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Analysis of Alkylamides in *Echinacea* Plant Materials and Dietary Supplements by Ultrafast Liquid Chromatography with Diode Array and Mass Spectrometric Detection

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ABSTRACT: Alkylamides are a class of compounds present in plants of the genus *Echinacea* (Asteraceae), which have been shown to have high bioavailability and immunomodulatory effects. Fast analysis to identify these components in a variety of products is essential to profile products used in clinical trials and for quality control of these products. A method based on ultrafast liquid chromatography (UFLC) coupled with diode array detection and electrospray ionization mass spectrometry was developed for the analysis of alkylamides from the roots of *Echinacea angustifolia* (DC.) Hell., *Echinacea purpurea* (L.) Moench, and commercial dietary supplements. A total of 24 alkylamides were identified by LC-MS. The analysis time for these components is 15 min. Compared to the alkylamide profiles determined in the *Echinacea* root materials, the commercial products showed a more complex profile due to the blending of root and aerial parts of *E. purpurea*. This versatile method allows for the identification of alkylamides in a variety of *Echinacea* products and presents the most extensive characterization of alkylamides in *E. angustifolia* roots so far.

KEYWORDS: *Echinacea* spp., ultrafast liquid chromatography, mass spectrometry, alkylamides, dietary supplements

INTRODUCTION

Echinacea products have consistently been among the top-selling herbal dietary supplements in the United States.[1,2] These products are available in Canada as natural health products and in Europe as phytomedicines and have also been classified as herbal medicinal products, which are used for both the treatment and prevention of common colds, flu, and upper respiratory tract infections. Traditionally, there are only three species of *Echinacea* that are used in commercial medicinal preparations, which are *Echinacea purpurea* (L.) Moench., *Echinacea angustifolia* (DC.) Hell., and *Echinacea pallida* (Nutt.) Nutt. Binns et al. have revised the classification of *Echinacea* into two subgenera and four species.[3] This revision indicates that *E. angustifolia* and *E. pallida* are both varieties of *E. pallida* (*E. pallida* var. *angustifolia* and *E. pallida* var. *pallida*). *E. purpurea* is a separate species still referred to as *E. purpurea*. To be consistent with plant species labeled in dietary supplements, in this paper we will still refer to as three species. The immunomodulatory effects of *Echinacea* species have been associated with several classes of constituents including caffeic acid derivatives, alkylamides, glycoproteins, and polysaccharides. As a result of differences in solubility of these constituents and varying extraction techniques, *Echinacea* preparations containing entirely different bioactive compounds are found on the market. Commercial *Echinacea* products are available in a variety of forms, including dried plant materials, liquid tinctures, dried extracts, tablets, and softgels. Because of the numerous factors that can affect the levels of the active constituents in the final products, such as growth conditions, postharvesting handling, storage, extraction techniques, and others, a high amount of variability in the composition of *Echinacea* products occurs.[4−6]

The outcomes of clinical trials have been evaluated through meta-analyses and have shown inconclusive results for the treatment and prevention of colds and flu.[7,8] Most clinical trials do not report on the phytochemical composition of the extracts or plant materials evaluated. However, this information is valuable in determining efficacy as it is well-known that *Echinacea* products vary considerably. Therefore, it is crucial to evaluate and report the phytochemical profiles of *Echinacea* dietary supplements and plant materials used in clinical trials.

Alkylamides are one of the main constituents present in ethanol−water extracts of *Echinacea* plant materials. The degree of unsaturation of the aliphatic chains varies significantly, resulting in over 20 different alkylamides that have been identified in *Echinacea* species. Figure 1 depicts the structures of alkylamides identified in *E. angustifolia* and *E. purpurea* roots. There are several isomeric pairs of alkylamides found in *Echinacea*, the structures differing solely by the double-bond configuration. Of the *Echinacea* plants that are used in herbal supplements, alkylamides are most abundant in the roots of *E. angustifolia* and *E. purpurea*. Significantly lower amounts have been found in the aerial parts of these plants and in *E. pallida* roots.[9,10] Alkylamides have been shown to induce anti-inflammatory responses in mouse macrophages,[11] interact with the endocannabinoid system,[12] and inhibit COX-2 activity,[13] confirming the in vitro effect of these compounds on immune response. Further investigations have proven that alkylamides are bioavailable as they have been found in the plasma of patients within 10 min after oral administration of the herb tincture.[14] Their high bioavailability compared to other chemical constituents suggests

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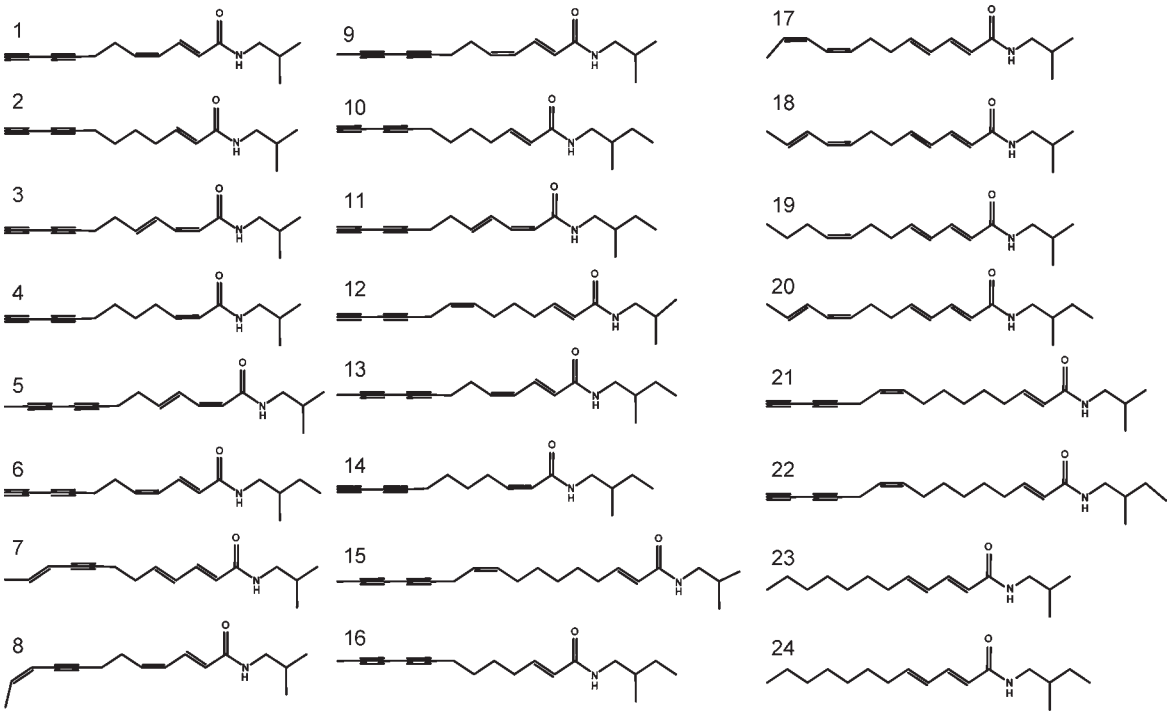

Figure 1. Structures of alkylamides found in *Echinacea angustifolia* and *Echinacea purpurea* roots. Compound numbering is based on the elution order using the UFLC method.

that these compounds play a role in the immune stimulatory response. To date, there have been several reports on the identification of alkylamides using HPLC with ultraviolet and/or mass spectrometric detection.[9,10,15−21] Most of these methods focus on the identification of alkylamides in the roots, extracts, and achenes of *E. purpurea*, whereas only a few of them report on *E. angustifolia* or *E. pallida*. Reversed-phase C18 columns have been used with mobile phase compositions of an acidic aqueous solution and acetonitrile.[9,10,15−21] Because of the long run times (30−45 min), these methods are time-consuming and have a low sample throughput. A quality control method using NIR spectroscopy was developed for the identification of *E. purpurea* roots.[22] Although this method is fast, it is limited to the identification of *E. purpurea* roots and does not allow for individual component profiling.

Recently, there has been a lot of interest in the development of fast methods for the analysis of bioactive compounds. With the development of sub-2-μm particle size columns and liquid chromatography systems that operate at pressures higher than conventional HPLC instruments, fast LC methods are currently being developed for the analysis of active components in dietary supplements. The benefits of fast LC methods include increased resolution and sensitivity and decreased consumption of solvents.[23] Furthermore, fast LC also significantly enhances MS detection.[24]

We here report a method for the fast analysis of alkylamides present in the three main *Echinacea* species and several commercial dietary supplements using UFLC-DAD-ESI-MS/MS. Due to the complexity of the structures of the alkylamides and the presence of isomeric pairs in *Echinacea* spp., there is a possibility for coelution to occur that may not be detected using ultraviolet detection. In contrast, the use of mass spectrometry allows the identification of these minor coeluting alkylamides.

MATERIALS AND METHODS

Solvents and Chemicals. Water, acetonitrile, and methanol were of HPLC grade and were purchased from Fisher Scientific (Ottawa, ON, Canada). The solvents used as mobile phases were filtered with a 0.2 μm nylon filter using a Milli-Q solvent filtration system. Formic acid (99%) was of analytical grade and purchased from Fisher Scientific.

Plant Material and Sample Preparation. Roots of *E. angustifolia* and *E. purpurea* harvested in 2009 were supplied as a powdered material from Three Feathers Farms Ltd. (Sherwood Park, AB, Canada). The roots of *E. angustifolia*, *E. purpurea*, and *E. pallida* were harvested in 2008 under the supervision of Dr. 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, vouchers 217, 218, and 216, respectively. Commercial dietary supplements, including capsules, tinctures, and softgels, were purchased from local supermarkets and natural health stores. The extraction of the alkylamides varied depending on the sample matrix. Two hundred milligrams of the plant material was extracted with 8 mL of MeOH/water (70:30, v/v) under sonication for 30 min at ambient temperature (21 °C). Three hundred milligrams of the contents of the capsules and softgels was extracted under the same conditions. For the commercial tinctures, 1 mL of MeOH/water (70:30, v/v) was added to a 1 mL aliquot of the liquid tincture and mixed thoroughly. Replicate samples were filtered using a 0.2 μm nylon filter into a HPLC vial for UFLC analysis.

Purification of Standards. Alkylamides were extracted from *E. angustifolia* roots by maceration with hexane. The hexane extract was dried under vacuum. For the isolation of alkylamides, 250 mg of the dried extract was subjected to high-speed counter-current chromatography (HSCCC) as described previously.[25] Three alkylamides, dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide, dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide, and dodeca-2*E*,4*E*-dienoic acid isobutylamide, were isolated at 97, 92, and 99% purity, respectively. Their structures

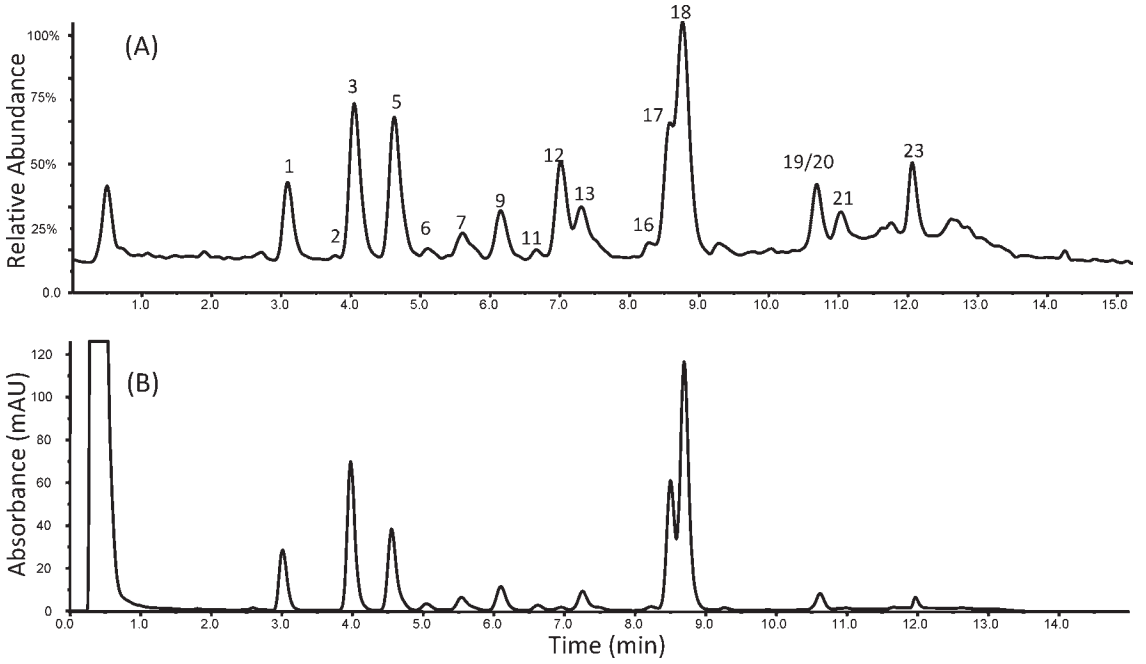

Figure 2. Separation of alkylamides from the root extract of *Echinacea purpurea* grown in Alberta by UFLC: TIC (A) and UV (B) at 254 nm. For peak assignment see Table 1.

were confirmed by ESI-MS and ^{1}H NMR and ^{13}C NMR spectroscopy.[26,27] The standards were dissolved in methanol and used for identification of the alkylamides in *Echinacea* products.

Instrumentation. A Shimadzu (Tokyo, Japan) Prominence UFLCXR liquid chromatographic system equipped with a CBM-20A communication bus module, two LC-20AD XR pumps, a DGU-20A3 vacuum degasser, an SIL-20AC XR autosampler, a CTO-20AC column oven, and an SPD-M20A diode array detector was used. The UFLC was coupled to a 4000 QTRAP LC-MS/MS System (ABSciex, Streetsville, ON, Canada). The chromatographic separation was performed on a VisionHT C18 HL 1.5 μm, 50 × 2.0 mm column (Mandel Scientific, Guelph, ON, Canada). The mobile phase was composed of (A) water containing 0.1% formic acid and (B) acetonitrile. The gradient program was as follows: 0−4 min, 39−40% B; 4−8 min, 40−50% B; 8−10.5 min, 50−70% B; 10.5−11.5 min, 70% B; 11.5−12 min, 70−39% B; 12−15 min, 39% B. The flow rate was 0.5 mL/min, and the column temperature was 20 °C. The injection volume was 2 μL. UV spectra were collected from 200 to 400 nm, whereas 254 nm was used for monitoring the alkylamides. The mass spectrometer was equipped with an electrospray ion source operating in positive ionization mode. Nitrogen gas (>99%) was used as nebulizing (gas 1, GS1) and collision gas (gas 2, GS2). The mass spectrometer sensitivity was optimized using the purified alkylamide dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide by direct injection. The values for optimum spray voltage, source temperature, GS1, GS2, and curtain gases were 4.5 kV, 600 °C, 50 psi, 50 psi, and 30 psi, respectively. The declustering potential was 66.0 V, the collision energy was 23.0 eV, and the collision exit potential was 10.0 V. The spectra were obtained over a mass range of m/z 50−500. MS/MS analyses were performed using the information-dependent acquisition (IDA) method whereby the mass spectrometer alternates between the enhanced MS scan for the full scan and the enhanced product ion scan and generates MS/MS data on the eight most intense peaks. This allows for the identification of both the major and minor components eluting from the column. In this method, extracted ion chromatograms and collision-induced dissociation spectra are simultaneously acquired and high selectivity is obtained. The data collection was performed using Analyst 1.5 software (ABSciex).

RESULTS AND DISCUSSION

Separation of Alkylamides by UFLC. Several methods for the HPLC separation of alkylamides with analysis times ranging from 30 to 45 min have been reported.[9,10,15−21] The UFLC method described here allows the separation of alkylamides in 15 min. A high-load C18 column was selected for the separation of the several isomeric pairs of alkylamides that are present in *Echinacea*. An improvement in the separation of dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide was achieved compared to previously published methods.[15,17,19,21] The UFLC method was optimized by variation of the gradient program, flow rates, and column temperatures. Using the conditions described under Materials and Methods, 15 peaks were observed at 254 nm in the *E. purpurea* roots (Figure 2) and 17 peaks in the *E. angustifolia* roots (Figure 3). The intense peak eluting in the beginning of the chromatogram contains the coextracted phenolic compounds. Their analysis was not considered in this study.

Identification of Alkylamides in *Echinacea* Plant Materials. The separation of alkylamides in *E. purpurea* roots is shown in Figure 2. The 17 alkylamides that have been identified on the basis of their mass spectra are listed in Table 1. The three standards purified by HSCCC were used to identify the compounds eluting at the same retention time, and their structures were confirmed by their molecular ion and MS/MS fragmentation as dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide, dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide, and dodeca-2*E*,4*E*-dienoic acid isobutylamide. For all other alkylamides, identification was performed by comparing the molecular ion, fragmentation pattern, and elution order published on alkylamides in *E. purpurea*.[15,17,19,21] Because the HSCCC method was capable of separating the two isomers of dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide, the *E* isomer was isolated with 97% purity and its structure was confirmed by NMR.[25] Using this standard, it was confirmed that the elution order of the isomeric

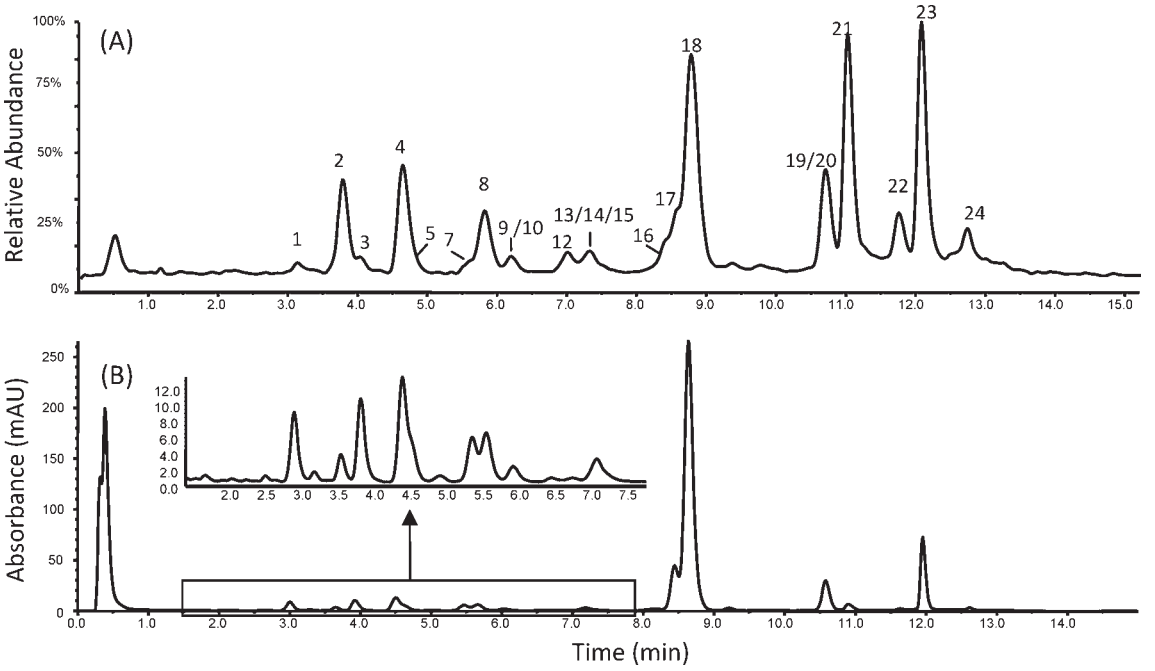

Figure 3. Separation of alkylamides from the root extract of *Echinacea angustifolia* from plants grown in Alberta by UFLC: TIC (A) and UV (B) at 254 nm. For peak assignment see Table 1.

Table 1. Assignment of Alkylamides in *Echinacea angustifolia* and *Echinacea purpurea* Roots

peak	t_R (min)	precursor $[M + H]^+$ (m/z)	product ions (m/z)	compound	present in *E. purpurea*	present in *E. angustifolia*
1	3.0	230	174, 167, 157, 129, 128, 116, 91	undeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide	X	X
2	3.6	232	176, 159, 131, 105, 91, 79	undeca-2*E*-ene-8,10-diynoic acid isobutylamide	X	X
3	4.0	230	188, 174, 166, 157, 146, 129, 116, 105, 91	undeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide	X	X
4	4.5	232	176, 159, 141, 133, 91, 79	undeca-2*Z*-ene-8,10-diynoic acid isobutylamide		X
5	4.6	244	188, 171, 167, 145, 117, 105,	dodeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide	X	X
6	5.0	244	174, 157, 131, 129, 116, 91	undeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide	X	
7	5.5	246	190, 173, 145, 119, 105, 91, 79	dodeca-2*E*,4*E*,10*E*-trien-8-ynoic acid isobutylamide	X	X
8	5.7	246	190, 173, 147, 143, 119, 105, 91, 79	dodeca-2*E*,4*Z*,10*Z*-trien-8-ynoic acid isobutylamide		X
9	6.1	244	188, 171, 148, 128, 117, 105	dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide	X	X
10	6.1	246	176, 148, 133, 131, 105, 91, 77	undeca-2*E*-ene-8,10-diynoic acid 2-methylbutylamide		X
11	6.6	244	180, 174, 157, 146, 131, 129, 117, 91	undeca-2*Z*,4*E*-diene-8,10-diynoic acid 2-methylbutylamide	X	
12	6.9	258	202, 157, 131, 117, 91	trideca-2*E*,7*Z*-diene-10,12-diynoic acid isobutylamide	X	X
13	7.2	258	188, 171, 160, 143, 129, 128, 117, 105	dodeca-2*E*,4*Z*-diene-8,10-diynioc acid 2-methylbutylamide	X	X
14	7.2	246	218, 176, 159, 131,105, 91	undeca-2*Z*-ene-8,10-diynoic acid 2-methylbutylamide		X
15	7.2	300	244, 227, 199, 166, 153, 91, 77	hexadeca-2*E*,9*Z*-diene-12,14-diynoic acid isobutylamide		X
16	8.2	260	190, 173, 147, 145, 105, 91	dodeca-2*E*-ene-8,10-diynoic acid 2-methylbutylamide	X	X
17	8.5	248	192, 175, 167, 166, 152, 149, 107, 79	dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide	X	X
18	8.7	248	192, 175, 167, 166, 152, 107, 79	dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide	X	X
19	10.6	250	194, 177, 167, 152, 109, 95	dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide	X	X
20	10.65	262	180, 166, 145, 107, 79	dodeca-2,4,8,10-tetraenoic acid 2-methylbutylamide	X	X
21	10.9	286	230, 213, 171, 145, 143, 105, 91	pentadeca-2*E*,9*Z*-diene-12,14-diynoic acid isobutylamide	X[a]	X
22	11.6	300	230, 213,185, 171, 159, 145, 105, 91	pentadeca-2*E*,9*Z*-diene-12,14-diynoic acid 2-methylbutylamide		X
23	11.9	252	196, 179, 161, 95	dodeca-2*E*,4*E*-dienoic acid isobutylamide	X	X
24	12.6	266	210, 196, 179, 133, 109, 95	dodeca-2*E*,4*E*-dienoic acid 2-methylbutylamide		X

[a] Detected only in *Echinacea purpurea* roots grown in Alberta.

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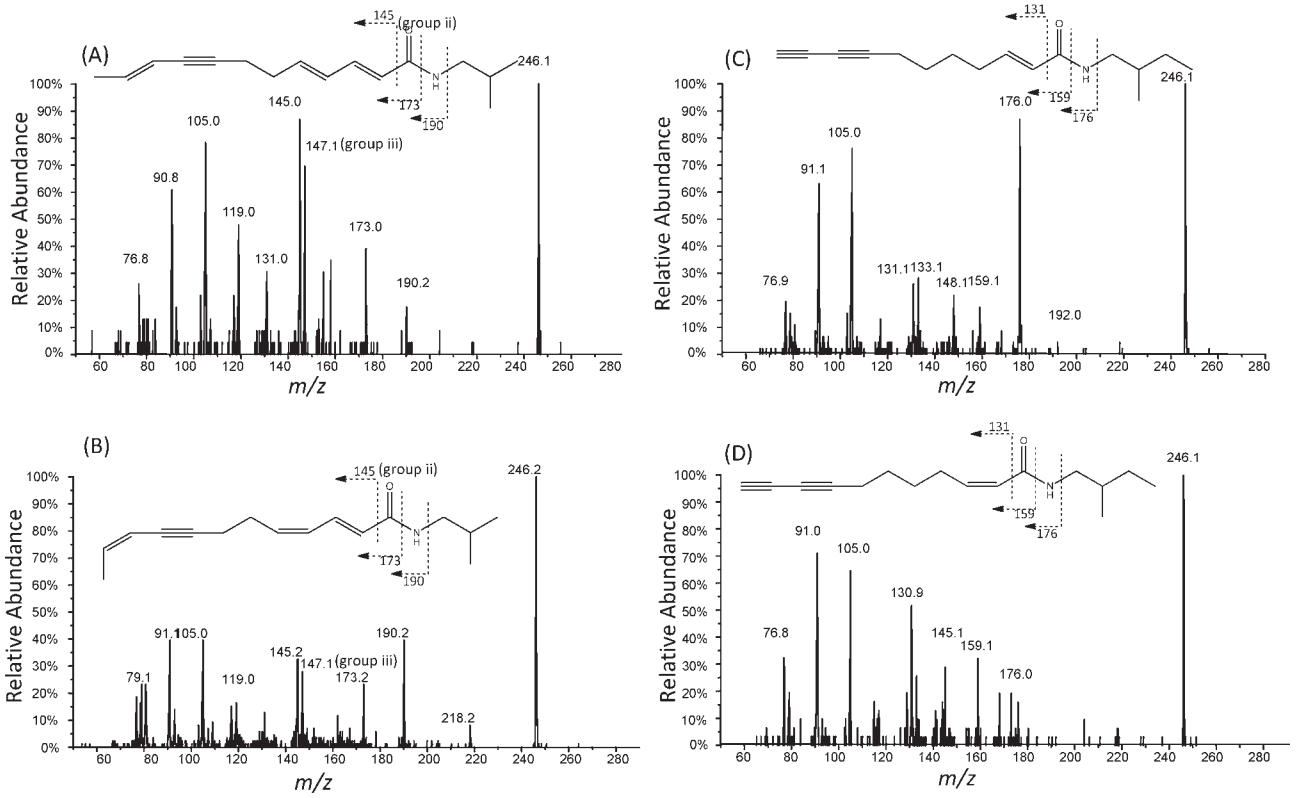

Figure 4. MS/MS spectra of isomeric alkylamide pairs with the molecular ion of *m/z* 246: (A) an isobutylamide eluting at 5.5 min; (B) an isobutylamide eluting at 5.7 min; (C) a 2-methylbutylamide eluting at 6.1 min; (D) a 2-methylbutylamide eluting at 7.2 min.

pair is the *Z* isomer followed by the *E* isomer. This is consistent with previously published work by Perry et al.[10]

It has been reported that a total of 17 alkylamides are present in *E. purpurea* roots,[21] although only 15 of the alkylamides reported in this publication could be identified in the *E. purpurea* root materials evaluated in the present study. Undeca-2*E*-ene-8,10-diynoic acid isobutylamide was observed, but dodeca-2*E*-ene-8,10-diynoic acid isobutylamide was not. This is contradictory to recently published data[21] and might be due to different genotypes and growing conditions. There are two additional alkylamides that have been identified in *E. purpurea* roots. Dodeca-2*E*-ene-8,10-diynoic acid 2-methylbutylamide was detected in both samples of *E. purpurea* roots and pentadeca-2*E*,9*Z*-diene-12,14-diynoic acid isobutylamide was found only in the roots of *E. purpurea* from the plants grown in Alberta. Both alkylamides have not previously been identified in *E. purpurea* roots.

Alkylamides are most abundant in *E. angustifolia* roots, and there is more variation in the different structures compared to *E. purpurea*.[9,26] To date, LC-MS methods of alkylamides only report on *E. purpurea*, whereas alkylamides identified using HPLC-UV methods of *E. angustifolia* have not previously been confirmed by mass spectrometry. As shown in Figure 3, only 17 alkylamides were observed at 254 nm, whereas MS detection allowed the identification of 22 alkylamides. The additional five alkylamides that coeluted could easily be distinguished on the basis of their mass spectrometric data (Table 1). The MS allowed for enough sensitivity to identify minor alkylamides, which coeluted with other more abundant alkylamides. For example, alkylamide peaks 13−15 all had retention times of 7.2 min with very low absorbance at 254 nm, but could be identified using mass spectrometric detection.

The identification of several alkylamides was achieved by comparing the retention times, molecular ions, and MS/MS fragmentation patterns to published data on *E. purpurea*.[15,17,19,21] The alkylamides that were identified only in *E. angustifolia* were compared with those reported by Bauer et al.[26] As with *E. purpurea*, the three pure standards were used to confirm the structures of the compounds eluting at the same retention time by comparing their molecular ions and fragmentation patterns.

Two alkylamides (peaks 7 and 8 in Figure 3) in *E. angustifolia* had very close retention times at 5.5 and 5.7 min and could not be completely separated. The molecular ion observed in both peaks was *m/z* 246 $[M + H]^+$. The fragmentation patterns of these two compounds were also identical as shown in Figure 4A,B. The fragment *m/z* 190 corresponds to the loss of the isobutyl group (−56 Da), and the fragment *m/z* 173 corresponds to the loss of the isobutyl amine (−73 Da), which indicates that these two alkylamides are isobutylamides. With the molecular ion of *m/z* 246 $[M + H]^+$, the molecular formula is determined as $C_{16}H_{23}NO$, which requires a degree of unsaturation of 6 and an alkyl chain of 12 carbons. The alkyl chain could contain either three double bonds and a triple bond or two triple bonds and one double bond. Spelman et al.[21] proposed that the MS/MS fragmentation pattern can be used in the structure elucidation to differentiate between diene and monoene alkylamides. The presence of two fragments separated by 2 mass units would represent the fragmentation that occurs between carbons 1 and 2 of the alkyl chain. The fragments observed are the alkyl chain (group ii) and the alkyl chain with the saturation of one of the double bonds (group iii), whereas the other double bond shifts to the 3-position, as shown in Figure 4A,B.[21,28] If the alkylamides were a diene, the group ii fragment would be *m/z* 145 and the

group iii fragment would be m/z 147. MS/MS analysis of these two alkylamides resulted in these two fragments, confirming that they are dienes, and therefore the alkyl chain must contain three double bonds and a triple bond (dodeca-2,4,10-trien-8-ynoic acid isobutylamide). The alkylamide at 5.5 min has been found in *E. purpurea*, whereas the alkylamide at 5.7 min was present only in *E. angustifolia*. The configuration of the double bonds cannot be confirmed with mass spectrometry, but on the basis of previously published data obtained by NMR spectroscopy, an alkylamide in *E. purpurea* has the structure of dodeca-2*E*,4*E*,10*E*-trien-8-ynoic acid isobutylamide.[27] According to Bauer et al.,[26] an alkylamide with the structure of dodeca-2*E*,4*Z*,10*Z*-trien-8-ynoic acid isobutylamide has been confirmed by NMR spectroscopy in *E. angustifolia* roots. Therefore, the alkylamide eluting at 5.7 min has been tentatively identified as dodeca-2*E*,4*Z*,10*Z*-trien-8-ynoic acid isobutylamide.

Two other alkylamides with the molecular ion of m/z 246 were identified in *E. angustifolia* roots with retention times of 6.1 and 7.2 min (peaks 10 and 14 in Figure 3). As shown in Figure 4C,D, the fragment m/z 176 corresponds to the loss of the 2-methylbutyl group (−70 Da) and the fragment m/z 159 to the loss of the 2-methylbutyl amine (−87 Da). This confirms that these alkylamides are 2-methylbutylamides and the alkyl chain contains 11 carbons. Because both alkylamides have similar fragmentation patterns, the structure must differ by double-bond configuration. For the previously identified monoene isomeric pair (peaks 2 and 4 in Figure 3), the elution order has been established as the *E* isomer eluting before the *Z* isomer of undeca-2-ene-8,10-diynoic acid isobutylamide.[21] Therefore, the same elution order can reasonably be assigned for the alkylamide (peak 10) eluting at 6.1 min as undeca-2*E*-ene-8,10-diynoic acid 2-methylbutylamide and the alkylamide (peak 14) eluting at 7.2 min as undeca-2*Z*-ene-8,10-diynoic acid 2-methylbutylamide.

An alkylamide eluting at 4.5 min had a molecular ion of m/z 232 $[M + H]^+$ and the fragmentation pattern was identical to that of undeca-2*E*-ene-8,10-diynoic acid isobutylamide. Therefore, this alkylamide was assigned to undeca-2*Z*-ene-8,10-diynoic acid isobutylamide.

Two alkylamides were detected with molecular ions of m/z 300 $[M + H]^+$, the first eluting at 7.2 min and the second at 11.6 min. The MS/MS fragmentation of the early-eluting alkylamide has fragments at m/z 244 and 227, which correspond to the loss of the isobutyl group and isobutyl amine, respectively. The fragmentation between carbons 1 and 2 of the alkyl chain produced a fragment at m/z 199. The formula based on the molecular ion would be $C_{20}H_{29}NO$, where the alkyl chain should contain 16 carbons with a degree of unsaturation of 7. Therefore, the alkylamide hexadeca-2*E*,9*Z*-diene-12,14-diynoic acid isobutylamide, previously isolated by Bauer et al.,[26] has been tentatively identified, although the double-bond configuration cannot be confirmed solely with the MS/MS fragmentation. The MS/MS fragmentation pattern for the later eluting alkylamide with the same molecular ion of m/z 300 $[M + H]^+$ has fragments at m/z 230 and 213, corresponding to the loss of the 2-methylbutyl group and the 2-methylbutyl amine, thus confirming the structure to be a 2-methylbutylamide. The fragment at m/z 185 corresponds to the fragmentation between carbons 1 and 2, and the molecular formula requires that this alkylamide contains an alkyl chain with 15 carbons and a degree of unsaturation of 7. The tentative assignment of this structure is pentadeca-2*E*,9*Z*-diene-12,14-diynoic acid 2-methylbutylamide, the locations of the double and triple bonds being in agreement with alkylamides previously identified in *E. angustifolia*.[26] This alkylamide has been found in an *Echinacea* extract containing a mixture of *E. angustifolia* and *E. purpurea* roots; however, no structural information was provided.[29] The absence of the group iii fragment also confirms that these two alkylamides are not conjugated 2,4-dienes.

An alkylamide with a molecular ion of m/z 260 $[M + H]^+$ was detected in both root materials and was characterized as a 2-methylbutylamide due to the presence of the fragments m/z 190 and 173, corresponding to the loss of the 2-methylbutyl and 2-methylbutyl amine. On the basis of the molecular formula of $C_{17}H_{25}NO$ and a degree of unsaturation of 6, this alkylamide was tentatively assigned as dodeca-2*E*-ene-8,10-diynoic acid 2-methylbutylamide, which has been isolated from *E. angustifolia* roots.[26] The other alkylamide found in both root materials had a molecular ion of m/z 286 $[M + H]^+$. The MS/MS fragments of m/z 230 and 213 confirmed the loss of the isobutyl and isobutyl amine, respectively. The molecular formula of $C_{19}H_{27}NO$ and a degree of unsaturation of 7 were used to confirm the structure as pentadeca-2*E*,9*Z*-diene-12,14-diynoic acid isobutylamide. This compound has also been identified in *E. angustifolia* roots.[26] The final alkylamide eluting at 12.6 min has a molecular ion of m/z 266 $[M + H]^+$. The fragments m/z 196 and 179 confirm the structure as a 2-methylbutylamide. This compound has recently been isolated and its structure confirmed using NMR spectroscopy as dodeca-2*E*,4*E*-dienoic acid 2-methylbutylamide.[25]

E. pallida roots were also analyzed for their alkylamide profile. Previous papers have identified very low levels of alkylamides or none at all.[9,30] Using the newly developed method, no alkylamides could be detected by LC-MS analysis.

There are significant differences in the alkylamide profile of *E. angustifolia* roots and *E. purpurea* roots, as shown in Table 1. The distinct chromatograms that were obtained by UFLC-DAD analysis can be used to differentiate between the two plant materials. *E. purpurea* roots contain more intense peaks for the acetylenic alkylamides and conjugated 2,4-dienoic acid amides, mainly the two alkylamides undeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide and dodeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide eluting at 4.0 and 4.5 min. Furthermore, there are much less intense peaks of the olefinic alkylamides eluting after the tetraenoic alkylamides (peaks 17 and 18 in Figure 2) compared with *E. angustifolia* roots. In the *E. angustifolia* root profile, the monoenoic acetylenic amides predominate. The alkylamides undeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide and undeca-2*Z*,4*E*-diene-8,10-diynoic acid 2-methylbutylamide were not detected and the isomeric pair dodeca-2,4,10-trien-8-ynoic acid isobutylamide was observed at 5.5 and 5.7 min. The other alkylamides detected only in *E. angustifolia* roots were the isobutylamides, undeca-2*Z*-ene-8,10-diynoic acid isobutylamide and hexadeca-2*E*,9*Z*-diene-12,14-diynoic acid isobutylamide, and the 2-methylbutylamides, undeca-2*E*-ene-8,10-diynoic acid 2-methylbutylamide, undeca-2*Z*-ene-8,10-diynoic acid 2-methylbutylamide, pentadeca-2*E*,9*Z*-diene-12,14-diynoic acid 2-methylbutylamide, and dodeca-2*E*,4*E*-dienoic acid 2-methylbutylamide.

Identification of Alkylamides in Commercial *Echinacea* Dietary Supplements. The commercial *Echinacea* dietary supplements that were purchased from local grocery and health food stores are listed in Table 2. The plant materials used in these products include aerial parts and roots from *E. purpurea* and *E. angustifolia* roots, although the exact compositions of each differ significantly. For example, two of the tinctures contain

E. purpurea flower and root extracts, whereas another tincture contains root extracts of both *E. purpurea* and *E. angustifolia*.

The alkylamides in each of the commercial products were identified on the basis of their fragmentation patterns in MS/MS analysis and are summarized in Table 3. With the exception of one product, the isomeric pair of dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide was detected in all samples investigated. The product that does not contain the tetraenoic alkylamides was produced only from *E. purpurea* aerial parts, according to the product label. From Table 3 it becomes evident that the alkylamides present in the dietary supplements vary considerably.

Table 2. Label Specifications of Commercial Products Analyzed for Alkylamides Using UFLC-DAD-MS/MS

no.	product type	contents
1	liquid tincture	*Echinacea purpurea* flower and root
2	liquid tincture	*Echinacea purpurea* flower and root
3	liquid tincture	*Echinacea purpurea* and *Echinacea angustifolia* roots
4	capsule, dried extract	extract of *Echinacea purpurea* aerial parts
5	capsule, dried extract	extract of *Echinacea purpurea* roots
6	softgel, extract	extract of *Echinacea purpurea* aerial parts and root
7	softgel, extract	extract of *Echinacea purpurea* aerial parts and roots
8	softgel, extract	extract of *Echinacea angustifolia* roots
9	Softgel, extract	extract of *Echinacea angustifolia*

An alkylamide with a retention time of 9.2 min that was detected in the commercial products but was not found in the plant material had a molecular ion of m/z 248. The fragment m/z 175 represents the loss of the isobutyl amine from the alkyl chain, making its structure an isobutylamide. The MS/MS experiment yielded two distinct fragments at m/z 152 and 166. The fragment m/z 166 represents the loss of the last six carbons on the alkyl chain, and the fragment m/z 152 represents the loss of the last seven carbons on the alkyl chain. The MS/MS fragmentation pattern is also identical to that of the tetraenoic alkylamides. Therefore, the structure is in agreement with the two main tetraene alkylamides, dodeca-2,4,8,10-tetraenoic acid isobutylamides, but with different stereochemistries. Previously, the alkylamide dodeca-2*E*,4*E*,8*E*,10*Z*-tetraenoic acid isobutylamide has been reported in *E. purpurea* roots, but the authors were unable to separate it using reversed-phase HPLC.[21] The exact configuration of this compound could not be assigned solely by mass spectrometry.

According to the labels, two dietary supplements (8 and 9) were said to contain *E. angustifolia* roots. Using mass spectrometry as a potential diagnostic tool to determine the plant species used in these two products, it was possible to confirm the presence of the same alkylamides that were found in the *E. angustifolia* roots analyzed in this study, which gives a strong indication that *E. angustifolia* roots were used in these two products. The acetylenic alkylamides undeca-2*Z*-ene-8,10-diynoic

Table 3. Assignment of Alkylamides in Commercial *Echinacea* Products

		presence in *Echinacea* commercial products								
peak[a]	alkylamide	1	2	3	4	5	6	7	8	9
1	undeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide	X	X	X	X	X	X	X	X	X
2	undeca-2*E*-ene-8,10-diynoic acid isobutylamide	X	X	X		X			X	X
3	undeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide	X	X	X		X	X		X	X
4	undeca-2*Z*-ene-8,10-diynoic acid isobutylamide			X					X	X
5	dodeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide	X	X	X		X			X	
6	undeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide	X	X	X		X				
7	dodeca-2*E*,4*E*,10*E*-trien-8-ynoic acid isobutylamide	X	X	X		X			X	X
8	dodeca-2*E*,4*Z*,10*Z*-trien-8-ynoic acid isobutylamide			X					X	X
9	dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide	X		X		X				
10	undeca-2*E*-ene-8,10-diynoic acid 2-methylbutylamide									
11	undeca-2*Z*,4*E*-diene-8,10-diynoic acid 2-methylbutylamide									
12	trideca-2*E*,7*Z*-diene-10,12-diynoic acid isobutylamide	X	X	X						
13	dodeca-2*E*,4*Z*-diene-8,10-diynioc acid 2-methylbutylamide			X		X				
14	undeca-2*Z*-ene-8,10-diynoic acid 2-methylbutylamide								X	X
15	hexadeca-2*E*,9*Z*-diene-12,14-diynoic acid isobutylamide									
16	dodeca-2*E*-ene-8,10-diynoic acid 2-methylbutylamide			X						
17	dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide	X	X	X		X	X	X	X	X
18	dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide	X	X	X		X	X	X	X	X
19	dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide	X	X	X		X	X	X	X	X
20	dodeca-2,4,8,10-tetraenoic acid 2-methylbutylamide	X	X						X	X
21	pentadeca-2*E*,9*Z*-diene-12,14-diynoic acid isobutylamide			X					X	
22	pentadeca-2*E*,9*Z*-diene-12,14-diynoic acid 2-methylbutylamide								X	X
23	dodeca-2*E*,4*E*-dienoic acid isobutylamide	X	X	X		X	X	X	X	X
24	dodeca-2*E*,4*E*-dienoic acid 2-methylbutylamide								X	X
n/a[b]	dodeca-2*E*,4*E*,8*E*,10*Z*-tetraenoic acid isobutylamide	X	X	X						

[a] Peak number presented in Table 1 [b] Found only in *Echinacea* dietary supplements; no number given in Table 1

acid isobutylamide and dodeca-2*E*,4*Z*,10*Z*-trien-8-ynoic acid isobutylamide were detected and confirmed on the basis of their fragmentation patterns. Also, pentadeca-2*E*,9*Z*-diene-12,14-diynoic acid 2-methylbutylamide (m/z 300) and dodeca-2*E*,4*E*-dienoic acid 2-methylbutylamide (m/z 266) were identified.

The products containing *E. purpurea* roots were usually blended with the aerial parts of the plant. One product contained only a root extract of *E. purpurea*. The alkylamides identified are consistent with those in the root material analyzed previously. Of the two alkylamides found only in *E. purpurea* roots, undeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide was identified in this commercial product. This was confirmed by comparing the MS/MS fragmentation of the same alkylamide in the plant material. On the basis of the range of products used in this study, the most common product available in the Canadian marketplace appears to be the blended *E. purpurea* roots and aerial parts. After blending, the presence and levels of alkylamides vary significantly between different products.

In conclusion, a UFLC-DAD-MS/MS method was developed for the qualitative analysis of alkylamides in *Echinacea* root materials and commercial products. This method is successful in separating and identifying alkylamides in two different plant species, giving insight into the alkylamide profile that could be used in the future as a diagnostic tool for species differentiation. It will be necessary to analyze additional samples and build a database representing the chemical diversity of *E. angustifolia* and *E. purpurea*. The main drawback of this method is that the double-bond configuration cannot be verified by mass spectrometry, although on the basis of elution order and previously published data, the alkylamides that have not previously been reported using LC-MS have been tentatively identified. This method is superior to previously published methods as the alkylamide profile in *E. angustifolia* is presented, which has only previously been characterized by LC-DAD, allowing for minor and coeluting alkylamides to be identified. The alkylamides present in the commercial products that contained pure root extracts were comparable with those in the root materials.

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