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Simultaneous analysis of caffeic acid derivatives and alkamides in roots and extracts of *Echinacea purpurea* by high-performance liquid chromatography–photodiode array detection–electrospray mass spectrometry

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Abstract

High-performance liquid chromatography (HPLC) coupled with UV photodiode-array detection and electrospray ionization mass spectrometry was developed for the simultaneous analysis of caffeic acid derivatives and alkamides in the roots and extracts of *Echinacea purpurea*. Caffeic acid derivatives and alkamides produced very abundant peaks in the total ion current chromatogram during negative and positive cone voltage switching. Cichoric acid and the isomer pair, dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide, were used as a standard for quantification of caffeic acid derivatives and alkamides in *E. purpurea*. This novel method surpasses previously published ones in product quality control and providing the HPLC chromatographic fingerprints of biological active components in *E. purpurea*.

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1. Introduction

Extracts of *Echinacea* species, primarily *E. purpurea* (L.) Moench., *E. angustifolia* DC., and *E. pallida* (Nutt.) Nutt, are known to exhibit immunostimulating activities and thus have been widely used for pharmaceutical preparations in Europe, North America and Australia. *Echinacea* preparations are among the best selling medicinal herb products in health food stores in the USA [1]. It has been reported that the immunostimulating properties of *Echinacea* species have been attributed to both the lipophilic and polar fractions of the extracts, including caffeic acid derivatives, alkamides, glycoproteins, and polysaccharides [2,3]. Among caffeic acid derivatives, cichoric acid has been shown to inhibit the replication of hyaluronidase and human immunodeficiency virus type 1 integrase, to protect collagen type from free radical induced degradation and to possess phagocytosis stimulatory activity in vitro and in vivo and antiviral activity [4–10]. Alkamides also exhibit immunostimulating activity and cause inhibi-

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tion of cycloxygenase and 5-lipoxygenase as anti-inflammatory agents [11–14]. Therefore, alkamides and cichoric acid are generally used as indices of quality control for standardized *Echinacea* extract [15]. Fig. 1 depicts the chemical structures of four caffeic acid derivatives and 11 alkamides.

Many HPLC methods have been reported for analyses of *Echinacea* [16–28]. Bauer et al. [16] pioneered the use of reversed-phase HPLC analysis for several caffeic acid derivatives in *Echinacea*; similar HPLC conditions but different extraction methods have also been reported by other researchers [17–21]. Alkamides in *Echinacea* have been isolated and identified by using HPLC or thin-layer chromatography [22–26]. However, the simultaneous analysis of caffeic acid derivatives and alkamides has seldom been reported in the literature. Laasonen et al. [27] analyzed alkamides and caffeic acid derivatives by HPLC, but could not provide molecular mass and structural information for these analytes. Sloley et al. [28] proposed an HPLC method directly coupled to ultraviolet absorbance and electrospray mass spectrometric detectors to analyze different *Echinacea* species. In their method, trifluoroacetic acid was used as mobile modifier, but it can pollute the polyether ether ketone (PEEK) tube of HPLC and ion source of mass spectrometry. In addition, the C_8 column was used as an analytical column; alkamides could not be separated completely.

In the present study, we aimed at developing a novel HPLC–UV photodiode array detection (DAD)–electrospray ionization mass spectrometry (ESI-MS) method for simultaneous analysis of caffeic acid derivatives and alkamides. Caffeic acid derivatives and alkamides were quantified by DAD, while the structure identification was completed by ESI-MS. The method is better than previously published techniques as regards product quality control, and can provide HPLC chromatographic fingerprints of bio-active components in *Echinacea purpurea*.

2. Experimental

2.1. Instrumentation

A Waters (Milford, MA, USA) Alliance 2695 liquid chromatographic system interfaced to a 996 DAD system and a Micromass ZQ 2000 electrospray mass spectrometer was equipped with a Johnson (Dalian, China) Spherigel analytical column (250× 4.6 mm) packed with 5 μm C_{18} silica. Water containing 0.1% formic acid (A) and acetonitrile (B) were used as chromatographic eluents. The gradient elution was programmed as follows: 0–9 min, 10–18.5% B; 9–9.5 min, 18.5–45% B; 9.50–39.50 min, 45–80% B; 39.5–42.0 min, 80–100% B; 42.0–45.0 min, 100–10% B. The flow rate was 1 ml/min. The column temperature was 30 °C. UV spectra recorded were in the range 200–400 nm, while 330 nm was used for quantification of caffeic acid derivatives and 254 nm for alkamides. The Micromass quadrupole mass spectrometer equipped with an electrospray source working at 103 °C was operated in the positive ion mode to generate $[M+H]^+$ and $[M+Na]^+$ ions of alkamides, and in the negative ion mode to generate $[M-H]^-$ ions of caffeic acid derivatives. Nitrogen was used as desolvation gas at a flow rate of 200 l/h. The desolvation temperature was 160 °C. Mass values of 150–800 u were measured. Capillary and cone voltages were 3850 and 60 V for ESI+, and 3050 and 35 V for ESI−, respectively. The eluent was split at the HPLC column end to allow 20% eluent to flow into the mass spectrometer.

2.2. Solvents and chemicals

Water, acetonitrile and methanol (Tedia, USA) of HPLC grade were used for HPLC analysis. Formic acid and phosphoric acid of analytical grade were used as mobile phase modifiers. Reagent-grade chloroform, methanol, ethanol, hexane, and ethyl acetate were used for extraction.

2.3. Plant material and sample preparation

Roots and extracts of *E. purpurea* were commercially obtained (Changsha, China). The plant materials were identified by Professor Jian-Zhong Li, Department of Botany, Hunan Normal University. A 1-g aliquot of each sample was ground in a mill, and then treated ultrasonically twice in methanol–0.1% phosphoric acid (70:30, v/v; 10 ml for each) for 20 min. A 2-ml aliquot of the extract after centrifu-

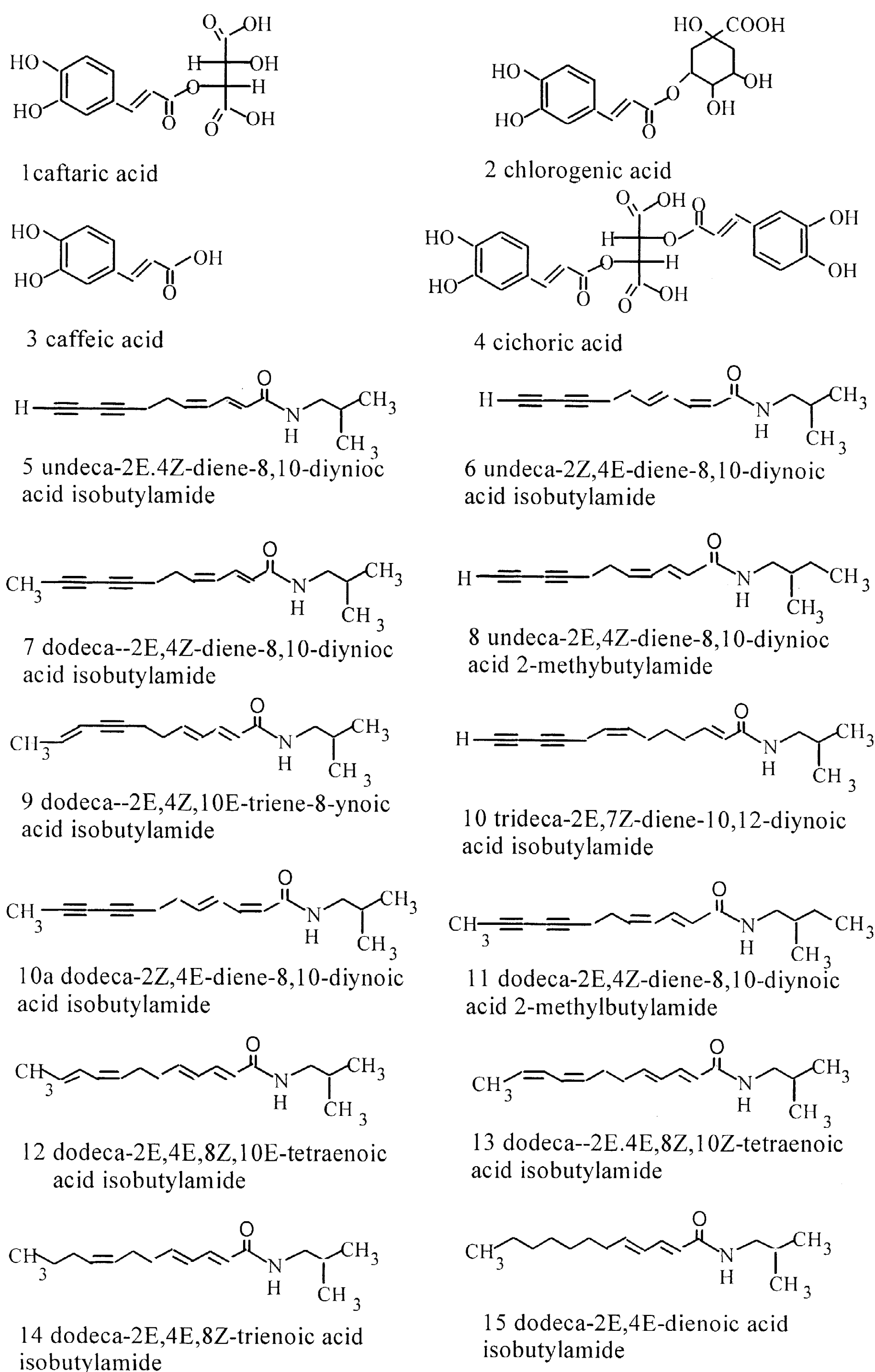

Fig. 1. Chemical structures of typical caffeic acid derivatives and alkamides in *E. purpurea*.

gation was filtered through a 0.45-μm PTFE filter into an HPLC vial for future HPLC analysis.

2.4. Purification of standards of cichoric acid and dodeca-2E,4E,8E,10E/Z-tetraenoic acid isobutylamide

Cichoric acid was isolated from *E. purpurea* extracts. Then 1 g of extracts of *E. purpurea* was ultrasonically treated in 10 ml of 0.1% formic acid aqueous solution. The supernatant after centrifugation was extracted with ethyl acetate (3×30 ml) and a gum obtained after solvent evaporation. The gum was allowed to dissolve in the mobile phase solvent, and the solution was injected into preparative reversed-phase HPLC. Cichoric acid was separated on a Prep Nova-Pak HR C_{18} column of 300×7.8 mm; a mobile phase of acetonitrile–0.1% formic acid aqueous solution (85:15) at 4 ml/min was used. Further purification was conducted by recrystallization in water, and cichoric acid was finally obtained as off-white crystals (98.4% pure by HPLC) [19].

Nusslein et al. [29] reported that cichoric acid is highly susceptible to enzymatic, acidic and/or temperature degradation during its purification and storing. In our experiments, the fraction of cichoric acid collected from preparative HPLC was freeze-dried, and the formic acid was then volatilized in vacuum. Cichoric acid was thus obtained without degradation. However, when phosphoric acid was used as a mobile phase modifier in preparative HPLC, we failed to obtain solid cichoric acid even when the cichoric acid fraction was freeze-dried. This may be ascribed to the acidic degradation effect of cichoric acid, as a synchronous concentration of the non-volatilizable phosphoric acid should increase the mixture acidity to a significant extent during and after freeze-drying.

Alkamides were isolated by combination of silica gel and reversed-phase chromatography using a modified version of the method described by He et al. [25]. Compounds 12 and 13 were separated from a mixture fraction by preparative HPLC (Prep Nova-Pak HR C_{18} column, 300×7.8 nm). The mobile phase was acetonitrile–water (1:1) at flow rate of 4 ml/min. The detection wavelength was 254 nm. The alkamide collection was recrystallized through hexane, giving finally a mixture of 12 and 13 as needles (95.7% pure by HPLC), with UV and MS measurements in accordance with the literature data [30]. Then 2.1 mg of compounds 12 and 13 and 1.9 mg of cichoric acid were dissolved in 10 ml methanol as standard solution, respectively.

3. Results and discussion

3.1. Specification of the method

Fig. 2 shows typical HPLC profiles for caffeic acid derivatives and alkamides in one run under these conditions. Caffeic acid derivatives and alkamides exhibit good responses at 330 and 254 nm, respectively. Under the selected HPLC conditions (Section 2.1) the two kinds of compounds also exhibit a good response of total ion current. A total of 18 peaks numbered as A–R were well separated from one another. Peaks A–E (Table 1) were identified as caffeic acid derivatives, with a retention order identical to the reported HPLC profiles [17,21,31]. Peaks F–R were alkamides, also with retention order in agreement with the reports [22,25], except for peak K which was new. In addition, the retention order of peaks J–M agrees with other reports [24,32]. There are a number of alkamide isomeric pairs in *E. purpurea*. Identification of the isomer is based on the retention order already published [22,25]. Information on identification of peaks A–E is shown in Table 1.

The absorbance at 330 nm responded linearly to the mass of cichoric acid from 600 to 1200 ng, with a detection limit of 40 ng ($S/N=3$). Mean recovery was 98.7% (five parallel trials of additions of 101 μg/g of cichoric acid to *E. purpurea* root extracts). The absorbance at 254 nm was also linear to the mass of compounds 12 and 13 from 300 to 900 ng, with a detection limit of 35 ng ($S/N=3$). Mean recovery was was 95.7% (five parallel trials of additions of 98 μg/g of compounds 12 and 13 to *E. purpurea* root extracts).

3.2. Optimization of HPLC conditions

Formic acid was used as a mobile phase modifier as it significantly restrained the peak tailing of caffeic acid derivatives in our experiments. A similar tailing-reduced effect was found with phosphoric acid, but we avoided its use because of its corrosive

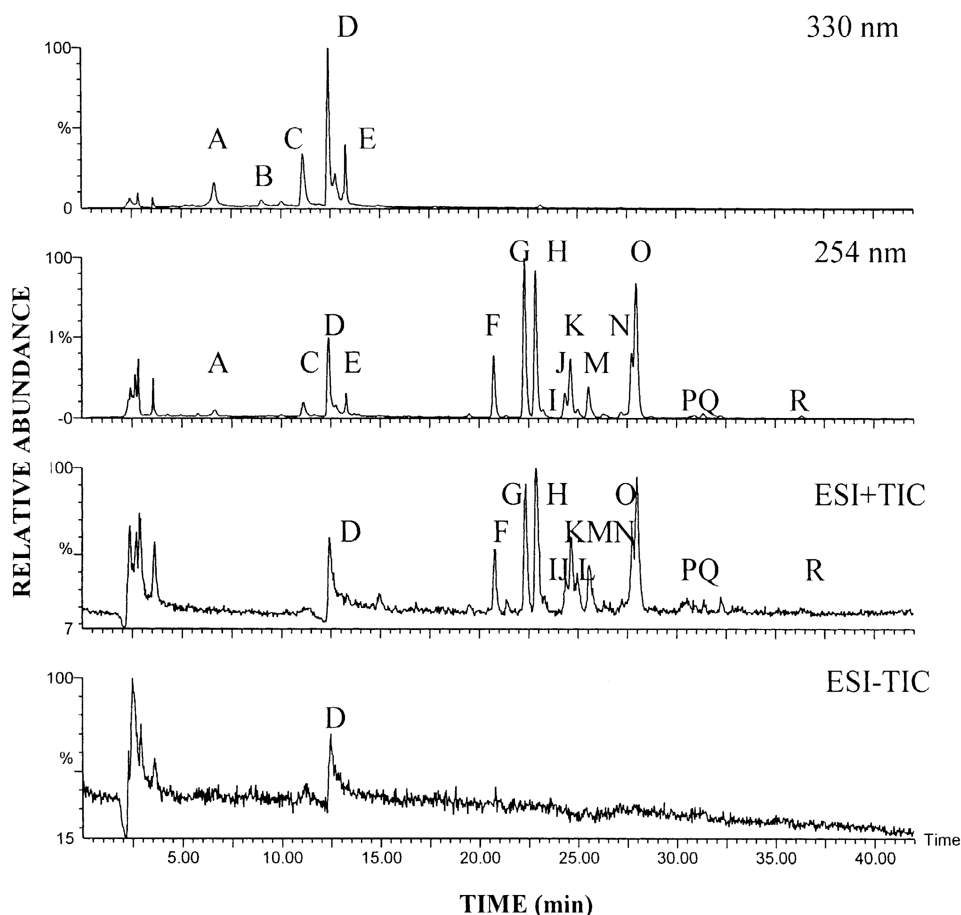

Fig. 2. HPLC–UV–ESI-MS-total ion current (TIC) chromatograms of *E. purpurea* root, with a 1:5 post-column stream splitting. Chromatographic conditions are described in Section 2.1. The peak identifications are given in Table 1.

and polluting effect on the ion source of mass spectrometry.

To determine the optimal elution conditions for the separation and quantification of alkamides and caffeic acid derivatives, various linear gradients of acetonitrile and 0.1% formic acid aqueous solution at a flow rate of 1 ml/min were employed. Under these gradient conditions (0–9 min, 10–18.5% B; 9–9.5 min, 18.5–45% B; 9.5–39.5 min, 45–80% B; 39.5–42 min, 80–100% B; 42–45 min, 100–10% B), peaks A–R could be well separated in a short time, and thus the simultaneous analyses of alkamides and caffeic acid derivatives in the *E. purpurea* roots and extracts could be achieved. The fingerprint of two types of active compounds could also be obtained clearly, as shown in Fig. 2. Other gradient conditions caused a poor separation of some peaks or extended run time.

3.3. Discussion of ESI− and ESI+ for MS identifications of compounds

Table 1 lists the retention times (t_R), maximum absorbance wavelength (λ_{max}), molecular ions and assignment of HPLC peaks. These peaks were identified based on a comparison of the on-line UV and MS information with literature data or with our purified standards.

In addition, selected ion recording (SIR) was investigated in our experiments. The SIR technique was more sensitive than full scanning. Fig. 3 shows the SIR chromatograms of m/z 501, 473 and 311 in

Table 1
Assignment of peaks in Figs. 2–4

Peak No. (Fig. 2)	t_R (min)	m/z $[M-H]^-$	m/z $[M+H]^+$	m/z $[M+Na]^+$	λ_{max} (nm)	Compound (Fig. 1)
A	6.65	311	–	–	329	1
B	9.02	353	–	–	330	2
C	11.12	179	–	–	329	3
D	12.42	473	–	–	330	4
E	13.29	501	–	–	329	N.I.
F	20.75	–	230	252	260	5
G	22.30	–	230	252	258	6
H	22.87	–	244	266	263	7
I	23.24	–	244	266	263	8
J	24.35	–	246	268	263	9
K	24.46	–	244	266	258	10a
L	25.00	–	258	280	263	10
M	25.55	–	258	280	263	11
N	27.74	–	248	270	235, 260	12
O	27.97	–	248	270	235, 260	13
P	30.90	–	262	–	N.D.	N.I.
Q	32.24	–	250	–	N.D.	14
R	36.35	–	252	–	N.D.	15

N.D., not detectable; N.I., not identified.

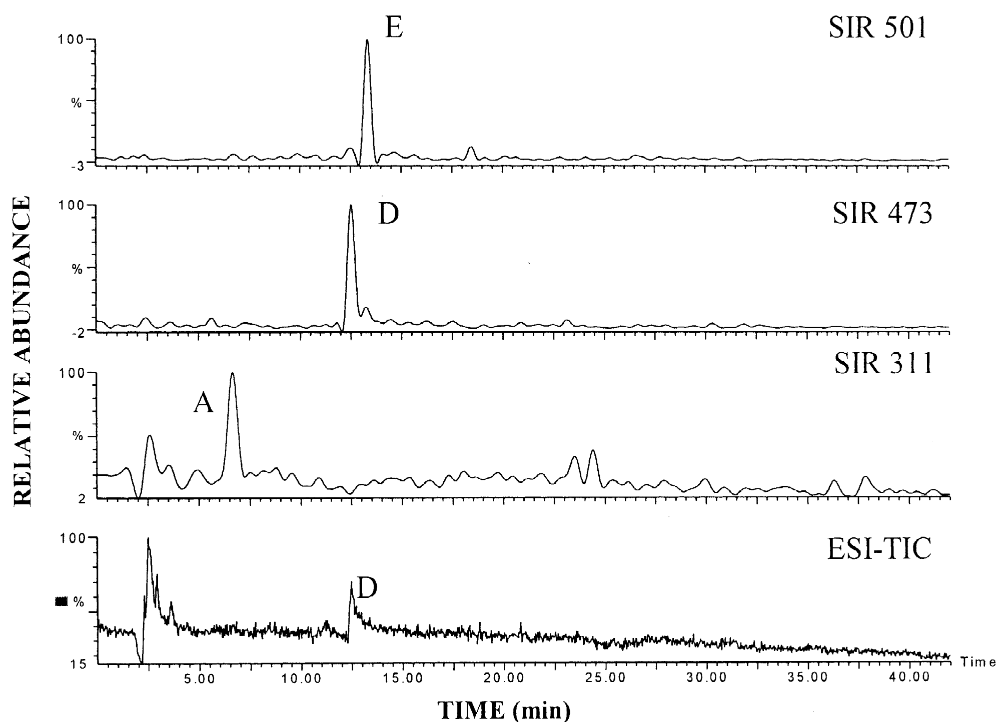

Fig. 3. HPLC–ESI-MS-TIC and HPLC–MS-SIR chromatograms of caffeic acid derivatives of *E. purpurea* root, with a 1:5 post-column stream splitting. Other conditions are the same as in Fig. 2.

the negative ion mode, and Fig. 4 shows the SIR chromatograms of m/z 258, 252, 248, 246, 244 and 230 in the positive ion mode, both with results from the full scanning method for comparison. Obviously, the signal-to-noise ratio value indicates that the SIR mode offered a better sensitivity than the full scanning mode. The MS response signals should come from $[M+H]^+$ or $[M+Na]^+$ ions for the positive ion mode, and $[M-H]^-$ ions for the negative ion mode. We have successfully assigned chromatograms of m/z 473, 311, 258, 252, 248, 246, 244, and 230, but that of m/z 501 remains unknown at present.

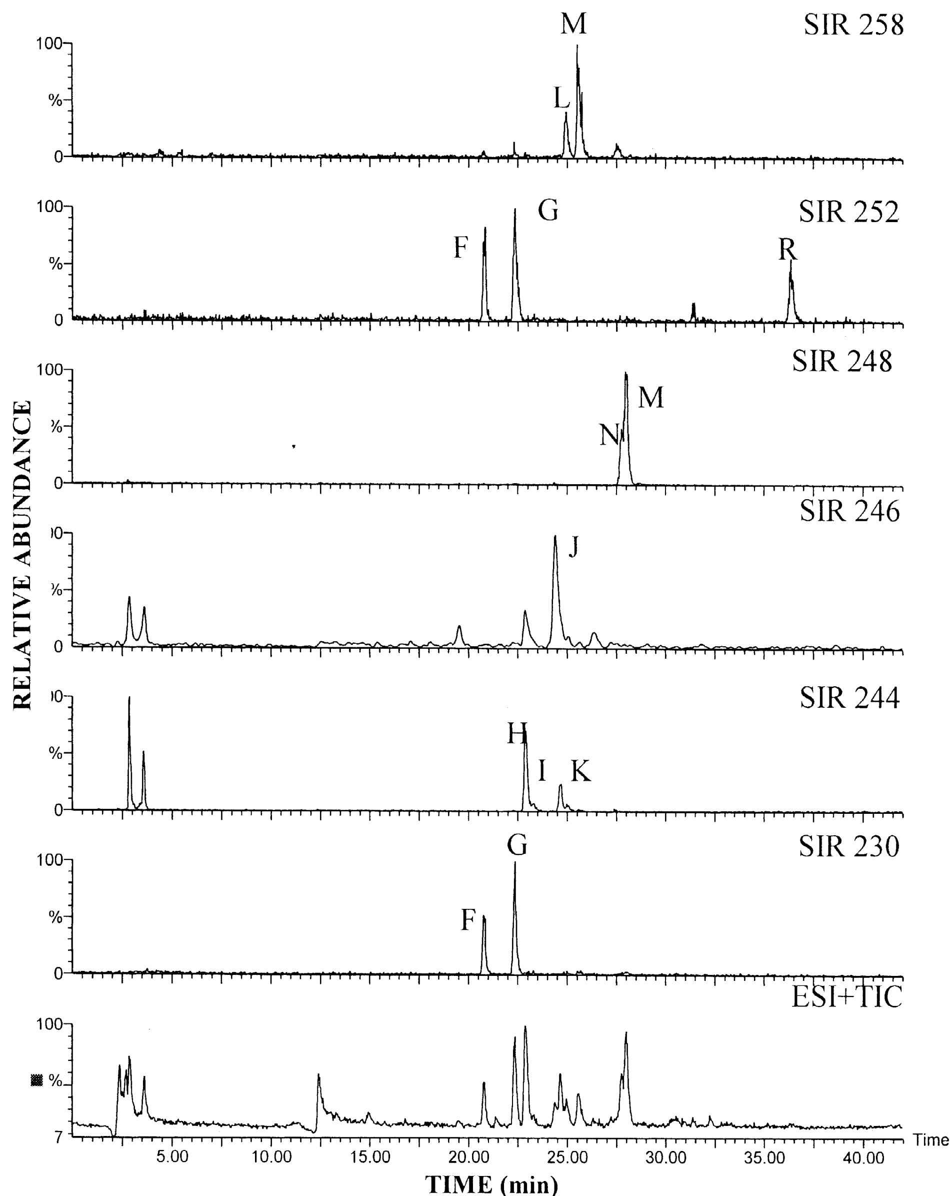

Fig. 4. HPLC–ESI-MS-TIC and HPLC–MS-SIR chromatogram of alkamides of *E. purpurea* root, with a 1:5 post-column stream splitting. Other conditions are the same as in Fig. 2.

Table 2
Effects of different extraction methods for roots and extracts of *E. purpurea*

Extraction solvent (v/v)	Extraction ratio of cichoric acid in extracts (%)	Extraction ratio of cichoric acid in roots (%)	Extraction ratio of compounds 12 and 13 in roots (%)
Water	95.3	90.1	<0.1
Chloroform	0.8	8.2	89.4
Acetonitrile	0.2	17.6	93.8
Ethanol	0.3	58.3	87.5
Methanol	0.1	54.2	95.6
Methanol–0.1% H_3PO_4 solution (8:2)	45.2	64.3	90.2
Methanol–0.1% H_3PO_4 solution (7:3)	65.3	89.5	88.2
Methanol–0.1% H_3PO_4 solution (6:4)	79.2	57.2	27.1
Methanol–0.1% H_3PO_4 solution (5:5)	83.6	44.6	18.3

3.4. Effects of different extraction methods for roots and extracts

A 1-g sample of *E. purpurea* powder or *E. purpurea* extract was ultrasonicated once in 10 ml water, methanol, ethanol, chloroform, acetonitrile and methanol–0.1% phosphoric acid aqueous solution in different ratios (8:2, 7:3, 6:4 or 5:5, v/v) for 20 min. The determination results were compared with those described in Section 2.3. As shown in Table 2, the ultrasonic extraction of dried samples with methanol–0.1% phosphoric acid aqueous solution (7:3, v/v) provided good yields for both cichoric acid and compounds 12 and 13.

When a methanol–water (7:3, v/v) mixture was used as a extraction solvent, caffeic acid derivatives and alkamides could be extracted from *Echinacea* solid dosage forms [33]. We conducted a comparison study on the extraction efficiency using the reported methanol–water mixture with a mixture of methanol–0.1% H_3PO_4 (7:3, v/v). We found ~20% higher extraction efficiency when the later solvent was used for extracts. Therefore, methanol–0.1% H_3PO_4 was used in this work.

3.5. Sample analysis

Eight samples of roots and extracts of *E. purpurea* were analyzed and the results are summarized in Table 3.

For extracts, cichoric acid level was 60–70 mg/g, and alkamides were less than 0.01 mg/g. One can see from the results that the methods of manufacturing *E. purpurea* extracts were problematic as al-

Table 3
Analyses of cichoric acid and compounds 12 and 13 in *E. purpurea* roots and extracts

Sample	Cichoric acid[a] (mg/g, dry mass)	Compounds 12 and 13[a] (mg/g, dry mass)
E. purpurea root (China)	11.02	0.35
E. purpurea root (USA1)	10.12	0.39
E. purpurea root (USA2)	20.82	1.43
E. purpurea root (USA3)	17.23	1.02
E. purpurea root (USA4)	19.12	1.16
E. purpurea extract (1)	60.21	<0.01
E. purpurea extract (2)	70.14	<0.01
E. purpurea extract (3)	65.74	<0.01

[a] Average values of five parallel measurements.

kamides were almost lost in the commercially available extracts.

For dried root samples, newly imported (August, 2002) USA2–4 contained cichoric acid and compounds 12 and 13 quantitatively comparable with literature data [19,33]. However, the levels of both cichoric acid and compounds 12 and 13 in USA1 are significantly low, which may be attributed to its six-month storage at room temperature and thus possible loss of these species.

We also compared herbs planted in Changsha and in the USA. Although the compositions found were very similar, the contents of both cichoric acid and alkamides 12 and 13 in the plant samples grown in Changsha were significantly lower than those grown in the USA, implying that the grown environment affects the levels of the compounds. In fact, some USA plants are not able to grow here in Changsha, e.g. *E. angustifolia* DC. cannot be cultivated successfully.

4. Conclusions

An HPLC–DAD–MS method for simultaneous analyses of caffeic acid derivatives and alkamides in *E. purpurea* has been developed. Simultaneous ionization was achieved for the two kinds of bio-active components leading to both their analyses in a single run, resulting in significant saving of time and sample. This novel method is more suitable for quality control for standardized *Echinacea* extract and for providing biological active HPLC chromatographic fingerprints of bio-active compounds in *E. purpurea* than for other analytical methods.

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References

[1] P. Brevoort, Herbalgram 36 (1996) 49.
[2] R. Bauer, Z. Arztliche Fortbild. 90 (1996) 111.
[3] R. Bauer, in: H. Wagner, N.R. Farnsworth (Eds.), Economic and Medicinal Plant Research, Vol. 5, Academic Press, New York, 1991, p. 253.
[4] R. Bauer, P. Remiger, K. Jurcic, H. Wagner, Z. Phytother. 10 (1989) 43.
[5] R.M. Facino, M. Carini, G. Aldini, C. Marinello, IL Farmaco 48 (1993) 1447.
[6] R.M. Facino, M. Carini, G. Aldini, L. Saibene, P. Pietta, P. Mauri, Planta Med. 61 (1995) 510.
[7] W.E. Ronison Jr., M.G. Reinecke, S. Abdel-malek, Q. Jia, A. Chow, Proc. Natl. Acad. Sci. USA 93 (1996) 6326.
[8] P.J. King, W.E. Robinson Jr., J. Virol. 72 (1998) 8420.
[9] P.J. King, G. Ma, W. Miao, Q. Jia, B.R. McDougall, M.G. Reinecke, C. Cornell, J. Kuan, T.R. Kim, W.E. Robinson Jr., J. Med. Chem. 42 (1999) 497.
[10] A. Cheminat, R. Zawatzky, H. Becker, R. Brouillard, Phytochemistry 27 (1988) 2787.
[11] R. Bauer, K. Jurcic, J. Puhlmann, H. Wagner, Drug Res. 38 (1988) 276.
[12] H. Wagner, W. Breu, F. Willer, M. Wierer, P. Remiger, G. Schwenker, Planta Med. 55 (1989) 566.
[13] B. Muller-Jakic, W. Breu, A. Prostle, K. Redl, H. Greger, R. Rauer, Planta Med. 60 (1994) 37.
[14] L.J. Clifford, M.G. Nair, J. Rana, D.L. Dewitt, Phytomedicine 9 (2002) 249.
[15] R. Bauer, J. Herbs, Spices & Med. Plants 6 (1999) 51.
[16] R. Bauer, P. Remiger, H. Wagner, Dtsch. Apoth. Ztg. 128 (1988) 174.
[17] R. Bauer, Z. Phytother. 18 (1997) 270.
[18] R. Bauer, S. Foster, Planta Med. 57 (1991) 447.
[19] N.B. Perry, J.B. Elaine, V.L. Glennie, J. Agric. Food Chem. 49 (2001) 1702.
[20] C. Hu, D.D. Kitts, J. Agric. Food Chem. 48 (2000) 1466.
[21] R.E. Snook, R.E. Lynch, A.K. Culbreath, C.E. Costello, J. Agric. Food Chem. 42 (1994) 1572.
[22] R. Rauer, P. Remiger, Planta Med. 55 (1989) 367.
[23] B.H. Schulthess, E. Giger, T.W. Baumann, Planta Med. 57 (1991) 384.
[24] H.O. Kim, T.D. Durance, C.H. Scaman, D.D. Kitts, J. Agric. Food Chem. 48 (2000) 4182.
[25] X.G. He, L.Z. Lin, M.W. Bernart, L. Lian, J. Chromatogr. A 815 (1998) 205.
[26] B. Dietz, J. Heilmann, R. Bauer, Planta Med. 67 (2001) 863.
[27] M. Laasonen, T. Wennberg, T. Harmia-Pulkkinen, H. Vuorela, Planta Med. 68 (2002) 572.
[28] B.D. Sloley, L.J. Urichuk, C. Tywin, R.T. Coutts, P.K. Pang, J.J. Shan, J. Pharm. Pharmacol. 53 (2001) 849.
[29] B. Nusslein, M. Kurzmann, R. Bauer, W. Kreis, J. Nat. Prod. 63 (2000) 1615.
[30] R. Bauer, P. Remiger, H. Wagner, Phytochemistry 27 (1988) 2339.
[31] H.O. Kim, T.D. Durance, C.H. Scaman, D.D. Kitts, J. Agric. Food Chem. 48 (2000) 4187.
[32] N.B. Perry, J.W. van Klink, E.J. Burgess, G.A. Parmenter, Planta Med. 63 (1997) 58.
[33] G.W. Schieffer, J. Am. Nutraceutical Assoc. 3 (2000) 67.