Received: 20 October 2017 | Revised: 29 January 2018 | Accepted: 8 February 2018

DOI: 10.1002/pca.2761

RESEARCH ARTICLE

WILEY Phytochemical Analysis

Chromatographic characterisation of 11 phytocannabinoids: Quantitative and fit-to-purpose performance as a function of extra-column variance

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Funding information
MITACS Accelerate programme; University Graduate Fellowship; Walter C. Sumner Memorial Fellowship

Abstract

Introduction: *Cannabis sativa* L. (cannabis) is utilised as a therapeutic and recreational drug. With the legalisation of cannabis in many countries and the anticipated regulation of potency that will accompany legalisation, analytical testing facilities will require a broadly applicable, quantitative, high throughput method to meet increased demand. Current analytical methods for the biologically active components of cannabis (phytocannabinoids) suffer from low throughput and/or an incomplete complement of relevant phytocannabinoids.

Objective: To develop a rapid, quantitative and broadly applicable liquid chromatography–tandem mass spectrometry analytical method for 11 phytocannabinoids in cannabis with acidic and neutral character.

Methodology: Bulk diffusion coefficients were calculated using the Taylor–Aris open tubular method, with four reference compounds used to validate the experimental set-up. Three columns were quantitatively evaluated using van Deemter plots and fit-to-purpose performance metrics. Low (1.2 μL^2) and standard (3.6 μL^2) extra-column variance ultra-high pressure liquid chromatography (UPLC) configurations were contrasted. Method performance was demonstrated with methanolic cannabis flower extracts.

Results: Bulk diffusion coefficients and van Deemter plots for 11 phytocannabinoids are reported. The developed chromatographic method includes the challenging Δ^8/Δ^9-tetrahydrocannabinol isobars and, at 6.5 min, is faster than existing methods targeting similar panels of biologically active phytocannabinoids.

Conclusions: The bulk diffusion coefficients and van Deemter curves informed the development of a rapid quantitative method and will facilitate potential expansion to include additional compounds, including synthetic cannabinoids. The developed method can be implemented with low or standard extra-column variance UPLC configurations.

KEYWORDS

Cannabis, extra-column variance, method development, van Deemter

1 | INTRODUCTION

The use of *Cannabis sativa* L. (cannabis; ssp *sativa* and *indica*) has been suggested as a treatment for a variety of medical conditions in recent reviews, including chronic pain, asthma, nausea associated with chemotherapy, multiple sclerosis and glaucoma.[1-4] Phytocannabinoids are putatively believed to be the primary bioactive constituents, although terpenes and flavonoids may also contribute to the overall bioactivity of cannabis.[1] A subset of the more than 100 phytocannabinoids found in cannabis[5] have demonstrable biological effects. These include (Figure 1): Δ^9-tetrahydrocannabinol (Δ^9-THC), Δ^8-tetrahydrocannabinol (Δ^8-THC), cannabinol (CBN), tetrahydrocannabivarin (THCV), Δ^9-tetrahydrocannabinolic acid (THCA), cannabidivarin (CBDV), cannabichromene (CBC), cannabidiol (CBD), cannabigerol (CBG), cannabidiolic acid (CBDA) and cannabigerolic acid (CBGA). Twenty-nine US states (plus the District of Columbia) and all Canadian provinces currently have medical cannabis legislation.[4] More recently, four US states have legalised the recreational sale and use of cannabis and cannabis-infused products and Canada is expected to legalise recreational sale and use in 2018. These changes are leading to an increase in the number of licenced cannabis producers.[4,6] As the number of licenced producers increases and potency testing becomes mandatory, analytical testing facilities will see an increased sample load that will require a quantitative, high throughput method that can be broadly implemented.

Many chromatographic methods exist for the analysis of cannabinoids in a variety of matrices (e.g. extracts, edibles, blood, hair, urine), including methods for potency testing. Most of these methods can be generally divided into those using chromatographic separation with optical[7-12] or mass spectrometry (MS) detection.[11,13-16] Despite the abundance of analytical methods, challenges persist. These include low throughput and/or an incomplete complement of phytocannabinoids with demonstrated biological activity. For example, many published methods have the notable absence of Δ^8-THC, which is likely avoided due to the chromatographic difficulties associated with separating it from Δ^9-THC. Achieving resolution of these compounds requires long run times (17–36 min) that impact throughput.[7,8,14] Further complicating these analyses are the presence of acidic and neutral cannabinoids that are integral to the

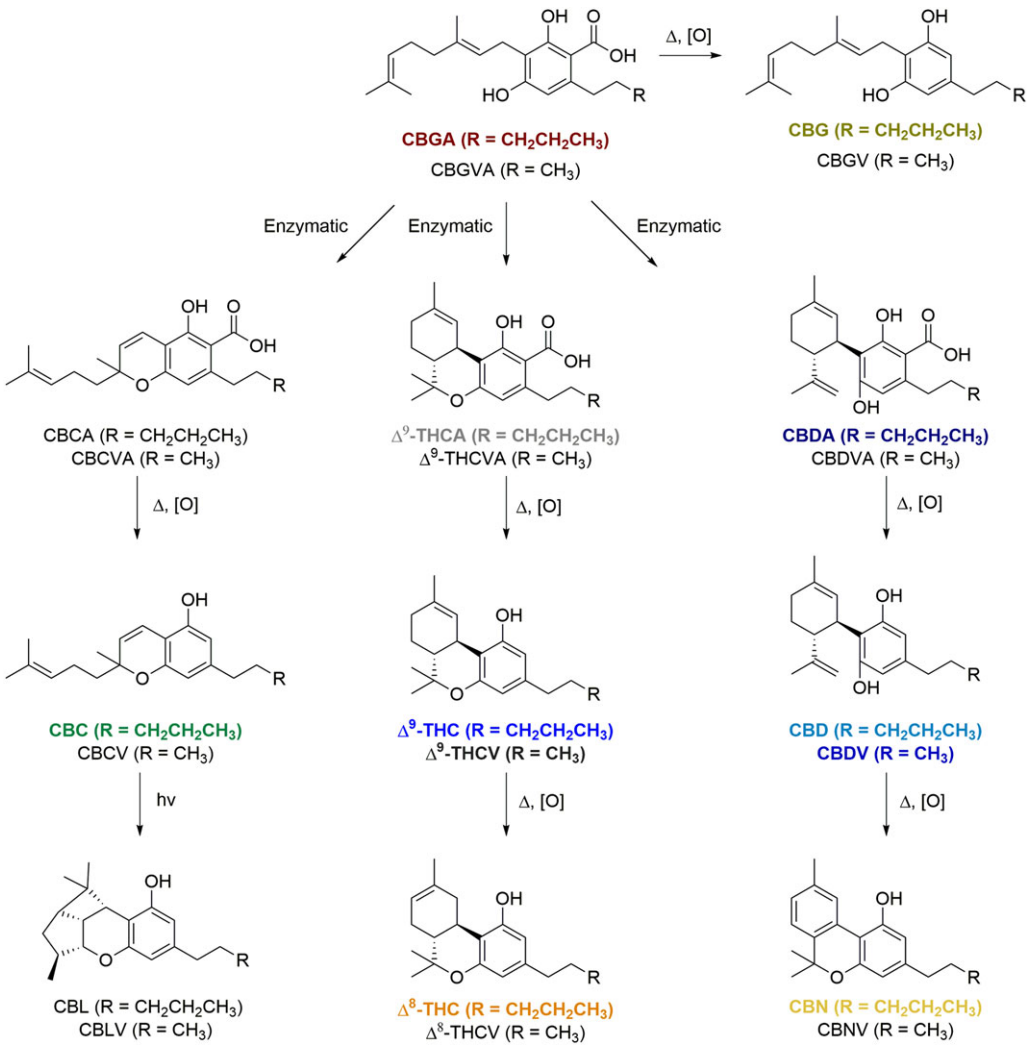

FIGURE 1 Structures of the phytocannabinoids used in the current study and some of their related derivatives present in lower abundance in cannabis. Known pathways for formation are shown, with those products showing *Δ, [O]* generally formed through combustion. Labels in bold were analysed in this study. Colour coding of labels were matched to the chromatogram colours in Figure 3 [Colour figure can be viewed at wileyonlinelibrary.com]

biological activity of cannabis, but generally have different requirements to achieve adequate chromatographic resolution.

Developing a rapid, quantitative method for cannabis potency that covers a range of phytocannabinoids must include the retention and separation of critical pairs (i.e. Δ^8-THC and Δ^9-THC), as well as acidic and neutral phytocannabinoids. Given the stated objective of a high-throughput method, ultra-high-pressure liquid chromatography (UPLC) was selected to separate the selected phytocannabinoids. A rigorous and objective approach using the van Deemter equation[17] was chosen to guide method development. This required evaluating phytocannabinoid behaviour using a UPLC configuration where extra-column variance was minimised, which was requisite to achieve maximum performance from small particle size (fully or superficially porous) UPLC columns. In addition, method performance was also evaluated using the default UPLC configuration provided by the manufacturer, which does not stringently control for extra-column variance. While not optimal, this latter case represents a scenario often encountered, since reducing extra-column variance can be overlooked and may be cost prohibitive to implement and maintain. Evaluating chromatographic performance under these two instrument configurations will extend the applicability of the developed method. The use of van Deemter curves for this comparison will provide objective and quantitative comparisons of the two configurations. Finally, quantitative, theory-based assessments of column performance should be balanced against practical considerations when implementing a method in a production environment. As such, fit-to-purpose metrics including throughput, chromatographic selectivity and solvent consumption were combined with rigorous theoretical assessments of three UPLC columns to develop a rapid chromatographic method that included 11 phytocannabinoids.

In addition to suitable chromatography, sufficient sensitivity to detect lower abundance compounds was also requisite, since, although present at lower concentrations than the dominant Δ^9-THC and CBD phytocannabinoids, the less abundant compounds still possess biological activity. To address the disparity in expected phytocannabinoid concentrations, tandem mass-spectrometry (MS/MS) was utilised, as it was expected to have the requisite sensitivity and dynamic range for the analytes of interest (Figure 1).

2 | EXPERIMENTAL

2.1 | Chemicals and general details

HPLC-grade methanol, acetonitrile, hexane, ethyl acetate and isopropanol, toluene, ascorbic acid, phenol and thiourea were purchased from Sigma-Aldrich (Saint Louis, MO, USA) and used as received. The following phytocannabinoid standards were purchased from Cerilliant (Round Rock, TX, USA) as 1.00 mg/mL methanol solutions and used as received: CBDV, THCV, CBN, CBC, CBD, Δ^8-THC, Δ^9-THC, CBG, CBDA, THCA and CBGA. The internal standard (ISTD), d_3-Δ^9-tetrahydrocannabinol was purchased from Cerilliant as a 100 µg/mL methanol solution and used as received. Water (16 MΩ) was provided by a Barnstead E-Pure water purification system (Thermo Fisher Scientific, Waltham, MA, USA). Weighing was performed using a Mettler Toledo ME204E analytical balance (Thermo Fisher Scientific).

2.2 | Instrumentation and software

Standard configuration UPLC (3.6 μL^2) experiments were performed using an Agilent 1290 Infinity system (Agilent Technologies, Santa Clara, CA, USA) equipped with a 1290 Infinity binary pump, 1290 Infinity autosampler, 1290 Infinity thermostatted column compartment and a 1290 Infinity photodiode array detector (DAD). Low extra-column dispersion (1.2 μL^2) UPLC experiments were performed using a Waters Acquity I-Class UPLC (Waters Corporation, Milford, MA, USA) equipped with a binary solvent manager, sample manager with 10 µL sample loop, thermostatted column compartment (with active mobile phase preheater) and DAD (500 nL flow-cell). MS/MS was performed on a SCIEX QTRAP® 6500$^+$ mass spectrometer (SCIEX, Framingham, MA, USA). UPLC-DAD experiments were performed separately from UPLC–MS/MS experiments.

UPLC-DAD data acquisition and processing were carried out using the MassHunter Workstation software suite (Agilent Technologies, Data Acquisition Workstation v B.06.01, Service Pack 1, Qualitative Analysis v B.07.00, Service Pack 2) and Empower 3 chromatography data software (Waters Corporation). UPLC–MS/MS data was analysed using the PeakView® (v 2.2) and MultiQuant™ (v 3.0.2) softwares (SCIEX). Data reduction and visualisation was performed using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

2.3 | Column metrics

Pycnometry was adapted from the work of McCormick and Karger.[18] Briefly, columns were equilibrated with either hexane or ethyl acetate at a flow-rate of 500 µL/min. At 30 min intervals, the columns were weighed until three consecutive values agreed to within 1% relative difference. The weights were averaged and the void volume (V_0) of each column was calculated per the following equation:

$$V_0 = \frac{\left(\overline{m}_{\text{Hexane}} - \overline{m}_{\text{Ethyl Acetate}}\right)}{\left(\rho_{\text{Hexane}} - \rho_{\text{Ethyl Acetate}}\right)} \quad (1)$$

where $\overline{m}_x$ represents the average weight (in grams) and ρ_x is the specific gravity (in g/mL). The specific gravities for hexane (0.6591 g/mL [d_4^{20}]) and ethyl acetate (0.9020 g/mL [d_4^{20}]) were not corrected for room temperature (22°C). Raw data and calculations are shown in Supporting Information Table S1.

Bulk molecular diffusion coefficients (D_m values) were calculated at 30, 40 and 50°C (depending on the column and UPLC used, Table 1) with the open tubular method described by Song *et al.*[19] using equation 2:

$$D_m = \frac{d_t^2 \times t}{96 \times \sigma_t^2} \quad (2)$$

where d_t is the capillary tube diameter, t is the residence time in the capillary and σ_t is the chromatographic peak variance (Table S2). The D_m data was collected at 100 µL/min in 55% acetonitrile, 0.1% formic acid using 10.668 m × 100 µm PEEK tubing and a 1 µL injection volume.

TABLE 1 UPLC column parameters

Column	Particle size (μm)	SA[a] (m^2/g)	Carbon[a] (%)	v_o (μL)	Temperature (°C)	F (μL/min)
Kinetex	1.3	200	12	96.47	50	50–1100
Luna Omega	1.6	260	11	102.4	50	100–1800
Kinetex	2.6	200	12	124.0	40	50–2000

Note: All columns were C18, 50 mm × 2.1 mm with 100 Å pores. F is the range of volumetric flow-rates evaluated for each column, v_o is the void volume determined using pycnometry (Table S1) and SA is the total particulate surface area. Columns were heated to 30°C for experiments performed with low extra-column dispersion.

[a]Manufacturer's specifications.

Retention times and peak widths used to construct van Deemter plots were calculated automatically in MassHunter Qualitative Analysis and Empower 3 softwares. All peak widths were calculated at full width half maximum (FWHM) intensity. Triplicate injections at all flow-rates were averaged for the construction of van Deemter plots and other column performance metrics. Linear velocity (u) and reduced linear velocity (v) were calculated per the following equations:

$$u = \frac{L}{v_o / F} \tag{3}$$

$$v = \frac{d_p \times u}{D_m} \tag{4}$$

where L is column length, F is the volumetric flow-rate and d_p is the particle diameter. Height equivalent to theoretical plate values (H, equation 5) were divided by d_p to obtain the reduced plate height (h).

$$H = \frac{L}{N} \tag{5}$$

$$N = 5.54 \times \left(\frac{t_r}{\mathrm{FWHM}}\right)^2 \tag{6}$$

Chromatographic resolution (R_s, equation 7) was calculated using FWHM values. Extra-column variance ($\sigma^2_{v,ext}$) was estimated according to equation 8 at the optimal van Deemter flow-rate for each cannabinoid, on each column.[20]

$$R_s = \frac{\Delta t_r}{0.5 \times (\mathrm{FWHM}_1 + \mathrm{FWHM}_2)} \tag{7}$$

$$\sigma^2_{v,total} = \frac{V_R^2}{N} + \sigma^2_{v,ext} \tag{8}$$

where $\sigma^2_{v,total}$ is the chromatographic peak variance (i.e. total system variance) and V_R^2 is the squared retention volume. A linear regression of $\sigma^2_{v,total}$ versus V_R^2/N yields $\sigma^2_{v,ext}$ as the intercept.

2.4 | UPLC-DAD and UPLC-MS/MS

For UPLC-DAD and UPLC-MS/MS analyses the aqueous mobile phase was 0.1% formic acid and the organic mobile phase was 0.1% formic acid in acetonitrile, with all elutions carried out isocratically at 55% organic mobile phase. Under the standard UPLC configuration a 10 μL injection volume was used. For the low dispersion UPLC configuration a 5 μL partial-loop injection was used, with 9:1 acetonitrile:water and 1:20 acetonitrile:water as the strong and weak wash solvents, respectively.[21] Individual and mixed cannabinoid standards were analysed at 1.00 or 10.0 μg/mL in 50% methanol. Analytes were detected at 280 nm, with a 4 nm slit width, a 360 ± 100 nm reference wavelength and a data acquisition rate of 2.5 Hz (80 Hz on Acquity I-Class). UPLC operating pressures did not exceed 1000 bar. The van Deemter analyses were performed on three columns (Table 1) from Phenomenex (Torrance, CA, USA): Luna® Omega C18 1.6 μm (50 mm × 2.1 mm); Kinetex® 1.3 μm C18 (50 mm × 2.1 mm); and Kinetex® C18 2.6 μm (50 mm × 2.1 mm). Flow-rates ranged from 50 to 2000 μL/min and the column temperature was 40°C or 50°C (Table 1).

For UPLC-MS/MS analysis elution was performed at 1000 μL/min on a Kinetex® 1.3 μm C18 (50 mm × 2.1 mm) column at 50°C. Analyses were performed using electrospray ionisation (ESI) with polarity switching (500 ms cycle time, Tables S3 and S4).

2.5 | Method performance

Eleven LC-MS/MS calibration standards were prepared in 50% methanol from 0.1 to 250 μg/L, with the ISTD added at 5 μg/L. Calibration curves were fit using linear regression and inverse concentration weighting from triplicate injections at each calibration level. The limit of detection (LOD) and limit of quantitation (LOQ) were calculated per International Conference on Harmonisation (ICH) guidelines as follows:

$$\mathrm{LOD} = \frac{3.3 \times s_n}{m_i} \tag{9}$$

$$\mathrm{LOQ} = \frac{10 \times s_n}{m_i} \tag{10}$$

where s_n is the standard deviation from n sample replicates near the estimated LOD and m_i is the slope of the calibration curve for compound i.[22] Eight sample replicates were prepared at 0.10, 0.25 and 0.50 μg/L in 50% methanol to evaluate LODs and LOQs. The concentration used to calculate the LOD/LOQ values was the lowest concentration where the qualifying ion was present with a signal-to-noise >3 (peak-to-peak) and all calculated LOQs were within their respective calibration ranges.

Cannabis extracts were prepared at a licenced facility by adding dried and homogenised cannabis flower (100 mg) to methanol (30 mL). After sonication [Branson 2510 Ultrasonic Cleaner, Branson (Danbury, CT, USA)] for 10 min at 30°C the samples were centrifuged for 5 min at 2550×g [C3-Select, CME (Warwick, RI, USA)]. The supernatant was diluted (100-fold and 10000-fold) with 50% methanol, ISTD was added (5 μg/L) and the extracts were analysed without further work-up.

3 | RESULTS AND DISCUSSION

Evaluation of cannabis potency is generally focused on Δ^9-THC and CBD, as they are present at a high weight percentage and contribute heavily to the biological activity of cannabis. But there are other phytocannabinoids with demonstrated biological effects, even though they are found at lower concentrations (Figure 1). Therefore, a panel of 11 phytocannabinoids, including Δ^9-THC and CBD, was investigated in this study. Other low-concentration phytocannabinoids [i.e. cannabigerovarin (CBGV), cannabicyclol (CBL) and Δ^9-tetrahydrocannabivarinic acid (THCVA)] also elicit biological effects,[23] but analytical standards were not available at the time of this study.

Initial method development to establish a chromatographic method capable of separating a critical pair (Δ^8- and Δ^9-THC) was performed on a Kinetex® 2.6 µm particle diameter C18 column (data not shown). Separation was best realised with isocratic elution in 55% acetonitrile with 0.1% formic acid, as the higher eluotropic strength was needed to elute the more hydrophobic phytocannabinoids, while the overall selectivity and retentivity still provided adequate resolution of, in particular, Δ^8- and Δ^9-THC. Subsequent to this work, two additional C18-based stationary phases were evaluated (Table 1). The UPLC columns selected represented superficially porous particles (2.6 and 1.3 µm) and sub-2 µm fully porous particles (1.6 µm). To quantitatively establish the chromatographic performance of each column, reduced van Deemter plots for all 11 phytocannabinoids were constructed.

To calculate the reduced linear velocity, it was necessary to know the phytocannabinoid bulk diffusion coefficients (D_m; Table 2, Table S2). The D_m values were calculated using the Taylor–Aris open tubular method summarised by Song *et al.*[19] Since the capillary dimensions used in the present study were different than Song *et al*., the contributions of axial diffusion (equations 10–13) and radial concentration equilibration (equation 14) to the observed band-broadening were evaluated:

$$\sigma^2_{t,diff} \ll \sigma^2_{t,Taylor-Aris} \tag{11}$$

$$\sigma^2_{t,diff} = \frac{2\times D_m \times t_r}{u^2} \tag{12}$$

$$\sigma^2_{t,Taylor-Aris} = \left(\frac{FWHM}{2.35}\right)^2 \tag{13}$$

$$L \gg \frac{d_t^2 \times u}{60 \times D_m} \tag{14}$$

with $\sigma^2_{t,diff}$ the peak variance due to axial diffusion, $\sigma^2_{t,Taylor-Aris}$ the standard Gaussian peak variance, t_r the retention time of the solute in the open tubular capillary, u the linear velocity, L the length and d_t^2 the diameter of the capillary. For all compounds, the axial diffusion and radial concentration contributions to band-broadening were found to be negligible (Table S2). A series of reference compounds were analysed to further validate the experimental set up. The values obtained for toluene (9.06×10^{-10} m^2/s), ascorbic acid (8.68×10^{-10} m^2/s), phenol (1.05×10^{-9} m^2/s) and thiourea (1.40×10^{-9} m^2/s) were consistent with reported data.[19] Having demonstrated the validity of the experimental set-up, phytocannabinoid D_m values were calculated as a function of temperature (30, 40, 50°C). The observed range 0.854–0.912 $\times 10^{-9}$ m^2/s at 50°C (Tables 2 and S2), while narrow, was reasonable when the structural similarity of all the phytocannabinoids was considered. The absolute phytocannabinoid D_m values reported herein are consistent with recently published D_m values for reference compounds with analogous lipophilicity and size.[19,24] Having these physical constants will facilitate future expansion of the developed method to include additional phytocannabinoids, or potentially synthetic cannabinoids.

TABLE 2 Summary of phytocannabinoid physical and method performance parameters

Compound	D_m[a] ($\times 10^{-9}$ m^2/s)	LOD (ng/g)	LOQ (ng/g)	LDR	*r*
CBDV	0.858	47.0	142.	3.4	0.9985
THCV	0.904	23.7	71.9	3.0	0.9989
CBDA	0.871	44.3	134.	3.4	0.9988
CBD	0.881	50.4	153.	3.4	0.9991
CBGA	0.858	50.2	152.	3.4	0.9992
CBG	0.901	48.1	146.	3.4	0.9982
CBN	0.880	40.6	123.	3.0	0.9990
Δ^9-THC	0.909	55.9	169.	3.0	0.9993
Δ^8-THC	0.915	20.4	61.9	3.0	0.9989
THCA	0.854	49.1	149.	3.4	0.9993
CBC	0.912	18.7	56.7	3.0	0.9995

Note: Complete calculations for validation of the bulk diffusion coefficients (D_m) are in Table S2. LOD, limit of detection; LOQ, limit of quantitation; LDR, linear dynamic range. Method performance data was determined using 50% methanol as a surrogate for a 10000-fold diluted cannabis extract.

[a]Calculated at 50°C.

Using the calculated D_m values, reduced van Deemter plots for the phytocannabinoids were constructed for the low $\sigma^2_{v,ext}$ UPLC configuration, which included the performance optimising injection sequence (POISe) to mitigate band broadening due to the injection system.[21] Comparing the reduced plate heights (h) obtained for the phytocannabinoids (h_{min} = 1.9–3.0) to those for butylparaben ($h_{min,\ 1.3\ \mu m}$ = 1.50 and $h_{min,\ 2.6\ \mu m}$ = 1.59)[25] and naphthalene ($h_{min,\ 1.3\ \mu m}$ = 2.5–3.3)[26] revealed suitable agreement (Figures 2, S1 and S2). This was expected since $\sigma^2_{v,ext}$ for the low dispersion UPLC system (1.2 µL^2) was similar to that reported for the evaluation of butylparaben and naphthalene (0.5–2.5 µL^2).[27]

The Kinetex 1.3 µm column did not display minima in the van Deemter plots for all compounds, or when it did (e.g. CBDV), the minimum was subtle and located at high v. Fekete and Guillarme[25] reported a similar result, which they attributed to the low permeability of this phase leading to high pressure drop under the conditions studied. This provides a plausible explanation why the Kinetex 2.6 µm (280000 plates/m) column yielded the highest efficiency for all column and linear velocity combinations examined with the low $\sigma^2_{v,ext}$ UPLC configuration, despite 500000 plates/m reported for the Kinetex 1.3 µm column.[25] Given their agreement with reported reduced plate heights on similar columns and the low $\sigma^2_{v,ext}$ UPLC configuration used, the reduced plate heights reported herein are benchmarks for the chromatographic efficiency of phytocannabinoid separations.

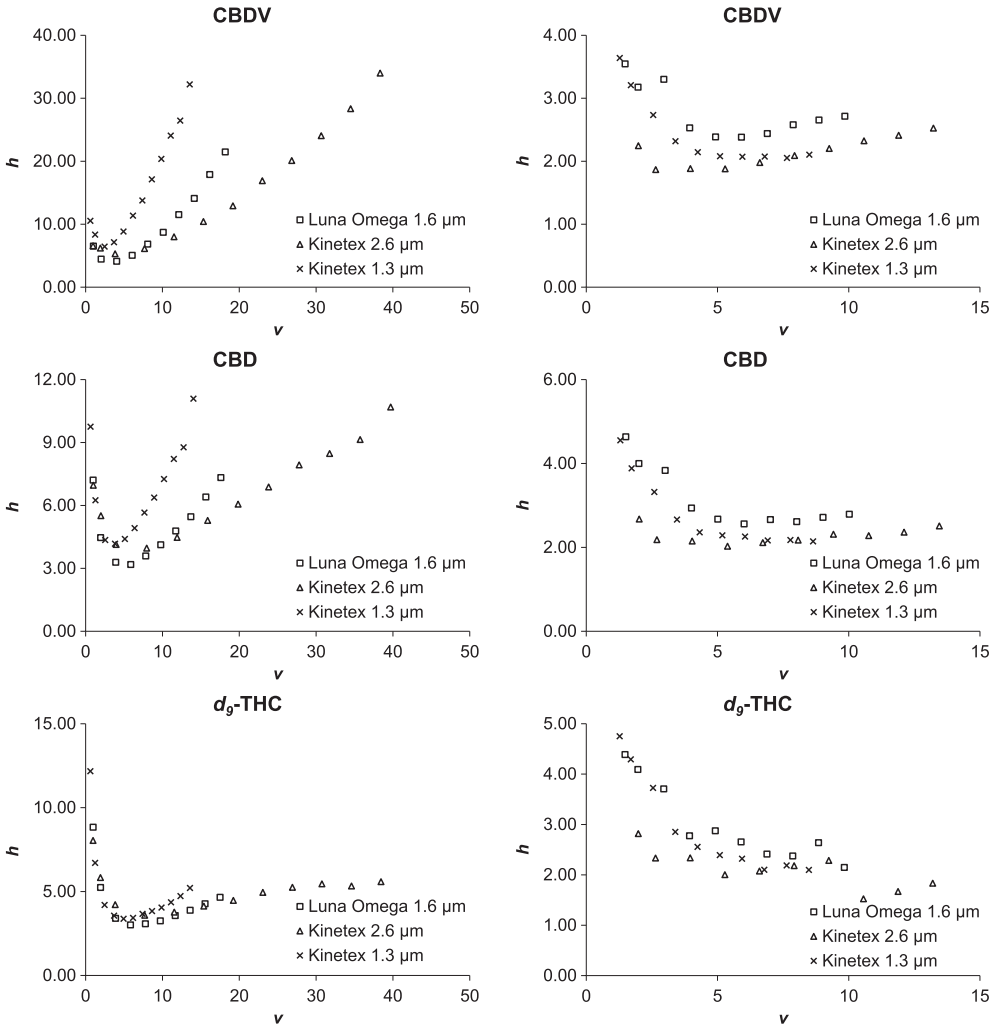

FIGURE 2 Column efficiency (h) for selected phytocannabinoids as a function of reduced linear velocity (v) under standard (left) and optimal (right) UPLC configurations. The van Deemter plots were constructed by averaging replicate analyses at each linear velocity (n = 3)

While a UPLC configuration that minimises $\sigma^2_{v,ext}$ is requisite when targeting optimal chromatographic performance from small particle diameter columns (fully and superficially porous), rigid adherence to such configurations may be deemed non-critical when weighed against other data quality objectives. For instance, in a production environment the stringent UPLC configurations that are used in the pursuit of benchmark chromatographic research may be overlooked, or not used due to the cost associated with implementation and upkeep. Therefore, to expand the scope of this performance evaluation, van Deemter plots were also prepared using a standard UPLC configuration with $\sigma^2_{v,ext}$ equal to what was provided during installation by the manufacturer (3.6 μL^2). The authors recognise that alternative chromatographic solutions exist to maximise performance on UPLC systems with high $\sigma^2_{v,ext}$ (e.g. selecting correctly matched column and particle dimensions). However, the empirical selection of chromatographic parameters (*vide infra*) performed in many laboratories that operate UPLC instruments with unoptimised $\sigma^2_{v,ext}$ underscores the value of the comparison reported herein.

The reduced van Deemter data from the standard UPLC configuration showed decreased chromatographic efficiency compared to the optimised data (Figures 2, S1 and S2). This was expected since $\sigma^2_{v,ext}$ impacts chromatographic efficiency when using sub-2 µm fully porous and sub-3 µm superficially porous particles.[20,28] It was also observed that the early eluting compounds displayed a sharp decrease in efficiency at higher linear velocities. This too was expected, as the impact of $\sigma^2_{v,ext}$ is more prominent for early eluting compounds.[25] This effect was most apparent for CBDV, which had the lowest retention factor across all conditions.

Notwithstanding these expected performance decreases, the standard UPLC configuration data yielded interesting results. For instance, independent of retention factors and reduced linear velocity, the highest chromatographic efficiencies were obtained from the Luna Omega 1.6 µm column, while the Kinetex 1.3 µm column was the least efficient. This trend was the opposite of that observed on the low $\sigma^2_{v,ext}$ UPLC configuration (Figure 2). This result was atypical (but not surprising in light of the known impact of $\sigma^2_{v,ext}$[26,29,30]) since superficially porous particles are more efficient, especially at higher flow-rates, compared to fully porous particles. This deviation was undoubtedly associated with the higher $\sigma^2_{v,ext}$, as the 1.3 µm superficially porous particles exaggerate the influence of $\sigma^2_{v,ext}$ compared to 2.6 µm superficially porous particles. The calculated v_o values

(Table 1) confirm this conclusion, with the impact of $\sigma^2_{v,ext}$ expected to be the greatest for the Kinetex 1.3 µm column and the least for the Kinetex 2.6 µm column (based on the ratio of $\sigma^2_{v,ext}$:v_o, where values <1 are optimal).

The deviation from anticipated behaviour indicates that care must be taken when interpreting experimental results acquired using a system with higher than optimal $\sigma^2_{v,ext}$. This is especially true when using sub-2 µm fully porous or sub-3 µm superficially porous particles under UPLC conditions.

Based on the reduced van Deemter evaluation under the standard UPLC configuration, the fully porous Luna Omega 1.6 µm column would be the best choice if high chromatographic efficiency were the sole benchmark for a suitable phytocannabinoid potency method. However, the practical considerations of a fit-to-purpose method and those concerning cost and subtle chromatographic differences between column phases should also be considered. This is especially true in fee-for-service testing environments, where cost and throughput are often paramount considerations.

A comparison of the chromatograms at the maximum flow-rate analysed for each column demonstrated differences in chromatographic performance that influenced the final column selection (Figure 3). A visual survey showed minor differences in selectivity for the early eluting compounds, as well as THCA and CBC. But these differences were not significant since the critical isobars in these regions were still resolved. A more pertinent performance difference was the achievable resolution (R_s) of Δ^8-THC and Δ^9-THC (Figure 4). The Kinetex 2.6 µm column demonstrated poor performance in this regard, with an $R_{s,\ max}$ = 1.16 at a reduced linear velocity of 7.7. The other two columns deviated from their order of chromatographic efficiency (*vide supra*), with the Kinetex 1.3 µm having an $R_{s,\ max}$ = 1.69 and the Luna Omega 1.6 µm having an $R_{s,\ max}$ = 1.45 at reduced linear velocities of 6.2 and 6.1, respectively. The carbon loads and effective surface areas (Table 1) were identical for the Kinetex 1.3 µm and 2.6 µm columns. As such, it seemed that the driving force behind the resolution difference was band broadening. For example, the Kinetex 1.3 µm column had a FWHM for Δ^8-THC = 0.068 min and Δ^9-THC = 0.063 min, while the Kinetex 2.6 µm column has FWHM for Δ^8-THC = 0.075 min and Δ^9-THC = 0.073 min. This result was expected given the relationship between particle size and band-broadening described by the van Deemter equation. However, the relative performance of the Luna Omega 1.6 µm and Kinetex 1.3 µm columns was surprising given that the Luna Omega column had the highest efficiency independent of retention factor or reduced linear velocity. Based on FWHM data for the Luna Omega 1.6 µm column, where Δ^8-THC = 0.080 min and Δ^9-THC = 0.077 min, it seems that this difference was also due to band broadening.

While the Kinetex 1.3 µm column showed the highest $R_{s,\ max}$ for Δ^8-THC and Δ^9-THC, it also displayed the sharpest drop in resolution at higher flow-rates for the standard UPLC configuration. This effect was attributed to the increased $\sigma^2_{v,ext}$ of the standard UPLC configuration, as a similar trend was not observed for the low dispersion system (Figure 4). Despite this effect, the resolution was still 1.38 at a reduced linear velocity of 12.3. While not the ideal of $R_s \geq 1.5$ or the value of 1.96 (at v = 8.6) from the low $\sigma^2_{v,ext}$ system, this

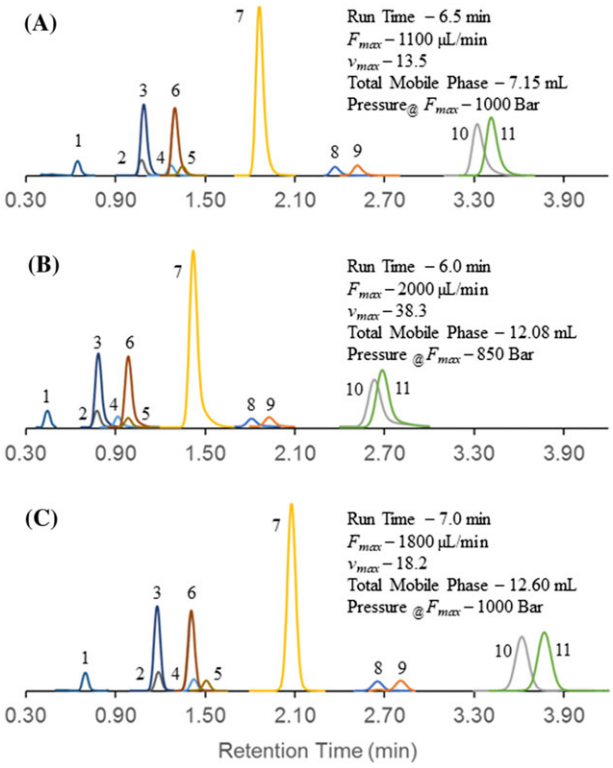

FIGURE 3 Chromatographic comparison of results at F_{max} on Kinetex 1.3 µm (A), Kinetex 2.6 µm (B) and Luna Omega 1.6 µm (C) columns acquired using the standard UPLC configuration. Total run time, solvent consumption and pressure at F_{max}/v_{max} are shown inset. (1) cannabidivarin (CBDV); (2) tetrahydrocannabivarin (THCV); (3) cannabidiolic acid (CBDA); (4) cannabidiol (CBD); (5) cannabigerol (CBG); (6) cannabigerolic acid (CBGA); (7) cannabinol (CBN); (8) Δ^9-tetrahydrocannabinol (Δ^9-THC); (9) Δ^8-tetrahydrocannabinol (Δ^8-THC); (10) Δ^9-tetrahydrocannabinolic acid (THCA); (11) cannabichromene (CBC) [Colour figure can be viewed at wileyonlinelibrary.com]

corresponded to <1.5% overlap for Gaussian peaks, which was suitable for a routine quantitative analytical method. Moreover, the Kinetex 1.3 µm resolution was superior to the Kinetex 2.6 µm at all reduced linear velocities. These data suggest that if column selection were based on Δ^8-THC and Δ^9-THC resolution, the Kinetex 1.3 µm column would be the best choice.

On a cost basis, the method run time and the volumetric flow-rate were also pertinent performance metrics (Figure 3). Owing to the low pressure drop, the Kinetex 2.6 µm column permitted the highest reduced linear velocities. Indeed, at v = 38 the pressure was 850 bar, suggesting that method run times closer to 5 min might be possible. This contrasts with the Kinetex 1.3 µm and Luna Omega 1.6 µm columns that yielded 6.5 and 7.0 min run times, respectively, with both columns operating at 1000 bar despite a column temperature of 50°C (versus 40°C for the Kinetex 2.6 µm). However, at v = 38 the Kinetex 2.6 µm R_s for Δ^8-THC and Δ^9-THC was only 0.96. Moreover, v = 38 represents a volumetric flow-rate of at 2000 µL/min, which would adversely impact MS ionisation efficiency (i.e. sensitivity) due to incomplete desolvation, without the introduction of a flow-splitter to reduce the work load on the desolvation system. In contrast, v = 13.5 (volumetric flow-rate of 1100 µL/min) was more likely to be directly compatible with a range of MS

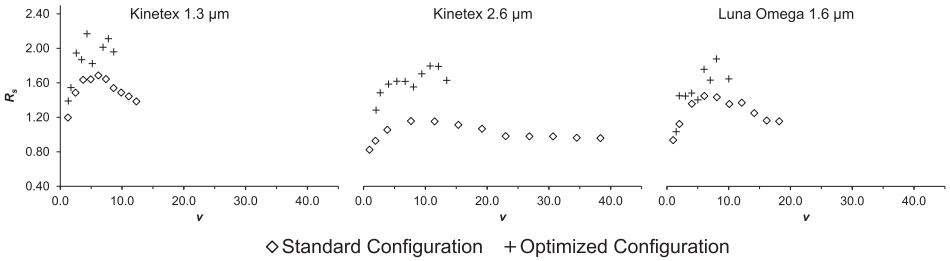

FIGURE 4 Chromatographic resolution of Δ^8-tetrahydrocannabinol (Δ^8-THC) and Δ^9-tetrahydrocannabinol (Δ^9-THC) as a function of reduced linear velocity for the standard and optimized UPLC configurations. All plots share the same *y*-axes

ionisation sources, making the Kinetex 1.3 µm column an attractive option. Another advantageous feature of the Kinetex 1.3 µm column was a solvent consumption of 7.15 mL per sample (Figure 3). This was 40% less than the Kinetex 2.6 µm at 12.6 mL.

Combining these considerations led to selecting the Kinetex 1.3 µm column for method performance evaluation. While the absolute chromatographic efficiency that guided this selection was not maximised (i.e. $\sigma^2_{v,ext}$ was not optimal), from a fit-to-purpose perspective the efficiency loses were acceptable considering the low mobile phase consumption, mass spectrometry-compatible volumetric flow-rate and suitable Δ^8-THC/ Δ^9-THC chromatographic resolution.

Method performance was demonstrated using a SCIEX 6500$^+$ QTRAP® with ESI and polarity switching. Dedicated positive mode was evaluated, but all the acidic phytocannabinoids displayed improved sensitivity in negative mode when using 0.1% formic acid as the mobile phase modifier (data not shown). All compounds were analysed from 0.1 to 250 µg/L in 50% methanol to establish the linear range, LOD and LOQ (Table 2). Linearity was excellent, with correlations >0.99 for all compounds and linear dynamic ranges of 3.0 to 3.4.

The high levels of some phytocannabinoids in cannabis extracts (i.e. THCA and CBD) made spike-recovery studies to establish method performance at low analyte concentrations challenging. To avoid these challenges, samples were prepared in 50% methanol for calculating LOD and LOQ. Background levels in 50% methanol and a 10000-fold diluted cannabis extracts appeared similar, so the validity of the resulting LOD/LOQ values should hold in 50% methanol or in a 10000-fold diluted cannabis extract (Figure S3). In the present study, LOD and LOQ were calculated per ICH guidelines (Table 2). The results show that the method is suitably sensitive for the analysis of the 11 phytocannabinoids evaluated in the present study, with LOQs <170 ng/g of dry cannabis flowers. The reported method was applied to the analysis of a cannabis extract (Figure 5). Owing to the very high levels of THCA relative to other phytocannabinoids the sample was analysed at 100-fold and 10000-fold dilutions. The specificity afforded by MS/MS detection was evident due to the absence of isobaric interferences in all chromatograms except CBDV (< LOD) and CBD. The presence of isobars for the primary transition of CBD could be problematic, even with

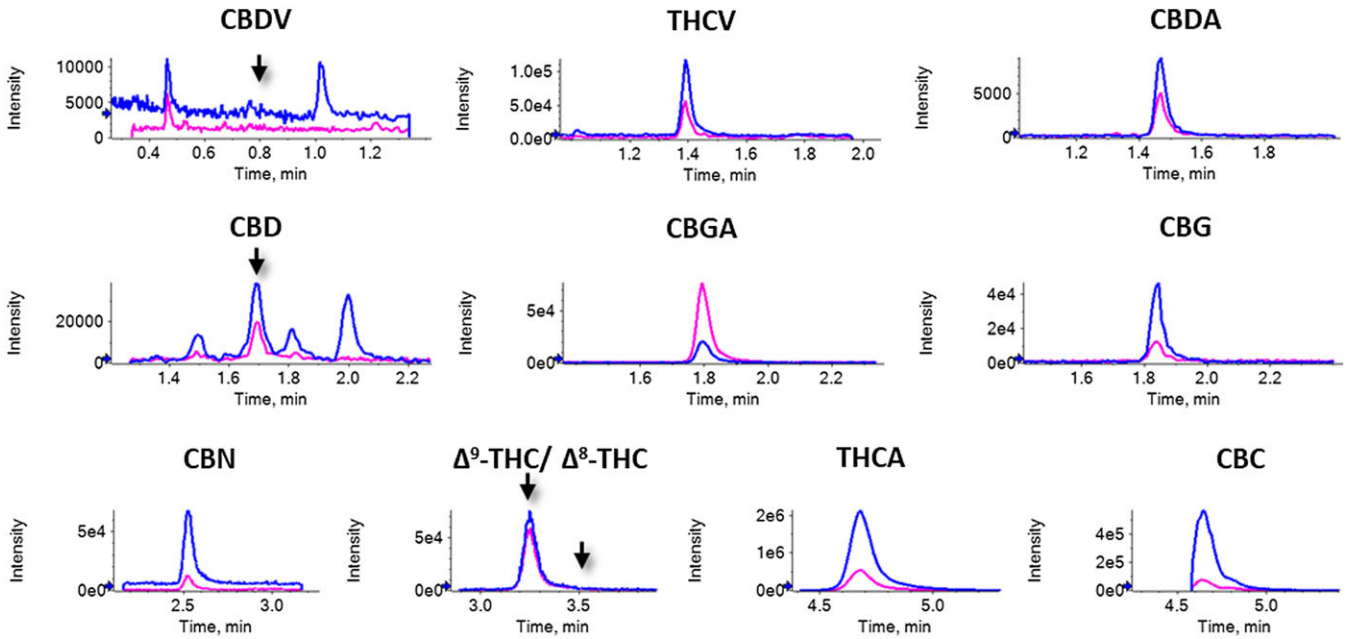

FIGURE 5 Sample chromatogram of a cannabis extract. Chromatography was carried out using the Kinetex C18, 50 mm × 2.1 mm, 1.3 µm column. Primary transitions (blue) are shown overlaid with secondary transitions (pink). Where ambiguity exists, an arrow indicates the expected retention time. Cannabidivarin (CBDV), tetrahydrocannabivarin (THCV), cannabidiol (CBD), cannabinol (CBN) and cannabichromene (CBC) are shown from 100-fold dilutions, while all other compounds are shown from 10000-fold dilutions [Colour figure can be viewed at wileyonlinelibrary.com]

retention time matching. The use of a secondary transition and ion response ratios afforded added confidence in the identification of the peak corresponding to CBD in the cannabis extract.

ACKNOWLEDGEMENTS

MN and WFZ would like to thank SCIEX for the use of their instrumentation and Supra Research and Development for supplying phytocannabinoid standards. MN acknowledges the MITACS Accelerate programme, University Graduate Fellowship and the Walter C. Sumner Memorial Fellowship for funding.

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SUPPORTING INFORMATION

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How to cite this article: Noestheden M, Friedlander G, Anspach J, Krepich S, Hyland KC, Zandberg WF. Chromatographic characterisation of 11 phytocannabinoids: Quantitative and fit-to-purpose performance as a function of extra-column variance. *Phytochemical Analysis*. 2018;1-9. https://doi.org/10.1002/pca.2761