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Purification of Alkylamides from *Echinacea angustifolia* (DC.) Hell. Roots by High-Speed Countercurrent Chromatography

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High-speed countercurrent chromatography (HSCCC) was used for the separation of alkylamides from the roots of *Echinacea angustifolia* (DC.) Hell. For this purpose, the alkylamides were extracted with hexane and subjected to semipreparative HSCCC using a two-phase solvent system consisting of *n*-hexane, ethyl acetate, methanol, and water (4:1:2:1). The lower aqueous phase was used as the mobile phase at a flow rate of 3 mL/min and a rotary speed of 1000 rpm. This procedure led to the isolation of four pure alkylamides, that is, dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide (38.9 mg, 97% purity), dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide (4.4 mg, 92% purity), dodeca-2*E*,4*E*-dienoic acid isobutylamide (3.2 mg, 99% purity), and dodeca-2*E*,4*E*-dienoic acid 2-methylbutylamide (0.3 mg, 92% purity). The identity and purity of the isolated alkylamides were confirmed by LC-ESI-MS and ^{1}H NMR and ^{13}C NMR data. To the best of the authors' knowledge, this is the first report of dodeca-2*E*,4*E*-dienoic acid 2-methylbutylamide in *E. angustifolia* roots.

KEYWORDS: *Echinacea angustifolia* (DC.) Hell.; high-speed countercurrent chromatography; alkylamides; LC-MS; NMR spectroscopy

INTRODUCTION

Echinacea spp. are indigenous to North America, and roots of these plants were used by Native Americans for the treatment of a wide variety of conditions. In the United States and Europe, various *Echinacea* preparations have been popular herbal medicines for preventing and treating the common cold, flu, and upper respiratory tract infections. The plants are cultivated in Europe, mainly Germany, and in Canada and the United States for use as a phytomedicine, natural health product, and dietary supplement, respectively. Aerial parts and roots of *Echinacea angustifolia* (DC.) Hell., *Echinacea purpurea* (L.) Moench, and *Echinacea pallida* (Nutt.) Nutt. in powder form or as extracts are considered potent immunostimulants (*1*, *2*). The major proposed active compounds in the *Echinacea* species are alkylamides and caffeic acid derivatives, together with polysaccharides and glycoproteins. Comprehensive investigations of *Echinacea* plants and products have resulted in the characterization of more than 20 alkylamides, most of which are isobutylamides of $C_{11}-C_{16}$ long-chain unsaturated fatty acids, with a mixture of isomeric dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamides as the predominant components. The roots contain higher amounts of alkylamides than other parts, and the highest concentrations are found in *E. angustifolia* (*3*). This class of compounds is known to exhibit a wide range of pharmacological activities in vitro such as anti-inflammatory, immunomodulatory, antimicrobial, and antioxidant properties (*4*).

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Typically, isolation of *Echinacea* alkylamides is achieved in several steps, including fractionation by silica gel column chromatography and purification by preparative HPLC and MPLC on reversed-phase stationary phases (*5*, *6*). However, basic amides can suffer from peak tailing and poor separation on silica-based columns, making it very difficult to obtain pure compounds using conventional methods such as preparative thin-layer chromatography and column chromatography. Furthermore, purification by HPLC and MPLC is very time-consuming (*5*, *6*).

During our recent investigations of commercial *Echinacea* products it became evident there is a paucity of commercially available alkylamide reference materials of known purity and stability. However, the availability of reference compounds would be highly desirable to develop analytical methods on the one hand and to facilitate studies of their biological activities on the other. In particular, it would allow bioassays using individual components rather than crude extracts. In continuation of our previous work on the isolation of carotenoids from vegetables and dietary supplements (*7*), hydroxycinnamoyltartaric acids from grapes (*8*), and gallotannins from mango kernels (*9*), we herein report the purification of alkylamides from *E. angustifolia* roots using high-speed countercurrent chromatography (HSCCC). This technique relies on the partition of a component between two immiscible solvent systems to achieve separation. The absence of a solid support matrix eliminates the irreversible loss of samples caused by adsorption to the stationary phase (*10*).

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Table 1. Two-Phase Solvent Systems Developed for the Separation of Alkylamides by HSCCC

		partition coefficient[b]						percentage	
system	solvent ratio[a]	15.1 min[c]	17.5 min[c]	20.1 min[c]	22.9 min[c]	27.1 min[c]	29.9 min[c]	UP	LP
33	9:1:2:1	0.09	0.49	0.86	1.68	3.05	7.01	76	24
54	6:1:2:1	0.19	0.64	1.34	2.49	3.76	8.10	67	33
71	4:1:2:1	0.15	0.54	1.18	2.06	3.41	7.29	57	43

[a] Two-phase solvent system hexane/ethyl acetate/methanol/water. [b] $K_{UP/LP}$ = partition coefficient determined by the ratio of HPLC peak areas; [c] Retention times (min) of alkylamides in HPLC-DAD analysis.

MATERIALS AND METHODS

Solvents and Reagents. All organic solvents used for HSCCC were of analytical grade and purchased from Fisher Scientific (Ottawa, ON, Canada). HPLC grade water, acetonitrile, and formic acid (99%) were also purchased from Fisher Scientific.

Preparation of Crude Hexane Extracts from *E. angustifolia*. Roots of *E. angustifolia* harvested in 2009 were supplied to our laboratory as powdered material by Three Feathers Farms Ltd. (Sherwood Park, AB, Canada), and roots of *E. angustifolia* collected in 2008 under the supervision of Dr. Wendy Applequist (Missouri Botanical Gardens, St. Louis, MO) were provided by Naturex (South Hackensack, NJ); the herbarium specimens for this collection were deposited with the Missouri Botanical Garden Herbarium (voucher 217). Samples were stored at −20 °C, protected from light and moisture, until analysis. Alkylamides were obtained by Soxhlet extraction for 6 h using 55 g of dried root powder and 400 mL of hexane to yield 1.21% (w/w) of crude extract from *E. angustifolia* grown in Alberta and 0.53% (w/w) from plant material grown in the United States and provided by Naturex.

HSCCC Apparatus. The separation of alkylamides was performed using a model TBE-300B high-speed countercurrent chromatograph (Tauto Biotech, Shanghai, China) supplied by Cherry Instruments (Chicago, IL). The specifications including pump, detector, and fraction collector were reported previously (*9*).

Selection of the Solvent System for HSCCC. The selection of two-phase solvent systems used as stationary and mobile phases, respectively, was based on the partition coefficient (K) of alkylamides ($0.5 \leq K \leq 1.0$), settling time (< 20 s), 1:1 phase volume ratio, and satisfactory retention of the stationary phase in the column (*10*). For the screening of the solvent systems, 2 mg of the crude extract was extracted with 2 mL (each) of upper and lower phases in a test tube by thorough vortex mixing. Aliquots of 10 μL of each phase were analyzed by HPLC, and peak area count (mAU) was selected to calculate the partition coefficient. The solvent systems evaluated consisted of a mixture of *n*-hexane, ethyl acetate, methanol, and water that was selected by adjusting the hydrophobicity in the organic phase. The most suitable solvent systems developed for the separation of alkylamides are listed in **Table 1**.

Separation of Alkylamides by HSCCC. The sample solution was prepared by dissolving 250 mg of the crude hexane extract in 5 mL (each) of upper and lower phases. The separation of the alkylamides was performed in one step using a two-phase solvent system consisting of *n*-hexane, ethyl acetate, methanol, and water (4:1:2:1). The coil was entirely filled with the upper phase, and the apparatus was rotated at 1000 rpm while the lower phase of the same solvent system was pumped into the column at a flow rate of 3 mL/min in the head-to-tail mode, resulting in 72.8% retention of stationary phase. After the mobile phase front emerged and the hydrodynamic equilibrium was established in the coil, the sample was injected through the injection valve. The mobile phase eluting from the chromatographic column was collected in fractions of 9 mL each. After 40 fractions (2 h) had been collected, the valve was switched and the upper phase was pumped into the column for 2 h. The rotation was stopped, and the fractions were pooled according to their HPLC profile.

HPLC-DAD Analysis. The profile of alkylamides present in the crude hexane extracts and HSCCC fractions was determined by HPLC using a series 1200 liquid chromatograph (Agilent, Mississauga, ON, Canada). For this purpose, 1 mg of crude extract was dissolved in 1 mL of methanol and filtered through a 0.45 μm nylon filter into an HPLC vial, and a 10 μL aliquot was injected. Solutions of pure alkylamides isolated by HSCCC had a concentration of 100 μg/mL. The separation was performed using a Nova-Pack C_{18} column (150 × 3.9 mm i.d., 4 μm particle size) from Waters (Wexford, Ireland) and water containing 0.1% formic acid (A) and acetonitrile (B) as the mobile phase with a gradient elution of 0−9 min (10−18.5% B), 9−9.5 min (18.5−45% B), 9.5−39.50 min (45−80% B), 39.5−42.0 min (80−100% B), 42.0−45.0 min (100−10% B). The flow rate was 1 mL/min, and the column was kept at 30 °C (*11*). Alkylamides were detected at 254 nm.

For the determination of the alkylamide profile in the powdered plant material, 1 g was added to 10 mL of methanol/0.1% phosphoric acid (70:30, v/v) and sonicated twice for 20 min (*11*). Extracts were transferred to a volumetric flask, the volume was made up to 25 mL, and 2 mL of the extract was filtered through a 0.45 μm nylon filter into an HPLC vial. HPLC analyses were performed as described above.

Structure Elucidation of Alkylamides. The structure of the alkylamides isolated by HSCCC was characterized using LC-ESI/MS and NMR spectroscopy. LC-MS analyses were performed using the HPLC unit described above, which was connected to a 4000 QTRAP LC-MS/MS System (Applied Biosystems, Streetsville, ON, Canada). Dodeca-2*E*,4*E*,8*Z*,10(*Z*/*E*)-tetraenoic acid isobutylamide (purified by HSCCC as described previously) was used to adjust mass spectrometer sensitivity in the positive ionization mode. The HPLC conditions were as described above. The flow rate was maintained at 1 mL min^{-1} with the pneumatically assisted electrospray probe using high-purity nitrogen gas (99.995%) as the nebulizing (GS1) and heating gas (GS2). The values for optimum spray voltage, source temperature, GS1, GS2, and curtain gases were at +3.5 kV, 400 °C, and 50, 30, and 15 psi, respectively. An information-dependent acquisition (IDA) method was used to profile the alkylamides. Both Q1 and Q3 were operated at low and unit mass resolution. The spectra were obtained over a scan range from m/z 50 to 500 in 2 s. Linear ion trap (LIT) fill time was set at 20 ms. The IDA threshold was set at 100 cps for collecting enhanced product ion (EPI) spectra from the eight most intense peaks. The EPI scan rate was 4000 amu/s. The enhanced MS (EMS) scan rate was 1000 amu/s. Collision-induced dissociation (CID) spectra were acquired using nitrogen as the collision gas. The collision energy (CE) was 23 eV. The other MS parameters used were as follows: declustering potential (DP), 66 V; entrance potential, 10 V; and collision exit potential, 10 V. Data acquisition was performed using Analyst 1.5 (Applied Biosystems). One-dimensional ^{1}H NMR and ^{13}C NMR spectra were measured on an Advance DMX 500 NMR spectrometer (Bruker, Rheinstetten, Germany) at 499.82 and 125.69 MHz, respectively. Chloroform ($CDCl_3$) was used as the solvent and tetramethylsilane (TMS) as the internal standard.

Compound **1** (dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide): colorless amorphous; ESI-MS (m/z) 248 $[M + H]^+$ (100), 270 $[M + Na]^+$ (62), 192 (2), 175 (11), 166 (48), 149 (49), 147 (18), 142 (15), 133 (12), 121 (9), 107 (14), 105 (12), and 93 (3); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.76 d (J = 15 Hz, H-2), 7.19 dd (J = 15, 13 Hz, H-3), 6.16 dd (J = 15, 13 Hz, H-4), 6.08 dt (J = 15, 6 Hz, H-5), 2.25 m (H-6), 2.30 m (H-7), 5.25 dt (J = 10, 6 Hz, H-8), 5.97 t (J = 10 Hz, H-9), 6.30 dd (J = 15, 13 Hz, H-10), 5.69 m (H-11), 1.78 dd (J = 6, 1 Hz, H-12), 5.46 br s (NH), 3.17 dd (J = 6, 6 Hz, H-1′), 1.80 m (H-2′), and 0.93 d (J = 6 Hz, H-3′,4′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 166.3 (C-1), 142.1 (C-5), 141.2 (C-3), 129.9 (C-11), 129.4 (C-9), 128.7 (C-4), 128.0 (C-8), 126.8 (C-10), 122.1 (C-2), 47.0 (C-1′), 33.1 (C-6), 28.7 (C-2′), 27.0 (C-7), 20.2 (C-3′,4′), and 18.3 (C-12).

Compound **2** (dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide): colorless oil; ESI-MS (m/z) 250 $[M + H]^+$ (100), 272 $[M + Na]^+$ (52), 194 (2), 177 (4), 167 (18), 152 (16), 149 (5), 135 (5), 121 (3), 109 (11), and 95 (6); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.75 d (J = 15 Hz, H-2), 7.18 dd (J = 15, 10 Hz, H-3), 6.14 dd (J = 15, 10 Hz, H-4), 6.08 dt (J = 15, 6 Hz, H-5), 2.18 m (H-6,7), 5.36 m (H-8,9), 2.00 dt (J = 7, 7 Hz, H-10), 1.37 m (H-11), 0.90 t (J = 7 Hz, H-12), 5.46 br s (NH), 3.17 dd (J = 7, 7 Hz, H-1′), 1.80 m (H-2′), and 0.93 d (J = 7 Hz, H-3′,4′); ^{13}C NMR ($CDCl_3$, 125 MHz)

δ 166.4 (C-1), 142.2 (C-5), 141.3 (C-3), 130.8 (C-9), 128.6 (C-4), 128.5 (C-8), 122.0 (C-2), 47.0 (C-1′), 33.1 (C-6), 29.4 (C-10), 28.7 (C-2′), 26.6 (C-7), 22.8 (C-11), 20.2 (C-3′,4′), and 13.8 (C-12).

Compound **3** (dodeca-2*E*,4*E*-dienoic acid isobutylamide): colorless needles; ESI-MS (*m*/*z*) 252 $[M + H]^+$ (100), 274 $[M + Na]^+$ (46), 196 (11), 179 (8), 161 (4), 137 (3), 119 (2), 105 (4), and 95 (6); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.76 d (*J* = 16 Hz, H-2), 7.20 dd (*J* = 16, 11 Hz, H-3), 6.14 dd (*J* = 15, 11 Hz, H-4), 6.06 dt (*J* = 15, 6 Hz, H-5), 2.14 m (H-6), 1.28 m (H-7,8,9,10,11), 0.88 t (*J* = 6 Hz, H-12), 5.49 br s (NH), 3.17 t (*J* = 6 Hz, H-1′), 1.81 m (H-2′), and 0.93 d (*J* = 6 Hz, H-3′, 4′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 166.6 (C-1), 143.5 (C-5), 141.6 (C-3), 128.4 (C-4), 121.9 (C-2), 47.2 (C-1′), 33.2 (C-6), 32.0, 29.4, 29.3, 29.0, 28.9 (C-2′), 22.8 (C-11), 20.3 (C-3′,4′), and 14.3 (C-12).

Compound **4** (dodeca-2*E*,4*E*-dienoic acid 2-methylbutylamide): colorless amorphous; ESI-MS (*m*/*z*) 266 $[M + H]^+$ (100), 288 $[M + Na]^+$ (16), 196 (10), 179 (4), 161 (12), 133 (11), 119 (3), 109 (9), and 95 (6); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.76 d (*J* = 16 Hz, H-2), 7.21 dd (*J* = 16, 11 Hz, H-3), 6.12 dd (*J* = 15, 11 Hz, H-4), 6.07 dt (*J* = 15, 6 Hz, H-5), 2.16 m (H-6), 1.28 m (H-7,8,9,10,11), 0.88 t (*J* = 6 Hz, H-12), 5.48 br s (NH), 3.18 m (H-1′), 1.56 m (H-2′), 1.41/1.15 m (H-3′), 0.91 t (*J* = 6 Hz, H-4′,5′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 166.6 (C-1), 143.6 (C-5), 141.8 (C-3), 128.2 (C-4), 121.4 (C-2), 45.4 (C-1′), 35.1 (C-2′), 33.0 (C-6), 31.8, 29.8, 29.2, 29.0, 27.1 (C-3′), 22.7 (C-11), 17.2 (C-5′), 14.1 (C-12), and 11.3 (C-4′).

RESULTS AND DISCUSSION

Preliminary attempts to purify the alkylamides by silica gel preparative thin-layer chromatography and column chromatography demonstrated that these techniques were not effective for obtaining good separations. The use of high-speed countercurrent chromatography was necessary to exploit the small differences in the alkylamide physicochemical properties. With gradual changes in the hydrophobicity of the organic phase, suitable two-solvent systems were obtained. The different partition coefficients obtained with systems composed of *n*-hexane, ethyl acetate, methanol, and water indicated that they were selective for the separation of the targeted alkylamides (**Table 1**). Because of the more favorable volume ratio between upper and lower phases and higher retention of stationary phase (73%), solvent system 71 was selected for HSCCC fractionation.

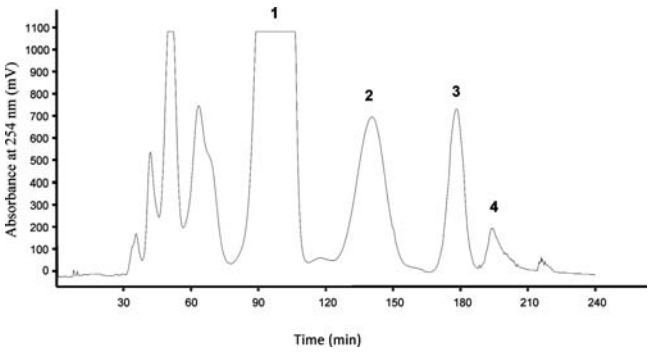

Figure 1. HSCCC profile of an *Echinacea* crude hexane extract using solvent system 71 *n*-hexane/ethyl acetate/methanol/water (4:1:2:1) in the head-to-tail elution mode: retention of the stationary phase, 73%; sample size, 250 mg; flow rate, 3 mL/min; temperature, 20 °C; revolution speed, 1000 rpm; detection wavelength, 254 nm.

The alkylamide profile of the crude hexane extract of *E. angustifolia* roots grown in Alberta was determined by HPLC-DAD analysis (data not shown). Using 0.25 g of the extract, the separation of alkylamides by semipreparative HSCCC with solvent system 71 composed of *n*-hexane, ethyl acetate, methanol, and water (4:1:2:1) was accomplished and yielded six main fractions (**Figure 1**; **Table 2**). Fraction 34−37 (38.9 mg) was found to contain the most abundant alkylamide, which was identified as dodeca-2*E*,4*E*,8*Z*,10(*Z*/*E*)-tetraenoic acid isobutylamide and determined chromatographically to be 97% pure for the *E*-isomer. These geometric isomers have recently been separated by preparative argentation reversed-phase HPLC (*12*). Compound **1** was a colorless amorphous substance. The MS, ^{1}H NMR, and ^{13}C NMR data are in agreement with those of dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide described in the literature (*6*, *12*−*14*).

Fraction 43−46 (2.5 mg) was composed of an isomeric mixture of alkylamides eluting at around 23 min in HPLC analysis. At this point, the selection of plant material containing only one isomer was necessary. For this purpose, the crude hexane extract (0.25 g) of the *E. angustifolia* roots grown in the United States was fractionated by HSCCC under the same conditions, and 4.4 mg of dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide (RT 22.9 min, 92% purity) was successfully obtained. This substance was a colorless oil. A comparison with data reported earlier revealed that the MS and ^{1}H NMR data are in agreement with those of dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide in the literature (*6*, *13*). No ^{13}C NMR data were found in the literature for this compound. Therefore, the ^{13}C NMR assignments were possible by comparison with the chemical shifts of the other alkylamides isolated and described herein.

Fraction 58−62 (3.2 mg), isolated from Alberta *E. angustifolia* roots, contained a pure alkylamide, dodeca-2*E*,4*E*-dienoic acid isobutylamide, and was determined to be 99% pure. The MS, ^{1}H NMR, and ^{13}C NMR data are in agreement with those of dodeca-2*E*,4*E*-dienoic acid isobutylamide described in the literature (*5*, *13*, *15*).

Fraction 65 (0.3 mg) isolated from *E. angustifolia* roots grown in Alberta contained dodeca-2*E*,4*E*-dienoic acid 2-methylbutylamide with 92% purity. This compound was a colorless amorphous substance. The molecular weight was 265 and was consistent with the molecular formula $C_{17}H_{31}NO$. Fragment *m*/*z* 196 is formed by dissociation of the C−N bond of a 2-methylbutylamide to lose the alkyl group directly attached to the amine ($MH^+ - 70$). Fragment *m*/*z* 179 is formed by dissociation of the C−N bond of a 2-methylbutylamide to lose the entire amine functional group ($MH^+ - 87$). The MS and ^{1}H NMR data are in agreement with those of dodeca-2*E*,4*E*-dienoic acid isobutylamide and dodeca-2*E*-ene-8,10-diynoic acid 2-methylbutylamide (*5*, *13*). The assignments of ^{13}C NMR signals were accomplished by comparison with the

Table 2. Alkylamides Isolated by HSCCC from Crude Hexane Extracts (0.25 g, Solvent System 71, Head-to-Tail Mode)

fraction	retention time[a] (min)	peak area[b] (%)	sample weight (mg)	compound
22	15.1	74.1	9.4	undeca-2-ene-8,10-diynoic acid isobutylamide[c]
28−29	17.5	74.9	10.1	dodeca-2,4,10-trien-8-ynoic acid isobutylamide[c]
34−37	20.2	96.8	38.9	isomeric dodeca-2*E*,4*E*,8*Z*,10(*Z*/*E*)-tetraenoic acid isobutylamide
43−46	22.9	77.3	2.5	dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide
40 (U.S. sample)	22.9	92.1	4.4	
58−62	27.1	99.4	3.2	dodeca-2*E*,4*E*-dienoic acid isobutylamide
65	30.1	91.7	0.3	dodeca-2*E*,4*E*-dienoic acid 2-methylbutylamide

[a] Retention times (min) of alkylamides in HPLC-DAD analysis. [b] Purity based on HPLC analyses. [c] Tentatively identified by mass spectrometry.

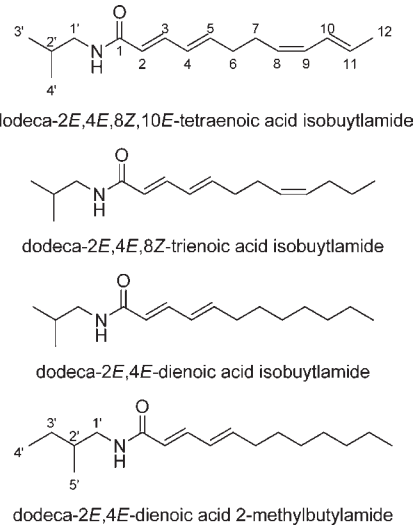

Figure 2. Chemical structures of four olefinic alkylamides purified from *Echinacea angustifolia* roots by HSCCC.

chemical shifts of dodeca-2*E*,4*E*-dienoic acid isobutylamide isolated and described herein and dodeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide (*15*, *16*). The structures of the four alkylamides purified by HSCCC are shown in **Figure 2**.

Two acetylenic alkylamides were separated but with low purity (≤75%) due to the presence of coeluting geometric isomers. Their chemical structures were tentatively assigned by comparison of their mass spectrometric data with previously published results (*5*, *13*). The first, dodeca-2,4,10-trien-8-ynoic acid isobutylamide, showed a molecular weight of 245, consistent with the molecular formula $C_{16}H_{23}NO$: ESI-MS (*m*/*z*) 246 $[M + H]^+$ (58), 268 $[M + Na]^+$ (100), 190 (65), 173 (21), 147 (58), 145 (28), 131 (24), 119 (21), 117 (20), and 105 (58). Fragment *m*/*z* 190 is formed by dissociation of the C−N bond of an isobutylamide to lose the alkyl group directly attached to the amine ($MH^+ - 56$), whereas fragment *m*/*z* 173 is formed by dissociation of the isobutylamide C−N bond to lose the entire amine functional group ($MH^+ - 73$). The second acetylenic alkylamide was assigned to undeca-2-ene-8,10-diynoic acid isobutylamide with ESI-MS (*m*/*z*) 232 $[M + H]^+$ (94), 254 $[M + Na]^+$ (81), 176 (58), 159 (41), 148 (14), 133 (54), 131 (73), 117 (21), 105 (100), and 95 (2). It showed a molecular weight of 231, consistent with the molecular formula $C_{15}H_{21}NO$. Fragment *m*/*z* 176 is formed by dissociation of the isobutylamide C−N bond to lose the alkyl group directly attached to the amine ($MH^+ - 56$). Fragment *m*/*z* 159 is formed by dissociation of the C−N bond of an isobutylamide to lose the entire amine functional group ($MH^+ - 73$). Undeca-2-ene-8,10-diynoic acid isobutylamide and dodeca-2,4,10-trien-8-ynoic acid isobutylamide have been previously identified in *E. angustifolia* extracts (*5*, *6*).

The elaborated two-phase solvent system was successful in the isolation and purification of olefinic alkylamides from *E. angustifolia* roots in one-step. The isolated compounds have small structural differences such as one or two additional double bonds and isobutyl or 2-methylbutyl groups. High-speed countercurrent chromatography was found to be a highly efficient method to resolve the alkylamide mixture and does not display the undesirable adsorption phenomenon and sample loss associated with other chromatographic techniques. The successful purification of alkylamides depends on the presence or absence of respective geometric isomers. Therefore, a preliminary evaluation of the alkylamide distribution in different *Echinacea* accessions by HPLC is recommended.

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