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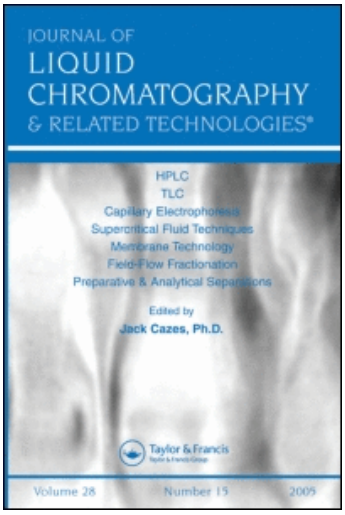

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Evaluation of Major Caffeic Acid Derivatives in *Echinacea purpurea* Dietary Supplements by HPLC with Photodiode Array Detection and Electrospray Mass Spectrometry

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ABSTRACT

An RP-HPLC method with photodiode array detection and electrospray ionization mass spectrometry was established for the determination of

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major caffeic acid derivatives (caftaric acid, chlorogenic acid, cynarin, and cichoric acid) in commercial *Echinacea purpurea* dietary supplements. The samples were extracted with 60% methanol (3 × 15 mL) by means of sonication at room temperature. The components of interest were separated on a RP-18 chromatography column using a 20-min water–methanol–trifluoroacetic acid (TFA) gradient, identified by photodiode array detection, and further confirmed by LC-ESI-MS. The quantification was performed using external standards. The sample preparations and stability of the methanolic extracts were extensively explored. Analyses of 16 commercial *E. purpurea* products revealed that there is a considerable variability in the content of the caffeic acid derivatives among the products tested. The current method may serve as a valuable tool for the Quality assurance (QA)/Quality control (QC) of echinacea dietary supplements.

Key Words: Echinacea; *Echinacea purpurea*; Caffeic acid derivatives; HPLC; PDA; LC-MS.

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INTRODUCTION

As one of the most important North American herbs, echinacea was used by Native Americans for the treatment of wounds, burns, snakebites, insect bites, colds, infections, inflammation, etc., and subsequently adopted by European settlers in the New World.[1,2] Pharmacological research and limited clinical trials have demonstrated the efficacy of echinacea (mostly *Echinacea purpurea*) preparations as immuno-stimulants and antioxidants for human.[1–4] In Germany, the fresh plant juice and its galenical preparations are internally used as an adjuvant therapy for relapsing infections of the respiratory tract and urinary tract, and externally for poorly healing superficial wounds.[5] In the USA, echinacea and its products have become one of the top ten botanical dietary supplements.[6] At least two studies have been carried out for the investigation of chemical constituents in echinacea.[7,8] Besides alkamides and polysaccharides, *E. purpurea* mainly contains a group of caffeic acid derivatives, including caftaric acid, chlorogenic acid, cynarin, and cichoric acid, and these constituents are believed to be mainly responsible for the biological activity of the preparations. As an example, cichoric acid has been shown to stimulate phagocyte activity *in vitro* and *in vivo*, and protect collagen from free radical-induced degradation.[9,10] Interestingly, cichoric acid has been reported to possess a considerable antiviral activity[7] and was observed to inhibit HIV-1 replication.[11–15]

E. purpurea and its products are commercially available as dried powders, liquid extracts, and formulated into tablets, capsules, caplets, soft gels, etc., and are classified as dietary supplements in the USA under the Dietary

Supplement Health and Education Act (DSHEA) of 1994. *E. purpurea* products are normally labeled with the content of milligram of *E. purpurea* whole plant, aerial parts or powdered extract, or milliliter of extract, but have not been subjected to mandated quality assurance (QA) and quality control (QC) standards. For the past decades, HPLC determination of major caffeic acid derivatives (mainly cichoric acid) in raw materials and manufactured products of *E. purpurea* have been carried out in Germany,[16,17] Australia,[18,19] New Zealand,[20] Poland,[21] Canada,[22–24] Denmark,[25] and China.[26] Considerable variations in the content of these constituents, especially cichoric acid, have been reported.[16–26] Still, there is a need for a rapid and reliable analytical method for the QA and QC of commercial *E. purpurea* products. Few methods have been validated for the sample preparation, reproducibility, recovery, and stability.

In continuing our investigation of botanical dietary supplements in the USA market,[27,28] we developed a high performance liquid chromatographic method with photodiode array detection and electrospray mass spectrometry (ESI-MS) confirmation for the determination of caffeic acid derivatives, including caftaric acid, chlorogenic acid, cynarin, and cichoric acid, in commercial *E. purpurea* products. Comprehensive investigations were carried out for sample preparation, stability, and reproducibility. By using mass spectrometry in negative electrospray ionization mode, the fragmentation pathways of caffeic acid derivatives were explored.

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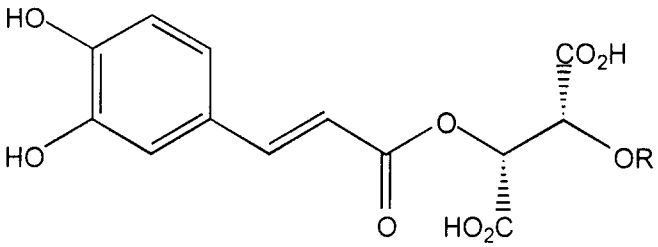
EXPERIMENTAL

Solvents

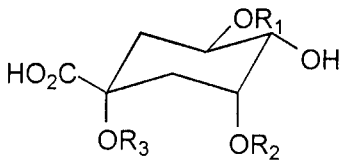
Methanol, trifluoroacetic acid (TFA), and formic acid (FA) were HPLC grade from Fisher Scientific Co. (Fair Lawn, NJ). Deionized water was obtained from an in-house Nano-pure® water system (Barnstead, Newton, MA).

Standards

Reference standards, caftaric acid, chlorogenic acid, cynarin, and cichoric acid were obtained from Chromadex Inc. (Laguna Hills, CA) (Fig. 1), and were accurately weighed (1 mg each) into a 10-mL volumetric flask and dissolved in methanol to make a stock solution. The stock solution was stored at -20°C and brought to room temperature before use. Calibration standard working solutions were freshly prepared by diluting the stock solution with methanol in appropriate quantities. In the same way, three sets of QC solutions



Caftaric acid, R = H
Cichoric acid, R = Caffeate



Chlorogenic acid, R_1 = Caffeate, R_2 = R_3 = H
Cynarin, R_1 = H, R_2 = R_3= Caffeate

Figure 1. Structures of reference standards.

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for caftaric acid, chlorogenic acid, cynarin, and cichoric acid were prepared from a separate stock, so as to lie in the lowest, middle, and highest regions of the calibration curves.

Samples

Commercial *E. purpurea* products, in the form of capsules, tablets, caplets and liquid extracts, were obtained from local pharmacies, Chicago, IL, and labeled **1–16**.

Sample Extraction

Initial analyses were carried out with the sample solutions prepared by sonicating *E. purpurea* dried powder (~330 mg) in a 20-mL PTFE-capped sample vial, using 15 mL of aqueous methanol with varying concentrations

(20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%, v/v) at room temperature for 30 min. After cooling, the resultant mixture was filtered through a filter paper (Whatman #1) into a 250-mL round-bottom flask. The residue was washed with methanol (3 × 10 mL) while on the filter. The combined extracts were transferred into a 50-mL volumetric flask and made up to the volume with methanol. After centrifugation, 10 μL each of above sample solutions was subjected to HPLC analysis as described below. The concentrations of the major caffeic acid derivatives, in the resultant sample solutions, were calculated based on the equations for the calibration curves. After comparing the content (%, w/w) of these constituents found in the above sample solutions, the method with the highest yield (%) was subjected to an extraction efficiency study to optimize the extraction procedure.

For comparison, sample solutions were also prepared by refluxing the same amount of *E. purpurea* dried powder (~330 mg) with 15 mL of aqueous methanol with varying concentrations (20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%, v/v) for 30 min. After cooling, the resultant mixture was filtered through a filter paper (Whatman #1) into a 250-mL round-bottom flask. The residue was washed with methanol (3 × 10 mL) while on the filter. Each of the combined extracts was transferred into a 50-mL volumetric flask and made up to the volume with methanol. After centrifugation, 10 μL each of the above sample solutions was subjected to HPLC analysis as described below.

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Extraction Efficiency

E. purpurea dried powder (~330 mg) was exactly weighed into a 20-mL PTFE-capped sample vial and sonicated with 15 mL of the solvent, which gave the highest yield (%) of the constituents of interest in the initial sample analysis as described above, at room temperature for 30 min. After cooling, the resultant mixture was filtered through a filter paper (Whatman #1) into a 250-mL round-bottom flask. The residue was washed with methanol (3 × 10 mL) while on the filter. The combined extracts were transferred into a 50-mL volumetric flask and made up to the volume with methanol (Ia). The above procedure was repeated two more times to make sample solutions (Ib, Ic).

For comparison, *E. purpurea* dried powder (~330 mg) was exactly weighed and refluxed with 15 mL of the solvent, which gave the highest yield (%) of the constituents of interest in the initial sample analysis as described above, for 30 min. After cooling, the resultant mixture was filtered through a filter paper (Whatman #1) into a 250-mL round-bottom flask. The residue was washed with methanol (3 × 10 mL) while on the filter.

The combined extracts were transferred into a 50-mL volumetric flask and made up to the volume with methanol (IIa). The above procedure was repeated two more times to make sample solutions (IIb, IIc).

After centrifugation, 10 μL each of above sample solutions was subjected to HPLC analysis as described below. The concentrations of the major caffeic acid derivatives in the above sample solutions (Ia, Ib, Ic and IIa, IIb, IIc) were calculated based on the equations for calibration curves, and the extraction efficiency was compared.

HPLC-PDA Analysis

The HPLC-PDA analysis was carried out using a Waters Alliance 2690 liquid chromatograph and a photodiode array (996) detector (Waters, Milford, MA). The chromatographic data were recorded and processed using Waters Millennium 2000 software. The measurements were carried out on a Supelco Discovery RP-18 column (250 × 4.6 mm, 5 μm particle size, Supelco, Bellefonte, PA) protected by a Waters Delta-Pak RP-18 guard column and set at 20°C. The mobile phases used were water (containing 5% methanol and 0.1% TFA), solvent A; and methanol (containing 0.1% TFA), solvent B. All injections were 10 μL in volume. The chromatography followed a linear gradient program of solvent B from 8% to 68% over 20 min at a flow rate of 1 mL/min. After 20 min, the column was washed with 90% B for 10 min and re-equilibrated with the starting mobile phase (8% B) over 20 min. The detection was carried out with a detection wavelength set at 200–400 nm to obtain UV spectrum, and at 330 nm for quantification of the compounds of interest.

LC-MS Analysis

LC-MS analysis was performed on a Waters 2690 HPLC system coupled with a Micromass Quattro II triple quadrupole mass spectrometer equipped with a Z-spray electrospray ionization source (Micromass, Manchester, UK). The mass spectrometer was operated in the negative ionization mode. The ion source parameters were optimized for the formation of $[M - H]^-$ ions of the reference standards with source temperature of 150°C, capillary voltage of −3.2 kV, and cone voltage of −25 V. Nitrogen was used both as a nebulizing gas and a drying gas at a flow of 20 and 450 L/hr, respectively. ESI-MS full scans were acquired from m/z 100 to 700. Product ion MS-MS spectra of the deprotonated molecules of the reference standards were obtained using argon as the collision gas at a pressure of 1×10^{-3} mBar.

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In the LC-MS, the same chromatographic gradient described as above was used, but with FA (0.1%) replacing TFA as the modifier. The injection volume was 10 μL. The column effluent was split, so that approximately 15% was transferred to the mass spectrometer. MassLynx software was used to collect and process the ESI-MS data.

Identification and Peak Purity

Peaks in HPLC-PDA were tentatively identified by comparison of the retention times and UV spectra of the peaks in the sample solutions with those of reference standards, and by the method of reference standards addition to the sample solutions. Identification was subsequently confirmed by LC-ESI-MS analysis. The purity of each peak was checked by using the Waters Millennium PDA software routines and by examination of the MS spectra.

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Stability

E. purpurea dried powder (~330 mg) was sonicated with 60% methanol (3 × 15 mL) as described above. After cooling, the combined extracts were evaporated under reduced pressure at 40–45°C, and resultant residue was dissolved into a 50-mL volumetric flask with methanol and 60% methanol, respectively. Triplicate sample solutions were prepared, and put in the dark at room temperature, and analyzed on 30 consecutive days to observe the stability of the sample solutions.

Quantification

Fine powder from a capsule, or one unit of finely pulverized tablet or caplet of *E. purpurea* product, was extracted with 60% methanol (3 × 15 mL) by means of sonication as above. After cooling, the extracts were combined into a 50-mL volumetric flask and made up to the volume with methanol. For the liquid product, 1 mL of sample was diluted to 2–10 mL using water. Duplicate sample solutions were prepared for each product. After centrifugation, 10 μL of the supernatant was subjected to HPLC analysis, and the content of each major caffeic acid derivative was calculated based on the respective calibration curve.

RESULTS AND DISCUSSION

HPLC-PDA Analysis

Under the current HPLC gradient conditions, all constituents in *E. purpurea* were eluted within 20 min. Figure 2 shows a typical chromatogram of a methanolic extract of an *E. purpurea* sample at 330 nm. The method was validated for linearity, sensitivity, reproducibility, and recovery.

The linearity was based on the duplicate analysis of calibration working standard solutions at five concentration levels on three consecutive days for caftaric acid, chlorogenic acid, cynarin, and cichoric acid (2–100 μg/mL) with regression coefficients (r^2) better than 0.998.

Under the current chromatographic conditions, the limit of detection (LOD) and limit of quantification (LOQ) were determined to be 100 ($S/N > 5$) and 200 ng/mL ($S/N > 10$), respectively, for caftaric acid, chlorogenic acid, cynarin, and cichoric acid.

The reproducibility of the method was evaluated by analyzing three sets of controls ($n = 3$) on three separate days ($n = 3$), and calculating the RSD (%) and relative error (%). As shown in Table 1, the RSD (%) and the relative error (%) were found to be less than 2.56 and 6.51, respectively. In addition, six sample solutions were prepared from the same batch and analyzed by HPLC-PDA, and the content of constituents of interest was evaluated by calculating the RSD (%). The following data were obtained: caftaric acid 2.15%, chlorogenic acid 3.87%, cynarin 4.23%, and cichoric acid 2.24%, indicating the extraction method is highly reproducible.

The recovery was assessed by adding 500 μg of each standard to 300 mg of powdered samples. After thorough mixing, samples were extracted using the optimized extraction method as described. The resultant extracts were combined into a 50-mL volumetric flask and made up to the volume with methanol. Triplicate sample solutions were prepared and analyzed. In the same way, triplicate sample solutions were prepared without spiking the standards and analyzed for comparison. The average recoveries of added caftaric acid, chlorogenic acid, cynarin, and cichoric acid were 95.6%, 98.9%, 97.4%, and 101.2% ($n = 3$), respectively.

LC-MS Analysis

Sodiated and protonated molecular ion species, $[M + Na]^+$, $[M + H]^+$, of cichoric acid, cynarin, and chlorogenic acid, from echinacea have been observed with limited structural information by positive electrospray ionization mass spectrometry.[4] For example, beside the sodiated and protonated

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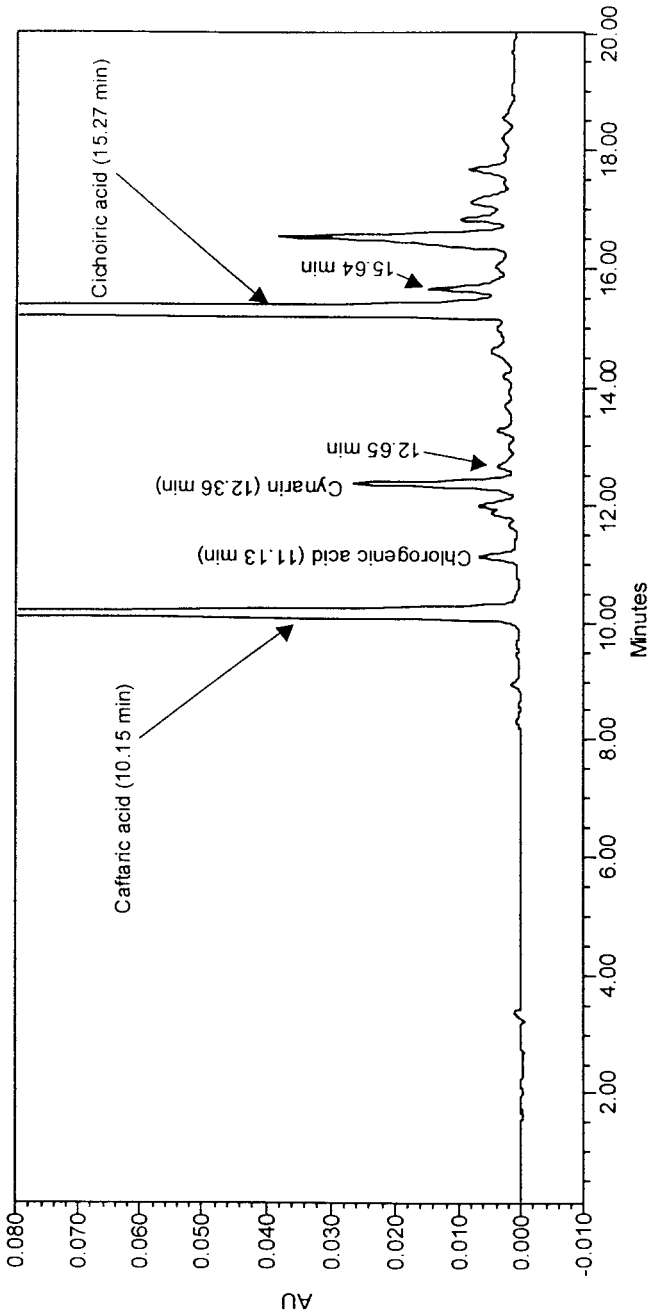

Figure 2. Chromatogram profile of the methanolic extract of *E. purpurea* with the LC-MS attributions of the components detected: caftaric acid (10.15 min), chlorogenic acid (11.13 min), cynarin (12.36 min), and cichoric acid (15.27 min). The peaks at 12.65 and 15.64 min were tentatively identified to be the analogues of cynarin and cichoric acid, respectively.

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Table 1. Reproducibility.

Compound	Day-1			Day-2			Day-3		
	Observed value (μg/mL)[a]	RSD (%)	RE (%)	Observed value (μg/mL)[a]	RSD (%)	RE (%)	Observed value (μg/mL)[a]	RSD (%)	RE (%)
Caftaric acid									
QC-1	9.86 ± 0.16	1.63	−1.04	9.48 ± 0.24	2.56	−4.85	9.77 ± 0.04	0.38	−1.87
QC-2	384.80 ± 0.07	0.18	−3.41	40.70 ± 0.25	0.63	2.17	41.22 ± 0.24	0.59	3.47
QC-3	76.09 ± 0.28	0.36	−4.50	80.72 ± 0.61	0.76	1.30	81.02 ± 0.50	0.62	1.68
Chlorogenic acid									
QC-1	9.94 ± 0.09	0.90	−0.61	9.80 ± 0.14	1.38	−2.07	9.82 ± 0.03	0.35	−1.82
QC-2	3.85 ± 0.12	0.31	−3.89	40.87 ± 0.27	0.65	2.12	40.73 ± 0.25	0.62	1.78
QC-3	78.65 ± 0.44	0.56	−1.73	83.66 ± 0.55	0.66	4.53	83.26 ± 0.47	0.56	4.02
Cynarin									
QC-1	10.11 ± 0.08	0.83	0.16	9.97 ± 0.12	1.16	−1.21	10.00 ± 0.05	0.47	−0.99
QC-2	38.84 ± 0.08	0.22	−3.83	41.32 ± 0.19	0.47	2.31	41.15 ± 0.27	0.65	1.88
QC-3	79.51 ± 0.49	0.62	−1.57	84.47 ± 0.46	0.54	4.58	84.01 ± 0.46	0.54	4.00
Cichoric acid									
QC-1	9.58 ± 0.05	0.56	−3.52	9.28 ± 0.06	0.69	−6.51	9.37 ± 0.02	0.19	−5.56
QC-2	38.30 ± 0.08	0.20	−3.53	40.48 ± 0.13	0.33	1.97	40.54 ± 0.12	0.30	2.12
QC-3	76.55 ± 0.43	0.56	−3.59	81.03 ± 0.38	0.46	2.06	80.03 ± 0.36	0.45	0.79

[a]The data represent the mean ± SD of three observations.

molecular ions, only $[M + H - H_2O]^+$, $[M + H - H_2O - \text{caffeic acid}]^+$, $[M + H - \text{caffeic acid}]^+$, and $[M + H - \text{caffeic acid} - H_2O]^+$ fragment ions for cichoric acid, $[M + H - H_2O]^+$ fragment ion for cynarin were observed, no fragment ion was reported for chlorogenic acid.[4] Deprotonated species, $[M - H]^-$, of caftaric acid, chlorogenic acid, and cichoric acid have been presented recently, but without structural information.[26] In the present study, the fragmentation of the deprotonated molecules, $[M - H]^-$, of these compounds were examined by direct infusion electrospray mass spectrometry and tandem mass spectrometry in order to obtain characteristic ions and fragmentation patterns. Figure 3 shows the MS-MS spectra obtained from caftaric acid (Fig. 3A), chlorogenic acid (Fig. 3B), cynarin (Fig. 3C), and cichoric acid (Fig. 3D) anions indicative of molecular ions and structurally characteristic fragments. The negative ionization mass spectrometry was found to be very useful for the characterization of caffeic acid derivatives in a plant matrix.

As shown in Fig. 3A, the MS-MS spectrum of the deprotonated molecule of caftaric acid (m/z 311) produced a base peak at m/z 149 due to the loss of caffeoyl group with the transfer of hydrogen. Another abundant fragment ion was observed at m/z 179 corresponding for caffeoate. The deprotonated molecule of chlorogenic acid (m/z 353) also eliminated the caffeoyl group with the transfer of hydrogen to form base peak at m/z 191 (Fig. 3B). However, the abundance of the fragment ion of m/z 179 corresponding to caffeoate was much lower than the ion of m/z 191. The product ion spectrum of the deprotonated molecule of cynarin (m/z 515) gave base peak at m/z 353 (Fig. 3C) due to the loss of caffeoyl group with the transfer of hydrogen. The elimination of the second caffeoyl group with the transfer of hydrogen formed a fragment ion of m/z 191. Other fragment ions were observed at m/z 335 and 155, due to consequent loss of caffeic acid from the deprotonated molecule (m/z 515). Again, the fragment ion corresponding to caffeoate was also observed at m/z 179. The product ion tandem mass spectrum of the deprotonated molecule of cichoric acid at m/z 473 (Fig. 3D) showed a fragmentation pattern that was similar to cynarin. For example, cleavage of the ester bond with the transfer of hydrogen formed the ion (base peak) at m/z 311, and the loss of an additional caffeoyl group with the transfer of hydrogen produced the ion of m/z 149 with relative lower abundance. Other abundant fragment ions were observed at m/z 293 and 113, due to consecutive loss of caffeic acids from the deprotonated molecule. Like cynarin, caffeoate was observed at m/z 179.

The peaks in HPLC-PDA chromatogram with retention times of 10.15, 11.13, 12.36, and 15.27 min, were confirmed by LC-MS to be caftaric acid, chlorogenic acid, cynarin, and cichoric acid, respectively, with the retention time order similar with that reported by Perry et al.[20] Based on HPLC retention time and the corresponding ESI-MS spectrum, the peak at 12.65 min was

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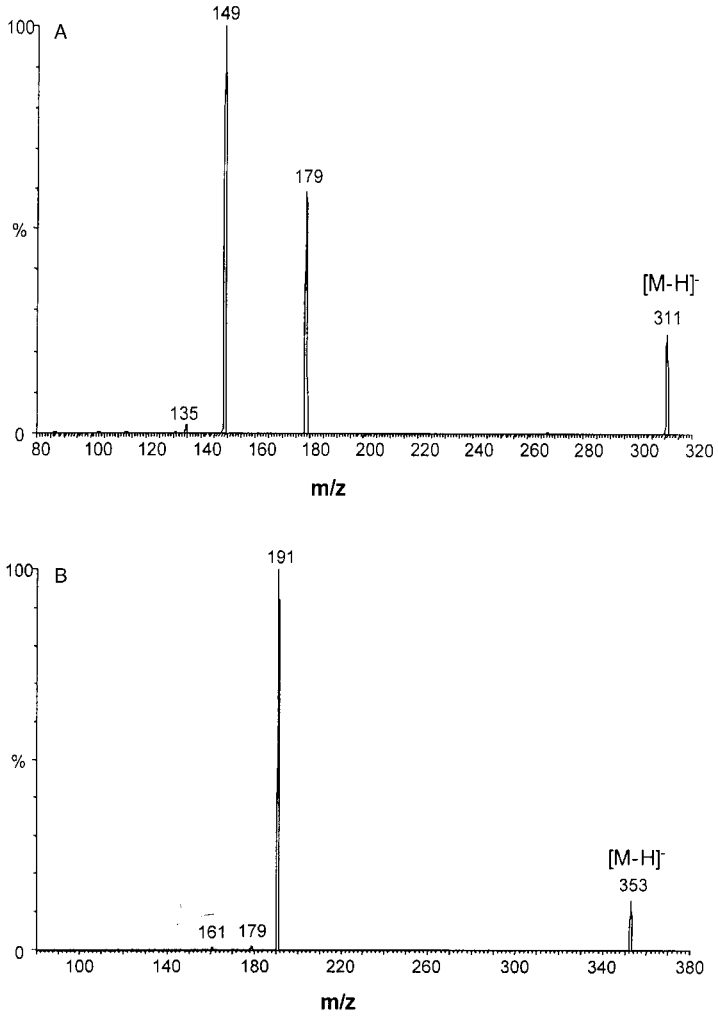

Figure 3. Mass spectra of caftaric acid (A), chlorogenic acid (B), cynarin (C), and cichoric acid (D).

(continued)

tentatively identified to be an isomer of cynarin. As shown in Fig. 4A, the ESI-MS spectral data were consistent with this interpretation with $[M - H]^-$ observed at m/z 515 and major fragment ions of m/z 353 and 191, respectively, due to consecutive loss of caffeoyl group with the transfer of hydrogen. The fragment ion corresponding to caffeoate was observed at m/z 179 with

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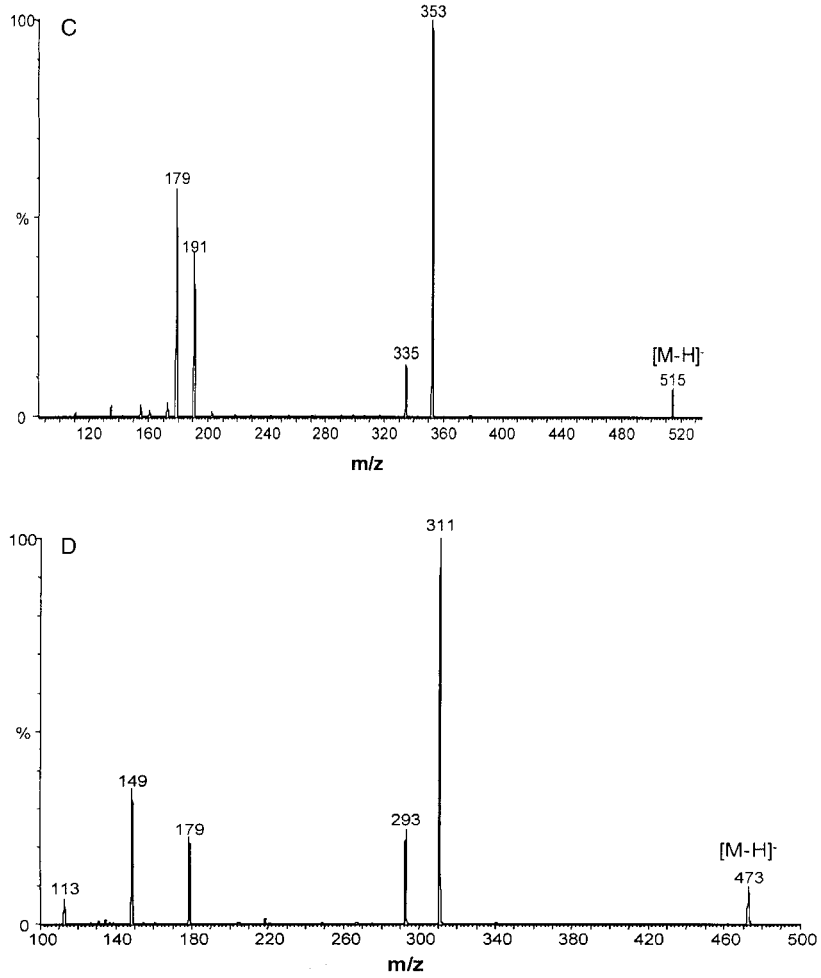

Figure 3. Continued.

very low abundance. Similarly, the peak at 15.64 min gave deprotonated molecular ion of m/z 473 and fragment ions of m/z 311 and 293 in the ESI-MS spectrum, due to consecutive loss of caffeoyl group with the transfer of hydrogen. The fragment ions at m/z 293 and 113 were also observed due to consecutive neutral loss of caffeic acid from deprotonated molecule. The overall MS pattern of this peak (Fig. 4B) is very close to that of cichoric acid. The identity of the peaks at 12.65 and 15.64 min is being investigated.

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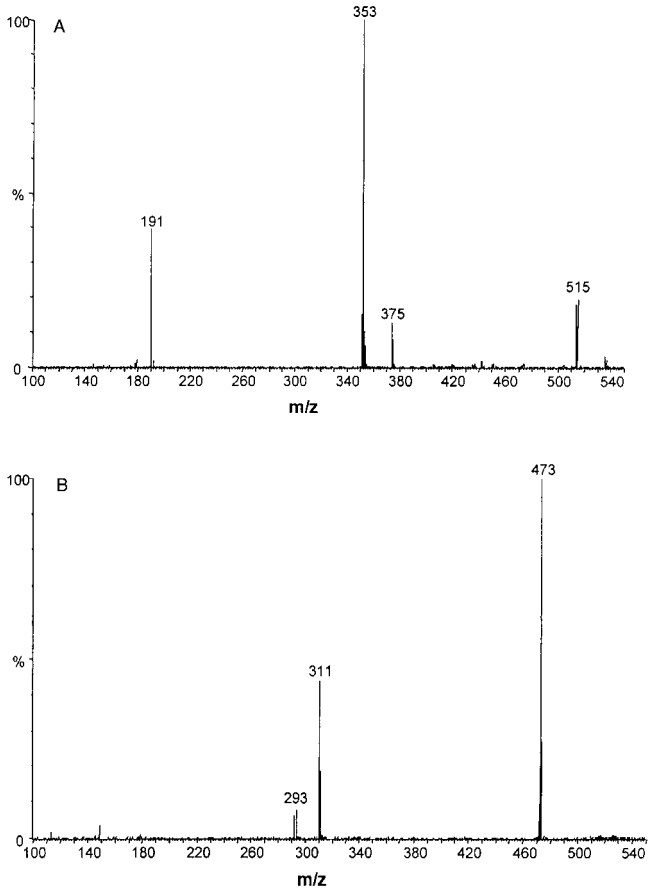

Figure 4. Mass spectra of the peaks corresponding to the retention times of 12.65 (A) and 15.64 (B) min, respectively.

Evaluation of Extraction Method and Extraction Efficiency

To evaluate the extraction efficiency, sample solutions were prepared by sonicating the same lot of *E. purpurea* dried powder (~330 mg) with 15 mL of aqueous methanol with various concentrations (20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%, v/v) for 30 min. The content (%, w/w) of the caffeic acid derivatives is summarized in Table 2. Obviously, sonicating the sample with 60% methanol yielded the highest content of caffeic acid derivatives in the resultant extract with the total content of 1.29% (w/w). Compared

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Table 2. The content (%, ±SD) (w/w) of caffeic acid derivatives found in the sample solutions prepared from the same lot of *E. purpurea* powder by means of sonication with various concentrations of methanol in water ($n = 3$).

Compound	MeOH	MeOH (90%)	MeOH (80%)	MeOH (70%)	MeOH (60%)
Caftaric acid	0.055 ± 0.006	0.176 ± 0.030	0.227 ± 0.015	0.359 ± 0.017	0.395 ± 0.005
Chlorogenic acid	0.018 ± 0.002	0.022 ± 0.01	0.021 ± 0.0008	0.025 ± 0.002	0.029 ± 0.001
Cynarin	0.020 ± 0.002	0.045 ± 0.007	0.056 ± 0.002	0.074 ± 0.002	0.080 ± 0.001
Cichoric acid	0.281 ± 0.029	0.570 ± 0.068	0.647 ± 0.008	0.768 ± 0.006	0.788 ± 0.014

Compound	MeOH (50%)	MeOH (40%)	MeOH (30%)	MeOH (20%)
Caftaric acid	0.382 ± 0.003	0.378 ± 0.003	0.356 ± 0.014	0.333 ± 0.013
Chlorogenic acid	0.026 ± 0.0003	0.024 ± 0.002	0.020 ± 0.001	0.022 ± 0.0004
Cynarin	0.078 ± 0.001	0.079 ± 0.001	0.076 ± 0.004	0.078 ± 0.001
Cichoric acid	0.769 ± 0.005	0.714 ± 0.011	0.645 ± 0.036	0.542 ± 0.019

with sonication, refluxing generally gave a higher yield of the above constituents in the resultant extract with 40% methanol giving the highest yield (1.40%) (Table 3). However, three-steps of sonication yielded a similar total content of above constituents (1.47%) in the resultant extracts when compared with refluxing (1.50%) (Table 4). Taking into consideration the simplicity, sonication was preferred for routine sample preparation. But one or two steps of sonication could not, as shown in Table 4, recover 100% of the constituents of interest.[18,19]

Stability

The stability of cichoric acid in dried *E. purpurea* sample and dried extract has been previously examined.[29] The current analysis revealed that the major constituents of *E. purpurea* are stable with RSD (%) of 4.15, 3.61, 3.71, and 3.65 for caftaric acid, chlorogenic acid, cynarin, and cichoric acid over 30 days, respectively, when extract was stored in aqueous methanol (60%). However, when extract was stored in methanol (100%), the above numbers increased to 6.21, 5.72, 9.58, and 6.63 for caftaric acid, chlorogenic acid, cynarin, and cichoric acid, respectively, with the tendency towards decreasing, especially for cynarin and cichoric acid. This needs a further investigation.

Sample Analysis

A typical HPLC chromatogram is shown in Fig. 2. Sixteen sets of commercial *E. purpurea* products were analyzed in duplicate according to the method described above. The average content of caftaric acid, cichoric acid, cynarin, and chlorogenic acid in these products are summarized in Table 5. As shown in Table 5, all products tested were found to contain caftaric acid, chlorogenic acid, cynarin, and cichoric acid, with the amount of total caffeic acid derivatives varying between 0.50 and 40.12 mg/serving ($n = 16$), indicating that there is a remarkable product-to-product difference and variability. Among the products with content claims, products **2**, **8**, and **15** meet the expectation for good quality based on their label claims. But for products **7** and **11**, the amount of total caffeic acid derivatives was found to be half or less of the label claims. However, since there is no publication comparing the efficacy of different *E. purpurea* products and since no dose–response studies have been reported, it is difficult to relate possible beneficial effects of *E. purpurea* products to the composition of caffeic acid derivatives. On the other hand, it is well known that *E. purpurea* contains other groups of natural products other than caffeic acid derivatives, such as

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Table 3. The content (%, ±SD) (w/w) of caffeic acid derivatives found in the sample solutions prepared from the same lot of *E. purpurea* powder by means of refluxing with various concentrations of methanol in water ($n = 3$).

Compound	MeOH	MeOH (90%)	MeOH (80%)	MeOH (70%)	MeOH (60%)
Caftaric acid	0.077 ± 0.006	0.167 ± 0.008	0.350 ± 0.011	0.400 ± 0.014	0.431 ± 0.011
Chlorogenic acid	0.020 ± 0.01	0.028 ± 0.001	0.026 ± 0.0003	0.029 ± 0.001	0.029 ± 0.001
Cynarin	0.025 ± 0.002	0.048 ± 0.002	0.075 ± 0.001	0.080 ± 0.003	0.084 ± 0.001
Cichoric acid	0.336 ± 0.015	0.573 ± 0.015	0.793 ± 0.005	0.775 ± 0.027	0.779 ± 0.031

	MeOH (50%)	MeOH (40%)	MeOH (30%)	MeOH (20%)
Caftaric acid	0.451 ± 0.006	0.464 ± 0.013	0.451 ± 0.009	0.448 ± 0.010
Chlorogenic acid	0.026 ± 0.001	0.030 ± 0.001	0.028 ± 0.001	0.023 ± 0.002
Cynarin	0.085 ± 0.001	0.088 ± 0.003	0.086 ± 0.0004	0.087 ± 0.003
Cichoric acid	0.813 ± 0.009	0.817 ± 0.010	0.758 ± 0.041	0.748 ± 0.033

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Table 4. The percentages of major constituents found in consecutively extracted methanolic solutions of *E. purpurea* ($n = 3$).

	Sonication with 60% methanol				Refluxing with 40% methanol			
	Caftaric acid	Chlorogenic acid	Cynarin	Cichoric acid	Caftaric acid	Chlorogenic acid	Cynarin	Cichoric acid
First extraction (%)	80.66	60.08	84.75	90.55	90.41	74.98	89.67	93.68
Second extraction (%)	14.49	25.83	15.25	7.13	6.48	25.02	10.33	4.22
Third extraction (%)	4.85	14.09	0	2.32	3.11	0	0	2.10
Total content found (%, w/w)	0.477	0.048	0.094	0.855	0.507	0.039	0.098	0.856

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Table 5. Content of caffeic acid derivatives in commercial *E. purpurea* products ($n = 2$).

#	Type	Unit (per serving)	Label claim[a]	Caftaric acid (mg/serving)	Chlorogenic acid (mg/serving)	Cynarin (mg/serving)	Cichoric acid (mg/serving)	Total caffeic acid derivatives content (mg/serving)
1	Capsule	3	N/A	4.782 ± 0.105	1.958 ± 0.016	0.845 ± 0.012	8.959 ± 0.094	16.545 ± 0.194
2	Capsule	1	2	1.184 ± 0.012	0.102 ± 0.0001	0.243 ± 0.002	2.705 ± 0.0003	4.234 ± 0.013
3	Capsule	2	N/A	0.769 ± 0.021	4.179 ± 0.096	0.292 ± 0.022	1.842 ± 0.028	7.083 ± 0.167
4	Capsule	3	N/A	4.557 ± 0.453	0.217 ± 0.004	0.964 ± 0.047	9.895 ± 0.331	15.633 ± 0.836
5	Caplet	2	N/A	2.949 ± 0.124	0.107 ± 0.001	0.612 ± 0.018	6.276 ± 0.094	9.943 ± 0.237
6	Capsule	1	N/A	1.658 ± 0.166	0.105 ± 0.001	0.377 ± 0.029	4.077 ± 0.089	6.217 ± 0.482
7	Capsule	1	4	0.533 ± 0.002	0.035 ± 0.01	0.128 ± 0.001	0.429 ± 0.003	1.125 ± 0.007
8	Caplet	1	3	0.897 ± 0.17	0.216 ± 0.003	0.188 ± 0.001	1.708 ± 0.021	3.009 ± 0.042
9	Tablet	2	N/A	0.153 ± 0.005	0.065 ± 0.001	0.066 ± 0.001	0.218 ± 0.007	0.502 ± 0.014
10	Capsule	1	N/A	1.875 ± 0.054	0.076 ± 0.001	0.381 ± 0.008	3.964 ± 0.077	6.295 ± 0.140
11	Tablet	1	5	0.781 ± 0.007	0.046 ± 0.0002	0.148 ± 0.001	1.597 ± 0.001	2.572 ± 0.009
12	Liquid	1 mL	N/A	5.660 ± 0.007	0.261 ± 0.004	4.704 ± 0.001	5.181 ± 0.084	15.806 ± 0.082
13	Liquid	1 mL	N/A	8.483 ± 0.055	0.457 ± 0.004	0.659 ± 0.007	7.448 ± 0.011	17.048 ± 0.055
14	Liquid	1 mL	N/A	7.113 ± 0.016	0.820 ± 0.022	1.708 ± 0.003	7.645 ± 0.071	17.285 ± 0.111
15	Liquid	5 mL	4.8	1.138 ± 0.001	0.672 ± 0.002	1.706 ± 0.004	1.556 ± 0.003	5.073 ± 0.001
16	Liquid	1 mL	N/A	27.279 ± 0.025	0.499 ± 0.0004	3.010 ± 0.005	9.332 ± 0.003	40.120 ± 0.027

[a]Milligrams of total caffeic acid derivatives.

alkamides, polysaccharides, that also have shown biological activity.[30,31] The optimal biological activity of *E. purpurea* might be due to the presence of the whole mixture of its constituents, rather than a single compound.

CONCLUSIONS

A high-performance liquid chromatography method has been developed for the detection and quantitation of major caffeic acid derivatives of *E. purpurea* products with photodiode array detection and ESI-MS confirmation. With this method, caftaric acid, cichoric acid, cynarin, and chlorogenic acid were successfully quantitated, using standard calibration curves. The sample preparation method was extensively explored. By using LC-MS, two possible cynarin and cichoric acid isomers were detected. The current method was found to be specific and suitable for routine analysis because of its simplicity, specificity, accuracy, and reproducibility.

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