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RESEARCH PAPER

A novel approach to the production of Δ^9-THC and CBN certified reference materials

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Abstract

Reliable and traceable quantification of cannabinoids is essential for forensic, regulatory, and quality control applications involving cannabis-derived products. However, the limited availability of certified reference materials (CRMs), particularly for minor cannabinoids such as cannabinol (CBN), remains a major analytical challenge. In this work, an integrated analytical and preparative strategy is presented for the production and characterization of Δ^9-tetrahydrocannabinol (Δ^9-THC) and CBN CRM candidates. Quantification of cannabinoids was performed by ^{1}H quantitative NMR using the PULCON method, providing traceable mass fraction values with well-defined uncertainties. To overcome the low natural abundance of CBN, a simple iodine-mediated oxidative conversion of Δ^9-THC to CBN was developed directly in cannabis extracts, enabling substantial enrichment of CBN and facilitating its subsequent isolation. The impact of this strategy was demonstrated by a more than one order of magnitude increase in isolated CBN yields compared to direct plant-based extraction. The feasibility of producing cannabinoid and plant-based cannabis CRMs was evaluated through homogeneity and transport stability studies conducted within a metrological framework. Overall, this work establishes a practical and metrologically sound framework to produce cannabinoid and plant-based cannabis reference materials, supporting reliable and comparable cannabinoid measurements suitable for forensic and regulatory applications.

Keywords Metrological traceability · Quantitative NMR · Cannabis analysis · Forensic drug analysis

Introduction

Cannabis-derived products are among the most widely used and seized drugs worldwide, creating persistent analytical and forensic challenges [1]. In legal and regulatory contexts, the reliability of cannabinoid identification and quantification is critical, as analytical results lacking metrological traceability may compromise judicial decisions. The use of certified reference materials (CRMs) is therefore essential to ensure measurement traceability, comparability of results, and compliance with international standards such as ISO/IEC 17025 [2–6].

Cannabis plants exhibit a complex chemical composition, in which Phyto cannabinoids are the most relevant markers for both identification and legal classification. Among them, Δ^9-tetrahydrocannabinol (Δ^9-THC) is the main psychoactive constituent and the primary target in forensic analysis, while cannabinol (CBN) is widely used as a degradation marker due to its formation by oxidative aromatization of Δ^9-THC during storage and exposure to environmental

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factors (Fig. 1) [7–9]. Because of its chemical stability and direct structural relationship with Δ^9-THC, CBN plays a key role in distinguishing aged or degraded cannabis materials [10–14].

In forensic practice, the distinction between illicit drug-type cannabis and legally permitted products, such as low-THC hemp or cannabidiol-rich materials, relies heavily on accurate and traceable cannabinoid measurements [15]. The lack of well-characterized reference materials, particularly for minor cannabinoids used as regulatory or degradation markers, increases uncertainty in reported results and may lead to legal disputes. These challenges underscore the need for analytical workflows that are supported by robust and traceable CRMs.

Despite its analytical relevance, CBN occurs naturally at low levels in cannabis plants, rendering its direct isolation inefficient and costly. Conventional synthetic routes to CBN typically rely on multistep procedures, limiting their applicability for the routine production of reference materials [16, 17]. As a result, the limited availability of CBN remains a challenge for the development of cannabinoid CRMs.

From an analytical perspective, nuclear magnetic resonance (NMR) spectroscopy has become a cornerstone technique in metrology due to its inherent traceability to the International System of Units (SI) and its ability to provide accurate quantification without the need for analyte-specific standards. In particular, quantitative NMR using external calibration with pulse length correction (PULCON) has emerged as a robust approach for high-accuracy determination of organic compounds, including in complex matrices [18].

In this work, we present an integrated analytical and preparative approach designed to address key limitations in the characterization of cannabis and its cannabinoids. The study focuses on the development of a simple and efficient strategy to increase the availability of cannabinol through the conversion of Δ^9-THC to CBN directly in cannabis extracts, combined with traceable quantification by ^{1}H quantitative NMR using the PULCON method. In parallel, the feasibility of producing cannabinoid and plant-based Cannabis reference materials is investigated through homogeneity and transport stability studies within a metrological framework relevant to forensic and regulatory applications.

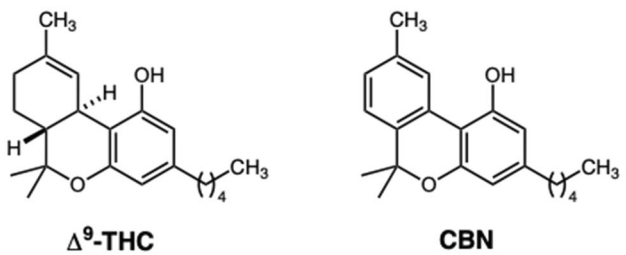

Fig. 1 Chemical structures of Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabinol (CBN)

Material and methods

Materials, sample preparation, and cannabinoid isolation

Cannabis plant material was supplied by the Brazilian Federal Police under judicial authorization and handled in accordance with institutional and legal requirements. Reference standards of Δ^9-THC, CBN, and other cannabinoids, as well as certified reference materials used for qNMR calibration, were obtained from accredited suppliers. All solvents were of HPLC or spectroscopic grade.

Prior to extraction, plant material was dried, ground, sieved, and homogenized to ensure sample representativeness. Cannabinoids were extracted using ultrasound-assisted extraction in a methanolic medium, followed by solvent removal under reduced pressure. Δ^9-THC and CBN were isolated either directly from crude plant extracts or from CBN-enriched extracts using preparative reversed-phase liquid chromatography.

Detailed information on reagents, suppliers, extraction conditions, chromatographic parameters, and fraction collection procedures is provided in the Supporting Information (SI, Sections S1-6).

Δ^9-THC-to-CBN conversion incannabis extracts

To overcome the low natural abundance of cannabinol, an iodine-mediated oxidative aromatization of Δ^9-THC was performed directly in Δ^9-THC-rich cannabis extracts. Reactions were carried out under reflux using iodine as the sole oxidant, enabling conversion without prior purification of Δ^9-THC. Reaction progress and conversion efficiency were monitored by ^{1}H quantitative NMR.

Optimization experiments, including reagent ratios, reaction times, and yield determination, are described in detail in the Supporting Information (SI, Sections S5.1–5.4).

Quantitative NMR analysis and method performance

Cannabinoid quantification was performed by ^{1}H quantitative NMR using the PULCON (PUlse Length-based CONcentration determination) method with external calibration [19]. Gravimetric sample preparation and certified reference materials ensured direct traceability to the International System of Units (SI). Cannabinoid mass fractions were calculated from integrated proton signals selected to avoid overlap and minimize systematic bias.

The fitness-for-purpose of the qNMR-PULCON approach was evaluated using a matrix-based model system (yerba mate extract) and compared to gas chromatography–mass spectrometry employing standard addition with coordinate swapping. Agreement between methods was assessed using normalized error analysis.

NMR acquisition parameters, signal selection criteria, validation data, equations, and uncertainty calculations are reported in the Supporting Information (SI, Sections S2-4 and S5.5).

Preparation and evaluation of Cannabinoid and Cannabis CRM candidates

Cannabinoid and plant-based Cannabis CRM candidates were prepared following internationally recognized metrological principles [20]. Isolated Δ^9-THC and CBN were evaluated as pure-substance CRM candidates, while a homogenized cannabis plant material was prepared as a matrix-based CRM candidate. Characterization focused on the assignment of cannabinoid mass fractions by qNMR-PULCON.

Between-unit and within-unit homogeneity of the Cannabinoid and the Cannabis CRM candidates were assessed using an isochronous experimental design. Transport stability was evaluated under refrigerated and ambient conditions using linear regression to identify statistically significant trends and associated uncertainty contributions.

Material preparation procedures, sampling strategies, statistical models, acceptance criteria, and uncertainty propagation methods are described in detail in the Supporting Information (SI, Sections S6).

Results and discussion

Traceable quantification of cannabinoids by qNMR-PULCON

To ensure reliable and metrologically traceable quantification of cannabinoids in complex cannabis matrices, quantitative ^{1}H NMR (qNMR) using external calibration with pulse length correction (PULCON) was employed throughout this study (see Supporting Information, Sections S2.3 and S3.4). qNMR has been widely recognized as a primary ratio method when appropriate calibration strategies are applied, as it provides direct traceability to the SI and does not require compound-specific calibration standards. These characteristics make qNMR particularly suitable for applications involving limited availability of reference materials, such as minor cannabinoids.

Prior to its application to cannabis extracts, the qNMR-PULCON approach was validated to demonstrate fitness for purpose. Method performance was assessed by comparing it with gas chromatography-mass spectrometry (GC–MS) using standard addition with coordinate swapping, which was selected as the reference method [21]. To avoid bias related to extraction efficiency, both techniques were applied to the same yerba mate extracts for caffeine determination (see Supporting Information, Section S4). Agreement between methods was evaluated using the normalized error approach, considering expanded uncertainties ($k = 2$).

The results demonstrated equivalence between qNMR-PULCON and GC–MS for all samples analyzed, with normalized error values below one (Table 1). These findings confirm that qNMR-PULCON provides accurate and unbiased quantification in complex plant matrices, supporting its suitability for cannabinoid determination in cannabis extracts.

Based on this validation, qNMR-*PULCON* was applied to the quantification of Δ^9-THC and CBN in cannabis extracts (Table 2). Cannabinoid mass fractions were determined gravimetrically using dimethylsulfone as a certified reference material for external calibration. Signal selection was based on well-resolved, non-overlapping resonances characteristic of each cannabinoid, and quantification was performed using multiple independent signals to improve robustness. The use of fixed receiver gain and repetition times exceeding seven times the longest measured T_1 ensured accurate integration and minimized systematic bias.

The qNMR-PULCON approach enabled direct determination of cannabinoid mass fractions without the need for compound-specific calibration curves or matrix-matched standards. Importantly, the method provided traceable measurement results accompanied by well-defined uncertainty estimates, which are essential requirements for the characterization of certified reference material candidates. These features allowed qNMR-PULCON to be consistently

Table 1 Comparison of caffeine concentrations in yerba mate extracts determined by qNMR-PULCON and GC–MS

Sample	GC–MS result in µg/g	GC–MS expanded uncertainty in µg/g (U)	qNMR- PULCON result in µg/g	qNMR-PULCON expanded uncertainty in µg/g (U)	Normalized error
Extract 1	122.6	8.9	130.4	3.3	0.821
Extract 2	173.2	10	176.8	2.9	0.343
Extract 3	147.0	9.5	153.5	1.7	0.654

Table 2 Quantification of Δ^9-THC and CBN in different extracts by ^{1}H-qNMR-PULCON

Extract (*Cannabis sativa* L. genotypes)	Mass fraction CBN (g/100 g)	Expanded uncertainty CBN ($k=2$; 95%) (g/100 g)	Mass fraction Δ^9-THC (g/100 g)	Expanded uncertainty Δ^9-THC (k = 2; 95%) (g/100 g)
Preta Kush (1)	2.70	0.02	19.4	0.15
Barbara Kush (2)	0.91	0.01	14.5	0.11
White Window (3)	1.57	0.01	10.8	0.10
Nightingale (4)	6.24	0.05	16.9	0.13
Sano 7 (5)	8.71	0.16	26.6	0.50
Peach Pure (6)	8.59	0.10	24.3	0.27
N.D. (7)[a]	4.75	1.27[b]	31.23	1.27[b]

[a]Obtained from a seized *Cannabis sativa* L. plant, provided by the Brazilian Federal Police, genotype unknown. [b]Relative combined uncertainty (u_c rel%)

applied across different stages of the study, including extract characterization, monitoring of Δ^9-THC conversion to CBN, and assignment of property values to candidate Cannabis reference materials.

Direct conversion of Δ^9-THC to *CBN* in cannabis extracts

Given the low natural abundance of cannabinol in cannabis plant material and the inherent difficulty of isolating Phyto cannabinoids from complex matrices, a direct chemical conversion of Δ^9-THC to CBN was investigated as a strategy to increase CBN availability in complex extracts. CBN is formed through the oxidative aromatization of Δ^9-THC, and previous studies have shown that iodine-mediated oxidation provides an efficient semisynthetic route for this transformation [22]. On this basis, the feasibility of carrying out the conversion directly in Δ^9-THC-rich cannabis extracts was evaluated.

Initial experiments were carried out using isolated Δ^9-THC to establish suitable reaction conditions (see Supporting Information, Section S5.2). Different oxidizing systems and reaction times were screened, revealing that iodine alone was more effective than combinations of iodine and DDQ as oxidants [23]. Optimal conversion was achieved under reflux in toluene using only iodine with a reaction time of 4 h leading to CBN in 52% isolated yield. Shorter reaction times resulted in incomplete conversion, while longer times led to lower yields, likely due to side reactions or degradation. These conditions were therefore selected for subsequent experiments.

The optimized reaction was then applied directly to cannabis extracts of different origins and cannabinoid compositions (see Supporting Information, Section S5.3). In all cases, the conversion led to a marked increase in CBN content accompanied by the complete disappearance of Δ^9-THC signals in the ^{1}H NMR spectra. Quantification by qNMR-PULCON confirmed that efficient conversion was achieved across extracts with varying initial Δ^9-THC mass fractions, demonstrating the robustness of the approach with respect to matrix composition.

To assess the practicality of the method, reactions were performed using fixed amounts of iodine calculated based on the total extract mass rather than on prior knowledge of Δ^9-THC content (Table 3 Characterization of Cannabis CRM candidate by 1H-qNMR-PULCONCannabis ExtractMass fraction CBN (g/100 g)Expanded uncertainty CBN (k = 2; 95%) (g/100 g)Mass fraction Δ9-THC (g/100 g)Expanded uncertainty Δ9-THC (k = 2; 95%)(g/100 g)Replicate A5.170.0430.140.23Replicate B5.150.0429.580.25Replicate C4.990.0428.930.213). Comparable CBN yields were obtained under these simplified conditions, indicating that precise pre-quantification of Δ^9-THC is not strictly required. This feature significantly enhances the applicability of the method for routine preparation of CBN-enriched extracts.

These results provide a general guideline for CBN enrichment in cannabis extracts. After optimization of the reaction conditions, we established that, in general, each 50 mg of *Cannabis sativa* L. extract can be treated with 19.7 mg of iodine in 10 mL of toluene and maintained under reflux (approximately 110 °C) with magnetic stirring for 4 h. Upon completion, the reaction mixture should be treated with 5 mL of a 5% aqueous sodium thiosulfate solution, extracted with 5 mL of ethyl acetate, dried over magnesium sulfate, filtered, and the solvent evaporated prior to purification.

Overall, the iodine-mediated oxidative conversion of Δ^9-THC directly in cannabis extracts provides a simple, efficient, and reproducible route to enrich CBN in complex plant matrices. This strategy circumvents the limitations associated with the direct isolation of CBN from cannabis and establishes a practical foundation for its subsequent purification and use in the preparation of cannabinoid reference materials.

Efficient access to CBN from cannabis extracts

The impact of the direct conversion strategy on CBN availability was evaluated by comparing the isolation of CBN

Table 3 CBN-enriched extracts using decreasing amounts of iodine[a]

Extract	Mass fraction of Δ^9-THC in the extracts (g/100 g)[b]	I_2 (mg)	Mass fraction of CBN after reaction (g/100 g)[b]	Uc rel%
1	19.4	39.95	22.45	1.16
		19.7	19.79	1.07
		9.8	24.31	1.07
2	14.5	39.95	22.49	1.09
		19.7	15.85	1.06
		9.8	18.75	1.07
3	10.8	39.95	12.61	1.05
		19.7	12.93	1.06
		9.8	9.98	1.05
4	16.9	39.95	26.95	1.06
		19.7	23.36	1.06
		9.8	23.64	1.07
5	26.6	39.95	33.65	1.08
		19.7	33.07	1.29
		9.8	24.17	1.28
6	24.3	39.95	28.12	1.27
		19.7	27.81	1.28
		9.8	20.92	1.29
7	31.23	39.95	30.91	1.35
		19.7	28.01	1.30
		9.8	15.86	1.32

[a]Reaction conditions: all reactions were done using 50 mg of extract in toluene under reflux for 4 h. [b]Quantification by ^{1}H-qNMR-PULCON

from untreated cannabis plant extracts with that obtained after Δ^9-THC conversion. Owing to its low natural abundance and chromatographic behaviour similar to other Phyto cannabinoids, direct isolation of CBN from plant material proved inefficient, requiring large amounts of extract to obtain limited quantities of purified compound.

In contrast, iodine-mediated conversion of Δ^9-THC in Δ^9-THC-rich extracts led to substantial enrichment of CBN prior to purification (Fig. 2 and Supporting Information, Section S5.4). As a result, significantly higher amounts of CBN were recovered after preparative chromatographic isolation, even when smaller quantities of starting material were used.

Cannabinoid extraction from the candidate cannabis plant CRM using methanolic direct pulsed ultrasound and ultrasonic bath proved efficient and reproducible, yielding 18% and 19% of crude extract, respectively, under identical operating conditions (60 W, 30 min). Preparative liquid chromatography enabled the isolation of Δ^9-THC and CBN at retention times of 8.75 and 7.57 min, respectively. From 12 g of crude plant extract, isolated yields of Δ^9-THC and CBN were 4.74% (568.80 mg) and 1.48% (177.60 mg). In contrast, isolation from 586.51 mg of CBN-enriched extract obtained via Δ^9-THC conversion afforded CBN in 24.05% yield (141.06 mg), despite the substantially lower amount of starting material.

Analysis of cannabinoids extracts by HPLC–PDA-MS/MS using multiple reaction monitoring (Supporting Information, Section S5.5) revealed trace amounts of Δ^9-THC in the CBN fraction isolated from cannabis plant extracts and trace amounts of CBN in the Δ^9-THC fraction, reflecting the similar polarity and chromatographic behavior of Phyto cannabinoids. In contrast, CBN obtained from Δ^9-THC-rich *Cannabis sativa* extracts subjected to the optimized synthetic conversion showed no detectable Δ^9-THC or other impurities under the conditions employed, demonstrating the advantage of producing CBN via controlled conversion of THC rather than direct isolation from the complex plant matrix.

Overall, enrichment of cannabis extracts through Δ^9-THC conversion increased isolated CBN yields from approximately 1–2% of the crude extract to values exceeding 20% in the converted material, corresponding to an improvement of more than one order of magnitude in practical CBN recovery. Beyond yield enhancement, the conversion strategy simplified downstream purification by reducing matrix complexity and minimizing co-elution of structurally related cannabinoids.

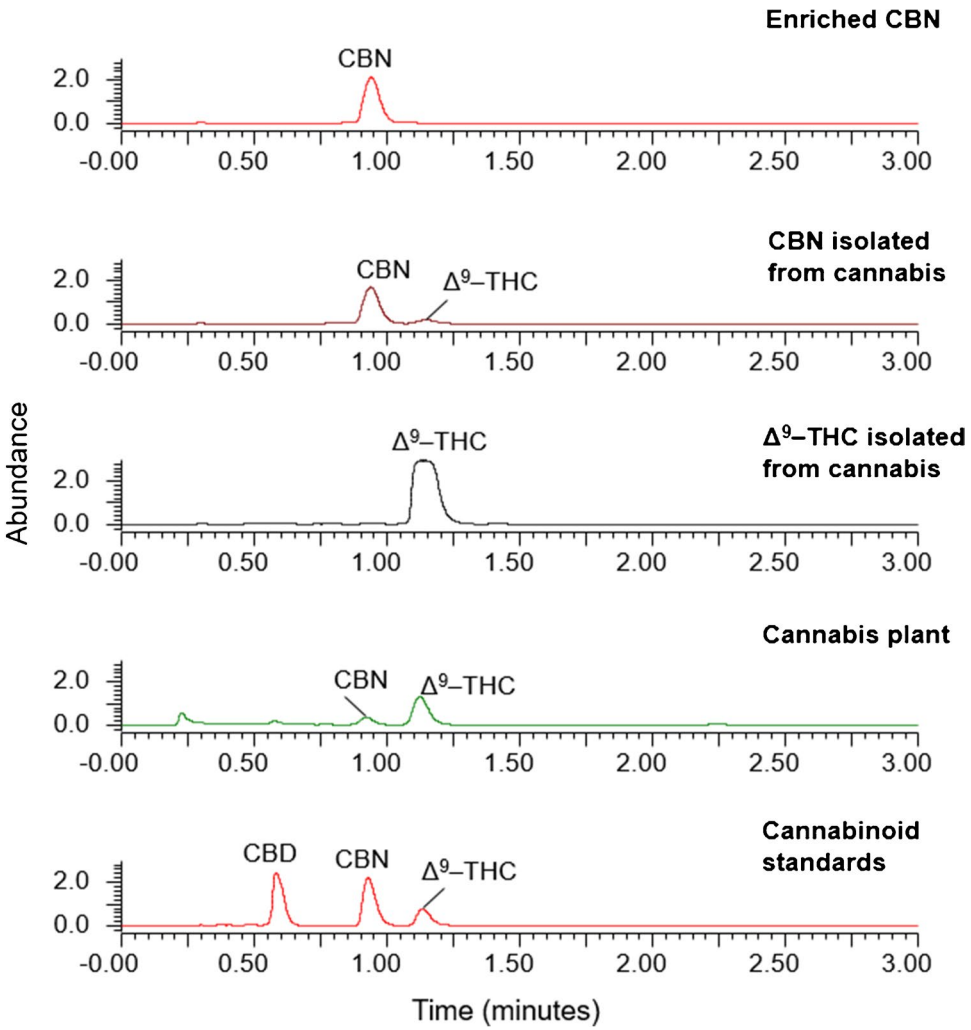

Fig. 2 HPLC–DAD-MS/MS chromatograms of enriched CBN, CBN and Δ^9-THC isolated from cannabis, Cannabis plant extracts, and cannabinoid standards at 210 nm

These results demonstrate that direct conversion of Δ^9-THC to CBN prior to isolation offers a clear advantage over direct plant-based extraction. This approach enables efficient access to CBN in quantities suitable for analytical and metrological applications, particularly for the preparation of cannabinoid reference materials, where yield, reproducibility, and material availability are critical considerations.

Cannabis and cannabinoid certified reference materials

The availability of certified reference materials (CRMs) is a fundamental requirement for ensuring the reliability, traceability, and comparability of analytical results in forensic and regulatory cannabis analysis. However, the complex chemical composition of cannabis and the limited availability of certain cannabinoids pose significant challenges for CRM production. In this study, Cannabis and cannabinoid CRM candidates were produced and evaluated following internationally recognized metrological principles. Isolated Δ^9-THC and CBN obtained either directly from plant material or via Δ^9-THC conversion was considered as potential pure-substance CRM candidates. In parallel, a plant-based Cannabis CRM candidate was prepared to support matrix-matched quality control and method validation. For both types of materials, traceable characterization by ^{1}H quantitative NMR using the PULCON method provided mass fraction values accompanied by well-defined measurement uncertainties.

For the plant-based Cannabis CRM candidate, particular attention was given to material preparation to ensure representativeness and reproducibility. After drying, grinding, sieving, and homogenization, the material was stored under controlled conditions prior to characterization. Cannabinoid extraction using methanolic ultrasonic techniques proved efficient and reproducible, enabling consistent analytical assessment of the candidate material. Quantification by qNMR-PULCON yielded mass fractions of approximately 5.1 g/100 g for CBN and 29.6 g/100 g for Δ^9-THC in the cannabis extract, corresponding to

approximately 0.9 g/100 g of CBN and 5.4 g/100 g of Δ^9-THC in the plant material, when the extraction yield was considered (Table 3 and Supporting Information, Section S5.5).

Homogeneity of the Cannabis CRM candidate was assessed through between-unit and within-unit studies using an isochronous experimental design (Fig. 3). No systematic trends were observed with respect to filling order or chromatographic injection sequence, indicating adequate control of sample preparation and analysis.

Evaluation by one-way analysis of variance showed that the relative uncertainty contributions associated with between-unit heterogeneity were 0.43% for CBN and 0.56% for Δ^9-THC, while within-unit contributions were 0.58% for CBN and 0.66% for Δ^9-THC. When combined, the overall uncertainty associated with material heterogeneity was 0.72% for CBN and 0.86% for Δ^9-THC.

These values are well below the predefined acceptance limit of 5% established during batch planning, demonstrating that the applied material preparation, homogenization, and bottling procedures yielded a sufficiently homogeneous batch suitable for use as a certified reference material.

Transport stability was evaluated to assess the robustness of the Cannabis CRM candidate under realistic distribution conditions. Short-term stability studies were conducted at 4 ºC and 20 ºC using an isochronous design, and results were evaluated based on corrected analytical responses and linear regression analysis (Fig. 4).

Under refrigerated conditions (4 °C), the material remained stable for up to 28 days. No statistically significant

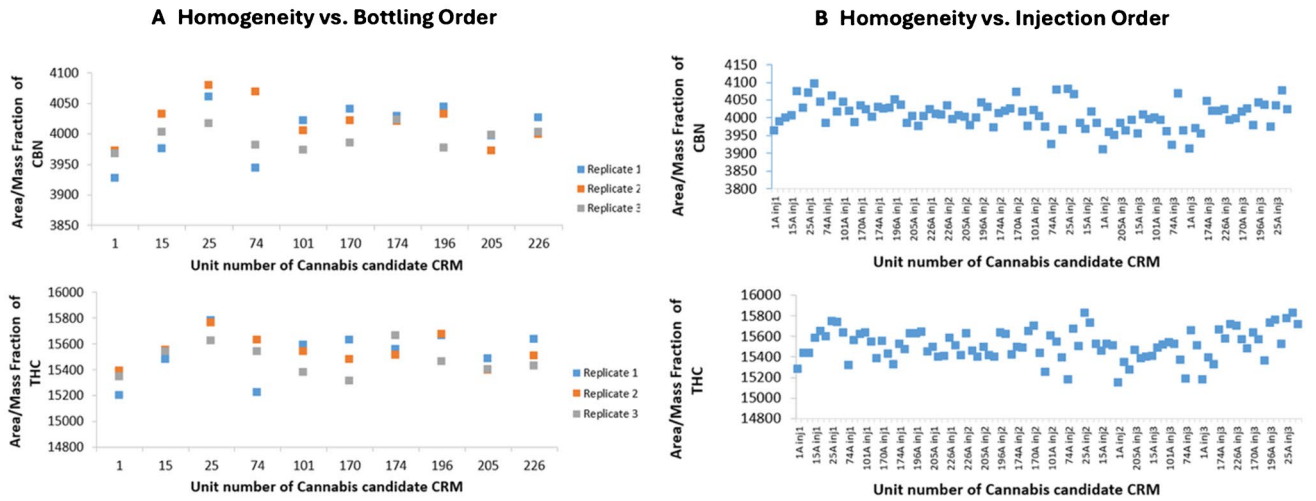

Fig. 3 Homogeneity study of the Cannabis CRM candidate

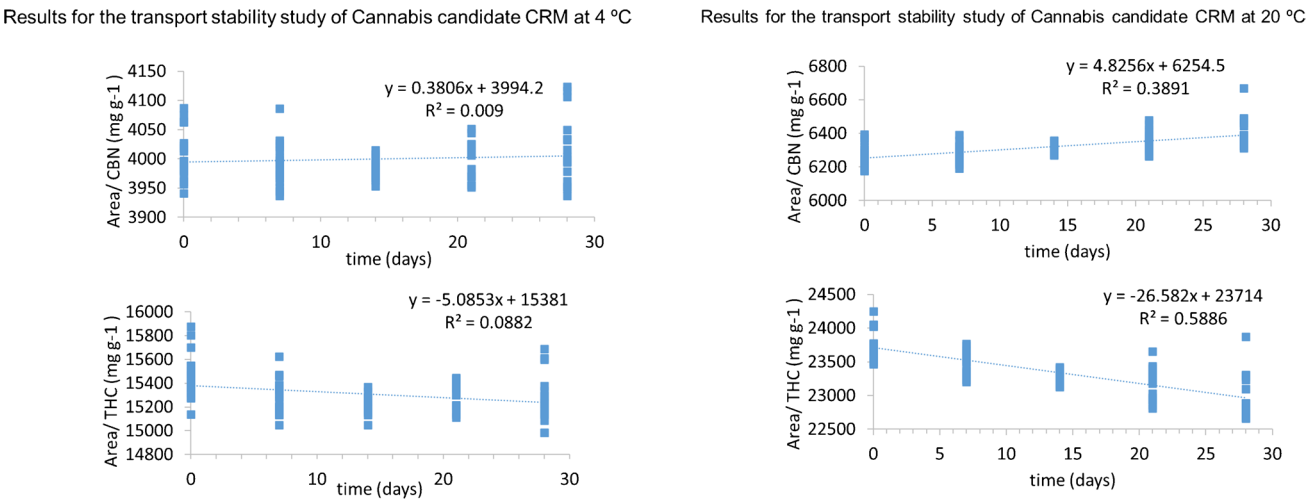

Fig. 4 Transport stability study of the Cannabis CRM candidate

trend was observed for CBN, whereas a significant slope was detected for Δ^9-THC, consistent with its known susceptibility to oxidative degradation. To ensure that potential transport-related changes did not bias the certified value, an additional uncertainty component associated with curve extrapolation was included in the uncertainty budget for Δ^9-THC. Even with this contribution, the material was considered suitable for transport at 4 ºC for the full study period.

At 20 °C, statistically significant trends were observed for both CBN and Δ^9-THC. These trends reflect the oxidative conversion of Δ^9-THC to CBN under ambient conditions, resulting in a decrease in Δ^9-THC accompanied by a corresponding increase in CBN.

Based on these results, the maximum allowable transportation time at room temperature was limited to 15 days. The combined stability-related uncertainty contributions under transport conditions at 20 ºC were estimated as 0.31% for CBN and 0.58% for Δ^9-THC. These values are well within the predefined target uncertainty for the material (25%, expanded uncertainty, $k = 2$) and therefore do not compromise the certification of the CRM.

Overall, the candidate Cannabis CRM was demonstrated to be stable for transport under refrigerated conditions (2–8 °C) for up to 28 days and at room temperature (≤ 25 ºC) for up to 15 days.

Conclusion

This study demonstrates the feasibility of an integrated analytical and preparative approach for addressing key challenges in the production of Cannabis and cannabinoid certified reference materials. The use of ^{1}H qNMR with the PULCON method enabled traceable and accurate quantification of cannabinoids in complex plant matrices, supporting the assignment of mass fraction values with well-defined uncertainties.

The iodine-mediated oxidative conversion of Δ^9-THC to CBN directly in cannabis extracts proved to be a simple and robust strategy to overcome the limited natural availability of CBN. This approach enabled substantial enrichment of CBN prior to purification, resulting in a more than one order of magnitude improvement in isolated CBN yields compared to direct plant-based extraction, while also simplifying subsequent purification steps. Following optimization of the reaction conditions, a general and standardized protocol for CBN enrichment in *Cannabis sativa* L. extracts was defined, providing a practical guideline for routine application.

In addition, the preparation and evaluation of cannabinoid and plant-based Cannabis CRMs candidates demonstrated that the materials met essential metrological requirements, including adequate homogeneity and transport stability under realistic distribution conditions. The associated uncertainty contributions remained within predefined acceptance limits, confirming the suitability of them for use as a reference material candidate.

Overall, the combination of Δ^9-THC-to-CBN conversion, efficient isolation strategies, and traceable qNMR quantification provides a practical and transferable framework for expanding the availability of cannabinoid reference materials. This approach is particularly relevant for forensic and regulatory laboratories, where matrix effects, measurement uncertainty, and traceability are critical to the interpretation and comparability of analytical results.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00216-026-06325-4.

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Data availability The data underlying this study are available in the published article and its Supporting Information.

Declarations

Competing of interest The authors declare that they have no competing interests.

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