Development and Validation of RP-HPLC Analysis Method for Determination of Total Alkaloid Content of Soursop (Annona muricata L.) Leaf Extract

Lindawati Setyaningrum\(^1\), Dyah Purwaningtyas\(^1\), Ayik Rosita\(^2\), Dyan Wigati\(^3\), Mohammad Rofik Usman\(^1\), Maasyitoh Sari Latifah\(^3\)

\(^1\)Faculty of Health Science, dr. Soebandari University, Jember, Indonesia
\(^2\)Faculty of Pharmacy, Jember University, Jember, Indonesia
\(^3\)Faculty of Vocation, Airlangga University, Surabaya, Indonesia

Abstract. Soursop leaves (Annona muricata L.) contain alkaloids that have pharmacological effects. This study aims to determine the total alkaloid content of soursop leaf ethanol extract using a validated HPLC method. Extraction was carried out by soxhletation followed by liquid-liquid extraction using chloroform. Optimizing HPLC conditions on the mobile phase, flow rate, concentration and wavelength, further testing the validation of the analytical method. Optimizing HPLC conditions obtained optimal results at a concentration of 500 ppm with a flow rate of 1.0 mL/min using acetonitrile: methanol: water (80:5:15) at a wavelength of 272 nm. These results have met the validation requirements of analytical methods including System Suitability Test (UKS), selectivity, linearity with \(y = 2.01914x - 219.97226\), correlation coefficient value \((r) = 0.9995\) and Vx0 value of 0.11201%. The limit of detection and limit of quantization obtained were 8,785.25 ppm and 29,284.18 ppm, respectively. The RSD percentage of the precision test is 1.0453%, and the accuracy test is obtained from the recovery of 98-102%. The result of determining the total alkaloid content of the sample was 0.0862 ± 0.004 % (%w/w) of the total alkaloid content (TALC) triple replication. The conclusion of this research is that the analytical method using the HPLC system for determining the total alkaloid content of the ethanolic extract of soursop leaves is validate.

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1. Introduction
Soursop is one of the plants developed into medicinal plants, including as antimicrobials, \cite{1}–\cite{4} and anticancer. The content of acetoginin has the ability to kill cancer cells, performed from normal cell to death cell, and showed cytotoxic activity \cite{5}–\cite{8}. According to \cite{9}, soursoup also has the ability as an antimalarial agent by antiplasmodial activity testing. In addition, soursop leaf extract potential as an anti-inflammatory in the cyclooxygenase-2 enzyme inhibition test \cite{10}. The soursop leaf aqueous extract showed an analgesic effect in white mice induced by 1% acetate \cite{11}.

Alkaloids is one of the secondary metabolites reported in family Annonaceae \cite{12}–\cite{14}. According to a study conducted by \cite{15}, qualitative and quantitative analysis reported positive results for alkaloids, and flavonoids in aqueous and ethanol extracts using the maceration method. \cite{16} reported that the ethanolic extract of soursop leaves contained alkaloids, flavonoids, terpenoids, tannins, saponins, steroids as acetylcholinesterase inhibitors. Research conducted by \cite{17}, showed that the phytochemical content of the isolated \textit{Annona muricata} L roots contained 5 alkaloids. The presence of alkaloids from soursop leaves allows researchers to analyze the total levels of these compounds and find out how much the ability of alkaloids as a drug.

For the analysis of total alkaloids, in addition to qualitative identification, it is also necessary to determine the appropriate analytical method for quantitative determination of compound content in \textit{Annona muricata} L. \cite{18}. Analytical methods include using chemometrics, HPLC, UV Spectrophotometer -Vis for determination of total alkaloid content was carried out on various plant extracts \cite{19}–\cite{25}. Therefore, an analysis of total alkaloids was developed using reverse phase HPLC on soursop leaf ethanol extract samples using caffeine standard as a comparison. This research was conducted by developing a highly sensitive and selective reverse phase HPLC analysis method on samples of soursop leaf ethanol extract for determination of total alkaloid content.

In the development of the reverse phase HPLC analysis method that has been carried out by \cite{26} that is carried out by optimizing the extractor, reconstitution solvent, mobile phase, column elution, and flow rate, then determined for analytical conditions that can produce separate chromatogram peaks between analyte and has a fairly good peak profile. In the Gabriela 2020 study, the determination of the total alkaloid content of Soursop Fruit (\textit{Annona muricata} L.) using ultrasound in extracting alkaloid compounds from other complex compounds was able to separate well. The soxhletation technique has also been developed to separate alkaloids from other metabolites.

\cite{19} reported that optimization under HPLC conditions in the analysis of alkaloids in herbal plants using acetonitrile (A) and 0.1% triethylamine as the mobile phase in gradient as 30% A (0 – 21 min), 30% A – 45% A (21–30 min), 45% A–55% A (30–55 min) at a flow rate of 1.0 mL/min. In Guo's research, 2016 reported an analysis of the isoquinoline alkaloid group using GCMS under conditions of the organic phase was kerosene with rubber emulsifier (1:15.25, v:v), acetic acid (36%) as carrier. The strip phase was 0.1 M of sulfuric acid aqueous solution and the volume ratio of strip phase to organic phase was 3/7. In addition, the study \cite{27} also explained that optimization was carried out on the mobile phase and variations in the mobile phase to produce analyte separation from other compounds using HPLC. From the selected conditions above, validation of the method is then carried out in order to obtain a validity based on the appropriate parameters.

This research will determine the total alkaloid content of soursop leaf extract by selecting the extraction technique using soxhletation and analysis using HPLC. Research of \cite{28} was developed and validated the TLC-Densitometry analysis method for the standardization of soursop leaf extract.
with quercetin which yielded optimum results for testing. According to [29] the selected analytical method is then validated first to ensure that the developed method is considered valid for routine testing if it meets the parameters of linearity, precision, accuracy, detection limit and quantitation limit. The application of the reverse phase HPLC analysis method was carried out to determine the total alkaloid content in the ethanol extract of soursop leaves. By knowing the total alkaloid content of soursop leaf extract this can be used as a reference to developed as a candidate for medicinal plants according to their therapeutic activity.

2. Experimental Section

![Diagram of experimental design]

**Figure 1.** Design Eksperimental

2.1 Tools
High Performance Liquid Chromatography (Agilent 1220 Infinity) isocratic LC system, Poroshell column 120 EC-C18 with diameter 4.6 µm, 150 nm with particle size 4 m PDA Detector (Agilent), Uv-Vis Spectrophotometer (Shimadzu), soxhletation apparatus (duran), Analytical balance (Ohaus), 0.45 m nylon filter membrane (whatman), separating funnel, 0.22 m Microsyringe, ultrasonic cleaner, and glassware
2.2 Materials
Caffeine standard (pa), sample of soursop leaf ethanol extract, ethanol 96% (Brataco), methanol pa (Merck), methanol pro HPLC (Merck), aqua bidestillata, NaOH pa (Merck), acetonitrile pro HPLC (Merck), syringe 3 cc, chloroform pa (Merck), HCl pa (Merck), Na₂HPO₄ (Merck), citric acid (Merck).

2.3 Extract Preparation
Soursop leaf powder (20 grams) wrapped in filter paper, placed into a soxhlet then in a round bottom flask was added 150 mL of ethanol. The extractor is started and stopped when the solvent color returns to its original state. The filtrate was concentrated using a water bath at a temperature of 50°C. The resulting concentrated extract was calculated the yield.

2.4 Sample Preparation
Soursop leaf ethanol extract (50.0 mg) dissolved with 2 N hydrochloric acid (HCl) then filtered. Furthermore, washing three times using chloroform, where the water phase is collected and added NaOH until the pH is neutral. The test solution was taken and added with 5 mL of phosphate buffer pH 4.7. The mixture was shaken with a separating funnel and extracted with 5 mL of chloroform. The chloroform phase was selected, then evaporated with nitrogen gas and reconstituted with methanol: aqua bidestillata (50:50) to 10.0 ml, then filtered and injected into the HPLC system. The sample solution was replicated three times [30].

2.5 Mobile Phase Preparation
In the development of the mobile phase is done by mixing acetonitrile: methanol: aquabidest (80: 5: 15). The mixture was sonicated for 10 minutes and filtered using a vacuum filter [31].

2.6 Solvent Preparation
The solvent in this study was carried out by mixing methanol and water (50:50), the mixture was sonicated for 10 minutes [32].

2.7 Preparation of Phosphate Buffer pH 4.7
A 0.2 M sodium phosphate (Na₂HPO₄) solution was made mixed with 0.2 M citric acid (C₆H₈O₇) then adjusted to pH 4.7 [30].

2.8 Preparation of Caffeine Standard Solution
Caffeine stock solution with a concentration of 2000 g/mL was prepared by weighing 100.0 mg in a 50.0 mL volumetric flask with solvent added and homogenized. The intermediate solution was made by pipetting 5.0 mL of stock solution, put into a 10.0 mL volumetric flask, added solvent to the mark and homogenized [32].

2.9 Development of HPLC System Conditions
a. Development of Flow Rates
Development of flow rates was operated with optimization of speed variations from 1 to 1.4 mL per minute was carried out to optimize the analyte separation conditions. By decreasing retention time is an increase in flow velocity from the system until a good peak is obtained[33].

b. Determination of Concentration and Wavelength
Determination of the optimal caffeine concentration varies from 100.0 to 500.0 ppm and then measured at a wavelength of 200-800 nm using a UV-Vis spectrophotometer [32].

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c. **Determination of Mobile Phase**

Determination of the optimal mobile phase by comparing the best peak profile using mobile phase optimization, namely Acetonitrile: methanol: aqua bidestillata (80:5:15), Methanol: aqua bidestillata (50:50), Methanol: aqua bidestillata: acetate acetate 2% (50 :48:2) [34].

### 2.10 Validation of Analytical Method

At the pre-validation stage, a System Conformity Test was carried out on HPLC. This test was carried out with six injections of the system which had been adjusted for the optimum analysis conditions. The system that shows the retention time and repeated area to meet the acceptance value requirements, namely RSD < 2%, is a confirmation of the selected condition [35].

The next stage is the validation of the analytical method by testing the following parameters:

- **Selectivity/specificity** is carried out to see the ability to recognize analyte responses and be able to distinguish analytes from other compounds, namely by testing purity and seeing the resolution results obtained from injection results [36].
- **Determination of linearity** is seen if there is a linear relationship from the regression line in the concentration range of 50-120% caffeine standard solution with nine points (USP, 2016) with the correlation coefficient parameter (r). The residual standard deviation (Sy) is also taken into account.
- **Limit of detection (LOD)** and **limit of quantitation (LOQ)** are determined by making a standard curve made with the smallest six points of the concentration that gives a signal to the detector from the caffeine standard solution so that the residual standard deviation and slope values of the calibration curve results are obtained, then plugged into the equation:

\[
LOD = 3 \times Sy / b \quad \text{(Equation 1.1)}
\]
\[
LOQ = 10 \times Sy / b \quad \text{(Equation 1.2)}
\]

(Sy is the standard deviation of the residual and b slope)

Precision is the repetition of six replications with a concentration level range between 80%, 100%, and 120% of the target concentration and then the RSD value < 2% is calculated, which indicates the closeness of the test results in each repetition at the concentration variation. The closeness of the analyte content to the actual concentration can be determined based on the accuracy parameter. The accuracy value was obtained by preparing the analyte concentration in the concentrated extract sample, namely 0.01% plus caffeine with various concentrations of 80%, 100%, and 120% and then analyzed (addition method). The three addition samples were replicated three times. The recovery parameters from the test data should not exceed the range of 85-110% [37].

### 3. Results and Discussion

The extraction process carried out by soxhletation has the advantage that the extraction time is relatively fast and the solvent used is small. Soxhlet also requires an optimum temperature to attract the alkaloids, so that they are able to bind the alkaloids to the appropriate solvent during the extraction process. The yield of the extract was 5.96%.

#### 3.1 Optimization of HPLC Conditions

Optimization was carried out on HPLC instruments to obtain the selected optimum conditions, including the development of flow rates. At a flow rate of 1.0 mL/minute, a shorter retention time and peak symmetry were obtained than the flow rates of 1.2 and 1.4 mL/minute. This is due to an increase in pressure from the pump so that the analyte can be retained longer in the column, the increase in pressure is caused by the large load carried by the mobile phase in the HPLC system. The choice of a flow rate of 1.0 mL/minute can be seen from the results of the analysis at a value of N > 2000. Other data are described in table 1 [38].
Table 1. The Results of The Development of Flow Velocity in HPLC

| No. | Water Speed | Symmetry | N > 2000 | Rt (menit) |
|-----|-------------|----------|----------|------------|
| 1   | 1.0         | 0.81     | 3244     | 1.4        |
| 2   | 1.2         | 0.36     | 1006     | 1.9        |
| 3   | 1.4         | 0.24     | 673      | 3.8        |

To determine the optimum wavelength, measurements were made at the maximum absorption of the caffeine standard (Denis, 2018). The measurement results show the highest absorption of caffeine at a wavelength of 272 nm, measurements were made using a UV-Vis spectrophotometer and analyzed and selected the maximum absorbance results which were still in the absorbance range of 0.2-0.8 [39]. From the test results, there is a 1 nm wavelength shift, but this result is still accepted because according to [40], it is stated that the maximum wavelength absorption shift should not be more than 3 nm.

Figure 2. The Wavelength Spectrum of the Caffeine Standard
(A) 500 ppm, (B) 300 ppm, (C) 100 ppm

The next optimization is in the form of a mobile phase. The research was conducted by injecting 500 ppm caffeine standard in variations in the composition of the mobile phase, including methanol : aqua bidestillata (50 : 50). The chromatogram peak results were not good because tailings occurred even though the retention time was < 10 minutes (Figure 2A). The next mobile phase is methanol : aqua bidestillata : acetic acid 2% (50 : 48 : 2) resulting in a retention time of 2.00 minutes but widening of the chromatogram peaks (Figure 2B). The third mobile phase was acetonitrile : methanol : aqua bidestillata (80 : 5 : 15) and a sharp chromatogram peak was obtained with a faster retention time of 1.49 minutes (Figure 2C).
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3.1 Development and Validation of RP-HPLC Analysis Method

The results of the system suitability test that have been carried out by injecting six times and looking at the repeatability parameters are each for retention time producing an RSD value of 0.253% and area area yielding an RSD of 0.3%. The results of the repeatability parameters of retention time and area area meet the acceptance requirements, namely RSD < 2% [35].

Selectivity is the ability to distinguish a number of compounds from one another or to separate the analyte from other compounds such as degradation products, metabolites and impurities accurately. The selectivity was determined by injecting the caffeine standard (figure 3a) measured by the HPLC system, the soursop leaf ethanol extract sample (figure 3b) was measured by the HPLC system, the caffeine standard plus other analytes (figure 3c) was measured by the HPLC system, and the sample of the leaf ethanol extract. soursop plus other analytes (3d image) was measured by HPLC system. Then the results of the chromatogram profile were seen for the resolution value (Rs) 1.5 [37]. The results of the selectivity determination are shown in Table 3.

| No. | Test Name                        | Rs  |
|-----|----------------------------------|-----|
| 1   | Standard without Distraction     | -   |
| 2   | Sample Without Distraction       | -   |
| 3   | Standard with other value        | 3.54|
| 4   | Sample With other value          | 2.87|

From the results above, it shows that the selectivity test that has been carried out has met the acceptance requirements with the resolution value of caffeine in the soursop leaf ethanol extract sample and the standard meets the resolution criteria of 1.5 so it is said that the analytical method used is able to separate caffeine from other (selective) analytes [7]. The results above shows with match factor value > 990.
The linear regression equation from the linearity test is $y = 219.97226x - 2.01914$. The correlation coefficient ($r$) > 0.9995 and the value of $Vx0$ is less than 5%, so it can be concluded that the developed method shows a linear relationship based on the required parameters [36].

LOD and LOQ tests showed that the values were 8.78525 ppm and 29.28418 ppm, respectively, using the calibration curve method. Meanwhile, in the precision test by adopting the determination of accuracy with the standard addition of caffeine with concentrations of 80%, 100%, and 120% in the test sample and then replicated six times, the coefficient of variation obtained is 1.0453%, which is less than 2% and meet the acceptance requirements of the precision parameter [37].
The last test on the validation of the analytical method was the accuracy determined by the standard addition of caffeine in various concentrations. The percent recurrence value is calculated based on the formula below, and is listed in table 4.

\[
\% \text{Recovery} = \frac{CF - CA}{C \times A} \times 100\%
\]

CF : pen sample concentration  
CA : actual sample concentration  
C*A : concentration of added analyte

| Concentration | Replication | CA         | C*A        | CF         | % Recovery |
|---------------|-------------|------------|------------|------------|------------|
| 80%           | 1           | 500 ppm    | 401,06 ppm | 895,29 ppm | 98,56%     |
|               | 2           | 500 ppm    | 405,03 ppm | 857,16 ppm | 88,18%     |
|               | 3           | 500 ppm    | 403,02 ppm | 873,09 ppm | 92,57%     |
| 100%          | 1           | 500 ppm    | 502,08 ppm | 954,57 ppm | 90,54%     |
|               | 2           | 500 ppm    | 508,02 ppm | 976,45 ppm | 93,79%     |
|               | 3           | 500 ppm    | 510,04 ppm | 982,09 ppm | 94,52%     |
| 120%          | 1           | 500 ppm    | 600,18 ppm | 1020,15 ppm | 86,67%     |
|               | 2           | 500 ppm    | 600,09 ppm | 1080,03 ppm | 96,66%     |
|               | 3           | 500 ppm    | 600,11 ppm | 1057,19 ppm | 92,85%     |

From the table 4, the average recovery (%) meets the specified range, which is 85-110% for the analyte in the sample with a concentration of 0.01% [37]. Each analytical method development requires further validation, to ensure that the method to be applied meets the validation requirements in evaluating the quality assurance of a product. The application of validation of the HPLC analysis method for alkaloids in various samples was also carried out in research conducted by [41] and [42]. The validation results above meet the validation parameters according to research conducted [43], [44] on the validation of the method of determining caffeine content in coffee grounds. using HPLC get results that meet the criteria for validation parameters including selectivity, specificity, linearity, accuracy, precision, LOD and LOQ.

### 3.3 Determination of Caffeine Content using the HPLC Method

Determination of total alkaloid content in samples of soursop leaf ethanol extract was determined when the validation results of the analytical method met the acceptance of each parameter. Determination of total alkaloid content was carried out under selected conditions from reversed phase HPLC using acetonitrile: methanol: aqua bidestillata (80:5:15), porosshell column 120 EC C-18 as stationary phase, flow rate 1.0 mL/min, Photodiode Array Detector (PDA) at 272 nm as detector. The results of the determination of total alkaloids were calculated using a calibration curve with the equation from the figure below.
The results of the calculation using the equation $y = 0.75852x + 106.19513$ with $r$ close to 1, namely the obtained levels of $0.0862 \pm 0.004 \% \ (w/w)$ of 100% standard caffeine with three replications. In this study, caffeine was used as the standard because it was known from previous research conducted by [45] that caffeine is one of the alkaloid groups with the chemical formula C8H10NO2. The presence of alkaloids in the ethanol extract of the soursop cycle shows that it has potential as a traditional medicinal plant that can be developed. According to [25], that alkaloids have C-N main bonds which are divided into three types, namely pseudoalkaloids, true alkaloids and protoalkaloids. Included in the pseudoalkaloids are xanthines (theophylline, caffeine and theobromine). The true alkaloids are found in the form of N-oxides, salts, or free including cocaine, morphine, nicotine and quinine. While those included in the protoalkaloids or simple alkaloids are mescaline, ephedrine and hordenine.

4. Conclusion
This reverse phase high performance liquid chromatography analysis method was developed for the determination of total alkaloid content in soursop leaf ethanol extract using a mobile phase contain acetonitrile: methanol: aqua bidestillata (80:5:15), poroshell column 120 EC C-18 as the stationary phase, the flow rate is 1.0 mL/min. Photodiode Array Detector (PAD) at a wavelength of 272 nm as a detector and an optimum concentration of 500 ppm. The selected conditions are then validated by analytical methods and it can be concluded that these conditions are in accordance with the acceptance requirements including System Conformity Test (UKS), selectivity, linearity, detection limit (LOD), quantization limit (LOQ), precision, and accuracy and can be used to determine levels compound in the sample. The total alkaloids in the ethanolic extract of soursop leaves was $0.0862 \pm 0.004 \% \ (w/w)$ of the total alkaloid content (TALC).

5. Acknowledgement
Acknowledgement to dr. Soebandi University for the grant funds, all research team who have collaborated during this research. Thank you to researchers from the Faculty of Pharmacy, University of Jember for their contribution to the development of this research.

References
[1] M. K. Simo, M. D. Nguepi, M. L. Sameza, R. K. Toghueo, F. B. Fekam, and G. Froldi. (2018) Cameroonian medicinal plants belonging to Annonaceae family: radical scavenging and antifungal activities. *Natural Product Research*, vol. 32, no. 17, pp. 2092–2095.
[2] T. Abdel-Rahman, A. S. Hussein, S. Beshir, A. R. Hamed, E. Ali, and S. S. El-Tanany. (2019). Antimicrobial activity of terpenoids extracted from *Annona muricata* seeds and its endophytic

http://www.eksakta.ppj.unp.ac.id/index.php/eksakta
Development and Validation of RP-HPLC Analysis Method for Determination of Total Alkaloid Content of Soursop (Annona muricata L.) Leaf Extract
[19] C. Z. Lin, S. M. Xie, C. C. Zhu, Z. D. Bairu, S. Q. Kangsa, and D. Zhu. (2013). Simultaneous determination of five diterpenoid alkaloids in Herba Delphinii by HPLC/ELSD. _Journal of Pharmaceutical Analysis_, vol. 3, no. 6, pp. 447–451.

[20] I. Ivanov, S. Berkov, and A. Pavlov. (2009). Improved hplc metod for the determination of amaryllidaceae alkaloids. _Biotechnology and Biotechnological Equipment_, vol. 23, pp. 809–813

[21] L. Li, W. Long, X. Wan, Q. Ding, F. Zhang, and D. Wan. (2015). Studies on quantitative determination of total alkaloids and berberine in five origins of crude medicine ‘sankezhen’. _Journal of Chromatographic Science_, vol. 53, no. 2, pp. 307–311

[22] N. Aini. (2016). Penentuan kadar alkaloid pada ekstrak daun tanaman menggunakan metode nir dan kemometrik. _Skripsi_. Universitas Jember, Jember, Jawa Timur

[23] J. Xiao et al., (2019). High performance liquid chromatography determination and optimization of the extraction process for the total alkaloids from traditional herb stephania cepharantha hayata. _Molecules_, vol. 24, no. 3

[24] S. Ahmed, A. Kadhim Alanbari, and H. R. R. Al-Taai. (2019). HPLC profiles of some bioactive compounds in _Annona muricata_ L. and Garcinia mangostana L. peels. _International Conference on Food, Nutrition and Agriculture (ICFNA19)_ Istanbul, Turkey.

[25] S. Wahyu and P. Marpaung. (2020). Determination of Total Alkaloid Levels Extracts of Akar Kuning (Fibraurea chloroleuca Miers) Based on The Differences of Ethanol Concentrations by Spectrofotometry UV-Vis Method. _Dalton: Jurnal Pendidikan Kimia dan Ilmu Kimia_, Volume 3 Nomor 2.

[26] R. Ayuningtyas, R. Primaharinastiti, M. Yuwono. (2020). Optimasi dan Validasi Metode KCKT untuk Identifikasi dan Penetapan Kadar Metabolit Nitrofuran dalam Bakso Udang the HPLC Methods for Identificaton and Determination of Nitrofuran Metabolite Levels in Shrimps Meatballs. _Journal Farmasi Dan Ilmu Kefarmasian Indonesia_, vol. 8, no. 2, p. 117

[27] D. Wulandaria, G. Gusrizala, and T. A. Zaharaha. (2020). Optimasi dan Validasi Metode Penentuan Kadar Asam Glikolat dan Asam Laktat Dalam Krim Menggunakan Kromatografi Cair Kinerja Tinggi, _ALCHEMY Jurnal Penelitian Kimia_, vol. 16, no. 1, pp. 10–24

[28] S. Sudjarwo, F. -Annuryanti, M. Nur Alfi, and F. Annuryanti. (2019). Validation and Development of Tlc-Densitometry Method For Standardization Of Soursop Leaf Extract (Annona Muricata Linn.) With Quercetin The Effectiveness And Toxicity Of Glucomannan In Forang And Konjac Powder For Niddm In Mus Musculus View Project Validation And Development Of Tlc-Densitometry Method For Standardization Of Soursop Leaf Extract (Annona Muricata Linn.) With Quercetin. _International Journal of Pharmaceutical Sciences and Research_, vol. 10, no. 2, p. 686

[29] N. S. D. Brahmana. (2020). Kemampuan Daya Hambat Beberapa Produk Sabun Cair Antibakteri Terhadap Bakteri _Staphylococcus aureus_. _Skripsi_. Universitas Medan Area. Medan

[30] R. K. Patel, J. B. Patel, and P. D. Trivedi. (2015). Spectrophotometric method for the estimation of total alkaloids in the _Tinospora cordifolia_ M. and its herbal formulations. _Int J Pharm Pharm Sci_, vol. 7, no. 10, pp. 249–251

[31] P. N. Patil (2012). Caffeine in various samples and their analysis with HPLC-A review. _International Journal of Pharmaceutical Sciences Review and Research_ 16(2):76-83.

[32] S. Chowdhury, M. Maleque, and M. H. Shihan. (2012). Development and Validation of a Simple RP-HPLC Method for Determination of Caffeine in Pharmaceutical Dosage Forms. _Asian Journal of Pharmaceutical Analysis_, vol. 2, pp. 1–4

[33] A. A. Rahim, S. Nofrizal, and B. Saad. (2014). Rapid tea catechins and caffeine determination by HPLC using microwave-assisted extraction and silica monolithic column. _Food Chemistry_, vol. 147, pp. 262–268.
Development and Validation of RP-HPLC Analysis Method for Determination of Total Alkaloid Content of Soursop (Annona muricata L.) Leaf Extract

[34] T. S. Ningtyas and I. Fajriati. (2020). Analisis Pemanis buatan Natrium Siklamat pada minuman ringan dengan Kromatografi cair kinerja tinggi. Indonesian Journal of Halal Science, vol. 1, no. 1, pp. 30–35
[35] I. G. Gandjar and A. Rohman. (2007). Kimia farmasi analisis. Pustaka Pelajar, Yogyakarta
[36] M. Yuwono and G. Indrayanto. (2005). Validation of chromatographic methods of analysis. Profiles of drug substances, excipients and related methodology, vol. 32, pp. 243–259.
[37] Association of Official Analytical Chemists. (2013). AOAC Appendix K: Guidelines for Dietary Supplements and Botanicals. pp. 1–32
[38] A.C. Moffat M.D. Osselton B. Widdop J. Watts. (2011). Review of: Clarke’s Analysis of Drugs and Poisons, 4th edn. Journal of Analytical Toxicology, Volume 36, Issue 5
[39] N. H. Fajriana and I. Fajriati. (2018). Analisis Kadar Kafein Kopi Arabika (Coffea arabica L.) pada Variasi Temperatur Sangrai secara Spektrofotometri Ultra Violet. Analit: Analytical and Environmental Chemistry, vol. 3, no. 2
[40] L. R. Snyder, J. J. Kirkland, and J. W. Dolan. (2011). Introduction to modern liquid chromatography. John Wiley & Sons
[41] L. Dhooghe et al. (2008). Development and validation of an HPLC-method for the determination of alkaloids in the stem bark extract of Nauclea pobeguinii, Talanta, vol. 76, no. 2, pp. 462–468
[42] M. Cao et al. (2020). HPLC-based qualitative and quantitative analyses of alkaloids in chewable areca products from different geographic regions. J AOAC Int, vol. 103, no. 5, pp. 1400–1405
[43] P. Naveen, H. Lingaraju, M. Deepak, B. Medhini, and K. Prasad. (2018). Method Development and Validation for the Determination of Caffeine: An Alkaloid from Coffea arabica by High-performance Liquid Chromatography Method. Pharmacognosy Research, vol. 10, no. 1, pp. 88–91
[44] R. Saad and H. Bustan. (2016). Validation and Determination of Caffeine Contents in Energy Drinks Available on the Iraqi Market by Using High Performance liquid chromatography (HPLC). International Journal of Pharmacy & Pharmaceutical Research. Vol 6, issue 1
[45] D. Sylvi, F. Azima, and S. Anggini. (2021). The Effect of Additional Fruit Eggplant (Solanum betaceum cav.) Juice on the Characteristics of Black Tea (Camelia sinensis) Beverage. EKSAKTA :Berkala Ilmiah Bidang MIPA. vol. 22, pp. 270–283