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^{1}H-NMR and UPLC-MS metabolomics: Functional tools for exploring chemotypic variation in *Sceletium tortuosum* from two provinces in South Africa

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ABSTRACT

Sceletium tortuosum (Aizoaceae) is widely recognised for the treatment of stress, anxiety and depression, as well as for obsessive compulsive disorders. A comprehensive intraspecies chemotypic variation study, using samples harvested from two distinct regions of South Africa, was done using both proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy of methanol extracts (N = 145) and ultra performance liquid chromatography-mass spectrometry (UPLC-MS) of acid/base extracts (N = 289). Chemometric analysis of the ^{1}H-NMR data indicated two main clusters that were region-specific (Northern Cape and Western Cape provinces). Two dimensional (2D) NMR was used to identify analytes that contributed to the clustering as revealed by the S-plot. The sceletium alkaloids, pinitol and two alkylamines, herein reported for the first time from *S. tortuosum*, were identified as markers that distinguished the two groups. Relative quantification of the marker analytes conducted by qNMR indicated that samples from the Northern Cape generally contained higher concentrations of all the markers than samples from the Western Cape. Quantitative analysis of the four mesembrine alkaloids using a validated UPLC-photo diode array (PDA) detection method confirmed the results of qNMR with regard to the total alkaloid concentrations. Samples from the Northern Cape Province were found to contain, on average, very high total alkaloids, ranging from 4938.0 to 9376.8 mg/kg dry w. Regarding the Western Cape samples, the total yields of the four mesembrine alkaloids were substantially lower (averages 16.4–4143.2 mg/kg). Hierarchical cluster analysis of the UPLC-MS data, based on the alkaloid chemistry, revealed three branches, with one branch comprising samples primarily from the Northern Cape that seemed somewhat chemically conserved, while the other two branches represented mainly samples from the Western Cape. The construction of an orthogonal projections to latent structures-discriminant analysis model and subsequent loadings plot, allowed alkaloid markers to be identified for each cluster. The diverse sceletium alkaloid chemistry of samples from the three clusters may facilitate the recognition of alkaloid profiles, rather than individual compounds, that exert targeted effects on various brain receptors involved in stress, anxiety or depression.

1. Introduction

Sceletium tortuosum (L.) N.E. Br. (Aizoaceae) is the only one of eight species in the genus *Sceletium* that has been commercialised. The psychoactive properties of the plant have been attributed mainly to the presence of four mesembrine alkaloids (Fig. 1), particularly mesembrine and mesembrenone (Krstenansky, 2017).

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Abbreviations

COSY	^{1}H-^{1}H correlation spectroscopy
dry w.	dry weight
FIDs	free induction delays
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
HCA	hierarchical cluster analysis
HMBC	^{1}H-^{13}C heteronuclear multiple bond coherence
^{1}H-NMR	proton nuclear magnetic resonance
HSQC	^{1}H-^{13}C heteronuclear single quantum coherence
HSQC-TOCSY	heteronuclear single quantum coherence-total correlation spectroscopy
ICH	International Conference on Harmonisation
LC-UV/MS	liquid chromatography-ultraviolet detection/mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
MS	mass spectrometer
m/z	mass-to-charge ratio
N	number of specimens/samples
NC	Northern Cape
nd.	not detected
NMR	nuclear magnetic resonance
OPLS-DA	orthogonal projections to latent structures-discriminant analysis
PC	principal component
PCA	principal component analysis
PDA	photodiode array
qNMR	quantitative nuclear magnetic resonance
RSD	relative standard deviation
Rt	retention time
TOCSY	total correlation spectroscopy
TSP	sodium 3-trimethylsilyl [2,2,3,3-^{2}H4] propanoate
UPLC-MS	ultra performance liquid chromatography-mass spectrometry
UPLC-PDA	ultra performance liquid chromatography coupled to photodiode array detection
UPLC-PDA/MS	ultra-performance liquid chromatography coupled to photodiode array detection and tandem mass spectrometry
UPLC-QToF-MS	ultra performance liquid chromatography-quadrupole time-of- flight-mass spectrometry
UV	ultraviolet
WC	Western Cape

A patent (USA patent 6,288,104) was filed in 1997 for the use of mesembrine and related compounds for treating a range of psychiatric and psychological conditions, including depression and drug dependence (Gericke and Van Wyk, 1999). The best researched commercial product is marketed under the trade name Zembrin® and consists of a standardised hydroalcoholic extract of the aerial parts of the plant (Murbach et al., 2014).

Sceletium tortuosum occurs naturally in the Western, Eastern and Northern Cape provinces of South Africa, where small-scale cultivation for commercial use is currently practised. The plant is particularly abundant in the Knersvlakte and Upper Karoo regions (Northern Cape), the Great Karoo and Ceres Karoo and more moist parts of the Western Cape Province, and is also found in the Namaqualand Rocky Hills (spanning the Western and Northern Cape provinces) (Gerbaulet, 1996). Populations native to the southern regions of Africa, including the Khoikhoi and San, have valued the beneficial properties of *Sceletium* species for centuries (Ilardi et al., 2009). Dried leaves of *S. tortuosum* are used in traditional practices to elevate mood and to alleviate anxiety and tension. Plant material is usually chewed, but is also smoked or taken as an infusion (Van Wyk and Gericke, 2003).

Shikanga et al. (2012) established the variability in the mesembrine-type alkaloid content of 151 specimens of wild *S. tortuosum* specimens, harvested from 31 localities in the Western Cape region of South Africa, using gas chromatography-mass spectrometry (GC-MS) analysis of the acid/base extracts. Five chemotypes were identified, based on the mesembrine-type alkaloid composition, using hierarchical cluster analysis (HCA) and principal component analysis (PCA) of the GC-MS data. They reported that individual chemotypes were not restricted to particular

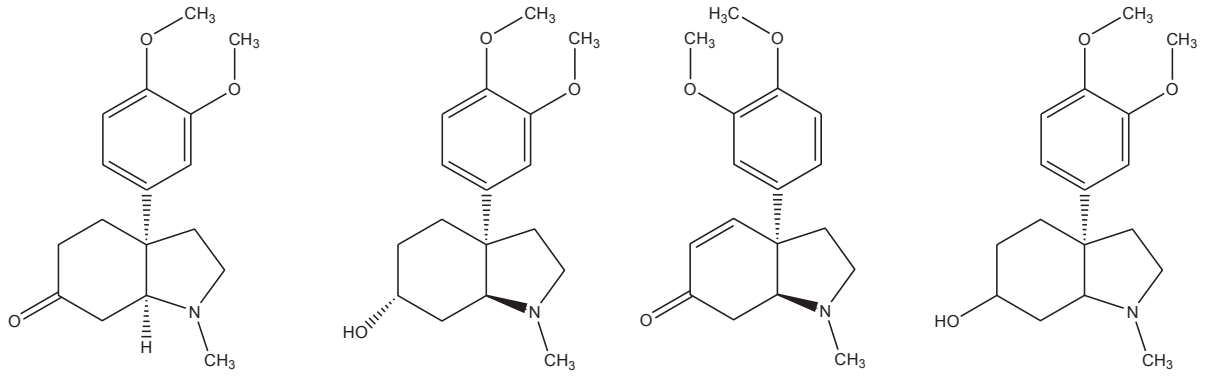

Fig. 1. Structures of the four mesembrine alkaloids associated with the central nervous system effects of *Sceletium tortuosum*.

geographical areas. However, the lack of data from plants growing in the Northern Cape Province necessitates further studies into the intraspecies chemotypic variation. This region is important from a historical perspective since the plant is widely used by the inhabitants (Gericke and Viljoen, 2008). Rapid progress in instrumentation and software has made nuclear magnetic resonance (NMR) spectroscopy one of the most powerful analytical methods in metabolomics (Larive et al., 2015). This technique can provide a wealth of accurate qualitative and quantitative information regarding the components of a mixture. In contrast to GC-MS and liquid chromatography coupled to ultraviolet and mass spectrometry (LC-UV/MS), the NMR approach does not rely on chromatographic separation. Hence, the loss of the whole metabolite information caused by the filtration through the chromatographic column can be avoided (Hall, 2006). Proton (^{1}H)-NMR can simultaneously detect all proton-bearing compounds in a sample, which includes most organic compounds in plant tissues. Thus, NMR spectra of crude extracts of plants may provide a relatively unbiased fingerprint, consisting of overlapping signals of the majority of the metabolites present in the sample (Ward et al., 2003). Factors that play a role in liquid chromatography analysis coupled to UV-radiation-based detectors or mass spectrometers, such as dependence on the absorbance of functional groups present and the ability of molecules to ionise, are not limiting factors in NMR analyses. Nevertheless, GC-MS and LC-UV/MS remain the techniques of choice for analysis of raw materials intended for herbal drugs, since these techniques result in complete separation and unequivocal identification of compounds in a mixture. For this reason, the chemical variation within a large number of *S. tortuosum* samples from two distinct geographical regions in South Africa was investigated using both ^{1}H-NMR spectroscopy and ultra performance liquid chromatography-mass spectrometry (UPLC-MS).

2. Results and discussion

2.1. Multivariate classification based on ^{1}H-NMR data

The ^{1}H-NMR spectra (Fig. 2) of *S. tortuosum* samples (two originating from the Northern Cape and three from the Western Cape provinces) illustrate compositional variation among the samples. It is evident that the variation is not limited to between the groups, but also within the groups.

Principal component analysis (PCA) was applied to visualize the data, allowing for a more holistic understanding of the clustering patterns displayed by the samples. The ^{1}H-NMR data derived from the 145 samples were Pareto-scaled and used to construct a PCA model, in which 71% ($R^2X_{cum} = 0.71$) of the variation within the data was explained by the first three principal components (PCs) with R^2X values of 0.51, 0.14 and 0.06 for PC1, PC2 and PC3, respectively. The PCA scores plot (PC1 *vs* PC3) demonstrates separation of the *Sceletium* samples from different provinces (Fig. 3A). Two main clusters were observed on the plot and the clustering was province-specific. Samples from the Northern Cape grouped together, and so did the Western Cape samples, indicating that within the province, the samples have similar chemical profiles and that they are conserved for the samples from a geographical region.

To identify the marker NMR signals that are responsible for the discrimination of samples between the Northern Cape and the Western Cape provinces, a supervised algorithm, orthogonal projections to latent structures-discriminant analysis (OPLS-DA), was applied. This supervised model could be used to reveal the NMR signal variation between the two assigned classes, as reflected by separation in the first predictive component (tp1), while the within-class variation was indicated by the first orthogonal component (to1). The OPLS-DA scores plot (Fig. 3B) indicates a clear separation of the samples from the two provinces along the tp1. A greater variation within the Western Cape samples can be observed, since the samples are spread across both the positive and negative orthogonal component (to1) in a wider range. The high R^2_{cum} value (93.6%) for the overall goodness of fit, and the high Q^2_{cum} value (92.1%), evaluated by cross-validation, demonstrates that the constructed model has good predictive ability to classify the samples into the two groups. The S-plot from the model (Fig. 3C), reveals the marker signals in the chemical shift regions of δ_H 3.80–3.88, δ_H 3.90–3.94, δ_H 3.60–3.64, δ_H 6.96–7.00, δ_H 3.04–3.08, δ_H 0.88–1.04, and δ_H 2.24–2.36 ppm, which reflects significant and reliable contributions to the differentiation between the two sample groups. In the S-plot, variables on the extremities are highly discriminant and depending on the position, a correlation between the variables and observations at the corresponding position in the scores plot can be established. Conversely, variables at the centre of the plot are non-discriminatory and occur in almost all the samples. The positions of the most pronounced marker signals in the S-plot corresponded to the Northern Cape region in the OPLS-DA scores plot, indicating that these signals are predominant in the Northern Cape samples and play a significant role in differentiating samples from the two geographic regions.

For spectral interpretation and signal assignment, 2D NMR techniques including ^{1}H-^{1}H correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC), ^{1}H-^{13}C heteronuclear multiple bond coherence (HMBC) and heteronuclear single quantum coherence-total correlation spectroscopy (HSQC-TOCSY) were applied to study the representative samples selected from the groups. In addition, the standard compounds and the proton/carbon NMR databases (ACD/NMR database) were used as references to assist with interpretation and to confirm assignments of the NMR spectral signals. The marker signals in the region of δ_H 3.80–3.88 ppm displayed high intensities for most of the samples from the Northern Cape group. They were singlet peaks that showed no COSY correlations with any other resonance peaks. The multiplicity-edited HSQC spectrum revealed that the signals accounted for methyl protons attaching to the carbons with chemical shifts at around δ_C 55 ppm. These signals could be further assigned to the protons of methoxy groups on phenyl rings, based on the HMBC spectrum, in which these protons showed correlations only to the carbons at δ_C 148–149 ppm. The ^{1}H-NMR spectrum also showed relatively strong signals from aromatic protons at the δ_H 6.96–7.00 ppm region. From the HMBC spectrum, the correlations of these aromatic protons to the carbons at δ_C 148–149 ppm indicate that these protons belong to the phenyl rings to which the methoxy groups are attached. The fact that the alkaloids in *S. tortuosum* contain characteristic methoxy-phenyl rings in their structures (Fig. 1) suggest that those signals in the chemical shift ranges of δ_H 3.80–3.88 ppm and δ_H 6.96–7.00 ppm are characteristic resonances arising from the sceletium alkaloids. The marker signals in the regions of δ_H 3.04–3.08 ppm and δ_H 2.24–2.36 ppm, which were revealed along with those in the regions of δ_H 3.80–3.88 ppm and δ_H 6.96–7.00 ppm in the S-plot of the OPLS-DA model, could be assigned to the resonances of the protons on the N-methyl groups of sceletium alkaloids. Spiking experiments, in which the intensity increases of those marker signals were observed after the standard references mesembrenone and mesembrine were added to the original NMR solution, confirmed the assignments.

When examining the marker signals in the chemical shift regions of δ_H 3.90–3.94 and δ_H 3.60–3.64 ppm by running 1D and 2D NMR, a doublet peak and a singlet peak, both with strong intensity, were observed in each respective region. The HSQC spectrum indicated that the doublet peak had correlations to two carbon

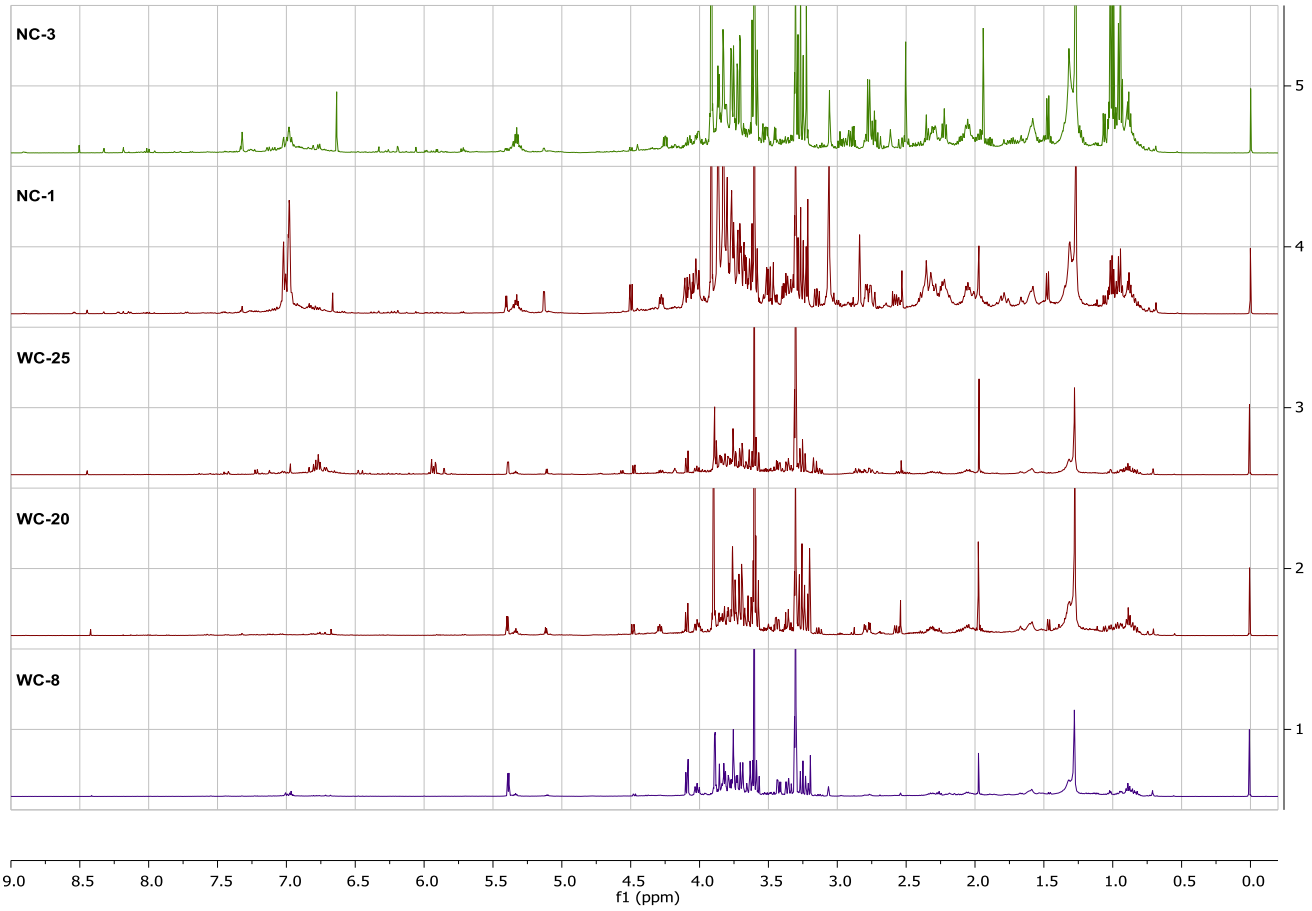

Fig. 2. ^{1}H-NMR spectra of representative *Sceletium tortuosum* specimens collected from the Northern Cape (NC-1 and NC-3) and Western Cape provinces (WC-8, WC-20 and WC-25).

signals at δ_C 71.9 and 72.1 ppm, indicating that the doublet was attributed to two oxygenated methine protons. The 2D NMR experiments, together with the HSQC-TOCSY experiment, revealed the existence of a cyclohexane-1,2,3,4,5,6-hexol structural unit. Furthermore, from the HSQC and HMBC spectra, it was deduced that the singlet peak in the region of δ_H 3.60–3.64 ppm was attributed to a methoxy group (δ_C 59.3 ppm) attaching to that cyclohexanhexol unit. By comparing the NMR data (^{1}H and ^{13}C chemical shifts and ^{1}H-^{1}H coupling constants) with those in the database and literature, it was found that pinitol, a reported compound from *S. tortuosum*, accounted for these marker signals. In addition, all the ^{1}H and ^{13}C data observed in this study for pinitol were in agreement with the reported ones (Raya-Gonzalez et al., 2008; SetShedi et al., 2010).

The signals in the region of δ_H 0.88–1.04 ppm, which were also revealed as markers in the S-plot from the OPLS-DA model, were found to represent mainly one triplet and three doublet peaks (δ_H 0.98–1.03 ppm). According to the 2D NMR analyses, they were the resonances from the protons of methyl groups. The COSY, HSQC and HMBC spectra further revealed that the signals were attributed to the methyl protons of two compounds, isobutylamine and 2-methyl-butanamine. The assignments were confirmed by using NMR spiking experiments, in which an increase of the signal intensity was observed after addition of standards of isobutylamine and 2-methyl-butanamine to the original NMR solution. The assignment was further confirmed by GC-MS analysis, in which the two compounds were identified in the total ion chromatogram. To the best of our knowledge, this is the first report on the presence of these two alkylamines in *S. tortuosum*.

2.2. Quantitative NMR results

On the basis of the OPLS-DA model and constituent identity analyses, it was clear that the sceletium alkaloids, pinitol, and alkylamines (isobutylamine and 2-methyl-butanamine) are the marker constituents that make the greatest contribution to the separation of the Northern Cape group from the Western Cape group in the scores plot. To explore the quantitative information concerning these marker compounds in the two different locality groups, quantitative NMR (qNMR) analyses were conducted in this study. The amounts of the constituents in the extracts were measured based on the integration of the characteristic peak in the ^{1}H-NMR spectra and expressed as relative intensity against that of the internal standard sodium 3-trimethylsilyl [2,2,3,3-^{2}H4] propanoate (TSP) at δ 0.00 ppm. The content level for a constituent was determined as relative intensity using the following equation:

$$C_x = I_x/I_{std} \times N_{std}/N_x$$

where I_x and I_{std} are the intensities (area integration) of appropriate signals of the constituent(s) and the internal standard (TSP), respectively, while N_x and N_{std} are the number of proton contributing to the resonances (Zhao et al., 2012).

Various alkaloids have been reported from *S. tortuosum*. Most of them possess the characteristic methoxy-phenyl moiety in their structures, and they are also the dominant alkaloids in the plant (Gericke and Viljoen, 2008). In the present study, for comparing the differences in sceletium alkaloid content in the samples from the Northern Cape and Western Cape provinces, the qNMR analysis

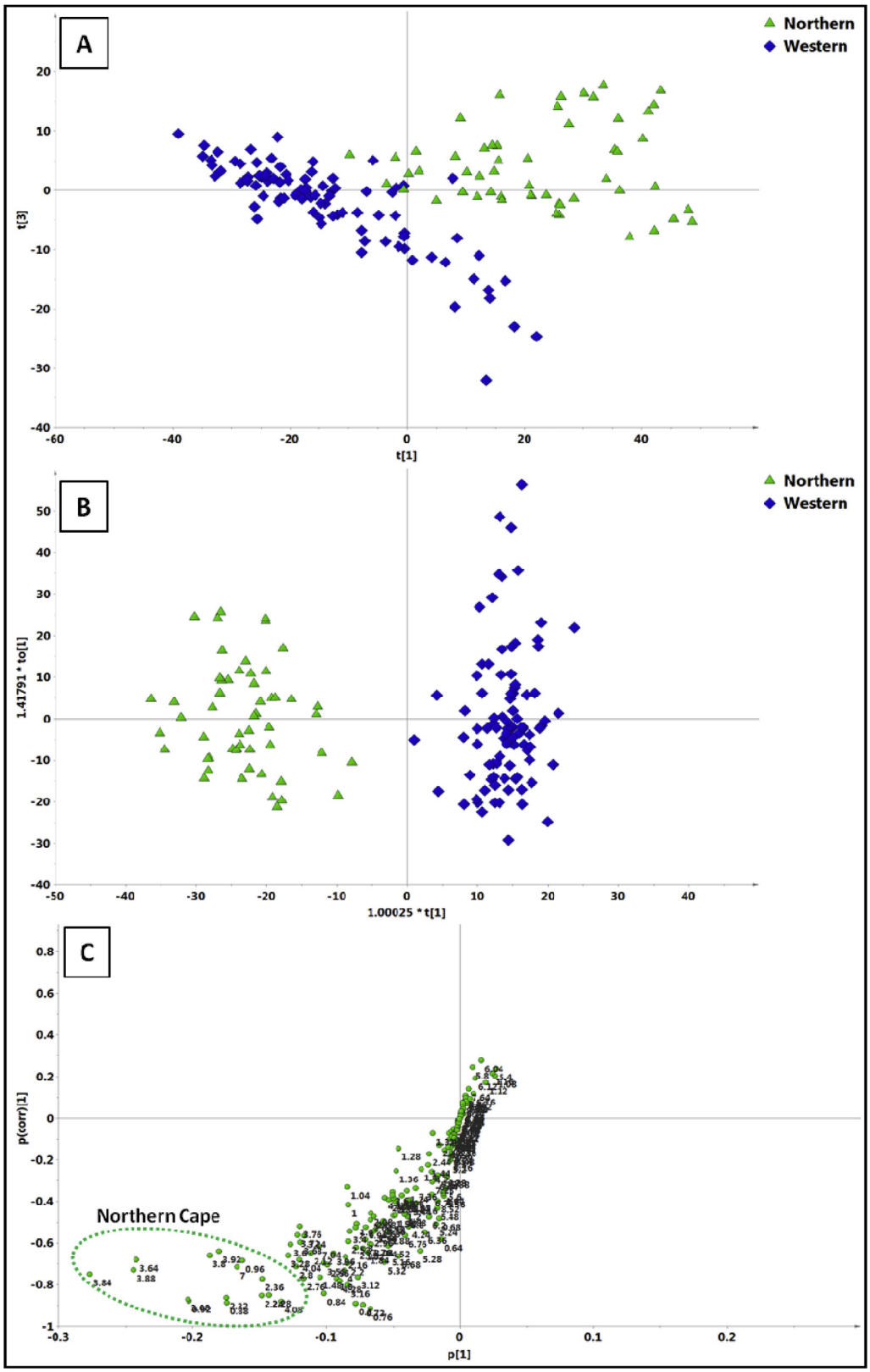

Fig. 3. A) PCA scores plot, B) OPLS-DA scores plot indicating clear distinction between the chemical profiles of samples from the Northern Cape (green triangles) and Western Cape (blue diamonds) provinces, and C) OPLS-DA S-plot indicating variables responsible for the differentiation between the samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

was performed by using the integration of the characteristic methoxy signals in the region of δ_H 3.80–3.88 ppm. As indicated in Fig. 4A, the average content (mean ± standard deviation) of sceletium alkaloids in the samples from the Northern Cape (27.7 ± 9.5) was higher than that from Western Cape (12.1 ± 5.6). Some samples collected from Steilsdrift (Western Cape) were found to contain more alkaloids than the other samples from the Western Cape. Samples from Vrolijkheid Nature Reserve, Vanwyksdorp, Beaufort West, Kontiki and Botmaskloof contained relatively lower alkaloid content when compared to samples from the Northern Cape Province. Among all the analysed samples, those collected from Paulshoek (Northern Cape) were on average the richest in sceletium alkaloids.

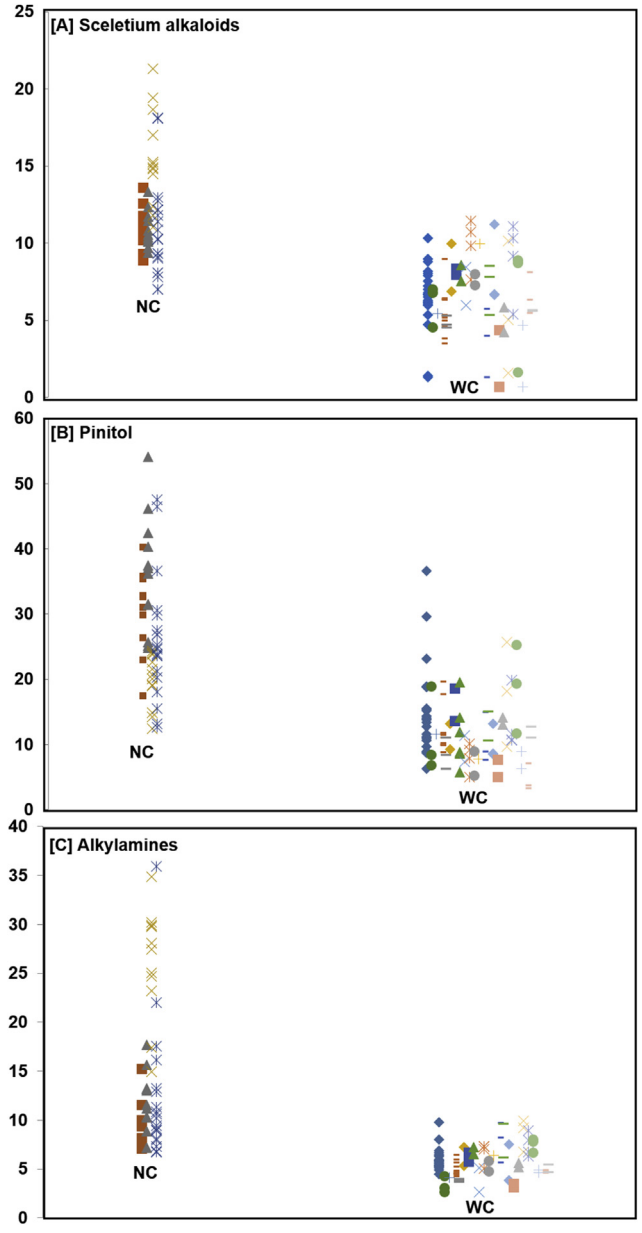

Fig. 4. Relative concentrations of analytes in *Sceletium tortuosum* samples collected from the Northern Cape (NC) and Western Cape (WC) provinces as determined by qNMR. A) sceletium alkaloid content, B) pinitol content and C) alkylamines content. *The relative concentration of analyte is expressed as the relative integration ratio of the characteristic peak of analyte to the peak of the internal standard (TSP) at δ 0.00 ppm (the number of protons contributing to the peak is taken into consideration).

The doublet peak in the region of δ_H 3.90–3.94 ppm was used as the characteristic peak for pinitol qNMR analysis. Fig. 4B indicates the content distribution of pinitol in the samples from both the Northern Cape and Western Cape groups. It is clearly demonstrated that the Northern Cape samples contained more pinitol than their Western Cape counterparts. The average content of pinitol (11.9 ± 3.2) in the former was nearly double (6.4 ± 2.4) that in the latter samples. The Student t-test revealed a significant difference ($p < 0.00001$) in the pinitol content of the two groups. Samples from Nourivier (Northern Cape Province) contained the most pinitol.

The qNMR determination of the alkylamine content was performed based on the integration of the total characteristic peaks in the region of δ_H 0.88–1.04 ppm, corresponding to the two methyl groups (δ_H 0.99–1.03 ppm) of isobutylamine and two methyl groups (δ_H 0.91–0.96 and 0.98–1.02 ppm) of 2-methyl-butanamine. The average content (14.5 ± 8.1) of the alkylamines in samples from the Northern Cape was higher than for those samples in the Western Cape group (5.8 ± 1.6), showing significant differences ($p < 0.00001$, *t*-test). Notably, the samples from Nourivier (Northern Cape) were found to possess not only the highest content of pinitol, but also the highest content of alkylamines (Fig. 4C). A Pearson's correlation analysis indicated a highly positive correlation (r = 0.80) between the content level of pinitol and alkylamine for all the samples. However, the correlation coefficients for both the pinitol and the alkaloid pair (r = 0.54), and the alkylamine and alkaloid pair (r = 0.41), were low. It is not clear what biological relationship or mechanism is behind the positive correlation between pinitol and the alkylamines, since they belong to two different metabolite classes. Nevertheless, based on the qNMR results, it is likely that external and internal factors including soil, climate, precipitation and/or genetic factors, might contribute to the variation and correlation of the content levels for all the metabolites in *S. tortuosum*.

The intra-population variation of chemical profiles within both the Northern and Western groups was investigated further by creating two separate PCA models for the samples in each group. The Northern Cape samples (Supplementary S1) clustered according to different collection localities, with the exception of the samples from Ratelkraal that spread throughout the scores plot range and overlapped with others. Garing samples generally clustered on the positive side of PC1 and were separated along the PC1 axis from the Nourivier samples, which clustered on the negative side of PC1. The Paulshoek samples also showed a diagonal separation along the PC2 axis from the Nourivier samples. The dendrogram from hierarchical cluster analysis (HCA) (Fig. 5A) revealed that the samples could be clearly grouped into three classes, suggesting the occurrence of three major chemotypes in Northern Cape samples. The NMR signals that made a significant contribution towards the separation of the groups were revealed from by the corresponding loadings plot of the PCA model. Resonances derived from pinitol (3.92 and 3.64 ppm), alkylamines (0.88–1.04 ppm), and fatty acids (1.28–1.32 ppm), made the greatest contributions to the separation of Nourivier samples from the others, while the resonances of alkaloids (3.84–3.88 ppm) played a significant role in the separation of Paulshoek samples from Nourivier samples.

The PCA model for the Western Cape samples indicated that there are no clear class separations for the groups according to locality (Supplementary S2). However, the HCA dendrogram (Fig. 5B) revealed that the samples were clearly grouped into two classes with a high Euclidean distance value (Fig. 5B), suggesting that two major chemotypes (type 1 and 2) can be distinguished for the Western Cape samples. Through analysis of the loadings plot it was found that fatty acids, alkaloids and pinitol, which were abundant in type 1 samples, contributed to the differences in the two chemotypes.

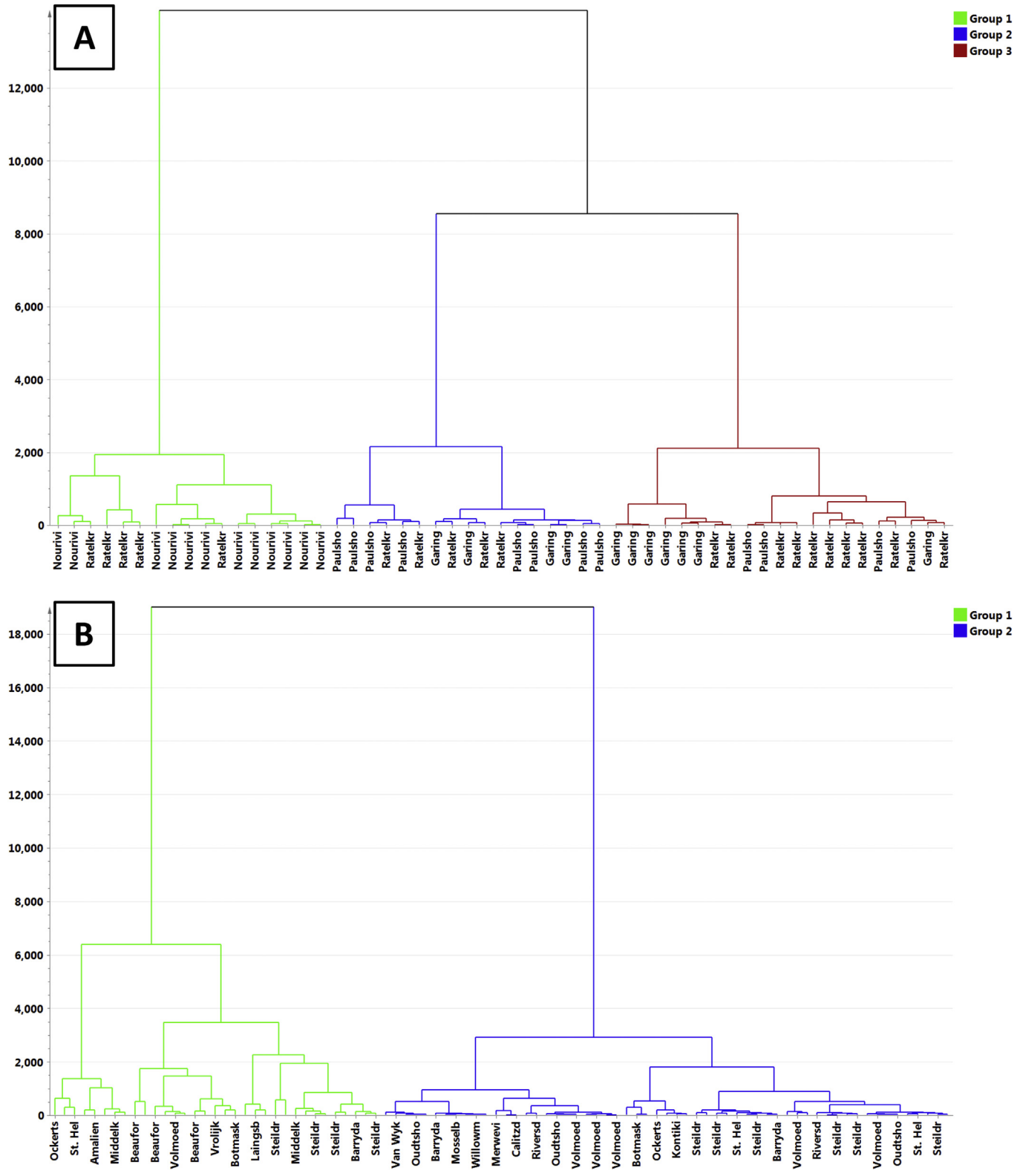

Fig. 5. Dendrograms showing chemical variation within samples from A) the Northern Cape and B) Western Cape provinces, based on ^{1}H-NMR data.

2.3. Multivariate classification based on UPLC-MS data

To observe clearly the relationships among samples in the scores plot, hierarchical cluster analysis (HCA) was performed and a dendrogram was constructed for the UPLC-MS data set. The interactive nature of the dendrogram and the scores plot allowed for easy identification and colour-coding of the different chemotypes in the data. To investigate the variation responsible for separation into the different classes, OPLS-DA was applied, based on the classes generated using HCA.

The dendrogram of the UPLC-MS data indicates clear separation of the entire set of *Sceletium* samples (N = 289) into three branches; Cluster 1-Western Cape 1; Cluster 2-Northern Cape and Cluster 3 - Western Cape 2 (Fig. 6A). These groups are displayed on the corresponding PCA scores plot (PC1 *vs* PC2) by using the colour groups from the dendrogram (Fig. 6B). Due to the highly variable alkaloid profiles of Western Cape samples, principal component analysis revealed two chemotypes in clusters 1 and 3. Cluster 1 (WC) samples were obtained from Ladismith, Calitzdorp, Merweville, Beaufort West, St Helena, Klaarstroom, Botmaskloof and Vrolijkheid. Cluster 3 (WC), which was more variable, comprised mainly samples from Steilsdrift, Willowmore, Amalienstein, Oudtshoorn, Barrydale, Mosselbay, Volmoed, Van Wyksdorp, Ockertskraal and Kontiki. The results concur with the NMR data where two chemotypes were also identified independently, following multivariate analysis of the Western Cape samples. Cluster 2 (NC) spread along the positive PC1 comprised samples predominantly from the Northern Cape Province. The tight clustering observed confirms the ^{1}H-NMR results indicating that samples from the Northern Cape have a somewhat conserved chemical profile.

To further investigate marker molecules responsible for the observed clusters, an OPLS-DA model of the three clusters (groups) was performed. Fig. 6C is the OPLS-DA scores plot of the observations, clearly indicating separation of the three pre-defined clusters. Chemical variation modelled along the first predictive component (Pp1) separated cluster 1 (WC1) and cluster 2 (NC) by 16.8% chemical variation. The second predictive component (Pp2 = 0.094), with a 9% modelled variation, positioned cluster 3 (WC2) on the Pp2 (+), while the other two clusters occupied Pp2 (−). In order to correlate the clustering patterns to the chemical profiles, a loadings plot of the variables was constructed (Fig. 6D). Distinctive discriminant variables, colour-coded to match the corresponding clusters in the scores plot, demonstrate that a typical Northern Cape chemical profile has high levels of compounds eluting at retention times (Rt) 3.3 and 4.7 min (Figs. 6D and 7A), identified as Δ^{7}-mesembrenone and mesembrine, respectively (Table 1). Δ^{7}-Mesembrenone is known to be produced by *S. tortuosum* (Swart and Smith, 2016). Visual inspection of the chromatograms confirmed the presence of mesembrine as the major peak in the samples, while Δ^{7}-mesembrenone was also visible as illustrated in Fig. 7A. The loadings plot also displayed a compound at Rt 5.52 min as a distinct chemical marker for Western Cape 1 (Fig. 6D). This marker compound is visibly dominant in the total ion chromatogram of Western Cape 1 and was identified as *N*-demethyl-*N*-formyl mesembrenone (Fig. 7B, Table 1). The loadings plot also filtered out compounds eluting at Rt 3.5, 3.7, 3.9 and 5.1 min as important variables in the classification of Western Cape 2 samples (Fig. 6D). The compounds were identified as mesembrenol, mesembranol, mesembrenone and sceletium alkaloid A4, respectively (Table 1). A typical total ion chromatogram of the samples displaying the four alkaloids is presented in Fig. 7C, indicating the marker compounds as identified in the loadings plot. Sceletium alkaloid A4, first isolated from *S. namaquense* (Jeffs et al., 1971) has been reported from *S. tortuosum* (Snyckers et al., 1971). However, *N*-demethyl-*N*-formyl mesembrenone, previously isolated from *Sceletium strictum* (Karle, 1977), has not been reported from *Sceletium tortuosum*. Since a standard for this compound was not available, the identity remains tentative. Samples from Cluster 1 were found to contain very low concentrations of the four mesemembrine alkaloids as determined by UPLC-PDA analysis.

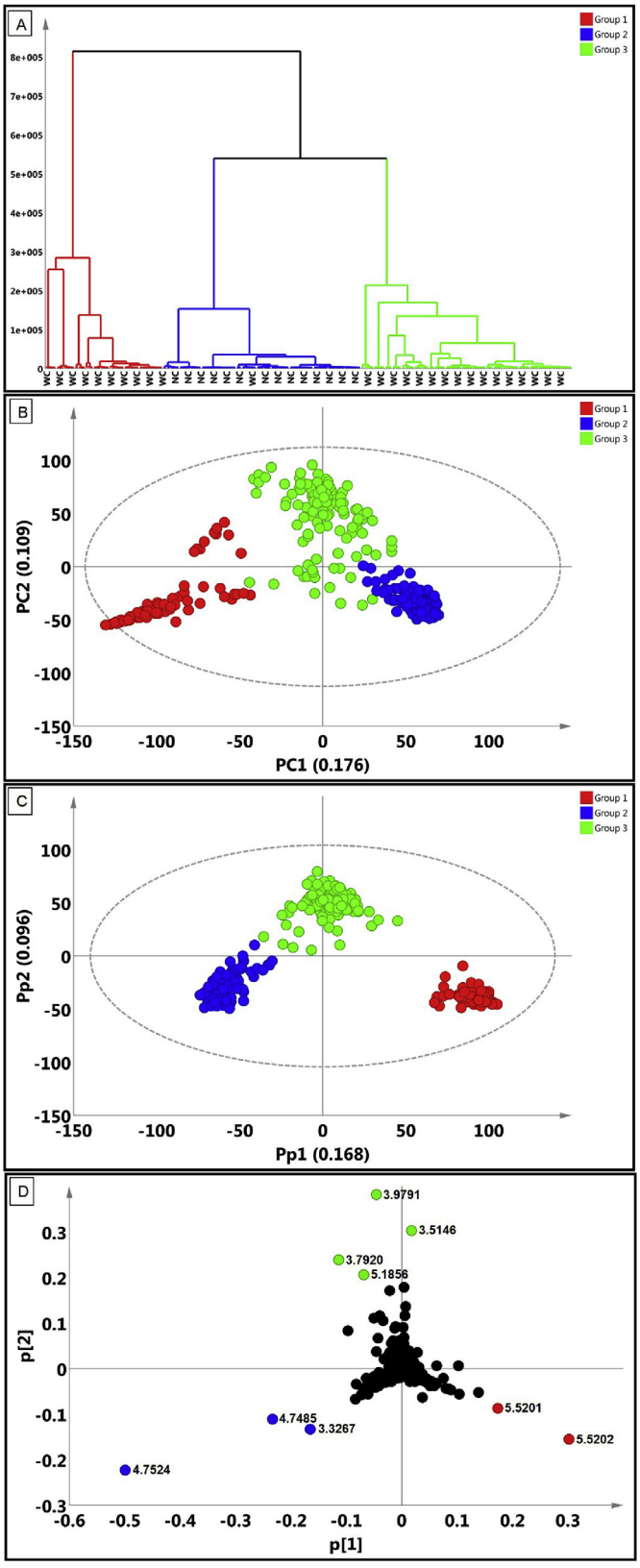

Fig. 6. A) Dendrogram showing separation of *Sceletium tortuosum* samples into three distinct groups, B) the corresponding PCA scores plot, C) OPLS-DA scores plot separated three chemotypes based on predictive variation and D) loadings scatter plot highlighting variables highly correlated to each cluster, based on UPLC-MS data.

2.4. Quantitative UPLC-PDA results

The detector was the most sensitive towards mesembrenone, as reflected by the steeper slope of the calibration plot for the alkaloid

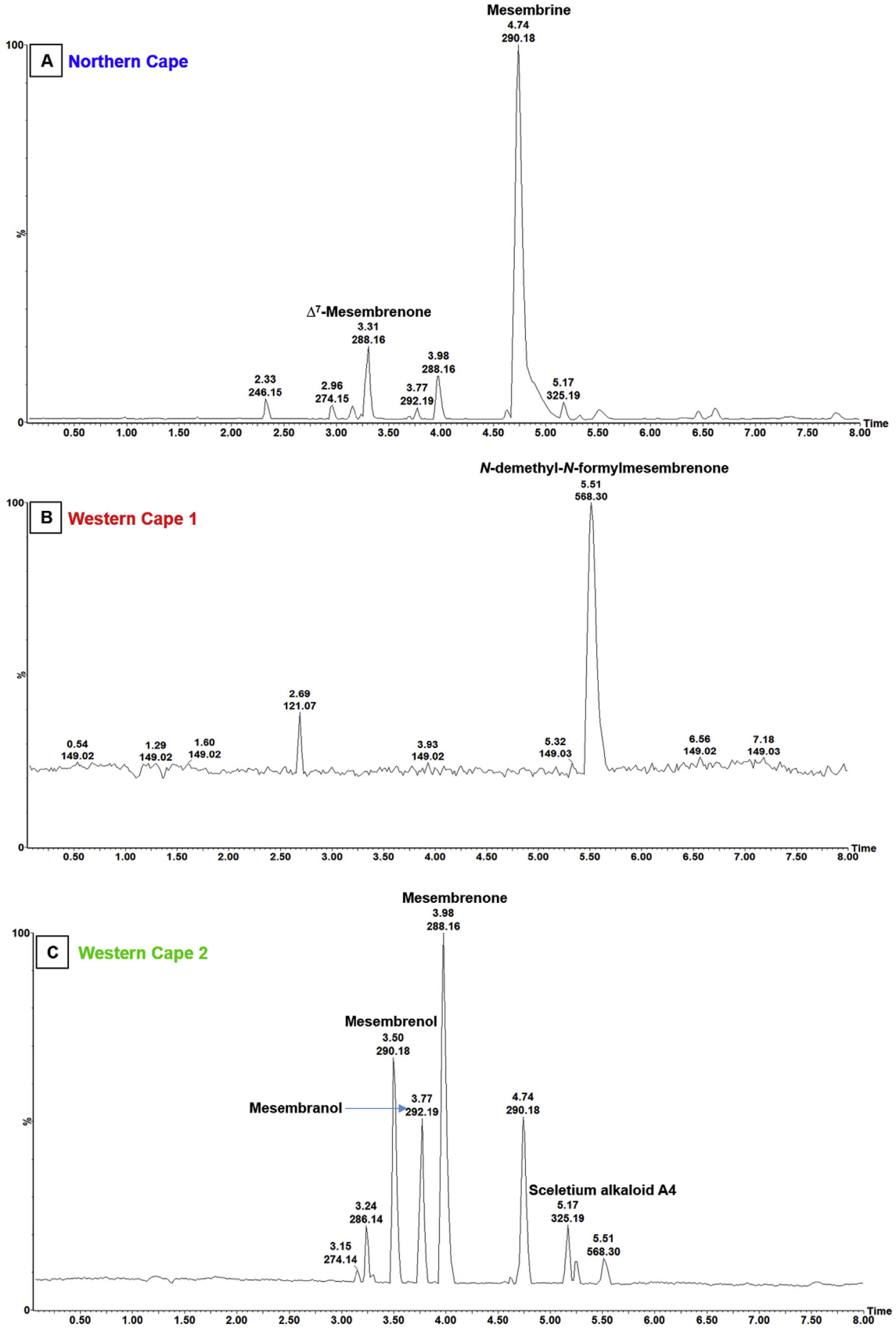

Fig. 7. UPLC-MS total ion chromatograms of A) Northern Cape, B) Western Cape chemotype 1 and C) Western Cape chemotype 2 samples, illustrating marker compounds for each cluster.

Table 1
Variables and the corresponding compounds highly correlated to the clustering pattern observed in *Sceletium tortuosum* samples based on UPLC-MS data.

Retention time (min)	Pseudo-molecular ion [M+H]+ (*m*/*z*)	Compound
Cluster 1 - WC		
5.5201	568.297; 569.299	*N*-demethyl-*N*-formyl mesembrenone
Cluster 2 – NC		
4.7524	290.178; 291.18	Mesembrine
3.3267	288.161	Δ^7-Mesembrenone
Cluster 3 – WC		
3.9791	288.161	Mesembrenone
3.7920	292.192	Mesembranol
3.5146	290.177	Mesembrenol
5.1856	325.192	Sceletium alkaloid A4

(Table 2), when compared to the others. The linearity of the calibration curves over the concentrations range (1.00–100 μg/mL) was confirmed by the high coefficients of determination (R^2) obtained (≥0.995). The LODs for the four alkaloids were similar and ranged from 0.101 μg/mL (mesembranol) to 0.166 μg/mL (mesembrenone). With the exception of mesembranol (84.9%), the recovery of the method was good (96.7–103% recovery). The small percentage RSDs (relative standard deviations) obtained for analyses conducted on the same, and consecutive days, indicated that the instrument yields repeatable results. These results indicate that the method was appropriate for the analysis of the *S. tortuosum* samples to determine the concentrations of the four alkaloids in the plant material.

The actual concentration (mg/kg dry w.) of each alkaloid in the plant material was established from the line formula of the calibration curve (Table 3). Samples from all four Northern Cape Province sites were found to contain, on average, very high total concentrations of alkaloids, ranging from 4465.6 ± 1095.7 to 9382.0 ± 2514.2 mg/kg dry w., with the lowest total alkaloid content measured as 1260.5 mg/kg dry w. in a sample from Ratelkraal. However, the total alkaloid yields of samples from the Western Cape Province were substantially lower, with the lowest average of 16.4 ± 12.5 mg/kg (Ladismith; N = 4) and the highest averages of 4143 ± 3586 mg/kg (Laingsburg; N = 7) and 3454 ± 1102 mg/kg dry w. (Amalienstein; N = 6). The majority of samples from this province contained less than 1000 mg/kg total alkaloids. These results correspond to those of qNMR. The production of alkaloids seems to be related to genetic make-up, rather than to climate, since samples from areas in close proximity to each other, with similar climates, produced variable amounts.

The relative percentage concentrations of mesembrenol, mesembranol, mesembrenone and mesembrine were obtained by expressing each peak area as a percentage of the sum of the peak areas of all four alkaloids (Supplementary S3). Mesembrine was the dominant alkaloid in all 100 samples from the Northern Cape Province (Table 3; Supplementary S3). The average relative percentage of mesembrine ranged from 87.3 ± 8.8% (Ratelkraal) to 95.6 ± 1.2% (Nourivier), while the corresponding average concentrations of mesembrine ranged from 4272.8 ± 1086.7 mg/kg (Nourivier) to 8651.7.0 ± 2451.2 mg/kg dry w. (Paulshoek). These results correspond to those from qNMR that indicated that these sites have the lowest and the highest alkaloid concentrations of samples from this province, respectively. This chemistry corresponds to chemotype C, which is characterised by high levels of mesembrine, as classified by Shikanga et al. (2012) in samples from the Western Cape Province. In that study, 25 of the 151 specimens (16.7%) contained mesembrine as the dominant alkaloid, while in the present study only 23 of the 189 samples (12.1%) from the Western Cape contained at least 50% mesembrine, relative to the other three alkaloids (Supplementary S3). The Middelkop region of the Western Cape Province (N = 6), on average, produced the most mesembrine (1797.9 ± 665.3 mg/kg), but this was substantially less than the concentrations in the Northern Cape samples (Table 3). Mesembrine is known to be a serotonin reuptake inhibitor, indicating that the compound could act as an antidepressant (Gericke and Viljoen, 2008; Harvey et al., 2011). Wild-harvested plants from the Northern Cape Province are an excellent and consistent source of *S. tortuosum* for commercial propagation to obtain botanical material destined for use as an antidepressant, since specimens from this region produce high levels of mesembrine and contain very low concentrations of the other three alkaloids. Low levels of mesembrenol (average concentrations 5.4–9.5 mg/kg dry w.) and mesembranol (average concentrations 10.9–19.0 mg/kg dry w.) were produced by the Northern Cape samples (Table 3).

In contrast to the samples from the Northern Cape Province, which were very consistent in their alkaloid profiles, the chemical compositions and yields of samples from the Western Cape Province were extremely variable. The effect of genetics on alkaloid production is also evident in that specimens differed greatly in the relative amounts of the four alkaloids. Samples from Beaufort West displayed huge variation with two of the ten samples producing exclusively mesembrenol, one producing only mesembranol, three only mesembrenone, and the remainder various ratios of two alkaloids. One sample did not produce any of the four alkaloids. Of all 289 samples analysed, eight samples (2.8%) did not produce any of the four alkaloids and these were all from the Western Cape, but from several sites. This chemical profile corresponds to chemotype A determined by Shikanga et al. (2012) in 19.9% of the samples from the Western Cape. Only 4.2% of our samples from the Western Cape were devoid of alkaloids.

Samples from Amalienstein (N = 6) were the only samples to produce large quantities of mesembrenol (3170.3 ± 1021.4 mg/kg) and mesembranol (6340.7 ± 2043.5 mg/kg) (Table 3). All six samples from this population produced mesembrenol and mesembranol in an approximately 1:2 ratio. The best producers of mesembrenone (1906.0 ± 1085.2 mg/kg) were from the Riversdale area (N = 5), while the best producers of mesembrine were from the Laingsburg region (N = 7; 2794.3 ± 3318.9 mg/kg dry w.). The highly variable nature of plant material from the Western Cape makes a strong case for only obtaining material for commercial use from cultivated stock. Although the earlier study of Shikanga et al. (2012) also indicated that Western Cape samples could not be distinguished by their geographical location, this finding is not true

Table 2
Regression equation, coefficient of determination (R^2), limits of detection (LOD) and quantification (LOQ), accuracy and inter-and intraday precision, as established for the developed UPLC-PDA method of analysis for four mesembrine alkaloids.

Compound	Regression equation	R^2	LOD (ug/mL)	LOQ (ug/mL)	Accuracy (%)	Intraday precision %RSD	Interday precision %RSD
Mesembrenone	$y = 6981x-1496$	0.995	0.166	0.549	101	0.42	0.76
Mesembrenol	$y = 3262x-642$	0.996	0.124	0.408	96.7	0.54	0.60
Mesembrine	$y = 3190x-800$	0.995	0.114	0.378	103	0.60	1.20
Mesembranol	$y = 3292x-1017$	0.995	0.101	0.333	84.9	0.23	0.44

Table 3
Number of specimens (N), average concentration (Ave), highest (H) and lowest (L) concentration, and total alkaloid concentration, based on the acid/base extract, (all expressed as mg/kg dry w.) of four mesembrine alkaloids determined in specimens collected from four localities in the Northern Cape Province (grey fill) and from 23 localities in the Western Cape Province.

Locality	N	Mesembrenol			Mesembranol			Mesembrenone			Mesembrine			Total alkaloid concentration		
		Ave	H	L	Ave	H	L	Ave	H	L	Ave	H	L	Ave	H	L
Garing	20	6.2	26.8	Nd	12.4	53.6	nd	167.9	849.3	34.3	6287.9	11256.0	2523.8	6786.8	12013.1	2769.4
Nourivier	19	9.5	18.7	Nd	19.0	44.8	nd	140.1	189.7	74.5	4272.8	5285.3	2217.6	4465.6	5844.8	2345.2
Paulshoek	19	5.4	49.8	Nd	10.9	99.6	nd	274.2	475.9	116.8	8651.7	14864.0	4455.2	9382.0	15613.1	4926.9
Ratelkraal/KV	42	7.6	34.8	Nd	15.2	69.5	nd	297.0	1355.9	38.3	5883.6	14518.9	639.2	6516.9	15162.0	1260.6
Amalienstein	6	3170.3	4786.3	1972.0	6340.7	9572.6	3942.3	166.0	381.5	64.5	26.0	45.8	nd	3453.7	5112.7	2187.6
Barrydale	12	7.5	41.9	nd	15.1	83.7	nd	11.7	20.9	nd	97.2	403.8	nd	207.6	632.6	nd
Beaufort West	10	3.6	21.3	nd	7.2	42.6	nd	6.9	15.0	nd	8.3	54.8	nd	27.0	68.5	nd
Botmaskloof	6	19.9	30.1	8.9	39.7	60.2	17.7	7.4	11.5	5.6	10.5	13.7	7.1	41.3	45.1	23.0
Calitzdorp	6	16.1	40.9	nd	32.2	81.8	nd	nd	nd	nd	19.9	44.1	nd	96.1	149.9	41.9
Klaarstroom	6	4.6	17.1	nd	9.1	34.1	nd	10.7	46.2	nd	22.1	56.4	nd	99.9	441.2	nd
Kontiki	5	323.7	422.8	156.6	647.3	845.7	309.1	58.6	102.3	40.9	297.0	624.7	44.2	909.3	1159.4	695.2
Ladismith	4	6.5	13.7	nd	13.1	27.3	nd	9.9	13.7	nd	nd	nd	nd	16.4	27.3	nd
Laingsburg	7	159.4	406.7	30.2	318.8	813.4	138.5	207.7	431.3	15.6	2794.3	9877.8	207.4	4143.2	11587.0	958.1
Matjiesvlei	6	59.4	83.4	41.3	118.7	166.8	82.6	567.5	970.2	205.2	1489.2	3523.1	530.6	2583.7	4764.8	1628.8
Merweville	4	11.0	22.1	nd	22.0	44.2	nd	7.6	15.9	nd	nd	nd	nd	26.4	39.7	nd
Middelkop	6	33.1	145.0	nd	66.2	289.9	nd	235.3	996.6	29.0	1797.9	2717.2	33.1	2353.7	3633.1	1450.0
Mossel Bay	6	233.7	880.9	52.0	467.3	1761.8	104.0	303.7	408.5	104.1	103.2	128.5	78.0	753.6	1456.8	537.5
Ockertskraal	7	9.8	22.8	nd	19.5	45.6	nd	3.3	22.8	nd	395.1	845.8	68.0	560.7	1042.7	184.5
Oudshoorn	6	182.2	445.4	30.7	364.4	890.8	61.3	377.9	584.5	123.6	172.5	418.2	81.2	869.2	1411.2	722.7
Oud/MB	6	110.8	232.0	68.8	221.5	464.0	137.6	639.0	804.4	343.4	290.4	450.7	183.1	1268.8	1571.9	816.4
Riversdale	5	353.0	666.1	106.6	705.9	1332.1	213.1	1906.0	3676.4	858.4	385.9	796.7	77.0	2899.9	5426.4	1172.2
Steilsdrift	39	371.9	1195.5	13.0	743.9	2391.1	25.9	559.6	1156.4	114.9	189.4	932.2	60.6	1316.1	2613.3	348.1
St Helena	11	47.5	90.1	20.1	95.0	180.2	40.2	24.3	117.1	nd	35.5	144.2	nd	169.8	684.8	68.0
Vanwyksdorp	3	389.1	844.6	105.4	778.2	1689.1	210.9	100.7	114.3	79.1	30.1	33.0	25.6	639.2	1231.9	276.8
Volmoed	18	77.8	520.8	nd	155.7	1041.6	nd	330.6	1031.0	nd	234.0	715.1	nd	770.8	2177.6	nd
Vrolijkheid	7	7.4	38.8	nd	14.8	77.6	nd	0.8	5.7	nd	6.0	31.9	nd	17.4	38.8	nd
Willowmore	3	10.4	15.7	nd	20.8	31.4	nd	53.5	108.8	20.4	50.1	88.1	20.4	305.7	502.5	147.9

Ratelkraal/KV = Ratelkraal/Kougelvlakte; Oud/MB = Oudshoorn/Mossel Bay; nd = not detected

for specimens from the Northern Cape. The results obtained in the current study confirm that climate is not a determining factor in the chemical profiles of the plant samples. The climate of the Northern Cape Province is dry and semi-arid with summer rainfall, while some of the Western Cape samples also originated from a similar climate (dry, semi desert zones, characterised by summer rainfall) and others came from more moist areas that experience both summer and winter rainfall.

3. Conclusions

The ^{1}H-NMR analysis of 145 samples collected from the different geographic locations revealed variations in their chemical profiles. Pinitol, alkaloids and alkylamines were identified as marker compounds that differentiate Northern Cape and Western Cape samples. qNMR analysis indicated significant differences in the contents of the marker compounds in samples of the two groups. In addition, the study revealed the occurrences of three and two chemotypes for Northern Cape and Western Cape samples, respectively.

Chemometric analysis of the UPLC-MS data indicated the presence of two main groups corresponding to the provinces of origin. Samples from the Northern Cape Province produced substantially higher levels of the alkaloids and specifically mesembrine, which is regarded as of importance for the CNS effects. Δ^7-Mesembrenone was identified as a second marker compound for this cluster. A greater degree of chemical variability was displayed by the Western Cape samples, dividing them further into two clusters. Mesembrenone, mesembranol and mesembrenol were identified as markers for Cluster 2, in addition to sceletium alkaloid A4. A compound, tentatively identified as *N*-demethyl-*N*-formyl mesembrenone, characterised samples from Cluster 3. This compound has not been reported from *S. tortuosum* before.

This study has demonstrated that the NMR methodological approach, together with chemometric analysis, could be an effective tool for investigating metabolite variation in medicinal plants. Furthermore, the findings from this study could add value to the cultivation and better utilization of this indigenous medicinal plant from South Africa.

4. Experimental

4.1. Plant collection and identification

Specimens (N = 289) of *Sceletium tortuosum* (L.) N.E. Br. (Aizoaceae) were collected from the Northern (N = 100) and Western Cape (N = 189) provinces of South Africa (Table 3; Supplementary S4) at the end of a dry winter season in August

2013. Species identification was confirmed by taxonomists based at the South African National Biodiversity Institute (Pretoria, South Africa). Voucher specimens have been retained in the Department of Pharmaceutical Sciences, Tshwane University of Technology (Pretoria, South Africa).

4.2. Sample preparation

A representative subset (145) of the samples were analysed by NMR spectroscopy. The powdered samples (200 mg each) were extracted using 500 μL deuterated methanol (CD_3OD, 99.8% D) obtained from Cambridge Isotope Laboratories, Inc. After being vortexed, the samples were sonicated at 25 °C for 15 min and left to stand for 60 min. Thereafter, they were centrifuged at 13,400 rpm for 9 min using a high speed mini-centrifuge, and the supernatants were transferred to NMR tubes for analysis. The methanolic extract for GC-MS analysis was obtained in the same way as described, but methanol was used instead of deuterated methanol.

For UPLC analysis, all the solvents and reagents used, including ammonia (25% w/w), dichloromethane, methanol and sulfuric acid, (98% w/w) were AR grade and were purchased from Merck (Germany). Initially the methanol extracts were analysed by UPLC, but the alkaloid concentrations were low and it was therefore decided to extract the alkaloids from all 289 samples using typical acid/base extraction as described by Alali et al. (2008), with modifications. Briefly, 60 mL of 0.25 M sulfuric acid was added to 5.00 g of the powdered plant material. After manual shaking, the mixture was filtered and subsequently basified by adding 30 mL of a 20% (v/v) ammonia solution. Alkaloids were extracted into dichloromethane (2 × 35 mL). The combined extracts were evaporated to dryness. Prior to UPLC analysis, the dried extract was re-dissolved in methanol (1 mg/mL) and filtered through a 0.2 μm syringe filter (Bonna-Agela Technologies, USA).

4.3. Reference standards

Four alkaloid standards (mesembrine, mesembrenol, mesembranol and mesembrenone) were previously isolated from *S. tortuosum*, and their purities determined, as described in Shikanga et al. (2011). Sodium 3-trimethylsilyl [2,2,3,3-^{2}H4] propanoate (TSP), isobutylamine and 2-methyl-butanamine were obtained from Sigma-Aldrich, Inc.

4.4. ^{1}H-NMR analysis

Spectra were recorded using an Agilent DD2-500 NMR spectrometer. The instrument was equipped with a One NMR probe operating at 499.79 MHz for ^{1}H and 125.67 MHz for ^{13}C. The PRESAT pulse program in the VnmrJ 4.0 software (Agilent, Santa Clara, CA, USA) was used to suppress the residual water signal. For each sample, 256 transients were collected. The spectra were recorded with the following parameters: spectral width = 8000 Hz, acquisition time = 2.60 s, relaxation delay = 5 s and pulse width = 7.20 μs (90°). Free induction delays (FIDs) were Fourier transformed with 0.3 Hz line broadening. The spectra were manually phased and baseline corrected using Mnova software v. 9.0 (Mestrelab Research S.L.), while TSP was used as the internal standard reference. Two dimensional NMR analysis, including COSY, TOCSY, HSQC, HMBC and HSQC-TOCSY were recorded on selected samples. The measurements of 2D spectra were performed using the standard pulse sequences of VnmrJ 4.0. The optimized coupling constants for HSQC and HMBC were 145 and 8 Hz, respectively. A mixing time of 80 ms was used for the HSQC-TOCSY experiment.

4.5. Gas chromatography-mass spectrometry analysis

Methanolic extracts of the selected samples were analysed by GC-MS using an Agilent 7890B GC system (Agilent Technologies, Santa Clara, CA, USA), coupled to an Agilent 7693 autosampler and an Agilent 5975C quadrupole mass spectrometer. The injector temperature was 280 °C. A 1 μL sample volume was injected, applying a split ratio of 20:1. The system was fitted with an Agilent J&W DB-5MS (5%-phenyl-methylpolysiloxane) fused silica capillary column (30 m × 0.25 mm i. d x 0.25 μm film thickness). Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The oven temperature program started at 40 °C (held for 5 min), where after the temperature was increased to 100 °C at a rate of 1.0 °C/min and then to 280 °C at a rate of 15 °C/min and held for 15 min. Mass spectra were collected in scan mode over the range *m/z* 40 to 500. An ionization voltage of 70 eV was applied. The transfer line and ion source temperatures were programmed at 280 °C and 230 °C, respectively, while the MS quadrupole temperature was 150 °C. Data acquisition was performed using Agilent MassHunter Workstation software (version B.07.03). The components of the sample were identified by matching the compound mass spectral fragmentation patterns with the standard databases (Wiley 09 and NIST 11), using a probability-based matching algorithm. Further identification was made by comparison with the reference standards.

4.6. Ultra performance liquid chromatography analysis

A 1 μL portion of each acid/base extract was introduced by full-loop injection into a Waters Acquity Ultra Performance Liquid Chromatographic system with a PDA detector (Waters, Milford, MA, USA). Separation was achieved on an Acquity UPLC BEH C_{18} column (150 mm × 2.1 mm, i. d., 1.7 μm particle size, Waters), maintained at 30 °C. The mobile phase consisted of 0.1% ammonium hydroxide (Solvent A) and 90% acetonitrile (Solvent B; ultra grade, Romil, USA) at a flow rate of 0.3 mL/min. Gradient elution was applied as follows: 80% A:20% B to 60% A:40% B in 2 min, changed to 50% A:50% B in 4.5 min, returning to the initial ratio in 0.2 min. The UPLC system was interfaced with a Xevo QTOF-MS (Waters, USA). The same column, elution gradient and flow rate were used for the UPLC-MS analysis. Mass spectrometry was carried out in the positive ion electrospray mode. Nitrogen was used as the desolvation gas at a flow rate of 500 L/h, while maintaining a desolvation temperature of 350 °C. The source temperature was 100 °C and the capillary and cone voltages were set to 3000 and 38 V, respectively. Data, collected over the range *m/z* 100 to 1000, were centroided during acquisition using independent reference lock-mass ions *via* the LockSpray™ interface to ensure mass accuracy and reproducibility.

The PDA detector was used for quantitative analysis of the four mesembrine alkaloids in the plant extracts, while the qToF-MS was used for confirmation of identification. Method validation was carried out according to the ICH Harmonised Tripartite Guidelines (ICH, 1994). The linearity of the calibration curve for each alkaloid standard was determined by triplicate analysis of seven calibration standards with concentrations ranging from 1.0 to 100 μg/mL in methanol. The accuracy of the method was determined by comparing the average alkaloid concentrations obtained for the 2.50, 25.0 and 100 μg/mL calibration standards measured from six replicate analyses. Intra- and inter-day precision of the method was established by repeatedly analysing a sample containing known concentrations of the four mesembrine alkaloids. On the first day (intra-day) the sample was analysed six times. For interday variation, the samples were analysed three times a day for three days, after fixed time intervals. The results were expressed as the %RSD of the measurements. In addition, the limits of detection (LOD) and

quantification (LOQ) for each alkaloid were established based on the standard deviation of the response and the slope of the calibration plot (Miller and Miller, 2010).

4.7. Multivariate data analysis

To investigate chemical variation within *S. tortuosum* samples from different geographical locations, NMR spectroscopy and UPLC-MS were used to generate two data sets based on the methanol and acid/base extracts, respectively. Due to the multivariate nature of the data obtained, chemometric tools were applied to extract chemical variability information from these large data sets. The NMR spectra were bucketed with an equal bin width of 0.04 ppm over the region δ 0.6–8.6 ppm, after performing the calibration and normalization using the signal of TSP at chemical shift $\delta_H = 0.00$ ppm. The spectral regions from δ 4.60–5.10 ppm and 3.29–3.31 ppm were excluded to eliminate the effects of water suppression and residual methanol signal. The data set was exported to SIMCA-P+14.0 (Umetrics AB, Umeå, Sweden) for chemometric analysis. The UPLC-MS data was pre-processed using MarkerLynx™ v4.1 (Waters Cooperation, USA), a software that allows comprehensive analysis of 'all' chromatographic peaks in a non-selective manner. During data pre-processing, parameters were set to enable baseline correction, noise elimination, peak selection and spectral alignment of all mass fragments across the sample set. The resulting pre-processed data comprising retention time, mass pairs (variables) and the corresponding amplitudes across the samples was exported to SIMCA-P+14.0.

Multivariate analysis tools that reveal clustering patterns in a dataset were employed to investigate chemical relationships among *S. tortuosum* samples. Initially, unsupervised PCA was applied to the data to investigate the direction of maximum variation. Various scaling methods were investigated and the method that generated the best statistical parameters was selected for further model development. The results for each dataset were analysed in a scores plot, which reveals clustering patterns, and a loadings plot that displays chemical variables that contribute to the patterns observed. To observe clearly the relationships among samples in the scores plots obtained, HCA was performed and dendrograms were constructed.

To investigate further the variation responsible for separation into the different classes, a supervised algorithm, OPLS-DA, was applied. Predictive variation was modelled separately from orthogonal variation, thus clearly demonstrating variation between the classes. The loadings plots obtained from the various models were used to identify variables correlated to the different chemotypes observed. A group to average comparison of the variables for each chemotype was performed to confirm the results of the loadings plots. The model performances were evaluated by considering the cumulative variation within X (R^2Xcum) and the predictive ability of the model (Q^2cum).

Authors' contributions

JZ designed and performed the NMR experiments and analysed the data, WC developed the UPLC-MS method and performed the experiments, MS performed multivariate data analysis of the UPLC data, SC analysed and provided all the quantitative UPLC data. All the mentioned authors contributed to the writing of the manuscript. IK and AV conceived the concept, designed the experiments and contributed to editing of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.phytochem.2018.03.013.

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