Echinacea Standardization: Analytical Methods for Phenolic Compounds and Typical Levels in Medicinal Species

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A proposed standard extraction and HPLC analysis method has been used to measure typical levels of various phenolic compounds in the medicinally used *Echinacea* species. Chicoric acid was the main phenolic in *E. purpurea* roots (mean 2.27% summer, 1.68% autumn) and tops (2.02% summer, 0.52% autumn), and echinacoside was the main phenolic in *E. angustifolia* (1.04%) and *E. pallida* roots (0.34%). Caftaric acid was the other main phenolic compound in *E. purpurea* roots (0.40% summer, 0.35% autumn) and tops (0.82% summer, 0.18% autumn), and cynarin was a characteristic component of *E. angustifolia* roots (0.12%). Enzymatic browning during extraction could reduce the measured levels of phenolic compounds by >50%. Colorimetric analyses for total phenolics correlated well with the HPLC results for *E. purpurea* and *E. angustifolia*, but the colorimetric method gave higher values.

Keywords: *Echinacea; phenolics; caffeic acid derivatives; high performance liquid chromatography; colorimetric analysis; medicinal herbs*

INTRODUCTION

Echinacea preparations, used as immunostimulants, are best-selling herbal medicines in the United States and Europe (*1*, *2*). The U.S. National Center for Complementary and Alternative Medicine (National Institutes of Health) has identified *Echinacea* as an herb that warrants further testing in Phase II and/or Phase III clinical trials (*3*). Issues to be clarified before such trials include method of extraction and chemical standardization. Bauer (*4*) has suggested that *Echinacea* preparations should be standardized for their contents on the lipophilic alkamides and the more polar phenolic compounds.

Three species of *Echinacea* (family Asteraceae) are generally used medicinally: *E. angustifolia* DC, roots (underground parts); *E. pallida* (Nutt.) Nutt., roots; and *E. purpurea* (L.) Moench, roots and tops (aerial parts) (*5*). The main phenolic compounds are caffeic acid derivatives (**1**−**5**, Figure 1), but there is some confusion about the different compounds found in the different medicinal species. Chicoric acid (dicaffeoyltartaric acid, also known as cichoric acid) (**1**) is the main phenolic in *E. purpurea* roots and tops, and echinacoside (**2**) is the main phenolic in *E. angustifolia* and *E. pallida* (*6*). Chicoric acid (**1**) has biological activities that could contribute to immunostimulatory activity, but echinacoside (**2**) does not seem to have any immunomodulatory relevance (*4*). Typical levels of **1** and **2** are given by Bauer and Wagner (*6*) but not levels of the minor compounds.

Bauer et al. (*7*) pioneered the use of reversed-phase HPLC for the analysis of individual phenolic compounds in *Echinacea*. Other groups have used different extraction methods but similar HPLC conditions (*8*−*12*). Colorimetric analyses of "total phenolics" (*13*, *14*) in *Echinacea* have been used as a more rapid method for standardization, but we have not seen any published reports on this approach.

In this paper we compare results from a proposed standard method from the Institute for Nutraceutical Advancement (a U.S. industry organization) for phenolics in *Echinacea* by HPLC (*15*) with a colorimetric (Folin−Denis) method, using high and low phenolics samples of *E. purpurea* tops and roots from a seasonal study plus *E. angustifolia* and *E. pallida* roots. We show that the ratio of chicoric acid (**1**) to caftaric acid (caffeoyltartaric acid, **3**) differs between *E. purpurea* tops and roots and point out potential problems with extraction methods leading to enzymatic browning. Cynarin (**4**) needs to be included in the INA method for analyses of *E. angustifolia* roots.

MATERIALS AND METHODS

Plant Samples and Reference Compounds. *E. purpurea* tops and roots (15−18 months old) were harvested in January and April 1999 at Lincoln on the South Island of New Zealand. Four replicate plots of eight plants each were harvested at each date from a randomized block design. Tops were cut off at 10 cm above soil level, and roots comprised the rest of the plant. Samples were dried at 30 °C to moisture contents of about 10%, ground, and stored at −70 °C. Eight samples each of roots and tops were analyzed.

E. angustifolia roots (18−21 months old) were harvested in May 2000 at Lincoln and Mosgiel on the South Island of New Zealand. Samples consisted of about 12 plants each. Samples were dried at 30 °C to moisture contents of about 10%, ground, and stored at −70 °C. Seven samples were analyzed.

E. pallida roots (12 months old) were harvested in August 1999 at Gisborne on the North Island of New Zealand. Dried roots (moisture contents of about 10%) were stored at −70 °C. Eight individual roots were analyzed.

Chlorogenic acid (**5**) was bought from Sigma. Echinacoside (**2**, impure) was donated by Prof. R. Brouillard (University Louis Pasteur, Strasbourg).

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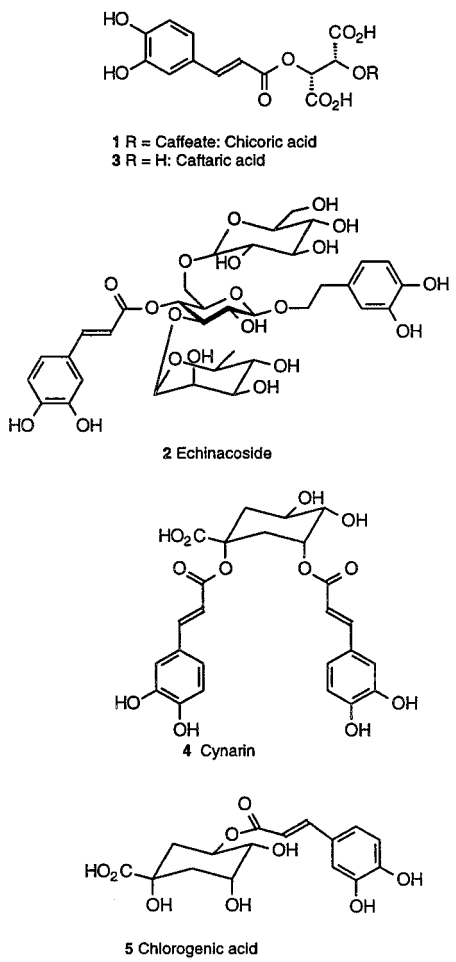

Figure 1. Structures of phenolic compounds in *Echinacea*.

Chicoric acid (**1**) was isolated from dried ground *E. purpurea* roots (100 g) extracted with chloroform (1 l, 3 h) in a Soxhlet apparatus and then with methanol (1 L, 24 h). The methanol extract was evaporated in vacuo to give a yellow gum (10 g). This was dissolved in water (50 mL) containing formic acid (0.5 mL) and then extracted into ethyl acetate (3 × 300 mL) to give a gum (2 g) after evaporation of ethyl acetate. Preparative reversed phase HPLC on this (Merck LichroCart C18, 10 × 250 mm, mobile phase 4 mL/min 7:3 water: acetonitrile containing 0.1% formic acid) gave chicoric acid (**1**) (5.95−7.65 min, 254 nm detection, 120 mg from 400 mg injected in 20 lots). Final purification was by recrystallization from water to give chicoric acid (**1**) as off-white crystals (70 mg from 120 mg): mp 204−210 °C (browned and decomposed, lit. value 206 °C (*16*)); $[\alpha]_D$ (c = 0.2 g/100 mL of MeOH) −381° (lit. value −384.2° (*16*)); UV (MeOH) λ_{max} 330 nm, ϵ 36 600. We could not find a value for the extinction coefficient of **1** anywhere in the literature. Analyses of a sample of 0.1 mg/mL of chicoric acid (>95% pure by HPLC) gave an average calculated value of 0.12 mg/mL of chicoric acid (n = 3, standard deviation ±0.01 mg/mL) by the INA method.

Cynarin (**4**) was isolated from dried ground *E. angustifolia* roots (200 g) extracted with chloroform (1 L, 3 h) in a Soxhlet apparatus and then with methanol (1 L, 24 h). The methanol extract was evaporated in vacuo to give a yellow gum (25 g). Lipophilic materials were removed from this by filtering through reversed phase C18 material (Sigma) eluting with water. Preparative reversed phase HPLC was then used: column Merck LichroCart C18, 10 × 250 mm; mobile phases water (containing 0.1% formic acid, solvent A) and acetonitrile (solvent B); initial 10% B, linear gradient to 25% B in 30 min, to 50% B in 5 min, recycle to initial conditions in 5 min, hold for 5 min; flow rate 5 mL/min; 330 nm detection. These conditions gave resolved peaks for echinacoside and cynarin at about 15.6 min, with cynarin (**4**) eluting first (1 mg from 20 mg injected in 4 lots, >95% pure by HPLC). The ^{1}H NMR spectrum in CD_3OD matched that reported by Horman et al. (*17*).

Phenolics in *Echinacea* by the INA Method. This method follows that given on the INA Web site on May 23, 2000 (*15*).

Extraction. Plant material was ground to pass through a 40-mesh screen (Tyler Equivalent 35 mesh or 0.0165 in.). An accurately weighed sample (ca. 0.125 g) was extracted with 25.0 mL of ethanol:water (70:30) for 15 min on an orbital shaker. The extract was then centrifuged and a 4 mL aliquot filtered through a 0.45 μm PTFE filter into an HPLC vial and capped.

Standards. Chlorogenic acid (10.0 mg) was dissolved in ethanol:water (70:30) with the aid of sonication and made up to a stock concentration of 1.0 mg/mL. Further dilutions gave standard solutions for a three-point calibration curve of 0.4, 0.04, and 0.008 mg/mL. Linear regression on the HPLC analyses gave R^2 values of 0.999 or better.

Chromatography. A Phenomenex Prodigy column (ODS^3, 5 μm, 100 Å, 4.6 × 250 mm) was used in conjunction with a Phenomenex security guard cartridge (4 × 2 mm). The column temperature was 35 °C. The mobile phases were water (containing 0.1% phosphoric acid, solvent A) and acetonitrile (Far UV Hypersolv, solvent B) in the following gradient system: initial 10% B; linear gradient to 22% B in 13 min; then to 40% B in 1 min; hold at 40% B for 0.5 min; recycle to initial conditions in 0.5 min; and hold for 5 min.

The flow rate was 1.5 mL/min, injection of $NaNO_3$ gave a t_m value of 3.89 min, injection volume was 5 μL and detection was at 330 nm. The retention times of the phenolic compounds using these conditions, followed by the INA correction factors, were caftaric acid 7.5 min, 0.888; chlorogenic acid 8.2 min, 1.00; cynarin 12.2 min, 0.729 (our value); echinacoside 12.5 min, 2.22; chicoric acid 18.2 min, 0.695.

Each phenolic compound was quantified using the following equation:

$$\%\ \text{w/w (of dried plant material) individual phenolic compound} = (C \times \text{FV} \times F \times 100)/W$$

where C is the concentration of the compound (mg/mL) in the analyzed extract, calculated as chlorogenic acid from peak areas and linear regression; FV is the final volume of the analyzed extract; W is the sample weight extracted (mg); and F is the correction factor for the compound's response against chlorogenic acid. An Excel spreadsheet was used for these calculations. A previously analyzed sample was run with each batch as an internal check.

Colorimetric Analyses for Total Phenolics. *Extraction.* Ground plant material (1.0 g) was sonicated with ethanol: water (70:30, 80 mL) for 2 h, and then the cooled volume was adjusted to 100 mL. After the extract had settled it was centrifuged prior to spectroscopic measurements.

Standards and Reagents. Chlorogenic acid (10, 30, and 50 mg) was dissolved in ethanol:water (70:30, 100 mL). The Folin−Denis reagent was prepared by mixing H_2O (750 mL), $Na_2WO_4 \cdot 2H_2O$ (100 g), phosphomolybdic acid ($H_3PMo_{12}O_{40}$, 20 g), and phosphoric acid (85%, 50 mL), heating to reflux for 2 h, cooling, and making the solution up to 1 L. The solution was stored in an amber bottle.

Spectroscopy. Folin−Denis reagent (10 mL) was added to either 1 mL of extract solution or 1 mL of standard solutions. After 3 min 35% sodium carbonate solution (10 mL) was added and the test solution was made up to 100 mL with H_2O and mixed. After 45 min an aliquot was centrifuged; then the clear solution was transferred into a cuvette and the absorption coefficient measured at 745 nm. The standard chlorogenic acid solutions were taken as equivalent to 1, 3, and 5% total phenolics calculated as chlorogenic acid. The total phenolics contents of the extracts were calculated using the linear regression coefficient from the standards.

Statistical Analyses. Compound levels (% weight/weight) and ratios between selected compounds and between phenolics

Table 1. Phenolic Compound Levels in Medicinal *Echinacea* by the INA (*15*) HPLC Method and Ratio to Total Phenolic Levels by a Colorimetric (FD) Method

		phenolic compound[a]						ratios	
		caftaric acid (**3**)	chlorogenic acid (**5**)	cynarin (**4**)	echinacoside (**2**)	chicoric acid (**1**)	total (**1**−**5**)	caftaric/ chicoric	totals, INA/FD
purpurea, tops	Jan	0.82	<0.01	<0.01	<0.01	2.02	2.84	0.41	0.49
	April	0.18	<0.01	<0.01	<0.01	0.52	0.70	0.36	0.42
purpurea, roots	Jan	0.41	<0.01	<0.01	<0.01	2.27	2.68	0.18	0.57
	April	0.35	<0.01	<0.01	<0.01	1.68	2.04	0.22	0.52
lsd[b] (df = 12)		0.09				0.50	0.57	0.05	0.13
angustifolia, roots	May	<0.01	0.15	0.12	1.04	0.09	1.40	<0.01	0.59
C.I.[c] (df = 6)			0.07	0.07	0.26	0.02	0.37		0.07
pallida, roots	August	0.04	0.03	<0.01	0.34	?[d]	0.44	?[d]	0.36
C.I.[c] (df = 7)		0.04	0.04		0.28		0.28		0.18

[a] Mean % w/w of dried plant material, calculated on the basis of chlorogenic acid as external standard. [b] Least significant difference between two means at the 5% level calculated from analysis of variance (df = associated degrees of freedom). [c] The mean ± this value gives a 95% confidence interval for the mean (df = associated degrees of freedom). [d] Several peaks overlapped around the retention time of chicoric acid.

by different analytical methods were examined by analysis of variance using Genstat 5 (Rothamsted Experimental Station, England).

RESULTS AND DISCUSSION

We used the method of the Institute for Nutraceutical Advancement (*15*) to extract and analyze the phenolic compounds in the medicinal species *E. angustifolia*, *E. pallida*, and *E. purpurea*. The HPLC peaks of chicoric acid (**1**) and echinacoside (**2**) were identified using reference samples. Repeat extractions and analyses of an *E. purpurea* check sample gave an average chicoric acid level of 1.15% w/w with coefficient of variation of 0.02 (n = 4). An *E. angustifolia* sample gave an echinacoside level of 0.61% w/w with coefficient of variation of 0.04 (n = 4).

E. purpurea roots and tops showed two main phenolic compounds, chicoric (**1**) and caftaric (**3**) acids (Table 1). These cold ethanol:water extracts did not show an extra peak eluting just after the main chicoric acid peak, which we have seen in extracts produced by the hot methanol extraction method (*7*). This extra peak could be due to the isomerization to *meso* chicoric acid mentioned by Snook et al (*18*).

We have found that chicoric acid (**1**) levels in *E. purpurea* roots and tops vary significantly through the growing season (unpublished results). Therefore, we analyzed samples from high (summer) and low (autumn) chicoric acid harvests to determine the full range of phenolic compound levels by this INA method (Table 1 and Figure 2). There was a significant (P < 0.05) decline in chicoric acid levels from January to April, by almost 75% for tops (2.02 to 0.52% w/w) and by 25% for roots (2.27 to 1.68% w/w). Stuart and Wills (*12*) have also found significant drops in chicoric acid levels for *E. purpurea* roots and tops in going from flowering to senescence. Our chicoric acid levels (Table 1) were similar to those reported by Bauer and Wagner (*6*) of 0.2−3.1% w/w in tops and 0.6−2.0% w/w in roots (location and harvest season not specified).

Caftaric acid (**3**) levels in *E. purpurea* also declined from January to April, but this decline was only significant (P < 0.05) for tops (0.82 to 0.18% w/w, Table 1 and Figure 2). The only previous report of caftaric acid (**3**) levels in *E. purpurea* found 0.256% w/w in freeze-dried flowers (*9*). The ratio of caftaric to chicoric acid levels differed significantly (P < 0.001) between roots (mean ratio 0.2) and tops (ratio 0.4, Table 1 and Figure 2). This ratio could distinguish *E. purpurea* root extracts from extracts of tops, but the alkamide composition is much more distinctive (*19*).

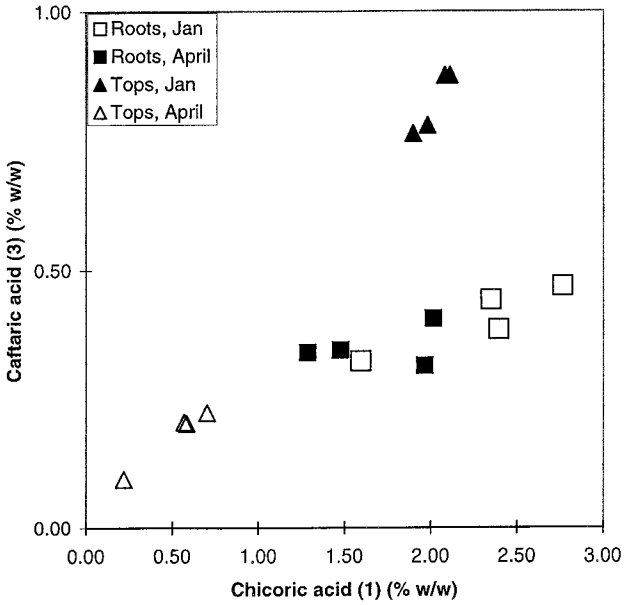

Figure 2. Phenolic compound levels in *E. purpurea* by INA (*15*) HPLC method.

E. angustifolia roots had echinacoside (**2**) as the major phenolic compound (Table 1) with levels in the range of 0.3−1.3% w/w reported by Bauer and Wagner (*6*). All of our samples showed a peak not identified in the INA method, eluting just before echinacoside. We purified the compound responsible for this peak by preparative HPLC. Its ^{1}H NMR spectrum matched that reported for cynarin (**4**) (*17*). Bauer and Wagner (*6*) report that cynarin is a characteristic component of *E. angustifolia* roots but did not give levels. We used a correction factor based on the relative molecular weights of chlorogenic acid (**5**) and cynarin (**4**), and the presence of two caffeate groups in cynarin, to give the levels shown in Table 1. We found that cynarin and echinacoside were not resolved if the HPLC loading was too high. Bergeron et al. (*8*) have reported that the relative retention of these compounds is dependent on the pH of the mobile phase. Including the cynarin peak area with echinacoside could give a >30% overestimate of the echinacoside level. We found a mean of 0.08% w/w chicoric acid (**1**) in our *E. angustifolia* roots (Table 1) whereas Bauer and Wagner (*6*) reported very low levels. Bergeron et al. (*8*) have

reported an average of 0.3% w/w chicoric acid in their *E. angustifolia* root samples.

Echinacoside (**2**) was the main phenolic compound in *E. pallida* roots (Table 1) with no detectable cynarin. This agrees with Bauer and Wagner (*6*) who suggest that the presence/absence of cynarin can be used to distinguish *E. angustifolia* and *E. pallida* roots. The echinacoside level found by us, mean 0.34% w/w, was at the low end of the 0.4−1.7% w/w range reported by Bauer and Wagner (*6*) but in the 0.2−0.8% w/w range reported by Schenk and Franke (*10*). The levels of phenolic compounds in *E. pallida* and *E. angustifolia* are likely to show seasonal variations similar to those of *E. purpurea*, but we are not aware of any seasonal studies on these species.

The INA method requires extraction of plant material with premixed 70:30 ethanol:water (*15*). It was determined that addition of water to ground *E. purpurea* roots prior to adding ethanol resulted in the extract going brown within seconds. HPLC analysis showed loss of >50% of both chicoric acid (**1**) and caftaric acid (**3**). Similar losses of >50% of these phenolic compounds were found upon adding water to *E. purpurea* tops. Adding water to *E. pallida* and *E. angustifolia* roots gave >50% losses of the other phenolic compounds, **2**, **4**, and **5**, in these species. We believe that these losses are due to enzymatic browning, in which a polyphenol oxidase (PPO) converts *o*-diphenols, such as caffeates, into *o*-quinones. These reactive *o*-quinones then form polymeric brown pigments (*20*). Kreis et al. (*21*) have characterized a PPO from *E. purpurea* tops which had high affinity toward caffeic acid and also accepted chicoric acid (**1**). Extraction with 70% ethanol serves to denature this enzyme. Wills and Stuart (1999) found that chicoric acid was lost if powdered *E. purpurea* got damp, but heating to denature the PPO enzyme could prevent these losses. Our results suggest that similar losses could occur for the other medicinal *Echinacea* species if they are not dried and stored carefully.

We compared the results of colorimetric (Folin−Denis) analyses of total phenolics levels in the different *Echinacea* samples with results from the INA HPLC method (Table 1 and Figure 3). There was a good correlation ($R^2 = 0.96$) between the two methods for *E. purpurea* roots and tops, but the absolute values were different (response ratio not equal to 1). The response ratios differed slightly but significantly ($P < 0.05$) between *E. purpurea* roots (mean ratio 0.53) and tops (0.47), probably because of the presence of other phenolic compounds. There was also a good correlation between the two methods for *E. angustifolia* roots (response ratio 0.59 ± 0.07, Table 1 and Figure 3), but the correlation was less good for *E. pallida* ($R^2 = 0.54$, response ratio 0.36 ± 0.18, Table 1 and Figure 3), perhaps because of varying levels of other phenolic compounds not included in the HPLC method.

In conclusion, we have used the INA HPLC method for total phenolics in *Echinacea* to measure typical levels of major and minor compounds in the medicinally used species (Table 1). The extraction method is faster than the two stage Soxhlet extraction of Bauer et al. (*7*) and seems to give less isomerization of chicoric acid. Cynarin (**4**) needs to be included in the method for analyses of *E. angustifolia* roots. Care must be taken to avoid enzymatic browning, which can dramatically reduce the measured levels of phenolic compounds. Results from colorimetric analyses for total phenolics correlate well with HPLC results, but the HPLC method is more informative in identifying the compounds characteristic of different species. Therefore, this INA method (*15*) is recommended as a method for standardization of *Echinacea* preparations, in combination with analyses of the alkamide levels (*19*). The major differences in phenolic compounds reported above, influenced by a wide range of factors, emphasize the need for careful standardization before investing in major clinical trials (*3*).

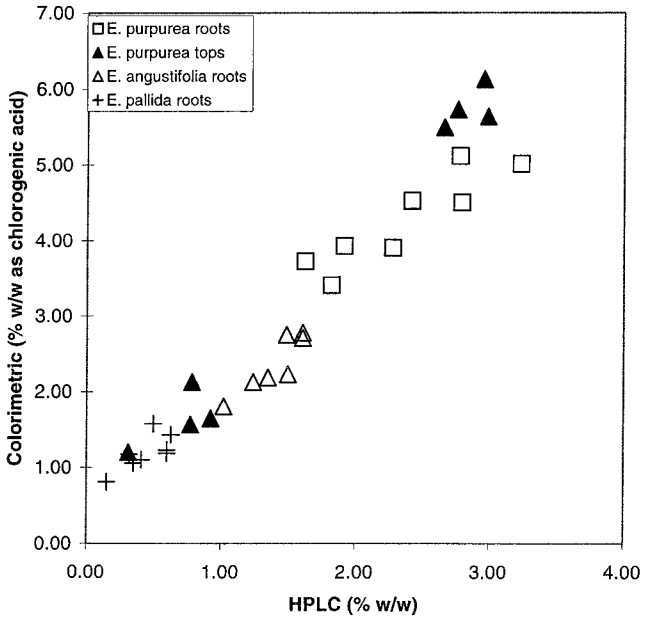

Figure 3. Total phenolic levels in medicinal *Echinacea* by INA (*15*) HPLC and colorimetric (Folin−Denis) methods.

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