High performance liquid chromatography/electrospray ionization mass spectrometry for simultaneous analysis of alkamides and caffeic acid derivatives from *Echinacea purpurea* extracts

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Abstract:

Extracts of the plant *Echinacea purpurea* are widely used for medicinal purposes. Effective quality control of these extracts requires rapid methods to determine their chemical composition. A new method for analysis of caffeic acid derivatives and alkamides from *Echinacea* extracts has been developed. With this method, isomeric isobutylamides and 2-methylbutylamides can be distinguished, a capability that previously published methods have lacked. Quantitative analyses carried out with this method on *E. purpurea* extracts that have been stored for 18 months indicate that they contain caftaric acid, cichoric acid, and undeca-2Z,4E-diene-8,10-diynoic acid isobutylamide at concentrations of 0.7, 0.71 and 2.0 mg/mL, respectively.

Keywords: Echinacea purpurea | Phenolics | Alkamides | Alkylamides | Isobutylamides | Caffeic acid derivatives | Cichoric acid | Caftaric acid | Dodeca-2E,4E,8Z,10Z-tetraenoic acid | HPLC | Electrospray ionization mass spectrometry | ESI-MS | Quadrupole ion trap

Article:

1. Introduction

Preparations of the medicinal plant *Echinacea purpurea* are widely used for the treatment and prevention of upper respiratory infections [1]. It is estimated that approximately $300 million are spent on such preparations annually in the US alone [2]. *Echinacea* is believed to act by modulating the activity of the immune system. A number of constituent classes may be responsible for this immunomodulatory activity, including polysaccharides and glycoproteins, alkamides, and caffeic acid derivatives [3]. Recent studies also suggest that the compound melanin may contribute to the activity of *Echinacea* [4]. Of the compounds present in *Echinacea*, alkamides (also known as alkylamides) are of particular importance because they are the major constituents of the ethanol/water extracts of *Echinacea* that are widely used for therapeutic purposes. These constituents are also of interest because they are likely to cross the intestinal barrier [5], and because they are found in the bloodstream of patients who ingest *Echinacea* extracts [6]. Furthermore, recent studies suggest the involvement of alkamides in the in vitro [7] and in vivo [8] immunomodulatory activity of *Echinacea* extracts. Caffeic acid derivatives are also found in ethanol/water extracts of *E. purpurea*. These constituents are worthy of study because they are unique to particular species of *Echinacea* and can, therefore, be used for authentication and quality control of extracts and plant samples[9]. It has also been proposed that they play a role in the anti-inflammatory activity of *Echinacea* preparations[3].

A number of methods have previously been developed for the analysis of constituents from *E. purpurea*extracts. Typically, these techniques require two separate analyses for the determination of these two different constituents classes. For example, investigators have employed reversed phase HPLC coupled to UV spectrophotometric detection [10], coulometric electrochemical detection [11], or electrospray ionization mass spectrometric detection [12] for the determination of alkamides. Caffeic acid derivatives from *E. purpurea* extracts have been analyzed using reversed phase HPLC [13] or capillary electrophoresis [14] with photodiode array UV spectrophotometric detection.

Recently, methods to determine both caffeic acid derivatives and alkamides in a single analysis have been developed. While it is more difficult to optimize the separation of such diverse constituents in one analysis, methods for the simultaneous determination of caffeic acid derivatives and alkamides are advantageous because they reduce both the time and sample size required for the analyses. The simultaneous analysis of caffeic acid derivatives and alkamides from *E. purpurea* has been accomplished using gradient elution reversed phase HPLC with photodiode array UV spectrophotometric detection [9] and [15] or electrospray ionization mass spectrometric detection [16]. Capillary electrophoresis using photodiode array UV spectrophotometric detection has also been employed for the simultaneous analysis of caffeic acid derivatives and alkamides from *E. purpurea* extracts [17]. To accomplish separation of these constituents with capillary electrophoresis, sodium dodecyl sulfate and hydroxypropyl-β-cycoldetrin were used as additives, and a Britton–Robinson buffer (10 mM, pH 8.0) was employed [17].

Like some of the methods that have formerly been published, the method presented in this paper facilitates the determination of both caffeic acid derivatives and alkamides with a single reversed phase HPLC separation. This method is novel in several ways. First, a two-stage gradient elution is employed so that the caffeic acid derivatives elute during the first stage of the analysis, followed by the alkamides. The sequential elution of these two different constituent classes is advantageous because it allows the parameters of the mass spectrometer to be optimized for these structurally diverse compounds during the time period in which they elute. The previously published HPLC/electrospray ionization mass spectrometry method for simultaneous analysis of caffeic acid derivatives and alkamides required switching between positive and negative cone voltages throughout the analysis [16], which can lead to losses in stability and sensitivity. A second novel aspect of the method presented here is that identifications of constituents made on the basis of retention time and mass-to-charge ratio (*m*/*z*) of the molecular ion are confirmed using MS–MS fragmentation patterns. This is possible because the mass spectrometer used in the work described here employs an ion trap mass analyzer, which provides the capability to generate fragmentation patterns of selected precursor ions by collisionally induced dissociation. Previous studies used a mass spectrometer with a single quadrupole mass analyzer [12] and [16], which does not provide MS–MS spectra. The structures and masses of characteristic caffeic acid derivative and alkamide MS–MS fragments can be used for structural elucidation of constituents from unknown samples, and provide more conclusive structural information than was possible with previously published HPLC/ESI-MS methods [12] and [16].

2. Experimental

2.1. Preparation and storage of extracts

Ethanol/water extracts of fresh *E. purpurea* roots were obtained from Horizon Herbs (Williams, OR). These extracts were prepared at the Horizon Herbs facility as follows. Fresh dormant roots of authentic *E. purpurea*plants (cultivated on the Horizon Herbs farm) were washed thoroughly and weighed to the nearest 1 g. A 1:2 extract (1 g of plant material: 2 mL solvent) was prepared with a final ethanol content of 50% (accounting for water from the plant material as well as the solvent). The solvent used for the extraction consisted of 75% grain alcohol (190 proof or 95% pharmaceutical grade ethanol) and 25% filtered water. The plant material and solvent were thoroughly blended using a commercial grade blender and allowed to macerate for a period of 14 days. The extract was then expressed from the plant material using a hydraulic press and filtered. Small aliquots of this extract were sent to the analytical laboratory at the University of North Carolina Greensboro for analysis. Two batches of extracts, one prepared in the fall of 2002 and one in the fall of 2003, were analyzed. Prior to analysis, the extracts were stored under ambient light in amber bottles with screw caps at room temperature (approximately 22 °C). These storage conditions were chosen to match as closely as possible those used for storage of extracts that are used for medicinal purposes. At the time of analysis, one extract had been stored for 6 months and the other for 18 months.

2.2. Analysis of extracts with HPLC and ESI-MS

An aliquot (1 mL) of each extract was centrifuged to pellet particulate matter and the supernatant was filtered with a 0.2 μm filter prior to analysis. The extract was then diluted 10-fold in a solvent of 50% ethanol (95% HPLC grade ethanol from Fisher Scientific) and 50% nanopure water (filtered with a nanodiamond water purification system from Barnstead, Dubuque, IA, USA). The content of this dilution solvent was chosen to match that of the extract to minimize precipitation.

The analysis was carried out with reversed phase HPLC coupled to electrospray ionization mass spectrometry (ESI-MS). The HPLC system used for the separation was an HP1100 (Agilent, Palo Alto, CA, USA) with a short, narrow bore C18 column (50 mm × 2.1 mm, 3 μm particle size, 110 Å pore size, Prevail packing, Alltech, Deerfield, IL, USA). A 0.5 μm precolumn filter (MacMod Analytical, Chadds Ford, PA, USA) was attached to the column inlet to remove any particulate caused by precipitation of the samples in the HPLC solvents. All samples were run in triplicate with an injection volume of 5 μL. The flow rate was set to 0.2 mL/min and the gradient used was as follows (where A = 1% acetic acid in nanopure water and B = HPLC grade acetonitrile): t = 0–4 min, 90% A (10% B); t = 4–15 min, 90–60% A (10–40% B); t = 15–30 min, 60–40% A (40–60% B); t = 30.1–35 min, 0% A (100% B); t = 35.1–43 min, 90% A (10% B).

The outlet of the HPLC column was directly connected to the electrospray ionization source of an ion trap mass spectrometer (LCQ Advantage, Thermofinnigan, San Jose, CA, USA). The mass spectrometer was operated with a scan range of 150–1000 m/z, a capillary temperature of 275 °C, a sheath gas pressure of 40 arb, and spray, capillary, and tube lens voltages of 4.5 kV, 10 V and 50 V, respectively. It was tuned in the positive ion mode using a solution of caffeine (m/z 195). The mass spectrometer was operated in the negative ion mode the first 15 min of the analysis, then switched to the positive ion mode for the remainder. The total analysis time was 43 min.

MS–MS analyses were accomplished using the data dependent acquisition capabilities of the Xcalibur data system (Thermo Finnigan, San Jose, CA, USA, version 1.2). With this method of MS–MS, the scan mode alternates between full scan MS and MS–MS of the most intense peak from the full scan mass spectrum. In this way, both MS and MS–MS spectra are obtained for each major component of the extract as it elutes from the column. The instrument was again operated in the negative ion mode for the first 15 min of the analysis and then switched to positive ion mode. The collision energy was set at 35%.

2.3. Isolation of an alkamide standard

The standard to be used for quantification of alkamides was isolated from a 100% ethanol *Echinacea* extract using HPLC with fraction collection. An HPLC (Shimadzu, USA, LC10AT vp) with a UV photodiode array detector and a Haisil 100 C18 column

(150 mm × 100 mm, 5 μm particle size, 100 Å pore size, Higgins Analytical, Mountain View, CA, USA) was used. The injection volume for this analysis was 250 μL and the flow rate was 1.5 mL/min. The separation was accomplished isocratically with a solvent composition of 50% A, 50% B (where A = 1% trifluoroacetic acid in water and B = 1% trifluoroacetic acid in acetonitrile). The peak that eluted from the column at 16.5 min was collected and analyzed using the same HPLC/ESI-MS method described for analysis of alkamides above. One major peak was observed in the chromatogram resulting from the HPLC/ESI-MS analysis of the standard compound with a molecular ion at a *m*/*z* value of 230.2. This peak was identified on the basis of previous literature [3] and [12] as undeca-2Z,4E-diene-8,10-diyonic acid isobutylamide (alkamide 4 in Fig. 1). The collected fractions from several separations were combined in a preweighed centrifuge tube and the solvent was removed under vacuum to determine dry weight. They were then redissolved in ethanol at a concentration of 1 mg/mL (4.3×10^{-3} M).

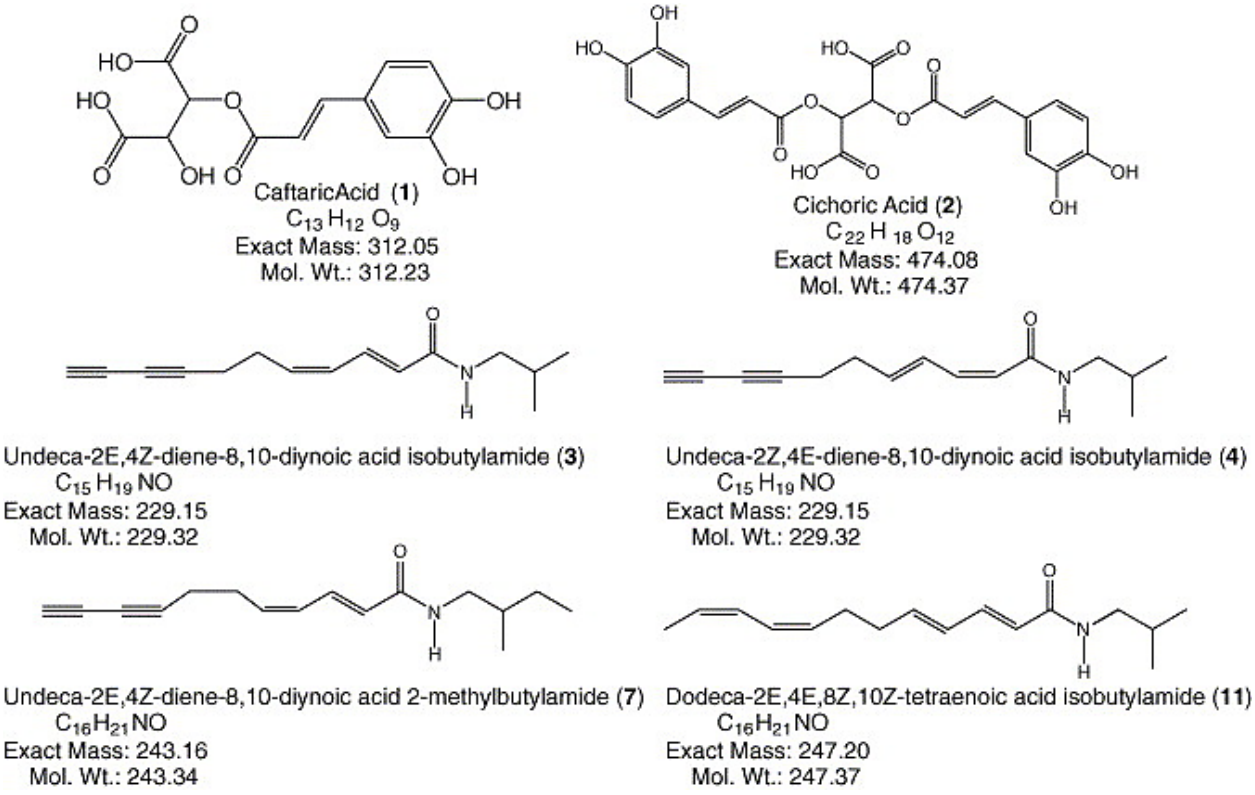

Fig. 1. Structures of selected caffeic acid derivatives and alkamides present in *Echinacea purpurea* extracts. A more comprehensive list of compounds identified can be found in Table 1.

2.4. Preparation and analysis of standards

Standards for the caffeic acid derivative compounds caftaric acid, chlorogenic acid, and cichoric acid (see structures in Fig. 1), all of which are found in *E. purpurea*, were purchased from Chromadex (Santa Ana, CA, USA). Stock solutions of caftaric acid and chlorogenic acid were prepared at concentrations of 1×10^{-2} M in ethanol. A stock solution of cichoric acid, which has

poor solubility in neat methanol, was prepared at a concentration of 5×10^{-3} M in 50% ethanol (reagent grade alcohol, Fisher Scientific, USA) and 50% nanopure water. Using these stock solutions and the stock solution of the alkamide undeca-2Z,4E-diene-8,10-diyonic acid isobutylamide (isolated from *E. purpurea* as described in Section 3.3), an equimolar mixture of standards was prepared at a concentration of 5×10^{-4} M. This stock solution was stored in a polypropylene centrifuge tube at −20 °C until time of use, when it was serially diluted to make five standard mixtures at concentrations of 2×10^{-4} M, 1×10^{-4} M, 5×10^{-5} M, 2×10^{-5} M and 1×10^{-5} M.

The five calibration solution mixtures were analyzed using the methods described for analysis of extracts in Section 3.2. The standards were analyzed on the same day as the extracts to minimize errors that might be introduced due to day-to-day variability in instrumental response. Using the data obtained from these analyses, calibration curves were prepared for caftaric acid (compound **1**, Fig. 1), cichoric acid (compound **2**, Fig. 1), and undeca-2Z,4E-diene-8,10-diynoic acid isobutylamide (compound **4**, Fig. 1). These curves were plotted as peak area of the selected ion chromatogram for the ion of interest versus concentration.

A stock solution of standard dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide (Chromadex, Santa Ana, CA, USA) was also prepared at a concentration of 2.0×10^{-4} M in ethanol. This solution was stored at +4 °C in a polypropylene centrifuge tube, then diluted to a concentration of 1.0×10^{-5} M prior to analysis. Although this compound was not included in the mixture used for quantification, it was used for identification of one of the alkamides in the extract as discussed later.

3. Results and discussion

3.1. Identification of constituents of *E. purpurea* extracts

Two different caffeic acid derivatives and 11 different alkamides were identified in the *E. purpurea* extracts. It is relevant to note all of these compounds were identified in both extracts, even though at the time of analysis, one extract was an entire year older than the other. Structures of the caffeic acid derivatives identified and of several representative alkamides are shown in Fig. 1. A more comprehensive identification is provided in Table 1, along with the masses of ions observed with ESI-MS and MS–MS. Standards were available for caftaric acid (compound **1**), cichoric acid (compound **2**) and dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide (compound **11**), therefore, the identities of the peaks representing these compounds in Fig. 2 were confirmed by matching the MS–MS fragmentation patterns and retention times of the compounds in the extract with those of the standards. Both the masses and the relative intensities for fragments of these standards were identical to those obtained for the compounds from the extracts (see Table 1). For the 10 alkamides for which standards were not available, the identities of the compounds were assigned by comparison of retention time order

and molecular weight of constituents in the *E. purpurea* extracts with previously published literature [12], [16], [18] and [19], and by interpretation of MS–MS spectra, as discussed later on.

Table 1. Assignment of identities of constituents of *E. purpurea* extracts

Number	Compound	Precursor ion (*m*/*z*)	Product ions (*m*/*z*)
1	Caftaric acid	311.1 ($M - H^-$)	149, 179, 135
2	Cichoric acid	473.1 ($M - H^-$)	311, 293, 149, 179, 341
3	Undeca-2E,4Z-diene-8,10-diynoic acid isobutylamide	230.1 ($M + H^+$)	131, 129, 174[a], 91, 157[b]
4	Undeca-2Z,4E-diene-8,10-diynoic acid isobutylamide	230.1 ($M + H^+$)	131, 174[a], 129, 146, 157[b]
5	*Dodeca-2Z,4E-diene-8,10-diynoic acid isobutylamide*	244.1 ($M + H^+$)	145, 188[a], 117, 143, 171[b]
6	Dodeca-2E,4Z,10E-triene-8-ynoic acid isobutylamide	246.1 ($M + H^+$)	147, 145, 173[b], 131, 119, 105, 190[a]
7	Undeca-2E,4Z-diene-8,10-diynoic acid 2-methylbutylamide	244.1 ($M + H^+$)	131, 174[c] 145, 129, 117, 157[d]
8	Dodeca-2Z,4E-diene-8,10-diynoic acid isobutylamide	244.1 ($M + H^+$)	145, 117, 171[b], 143, 188[a]
9	*Dodeca-2,4-diene-8,10-diynoic acid 2-methylbutylamide*	258.1 ($M + H^+$)	145, 117, 188[c] 147, 171[d]
10	Dodeca-2E,4Z-diene-8,10-diynoic acid 2-methylbutylamide	258.1 ($M + H^+$)	145, 117, 171[d],143, 188[c], 128, 202
11	Dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide	248.2 ($M + H^+$)	149, 142, 147, 175[b], 107, 192[a]
12	N.I.	248.2 ($M + H^+$)	166, 149, 167, 107, 175, 93, 121
13	N.I.	262.2	149, 156, 147, 175, 133

		$(M + H^+)$	
14	Dodeca-2E,4E,8Z-trienoic acid isobutylamide	250.2 $(M + H^+)$	167, 177[b], 95, 149, 109, 177, 194[a]
15	Dodeca-2E,4E-dienoic acid isobutylamide	252.1 $(M + H^+)$	179[b], 196[a], 161, 95, 119

Fragments are shown in order of relative intensity in MS–MS spectra. Compounds in italics have been assigned different structures than in previous literature on the basis of MS–MS spectra. E/Z stereochemistry is indicated here in accordance with existing literature [12], [16], [18] and [19], but it should be acknowledged that without conformational NMR spectra, it is not possible to conclusively distinguish between E and Z isomers. [a] Fragment formed by dissociation of the C–N bond of an *isobutylamide* to lose the alkyl group directly attached to the amine ($MH^+ - 56$). [b] Fragment formed by dissociation of the C–N bond of an *isobutylamide* to lose the entire amine functional group ($MH^+ - 73$). [c] Fragment formed by dissociation of the C–N bond of a *2-methylbutlyamide* to lose the alkyl group directly attached to the amine ($MH^+ - 70$). [d] Fragment formed by dissociation of the C–N bond of a *2-methylbutylamide* to lose the entire amine functional group ($MH^+ - 87$).

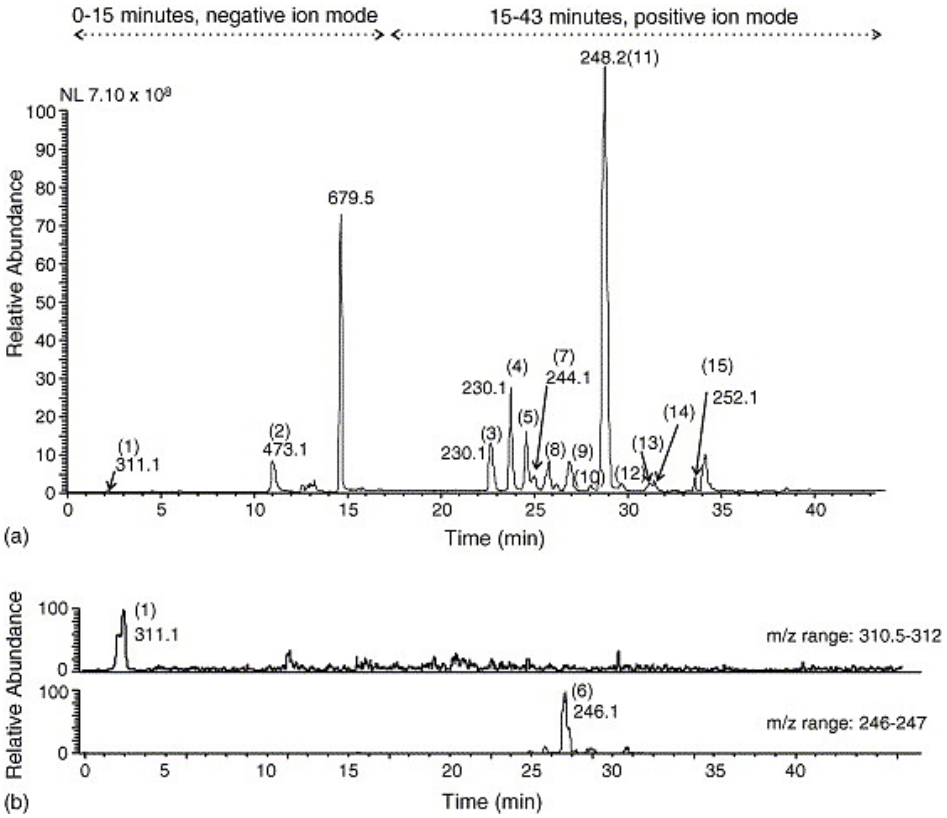

Fig. 2. (a) A typical base peak chromatogram obtained using the method presented in this paper. The mass of the base peak in the mass spectrum is labeled above the major peaks in the

chromatogram. The peak numbers correspond to the compounds in Table 1. The signal during the first 15 min represents the intensity of *negative* ions striking the detector in the mass spectrometer, while the signal between 15 and 43 min represents the intensity of *positive* ions striking the detector. (b) Selected ion chromatograms for some of the minor constituents of the *Echinacea purpurea* extracts. These ions are shown with selected ion chromatograms because their signals are either too weak to be distinguished in the total ion chromatogram (compound **1**) or because they co-elute with other constituents in the *Echinacea purpurea* extract (compound **6**).

Fig. 2 shows a typical base peak chromatogram obtained from an *E. purpurea* extract analyzed using the method presented here. Peaks 1 and 2 represent caffeic acid derivatives, which elute first in the reversed phase HPLC separation because they are more polar than the alkamides. The remaining peaks represent various alkamides. The mass spectrometer is set in negative ion mode for the first stage of the analysis (0–15 min), during which time the caffeic acid derivatives elute from the column. These compounds are detected in the negative ion mode as the deprotonated molecular ion due to their acidic functionality (see Fig. 1). For the second stage of the analysis (15–43 min), the mass spectrometer is operated in the positive ion mode to facilitate detection of the alkamides. These compounds are slightly basic, and are detected in the positive ion mode as protonated molecular ions.

3.2. Mass spectra for the identification of caffeic acid derivatives

Three caffeic acid derivatives are commonly found in *E. purpurea*, caftaric acid, chlorogenic acid, and cichoric acid [3]. Of these, caftaric acid and cichoric acid were identified in the *E. purpurea* extracts studied here. The detection limit for standard chlorogenic acid with this method was approximately 1×10^{-4} M (35 μg/mL). Thus, it can be concluded that chlorogenic acid was not present in the extracts at a concentration above approximately 35 μg/mL.

The electrospray ionization mass spectrum of a typical caffeic acid derivative consists of two peaks, one for the deprotonated molecular ion and another for a proton bound dimer of this compound. For example, Fig. 3a is a mass spectrum corresponding to chromatographic peak (1) in Fig. 2. There are two major ions present in this mass spectrum, one at *m*/*z* 311.2, which corresponds to deprotonated caftaric acid (see proposed structure, Fig. 3a) and another at *m*/*z* 623.1, which corresponds to a proton bound dimer of caftaric acid (a cluster consisting of one neutral caftaric acid molecule bound to one deprotonated caftaric acid molecule).

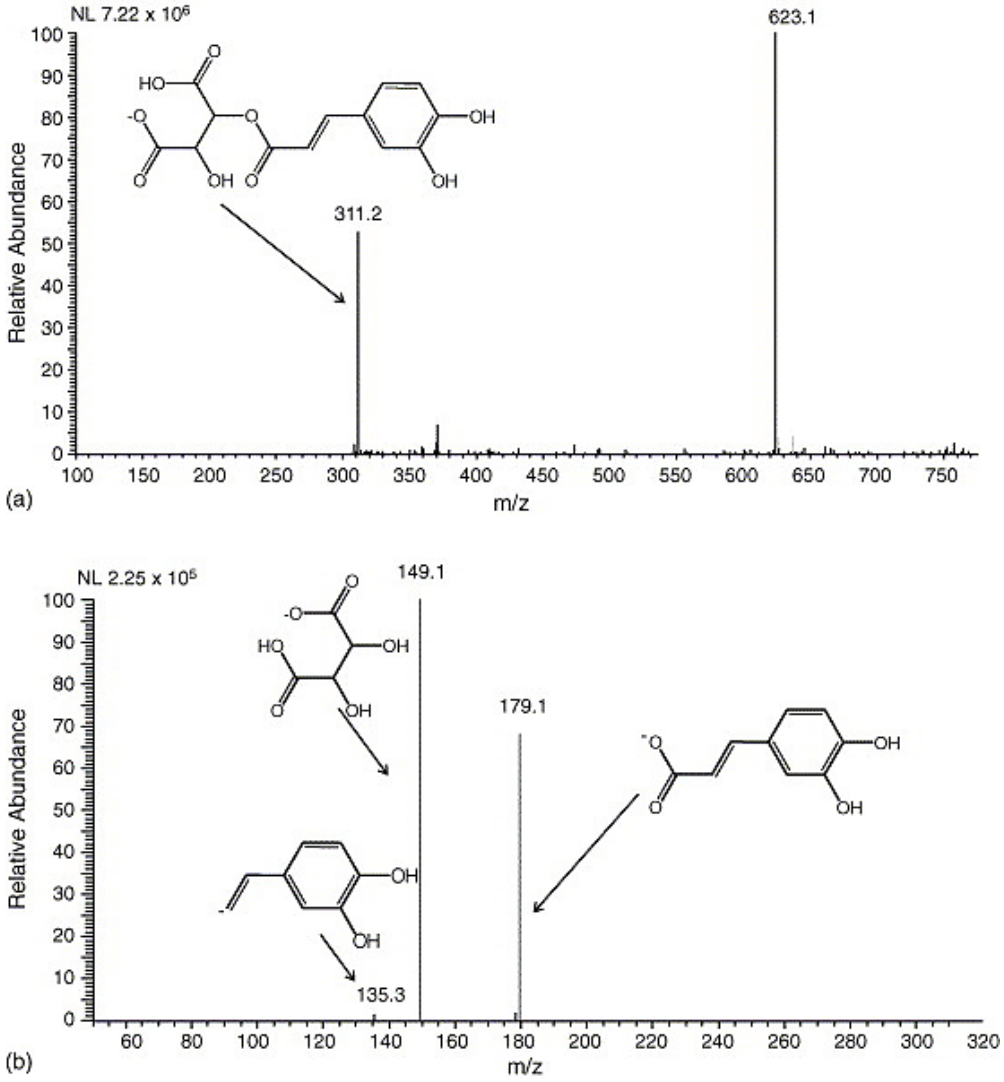

Fig. 3. (a) A mass spectrum of a standard solution of caftaric acid (Chromadex, Santa Ana, CA). This spectrum was obtained using the HPLC method as described in Section 2, using a 5 μL injection of a solution of 1.0×10^{-5} M caftaric acid in ethanol. The mass spectrum consists of two peaks, the first representing deprotonated caftaric acid ($M - H^-$ at *m*/*z* 311.1), and the second a proton bound dimer of two caftaric acid molecules ($2M - H^-$ at *m*/*z* 623.1). (b) The MS–MS spectrum obtained by fragmentation of the ion in spectrum (a) with *m*/*z* of 311.1. This spectrum consists of two major peaks, the tentative structures of which are assigned in the figure. Structural interpretation was accomplished using Mass Frontier Software (Thermo Finnigan, San Jose, CA). The structures of the fragments correspond to the expected results of fragmentation of the weakest bonds in the molecule with collisionally induced dissociation (CID).

The MS–MS fragmentation spectrum obtained from the $M - H^-$ ion of caftaric at *m*/*z* 311.2 is shown in Fig. 3b. The fragments in Fig. 3b are produced by collisionally induced dissociation within the ion trap mass analyzer of the mass spectrometer. Proposed structures of the caftaric acid fragments are shown in Fig. 3b. These structures were assigned with the help of the Mass

Frontier Software (Thermo Finnigan, San Jose, CA). The MS–MS spectra for caftaric acid and cichoric acid from the *E. purpurea* extract (peaks 1 and 2, Fig. 2) matched the MS–MS spectra obtained from standards of these compounds in terms of both relative intensities and *m*/*z* values of fragment ions. See Table 1 for a complete list of the major fragments produced by collisionally induced dissociation of caftaric acid and cichoric acid.

3.3. Mass spectra for the identification of alkamides

Alkamides also produce characteristic mass spectra, as shown in Fig. 4a, which displays the mass spectrum corresponding to peak 11 in Fig. 2. The major ions observed in this mass spectrum correspond to protonated dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide (the structure of the Z isomer is shown) and its proton bound dimer (a cluster of two neutral dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide molecules sharing a single proton). It has previously been reported that it is difficult to separate the E and Z isomers of dodeca-2E,4E,8Z,10E/Z-tetranoic acid isobutlyamide [12]; thus, it is possible that peak 11 in Fig. 2 represents a mixture of these isomers, and it is not possible to say which isomer is represented in the mass spectrum in Fig. 4a. Standard dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide (Chromadex, Santa Ana, CA, USA) was analyzed, and its mass spectrum, MS–MS spectrum, and retention time matched that of peak 11 in Fig. 2.

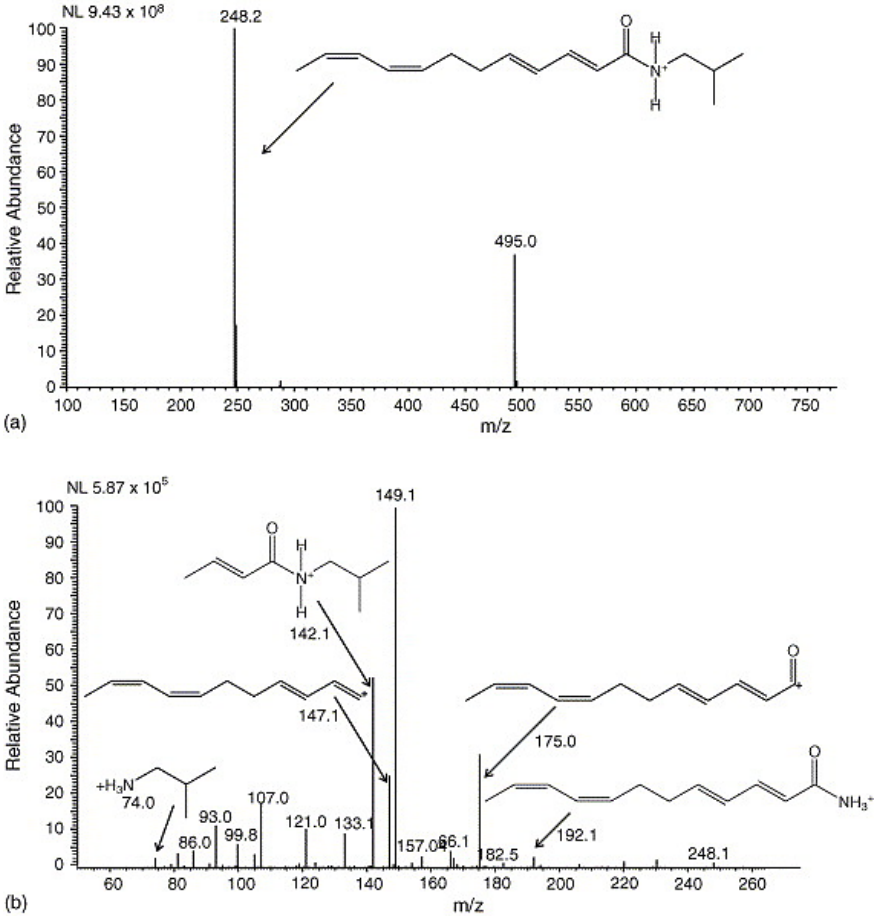

Fig. 4. (a) The mass spectrum of peak 11 in Fig. 2 analyzed with the HPLC/ESI-MS method described in Section 2. The peaks in the spectrum correspond to the protonated form of the compound ($M + H^+$) at *m*/*z* 248.1 and a proton bound dimer ($2M + H^+$) at *m*/*z*495.9. The *m*/*z* values of the protonated molecular ion and the dimer match exactly with those obtained from analysis of a standard of dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide (Chromadex). (b) An MS–MS spectrum obtained from the precursor ion with *m*/*z* 248.2 from an *Echinacea purpurea* extract, the mass spectrum of which is shown in part (a). Both the *m*/*z* values and relative intensities of these fragments match exactly with those obtained by fragmenting the protonated molecular ion of standard dodeca-2E,4E,8Z,10Z-tetraenoic acid isobutylamide at *m*/*z* 248.2. The tentative structures of the fragments were generated using Mass Frontier Software based on the proposed structure of the parent compound. Major fragments are observed due to dissociation of the C–N bonds corresponding to loss of the isobutyl group attached to the nitrogen and loss of the entire amine functionality (see structures in the figure).

The MS–MS fragmentation spectrum produced upon collisionally induced dissociation of the ion with *m*/*z*248 is shown in Fig. 4b. Major sites of fragmentation are the CN bonds of the amide functional group. Fragments corresponding to loss of the alkyl group attached to the nitrogen (*m*/*z* 192 in Fig. 4b) and loss of the entire amine portion of the molecule (*m*/*z* 175 in Fig. 4b) are observed. The structures of these fragments are shown in the figure.

The fragments that result from dissociation of the CN bonds can be expected for any alkamide, and can be used in conjunction with retention order and *m*/*z* of the protonated molecular ion to obtain structural information. There are two types of alkamides that have been identified in *Echinacea*, isobutylamides, in which an isobutyl group is attached to the amide nitrogen (for example, compounds **3**, **4** and **11** in Fig. 1), and 2-methylbutylamides, in which a 2-methylbutyl group is present in place of the isobutyl group (for example, compound **7** in Fig. 1). In many cases, both the isobutyl and 2-methylbutyl amide isomers of a given alkamide are present in the extract. By definition, these isomers have the same mass and cannot be distinguished on the basis of molecular weight of the protonated molecular ion. Therefore, MS–MS spectra are invaluable for distinguishing between them.

MS–MS spectra can be used to distinguish isobutylamides and 2-methylbutlyamides. For any isobutylamide, fragments should be observed in the MS–MS spectrum corresponding to a loss of 56 (for the isobutyl group) and a loss of 73 (for the isobutyl amine). Examples of MS–MS spectra that include the characteristic −56 and −73 fragments are displayed in Figs. 4b and 5a, which represent fragmentation of dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide and dodeca-2,4-diene-8,10-diynoic acid isobutylamide, respectively. For 2-methylbutylamides, fragments corresponding to a loss of 70 (for the 2-methylbutyl group) and a loss of 87 (for the 2-methylbutyl amine) will be observed. An example 2-methylbutylamide MS–MS spectrum that displays these fragments is shown in Fig. 5b.

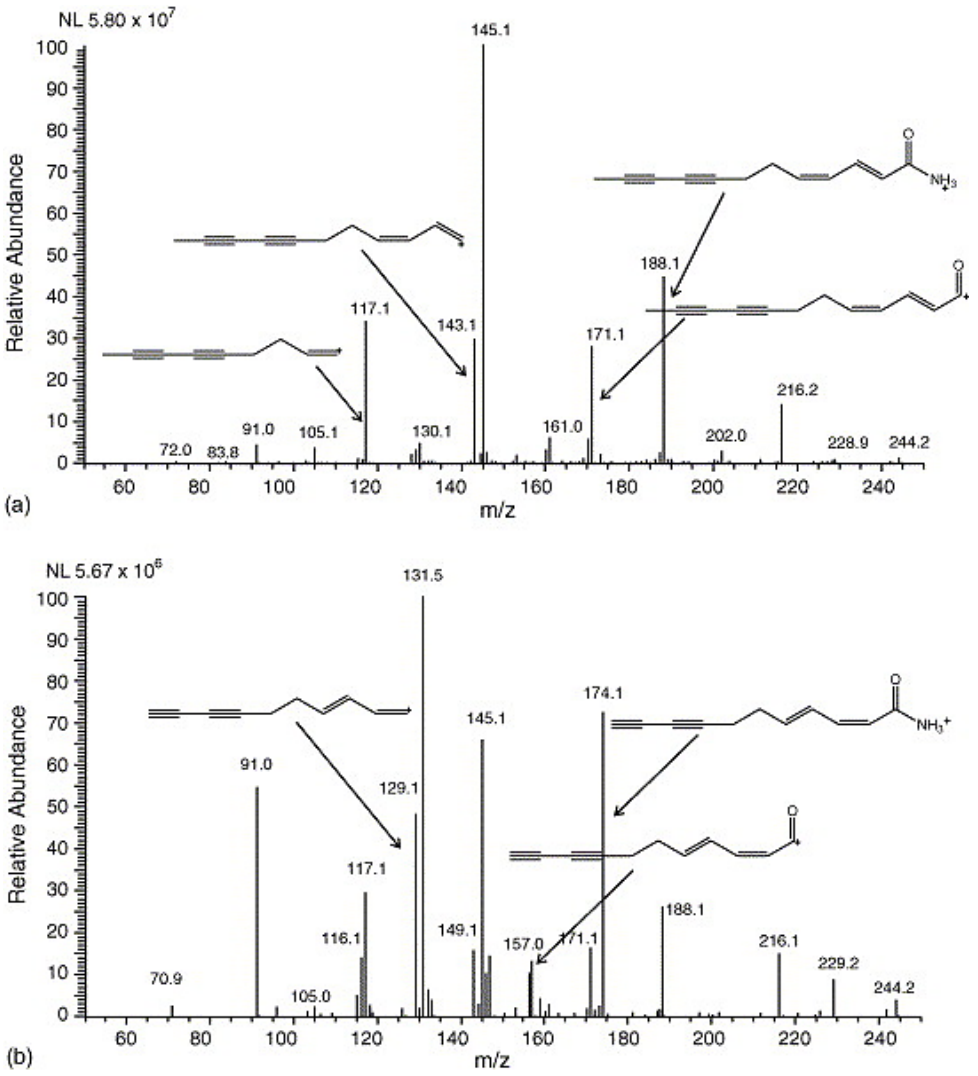

Fig. 5. (a) MS–MS spectrum obtained from the MH^+ ion at *m*/*z* 244 (peak 5 in Fig. 2). This spectrum represents fragmentation of dodeca 2Z,4E-diene-8,10-diynoic acid isobutylamide. Fragments that aid particularly in its identification are the $MH^+ - 56$ ion at *m*/*z* 188 and the $MH^+ - 73$ ion at *m*/*z* 171. With the MS–MS spectrum, this compound can easily be distinguished from the 2-methylbutylamide from *Echinacea purpurea* with the same parent mass (Fig. 5b). (b) MS–MS spectrum obtained from the $M + H^+$ion at *m*/*z* 244 (peak 7 in Fig. 2). This compound can be identified as undeca-2E,4Z-diene-8,10-diynoic acid 2-methylbutylamide. The ions that are particularly helpful in this identification are the $MH^+ - 70$ ion at *m*/*z* 174 and the $MH^+ - 87$ ion at *m*/*z* 157.

It is important to note that the fragments produced by dissociation of the CN bonds are not the only fragments observed in MS–MS spectra of alkamides from *Echinacea*. Quite on the contrary, these compounds produce complex MS–MS spectra (Fig. 4 and Fig. 5) with many peaks that likely arise due to fragmentation of the main carbon chain. The main carbon chains of alkamides have many sites of unsaturation (Fig. 1) and, consequently, would be expected to produce

multiple stable cations upon collisionally induced dissociation. The structures of some of these cations are shown in Fig. 4 and Fig. 5.

The MS–MS spectra generated with the method described herein can be used to correctly identify compounds that have been misidentified in other studies. For example, peak 5 in Fig. 2, which corresponds to an ion with *m*/*z* 244, has been assigned to undeca-2E,4Z-diene-8,10-diynoic acid 2-methylbutylamide in published work [12] and [16]. However, its MS–MS fragments (Fig. 5a) suggest that the true identity of this compound is dodeca-2Z,4E-diene-8,10-diynoic acid isobutylamide, the most recent isobutylamide to be discovered in *E. purpurea* extracts [19]. Fragments of this compound are observed at *m*/*z* 171, representing loss of an isobutyl group, and 188, representing the loss of the isobutylamine. The fragments that would be expected if this compound were a 2-methylbutylamide are absent from its MS–MS spectrum. The MS–MS spectrum for peak 7 in Fig. 2 (Fig. 5b) does demonstrate the expected fragments for undeca-2E,4Z-diene-8,10-diynoic acid 2-methylbutylamide. Peaks at *m*/*z* 174 (for loss of the 2-methylbutyl group) and 157 (for loss of the entire amine) are observed. Thus, on the basis of MS–MS spectra, peak 7 is assigned to undeca-2E,4Z-diene-8,10-diynoic acid 2-methylbutylamide and peak 5 is assigned to dodeca-2Z,4E-diene-8,10-diynoic acid isobutylamide (Table 1).

Another example of the usefulness of MS–MS spectra for the purposes of identity confirmation is provided for the case of compound **9** (Table 1). In a recent publication [16], this compound was identified as trideca-2E,7Z-diene-10,12-diynoic acid isobutylamide. The fragments observed in the MS–MS spectra (Table 1), however, clearly show that this compound is a 2-methylbutylamide and not an isobutylamide. Fragments are observed at *m*/*z* 188 (−70, loss of a 2-methybutly group) and *m*/*z* 171 (−87, loss of 2-methylbutylamine). For this reason, the compound has been tentatively identified here as an isomer of compound **10**, dodeca-2,4-diene-8,10-diynoic acid 2-methylbutylamide.

3.4. Quantification of constituents of *E. purpurea* extracts

Calibration curves were prepared for caftaric acid, cichoric acid (from Chromadex) and undeca-2Z,4E- (isolated from an *E. purpurea* extract). These calibration curves were plotted as area of the selected ion chromatogram for the protonated molecular ion of the compound of interest versus concentration. The linear regression equations and statistical data for the three calibration curves are reported in Table 2. The calibration curves were linear over a range of approximately 2×10^{-5} to 5×10^{-4} M. Above this concentration, loss of linearity was observed.

Table 2. Calibration curve data for standard caffeic acid derivative and alkamides

Compound	Slope (±standard deviation)	Intercept (±standard deviation)	Standard error	Concentration range (M)

Caftaric acid (**1**)	$1.43 \times 10^{11}(\pm 8 \times 10^{9})$	-2.9×10^{6} $(\pm 2 \times 10^{6})$	3.2×10^{6}	2×10^{-5} to 5×10^{-4}
Cichoric acid (**2**)	$1.69 \times 10^{12}(\pm 2 \times 10^{10})$	-1.5×10^{7} $(\pm 2 \times 10^{6})$	3.3×10^{6}	1×10^{-5} to 2×10^{-4}
Undeca-2Z,4E-diene-8,10-diynoic acid isobutylamide (**4**)	$7.35 \times 10^{11}(\pm 3 \times 10^{10})$	6.0×10^{6} $(\pm 3 \times 10^{6})$	4.0×10^{6}	1×10^{-5} to 2×10^{-4}

Calibration curves were plotted as peak area of the selected in chromatogram for the ion of interest vs. concentration (M). Each calibration curve included five data points ($n = 5$).

Instrumental factors such as overfilling of the ion trap mass analyzer or saturation in the ionization process are likely to be major contributors to the loss of linearity observed in calibration curves at high concentrations. Another factor in this loss of linearity is the formation of dimer ions. As shown in Fig. 3 and Fig. 4, dimer ions are commonly observed in the electrospray ionization mass spectra of both caffeic acid derivatives and alkamides. The relative intensities of the ions representing the monomer and dimer vary depending on the concentration of the solution. The dimer ion may be completely absent in very dilute solutions, but its intensity increases dramatically as the solution concentration is increased. Even if the signal for the dimer ion is included in the overall peak area, discrimination by the mass analyzer or differences in ionization efficiency of the monomer versus the dimer could contribute to a loss of linearity at high concentrations where dimer formation is significant. For this reason, it is advisable to dilute extracts prior to analysis such that the concentrations of the compounds of interest fall within the linear range of the calibration curve. Of course, attention must be paid that dilution does not cause minor constituents to become too dilute to be detected. In this study, extracts were diluted 10-fold prior to analysis to achieve responses within the linear dynamic range of the calibration curves.

Quantitative analysis was performed on two different *Echinacea* extracts that had been stored for various lengths of time. The two extract batches were both prepared using the same method, as described in Section 2.1, but extract 1 was prepared in 2002 and extract 2 was prepared in 2003, and they were prepared using different batches of *Echinacea* roots. At the time of analysis, extract 1 had been stored for 12 months and extract 2 had been stored for 6 months (see Section 2.1 for storage conditions and details on methods of analysis).

The results of the quantitative analyses of the extracts are shown in Table 3. As mentioned in the previous section, all of the same alkamides and caffeic acid derivatives listed in Table 1 were identified in both of the extracts. Quantitative data is, however, only provided for the two caffeic acid derivatives and one alkamide for which standards were available at the time of analysis. Because extracts 1 and 2 were prepared from different plant samples in different years, one

would expect to observe some difference in the concentrations of active constituents. This variation in concentration could result from a number of variables besides extract age, including differences in plant water content or in concentration of constituents among different genotypes or phenotypes of *E. purpurea* plants used to prepare the extracts. Given all of these variables, it is remarkable that the profile of constituent concentrations in the two extracts (column 2 versus column 3 in Table 3) is quite similar. It can be concluded from these data that significant concentrations of alkamides and caffeic acid derivatives are present in ethanol/water extracts of *E. purpurea* even after storage for periods of longer than 1 year. However, these data do not rule out the possibility that these constituents degrade somewhat over time. The extent to which degradation of caffeic acid derivatives and alkamides occur in *E. purpurea* extracts could be the subject of future studies. Because extracts of *E. purpurea* that are used for medicinal purposes are commonly stored at room temperature in glass bottles for some time prior to use by the consumer, such studies would seem quite relevant.

Table 3. Quantities of alkamides and caffeic acid derivatives in *E. purpurea* extracts

Compound	Extract #1 (stored for 18 months)		Extract #2 (stored for 6 months)	
	Concentration (±*sx*) (mg/mL)	Concentration (±*sx*) (M)	Concentration (±*sx*) (mg/mL)	Concentration (±*sx*) (M)
Caftaric acid **(1)**	0.7 (±0.2)	$2.4 \times 10^{-3}(\pm 8 \times 10^{-4})$	0.52 (±0.07)	$1.7 \times 10^{-3}(\pm 2 \times 10^{-4})$
Cichoric acid **(2)**	0.71 (±0.09)	$1.5 \times 10^{-3}(\pm 2 \times 10^{-4})$	0.54 (±0.05)	$1.1 \times 10^{-3}(\pm 1 \times 10^{-4})$
Undeca-2Z,4E-diene-8,10-diynoic acid isobutylamide (**4**)	2.0 (±0.07)	$8.7 \times 10^{-3}(\pm 3 \times 10^{-4})$	1.60 (± 0.05)	$7.0 \times 10^{-3}(\pm 2 \times 10^{-4})$

Quantitative analysis was accomplished using the calibration curve data from Table 2. The extracts were diluted 10-fold prior to analysis to adjust the concentrations to within the calibration curve concentration ranges. Standard deviations are for three replicate analyses.

4. Conclusion

In this paper, a novel HPLC/ESI-MS–MS method is presented for the quantitative and qualitative analysis of*E. purpurea* extracts. This method is an improvement on existing methods because MS–MS fragmentation patterns of alkamides and caffeic acid derivatives are taken into consideration when making identifications. Because *E. purpurea* extracts contain many isomeric alkamides, the additional level of certainty in structural elucidation provided by the use of MS–

MS spectra is invaluable. Previous methods of identification that relied solely on the retention time and *m*/*z* value of the protonated molecular ion were more prone to misidentification than is the method presented here.

The results obtained from these studies should be useful to researchers studying the chemical composition and biological activity of *E. purpurea* extracts in several ways. First, new data is provided about the structure of alkamides present in these extracts, and several previously published misidentifications are corrected. Second, the quantitative analyses of *E. purpurea* extracts of various ages published here indicate that alkamides and caffeic acids are still present at significant concentrations in ethanol/water extracts of this plant stored for longer than one year. These findings are important because alkamides and caffeic acid derivatives have been identified as possible active constituents of *E. purpurea*. However, more in-depth studies about stability of alkamides in extracts of *E. purpurea* are warranted. Ideally, these studies would employ a method validated for quantification of the constituents from *E. purpurea* extracts to analyze a single extract at the time it was prepared and then after storage for under a variety of conditions for various lengths of time.

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