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Research Article

# Development of a Simplified Heart-Cutting Two-Dimensional Liquid Chromatography Method for Green Analysis of Four Polyphenols in *Aerides* Plants

Pornpoom Angsuwattana, Wisut Wichitnithad, Yanyong Punpreuk, Hasseri Halim, Boonchoo Sritularak, Worathat Thitikornpong,* and Pornchai Rojsitthisak

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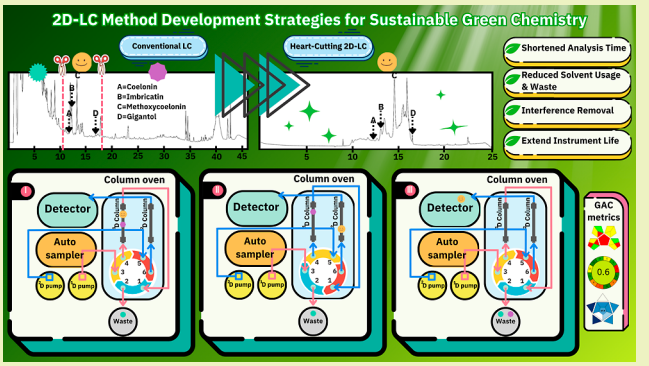
**ABSTRACT:** Thai traditional medicine utilizes herbs to treat various ailments, but robust quality control measures are crucial to ensuring their safety and efficacy. Quantifying the active compounds is a key approach for achieving this. *Aerides* plants, distributed in southeast Asia, are epiphytic orchids containing various bioactive compounds, including alkaloids, coumarins, flavonoids, terpenoids, and polyphenols. Various polyphenols such as coelonin, imbricatin, 6-methoxycoelonin, and gigantol are present in *Aerides* species and can act as analytical markers for orchid plants. This study proposes an innovative strategy using single heart-cutting 2D-LC to quantify phytochemicals in *Aerides* plants. In addition, the developed method emphasizes the importance of integrating green analytical chemistry metrics into analytical method development, thereby offering insights for potential enhancements. The method utilized BetaSil (C18, 4.6 × 100 mm, 3.0 $\mu$m) and HALO (C18, 4.6 × 100 mm, 5.0 $\mu$m) columns for chromatographic separation employing gradient elution, with a mobile phase comprising 0.1% formic acid in water and 0.1% formic acid in acetonitrile. These chromatographic conditions gave excellent peak shape and resolution. This method was validated following the ICH Q2(R1) and AOAC guidelines. The method demonstrated linearity within the range of 0.12−2.40 $\mu$g/mL, with a coefficient of determination ($r^2$) exceeding 0.995. The method's accuracy, indicated by % recovery, varied between 90% and 110%. Its precision, represented by % CV, was less than 7.4%. Furthermore, the environmental sustainability of the proposed single heart-cutting technique in 2D-LC was evaluated by using in-silico tools such as GAPI, AGREE, and BAGI software. This developed method also emphasizes environmental sustainability to reduce adverse environmental impacts.




**KEYWORDS:** *green analytical chemistry, two-dimensional chromatography, phytochemical analysis, chromatographic optimization, Aerides, orchids*



## 1. INTRODUCTION

In plant-based research, analyzing phytochemicals is crucial to understanding the bioactive compounds that contribute to the therapeutic efficacy of plant-derived products.[1,2] High-performance liquid chromatography (HPLC) is vital for precisely separating, identifying, and quantifying complex phytochemical matrices.[3] However, traditional HPLC faces challenges due to the diversity and dynamism of plant compounds,[4,5] highlighting the need for advanced techniques and sustainable practices. The ecological impact of conventional HPLC methods, primarily due to organic solvents, is concerning, necessitating the integration of green chemistry principles to minimize chemical waste, reduce solvent use, and enhance sustainability.[6] The emergence of Green Chemistry nearly three decades ago and, subsequently, Green Analytical Chemistry (GAC) in 2000 has significantly influenced sustainable analytical laboratory practices.[7,8] New analytical methods should focus on enhancing both the analytical capabilities and environmental friendliness.

To enhance the analytical capabilities of traditional HPLC in phytochemical research, several innovative techniques have been developed.[9,10] Micro-HPLC and advanced detection systems like mass spectrometry not only reduce solvent usage but also improve the identification of phytochemicals,[11] while the use of greener, biodegradable solvents and automated

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systems enhances reproducibility and sustainability. The advent of ultrahigh performance liquid chromatography (UHPLC) and two-dimensional liquid chromatography (2D-LC), including the heart-cutting technique, has significantly improved separation efficiency and accuracy, minimizing environmental impact by decreasing solvent consumption.[12−14] Additionally, the evolution from offline to online 2D-LC systems has further reduced sample contamination and loss, proving especially effective in reducing matrix interference through the LC−LC heart-cutting approach.[15,16] This approach simplifies sample preparation, requires smaller sample volumes, and maintains or improves detection limits, as evidenced by a study on the rapid, efficient assessment of bioactive markers in plants, setting new benchmarks for sustainable analytical practices.[17]

Allowing comprehensive comparisons to select the most suitable methodologies, the selected methods should be evaluated to ensure their adherence to GAC metrics.[18] In this evaluation, three open-source platforms are utilized to assess the environmental impact of analytical methods: the Green Analytical Procedure Index (GAPI),[19] the Analytical GREEnness Calculator (AGREE),[20] and the Blue Application Grade Index (BAGI).[21] GAPI evaluates the environmental sustainability of an entire analytical methodology, facilitating comparisons with other procedures but does not consider the synthesis part before sample preparation, lacks information on the structure of hazards, and does not cover all 12 principles of GAC. AGREE addresses all perspectives within the GAC principles and offers easily interpretable results, making it adaptable to different contexts and needs, but it also overlooks the synthesis part and the practicality of the methods. BAGI focuses on the practicality of the method and adjusts biases within realistic ranges by considering the field of application, though it provides insufficient quantitative analysis of the method's environmental attributes. Together, these tools help researchers evaluate and select the most suitable methodologies by balancing analytical capabilities and environmental friendliness.

To demonstrate the transformative impact of green chemistry in phytochemical studies and establish new sustainability standards, we developed a novel analytical method employing two-dimensional liquid chromatography (2D-LC) with a heart-cutting technique. This method is meticulously optimized for analyzing four phytochemicals in *Aerides* orchids using UHPLC to enhance the analytical efficiency and minimize the environmental impact. The targeted phytochemicals, coelonin, imbricatin, 6-methoxycoelonin, and gigantol (Figure 1), are polyphenolic compounds known for their antioxidant and anti-inflammatory properties.[1,2,22−24] The selection of coelonin, gigantol, imbricatin, and 6-methoxycoelonin as bioactive markers is supported by previous studies that have identified and quantified these compounds in *Aerides* orchids and other related species.[22−26] Their abundance in these plants, along with their notable pharmacological activities, underscores their suitability as key phytochemical markers for the quality control and phytochemical profiling of *Aerides* species. Currently, there is a lack of robust analytical methods that combine enhanced efficiency with minimized environmental impact for the quantification of these bioactive markers. In this study, we bridge this gap by developing a simplified heart-cutting 2D-LC method that effectively overcomes these challenges. Compared to conventional HPLC-based methods for analyzing orchid extracts, such as those reported by Cakova et al. and Wang et al.,[24,27] our approach offers superior specificity and sensitivity, significantly reduces solvent consumption, and shortens the analysis time by nearly 50%. This novel method demonstrates its potential as a robust and sustainable alternative for phytochemical analysis, addressing the limitations of existing methodologies.

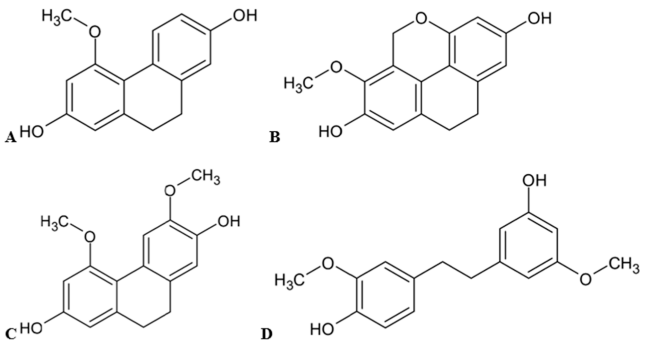


**Figure 1.** Structure of the bioactive compounds. (A) coelonin, (B) imbricatin, (C) 6-methoxycoelonin, and (D) gigantol.

## 2. EXPERIMENTAL SECTION

**2.1. Chemicals and Reagents.** Standard compounds of coelonin, 6-methoxycoelonin, imbricatin, and gigantol (purity ≥99%) were generously provided by Associate Professor Dr. Boonchoo Sritularak from the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University (Bangkok, Thailand). HPLC-grade acetonitrile and methanol were obtained from Fisher (Seoul, Korea). Formic acid was sourced from Carlo Erba (Cornaredo, Italy). High-purity water was prepared in-house using a Purelab Flex 2 system (ELGA LabWater, IL, USA).

**2.2. Plant Material and Sample Preparation.** The root of *Aerides houlletiana* and the root and leaves of *Aerides krabiense* were purchased from local markets in Bangkok, Thailand, and authenticated by the experts, Associate Professor Dr. Boonchoo Sritularak and Mr. Yanyong Punpreuk. The voucher specimens were deposited at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. In addition, the root of *A. krabiense* was selected as a representative sample due to its comparatively low concentration of the phytochemical compounds of interest and high matrix effect, making it a suitable surrogate matrix for the validation study. All samples were dried at 60 °C and then ground to powder, passed through a 40-mesh screen sieve before extraction. An amount of 250 mg of each powdered plant sample was accurately weighed in a 15 mL screw-cap test tube. Subsequently, 5 mL of methanol was added, and the sample was macerated at 50 °C for 60 min. After that, the mixture was subsequently sonicated for 45 min and centrifuged at 5500 rpm and 20 °C for 10 min. The supernatant was filtered through a 0.22 μm nylon membrane filter before analysis.

**2.3. Instruments and Software.** The Thermo Scientific Vanquish UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) was employed for method development and validation. The system comprised a dual pump with binary solvent capability, a column oven with two six-port valves, an autosampler, and a PDA detector. Instrument control and data acquisition were managed by using Chromeleon 7.3.2 software. The first-dimensional ($^1$D) analytical column was BetaSil (C18, 4.6 × 100 mm, 3.0 μm), and the second-dimensional ($^2$D) analytical column was HALO (C18, 4.6 × 100 mm, 5.0 μm).

**2.4. Preparation of a System Suitability Solution.** Four standard stock solutions of coelonin, 6-methoxycoelonin, imbricatin, and gigantol were individually prepared by dissolving 5 mg of each compound in methanol within 25 mL volumetric flasks (200 μg/mL). An aliquot of 0.6 mL from the standard stock solution was transferred to a 5 mL volumetric flask and diluted with methanol to prepare a


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working standard solution of each compound at a concentration of 24 μg/mL.

A system suitability solution was generated by cospiking standard solutions of coelonin, 6-methoxycoelonin, imbricatin, and gigantol to achieve a mixture concentration of 2.4 μg/mL. The system suitability solution was subjected to filtration using a 0.22 μm nylon membrane filter before analysis.

**2.5. Calibration Curve Preparation.** A five-point calibration curve for all compounds was constructed between the range 0.12−2.40 μg/mL. Calibration samples were obtained by diluting the standard stock solution of each component. The solution was subjected to filtering utilizing a 0.22 μm nylon membrane filter prior to analysis.

**2.6. Chromatographic Conditions.** The chromatographic system utilized two solvents: solvent A (0.1%, v/v, formic acid in water) and solvent B (0.1%, v/v, formic acid in acetonitrile). The gradient program and valve switching times were optimized for effective chromatographic separation.

Initially, both pumps were operated at a flow rate of 1.0 mL/min. The $^{1}$D pump started at 20% solvent B, increasing to 30% at 3 min, 33% at 8 min, and reducing to 0.2 mL/min at 8.1 min while maintaining 33% solvent B. At this point, the valve position switched from 1_2 to 6_1 to transfer the target analytes to the second column. The $^{1}$D pump maintained a flow rate of 0.2 mL/min, while the $^{2}$D pump operated at 1.0 mL/min with 30% solvent B. From 11 min onward, the $^{1}$D pump continued at 0.2 mL/min without solvent B, while the $^{2}$D pump remained at 1.0 mL/min with 30% solvent B, switching to 40% at 13 min. At 15 min, the valve switched back to position 1_2, and the $^{1}$D pump flow rate returned to 1.0 mL/min with 100% solvent B. The $^{2}$D pump did not use solvent B until 19 min, when both pumps operated at 1.0 mL/min with 20% solvent B in the $^{1}$D pump and no solvent B in the $^{2}$D pump. At 23 min, the $^{2}$D pump reached 100% solvent B, and by 25 min, both pumps returned to their initial conditions: the $^{1}$D pump with 20% solvent B at 1.0 mL/min and the $^{2}$D pump with 30% solvent B at 1.0 mL/min. Valve positions were strategically shifted between 1_2 and 6_1 at designated times to optimize phase transitions and enhance analytical performance. The detailed control of solvent gradients and valve timing is summarized in Table S3 of the Supporting Information.

**2.7. Method Validation.** The developed method was validated following the ICH Q2(R1) and AOAC guidelines, which provide criteria for validating analytical procedures.[28,29] Validation parameters included system suitability, specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), robustness, and stability.

*2.7.1. System Suitability.* System suitability tests were conducted to assess the repeatability of injections and the performance of the chromatographic system before validation experiments and routine analyses. Five replicates of a system suitability solution containing a mixture of coelonin, imbricatin, 6-methoxycoelonin, and gigantol at a concentration of 2.4 μg/mL were injected to evaluate repeatability and chromatographic performance. System reproducibility was determined by variations (% CV ≤ 2%) in retention time and peak area, indicating the precision of injections.[30] The performance of the chromatographic system was further evaluated through parameters such as resolution ($R > 2$) between adjacent peaks, USP tailing factor ($T < 2$), and the number of theoretical plates ($N > 2000$), demonstrating the efficiency of chromatographic separation and column performance.

*2.7.2. Specificity.* Specificity was assessed by injecting individual solutions of (1) the diluent (methanol), (2) standard solutions of each compound, and (3) a mixture of standard compounds spiked with the matrix along with (4) the matrix solution itself. Specificity was evaluated by checking for the absence of interference peaks at the same retention times as those of coelonin, imbricatin, 6-methoxycoelonin, and gigantol. The root of *A. krabiense* was chosen as the surrogate matrix for these experiments to ensure there was no interference from the matrix components.

*2.7.3. Linearity and Range.* Linearity was investigated by establishing five-point calibration curves over a concentration range of 0.12−2.40 μg/mL through three independent experiments conducted on separate days. Analyte peak responses ($y$) were plotted against varying concentrations ($x$), and a linear least-squares model was employed to derive linear equations. Linearity was confirmed through a coefficient of determination ($r^2 \geq 0.995$).[29] Statistical parameters such as $p$-values and $F$-values were computed to evaluate the linear regression.[31,32] The method's range was established by analyzing standard mixed spiked samples covering the same concentration range.

*2.7.4. Accuracy and Precision.* Accuracy requires a minimum of nine determinations across at least three concentration levels spanning the method's range. Precision experiments involved nine determinations across the range or six independent preparations at 100% of the specified amount within a sample. Accuracy was gauged by the % recovery, which should fall within 80−115%, and precision was measured by the %CV of the % recovery. Accuracy and precision were assessed through triplicate analyses of three concentration levels of mixed spiked standard samples for both repeatability (intraday) and intermediate precision (interday).

*2.7.5. Limit of Detection and Limit of Quantitation.* LOD and LOQ were determined by analyzing the signal-to-noise ratio ($S/N > 3$ for LOD and $S/N > 10$ for LOQ) from six replicate injections of standard solutions. The LOQ was further validated by achieving 80−115% recovery with %CV < 11 from six replicate injections of spiked samples. In this study, LOD and LOQ were assessed at nominal concentrations of 0.060 and 0.120 μg/mL, respectively.

*2.7.6. Robustness.* The robustness of the analytical method was tested by introducing slight variations in the chromatographic parameters. The formic acid content in the mobile phase was adjusted within a narrow range of ±0.01% from the baseline concentration of 0.1% v/v. Similarly, the column temperature was altered by ±1 °C from the standard setting of 30 °C. Precision measurements, including peak response and retention time, were recorded for the four compounds after these adjustments to ensure the method's reliability under varied conditions.

*2.7.7. Stability.* For stability assessment, the autosampler was set at 15 °C. Samples comprising both spiked and standard mixtures were injected at time intervals of 0, 4, 8, 16, and 24 h. The retention time and peak response were monitored with allowable deviations set at no more than 1%. This ensured that the compounds remained stable under the specified conditions for the duration of the analysis.

**2.8. Methodology for the Evaluation of Green Analytical Chemistry Metrics.** To ensure that the developed chromatographic method adheres to the principles of GAC, three key metrics were employed: the GAPI, AGREE metric, and BAGI. These metrics provide a comprehensive evaluation of the environmental impact, safety, and practicality of the analytical procedure. The GAPI, AGREE, and BAGI evaluations were interpreted to offer a thorough assessment of the method's adherence to green chemistry principles. The GAPI star diagram, AGREE circular diagram, and BAGI composite score visually represent and compare the method's greenness, comprehensiveness, and practicality, ensuring it meets the goals of sustainable and environmentally friendly analytical practices.

*2.8.1. Evaluation of the Green Analytical Procedure Index.* GAPI is a comprehensive tool used to evaluate the environmental sustainability of an analytical method. The GAPI assessment involves considering various aspects of the procedure, from sample preparation to final determination. Each step of the method is assigned a score based on its environmental impact, with lower scores indicating greener practices. Key factors assessed include the type and quantity of solvents used, energy consumption, waste generation, and potential hazards associated with the chemicals involved. The GAPI methodology involves creating a GAPI star diagram, which visually represents the greenness of the analytical method across multiple dimensions.

*2.8.2. Evaluation of the Analytical GREEnness (AGREE) Metric.* The AGREE metric provides a comprehensive evaluation of the environmental friendliness of the analytical procedure, covering all 12 principles of green chemistry and adapting to various analytical contexts.[20] Each principle is quantitatively assessed based on its


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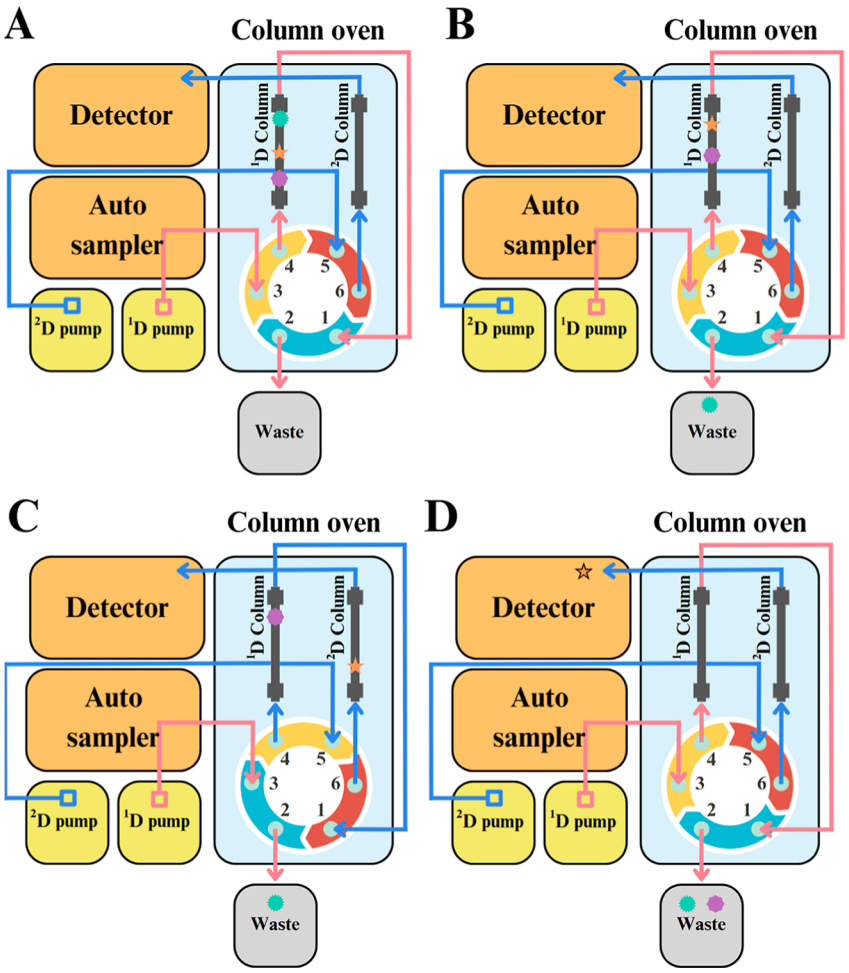


**Figure 2.** Panel (A) depicts the entirety of compounds eluted into the $^{1}$D column during the initial phase. The orange star symbolizes analytes of interest (in this case, it represents the four phytochemicals), while the green and purple entities denote impurity substances. Panel (B) illustrates the swift elution of unwanted polar matrix peaks from the $^{1}$D column into the waste stream. Panel (C) demonstrates that all intended analytes were successfully transferred to the second column, leading to the valve's activation to transition to position 1_6 for connecting the $^{1}$D column with the $^{2}$D column. Panel (D) illustrates that after the complete elution of all four phytochemical compounds (orange star) to the detector, the valve was returned to its original position (1_2) to prevent further impurities (purple entity) from entering the $^{2}$D column and UV detector.

implementation in the method, with scores assigned according to predefined scales. These scores are then aggregated to form an overall greenness score represented on a circular AGREE diagram. The final AGREE score, ranging from 0 to 1 (with 1 indicating the highest degree of greenness), is calculated by averaging the scores of all criteria.

*2.8.3. Evaluation of the Blue Applicability Grade Index.* BAGI focuses on the practicality and applicability of the analytical method in routine laboratory settings.[21] This metric evaluates the method based on criteria such as ease of implementation, cost-effectiveness, and compatibility with existing laboratory infrastructure. BAGI assigns scores to different aspects of the method, including the complexity of the procedure, availability of required equipment and reagents, and the method's robustness. The BAGI evaluation results in a composite score that reflects the overall applicability and practicality of the method, making it a valuable tool for routine analytical laboratories.

## 3. RESULTS AND DISCUSSION

**3.1. Method Development of the Heart-Cutting Approach.** Initially, a chromatographic condition sourced from the research literature was employed to obtain a comprehensive overview of the chromatogram.[24] These conditions were then optimized to distinguish between the desired and interfering peaks. Characterizing the chromatographic profile is crucial in determining the optimal mode for 2D-LC approaches. The heart-cutting approach was selected for this study due to its suitability, as detailed earlier.

In the heart-cutting 2D-LC method, the system employs a valve-switching mechanism to transfer specific fractions from the first to second dimension for further separation. This process improves the resolution and sensitivity while minimizing matrix interference. The valve positions are crucial for directing the flow of the sample: position 1_2 connects the first column to waste, diverting undesired components, while position 1_6 connects the first column to the second column, enabling the transfer of target analytes for further separation (Figure 2).

Once the sample was injected into the first column (Figure 2A) using the right pump ($^{1}$D pump), undesired polar matrices were quickly eluted to waste (position 1_2), as shown in Figure 2B. After 8 min, the desired analytes were transferred to the second column, prompting the valve to switch to position 1_6, as shown in Figure 2C. The $^{1}$D pump flow rate was reduced to 0.2 mL/min to conserve solvents. Once the phytochemicals were fully eluted onto the second column, the valve reverted to position 1_2 to prevent additional impurities


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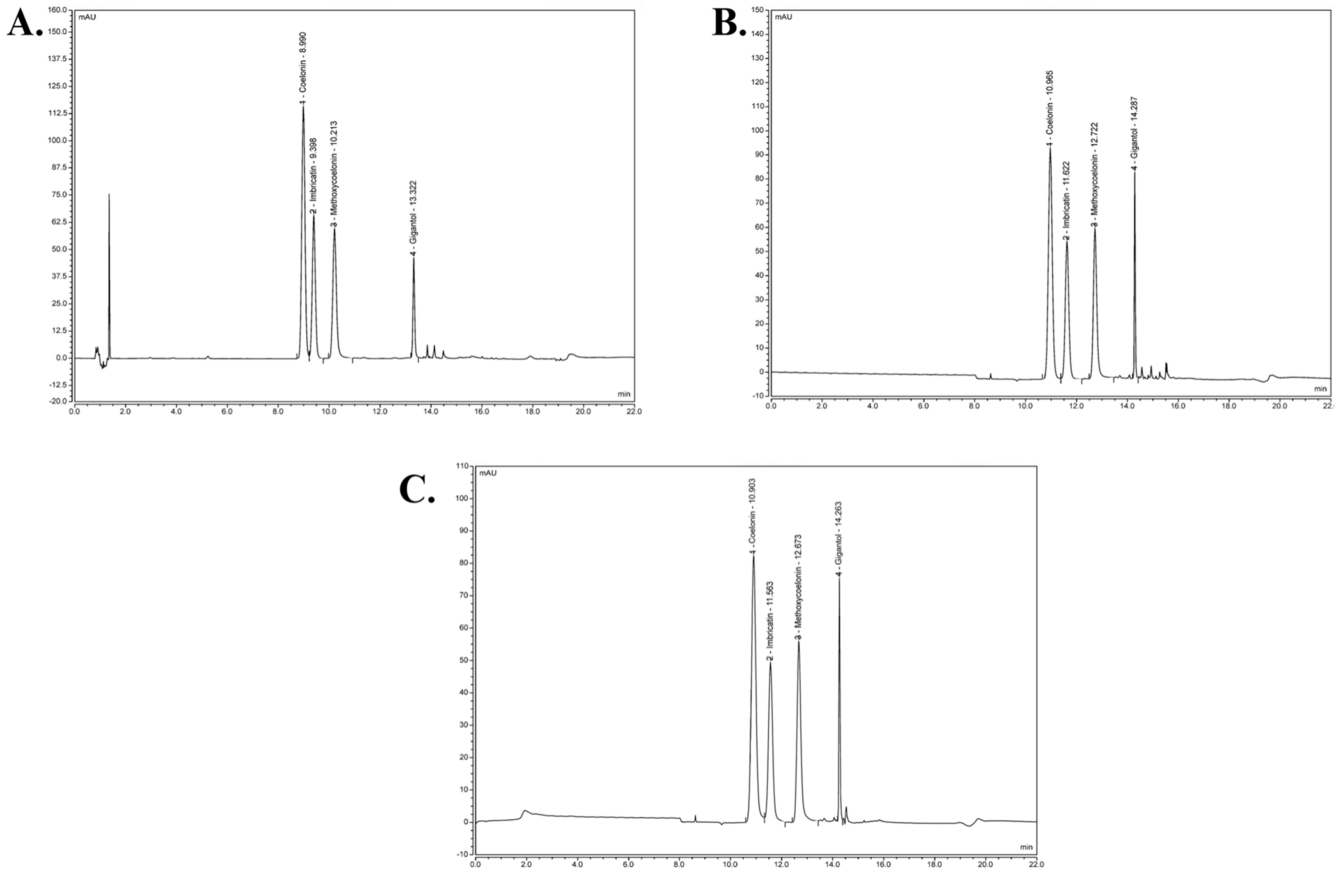


**Figure 3.** Chromatograms of standard mixed solution using (A) BetaSil (C18, 4.6 × 100 mm, 3.0 $\mu$m) as the $^{1}$D column connecting directly to a UV detector and (B) BetaSil (C18, 4.6 × 100 mm, 3.0 $\mu$m) and HALO (C18, 4.6 × 100 mm, 5.0 $\mu$m) as the $^{1}$D and $^{2}$D columns, respectively. (C) Chromatogram illustrates the enhanced chromatogram resulting from the accelerated valve switching. The preliminary chromatographic condition was modified from the research literature.[24]

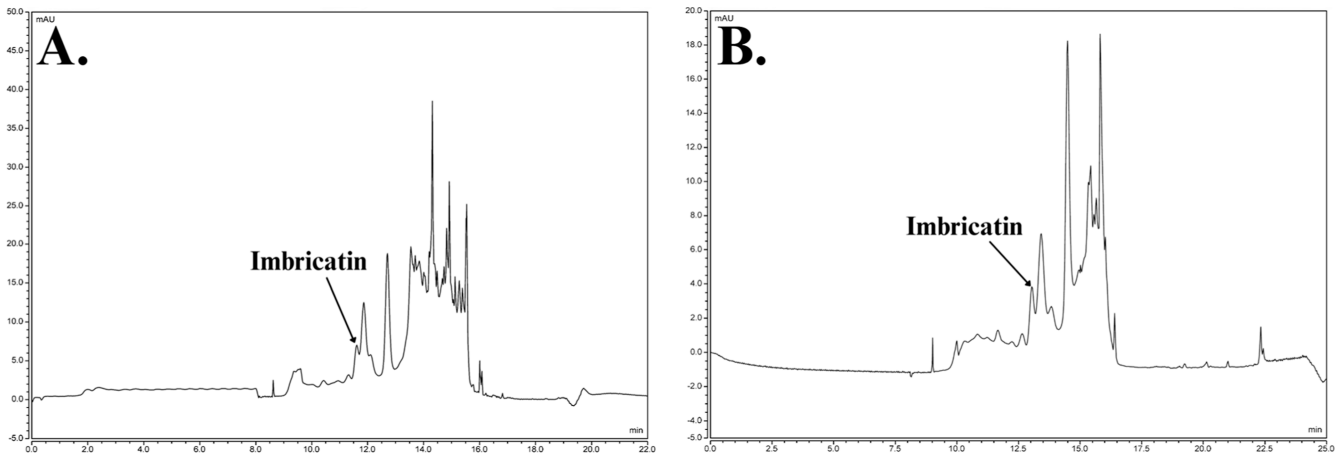


**Figure 4.** (A) Chromatogram of the matrix sample reveals a coelution peak alongside the imbricatin peak. (B) Chromatogram of the matrix sample employing the latest developed version of the gradient program illustrates the improvement of separation of imbricatin and coeluted compounds. Although a clear separation of the coeluting peak was not achieved, the analytical method could be successfully validated using perpendicular drop integration for the integration of imbricatin.

from entering the second column and UV detector, as shown in Figure 2D.

Method development commenced with optimization of the $^{1}$D column and the corresponding chromatographic conditions. The primary objective in the initial phase was the separation of impurities, which migrate faster in the column compared to the analytes. This separation was achieved using BetaSil (C18, 4.6 mm × 100 mm, 3.0 $\mu$m) as the $^{1}$D column (Figure 3). The $^{1}$D column was directly connected to the UV detector via a viper capillary, bypassing the six-port valves. The solvent gradient program was refined until a satisfactory resolution was achieved within a short analysis time. The preliminary assessment revealed that the first peak attributed to coelonin appeared at a retention time of 8.990 min, with a


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**Table 1. Summary of the System Suitability Results**

| compound | % CV ($n$ = 5) | | retention time | resolution | tailing factor | theoretical plate |
|---|---|---|---|---|---|---|
| | retention time | peak response | | | | |
| coelonin | 0.13 | 0.3 | 12.17 | 2.4 | 1.2 | 21,196 |
| imbricatin | 0.15 | 0.3 | 13.05 | 4.8 | 1.2 | 22,602 |
| 6-methoxycoelonin | 0.07 | 0.5 | 14.05 | 10.9 | 1.3 | 50,513 |
| gigantol | 0.01 | 0.5 | 16.41 | - | 1.2 | 515,357 |

**Table 2. Summary of Regression Equations, Correlation Coefficients, Signal-To-Noise of Detection, and Quantification Limit and Intra-Day and Inter-Day Accuracy and Precision Values of Polyphenols in *Aerides* Plants**

| compound | linearity ($n$ = 3) | | LOD S/$N$ ($n$ = 6) | LOQ S/$N$ ($n$ = 6) | concentration ($\mu$g/mL) | accuracy (% recovery) | | precision (% CV) | |
|---|---|---|---|---|---|---|---|---|---|
| | regression equation | $r^2$ | | | | intraday ($n$ = 3) | interday ($n$ = 3) | intraday ($n$ = 3) | interday ($n$ = 3) |
| coelonin | y = 0.755x − 0.00599 | 0.9999 | 12 | 31 | 0.12 | 103.6 | 103.9 | 0.9 | 3.2 |
| | | | | | 0.60 | 92.7 | 96.6 | 1.2 | 1.1 |
| | | | | | 2.40 | 94.2 | 94.9 | 2.6 | 1.4 |
| imbricatin | y = 0.424x − 0.0184 | 0.9997 | 6 | 14 | 0.12 | 91.7 | 100.2 | 9.3 | 1.9 |
| | | | | | 0.60 | 91.0 | 88.9 | 3.7 | 2.8 |
| | | | | | 2.40 | 94.1 | 89.2 | 0.7 | 2.0 |
| 6-methoxycoelonin | y = 0.457x − 0.0284 | 0.9996 | 8 | 19 | 0.12 | 103.7 | 109.4 | 5.3 | 4.2 |
| | | | | | 0.60 | 100.3 | 94.2 | 3.8 | 1.8 |
| | | | | | 2.40 | 98.1 | 97.2 | 1.6 | 1.4 |
| gigantol | y = 0.172x + 0.00131 | 0.9997 | 11 | 23 | 0.12 | 106.1 | 95.8 | 2.6 | 3.3 |
| | | | | | 0.60 | 97.9 | 95.5 | 2.4 | 0.7 |
| | | | | | 2.40 | 97.3 | 97.4 | 0.5 | 0.5 |

**Table 3. Robustness Tests Under the Variations of Column Temperature and % Formic Acid**

| compound | parameter change | % CV ($n$ = 5) | | retention time (min) | resolution | tailing factor | plate count |
|---|---|---|---|---|---|---|---|
| | | retention time | peak response | | | | |
| coelonin | no change | 0.06 | 0.28 | 12.3 | 2.3 | 1.1 | 20,750 |
| imbricatin | | 0.07 | 0.33 | 13.1 | 4.8 | 1.1 | 22,302 |
| 6-methoxycoelonin | | 0.03 | 0.48 | 14.5 | 10.7 | 1.3 | 49,011 |
| gigantol | | 0.01 | 0.32 | 16.4 | - | 1.2 | 500,259 |
| coelonin | column temperature (29 °C) | 0.15 | 0.21 | 12.4 | 2.4 | 1.2 | 21,112 |
| imbricatin | | 0.13 | 0.33 | 13.2 | 4.7 | 1.1 | 22,615 |
| 6-methoxycoelonin | | 0.07 | 0.52 | 14.6 | 10.8 | 1.3 | 53,873 |
| gigantol | | 0.02 | 0.32 | 16.4 | - | 1.2 | 512,410 |
| coelonin | column temperature (31 °C) | 0.14 | 0.30 | 12.1 | 2.4 | 1.1 | 20,848 |
| imbricatin | | 0.11 | 0.24 | 12.9 | 4.9 | 1.2 | 22,338 |
| 6-methoxycoelonin | | 0.06 | 0.41 | 14.4 | 10.7 | 1.3 | 44,309 |
| gigantol | | 0.02 | 0.13 | 16.4 | - | 1.3 | 502,700 |
| coelonin | percentage of acid (0.09% formic acid) | 0.16 | 0.50 | 12.3 | 2.3 | 1.1 | 20,573 |
| imbricatin | | 0.15 | 0.48 | 13.1 | 4.7 | 1.1 | 21,934 |
| 6-methoxycoelonin | | 0.08 | 0.42 | 14.5 | 10.7 | 1.3 | 48,871 |
| gigantol | | 0.02 | 0.35 | 16.4 | - | 1.2 | 500,811 |
| coelonin | percentage of acid (0.11% formic acid) | 0.22 | 0.23 | 12.2 | 2.3 | 1.2 | 20,661 |
| imbricatin | | 0.20 | 0.67 | 13.0 | 4.8 | 1.2 | 22,276 |
| 6-methoxycoelonin | | 0.10 | 0.23 | 14.5 | 10.7 | 1.2 | 48,541 |
| gigantol | | 0.02 | 0.29 | 16.4 | - | 1.2 | 500,781 |

resolution of 1.78 (Figure 3A). At this retention time, the proportion of solvent B was approximately 33% v/v. Therefore, the valve position should switch from 1_2 to 1_6 before the coelonin peak emerges, but it should remain at 1_2 long enough to divert undesired polar matrix peaks to waste.

After establishing the appropriate $^1$D column and chromatographic conditions, the next phase involved selecting the optimal $^2$D column and finalizing the chromatographic conditions. Various $^2$D columns were evaluated, including HALO (C18, 4.6 × 50 mm, 2.7 $\mu$m), Inertsil (C8−3, 4.6 × 100 mm, 3.0 $\mu$m), and Inertsil (Ph-3, 3.0 × 100 mm, 5.0 $\mu$m). The chromatograms are shown in Figure S1 of the Supporting Information. Despite these efforts, the resolution between coelonin and imbricatin remained below 2.0. Ultimately,


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HALO (C18, 4.6 × 100 mm, 5.0 μm) was selected for the second column (Figure 3B). Efforts were made to minimize impurity peaks trailing behind gigantol by adjusting the valve's turnaround time as shown in Figure 3C. Peaks emerging last showed increased intensity after the valve switched back to position 1_2, indicating improved sensitivity for gigantol. However, a coelution peak was observed with imbricatin in a matrix sample, as shown in Figure 4A. Despite meticulous optimization of the gradient program, clear separation of the coeluting peak was not achieved (Figure 4B), likely due to similar chemical structures or properties of secondary metabolites in plants. Nonetheless, integration of the imbricatin peak was calculated using perpendicular drop integration.

**3.2. Analytical Method Validation.** The validated heart-cutting 2D-LC method offers significant advantages over conventional 1D-LC for analyzing complex matrices such as *Aerides* orchid extracts. By employing a two-step separation process, it effectively isolates the target analytes—coelonin, gigantol, imbricatin, and 6-methoxycoelonin—while minimizing matrix interferences. System suitability results (Table 1) confirm the method's robustness, demonstrating high resolution ($R$ > 2), low tailing factors (<1.3), and superior theoretical plate counts (>5000). These optimized chromatographic conditions ensure accurate and reliable separation, in accordance with ICH and AOAC validation guidelines. As shown in Table 2, the concentration range for linearity spanned from 0.12 to 2.40 μg/mL, exhibiting a mean slope (±standard error of mean) of 0.755 ± 0.016, 0.424 ± 0.011, 0.457 ± 0.011, and 0.171 ± 0.003 for coelonin, imbricatin, 6-methoxycoelonin, and gigantol, respectively, with the coefficient of determination ($r^2$) values ranging from 0.9996 to 0.9999. Accuracy and precision of the proposed method were evaluated at 0.12, 0.60, and 2.40 μg/mL by calculating the % recovery and %CV of three replicates for each compound, yielding values of 88.9−109.4% for %recovery and 0.5−9.3% for %CV, respectively. Additionally, the concentration of 0.12 μg/mL (LOQ) exhibited a signal-to-noise ratio within the range of 14−31, surpassing the threshold of 10, thus indicating acceptability for the lower level of quantification. Furthermore, the developed method demonstrated robustness in response to variations in the temperature and acid content (Table 3). Both standard and sample solutions exhibited good stability when stored in the autosampler at 15 °C for 24 h. For additional detailed information on the validation results, please refer to the Supporting Information.

**3.3. Application for Phytochemical Compounds Determination in an Orchid Sample.** The roots of *A. krabiense* (AKR) and *A. houlletiana* (AHR), along with the leaves of *A. krabiense* (AKL), were quantified by utilizing the validated method. The findings of the analysis are presented in Table 4. Notably, coelonin and imbricatin were not detected in the AHR sample. However, the concentration of 6-methoxycolonin detected in both the AKR and AKL samples exceeded the validated range. Consequently, it was recommended to dilute the AKR and AKL samples by a factor of 2 before quantification.

The method's ability to resolve complex matrices with high specificity and sensitivity makes it suitable for analyzing a wide range of phytochemicals, such as alkaloids, flavonoids, and terpenoids. With minor adjustments to chromatographic conditions, including column selection and mobile phase composition, the method could be tailored to target compounds with varying polarities and chemical properties. Future studies could explore this versatility to further establish the method's applicability to diverse classes of secondary metabolites, supporting its use in phytochemical research and quality control of herbal products.

**Table 4. Phytochemical Compound Determination in *Aerides* Plant Samples[a]**

| name | part | amount (μg/mL) | | | |
|---|---|---|---|---|---|
| | | coelonin | imbricatin | 6-methoxycoelonin | gigantol |
| *Aerides krabiense* | root | - | 1.583 | 5.681 | 0.735 |
| *Aerides krabiense* | leaf | - | 2.482 | 2.979 | - |
| *Aerides houlletiana* | root | - | - | 0.774 | 0.691 |

[a]-means a compound is not detected.

**3.4. Evaluation of Green Analytical Chemistry Metrics.** *3.4.1. Evaluation of the Green Analytical Procedure Index.* The plants were sourced from a specific location, thus constituting an off-line collection method. Subsequently, the plants underwent grinding and drying before being transported to the analytical laboratory without preservation. The resultant plant powders were stored under normal conditions. Extraction of the plant powders was conducted via heat-mashing in methanol, which is recognized as an environmentally friendly solvent. The total volume of solvents utilized in sample preparation and analysis did not exceed 100 mL. Notably, the primary hazardous reagent employed was formic acid, possessing an National Fire Protection Association (NFPA) health hazard score of 3. Acetonitrile and methanol, both with an NFPA flammability score of 3, represented the most flammable components used. As indicated by Agilent Technologies Inc., energy consumption per sample was estimated to be below 1.5 kWh.33 Waste generation, primarily from mobile phase elution, exceeded 10 mL and was appropriately managed through outsourcing for proper treatment. Figure 5A illustrates the GAPI assessment of the environmental sustainability of the developed analytical method for detecting specific phytochemical compounds in the orchid.

GAPI offers a comprehensive environmental assessment of the entire analytical process visually represented through a star diagram. However, it does not consider the practical applicability of the method in routine laboratories, particularly in terms of cost-effectiveness, accessibility, and infrastructure compatibility, which are critical factors for widespread adoption.

*3.4.2. Evaluation of the Analytical GREEnness Metric.* The plants were initially obtained from a specific location and subsequently transported to the analytical laboratory, constituting an off-line analysis. A sample weighing 0.25 g of plant powder was utilized in the preparation process. This off-line measurement involved a three-step sample preparation procedure: heat maceration, sonication, and centrifugation. While the procedure was semiautomated, it was not miniaturized. Derivatization agents were not utilized in the analysis. Analytical wastes comprised the sample itself (0.25 g), 5 mL of methanol, 50 mL of acetonitrile, and 0.1 mL of formic acid; however, the usage of pure water was not considered to be waste. Four analytes were determined in a single run, and the analytical throughput was single sample $h^{-1}$ as heat


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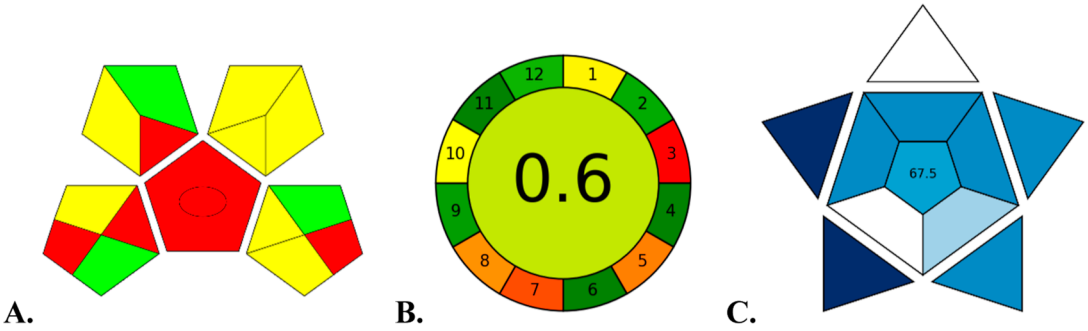


**Figure 5.** Outcomes of the greenness assessment conducted on the developed analytical method employing GAC metrics: (A) GAPI, (B) AGREE, and (C) BAGI.

maceration extraction took 60 min. The estimated power consumption for a single analysis was approximately 0.25 kW h. Some of the reagents, such as alcohols, could potentially be sourced from biobased origins. The method abstained from employing toxic reagents and solvents. Notably, methanol and acetonitrile were classified as highly flammable based on their high NFPA flammability scores. Therefore, the assigned AGREE score for the developed method was 0.6 (Figure 5B).

AGREE addresses all 12 principles of GAC into a single adaptable scoring system, providing a broad assessment of a method's sustainability. However, it lacks detailed quantitative metrics to evaluate the toxicity of reagents and solvents, making certain aspects of the assessment somewhat subjective and dependent on interpretation rather than precise quantification.

*3.4.3. Evaluation of the Blue Applicability Grade Index.* The developed method was employed to quantify four phytochemicals in orchid extracts. This determination facilitated the quantification of four compounds spanning three distinct classes: coelonin and 6-methoxycoelonin, classified as phenanthrenes; imbricatin, categorized as a phenanthropyrans; and gigantol, designated as bibenzyles.[23] Utilizing 2D-LC instruments, which are not widely available in most laboratories, the method assumed simultaneous sample preparation for approximately 10 samples. In contrast to AGREE, the sample throughput of BAGI was computed from sample preparation (70 min) to the end of analysis (25 min) for a single sample, resulting in a sample throughput of 0.63 $h^{-1}$. Common commercially available reagents were utilized, and no preconcentration was necessary. The use of an autosampler enabled semiautomation. A sample weighing 250 mg of plant powder was utilized. The assigned BAGI score for the developed method was 67.5 (Figure 5C), affirming its practicality and relevance.

BAGI is particularly useful for assessing the practicality and applicability of analytical methods in routine laboratory settings. It incorporates factors such as cost-effectiveness, ease of implementation, and compatibility with existing infrastructure, making it a valuable tool for real-world applications. However, BAGI relies on subjective scoring criteria for attributes like practical feasibility and ease of use, which may introduce bias based on the scorer's experience and familiarity with the technique.

## 4. CONCLUSIONS

This study successfully developed a novel heart-cutting 2D-LC method for the simultaneous quantification of four key polyphenols in *Aerides* orchids, a species that is significant in Thai traditional medicine. The method demonstrates high sensitivity, selectivity, and matrix interference reduction, making it adaptable to other orchid species and medicinal plants with similar phytochemical compositions. By adhering to ICH Q2(R1) and AOAC guidelines, it provides a robust foundation for quality control and herbal product standardization, ensuring batch-to-batch consistency in herbal formulations. This targeted approach is particularly valuable in regulatory frameworks, where precise bioactive marker quantification supports herbal medicine authentication, safety, and efficacy assurance. Additionally, this study emphasizes the importance of evaluating GAC metrics for sustainable analytical method development and serves as a reference for future improvements in phytochemical analysis and regulatory compliance.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.4c10145.

Chromatographic conditions, green analysis parameters, and analytical method validation results (PDF)

## ■ AUTHOR INFORMATION

### Corresponding Author

**Worathat Thitikornpong** − *Center of Excellence in Natural Products for Ageing and Chronic Diseases, Chulalongkorn University, Bangkok 10330, Thailand; Department of Food and Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand;* orcid.org/0000-0002-3537-3646; Email: Worathat.t@pharm.chula.ac.th

### Authors

**Pornpoom Angsuwattana** − *Center of Excellence in Natural Products for Ageing and Chronic Diseases, Chulalongkorn University, Bangkok 10330, Thailand; Department of Analytical Development, Pharma Nueva Co., Ltd., Bangkok 10900, Thailand*

**Wisut Wichitnithad** − *Department of Analytical Development, Pharma Nueva Co., Ltd., Bangkok 10900, Thailand*

**Yanyong Punpreuk** − *Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok 10200, Thailand*

**Hasseri Halim** − *Integrative Pharmacogenomics Institute (iPROMISE), Universiti Teknologi MARA Selangor, Bandar Puncak Alam 42300 Selangor, Malaysia; Faculty of Pharmacy, Universiti Teknologi MARA Selangor, Bandar Puncak Alam 42300 Selangor, Malaysia*



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**Boonchoo Sritularak** − *Center of Excellence in Natural Products for Ageing and Chronic Diseases, Chulalongkorn University, Bangkok 10330, Thailand; Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand;* orcid.org/0000-0001-8352-4122

**Pornchai Rojsitthisak** − *Center of Excellence in Natural Products for Ageing and Chronic Diseases, Chulalongkorn University, Bangkok 10330, Thailand; Department of Food and Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand;* orcid.org/0000-0003-1391-6993

Complete contact information is available at: https://pubs.acs.org/10.1021/acssuschemeng.4c10145

### Author Contributions

P.A. Methodology, investigation, formal analysis, and writing—original draft. W.W. Methodology, investigation, formal analysis, and writing—review and editing. Y.P. Resources. H.H. Investigation and writing—review and editing. B.S. Investigation and writing—review and editing. W.T. Conceptualization, methodology, investigation, formal analysis, supervision, resources, project administration, and writing—review and editing. P.R. Conceptualization, supervision, methodology, formal analysis, and writing—review and editing.

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

NFPA, national fire protection association; GAC, green analytical chemistry; 2D-LC, two-dimensional liquid chromatography; AGREE, analytical GREEnness calculator; BAGI, blue applicability grade index; GAPI, green analytical procedure index

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