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Determination of Major Phenolic Compounds in *Echinacea* spp. Raw Materials and Finished Products by High-Performance Liquid Chromatography with Ultraviolet Detection: Single-Laboratory Validation Matrix Extension

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Abstract

A method previously validated to determine caftaric acid, chlorogenic acid, cynarin, echinacoside, and cichoric acid in echinacea raw materials has been successfully applied to dry extract and liquid tincture products in response to North American consumer needs. Single-laboratory validation was used to assess the repeatability, accuracy, selectivity, LOD, LOQ, analyte stability (ruggedness), and linearity of the method, with emphasis on finished products. Repeatability precision for each phenolic compound was between 1.04 and 5.65% RSD, with HorRat values between 0.30 and 1.39 for raw and dry extract finished products. HorRat values for tinctures were between 0.09 and 1.10. Accuracy of the method was determined through spike recovery studies. Recovery of each compound from raw material negative control (ginseng) was between 90 and 114%, while recovery from the finished product negative control (maltodextrin and magnesium stearate) was between 97 and 103%. A study was conducted to determine if cichoric acid, a major phenolic component of *Echinacea purpurea* (L.) Moench and *E. angustifolia* DC, degrades during sample preparation (extraction) and HPLC analysis. No significant degradation was observed over an extended testing period using the validated method.

Echinacea is a genus of flowering plants endemic to North America; species used medicinally include *Echinacea angustifolia* DC, *E. pallida* (Nutt.), *and E. purpurea* (L.) Moench (1). These species and their extracts are most often used for the prevention and treatment of upper respiratory tract infections, such as colds and flu, and as an immune stimulant (2). Phytochemical constituents include the caffeic acid derivatives (also called phenolics), alkamides, glycoproteins, and polysaccharides (3, 4). Root and aerial plant parts may be used raw or in formulations, while homeopathic remedies may include the whole plant (5). According to a 2002 U.S. Food and Drug Administration (FDA) survey, echinacea was the most used nonvitamin/nonmineral dietary supplement (6). In a 2007 National Center for Complementary and Alternative Medicine survey, echinacea was the third most commonly used nonvitamin, nonmineral natural product among adults, and the most

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administered natural product in children in the United States (7). In 2009, it was still the fifth-highest-selling herbal dietary supplement (8). It is also the second most used Natural Health Product in Canada (9, 10). This popularity has resulted in production and availability of a wide variety of products for which analytical methods to support quality assessments by manufacturers and regulators are needed.

Echinacea commercial products have diversified recently to include capsules, tablets, powders, tinctures, teas, and other beverages, as well as personal care products. Many capsules and tinctures incorporate other popular immune-boosting ingredients such as goldenseal (*Hydrastis canadensis* L.), zinc, and vitamin C, which may interfere with extraction and analysis of echinacea components. Up to 80% of commercial echinacea products contain *E. purpurea* (11), and many incorporate both root and aerial parts. Cichoric acid is the most prominent phenolic component of *E. purpurea* root (12, 13), making it an important marker for possible standardization and QC purposes (3, 14). It, along with the other phenolic compounds, may play a critical role in stimulating the immune system; these are often of most importance during the quantification and qualification of echinacea products (2, 15–18). However, it has been reported that the cichoric acid is especially susceptible to degradation by endogenous enzymes during the processing of fresh echinacea plant material (19). There is also concern that other phenolic components may undergo similar degradation during preparation of plant materials for analysis (20, 21). Because cichoric acid and alkamides are sensitive to extraction conditions, they may be good indicators of reproducible extract production (3, 5, 17).

Although recent animal research suggests *E. purpurea* may be an effective immunomodulator (22, 23), clinical trials have not been able to conclusively demonstrate efficacy or nonefficacy (24–34). In an attempt to derive meaning from these trials, several meta-analyses were conducted (35–38), and flaws in study designs were noted by the authors of those analyses. These included poorly defined outcome measures, inconsistencies in clinical intent (prevention versus treatment, natural infection versus rhinovirus challenge, dose), and limited quality of evidence (39). Another major issue is a general lack of adequate chemical and botanical descriptions of test articles used in botanical clinical trials (40, 41). Manufacturers and consumers of echinacea preparations are faced with similar issues. Nonstandardized material can and does result in the production of batches of the same product that have completely different phytochemical composition (42). There have also been numerous cases of misidentification and/or adulteration involving both commercial products and research materials (2, 14).

Although alkamides, polysaccharides, glycoproteins, and phenolics have all been hypothesized as potential active ingredients of echinacea, most of the published methods for ensuring identity and potency were developed to quantify the phenolic marker compounds, cichoric acid, echinacoside, and/or total phenolics (12, 16, 17, 43–47). Until recently (48), none of these methods had been validated according to the guidelines published by AOAC INTERNATIONAL (49). To address this issue, an AOAC expert review panel convened in 2005 to identify a published echinacea phenolics method with potential to be widely implemented by industry. The Institute for Nutraceutical Advancement method (15), reviewed by Perry et al. (17) and included in the American Herbal Pharmacopeia monograph for quantifying phenolic marker compounds in raw materials and extracts (2), was chosen for further optimization and single-laboratory validation (SLV). The resulting study, published by Brown et al. (48), was conducted only on echinacea biomass and not on extract raw materials and finished products containing echinacea as well as other ingredients.

This paper describes a matrix extension SLV study of the HPLC method for the quantification of the major phenolic compounds in echinacea of Brown et al. (48), conducted according to AOAC INTERNATIONAL guidelines (49). The new matrixes include echinacea raw materials and finished products with goldenseal, vitamin C, zinc, and other possible interfering species. It also presents key details of an analyte stability study (21) designed to determine if cichoric acid degrades during extraction and HPLC analysis.

Experimental

Principle

This HPLC method is used to detect and quantify five phenolic compounds commonly found in *Echinacea* spp. raw materials, powdered extract finished products, and tinctures. The phenolic compounds are caftaric acid, chlorogenic acid, cichoric acid, cynarin, and echinacoside.

Test Materials

All test materials were stored at room temperature. Whole root and aerial parts of *E. angustifolia*, *E. purpurea*, and *E. pallida* were harvested in 2008 under the supervision of Wendy Applequist (Missouri Botanical Gardens, St. Louis, MO) and provided by Naturex (South Hackensack, NJ). The herbarium specimens for these three species were deposited with the Missouri Botanical Garden Herbarium, Voucher Nos. 217, 218, and 216, respectively.

- **a.** *E. purpurea whole root*.—Naturex.
- **b.** *E. purpurea aerial parts*.—Naturex.
- **c.** *E. angustifolia whole root*.—Naturex.
- **d.** *E. angustifolia aerial parts*.—Naturex.
- **e.** *E. pallida whole root*.—Naturex.
- **f.** *E. purpurea aerials powdered extract*.—Naturex.
- **g.** *E. angustifolia root powdered extract*.—Naturex.
- **h.** *E. angustifolia root powdered extract in softgel capsule*.—Supplied by commercial manufacturer.
- **i.** *E. purpurea aerials powdered extract*.—Supplied by commercial manufacturer.
- **j.** *E. purpurea root glycerite tincture*.—Supplied by commercial manufacturer.
- **k.** *E. angustifolia aerials tincture*.—Supplied by commercial manufacturer.
- **l.** *E. angustifolia/E. purpurea roots and aerials tincture*.—Supplied by commercial manufacturer.
- **m.** *E. purpurea leaf, stem, and flower in combination with elderberry and zinc in hardshell capsule*.—Supplied by commercial manufacturer.
- **n.** *E. purpurea leaf, stem, flower in combination with Astragalus and Reishi in hardshell capsule*.—Supplied by commercial manufacturer.
- **o.** *E. purpurea leaf, stem, flower in combination with vitamin C in hardshell capsule*.—Supplied by commercial manufacturer.

Reagents and Supplies

a. *Acetonitrile (C_2H_3N).*—Formula weight (FW): 41.05, CAS No. 75-05-08, purity: ≥99.8% (GC), HPLC grade or equivalent. Colorless liquid, flammable, and poisonous (Fisher Scientific, Ottawa, ON, Canada).

b. *Methanol (CH_3OH).*—FW: 32.04, CAS No. 67-56-1, purity: 99.97%, HPLC grade or equivalent. Colorless liquid, volatile, flammable, and poisonous (Fisher Scientific).

c. *Water (H_2O).*—FW: 18.01, CAS No. 7732-18-5, purity: submicron filtered, HPLC grade or equivalent. Colorless liquid (Fisher Scientific).

d. *o-Phosphoric acid (H_3PO_4).*—FW: 98.00, CAS No. 7664-38-2, purity: ≥85.0%, HPLC grade or equivalent. Colorless liquid, irritant, and poisonous (Fisher Scientific).

e. *Magnesium stearate ($C_{36}H_{70}MgO_4$).*—FW: 594.27, CAS No. 557-04-0, vegetable grade or equivalent. White powder (Fisher Scientific).

f. *Maltodextrin ($C_{6n}H_{(10n+2)}O_{(5n+1)}$).*—FW: variable, CAS No. 9050-36-6, Maltrin M100 (grain derived) or equivalent. Hygroscopic white powder provided by Natural Factors Nutritional Products Ltd (Coquitlam, BC, Canada).

g. *Panax quinquefolius L. root.*—Obtained from the Ontario Ginseng Grower's Association (Simcoe, ON, Canada) for use as a matrix blank (negative control) for spiking studies.

Solutions

a. *Extraction solvent.*—Methanol–water (60 + 40).

b. *Mobile phase A.*—0.1% *o*-Phosphoric acid in water (filtered through 0.2 μm nylon filter).

c. *Mobile phase B.*—Acetonitrile.

Reference Standards

For caftaric acid, cichoric acid, and echinacoside, 1000 ppm stock solutions were prepared by dissolving individual reference materials in extraction solvent. Chlorogenic acid and cynarin stock solutions were also prepared at 1000 ppm, but then diluted to 200 and 100 ppm, respectively. These stock concentrations were then diluted to appropriate concentrations to establish retention time and combined at different concentration levels for external calibration.

a. *Caftaric acid ($C_{13}H_{12}O_9$).*—FW: 312.23, CAS No. 67879-58-7, purity: 88.6%, acquired from ChromaDex (Irvine, CA), Part No. 3028, stored at −20°C and desiccated. Purity was adjusted for HPLC and presence of water and other solvents.

b. *Chlorogenic acid ($C_{16}H_{18}O_9$).*—FW: 354.31, CAS No. 327-325-6, purity: 93.9%, acquired from ChromaDex, Part No. 3450, stored at room temperature and desiccated.

c. *Cichoric acid ($C_{22}H_{18}O_{12}$).*—FW: 474.37, CAS No. 70831-56-0) purity: 97.0%, acquired from ChromaDex, Part No. 3640, stored at −20°C and desiccated.

d. *Cynarin ($C_{25}H_{24}O_{12}$).*—FW: 516.47, CAS No. 1182-34-9, purity: 95.44%, acquired from ChromaDex, Part No. 3990, stored at −20°C and desiccated.

e. *Echinacoside ($C_{35}H_{46}O_{20}$).*—FW: 786.74, CAS No. 82854-37-3, purity: 97.0%, acquired from ChromaDex, Part No. 5020, stored at −20°C and desiccated.

Apparatus

a. *Centrifugal mill or grinder.*—Retsch Ultra centrifugal mill ZM 100 (Retsch GmbH, Haan, Germany) or equivalent mill capable of grinding root samples to 60 mesh.

b. *Analytical balance.*—Mettler Toledo AE 260 analytical range (±0.1 mg; VWR International, Edmonton, AB, Canada) or equivalent.

c. *Centrifuge.*—Eppendorf 5804 table top centrifuge (VWR International) or equivalent

d. *Wrist action shaker.*—Burrell Model BT wrist action shaker (VWR International) or equivalent.

e. *Syringes.*—3 mL Luer-lok® fitted with PTFE filter, 0.45 and 0.2 μm pore size, 25 mm diameter (Fisher Scientific).

f. *Vortex mixer.*—Table top (VWR International) or equivalent.

g. *Micropipets.*—Eppendorf Reference Series, 100, 200, and 1000 μL (VWR International) or equivalent.

h. *HPLC system.*—Agilent 1100 Series liquid chromatograph equipped with quaternary pump and degasser (G1354A), temperature-controlled column compartment (G1316A), temperature-controlled autosampler (G1327A), standard flow-cell 10 mm, 13 μL, 120 bar (G1315-60012), diode-array detector (G1315B), HPLC 2D ChemStation software (G2175AA), and online degasser (1322A; Agilent Technologies, Mississauga, ON, Canada) or equivalent.

HPLC Conditions

a. *Autosampler temperature.*—5°C.

b. *Analytical column.*—Cosmosil 5C18-AR-II, 150 × 4.6 mm id.

c. *Column temperature.*—25°C.

d. *Detector conditions.*—Monitor at 330 nm (8 nm bandwidth), no reference.

e. *Flow rate.*—1.5 mL/min.

f. *Run time.*—14.5 min with 3.5 min post time for column equilibration.

g. *Injection volume.*—5 μL.

h. *Gradient conditions.*—*See* Table 1.

Calculations

The equations used to determine the average weight of finished products, based on 20 capsules, are as follows:

Average capsule fill weight (g): AFW (g) = (C − S)/20

where C = weight of capsule with shell and fill content (g), and S = weight of empty shell (g).

Average tablet weight (g): ATW,

g = weight of 20 tablets/20

The calculation used to determine phenolic concentration is as follows:

Concentration (μg/mL): C, μg/mL = (A − B)/D

where A = peak area (mAu × s), B = intercept of the calibration curve, and D = slope of the calibration curve.

To quantify the individual phenolic compounds on a % (w/w) basis, the following calculation was used:

Phenolic, % (w/w) = [(C)(FV)(D)(100%)]/(W)

where C = concentration (μg/mL) from linear regression analysis, FV = the final volume (mL) of the sample preparation, D = the dilution factor of the sample preparation, and W = the sample weight (mg).

To quantify the individual phenolic compounds on a part per million (μg/mL) basis for tinctures, the following calculation was used:

Phenolic in tincture, μg/mL = [(C)(FV)(D)]

where C = concentration (μg/mL) from linear regression analysis, FV = the final volume (mL) of the sample preparation, D = the dilution factor of the sample preparation (25), and W = the sample weight (mg).

For the validation study, the following equations were used for evaluating precision:

RSD_r (found, %): $RSD_r = SD(r)/mean \times 100$

where SD(r) = population SD (σ/n, where σ = sum of squares and n = number of replicates).

$PRSD_r$ (RSD_r calculated, %): $PRSD_r = 2 \times C^{-0.15}$

HorRat value: HorRat = RSD_r (found, %)/PRSDr (calculated, %)

Within-day: average and SDs of four data points within-day.

Within-laboratory: average and SDs of 12 data points over 3 days (separate batches on 3 days).

Chromatographic resolution, Rs, was calculated using the following equation:

$Rs = 2[(t_R)_A - (t_R)_B]/(W_A + W_B)$

where t_R = retention time, min, W = width of peak at baseline, min, A = earlier-eluting peak, and B = later-eluting peak. Baseline resolution requires an Rs >1.5.

Preparation of Test Materials

a. *Raw materials and powdered extracts.*—

1. Raw materials (root, aerials) were ground to 60 mesh powder in a grinding mill, homogenized, and stored in separate plastic bags. Extract powders were mixed to ensure homogeneity.
2. 125 mg (±0.1 mg) of each powdered test material was weighed into separate 50 mL conical tubes.
3. Using volumetric pipets, 25 mL extraction solvent (60% aqueous methanol solution) was added to each conical tube containing the test materials.

4. Each sample tube was then mixed with a wrist action shaker for 30 min at room temperature.
5. Tubes were centrifuged at 5000 rpm for 5 min.
6. A portion of the supernatant was filtered through a 3 mm syringe fitted with a 0.45 μm Teflon membrane filter into an amber glass HPLC vial and capped.

b. *Soft shell dry filled capsule test materials.*—

1. The total capsule content weight was determined by weighing 20 capsules. The 20 capsules were cut open with a suitable instrument.
2. The capsule contents were emptied and combined in a conical tube. Weights of the capsule contents and the empty capsule shells were obtained and recorded. The average fill weight/capsule was calculated.
3. The capsule contents were transferred into a 50 mL conical tube and mixed using a spatula to homogenize the samples.
4. The same procedure described in the *Raw Materials and Powdered Extracts* section, Steps (*2*) to (*6*), was followed.

c. *Tinctures (ethanol and glycerite types).*—

1. Tincture vessels were inverted several times to ensure homogeneity before aliquotting.
2. Using a volumetric pipet, 1 mL tincture was accurately transferred into a 50 mL conical tube.
3. Using volumetric pipets, 24 mL extraction solvent (60% aqueous methanol solution) was added the conical tube.
4. Tubes were vortexed for 30 s to mix.
5. Tubes were centrifuged at 5000 rpm for 5 min.
6. A portion of the solution was filtered through a 3 mm syringe fitted with a 0.45 μm Teflon membrane filter into an amber glass HPLC vial and capped.

Preparation of Calibration Solutions

Refer to Table 2 for the approximate concentration of the individual phenolic compounds for each linearity determination (calibration level). All solutions not immediately used were stored at −20°C.

a. *Linearity 1.*—Refer to Table 3 for preparation instructions.

b. *Linearity 2.*—Refer to Table 3 for preparation instructions.

c. *Linearity 3.*—Refer to Table 3 for preparation instructions.

d. *Linearity 4.*—Refer to Table 3 for preparation instructions.

e. *Linearity 5.*—100 μL of Linearity 2 solution was diluted with 900 μL extraction solvent and mixed well.

f. *Linearity 6.*—100 μL Linearity 3 solution was diluted with 900 μL extraction solvent and mixed well.

g. *Linearity 7.*—100 μL Linearity 5 solution was diluted with 900 μL extraction solvent and mixed well.

SLV Parameters—Matrix Extension

This method was validated according to AOAC INTERNATIONAL guidelines for conducting an SLV (49).

a. *Selectivity.*—The selectivity of the method for the targeted phenolic compounds was established by injecting each individual reference analyte and comparing to the chromatographic profile of the test articles to establish that resolution between each analyte was achieved. An Rs of >1.5 between closely eluting components was deemed acceptable.

b. *Linearity.*—The linearity for each analyte was evaluated using seven-point standard calibration curves. Calibration solutions for constructing calibration curves were prepared as described above. The calibration curves for each phenolic compound were plotted, and simple linear regression used to determine the slope and *y*-intercept of each curve for each analyte. Calibration curves were visually inspected to confirm linearity and r^2 values for the regression curves were calculated for each quantified phenolic compound. An r^2 of ≥99.5% was deemed acceptable for quantification.

c. *Method detection limit (MDL) and LOQ.*—The absence of a suitable sample blank precluded the use of the International Union for Pure and Applied Chemistry method for determination of detection limits for the analytes. As an alternative, the detection limit for each analyte was determined using the U.S. Environmental Protection Agency MDL protocol (50). The MDL is defined as the minimum concentration of substance that can be measured and reported with 99% confidence that the analyte concentration is greater than zero. Seven replicates of a standard solution containing very low concentrations of each analyte were used to determine the MDL. The concentration of each replicate was calculated. The LOQ was calculated as 10 times the sample SD of the replicates used to determine MDL.

d. *Precision.*—Precision was evaluated by analyzing multiple replicates of each test sample. Four replicates of each material were prepared and analyzed on each of 3 separate days, resulting in 12 replicates for each test material. The within-day, between-day, and total SDs were calculated for each individual phenolic compound for each of the 15 materials using single factor analysis of variance (ANOVA) with an α-value of 0.5. In all cases, null hypothesis (H_0) for the analysis was that determination of analyte content does not differ between days; the alternative hypothesis (H_A) was that determination of analyte content does differ between days. A “pass” under ANOVA indicates acceptance of H_0. Finally, the HorRat value for each analyte in each material was calculated (51, 52). Values between 0.3 and 1.3 were considered acceptable for SLV.

e. *Accuracy.*—Method extraction efficiency in raw materials was evaluated by performing a spike recovery study at three levels of the five analytes [1.8, 4.7, and 8.6% (w/w) total phenolics] onto 125 mg of *P. quinquefolius* L. root powder ground to 60 mesh. Seven replicates were prepared/level. As a check on extraction efficiency from a commercial extract, a matrix model of 99% maltodextran and 1% magnesium stearate was used. Three different levels each of caftaric acid, chlorogenic acid, cynarin, echinacoside, and cichoric acid were spiked onto 125 mg of a negative control material consisting of 99% maltodextran and 1% magnesium stearate. While some commercially available echinacea extracts (loose or in capsule form) claim to contain as much as 4% “echinacosides,” our laboratory has observed

actual levels between 1.0 and 2.8% (w/w) total phenolics. Typical extracts contain *E. purpurea* root (no cynarin or echinacoside) or aerials (no cynarin), and occasionally *E. angustifolia* aerial parts (all analytes). The spike study was meant to capture the range of 0.9–3.8% (w/w) total phenolics.

f. *Stability.*—To determine if cichoric acid degrades during extraction and analysis, dried root samples of *E. angustifolia* DC and *E. purpurea* (L.) Moench were ground to 60 mesh. Each was extracted four times with four different extraction solvents: 100% water; methanol–water (60 + 40); methanol–water (60 + 40), with 1 mM ascorbic acid; and ethanol–water (20 + 80). Following the validated method, 25 mL extraction solvent was added to 125 mg ground echinacea and vortex-mixed for 30 s. Exactly 1 mL suspension was removed, filtered through a 0.2 μm syringe filter into an HPLC vial, and immediately analyzed by HPLC (time 0). Exactly 1 mL extraction solvent was added to the tube to replace the lost volume, and the mixture was shaken for 20 min on a wrist-action shaker at 385 osc./min. Again, 1 mL suspension was removed, filtered into an HPLC vial, and analyzed immediately (time 20). Another 1 mL of fresh extraction solvent was added back to the tube. This process was repeated at 20 min intervals until aliquots from 0, 20, 40, 60, and 80 min of shaking were prepared for HPLC analysis. Cichoric acid was quantified against an 11-point external standard calibration curve ranging from 1 to 200 μg/mL cichoric acid prepared in methanol.

In a separate experiment, stability of extracted test solutions was assessed by combining 125 mg *E. purpurea* with 25 mL methanol–water (60 + 40), vortexing 30 s, and shaking for 20 min on a wrist-action shaker. A portion of the final extracted solution was filtered into seven separate HPLC vials kept at room temperature. From six of these vials, 30 consecutive HPLC injections were made over the course of 9 h. The seventh vial was stored at room temperature for 6 days and then analyzed. The concentration for each of the five analytes was calculated for each sample. Degradation would be indicated if the actual concentrations were less than the expected concentrations.

Results and Discussion

Method Validation Results—Performance Characteristics

Identification of analytes in test materials was performed by comparing peak t_R values and UV profiles to the individual reference standards diluted to within the method calibration curve concentrations. A gradient elution was used for the analysis of the five major phenolic compounds in *Echinacea* spp. (Table 1). The order of elution was caftaric acid (4.18–4.23 min), chlorogenic acid (4.50–4.57 min), cynarin (7.57–7.69 min), echinacoside (7.81–7.92 min), and cichoric acid (12.96–13.13 min). A representative chromatogram of a mixed calibration standard illustrating this elution order can be seen in Figure 1.

Quantification of the analytes was carried out by linear regression analysis using quadruplicate samples prepared on three separate days at seven concentration levels. The analytical range used for each phenolic is listed in the section *Preparation of Calibration Solutions*. Test matrixes included *E. purpurea*, *E. angustifolia*, and *E. pallida* in root and/or aerial raw materials; *E. purpurea* and *E. angustifolia* in powdered extract (loose, in capsule as single botanical ingredient, or in capsule with other botanical ingredients); and *E. purpurea* and/or *E. angustifolia* in tincture (ethanol or glycerite).

Selectivity

Baseline resolution (R_s >1.5) was achieved for each analyte within the calibration range. R_s >4.0 and R_s >3.0 was achieved between caftaric acid/chlorogenic acid and cynarin/

echinacoside peaks, respectively. There was no evidence of chromatographic interference with analytes of interest by goldenseal, zinc, vitamin C, reishi, astragalus, or elderberry in formulations.

Linearity

All of the calibration curves generated over the course of the study appeared linear upon visual inspection. All of the RSDs were above 99.5%. These results confirm that the curves were linear over the expected concentration range for echinacea materials.

MDL and LOQ

Variance checks showed that the method used was applicable for the analytes. The MDL and LOQ for each of the analytes are reported in Table 4.

Precision

For some of the test materials, chlorogenic acid, cynarin, and echinacoside were not detected; for that reason, no response values were reported for these analytes, and no precision analysis was performed. The responses observed for all other analytes, in all test articles, were above the detection limit of the method and were thus reported as detected. For all reported materials, ANOVA indicated no significant differences for between-day precision. Average HorRat values for raw materials and dry finished products (Tables 5a and b) were 0.49 and 0.55, respectively, while average HorRat values for the tinctures (Table 5c) were lower than expected at 0.19. The low tincture HorRat values could be attributed to the use of volumetric glassware and high within-laboratory precision since they were consistently low among all analytes.

Accuracy

Two spike recovery studies were conducted to determine method accuracy. The first study was designed to emulate raw *Echinacea* spp. materials containing 1.8, 4.7, and 8.6% (w/w) total phenolics (sum of all five analytes) spiked onto *P. quinquefolius* L. root powder. Recovery (Table 6) over the three levels, averaged over all samples, was 99.8%. A second study used materials designed to resemble commercial *Echinacea* spp. extracts containing 0.9, 1.8, and 3.8% (w/w) total phenolics. The average recovery (Tables 7a–c) over these three levels was 100.0% (1.39% RSD).

Stability

Significant cichoric acid degradation was observed when *E. purpurea* and *E. angustifolia* root samples were extracted with highly aqueous extraction solvents [ethanol–water (20 + 80)]. No advantage was observed when 1 mM ascorbic acid was added to the methanolic extraction solution. The cichoric acid concentrations in liquid extracts of echinacea materials prepared by the validated method [methanol–water (60 + 40)] did not degrade over the entire 100 min test period.

Concentrations of each of the five analytes in an *E. purpurea* root sample extracted with methanol–water (60 + 40) were found to be stable through 30 injections over a 9 h period, based on a ≤4% difference in peak areas. A sample of this solution stored at room temperature for 6 days did not show any significant degradation of any analyte.

Conclusions

The reported method, previously validated for echinacea raw materials, was extended to extracts, extract in capsules, and tinctures, and subjected to an SLV study, according to

AOAC guidelines. All parameters investigated were found to be in compliance with those guidelines. As such, the described method is considered suitable for the purpose of determining caftaric acid, chlorogenic acid, cynarin, echinacoside, and cichoric acid in *E. purpurea, E. angustifolia*, and *E. pallida* powdered commercial extracts alone or in combination with *H. canadensis* L., zinc, and ascorbic acid (Vitamin C), and extracts in ethanolic or glycerite tinctures alone or in combination with other ingredients. In the interest of establishing an *Official Method of Analysis*SM for determination of phenolic compounds in *Echinacea* spp., a collaborative study of the described method is planned.

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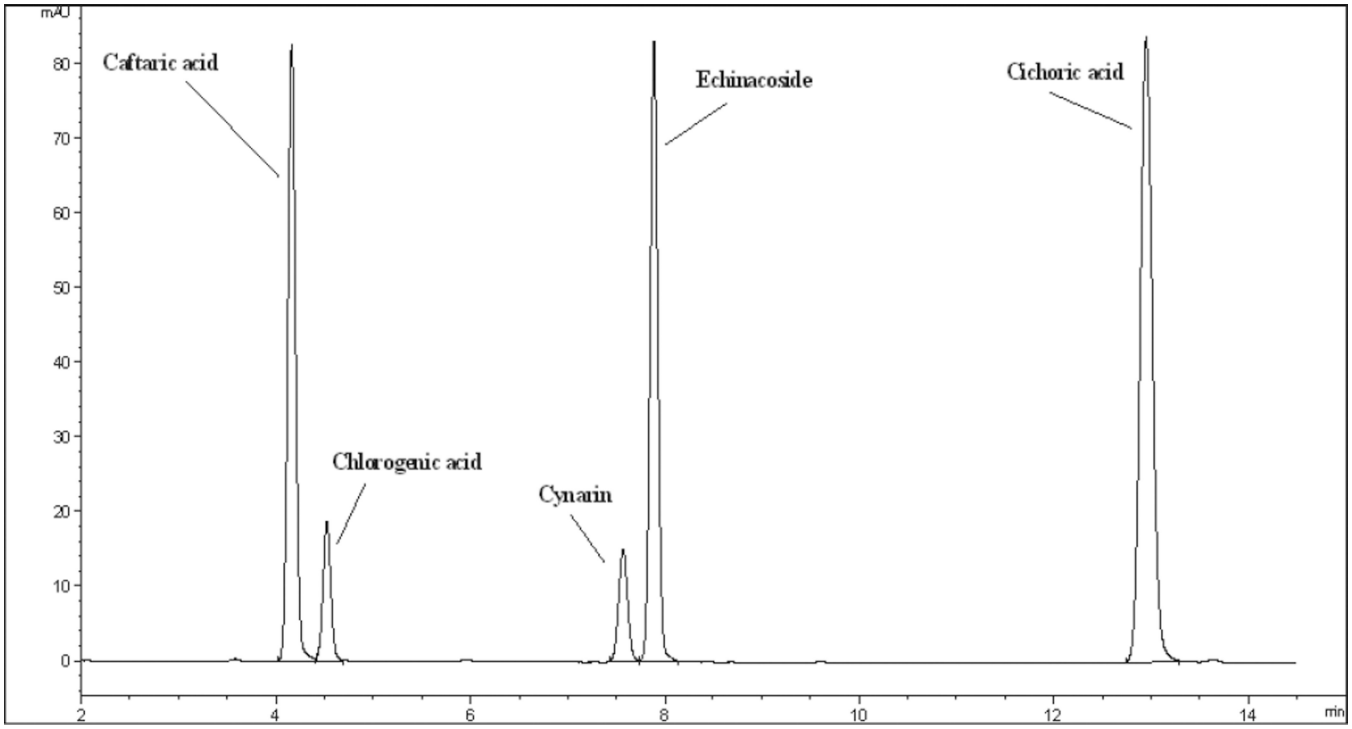

Figure 1.
Representative chromatogram of a mixed calibration standard containing the five phenolic compounds quantified using this analytical method.

Table 1

Gradient conditions for the separation of five phenolic compounds in echinacea test samples

Time, min	Mobile phase B, %
0	10
13	22
14	40
14.5	40

Table 2

Approximate concentrations of the phenolic compounds at each linearity (Lin) level

Phenolic compound	Approximate concentration, µg/mL						
	Lin 1	Lin 2	Lin 3	Lin 4	Lin 5	Lin 6	Lin 7
Caftaric acid	150	100	50	25	10	5	1
Chlorogenic acid	50	20	10	5	2	1	0.2
Cynarin	50	20	10	5	2	1	0.2
Echinacoside	300	200	100	50	20	10	2
Cichoric acid	200	100	50	25	10	5	1

Table 3

Mixed calibration standard (linearity) preparation details

Phenolic compound	Calibration std ID	Lin 1	Lin 2	Lin 3	Lin 4
Caftaric acid	Stock solution ID[a]	Caf 1000	Caf 1000	Caf 1000	Caf 1000
	Volume, µL	150	100	50	25
Chlorogenic acid	Stock solution ID	Chloro 1000	Chloro 200	Chloro 200	Chloro 200
	Volume, µL	50	100	50	25
Cichoric acid	Stock solution ID	Cich 1000	Cich 1000	Cich 1000	Cich 1000
	Volume, µL	200	100	50	25
Cynarin	Stock solution ID	Cyn 1000	Cyn 100	Cyn 100	Cyn 100
	Volume, µL	50	200	100	50
Echinacoside	Stock solution ID	Ech 1000	Ech 1000	Ech 1000	Ech 1000
	Volume, µL	300	200	100	50
Extraction solvent volume, µL		250	300	650	825

[a]ID = Identification code.

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Table 4

Method detection limit (MDL) and LOQ values calculated for each of the analytes

Analyte	MDL, μg/mL	LOQ, μg/mL
Caftaric acid	0.044	0.44
Chlorogenic acid	0.015	0.15
Cynarin	0.025	0.25
Echinacoside	0.050	0.50
Cichoric acid	0.070	0.70

Table 5

a. Precision results summary of echinacea raw material test samples

Matrix	Analyte	ANOVA	HorRat	Mean, % (w/w)
E. purpurea root (BCIT-ECH-002-09)	Caftaric acid	Pass	0.38	0.56
	Chlorogenic acid	Pass	0.61	0.02
	Cynarin	NA[a]	NA	Not detected
	Echinacoside	NA	NA	Not detected
	Cichoric acid	Pass	1.01	2.78
E. purpurea aerial parts (BCIT-ECH-004-09)	Caftaric acid	Pass	0.41	0.85
	Chlorogenic acid	Pass	0.36	0.01
	Cynarin	NA	NA	Not detected
	Echinacoside	NA	NA	Not detected
	Cichoric acid	Pass	0.71	2.20
E. angustifolia root (BCIT-ECH-003-09)	Caftaric acid	Pass	0.61	0.02
	Chlorogenic acid	Pass	0.34	0.02
	Cynarin	Pass	0.42	0.07
	Echinacoside	Pass	0.35	1.55
	Cichoric acid	Pass	0.43	0.03
E. angustifolia aerial parts (BCIT-ECH-005-09)	Caftaric acid	Pass	0.32	0.39
	Chlorogenic acid	Pass	0.42	0.08
	Cynarin	NA	NA	Detected
	Echinacoside	Pass	0.63	0.11
	Cichoric acid	Pass	0.32	0.78
E. pallida root (BCIT-ECH-001-09)	Caftaric acid	Pass	0.36	0.01
	Chlorogenic acid	NA	NA	Not detected
	Cynarin	NA	NA	Not detected
	Echinacoside	Pass	0.43	0.13
	Cichoric acid	Pass	0.65	0.02

b. Precision results summary of echinacea dry finished product test samples

Matrix	Analyte	ANOVA	HorRat	Mean, % (w/w)
E. purpurea aerial powdered extract (BCIT-ECH-006-09)	Caftaric acid	Pass	0.30	2.31
	Chlorogenic acid	Pass	0.46	0.03
	Cynarin	NA	NA	Not detected
	Echinacoside	Pass	1.39	0.11
	Cichoric acid	Pass	0.31	3.15
E. angustifolia aerial powdered extract (BCIT-ECH-007-09)	Caftaric acid	Pass	0.42	0.03
	Chlorogenic acid	Pass	0.32	0.03
	Cynarin	Pass	0.38	0.13
	Echinacoside	Pass	0.81	2.49
	Cichoric acid	Pass	1.33	0.03

b. Precision results summary of echinacea dry finished product test samples

Matrix	Analyte	ANOVA	HorRat	Mean, % (w/w)
E. purpurea leaf/stem/flower capsule with elderberry, goldenseal, vitamin C, and Zn (BCIT-ECH-009-09)	Caftaric acid	Pass	0.38	0.34
	Chlorogenic acid	Pass	0.48	0.04
	Cynarin	NA	NA	Not detected
	Echinacoside	NA	NA	Not detected
	Cichoric acid	Pass	0.39	0.61
E. purpurea leaf/stem/flower capsule with Astralagus, Reishi (BCIT-ECH-011-09)	Caftaric acid	Pass	0.34	0.36
	Chlorogenic acid	Pass	0.69	0.01
	Cynarin	NA	NA	Not detected
	Echinacoside	NA	NA	Not detected
	Cichoric acid	Pass	0.26	0.64
E. purpurea leaf/stem/flower with vitamin C (BCIT-ECH-012-09)	Caftaric acid	Pass	0.45	0.44
	Chlorogenic acid	Pass	0.50	0.01
	Cynarin	NA	NA	Not detected
	Echinacoside	NA	NA	Not detected
	Cichoric acid	Pass	0.33	0.81
E. purpurea powdered extract, 4% polyphenols (BCIT-085-012)	Caftaric acid	Pass	0.44	1.07
	Chlorogenic acid	Pass	0.49	0.04
	Cynarin	NA	NA	Detected
	Echinacoside	NA	NA	Not detected
	Cichoric acid	Pass	0.24	1.52
E. purpurea powdered extract in capsule (BCIT-001-08)	Caftaric acid	Pass	0.30	0.54
	Chlorogenic acid	Pass	0.79	0.03
	Cynarin	NA	NA	Detected
	Echinacoside	NA	NA	Not detected
	Cichoric acid	Pass	0.73	0.97

c. Precision results summary of echinacea tincture test samples

Matrix	Analyte	ANOVA	HorRat	Mean, μg/g
E. purpurea root glycerite (BCIT-ECH-008-09)	Caftaric acid	Pass	0.20	200
	Chlorogenic acid	NA	NA	Not detected
	Cynarin	NA	NA	Not detected
	Echinacoside	NA	NA	Not detected
	Cichoric acid	Pass	0.18	258
E. angustifolia aerial (BCIT-ECH-005-08)	Caftaric acid	Pass	0.13	611
	Chlorogenic acid	Pass	0.22	221
	Cynarin	Pass	0.28	73
	Echinacoside	Pass	0.15	475
	Cichoric acid	Pass	0.10	405
E. angustifolia/purpurea root/aerial (BCIT-ECH-006-08)	Caftaric acid	Pass	0.09	642
	Chlorogenic acid	Pass	0.34	32
	Cynarin	NA	NA	Not detected

c. Precision results summary of echinacea tincture test samples

Matrix	Analyte	ANOVA	HorRat	Mean, μg/g
E. purpurea root/aerial with *H. canadensis* L. (BCIT-GOLD-002-08)	Echinacoside	Pass	0.30	73
	Cichoric acid	Pass	0.10	1017
	Caftaric acid	Pass	0.61	4659
	Chlorogenic acid	NA	1.10	502
	Cynarin	NA	NA	Not detected
	Echinacoside	NA	NA	Not detected
	Cichoric acid	Pass	1.09	3371

[a]NA = Not applicable.

Table 6

Raw material negative control spike recovery results[a]

Analyte	Mean expected concentration, % (w/w)	Mean recovery, %	RSD, %
Caftaric acid	35.8	108	10.5
Chlorogenic acid	4.50	114	9.80
Cynarin	8.90	94.9	7.60
Echinacoside	53.3	91.7	9.50
Cichoric acid	51.9	90.0	3.50
Overall recovery for all analytes, %		99.8	—

[a]125 mg *P. quinquefolius* L. (North American ginseng) powder.

Table 7

a. Powdered extract negative control spike recovery results (50% level)[a]

Level 1 (0.9%, w/w, total phenolics)			
Analyte	**Spiked concentration, µg/mL**	**Recovery, %**	**RSD, %**
Caftaric acid	0.44	103	0.37
Chlorogenic acid	0.97	100	0.86
Cynarin	0.49	100	3.77
Echinacoside	23.9	98.5	0.46
Cichoric acid	18.7	98.3	0.39

b. Powdered extract negative control spike recovery results (100% level)[a]

Level 2 (1.8%, w/w, total phenolics)			
Analyte	**Spiked concentration, µg/mL**	**Recovery, %**	**RSD, %**
Caftaric acid	0.88	98	4.44
Chlorogenic acid	1.93	97	0.77
Cynarin	0.97	103	1.40
Echinacoside	47.7	99.1	0.82
Cichoric acid	37.4	99.4	0.37

c. Powdered extract negative control spike recovery results (200% level)[a]

Level 3 (3.8%, w/w, total phenolics)			
Analyte	**Spiked concentration, µg/mL**	**Recovery, %**	**RSD, %**
Caftaric acid	1.75	101	3.79
Chlorogenic acid	3.87	99.5	1.03
Cynarin	1.95	103	1.32
Echinacoside	105	98.3	0.35
Cichoric acid	74.8	102	0.75

[a] 125 mg of 99% maltodextran, 1% magnesium stearate.