Supplemental Online Content

Cohen PA, Avula B, Wang Y-H, Katragunta K, Khan I. Quantity of melatonin and CBD in melatonin gummies sold in the US. *JAMA.* Published April 25, 2023. doi:10.1001/jama.2023.2296

Supplement 1. **eAppendix.** Supplemental Methods

eReferences

Supplement 2. **Data Sharing Statement**

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eAppendix. Supplemental Methods

1. Chemicals and reagents

High performance liquid chromatography (HPLC) grade acetonitrile, HPLC grade methanol, and liquid chromatography-mass spectrometry (LC-MS) grade formic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Water was obtained using a milliQ-Gradient system (Millipore, Billerica, USA). The reference compounds, melatonin, cannabidiol (CBD), cannabidiolic acid (CBDA), cannabigerolic acid (CBGA), cannabigerol (CBG), cannabinol (CBN), Δ^9-tetrahydrocannabinol (Δ^9-THC), and Δ^9-tetrahydrocannabinolic acid-A (THCAA) were purchased from Sigma (St. Louis, MO, USA). The purities of all reference standards are >99%. All dietary supplements were purchased online from Amazon (www.amazon.com), Walmart (www.walmart.com), GNC (www.gnc.com), Walgreens (www.walgreens.com) and other retailers' websites.

2. Preparation of reference materials and samples

Preparation of melatonin standard solutions

A stock solution of the melatonin was prepared at a concentration of 1 mg/mL in methanol. The calibration curve was prepared in methanol at five different concentrations, ranging from 1 to 100 µg/mL.

Preparation of CBD and related cannabinoids standards

Stock solutions of the individual standard including CBD, CBDA, CBGA, CBG, CBN, Δ^9-THC, and THCAA were 1 mg/mL in methanol. The calibration curve of each analyte was prepared in a mixture of acetonitrile and methanol (80:20, v/v) at nine different concentrations ranging from 1 - 100 µg/mL.

Preparation of the purchased supplements (gummies)

Melatonin analysis: The dietary supplements were in the form of gummies. Five gummies were weighed, cut into small pieces and uniformly mixed. Next, about one-fourth weight of one gummy of the homogenized samples were weighed into centrifuge tubes and resuspended in 2.5 mL of methanol for 30 minutes sonication followed by centrifugation for 15 minutes at 959 ×g. The supernatant, consisting of a clear solution, was transferred to a 10 mL volumetric flask. The procedure was repeated three times and the obtained supernatants were combined. The final volume was subsequently adjusted to 10 mL with methanol and mixed thoroughly. Prior to injection, all samples were filtered through a 0.45µm polytetrafluoroethylene (PTFE) membrane filter. The first 1.0 mL was discarded and the remaining volume was collected in a sample vial.

CBD and related cannabinoids analysis: One gummy was weighed and completely dissolved in water in a ratio of 1 gram of gummy:2 mL of water. In a 15 mL centrifuge tube, 140-160 µL of gummy water solution was weighed, then 1.8 mL of water and 6.0 mL of extraction solvents (acetonitrile/methanol = 80:20, v/v) were added to the content of the centrifuge tube. The sample was extracted in ultrasonic equipment with a water bath for 30 min and followed by centrifugation at 959 ×g for 15 min. The clear solution was transferred to a 10 mL volumetric flask. The extraction procedure was repeated using 2.0 mL of extraction solvents. All extracts were combined. The final volume was justified to 10 mL using extraction solvents. After mixing thoroughly, the sample was filtered using a 0.45µm PTFE membrane filter prior to LC analysis.

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3. Instrumental conditions

Ultra-High-Performance Liquid Chromatography-Photodiode Array (UHPLC-PDA) Analysis

Melatonin analysis: All analyses were performed on a Waters Acquity UPLC™ H-Class system (Waters Corp., Milford, MA, USA) including quaternary solvent manager, sampler manager-flow through needle, column heater, and photo-diode array (PDA) detector connected to Waters Empower 3.7.0 data station. An Acquity UPLC™ BEH Shield RP18 column (100mm×2.1mm I.D., 1.7µm) also from Waters was used. The column and sample temperature were maintained at 40 °C and 15 °C, respectively. The column was equipped with a LC-18 guard column (Vanguard 2.1 x 5 mm, Waters Corp., Milford, MA, USA). The mobile phase consisted of water (0.1 % formic acid) (A), acetonitrile (B) (0.1 % formic acid) at a flow rate of 0.23 mL/min, which were applied in the following linear gradient elution: 0-3 min, 70 % A:30 % B to 100 % B. Separation was followed by a 2 min column washing procedure with 100 % B and a re-equilibration period of 4.5 min at the starting conditions. A strong needle wash solution (95/5; acetonitrile/water) and a weak needle wash solution (10/90; acetonitrile/water) were used. All solutions were filtered via 0.45 µm membrane filters and degassed before their usage. The total run time for analysis was 3 minutes. The injection volume was 2 µL. The detection wavelength for melatonin was 220 nm. Peak identity for melatonin was assigned by analysis of a reference standard and comparing the retention time and ultra-violet spectrum with samples fortified with the reference standard.

CBD and related cannabinoids analysis: The analysis was conducted following a previously published methodology [1]. In brief, all analyses were carried out on a Waters Acquity UPLC system (Waters Corp., Milford, MA, USA) consisting of a binary solvent manager, sample manager, heated column compartment, and photodiode array (PDA) detector. A CORTECS UPLC C18 column (100mm× 2.1mm I.D., 1.6 µm) from Waters was used. The column and sample temperature were set at 35°C and 15°C, respectively. The mobile phase was composed of water containing 0.05% formic acid (A) and acetonitrile with 0.05% formic acid (B) using the following gradient elution at a flow rate of 0.25 mL/min: 0–8 min, 70 to 80% B; 8–9 min, 80 to 100% B. The injection volume was 2 µL. The PDA detection wavelength was 220 nm.

4. Validation procedure

The newly developed UHPLC-PDA method for melatonin was validated with respect to selectivity, sensitivity, limit of detection (LOD), limit of quantification (LOQ), stability, precision, accuracy, specificity and linearity according to International Council for Harmonization (ICH) guidelines.[2]

The specificity and selectivity of the method was confirmed by (a) comparing chromatograms of blanks (i.e., methanol and matrix without melatonin) with blanks containing melatonin, and (b) comparing chromatograms of L and U with samples L and U spiked with melatonin.

The LOD and LOQ were determined by injecting a series of dilute solutions with known concentrations for each standard. LOD and LOQ were defined as the signal-to-noise ratio equal to 3 and 10, respectively. A five-point calibration curve for melatonin showed a linear correlation between concentration and peak area. Calibration data indicated the linearity (r^2>0.99) of the detector response. The LOD and LOQ were found to be 100 and 300 ng/mL, respectively. All samples and standard solutions were injected in triplicate.

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The accuracy of the assay method was evaluated by spiking two products (product code L, U) in triplicate using concentration level of 1 µg/mL, 50 µg/mL and 100 µg/mL. The accuracy of the method was determined for the related compound by spiking sample (brand code L, U) with a known amount of melatonin standard. These samples spiked with known amounts of the standard compound mixture were extracted four times under optimized conditions. The percentage recovery of these samples ranged from 95-103%.

Precision of a method is the degree of agreement among individual analytical results when the procedure is applied repeatedly to multiple samples of each product. The intra- and inter-day precision were estimated by analyzing multiple replicates of two products (product code L, U). The intra-day precision of the assay was estimated by calculating the relative standard deviation (RSD) for the analysis of samples in three replicates (n=3) of each product and inter-day precision was determined by the analysis of three replicates each of same product on three consecutive days. The intra-day RSD for the replicates were between 0.3 and 1.0% and RSD for the day to day replicates were between 0.4-1.6 %. From the measured standard deviation (SD) and mean values, precision as relative standard deviation (% RSD) is calculated as % RSD = SD/mean×100

The sample solution (product code A, F, L, U) and standard solutions (10, 50 µg/mL) were prepared as per the proposed method and subjected to stability study at room temperature for 72h. The sample solution was analyzed at initial and at three time intervals up to 72h. No significant changes were observed in the concentrations of the components analyzed with respect to time.

Screening for the presence of serotonin

The supplements were screened for synthetic or hidden compounds using Agilent MassHunter Forensics and Toxicology (9203 compounds) Personal Compound Database (PCD). During this screening, serotonin was not detected.

eReferences

[1] Yan-Hong Wang, Bharathi Avula, Mohmoud A ElSohyly, Mohamed M. Radwan, Mei Wang, Amira S. Wanas, Zlatko Mehmedic, and Ikhlas A. Khan, Quantitative determination of Δ^9-THC, CBG, CBD, their acid precursors and five other neutral cannabinoids by UHPLC-UV-MS. *Planta Medica*, 2018, 84: 260-266

[2] ICH. Validation of Analytical Procedures: Text and Methodology. ICH Harmonized Tripartite Guidelines Nov. 2005

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