Quality control of cannabis inflorescence and oil products: Response factors for the cost-efficient determination of ten cannabinoids by HPLC

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ARTICLE INFO

ABSTRACT

The quality control of medicinal cannabis should include quantification of as many cannabinoids as practicable in a routine analytical lab, to accurately reflect the quality of the product. However, the cost and availability of some cannabinoid standards is an impediment to their routine use. This work seeks to overcome this obstacle by analysing samples using relative retention times (*RRT*) and relative response factors (*RRF*), relative to CBD and CBDA reference standards which are readily available. A high-performance liquid chromatography-photodiode array method was developed to quantify ten cannabinoids (Δ^9-THC, Δ^8-THC, THCA-A, CBN, CBD, CDBA, CBC, CBDV, CBG, and CBGA) in dried cannabis inflorescence and cannabis oil. This method was validated according to ICH guidelines. The proposed method has detection limits ranging from 20 to 78 μg/g, which provided sufficient sensitivity for the panel of cannabinoids. Non-cannabinoid surrogate matrices were used for spike recovery studies to determine method accuracy – analyte recoveries for the inflorescence and oil ranged from 90.1 to 109.3% (inflorescence mean, 100.9%; oil mean, 99.6%). The *RRT* and *RRF* values determined independently by three analysts were comparable, indicating the method is robust. The validity of analysis using *RRT* and *RRF* was further confirmed by testing six inflorescence samples, as it was found that concentrations above the order of magnitude of the LoQ agreed satisfactorily (range, 95.0 to 111.9%; mean, 100.0%) with the concentrations obtained through the conventional approach of multipoint calibration using pure standards. The proposed method is therefore suitable for the rapid and simple determination of a panel of ten cannabinoids without having to repeatedly purchase every expensive pure standard. Accordingly, analysts in the medicinal cannabis field may explore the use of RRF and RRT for their methods and instruments.

Introduction

Despite an extensive history of use as a medicinal plant spanning ancient cultures [1–3], cannabis use is contentious in many jurisdictions as it has been considered a social drug of abuse since the mid-1930s [4, 5]. Over the last two decades, meaningful legal, sociocultural and economic change has led to the establishment of medicinal cannabis research programs in several countries, which have validated the therapeutic use of cannabis for indications including: chronic neuropathic pain, certain intractable epilepsies, the vomiting and spasticity of multiple sclerosis, and chemotherapy-induced nausea [6]. Further to this, the use of medicinal cannabis has expanded into paediatric and vulnerable patient groups [7–9] and regulated markets for recreational use have developed in some jurisdictions. Accordingly, quality control across the supply chain is increasingly important to ensure that cannabis products are safe and have well-defined chemical and therapeutic profiles.

Critically, the complex relationship between chemical profiles and

therapeutic activity requires further exploration. Presently, the activities of the three most abundant neutral cannabinoids – Δ^9-THC, CBD, and CBG – have been studied closely, exhibiting properties including: analgesic, anticonvulsant, and anti-inflammatory [6,10]. However, the full potential of medicinal cannabis may not be realised without leveraging the full diversity of cannabinoids. Over 140 cannabinoids have been identified, many of which have their own inherent pharmacological properties [11,12]. This includes the acidic cannabinoids which have significant anticonvulsant activities, contrary to the historical perspective that they were inert precursors which only acquired activity after decarboxylating into the neutral cannabinoids [13]. Furthermore, the complexity of cannabis increases geometrically under the 'entourage effect', which postulates that cannabinoids interact to modulate their therapeutic effects [14,15].

An experimental basis for the entourage effect is provided by murine studies, which have demonstrated that binary combinations with acidic cannabinoids increase bioavailability, potency and efficacy of neutral cannabinoids in epilepsy models [16–18]. Even authors exclusively preoccupied with neutral cannabinoids have demonstrated synergistic binary combinations [19–21]. Clinical evidence is also mounting, with a recent meta-analysis on observational studies of epileptic patients concluding that crude cannabis extracts yielded a greater reduction in seizure frequency and had fewer side-effects than equivalent doses of purified CBD [22]. However, as most extracts were only characterised to the extent of standardising the CBD dose, information about other cannabinoids was absent or based on inference. Consequently, the authors' attribution of the differences between the extracts and purified CBD to the entourage effect was speculative. It was not possible to evaluate if the effects of the other cannabinoids added together, comparable to merely increasing the dose of CBD, or if they magnified the effect to surpass what CBD could achieve alone. Evidently, to progress beyond studies of binary combinations or poorly characterised extracts, routine analyses capable of quantifying panels of cannabinoids could help to better inform the design and interpretation of future studies that investigate the entourage effect. A clinical understanding of this effect might subsequently inform the extent to which cannabinoids are screened during cannabis product quality control.

Several published methods are available for the separation and quantification of cannabinoids, with a variety of limitations which constrain their routine use. For the analysis of neutral cannabinoids, GC is simple, sensitive, and provides acceptable resolution [23]. However, GC is not immediately suitable for acidic cannabinoids, as they are poorly volatilised and rapidly undergo thermal decarboxylation into neutral cannabinoids [24]. Fortunately, this limitation can be surmounted by trimethylsilyl derivatisation of the labile acid group [24, 25]. Alternatively, some analysts have adopted LC for the separation of cannabinoids in medicinal cannabis. Following separation by LC, detection can be achieved by MS or by PDA. The MS detector enables the peak identity confirmation from their fragmentation patterns and relative ratios [26], and is sufficiently specific to recognise coeluting impurities in complex matrices [24]. However, the required technical expertise, operation, and maintenance costs prohibit the use of MS for the routine analysis of cannabinoids. The UV-Vis PDA detectors are much cheaper, require less operator expertise, and are widely available. Since cannabinoids contain UV chromophores [27], they are amenable to PDA detection. Moreover, the UV spectra may assist with compound identity confirmation and the measurement of peak purity, which aids in quantification.

Whichever detector is used, the elevated cost and limited availability of certified analytical reference standards for some cannabinoids remain impediments to their analysis. The cost can exceed $200 AUD per mg, and newly identified pharmacological leads in cannabis are possibly more expensive with significantly longer shipping times. To surmount this, some analysts have performed stereoselective microscales syntheses to obtain cannabinoids in a timelier manner [28,29], but this is beyond the remit of a typical QC lab. If the analysis of such cannabinoids

is to become routine, the cost for their quantification must be mitigated.

To this end, this study aimed to develop and validate a HPLC-PDA method for the determination of ten cannabinoids in medicinal cannabis inflorescence and oil and to explore the feasibility of using *RRT* for peak identification and *RRF* for their quantification. By this approach, an initial once-off purchase of all the standards was required to establish the *RRT* and *RRF* between the cannabinoids and the reference compounds: CBD as a reference for neutral cannabinoids, and CBDA as a reference for acidic cannabinoids; chosen as they are cheaper and available in many jurisdictions. Subsequently, the method may be routinely used in QC laboratories for the quantification of a panel of ten cannabinoids, requiring only sparing amounts of the reference compounds.

Materials and methods

Reagents and standards

LC grade acetonitrile and methanol (purity >99.9%) were sourced from Honeywell (North Ryde, NSW, AU). Formic acid (>98%), ammonium formate (>98%), and dichloromethane (>99.8%) were sourced from Sigma-Aldrich (Castle Hill, NSW, AU). Ultra-pure water (resistivity >18.0 MΩ.cm) was obtained from a Milli-Q Direct 9 system (Sigma-Aldrich). A mixed 250 µg/mL standard of neutral (Δ^9-THC, Δ^8-THC, CBN, CBD, CBC, CBDV, CBG) and acidic (THCA-A, CBDA, CBGA) cannabinoids in acetonitrile was produced by Cayman Chemical Company (Ann arbor, MI, USA) and distributed by Sapphire Bioscience (Redfern, NSW, AU). Primary standards of single cannabinoids were also from the Cayman Chemical Company: CBN, THCA-A and CBDA were obtained as 1000 µg/mL acetonitrile solutions; CBDV, THCV and CBC were obtained as 1000 µg/mL methanol solutions; and CBD, Δ^9-THC, Δ^8-THC, CBGA and CBG were sourced as anhydrous solids. All standards were stored at −20 °C and allowed to come to room temperature in a desiccator before use.

An extraction solvent of acetonitrile:methanol (4:1 *v/v*) was prepared fresh daily, as required for the dilution of standards and the extraction of samples. For the conventional multipoint calibration curve, a 50 µg/mL dilution was prepared directly from the original 250 µg/mL mixed standard, and serial dilutions with a factor of two covered the concentration range of 1.6 to 25 µg/mL. For the quantification by *RRF*, a 25 µg/mL working standard of CBD and CBDA was prepared from their primary standards. This working standard concentration was chosen to be in the same order of magnitude as the sample concentration obtained when a 10 mg/g CBD oil is extracted according to the 'sample preparation' section. All dilutions were prepared in the extraction solvent.

Sample preparation

Six air-dried and coarsely ground cannabis inflorescences, denoted as samples A-F, were provided by Little Green Pharma (Kings Park, WA, Australia). To ensure representative sampling of the biomass, the inflorescences were mechanically processed to pass through a 24-mesh sieve (710 µm openings). Residual water content (mean 5.5% *w/w*; CV <3%) was verified by drying triplicate sub-samples (~ 800 mg) of the air-dried inflorescence over phosphorous pentoxide in a vacuum desiccator; drying was to a constant mass (<2 mg difference between days 7 and 8). For the analysis of cannabinoids, air-dried samples (400 mg) prepared in extraction solvent (25 mL) were sonicated in a Powersonic 420 ultrasonic bath (Thermoline Scientific; Sydney, NSW, Australia) on low power at room temperature for 30 min. Extracts were passed through 0.45 µm Nylon syringe filters into 2 mL HPLC autosampler vials. Where necessary, the extraction solvent was used to prepare 1:9 *v/v* dilutions of these extracts, so that cannabinoids with high concentrations (15–150 mg/g) could be analysed in the same batch as those at low concentrations (0.1–15 mg/g). Both the undiluted and the diluted extract solutions were analysed.

Medicinal cannabis oil (Cannimed; Saskatoon, Saskatchewan, Canada) was supplied by Health House International (Perth, WA, AU). The claimed composition was 10 mg/g total THC and 10 mg/g total CBD. These totals are corrected for the mass loss due to decarboxylation to report the concentrations in terms of neutral cannabinoid equivalents: CBD_{Total} (mg/g) = CBD (mg/g) + 0.877 × CBDA (mg/g); and THC_{Total}(mg/g) = Δ^9-THC (mg/g) + 0.877 × THCA-A (mg/g). Oil samples were prepared for analysis by dissolving 50 μg in dichloromethane (1 mL), which made it miscible with the extraction solvent (total, 25 mL), and was subsequently sonicated on low power for 10 min. Extracts were passed through 0.45 μm Nylon syringe filters before analysis.

Instrumentation and analytical method

A Shimadzu Prominence-i LC-2030C 3D Plus HPLC-PDA (Rydalmere, NSW, AU), comprising of a low-pressure quaternary solvent system, an auto sampler and a PDA detector, was used. Shimadzu LabSolutions (v5.93A) was used for instrument control, data acquisition and processing. A Phenomenex Luna C18(2) (150 × 4.6 mm × 5 μm) analytical column with a Security C18 (20 × 4.6 mm × 5 μm) guard column (Lane Cove West, NSW, AU) was employed to achieve reversed phase separation. The column was maintained at 40 °C, with a mobile phase flow rate of 2.5 mL/min. The injection volume was 10 μL, and all standards and samples were injected in duplicate. Gradient elution employing mobile phase A (Milli-Q water buffered with 20 mM ammonium formate and 0.1% formic acid), mobile phase B (acetonitrile) and mobile phase C (methanol buffered with 10 mM ammonium formate and 0.05% formic acid) was used. The gradient program is summarised in Table 1, which includes 2 min of column rinse with the organic phases and 2 min of re-equilibration at the starting condition. During each run, the PDA was set to acquire data from 190 to 800 nm and the chromatograms was visualised at 232 nm.

System suitability

System suitability criteria were established to routinely ensure that the chromatographic system functioned as specified for each batch. To this end, a minimum of six injections of the CBD and CBDA 25 μg/mL working standard was made throughout each batch. To pass, the CV of the standard retention times and peak areas of the six injections must be <2%.

Analysis by relative retention times and relative response factors

Standard values for the *RRT* and *RRF* were determined from three independent analysts who each performed six replicate injections of the mixed cannabinoid at the working standard concentration. The reference standards were CBDA for acidic cannabinoids and CBD for neutral cannabinoids. Adjusted retention time (t_R') is the difference between the analyte retention time (t_R) and the void time (t_{void}); t_R'=t_R-t_{void}. The *RRT* of a generic cannabinoid denoted as 'a', relative to the reference, is the ratio of their adjusted retention times; t_R'(a)/t_R'(reference). Likewise,

Table 1
Gradient program for the HPLC method.[a]

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Mobile phase C (%)
0.00	50	50	0
17.00	47	53	0
30.00	25	30	45
30.01	10	45	45
32.00	10	45	45
32.01	50	50	0
34.00	50	50	0

[a] Mobile phase A (Milli-Q water buffered with 20 mM ammonium formate and 0.1% formic acid), mobile phase B (acetonitrile) and mobile phase C (methanol buffered with 10 mM ammonium formate and 0.05% formic acid).

the response factor (*RF*) is the cannabinoid peak area (*A*) divided by its concentration (*C*); $RF = A/C$. The *RRF* of a generic cannabinoid denoted as 'a', relative to the reference, is the ratio of their response factors; RF(a)/RF(reference).

Quantitative analyses using *RRT* and *RRF* values required that the CBD and CBDA working standards be tested in every batch of analysis. Using the reference retention times and the known *RRT* values, the expected retention time for cannabinoid 'a' is determined; t_R(a, expected) = [t_R(reference, standard) − t_0] × RRT(a) + t_0. UV-vis spectra can verify this peak identification. Subsequently, the unknown concentration of cannabinoid 'a' can be calculated; C(a) = [A(a, sample)/A(reference, standard)] × [1/RRF(a)] × C(reference, standard). This represents the concentration in the extract solution which, using the precise mass extracted in the known volumetric flask, is converted into the concentration of the original sample; reported as the mass of the cannabinoid (mg or μg) relative to the mass of the air-dried inflorescence or oil sample (/g).

Method validation

Analytical method validation was informed by the International Conference on Harmonisation [30]. For the dried inflorescence sample, validation was performed for the quantification of all ten cannabinoids. However, for the oil matrix, validation was only performed for the cannabinoids present in the preparation, namely Δ^9-THC, THCA-A, CBD, and CBDA.

Linearity of the detector response was evaluated from calibration curves, considering both the R^2 and the significance of the intercept. The detection and quantification limits were determined as LoD = 3.3 s / m and LoQ = 10 s / m, respectively; where s is the sample standard deviation of the lowest linear concentration (3.1 μg/mL), and m is the calibration slope. The instrument and method precision were determined as the *CV* from six replicate standard injections and sample preparations, respectively. Intermediate precision was evaluated from the pooled *CV* between three analysts who independently prepared the same samples six times on separate days. The accuracy of the method was determined by a recovery study, see the 'Accuracy protocol' section. The stability of the standard and sample extracts was tested for up to 24 h and 48 h, respectively. Finally, the quantitative results obtained using the multi-point calibration curve and *RRF* method were compared.

Accuracy protocol

Recoveries of the ten cannabinoids were tested using chamomile as a surrogate matrix for the cannabis inflorescence. For the cannabis oil, the recoveries of Δ^9-THC, THCA-A, CBD, and CBDA were tested using olive oil as a surrogate matrix. These surrogate matrices were extracted according to the 'Sample preparation' section to confirm that they did not give rise to peaks at the retention times of interest. Triplicate preparations of the surrogate matrices were spiked with the individual cannabinoid standards at levels relative to a representative sample for each matrix: Δ^9-THC, CBD and CBGA were spiked at 50%, 100% and 200% levels; THCA-A, CBDA and CBG were spiked at the 100% level due to limited supply; and all other cannabinoids were spiked at their *LoQ*.

Results and discussion

Method development

To optimise sample preparation, a variety of extraction solvents were tested with duplicate extractions. The solvents trailed were methanol, ethanol, acetonitrile, ethyl acetate, methanol:water (1:1 *v/v*), and acetonitrile:methanol (4:1 *v/v*). Cannabinoid peak areas were maximised by ethyl acetate and acetonitrile:methanol. However, due to markedly mismatching the initial mobile phase condition, ethyl acetate gave rise to significant band broadening. Thus, acetonitrile:methanol

(4:1 *v/v*) was selected as the extraction solvent, which is consistent with other extraction optimisation reports [25,31].

The *CV* contribution of the method preparation procedure to the total uncertainty was determined by performing six replicate extractions and analysis of a single cannabis inflorescence sample. Grinding the inflorescence to pass through $a < 710$ µm sieve before subsampling achieved a *CV* range of 1.2 to 3.6%. When subsampling without grinding, the *CV* unacceptably ranged from 7.6 to 23.6%, thus indicating the importance of preparing a homogeneous sample.

To optimise the chromatographic conditions, the method was iteratively developed. Baseline separation was achieved for eight of the ten cannabinoid standards (resolution > 2.0), however, the CBD and CBG standard peaks overlapped slightly (resolution > 1.5, in all batches), as shown in Fig. 1A. Likewise, acceptable separation of cannabinoids in the extracts of cannabis inflorescence and cannabis oil were demonstrated in Fig. 1B and C, respectively. Whilst most matrix components eluted before the cannabinoids, a compound in inflorescence samples was observed to elute between CBDA and CBGA. This peak was identified to be tetrahydrocannabivarin (THCV, t_R 13.70 min) by comparison with the UV spectrum and retention time obtained for the THCV standard. THCV was not included in the present method validation study as it was not part of the original selected set of analytes. When the analytes were sufficiently abundant in the sample, the UV spectra of their peaks were compared to that of the standard. As shown in Fig. 2, spectra superimposed closely, indicating good peak purity.

To optimise PDA detection, wavelengths corresponding to the λ_{max} of the different cannabinoids, specifically 210, 232, and 270 nm, were considered. Whilst 210 nm has been used in other studies [24,25,32], it produced a sloping baseline in the present study due to the use of methanol (UV cut-off 210 nm) rather than exclusively using acetonitrile (UV cut-off 190 nm) as the organic component of the mobile phase. Instead, it was found that visualising the chromatogram at 232 nm gave the best compromise between sensitivity and baseline noise. Some studies used 270 nm to improve sensitivity for the acidic cannabinoids [33], but this higher sensitivity is not required due to their relatively high abundance in the inflorescence samples. This high abundance was anticipated as acidic cannabinoids are the secondary metabolites synthesised in cannabis, whereas the neutral forms are produced by spontaneous decarboxylation [34].

Linearity and calibration range

Considering the seven mixed-standard dilutions prepared over the 1.6 to 250 µg/mL range, replicates at the lowest concentration for most cannabinoids deviated from their mean by >5%, indicating significant baseline noise at this level. Accordingly, calibration curves were

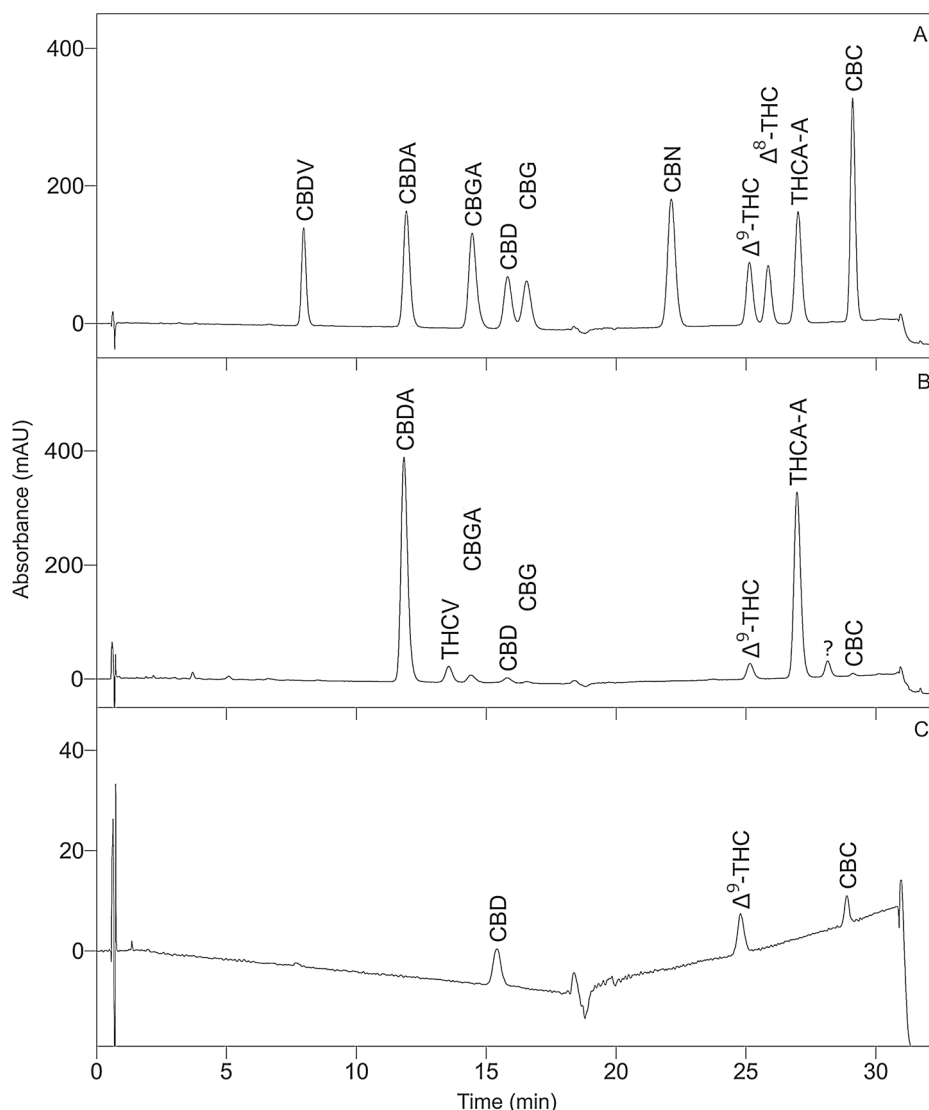**Fig. 1.** Representative chromatograms at 232 nm: A, mixed cannabinoid standard; B, cannabis inflorescence; C, cannabis oil.
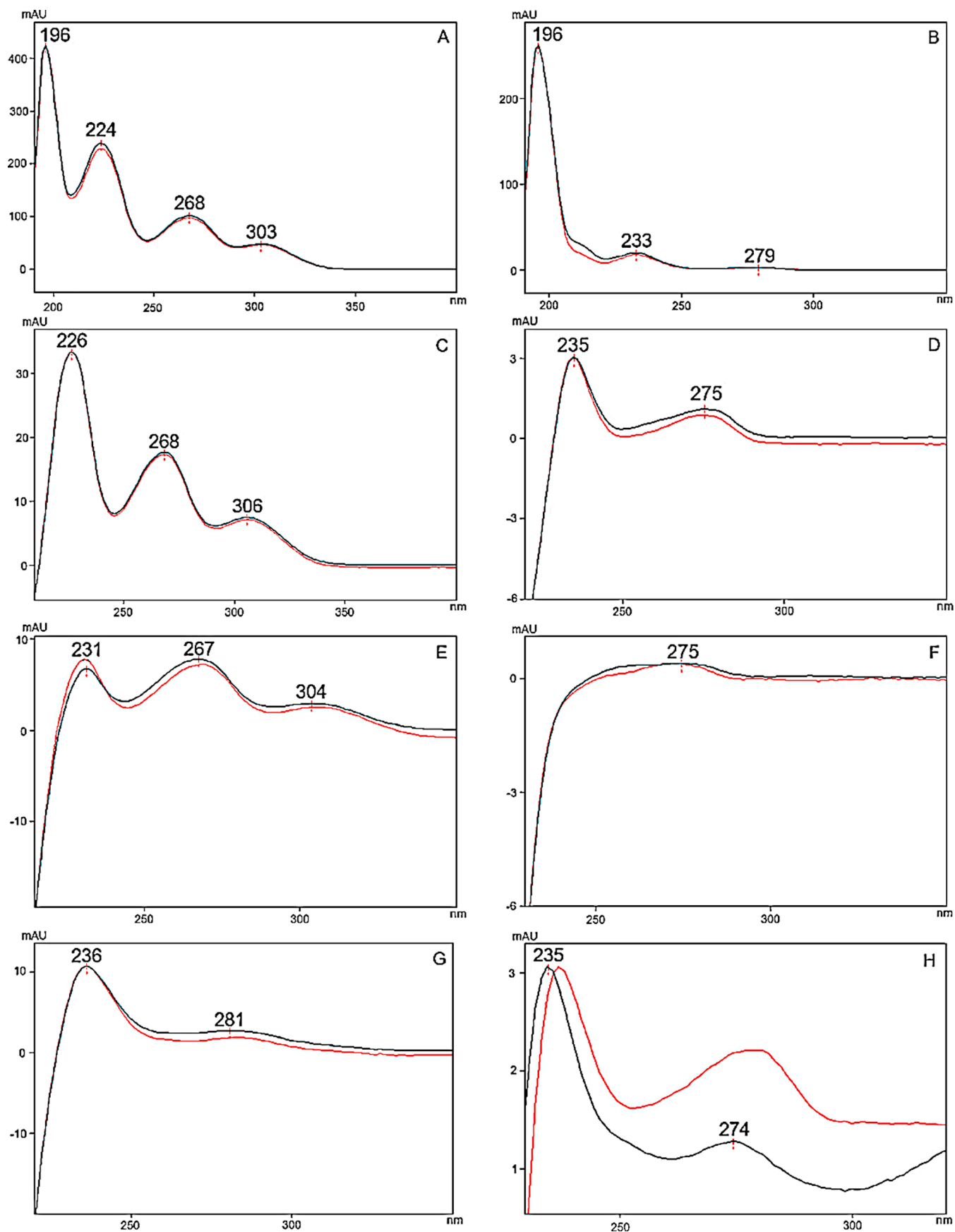
Fig. 2. Spectra of cannabinoid peaks recorded from the standards (black) and inflorescence sample (red): A, THCA-A; B, Δ^9-THC; C, CBDA; D, CBD; E, CBGA; F, CBG; G, CBC; H, Δ^9-THCV [a]. [a] Different abundances relative to an uneven baseline absorbence accounts for the imperfect overlap.

constructed using the six standards from 3.1 to 250 µg/mL. The corresponding linear equations and their R^2 are summarised in Table 2. Good linearity was obtained, with $R^2 > 0.9999$ for all the analytes. The magnitudes of the intercepts were compared to the integrations at the working standard concentration, and all were deemed insignificant as 3% of the working standard integration was greater than the absolute value of the intercept. Thus, the calibration equations were linear and passed sufficiently close to the origin for *RRF* values determined from the working standard concentration to be reliable.

Retention times compared to relative retention times

Retention times pooled from the three analysts are reported in Table 3. The *CV* in the retention times for each cannabinoid ranged from 0.18% to 0.56%, demonstrating an excellent inter-batch repeatability. For the cannabinoids detected in the available inflorescence samples, the retention times observed for the sample peaks deviated by <1% from the standard retention times.

To formalise the peak identification, and to demonstrate further gains in the inter-batch repeatability, the *RRT* were also pooled from the three analysts and were appended to Table 3. *RRT* should correct for inter-batch variabilities in retention times, provided that the variation in conditions proportionally affected all of the closely related analytes being studied [27]. As anticipated, the pooled *RRT* values for each cannabinoid had *CV* which ranged from 0.04 to 0.34%. This represents a modest gain in repeatability, which should be maintained even if the retention times start to shift by >1%. Critically, it was also shown that the range of *RRT* values for each cannabinoid did not overlap. This means that analysts reported comparable values for the *RRT*, and that these values were unique for each cannabinoid. Thus, cannabinoid peaks in samples may be identified from their *RRT* values relative to the retention time of the CBD or CBDA from the working standard tested in the same batch of analysis.

Relative response factors

From the same replicate working standard injections performed by the three analysts, the pooled *RRF* were determined and appended to Table 3. The *CV* ranged from 1.29 to 2.67%, acceptably within the 3% criteria. Agreement was demonstrated between the *RRF* values calculated by each analyst. Thus, with an acceptably small error, cannabinoids can be quantified using the *RRF* values relative to response factors for CBD or CBDA from the working standard tested in the same batch of analysis. This use of *RRF* for the quantification of selected cannabinoids in cannabis products is a novel contribution of the present study, which eliminates the need for the expensive cannabinoid standards during routine analysis.

Table 2
Linearity of calibration curves including the calibration range, calibration equation, and coefficient of determination.

Compound	Calibration range (µg/mL)	Equation [a]	R^2
Δ^9-THC	3.0 - 240	$y = 6294\,x + 2692$	0.999 93
Δ^8-THC	3.1 - 250	$y = 5458\,x + 3194$	0.999 98
THCA-A	3.0 - 240	$y = 11{,}712\,x + 4031$	1.000 00
CBD	3.0 - 240	$y = 6687\,x + 1062$	0.999 98
CBN	2.8 - 220	$y = 17{,}792\,x + 9181$	0.999 99
CBDV	3.1 - 240	$y = 7225\,x + 4889$	0.999 98
CBC	3.1 - 250	$y = 17{,}498\,x + 5227$	0.999 99
CBDA	2.8 - 230	$y = 13{,}059\,x + 4836$	0.999 98
CBG	2.9 - 230	$y = 6452\,x - 881$	0.999 96
CBGA	3.0 - 240	$y = 12{,}006\,x + 2247$	0.999 98

[a] Where: y represents the peak integration (mV.min), and x is the concentration (µg/mL).

Table 3
Retention time (tR), relative retention time (RRT), and relative response factors (RRF) for the ten cannabinoids, calculated relative to CBD (for neutral cannabinoids) or CBDA (for acidic cannabinoids); determined as the mean ± standard deviation (CV%) from 18 runs of the mixed standard (6 extractions and analysis × 3 analysts).

Compound	t_R (min)	*RRT* (unitless)	*RRF* (unitless)
Δ^9-THC	24.88 ± 0.06 (0.24)	1.631 ± 0.004 (0.26)	0.944 ± 0.025 (2.67)
Δ^8-THC	25.60 ± 0.05 (0.21)	1.679 ± 0.005 (0.29)	0.821 ± 0.020 (2.43)
THCA-A [a]	26.72 ± 0.07 (0.26)	2.370 ± 0.006 (0.24)	0.931 ± 0.014 (1.46)
CBD	15.53 ± 0.07 (0.46)	1.000	1.000
CBN	21.84 ± 0.07 (0.33)	1.4258 ± 0.0024 (0.17)	2.23 ± 0.04 (1.91)
CBDV	7.82 ± 0.04 (0.56)	0.4805 ± 0.0007 (0.15)	1.037 ± 0.020 (1.93)
CBC	28.91 ± 0.05 (0.18)	1.903 ± 0.006 (0.34)	2.39 ± 0.03 (1.29)
CBDA [a]	11.68 ± 0.03 (0.24)	1.000	1.000
CBG	16.27 ± 0.08 (0.49)	1.0501 ± 0.0004 (0.04)	0.991 ± 0.025 (2.57)
CBGA [a]	14.20 ± 0.04 (0.29)	1.2298 ± 0.0011 (0.09)	1.003 ± 0.021 (2.10)

[a] Acidic cannabinoids.

Detection and quantification limits

Detection and quantification limits for the cannabinoids are presented in Table 4. The *LoD* ranged from 20 to 78 µg/g and the *LoQ* ranged from 60 to 238 µg/g, relative to the inflorescence sample preparation. These limits are sufficiently low to enable the quantification of the studied cannabinoids in cannabis biomass and, observing that even relatively small amounts in crude biomass can be extracted and concentrated to therapeutically relevant concentrations in final products, these limits are suitable for quality control throughout the supply chain. However, with the quantification limits in the determined order of magnitude, it is unlikely that this method could be adapted for the analysis of the recently identified trace cannabinoids with heptyl side-chains (denoted with the suffix *-phoryl*) [28]. This includes THCP, which, by a published MS method, was identified in the inflorescences of THC dominant chemovars at concentrations routinely less than 140 µg/g and was undetected in CBD dominant chemovars [35].

Instrument, method and intermediate precision

Instrument and method precision results are collated in Table 5. Instrument precision ranged from 0.10 to 2.00%, which was acceptedly below 2%. Method precision ranged from 1.15 to 3.58% for the inflorescence and from 1.27 to 1.32% for the oil, acceptably within 5% for both sample matrices. Concentrations reported by the independent

Table 4
Cannabinoid limit of detection and quantification, relative to the inflorescence sample preparation.

Compound	*LoD* (µg/g)	*LoQ* (µg/g)
Δ^9-THC	78	238
Δ^8-THC	48	144
THCA-A	32	96
CBD	35	107
CBN	55	167
CBDV	39	118
CBC	20	60
CBDA	41	123
CBG	57	172
CBGA	31	94

Table 5

Instrumental precision for the determination of cannabinoids in the standard mixture, and method precision for the inflorescence and oil preparations.

Compound	Instrument precision	Method precision	
	CV (%), standard	CV (%), inflorescence	CV (%), oil
Δ^9-THC	1.21	1.23	1.32
Δ^8-THC	1.14	<LoD	–
THCA-A	0.68	2.09	<LoD
CBD	1.49	1.15	1.27
CBN	0.38	3.58	–
CBDV	0.44	<LoD	–
CBC	0.32	1.41	–
CBDA	0.10	1.24	<LoD
CBG	2.00	1.61	–
CBGA	0.68	1.76	–

'–' = Not analysed for the oil sample matrix.

analysts for the samples tested on separate days and the intermediate precision were reported in Table 6. The results of the analysts agreed, with the intermediate precision ranging from 0.67 to 4.58% for the inflorescence and from 1.28 to 1.60% for the oil. Though two of the analysts had no prior exposure to the method, an acceptable precision of <5% was achieved for both sample matrices, thus demonstrating the robustness of the method.

Accuracy and recovery

Accuracy of the method was evaluated from the recoveries of analytes spiked onto surrogate matrices, as presented in Table 7. For the cannabis inflorescence and oil, the spike recoveries from the surrogate matrices ranged from 90.1 to 109.3% (mean 100.9%) and from 95.4 to 103.1% (mean 99.6%), respectively. Most recoveries were within 5% of the nominal concentration and the only two recoveries which were outside of this criterion had been spiked at the quantification limit, so their recoveries within 10% were acceptable. The precision of the recoveries was also acceptable, except at the *LoQ* of Δ^8-THC and CBDV which were only precise to 12%. Therefore, the method for the quantification of cannabinoids has acceptable accuracy.

In this study, chamomile was selected as surrogate matrix for cannabis inflorescence as it was floral, available at little cost and, with the exception of the cannabinoids, shared phytochemical classes such as fragrant terpenes and flavonoids [10,36]. Other published articles have used *Urtica dioica* (stinging nettle) [37,38] or *Humulus lupulus* (beer hops) [24], with justifications based on tracing their phylogenies relative to *Cannabis sativa*. Whilst sharing botanical orders or even families does not necessarily provide better matrix matching, it may be a reasonable approximation. Likewise, for cannabis oil, the choice of olive oil as a surrogate matrix had precedent from previous publications [39].

Indeed, some cannabis oil products contain refined resins or even crude inflorescence extracted into an olive oil base [40], making its choice as the surrogate matric reasonable for such products. The appearance of publications employing surrogate matrices is being increasingly accepted as a cost-reduction strategy during method development, which is a clear advantage over articles which did not conduct recovery studies at all [41–43]. Analysts in some jurisdictions may also find it pertinent to consider the use of surrogate matrices if licencing requirements preclude the use of the amount of cannabis material which would be required for the complete spike-recovery protocol on the true matrices.

Standard and sample stability

Response factors were determined from six replicate injections of a freshly diluted CBD and CBDA working standard. The standards were stored for 24 h in a resealing vial within the autosampler at 10 °C. Subsequently, another six injections were made, and the response factors from the original determination were used to calculate that CBD and CBDA were 101.5% and 98.1% of their original concentrations, respectively. Observing that the changes in concentration were less than the 2% criteria used to validate instrument precision, the working standard was deemed stable. The stability of cannabinoids in the cannabis oil extract was evaluated at 48 h. At this timepoint, Δ^9-THC and CBD were 98.4% and 97.3% of their original concentrations, respectively. Accordingly, compared to the 5% criteria utilised to validate the method precision, the oil sample extracts were deemed stable. This extended stability relieves the pressure on laboratories to analyse the samples quickly after extraction.

Comparing quantification methods

Cannabinoid concentrations in six different inflorescence samples were determined by conventional multipoint calibrations and the *RRF* method, as reported in Fig. 3. For cannabinoids above the order of magnitude of the *LoQ*, concentrations determined by the two methods agreed satisfactorily (range, 95.0 to 111.9%; mean, 100.0%). The only cannabinoid above the *LoQ* which differed between quantifications by more than 5% was CBC but, relative to its low concentrations, the absolute differences was always acceptably less than 80 μg/g. The good agreement between the results obtained using the two different quantification methods applied to real samples demonstrates that the use of *RRF* for quantification is a valid alternative with its concomitant cost saving.

Considering the cannabinoid profiles of the inflorescence samples, the high ratios of acidic to neutral cannabinoids were indicative of good drying and storage conditions. Furthermore, samples A and B were classified as having moderately high total THC (~100 mg/g) and low

Table 6

Intermediate precision of analytical method.

Sample	Compound	Analyte concentration (mg/g)				Inter-analyst CV (%)
		Analyst 1	Analyst 2	Analyst 3	Mean	
Inflorescence	Δ^9-THC	24.6	25.3	24.2	24.7	2.34
	Δ^8-THC	<LoD	<LoD	<LoD	<LoD	–
	THCA-A	29.8	31.9	29.8	30.5	3.97
	CBD	16.9	17.2	17.0	17.0	0.67
	CBN	0.514	0.543	0.563	0.540	4.58
	CBDV	<LoD	<LoD	<LoD	<LoD	–
	CBC	1.45	1.46	1.42	1.44	1.23
	CBDA	79.8	83.7	79.3	80.9	3.01
	CBG	1.16	1.20	1.18	1.18	1.76
	CBGA	2.39	2.47	2.42	2.42	1.70
Oil	Δ^9-THC	10.6	10.5	10.7	10.6	1.28
	THCA-A	<LoD	<LoD	<LoD	<LoD	–
	CBD	11.1	10.8	11.2	11.0	1.60
	CBDA	<LoD	<LoD	<LoD	<LoD	–

Table 7

Recoveries of cannabinoids spiked into surrogate matrices (n = 3 replicate preparation at each level × 2 replicate injections).

Sample	Compound	Spike level (%)	Spiked concentration (mg/g)	Recovered concentration (mg/g)	Recovery (%)	CV (%)
Inflorescence control	Δ^9-THC	50	0.500	0.514	102.7	1.0
(*camomile tea*)		100	1.000	1.051	105.1	2.4
		200	2.000	1.999	100.0	1.4
	Δ^8-THC	*LoQ*	0.167	0.151	90.1	12.7
	THCA-A	100	5.500	5.328	96.9	1.8
	CBD	100	0.402	0.411	102.4	4.2
		200	0.803	0.765	95.3	5.4
	CBN	*LoQ*	0.188	0.193	102.9	6.1
	CBDV	*LoQ*	0.187	0.193	103.1	12.4
	CBC	*LoQ*	0.400	0.403	100.8	1.5
	CBDA	100	5.500	5.633	102.4	2.5
	CBG	100	0.900	0.858	95.4	4.1
	CBGA	100	0.088	0.096	109.3	6.6
		200	0.176	0.173	97.9	7.0
Oil control	Δ^9-THC	50	0.625	0.614	98.2	2.0
(*olive oil*)		100	1.250	1.221	97.7	1.9
		200	2.500	2.384	95.4	0.6
	THCA-A	*LoQ*	0.100	0.101	100.7	2.1
	CBD	50	0.603	0.622	103.1	4.5
		100	1.206	1.224	101.5	1.8
		200	2.411	2.368	98.2	3.1
	CBDA	*LoQ*	0.100	0.102	101.9	5.0
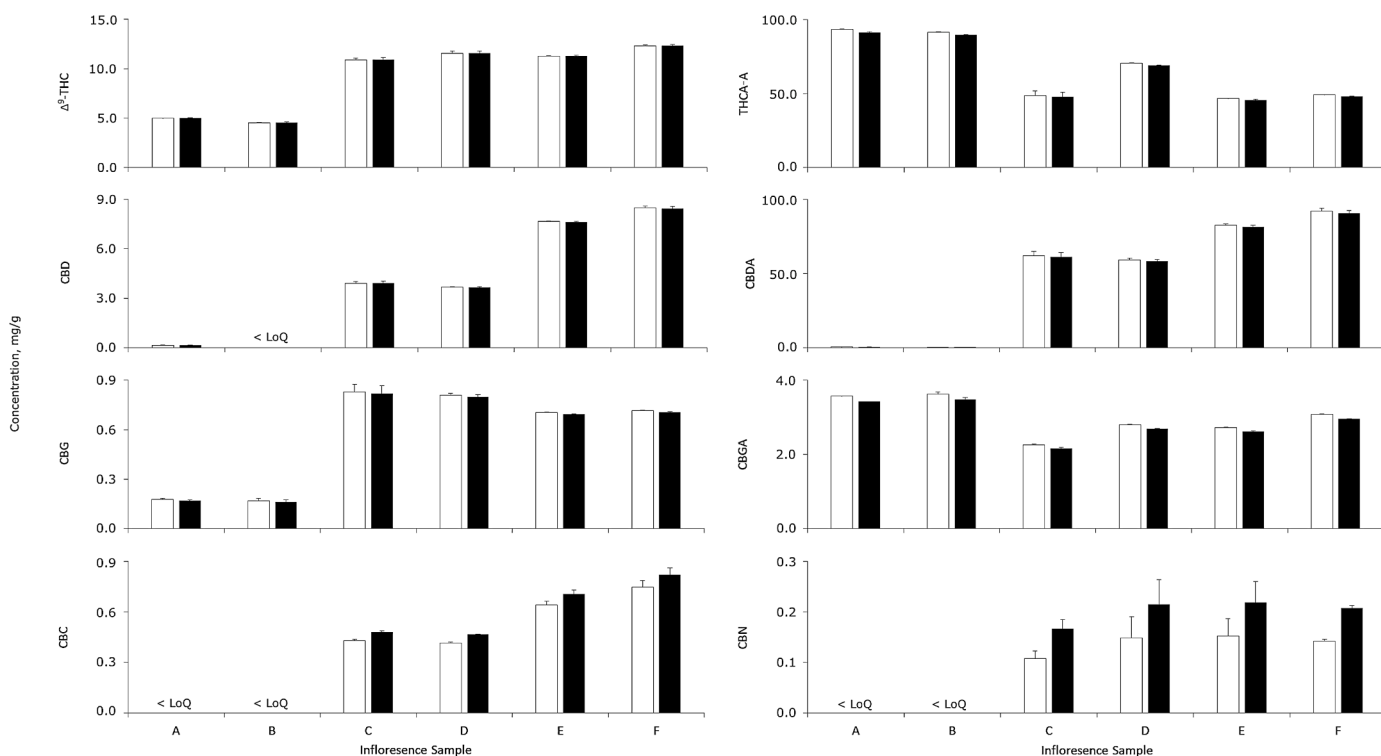
Fig. 3. Cannabinoid concentrations for inflorescence samples A to F, as reported by the multipoint calibration (white) against the RRF calibration (black); mean ± sample standard deviation (n = 2 × 2, sample preparations × injections). No sample contained detectable amounts of Δ^8-THC or CBDV.

total CBD (<1 mg/g), whilst samples C to F had moderate amounts of both (~60 to 90 mg/g). Beyond these observed concentrations, the proposed method is appropriate to analyse most samples with even greater levels of cannabinoids, as very few inflorescences exceed 200 mg/g total THC [34]. Other cannabinoids such as CBC and CBN were also quantifiable, but Δ^8-THC was not detected in any sample. However, other authors have reportedly identified inflorescence samples with Δ^8-THC concentrations up to 4.9 mg/g [44], well above the *LoQ* of the present method. Accordingly, the present method has sufficient dynamic range to quantify cannabinoids at their various native concentrations.

Conclusion

A simple HPLC-PDA method has been developed for the analysis of Δ^9-THC, Δ^8-THC, THCA-A, CBD, CBDA, CBG, CBGA, CBN, CBDV, and CBC in the inflorescence and oil of medicinal cannabis. This method was validated according to ICH guidelines. During the validation process, surrogate matrices were shown to be viable substitutions when costs prohibit the required replicates for spiking onto the true matrices. Considering the *RRT* and *RRF* values, they were consistent between batches independently performed by three analysts. Moreover, the validity of using *RRT* and *RRF* was demonstrated as the quantifications of

cannabinoids in six inflorescence samples agreed with the conventional approach of multipoint calibration. Collectively, analysts in the medicinal cannabis field are encouraged to Before use, analysts need to validate the *RRF* quantification for their existing methods and for any new methods that they design; potentially including methods capable of analysing broader panels than the present ten cannabinoids. In doing so, cost barriers for the analysis of panels of cannabinoids can be overcome, such that a diversity of cannabinoids can be analysed as a part of routine quality control, with results that reflect the therapeutic efficacy for the consumer.

Funding

Acknowledgments

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