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Research Article

Cyclodextrin-modified capillary electrophoresis for achiral and chiral separation of ergostane and lanostane compounds extracted from the fruiting body of *Antrodia camphorata*

A CD-modified capillary electrophoretic method has been developed for achiral and chiral analysis of seven bioactive compounds isolated from the fruiting body of *Antrodia camphorata*. Such important target analytes exhibit similar chemical structures and are known for their diverse properties including antioxidant and anticancer effects. The analytes were separated in 25 min using a pH 9.3, 20 mM sodium borate buffer containing 20 mM methyl-β-CD and 30 mM sulfobutylether-β-CD. With the exception of the optical isomer pairs (antcin B or zhankuic acid A, zhankuic acid C, and antcin A), the remaining bioactive compounds including the chiral pair antcin C were baseline-separated. Analysis time was noticeably longer to baseline separate all of the above chiral pairs (~38 min) by adding 5% DMF to the running buffer. The migration order was reversed compared with the HPLC elution. More hydrophobic compounds complexed favorably with methyl-β-CD and emerged earlier in the electropherogram than their more hydrophilic counterparts which were strongly associated with sulfobutylether-β-CD. The simple capillary electrophoretic method developed was applicable for rapid separation and characterization of several important bioactive compounds isolated from the fruiting body of *A. camphorata*.

Keywords:
Antrodia camphorata / Bioactive compounds / Ergostane / Fruiting body / Lanostane
DOI 10.1002/elps.200800758

1 Introduction

Antrodia camphorata (syn. *Taiwanofungus camphoratus*), a rare *Ganoderma*-like fungus, has been used to manage various diseases such as abdominal pain, diarrhea, drug intoxication, hypertension, and skin itching. It has also been used in the formulation of nutraceuticals and functional foods in Taiwan to enhance the immune system and liver function [1]. To date, this edible fungus exhibits several important biological activities: anticancer [2–5], antihepatotoxic [6, 7], antihypertensive [8], anti-inflammatory [9, 10], antioxidant [11], and neuroprotective activities [12]. *A. camphorata* inhibits lipopolysaccharide/inteferon (LPS/IFN)-γ-induced inflammatory mediator production in macrophages, as well as tumor cell proliferation [13]. Previous phytochemical investigations on *A. camphorata* have resulted in the isolation of diterpenoids, triterpenoids, sesquiterpene lactone, benzenoids, and polysaccharides [10, 12, 14–20]. To date, three triterpenoids named antcin A (AC6), antcin B (AC3), and antcin C (AC7) with ergostane skeletons in the fruiting body of *A. camphorata* were first isolated and identified [16]. The extract of the fruiting body obtained by use of ethyl alcohol has also three triterpenoids named antcin B or zhankuic acid A, zhankuic acid B, and zhankuic acid C (AC5) [14]. Four triterpenoids named antcin E, antcin F, methyl antcinate G, and methyl antcinate H were then isolated from *A. camphorata* [17]. Another two new compounds having ergostane skeletons named zhankuic acid D (AC1) and zhankuic acid E, and three compounds having lanostane skeletons named 15 α-acetyl-dehydrosulfurenic acid, dehydroeburicoic acid

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Abbreviations: AC1, zhankuic acid D or methyl antcinate B; **AC2,** dehydroeburicoic acid; **AC3,** antcin B or zhankuic acid A; **AC4,** dehydrasulfurenic acid; **AC5,** zhankuic acid C; **AC6,** antcin A; **AC7,** antcin C; **CC,** column chromatography; **MECD,** methyl-β-CD; **SBCD,** sulfobutylether-β-CD

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(AC2), and dehydrasulfurenic acid (AC4) have also been purified and identified [18].

A. camphorata extract could inhibit tumor development; hence, it would be desirable if such an active composition purified from *A. camphorata* extract exhibits a certain effect on human cancers. The analysis of bioactive compounds in a variety of matrices is therefore necessary to unambiguously identify and monitor the presence and/or effect of these chemicals in diversified applications. The separation of bioactive compounds is challenging for several chromatographic procedures including CE and HPLC because their respective molecular mass and chemical structure are extremely similar. In addition, some bioactive compounds might be chiral, thus they must be placed in a chiral environment. Modified CE methods that can separate uncharged and hydrophobic molecules have also emerged, most notably MEKC [21] and CD-modified CE using a mixture of neutral and charged CDs [22, 23]. Separation will be effected, provided neutral molecules differentially partition between the buffer and micelle or CD phases.

This paper describes the application of CD-modified CE to the analysis of bioactive compounds isolated from *A. camphorata* as an alternative to existing HPLC methods to develop a simple method using nonhazardous chemicals and small sample volumes. Representatives of seven compounds, including four chiral compounds, commonly isolated from *A. camphorata*, are chosen. In particular, three of the selected compounds are very potent in terms of toxicity [24]. The procedure employs a mixture of negatively charged sulfobutylether-β-CD (SBCD) ($pK_a = 2$) and neutral methyl-β-CD (MBCD) to effect differential distribution (partitioning) of the bioactive compounds between the buffer and CD phases. A rationale behind such differential partitioning is proposed and corroborated by HPLC data. To our knowledge, this is the first report on the use of CD-modified CE for achiral and chiral separation of these important bioactive compounds. The present research is a continuation of our previous contributions to the area of CE using CDs to expand the scope of applicability of this technique for analysis of chemically similar water-insoluble compounds.

2 Materials and methods

2.1 Materials

MBCD (MW 1310, average degree of substitution of 0.8 methyl groups *per* CD ring) and SBCD (MW 2005, degree of substitution = 4) were purchased from Aldrich (St. Louis, MO) and Cydex (Overland Park, KS), respectively. Mono and di basic potassium phosphate salts were purchased from EM Science (Gibbstown, NJ). DMF, boric acid, and sodium borate were purchased from Anachemia (Montreal, QC, Canada), whereas ethanol was obtained from Commercial Alcohols (Brampton, ON, Canada). Mesityl oxide (98% purity) was purchased from Aldrich. Test compounds/standards: AC1; AC2; AC3; AC4; AC5; AC6 and AC7 were prepared as described previously by Male *et al.* [24] and Cherng *et al.* [16]. The purity (>95%) of the compounds AC1–7 was confirmed from their sharp melting points (Buchi-540 melting point apparatus), TLC on silica gel (one spot) as well as NMR (Varian Unity Inova-600 VXR-300/51) studies. All solutions were prepared using Milli-Q A-10 gradient deionized water (18 MΩ cm) and filtered through a 0.22 μm Millipore millex GV4 syringe filter (Millipore, Bedford, MA).

2.2 Instrumentation

All CE experiments were performed on an HP3D (Agilent Technologies, Santa Clara, CA) CD system interfaced to a computer loaded with ChemStation software for data acquisition and analysis. Fused-silica capillaries with effective lengths of 46 cm (50 μm id and 360 μm od) were installed inside a cassette and treated with 1 M NaOH for 30 min followed by washing with water and buffer, respectively, for 30 min each. On a daily basis, capillaries were treated with 0.1 M NaOH for 3 min followed by washing with deionized water and buffer for 3 min each. Further conditioning was performed by applying a +15 kV potential for 3 min before each run. Samples were kept at ambient temperature in the auto-sampler and injected in hydrodynamic mode at 5 mbar positive pressure for 5 s. Separation was performed at +15 kV at room temperature (22–23°C) with the analytes monitored by UV detection at 243 nm. UV absorption measurement of the bioactive compounds was performed on a Beckman spectrophotometer (DU 640, Fullerton, CA) with a 1 cm quartz cuvette.

2.3 Sample preparation and operation procedure

The running buffer consisted of a 20 mM phosphate buffer at pH 7 or 20 mM borate buffer at pH 9.3. Since the electrophoretic process alters the running buffer pH by electrolysis and subsequently affects migration times, the separation buffer was refreshed regularly. MECD and/or SBCD were weighed and added to the buffer to the desired concentration. For sample preparation, stocks (~25–50 mM) of each bioactive compound (Fig. 1) were prepared by dissolving ~1 mg in 20–40 μL of ethanol at room temperature and then the exact concentrations of the stocks were determined based on the absorption coefficients of each compound as shown in Table 1. A stock sample mixture (AC1–7 ranging from 0.2–2.0 mM) was then prepared in the running buffer by the addition of appropriate amounts of each individual stock. All the compounds were soluble in ethanol except for compound 2 which had to be dissolved in DMF. The sample mixture was then diluted in the running buffer to obtain lower concentrations for calibration purposes. Although the wavelength of maximum absorbance of each compound is different, the on-line UV

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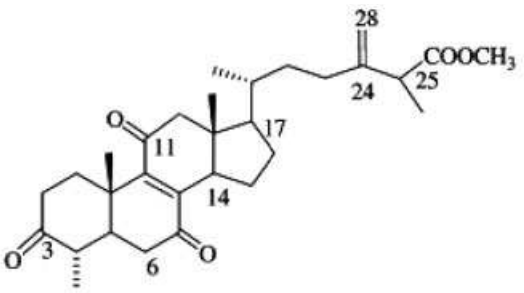
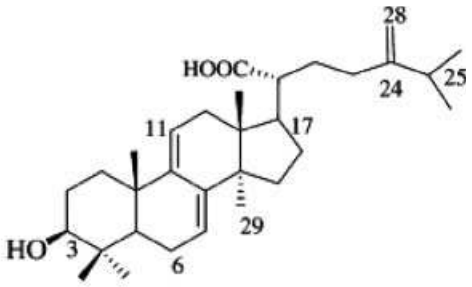
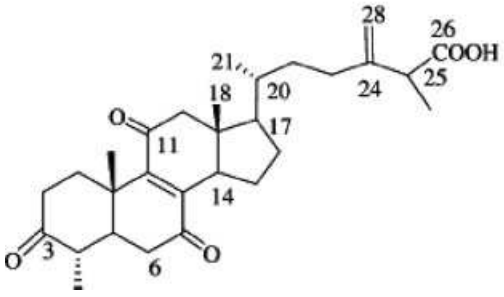


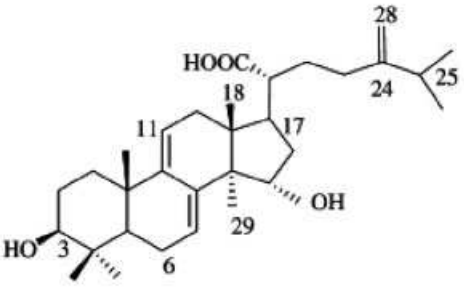
Methyl antcinate B (or)
Zhankuic acid D (**AC1**), MW 482



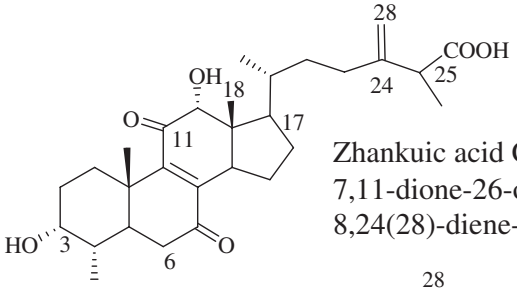
Dehydroeburicoic acid (or) 24-methylenelanost-7,9 (11)-diene-3β-ol-21-oic acid (**AC2**), MW 468



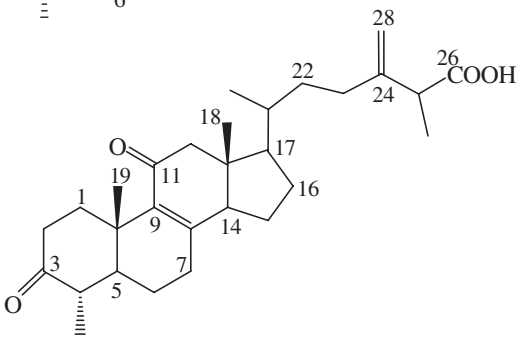
Antcin B (or) 2,4α-methylergosta-8,4(8)-diene-3,7,11-trion-26-oic acid, **AC3**, MW 468



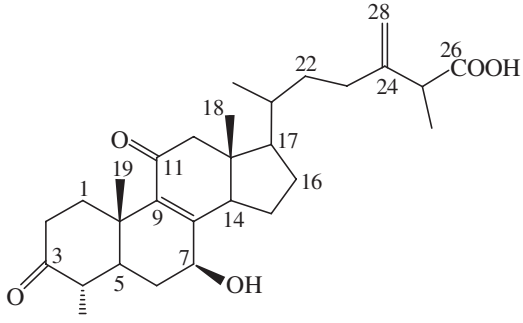
Dehydroesulfurenic acid (or) 24-methylenelanosta-7,9 (11)-diene-3β-15a-diol-21-oic acid, **AC4**, MW 484



Zhankuic acid C (or) [3,12-dihydroxy-4-methylergosta-8,24(28)diene-7,11-dione-26-oic acid, (or) 3α,12α-dihydroxy-4α-methylergosta-8,24(28)-diene-7,11-dione-26-oic acid, **AC5**, MW 486



Antcin A (or) (1, 4α-methylergosta- 8,4(8)-diene-3,11-dion-26-oic acid), **AC6**, MW 454



Antcin C (or) (3, 7β-hydroxy-4α-methylergosta-8, 4(8)-diene-3,11-dion-26-oic acid), **AC7**, MW 470

Figure 1. Chemical structures of the seven isolates from *A. camphorata*.

detector was set at 243 nm since all compounds absorbed strongly at this wavelength. The diluted samples were prepared from their ethanol stocks fresh each time since precipitation occurred within 24 h after preparation. However, once diluted over tenfold the samples remained stable for several weeks.

2.4 HPLC

HPLC experiments were carried out at room temperature, using a TSP SpectraSystem (Thermo Electron, Altrincham, Cheshire, UK) equipped with an AS 1000 auto-sampler, a UV 1000 single wavelength detector (set at 254 nm), a P4000

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Table 1. Absorption coefficients in ethanol of 100 μM *A. camphorata* isolates (AC1–7)

Compound	Wavelength λ (nm)	Absorption coefficient α (cm^{-1}/mol)
1	267	10 232
2	242	8150
3	267	14 075
4	251	11 486
5	270	11 117
6	253	11 796
7	252	8568

quaternary pump, a 5 μL injection loop, and a 2.7 μm particle size, $L \times$ id 5 cm $\times$ 4.6 mm column (Accentis Express^{R} C8, Supelco, Sigma-Aldrich, St. Louis, MO). The chromatogram was recorded from ChromQuest® version 3.0 CDS. Gradient elution of the bioactive isolates was carried out at 1.5 mL/min by linearly changing the 0.1% formic acid in ACN:0.1% formic acid mobile phase from 40:60 to 70:30 v/v over the first 10 min of the separation and then maintaining this condition for the remaining separation. The stock mobile phase was purged with helium before and during use. Spiking with individual components together with the elution time identified the chromatogram peaks. All seven compounds were dissolved in 0.1% formic acid in ACN and filtered through a non-sterile Millex (syringe driven) filter.

2.5 Extraction and isolation of isolates from *A. camphorata*

The fruiting bodies of *A. camphorata* were obtained through a solid state cultivation process (accession numbers G908AC for the microorganism *A. camphorata,* which is deposited in Chaoyang University of Technology). The compounds AC1–5 [24] and AC6 and AC7 [16] were isolated from *A. camphorata* according to the extraction and isolation procedures as described previously. Briefly, an air-dried powder of *A. camphorata* was extracted with $CHCl_3$ using a Soxhlet extractor. After solvent evaporation, the residue was subject to silica gel column chromatography (CC), and eluted with increasing polarity using mixtures of *n*-hexane/EtOAc. Following the TLC analysis, eluates of similar profiles were combined to give six fractions (A–F). Fraction B was purified by CC to afford AC1 and AC2. From the fraction C, AC3 was obtained. Fraction D was further separated using a silica gel column eluting with a gradient of *n*-hexane/EtOAc, to afford five subfractions. AC4 and AC5 were obtained from subfractions D–3 and D–4, respectively. Fraction F was further purified by silica gel CC using $CHCl_3$/MeOH from 100% $CHCl_3$ to 20% MeOH to yield AC6 and a mixture of triterpenes that was further separated by CC using $CHCl_3$/MeOH elution (9:1) to give AC7. The structures of compounds AC1–5 [24] and AC6–7 [16] were determined by ^{1}H and ^{13}C NMR spectroscopy and by comparison of the spectral data with those published values [4, 14, 18, 20].

2.6 Analysis of samples of *A. camphorata*

Three different *A. camphorata* mushroom samples were cut into very small pieces (1 mm $\times$ 2 mm) and soaked in 10 mL ethanol followed by sonication until an identical absorption spectrum was obtained, indicating the maximum extent of extraction (about 30 min of sonication). The extracts were filtered through a piece of cotton with a 10 mL plastic syringe followed by filtration through a 0.22 μm Millipore filter. The extracts were diluted fivefold with the running buffer before being injected for CE separation.

3 Results and discussion

3.1 Effect of CDs on separation

To illustrate the applicability of separation through the use of a CD mixture, a series of experiments was first conducted to examine the effect of MECD and/or SBCD on the separation of *A. camphorata* isolates. In brief, the experimental data confirmed that the combined concentration of the two CDs has to be over 40 mM; equally contributed by each CD in order to induce a significant difference in selectivity. As an example, without MECD or SBCD as the chiral selector in a 20 mM, pH 9.3 borate buffer containing 5% DMF, the electropherogram obtained for the optical isomer AC3 only exhibited a single peak with a migration time of 7.9 min (Fig. 2A). DMF had to be added to the running buffer to support the solubility of this analyte. The addition of 10–30 mM MECD in the same running buffer resulted in better solubility of AC3, as reflected by a higher and sharper peak at 9.0 min in the electropherogram (Fig. 2B). Although MECD exhibited high aqueous solubility (over 250 g/L), its addition to the running buffer was still anticipated to noticeably increase the viscosity of the running buffer, which in turn affected the migration time of AC3 (7.9 *versus* 9.0 min). For better solubility, 20 mM MECD was used for all subsequent experiments. Baseline separation of this optical pair was obtained by using the running buffer consisting of 5% DMF, 20 mM MECD, and 30 mM SBCD (Fig. 2C) with the optical isomer pair emerging after 32 min. Such a behavior also illustrated that AC3 was favorably associated with SBCD under this operating condition. The peak assignment of this chiral pair was not possible due to the lack of commercial standards. Chiral resolution of neutral and cationic species can be enhanced by addition of anionic SBCD [25]. Apparently, a combination of the two CDs was necessary to create a chiral environment that modified the physicochemical properties of the two optical isomers, transforming enantiomers to diastereoisomers. Experimental data revealed that 20 mM SBCD was insufficient for complete peak resolution, whereas 40 mM SBCD was accompanied by higher noise due to high Joule heating (figure not shown). Under normal electrophoretic conditions, this negatively charged CD migrates at a slower rate than the buffer phase

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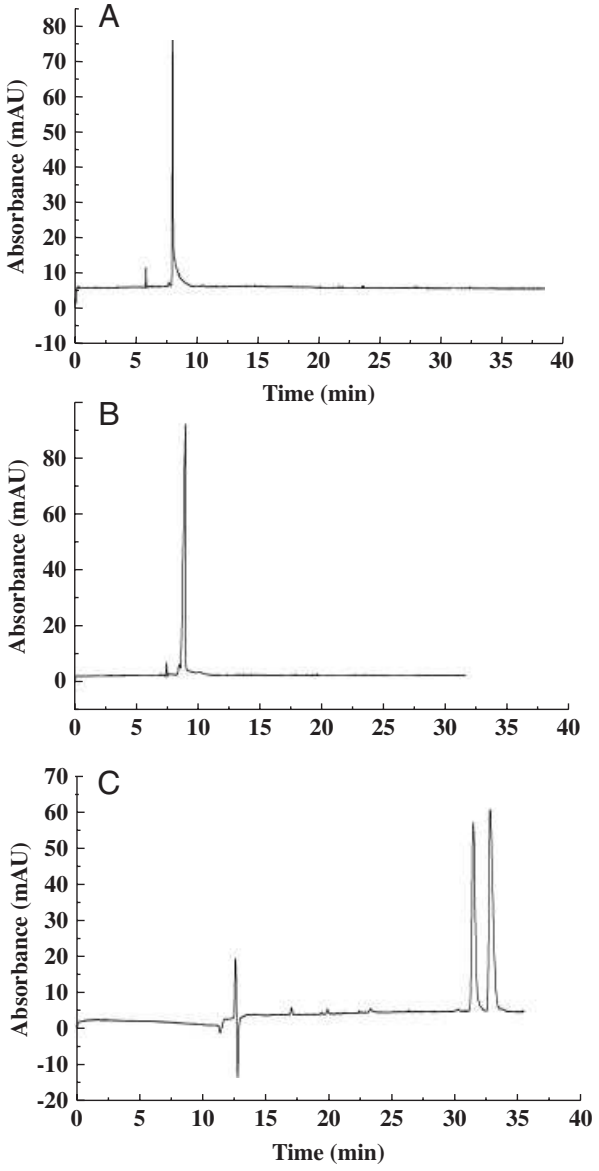

Figure 2. Separation of the AC3 optical isomer pair (1640 μg/mL) using (A) 20 mM sodium borate buffer, pH 9.3 and 5% DMF, (B) 20 mM sodium borate buffer, pH 9.3, 5 % DMF and containing 20 mM MECD, (C) 20 mM sodium borate buffer, pH 9.3, 5% DMF and consisting of 20 mM MECD and 30 mM SBCD. Note that DMF had to be added to the running buffer to support the solubility of AC3.

and can function as a pseudostationary phase in analogy with MEKC. Apparently, SBCD, the effective chiral selector of the separation system, was capable of interacting enantioselectively with the AC3 pair, affecting their electrophoretic mobility. Both MECD and SBCD are chiral because of the presence of asymmetric carbons on the glucose units. Similar effect of CDs on the separation of all the isolates individually and together was observed (figure not shown).

An interesting feature was noted from the electropherogram using SBCD in the running buffer. The ethanol (used to solubilize the isolate) was anticipated to associate sufficiently with this negatively charged SBCD, therefore, its migration was retarded relative to water. For samples containing neutral CD in water, buffers containing negative SBCD also retarded neutral MECD migration relative to water. As shown in Fig. 2C, a positive peak appeared in the absorbance plot corresponding to the arrival of water at the detector (~12.4 min), followed by a negative peak due to ethanol. The absorbance of ethanol is actually less than that of the buffer, and a negative absorbance peak was expected. The positive peak must have been the result of an optical effect in the detector, probably a result of the changing refractive index in a cylindrical section of the capillary column in the detector. Thus, this feature could be used for determination of the EOF since the buffer composition has been known to affect the electroosmotic migration time [26]. Such behavior was also reported by Szolar *et al.* [22]. An independent experiment using metisyl oxide as a neutral marker confirmed the afore-mentioned value of EOF (12.2 ± 0.24 min with $n = 5$).

3.2 Effect of buffer, pH, and organic modifier on separation

Except for AC1, the remaining six *A. camphorata* isolates contain –OH and/or –COOH groups, thus, the operating pH would play an important role in separation of these compounds. The separation at pH 4 (20 mM MECD, 30 mM SBCD in 20 mM phosphate, 5% DMF) took well over an hour due to very slow EOF (figure not shown). At pH 7 (20 mM MECD, 30 mM SBCD in 20 mM phosphate, 5% DMF) the run was complete within 26 min, however, the AC6 optical isomer was not baseline separated (Fig. 3A) and practically co-migrated with AC4. The addition of 5% ACN or ethanol in the separation buffer was unable to provide adequate chiral separation of this optical pair, probably a result of solvent disruption of the electrophoretic properties of the system. In general, organic modifiers are often used in MEKC to decrease the affinity of hydrophobic solutes for the micellar phase. As a result, resolution of hydrophobic compounds in MEKC is enhanced, whereas the capacity factor decreases with increasing concentration of the organic modifier.

Even at pH 9.3 (20 mM borate buffer consisting of 20 mM MECD and 30 mM SBCD), baseline separation of the optical isomer AC6 was still not achievable and there was a switch of the migration order between AC1 and AC4 (Fig. 3B). In order to achieve baseline separation for the optical isomers (AC6, AC3, and AC5), 5% DMF had to be added to the running buffer and the run was noticeably longer as expected owing to the presence of DMF (Fig. 3C). Like other organic modifiers such as methanol, propanol, and ACN, the addition of DMF to the running buffer mainly modifies the charge (zeta potential), particularly at high pH [27] and hydrophobicity of the capillary inner wall [27], leading to reduced EOF and an extension of the migration time window. In addition, the organic modifiers can also

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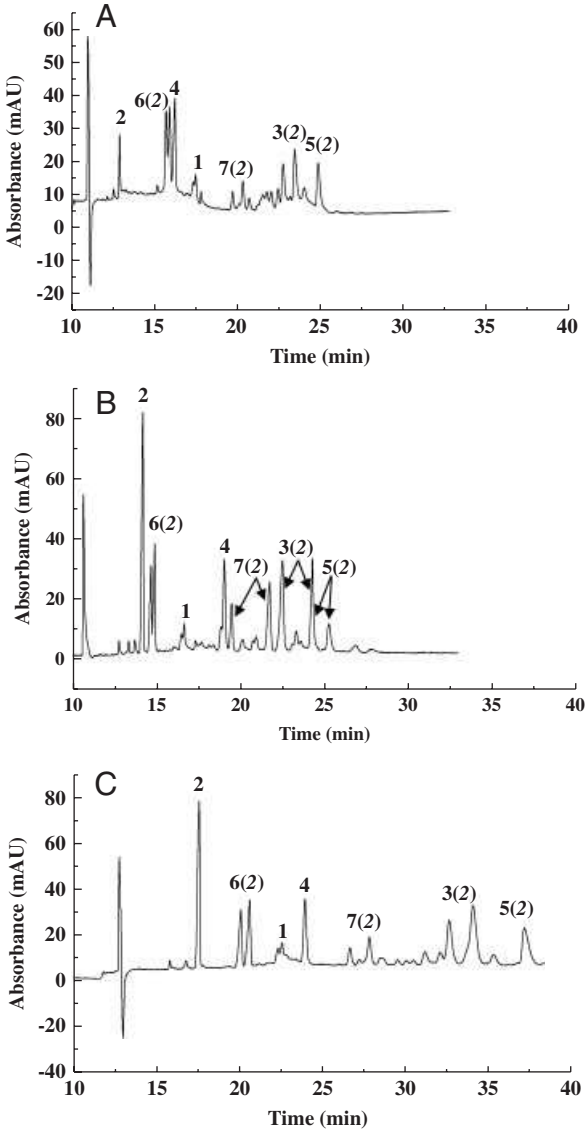

Figure 3. Separation of the seven bioactive compounds isolated and purified from the fruiting body of *A. camphorata* using (A) 20 mM sodium phosphate buffer pH 7, 5% DMF and consisting of 20 mM MECD and 30 mM SBCD, (B) 20 mM sodium borate buffer, pH 9.3 consisting of 20 mM MECD and 30 mM SBCD, (C) 20 mM sodium borate buffer, pH 9.3 consisting of 20 mM MECD, 30 mM SBCD, and 5% DMF. The concentration of each bioactive isolate is expressed in μg/mL. AC1 = 482, AC2 = 468, AC3 = 468, AC4 = 242, AC5 = 365, AC6 = 908, and AC7 = 94.

affect the binding constant of the analyte with MECD and SBCD. The use of other organic modifiers including ethanol and ACN was less effective. The use of organic buffers in CE separation is somewhat unpredictable, depending upon the composition of the separation buffer and the chemical structures of the target analytes. In this work, the addition of ACN did not provide any beneficial effects, whereas ACN has been reported to exhibit a positive effect for the separation of a pair of clinically significant positional isomers (coproporphyrin isomers) in urine samples [28]. SBCD has four modified primary hydroxyl groups (position 6 of the four glucose units) with a butyl chain and sulfonic groups. This feature allows its application as a charged chiral selector over a wide pH range [2–12]. Except for the switching between AC1 and AC4, increasing pH from 7 to 9.3 largely unaffected the migration order of the other isolates. The repeatability of the migration time (16.85 ± 0.35 min for AC2 (1 mM) was within 2% at a 95% confidence interval ($n = 7$), whereas the repeatability of the integrated peak area (503 ± 4.0 mAU min/mM) was within 1%. The addition of both MECD and SBCD to the running buffer had an important utility for complete achiral and chiral separation of these seven active compounds. Under this separation scheme, the active compounds with slightly different chemical structures were anticipated to have different association/partition constants for the hydrophobic core of the ring-shaped structure of the additives.

It should be important to note that α-configuration has the hydroxyl or other substitution pointing down to the plane (equatorial, below the plane, the symbolin the structure), and usually the protons attached to this carbon give singlets or doublets with smaller coupling constant (J) in ^{1}H NMR spectrum. The β-configuration has the hydroxyl or other substitution pointing up to the plane (axial, above the plane, the symbolin the structure), and usually the protons attached to this carbon give multiplets with higher coupling constant (J) in ^{1}H NMR. In general, the two forms are stable solids (even for NMR recording which used deuterrated solvents, so that no variation was observed in the spectra). However, in solution, as time passes, some of the pure α-form in solution will be converted into the β-form by the process mutarotation until a stable equilibrium is established between the two. Therefore, the bioactive compounds may behave like stereoisomers (keeping the same atoms, but altering the way they are bonded to each other creating isomers), which only differ in the three-dimensional arrangement of the groups bonded to one or more atoms in the molecule. Although the isolated compounds were pure, because of their chiral nature (having asymmetric carbons; for example, C-4 and 20 in AC3; C-3,4,12 and 20 in AC5, Fig. 1), double peaks in CE separation were observed.

All experiments were conducted at 15 kV since higher voltages were associated with higher noise whereas the run became very long at lower separation potentials. The calibration curves were constructed for all seven compounds (data not shown) to provide a detection limit ranging between 1 and 9 μM. This range corresponds to the inhibitory level of *A. camphorata* isolates on insect cells [24]. Among these isolates, AC1, AC3, and AC5 were chiral and most potent with respect to inhibition. The detection limit and sensitivity for each compound are summarized in Table 2. The highest theoretical plate was observed for AC6 (89 508–103 460), followed by AC7 (73 760–83 670), AC1 (71 930), AC3 (54 090–66 500), AC2 (63 730), AC5 (46 680–49 580), and AC4 (17 470).

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Table 2. Sensitivities and detection limits from calibration curves (10–1000 mM) of *A. camphorata* isolates

Compound	Sensitivity (mAUmin/μM), $n = 7$	Detection limit (μM), $S/N = 3$	R^2
AC1	0.264 ± 0.01	8.9	0.9996
AC2	0.498 ± 0.01	2	0.9998
AC3 (peak 1)	0.530 ± 0.04	2.4	0.9962
AC3 (peak 2)	0.714 ± 0.04	2.5	0.9976
AC4	0.637 ± 0.02	2.9	0.9994
AC5 (peak 1)	0.233 ± 0.03	6.2	0.9931
AC5 (peak 2)	1.105 ± 0.04	1.3	0.9989
AC6 (peak 1)	0.962 ± 0.0	1.4	0.9997
AC6 (peak 2)	1.566 ± 0.04	0.9	0.9995
AC7 (peak 1)	1.109 ± 0.02	0.9	0.9996
AC7 (peak 2)	1.273 ± 0.04	0.8	0.9995

3.3 Migration order

The isolates emerged as three distinct clusters in the electropherogram. In the first cluster, the most water-insoluble AC2 migrated first followed by the optical isomer AC6. AC1 stood alone in the second cluster. The third cluster was formed by AC4 and three optical isomers, AC7, AC3, and AC5, indicating their strong interaction with SBCD. In view of the negative charge for SBCD and these three optical isomers, the former would likely form inclusion complexes with the COOH moiety of these isolates to minimize negative charge repulsions. Upon complexation, the polar –COOH moiety of the hydrophobic compound is anticipated to form hydrogen bonding with MECD and/or SBCD to stabilize the inclusion complex. The presence of the –COOH group (position 26) appeared to render AC6, AC7, AC3, and AC5 (migration order) more negatively charged compared with AC2 and AC1 (without the COOH group) as reflected by the migration order. The most hydrophobic AC2 (with seven methyl groups and insoluble in ethanol) was reasoned to emerge as the first peak due to its strong interaction with MECD *via* the isopropyl moiety. In addition, except for AC6 as the smallest compound (MW = 454), the isolate with higher methyl groups (seven for AC2 and AC4, six for AC1) appeared to be favorably complexed with MECD. In contrast, AC7, AC3 and AC5 with only five methyl groups appeared to complex strongly with SBCD and emerged later in the electropherogram. Considering structurally similar compounds, AC7 should migrate after AC6 because of the presence of the hydroxyl group in its structure (at C7). Similarly, AC2 with one hydroxyl group emerged before AC4 with two hydroxyl groups. A similar argument could be used to explain the reason why AC3 with no hydroxyl group emerged before AC5 since the latter contains two hydroxyl groups in its structure. In the case of AC5, the possible intramolecular hydrogen bonding between the adjacent keto (at C11) and hydroxyl group (at C12) may also explain why it has the strongest interaction with SBCD.

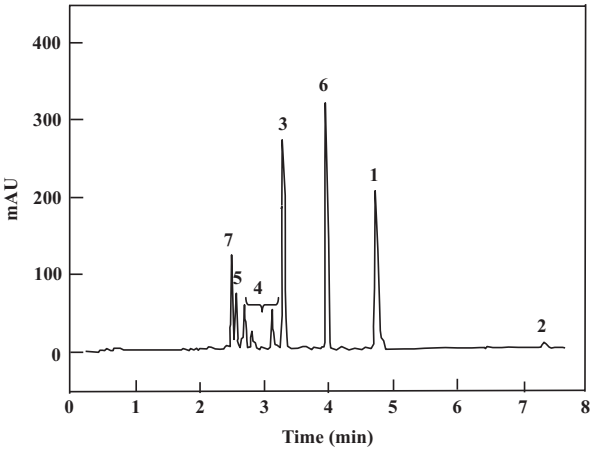

Figure 4. The elution order of the seven bioactive compounds separated by HPLC using a C8 column. The concentration of each bioactive isolate is expressed in μg/mL. AC1 = 84, AC2 = 60, AC3 = 60, AC4 = 20, AC5 = 30, AC6 = 10, and AC7 = 60.

Of particular interest was the HPLC elution order obtained by a C8 column of the seven isolates. AC2 emerged as the first peak in the electropherogram, but was eluted as the last peak in the chromatogram, indicating its strongest hydrophobicity (Fig. 4). The two early emerged peaks in the electropherogram (AC6 and AC1) were eluted just before the last peak (AC2) in the chromatogram. The third cluster group (AC7, AC5, AC4, and AC3) emerged very late in the electropherogram and was eluted as the first group in the HPLC chromatogram. Such results thus revealed that more hydrophobic compounds complexed favorably with MECD, whereas more hydrophilic counterparts were strongly associated with SBCD.

3.4 Analysis of natural samples of *A. camphorata*

Figure 5 shows the electropherograms of the extracts obtained from three different samples. Samples A and B were obtained from fruiting bodies of multi-year grown wild and two year grown wood cultivated *A. camphorata*, respectively. Sample C was obtained from plates of a one year cultivation on wood. Electropherogram peaks were identified by their order of elution and migration times. Confirmation was determined by spiking the sample with the individual isolate and observing an increase in the peak intensity. Table 3 indicates the identified compounds and their respective concentration in the extract for samples A, B, and C. Except for AC1 found in the B sample, the A and B samples appeared to have very similar components (AC2, AC4, and three optical isomers) since both of them are from fruiting bodies. This was considered as an important finding since AC1 is the most potent compound in terms of toxicity [24]. Notice also that some unknown peaks could not be identified due to the lack of chemical standards available to us during the

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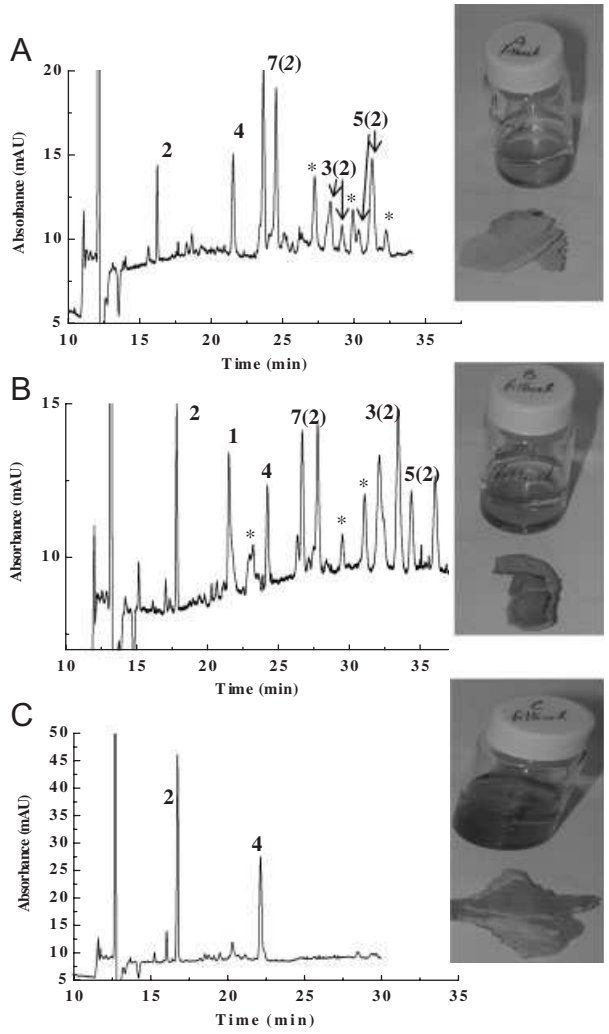

Figure 5. Electrophoretic analysis of bioactive compounds from the extract of three different types of *A. camphorata*. The peaks were numbered [1–7] based on spiking experiments with the pure isolates (AC1–AC7) and unknown peaks were labeled with an asterix (*). (A) Sample A was obtained from fruiting bodies of multi-year grown wild. (B) Sample B was obtained from two year grown wood cultivated *A. camphorata*. (C) Sample C was obtained from plates of a one year cultivation of *A. camphorata* on wood.

course of this study and were indicated by an asterix (*). The fruiting body of *A. camphorata* has been known to consist of several components including steroids, triterpenoids, sesquiterpenes, lignoids, fatty acids, phenyl methanoids, and its dimers [14,16–17, 20, 29–32]. Then, four new compounds, including three new benzenoids, antrocamphin A, antrocamphin B, and 2,3,4,5-tetramethoxybenzoyl chloride, and a new 1,3-dioxolan-2-one derivative, antrodioxolanone, together with 13 known compounds have been isolated from the fruiting body of *A. camphorata* [10]. In addition, several new compounds including 10-hydroxy-γ-dodecalactone, 11-hydroxy-γ-dodecalactone, 2-(2-hydroxyethyl)phenol, 12-hydroxydodecanoic acid methyl ester [33], and antroquinonol [34] were recently isolated from the submerged whole broth of *A. camphorata*. Sample C with one year of cultivation contains only two isolates (AC2 and AC4), neither of which are optical pairs. AC6 was not found in such three samples. A comparison of the absorbance intensity of AC2 and AC4 obtained in Fig. 5 implied that they were the two original compounds synthesized by *A. camphorata* that underwent photochemical and enzymatic transformation to other isolates. The purification process using different solvents might also modify the original structures of these two original isolates.

Table 3. Concentration of *A. camphorata* isolate in extracts and mushrooms

Compound	Identified compounds	Concentration	
		In extract (mM) ($n = 3$)	In mushroom (mg/g)
A	AC2	0.37 ± 0.02	5.8
	AC3	0.54 ± 0.04	7.2
	AC4	0.53 ± 0.02	8.6
	AC5	1.08 ± 0.09	17.7
	AC7	1.04 ± 0.08	16.4
B	AC1	1.03 ± 0.05	16.8
	AC2	0.59 ± 0.02	9.3
	AC3	1.84 ± 0.11	29.2
	AC4	0.29 ± 0.02	4.7
	AC5	0.53 ± 0.03	8.6
	AC7	0.50 ± 0.03	7.9
C	AC2	2.26 ± 0.09	36.8
	AC4	1.69 ± 0.08	28.5

4 Concluding remarks

In brief, a mixture of neutral and anionic CDs can be used as CE buffer modifiers to provide efficient achiral and chiral separation of bioactive compounds isolated from *A. camphorata* in one single run. The method development for this system is therefore quite straightforward, since the only choices to be made are the CD derivatives and concentrations to be used. The identification of chirality of several isolates also raises an important question about the relationship between chirality and toxicity. Apparently, such chiral compounds must be separated and identified followed by toxicity studies. After the catastrophic event of thalidomide [35], some optically active substances (eutomers) are proven superior to their enantiomers (distomers) regarding the pharmacological, toxicological, or pharmacodynamic effects.

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