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Simultaneous Quantification of *Echinacea* Species, *Flos Lonicerae*, *Radix Scutellaria* and *Fructus Forsythiae* Combinations by Rapid Resolution Liquid Chromatography

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Echinacea angustifolia and *E. purpurea* are commonly used in North America for their anti-bacterial effects. *Flos Lonicerae*, *Radix Scutellaria* and *Fructus Forsythiae* are traditional Chinese medicinal herbs commonly used for the treatment of complaints such as pneumonia, acute upper respiratory tract infection, and acute bronchitis. A reproducible, simple, and reliable rapid resolution liquid chromatographic (RRLC) method has been developed to analyze extracts of products formulated containing *E. angustifolia*, *E. purpurea*, *Flos Lonicerae*, *Radix Scutellariae* and *Fructus Forsythiae* simultaneously in one run in less than 6 minutes. The method uses a C18-HST column, a mobile phase consisting of 0.1% aqueous phosphoric acid solution and acetonitrile, and UV detection at 327 nm and 229 nm. A stability test was performed that revealed that chlorogenic acid is more stable in acidic pH, and hence it is best to keep the extract of *E. augustifolia*, *E. purpurea*, *Flos Lonicerae*, *Radix Scutellariae* and *Fructus Forsythiae* in mild acidic conditions at approximately pH 5.

Keywords: *Echinacea, Flos Lonicerae*, chlorogenic acid, RRLC, stability testing, HPLC profile.

Echinacea species (Asteraceae) are perennial plants that are native to North America ranging throughout the US Great Plains, Canadian Prairies, Appalachian uplands and coastal plains [1]. There are nine *Echinacea* species of which three are used clinically, namely, *E. angustifolia* DC. var. *angustifolia, E. purpurea* (L.) Moench and *E. pallid*a (Nutt.) Nutt. [1,2]. *E. angustifolia* has adapted naturally to different habitats from Texas to Saskatchewan, but is one of the more difficult *Echinacea* species to cultivate. *E. purpurea* represents about 80% of commercial products, such as extracts and tinctures, produced according to general manufacturing practices (GMPs) [2]. Traditionally, *Echinacea* species were used extensively by native Americans of the Great Plains region [3,4], but from the 1930s to 1970s, due to antibiotic development, there was a great decline in the use of *Echinacea*, but in the 1980s, the increasing awareness of herbal medicine led to renewed interest in these species. In the last 20 years, research has shown *Echinacea* species to have anti-inflammatory, wound-healing and immune system stimulating effects against bacterial and viral infections [5-8]. Treatment of the common cold using *E. purpurea* was able to shorten effectively the healing time [9]. Many traditional Chinese formulas, namely, Yi-Qing capsules, Niu-Huang-shang-Qing-Wan, Ba-Du-Gao, and Shuang-Huang-Lian (SHL), comprised of *Flos Lonicerae, Radix Scutellariae* and *Fructus Forsythia*e in different percentages, are also used for viral and bacterial infections. They are often taken to treat respiratory infections such as, tonsillitis, pharyngitis, and pneumonia, and for acute enteritis and viral dysentery caused by viral and bacterial infections [10,11].

Echinacea species contain polysaccharides, flavonoids, caffeic acid derivatives, essential oils, polyacetylenes, alkylamides and miscellaneous chemicals [12]. The USP reports that caffeic acid, cichoric acid, chlorogenic acid, dicaffeoylquinic acids, echinacoside and dodecatetraenoic acid isobutylamides are found in *Echinacea* species [13], and the HPLC profile of these can be used for identification of *Echinacea* and *Echinacea*-formulated products. Among these active ingredients, echinacoside and cichoric acid, a caffeic acid derivative, are commonly used as markers for the identification of *E. angustifolia* and *E. purpurea*, respectively [14,15]. Echinacoside is commonly used in the Natural Health Product industry as a marker for the quality control of *Echinacea* preparations, but it is not found in *E. purpurea*. It is most popular for its antibacterial and antiviral effects, as well as for its ability to protect against reactive oxygen species [15,16].

Cichoric acid, the major active compound found in *E. purpurea*, is known for its *in vitro* and *in vivo* immunostimulatory properties, and its ability to inhibit the enzyme hyaluronidase. This enzyme is involved in bacterial infections and is an inhibitor of integrase, an enzyme required during human immunodeficiency virus-1 replication to integrate the double-stranded DNA copy of the viral genome into host cells [15,17].

Chlorogenic acid, baicalin and forsythin are the main active compounds of *Flos Lonicerae*, *Radix Scutellariae* and *Fructus Forsythiae*, respectively [18]. Studies showed that chlorogenic acid has anti-inflammatory, antipyretic, analgesic, antimutagenic and anticancer effects [19-23]. Chlorogenic acid, kryptochlorogenic acid and neochlorogenic acids, also known as 3-*O*-, 4-*O*- and 5-*O*-caffeoylquinic acids, are isomers of each other and are believed to have different pharmacological effects. It is, therefore, important to analyze and quantify each compound separately [24].

Recently, many analytical methods, including miceller electrokinetic chromatography [25], GC-MS [26], LC-UV [10,27-30], LC-MS [31] and capillary zone electrophoresis [32] have been used for *E. angustifolia*, *E. purpurea, Flos Lonicerae, Radix Scutellariae*, and *Fructus Forsythiae* herbs. Unfortunately, all these methods either require a large consumption of organic solvents or a long analysis time, and, therefore, a rapid resolution liquid chromatographic (RRLC) method that was highly sensitive, provided high-speed detection and had reduced analytical costs, would be ideal for both qualitative and quantitative analysis.

In recent years *Echinacea* species, typical North American herbs, and *Flos Lonicerae*, *Radix Scutellariae* and *Fructus Forsythiae*, traditional Chinese drugs, have been used in conjuction. This formula has been very popular in both the North American and Asian markets, and, therefore, it is important to have a method that can analyze the compounds in these herbs simultaneously, both quantitatively and qualitatively. It is a challenge today in the natural health product market to analyze the major active compounds and establish an HPLC profile which can effectively reflect each original herbal product in the formulated products.

The original method described by Ma *et al*. is well established for the Shuang – Huang – Lian formula, but, with the addition of *Echinacea*, baseline separation of echinacoside and cichoric acid could not be achieved and hence the need for a new analytical procedure for products containing *Echinacea* and honeysuckle (*Lonicera*) [24]. UV absorptions for each of the extracts of the five herbs under consideration in this study give characteristic peaks at 329 and 229 nm, but for *Echinacea* plants at 330 nm. The major active compounds in *Echinacea* species have a relatively stronger absorption due to their aromatic nature, and, therefore, detection at 329 nm and 229 nm were selected to analyze the bioactive compounds of the five herbs in the mixture.

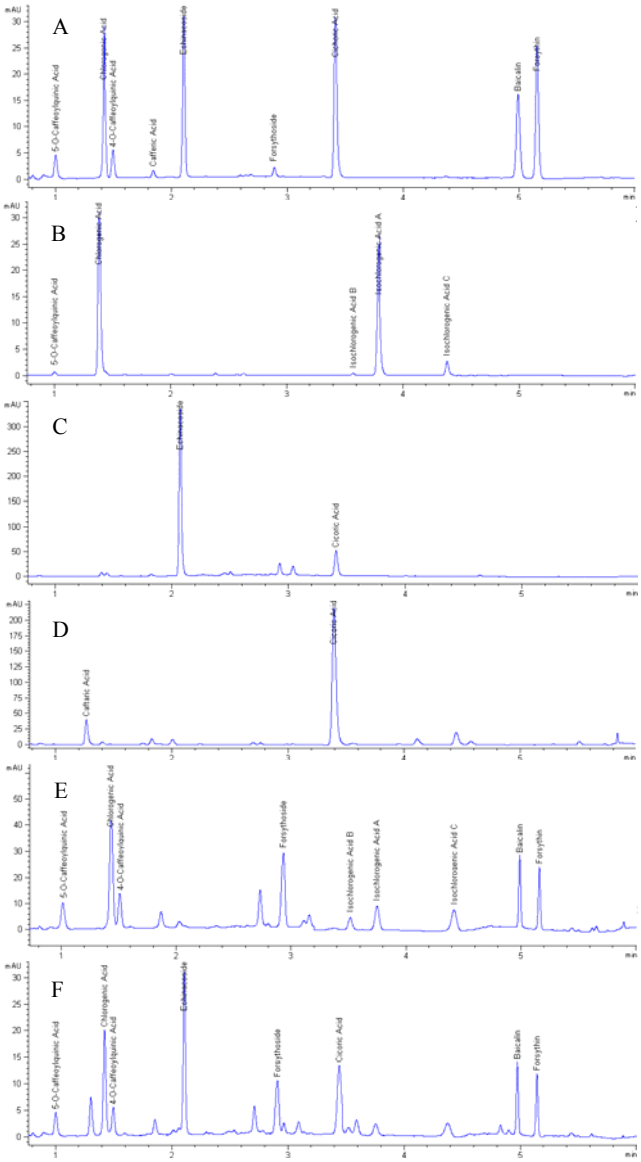

Figure 1: HPLC Chromatograms of (A) Chlorogenic acid, echinacoside, cichoric acid, baicalin and forsythin reference standards (B) *Flos Lonicerae* herb (C) *E. augustifolia* extract (D) *E. purpurea* extract (E) *Flos Lonicerae* + *Radix Scutellariae* + *Fructus Forsythiae* extract (F) *E. augustifolia* + *E. purpurea* + *Flos Lonicerae* + *Radix Scutellariae* + *Fructus Forsythiae* extract analysis performed at 327 nm from 0 – 4.8 mins and 229 nm from 4.8 – 6 mins.

The linearity for echinacoside, cichoric acid, chlorogenic acid, forsythin, forsythoside and baicalin were established by plotting the peak area (Y) versus concentration (X), expressed by the equations given in Table 1. Correlation studies verified the linearity of the calibration curves, and the correlation coefficients were all above 0.999. Peaks were assigned by comparing the retention time of samples with the appropriate reference standards and were represented over a range of 0.37 – 1000 µg/mL. The Limit of Detection (LOD) and Limit of Quantification (LOQ) for echinacoside, cichoric acid, chlorogenic acid, forsythin,

forsythoside and baicalin were estimated at signal-to-noise ratios (*S*/N) of 3 and 10, respectively. The LOD and LOQ for the bioactive compounds were ≤ 0.56 μg/mL and ≤ 1.85 μg/mL, respectively. The original HPLC profile of each herb is shown in Figure 1. Figure 1A shows the separation of mixed marker compounds: caftaric acid, echinacoside, cichoric acid, chlorogenic acid and forsythoside analyzed at 327 nm, and forsythin and baicalin at 229 nm. Forsythoside and caftaric acid were identified by use of reference standards using retention times. The mixed reference standard solution was analyzed in triplicate to determine the reproducibility of the method developed. Figure 1B shows the profile of the *Flos Lonicerae* herb. Figures 1C and 1D show the typical profiles of *E. augustifolia* and *E. purpurea,* respectively, which are identical to that shown in the USP [13]. Figure 1E shows the chromatograph of *Flos Lonicerae*, *Radix Scutellariae* and *Fructus Forsythiae* extract, and Figure 1F that of the extract of *E. angustifolia*, *E. purpurea*, *Flos Lonicerae*, *Radix Scutellariae* and *Fructus Forsythiae*. Table 1 shows the Relative Standard Deviation (RSD) for both retention time and recovery rate. It is less than 1.88%. This shows that the method is precise for the analysis of the extract of *E. angustifolia*, *E. purpurea*, *Flos Lonicerae*, *Radix Scutellariae* and *Fructus Forsythiae* herbs. A recovery test was used for determining accuracy. Known quantities of the reference standards were spiked into 1 mL of a sample of the combined extract of *E. angustifolia*, *E. purpurea*, *Flos Lonicerae*, *Radix Scutellariae* and *Fructus Forsythiae*. The sample was then diluted and analyzed in triplicate. The results were compared with the original extract samples of *E. angustifolia*, *E. purpurea*, *Flos Lonicerae*, *Radix Scutellariae* and *Fructus Forsythiae*. The mean recoveries were from 98.3% to 100.4% with RSD less than 1.88% for the 12 bioactive compounds. Therefore, these results show precision, accuracy, recovery and good repeatability.

Two analysts performed the ruggedness test on samples of the extract of *E. angustifolia*, *E. purpurea*, *Flos Lonicerae*, *Radix Scutellariae* and *Fructus Forsythiae* Three different lot numbers of each herbal extract, prepared in duplicate, were analyzed by each analyst. The RSD of chlorogenic acid, baicalin, forsythin, echinacoside and cichoric acid concentrations in all of the samples was less than 1.6%, indicating acceptable ruggedness.

Chromatographic parameters are important factors to consider for the separation of *E. augustifolia*, *E. purpurea*, *Flos Lonicerae*, *Radix Scutellariae* and *Fructus Forsythiae* extract samples. Different conditions were observed, but only one factor was changed each time, while the others were kept constant. Different pH values of the mobile phase A (1.8, 2.0, and 2.2), flow rates of 0.95, 1.0 and 1.05 mL/min, and column temperatures of 38, 40 and 42°C were considered. The results obtained are summarized in Table 2. Satisfactory separation, symmetrical peaks and minimum baseline resolution were all achieved using the above conditions, indicating the robustness of this method in the tested range.

The extract of *E. purpurea, E. angustifolia*, *Flos Lonicerae*, and *Fructus Forsythiae* was examined. Three different pH values were considered for stability testing, namely, pH 5.06, 5.36 and 7.00. At pH 5.36, no pH adjustments were made, for pH 5.06, a small amount of HCL was added and for pH 7.00, NaOH was added. At pH 5.36 and 5.06, baicalin did not dissolve completely, but was suspended in the extract, but at pH 7.00, it dissolved fully. After 7 days of stability testing in the Equity chamber at pH 7 (equivalent to over one month's storage at room temperature), chlorogenic acid had isomerized by 12.6%, determined through calculation of peak area. This decrease in the concentration of chlorogenic acid resulted in an equivalent increase in the concentrations of 4-*O* and

Table 1: Linearity, regression equation, LOD, LOQ, retention time, and peak area for the reference compounds using the Phenomenex Luna column.

Compounds	Regression Equation	r^2	LOD (μg/mL)	LOQ (μg/mL)	t_R (min)	RSD%	Recovery	RSD%
Chlorogenic Acid	913.92X + 1.63	0.9999	0.0044	0.01295	1.428	0.25	100.4	1.15
Echinacoside	529.98X – 0.08	1.0000	0.37	1.11	2.111	0.42	98.3	1.88
Cichoric Acid	490.98X – 0.10	1.0000	0.56	1.85	3.353	0.40	98.9	1.45
Baicalin	362.90x – 0.63	0.9998	0.010	0.030	4.962	0.17	98.6	1.42
Forsythin	597.91X + 2.98	0.9999	0.0099	0.029	5.218	0.15	99.5	1.36

R^2, Correlation coefficient of regression equations; LOD, limit of detection (*S*/N = 3); LOQ, limit of quantification (*S*/N = 10)

Table 2: Summary of resolution values for method robustness testing for chlorogenic acid, echinacoside, cichoric acid, baicalin and forsythin.

Method parameter	Chlorogenic acid	Echinacoside	Cichoric Acid	Baicalin	Forsythin
Column Temp. (°C)					
38	1.62	6.51	1.51	6.46	4.69
40	1.60	5.45	1.50	5.16	3.53
42	1.37	5.13	1.30	5.08	3.12
pH of mobile phase A					
1.8	1.57	5.49	1.48	5.18	3.46
2.0	1.60	5.45	1.50	5.16	3.53
2.2	1.43	4.53	1.29	4.80	3.62
Flow rate (mL/min)					
0.95	1.28	3.48	1.20	3.19	3.48
1.00	1.60	5.45	1.50	5.16	3.53
1.05	1.38	3.56	1.31	3.21	3.21

5-*O*-caffeoylquinic acid. After 7 days at pH 5.36 (the original state), minor degradation of chlorogenic acid was observed (around 5.1%), but echinacoside, cichoric acid, baicalin and forsythin concentrations showed no significant changes. At pH 5.06, the concentration stayed the same for chlorogenic acid, echinacoside, cichoric acid, baicalin and forsythin. After 14 days of storage (equivalent to about nine weeks of storage at room temperature) in a stability chamber at pH 7.00, 15.6% isomerization of chlorogenic acid to 4-*O*- and 5-*O*-caffeoylquinic acid was recorded, and at pH 5.36, 12.3%. At pH 5.06, all active compounds were relatively stable. At pH 7 or greater, although baicalin was dissolved fully, chlorogenic acid was not stable. At pH 5.06, although baicalin is not fully dissolved and remained partly in suspension, it was shown to be stable, as was chlorogenic acid. From these results, it can be concluded that the extract of *E. angustifolia*, *E. purpurea*, *Flos Lonicerae*, *Radix Scutellariae* and *Fructus Forsythiae* should be stored in mild acidic conditions at around pH 5.

Experimental

Materials and reagents: Acetonitrile and methanol of HPLC grade were purchased from Fisher Scientific Canada. Deionized water was purified using Barnstead's Nanapure ultrapure water system (Barnstead, USA). HPLC grade phosphoric acid (85%) (H_3PO_4) was obtained from EM Science (Darmstadt, Germany). Echinacoside and cichoric acid standards were purchased from Chromadex (CA, USA), and chlorogenic acid, baicalin and forsythin from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Standards of forsythoside, caftaric acid, cafferic acid, 5-*O*- and 4-*O*-caffeoylquinic acid, and isochlorogenic acids A, B and C were provided by Dr Yu-Xin Zhou (Beijing, China). *Flos Lonicerae*, *Fructus Forsythiae* and *Radix Scutellariae* raw herbs were collected from Shandong, Hunan and Heilongjiang Provinces (China). *E. angustifolia* and *E. purpurea* were obtained from local growers. Membrane filters (0.20 μm) were purchased from VWR International (Mississauga, Canada).

Sample and reference standard solution preparation: Standard stock solutions of echinacoside, cichoric acid, chlorogenic acid, forsythin, forsythoside and baicalin were prepared by dissolving the required amounts in 50% aqueous MeOH to a final concentration of 1 mg/mL and refrigerated at 4°C.

Approximately 5 g of dried raw herbs of *E. angustifolia*, *E. purpurea*, *Flos Lonicerae*, *Radix Scutellariae* and *Fructus Forsythiae* were ground into fine powder and 0.10 g was accurately weighed in a 10 μL volumetric flask and mixed with 10 mL of 100% MeOH and ultra-sonicated at 37°C for 15 mins. After sonication, the volume was adjusted to 10 mL with 100% MeOH. The extract solutions were cooled to room temperature, the supernatant collected, and passed through a membrane filter (0.20 μm) for further analysis. Each sample was injected in triplicate and the RSD calculated for all the samples. Each of the prepared solutions (1 μL) was injected into the RRLC system. All analyses were performed on an Agilent 1200 Series Rapid Resolution Liquid Chromatography (RRLC) system (Agilent, CA, USA) equipped with a binary pump SL, vacuum degasser from a high-speed, high-performance auto plate-sampler SL for high area precision, and a thermostated column compartment SL that reaches up to 100°C. This system was controlled by an Agilent ChemStation, revision B01.01. A Phenomenex Luna 2.5 μm C18 (2)-HST (3.0 mm x 100 mm) column (Torrance, CA, USA) was employed at 40°C. Gradient elution with a mobile phase of A (H_2O + 0.1% phosphoric acid) and B (acetonitrile, CH_3CN) was used at a flow rate of 1.00 mL/min. Gradient elution was employed as follows: 12% B at 0 - 2 min; 20% B at 2 - 3 min; 21% B at 3 – 3.5 min; 20% B at 3.5 - 4 min; 30% B at 4 - 5 min; 38% B at 5 - 6 min; and 100% B at 6 min. Simultaneous wavelength monitoring was performed at 327 nm and 229 nm

All samples for stability testing were prepared, tested in triplicate and stored in a Testequity 1000H series temperature/humidity chamber (Moorpark, USA) at 40°C and 75% relative humidity. RRLC analysis was performed after 7 and 14 days of storage using the above indicated method.

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