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Development and validation of a reliable reverse-phase high-performance liquid chromatography method for quantifying triterpenes in *Centella asiatica*: A step towards quality control of herbal products

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ABSTRACT

Centella asiatica (C. asiatica), a medicinal plant with diverse pharmacological properties, contains triterpenes with pharmaceutical activity, including madecassoside (MC), asiaticoside (AC), madecassic acid (MA), and asiatic acid (AA). However, the current assay for these triterpenes in the United States Pharmacopeia 36–National Formulary 31 could be enhanced with improved compound separation and shorter analysis times. A new reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated, which utilized a C18 column with low-pressure gradient elution and a rapidly altered mobile phase ratio (acetonitrile and 0.3% v/v phosphoric acid in water). This method offered significantly reduced analysis times and suitable peak shapes for all triterpenes. The retention times were 11.49, 11.84, 14.67, and 15.74 min for MC, AC, MA, and AA, respectively. The method displayed linearity ($R^2 > 0.9907$) across a 0.01–0.25 mg/mL range, and its accuracy was confirmed by spiked sample recoveries of 90.60%–111.50%. The repeatability and intermediate precision were outstanding, with percentage of relative standard deviations lower than 1.31% for all triterpenes. This validated RP-HPLC method offers an accurate and time-saving alternative for analyzing *C. asiatica* triterpenes, providing a practical solution for the pharma-ceutical and herbal product industries.

Keywords: *Centella asiatica*; reverse-phase liquid chromatography; method validation

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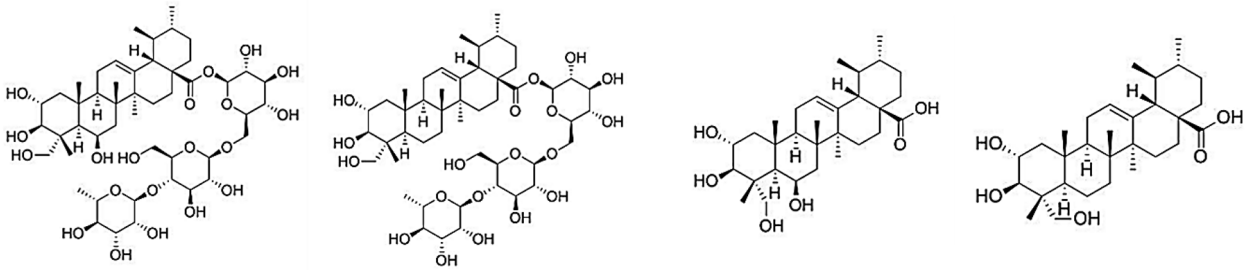
1. INTRODUCTION

Disease is a problem in public health and can cause pain, *Centella asiatica* (L.) has significance in the traditional herbal pharmacopeias of various Asian cultures, boasting a rich history of medicinal use spanning centuries. Triterpenes from *C. asiatica* are key bioactive constituents responsible for many of the observed pharmacological effects of the plant. These triterpenes possess potent anti-inflammatory effects, which are mediated through the modulation of various inflammatory pathways and cytokine production. Additionally, *C. asiatica* triterpenes exert effects against oxidative stress, scavenging free radicals and protecting against oxidative damage in vitro and in vivo. Moreover, research has demonstrated the anti-apoptotic effects of *C. asiatica*, with studies highlighting its roles in preserving cellular integrity and promoting tissue regeneration. Furthermore, emerging evidence suggests that *C. asiatica* can exert beneficial effects on mitochondrial function, enhancing cellular energy production and metabolic homeostasis.

C. asiatica has abundant levels of bioactive triterpenes, including madecassoside (MC), asiaticoside (AC), madecassic acid (MA), and asiatic acid (AA) as critical constituents (Bansal et al., 2024; He et al., 2023; Monton et al., 2018; Suksaeree et al., 2022). Figure 1 illustrates the complex structural intricacies of these compounds, highlighting the structural similarities between MC and AC and between MA and AA. Specifically, these compounds differ in the position of their hydroxyl (-OH) groups at C6, as well as the presence (or absence) of a glycoside molecule at C28, leading to distinct chemical and biological properties.

The bioactive compounds in *C. asiatica* are primarily triterpenoid saponins. These are complex molecules where aglycones (the non-sugar core) is linked to sugar chain units. Because of their intricate structural nature, it can be challenging to separate and quantify these compounds, as they are usually found as mixtures of closely related forms with similar polarities within plant extracts (Wang et al., 2020). As such, robust methodologies are needed to effectively separate and quantify the diverse pharmacological activities and toxicity profiles of triterpenes isolated from *C. asiatica* (Rachpirom et al., 2023). Reverse-phase high-performance liquid chromatography (RP-HPLC), leveraging C18 as the stationary phase together with UV detection, has emerged as the gold standard for triterpene analysis in plant extracts (Kuo et al., 2020; Lü et al., 2020; Yuan et al., 2020). Although RP-HPLC techniques have been widely adopted, previous methodologies, such as the United States Pharmacopeia 36–National Formulary 31 (USP36-NF31) assay (USP, 2012), are limited in their ability to effectively distinguish between structurally similar triterpenes. This challenge is exacerbated by the highly polar nature of MC and AC. The structural and polar differences between aglycones are difficult to discern because of the presence of highly polar sugar chain units (Masi et al., 2022; Wang et al., 2020).

In response to this gap in analytical methodologies, our study addressed the intricacies of triterpene separation and quantification. Specifically, we explored RP-HPLC-based separation techniques targeting MC, AC, MA, and AA. Through systematic investigations of the effects of gradient elution and the mobile phase composition on the separation of these triterpenes, we aimed to elucidate the optimal conditions for their distinct identification and quantification. Furthermore, recognizing the practical implications of our research within the broader context of herbal medicine and pharmaceutical product development, we developed a tailored RP-HPLC method optimized for the precise quantification of MC, AC, MA, and AA in *C. asiatica* products. Finally, we undertook rigorous method validation to ensure the reliability and accuracy of our findings. Method validation is a crucial step in analytical research that verifies a method's suitability for its intended purpose. In this study, the RP-HPLC method for the quantification of triterpenes in *C. asiatica* products was validated according to protocols assessing parameters such as specificity, linearity, range, accuracy, and precision. By adhering to internationally recognized guidelines and standards, such as those from the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) (ICH Guideline, 2022), our validated methodology provides a solid foundation for the precise and reproducible analysis of these bioactive compounds. This commitment to method validation enhances the credibility of our research and reinforces its utility in supporting quality control and standardization efforts within the herbal medicine industry.



Madecassoside (MC) Asiaticoside (AC) Madecassic acid (MA) Asiatic acid (AA)

Figure 1. Chemical structures of MC, AC, MA, and AA

2. MATERIALS AND METHODS

2.1 Materials

Standard MC (CAS: 464-92-6, purity ≥ 98%), standard AC (CAS: 16830-15-2, purity ≥ 98%), standard MA (CAS: 18449-41-7, purity ≥ 98%), and standard AA (CAS: 3450-22-2, purity ≥ 98%) were purchased from Wuhan ChemFaces Biochemical Co., Ltd. (Hubei, China). Ortho-phosphoric acid was purchased from QRëC (Chonburi, Thailand). Acetonitrile was purchased from RCI Labscan Co., Ltd. (Bangkok, Thailand). All other chemicals and reagents were of analytical grade.

2.2 Instrumentation

Chromatographic analysis was performed on an HPLC system (DGU-20A5R, Shimadzu, Kyoto, Japan) equipped with a quaternary gradient pump, an online degasser, an autosampler, and a temperature-controlled column. Chromatographic separation and detection were achieved with a photodiode array detector, which permitted the simul-taneous measurement of multiple wavelengths and provided spectral data for peak identification. Data were acquired and processed, using LabSolutions software.

2.3 Chromatographic conditions

The performance of the developed RP-HPLC method was benchmarked against the official USP36-NF31 assay for quantitative determination of four triterpenes (MC, AC, MA, and AA) in *C. asiatica* extract. Both methods utilized a symmetry C18 column (25 cm × 4.6 mm, 5 µm particle size, ACE 5 C18, ACE, Reading, UK) as the stationary phase. The column was maintained at 25°C in a temperature-controlled oven. The chromatographic conditions were the same for both the USP36-NF31 assay and the developed method. The mobile phase consisted of acetonitrile and 0.3% (v/v) phosphoric acid in deionized water, pumped at a flow rate of 1 mL/min. A low-pressure gradient elution was used for both methods, with the specific gradient programs detailed in Table 1 (USP36-NF31) and Table 2 (developed method). The injection volume was 20 µL, and detection was conducted at a wavelength of 205 nm to optimize the simultaneous determination of MC, AC, MA, and AA.

Table 1. Low-pressure gradient elution protocol for RP-HPLC in the USP36-NF31 assay

Time (min)	Acetonitrile (%)	0.3% (v/v) phosphoric acid in deionized water (%)
0.00	33	78
65.00	55	45
66.00	95	5
75.00	95	5
76.00	22	78
85.00	22	78

Table 2. Low-pressure gradient elution protocol for RP-HPLC in the developed method

Time (min)	Acetonitrile (%)	0.3% v/v phosphoric acid in DI water (%)
0.00	30	70
3.00	30	70
11.00	70	30
25.00	70	30
27.00	30	70
32.00	30	70

2.4 Standard stock solution preparation

Individual stock solutions of standard MC, AC, MA, and AA (1 mg/mL) were prepared by dissolving 10 mg of each standard powders in 10-mL volumetric flasks and diluting to volume with methanol.

2.5 Standard solution preparation

Calibration standard of MC, AC, MA, and AA, ranging from 0.01 to 0.25 mg/mL, were prepared by diluting the stock solutions with methanol. Before injection, each standard solution was filtered through a 0.45-µm nylon filter to remove any particulate matter and ensure sample clarity.

2.6 Spiked standard solution preparation

Standard solutions (2 mL each) of MC, AC, MA, and AA were prepared with concentrations ranging from 0.01 to 0.25 mg/mL. Subsequently, a 20-µL aliquot of a 0.25 mg/mL standard solution was spiked into the each of these solutions, giving a total volume of 2.02 mL. Before injection, the resulting spiked standard solutions were filtered through a 0.45-µm nylon filter to remove any particulate matter and ensure sample clarity.

2.7 Sample solution preparation

C. asiatica dry powder was dissolved in methanol (5 mg/mL) and sonicated for 10 min to ensure proper dissolution. Prior to injection, the resulting sample solution was filtered through a 0.45-µm nylon filter to remove any particulate matter and ensure sample clarity.

3. RESULTS AND DISCUSSION

3.1 Chromatographic development

The peaks corresponding to triterpenes were not adequately resolved when analyzed using the official USP36-NF31 assays for *C. asiatica*, (Figure 2a). This lack of resolution could be attributed to the slow gradient ramp rate of the mobile phase (Figure 2b), resulting in insufficient separation of structurally similar compounds like MC and AC, as well as MA and AA. Consequently, this condition proved unsuitable for the precise quantification of triterpenes because the selected mobile phase composition and gradient elution profile were not optimized to effectively resolve the triterpenes in the sample matrix. Additionally,

the chromatographic conditions might have needed to adequately account for factors such as column selectivity and temperature, which are crucial for optimizing resolution in complex mixtures. Therefore, the accurate quantification of triterpenes was hindered by compromised separation and the resulting peak overlap.

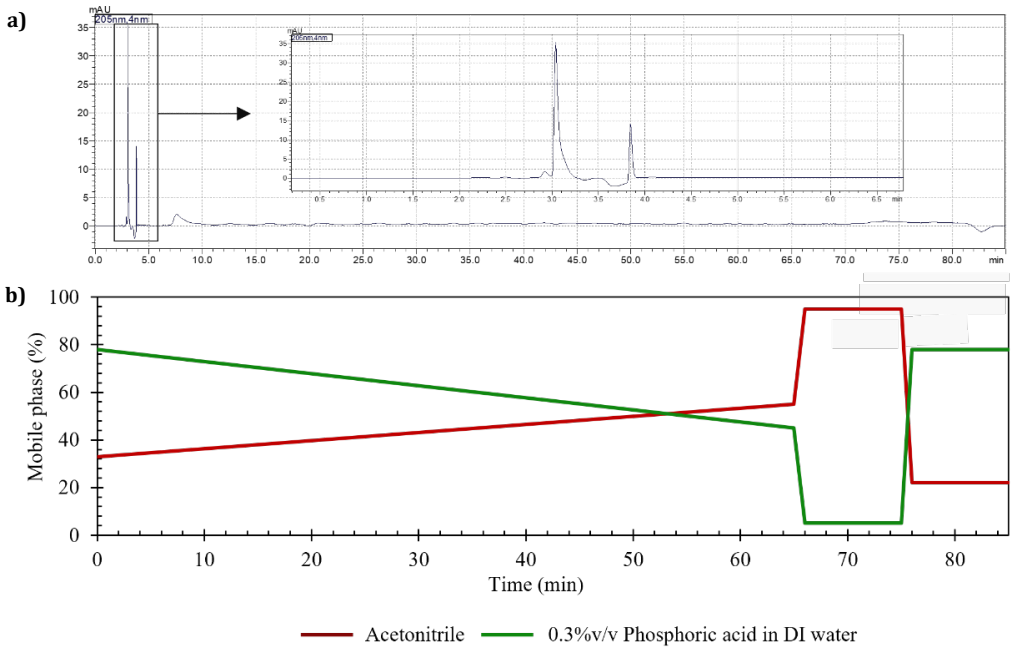


Figure 2. (a) Chromatogram of the standard mixture solution, and (b) low-pressure gradient elution protocol for official USP36-NF31 assays

To address this issue, the chromatogram illustrated in Figure 3a was developed under optimized low-pressure gradient elution conditions (Figure 3b) for quantitative analysis and specificity assessment. To achieve sufficient separation of MC, AC, MA, and AA, the RP-HPLC conditions were optimized by testing various low-pressure gradient elution profiles using a mobile phase composed of acetonitrile and 0.3% (v/v) phosphoric acid in deionized water, while ensuring adequate column retention. Parameters such as the total number of peaks, the resolution between the analytes, and the overall analysis time were assessed to determine the optimal conditions for the analysis. A consistent flow rate of 1 mL/min, a detection wavelength of 205 nm, and a column temperature of 25°C were maintained throughout the optimization process. The gradient was initiated with a 3-min isocratic hold at a high aqueous composition to facilitate elution of the water-soluble constituents. Subsequently, the proportion of acetonitrile gradually increased over the next 11 min to resolve MA and AA while ensuring their retention on the column. This adjustment in the mobile phase composition was meticulously calibrated to achieve complete and clear separation of the triterpene compounds by exploiting their subtle physicochemical disparities. With a stepwise increase in the content of the organic modifier (acetonitrile), the polarity of the mobile phase gradually decreased. This strategically created a controlled gradient that permitted the differential elution of closely related compounds such as MA and AA based on their slight differences in affinity for the stationary phase (Wolfender et al., 2010). No observable interferences were present in the chromatogram. Then, a constant mobile phase composition was used for 25 min to achieve baseline separation of MA and AA. The system was then returned to the initial mobile phase composition over 2 min (from 25–27 min), followed by a 5-min column re-equilibration period (from 27–32 min) prior to the next injection. The effective separation of the MA and AA chromatogram peaks might be attributable to the controlled gradient elution creating a dynamic range of polarities within the column, enabling the gradual separation of these structurally similar compounds (Jandera, 2004). These refinements of the analytical conditions significantly enhanced the precision and reliability of triterpene analysis in *C. asiatica*. Additionally, this condition was also suitable for analyzing *C. asiatica* samples (Figure 4), as sufficient separation of MC, AC, MA, and AA was achieved in the *C. asiatica* extract. Additionally, this method utilizes a mobile phase that is generally used in laboratory settings, and shorter analysis times were achieved compared with those reported in previous studies (Rafi et al., 2018; Schaneberg et al., 2003; Xing et al., 2009).

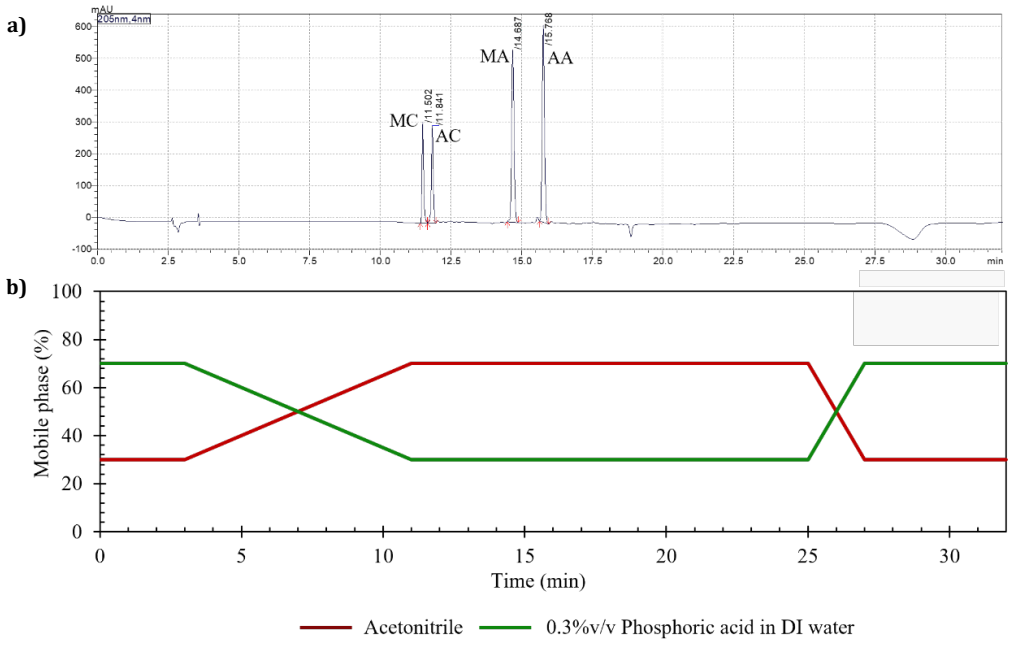


Figure 3. (a) Chromatogram of the standard mixture solution under optimized conditions, and (b) the low-pressure gradient elution profile used for the separation

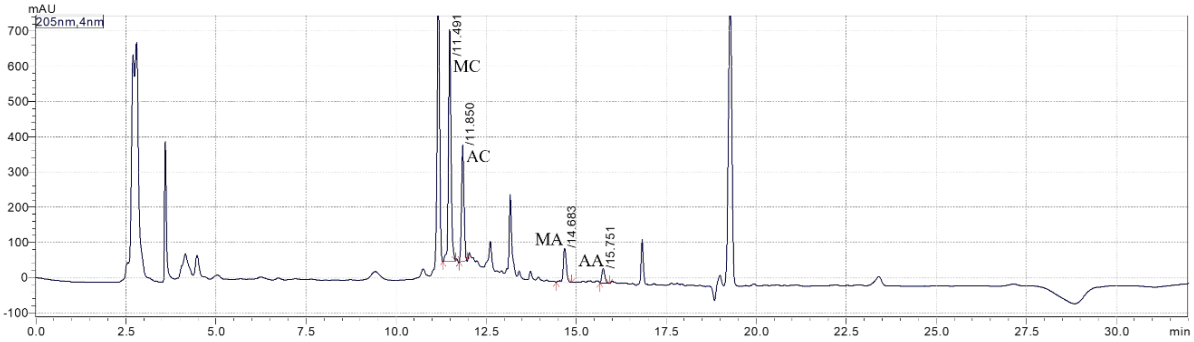


Figure 4. Chromatogram of 5% w/v *C. asiatica* extract

3.2 Method validation

The assay was validated following the ICH Q2(R1) guideline, which encompassed a comprehensive examination of various parameters, including specificity, linearity, range, accuracy, and precision (ICH guideline, 2022). The acceptance criteria were determined meticulously in accordance with the rigorous standards outlined in the AOAC 2016 guideline (Latimer & Horwitz, 2016) and USP36-NF31 (USP, 2012).

3.3 Specificity

Specificity refers to the precise determination of a substance amidst matrix effects and additives, ensuring the accurate identification of the analytes of interest. To assess the specificity of the RP-HPLC assay, blank solutions, individual standard solutions, and standard mixture solutions were analyzed, and the peaks corresponding to each solution were evaluated to determine the retention times of MC, AC, MA, and AA. The standard solutions were analyzed at 205 nm, and the retention times of the standard mixture solution were comparable to those of the separately analyzed individual standard solutions (Figure 5). The average retention times for separate injections of MC, AC, MA, and AA were 11.49, 11.84, 14.67, and 15.74 min, respectively. The RP-HPLC chromatogram of the mixed standard solution (Figure 4) revealed complete peak resolution, indicating satisfactory separation of the standard mixture solution. According to FDA guidelines, well-separated peaks with a resolution (Rs) greater than 2 between the closest eluting peak are considered reliable for quantification (Center for Drug Evaluation and Research, 1994). This confirms the specificity of the method for analyzing triterpenes in *C. asiatica* samples.

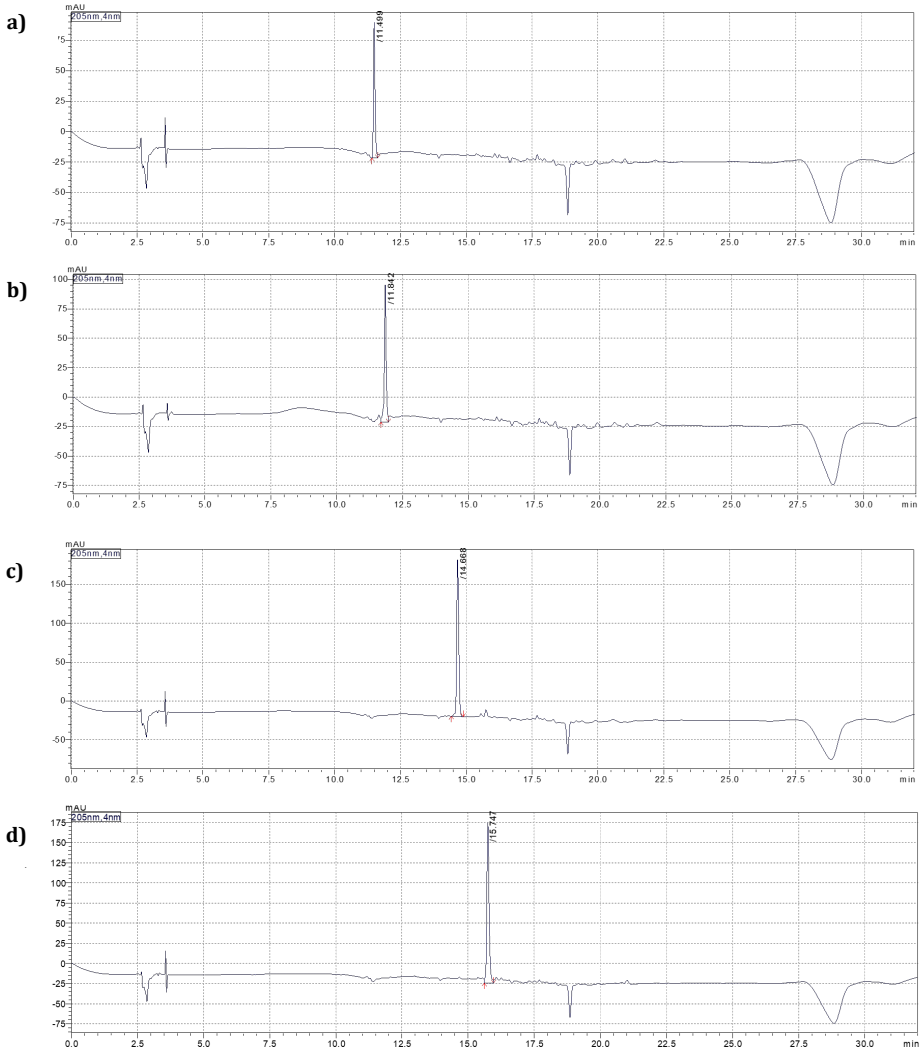


Figure 5. Individual chromatograms of (a) MC, (b) AC, (c) MA, and (d) AA standard solutions

3.4 Linearity and range

Linearity and range are crucial parameters in the validation of analytical methods, particularly in chromatographic analysis. Linearity refers to the ability of the method to produce test results that are directly proportional to the concentration of the analyte in the sample within a specified range. Range, on the other hand, defines the interval between the upper and lower concentration levels of analyte within which the method demonstrates acceptable linearity, accuracy, and precision. In this study, various concentrations of the standard solutions were prepared to establish the linearity and range, and the calibration curve was generated by plotting the peak area against the concentration of the standard solutions. Calibration curves, accompanied by their respective correlation coefficients (R^2) values of 0.9938, 0.9940, 0.9963, and 0.9907, were obtained over seven standard concentrations (0.01 to 0.25 mg/mL) for MC, AC, MA, and AA. These calibration curves are depicted in Figure 6a, 6b, 6c, and 6d, respectively. According to the criterion of linearity outlined in USP36-NF31, R^2 values should not fall below 0.9900 (USP, 2012). As illustrated in Figure 6, all R^2 values exceeded this threshold, indicating that the analytical method demonstrated linearity within this concentration range. This robust linearity ensures the reliable and accurate quantification of triterpenes in *C. asiatica* samples across the defined range.

3.5 Accuracy

Accuracy is a critical parameter that assesses the closeness of measured values to the true or reference values. In this study, the accuracy of the method was evaluated through recovery experiments utilizing spiked standard solutions. Known amounts of standard MC, AC, MA, and AA were added to the standard solution, covering concentrations ranging from 0.01 to 0.25 mg/mL for each compound. The concentrations of the substances in each sample were determined using the calibration curve, and the percent recovery was calculated (Table 3). The percent recovery ranged 93.81%–111.50% for the spiked MC samples, 91.07%–109.75% for the spiked AC samples, 90.60%–109.18% for the spiked MA samples, and 92.79%–111.08% for the spiked AA samples. Notably, all recoveries fell within the acceptable range of 80%–120%, as specified by the AOAC 2016 standards (Latimer & Horwitz, 2016). Therefore, based on the experimental results, the analytical method demonstrated accuracy across all investigated related compounds, confirming its reliability for the quantitative analysis of triterpenes in *C. asiatica* samples.

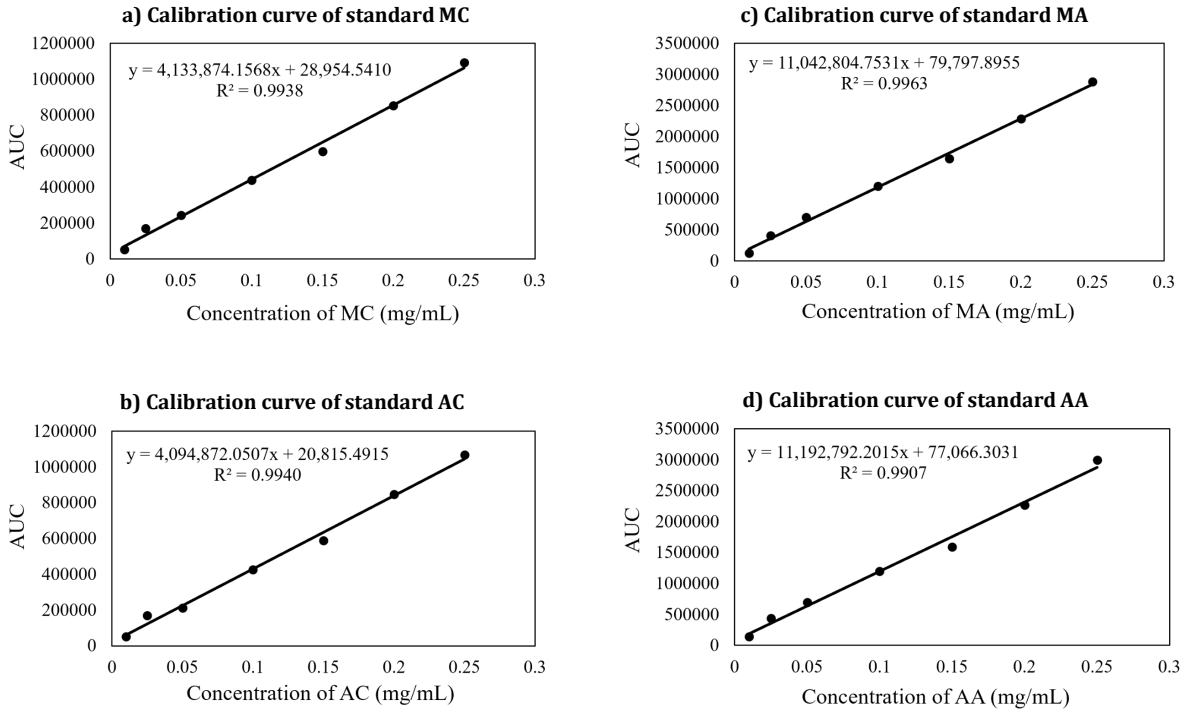


Figure 6. Calibration curves of (a) MC, (b) AC, (c) MA, and (d) and AA

Table 3. Theoretical spiked concentrations, average actual determined concentrations, and percent recoveries of MC, AC, MA, and AA

Reference standard	Theoretical concentration spiked (mg/mL)	Actual determined concentration (mg/mL)	Percent recovery (%)
MC	0.0119	0.0124	95.98
	0.0304	0.0272	111.50
	0.0530	0.0520	101.87
	0.1064	0.1015	104.82
	0.1416	0.1510	93.81
	0.2092	0.2005	104.34
	0.2704	0.2500	108.17
AC	0.0113	0.0124	91.07
	0.0291	0.0272	106.85
	0.0495	0.0520	95.17
	0.1102	0.1015	108.59
	0.1475	0.1510	97.66
	0.2091	0.2005	104.28
	0.2744	0.2500	109.75
MA	0.0112	0.0124	90.60
	0.0252	0.0272	92.64
	0.0568	0.0520	109.18
	0.1097	0.1015	108.06
	0.1471	0.1510	97.45
	0.2149	0.2005	107.21
	0.2687	0.2500	107.48
AA	0.0115	0.0124	92.79
	0.0290	0.0272	106.56
	0.0577	0.0520	111.08
	0.1092	0.1015	107.64
	0.1410	0.1510	93.36
	0.2076	0.2005	103.56
	0.2744	0.2500	109.77

3.6 Precision

Precision is a fundamental aspect that evaluates the consistency and reproducibility of analytical results. In this study, the precision of the RP-HPLC assay was thoroughly examined by measuring the area under the curve of the standard peaks obtained from a standard solution. Repeatability was assessed by injecting the standard solution six times consecutively, with each prepared separately. The intermediate precision was evaluated by injecting the standard solutions six times on different days. As presented in Table 4, the percent relative standard deviation (%RSD) values for repeatability were 1.00%, 1.31%, 0.53%, and 0.29% for MC, AC, MA, and AA, respectively. Similarly, the %RSD values for intermediate precision were 0.80%, 0.92%, 0.22%, and 0.27% for MC, AC, MA, and AA, respectively. Notably, all acquired %RSD values were lower than 7.30%, indicating that the assay met the precision criteria outlined in the 2016 AOAC standard (Latimer & Horwitz, 2016). These results highlight the reliability and consistency of the RP-HPLC assay for the precise quantification of triterpenes in *C. asiatica* samples.

Table 4. %RSD of MC, AC, MA, and AA

Reference standard	%RSD	
	Repeatability	Intermediate precision
MC	1.00	0.80
AC	1.31	0.92
MA	0.53	0.22
AA	0.29	0.27

4. CONCLUSION

Our developed RP-HPLC method represents a significant achievement in the simultaneous analysis of triterpenes in *C. asiatica*. The successful separation of MC, AC, MA, and AA was achieved using a symmetric C18 column with a low-pressure gradient elution and a rapid alteration of the mobile phase, which consisted of acetonitrile and 0.3% (v/v) phosphoric acid in deionized water. This condition allowed the closely related triterpenes to be resolved effectively. Validation studies conducted following the ICH and AOAC guidelines confirmed the suggested method's specificity, linearity, range, accuracy, and precision, further reinforcing its reliability. This efficient and reliable RP-HPLC method provides a valuable tool for the quality control of *C. asiatica* extracts. Furthermore, our methodology holds promise for future adaptation to the separation and analysis of other similar compounds in *C. asiatica* and other herbal products. This robust methodological framework enhances the assessment of *C. asiatica* extracts and opens avenues for broader applications in analyzing similar complex mixtures in herbal medicine and related fields. Its comprehensive validation, specificity, and precision underscore its suitability for routine use in pharmaceutical and herbal product development, ensuring consistent quality and efficacy.

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