3.1.3 Hypothesis

It was hypothesized that:

- *S. frutescens* grown in the natural field and cultivated field groups will have an increase in the number and concentrations of sutherlandins 3 and 4 and sutherlandiosides B and D compared to that of the greenhouse group.
- The application of the iBatech^{TM} product will increase the concentrations of sutherlandins 3 and 4 and sutherlandiosides B and D.

3.2 The study approach

To realize the above-mentioned objectives, the following was done.

3.2.1 Identification of sites where *S. frutescens* is grown and the pertinent growth factors

Various sites where *S. frutescens* is grown where visited and broadly categorized into environmental categories, based on the pertinent environmental growth factors. The weather conditions at the time of harvest, the factors that we assumed could limit the synthesis of secondary metabolites, and plant heights were documented. This environmental growth factor identification was done to be able to correlate the environmental categories with the concentrations of sutherlandins 3 and 4 and sutherlandiosides B and D.

3.2.2 Determination of sutherlandins 3 and 4 and sutherlandiosides B and D profiles and concentrations in *S. frutescens* plants from different growth sites.

To determine the sutherlandins 3 and 4 and sutherlandiosides B and D profiles and concentrations HPLC was used. It is appropriate, accurate and precise and has the ability to provide scientifically acceptable results. It is sensitive, to easily detect compounds and allows for the simultaneous analysis of flavonoids and terpenoids. A new method will be developed based on a method developed by Avula *et. al.,* (2010) and will be validated according to the guidelines as set out by the International Conference for Harmonization (ICH), to assay for sutherlandins 3 and 4 and sutherlandiosides B and D using HPLC - DAD. This new HPLC method will be used to determine the profiles and average levels of sutherlandins 3 and 4 and sutherlandiosides B and D of *S. frutescens* collected from the different sites, and that treated with the iBatech™ product. The *Sutherlandia frutescens* Afriplex™ sample was used as the standard plant material.

3.2.3 Determination of the effects of iBatech™ treatment on the profiles of sutherlandins 3 and 4 and sutherlandiosides B and D

According to the manufacturers, the iBatech™ product, in previous studies has shown to increase overall polyphenol count in *Lycopersicon* agricultural crops. In these studies, general polyphenols were assayed for but specific classes of compounds were not. Only one HPLC assay was run on samples treated with the iBatech™ product and thus, a thorough understanding of the chemical changes taking place in these plant samples are not fully understood. The use of HPLC-DAD would provide a better understanding of the

type of chemical changes taking place in the plants when treated with the iBatech™ product at varying concentrations.

CHAPTER 4

Methods

This chapter describes the chemicals, materials, equipment and methods used. The identification of the sites where *Sutherlandia* is grown, with their relative pertinent growth factors, are documented, the collection, preparation and storage of plant material are described. The iBatech™ treatment experiment, the development and validation of an HPLC assay for the identification and profiling of sutherlandins 3 and 4 and sutherlandiosides B and D and the concentrations of the latter of *S. frutescens* from the varying environmental growth categories are presented here.

4.1 Chemicals, products and equipment

4.1.1 Chemicals and products

The methanol used for extractions and acetonitrile (HPLC grade) used for HPLC assays were purchased from Merck chemicals, SA. The distilled water also used for the HPLC assays was prepared in the pharmacology laboratory and purified using a (Glass Chem WS8Lc, Waterstill 8 L/H, 220 V). The plant material was collected from the wild, donated by Kirstenbosch botanical gardens, the University of the Western Cape greenhouse and local farmlands from the Western Cape area growing *S. frutescens* for medicinal use. The patent holder, Dr. J. Klaasen Department of Medical BioSciences, University of the Western Cape, provided the iBatech™ product used in this experiment (Patent reference number PA149863/P, batch number IF/UWC – T70023). The sutherlandiosides B and D ratio used as the standard, to construct the calibration curves,

were obtained from the University of Mississippi. The consumables used were; test tubes *(Cellstar 15 ml, PP, graduated, sterile, blue cap),* airtight plastic bags *(Packit 1 BRC 2536, SA),* millipore filters *(Millipore Millex-HV, hydrophilic PVDF 0.45 um),* syringes *(K 12 5ml)* and amber HPLC vials *(Supelco, conv pack 8/425, PTFE/ Silicone/PTFE Septa, USA).*

4.1.2 Equipment

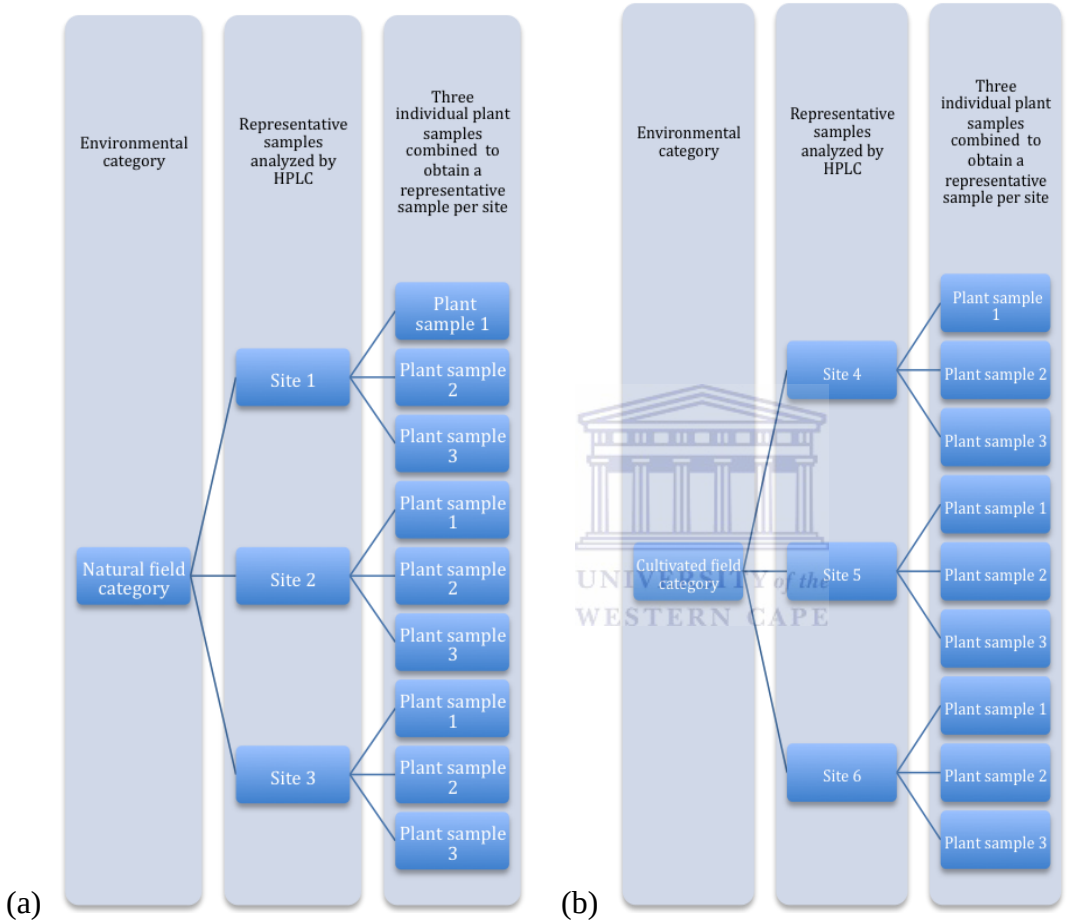
A ventilated oven *(Memmert, 854 Schwabach, Western Germany),* a coffee grinder *(Philips Cucina coffee grinder Type HR 1737, Brazil),* a weighing balance *(AR 2140 Max cap. 210 g, China),* a vortex *(Vortex Genie 2, model no. G-560E, Scientific industries, USA),* a centrifuge *(Labofuge 200 Heraeus Sepatech 1,2 kg/dm^3 Germany)* and a pipette *(Finnpipette 773676 thermo Electronic 100-1000 ul)* were used in this experiment.

4.2 Location of the sites in which *S. frutescens* is grown in the Western Cape area, collection, preparation and storage of plant material

Sites in the Western Cape area where *S. frutescens* are grown were visited during a thirty-day period from September to October 2009 during early spring. Eventually eight *S. frutescens* sites were identified and broadly categorized into natural field, cultivated field and greenhouse based on the location and the conditions under which the plants were growing. For each site the pertinent environmental growth factors, which we assume may affect the synthesis of secondary metabolites were identified and documented.

To determine the effects of the growth factors collectively on sutherlandins 3 and 4 and sutherlandiosides B and D in *S. frutescens,* plant samples were collected in the following order. The natural field and cultivated field categories plant samples were collected from three sites each, while for the greenhouse category, which is not common practise for the growth of *S. frutescens*, samples were only collected from two sites. Thus, plant samples from eight *Sutherlandia* sites were collected for this study. From each of the eight, selected *Sutherlandia* sites, a minimum of three samples of leaf and stem material per site were collected. All samples were oven-dried at 40 ˚C for 72 hours after collection and thereafter the flowers and seedpods removed. The leaves and stems were then ground into a powder and put into appropriately labelled airtight plastic bags. In order to prevent moisture, light and oxidative stress from compromising the quality of the *S. frutescens* powdered material; the bags were placed inside a brown cardboard box and placed inside a cool, dry cupboard for storage until analysis.

In preparation for HPLC analysis, the three samples from each site were weighed individually to obtain 1 g per sample, these were then combined, shaken, and 1 g of this homogenized material taken as a representative sample for the site. In addition, one further sample from each site was collected and used by a botanist, for species verification. At the time of sampling, the size of the plant, the weather conditions, the presence of flowers and/ or seedpods were also determined and recorded.



Figures 4.1: The collection of plant samples from (a) the natural field, (b) cultivated field and (c) greenhouse environmental categories

individual plant, of three plants of each experimental group were taken as an individual sample, resulting in nine samples. These nine samples were dried and stored in the same manner as previously described in section 4.2.

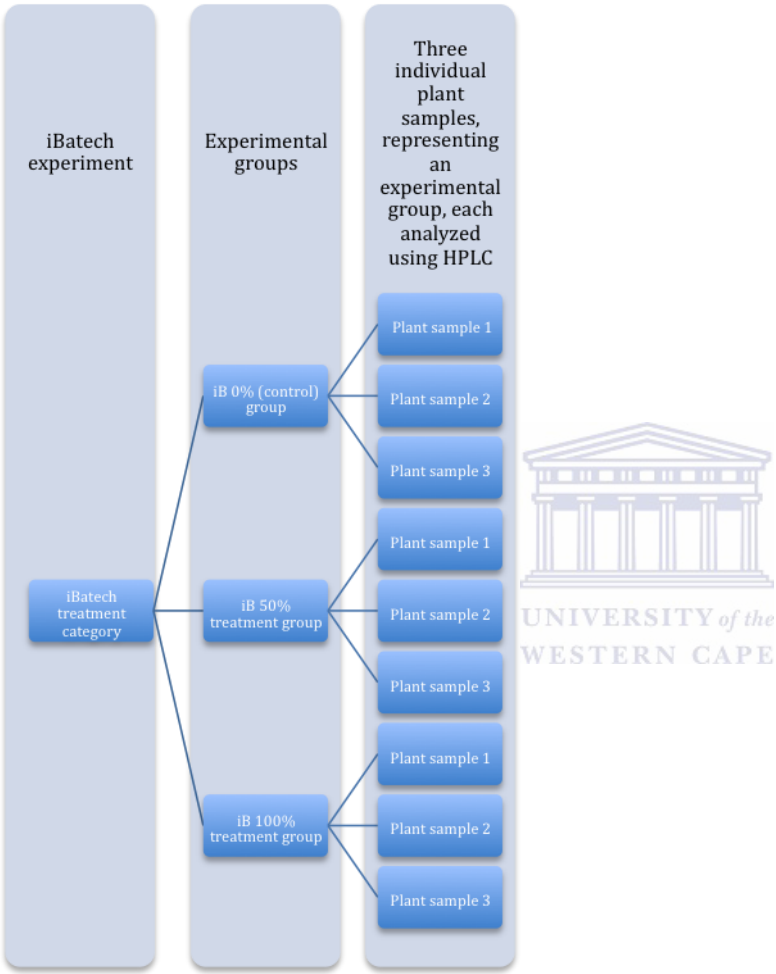


Figure 4.2: Plant sample collection for the iBatech™ experiment

4.5 Confirmation of the identified peaks, using Liquid Chromatography – Mass Spectrometry

Fractions of the sutherlandiosides B and D were collected and pooled together, then subjected to liquid chromatography - mass spectrometric (LC-MS) analysis to confirm their identity. The LC-MS was performed at Stellenbosch University, Central Analytical Facility, on a Waters API Q-TOF Ultima LC-MS instrument. The sample was diluted 10 times with 1 ml 0.1% formic acid and 50% acetonitrile using an ultrasonic bath for 20 minutes then a 2 µL injection volume was injected onto the column (Waters BEH C18, 2.1x50 mm). The mobile phase consisted of solvent A: water and solvent B: acetonitrile, with a gradient of 0 minutes to 0.5 minutes solvent A was 100%, from 0.5 to 6 minutes solvent A decreased steadily to 0% from 6 minutes to 8 minutes, solvent B remained at 100% at 8.01 minutes and the system was re-equilibrated to 100% of solvent A with a total run-time of 15 minutes and a flow rate of 0.35 ml /minute.

The detection conditions were as follows: source ESI +, capillary voltage set at 3.5 kV, cone voltage 35, RF1 40, source temperature 100 °C, desolvation temperature 350 °C, desolvation gas flow 350 L/h and cone gas flow 50 L/h. The mass to charge ratio (*m/z*) of the LC-MS data was noted and compared to existing data in the literature to confirm the identity of the compounds.

4.6 Identification and quantification of sutherlandiosides B and D.

The sutherlandiosides B and D from the samples were identified based on retention times and spectral analysis, and compared to that obtained from a standard mixture of

sutherlandiosides B and D, isolated and purified by the University of Mississippi. The serial dilutions containing 0.465 mg /ml, 0.931 mg /ml, 1.862 mg /ml, 3.725 mg /ml, 7.45 mg /ml, were injected in triplicate. Standard curves of the concentrations vs. peak area were obtained and used to quantify sutherlandiosides B and D in all plant samples, using linear regression and the Graphpad prism5 software.

4.7 Statistical analysis

The statistical analysis was performed using the GraphPad Prism 5 software at a significance level of 0.05, in the following manner. The Kruskal-Wallis test was used to determine if there were any significant differences in the mean levels of sutherlandins 3 and 4 and sutherlandiosides B and D, among the experimental groups and was applied to both the environmental group data and iBatech™ experiment data. Furthermore, groups that showed to be significantly different, was subjected to the post ANOVA, Dunn's Multiple Comparison test. The Mann-Whitney, two-tail, t-test was used to compare the mean levels of each experimental group to the standard and was only applied to the environmental group data. Lastly, the column statistics was done to determine whether there were any significant differences among samples from the same group.

CHAPTER 5

Results and discussion

The results obtained in the location of the sites where *Sutherlandia* is grown, the determination of the profiles and the average peak areas of sutherlandins 3 and 4 and sutherlandiosides B and D, the effects of the iBatech^{TM} product treatment on the profiles and average peak areas of sutherlandins 3 and 4 and sutherlandiosides B and D, and the quantification of sutherlandiosides B and D, are presented and discussed here.

5.1 Growth environments and locations in which *Sutherlandia* is grown in the Western Cape area used in this study

To determine the growth environments in which *Sutherlandia* is grown in the Western Cape area, various sites were visited and analyzed for their relative collective growth conditions, broadly categorized into the 1. natural field, 2. cultivated field or 3. greenhouse environmental categories and allocated a site number which represents a specific location from which the plant material was collected and will be referred to from here forward. The weather conditions at the time of collection, the presence or absence of flowers and/ or seedpods and the height were documented and are presented in Table 5.1 below. Site 5 and 6 samples were donated and therefore the variables are unknown.

Table 5.1: The environmental category, sample name, weather conditions at time of collection, presence or absence of flowers or seedpods and height

Environmental category	Samples	Conditions at collection	Presence of flowers	Presence of seed pods	Height cm
Natural field	Site 1	Sunny/ windy	No	Yes	10-20
	Site 2	Cold/ snowy	Yes	No	10-20
	Site 3	Sunny	Yes	Yes	20-30
Cultivated field	Site 4	Sunny	No	No	40-60
	Site 5	Unknown	Unknown	Unknown	Unknown
	Site 6	Unknown	Unknown	Unknown	Unknown
Greenhouse	Site 7	Shady	No	No	40-60
	Site 8	Shady	No	No	30-40

From the range of weather conditions at the time of collection, the greenhouse category appears more uniform. As covered in the literature, environmental growth conditions play a fundamental role in the synthesis of secondary metabolites (Mosaleeyanon *et. al*., 2005). The natural field category samples i.e. Site 1, Site 2, and Site 3, were collected from the Western Cape's natural *Fynbos* locations. From the literature reviewed, the Western Cape *Fynbos* region should be experiencing the summer drought period, after the long wet winter (Aschmann, 1973). From the conditions at harvest column, it is evident that the weather patterns are not true to that described in the literature. The observations made here were only performed on the day of collection and therefore cannot be generalized for the season, however, these environmental factors, when experienced for a prolonged period, could affect the concentrations (Wink, 1999) of sutherlandins 3 and 4, and sutherlandiosides B and D.

The presence and or absence of flowers and seedpods provide an indication as to which stage of development the plant may be in. Previous studies suggest that the stage of development also greatly affects the synthesis of secondary metabolites (Whisgary *et. al*., unpublished data), as it is believed that during the early stages of development the plant allocates assimilated carbon and nitrogen to primary growth (Bourgaud *et. al*., 2001) and once growth plateaus, assimilated carbon and nitrogen can be allocated to secondary growth and the synthesis of secondary metabolites. In the case of flowering, the plant has reached a secondary growth stage, however, assimilated carbon and nitrogen is now allocated to flowering and the development of seeds, thus reducing the concentrations of secondary metabolites. In the natural field flowers and seedpods are present and therefore

the plant is in the developmental stage to be able to produce secondary metabolites such as sutherlandins 3 and 4 and sutherlandiosides B and D. The fact that flowers and seedpods are present could also mean that the concentrations of these secondary metabolites could have been compromised at the time of collection and may be increased in the stages before and after flowering. The absence of flowers and seedpods in the greenhouse category can be attributed to the controlled environment under which these plants are grown (Zobayed *et. al*., 2004). In the natural field and cultivated field categories plants are exposed to natural seasonal cycles and therefore should be flowering as per normal, at the time of harvest (early spring) (Pienaar, 2001). The greenhouse category however, maintains fairly constant growth conditions throughout the year, regardless of season, thus the plant no longer follows its natural growth cycle.

The two samples in the greenhouse category are both shady and this could explain for the height being greater than that of the other categories. When plants are in competition for light, they tend to invest more energy into longitudinal growth than latitudinal growth; however, plant biomass cannot necessarily be used as an indication for secondary metabolite concentrations. It is often observed, that smaller, more compact plants have higher secondary metabolite yields compared to that of taller plants. Overall, the greenhouse category shows more uniform characteristics whereas the natural field and cultivated field categories have considerably varying characteristics.

5.2 Determination of the profiles and peak areas of sutherlandins 3 and 4 and sutherlandiosides B and D of *S. frutescens* samples from the three environmental categories, the *S. frutescens* Afriplex™ standard and the iBatech™ treatment

The aim was to determine if there were any variations in the profiles and average peak area values of Su3, Su4, SuB and SuD in the plant samples collected from the natural field, cultivated field, greenhouse categories when compared to the *S. frutescens* Afriplex™ standard sample, and to determine any variations in average peak area values among the iBatech™ treatment groups, using HPLC.

5.2.1 HPLC assay validation

To validate the HPLC assay used for the analysis of Su3, Su4, SuB and SuD in the *S. frutescens* samples, inter-day variations, intra-day variations and the construction of calibration curves were used according to the ICH guidelines for analytical methods. In Figure 5.1 is a representative chromatogram of *S. frutescens* obtained from the analysis of the Afriplex™ sample, which is being used in this study, as a standard to compare the natural field, cultivated field and greenhouse categories to. The fingerprint obtained, illustrates the presence of a number of metabolites however for this study only sutherlandins 3 and 4 (Su3 and Su4) and sutherlandiosides B and D (SuB and SuD) were monitored. For inter-day and intra-day analysis (Appendix Table 1), the *S. frutescens* Afriplex™ standard plant material was used, whereas the isolated standard SuB/ SuD ratio was used to construct the calibration curves.

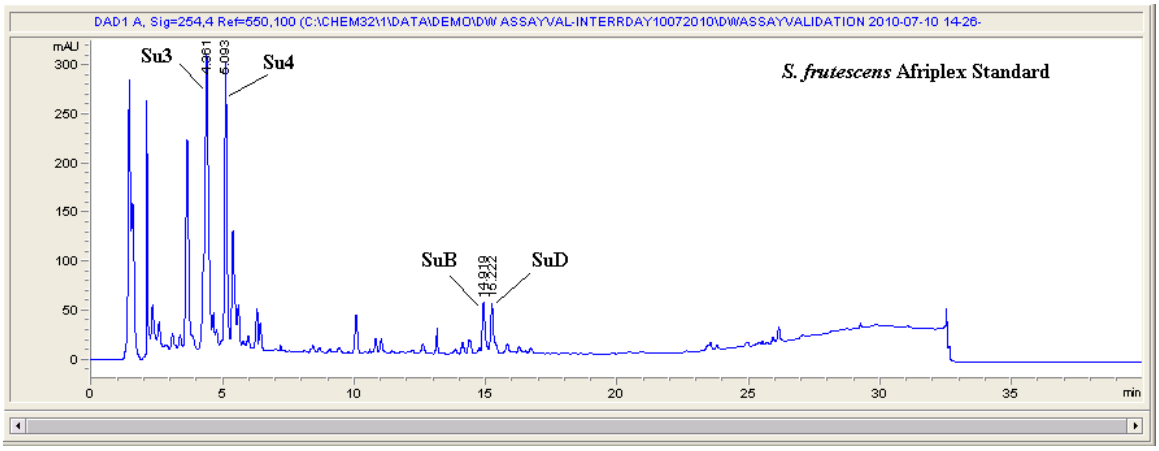


Figure 5.1: HPLC chromatographic fingerprint of *S. frutescens* Afriplex™ sample used as plant sample standard

The spectral data was obtained (Figure 5.2) and found to conform to those of flavonoids and terpenoids. The flavonoids Su3 and Su4, were compared to the spectral data obtained by Hess *et. al., (unpublished data)* with the relative retention times of 4.361 and 5.093 respectively and the terpenoids SuB and SuD, were compared to the spectral data obtained by Avula *et. al.*, (2010) with the relative retention times of 14.919 and 15.222 respectively, to verify their identity.

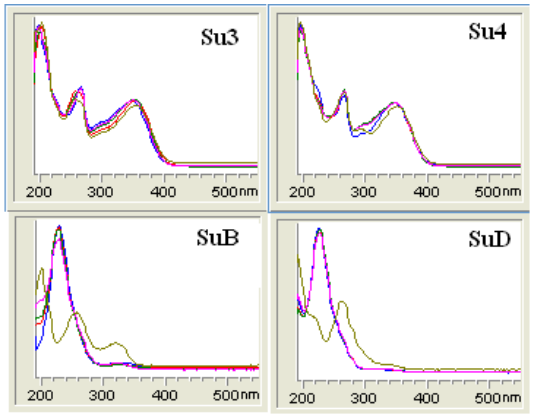


Figure 5.2: The spectral data for Su3, Su4, SuB and SuD

Serial dilutions of the sutherlandiosides B and D standard were prepared and a 10*u*L injection was made onto the HPLC column. The following chromatogram (Figure 5.3) is a representation of the SuB and SuD standard ratio and also provides the spectral data as well as the relative retention times. This was then used for accurate identification of sutherlandiosides B and D in all the plant samples analyzed with HPLC.

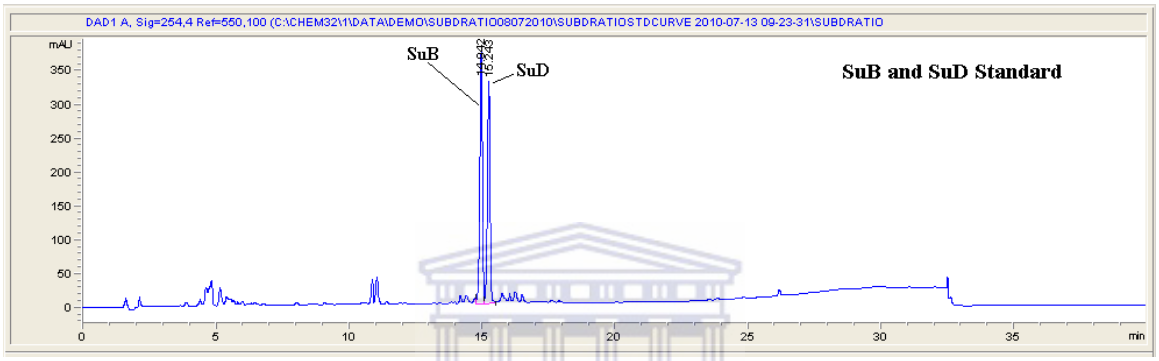


Figure 5.3: Representative chromatogram of SuB and SuD standard ratio

5.2.2 Profiles and average peak area values of sutherlandins 3 and 4 and sutherlandiosides B and D of *S. frutescens* samples from the Afriplex^{TM} standard, natural field, cultivated field and greenhouse environmental categories

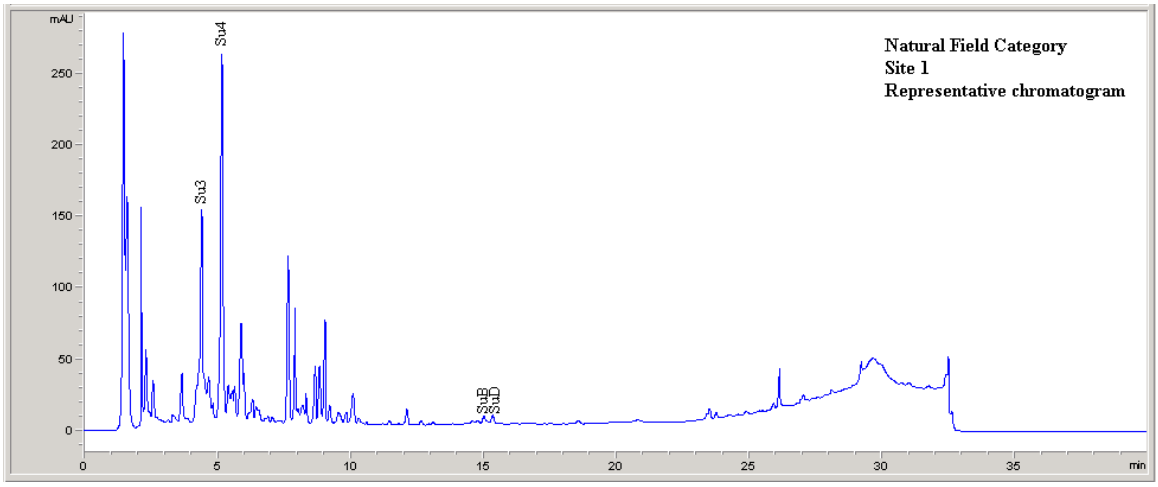
The aim was to determine the qualitative variations, if any, in the profiles of sutherlandins 3 and 4 and sutherlandiosides B and D. The following chromatograms serve as representatives for all of the samples analyzed from the same environmental category. The samples collected from the various sites and categorized into the environmental categories, were allocated a name with the relative environmental category in brackets e.g. Site 1 (NF), and will be referred to from here forth. One gram of representative plant sample from each site was extracted with 10 ml MeOH. A 10 µL sample was injected to

obtain the chromatograms as described in method 4.3. Sutherlandin 3 (Su3), sutherlandin 4 (Su4), sutherlandioside B (SuB) and sutherlandioside D (SuD) were monitored.

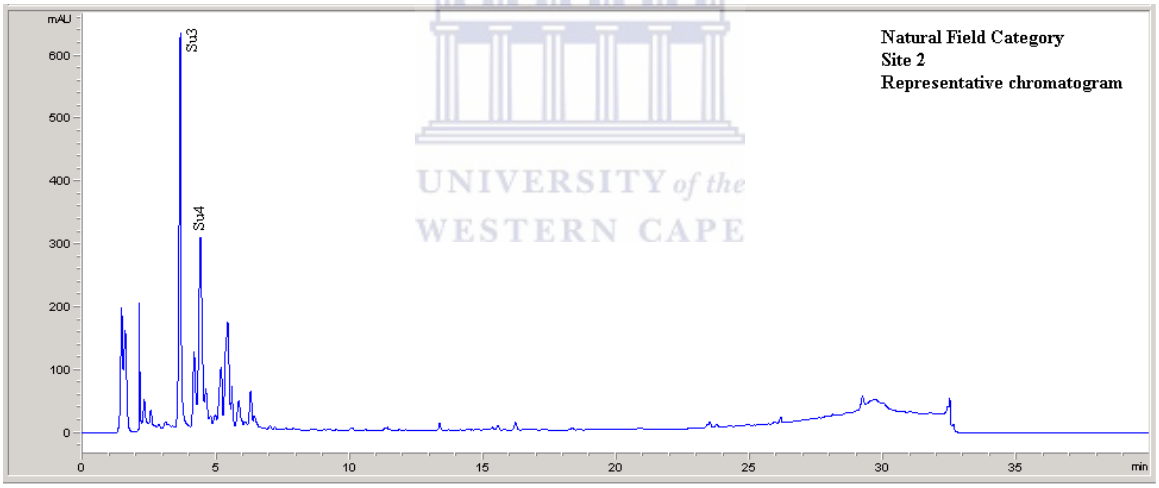
Figure 5.4 represents the natural field (NF) group. Sutherlandins 3 and 4 quantities are minimal in the Site 1 (NF) sample and considerably increased in the Site 2 (NF) and Site 3 (NF) samples. In Site 2 (NF) sutherlandin 3 is greater than 4 and vice versa in the Site 3 (NF) sample. Sutherlandiosides B and D are also in greater quantity in the Site 3 (NF) sample and unidentifiable in the Site 1 (NF) and Site 2 (NF) samples.

Figure 5.5 shows dramatic variations in the quantities of Su3, Su4, SuB and SuD among all samples. The Site 4 (CF) sample shows the lowest quantities whereas the Site 5 (CF) shows higher quantities and Site 6 (CF) dramatically higher quantities especially in SuB and SuD when compared to all other samples analyzed across all environmental categories. Figure 5.6 shows Su3, Su4, SuB and SuD profiles with no visible variations in quantities between the Site 7 (GH) and Site 8 (GH) samples.

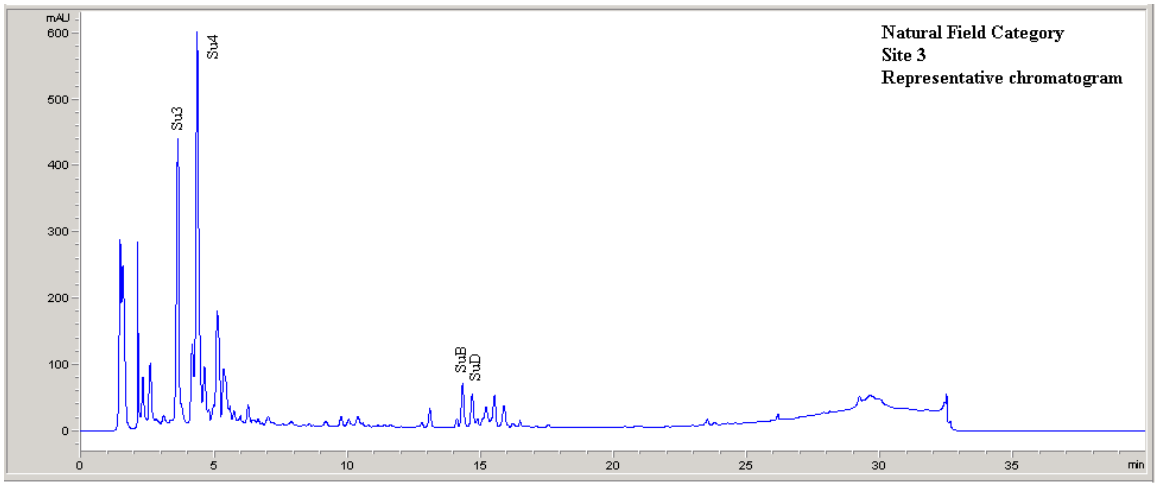
a)



b)

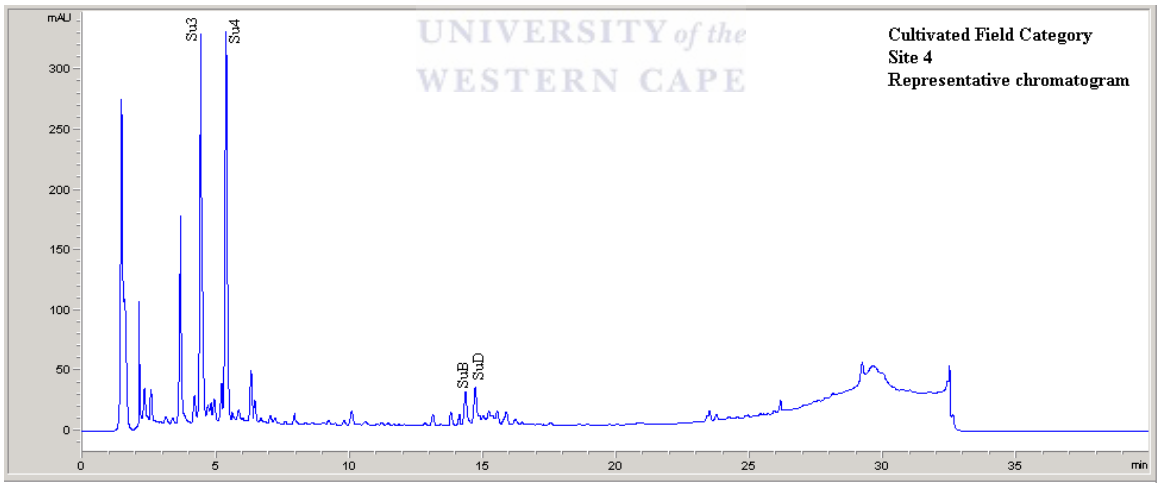


c)

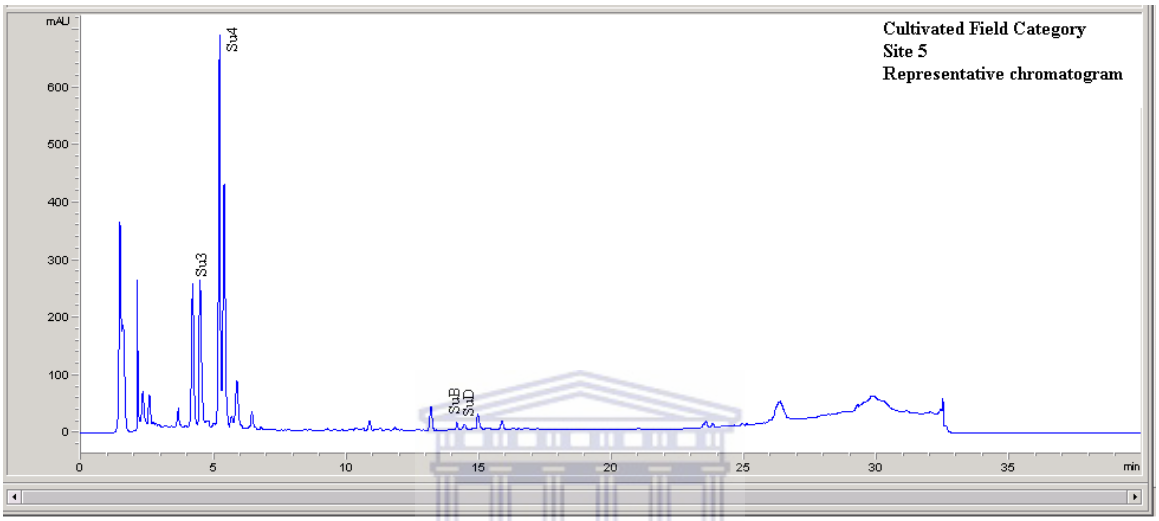


Figures 5.4 a , b and c: Representative HPLC chromatograms for the natural field category samples.

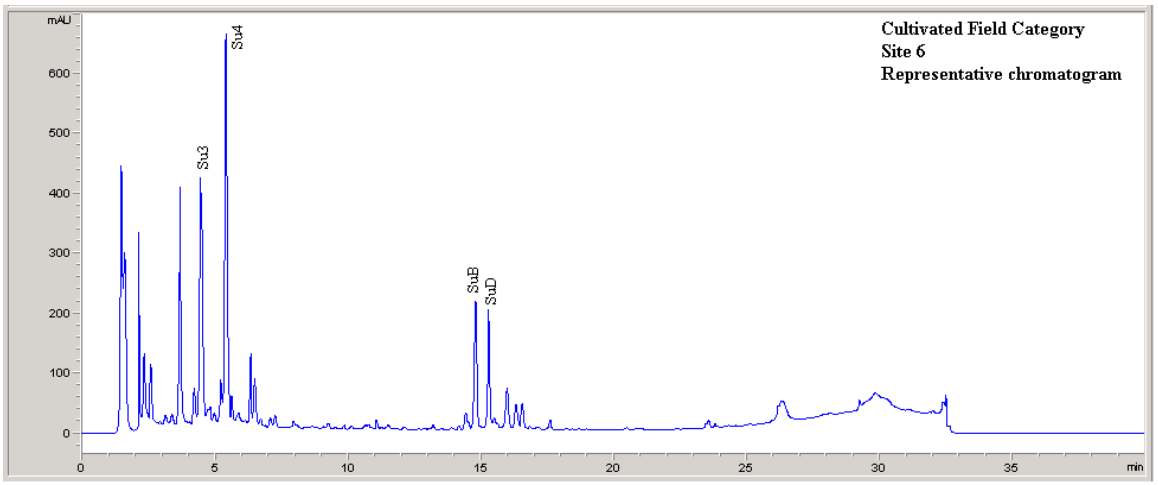
a)



b)

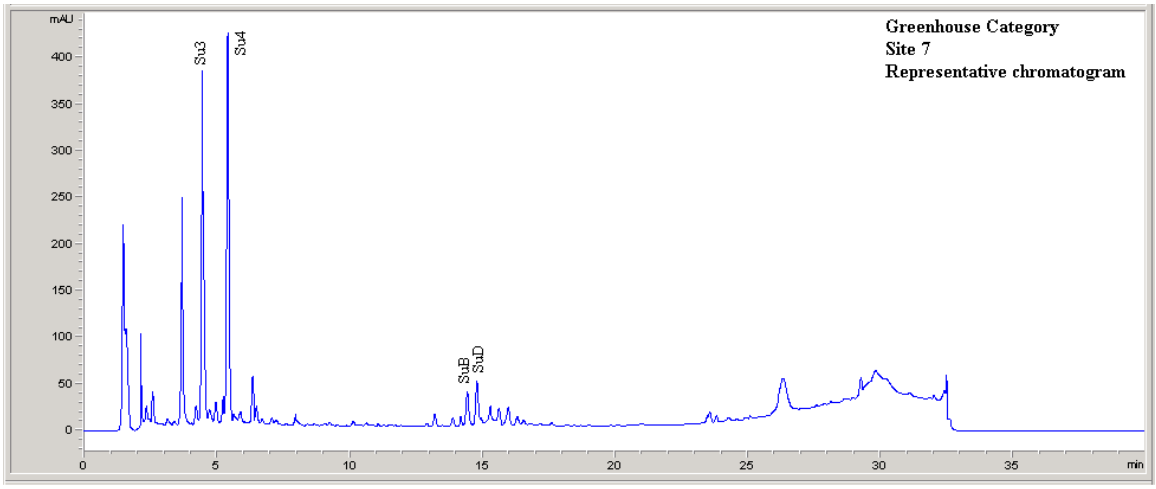


c)

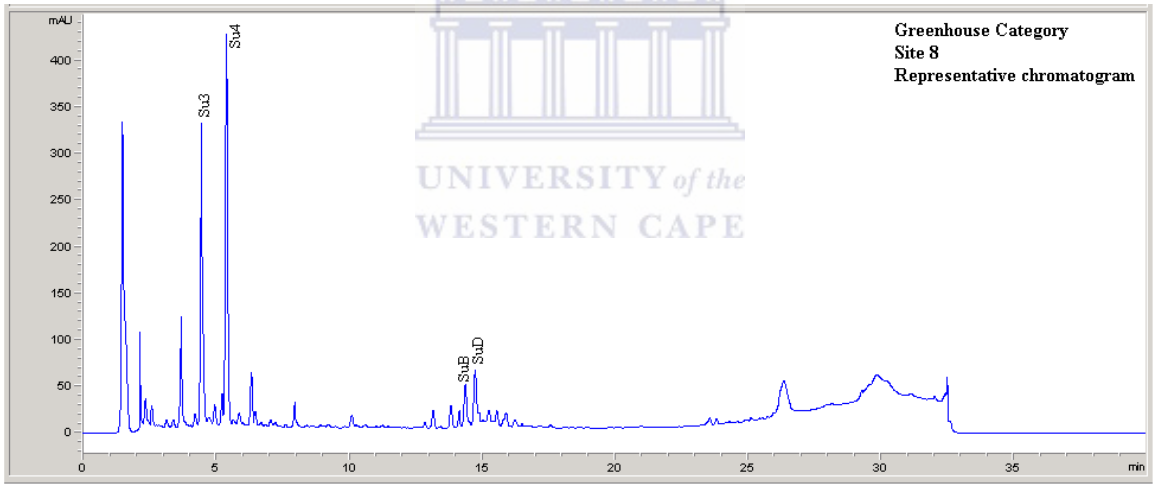


Figures 5.5 a, b and c: Representative HPLC chromatograms for the cultivated field category samples.

a)



b)



Figures 5.6 a and b: Representative HPLC chromatograms for the greenhouse category samples.

Figures 5.7, 5.8, 5.9, and 5.10, represent the average peak area values of Su3, Su4, SuB and SuD respectively and compares the average peak area values for the natural field (NF), cultivated field (CF) and greenhouse (GH) environmental categories to that of the

Afriplex™ *S. frutescens* plant material (Std.) used as the standard. The error bars plotted illustrate the standard deviation among three homogenized samples collected from the different sites but in the same environmental category. Due to the unavailability of a third sample from the greenhouse category, the error bar represents the standard deviation of two samples.

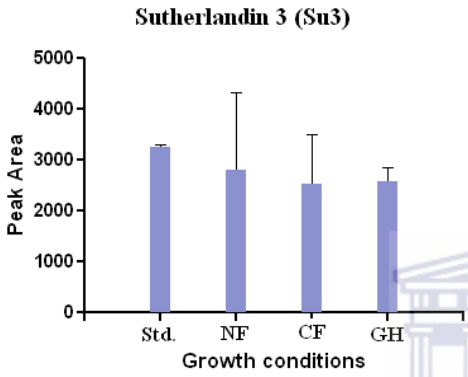


Figure 5.7: Sutherlandin 3 (Su3)

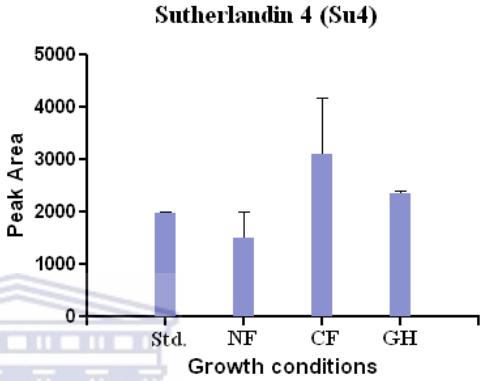


Figure 5.8: Sutherlandin 4 (Su4)

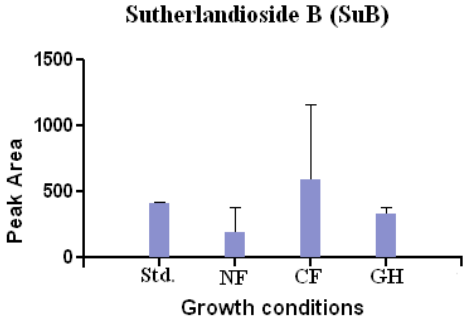


Figure 5.9: Sutherlandioside B (SuB)

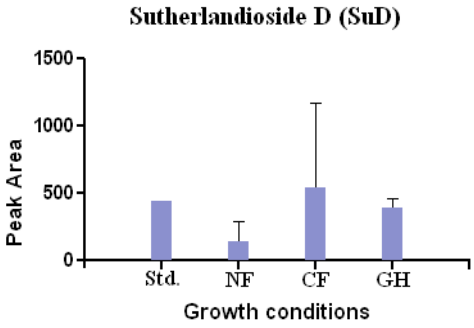
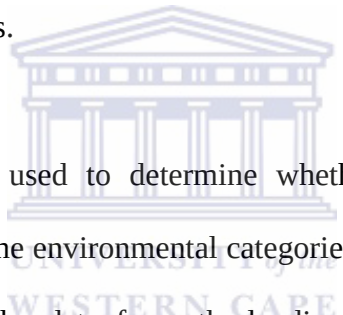


Figure 5.10: Sutherlandioside D (SuD)

Figure 5.7 (sutherlandin 3) illustrates the average peak area values of Su3 for the Afriplex™ (Std.) 2495.08, the natural field (NF) 2810.33, the cultivated field (CF)

2519.81 and the greenhouse (GH) 2580.25 categories. Figure 5.8 (sutherlandin 4) illustrates the average peak area values of (Std.) 2065.18, (NF) 1495.67, (CF) 3114.42 and (GH) 2361.72, with the CF group showing the highest average peak area values of Su4 and the NF showing the lowest. The GH group shows higher average peak area values of sutherlandin 4 than that of the Std. Figure 5.9 (sutherlandioside B) illustrates the average peak area values of (Std.) 236.95, (NF) 189.7, (CF) 594.56 and (GH) 326.72, however, the CF group shows the highest average peak area values for SuB. Figure 5.10 (sutherlandioside D) illustrates the average peak area values of the (Std.) 255.38, (NF) 144.1, (CF) 544.37 and (GH) 387.49 groups for SuD, with the NF category having the lowest average peak area values.



The Kruskal-Wallis test was used to determine whether there are any statistically significant differences among the environmental categories, for sutherlandins 3 and 4 and sutherlandiosides B and D. The data for sutherlandin 4 (Su4) was significant *(P = 0.0021)* and therefore the post ANOVA, Dunn's Multiple Comparison test was performed to determine which groups were significantly different. The results showed that the natural field group was significantly different to both the cultivated field and greenhouse groups.

The Mann-Whitney, two-tail, t-test was used to analyze the data for Su3, Su4, SuB and SuD, to compare each environmental category to the standard, however, no significant differences were found.

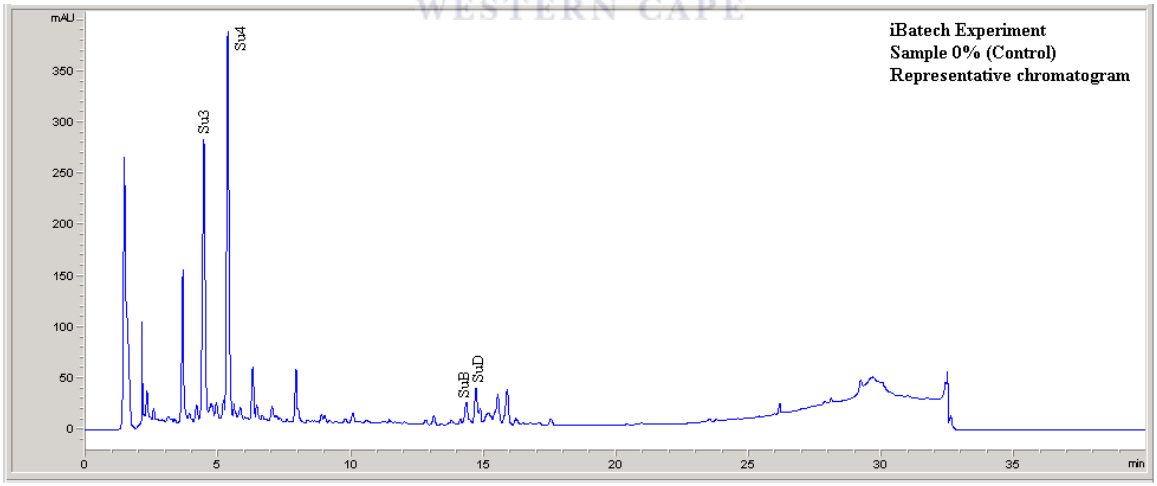
The column statistics of the raw data was analyzed to determine if there were any significant differences among samples from the same environmental category. Sutherlandin 3 (Figure 5.7), illustrated significant differences in Su3 concentrations among the samples from the same environmental category i.e. NF (*P = 0.0005*), CF (*P = 0.0001*) and GH (*P = 0.0001*). Sutherlandin 4 (figure 5.8), illustrated significant differences in Su4 concentrations among samples when analyzing the NF (*P = 0.0001*), CF (*P = 0.0001*) and GH (*P = 0.0001*) groups. Sutherlandioside B (figure 5.9), illustrated significant differences in SuB concentrations for NF (*P = 0.0189*), CF (*P = 0.0140*) and GH (*P = 0.0001*) among samples from the same group. Sutherlandioside D (figure 5.10), had significant differences in SuD concentrations among samples for the NF (*P = 0.0192*), CF (*P = 0.0308*) and GH (*P = 0.0001*) groups.

In summary, the data shows that only sutherlandin 4 (Su4) was significantly different. The natural field group was significantly different to both the cultivated field and greenhouse groups. When comparing all groups to the standard, no significance was found, however, significant differences were found among samples from the same environmental group. These significant differences among samples from the same group can therefore be responsible for the lack of significant differences among environmental categories due to the wide range of data.

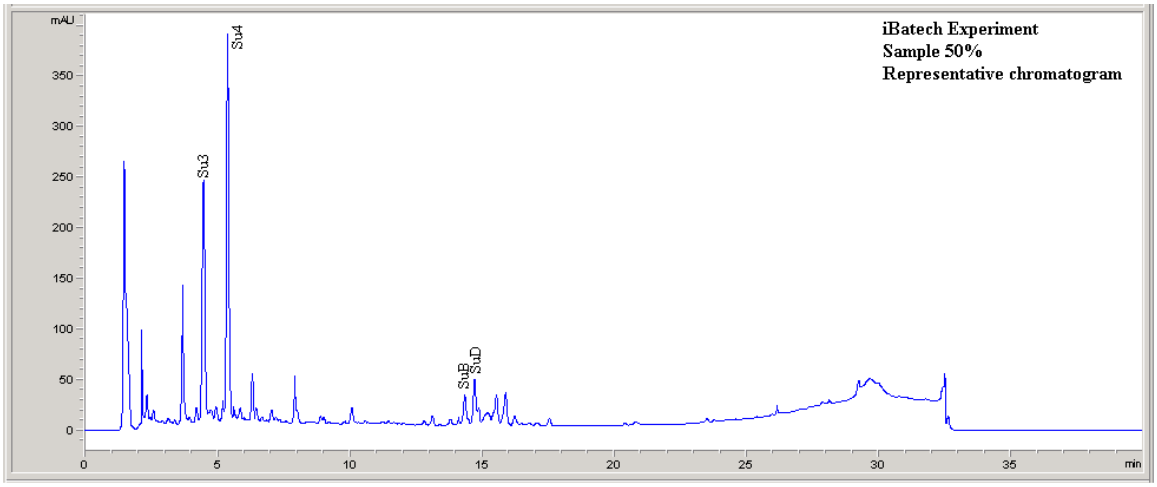
5.2.3 Profiles and average peak area values of sutherlandins 3 and 4 and sutherlandiosides B and D of *S. frutescens* samples from the iBatech™ treatment experiment

The chromatograms presented in Figure 5.11 illustrate minimal visual differences in Su3, Su4, SuB and SuD profiles when comparing the control group, 50% treatment group and 100% treatment group. However, with closer examination of the average peak area values, a concentration dependant increase in Su3, Su4, SuB and SuD are evident. One gram of representative plant sample for each site was extracted with 10 ml MeOH. A 10 µL sample was injected to obtain the chromatograms as described in method 4.3. Sutherlandin 3 (Su3), sutherlandin 4 (Su4), sutherlandioside B (SuB) and sutherlandioside D (SuD) were monitored.

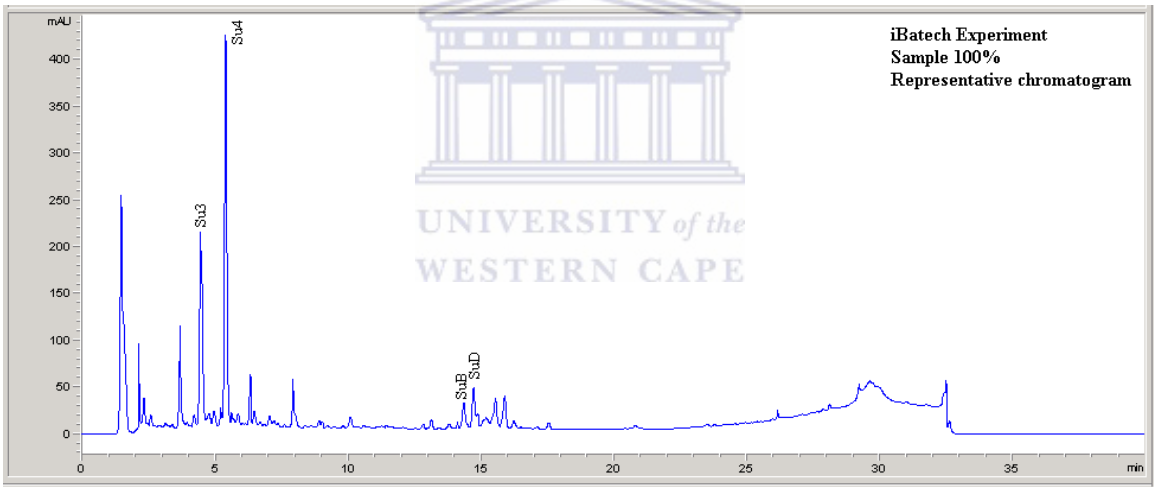
a)



b)

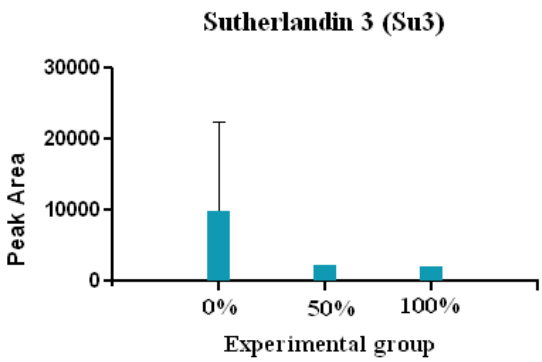


c)



Figures 5.11 a, b and c: Representative HPLC chromatograms for the iBatech™ samples.

Figures 5.12, 5.13, 5.14 and 5.15 illustrate the average peak area values of Su3, Su4, SuB and SuD for the plants subjected to the iBatech™ product treatment, with a control 0%, 50% and 100% treatment concentrations. The error bars plotted represent three plant samples from the same product treatment concentration.



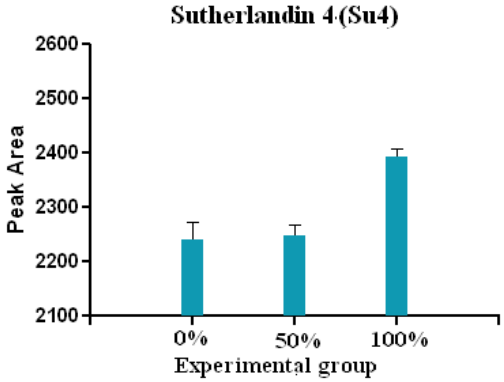
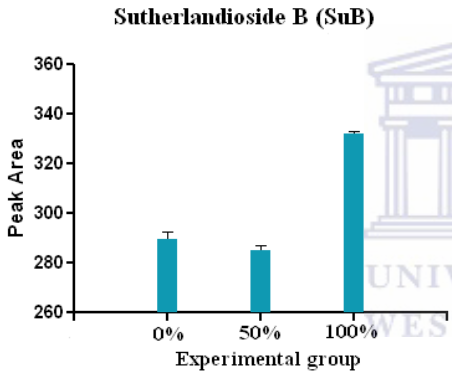


Figure 5.12: Sutherlandin 3 (Su3)

Figure 5.13: Sutherlandin 4 (Su4)



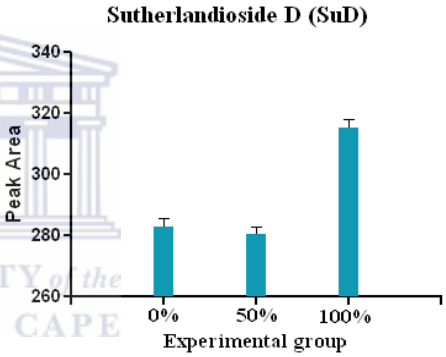


Figure 5.14: Sutherlandioside B (SuB)

Figure 5.15: Sutherlandioside D (SuD)

Figure 5.12 (sutherlandin 3) illustrates the average peak area values of the (control) 9758.43, the (50%) 2232.63 and the (100%) 2031.97 treatment groups. Figure 5.13 (sutherlandin 4) illustrates the average peak area values of the (control) 2241.63, the (50%) 2247.47 and the (100%) 2392.60 treatment groups, with the 100% treatment group having the highest average peak area values of Su4. Figure 5.14 (sutherlandioside B) illustrates the average peak area values of the (control) 289.66, the (50%) 284.93 and the (100%) 332.30 treatment groups. Figure 5.15 (sutherlandioside D) illustrates the average

peak area values of the (control) 282.77, the (50%) 280.60 and the (100%) 315.13 treatment groups, with the 100% treatment group having the highest average peak area values for SuD.

The Kruskal-Wallis test was used to determine whether there are any variations among the iBatech^{TM} experimental groups for sutherlandins 3 and 4 and sutherlandiosides B and D. The data for sutherlandioside B (SuB) was significant *(P = 0.0390)* and therefore the post ANOVA, Dunn's Multiple Comparison test was performed to determine which groups were significantly different. The results showed that the 50% treatment group was significantly different to the 100% treatment group.

The column statistics of the raw data was analyzed to determine if there were any significant differences among samples from the same iBatech^{TM} treatment group. Sutherlandin 3 (Figure 5.12) showed significant differences among samples from the 50% (*P = 0.0001*) and 100% (*P = 0.0001*) treatment groups but not from the control (*P = 0.3158*) group. Sutherlandin 4 (Figure 5.13) had significant differences among samples from the control (*P = 0.0001*), 50% (*P = 0.0001*) and 100% (*P = 0.0001*) treatment groups. Sutherlandioside B (Figure 5.14) had significant differences among the samples from the control (*P = 0.0001*), 50% (*P = 0.0001*) and 100% (*P = 0.0001*) treatment groups. Sutherlandioside D (Figure 5.15) had significant differences among the samples from the control, 50% and 100% treatment groups.

In summary, sutherlandioside B was found to have significant differences when comparing the 50% and 100% treatment groups. It is important to note that there were significant differences among all samples from all of the iBatech^{TM} treatment groups, except for sutherlandin 3 in the control group. This means that the data had a wide range and could explain why significant differences could not be found when comparing the different treatment groups.

5.3 Liquid Chromatography – Mass Spectrometry

HPLC was performed on *Sutherlandia frutescens* Afriplex^{TM} plant material as described in section 4.2 and fractions of the SuB and SuD collected and pooled. Due to the nature of sutherlandiosides B and D i.e. the compounds elute with less than a minute apart, which complicates collecting them as separate fractions. The pooled collected fractions were used for liquid chromatography – mass spectrometric (LC-MS) analysis. The standard SuB and SuD fraction obtained from the University of Mississippi was also a ratio between the two compounds.

The fractions collected from the *Sutherlandia frutescens* Afriplex^{TM} sample with the spectral data conforming to those of sutherlandiosides B and D (SuB and SuD) as described by Avula *et. al.* (2010), were subjected to mass spectrometry for verification of their identities. The prominent compound in the collected fraction (Figure 5.16 and Figure 5.17) was eluted at retention time 7.23 minutes and had a mass of 635.4412.

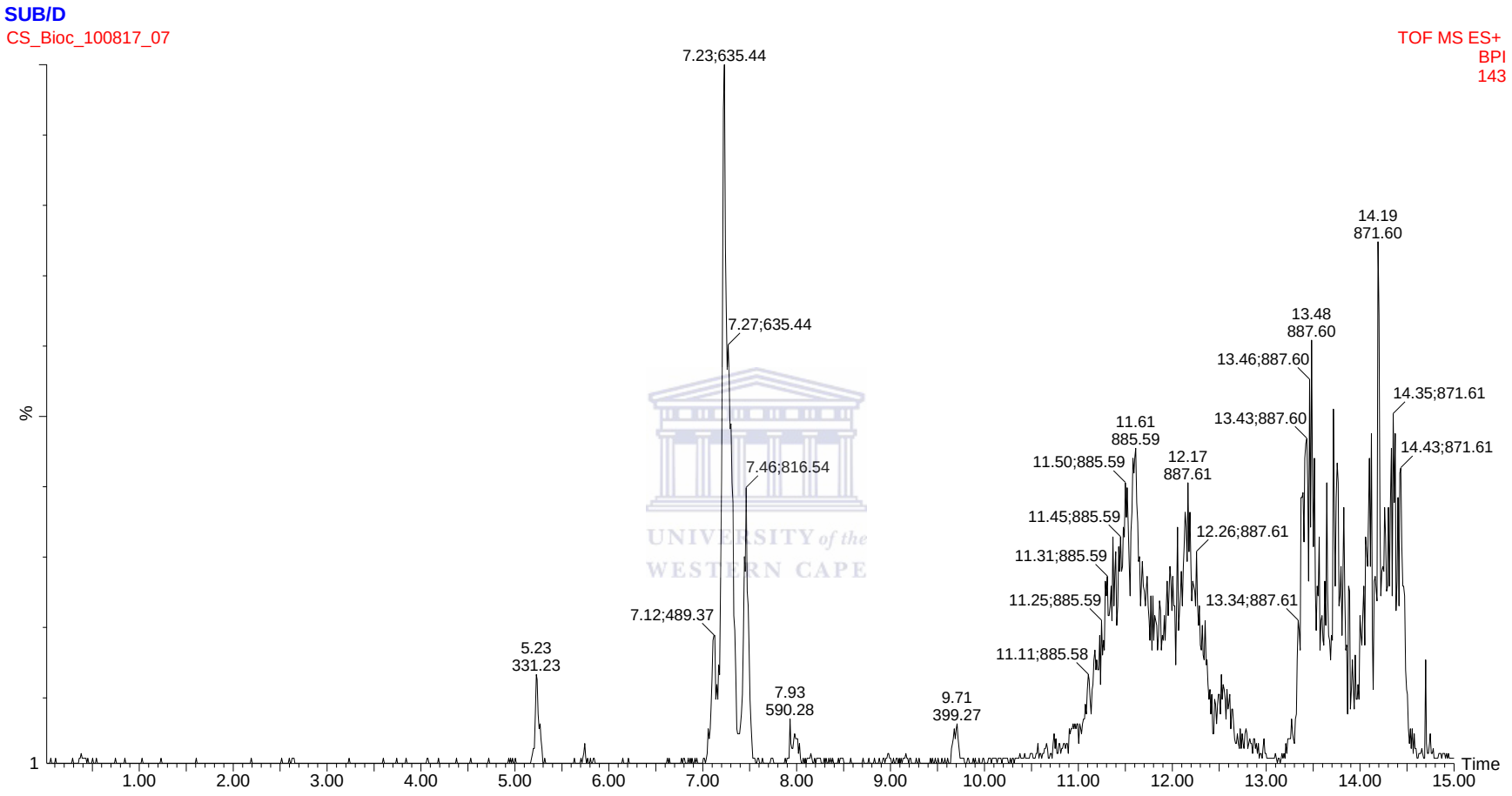


Figure 5.16: An LC-MS chromatogram of the collected SuB/ SuD pooled fraction

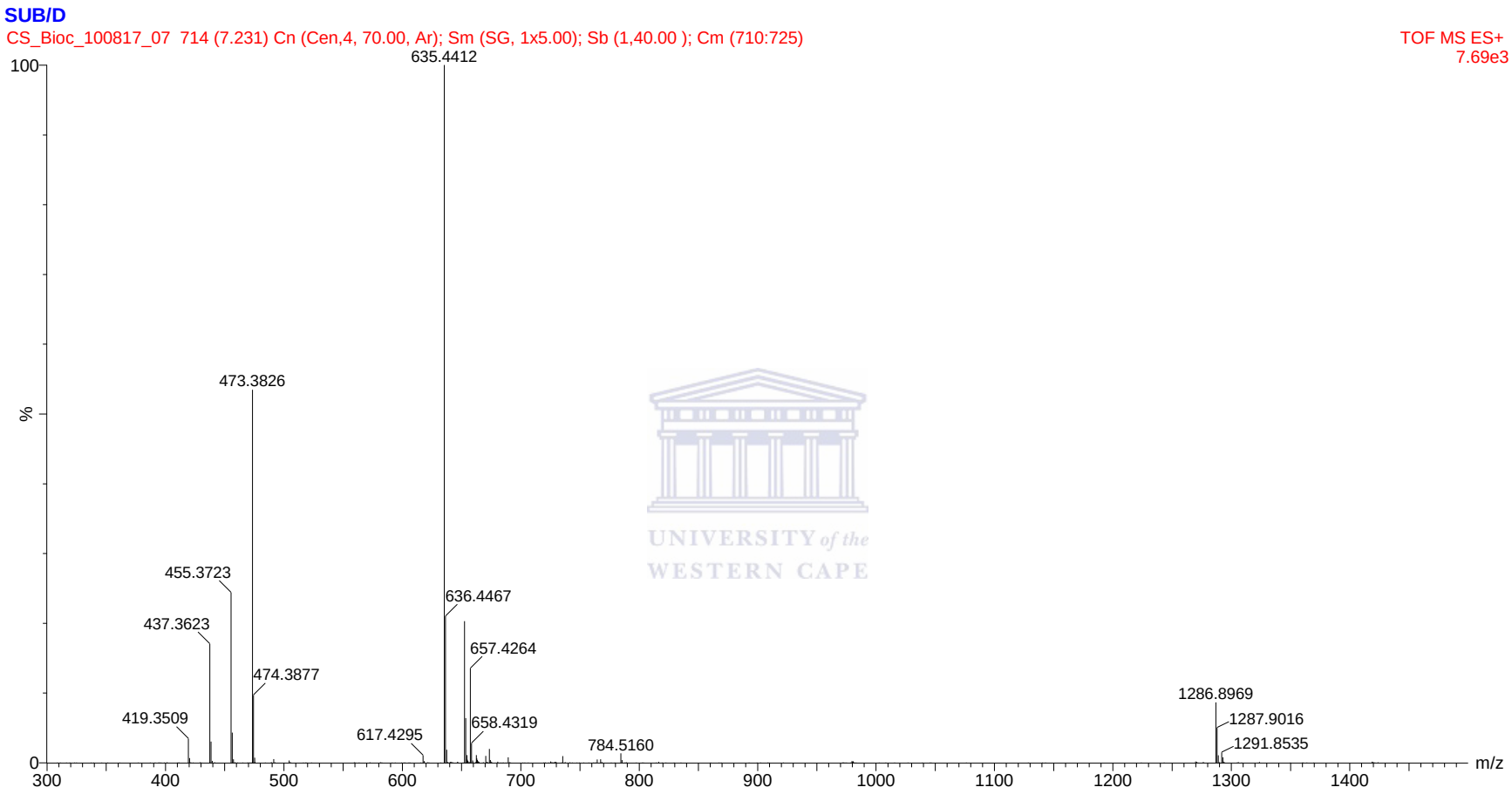


Figure 5.17: LC-MS mass spectral chromatogram for the collected SuB/ SuD fraction

Table 5.2: The concentrations of sutherlandiosides B and D in mg/ml in all plant samples

		SuB		SuD	
Environmental category	**Sample Name**	**Peak Area**	**Concentration mg/ml**	**Peak Area**	**Concentration mg/ml**
Natural field category	**Site 1**	53.566	0.15	43.066	0.09
	Site 2	67.233	0.19	48.100	0.11
	Site 3	448.300	1.43	341.133	1.15
Cultivated field category	**Site 4**	216.433	0.68	231.800	0.76
	Site 5	212.733	0.66	32.766	0.05
	Site 6	1354.500	4.36	1368.533	4.80
Greenhouse category	**Site 7**	275.500	0.87	321.900	1.08
	Site 8	377.933	1.20	453.066	1.55
iBatech experiment	**iBatech 0% (control)**	289.666	0.91	282.766	0.94
	iBatech 50% treatment	284.933	0.90	280.600	0.93
	iBatech 100% treatment	332.300	1.05	315.133	1.06

5.4 Quantification: concentrations of sutherlandiosides B and D in samples collected from the natural field group, cultivated field group, greenhouse group and the iBatech™ experiment

In both the NF and CF groups (Table 5.2) there appears to be dramatic variations in the concentrations of SuB and SuD. The Site 3 (CF) and Site 6 (CF) samples have a five and six fold increase respectively in SuB and SuD concentrations when compared to the Site 1 (NF), Site 2 (NF), Site 4 (CF) and Site 5 (CF) samples. The GH group also had differences among samples from the same group but appear relatively uniform when compared to the iBatech™ control group and iBatech™ 50% treated group that were also grown in a greenhouse. The iBatech™ 100% treated group shows an increase in SuB and SuD concentrations to that of the iBatech™ control and iBatech™ 50% treated groups.

In conclusion, there were dramatic variations in the environmental factors of samples from the same environmental category; however the environmental factors in the greenhouse group, appeared more uniform. Significant differences were found in sutherlandin 4 when comparing the different environmental categories and sutherlandioside B when comparing the 50% and 100% treatment groups in the iBatech™ experiment. Furthermore, significant differences were found in metabolite profiles and peak area values of Su3, Su4, SuB and SuD, among samples from the same environmental category as well as among samples from the iBatech™ experiment. The LC-MS results obtained on the collected fractions confirm that it was SuB and SuD as described by Avula *et. al*. (2010) and the SuB and SuD quantification results suggest no

relationship between environmental category or iBatech^{TM} treatment group and the synthesis of these metabolites.

Chapter 6

Conclusions and recommendations

This study was aimed at to locate sites where *S. frutescens* is grown, broadly categorize the sites into environmental groups based on the pertinent environmental growth factors, to develop and validate a high-performance liquid chromatography method for Su3, Su4, SuB and SuD analysis, to determine and compare the profiles and levels of Su3, Su4, SuB and SuD *S. frutescens* grown in different environmental growth sites and to determine whether the application of the iBatech™ product increases the number and concentrations of secondary metabolites in *S. frutescens*. To achieve the aim of this study, a minimum of three *S. frutescens* samples (homogenized) per site, were collected from eight different sites and broadly categorized into three environmental categories viz. 1. natural field, 2. cultivated field and 3. greenhouse. *S. frutescens* plants were also grown in a greenhouse and treated with the iBatech™ product, with a 0% control, 50% product concentration and 100% product concentration. A high performance liquid chromatography (HPLC) method was developed and validated, using the ICH guidelines, for the simultaneous analysis of flavonoids and terpenoids in *S. frutescens* and used for the profiling and determination of the average peak area values of sutherlandins 3 and 4 and sutherlandiosides B and D. Liquid chromatography – mass spectrometry (LC-MS) was used to confirm the identity of the isolated and collected SuB and SuD pooled fractions. Furthermore, HPLC was also used for the construction of calibration curves, for the quantification of sutherlandiosides B and D (SuB and SuD) in all samples.

The specific objectives were: to determine and describe sites where *Sutherlandia frutescens* is grown, which could be broadly categorized into, 1. natural field, 2. cultivated field and 3. greenhouse and the pertinent growth factors which we assume could affect secondary metabolite synthesis, to determine sutherlandins 3 and 4 (Su3 and Su4) and sutherlandiosides B and D (SuB and SuD) profiles and average peak area values in *S. frutescens* collected from the eight sites, to determine the effects of the iBatech™ product treatment on sutherlandins 3 and 4 sutherlandiosides B and D profiles and average peak area values, and finally, to quantify the concentrations of sutherlandiosides B and D in all samples.

From the results obtained in this study the following conclusions can be drawn:

1. The sites that were categorized into three environmental categories illustrate a range of conditions at the time of collection, possible secondary metabolite synthesis-limiting factors and variations in plant size. The Su3, Su4, SuB and SuD profiles, average peak area values and concentrations of SuB and SuD can therefore not be correlated with the environmental categories.

2. The sutherlandins 3 and 4 and sutherlandiosides B and D profiles and average peak area values vary among the different environmental growth categories. Variations in the profiles and peak area values are also visible among sites from the same environmental growth category. These variations can be attributed to the specific prevailing environmental conditions (micro-climates) at each site. The greenhouse (GH) group,

although not having significantly different, or the highest values for average peak areas of sutherlandins 3 and 4 and sutherlandiosides B and D, appear more uniform in the synthesis of these metabolites and can be attributed to the control of the environmental growth factors, which is the nature of a greenhouse.

3. The iBatech^{TM} product appears to have had a concentration related effect; however these differences were not significant. To date, the iBatech^{TM} product has not been thoroughly investigated, thus its mechanism of action is unknown. Mass spectrometry results on the iBatech^{TM} product performed by the manufacturers reveal that although containing a wide variety of flavonoids and terpenoids it does not contain the specific compounds evaluated in this study. However, some of the compounds revealed by mass spectrometric analysis are known to act as precursor compounds. It is therefore plausible to deduce that in this experiment, these compounds acted as precursors and promoted the synthesis of sutherlandins 3 and 4 and sutherlandiosides B and D.

4. The results obtained from the sutherlandiosides B and D (SuB and SuD) quantification, show two samples from the natural field (NF) and the cultivated field (CF) groups each with higher concentrations of SuB and SuD, when compared to the other samples. The greenhouse (GH), iBatech^{TM} control and iBatech^{TM} 50% treatment groups, which were all grown under greenhouse environmental conditions, show more stable synthesis of SuB and SuD.

From these results, we can conclude that the greenhouse growth environment, although not producing the significantly highest concentrations of sutherlandins 3 and 4 and sutherlandiosides B and D, have more consistent/ uniform results with regard to environmental factors, metabolite profiles, average peak area values and the concentrations of sutherlandiosides B and D. I therefore recommend that *S. frutescens* grown for medicinal purposes, for the yield of sutherlandins 3 and 4 and sutherlandiosides B and D, be grown under greenhouse conditions in conjunction with the application of the iBatech^{TM} product to produce consistent concentrations of Su3, Su4, SuB and SuD. As we have covered in the literature review, the environmental growth factors can be controlled in the greenhouse. It would therefore be beneficial to more closely investigate the environmental conditions in the sites from the natural field (NF) and cultivated field (CF) groups, which show the highest concentrations of sutherlandins 3 and 4 and sutherlandiosides B and D, and mimic those conditions in the greenhouse.

The results of this study are important in understanding the best suited environmental growth factors, applied collectively, in which to grow *S. frutescens* for greater yields of sutherlandins 3 and 4 and sutherlandiosides B and D, when growing *S. frutescens* for medicinal purposes.

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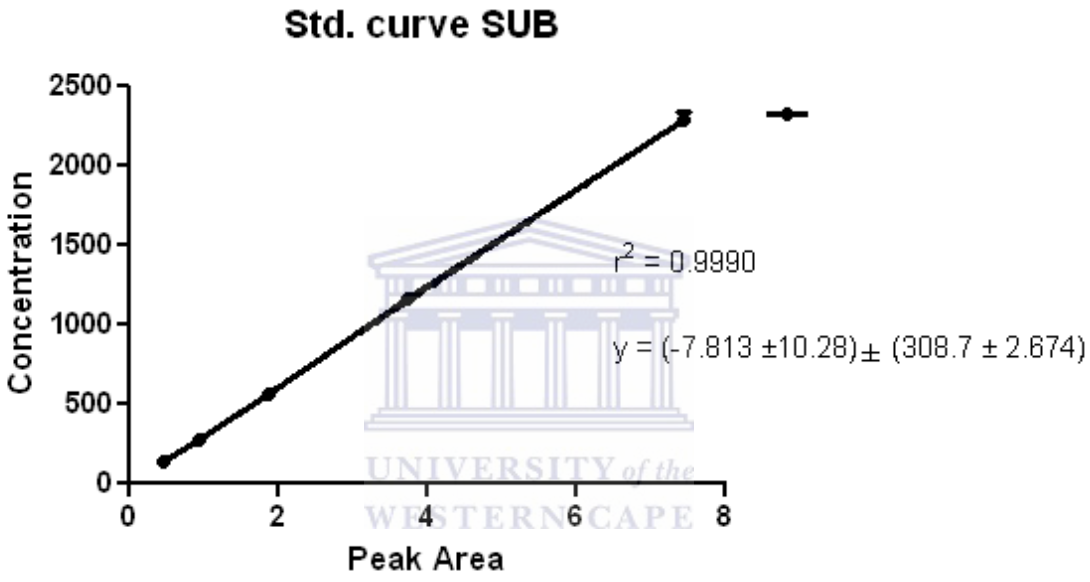
Appendixes

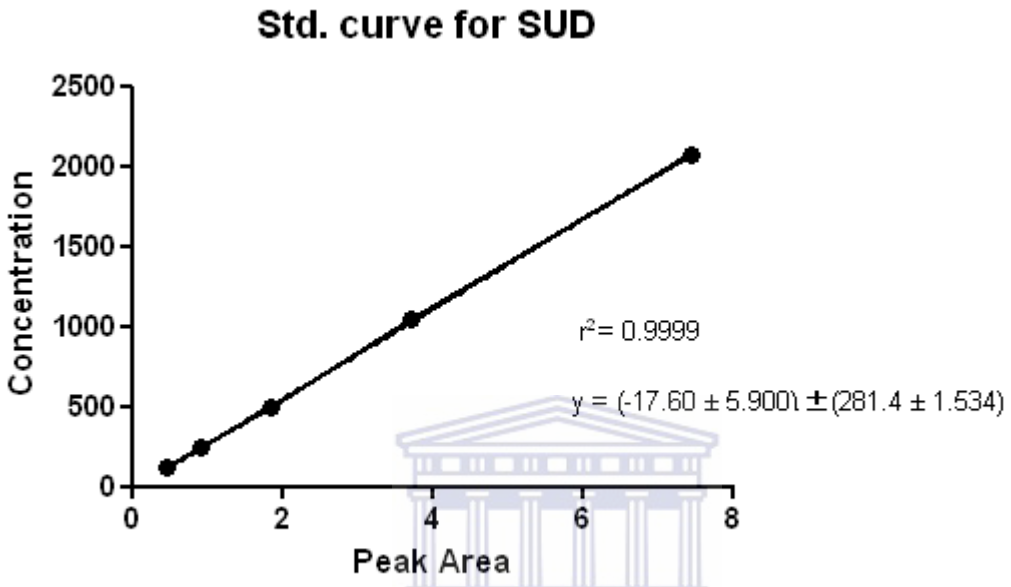
Appendix 1:

Assay validation data: SuB std. curve linear regression



Appendix 2:

Assay validation data: SuD std. curve linear regression



Appendix 3: The values for standard deviation, the averages and percentage RSD of the intra and inter-day variations for assay validation

		Su3	Su4	SuB	SuD
Intra-day	STDeviation	46.56798	21.30078	5.345789	6.5292
	Average	3220.178	1981.207	402.6148	427.3259
	%RSD	1.44	1.07	1.32	1.52
		Su3	**Su4**	**SuB**	**SuD**
Inter-day	STDeviation	48.15179	22.38726	9.00924	35.61073
	Average	3250.373	1991.1	426.0444	406.2852
	%RSD	1.48		2.11	8.76

Appendix 4: Retention time and peak area averages and standard deviation values for Sites 1-8 and iBatech™ experiment.

Plant Samples		sutherlandin 3		sutherlandin 4		sutherlandioside B		sutherlandioside D	
		Rt (mins)	Area (mAU)	Rt (mins)	Area (mAU)	Rt (mins)	Area (mAU)	Rt (mins)	Area (mAU)
Natural field	Site 1	4.355 ± 0.014	1527.9 ± 6.286	5.115 ± 0.013	2045.03 ± 6.785	15.005 ± 0.005	53.567 ± 0.251	15.341 ± 0.003	43.067 ± 0.450
	Site 2	4.312 ± 0.128	2330.17 ± 1314.352	5.1567 ± 0.009	907.867 ± 6.092	15.326 ± 0.003	67.23 ± 2.112	15.533 ± 0.003	48.1 ± 0.7
	Site 3	4.338 ± 0.013	4572.93 ± 2.055	5.098 ± 0.016	1534.1 ± 2.951	14.329 ± 0.020	448.3 ± 2.271	14.692 ± 0.019	341.133 ± 2.490
Cultivated field	Site 4	4.417 ± 0.025	2444.5 ± 23.612	5.3777 ± 0.027	1876.5 ± 16.802	14.357 ± 0.027	216.433 ± 2.350	14.725 ± 0.027	231.8 ± 1.824
	Site 5	4.49 ± 0.019	1747.27 ± 29.286	5.254 ± 0.126	3433 ± 847.971	14.944 ± 0.027	212.733 ± 2.335	15.299 ± 0.023	32.767 ± 0.230
	Site 6	4.430 ± 0.036	3367.67 ± 1360.339	5.377 ± 0.017	4033.77 ± 5.834	14.756 ± 0.027	1354.5 ± 2.497	15.262 ± 0.026	1368.53 ± 2.948
Green-house	Site 7	4.407 ± 0.008	2826.2 ± 3.716	5.369 ± 0.011	2390.43 ± 0.550	14.364 ± 0.034	275.5 ± 6.337	14.731 ± 0.034	321.9 ± 3.704
	Site 8	4.406 ± 0.004	2334.3 ± 3.831	5.363 ± 0.001	2333 ± 2.951	14.350 ± 0.006	377.933 ± 5.481	14.721 ± 0.007	453.067 ± 3.444
iBatech experiment	iBatech 0%	4.463 ± 0.017	9758.433 ± 12738.012	5.3555 ± 0.021	2241.633 ± 29.885	15.525 ± 0.009	289.667 ± 3.008	15.880 ± 0.008	282.767 ± 2.843
	iBatech 50%	4.45 ± 0.010	2232.633 ± 22.292	5.346 ± 0.009	2247.467 ± 21.096	15.518 ± 0.007	284.933 ± 2.150	15.873± 0.008	280.6 ± 2.151
	iBatech 100%	4.406 ± 0.011	2031.967 ± 12.859	5.364 ± 0.005	2392.6 ± 15.561	15.521 ± 0.010	332.3 ± 0.624	15.875 ± 0.010	315.133 ± 2.676

Appendix 5: Unprocessed HPLC data for the iBatech™ experiment and Sites 1-8.

SAMPLE	sutherlandin 3				sutherlandin 4				sutherlandioside B				sutherlandioside D			
	Rt	Area	Height	Width	Rt	Area	Height	Width	Rt	Area	Height	Width	Rt	Area	Height	Width
iBatech Control	4.453	2426.9	283.5	0.1179	5.343	2250.2	387.8	0.0857	15.52	291.6	30.6	0.1332	15.875	285.1	35.8	0.117
iBatech Control	4.452	2381.4	278.5	0.1198	5.344	2208.4	378.3	0.0861	15.519	286.2	29.9	0.1338	15.874	279.6	35.1	0.117
iBatech Control	4.483	24467	286.4	0.1177	5.38	2266.3	390.6	0.0857	15.536	291.2	30.7	0.1328	15.89	283.6	35.8	0.1164
iBatech 50%	4.459	2258.3	391.7	0.0877	5.354	2271.7	391.7	0.0877	15.514	287.1	31.2	0.1295	15.868	282.7	33.4	0.1207
iBatech 50%	4.452	2221.5	239.4	0.1262	5.348	2237.5	392.2	0.0846	15.527	284.9	30.7	0.1322	15.882	280.7	32.9	0.1236
iBatech 50%	4.439	2218.1	239.7	0.1239	5.336	2233.2	388.4	0.0871	15.513	282.8	30.5	0.13	15.868	278.4	32.8	0.1232
iBatech 100%	4.414	2019.4	215	0.1256	5.369	2377.4	425.7	0.0852	15.517	332.1	34.2	0.1353	15.869	318.1	37.2	0.1237
iBatech 100%	4.412	2031.4	217.5	0.127	5.366	2391.9	425.6	0.0856	15.513	331.8	34.1	0.1355	15.868	312.9	37.2	0.1221
iBatech 100%	4.394	2045.1	213.6	0.1297	5.358	2408.5	425.6	0.086	15.532	333	34.5	0.1346	15.887	314.4	37.3	0.1202
SAMPLE	sutherlandin 3				sutherlandin 4				sutherlandioside B				sutherlandioside D			
	Rt	Area	Height	Width	Rt	Area	Height	Width	Rt	Area	Height	Width	Rt	Area	Height	Width
NA	4.365	1529.1	154.2	0.1357	5.125	2048.8	263.1	0.1109	15.01	53.6	5.4	0.1418	15.344	43.5	6.1	0.1094
NA	4.338	1521.1	153	0.138	5.099	2037.2	261.9	0.1107	14.999	53.3	5.3	0.1455	15.337	42.6	5.9	0.1116
NA	4.362	1533.5	154.2	0.136	5.12	2049.1	265.4	0.1101	15.007	53.8	5.4	0.1426	15.343	43.1	6	0.1116
NB	4.376	3072.7	310.2	0.1376	5.148	900.9	103.3	0.1218	15.328	68.3	5	0.1904	15.535	48.1	8.2	0.0928
NB	4.395	3105.2	314	0.1374	5.167	910.5	106	0.1202	15.328	68.6	5	0.1887	15.536	48.8	8.3	0.0907
NB	4.164	812.6	128.5	0.0938	5.155	912.2	105.5	0.1228	15.322	64.8	4.9	0.1834	15.529	47.4	8.2	0.0912

NC	4.324	4575.3	602.3	0.1086	5.082	1532.6	179.6	0.1356	14.306	447.3	67.7	0.0991	14.669	339.9	51.3	0.1014
NC	4.351	4571.6	599.6	0.1089	5.114	1537.5	178.9	0.1382	14.342	450.9	67.9	0.0995	14.704	344	51.8	0.1015
NC	4.338	4571.9	602.2	0.1086	5.099	1532.2	179.2	0.1378	14.34	446.7	68	0.1007	14.702	339.5	51.4	0.099
SAMPLE	**sutherlandin 3**				**sutherlandin 4**				**sutherlandioside B**				**sutherlandioside D**			
	Rt	**Area**	**Height**	**Width**	**Rt**	**Area**	**Height**	**Width**	**Rt**	**Area**	**Height**	**Width**	**Rt**	**Area**	**Height**	**Width**
CA	4.404	2418.1	328.9	0.1038	5.365	1857.5	330.7	0.0855	14.334	216.4	28.3	0.1113	14.702	230.9	32.2	0.1076
CA	4.401	2451.8	334.6	0.1035	5.359	1882.6	336.8	0.0832	14.349	214.1	28.3	0.1124	14.719	230.6	32.3	0.1073
CA	4.446	2463.6	339.6	0.1026	5.409	1889.4	338.5	0.0851	14.387	218.8	28.7	0.113	14.755	233.9	32.6	0.1077
CB	4.483	1754	265.6	0.097	5.189	3959.5	691.1	0.0868	14.96	213.2	27.5	0.1124	15.225	32.9	4.4	0.1065
CB	4.475	1715.2	264.6	0.0956	5.173	3884.7	679.4	0.0867	14.912	210.2	27.3	0.112	15.208	32.5	4.4	0.1081
CB	4.512	1772.6	271.8	0.0961	5.399	2454.8	434.5	0.0839	14.959	214.8	27.9	0.1119	15.254	32.9	4.5	0.1061
CC	4.416	4146.6	426	0.1316	5.379	4027.6	666.3	0.0905	14.77	1352.5	216.5	0.0949	15.275	1365.2	202.4	0.1027
CC	4.471	1796.9	400	0.0749	5.393	4034.5	676.7	0.0896	14.774	1357.3	218.2	0.0966	15.279	1369.6	204.1	0.1023
CC	4.402	4159.5	423.5	0.1326	5.359	4039.2	666.8	0.0907	14.724	1353.7	216.8	0.0948	15.232	1370.8	202	0.1032
SAMPLE	**sutherlandin 3**				**sutherlandin 4**				**sutherlandioside B**				**sutherlandioside D**			
	Rt	**Area**	**Height**	**Width**	**Rt**	**Area**	**Height**	**Width**	**Rt**	**Area**	**Height**	**Width**	**Rt**	**Area**	**Height**	**Width**
GA	4.416	2829.7	384.4	0.1059	5.382	2389.9	425	0.0856	14.404	268.2	37.7	0.1069	14.77	317.7	48.2	0.0989
GA	4.406	2822.3	384.2	0.1037	5.364	2390.4	426.8	0.0853	14.341	279.6	38.7	0.1084	14.705	323.3	47.7	0.1031
GA	4.4	2826.6	384	0.1039	5.362	2391	428.2	0.0851	14.348	278.7	37.9	0.1079	14.719	324.7	48.5	0.102
GB	4.409	2331.7	331.6	0.102	5.362	2331.1	426.9	0.0817	14.345	371.7	47.9	0.1146	14.715	449.1	63	0.1071
GB	4.407	2338.7	335.3	0.1014	5.363	2336.4	422.2	0.0846	14.357	380.1	48.4	0.1155	14.729	454.8	62.8	0.1085
GB	4.401	2332.5	334.2	0.0994	5.364	2331.5	419.4	0.0849	14.347	382	48.4	0.1162	14.718	455.3	62.7	0.1087

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