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LC–MS-based metabolomics assists with quality assessment and traceability of wild and cultivated plants of *Sutherlandia frutescens* (Fabaceae)

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Abstract

The metabolite profiles of *Sutherlandia frutescens* populations may vary depending on their geographical location, affecting the quality of plant-based pharmaceutical products generated from this species. This paper aims at using metabolic profiling through liquid chromatography mass spectrometry (LC–MS) to assess the metabolite content of seed pods from populations of the medicinal plant *S. frutescens* growing in geographically different environments. Terpenoid (retention time: 7.5–9.0 min) and flavonoid (retention time: 15.0–19.0 min) regions of the chromatograms were useful in distinguishing between samples and five distinct clusters were revealed after principal component analysis (PCA). This may assist in tracing the region where plants actually come from and the identification at the subspecies level. To increase the class separation and simplify interpretation, we focused on those populations from the arid Karoo and the coastal Gansbaai area, applying orthogonal partial least squares discriminant analysis (OPLS-DA) to organize these into two clear groups. The presence or absence of sutherlandioside B (SU1) and its derivatives contributed significantly to the separation of the Karoo plants from those from the Gansbaai cluster. Processing procedures of herbal products require standardization, but this becomes challenging when plants do not contain key chemical principles. Extracts from the Gansbaai population had virtually undetectable levels of SU1; consequently products manufactured from farmed plants originating from this region may lack these compounds, which are now proposed to be anti-cancerous. There were several sutherlandioside-type metabolites that distinguished populations from each other. These chemicals may add new knowledge in terms of the broader metabolomic understanding of Sutherlandia populations and their potential pharmacological action. *In vitro* plants generated as part of a commercialization–conservation strategy had a similar metabolite profile to non-propagated plants. In fact, these plants could be traced to the West Coast populations, further confirming their identity. This study highlighted that SU1 cannot be used as the only quality control marker for Sutherlandia products, since it does not occur in all populations and there is no conclusive evidence that it is the main active ingredient of the plant. The effect of α-naphthalene acetic acid (NAA) at 1 mg l^{-1} was tested on *in vitro* plants. Sutherlandiosides and sutherlandins were detectable in treated plants. Although the treatment had impacts on the growth capacity of plants, SU1 did not accumulate at higher levels in auxin-treated plantlets. The similarity of micropropagated plants to wild plants proved that tissue culture does not have deleterious effects on the chemistry of Sutherlandia plants. Metabolomic approaches using LC–MS are thus an important feature as a diagnostic tool and should be integrated into the herbal product manufacturing process utilizing Sutherlandia and its extracts.

Keywords: Cancer bush; Medicinal plant; MS^E; Principal component analysis; Sutherlandioside

1. Introduction

The leguminous plant *Sutherlandia frutescens* (syn. *Lessertia frutescens*, Fabaceae) is the only indigenous, South African medicinal plant that is known as the "cancer bush" (Van Wyk et al., 2009). It displays bright red to orange flowers (Fig. 1A–B), one of the distinguishing features of this plant. Even though

Abbreviations: DM, dry mass; GABA, gamma-aminobutyric acid; LC–MS, liquid chromatography mass spectrometry; NAA, α-naphthalene acetic acid; OPLS DA, orthogonal partial least squares discriminant analysis; PCA, principal component analysis; SU1, sutherlandioside B.

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claims on phyto-extractions date back more than 100 years to 1895 when Smith reported that these plants can 'cure malignant tumours, delaying the progress of true cancer' (Smith, 1895), the debate on this aspect is still ongoing. Although reports based on its cancer preventing and therapeutic capacity are often conflicting, *S. frutescens* has become a highly-valued commercialized plant for the production of a variety of phytopharmaceutical products (Van Wyk and Albrecht, 2008). It is well known as a 'multi-functional, all-purpose' herbal remedy that stands inextricably linked to the anthropology of Nama, Khoi and San populations (Fernandes et al., 2004). It is taken as a supportive treatment for a wide array of ailments, suggesting a plethora of metabolites with pharmacological activity (Van Wyk and Albrecht, 2008). Sutherlandia is thus consumed for stomach problems, diabetes, anxiety and stress, inflammation, and microbe-related diseases, amongst a range of others (Moshe et al., 1998; Fernandes et al., 2004). It is also sold to assist with the control of sugar homeostasis in diabetics due to its proven hypoglycemic action (Chadwick et al., 2007). The latest research on Sutherlandia extracts using animal models shows how it regulates insulin and fatty acid metabolism for purposes of controlling Type 2 diabetes (MacKenzie et al., 2009, 2012). It has a growing reputation as an adoptogen (Van Wyk, 2012) and has been highlighted as an appetite-stimulant, helping to reduce muscle atrophy. It is

Fig. 1. A) Characteristic red flowers of *Sutherlandia frutescens* of a plant from the West Coast area of Melkbostrand; B) Plants in flower growing in a coastal area; C) Seed pod of a 'garden-grown' *S. frutescens* plant (origin unknown) with seed inside; D) Seed pod of a wild plant that is undergoing colourimetric changes during its development in a sandy soil habitat.

claimed to positively promote health in terminally-ill patients through its immune-boosting effects (Fernandes et al., 2004; Van Wyk and Albrecht, 2008).

The response to the chemical identification and quantification of secondary metabolites and broader metabolomic information on this herb (Tai et al., 2004; Olivier et al., 2009; Avula et al., 2010) has been slow, despite it featuring prominently in the commercial range of phytopharmaceutical products in South Africa. Consequently, information on the chemical composition, both quantitative and qualitative aspects, remains fragmented and disparate. The amino acid chemistry has received better attention than the secondary metabolites and so several amino acids have been used in the past for quality control purposes (consult Mncwangi and Viljoen, this issue). A growing body of evidence confirming its anti-proliferating and apoptotic effects using cancer cell lines and leaf extracts is now available. For examples refer to the work of Tai et al. (2004; human leukemia and breast cancer); Chinkwo (2005; cervical cancer); Stander et al. (2007; 2009); Vorster et al. (2012; breast cancer); and Skerman et al. (2011; oesaphageal cancer). Whereas most of these studies showed some anti-cancer activity at the concentrations tested, it is highly debatable whether these concentrations have physiological efficacy or not. Most dietary supplements, in the form of capsules or tablets, contain SU1 at 1–5 mg g^{-1} average weight of sample (Avula et al., 2010). Sutherlandia plants are found in varying biomes in different geoclimactic locations and are thus tolerant to different environments. This invariably leads to a tremendous amount of chemical heterogeneity, particularly between different populations. Recent focus on understanding key metabolites that may feature in the broad bioactivity of these plants has led to the appreciation of its complex chemistry being a cocktail of flavonoids, triterpenes, saponins and other plant steroids (Van Wyk and Albrecht, 2008 and references therein). Natural genetic hybridization changes the chemotypic expression in plants and this ultimately has a profound influence on the metabolome (Kuhajek et al., 2004). Some consider *S. frutescens* to be a species complex as it shows a mosaic of highly variable morphological and chemical characteristics that are coupled to distinct, discontinuous geographical localities (Van Wyk and Albrecht, 2008). Furthermore, it has been postulated that Sutherlandia undergoes continuous and extreme metabolomic variation and that certain extremely unlikely metabolite patterns, particularly with regards to the flavonoids and triterpenoids, contribute significantly to pharmacological mixtures that have the capability to induce tumour-cell death selectively (Albrecht, 2008). Chemical signatures that characterize these plants are thus required, particularly for assessing the quality of herbal products and to assist with standardization. The recent isolation and structural elucidation of unique chemicals, aptly named sutherlandiosides (terpenoids/cycloartanol glycosides) (Fu et al., 2008, 2010) and sutherlandins (quercetin and kaempferol-derived flavonoids) (Avula et al., 2010) has been a major breakthrough. This provides us with an opportunity to exploit these compounds, solely synthesized by *S. frutescens*, for metabolite fingerprinting purposes as these are now thought to be linked to the inhibitory effects of extracts on cancer cell lines.

Imminent commercialization within the international natural products industries, which has rigorous quality standards, requires quality-assured plant material produced using high-level manufacturing practices. Like so many other medicinal plants, Sutherlandia is often collected from wild populations as few farmers are actively engaged in cultivating this plant as a crop (Van Wyk and Albrecht, 2008). The highly variable nature of Sutherlandia plants thus presents a quality control challenge. In general, farming of medicinal plants in South Africa is limited. The current level of farm production does not meet commercial demand, even with developments of large-scale farming by individual companies since the late 1990s (Van Wyk, 2012). Alternative strategies to access this biodiversity that are ecologically friendly and commercially sustainable are required. With this in mind, we generated plants in tissue culture (Colling et al., 2010) as part of a germplasm conservation practice to reduce harvesting pressures. This would also provide large volumes of quality stock for an ever-growing pharmaceutical industry. Wild plant populations exhibit inherent genetic-to-chemical heterogeneity with some populations being superior to others, *in vitro* culture offers a method of fast preserving desired chemo-elite types. Cultivation of Sutherlandia through seed technology is not a feasible option. With seed germination being precarious and unpredictable, requiring chemical treatments to break dormancy (Shaik et al., 2008), *in vitro* propagation has been explored by Colling et al. (2010) and Shaik et al. (2010, 2011). There are many advantages to tissue culture for Sutherlandia, including: 1) it allows for the rapid production of disease-free plants using leaf material, which is an easily renewable explant source for culture induction; 2) propagules may fit into a phytopharmaceuticals supply chain platform using *in vitro*-derived plants to produce a cultivated field crop; 3) as plants are produced under aseptic conditions, this reduces quarantine restriction related to international export of biomaterial; and, 4) as plants are available all year round this negates problems associated with inaccessibility in the case of plant varieties that are adapted to unique niche environments (Nigro et al., 2004; Fennell et al., 2006).

Plant tissue culture has been part of biotechnological approaches that have fast-tracked our overall understanding of plant biosynthetic pathways and their regulation. Micro-propagation techniques have aided in cultivating new genetic variants, leading to establishment of superior plant cultivars that are now in agricultural cultivation. Moyo et al. (2011) emphasized the role of mass micropropagation of medicinal cultures synthesizing active principles using bioreactors as one direction to 'innovate the concept of indigenous medicinal plant knowledge in South Africa', creating new economic growth opportunities. Several bioreactor protocols have been described for Sutherlandia, which can assist with standardization (Shaik et al., 2011).

Here, a comparative metabolomic study using high-throughput liquid chromatography–mass spectroscopy (LC–MS) of a set of plant samples from distinct geographic locations (Table 1) was explored. It was hoped that this would reveal extremely rare chemo-variants, with higher levels of sutherlandiosides and sutherlandins, which in the future would be worth evaluating

Table 1
Geographic locations of Sutherlandia species collections sampled analysed with targetted and non-targetted LC–MS. The concentration of sutherlandiose B (SU1) is represented in percentage (%) dry mass (DM).

No.	Facile name	Details of location	GPS S (south)	GPS E (east)	SU1 (% DM)
1	Garden grown-A	Spontaneous appearance in 2010	35° 51′ 32.12′	18° 39′ 45.69′	0.002 a
2	Garden grown-B	Growth continuation in 2011	35° 51′ 32.12′	18° 39′ 45.69′	0.001 a
3	Karoo	Winifred Grobler, Murraysburg	31° 57′ 46.31′	23° 45′ 36.20′	0.993 g
4	Albertina	Near Albertina	34° 11′ 58.89′	21° 35′ 00.29′	0.642 d
5	Stanford	Between Hermanus and Gansbaai	34° 26′ 29.67′	19° 27′ 19.84′	0.008 a
6	Grootbos	Between Stanford and Gansbaai	34° 32′ 20.42′	19° 24′ 46.97′	0.002 a
7	Blomerus	Beyond Grootbos	34° 35′ 31.59′	19° 32′ 13.96′	0.005 a
8	Gansbaai	Four km from town towards Stanford	34° 33′ 03.61′	19° 23′ 38.74′	0.000 a
9	Franskraal	Between Kleinbaai and Franskraal	34° 37′ 36.54′	19° 28′ 14.30′	0.006 a
10	Uilkraalmond	Walker Bay Nature Reserve	34° 37′ 02.55′	19° 26′ 21.09′	0.000 a
11	Blouberg	Used by VULA Pty Ltd to bind sand	33° 47′ 35.63′	18° 27′ 38.35′	0.170 b
12	Namaqualand	Unknown			0.526 c
13	Weskus	Near Mykonos	33° 02′ 29.19′	18° 02′ 37.04′	0.836 f
14	Yzerfontein	Between N7 and Yzerfontein	33° 00′ 02.74′	18° 14′ 24.80′	0.198 b
15	Duwisib	Duwisib Castle South Namibia	25° 15′ 27.65′	16° 32′ 04.48′	0.855 e

All letters that are different represent statistical significance using the Tukey's HSD test ($p \leq 0.5$).

for their pharmacological potential. To this end, dried pods (Fig. 1C–D) from 13 different geographical locations plus two cultivated garden specimens were studied as very little, if any, work has been done on Sutherlandia pods. Both leaves and seed pods are used in traditional medicine (Van Wyk and Albrecht, 2008), however, only leaf extracts have previously been studied. We were also interested in assessing the presence of triterpenoids and flavonoids in the pods, as this may have therapeutic implications. Additionally, populations from various geo-spatial regions were easily distinguishable grouping into distinct chemical lineages using unsupervised chemometric-based clustering. *In vitro* cultured plants could thus be easily traced to populations of coastal origin, meeting the second aim of this study of using LC–MS as a metabolomic diagnostic tool. Using this feature to characterize both the quality and quantitative composition of *in vitro*-generated plants, we could confirm that the tissue culture method was appropriate and that the propagules remain true to their source chemotype. The effect of auxin, a plant growth regulator in the form of NAA, was used *in vitro* to increase plant biomass. This tissue culture application was able to generate large, quality-assured, commercializable volumes of *S. frutescens* microplants. Sutherlandins and sutherlandiosides were ideal quality marker candidates assisting with the visualization of mass spectrometry data.

2. Materials and methods

2.1. Plant material

2.1.1. Wild collections

The *S. frutescens* wild specimens were collected from different sites throughout the Western Cape and Northern Cape Provinces (South Africa). The collection sites are listed in Table 1 and plant collections were assigned numbers 1 to 15. Samples are denoted according to the geographic locality where they were harvested and characteristic taxonomic features such as bright red flowers and pods (Fig. 1) were used by Carl F Albrecht to identify material. Plants collected (in 2009) from populations growing in the Melkbostrand (West Coast) for *in vitro* propagation are deposited as voucher specimens in the Department of Botany and Zoology Herbarium (Stellenbosch University).

2.1.2. In vitro culture

In vitro cultures of *S. frutescens* were generated as described by Colling et al. (2010). These plants are kept in a continuous culture system by subculturing nodal explants onto full-strength Murashige and Skoog (1962) medium (Highveld Biological, South Africa), supplemented with 30 g l^{-1} sucrose, 0.1 g l^{-1} myo-inositol and 10 g l^{-1} agar–agar (Merck, Germany). The pH of the medium was adjusted to 5.8 using 1 M NaOH or 1 M HCl prior to the addition of the agar. Media were sterilized by autoclaving for 20 min at 122 kPa and 120 °C. Before aseptic transfer under laminar flow conditions, 10 ml medium was poured into glass test tubes (7 cm × 2 cm). Routine subculture took place at the end of four weeks, by transferring 1–2 cm nodal explants (with one axillary bud) to glass test tubes (7 cm × 2 cm). Plastic caps were used as test tube closures and were secured with a strip of Parafilm (American National Can™, USA). Plantlets were left to develop *in vitro* inside a growth room with a photoperiod of 16 h light and 8 h of darkness daily. The room was fitted with cool white fluorescent tubes (Osram model L75W/20X [F96712], USA), providing a photosynthetic photon flux density of 50 µmol m^{-2} s^{-1}. The temperature was thermostastically controlled at 23 ± 2 °C.

To test the effect of 1 mg l^{-1} α-naphthalene acetic acid (NAA) on growth and secondary metabolite profiles, 15 explants were used. One individual explant was cultured per test tube, representing one replicate. Fifteen replicates were used and a randomized design was applied. As a control, NAA was omitted from the culture medium. Plants were left to grow for 30 days. Upon harvesting, the fresh and dry mass of the shoots was determined, the length of each shoot was measured and the number of axillary bud outgrowths recorded. For metabolite extraction, samples were freeze-dried for 24 h (Virtis Benchtop K; SP Scientific, USA) and then stored at −80 °C until metabolite analysis.

2.2. Statistical analysis: In vitro culture experiment

All *in vitro* culture experiments were conducted at least three times for 28 days to generate growth data. To reiterate, fifteen samples were used in each treatment for growth experiments. For quantification of SU1 in the shoots, five replicates were used to measure the SU1. All data were subjected to normality testing (Shapiro Wilk's W test), prior to analysis of variance (ANOVA). For the data which were not normally distributed, Kruskal–Wallis analysis was used to separate means. When the data were normally distributed, a Tukey's Honestly Significant Difference (HSD) test was applied. Arcsine transformation for all percentage data were used before ANOVA. All tests were conducted at the 95% confidence level using the Statistica version 8 software programme (Statsoft Inc. 2007).

2.3. Sample preparation: Metabolite profiling

All plant material was ground to a fine powder and 50 mg from each sample was extracted with 50% (v/v) acetonitrile (Romil far UV grade; Microsep, South Africa) containing 0.1% (v/v) formic acid (Sigma-Aldrich, South Africa). In all cases, ultrapure water (MilliQ) was used as a diluent. For each extraction, 2 ml of extraction solvent was added to the plant powder and all samples were sonicated in an ultrasonic bath for 30 min. Samples were left for 16 h in the solvent at room temperature before analysis. To remove cell debris, samples were filtered and diluted ten-fold before LC–MS analysis. Extractions were performed in triplicate for statistical processing and all samples were randomized to eliminate changes in the instrument conditions over time.

2.4. Liquid chromatography mass spectrometry (LC–MS) analysis

LC–MS and LC–MS/MS analysis were conducted on a Waters Synapt G2 quadrupole time-of-flight mass spectrometer (Milford, MA, USA) (Fig. 2). The instrument was connected to a Waters Acquity ultra-performance liquid chromatograph (UPLC) and Acquity photo diode array (PDA) detector. Ionisation was achieved with an electrospray source using a cone voltage of 15 V and capillary voltage of 2.5 kV and both positive and negative modes were utilized. Nitrogen was used as the desolvation gas at 650 l h^{-1} and the desolvation temperature was set to 275 °C.

2.4.1. LC Method 1

A Waters UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm particle size) was used and 3 μl was injected for each analysis. The gradient started with 100% using 0.1% (v/v) formic acid (solvent A) and this was kept 100% for 0.5 min, followed by a linear gradient to 22% acetonitrile (solvent B) over 2.5 min, 44% solvent B over 4 min and finally to 100% solvent B over 5 min. The column was subjected to 100% solvent B for an additional 2 min. The column was then re-equilibrated over 1 min to yield a total run time of 15 min. A flow rate of 0.4 ml min^{-1} was applied. Data generated from these runs were subjected to principal component analysis.

2.4.2. LC Method 2

A longer column with the same packing: Waters UPLC BEH C18 column (2.1 × 100 mm, 1.7 μm particle size) was used. The injection volume (3 μl) was kept the same as described but

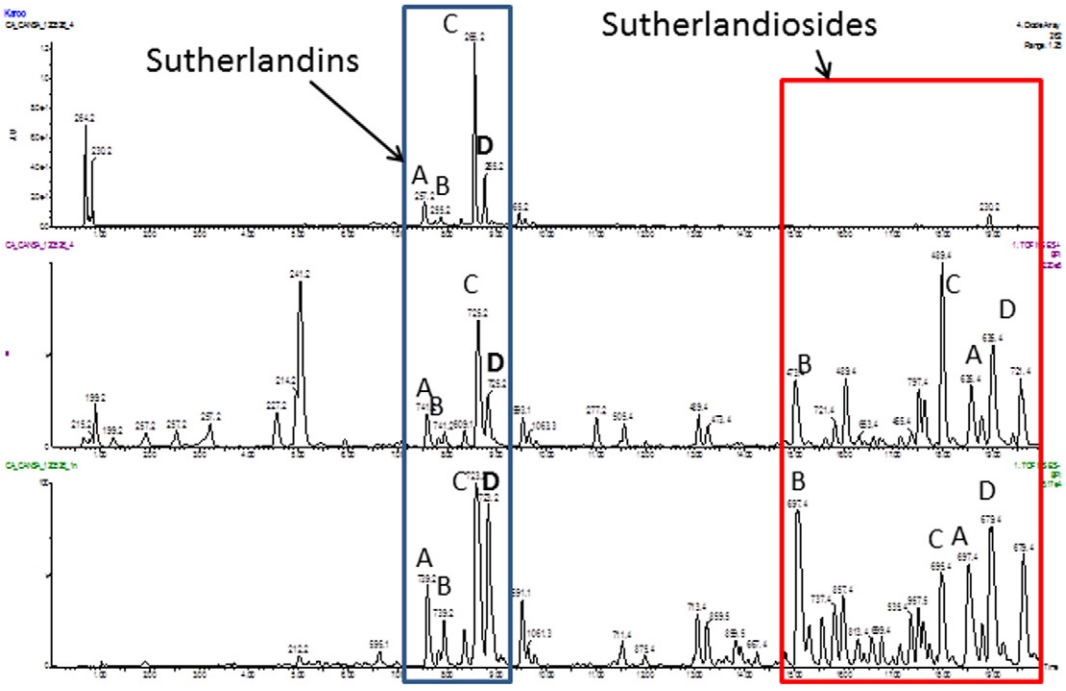

Fig. 2. LC–MS chromatogram of the Karoo extract of Sutherlandia showing the UV trace at 260 nm (top), electro-spray ionisation in the positive mode (middle) and negative mode (bottom).

Table 2
LC–MS of sutherlandins and sutherlandiosides identified using positive and negative ESI scanning and key fragment analysis.

A

	Exact mass	ESI negative	ESI positive			
		$[M-H]^-$ (Base peak)	$[M+H]^+$ (Base peak)	$[M+H\text{-sugar}]^+$	Fragment	UV max
Sutherlandin A	740.1800	739.1722	741.1877	609.1433	303.0491	263.2; 351.2
Sutherlandin B	740.1800	739.1725	741.1884	609.1457	303.0496	255.2; 350.2
Sutherlandin C	724.1851	723.1785	725.1946	593.1517	287.0547	265.2; 351.2
Sutherlandin D	724.1851	723.1782	725.1941	593.1516	287.0558	265.2; 345.2

B

	Exact mass	ESI negative		ESI positive						
			$[M+\text{formate}]^-$ (Base peak)	$[M+H]^+$	$[M+H\text{-}H2O]^+$	$[M+H\text{-glu}]^+$	$[M+H\text{-glu-}H_2O]^+$	$[M+H\text{-glu-}2H_2O]^+$	$[M+H\text{-glu-}3H_2O]^+$	$[M+H\text{-glu-}4H_2O]^+$
Sutherlandioside A	652.4186	651.4086	697.4169	653.4161	635.4272 (Base peak)	491.374	473.3629	455.3516	437.3416	419.3305
Sutherlandioside B	652.4186	651.408	697.4161	653.4281	635.4281 (Base peak)	491.3741	473.3635	455.3532	437.3417	419.3275
Sutherlandioside C	650.4030	649.3942	695.3993	651.4123	633.4034	489.3588 (Base peak)	471.3475			
Sutherlandioside D	634.4081	633.3932	679.4061	635.4161 (Base peak)	617.4049	473.3629	455.352	437.3412	419.3308	

the gradient started with 80% of solvent A which preceded a holding step of 0.5 min. This was then followed by a linear gradient to 44% solvent B over 14.5 min leading to 100% solvent B (1 min). The column was kept at 100% solvent B for another minute followed by re-equilibration over 4 min to yield a total run time of 22 min. A flow rate of 0.35 ml min^{-1} was applied. The MS data were acquired by using a low energy of 6 V for LC–MS data and the trap collision energy was ramped from 15 to 60 V to generate fragmentation data (MS^E). MS^E is a powerful technique which enables the collection of MS fragmentation data at all times for all compounds eluting off the column, whereas normal LC–MS/MS techniques only perform MS/MS fragmentation on predefined ions or criteria.

2.5. Sutherlandioside and sutherlandin analysis

A reference standard of SU1 was used for quantification of this chemical in both seed pods of wild plants, seed pods of cultivated plants grown in the garden and *in vitro* leaves. This standard was isolated by V. Gabrielse (refer also to Van Wyk and Albrecht, 2008). The SU1 calibration curve had a linear range of 0 to 50 ppm ($R^2 = 0.998$) with a detection limit of 10 ppb. The relative standard deviation (%RSD) between the 3 replicate injections was less than 3% for all samples. The other sutherlandiosides (A, C, and D) together with the sutherlandins (A, B, C and D), were identified using the techniques described in detail by Avula et al. (2010). In conjunction with the

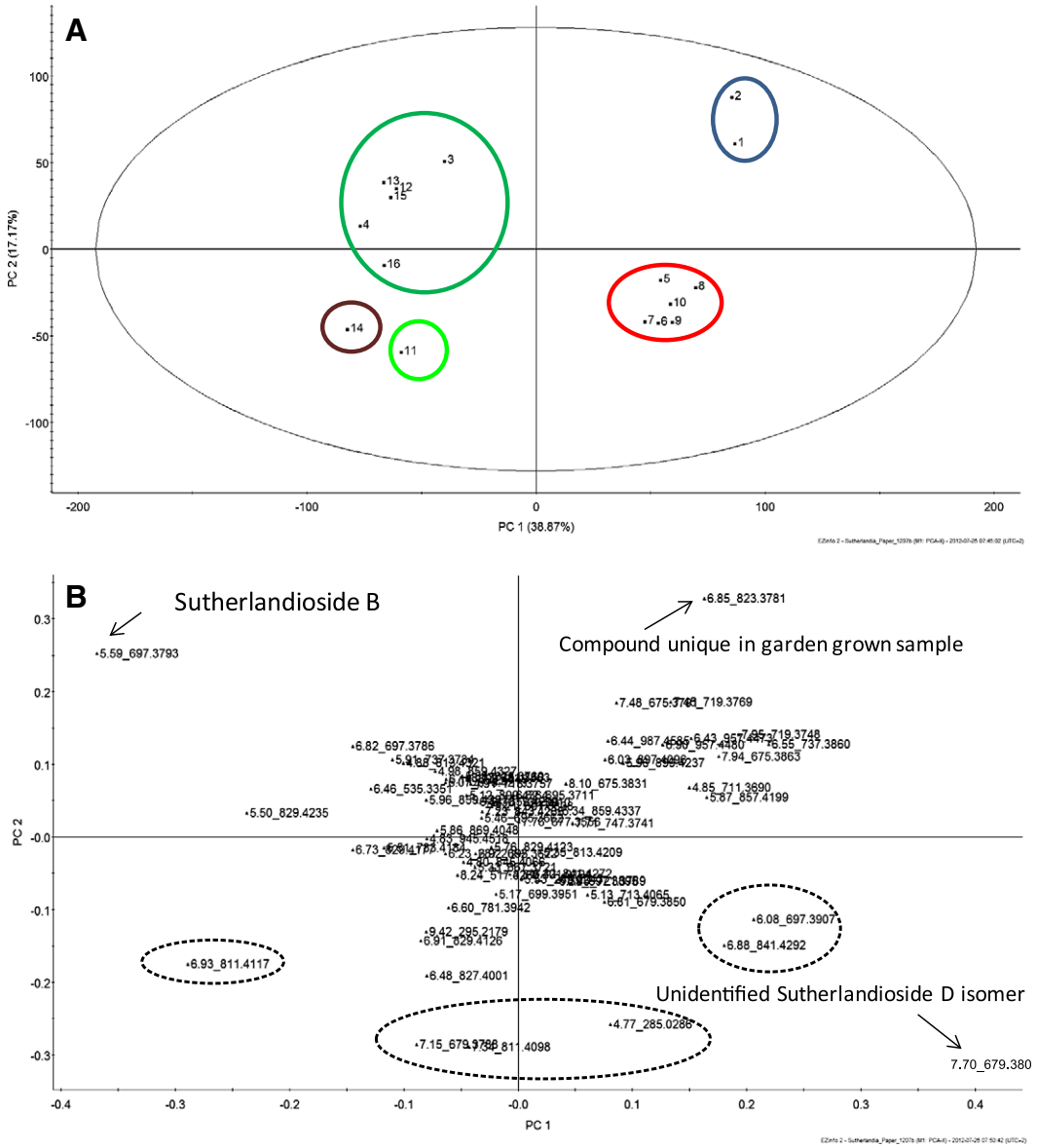

Fig. 3. A) Score plot of principal component analysis based on LC–MS spectra of wild and garden-grown plants of Sutherlandia. Populations are labelled according to Table 1. Three replicates were represented for each sample set but for clarity of points, only one is shown. B) Loading of principal component analysis analysis based on LC–MS spectra of wild and garden-grown plants of Sutherlandia. Arrows indicate sutherlandioside B and sutherlandioside-like terpenoids. Circles are assigned to unknown compounds.

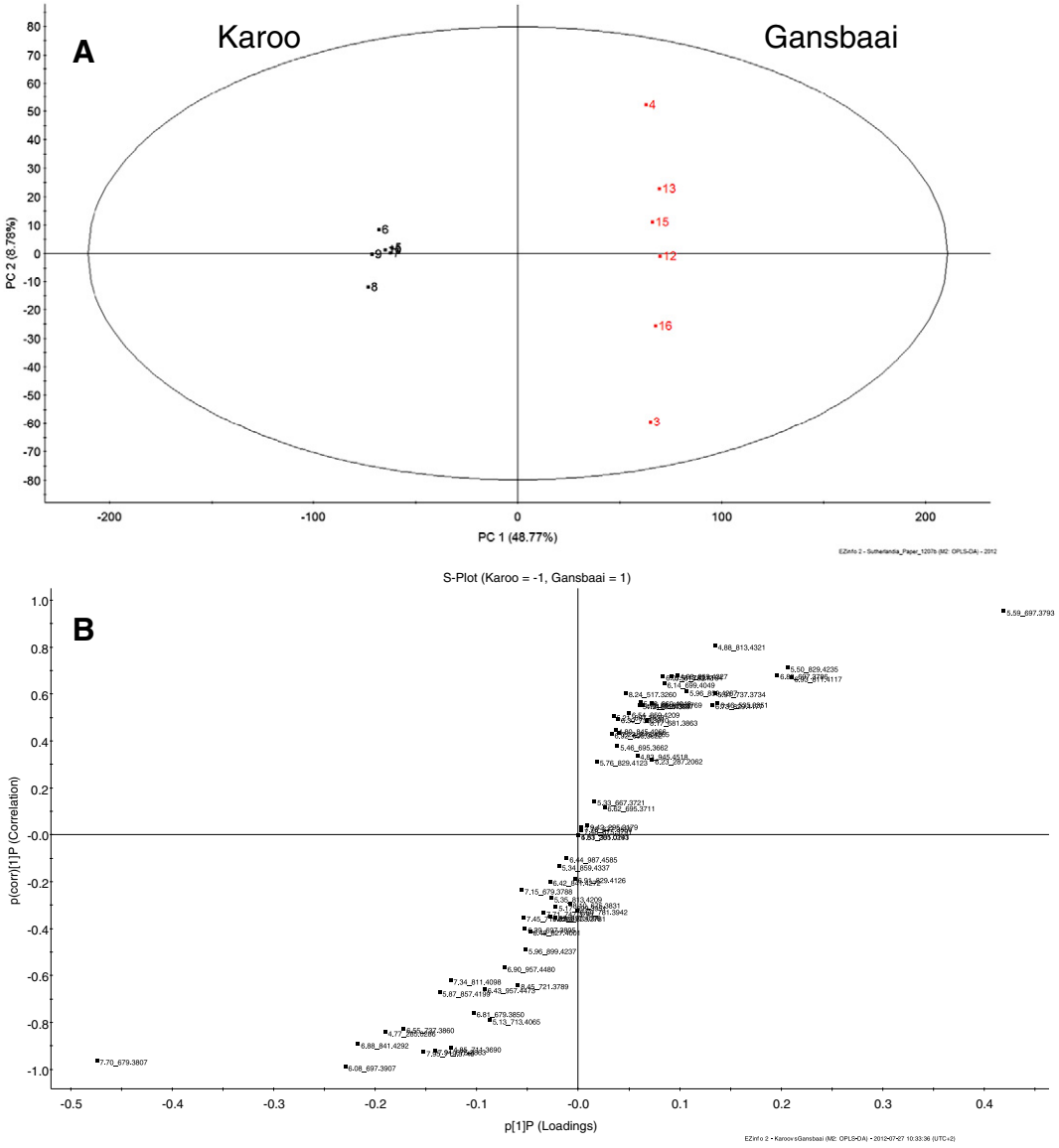

Fig. 4. A) Score plot of orthogonal partial least squares discriminant analysis of LC–MS spectra of Gansbaai and Karoo plant populations of Sutherlandia. Three replicates are represented for each sample set. B) Score plot of orthogonal partial least squares discriminant analysis of LC–MS spectra of Gansbaai and garden-grown plant populations of Sutherlandia. Three replicates were analysed for each sample set but for simplicity only one is shown.

presented chemical structures (Avula et al., 2010), the identification was based on UV spectra (supplementary data), the retention time (Fig. 2) and MS data (Table 2; supplementary data).

2.6. Chemometric data processing

The Masslynx version 4.1 (Waters) was used to acquire and process the data. Targetlynx and Markerlynx were the application managers within Masslynx software that were used to quantify SU1 for *ex vitro* wildtypes (Table 1, Figs. 3–4) and *in vitro* cultured plantlets and to perform the multivariate analysis, respectively. The Markerlynx method was set to process data from a retention time of 2.1 to 9.8 min, over the mass range 250 to 1200 Da and a mass window of 0.01 Da. Smoothing and noise elimination level of 1000 were used. The PCA-X model with pareto scaling was used as an unsupervised multivariate cluster technique. Pareto scaling increases the contributory effects of low concentration metabolites but does not amplify noise and artefacts linked to metabolomics data (Cloarec et al., 2005). Therefore, this simplifies the interpretation of loading score plots (Heyman and Meyer, 2012). Inter-relationships were revealed through PCA groupings, clusters and outliers amongst 16 observations for 67 variables based on 3 components (MS data, relative abundances and spatial distribution) for the test populations. Three biological replicates and three technical replicates were injected randomly for each test sample. The instrument variation was negligible and replicates clustered close to each other (supplementary data), therefore only one representative is shown to simplify the

data (Fig. 3). Intra-replicate patterns are shown for Fig. 4A depicting the OPLS-DA groups. Separation is based on 16 observations and 67 variables for Fig. 4A. Pareto scaling was also used for the OPLS-DA test.

3. Results and discussion

3.1. Profiling of wild and garden-grown populations

The detection of sutherlandins and sutherlandiosides in extracts of Sutherlandia is being developed as a quality control protocol and here we were able to successfully identify some of these flavonoid and terpenoid compounds using various LC–MS modes (using positive and negative ESI, and MS^E). In fact, four sutherlandins and four sutherlandiosides were identified through integrating the retention time, UV spectra and MS fragmentation data for accurate mass-based identification (Table 2). The LC spectral traces and representative structures are presented in Fig. 2. The LC-ESI–MS data from positive and negative modes is presented in Table 2 and the fragmentation patterns for the eight biomarkers were very similar to those published by Avula et al. (2010). For example, sutherlandin A, showing the *m/z* 303.0491 fragment indicative of quercetin and sutherlandin D, showing the *m/z* 287.0558 fragment indicative of the kaempferol moiety, were detected using ESI^+ (Table 2; Supplemental data). The theoretical exact mass of SU1 is 652.4186. This corresponds to the following ions detected using electrospray ionisation in the negative mode (ESI^-): *m/z* 651.4080 $[M\text{-}H]^-$; and *m/z* 697.4161 $[M+\text{formate}]^-$. In electrospray ionisation in the positive mode (ESI^+): the following ions were identified: *m/z* 653.4281 $[M+H]^+$; *m/z* 635.4281 $[M+H\text{-}H_2O]^+$; *m/z* 491.3741 $[M+H\text{-glu}]^+$; *m/z* 473.3635 $[M+H\text{-glu-}H_2O]^+$; *m/z* 455.3532 $[M+H\text{-glu-}2H_2O]^+$; *m/z* 437.3417 $[M+H\text{-glu-}3H_2O]^+$ and *m/z* 419.3275 $[M+H\text{-glu-}4H_2O]^+$. These compounds were detectable with the short LC–MS method, although the longer method (termed LC–MS method 2) resulted in better peak-to-peak separation. These compounds are labelled as denoted by Avula et al. (2010) and they are in the retention time range of 7.5–9.0 min and 15.0–19.0 min, respectively. To assess and quantify the level of chemotype variation between the various populations of Sutherlandia, we subjected the spectra to multivariate analysis. The PCA analysis of the LC–MS spectra is presented in Fig. 3A. The PC 1 and PC 2 explain 38.87 and 17.17% of the variance and this enabled good statistical separation amongst the different populations according to their spatial (regional) identities. Bearing in mind that those samples with the greatest variance are displayed in PC 1 (Van der Kooy et al., 2008), with this data we could develop the concept of there being at least two major groups of *S. frutescens*. Those plants containing the SU1

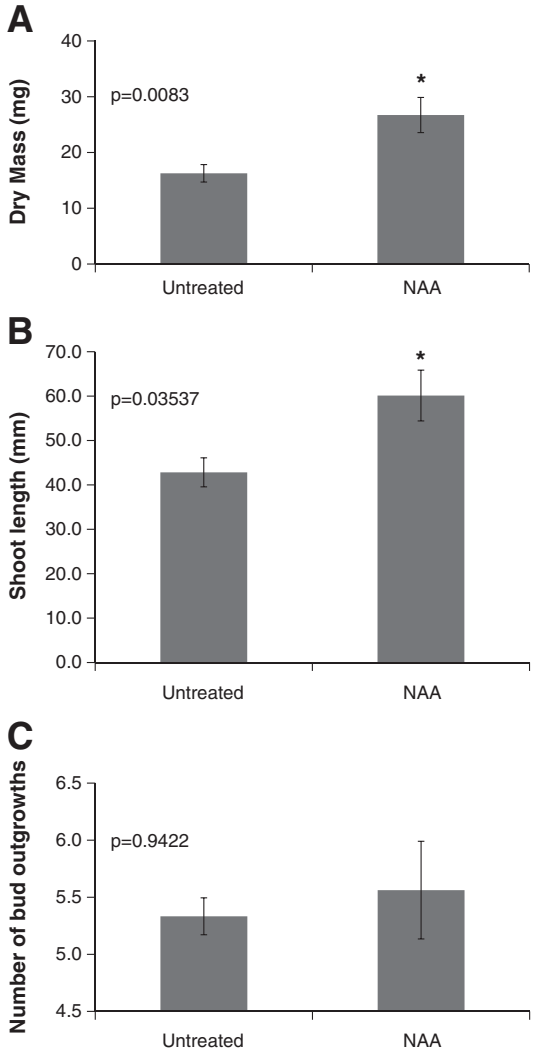

Fig. 5. Effect of 1 mg l^{-1} NAA on *in vitro* plants of Sutherlandia. A) Dry mass; B) shoot length and C) number of axillary bud outgrowths. * Indicates statistical significance ($p \leq 0.05$; N=18 for the untreated control and 16 for the NAA-treated plants).

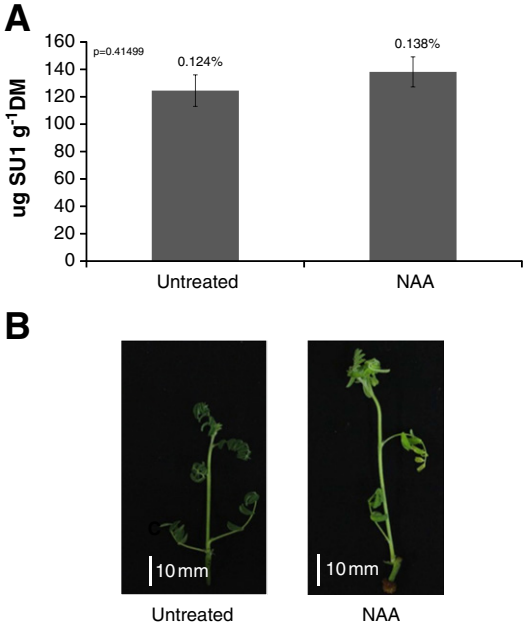

Fig. 6. A) Sutherlandioside B (SU1) content in propagated cultures of Sutherlandia. The percentage value of SU1 is indicated above the bars. B) Examples of *in vitro* untreated and treated (1 mg l^{-1} NAA) plants of Sutherlandia. N=5 for the untreated control and 5 for the NAA-treated plants.

triterpenoid were found in arid areas such as the Karoo, whereas the other group (Fig. 3B), with virtually no SU1, was composed of plants that are spatially situated in the Gansbaai area. Although speculative, the first group is most probably *S. frutescens* sub-species *microphylla* and the second set is likely to be *S. frutescens* sub-species *incana*, respectively. Those plants from the south-western Overberg region (termed here as the Gansbaai population) were associated in closer proximity on the positive vector of PC 1, suggesting similarities in their chemical makeup.

The data presented in Table 1 corroborate this notion as SU1 was only detectable in some spatial variants. The highest level of this chemical was recorded for the Karoo pods at 0.993% (DM) but these were similar to the West Coast plants (Table 1), further substantiating the PCA data. The garden grown material is interesting as it contains some unique metabolites. These plants were obviously not closely related to either the Karoo or Gansbaai groups. The superior resolving power of LC–MS-PCA analysis is artistically illustrated in this study because

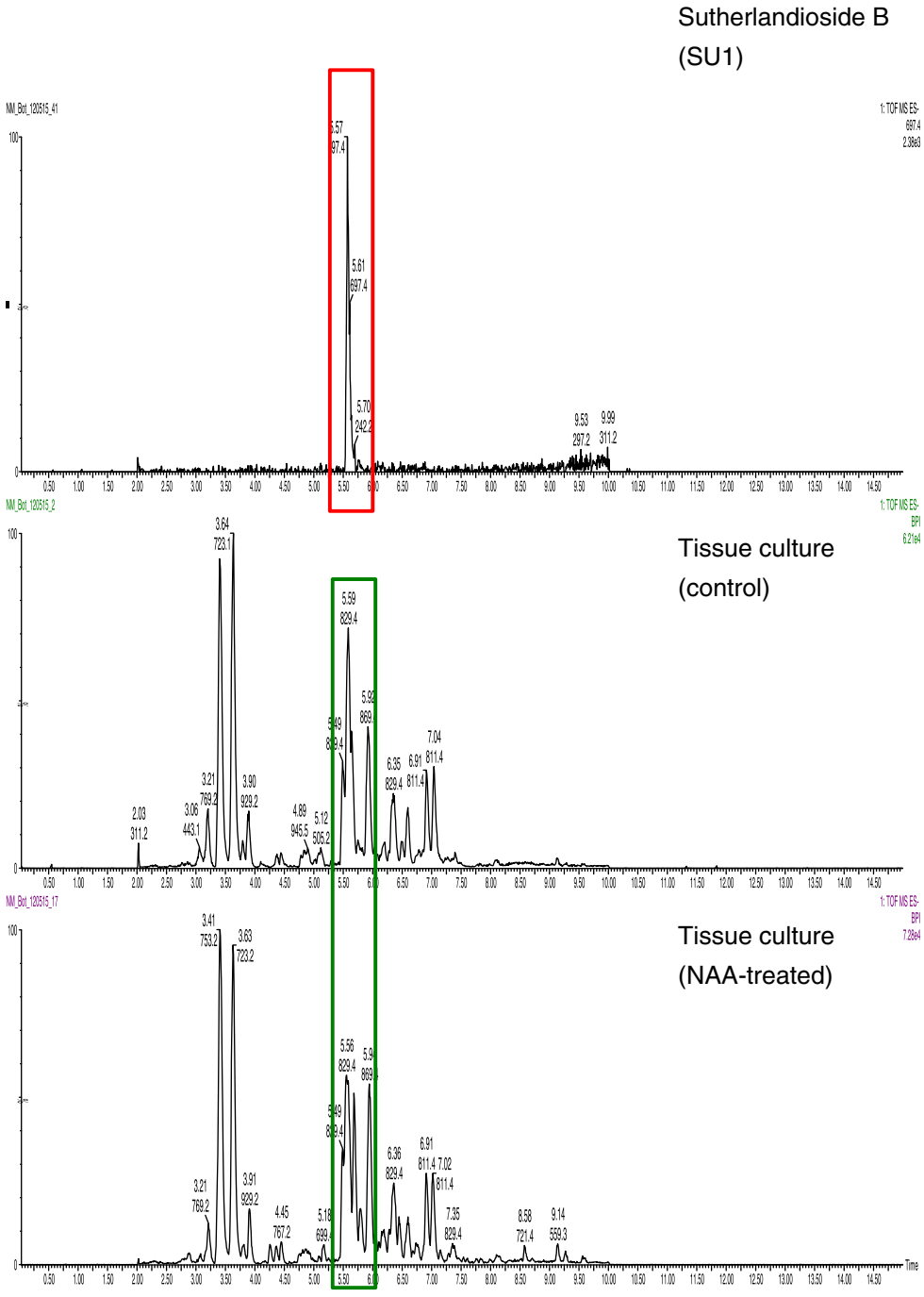

Fig. 7. LC−MS spectra for plants growing *in vitro*. Plants were either untreated (centre) or treated with 1 mg l^{-1} NAA (bottom). The spectrum for Sutherlandioside B is shown at the top.

it allows for even low quantity metabolites to be used for constructing population relationships and quality assessments (Van der Kooy et al., 2009). This may suggest similar environmental challenges which may tightly control expression of the SU1 phenotype (such as water deprivation), but genetic structure cannot be dismissed. To confirm our suspicions, it may thus be interesting to attempt to correlate metabolomic data with geographical distribution and phylogenetic data to determine whether metabolic characters are largely dependent on the genome or rather the influence of the environment. Unfortunately, few phylogenetic studies have focused solely on Sutherlandia, and to date, these have often been unable to provide resolute genetic differentiation (Van Wyk and Albrecht, 2008). An in-depth phylogenetic-metabolomic analysis is thus long overdue for this species and its relatives.

Overall the chemistry of Sutherlandia was once more confirmed to be complex with many constituents (close to 67 variables) being similar amongst the different populations (Fig. 3B). Several unidentified compounds were implicated in the separation amongst wild-collected and cultivated garden plants. A unique compound (indicated by an arrow, Fig. 3B) contributed to isolating the garden-grown specimens (ESI^- *m/z* 823.3781 refer to the score loading plot). At this stage this compound remains unidentified. The true identity of those chemicals that are in the negative component of PC 2 (Fig. 3B) has not been resolved in this study. However, even though they seem to be minor constituents of the overall profile of Sutherlandia, they may be useful markers to characterize those plants coming from the Yzerfontein and Blouberg regions (West Coast). The phenomenon of the Karoo-chemotype or Gansbaai-chemotype is further emphasized through supervised comparison by subjecting data to OPLS-DA (Fig. 4). This method of cluster analysis validates our interpretation of there being two major groups (variance is recorded at the 8.78% level).

3.2. Profiling of micropropagated plants

For commercialized medicinal herbs, a more targeted approach is generally employed which focuses on known bioactives and so we quantified the levels of SU1 in auxin-treated and untreated (control) *in vitro* plants of Sutherlandia. Although this plant growth regulator produced more plants with longer shoots (Fig. 5), the levels of SU1 detected in the *in vitro* plants irrespective of treatment were similar (120 mg g^{-1} dry mass; Fig. 6A). Consequently, NAA could be applied as a growth regulator in tissue culture to generate a higher biomass as it may produce a greater number of plantlets for *ex vitro* planting. The number of axillary buds influences the exponential potential of the tissue culture system for bulking up stock plants, as each node has the capacity for shoot regeneration.

In our environment, we normally do not expose the propagules to plant growth regulators as the plants produced *in vitro* are generally healthy and sufficient endogenous phytohormones are available to maintain a high-yielding, continuous stock culture system. Overall, treated plants had similar chemical profiles to control plants (Fig. 7) explaining the similar levels in SU1 content. With the LC–MS method used here, chromatograph traces of micropropagated plants confirmed the chemotype of starter material as originating in the West Coast. The SU1 elutes at the 5.50–6.0 min of the spectra (Fig. 7). Although several different types of metabolites have been detected in Sutherlandia, none of these have been irrevocably been linked to its anti-tumourigenic effects. Presently, it is postulated that terpenoids and flavonoids are the secondary metabolites that are responsible for Sutherlandia's anti-cancer properties. High levels of somaclonal variation arising from inappropriate tissue culture regimes may be problematic when considering micropropagated plants for field cultivation, as these changes may prevent acclimation and subsequent development as field crops. As a rapid test, we used a metabolite profiling approach to detect changes to plant metabolism. Although not surprising, there were no apparent abnormal chemical changes. Instead, plants resembled those from the West Coast area of Yzerfontein, which is in close proximity and has a similar microclimactic environment to Melkbosstrand. This may suggest that the chemical constituents of Sutherlandia are more closely controlled by genetic factors as opposed to environmental influences when conditions are optimal. This adds impetus for a phylogenetic revision which is linked to chemotyping. There were no major qualitative differences in the terpenoid and phenolic components of auxin-treated and untreated (control) plants but quantitative differences for SU1 were evident when comparing these to detected levels on wild growing populations. Lower levels of SU1 were detected in the *in vitro* plants (Fig. 6A). Several reasons may explain this result. Firstly, *in vitro* grown plants are not subjected to the same considerable abiotic and biotic stresses as plants from natural populations, where secondary metabolite synthesis of defense chemicals drives the phenotypic plasticity associated with spatial and environmental fluctuations. Secondly, *in vitro* grown plants may be considered as being young compared with plants growing outside. Temporal control of secondary metabolism is a well-recognized phenomenon, with older plants often exhibiting better chemical differentiation and higher levels of key bioactive principles. Finally, the differences observed may also be attributable to tissue-specific SU1 accumulation and the different plant parts used (seed pods versus *in vitro* leaves). It is thus possible that the plants grown *in vitro* accumulate quantitatively comparable levels of sutherlandins and sutherlandiosides when transferred *ex vitro*. Accumulation of industrially important primary metabolic biomarkers in acclimated, micropropagated plants has been reported by several authors who detected canavanine, gamma amino butyric acid (GABA), asparagine, aspartic acid and pinitol (Colling et al., 2010; Shaik et al., 2010, 2011), but the detection of these putative anti-cancer terpenoids and flavonoids in micropropagules is described and quantified here for the first time.

4. Conclusions

Integrative LC–MS-PCA is an extremely powerful resolution technique that can be used to process and analyse data in order to assess quality, traceability and novelty. With this

method, populations of Sutherlandia were shown to differ chemically according to their environmental lineages. Using this approach, we identified the 'garden-grown' plants as being chemically distinct and so extracts of this seemingly unique Sutherlandia will be subjected to testing to ascertain anti-cancer capability. Plant tissue culture did not elicit unwarranted epigenetic mutations that could adversely affect the chemical constitution of plants, ensuring that this propagation technique is industrially suitable for product manufacturing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.sajb.2012.07.018.

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