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PCR and PCR–RFLP of the 5S-rRNA-NTS region and salvinorin A analyses for the rapid and unequivocal determination of *Salvia divinorum*

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Abstract

Salvia divinorum Epling & Játiva-M. is a perennial herb belonging to the Lamiaceae family; its active ingredient, the neoclerodane diterpene salvinorin A, is a psychotropic molecule that produces hallucinations. A comparative evaluation of *S. divinorum* fresh and dried leaves, *S. officinalis* fresh leaves, and dried powdered leaves claimed to be *S. divinorum* was done. HPLC–MS data confirmed the presence of salvinorin A in both *S. divinorun* leaf extracts and the powdered leaves, whereas no salvinorin A was found in *S. officinalis*. The non-transcribed spacer (NTS) in the 5S-rRNA gene of all leaf samples and the dried powdered leaves was amplified by PCR using a pair of primers located at the 3′ and 5′ ends of the coding sequence of 5S-rRNA gene. The resulting PCR products (about 500 bp for *S. divinorum* and 300 bp for *S. officinalis*) were gel purified, subcloned into pGEM®-T Easy vector and sequenced. By aligning the isolated nucleotide sequences, great diversities were found in the spacer region of the two species. Specific *S. divinorum* primers were designed on the sequence of the 5S-rRNA gene spacer region. In addition, a PCR–restriction fragment length polymorphism (PCR–RFLP) method was applied using *Nde*I and *Taq*I restriction enzymes. An *Nde*I site, absent in *S. officinalis,* was found in *S. divinorum* NTS region at 428–433 bp. For *Taq*I, multiple sites (161–164, 170–173, and 217–220 bp) were found in *S. officinalis,* whereas a unique site was found in *S. divinorum* (235–238 bp). The results of this work show that the combined use of analytical chemical (HPLC–MS) and molecular (DNA fingerprinting) methods lead to the precise and unequivocal identification of *S. divinorum*.

Keywords: *Salvia divinorum* Epling & Játiva-M.; Lamiaceae; Diviner's sage; Salvinorin A; 5S-rRNA spacer region; DNA sequence analysis; *Nde*I and *Taq*I site PCR–RFLP; *S. divinorun* specific primer design

1. Introduction

Salvia divinorum Epling & Játiva-M. is a perennial herb belonging to the Lamiaceae family and is most recognized for its hallucinogenic properties (for reviews, see Valdes et al., 1983; Sheffler and Roth, 2003). The current distribution of *S. divinorum* suggests that all existing stands of the plant have been intentionally cultivated by humans; no clearly wild populations of the species have been identified and it has been proposed that *S. divinorum* may in fact be a hybrid, resulting in substantially reduced fertility within the species (Reisfield, 1993). The active ingredient of

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S. divinorum is the neoclerodane diterpene salvinorin A, a psychotropic molecule that produces hallucinations (Yan and Roth, 2004; Babu et al., 2005). For this reason, *S. divinorum* is a frequently used hallucinogen (Giroud et al., 2000), with similar potency as LSD in producing hallucination (Siebert, 1994). *S. divinorum* is currently non-scheduled (i.e., legal) in the United States (Roth et al., 2004). At present, in almost all countries, the use of *S. divinorum* is not banned because neither the plant nor or any of its constituents is listed in the controlled substance lists. In August 2004, the plant and its main active principle, salvinorin A, were added to Lists of Controlled Illicit Substances in Italy and sales are also prohibited in Spain, Finland, Denmark and Australia (Bücheler et al., 2005; Pichini et al., 2005). A recent pivotal study reported that salvinorin A was a selective, high efficacy κ-agonist in cloned human κ-receptors, and in guinea pig brain (Roth et al., 2002). Recently, it has been reported that salvinorin A produces discriminative stimulus effects similar to those of a high efficacy κ-agonist also in non-human primates (Butelman et al., 2004). Synthetic κ-agonists administered systemically to humans are known to produce a variety of dose-dependent and reversible subjective/interoceptive effects, including sedation, dysphoria and "psychotomimetic" effects (Pfeiffer et al., 1986; Ur et al., 1997; Walsh et al., 2001). Salvinorin A is also the only known lipid-like molecule that selectively and potently activates a G-protein coupled receptor (GPCR), which has as its endogenous agonist a peptide; salvinorin A and a few C(4)- and C(2)-modified salvinorin A analogues are the only known non-nitrogenous opioid receptor agonist (Lee et al., 2005a,b; Béguin et al., 2005; Yan et al., 2005). HPLC (Gruber et al., 1999; Schmidt et al., 2005) and GC/MS (Pichini et al., 2005) methodologies to quantify salvinorin A in *S. divinorum* leaves have been developed in conventional and non-conventional biological matrices; whereas salvinorin A structure has been determined using ^{1}H nuclear magnetic resonance (H NMR) and by independent single-crystal X-ray studies (Sheffler and Roth, 2003). Moreover, a straightforward synthesis of a deuterium labeled analog of salvinorin A and its utility as an internal standard for the detection of salvinorin A and its metabolites in biological fluids by LC–MS have been described (Tidgewell et al., 2004). Finally, a microscopic survey of the *S. divinorum* plant was performed to examine the various types of trichomes present. Salvinorin A and related compounds have been found secreted as components of a complex resin that accumulates in the subcuticular space of peltate glandular trichomes (Siebert, 2004).

The concentration of salvinorin A in leaves at various stages of development on an individual plant is often remarkably consistent. However, leaves collected from separate plants can vary considerably, even when they are genetically identical (Gruber et al., 1999). Often *S. divinorum* is sold as a powder that can be easily adultered by adding dried leaves of other species, thus making hard to establish the purity of the samples. The general approaches to herbal identification depend on morphological, anatomical, and chemical analyses, but these characteristics are often affected both by the environmental and/or developmental factors during plant growth (Cai et al., 1999) and the status of samples.

Molecular genetic methods have several advantages over classical morphological and chemical analyses. For instance, the genetic method requires genotype instead than phenotype, therefore DNA based experiments have become widely employed techniques for a rapid identification of herbal medicine. We recently demonstrated that molecular approaches represent a powerful tool to distinguish the *Acorus calamus* diploid β-asarone-free cytotype from the other cytotypes which contain this dangerous molecule (Bertea et al., 2005). Since by using PCR approaches, only nanogram quantities of DNA are required to amplify and yield sufficient amounts of template DNA for molecular genetic analysis, we used biomolecular approaches to create a molecular fingerprinting allowing the rapid and precise identification of *S. divinorum*. Owing to the increasing awareness of the dangerous use of this plant, the results presented here represent a powerful tool for the rapid identification of *S. divinorum* samples for phytochemical, forensic and toxicological investigations aimed to detect and quantify this plant.

2. Results and discussion

The chemical characterization of the neoclerodane diterpene salvinorin A is the only available method, besides morphological identification of the plant, when possible, used to distinguish samples of *S. divinorum*. In order to make a direct and comparative analysis of known and unknown samples, we extracted dried and fresh leaves of *S. divinorum* and *S. officinalis* as well as a sample consisting of dried powdered leaves claimed to be *S. divinorum*. HPLC–MS was performed on authentic salvinorin A, which was used as a reference standard for the chemical identification of this compound in all extracts. The elution profile of the authentic standard, as recorded at 250 nm by the UV DAD detector, showed a tiny peak of salvinorin A. By using mass spectrometry in full scan mode from 50 to 700 amu it was possible to better identify the salvinorin A standard as a clear peak. By measuring only the daughter ion 373 in an ms/ms analysis it was possible to use the peak area for the quantitative determination of the salvinorin A standard, which in our case was 10 ppm. When a sample of fresh *S. divinorum* leaves was analyzed, the same method was used and the salvinorin A was quantified. The content of salvinorin A in dried *S. divinorum* leaves was found to be 12.18 mg g^{-1} d. wt (± 0.21), in fresh *S. divinorum* leaves the content was 1.14 mg g^{-1} d. wt (± 0.01), whereas in the dried powdered leaves claimed to be *S. divinorum* the content of salvinorin A was 45.37 mg g^{-1} d. wt (± 1.64). The latter analysis confirmed that the dried powdered leaves contained salvinorin A

which was possibly indicating the presence of *S. divinorum*. However, analysis of *S. officinalis* leaves adulterated with exogenous salvinorin A may lead to the same conclusions. HPLC–MS analyses performed on leaf extracts of *S. officinalis* did not show any salvinorin A content, thus *S. officinalis* was used as a further control for molecular biology studies. The content of salvinorin A detected in our fresh *S. divinorum* samples was in line with the most recent findings (Gruber et al., 1999; Munro and Rizzacasa, 2003; Siebert, 2004), whereas the content in dried leaves and in the powdered unknown sample was remarkably high. However, high contents of salvinorin A have already been reported (Pichini et al., 2005) and variability in salvinorin A content in different *S. divinorum* plant samples has been documented (Siebert, 2004).

In higher eukaryotes, the 5S-rRNA gene is separated by simple spacers. The gene occurs as a tandem repeated unit consisting of a ~120 bp coding region separated by a spacer region of various size. Although the coding region is highly conserved, the spacer regions are variable in different species. Thus, the diversity of the spacer region can be used as an identification basis (Cai et al., 1999). Here, two primers flanking the spacer region of 5S-rRNA, already employed for differing *A. calamus* chemotypes (Sugimoto et al., 1999) and *A. calamus* cytotypes (Bertea et al., 2005) were used in PCR analysis of genomic DNA isolated from fresh and dried leaves of *S. divinorum* and the unknown powdered leaf material claimed to be *S. divinorum*. A single fragment of approximately 500 bp was produced from each *S. divinorum* samples (Fig. 1, lanes 1–3), whereas a single fragment of about 300 bp was produced for *S. officinalis* (Fig. 1, lane 4). Fragments derived from both species were ligated into pGEM®-T Easy vector and the nucleotide sequence was determined. The sequenced region spans 487 bp for *S. divinorum* (NCBI GenBank Accession No. DQ230979) and 308 bp for *S. officinalis* (NCBI GenBank Accession No. DQ230980). When DNA templates were isolated either from fresh or dried leaves or powdered leaf material, identical sequences were obtained from all *S. divinorum* tested samples, including the unknown powder.

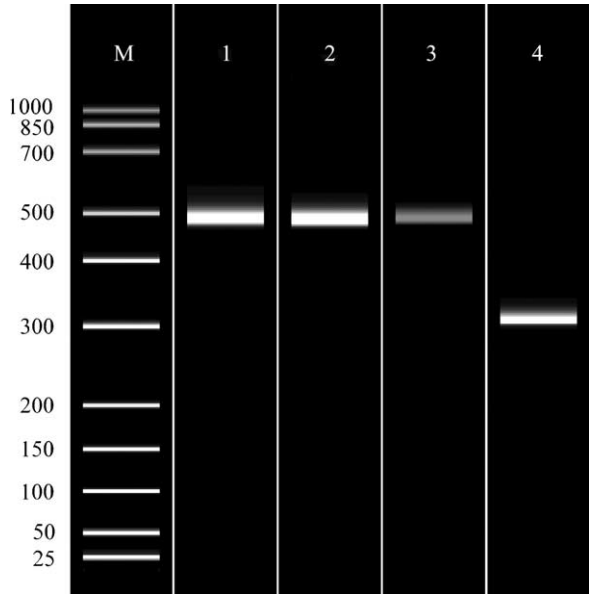

Fig. 1. PCR products generated by primers (5S-P1 and 5S-P2) flanking the spacer region of 5S-rRNA gene using DNAs from *S. divinorum* fresh leaves, dried leaves and powder and *S. officinalis* fresh leaves. A single fragment of approximately 500 bp was produced from each *S. divinorum* samples, whereas a single fragment of about 300 bp was produced for *S. officinalis*. M = bp markers. The PCR products were separated by using the Agilent 2100 Bioanalyzer and the DNA 1000 LabChip® Kit (Agilent Technologies).

Sequence alignment of the 5S-rRNA spacer region flanked by the 3′- and 5′-ends of the coding region is shown in Fig. 2. Surprisingly, *S. officinalis* presented a difference of 179 nucleotides with respect to *S. divinorum*. The identity between *S. divinorum* and *S. officinalis* 5S-rRNA spacer is very low, since the two sequences align only at the regions flanking the spacer domain (Fig. 2).

In order to characterize better *S. divinorum* and to simplify the identification method, nucleotide sequences of the 5S-rRNA gene spacer region were used to design four specific primers (Fig. 3).

PCR products derived from all possible combinations of *S. divinorum* specific primers also used with the primers designed on the coding regions of the plant 5S-rRNA gene, were analyzed. A single fragment of 265 bp in length was amplified using the primer SD1 in combination with the primer 5S-P1 (Fig. 4, lane 1), whereas the second (5S-P1 and SD2, Fig. 4, lane 2) and the third (5S-P1 and SD3, Fig. 4, lane 3) primer set amplified a fragment of 371 and 466 bp, respectively (see also Fig. 3). The use of the specific forward primer SDF in combination with SD1, SD2, SD3 and 5S-P1 produced single fragments of 127 bp (Fig. 4, lane 5), 233 bp (Fig. 4, lane 6), 327 bp (Fig. 4, lane 7) and 349 bp (Fig. 4, lane 8), respectively. All amplifications occurred only in *S. divinorum* and no PCR products were detected when *S. officinalis* DNA was employed as a template (data not shown), indicating that this approach can be used to identify easily *S. divinorum*.

In addition, a PCR–RFLP method was applied. From the identified sequences, a *Nde*I site, absent in *S. officinalis,* could be found in *S. divinorum* 5S-rRNA spacer region at 428 bp position (Fig. 2, shaded square box). Purified PCR products obtained by using 5S-P1 and 5S-P2 primers were digested with *Nde*I. As expected, PCR products from *S. divinorum* could be digested by *Nde*I. Two fragments of 428 and 59 bp were created from digested *S. divinorum* DNA (Fig. 5, lane 2). When purified PCR products from both *S. divinorum* and *S. officinalis* were digested using *Taq*I, a completely different RFLP profile was observed. *Taq*I cleaved *S. divinorum* 5S-rRNA spacer region at 235 bp position, whereas in *S. officinalis* cleavages were at 161, 170 and 217 bp (Fig. 2, empty square boxes). In *S. divinorum* two fragments of 235 and 252 bp were created (Fig. 5, lane 3), whereas in *S. officinalis* the same digestion produced four fragments: 9 (not visible), 47, 91 and 161 bp

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S. divinorum    GTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCCCCTGGGAAGTCCTC   50
S. officinalis  GTGCTTGGGCGAGAGTAGTACTAGGATGGGTGACCCCCTGGGAAGTCCTC   50
                **************************************************

S.divinorum     GTGTTGCACCCCTTTTT---------------------------------   67
S.officinalis   GTGTTGCACCCCTTTTTTGCTGCTTCGCGGTCTCTATTTCTTATATTCCT  100
                *****************

S. divinorum    -----------GTCAGTTTTTTTTGTTTTCTTTATTTATTATTTGTCGGT  106
S. officinalis  TCCCGATTTTT---------------------------------------  111

S. divinorum    TAACTTGCTCAATTCTATGAACCCAAATATATTGGAAGTCAGTCAGAGGG  156
S. officinalis  --------------------------------------------------   -

S. divinorum    ATTGTGGTACGTAGTCGTTGTGTTGATAAACGAGCGCGTCCCGCGATCTT  206
S. officinalis  --------------------------------------------------   -

S. divinorum    TGAAGTTGCCGACAATCTGTTTGTAATATCGAAATATGCCCGAAATGGCT  256
S. officinalis  --------------------------------------------------   -

S. divinorum    CAAAACGCTTATTTTTTTGGTGTCATATCACCCCCAACGGGCTACGCCGG  306
S. officinalis  -------------------------------GCTTTTCCGGCAATTCTAA  130
                                                *    ***** *  *

S. divinorum    TTGGCTACGTGGTCGGGCTCGGCGAGAGCTTCGCGATGAT--ATGCTGTG  354
S. officinalis  TAGACGATCTAGTACAAATTGTGTAAAATCTCGAGTTGCTCGATGTACGC  180
                * * * *  * **     * *   * *   *** * ** *  ***

S. divinorum    GCTCCCGTAACTCCTATCGGGTTAAAAGTTATGCCCGTACGAAGGTTGGA  404
S. officinalis  GCACCAATGTCTCCTTCGTGG--GACCCACATGTACTTTCGAAGATT--A  226
                ** **  *  *****     **   *       *  * * ***** **  *

S. divinorum    AGATGTTCACCTGTTCACTGGCGCATATGCTATAAAGGAATACATTGCGG  454
S. officinalis  TGAT-CCAACTGGAGCCCCACCGCATCATCTATAAGAGAATAGAACGCGG  275
                  **    **  *  * *   *****   ******   **** *  ****

S. divinorum    TGGACATGATGGGTGCGATCATACCAGCACTAA  487
S. officinalis  TGTTATTGATGGGTGCGATCATACCAGCACTAA  308
                **    ***************************
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Fig. 2. Sequence aligment of the spacer regions of 5S-rRNA genes from *S. divinorum* and *S. officinalis*. Primer sequences (5S-P1 and 5S-P2) used for amplification are in bold. Identical sequences are indicated by (*). Gaps (-) are introduced for the best alignment. *Nde*I site is evidenced by the shaded squared box, whereas *Taq*I sites are indicated by empty squared boxes.

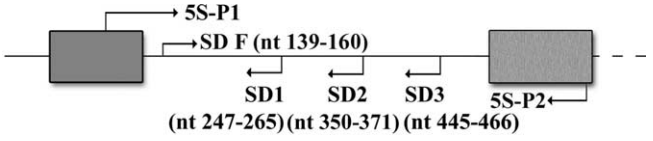

Fig. 3. Position of the primers (5S-P1 and 5S-P2) flanking the spacer region of 5S-rRNA gene and forward (SDF) and reverse (SD1, SD2 and SD3) specific primers used for PCR amplification of the 5S-rRNA spacer region of *S. divinorum*.

long (Fig. 5, lane 6). When *Taq*I digested products from *S. divinorum* were digested with *Nde*I, four fragments were found: one at about 60 bp and one at about 190 bp, deriving from the cleavage of the former fragment at 252 bp, one of uncleaved product at about 235 bp, and the fourth at about 250 bp indicating an incomplete cleavage of the former 252 bp fragment (Fig. 5, lane 4). The PCR–RFLP approach represents a powerful tool for plant material identification, and it was also successfully employed for the identification of a β-asarone-free *A. calamus* cytotype (Bertea et al., 2005) and for the discrimination of *Fritillaria* species (Cai et al., 1999).

In conclusion, the results of this work demonstrated that the combined use of analytical chemical (HPLC–MS) and molecular (DNA fingerprinting) methods lead to the precise and unequivocal identification of *S. divinorum* when analyzed in fresh, dried leaves or even when the morphological identification is not possible, as in the case of powdered dried material. Furthermore, here we have developed a rapid and precise method of identification of *S. divinorum* based on its 5S-rRNA spacer region sequence. Finally, we have shown that a unique *Nde*I site on this sequence can

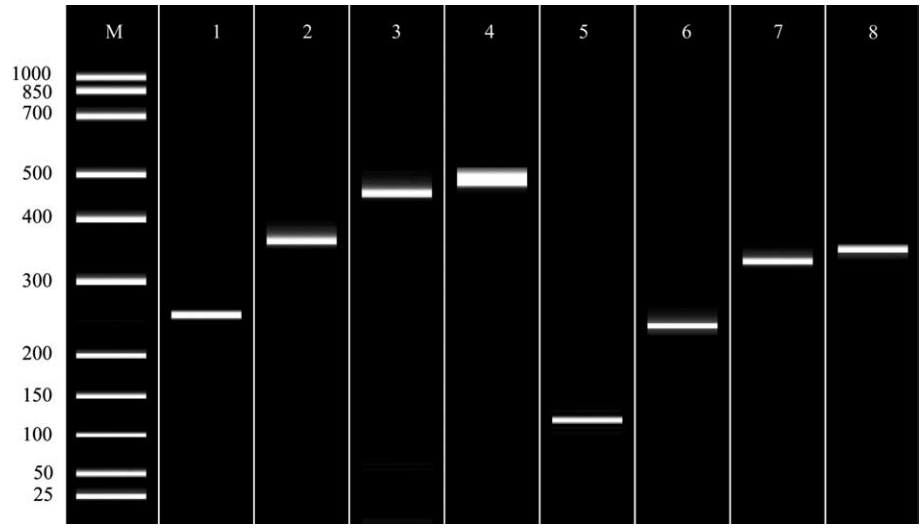

Fig. 4. *S. divinorum* PCR products generated by primers 5S-P1 → SD1 (lane 1); 5S-P1 → SD2 (lane 2); 5S-P1 →SD3 (lane 3); PCR products generated by primers (5S-P1 and 5S-P2) flanking the spacer region of 5S-rRNA gene using DNAs from fresh leaves (lane 4); PCR products generated by primers SD F → SD1 (lane 5); SD F → SD2 (lane 6); SD F → SD3 (lane 7); SD F → 5S-P2 (lane 8); M = bp markers. See also Fig. 3 for primers design. The PCR products were separated by using the Agilent 2100 Bioanalyzer and the DNA 1000 LabChip® Kit (Agilent Technologies).

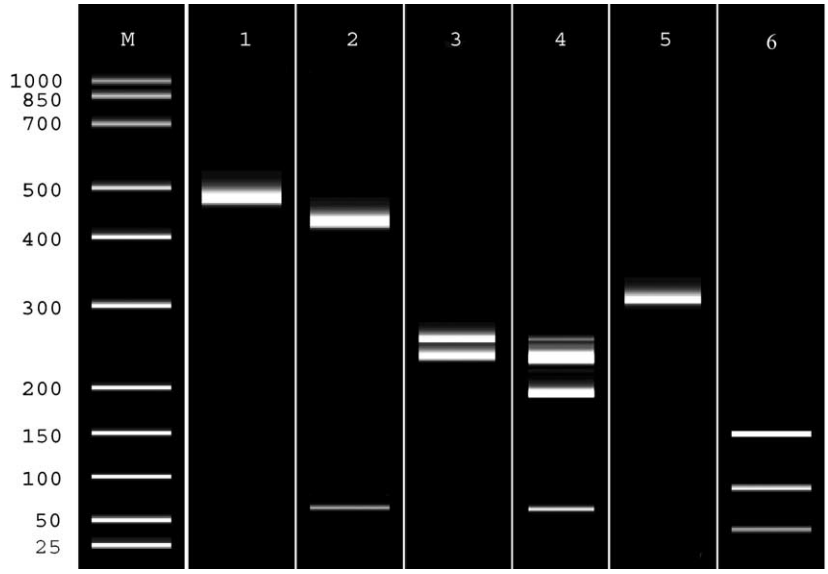

Fig. 5. PCR–RFLP analysis using *Nde*I and *Taq*I. *S. divinorum* undigested PCR products (lane 1); *S. divinorum Nde*I digested PCR products (lane 2); *S. divinorum Taq*I PCR digested products (lane 3); *S. divinorumTaq*I PCR digested products subsequently digested with *Nde*I (lane 4); *S. officinalis* undigested PCR products (lane 5); *S. officinalis Taq*I digested PCR products (lane 6). M = bp markers. The digested and undigested products were separated by using the Agilent 2100 Bioanalyzer and the DNA 1000 LabChip® Kit (Agilent Technologies).

easily discriminate *S. divinorum* from any other plant, whereas *Taq*I generates distinctive fragments in *S. divinorum* and in *S. officinalis*, respectively. In this context only *S. officinalis* was used as a direct comparison, but many other species are under study and will be reported soon. However a blast search revealed that even though the 5S-rRNA gene is highly conserved, the NTS region is highly variable from species to species, allowing its use as a molecular marker (in accordance with Ma et al., 2000). These results are particularly applicable in all cases when a rapid, precise and unequivocal identification of *S. divinorum* is needed, even when traces of *S. divinorum* are present in drug samples. This newly developed method is particularly suitable for phytochemical, forensic and toxicological investigations.

3. Experimental

3.1. Plant material

Healthy plants of *S. divinorum* were obtained from a kind permission of use by the judge for preliminary investigations GIP Dr Ferraro from samples used during the

criminal proceeding 10061/05 RGPM. Plants were identified by taxonomists of the Department of Plant Biology of Turin. Plants of *S. officinalis* were grown for several years in the experimental plots of the Botanical Garden of the University of Turin. Dried powdered leaf material claimed to be *S. divinorum* was provided by the Interregional Department of Scientific Police – Piedmont and Aosta Valley – Questura di Torino, following a seizure by the Police Narcotics Section. A voucher specimen is deposited at the Herbarium Taurinensis of the Department of Plant Biology of the University of Turin.

3.2. HPLC–MS

Fresh an dried leaves of *S. divinorum*, fresh leaves of *S. officinalis* and the unknown dried powdered leaves were crushed and homogenated in a mortar in presence of acetonitrile/water 50/50 extraction mixture with a ratio of 5 mg ml^{-1} for fresh leaves and 2 mg ml^{-1} for died leaves and powder. The resulting extracts were filtered by a first passage through 8 layers of cheesecloth and then by filtering through 0.45 μm filters. The salvinorin A standard was isolated from *S. divinorum* leaves and kindly provided by Rizzacasa and Munro (Melbourne University); the compound was dissolved in methanol and then diluted to 10 ppm with methanol/water 50/50 mixture. HPLC grade water was obtained from MilliQ System Academic (Waters, Millipore) and methanol HPLC grade was preventively filtered through 0.45 μm filters.

HPLC–MS analyses were performed using a Thermo-Finnigan Surveyor equipped with a diode array UV detector PDA-UV 6000 LP and coupled with the mass spectrometer LCQ Deca XP plus equipped with an electrospray interface and an ion trap as mass analyzer. The chromatographic separations were performed on a Phenomenex Luna C-18 column (150 × 2 mm, 3 μm particle size). Injection volume was 10 μl and the flow rate was 200 μl min^{-1}. The gradient for the mobile phase was: 80/20 to 0/100, 0.05% formic acid in water/acetonitrile in 40 min. Solvents and reagents were purchased from Sigma–Aldrich (Milan, Italy). The LC column effluent was delivered into the ion source using nitrogen as sheath and auxiliary gas (Claind Nitrogen Generator apparatus). The source voltage was set at the 4.5 kV value in positive mode and 4.0 kV in negative mode. The heated capillary value was maintained at 300 °C. The acquisition method used was previously optimized in the tuning section for the parent compound (capillary, magnetic lenses and collimating octapoles voltages) in order to achieve the maximum sensitivity. The tuning parameters adopted for ESI source were set as follows: source current 5.0 μA, capillary voltage 39.0 V, tube lens −20 V; for ions optics: multipole 1 offset −6.75 V, inter multipole lens voltage −16.0 V, multipole 2 offset −10.50 V.

Peak identification and quantification was performed using an external standard approach and performing MS/MS analysis of the 373 amu daughter ion generated from 433 amu ion and comparing the resulting chromatogram with those obtained in unknown samples. Mass spectra (*m/z* and, in brackets, abundance) data for salvinorin A: 373(4.9), 355(15.7), 341(13.5), 313(17.1), 299(2.8), 295(2.3), 267(1.2), 247(1.9), 239(2.6), 221(1.6), 173(0.9).

3.3. Genomic DNA extraction

Plant material (200 mg of fresh leaves or 60 mg of dried leaves or powdered material) was frozen in liquid nitrogen and ground to a fine powder in a chilled mortar.

Genomic DNA was extracted from the ground powder by using DNeasy® Plant Mini Kit (Qiagen) following manufacturer's instruction. The quantity and quality of the DNA were assessed by both spectrophotometric analysis using a GeneRay UV-Photometer (Biometra®) and gel electrophoresis.

3.4. PCR amplification, subcloning and sequencing

Approximately 20 ng of genomic DNA isolated from fresh, dried leaves and powdered leaf material of *S. divinorum* and fresh leaves of *S. officinalis* were used as a template for PCR amplification with forward primer 5S-P1 (5′-GTGCTTGGGCGAGAGTAGTA-3′) and reverse primer 5S-P2 (5′-TTAGTGCTGGTATGATCGCA-3′) flanking the NTS of 5S-rRNA gene (Sugimoto et al., 1999; Bertea et al., 2005). The amplification was carried out in a 50 μl reaction mixture containing 5 μl 10× PCR reaction buffer (Fermentas), 0.2 mM dNTPs, 20 pmol forward and reverse primers and 0.5 U of *Taq* DNA polymerase (Fermentas). The PCR reactions were carried out in a Whatman Biometra® T-Gradient Thermocycler. Cycling conditions consisted of an initial 2 min at 94 °C followed by 1 min denaturing at 94 °C, 1 min annealing at 56 °C and 2 min elongation at 72 °C repeated for 50 cycles and with 5 min extension at 72 °C.

One microliter of the amplification reaction was analyzed by using the Agilent 2100 Bioanalyzer (Agilent Technologies) and the DNA 1000 LabChip® Kit (Agilent Technologies) following manufacturer's instructions. The DNA 1000 LabChip® kit provides sizing and quantitation of dsDNA fragments ranging from 25 to 1000 bp. PCR products were also analyzed by a 2% agarose gel electrophoresis and visualized by ethidium bromide staining under UV. From this gel a band of about 500 bp for *S. divinorum* and about 300 bp for *S. officinalis* was purified by using GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences) and then subcloned into pGEM®-T Easy vector (Promega). The ligated products were transformed into the *Escherichia coli* Subcloning DH5α Efficiency Competent Cells (Invitrogen). Colonies containing DNA inserts of the correct size were picked and grown overnight in 3 ml of Luria–Bertani (LB) liquid medium. The mini-preparation of plasmid DNAs were performed using QIAprep® Spin Miniprep Kit, following

manufacturer's instructions. The plasmid DNAs were employed as a template for sequencing. ABI Prism, BigDye Terminator and Cycle Sequencing Ready Reaction Kit were used for sequence reaction with T7 and SP6 primers (Applied Biosystems). Sequences were detected by an ABI 377 automated sequencer according to the manufacturer's protocol (Applied Biosystems). Both strands of DNA were sequenced at least twice and the sequences were aligned by using ClustalX software.

3.5. PCR amplification using specific primers for S. divinorum

The sequences derived from fresh, dried leaves and the powdered leaf material of *S. divinorum* were aligned in a unique sequence that allowed the design of one forward specific primer SDF 5′-TGGAAGTCAGTCAGAGGGATTG-3′ and three reverse specific primers: SD1 5′-AGCGTTTTGAGCCATTTCG-3′, SD2 5′-ATAGGAGTTACGGGAGCCACAG-3′ and SD3 5′-CCATCATGTCCACCGCAATGT-3′, which corresponded respectively to nucleotides 139–160, 247–265, 350–371, and 445–466 of the *S. divinorum* non-transcribed spacer (NTS) sequence. The specific primers were used for amplification also in combination with primer 5S-P1 and 5S-P2. The conditions of the PCR reactions were the same as mentioned above.

One microliter of the amplification products were separated with the Agilent 2100 Bioanalyzer (Agilent Technologies) and DNA 1000 LabChip® Kit (Agilent Technologies) following manufacturer's instructions.

3.6. PCR–RFLP

The purified PCR products of the 5S-rRNA gene spacer region of *S. divinorum* were digested with 10 U of *Nde*I (Promega) at 37 °C for 1 h. The purified PCR products of the 5 S-rRNA gene spacer region of *S. divinorum* and *S. officinalis* were also digested with 10 U of *Taq*I (Promega) at 65 °C for 1 h. One microliter of both digestion reactions was fractionated by using the Agilent 2100 Bioanalyzer (Agilent Technologies) and DNA 1000 LabChip® Kit (Agilent Technologies) following manufacturer's instructions.

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