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PII: S0731-7085(17)32236-7
DOI: https://doi.org/10.1016/j.jpba.2017.11.044
Reference: PBA 11618

To appear in: *Journal of Pharmaceutical and Biomedical Analysis*

Received date: 4-9-2017
Revised date: 14-11-2017
Accepted date: 15-11-2017

Please cite this article as: Cinzia Citti, Barbara Pacchetti, Maria Angela Vandelli, Flavio Forni, Giuseppe Cannazza, Analysis of cannabinoids in commercial hemp seed oil and decarboxylation kinetics studies of cannabidiolic acid (CBDA), Journal of Pharmaceutical and Biomedical Analysis https://doi.org/10.1016/j.jpba.2017.11.044

Analysis of cannabinoids in commercial hemp seed oil and decarboxylation kinetics studies of cannabidiolic acid (CBDA)

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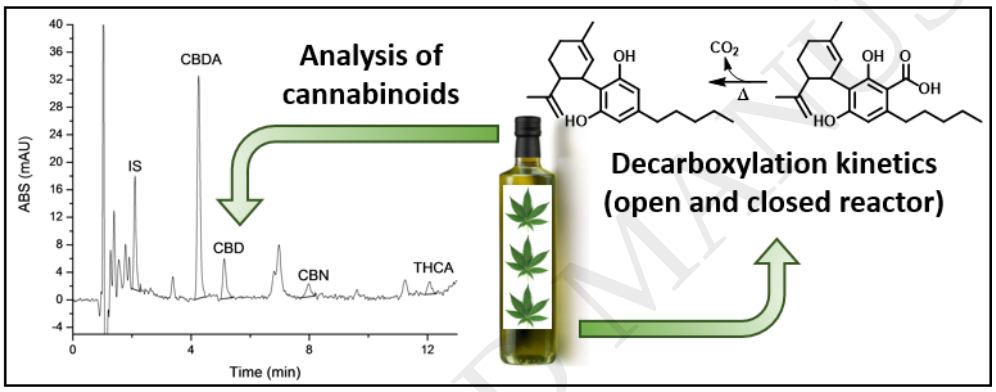
Graphical abstract



Highlights

- A sensitive, selective and rapid HPLC-UV method was developed and validated for the determination of the main cannabinoids in hemp seed oils
- High-resolution tandem mass spectrometry was employed to confirm cannabinoids identity
- CBDA to CBD ratio is a marker of cold pressing and good storage conditions
- Decarboxylation studies of CBDA were carried out in open and closed reactor
- Stability of hemp seed oil was evaluated through the kinetic parameters

Abstract

Hemp seed oil from *Cannabis sativa* L. is a very rich natural source of important nutrients, not only polyunsaturated fatty acids and proteins, but also terpenes and cannabinoids, which contribute to the overall beneficial effects of the oil. Hence, it is important to have an analytical method for the determination of these components in commercial samples. At the same time, it is also important to

assess the safety of the product in terms of amount of any psychoactive cannabinoid present therein. This work presents the development and validation of a highly sensitive, selective and rapid HPLC-UV method for the qualitative and quantitative determination of the main cannabinoids, namely cannabidiolic acid (CBDA), tetrahydrocannabinolic acid (THCA), cannabidiol (CBD), tetrahydrocannabinol (THC), cannabinol (CBN), cannabigerol (CBG) and cannabidivarin (CBDV), present in 13 commercial hemp seed oils. Moreover, since decomposition of cannabinoid acids generally occurs with light, air and heat, decarboxylation studies of the most abundant acid (CBDA) were carried out in both open and closed reactor and the kinetics parameters were evaluated at different temperatures in order to evaluate the stability of hemp seed oil in different storage conditions.

.***Keywords****: hemp seed oil, cannabidiol, liquid chromatography, decarboxylation kinetics*

1. Introduction

Cannabis sativa L. produces small fruits, botanically defined as "achenes", although they are usually named "seeds", which are variable in size depending on the cultivar. The actual "seed" is enclosed in the pericarp, which represent the protective "shell". Most of the seed consists of an embryo, mainly the two cotyledons (embryonic leaves), rich in oils, proteins and carbohydrates, that represents the nourishment of the plant during germination [1].

According to an old legend, Buddha (Prince Siddharta Gautama), founder of Buddhism, was able to survive eating only one hemp seed each day for six years. This apocryphal story reveals a seed of truth since hemp seeds possess an extremely high nutritional value due to a high content of unsaturated fatty acids (about 80% of fatty acids) and proteins (about 25%) [2]. Moreover, it has been suggested that other minor components, such as terpenes and cannabinoids, could contribute to the surprising beneficial effects of hemp seeds [3].

After removal of the bracts, the seed is squashed or macerated, and finally pressed at high pressure to give the hemp seed oil and a mushy residue, the "husk", which is used as fertilizer or compressed into tablets and used for cattle feed. The hemp seed oil obtained by cold pressing finds its main use as food. The hemp seed oil has a pleasant nutty flavour, sometimes with a hint of a bitter aftertaste. Polyunsaturated fatty acids (PUFA) constitute about the 80% of the total fatty acids, including linoleic, α- and γ-linolenic acid, which offer the best omega-6 to omega-3 PUFA ratio considered optimal for nutrition [4]. Cold-pressed hemp seed oil may also serve as dietary source of natural antioxidants (γ-tocopherol, vitamin E, etc.) for disease prevention and health promotion [4, 5]. Therefore, the presence of antioxidants is a warranty of a cold-pressed oil, thus of chemical stability against oxidative rancidity and a consequent relatively short shelf life.

Although hemp seeds do not contain any cannabinoid, their contact with the resin secreted by the epidermal glands located on flowers and leaves and/or a bad selection of the bracts of the perigonium, which have the highest cannabinoid content, can cause the presence of the latter in the hemp oil. Hence, cannabinoids actually represent "impurities" of the hemp seed oil. Their concentration depends from both the cultivar and the cleaning process of the seed. The concentrations of cannabinoids can be extremely variable among different oil varieties. Cultivars of *Cannabis sativa* (CS) that can be used in Europe for seed production are those authorized with a level of tetrahydrocannabinol (THC) lower than 0.2%. As a result, THC contamination in hemp seed oil is generally extremely low and only exceptionally it exceeds the limit of 5 mg/kg (maximum THC limit in food imposed by German legislation in 2000) [6-8]. It is reasonable that such limit can be met in

northern Europe, but it is known that thermal stress that occurs in southern Europe could easily raise the value of 5 mg/kg. In fact, the European Industrial Hemp Association has proposed a less strict THC limit of 10 mg/kg in hemp seed oil and specified that the oil must be consumed without heating, since heat would raise the THC content [8].

CS cultivars employed for seed production with a low THC level generally contain a high concentration of cannabidiol (CBD). The latter is void of the THC psychotropic activity and is one of the most studied cannabinoids with proven anti-inflammatory, antiepileptic, analgesic, neuroprotective, antipsychotic and anticonvulsant efficacy [9-13]. Besides, it is also a potent antioxidant [14]. It has been hypothesized that the nutraceutical properties of hemp seed oil are not due only to its equilibrated PUFA content but mainly to the presence of CBD [3].

Anyway, a purely chemical clarification is needed: CBD is not a biosynthetic product of CS. In fact, CS plant synthesises cannabigerolic acid (CBGA) from olivetolic acid and geranyl pyrophosphate [15]. CBGA can undergo transformations catalysed by enzymes such as tetrehydrocannabinolic acid-synthase (THCA-synthase), which leads to tetrahydrocannabinolic acid (THCA), and cannabidiolic acid-synthase (CBDA-synthase), which leads to cannabidiolic acid (CBDA) [15]. There exist varieties of CS selected for the lack of THCA-synthase, which produce only CBDA. They are called "legal", thus they can be cultivated for fibre and seed production.

Cannabinoid acids like THCA, CBDA, CBGA, etc. undergo a decarboxylation process whose rate depends on different factors, but mainly the higher the temperature the faster the process. This decarboxylation finally leads to the formation of the corresponding neutral cannabinoids, THC, CBD, CBG, etc., which is not due to the action of enzymes, but to a simple chemical reaction or, more precisely, to a decomposition catalysed by heat. The conversion also takes place at room temperature, but it is much slower.

Anyway, the ratio between CBDA and CBD concentration in hemp seed oil could represent a valuable marker of both the storage conditions and the production process. To this end, 13 different commercial hemp seed oils obtained by cold pressing were analysed in order to determine the content of CBDA, CBD, cannabinol (CBN), cannabidivarin (CBDV), cannabigerol (CBG), THC and THCA. CBN originates from cannabinolic acid (CBNA), which in turn derives from the oxidation of THCA as a consequence of an incorrect storage (exposure to air) [16].

The analysis of cannabinoids in hemp food products is of great importance in order to guarantee the absence of any psychotropic or intoxicant component for human health. The analysis can be performed by several methods, but the most widely employed are gas chromatography coupled to flame ionization detector (GC-FID) or mass spectrometry detector (GC-MS) and high performance liquid chromatography coupled to UV (HPLC-UV) or mass spectrometry detector (HPLC-MS) [17]. In particular, very few works have been published on the analysis of cannabinoids in hemp food products, most of which carried out by GC-MS [18-21].

The present work focuses on the development and validation of a rapid and sensitive HPLC-UV for the identification and quantification of the main cannabinoids, namely CBDA, THCA, CBD, THC, CBG, CBN and CBDV, in commercially available hemp seed oils. The molecular structure of all the aforementioned cannabinoids and IS is reported in Figure 1. Moreover, since CBDA to CBD ratio can be taken as a marker of cold pressing and good storage conditions, the decarboxylation kinetics of CBDA to CBD in hemp seed oil has been investigated in both open and closed reactor.

2. Materials and methods

2.1 Chemicals and reagents

Acetonitrile, water, 2-propanol, formic acid LC-MS grade were purchased from Carlo Erba (Milan, Italy). THCA, CBDA, CBDV, THC, CBD, CBG and CBN were purchased from Cerilliant (Sigma Aldrich, Round Rock, Texas). Organic hemp seed oils were bought from the normal commercial cycle of the Italian territory and numbered from H1 to H13.

2.2 HPLC-UV and HPLC-MS analysis

HPLC analyses were performed on an Agilent Technologies (Waldbronn, Germany) modular model 1200 system, consisting of a vacuum degasser, a binary pump, a thermostated autosampler, a thermostated column compartment and a diode array detector (DAD). A Poroshell 120 EC-C18 column (3.0 × 100 mm, 2.7 µm, Agilent, Milan, Italy) was used with a mobile phase composed of 0.1% formic acid in both (A) water and (B) acetonitrile. The chromatographic conditions were set as follows: 0.0-10.0 min linear gradient from 70 to 80% B; 10.0-10.1 min from 80 to 95% B; 10.1-11.0 min isocratic elution with 95% B; 11.0-11.1 min from 95 to 70% B for equilibration of the column with the initial conditions. The total run time was 15 min. The flow rate was set at 0.4 mL/min. The column temperature was set at 25° C. The sample injection volume was 5 µL. The UV/DAD acquisitions were carried out in the range 190-500 nm and chromatograms were acquired at 228 nm. Three injections were performed for each sample.

HPLC-MS analyses were carried out in order to ensure the identity of the peaks and were performed on an Agilent Technologies (Waldbronn, Germany) modular model 1200 system, consisting of a vacuum degasser, a binary pump, a thermostated autosampler, a thermostated column compartment and a 6540 quadrupole time of flight (QToF) mass spectrometer operating in both positive (ESI+) and negative ionization (ESI–) mode. The mass spectrometer experimental parameters were set as follows: the capillary voltage was 3.5 kV, the nebulizer (N_2) pressure was 35 psi, the drying gas temperature was 350 °C, the drying gas flow was 11 L/min and the skimmer voltage was 40 V. Data were acquired by Agilent Mass Hunter system software (version 6.0). The mass spectrometer was operated in full-scan mode in the *m/z* range 50-700. MS/MS spectra were automatically performed with nitrogen as the collision gas in the *m/z* range 50-700, using the auto MS/MS function and a collision energy of 20 eV. Extracted ion chromatograms (EICs) were obtained with an accuracy of 10 ppm *m/z* from total ion chromatogram (TIC) employing the *m/z* corresponding to the molecular ions $[M+H]^+$ 359.2217 for CBDA and THCA, 315.2300 for CBD and THC, 287.1998 for CBDV, 317.2468 for CBG and 311.2024 for CBN. In ESI– the molecular ions $[M–H]^–$ considered were 357.2164 for CBDA and THCA, 313.2012 for CBD and THC, 285.1830 for CBDV, 315.2385 for CBG and 309.1902 for CBN.

2.3 Preparation of standard solutions

Blank matrix was obtained by washing hemp seeds (22 g cleared of bracts) with ethyl alcohol (3 × 100 mL) in order to remove cannabinoids. Then the seeds were cold squeezed to obtain 4 mL of cannabinoid-free hemp seed oil (concentration of cannabinoids <LOD). The final blank matrix (20 mL) was obtained by diluting the oil with 16 mL of 2-propanol. A stock solution of IS (5000 µg/mL) was prepared by dissolving 50 mg in 10 mL of 2-propanol. IS working solution was prepared by further diluting the stock solution in mobile phase to 500 µg/mL. Stock solution of CBDV, CBDA,

CBG, CBD, CBN, THC and THCA (1000 µg/mL) in methanol were properly diluted in blank matrix to obtain samples of 10, 25, 50, 75 and 100 µg/mL. 100 µL of each sample were diluted in 890 µL of blank matrix and 10 µL of IS (500 µg/mL) to the final concentrations of 1.0, 2.5, 5.0, 7.5 and 10 µg/mL for CBDV, CBDA, CBG, CBD, CBN, THC and THCA and 5 µg/mL for IS. Independently prepared CBDV, CBDA, CBG, CBD, CBN, THC and THCA stock solutions of 12.5, 50 and 80 µg/mL were used for the preparation of low concentration quality control (LQC) (1.25 µg/mL), medium concentration quality control (MQC) (5.00 µg/mL), and high concentration quality control (HQC) (8.00 µg/mL) samples, respectively. QC samples were prepared as for calibration standards.

2.4 Method validation

In order to demonstrate the reliability and robustness of the HPLC-UV method, full method validation was carried out based on EMEA guidelines and in agreement with international guidelines for analytical techniques for the quality control of pharmaceuticals (ICH guidelines) [22]. Specifically, the method was validated in terms of specificity and selectivity, linearity, limit of detection (LOD) and limit of quantification (LOQ), precision and accuracy, recovery and matrix effect, dilution integrity and stability.

2.4.1 Specificity and selectivity

Moreover, identification was assessed by comparing accurate (within 5 ppm error) *m/z* of $[M+H]^+$ and MS/MS spectra of authentic standards with those obtained by HPLC-MS and MS/MS of hemp seed oil samples.

2.4.2 Linearity

Calibration curve was constructed at five calibration levels 1.0, 2.5, 5.0, 7.5 and 10 µg/mL of CBDV, CBDA, CBG, CBD, CBN, THC and THCA, and 5 µg/mL of ibuprofen (IS). Peak area ratios of cannabinoid-to-IS were plotted *versus* nominal concentrations. Calibration curve was evaluated at the beginning of each validation day of five consecutive days (n = 5). Linearity was assessed through evaluation of the coefficient of determination (R^2), which should be greater than 0.998 using weighted regression method (1/x).

2.4.3 Limit of detection (LOD) and limit of quantification (LOQ)

Limit of detection (LOD) was estimated based on a 3:1 signal-to-noise ratio. Standard stock solutions of all analytes were appropriately diluted at the levels of these estimated values and were analysed repeatedly six times. Limit of quantification (LOQ) was estimated based on a 10:1 signal-to-noise ratio. Standard stock solutions of all analytes were appropriately diluted at the levels of these estimated values and were analysed repeatedly six times.

2.4.4 Precision and accuracy

The precision and accuracy were evaluated at three levels, LQC (1.25 µg/mL), MQC (5.00 µg/mL), and HQC (8.00 µg/mL). Each sample was analysed repeatedly three times within a single day to determine the intra-day precision and accuracy, and three times a day for five successive days (n = 15) to determine the inter-day precision and accuracy. The precision was expressed as the coefficient of variance (RSD), and the accuracy was expressed as the percentage of mean calculated to nominal concentration.

2.4.5 Recovery

Recovery was evaluated using authentic hemp seed oil samples spiked at three different levels. To this end, a standard solution at three different concentrations was added to the authentic oil sample. Recovery (*R*) was calculated by the following formula:

$$R = 100 \times [(C_S - C_H)/C_{STD}]$$

where C_S is the concentration of the sample after spiking with the standard solution, C_H is the concentration without spiking the standard solution and C_{STD} is the concentration of the standard solution.

2.4.6 Matrix effect

Matrix effect was evaluated for each analyte analyzing the QC samples spiked at low, medium and high concentrations. ME values were calculated by dividing the analyte peak area in QC samples spiked after the SPE procedure (post-extraction addition) to the response for neat standards. Matrix effect was calculated as matrix factor (*MF*) by the formula:

$$MF\% = \left(\frac{A_M - A_S}{A_S}\right) x\ 100$$

Where A_M is the area of the peak (normalized for the area of the IS) in the matrix and A_S is the area of the peak (normalized for the area of the IS) of neat standards.

2.4.7 Dilution integrity

Dilution integrity was demonstrated by spiking the blank matrix at 2, 10 and 25 times the HQC concentration (16, 80 and 200 µg/mL, respectively). Six replicates per dilution factor were prepared and diluted with blank matrix. The concentrations were calculated by applying the dilution factor 2, 10 and 25 against freshly prepared calibration curve.

2.4.8 Stability

The autosampler stability was determined for LQC and HQC samples in blank matrix diluted samples for 24 h at 10 °C (n = 3). The drugs were considered stable if the mean concentration was within ±10% of the original concentration.

2.5 Authentic hemp seed oil sample preparation

100 µL of hemp seed oil were diluted with 395 µL of 2-propanol and 5 µL of IS solution. The solution was vortex mixed for 1 min at maximum speed and directly injected into the HPLC system.

2.6 Decarboxylation studies

2.6.1 Open reactor

500 µL of hemp seed oil was placed in 10 open vials and put in an oven at 80 °C. One vial was taken out every 15 min. The oil was let to cool down to room temperature and 100 µL were diluted as descripted above and analysed by HPLC-UV (n = 3). The experiment was repeated at 90, 100, 110 and 120 °C.

2.6.2 Closed reactor

500 µL of hemp seed oil was placed in 10 ampoules, which were flame sealed and put in an oven at 120 °C. One ampoule was taken out every 15 min. The oil was let to cool down to room temperature and 100 µL were diluted as descripted above and analysed by HPLC-UV (n = 3).

2.6.3 Calculation of kinetic parameters and free energy barriers of decarboxylation

The rate constant of decarboxylation (*k*) refers to a first-order kinetic process. Therefore, *k* was calculated by plotting the linear logarithm values of unconverted CBDA (in percentage) (*x* axis) *versus* time (*y* axis), according to the following equation Eq. (1):

$$\ln[CBDA] = -kt \quad \text{Eq. (1)}$$

Where *k* is the decarboxylation rate constant (s^{-1}) and *t* is the decarboxylation time (s). The slope of linear equation corresponds to –*k*. The half-life of decarboxylation is the time in which 50% of the molecules of CBDA are decarboxylated into CBD (CBDA concentration is reduced by 50%). For a first-order reaction the half-life can be calculated by the Eq. (2):

$$t_{1/2} = \frac{\ln 2}{k} \quad \text{Eq. (2)}$$

By plotting the logarithmic values of *k* on the *y* axis and 1/*T* (K^{-1}) on the *x* axis, the slope of the equation corresponds to $-\frac{E_A}{R}$, where E_A is the activation energy and *R* the universal gas constant (8.31441 J/mol·K), and the intercept is ln*A*, where *A* is the pre-exponential or frequency factor. The corresponding equation is:

$$\ln k = \ln A - \frac{E_A}{RT} \quad \text{Eq. (3)}$$

In the transition-state theory, the influence of the temperature on reaction rates can be expressed by Eq. (4):

$$k = \left(\nu\, e^{\frac{\Delta S}{R}}\right) e^{-\frac{\Delta E_A}{RT}} \quad \text{Eq. (4)}$$

where the frequency of decomposition of the transition-state complex, *v*, may vary depending on the nature of the reactants and is calculated by the following Eq. (5):

$$\nu = \frac{RT}{Nh} \quad \text{Eq. (5)}$$

where *N* is the Avogadro's constant (6.0221409×10^{23}) and *h* is the Plank's constant (6.626176×10^{-34} J·s). In this theory, the Arrhenius factor *A* is related to the entropy ($\Delta S^{\#}$) of activation of the transition state:

$$A = \nu e^{\frac{\Delta S}{R}}$$

Eq. (6)

The Arrhenius equation was also applied for the extrapolation of k and $t_{1/2}$ at 25, 20 and 5 °C.

From the kinetic rate constants obtained for all the temperatures, the corresponding activation energies of decarboxylation (free energy barriers, $\Delta G^{\#}(T)$) can be calculated by the Eyring equation (Eq. (7)):

$$\Delta G^{\#}(T) = -RT \ln\left(\frac{kh}{\kappa k_B T}\right)$$

Eq. (7)

Where k_B is the Boltzmann's constant (1.380662×10^{-23} J·K^{-1}), and κ is the transmission coefficient (1 for irreversible microscopic interconversion, as in this case, and 0.5 for reversible microscopic interconversion). In accordance with the Eyring equation (Eq. (8)), the enthalpic and entropic terms can be calculated by plotting the logarithmic values of k/T on the y axis and $1/T$ on the x axis:

$$\ln\left(\frac{k}{T}\right) = -\frac{\Delta H}{RT} + \ln\left(\frac{k_B}{h}\right) + \frac{\Delta S}{R}$$

Eq. (8)

3. Results and discussion

3.1 Chromatographic conditions

One aim of the present work was to develop a rapid and sensitive analytical method for the determination of the main cannabinoids in hemp seed oil. The fused core technology provides better chromatographic performance with respect to fully porous stationary phases for the separation of this class of compounds, as demonstrated by our previous work [23]. Therefore, in a first attempt, Poroshell 120 EC-C18 (3.0 × 50 mm, 2.7 μm) was used for the experiments. Using a mixture of analytical standards (CBDV, CBDA, CBG, CBD, CBN, THC and THCA) diluted in blank matrix at the concentration of 5 μg/mL, an isocratic elution with 70% B at a flow rate of 0.5 mL/min was attempted. In these conditions, a good separation was obtained for CBN, THC and THCA, as expected, but no separation was obtained between CBDA, CBG and CBD. By varying the amount of the strong solvent and the flow rate other isocratic conditions were tried, but with no significant improvements. Hence, different gradient elution conditions were attempted. The optimal conditions were set as follows: linear gradient from 70 to 80% B in 10 min, rapid increase of B from 80 to 95% in 0.1 min, held for 1 min, and then back to the initial conditions in 0.1 min (70% B), leaving an equilibration time of 4 min. The flow rate was maintained at 0.4 mL/min throughout the chromatographic run. By switching to a longer Poroshell column (3.0 × 100 mm, 2.7 μm), a significant improvement of the chromatographic performance was achieved in terms of symmetry, resolution and number of theoretical plates of the main chromatographic peaks, especially of the co-eluting cannabinoids (CBDA, CBG and CBD). Therefore, it was chosen for this study. The data of retention time and maximum absorption wavelength (λ_{max}) are reported in the Supplementary Material (Table S1). The spectroscopic data obtained for CBDA, CBD, CBN, THC and THCA in our previous work were confirmed in this study [23].

3.2 Method validation

As shown in Table S2, linear calibration curves were obtained where the coefficient of determination (R^2) was greater than 0.994 over the concentration range 1-10 μg/mL for CBDV, CBDA, CBG, CBD, CBN, THC and THCA in blank matrix. 0.2 μg/mL and 1 μg/mL were set as the LOD and LOQ respectively. LOD and LOQ refer to the actual limits of the oil as sold (before sample preparation). Representative chromatograms of a blank sample spiked with IS (5 μg/mL) and of a blank sample spiked with IS (5 μg/mL) and analytes standards at LOQ (1 μg/mL) are reported in Figure 2 in panels A and B, respectively. All the other details about method validation are reported in the Supllementary Material.

3.3 Authentic hemp seed oil sample preparation and analysis

The concentrations of cannabinoids can be extremely variable among different oil varieties. Cultivars of CS that can be used in Europe for seed production are those authorized with a level of THC lower than 0.2% (w/w). As a result, THC contamination in hemp seed oil should be sufficiently low to not have intoxicating effect. Only three countries so far have imposed a national limit for THC in food. The lowest THC limit in Europe of 5 mg/kg is recommended by Germany [6-8], whereas Switzerland and Belgium maintain higher levels of THC, 20 and 10 mg/kg, respectively [8]. In this context, it is important to develop a highly sensitive, but at the same time rapid method in order to detect even low traces of cannabinoids. In the present work, 13 different hemp seed oils were analysed for their cannabinoid content. In order to achieve a high sensitivity with a photodiode array detector, we attempted different dilution options. Our previously developed method for medicinal cannabis was not suitable for this purpose since the sensitivity reached was only of 180 mg/kg [23], which is far beyond the European average limit. Therefore, by gradual addition of 100 μL aliquots of 2-propanol to 100 μL of hemp seed oil, the optimal dilution resulted to be a 1:5 (v/v) ratio of oil:2-propanol (400 μL total volume of 2-propanol) to reach a sensitivity of 1 mg/kg in real oil sample. By injecting 5 μL of the oil/alcohol solution into the HPLC system a good peak shape was obtained. The results of the analyses of the cannabinoids in hemp seed oils are reported in Table 1. A representative chromatogram of an authentic hemp seed oil sample spiked with IS is reported in Figure 2 panel C. The data obtained indicated that oils H1, H4, H5, H6, H7 and H9 contained a low amount of CBDA and CBD, which means an amount of total CBD [calculated as (CBDA × 0.877)+CBD] lower than 5 mg/kg. On the other hand, these oils, with the exception of H9, which is poor of cannabinoids, contained between 5 and 10 mg/kg of total THC [calculated as (THCA × 0.877)+THC]. These values exceeded the German THC limits in hemp seed oil, but only if the limit refers to the sum of THC and THCA. The abovementioned oils did not contain a high content of neutral cannabinoid THC (<2 mg/kg), but a high content of THCA, which should not be considered intoxicating if the oil is consumed as such (without heating). H2, H3, H8, H10, H11, H12 and H13 resulted with a high CBDA and CBD content and an amount of total THC below the LOQ. Looking at the CBDA/CBD ratio, which can be considered as a marker of cold pressing and good storage conditions, most oils showed a low value in the average of 3.7:1. Conversely, H4, H5 and H6 showed a very high CBDA/CBD ratio in the average of 14:1. H11, in particular, was stored for one year at room temperature; it fact, it displayed a CBDA/CBD value below 1. These results suggested a correlation between CBDA/CBD ratio and the oil age, but further investigations are needed to have the scientific proof.

3.4 Decarboxylation studies

Very few studies have been reported in the literature on the decarboxylation kinetics of cannabinoids in CS derived products. The decarboxylation kinetics of THCA in *Cannabis Flos* has been studied by Perrotin-Brunel *et al.* in a vacuum system [24]. The reaction was described as a pseudo-first order catalysed by formic acid, which encounters a keto-enol structure in the transition state. The decarboxylation of THCA and CBDA was also investigated in open reactor in the absence of solvent by T. Veress in 1990 [25]. In order to give an estimation of the stability of cannabinoid acids in hemp seed oil, a part of the work was focused on the evaluation of the influence of the temperature on the decarboxylation rate in this matrix. Given the very low amount of THCA or any other cannabinoid acid in hemp seed oil, we focused our attention on the most abundant of this class of compounds, CBDA. Oil H5 was chosen for the experiments as it contained a CBD amount below the LOD. Decarboxylation studies were conducted in both open and closed reactor. As shown in Figure 3, while the decrease of CBDA due to the decarboxylation process can be assumed as a pseudo-first order reaction, the consequent formation of CBD does not appear to follow the same trend as its precursor. In details, at temperatures below 100 °C the sum of CBD and CBDA in concentration remained constant (the registered loss was only 1% and 2% for the decarboxylation of CBDA at 80 and 90 °C respectively). It could be hypothesised that below this temperature the decarboxylation of CBDA leads only to the formation of CBD. At 100 °C or above, other processes could be involved, including either the formation of unknown side products or the evaporation of the neutral cannabinoid. In fact, the higher the temperature the lower the CBD formed (and the sum of the two forms). At 100 °C the loss of the total molar concentration was about 20%. At 110 and 120 °C in open reactor, there was no linear increase of CBD or decrease of CBDA, suggesting a more complex chemistry. The loss of the total molar concentration was about 30% at 110 °C, whereas it was two times higher at 120 °C. Decarboxylation in closed reactor displayed a linear decrease of CBDA and simultaneous formation of CBD, but the sum of the concentrations showed a loss of about 11%. Figure 4 represents the linear regressions of the decarboxylation kinetics at each temperature in open reactor (80, 90, 100, 110 and 120 °C) and at 120 °C in closed reactor (sealed ampoules). From the obtained equations, the kinetic parameters have been calculated as described in the methods section and reported in Table 2.

The Eyring equation (Eq. (5)) was then employed to calculated the thermodynamic parameters $\Delta H^{\#}$ and $\Delta S^{\#}$ (enthalpy and entropy) of the decarboxylation process in open reactor, which resulted to be about 86.4±3.4 kJ·mol^{-1} and -85.9±9.7 J·mol^{-1}K^{-1}, respectively. The contribution of $\Delta S^{\#}$ is very low (negative value) with respect to that of $\Delta H^{\#}$, indicating that the free energy barrier depends exclusively on the enthalpic factor (Figure 5).

The Arrhenius plot was built to calculate the activation energy, which resulted 89.5±3.6 kJ/mol, and *A*, which was 6.88×10^{8} s^{-1} (Eq. (3)).

Our results were compared with those reported by Perrotin-Brunel *et al.* for THCA [24]. The rate constant *k* at 100 °C and 120 °C was about 5×10^{-4} s^{-1} and 21×10^{-4}, indicating a decarboxylation rate respectively twice and three times higher than those obtained for CBDA in this study. Perrotin-Brunel *et al.* [24] and Wang *et al.* [26] reported a value of *A* of 3.7×10^{8} s^{-1} and 8.7×10^{8} s^{-1}, respectively. Our results for *A* (6.88×10^{8} s^{-1}) confirmed that the lower rate constant for the decarboxylation of CBDA is not due to the entropy factor (to which *A* is related). The data obtained for *k* of CBDA decarboxylation are also in accordance with the values reported by Wang *et al.* [26] at the temperatures considered.

The Arrhenius plot also allowed to extrapolate k and $t_{1/2}$ of decarboxylation at 25, 20 and 5 °C in order to provide an indication of stability of CBDA in hemp seed oil during common storage conditions (Figure 6). The decarboxylation rate constant at 25 °C $k(25)$ was $1.66 \pm 0.99 \times 10^{-7}$ s^{-1} and the $t_{1/2}(25)$ was about 49 days (less than 2 months). Decreasing the temperature by only 5 °C, the resulting $k(20)$ was one order of magnitude lower than $k(25)$, $9.20 \pm 5.97 \times 10^{-8}$, and $t_{1/2}(20)$ was about two times higher than that at 25 °C, i.e. 87 days (about 3 months). If the hemp seed oil is stored in the fridge (5 °C), the decarboxylation rate constant $k(5)$ becomes $1.37 \pm 1.07 \times 10^{-8}$, which is translated in a $t_{1/2}(5)$ of 587 days (about 20 months).

4. Conclusions

A highly sensitive and rapid HPLC-UV method was developed and validated for the qualitative and quantitative determination of the main cannabinoids present in commercial hemp seed oils. The developed method could be easily employed by any analytical laboratory to assess the safety and quality of hemp seed oil before its commercialization. The analysis can provide not only quantitative data of the cannabinoids content, but also qualitative information about the purity of the oil (e.g. CBDA/CBD ratio can be taken as a marker of cold pressing and good storage conditions).

The decarboxylation kinetics studies of CBDA, which is the main acid cannabinoid present in hemp seed oil, provided useful suggestions for the best storage conditions (5 °C) in order to preserve the stability of hemp seed oil for longer time.

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LEGEND TO FIGURES

Figure 1. Molecular structure of ibuprofen (IS), cannabidivarin (CBDV), cannabidiolic acid (CBDA), cannabigerol (CBG), cannabidiol (CBD), cannabinol (CBN), tetrahydrocannabinol (THC) and tetrehydrocannabinolic acid (THCA).

Figure 2. High-resolution MS/MS spectra of the cannabinoids under investigation acquired in ESI+.

Figure 3. High-resolution MS/MS spectra of the cannabinoids under investigation acquired in ESI–.

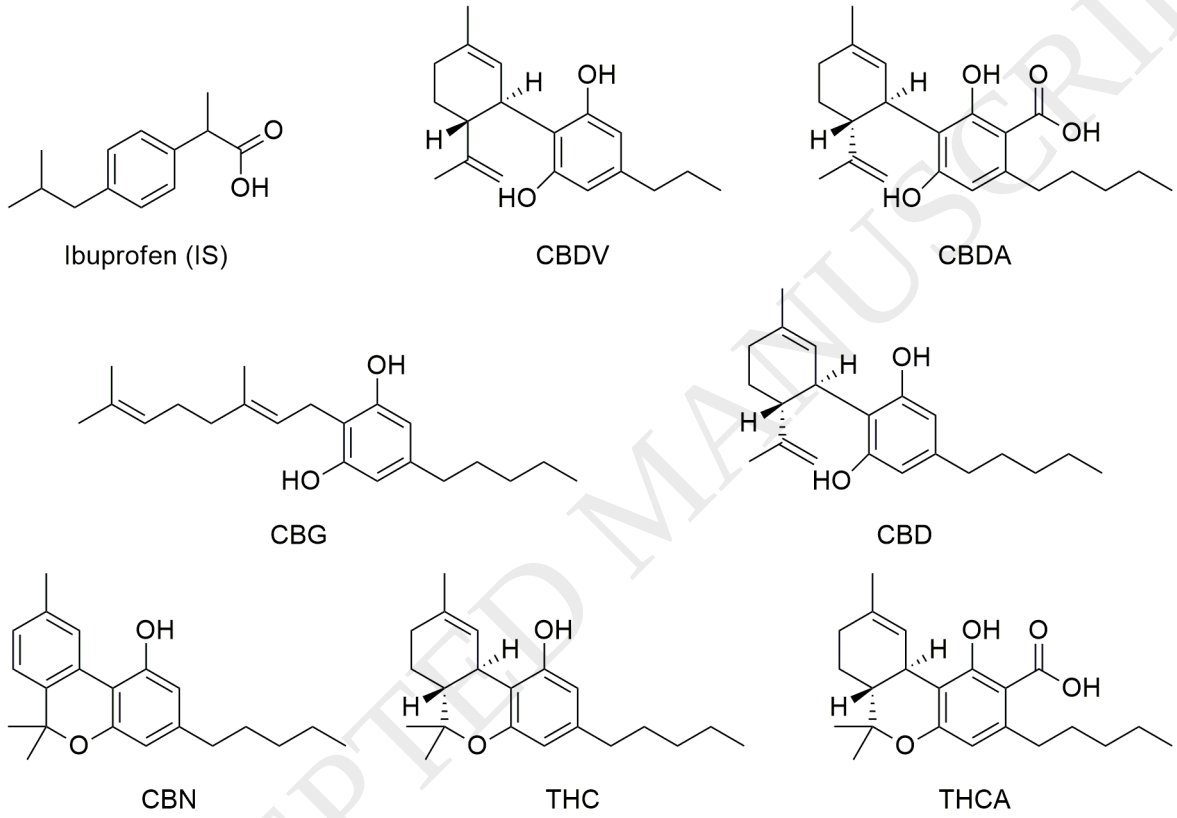
Figure 4. A) HPLC-UV chromatogram of blank sample spiked with IS (5 µg/mL). **B**) HPLC-UV chromatogram of analytical standards and IS mixture at LOQ (IS 5 µg/mL, analytes standards 1 µg/mL). **C**) HPLC-UV chromatogram of an authentic hemp seed oil sample (H8).

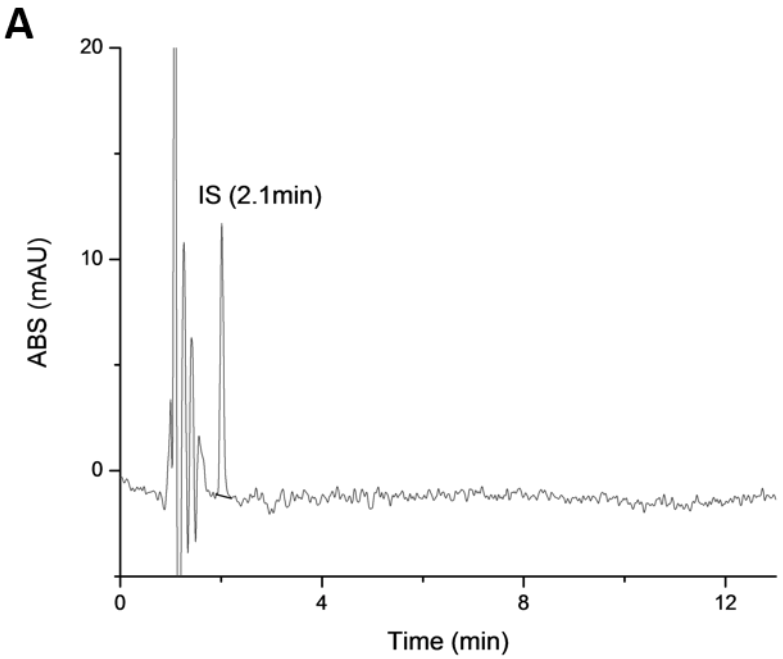
Figure 5. Decarboxylation of CBDA at 80 (**A**), 90 (**B**), 100 (**C**), 110 (**D**) and 120 °C (**E**) in open reactor and at 120 °C in closed reactor (**F**). Values are expressed as concentration (µg/mL) *vs* time (min). Non-linear fitting curves of CBD and sum of CBDA and CBD are also shown.

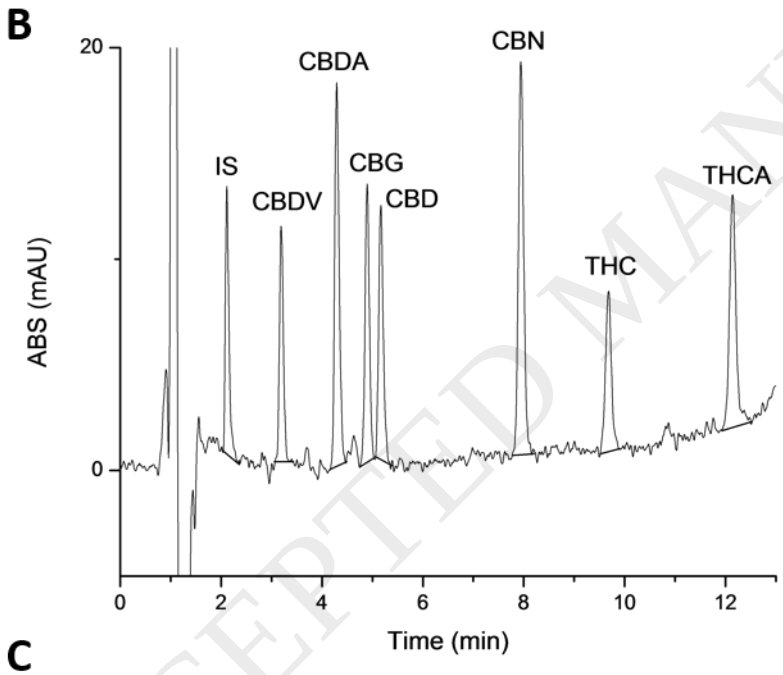
Figure 6. Decarboxylation kinetics of CBDA in hemp seed oil in open and closed reactor.

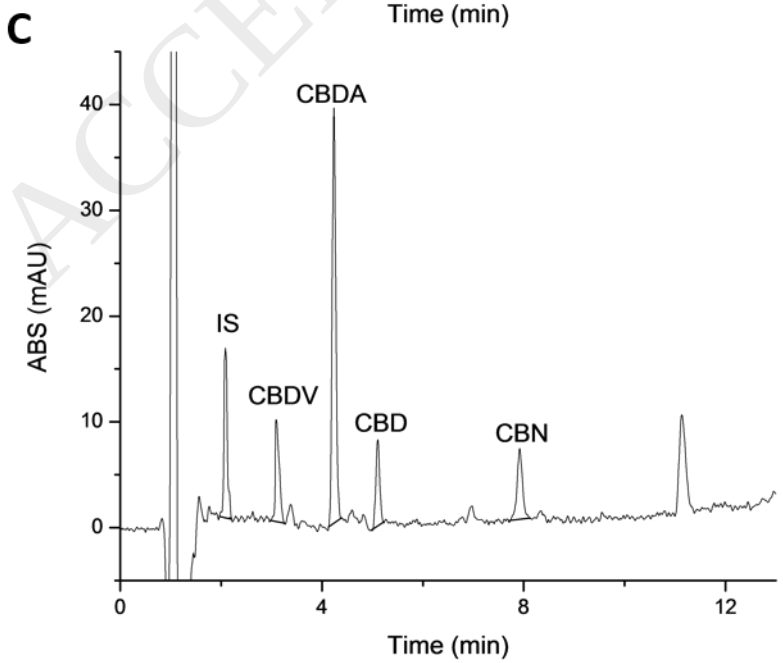
Figure 7. Eyring plot for the calculation of the thermodynamic parameters of CBDA decarboxylation.

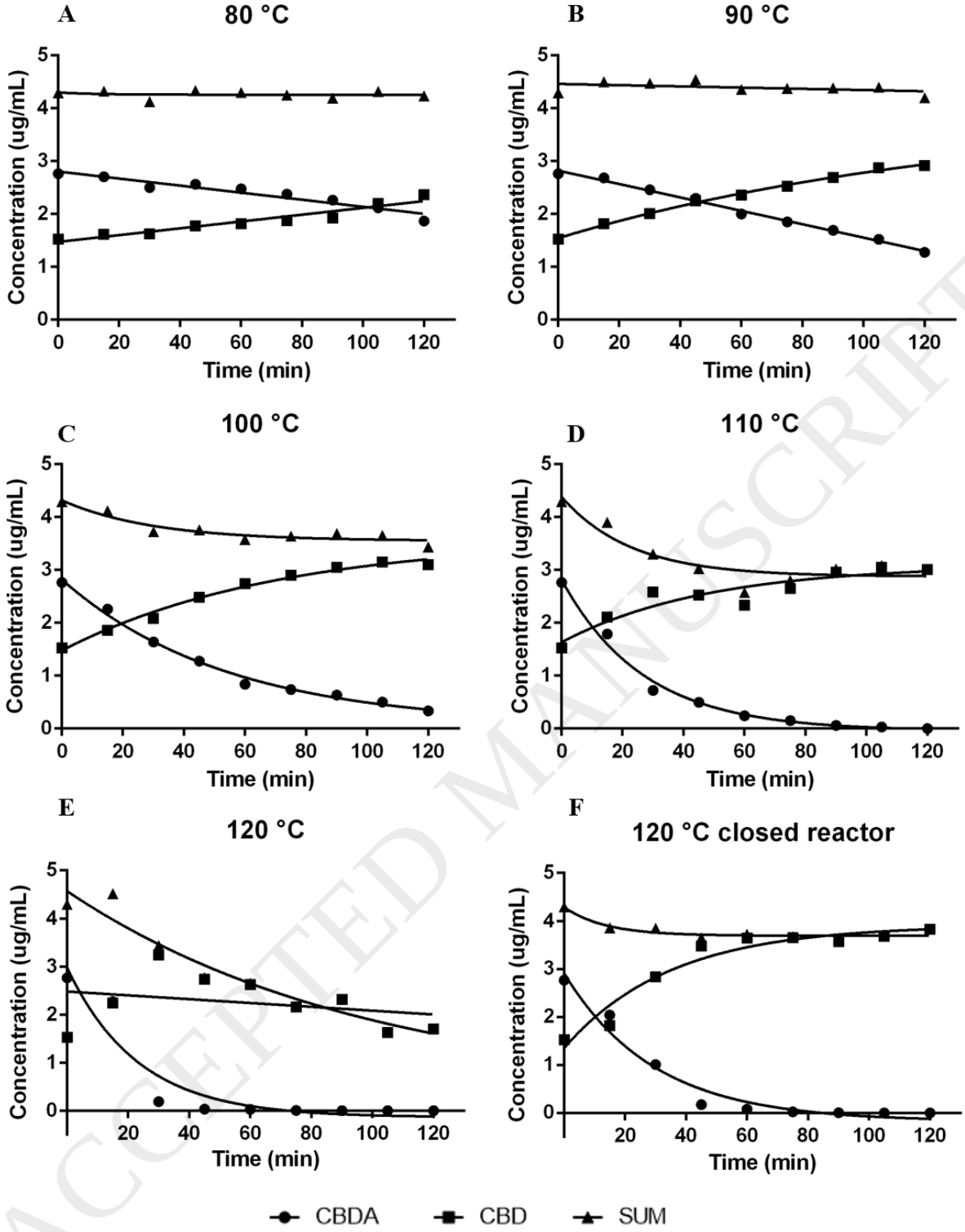
Figure 8. Arrhenius plot for the calculation of the activation energy and the extrapolation of *k* at 25, 20 and 5 °C.

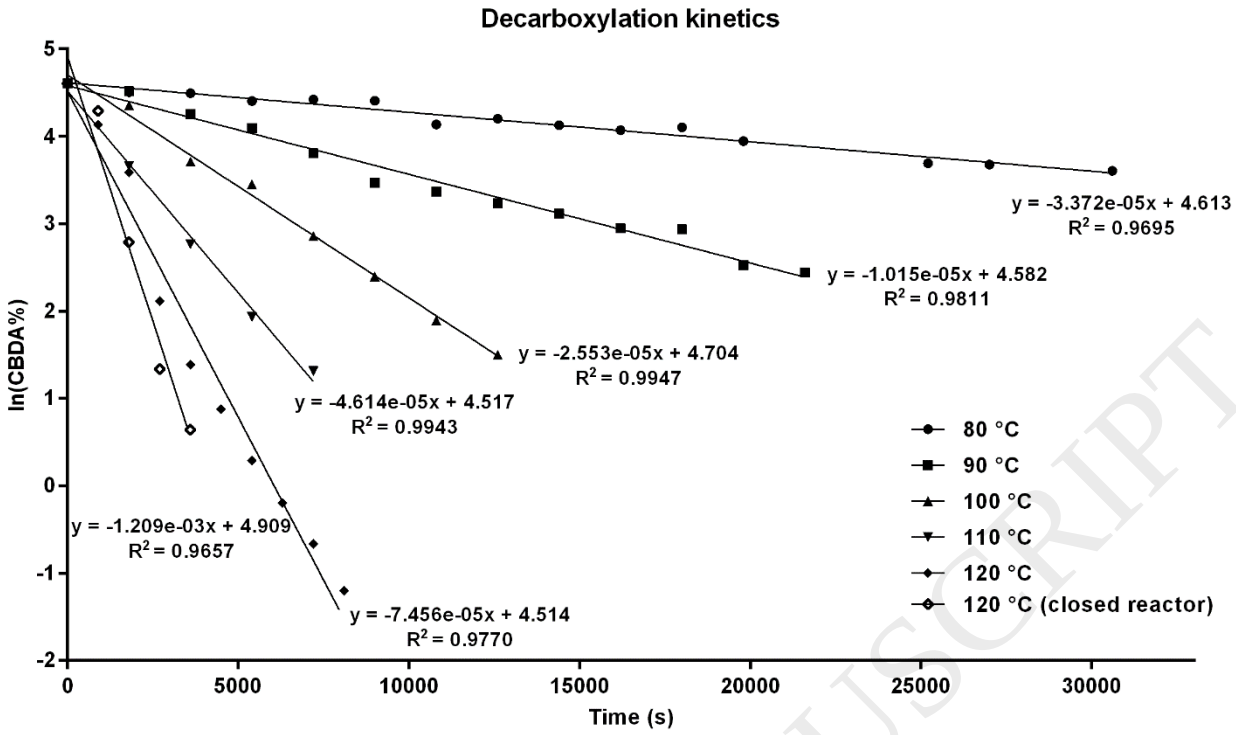


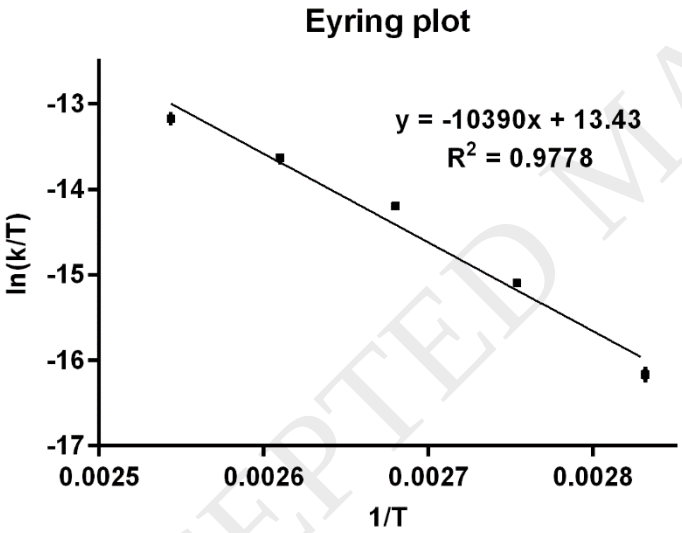


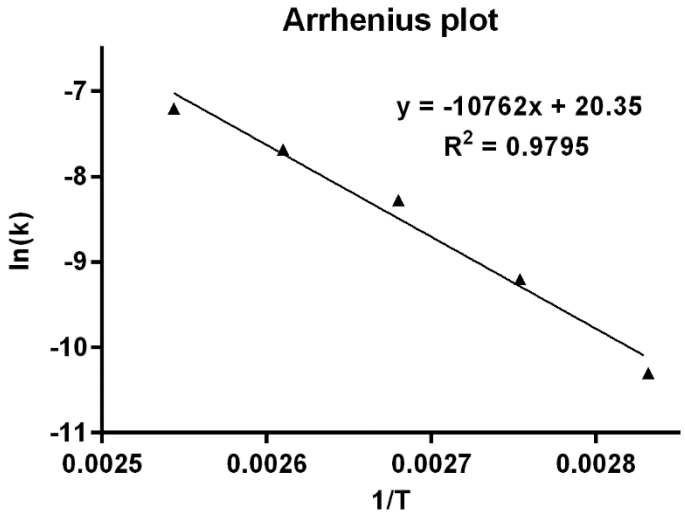












Tables

Table 1. *Amount of the main cannabinoids in 13 different commercial hemp seed oils. The values are expressed in ppm as mean ± standard deviation (n = 3).*

Oil	mg/Kg							
	CBDV	**CBDA**	**CBG**	**CBD**	**CBN**	**THC**	**THCA**	**CBDA/CBD**
H1	8.769±0.080	<LOQ	<LOD	<LOD	1.004±0.199	1.429±0.117	5.525±0.113	2.3±0.5[a]
H2	5.984±0.052	31.93±1.051	<LOD	4.801±0.270	1.460±0.159	<LOQ	<LOQ	6.7±0.5
H3	6.061±0.015	41.59±0.506	<LOD	8.658±0.410	12.41±0.850	<LOD	<LOQ	4.8±0.3
H4	7.741±0.057	2.575±0.442	<LOD	<LOD	2.060±0.340	1.804±0.317	8.883±0.408	12.9±2.2[a]
H5	6.273±0.023	3.641±0.128	<LOD	<LOD	3.227±0.230	<LOQ	4.913±0.674	18.2±0.6[a]
H6	6.352±0.037	2.265±0.203	<LOD	<LOD	1.642±0.134	1.042±0.036	7.074±1.253	11.3±1.0[a]
H7	6.770±0.035	3.178±0.243	<LOD	1.545±0.387	2.046±0.455	<LOQ	9.462±0.514	2.1±0.5
H8	<LOQ	24.33±0.387	<LOD	5.743±0.348	1.217±0.054	<LOQ	1.473±0.093	4.3±0.3
H9	7.448±0.009	3.509±0.151	<LOD	1.637±0.091	<LOQ	<LOQ	<LOQ	2.2±0.2
H10	6.370±0.112	22.26±0.696	<LOQ	6.525±0.027	8.398±0.757	<LOQ	<LOQ	3.4±0.1
H11	75.39±0.596	821.1±13.22	<LOQ	1056±21.84	8.470±0.466	<LOQ	<LOQ	0.7±0.0
H12	64.89±0.156	233.8±10.85	1.283±0.960	60.26±2.716	12.05±1.335	<LOQ	<LOQ	3.9±0.1
H13	66.27±0.345	195.7±2.987	1.381±0.044	56.21±3.985	8.956±0.468	<LOQ	<LOQ	3.5±0.2

[a] When CBD concentration was too low, we considered the LOD value to calculate the CBDA/CBD ratio.

Table 2. *Kinetic parameters (k and $t_{1/2}$), linear regressions with R^2, free energy barriers ($\Delta G^{\#}(T)$) and activation entropy ($\Delta S\#(T)$) of the decarboxylation of CBDA in hemp seed oil at each temperature in open reactor (80, 90, 100, 110 and 120 °C) and at 120 °C in closed reactor.*

T (°C)	Linear regression	R^2	k (s^{-1})	$t_{1/2}$ (min)	$\Delta G^{\#}(T)$ (kJ·mol^{-1})	$\Delta S^{\#}(T)$ (J·K^{-1}·mol^{-1})
80	$y = -3.37\times10^{-5}x + 4.61$	0.9695	$3.37\pm0.20 \times 10^{-5}$	385	117.2±0.2	–77.1±9.6
90	$y = -1.02\times10^{-4}x + 4.58$	0.9811	$1.01\pm0.04 \times 10^{-4}$	115	117.3±0.1	–77.4±9.6

100	$y = -2.55\times10^{-4}x + 4.70$	0.9947	$2.55\pm0.08 \times 10^{-4}$	38	117.8±0.1	–77.6±9.6
110	$y = -4.61\times10^{-4}x + 4.52$	0.9943	$4.61\pm0.20 \times 10^{-4}$	23	119.1±0.1	–77.8±9.6
120	$y = -7.46\times10^{-4}x + 4.51$	0.9770	$7.46\pm0.41 \times 10^{-4}$	14	120.7±0.2	–78.0±9.6
120[a]	$y = -1.21\times10^{-3}x + 4.91$	0.9657	$1.21\pm0.91 \times 10^{-4}$	14	119.2±0.4	-

[a] Experiment carried out in closed reactor.