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Journal of Pharmaceutical and Biomedical Analysis 37 (2005) 885–891
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

High-resolution method for regulatory control of *Echinacea* species in Nutraceuticals by CD-MEKC

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Received 20 May 2004; received in revised form 4 October 2004; accepted 4 October 2004
Available online 23 November 2004

Abstract

One problem in the international regulatory control of *Echinacea*, a therapeutic Nutraceutical, is recognition of caffeoyl solutes and alkamides in different products. Cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC) has been applied to *Echinacea* spp. in combination with pattern recognition of some caffeoyl solutes. A novel metric based on relative migration time (RMT) data has been developed in CE to address the problem of variable reported migration time.

The CD-MEKC method of Gotti's group using hydroxypropyl-β-cyclodexrin (HP-β-CD; 100 mM) with sodium dodecyl sulphate (SDS; 110 mM), in a triacid background electrolyte (10 mM, pH 8) under 19 kV was adapted to identify two key hydrophilic solutes: chlorogenic acid and cichoric acid present in all commercial products. Two internal markers were taken as reference points to calculate the RMT of any target peak: $\mathrm{RMT} = t_{\mathrm{m\ (target)}}/t_{\mathrm{m\ (marker)}}$.

The RMT method was robust to temperature change from 20 to 40 °C, but sensitive to pH. The lateral shift and reproducibility of the target peak $t_{\mathrm{m\ (target)}}$ were significantly reduced by this novel transformation. In the worst cases migration time variability ranged up to 12% ($n = 6$); the RMT algorithm reduced this to less than 1%. In general, the RMT transformation reduced the variability of migration time data by a factor of 2–5.

For systematic comparison of electrophoretic profiles for test sample and standard, a new pattern recognition algorithm permits sequential peak-by-peak comparison using specified segments of the electropherograms for comparison of test and *Echinacea purpurea* (root product) as a standard. This algorithm was capable of rapidly characterising the similarity of target peaks in a test sample relative to those in the reference standard. Combination of the RMT algorithm and pattern recognition in CE is expected to offer a robust approach to international regulatory characterisation and control of Nutraceuticals.

Keywords: CD-MEKC; *Echinacea* spp.; Relative migration time; Herbal medicines

1. Introduction

In the world of Nutraceuticals *Echinacea* spp. are widely used for medicinal purposes. *Echinacea* is a member of the Compositae (daisy) family, known as coneflower. Three species of *Echinacea* are in use medicinally: *Echinacea angustifolia* (narrow-leafed purple coneflower), *Echinacea purpurea*(L.) Moench (commonly known as broad-leafed purple coneflower), *Echinacea pallida (Nutt)* (pale purple coneflower) [1].

The active constituents of *Echinacea* spp. can be divided into three major groups, namely polysaccharides, phenolic compounds (hydrophilic compounds also known as caffeoyl phenols) and lipophilic compounds including alkamides and ketoalken/ynes [2]. However, the distribution of the key compounds varies within the same plant (roots, rhizomes, stems, leaves and flowers [2–4]) and also between the different

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doi:10.1016/j.jpba.2004.10.015

species of *Echinacea*. For instance, different levels of alkamides and caffeic acid derivatives were reported in *E. purpurea*, *angustifolia* and *pallida* [5] and in *Echinacea simulata* and *paradoxa* [6].

E. purpurea has become the most cultivated of all the species, because the entire plant can be used (root, leaf, flower, seed) and also it is more easily cultivated. The root and rhizome of *E. angustifolia* and *pallida* are also used medicinally [1]. Many compounds can be extracted from *Echinacea* spp. that are claimed to have immunostimulant and antitumoral effects [7]. In fact it was reported that *Echinacea* is used as a prophylactic agent against common cold and some other viral diseases; however, their mechanism of action is not well understood [8]. Moreover, *Echinacea* has found application in a range of mainstream manufactured products such as lip balms, toothpaste, and skin and hair care products, including facial toners, creams and lotions especially for damaged skin [9].

Due to this wide commercial use, there is serious industrial interest in the potential therapeutic agents that could be based on extracts of plants such as *Echinacea* spp. However, producing a homogenous high-quality product presents a significant challenge. There is strong evidence for the need for better regulatory control of *Echinacea* products at the international level [10].

Previous work on the separation of *Echinacea* compounds using HPLC for the quality assurance of *Echinacea* products includes that of Bergeron et al. [10], who separated alkamides and phenolic compounds, and assessed the extraction efficiency of phytochemicals from the aerial parts and the roots of *Echinacea* species (*purpurea* and *angustifolia*). Perry et al. [11] proposed a standard extraction and HPLC method for the assay of phenolics in *Echinacea* species for medicinal purposes. Other HPLC methods [12–16] include that of Pellati et al. [15], who recently published a comprehensive HPLC study on the hydrophilic constituents of *Echinacea* spp. in commercial and bulk plant samples.

Capillary electrophoresis (CE) is a competitive technique for high-efficiency separation of both large and small molecules. Various CE methods have been published for the separation of phenolics in *Echinacea* spp. [17,18]. Gotti et al. have separated alkamides by micellar electrokinetic chromatography (MEKC) and CD-MEKC [19]. The simultaneous separation of phenolics and alkamides was also demonstrated [20].

In this study, commercial products based on *E. purpurea* were examined using a root extract as a reference standard, since it contains dried plant material and is excipient free.

The CD-MEKC method of Gotti [20] was adapted to separate the complex mixture of solutes present in market products of *Echinacea* spp. Two key hydrophilic solutes were identified: chlorogenic acid and cichoric acid. However, variability in migration time (t_m) was observed and this made routine identification of key solutes difficult. A novel metric based on relative migration time (RMT) has been developed to enable comparison of target peaks present in a test sample with those in a reference standard. Its robustness has been studied.

Furthermore, a method for sequential peak-by-peak comparison between the reference standard selected and the test articles was developed using pattern recognition software selecting specified segments of the electropherograms.

2. Experimental

2.1. Chemicals

Boric acid, phosphoric acid and chlorogenic acid were from Sigma–Aldrich, Dorset, UK. Acetic acid, sodium hydroxide and methanol were from Fisher Scientific, Loughborough, UK. 2-Hydroxypropyl-β- cyclodextrin and sodium dedocyl sulphate were from Fluka, UK. Cichoric acid was from LGC Promochem, Hatfield, UK. Deionised water was obtained using a Milli Q water system (Millipore Corporation, Massachusetts, USA).

2.2. Solutions

The run buffer consisted of a triacid background electrolyte comprising orthophosphoric acid (10 mM), acetic acid (10 mM), boric acid (10 mM) and was adjusted to pH 8 using NaOH.

In addition to this, surfactant sodium dodecyl sulphate (SDS, 110 mM) and 2-hydroxypropyl-β-cyclodexrin (HP-β-CD, 100 mM) were added.

2.3. Surrogate standard

A commercial product "Good'n Natural *Echinacea*" (Holland and Barrett capsules, UK) was taken as a surrogate reference standard, since these capsules contain pure dried root extract from *E. purpurea* with no additives.

The content of three capsules (1.200 g) was extracted with 4 ml of methanol-water (70:30 v/v), sonicated for 30 min and centrifuged for a period of 20 min to produce a solution containing the equivalent of 300 mg/ml extracted root material. This was diluted 10 times with the triacid background electrolyte 10 mM, pH 8, to yield a final working standard of 30 mg/mL root extract.

2.4. Sample preparation

2.4.1. Echinaforce (Bioforce AG tincture, Switzerland)

A volume of the tincture (50 μL) was diluted to 500 μL with the background electrolyte (10 mM, pH 8).

2.4.2. Echinacea (Sainsbury's tablets, UK)

Two tablets were ground with a pestle and a glass mortar and sonicated in 2 mL methanol-water (70:30 v/v). One volume of 50 μL was then diluted to 500 μL with the background electrolyte (10 mM, pH 8).

2.5. Capillary electrophoresis apparatus and conditioning

The CE system employed for these experiments was a P/ACE System MDQ (Beckman Instruments Inc., Fullerton, USA) equipped with ultraviolet (UV) detector and photodiodarray (PDA) detector. The data were collected on a PC using Beckman P/ACE System MDQ software (version 2.3).

The fused silica capillaries (Composite Metal Services Ltd., UK) used were 50 cm in total length (40 cm effective length) × 50 μm i.d. When the capillary was new, it was first conditioned by rinsing according to the following regime:

NaOH 1 M for 15 min, followed by Milli Q water for 3 min, NaOH 0.1 M for 5 min, Milli Q water for 3 min, methanol for 5 min, Milli Q water for 5 min and finally a voltage of 1 kV was applied with the run buffer in the capillary for 1 min.

At the start of each day, the capillary was first rinsed with NaOH 0.1 M for 3 min and with the run buffer for 3 min, and then a hydrodynamic injection of buffer was made, followed by 30 min electrophoresis under a voltage of 1 kV.

Prior to each separation run, the capillary was rinsed with NaOH (0.1 M) for 3 min, then with the run buffer for 3 min under a pressure of 20 psi. The sample was injected hydrodynamically (0.5 psi, for 30 s), and then electrophoresis was performed applying an appropriate voltage, typically 19 kV, for 20 min at 30 °C ± 1 °C, with detection at 320 nm using the diode-array detector.

3. Chemistry

As mentioned above, *Echinacea* spp. contains two main groups of compounds: the alkamides which are lipophilic and chemically defined as alkyl isobutylamides (1–3, 5, 6, 8, and 9) and alkyl methyl-butylamides [21]; and the caffeoyl conjugates, also called caffeic acid derivatives, which are hydrophilic and typically include caffeic acid, caftaric acid, chlorogenic acid, cichoric acid, cynarin, and echinacoside. In this study chlorogenic acid and cichoric acid were selected for examination (Fig. 1).

4. Results and discussion

4.1. Relative migration time (RMT)

Classical methods in GLC and HPLC exploit retention relationships defined relative to a reference or marker peak within the elution range, in order to reduce variations in observed retention properties. A novel measure for migration time (t_m) in CE has been developed, based on the relative migration time (RMT) of a target peak eluting at t_m (target) with respect to the elution position t_m (marker) of a selected reference or marker peak, *P* (see Fig. 2). Thus:

$$\mathrm{RMT} = \left[\frac{t_m(\mathrm{target})}{t_m(\mathrm{marker})}\right]$$

In principle, the RMT function should be robust to changes in observed migration time of a specified analyte from run to run, provided that it can be assumed that the process of migration within an electropherogram is uniform and continuous. Its dependence on temperature and certain operating conditions (e.g. pH) needs to be defined.

4.2. Experimental exploration of the RMT function for Echinacea spp.

The use of RMT for direct comparison of data from a test sample with a control system was assessed in order to establish the potential benefit for qualitative data in CE for recognition purposes.

The statistical performance was assessed for RMT in relation to chlorogenic acid, cichoric acid and a system internal standard (SIS) marker peak "*P*": namely, peak area precision and corrected peak area precision (normalised w.r.t. migration time). This assessment was carried out on

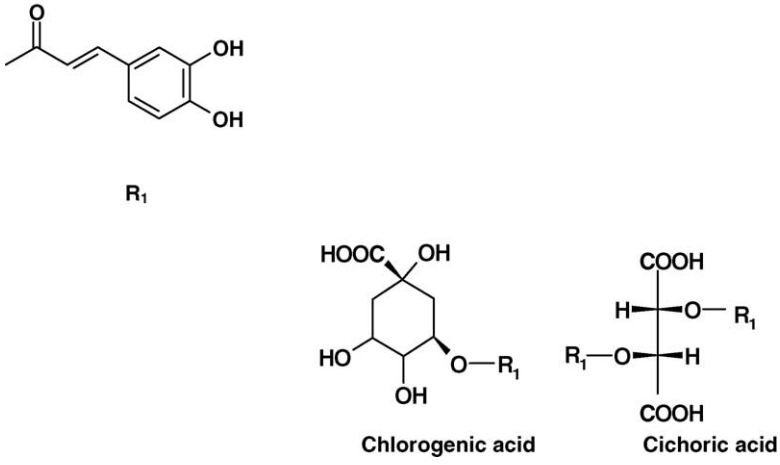

Fig. 1. Chemical structure of hydrophilic compounds: chlorogenic and cichoric acid.

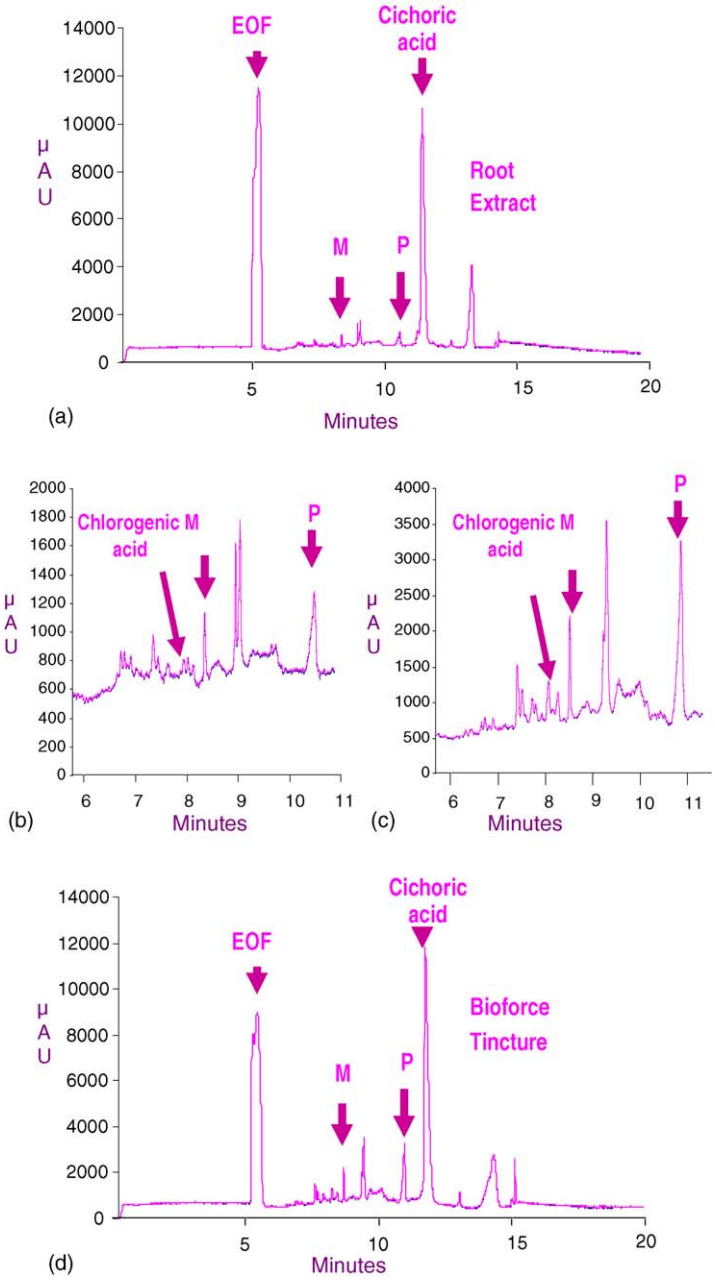

Fig. 2. Constituents of *E. purpurea* root extract and Bioforce tincture: pH 8; temperature 30 ° C. (a) Root extract electropherogram. (b) Expanded CE of root extract. (c) Expanded CE of Bioforce tincture. (d) Bioforce tincture electropherogram.

E. purpurea in Bioforce tincture and the surrogate standard root extract. Thus, RMT is defined as the migration time of a target peak/migration time of the SIS or marker peak.

The results in Tables 1 and 2 are based on analysis ($n = 6$) of four individual batches of the material.

According to these data (Tables 1 and 2), the use of RMT reduces the error marginally in reporting peak position. How-

Table 1
Precision ($n = 6$) data for four batches: migration time, relative migration time, peak area, and corrected area for chlorogenic acid, cichoric acid and marker (peak "*P*"), respectively, in the root extract product (Holland & Barrett's "Good'n Natural *Echinacea*")

Standards	Mean migration time (min)	R.S.D. of migration time (%)	R.S.D. of RMT (%)	R.S.D. of peak area (%)	R.S.D. of corrected area (%)
Chlorogenic acid	8.16	0.12–0.23	0.15–0.21	6.20–13.77	6.18–13.63
Cichoric acid	11.79	0.19–0.30	0.04–0.10	3.26–4.64	3.18–4.66
Peak *P*	10.94	0.16–0.28		2.58–9.14	2.54–9.06

Table 2
Precision ($n = 6$) data for four batches: migration time, relative migration time, peak area, and corrected area for chlorogenic acid, cichoric acid and marker (peak "*P*"), respectively, in Bioforce tincture

Standards	Mean migration time (min)	R.S.D. of migration time (%)	R.S.D. of RMT (%)	R.S.D. of peak area (%)	R.S.D. of corrected area (%)
Chlorogenic acid	8.22	0.16–0.18	0.18–0.28	2.90–5.00	3.00–5.12
Cichoric acid	11.82	0.17–0.54	0.06–0.14	1.70–2.10	1.73–2.61
Peak *P*	11.00	0.19–0.40		3.82–6.50	3.60–6.43

ever, in one measurement series, t_m for cichoric acid in the reference sample (root extract) varied from 11.9 to 12.1 min (range 1.5%; $n = 6$). Also t_m for cichoric acid in a test sample (Bioforce tincture) varied from 11.2 to 12.6 min (range 12%; $n = 6$), making identification difficult. The corresponding RMT (cichoric acid/peak P) for Bioforce Tincture was 1.07 (R.S.D. 0.49%). This is very close to that for the reference root extract, RMT = 1.08 (R.S.D. 0.05%).

Highly consistent data were also found for the RMT of chlorogenic acid in these samples: 0.75 (±1.4%) in Bioforce Tincture and 0.74 (±0.21%) in the reference root extract.

The reasons for the unusually big shift in observed migration time could be attributable to incomplete conditioning of the capillary, and/or other mechanical/electronic sources of variation affecting the applied voltage, the temperature and the sample introduction. In fact, calculation of the position of a peak relative to that of an assumed standard forms part of the classical repertoire of solutions developed in earlier years for other forms of separation where variability in peak position was a problem, as in gas chromatography [21].

By comparison with the precision of the migration time data, that for the corrected peak areas (Tables 1 and 2) was much poorer. Reasons for this could be associated with the imprecision of the integration software, and/or the possible variation introduced by the extraction procedure itself, even though the same protocol was used for each sample.

In CE in general, good reproducibility of t_m is found (R.S.D. < 1%) within and between runs. However, where peak position variability does occur, the RMT algorithm yields a robust parameter that clearly reduces variability to about R.S.D. 1% for recognition purposes.

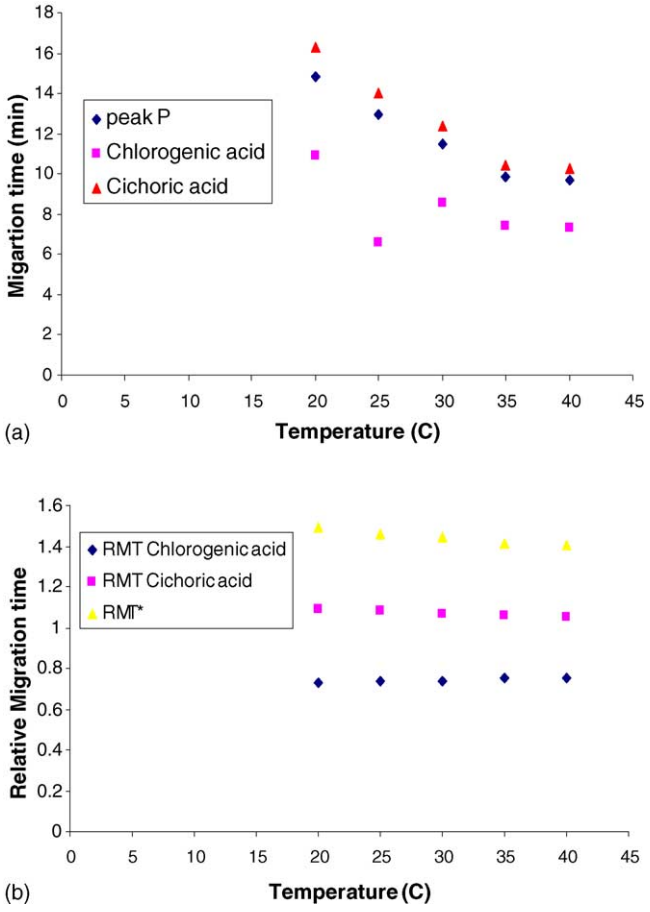

Fig. 3. Effect of temperature on migration time. (a) migration time of chlorogenic acid, cichoric acid, and marker peak *P* in Bioforce tincture. (b) RMT of chlorogenic acid, cichoric acid and on RMT* in Bioforce tincture.

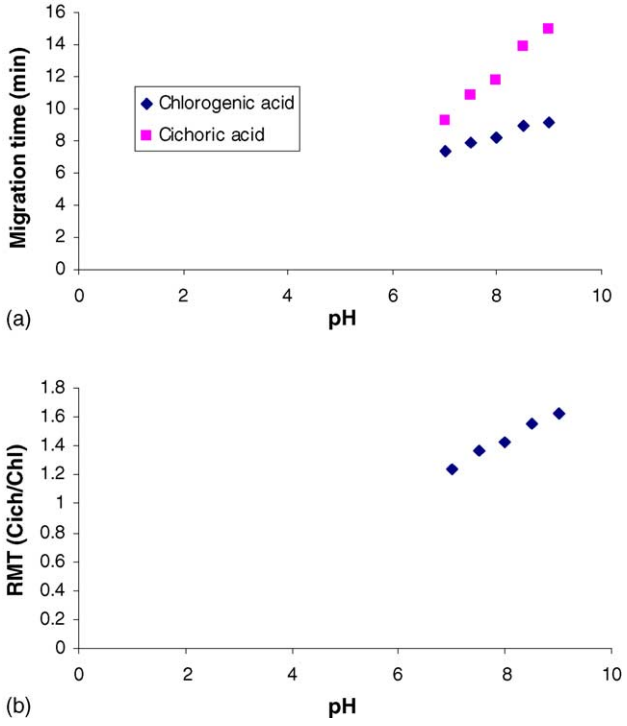

Fig. 4. The effect of pH at 30 °C. (a) the effect on t_m chlorogenic and cichoric acid in Bioforce tincture. (b) The effect on RMT* (t_m cichoric acid/t_m chlorogenic acid).

Table 3
RMT and t_m of chlorogenic acid, cichoric acid and peak *P* and RMT* at 20, 25, 30, 35, and 40 ° C for Bioforce product (pH 8.0) ($n = 3$)

	20.0 °C	25.0 °C	30.0 °C	35.0 °C	40.0 °C
t_m chlorogenic acid (min)	10.90	6.60	8.60	7.40	7.30
t_m cichoric acid (min)	16.30	14.00	12.40	10.40	10.20
t_m peak *P* (min)	14.90	13.00	11.50	9.80	9.70
RMT chlorogenic acid	0.73	0.74	0.74	0.75	0.75
RMT cichoric acid	1.09	1.08	1.07	1.06	1.05
RMT* cichoric acid/chlorogenic acid	1.49	1.46	1.45	1.41	1.40

4.3. Effect of temperature variation on the relative migration time

Using Bioforce tincture as an example, run under the same conditions (Section 2.5), the effect of temperature (20, 25, 30, 35, 40 °C) on RMT was examined at pH 8.

Table 3 and Fig. 3 express the mean change ($n = 3$) in migration time of chlorogenic acid, cichoric acid and marker peak "*P*" at different temperatures, and the relative migration time for chlorogenic acid and cichoric acid with respect to internal marker peak "*P*". A second parameter, RMT*, is also defined as the ratio of cichoric acid to chlorogenic acid, for comparison purposes.

The t_m values for both cichoric acid and chlorogenic acid decreased significantly (ca. 40%) with temperature over 20–40 °C (Fig. 3a). The corresponding RMT data (with reference to peak *P*) were found to be robust, varying by about 3% from 20 to 40 °C (Fig. 3b). Therefore, the relative migration time for these particular solutes is robust to temperature change.

4.4. Effect of pH variation on the relative migration time

It was difficult to identify the marker peak "*P*" unequivocally at pH values other than 8.0 due to changes in peak shape and position at other pH values. Thus, the relative migration time was redefined as RMT*, in terms of the ratio between the standards themselves, namely cichoric acid/chlorogenic acid, as noted in Section 4.3 above. Table 4 and Fig. 4 show the variation in t_m and RMT* for cichoric acid and chlorogenic acid over the pH range: 7.0–9.0. The other conditions including temperature (30 °C) were kept constant.

Under these conditions, the gradient of each solute t_m response was found to differ over the pH range 7–9 (Fig. 4a).

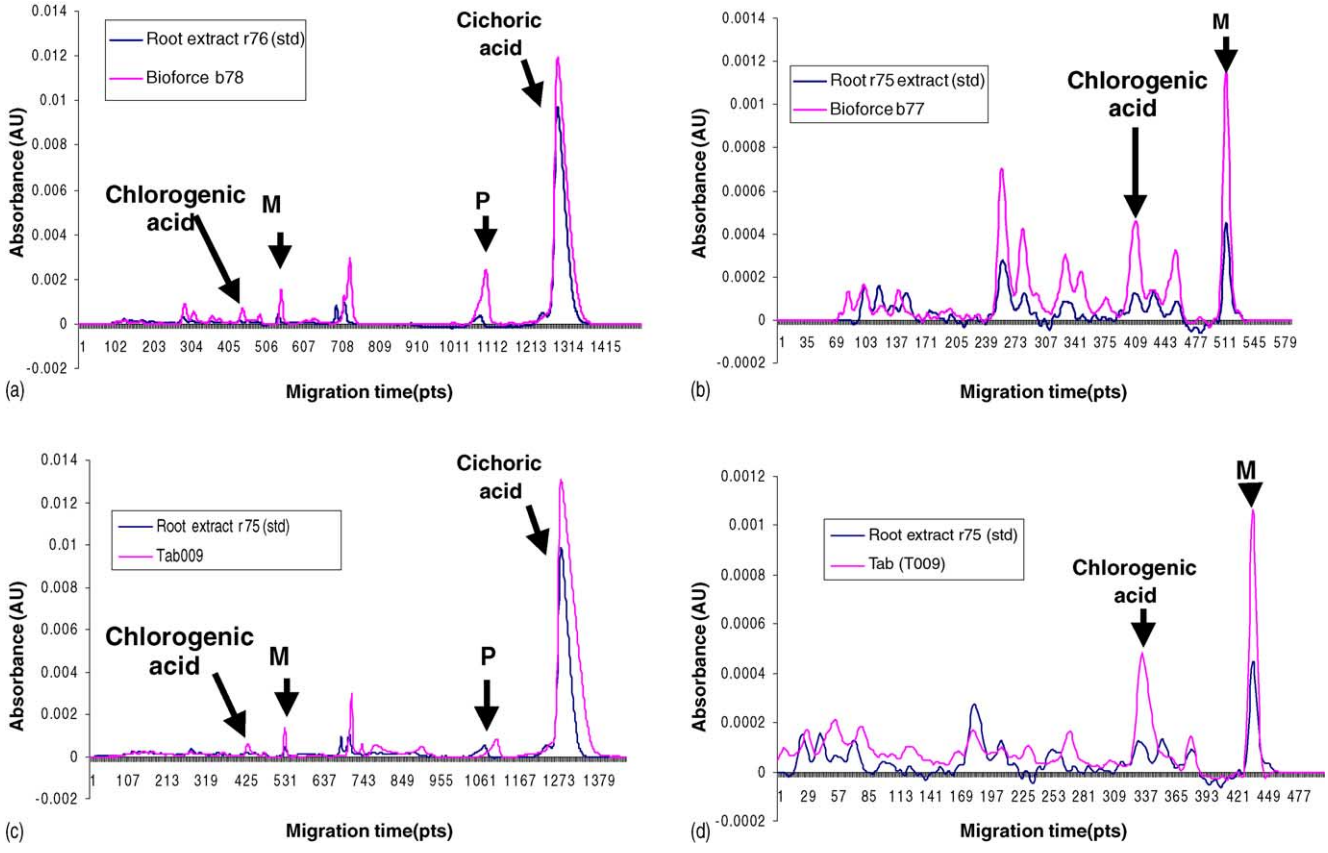

Fig. 5. Performance of peak matching software. (a) Root extract as reference for matching peaks in Bioforce segment (EOF–cichoric acid). (b) Root extract as reference for matching peaks in Bioforce segment (EOF–Peak M). (c) Root extract as reference for matching peaks in Sainsbury's tablet segment (EOF–cichoric acid). (d) Root extract as reference for matching peaks in Sainsbury's tablet segment (EOF–peak *M*).

Table 4
Effect of pH variations on the relative migration time (cichoric acid/chlorogenic acid)

pH	RMT* cichoric acid/ chlorogenic acid	t_m Cichoric acid (min)	t_m Chlorogenic acid (min)
7.0	1.24	9.25	7.42
7.5	1.37	10.84	7.89
8.0	1.43	11.83	8.24
8.5	1.55	13.95	8.96
9.0	1.62	15.00	9.20

Thus the RMT and RMT* would be expected to vary significantly. In fact, RMT* shows marked dependence on pH (Fig. 4b) due to the differential electrophoretic behaviour of these solutes.

4.5. Pattern recognition software

The pattern recognition algorithm developed enables sequential peak-by-peak comparison using specified segments of CD-MEKC electropherograms of *E. purpurea*. The pattern recognition software first selects an initial peak in each of the two segments of data (e.g. the EOF). The last data point is then selected (e.g. cichoric acid or peak *M*). The first and last matching points are aligned, so that the position of any solute within the segment (and above a given threshold) can be adjusted using a cubic spline interpolation operating under Lab Windows (National Instruments).

A linear correlation is assumed between observed migration time (t_m1) and aligned migration time (t_m2) within the segment:

$$t_m2 = t_m1 \cdot a + i$$

where a = slope and i = intercept

In the results in Fig. 5 above, good matching is observed, especially for a smaller segment of the electropherogram.

Commercial capsules (Holland and Barrett) of dried roots free of excipient were extracted to generate the surrogate reference standard, "root extract". Two market products, namely Echinaforce (Bioforce AG tincture, Switzerland) and *Echinacea* (Sainsbury's tablets, UK) were used as test samples, for comparison with root extract (Fig. 5). The reference electropherogram of root extract revealed chlorogenic acid and cichoric acid (identified by spiking), plus the EOF and two unknown (constant) internal marker peaks, *P* and *M*. This compared well with similar peaks in the test electropherograms.

5. Conclusions

Variability in migration time data for cichoric acid and chlorogenic acid due to local factors (e.g. conditioning of capillary, contamination of electrode, temperature variation) can be significantly reduced by converting raw t_m data to relative migration time (RMT), with reference to any other defined peak in the data window.

RMT is robust to changes of ±10 °C above and below 30 °C in the CD-MEKC separation of *E. purpurea* examined. However, both t_m and RMT for peaks in this system are strongly sensitive to changes in pH from 7 to 9, because of differing electrophoretic behaviour of solutes.

The pattern recognition algorithm performed reasonably well in characterising *E. purpurea* in two commercial products. The RMT offers a new, robust metric for characterisation of peaks in CE; combination of RMT with pattern recognition algorithms should enhance the robustness of regulatory controls for commercial Nutraceuticals.

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