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Capillary Electrochromatography of Cannabinoids

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The applicability of capillary electrochromatography (CEC) with photodiode array UV detection for the analysis of cannabinoids is presented. Baseline separation of seven cannabinoids (cannabigerol, cannabidiol, cannabinol, Δ-9-tetrahydrocannabinol, Δ-8-tetrahydrocannabinol, cannabichromene, Δ-9-tetrahydrocannabinolic acid) is obtained using a 3-μm CEC Hypersil C18 capillary with an acetonitrile/phosphate (pH 2.57) mobile phase. The effects of acetonitrile concentration, buffer concentration, voltage, temperature, stationary phase, and column length on the separation of the cannabinoids were investigated. Good short- and long-term precision in retention times are observed, with significant improvement obtained using relative retention times with cannabinol as reference compound. Although short- and long-term peak area precisions are poor, satisfactory reproducibility is obtained using relative peak areas with cannabinol as reference compound. The applicability of the CEC methodology to drug seizures was demonstrated on marijuana and hashish. Using a high-sensitivity UV flow cell with an extended path length of 1.2 mm, concentration sensitivities approaching HPLC were obtained.

Cannabinoids are the major constituents of marijuana, hashish (hash), and hash oil, which are the most abused illicit drugs in the world.[1] Due to their complexity, the in-depth analysis ("fingerprinting") of these exhibits for either strategic or tactical intelligence represents a major analytical challenge to the forensic chemist. [Strategic intelligence involves the determination of country or region of origin, while tactical intelligence determines whether two or more exhibits came from a common source (e.g., the same batch of hash.)[2] Tactical intelligence is therefore important for conspiracy cases, i.e., determining whether two or more exhibits came from the same distributor.]

Both packed [3,4] and capillary[5−7] gas chromatography (GC) and mass spectrometry (MS),[8] GC/MS,[7] high-performance liquid chromatography (HPLC)[1,4,6,7,9], HPLC/MS,[10] and random amplification of polymorphic DNA (RAPD)[11,12] have been previously used to fingerprint cannabis products.

GC and GC/MS are both high-resolution techniques which have been utilized for the analysis of both the cannabinoids and noncannabinoids (such as terpenes, alkanes, and spiroindans[5,7]) in cannabis-derived products. However, to analyze thermolabile and highly polar constituents, derivatization is required. These compounds include cannabinoid carboxylic acids and dihydroxylated noncannabinoid phenols. HPLC is a viable technique for the analysis of these latter compounds, as well as the less acidic cannabinoids (phenols). However, due to pressure drop considerations and laminar flow, conventional HPLC is limited in the separation efficiency obtainable in a reasonable time frame. For example, Lehmann and Brenneisen were not able to adequately resolve many compounds found in cannabis products[1] using a 3-μm ODS column and gradient elution (60-min run). For complete profiling of samples, therefore, a combination of GC and a high-resolution HPLC-type procedure (with the ability to handle thermolabile and highly polar compounds) is needed. Capillary electrophoresis under standard conditions does not appear to be a useful complementary technique. Due to a strong affinity for the SDS micelle, a poor separation of weakly acidic cannabinoids is obtained using micellar electrokinetic capillary chromatography (MECC).[13] Capillary zone electrophoresis (CZE) at high pH does not appear viable due to the similar charge-to-size ratios of many of the cannabinoids (e.g., Δ-9- and Δ-8-tetrahydrocannabinol).

Capillary electrochromatography (CEC), which combines the best features of CE (i.e., separation efficiency) with the best features of HPLC (i.e., well-characterized retention and selectivity mechanisms, ability to handle thermally labile solutes and highly polar compounds, and increased sample capacity), appears well suited for the analysis of cannabinoids. Furthermore, electrically driven reversed-phase capillary electrochromatography (RPCEC) has the potential to give efficiencies 5−10 times higher than standard pressure-driven RP HPLC.[14] This occurs in part because the main driving force for transporting mobile phase and solute in CEC is electrosmotic flow, which gives a pluglike velocity profile. This is in contrast to HPLC, where laminar flow gives

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rise to a parabolic velocity profile. In addition, the lack of a pressure drop in CEC allows the use of smaller particle sizes and longer columns than is possible in HPLC.

This paper describes the analysis and the effects of varying several chromatographic parameters on the CEC separation of cannabinoids. The applicability of CEC for the profiling of cannabis products is presented. To the authors' knowledge, this is the first reported application of CEC for the analysis of seized drugs.

EXPERIMENTAL SECTION

Instrumentation. A Hewlett-Packard Model HP^{3D}CE capillary electrophoresis system (Waldbronn, Germany) was used for all CEC studies.

Columns, 100-μm i.d. and 350-μm o.d. with packed-bed lengths of 25 (CEC Hypersil C_{18}, 3 μm and CEC Hypersil C_8, 3 μm) and 40 cm (CEC Hypersil C_{18}, 3 μm), respectively, were obtained from Hewlett-Packard. A 40-cm CEC Hypersil C_{18}, 3-μm column fitted to a high-sensitivity UV cell was also obtained from Hewlett-Packard. For all separations, the total column length was packed-bed length plus 8.5 cm of polyimide-coated fused-silica tubing.

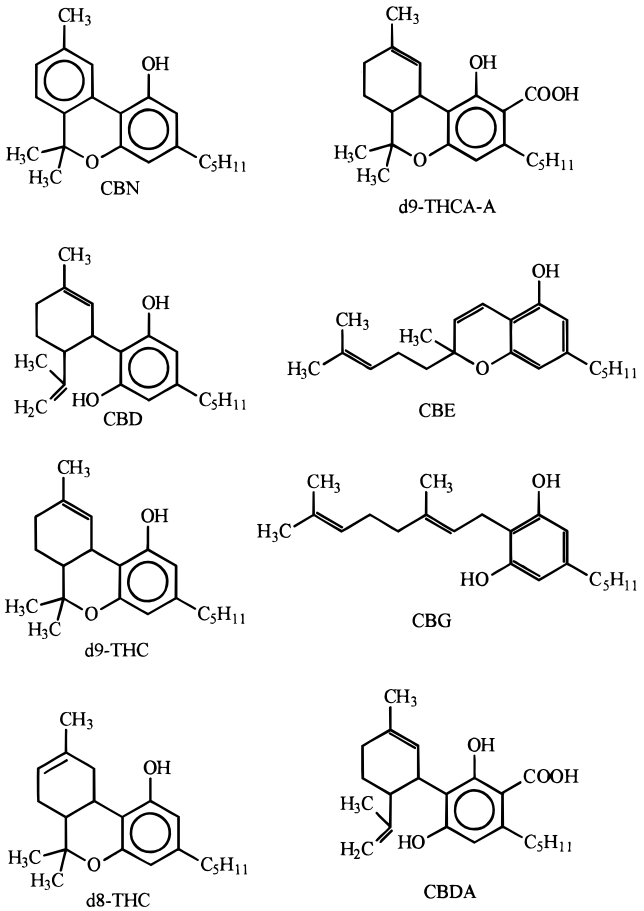
The columns were conditioned with mobile phase by first pressurizing the inlet at 10 bar and ramping the voltage to 25 kV over a 30-min period. Both the inlet and outlet were pressurized at 10 bar, and the voltage was maintained at 25 kV for another 30 min. Changing mobile phases was also accomplished electroosmotically with pressurization of the inlet and outlet to 10 bar.

Reagents. Tris(hydroxymethyl)aminomethane (Tris) buffer was obtained from Sigma (St. Louis, MO), and sodium phosphate monobasic buffer was acquired from Fisher (Fairlawn, NJ). The Tris buffer was adjusted to pH 9.0 using hydrochloric acid obtained from Baker (Phillipsburg, NJ), and the phosphate buffer was adjusted to pH 2.57 using phosphoric acid acquired from Mallinckrodt (Paris, KY). Mobile phases were prepared by first adjusting the pH of the buffer and then mixing with acetonitrile (ACN) obtained from Burdick and Jackson (Muskegon, MI). For 100% aqueous buffer solutions, the pH was measured by a pH meter; pH paper was used to estimate the pH for the buffer containing acetonitrile. The cannabinoid standards cannabidiol (CBD), Δ-9-tetrahydrocannabinol (d9-THC), Δ-8-tetrahydrocannabinol (d8-THC), and cannabichromene (CBE) were obtained from the reference collection of the Drug Enforcement Administration's Special Testing and Research Laboratory. Cannabigerol (CBG) and cannabinol (CBN) were acquired from RTI (Research Triangle, NC). Δ-9-Tetrahydrocannabinolic acid A (d9-THCA-A) was obtained from the Research Institute of Pharmaceutical Sciences, School of Pharmacy, The University of Mississippi (University, MS). The structures of the major cannabinoids for which CEC was performed are presented in Table 1. Dimethyl sulfoxide (DMSO), acquired from Burdick and Jackson, and thiourea, acquired from Baker, were used as neutral markers.

Procedures. Standard solutions consisting of mixtures of ~0.10 mg/mL of each solute in mobile phase were used.

Marijuana and hashish samples were extracted into methanol/chloroform (9:1), evaporated to dryness and reconstituted in 1.0 mL of mobile phase to give a final d9-THC concentration of ~1.0 mg/mL.

Table 1. Structures of Major Cannabinoids



RESULTS AND DISCUSSION

CEC of Cannabinoids. (a) Effects of pH. CEC was performed on a standard mixture of cannabinoids using a Hypersil C18 column (25 cm to detector) and a mobile phase consisting of acetonitrile/25 mM Tris-HCl pH 8.0, 65:35. Good separation was obtained for the weakly acidic cannabinoids such as CBD, CBN, d9-THC, and d8-THC. However, the more strongly acidic d9-THCA-A exhibited peak splitting. This poor chromatographic performance was due to the interaction of the fully dissociated carboxylic acid with unbonded silanol groups (which bind acids).[15]

As shown in Figure 1, good chromatographic performance was obtained for the same cannabinoids, including d9-THCA-A, using a Hypersil C18 column (25 cm to detector) and a mobile phase containing acetonitrile-25 mM phosphate pH 2.57, 75/25. In the pH 2.57 buffer, all cannabinoids (including d9-THCA-A) are essentially un-ionized, which minimizes any silanophilic interactions. Performing CEC at low pH with a phosphate buffer has been previously shown to improve chromatographic performance for carboxylic acids.[16] There is still appreciable osmotic flow with the lower pH buffer. This is manifested by a t_o of approximately

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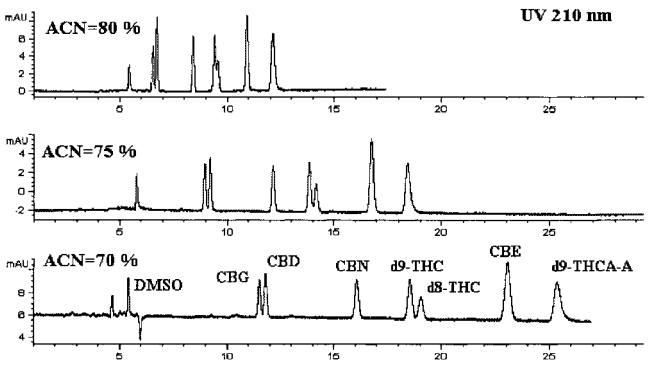

Figure 1. Effect of percent ACN on the CEC separation of standard cannabinoids. Conditions: acetonitrile/25 mM phosphate buffer pH 2.57 with voltage 25 kV and temperature 20 °C. A Hypersil C18, 3-μm [100 μm × 34 cm (25 cm length to detector)] column is used. Electrokinetic injections of 3.0 s at 5.0 kV are used.

5 min for the neutral marker DMSO. [DMSO gives an identical retention time with a previously reported[14] neutral marker (thiourea).] There are several factors that could be contributing to this result. The "apparent" pH of the mobile phase (~5) appears to be high enough to support a reasonable osmotic flow. In addition, the effective buffer concentration (6.25 mM phosphate) of the buffer mixed with acetonitrile is relatively low, which (again) would support a reasonable zeta potential (ζ). In addition, as shown by Dorsey et al.,[17] acetonitrile has a high ζ. For these authors,[17] a reasonably high ζ was obtained for CEC with a 65:35 acetonitrile/water mobile phase and a 5-μm ODS Hypersil capillary. Consistent with a previous study,[14] the osmotic flow for the present work at buffer pH 2.57 is approximately half that obtained at buffer pH 8.0.

(b) Effect of Acetonitrile Concentration. The retention, selectivity, and resolution of standard cannabinoids change with acetonitrile concentration (see Figure 1). Retention times and k' values decrease with increasing acetonitrile concentration, mainly due to changes in partition coefficient. Smaller changes in α and N values also contribute to changes in resolution. As shown in Figure 1, there is little change in osmotic flow (retention time of neutral marker) with varying acetonitrile concentration. Both increases and decreases of electroosmotic flow with decreasing acetonitrile concentration have been previously observed.[14] Taking into account the resolution of the most critical pair (CBG and CBD) and speed of analysis, 75% acetonitrile was used for additional experimentation.

(c) Effect of Phosphate Concentration. The effects of phosphate concentration on the separation of standard cannabinoids are shown in Figures 2 and 3. As shown in Figure 2A, k' decreases with increasing buffer concentration while for the most part α remains relatively constant. Since surface tension increases with increasing buffer concentration, an increase in k' would have been expected for un-ionized species.[18] Such an effect was observed for HPLC. At present, no explanation can be given for this apparent anomaly. Due to a decrease in osmotic flow with the increase in buffer concentration, the retention times of all

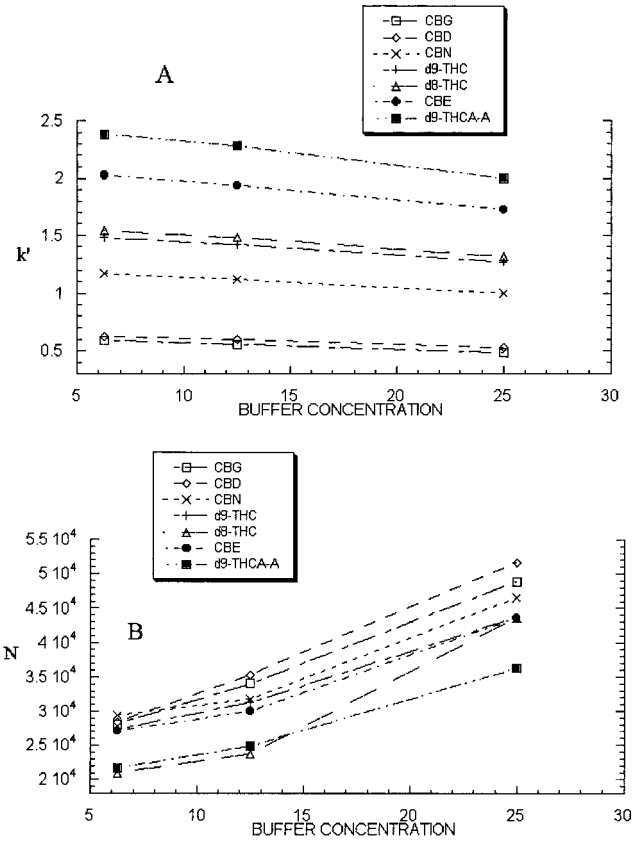

Figure 2. (A) K' versus buffer (phosphate) concentration and (B) N versus buffer concentration for standard cannabinoids. Conditions: acetonitrile/phosphate pH 2.57, 75:25 with other experimental parameters identical to Figure 1.

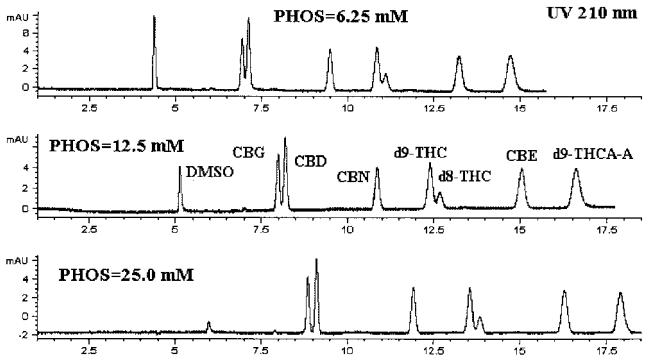

Figure 3. Effect of phosphate (PHOS) concentration on the CEC separation of standard cannabinoids. Conditions identical to Figure 1 except for 75% ACN and phosphate concentration.

solutes increased (see Figure 3). Resolution increases with increasing phosphate concentration due primarily to the increase in plate counts (N) (see Figures 2B and 3). A reduction in double-layer overlap[19] and lower silanophilic interactions are also possible consequences of a higher run buffer ionic strength, which could lead to higher plate counts. However, use of even higher phosphate concentrations (i.e., than those used in this study) would not only lead to longer retention times but also result in higher currents, which could cause band spreading or possible bubble formation.

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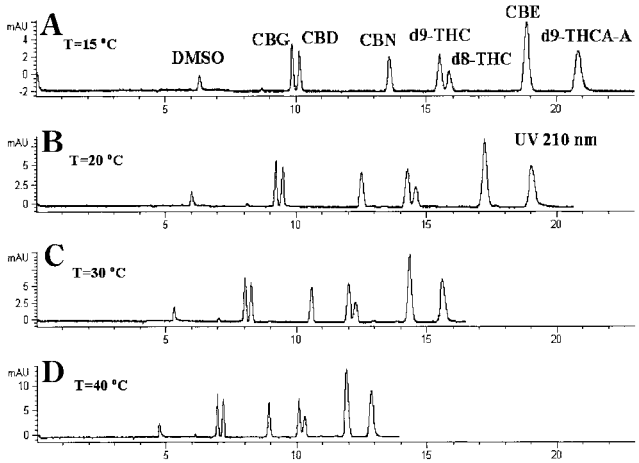

Figure 4. Effect of temperature (*T*) on the CEC separation of standard cannabinoids. Conditions identical to Figure 1 except for 75% ACN and temperatures as noted.

(d) Effect of Voltage and Temperature. Due to the expected increase in electroosmotic velocity, retention times decrease with increases in voltage (15−30 kV). However, resolution does not change with voltage, which indicates there are no adverse effects of Joule heating occurring. Excessive Joule heating could alter partition coefficients and/or cause band spreading. For the cannabinoid standards, k', α, and N values change little with voltage. A linear Ohm's law plot is further evidence that excessive Joule heating did not occur.

The effects of temperature on the separation of standard cannabinoids are shown in Figure 4. Retention times decrease with increasing temperature, due to an increase in electroosmotic velocity as a result of a decrease in viscosity. An increase in temperature from 15 to 40 °C resulted in a 40% reduction in analysis time. As also shown in Figure 4, overall resolution remained relatively constant with changes in temperature.

Similar to HPLC,[18] k' values decrease with increasing temperature. Small changes in α (either positive or negative) can occur with increasing temperature. In contrast to HPLC,[18] however, N does not increase with increasing temperature, but rather remains relatively constant. A possible explanation for this effect may be the greater effect of the mobile-phase diffusion coefficient (D_m) on the C term of the Van Deemter equation in HPLC versus CEC. In HPLC, $H_m \sim {}^1/_{24}D_m$, while in CEC, $H_m \sim {}^1/_4D_m$.[20] D_m increases with increasing temperature due to a decrease in viscosity.

By combining high voltage (30 kV) and high temperature (40 °C), the standard cannabinoids are separated in under 11 min with little loss in resolution compared with Figure 1B.

(e) Effect of Stationary-Phase Type and Column Length. A comparison of the CEC separations of standard cannabinoids using C18 and C8 stationary phases under identical chromatographic conditions is shown in Figure 5. The C18 stationary phase used in this study is clearly advantageous versus the Hypersil C8 phase. Under conditions of approximately equal electroosmotic velocity, the C18 column exhibits shorter retention times and better overall resolution. In addition, the C18 stationary phase gives better peak shape for the more strongly acidic d9-THCA-A.

(20) Dittman, M. M.; Wienand, K.; Bek, F.; Rozing, G. P. *LC-GC* **1995**, *13*, 800−14.

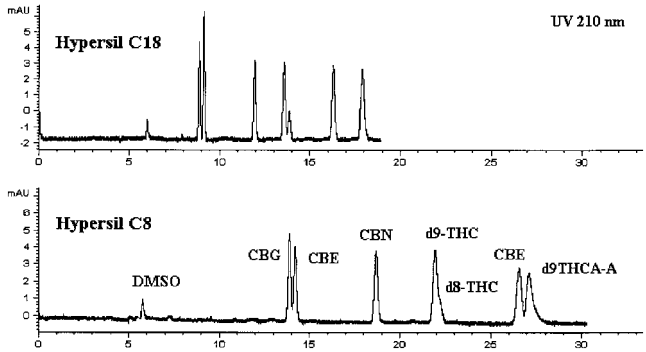

Figure 5. Effect of stationary phase on the CEC separation of cannabinoids. Conditions identical to Figure 1 except for 75% ACN and stationary phase.

Increasing the length to detector for the C18 column from 25 to 40 cm [at approximately the same field strength (600 V/cm)] resulted in nearly proportionate increases in retention time (1.6×) and theoretical plates (1.4×), and a 1.2× increase in resolution. Using a longer column (see Figure 6B), the seven cannabinoid standards were baseline resolved in under 40 min. For this separation, an average plate count of 63 000 is obtained. Since cannabis products are highly complex mixtures, this more efficient column was used for subsequent experiments.

(f) Short- and Long-Term Reproducibility. Short- and long-term reproducibility of retention time (RT), relative retention time (RRT), and area and relative area (Rarea), with CBN as a reference compound, are shown in Table 2.

As shown in Table 2, the short-term RT reproducibility is good (RSD ≤ 0.58%) when using electrokinetic injection, but the area reproducibility is poor (RSD ≥ 37%). Dramatic improvements in RT and area precision are obtained by using a reference compound, which suggests the need for an internal standard, especially when performing quantitative analysis. RSD values for RRT of ≤0.10% were obtained for most solutes, while RSDs for RArea were ≤4.6% for the cannabinoids. It is not clear why d8-THC exhibits a much higher RSD value of 0.67% for RRT.

As also depicted in Table 2, the long-term RT and RRT reproducibilities are good, with RSD values of ≤2.0 and ≤0.93%, respectively. The long-term reproducibility again points out the need for an internal standard, with RSD values of ≤49.8 and ≤8.10%, respectively, for area and relative area. At the end of a 10-day period, there was noticeable loss in plates counts (∼10%) and the appearance of tailing (probably due to the presence of a void). According to Hewlett-Packard, flushing the capillary with mobile phase (using an HPLC pump) in such cases should restore performance.[21]

(g) Application to Drug Seizures. Chromatograms of concentrated extracts of hashish and marijuana and a standard mixture of cannabinoids are shown in Figure 6. Methanol/chloroform (9:1) was used to extract the samples; this solvent pair had been previously shown to be an effective solvent for the recovery of the cannabinoids.[22] Analyses were carried out at 20 °C; higher temperatures were not possible because of incomplete resolution between CBDA and CBG.

(21) Care and Use Information for Hewlett-Packard Capillary Columns for Capillary Electrochromatography Packed with CEC−Hypersil C18; Hewlett-Packard, Waldbromm, Germany, 1997.

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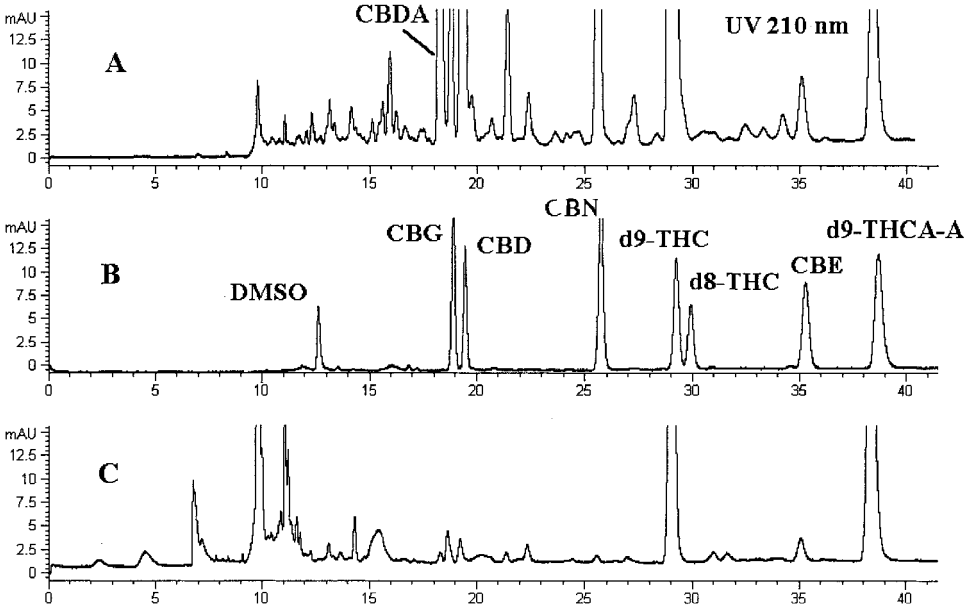

Figure 6. CEC of (A) concentrated hashish extract, (B) standard mixture of cannabinoids, and (C) concentrated marijuana extract. Conditions identical to Table 2.

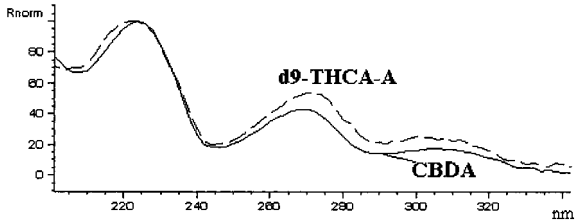

Figure 7. PDA-UV spectra of d9-THCA-A and CBDA, respectively, obtained from the CEC separation depicted in Figure 6A.

Table 2. Short-Term and Long-Term Reproducibility (RSD, %, $n = 7$) of the Retention Time (RT), Relative Retention Time (RRT), Area, and Relative Area (RArea) of Cannabinoids[a]

	RT		RRT[b]		area		RArea[b]	
solute	short	long	short	long	short	long	short	long
CBG	0.50	1.49	0.06	0.07	38.6	48.9	2.33	1.65
CBD	0.51	1.91	0.04	0.61	38.0	49.2	2.45	8.10
CBN	0.52	1.52	0.00	0.00	39.4	48.1	0.00	0.00
d9-THC	0.49	2.03	0.05	0.93	39.2	44.8	3.95	5.83
d8-THC	0.50	1.68	0.67	0.20	40.6	47.0	3.22	2.02
CBE	0.50	1.70	0.04	0.19	37.4	49.8	2.95	4.39
d9-THCA-A	0.58	1.67	0.09	0.16	42.2	48.2	4.61	2.32

[a] Conditions identical to Figure 1 except for 75% ACN, voltage 30 kV, column length 49 cm (40 cm to detector) and injections of 8.0 s at 5.0 kV. [b] Relative to CBN.

The presence of certain cannabinoids in the extracts was confirmed by matching retention times and PDA UV spectra with those from the standard mixture. It is interesting that a major peak present in hash (18.2 min) could be tentatively identified as cannabidiolic acid (CBDA) by its UV spectra. As shown in Figure 7, a spectrum similar to that for d9-THCA-A is obtained, which is very similar to a published spectrum for CBDA.[1] The close similarity of the spectra of the two acids is not surprising, considering that the major UV chromophore (substituted benzene ring) is nearly identical in both structures (see Table 1).

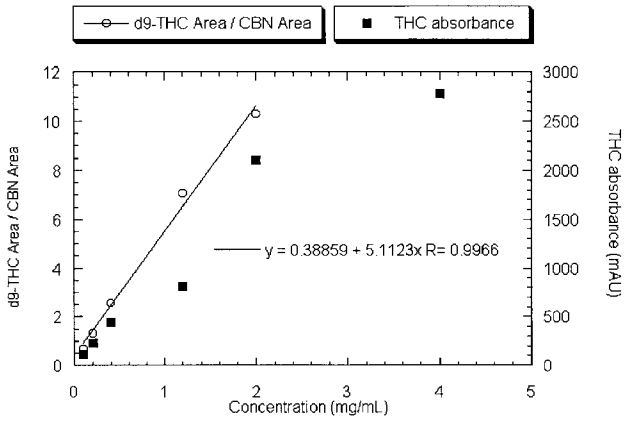

Figure 8. d9-THC area/CBN area versus d9-THC concentration, and THC absorbance versus d9-THC concentration. Conditions identical to Figure 6, except for use of high-sensitivity UV detection cell.

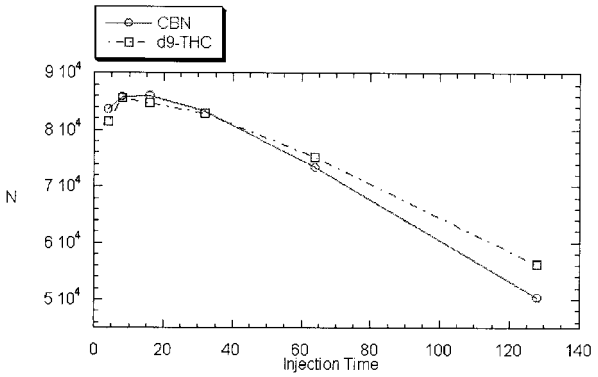

Figure 9. *N* versus injection time for CBN and d9-THC. Conditions identical to Figure 8.

A major disadvantage of CEC is limited concentration sensitivity. Several means for overcoming this limitation that were used in this study included increasing both detection path length and injection size. Use of a high-sensitivity UV detection cell (with an extended path length of 1.2 mm) for the analysis of a hash

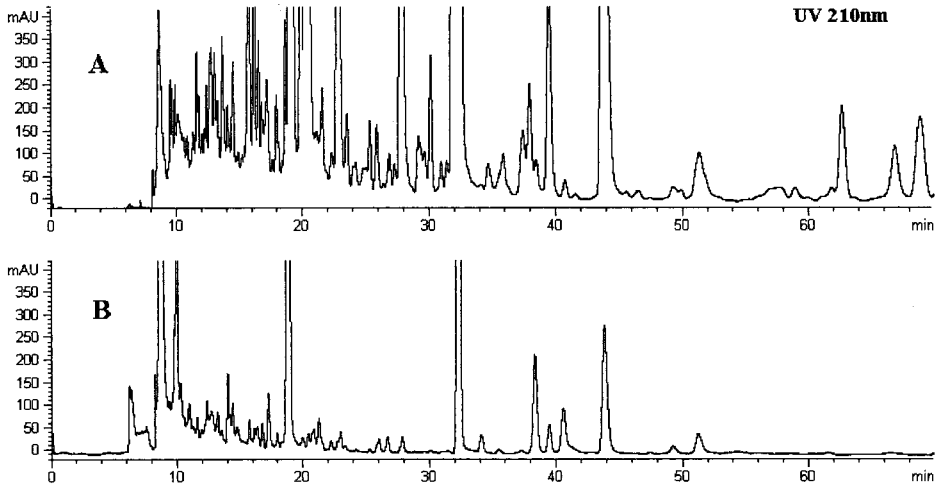

Figure 10. CEC of (A) concentrated hashish extract and (B) concentrated marijuana extract. Conditions identical to Figure 6, except for injections of 32.0 s at 5.0 kV and use of a high-sensitivity UV detection cell.

extract resulted in an 8× increase in signal-to-noise ratio with little (if any) loss in resolution. Another advantage of the high-sensitivity cell, as shown in Figure 8, is extended linearity to greater than 2000 mAU.

Larger injection sizes can be accomplished instrumentally, by increasing either the voltage or the injection time. As shown in Figure 9, using a fixed voltage of 5 kV, the injection time can be as high as 32 s without an appreciable loss in column efficiency. It should be noted (see Figure 9) that over 200 000 plates/m was achieved with the 3-μm Hypersil C18 column coupled to a high-sensitivity cell. Using a 32-s injection time and a high-sensitivity UV cell, the limit of detection for d9-THC (3× signal to noise) was 500 ng/mL.

A combination of a larger injection size and the high-sensitivity cell greatly enhanced the ability to fingerprint cannabis samples (cf. Figures 6 and 10). The CEC isocratic procedure resolved ~50% more peaks for a hash sample in comparison to a gradient HPLC procedure with a similar run time.[1] CEC with smaller particle size packing materials and/or gradient elution could provide even greater peak capacities. Gradient elution could increase peak capacity by providing better resolution of the earlier eluting solutes while still maintaining resolution of the latter eluting compounds.

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