Forensic Science International 227 (2013) 33–41

Contents lists available at SciVerse ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

DNA sequence analyses of blended herbal products including synthetic cannabinoids as designer drugs☆

Jun Ogata, Nahoko Uchiyama, Ruri Kikura-Hanajiri, Yukihiro Goda *

National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

ARTICLE INFO

Article history:
Available online 23 October 2012

Keywords:
BLAST search
DNA analysis
DNA barcode
GC–MS
Herbal product
LC–MS

ABSTRACT

In recent years, various herbal products adulterated with synthetic cannabinoids have been distributed worldwide via the Internet. These herbal products are mostly sold as incense, and advertised as not for human consumption. Although their labels indicate that they contain mixtures of several potentially psychoactive plants, and numerous studies have reported that they contain a variety of synthetic cannabinoids, their exact botanical contents are not always clear. In this study, we investigated the origins of botanical materials in 62 Spice-like herbal products distributed on the illegal drug market in Japan, by DNA sequence analyses and BLAST searches. The nucleotide sequences of four regions were analyzed to identify the origins of each plant species in the herbal mixtures. The sequences of "Damiana" (*Turnera diffusa*) and Lamiaceae herbs (*Mellissa*, *Mentha* and *Thymus*) were frequently detected in a number of products. However, the sequences of other plant species indicated on the packaging labels were not detected. In a few products, DNA fragments of potent psychotropic plants were found, including marijuana (*Cannabis sativa*), "Diviner's Sage" (*Salvia divinorum*) and "Kratom" (*Mitragyna speciosa*). Their active constituents were also confirmed using gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS), although these plant names were never indicated on the labels. Most plant species identified in the products were different from the plants indicated on the labels. The plant materials would be used mainly as diluents for the psychoactive synthetic compounds, because no reliable psychoactive effects have been reported for most of the identified plants, with the exception of the psychotropic plants named above.

1. Introduction

In recent years, various herbal products (dried leaves, stems, petals and seeds mixtures) including the synthetic marijuana product "Spice," have been distributed around the world via the Internet [1–12]. In Japan, these products are readily available as herbal incense via the Internet and in "head shop". However, anonymous posters on various Internet web sites have reported experiencing cannabis-like effects after smoking these herbal products.

In 2009, our group and a group in Germany were the first to detect and identify the synthetic cannabinoids cannabicyclohexanol (CCH) and JWH-018 in these herbal products [5–7]. Since those reports, more than 30 synthetic cannabinoids have been detected as psychoactive ingredients in these herbal products in various countries around the world [1–12]. In addition, our group identified two new-type synthetic cannabinoids [12]. In Japan, 23 of these synthetic cannabinoids were controlled as "Designated Substances" under the Pharmaceutical Affairs Law as of July 2012 [13,14].

The labels of these herbal products indicate that these products contain several potentially psychoactive plants. In some instances, the ingredients are listed as common plant names, such as "Baybean," "Blue lotus," "Pink lotus," "Dwarf skullcap," "Indian warrior," "Lion's tail," "Maconha brava," "Marshmallow," "Red clover," "Rose," "Siberian motherwort," and "Vanilla". However, there is no precise information on their contents, and the actual plant species included in these herbal products have never been determined.

The identification of plant spices by morphology requires botanical knowledge and a great deal of experience. In addition, morphology and anatomy rarely confirm the source of the plant, particularly for degraded and fragmented materials, unless some content information already exists. Recently, a new genetic analysis technique in plants has been widely used for species

☆ This paper is part of the special issue entitled "The 50th Annual Meeting of the International Association of Forensic Toxicologists (TIAFT)". June 3–8, 2012, Hamamatsu, Japan. Guest edited by Adjunct Professor Einosuke Tanaka and Associate Professor Masaru Terada.

* Corresponding author. Tel.: +81 3 3700 9154; fax: +81 3 3707 6950.
E-mail address: goda@nihs.go.jp (Y. Goda).

0379-0738/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.forsciint.2012.09.006

identification and the determination of phylogenetic relationships. The advances in molecular genetics over the last few years have provided genetic markers involved in the conservation of plant genetic resources for easy and reliable identification of plant species [15,16]. The methodology of DNA barcoding is used not only in botany but also in forensic science and herbal medicine. Moreover, the use of short orthologous standard DNA sequences as a tool for species identification with a standardized protocol, known as DNA barcoding, has been proposed and initiated to facilitate biodiversity and taxonomic studies [17]. A variety of loci have been recently suggested as potential DNA barcodes in plants, in both the nuclear and chloroplast genomes (e.g., the internal transcribed spacer (ITS), external transcribed spacer (ETS), *trnH-psbA*, *rpl16* intron, *matK*, *rbcL*, and *trnL-trnF* intron [17–26]). In addition, The Plant Working Group of the Consortium for the Barcode of Life (CBOL) has recently proposed a two-locus combination of *matK* and *rbcL* as the standard plant barcode [23]. CBOL is fostering the development of international research alliances to build a barcode library for all eukaryotic organisms. The main purpose of DNA barcoding is to provide rapid and accurate identification of unidentified plant organisms whose DNA barcodes have already been registered in a sequence library (DNA database; BLAST or BOLD systems).

In this study, we investigated the plant species (genus or family) of botanical materials included in herbal products by sequencing three regions of the chloroplast genome (*trnL-trnF*, *matK*, and *rbcL*) and one of the nuclear genome (an ITS combination), and positive matches in two or more regions were considered sufficient for identification of a particular species. Moreover, we analyzed some cannabinoids in an herbal product in which Cannabis genome sequences were detected by using gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS).

2. Materials and methods

2.1. Plant materials

Sixty-two herbal products being sold in Japan were purchased via the Internet from 2008 to 2011. All products had different names and were contained in different packages. We reported that various synthetic cannabinoids were contained as psychoactive ingredients in all these herbal products (see Table 1) [5,6,8–12]. Diviner's Sage (*Salvia divinorum*) was from a laboratory collection [27].

2.2. Chemicals and reagents

Δ^9-Tetrahydrocannabinol (Δ^9-THC) was purchased from Cirilliant (Round Rock, TX). Authentic CCH was isolated from herbal products and identified in our previous studies [5,6]. Cannabidiol (CBD) and cannabinol (CBN) were purchased from Sigma–Aldrich. All reagents and chemicals used were of analytical reagent grade or high-performance liquid chromatography (HPLC) grade.

2.3. DNA extraction, amplification and sequencing

Each herbal product (dry minced leaves, stems, petals and seed mixtures) was separated to obtain a single fragment (tissue) and then the surface of the tissue was rinsed with 100% ethanol. Each separated sample (ca. 10 mg) was transferred into a 2.0 mL reaction tube, and crushed in an MM-300 mixer mill (Qiagen, Germany) under liquid nitrogen. Total genomic DNA was isolated from the different types of tissue (dry minced leaves, stems, petals and seeds) with a QIAGEN DNeasy plant mini kit (Qiagen, Germany) following the manufacturer's guidelines. Using different sets of primers, we amplified the three regions of the chloroplast DNA, i.e., the *trnL-trnF* (comprised of the *trnL* intron with its 3′exon, and the *trnL-trnF* spacer), *matK*, and *rbcL* regions and the single region of nuclear rDNA, i.e., the ITS region (comprised of the internal transcribed spacer 1 between 18S rDNA and 5.8S rDNA, and 5.8S rDNA, and internal transcribed spacer 2 between 5.8S rDNA and 26S rDNA) by a polymerase chain reaction (PCR) using the respective genomic DNA samples as a template (Fig. 1). Approximately 1–5 ng of template DNA was used in a 20 μL PCR reaction consisting of 10 μL 2× Ampdirect plus (Shimadzu, Japan), 0.5 μM of each primer, and 0.5 units of Ex Taq (5 U/μL, Takara, Japan). Amplification was performed using the following protocol: an initial cycle at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 90 s, and a final extension step at 72 °C for 10 min. The universal primers were designed from each of the sequences conserved between the plant genes [18,28]. Their sequences were as follows: *trn*-forward primer, 5′-CGAAATCGGTAGACGCTACG-3′; *trn*-reverse primer, 5′-ATTTGAACTGGTGACACGAG-3′; *matK*-forward primer, 5′-CGTACAGTACTTTTGTGTTTACGAG-3′; *matK*-reverse primer, 5′-ACCCAGTCCATCTGGAAATCTTGGTTC-3′; *rbcL*-forward primer, 5′-ATGTCACCACAAACAGAGACTAAAGC-3′; *rbcL*-reverse primer, 5′-GTAAAATCAAGTCCACCRCG-3′; ITS-forward primer, 5′-CCTTATCATTTAGAGGAAGGAG-3′; ITS-reverse primer, 5′-TCCTCCGCTTATTGATATGC-3′. PCR products were confirmed by separation on 1% agarose gels containing 0.3 μg/mL of ethidium bromide. The single PCR product was purified and concentrated using polyethylene glycol precipitation, and direct sequencing of purified PCR products was carried out with each of the forward and reverse PCR primer pairs with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems). In the case of mixed PCR products, the DNA fragments were subcloned into the pMD20-T vector of a Mighty TA-cloning Kit (Takara), and insert DNA was amplified using M13 primers by colony PCR, and then was sequenced by the method described above. For sequence similarity we used the nucleotide BLAST programme (http://blast.ncbi.nlm.nih.gov/) [29,30] to identify each sample sequence.

2.4. Identification of plant species

When two different regions among the four (*trnL-trnF*, ITS, *rbcL* and *matK*) showed very high similarity (more than 99%) to the sequences of the registered reference species in GenBank, we defined the sample plant as belonging to the reference species for purposes of this report.

2.5. Preparation of samples from herbal products for GC and LC–MS analyses

Ten mg of the product sample was crunched and extracted with 1 mL of methanol by ultrasonication for 10 min. After centrifugation (5 min at 3000 rpm), the solution was passed through a centrifugal filter (Ultrafree-MC, 0.45 mm filter unit; Millipore, Bedford, MA).

2.6. GC–MS conditions

MS analysis was performed by GC–MS in electron impact (EI) mode at 70 eV electron energy. The GC–MS analysis was performed on a Hewlett-Packard 6890N GC with a 5975 mass selective detector using a capillary column (HP1-MS capillary; 30 m × 0.25 mm i.d., 0.25 μm film thickness) and helium gas as a carrier. The initial column temperature was 80 °C, and was increased at a rate of 5 °C/min to 190 °C followed by 10 °C/min to 310 °C. Data were obtained in full scan mode with a scan range of m/z 40–550. The analysis was performed using the established method and under conditions described in our previous reports [13,14].

2.7. LC–MS conditions

LC–MS analysis was conducted on an ultra-performance liquid chromatography–electrospray ionization-mass spectrometer (UPLC–ESI-MS), consisting of an ACQUITY UPLC system, a single-stage quadrupole detector and a photo diode array (PDA) detector (Waters, Milford, MA). The sample solutions were separated using an Atlantis HSS T3 column (100 mm × 2.1 mm i.d., particle size 1.8 μm; Waters) protected by a Van Guard column (5 mm × 2.1 mm i.d., 1.8 μm; Waters) at 40 °C. The mobile phase consisted of 0.1% formic acid in water (solvent A) and acetonitrile/MeOH (1:1) containing 0.1% formic acid (solvent B). The LC flow programme was: initially 95% A, linearly changed to 80% A in 15 min, then changed to 20% A at 25 min, and held for 25 min [A:B 95:5–80:20 (15 min) – 20:80 (25–50 min)]. The flow rate of the mobile phase was 0.3 mL/min and the injection volume was 1 μL. The wavelength of the PDA detector was set from 190 to 500 nm.

3. Results and discussion

3.1. DNA analyses of herbal products

We attempted to investigate the origins of plant species in each unknown herbal minced mixture (herbal product) using the DNA barcoding method. The central concept in species identification is to match the sequence of the applied sample to a reference sequence through DNA sequence similarity searches. If the sequence data of the applied sample is completely matched with the data in the GenBank database by a BLAST search, we can obtain the information of the correct plant species. In addition, even if no sequence data for the appropriate species have been published in GenBank, we can obtain the information of the correct genus or family affiliation from the database.

The contents printed on the herbal product packages included the plants Baybean (*Canavalia rosea*), Indian warrior (*Pedicularis*