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Development of an accelerated ageing protocol for the study of phytocannabinoid stability in *Cannabis sativa* L.

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ABSTRACT

Cannabis sativa L. is a plant belonging to the *Cannabaceae* family known primarily for its recreational use due to the psychoactive properties of Δ^9-tetrahydrocannabinol (THC). Despite this, several compounds belonging to the category of phytocannabinoids have shown in recent years a number of potentially promising therapeutic effects that have increased the interest in the pharmaceutical field towards this plant. However, the content of these compounds is very variable and influenced by different factors, such as growing conditions and time of the year. An indication of the status and age of Cannabis samples is provided by the content of CBN, a minor phytocannabinoid and degradation product of other phytocannabinoids, including THC. In this research work an innovative, solid state analytical approach has been developed to observe and evaluate the variations in the content of two phytocannabinoids (CBN and CBD) in Cannabis-derived products over time. In order to simulate the ageing of the Cannabis samples, an artificially accelerated ageing procedure has been developed and optimised by using high temperatures. The analyses were carried out using an innovative ATR-FTIR method for solid state analysis, enabling direct analysis of a solid sample without any pretreatment phase. This study has allowed the development of an innovative analytical approach for the evaluation of the age and state of conservation of Cannabis samples and may be a useful tool both in the industrial, pharmaceutical and forensic fields.

1. Introduction

Cannabis sativa L. is a versatile plant known for its varied applications in textiles, food, cosmetics, construction, and pharmaceutical fields. The chemical composition of Cannabis is largely determined by plant genetics, but is also influenced by cultivation practices, harvesting time, drying and other external factors [1].

More than 500 chemical compounds have been identified in *Cannabis sativa* L., including terpenoids, flavonoids, alkaloids, and the specific active compounds of this plant, namely phytocannabinoids [2–4]. Phytocannabinoids are synthesized in the plant as carboxylic acids and are transformed into their neutral form by decarboxylation. This occurs when the material is exposed to high temperatures, to light sources, or simply through ageing. The most abundant (acidic and neutral) cannabinoids found in drug-type plants are Δ^9-tetrahydrocannabinolic acid (THCA) and Δ^9-tetrahydrocannabinol (THC), while fibre-type plants contain relatively high amounts of other phytocannabinoids, such as cannabidiolic acid (CBDA), cannabidiol (CBD), cannabigerolic acid (CBGA), cannabigerol (CBG) and cannabinol (CBN) [5]. CBN is a THC oxidation product that is formed after prolonged storage of the plant [3,6]: in fact, over time, the concentration of CBN tends to increase and that of THC to decrease.

Due to the psychoactive effects of THC [7], the cultivation and use of *Cannabis sativa* L. for recreational purposes is illegal and prohibited in many countries. However, THC has been proposed for medical applications, such as pain relief and appetite stimulation in cancer patients, and fot reducing chemotherapy-induced nausea [8]. In addition to THC, other phytocannabinoids such as CBD and CBN have shown interesting

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applications in the pharmaceutical field. CBD exhibits anticonvulsant, antispasmodic, anxiolytic, antinausea, analgesic, neuroprotective properties [9–12] while CBN has demonstrated emollient action on inflamed skin [13], increased appetite [14], neuroprotection [15] and possible anticonvulsant effects [16].

These innovative, current and prospective applications of *Cannabis sativa* L. in several areas have resulted in increased decriminalisation or legalisation [17] of its cultivation, trade and use in the medical and industrial fields. This has therefore led to a distinction between different varieties of the plant based on the CBD/THC ratio: the "Drug-type", used for recreational and medicinal purposes, having a CBD/THC ratio $\leq$ 0.005; the "CBD-type" where CBD content is predominant and the CBD/THC ratio is $\geq$ 15, and the "Fibre-type", a plant in which both THC and CBD concentrations are relatively low and their ratio is intermediate ($0.5 \leq$ CBD/THC ≤ 3) [18].

Following the expansion of the research performed on *Cannabis sativa* L., several studies have been carried out to investigate the stability of phytocannabinoids. These studies are important in particular to evaluate the proper storage (or lack thereof) of the plant and its derived products, allowing one to take all the necessary precautions to avoid phytocannabinoid content variations over time. During the natural ageing process of the plant, both transformation of acid phytocannabinoids into neutral ones, and interconversion phenomena among neutral phytocannabinoids can occur. Scientific studies show that these changes can be more or less pronounced, depending on storage conditions and time [19].

Of particular interest is the conversion of THC to CBN. This phenomenon, besides being observed in nature, has also been observed in stability studies. For example, after a year of plant storage at 4°C in the dark, 21.6 % of the THC content converts into CBN, with an average transformation rate of 4.4 % every three months. Interconversion is more pronounced when the sample is stored at higher temperatures and exposed to light [20]. To address these changes, this research aimed to design and develop an artificially accelerated ageing procedure for *Cannabis sativa* L. inflorescences. We monitored this process using a novel solid-state analysis method for CBN and CBD based on infrared (IR) spectroscopy. Multiple tests were conducted to determine the optimal conditions for accelerated ageing, with attenuated total reflectance – Fourier transform IR (ATR-FTIR) spectroscopy selected for its suitability in analysing "Fibre-type" *C. sativa* samples. Traditional analytical methods for cannabinoid determination typically involve chromatographic techniques such as high-performance liquid chromatography (HPLC) [21–24] or gas chromatography (GC) [25]. An alternative approach is solid-state analysis using infrared (IR) technologies [26], which offers rapid and high-throughput analysis directly on solid samples while maintaining robust data quality. This is particularly valuable given the growing market for *C. sativa* products, necessitating frequent, reliable, and feasible quality controls. This study presents the first example of an accelerated ageing procedure for evaluating product shelf life and cost-efficient quality control of Cannabis and its derivatives using ATR-FTIR, a non-destructive, portable and reliable methodology [18] that has been updated and improved in order to maximise analytical performance in the context of this application study for the investigation of Cannabis accelerated aging.

2. Materials and methods

2.1. Reagents and samples

HPLC grade acetone, methanol, and acetonitrile, used for cleaning the ATR-FTIR instrument, were supplied by Merck KGaA (Darmstadt, Germany). Ultrapure water (18.2 MΩ cm) was produced by a MilliQ apparatus from Millipore Merck.

For the study, samples of commercial "Fiber-type" *Cannabis sativa* L. were used, with a declared concentrations of CBD $<$ 18 %.

2.2. Instrumentation

The analysis of the solid-state samples was carried out using a Nicolet iS5 FTIR spectrometer from ThermoFisher Scientific (Waltham, MA, United States) with an iD5 ATR accessory equipped with a high-energy efficiency laminated diamond crystal (single-bounce ATR; crystal type: diamond with ZnSe lens; sampling area: 1.5 mm). OMNIC Spectra software was used for the acquisition and processing of spectral data. TQ Analyst software was used for the quantification of phytocannabinoids. For the analysis of Cannabis inflorescence samples, ground aliquots of floret samples were placed on the diamond crystal sample area and squashed with the calibrated screw to keep the sample in place and apply equal pressure to each sample, thus improving reproducibility. All the samples were analyzed in triplicate in the $4000-400\ cm^{-1}$ range, and 32 scans were acquired for each spectrum with a resolution of $4\ cm^{-1}$, then spectral data was averaged to produce one result per sample for quantitative analysis. Before every analysis session, automatic iD5 ATR accessory detection, performance verification and system suitability tests with internal NIST-traceable polystyrene film (1.5 mil thickness) were carried out for complete system performance assurance. All measurements were carried out under standard room conditions (22.5 ± 2.5 °C, RH 40 ± 10 %).

2.3. Quantitative analysis of phytocannabinoids by ATR-FTIR

For the quantitation of CBN and CBD via ATR-FTIR, a previously developed and validated method for the analysis of phytocannabinoids in the solid state was used [18]. Briefly, an initial comparison was made between the reference spectra of pure phytocannabinoids with the spectra of different Cannabis varieties to identify the characteristic bands of analytes. The band of the IR spectrum selected as peculiar to the CBN and used for its quantification was the one between $910\ cm^{-1}$ and $872\ cm^{-1}$. For CBD, the spectrum band between $1036\ cm^{-1}$ and $1001\ cm^{-1}$ was chosen.

The spectra were replicated ($n = 5$) for each type of sample. Qualitative analysis was also carried out by observing the spectra obtained and comparing them with the standard spectra of the phytocannabinoids of interest, in order to confirm the presence of the characteristic CBN and CBD bands. The concentration of phytocannabinoids was determined by the TQ Analyst software, exploiting a quantitative approach using updated and refined calibration lines starting from those developed for CBN and CBD in the above method [18].

2.4. Development of accelerated sample ageing protocols

To simulate long-term stability studies, the accelerated ageing of the plant was induced through various *ad hoc* optimised methods. The Cannabis sample was first ground in a blade mill and then subjected to the following procedures. After each treatment, the change in CBN and CBD content was evaluated over time by ATR-FTIR analysis.

2.4.1. Microwave ageing

The use of microwaves (MW) for accelerated ageing of Cannabis has been evaluated by testing different conditions in terms of microwave oven power (600–1000 W) and treatment time (1–600 s), with ATR-FTIR analyses carried out at 120 s intervals. A volume of 100 mL of ultrapure water in an open borosilicate glass heatproof container was placed close to the sample to create the humid environment required in the development of accelerated ageing protocols (humidity not measured).

2.4.2. Temperature ageing

The Cannabis sample was subjected to several procedures, using an oven set at different temperatures between 50°C and 70°C. In this case, an open borosilicate glass heatproof container with 100 mL of ultrapure water was placed close to the sample, and both were put within a container closed with a non-airtight glass lid, to recreate a uniformly humid environment (RH 75 ± 5 %). Initially, the sample was heated for a total of 32 h and ATR-FTIR analyses were carried out after 0, 6, 16, 24 and 32 h. Once the treatment conditions were optimised, the procedure was repeated in triplicate for a period of 32 h, performing the ATR-FTIR analysis at 4-h intervals.

2.5. Comparison with natural product ageing

Different aliquots of the same samples subjected to the experimental accelerated ageing protocols were also subjected to natural ageing. The products were kept at room temperature for 10 months and analysed at regular intervals (every 30 d) during this period. The CBN and CBD contents of the naturally aged samples were compared to those aged in an accelerated manner to try and find any possible relationships between the real time elapsed and the accelerated procedure time that produced corresponding analyte levels.

3. Results and discussion

3.1. Quantitative analysis by ATR-FTIR

The concentration of phytocannabinoids was determined by the TQ Analyst software, exploiting a quantitative approach using updated and refined calibration lines starting from those developed for CBN and CBD in the above method [18]. Briefly, 12 calibration samples were analysed both by ATR-FTIR and LC–MS/MS. The cannabinoid amount found in each sample by LC–MS/MS was used to set up ATR-FTIR calibration curves. In total, 12 calibration samples were analysed and improved r^2 values were obtained: 0.9999 for CBD and 0.9982 for CBN. Back-calculated concentrations were very close to the real ones, in the 86–102 % range. Improved sensitivity (calculated as LOQ = 10 σ/S, where σ is the standard deviation of the response and S is the slope of the calibration curve) was also observed: 0.7 mg/g for CBD and 0.5 mg/g for CBN. The same set of calibration samples were also evaluated by both ATR-FTIR and LC–MS/MS after application of the optimised accelerated aging protocol: in this case, the accuracy of back-calculated concentrations was in the 84–104 % range, demonstrating good robustness of the model taking into account the aging process of the sample and thus the degradation of the phytocannabinoids and possible changes in sample matrix.

Moreover, in order to minimise potential precision issues due to sample inhomogeneity, a preliminary step has been introduced by finely cutting Cannabis floret samples by means of a blade mill. This has allowed to improve the precision of the measurements: from an intra-floret inhomogeneity < 11.0 % (as RSD%, n=6) to RSD% < 7.8 and from an inter-floret inhomogeneity < 13.9 % to < 10.2 %.

Finally, IR spectrum bands selected as characteristic to the target analytes and used for their quantitation were investigated and refined to take into account potential changes in the spectra, due to the ageing protocols applied to the samples, resulting from matrix modifications especially in the proximity of the analyte signals. The ranges thus optimised and exploited were between 910 cm^{-1} and 872 cm^{-1} for CBN (possibly attributable to double bond CH_2 wagging vibrations [27]) and between 1036 cm^{-1} and 1001 cm^{-1} for CBD (likely from the alkyl carbon-*O*-aryl carbon vibrational mode [28]).

3.2. Accelerated ageing procedures by microwaves

For the development of accelerated plant ageing protocols by microwaves (MW), some methods present in the scientific literature were considered as starting points. Based on the paper by Rêkas *et al.* [29], two procedures at different MW potency were developed.

In the two procedures, of 10 mg of Cannabis inflorescence were inserted into a beaker inside the MW oven and treated at two different power levels: 600 W and 1000 W. Both samples were exposed to MW irradiation for 10 min and ATR-FTIR analyses were carried out every 2 min. Since the ATR-FTIR methodology was developed to be applied in a non-destructive manner, the various accelerated aging and analysis experiments were conducted longitudinally on the same samples, without any prior sample preparation. The spectra collected by ATR-FTIR are reported in Fig. 1 and changes in analyte signals can be observed before and after the two MW procedures (10 min).

Quantitative results are shown in Fig. 2, which reports the trends in CBN and CBD content during the assay.

The MW treatment of plant samples at 600 W led to a significant change in CBN concentration, which after an initial decrease tends to increase rapidly and then to stabilise over time. CBD shows smaller variations over time. A decrease is initially observed, probably due to its initial degradation, followed by a relative increase, possibly due to the partial decarboxylation of CBDA into CBD.

The MW irradiation procedure at 1000 W produced different changes in phytocannabinoid contents. In this case, CBN immediately increases and then stabilises quite soon. On the other hand, CBD again showed an initial small (but more marked) decrease and then a relative increase; finally, a further decrease was observed, possibly due to degradation processes overcoming CBDA decarboxylation.

The results obtained from these assays show phytocannabinoid concentration variations in the plant with uneven trends over time and not easily controlled. Thus, it seems that the effects of MW on phytocannabinoids are difficult to predict and control, and that such a fast

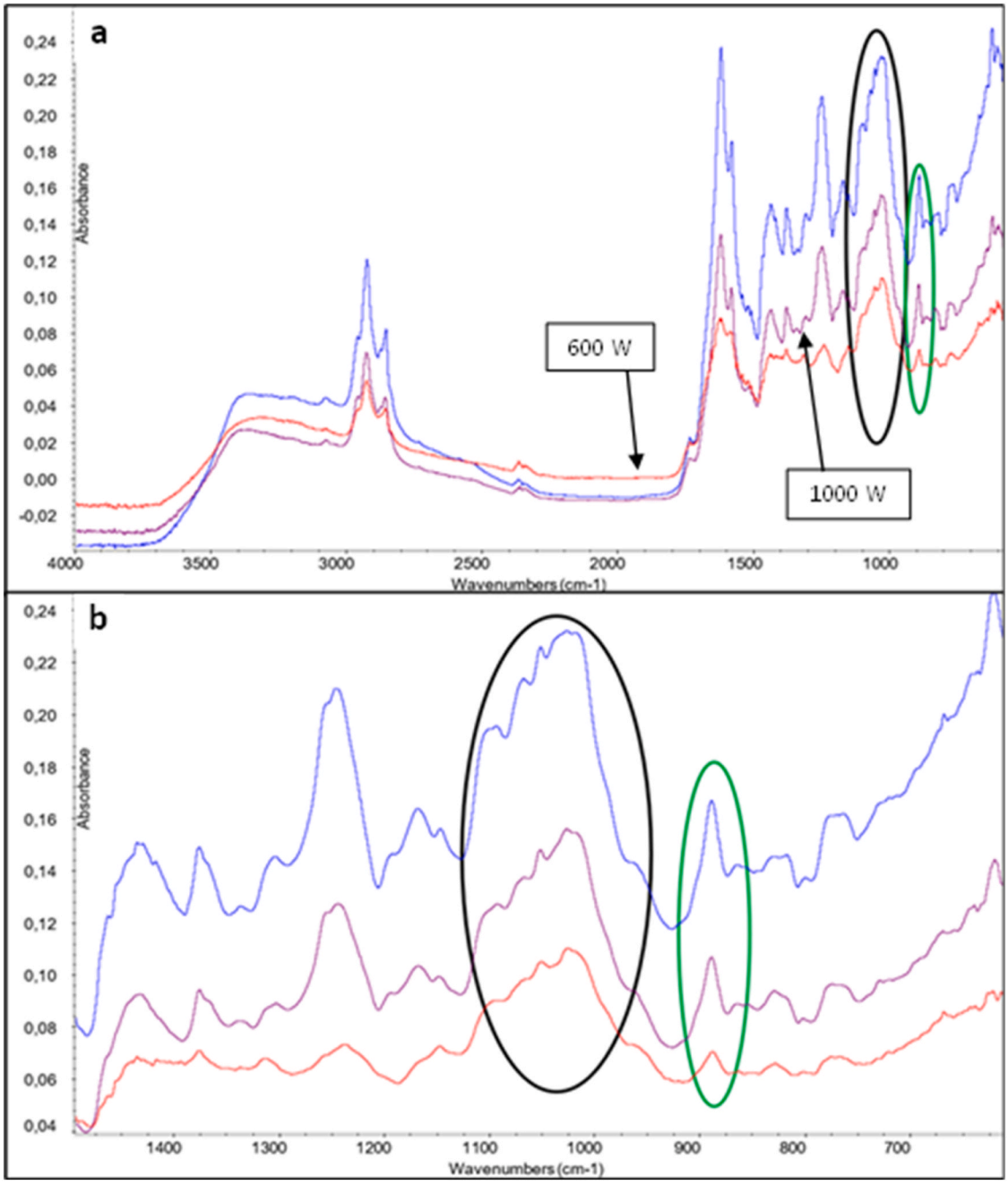

Fig. 1. Comparison between the spectra of a *Cannabis sativa* L. inflorescence sample before (blue) and after microwave accelerated aging at 600 W (purple) and 1000 W (red): (a) full spectra, (b) zoom of the 600–1500 cm^{-1} range including the characteristic peaks of CBD (1036–1001 cm^{-1}, black oval) and CBN (910–872 cm^{-1}, green oval).

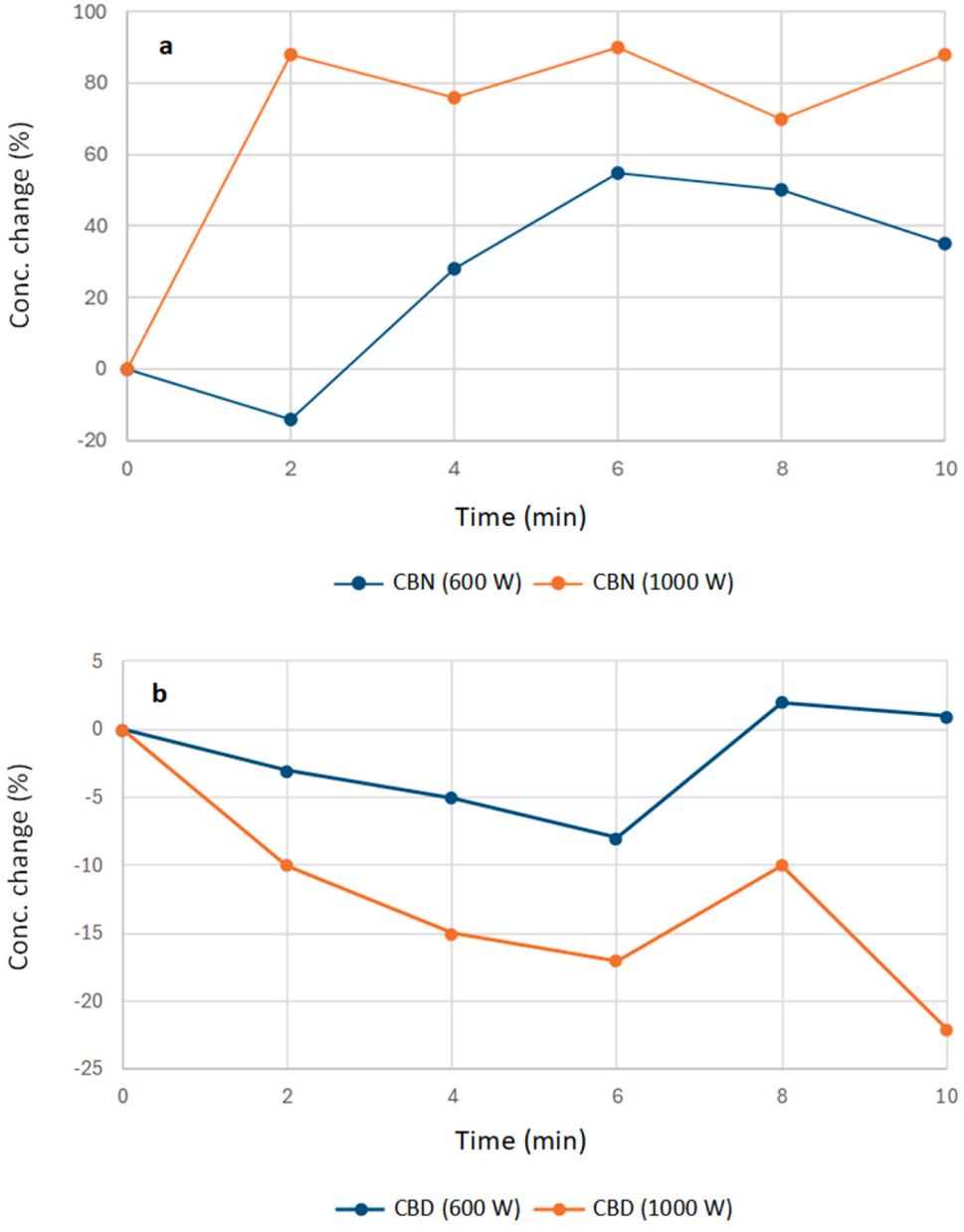

Fig. 2. Percentage changes in a) CBN and b) CBD content obtained from the microwave accelerated ageing procedure.

sample degradation can be non-monotonic, scarcely reproducible. Such trends are scarcely useful to evaluate sample stability over time.

3.3. *Accelerated ageing procedures by temperature*

For the development of the accelerated ageing protocol by temperature, it was necessary to define different parameters such as temperature itself, time, and method of sample exposure. The paper by Jaidee *et al.* [30] on Cannabis resins exposed to different temperatures has led us to develop and test procedures of accelerated ageing through heating.

The first variant involved the use of an electric oven, where two open borosilicate glass heatproof containers were placed, one containing 10 mg of Cannabis inflorescence sample and the other containing 100 mL of ultrapure water.

This procedure was tested between 50 and 80°C (namely, at 50, 60, 70 and 80°C), and the sample was kept in the oven for a total of 32 h and analysed at regular intervals by ATR-FTIR. The results obtained from this protocol are reported in Table 1, which expresses the percentage changes (with respect to the initial values) in CBN and CBD at each temperature.

From the data reported in Table 1, it is possible to observe a larger and faster variation in the concentration of the two analytes at 70°C than at lower temperatures; at 80°C, signs of degradation started to appear, with modifications in IR spectra outlines and scarcely detectable analyte signals (data not shown). The trend for the concentration variations of each analyte over time is, however, similar at all temperatures. On the

Table 1
Mean values of changes in CBN and CBD content over time in *Cannabis sativa* L. obtained from the oven accelerated ageing procedure between 50°C and 70°C.

Time (h)	Change in CBN concentration (%)			Change in CBD concentration (%)		
	50°C	60°C	70°C	50°C	60°C	70°C
0	0	0	0	0	0	0
6	+4	+15	+47	+3	+3	+5
16	+18	+47	+92	−5	−7	+18
24	+20	+48	+94	−10	−15	+30
32	+21	+49	+93	−12	−19	+35

one hand, CBN levels increase almost constantly for 16 h and then reach a plateau; on the other hand, CBD follows a non-monotonic trend with an initial, limited increase in the first 6 h, followed by a more marked decrease until the end of the observation period.

The same data is visualised in Fig. 3, where it is possible to observe how the different temperatures lead to a different sample ageing state; in particular, as expected, higher temperatures induce faster changes in cannabinoid concentration.

Based on the results obtained, it was decided to carry out the procedure at 70°C, since it was found to induce the most accelerated ageing rate, without producing any significant sample degradation. To obtain more uniform humidity conditions, a second protocol was devised, where the sample and the water beaker were put together in the oven within another, larger and taller glass container that was then non-hermetically closed with a glass lid.

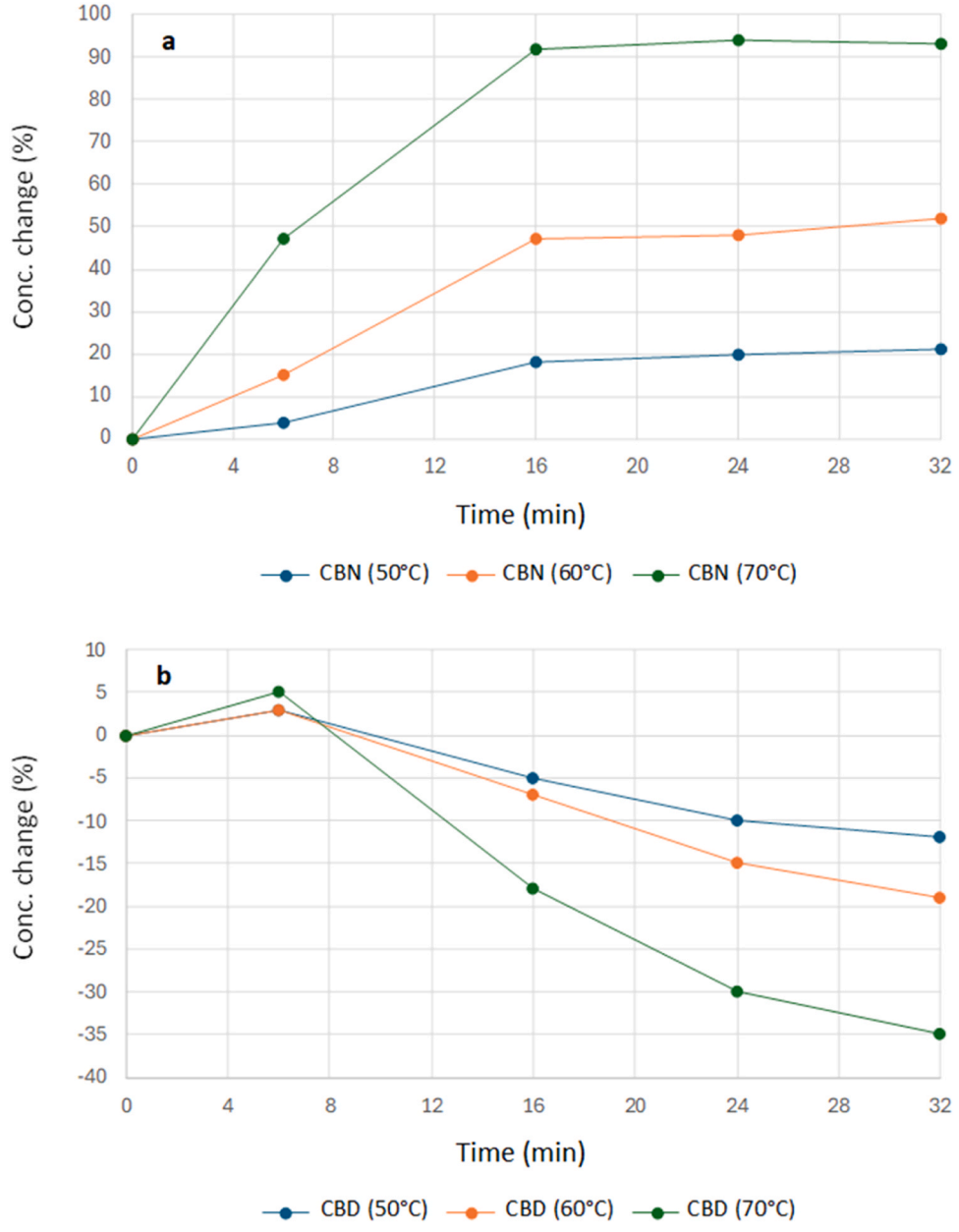

Fig. 3. Percentage changes in a) CBN and b) CBD content obtained from the oven accelerated ageing procedure.

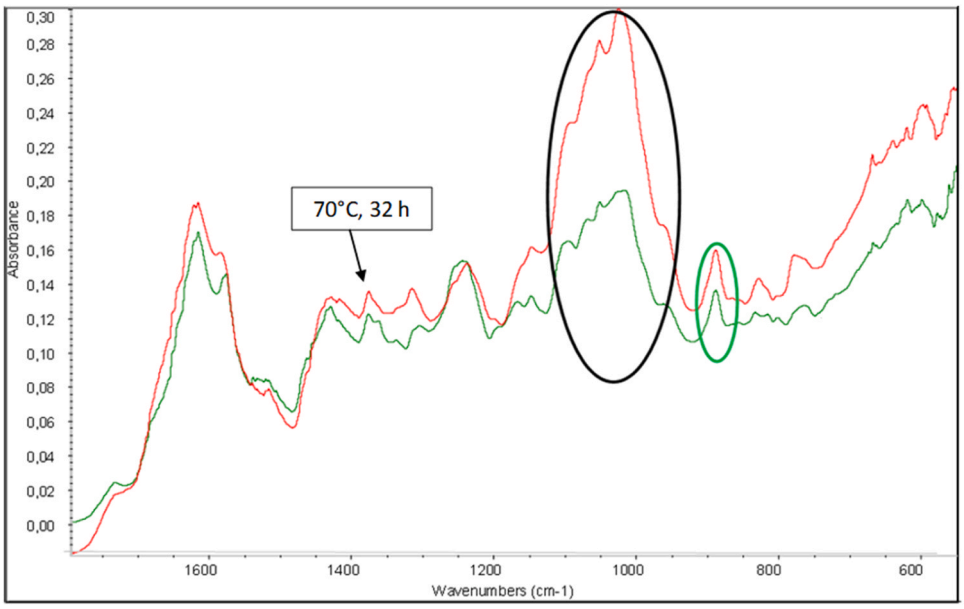

Fig. 4. Comparison between the spectra of a *Cannabis sativa* L. inflorescence sample before (green) and after (red) the procedure of accelerated aging with high temperature optimised at 70°C. Highlights show characteristic peaks of CBD (1036 cm^{-1} – 1001 cm^{-1}, black oval) and CBN (910 cm^{-1} – 872 cm^{-1}, green oval).

Fig. 4 shows the ATR-FTIR spectra obtained before and after sample treatment (32 h) by the same procedure. In the spectra, it is possible to observe changes in the intensities of the characteristic analyte signals before and after the treatment, consistent with the obtained quantitative results.

This protocol was carried out in triplicate and the trends of percent content changes of the two individual phytocannabinoids were elaborated, as shown in Fig. 5 (SD ranges were 12.2 % - 22.0 % for CBN and 2.1 % - 5.9 % for CBD). The graphs show that the behaviour of CBN is remarkably similar to the one obtained previously, while the concentration evolution of CBD changed visibly, approaching a monotonic shape. On the other hand, very similar trends over time were observed among the three replicates for each analyte, showing how the procedure is satisfactorily reproducible. CBN, in these conditions, varies with a relatively sharp increase at the beginning, and then stabilises over time (after 16 h). CBD, on the other hand, decreases less sharply and with a less regular trend at the beginning. After about 10 h, the decrease becomes almost linear and does not reach any plateau within 32 h.

The results obtained show how the heating procedure developed and optimised here has allowed to obtain a variation in time of the content of the two phytocannabinoids, which is more stable and controlled compared to the procedures using MW.

3.4. Comparison with the literature

Despite the limited information and data found in the scientific literature, it has been possible to establish a correlation between the accelerated ageing time in the oven at 70°C and the natural ageing time at RT. In the paper by I.G. Trofin *et al.* [31], the variation of some phytocannabinoids in the natural ageing of Cannabis resins was studied. Through the data provided by this article, it is possible to hypothesize a correspondence between the CBN and CBD variations observed in the natural ageing of the plant product (exposed to light and at RT) and those recorded in this study with artificial ageing induced in an oven at 70°C (Table 2). Comparing the data of the two experiments, one can see how in a natural ageing time of 3 months, there is an increase in CBN comparable to what is obtained after treatment at 70°C for 4 h. After 16 h, the variation in CBN is similar to that observed after 6 months of natural ageing.

After that, the CBN trend loses significance, since its levels reach a plateau. However, the decay of CBD can be used as a less sensitive but longer-lasting marker of aging: its decrease during accelerated ageing is surely less pronounced, but it continues for a longer time, without plateauing. In particular, CBD decrease percentages after 16 and 24 h closely approach those observed after 6 and 9 months of natural ageing, respectively.

3.5. Comparison with natural ageing

To further confirm the obtained results, a Cannabis inflorescence sample was left to age naturally, i.e. exposed to light and at RT (25 ± 2°C, RH 40 ± 5 %, standard laboratory fluorescent light under 12 h light/12 h dark cycles). It was analysed by ATR-FTIR every month for up to 12 months. As can be seen from Table 2, the results obtained are

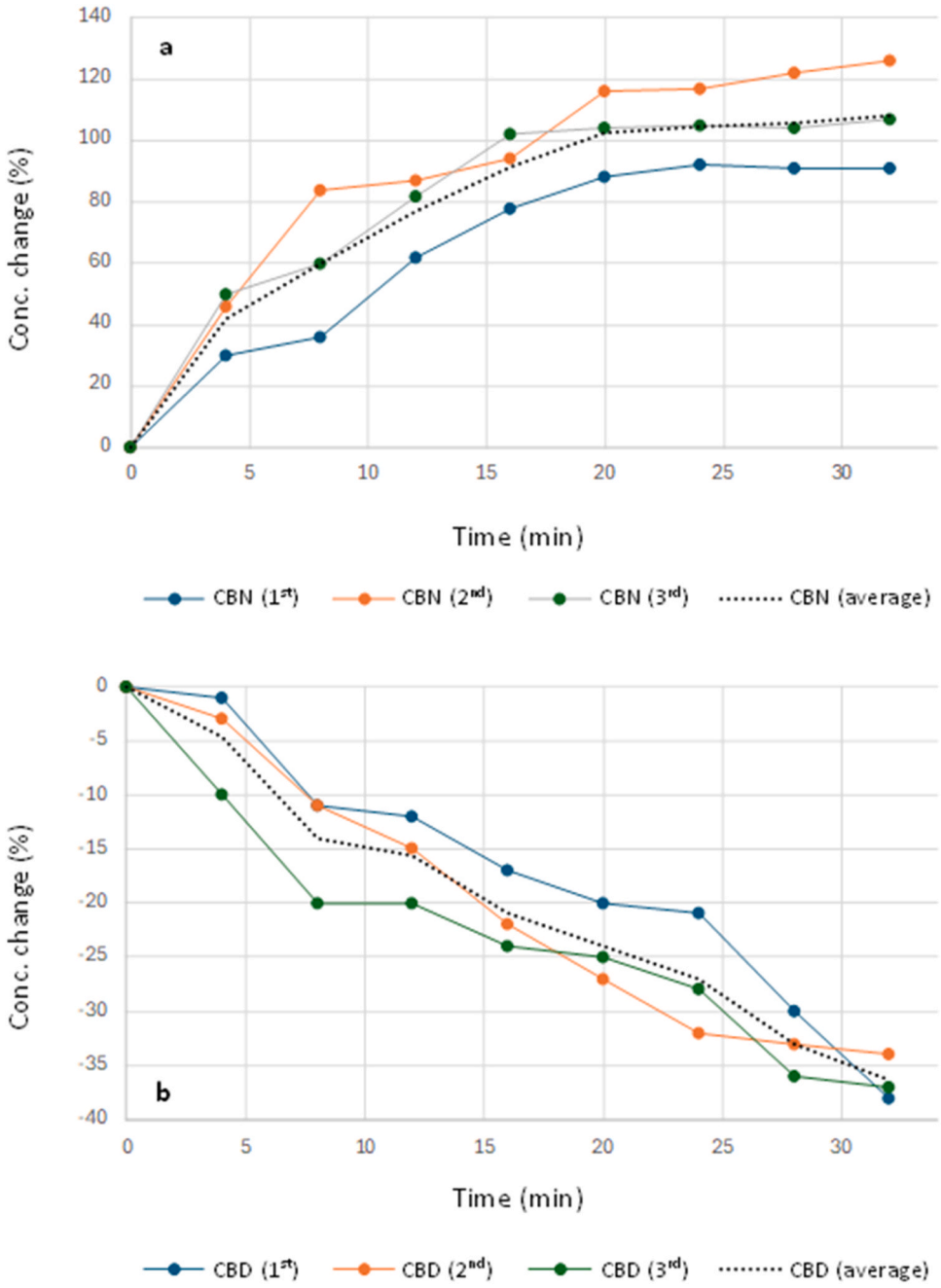

Fig. 5. Percentage changes in a) CBN and b) CBD content obtained from three repetitions of the optimised oven accelerated ageing procedure.

Table 2
Variations in analyte content during exposure to light and room temperature according to the literature [29] and during exposure to a natural ageing procedure carried out within the present study.

Time (months)	Change in CBN concentration (%)		Change in CBD concentration (%)		Accelerated ageing correspondence
	Literature [29]	Present study	Literature [29]	Present study	
1	–	+15	–	−5	
2	–	+28	–	−12	
3	+45	+48	−18	−16	CBN 4 h
4	–	+56	–	−18	
5	–	+66	–	−20	CBN 12 h
6	+93	+90	−21	−22	CBN 16 hCBD 16 h
7	–	+113	–	−23	
8	–	+127	–	−25	
9	+141	+150	−31	−29	CBD 24 h
10	–	+169	–	−30	
12	+188	+180	−33	−35	CBD 32 h

closely matching those from the literature (and thus, those obtained by the accelerated ageing procedure). This confirms that the trend correspondence between the natural and the accelerated protocol is not random, and that within specific timespans the accelerated protocol has quantitative effects on CBN and CBD similar to natural ageing, but within much shorter times.

4. Conclusion

In this paper, we have studied and optimised an accelerated ageing procedure of "Fibre-type" *Cannabis* inflorescences. Several protocols using microwaves or high temperatures were tested, and changes in CBN and CBD concentrations were observed over time to evaluate the best protocol. The determination of the two phytocannabinoids was carried out using an innovative solid state ATR-FTIR method. This allowed to skip all laborious steps necessary for sample treatment, as the analyses have been carried out directly on solid, intact samples. The most promising ageing procedure has been proven to be heating at 70°C in a humid environment. The CBN and CBD trends observed over time have been consistent with the expectations and current scientific theories, thus allowing to carry out accelerated studies of stability and conservation of phytocannabinoids in Cannabis inflorescences. Moreover, the analysis technique is non-destructive. The method developed and proposed herein may be useful to assess the state and conditions of storage of harvested Cannabis plants and to evaluate their stability. In the industrial field, the method could be used for both the quality control of Cannabis products before commercialisation and the evaluation of shelf life after commercialisation. In addition, this approach can help in the evaluation of the best product storage conditions in order to reduce the variability of phytocannabinoid content in *Cannabis sativa* L.

CRediT authorship contribution statement

Bruno Saladini: Software, Methodology, Data curation. **Stefano Girotti:** Supervision, Funding acquisition. **Přemysl Mladěnka:** Resources, Project administration, Funding acquisition. **Michele Protti:** Writing – review & editing, Visualization, Supervision, Methodology, Data curation, Conceptualization. **Laura Mercolini:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization. **Roberto Mandrioli:** Writing – review & editing, Visualization, Supervision, Methodology, Data curation, Conceptualization. **Marco Cirrincione:** Writing – original draft, Validation, Investigation, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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