H NMR quantification of cannabidiol (CBD) in industrial products derived from Cannabis sativa L. (hemp) seeds

C Siciliano¹*, Lucia Bartella², F. Mazzotti², D Aiello³, A Napoli², P De Luca¹ and A Temperini⁴
¹Dipartimento di Farmacia e Scienze della Salute e della Nutrizione, Università della Calabria, I-87036 Arcavacata di Rende (CS), Italy
²Dipartimento di Chimica e Tecnologie Chimiche (CTC), Cubo 12/D, Università della Calabria, I-87036 Arcavacata di Rende (CS), Italy
³Dipartimento di Ingegneria Meccanica, Energetica e Gestionale, Università della Calabria, I-87036 Arcavacata di Rende (CS), Italy
⁴Dipartimento di Scienze Farmaceutiche, Università di Perugia, Via del Liceo 1, 06123 Perugia, Italy

E-mail: carlo.siciliano@unical.it

Abstract. A practicable and reliable quantitative proton nuclear magnetic resonance (¹H qNMR) method was developed and evaluated for the qualitative and quantitative determination of cannabidiol (CBD), the principal and most important among cannabinoids in Cannabis sativa L. (hemp), and present in food products and animal feeding derived from the industrial processing of hemp seeds. Specificity, sensitivity, linearity range, precision, accuracy, LOD and LOQ of the method proved to be entirely satisfactory. This spectroscopic method uses the unlabelled residual solvent of CDCl₃ as the “intrinsic” internal standard. The development procedure might also be applied to measure levels of all the other lawful natural cannabinoids in commercial productions obtained from hemp seeds. Moreover, the rapid and relatively economical quantification of CBD could be of great importance, because it is possible to candidate this cannabinoid to the role of a molecular marker attesting food processing quality.

1. Introduction
The research for markers that could be employed to characterize industrial food productions, and that can correlate the final products with the starting raw material, plays an increasingly important role. As a consequence, there is the need for new and innovative instrumental techniques, which can be capable of rapidly analyzing different classes of compounds. In this context, high-resolution nuclear magnetic resonance (NMR) spectroscopy plays a privileged role [1-4]. NMR techniques are mostly used for structural analysis of synthetic and biosynthetic organic and bioorganic compounds [5-12], natural products [13-17], as well as for the identification of single or multiple components in complex matrices [18-27]. NMR spectroscopy represents also one of the most robust analytical tools for the qualitative and quantitative analysis in biological fluids of endogenous low molecular weight metabolites produced by pharmaceuticals and abuse drugs [28-33]. Nowadays, there is a growing trend in using high resolution NMR spectroscopy in food science [34-36]. This technique has extensively been applied to the direct investigation of the molecular profiling of foods and beverages [37, 38], by establishing quality markers and criteria [39-43]. One of the most important advantages of
this technique relies in the direct analysis of food products, avoiding the drawbacks connected with the chemical and physical pre-treatment of samples, which often causes modification of the molecular composition. The times for a complete analysis are very short, and a single spectrum is capable to deliver all the required information. Quantitative NMR (qNMR) is directly related to the measurement of the amount of target compounds in solution [44]. It is a robust analytical tool, and it is considered a primary analytical method, because the basic principle consists in the direct proportionality between the signal intensities and the number of magnetically active nuclei, e.g. protons, generating them. This proportionality strictly depends on the setting of the most appropriate instrumental and analysis conditions, which must remain constant along the series of experiments. Other advantages of qNMR are the high specificity, linearity, precision, accuracy, repeatability, intra- and infra-day reproducibility, and high dynamic range. Furthermore, chemical and physical manipulation during sampling are unnecessary, thus it is possible to avoid loss of low concentrated analytes. It is possible to obtain an easy, rapid, accurate and precise quantitation of organic compounds by $^1$H NMR methods, in which the analyte amounts can be measured from the absolute integration of an external reference standard of known concentration and the spectrum generated by the analyte. These method works well only when the NMR instrument is set to be highly stable and linear. Calibration is carried out by measuring samples with concentrations ranging from 10 to 100 mM. The sample must contain a single narrow signal or a group of well-resolved resonances with no overlap with the other signals. Moreover, it is mandatory to know the number of protons for each signal which can be used for calibration. The highest levels of precision and accuracy are obtained by calibrating the system before each analysis, in order to balance little variations of instrumental stability, which are often due to possible variations of the laboratory environment. A good set of the initial operative conditions allows the selection of the most appropriate instrumental parameters for the tuning and matching of the probe, and the magnet shimming. At this point, it should be required only to maintain the same signal amplification for all samples. The use of appropriate internal and/or external standards [45-47] is a desirable feature of laboratory routine qNMR investigations. These reference compounds aid quantification of analytes, without altering the sample composition or interfering with the components of a complex mixture. The introduction of calibrating compounds could be most challenging if samples already contain a marker which can generate a unique, common and detectable signal. Although the internal standard method can deliver accurate quantifications, the preparation of standard solutions and real samples is often laborious. Moreover, organic compounds chosen as the internal standards can introduce sample contamination, not preserving the analysis from uncertainties and untrue results. As well as for mass spectrometric techniques [48-53], in these cases it is possible to refer the molecular composition, and/or the measure for a single or more than one analyte, to the intensity of a particular signal generated by a calibrating substance totally not interfering with samples. More amazingly, if the internal standard is a compound which can be quantified without preparing additional stock solutions.

With the last point in mind, we designed a simple and innovative qNMR method for the rapid quantification of cannabidiol, the principal cannabinoid contained in legal Cannabis sativa L. (hemp), an annual dioecious plant which grows in every developed world country. Hemp is an economically valuable and important source for a series of industrial products, whose chemical composition are object of continuous investigations. In particular, hemp seeds represent an interesting source of food, fiber, and pharmaceuticals [54, 55]. Hemp seeds have a rich nutritional profile and provide a range of health benefits [56-61]. The growing demand for vegetables oils, special ingredients, functional foods and animal feedings, has made hemp seeds a target for the innovative industrial use of its components. Hence, there is an increasing demand for rapid, reliable and effective analytical methods to detect and identify known compounds of biological and nutritional importance in hemp derived products which continually appear on the market.

Our attention was attracted by the application of a smart and convenient qNMR method for the determination of CBD contents in hemp seed flour, widely employed as a valuable alternative to other common flours, and cakes which are considered important sources of dietary fibre, with the added
benefits of essential fatty acids [62]. A wide variety of analytical techniques have been described for the qualitative and quantitative determination of cannabinoids in Cannabis sativa L. plant extracts, seed oil, materials and preparations [63-69]. CBD and the other legal and illegal cannabinoids have generally been quantified in biological matrices by conventional methods [70-73] based on chromatographic separations (HPLC, GC) coupled to different arrays (UV, diodes, FID), or mass spectrometric detectors. The literature reports on other experimental quantitative protocols which did not use chemical standards in order to quantitate the desired analyte. In these methods (ERETIC, PIG, ARTSI, QUANTAS), a reference electronic signal is artificially generated to correlate and measure the intensities of analyte signals [74-77]. However, these techniques were successful only in a limited number of applications, due to the need for complex mixture fractionations, time-consuming pre-analysis chemical derivatization steps, instrument conditioning, high possibility of error due to the thermal conversion of cannabinoid structures, [78]. HPLC is limited by the matrix solubility, and serious sample chemical handling cannot often be avoided in order to obtain an efficient separation of all the major cannabinoids [79]. Therefore, there is a lack of innovative analytical solutions which enable the determination of metabolite profiles, especially when their reference standards are not available. Direct 1H NMR analysis has been used to determine and quantitate natural and synthetic psychotropic cannabinoids in illegal herbal blends and tinctures derived from hemp [80]. Proton 1D and 2D NMR techniques have been used for Cannabis sativa L. chemotype distinction, extract analysis, profiling and specification [81-83], and to differentiate Cannabis sativa L. cultivars [84]. 1H NMR has successfully been employed coupled to mass spectrometry or conventional analytical methods in metabolomics studies [85-88]. Notwithstanding, only limited qNMR characterizations of hemp seed derived products have been reported in the literature until today. We investigated the power of high-resolution qNMR spectroscopy to quickly and accurately determine the amounts of CBD in widely consumed foodstuffs and feedings derived from hemp seeds. The qNMR method here presented took advantage from the use of the calculable amount of unlabeled residual chloroform contained in commercially available deuterated chloroform. Detailed information was delivered by spectral data, without the need for chemical and/or physical manipulations during matrix sampling.

2. Materials and methods

2.1. Chemicals and materials
Chloroform (analytical grade) for extraction of hemp seed flour and powdered cakes, and deuterated chloroform (99.5 % isotopically pure, 0.01% TMS) were purchased from Sigma-Aldrich (Milano, Italy). Hemp seed flour, cakes, and oil were obtained from a local industry. The presence of THC and other illegal cannabinoids in all samples was excluded on the basis of the factory declaration. Crystalline CBD (99.9% pure) was purchased from a local retailer.

2.2. 1H NMR spectroscopy
Spectra were recorded at 300.132 MHz with a Bruker Advance 300 Ultrashielded instrument, equipped with a 5 mm BBO probe with Z-axis gradient coils and a temperature control unit. NMR 5 mm tubes (Wilmad) were used for all samples. Spectra were obtained by setting the temperature at 25, 35, and 45 °C, with a variation of ± 0.1 °C. Chemical shift were expressed in ppm and referred to the signal of unlabeled residual chloroform (7.25 ppm) present in CDCl3. Acquisition and elaboration parameters were as elsewhere published [89-92].

2.3. Spectroscopic analysis of CBD
A solution of pure CBD (10 mg) in CDCl3 (1 mL) was prepared. Analysis was carried out by varying the probe temperature, and three spectra were obtained at 25, 35, and 45 °C. Fluctuation of the sample temperature was in the range of ± 0.1 °C. The best signal resolution and line shape were observed at
45 °C. Signals were attributed to the corresponding protons by homonuclear decoupling experiments, and confirmed according to the literature [93].

2.4. Preparation of CBD stock solution. Calibration curve
Commercially available crystalline CBD was used to prepare a stock solution in CDCl$_3$ at the final concentration of 5 mg/mL. Aliquots of this solution were further diluted in NMR tubes, using CDCl$_3$ until a final volume of 1 mL. The final concentrations of samples were 0.05, 0.24, 0.87, 1.74, 2.32, 2.98, and 3.48 mg/mL. A $^1$H NMR spectrum was recorded in triplicate for each sample. Signals of the unlabeled residual chloroform (a singlet at 7.25 ppm), and the pair of aromatic protons H-2‘ and H-6’ (a singlet at 6.22 ppm) were repeatedly integrated seven times. The ratios between the intensity of the singlet due to the aromatic protons, and that of the singlet due to CHCl$_3$ were plotted against the concentrations of the respective standard CBD samples. The calibration curve displayed in Figure 1 was calculated by applying the mean squares linear regression model. The equation $y = 9.2504 + 3.5302$ was calculated. Linearity of the method, in the selected concentration range, was assessed by the correlation coefficient $R^2$ which resulted to be 0.9998.

![Figure 1. Calibration curve for CBD.](image)

2.5. Preparation of real samples and blank matrix
Weighed amount (200 mg) of hemp seed flour and ground cakes were charged in sealed vials, and suspended in CHCl$_3$ (5 mL). The heterogeneous mixtures were magnetically stirred at room temperature for 15 minutes, then filtered by using a 10 mL syringe packed with a short silica gel pad. Further CHCl$_3$ (5 mL) was used to wash the solid layer, and the organic solution was evaporated to dryness, affording a residue which was re-dissolved in a NMR tube by adding CDCl$_3$ (1 mL). In order to calculate the recovery of the method, a blank matrix was prepared with wheat flour (200 mg), and spiked with CBD at a known concentration (2.5 mg/mL). The mixture was treated as described for real samples, and the recovery was determined by the calculated calibration curve. Hemp seed oil (100 mg) was dissolved in CDCl$_3$ (1 mL) and the sample was used as reference for signals generated by essential fatty acid acyl chains.

3. Results and discussions
Hemp seed flour, cakes and oil were the matrices investigated for CBD quantification. Cakes are obtained as a by-product from the extraction of oil from seeds, and they consist of fibers and oily residues composed in form of brownish solid little sticks. Both the products can contain antioxidants...
and many other low molecular weight compounds characterized by different kinds and levels of bioactivities. These products can contain non-psycotropic cannabinoids, with the powerful antineoplastic and antioxid ant CBD being the most representative and concentrated among them. Since flour and cakes are industrially produced starting from seeds after extraction of oil, it was expected that only a minor part of CBD is transferred to these products. In fact, it should be thought that lipophilic CBD and the other cannabinoids can be extracted together with the triacylglycerol fraction from seeds during the oil extraction [94]. Due to the well-known bioactivity exerted by CBD, we reputed desirable to determine the remaining amounts of this important natural cannabinoid in flour and cakes.

The basic idea of our 1H qNMR method was to use the proton resonance generated by the residual unlabeled chloroform which is contained in the commercially available CDCl₃ for spectroscopic applications. Literature refers on a sparingly use of the residual solvent resonance as the internal standard, a unique case being reported for deuterated dimethylsulfoxide [95]. If a deuterated chloroform with purity less than 100% is employed, the declared percentage of unlabeled solvent can be quantified. In our experiments, CDCl₃ with an isotopic purity of 99.5% was used to prepare the CBD stock solution, samples, blank matrix, for the qNMR analysis, and to check the accuracy of the method. Therefore, the 0.5% of unlabeled residual CHCl₃ present in the deuterated solvent can be quantified in terms of weight dissolved in samples, and used either as the internal standard for calibrating spectra and, at the same time, to measure the amounts of CBD contained in each sample. Thus, if samples are prepared for the analysis by dissolving them in a constant volume of deuterated solvent, all of them will contain a fixed amount of unlabeled residual CHCl₃. Chloroform will generate a signal in form a singlet at 7.25 ppm, which can be integrated together with all detectable and well-resolved signals of the analyte under investigation. It will be sufficient to prepare a stock solution of the pure standard analyte dissolved in deuterated chloroform, to calculate a calibration curve which will be employed to determine the linearity of the method together with the unknown concentrations of the analyte in the sample series. The advantages of this method are evident: (a) there is no use of organic substances which can interfere with the molecular composition of the sample, (b) it is possible to avoid the preparation of stock solutions of the internal/external standard, (c) the same NMR solvent can be used to extract little amounts of matrices and recover all soluble analytes, (d) a compound having only one proton to be correlated with the signal generated by a single proton or groups of nuclei in the structure of the analyte, (e) short times of analysis, and (f) reasonably diminished laboratory costs. We exploited the power and analytical characteristics of this qNMR method based on the use of an “intrinsic” internal standard, for the complete analysis of the primary biological active constituent CBD in industrial products obtained from hemp seeds. To the best of our knowledge, the unlabeled residual CHCl₃ in the commercially available CDCl₃ has never been proposed as an “intrinsic” internal standard to quantify a single or more than one analyte in complex mixtures.

CBD is a compound belonging to the chemical class of aromatic terpenes. Its structure (Figure 2) features functional groups which can confer a balanced character between hydrophilicity (the phenolic
ring) and lipophilicity (the terpene structure). Starting our study, some solubility tests were carried out. Amounts of 100 mg of pure CBD were solubilized in 1 mL of some of the most commonly used organic solvents. CBD dissolved smoothly in methanol and chloroform. For the economical and spectroscopic characteristics of the two solvents, chloroform was chosen for spectral analysis.

Figure 3. Variable temperature analysis for a sample of standard CBD. Spectrum were obtained at 25, 35 and 45 °C (plots A, B, and C, respectively).
Our investigation started by recording a spectrum for a pure CBD sample. An interference in selecting the appropriate signals, and determining the integration regions for quantitation, due to the presence of minor peaks appearing close to the most intense signals, was observed and investigated. Furthermore, the CBD spectrum recorded at room temperature, displayed signals not well-resolved and with a detectable line shape broadening. This was correlated to the possible presence of conformational equilibria in solution [96-98]. Thus, a study based on the variable temperature application was started. Influences on signals exerted by the variable temperature are evidenced in Figure 3.

The attention was pointed out on the two signals in the region between 5.80 and 6.30 ppm. A series of spectra were acquired by varying the sample temperature. The spectrum recorded at 25 °C (Figure 3A) showed two distinct broad singlets at 5.96 and 6.22 ppm, which were attributed to the pair of aromatic protons H-2' and H-6'. These protons generated two different unresolved peaks, although the phenyl ring is symmetric. The effect is reasonably due to thermal equilibria, involving the single bond between the phenyl carbon C-1' and the carbon C-1 of the terpene moiety, which can be operative in solution giving rise to a mixture of rotamers, similarly to the cases of the co-existence of different atropisomers [99]. As the experiments highlighted, these two signals showed the correct integrals and line shapes at 45 °C. These thermal conditions were selected in order to obtain calibration curves for the method, and analyze real samples and blank matrix extracts. The spectrum obtained at 45 °C showed two signals well-distinguishable and with the appropriate characteristics as required for their integrations. Starting with the quantitative NMR investigation, the first point was to assign all signals to the respective protons in the molecular structure of CBD. The coalescence of the pair of signals at 45 °C generated a unique narrow singlet, confirming the above hypothesis. The other broad singlet at 4.74 ppm, which partially overlapped the signal assigned to the H-9 trans proton in the exocyclic carbon-carbon double bond, was due to the resonances of the aromatic OH groups. At 45 °C, this signal totally overlapped that of trans proton, making not useful the olefinic proton signals for quantitative scopes. Both the two singlets appearing at 5.56 ppm and 6.22 ppm, were tentatively selected to fulfill our aim. A previously reported method for the quantification of CBD, based on the 1H NMR spectroscopy, used signals in the spectral window between 3.50 and 6.50 ppm [79]. Unfortunately, the signals attributable to the double bond methylene protons were not useful, due to the oscillation of their ppm values which were found strongly influenced by the overlap with the signals of the OH protons, as we initially confirmed. Thus, only the signal appearing at 5.56 ppm has previously been reported as the reference peak for quantitation.

![Figure 4](image-url)

**Figure 4.** Full spectra of standard CBD (left), and hemp seed oil (right). [(A): Full spectrum of hemp seed oil; (B): spectral window showing signals of CBD aromatic protons and H-2; (C) signals of CBD double bond methylene protons].

A full spectrum of CBD (Figure 4a) was recorded at 45 °C, and taken as the reference to correlate all detectable resonance signals with protons in the cannabinoid structure. Assignment was done by
using the integral values of each signal, and on the basis of extended homonuclear decoupling experiments. The signal-structure correlation was definitively confirmed by the literature reports [89]. All signals were well-resolved, showing the appropriate line shape, and appeared distributed in two main spectral regions (0.65-2.75 ppm and 3.50-6.50 ppm). The high-field region (0.65-2.75 ppm) contained signals generated by the resonances of the protons belonging to the linear alkyl chain, methyl groups, and terpene moiety. The multiplet centered at 3.86 ppm was assigned to the H-1 proton. The two singlets appearing at 4.57 and 4.66 ppm were definitively attributed to the resonances of cis and trans, respectively, methylene protons of the terpenic double bond. The singlets at 5.56 ppm and 6.22 ppm were generated by the terpenic H-2 and the pair of two aryl protons H-2’ and H-6’, respectively. All integrals were consistent with the expected number of protons generating the corresponding signal. A sample of hemp seed oil was also analyzed. The spectrum recorded at 45 °C (Figure 4b) showed signals which were typical of the essential fatty acid components. Signals of CBD were detected in the sub-spectrum. All peaks were used to determine the presence of fatty acid chains in the extracts of flour and cakes, which were expected contain variable amount of glycerides relating these hemp seed products to the raw material used for their manufactures.

A calibration curve for CBD was thus obtained by acquiring spectra of seven samples (C_1-C_7), prepared at different concentrations. Figure 5 shows the stacked plot of the spectral analysis used to calculate the calibration curve. Experiments were carried out at 45 °C because at that temperature the coalescence of signals assigned to the aromatic protons was observed. This signal, counting for two protons, was integrated together with that of residual CHCl_3, and a coefficient of 0.5 was used to multiply aromatic integrals in order to calculate the calibration curve. Under these conditions, linearity of the method in the considered concentration range was assessed by a correlation coefficient (R^2) equal to 0.9998.

![Figure 5. Stacked plot of spectra used to calculate the CBD calibration curve.](image)

Parameters of the quantitative method were calculated before analyzing real samples. Limit of detection (LOD) was estimated based on a 3:1 signal-to-noise (S/N) ratio. An aliquot of the standard CBD stock solution was appropriately diluted at the levels of this S/N estimated value, and was analyzed in triplicate. Limit of quantification (LOQ) was estimated based on a 10:1 S/N ratio, and three samples of standard CBD stock solution were prepared reaching the level of this estimated S/N
value. Our investigation found that the LOD for CBD in CDCl₃ was fixed at 0.003 mg/mL (the corresponding S/N ratio was 2.98:1), and the LOQ at 0.008 mg/mL (with a S/N ratio of 9.71:1).

The necessary second step was to test the method for accuracy and recovery, prior to analyze samples prepared by extraction of hemp seed flour and powdered cakes. For similarity with these products, commercial wheat flour was chosen as a blank matrix not containing cannabinoids. Three samples of wheat flour (500 mg) were spiked with a known amount of CBD stock solution (2.5 mg/mL), and subjected to the extractive procedure with chloroform. Recovery of CBD was quantitative (> 99%) in all cases, as highlighted by the spectral investigation and calculated from the CBD calibration curve.

Table 1. Values obtained for accuracy.

| Spiked sample | CBD added (mg/mL) | CBD found (mg/mL) | Accuracy (%) | RSD (%) |
|---------------|-------------------|-------------------|--------------|---------|
| S1            | 0.07              | 0.073 ± 0.003     | 104.3        | 4.1     |
| S2            | 3.20              | 3.250 ± 0.065     | 101.5        | 2.0     |

Accuracy was estimated by obtaining spectra of spiked blank matrix extracts in triplicate. Two samples were prepared by adding standard CBD stock solution to a fixed amount of wheat flour, reaching final concentrations in cannabinoid which were representative of the calibration curve edges. Referring to Table 1, the obtained results indicated that the qNMR method can provide an ideal accuracy for CBD determination.

Figure 6. High-resolution ¹H NMR spectrum of a sample of hemp seed cake extract. (A) Full spectrum, and (B) spectral window between 3.00 and 7.00 ppm of the sub-spectrum, showing signals generated by CBD at 4.56, 4.64, and 6.21 ppm.
Finally, performance of the method was tested by analyzing real samples of hemp seed flour, cakes, and oil. Experiments were repeated in triplicate for each product, at 45 °C. Initially, an extract obtained from hemp seed cakes was dissolved in CDCl₃ and prepared for analysis. The full spectrum (Figure 6a) displayed a set of high intensity signals (0.70-3.0 ppm), and a low field region where it was possible to detect a single intense peak centered at 5.35 ppm. Peaks appearing in the window between 0.70 and 3.00 ppm were assigned to resonances of free fatty acid chains, on the basis of an experimental spectrum recorded for a sample of hemp seed oil, and as found in previously published literature reports [100]. Other signals were not visible when spectrum was scaled and plotted. Differently, the spectral windows of the sub-spectrum between 3.00 and 5.00 ppm, and 5.50-7.00 ppm showed a more interesting series of low intensity peaks. In particular, the presence of the three singlets at 4.56, 4.66, and 6.21 ppm confirmed the presence of CBD in the samples. The apex of the singlet attributable to the H-2 proton of CBD appeared at 5.56 ppm, as a should of the main peak centered at 5.35 ppm. This proton coincided with the proton used in the already reported method [79] for the quantification of CBD together with other natural cannabinoids, however it was impossible to use it in the case of cake extract. Double bond methylene protons also cannot successfully be used, due to the overlapping with less intense signals attributable to minor components of the extract. Thus, the choice of aromatic protons as reference signal for quantification was reasonable. Concentration of CBD in cake extracts was found to be 1.65 ± 0.07 mg/mL, from the integral value of the reference signal, and based on the calculated calibration curve.

Figure 7. High-resolution ¹H NMR spectrum of a sample of hemp seed flour extract. (A) Full spectrum, and (B) spectral window between 3.00 and 7.00 ppm of the sub-spectrum, showing signals generated by CBD at 4.55, 4.64, and 6.20 ppm.

The spectral plots recorded for a sample of hemp flour extract are depicted in Figure 7. The full spectrum showed peaks distributed along two principal regions, in a strict analogy with the spectrum obtained for cake extracts. The sub-spectrum between 3.00 and 7.00 showed the set of singlets generated by the CBD proton resonances. Referring to the singlet at 6.20 ppm, and to the calculated
calibration curve, CBD was determined to be present in samples at a concentration of 1.28 ± 0.04 mg/mL. Further, for samples of hemp seed oil, a CBD level of 2.16 ± 0.06 mg/mL was obtained.

4. Conclusions
A novel, fast and reliable method of extracting and estimating the biologically and pharmacologically important cannabinoid CBD in Cannabis sativa L. (hemp) seed derived products was planned and developed. High-resolution 1H NMR spectroscopy was used to quantify the natural CBD in flour, cakes, and oil obtained from the industrial processing of hemp seeds. The quantitative spectroscopic method required only the direct solvent extraction of the pair of matrices, without any further physical and/or chemical manipulation evaporation, or chromatographic steps. Oil is analyzed as it is. Temperature of the samples during analysis resulted to be the only one critical factor, because the thermal conditions applied to record NMR spectra might affect signal resolution, line shape, and integration. The correct setting of instrumental parameters and temperature control unit did not limit the validity of the disclosed method. Quantitative 1H NMR experiments were performed in CDCl3, and the residual amount of unlabeled chloroform was used as the “intrinsic” internal standard, avoiding addition of different organic compounds as internal standards which must appropriately be chosen for zeroing difficult in detection and quantification of single analytes in complex matrices. The results showed that the analytical parameters of the spectroscopic method (specificity, linearity range, precision, accuracy, LOD and LOQ) proved to be entirely satisfactory.

5. References
[1] Markley J L, Brünschweiler R, Edison A S, Eghbalnia H R, Powers Raftery R D and Wishart D S 2017 Current Opinion in Biotechnology 43 34-40
[2] Fan T W-M and Lane A N 2016 Progress in Nuclear Magnetic Resonance Spectroscopy 92-93 18-53
[3] Gouk S W, Cheng S F, Malon M, Ong A S H and Chuah C H 2013 Analytical Methods 5 2064-2073
[4] Bharti S K and Roy R 2012 Trends in Analytical Chemistry 35 5-26
[5] Henrig J, Warner L R, Simon B, Geerlof A, Mackeret C D and Sattler M 2015 Methods in Enzymology 558 333-362
[6] Minuti L, Ballerini E, Barattucci A, Bonaccorsi P M, Di Gioia M L, Leggio A, Siciliano C and Temperini A 2015, Tetrahedron 71 3253-3262
[7] Göbl C, Madi T, Simon B and Sattler M 2014 Progress in Nuclear Magnetic Resonance Spectroscopy 80 26-63
[8] Kim N-K, Nam Y S and Lee K-B 2014 Journal of the Korean magnetic resonance Society 18 5-9
[9] Barattucci A, Di Gioia M L, Leggio A, Minuti L, Papalia T, Siciliano C, Temperini A and Bonaccorsi P 2014 European Journal of Organic Chemistry 2014 2099-2104
[10] Espina R, Yu L, Wang J, Tong Z, Vashishtha S, Talaat R, Scatina J and Mutlib A 2009 Chemical Research in Toxicology 22 299-310
[11] Siciliano C, De Marco R, Guidi L E, Spinella M and Liguori A 2012 Journal of Organic Chemistry 77 10575-10582
[12] Forseth R and Schroeder F C Current Opinion in Chemical Biology 15 38-47
[13] Fan T W-M and Lane N A 2016 Progress in Nuclear Magnetic Resonance Spectroscopy 92-93 18-53
[14] Clendinen C S, Stupp G S, Ajredini R, Lee-McMullen B, Beecher C and Edison A S 2015 Frontiers in Plant Science 6 611
[15] Gödecke T, Napolitano J G, Rodriguez-Brasco M F, Chen S-N, Jakia B U, Lankin D C and Pauli G F 2013 Phytochemical Analysis 24 581-597
[16] Pauli G F, Gödecke T, Jaki B U and Lankin D C 2012 Journal of Natural Products 75 834-851
[17] Reynolds W F and Enriquez R G 2002 Journal of Natural Products 65 221-244
Wolfender J-L, Marti G, Thomas A and Bertrand S 2015 Journal of Chromatography A 1382 136-164

Crockford D J, Keun H C, Smith L M, Holmes E and Nicholson J K 2005 Analytical Chemistry 77 4556-4562

De Luca P, Carbone I and Nagy J B 2017 Journal of Green Building 12 141-161

De Luca P, Nappo G, Siciliano C and Nagy J B 2018 Journal of Porous Materials 25 283-296

Nagana Gowda G A and Raftery D 2015 Journal of Magnetic Resonance 260 144-160

Larive C K, Barding G A, Jr. and Dinges M M 2015 Analytical Chemistry 87 133-146

Clendinnen C S, Lee-McMullen B, Williams C M, Stupp G S, Vandenbome K, Hahn D A, Walter G A and Edison A S 2014 Analytical Chemistry 86 9242-9250

Bothwell J H F and Griffin J L 2011 Biological Reviews 86 493-510

Lewis I A, Schommer S C and Markley J L 2009 Magnetic Resonance in Chemistry 47 S123-S126

Putri S P, Nakayama Y, Matsuda F, Uchikata T, Kobayashi S, Matsubara A and Fukusaki E 2013 Journal of Bioscience and Bioengineering 115 579-589

Duarte I F, Diaz S O and Gil A M 2014 Journal of Pharmaceutical and Biomedical Analysis 93 17-26

Emwas A-H M, Salek R M, Griffin J L and Merzaban J 2013 Metabolomics 9 1048-1072

Gebregiorgis T and Powers R 2012 Combinatorial Chemistry and High Throughput Screening 15 595-610

Holzgrabe U 2010 Progress in Nuclear Magnetic Resonance Spectroscopy 57 229-240

Holzgrabe U, Deubner R, Schollmayer C and Waibel B 2005 Journal of Pharmaceutical and Biomedical Analysis 38 806-812

Pauli G F, Jaki B U and Lankin D C 2005 Journal of Natural Products 68 133-149

Hatzakis E 2019 Comprehensive Reviews in Food Science and Food Safety 18 189-220

Laghi L, Picone G and Capozzi F 2014 Trends in Analytical Chemistry 59 93-102

Rodrigues J E and Gil A M 2011 Magnetic Resonance in Chemistry 49 S37-S45

Trimagno A, Cesare Marincola F, Dellarosa N, Picone G and Laghi L 2015 Current Opinion in Food Science 4 99-104

Sugimoto N, Tada A, Suematsu T and Arifuku K 2010 Foods and Food Ingredients Journal of Japan 215 129-136

Martinez-Yusta A, Goicocecha E and Guillén M D 2014 Comprehensive reviews in Food Science and Food Safety 13 838-859

Marcone M F, Wang S, Albabish W, Nie S and Somnarain D 2013 Food research International 51 729-747

Erikson U, I. Standal B, Aursand I G, Veliyulin E and Aursand M 2012 Magnetic resonance in Chemistry 50 471-480

Cevallos-Cevallos J, Reyes-De-Curcuera J I, Etxeberria E, Danyliuk M D and Rodrick G E 2009 Trends in Food Science and Technology 20 557-566

Sundekilde U, Larsen L and Bertram H 2013 Metabolites 3 204-222

Chauke S K, Sharma R J, Aqil F, Gupta R C and Singh I P 2012 Phytochemical Analysis 23 689-696

Huang Y, Su B-N, Ye Q, Palaniswamy V A, Bolgar M S and Raglione T V 2014 Journal of Pharmaceutical and Biomedical Analysis 88 1-6

Rundlöf T, Mathiasson M, Bekiroglu S, Hakkarainen B, Bowden T and Arvidsson T 2010 Journal of Pharmaceutical and Biomedical Analysis 52 645-651

Burton I W, Quilliam M A and Walter J A 2005 Analytical Chemistry 77 3123-3131

Aiello D, Giambona A, Leto F, Passarello C, Damiani G, Maggio A, Siciliano C and Napoli A 2018 Scientific Reports 8 10973

Aiello D, Materazzi S, Risoluti R, Thangavel H, Di Donna L, Mazzotti F, Casadonte F, Siciliano C, Sindona G and Napoli A 2015 Molecular. BioSystems 11 2373-2382
[50] Marshall D D, Lei S, Worley B, Huang Y, Garcia-Garcia A, Franco R, Dodds E D and Powers R 2015 *Metabolomics* 11 391-402

[51] Mazzotti F, Di Donna L, Napoli A, Aiello D, Siciliano C, Athanassopoulos C M and Sindona G 2014 *Journal of Mass Spectrometry* 49 802-810

[52] Crockford D J, Holmes E, Lindon J C, Plumb R S, Zirah S, Bruce S J, Rainville P, Stumpf C L and Nicholson J K 2006 *Analytical Chemistry* 78 363-371

[53] Fischedick J T, Hazekamp A, Erkelens T, Choi Y H and Verpoorte R 2010 *Phytochemistry* 71 2058-2073

[54] Aizpurua-Olaizola O, Soydaner U, Öztürk E, Schibano D, Simcir Y, Navarro P, Extebarria N and Usobiaga A 2016 *Journal of Natural Products* 79 324-331

[55] Pellati F, Borgonetti V, Brighenti V, Biagi M, Benvenuti S and Corsi L 2018 *Biomedical Research International* 2018 1-15

[56] Citti C, Braghiroli D, Vandelli M A and Cannazza G 2018 *Journal of Pharmaceutical and Biomedical Analysis* 147 565-579

[57] Smeriglio A, Galati E M, Monforte M T, Lanuzzo F, D’angelo V and Circosta C 2016 *Phytotherapy Research* 30 1298-1307

[58] Radwan M, Ross S, Slade D, Ahmed S, Zulfijar F and ElSohly M 2008 *Planta Medica* 74 267-272

[59] Zuardi A W, Crippa J A S, Hallak J E C, Moreira F A and Guimarães F S 2006 *Brazilian Journal of Medical and Biological Research* 39 421-429

[60] Ebanks L M, Rogers C J, Beuscher A E IV, Koob G F, Olson A J, Dickerson T J and Janda K D 2006 *Molecular Pharmaceutics* 3 773-777

[61] Lachenmeier D W, Kroener L, Musshoff F and Madea B 2004 *Analytical Bioanalytical Chemistry* 378 183-189

[62] Cittia C, Pacchetti B, Vandelli M A, Forni F and Cannazza G 2018 *Journal of Pharmaceutical and Biomedical Analysis* 149 532-540

[63] Poplawksa M, Blazewicz A, Kamiński K, Bednarek E, Fijalek Z and Kozerski L 2018 *Forensic Toxicology* 36 122-140

[64] Wang M, Wang Y -H, Avula B, Radwan M M, Wanas A S, Mehmedic Z, van Antwerp J, ElSohly M A and Khan I A 2017 *Journal of Forensic Science* 62 602-611

[65] De Backer B, Debrus B, Lebrun P, Theunis L, Dubois N, Decock L, Verstraete A, Hubert P and Charlier C 2009 *Journal of Chromatography B* 877 4115-4124

[66] Lopes de Oliveira, Voloch M H, Sztulman G B, Negrini Neto O and Yonamine M 2008 *Forensic Toxicology* 26 31-35

[67] Lachenmeier D W, Kroener L, Musshoff F and Madea B 2004 *Analytical Bioanalytical Chemistry* 378 183-189

[68] Gambare V, Dell’acqua L, Faré F, Froldi R, Saligari E and Tassoni G 2002 *Analytica Chimica Acta* 468 245-254

[69] Fowler F, Voyer B, Marino M, Finzel J, Veltri M, Wachtera N M and Huang L 2015 *Analytical Methods* 7 7907-7916

[70] Langer N, Lindigkiet R, Schiegel H -M, Papke U, Ernst L and Beuerle T 2016 *Forensic Science International* 269 31-41

[71] Materazzi S, Peluso G, Ripani L, Risoliti R 2017 *Microchemical Journal* 134 277-283

[72] Brighenti V, Pellati F, Steinbach M, Maran D and Benvenuti S 2017 *Journal of Pharmaceutical and Biomedical Analysis* 143 228-236

[73] Evangelidis T, Nerli S, Nováček J, Brereton A E, Karplus P A, Dotas R R, Venditti V, Sgourakis N G and Tripianes K 2018 *Nature Communications* 9 384

[74] Farrant R D, Hollerton J C, Lynn S M, Provera S, Sidebottom P J and Upton R J 2010 *Magnetic resonance in Chemistry* 48 753-762

[75] Albers M J, Butler T N, Rahwa I, Bao N, Keshari K R, Swanson M G and Kurhanewicz J 2009 *Magnetic Resonance in Medicine* 61 525-532
The work was supported by grants from the Ministero dell’Istruzione, dell’Università e della Ricerca.

Acknowledgments
The work was supported by grants from the Ministero dell’Istruzione, dell’Università e della Ricerca.