Single-run separation and quantification of 14 cannabinoids using affinity non-aqueous capillary electrophoresis

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Research Article

Keywords: cannabis, high-pressure liquid chromatography (HPLC).

DOI: https://doi.org/10.21203/rs.3.rs-129441/v1

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Abstract

The legalization of cannabis has magnified the importance of determining the quantity and identification of cannabinoids. Both industry and consumers are highly interested in the content of cannabinoids available in their products, while health care professionals and regulators are concerned with the safety of cannabis. Quantification of major cannabinoids in products answers some of these concerns. Currently, popular methods of quantifying cannabinoids use high-pressure liquid chromatography (HPLC). Still, these HPLC methods are limited to quantifying a small number of cannabinoids unless more powerful but more costly instruments are employed to achieve better analysis, such as UHPLC and mass spectrometry. We propose a quick method that successfully separates and quantifies 14 cannabinoids in a single run using capillary electrophoresis (CE) coupled with a UV detector in 25 minutes. Our CE method demonstrated the limit of detection between 1.2–1.8 µg/mL, with the detection range reaching up to 50 µg/mL.

Introduction

Cannabis sativa L. is a flowering plant in the family Cannabaceae and its taxonomy has been widely debated with some scientists considering the plant to be a polytypic genus with three subspecies, sativa, indica and ruderalis, and others considering these taxa to be different species. In addition, the continual cross-breeding of C. sativa- and C. indica-like plants has led to many perceiving the group as one species, C. sativa. Phytochemically, C. sativa is recognized for its production of cannabinoids, which are unique to cannabis and underlie its therapeutic potential, including management of anxiety and stress-related symptoms, stimulation of appetite, pain relief, and promotion of sleep.

The Cannabis Act (Bill C-45) legalized adult access to cannabis in Canada on October 17, 2018. It permits the selling of fresh and dried cannabis, cannabis plants and seeds, and cannabis oil by an authorized entity with subsequent regulation of commercial edible products and concentrates the year following. In terms of the cannabinoid content, the regulation requires quantification of only THC, THCA, CBD and CBDA.

In the USA, cannabis legalization is more nuanced. At the federal level, cannabis is recognized under the Controlled Substances act as a Schedule 1 substance, where it is considered to have a high potential for dependency and no accepted medical use. However, the medical use of cannabis is legalized in 33 states, with 11 states also legalized for recreational use. In Europe, recreational cannabis is illegal, although several countries have legalized it for medical use or decriminalized it, for instance, in the Netherlands.

Phytocannabinoids are products naturally found in C. sativa that share a typical C21 terpenophenolic skeleton. The plant synthesizes cannabinoids as acids, such as THCA, where decarboxylation of the 2-COOH by heat or light leads to the neutral cannabinoids, such as THC. This thermal conversion is a process common to all acid-form cannabinoids. To date, 120 cannabinoids have been isolated, which has led to classification by 11 types: (-)-Δ9-trans-tetrahydrocannabinol (Δ9-THC), (-)-Δ8-trans-
tetrahydrocannabinol (Δ8-THC), cannabigerol (CBG), cannabichromene (CBC), cannabidiol (CBD), cannabidiol (CBND), cannabielsoin (CBE), cannabicyclol (CBL), cannabionin (CBN), cannabitriol (CBT), and miscellaneous.

Current analytical methods for the quantification of cannabis are mostly based on high-performance liquid chromatography (HPLC)\textsuperscript{13}; however, they are costly, especially ultra-high-performance liquid chromatography UHPLC systems. Capillary electrophoresis (CE) is an alternative separation technique that exploits intrinsic charges present on compounds and uses their different electrophoretic mobilities to separate them inside a narrow (75 µm internal diameter). In this work, we employed non-aqueous affinity capillary electrophoresis (NACE), which relies on the acetonitrile-based background electrolyte in the presence of β-cyclodextrin (βCD). Cannabinoids are poorly soluble in water, requiring acetonitrile presence in the background electrolyte (BGE) for optimal solubility and separation. Additionally, cannabinoids share very similar ring structures and similar charges, rendering electrokinetic separation either difficult to achieve or making separation time excessively long.

Accordingly, we added βCD, which brings an orthogonal separation media by transiently interacting with compounds based on their geometry and polarity. In addition, the CE technique is associated with extremely small sample consumption, where one injection in a particular case of this study is only 4 nL. While separation of these compounds is possible in the absence of βCD, its presence shortens the analysis time by ~10 minutes leading to better resolution due to a lesser extent of longitudinal diffusion. A drawback of our method is the low buffering capacity of BGE. Acetonitrile in our electrolyte tends to evaporate fast once the vial has been opened, leading to fast changes in pH. Using fresh BGE is mandatory for reproducible data; reusing the same one twice is not recommended, as the pH will be different, as well as data.

We propose a cost-effective, high throughput method using NACE for the accurate quantification of 14 cannabinoids (THC, CBD, THCV, CBDV, CBG, CBN, CBC, and their respective acidic forms, see their structures in Figure S1) with detection by a UV detector at 230 nm. We separated deprotonated forms of these cannabinoids in highly basic conditions (pH ~12); furthermore, we reduced the analysis time to under 20 minutes with the aid of β-cyclodextrin.

**Materials And Methods**

**Plant extraction.** Lyophilized cannabis flowers were ground up with a hand grinder and extracted immediately. 5 mg of ground cannabis flower material was extracted in 1 mL of methanol (Optima™ LC/MS Grade methanol, A456212) for 30 minutes on a shaker at 200 rpm at room temperature. The extracted plant material was removed by filtering through a 0.22 µm PTFE membrane syringe filter (Whatman® Puradisc 25, WHA67842502), and the filtrate was stored in an HPLC vial at -20°C until further analysis.
Capillary electrophoresis. New bare silica capillaries (75µm ID, Polymicro, 1068150019) were washed and conditioned at 2000 mbar pressure as following: 5 minutes wash with 1M HCl, followed by 2 minutes wash with ddH2O, 10 minutes wash with 0.1M NaOH, 2 minutes wash with ddH2O and 5 minutes with BGE (6mM NaOH and 25µM β-cyclodextrin in 60/40 acetonitrile/water). Samples were injected into the capillary electrophoresis system (Lumex Capel 205) at 10 mbar for 3 seconds, forming a 2 mm plug of approximately 4 nL volume. 450 V/cm electric field was applied for 19 minutes to separate. Cannabinoid standards were diluted in methanol and injected the same way as the samples. Standards were purchased from Cayman chemicals (THC, 12068; THCA, ISO60175, CBD, 90080; CBDA, 18090; CBG, 15293; CBGA, 20019; CBC, 26252; CBCA, 30879; CBN, 25495; THCV, 18091; CBDV, 29117; CBGVA, 29787).

HPLC. Extracts were analyzed using an HPLC system (Agilent 1100 HPLC) coupled with a diode array detector (DAD, series G1315) and an autosampler (series G1313). Chromatography was performed on a Kinetex® 2.6 µm C18 150 x 2.1mm column (Phenomenex) using water + 0.1% TFA (A) and methanol + 0.1% TFA (B) as the mobile phase with a linear gradient from 68% to 85% B in 13 minutes followed by seven minutes of isocratic conditions (85% B). The flow rate was set at 0.25 mL/min, and the column temperature was maintained at 60°C with DAD monitoring at a wavelength of 230 nm.

Reproducibility. Precision was determined as a measure of how close the repeated injections are to each other in terms of peak areas. The coefficient of variation was calculated to assess precision according to the formula: CV = (SD/x) * 100, where x is the arithmetic mean of all injections.

Limit of detection and quantification. The limit of detection (LOD) was defined as the lowest concentration of an analyte that can still be detected by the instrument. We used a method based on standard deviation (SD) of multiple injections of a standard mix (in our case, 7 injections of 5 µg/mL), where LOD = (3.3 * SD)/m, where m is the slope of the linear curve. The limit of quantification (LOQ) was calculated in a similar manner; however, instead of a coefficient of 3.3, a coefficient of 10 was used.

Results And Discussion

Method development. The purpose of this study was to develop a reliable CE method for the separation and quantification of 14 cannabinoids from extracted cannabis samples. CE is a technique that separates compounds based on their charge; therefore, we used highly basic (apparent pH ~12, measured with a pH meter) conditions to deprotonate all 14 standards. Cannabinoids share a highly similar ring structure and exhibit similar migration time in the capillary, causing overlapping peaks if the conditions are inadequate (incorrect pH, for instance).

Sodium hydroxide and high pH alone resulted in adequate separation; however, the analysis time approached 30 minutes, which is not optimal for a high throughput technique. We have tested various concentrations of sodium dodecyl sulphate (SDS) in BGE ranging from fully aqueous to complete non-aqueous; however, most additives resulted in a loss of separation of these 14 compounds, with some of them inevitably coeluting and not properly resolving.
βCD, normally used as a chiral selector in CE applications, demonstrated very positive effects on the separation of 14 cannabinoids. Since it is an inclusion complex with a hydrophobic core, the cannabinoids would transition between βCD and BGE itself throughout the whole run. As a result, cannabinoids that elute the last closer to 30 minutes would be “carried” by βCD and elute slightly earlier. Indeed, with βCD added to the background electrolyte, all cannabinoids still showed great separation in a much shorter time (around 10 minutes less), with the exception of THC and CBG, which, although partially resolved, had an overlap close to the baseline.

We found that a combination of βCD and highly basic conditions resulted in a reproducible separation of all 14 compounds (Fig. 1) in a 60 cm capillary, enabling further quantification. Only two compounds (CBG and THC) were not resolved to a baseline and had a resolution of Rs = 1; all other peaks were well resolved to a baseline. Shorter capillary length, while feasible for separation of most compounds, results in lower resolution of CBG and THC and loss of separation.

Individual standards demonstrated high retention time reproducibility when compared to the more complex mixture (Fig. 1), allowing for the identification of each individual peak. All decarboxylated cannabinoids (CBD, CBDV, CBG, THC, THCV, CBN, CBC, in this order) tend to elute before the acidic forms (CBCA, THCA, CBNA, THCVA, CBDA, CBDVA, CBGA, in this order), which can be attributed to the higher overall negative charge on acidic cannabinoids due to their carboxyl group, and longer migration time as a result.

We performed HPLC analysis to compare with our CE method (Fig. 2), which provided comparable separation over a similar run time. However, some compounds were not fully resolved with this method (THCV, CBD and CBG; CBGA and CBN; THC and THCV). Reducing the particle size of the column and increasing pressure may result in better separation and a more efficient method than the one we present in the paper; in addition, more expensive UHPLC systems can be used to further improve the separation and analysis time.

**Quantitation of cannabinoids in samples.** We demonstrated the applicability of the CE technique for cannabinoids separation in a mixture of synthetic standards. Extracts of a real cannabis flower are rich in THCA and CBDA compared to other classes of cannabinoids. This situation sometimes leads to results where the peaks representing these 4 cannabinoids are magnitudes larger than anything else present in the sample (Fig. 3). These peaks may, in some cases, overlap adjacent peaks representing low abundant cannabinoids, especially in the case of THCA, which can overlap CBCA and CBNA. However, dilution of the sample may result in loss of detection of low abundant cannabinoids. To solve the problem, two injections may be necessary for the detection and quantification of all 14 compounds: injection of concentrated sample and injection of its 10-fold dilution. In the case of our sample, this was not necessary.

**Table 1.** Limits of detection (LOD) and quantification (LOQ) for each cannabinoid compound (n = 7).
| Compound | LOD (µg/mL) | LOQ (µg/mL) |
|----------|-------------|-------------|
| CBD      | 1.3         | 3.8         |
| CBG      | 1.5         | 4.6         |
| THC      | 1.2         | 3.7         |
| THCV     | 1.3         | 4.0         |
| CBN      | 1.2         | 3.7         |
| CBC      | 1.4         | 4.1         |
| CBCA     | 1.4         | 4.3         |
| THCA     | 1.3         | 3.8         |
| CBNA     | 1.4         | 4.3         |
| THCVA    | 1.6         | 4.8         |
| CBDA     | 1.4         | 4.1         |
| CBDVA    | 1.8         | 5.4         |
| CBGA     | 1.7         | 5.0         |

For all our calibration curves, we injected each concentration 3 times, with the exception of 5 µg/mL, which we injected 7 times for accurate limits estimations (Fig. S2). The CE method demonstrated the limit of detection between 1.2-1.8 µg/mL, the limit of quantitation between 3.7 – 5.4 µg/mL and with detection range reaching up to 50 µg/mL (Table 1). Our standard mix showed decent precision at all concentrations except 10 µg/mL, where it had around 30% variation (Fig. 4). All 14 compounds showed highly similar CV values, with more variation between concentrations rather than between compounds. In the upper ranges, we observed some loss of precision, likely due to the higher ionic strength of the mixture injected. In complex sample mixtures, however, other compounds may be eluting simultaneously with the compounds of interest, interfering with quantification. We demonstrated that our CE method also provided reliable means of detection and quantitation of cannabinoids in real samples as opposed to a standard mix (Fig. 3). Our extracted sample was analyzed using NACE. We quantified all detected cannabinoids in the sample and reported our findings in Figure 5 in quantities of each compound per gram of dry flower.

While the standard mix shows detection of all 14 compounds, in our samples, we found no traces of THCV, CBDV, THCVA and CBDVA. We hypothesize that there may be two underlying reasons for this. First, our samples may be completely devoid of these 4 compounds. In this case, different strains with the presence of these compounds should be analyzed for their detection. The second reason, which we think to be the case, is the very low abundance of these compounds in the samples. In this case, more sensitive
detection methods should be employed. While it is possible to use a wider capillary to increase the length of the UV detection path, more sensitive detectors such as mass spectrometers should be employed to detect and quantify these 4 compounds in the samples.

**Conclusion**

Our method extends to the previous work done in this area. A study dating back to 2002 performed similar NACE separation of cannabinoids extracted from hair\textsuperscript{14}. The method described separated four cannabinoids (THC, CBD, CBN, THCA) using a very similar running background electrolyte with sodium hydroxide as an electrolyte, however, in a fully non-aqueous environment, and with electrochemical detection in contrast to UV absorption. Another study with a similar method separated only THC and CBD in oral fluids; however, they employed LED-induced fluorescence for detection, with 280 nm excitation and 307 nm emission \textsuperscript{15}. A different approach was picked in a study from 1998, where the stationary phase was involved in capillary electrochromatography to facilitate the separation of structurally similar cannabinoids\textsuperscript{16}. 7 cannabinoids (6 decarboxylated and one acidic) were separated on a capillary packed with 3 µm C18 beads. The stationary phase helps separation due to the presence of the same charge on all cannabinoids in addition to very similar polarity. Another approach with the exploitation of the hydrophobic nature of cannabinoids was undertaken in a study from 2012, where micellar electrokinetic chromatography with SDS was used to separate 10 cannabinoids\textsuperscript{17}. The study demonstrated separation of 10 cannabinoids in one run, which was not achieved prior to that.

Crude flower extracts sometimes yield very low amounts of certain cannabinoids, in contrast to large amounts of THC and sometimes CBD, making low concentrated compounds difficult to analyze with a UV detector. A study demonstrated a potential solution to that by introducing stacking in the presence of SDS to suppress electroosmotic flow\textsuperscript{18}. They reported over a 2000-fold increase in sensitivity, overcoming one of the greatest problems of CE, which is low injection volumes.

We propose a fast and cost-efficient method of separation and quantitation of 14 cannabinoids both in refined products and crude extracts. Capillary electrophoresis is a powerful tool in analytical chemistry; however, only a handful of studies employing this technique exist, with fewer cannabinoids detected and separated than the current study. This study covers 7 major classes of cannabinoids and their acidic forms, which has only been previously performed on a costly UHPLC system. Thus, we propose CE as an alternative and demonstrate its feasibility.

**Declarations**

**Funding**

This work was funded by an NSERC Collaborative Research and Development grant (grant number CRDPJ/ 491434-2016). E.Z. was supported by Queen Elizabeth II Graduate Scholarship in Science & Technology and funded by the Government of Ontario (Canada) and Lumex Instruments Canada Inc.
Conflict of Interest

One of the authors A.E. is an employee of Lumex Instruments Canada. The rest of the authors declares no conflict of interest.

Compliance with ethical standards. This article does not contain any studies with human participants or animals performed by any of the authors.

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