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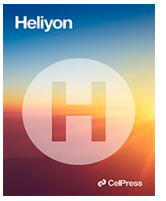

Research article

Development of a high-speed and ultrasensitive UV/Vis-CM for detecting total triterpenes in traditional Chinese medicine and its application

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ABSTRACT

This study proposes a novel colorimetric method based on the ultraviolet/visible spectrophotometry-colorimetric method (UV/Vis-CM) for detecting and quantifying total triterpenoids in traditional Chinese medicine. By incorporating the colourants 2-hydroxy-5-methylbenzaldehyde and concentrated sulfuric acid, triterpenoid compounds colour development became more sensitive, and the detection accuracy was significantly improved. 2-hydroxy-5-methylbenzaldehyde and concentrated sulfuric acid were incorporated in a 1:3 vol ratio at room temperature to react with the total triterpenes for 25 min, incorporated to an ice bath for 5 min, and then detected at the optimal absorption wavelength. The accuracy and reliability of this method were verified by comparison with high-performance liquid chromatography and four other colorimetric methods. Additionally, this approach has the advantages of not requiring heating during operation, high sensitivity, short usage time, low solvent usage, and low equipment costs. This study not only offers a reliable method for detecting total triterpenes in traditional Chinese medicine but also offers a rapid detection tool for on-site testing and large-scale screening, laying a foundation for the modernization of traditional Chinese medicine research, quality control, and drug development.

1. Introduction

Traditional Chinese Medicine (TCM) plays a crucial role in the Chinese medical system. Its rich, complex composition and wide-ranging pharmacological properties, characterized by multi-target and multi-pathway effects, enable TCM to offer holistic and harmonizing interventions for a variety of ailments. This unique approach allows TCM to holistically regulate the body to address health issues, contrasting sharply with the more focused interventions typical of Western medicine [1]. In contrast to the single-component, single-target mode of action of Western medicine, the complexity of the multi-component and multi-target action of TCM makes its research extremely complicated. The study of the mechanism of action and safety of TCM necessitates the utilisation of a diverse range of professional knowledge, including TCM chemistry, drug analysis, pharmacotoxicology, and pharmacokinetics. Among these disciplines, compositional analysis (comprising qualitative and quantitative analyses) of TCM chemistry serves as the fundamental basis for studying TCM; therefore, it is crucial to establish an accurate and appropriate method for detecting and analysing the chemical constituents present in TCM. TCM (including Chinese medicinal materials, decoction pieces, and proprietary Chinese

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medicines) contains several effective ingredients, including flavonoids [2], phenylpropanoids [3], anthraquinones [4], terpenoids [5], triterpenoids [6] and their classes, steroids and their glycosides, alkaloids [7]. Their structures and properties differ; therefore, it is essential to design appropriate detection and analysis methods for diverse components.

Triterpenoids are extensively present in plants, including licorice [8], ginseng [9], hawthorn, bupleurum [10], animals and fungi [11]. Most contain 30 carbon atoms derived from methylglutaric acid with six isoprene units [12]. Studies have demonstrated that triterpenoids predominantly have pharmacological activities [13], including antitumor[[14,15]], anti-inflammatory [16], antiviral [17], blood lipid reduction [18], antibacterial activity [19] and liver protection [20]. Currently, the primary methods for determining total triterpene content include ultraviolet/visible spectrophotometry - colorimetry (UV/Vis-CM), high-performance liquid chromatography-diode array detector (HPLC-DAD) [21], and high-performance liquid chromatography-evaporative light scattering detection (HPLC-ELSD) [22]. Among these techniques, UV/Vis-CM is an extensively used classical approach for analysing traditional Chinese medicine active components [23], which typically involves a reaction between the substance to be measured and one or more reagents to generate a highly colored product. The absorbance of the product was directly proportional to the target substance concentration in the original solution [24]. By selecting an appropriate wavelength that corresponds to the specific target substance absorption, its concentration or mass in an unknown sample can be computed by comparing its absorbance to that of a standard solution. High-performance liquid chromatography (HPLC) is extensively employed for detecting individual compounds due to its exceptional sensitivity, accuracy, and reproducibility. When using HPLC to analyse specific components within a mixture, it is crucial for these components to achieve baseline separation and exhibit peak heights at least three times higher than the background noise [25]. In cases where baseline separation cannot be achieved, or low-level components are difficult to detect owing to structural or polarity similarities among diverse analytes, HPLC may yield lower detection values than UV/Vis. Compared to UV/Vis, HPLC has some disadvantages, including a longer HPLC time, more reagents, and a higher cost of equipment and consumables. Conversely, UV/Vis stands out for its simplicity and rapidity, as it not only uses fewer reagents but also has relatively low equipment and consumer costs. Therefore, when selecting the detection method, it is essential to comprehensively consider the experimental conditions, sample properties and economic costs, and other factors to determine the most suitable detection scheme. Owing to some of the limitations of HPLC in practical applications, UV–Vis remains a competitive alternative.

Currently, there are four major colour development methods for UV/Vis-CM detection of total triterpenes: sulfuric [26], vanillin-sulfuric [27], perchloric [28] and vanillin-perchloric acid [29]. Kurkin et al. [26] used the sulfuric acid method (70 %) to determine the total saponin content in ginseng syrup. Although this method was successfully used for content determination, the detection results were low, indicating that there is still room for improvement. Marija et al. [30] used the vanillin perchloric acid method to determine the terpenoid content in diverse moss extracts and subsequently conducted pharmacological activity studies on them; Aguiara et al. [31]used vanillin sulfuric acid spectrophotometry to determine the total saponins in *Ephedra equisetifolia* leaves. Although this method was successfully applied in the experiment, the data stabilised only when the cooling time reached 180 min, thereby exposing the shortcomings of this method in terms of stability. According to the 2020 edition of the Chinese Pharmacopoeia, the vanillin-perchloric acid method was used for determining total saponins in Chinese medicinal materials *Ganoderma lucidum* and ginseng, the vanillin-sulfuric acid method was used for determining triterpenoids in the Chinese patented medicine Xinyuet capsule, and the perchloric acid method was utilised for determining triterpenoids in ophiopogon (Chinese medicinal materials and decoction pieces) [32]. The vanillal-perchloric acid method is the most used for determining the total triterpene content reported in the literature. The proposed principle behind this colour reaction suggests that under strongly acidic conditions, the hydroxyl groups within triterpenoids may undergo oxidation to increase the double-bond structures. Subsequently, they condense with aldehyde compounds to form a conjugated diene system responsible for colour development. We used the above four methods for colour development, and the detection results were significantly different from those of the HPLC area normalisation method; the measured values of the vanillin perchloric acid method were much smaller than those of the HPLC area normalisation method. To better develop and utilise triterpenoid-containing resources and control the quality of their formulations, there is an urgent need to develop a method with high accuracy and sensitivity for detecting total triterpenoids in TCM. This study used the 2-hydroxy-5-methylbenzaldehyde sulfuric acid method to determine the total triterpene content. We determined the total triterpene content in hawthorn leaves and liquorice, and compared it with four reported colorimetric methods. We validated the rationality of the method using total triterpenes prepared with triterpenoid compound standards and HPLC area normalisation. This study proposes an innovative analytical method for rapid and accurate quantification of total triterpenoids in herbal medicines, providing a robust assurance for patient medication safety. Not only does it significantly enhance the precision of quality control in TCM, but it also offers powerful technical support for the modernization and standardization of Chinese herbal production.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Perchloric acid, sulfuric acid, 2-hydroxy-5-methylbenzaldehyde, vanillin, glacial acetic acid, ethanol, methanol, chromatographic methanol, and other reagents are MREDA chemical plant products, purity $\geq$98 %.

2.1.2. Test drug and control product

Chinese herbal medicine: Hawthorn leaves (iron hawthorn leaves) from Hebei Province (Chengde), Xinglong Hawthorn Industrial Technology Research Institute, and liquorice were purchased from Beijing Tontontang Pharmaceutical Store. Reference products

ursolic, oleanolic, betulinic, corosolic, hawthorn, 2α,3β,19α-trihydroxyursolic, snow oxalic (note: the above triterpenoids are contained in hawthorn leaves), and standard glycyrrhizic acid (note; Glycyrrhizic acid is the major triterpenoid compound contained in liquorice) purchased from Shanghai Tongtian Biotechnology Co., Ltd. and self-made by our laboratory, purity ≥98 %. Total liquorice triterpenes isolated and refined from liquorice were prepared in our laboratory and detected by HPLC-DAD as liquorice triterpenes, purity ≥98 %.

2.2. Preparation of test solution

2.2.1. Preparation of reference solution

The reference product was 5 mg in a 25 mL volumetric bottle and a reference solution was prepared with methanol at a 0.20 mg/mL concentration.

2.2.2. Preparation of total triterpene crude solution from Chinese medicinal materials by separation and purification

The dried hawthorn leaves (10 g) were placed in a 250 mL round-bottom flask, and an appropriate ethanol-aqueous solution (55:45, v/v) was incorporated for extraction under the conditions of suitable ultrasonic power (500 W), temperature (65 °C), time (1 h), and solid-liquid ratio (1:25, m/v). Table 1 summarizes the extraction process parameters. These parameters have been proven effective and optimized in previous studies by our research group. However, since the results have not yet been published, this article will not elaborate further on them.

The filtrate was used as the total extract of hawthorn leaves. First, remove the ethanol from the total extraction solution (to form a clear two-phase in subsequent separation), and then add the petroleum ether extraction aqueous solution. The petroleum ether layer was removed (to remove non-triterpene lipophilic impurities), and the water layer was further extracted with n-butanol and an appropriate amount of alkaline water (pH 11). The upper layer of the extraction layer was removed and stored to obtain the purified total triterpene solution. The preparation solution of crude total triterpenoids for isolation and purification from hawthorn leaves is shown in Fig. 1.

Similarly, an ethanol-aqueous solution (40:60, v/v) was used as the extraction solvent to prepare a crude total triterpenes from liquorice. The other preparation methods were similar to those used for hawthorn leaves, as depicted in Table 1 and Fig. 1.

2.2.3. Preparation of total triterpene pure solution

Ursolic, oleanolic, betulinic, corosolic, cratonic, 2α,3β,19α-trihydroxy-ursolic, and cystoxalic acid were each approximately 1.5 mg in a 10 mL volume bottle, and ethanol was incorporated to the scale, which was the total triterpene pure solution prepared with hawthorn leaves, the concentration was 1.07 mg/mL.

Herein, 5.1 mg of the refined liquorice triterpene was placed into a 10 mL volumetric bottle, and ethanol was incorporated to the scale, i.e., the purified liquorice total triterpene solution at a concentration of 0.50 mg/mL.

2.3. Colorimetric analysis method

2.3.1. 2-Hydroxy-5-methylbenzaldehyde - sulfuric acid method

The prepared test solution was evaporated using a rotary evaporator, 1 mL of a 5 % 2-hydroxy-5-methylbenzaldehyde ethanol solution was incorporated and dissolved after cooling to room temperature. Then, 3 mL of concentrated sulfuric acid was incorporated to allow it to colour at room temperature for 25 min and immediately cooled with flowing water for 5 min. The samples were shaken well and analysed using a UV/visible spectrophotometer. The sample solution was replaced with 60 % ethanol as the blank control. The operational process of the 2-hydroxy-5-methylbenzaldehyde - sulfuric acid method is depicted in Fig. 2a.

2.3.2. Sulfuric acid method

A rotary evaporator was used to evaporate the prepared test solution, 1 mL of anhydrous methanol was incorporated after cooling to room temperature. We waited for it to dissolve and then incorporated 6 mL of concentrated sulfuric acid, heated in a water bath at 60 °C for 20 min, and removed from the ice bath for 5 min. The detection and blank controls were the same as those described in Section 2.3.1. The contents of hawthorn leaves and the liquorice system were determined at 307 and 400 nm, respectively. The operation of the sulfuric acid method is illustrated in Fig. 2b.

2.3.3. Vanillin-sulfuric acid method

A rotary evaporator was used to evaporate the prepared sample solution and an 8 % vanillin ethanol solution (0.5 mL) was incorporated post cooling to room temperature. Subsequently, 5 mL of 80 % sulfuric acid was incorporated and heated in a 60 °C water bath for 15 min, followed by an ice bath for 15 min. The detection and blank controls were the same as those described in Section 2.3.1. The contents of the hawthorn leaves and liquorice system were determined at 543 and 558 nm, respectively. The anillin–sulfuric acid

Table 1
The optimized experimental conditions.

Condition	Ultrasonic power	Temperature	Time	Solid-liquid ratio	Ethanol-aqueous solution
	500 W	65 °C	1 h	1:25, m/v	55:45(or 40:60), v/v

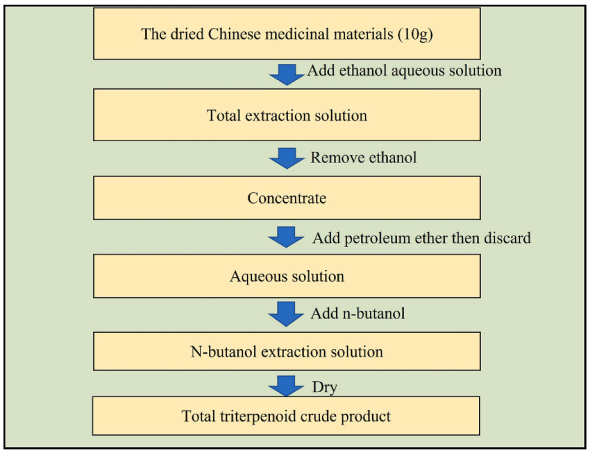

Fig. 1. Preparation solution of total triterpenoid crude for separation and purification from traditional Chinese medicine.

method is depicted in Fig. 2c.

2.3.4. Perchloric acid method

A rotary evaporator was used to evaporate the prepared sample solution, cooled to room temperature, and then 4 mL perchloric acid was incorporated, heated in a water bath at 60 °C for 15 min, and removed from the ice bath for 5 min. The detection and blank controls were the same as those described in Section 2.3.1. The contents of the hawthorn leaves and the liquorice system were determined at 304 and 386 nm, respectively. The operation process of perchloric acid method is illustrated in Fig. 2d.

2.3.5. Vanillal-perchloric acid method

A rotary evaporator was used to evaporate the prepared test solution, cooled to room temperature, and then 0.2 mL 5 % vanillin ice acetic acid solution was incorporated to dissolve. Then, 0.8 mL of perchloric acid was incorporated again, then heated in a 65 °C water bath for 15 min and an ice bath for 5 min. Then, 4 mL glacial acetic acid was incorporated to the mixture. The detection and blank controls were the same as those described in Section 2.3.1. The contents of the hawthorn leaves and liquorice system were determined at 550 and 543 nm, respectively. The operational process of the vanillin–perchloric acid method is illustrated in Fig. 2e.

Here's the translation.

2.4. Liquid chromatography conditions

The chromatography column used in this study was a Durashell C18(L) column (250 mm × 4.6 mm, 5 μm); mobile phase: B-methanol, A-water with 0.1 % phosphoric acid; Detection wavelength: 210 nm; Flow rate: 0.8 mL/min; Elution gradient: 0–18 min, 71 % B; 18–20 min, 71–75 % B; 20–24 min, 75 → 79 % B; 24–44 min, 79 → 82 % B; 44–46 min, 82 → 83 % B; 46–68 min, 83 % B; 70–110 min, 100 % B. Column temperature: 30 °C; Injection volume: 10 μL.

2.5. Analysis of traditional Chinese medicine specimens

The quantification of total crude and refined triterpenes extracted from hawthorn leaves and glycyrrhiza was conducted using the 2-hydroxy-5-methylbenzaldehyde-sulfuric acid method that we developed and compared against four other reported UV/Vis-CMs, including the HPLC area normalisation method. The comparison was based on plotting the results of the HPLC area-normalisation method on the X-axis against those obtained from UV/Vis-CM on the Y-axis. The correlation between the two sets of results was evaluated using regression analysis to derive an equation and correlation coefficient. A Bland–Altman analysis was conducted to evaluate the agreement between the methods. This involved plotting the mean values obtained from both methods on the x-axis against their relative differences (expressed as the ratio of the difference to the mean) on the Y-axis [33]. This analysis facilitated the determination of the mean relative error and 95 % confidence interval, offering insights into the consistency and reliability of the UV/Vis-CM method compared to the standard HPLC approach.

2.6. Data analysis

All statistical analyses were conducted using Origin software. The total triterpene content is expressed as a percentage, and all data are the averages of three parallel operations.

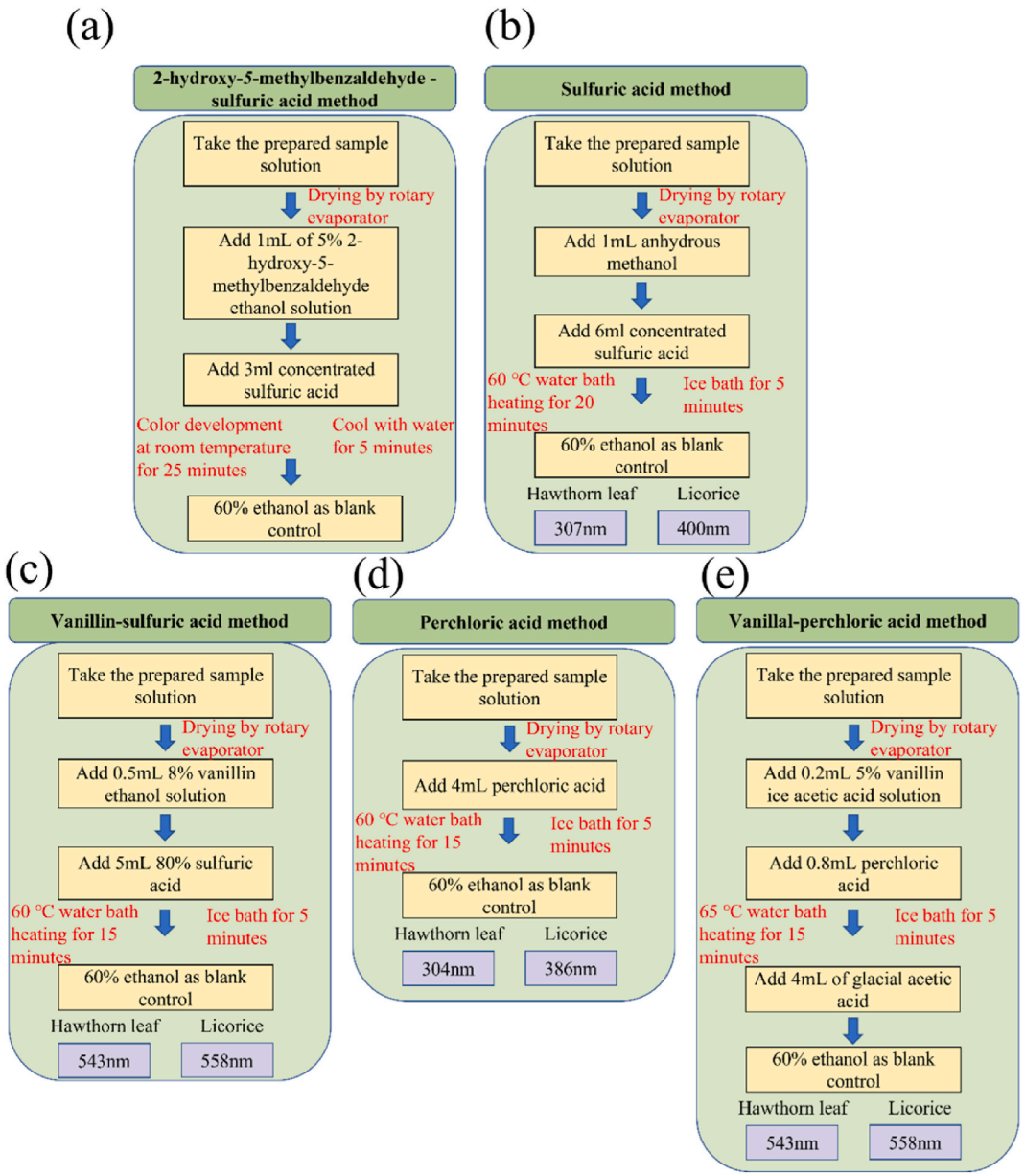

Fig. 2. Operation process of different colorimetric methods.
((**a**) is 2-hydroxy-5-methylbenzaldehyde - sulfuric acid method. (**b**) is sulfuric acid method. (**c**) is vanillin-sulfuric acid method. (**d**) is perchloric acid method. (**e**) is vanillal-perchloric acid method.).

3. Results and discussion

Before developing the new method, four studies selected reagents based on the following reaction principles: 2-hydroxy-5-methylbenzaldehyde, 3,5-dinitrobenzoic acid, 3,5-dinitrobenzaldehyde, and 4-hydroxybenzaldehyde, and their applicability was evaluated. The experimental results revealed that 2-hydroxy-5-methylbenzaldehyde underwent a distinct chromogenic reaction with sulfuric acid at room temperature, indicating its potential for further methodological development. Conversely, both 3,5-dinitrobenzoic acid and 3,5-dinitrobenzaldehyde demonstrated no colour change when heated with sulfuric acid at 65 °C, suggesting a low likelihood of reaction under these conditions. The reaction between 4-hydroxybenzaldehyde and sulfuric acid was vigorous and produced dense smoke, indicating that this combination is unsuitable in laboratory settings due to safety concerns and potential hazards to personnel. Therefore, considering its safety and reaction efficacy, 2-hydroxy-5-methylbenzaldehyde was selected for the subsequent investigations.

3.1. Complete wavelength scanning of 2-hydroxy-5-methylbenzaldehyde - sulfuric acid method to determine the detection wavelength

Full-wavelength scanning is a pivotal technique for elucidating the spectral properties of compounds and offering insight into their absorption across diverse wavelengths. This facilitated the identification and analysis of sample components. Herein, we employed the 2-hydroxy-5-methylbenzaldehyde - sulfuric acid method for full wavelength scanning to evaluate total and major triterpenoids in hawthorn leaves and liquorice at 200–800 nm range. Ultimately, the 500–700 nm band was selected for prominent expression due to its pronounced absorption peaks, with findings illustrated in Fig. 3.

Fig. 3a depicts the scanning outcomes for the hawthorn leaf triterpene samples across the entire wavelength spectrum. Under meticulously controlled conditions, the maximum absorption of both the crude and purified total triterpene extracts, including the pure standard-derived total triterpenes, was observed at 543 nm. This peak aligns with the known maximum absorption wavelengths of specific triterpenoids, including ursolic, betulinic, and oleanolic acid, affirming that 543 nm is the optimal detection wavelength for hawthorn leaf triterpenoids. Fig. 3b depicts the scanning results for liquorice triterpenes, where both crude and purified total triterpene extracts from liquorice exhibited maximum absorption at 547 nm. This observation corroborates the absorption characteristics of glycyrrhizic acid, thereby validating the accuracy of the triterpenoid detection method.

3.2. Investigation of 2-hydroxy-5-methylbenzaldehyde-sulfuric acid method

3.2.1. Optimization of additive condition of colour developer

The amount of the colour-developing agent has an important influence on the colour-developing reaction. Herein, 200 μl ursolic acid reference solution was absorbed, the solvent was dried, concentrated sulfuric acid and 5 % 2-hydroxy-5-methylbenzaldehyde (volume ratio 1:1, 2:1, 3:1, 4:1, 5:1) was incorporated, and then absorbance value at 543 nm was determined post colour development at room temperature (blank control under each content was set at the same time, parallel operation for three times, the average value was considered). The volume ratio of concentrated sulfuric acid to 5 % 2-hydroxy-5-methylbenzaldehyde was considered as the x-axis, and the total triterpene absorbance of liquorice was considered as the y-axis. The results are depicted in Fig. 4. When the volume ratio of concentrated sulfuric acid to 5 % 2-hydroxy-5-methylbenzaldehyde was 3:1, the absorbance value was the largest; therefore, the colour-developing agent was determined to be sulfuric acid and the 5 % 2-hydroxy-5-methylbenzaldehyde volume fraction was 3:1.

3.2.2. Optimization of colour development time

Optimising colour development time is essential for enhancing the outcome, sensitivity, accuracy, repeatability, and efficiency of the colour reaction. After precisely measuring 200 μL of the control solution and evaporating the solvent, we incorporated 1 mL of a freshly prepared 5 % 2-hydroxy-5-methylbenzaldehyde solution and 3 mL of concentrated sulfuric acid. The absorbance was recorded at 543 nm at room temperature after varying the colour development times (2, 5, 10, 15, 20, 25, 30, and 35 min), with parallel operations conducted thrice for averaging. The colour development time was plotted on the x-axis against the total triterpene absorbance on the y-axis, as depicted in Fig. 5. The absorbance values plateaued after 25 min of colour development, followed by immediate cooling in ice water for 5 min.

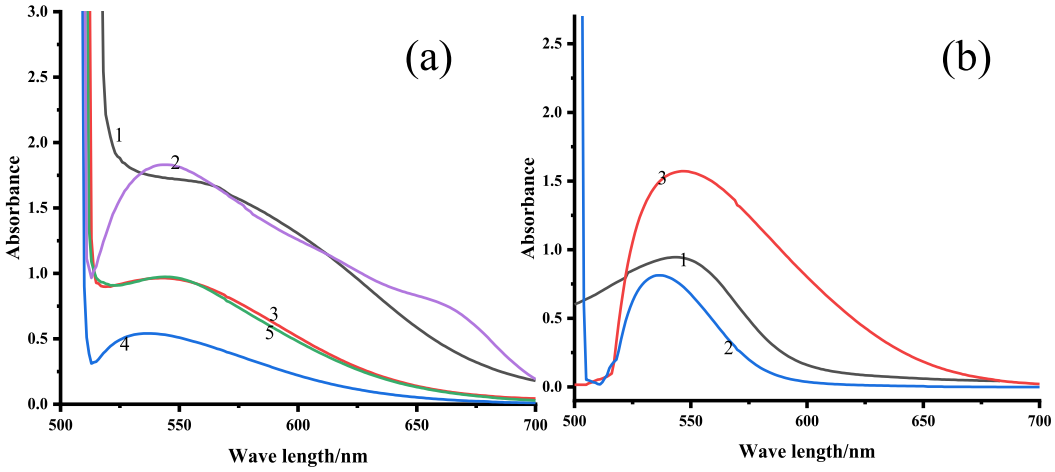

Fig. 3. Complete wavelength scanning of 2-hydroxy-5-methylbenzaldehyde in hawthorn leaves and liquorice
((**a**) is a hawthorn leaf and (**b**) is liquorice. (**a**), 1–5 represent the total triterpene crude product isolated and purified from hawthorn leaves, the total triterpene pure product prepared from hawthorn leaves, ursolic, betulinic, and oleanolic acid, respectively. (**b**), 1–3 represent the total crude triterpene product isolated and purified from liquorice, the total pure triterpene product isolated and refined from liquorice, and glycyrrhizic acid, respectively.).

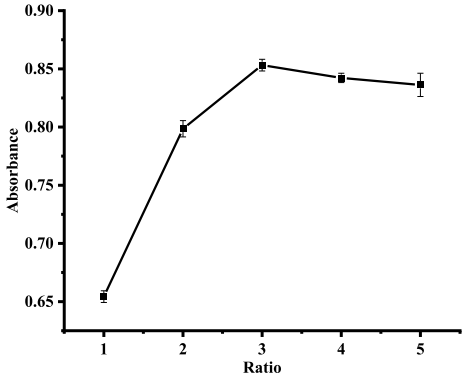

Fig. 4. Absorbance-volume ratio graph.

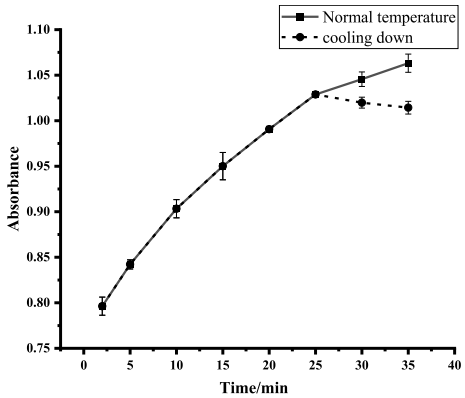

Fig. 5. Absorbance-chromogenic time graph.

3.2.3. Stability test

We precisely measured 200 μL of standard solution, evaporated the solvent, and added concentrated sulfuric acid and 5 % 2-hydroxy-5-methylbenzaldehyde in a 3:1 ratio. After coloring at room temperature for 25 min, the mixture was placed in ice water. Measure the absorbance of the mixture at 543 nm every 10 min (setting up a blank control for each time point, repeating the process three times, and taking the average value). The results in Table 2 show that the standard is stable in ice water for at least 1 h (average absorbance value of 1.0242, RSD 1.10 %), but present significant variability at room temperature. Therefore, measurements should be conducted within 1 h in ice bath.

3.2.4. Precision test

We precisely measured five samples of pure total triterpene solutions prepared from the hawthorn leaf series reference products. After drying the solution, absorbance measurements were conducted as described in Section 2.3.1. The results presented in Table 3 depicts an RSD of 0.3 %, indicating the high precision of the method.

3.3. Investigation of linear relationship

3.3.1. Linear relationship of ursolic acid

A standard curve was obtained through the absorbance of a series of gradient-diluted ursolic acid mother solutions. The UV/Vis CM detection standard curves of ursolic acid were plotted with mass as the horizontal coordinate and absorbance as the vertical coordinate. The 2-hydroxy-5-methylbenzaldehyde sulfuric acid method (543 nm): $y = 4.5588x + 0.2597$, $R^2 = 0.9881$; sulfuric acid method (307 nm): $y = 0.0109x-0.1956$, $R^2 = 0.9989$; vanillal-sulfuric acid method (543 nm): $y = 0.0088x-0.1072$, $R^2 = 0.9987$; perchloric acid method (304 nm): $y = 0.0088x-0.1163$, $R^2 = 0.9987$; Vanillal-perchloric acid method (550 nm): $y = 7.1098x + 0.0597$,

Table 2
Stability test.

Time	0	10	20	30	40	50	60	RSD/%
Abs	1.0057	1.0133	1.0189	1.0278	1.0285	1.0367	1.0388	1.10

Table 3
Precision analysis.

NO.	1	2	3	4	5	Average	RSD/%
Abs	0.7045	0.7033	0.7005	0.7081	0.7063	0.7045	0.3

$R^2 = 0.9982$, the linear range was 20–150 μg. Using ursolic acid content as horizontal coordinate and peak area as vertical coordinate, the linear equation of HPLC standard curve of ursolic acid was obtained as follows: y = 606677x +18148, linear correlation coefficient $R^2 = 0.9997$, linear range 32.25–500 μg, and the linear relationship of standard curve was good. The relevant linear relationships are presented in Table 4.

3.3.2. Linear relationship of glycyrrhizic acid

The standard curve for the UV/Vis-CM detection of glycyrrhizic acid was plotted using mass as the horizontal coordinate and absorbance value as the vertical coordinate. 2-hydroxy-5-methylbenzaldehyde sulfuric acid method (547 nm): y = 1.0294x+0.0497, $R^2 = 0.9994$. Sulfuric acid method (400 nm): y = 2.7104x+0.0466, $R^2 = 0.9985$; Vanillal-sulfuric acid method (558 nm): y = 5.4338x-0.0429, $R^2 = 0.9981$; Perchloric acid method (386 nm): y = 2.1941x-0.0355, $R^2 = 0.9995$; Vanillal-perchloric acid method (543 nm): y = 2.835x+0.0895, $R^2 = 0.9986$, linear range of 20–150 μg; Using glycyrrhizic acid content as horizontal coordinate and peak area as vertical coordinate, the linear equation of glycyrrhizic acid HPLC standard curve was obtained as follows: y = 825700x +48372, linear correlation coefficient $R^2 = 0.9996$, linear range 32.25–500 μg, and the linear relationship of standard curve was good. The relevant linear relationships are presented in Table 5.

3.4. Determination of total triterpenes by HPLC area normalisation method

HPLC chromatograms are indispensable in modern scientific research, HPLC chromatograms play a crucial role in diverse fields, including chemical analysis, quality control, and drug development, particularly in qualitative and quantitative analyses. Fig. 6 depicts the HPLC chromatogram of the total triterpene crude solution isolated and purified from hawthorn leaves. As depicted in Figs. 6a and 10 peaks reached baseline separation. In comparison to the retention times of the standard products, peaks 2, 4, 5, 7, and 8 correspond to snow oxalic, hawthorn, corosolic, oleanolic, and ursolic acid, respectively. According to the 3D map of DAD detection in Fig. 6b, the UV absorption peaks of peaks 1 to 8 were very similar, and they are absorbed at 190–230 nm. These peaks correspond to triterpenoids. However, at 35–40 min, some overlapping peaks did not reach baseline separation and could not be enumerated, indicating that the triterpenoid content may be low. Using ursolic acid as the control, the peak area of each triterpene was determined and converted into a standard curve to determine the total triterpene content of the sample solution. The results are summarised in Table 6.

Fig. 7 depicts the HPLC chromatogram of the total triterpene purified solution isolated and refined from liquorice. It can be seen from Fig. 7a that eight peaks reached baseline separation, and peak 2 corresponded to glycyrrhizin compared to the retention time of the standard product. According to the DAD detection 3D map in Fig. 7b, the UV absorption peaks of peaks 1 to 8 were very similar, with maximum absorption at 254 nm. These peaks correspond to triterpenoids. Since all peaks reached baseline separation, it was reasonable to verify this using the HPLC area normalisation method. Using glycyrrhizic acid as the control substance, the peak area of each triterpene was detected and incorporated to the standard curve to determine the total triterpene content in the sample solution. The results are summarised in Table 6.

3.5. The analytical capability of the 2-hydroxy-5-methylbenzaldehyde-sulfuric acid method

3.5.1. Comparison of the contents of total triterpenes in Chinese medicine

Total triterpenes were determined using the colorimetric and HPLC area normalisation methods, and the corresponding standard curve was substituted to compute the content. Three independent experiments were conducted for each sample to ensure reliability of the results. The average values for each group of data were obtained, and the results are depicted in Table 6.

Nageswara [34] provides an extensive review of the literature published in various analytical and medicinal chemistry journals, focusing on the development and application of HPLC methods for the determination of process-relevant ingredients in pharmaceutical products. HPLC separates the compounds in the test sample solution through a column such that the diverse components gradually separate and peak at diverse time points, and the detector can quantify the individual components more accurately. As a

Table 4
Linear relationship of ursolic acid.

Method	Linear regression	Variance
2-hydroxy-5-methylbenzaldehyde sulfuric acid method (543 nm)	y = 4.5588x + 0.2597	$R^2 = 0.9991$
Sulfuric acid method (307 nm)	y = 0.0109x-0.1956	$R^2 = 0.9989$
Vanillal-sulfuric acid method (543 nm)	y = 0.0088x-0.1072	$R^2 = 0.9987$
Perchloric acid method (304 nm)	y = 0.0088x-0.1163	$R^2 = 0.9987$
Vanillal-perchloric acid method (550 nm)	y = 7.1098x + 0.0597	$R^2 = 0.9982$
HPLC	y = 606677x +18148	$R^2 = 0.9997$

Table 5
Linear relationship of glycyrrhizic acid.

Method	Linear regression	Variance
2-hydroxy-5-methylbenzaldehyde sulfuric acid method (547 nm)	y = 1.0294x+0.0497	R^2 = 0.9994
Sulfuric acid method (400 nm)	y = 2.7104x+0.0466	R^2 = 0.9985
Vanillal-sulfuric acid method (558 nm)	y = 5.4338x - 0.0429	R^2 = 0.9981
Perchloric acid method (386 nm)	y = 2.1941x - 0.0355	R^2 = 0.9995
Vanillal-perchloric acid method (543 nm)	y = 2.835x + 0.0895	R^2 = 0.9986
HPLC	y = 825700x +48372	R^2 = 0.9996

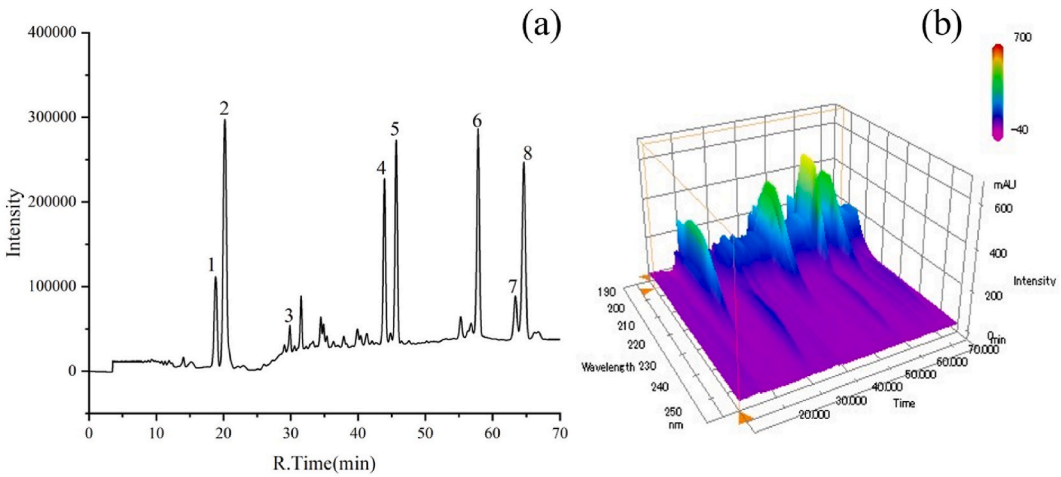

Fig. 6. HPLC chromatogram of total triterpene solution of hawthorn leaves
((a) is the peak sequence spectrum of total triterpenoids HPLC in hawthorn leaves at 210 nm, and (b) is the HPLC 3D view of total triterpenoids in hawthorn leaves).

Table 6
Determination results of total triterpene content by different methods(n = 3).

Sample for test	HPLC Area normalisation method	2-hydroxy-5-methylbenzaldehyde sulfuric acid method	Sulfuric acid method	Vanillin sulfuric acid method	Perchloric acid method	Vanillin perchloric acid method
Isolated and purified crude triterpenes from hawthorn leaves	74.41 %	76.69 %	66.14 %	64.05 %	60.36 %	38.71 %
Pure total triterpenes from hawthorn leaves formulated with reference substance	98.5 %	90.74 %	87.71 %	80.37 %	67.96 %	44.09 %
Isolated and purified crude licorice total triterpenes	48.80 %	50.58 %	45.55 %	42.53 %	41.48 %	28.27 %
Separation of delicate liquorice total triterpene pure products	97.68 %	96.39 %	91.39 %	85.96 %	58.42 %	48.30 %

high-resolution and high-sensitivity analytical technique, if the chromatographic peaks of each compound reach baseline separation, the HPLC area normalisation method can be used to verify the content determination results accuracy using diverse colorimetric methods [35]. Herein, the results of extraction and purification of total triterpenes from the Chinese herb hawthorn leaves and liquorice using sulfuric, vanillal-sulfuric, and perchloric acid methods were different from those of the HPLC area normalisation method. The vanillal-perchloric acid method, which has been extensively used in the literature, had the largest deviation compared to the HPLC method. The results of the UV/Vis-CM determination of 2-hydroxy-5-methylbenzaldehyde-sulfuric acid colour development were the closest to those of the HPLC area normalisation method. For determining total triterpenes in the two crude products, the results obtained by UV/Vis-CM using 2-hydroxy-5-methylbenzaldehyde-sulfuric acid were slightly higher than those obtained by the area normalisation method using HPLC. Analysis of this difference demonstrated that when measured using HPLC, only triterpene components that were high in content and reached baseline separation were enumerated. Triterpene components that failed to achieve baseline separation and had noise peaks less than 3x were excluded from the measurements. Conversely, when the total triterpene

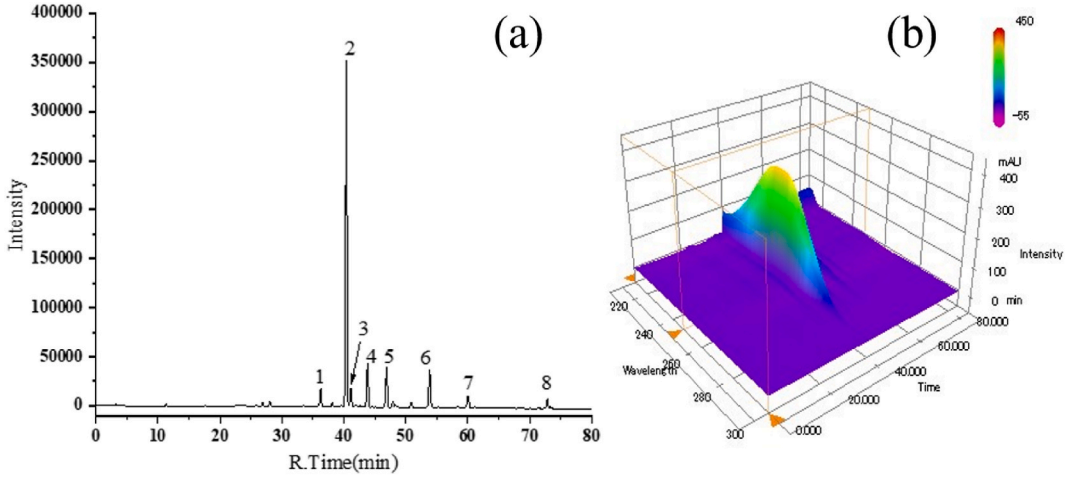

Fig. 7. HPLC chromatogram of total triterpene solution of Licorice
((a) is the peak sequence spectrum of total triterpenoids HPLC in Licorice at 254 nm, and (b) is the HPLC 3D view of total triterpenoids in Licorice).

content was determined by UV/Vis-CM using the 2-hydroxy-5-methylbenzaldehyde-sulfuric acid chromogenic method, all triterpene components were detected regardless of their content.

3.5.2. Recovery

Herein, 0.1 and 0.05 mL of total triterpenoid solution prepared with a known content of reference substance (contained in hawthorn leaves) was accurately absorbed, placed in stoppered test tubes, and 0.1 and 0.05 mL ursolic acid reference solution 1 mg/mL was incorporated, respectively. Absorbance was measured according to the method described in Section 2.3.1. As depicted in Table 7, the average recovery was 96.93 %, and the RSD was 2.78 %.

3.6. Consistency comparison between 2-hydroxy-5-methylbenzaldehyde sulfuric acid UV/Vis-CM and HPLC area normalisation method

To further evaluate the feasibility of 2-hydroxy-5-methylbenzaldehyde-sulfuric acid for determining total triterpenes in traditional Chinese medicine, this method was used to detect total triterpenes in 12 of the four samples under item 3.5.3, with three parallel samples in each sample, and was compared with the HPLC area normalisation method. As depicted in Fig. 8, the linear regression analysis demonstrated that the UV/Vis-CM and HPLC area normalisation methods were highly consistent. The linear regression equation was $y = 0.8221x+12.1598$, and the coefficient of determination R^2 was 0.9775. Bland-Altman analysis was conducted to assess consistency. As depicted in Fig. 9, the Mean Difference of 0.0189 was close to zero, indicating that there is no significant systematic error between the two methods. The Limits of Agreement (LoA) are +0.10497 (upper bound) and −0.06712 (lower bound), and 95 % of the measurement differences fell within this range, with no significant scale error. There was good consistency between the two methods, and no significant systematic deviation or proportional error was observed, making them suitable for detecting total triterpenes in Chinese medicine.

The above research results indicate that our UV/Vis CM with 2-hydroxy-5-methylbenzaldehyde sulfur acid colour rendering for detecting total triterpenes in traditional Chinese medicine has the following advantages: on the one hand, the method not only simplifies the operation steps but also eliminates cumbersome procedures such as heating, and so on,significantly improving the detection sensitivity. This is particularly important for detecting triterpenes with similar structures and lower concentrations in complex mixtures. It is worth mentioning that the innovation of this method lies in its accuracy comparable to the area normalisation method of high-performance liquid chromatography, providing a reliable, economical, and convenient new technology for the detection of total triterpenoids in traditional Chinese medicine quality evaluation and monitoring. This provides favorable technical support for the modernization research of traditional Chinese medicine. On the other hand, this method has significant advantages in analysis speed, ease of operation, and cost-effectiveness. In future work, this technology is expected to be promoted in a wider range of application scenarios, including on-site rapid detection and large-scale screening, which will help promote the development of traditional Chinese medicine to a new stage. This not only has a profound impact on the development of new drugs and the sustainable development of the health industry, but also provides reliable analytical tools for related fields. This method has important practical value and broad application prospects.

4. Conclusion

This study has successfully developed a new UV/Vis-CM with 2-hydroxy-5-methylbenzaldehyde-sulfuric acid colour rendering to detect total triterpenes. This method is as accurate as HPLC area normalisation technology, particularly, does not require heating for colour development, with high sensitivity and low cost in materials and equipment, and is suitable for the detection of total

Table 7
Analysis of recovery.

Content of triterpenoids/ mg	The amount of ursolic acid/ mg	Total triterpenoid content after addition/mg	Sample recovery/ %	Average recovery rate/%	RSD/ %
0.0905	0.1	0.1817	91.20	96.93	2.78
0.0877	0.1	0.1870	99.23		
0.0952	0.1	0.1920	96.77		
0.042	0.05	0.0908	97.56		
0.041	0.05	0.0912	99.06		
0.040	0.05	0.0884	97.74		

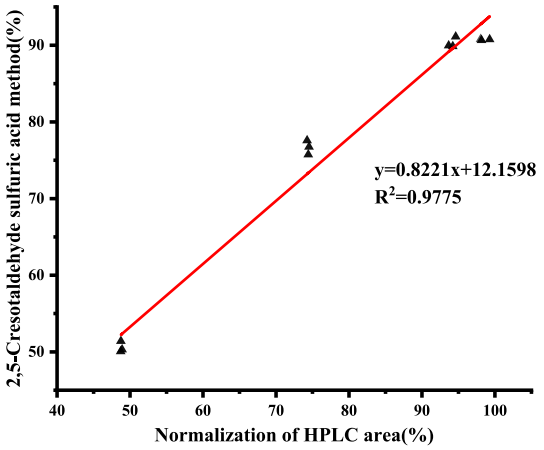

Fig. 8. Correlation between 2-hydroxy-5-methylbenzaldehyde-sulfuric acid and HPLC area normalisation method normalisation method.

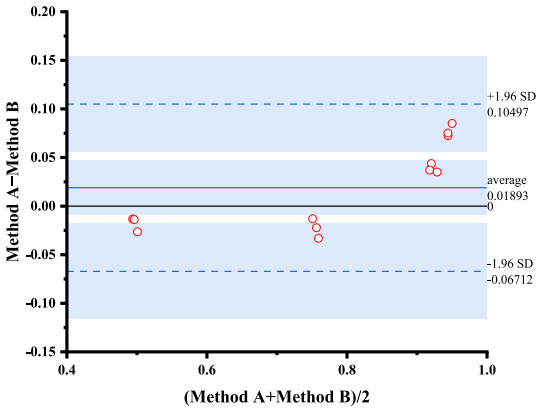

Fig. 9. Bland-Altman analysis of 2-hydroxy-5-methylbenzaldehyde sulfuric acid and HPLC area normalisation method.

triterpenoids in complex TCM. In addition, this method has wider application prospects in rapid on-site detection and large-scale screening.

Data availability statement

Data associated with the study has not been deposited into a publicly available repository, the raw data supporting the findings of this study are available from the corresponding author at request.

CRediT authorship contribution statement

Yuanyuan Guo: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Zhe Han:** Investigation. **Jingwei Zhang:** Investigation. **Yue Lu:** Investigation. **Chunfeng Li:** Investigation. **Guiyan**

Liu: Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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