Simple isolation method of cannabinoI from *Cannabis sativa* to produce secondary reference standard analysis material

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**Abstract.** The simple isolation method of Cannabinol compounds from cannabis plants by maceration combined with ultrasonication assisted extraction, fractionation, separation and purification was carried out to obtain Cannabinol isolates which can be used as a reference standard analysis material. Ultrasonication was proven to shorten the extraction time, where extraction for 5 h with ultrasonication assisted for 15 min produced 5.536 % yield greater than the 24 h extraction. The results of extraction were fractionated using n-hexane and then chromatographically isolated with a column containing Silica Gel 60, with 2.5 cm diameter and a 15.3 cm height, eluted by n-hexane-ethyl acetate (90:10) solution. The fraction containing Cannabinol was purified using HPTLC preparative with eluent n-hexane-ethyl acetate (80:20). The purification of the Cannabinol isolate was further characterized by Spectrophotometer UV, FTIR, DCS, GCMS and LCMSMS and compared its profile to the reference standard of Cannabinol from Lipomed. The characterization results showed that the purified isolates had UV spectra with \( \lambda_{\text{max}} \) at 219.0 nm and 284.5 nm, FTIR spectra at wave numbers 1620.21 cm\(^{-1}\), 1051.20 cm\(^{-1}\), 1581.63 cm\(^{-1}\), 1026.13 cm\(^{-1}\), 1128.36 cm\(^{-1}\) and 1232.51 cm\(^{-1}\). The DSC thermogram shows the melting point of compound is 74.36 °C with 99.35 % purity, GCMS fragmentation at m/z 295, 296, 238 and 310, LCMSMS with [M + H]\(^+\) at 311.1 and MS\(^2\) at 222.95, 292.95 and 240.95 confirmed the chemical structure of the compound. The results of the characterization of pure isolates indicate that the compound produced was Cannabinol in accordance with the standard characterization profile of the reference standard of Cannabinol used.

**Keywords:** Cannabinol, isolation, ultrasonication assisted extraction, purification, reference standard

1. **Introduction**

Reference standard analysis material is used to guarantee of the validity of the testing method. Narcotics reference standard material is difficult to obtain in Indonesia, because of the high price and import bureaucracy. So far, the evidence from abuse and illegal trafficking cases of cannabis have been destroyed after the case is finished, even though it can be used as a raw material to produce some reference standard material for cannabis testing. The aging and improperly storing condition of cannabis evidence causes the \( \Delta^2 \)-Tetrahydrocannabinol acid as the major compound of cannabis
undergoes a decarboxylation then oxidized to Cannabinol [1]. Cannabinol is one of marker testing of the cannabis compound that recommended by United Nations Office on drugs and Crime (UNODC) [2]. So, the aging cannabis evidence is potential to be applied as raw material to produce Cannabinol secondary reference standard analysis material.

Nowadays, isolation of cannabinoid compounds by Supercritical Fluid Extraction (SFE) extraction techniques continued by HPLC sample preparative are widely used because they have advantages including selective, no residual solvent, and automation. However, these techniques have drawbacks including very expensive equipment, and complex operating systems [3, 4]. Conventional techniques for extraction, such as maceration are widely used and easy to do, but require large amounts of solvents and time consuming, while Ultrasonication Assisted Extraction (UAE) technique gives significantly different results [5, 6]. For dry biological material, alcoholic solvents are used because it can break down the cell membranes and extract more endocellular materials [7]. Accordingly, it requires simple and safe extraction method to get good yields in a shorter time of extraction. The extract of cannabinoid must be purified to get almost 100 % purity of isolate. Purification of natural product using column chromatography and High Performed Thin Layer Chromatography (HPTLC) Preparative have been reported to have a satisfactory result [8, 9].

In this study, isolation method of Cannabinol compounds from cannabis plants by maceration combined with ultrasonication assisted extraction, fractionation, separation and purification was carried out to obtain Cannabinol isolates which can be used as a reference standard analysis material. The purified Cannabinol isolate was further characterized by UV Densitometer, FTIR, DCS, GCMS and LCMSMS and compared its profile to the reference standard of Cannabinol from Lipomed.

2. Materials and method

2.1. Materials

Methanol LC grade, acetonitrile LCMS grade, formic acid, n-hexane, toluene, and ethyl acetate were purchased from Merck, Germany. TLC aluminum Plate 20 × 20 cm and HPTLC glass plate 5 × 10 cm containing Silica gel 60 F254 and Silica gel 60 were obtained from Merck. Cannabinol RS from Lipomed and raw material dry cannabis (retained sample from Police Department of Aceh, Mei 2018) were obtained from National Agency of Drug and Food Control-Republic of Indonesia. Glass Column 2.5 cm id, Ultrasonic Branson 5510, Mechanical shaker Combi shaker NB-101 MT. LCMS column × Select HSS T3 Waters 1.8 μm, 1 mm × 100 mm, Elite-5 MS Perkin Elmer 30 m × 0.25 mm id × 0.25 μm df. The instruments used in this study were UV Densitometer (Camag TLC Scanner 3), FTIR (Shimadzu IR Prestige-21), DSC (Shimadzu-60A), GCMS (Agilent 5977B MSD) and LCMSMS (Waters Acquity).

2.2. Maceration combined with ultrasonication assisted extraction of cannabino (CBN)

Dry cannabis was grounded. Each 1 g cannabis powder was added with 30 mL methanol, sonicated for 5, 10, 15, 20, 25, 30 min, respectively, then macerated under mechanical shaker at 150 rpm for 5 h. For control solution, each 1 g cannabis powder was added with 30 mL methanol then macerated under mechanical shaker with 150 rpm for 5 h and 24 h. The extracts were spotted on TLC plate, then developed using n-hexane-ethyl acetate (90:10) to a height of 8 cm. UV Densitometer was used for plate analysis [10].

2.3. Isolation and purification of cannabino

CBN extracts were fractionated by water-n-hexane (50:50). N-hexane fraction was then allowed to dry. Each 1 g dry n-hexane fraction was separated using column chromatography containing the silica gel 60, with 2.5 cm diameter and 15.3 cm height and n-hexane-ethyl acetate (90:10) as eluent. Fraction was collected at each 7 mL, until 30 fraction. The purification of CBN fraction was performed using
HPTLC plate with n-hexane-ethyl acetate (80:20). Then, the spots of CBN were collected and dissolved in methanol, filtered then the solvent was evaporated.

2.4. Characterization of CBN isolate
CBN isolate was characterized using UV Densitometer which was scanned from 200 to 400 nm. FTIR were recorded in the range of 4000–400 cm\(^{-1}\) using dried KBr powder into a mortar with 1 % (w/w) of the sample. The melting point and purity of the sample were determined by DCS. The molecular weight was confirmed by GCMS and LCMSMS. GCMS scan mode using HP-5 MS column 30 m × 250 mm × 0.25 mm, with injection temperature of 290 °C, MSD transfer line 290 °C, gas flow 1.5 mL/min, column temperature 100 °C with 1 min hold, rate 10 °C/min until 290 °C and held for 5 min. The analysis was conducted for 25 min with mass scan of 40–550. LCMSMS, using HSS T3 column, mobile phase A (Water containing 0.1 % formic acid) and B (acetonitrile containing 0.1 % formic acid), with the composition of solution A start on 80 % to 15 % during 6 min of the running analysis. The mass spectrometer was operated in MRM and Daughter Scan Mode, with capillary voltage at 3.5 kV ESI positive ion mode.

3. Results and discussion

3.1. Maceration combined with ultrasonication assisted extraction of cannabinol (CBN)
TLC and densitometer UV profile of CBN extract by maceration combined with ultrasonication assisted extraction is shown in figure 1. Based on figure 1 and table 1, it shows that ultrasonication can shorter the extraction time, because of the solvent cavitation production, which can accelerate dissolution and diffusion of solutes and heat transfer, which increases the extraction efficiency [11].

Result of maceration combined with ultrasonication assisted extraction of CBN is shown in figure 1. Based on figure 1 and table 1, it shows the yield is increased by adding sonication in maceration technique.

Figure 2 shows that yield of extraction is increased by ultrasonication assisted, which 15 min ultrasonication before 5 h maceration can increase 5.536 % yield, compared by 24 h maceration without sonication. Ultrasonication can shorter the extraction time, that was because of producing solvent cavitation which can accelerate dissolution and diffusion of solutes and heat transfer, which increases extraction efficiency [11]. The result after 15 min sonication is not significantly different which indicates an equilibrium was reached. The increasing time of sonication will not affect the extraction after the equilibrium of the solute is reached inside and outside the solid material [11].

![Figure 1](image1.png)

(a) (b)

**Figure 1.** (a) TLC and (b) densitometer UV profiles of extract CBN.
Table 1. Effect of sonication in CBN extraction.

| No. spot | Time of maceration (M) and sonication (S) | Concentration (g/mL) | Spotted volume (µL) | Rf | Height (AU) | Average height (AU) | Increasing yield (%) |
|----------|------------------------------------------|----------------------|---------------------|----|-------------|---------------------|---------------------|
| 1        | 24 h M                                   | 0.17                 | 20                  | 0.49 | 552.0       | 544.6               |                     |
| 2        | 25 h M                                   | 0.17                 | 20                  | 0.49 | 537.2       |                     |                     |
| 3        | 5 h M 0 min S                            | 0.17                 | 20                  | 0.49 | 537.2       | 538.6               | -1.111              |
| 4        | 5 h M 5 min S                            | 0.17                 | 20                  | 0.50 | 565.0       | 565.2               | 3.783               |
| 5        | 5 h M 10 min S                           | 0.17                 | 20                  | 0.50 | 565.4       | 562.8               | 3.342               |
| 6        | 5 h M 15 min S                           | 0.17                 | 20                  | 0.50 | 560.2       |                     |                     |
| 7        | 5 h M 20 min S                           | 0.17                 | 20                  | 0.50 | 574.0       | 574.8               | 5.536               |
| 8        | 5 h M 25 min S                           | 0.17                 | 20                  | 0.50 | 575.5       |                     |                     |
| 9        | 5 h M 30 min S                           | 0.17                 | 20                  | 0.50 | 574.5       | 574.5               | 5.481               |
| 10       | 5 h M 35 min S                           | 0.17                 | 20                  | 0.50 | 574.4       |                     |                     |
| 11       | 5 h M 40 min S                           | 0.17                 | 20                  | 0.49 | 575.7       | 574.0               | 5.389               |
| 12       | 5 h M 45 min S                           | 0.17                 | 20                  | 0.49 | 572.2       |                     |                     |
| 13       | 5 h M 50 min S                           | 0.17                 | 20                  | 0.49 | 570.3       | 570.7               | 4.783               |
| 14       | 5 h M 55 min S                           | 0.17                 | 20                  | 0.49 | 571.0       |                     |                     |

Figure 2. Yield increasing by ultrasonication.

3.2. Isolation and purification of cannabinol
CBN extract was fractionated with n-hexane-water (50:50). Because CBN is insoluble in water, so CBN will be dissolved in n-hexane fraction [2]. CBN in n-hexane fraction was then isolated by column chromatography. Silica gel column is a polar stationary phase with eluent n-hexane-ethyl acetate (90:10) which is less polar. The analytes are separated on the basis of their distribution coefficients as the eluent flows through the column [12]. CBN is eluted in the beginning and more polar compound stays longer in the column. Fraction was collected at each 7 mL until 30 fraction. Total eluent volume was about three times of the volume column, to ensure the target compound is
eluted properly [13]. Figure 3 shows the TLC profile of fractions. CBN compounds were in the fraction no. 10–14.

Based on the TLC profile, fraction no. 10–14 still contain another compound, therefore, purification was carried out to get the required purity compound as a reference standard material. HPLC Preparative was selected as the purification technique, because it is easy to do, low solvent consumption and quiet efficient. Figure 4 shows a different profile of CBN isolate before and after purification. After purification with HPTLC Preparative CBN isolate only has one spot indicates an increasing purity of the CBN compound.

(a) (b)

Figure 3. TLC Profile of fractions (a) fraction no. 1–19 and (b) fraction no. 12–30.

(a) (b) (c)

Figure 4. TLC profile (a) isolate CBN from separation in column chromatography, (b) purification by HPTLC preparative and (c) pure CBN isolate.
3.3. Characterization of CBN isolate

The purified CBN isolate was then characterized using UV Densitometer, FTIR, DCS, GCMS and LCMSMS and compared its profile with the reference standard of Cannabinol from Lipomed, except for DSC analysis. Figure 5 shows the UV spectrum of purified CBN isolate and CBN Lipomed as the reference standard. Table 2 shows $\lambda_{\text{max}}$ of the compound.

Purified CBN isolate and CBN Lipomed have a similar $\lambda_{\text{max}}$, with less than 2 nm difference indicating that both are similar compounds [14, 15].

FTIR spectrum of purified CBN isolate and CBN lipomed is shown in figure 6. FTIR profile indicates that both compounds have the same constituent functional group.

![Figure 5. UV Spectrum of CBN purified isolate and CBN lipomed.](image)

| $\lambda_{\text{max}}$ (nm) | CBN lipomed | Absorbance | $\lambda_{\text{max}}$ purified CBN isolate | Absorbance |
|-----------------------------|-------------|------------|--------------------------------------------|------------|
| 284.5                       | 0.660       | 284.5      | 0.737                                      |
| 218.5                       | 1.255       | 219.0      | 1.484                                      |

![Figure 6. FTIR spectrum of CBN purified isolate and CBN lipomed.](image)
Based on figure 6 and table 3, FTIR profile of purified CBN isolate, functional group of -C=C- of the aromatic compound that appears at the wave number area 1620.21 cm\(^{-1}\), wave number 1581.63 cm\(^{-1}\) of -C-O- bond, wave number 1026.13 cm\(^{-1}\) of -C-O-C- symmetric and 1232.51 cm\(^{-1}\) of -C-O-C- asymmetric, that confirmed with CBN Lipomed and CBN literature profile, indicated the similar structure of purified CBN isolate and CBN Lipomed. Figure 7 shows the melting point of purified CBN isolate, which the melting point of compound is 74.36 °C and 99.35 % purity.

Confirmation of the structure and molecular weight were determined by GCMS and LCMS/MS. Based on figure 8, the purified CBN isolate has only one peak appears in retention time 18.307 min, same as the retention time of CBN peak from CBN lipomed. Fragmentation of the compound, m/z 310.1, 296.2, 295.2, 251.1, 239.1, 238.1, 223.1 and base peak at 295.2 are similar between purified CBN isolate and CBN Lipomed, indicating a structure similarity of the compounds, that is CBN with 310 molecular weight.

Relative abundance of the m/z fragmentation is also used as qualification of identification of the compound [17]. Based on table 4, the relative abundance of fragmentation of purified CBN isolate is similar to the CBN Lipomed, which is slightly different at m/z 296.2, with the difference value of 2.17 point, indeed it is still in range of tolerance value.

LCMSMS data in figure 9 show that molecular weight of purified CBN isolate is 310, confirmed with daughters scan result, that shows [M+1] m/z 311 fragmented to 293, 241, 223, 222, 195, 169, 119 and 71, similar profile with CBN Lipomed. MRM Data in table 5 show that relative abundance of MSMS fragmentation are in accordance to the qualification of identification point [17].

### Table 3. λ max of CBN purified isolate and CBN lipomed.

| CBN lipomed | Purified CBN isolate | Wave number literature (cm\(^{-1}\)) [16] | Functional grup [15] |
|-------------|----------------------|-------------------------------------------|----------------------|
| 1620.21     | 1620.21              | 1620                                      | -C=C-, Aromatic      |
| 1051.20     | 1051.20              | 1050                                      |                     |
| 1581.63     | 1581.63              | 1580                                      | -C-O-                |
| 1026.13     | 1026.13              | 1030                                      | -C-O-C- symmetric    |
| 1128.36     | 1128.36              | 1120                                      |                     |
| 1232.51     | 1232.51              | 1228                                      | -C-O-C- asymmetric   |

![Figure 7. Thermogram of CBN purified isolate.](image)
Figure 8. GCMS profile (a) chromatogram of CBN lipomed, (b) chromatogram of purified CBN isolate, (c) fragmentation of CBN lipomed, (d) fragmentation of purified CBN isolate.
Table 4. GCMS Fragmentation and m/z abundance of CBN purified isolate and CBN lipomed.

| m/z | CBN Lipomed Relative abundance (%) | m/z | Purified CBN Isolate Relative abundance (%) | Tolerance of relative abundance different [17] |
|-----|-----------------------------------|-----|--------------------------------------------|---------------------------------------------|
| 223.1 | 1653.54 4.84 | 223.1 | 88809.41 4.54 | ± 50 % |
| 238.1 | 5220.6 15.28 | 238.1 | 296073.97 15.14 | ± 20 % |
| 239.1 | 1283.69 3.76 | 239.1 | 72898.01 3.73 | ± 50 % |
| 251.1 | 1554.73 4.55 | 251.1 | 85172.15 4.35 | ± 50 % |
| 295.2 | 34160.38 100 | 295.2 | 1956120.4 100 | ± 15 % |
| 296.2 | 7681.78 22.49 | 296.2 | 482341.88 24.66 | ± 20 % |
| 310.2 | 3409.84 9.98 | 310.2 | 252629.33 12.91 | ± 20 % |

Figure 9. LCMSMS profile (a) parent scan of CBN lipomed, (b) parent scan of purified CBN isolate, (c) daughter scan of CBN lipomed, (d) daughter scan of purified CBN isolate.

Table 5. LCMSMS MRM mode: MSMS fragmentation profile and m/z abundance of purified CBN isolate and CBN lipomed.

| m/z  | CBN Lipomed Abundance | Relative abundance (%) | m/z  | Purified CBN Isolate Abundance | Relative abundance (%) | Tolerance of relative abundance different [17] |
|------|-----------------------|------------------------|------|-------------------------------|------------------------|---------------------------------------------|
| 222.95 | 216756                | 100                    | 222.95 | 58375                         | 100                    | ± 20 % |
| 292.95 | 122415                | 56.48                  | 292.95 | 33688                         | 57.71                  | ± 50 % |
| 240.95 | 77707                 | 38.35                  | 240.95 | 22389                         | 38.35                  | ± 25 % |
| 194.95 | 80558                 | 37.17                  | 194.95 | 22601                         | 38.72                  | ± 25 % |
4. Conclusion
CBN Compound has been extracted from cannabis plants by maceration combined with ultrasonication assisted extraction. Extraction time reduced from 24 h to 5 h by adding ultrasonication before maceration, while 15 min ultrasonication before 5 h maceration could increase 5.536 % yield relative compared to 24 h maceration. The isolation of CBN compound from CBN extract by column chromatography with n-hexane-ethyl acetate (90:10) eluent and purification by HPTLC preparative with n-hexane-ethyl acetate (80:20) eluent produce a CBN isolate with high purity, indicating by single spot of CBN in HPTLC system. The purified CBN isolate was characterized using Spectrophotometer UV, FTIR, DCS, GCMS and LCMSMS and compared the profile with the reference standard of Cannabinol from Lipomed. The characterization results showed that the resulting isolate was Cannabinol with molecular weight of 310, melting point 74.36 °C and purity 99.35 %, therefore, it could be a candidate for Cannabinol secondary reference standard material.

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