Isolation and determination of FUB-AMB in synthetic cannabinoids by gas chromatography-mass spectrometry

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Abstract. N-[[1-[(4-fluorophenyl)methyl]-1H-indazol-3-yl]carbonyl]-L-valine, methyl ester (FUB-AMB) is the most recent psychoactive substance in Vietnam. FUB-AMB is a synthetic cannabinoid (SC) with has similar biological effects to tetrahydrocannabinol (THC), the main active component of natural cannabis. The previous study exhibits a harmful SC compound, which may cause many threatening to consumer health and social security due to the intensive effect of this compound on the mental status and behavior of the consumer. In this study, FUB-AMB was isolated the first time in Vietnam (1 g) with a purity of 98.34% (determined by High Performance Liquid Chromatography). Spectroscopic data (ultraviolet, infraRed 1Hydro - nuclear magnetic resonance, 13carbon - nuclear magnetic resonance) confirmed the structure of that isolated compound. The isolated FUB-AMB was used as a reference standard to develop an analytical method to detect drugs in the general marijuana group. Chromatography separation was achieved using a diphenyl dimethyl polysiloxane Gas chromatography (GC) column (30m×0.25mm×0.25µm) with a total run time of 10 minutes. The limits of detection and quantification for FUB-AMB was 0.15 µg/mL and 0.5 µg/mL, respectively. The developed method was used to detect FUB-AMB in synthetic cannabinoids in 30 samples. The results showed that the content of FUB-AMB ranged from 3.4-59.2 mg/g in 30 synthetic cannabinoids. The advanced GC-MS method is simple, sensitive, accurate and practically useful for the determination of FUB-AMB in synthetic cannabinoids, which supports the authorities’ crime handling.

1. Introduction

Synthetic cannabinoids (SCs) are referred to substances possessing structure that allows binding to one of the known cannabinoids receptors (CB), i.e., CB1 or CB2, present in human cells. Among these receptors, the CB1 receptor is mainly present in the brain and spinal cord, responsible for respiratory, cardiovascular function, and especially psychotropic effect similar to marijuana, the natural cannabinoid. However, the main compositions of marijuana belong to Δ9 - tetrahydrocannabinol and cannabidiol skeleton. Meanwhile, SCs have a variety of structure which has an affinity to one of the cannabinoid receptors. Synthetic cannabinoids (SCs) constitute a wide category of New Psychoactive
Substances (NPS) substances. They are called as "legal alternative to (illegal) cannabis" because they imitate the psychoactive effects (elevated mood, relaxation, altered perception, and psychotic symptoms) of the principal psychoactive component of cannabis – Δ9-tetrahydrocannabinol [1].

Most patients with SCs experience adverse symptoms such as vomiting, irritability, race feelings, palpitations, fear, hysteria and hallucinations, chest pain, severe kidney failure, ischemic stroke, myocardial infarction and myocardial ischemia. A disturbing increase in deaths following inhalation of SCs has been recorded over the last few years [2–4], and in some cases, information on forensic autopsies was received [5, 6]. Pathological findings included pulmonary edema and visceral congestion, acute oxygen deficiency, cerebral edema and hypoxic-ischemic damage of the central nervous system (CNS) [7]. The effects of synthetic marijuana on humans are similar to those of natural marijuana, including excitement, distraction, feelings of suffering, confusion, anxiety, and fear. The overdose of synthetic marijuana will lead to hallucinations, paranoia, psychotic syndrome, increased heart rate, nausea, seizures, and movement disorders. In particular, the synthetic marijuana effect is considered to be more toxic than natural marijuana. The synthetic marijuana causes toxicity at lower doses, more likely to cause overdose, causing more serious toxicity than natural marijuana. Langer et al. also suggest that the misuse of synthetic marijuana can lead to life-threatening symptoms such as seizures, acute kidney failure, and sudden death [7].

From 2016-2019, the National Institute of Criminal Sciences had detected many new SCs, and FUB-AMB is the most recent agent in this group, which was synthesized by Ingrid et al [8]. This compound was detected in a product named "Train Wreck 2" at Louisiana, USA. Immediately, this compound was abandoned by the local Government. FUB-AMB other SCs have been considered as the replacement for classical drugs such as amphetamine, heroin, cocaine and natural marijuana [9]. This substance causes hallucinations and has toxic effects, including respiratory depression, cardiac arrest, cerebral ischemia, and convulsions. In 2015, more than 20 new synthetic marijuana compounds reported by the European Police Department. In 2016, FUB-AMB appeared in a synthetic marijuana product found in New York, resulting in 33 hospitalizations (Figure ). In 2016, in Brooklyn (USA), appeared dozens of people in the subway station has the same morale and behavior as "zombies". The report showed that the victims had overdosed of synthetic marijuana called FUB-AMB, which was 85 times more toxic than natural marijuana [10]. In 2017, in New Zealand, at least 20 deaths were related to FUB-AMB. In particular, the concentration of FUB-AMB in confiscated products is 2 to 25 times higher than reported in New York [11]. In 2018, FUB-AMB was discovered increasingly popular in Europe, the United States, and New Zealand. More than 800 new psychotropic substances have been reported to the United Nations from 111 countries and territories, of which FUB-AMB has been listed on the list of legal oversight in China and many other countries [12, 15]. In 2019, in Bulgaria, an 18-year-old male victim died from a long-term combination of marijuana use, substance abuse within 48 hours. The cause of death was determined by acute respiratory failure. Samples of samples were assessed, resulting in FUB-AMB and 5F – ADB [14].

![Figure 1. Evolution of Synthetic Cannabinoid Structures.](image-url)
In Vietnam, FUB-AMB has a powerful effect in stimulating nerves and hallucinations compared to the active ingredient Delta9-THC in plant cannabis. The use of these drugs is considered hazardous, which causes the loss of the ability to control behavior, give rise to violations of law and violate to commune ethical standards guilds. Therefore, the Vietnamese Government issued the Government's Decree No. 73/2018 / ND-CP, which listed controlled narcotic substances, including the FUB-AMB.

One difficulty for dealing with violations, investigations, prosecution, and adjudication of drug cases in Vietnam is that there has not been much research on the analysis of synthetic marijuana, especially FUB-AMB leads to more drug substances in cases from the end of 2016 to the beginning of 2019. Due to these mentioned reasons, this study was conducted at the Ho Chi Minh City Institute of Criminal Science. This study aims to isolate and develop an analytical method for quantitative determination FUB-AMB in a synthetic cannabis sample.

2. Materials and Methods

2.1. Chemicals and reagents
All chemicals used for isolation were of analytical grade. Solvents used for analytical purposes are chromatography grade. Benzydamine hydrochloride, ethyl acetate (EtOAc), acetonitrile (MeCN), chloroform-d$_1$ (CDCl$_3$), chloroform, and dichloromethane were purchased from Sigma-Aldrich. Methanol (MeOH) was from J.T. Baker (Deventer, Netherlands). The silica gel 60 used for column chromatography and thin-layer chromatography (TLC) silica gel 60 F 254 plate was purchased from Merck (Germany).

2.2. Plant material and extraction
Samples of synthetic marijuana are exhibits collected from 2016-2019 in southern Vietnam. The samples were dried in temperature under 60°C by Memmert UN55 machine and pulverized to achieve powder with particle size under 1 mm (Hitech-2T350). Sample powder was triplicated extract by ultrasound using methanol for 20 min at 50 °C (Elmasonic S 100H). The extracts were combined, filtered, and evaporated to dryness under reduced pressure to obtain 15.58 g of total MeOH extract.

2.3. Isolation
MeOH extract was fractionated by flash column chromatography using silica gel (40-63 µm) as stationary phase to obtain hexane, CHCl$_3$ and hexane-ethyl acetate (8:2) fraction. The hexane-ethyl acetate (8:2) fraction was further purified using a MeOH mixture to yield pure FUB-AMB (1 g) and used for further spectroscopic characterization. Purity and authenticity of FUB-AMB was confirmed by NMR, UV-Vis, IR spectra, and HPLC-DAD, GC–EI-MS analysis [15].

2.4. Analysis of synthetic cannabinoids
Homogenized herbal sample (100 mg) was mixed with MeOH (10 mL), vortexed for 1 min and then extracted by ultrasound for 20 min. The extract was centrifuged and filter through 0.45 µM syringe filter. Internal standard (benzylamine hydrochloride, 0.5 g) was added. The mixture was then transferred to 10 mL volumetric flask and filled with MeOH. GCMS analysis of the extracts was carried out using a gas chromatography system of the 7890B series coupled with a mass selective detector of the series 5977B (Agilent Technologies, USA). The GC conditions including split (1:5) injection; column: HP-5-MS capillary (30m x 0.25mm ID, 0.25 mm film thickness); injection port temperature, 270°C; carrier gas, helium; flow rate, 1.2 mL/min; oven temperature, initially 150 °C (kept for 1 min), ramped to 290 °C at 30 K/min (290 °C kept for 4 min). The MS conditions follows: transfer line heater, 280 °C; ion source temperature, 230 °C; electron impact ionization (EI) mode; ionization energy, 70 eV. The analysis was performed in full-scan mode from 50 to 550 amu at a speed of 1.5 scans per second. The
solvent delay was set to 2.5 min. Compounds were defined through comparison with Cayman Spectral Library and NIST MS library mass spectral reference data [16].

3. Results and Discussions

3.1. Extraction and isolation of FUB-AMB

Figure 2. "American Grass" sample mixture. (a) 2016; (b) 2017; (c) 2018; (d) 2019

Figure 2 illustrated the American Grass sample mixture, which are obtained at the Ho Chi Minh City Institute of Criminal Science. Synthetic cannabinoids (SCs) or "American Grass" is the herbals infused with synthetic compounds that possess similar bioactivity as delta 9-THC from natural marijuana. About 30 samples were aliquoted and mixed to obtain the present sample used for the chemical study. The presence of FUB-AMB in the raw material was confirmed by using GC-MS. The primary component was found at the retention time of 7.8 min in the total ion chromatogram with fractions of m/z 109, 253, 324, 269, 383 (Figure 3).

Figure 3. GC-MS total ion chromatogram of sample mixture.
Four solvents (methanol, ethyl acetate, acetonitrile, and dichloromethane) were tested for extraction capacity. The results showed no significant difference between the yield of the extract of each solvent (Table 1). Therefore, methanol was chosen for the extraction due to its polarity, low price, and less toxicity. The extraction procedure was optimized with the parameters of solvent volume (5 mL, 10 mL, 20 mL), temperature (40 °C; 50 °C; 60 °C), duration (10 min; 15 min; 20 min), and replication time (1 time; 2 times; 3 times). GC-MS analyzed the FUB-AMB content of each experiment, and the peak area was compared (Figure 3). Figure 4 compares extraction yield of different extraction conditions to optimize extraction parameters achieved at solvent volume 10 mL, temperature 50 °C, duration 15 min, and replication time two times.

Table 1. The yield of the extract of methanol, ethyl acetate, acetonitrile, and dichloromethane.

| Solvent          | Weight (g) | Area peak (µV.min) |
|------------------|------------|--------------------|
| Methanol         | 0.0104     | 213527 18.53       |
| Ethyl acetate    | 0.0105     | 185197 07.73       |
| Acetonitrile     | 0.0104     | 150936 83.49       |
| Dicloromethane   | 0.0106     | 741844 1.01        |

Table 2. Parameters for extraction procedure optimization

| Parameters       | Analyzed condition | Fixed condition                  |
|------------------|--------------------|-----------------------------------|
| Solvent volume   | 5 mL; 10 mL; 20 mL | 40 °C; 10 min; no replicate       |
| Temperature      | 40 °C; 50 °C; 60 °C| 10 mL; 10 min; no replicate       |
| Duration         | 10 min; 15 min; 20 min| 10 mL; 50 °C; no replicate       |
| Replication time | 1 time; 2 times; 3 times| 10 mL; 50 °C; 10 min          |

Figure 4. Comparison of extraction yield of different extraction conditions.

MeOH total extract was evaporated under vacuum to achieve dry extract and then subjected to normal-phase column chromatography. The column was eluted by 100% n-hexane, 100% CHCl₃, followed by n-hexane-EA (8:2) to achieved three fractions. The final fraction was recrystallized by
MeOH to obtain pure FUB-AMB (Figure 5). Based on $^1$H and $^{13}$C NMR spectra of isolated FUB-AMB (Figure 6) and $^1$H and $^{13}$C NMR data of isolated FUB-AMB (Table 3), the structure of the isolated compound was confirmed by NMR spectrometry with the name of methyl-(2S)-2-[[1-[(4-fluorophenyl)methyl]indazol-3-carbonyl] amino]-3-metylbutanoate.

Figure 5. (a) Recrystallized pure FUB-AMB. (b) Structural formula of FUB – AMB

Figure 6. $^1$H and $^{13}$C NMR spectra of isolated FUB-AMB.
Table 3. $^1$H and $^{13}$C NMR data of isolated FUB-AMB

| Carbon no. | $\delta$ $^1$H-NMR (ppm) | $\delta$ $^{13}$C-NMR (ppm) | IR |
|------------|---------------------------|----------------------------|----|
| 1'         | -                         | 162.6                      |    |
| 3          | -                         | 136.9                      |    |
| 3a         | -                         | 122.4                      |    |
| 4          | 8.15, 1H, J = 8 Hz         | 121.7                      |    |
| 5          | 7.29, 1H, t, J = 7 Hz      | 122.7                      |    |
| 6          | 7.45, 1H, m               | 127.0                      |    |
| 7          | 7.78, 1H, J = 8.5 Hz       | 110.5                      |    |
| 7a         | -                         | 140.5                      | 3422 (w) |
| 1''        | 5.78, 2H, s               | 51.8                       | 1740 (s) |
| 2''        | -                         | 132.9                      | 1674 (s) |
| 3''/7''    | 7.32 – 7.35, 2H, m        | 129.4                      | 1534 (s) |
| 4''/6''    | 7.14 – 7.18, 2H, m        | 115.5                      | 1218 (s) |
| 5''        | -                         | 161.2                      | 1181 (s) |
| 1'''       | -                         | 171.9                      | 781 (m) |
| 2''''      | 4.45, 1H, dd, J = 7, 1 Hz | 57.3                       |    |
| 3'''       | 2.26, 1H, m               | 29.9                       |    |
| 4'''       | 0.97, 3H, d, J = 6.5 Hz   | 19.0                       |    |
| 5'''       | 0.95, 3H, d, J = 6.5 Hz   | 18.6                       |    |
| OCH$_3$    | 0.945 – 0.977, 3H, m      | 51.6                       |    |
| CONH       | 7.78, 1H, J = 8.5 Hz      |                            |    |

3.2. Qualitative and quantitative analysis of FUB-AMB by GC-MS method

For qualitative and quantitative analysis of FUB-AMB in "American Grass" samples, a GC-MS method was developed and optimized. In GC analysis, the temperature program plays a crucial role in separating the compound of interest from the matrix. FUB-AMB was eluted at 7.8 min with the asymmetry index of 1.18 and separated with the other peaks. In this study, the initial temperature was set to 150 °C, which allowed to shorten the analysis duration to 10 mins. In the previous research carried out by Michelle et al. 2017, a GC-MS method was developed to determine FUB-AMB in electric cigarettes with a lower initial temperature of 120°C. As the results, the analysis duration is 30 min, three times longer than our methods [17]. To detect FUB-AMB eluted from column, a mass spectrometry detector was used. The ionization FUB-AMB could be conducted using various technologies such as electrical ionization (EI), chemical ionization, and electrospray ionization (ESI+). In this study, EI was used to ionized the compound of interest. EI’s advantage is that the signal response is not affected by the structures and could produce a large number of characterized fractions. Also, the use of ion traps in scan mode could detect all the compounds without choosing a characteristic ion during data acquisition. Previously, Marisol et al. used the Time of Flight (QtoF-MS) to detect FUB-AMB in urine samples, which required a highly sensitive and selective technique with complicated and expensive apparatus [13]. Beside GC-MS, High Performance Liquid Chromatography (HPLC) coupled with UV-Vis or photodiode array (PDA) detector is also utilized to determine SCs. Particularly, Barry K. Logan et al. used HPLC-PDA to detect SCs with a mobile phase consisting of acetonitrile – 0.1% trifluoroacetic acid. However, the selectivity and sensitivity of this method were not as much as GC-MS.

The developed GC-MS method was validated, and the result showed that FUB-AMB has a wide linear range from 0.5-200 µg/mL, achieving precision with %RSD of 0.26% and accuracy with recovery ranged from 99.36-101%. The limit of detection (LOD) and Limit of Quantitation (LOQ) values are 0.15 µg/mL and 0.5 µg/mL, respectively. Therefore, the methods achieved proper sensitivity for detecting and quantifying such shallow content of FUB-AMB in "American Grass" samples. The verification results also show that the method meets all ICH guidelines requirements, ensuring reliability, selectivity, accuracy, and accuracy. Hence, our developed GC-MS method is sensitive and
reliable for the qualitative and quantitative determination of the shallow content of FUB-AMB found in "American grass" samples.

Developed GC-MS was applied to detect FUB-AMB in 30 "American Grass" samples collect from 2016-2018 (Table 4). The result exhibits this compound’s presence in 24/30 samples with an average content of 29.6 (mg/g). Seriously, there were two samples with high warning content of 52.4 mg/g and 59.6 mg/g. There was also a large variety of FUB-AMB among samples, about 10 times difference in particular. On the other hand, the toxicity of FUB-AMB has been reported to be 75-85 times more than that of tetrahydrocannabinol in natural cannabis and 50 times more than of JWH-018, another synthetic narcotic drug. Hence due to the high toxicity and variety of FUB-AMB in "American Grass" circulating on the market, the consumption of these top risk products could cause fatal consequences for the health of consumers as well as give rise to threatening criminal acts.

Table 4. FUB-AMB content in 30 “American Grass” samples

| Sample | Content (mg/g) | Sample | Content (mg/g) | Sample | Content (mg/g) |
|--------|----------------|--------|----------------|--------|----------------|
| A1     | 39.0           | B1     | 42.3           | C1     | 41.3           |
| A2     | 27.3           | B2     | 13.1           | C2     | 59.2           |
| A3     | 39.0           | B3     | 45.5           | C3     | 11.2           |
| A4     | 30.3           | B4     | 42.0           | C4     | 7.1            |
| A5     | –              | B5     | 52.4           | C5     | 3.4            |
| A6     | –              | B6     | 7.9            | C6     | 29.5           |
| A7     | –              | B7     | 25.8           | C7     | 6.9            |
| A8     | 22.6           | B8     | 33.6           | C8     | 7.2            |
| A9     | 31.1           | B9     | –              | C9     | –              |
| A10    | –              | B10    | 52.6           | C10    | 40.0           |

(–) Not detected

4. Conclusions
In our study, the compound FUB-AMB (1g) was isolated and identified by spectrum data with 98.34% purity. The total yields were dependent on the extraction conditions. The optimized extraction parameters were at 10 mL of solvent volume, temperature at 50 °C, duration of 15 min, and two replication times. From this result, FUB-AMB can be used as a standard in testing, contributing to the method development for analyzing drug substances in the general marijuana group to support the authorities’ crime handling. Using this compound as a reference standard, a GC-MS method was developed and validated for the qualitative and quantitative determination of FUB-AMB in 30 "American Grass" samples (from 3.4 mg/g to 59.6 mg/g). The limits of detection and quantification of FUB-AMB was 0.15 μg/mL and 0.5 μg/mL, respectively.

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