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Simultaneous analysis of the lipophilic and hydrophilic markers of *Echinacea* plant extracts by capillary electrophoresis

The phytochemical profile of *Echinacea* plant extracts has been obtained by a CD-MEKC method using sodium dodecyl sulfate (SDS) as surfactant and hydroxypropyl-β-cyclodextrin (HP-β-CD). The separation of the major lipophilic markers of *E. purpurea* (alkyl isobutylamides and alkyl methylbutylamides; designated as alkamides) was affected by the nature and concentration of both SDS and CD, while the nature, concentration, and pH of running buffer exerted little influence on the migration of alkamides. On the other hand, the separation of hydrophilic compounds of the different *Echinacea* species (caffeoyl conjugates, namely: echinacoside, cynarin, chlorogenic acid, caffeic acid, caftaric acid, and cichoric acid) were strongly influenced by the nature, concentration, and pH of running buffer. Thus a step-by-step optimization procedure was carried out in order to choose the best BGE able to provide simultaneous separation of both alkamides and phenolic compounds. By using a SDS/HP-β-CD mixture of concentration 110 mM/100 mM in a BGE consisting of Britton-Robinson buffer (10 mM, pH 8.0), complete separation of all the principal phytochemical markers of *Echinacea* species was achieved in less than 10 min. The proposed conditions were applied to the evaluation of the phytochemical profile of *Echinacea purpurea* plant extract; quantitative determination was performed on the hydrophilic caffeoyl conjugates in commercially available phytopharmaceutical products.

Key Words: *Echinacea* extracts; CD-MEKC analysis; Phenolic compounds; Alkamides; BGE nature

Received: June 18, 2002; revised: August 2, 2002; accepted: August 5, 2002

1 Introduction

Echinacea extracts have attracted continuous and increasing interest as a source of important plant metabolites responsible for the pharmacological activity ascribed to this medicinal species [1–3]. Both the hydrophilic and lipophilic components of the plant tissue extracts possess therapeutic properties; the former due especially for the presence of phenolic derivatives (caffeoyl conjugates, **Figure 1**) and the latter for the relative high content of alkamides (**Figure 2**) and polyacetylenes. Structurally, the most important alkamides are chemically defined as alkyl isobutylamides (**1**–**3**, **5**, **6**, **8**, and **9**) and alkyl methylbutylamides (**4** and **7**). The use of alkamides as immunostimulants and as anti-inflammatories has been widely reported, whereas the polar constituents are believed to have anti-inflammatory and antimicrobial actions. In *Echinacea*, alkamides are mainly found in *purpurea* and *angustifolia* species, with *E. pallida* being especially rich in polyacetylenes. On the other hand, among the hydrophilic markers, cynarin was found to be characteristic of *E. angustifolia,* while cichoric and caftaric acid can be found at high levels in *E. purpurea*. Finally, echinacoside, which is the primary antimicrobial component of *Echinacea,* is almost exclusively present in *angustifolia* and *pallida* roots [1–4]. Reversed phase HPLC with UV detection is commonly applied for the analysis of the phytochemical markers of *Echinacea* species [4–8] and it constitutes the reference method of Pharmacopeial Forum [9]. A GC-MS method has recently been used to evaluate the phytochemical impact of virus infections on the *E. purpurea* lipophilic phytochemicals [10]. Applications of capillary electrophoresis (CE) to the evaluation of phytochemical profile of *Echinacea* were proposed for the analysis of phenolic compounds by means of micellar electrokinetic chromatography (MEKC) [11, 12].

Recently, the separation of the nine most important closely related alkamides from *E. purpurea* extracts was approached by means of cyclodextrin-modified micellar electrokinetic capillary chromatography (CD-MEKC) [13].

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Abbreviations: BGE, background electrolyte; HP-β-CD, hydroxypropyl-β-cyclodextrin; *E*, *Echinacea*; CD-MEKC, cyclodextrin-modified micellar electrokinetic chromatography

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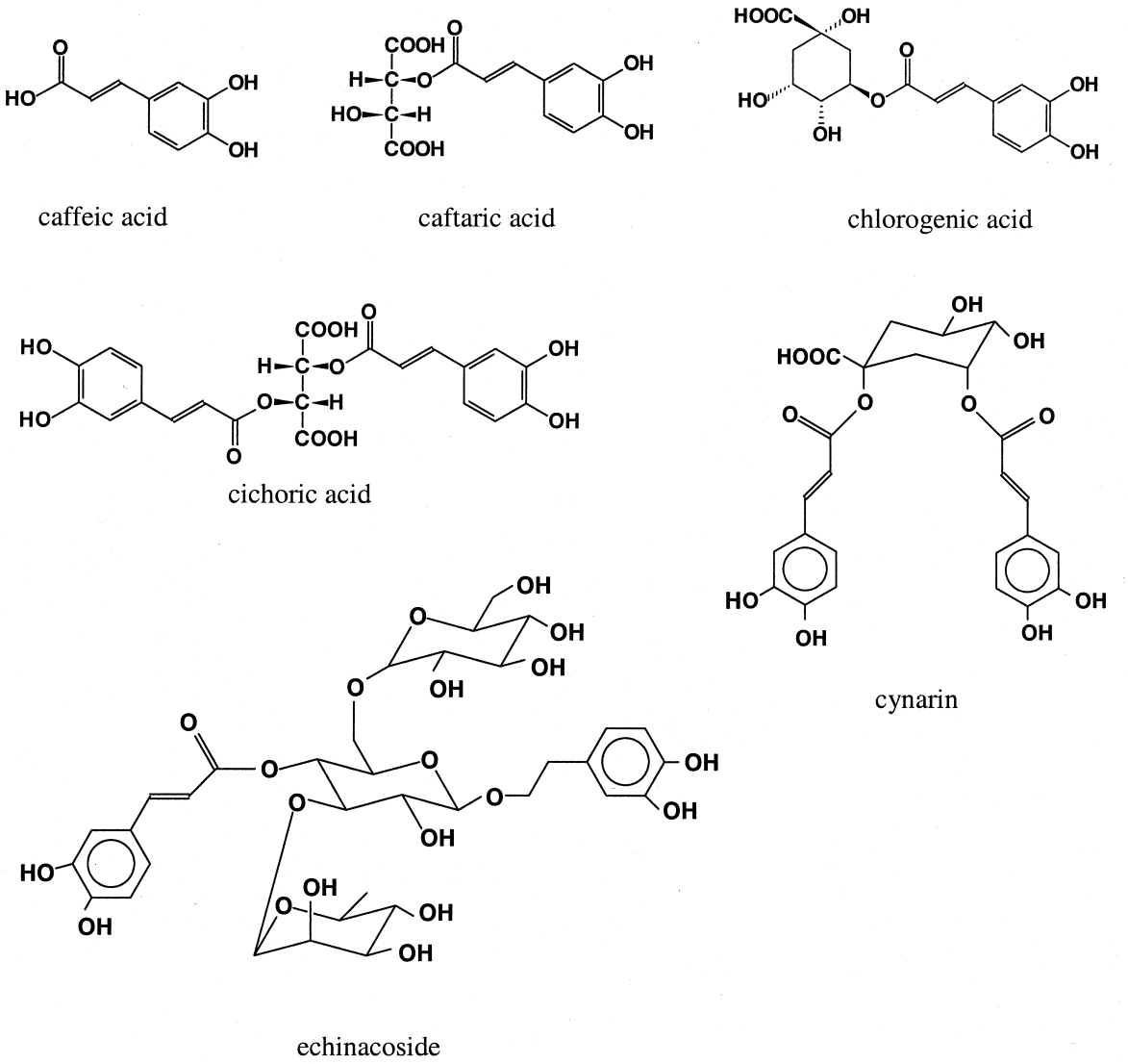


Figure 1. Structures of the caffeoyl conjugates of *Echinacea* species.

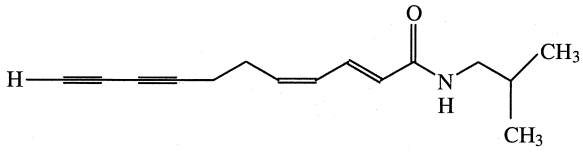
In this technique, the addition of a suitable cyclodextrin to the micelle solution can significantly affect the selectivity of separation because the inclusion of analytes in the cavity of cyclodextrin can favourably change the capacity factor of solute/micelle [14, 15]. Using this approach the best results in terms of separation of alkamides were obtained with a combination of sodium dodecyl sulfate (SDS) and hydroxypropyl-β-cyclodextrin (HP-β-CD) 110 mM/100 mM under alkaline conditions (phosphate buffer, pH 8.0) [13], but the method application was restricted to the lipophilic alkamide fraction.

The aim of the present study has been to provide the appropriate electrophoretic conditions for a simultaneous analysis of both the lipophilic (alkamides) and hydrophilic (caffeoyl conjugates) components of *Echinacea* plant extracts. For this purpose, the previous CD-MEKC method was modified and improved by investigating the effects of the nature of the BGE, and the concentration and pH of the buffer on the separation of the potentially present phytochemical markers of *Echinacea*. As a result, the developed method proved to be of general applicability, suitable for the discrimination of the three *Echinacea* species (*purpurea, pallida, angustifolia*) and for the analytical control of commercial products containing *Echinacea* active components.

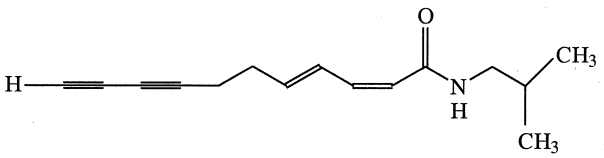
2 Experimental

2.1 Materials

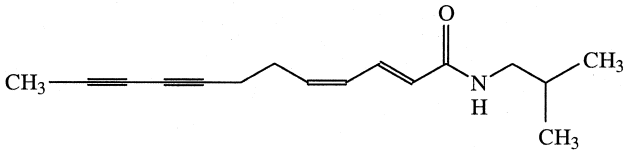
Sodium phosphate, boric acid, acetic acid, phosphoric acid, and sodium tetraborate decahydrate were from



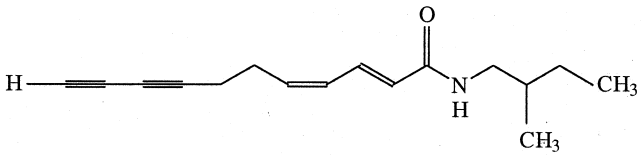
Undeca-2E, 4Z-diene-8, 10-diynoic acid isobutylamide (**1**)



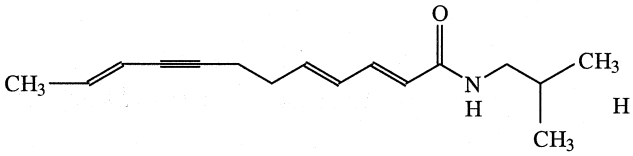
Undeca-2Z, 4E-diene-8, 10-diynoic acid isobutylamide (**2**)



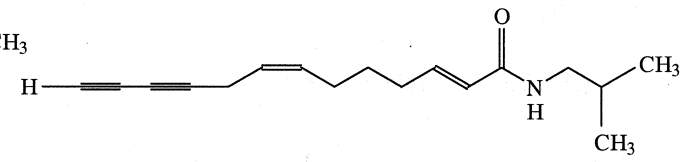
Dodeca-2E, 4Z-diene-8, 10-diynoic acid isobutylamide (**3**)



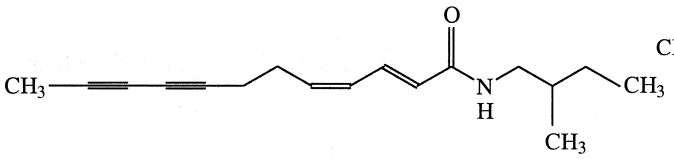
Undeca-2E, 4Z-diene-8, 10-diynoic acid 2-methylbutylamide (**4**)



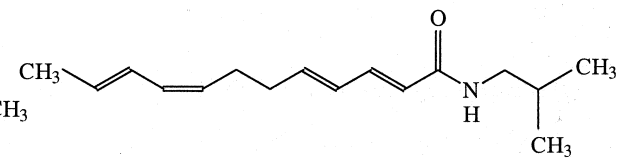
Dodeca-2E, 4E, 10E-triene-8-ynoic acid isobutylamide (**5**)



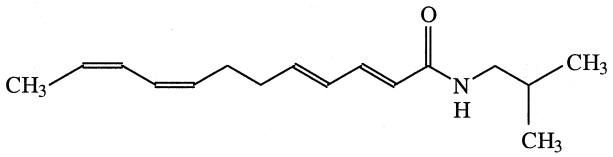
Trideca-2E, 7Z-diene-10, 12-diynoic acid isobutylamide (**6**)



Dodeca-2E, 4Z-diene-8, 10-diynoic acid 2-methylbutylamide (**7**)



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



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

Figure 2. Structures of alkamides from *Echinacea* purpurea.

Carlo Erba Reagenti (Milan, Italy); sodium dodecyl sulfate (SDS), hydroxypropyl-β-cyclodextrin (HP-β-CD) were purchased from Fluka (Buchs, Switzerland).

Echinacoside was a kind gift from Indena (Milan, Italy); cynarin, cichoric acid, and caftaric acid were from ChromaDex (Laguna Hills, CA, USA); chlorogenic acid was from Fluka (Buchs, Switzerland), and caffeic acid was from Sigma (Milan, Italy).

Other reagents were of analytical grade and were used as received. Deionized water was obtained with a Milli-RX system (Millipore, Milford, MA, USA).

2.2 CE system

All separations were carried out using a ^{3D}CE Capillary Electrophoresis system (Agilent Technologies, Waldbronn, Germany), equipped with a diode array detector. The data were collected on a PC using the ^{3D}CE-ChemStation software ver. A 06. New fused-silica capillaries (Agilent Technologies) 48.5 cm in length (40 cm effective length) × 50 μm ID were used after a conditioning program consisting of rinsing with 1 M NaOH (10 min), 0.1 M NaOH (10 min), and finally deionized water (10 min). Prior to each run, a brief rinse (3 min) of the capillary with the separation buffer provided excellent repeatability of migration times. Hydrodynamic injections were performed at 25 mbar for 3 s and the voltage was 20 kV; the separations were performed under controlled temperature (30°C); detection was carried out employing a diode-array detector recording the UV signal at 260 and 320 nm.

2.3 Solutions

The running buffers were freshly prepared every day at a concentration of SDS and HP-β-CD corresponding to 110 mM and 100 mM, respectively, in the BGE solution. Phosphate, tetraborate, and Britton-Robinson buffer solutions were prepared according to standard methods and used in a concentration range of 5–30 mM. The BGE pH value was investigated by preparing solutions at pH 7.0, 8.0, 9.0, and 10.0 for each tested buffer using sodium hydroxide or phosphoric acid to adjust the acidity to the desired value. Before the injection into the CE system, each solution (running buffer and sample solutions) was filtered through a 0.45 μm GyroDisc (Orange Scientific, Waterloo, Belgium) membrane.

2.4 Sample preparation

2.4.1 Plant materials

About 1–2 kg of plant material (roots) of *E. purpurea* plants grown in an open field at the Herb Garden of Casola Valsenio (Ravenna, Italy) were collected in October 2000 at almost full growth. Root materials were separated, washed, cut into small parts, air-dried, and ground into small particles (0.5 mm) just before extraction.

2.4.2 Plant extraction procedure

Accurately weighed amounts (about 1 g) of dried ground root materials were extracted in an ultrasonic bath (Bandelin Sonorex super RK 103H, Berlin, Germany) for 6 min at room temperature, three times with 8 mL solvent (70% *v/v* methanol in water). The extracts ($n = 3$) were collected, filtered through a 0.20 μm membrane filter, and the volume was made up to 25 mL, giving a final concentration equivalent to 0.04 g roots per mL.

Aliquots of this solution were used for the simultaneous direct analysis of the polar caffeoyl derivatives and for the lipophilic alkamide components.

2.4.3 Identification of alkamides

Identification of the different alkamides was realized by a combination of RP-HPLC and GC/MS analyses as previously described [13].

The identification of caffeoyl conjugates contained in the total extract of *Echinacea* was simply accomplished by comparing their migration times with those of available standard compounds; further confirmation of their identity was accomplished by fortifying the extracts with the standard compounds and by a comparison of the UV spectra of the electrophoretic peaks recorded on-line by a DAD detector.

2.5 Calibration graphs

Stock solutions (1 mg/mL) of each phenolic compound were prepared in methanol. The linearity of the response was evaluated by analyzing standard solutions of echinacoside (59–178 μg/mL), chlorogenic acid (5.4–16.2 μg/mL), caffeic acid (5.1–15.3 μg/mL), cynarin (5.0–15.0 μg/mL), cichoric acid (50–90 μg/mL), and caftaric acid (30–70 μg/mL) in water-methanol 90:10 (*v/v*). Triplicate injections were performed for each standard solution and the corrected peak area (area/migration time) of the analyte was plotted against the concentration to obtain the calibration graphs.

2.6 Commercial product analysis

The developed MEKC method was applied to the analysis of different commercial phytoproducts: *E. purpurea*-based tablets (Echinaforce®, Biohorma Italia, Pomezia, Italy), *E. angustifolia*-based capsules (Echinacea-body spring®, Body Spring s.r.l., Verona, Italy) and *E. pallida*-based hydroalcoholic solution (Echinacea-Aboca®, ABOCA, Arezzo, Italy).

2.6.1 Extraction procedure

The analysis of tablets and capsules was performed after a simple extraction procedure: about 1 g accurately weighed amounts of powdered commercial tablets (or of the capsule contents) were extracted three times in an ultrasonic bath for 5 min at room temperature, with 8 mL of 70% methanol in water. The extracts ($n = 3$) were collected, filtered through 0.45 μm pore size membrane filter, and the volume was made up to 25 mL.

2.6.2 Assay procedure

The obtained solution from a formulation based on *E. purpurea* (tablets) was filtered and directly subjected to the CD-MEKC analysis; the extract from a formulation based on *E. angustifolia* (capsules) was diluted 1 : 1 with a mixture methanol-water (70 : 30 *v/v*) prior to the injection. Finally, the analysis of the hydroalcoholic solution of *E. pallida* was performed after dilution of 1 mL aliquot of the commercial product to 20 mL volume with methanol : water 70 : 30 (*v/v*) mixture. The sample solutions were subjected to the CD-MEKC analysis and the content of the main caffeoyl conjugate components was determined by comparison with appropriate standard solutions.

3 Results and discussion

3.1 Method development and optimization

In previous work the neutral alkamides shown in Figure 2 were completely separated by a CD-MEKC method using a combination of SDS and HP-β-CD (110 mM and 100 mM, respectively) in the presence of a BGE constituted of 10 mM phosphate solution (pH 8.0) [13]. The samples subjected to analysis were obtained from a total *Echinacea purpurea* root extract after a liquid-liquid extraction able to eliminate the hydrophilic compounds (caffeoyl conjugates) potentially interfering with the complex separation profile of the alkamides.

However, the need for a simple CE method able to provide fast separation of all the phytochemical markers of *Echinacea* species prompted us to improve the previously developed CD-MEKC system by extending its applicability to the total plant extracts (hydrophilic and lipophilic components).

Among the phytochemical markers of *Echinacea*, the neutral alkamides exhibited a migration profile which should be hardly affected by variation of the nature, concentration, and pH value of the BGE (over pH value of 7.0); in contrast, the separation of the potentially present phenolic compounds is greatly influenced by the above mentioned BGE characteristics. On this basis, step-by-step optimization of the BGE composition was performed to find electrophoretic conditions suitable for a complete and simultaneous separation of alkamides and phenolic compounds.

3.1.1 Effect of nature and concentration of the running buffer

The conditions previously optimized for separation of alkamides from *E. purpurea* (SDS/HP-β-CD, 110 mM/100 mM) were based on the use of a phosphate running buffer (10 mM, pH 8.0). However, these conditions did not allow satisfactory separation of a standard mixture of the six studied phenolic compounds, namely: echinacoside, cynarin, caffeic acid, caftaric acid, chlorogenic acid, and cichoric acid. Since borate buffers are well documented as suitable media for the electrophoretic separation of polyhydroxy compounds [12, 15, 16], attention was focused on borate and Britton-Robinson BGEs. Working at pH 8.0 and using a concentration of 10 mM, good and rapid separations of the phenolic compounds were obtained employing Britton-Robinson BGE (**Figure 3.a**), whereas in the presence of borate buffer a co-migration of caffeic and cichoric acid was observed (**Figure 3.b**).

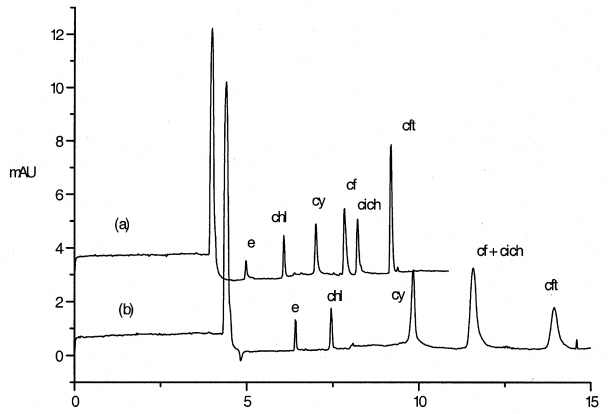


Figure 3. Electropherograms of a standard mixture of the six analysed caffeoyl conjugates. Conditions: running buffer constituted of SDS/HP-β-CD (110 mM/100 mM) dissolved in (a) Britton-Robinson buffer (10 mM, pH 8.0) and (b) tetraborate buffer (10 mM, pH 8.0). Other conditions: fused-silica capillary 48.5 cm total length (40 cm to detector) × 50 μm. Voltage: 20 kV. Temperature: 30°C. Hydrodynamic injection: 2.5 kPa × 3 s. Detection at 320 nm. Symbols. e: echinacoside; chl: chlorogenic acid; cy: cynarin; cf: caffeic acid; cich: cichoric acid; cft: caftaric acid.

The influence of the concentration of pH 8.0 Britton-Robinson running buffer on the separation of the used standard mixture was investigated in the range 5–30 mM. Only small differences in the migration of phenolic compounds were observed and a 10 mM solution was chosen as an optimum, especially to keep current values acceptably low (50 μA).

3.1.2 Effect of running buffer pH

Using a fixed concentration of Britton-Robinson buffer of 10 mM, the acidity of the solutions was varied by adjusting the pH with sodium hydroxide; solutions of pH 7.0, 8.0, 9.0, and 10.0 were used. While the electroosmotic flow remained substantially constant in the considered pH range, significant differences were observed in the separation profile of the standard mixture of phenolic compounds. Specifically, the migration of echinacoside, chlorogenic acid, cynarin, and caffeic acid was hardly affected by the pH variation up to pH 8.0, whereas at pH 9.0–10.0, a co-migration of cichoric and caftaric acid was observed. The complete separation of the phenolic compounds obtained at pH 8.0 encouraged us to evaluate the behaviour, under the same conditions, of the alkamides from *E. purpurea.* As a sample, a plant extract from *E. purpurea* (originally containing alkamides, chlorogenic acid, caftaric acid, and cichoric acid) was supplemented with echinacoside and cynarin (typical phytochemical markers of *E. angustifolia* and *E. pallida*) by spiking the extract with the corresponding analyte aqueous solutions. As shown in **Figure 4**, the chosen electrophoretic conditions allowed complete separation of all the phenolic compounds potentially present in the principal *Echinacea* species (*purpurea, pallida,* and *angustifolia*) and, simultaneously, of the major alkamides (from *E. purpurea* and *angustifolia*). In this context, the following observations warrant mention. First of all, the advantages of using a DAD detector should be pointed out: chlorogenic acid (detected at 320 nm) hardly affects the separation profile of alkamides on using detection at 260 nm (Figure 4). On the other hand, the most critical alkamides showing migration times close to that of chlorogenic acid (namely alkamide **4**, **5**, and **7**) are present in only very small amounts in the *E. purpurea* and *angustifolia* extracts. In contrast, alkamide **2** and the *cis-trans* isomers **8** and **9**, which are the most diagnostically useful lipophilic markers [2], migrate in a range not affected by the presence of phenolic compounds. Therefore, the proposed final conditions can be useful for general applications addressed to the characterization of the profile of the major secondary *Echinacea* metabolites.

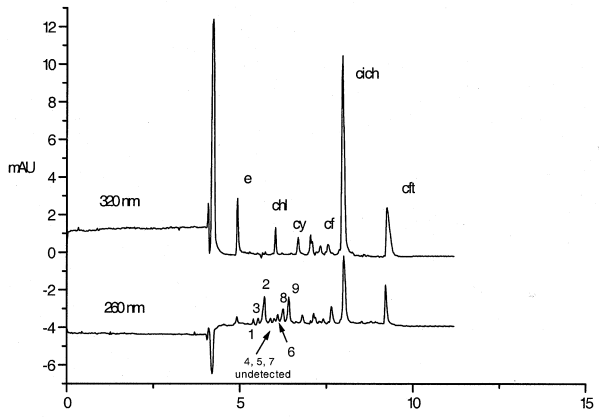


Figure 4. Electropherogram of *Echinacea purpurea* root extract spiked with echinacoside and cynarin, recorded at two different wavelength (260 nm to detect alkamides and 320 nm to detect phenolic compounds). Running buffer: SDS/HP-β-CD (110 mM/100 mM) dissolved in Britton-Robinson buffer (10 mM, pH 8.0). Other conditions and symbols as in Figure 3.

3.2 Method validation and application to commercial phytoproducts

The developed and optimized method (SDS/HP-β-CD, 110 mM/100 mM in a pH 8.0 Britton-Robinson 10 mM buffer) was applied to the identification and quantification of the caffeoyl conjugates in commercial phytoproducts. An excellent intraday and interday repeatability of the separation was observed (Table 1), suggesting the possibility of identifying the analytes on the basis of their migration times. Nevertheless, further confirmation of the identity of each compound was provided by spiking experiments and by the spectra recorded on-line from the DAD detector; in fact, the UV spectra offer useful support by confirming the presence of a 3,4-dihydroxycinnamic moiety [18]. Using the described procedures, the proposed method proved to be highly selective and able to discriminate between caffeoyl conjugates and, simultaneously, between closely related alkamides (notable the separation of *cis/trans* isomeric alkamides **8/9**).

For quantitative purposes, the linearity of the response was verified for each studied caffeoyl conjugate. In Table 2, the reported regression data were obtained by plotting the corrected peak areas against the corresponding analyte concentrations; at the same time the detection sensitivities (LOD) were extrapolated according to the ratio $3.3\,\sigma/S$, where σ is the standard deviation of the *Y*-intercept and *S* is the slope of the calibration graph. The limit of quantitation (LOQ) was evaluated for each analyte by using the ratio $10\,\sigma/S$, by analogy with the determination of LOD [19]. The reported values suggest the suitability of the method for applications to real samples.

The considered commercially available products were labelled as containing extracts from *E. purpurea, E. pallida,* and *E. angustifolia*, respectively, thus exhibiting different and specific phytochemical profiles. Actually, the preparation (tablets) based on *E. purpurea* extract was analysed after storage at room temperature for one year and the concentration levels of cichoric and caftaric acids were 0.13 mg/g (RSD 2.84%, $n = 5$) and 0.14 mg/g (RSD 3.07%, $n = 5$), respectively. The analysed hydroalcoholic solution based on *E. pallida* was found to contain only echinacoside as caffeoyl conjugate at a concentration level of 1.36 mg/mL (RSD 2.80, $n = 5$) (**Figure 5.B** and **Figure 5.C**). These results are in agreement with the phyto-

Table 1. Intraday and interday precision of the migration time and peak area (RSD, $n = 10$) for the studied phenolic compounds (concentration: 10 μg/mL)[a].

Analyte	Intraday precision		Interday precision	
	t_m (min) (RSD%)	Corrected peak area (RSD%)	t_m (min) (RSD%)	Corrected peak area (RSD%)
Echinacoside	4.93 (0.594)	1.69 (0.661)	4.91 (0.622)	1.68 (0.851)
Chlorogenic acid	6.02 (1.52)	0.610 (0.965)	6.00 (1.00)	0.610 (1.27)
Cynarin	6.71 (1.46)	0.422 (1.02)	6.70 (1.55)	0.430 (1.92)
Caffeic acid	7.55 (1.10)	0.257 (0.687)	7.50 (1.60)	0.260 (0.961)
Cichoric acid	7.95 (0.972)	6.53 (0.581)	7.93 (1.20)	6.48 (1.26)
Caftaric acid	9.28 (1.35)	2.05 (0.655)	9.26 (1.88)	2.06 (1.75)

[a] Experimental conditions: SDS/HP-β-CD (110 mM/100 mM) dissolved in Britton-Robinson buffer (10 mM, pH 8.0). Fused-silica capillary 50 μm (48.5 cm total length, 40 cm to detector). Voltage 20 kV. Temperature 30°C. Hydrodynamic injection at 25 mbar × 3 s. Detection at 320 nm.

Table 2. Regression curve data[a] and sensitivity data for the studied analytes.

Analyte	Concentration range (μg/mL)	*b*	*a*	r^2	LOD (μg/mL)	LOQ (μg/mL)
Echinacoside	59–178	−0.0583 (±0.0339)	0.0249 (±0.0003)	0.999	4.49	13.61
Chlorogenic acid	5.4–16.2	−0.0877 (±0.0078)	0.0729 (±0.0007)	0.999	0.35	1.07
Cynarin	5–15	−0.1890 (±0.0116)	0.0628 (±0.0011)	0.999	0.61	1.85
Caffeic acid	5.1–15.3	−0.0532 (±0.0309)	0.1143 (±0.0029)	0.999	0.90	2.70
Cichoric acid	50–90	−1.9603 (±0.1330)	0.1006 (±0.0019)	0.999	4.36	13.22
Caftaric acid	30–70	−0.3113 (±0.0674)	0.0478 (±0.0013)	0.999	4.65	14.10

[a] Regression curve data for five calibration points. $y = ax + b$, where y is the corrected peak area (area/migration time), x is the concentration (μg/mL), a is the slope, b is the intercept, and r^2 is the correlation coefficient. Experimental conditions as in Table 1.

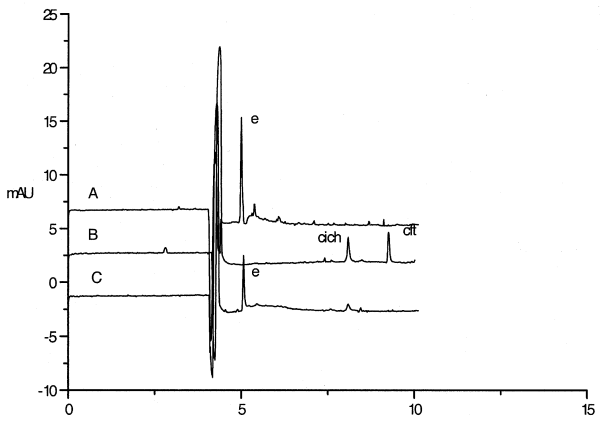


Figure 5. Electropherograms of phenolic compounds contained in commercially available phytopharmaceutical products based on the extracts from *E. angustifolia* (A), *E. purpurea* (B), and *E. pallida* (C). Detection wavelength 320 nm. Conditions and symbols as in Figure 3

chemical profile of the two considered *Echinacea* species. Concerning the product based on *E. angustifolia* (capsules), the expected echinacoside was found to be present at 8.20 mg/g level (RSD 2.76%, $n = 5$), while no evidence of the others minor markers chlorogenic acid and cynarin was found (**Figure 5.A**). These data, however, may result from environmental and genetic variation as well as variation in plant parts used and in preservation after harvesting [7, 8]. Enzymatic browning can be also responsible for marked losses of phenolic compounds [8]. The echinacoside content found (1.36 mg/mL) in the commercial *E. pallida* based hydroalcholic solution was in agreement with the declared quantity. Concerning the accuracy of the method, the preparation based on hydroalcoholic solution was only diluted prior to injection and no extraction steps were involved. Preparations based on capsules and tablets (the latter containing lactose, magnesium stearate, and gum arabic as excipients) were extracted with the binary mixture methanol-water 70 : 30 (*v/v*), which was found to provide the best recovery (about 90%) of cichoric and caftaric acids [7, 12] as well as of echinacoside [7]. On the other hand, method accuracy constitutes a real problem in phytochemical analysis due to the labile nature and often poor availability of standard compounds.

Concerning the practical applications of the method, the high stability of the interday and intraday migration times and repeatability of corrected peak area obtained by two different operators should be underscored. These results

indicate an adequate robustness of the new CE method, which proved to be especially informative in identifying the compounds characteristic of different *Echinacea* species.

4 Concluding remarks

The influence of the nature, concentration, and pH of the BGE has been evaluated with the goal of developing a CD-MEKC method suitable for the simultaneous analysis of both the hydrophilic (phenolic compounds) and the lipophilic phytochemical markers (alkamides) potentially present in the major *Echinacea* species. The resulting method offers qualitative and quantitative applications. The different *Echinacea* species (*E. purpurea*, *angustifolia,* and *pallida*) can be unambiguously identified and all the caffeoyl conjugates (hydrophilic markers) can be selectively and rapidly determined. The proposed CD-MEKC method can constitute a useful alternative to existing HPLC methods and, due to its particular selectivity, it can offer a complementary tool for identifying and determining the quality of phytoproducts. Furthermore, the feasibility and reliability displayed by the proposed method suggests a further extension to the quantitative determination of the alkamide components in *Echinacea* species extracts.

Acknowledgements

This work was supported by a grant from MURST (cofin. 2000), Rome, Italy. Thanks are due to Miss Marina Naldi for her valuable technical assistance.

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