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ORIGINAL ARTICLE

Smart drugs: green shuttle or real drug?

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Abstract We have combined morphological, molecular, and chemical techniques in order to identify the plant and chemical composition of some last-generation smart drugs, present on the market under the following names: Jungle Mistic Incense, B-52, Blendz, and Kratom 10x. Micromorphological analyses of botanical fragments allowed identification of epidermal cells, stomata, trichomes, starch, crystals, and pollen. DNA barcoding was carried out by the plastidial gene *rbcL* and the spacer *trnH-psbA* as universal markers. The combination of morphological and molecular data revealed a mixture of plants from different families, including aromatic species, viz., Lamiaceae and Turneraceae. GC-MS and LC-MS analyses on ethanol or methanol extracts showed the presence of synthetic cannabinoids, including JWH-250 in Jungle, JWH-122 in B-52, and JWH-073 and JWH-018 in Blendz. In Kratom 10x, only the indole alkaloid mitragynine was detected. All the identified synthetic cannabinoids, apart from mitragynine, are under the restriction of law in Italy (TU 309/90). Synthetic cannabinoid crystals were also identified by scanning electron microscopy and energy dispersive X-ray spectroscopy, which also detected other foreign organic chemicals, probably preservatives or antimycotics. In Kratom only leaf fragments from *Mitragyna speciosa*, containing the alkaloid mitragynine, were found. In the remaining products, aromatic plant species have mainly the role of hiding synthetic cannabinoids, thus acting as a "green shuttle" rather than as real drugs. Such a multidisciplinary approach is proposed as a method for the identification of herbal blends of uncertain composition, which are widely marketed in "headshops" and on the Internet, and represent a serious hazard to public health.

Keywords Smart drugs · Cannabinoids · DNA barcoding · Micromorphology · GC-MS analysis · SEM-EDS

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Introduction

A vast array of substances marketed as "legal highs" and known as smart drugs are available online and from high street retailers in many countries of the European Union [1]. These products are available in a variety of formulations such as pills or tablets, smoking blends, single plant material or single plant extracts, powders, liquids, orodispersible strips, and chewing gum [2]. Several of these herbal smoking mixes are marketed as legal alternatives to cannabis and consist of plants rich in alkaloids or stimulant substances. On the other hand, some herbal mixtures also contain added synthetic cannabinoids [3, 4]; in these cases, the plant mixtures play mainly the role of "shuttle," hiding illegal chemicals and allowing them to be transported and marketed through different countries [5].

The authorities are seeing an increasing use of synthetic drugs which are disguised as bath salts, potpourri, incense, foods of plant origin, and other products. At the present time, many of these drugs are not illegal because manufacturers are changing the chemical makeup to achieve an alteration for these products wide enough to avoid legal restriction. For example, in many Western European countries, products known as "Spice" and analogous herbal blends are sold as incense not suitable for human consumption; however, they are normally smoked as drugs, much like cannabis [1]. Only

recently, a number of countries in Europe, as well as the USA and Canada, have banned the use of these substances. However, the main problem concerning these herbal blends resides in their identification.

Most herbal products do not list ingredients, and the complex composition of these preparations is often lacking. Consequently, it is difficult to understand whether a herbal mixture contains (a) species with natural toxic metabolites, such as alkaloids dangerous for human health; (b) synthetic toxic compounds, such as synthetic cannabinoids added to herbal mixtures [6]; and (c) simple aromatic plants (i.e., species of Lamiaceae) which are sometimes edible (i.e., *Salvia officinalis*; *Ocimum basilicum*) or ornamental (i.e., *Salvia splendens*; *Lavandula spicata*) without negative effects to human health. The morphological identification of each plant species in the herbal blend is difficult because the mixture is usually composed of dried and fragmented material. Only a micromorphological analysis based on the evaluation of histological structures and cells may help to identify different taxa. At the same time, chemical identification requires the availability of suitable analytical procedures as well as an updated reference database describing natural and synthetic drugs [1].

Recently, a new method for plant identification based on a molecular technique, known as DNA barcoding, has been proposed. This consists in the analysis of a short, standardized DNA region, known as the DNA barcode [7, 8], which is able to univocally identify species. In practice, a DNA sequence from such a region can be generated from a small tissue sample and be compared to a library of reference sequences from the described species. A match of the sequence from the unknown organism(s) to one of the reference sequences can provide a rapid and reproducible identification [8, 9].

The aim of our study was to develop a multidisciplinary approach enabling the identification of ingredients in commercial plant mixtures, based on DNA barcoding analysis combined with micromorphological characterization. In addition, we also analyzed the chemical composition of these products to identify synthetic and natural psychoactive compounds, which could allow us to distinguish between real drugs, i.e., plants containing natural bioactive compounds, and green shuttle, consisting of aromatic herbs augmented with synthetic drugs.

Materials and methods

Herbal blends and reference plant species

Four common European herbal blends were examined: "Jungle Mistic Incense", labeled as potpourri of dried herbs and flowers and commercialized as natural air freshener; "Blendz" and "B-52" labeled as aromatic herbal mixtures and declared as herbal tea; and "Kratom 10x", enriched with a Kratom extract, labeled with the scientific name *Mitragyna speciosa* and commercialized as incense. The first three samples consisted of dried plant material, while Kratom was in the form of solid resinous agglomerates.

Labels on packaging indicated that the products contained 1–2 g of the material, declared not for human consumption. These mixtures were seized by the Police Department of Genoa, Italy, from August to October 2010, during investigations on illicit drug market activities.

A list of medicinal and aromatic plants was used as reference species. These were selected on the basis of bibliographic data concerning the composition of common herbal blends. Reference plant samples were obtained from the firm A. Minardi & Figli s.r.l. (Bagnacavallo, Ravenna, Italy), from the Botanical Garden of the Genoa University, and from the "Clelia Durazzo Grimaldi" Botanical Garden, Genoa, Italy. Reference species were vouchered as "MIB:ZPL" following the protocols specified by the Registry of Biological Repositories (www.biorepositories.org) and the data standards for barcode records in INSDC (http://barcoding.si.edu/PDF/DWG_data_standards-Final.pdf). A complete list of samples and voucher names has been included in Table 1.

Molecular analysis

Herbal blends and reference plant species (Table 1) were used for DNA analysis. DNA was isolated by using the DNeasy Plant Mini kit (Qiagen, Milan, Italy) to obtain high-quality DNA, free of polysaccharides or other metabolites that might interfere with DNA amplification. The concentration of extracted DNA for each sample was estimated fluorometrically.

DNA barcoding analysis was performed with the *rbcL* and *trnH-psbA* regions. PCR for each candidate marker was performed using puReTaq Ready-To-Go PCR beads (Amersham Bioscience, Italy) in a 25-μl reaction, according to the manufacturer's instructions. PCR cycles consisted of an initial denaturation for 7 min at 94 °C, 35 cycles of denaturation (45 s at 94 °C), annealing (30 s at 50 °C for *rbcL* and at 53 °C for *trnH-psbA*), extension (1 min at 72 °C), and a final extension at 72 °C for 7 min. The *rbcL* was amplified using primers *rbcL1F* and *rbcL724R* [10], while for the non-coding region *trnH-psbA*, the primer pair *psbA-trnH* was used [11].

The PCR products obtained from reference species were submitted for sequence analysis to Macrogen (www.macrogen.com). PCR fragments obtained from herbal blends were cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA). For each of the four analyzed samples, a total of 50 clones was randomly selected and the DNA inserts were sequenced. The heavy DNA strands were bidirectionally sequenced using an ABI 155 3730XL automated sequencer at Macrogen Inc., Korea. Manual editing of raw traces and subsequent alignments of forward and reverse sequences enabled us to assign edited sequences for most species. The 3′ and 5′ terminals were clipped

Table 1 List of reference *species*

Species name	Voucher number	*rbcL*	*trnH-psbA*
Artemisia absinthium	MIB:ZPL:03238	HE963336	HE966491
Asperula aristata	MIB:ZPL:03506	HF567848	HF567846
Borago officinalis	MIB:ZPL:03527	HE963354	HE966511
Cannabis sativa	MIB:ZPL:06919	HF565316	HF565307
Carpinus betulus	MIB:ZPL:03338	HF567847	HF567845
Carpinus orientalis subsp. *orientalis*	MIB:ZPL:03043	HE963386	HE966543
Cichorium intybus	MIB:ZPL:03382	HE963414	HE966571
Citrus aurantium	MIB:ZPL:04527	HF565319	HF565310
Crataegus monogyna	MIB:ZPL:03041	HE963432	HE966585
Erica carnea subsp. *carnea*	MIB:ZPL:04506	FR865145	FR865096
Galium aparine	MIB:ZPL:03129	HE963496	HE966646
Laurus nobilis	MIB:ZPL:03036	HE963536	HE966685
Lavandula angustifolia	MIB:ZPL:03990	HE963537	HE966686
Leonotis leonurus	MIB:ZPL:04526	HF565318	HF565309
Malva sylvestris subsp. *sylvestris*	MIB:ZPL:03184	HE963550	HE966699
Melissa officinalis	MIB:ZPL:03435	HF565314	HF565305
Mentha aquatica	MIB:ZPL:03782	FR720533	FR726100
Mentha longifolia	MIB:ZPL:03237	HE963563	HE966712
Mentha piperita	MIB:ZPL:03780	FR720531	FR726098
Mentha pulegium subsp. *pulegium*	MIB:ZPL:03125	HE963564	HE966713
Mentha spicata	MIB:ZPL:03783	FR726101	FR720534
Myrtus communis	MIB:ZPL:03543	HE963567	HE966716
Ocimum basilicum	MIB:ZPL:02884	FR720547	FR726114
Origanum heracleoticum	MIB:ZPL:03796	FR720565	FR726138
Origanum majorana	MIB:ZPL:03791	FR720558	FR726125
Origanum vulgare	MIB:ZPL:03794	FR720563	FR726133
Panax sp.	MIB:ZPL:05513	HF565320	HF565311
Passiflora incarnata	MIB:ZPL:05514	HF565321	HF565312
Petroselinum crispum	MIB:ZPL:04520	HF565315	HF565306
Piper nigrum	MIB:ZPL:04524	HF565317	HF565308
Rosa canina	MIB:ZPL:03103	HE963633	HE966777
Rosmarinus officinalis	MIB:ZPL:03403	HE963635	HE966779
Rubia peregrina	MIB:ZPL:03399	HE963636	HE966780
Salvia officinalis	MIB:ZPL:03572	HE963643	HE966789
Salvia pratensis subsp. *pratensis*	MIB:ZPL:03556	HE963644	HE966790
Salvia rutilans	MIB:ZPL:03802	FR720575	FR726142
Salvia uliginosa	MIB:ZPL:03804	FR720577	FR726144
Sherardia arvensis	MIB:ZPL:03077	HE963666	HE966811
Syringa vulgaris	MIB:ZPL:03260	HE963698	HE966838
Taraxacum officinale	MIB:ZPL:03842	HE963702	HE966842
Thymus vulgaris	MIB:ZPL:03807	FR720581	FR726148
Tilia cordata	MIB:ZPL:03537	HE963708	HE966847
Urtica dioica subsp. *pubescens*	MIB:ZPL:03088	HE963719	HE966857
Verbascum chaixii subsp. *chaixii*	MIB:ZPL:03117	HE963721	HE966859
Verbascum nigrum	MIB:ZPL:04321	HF565313	HF565304
Verbascum phlomoides	MIB:ZPL:03550	HE963722	HE966860

For each taxon, species name, voucher number, and accession number, corresponding to the DNA sequences of the two analyzed markers (*rbcL* and *trnH-psbA*), are reported

to generate consensus sequences for each taxon. To define herbal blend composition, the 50 sequences were firstly analyzed by ClustalW 2.1 [12] alignments to obtain the number of Molecular Operational Taxonomic Units (MOTUs). The resulting sequences were used to identify the plant composition of different herbal blends by comparison with the plant reference

group (Table 1) and with the public DNA database (GenBank), using Basic Local Alignment Search Tool (BLAST) analysis [13]. Each MOTU was identified with the species name showing the nearest matches (maximum identity) according to Barcode of Life Database Identification System (BOLD-IDS) guidelines. Identification results were provided as the species name showing the maximum nearest matches (maximum identity) according to BOLD-IDS guidelines [14]. When the value of identity matches was lower than 95 %, the MOTU was considered not identifiable (NI). The analysis was performed separately for each of the two tested markers.

Micromorphological analysis

Morphological analysis was used to identify the distinctive anatomical features of the four finely ground herbal mixtures. Based on the characteristics of plant materials (fragments of leaves, buds, petioles, small stems, flowers, fruits, and pollen grains), the comparison with microscopy atlases and studies concerning medicinal plants and spice [15–19], or with palynological database and studies [20–23], allowed us to define the plant species composition of each examined sample.

Light microscope observations were carried out by a Leica M205 C stereomicroscope, coupled to EC3 camera and LAS EZ V1.6.0 image analysis software, and by a Leica DM 2000 transmission-light microscope, coupled to DFC 320 camera and IM 1000 and QWin software. Polarization optics were also used for crystal types and locations (Leica Microsystems, Wetzlar, Germany). For light transmission analyses, two sample-clearing processes were carried out: (a) a standard method, consisting of an aqueous solution of sodium hypochlorite (5 %) for 15–30 min, followed by rinsing in distilled water and immediate microscopic examination; (b) an alternative method, according to Lersten and Horner [24], for clearing dehydrated leaves, particularly indicated for crystals observation. This latter method consists of rehydration of leaf portions in 50 % ethanol, followed by rinsing in distilled water and immersion for 20 min to 2 h in full-strength household bleach. The material was then dehydrated in an ethanol series, transferred to a solution of 1:1 ethanol/xylene, followed by two changes in pure xylene, and finally mounted on a slide using Eukitt medium.

Scanning electron microscopy observations were carried out using a scanning electron microscope Vega3 Tescan type LMU, equipped with X-ray energy dispersive system EDS Apollo XSD (Tescan USA Inc. Cranberry Twp, PA, USA). Samples were fixed with FAA (formalin/acetic acid/ethanol) for 24 h, according to Jensen [25], followed by dehydration through an ethanol series. Samples were then transferred to ultrapure 100 % ethanol, left to evaporate at room temperature, and placed on aluminum SEM stubs. Stubs were sputter-coated with about 10-nm gold or carbon. Plant fragments and sections with crystalline idioblasts and concretions were elementally analyzed by EDS to verify crystal composition. Herbal authentication was supported by a comparison with SEM data on plant anatomy, reported in scientific publications and atlases [26–30].

Chemical analysis

Chemical analysis was performed to evaluate the presence of synthetic cannabinoids or natural psychoactive substances. All reagents were of analytical grade or high-performance liquid chromatography (HPLC) grade. Approximately 40 mg of Jungle, Blendz, and B-52 herbal products were crushed into powder and extracted with 2 ml of ethanol under ultrasonication for 20 min. In the case of Kratom, a total of 150 mg of pulverized leaves was extracted with 3 ml of methanol, under ultrasonication for 20 min. After centrifugation (5 min, 1,000×*g*), the supernatant solution was passed through a syringe filter (Phenex PTFE membrane 0.45 μ–15 mm d; Phenomenex, Castel Maggiore, Bologna, Italy). A volume of 1 μl was then injected into the gas chromatography–mass spectrometry (GC-MS) system. The sample solutions were qualitatively analyzed by GC-MS, using an Agilent Technologies 6890 GC system, equipped with 5973 mass selective detector, with electron ionization mode at 70 eV. Samples were separated with a Restek capillary column XTI-5 (30 m, 0.25 mm i.d., 0.25 μm film thickness) with helium gas as a carrier at 0.9 ml/min. The conditions were as follows: injector temperature, 250 °C; injection, splitless mode; oven temperature program, 100 °C (3-min hold) and increase at a rate of 30 °C/min to 220 °C (2-min hold), followed by increase at 30 °C/min up to 300 °C (13-min hold); mass selective detector temperature, 230 °C; scan range, *m/z* 50–450. Compound identification was achieved by comparison with data reported by Uchiyama et al. [31] and with the alerts of the Department for Antidrug Policies [32]. Further investigation was also carried out with an Agilent Technologies 1100 HPLC equipped with MSD Trap SL, in the laboratories of the Science Police Service (DAC-SPS), Rome, Italy, and comparing GC-MS data with the Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) mass spectral library, version 1.6 [33].

Results

Use of DNA barcoding markers in herbal blend identification

DNA extraction resulted in high-quality samples and good yield (i.e., 30–50 ng/μl) for all the 46 reference samples and herbal blends, with the exception of Kratom, for which the yields were modest (5 ng/μl). High amplification success, with standard primer pairs and thermal conditions, was obtained from both *rbcL* and *trnH-psbA* markers, with the exclusion of Kratom, for which only *rbcL* showed a clear PCR product.

PCR products obtained from reference species were directly sequenced. Accession numbers for each sequence are provided in Table 1. These data were used as a reference database (RD) for plant species identification in herbal blends. For herbal blends, a total of 50 clones was correctly sequenced for each of the two markers. Multiple sequence alignment showed more than two MOTUs for all samples, with the exclusion of Kratom (Table 2). These data suggest that commercial herbal blends consist of different species. However, the number of clones obtained by the MOTU does not account for the abundance of each species in the samples.

To identify plant species, the obtained MOTUs were compared to the RD database and with available DNA barcode in GenBank (November 2012). High ID values were obtained for *rbcL* data (Table 2) ranging from 97 to 99 % of identity. In the case of *trnH-psbA* sequences, the majority of MOTUs did not match any DNA sequence of the RD database. In these cases, the identification was only based on GenBank (see Table 2). The combination between *rbcL* and *trnH-psbA* data allowed identification of the plant composition of herbal blends only at the family level (Table 3).

Microscopic characterization of herbal mixtures

The results of light and scanning electron microscope analyses on herbal blends are summarized in Table 3. For each sample the main diagnostic features have been highlighted, such as cell types, calcium oxalate crystals, trichomes, starch grains, and the presence of pollen.

Stereomicroscopic observations revealed that Jungle, Blendz, and B-52 consist of plant material: mainly fragmented leaves and buds, covered by a dense indumentum, followed by small portions from petioles, stems, flowers, and fruits (Figs. 1a, 2a, and 3a). In Kratom (Fig. 4), fragments of leaves with the appearance of caked material were detected (Fig. 4a).

Observations carried out by optical and scanning electron microscopy confirmed that the four herbal blends were largely made up of leaf fragments. The most frequent histological structures were epidermis with stomata, parenchyma, mechanical tissue, and small-size vascular elements. In many cases abundant starch was present (Fig. 1b, arrow). In addition, covering and glandular trichomes (Fig. 1d, f) and calcium oxalate crystals (arrows in Figs. 2b, 3b and 4b–d) were observed, while also "exogenous" crystal aggregates, probably added to herbal mixtures, were detected (Fig. 1c, arrows and Fig. 5b–d).

The combination of histological and cytological elements allowed the identification of plants at the family level or, in a few cases, at the genus level. In particular, the occurrence of Malvaceae was detected by the dense indumentum of stellate trichomes (Figs. 2c and 3b, e), the abundance of oxalate calcium druses (Fig. 3b, arrows) and pollen grains up to 100 μm in diameter, pantoporate, spheroidal, showing exine

Table 2 Results of BLAST search analysis performed on *rbcL* and *trnH-psbA* barcode sequences in the four herbal blends

Samples	*rbcL* molecular identification				*trnH-psbA* molecular identification				Overall represented taxa
	n MOTUs	MOTUs (*n* clones)	Reference database (ID %)	GenBank (ID %)	*n* MOTUs	MOTUs (*n* clones)	Reference database (ID %)	GenBank (ID %)	
Jungle	4	MOTU 1 (8)	*Cichorium intybus* (98)	*Calendula arvensis* L.(99)	3	MOTU 1 (35)	*Verbascum nigrum* (97)	*Verbascum nigrum* (97)	Malvaceae, Scrophulariaceae, Lamiaceae, Asteraceae
		MOTU 2 (20)	*Verbascum phlomoides* (100)	*Verbascum thapsus* (100)		MOTU 2 (10)	*Thymus vulgaris* (100)	*Thymus vulgaris* (100)	
		MOTU 3 (17)	*Leonotis leonurus* (97)	*Origanum vulgare* (99)					
		MOTU 4 (5)	*Malva sylvestris* (99)	*Malva pusilla* (99)		MOTU 3 (5)	No match	*Althaea officinalis* (100)	
Blendz	2	MOTU 1 (37)	*Malva sylvestris* (99)	*Malva pusilla* (99)	1	MOTU 1 (50)	No match	*Althaea officinalis* (100)	Malvaceae, Turneraceae
		MOTU 1 (17)	No match	*Turnera diffusa* (99)					
B-52	3	MOTU 1 (43)	*Malva sylvestris* (99)	*Malva pusilla* (99)	2	MOTU 1 (3)	No match	*Althaea officinalis* (99)	Malvaceae, Scrophulariaceae, Turneraceae
		MOTU 2 (2)	*Verbascum phlomoides* (100)	*Verbascum thapsus* (98)		MOTU 2 (47)	*Verbascum nigrum* (97)	*Verbascum thapsus* (100)	
		MOTU 5 (5)	No match	*Turnera diffusa* (99)					
Kratom	1	MOTU 1 (50)	No match	*Mitragyna speciosa* (100)	0	–	–	–	Rubiaceae

For each sample, the number of detected MOTUs (molecular operational taxonomic units) and the number of clones for each MOTU are indicated for both markers. The obtained sequences were used for BLAST analysis performed on Reference Database and GenBank. For each MOTU, the name of species with nearest matches (*ID* maximum identity value) is indicated. Values below 90 % of ID have been considered "No match"

Table 3 Plant identification and chemical composition of the herbal blends, obtained by microscopic, molecular, and chemical analyses

Samples	Commercial labels	Macroscopic features	Micromorphological identifications	DNA barcoding data	Psychoactive compounds identified
Jungle Mistic Incense	Potpourri of dried herbs and flowers	Plant material, mainly in form of sharp-edged fragments and small leaf portions, covered by a dense indumentum	Scrophulariaceae, Lamiaceae, Malvaceae, Asteraceae	Scrophulariaceae (all markers) Lamiaceae (all markers), Malvaceae (all markers), Asteraceae (only *rbcL*)	JWH-250 (2-(2-methoxyphenyl)-1-(1-pentyl-1*H*-indol-3-yl)ethanone)
Blendz	Aromatic mixture with extracts of vegetable products	Small aggregates of plant material; mainly small leaf portions and buds, covered by a dense indumentum	Malvaceae; Turneraceae, cfr. *T. diffusa*	Malvaceae (all markers), Turneraceae (only *rbcL*)	JWH-073 (1-naphthalenyl(1-butyl-1*H*-indol-3-yl)methanone); JWH-018 (naphthalen-1-yl-(1-pentylindol-3-yl) methanone)
B-52	Aromatic mixture with extracts of vegetable products	Plant material covered by a dense indumentum, extremely fragmented and very dehydrated	Scrophulariaceae; Malvaceae; Turneraceae, cfr. *T. diffusa*; traces of Asteraceae	Scrophulariaceae (all markers), Malvaceae (all markers), Turneraceae (only *rbcL*)	JWH-122 (1-pentyl-3-(4-methyl-1-naphtoyl)indole)
Kratom 10x extract	*Mitragyna speciosa*	Fragments of leaves and small masses of dark brown material	Rubiaceae, *Mitragyna* sp.	Rubiaceae (*Mitragyna* sp.) (only *rbcL*)	Mitragynine (E)-2-[(2*S*,3*S*)-3-ethyl-8-methoxy-1,2,3,4,6,7,12,12*b*-octahydroindolo[3,2-h]quinolizin-2-yl]-3-methoxyprop-2-enoic acid methyl ester

overspread with echini of variable height and width (Fig. 3d). Scrophulariaceae, probably represented by the genus *Verbascum*, were identified by anomocytic stomata, spheroidal starch grains, typical candelabrum-like or dendroid trichomes (Figs. 1b and 3c), and short capitate glandular trichomes. Lamiaceae showed the typical glandular capitate and peltate trichomes and diacytic stomata (Fig. 1d, f). The presence of Asteraceae was revealed by achenium portions, fragments of corolla from ligulate florets with biseriate, multicellular covering trichomes, and the occurrence of trizonocolporate, echinate pollen (Fig. 1e). A dense indumentum of unicellular, warty, somewhat bent, and thick-walled covering trichomes, and anomocytic stomata on the lower surface only, indicated the occurrence of Turneraceae (Figs. 2d and 3f); in addition, numerous calcium oxalate crystals were also found in the mesophyll. For Rubiaceae, the presence or absence of typical crystals is a distinctive feature and is also useful to distinguish among subfamilies [34, 35]. In samples of Kratom, the genus *Mitragyna* was identified on the base of spheroidal calcium oxalate concretions (Fig. 4b–d), a unique feature of four species of *Mitragyna* belonging to Mitragynineae, as recently reported by Lersten and Horner [24].

The composition of the different crystals found in all samples (Fig. 5, spectra) was verified by using elemental analysis (SEM/EDS). Most commonly, the inclusions were calcium oxalate crystals in general, occurring as styloids, druses, or as the typical spheroidal concretions in Kratom (Fig. 5a, spectrum). In this last sample, the presence of small crystal aggregates was also found, formed by potassium and sulfur salts (Fig. 5b, spectrum). These aggregates formed a crust layer on plant fragments, whose presence might be in relation with the addition of food preservatives or antimycotic agents. The presence of other exogenous chemicals in three of the herbal blends was detected by SEM observations, occasionally as isolated crystals (Fig. 5c), or more frequently in the form of a dense crystalline crust on leaf fragments and on the dense indumentum of covering trichomes (Fig. 5d). The elemental composition of this material, revealed by EDS analysis, showed the presence of carbon, nitrogen, and oxygen, and the absence of calcium (Fig. 5c–d, spectra). These results indicated that this crystalline material was probably formed by synthetic cannabinoids.

Overall, the combination of micromorphological and DNA barcoding analyses allowed the identification of almost all taxa present in herbal blends. However, microscopic analysis, but not DNA barcoding, allowed us to identify traces of Asteraceae in B-52.

Identification of cannabinoids

In Jungle, Blendz, and B-52, four cannabimimetic compounds with an aminoalkylindole structure, commonly referred to as "JWH", were identified as adulterants (Table 3).

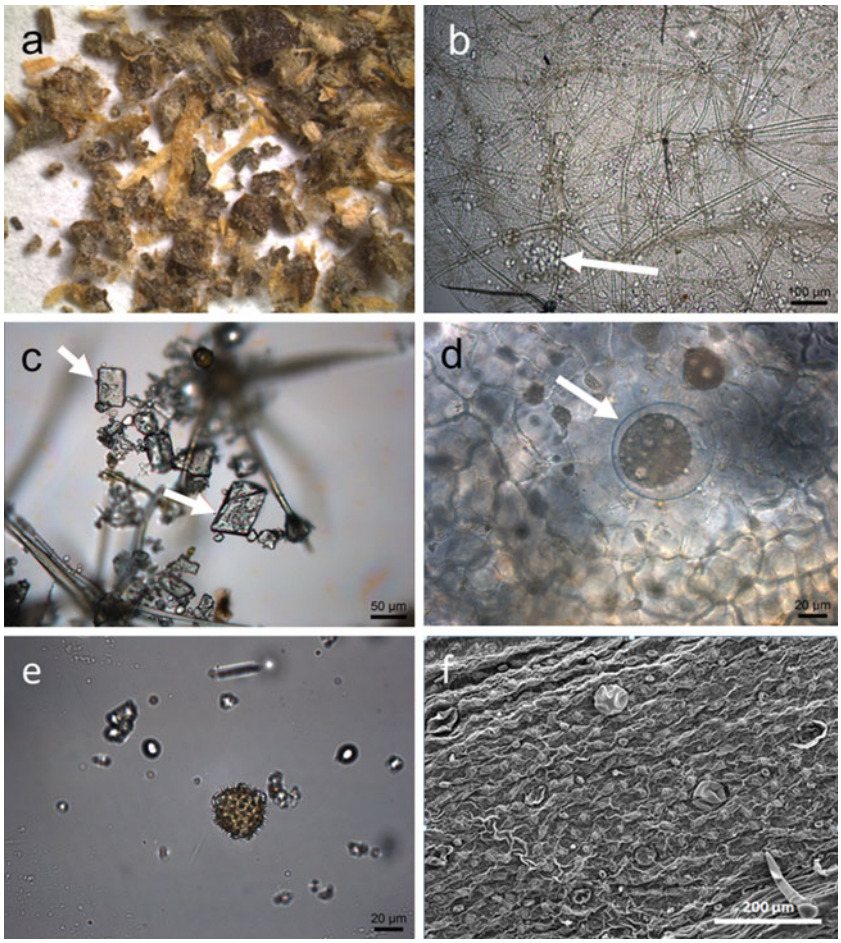

Fig. 1 Jungle herbal blend. Stereomicroscope image showing fragmented plant material (**a**). Transmission-light micrographs (**b**–**e**) of typical *Verbascum* candelabrum-like trichomes and abundant spheroidal starch grains (*arrow*) (b); exogenous crystals (*arrows*) mixed with stellate trichomes from Malvaceae indumentum (c); typical peltate glandular trichome (*arrow*) of Lamiaceae (d); triangular, trizonocolporate, echinate pollen of Asteraceae (e). SEM micrograph (f) of fragment of Lamiaceae leaf showing lower epidermis with stomata, peltate glandular trichomes, and a few unicellular covering trichomes on the vein

Ethanol extracts of the three herbal samples were analyzed by gas chromatography coupled to electron ionization (EI) single-quadrupole mass spectrometry. The total ion current plot (TIC) of Jungle (Fig. 6a) showed a peak appearing at 15.06 min and the fragmentation spectrum revealed a major signal at *m/z* 214, a relatively large signal at *m/z* 144 and a signal at *m/z* 335 as well,

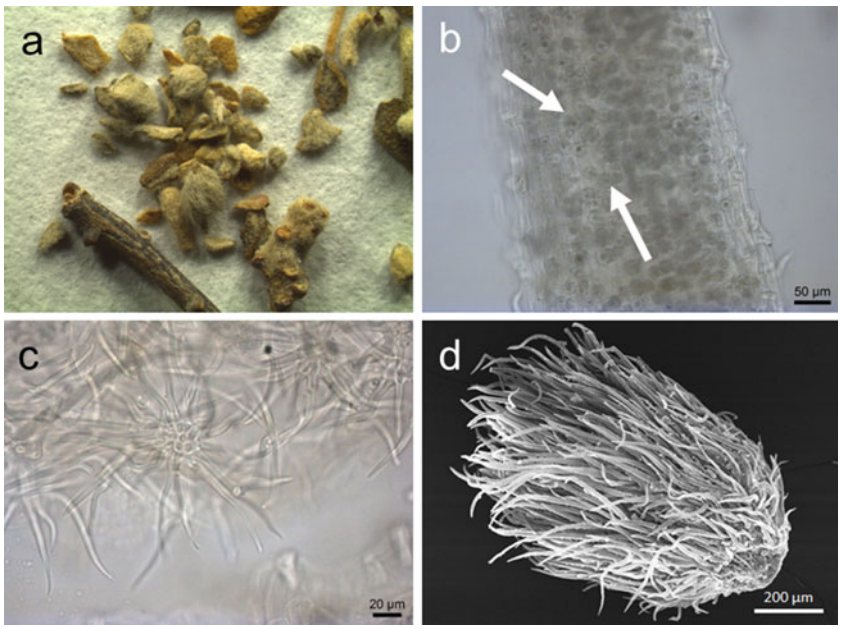

Fig. 2 Blendz herbal blend. Stereomicroscope image showing fragmented plant material (**a**). Transmission-light micrographs (**b**–**c**) of abundant calcium oxalate druses (*arrows*) along the leaf vein (b) and stellate trichomes (c), typical of Malvaceae. SEM micrograph (**d**) of bud with a dense indumentum of unicellular, warty, covering trichomes of Turneraceae

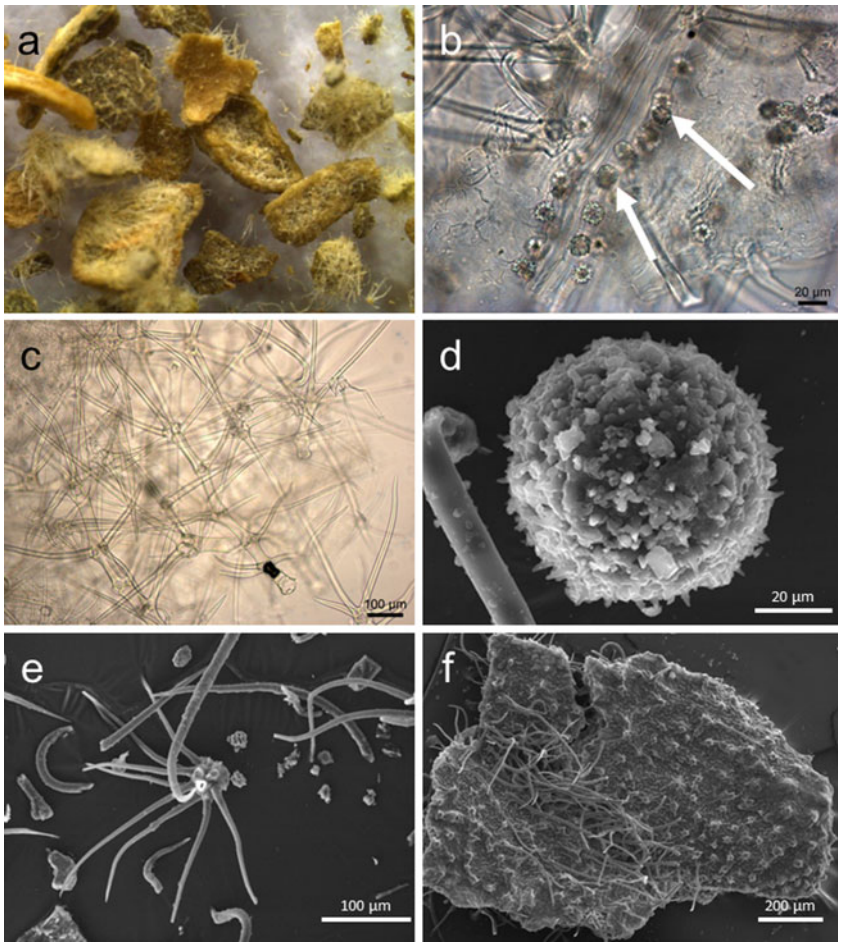

Fig. 3 B-52 herbal blend. Stereomicroscope image showing fragmented plant material (**a**). Transmission-light micrographs of abundant calcium oxalate druses along the leaf vein (b, *arrows*) and stellate trichomes typical of Malvaceae (**b**); candelabrum-like trichomes of Scrophulariaceae (**c**). SEM micrographs (**d**–**f**) of a spheroidal pollen grain, showing exine overspread with echini of variable height and width (d), and stellate trichomes (e), both typical of Malvaceae; leaf epidermis with cuticular protuberances and unicellular, straight or slightly curved, warty, covering trichomes of Turneraceae (f)

probably corresponding to the molecular ion peak. These results revealed the presence of JWH-250 (2-(2-methoxyphenyl)-1-(1-pentyl-1*H*-indol-3-yl)ethanone) (Fig. 6b).

The TIC of B-52 (Fig. 6c) showed a peak appearing at 19.19 min and the fragmentation spectrum revealed peaks at *m/z* 214 and *m/z* 144, similar to those found in Jungle. In this

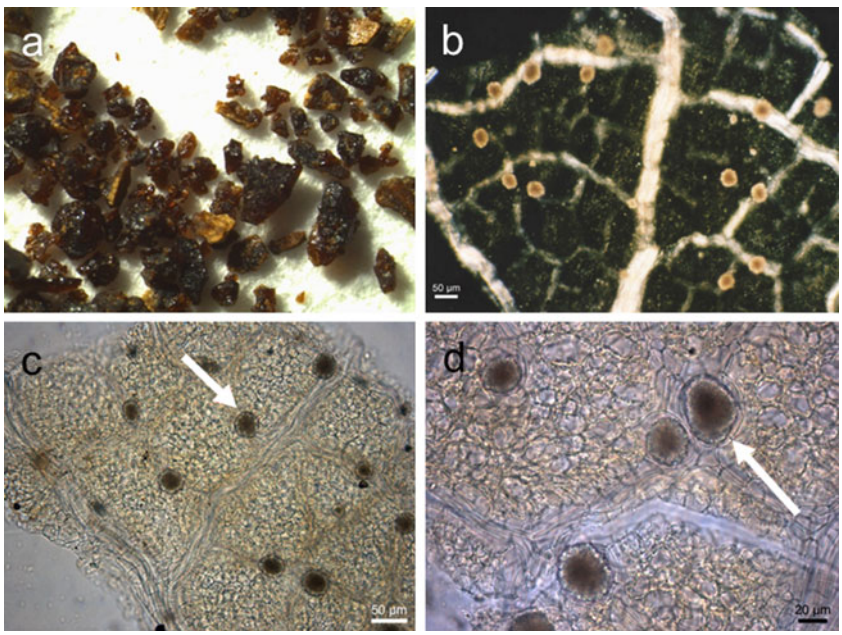

Fig. 4 Kratom herbal blend. Stereomicroscope image showing fragmented plant material (**a**). Transmission-light micrographs (**b**–**d**) of calcium oxalate crystal aggregates, appearing birefringent when viewed with polarization optics (b); typical spheroidal calcium oxalate concretions of *Mitragyna* (Rubiaceae) distributed along leaf veins (c–d, *arrows*)

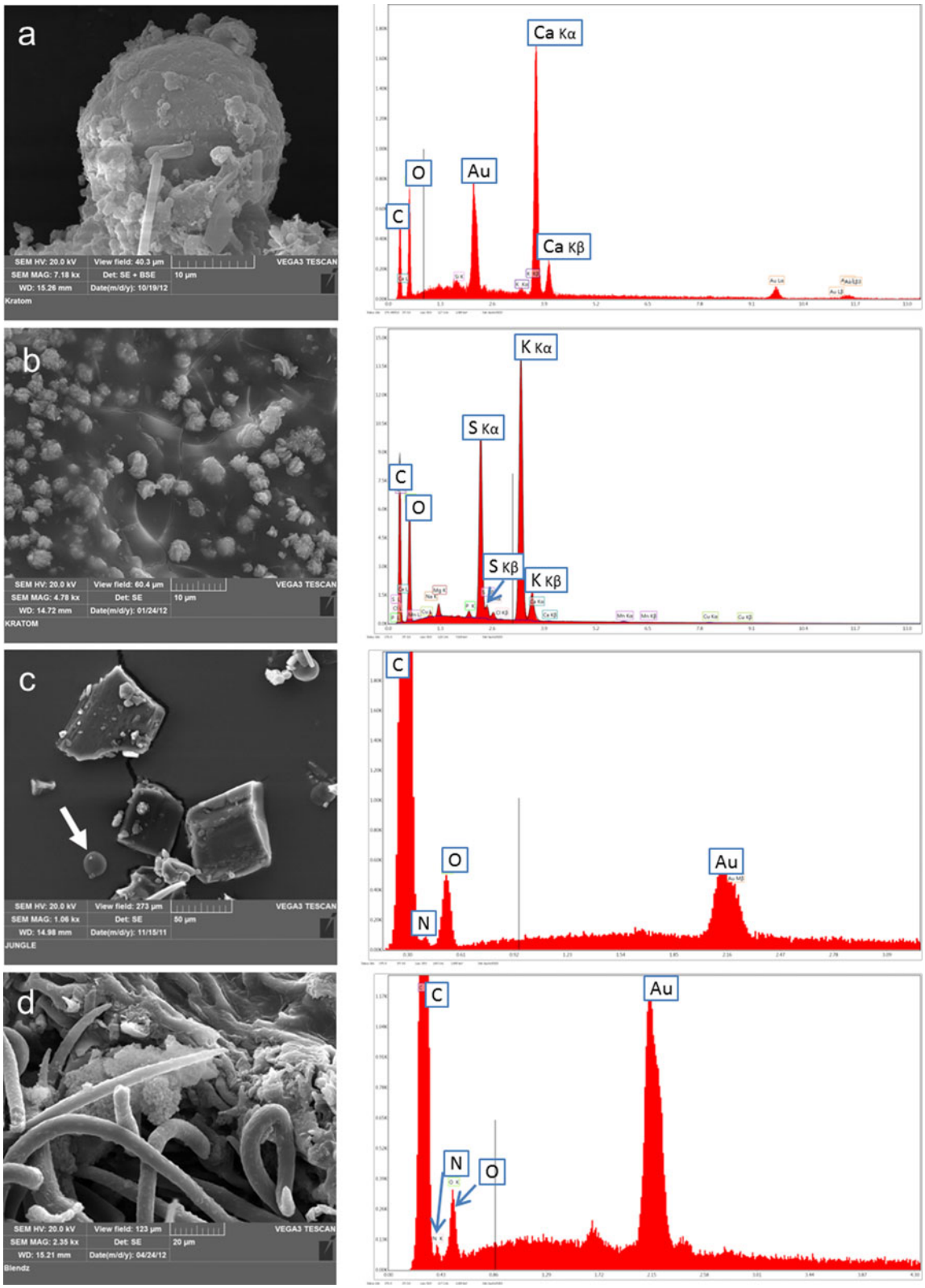

Fig. 5 Kratom (**a**–**b**), Jungle (**c**), and Blendz (**d**) herbal blends. SEM–EDS analyses of typical spheroidal calcium oxalate concretions show a high calcium content (a, spectrum), while in small crystal aggregates, potassium and sulfur are detected (b, spectrum). Other crystalline substances (c, Jungle; d, Blendz), are occasionally found as isolated crystals mixed among trichomes or starch (*arrow*) (c) or, more frequently, as a dense crystalline crust on the leaf covering trichomes (d). The elemental composition of this material shows the presence of carbon, nitrogen, and oxygen, and the absence of calcium (c–d spectra)

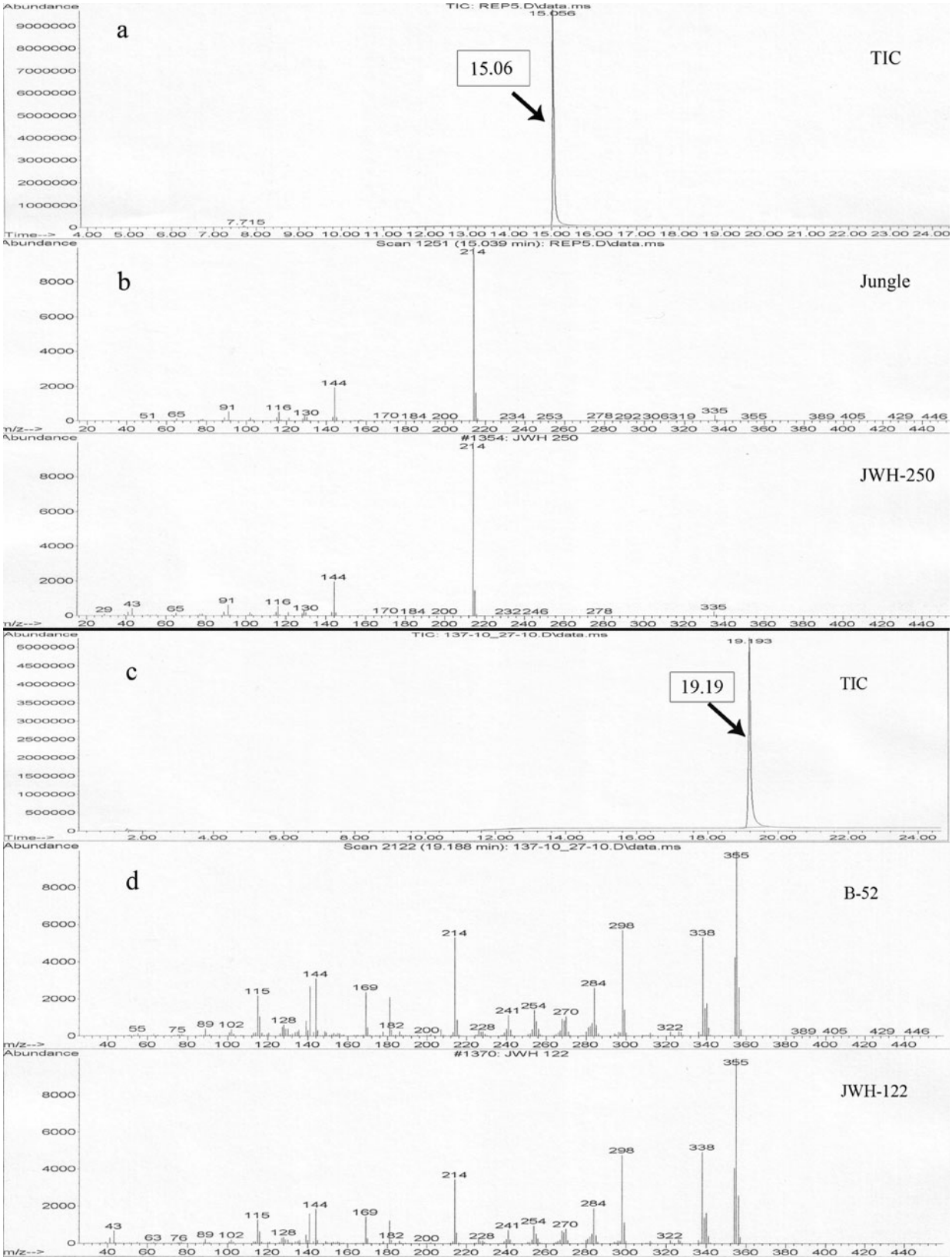

Fig. 6 GC–MS analysis of Jungle and B-52. Total ion current plot (TIC) of Jungle (**a**) and electron ionization (EI) mass spectrum of detected peak at the retention time of 15.06 (**b**) showing the presence of JWH-250. The TIC of B-52 (**c**) and EI mass spectrum of detected peak at the retention time of 19.19 (**d**) showing the presence of JWH-122. The identification of two cannabinoids is confirmed by comparison with the SWGDRUG mass spectral library (99 % match)

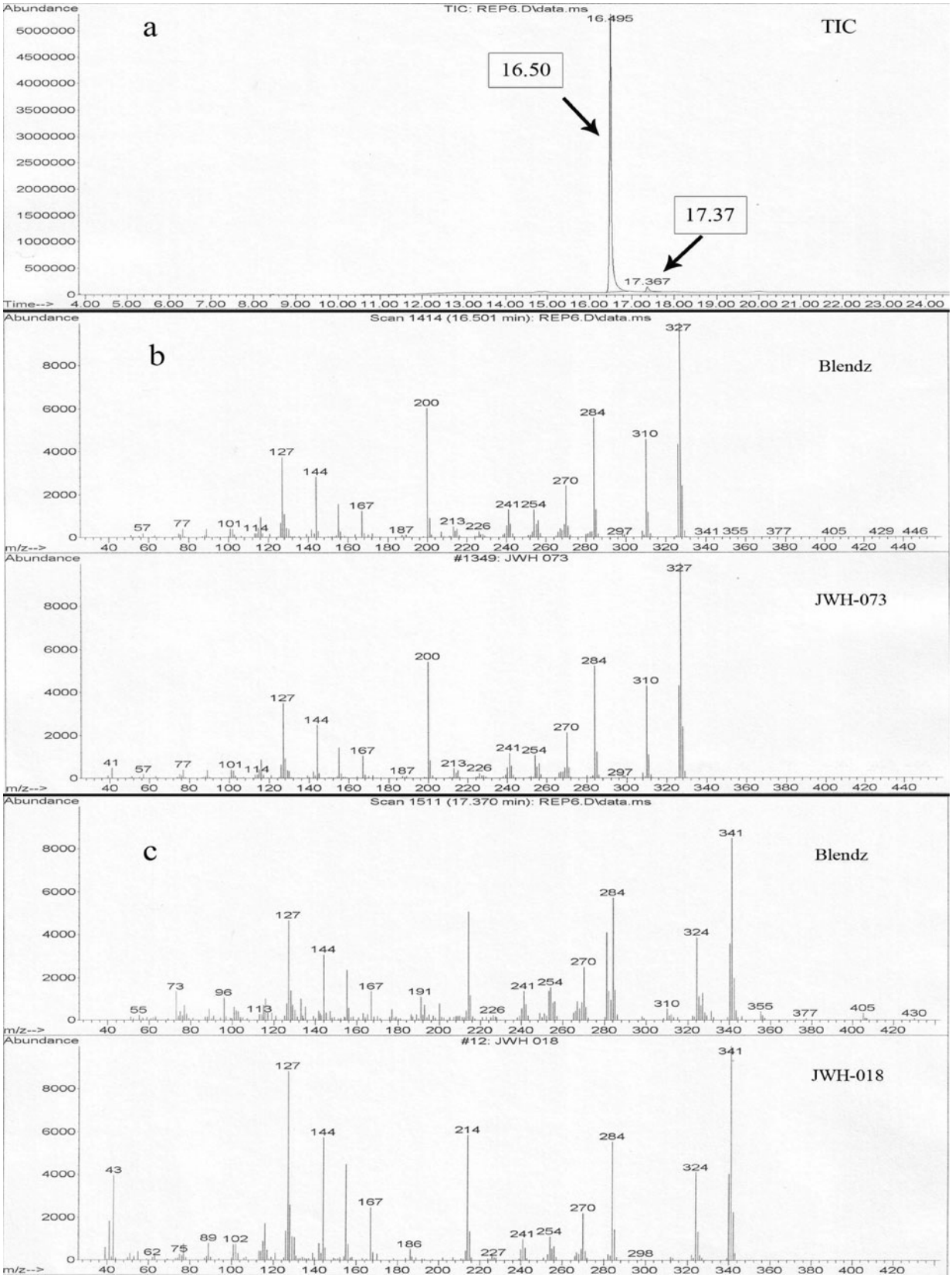

Fig. 7 GC–MS analysis of Blendz. TIC (**a**) and EI mass spectra of detected peaks at the retention time of 16.50 (**b**) and of 17.40 (**c**), assigned to JWH-073 (b) and JWH-018 (c). The identification of two cannabinoids is confirmed by comparison with the SWGDRUG mass spectral library (>95 % match)

case, the basal peak was at *m/z* 355, probably corresponding to the molecular ion. Fragmentation patterns showed the fragment at *m/z* 214 that, due to the subsequent loss of the alkyl chain, formed the fragment *m/z* 144. The mass spectrum of the compound isolated in B-52 was attributed to JWH-122 (1-pentyl-3-(4-methyl-1-naphtoyl)indole) (Fig. 6d).

In Blendz (Fig. 7a), two peaks were observed in the TIC (peak 1 RT 16.50 min, peak 2 RT 17.37 min), assigned respectively to JWH-073 (1-naphthalenyl (1-butyl-1*H*-indole-3-yl)methanone) (peak 1) and to JWH-018 (naphthalen-1-yl-(1-pentylindol-3-yl)methanone) (peak 2). For JWH-073 (Fig. 7b), the most important ions occur at *m/z* 327, 310, 284, 270, 254, 241, 226, 213, 200, 167, 155, 144, 127, 116, 101, 89, and 77, while for JWH-018 (Fig. 7c) at *m/z* 341, 324, 310, 284, 270, 254, 241, 226, 214, 200, 191, 167, 155, 144, 127, 116, and 102.

Further analyses performed by ion trap LC-MS confirmed the identification of synthetic cannabinoids from the protonated pseudo-molecular ion $[M+H]^+$. In addition, through this technique we collected the TIC, MS, and MS/MS spectra of each identified analyte. The data obtained matched with MS and MS/MS spectra reported by Kennedy and Collin [36]. The identification of synthetic cannabinoids was then validated comparing our data with the information reported on the alerts of DPA (Italian Department for Anti-Drug Policies) [32] and by comparison of our GC/MS spectra with the SWGDRUG mass spectral library [33].

Regarding Kratom, the TIC of the methanolic extract showed a peak at a retention time of 19.24 min (Fig. 8a), revealing the presence of mitragynine, (E)-2-[(2*S*,3*S*)-3-ethyl-8-methoxy-1,2,3,4,6,7,12,12*b*-octahydroindolo[3,2-h]quinolizin-2-yl]-3-methoxyprop-2-enoic acid methyl ester (Fig. 8b). The identification of this molecule was obtained by comparison with the SWGDRUG mass spectral library [33].

The chemical compounds found in the examined commercial products, except mitragynine, are currently restricted by Italian law, according to TU 309/90 [37].

Discussion

Most herbal blends contain finely minced plant material, including *Leonotis leonurus*, *Nymphaea caerulea*, and *Pedicularis densiflora*, claimed to be able to produce cannabis-like effects due to their constituents [38]. However, starting from the end of 2008, several laboratories have identified synthetic additives in these products which mimic the pharmacological activity of cannabinoids [5, 39]. Indeed, it is

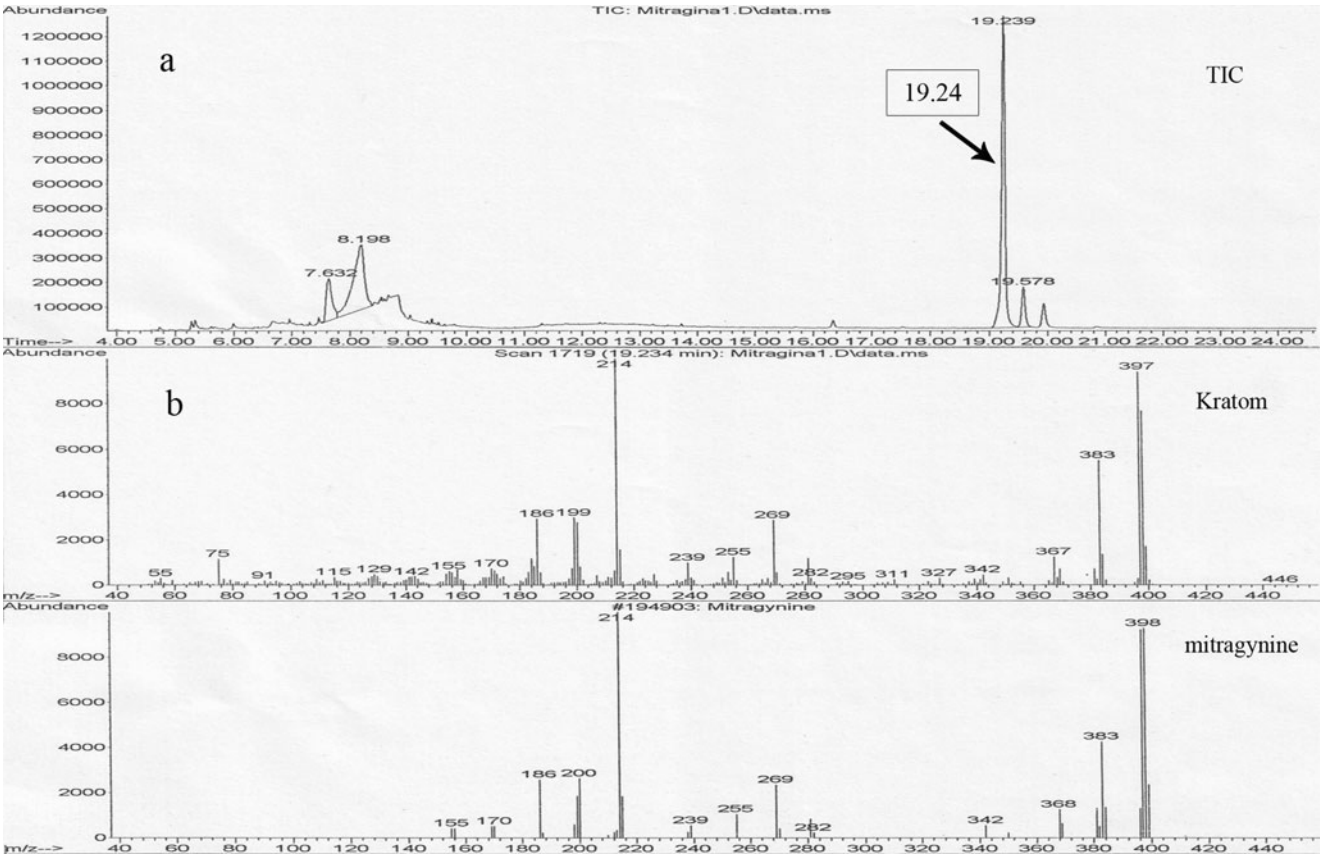

Fig. 8 GC–MS analysis of Kratom. TIC (**a**) and EI mass spectrum of detected peak at the retention time of 19.24 allowing to identify mitragynine (**b**). The identification is confirmed by comparison with the SWGDRUG mass spectral library (99 % match)

currently well known that synthetic agonists of cannabinoid receptors are sprayed onto many of the commercial "smokable herbs" [40, 41].

In our study, three of the examined blends, viz., Jungle Mistic Incense, Blendz, and B-52, contained plant material included as diluent (green shuttles) for synthetic cannabinoids, giving these products the appearance of "legal-drugs" [6, 42]. However, in Blendz and B-52, the presence of *Turnera diffusa* Willd. (known as "damiana") was detected. This is an aromatic plant marketed as "Mexican tea" and approved by the US FDA [43]. Conversely, the product marketed as Kratom is a real drug, i.e., the active compound derives from a plant species, *M. speciosa*, with psychoactive properties.

Our chemical analyses allowed us to identify cannabinoid-like JWH-073 and JWH-018 in Blendz, JWH-250 in Jungle Mistic Incense, and JWH-122 in B-52. These products are known to cause acute intoxications, and consequently, like any structural derivative of 3-phenylacetylindole and 3-(1-naphthoyl)-indole, have been included as illegal drugs in the above schedule [37] by the Italian Ministry of Health [44, 45].

The Kratom product, reporting on the label the indication "*M. speciosa* 10x", consisted of powdered leaves enriched with an extract of this plant and did not contain synthetic cannabinoids. Kratom is an important drug for traditional Thai medicine and is also used as an opium substitute in Asian countries. Nowadays, it is banned in the countries of origin; while in Italy, neither the whole plant, nor its alkaloids, are listed in any of the schedules of DPR 309/90 [37]. The main alkaloids of the plant are mitragynine and 7-hydroxy-mitragynine, while its psychoactive properties are paradoxical, since it is simultaneously stimulating like cocaine and soothing like morphine [46].

Besides chemical analyses, the determination of plant material is essential for a correct classification of herbal blends. The most common botanical dichotomous keys are not useful for taxonomic identifications because the plants' morphological traits are not easily detectable on fragmented materials. Therefore we have introduced here a new approach, consisting in the combination of DNA barcoding with micromorphological analysis of fragmented material. Our results have shown that the preparation of herbal blends (e.g., crumbling, drying) does not affect the success rate of DNA fingerprinting. Only processed materials such as Kratom could affect DNA quality and amplification yield. Since the commercial herbal mixtures are composed of different plant species, an ideal DNA fingerprint approach must be based on universal tools capable of amplifying a large number of taxa under standard PCR conditions. In this context, DNA barcoding fits well with this need [8]. Both *rbcL* and *trnH-psbA* were found to be universal tools able to identify the plant composition of the tested samples. In addition, this method allows the identification of plant species starting from small fragments [47]. However, a limit of the plant DNA barcoding approach is the definition of reference databases. The number of accessions deposited in BOLD is still incomplete, while the DNA barcode entry of GenBank does not guarantee all BOLD descriptors for correct species identification [48].

Hence, analysis of microscopic details become essential when molecular approaches cannot distinguish closely related species. In our study, the micromorphological analysis contributed to the identification of blends based on histological structures (e.g., parenchyma, collenchyma, fibers, stone cells, vessels, trichomes, secretory tissues, epidermal cells, and stomata types). Moreover, in the presence of finely fragmented or powdered plants, cell inclusions like cluster crystals and prisms of calcium oxalate and silica, and starch granules, were also useful for taxonomical identification [49]. The role of microscopic analyses is well exemplified in the case of Rubiaceae, in which the different types of crystal inclusions have taxonomic significance. In our study, SEM/EDS microanalysis showed that these kinds of crystals consisted of calcium oxalate and also highlighted the presence of potassium and sulfur salts in Kratom, indicating the addition of preservatives.

In conclusion, the present research is a first multidisciplinary approach to the study of smart drugs, subdivided into taxonomic determination of plant material and chemical characterization of botanical or synthetic compounds. Taxonomical determinations were based on microscopic examination of diagnostic features considered as botanical biomarkers, coupled to DNA barcoding analysis. In addition, microscopic analyses allowed us to discriminate between inorganic crystals typical of many plants and crystalline substances added to the herbal blends. In line with these findings, chemical analyses discriminated between blends based on plant alkaloid principles (Kratom) and those in which botanicals play a role of green shuttle for synthetic compounds with psychotropic effects (Blendz, Jungle Mistic Incense, and B-52). Our integrated approach is proposed as a method for the accurate classification of new herbal blends of uncertain composition that are put in the market. The implementation of this kind of data in a web repository (e.g., made available by EMCDDA, European Monitoring Centre for Drugs and Drug Addiction), could speed up the risk assessment of herbal products and smart drugs and contribute to the protection of public health.

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