PHYTOCHEMICAL ANALYSIS
Phytochem. Anal. **11**, 207–215, (2000)

A Quantitative HPLC Method for the Quality Assurance of *Echinacea* Products on the North American Market

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A rapid and quantitative method of quality assurance for marker phytochemicals in products containing material derived from *Echinacea* species has been developed. In order to assess the efficiency of extraction of phytochemicals from the roots and aerial parts of *Echinacea purpurea* and *E. angustifolia*, a study of solvent mixtures and extraction methods was carried out to determine the recovery of known compounds from plant materials. Ultrasonic extraction of dried samples with methanol:water (7:3) or ethanol:water (7:3) gave good yields of cichoric acid, echinacoside and the alkamides, undeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide and a mixture of dodeca-2*E*,4*E*,8*Z*,10*E/Z*-tetraenoic acid isobutylamides (recoveries of 89%, 85%, 80% and 90%, respectively). The HPLC separation of the phenolic compounds cichoric acid, chlorogenic acid and echinacoside was also improved by careful attention to the pH of the mobile phase. A shortened HPLC column allowed turnaround times of 22 min for phenolic components and 15 min for alkamides with lower solvent use. Assessment of commercial raw materials from the North American market using the new method was useful for confirmation of species and showed a very large variation in concentration of markers in the products sold in this market. Copyright © 2000 John Wiley & Sons, Ltd.

INTRODUCTION

Herbal medicinal products and dietary supplements derived from *Echinacea* spp. are widely used for the treatment of colds and influenza. They have recently become the top-selling herbal products in North America, representing 9.9% of sales (Brevoort, 1995). The same survey showed that the market for herbal products is increasing by at least 15% per year and that there is a demand for higher quality, standardised products along the phytopharmaceutical model.

Echinacea spp. contain a variety of components with demonstrated biological activities, which are suitable phytochemical markers for quality assurance. Several caffeic acid derivatives have been isolated (Cheminat *et al.*, 1988) from hydrophilic fractions of *Echinacea* extracts, including cichoric acid, which is known to have *in vitro* and *in vivo* immunostimulatory properties and also inhibits hyaluronidase, a key enzyme involved in bacterial infection (Bauer, 1998). Cichoric acid was also shown to act *in vitro* as an inhibitor of the enzyme integrase, which is required during human immunodeficiency virus-1 replication to integrate the double-stranded DNA copy of the viral genome into host cells (Robinson *et al.*, 1996). Another caffeic acid derivative, echinacoside, is commonly used by the herbal industry as a marker for *Echinacea* preparations, but this compound does not occur in *Echinacea purpurea* in appreciable concentrations. It does not possess immunostimulant activity, but has weak antibacterial and antiviral effects (Bauer, 1998) and is a protectant against reactive oxygen species (Facino *et al.*, 1995). The lipophilic fraction of *Echinacea* preparations, especially roots, contains numerous alkamides or ketoalken/ynes and polyacetylenes. This fraction also strongly stimulates the immune system *in vitro* and *in vivo* by increasing the phagocytic activity of granulocytes (Bauer, 1998). Recently the lipophilic fraction has been shown to possess a novel light activated toxicity to various *Candida* spp. (Binns *et al.*, 2000). Several alkamides from *E. angustifolia* have been shown to act as inhibitors of cyclooxygenase and lipoxygenase (Müller-Jakic *et al.*,1994), providing a mechanism for the known anti-inflammatory activity of *Echinacea*.

A complication in the phytochemistry of *Echinacea* is that three species (*E. purpurea*, *E. pallida*, and *E. angustifolia*) are commonly used as herbal products and phytomedicines, and either tops or roots may be used. Each of these species has a different phytochemical profile in the roots, leaves and inflorescences (Bauer and Wagner, 1991; Perry *et al.*, 1997). Bauer and Reminger (1989) have provided excellent TLC and HPLC methods

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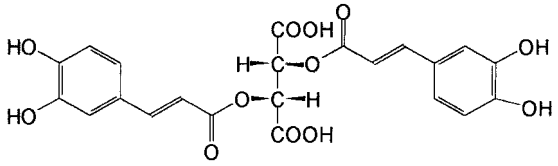
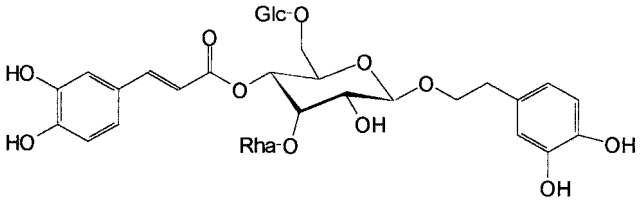
Contract/grant sponsor: Trout Lake Farm, USA. Contract/grant sponsor: Amway Corp, USA. Contract/grant sponsor: The Natural Sciences, Engineering and Council of Canada (NSERC). Contract/grant sponsor: Agriculture and Agrifood Canada.

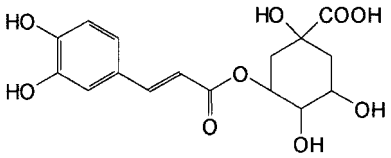
Received 1 February 1999
Revised 28 June 1999
Accepted 1 July 1999



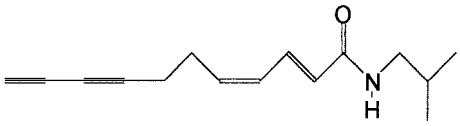
Cichoric acid



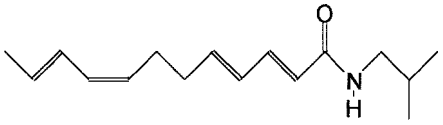
Echinacoside



Chlorogenic acid



1. Undeca-2*E*,4*Z*-dien-8,10-diynoic acid isobutylamide



8. Dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide

9. Dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide

18. Pentadeca-2*E*,9*Z*-dien-12,14-diynoic acid isobutylamide

for differentiating the species and plant parts, and Perry *et al.*, (1997) have examined alkamide levels in different plant parts of *E. purpurea* grown in New Zealand. A GC-MS method (Leinert *et al.*, 1998) and a micellar electrokinetic chromatography method (Pietta *et al.*, 1998) have been developed that can also be used to fingerprint *Echinacea* preparations. Recently Bauer (1997) has examined cichoric acid and alkamide levels in expressed juice of *E. purpurea* obtained from European manufacturers. However, little information is available on currently used commercial *Echinacea* materials (mostly *E. angustifolia* and *E. purpurea*) sold on the North American market.

In the present work, a modern, rapid and fully verified quantitative method for the determination of the amounts of marker phytochemicals in herb and roots of *Echinacea purpurea* and in roots of *E. angustifolia* is provided, together with information on the analysis of commercial raw materials in current use in North America.

EXPERIMENTAL

Plant material. Authentic plant material of *E. angustifolia* and roots of *E. purpurea* were provided by Trout Lake Farm (Trout Lake, WA, USA) from 3-year-old plants. Additional aerial parts of *E. purpurea* were provided by Amway Corporation (Ada, MI, USA). The material was analysed within 6–9 months of the fall harvest in 1998. Species identification of plant material was confirmed by S. Binns (University of Ottawa, Canada) and B. Baum (Agriculture and Agrifood Canada, Ottawa, Canada) using existing taxonomic keys. The dried plants were ground on a La Minerva grinder (Bologna, Italy) and ultrasonic extractions were performed in a Branson 200 sonicator (Danbury, CT USA). Additional raw materials (seven samples of *E. purpurea* roots, 20 samples of *E. purpurea* tops and nine samples of *E. angustifolia* roots) were obtained from commercial suppliers in the North American market. These samples were also from the 1998 harvest.

Extraction. Extraction efficiencies were assessed in various concentrations (50, 60, 70, 80, 90 and 100%) of methanol or ethanol in water. Powdered samples of *E. purpurea* or *E. angustifolia* (0.5 g of leaves or roots) were extracted three times with 8 mL solvent using 5 min ultrasound treatments for each aliquot. Successive aliquots from each sample were pooled and the volume was adjusted to 25 mL. Samples were filtered through 0.22 μm PTFE membranes (Chromatographic Specialities, Brockville, Canada) prior to injection of 5 μL into the HPLC column.

HPLC-UV analysis. The HPLC was performed using a Beckman (Missisauga, Canada) System Gold with a model 502 autosampler, model 126 pump, and model 168 photodiode array detector. The analytical column was a SuperSpher® 100, RP-18 cartridge (75 × 4.6 mm i.d.; 3 μm; BDH, Toronto, Canada) with a SuperSpher 100, RP-18 guard cartridge (4 × 4.6 mm i.d.; 5 μm). Chromatographic conditions for the analysis of alkamides were adapted from Bauer and Reminger (1989) to reflect the shorter column used here. The elution profile consisted of a linear gradient of acetonitrile and water rising from 40% to 80% acetonitrile in 15 min, declining from 80% to 40% acetonitrile in 1 min, followed by a 6 min

equilibration at 40%: the flow rate was 1.0 mL/min. Eluting peaks were monitored simultaneously at 210 and 260 nm, and online UV spectra were collected from 200 to 400 nm for each peak.

The separation of the phenolics was performed on the same column as the alkamides, using the same sample extract, but the mobile phase consisted of 50 mm sodium dihydrogen phosphate adjusted to pH 2.80 with phosphoric acid (solvent A), and 1% 0.1 M phosphoric acid in acetonitrile (solvent B). The elution profile was a linear gradient of 5% to 25% B in 7 min, held at 25% for 2 min, 25% to 5% B in 1 min, and a 5 min equilibration with 5% B: the flow-rate was 1.5 mL/min with detection at 320 nm.

In order to assess recoveries and linearity using these methods, known amounts, as indicated in the figures, of purified standards were added to powdered samples before extraction and the determinations were compared with those produced from unspiked aliquots of the same sample.

The reproducibility of the method was evaluated by examining triplicate aliquots (during studies concerning the optimisation of the extraction solvent) and determination of the coefficient of variation.

Isolation of standards. Dried ground flowers of *E. purpurea* (501 g) were extracted three times with 1.25 L of 70% ethanol in the ultrasonic bath and the bulked extracts evaporated to dryness. The residue was then partitioned between hexane and water. The hexane extract (2 g) was further fractionated by column chromatography (20 × 2 cm i.d.) using silica gel (63–200 μm; Merck, Darmstadt, Germany) and gradient elution (hexane:ethyl acetate; 9:1 to 0:1, using nine step gradients of 70 mL). Undeca-2*E*,4*Z*-dien-8,10-diynoic acid isobutylamide, **1** (cf. Bauer and Reminger, 1989) (30 mg), and pentadeca-2*E*,9*Z*-dien-12,14-diynoic acid isobutylamide, **18** (35 mg), were obtained from fractions IV and III, respectively, whilst fraction V (73 mg) was further fractionated on a column (40 × 1 cm i.d.) of Sephadex LH-20 using methanol (150 mL) as solvent to give dodeca-2*E*,4*E*,8*Z*,10*E/Z*-tetraenoic acid isobutylamide, **8** and **9** (39 mg).

A methanolic extract of the roots of *E. angustifolia* (900 g) was fractionated on an RP-18 open column (10 × 1 cm i.d.) using a methanol:water step gradient (25:75 to 30:70 in 400 mL) to give echinacoside (184 mg). Dried ground leaves of *E. purpurea* (519 g) were extracted with methanol and the extract evaporated to dryness. The residue was dissolved in equal volumes (20 mL) of ethyl acetate and water. The ethyl acetate layer, containing the lipophilic compounds, was removed, the water layer was acidified to a pH 3.5 and an equal volume of ethyl acetate (20 mL) was added. The cichoric acid (80 mg) was found in the ethyl acetate layer.

The purities of all compounds were checked by HPLC using the conditions described above. ^{1}H- and ^{13}C-NMR spectra were measured at 200 and 75 MHz, respectively, in deuterated methanol (cichoric acid and echinacoside) and deuterated chloroform (alkamides) using the solvent peak as reference. Identities were confirmed by comparison of spectral data (^{1}H- and ^{13}C-NMR) with previous literature reports for **1** (Perry *et al.*, 1997), **8** and **9** (Yasuda *et al.*, 1981), echinacoside (Kobayashi *et al.*, 1984), and cichoric acid (Cheminat *et al.*, 1988).

RESULTS AND DISCUSSION

Choice and purity of markers

The specific markers employed were chosen for several reasons. Echinacoside is used to standardise *Echinacea* products on the commercial market by many manufacturers, whilst cichoric acid is recognised to have immunostimulatory properties (Bauer, 1998) and also allowed distinction between *E. angustifolia* and *E. purpurea* species (Bauer and Wagner, 1991). The alkamides **8** and **9** are major compounds in both species, whilst **1** is a major component of the roots of *E. purpurea*, and **18** is a major component of the alkamide fraction of roots of *E. angustifolia* (Bauer and Reminger, 1989). These compounds appear respectively at the beginning (**1**), middle (**8** and **9**) and near the end (**18**) of the elution of the alkamide peaks, thereby providing reference points for the alkamide profile. Alkamide, **18**, was used only as an authentic chromatographic marker and not for recoveries. When the purity of the compounds was assessed by the HPLC method described above, in each case the standard appeared as a single peak on the chromatograms representing >95% of the total area percent of the chromatogram.

Development of an improved extraction method

Experiments showed that the ultrasonic method employed [i.e. sequential 5 min ultrasound treatments of plant material in ethanol:water (7:3)] provided as good, or better, yields of the marker phytochemicals as the standard exhaustive Soxhlet extraction (i.e. 100% methanol at 60 °C for 1 h). The ultrasound method is an improvement because Soxhlet is time consuming, equipment and supply intensive and has the potential to degrade labile phytochemicals. The comparison of Soxhlet and ultrasound extraction at 70% methanol and 100% methanol is provided (Fig. 1) for **8** and **9** and cichoric acid in root and tops of *E. purpurea*, and for **8** and **9** and echinacoside in roots of *E. angustifolia*. Clearly the ultrasound method is efficient for all of these combinations and also leads to improved yields of cichoric acid. Because ultrasound and Soxhlet are equally efficient for other markers, the results suggest that the Soxhlet procedure either poorly extracts, or degrades, a portion of the cichoric acid. The reproducibility of the extractions was also better using the ultrasound method than with the Soxhlet method for all tissues and marker combinations. Reproducibility was especially improved for cichoric acid by the ultrasound method (Table 1).

To arrive at the choice of 70% methanol or ethanol for ultrasound extraction, several solvent concentrations were evaluated in order to determine the best extraction methods for alkamide and phenolic markers (Fig. 2). For the phenolic markers, extraction does not vary between 40% and 70% alcohol and is similar if methanol or ethanol is used. Above 70% alcohol, organic extraction efficiency declines and is less efficient with ethanol than with methanol. For the alkamide markers, 50–70% alcohol content gave the best extraction (Fig. 2). Ethanol:water (7:3) or methanol:water (7:3) mixtures were most effective in isolating both classes of markers. The ethanol extraction was observed to have a lower recovery of polysaccharides, materials

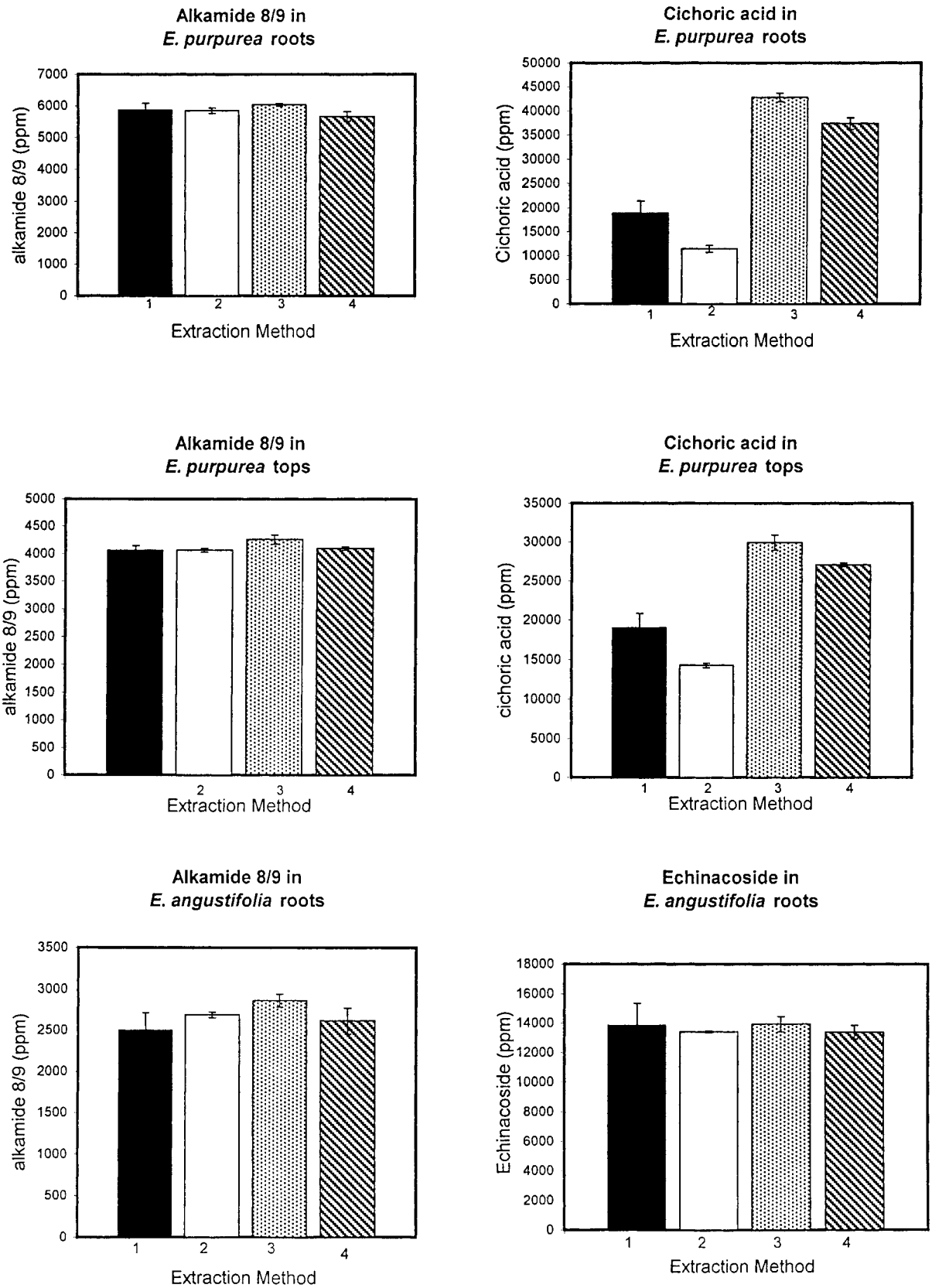


Figure 1. Extraction efficiency of marker phytochemicals (cichoric acid, echinacoside and alkamides **8** and **9**) from various tissues of *E. purpurea* and *E. angustifolia* by different solvents and procedures. 1, Soxhlet with 100% methanol for 60 min; 2, ultrasound with 100% methanol, 3 × 5 min treatments; 3, ultrasound with 70% methanol, 3 × 5 min treatments; 4, ultrasound with 70% ethanol, 3 × 5 min treatments.

Table 1. Coefficient of variation (%) for replicate analyses of cichoric acid, echinacoside and alkamides 8 and 9 by the ultrasound vs. the Soxhlet method using 70% ethanol

	Coefficient of variation (%)					
	Cichoric acid		Echinacoside		Alkamides 8 and 9	
	Soxhlet	Ultrasound	Soxhlet	Ultrasound	Soxhlet	Ultrasound
E. angustiolia root			10.9	3.5	8.5	6.0
E. purpurea root	13.7	3.3			3.9	2.7
E. purpurea top	9.7	0.9			2.2	0.8

which may eventually foul the HPLC column. These extraction results should have implications for manufacturers attempting to formulate alcohol extracts of *Echinacea*.

Other solvents have been successfully used to extract alkamides including chloroform (Bauer and Reminger, 1989) and acetonitrile Perry *et al.* (1997). However, in the present work ethanol was preferred because of its lower potential toxicity to laboratory personnel, and comparable recoveries (determined below) to the other solvents.

Improvement of chromatographic conditions

The use of a short C-18 column with small particle size (75 × 4.6 mm i.d.; 3 m) allowed us to improve the separation and shorten the analysis time for both the phenolic and the alkamide constituents of *Echinacea* (Figs 3 and 4) compared to previous reports. When using the Bauer and Reminger (1989) chromatographic method for phenolic constituents, excellent separations were obtained but occasionally poor peak shapes resulted or no cichoric acid could be observed from

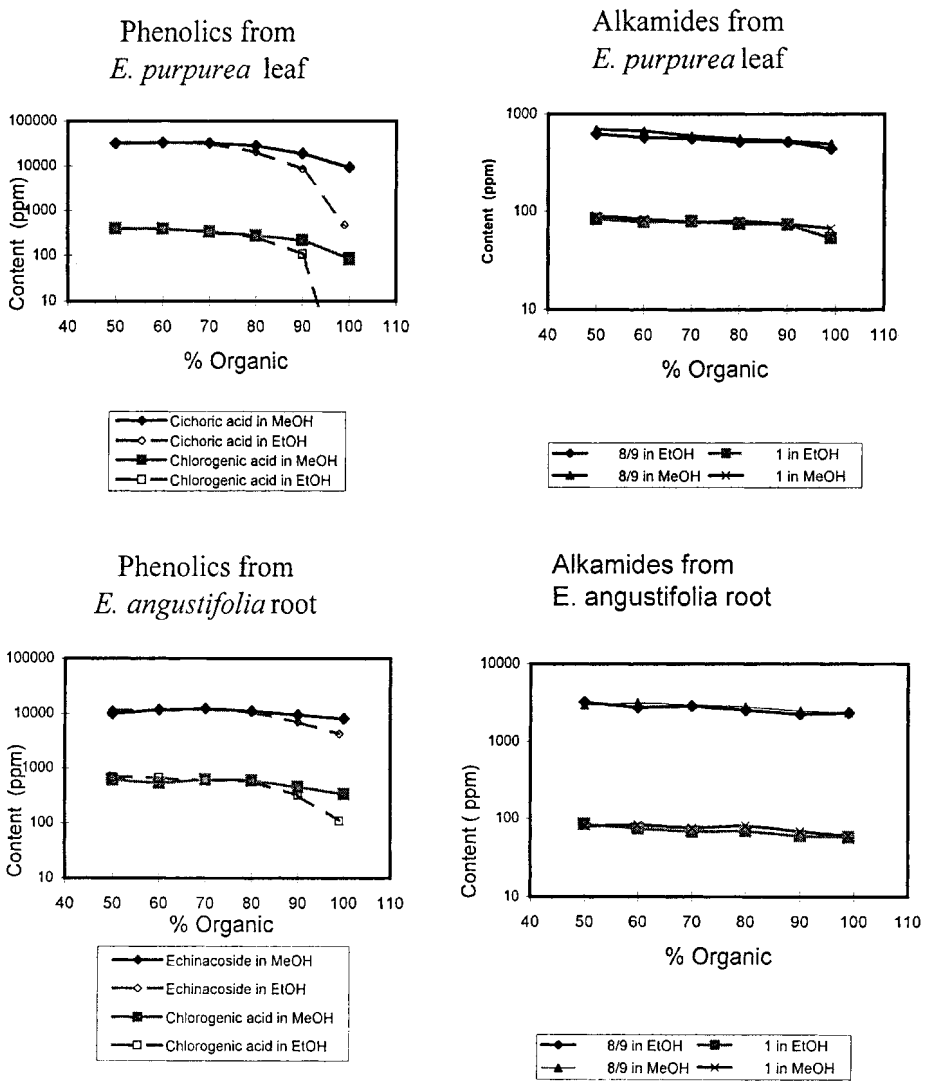


Figure 2. Optimisation of the extraction of marker phytochemicals (phenolics and alkamides) from *E. purpurea* and *E. angustifolia* with the ultrasonic method (3 × 5 min treatments) using different mixtures of ethanol:water or methanol:water.

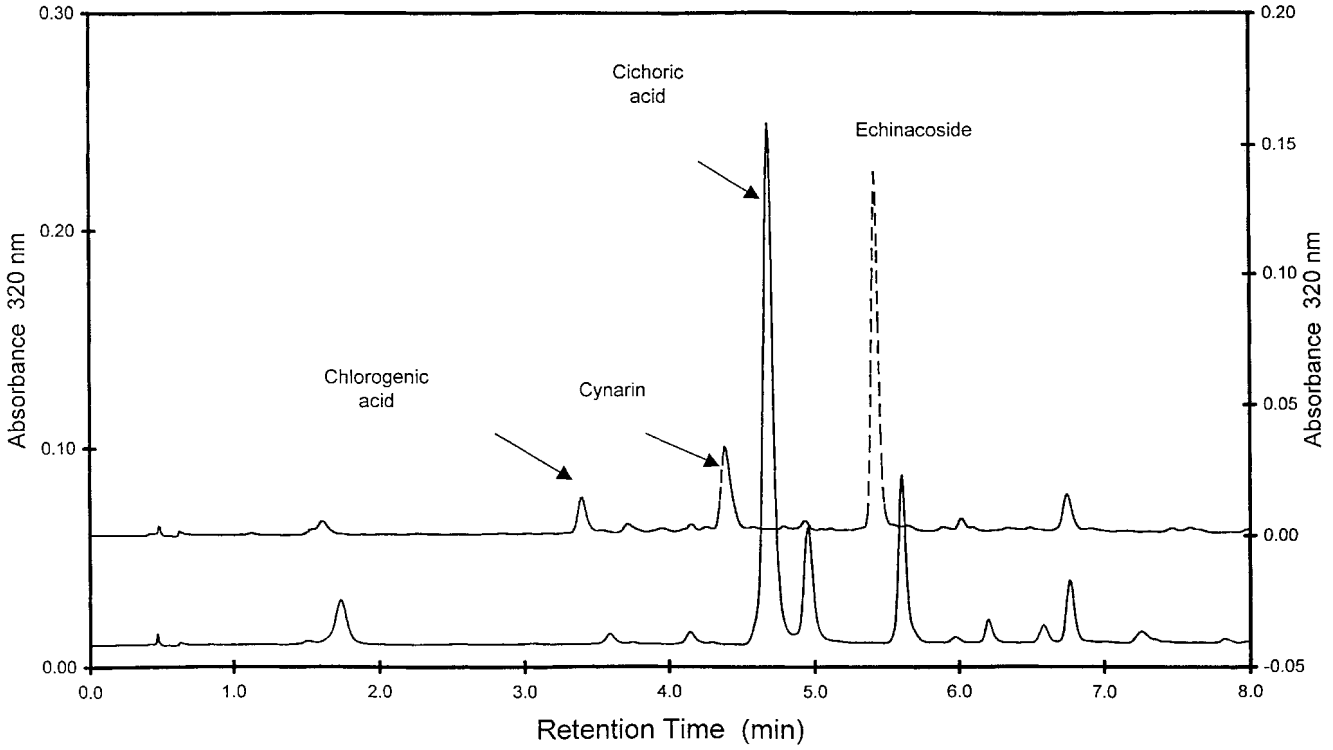


Figure 3. HPLC chromatograms of phenolic compounds derived from roots of *E. angustifolia* (dashed line) and of *E. purpurea* (solid line) detected at 320 nm. (For chromatographic protocols see the Experimental section.)

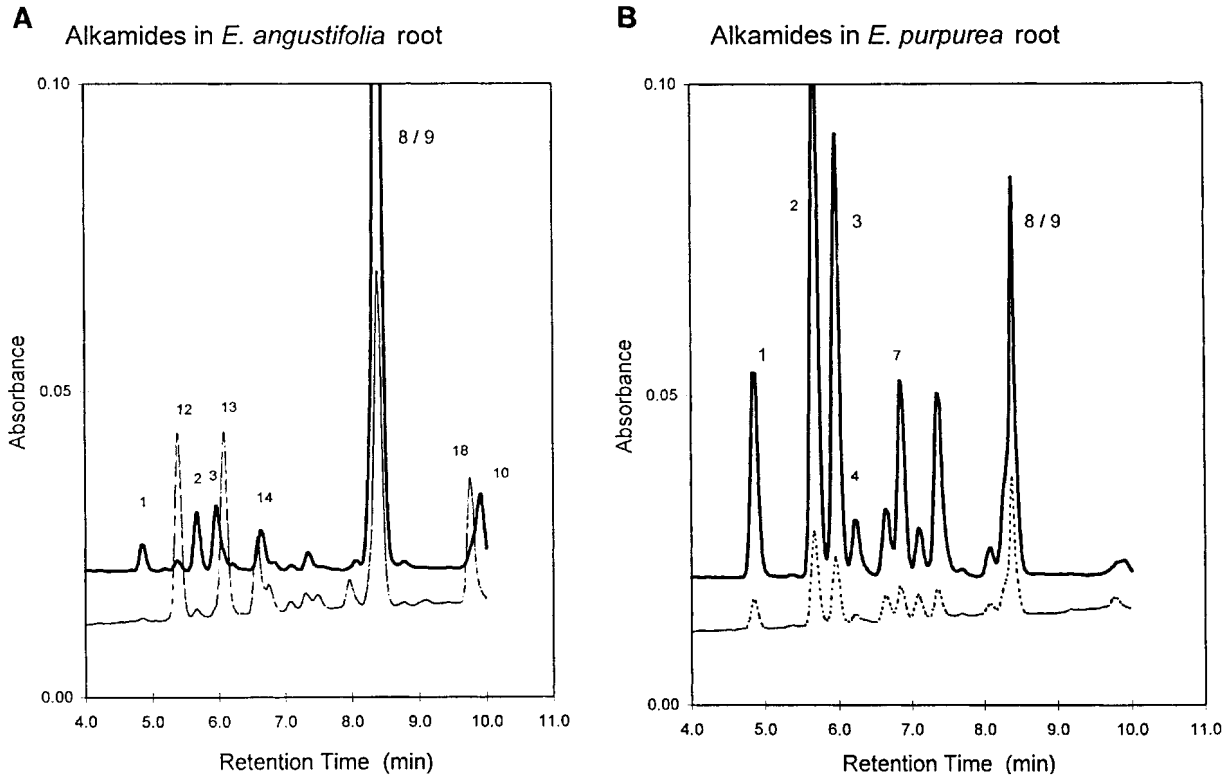


Figure 4. HPLC chromatograms of alkamides from roots of (A) *E. angustifolia* and (B) *E. purpurea*: detection at 210 nm (dashed line) and 260 nm (solid line). Key to peak identity: **1**, undeca-2E, 4Z-dien-8,10-diynoic acid isobutylamide; **2**, undeca-2Z, 4E-dien-8,10-diynoic acid isobutylamide; **3**, dodeca-2E, 4Z-dien-8,10-diynoic acid isobutylamide; **4**, undeca-2E, 4Z-dien-8,10-diynoic acid 2-methyl-butylamide; **7**, dodeca-2E, 4Z-dien-8,10-diynoic acid 2-methyl-butylamide; **8**, dodeca-2E, 4E, 8Z, 10E-tetraenoic acid isobutylamide; **9**, dodeca-2E, 4E, 8Z, 10Z-tetraenoic acid isobutylamide; **10**, dodeca-2E, 4E, 8Z, trienoic acid isobutylamide; **12**, undeca-2E-en-8, 10-diynoic acid isobutylamide; **13**, undeca-2Z-en-8, 10-diynoic acid isobutylamide; **14**, dodeca-2E-en-8, 10-diynoic acid isobutylamide; and **18**, pentadeca-2E, 9Z-dien-12,14-dienoic acid isobutylamide. (For chromatographic protocols see the Experimental section).

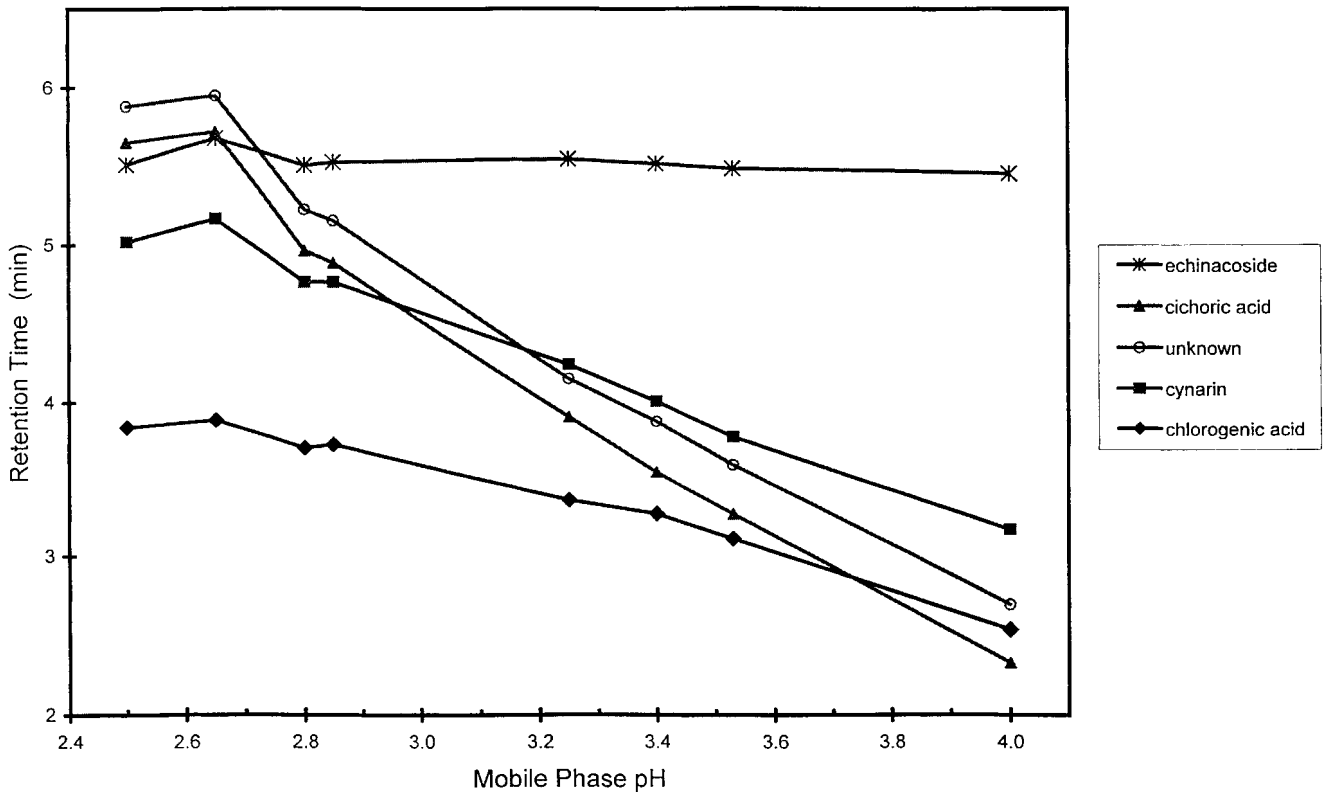


Figure 5. Effect of changes of the pH of the mobile phase (solvent A) on retention time of phenolics derived from the root of *E. angustifolia*. The best separation was at pH 2.80. (For chromatographic protocols see the Experimental section).

samples which ought to contain large amounts. The problem was traced to variable amounts of acids in the experimental extracts. In order to obtain reproducible chromatograms, it is necessary to buffer the mobile phase against the acidic extracts: 50 mm phosphate buffer is sufficient to buffer the acids found in a 5 μL injection. Because it has two carboxylic acid moieties, the retention time of cichoric acid can be adjusted relative to other phenolic monocarboxylic acids in the extracts by varying the pH of the mobile phase (Fig. 5). A pH of 2.80 was chosen since it provided good peak shapes (minimal tailing) and satisfactory resolution from potentially interfering peaks. In particular, samples of *E. angustifolia* normally contained a peak tentatively identified as cynarin (based on the on-line spectrum and relative retention time), which is eluted (when using pH 2.80) just before cichoric acid. Since either or both may be present in a given sample, it is important to maintain good separation between these peaks. From Fig. 5 it may be seen that the retention time of echinacoside is not pH-dependent, since echinacoside is not as readily ionised as carboxylic acids. Cichoric acid has two carboxylic acid moieties which are more ionized as the pH is raised from 2.5 to 4.0. The additional negative charge dramatically reduces the retention time of cichoric acid on the C18 column. Chlorogenic acid and cynarin both have a single carboxylic acid group, and exhibit retention time displacements induced by pH similar to, but less than, those shown by cichoric acid.

Identity assurance of markers by spiking experiment

The identities of putative markers in the chromatograms were confirmed by spiking experiments in which the addition of (for example) authentic echinacoside to an *E. angustifolia* extract showed increase of the marker peak without the appearance of shoulders or split peaks.

Linearity and recoveries

The relationship between peak area and the concentration of the standard injected was found to be highly linear for the **1** at 210 nm, **8** and **9** at 260 nm, and echinacoside and cichoric acid at 320 nm, all with r^2 values >0.99 [Fig. 6(A–C)].

The recovery experiment is critical because of the labile nature of the phytochemical markers. It is the most often neglected aspect of phytochemical analysis due to the small amounts of standards that are normally available owing to difficulties of isolation and purification. Reliable recovery estimates over a range of concentrations were made by adding different amounts of the standard to the plant preparation [Fig. 6 (D–F)]. Recoveries were 89% for cichoric acid, 85% for echinacoside, 80% for **1**, and 90% for **8** and **9**.

Evaluation of commercial samples

Because of the availability of the three alkamide standards, the whole alkamide profile of roots could be recognised with certainty, allowing separation of *E. angustifolia, E. purpurea* (and *E. pallida*) for various commercially grown North American raw materials submitted to our laboratory for analysis. A few samples gave alkamide profiles that were not consistent with their designated species and subsequent examination of the plant voucher confirmed that the species assignment was

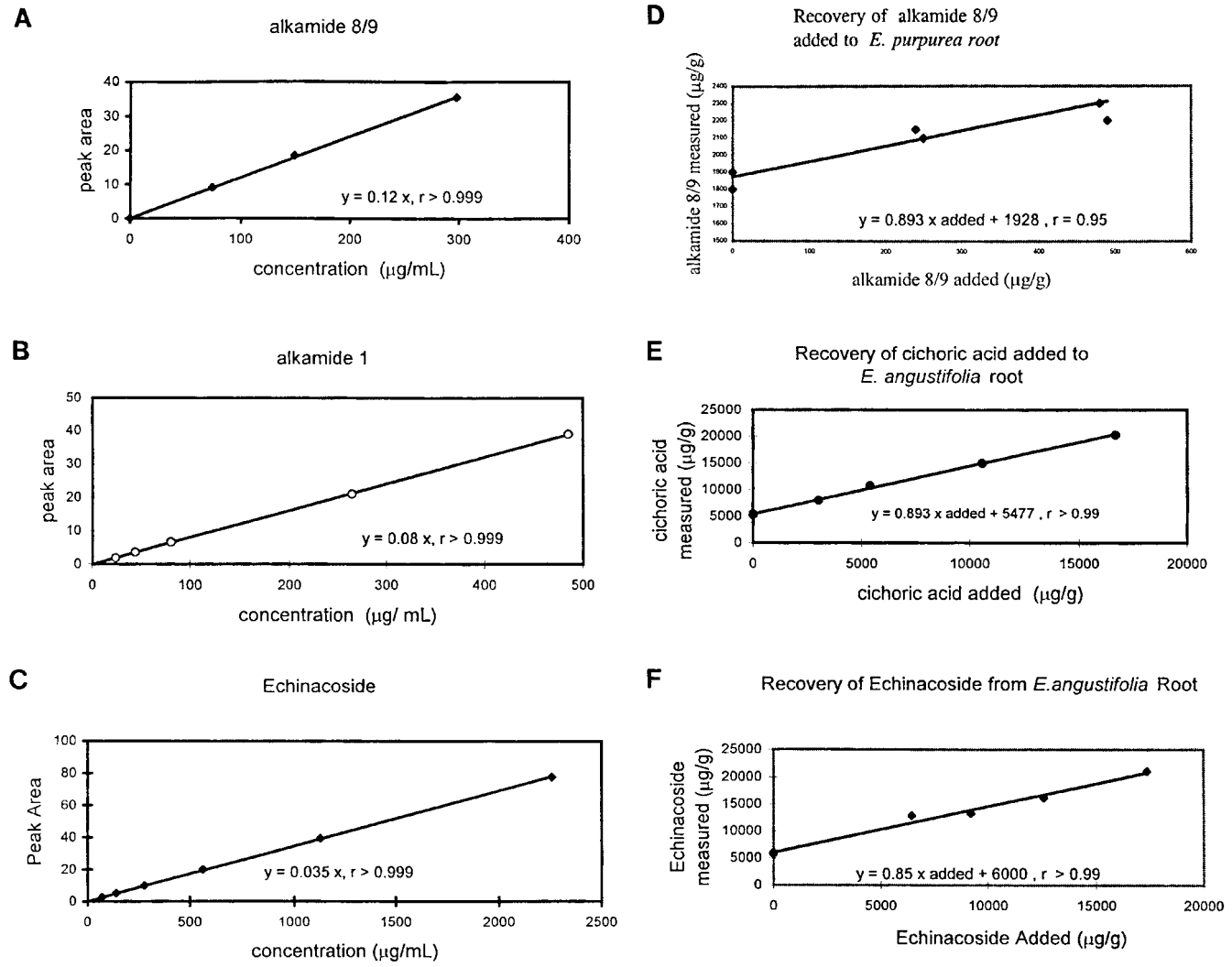


Figure 6. (A–C) The linearity of peak area vs. concentration for the standard alkamides **8** and **9**, and **1** and for standard echinacoside, respectively. (D–F) Recovery experiments illustrated for different amounts of alkamides **8** and **9** added to a root preparation of *E. purpurea*, and of cichoric acid and echinacoside added to a root preparation of *E. angustifolia*, respectively.

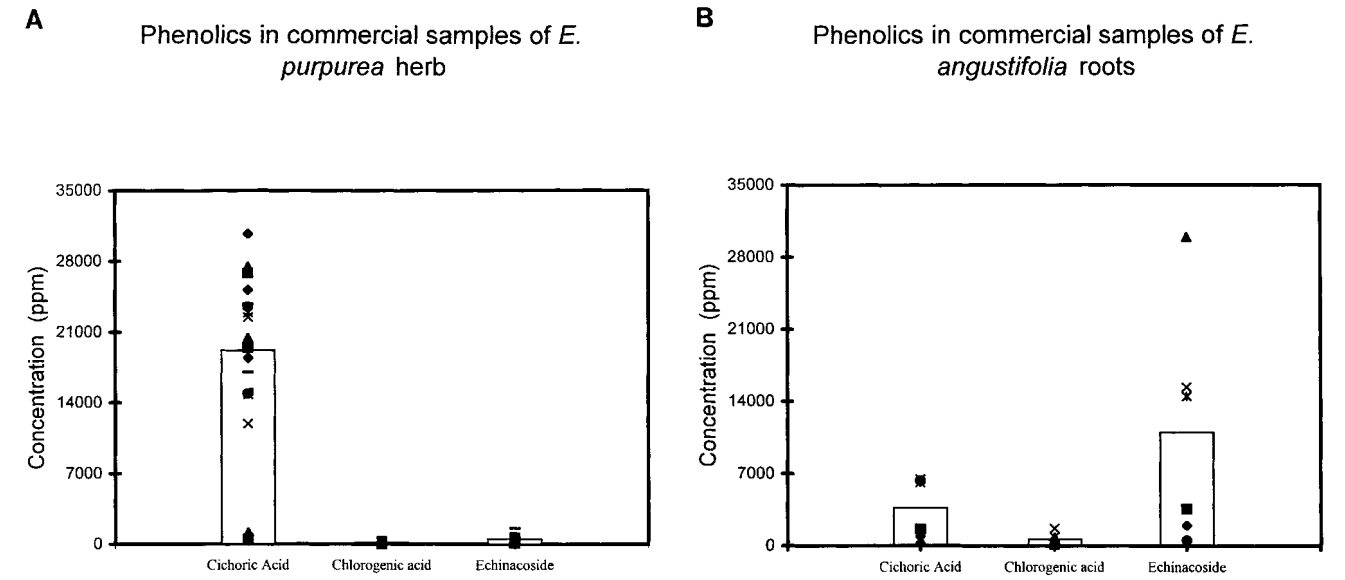


Figure 7. Distribution of content of marker phenolics in various commercial samples of (A) *E. purpurea* herb and (B) *E. angustifolia* roots from the North American market.

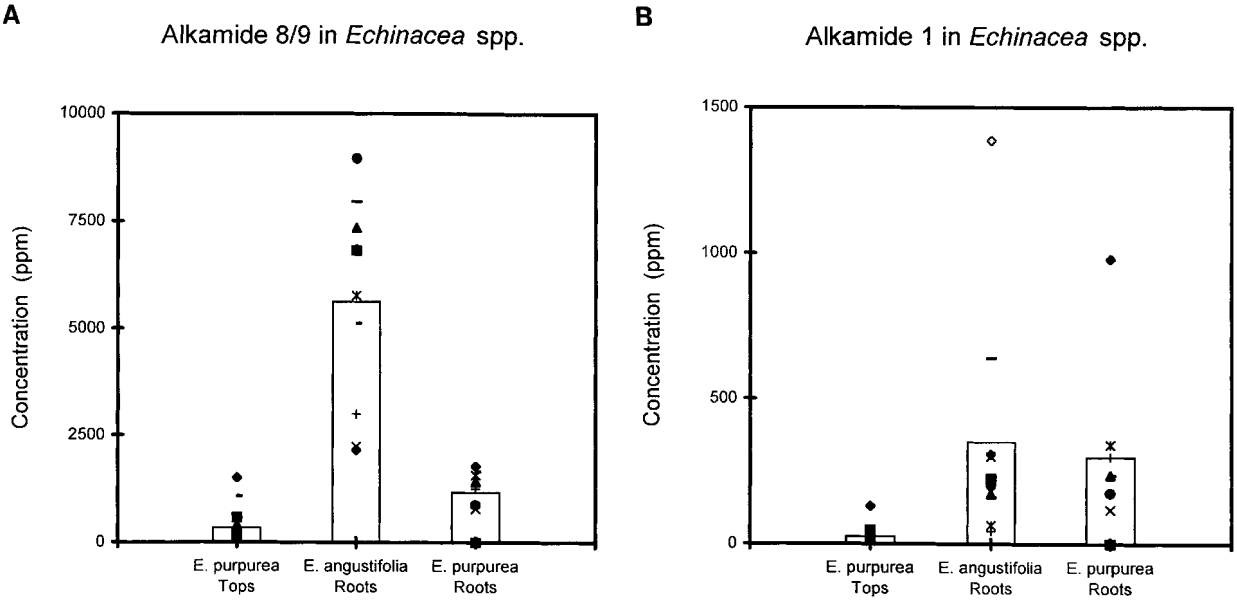


Figure 8. Distribution of content of marker alkamides (A, alkamide **8** and **9**; B, alkamide **1**) in various commercial samples of *E. purpurea* herb and roots and *E. angustifolia* roots.

wrong. Conversely, some authentic *E. angustifolia* roots contained small amounts of cichoric acid, tentatively identified by spiking and online UV comparisons with standards. Cichoric acid is not previously reported from this source and warrants further study in North American accessions. After removal of the misidentified samples, analysis of representative commercial dried plant samples from the North American market was completed using the methodology developed here, and the results are presented in Figs 7 and 8. There is a very large range of concentrations for each of the markers not only between species, but within species as well. This may result from environmental or genetic variation, variation in plant parts used and in preservation of principles after harvest. It is clear that the North American market is far from producing a homogenous high quality product at this early stage of development. In Europe, Bauer (1997) also found a large range in the concentration of cichoric acid (0–0.4%) and alkamides (0.1–1.8 mg/mL) in expressed juice products. Taken together, Bauer's study and our results show the need for better quality control of *Echinacea* products.

Acknowledgements

This study was supported by Trout Lake Farm, Amway Corp., the Natural Sciences Engineering and Council of Canada (NSERC) and Agriculture and Agrifood Canada.

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