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OPTIMIZATION AND VALIDATION OF QUERCETIN ANALYSIS METHOD IN NON-BIOLOGICAL MATRIX USING HPLC

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Abstract

Quercetin is a natural bioactive found in syncthetic form, is useful as an antioxidant, anti-inflammatory and antibacterial. High-perfomance liquid chromatography is a method used for analyzing bioactive compounds. This research aims to be a standard reference for the bioactive extraction of natural quercetin. The exporting method involves collecting five groups of samples and performing five repetitions. The data anaylisis is descriptive and based on partial validation parameters issued by the Food and Drug Administration (FDA). The results show that a C18 column with an optimal wavelength of 375 nm and a solvent ratio of 60:40 using methanol and water is optimal for quercetin analysis. However, the selectivity test results are good, with an average score of 5.6 out of 1.5. The precision test results are less corresponding, with an average score of 7% from the maxium of 2%. The linearity test results are less good, with an average score of 0.901 out of 0.999. The sensitivity results are 0.23 for LOD and 0.878 for LOQ.

Keywords: Quercetin, Bioactive, Optimization, Validation, Chromatography

Introduction

Quercetin is the alkaloid part among flavonoids that are useful as antioxidants, anti-inflammatory, antidiabetic and the adsorption- and partition-based HPLC technique (Guo, 2015) can be used to separate certain phytochemical compounds in a sample. For example, in a antibacterial (Reyes-Farias *et al.*, 2019) as well as anti-cancer (Vafadar *et al*., 2020). High Performance Liquid Chromatography (HPLC) is a technique of separating components from tested samples, which can be used for analysis, identification, purification and determination of concentration on samples phyto-chemical test, the HPLC technique can be applied to separate flavonoid, alkaloid and quercetin compound in a plant sample (Purba *et al*., 2019). Synthetic quercetin with 95% purity is used as a standard reference on high-performance liquid chromatography devices (HPLC) (Chu *et al.*, 2021), while non-biological (Rubiyanto, 2016). matrix quercetin serves as standard specificity, linearity, precision, and references in analysing and identifying naturally occurring quarsetin compounds for withdrawal using HPLC devices. HPLC optimization is intended to find the best conditions in the analysis of samples used for subsequent validation (Deshmukhe, 2021). HPLC optimization includes the determination of movement phase fractions with thin layered chromatography (Rahmawati *et al.*, 2019), solvent determination "like dissolve like" (Mariana *et al.*, 2018), column chromatographic determination (Aulia *et al.*, 2016) and optimum wavelength determination (Nurhamidah, 2005). Validation is the process of establishing a new method discovered, aimed at ensuring that the new

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method can be applied. The FDA (2018) describes the existence of a partial validation method, a modification to a valid bioanalysis method, in which samples are said to be valid when at least three parameters have been met. Parameters that can be met include sensitivity.

Method

This research is using exploratory research, that used to analyze or describe the results of the subject, but is not intended to give wider implications. Explorative method research is used to find optimization and validation on non- biological matrix queries with HPLC devices. The synthetic quercetin samples used were produced by a chemical company with sample code Q495, formula C15H10O7 and formula weight 302.24 g/mol with a purity of 95%. The sample size was divided into five groups with five repetitions. The sample is prepared by making a 10 ppm quercetin root solution by dissolving 1 mg of quercetine powder sample and inserting in 100 ml of methanol for chromatography. The primary solution will be the basis of the secondary solution, the concentration of which will be reduced to 0.5 ppm; 1 ppm, 2 ppm, 3 ppm and 4 ppm. Secondary solution is the solution to because they are both polar, selection of be tested at the HPLC.

The contents of the research results reveal research findings based on field data obtained using questionnaires, surveys, documents, interviews, observations and other data collection techniques. The results at least contain an element of what/how, whether the data presented has been processed (not raw data), presented in the form of tables or pictures (choose one), or descriptive research and given information that is easy to understand. Write down the findings, but don't discuss the discussion here.

Table 1 Quercetin Sample Groups

Group	Sample
1	0,5 ppm
2	1 ppm
3	2 ppm
4	3 ppm
5	4 ppm

Optimization of HPLC is done with the selection of methanol and water solvents with because they are both polar, selection of non-polar C18 columns, choice of the fraction of methane eluen versus water a ratio of 95:5; 90:10; 80:20; 70:30; 60:40 and 50:50, as well as selection of wavelengths based on the optimal wave absorbed with UV-Vis spectroscopy. After optimization and samples are ready, further testing of samples with each concentration and the results are validated.

Table 2 Determining Partial Validation Value

Validation	Determined	Note
Selectivity	Resolution Formula $Rs = \frac{1{,}18\ (t2 - t2)}{W_{0,5(1)} + W_{0,5(2)}}$	Rs, Resolution t1, first peak retention time t2, second peak retention time W0,5(1), width half the height of the first peak W0,5(2), width half the height of the second peak
Linearity	Correlation coefficient value (r)	The condition for a good correlation coefficient value (r) is ≥0,999
Precision	Variation Coefficient (VC)	The condition for a good variation coefficient if the result of %VC ≤2%

Sensitivity	LOD Formula **LOD** = $\frac{3.S_{y/x}}{b}$ LOQ Formula LOQ = $\frac{10.S_{y/x}}{b}$	The smaller the value, the better the result

The analysis of the data obtained will be done in a descriptive manner and presented in the form of tables, narratives and discussions of the conclusions on the optimization already done.

Results

Optimization

The results of the optimization show an array of non-biological metrix results using the HPLC device, which are the use of methanol solvents for chromatography and water for Chromatography with a concentration of 60% methanols and 40% water, using the C18 column and the selected wavelength of 375 nm (Table 3).

Table 3 Quercetin Parameter Optimization Results Using HPLC Devices

Optimization Parameters	Optimization Results
Solvent	Methanol for chromatography and Water for chromatography
Solvent fraction percentage	60% Methanol : 40% Water
HPLC column	C_{18}
Optimum wavelenght	375 nm

The lower the Rf value, the higher the polarity at the tested motion phase (Arifah dkk., 2023), so this is in line with the principle of "like dissolve like". Agustin dkk. (2021) in his research stated that samples were categorized well when the difference value of Rf obtained the result ≤ 0,05. The results of the study showed that the ratio of 60:40 resulted in the difference Rf value of 0,04 which means this ratio is the best ratio for conducting analysis of quercetin compounds (Table 4).

Table 4 Chromatography Thin Layered Test Results (Solvent Fraction Percentage)

Comparison (Methanol/Water)	Mileage of Quercetin	Rf (cm)	Differentiatial Rf
90 : 10	5.18	0.24	0.09
80 : 20	7.48	0.17	0.23
70 : 30	7.63	0.07	0.05
60 : 40	7.03	0.02	0.04
50 : 50	6.95	0.06	0.05

Validation

The results of the optimization average obtained from partial validation parameters based on FDA validation standards can be seen in Table 5. electivity has a result on the average of 5.7, the standard should be ≥1.5 whereas at accuracy it has an outcome on the average of 7% of what should have been ≤ 2%. Linearity has the output average 0.901 of which should ≥ 0.999. Whereas LOD and LOQ showed the average result of 0.263 and 0.878.

Table 5 Partial Validation Test Results

Samples (ppm)	Selectivity (Resolution)	Precision (VC)	Linearity (r)	Sensitivity	
				LOD	LOQ
0.5	6.510	3%	39.862		
1	5.542	3%	40.063		
2	5.180	3%	42.929		
3	5.326	12%	49.023		
4	5.826	5%	46.959		
Mean	5.7	7%	0.901	0.263	0.878
Standard Value	≥1.5	≤2%	≥0.999		

The result of the linearity test obtained on HPLC optimization of quercetin compounds is 0.901 with a graph of linearities. The graphic results show an inconsistent increase in the sample of 3 ppm and 4 ppm (Figure 1). This is due to the presence of non-quercetin samples that accidentally entered and contaminated the samples of 3 and 4ppm. The contaminator in the movement phase HPLC is greatly affects the result of the chromatogram produced so the optimal condition must always beined (Widyaratna, 2015).

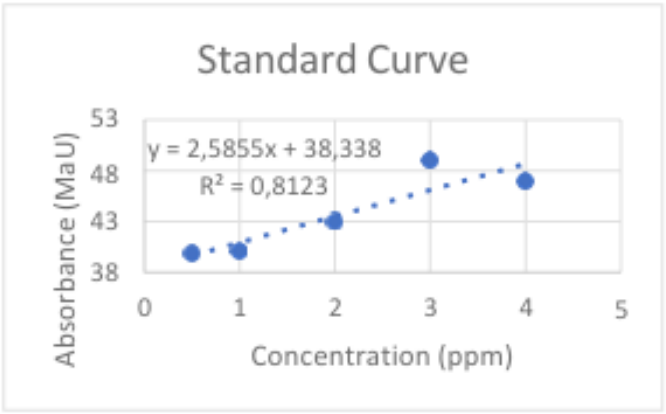


Figure 1. Lienarity Test Result

Discussion

Optimization

The principle of reverse phase HPLC is the quiet phase will be more nonpolar than the motion phase (Syallah, 2021). The C18 column has a large amount of carbon so it can be categorized as a non-polar silent phase (Saputra, 2017). A polar column produces good separation in quercetin analysis. So the C18 Column can carefully separate quercetin using the methanol and water motion phases with a 60:40 ratio. Determination The optimal wavelength can be obtained using spectrophotometry, because the Diode Array Detection (DAD) light absorbed by the quercetin sample (Markkinen, 2024). Dalming *et al.* (2022) in his research stated that the optimum wavelength quercetin obtained is 373 nm. The optimum wave length can be influenced by several factors, such as the condition and use of the instrument (Fadhilah *et al.*, 2022), so the difference in the optimal length may be due to the existence of differences spectrophotometry that used.

Validation

Selectivity test is aimed to looking at the ability of a method to identify a matrix with the possibility of the presence of other matrix components (Husnia and Budiarti, 2021). Good selectivity results if the resolution value is ≥1.5 (FDA, 2018). The results of this study obtained a resolution ratio of 5.7, which indicates that the separation between the quercetin chromatogram peak and other compounds can be clearly distinguished so that it can be stated that this method of research has proven to be able to carefully and accurately identify the quecetin compound (≥1.5). Precision test is aimed to determine the measurement method produces a consistent result in each test (Fatmawati *et al.*, 2023).

Precision test has a requirement of at least three repetitions of the sample to be tested (FDA, 2018). The precision test results are said to be good when the variation coefficient value (VC) is ≤2%. The result obtained from the precision test of quercetin compounds in this study is 7%. The percentage result is very far from the desired value, this is due to the fragility of the sample 3 ppm and 4 ppm obtaining 12% and 5%, because when performing the test there are non-quercetin samples that have been tested previously so that it becomes the contaminator of the series of optimal conditions in the HPLC, proved by the presence of a boost in the sampel 3 pppm and begins to decrease in sample 4 ppm.

The linearity test aims to see the ability of an analysis method to obtain the test results of each concentration of the analyite with a certain range (Anggraena, 2018). The linearity test is calculated using the correlation coefficient (r) formula and can be said well when the result is ≥0.999 (FDA, 2018).

The sensitivity test is divided into two categories, Limit of Detection (LOD) and Limit of Quantification (LOQ). The LOD is the smallest concentration of the internal analyte of the sample that still shows the absorption or absorbance value, while the LOQ is the smallest amount of analyte in a sample which can still be measured accurately and precisely by the HPLC device (Sumarno dan Kusumaningtyas, 2018). The smaller the LOD and LOQ results, the more sensitive the analytical methods are created (Zakkula et al., 2019). Husnia and Budiarti (2021) in their research obtained LOD and LOQ test results of 0.355 ug/mL and 1.185 ug/ml respectively. This suggests that the results of the optimization method performed in this study are more sensitive than previous studies.

Conclusion

The most suitable optimization for quercetin analysis in non-biological matrix is the use of a combination of solvent methanol for chromatography and water for chromatography with a 60:40 ratio, a C18 column and an optimal wavelength of 375 nm. This is demonstrated by partial validation tests with selectivity test parameters that meet the standard, precision and linearity tests close to the standard as well as sensitivity tests that are declared to be more sensitive than previous studies.

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