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Analytical methods for determination of magnoflorine and saponins from roots of *Caulophyllum thalictroides* (L.) Michx. Using UPLC, HPLC and HPTLC

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ABSTRACT

Analytical methods including HPLC, UPLC and HPTLC are presented for the determination of major alkaloid and triterpene saponins from the roots of *Caulophyllum thalictroides* (L.) Michx. (blue cohosh) and dietary supplements claiming to contain blue cohosh. A separation by LC was achieved using a reversed phase column, PDA with ELS detection, and ammonium acetate/acetonitrile gradient as the mobile phase. Owing to their low UV absorption, the triterpene saponins were detected by evaporative light scattering. The eight triterpene saponins (cauloside H, leonticin D, cauloside G, cauloside D, cauloside B, cauloside C, cauloside A and saponin PE) and the alkaloid magnoflorine could be separated within 35 min using HPLC method and within 8.0 min using UPLC method with detection limits of 10 μg/mL for saponins and 1 μg/mL for magnoflorine. The detection wavelength was 320 nm for magnoflorine and ELS detection was used for the eight saponins. The methods were also successfully applied to analyze different dietary supplements. For the products claiming to contain blue cohosh, there was a significant variability in the amounts of triterpene saponins detected. Calculations based on the analysis results for dietary supplements showed that maximum daily intake of alkaloid and saponins vary with the form (solids/liquids) and recommended doses according to the products label. Intakes varied from 0.57 to 15.8 mg/day for magnoflorine and from 5.97 to 302.4 mg/day for total saponins. LC–mass spectrometry coupled with electrospray ionization (ESI) method is described for the identification and confirmation of nine compounds in plant samples and dietary products. A HPTLC method was also developed for the fast chemical fingerprint analysis of *C. thalictroides* samples.

1. Introduction

Caulophyllum thalictroides (L.) Michx. (Berberidaceae), known as blue cohosh, is an indigenous perennial herb commonly found in eastern North America where the roots are used in diuretic, uterine antispasmodic and laxative preparations [1]. Traditionally the roots and rhizomes of *C. thalictroides* are used for the treatment of menstrual difficulties and inducing uterine contractions [2]. Earlier studies have revealed blue cohosh rhizomes and roots contain two types of saponins, caulosaponins and caulophyllosaponins [3–6]. The caulosaponins (caulosides A, C, D, G) contain caulosapogenin (later corrected to hederagenin [3]) as aglycon [4,5] whereas caulophyllosaponins (caulosides B, H, leonticin D) show caulophyllosapogenin as aglycon [4–6]. The saponins in blue cohosh are considered to be responsible for the uterine stimulant effects together with teratogenic alkaloids [2]. Between 1882 and 1905, blue cohosh was listed in the *United States Pharmacopoeia* as a labor inducer [6] and sold as an herbal supplement that can aid in childbirth. There is considerable concern about the safety of blue cohosh with reports of new born babies having heart attacks or strokes after the mother consumed blue cohosh to induce labor [6–9]. Blue cohosh is avoided during the first trimester (three months) of pregnancy [6]. In addition to the quinolizidines, the aporphine alkaloid magnoflorine is found in substantial quantities [10,11] which have been implicated as teratogens. Based on available scientific evidence, there is no proven safe or effective dose for blue cohosh [6].

Few published analytical methods have been reported for the analysis of alkaloids alone or for alkaloids and saponins using GC, HPLC and densitometry for blue cohosh [1,2,12,13]. Levels of the major quinolizidine alkaloids in herbal preparations have been determined by gas chromatography, HPLC and densitometry [1,12]. Betz et al. [12], developed a GC methodology for the quantitative determination of alkaloids from the roots of *C. thalictroides* L. Woldemariam et al. [1], developed a TLC densitometric method and

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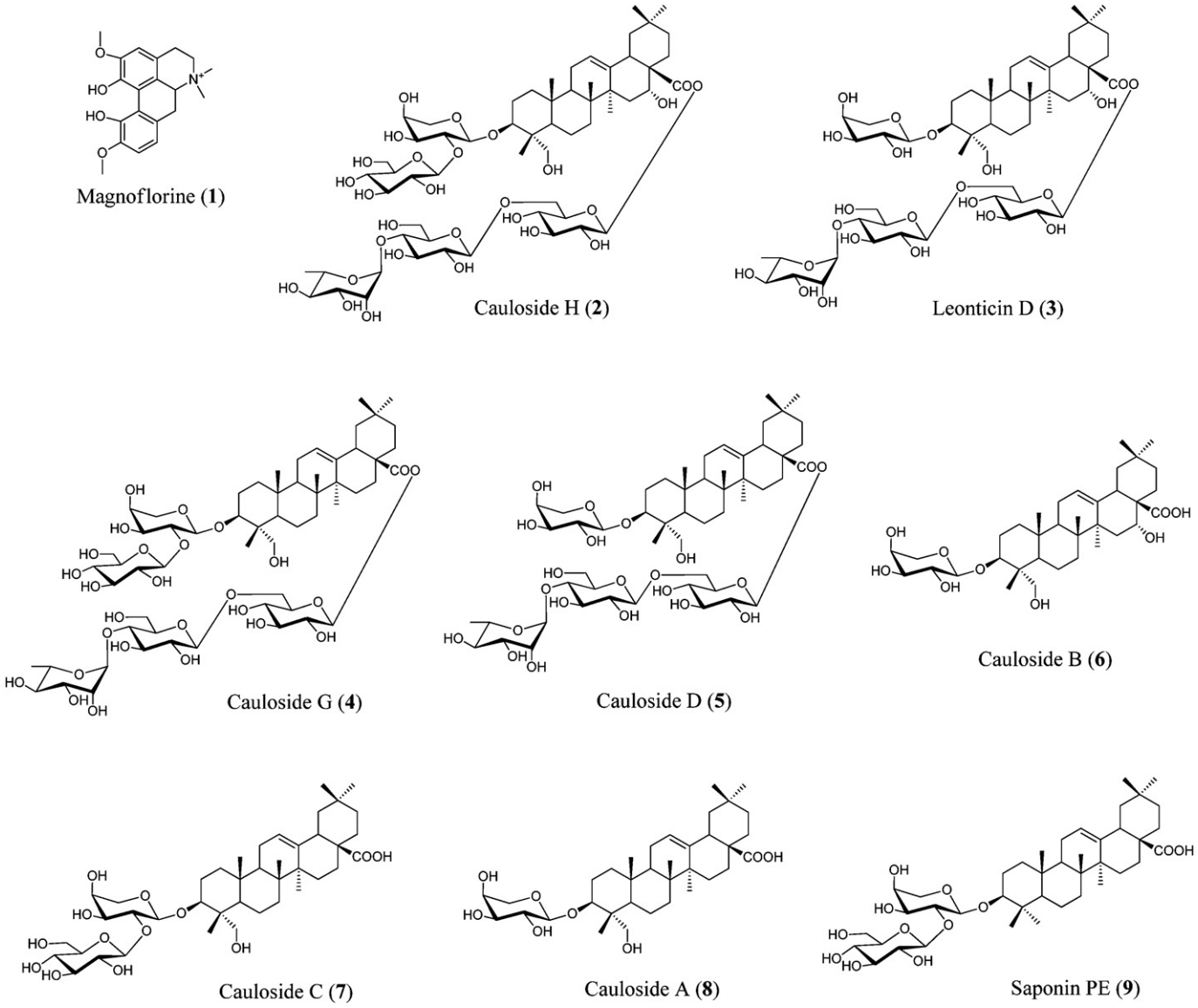

Fig. 1. Structure of magnoflorine and saponins from *Caulophyllum thalictroides* (L.) Michx.

an HPLC method for the quantification of alkaloids from extracts of *C. thalictroides* L. roots. The studies by Woldemariam et al. [1], and Betz et al. [12], focused on isolation and determination of alkaloids including N-methylcystisine, baptifoline, anagyrine and magnoflorine. The saponins present in *C. thalictroides* L. have received less attention than the alkaloids. Ganzera et al. [2], developed an HPLC method for the quantitative analysis of primary alkaloids and saponins from *C. thalictroides* L. roots. Subramaniam et al. [13], also developed a HPLC method for the separation and quantification of three alkaloids and three saponins from extracts of blue cohosh roots and dietary supplements. The newly developed UPLC method for quantitative determination of one major alkaloid [magnoflorine (**1**)] and eight triterpene saponins [cauloside H (**2**), leonticin D (**3**), cauloside G (**4**), cauloside D (**5**), cauloside B (**6**), cauloside C (**7**), cauloside A (**8**) and saponin PE (**9**)] (Fig. 1) from the roots of *C. thalictroides* is found to be capable of giving shorter retention times while maintaining good resolution and sensitivity. Detection of the saponins was achieved with the use of an ELS detector. The compounds were numbered by the order of elution using LC–UV–ELSD method. A comparison of chromatographic performance of HPLC and UPLC was performed. A simple and fast HPTLC method was also developed for the chemical fingerprint analysis of alkaloid (magnoflorine) and saponins. The highly sensitive UPLC–MS method was used to identify and confirm the compounds in blue cohosh root samples and dietary supplements that claim to contain *C. thalictroides*. These methods are useful in establishing the quality and safety of herbal products claiming to contain *C. thalictroides*.

2. Materials and methods

2.1. Instrumentation and chromatographic conditions

2.1.1. UPLC–UV–ELSD

All analyses were performed on a Waters Acquity UPLC™ system (Waters Corp., Milford, MA, USA) including binary solvent manager, sampler manager, column compartment, PDA (Waters

Acquity model code UPD), ELS detector (Waters Acquity model code UPE), and MS detector (Waters Acquity model code SQD), all connected to Waters Empower 2 data station. An Acquity UPLC™ BEH Shield RP18 column (50 mm × 2.1 mm I.D., 1.7 μm) also from Waters was used. The column and sample temperature were maintained at 35 °C and 25 °C, respectively. The column was equipped with a LC-18 guard column (Vanguard 2.1 × 5 mm, Waters Corp., Milford, MA, USA). The mobile phase consisted of ammonium acetate (50 mM) (A), acetonitrile (B) at a flow rate of 0.25 mL/min, which were applied in the following gradient elution: 0 min, 93% A: 7% B in next 2 min to 72% A: 28% B, then for 2 min 60% A: 40% B and to 23% A: 77% B in next 4 min using a slightly concave gradient profile (Waters curve type 7). Separation was followed by a 2 min washing procedure with 100% B and re-equilibration period of 3.5 min. A strong needle wash solution (95/5; acetonitrile/water) and weak needle wash solution (10/90; acetonitrile/water) were used. All solutions were filtered via 0.20 μm membrane filters and degassed before their usage. The total run time for analysis was 8 min. The injection volume was 2 μL. The detection wavelength was 320 nm for magnoflorine and ELS detection was used for the eight saponins. The ELS detector was set at 50 °C, gain 250 and the nitrogen pressure was adjusted to 45 psi. Peaks were assigned by spiking the samples with standard compounds and comparison of retention times. A UV comparison was also done for magnoflorine.

The effluent from the LC column was directed into the ESI probe. Mass spectrometer conditions were optimized to obtain maximal sensitivity. The source temperature and the desolvation temperature were maintained at 150 and 350 °C, respectively. The probe voltage (capillary voltage), cone voltage and extractor voltage were fixed at 1.5 kV, 50 V and 3 V, respectively. Nitrogen was used as the source of desolvation gas (650 L/h) and drying gas (25 L/h). Compounds were confirmed in selected ion recording (SIR) mode. Mass spectra were obtained at a dwell time of 0.1 s in SIR and 500 Da/s of scan rate.

2.1.2. HPLC–UV–ELSD

The HPLC system consisted of a Waters Alliance 2695 HPLC system, equipped with a 996 photodiode array detector (Waters Corp., Milford, MA, USA) and ELSD. A computerized data station equipped with Waters Empower-2 software and a Sedex (SEDERE, Alfortuille, France) model 75 ELSD operated at 43 °C, gain 10 and 3.5 bar nitrogen. Separation was achieved on a Synergi MAX RP (Phenomenex, 150 × 4.6 mm; 4 μm particle size; Phenomenex Inc., Torrance, CA, USA) and the temperature was maintained at 35 °C. The column was equipped with a 2 cm LC-18 guard column (Phenomenex, Torrance, CA, USA). The mobile phase consisted of ammonium acetate (50 mM) pH 7.2 (A) and acetonitrile (B) at a flow rate of 1.0 mL/min. Analysis was performed using the following gradient elution: 0 min, 97% A: 3% B in next 13 min to 77% A: 23% B, for next 12 min to 55% A: 45% B and then to 23% A: 77% B for 10 min using a slightly concave gradient profile (Waters curve type 7). Each run was followed by a 5 min wash with 100% B and an equilibration period of 15 min. The detection wavelength was 320 nm for magnoflorine and ELS detection for eight saponins. Ten microliters of the sample solution was injected and peaks were assigned by spiking the samples with standard compounds, comparison of the UV spectra for magnoflorine and retention times.

2.1.3. HPTLC

The chromatographic equipment used for HPTLC system is Linomat 5 automatic applicator, TLC scanner 3, reprostar 3 along with winCATS 4 software ver. 1.3.2 (CAMAG, Switzerland).

2.1.3.1. HPTLC plates. Glass plates (Merck, Darmstadt, Germany) with silica gel $60F_{254}$ (20 × 10 cm). Before use plates were prewashed with methanol and dried in oven for 3 min at 100 °C.

2.1.3.2. Sample application. Sample application with CAMAG Linomat 5, application position from the lower edge of the HPTLC plate is 8 mm at dosing rate 90 nL/s. The plate was accommodated with 12 tracks and all samples were applied according to the following settings: 8 mm from the bottom of the plate, band width 8 mm; distance between bands 10 mm; application volume 2–5 μL. All remaining measurement parameters were left at default settings.

2.1.3.3. Development. Chamber saturation was done using 20 × 10 cm Whatman filter paper for 20 min. Development solvent was the lower layer of chloroform:methanol:water (65:35:10.5, v/v/v) till 80 mm from the lower edge of the plate. Temperature at 20–25 °C and relative humidity at 55–65% were measured by an acurite instrument.

2.1.3.4. Derivatization reagent. Developed plates were immersed for 2 s in 5% sulfuric acid reagent in ethanol, and dried in oven for 5 min at 100 °C.

2.1.3.5. Documentation. CAMAG DigiStore2 digital system with winCATS software 1.4.3 was used for documentation of derivatized plates under white light. The images were also taken at 366 nm before dipping in 5% sulfuric acid reagent in ethanol.

2.2. Chemicals and plant materials

The standard compounds [magnoflorine (**1**) cauloside H (**2**), leonticin D (**3**), cauloside G (**4**), cauloside D (**5**), cauloside B (**6**), cauloside C (**7**), cauloside A (**8**) and saponin PE (**9**) (Fig. 1)] were isolated at the NCNPR, the identity and purity was confirmed by chromatographic (TLC, HPLC) methods, by the analysis of the spectroscopic data (IR, 1D- and 2D-NMR, HR–ESI–MS) and comparison with published spectroscopic data [14].

Acetonitrile, water, ammonium acetate and methanol were of HPLC grade, purchased from Fisher Scientific (Fair Lawn, NJ, USA).

The roots of *C. thalictroides* (L.) Michx. (NCNPR code #2972, 2973) (BC-1, BC-2) were purchased from Mountain Rose Herbs™ (www.mountainherbs.com) in 2006. All samples were deposited at the NCNPR repository. Dietary supplements (BC-3 to BC-11) claiming to contain blue cohosh were purchased online.

2.3. Sample preparation

The dietary supplements analyzed in this work were in multiple dosage forms including capsules and liquids. In order to perform the determinations on these different matrices an extraction protocol was developed that was specific for each class of formulation.

2.3.1. For capsules

Five (5) capsules were weighed, opened and the contents were emptied, then mixed and triturated in a mortar and pestle.

Dry plant samples (0.1 g) or an adequate amount of capsule contents were weighed (about 100 mg) and sonicated in 2 mL of methanol for 30 min followed by centrifugation for 15 min at 4000 rpm. The supernatant was transferred to a 10 mL volumetric flask. The procedure was repeated thrice and respective supernatants combined. The final volume was adjusted to 10 mL with methanol and mixed thoroughly. Prior to injection, an adequate volume (ca. 2 mL) was passed through a 0.2 μm nylon membrane filter. The first 1.0 mL was discarded and the remaining volume was

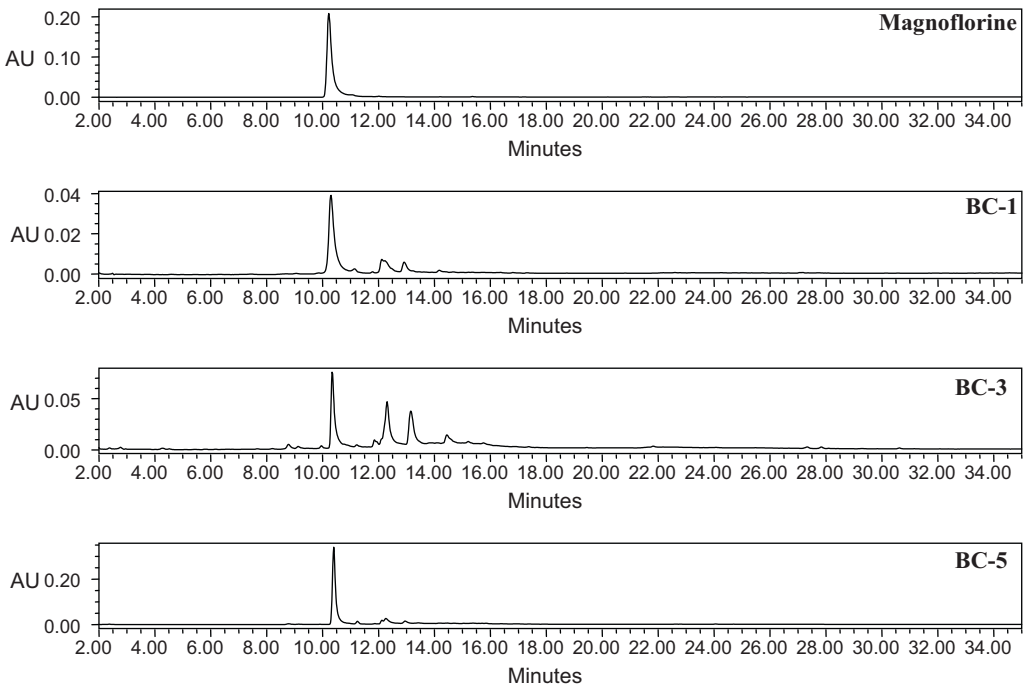

Fig. 2. HPLC–UV chromatograms of standard magnoflorine, plant sample (BC-1) and a dietary supplements (BC-3 and BC-5) at 320 nm.

collected in an LC sample vial. Each sample solution was injected in triplicate.

2.3.2. For liquids

1 mL of solution was mixed with 1.0 mL of methanol, vortex for 30 s and sonicated for 30 min, vortexed for 30 s and centrifuge for 10 min at 4000 rpm. The clear supernatant solution was used for analysis. For BC-4 to BC-8, the samples required further dilution to 50 fold.

2.4. Standard prepration

2.4.1. Preparation of standard magnoflorine solution

Individual stock solution of the standard compound was prepared at a concentration of 1.0 mg/mL in methanol. The calibration curves were prepared at five different concentration levels. The range of the calibration curves was 1.0–500 μg/mL for LC–UV analysis.

2.4.2. Preparation of standard saponins solution

An individual stock solution of the standard compound was prepared at a concentration of 1.0 mg/mL in methanol. The calibration curves were prepared at five different concentration levels. The range of the calibration curves was 25–250 μg/mL for LC–ELSD analysis.

2.5. Validation procedure

The HPLC and newly developed UPLC method were validated in terms of precision, accuracy, and linearity according to ICH guidelines [15]. The limit of detection (LOD) and limit of quantification (LOQ) were determined by injecting a series of dilute solutions with known concentrations. Limit of detection (LOD) and limit of quantification (LOQ) were defined as the signal-to-noise ratio equal to 2 or 3 and 10, respectively. The accuracy of the assay method was evaluated in triplicate using two concentration levels of 25 and 50 μg/ml. Intra- and inter-day variation of the assay was determined on 3 consecutive days with 3 repetitions each.

3. Results and discussion

3.1. Chromatographic conditions optimization

Optimal chromatographic conditions were obtained after running different mobile phases with a reversed phase C18 column. The different columns tried for UPLC were Acquity UPLC BEH C18 (100 mm × 2.1 mm I.D., 1.7 μm), Acquity UPLC BEH C18 (50 mm × 2.1 mm I.D., 1.7 μm) and Acquity UPLC BEH Shield RP18. The best results were observed with BEH shield RP18 column (50 mm × 2.1 mm I.D., 1.7 μm) using ammonium acetate (pH 7.2) and acetonitrile as the mobile phase. Acetonitrile was preferred over methanol as the mobile phase as its use resulted in improved separation as well as a significantly reduced column back pressure. Five different gradient systems of mobile phase were tried for the best separation of peaks.

Stationary phases of different columns (Luna 5 μ C18 (2), Synergi 4 μ Max-RP 80 A, Lichrospher 5 RP18 and Gemini 5 μ C18) have been screened for the HPLC system and most of the column materials tested could not resolve compounds satisfactorily. The best results were obtained with Synergi 4 μ Max-RP stationary phase from Phenomenex. Optimal chromatographic conditions were obtained after running different mobile phases with a reversed phase C12 column. A chromatogram of samples and dietary supplements claiming to contain *C. thalictroides* is shown in Figs. 2 and 3.

3.2. Comparison study of chromatographic performance

The analytical conditions for the tested columns were optimized with consideration that the method will be frequently used in routine analysis therefore a priority was placed on maintaining the speed, sensitivity, and resolution of analysis. The developed HPLC method took about 35 min and is considered a long run time for a series of routine analyses whereas the developed UPLC method was completed within 8 min. The UPLC method allowed for a shortened analysis time up to 4.5-fold compared to that of HPLC method therefore, the UPLC method was selected in order to save time and solvent consumption. The sensitivity of UPLC

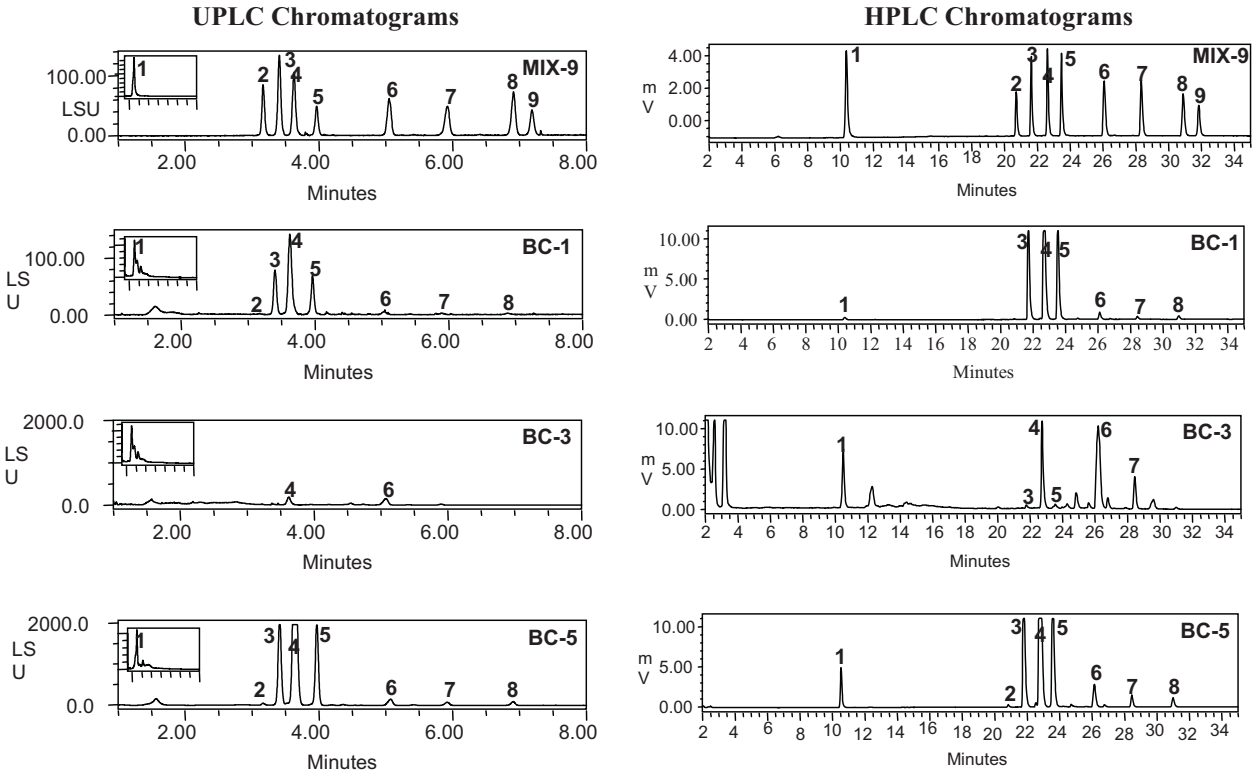

Fig. 3. UPLC and HPLC chromatograms of standard mix, plant sample (BC-1) and a dietary supplements (BC-3, BC-5) using ELS detector (**1**) magnoflorine, (**2**) cauloside H, (**3**) Leonticin D, (**4**) cauloside G, (**5**) cauloside D, (**6**) cauloside B, (**7**) cauloside C, (**8**) cauloside A, (**9**) saponin PE.

system was good even for such low injection volumes as 2 μL. The limits of detection for the saponins were between 10 and 35 μg/mL using HPLC–ELSD as compared to the UPLC–ELSD system which was in the range 7–10 μg/mL, whereas mass detector limits were as low as 0.1–0.25 μg/mL. Since the UPLC operates according to the chromatographic principles and separation mechanism similar to that of HPLC, method transfer and revalidation were quite easy and provided significant time saving. The typical chromatograms obtained from final HPLC and UPLC conditions of chemical fingerprint analysis are depicted in Fig. 3. The developed HPTLC method provides a simple, fast and high throughput technique that can be employed where inexpensive analysis is required. In addition multiple samples and standards can be spotted on the same HPTLC plate and developed in parallel making HPTLC an excellent application where many samples are routinely analyzed. This eliminates any variation that may occur when the sample and standards are run sequentially. Compared to UPLC or HPLC chromatograms, such fingerprints usually show less resolution but are still sensitive enough to monitor the differences between two samples and can be used for routine quality control analyses.

3.3. Method validation

The validation study allowed the evaluation of the method for its suitability for routine analysis.

3.3.1. Linearity range, LOD and LOQ

The five-point calibration curve for magnoflorine showed a linear correlation between concentration and peak area. Table 1 shows the calibration data for all nine compounds, including the regression equation, correlation coefficient ($r^2 > 0.999$), LOD and LOQ. Using the regression analysis, calibration curves for one alkaloid (linear range from 1 to 500 μg/mL) and saponins (25–250 μg/mL) was established, UV signals followed a linear response between concentration and peak area. The ELSD response was exponential relationship (log of response versus log of concentration was linear) and is a function of the mass of the observed compound. The limits

Table 1
Regression equation, correlation coefficient (r^2), limit of detection (LOD) and limit of quantification (LOQ) for magnoflorine and saponins from *C. thalictroides* by LC–UV/ELSD methods.

Compounds	Regression Equation		r^2		LOD (μg/mL)	LOQ (μg/mL)
	UPLC	HPLC	UPLC	HPLC		
1	$Y = 7.90e+003X - 2.97e+004$	$Y = 9.84e+003X - 1.32e+004$	0.9996	0.9998	1	5
2	$Y = 1.54e+000X + 1.87e+000$	$Y = 1.59e+000X + 1.05e+000$	0.9986	0.9999	10	25
3	$Y = 1.60e+000X + 1.93e+000$	$Y = 1.62e+000X + 1.22e+000$	0.9992	0.9999	10	25
4	$Y = 1.55e+000X + 1.99e+000$	$Y = 1.62e+000X + 1.21e+000$	0.9990	0.9999	10	25
5	$Y = 1.58e+000X + 1.60e+000$	$Y = 1.77e+000X + 1.23e+000$	0.9995	0.9996	10	25
6	$Y = 1.25e+000X + 2.52e+000$	$Y = 1.52e+000X + 1.30e+000$	0.9989	0.9998	10	25
7	$Y = 1.27e+000X + 2.46e+000$	$Y = 1.28e+000X + 1.37e+000$	0.9993	0.9999	10	25
8	$Y = 1.33e+000X + 2.42e+000$	$Y = 1.31e+000X + 1.72e+000$	0.9990	0.9999	10	25
9	$Y = 1.22e+000X + 2.44e+000$	$Y = 1.26e+000X + 1.61e+000$	0.9987	0.9999	7	25

Table 2
Intra- and inter-day precision of plant sample BC-1 assayed under optimized conditions for compounds **1**–**9** using HPLC–UV–ELSD and UPLC–UV–ELSD methods.

Compounds	Intra-day ($n = 3$)						Inter-day ($n = 9$)	
	Day-1		Day-2		Day-3		HPLC	UPLC
	HPLC	UPLC	HPLC	UPLC	HPLC	UPLC		
1	0.355 ± 0.18	0.355 ± 0.23	0.354 ± 0.11	0.356 ± 0.15	0.354 ± 0.12	0.356 ± 0.17	0.354 (0.13)	0.356 (0.18)
2	0.0255 ± 0.79	0.024 ± 3.65	0.0252 ± 0.65	0.026 ± 3.89	0.0253 ± 1.23	0.025 ± 4.19	0.0253 (0.89)	0.025 (3.91)
3	0.313 ± 0.48	0.310 ± 0.53	0.311 ± 0.50	0.313 ± 0.76	0.312 ± 0.49	0.312 ± 0.66	0.312 (0.49)	0.312 (0.65)
4	0.559 ± 0.19	0.555 ± 0.43	0.561 ± 0.25	0.559 ± 0.63	0.560 ± 0.43	0.557 ± 0.50	0.560 (0.29)	0.557 (0.52)
5	0.472 ± 0.059	0.473 ± 0.33	0.472 ± 0.075	0.471 ± 0.26	0.472 ± 0.066	0.472 ± 0.28	0.472 (0.07)	0.47 (0.29)
6	0.0734 ± 3.61	0.069 ± 1.45	0.0697 ± 3.45	0.071 ± 1.47	0.0638 ± 3.54	0.070 ± 1.46	0.072 (3.53)	0.069 (1.45)
7	0.0648 ± 2.11	0.061 ± 1.54	0.0629 ± 2.08	0.062 ± 1.59	0.0638 ± 2.09	0.062 ± 1.55	0.064 (2.09)	0.062 (1.56)
8	0.0337 ± 2.77	0.035 ± 3.43	0.0350 ± 2.67	0.033 ± 3.25	0.0344 ± 2.73	0.034 ± 3.34	0.034 (2.72)	0.034 (3.34)
9	DUL	DUL	DUL	DUL	DUL	DUL	DUL	DUL

Values in mg/100 mg of plant sample; relative standard deviation (% CV) are given in parentheses.

of detection and limits of quantification for all nine **(1–9)** markers were found to be in the range from 1 to 10 μg/mL and 5–25 μg/mL, respectively. The All standards and samples were injected in triplicate.

3.3.2. Specificity

The specificity of the method was determined by injecting individual samples, wherein no interference was observed for any of the components. The chromatograms were checked for the appearance of any extra peaks. The purity of the chromatographic peaks was found to be satisfactory.

3.3.3. Precision

Intra- and inter-day variation of the analysis was determined for sample BC-1 and was lower than 5%, with a maximum RSD of 3.77%. It was performed three times on three different days and each run was repeated in triplicate. Multiple injections showed that the results are highly reproducible and displayed a low standard error. The intra-day RSD for the replicates were between 0.67 and 3.77% for compounds **1**–**9** using HPLC and UPLC methods. The RSD for the day to day replicates were between 0.07 and 3.53% using UPLC method and 0.18–3.91% using HPLC method for compounds **1**–**9** (Table 2).

3.3.4. Accuracy

The accuracy of the method was determined for the related compound by spiking sample (BC-1) with a known amount of magnoflorine and saponins. Plant sample was exhaustively extracted with four times and dried then spiked with known amounts of the standard compounds at two different concentrations, extracted again and analyzed under optimized conditions. The accuracy of the assay method was evaluated in triplicate at two concentration levels, 25 and 50 μg/ml of standards in the sample. The percentage recovery in samples ranged from 96.0 to 103.0%.

3.3.5. Analysis of plant samples and dietary supplements

3.3.5.1. LC–UV–ELSD. The LC–UV–ELSD data and the LC–MS chromatograms show the presence or absence of compounds in plant samples (BC-1, BC-2), and dietary supplements (BC-3 to BC-11). The UPLC and HPLC methods were applied for quantification of one alkaloid and eight saponins in various plant samples and commercial products.

Dietary supplements (BC-3 to BC-11) claiming to contain *C. thalictroides* are available as root/rhizome powders (fresh or dry) in liquid extracts (BC-3 to BC-8) and capsules (BC-9 to BC-11). For most of the products the information provided on the labels generally included the part of the plant used, other ingredients like alcohol (for liquids) and gelatin (for capsules), recommended daily intake, and suggested use. All the product labels carried statements warning against use during pregnancy or lactation (e.g., "not to be used during pregnancy", "seek expert medical advice before taking during pregnancy", "do not use if you are pregnant or may become pregnant", "do not use if pregnant or nursing"). Blue cohosh supplements were labeled simply as herbal dietary supplement. Suggested uses included use as a woman's tonic, to ease menstruation, to balance uterine function, and for smooth muscle relaxation. Recommended intakes were expressed as number of capsules/day (for capsules), and number of drops or mL/day (for extracts). All the products contained only *C. thalictroides* as the botanical ingredient.

Estimated maximum daily intake (mg/day) = to the weight of alkaloid or saponin (mg) × dilution factor × the suggested maximum daily intake in capsules or drops/weight (mg) of content in capsules or drops. Total amounts of alkaloids and saponins that might be consumed daily were highest in BC-4 and lowest in BC-3 samples (Table 4). Magnoflorine content that might be consumed daily ranged from 0.6 to 15.8 mg for all the products (BC-3 to BC-11) and about 0.36% in plant samples (BC-1, BC-2). Total saponins that might be consumed daily ranged from 5.87 to 302.4 mg for all products (BC-3 to BC-11) and 1.5 and 1.3% for BC-1 and BC-2 root samples, respectively. Compound **9** was detected under limits of quantification for samples BC-1, BC-2, BC-5 to BC-9 and not detected in samples BC-3, BC-10 and BC-11. Compounds **2**, **8** and **9** were not detected in sample BC-3. Figs. 2 and 3, show the HPLC and UPLC chromatograms of plant sample and commercial products. Table 3 shows the % content of alkaloid and saponins in plant samples. Table 4 shows the different nature of information contained on the labels for the supplements studied as compared to the mg/day of dosage form. Nine commercially available dietary supplements were tested (BC-3 to BC-11) for the presence of magnoflorine and saponins. Six products (out of 9 products) contained all the compounds. Compounds **1**, 3–**5** were found to be in major amounts. One product (BC-3) did not show the presence of compounds **2**, **8** and **9** and three products (BC-3, BC-10 and BC-11) did not contain compound **9** by all three methods (HPLC, UPLC and HPTLC).

By the LC–UV–ELSD methods, the identification of the compounds in plant samples and dietary supplements was based on the retention times and the comparison of UV spectra for magnoflorine or by spiking the extracts with the standard compounds.

Different plant samples and commercial products have been analyzed using the described methods, and remarkable qualitative and quantitative variations were revealed. Comparing the daily uptake of total saponins, a difference of greater than 70-fold was observed with in the various products.

3.3.5.2. TLC densitometry. Different proportions chloroform:methanol:water were tried as mobile phase to achieve the separation of compounds **1**–**9** on silica gel HPTLC plates. Lower layer of chloroform: methanol: water in a ratio of 65:35:10.5 (v/v/v)

Table 3
Content of magnoflorine and saponins from roots of *C. thalictroides* (L.) Michx. (%, w/w) and dietary supplements [mg/mL or mg/average wt of sample (mg)] using (a) UPLC–UV–ELSD and (b) HPLC–UV–ELSD methods.

#	Plant sample (BC-1) (%, *w/w*)	Plant sample (BC-2) (%, *w/w*)	Liquids (mg/mL)						Capsules (%, w/w)		
			BC-3	BC-4	BC-5	BC-6	BC-7	BC-8	BC-9	BC-10	BC-11
(a) UPLC–UV–ELSD method											
1	0.36 ± 0.18	0.36 ± 0.007	0.75 ± 0.005	3.95 ± 0.13	3.25 ± 0.15	2.94 ± 0.14	1.36 ± 0.28	0.19 ± 0.37	1.12 ± 0.29	1.25 ± 0.05	1.06 ± 0.06
2	0.025 ± 3.91	0.02 ± 1.42	ND	1.21 ± 0.34	0.73 ± 0.39	0.65 ± 0.87	0.72 ± 0.27	0.63 ± 0.83	0.19 ± 1.87	0.16 ± 0.01	0.22 ± 3.11
3	0.312 ± 0.65	0.23 ± 1.67	0.095 ± 0.19	15.28 ± 0.02	6.25 ± 0.06	6.03 ± 0.08	5.86 ± 0.03	4.57 ± 0.02	2.99 ± 0.07	1.91 ± 0.09	2.87 ± 0.11
4	0.56 ± 0.52	0.45 ± 0.14	0.62 ± 0.14	24.73 ± 0.01	17.19 ± 0.02	12.54 ± 0.03	9.25 ± 0.03	7.09 ± 0.04	4.31 ± 0.07	4.54 ± 0.002	4.19 ± 0.18
5	0.47 ± 0.29	0.38 ± 0.12	0.09 ± 0.06	27.41 ± 0.02	9.95 ± 0.03	10.51 ± 0.04	9.69 ± 0.06	7.36 ± 0.06	5.45 ± 0.05	3.67 ± 1.06	5.05 ± 0.04
6	0.07 ± 1.45	0.07 ± 3.95	0.99 ± 0.12	3.42 ± 0.08	2.18 ± 0.27	1.56 ± 0.21	1.37 ± 0.27	1.06 ± 0.34	0.47 ± 1.18	0.48 ± 0.35	0.53 ± 1.46
7	0.062 ± 1.56	0.058 ± 0.43	0.188 ± 0.66	1.46 ± 0.23	1.85 ± 0.27	0.83 ± 0.72	1.05 ± 0.31	0.69 ± 0.33	0.34 ± 0.76	0.48 ± 1.91	0.38 ± 1.02
8	0.034 ± 3.34	0.04 ± 1.97	ND	1.99 ± 0.07	1.45 ± 0.39	1.03 ± 0.34	0.80 ± 0.48	0.65 ± 0.55	0.28 ± 2.39	0.33 ± 0.03	0.37 ± 3.33
9	DUL	DUL	ND	0.093 ± 4.44	DUL	DUL	DUL	DUL	DUL	ND	ND

#	Plant sample (BC-1) (%, *w/w*)	Plant sample (BC-2) (%, *w/w*)	Liquids (mg/mL)						Capsules (%, w/w)		
			BC-3	BC-4	BC-5	BC-6	BC-7	BC-8	BC-9	BC-10	BC-11
(b) HPLC–UV–ELSD method											
1	0.35 ± 0.13	0.36 ± 0.08	0.75 ± 0.11	3.93 ± 0.15	3.253 ± 0.11	3.01 ± 4.26	1.361 ± 0.21	0.194 ± 0.37	1.12 ± 0.14	1.26 ± 0.08	1.05 ± 0.21
2	0.025 ± 0.89	0.02 ± 2.05	ND	1.211 ± 0.43	0.74 ± 0.26	0.654 ± 1.03	0.71 ± 0.09	0.64 ± 1.35	0.18 ± 0.55	0.158 ± 2.89	0.22 ± 0.57
3	0.31 ± 0.49	0.23 ± 0.15	0.095 ± 1.26	15.29 ± 0.04	6.21 ± 0.07	6.04 ± 0.09	5.90 ± 0.04	4.56 ± 1.23	2.98 ± 0.05	1.94 ± 0.77	2.87 ± 0.11
4	0.56 ± 0.29	0.45 ± 0.15	0.62 ± 0.04	24.72 ± 0.02	17.18 ± 0.002	12.52 ± 0.01	9.36 ± 0.04	6.88 ± 0.41	4.29 ± 0.07	4.50 ± 0.035	4.20 ± 0.10
5	0.47 ± 0.07	0.38 ± 0.29	0.094 ± 0.67	27.40 ± 0.02	9.94 ± 0.04	10.53 ± 0.05	9.75 ± 0.05	7.23 ± 0.49	5.45 ± 0.12	3.666 ± 0.28	5.04 ± 0.05
6	0.07 ± 3.53	0.07 ± 0.39	0.988 ± 0.02	3.40 ± 0.10	2.18 ± 0.065	1.549 ± 0.37	1.39 ± 0.25	1.03 ± 4.45	0.47 ± 0.94	0.49 ± 0.32	0.52 ± 0.33
7	0.064 ± 2.09	0.06 ± 1.94	0.187 ± 1.51	1.45 ± 0.29	1.825 ± 1.94	0.84 ± 0.34	1.06 ± 0.60	0.79 ± 0.44	0.34 ± 1.31	0.499 ± 1.44	0.38 ± 0.44
8	0.034 ± 2.72	0.038 ± 0.92	ND	1.99 ± 0.07	1.447 ± 0.24	1.04 ± 0.34	0.79 ± 0.45	0.653 ± 0.49	0.27 ± 0.52	0.34 ± 1.58	0.37 ± 0.06
9	DUL	DUL	ND	0.091 ± 2.13	DUL	DUL	DUL	DUL	DUL	ND	ND

Abbreviations: ND, not detected; DUL, detected under limits of quantification.
*Mean Values (n = 3) ± RSD.

Table 4
Content (mg) of alkaloid and total saponins in various blue cohosh products and estimated maximum daily intakes to information provided on product labels.

Product code	Wt used for analysis	Serving size	Content/serving size	Label claim	Magnoflorine content	Total saponins content
BC-1 (dry ground roots)	100.0 mg	NA	NA	NA	0.356%	1.53%
BC-2 (dry ground roots)	100.2 mg	NA	NA	NA	0.359%	1.25%
BC-3 (fresh wild crafted roots)	1 mL = 30 drops	30 drops	1000 mg	0.5–1 mL thrice daily	2.24 mg	5.97 mg
BC-4 (roots)	1 mL = 30 drops	30 drops	500 mg	1 mL (3–4 times daily)	15.8 mg	302.4 mg
BC-5 (dried roots)	1 mL = 20 drops	20 drops	–	0.5–1 mL (3 times daily)	9.75 mg	118.8 mg
BC-6 (wild harvested roots)	1 mL = 30 drops	30 drops	333 mg	5–20 drops (2–3 times daily)	5.88 mg	66.32 mg
BC-7 (hand harvested dried roots & rhizomes)	1 mL	–	–	30–40 drops (2–4 times a day)	5.46 mg	114.92 mg
BC-8 (fresh wild harvested roots)	1 mL = 20 drops	20 drops	–	5–20 drops (3 times a day)	0.57 mg	66.15 mg
BC-9 (roots)	100.4 mg	1 capsule	500 mg	2 servings/day	11.2 mg	140.3 mg
BC-10 (roots)	100.0 mg	1 capsule	500 mg	2 servings/day	12.55 mg	115.6 mg
BC-11 (fresh roots)	100.4 mg	–	595 mg	2 capsules, one–two times a day	12.61 mg	161.84 mg

Abbreviations: NA, not applicable, bulk plant materials (no label claim); "–", not mentioned.

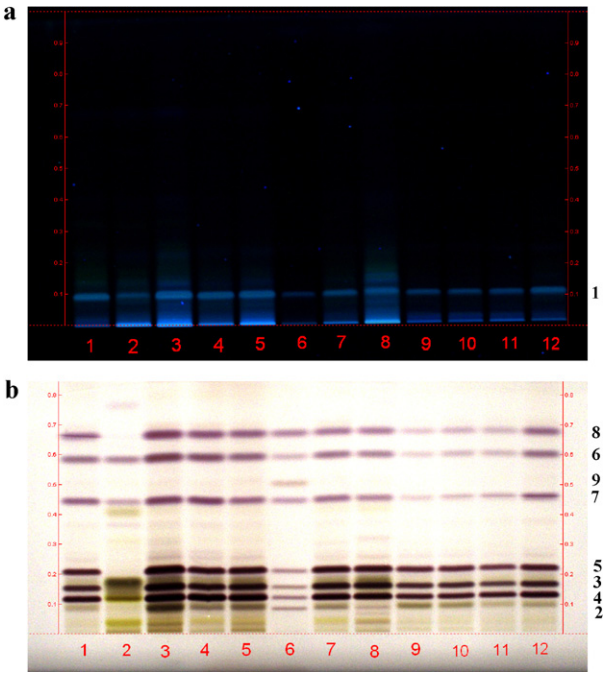

Fig. 4. HPTLC fingerprints of various products of blue cohosh (a) Before derivatization for magnoflorine at UV 366 nm; (b) After derivatization (white light) for saponins. Tracks 1 and 12, Plant sample (BC-1); tracks 2–5 and 7–11, dietary supplements (BC-3 to BC-11); track 6, standard mixture of nine in ascending order [cauloside H (**2**), cauloside G (**4**), magnoflorine (**1**), leonticin D (**3**), cauloside D (**5**), cauloside C (**7**), saponin PE (**9**), cauloside B (**6**), cauloside A (**8**)].

was found to give good resolution without prechromatographic derivatization or impregnation of the thin layer chromatography of silica gel plates. The fingerprinting can be described using **1**–**9** as standard compounds after derivatization with 5% sulfuric acid reagent (Fig. 1S). The analysis of nine dietary products showed variation in the relative intensities of the separated zones, but the profile was found to be same in comparison with the plant samples (Fig. 4). A thin layer chromatography–densitometric method has been developed for chemical fingerprint analysis. The method is suitable for rapid, decisive authentication, and visual comparison of the differences among samples from various plant sources. The developed HPTLC method provides a faster, cost effective qualitative analysis that can be useful in the authentication of *C. thalictroides* and for the screening of blue cohosh products.

3.3.5.3. UPLC–MS. This method involved the use of $[M]^+$, and $[M+Na]^+$ ions of compounds **1**–**9** which were observed for standard compounds in the positive ion mode with selected ion recording (SIR) at *m/z* 342.1 $[M]^+$, 1275.5 $[M+Na]^+$, 1113.5 $[M+Na]^+$, 1259.5 $[M+Na]^+$, 1097.5 $[M+Na]^+$, 643.3 $[M+Na]^+$, 789.4 $[M+Na]^+$, 627.4 $[M+Na]^+$ and 773.4 $[M+Na]^+$, respectively. No interfering peaks were found at the retention time of interest. Further, the fragmentation patterns observed in the mass spectrum were useful in characterization of the compounds. Leonticin D (**3**), cauloside G (**4**) and cauloside D (**5**) were found to be the major compounds among the analyzed saponins. Fragments detected for leonticin D (**3**), cauloside B (**6**) of two root samples and the dietary supplements that claimed to contain *C. thalictroides* are 453.3 $[\text{aglycone}+H-2H_2O]^+$ and 435.2 $[\text{aglycone}+H-3H_2O]^+$. The key fragments detected for compounds 4, 5, 7 and 9 were 455.3 $[\text{aglycone}+H-H_2O]^+$, and 437.3 $[\text{aglycone}+H-2H_2O]^+$ (Fig. 2S) (Table 4). Compounds **4**, **5**, **7** and **8** have hederagenin as their aglycone (Fig. 1) with the sugar moiety positioned at C-3. Compounds **4**, **5** also showed the presence of sugar moieties at C-28 position. The identities, retention time $[M+Na]^+$, and characteristic

Table 5
Peak assignment for the analysis of *C. thalictroides* roots by UPLC–MS Method.

Peak	RT	$[M]^+$ or $[M+Na]^+$ (*m/z*)	MS fragment ions (*m/z*)	Identities
1	1.91	342.1 $[M]^+$	297 $[M^+–OCH_3–CH_2]$	Magnoflorine
2	3.24	1275.5	976.5, 635.3, 471.2 $[aglycone+H–H_2O]^+$	Cauloside H
3	3.46	1113.5	453.3 $[aglycone+H–2H_2O]^+$, 435.2 $[aglycone+H–3H_2O]^+$	Leonticin D
4	3.68	1259.5	960.5, 455.3 $[aglycone+H–H_2O]^+$, 437.3 $[aglycone+H–2H_2O]^+$	Cauloside G
5	4.05	1097.5	455.3 $[aglycone+H–H_2O]^+$, 437.3 $[aglycone+H–2H_2O]^+$	Cauloside D
6	5.08	643.3	471.3 $[aglycone+H–H_2O]^+$, 453.3 $[aglycone+H–2H_2O]^+$, 435.2 $[aglycone+H–3H_2O]^+$	Cauloside B
7	5.97	789.4	455.3 $[aglycone+H–H_2O]^+$, 437.3 $[aglycone+H–2H_2O]^+$	Cauloside C
8	7.00	627.4	455.3 $[aglycone+H–H_2O]^+$, 437.3 $[aglycone+H–2H_2O]^+$, 409.3 $[aglycone+H–2H_2O–CO]^+$	Cauloside A
9	7.26	773.4	439.3	Saponin PE

fragment ions for individual peaks are presented in Table 5. Compounds **1**–**9** in *C. thalictroides* and in the dietary supplements that contained *C. thalictroides* were identified by comparison of the retention time and mass spectral data with those of the standards (Table 5).

4. Conclusion

The newly developed UPLC method for the chemical analysis of nine compounds from blue cohosh was found to be capable of providing short retention times while maintaining good resolution as compared to conventional HPLC. The new UPLC technique allowed for the reduction in the mobile phase flow rate and an increase in acquisition rate with the benefit of decreased injection volumes to achieve good peak shapes. The method is suitable for rapid analysis of magnoflorine and saponins and for chemical fingerprint analysis of blue cohosh samples. The developed method was validated for all the parameters tested and successfully applied to the identification of nine compounds in two samples of blue cohosh and nine dietary supplements which claim to contain *C. thalictroides*. All samples of *C. thalictroides* and the nine dietary supplements were found to contain compounds **1**, and **3**–**7**. Different sample matrices were successfully analyzed, including capsules and liquids. LC–mass spectrometry coupled with electrospray ionization (ESI) method is described for the identification and confirmation of compounds in various plant samples. The HPTLC fingerprints developed in this study are based on multiple detection method for the same plate which can provide a very characteristic visual impression of a sequence of (colored) zones that will aid in the identication of blue cohosh samples. An important feature of developed HPTLC method is its ability to identify a large number of samples in parallel thus affording rapid results. Finally it can be concluded that because of the simple sample preparation, short analysis time and high reproducibility, the methods presented in this paper can provide a useful tool for the routine analysis of magnoflorine and saponins from plant samples as well as commercial preparations and help in assuring safety and quality control of blue cohosh preparations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2011.07.028.

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