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Development of Green HPLC-PDA Method for Quantification and Extraction Optimization of Dihydromyricetin, Vitexin, and Isovitexin in the LCD20 Herbal Remedy

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Cite This: *ACS Omega* 2026, 11, 30926−30935

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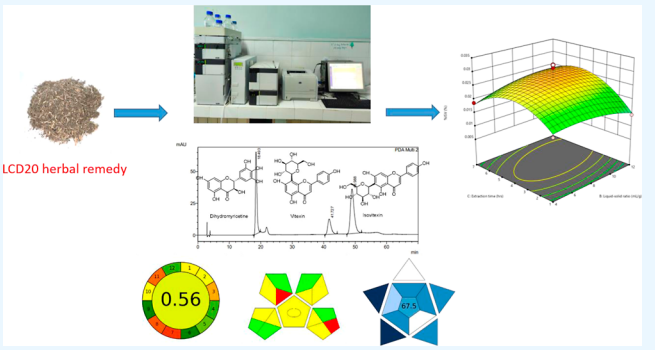
ABSTRACT: Dihydromyricetin, vitexin, and isovitexin are bioactive compounds in the LCD20 herbal remedy, valued for their role in peptic ulcer treatment. This study presents the development and validation of a green HPLC-PDA method for the simultaneous quantification of these three compounds in the remedy. Chromatographic separation was achieved using an InertSustain C18 column (4.6 × 250 mm, 5 μm) with a mobile phase of acetonitrile and 0.1% formic acid (13:87, v/v) and detection at 330 nm. The method demonstrated excellent linearity with ranges of 25−1000 μg/mL for dihydromyricetin and 5−200 μg/mL for vitexin and isovitexin. The method demonstrated high sensitivity with low limits of detection and quantification. The method also exhibited satisfactory specificity, accuracy, precision, sensitivity, and stability. Greenness assessment confirmed the method's alignment with green analytical chemistry, with AGREE (0.56), Eco-Scale (77), ComplexGAPI classification as low risk, and a BAGI score of 67.5. Finally, the validated method was utilized to optimize the manufacturing process of the herbal extract via response surface methodology; conditions of 84.3% ethanol, 11.4 mL/g solvent-to-solid ratio, and 4.86 h were established for maximum efficiency. This approach ensures reliable quantification and extraction, aiding the standardization of LCD20 for pharmaceutical use.



1. INTRODUCTION

Peptic ulcer disease (PUD) is characterized as an acid-induced lesion of the digestive tract and is typically found in the stomach or proximal duodenum. It is defined by the presence of a denuded mucosa, with the ulcer extending into the submucosa or muscularis propria.[1,2] The prevalence of PUD in the general population is estimated to be between 5% and 10%, with an annual incidence ranging from 0.1% to 0.3%.[3,4] The primary focus of treatment strategies for PUD is on symptom relief and promotion of mucosal healing. Recently, herbal medicine has gained attention as a viable treatment option due to its multitarget effects and relatively low side effects.[1]

The LCD20 herbal remedy has been developed by traditional medicine practitioners at the Traditional Medicine Hospital of Hue City, Vietnam. The LCD20 herbal remedy is formulated with seven traditional ingredients prepared based on the principles and methods of traditional medicine, such as *Ardisia silvestris*, Pit leaf 25 g; *Hedyotis capitellata*, aerial part 20 g; *Ampelopsis cantoniensis* (Hook. and Arn.) K. Koch, aerial part 20 g; *Lactuca indica* L., aerial part 16 g, *Croton tonkinensis* Gagnep, leaf 16 g; *Curcuma longa* L., rhizome 10 g; and *Sepia esculenta*, cuttlebone 27 g. Research indicates that several of these herbs possess significant biological activities, including anti-inflammatory, antibacterial, and antioxidant properties, which are essential for mitigating mucosal damage and preventing complications associated with PUD.[1]

Among the active compounds in this herbal mixture, dihydromyricetin (DHM), vitexin (VTX), and isovitexin (ISV) (Figure 1) stand out due to their significant biological activities, including anti-inflammatory, antioxidant, anticancer, and antibacterial effects. To the best of our knowledge, an HPLC−PDA method has not yet been reported for the LCD20 herbal remedy that enables the simultaneous quantification of DHM, VTX, and ISV within a single chromatographic run. Most published studies have focused on either single-analyte determination or two-analyte assays in single-herb matrices, often involving multistep sample preparation and/or instrument-intensive platforms such as

Received: January 5, 2026
Revised: May 4, 2026
Accepted: May 6, 2026
Published: May 19, 2026

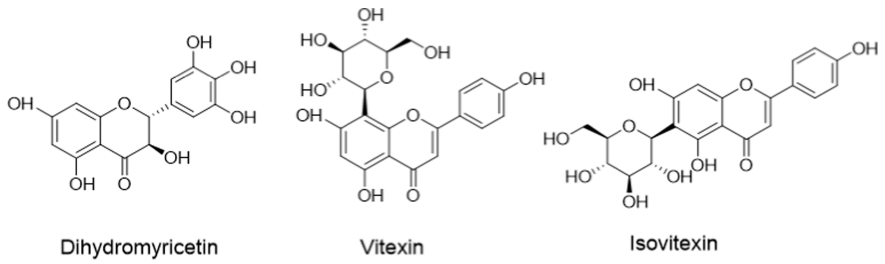


Figure 1. Structures of DHM, VTX, and ISV.

Table 1. Summary of Validation Result[a]

parameter	DHM	VTX	ISV
system suitability[b]			
t_R (min)	18.278 (RSD 0.69%)	40.818 (RSD1.35%)	47.831 (RSD 1.46%)
S (mAU.s)	2245241 (RSD 0.94%)	898027 (RSD 0.49%)	2947595 (RSD 0.73%)
R_s	2.85	11.58	3.54
N	7272	7636	8338
T_f	1.47	1.47	1.39
linearity			
range (μg/mL)	25−1000	5−200	5−200
r^2	0.9985	0.9981	0.9986
slope ±SD	4948.9 ± 95.9	9561.1 ± 207.8	31386.9 ± 589.2
intercept ±SD	−110610 ± 50878	−49421 ± 22053	−149480 ± 62518
$F_{statistic}$	2663.0	2116.3	2837.6
$F_{critical}$	7.71	7.71	7.71
precision			
intraday[c]			
mean (μg/mL)	509.130	135.426	129.680
RSD %	2.36	3.49	2.30
interday[c]			
mean (μg/mL)	518.118	163.325	138.504
RSD %	3.40	3.35	3.05
accuracy[d]			
% recovery	101.33	100.52	100.48
RSD %	1.25	1.45	1.46
LOD (μg/mL)	0.78	1.25	0.31
LOQ (μg/mL)	1.56	5.00	1.25

[a] $Y = ax + b$, where y is the corrected peak area and x is the concentration (μg/mL). t_R, retention time; S, peak area; R_s, resolution; N, theoretical plate number; T_f, tailing factor; and RSD, relative standard deviation. [b] $n = 6$. [c] $n = 6$. [d] $n = 9$.

UHPLC or HPTLC.[5−10] Accordingly, an unmet need remains for a fit-for-purpose, matrix-specific HPLC method capable of concurrently quantifying these three markers in a complex, multiherb formulation such as LCD20.

In recent years, the integration of sustainability principles into analytical science has gained increasing importance, particularly in the quality control of herbal medicines. Green Analytical Chemistry (GAC) promotes the development of methodologies that not only meet analytical performance standards but also minimize environmental impact.[11] To support this objective, a range of advanced evaluation tools have been introduced. These frameworks offer complementary perspectives on method sustainability by assessing criteria such as solvent toxicity, energy consumption, waste generation, reagent safety, and overall procedural greenness.[12−16]

Building on these developments, the current study aims to develop and validate a method for the simultaneous quantitative analysis of DHM, VTX, and ISV in the LCD20 herbal remedy using high-performance liquid chromatography with photodiode array detection (HPLC-PDA). This approach not only enables accurate and reliable quantification of key bioactive compounds but also incorporates a systematic evaluation of the method's ecological impact. By incorporating green analytical chemistry principles into both the extraction procedure and method validation process, this work contributes to the broader objective of standardizing traditional herbal remedies in a sustainable and scientifically rigorous manner, ensuring both therapeutic efficacy and environmental responsibility.

2. RESULTS AND DISCUSSION

2.1. Optimization of Chromatographic Conditions

Initially, chromatographic conditions were optimized with reference to previously published methods,[5−9,17,18] while the injection volume, flow rate, column temperature, detector, and detection wavelength were fixed at 10 μL, 1.0 mL/min, 30 °C, PDA, and 330 nm, respectively. Method optimization focused on the selection of the stationary phase and mobile phase to obtain complete elution of DHM, VTX, and ISV with acceptable peak shape, sufficient resolution, and no interfering peaks.

https://doi.org/10.1021/acsomega.6c00078
ACS Omega 2026, 11, 30926−30935

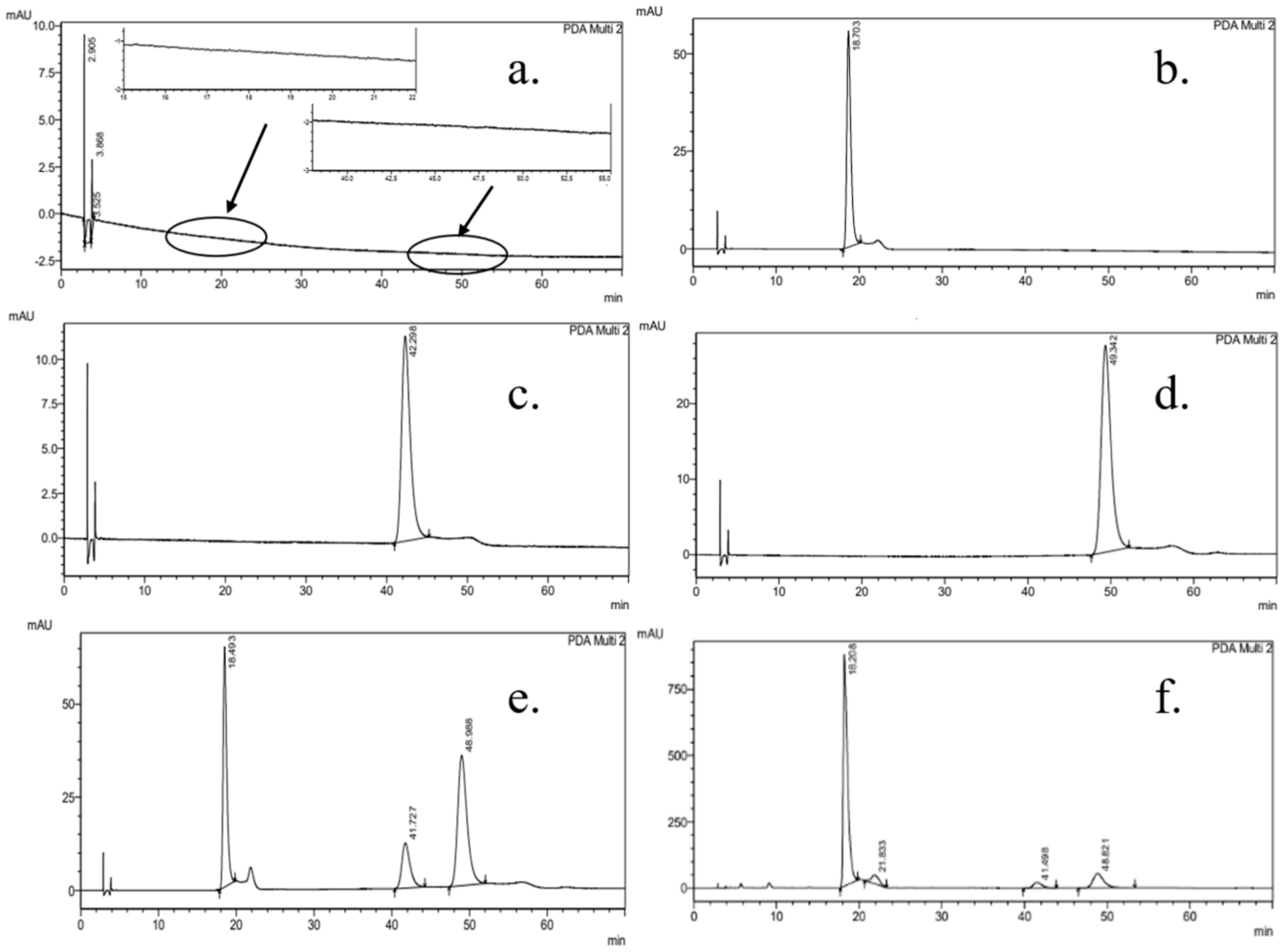


Figure 2. Chromatograms of blank (a), DHM (b), VTX (c), ISV (d), mixture of standard (e), and sample solution (f).

The Agilent Eclipse C18 column (4.6 × 150 mm, 5 μm) was first tested with ACN−H_2O−1% acetic acid and ACN−1% acetic acid. Both systems resulted in incomplete peak appearance, broad peaks, poor symmetry, and insufficient separation. ACN−1% acetic acid at 10:90 (v/v) improved the resolution between vitexin and isovitexin, but peak broadening and asymmetry were still observed. These results indicated that the Agilent Eclipse C18 column was not suitable for reliable simultaneous determination of the three flavonoids under the investigated conditions.

Further optimization was performed on the InertSustain C18 column (4.6 × 250 mm, 5 μm), which showed improved peak shape and separation. ACN−1% acetic acid, ACN−0.1% TFA, ACN−0.05% formic acid, ACN−phosphate buffer (pH 2), and ACN−0.1% formic acid were then examined as mobile phases. Acidic conditions gave better chromatographic performance for the target flavonoids, particularly in terms of peak symmetry and resolution.

Among the investigated mobile phase systems, ACN−0.1% formic acid showed the most suitable chromatographic behavior. At a ratio of 13:87 (v/v), dihydromyricetin, vitexin, and isovitexin were completely eluted with acceptable peak symmetry and resolution, and no adjacent interfering peaks were observed. In contrast, the acetic acid system was still associated with peak broadening and splitting, the TFA system produced interfering peaks close to the analyte peaks, the phosphate buffer system was less favorable in terms of pH control and analytical robustness, and ACN−0.05% formic acid gave a lower vitexin response in the herbal sample. Based on these results, ACN−0.1% formic acid (13:87, v/v) on the InertSustain C18 column was selected as the final chromatographic condition. The chromatograms shown in the Supporting Information (Figures S1−S3) further illustrate the improvement in peak shape and separation achieved under the optimized conditions.

The final optimized chromatographic conditions included an InertSustain C18 (4.6 × 250 mm, 5 μm) column, ACN - 0.1% formic acid (13:87, *v/v*) as the mobile phase, 10 μL injection volume, 1 mL/min flow rate, 30 °C column temperature, and PDA detection at 330 nm.

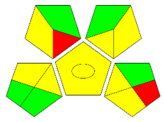
The system suitability data are summarized in Table 1. The results met the predefined acceptance criteria, confirming the suitability of the optimized conditions for quantitative analysis.

2.2. Method Validation

2.2.1. Specificity. No interference was found in the retention time of analytes in the blank solution by visual inspection of the chromatograms (Figure 2). Specificity was further supported by the similarity of the UV spectra of DHM, VTX, and ISV in the sample and the corresponding individual standard solutions, as well as by PDA peak purity analysis of the analyte peaks (Figures S4 and S5, Supporting Information).

https://doi.org/10.1021/acsomega.6c00078
ACS Omega 2026, 11, 30926−30935

Table 2. Evaluated Results of Green HPLC Method by Analytical Score, AGREE, Complex-GAPI, and BAGI

Eco-Scale score		AGREE	ComplexGAPI	BAGI
Reagent	Penalty points			
Methanol, 10 mL	6*1 = 6			
Acetonitrile, 9.1 mL	4*1 = 4			
Formic acid, 0.06 mL	4*1 = 4			
Water, 60.84 mL	0*2 = 0			
HPLC-PDA	1			
Occupational hazards	0			
Waste (70 mL)	5			
No treatment	3			
Total penalty points	23			
Eco-Scale Score	77			

2.2.2. Linearity. The result of the linearity test in Table 1 demonstrated a direct proportional relationship between concentration and peak area within the specified ranges: 25−1000 μg/mL for DHM and 5−200 μg/mL for both VTX and ISV. The obtained correlation coefficients ($r^2 > 0.995$) confirm the strong linearity of the proposed analytical method.

2.2.3. Precision. The data obtained from the precision are given in Table 1. Precision was evaluated in accordance with ICH Q2(R2) through repeatability (intraday) and intermediate precision (interday).[19] The %RSD values for DHM, VTX, and ISV were ≤3.7%, indicating acceptable precision. Given the estimated analyte levels in the herbal, the observed precision is consistent with the concentration-dependent expectations described in AOAC Appendix K.[20]

2.2.4. Accuracy. Accuracy assessed by standard addition showed recoveries of 95−105% for DHM, VTX, and ISV (Table 1), meeting the predefined criteria for the intended application (ICH Q2(R2)). These recoveries are also within the recovery ranges recommended in AOAC Appendix K.[19,20]

2.2.5. Limit of Detection and Limit of Quantification. LOD/LOQ were established using a signal-to-noise approach consistent with ICH Q2(R2) and USP <621>.[19,21] The LOD/LOQ values were 0.78/1.56 μg/mL (DHM), 1.25/5.00 μg/mL (VTX), and 0.31/1.25 μg/mL (ISV). Precision at the LOQ level was verified, with %RSD <7.3% for all analytes (Table 1), supporting quantitative suitability at the LOQ.

2.2.6. Robustness. The method's robustness was evaluated by intentionally altering critical parameters, such as flow rate, mobile phase composition, and column temperature. The results indicated that the analytical outcomes are significantly affected by deliberate, minor variations in the method parameters.

2.2.7. Solution Stability. Each sample solution was analyzed in triplicate (n = 3) at each time point (0, 24, and 48 h). Statistical evaluation was performed to assess variations in retention time and peak area over time. The results (Table S6, Supporting Information) indicated that, within each time point, the %RSD values for both retention time and peak area were ≤2.0% for all analytes. Between time points, a statistical comparison (α = 0.05) showed a significant difference ($p < 0.05$) in the mean peak areas of VTX and ISV at 48 h (day 3) relative to 0 h (day 1), whereas DHM did not show a significant change. As a result, the sample solution was considered stable up to 24 h but not at 48 h under the studied storage conditions.

2.3. The Greenness of the HPLC-PDA Methods

The greenness assessment results of the developed HPLC method using the Analytical Eco-Scale, AGREE, Complex-GAPI, and BAGI tools are presented in Table 2.

The analytical Eco-Scale evaluation revealed particularly promising results in Table 2, with the method achieving an excellent score of 77 out of 100. This semiquantitative assessment considered the hazards and amounts of the reagents used, solvent consumption, energy requirements, and waste generation during the analytical procedure. Although methanol, acetonitrile, and formic acid contributed penalty points, the overall score remained favorable because the method involved a relatively simple sample preparation procedure, no derivatization step, and moderate instrumental operating conditions.[12]

The AGREE assessment further supported the greenness profile of the method, giving a score of 0.56 (Table 2). This result indicates moderate compliance with the principles of green analytical chemistry. The AGREE evaluation reflected the actual analytical workflow described in the paper, including the sample preparation procedure and chromatographic conditions. The score was mainly influenced by the use of organic solvents and waste generation, while the simultaneous determination of three analytes in a single run contributed positively to the overall assessment.[13]

The ComplexGAPI assessment reflected the sample preparation and analysis stages of the method. No dedicated preanalysis process was involved in the present study; therefore, the preanalysis field was not included in the evaluation. The ComplexGAPI pictogram contained 5 green, 8 yellow, and 2 red fields, indicating that the main environmental burdens were associated with extraction-based

https://doi.org/10.1021/acsomega.6c00078
ACS Omega 2026, 11, 30926−30935

sample preparation, the use of organic solvents, and waste generation.[14,16]

The practical applicability of the method was evaluated by using the BAGI metric. The BAGI score was 67.5 (Table 2), exceeding the 60-point threshold, which is generally considered indicative of acceptable practical applicability. This evaluation reflected the actual analytical workflow of the method and also showed limitations related to the sample preparation complexity and analytical throughput. Nevertheless, the simultaneous determination of DHM, VTX, and ISV in a single chromatographic run contributed positively to the overall applicability of the method.[15]

Overall, the combined greenness assessment indicates that the developed HPLC-PDA method offers a reasonable balance among analytical performance, environmental impact, and practical applicability for the determination of DHM, VTX, and ISV in the LCD20 remedy. Despite the use of organic solvents and multistep sample preparation, the simultaneous determination of three analytes under a single optimized condition supports its practical value.

2.4. Application of the Validated Method to the Manufacturing Process of LCD20 Extract

2.4.1. Investigation of Extraction Methods. The preliminary investigation focused on optimizing extraction conditions for the LCD20 herbal powder using two methods: reflux extraction and exhaustive percolation. For reflux extraction, various factors were examined, including ethanol concentration, liquid-to-solid ratio, extraction temperature, extraction time, and the number of extraction cycles. The selected reflux conditions were 90% ethanol, a liquid-to-solid ratio of 12 mL/g, 50 °C, 1 h, and two extraction cycles. These conditions yielded the highest content of DHM (0.9480%), VTX (0.0083%), and ISV (0.0321%).

For exhaustive percolation, the ethanol concentration, liquid-to-solid ratio, and soaking duration were investigated as key process variables. The selected conditions were 90% ethanol, a liquid-to-solid ratio of 8 mL/g, and 4 h of cold-soaking. These conditions resulted in the highest contents of DHM (1.1123%), VTX (0.0095%), and ISV (0.0338%).

A triplicate comparison was then performed between reflux extraction and exhaustive percolation using the selected preliminary conditions (Figure 3). The findings revealed that utilizing exhaustive percolation provided a better average DHM recovery rate of 1.1376%, outperforming the 0.9746% recorded for reflux extraction. Similarly, the VTX content was greater in exhaustive percolation (0.0099%) than in reflux extraction (0.0071%), with a statistically significant difference ($p < 0.05$). Conversely, based on *t*-test results, the variation in ISV content—0.0260% via percolation and 0.0331% via reflux—was not statistically significant ($p > 0.05$).

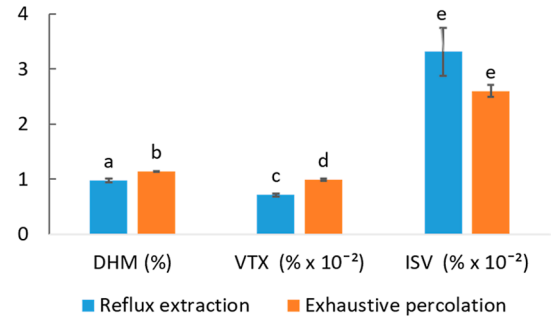


Figure 3. Extraction performance of reflux extraction and exhaustive percolation. Different letters denote statistically significant differences ($p < 0.05$).

Exhaustive percolation was therefore selected for further optimization because it provided better extraction of the target markers.

2.4.2. RSM-Assisted Extraction Optimization. A Box–Behnken design within the RSM framework was used to optimize the extraction of DHM, VTX, and ISV from LCD20. Ethanol concentration (A), liquid-to-solid ratio (B), and extraction time (C) were selected as independent variables, and the contents of the three analytes were used as responses. The factor levels were chosen based on preliminary single-factor experiments and are listed in Table 3.

Table 3. Coded and Actual Levels of the Independent Variables Used in the BBD Optimization Design

variables	coded levels of variables		
	−1	0	+1
ethanol concentration (%) (A)	80.5	90	99.5
liquid−solid ratio (mL/g) (B)	4	8	12
extraction time (hrs) (C)	1	4	7

A series of 15 experimental trials were carried out, and the subsequent analytical results are recorded in Table 4.

Table 4. Experimental Matrix and the Corresponding Responses for the Extraction Optimization of DHM, VTX, and ISV

no.	A	B	C	DHM content (%) X_1	VTX content ($\times 10^{-2}$%) X_2	ISV content ($\times 10^{-2}$%) X_3
1	99.5	12	4	1.108	0.544	0.831
2	80.5	4	4	1.012	0.931	2.092
3	99.5	8	7	1.020	0.392	0.894
4	90	4	1	1.147	0.856	1.721
5	90	12	7	1.334	0.789	2.463
6	80.5	8	1	1.190	0.856	1.802
7	80.5	12	4	1.264	1.022	2.819
8	90	8	4	1.295	1.016	3.133
9	99.5	8	1	1.134	0.509	0.639
10	90	4	7	1.123	0.954	1.874
11	80.5	8	7	1.174	0.976	1.967
12	90	8	4	1.329	1.003	2.422
13	90	8	4	1.281	0.994	2.568
14	99.5	4	4	1.021	0.572	1.406
15	90	12	1	1.403	0.924	1.432

The ANOVA results for DHM extraction are provided in Table S7 (Supporting Information). The model was statistically significant, with a model *p*-value of <0.05, whereas the lack-of-fit test was not significant ($p = 0.4272 > 0.05$), confirming that the fitted model adequately described the experimental data. The independent variables (A, B, and C), interaction term (AB), and quadratic terms (A^2 and B^2) were significant ($p < 0.05$), indicating their important effects on DHM content at the 95% confidence level.

The regression model showed satisfactory goodness of fit and predictive performance, as reflected by $R^2 = 0.9820$, adjusted $R^2 = 0.9495$, and predicted $R^2 = 0.7884$. These results

https://doi.org/10.1021/acsomega.6c00078
ACS Omega 2026, 11, 30926−30935

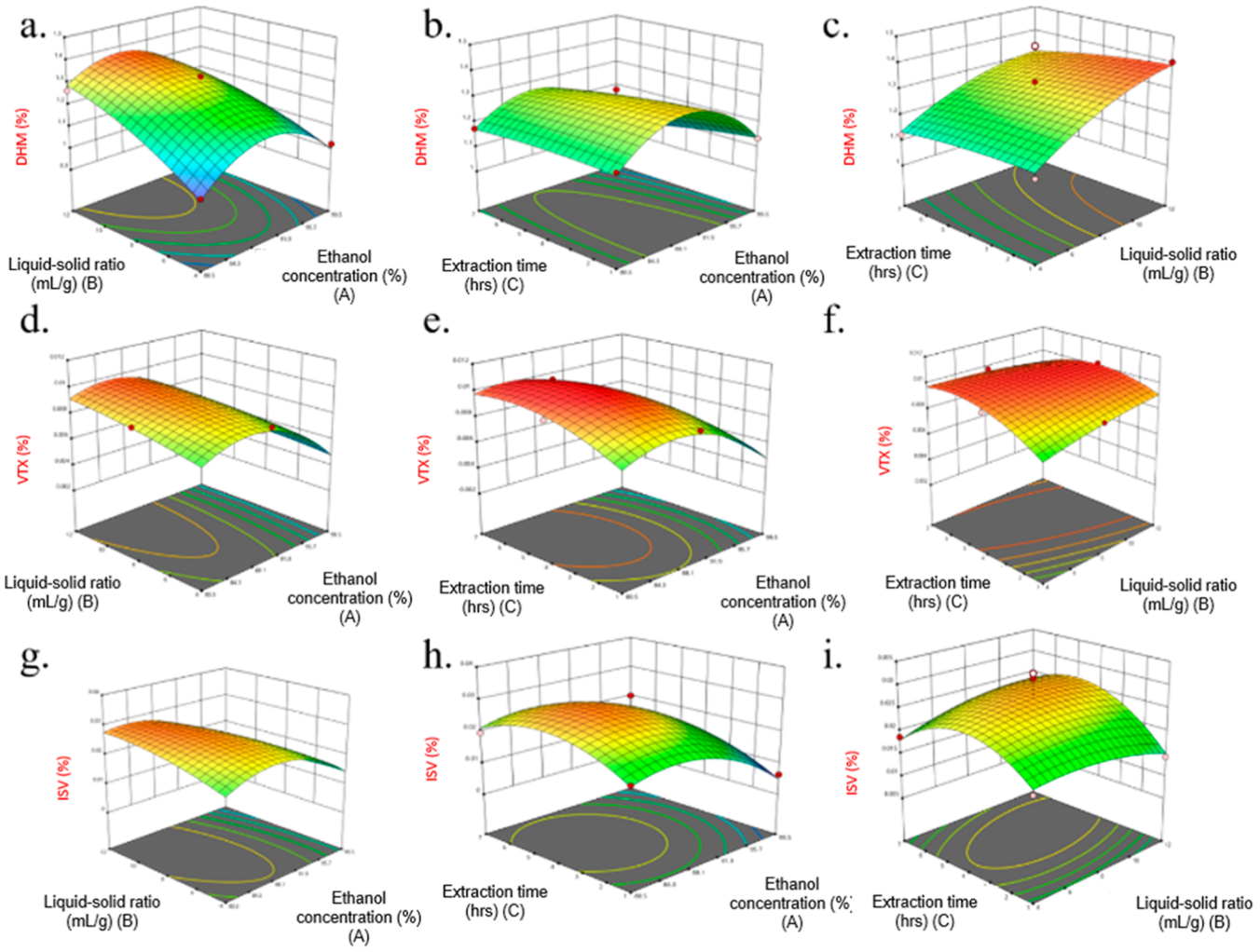


Figure 4. Response surface plots showing the effects of variable interactions on the extraction of DHM (a–c), VTX (d–f), and ISV (g–i) from LCD20 remedy.

indicate that the model could be used to estimate the conditions providing the highest DHM content in the herbal matrix. The fitted polynomial equation describing the effects of the process variables on DHM content (X_1) is given below

$$X_1 = -14.20902 + 0.329027 \times A + 0.166342 \times B + 0.083994 \times C - 0.001092 \times AB - 0.000848 \times AC - 0.000947 \times BC - 0.001787 \times A^2 - 0.002445 \times B^2 - 0.001174 \times C^2 \quad (1)$$

The ANOVA results for VTX extraction are provided in Table S8 (Supporting Information). The model was statistically significant, with a model p-value of <0.0001, whereas the lack-of-fit test was not significant ($p = 0.0796 > 0.05$), confirming that the fitted model adequately described the experimental data. The independent variable A, interaction terms AC and BC, and quadratic terms A^2 and C^2 were significant ($p < 0.05$), indicating their important effects on VTX content at the 95% confidence level.

The regression model showed satisfactory goodness of fit and predictive performance, as reflected by $R^2 = 0.9924$, adjusted $R^2 = 0.9787$, and predicted $R^2 = 0.8840$. These results indicate that the model could be used to estimate the conditions providing the highest VTX content in the herbal matrix. The fitted polynomial equation describing the effects of the process variables on VTX content (X_2) is given below

$$X_2 = -0.181375 + 0.004251 \times A + 0.001087 \times B + 0.003166 \times C - 0.00000783801 \times AB - 0.000021 \times AC - 0.000048 \times BC - 0.000024 \times A^2 - 0.000012 \times B^2 - 0.000115 \times C^2 \quad (2)$$

The ANOVA results for ISV extraction are provided in Table S9 (Supporting Information). The model was statistically significant, with a model p-value of <0.05, whereas the lack-of-fit test was not significant ($p = 0.8663 > 0.05$), confirming that the fitted model adequately described the experimental data. The independent variable A and quadratic terms A^2 and C^2 were significant ($p < 0.05$), indicating their important effects on ISV content at the 95% confidence level.

The regression model showed satisfactory goodness of fit and predictive performance, as reflected by $R^2 = 0.9503$, adjusted $R^2 = 0.8608$, and predicted $R^2 = 0.7094$. These results indicate that the model could be used to estimate the conditions providing the highest ISV content in the herbal

https://doi.org/10.1021/acsomega.6c00078
ACS Omega 2026, 11, 30926–30935

matrix. The fitted polynomial equation describing the effects of the process variables on ISV content (X_3) is given below

$$X_3 = -0.649348 + 0.014648 \times A + 0.008988 \times B + 0.004257 \times C - 0.000086 \times AB + 0.00000791204 \times AC + 0.000183 \times BC - 0.000081 \times A^2 - 0.000117 \times B^2 - 0.000721 \times C^2 \quad (3)$$

Response surface plots were generated to visualize the pairwise effects of the extraction variables on the extraction efficiency while maintaining the third variable at a fixed level (Figure 4).

Based on the 15 experimental runs generated by the Box−Behnken design, the predicted optimal extraction conditions were 84.289% ethanol, a liquid-to-solid ratio of 11.368 mL/g, and an extraction time of 4.86 h. For practical implementation during method verification, ethanol concentration and liquid-to-solid ratio were adjusted to 84.3% and 11.4 mL/g, respectively, while the extraction time was maintained at 4.86 h. Under these conditions, the contents of DHM, VTX, and ISV were 1.357 ± 0.083%, 0.012 ± 0.002%, and 0.033 ± 0.004%, respectively. A *t*-test indicated that the predicted and experimental extraction efficiencies were not significantly different statistically (p-value >0.05, 95% confidence level). These findings substantiate good agreement between the established optimal extraction conditions and the empirical results obtained.

3. CONCLUSIONS

A green and reliable HPLC-PDA method was successfully developed and validated for the simultaneous quantification of DHM, VTX, and ISV in the LCD20 herbal formulation. This work further supports multimarker analysis by providing a matrix-specific assay for three targets within a single chromatographic run. The method fulfilled essential validation parameters and was effectively applied to optimize the extraction process using response surface methodology. Optimal extraction conditions yielded satisfactory concentrations of all target compounds. Additionally, the method's greenness was confirmed through assessments using Complex-GAPI, AGREE, BAGI, and Eco-Scale, demonstrating strong compliance with green analytical chemistry principles. Overall, the method can be applied to monitor extraction performance, support standardization of LCD20, and enable quality control of derived products.

4. MATERIALS AND METHODS

4.1. Materials

Ingredients of the LCD20 include eight medicinal herbs that were purchased from Cong Thanh Investment and Development Joint Stock Company, Hue City, Vietnam, which has achieved certification under the TCVN 11892-1:2017 standard on GAP (no. 08/VietGAP.TT.01.21) and ISO 22000:2018 (certification no. 2202140068) for primary processing and packaging of medicinal herbs. All of these medicinal herbs were authenticated by Dr. Minh Van Doan (Faculty of Traditional Medicine, University of Medicine and Pharmacy, Hue University, Vietnam). In brief, the herbs in the LCD20 remedy were mixed according to the above-mentioned formulation ratio and ground through a 60-mesh sieve to obtain a fine uniform powder. This powder was used for experiments in this study.

4.2. Reference Compounds

Reference standards of DHM (98%, batch no. PRF9090746), VTX (98%, batch no. PRF10102941), and ISV (98%, batch no. PRF20111321) were obtained from Chengdu Biopurify Phytochemicals Ltd. (China).

4.3. Chemicals and Solvents

The chemical reagents and solvents used in the study included acetonitrile (HPLC grade, Merck), distilled water, glacial acetic acid, formic acid, trifluoroacetic acid (TFA), petroleum ether, solid-phase extraction (SPE) C18 columns, silica gel, and other necessary reagents.

4.4. Instrumentation

Chromatographic analysis was performed using a Shimadzu LC-20AD system (Japan) equipped with an SPD-M20A PDA detector, a CTO-20A column oven, a SIL-20AC autosampler, and a DGU-20A degasser. Sample weighing was carried out on a Sartorius Quintix 125D-1S analytical balance with a readability of 0.1 mg. pH monitoring was carried out with a Hanna Instruments HI 2550-02 pH meter (USA).

4.5. Preparation of Standard Solutions

DHM, VTX, and ISV stock solutions were prepared by accurately weighing 10 mg of each standard into 5 mL volumetric flasks, dissolving in methanol, and sonicating for 30 min until complete dissolution.

For the calibration curve, the mixed standard solution was prepared by combining the stock solutions to achieve final concentrations of 25−1000 μg/mL for DHM, 5−200 μg/mL for VTX, and 5−200 μg/mL for ISV.

4.6. Preparation of Sample Solutions

For HPLC method development, 2.5 g of herbal powder was accurately weighed into a Falcon tube. Methanol (10 mL) was added, and the mixture was vortexed for 10 min and sonicated at room temperature for 30 min. The extract was then filtered through a 5 μm membrane filter.

4.7. Optimization of Chromatographic Conditions

The chromatographic method was developed for the simultaneous quantification of DHM, VTX, and ISV in the LCD20 formulation through systematic experimental optimization based on previous studies and the experimental screening of relevant chromatographic parameters. System suitability was assessed prior to sample analysis using replicate injections of a mixed standard, with evaluation based on the % RSD of peak areas and retention times, resolution of critical peak pairs, peak tailing, and theoretical plate number (N).

Initial trials were performed under conditions adapted from previous studies.[5−9,17,18] Throughout chromatographic screening, the injection volume was fixed at 10 μL, the flow rate at 1.0 mL/min, the column oven temperature at 30 °C, and PDA detection at 330 nm. The starting conditions included the use of an Agilent Eclipse C18 column (4.6 × 150 mm, 5 μm) with ACN−H_2O−1% acetic acid and ACN−1% acetic acid as the initial mobile phase systems.

To obtain a suitable chromatographic separation, several parameters were systematically evaluated. Two reversed-phase columns were assessed: Agilent Eclipse C18 (4.6 × 150 mm, 5 μm) and InertSustain C18 (4.6 × 250 mm, 5 μm). Several mobile phase compositions were investigated, including ACN−H_2O−1% acetic acid, ACN−1% acetic acid, ACN−0.1% trifluoroacetic acid (TFA), ACN−0.1% formic acid, ACN−phosphate buffer (pH 2), and ACN−0.05% formic acid. Various solvent ratios and gradient elution programs were tested to identify conditions providing acceptable peak shape, symmetry, resolution, and freedom from adjacent interfering peaks. Representative chromatograms obtained under the initial and optimized chromatographic conditions are provided in the Supporting Information (Figures S1−S3).

4.8. Method Validation

The optimized condition was evaluated according to the ICH Q2(R2) and AOAC guidelines for the specificity, linearity, precision, accuracy,

https://doi.org/10.1021/acsomega.6c00078
ACS Omega 2026, 11, 30926−30935

limits of detection (LOD) and quantitation (LOQ), robustness, and solution stability.[19,20]

Specificity was assessed by comparing the chromatograms of the blank, standard, sample, and spiked sample solutions. In addition, specificity was further assessed by comparison of the UV spectra of the analyte peaks in the sample to those of the corresponding individual standard solutions and by PDA peak purity analysis.

Linearity was established at six different concentrations in the range of 25−1000 μg/mL for DHM and 5−200 μg/mL for VTX and ISV. Calibration curves were constructed from peak area versus concentration, and linear regression was used to calculate the coefficients of determination (r^2).

Precision was assessed through repeatability (intraday) and intermediate precision (interday). Repeatability (intraday precision) was determined by analyzing six replicates of both the original sample and a 10-fold diluted sample under identical conditions. Intermediate precision (interday) was assessed by repeating the analysis on another day.

Accuracy was performed by spiking known amounts of DHM (into diluted samples), VTX, and ISV (into original samples) at 110%, 120%, and 150% of their expected concentrations. Each level was analyzed in triplicate, and recovery percentages were calculated.

LOD and LOQ were determined using a signal-to-noise approach consistent with USP practice (USP <621>),[21] with LOD established at S/N ≈ 3 and LOQ at S/N ≈ 10. The S/N ratio was evaluated from the chromatograms as the analyte peak height relative to the peak-to-peak baseline noise measured in a defined blank/baseline region adjacent to each analyte peak. Precision at LOQ was verified by calculating the %RSD of replicate measurements of analyte concentrations.

The robustness of the analytical method was evaluated by introducing intentional minor variations in critical parameters, including flow rate (±0.2 mL/min), mobile phase composition (±2%, v/v), and column temperature (±2 °C) around the optimized values. The impact of these modifications was assessed by calculating the %RSD of retention time, tailing factor, and resolution for DHM, VTX, and ISV.

Solution stability was examined using model sample solutions maintained at room temperature in tightly closed containers protected from light. Injections were performed at 0, 24, and 48 h (day 1−day 3). Stability was evaluated using predefined criteria: within each time point, %RSD of retention time and peak area from replicate injections was required to be ≤2.0%; between time points, mean peak areas (or concentrations) were compared against day 1 (0 h) using a significance test (α = 0.05). Solutions were considered stable when no significant difference was observed (p > 0.05).

4.9. Greenness Evaluation of the Analytical Method

This research employed a comprehensive multitool approach to rigorously evaluate the environmental sustainability of the developed HPLC-PDA analytical method. Four distinct yet complementary green chemistry assessment tools were utilized: the Analytical Eco-Scale,[12] Analytical Greenness Calculator (AGREE),[13] Complementary Green Analytical Procedure Index (ComplexGAPI),[14,16] and the Blue Applicability Grade Index (BAGI).[15] Each tool provided unique insights into different aspects of the method's environmental impact, allowing for a holistic evaluation of its green credentials. The selection of these particular assessment frameworks was deliberate, as they collectively address both the theoretical principles and practical applications of green analytical chemistry while overcoming individual limitations through their combined use.

The Analytical Eco-Scale provides a semiquantitative score (maximum of 100) by deducting penalty points based on hazardous reagents, energy consumption, and waste generation. Higher scores reflect greater environmental compatibility and identify critical parameters for a potential improvement.

The analytical greenness (AGREE) metric applies the 12 principles of green analytical chemistry to generate a consolidated score ranging from 0 (least green) to 1 (most green). It further visualizes performance through a radial chart, offering both qualitative and quantitative feedback on factors such as miniaturization, automation, and safety.

The Complex Green Analytical Procedure Index (ComplexGAPI) evaluates various aspects of the method's environmental footprint, such as sample preparation, solvent toxicity, reagent usage, energy demand, and waste management. Results are presented using a color scale—green indicating minimal environmental risk, red indicating high impact—allowing rapid identification of strengths and limitations.

The blue applicability grade index (BAGI) assesses the practical utility of analytical methods across laboratory settings. With scores between 25 and 100, BAGI evaluates user friendliness, transferability, and routine applicability, supplementing the environmental focus of the other tools.

4.10. Application of the Validated Method to the Manufacturing Process of LCD20 Extract

4.10.1. Investigation of Extraction Methods. The quantification procedure was applied to determine the active ingredient content of the LCD20 herbal remedy. The study evaluated two extraction methods for LCD20 powder (approximately 5 g): reflux extraction and exhaustive percolation.

For reflux extraction, the powder was extracted under various conditions. The resulting extract was filtered, adjusted to the target volume, and passed through a 0.45 μm membrane filter into HPLC vials. The investigated conditions included ethanol concentration (0, 30, 50, 70, and 90% (v/v)), liquid-to-solid ratio (6, 8, 10, 12, and 16 mL/g), extraction temperature (40, 50, 60, and 70 °C), extraction time (0.5, 1, 2, 3, 4, and 5 h), and number of extractions (1, 2, and 3 times).

For exhaustive percolation, the powder was moistened with solvent, allowed to swell for 2 h and then subjected to percolation at a flow rate of 0.5−1 mL/min. The collected extract was diluted to the target volume and passed through a 0.45 μm membrane filter. The investigated conditions included ethanol concentration (0, 30, 50, 80, 90, and 99.5% (v/v)), liquid-to-solid ratio (4, 8, 10, 12, and 16 mL/g), and cold soaking time (1, 2, 3, 4, 6, 7, and 24 h).

To select the optimal extraction method, the yields of DHM, VTX, and ISV obtained from both methods under the preliminary optimized conditions were compared. A t-test was used to determine whether the mean differences between the two methods were statistically significant (p < 0.05). This comparison aimed to identify the most effective extraction method for further optimization.

4.10.2. Response Surface Methodology Experimental Design. The optimization of the LCD20 extraction process was achieved by using quantitative methods. Experimental design and statistical methods were applied to model and analyze situations in which multiple variables impact the outcome. Design of experiments (DOE) provides a systematic approach for identifying optimal process conditions. Response surface methodology (RSM) is an effective optimization strategy that utilizes mathematical and statistical techniques to create experimental models.

A Box−Behnken design (BBD) based on RSM was used to optimize ethanol concentration (A), liquid-to-solid ratio (B), and extraction time (C). BBD was preferred over a central composite design (CCD) to avoid axial runs that could push ethanol concentration beyond feasible limits. The extraction yield of DHM, VTX, and ISV from the LCD20 herbal remedy served as the dependent responses. Each parameter was evaluated at three levels. Based on the experimental design, a total of 15 experiments were performed, including three replicates at the center point and 12 edge points. Extraction conditions for DHM, VTX, and ISV were optimized using Design-Expert 13 software. This methodical approach offers an efficient strategy for optimizing the extraction of bioactive compounds from the LCD20 remedy.

4.11. Data Processing

Microsoft Excel was used for data analysis, and differences were considered significant at p < 0.05. Extraction conditions were optimized using Design-Expert software (ver. 13.0, Stat-Ease Inc., Minnesota, USA).

https://doi.org/10.1021/acsomega.6c00078
ACS Omega 2026, 11, 30926−30935

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.6c00078.

Method development chromatograms, validation data, and extraction ANOVA analyses for DHM, VTX, and ISV (PDF)

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Funding

Ministry of Education and Training of Vietnam (grant no. B2024-DHH-03).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was funded by the Ministry of Education and Training of Vietnam under grant no. B2024-DHH-03.

■ ABBREVIATIONS

ANOVA	analysis of variance
AGREE	analytical greenness calculator
AOAC	association of official analytical chemists
BAGI	blue applicability grade index
BBD	Box−Behnken design
CCD	central composite design
ComplexGAPI	complementary green analytical procedure index
DOE	design of experiments
GAC	green analytical chemistry
HPLC	high-performance liquid chromatography
HPTLC	high-performance thin layer chromatography
ICH	international conference on harmonization
RSD	relative standard deviation
PDA	photodiode array.

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