DNA internal standard for the quantitative determination of hallucinogenic plants in plant mixtures

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Abstract

Here, we show a new, simple, and rapid SYBR Green-based Real-Time PCR assay for the quantification of hallucinogenic plants in plant mixtures. As a test plant, *Salvia divinorum* Epling & Játiva-M., a perennial herb belonging to the Lamiaceae family able to induce hallucinations, changes in perception, or other psychologically induced changes with similar potency as LSD, was used. The method was tested on seven mixtures 100/0%, 80/20%, 60/40%, 40/60%, 20/80%, 10/90%, 0/100% (w/w) *S. divinorum* versus a non-hallucinogenic plant, *Salvia officinalis*. Total DNA was extracted from samples and quantified by Real-Time PCR. *Arabidopsis thaliana* genomic DNA was added, as internal standard, at the beginning of each extraction. A new formula for the interpretation of Real-Time PCR data, based on the relative quantification of DNA extracted from mixture versus a reference DNA extracted from a known amount of pure *S. divinorum*, was developed. The results of this work show an almost perfect correspondence between Real-Time PCR-calculated weight and the weight estimated by an analytical weighted method, proving the effectiveness of this method for the quantitative analysis of a given species in a plant mixture.
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1. Introduction

Morphological, anatomical and chemical analyses aimed to detect and quantify plant samples are affected by environmental and/or developmental factors or by method of sample storage. Very often, the identification of plant samples in a mixture is difficult to achieve and this problem is particularly exacerbated when plant mixtures are powdered. When toxic/hallucinogenic plants are only present in powder, plant identification is usually achieved by the determination and quantification of active compounds, which often requires time and GC- or LC–mass spectra determination ([1] and references therein). By contrast, DNA analysis is relatively fast and the presence of molecules with higher stability makes quantification easier, provided that specific primers for target genes can be used. *Salvia divinorum* Epling & Játiva-M. is a perennial herb belonging to the Lamiaceae family and is most recognized

for its hallucinogenic properties [2]. The psychoactive principle of the plant has been identified as the neoclerodane diterpene salvinorin A [3], a psychotropic molecule that produces hallucinations [4]. For this reason, *S. divinorum* is a frequently used hallucinogen [5], with similar potency as LSD in producing hallucination [3]. *S. divinorum* is often sold, in legal or illegal markets, as a powder that can be easily adulterated by adding dried leaves of other species, thus making hard to establish the purity of samples [6].

On continuation of our previous work, we used specific *S. divinorum* primers designed on the sequence of the 5S-rRNA gene spacer region [1] and developed a Real-Time PCR-based mathematical model for the quantification of *S. divinorum* in plant mixtures. Real-Time PCR detection strategies rely on continuous measurements of the increments in the fluorescence generated during the PCR [7,8]. Moreover, Real-Time PCR-based methods allow a reliable quantification of the amplicons produced during the exponential phase of amplification, present a broad dynamic range, and low intra- and inter-assay variability, allow a high sample throughput, and avoid the need of post-PCR manipulation [9].

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Here, we show a new mathematical model for the quantitative analysis of *S. divinorum* in a biological mixture by quantifying DNA by means of SYBR Green I fluorescence dye quantitative Real-Time PCR [10]. This model is based on relative quantification of DNA extracted from a mixture versus a reference DNA extracted from a known amount of the pure species. Furthermore, to avoid eventual errors derived from laboratory manipulation and other factors which affect the yield of extraction, an *Arabidopsis thaliana* external genomic DNA serving as internal standard was added to each sample before DNA extraction. To validate the method, seven mixtures (100/0%, 80/20%, 60/40%, 40/60%, 20/80%, 10/90%, 0/100%, w/w) of *S. divinorum* versus *Salvia officinalis* were extracted.

The method presented here represents a powerful tool for the rapid and accurate quantification of *S. divinorum* and other drugs in unknown samples for phytochemical, forensic, and toxicological investigations.

2. Materials and methods

2.1. Plant material

Healthy plants of *S. divinorum* were obtained with the kind permission of GIP Dr Ferraro, the judge for preliminary investigations. The samples have been seized as part a of criminal investigations (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.

Seeds of *A. thaliana* ecotype Columbia 0 were sown in pots containing peat moss soil, some vermiculite for aeration, and Osmocote and Triabon fertilizers (1 g/l) and then placed in a growth chamber.

The growth conditions were as follows: 16-h-light $(130 \ \mu mol \ m^{-2} \ s^{-1})$:8-h-dark cycle, temperature of 22 °C (day/night), and relative humidity of $65 \pm 10\%$. Leaves of *S. divinorum*, *S. officinalis* and *A. thaliana* were dried for 4 h at 65 °C (up to dry steady weight). The dried samples were then pulverized and sieved with a 1 mm mesh sieve.

2.2. Genomic DNA extraction

With the obtained powder, 10 mg of seven mixtures 100/0%, 80/20%, 60/40%, 40/60%, 20/80%, 10/90%, 0/100% (w/w) *S. divinorum*/*S. officinalis*, respectively, were prepared.

Ten minutes before extraction, 50 μl of *A. thaliana* DNA solution (0.02 μg/μl) was added, to each sample, as an internal standard.

To each sample, prepared as described above, 900 μl of cetyltrimethylammonium bromide (CTAB) extraction buffer (2% CTAB; 50 mM Tris–HCl, pH 8.0; 10 mM EDTA pH 8.0; 0.7 M NaCl; 0.2% 2-mercaptoethanol) was added [11]. The suspension was incubated in a water bath at 65 °C for 2 h with occasional shaking, then cooled to room temperature and extracted with 1 V of aqueous phenol/chloroform/isoamyl alcohol solution (25:24:1). This step was repeated twice. After

centrifugation at $12,000 \times g$ for 10 min, 0.7 V of 2-propanol was added to the upper aqueous phase and incubated at −20 °C for 30 min. DNA was pelleted by centrifugation at $12,000 \times g$ for 10 min. The pellet was resuspended in 500 μl of sterile water and 2 V of ethanol, and 0.1 V of 0.2 M sodium acetate pH 5.2 were added to the solution. The mixture was incubated for 24 h at −20 °C and the pellets were collected after centrifugation ($12,000 \times g$ for 10 min). These last two steps were repeated twice in order to remove phenolic compounds that could interfere with the PCR analyses. The pellet was then rinsed with 100 μl of 70% aqueous ethanol and centrifuged for 10 min at $12,000 \times g$. After drying the pellet at room temperature, the DNA was resuspended with 500 μl of sterile water.

Genomic DNA of *A. thaliana* was extracted from 200 mg of leaves using the above CTAB procedure.

The quantity of DNA was assessed by using a GeneRay UV-Photometer (Biometra®).

2.3. Primer design and selection

S. divinorum-specific forward primers SDF (TGGAAGT-CAGTCAGAGGGATTG) and SD1 (AGCGTTTTGAGC-CATTTCG)-specific reverse primers were already obtained and tested in a previous work [1]. Primers for *A. thaliana* were designed on the cDNA sequence of gibberellin 2-beta-dioxygenase (*GA2OX2*, GenBank accession number NM_100121) [12] (GA2OX2F Forward: GCTCGGCGAC-GAATGATTAC; GA2OX2R Reverse: CGGTGCTGGTGGA-TAGTGAT) using the Primer 3 Software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

Approximately 20 ng of genomic DNA isolated from *S. divinorum* and *S. officinalis* were used as a template for PCR amplification with GA2OX2F and GA2OX2R in order to check primer specificity.

All amplifications were carried out in a 50 μl reaction mixture containing: 5 μl 10X PCR reaction buffer (Fermentas), 0.2 mM dNTPs, 20 pmol forward and reverse primers and 0.5 units of *Taq* DNA polymerase (Fermentas). PCR products were separated by 1% agarose gel electrophoresis with the addition of 1% ethidium bromide and visualized using a UV transilluminator.

2.4. Real-Time PCR

Real-Time PCR assays of each DNA sample were performed in two separate reactions; one for SDF/SD1 primer pairs (*S. divinorum* DNA) and the other for *GA2OX2* (*A. thaliana* DNA) by using a Stratagene MX3000P Real-Time PCR System (Stratagene, La Jolla, CA). The process was performed with 25 μl of reaction mixture consisting of 12.5 μl of 2X Brilliant SYBR Green QPCR Master Mix (Stratagene), 1 μl DNA, 100 nM primers, and 30 nM ROX as a reference dye. For all amplifications, thermal cycling conditions begun with an initial step at 95 °C (10 min) followed by 40 cycles at 95 °C (10 s), 56 °C (25 s) and 72 °C (20 s). In each cycle, fluorescence was detected after annealing at 56 °C. A final dissociation stage

(56–95 °C with a heating rate 0.1 °C/s and a continuous fluorescence measurement) was run to generate a melting curve for verification of amplification product specificity.

Real-Time PCR efficiencies were calculated from the given slopes with the LightCycler software, according to the equation $E = 10^{-1/\text{slope}}$. Standard errors were calculated.

2.5. Mathematical model

The equation that expresses the exponential relationship between the copies of target at the beginning of PCR (N_0) and the quantity of amplicon product (N) at every circle (n) [8,13,14] is:

$$N = N_0(E + 1)^n \tag{1}$$

or by resolving Eq. (1) for N_0:

$$N_0 = \frac{N}{(E + 1)^n} \tag{2}$$

The quantity of a particular product is equal at the C_t point [15] and the term N becomes a constant (called N_t) in a way that Eq. (2) for given a sample becomes:

$$N_{0\,\text{sample}} = \frac{N_t}{(E_{\text{sample}} + 1)^{C_{t\,\text{sample}}}} \tag{3}$$

The yield of DNA extracted (YE) from each sample can then be calculated from the ratio between the number of copies of the internal standard in each sample at the beginning of PCR ($N_{0\,\text{standard}}$) and the number of copies added to the same sample before the extraction ($N_{0\,\text{standard}}^*$).

$$\text{YE} = \frac{N_{0\,\text{standard}}}{N_{0\,\text{standard}}^*} \tag{4}$$

or by using Eq. (3)

$$\text{YE} = \frac{N_{t\,\text{standard}}/(E_{\text{standard}} + 1)^{C_{t\,\text{standard}}}}{N_{t\,\text{standard}}^*/(E_{\text{standard}}^* + 1)^{C^*}} \tag{5}$$

or, owing to the identical values of $N_{t\,\text{standard}}$ and $N_{t\,\text{standard}}^*$

$$\text{YE} = (E_{\text{standard}} + 1)^{(C_{t\,\text{standard}}^* - C_{t\,\text{standard}})} \tag{6}$$

The effective amount of DNA in a sample (N_0^*) before the DNA extraction can be calculated by the following ratio:

$$N_{0\,\text{sample}}^* = \frac{N_{0\,\text{sample}}}{(E_{\text{standard}} + 1)^{(C_{t\,\text{standard}}^* - C_{t\,\text{standard}})}} \tag{7}$$

where RQ ($= N_{0\,\text{mixture}}^*/N_{0\,\text{ref}}^*$) is calculated according to Eqs. (3) and (7) and the two terms $N_{t\,\text{standard}}$ can be simplified, as well as the two terms $N_{t\,\text{mixture}}$ because they were already calculated at the threshold cycle. Moreover, the $C_{t\,\text{standard}}^*$ terms can be simplified because the same amount of internal standard was added to all samples.

3. Results and discussion

3.1. Mathematical model

The number of PCR cycles necessary to generate a statistically significant signal above the background "noise" is taken as a quantitative measure and is called threshold cycle (C_t). For data analysis, the individual Real-Time PCR efficiency (E) and the C_t values must be known. In order to determine the target effective copy number in the sample before the DNA extraction, the copies of sample target at the beginning of PCR ($N_{0\,\text{sample}}$) must be standardized ($N_{0\,\text{sample}}^*$; in which the asterisk indicates before extraction) with respect to the extraction yield.

Normally, the measurement of the gene of interest (GOI) is performed relative to at least one internal standard (sometimes also called endogenous control): an "unregulated" housekeeping gene (HKG) [8]. However, HKG have been found to be regulated and vary under experimental conditions [16]. Otherwise, an absolute quantification can be based either on an internal or an external calibration curve [10,17]. But even in this case the method requires identical PCR efficiencies for standard material and target cDNA [18]. As an alternative method, we decided to add to each sample at the beginning of DNA extraction an exogenous DNA as an internal standard.

Our aim was the quantification of *S. divinorum* DNA extracted from a mixture containing other plant samples versus a reference DNA extracted from a known amount of pure (100%) *S. divinorum*. If we consider the relative quantification of a plant mixture ($N_{0\,\text{mixture}}^*$) with respect to the values of the reference DNA extracted from a known amount of pure (100%) *S. divinorum* ($N_{0\,\text{ref}}^*$) we obtain the following ratio:

$$\text{RQ} = \frac{N_{0\,\text{mixture}}^*}{N_{0\,\text{ref}}^*} \tag{8}$$

where RQ expresses the DNA relative quantification from an unknown sample with respect to a known sample.

Eq. (8) can also be written as follows:

$$\frac{N_{0\,\text{mixture}}^*}{N_{0\,\text{ref}}^*} = \frac{(E_{\text{target}} + 1)^{(C_{t\,\text{ref}} - C_{t\,\text{mixture}})_{\text{target}}}}{(E_{\text{standard}} + 1)^{(C_{t\,\text{ref}} - C_{t\,\text{mixture}})_{\text{standard}}}} \tag{9}$$

where E_{target} indicates the efficiency of the PCR reaction of the selected primers (e.g. SDF and SD1) and E_{standard} indicates the efficiency of the PCR reaction of the selected internal standard primers (e.g. a specific primer for an *A. thaliana* gene). Supposing to have a constant concentration of DNA on dry weight biological material, the amount of the inquired species can be estimated by multiplying the relative amount of DNA for the dry weight of the reference sample:

weight of target in mixture = weight of reference × RQ

3.2. Validation of the model

To validate the mathematical model, we extracted DNA from a mixture of dried powdered leaves containing *S.*

divinorum and *S. officinalis* in a 100/0; 80/20; 60/40; 40/60; 20/80; 10/90 and 0/100 (w/w) ratio, respectively, as described in Section 2. Instead of using an unregulated HKG, we used as internal standard a given amount of genomic DNA extracted from *A. thaliana*, which was added to each sample before DNA extraction, just like a known amount of a compound is added as internal standard during phytochemical analyses. Specificity of primers was documented with gel electrophoresis, resulting in a single product for reaction with desired length, and melting curve analysis resulted in single product-specific melting temperatures ($T_m = 79.95\,°C$; $T_m = 79.35\,°C$). In addition, no PCR amplicons were detected after 40 Real-Time PCR cycles with the combinations SDF/SD1 on $DNA_{A.\ thaliana}$ as well as on $DNA_{S.\ officinalis}$, thus confirming the specificity of the primers for their targets.

After several triplicate trials and Real-Time PCR analyses, C_t values were calculated (see supplementary Table 1) and the obtained RQ values (obtained from Eqs. (8) or (9)) were plotted against increasing percentages of *S. divinorum* versus *S. officinalis*. Fig. 1 shows the almost perfect correlation between RQ values and the percentage of *S. divinorum* present in the mixture; moreover, the regression curve indicates a highly significant correlation of data ($R^2 = 0.99$) and a slope near to unit ($m = 1.02$, indicating an almost perfect correspondence between the calculated weight in Real-Time PCR and the weight measured with the use on an analytical balance). Fig. 1 also shows the coefficients of variation (CV) determined from independent triplicate repetitions in three different LightCycler runs. The accuracy of the method can be evaluated by the relative low CVs (ranging from 1.09 to 9.36), with the only exception for the mixtures containing 10% *S. divinorum* (CV = 19.12). In order to understand whether this CV value was depending on the accuracy of the method or to the technical limits of the weighting method, several dilutions (1:10, 1:100, 1:1,000, 1:10,000) were carried out with LightCycler runs. When threshold cycle values of both *S. divinorum* SD primer and *A. thaliana GA2OX2* were plotted against log mg *S.*
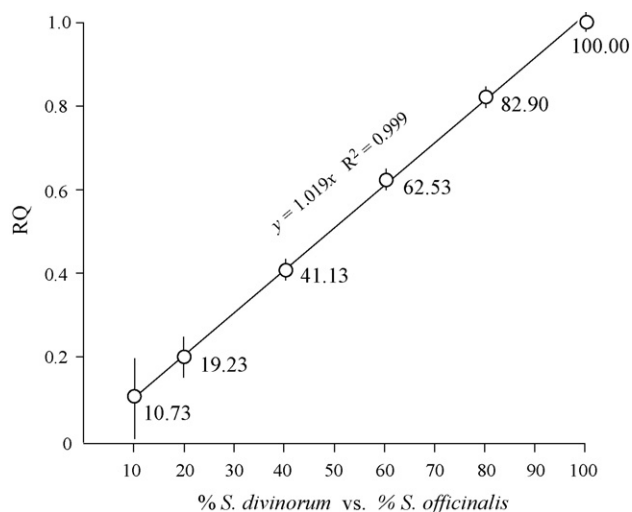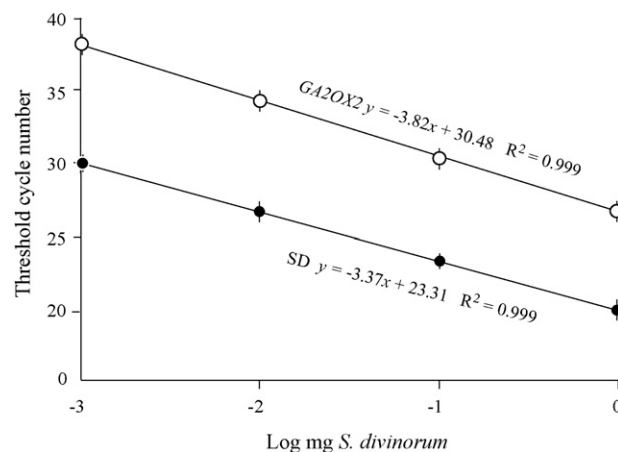
Fig. 2. Threshold cycle values using primers *S. divinorum* SD and *A. thaliana GA2OX2* plotted against log mg *S. divinorum*.

divinorum we found that the lower quantification limit was 10^{-6} g (Fig. 2), a value that is much lower than that achievable with conventional analytical balances. The data of Fig. 2, by confirming the accuracy of the method, indicate that the higher CV values found for 10% *S. divinorum* were probably depending on the accuracy limits of the analytical balance used.

The variability of extraction efficiency has been well studied and documented [19]. A quantitative approach based on the absolute quantification and co-extraction with standard DNA has been used to overcome the bias in extraction of DNA from Baltic Sea sediment samples [20] and quantification of human Herpesvirus 8 DNA in biological fluids [21]. In both cases, the co-extraction method was found to be more accurate and more precise for the quantification by PCR. Furthermore, quantitative PCR technology, incorporating fluorigenic 5′ nuclease (TaqMan) chemistry was utilized for the specific detection and quantification of six pathogenic species of *Candida* in water samples [22] and harmful algal species in field samples [23]. A close analogy can be observed between Eqs. (9) and (1) quoted by Pfaffl [17]:

$$\text{ratio} = \frac{(E_{\text{target}})^{\Delta CP_{\text{target}}(\text{control-sample})}}{(E_{\text{ref}})^{\Delta CP_{\text{ref}}(\text{control-sample})}} \tag{10}$$

The housekeeping gene (shown as reference in Eq. (10)) is replaced in our Eq. (9) by the *A. thaliana* genomic DNA used as internal standard, while a known amount (in our work 10 mg) of species to be quantified constitutes the control sample.

The data presented in this work prove the possibility to quantify the weight of a given plant species in an unknown sample based on DNA analysis only, and this method can be extended to any other organism. The use of an external genomic DNA as an internal standard was successful, also considering the particular *A. thaliana* gene selected (*GA2OX2* [12]).

Once a specific primer is designed for a given species, the presented method becomes a powerful tool for the quantification of that given species in biological mixtures.

Fig. 1. Plot of increasing percentages of *S. divinorum* vs. *S. officinalis* as a function of RQ. Bars indicate coefficient of variation.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fsigen.2007.06.003.

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