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DEVELOPMENT OF ISOCRATIC HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR SIMULTANEOUS ANALYSIS OF THE FLAVONOL FLAVONOIDS

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ABSTRACT
Quercetin, rutin, and myricetin are flavonoids belonging to the flavonol subclass, commonly found in a variety of fruits, vegetables, and medicinal plants. However, a validated isocratic High Performance Liquid Chromatography (HPLC) method for the simultaneous analysis of these compounds has not yet been established. Therefore, the development of a robust isocratic HPLC method enabling the concurrent quantification of quercetin, rutin, and myricetin within a relatively short analysis time is warranted. The method development process necessitates an initial optimization phase to establish ideal analytical conditions. Therefore, the development of analytical methods in this research begins with the optimization stage aimed at producing ideal conditions during the analysis process. This study commenced with the determination of the optimal detection wavelength for the HPLC detector, followed by the optimization of mobile phase compositions and their respective ratios, as well as the evaluation of acceptance criteria based on column efficiency parameters. The mobile phase consisting of methanol:0.1% phosphoric acid (70:30), at a flow rate of 1 mL/min, produced chromatograms with satisfactory separation. This was evidenced by the resolution factor (R) and the number of theoretical plates (N), both of which met the established acceptance criteria, resolution factor exceeding 1.5 and theoretical plate number above 2000, along with relatively short retention times (Tr). Furthermore, the peak symmetry factors for myricetin and quercetin were within acceptable limits, approaching the ideal value of 1. However, the peak symmetry of rutin requires further refinement to attain an optimal symmetry factor. The mobile phase of methanol:0.1% phosphoric acid (70:30) with a rate of 1 ml/min is most suitable for the simultaneous analysis of quercetin, rutin, and myricetin in the isocratic HPLC method.

Keywords: flavonoid; flavonol; high performance liquid chromatography; isocratic

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INTRODUCTION

Flavonoids, a class of naturally occurring phenolic compounds, are widely distributed throughout various parts of plants and are classified as aromatic phytochemicals. Numerous studies have investigated the development of natural products as therapeutic agents and health supplements, particularly emphasizing the therapeutic potential attributed to the phenolic constituents of flavonoids. This interest is primarily due to their notable antioxidant properties. The functional hydroxyl groups present on the core flavonoid structure contribute to antioxidant activity by scavenging free radicals. According to Ekalu and Habila (2020), the biological activities associated with flavonoids include antioxidant (30%), antimicrobial (18%), anticancer (18%), antiedematogenic (9%), anti-inflammatory (9%), antiviral (5%), antiulcer (5%), antifungal (2%), antinociceptive (2%), and antihistaminic (2%) effects.

The biological activity of flavonoids is largely determined by their chemical structure. These compounds are characterized by a C6-C3-C6 carbon skeleton, and structural variations result in a diverse array of flavonoid subclasses, including flavonols, flavones, isoflavones, flavanols, flavanones, chalcones, and anthocyanidins (Dimcheva et al., 2019; Ekalu & Habila, 2020). Among these, flavonols represent the most abundant subclass and are commonly found in foods—especially fruits and vegetables—as well as in various medicinal plants (Kumar & Pandey, 2013; Panche et al.,

2016). Due to their prevalence, flavonols are often employed as marker compounds in the development of chromatographic fingerprinting methods, which are essential for the standardization and quality control of herbal medicines and their derivative products. Sudrajat et al. (2020) reported that High-Performance Liquid Chromatography (HPLC) is the most widely used technique for chromatographic fingerprinting, owing to its ability to separate and detect multiple compounds with high selectivity and sensitivity. Furthermore, HPLC can generate chromatographic profiles for both identified marker compounds and unidentified constituents within complex sample matrices.

Multicomponent analysis of polyphenols, including flavonoids, involving seven to nine compounds has been developed using gradient HPLC methods. However, these methods generally require prolonged elution times ranging from 30 to 60 minutes (Dimcheva et al., 2019; Lee et al., 2020; Tang et al., 2010). In contrast, isocratic HPLC methods for the simultaneous analysis of flavonol-type flavonoids have been limited primarily to two-compound systems. These include quercetin–rutin with elution times 10–15 minutes (Moghaddasian et al., 2013; Suzery et al., 2019), quercetin–kaempferol with elution times 30–35 minutes (Wang et al., 2014), and a shorter version of quercetin–kaempferol analysis with elution times under 6 minutes (Rifai et al., 2015). Additionally, isocratic HPLC analysis have been reported for quercetin–rutin–kaempferol up to 35 minutes (Nongalleima et al., 2017) and quercetin–kaempferol–myricetin within 10 minutes (Shervington et al., 2018). However, to date, no isocratic HPLC method has been reported for the simultaneous analysis of quercetin, rutin, and myricetin.

Rutin, myricetin, and quercetin are flavonols frequently reported to coexist in various fruits, vegetables, and medicinal plants, such as onions, apples, grapes, berries, tomatoes, and ginger (Kumar & Pandey, 2013; Panche et al., 2016), as well as in bitter melon (Oliveira et al., 2018), purslane (including leaves, stems, and flowers) (Husein et al., 2021), Muntingia calabura fruit (Nurholis & Saleh, 2019), broccoli, green leafy vegetables, and numerous members of the Asteraceae family (Ekalu & Habila, 2020). Despite this, no isocratic HPLC method for the simultaneous determination of these three compounds has been reported. Consequently, it is necessary to develop an isocratic HPLC method capable of concurrently analyzing quercetin, rutin, and myricetin within a relatively short analysis time. Isocratic HPLC is preferable due to its simplicity and practicality, as it does not require gradient modifications of the mobile phase during the analytical process.

In the early stages of analytical method development, a range of parameters must be optimized to establish robust and reproducible conditions. Therefore, this research was initiated with an optimization phase to identify ideal analytical conditions. The optimization process followed a laboratory-based univariate approach, where one variable was altered while maintaining the others constant, and the outcomes were subsequently assessed. This method facilitates a comprehensive understanding of the analytical principles, theoretical foundations, and potential interactions among variables. The variables subjected to optimization in this study were the type and composition of the mobile phase delivered isocratically. The analytical method was considered optimal if it could produce chromatograms exhibiting well-resolved, symmetric peaks within a relatively short analytical time. This study aims to obtain the best type and composition of mobile phase in an isocratic flow system for the analysis of rutin, myricetin, and quercetin.

METHOD

The maximum absorption wavelengths of each rutin, myricetin, and quercetin stock solutions were determined by measuring the absorbance of their respective working solutions using a UV-Vis spectrophotometer across the wavelength range of 200–400 nm. Method optimization was performed by evaluating three different mobile phase compositions at a flow rate 1 ml/min, as presented in Table 1. The most suitable mobile phase was then further optimized by adjusting the proportion of its constituent solvents.

Table 1.
Variation of mobile phase

Code	Mobile Phase
A	Methanol : Acetonitrile : Water : Acetic Acid (30:20:49:1)
B	Methanol : Phosphoric Acid 0,1% (65:35)
C	Methanol : Water (50:50)

The HPLC system was operated using an ODS (250 mm × 4.6 mm) stationary phase column and a mobile phase delivered isocratically in ratios as specified in Table 1, with a flow rate 1,0 ml/min. The injection volume was set to 20 µL, corresponding to the capacity of the manual injector valve. The detector was set at the maximum absorption wavelength identified earlier. Filtered and degassed individual solutions, mixed solutions, and methanol blanks were each injected (20 µL) into the HPLC system. The analytical method was considered optimal if it achieved the simultaneous separation of quercetin, rutin, and myricetin with a minimum resolution (R) of 1.5, an asymmetry factor (As) approaching 1 (within the range of 0.9–1.1), a number of theoretical plates (N) exceeding 2000, and relatively short retention times (Snyder et al., 2010; USP, 2021).

RESULT

In the initial stage, the determination of maximum absorption wavelengths was carried out, with the results presented in Table 2, while the absorption spectra are shown in Figure 1.

Table 2.
Results of determining the maximum wavelength

Flavonoid	Benzoyl		Cinnamoyl	
	λ (nm)	Abs	λ (nm)	Abs
Rutin	267	0,203	364	0,259
Myricetin	254	0,381	376	0,462
Quercetin	255	0,146	371	0,148

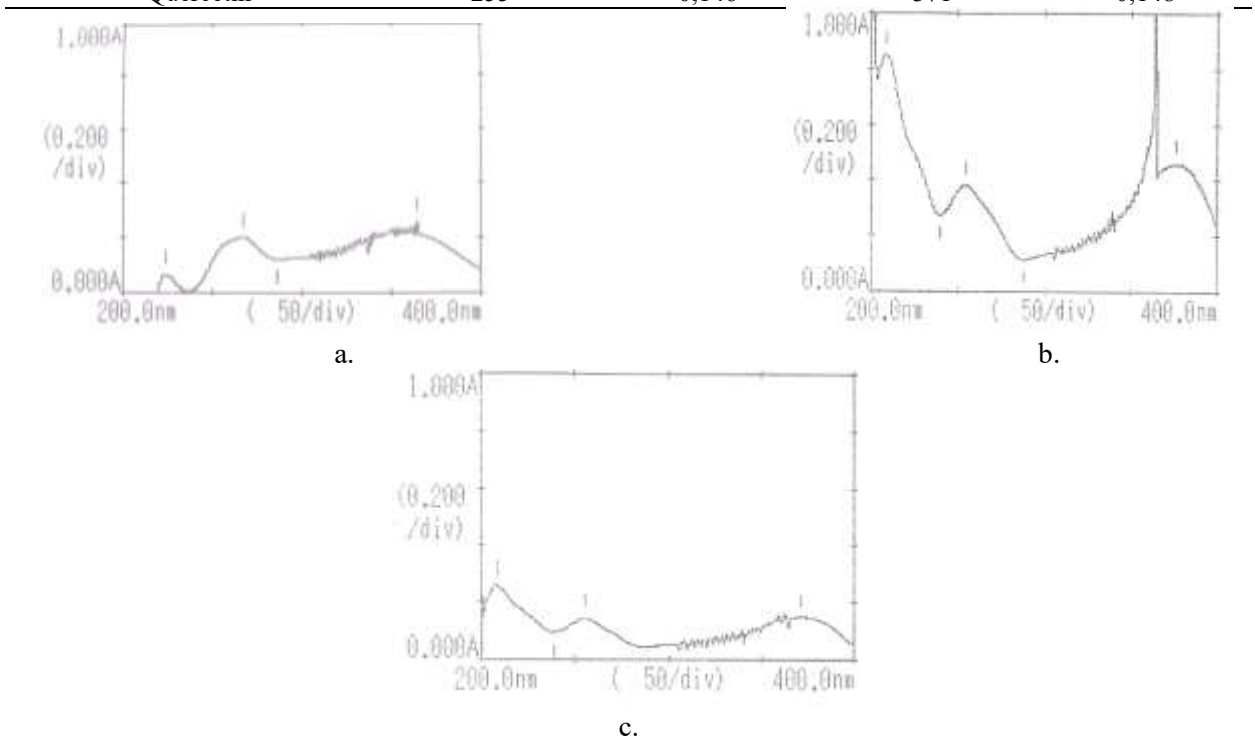


Figure 1. Spectrum of rutin (a), myricetin (b), quercetin (c) in methanol

The optimization of the isocratic HPLC method for the simultaneous analysis of flavonoid marker compounds was conducted using three types of mobile phases at a flow rate 1,0 ml/min, designated as A, B, and C, with the results summarized in Table 3.

Table 3.
Retention times in various mobile phase

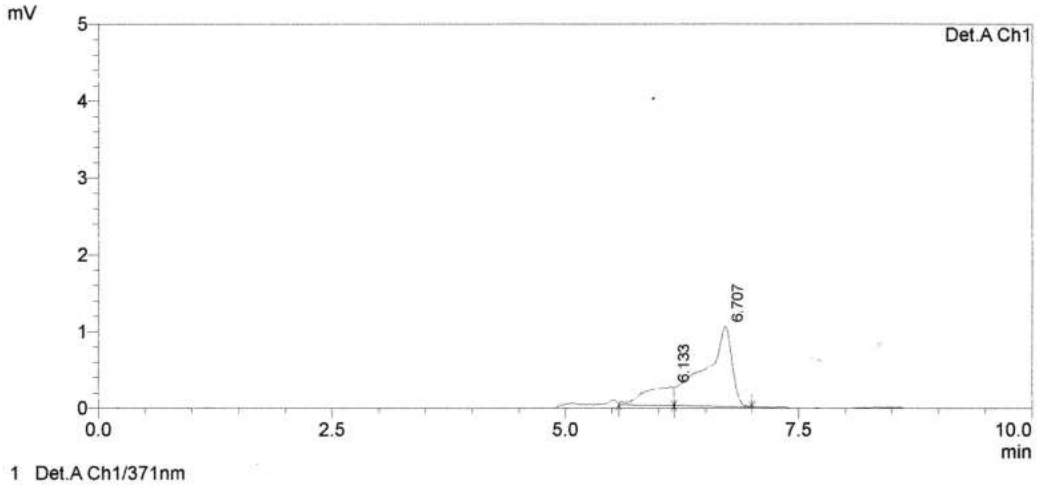
Code	Mobile phase	Time of retention (Tr)		
		Rutin	Myricetin	Quercetin
A	Methanol : Acetonitrile : Water : Acetic Acid (30:20:49:1)	4,258	4,719	6,283
B	Methanol : Phosphoric Acid 0,1% (65:35)	6,707	8,402	8,845
C	Methanol : Water (50:50)	8,642	8,973	9,073

Subsequent optimization was conducted by varying the composition of mobile phase B at a flow rate 1,0 ml/min, with the results shown in Table 4 and Figures 2 to 4.

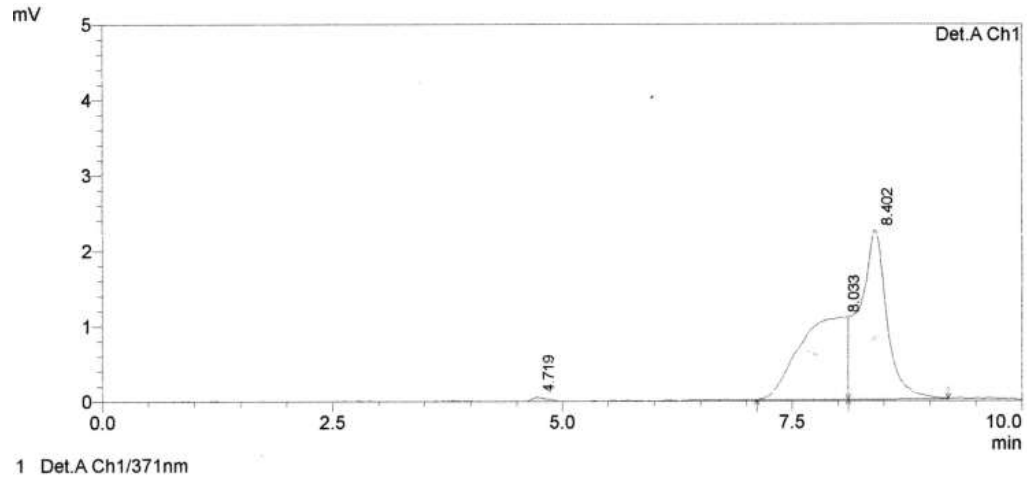
Table 4. Retention time in various composition ratios of the mobile phase

Code	Methanol : Phosphoric Acid 0,1%	Time of retention (Tr)		
		Rutin	Myricetin	Quercetin
B1	65:35	4,258	4,719	6,283
B2	70:30	6,707	8,402	8,845
B3	75:25	8,642	8,973	9,073

a. Rutin



b. Myricetin



c. Quercetin

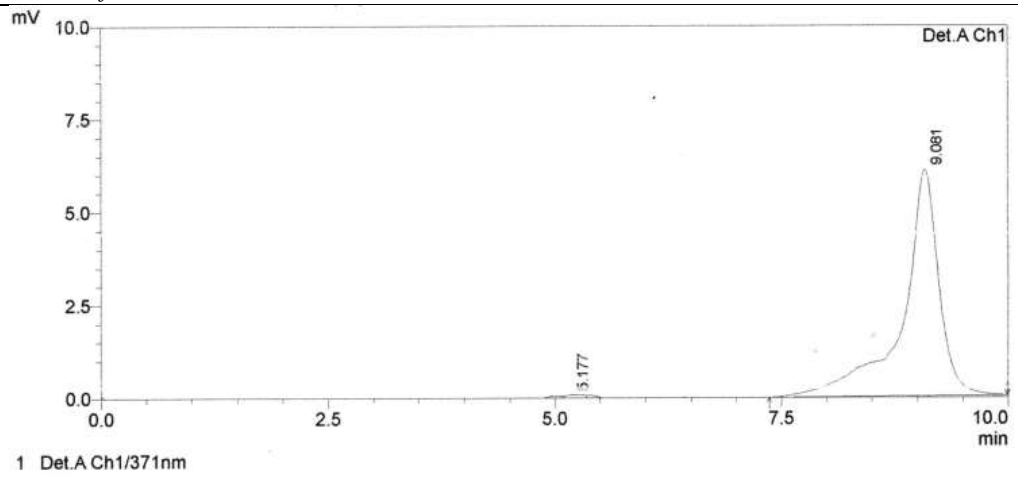
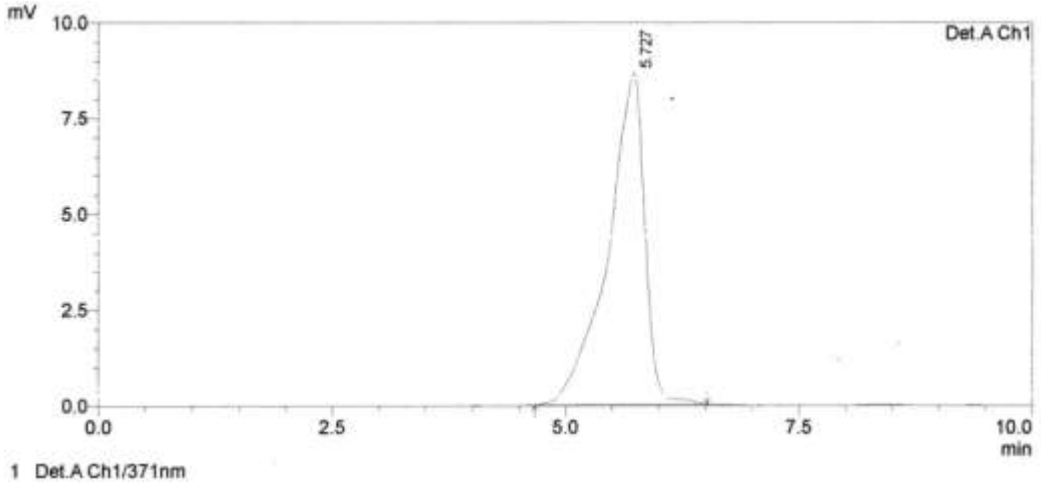
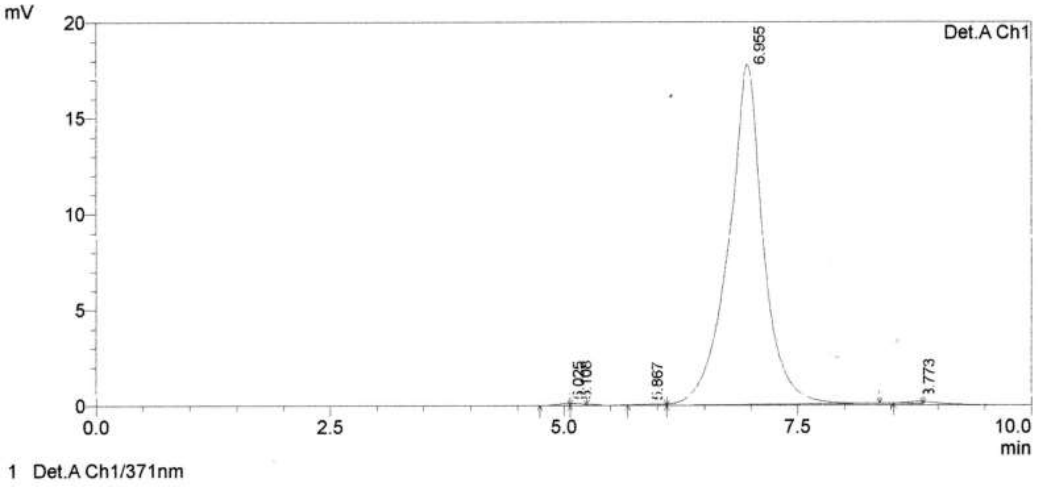


Figure 2. Chromatogram of rutin (a), myricetin (b), quercetin (c) in methanol solution by methanol:phosphoric acid 0,1% (65:35) as mobile phase at a flow rate 1 ml/minute

a. Rutin



b. Myiricetin



c. Quercetin

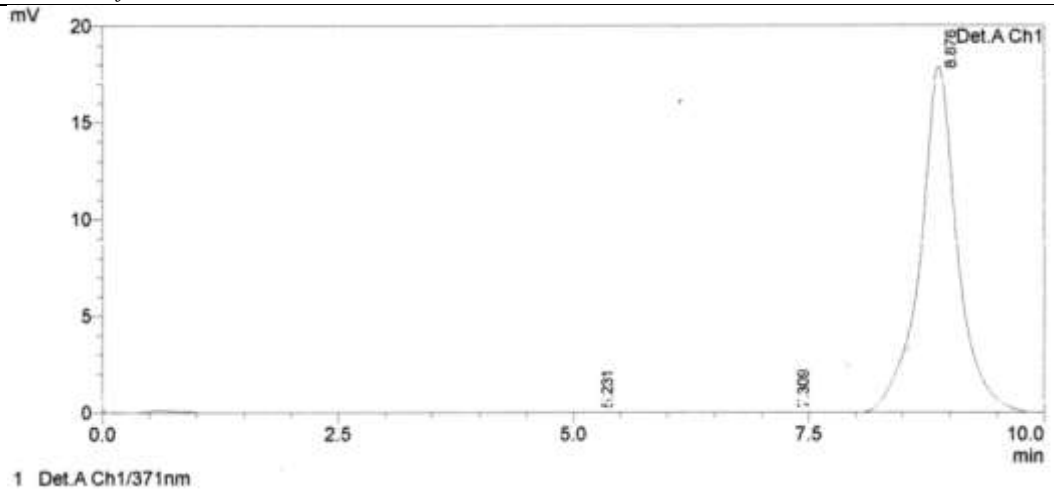
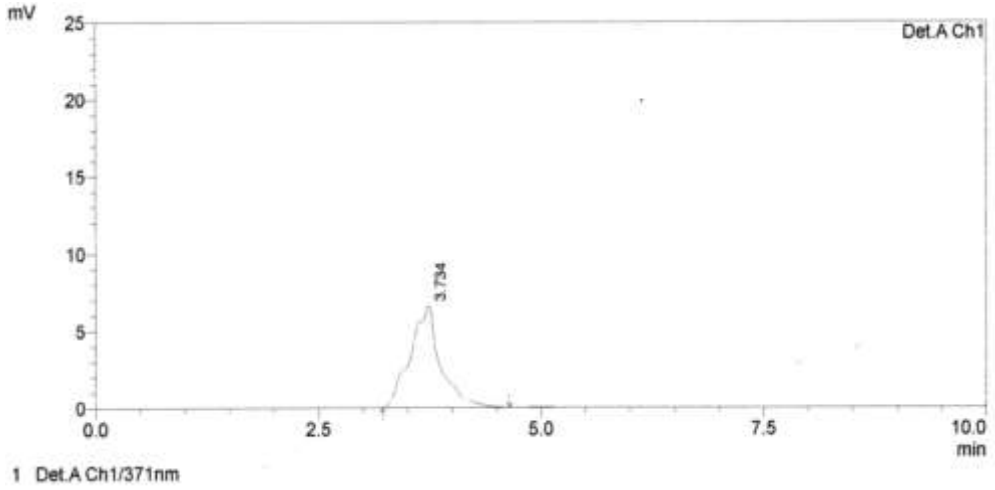
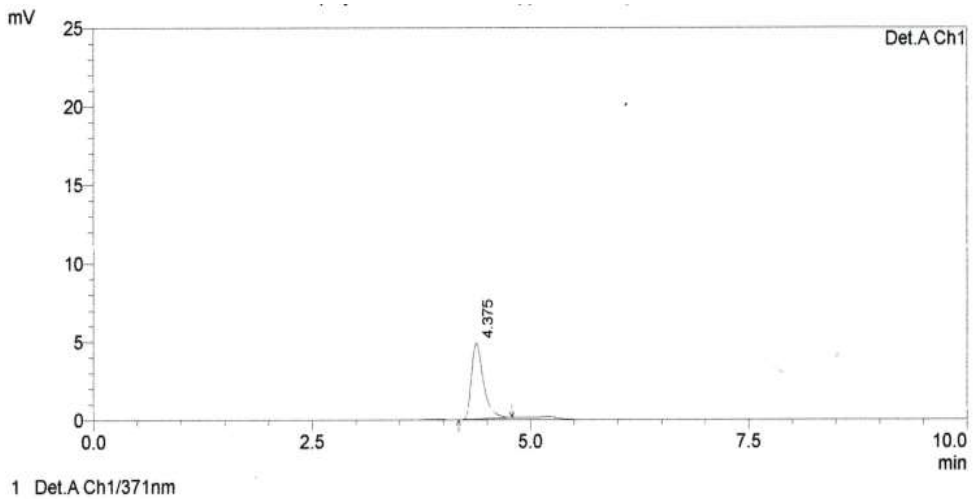


Figure 3. Chromatogram of rutin (a), myricetin (b), quercetin (c) in methanol solution by methanol:phosphoric acid 0,1% (70:30) as mobile phase at a flow rate 1 ml/minute

a. Rutin



b. Myricetin



c. Quercetin

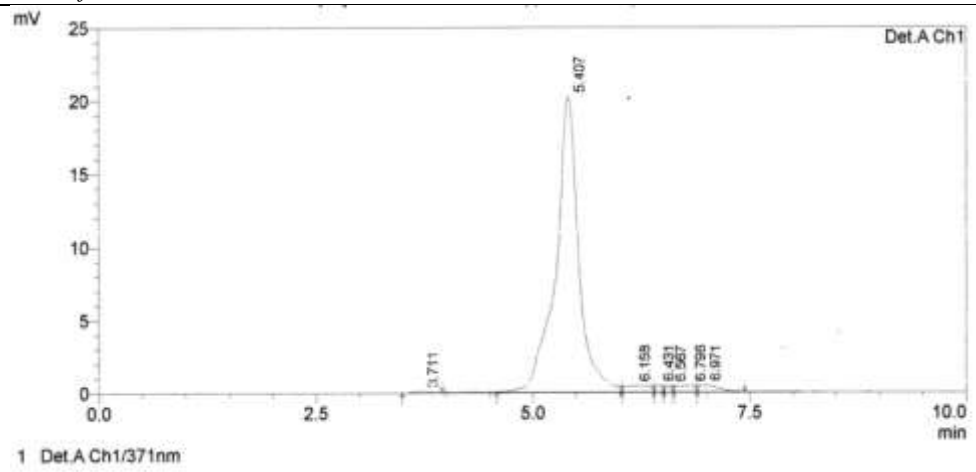


Figure 4. Chromatogram of rutin (a), myricetin (b), quercetin (c) in methanol solution by methanol:phosphoric acid 0,1% (75:25) as mobile phase at a flow rate 1 ml/minute

Table 5. The results of the method optimization acceptance measurement using a mobile phase of methanol: 0.1% phosphoric acid (70:30) at a rate of 1 ml/minute

Flavonoid	Parameter Optimasi Metode			
	Tr	R	As	N
Rutin	6,145	5,183	1,577	20443
Mirisetin	7,085	2,527	1,049	17088
Kuersetin	8,913	3,130	1,007	11432

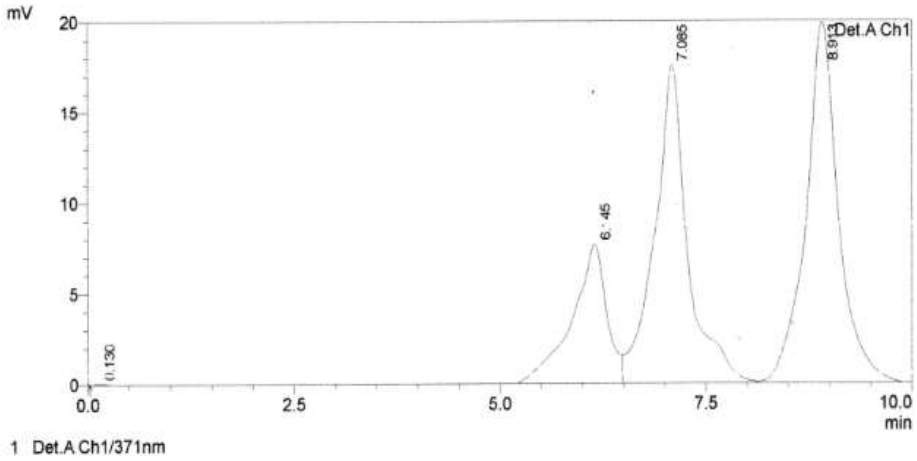


Figure 5. Chromatogram of rutin (a), myricetin (b), and quercetin (c) in methanol with a mobile phase of methanol:0.1% phosphoric acid (70:30) at a rate of 1 ml/min

DISCUSSION

Due to the cinnamoyl band exhibiting a higher absorbance than the benzoyl band, the cinnamoyl band wavelength was selected as the detection wavelength for the HPLC system. The HPLC detector was set at 371 nm, corresponding to the maximum absorption wavelength of quercetin, which lies between the absorption maxima of rutin and myricetin.

Mobile phase B yielded a faster retention time than mobile phase C while providing superior peak separation. Although mobile phase A offered faster retention times than mobile phase B, the chromatographic profile produced by mobile phase B was superior to that of both A and C. Therefore, mobile phase B was selected for further optimization.

Mobile phase B2 produced a faster retention time than B1 and better peak separation. While mobile phase B3 yielded even shorter retention times than B2, the chromatographic profile generated by B2 was superior to those of B1 and B3, as illustrated in Figures 2 to 4. Consequently, mobile phase B2 was selected to assess the acceptance parameters of the analytical method.

Increasing the proportion of methanol in the mobile phase composition improved the polarity compatibility for flavonol-type flavonoid compounds, thus accelerating retention times. This effect is primarily attributed to methanol's capacity to enhance flavonoid solubility, particularly considering that methanol was also used as the solvent for the preparation of test solutions. Moreover, including 0.1% phosphoric acid in the mobile phase composition reduced non-equilibrium mass transfer, thereby improving peak shapes by minimizing tailing or leading. However, it was necessary to determine the most optimal composition between methanol and 0.1% phosphoric acid to achieve a shorter retention time while maintaining excellent peak profiles. This optimal composition was identified as methanol:0.1% phosphoric acid in a 70:30 ratio. Accordingly, this mobile phase assessed acceptance parameters in the method optimization stage.

The assessment of method optimization acceptance parameters was conducted to evaluate compliance with column efficiency criteria, including resolution (R), peak asymmetry factor (As), number of theoretical plates (N), and retention time (Tr) yielded by the selected mobile phase. The results are the basis for recommending further steps, including system suitability testing.

During the evaluation of acceptance parameters, retention time (Tr), resolution (R), peak asymmetry factor (As), and number of theoretical plates (N) were observed following the injection of the mixed standard solution eluted with the selected mobile phase. Based on the results presented in Table 5 and the chromatographic profile in Figure 5, it was established that the mobile phase consisting of methanol and 0.1% phosphoric acid (70:30), delivered at a flow rate of 1 mL/min, successfully produced well-separated chromatographic peaks. This was evidenced by resolution values exceeding 1.5, theoretical plate counts surpassing 2000, and relatively short retention times (as referenced by Maryanto, 2021; Purnomo, 2021). Furthermore, the peak asymmetry factors for myricetin and quercetin met the acceptance criteria, approaching a value of 1. However, the peak asymmetry for rutin did not yet meet the required standard and requires further improvement. Therefore, the optimized method may proceed to system suitability testing and method validation in subsequent research stages.

CONCLUSION

The mobile phase, composed of methanol and 0.1% phosphoric acid in a 70:30 ratio, with a flow rate of 1 mL/min, was capable of producing chromatograms with satisfactory separation, as evidenced by resolution (R) and theoretical plate number (N) values that fulfilled the acceptance criteria, along with relatively short retention times. The peak asymmetry factor (As) for myricetin and quercetin also met the acceptance criteria, whereas the peak asymmetry for rutin requires further improvement.

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REFERENCES

Dimcheva, V., Kaloyanov, N., Karsheva, M., Peycheva, N.F., dan Stoilova, N. (2019). HPLC-DAD Method for Simultaneous Determination of Natural Polyphenols. Analytical and Bioanalytical Chemistry, 3(1): 039-043. DOI: http://dx.doi.org/10.17352/ojabc.000009.

Ekalu, A., dan Habila, J.D. (2020). Flavonoids: Isolation, Characterization, and Health Benefits. Beny-Suef University Journal of Basic and Applied Sciences, 9:45. DOI: https://doi.org/10.1186/s43088-020-00065-9.

Husein, S.G., Sundalian, M., Husna, N. (2021). Review: Analisis Komponen Senyawa Kimia Krokot (Portulaca oleraceae L. dan Portulaca grandiflora Hook.). Jurnal Sains dan Kesehatan, 3(2): 317-327. DOI: https://doi.org/10.25026/jsk.v3i2.278.

Kumar, S. dan Pandey, A.K. (2013). Chemistry and Biological Activities of Flavonoids: An Overview. The Scientific World Journal, Vol. 2013. DOI: http://dx.doi.org/10.1155/2013/162750.

Lee, J.S., Paje, L.A., Choi, W.H., Cho, E.J., Kim, H.Y., Jacinto, S.D., dan Lee, S. (2020). Validation of an Optimized HPLC/UV Method for The Quantification of Flavonoids in Lotus. The Korean Society for Applied Biological Chemistry, 63(84):1-6, DOI: https://doi.org/10.1186/s13765-020-00568-0.

Maryanto, C.D. (2021). Optimasi Metode KCKT untuk Analisis Kadar Rutin dalam Ekstrak Etanol Daun Binahong (Anredera cordifolia (Ten.) Steenis). Skripsi. Yogyakarta: Fakultas Farmasi Universitas Sanata Dharma.

Moghaddasian, B., Eradatmand, A.D., dan Alaghemand, A. (2013). Simultaneous Determination of Rutin and Quercetin in Different Parts of Capparis spinosa. Bulletin of Enviroment, Pharmacology and Life Sciences, 2(2): 35-38.

Nongalleima, K., Ajungla, T., dan Singh, C.B. (2017). Determination of Antioxidant Activity and Simultaneous RP-HPLC Analysis of Quercetin, Rutin, Kaempferol in Citrus macroptera Montruz. Journal of Pharmacognosy and Phytochemistry, 6(3): 474-478.

Nurholis dan Saleh, I. (2019). Hubungan Karakteristik Morfofisiologi Tanaman Kersen (Muntingia calabura). AGROVIGOR, 12(2): 47-52.

Oliveira, M.S., Costa, W.A., Bezzera, F.W.F., Araujo, M.E., Ferreira, G.C., Carhalho, R.N. (2018). Phytochemical Profile and Biological Activities of Momordica charantia L. (Cucurbitaceae): A Review, African Journal of Biotechnology, 17(26): 829-846, DOI: 10.5897/AJB2017.16374.

Panche, A.N., Diwan, A.D., dan Chandra, S.R. (2016). Flavonoids: An Overview. Journal of Nutritional Science, 5(c47):1-15, DOI: 10.1017/jns.2016.41.

Purnomo, G.A. (2021). Validasi Metode Analisis Kromatografi Cair Kinerja Tinggi Fase Terbalik pada Penetapan Kadar Rutin dalam Ekstrak Etanol Daun Binahong. Skripsi. Yogyakarta: Fakultas Farmasi Universitas Sanata Dharma.

Rifai, A.A., Aqel, A., Awaad, A., dan Alothman, Z.A. (2015). Analysis of Quercetin and Kaempferol in An Alcoholic Extract of Convolvulus pilosellifolius using HPLC. Communications in Soil Science and Plant Analysis, 46(11): 1411-1418, DOI: https://doi.org/10.1080/00103624.2015.1043454.

Shervington, L.A., Li, B.S., Shervington, A.A., Alpan, N., Patel, R., Muttakin, U., dan Mulla, E. (2018). A Comparative HPLC Analysis of Myricetin, Quercetin and Kaempferol Flavonoids Isolated from Gambian and Indian Moringa oleifera Leaves. International Journal of Chemistry, 10(4): 28-37, DOI: https://doi.org/10.5539/ijc.v10n4p28.

Snyder, L.R., Kirkland, J.J., and Dolan, J.W. Editors. (2010). Introduction to Modern Liquid Chromatography. Third edition. New Jersey: John Wiley & Sons, Inc.

Sudrajat, F.R., Mutakin, Saputri, F.A., dan Shalihat, A. 2020. Review: Analisis Sidik Jari dalam Kontrol Kualitas Tumbuhan. Farmaka, 18(2): 95-104.

Suzery, M., Ningrum, A.N., Nudin, B., Mulyani, N.S., Cahyono, B. (2019). Determination of Quercetin and Rutin in Red Galangal Rhizomes (Alpinia purpurata) and White Galangal (Alpinia galanga) with High Performance Liquid Chromatography Method. International Conference of Food Science & Technology, 292(2019): 1-9, doi:10.1088/1755-1315/292/1/012064.

Tang, D., Yang, D., Tang, A., Gao, Y., Jiang, X., Mou, J., dan Yin, J. (2010). Simultaneous Chemical Fingerprint and Quantitative Analysis of Ginkgo biloba Extract by HPLC-DAD. Analytical and Bioanalytical Chemistry, 2010(396): 3087-3095, doi: 10.1007/s00216-010-3536-8.

USP. (2021). United State Pharmacopeia 44 – NF 39, US.

Wang, L., Mei, Q., dan Wan, D. (2014). Simultaneous Determination by HPLC of Quercetin and Kaempferol in Three Sedum Medical Plants Harvested in Different Seasons. Journal of Chromatographic Science, 2014(52): 334-338, doi:10.1093/chromsci/bmt035.