



Qualitative and quantitative measurement of cannabinoids in cannabis using modified HPLC/DAD method



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ABSTRACT

This study presents an accurate and high throughput method for the quantitative determination of various cannabinoids in cannabis plant material using high pressure liquid chromatography (HPLC) with a diode array detector (DAD). Sample extraction and chromatographic analysis conditions for the measurement of cannabinoids in the complex cannabis plant material matrix were optimized. The Agilent Poroshell 120 SB-C18 column provided high resolution for all target analytes with a short run time (10 minutes) given the core shell technology. The aqueous buffer mobile phase was optimized with ammonium acetate at pH 4.75. The change in the mobile phase and the new column ensured a separation between cannabidiol (CBD and cannabigerol (CBG) along with cannabigerol and tetrahydrocannabinolic acid (THCA), which were not well separated by previous publications, improved buffering capacity, and provided analytical performance stability. Moreover, baseline drifting was significantly minimized by the use of a low concentration buffer solution (25 mM ammonium acetate). In addition, evaporation and reconstitution of the sample residue with a methanol-organic pure (OP) water solution (65:35) significantly reduced the matrix interference. The modified extraction produced good recoveries (>91%) for each of the eight cannabinoids.

The optimized method was validated for specificity, linearity, sensitivity, precision, accuracy, and stability. The combined relative standard deviation (%RSD) for intra-day and inter-day precision for all eight analytes varied from 2.5% to 5.2% and 0.28% to 5.5%, respectively. The %RSD for the repeatability study varied from 1.1% to 5.5%. The recoveries from spiked cannabis matrix samples were greater than 90% for all analytes, except delta-8-tetrahydrocannabinol (Δ^8 -THC), which was 80%. The recoveries varied from 81% to 107% with a precision of 0.7–8.1%RSD. Delta-9-tetrahydrocannabinol (Δ^9 -THC) in all of the cannabis samples ($n=635$) was less than 10%, which is in compliance with the NJ Medicinal Marijuana regulation. Analysis of samples from two cultivars, which included ten individual samples, four composite samples, seven calibration standards, and four quality control standards, can be performed within 24 hours by this high throughput method.

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1. Introduction

Medical cannabis has rapidly become an important topic in today's society. As of January 2017, twenty-nine states and the District of Columbia have legalized the use of cannabis for medical purposes [6,5,2]. Sixteen additional states have limited laws allowing the use of cannabis extracts containing high CBD and low

Δ^9 -THC concentrations, primarily for children suffering seizure disorders. Eight states (Alaska, California, Colorado, Maine, Massachusetts, Nevada, Oregon, and Washington) and the District of Columbia have legalized cannabis for recreational use [6].

The New Jersey Compassionate Use Medical Marijuana Act was signed into law on January 18, 2010 [7], and the New Jersey Medicinal Marijuana Program (NJMMMP) was established in 2011 to enforce regulations of the law. The objectives of the NJMMMP are to ensure the compliance of regulation which requires Δ^9 -THC content to be less than 10% by weight, and to ensure that the products are free of molds, fungi, pesticides, and heavy metals. Therefore, an accurate quantitative method with high throughput was needed to meet the objectives of the NJMMMP and provide physicians and patients with

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reliable data for large number of samples with a short turnaround time.

High Pressure Liquid Chromatography (HPLC)-Ultraviolet Diode Array Detector (UV-DAD) methods for the measurement of cannabinoids in cannabis plant material were published previously [3,1,8] with the method published by De Backer being the most referred. This method employs HPLC-UV-DAD for the analysis of cannabis and is a fully validated method for qualitative and quantitative determination of cannabinoids. A LC method is better suited than a gas chromatography (GC) method for the measurement of cannabinoids because the LC method allows simultaneous analysis of both the acidic and neutral forms of each cannabinoid. The acids remain in their original form under the LC analytical conditions, whereas they decompose into their neutral analogs due to the hot inlet and oven conditions of GC methods. The conversion of acids to neutral compounds during the GC analysis is not quantitative. An additional derivatization step is necessary in order to properly quantitate the acids [4] by gas chromatography.

However, the above-mentioned LC methods are less suitable for the analysis of a large number of cannabis samples for numerous reasons. First, a relatively long run time of 25–36 minutes makes them unsuitable for a routine potency assay of cannabinoids with a large number of samples. Second, the resolution between CBD, CBG and THCA peaks reported in previous studies are inadequate for accurate determination. Third, the recoveries of cannabinoids from plant material with a single extraction are low for several target compounds (<80%). The low recoveries are partially due to the incomplete dissolution of cannabinoids upon reconstitution of extract residue in methanol:OP water (1:1). Fourth, a drifting UV detector baseline was observed during the chromatographic run. Thus, a study was needed to achieve better resolution by optimizing analytical conditions, including the column, the mobile phase composition, and the elution program.

The present work is a sensitive and accurate high throughput liquid chromatographic method for the measurement of eight cannabinoids in cannabis plant material. This study also monitored the potency and stability of cannabinoids in plant material stored at room temperature, which is important for understanding the optimum storage conditions for cannabis products. Moreover, the variation of cannabinoids by strain type and cultivation center has been examined.

2. Materials and methods

2.1. Reagents and materials

Concentrated stock standards of 1000 $\mu\text{g/mL}$ for THC, CBD, Cannabinol (CBN), Δ^8 -THC, THCA and CBG in methanol were purchased from Restek Corporation (Bellefonte, PA). Cannabigerolic Acid (CBGA) and Cannabidiolic Acid (CBDA) at 1000 $\mu\text{g/mL}$ in ethanol were obtained from Echo Pharmaceuticals BV (Jonkerbosplein, Netherlands). All eight cannabinoids were also obtained from Cerilliant Corporation (Round Rock, TX) to be used for quality control purposes. Intermediate mixed calibration standards containing each analyte at 5 and 50 $\mu\text{g/mL}$ in methanol:OP water (65:35) were prepared from the individual cannabinoids stock standards. Working calibration standards were prepared at concentrations of 0.25, 0.5, 1.0, 5.0, 10.0, 20.0 and 50 $\mu\text{g/mL}$ in methanol:OP water (65:35) from the intermediate mixed calibration standards. All standards were stored at -20°C .

Ammonium formate, ammonium acetate, formic acid, acetic acid (HPLC grade), and ibuprofen were obtained from Sigma Aldrich (Allentown, PA). Methanol (HPLC Grade), chloroform, and isopropyl alcohol (2-propanol, reagent grade) were obtained from Thomas Scientific (Swedesboro, NJ). Deionized organic pure (OP) water

(18 m Ω , TOC <5 ppm) was obtained from a Millipore Milli-Q Gradient A system (Millipore Corporation, Billerica, MA).

The internal standard stock solution of 10,000 $\mu\text{g/mL}$ was prepared by dissolving 100 mg of neat ibuprofen ($\geq 98\%$) in 10.0 mL of 65:35 methanol:OP water. The spiking internal standard solution of 200 $\mu\text{g/mL}$ was prepared by diluting 200 μL of the stock solution to 10.0 mL with 65:35 methanol:OP water.

2.2. Instrumentation

Analysis was performed using an Agilent 1100 (Agilent Technologies, Santa Clara, CA) Series LC system equipped with a degasser, binary pump, autosampler, thermo column compartment, UV DAD, and MSD trap (SL) detector. Agilent ChemStation software (Rev. B.01.03[204]) was used to control the instrument components and acquire, store, and reduce UV data. MS data were collected and analyzed using Bruker MSD trap software (Rev. 5.3 Build 22.14).

2.3. Sample preparation and analysis conditions

Various sample preparation methods and analytical conditions were tested and optimized. These modifications included sample extraction, the analytical column, ionic strength, pH and gradient program of the mobile phase, injection volume, and UV detection parameters. The specific conditions tested are presented below.

2.3.1. Cannabis sample collection and preparation

All cannabis cultivars were grown by the state permitted Alternate Treatment Centers (ATC) located in New Jersey.

Five individual cannabis flower samples (2.5 g each) per cultivar were randomly collected from the ATC to provide a representative selection of a complete harvest. They were collected by the personnel from the state Medicinal Marijuana Program and delivered to the laboratory for analysis. The samples collected were the same as the samples distributed to patients. They contain buds with high trichome content, a nominal amount of stem and no leaves. The collected samples were processed without alteration.

Finished packaged products were also collected for quality assurance (QA). One QA sample was tested with each cultivar. The QA sample was collected and stored under the same conditions as other products at the center and tested six months later to determine whether there were changes in potency or contamination due to packaging and storage.

Each individual cannabis flower sample (~ 2.5 g) was transferred into a clean, dry stainless steel Robot Coupe bowl (Thomas Scientific, Swedesboro, NJ). Cannabis flowers were ground with different amounts of dry ice for varying amounts of time in order to determine the optimal method of sufficiently grinding the sample. When grinding the cannabis plant material, it was determined that dry ice (roughly three times the weight of the individual sample) should be added to ensure that the sample was ground into finer particulates. Plant material should be pulsed twice for 30 seconds each time to ensure homogeneity in the granulation of the sample and reproducible results in the extraction process. Dry ice in the ground sample was allowed to sublime at room temperature for 30 minutes before transferring the samples into a clean pre-weighed amber glass jar.

After grinding, approximately 200 mg of the ground cannabis flowers were weighed accurately in a tared screw cap 50 mL propylene centrifuge tube from VWR. Each sample was extracted with 20.0 mL of a methanol:chloroform (9:1) solvent mixture, hand shaken for 30 seconds, vortexed for 30 seconds, and further shaken using a Burrell Wrist Action Shaker Model 75 (Burrell Scientific, LLC, Pittsburg, PA) for 30 minutes at speed 10. After centrifuging for ten minutes with a Damon IEC HN-SII centrifuge (International Equip-

ment Company, Needham Heights, MA) at 3000 rpm, the liquid extract was decanted into another clean 50 mL centrifuge tube.

Tests were conducted to optimize the sample extraction protocol. Each sample was extracted three times in series, and the first, second, and third supernatant extracts were kept separate. Each of them was analyzed separately by HPLC-DAD, and the recovery of each extract was calculated. The total recovery was examined after each extraction to determine the number of extractions needed to achieve a total recovery of 90% or greater. Analysis of individual extracts for a single sample was discontinued after the total number of extracts to reach a recovery of 90% or higher was determined.

The final extraction procedure includes the following: extract the 200 mg sample with 20 mL of methanol:chloroform (90:10) two times, combine both the supernatant extracts, vortex and filter 3 mL of extract with a 0.2 μm nylon membrane. The filtered extract was placed in a 2 mL clean amber glass vial and stored at -20°C if the analysis is not conducted immediately. 100 μL of sample extract was further diluted with 400 μL , 900 μL or 1900 μL methanol to produce 5x, 10x and 20x dilutions. The diluted extracts were analyzed by HPLC-DAD.

After extraction, approximately two mL of extract were filtered through a 0.2 μm nylon membrane into a clean 2 mL amber glass vial and stored at -20°C .

The filtered extract was diluted to 1:5 and 1:10 with methanol. These dilutions were performed to prevent overloading of the column and saturation of the UV detector. Dilutions were referred to in the De Backer (2009) method, but levels were not specified.

A good liquid chromatographic separation is achieved when the composition of the sample extract is similar and compatible to the initial composition of the mobile phase. This can be achieved by evaporation and reconstitution. After dilution, 200 μL of diluted extract was pipetted into a 12 \times 75 mm culture tube. Methanol and chloroform were evaporated off to dryness with nitrogen gas using a Turbo Vap LV (Caliper Lifesciences, Waltham, MA) at room temperature. Residues were reconstituted with different organic solvents as well as with different ratios of organic solvents to OP water to determine the best conditions for re-dissolving the analytes of interest. Isopropyl alcohol, methanol, and ethanol were tested in ratios of 60:40, 70:30 and 65:35 with OP water.

In an attempt to shorten sample preparation time, experiments were also performed to determine if evaporation and sample reconstitution steps were necessary. Samples that were defined as direct dilution samples did not go through the evaporation and reconstitution steps.

2.3.2. Chromatographic columns

Three different columns, which included the Waters XTerra MS C18 2.1 \times 250 mm \times 5 μm column, Agilent Poroshell 120 SB-C18 3.0 \times 75 mm \times 2.7 μm , and Agilent Poroshell 120 EC-C18 3.0 \times 75 mm \times 2.7 μm columns, were tested under different mobile phase and gradient conditions. These tests were conducted in an attempt to shorten the run time and to increase the separation of cannabinoids. The Waters column is an older column that has particles of size 3.5 μm . The Agilent columns contain a porous particle layer 0.5 μm thick and a particle size of 2.7 μm with a 1.7 μm solid core [9]. The difference between the SB and EC Agilent columns is that the SB column has a greater ruggedness for temperature and can be used with pH as low as 0.8. The EC column has a greater number of silanol groups endcapped to prevent negative charges from forming on the surface of the particles in the column.

2.3.3. Mobile phase and elution program

The mobile phases consisted of a 50 mM aqueous solution of ammonium formate, pH 5.19 (mobile phase A) and methanol (mobile phase B) in De Backer's method (2009). In addition, baseline drifting was observed when the gradient progressed from a

low to a high percentage of the solvent (mobile phase B). Drifting occurred due to a decrease in UV absorption, which affected automated integration of chromatographic peaks. To resolve the drifting problem, the ammonium formate concentration was lowered from 50 mM to 25 mM. Baseline separation between all peaks was achieved during initial analysis (Fig. 1a) using the SB-C18 Agilent column and 25 mM ammonium formate; however, the THCA peak was gradually eluted earlier and co-eluted with the CBG peak after 200–300 injections of standards and sample extracts. Although the column could be regenerated by flushing for six to twelve hours with methanol:water (95:5), the column had to be replaced frequently to reproduce the original chromatograms.

The pH of mobile phase A (containing 25 mM ammonium formate) was tested between 4.8 and 5.2 at increments of 0.1 to determine the optimum pH for baseline separation of CBG and THCA. Although a good separation between THCA and CBG was achieved at approximately pH 5, a fluctuation in retention times was still observed. To improve the separation, ammonium formate was replaced with ammonium acetate and the pH was reduced from 5.19 to 4.75. Since the optimum pH of a buffer is ± 1 the pK_a of its acid, it was expected that a solution of ammonium acetate (the pK_a of acetic acid is 4.75) at pH 4.75 would have a greater buffering capacity than a solution of ammonium formate (the pK_a of formic acid is 3.75) at pH 5.19. Therefore, comparison experiments were conducted using both 25 mM ammonium acetate and ammonium formate buffer solutions with pH of 4.75 and 5.19, respectively (Fig. 2a and b). Each solution was then titrated with 25 mM HCl and 25 mM NaOH. The pH was monitored with a Thermo electronics Orion 410A+ pH meter.

In order to select the optimum pH for separation of THCA and CBG, tests were conducted at different pH values (4.6, 4.7, 4.75, 4.8, 4.9, 5.0, 5.1, 5.2 and 5.3) of the mobile phase A ammonium acetate solution. Standards at concentrations of 1, 10 and 50 ppm, along with 5x and 10x dilutions of a cannabis sample extract were tested under these pH conditions.

In the De Backer et al. study (2009), the initial gradient setting was 68% methanol, with a linear increase to 90.5% over 25 min, then to 95% in 1 min. In this study, various gradient conditions were tested to determine the optimal conditions to achieve the best separation in a short run time.

2.4. Quantitation and calibration

Both external and internal standard methods were used in the analysis of samples. The external method was used initially, and the internal method was developed following the implementation of the external method. The internal standard method is preferable as it provides better performance control of the instrument during the analysis for better reproducibility and accuracy.

For both external and internal standard calibration, seven levels of calibration standards [0.25, 0.5, 1.0, 5.0, 10.0, 20.0 and 50.0 $\mu\text{g}/\text{mL}$ in methanol:OP water (65:35)] were prepared. Quantification was performed using a linear calibration curve forced through zero with equal weighting. R^2 values were greater than 0.995.

2.4.1. External standard method

Samples were prepared as per the sample preparation described in Section 2.3.1. The reconstituted extract was transferred into a 250 μL auto-sampler vial for HPLC analysis. Samples were quantitated directly based on the area counts for each cannabinoid.

2.4.2. Internal standard method

Since the cannabis sample matrix is complex and can cause variation in the instrument condition during analysis, the addition of the internal standard was implemented to ensure the ruggedness of the method. After adding 50 μL an ibuprofen internal standard

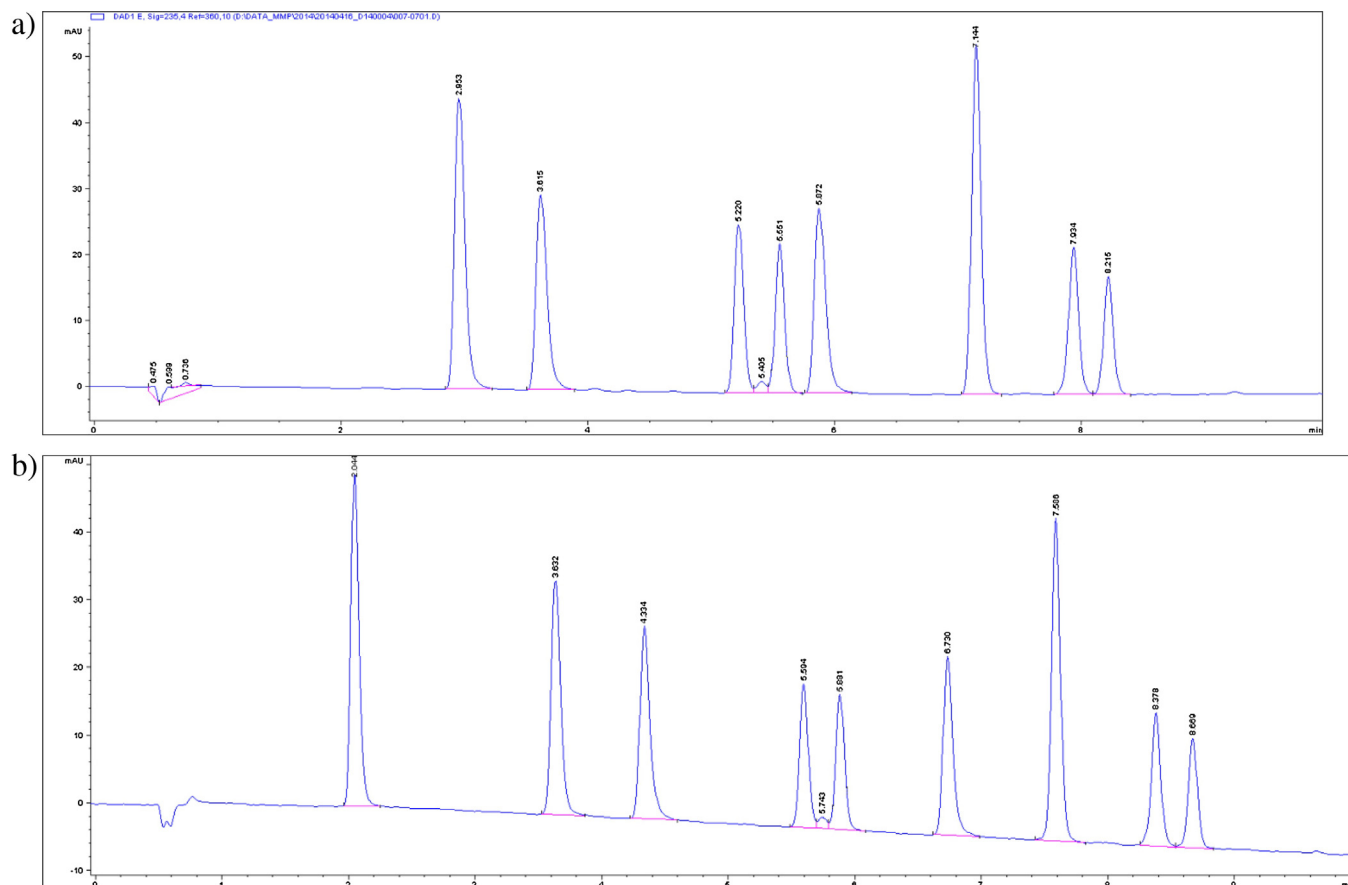


Fig. 1. (a) Chromatogram of 10 ppm cannabinoids standard-ammonium formate buffer pH 5.19 conditions. (b) Chromatogram of 10 ppm cannabinoids standard-ammonium acetate buffer pH 4.75 conditions.

(200 $\mu\text{g}/\text{mL}$ in methanol:OP water 65:35) to 200 μL of each working calibration standard, quality control check standard, blank, and sample extract in 250- μL auto sampler vials, each was mixed by vortexing before HPLC analysis. Samples were quantitated similarly to the external standard method, except the internal standard was utilized to correct the results for potential instrumental variation during the analysis. The software adjusted the values of every sample based on the variation of each sample's internal standard to that of the average.

2.4.3. DAD incidence wavelength

Our target analytes include both acid and neutral forms. The UV λ_{max} of the target analytes vary largely, ranging from 220 nm to 300 nm. The UV λ_{max} for neutral cannabinoids (CBD, CBG, delta 9-THC and delta 8-THC) are at ~ 220 nm and the UV absorption of cannabinoid acids (CBDA, CBGA and THCA) are at ~ 225 nm, ~ 260 nm and ~ 300 nm. CBN, a degradation product of THC, absorbs at ~ 220 nm and ~ 290 nm. Ibuprofen, an acid, was used as an internal standard for quantitation. It has UV λ_{max} of ~ 225 nm and has very minimal absorption at ~ 270 nm.

Tests were conducted to determine the optimal wavelength for quantitation. UV-DAD data at 230, 235, 240, 260 and 290 nm were collected at a 4 nm bandwidth using 360 nm as a reference and a 10 nm bandwidth for quantitation purpose. For cannabinoid identification, all spectrum scans were performed from 200 to 400 nm with a 2.0 nm step size, threshold at 1.0 mAU, auto balance at pre-run, peak width at > 0.1 min, and a slit of 4 nm. The software identified each analyte within a specified retention time window for each LC peak. Identified peaks were integrated and quantitated using linear regression forced through zero with equal weighting.

2.5. Validation

The method was validated through precision, repeatability, stability, and accuracy studies. Precision of the reported method was validated through inter-day and intra-day testing of the calibration standards. De Backer's calibration curve spanned 0.375 to 50 ppm, while this study tested a concentration range from 0.25 to 50 ppm. For the inter-day study, a 10 ppm cannabinoid standard was analyzed on five different days in a week. The intra-day study was performed using three different levels of calibration standards (0.5, 5.0 and 50.0 $\mu\text{g}/\text{mL}$) and the analysis was performed five times during a 24-hour period. The repeatability of the method was demonstrated through the extraction and analysis of seven replicates of a cannabis sample obtained from the New Jersey State Police.

Method accuracy was tested through spiking experiments. Most cannabinoid standards are not readily available in large quantities because they are expensive, illegal, and controlled substances in the US. Spiking standards at high concentrations were required due to the fact that cannabis plant material contains either THCA or CBDA at high levels. Since CBN and Δ^8 -THC are generally not found in newly harvested cannabis plant material, these two compounds (10 mg/mL) that are commercially available in large quantities were used as the spike solution. A concentrated spiking extract (CBDA, CBGA, CBD, CBG, THCA and Δ^9 -THC) for the other six cannabinoids was prepared by combining concentrated extracts of three different cultivars. The extract was utilized in spiking experiments to evaluate the accuracy of the method. Three levels of spiking, each in triplicate, in cannabis plant material, were performed.

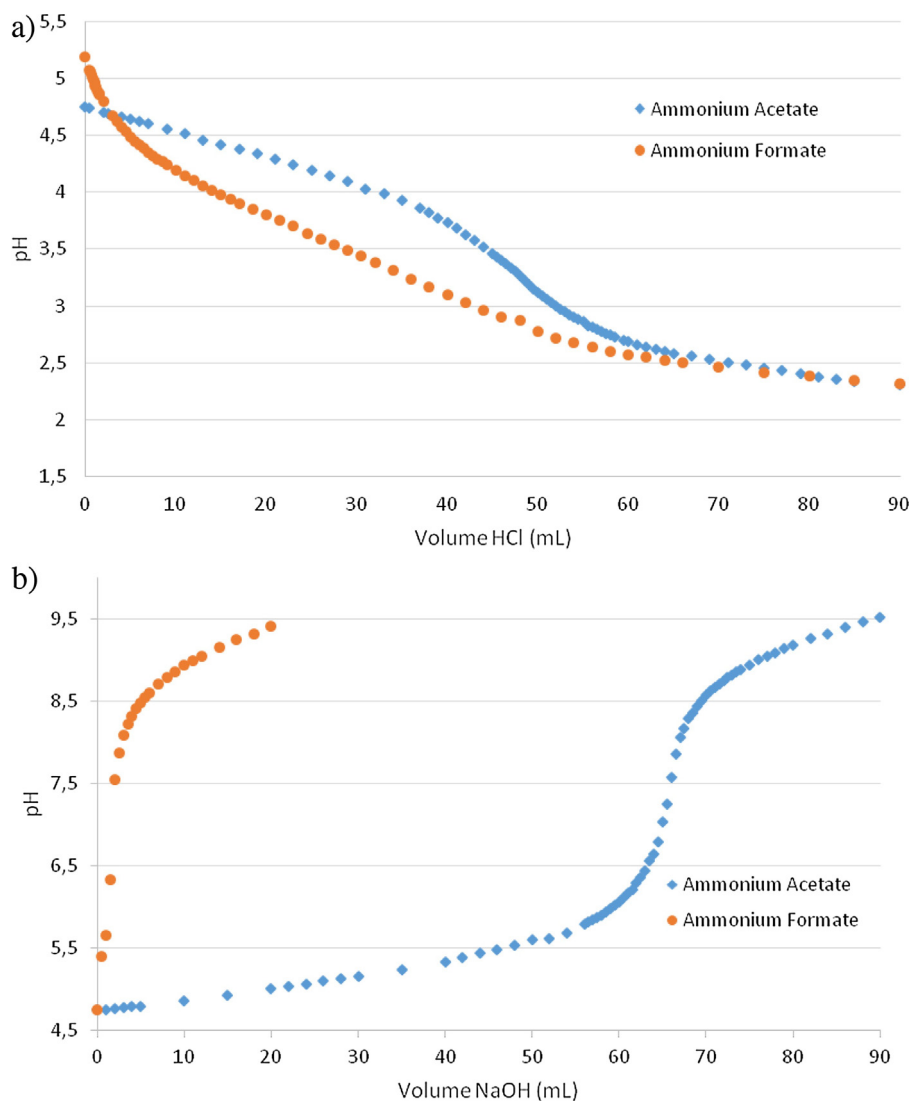


Fig. 2. (a) Titration of Ammonium Acetate and Ammonium Formate Buffer Solutions with 25 mM HCl. (b) Titration of ammonium acetate and ammonium formate buffer solutions with 25 mM NaOH.

The recovery was calculated using the following formula:

$$\% \text{Recovery} = [(C_f - C_i) / C_s] \times 100$$

C_f is the amount of cannabinoid determined in the spiked sample, C_i is the average amount of the cannabinoid found in the unspiked sample, and C_s is the spiked amount of a cannabinoid from the standard spike solution.

2.6. Application of the method

The optimized method has been used to analyze 635 cannabis samples in the past five years. These samples included 68 strain types submitted by five New Jersey Alternative Treatment Centers. Summary statistics were performed for the sample results and presented in the Results and Discussion Section 3.2.

3. Results and discussion

3.1. Modifications and optimization of sample preparation and analysis conditions

3.1.1. Sample preparation and extraction efficiency

Three extractions were performed to examine the recoveries. Recoveries of five analytes, CBGA, CBD, CBG, THCA and CBN, were

close to 80% or higher after the first extraction (Table 1). However, the recoveries of CBDA and Δ^9 -THC from the first extraction were only 62 and 72%, respectively. After the second extraction, the total recovery of each analyte was >91%. The third extraction amounted to additional recoveries of <2% for CBD, THCA and CBN, <3% for CBG, <7.5% for CBGA and <9% for Δ^9 -THC. Results demonstrated that two extractions produced acceptable results (>90% recovery), while the third extraction did not increase the recovery significantly with the increased amount of time spent. Therefore, only two extractions were performed in the final method.

3.1.2. Optimization of reconstitution

Methanol was first used for reconstitution. It provided the best recoveries of all the cannabinoids, but produced poor chromatographic separation and peak shapes. The methanol:OP water ratio (1:1) used by the De Backer method produced an acceptable chromatogram, but the (1:1) ratio produced only half the recoveries of methanol:OP water (65:35). The 65:35 ratio gave the best recoveries of all cannabinoids and produced the best chromatograms. The recoveries for all cannabinoids except CBGA were greater than 90%. CBGA was the most difficult compound to re-dissolve and recover with an overall recovery \approx 80% with methanol:OP water (65:35).

Table 1
Percent recoveries of cannabinoids for each extraction (n = 3).

Extraction #	CBDA	CBGA	CBD	CBG	THCA	CBN	Δ^9 -THC	Δ^8 -THC
1	62.1	79.8	89.5	87.8	86.9	85.6	72.2	0
2	29.6	13.0	9.1	9.5	11.6	12.6	19.2	0
3	8.3	7.2	1.4	2.7	1.4	1.7	8.6	0
Total recovery after two extractions	91.7	92.8	98.6	97.3	98.5	98.2	91.4	N/A

Table 2
System suitability testing results (n = 14).^a

Analytes	Retention time (min)		Symmetry		Tailing factor	
	RT	Std. Dev.	Average	% RSD	Average	% RSD
Ibuprofen	2.012	0.011	0.790	0.009	1.200	0.005
CBDA	3.552	0.019	0.851	0.077	1.055	0.126
CBGA	4.248	0.019	0.842	0.077	1.069	0.128
CBD	5.548	0.084	0.970	0.025	0.954	0.072
CBG	5.833	0.023	0.946	0.034	0.937	0.063
THCA	6.685	0.020	0.813	0.134	1.094	0.185
CBN	7.536	0.025	0.965	0.014	1.580	0.765
Δ^9 -THC	8.322	0.056	1.001	0.028	0.918	0.059
Δ^8 -THC	8.636	0.025	0.966	0.073	0.942	0.117

^a This data was taken from two sets of standards used to create separate calibration curves. The retention time, symmetry, and trailing factors were all taken from the report produced by the ChemStation software provided by Agilent Technologies.

Ethanol and isopropyl alcohol produced recoveries similar to those obtained with methanol when used as reconstitution solvents, but did not offer any additional advantages. Moreover, an experiment was performed to examine if reconstituting with mobile phase composition (35% 25 mM ammonium acetate pH 4.75 and 65% methanol) would enhance recovery. The recoveries of the acid cannabinoids (CBDA, CBGA and THCA) decreased because of the acidity of the aqueous solution (pH 4.75) compared to reconstituting with 65% methanol and 35% OP water (pH 7.0).

It was concluded that the optimal solvent composition for reconstitution was a mixture of methanol:OP water (65:35 pH 7.0). This solvent mixture gave the best recoveries for all cannabinoids as well as produced the best chromatograms.

To further maximize cannabinoid recovery after drying, sample wetting and vortexing studies were performed. A combination of wetting the sample for thirty seconds and vortexing for one minute was found to produce the best recovery.

The direct dilution samples described in Section 2.3.1 were analyzed with the instrument under the same conditions as the reconstituted samples. The chromatographic separation and peak shape were not reproducible and cannabinoid recoveries were much lower than those achieved with the reconstitution step. These results suggest that the reconstitution step removed the interferences from the plant matrix and is necessary.

3.1.3. Analytical column

Tests showed that the Agilent Poroshell 120 SB-C18 (3.0 × 75 mm ID × 2.7 μm particle size) core shell technology base column provided the best performance amongst the three columns tested. Compared to the Waters XTerra MSC18 column, the run time was shortened 20 minutes, and a significant improvement in separation and symmetry of chromatographic peaks was observed with a baseline separation between CBD and CBG (Fig. 1b). Core shell columns produce a fast, high efficiency separation and resolution given the C18 chemically bonded porous outer layer, narrow particle size distribution, and solid core. The internal structure of the column also limits diffusion distance and improves separation speed by reducing back pressure [10,9].

It is important to note that an unknown peak appeared between CBD and CBG peaks when a mixed standard was utilized. This unknown peak was separated from the two cannabinoids and did not interfere in the quantitation of CBD and CBG. This unknown

Table 3
Regression equation, correlation coefficient, linear range, LOD and LOQ.^a

Cannabinoid	R ²	Linear range μg/mL	LOD μg/mL	LOQ μg/mL
CBDA	0.9999	0.25-50	0.0625	0.25
CBGA	0.9997	0.25-50	0.0625	0.25
CBD	1.0000	0.25-50	0.0625	0.25
CBG	1.0000	0.25-50	0.0625	0.25
THCA	0.9997	0.25-50	0.0625	0.25
CBN	1.0000	0.25-50	0.0625	0.25
Δ^9 -THC	0.9998	0.25-50	0.0625	0.25
Δ^8 -THC	0.9997	0.25-50	0.0625	0.25

^a Data were derived from the average of 6 calibrations performed over a period of 6 months.

peak was due to the enhanced resolution between the CBD and CBG peaks that was seen in this method. When a fresh CBG standard from Echo Pharmaceuticals was injected, the same unknown peak was eluted and separated prior to the CBG peak. From its mass spectrum, it is likely related to CBG itself and originated in the CBG reference standard. It is not known whether this molecule is an impurity, an isomer of CBG, or simply an artifact. This peak was not observed in the analysis of cannabis plant material.

The injection volume was reduced from 30 μL to 10 μL to decrease column loading. Compared to De Backer’s method, this method has a shorter run time with sharper peaks due to both the Agilent Poroshell column and the smaller injection volume.

3.1.4. Gradient

The gradient elution was modified to the following, 0-8.25 min: 68.0-85.0% mobile phase B, 100% methanol, 8.2-9.0 min: 85.0-95.0% mobile phase B, 9.0-10.0 min: 95.0-68.0% mobile phase B. The flow rate was set at 0.70 mL/min and the column temperature maintained at 30 °C. This ensured the elution of all cannabinoids with a base line separation in a short run time of ten minutes. The last cannabinoid, Δ^8 -THC, was eluted at approximately 8.4 to 8.5 minutes (see Fig. 1a).

3.1.5. Mobile phase

The best resolution between all peaks was achieved when ammonium formate was replaced with 25 mM ammonium acetate (adjusted to pH 4.75 with acetic acid) in mobile phase A. Acetate acts as a better buffer than ammonium formate at the pH of inter-

Table 4
Precision, repeatability and stability for the eight cannabinoids.

Cannabinoid	Intra-day %RSD (N = 5)	Inter-day %RSD (0.5 µg/mL) (N = 5)	Inter-day %RSD ^b (5.0 µg/mL) (N = 5)	Inter-day %RSD ^b (50.0 µg/mL) (N = 5)	Repeatability ^a %RSD (N = 10)	Stability ^a %RSD (N = 7)
CBDA	2.50	2.40	2.49	3.32	1.50	0.81
CBGA	3.24	1.99	1.67	2.87	2.98	1.15
CBD	3.50	1.10	1.37	0.89	2.10	1.18
CBG	3.43	0.71	1.01	0.98	1.10	3.45
THCA	2.68	1.19	2.68	5.52	1.48	3.03
CBN	3.25	0.28	0.52	0.22	1.38	0.60
Δ ⁹ -THC	4.73	1.74	1.33	1.44	5.53	7.11
Δ ⁸ -THC	5.19	1.21	1.52	1.92	n/a	n/a

^a These studies were performed with the New Jersey state police samples.

Table 5
Accuracy (Spike recoveries for the eight cannabinoids) (N = 3).

Cannabinoid	Sample Concentration (mg/g)	Spiked amount (mg/g)	Total amount (mg/g)	Analyst 1		Analyst 2	
				% Recovery	% RSD	% Recovery	% RSD
CBDA	6.51	7.93	14.49	100.7	1.8	87.4	3.5
		15.85	21.98	97.6	1.5	93.0	1.0
		23.78	28.71	93.4	2.3	85.6	5.8
CBGA	0.90	0.81	1.76	106.6	1.7	87.2	3.6
		1.61	2.41	93.6	3.9	87.4	4.6
		2.42	3.27	97.9	6.3	80.0	4.2
CBD	1.50	1.95	3.59	107.0	1.3	89.6	3.1
		3.91	5.62	105.4	1.1	92.4	2.0
		5.86	7.39	100.5	2.5	87.6	7.5
CBG	0.54	0.28	0.79	88.9	2.4	103.9	2.0
		0.56	0.99	80.0	4.9	94.0	5.8
		0.84	1.33	93.6	8.1	87.7	3.1
THCA	33.78	12.63	45.74	94.7	0.8	75.9	3.8
		25.26	57.17	92.6	2.0	89.4	6.0
		37.90	69.34	93.8	1.8	79.0	4.4
CBN	5.30	2.62	7.72	92.4	1.1	73.7	2.0
		5.24	10.38	97.0	0.7	98.1	3.2
		7.86	13.32	102.1	1.7	95.7	2.3
Δ ⁹ -THC	8.60	3.24	11.63	93.5	0.7	89.7	3.8
		6.48	14.76	95.0	1.7	95.2	4.2
		9.72	17.88	95.4	3.9	87.6	5.4
Δ ⁸ -THC	0.12	2.50	2.15	81.2	3.8	97.0	4.6
		5.00	4.33	84.2	2.8	99.1	3.5
		7.50	6.82	89.3	1.2	102.7	2.8

est (4.75) for cannabinoid elution (Fig. 3a and 3b). Moreover, the THCA retention time was stabilized and the co-elution of THCA with CBG was eliminated (Fig. 1a, Table 2). When the pH was increased by 0.1 units, the retention time of the THCA peak decreased by 0.3 minutes, which is a significant change. It was concluded that the pH of the mobile phase A must be tightly controlled within ± 0.05 pH units. Moreover, the chromatographic separation using ammonium acetate was much more reproducible, reliable, and rugged when compared to the use of ammonium formate. Good separation between CBG and THCA during day to day analytical runs was maintained, and the chromatograph was not affected by sample matrix variation and accurate quantitation was achieved. Furthermore, the deterioration of the instrument performance was minimized with the modified mobile phase. Acetate buffer kept the matrix constituents solubilized during analysis and reduced clogging of LC valves. Multiple analyses of cannabis cultivars were run on the same column without using a guard column for more than eight months and no significant change in the chromatograph was observed. The preservation of the column's integrity was the biggest advantage as it saved time and resources by reducing instrument breakdowns and column replacements.

In addition, the new mobile phase composition (25 mM ammonium acetate in Mobile Phase A) improved MSD ion trap

chromatograms of all cannabinoids with improved peak shapes and sensitivity when compared to the former mobile phase composition (25 mM ammonium formate). The improvement in ion trap chromatograms was due to the increase of electrospray ionization of cannabinoids in negative ionization mode in the modified conditions.

3.1.6. Optimal wavelength for quantitation

The UV signal with wavelength of 235 nm was selected for quantitation purposes, because the UV cut off point of methanol is 220 nm. The UV background was significantly decreased by reducing the concentration of ammonium acetate from 50 mM to 25 mM for aqueous mobile phase A. The lower concentration in buffer salt helped to stabilize the drifting UV detector baseline significantly, achieve the optimum detection limits at 235 nm, and produce a coefficient of determination, R^2 , of more than 0.995 for all the analytes of interest. Typical retention time, symmetry, and tailing factors data of cannabinoids peaks are presented in Table 2.

3.2. Method validation

Method validation studies were conducted to determine method specificity, sensitivity, linearity, accuracy, precision and

Table 6
The summary statistics of the analysis results for the cannabis plant material.

Analyte	N	% Mean	SD	% Median	% Min	% Max
CBDA	144	1.24	3.38	0.04	ND	16.19
CBD	144	0.48	0.43	0.37	ND	3.29
CBGA	144	0.09	0.23	0.02	ND	2.13
CBG	144	0.08	0.06	0.07	ND	0.25
THCA	144	14.60	6.941	14.95	ND	30.58
Δ^9 -THC	144	0.16	1.01	0.00	ND	9.16
Δ^8 -THC	144	0.65	0.53	0.49	ND	3.02
CBN	144	0.02	0.16	0.00	ND	1.85

suitability. The calibration regression equation, correlation coefficient, linear range, LOD, and LOQ for each cannabinoid are presented in Table 3. Curve fit settings were linear with equal weighing.

3.2.1. Precision

Method precision was validated through inter-day and intra-day testing of the calibration standards. The results from an intra-day and inter-day study are shown in Table 4. The combined %RSD for all eight analytes varied from 2.5 to 5.2% and 0.28 to 5.5% respectively which is well below acceptable limits of 15%. This shows excellent method precision.

3.2.2. Repeatability

Data presented in Table 4 demonstrates extraordinary repeatability. The %RSD of the analyses for the repeatability experiment varied from only 1.1 to 5.5. In both studies, the largest variation of about 5% was observed for THCA. This is probably due to its much higher concentration as compared to other cannabinoids present in plant material.

3.2.3. Method accuracy

The recoveries of the eight cannabinoids are summarized in Table 5. The recoveries varied from 81 to 107%, with %RSD of 0.7–8.1%. These recovery levels and associated variations are acceptable. The variability may be partially due to the inhomogeneity of the ground cannabis plant material.

3.2.4. Stability of the method extracts

Method extract stability during the sample analysis by LC-DAD was tested and the results are presented in Table 4. The results varied from 0.6 to 7.11%, which indicates that the sample extract was stable at room temperature over a period of 24 hours. Moreover, data suggested that the prepared sample extracts may be stable for up to two days at room temperature with little variation. This allows for the re-analysis of extracts if the initial run fails.

3.3. Analysis of cannabis cultivars

A summary of results of 144 cannabis batches for 68 strain types collected from different growers between 2012 to 2017 is presented in Table 6. The Δ^9 -THC content in all the samples was lower than 10%, which was in compliance with the NJMMP statute. However, a variation in the concentration of the target cannabinoids were observed as expected among the samples analyzed (Table 6). THCA varied over 30% and CBDA varied over 16% between strains. This variation may be primarily due to different strain types and growth conditions utilized by each individual ATC. These results indicate that it is important to evaluate the cannabinoid profile for each batch so that accurate information can be provided to the physician and the patients by the growers. More in-depth statistical analyses, such as analysis of variation in cannabinoids concentrations for the same strain type produced by different growers, the

Table 7
The differences between two samples types by paired t-test and Wilcoxon signed rank-sum test for target analytes.

Analyte	Paired T-test	Wilcoxon Signed Rank-sum Test
CBDA	0.3082	0.1055
CBGA	0.9524	0.6074
CBD	0.4049	0.3906
CBG	0.2191	0.3555
THCA	0.3975	0.2754
CBN	0.3434	1.0000
Δ^9 -THC	0.2231	0.1934
Δ^8 -THC*	N/A	N/A

Note: The concentrations for Δ^8 -THC were very low, so statistical tests were not conducted.

same strain type produced by the same grower, etc., will be conducted when more samples are analyzed.

The cannabinoid concentrations in the five replicate individual samples collected from each batch were very similar. The %RSD was less than 15% for 115 batches of samples analyzed. In addition, paired t-tests and Wilcoxon signed rank-sum tests, non-parametric paired t-tests, were conducted for target analytes to compare the individual samples and the composite samples. The average of the five individual samples (N = 115 batches) and the average of the two composite samples was used for comparison. Test results are presented in Table 7. Results showed that there were no significant differences between the two sample types ($p > 0.05$), suggesting that the collection method was adequate and the samples collected were representative of each harvest.

Based on testing workflow, two cultivars (a total of ten individual samples and four composite samples), seven calibration standards, and four quality control standards could be analyzed within 24 hours. This meets the need for analyzing large numbers of cannabis samples within a short period of time.

4. Conclusions

Previously published chromatographic methods were less suitable for the analysis of a large number of cannabis samples due to their long run times (over 30 minutes) as well as their poor separation/resolution of CBD and CBG peaks. This study successfully optimized sample preparation and analysis conditions. All eight cannabinoids were well separated in less than 10 minutes with a base line resolution ($R > 2.0$) between CBD and CBG by using a core shell based column as well as the modified and optimized analytical conditions. An aqueous ammonium acetate mobile phase buffered at pH 4.75 maintained good, stable separation between CBG and THCA, dominant components of most cannabis cultivars, with a resolution $R > 5.0$. Moreover, the use of an acetate buffer in the mobile phase extended the longevity of the Poroshell column to about 30 cultivar analyses, while a formate buffer allowed only 10 cultivar analyses. The modified and optimized method was more robust compared to previous methods.

Specificity, linearity, sensitivity, accuracy, suitability, and recovery have all been tested to fully validate the modified method. The validation tests confirmed the precision and accuracy of this method. The consistent results achieved by employing this method for analysis of cannabinoids over four years demonstrated the ruggedness and quality of this method. The short run time made this method highly ideal for handling large number of samples.

Although all the samples were composed of less than 10% Δ^9 -THC, in compliance with the NJMMP regulation, a large variation in cannabinoids content was observed across cultivars. This suggested that establishing a cannabinoid profile for strains produced by different cultivators may be helpful to provide accurate information to the physicians and patients. However, the data are not

sufficient for conducting statistical analysis to examine the variation in cannabinoid distribution by strain type, grower, and time. Future analysis will be conducted after more samples are analyzed.

Research into the impurity associated with the CBG standard should be pursued. Since CBG concentrations are low (1–3% of plant mass) in recently harvested cannabis plant samples, the impurity detected in the CBG standard was not detected in UV/DAD chromatographs of cannabis samples. This should be further examined to differentiate between an isomer, another previously identified cannabinoid, a degradation product, or a novel compound.

Future work should also include the development of faster sample grinding and an automated extraction procedure to further decrease the sample turnaround time; especially as demand for medicinal cannabis and cannabis products continue to grow.

Disclaimer

The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the New Jersey Department of Health (NJDOH). Mention of any company or product does not constitute endorsement by the NJDOH.

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References

- [1] L. Ambach, F. Penitschka, A. Broillet, S. König, W. Weinmann, W. Bernhard, Simultaneous quantification of Delta-9-THC, THC-acid A CBN and CBD in seized drugs using HPLC-DAD, *Forensic Science International* 243 (2014) 107–111.
- [2] Ballotpedia, Marijuana on the ballot, 2017 (Accessed 11 January 2017) https://ballotpedia.org/Marijuana_on_the_ballot.
- [3] B. De Backer, B. Debrus, P. Lebrun, L. Theunis, N. Dubois, L. Decock, A. Verstraete, P. Hubert, C. Charlier, Innovative development and validation of an HPLC/DAD method for the qualitative and quantitative determination of major cannabinoids in cannabis plant material, *Journal of Chromatography B* 877 (2009) 4115–4124.
- [4] F.E. Dussy, C. Hamberg, M. Luginbühl, T. Schwerzmann, T.A. Briellmann, Isolation of Delta(9)-THCA-A from hemp and analytical aspects concerning the determination of Delta(9)-THC in cannabis products, *Forensic Science International* 149 (2005) 3.
- [5] Governing, State Marijuana Laws Map, 2016 (Accessed 25 May 16) <http://www.governing.com/gov-data/state-marijuana-laws-map-medical-recreational.html>.
- [6] NCSL, State Medical Marijuana Laws, 2016 (Accessed 29 September 2016) <http://www.ncsl.org/research/health/state-medical-marijuana-laws.aspx>.
- [7] NJ Compassionate Use Act of 2008. Pub. L. 2009, c.307.
- [8] W. Swift, A. Wong, K.M. Li, J.C. Arnold, I.S. McGregor, Analysis of Cannabis Seizures in NSW, Australia : Cannabis Potency and Cannabinoid Profile, *PLoS ONE* 8 (2013) e70052.
- [9] Agilent Technologies Inc, Perform Rugged, Fast LC with Confidence, 2015 (Accessed 3 February 2015) https://www.agilent.com/cs/library/brochures/5990-5951EN_Poroshell%20120.pdf.
- [10] L. Pereira, An Overview of Core Enhanced Technology for Fast, High Efficiency HPLC, 2012 (Accessed 1 March 2016) <https://tools.thermofisher.com/content/sfs/brochures/an-overview-of-core-enhanced-technology-for-fast-high-efficiency-hplc.pdf>.