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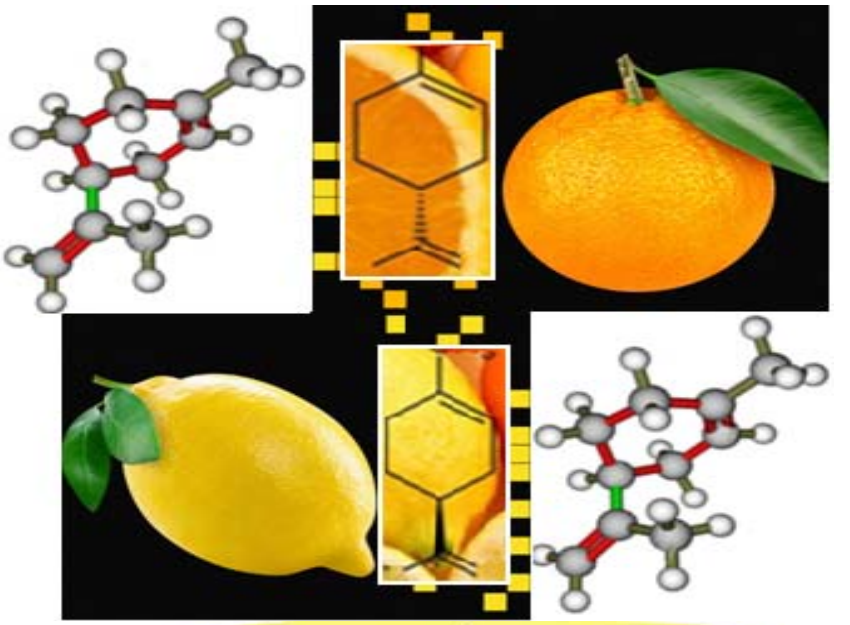

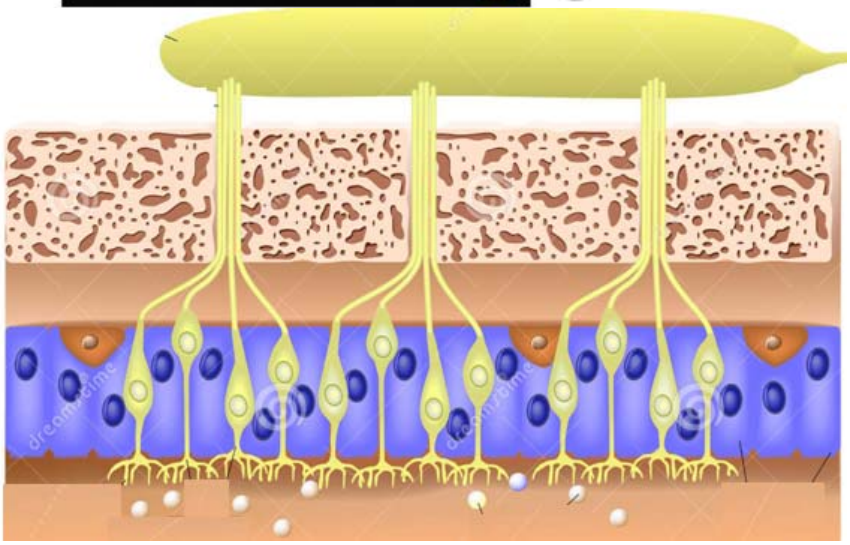

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Validation and exploratory application of a simple, rapid and economical procedure (MESQ) for the quantification of mescaline in fresh cactus tissue and aqueous cactus extracts

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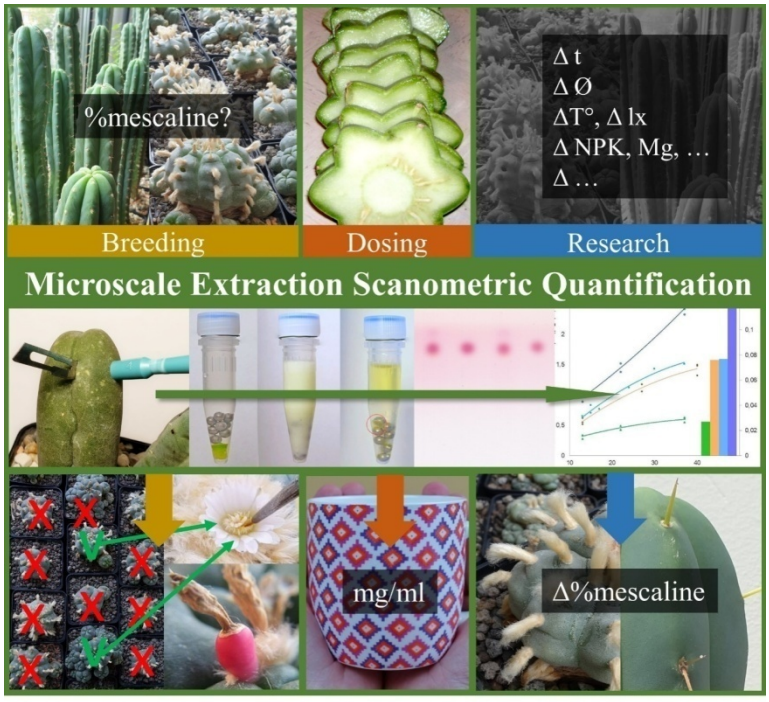
Abstract. ***Objective:*** *An effective way to protect the currently endangered wild populations of Lophophora williamsii is to select mescaline-rich specimens for selective propagation. Also, users of these and other entheogenic cacti are in need of an accessible way of dosing extracts. This research aims to introduce and validate an accessible procedure for the quantification of mescaline in fresh cactus tissue and aqueous cactus extracts, providing harm-reduction to entheogenic cactus populations and their users.*

Method: *Standardized biopsy and Microscale Extraction followed by Scanometric Quantitative thin-layer chromatography (MESQ) were applied to live specimens and the extracts of Lophophora williamsii, Echinopsis lageniformis, E. peruviana and E. pachanoi.*

Results: *The procedure was found to be valid for the intended applications. A previously undescribed radial mescaline gradient in L. williamsii and a longitudinal mescaline gradient in the Echinopsis species was thereby discovered. In the process, there also appeared to be a predictable correlation between the mescaline concentration in targeted biopsies and the strength of the overall cactus.*

Conclusion: *This accessible and minimally invasive procedure is valid for the quantification of mescaline in live cacti and aqueous cactus extracts for breeding and dosing purposes. In addition, the MESQ-procedure holds promise as a valuable research tool for further botanical and phytochemical research.*



Validation and exploratory application of a fast, simple and economical procedure (MESQ) for the quantification of mescaline in fresh cactus tissue and aqueous cactus extracts. Frederick Van Der Sypt, 2021

Key Words: *Mescaline, psychedelic, cactus, Peyote, San Pedro, harm-reduction, TLC*

1. INTRODUCTION

This article introduces a simple analytical procedure for the quantification of mescaline in fresh cactus tissue and aqueous extracts thereof. The procedure can be applied in the breeding of mescaline-containing cactus species and the dosing of their extracts. In this way, both the health of endangered cactus populations and that of their users can be protected. In addition, this minimally invasive procedure also allows for detailed studies to be carried out on living cactus specimens, opening new doors for further phytochemical and botanical research.

Mescaline-containing cacti have been used for thousands of years for religious and medicinal purposes [1-4]. In Mexico and the southern states of the U.S., the cactus *Lophophora williamsii* (Peyote) is used, where in South America it is mainly *Echinopsis pachanoi, peruviana* and *lageniformis*. For hundreds of thousands of people, these cacti are the sacrament within the practice of their religion [5].

However, the use of peyote as a sacrament is currently coming under pressure due to the excessive and erroneous harvesting of wild populations of *L. williamsii*, the plowing of their habitat, their processing into a variety of health products, and legislative restrictions on the cultivation of the cactus. As a result, the peyote cactus has now entered what is called a "conservation crisis"[6] and has been officially recognized as vulnerable by the IUCN[7]. *Echinopsis* populations are also under pressure by religious tourism [8] and the online trade of wild-harvested cactus despite restrictions imposed by CITES[9]. The current global trend towards regulation of psychoactive drugs will potentially further increase the demand for entheogenic cacti [10-12]. Therefore, the need to rebalance supply and demand of entheogenic cacti is becoming more compelling.

An obvious solution to this imbalance would be the cultivation of the above species [13]. However, even if the legal barriers were removed, the problem of the slow growth of cacti and the relatively high dose of active alkaloid required for an entheogenic experience (300-500mg of mescaline free base) remains. This is especially alarming for peyote, as even in optimal growing conditions it can take 5 plants 5 years to provide a single dose. In the wild, this can take up to 30 years [14, 5]. It also appears that after harvesting, the mescaline concentration is lower in the regrowth than in the original 'button' [15,16]. In addition, it has recently been shown that there is a genetic line of *Lophophora williamsii* that does not contain mescaline [17]. Selective propagation is therefore important to optimize the return on investment in biomass.

For entheogenic purposes, selective breeding relies on the efficient identification of mescaline-rich specimens in large populations. To date however, quantification of mescaline in cacti is done with labor-intensive extraction techniques on large biopsies or whole specimens, followed by analysis with often very specialized and expensive equipment [18-20]. Consequently, this possibility has remained inaccessible to cactus growers. To enable a sustainable supply of entheogenic cacti, there is a need for a simple, cheap and fast method to screen cactus populations for strong specimens and to compare them through absolute quantification based on a standardized protocol.

Apart from the spiritual potential of entheogenic substances, a psychedelic renaissance within the medical community has started, with entheogens being studied for several conditions for which they are also used therapeutically in traditional contexts, e.g., anxiety, mood disorders, and addiction [21-24]. Within the class of classical psychedelics (including DMT and psilocybin), substance use disorders are very rare and mescaline intoxications cause only mild symptoms with favorable outcomes [25]. Indeed, mescaline seems to be one of the least harmful psychoactive substances, especially considering its potentially profound effects. Very

recent research confirms the therapeutic and otherwise positive influences on life brought about by experiences with mescaline [26, 27] and in the wake of this research, the pharmaceutical industry has resumed the production of mescaline and other psychedelics [28, 29].
Despite their favorable safety profile, psychedelics should always be approached with respect. Any entheogenic experience that is inadequately framed can turn out to be subjectively overwhelming with the temporary occurrence of pronounced anxiety or other unwanted psychological reactions. Three elements are very important in obtaining safe and enriching experiences with these substances: first, the current psychological state and intention of the user; second, the immediate environment in which the drug is consumed and the support present therein; and third, the dose. The uncertainty regarding the dose of active substance is specific to plant derivatives. Even more so, in all of the mentioned cactus species, the range of mescaline content is very wide [30, 25, 5]. This can lead on the one hand to well-prepared users ingesting insufficient active ingredient for the intended experience, and on the other hand it can cause users being faced with an undesirably intense experience. Currently, there are no accessible means to allow for correct dosing. There is a need for a quick, simple, and inexpensive way to determine the strength of cactus extracts, thereby providing harm-reduction to their users.
To address the above questions, the MESQ procedure was developed consisting of aligned steps for standardized sampling, selective extraction, chromatographic separation, selective staining and digital quantification. This article describes the development and validation of the procedure and will also discuss some exploratory applications. Surprisingly, to date, hardly any research has been conducted on the distribution of mescaline in individual cactus specimens [31, 32]. Through application of the MESQ procedure, new facts are now emerging that are not only compelling on a biological and phytochemical level, but may also have a direct and important positive impact on the precarious situation of cactus populations and the safety of their users.

2. METHODS AND MATERIALS

Section 2.1 of this chapter first provides a precise description of the procedure. How it was developed is discussed later in the discussion. Next, section 2.2 discusses the methodology by which the procedure was validated. Finally, section 2.3 describes how the exploratory applications took place.

2.1 Presentation of the procedure

The MESQ (Microscale Extraction - Scanometric Quantification) procedure presented here is shown graphically in Figure 1. Three main phases can be distinguished within the procedure: the preparation of the sample and standards, a microscale liquid-liquid extraction and finally quantification by thin-layer chromatography.
Although the procedure only makes limited use of corrosive or otherwise harmful reagents, it is advised to wear suitable safety clothing and eye protection.

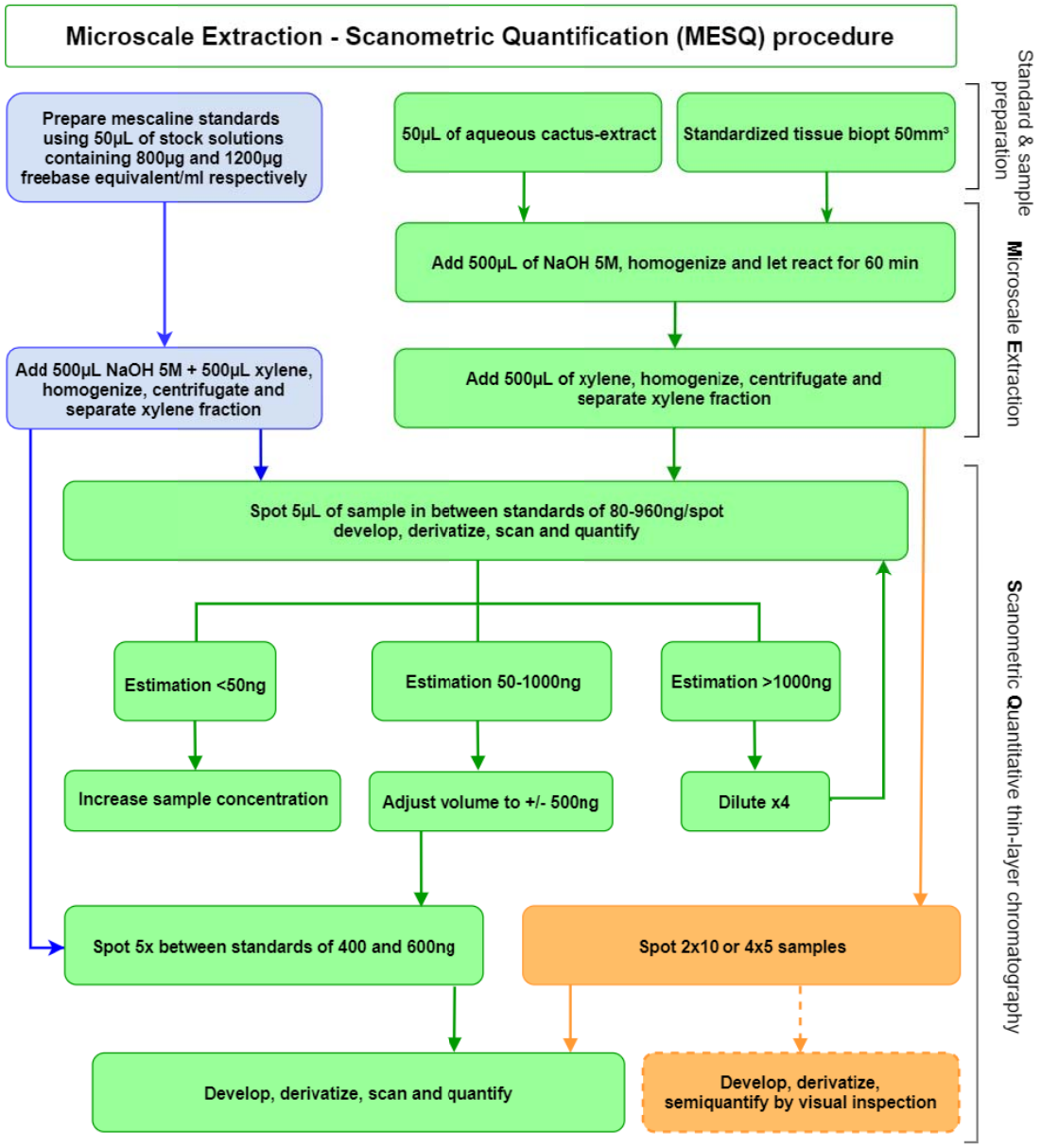


Figure 1: *Flow chart of the MESQ procedure. The 3 phases of the procedure are shown in the right margin. The blue frames and arrows describe the path of the mescaline standards. The green frames and arrows describe the path for absolute quantification of samples. The orange frames and arrows describe the path for relative quantification of samples, within which the distinction between scanometric and visual quantification (dotted line) is made.*

2.1.1 Preparation of samples and standards

Sampling of cactus tissue

The goal of this step is to cut away a precise and reproducible volume of cactus tissue. For this purpose, a skin punch with a diameter of 4 mm (Kai Medical) is used. The biopsy is best taken in homogeneous parenchymal tissue, i.e. at a sufficient distance from areoles. After disinfecting the insertion site with 70% ethanol, the punch is inserted in a circular motion until it reaches the stop. Only light pressure is used to minimize tissue deformation. Next, a

sharp blade (Swann-Morton no.11) is inserted from the side, perpendicular to the axis of the punch and directed towards the tip (see Figure 2). Inside the cactus, the piece of tissue is then cut loose from the surrounding matrix against the cutting edge of the punch.

Figure 2*: Standardized biopsy in E. lageniformis var. monstrose with skin punch and scalpel.*

Both instruments are removed after which, optionally, a very small amount of sulfur powder is blown into the biopsy site with a straw to further reduce the chance of infection. Then a metal threaded rod with pre-positioned stop nut is inserted from the back of the punch so that the sample is pushed out of the punch leaving exactly 4mm in it. The protruding portion is cut off with the scalpel and the remaining sample is now pushed out of the punch with another threaded rod into a 1.5ml reaction vessel with a screw cap (Figure 3).

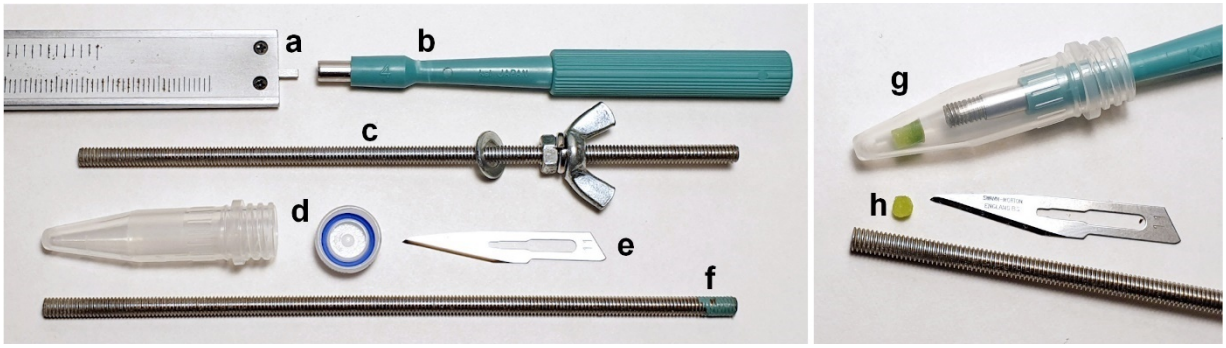


Figure 3: *Material for standardized biopsy. a: micrometer with depth probe; b: 4mm skin punch; c: threaded rod with stop nut; d: 1.5ml reaction vessel; e: scalpel; f: threaded rod without stopper; g: standardized sample in reaction vessel; h: cut portion of sample*

Sampling of aqueous cactus extract

Pre-determined portions of the fresh cactus are homogenized with a hand blender along with 15x its weight of demineralized water. This foaming mixture is boiled under an overpressure of 1 bar (15 psi) at 121°C for 1 hour after which it is sieved and then filtered through paper. The residue is squeezed out and boiled again under the same pressure in the same amount of water for 1 hour after which both extracts are combined and boiled down to approximately the fresh weight in milliliters. Then, using a microliter syringe (Unimetrics 4050), 50 µl of this extract is transferred into a 1.5ml reaction vessel with screw cap.

Preparation of standards

All standards used for the quantification of samples and validation of the method were prepared starting from an aqueous standard solution of mescaline sulfate dihydrate. This salt was isolated from 100g of fresh green parenchyma of *Echinopsis lageniformis* by acid-base extraction followed by washing with cold anhydrous acetone and triple recrystallization from acetone-water. Half of the obtained amount (+/-60mg) was sent to Energy Control. There, the purity was found to be >99% after analysis with LC-DAD-MS (Sample ID 54937, Appendix 1). The other half (60.0mg) was dissolved in demineralized water to a concentration of 4mg/ml. For easy comparison of results with other research, all concentrations in this article are expressed in units of mescaline free base. Taking into account their relative molecular masses [33], a conversion factor of 0.7816 is applied for the conversion of mescaline sulfate dihydrate to mescaline free base.

Using a microlitre syringe (Unimetrics 4050 50µL), 50µL of aqueous solution of a mescaline salt with corresponding free base concentrations of 800ng/µL and 1200ng/µL, respectively, are transferred into a 1.5ml screw-cap reaction vessel. To both vessels, 8 steel beads with diameter 3mm and 500µL of NaOH 5M are added (Figure 4 right).

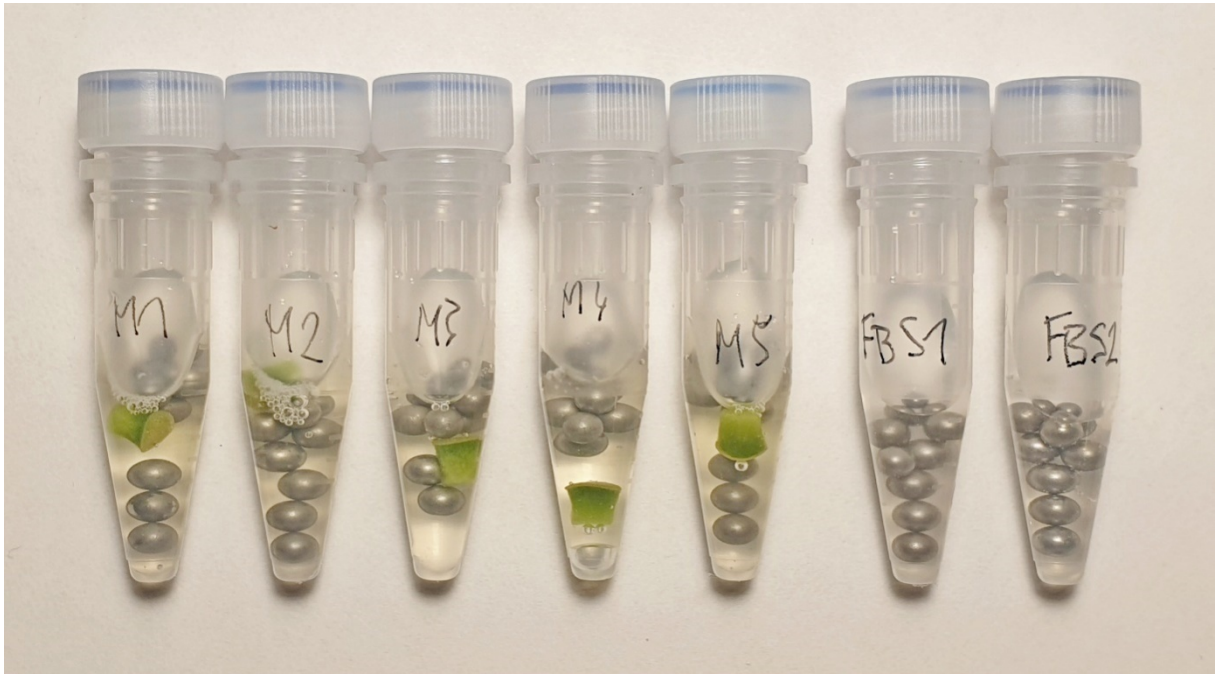


Figure 4: *The reaction vessels with the respective biopsies from five different specimens of E. lageniformis var. monstrose together with both reference standards. Throughout the procedure, samples and standards are always processed in parallel.*

Then, 500µL of xylene are added to the vessels containing standard solution and the vessels are shaken for 120 seconds at 2000 displacements/min over a distance of 20mm. For this purpose, an adapter was built that can be attached to a jigsaw and can handle up to 8 reaction vessels at a time (Figure 5). After shaking, the vials are centrifuged at 7000rpm for 2x3min using a microcentrifuge (KRACHT-D1008-BL). If needed, in order to obtain clean separation of layers, the vials can be rotated 180° around their longitudinal axis after the first 3min to compensate for the oblique position during centrifugation. After this, most of the xylene fraction is pipetted off into empty, xylene-resistant vials. From here, both standards will now be named S1 and S2 prepared from 800ng and 1200ng mescaline free base equivalents/µL of water, respectively. The concentrations of mescaline free base in the respective xylene fractions will then become 80ng/µL for S1 and 120ng/µL for S2 (assuming complete extraction, see discussion under Section 3.1).

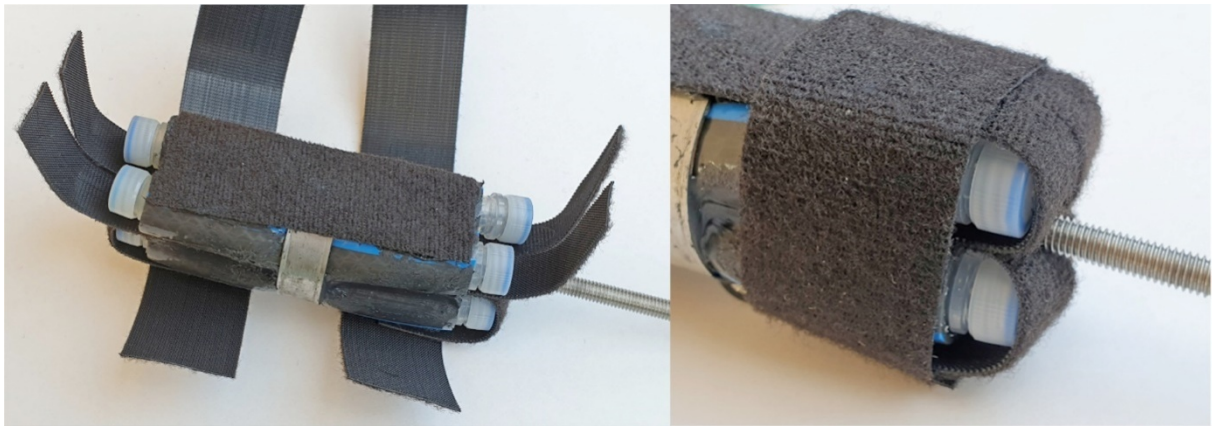


Figure 5: *The adapter was composed of threaded rod of 6mm diameter and 4 pieces of garden hose. These were attached together with glue and a metal clamping ring. Velcro strips were glued on top of these to hold the 8 reaction vessels securely in place during homogenization. The threaded rod was welded to a suitable saw blade that can be locked in the jigsaw.*

2.1.2 Microscale extraction

Mechanical and chemical lysis of the plant matrix and conversion to free base

To the reaction vessel are added 500µL NaOH 5M and 8 steel balls of 3mm diameter. The sample is then mechanically homogenized as described in the preparation of the standards. After homogenization, the vessel is kept at room temperature for at least 1 hour. If disturbing emulsion formation is to be expected, the reaction time can be extended by several hours according to need (see 3.2.6c).

Liquid-liquid extraction, centrifugation and separation

To the reaction vessel are now added 500µL of xylene after which the contents are homogenized and centrifuged following the same procedure as for the standards (Figure 6). With some matrices, emulsions occur which can usually be resolved by interrupting the centrifugation a few times and breaking up the emulsion layer by gentle shaking. Optionally, after centrifugation, a sufficient amount of xylene can be pipetted off into a separate vessel to prevent mixing with the raffinate during spotting. This is not necessary for most applications since only a very limited amount of xylene is used, thus leaving sufficient distance from the separation zone.

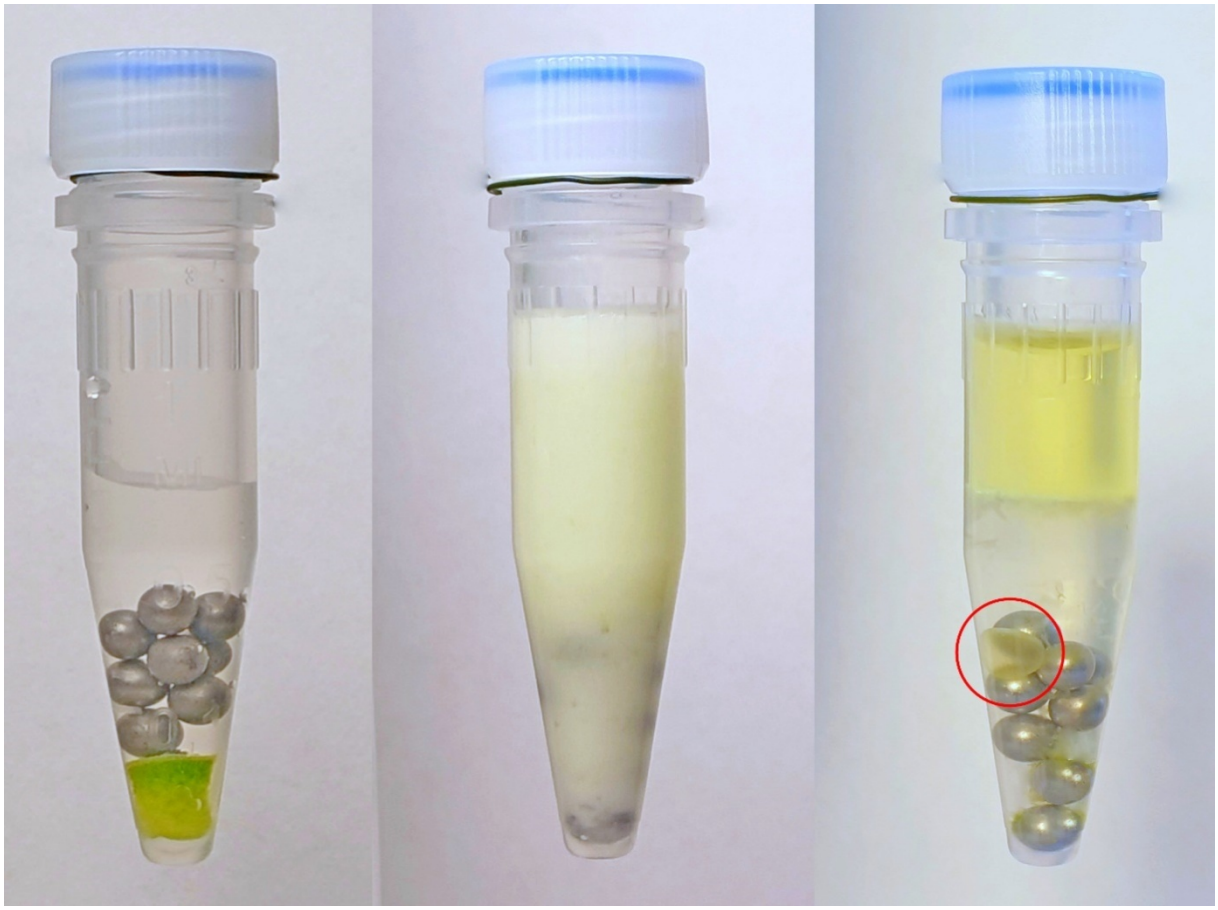


Figure 6: *Successive stages of microscale extraction. a: reaction vessel with sample, steel beads and NaOH solution; b: vessel after homogenization with xylene; c: vessel after centrifugation. The red circle shows the circular outer skin layer of Echinopsis species that often remains intact after homogenization.*

2.1.3 Planar thin-layer chromatography

Spotting

Macherey-Nagel ALUGRAM SIL G UV254, 20x20cm plates are used as the stationary phase. Each plate is cut into 3 plates of 20 x 6.6cm in advance. To avoid distortion and flaking of the plate, the scissor opening is kept as small as possible at the start and the scissors are slightly tilted clockwise during cutting. On the plates, 23 spots are placed next to each other at 10 mm from the bottom edge and 5 mm from both sides. Spotting is done manually with Hirschmann Ring caps 1/2/3/4/5µL micropipettes (precision CV </= 0.6%; accuracy R </= 0.3%). In order to avoid evaporation of xylene and thus artificial concentration of the solutions, aspiration of the xylene is done through the lid of a reaction vessel in which an opening of 2mm diameter is drilled at the center. Subsequently, the entire contents of the pipette are emptied onto the plate in one time. During spotting a powerful stream of hot air (60-70°C) is blown over the plate with a hair dryer (BaByliss 6500FRE).

Depending on the intended purpose, we distinguish 3 ways of spotting:

1) **For relative quantification of samples** (orange boxes in Figure 1), 10 samples (A-J) are spotted 2 times or 5 samples (A-E) are spotted 4 times, depending on the desired level of precision and according to the schemes below:

10 samples: A-A-B-C-D-E-F-G-H-I-J-A-B-C-D-E-F-G-H-I-J-A-A
Or
5 samples: A-A-B-C-D-E-A-B-C-D-E-A-B-C-D-E-A-B-C-D-E-A-A

At each position 5µL is spotted, or that volume known to be above the 67ng quantification limit (LOQ) (see 3.2.2) and preferably below 1µg/spot (because the response curve loses discriminatory sensitivity above this value, see 3.2.3).
No standards are spotted for relative quantification.

2) To ***estimate*** the absolute mescaline concentration in the sample, 5µL of 5 different samples (A-E) are spotted 2 times between different volumes of both standards S1 and S2 according to the scheme below:

1µLS1 - 1µLS1 - 5µLA - 3µLS1 - 5µLB - 4µLS2 - 5µLC - 9µLS1 - 5µLD - 8µLS2 - 5µLE - 1µLS1 - 5µLA - 3µLS1 - 5µLB - 4µLS2 - 5µLC - 9µLS1 - 5µLD - 8µLS2 - 5µLE - 1µLS1 - 1µLS1
This corresponds to mescaline free base amounts in the standard spots of 80, 240, 480, 720 and 960ng/spot, respectively (Figure 7, blue row).

3) For **absolute quantification** of 2 samples, adjusted sample volumes and/or concentrations based on prior estimation (A' and B') are each spotted 5 times between brackets of 5µL S1 and 5µL S2 according to the following scheme:

5µLS1 - 5µLS1 - A’ - 5µLS2 - B’- 5µLS1 - A’ - 5µLS2 - B’ -5µLS1 - A’ - 5µLS2 - B’ - 5µLS1 - A’ - 5µLS2 - B’-5µLS1 - A’ - 5µLS2 - B’- 5µLS1 - 5µLS1
In this manner, each sample spot is flanked by a 400ng and 600ng mescaline free base standard spot (Figure 7, red row).

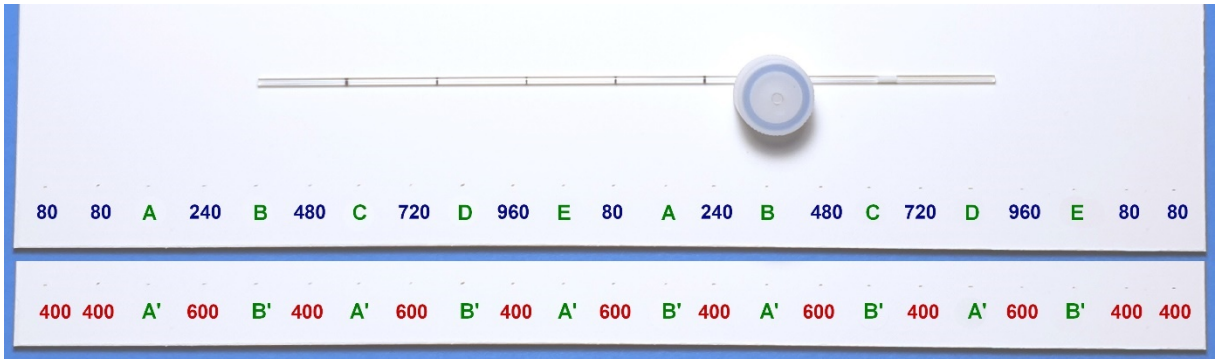


Figure 7: *Positions of standards and samples for estimation (top row, standard masses in blue, samples A-E) and absolute quantification (bottom row, standard masses in red, samples A' and B'). Also notice the perforated lid used during the spotting phase.*

After spotting, each plate is further dried for 2 minutes with a powerful stream of hot air (60-70°C, BaByliss 6500FRE).

Development

Development of the plates takes place in low, hermetically sealed chambers of polypropylene (22 x 16 x 7 cm) provided with sufficient filter paper to obtain rapid and complete saturation of the chamber with the eluent. Inside the chamber, the plates themselves are placed in a V-shaped aluminum recipient, closed at the sides, to ensure the most correct composition of fresh eluent. As the mobile phase, ethanol 99% is used (denatured with 1% methyl ethyl ketone (MEK)) to which +5% ammonia solution (25%) is added. After the chamber is saturated for at least 20 minutes, 4ml of eluent is poured into the V-shaped holder, the plate is inserted and kept at an 80° angle (Figure 8). Then the plate is developed until the eluent just reaches the top edge all along the plate. Subsequently, the plate is removed and immediately dried for 10 minutes with a powerful stream of hot air at 60-70°C.

Figure 8: *Development chamber made of polypropylene with aluminium holder for the plate and filter paper to saturate the chamber.*

Derivatization

After complete drying, the bottom 11mm of the plate are cut off. The plate is then derivatized in-situ in an identical development chamber in which the filter paper is now saturated with ethanol 99% (+1% MEK) to which +10% acetic acid 7% is added. In the V-shaped container 4 ml of eluent is poured. This eluent consists of ninhydrin (Roth Art.-No. 4378.1) 0.1% in ethanol 99% (+1% MEK) + 10% acetic acid 7%. After the eluent has reached the top of the plate, the plate is developed for an additional 5 minutes to allow for homogeneous saturation of the stationary phase with the eluent. Then the plate is simultaneously dried and derivatized by directing a powerful stream of hot air at 60-70°C (BaByliss 6500FRE) from a height of 10cm vertically onto the plate for 10 minutes. To promote uniform development, the stream is directed alternately for 10 seconds at a point 6 cm away from both plate edges.

2.1.4 Quantification

Video densitometry

The plate is scanned immediately after derivatization on a flatbed scanner (Epson Perfection V39) at 300dpi and uploaded to JustQuantify Free [34]. On the main screen, the 'spots' setting is chosen. After uploading, the spot number is set to 23 and noise level 1 is chosen for detecting and quantifying the spots (Figure 9).

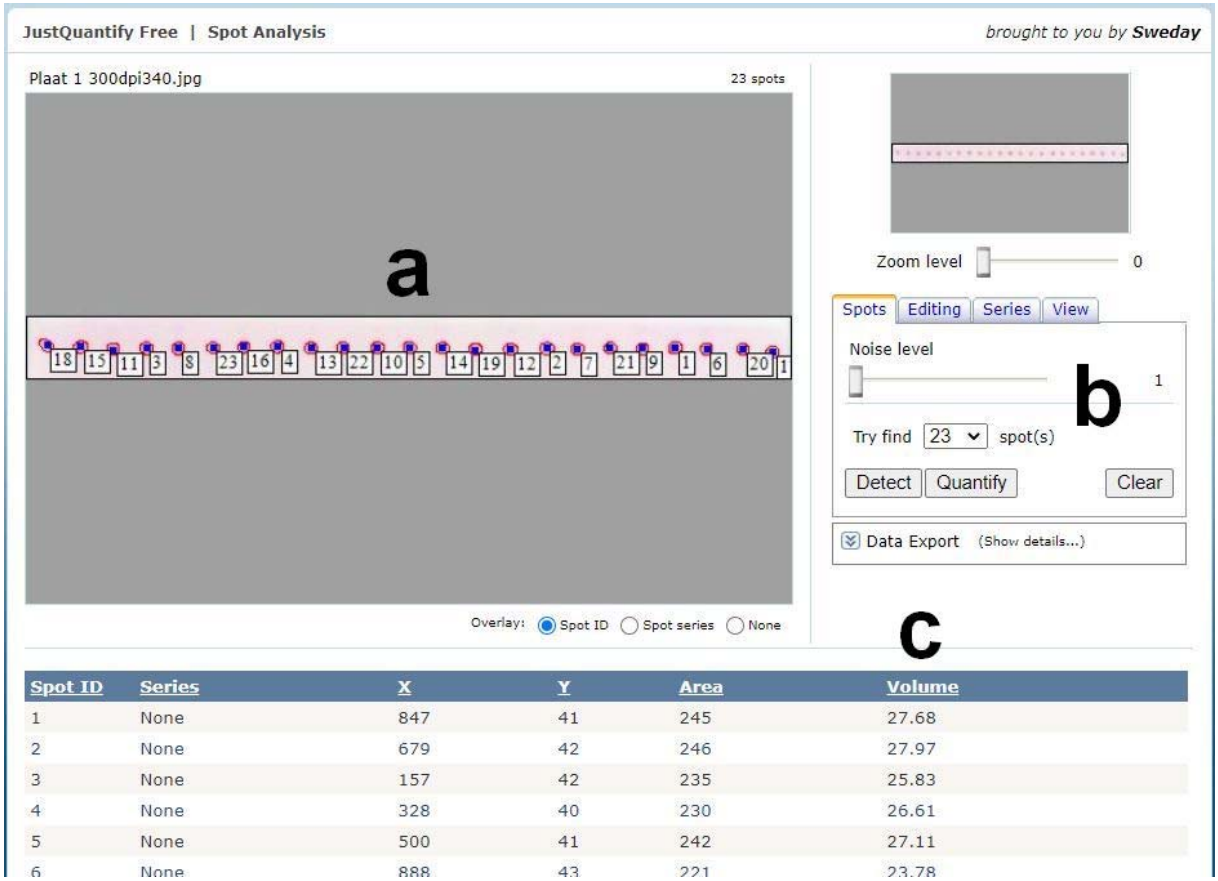


Figure 9: *Screenshot of JustQuantify Free. a: cutout of scanned plate; b: setting the number of spots and noise-level; c: 'volume' values used for quantification*

Calibration and quantification

The obtained densitometric output of the samples and standards is fed into a spreadsheet that places the samples either within a wide range of standards (80-960ng) for concentration estimation or between pairs of standards (400 and 600ng) for absolute quantification (green boxes in Figure 1). In the case of relative quantification, the densitometric output of Just Quantify Free is used directly to rank the samples according to mescaline content (orange boxes in Figure 1). This ranking can even be done by visual inspection without scanometry in case of large concentration differences between the samples. This route is marked in Figure 1 with dashed lines.

For calculating the concentration in the estimation step, a freely available spreadsheet is used: 'Quadratic calibration with error estimation' by Prof. Tom O'Haver [35]. This table draws the quadratic calibration curve, calculates its regression coefficient, residuals, the standard error

of the standard concentrations and, on entering the densitometric sample value, gives the correlating amount of mescaline in the spot. Figure 10 shows a screenshot of this spreadsheet.

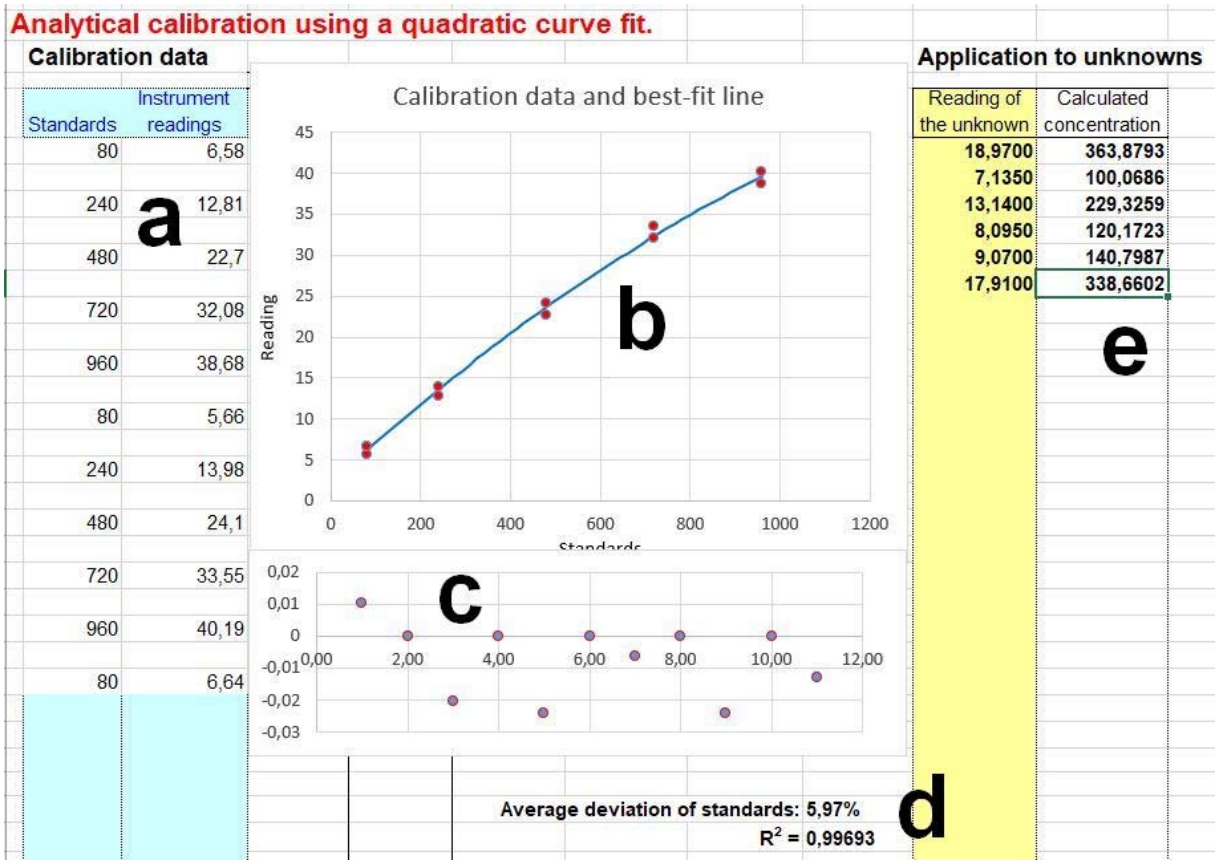


Figure 10: *Screenshot of the spreadsheet used for estimating mescaline concentration in samples. a: measured values of standards exported from JustQuantify Free; b. calibration curve; c. residual plot; d. regression coefficient of the standard curve; e. calculated sample concentrations based on measured values from JustQuantify Free.*

The spreadsheet used for absolute quantification was designed so that the 5 spot-values generated for each of the 2 samples per plate are converted to their corresponding mescaline mass by means of linear interpolation between 400ng and 600ng, using the neighbouring standards on the plate (data pair method). A screenshot of this spreadsheet and its interpretation is discussed further below in section 3.1.

2.2 Validation of the procedure

For the validation of the procedure, the guidelines of Eurachem [36], IUPAC [37], UNODC [38] and EMEA [39] were used as guidance. The latter, the ICH-Q2(R1), was translated by the AOAC into practical acceptance criteria for TLC [40], which were integrated into this validation.

2.2.1 Selectivity (Specificity)

Final selectivity was judged by the observation of sufficient separation of the mescaline band from surrounding bands on the derivatized chromatograms.

2.2.2 Limit of Detection (LOD) and Limit of Quantification (LOQ)

A distinction must be made here between the *instrument* LOD & LOQ on the one hand and the *method* LOD & LOQ on the other. In the latter case, any increase in spot volume should also be taken into account.

The instrument LOD & LOQ were both theoretically determined from a calibration curve of low concentrations and experimentally confirmed by additional concentration determinations near the theoretical LOD & LOQ.

2.2.3 Working range

Again, the distinction must be made between the *instrument* working range and the *method* working range. In the latter case, any dilution or volume increase of the spot solution should also be taken into account.

To determine the *instrument* working range, 11 exploratory concentrations of mescaline-FB were first spotted in the range 50-2000ng.

Within this range of interest, a linear working range was then delineated and validated by separately preparing and analyzing 8 evenly distributed standard concentrations over a range 40% wider than the working range itself. In addition to the regression coefficient, the residuals of the calibration curve were analyzed to confirm linearity.

If the procedure is used for estimation, the sample is spotted between standard spots within a wide range and then quantified using a quadratic calibration curve. To validate this step, the regression coefficient of the quadratic curve was evaluated.

2.2.4 Trueness

In this validation study, the recovery method was chosen to determine bias. In a first phase, 11 determinations were done on separately prepared samples based on pure standard divided over the entire method working range of 0.01% to 1% and each spotted 5 times. The spiked blanks of the lowest concentrations (0.01%, 0.02% and 0.04%) were prepared by gravimetric dilution (Kern ABJ 120-4NM).

Because the MESQ-procedure should be valid for the determination of mescaline in the presence of matrix components present in fresh cactus tissue and aqueous extract, in addition to the quantification of the spiked blanks, 28 recovery determinations were subsequently performed, divided between 2 fresh matrices and 2 extract matrices.

Matrix of fresh tissue of E. lageniformis

First, a sufficient amount of matrix was prepared by homogenizing approximately 800mg of green parenchyma from the tip of a 40cm tall specimen of *E. lageniformis* with 450µl of NaOH 5M using eight 3mm steel beads, allowing this mixture to digest for two hours and homogenizing again. After removal of the beads and reduction of the foam, the mescaline concentration of this solution was determined (0.128%) and used as matrix for the recovery determination. Subsequently, 4µl of this solution were pipetted into 5 separate vessels as the base-matrix for the determination of the lowest concentrations and 150µl into 5 other vessels as a matrix for the highest concentrations. This was done to ensure that any matrix effect would become apparent. A measured amount of mescaline standard solution was then added to all vials so that the total concentrations were evenly distributed over the intended range. Finally, to each vessel 550µl of NaOH 5M and 8 steel beads of 3mm diameter were added and the samples were further processed according to the above MESQ procedure. The recovery-values at each concentration were determined and plotted on a curve.

Matrix of fresh tissue of L. williamsii

Again, a sufficient amount of matrix was first prepared by homogenizing approximately 800mg of green parenchyma from the side of a *L. williamsii* specimen of 6cm diameter together with 450µl of NaOH 5M using eight 3mm steel beads, allowing this mixture to settle for 2 hours and homogenizing again. The beads were removed and an absolute quantification was then performed on 50µl of this mixture. The concentration was measured to be 0.091%. Next, 6µl of this mixture were brought into 5 separate vessels as a matrix for the lowest concentrations and 220µl into 5 other vessels as a matrix for the highest concentrations. Then a measured amount of mescaline standard solution was added to all the vials so that the total concentrations were evenly distributed over the intended range. Finally, to each vessel 550µl of NaOH 5M and 8 steel beads of 3mm diameter were added and the samples were further processed according to the above MESQ procedure. The recovery-values at each concentration were determined and plotted on a curve.

Matrix of aqueous extracts of E. lageniformis and L. williamsii

For this purpose, aqueous extracts of *E. lageniformis* and *L.* Williamsii, obtained from experiments 2.3.2 and 2.3.3 were used. These were prepared according to the method explained above in 2.1.1. The solutions were quantified and diluted to 1mg/ml. For each extract, 4 separate vials were prepared containing 25µl each. Next, another 4 vials for each extract were prepared, now containing 138µl and finally another 4 vials were prepared containing 250µl of each extract respectively. In this way, any matrix effects were co-amplified over the entire recovery range. Mescaline standard was then added to 3 of the 4 vials of each series of 4, so that the total concentrations were evenly distributed over the intended range. For a graphic illustration of this process, see Figure 33 in Section 3.2.4. The vials were then allowed to dry using a gentle unheated airflow after which 550µl of NaOH 5M were added to each vial. Subsequently, the mescaline concentration of each vial was determined using the MESQ procedure.

2.2.5 Precision

Precision of the standardized biopsy in Lophophora and Echinopsis

To determine the precision of the biopsy, the samples from the recovery study above as well as the samples from exploratory applications 2.3.2 and 2.3.3 were weighed to the nearest 0.1mg (Kern ABJ 120-4NM). Each series was excised with a new skin punch and all samples within one series were taken with the same skin punch. Before each sampling, the punch was rinsed in ethanol 99% to wash away residual tissue and disinfect the punch. For the biopsies taken with the same skin punch and rod length, the RSD was determined. This is a measure of repeatability within each series. Subsequently, the RSD of all biopsies combined was also determined. This value can give an indication of the intermediate precision where the parameters time and sample-differences due to punch-variations vary. In *Echinopsis* specimens, the relative standard deviation (RSD) of the weight of samples taken near the top versus other parts on the cactus stem was determined.

Precision of sample processing

The repeatability of the extraction step was examined by processing 3 separate samples each of 3 standard solutions with concentrations 0.01%, 0.02%, and 0.04%. Each of these 9 samples was quantified and the inter-individual standard deviation per concentration was determined. The outcome is the combined repeatability of the extraction, spotting and scanometric steps.

Precision of scanometry

The precision of the scanometric analysis itself was studied based on the data obtained through the recovery experiments on all matrices. In the MESQ procedure, for absolute quantification, each sample is spotted 5 times. Based on the standard deviation, the standard error of these 5 analyses was calculated for each sample. Within each set of 5, the standard error is a measure of repeatability while the average of standard errors of all sets of 5 can be seen as an indicator of intermediate precision, with time being the variable.

2.2.6 Robustness

a. The effect of ambient temperature on the liquid-liquid extraction

Identical 1200µg/ml standard solutions were prepared in 3 vials as described above in the MESQ procedure. Suspended in an insulating plate as a lid, these vials were placed in water baths (thermos beakers) with temperatures of 10°C, 25°C, and 50°C, respectively. The vials were left to equilibrate for 20 minutes during which time they were vigorously shaken and replaced every 5 minutes. After the final homogenization, they were centrifuged for 30 seconds and placed back into the beakers. At that time, the temperature of the respective baths was 12°C, 25°C and 44°C. Then 7 x 5µl of each solution were spotted in sets of 3 (data pairing) and further processed according to the MESQ procedure. Afterwards, the measured spot masses and their respective 95% confidence intervals were compared.

b. The volume ratio of the polar and non-polar phases during extraction

Identical 1200µg/ml standard solutions were prepared in 3 vials as described in the MESQ procedure. To the first vial, 450µl NaOH 5M was added, to the second 500µl, and 550µl to the third vessel. Then the vials were further processed according to the MESQ-procedure. The influence of these volume differences on the obtained measurements was investigated.

c. Degradation of mescaline in strong basic solutions

In this experiment, we investigated whether mescaline degrades in the highly basic solution of NaOH 5M. For this purpose, 2 samples of standard S1 (see 2.1.3) were prepared. In one vial, 500µl of NaOH 5M were added to the standard solution after which the mixture was kept at room temperature for 36 hours. After this time, the same amount of NaOH was also added to the second vial after which both samples were further processed together according to the MESQ procedure. Both samples were alternately spotted side by side (n=2x10) after which the spots were absolutely quantified. The error margins and corresponding 95% confidence intervals of the measured concentrations were determined.

d. Letting spots air dry versus forced drying with hot air

For this purpose, 2 series of 11 spots were alternately applied on 1 plate. When spotting the first series, the MESQ procedure was followed in which the spotting was done in a powerful stream of hot air. After cooling, the other series was spotted without any air flow. Subsequently, the plate was air dried for 2 hours and developed. The respective standard errors of the measured spot masses for both series were determined.

e. Spotting in one or two times

Two series of 11 spots were alternately applied on a plate. While spotting the first series, the MESQ procedure was followed and the pipette was emptied onto the plate in one time. In the second series, each spot was spotted in two times. After the pipette was emptied half way, the spot was blown dry with a stream of warm air for 10 seconds after which the rest of the pipette was emptied.

f. Saturation of the ethanol-ammonia development chamber
Identically spotted plates were developed in an unsaturated chamber on the one hand and a chamber saturated for 30 minutes on the other. The development of the plates in both chambers was compared.

g. Use of commonly available ethanol and ammonia
The eluent for development was prepared with readily available 96% ethanol and 12% ammonia solution. In this case, 10% instead of 5% ammonia solution was added to the ethanol.

h. Change of ammonia concentration in the eluent
The percentage of added ammonia solution 25% was varied and its effect on the Rf and shape of the spots was investigated.

i. Change of acetic acid concentration in the derivatization eluent
The percentage of acetic acid 7% added to the derivatization eluent was varied from 0% to 10% and the effect on plate development was studied.

j. Change of ninhydrin concentration
The concentration of ninhydrin was doubled to 0.2% and the effect on plate development was investigated.

k. Derivatization of chromatographic plates in a convection oven
It was investigated whether the derivatization of the plates in a convection oven affected the precision of the scanometric quantification compared to drying by means of a hot air flow. For this purpose, 2 identical plates were spotted with 600ng spots and developed according to the MESQ procedure. Immediately after the second elution, one plate was further dried and derivatized with a hot air stream as described in the procedure while the other plate was first dried in an unheated stream of air (Remington AC9096, room temperature 22°C) for 10 minutes. Subsequently, this dried plate was derivatized at 100°C for 15 minutes in the middle of a convection oven (BOSCH HBN330551) and further analyzed according to the MESQ procedure.

l. Using a smartphone instead of a flatbed scanner
To evaluate the usability of a smartphone versus a flatbed scanner for scanometry, the estimation plate from exploratory application 2.3.7 was used. Immediately after derivatization, the plate was photographed perpendicularly from a distance of about 50cm with a Samsung S9+ on automatic setting. The cutout of this picture was converted without further color, contrast or brightness editing to a size that could be uploaded to JustQuantify Free, in this case 2800x246 pixels. Immediately after taking the picture, the plate was scanned as described in Section 2.1.4. Both images were quantified and the calibration curves with corresponding regression coefficients and residual graphs were compared as a measure of precision of the measurements.

m. The use of other free software for scanometric analysis
Another program for analyzing the chromatograms was tested: Image J (Fiji) [41]. For this purpose, a plate for absolute quantification with 2 samples was processed and quantified with

JustQuantify Free (noise level 1) on the one hand and with Fiji on the other, where manual baseline correction was applied.

2.3 Exploratory applications: methods

The cacti used in Sections 2.3.1, 2.3.3, and 2.3.5 were obtained from the online retailer "Peyote Paradise" [43], the specimens in Section 2.3.7 from the online retailer "ikhebeencactus" [44], and the specimens in Sections 2.3.2, 2.3.4, and 2.4.6 were sourced from a private collection.

2.3.1 Mescaline concentration gradient in E. peruviana and E. pachanoi

In an initial exploration, one biopsy was taken near the top and one near the bottom in 3 specimens of *E. pachanoi* and 2 specimens of *E. peruviana*. The bottom biopsies were taken about 10 cm above the ground and the top biopsies about 5 centimeters from the top. The exact height of the specimens was not recorded in this exploratory application but varied between 60 and 90cm. The samples were quantified using the MESQ procedure.

2.3.2 Mescaline concentration gradient in E. lageniformis and its relationship to the total mescaline content of the specimen

In 2 genetically different specimens of *E. lageniformis* of equal size (Figure 11; height El1: 90cm and height El2: 89cm, both with varying stem diameters of 4-6cm), biopsies were taken at 3 different heights: 3 biopsies distributed concentrically at 3cm from the top, 1 biopsy at about 51cm above the ground and 1 at 10cm above the ground. The biopsies were then quantified using the MESQ procedure.

Figure 11: *Detail images of both specimens of E. lageniformis. Left: specimen El1, right: specimen El2.*

In a second phase, both cacti were cut off at 8cm above ground level and each one was divided into 3 sections. Top part: +/- 15cm, middle part: +/- 40cm, bottom part: +/- 25cm. From each of these 6 sections an aqueous extract was prepared using the method described in paragraph 2.1.1. These extracts were quantified by the MESQ procedure and correlated with their fresh weight and the mescaline concentration in the previously taken biopsies.

2.3.3 Mescaline concentration gradient in L. williamsii and its relationship to the total mescaline content of the specimen

Figure 12: L. williamsii specimens Lw1-Lw4.

According to the method described in paragraph 2.1.1, biopsies were taken from 4 different specimens of *Lophophora williamsii* with a diameter of about 6.5cm (Figure 12; Lw1-Lw3 were sold as '*L. williamsii var. decipiens*', Lw4 as '*L. williamsii*'). Table 1 specifies the location of these biopsies in relation to the center. The biopsies were quantified according to the MESQ procedure.

Table 1*: Location of biopsies in relation to the center in 4 different specimens of L. williamsii.*

Specimen	Circumference (mm)	Distance to center (mm)		
		Samples 1,2,3	Samples 4,5	Samples 6,7,8
Lw1	211	13	22	37
Lw2	208	13	22	37
Lw3	208	13	27	40
Lw4	210	13,15,15	17,24	28,30,37

.

Figure 13: *The examined specimens of L. williamsii were cut, retaining a thin ring of green parenchymal tissue to allow for regrowth.*

Then the 4 specimens were cut horizontally just above ground level so that a thin ring of green parenchyma remained (Figure 13: cut peyote). The peyote buttons were then extracted

as described in paragraph 2.1.1. Finally, the mescaline concentration of these extracts was determined and correlated with the fresh weight of the respective specimens as well as with the mescaline concentration in the biopsies.

2.3.4 Mescaline content of some L. williamsii specimens

In another collection, 10 specimens of *L. williamsii* with different genetic background and geographical origin were examined. Figure 14 shows these specimens and specifies their respective origins. Biopsies were taken at the side of each cactus according to the method described in paragraph 2.1.1 and analyzed according to the MESQ procedure. Based on an exploratory biopsy that showed weak concentrations of mescaline, a spotting volume of 15µl of each sample was applied to the plate.

These specimens were raised in a substrate of expanded clay pellets (Seramis). For the last 2 years, they received only inorganic nutrients (N-P-K-Mg 7-17-35-3 + trace elements sold by 'Peyote Paradise' [43]) monthly from march to september. The nutrient solution was prepared with rainwater and had a pH of 5.5 after preparation.

Figure 14*: The 10 tested specimens of L. williamsii. Lw5: 'Caespitosa La Perdida', Lw6: 'Caespitosa (unspecified origin)', Lw7: 'Cedral', Lw8: 'Cedral', Lw9: 'Huizache', Lw10-Lw13: unspecified, Lw14: 'Huizache'. The sowing date of Lw5, Lw7-Lw10 and Lw14 is 20/03/2006.*

An additional study was done on specimen Lw12, where the substrate had been changed since 1 year (5/2020-5/2021) to 2/3 Seramis, 1/3 worm castings + 1 tablespoon of lava flour per liter of substrate. Exactly 1 year later, another biopsy was performed, processed according to the MESQ procedure and compared with the sample that had been taken 1 year before.

2.3.5 Mescaline concentration in Lophophora jourdaniana

In 2 genetically different specimens of *L. jourdaniana* (Figure 15) with a diameter of about 6cm, biopsies were taken at 38mm distance from the center.

Figure 15: *One of the examined specimens of Lophophora jourdaniana.*

2.3.6 Mescaline quantification in a seedling of E. lageniformis

In a seedling of *E. lageniformis* measuring 53mm (Figure 16), a biopsy was taken with the center at 41mm above the soil. The sample was quantified using the MESQ procedure.

Figure 16: *Tested seedling of E. lageniformis.*

2.3.7 Mescaline quantification in E. lageniformis var. monstrose 'clone B'

In 5 specimens of *E. lageniformis var. monstrose* 'clone B', a biopsy was taken at 2 cm from the furrow at the apex in not actively growing shoots (see Figure 17).
The samples were further quantified using the MESQ procedure.

Figure 17: The 5 examined specimens of E. lageniformis var. monstrose Clone B (short form) with visible biopsy sites.

3. RESULTS AND DISCUSSION

3.1 Development of the MESQ procedure.

The objective of developing this procedure was to make an analytical method for mescaline quantification accessible to researchers, breeders and end-users of mescaline-containing cacti. For this reason, the procedure has to be simple enough to be performed even without formal training or scientific background. It also has to depend upon inexpensive, freely available and safe instruments and reagents. The following paragraphs will discuss, in analytical order, how the procedure was developed.
To circumvent the need for an expensive analytical scale as well as to increase the speed of sampling, limit tissue damage and standardize the kind and amount of parenchymal tissue, a volumetric sampling method using a skin punch was chosen. In this way, histologically similar tissue of reproducible volume is extracted from the outer parenchymal layer, where the highest concentration of alkaloids is located [32]. Initially, biopsies were taken by inserting the punch to the stop and then tilting it back and forth to pull the sample away from the surrounding tissue inside the cactus. This operation causes a lot of internal damage and is not always successful, resulting in the sample being stretched or left behind, which adversely affects the reproducibility of the sampling. Therefore, the scalpel technique was chosen which, although creating an additional incision, does not cause blunt internal trauma or deformation of the sample. The precision of this technique and areas for improvement are discussed further in paragraph 3.2.5. Despite the aforementioned disadvantages, biopsy without an additional incision may be justifiable for aesthetic reasons, for example, to avoid a visible scar in the top area of a *L. williamsii* specimen. Figure 18 shows, by way of illustration, the scars that remain after the biopsy of Figure 2.

Figure 18: *Scarring from a biopsy taken 3 months before.*

After taking dozens of biopsies during the study, an infection was seen in a specimen of *L. jourdaniana* that died as a result. Since then, the insertion site and the punch were always disinfected and also a small amount of sulfur powder was blown into the biopsy wound with a straw. Afterwards, no more infections occurred.

In order to minimize errors due to multiple extraction steps, the procedure was initially performed using 500µl of ethanol 99% (+1%MEK) as the only solvent. In this pure ethanol, the cactus matrix was homogenized and centrifuged after which the supernatant was spotted onto the plate without further purification. The use of ethanol ensures that no mucilage formation of the polysaccharides can take place as it does in aqueous medium and thus provides a non-viscous, easily pipettable extract. However, because of insufficient selectivity, it was sometimes necessary to perform 2 consecutive unidirectional elutions with ammoniacal ethanol to obtain sufficient separation of the spots. This added an additional time-consuming step to the procedure. And even then, not every matrix could be sufficiently separated into its components. It also appeared that the derivatization with ninhydrin of the sample spots and standard spots did not proceed simultaneously, possibly as a result of different salt forms of mescaline present in the matrices. These factors made mescaline quantification in some alcoholic cactus matrices impossible. Nevertheless, this variation of the procedure may be useful for the rapid relative quantification of specimens within the same cactus species, provided that the mescaline spots are sufficiently separated from other matrix components. Figure 19a shows a chromatogram of an alcoholic extract of *E. lageniformis 'huarazensis x lumberjack'*. Notice here the compression of spots that occurs due to the double unidirectional elution. On this chromatogram the selectivity is excellent and so the mescaline spots can be further analyzed scanometrically. The RSD of these determinations is similar to that from the MESQ procedure and consequently small enough to be effective for relative quantification between specimens. Figure 19b shows the development of an alcoholic extract of *E. lageniformis 'huarazensis x scopulicola'*. Here we notice a second matrix component visible as a spot above and separated from the mescaline spot. However, despite the double elution, we also see a third matrix component as a faint violet zone at the top of the mescaline spot.

Both zones are not separated from each other which consequently makes selective quantification impossible.

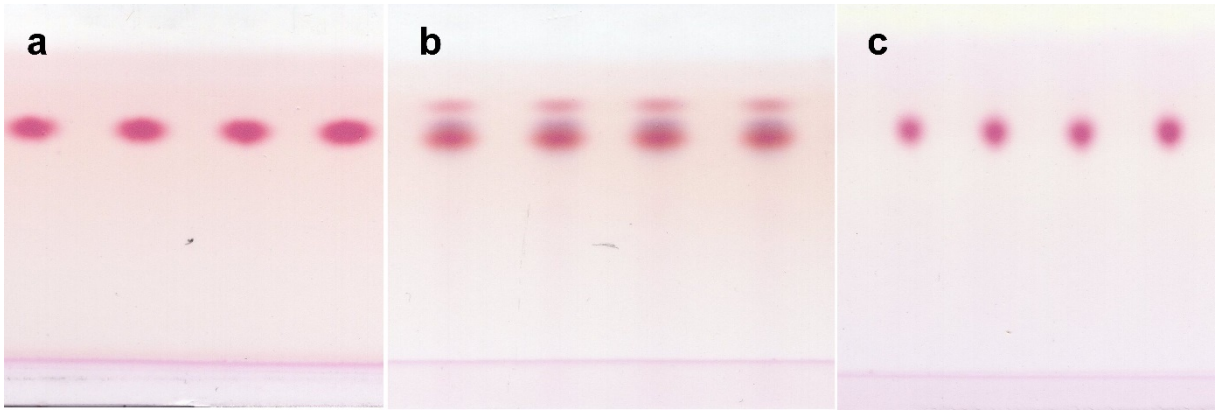


Figure 19: *Chromatograms of alcoholic sample extracts versus samples processed according to the MESQ procedure. a: chromatogram of an alcoholic extract of E. lageniformis 'Huarazensis' x Lumberjack' (double elution); b: chromatogram of an alcoholic extract of E. lageniformis 'Huarazensis x Scopulicola' (double elution); c: Same sample as in b, but now processed according to the MESQ procedure. Notice the increased specificity for the mescaline spot and the preservation of spot roundness due to the single basic elution.*

Despite the advantages of the alcohol method in terms of simplicity, speed and reagent safety, its lack of specificity with certain matrices prompted the search for a procedure that could circumvent this limitation. A single basic liquid-liquid extraction was explored because it allowed the entire extraction to take place within the same container, thus avoiding errors due to the transfer of extracts or raffinates. In addition, the introduction of a highly concentrated NaOH solution has several advantages: dissolving the cactus matrix, counteracting mucilage formation, decomposing interfering matrix components, increasing the ionic strength of the solution promoting better partitioning, counteracting emulsions after homogenization, increasing the density of the aqueous phase which favors separation during centrifugation, and importantly the conversion of the different mescaline salts to mescaline free base. The latter has the advantage of allowing uniform derivatization further down the line. A final advantage of using a highly concentrated NaOH solution is that a pH well above the pKa of mescaline (9.56) in the aqueous phase ensures an extraction-recovery in the non-polar phase approaching 100% [45-47]. And as shown in the robustness tests in paragraph 3.2.6, mescaline remains stable in the midst of this extreme pH. And it does so for a long time, which opens the possibility to let the NaOH solution act on the sample for a longer time and as a result digest more emulsifying components which greatly facilitates the centrifugation step. Since related primary amines that also react with ninhydrin are apparently less resistant to the action of concentrated NaOH solution, this also enhances the selectivity of the procedure compared to the alcoholic method. Figure 19c shows the chromatogram of the same specimen as in Figure 19b, but now processed by the MESQ procedure. Notice the now round, solitary mescaline spots after a single elution.

Both toluene and xylene were evaluated as the non-polar phase for the liquid-liquid extraction. Throughout the experiments, xylene was ultimately preferred because of its lower volatility, which limits the likelihood of falsely elevated sample concentrations due to evaporation.

The standards are processed in parallel with the samples throughout the procedure to avoid errors due to different analytical conditions. Standards are best prepared and stored as an aqueous solution of a mescaline salt. As part of the validation of this procedure, a minimal but essential amount of pure mescaline sulfate was isolated from cactus tissue (one quarter dose). For the absolute quantification of 2 samples as described in the procedure, a total of only 6.2µg of mescaline free base is required as a standard. This means that extraction of 5g of fresh green cactus parenchyma (with a mescaline concentration of about 0.1%) provides sufficient standard for the analysis of a 1000 samples. There are, however, technical and legal obstacles to isolating mescaline. In practice, these can be avoided by performing a relative quantification of the samples in reference to a standard prepared from a stable cactus extract. If absolute quantification is then required, this reference standard itself can be quantified by an external service. This is unnecessary if the reference solution is only intended for 'internal use' in the comparison of samples within the same setting but on different plates and/or at different times. And if all samples for relative quantification can be spotted on the same plate, a reference standard is not even needed. However, in the case of relative quantification, one can only conclude that one sample does or does not contain more mescaline than another. Because of the non-linearity of the response curve, no statement can be made about the ratio of these mescaline concentrations. Purely relative quantification of samples is much less labor-intensive than absolute quantification. Especially if this can be done on the basis of visual inspection. Consequently, this seems the appropriate way to identify strong specimens in large collections. The biopsies from these can then be absolutely quantified to allow comparison with specimens from other collections.

Theoretically, one could only use a single standard solution of which different volumes are spotted as brackets for absolute quantification. However, using 2 separately prepared standard solutions offers some advantages. First, it generates enough standard solution for the spotting of multiple plates, without having to use a recipient with larger volume than the sample-recipients, hereby assuring identical analytical conditions. Second, by using a spotting volume of 5mcl, one maximizes the precision of the pipettes. And third, the analysis of different standard solutions will indicate possible irregularities in their preparation, adding a safety-check to the procedure.

The thin-layer chromatographic step is technically simple, but to obtain precise results, the successive steps must be performed accurately. The best practices from current textbooks were adopted for this purpose [48, 49]. In this context, the choice of materials proved to be relevant. Of the different plastic containers tested for development, only polypropylene proved to be resistant to the ammoniacal alcohol. Also, Rf changes occurred where the elution front was delayed in spot areas where plastic had been deposited that had become dissolved due to prolonged action of xylene on the reaction vial after the extraction step (Figure 20). Therefore, it is best to test the resistance of the vessels to xylene in advance.

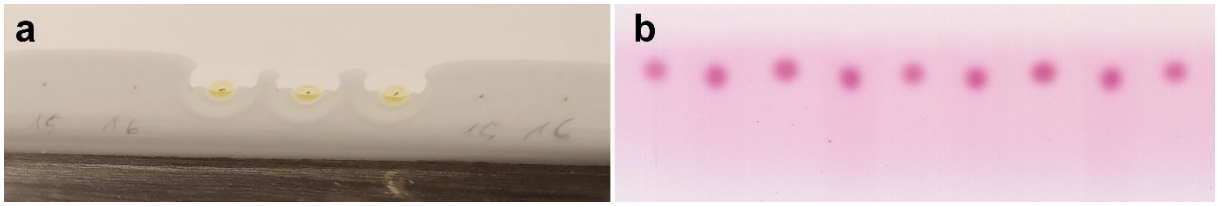


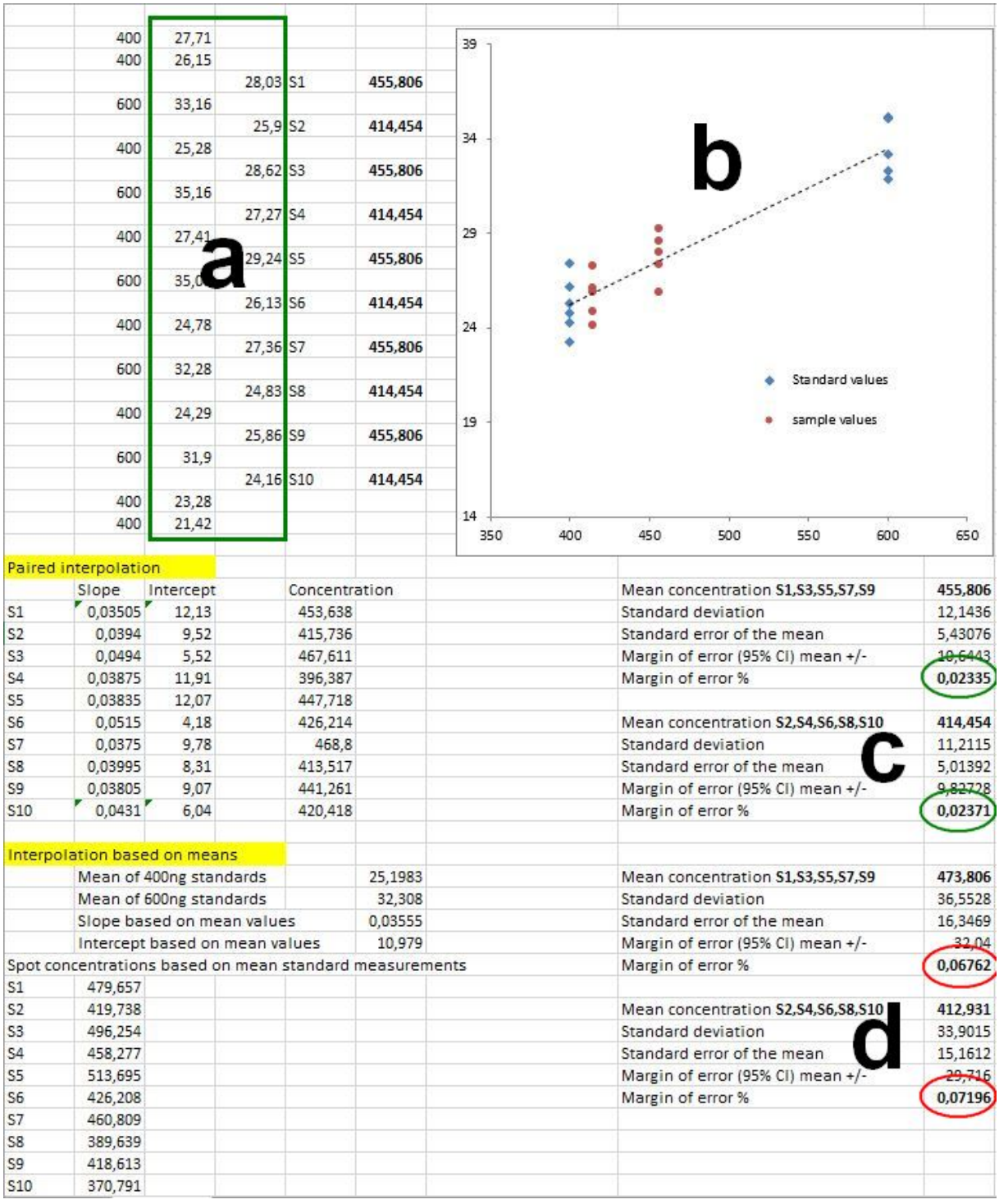
Figure 20: *Artifacts due to dissolution of the plastic reaction vessel in xylene. a: delay of the elution front during development. b: alternating Rf values for mescaline standards are visible on this other plate. Lower Rf values are associated with a longer reaction time between xylene and the vials prior to spotting (several hours).*

In the selection of a stationary phase, a UV-active plate was chosen which allowed visualization of the spots prior to derivatization. This choice was made in function of the development of the procedure. It also proved useful in verifying that each spot was applied prior to development. However, a fluorescent stationary phase is not necessary.
For the derivatization of the plates, ninhydrin was chosen as reagent because of its clear and sensitive staining capacities for primary amines and its important selectivity for mescaline within the cactus matrix [50]. Adding it to the eluent prevents spot distortion and uneven staining as can occur with spraying or dipping. Another study that incorporated ninhydrin into the eluent used a concentration of 0.4% [51]. However, in this procedure, 0.1% proved to be more than sufficient for staining. This makes that for absolute quantification of 2 samples or relative quantification of 10 samples using the MESQ-procedure, only 4mg of ninhydrin is required. In some experiments the residual eluent was recycled by adding fresh eluent to make up for the 2ml that were actually consumed by the plate. In doing so, no noticeable effect on development or derivatization was observed. If ninhydrin is the limiting factor in certain settings, recycling the eluent in this way allows for 3x10 samples or more to be relatively quantified with only 8mg.
During this research, the eluent was prepared by diluting a 1% stock solution of ninhydrin in ethanol 99% (+1%MEK) to 0.1%. The use of a stock solution avoids having to repeatedly weigh and dissolve ninhydrin. In practice, the stock solution remained stable for more than 1 month with no noticeable effect on derivatization qualities.
During elution, ninhydrin itself also interacts with the sorbent matrix of the chromatographic plate. This results in a front that is clearly visible on the developed plates. To further remove the mescaline spots from this front and thereby lower the chances of heterogeneous derivatization, the procedure involves trimming the plate by 11mm prior to derivatization. This has the added benefit of reducing the development time. This step however, is not strictly necessary.
Concurrently with the derivatization, an additional chromatographic separation by the acidic alcohol takes place, thereby further increasing the procedure's selectivity. At acetic acid concentrations below +4% and after drying of the plate, spots already derivatize at room temperature. However, the spots then exhibit both tailing and fronting. Increasing the concentration of acetic acid in the derivatization eluent causes an increase in spot focus, which greatly improves the precision of the scanometric measurements. On the other hand, adding more acid also causes an increase in Rf of the mescaline spots, bringing them closer to the ninhydrin front. It has not yet been determined if this increase is due to the added acid, water or both. After some experimentation, an addition of +10% acetic acid (7%) was selected at which the spots are well defined while still maintaining a safe distance from the ninhydrin front. At this concentration the spots no longer derivatize spontaneously at room temperature which also allows for better standardization of the process. Adding enough acetic acid also has the added benefit of converting residual free ammonia from the previous step to ammonium acetate before it can react with the ninhydrin. This keeps the eluent colorless, as most inorganic ammonium salts are much less likely to form Ruhemann's purple [52]. Nevertheless, the use of fresh eluent for each plate remains recommended.

To correct for uneven elution fronts despite good chamber saturation, during the first elution the plate is developed just until the front has reached the top edge all along the plate. This takes about 25 to 30 minutes. During the second elution, the plate is left in the chamber for an additional 5 minutes after reaching the top edge to allow the upper portion of the stationary phase to get evenly saturated with the derivatization agent.

How the plate is subsequently dried is an important point in quantitative thin-layer chromatography [53]. After some experimentation, the choice was made to dry the plate immediately with a powerful stream of hot air both after the first and second development. This has several advantages: it reduces the drying time of the plates from hours to minutes, it reduces diffusion of the spots, and it also provides a more thorough elimination of residual ammonia. This is important because the latter generates background noise during derivatization.
After the second elution, the heat from the hair dryer brings about the derivatization with ninhydrin. It was found that the hair dryer is best kept at a height of 10 cm above the mescaline front and held alternately above both halves of the plate. Although the standard deviation thus becomes small enough, there still seems to be room for optimization here. An automated laminar flow of hot air perpendicular to the entire mescaline front would certainly improve the precision, comfort and speed of the MESQ procedure.
Despite proper execution of the chromatographic steps, plate irregularities, proximity to the edge, and local differences in drying and derivatization conditions can still cause inaccuracies in quantification. To largely address these, the MESQ procedure uses a combination of linear interpolation-calibration coupled to a spotting sequence according to the 'data-pair' method [54]. In this way, each sample spot is quantified separately based on the neighbouring standard spots. From the individual values of these sample spots, the average is then taken as the final value. This technique reduces the standard error of the measurements in comparison with final sample values that are obtained by averaging the sample and standard values beforehand. Figure 21 shows the quantification of a plate which, due to uneven development, shows a relatively large spread of the respective standard and sample spots. The table shows how the standard error using the data-pair method provides a significantly lower standard error than the values obtained by prior reduction of the values to one average.

The use of a narrow calibration bracket not only ensures linearity in the calculations, but also ensures better scanometric precision because the 'noise-level' used for spot-intensity measurement can be tuned to this smaller interval. Indeed, the precision of the results generated by JustQuantify Free appear to depend on a good match between spot mass and noise-level at readout. At higher spot masses, higher noise levels were found to lead to more precise results. Therefore, a wide range of intensities measured using the same noise setting will be less precise. For estimation this is less important, but for absolute quantification a narrow calibration bracket therefore offers an advantage.
This whole scanometric analysis with inexpensive equipment and freely available software can compete with commercial systems in terms of performance [41, 55], and is therefore more than adequate for the intended applications. Interestingly, JustQuantify Free allows the export of measurements to a spreadsheet. This option has not been explored yet, but such automation could make the procedure a lot more time efficient, especially if all steps from photographing the plate to the final quantification could be integrated into a single smartphone application. See also 3.2.6.l.
To the extent that the necessary devices can be battery operated or mechanically driven (battery operated jigsaw, manual centrifuge, laptop/smartphone), the MESQ procedure can be applied during field research. For example, to map the strength of *L. williamsii* specimens in certain regions or microclimates.



	400	27,71			
	400	26,15			
			28,03	S1	455,806
	600	33,16			
			25,9	S2	414,454
	400	25,28			
			28,62	S3	455,806
	600	35,16			
			27,27	S4	414,454
	400	27,41			
			29,24	S5	455,806
	600	35,0			
			26,13	S6	414,454
	400	24,78			
			27,36	S7	455,806
	600	32,28			
			24,83	S8	414,454
	400	24,29			
			25,86	S9	455,806
	600	31,9			
			24,16	S10	414,454
	400	23,28			
	400	21,42			

a

b

Paired interpolation				
	Slope	Intercept	Concentration	
S1	0,03505	12,13	453,638	
S2	0,0394	9,52	415,736	
S3	0,0494	5,52	467,611	
S4	0,03875	11,91	396,387	
S5	0,03835	12,07	447,718	
S6	0,0515	4,18	426,214	
S7	0,0375	9,78	468,8	
S8	0,03995	8,31	413,517	
S9	0,03805	9,07	441,261	
S10	0,0431	6,04	420,418	

Mean concentration **S1,S3,S5,S7,S9**	**455,806**
Standard deviation	12,1436
Standard error of the mean	5,43076
Margin of error (95% CI) mean +/-	18,6443
Margin of error %	**0,02335**
Mean concentration **S2,S4,S6,S8,S10**	**414,454**
Standard deviation	11,2115
Standard error of the mean	5,01392
Margin of error (95% CI) mean +/-	9,82728
Margin of error %	**0,02371**

c

Interpolation based on means		
Mean of 400ng standards		25,1983
Mean of 600ng standards		32,308
Slope based on mean values		0,03555
Intercept based on mean values		10,979
Spot concentrations based on mean standard measurements		
S1	479,657	
S2	419,738	
S3	496,254	
S4	458,277	
S5	513,695	
S6	426,208	
S7	460,809	
S8	389,639	
S9	418,613	
S10	370,791	

Mean concentration **S1,S3,S5,S7,S9**	**473,806**
Standard deviation	36,5528
Standard error of the mean	16,3469
Margin of error (95% CI) mean +/-	32,04
Margin of error %	**0,06762**
Mean concentration **S2,S4,S6,S8,S10**	**412,931**
Standard deviation	33,9015
Standard error of the mean	15,1612
Margin of error (95% CI) mean +/-	29,716
Margin of error %	**0,07196**

d

Figure 21: *Spreadsheet for absolute quantification. a: standard and sample values exported from JustQuantify Free (green box); b.calibration curve with individual standard (blue) and sample values (red); c. calculated sample concentrations and error margins based on paired data (green ellipses); d. calculated sample concentrations and error margins based on averaged standard and sample values (red ellipses).*

3.2 Validation of the procedure

3.2.1 Selectivity/specificity

The MESQ procedure owes its excellent selectivity to several successive selection processes: the stability of mescaline free base in a concentrated NaOH solution, its solubility in xylene, the separation from other matrix components by elution with basic ethanol, the additional separation by elution with acidic ethanol and finally the selectivity of ninhydrin for mescaline compared to remaining matrix components [50]. These successive steps succeed in obtaining complete discrimination for the mescaline band. This has already been demonstrated above in Figure 19c. Figure 22 illustrates the selectivity of ninhydrin in a *Lophophora* matrix. In UV light, multiple spots and longitudinal zones are visible after ammoniacal development (a) that are no longer visible after derivatization (b). In some specimens of *Lophophora* and *Echinopsis*, a distinct other spot with slightly higher Rf often appears after ninhydrin derivatization. Further research can identify which matrix component (x) is involved.
Together with this high selectivity, the procedure guarantees correct identification by simultaneous spotting of pure mescaline standard.

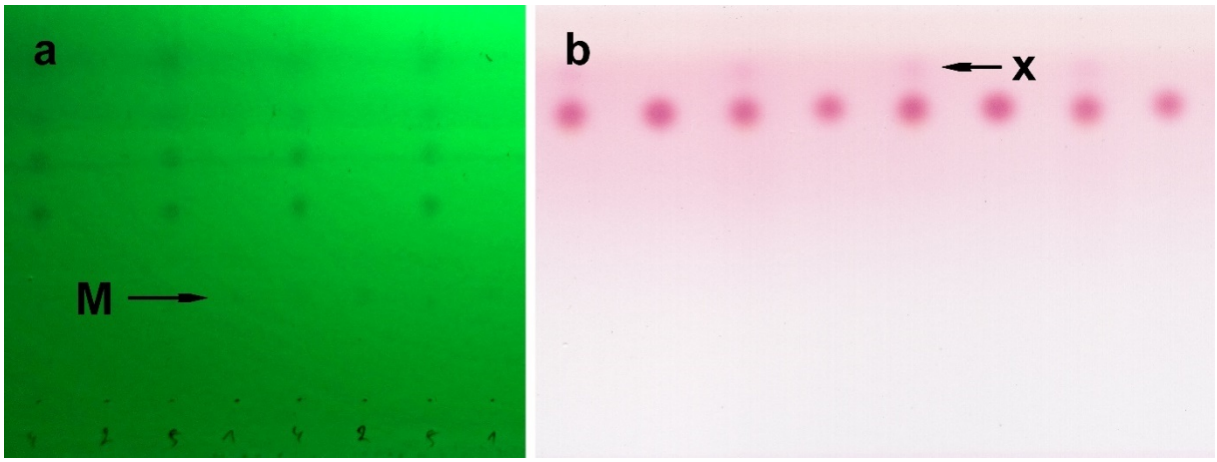


Figure 22: *Chromatogram of Lophophora williamsii before and after derivatization. Samples are alternated with standards. a: In UV light, different spots are visualized. The mescaline band is indicated (M). After derivatization, the specificity is greatly increased and sufficiently selective to allow for correct quantification. The position of matrix component 'x' is indicated. This is the most common additional spot after derivatization, both in the tested Echinopsis species and in L. williamsii.*

3.2.2 Limit of detection (LOD) and limit of quantification (LOQ)

Here we must distinguish between the *instrument* LOD and LOQ and the *method* LOD and LOQ. Indeed, the latter also depends on pre-analytical concentration or dilution of the samples.

Instrument LOD and LOQ

The instrument LOD and LOQ were determined based on a calibration curve of spiked blanks at low concentrations. The equation of the linear function was calculated to be y=0.0366x + 2.1108 (R^2=0.9945) with a standard deviation at the intercept of 0.3089. This resulted in:
LOD=3,3SD/m= 27,9ng/spot
LOQ=10SD/m= 84,4ng/spot
The calculated value for the instrument LOD was then experimentally confirmed by spotting 3 series of 11 spots of 32ng, 64ng and 96ng respectively and observing whether JustQuantify

Free was able to still distinguish the spots from the background noise. The RSD of these spot measurements was also noted. Just quantify was found to be able to distinguish all spots of 32ng from the background noise only at noise level 2. At other noise levels, 1 or more spots were no longer detected. This instrument LOD also agrees well with the LOD by visual inspection (Figure 23).

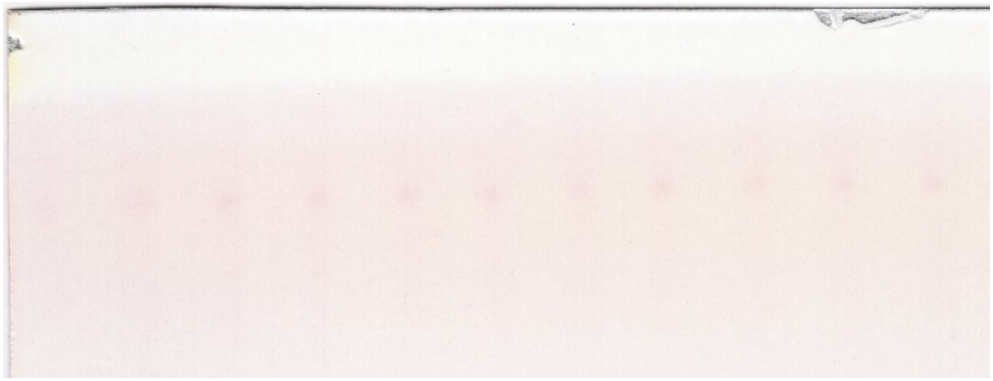


Figure 23: *Limit of detection (LOD). At 32ng mescaline free base per spot, the visual and instrumental LOD is reached.*

The RSD of the different spot values was also calculated and, based on a fitting polynomial, it could be deduced that the RSD becomes greater than 10% at spot masses lower than 67ng (Figure 24). This value was therefore retained as the experimentally determined LOQ.

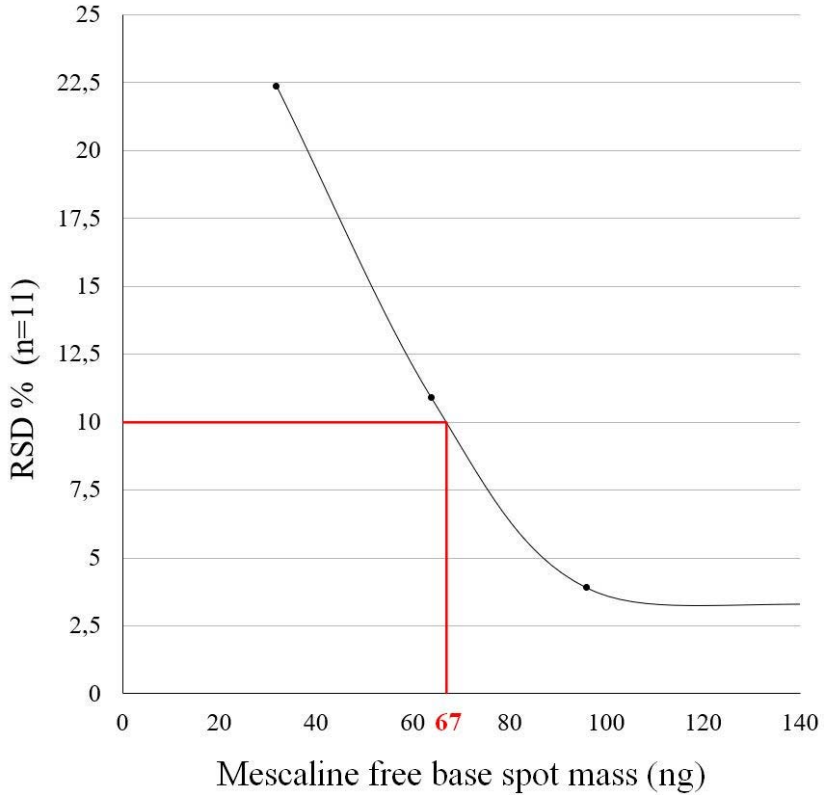


Figure 24: *The relationship of the RSD of spot values versus spot mass. At a cutoff point of 10% RSD, the experimentally determined LOQ is 67ng mescaline free base per spot.*

It is notable that the MESQ procedure possesses a much lower LOD than the one mentioned in the 1989 United Nations Test Manual (30ng vs 1µg/spot) [56], where the spots are derivatized by spraying with ninhydrin 10% in ethanol and heating at 120°C for 15 min. This could possibly be due to the higher background noise that occurs with such concentrations of ninhydrin and derivatizing in an oven rather than with a stream of hot air (see also results 3.2.6.d and g).

Method LOD and LOQ

These are determined by the pre-analytical processing of the sample and the spot volume on the plate. Assuming the standard procedure is followed as described in paragraph 2.1.1 and limiting the spot volume to 10x5µl for practical reasons, for an instrument LOD of 30ng we obtain a *method LOD* of 30ng/50µl xylene x 500µl xylene/50mg sample= 6ng/mg=0.0006%= *6ppm*. In the same way, the *method LOQ* can be determined to be: 400ng/50µl xylene x 500µl xylene/50mg sample = 80ng/mg = *80ppm*. This applies when the method LOQ is used for absolute quantification within the validated bracket of 400ng and 600ng. If the requirement is only that the spots must be quantifiable so that the relative strengths of samples can be determined, then it is sufficient that we stay above a spot mass of 80ng. In this case, the *method LOQ for relative quantification* = 80ng/50µl xylene x 500µl xylene/50mg sample = 16ng/mg = *16ppm*.

To lower the method LOQ for absolute quantification, a calibration bracket with lower values could be chosen, e.g. 100ng-200ng. As this is not necessary for the intended applications (for seedling selection, relative quantification is sufficient), this alternative was not validated in the current research.

During the development of the method, the sample concentration was occasionally increased up to 3x by introducing 3 biopsies or 150µL of standard solution into the vessel. In this case, 400µL of 6.25M NaOH solution were added to the vial after which it was further processed according to the standard MESQ procedure. In doing so, no peculiarities occurred. This concentration step was not validated, but is mentioned here in the context of a possible need to further lower the method LOD and LOQ.

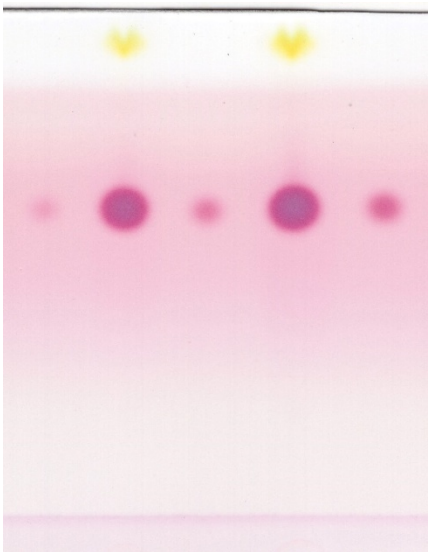


Figure 25*: Even 3-fold concentrated samples of E. lageniformis with large spot volumes of 32µl (left) and 50µl (right) maintain adequate spot focus, resolution and selectivity.*

A final way to lower the method LOD and LOQ is to further increase the spot volume. And while 10-fold spotting does not cause problems with spot focus or resolution (Figure 25), nor does it compromise precision (as demonstrated further below for the lowest concentrations in 3.2.4), spotting in this way becomes very labor intensive. For reference, spotting a plate of 2 samples for absolute quantification at the lower limits takes about 45 minutes. Compare this to 10-15 minutes for the spotting of a plate where the spots can be applied in one time, which is usually the case.

3.2.3 Working range

The various steps within the MESQ procedure are aligned so that the procedure is equally useful for the determination of very low tissue concentrations in seedlings (down to 0.01%) as well as for high concentrations in the quantification of high potency specimens (up to 1% in fresh tissue) [16, 30, 31, 57] and concentrated aqueous extracts. Quantification is therefore possible over the course of 2 orders of magnitude.

When an exploratory mass response curve was plotted, it became clear that it was second order and flattened out so significantly above the 2µg/spot that the sensitivity became too low for precise quantification (Figure 26).

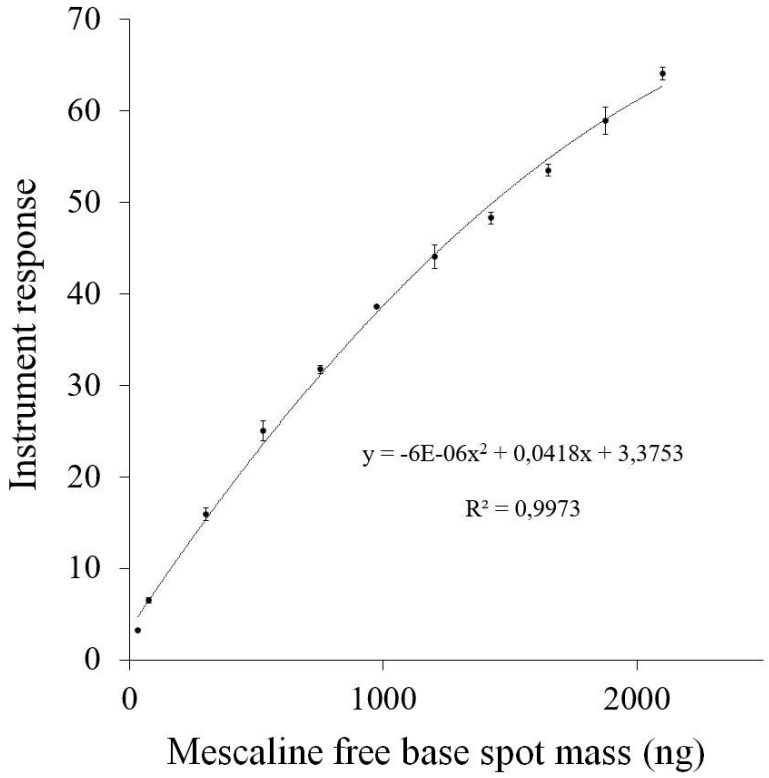


Figure 26: *Range of interest with instrument response plotted against spot mass.*

The response curve becomes more linear as the concentrations become lower. The direction of the curve and thus the analytical sensitivity also increases in this regard. However, the lower the spot quantities become, the more sensitive the analysis becomes to background noise, especially if the remaining ammonia is not sufficiently driven out of the plate. Taking these factors into account and in order to guarantee linearity as closely as possible, a relatively small instrument working range between 400ng and 600ng was chosen. This is therefore a ‘linear interpolation calibration’ or ‘bracket’ method that assumes that the precision of an

adjusted volumetric spotting combined with a small quasi-linear calibration interval is better than that of a constant spot volume coupled to a wide quadratic calibration range.
Linearity within this calibration bracket was confirmed by a regression coefficient of >0.99 (Figure 27) and a sufficiently random distribution of residuals (Figure 28).

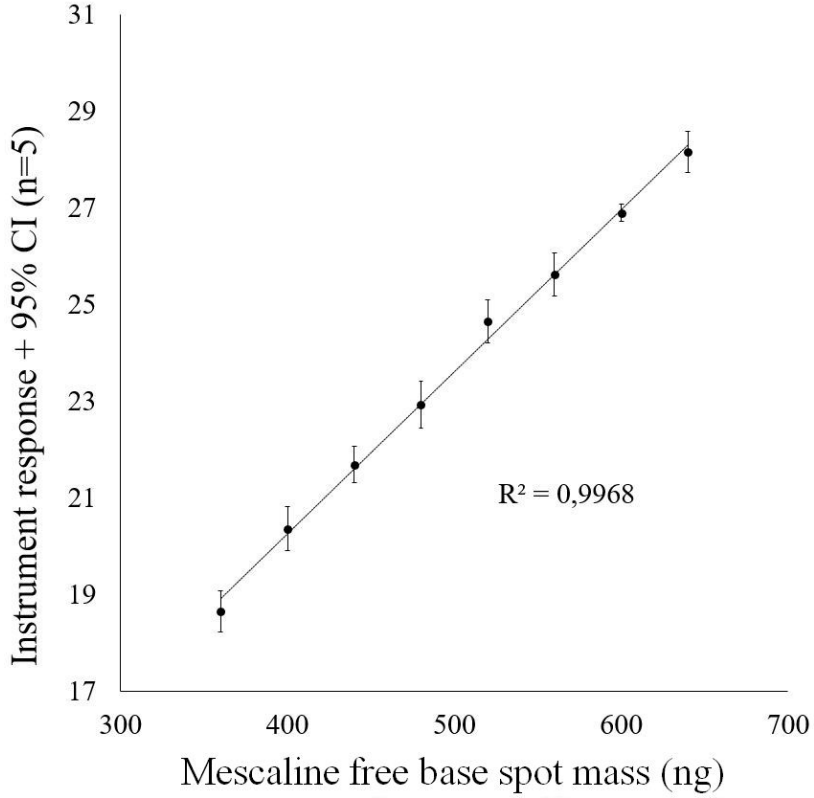


Figure 27: *Linear working range*

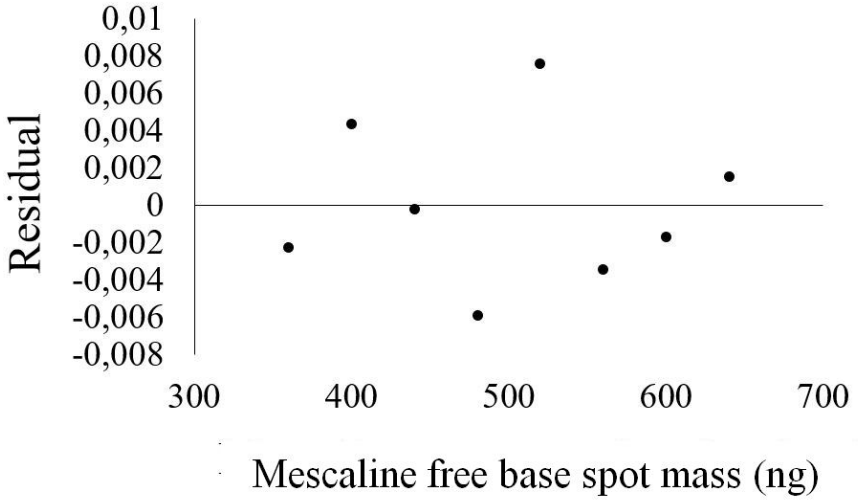


Figure 28: *Residual plot of the calibration curve within the working range. The absence of any trend confirms linearity.*

The definition of this bracket together with specifically adapted pre-analytical steps allows the MESQ procedure to handle the most common sample concentrations as easily and precisely as possible. For example, a full entheogenic dose of 500mg of mescaline free base, dissolved in 100ml to 1000ml aqueous solution, can be dosed without pre-analytical processing within the instrument working range using a spot volume of 1-10mcl. Similarly, for the analysis of fresh tissue, the chosen range is the most common. As an example, 12 of the 14 parenchymal tissue samples in the study by Ogunbodede et al [30] could have been analyzed with the MESQ procedure without sample concentration adjustments.
Usually, prior to absolute quantification, the mescaline concentration will have to be estimated in order to determine a suitable spot volume. Because wider margins of error are acceptable for this estimation, a constant spot volume (5µl) within a wide quadratic calibration range of 80-960ng is chosen here. Figure 29 shows the typical response curve of such an estimation plate. The polynomial based on 10 standard spots had a regression coefficient of >0.99 which justifies the applicability of this calibration curve.

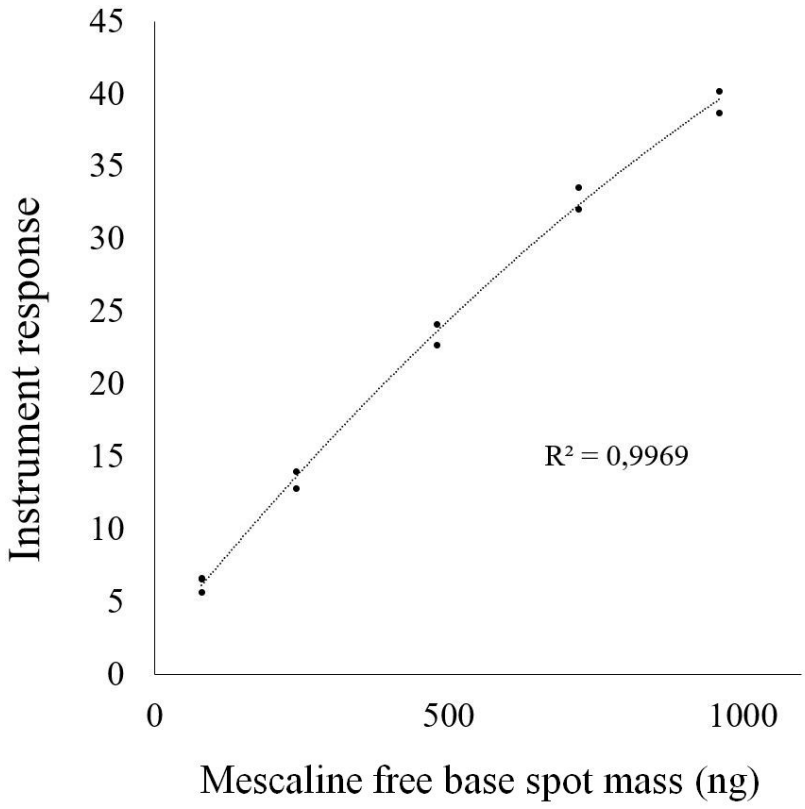


Figure 29: *Typical quadratic calibration curve within the 'estimation range'.*

3.2.4 Trueness

For the procedure to be fit for the intended applications, it must be valid within the various matrices over the entire working range.
First of all, a recovery study was made on 17 spiked blanks over the entire method working range of 0.01-1%. Figure 30 shows the recovery curve with direction 1.0118 and an intercept at 0.0005. The latter indicates the absence of a constant systematic error.

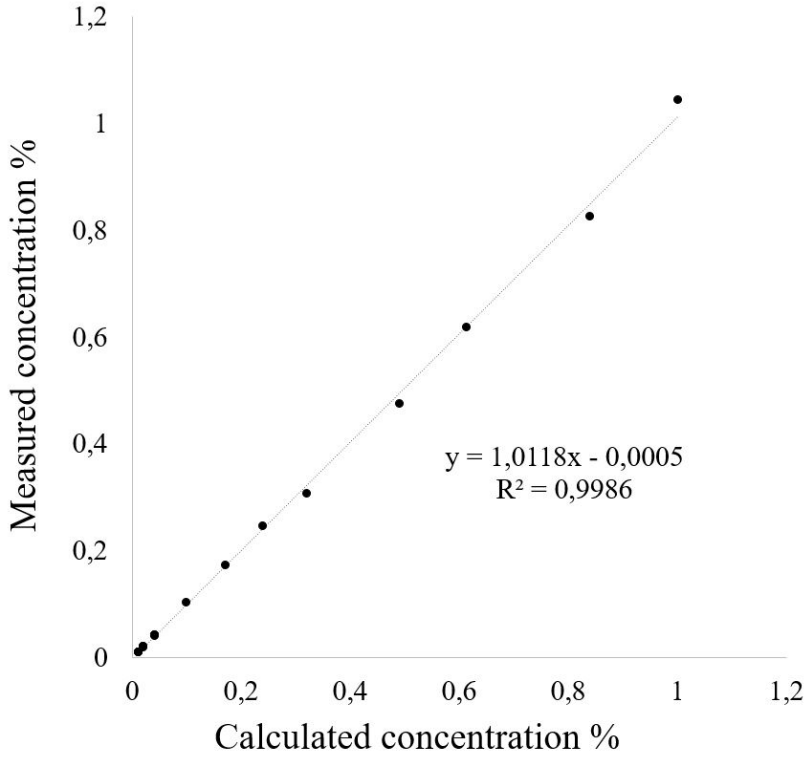


Figure 30: *Recovery function of spiked blanks in the method working range (0.01-1%)*

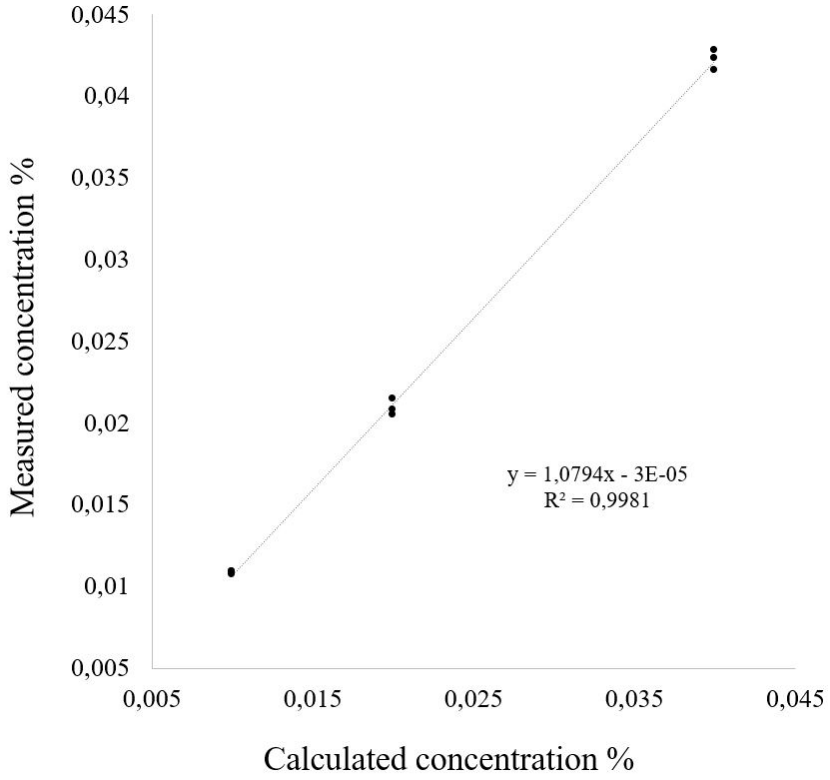


Figure 31: *Recovery function of 3x3 spiked blanks at low concentrations*

The average of all recoveries was 103.6% with an RSD of 3.9%. An examination of the individual recoveries here showed consistently high recoveries at the lowest concentrations. For the 3 lowest values, the direction of the curve is 1.0794 (Figure 31). Their average recovery is 106.6% with an RSD of 2.0%. For all higher concentrations (>0.05%) together, the average recovery is 100.3% with RSD 3.2%.

The cause of this systematic bias in the lowest concentrations may be due to the multiple pipetting at these larger spot volumes. Future research will show whether this phenomenon is reproducible and what the exact cause is. These increased recoveries are of little importance to the common application of the MESQ procedure since in this concentration range primarily relative quantification of seedlings will occur. The quantification of extracts will always be done at concentrations higher than 0.05% (500mg mescaline base/liter), in which range there is no more significant bias in spiked blanks. Next, the recovery experiments on the 2 fresh and 2 extraction matrices were analyzed. Figures 32 and 33 show the respective recovery functions of these measurements.

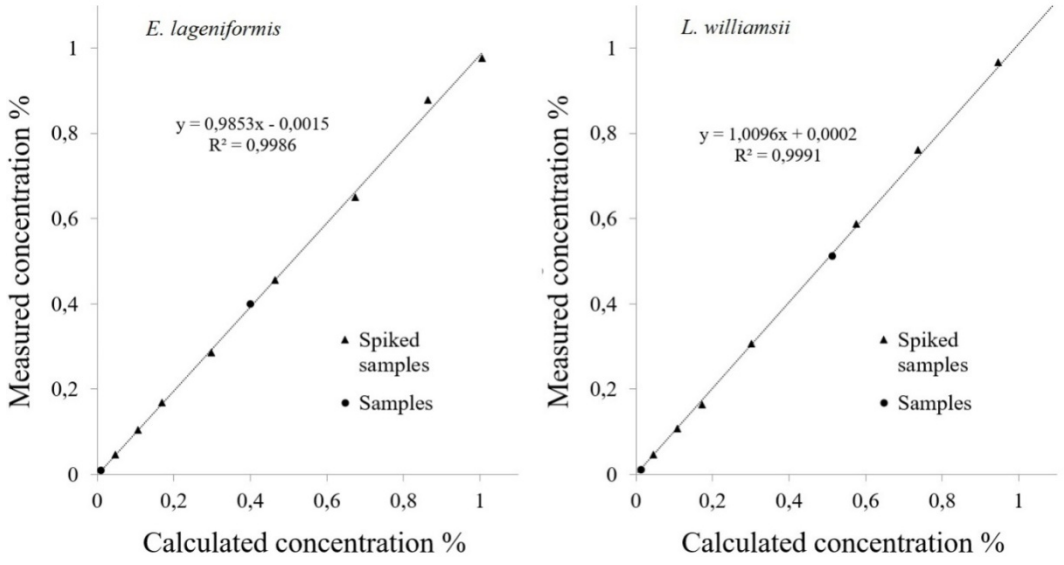


Figure 32: *Recovery functions in fresh matrices of E. lageniformis(left) and L. williamsii (right).*

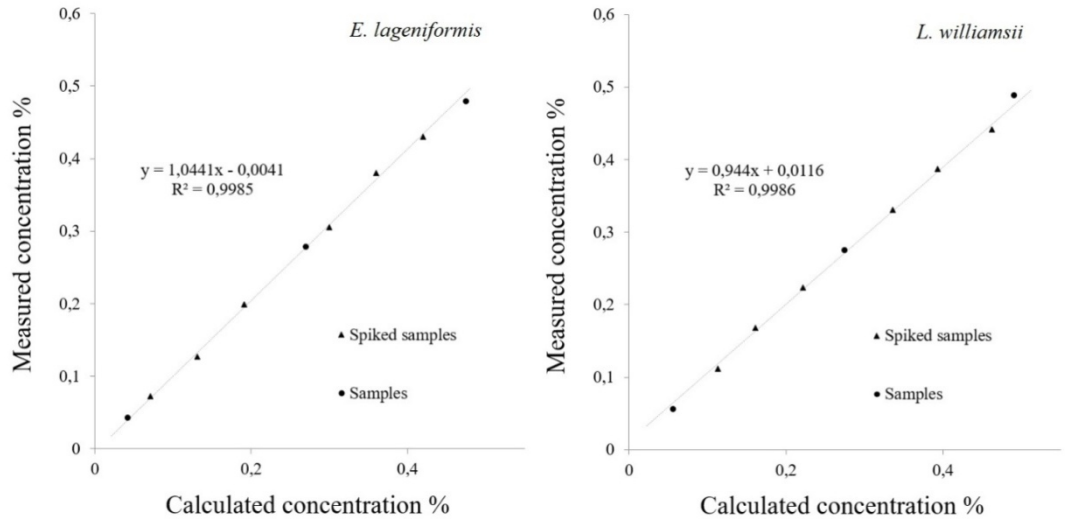


Figure 33: *Recovery functions in aqueous extracts of E. lageniformis (left) and L. williamsii (right).*

Table 2 shows the average recoveries in the 4 respective matrices. The lower limit for these determinations is set at 0.05%. This is because these lowest recovery determinations are the sum of the mescaline already present in the sample matrix and the added standard. This did not allow for the examination of the increased recoveries below 0.05% as observed for the spiked blanks. In any case, the table shows that the recoveries do not provide arguments for a systematic bias above 0.05%. The individual recoveries as well as their RSD are well within the ranges suggested by the literature [40].

Table 2: *Mean recoveries and their relative standard deviation by matrix.*

		Mean recovery	[RSD]
Fresh *E. lageniformis* matrix	0,05-1%	98,2%	[1,7%]
Fresh *L. williamsii* matrix	0,05-1%	99,5%	[3,2%]
E. lageniformis aqueous extract	0,05-0,5%	102,1%	[2,6%]
L. williamsii aqueous extract	0,05-0,5%	99,7%	[2,6%]

A problem with the recovery method may be that the added standard is less bound to the matrix than the mescaline already present in the tissue or extract. However, the similar results of the recoveries in the different spiked matrices and blanks shows that there seems to be no relevant matrix mismatch [58]. This can be expected given the mechanical and chemical matrix destruction and the subsequent conversion of the different mescaline salts to free base. For this reason, it can also be assumed that the procedure will show to be valid for similar determinations in (extracts of) *E. pachanoi* and *E. peruviana*. Indeed, when the procedure was applied to these matrices (see Section 3.3.1), no irregularities appeared either in the processing of the samples nor during the chromatographic development and quantification.

3.2.5 Precision

The precision of each of the individual steps within the MESQ procedure is discussed below. The main focus will be on the *repeatability* of the method, whether or not extended to the *intermediate precision* if several measurement series are pooled that were analyzed at different times. *Reproducibility* could not yet be studied due to the fact that the procedure was developed and executed by a single person in one research setting. It is up to future research to quantify this third precision parameter as well.

Precision of the standardized biopsy in Lophophora

The precision of the biopsies is determined in part by the accuracy with which the threaded rod can be adjusted to retain a sample of exactly 4mm length. This setting is itself dependent upon the accuracy of the micrometer. In this case it was 0.1mm (=2.5% of 4mm). This error is not important if samples are taken with the same punch and rod and if they will only be quantified relative to each other. However, when comparing samples that were not taken with the same punch and rod setting, this error must be included in the calculation.

An analysis of the 41 samples divided between 7 series gave an average weight of 46.2mg with an RSD of 5.8%. However, if we determine the RSD per series and take the average of these RSDs, it is only 2.5%. This confirms that it is best to take samples that are to be compared with each other using the same skin punch and rod setting. Investigating a way to set the biopsy length more accurately could further increase the precision of this step of the

MESQ procedure and thus better standardize the comparison of results between different researchers and/or at different times.

Precision of the standardized biopsy in Echinopsis

During the biopsies in *Echinopsis* specimens it was noticed that, unlike with *Lophophora*, there is a strong outer layer of skin that needs to be pierced. Also, the density and structure of the parenchyma is different according to the height at which the biopsy is taken. These factors have a significant impact on the precision of the sampling. This finding will be discussed further in section 3.3.2, but it can already be noted here that taking biopsies near the top of the specimen is more accurate than at lower parts of the stem. The analysis of the sample weights of 4 biopsy series with a total of 36 samples of which 9 were taken at the top showed an average RSD per series of 5.4% for all biopsies not taken at the top compared to an RSD of only 2.2% for the top biopsies.

Precision of sample preparation and microscale extraction

The next steps that can introduce inaccuracy into the MESQ procedure are the preparation of the standard solutions and the microscale extraction. It is difficult to assess these separately, and for this purpose the cumulative precision of these steps was calculated together with that of the subsequent scanometric quantification. For this particular precision study, the lowest concentrations within the working range were chosen because that is where the largest deviations are to be expected. Specifically, these are the analyses presented in Figure 31. These 9 determinations of 3 separately prepared spiked blanks per concentration had an average standard error of their respective 5 spot measurements of 3.9%. Thus, as can also be seen on the figure, the sample preparation and extraction steps within the MESQ procedure have low error margins, even at these low concentrations. The procedure was validated using a microliter syringe with an accuracy of only 1µl (=2% of 50µl). The precision of this step can easily be improved by the use of more accurate volumetric instruments.

Precision of the scanometric quantitative thin-layer chromatography

To quantify the precision of the spot measurements, the absolute mescaline quantifications of 38 samples were analyzed. Because in the MESQ procedure each sample is spotted 5 times and because the degree to which the measured spot concentrations deviate from the true mean value is more important than the relative deviation of the individual determinations, the standard error is preferably used as a measure of precision, rather than the standard deviation. Following this principle, the margin of error was calculated by means of the equation: margin of error = standard error x critical value. A confidence level of 95% is used for all measurements in this article. The margin of error of the scanometric determinations of all 38 samples was determined in this way to be 3.31%.

If one combines the error margins of all successive steps within the MESQ procedure including biopsies taken with the same punch and rod setting, one still stays below 6% in total. For the quantification of extracts, the total margin of error is less than 5% and depends largely upon the precision of the volumetric instruments.

What was striking about the analysis of the values was the large standard deviation of the individual error margins. This amounted to as much as 37%. This indicates that there are still uncontrolled variables present in this step. It is up to future research to identify these and thus further optimize the method. Nevertheless, the precision of the MESQ procedure as outlined above, is already sufficient for its intended applications.

3.2.6 Robustness

A key requirement of the MESQ procedure is that it has to be applicable in low-tech settings with varying environmental conditions. To this end, the procedure must be sufficiently robust. The impact of various changes in the method and environmental conditions was investigated.

a. The effect of ambient temperature on the liquid-liquid extraction

In liquid-liquid extraction, the partition coefficient is temperature dependent, and given the future use of the MESQ procedure in mostly warm conditions, it was important to investigate this factor. The measured concentrations and corresponding 95% CI of the solutions at 12°C, 25°C and 44°C were 623 +/- 19ng, 606 +/-9ng and 615ng +/- 14ng, respectively. Thus, there appeared to be no relevant difference between the measured concentrations of the solutions. This indicates that the ambient temperature during extraction does not seem to play a relevant role.

b. The volume ratio of the polar and non-polar phases during extraction

The measured concentrations and 95% CI of the samples with 450µl, 500µl and 550µl added NaOH 5M solution were 579 +/-17ng, 591 +/-7ng and 582 +/-13ng respectively. This shows that within this range of polar phase volume and possibly even beyond, there is no significant influence on the final result.

c. Degradation of mescaline in strong basic solutions

The stability of mescaline in NaOH 5M was confirmed as the absolute values of both determinations were not significantly different (412 +/- 15ng and 399 +/-11ng, 95% CI). This shows that the mescaline molecule is very stable in strong basic solutions and the time taken for chemical decomposition of the cactus matrix can be extended to at least 36h if necessary.

d. Letting spots air dry versus forced drying with hot air

Forced drying during spotting proved to have 2 major advantages over letting the spots air dry. Forced drying allows for a much faster finishing of the plate. In addition, this method was also found to reduce the margin of error significantly. The margin of error (n=11) for air drying was 2.36% while that of forced drying was only 1.04%.

e. Spotting in one or two times

Spotting in two times with the intention of making the spot less wide did not result in improved scanometric precision but, on the contrary, resulted in a larger margin of error (1.15%; n=11) than if the pipette was emptied in one time (0.74%).

f. Saturation of the ethanol-ammonia development chamber

Saturating the development chamber proved to have advantages. The development time shortens from 42 minutes to 27 minutes which significantly accelerates the workflow. Saturation also ensures that the elution front rises evenly across the full width of the plate. With unsaturated chambers, convex rising occurs, causing uneven development and derivatization conditions.

The observed Rf of mescaline in the saturated chamber was 0.34 versus 0.27 in the unsaturated chamber. No influence was observed on the size and shape of the spots.

g. Use of commonly available ethanol and ammonia

Using ethanol (96%) + 10% ammonia (12%) increased the development time from 27 to 37 minutes and the Rf from 0.34 to 0.43. Otherwise, no influence on derivatization and quantification was observed.

h. Change of ammonia concentration in the eluent

Figure 34 shows how changing the amount of ammonia solution (25%) added to the eluent gives rise to significant changes in the migration distance of the spots. At full chamber saturation, the Rf for mescaline increases from 0.17 to 0.54 when adding +0.5% to +10% ammonia, respectively. Adding an equal amount of less concentrated ammonia had no different effect on the Rf. Thus, it appears to be primarily the influence of the added water that increases the Rf. This assumption is confirmed by the results of experiment g.

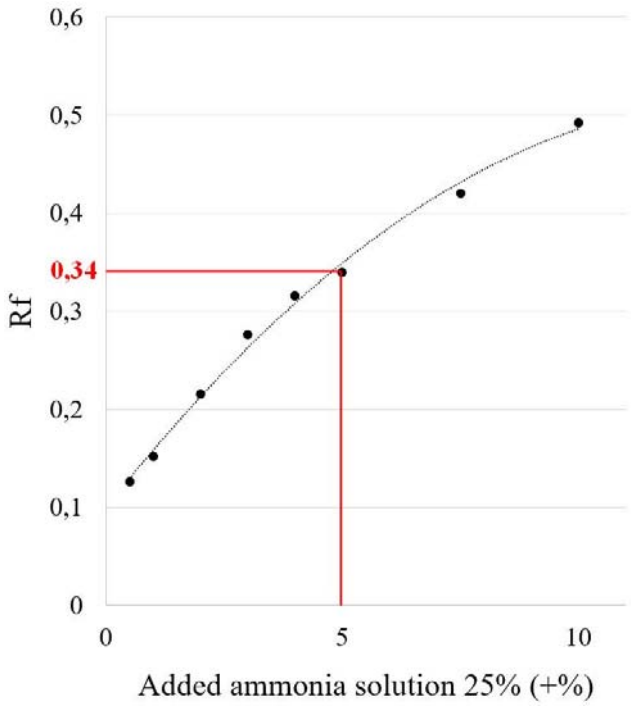


Figure 34: *Change in mescaline Rf at various ammonia concentrations.*

It was also observed that the mescaline spots became more focused as the concentration of added ammonia solution increased. Above +3%, no more fronting or tailing of the spots was observed.

i. Change of acetic acid concentration in the derivatization eluent

With increasing amounts of added acetic acid, the focus of the spots increased. Up to +5% there was interfering fronting. The Rf of the spots also increased, causing them to move further towards the ninhydrin front. This is not desirable because the derivatization of the spots in function of optimal precision should take place in a homogeneously concentrated stationary phase.

As also discussed in paragraph 3.1, increasing the concentration of acetic acid affects the heat sensitivity of the spots when derivatized with ninhydrin. At concentrations lower than 4%, the plates already develop at room temperature but at the expense of precision in scanometry.

Added amounts greater than 10% were not investigated. In exploratory experiments it was observed that this made derivatization of the plates too difficult.

j. Change of ninhydrin concentration

It was observed that a 0.1% ninhydrin concentration creates equally distinct spots with less background noise than a 0.2% concentration.

k. Derivatization of chromatographic plates in a convection oven

The margin of error for the plate developed in the oven was found to be much larger than that of the plate developed in a hot air stream (4.53% versus 0.92%, n=11 spots). This could be explained by the significantly higher background noise associated with oven drying (Figure 35).

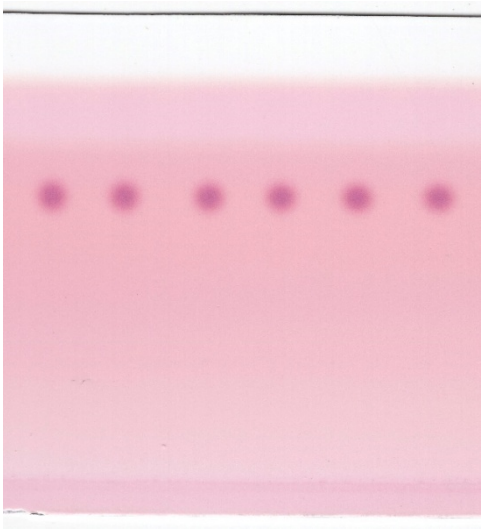


Figure 35: *Oven derivatization leads to substantially more background noise compared to drying in a hot air stream. For example, compare with Figure 36 below.*

l. Using a smartphone instead of a flatbed scanner

The comparison between the precision of scanometry with a smartphone versus a flatbed scanner yielded a surprising result. The cropped photograph made with the smartphone seems to suffer from more background noise (Figure 36) than the scanned image below, but on the calibration curves and residuals in figure 37, however, we see that the photograph achieves better precision than the scan.

Figure 36*: Smartphone photograph versus flatbed scan of the same plate (below).*

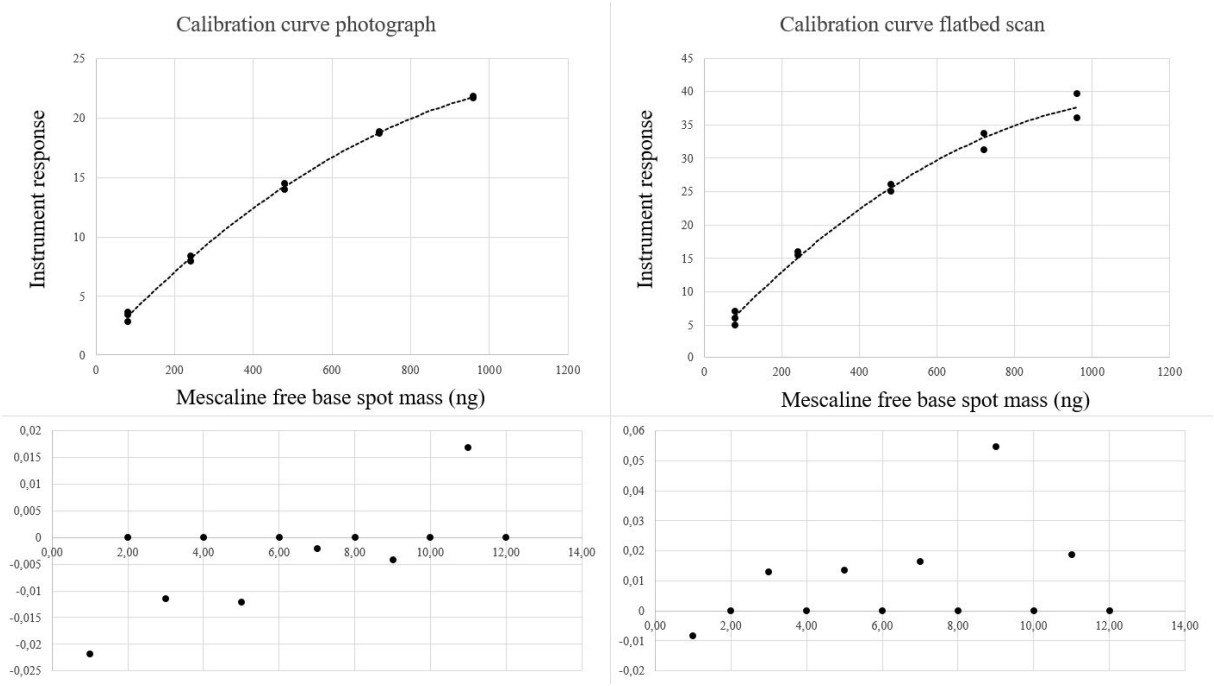


Figure 37: *Calibration curves based on the cutouts of figure 36. The left graph, based on the photograph, has a higher correlation coefficient (0,9989) than the graph based on the scan (0,9912), suggesting better measurement precision.*

This experiment shows that further research into the optimal scanometric method is certainly warranted. This was not investigated, but possibly the orientation of the plate on the scanner is important for uniform exposure. Standardization of photographic methods with a smartphone seems a promising avenue that could make the method even more accessible. Especially if this function could be directly coupled to quantification in a dedicated smartphone application.

m. The use of other free software for scanometric analysis

Figure 38 shows the processing of a scanned plate in ImageJ. The results are compared with those of JustQuantify Free in table 3. The measured values are very close, although the error margin of the measurements with Fiji is more than twice as large as with JustQuantify Free. Nevertheless, other freely available software appears capable of performing useful scanometric analyses_QuanTLC [42] also seems to be usable and, being open-source software, could be integrated into a complete application from scanning to quantification.

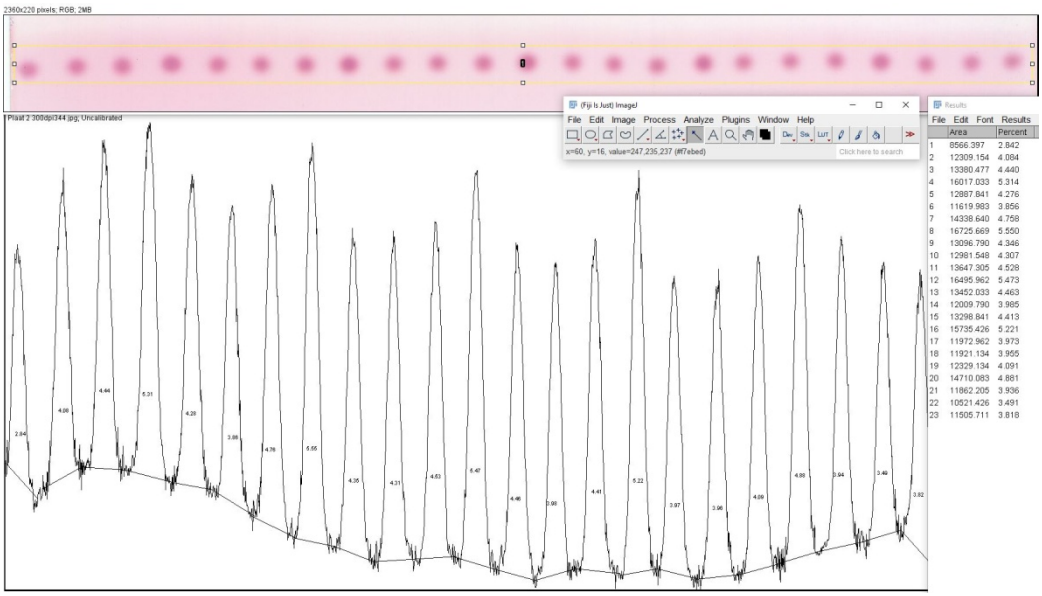


Figure 38: *Chromatogram analysis by Fiji.*

Table 3: *Comparison of spot masses and margins of error quantified by JustQuantify Free and Fiji.*

	Sample 1		Sample 2	
	Spot mass (ng)	Margin of error (%, n=5)	Spot mass (ng)	Margin of error (%, n=5)
Justquantify Free	456	2,3	414	2,4
Fiji (ImageJ)	460	5,8	439	6,3

3.3 Results and discussion of the exploratory applications

3.3.1 Mescaline concentration gradient in E. peruviana and E. pachanoi

Figure 39 shows the mescaline concentration in paired top and bottom biopsies in *E. peruviana* (blue) and *E. pachanoi* (green) specimens

Biopsies taken near the top contained significantly more mescaline. To date, only a decreasing concentration gradient from outside to inside was reported, but surprisingly no other mescaline gradients had been investigated so far. The discovery of this other gradient by application of the MESQ-procedure immediately raised some questions that were subsequently investigated in the following experiments.

3.3.2 Mescaline concentration gradient in E. lageniformis and its relationship to the total mescaline content of the specimen

In Figure 40, the mescaline concentrations of the biopsies in both specimens of *E. lageniformis* are plotted as a function of their height (dots). The mescaline concentrations of the respective segments (bottom, middle, top) of both specimens El1 and El2 are also plotted (columns where the width represents the length of the segment). At the right, the respective average mescaline concentrations of the complete specimens are shown. Notice the similar mescaline concentration ratio when comparing the top biopsies of both specimens.

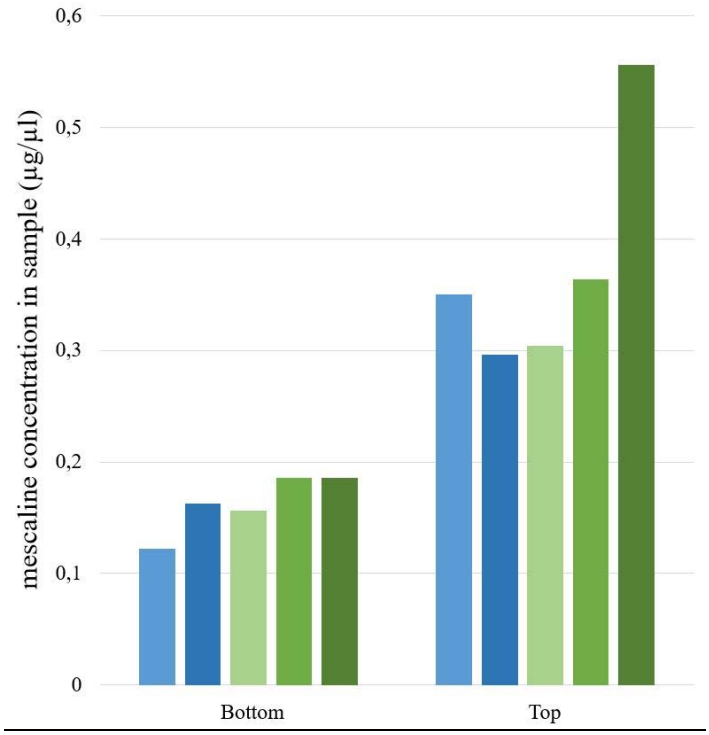


Figure 39: *Top versus bottom mescaline concentrations in paired samples of 2 specimens of E. peruviana and 3 specimens of E. pachanoi.*

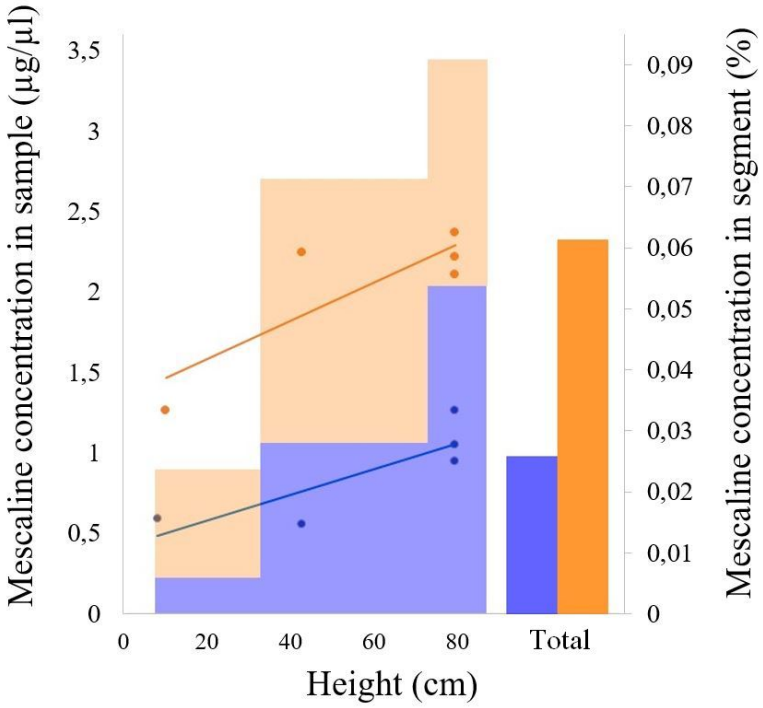


Figure 40: *Concentrations of mescaline in the biopsies (dots) and segment extracts (columns) of El1 (orange) and El2 (blue) plotted against height. The ratio of total mescaline content of both specimens (right) appears to be similar to that of the biopsy concentrations at their respective tops.*

This experiment confirmed the vertical mescaline concentration gradient in *E. lageniformis* and also demonstrated the correlation between the mescaline concentration in the top biopsies and the total mescaline content of the specimens. The difference in local concentration between higher and lower segments in the analyzed specimens was very high (3-10x).
This discovery has important implications on several levels. First of all, in terms of consumption: if a cactus is divided for distribution among users, this should preferably be done lengthwise. Only then will the strength of the pieces be comparable. Alternatively, the entire cactus could be processed after which the extract is distributed. Second, this finding has an impact on the use of the MESQ procedure in the context of breeding. Specifically, in order to compare specimens, one will have to take the biopsies in a similar location. During the experiments it became clear that this is best done at the top. It is here that the skin is not yet so thick and the parenchymal tissue is still homogeneous and undamaged. As already described in paragraph 3.2.5, the precision of sampling near the top is also consistently higher. In addition, the mescaline concentration is highest at the top, which facilitates comparison of specimens as early as possible in their growth. Future research should perform a detailed analysis of the concentration gradient near and at the top in order to determine the most appropriate position for standardized biopsy. Anyway, in order not to disrupt growth, biopsies should be taken at a safe distance from the growing tip.
A third and important consequence of this discovery is that previous research on mescaline concentration in *Echinopsis* specimens must be revisited in light of this newly discovered gradient. For example, the widely cited study by Ogunbodede et al. [30] does not specify where the samples were taken from the specimens. As this exploratory study with the MESQ procedure has now made clear, the results obtained in their article cannot possibly be compared since the concentrations can differ by an order of magnitude depending solely on the place where the sample was taken. Even in the recent study by Cameron et al [19], the height at which the 10x2cm segments were taken is not specified. Again, the results obtained must, with hindsight, be approached with great caution.
Further research into the characteristics of this gradient and how it evolves through the life of specimens will preferably be done at dedicated propagation facilities because they own plant (sub) populations for which the growing conditions are the same and any differences can therefore more easily be attributed to single factors or genetics. Because of its applicability in low-tech settings, the MESQ-procedure can be a valuable tool to conduct these investigations.

3.3.3 Mescaline concentration gradient in L. williamsii and its relationship to the total mescaline content of the specimen

Figure 41 summarizes the results from the exploratory investigation of specimens Lw1-Lw4.
This study is the first to investigate concentration differences of mescaline within the green parenchyma of a single specimen. The exploratory application of the MESQ-procedure shows that a consistent mescaline gradient is present in all of the four specimens studied. This gradient ascends from the center to the sides with the highest concentrations measured at the lateral side of the button. In addition, no circular or other gradient was observed, which means that the mescaline concentration at a given distance from the center is similar all around and that it does not matter on which side of the plant the biopsy is taken.
If we then compare the ratio of mescaline concentrations in the most lateral biopsies of the respective specimens to the total amount of mescaline obtained by aqueous extraction of the entire aboveground part, we see that they correlate very well for all 4 specimens studied. And although the limited number of specimens does not allow us to make statements about the entire species, this exploratory study provides strong arguments to believe that it is possible to

predict the mescaline concentration of an entire peyote button by means of just a single biopsy from its side.

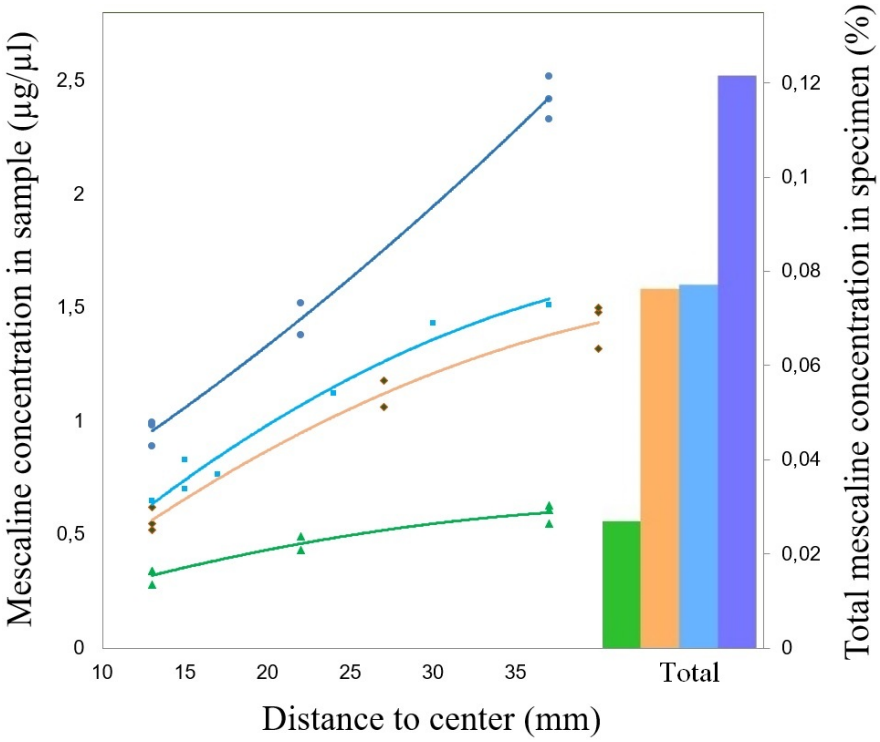


Figure 41: *Biopsy concentrations (dots) in relation to the distance from the center in 4 specimens of L. williamsii. The trend lines reveal the radial mescaline gradient. The most lateral measurements correlate well with the respective total mescaline concentrations in the aboveground part of the specimens (columns on the right).*

It should be mentioned that the mescaline content of the four specimens was determined by a simple aqueous extraction that was chosen for comparability with common and traditional preparation methods. However, such an extraction cannot be considered complete and future research may refine the absolute values given in Figure 41 using complete extraction techniques. Then some conversion factor may be determined to convert biopsy concentrations to total mescaline content for the examined specimen.

There are 5 good reasons for taking biopsies at the side of the button: first, the mescaline concentration appears to be highest there, which allows relative quantification to be applied as early as possible. Second, it increases the absolute concentration differences between specimens, which facilitates relative quantification. Third, there is no chance of damaging the central growth tip. Fourth, there is a lower chance of infection or decay from water that may enter and remain in an open biopsy cavity, and lastly, a scar in the side is aesthetically least disruptive, and the biopsy site will become invisible with further growth. Figure 42 shows how a lateral biopsy site flattens with further growth of the cactus. Eventually, the scar will be completely concealed by the folds of the outgrown cactus stem.

Figure 42 *: With continued growth, this biopsy site in Lophophora will become flattened and invisibly integrated into the lower part of the stem.*

This discovery of the uneven distribution of mescaline in the green parenchyma of a single specimen, as with the newly discovered gradient in *Echinopsis*, calls into question conclusions of previously published research. Studies using the entire *Lophophora* button for analysis may compare them correctly [31], but the results of studies that took only unspecified biopsies [16, 57] should be reinterpreted with caution in light of this new knowledge.
To reduce the multitude of variables that could potentially affect mescaline concentration, this study chose to examine specimens from the same grower. This ensured standardization of substrate, fertilizer, watering, lighting and aeration. In addition, specimens with the same diameter were chosen. Future research can focus on the relationship that exists between biopsy concentration and total button concentration in specimens of different size and age and under different growing conditions.

3.3.4 Mescaline content of some L. williamsii specimens

The results of this experiment were rather surprising. After the ammoniacal development of the plate, it appeared that under UV light only 3 of 10 specimens showed an observable amount of mescaline (Figure 43 left). After derivatization, these showed up as supersaturated spots (Figure 43 right). The other samples appeared to stain only very weakly in the mescaline band and even then sometimes as light brown instead of purple spots.

To better visualize this unexpected matrix composition, an even higher spotting volume was used. Figure 44 shows that in the 2 specimens on the left no mescaline could be detected, despite the higher spot volume of 25µl. Their purple spots have a higher Rf than mescaline and seem to color more violet than the purple-red of mescaline. Also notice the yellow-orange spots with just slightly higher Rf than mescaline. This is probably an iminium salt that was formed by the reaction of ninhydrin with a secondary amine. In specimen 3 (third from left), a trace of the dominant violet component of samples 1 and 2 appears to be present in addition to the clear mescaline spot (standard spot on the right).

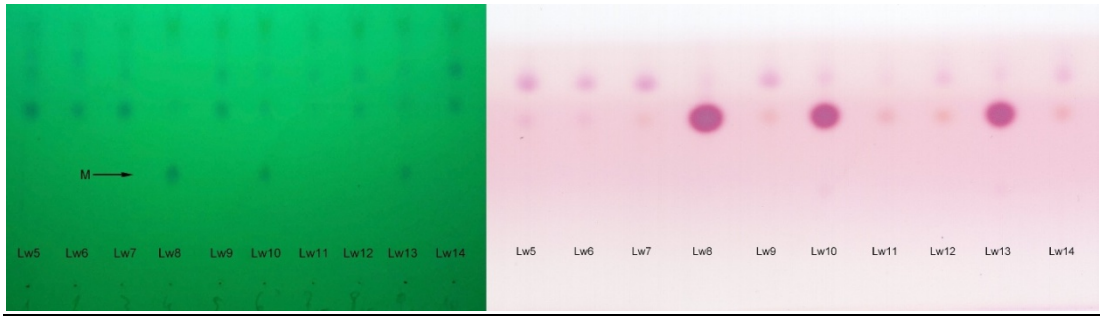


Figure 43: *Left: chromatogram of Lw5-Lw14 under UV light prior to derivatization, mescaline band indicated (M). Right: The same plate after derivatization.*

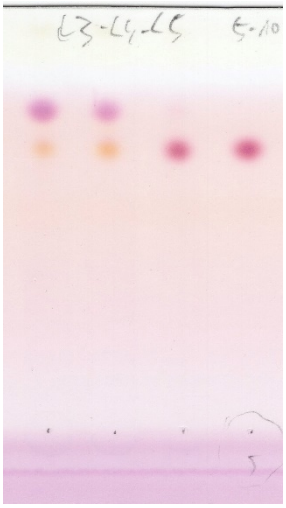


Figure 44: *Chromatogram of 2 L. williamsii specimens containing no detectable mescaline (left), 1 specimen containing a low amount of mescaline (grafted L. williamsii on E. peruviana) and a standard spot of mescaline on the far right for reference.*

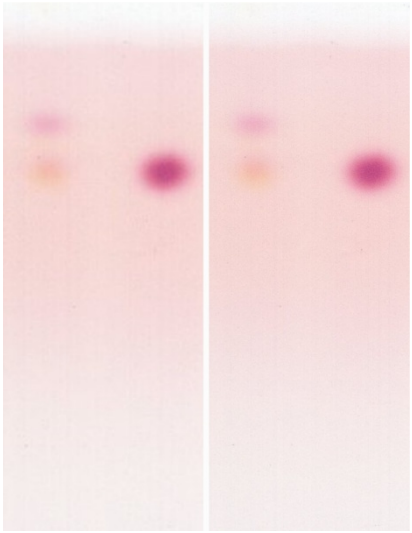


Figure 45: *MESQ chromatogram of Lw12 in Seramis (left) and after 1 year in enriched substrate (right). The standard spots do not contain the same mass. The Lophophora samples were taken and processed identically, however. The appearance of the spots did not change appreciably after 1 year.*

From the research of Aragane and Sasaki [17], we know that there is a genetically distinct lineage of *L. williamsii* that does not contain mescaline. This study now shows the absence of mescaline in specimens from geographically and presumably genetically different backgrounds. Given the specificity of the substrate used (pure Seramis clay) and the purely inorganic fertilizer, a blocked biosynthetic pathway based on micronutrient deficiency or bacterial symbiosis was also considered. Therefore, the substrate of Lw12 was adjusted as described in paragraph 2.3.4 and the chromatogram was repeated after 1 year. The cutouts in Figure 45 suggest that soil richness does not appear to be the limiting factor for mescaline production in the specimens studied.

This exploratory research indicates that there are subpopulations within *L. williamsii* that do not (always) produce detectable amounts of mescaline. The MESQ procedure appears to be a very useful method within large populations of *L. williamsii* for selecting those specimens that are very rich in mescaline but also those that do not contain mescaline. It is not inconceivable that mescaline-free genetic lines of *L. williamsii* could be given a different legal status analogous to that of cannabis/hemp legislation. In addition, there is also current interest in detecting possible links between the mescaline concentration of peyote cacti and their external characteristics such as the number of ribs [59]. For this kind of research as well, the MESQ procedure can prove to be a valuable tool because it allows the screening of large populations in a short time with few resources.

3.3.5 Mescaline concentration in Lophophora jourdaniana

The lateral biopsies from both specimens contained a similar mescaline concentration of about 0.02µg/µl (0.002%), this is 25x less than the weakest *L. williamsii* from this study. For this reason, *L. jourdaniana* appears to be unsuitable as an entheogen.

3.3.6 Mescaline quantification in a seedling of E. lageniformis

The concentration in the sample was determined to be 0.068µg/µl.
The fact that seedlings produce quantifiable amounts of mescaline at an early stage can be very important for the breeding process. Especially if further research shows that the relative strength of seedlings is maintained throughout their further growth. For this research, the MESQ procedure seems very suitable because it can monitor the evolution of the mescaline concentration in a certain parenchymal area in a minimally invasive way.

3.3.7 Mescaline quantification in E. lageniformis var. monstrose 'clone B'

Because the various specimens examined should be clones of a single *E. lageniformis*, they are expected to possess approximating mescaline tissue concentrations. Indeed, Figure 46 shows that these values are comparable. The absolute standard deviation of the values here is 0.06% (RSD 12%). This standard deviation is the sum of the standard deviation of the complete MESQ procedure (from sampling to quantification) and the standard deviation of the mescaline concentrations in the respective cactus parts.
At the same time, this experiment highlights the potency of this particular clone, with a ‘green tissue’ concentration of around 0,5% (m/m, fresh weight). This confirms the claims made in user reports [60]. See also Figure 40 for comparison with other *E. lageniformis* specimens.

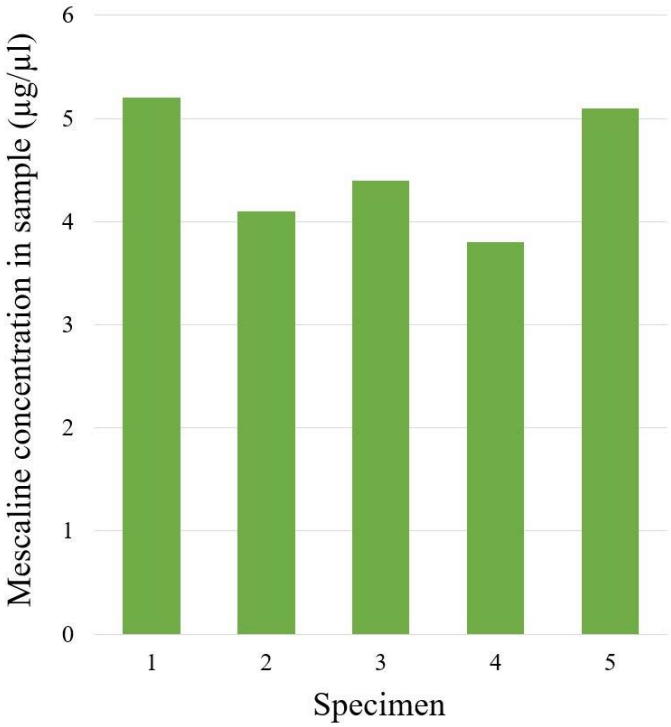


Figure 46: *Comparison of top biopsy concentrations in 5 specimens of E. lageniformis var. monstrose.*

4. CONCLUSION

The MESQ procedure is a validated and versatile tool for quantifying mescaline in cactus tissue and aqueous cactus extracts. It has various applications in the process from seedling to consumption. The procedure uses easily accessible and affordable materials, reagents and software. As a result, growers and end users of entheogenic cacti now have a tool for selection and correct dosing.

Exploratory application of the MESQ procedure led to the discovery of an unpublished mescaline gradient in specimens of *Lophophora williamsii* and *Echinopsis pachanoi, peruviana* and *lageniformis*. Further investigation then also demonstrated a correlation between the total mescaline content of specimens and the mescaline concentration in targeted biopsies. This observation has important implications for the breeding of the mentioned species and allows the MESQ-procedure to contribute to the protection of threatened wild *Lophophora* populations. At the same time, the results of this study call for a critical re-examination of previous research on mescaline concentrations in the discussed species that did not take into account this newly discovered gradient.

Several research questions present themselves based on these exploratory results, and here again the MESQ procedure can assume its role as a sufficiently precise and minimally invasive tool for the further scientific investigation of the impact of genetics, age, and growing conditions on mescaline concentration in individual cactus specimens as well as in larger populations.

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He would also like to extend a special thanks to biochemist Quinten Remijsen PhD for his valuable suggestions in critically proofreading the manuscript.

ABOUT THE AUTHOR AND THIS RESEARCH

Frederick Van Der Sypt MD, *has been providing medical care to socially vulnerable groups for more than 15 years. Psychiatric disorders are very prevalent amongst these groups, and the relationship between humans and the use of psychoactive substances became a particular point of interest. From there he started as an addiction specialist within various departments of specialized outpatient, residential and forensic addiction care. From this expertise, he taught substance-related disorders at the Faculty of Medicine of Ghent University and to this day to a wide audience of healthcare professionals.*

The confrontation of his academic background with day-to-day clinical experience raised many questions regarding the current legal status and public perception of many psychoactive substances. This discrepancy between the clinical and scientific realities of substance use on the one hand and the current level of control on the other is very aptly described by the Global Commission on Drug Policy in their 2019 report: "Classification of Psychoactive Substances: When Science Was Left Behind" [61]. In particular, the group of classical psychedelics is poorly understood, which has greatly hindered the research into their therapeutic and otherwise positive effects. The author believes in the holistic potential that these substances hold and therefore wants to contribute with this current article to a safe and sustainable renaissance of psychedelic science.

A second motive for this research was the recent global trend towards regulation of psychoactive substances. In the view of the author, this is a beneficial and necessary evolution that will promote individual as well as societal health. The scientific justification for this view is extensively covered in several reports of the Global Commission on Drug Policy and the author heartily recommends these texts as background for all health care and policy professionals dealing with psychoactive substances [12]. The regulation of classical psychedelics will make these substances more accessible to a broad public outside of protected research settings. Consequently, we need to provide contemporary and realistic frameworks that can facilitate the harmonious reintegration of these substances into our society. The author hopes that the development of the accessible dosing method presented in this article may contribute to such a harm-reducing framework. Not only to end users, but to populations of entheogenic plants as well.

This brings us to the third and final reason for starting this research: the critical situation in which L. williamsii finds itself and the fact that the trends described above could generate further pressure on its populations [62]. These contextual factors ask for timely and pragmatic solutions to safeguard one of the longest standing and most harmonious relationships between man and his substances. It is the hope of the author that the procedure presented in this article will create alternatives for the harmful harvesting of wild Lophophora populations.

Not using mescaline-containing cacti still remains the safest choice and the author does not want to encourage anyone to use them. But for those people who do choose to use them, the author hopes to have given them a tool for using them in a safer way.

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APPENDIX 1

International EnergyControl
Address: C/ Independencia 384, local bajos
08041 Barcelona Spain
e-mail: international@energycontrol.org

ANALYSIS REPORT

ORDER	2411
Item	undefined
Substance	mescalina
Date of purchase	2020-07-10

SAMPLE INFORMATION

Sample ID	54937
Type of analysis	Quantitative
Qualitative analysis instrument	LC-DAD-MS
Date of Analysis	Thu Dec 24 2020

RESULT:

Mescaline >99 %

DISCLAIMER

Our drug testing service is specifically designed for final users. The results we offer are only valid if the sample used is from the exact same batch as the sample analyzed. As such, none of our results should be used as a quality guarantee from any drug vendor or product.

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