JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Seasonal Variations in the Concentrations of Lipophilic Compounds and Phenolic Acids in the Roots of *Echinacea purpurea* and *Echinacea pallida*

Maria O. Thomsen,*[,†] Xavier C. Fretté,[‡] Kathrine B. Christensen,[‡] Lars P. Christensen,[‡] and Kai Grevsen[†]

[†]Department of Food Science, Faculty of Science and Technology, Aarhus University, Kirstinebjergvej 10, DK-5792 Aarslev, Denmark

[‡]Institute of Chemical Engineering, Biotechnology and Environmental Technology, Faculty of Engineering, University of Southern Denmark, Niels Bohrs Allé 1, DK-5230 Odense, Denmark

ABSTRACT: Roots of *Echinacea purpurea* and *Echinacea pallida* cultivated for 4 years in a North European climate were analyzed for seasonal variations in the concentrations of lipophilic constituents (alkamides, ketoalkenes, and ketoalkynes) and phenolic acids by harvesting five times during 1 year to establish the optimal time for harvest. A total of 16 alkamides, three ketoalkenes, two ketoalkynes, and four phenolic acids (echinacoside, cichoric acid, caftaric acid, and chlorogenic acid) were identified in aqueous ethanolic (70%) extracts by liquid chromatography−mass spectrometry and quantified by reverse-phase high-performance liquid chromatography. The major alkamides in the roots of *E. purpurea* were at their lowest concentration in the middle of autumn and early winter, and the total concentration of lipophilic compounds in *E. pallida* showed the same pattern. Moreover, all of the major phenolic acids in *E. purpurea* were at their highest concentrations in spring. The optimal harvest time in spring is in contrast to normal growing guidelines; hence, this specific information of seasonal variations in the concentrations of lipophilic and phenolic compounds in *E. purpurea* and *E. pallida* is valuable for research, farmers, and producers of medicinal preparations.

KEYWORDS: *Echinacea spp., plant development, harvest time, cultivation, phenolic acids, ketoalkenes, ketoalkynes, alkamides*

INTRODUCTION

The genus *Echinacea* Moench (Heliantheae, Asteraceae) originates from North America, and Native Americans have used the roots of *Echinacea* species for the treatment of a wide variety of diseases. In Europe and North America, various preparations of *Echinacea* are popular herbal medicines for preventing and treating the common cold, flu, upper respiratory tract infections, and a number of other diseases.[1−3] The plants are cultivated in Europe, the United States, and Canada for use as phytomedicines, dietary supplements, and natural health products, respectively. Commercial medicinal preparations of *Echinacea* are primarily made from the roots and aerial parts of *Echinacea purpurea* (L.) Moench, *Echinacea pallida* var. *pallida* (Nutt.), and *Echinacea angustifolia* (DC.) Hell. They are considered to have potent immunomodulatory effects as well as other pharmacological activities such as anti-inflammatory activity. Several major groups of active compounds in *Echinacea* species have been proposed, which include lipophilic compounds (alkamides, ketoalkenes, and ketoalkynes), phenolic acids, glycoproteins, and polysaccharides.[2−6]

Alkamides are the most studied group among the bioactive compounds in *Echinacea* species, and comprehensive investigations of *Echinacea* plants and products have resulted in the characterization of more than 25 alkamides, of which several are *E*/*Z* isomeric pairs. Approximately 2/3 of the alkamides found in *Echinacea* species are isobutylamides of C_{11}−C_{16} long-chain unsaturated fatty acids, whereas the rest are 2-methylbutylamides (Figure 1). The alkamides are most abundant in the roots of *E. purpurea* and *E. angustifolia*. Significantly lower amounts have been found in the aerial parts of these plants and in *E. pallida* roots.[7] Alkamides have been shown to induce anti-inflammatory responses in macrophages by inhibiting prostaglandin E_2 production[8] and to inhibit cyclooxygenase-2 (COX-2) activity.[9,10] In addition, alkamides have shown good bioavailability following oral administration of herbal medicine in humans.[11] The relatively high bioavailability of alkamides, which are most likely due to their lipophilic properties, suggests that these compounds may play a significant role in the immunostimulatory effect of *Echinacea* preparations.

Although the roots of *E. pallida* do not contain high amounts of alkamides, they are rich in highly lipophilic ketoalkenes and ketoalkynes (Figure 1).[5,7] Recent investigations of *n*-hexane extracts of *E. pallida* roots using bioassay-guided fractionation have shown that these compounds are responsible for the cytotoxicity on cancer cells and further demonstrated the potential bioavailability of these lipophilic compounds in humans when taken orally.[5] These results suggest that ketoalkenes and ketoalkynes may play an important role in the bioactivity of *Echinacea* preparations based on this plant.

Several phenolic acids, including cichoric acid and echinacoside (Figure 2), have been isolated from hydrophilic fractions of *Echinacea* extracts and are well-known for their antioxidant activity.[6] It has been reported that it is possible to distinguish between *E. purpurea* and *E. pallida* root material solely by their contents of echinacoside.[12] Echinacoside is the main phenolic

Received: July 31, 2012
Revised: November 1, 2012
Accepted: November 26, 2012
Published: November 26, 2012

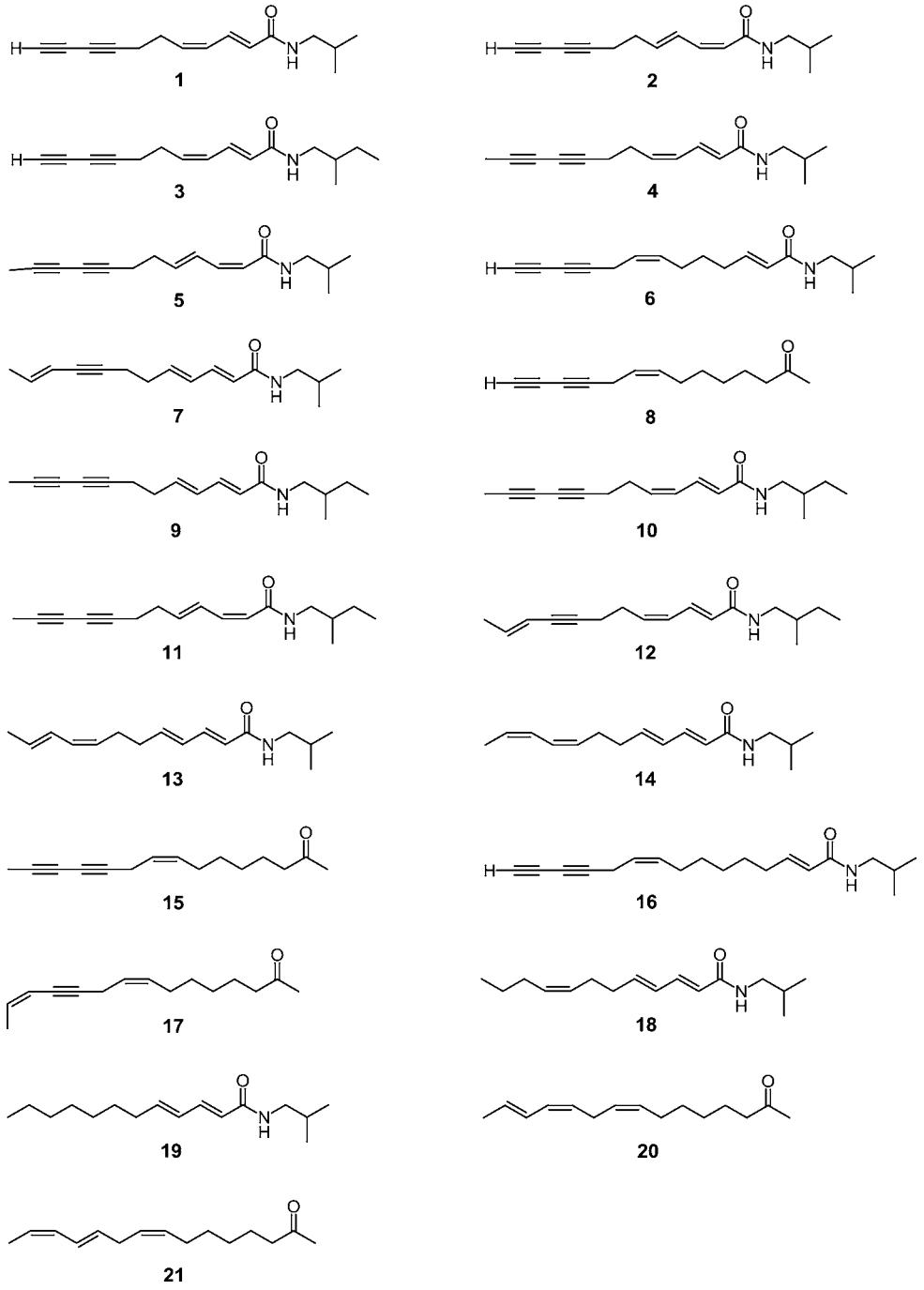
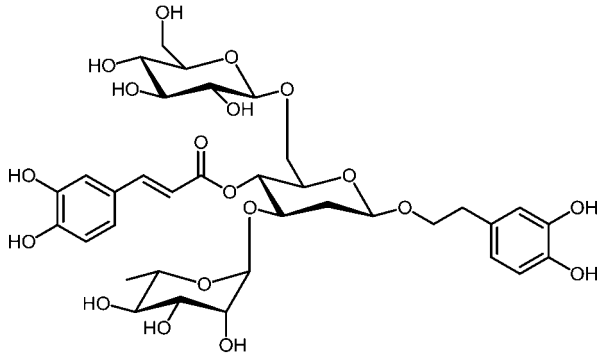


Figure 1. Chemical structures of lipophilic compounds (alkamides, ketoalkenes, and ketoalkynes) found in *E. purpurea* (**1**−**7**, **9**−**14**, **18**, and **19**) and *E. pallida* (**2**, **5**, **8**, **15**−**17**, **20**, and **21**) roots. Compound numbering is based on the elution order on RP-HPLC (Table 1).

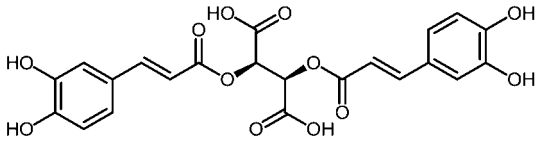
acid in *E. pallida* but is absent or occurs in relatively low concentrations in *E. purpurea*. Cichoric acid, which is the major phenolic acid in *E. purpurea*, has been shown to have potential immunostimulatory properties. However, as compared to the alkamides, it is probably not an important contributor to the modulation of the immune response in vivo due to its relatively low bioavailability.[9] Echinacoside on the other hand does not seem to possess immunostimulatory activity[13] but has shown cytoprotective activity in an animal model of Parkinson's disease.[14]

The concentration of secondary metabolites in plants may vary significantly throughout the year and with the developmental stages of the plant. Thus, it is important to establish the optimal time for harvest with respect to yield and concentration of lipophilic compounds and phenolic acids in *Echinacea* species, as their medicinal effects depend on the concentration of these compounds. Most investigations on compounds in roots of these perennials have been made on material from *Echinacea* plants harvested before or just after the first growing season;[12,15,16] however, most medicinal plant producers are harvesting *Echinacea* roots after the third or fourth growing season[17] when root yields are higher and flowers and aerial herbals have already been harvested and used for medicinal preparations for 2 or 3 years.

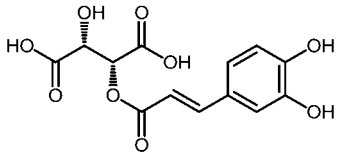
The total concentration of alkamides and the major phenolic acid, cichoric acid, in roots of *E. purpurea* decreases from the



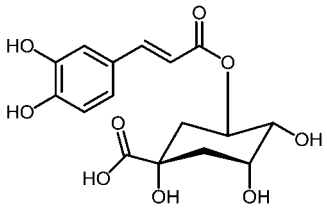
Echinacoside



Cichoric acid



Caftaric acid



Chlorogenic acid

Figure 2. Chemical structures of phenolic acids found in *E. purpurea* and *E. pallida* roots.

flower bud stage to the senescent stage.[15,16,18] However, few investigations have been conducted on the seasonal variations of compounds in roots of *Echinacea* species, and to our knowledge, no one has investigated the concentration of the individual lipophilic compounds during a whole cultivation year in *E. purpurea* or *E. pallida*. Liu et al.[15] investigated the concentration of phenolic acids in 1 year old roots of *E. purpurea* from March to November in Changsha, China, and they concluded that both the cichoric acid and the total concentration of phenolic acids decrease during spring/summer until the aerial parts flower (July), followed by a small increase in the concentration during autumn (July to November).

We hypothesized that the concentration and composition of secondary metabolites in roots of *Echinacea* species will vary with the developmental stages of the plant. This knowledge is important for the production of plant material for medicinal preparations and for research on their bioactivity. Therefore, we investigated the variation in the concentration of phenolic acids and lipophilic compounds in roots of *E. purpurea* and *E. pallida* plants in their third and fourth growth years, respectively, over a whole cultivation year in a North European temperate climate.

MATERIALS AND METHODS

Solvents and Chemicals. Acetonitrile (MeCN), methanol (MeOH), and ethanol (EtOH, 96%) [high-performance liquid chromatography (HPLC) grade for chromatography] were obtained from Fisher Scientific (Roskilde, Denmark). Triflouroacetic acid (TFA) of reagent quality was obtained from Prolabo (Leuven, Belgium). Dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide (purity >95%) and chlorogenic acid (purity >98%) were obtained from Phytolab GmbH & Co. KG (Vestenbergsgreuth, Germany), and cichoric acid (purity >95%), caftaric acid (purity >97%), and echinacoside (purity >98%) were obtained from Sigma-Aldrich (Steinheim, Germany). Milli-Q water was purified locally on own equipment on a SG Ultra Clear Basis (Barsbüttel, Germany).

Plant Material. *E. pallida* var. *pallida* (Nutt) and *E. purpurea* (L.) Moench plants were propagated from seeds purchased from the seed companies Impecta (Julita, Sweden) and Rieger Hoffmann (Blaufelden-Raboldshausen,Germany), respectively. The plantlets were raised in a greenhouse and transplanted in a sandy loam soil (Aarslev, Denmark; coordinates: 55.3° N, 10.5° E) in early June 2006 and 2007 (*E. pallida* and *E. purpurea*, respectively) with a plot size of 6 m^2 each and a plant density of 9 plants m^{-2}. The crop was fertilized with 50 kg N/ha, 8 kg P/ha, and 25 kg K/ha every spring. The flowers and 20 cm of the top of the plants were cut off every year (July/August) to mimic flower harvest.

From early winter 2009 to autumn 2010, samples of five roots of *E. pallida* and seven roots of *E. purpurea* were harvested five times. Root sampling days were as follows: December 15, 2009; March 23, 2010; May 25, 2010; August 13, 2010; and October 29, 2010). The five harvest dates were arranged with the purpose to represent the different periods and developmental stages in a cultivation year (Figure 3). The first harvest (December 15, 2009) was in early winter, just before the

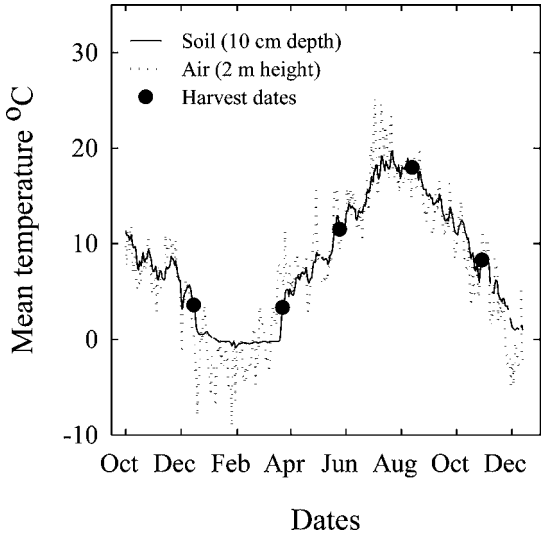


Figure 3. Daily air and soil temperatures (in °C) from October 2009 to December 2010 (Aarslev, Denmark; coordinates: 55.3° N, 10.5° E). Root sampling days were as follows: winter (December 15, 2009), root dormancy; early spring (March 23, 2010), frost-free soil, first signs of aerial parts; late spring (May 25, 2010), aerial parts growing fast; summer (August 13, 2010), flowers in bloom and starting to wilt; and midautumn (October 29, 2010), aerial parts wilting.

air frost period started; the aerial parts were wilted, and the roots were dormant. The second harvest (March 23, 2010) was in early spring, just after frost had left the soil, and the first signs of new aerial shoots were just visible. The third harvest (May 25, 2010) was in late spring, in the middle of the period with increasing temperatures, and the aerial parts were visible and growing fast. The fourth harvest (August 13, 2010) was during summertime; temperatures in both air and soil were high, the flowers were in bloom, and some had just started setting seeds. The last harvest (October 29, 2010) was in midautumn with fast declining temperatures, and the aerial parts were wilting and entering the senescent stage. The roots were washed, cut into pieces (<2 cm), and frozen instantly at −20 °C until further processing.

The Danish Meteorological Institute (DMI) monitored air temperature (at a height of 2 m) and soil temperature (at a depth of 10 cm) as a daily average (log at hourly intervals) in the experimental period (Figure 3). The average air temperature during the 12 months of root sampling in the experiment (December 1, 2009, to November 30, 2010) was 7.8 °C with a maximum of 25.2 °C in July and a minimum of −9.1 °C in January (Figure 3). The average soil temperature in the same period was 8.2 °C with a maximum of 19.7 °C in July and a minimum of −0.9 °C in January. There were approximately 80 days between each harvest, and only the second harvest was postponed to secure a root sample from frost-free soil.

Extraction Procedure. Frozen root material was lyophilized at −5 °C to total dryness (Crist Gamma 1−20), crushed, and homogenized using a Retsch ZM1 laboratory mill (Burladingen, Germany) to obtain a particle size of <500 μm, then vacuum-packed, and stored frozen (−20 °C) until analysis. The extraction of bioactive constituents from root material followed Stuart and Wills[19] methodology with modifications. Lipophilic constituents and phenolic acids were extracted from 1.0 g of ground plant root material with 15 mL of EtOH−H_2O (70:30) under shaking for 2 h. After extraction, the extract was filtered through filter paper (AGF 118, 15 cm, 15 μm) and washed with 2 mL of EtOH−H_2O (70:30). The exact weight of extract was noted and used to calculate the concentration. Extractions were replicated three times. The described extraction procedure ensured extraction of >95% of lipophilic compounds (alkamides, ketoalkenes, and ketoalkynes) and phenolic acids as demonstrated by successive extraction experiments of the same plant material.

Quantification of Alkamides, Ketoalkenes, and Ketoalkynes. Alkamides, ketoalkenes, and ketoalkynes were quantified in root extracts of *E. purpurea* and *E. pallida* by reverse-phase (RP) HPLC on a Dionex UltiMate 3000 HPLC system (Dionex Denmark A/S) consisting of an HPLC pump (LPG-3400SD), an autosampler [WPS-3000(T)SL Analytical], a column compartment (TCC-3000SD), and a diode array detector (DAD-3000) operating from 190 to 800 nm. Alkamides, ketoalkenes, and ketoalkynes were monitored at 210 and 254 nm, and UV spectra were recorded between 190 and 600 nm. Separations were obtained on a Purospher STAR RP-18 end-capped column (5 μm, 250 mm × 4.6 mm i.d.) from Merck (Hellerup, Denmark) equipped with a matching precolumn. The mobile phase consisted of H_2O (A) and MeOH (B), and separations were performed using the following solvent gradient: 0 (50% B), 35 (80% B), 41 (80% B), 45 (50% B), and 55 min (50% B). All changes of solvents were linear programmed. The column temperature was 40 °C, the flow rate was 1 mL/min, and the injection volume was 50 μL. Samples were filtered (Q-max syringe filters, nylon, 0.45 μm) prior to HPLC analysis. Identification of alkamides, ketoalkenes, and ketoalkynes was performed by liquid chromatography−diode array detection−atmospheric pressure chemical ionization−tandem mass spectrometry (LC-DAD-APCI-MS/MS) as described in Identification of Alkamides, Ketoalkenes, and Ketoalkynes. The alkamides **1**−**5**, **7**, **9**−**14**, **18**, and **19** were quantified using an external calibration curve of the authentic standard dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide (**13**/**14**) dissolved in 100% MeOH. Quantification of alkamides **6** and **16** as well as the ketoalkynes **8** and **15** was based on an external calibration curve of an authentic standard of compound **8**, and the quantification of compounds **17**, **20**, and **21** was based on an external calibration curve of an authentic standard of the ketoalkenes **20** and **21** (inseparable mixture). Calibration curves of all lipophilic standards were in the concentration ranges occurring in the plant material with correlation coefficients above R^2 > 0.99. Dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide (**13**/**14**) was used to validate the analytical HPLC method used for quantification of alkamides, ketoalkenes, and ketoalkynes in root extracts of *E. purpurea* and *E. pallida*. Mean recovery rates (∼accuracy) for dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide were >98% with a relative standard deviation <5% and were determined by spiking a known amount of the standard to *E. purpurea* root extract samples. Repeatability was determined by six injections of an *E. purpurea* extract sample in 1 day (intraday variability), and intermediate precision was determined by analyzing an *E. purpurea* extract sample on three different days (interday variability). The overall intraday and interday variations were found to be less than 5%.

Isolation of Standards for Quantification of Lipophilic Constituents in *E. purpurea* and *E. pallida* Roots. The standards used for quantification were not all commercially available, that is, compounds **8** and **20** and **21** were isolated from an *E. pallida* root extract by semipreparative HPLC. An UltiMate 3000 semipreparative LC system (Dionex Denmark A/S) consisting of an HPLC pump (HPG-3200P), autosampler (WPS-3000SL Semiprep), and a DAD (DAD-3000 RS) operating from 190 to 800 nm. Compounds were monitored at 210 and 254 nm, and UV spectra were recorded between 190 and 600 nm. Separations were performed on an Uptisphere 120 Å C18-ODB HPLC column (5 μm, 250 mm × 21.2 mm) from Interchim (Mountluçon Cedex, France), at 25 °C, using a linear-programmed solvent gradient with 0.05% TFA in H_2O (A) and 0.05% TFA in MeCN (B): 0 (50% B), 45 (100% B), 60 (100% B), and 70 min (50% B). The flow rate was 5 mL/min, and the injection volume was 1.5 mL (60 mg/mL) per separation. Compounds **8** and **20** and **21** eluted at approximately 39 and 50 min, respectively, and finally 3.5 mg of **8** and 4.5 mg of **20** and **21** were obtained as colorless oils with a purity >98%, dissolved in 100% MeOH, and stored at −80 °C until further use. The identity and purity of the isolated compounds were determined by LC-DAD-APCI-MS/MS (see Identification of Alkamides, Ketoalkenes, and Ketoalkynes).

Identification of Alkamides, Ketoalkenes, and Ketoalkynes. Alkamides, ketoalkenes, and ketoalkynes were identified by LC-DAD-APCI-MS/MS. LC-MS data were obtained using a Thermo Scientific LTQ XL (ESI-2D-iontrap) operated in APCI positive mode and hyphenated with an Accela HPLC Pump and a DAD operating from 200 to 600 nm. Compounds were monitored at 210 and 254 nm, and UV spectra were recorded between 200 and 600 nm. Settings for the mass spectrometer were 50, 5, and 5 (arbitrary units) for sheath, auxiliary, and sweep gas flow rates (N_2), respectively, a vaporizer temperature of 450 °C, a discharge current of 5 μA, a capillary temperature of 275 °C, a capillary voltage of 16 V, a tube lens of 35 V, and AGC target settings of 3×10^4 and 1×10^4 for full MS and MS/MS, respectively. Separations were obtained on a LiChrospher RP18 (5 μm; 250 mm × 4.6 mm, 100 Å) column from Phenomenex (Allerød, Denmark). The mobile phase consisted of 0.1% formic acid in H_2O (A) and 0.1% formic acid in MeOH (B), and separations were performed using the following linear-programmed solvent gradient: 0 (50% B), 35 (80% B), 45 (80% B), 50 (50% B), and 60 min (50% B). The flow was 1 mL/min, the temperature was 35 °C, and the injection volume was 10 μL.

Quantification of Phenolic Acids. Phenolic acids (caftaric acid, cichoric acid, chlorogenic acid, and echinacoside) were quantified in root extracts of *E. purpurea* and *E. pallida* by HPLC-DAD on a Dionex UltiMate 3000 HPLC system (Dionex Denmark A/S). Compounds were monitored at 330 nm, and UV spectra were recorded between 190 and 600 nm. Separations were obtained on a Purospher STAR RP-18 end-capped column (5 μm, 250 mm × 4.6 mm i.d.) equipped with a matching precolumn. The mobile phase consisted of 0.01% TFA in H_2O (A) and MeCN (B), and separations were performed using the following solvent gradient: 0 (5% B), 20 (25% B), 23 (100% B), 33 (100% B), 38 (5% B), and 50 min (5% B). All changes of solvents were linear programmed. The column temperature was 40 °C, the flow rate was 1 mL/min, and the injection volume was 50 μL. Samples were filtered (Q-max syringe filters, nylon, 0.45 μm) prior to

HPLC analysis. Identification of the individual phenolic acids in the extracts was performed by spiking with authentic standards (dissolved in 100% MeOH), and quantification was done using external calibration curves of authentic standards in the concentration ranges occurring in the plant material (correlation coefficient, $R^2 > 0.99$ for all phenolic acids). Cichoric acid was used to validate the analytical HPLC method described above. Mean recovery rates (~accuracy) for cichoric acid were >98% with a relative standard deviation <5% and were determined by spiking a known amount of this authentic standard to *E. purpurea* root extract samples. Repeatability was determined by six injections of an *E. purpurea* extract sample in 1 day (intraday variability), and intermediate precision was determined by analyzing an *E. purpurea* extract sample on three different days (interday variability). The overall intraday and interday variations were found to be less than 5%.

Identification of Phenolic Acids. Phenolic acids were identified by LC-DAD-APCI-MS/MS. LC-MS data were obtained using a Thermo Scientific LTQ XL (ESI-2D-iontrap) operated in APCI negative mode and hyphenated with an Accela HPLC Pump and a DAD operating from 200 to 600 nm. Compounds were monitored at 280 and 330 nm, and UV spectra were recorded between 200 and 600 nm. Settings for the mass spectrometer were 50, 5, and 5 (arbitrary units) for sheath, auxiliary, and sweep gas flow rates (N_2), respectively, a vaporizer temperature of 450 °C, a discharge current of 15 μA, a capillary temperature of 275 °C, a capillary voltage of −23 V, a tube lens of −110 V, and AGC target settings of 3×10^4 and 1×10^4 for full MS and MS/MS, respectively. Separations were obtained on a LiChrospher RP18 (5 μm; 250 mm × 4.6 mm, 100 Å) column. The mobile phase consisted of 0.01% TFA in H_2O (A) and MeCN (B), and separations were performed using the following linear-programmed solvent gradient: 0 (5% B), 20 (25% B), 23 (100% B), 33 (100% B), 38 (5% B), and 50 min (5% B). The column temperature was 40 °C, the flow rate was 1 mL/min, and the injection volume was 50 μL. The MS spectra of the identified phenolic acids (caftaric acid, cichoric acid, chlorogenic acid, and echinacoside) were compared with those of commercial available standards.

Statistics. The statistical design of the field experiment was as follows: two species ($n = 2$), five harvest dates ($n = 5$), and seven or five replicates (*E. purpurea* $n = 7$ plants, *E. pallida* $n = 5$ plants). Analyses of variance (ANOVA) were performed using the general linear models (GLM) procedure in the Statistical Analysis System (SAS Institute Inc., Cary, NC). If the statistical analysis (F test) showed significant effect ($p < 0.05$) of treatment (harvest date), means in tables were separated with the least significant difference ($LSD_{0.05}$) test ($p < 0.05$).

RESULTS AND DISCUSSION

Analysis of Alkamides, Ketoalkenes, and Ketoalkynes and Phenolic Acids in Roots of *Echinacea* Species. The separations of alkamides in extracts of *E. purpurea* roots and alkamides, ketoalkenes, and ketoalkynes in extracts of *E. pallida* roots by analytical RP-HPLC are shown in Figures 4 and 5, respectively. In total, 16 alkamides, two ketoalkenes, and three ketoalkynes were identified based on their mass and UV spectra (Table 1). By using LC-DAD-APCI-MS and MS/MS, it was possible to obtain the quasi-molecular precursor ion and product ions of the alkamides, which, in combination with UV data and retention time (t_R), made their identification very reliable. The identification of the alkamides was furthermore confirmed by comparing the MS, MS/MS, and UV data with literature.[7,20−23] The identification of ketoalkenes and ketoalkynes in *E. pallida* was performed by comparing the UV spectra and elution order on RP-HPLC published for these compounds[7,24] and was furthermore confirmed by MS and MS/MS (Table 1).

In the present investigation, 15 alkamides could be identified in the *E. purpurea* root material evaluated. In accordance with

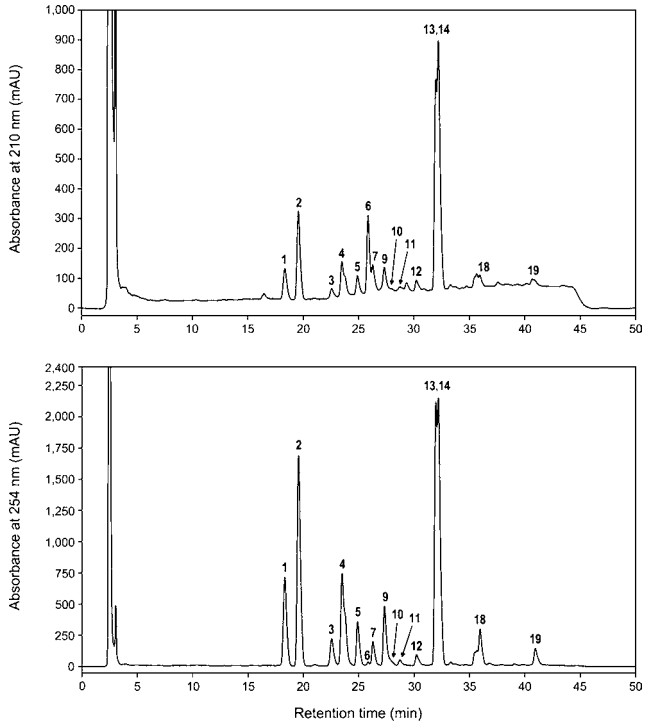


Figure 4. Separation of alkamides in a root extract of *E. purpurea* by analytical RP-HPLC at 210 (above) and 254 nm (below). Peak numbering on the HPLC chromatogram refers to the compound numbers given in Figure 1 and Table 1. The chromatogram is from the analysis of a root harvested August 13, 2010.

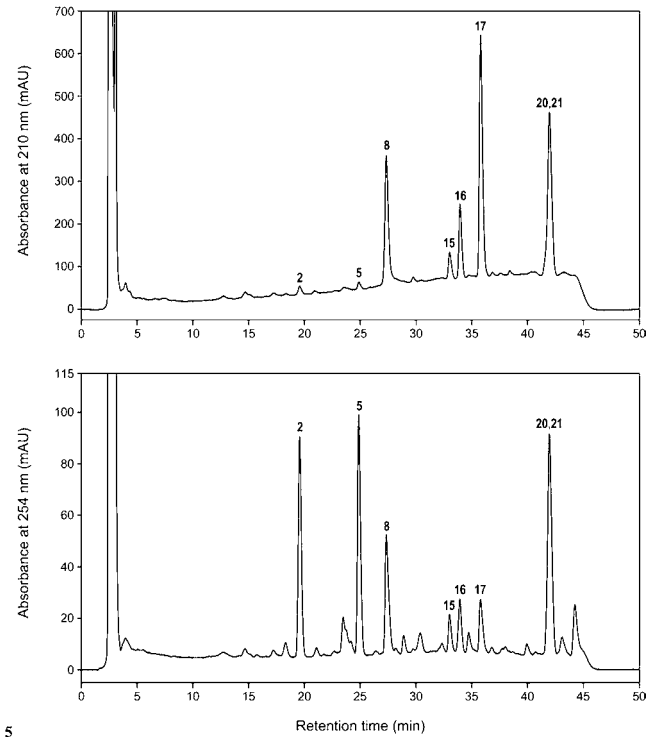


Figure 5. Separation of alkamides, ketoalkenes, and ketoalkynes in a root extract of *E. pallida* by analytical RP-HPLC at 210 (above) and 254 nm (below). Peak numbering on the HPLC chromatogram refers to the compound numbers given in Figure 1 and Table 1. The chromatogram is from the analysis of a root harvested October 29, 2010.

Table 1. Lipophilic Compounds (Alkamides, Ketoalkenes, and Ketoalkynes) Identified in *E. purpurea* and *E. pallida* Root Extracts by LC-DAD-APCI-MS/MS (APCI; Positive Mode)

t_R (min)[a]	compd/ peak no.	compd name[b]	present in *E. purpurea*	present in *E. pallida*	UV λ_{max} (nm)	quasi-molecular precursor ion (m/z)	product ions (m/z)
18.5	**1**	undeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide	×		262	230 $[M + H]^+$	202 (6), 188 (3) $[M + H - C_3H_6]^+$, 174 (35) $[M + H - C_4H_8]^+$, 157 (20) $[M + H - C_4H_{11}N]^+$, 146 (11), 131 (100), 129 (63) $[M + H - C_5H_{11}NO]^+$, 116 (24), 103 (6) $[M + H - C_7H_{13}NO]^+$, 91 (58)
19.8	**2**	undeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide	×	×	260	230 $[M + H]^+$	202 (19), 188 (25) $[M + H - C_3H_6]^+$, 174 (85) $[M + H - C_4H_8]^+$, 157 (30) $[M + H - C_4H_{11}N]^+$, 146 (40), 131 (100), 129 (58) $[M + H - C_5H_{11}NO]^+$, 116 (6), 103 (4) $[M + H - C_7H_{13}NO]^+$, 91 (18)
22.8	**3**	undeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutyl-amide	×		262	244 $[M + H]^+$	216 (6) $[M + H - C_2H_4]^+$, 174 (51) $[M + H - C_5H_{10}]^+$, 157 (19) $[M + H - C_5H_{13}N]^+$, 146 (10), 131 (100), 129 (45) $[M + H - C_6H_{13}NO]^+$, 116 (11), 91 (35) $[M + H - C_9H_{15}NO]^+$
23.7	**4**	dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide	×		263	244 $[M + H]^+$	216 (14), 202 (4) $[M + H - C_3H_6]^+$, 188 (48) $[M + H - C_4H_8]^+$, 171 (24) $[M + H - C_4H_{11}N]^+$, 145 (100), 143 (26) $[M + H - C_5H_{11}NO]^+$, 117 (42) $[M + H - C_7H_{13}NO]^+$, 91 (4) $[M + H - C_9H_{15}NO]^+$
25.1	**5**	dodeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide	×	×	260	244 $[M + H]^+$	216 (6), 202 (4) $[M + H - C_3H_6]^+$, 188 (28) $[M + H - C_4H_8]^+$, 171 (68) $[M + H - C_4H_{11}N]^+$, 145 (100), 143 (44) $[M + H - C_5H_{11}NO]^+$, 117 (91) $[M + H - C_7H_{13}NO]^+$, 91 (4) $[M + H - C_9H_{15}NO]^+$
26.0	**6**	trideca-2*E*,7*Z*-diene-10,12-diynoic acid isobutylamide	×		210	258 $[M + H]^+$	230 (15), 216 (9) $[M + H - C_3H_6]^+$, 202 (100) $[M + H - C_4H_8]^+$, 185 (36) $[M + H - C_4H_{11}N]^+$, 159 (44), 157 (53) $[M + H - C_5H_{11}NO]^+$, 131 (62) $[M + H - C_7H_{13}NO]^+$, 129 (35), 117 (68) $[M + H - C_8H_{15}NO]^+$, 103 (14) $[M + H - C_9H_{17}NO]^+$, 91 (15)
26.6	**7**	dodeca-2*E*,4*E*,10*E*-triene-8-ynoic acid isobutylamide	×		232, 263	246 $[M + H]^+$	190 (8) $[M + H - C_4H_8]^+$, 173 (50) $[M + H - C_4H_{11}N]^+$, 147 (100), 145 (55) $[M + H - C_5H_{11}NO]^+$, 131 (34), 119 (22) $[M + H - C_7H_{13}NO]^+$, 105 (22), 91 (8)
27.5	**8**	tetradeca-8*Z*-ene-11,13-diyn-2-one		×	198, 231, 244, 256	203 $[M + H]^+$	185 (100) $[M + H - H_2O]^+$, 145 (43) $[M + H - H_2O - C_3H_4]^+$, 133 (13) $[M + H - C_4H_6O]^+$, 119 (11) $[M + H - C_5H_8O]^+$, 105 (9) $[M + H - C_6H_{10}O]^+$
27.8	**9**	dodeca-2*E*,4*E*-diene-8,10-diynoic acid 2-methylbutylamide[c]	×		263	258 $[M + H]^+$	230 (15) $[M + H - C_2H_4]^+$, 202 (5) $[M + H - C_4H_8]^+$, 188 (49) $[M + H - C_5H_{10}]^+$, 171 (23) $[M + H - C_5H_{13}N]^+$, 145 (100), 143 (25) $[M + H - C_6H_{13}NO]^+$, 130 (6), 117 (39) $[M + H - C_8H_{15}NO]^+$, 105 (5), 91 (4) $[M + H - C_{10}H_{17}NO]^+$
28.2	**10**	dodeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide[c]	×		260	258 $[M + H]^+$	230 (16) $[M + H - C_2H_4]^+$, 202 (6) $[M + H - C_4H_8]^+$, 188 (40) $[M + H - C_5H_{10}]^+$, 171 (25) $[M + H - C_5H_{13}N]^+$, 145 (100), 143 (30) $[M + H - C_6H_{13}NO]^+$, 130 (8), 117 (43) $[M + H - C_8H_{15}NO]^+$, 105 (6), 91 (5) $[M + H - C_{10}H_{17}NO]^+$
29.1	**11**	dodeca-2*Z*,4*E*-diene-8,10-diynoic acid 2-methylbutylamide	×		260	258 $[M + H]^+$	230 (6) $[M + H - C_2H_4]^+$, 202 (5) $[M + H - C_4H_8]^+$, 188 (28) $[M + H - C_5H_{10}]^+$, 171 (66) $[M + H - C_5H_{13}N]^+$, 145 (100), 143 (44) $[M + H - C_6H_{13}NO]^+$, 130 (6), 117 (85) $[M + H - C_8H_{15}NO]^+$, 105 (6), 91 (3) $[M + H - C_{10}H_{17}NO]^+$
30.6	**12**	dodeca-2*E*,4*Z*,10*E*-triene-8-ynoic acid 2-methylbutylamide	×		232, 263	260 $[M + H]^+$	232 (3) $[M + H - C_4H_8]^+$, 190 (13) $[M + H - C_5H_{10}]^+$, 173 (53) $[M + H - C_5H_{13}N]^+$, 147 (100), 145 (56) $[M + H - C_6H_{13}NO]^+$, 131 (31), 119 (23) $[M + H - C_8H_{15}NO]^+$, 105 (22), 91 (8)
32.2	**13**, **14**	dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide	×		236, 261	248 $[M + H]^+$	192 (5) $[M + H - C_4H_8]^+$, 175 (29) $[M + H - C_4H_{11}N]^+$, 167 (8) $[M + H - C_6H_{10}]^+$, 166 (16), 149 (100), 147 (25) $[M + H - C_5H_{11}NO]^+$, 142 (49), 133 (14), 121 (13) $[M + H - C_7H_{13}NO]^+$, 107 (19), 100 (8), 93 (15)
33.1	**15**	pentadeca-8*Z*-ene-11,13-diyn-2-one		×	198, 231, 244, 256	217 $[M + H]^+$	199 (100) $[M + H - H_2O]^+$, 159 (27) $[M + H - H_2O - C_3H_4]^+$, 143 (11) $[M + H - C_4H_{10}O]^+$, 119 (5) $[M + H - C_6H_{10}O]^+$
34.1	**16**	pentadeca-2*E*,9*Z*-diene-12,14-diynoic acid isobutylamide		×	210	286 $[M + H]^+$	230 (100) $[M + H - C_4H_8]^+$, 213 (11) $[M + H - C_4H_{11}N]^+$, 185 (39) $[M + H - C_5H_{11}NO]^+$, 171 (38), 157 (17), 145 (43) $[M + H - C_8H_{15}NO]^+$, 131 (26) $[M + H - C_9H_{17}NO]^+$, 117 (22) $[M + H - C_{10}H_{19}NO]^+$, 105 (11), 95 (11)
35.9	**17**	pentadeca-8*Z*,13*Z*-dien-11-yn-2-one		×	225	219 $[M + H]^+$	201 (100) $[M + H - H_2O]^+$, 173 (17), 161 (13) $[M + H - H_2O - C_3H_4]^+$, 145 (7) $[M + H - C_4H_{10}O]^+$, 135 (6), 121 (5) $[M + H - C_6H_{10}O]^+$
36.1	**18**	dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide	×		261	250 $[M + H]^+$	194 (11) $[M + H - C_4H_8]^+$, 177 (21) $[M + H - C_4H_{11}N]^+$, 167 (100) $[M + H - C_6H_{10}]^+$, 152 (11), 149 (15) $[M + H - C_5H_{11}NO]^+$, 135 (12), 121 (8), 117 (6), 109 (23), 107 (14), 95 (38), 93 (12)
40.9	**19**	dodeca-2*E*,4*E*-dienoic acid isobutylamide	×		262	252 $[M + H]^+$	210 (6) $[M + H - C_3H_6]^+$, 196 (100) $[M + H - C_4H_8]^+$, 179 (85) $[M + H - C_4H_{11}N]^+$, 168 (15) $[M + H - C_6H_{10}]^+$, 161 (39), 154 (19) $[C_9H_{16}NO]^+$, 151 (8) $[M + H - C_5H_{11}NO]^+$, 137 (18), 133 (16), 119 (31), 109 (23), 105 (21), 97 (38), 95 (80)
42.1	**20**, **21**	pentadeca-8*Z*,11*Z*,13*E*-trien-2-one; pentadeca-8*Z*,11*E*,13*Z*-trien-2-one		×	233	221 $[M + H]^+$	203 (100) $[M + H - H_2O]^+$

[a] t_R = retention time on analytical RP-HPLC. [b] Identification based on t_R and UV, LC-MS, and MS/MS spectra. *E*/*Z* stereochemistry is indicated here in accordance with existing literature,[7,20−24] although without conformational NMR spectra, it is not possible to conclusively distinguish between *E* and *Z* isomers, and the identification is therefore considered to be tentative. [c] New *E*/*Z* isomer of the known dodeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide (**10**). Tentative identification based on t_R, UV, LC-MS, and MS/MS.

dx.doi.org/10.1021/jf303292t | J. Agric. Food Chem. 2012, 60, 12131−12141

previous investigations,[7,12,20−24] the major alkamides were undeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide (**1**, t_R = 18.5 min), undeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide (**2**, t_R = 19.8 min), dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide (**4**, t_R = 23.7 min), and dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide (**13** and **14**, t_R = 31.2 min). On the other hand, differences in the profile among the minor alkamides were observed as compared to other investigations of *E. purpurea* roots.[7,20−23] For example, the alkamides undeca-2*E*-ene-8,10-diynoic acid isobutylamide and undeca-2*Z*,4*E*-diene-8,10-diynoic acid 2-methylbutylamide previously reported to be present in *E. purpurea* roots were not detected in the present investigation.

Furthermore, the appearance of three alkamides eluting at t_R = 27.8 min (**9**), t_R = 28.2 min (**10**), and t_R = 29.1 min (**11**) (Figure 4 and Table 1) with the same quasi-molecular precursor ion 258 $[M + H]^+$ and MS/MS fragmentation patterns as well as similar UV spectra (Table 1) indicated the presence of three alkamide isomers and, thus, an alkamide profile not previously observed in *E. purpurea* roots. The fragment at *m*/*z* 188 corresponds to the loss of a 2-methylbutyl group (−70 Da), and the fragment *m*/*z* 171 corresponds to the loss of the 2-methylbutylamine (−87 Da), which clearly indicated that the alkamides **9**−**11** are 2-methylbutylamides (Figure 6).[20−23] On the basis of the quasi-molecular precursor ion of 258 $[M + H]^+$, the molecular formula was determined to be $C_{17}H_{23}NO$ for alkamides **9**−**11**, which requires a degree of unsaturation of 7 and an alkyl chain of 12 carbons. Thus, the alkyl chain could contain either four double bonds and a triple bond or two double bonds and two triple bonds. The UV spectra of **9**−**11** with λ_{max} around 260 nm clearly indicated that

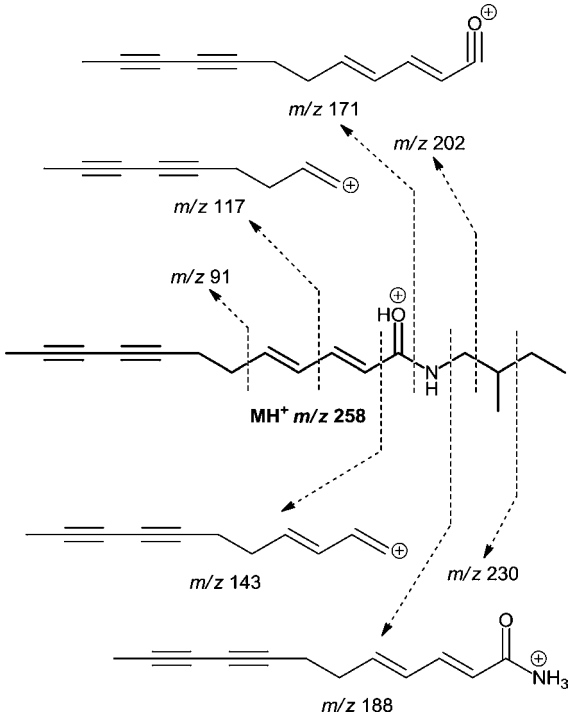


Figure 6. Proposed fragmentation pathway of the protonated molecular ion (MH^+) of dodeca-2*E*,4*E*-diene-8,10-diyne 2-methylbutylamide (**9**) as determined by MS/MS, including chemical structures of some of the most characteristic fragments. The fragmentation pathway of the 2*E*,4*Z*-isomer (**10**) and 2*Z*,4*E*-isomer (**11**) of compound **9** is the same as illustrated above.

these compounds were 2,4-diene alkamides.[7,22] This was also confirmed by the analysis of their MS/MS fragmentation patterns showing two fragments separated by two mass units at *m*/*z* 145 and *m*/*z* 143, respectively. The fragment at *m*/*z* 143 results from the loss of the amide portion (2-methylbutylamide) of the alkamides and corresponds to the alkyl chain of the 2,4-diene alkamides (Figure 6), whereas the fragment at *m*/*z* 145 is probably formed when a double bond is lost and the remaining double bond of the original 2,4-diene shifts to the 3-position, with a subsequent gain of two hydrogens.[21,23,25] Furthermore, the relative intensities of the fragments at *m*/*z* 145 (100%, base peak) and *m*/*z* 143 (25−44%) of the MS/MS fragmentation pattern were in agreement with alkamide **9**−**11** being 2,4-diene alkamides.[21,23] On the basis of the above information, it can be concluded that alkamide **9**−**11** must contain two double bonds and two triple bonds and, thus, are isomers of dodeca-2,4-diene-8,10-diyne 2-methylbutylamide.

The 2*E*,4*Z*-isomer of dodeca-2,4-diene-8,10-diyne 2-methylbutylamide (**10**) has previously been detected in roots of *E. purpurea* and *E. angustifolia*.[7,20−23] In addition, Cech et al.[20] have indicated the presence of another isomer of dodeca-2,4-diene-8,10-diyne 2-methylbutylamide in *E. purpurea*, although the *E*/*Z* stereochemistry was not determined. Thus, two of the detected isomers of dodeca-2,4-diene-8,10-diyne 2-methylbutylamide are to the best of our knowledge to be considered as new alkamides. The mass spectral data do not indicate stereochemistry or bond position; however, relative retention times have shown to be useful to distinguish alkamide isomers. For 2,4-diene isomers, it has been demonstrated that the 2*E*,4*Z*-isomers elute before 2*Z*,4*E*-isomers on RP-HPLC and that pure *E*-isomers elute before their corresponding *E*/*Z* isomers,[7,20−23] which was also demonstrated in the present study (Table 1). Thus, it is logical to assume a similar relationship in stereochemistry between the three isomers of dodeca-2,4-diene-8,10-diyne 2-methylbutylamide; hence, we propose the following structures for the new alkamides **9** and **11**: dodeca-2*E*,4*E*-diene-8,10-diyne 2-methylbutylamide (**9**) and dodeca-2*Z*,4*E*-diene-8,10-diyne 2-methylbutylamide (**11**). However, without conformational NMR data, it is not possible to conclusively distinguish between *E* and *Z* isomers, and the reported stereochemistry of the new alkamides is therefore only tentative as is the case for all lipophilic constituents identified in the present investigation (Table 1).

The roots of *E. purpurea* and *E. angustifolia* are characterized by their relatively high content of alkamides, while the content of alkamides in *E. pallida* roots is low.[7,12] On the other hand, *E. pallida* roots contain polyacetylenes and polyenes. So far, 12 ketoalkenes and ketoalkynes with 14 and 15 carbon atoms have been isolated and characterized from lipophilic root extracts of *E. pallida*, and their structures have been determined by means of spectroscopic (UV and NMR) and mass spectrometric techniques.[7,24] Analysis of *E. pallida* root extracts by HPLC-DAD, LC-DAD-APCI-MS, and MS/MS revealed the presence of three alkamides; undeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide (**2**, t_R = 19.8 min), dodeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide (**5**, t_R = 25.1 min), and pentadeca-2*E*,9*Z*-diene-12,14-diynoic acid isobutylamide (**16**, t_R = 34.1 min), of which the former two were also detected in *E. purpurea* in the present investigation. These alkamides are, however, well-known in *Echinacea* species and have also previously been isolated from roots of *E. pallida*.[7,12] As expected, the most abundant lipophilic compounds in the roots of *E. pallida* were ketoalkenes and ketoalkynes. Three ketoalkynes were detected

Table 2. Phenolic Acids Identified in *E. purpurea* and *E. pallida* Root Extracts by LC-DAD-APCI-MS/MS (APCI; Negative Mode)

		present in				
t_R (min)[a]	compd name[b]	*E. purpurea*	*E. pallida*	UV λ_{max} (nm)	quasi-molecular precursor ion (m/z)	product ions (m/z)[c]
11.8	caftaric acid	×	×	330	311 $[M - H]^-$	179 (72), 149 (25), 135 (100)
13.3	chlorogenic acid	×	×	325	353 $[M - H]^-$	191 (100)
16.9	echinacoside	×	×	330	785 $[M - H]^-$	623 (100), 459 (7), 313 (18)
22.8	cichoric acid	×	×	330	473 $[M - H]^-$	311 (13), 179 (100), 149 (46)

[a] t_R = retention time on analytical RP-HPLC. [b] Identification based on t_R and UV, LC-MS, and MS/MS spectra. [c] Percent of base peak in parentheses.

Table 3. Concentrations (mg/g DM ± SE) of Lipophilic Compounds (Alkamides, Ketoalkenes, and Ketoalkynes) in the Roots of *E. purpurea* and *E. pallida* at Different Harvest Dates

		harvest date					
compd	group[a]	December 15	March 23	May 25	August 13	October 29	average
				E. purpurea			
1	II	0.131 ± 0.020 c	0.153 ± 0.025 bc	0.279 ± 0.052 ab	0.389 ± 0.085 a	0.119 ± 0.018 c	0.21 ± 0.15
2	II	0.415 ± 0.056 c	0.545 ± 0.081 abc	0.665 ± 0.074 ab	0.774 ± 0.147 a	0.452 ± 0.069 bc	0.57 ± 0.26
3	II	0.046 ± 0.006 c	0.062 ± 0.016 bc	0.091 ± 0.014 ab	0.131 ± 0.024 a	0.046 ± 0.008 c	0.07 ± 0.05
4	III	0.291 ± 0.044 c	0.601 ± 0.088 a	0.473 ± 0.046 ab	0.388 ± 0.057 bc	0.327 ± 0.043 bc	0.42 ± 0.19
5	III	0.095 ± 0.020 c	0.254 ± 0.033 a	0.186 ± 0.036 ab	0.173 ± 0.026 b	0.094 ± 0.016 c	0.16 ± 0.09
6	II	0.072 ± 0.010 c	0.058 ± 0.014 bc	0.103 ± 0.012 ab	0.115 ± 0.013 a	0.061 ± 0.010 c	0.08 ± 0.05
7	II	0.041 ± 0.008 bc	0.061 ± 0.012 abc	0.062 ± 0.007 ab	0.072 ± 0.014 a	0.034 ± 0.007 c	0.05 ± 0.30
9	III	0.151 ± 0.022 c	0.399 ± 0.053 a	0.261 ± 0.035 b	0.241 ± 0.034 bc	0.175 ± 0.024 bc	0.25 ± 0.12
10[b]		NQ	NQ	NQ	NQ	NQ	NQ
11	III	0.022 ± 0.005 bc	0.066 ± 0.009 a	0.047 ± 0.014 ab	0.041 ± 0.009 abc	0.017 ± 0.004 c	0.04 ± 0.03
12	III	0.026 ± 0.004 bc	0.048 ± 0.007 a	0.039 ± 0.006 ab	0.050 ± 0.009 a	0.022 ± 0.005 c	0.04 ± 0.02
13, 14	I	1.639 ± 0.345	1.212 ± 0.368	1.917 ± 0.528	1.892 ± 0.328	0.693 ± 0.186	1.44 ± 1.00
18	I	0.076 ± 0.028	0.100 ± 0.031	0.105 ± 0.038	0.174 ± 0.078	0.042 ± 0.014	0.10 ± 0.11
19	I	0.040 ± 0.008	0.094 ± 0.025	0.059 ± 0.007	0.081 ± 0.020	0.033 ± 0.009	0.06 ± 0.05
total		2.881 ± 0.462 b	3.371 ± 0.496 a	4.265 ± 0.727 a	4.251 ± 0.466 a	2.108 ± 0.320 b	3.36 ± 1.53
				E. pallida			
2		0.078 ± 0.008	0.184 ± 0.048	0.099 ± 0.035	0.097 ± 0.025	0.118 ± 0.039	0.11 ± 0.08
6		0.054 ± 0.006	0.069 ± 0.019	0.032 ± 0.005	0.050 ± 0.021	0.074 ± 0.029	0.05 ± 0.04
8		4.333 ± 0.391	5.984 ± 0.335	4.626 ± 0.534	4.060 ± 0.261	3.687 ± 0.469	4.54 ± 1.39
15		0.693 ± 0.169	0.826 ± 0.132	0.649 ± 0.129	0.578 ± 0.153	0.412 ± 0.087	0.64 ± 0.34
16		1.246 ± 0.122	1.645 ± 0.519	0.871 ± 0.329	0.907 ± 0.189	0.485 ± 0.165	1.04 ± 0.67
17		5.657 ± 0.863 ab	6.881 ± 0.412 a	4.177 ± 0.597 bc	4.353 ± 0.608 bc	2.792 ± 0.571 c	4.77 ± 2.08
20, 21		1.387 ± 0.171	1.149 ± 0.241	1.165 ± 0.158	1.039 ± 0.254	1.190 ± 0.273	1.18 ± 0.67
total		13.22 ± 1.327 ab	16.41 ± 1.000 a	12.36 ± 1.235 b	10.74 ± 0.840 bc	8.148 ± 1.510 c	12.0 ± 4.21

[a] The groups that the lipophilic compound could be divided into according to effect of different harvest dates. [b] Compound **10** could not be quantified (NQ) due to lack of baseline separation and very low concentrations (<below limit of quantification).

and identified as tetradeca-8*Z*-ene-11,13-diyn-2-one (**8**, t_R = 27.5 min), pentadeca-8*Z*-ene-11,13-diyn-2-one (**15**, t_R = 33.1 min), and pentadeca-8*Z*,13*Z*-dien-11-yn-2-one (**17**, t_R = 35.9 min) as well as two ketoalkenes pentadeca-8*Z*,11*Z*,13*E*-trien-2-one (**20**) and pentadeca-8*Z*,11*E*,13*Z*-trien-2-one (**21**), which eluted at 42.1 min as an inseparable mixture. The identity of these ketoalkenes and ketoalkynes was verified by UV, MS, and MS/MS data. All compounds showed, beside a quasi-molecular precursor ion $[M + H]^+$, a characteristic fragment corresponding to $[M + H - H_2O]^+$ (base peak) in MS/MS (Table 1) in accordance with recently published mass spectrometric analyses of these compounds.[24] However, the ketoalkyne profile of the investigated *E. pallida* roots differed from other investigations of *E. pallida* root materials as we did not detect any allylic oxidized products of the ketoalkynes **8**, **15**, and **17**.[24,26] The allylic oxidation reaction is quite slow in *E. pallida* crude extracts but is rapid for the purified compounds.[24] Although the absence of the allylic oxidized products can be explained by genotypic differences, another explanation could be that they are formed during processing and extraction of the roots and therefore may be considered as artifacts.[24,26] To avoid oxidation of any compounds in the extracts, we performed the extraction on crushed freeze-dried root material protected from light and humidity and packed airtight and stored at −20 °C until extraction, followed by storage of the resulting extracts at −20 °C until analysis. However, no investigation was performed to verify that the allylic oxidized products detected in *E. pallida* roots in fact could be artifacts, as this was not the aim of the present study.

The analysis for hydrophilic constituents of the EtOH−H_2O (70:30) extracts of *E. purpurea* and *E. pallida* roots was performed in a separate analysis by HPLC-DAD and resulted in the identification of four phenolic acids: caftaric acid, cichoric acid, echinacoside, and chlorogenic acid, which are all well-known constituents of *Echinacea* species.[12,20,24,26−28] The identification of these phenolic acids was based on comparison with UV spectra and t_R on HPLC with authentic standards. The identification was furthermore confirmed by LC-DAD-MS

Table 4. Concentrations (mg/g DM ± SE) of Phenolic Acids in the Roots of *E. purpurea* and *E. pallida* at Different Harvest Dates

	harvest date					
compd	December 15	March 23	May 25	August 1	October 29	average
			E. purpurea			
caftaric acid	0.168 ± 0.023 b	0.128 ± 0.015 bc	0.230 ± 0.016 a	0.138 ± 0.007 bc	0.121 ± 0.007 c	0.15 ± 0.06
echinacoside	0.638 ± 0.092 b	1.618 ± 0.233 a	0.690 ± 0.120 b	0.779 ± 0.099 b	0.618 ± 0.044 b	0.88 ± 0.54
cichoric acid	2.906 ± 0.422 b	2.200 ± 0.229 b	3.943 ± 0.070 a	3.021 ± 0.205 b	2.379 ± 0.295 b	2.87 ± 0.96
chlor. acid[a]	0.050 ± 0.008	0.097 ± 0.043	0.040 ± 0.006	0.043 ± 0.009	0.053 ± 0.007	0.06 ± 0.05
total	3.762 ± 0.490 b	4.029 ± 0.303 ab	4.902 ± 0.156 a	3.970 ± 0.225 b	3.140 ± 0.333 b	3.94 ± 1.05
			E. pallida			
caftaric acid	0.033 ± 0.007	0.018 ± 0.005	0.042 ± 0.006	0.039 ± 0.004	0.037 ± 0.006	0.04 ± 0.02
echinacoside	0.532 ± 0.089 b	1.198 ± 0.653 a	1.162 ± 0.153 a	0.215 ± 0.048 b	0.503 ± 0.144 b	0.71 ± 0.73
cichoric acid	0.172 ± 0.049	0.146 ± 0.057	0.381 ± 0.097	0.257 ± 0.036	0.369 ± 0.058	0.27 ± 0.17
chlor. acid[a,b]	NQ	NQ	NQ	NQ	NQ	NQ
total	0.737 ± 0.118	1.364 ± 0.708	1.585 ± 0.210	0.551 ± 0.080	0.894 ± 0.183	1.00 ± 0.79

[a]Chlorogenic acid. [b]Chlorogenic acid in *E. pallida* could not be quantified (NQ) due to lack of baseline separation and very low concentrations (<below limit of quantification).

analysis (Table 2). All four phenolic acids were found in both *Echinacea* species; thus, chlorogenic acid could not be quantified in *E. pallida* due to very low concentrations. As expected, the most abundant phenolic acid in the roots of *E. purpurea* was cichoric acid, whereas echinacoside was the major phenolic acid in *E. pallida*.

Seasonal Variation in the Concentration of Alkamides, Ketoalkenes, and Ketoalkynes in *Echinacea* Roots. The concentration and composition of lipophilic compounds vary significantly between the two species of *Echinacea*, and it is possible to distinguish the root material of the two species solely from analysis of lipophilic compounds (Figures 4 and 5 and Table 1). The lipophilic compounds in roots of *E. purpurea* can be separated into three groups regarding their reaction to different harvest dates. Different harvest dates did not have a significant effect throughout the year on the concentration of the lipophilic compounds in the first group, which contains alkamides **13** and **14** as well as **18** and **19** (Table 3). The second group with compounds **1**−**3**, **6**, and **7** reached their significantly highest concentration during summer ($p < 0.05$) (Table 3), when the plants were blooming. The third group comprising compounds **4**, **5**, **9**, **11**, and **12** reached their significantly highest concentration in early spring ($p < 0.05$), where soil temperatures were just above 0 °C and the aerial parts were starting to sprout. An investigation from New South Wales, Australia,[16] demonstrated that the total amount of alkamides significantly decreases in roots in the period from preflowering to senescence stage. Unfortunately, the Australians did not identify all of their compounds and did not measure the concentration of the individual compounds.[16] The total concentration of lipophilic compounds in this investigation did decrease significantly from the flowering to senescent stage (August 13 to October 29, 2010) (Table 3), and the results from Australia seem to be in agreement with this with respect to the total concentration of lipophilic compounds.

Because the lipophilic compounds in *E. purpurea* roots are dominated by alkamides, while the lipophilic compounds in *E. pallida* are mostly ketoalkynes and ketoalkenes, the choice of plant species for cultivation is highly dependent on the compounds of interests. Moreover, the lipophilic compounds are found in a much higher concentration in *E. pallida* (12.0 ± 4.21 mg/g DM) than in *E. purpurea* (3.36 ± 1.53 mg/g DM). The lipophilic compound **17** in *E. pallida* was the only single compound that varied significantly with the different harvest times (Table 3), and the concentration was significantly the highest in early spring when the soil temperature was just above 0 °C and the aerial parts started to sprout. Although compounds **2**, **8**, and **15** and **16** showed no significant difference between different harvest times, there was a tendency to higher concentrations in early spring. The total concentration of lipophilic compounds was also significantly highest when roots were harvested in the cold period of the year (Table 3). Thus, when a high concentration of lipophilic compounds is desired, early spring is the most beneficial harvest time for roots of *E. pallida*.

In our experiment, the average concentration of alkamides **13** and **14** in *E. purpurea* roots over the season was 1.44 (0.69−1.97) mg/g DW (Table 3). As compared to results from other studies in different parts of the world, this concentration is average. An investigation from Ontario, Canada, on a natural population of *Echinacea* species reported a concentration of alkamides **13** and **14** of 2.79 mg/g DW in roots of 1 year old "germlings" grown in a greenhouse.[12] An investigation from the United States reported a concentration of 1.12 mg/g DW in 1/2 year old roots, and from Finland, a concentration of 1.73 mg/g DW was reported.[27,29] The composition of the different lipophilic compounds in our roots of *E. purpurea*, that is, the ratio of single compounds in relation to total concentration (Table 3), is dominated by 42% of compounds **13** and **14**. This is almost the same as reported in the investigation from New South Wales, Australia, where compounds **13** and **14** comprised 45% of the total concentration.[16]

The harvested root DW yield was approximately the same throughout the year for the 3−4 year old plants of both *E. purpurea* and *E. pallida* (data not shown). This corresponds with a guideline from Germany, which reports a near stable weight of roots after 3−4 years of growth of *E. purpurea*.[17] The most beneficial harvest time for 3−4 year old roots is therefore defined as when the concentration of the bioactive compounds is highest, since neither earlier nor later harvest results in a higher root yield (g) or higher yield per ha. The results of our investigation show that the most beneficial harvest time for the concentration of alkamides in roots of *E. purpurea* depends on the compounds of interest. In the future, when clinical effects of the individual lipophilic compounds in *Echinacea* are revealed, this will be important knowledge for farmers to be able to

supply high value raw material of roots for pharmacological products. Both harvesting in early spring and in summertime will have some advantages as the former gives the opportunity to use the field for new cash crop the same year and the latter opportunity to harvest the aerial parts as well. Whether harvest of the aerial parts will influence the concentration in the subsequent harvested roots will be investigated in our following work with *Echinacea*.

Seasonal Variation in the Concentration of Phenolic Acids in *Echinacea* Roots. The concentration and composition of the phenolic compounds varied considerably in the two species *E. purpurea and E. pallida* as was the case with the lipophilic compounds. The average total concentration of phenolic compounds was considerably higher in roots of *E. purpurea* (3.94 ± 1.05 mg/g DW) as compared to *E. pallida* (1.00 ± 0.79 mg/g DW). The two species had the same four phenolic acids as their major phenolic compounds, but echinacoside was the major phenolic compound found in roots of *E. pallida* (Table 4), while cichoric acid was the major phenolic compound in *E. purpurea*. The concentration of both caftaric acid and cichoric acid was very different in the two species, and only the concentration of echinacoside was nearly the same in roots of the two species. Chlorogenic acid was present in roots of both *E. purpurea* and *E. pallida* but in very low amounts. However, in *E. pallida*, the amounts of chlorogenic acid were <20 μg/g DM; therefore, a precise quantification was not possible (Table 4).

It has been reported that it should be possible to distinguish between the two species *E. purpurea* and *E. pallida* solely by their concentration of echinacoside.[12] Echinacoside should be the major compound in *E. pallida* but absent in *E. purpurea*.[12] Our results show, however, nearly the same concentration of echinacoside in both species, and this compound can in our view not be used to distinguish between the root material of *E. purpurea* and *E. pallida*. These results are supported by reports of echinacoside concentration in *E. purpurea* in other studies from both Denmark and China.[15,30]

In our experiments, the average concentrations of caftaric acid, echinacoside, and cichoric acid in roots of *E. purpurea* were 0.16, 0.81, and 2.89 mg/g DW, respectively. This can be compared to results from studies from other parts of the world. An investigation from Taiwan[31] reported concentrations of caftaric acid and cichoric acid of 0.23 and 0.71 mg/g DW, respectively, whereas an investigation from Canada[12] reported a concentration of caftaric acid of 2.39 mg/g DW and cichoric acid of 8.06 mg/g DW, and from Finland,[27] a concentration of cichoric acid of 9.4 mg/g DW was reported. In Australian grown *E. purpurea*, Stuart and Wills[32] found a much higher concentration of cichoric acid (30.6 mg/g DW), and even from another field experiment in Denmark, Mølgaard et al.[30] found a much higher concentration of cichoric acid (24 mg/g DW). Indeed, a considerable variation in the concentration of bioactive compounds in plants may be due to genetic differences between populations, climate, and cultivation conditions, as Mølgaard et al.[30] also comment, and especially for phenolic compounds.

All major phenolic acids in roots of *E. purpurea* reached their significantly highest concentration in spring ($p < 0.01$), with echinacoside in early spring ($p < 0.001$) when the aerial parts were about to sprout and cichoric acid and caftaric acid in late spring ($p < 0.01$) when the aerial parts were clearly visible and fast growing (Table 4).

In the Danish grown *E. purpurea* roots, the concentration of cichoric acid and total concentration of phenolic acid were highest in late spring (May) and decreased until the plants were in full flower (Table 4), followed by a nonsignificant increase in autumn. This corresponds to a Chinese investigation[15] on 1 year old root of *E. purpurea* grown in Changsha, China (latitude 29° N). The highest concentration of cichoric acid was also in spring, and the concentration decreased during spring/summer and was lowest when the Chinese plants were in full flower.[15]

In *E. pallida* roots, only echinacoside showed a significantly higher concentration in spring ($p < 0.05$) (Table 4). Caftaric acid also showed a tendency to higher concentration in late spring, and cichoric acid showed a tendency to higher concentration from late spring to midautumn. Although the concentration of phenolic acids varies a lot with climate, growing conditions, and genetic background in terms of concentration, the general recommendation for harvest time for highest concentration of phenolic acids from roots of *Echinacea* would be in spring.

In summary, this investigation of root extracts of *E. purpurea* and *E. pallida* by HPLC-DAD and LC-MS-DAD-APCI-MS/MS added new information to the metabolite profiles of the roots of these plants as compared to previous investigations. The most interesting observations in that respect were the presence of three isomers of dodeca-2,4-diene-8,10-diyne 2-methylbutylamide in *E. purpurea*, of which two are new alkamides, and the absence of allylic oxidation products of ketoalkynes in *E. pallida* as well as relatively high concentrations of echinacoside in *E. purpurea*. The present investigation furthermore showed that the contents of both lipophilic constituents and phenolic acids showed clear seasonal variations. The agronomic recommendation for harvest of roots of 3−4 year old *E. purpurea* has been stated as late autumn.[17] However, the results of the present study show that this is not always the best time, and the recommendation should be more specific depending on the species and compounds of interest. The major lipophilic compounds in roots of *E. purpurea* were at their lowest concentrations in midautumn and early winter (Table 3), and the total concentrations of lipophilic compounds in *E. pallida* showed the same pattern. Moreover, all of the major phenolic acids in *E. purpurea* were at their highest concentrations in spring. Harvesting of roots in spring will nevertheless allow for an alternative cash crop in the field. This specific information on seasonal variations in the concentrations of lipophilic and phenolic compounds in *E. purpurea* and *E. pallida* will be valuable for research, farmers, and producers of medicinal preparations.

AUTHOR INFORMATION

Corresponding Author

*Tel: +45-87158327. Fax: +45-87154812. E-mail: MariaObel.Thomsen@agrsci.dk.

Funding

We greatly acknowledge the financial support from the The Danish Counsil for Strategic Research (Project "Health-promoting bioactive compounds in plants" 2101-07-006).

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Grimm, W.; Müller, H. A randomized controlled trial of the effect of fluid extract of *Echinacea purpurea* on the incidence and severity of colds and respiratory infections. *Am. J. Med.* **1999**, *106*, 138−143.

(2) Woelkart, K.; Bauer, R. The role of alkamides as an active principle of *Echinacea*. *Planta Med.* **2007**, *73*, 615−623.

(3) Barnes, J.; Anderson, L.; Gibbons, S.; Phillipson, J. *Echinacea* species (*Echinacea angustifolia* (DC.) Hell., *Echinacea pallida* (Nutt.) Nutt., *Echinacea purpurea* (L.) Moench): A review of their chemistry, pharmacology and clinical properties. *J. Pharm. Pharmacol.* **2005**, *57*, 929−954.

(4) Clifford, L.; Nair, M.; Rana, J.; Dewitt, D. Bioactivity of alkamides isolated from *Echinacea purpurea* (L.) Moench. *Phytomedicine* **2002**, *9*, 249−253.

(5) Chicca, A.; Pellati, F.; Adinolfi, B.; Matthias, A.; Massarelli, I.; Benvenuti, S.; Martinotti, E.; Bianucci, A. M.; Bone, K.; Lehmann, R.; Nieri, P. Cytotoxic activity of polyacetylenes and polyenes isolated from the roots of *Echinacea pallida*. *Br. J. Pharmacol.* **2008**, *153*, 879−885.

(6) Pellati, F.; Benvenuti, S.; Magro, L.; Melagari, M.; Soragni, F. Analysis of phenolic compounds and radical scavenging activity of *Echinacea* spp. *J. Pharm. Biomed. Anal.* **2004**, *35*, 289−301.

(7) Bauer, R.; Remiger, P. TLC and HPLC analysis of alkamides in *Echinacea* drugs. *Planta Med.* **1982**, *55*, 367−371.

(8) LaLone, C. A.; Hammar, K. D. P.; Wu, L.; Bae, J.; Leyva, N.; Liu, Y.; Solco, A. K.; Kraus, G. A.; Murphy, P. A.; Wurtele, E. S.; Kim, O. K.; Seo, K. L.; Widrlechner, M. P.; Birt, D. F. *Echinacea* species and alkamides inhibit prostaglandin E_2 production in RAW264.7 mouse macrophage cells. *J. Agric. Food Chem.* **2007**, *55*, 7314−7322.

(9) Matthias, A.; Banbury, L.; Stevenson, L. M.; Bone, K. M.; Leach, D. N.; Lehmann, R. P. Alkylamides from *Echinacea* modulate induced immune responses in macrophages. *Immunol. Invest.* **2007**, *36*, 117−130.

(10) Hou, C. C.; Chen, C. H.; Yang, N. S.; Chen, Y. P.; Lo, C. P.; Wang, S. Y.; Tien, Y. J.; Tsai, P. W.; Shyur, L. F. Comparative metabolomics approach coupled with cell- and gene-based assays for species classification and anti-inflammatory bioactivity validation of *Echinacea* plants. *J. Nutr. Biochem.* **2010**, *21*, 1045−1059.

(11) Woelkart, K.; Koidl, C.; Grisold, A.; Gangemi, J. D.; Turner, R. B.; Marth, E.; Bauer, R. Bioavailability and pharmacokinetics of alkamides from the roots of *Echinacea angustifolia* in humans. *J. Clin. Pharmacol.* **2005**, *45*, 683−689.

(12) Binns, S.; Livesey, J.; Arnason, J.; Baum, B. Phytochemical variation in *Echinacea* from roots and flower heads of wild and cultivated populations. *J. Agric. Food Chem.* **2002**, *50*, 3673−3687.

(13) Rinniger, J. A.; Kickner, S.; Chigurupati, P.; McLean, A.; Franck, Z. Immunopharmacolo-gical activity of *Echinacea* preparations following simulated digestion on murine macrophages and human peripheral blood mononuclear cells. *J. Leukocyte Biol.* **2000**, *68*, 503−510.

(14) Geng, X.; Tian, X.; Tu, P.; Pu, X. Neuroprotective effects of echinacoside in the mouse MPTP model of Parkinson's disease. *Eur. J. Pharmacol.* **2007**, *564*, 66−74.

(15) Liu, Y.; Zeng, J.; Chen, B.; Yao, S. Investigation of phenolic constituents in *Echinacea purpurea* grown in China. *Planta Med.* **2007**, *73*, 1600−1605.

(16) Stuart, D.; Wills, R. Alkylamide and cichoric acid levels in *Echinacea purpurea* tissues during plant growth. *J. Herbs, Spices Med. Plants* **2000**, *7*, 91−101.

(17) Marquard, R.; Kroth, E. Purpur-Sonnenhut (*Echinacea purpurea* (L.) Moench), Schmalblättriger Sonnenhut (*E. angustifolia DC*) und Blasser Sonnenhut (*E. pallida Nutt.*). In *Anbau und Qualitätsanforderungen ausgewählter Arzneipflanzen*; Buchedition Agrimedia GmbH: Bergen, Germany, 2001; pp 261−274.

(18) Callan, N.; Yokelson, T.; Wall-Maclane, S.; Westcott, M. P.; Miller, J. B.; Ponder, G. Seasonal trends and plant density effects on cichoric acid in *Echinacea purpurea* (L.) Moench. *J. Herbs, Spices Med. Plants* **2005**, *11*, 35−46.

(19) Stuart, D.; Wills, R. Factors affecting the extraction of alkylamides and cichoric acid during ethanolic processing of *Echinacea purpurea* (L.) Moench. *Aust. J. Exp. Agric.* **2000**, *40*, 873−877.

(20) Cech, N. B.; Eleazer, M. S.; Shoffner, L. T.; Crosswhite, M. R.; Davis, A. C.; Mortenson, A. M. High performance liquid chromatography/electrospray ionization mass spectrometry for simultaneous analysis of alkamides and caffeic acid derivatives from *Echinacea purpurea* extracts. *J. Chromatogr., A* **2006**, *1103*, 219−228.

(21) Spelman, K.; Wetschler, M. H.; Cech, N. B. Comparison of alkylamide yield in ethanolic extracts prepared from fresh versus dry *Echinacea purpurea* utilizing HPLC-ESI-MS. *J. Pharm. Biomed. Anal.* **2009**, *49*, 1141−1149.

(22) Pellati, F.; Epifano, F.; Contaldo, N.; Orlandini, G.; Cavicchi, L.; Genovese, S.; Bertelli, D.; Benvenuti, S.; Curini, M.; Bertaccini, A.; Bellardi, M. G. Chromatographic methods for metabolite profiling of virus- and phytoplasma-infected plants of *Echinacea purpurea*. *J. Agric. Food Chem.* **2011**, *59*, 10425−10434.

(23) Mudge, E.; Loes-Lutz, D.; Brown, P.; Scheiber, A. Analysis of alkylamides in *Echinacea* plant materials and dietary supplements by ultrafast liquid chromatography with diode array and mass spectrometric detection. *J. Agric. Food Chem.* **2011**, *59*, 8086−8094.

(24) Pellati, F.; Orlandini, G.; Benvenuti, S. Simultaneous metabolite fingerprinting of hydrophilic and lipophilic compounds in *Echinacea pallida* by high-performance liquid chromatography with diode array and electrospray ionization-mass spectrometry detection. *J. Chromatogr., A* **2012**, *1242*, 43−58.

(25) Hiserodt, R. D.; Pope, B. M.; Cossette, M.; Dewis, M. L. Proposed mechanisms for the fragmentation of doubly allylic alkenamides (tingle compounds) by low energy collisional activation in a triple quadrupole mass spectrometer. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 1462−1470.

(26) Pellati, F.; Calò, S.; Benvenuti, S.; Adinolfi, B.; Nieri, P.; Melegari, M. Isolation and structure elucidation of cytotoxic polyacetylenes and polyenes from *Echinacea pallida*. *Phytochemistry* **2006**, *67*, 1359−1364.

(27) Laasonen, M.; Wennberg, T.; Harmia-Pulkkinen, T.; Vuorela, H. Simultaneous analysis of alkamides and caffeic acid derivatives for identification of *Echinacea purpurea*, *Echinacea angustifolia*, *Echinacea pallida* and *Pathenium integrifolium* roots. *Planta Med.* **2002**, *68*, 572−574.

(28) Luo, X. B.; Chen, B.; Yao, S. Z.; Zeng, J. G. Simultaneous analysis of caffeic acid derivatives and alkamides in roots and extracts of *Echinacea purpurea* by high-performance liquid chromatography-photodiode array detection-electrospray mass spectrometry. *J. Chromatogr., A* **2003**, *986*, 73−81.

(29) Qu, L.; Chen, Y.; Wang, X.; Scalzo, R. Patterns of variation in alkamides and cichoric acid in roots and aboveground parts of *Echinacea purpurea* (l.) Moench. *HortScience* **2005**, *40*, 1239−1242.

(30) Mølgaard, P.; Johnsen, S.; Christensen, P.; Cornett, C. HPLC method validated for the simultaneous analysis of cichoric acid and alkamides in *Echinacea purpurea* plants and products. *J. Agric. Food Chem.* **2003**, *51*, 6922−6933.

(31) Lin, S.; Sung, J.; Chen, C. Effect of drying and storage conditions on caffeic acid derivatives and total phenolics of *Echinacea purpurea* grown in Taiwan. *Food Chem.* **2011**, *125*, 226−231.

(32) Stuart, D.; Wills, R. Effect of drying temperature on alkamide and cichoric acid concentrations of *Echinacea purpurea*. *J. Agric. Food Chem.* **2003**, *51*, 1608−1610.