, methanol-0.1% formic acid, $NH_4H_2PO_4$ 0.01 mol/L, and methanol-0.1% orthophosphoric acid) were in examination and comparison in order to obtain good chromatographic behaviour. Finally, $NH_4H_2PO_4$ 0.01 mol/L pH. 2.67, methanol and 0.1% orthophosphoric acid (51 : 49, v/v) were chosen as gradian system, which had a best separation. The limitation of the gradient elution system is that ghost peaks can be formed as seen in the standard ascorbic acid (A) chromatogram and the detected sample chromatogram at 254 nm (Fig. 1). However, no ghost peaks were found on the standard chromatogram of quercetin (C) and chromatogram of the sample (D) which were detected at 370 nm. The "ghost peaks" can occur in HPLC due to contamination or impurities in the reagents involved in the mobile phase and sample dilution. Inorganic impurities, such as nitrites, organic substances contained in dissolved plastic containers or synthetic impurities in methanol and acetonitrile, and even detergent residues on glassware have the potential to cause ghost peaks (20). However, ghost peaks do not affect the separation and quantitative analysis of these two compounds.

3.1.3. Optimization of sample preparation conditions

In our experiment, liquid-liquid extraction (LLE) were performed for the sample preparation. Initially, we used the 0.5mL methanol dilute the quercetin and ascorbic acid. However, the results showed that the method good for quercetin, but not for ascorbic acid. In the separation process, ascorbic acid in methanol undergoes decomposition that causes its levels to decrease. Furthermore, ascorbic acid was dissolved using NH4H2PO4 0.01 mol / L which is also mobile phase A and gave good results in the form of relatively stable ascorbic acid. Furthermore, for combination examination, quercetin was first dissolved with 500 µl of methanol before it was combined with ascorbic acid in NH4H2PO4 0.01 mol / L pH 2.67. Afterwards, it was mixed up to 2.0 ml and stored as stock solution. However, this condition can only be maintained for a maximum of 4 hours. Thus, the samples must always be kept as fresh as possible for optimal results.

3.2. Method validation

The method was designed, developed, carried out in accordance with USP guidelines taking into account such factors as linearity, specificity, LOD, LOQ, precision, and accuracy.

3.2.1. Specificity

This study confirms that this method has met the established criteria. Typical chromatograms is obtained from samples and spiked matriks sample with the two analytes are shown in Fig. 2. As a result, the studies showed that the matriks of guava fruit extract had a bit interference because there were peaks adjacent to ascorbic acid and quercetin, so the specificity values of ascorbic acid and quercetin were 2.58 and 2.29, respectively.

3.2.2. Linearity, LOD, and LOQ

Relationships of the excellent linear are presented in Table 2, where *Y* is the peak-area ratio of analytes to IS and *X* is the analytes concentration in contains in extract samples, respectively. Three curves of calibration for quercetin and ascorbic acid were established at seven concentrations over the range of 1.11-31.78 μg/ml and 0.87-31.84μg/ml, respectively.

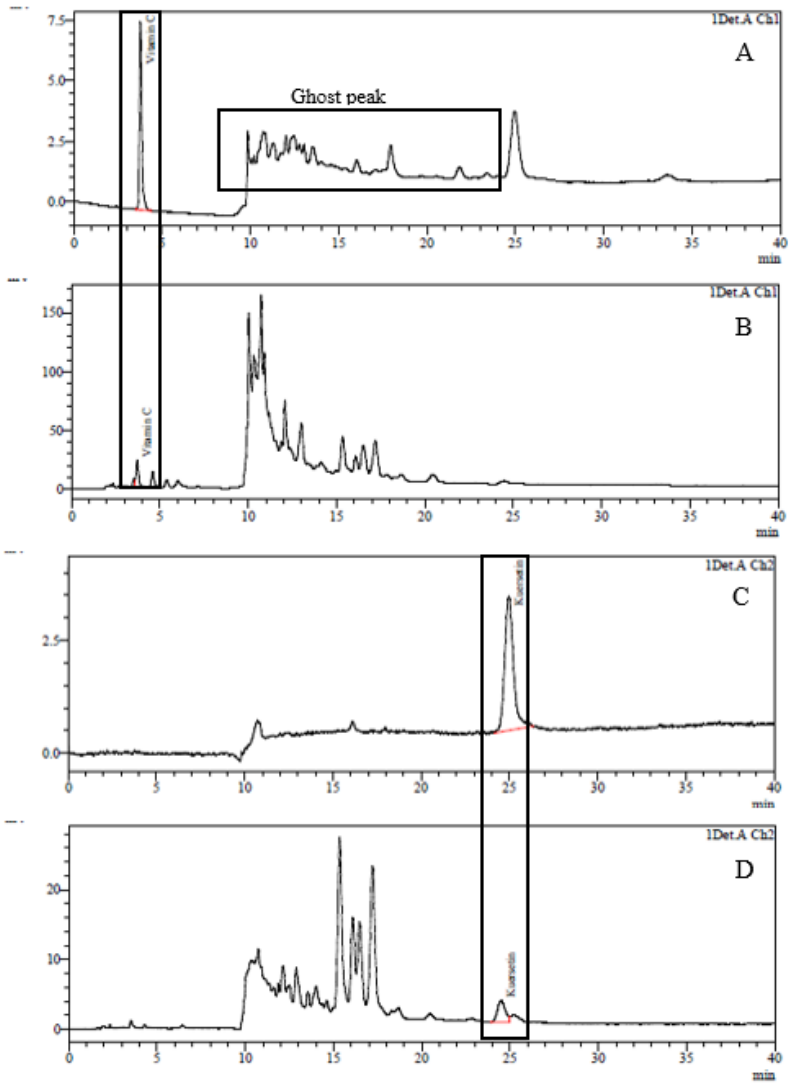


Fig. 2. Chromatogram of mix standard solution and sample. Ascorbic acid standard with 254 nm (A), Ascorbic in sample with 254 nm (B), Quercetin standard with 370 nm (C), and Quercetin in sample with 370 nm (D).

The LOD for two compounds was 0.01 μg/ml, and LOQ 0.04 μg/ml for ascorbic acid, and 0.02μg/ml for quercetin which are sensitive enough for the quantification studies of these compounds in guava fruit extracts.

3.2.3. Precision and accuracy

Precision test was carried out on a placebo-spiked sample with 100% levels of each active ingredient, with a concentration of 30 μg / ml ascorbic acid, and 30 μg / ml quercetin which was measured 6 times on the same day (interday precision). The criteria for the precision test are shown with a value of %RSD for six replicate injection <2%. Accuracy is seen based on the value of % recovery from extraction recoveries for quercetin, and ascorbic acid from guava fruit extract were determined at 80%, 100%, and 120% concentrations. are demonstrated in Table 3 and investigated by analysing QC samples. The precision of quercetin and ascorbic acid were 4.33-4.50% and 8.70-9.05%, with the RSD values less than 1.35%, and 1.65%, respectively. The mean extraction recoveries for quercetin, and ascorbic acid dalam extract following the requirements ranging from 80 to 110% [19]. These results indicated that the method which is being developed in this study is both accurate and precise.

3.3. Quantification of ascorbic acid and quercetin in guava fruit extract

The chromatogram from the validation and measurement results is illustrated in Fig. 2. The profile of the quantification results of the two compounds is portrayed in Fig. 3. It appears that the distribution of quercetin is mostly found in fruits with medium ripeness and then the levels decrease with the ripeness level. In unripe fruit, it is suspected that the quercetin content is less than tannins which is indicated by astringent taste. The levels of both decreased during the fruit ripening process and were thought to be associated with increased polymerization and hydrolysis reactions [21]. Meanwhile, ascorbic acid levels increase along with the ripeness of the guava fruit. This is in line with the study of Gull (2012) which reported that the highest levels of ascorbic acid were found in ripe fruit and had the highest antioxidant activity and the highest flavonoid content was in unripe fruit [22].

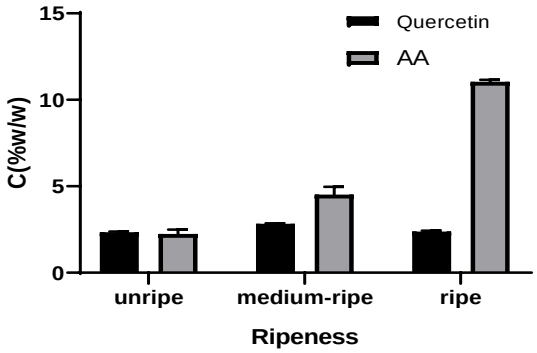


Fig. 3. Profile of quercetin and ascorbic acid level in guava fruit extract at three different ripening stage.

4. Conclusion

The present study described a specific, simple, and reliable HPLC-UV with IS method to simultaneously determine two compounds, ascorbic acid and quercetin. This is the first validated HPLC method for analysis of ascorbic acid and quercetin, in guava fruit extract, which was highly sensitive and accurate. The method has

also been successfully applied to quantify ascorbic acid and quercetin in three different ripening stages of guava. Therefore, to obtain optimal efficiency from the anti-inflammatory and antioxidant effects of guava fruit, it is advisable to choose guava fruit at medium ripeness because the content of these two compounds is at their best in this level of ripeness.

Conflicts of Interest

The authors have declared that there is no conflict of interest.

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