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ScienceDirect
Journal of Chromatography A, xxx (2007) xxx–xxx
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Capillary electrochromatography of selected phenolic compounds of *Chamomilla recutita*

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Received 3 January 2007; received in revised form 5 March 2007; accepted 13 March 2007

Abstract

This article explores the use of capillary electrochromatography for the analysis of chamomile (*Chamomilla recutita* L.) extracts. After a thorough study of analytical parameters such as mobile and stationary phase composition, applied voltage, and temperature, a methodology to determine 11 bioactive phenolic compounds (coumarins: herniarin, umbelliferone; phenylpropanoids: chlorogenic acid, caffeic acid; flavones: apigenin, apigenin-7-*O*-glucoside, luteolin, luteolin-7-*O*-glucoside; flavonols: quercetin, rutin and flavanone: naringenin) in chamomile extracts was proposed. The method was performed in a Hypersil SCX/C18 column with pH 2.8 phosphate buffer at 50 mmol L^{-1} containing 50% acetonitrile (pH adjusted before the addition of the organic solvent). All compounds were separated in less than 7.5 min under isocratic conditions. Figures of merit include linearity (peak area versus apigenin concentration) from 50.0–1000 μg/mL ($r^2 = 0.995$), and intra-day precision of retention time and peak area better than 1.3% CV and 15%, respectively. The limits of detection and quantification for apigenin were 35.0 μg/mL and 150.0 μg/mL, respectively. This article also describes an NMR ^{1}H study, carried out to monitor a new clean-up procedure for extracts containing propyleneglycol, whose components are poorly retained in conventional octadecyl silica cartridges.

Keywords: Capillary electrophoresis; μ-HPLC; *Chamomilla recutita*; Chamomile; Herbal extracts; Plant extracts; Sample clean-up; Flavonoids; Coumarins; Phenylpropanoids

1. Introduction

"German chamomile" (*Matricaria recutita*, L.; synonym *Chamomilla recutita*, L. Rausch; synonym *Matricaria Chamomila*, L.) is an herbaceous plant, native of southwest Europe and west Asia, and now spread throughout Europe, North America, Australia, and more recently throughout South America [1].

Chamomile extracts are used as anti-inflammatory, antiemetic, bactericide, fungicide and spasmolytic [1,2]. Such activities have been attributed to two classes of compounds: terpenes and flavonoids [3]. The phenolic fraction of chamomile extracts comprises up to 8% of flavonoids, *c.a.* 0.1% of coumarins, and phenylpropanoids, depending on the flower development [1,4–6].

Chamomile's phenolic fraction is most commonly analyzed by reverse-phase chromatography in gradient [4,7,8] or isocratic elution mode [9] and to a lesser extension, by UV-Vis spectroscopy [10], high performance thin layer chromatography (HPTLC) and thin layer chromatography (TLC) [9]. However, all methodologies can be rather slow when attempted to determine several phenolic sub-classes in a single run. In a previous work, [11,12] two different capillary electrophoresis (CE) methods to analyze the total phenolic fraction of chamomile's extracts were proposed. Although these methods were fast and reproducible, the resolution of similar compounds belonging to the same sub-class and with similar substitution patterns was troublesome. CE has been widely used as an analytical tool for the study of plant phenolics since early 90s [13,14], but it still faces some limitations regarding lack of reproducibility [14], specially when MEKC [15] based methods are employed.

Capillary electrochromatography (CEC) is a hybrid technique of capillary zone electrophoresis and μ-HPLC [16]. It bridges the advantages of both techniques, offering a unique separation mode, exploiting a combination of chromatographic

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doi:10.1016/j.chroma.2007.03.106

retention and electrophoretic mobility [17]. Lurie et al. [18] was the first group to explore the use of CEC to analyze herbal extracts. Using a CEC Hypersil C18 column, the authors described a method that was able to separate seven cannabinoids in *Cannabis sativa* and hashish extracts. In addition to Lurie, other authors successfully analyzed herbal phenolics using CEC as well. Kvasničková et al. [19] developed a new macroporous polyacrylamide column to separate four different lignans from seeds of *Schisandra chinensis*. Ding et al. [20] described a new methodology to pack homemade CEC columns and to analyze four different anthraquinones in rhubarb extracts. Cherkaoui et al. [21] developed a CEC–MS methodology to separate, identify and quantify three withanoides in *Lochroma gesnerioides* plant extracts. And more recently, Stoggl et al. described a CEC methodology to analyze selected biologically relevant flavonoids [22].

This present article explores the use of capillary electrochromatography for the analysis of chamomile (*Chamomilla recutita* L.) extracts. Method development was performed with 11 selected chamomile's phenolic compounds, from different sub-classes. These compounds were chosen based on their pharmacological role in chamomile extracts, although their unique structural characteristics also allow studying the interplay of electrophoretic and chromatographic mechanisms in the overall separation. The optimized methodology was further applied to the analysis of three different chamomile extracts originated from methanol, ethanol and propyleneglycol as extractor solvents. The methanolic extract was obtained from chamomile flowers cultivated in Brazil and the ethanolic along with the propyleneglycolic extracts were supplied by a Brazilian company which manufactures herbal extracts for cosmetic and pharmaceutical uses. In addition, a new procedure to clean-up herbal extracts that contain glycerine and propyleneglycol as main extractor liquids was described.

2. Material and methods

2.1. Chemicals

Reagent grade monobasic sodium phosphate and dibasic sodium phosphate were purchased from J. T. Baker (Phillisburg, NJ, USA). The phosphate buffer pH was adjusted to 2.8 using phosphoric acid (85%). Mobile phase was prepared first by adjusting the pH of the buffer and then by mixing it with acetonitrile (Fischer Scientific, Fair Lawn, NJ, USA). The water used throughout this work was purified and deionized with a Nanopure system (Barnstead, Boston, MA, USA). ChemServices (West Chester, PA, USA) supplied thiourea, benzaldehyde and biphenyl used to check column performance.

Umbelliferone (2), caffeic acid (3), chlorogenic acid (4), apigenin (5), luteolin (7), quercetin (9), rutin (10) and naringenin (11) were purchased from Sigma (St. Louis, MO, USA); apigenin-7-*O*-glucoside (6) and luteolin-7-*O*-glucoside (8) were supplied by Extrasynthèse (Genay, France) and herniarin (1) by Fluka (St. Louis, MO, USA).

2.2. Equipments

Experiments in CEC were carried out in a HP^{3D}CE unit (Agilent Technologies, Palo Alto, CA, USA) connected to a nitrogen cylinder to pressurize both the inlet and outlet vials up to 12 bar. This machine was equipped with a diode array detector set at 337 nm (*vide* Fig. 4 for spectra) and data was collected with a ChemStation platform (Agilent, v.4.01).

All μ-HPLC experiments were performed on a modified Hewlett-Packard model 1090 liquid chromatograph (Wilmington, DE, USA) equipped with a model DRV 5 high performance pumping system. In order to operate the instrument in the flow rate range required for capillary columns, the mobile phase flow was split after the injection valve by using a T fitting from Upchurch Scientific Inc. (Bellefonte, PA, USA) and a restrictor capillary. The column was attached at a right angle to the fluid inlet of the T piece, with the restrictor capillary placed opposite to the fluid inlet. A dual wavelength UV-Vis detector (Thermo Separations Products, model UV 2000, San Jose, CA, USA) was coupled to a cell (Thermo Separations Products, model 9550-0155) for on-column detection. Columns were placed in close proximity to the left hand side of the chromatograph at the same level as the injector valve setup in order to minimize the length of connecting tubing. Data was collected using ChemStation software (Agilent).

2.3. Sample preparation

2.3.1. Cultivation, harvest and drying process

Chamomilla recutita L. (Asteraceae) flowers were cultivated in an experimental field in Sao Paulo State. (Seara Farm, Jacupiranga, SP, Brazil). Seeds were purchased from Fazenda Demétria (Botucatu, Sao Paulo, Brazil). Professor Lin Chau Ming (Universidade Estadual Paulista, Botucatu, SP, Brazil) confirmed the authenticity of the samples.

Seeds were cultivated late in the fall of 1998 (first week of April). Once flower heads developed to a stage containing mainly lingulate and tubular flowers, they were harvested daily from 8:00 am to 12:00 pm. After harvesting, flowers were taken every hour to the drying facility and dried in an adapted air forced oven (40 °C), until constant weight.

2.3.2. Extracts

About 16 g of the dried material were extracted with 400 mL of 1:1 MeOH:H_2O in a sonic bath for 1 h. This procedure was repeated three times and all recovered fractions were filtered and evaporated, under vacuum. The residue was suspended in 24 mL MeOH, and 10 mL of this solution was diluted with 20 mL H_2O to give a stock solution. Exactly 2.5 g of the stock solution was diluted in 10 mL 1:1 MeOH:H_2O. Ethanolic (lot number FS65521098) and propyleneglycolic (lot number FS54481197) extracts were obtained from Farma Service Bioextract (Sao Paulo, Brazil) and diluted appropriately in 1:1 MeOH:H_2O (2.5 g extract to 10 mL solvent). According to the provider, approximately 10% (w/w) of floral heads were used in the preparation of these extracts. The extractor solvent of the so called "glycolic" extract consisted of propyleneglycol (92.7%, w/w), glycerine

(2%, w/w) and ethanol (5%, w/w). Alcoholic extracts were obtained with a 70% ethanolic-aqueous solution. In both cases, a mixture of methylparaben (0.2%, w/w) and propylparaben (0.1%, w/w) was used as preservative.

2.3.3. Sample clean-up

2.3.3.1. Methanolic and ethanolic extracts. Solid-phase extraction (SPE) cartridges Sep-Pak C18, 500 mg (Waters, Milford, MA, USA) were preliminary conditioned by 2 mL MeOH followed by 2 mL water. About 2 mL of filtered methanolic or ethanolic extracts were loaded into the column and washed with 2 mL of water in order to elute the most polar matrix components. The phenolic fraction, which was retained in the cartridge, was eluted with 1 mL of 3:7 MeOH/H_2O followed by the same amount of 5:5 MeOH/H_2O and finally 7:3 MeOH/H_2O; all fractions were combined together. Eluates were evaporated to dryness and residues were suspended in 125 μL of a 8:2 MeOH/H_2O mixture.

2.3.3.2. Glycolic extracts. The clean-up study for the glycolic extracts were done with HLB-OASIS cartridges, 500 mg (Waters), using the same eluotropic series described for the ethanolic extract. In all clean-up steps, the flow rate did not exceed 1 mL/min.

2.3.4. Standards

Stock solutions of the standard compounds at 1000 μg/mL concentration were prepared in 50 mmol L^{-1} phosphate buffer at pH 2.8 containing 75% ACN. Samples were prepared every other week and stored at −80 °C. Every week, 100 μL of each stock solution were mixed together and kept in opaque flasks, stored in freezer. This procedure intended to minimize phenylpropanoid isomerization as well as the degradation of other compounds such as rutin.

2.4. Capillary electrochromatography

2.4.1. Columns

CEC experiments were performed in two different columns. Method development was mainly done with an in-house packed column using Spherisorb ODS 1 (75 μm i.d., 5 μm diameter particle and 80 Å mean pore). The other column, a Hypersil Duet SCX/C18 Mixed-mode (50 μm i.d., 33.5 cm/25.0 cm of total and effective length, respectively, 3 μm particle and 80 Å mean pore) was purchased from Keystone Scientific Inc. (Bellefonte, PA, USA).

2.4.1.1. Packing procedure. Polyimide fused-silica capillaries with 75 μm inner diameter and 375 μm outer diameter were purchased from Quadrex (New Haven, CT, USA). Spherisorb ODS 1 as specified above was purchased from Waters.

A packing procedure described before [23] was adapted for the packing of Spherisorb ODS into 75 μm i.d. fused-silica capillaries. One end of a 500 mm long capillary was connected to a temporary frit (Parker Hannifin Corporation, Huntsville, AL, USA) and the other one to a cylindrical stainless steel reservoir (50 mm × 2 mm) containing a slurry solution of 1% (w/v) of stationary phase in THF. Acetone, used as packing solvent, was pumped through the reservoir by an Air Driven Liquid Pump Model MLP-46 (Haskel Inc., Burbank, CA, USA) at 10,000 p.s.i. until the packing bed reached at least 40 cm. The pressure was then slowly released to 7000 p.s.i. in order to stop the packing material to flow. The column was allowed to stay under 7000 p.s.i. for one more hour. The pump was then turned off and the pressure was allowed to decline slowly so that the packing bed would not be disturbed by a sudden release of pressure.

The column was disconnected from the reservoir, attached directly to the pump outlet by using pressure resistant ferrules (Upchurch Scientific Inc.) and flushed with water at 7000 p.s.i. for about 20 h, after which the outlet frit was synterized at 30–35 cm from the pump inlet using an electrically heated Ni–Cr wire looped around the capillary. Once the frit was done, the pump motor was turned off and the pressure was released slowly. After the temporary inlet frit was taken out, the column was attached in its reverse direction to the pump inlet. The packed bed was washed with water (1500 p.s.i.) for another 10 h, and while it was still under pressure, the permanent inlet frit was done at 25 cm from the outlet frit. After the pressure was released, the column was once more inverted and washed with ACN (1500 p.s.i.) for about 10 h in order to wash away the excess of stationary phase and to recondition the C18 chains after a long exposition to water. The inlet extremity of the capillary now empty was carefully cut off close the inlet frit. The detection window was made close to the outlet frit using a razor blade.

The very fist time this column was used in the CEC instrument, it was flushed with methanol with 10 kV for *ca.* 20 min prior to the conditioning regime. This procedure helped to release any loose particle of stationary phase that did not get completely fused when the outlet frit was done.

2.4.2. Analysis conditions

Mobile phase composition, applied voltage and temperature were studied in the method development and are described later. All analyses were carried out using 337 nm as wavelength. Sample injections were done hydrodynamically with 12 bar/0.15 min and 12 bar/0.26 min for Spherisorb ODS and SCX/C18, respectively.

2.4.3. Column conditioning protocol

In the beginning of a working day, columns were flushed in the μ-HPLC instrument (600 p.s.i.) with desired mobile phase for 2 h, and after that columns were carefully transferred to the CEC equipment in order to maintain both ends wet. Columns were then equilibrated electrokinetically with +15 kV, +20 kV and +25 kV (10 min each voltage) with its inlet and outlet pressurized with 12 bar nitrogen. Columns were ready to use when both baseline and current were stable. This protocol was also employed every time the running buffer was changed. During between-runs conditioning, columns were flushed with mobile phase under 12 bar applied to the inlet for 20 min, followed by a voltage step gradient of +5 kV, +15 kV and +25 kV, 10 min each.

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2.4.4. Linearity, LOD, LOQ and repeatability

For the calibration curves, appropriate aliquots of the standard stock solution of apigenin (1000 μg/mL) were transferred to separate 10 mL volumetric flasks and the volumes were completed with deionized water to yield final concentrations of 50.0 μg/mL, 100.0 μg/mL, 150.0 μg/mL, 250.0 μg/mL, 500.0 μg/mL, 750.0 μg/mL and 1000 μg/mL. All solutions were sonicated and filtered through a 0.22 μm filter (Millipore) prior to injection. Each solution was analyzed in triplicate, and the resulting peak areas were plotted against the respective apigenin concentration. The repeatability was determined by the injection of ten replicate samples of the standard solution sequentially in a single day.

2.5. μ-High performance liquid chromatography

The μ-HPLC experiments were performed with the same Spherisorb ODS 1 used in the CEC experiments. Columns were connected directly into one end of the T piece and the detector position was adjusted so that the column effluent was monitored immediately after the retaining frit at the outlet, while keeping the column straight. After installation, the column was flushed with the eluent at an inlet pressure of about 84 bar for 50–60 min until stable baseline was obtained at 337 nm. All μ-HPLC experiments were carried out at room temperature with flow rate of 0.05 mL/min, and a capillary with 30 cm/50 μm as a split. Applied pressure did not exceed 180 bar. Among several tested conditions, a flow rate of 0.03 mL/min with a split column of 60 cm length and 50 μm internal diameter was chosen.

2.6. Capillary zone electrophoresis

Experiments were conducted in a capillary electrophoresis system HP^{3D}CE (Agilent Technologies), equipped with a diode array detector set at 337 nm, and a data acquisition/treatment software (ChemStation, rev A.06.01). Analytical conditions were the same as described for CEC.

3. Results and discussion

3.1. Method development

CEC method development was mostly performed in a home-made column packed with Spherisorb ODS1 (5 μm particle) using a standard mixture containing 11 selected phenolic compounds present in chamomile and a mobile phase consisting of acetonitrile and pH 2.8 phosphate buffer. Although CEC is generally performed at high pH in order to maintain a fast EOF, a low pH was chosen in order to inhibit ionization of the acidic compounds and thus avoid any electrophoretic migration, which would resist the EOF.

The first parameter studied during method development was the concentration of phosphate buffer in the mobile phase, where solutions containing 20 mmol L^{-1}, 50 mmol L^{-1} and 70 mmol L^{-1} of pH 2.8 phosphate buffer were mixed to 50% ACN. As it can be seen in Fig. 1A–C, the electroosmotic flow (EOF) decreased when buffer concentration was increased, and consequently, the retention times of all solutes also increased. However, after 50 mmol L^{-1} with exception of the first eluting compounds (3, 6, 8 and 10), selectivity and retention remained relatively constant. These referred compounds are more acidic than the others, and consequently more susceptible to buffer concentration variations than the neutral ones.

Resolution of compounds 3, 6, 8 and 10 increased when phosphate concentration was also increased from 20 mmol L^{-1} to 50 mmol L^{-1}. However, at 70 mmol L^{-1}, these same compounds were co-eluted in a single peak, perhaps due to an inhibition of the partitioning caused by the excess of phosphate salt.

Also in Fig. 1A–C, it can be seen that compound 4 (chlorogenic acid) behaved in a very different fashion when compared to other acidic compounds of the same class, such as caffeic acid (3), eluting between compounds 3 and 2 in 20 mmol L^{-1} and 50 mmol L^{-1} buffer concentration and after compounds 7/9 in 70 mmol L^{-1} buffer concentration. That led us to speculate whether it was partially ionized at pH 2.8. The referred compound (4) was analyzed by capillary zone electrophoresis (CZE) using a capillary with the exactly same specifications, dimensions and analytical conditions as those described for the packed column, and have seen that indeed, it was ionized at pH 2.8 having an effective mobility equal to -0.67 mm^2 V^{-1} s^{-1} (Fig. 2B).

Although some authors have reported that N increases with the phosphate concentration [18], we have found that 70 mmol L^{-1} phosphate buffer had a deleterious effect. That may be explained by the fact that higher phosphate concentration may incur higher current values, which in turn could have caused some band spreading.

The effect of organic solvent content on the CEC separation of chamomile's phenolic compounds was also assessed by using 50 mmol L^{-1} phosphate buffer at pH 2.8 with 40%, 50% and 60% acetonitrile. Fig. 1D, B and E depicts the respective electrochromatograms.

While EOF velocity increased with ACN percentage increase in the mobile phase, selectivity and efficiency showed major variations. For most compounds, resolution improved as percentage of ACN decreased from 60% to 50% (Fig. 1E and B). Interestingly, at 40% acetonitrile (Fig. 1D), compounds 6, 8, and 10 but not 3, the most acidic ones, were completely co-eluted in a single peak as it happened in 70 mmol L^{-1} phosphate buffer (Fig. 1C). In order to verify if this behavior was due to electrophoretic of chromatographic forces, the standard mixture was analyzed by μ-HPLC using the same column used in the CEC analysis. Mobile phase composition and analytical conditions were kept constant of that of Fig. 1B. The resulting chromatogram is shown in Fig. 2A. The same elution pattern was observed, which confirms the fact that, except for compound 4, all others were separated chromatographically rather than electrophoretically.

Fig. 1F, B and G and Fig. 1H, B and I show the effect of applied voltage (from +25 kV to +30 kV) and temperature (15–45 °C), respectively in the CEC separation of the standard mixture. Resolution decreased as applied voltage and temperature increased. Increases in the capillary temperature led to a reduction of retention times most probably as a result of the decrease in partition coefficients, although mobile phase viscos-

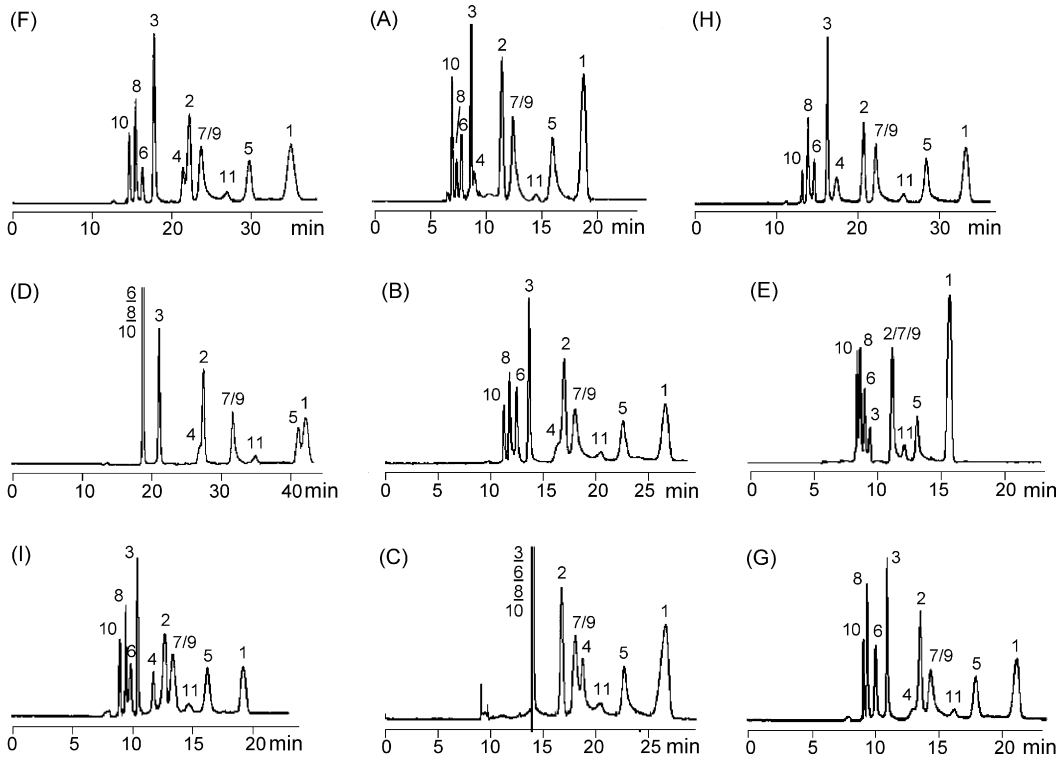

Fig. 1. Capillary electrochromatograms of chamomile's phenolic standard compounds illustrating the effect of: buffer concentration (A) 20 mmol L^{-1}, (B) 50 mmol L^{-1} and (C) 70 mmol L^{-1}; %ACN (D) 40%, (B) 50%, (E) 60%; applied voltage (F) 20 kV, (B) 25 kV, (G) 30 kV and temperature (H) 15 °C, (B) 25 °C, (I) 45 °C in pH 2.8 phosphate buffer. Column: Spherisorb ODS 1 (33.5 cm total/25 cm effective length), 75 μm i.d., 5 μm particle. Other conditions: injection: 12 bar/0.15 min; λ = 337 nm. Peak legend: herniarin (1), umbelliferone (2), caffeic acid (3), chlorogenic acid (4), apigenin (5), apigenin-7-*O*-glucoside (6), luteolin (7), luteolin-7-*O*-glucoside (8), quercetin (9), rutin (10) and naringenin(11).

ity also decreased. Towards higher values of applied voltage, temperature did not affect much the plate number (less than 25%; data shown for +25 kV only). Lurie et al. observed similar effect when analyzing cannabinoids by CEC [18], although this comes in contrast to what has been published for HPLC [24]. A possible explanation given by the authors was that the effect of the mobile phase diffusion coefficient (Dm), on the *C* term of the Van Deemter equation, might be greater in HPLC than in CEC [18].

By combining +25 kV and 45 °C (Fig. 1I), 10 out of 11 compounds were separated in the standard mixture in nearly 20 min under isocratic run. However, after nearly 10 runs at 45 °C, N decreased considerably and it was not possible to obtain a stable current anymore. Light microscopic examination of the column showed a 600 μm gap in the column packing close to the window extremity. This observation suggests that temperature can be used to aid stationary phase sedimentation during packing procedures.

Due to the column instability at 45 °C, we chose to continue our experiments using 50% acetonitrile in 50 mmol L^{-1} phosphate buffer at pH 2.8, 25 °C and +25 kV, as shown in the electrochromatogram of Fig. 1B. On that condition, we were able to successfully separate 10 out of 11 studied compounds in 28 min, but the EOF velocity was considerably low, μeof = 6.8 mm^2 V^{-1} s^{-1}, which accounted for 35% of the total analysis time. In order to increase EOF velocity, a commercially available Hypersil SCX/C18 column was tested. This column was packed with a "mixed-mode" stationary phase, which contains not only strong cation exchange sites that will remain ionized under low pH conditions, but also aliphatic chains to provide sites for partitioning and hydrophobic interactions.

Fig. 2C shows the dramatic improvement of the separation performed in the described column as compared to the Spherisorb ODS column (Fig. 1B), being possible to separate all 11 compounds, (including the critical pair 7/9, although partially) in less than 7.5 min (μeof = 1.8 mm^2 V^{-1} s^{-1}). However, and not surprisingly, compound 4 was less retained in SCX/C18 than in Spherisorb ODS forming a partially resolved pair with compound 3, rather than with 2, as it happened in the latter column. To our best knowledge, this is the first time that chamomile's phenolic compounds, including flavones, flavanols, flavanones, phenylpropanoids and coumarins were separated in less than 7.5 min, under isocratic conditions.

3.2. Sample "clean-up"

Herbal extracts are known to be rich in apolar compounds such as chlorophyll, fatty acids and steroids. Since these compounds are strongly retained by octadecyl silica, it is a common practice to clean-up an herbal extract sample prior to its injection into a ODS column. Examples for such procedures abound [25]. However, although literature examples dealing with sample clean-up for methanolic and ethanolic extracts are many, this

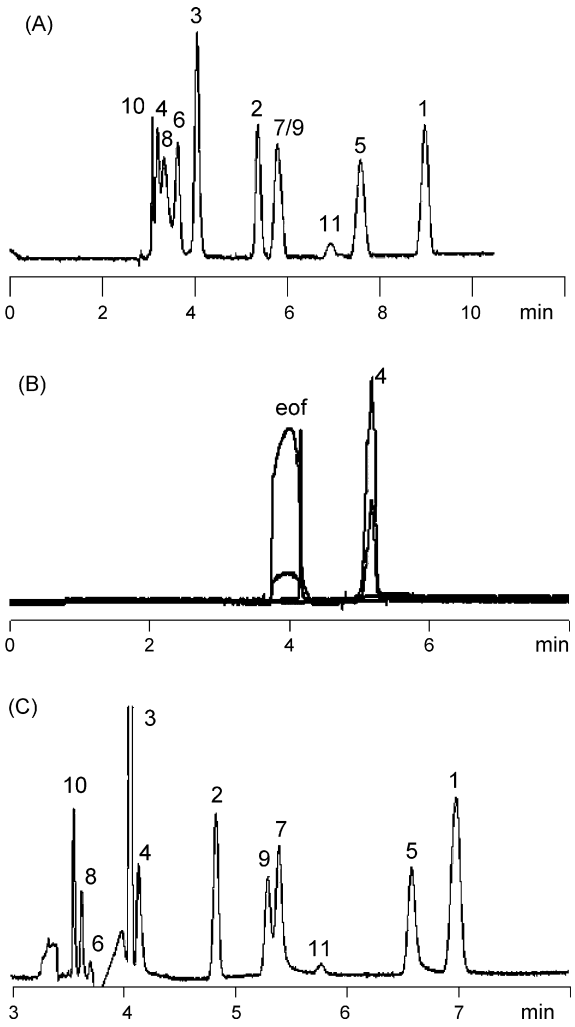

Fig. 2. Capillary chromatograms of chamomile's phenolic standard compounds in (A) μ-HPLC, (B) CZE and (C) SCX-C18 CEC. Mobile phase: pH 2.8 phosphate buffer at 50 mmol L^{-1} concentration containing 50%ACN. μ-HPLC conditions: column: Spherisorb ODS 1 (33.5 cm total/25 cm effective length), 75 μm i.d., 5 μm particle; split column: 30 cm (50 μm i.d.); flow rate: 0.05 mL/min; injection loop: 20 μL; temperature: 25 °C; λ = 337 nm. CZE conditions: 75 μm i.d. capillary; injection: 50 mbar/3 s; applied voltage: +25 kV; temperature: 25 °C; λ = 337 nm. CEC conditions: column: SCX/C18 (33.5 cm total/25 cm effective length), 50 μm i.d; 3 μm particle; injection: 12 bar/0.4 min; applied voltage: +25 kV; temperature: 25 °C; λ = 337 nm. Peak labels as in Fig. 1.

is not the case for of extracts containing propyleneglycol and/or glycerine as extractor liquids.

In a previous work [26], we have attempted to remove highly apolar compounds from chamomile's methanolic, ethanolic and glycolic extracts using traditional ODS solid-phase extraction cartridges. Our attempt consisted of conditioning the cartridge's stationary phase with a solvent of medium polarity in order to retain compounds with medium to low polarity. Cartridges were then washed with water in order to release all absorbed highly polar compounds. After this "washing step", medium-polarity compounds were eluted with 30%, 50%, 70% and 100% methanol in water and all collected fractions were subjected to analysis. Although this procedure enabled us to successfully prepurify the desired phenolics from both methanolic as well as ethanolic extracts (phenolic compounds were eluted on fractions

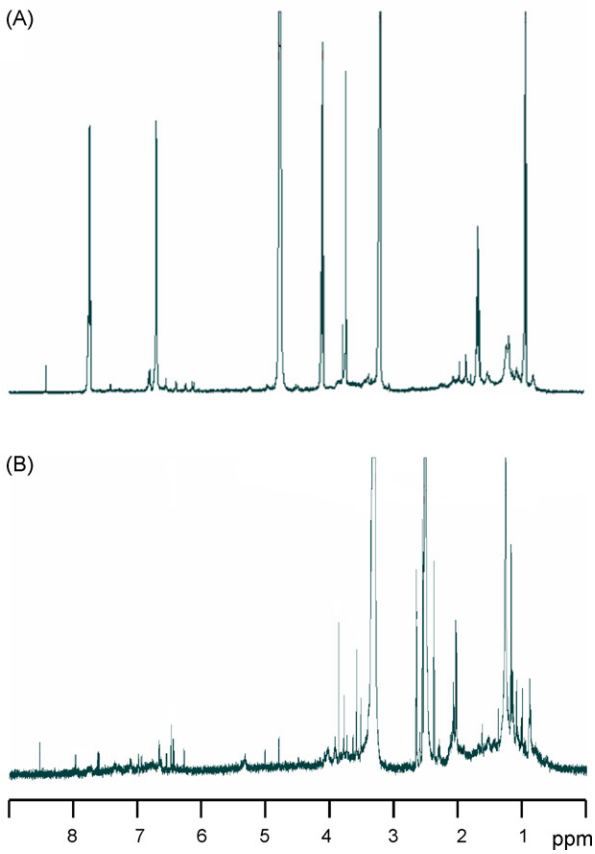

Fig. 3. NMR ^{1}H spectra of chamomile glycolic extract clean-up samples. (A) Deuterated methanolic combined fractions and (B) chloroform fraction.

containing 30%, 50% and 70% of methanol in water), it did not do so for glycolic ones. In this latter case, most compounds, including apolar ones, were readily eluted when cartridges were first washed with water. This was attributed to the presence of propyleneglycol and glycerine in the matrix, which would be inhibiting the absorption of the compounds to the stationary phase.

We hypothesized that a more hydrophobic stationary phase would perhaps increase the binding strength between the analytes and stationary phase to a point where both propyleneglycol and glycerine would not interfere with the separation.

In a second attempt to clean-up chamomile glycolic extracts, we used a polymeric stationary phase composed of polymerized *N*-vinylpyrrolidone and divinylbenzene commercialized as OASIS HLB. The new experiment followed the same rationale as before except by two points: the pH of the water used in the washing-step was adjusted with 3% (v/v) acetic acid and we included a final washing step with chloroform in order to remove any further retained compound, which were not eluted with methanol. All collected fractions were then submitted to NMR ^{1}H analysis. NMR technique was chosen because it would allow us to visualize fatty acids and steroids, which are generally non-optically active in the UV range.

All fractions from 30% to 100% of methanol showed characteristic protons of flavonoids and/or phenolic compounds (Fig. 3A), such as A-ring aromatic protons absorbing in the

range of 6.0–6.9 ppm, as well as B-ring protons appearing in the lower field from 6.7 to 7.9 ppm, along with characteristic singlets at approximately 3.8 ppm, indicative of methoxy groups in the B-ring. None of the methanolic fractions showed characteristic proton signals of fatty acids or steroids, which absorb in the range 0.8–2.5 ppm. On the other hand, these protons were the main feature of chloroformic fraction, depicted on Fig. 3B. We therefore grouped fractions 30–100% and used it in the subsequent CEC experiments.

It was possible to conclude at this point that the Oasis HLB stationary phase retained chamomile's phenolic compounds more strongly than ODS and this could be used advantageously to clean-up herbal extracts containing propyleneglycol and glycerine in their composition. However, more tests still need to be

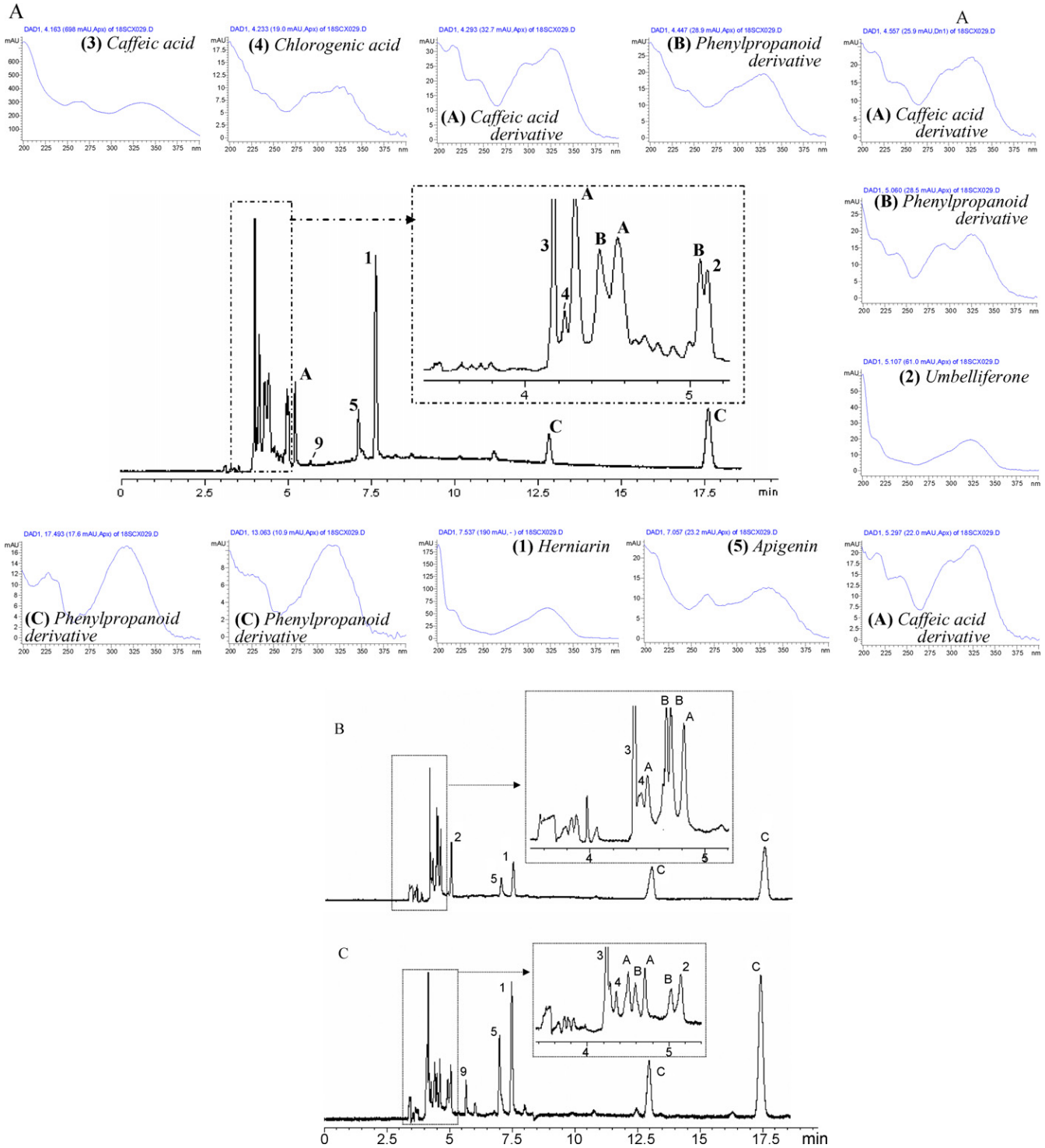

Fig. 4. Identification of phenolic compounds in chamomile methanolic (A), ethanolic (B) and glycolic (C) extracts. Column: SCX/C18. Analytical conditions as in Fig. 2C. Peak labels as in Fig. 1.

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done in order to accurately access the recovery of such extractions.

3.3. Extracts analysis

Fig. 4A shows a typical electrochromatogram of a methanolic extract along with UV spectra of its main peaks. Peak identities were assigned based on their UV spectra and also by spiking techniques.

We were able to identify caffeic (3) and cholorogenic (4) acids, apigenin (5), quercetin (9), umbeliferone (2) and herniarin (1). Although we were able to obtain baseline separation for the flavonoid glucosides (6, 8, 10) in the standard solution electrochromatograms (Fig. 2C), their identification in the extracts was troublesome due to the low intensity of the peaks eluting from 3.5 min to 4.0 min, region where these compounds were expected to be seen (see expansion of Fig. 4A).

It is interesting to note that all the major peaks in the methanolic extract electrochromatogram had characteristic spectra of hydroxycinnamic acid derivatives. Unfortunately we were not able to record the spectra of the several other smaller peaks, due their low abundance.

Since, in most cases, only the phenylpropanoid moiety of cinnamoyl conjugates contributes significantly to UV absorption, their spectra are usually similar to those of the corresponding free cinnamic acid. The long wave band of caffeoyl and feruloyl conjugates are generally recorded around 330–335 nm and 320–325 nm, respectively [27]. However, caffeic acid and its derivatives have characteristic absorption bands at 243 nm and 326 nm, with a distinctive shoulder at 300 nm to the long wave band [5]. Feruloyl conjugates also show this characteristic shoulder around 295–300 nm.

We classified the unidentified peaks in three groups based on their UV λ_{max} similarities. The group with peak labeled as 'A' (Fig. 4A) showed λ_{max} = 243, 300, 326 nm and thus these peaks were assigned as caffeic acid derivatives. Group 'B' showed λ_{max} at 245, 297, 333 nm and based on their similarity with caffeic acid's UV spectrum and also due to the presence of the shoulder at 297 nm we hypothesize that they might be derivatives of ferulic acid. However, due to the lack of more conclusive data, we labeled peaks as general phenylpropanoid derivatives. Group 'C' showed λ_{max} at 232 and 315 nm. However, the characteristic shoulder was absent. These spectra could indicate either *p*-coumaric derivatives or also hydroxycinnamic derivative conjugated at its 4-hydroxy position to other hydroxycinnamic acid. This type of conjugation is known to produce an important hypsochromic shift of the long-wave maximum [5] and has also been reported in chamomile flowers [8].

Fig. 4B and C shows the identification of the main peaks in ethanolic and glycolic extracts. Besides a very similar peak profile, UV spectra of main peaks were also similar to those recorded for methanolic extract. Relative abundance however, differed considerably among these extracts. Both glycolic and ethanolic extracts showed high concentration of hydroxycinnamic derivative "C", but on the other hand, quercetin content in ethanolic extract was much higher.

Table 1
Calibration, linearity and limits of detection and quantification for the CEC method developed in a SCX/C18 column

Parameter	Statistical data
Concentration range (μg/mL)	50.0–1000
Intercept	3.060
Slope	0.3154
Coefficient of determination (r^2)	0.995
Limit of detection (μg/mL)	35.0
Limit of quantification (μg/mL)	150.0

The presence of high amounts of phenylpropanoids in chamomile, especially in tubular florets, has already been described [8,10,26,28,29]. We have previously subjected this same batch of flowers to acidic hydrolysis in order to quantify its total apigenin content [12], and it was possible to detect a high concentration of caffeic acid and other phenylpropanoid acid, which by UV spectra evidences, led us to hypothesize as being ferulic acid. However, since flowers used in the manufacture of these three extracts were of different origins, further comparison would be mere speculation.

Matos et al. [30] analyzed the essential oil from Brazilian chamomile cultivars and found that they are qualitatively and quantitatively similar to the European ones, which were analyzed in the same study. Worldwide, most of the studies about phenolic content variation in chamomile flowers were based on their free apigenin and apigenin-7-*O*-glucoside derivatives [31,32] and in a less extension on their coumarin content [32]. In previous works, we have proposed capillary electrophoresis methodologies to analyze phenolic fraction of chamomile flowers grown in Brazil focusing on the quantification of free and total apigenin [11,12]. Mulinacci et al. [8] have described a semi-quantitative study on the flavonoids and phenypropanoid content of Italian chamomile flowers. In spite of all the work done to characterize flavonoidic fraction of chamomile flowers, there is still a lack of a comprehensive study regarding flavonoids, phenylpropanoids and coumarins in flowers from different origins.

3.4. Figures of merit

For standardization purposes of chamomile extracts, apigenin has been the flavonoid of choice to which several therapeutic properties have been associated [3,9,12]. A few figures of merit for the proposed method has been established for apigenin using the following optimized conditions: Column: SCX/C18 (33.5/25 cm; 50 μm i.d; 3 μm particle); Mobile phase: pH 2.8 phosphate buffer at 50 mmol L^{-1} concentration containing 50% ACN; injection: 12 bar/0.4 min; applied voltage: +25 kV; temperature: 25 °C; λ = 337 nm.

The interval of linear response covered the concentration range from 50.0 μg/mL to 1000 μg/mL. The proposed method also provided an intercept close to the origin [33,34], and good linearity (r^2 = 0.995) between peak area and apigenin concentration (Table 1).

Table 2
Repeatability (%CV, $n = 10$) of the retention time (RT), peak area (PA) and peak height (PH) of phenolic compounds in a SCX/C18 column

Compounds (elution order)	RT[a]	PA[b]	PH[c]
10	0.93	8.3	6.4
8	0.97	9.5	4.6
6	1.0	9.5	10
3	0.97	13	8.3
4	2.0	5.5	8.7
2	0.98	8.3	3.3
7	1.0	15	4.6
9	1.1	13	4.7
11	1.3	6.6	8.0
5	1.3	5.6	3.8
1	1.2	7.4	2.8

[a] Retention time.
[b] Peak area.
[c] Peak height.

LOD and LOQ were 35.0 μg/mL and 150.0 μg/mL, respectively (Table 1). The criteria used to determine these limits were based on the S/N ratio of 3 for LOD and 10 for LOQ. These numbers however were poor when compared with those from other techniques such as HPLC [26] or CE [11]. Low detectability is already known to be a major disadvantage of CEC and this can be attributed to the small light path in the capillary. Lurie et al. [18] reported that by using a high sensitivity UV detector cell (with an expanded path length of 1.2 mm) they were able to increase the signal-to-noise ratio eight times with little loss of resolution.

Repeatability in SCX-C18 column was determined by 10 consecutive injections of standard solution and it was expressed in terms of %CV as shown in Table 2. Even though retention time repeatability was satisfactory, it was highly dependable of the column conditioning procedure used between runs. If column was not submitted to a regular conditioning regime, we saw a clear drop of the EOF velocity and decrease of resolution of critical pair 7/9 (data not shown). Peak area and peak height repeatability were less than 15% CV and 10% CV, respectively. It is interesting to notice that peak height values were more uniform. Several factors such as poor detection, low sample load associated to low EOF repeatability might have contributed to result, which was also observed by Lurie et al. [18].

4. Conclusions

This study contributes to the concept that CEC is a powerful analytical technique with much potential to the analysis of herbal extracts, in particular to the various natural phenolic compounds. It offers high separation efficiency and resolution, and acceptable repeatability as long as column is properly conditioned.

However, in spite of such promising perspectives, CEC still faces serious problems that may still overshadow its many benefits. Two of the main problems are the column itself and the long time that is necessary to condition the column. Columns are very fragile, and this may have a large impact not only in the method robustness, but also in the overall analysis price. When low LOD/LOQ are necessary, in which case columns with expanded light path are required, the overall analysis price would be even higher. Polymer based monolithics stationary phase are a promising venue to overcome such problems.

Column conditioning routine, both in between runs and in the beginning of the day, was another major problem. The excessive time and labor required to obtain stable current/baseline and reproducible retention times, not only increases considerably the overall analysis price but also contributes to bubble formation, which is detrimental to baseline and current stabilization. Commercially available equipments capable of high pressurization would not only improve column conditioning time and avoidance of bubble formation, but also it would facilitate bubble removal from the column.

Acknowledgements

The authors want to express their deep appreciation to Prof. Csaba Horváth (in memoriam) for his unconditional support to this work allowing its entire development in his laboratory at Yale University (New Haven, CT, USA) as part of an exchange program between Yale University and University of Sao Paulo, financed by the Brazilian agency Fundação de Amparo à Pesquisa do Estado de São Paulo (Grant number: 98/06822). Also our appreciation to Prof. Lin Chau Ming from Universidade Estadual Paulista "Julho de Mesquita Filho" (Sao Paulo, SP, Brazil) for the identification of the flowers used for the methanolic extraction. The authors acknowledge Farma Service Bioextract for donation of the ethanolic and glycolic chamomile extracts. F.N. Fonseca also wishes to thank Mr. Langerton Neves da Cunha (in memoriam) for sharing his deep knowledge of phytotherapy, and also for overseeing the cultivation of chamomile.

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Please cite this article in press as: F.N. Fonseca et al., J. Chromatogr. A (2007), doi:10.1016/j.chroma.2007.03.106