QUALITY PROTOCOLS FOR NEMARIOC-AL AND NEMAFRIC-BL PHYTONEMATICIDES AND POTENTIAL CHEMICAL RESIDUES IN TOMATO FRUITS

by

KAGISO GIVEN SHADUNG

THESIS

Submitted in (partial) fulfilment of the

Requirements for the degree of

Doctor of Philosophy

in

Plant Production

in the

FACULTY OF SCIENCE AND AGRICULTURE

(School of Agricultural and Environmental Sciences)

at the

UNIVERSITY OF LIMPOPO

SUPERVISOR : PROF. P.W. MASHELA

CO-SUPERVISOR : DR M.S. MPHOSI

CO-SUPERVISOR : MR V.L. MULAUDZI

2016

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DECLARATION

I declare that the thesis hereby submitted to the University of Limpopo, for the degree Doctor of Philosophy in Agriculture (Plant Production) has not been submitted previously by me or anybody for a degree at this or any other University. Also, this is my work in design and in execution, and related materials contained herein had been duly acknowledged.

Candidate: Kagiso Given Shadung	Signature	Date
Supervisor: Prof. P.W. Mashela	Signature	Date
Co-Supervisor: Dr M.S. Mphosi	Signature	Date
Co-Supervisor: Mr V.L. Mulaudzi	Signature	Date

DEDICATION

To my boys, PraiseGod Tumisang Shadung and Kagiso Given Shadung Jnr and my lovely wife, Mmabatho Madula Shadung.

ACKNOWLEDGEMENTS

I wholeheartedly thank The Holy Trinity, God the Father, God the Son and God the Holy Spirit, for keeping this promise “I will never leave you nor forsake you”, which was evident throughout the execution of this PhD programme. To you belongs the Glory, the Honour and the Power. Forever and ever, in the Name of Jesus Christ of Nazareth. Amen. Jer 30:16-22 (Ke Maatla).

Sincerely, I would like to express my heartfelt gratitude to my supervisor, Prof. P.W. Mashela, for his unwavering support, encouragement, training in various concepts and for instilling in me the passion for hardwork with his ‘KUW’ philosophy. I am ready to serve. To my co-supervisory team members, Dr M.S. Mphosi and Mr V.L. Mulaudzi, you provided the much needed professional guidance on numerous areas of this research, along with continuous encouragements and prayers. Today, I wouldn’t be here had it not been for all of you. I had been greatly honoured to have worked under the Green Technologies Research Centre (GTRC), under the auspices of the Land Bank Chair of Agriculture - University of Limpopo, where I was capacitated in the incredible and awesome world of scientific research and writing, along with supervision of postgraduate students.

I am grateful to the University of Limpopo, the Technology Innovation Agency (TIA), the Land Bank Chair of Agriculture – University of Limpopo, the Flemish Interuniversity Council (VLIR) and the Agricultural Research Council - University Collaboration Centre for generously funding various aspects of my doctoral studies in the Green Technologies

Research Programme. Special gratitude to Prof. I. Ncube in the Biotechnology Unit, University of Limpopo, for providing the much needed technical expertise in using the HPLC and for creating bench space for me in his laboratory. Prof, I am just wordless for providing Mr Mahlale and Mrs Lekganyane to assist anytime I needed assistance. Also, Mr Mulaudzi in the Department of Chemistry, who found me aground and opened his laboratory and gave me his keys to access the laboratory anytime, I will forever remain grateful. I will never cease to appreciate the assistance rendered by Refiloe, Nombuso and Musa in your laboratory. Limpopo Agro-Food Technology Station (LATS) will forever remain a home away from home, the Managing Director, Dr Mphosi, who gave me the opportunity to pursue this doctoral study and also provided financial support. To all colleagues at LATS, especially Dr M.Y. Maila, Mr N. Mutshekwa, Ms V. Mogashoa and Mr M. Bohlale, I remain speechless for the various but indispensable contributions towards the successful completion of this project.

I would like to single-out LATS and the Land Bank Chair of Agriculture – University of Limpopo for sponsoring my conference trips during the PhD programme to (1) Entebbe, Uganda, where I presented two papers at the 11th African Crop Science Society Conference in 2013, (2) Kuching, Malaysia, where I presented two papers at the International Conference on Advances in Plant Sciences in 2014, (3) George, Western Cape, where I presented two papers at the Combined Congress in 2015, (4) Bloemfontein, Free State, where I presented three papers at the Combined Congress in 2016, (5) Johannesburg, Gauteng, where I presented two papers at 2016 Autumn Scientific Conference on Food Safety and Security in 2016 and (6) Athens, Greece, where

I presented one paper at the 9th Annual International Symposium on Agriculture in 2016. The Land Bank Chair of Agriculture also paid for my accommodation during my first year, thereby enabling me to stay on campus with my family near the research laboratories.

My love goes to my family, friends and the loved ones, especially my wife, Mmabatho and my sons (PraiseGod and Kagiso Jnr), my mother (Linah Shadung) and the in-laws (Dorothy and Paulos Mothoa), my sisters (Eva and Thembi) and brothers (Happy and Blessing) – for tirelessly cheering me up and supporting me, always. I definitely could not have been this far without my father-in-law, Pastor P Mothoa, who took over the pastoring roles, taking care of my two churches, when my PhD tight schedules called. All my pastors who understood my lengthy and continuous absences and willingly filled the gap, thank you a million times. May the Good God grant you all your desires. Special thank you to my research friends and colleagues at the Green Technologies Research Centre: Zakheleni Dube, Vivian Mathabatha, Thabo Nkuna, all postgraduate students and the service workers for any and all the help you generously offered. Your motivation drove me to this end.

Ke rutegile! To God be the Glory.

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ABSTRACT

Nemarioc-AL and Nemafric-BL phytonematicides are being researched and developed from fruits of wild cucumber (*Cucumis myriocarpus* Naude.) and wild watermelon (*Cucumis africanus* LF.), respectively. Both are indigenous to Limpopo Province, South Africa. The two products are used for managing nematode population densities on various crops. The products have the potential to serve as alternatives to the environment-unfriendly synthetic fumigant nematicides. The active ingredient in *C. myriocarpus* fruit is cucurbitacin A ($C_{32}H_{48}O_9$), which is unstable and is slightly soluble in water due to its partial polarity. Cucurbitacin A readily oxidises into cucumin ($C_{27}H_{40}O_9$) and leptodermin ($C_{27}H_{38}O_8$). In contrast, cucurbitacin B ($C_{32}H_{48}O_8$), is an active ingredient in *C. africanus* fruit, which is nonpolar and therefore, is insoluble in water. Quality (Q) is an intangible trait that is based on perception and can be depicted using the relation, Q = P/E, where Q = quality, P = performance and E = expectations. Product efficacy is characterised by its performance and when efficacy surpasses expectations, the product is viewed as being of high quality. However, information on quality protocols and chemical residues in fruits related to the two phytonematicides are not documented. Research and development of quality protocols and the potential chemical residues in crop produce are integral part of the development of pesticides. Quality protocols would ensure that the product retains its “true-to-type” efficacy characteristics whenever it is used. Therefore, the aim of the study was to develop quality protocols for Nemafric-BL and Nemarioc-AL phytonematicides with respect to fulfilling expectations with regard to consistency of the products and their related potential chemical residues in crops, all of which would be

indispensable in the registration and up-scaling of the two products. The objectives of the study were therefore, to:

1. Determine (a) whether the content of cucurbitacin B in all organs of *C. africanus* was equally distributed to allow for the use of whole plants in the production of Nemafric-BL phytonematicide and (b) whether the accumulation of cucurbitacin A and B during different developmental fruit stages in *C. myriocarpus* and *C. africanus* could enhance the establishment of optimum harvest times, thereby ensuring improved quality of Nemarioc-AL and Nemafric-BL phytonematicides, respectively.
2. Investigate whether sun-, shade- and freeze-drying methods relative to the oven-drying method at 52°C would affect concentrations of cucurbitacins A and B in fruit pieces of *C. myriocarpus* and *C. africanus,* respectively.
3. Determine whether increasing the drying temperatures relative to 52°C would affect the concentrations of cucurbitacin A and B in fruit pieces of *C. myriocarpus* and *C. africanus*, respectively.
4. Investigate (a) whether the storage of phyto-inventories for Nemarioc-AL and Nemafric-BL phytonematicides would influence the concentration of cucurbitacin A and B in sealed and unsealed containers at room temperature and (b) whether the storage of manufactured Nemarioc-AL and Nemafric-BL phytonematicides would affect the concentrations of cucurbitacin A and B, respectively.
5. Determine whether the location where *C. myriocarpus* and *C. africanus* fruits were harvested would affect the concentration of cucurbitacin A and B, respectively.

6. Investigate whether tomato plants treated with Nemarioc-AL and Nemafric-BL phytonematicides under field conditions would contain residues of cucurbitacin A and B with improved foliar essential mineral nutrients.

Field and laboratory experiments were conducted to achieve the stated objectives, with the experimental designs being completely randomised and randomised complete block designs, respectively. Data were analysed using either parametric or non-parametric tests. Unless stated otherwise, a parametric test was implied. Reliability of measured variables was ensured by using statistical levels of significance at 5% level of probability and the coefficients of determination (R^2), whereas validity was ensured by conducting the experiments at the same location over two seasons or using factorial experiments. The concentration of cucurbitacins were quantified using the isocratic elution Shimadzu HPLC Prominence with detection using Shimadzu CTO-20A diode array detector. Among fruit, leaf, root and vine of *C. africanus*, fruit had the highest concentration of cucurbitacin B, whereas the other organs contained equivalent concentrations. Relative to fruit, concentrations of cucurbitacin B in other organs were lower by 67-94%. In both *Cucumis* species, concentrations of cucurbitacins and the developmental stages of fruits exhibited density-dependent growth (DDG) patterns, with cucurbitacin A and B achieving optimum concentrations at 89 and 95 days after transplanting, respectively. In the selection of the drying method using the concentrations of cucurbitacin A and B, although there were overlaps, for the purpose of bulk-drying of the fruits, the oven-drying method was consistently better. In the investigation of the suitable temperatures for the preservation of cucurbitacins, cucurbitacin A could withstand drying temperatures up to 60°C, whereas cucurbitacin B could not withstand temperatures above 52°C. In either case, significant

declines in cucurbitacins over increasing temperatures occurred. Cucurbitacins in phyto-inventories of *C. myriocarpus* and *C. africanus* in sealed and unsealed containers over increasing storage periods exhibited significant DDG patterns. In sealed and unsealed containers, the optimum storage periods for cucurbitacin A were 4.20 and 4.22 months, respectively, whereas those for cucurbitacin B were still in the stimulation phase of the DDG patterns, resulting in failure to optimise. In Nemarioc-AL and Nemafric-BL phytonematicides, cucurbitacins against increasing storage periods exhibited DDG patterns, with optimisation occurring at 3.90 and 2.74 months for cucurbitacin A and B, respectively. Locations had significant effects on concentrations of cucurbitacin A and B when using non-parametric tests. Under field conditions, the application of Nemarioc-AL and Nemafric-BL phytonematicides at 3% for 16- and 18-day application intervals, respectively, did not result in traceable concentration of cucurbitacin A and B in fruit of tomato plants. However, the concentrations of certain nutrients in fruit were improved. In conclusion, the developed quality protocols demonstrated that pre- and post-harvest storage handling pesticides could play important roles in quality of Nemarioc-AL and Nemafric-BL phytonematicides.

PUBLICATIONS GENERATED FROM THE THESIS

A. Journal articles

1. Shadung, K.G., Mashela, P.W. and M.S. Mphosi. 2013. Optimisation of NPK fertiliser requirement for *Cucumis africanus*. *African Crop Science Journal Proceedings* 11:911-913.
2. Shadung, K.G. and P.W. Mashela. 2013. Responses of *Cucumis myriocarpus* as low-input food security vegetable to NPK fertilization. *African Crop Science Journal Proceedings* 11:851-854.
3. Shadung, K.G., Mashela, P.W., Mphosi, M.S. Mulaudzi, L.V. and I. Ncube. 2015. Optimum harvest time of *Cucumis africanus* fruit using concentration of cucurbitacin B as a maturity standard. *Journal of Agricultural Science* 7(10):181-186.
4. Shadung, K.G. and P.W. Mashela. 2016. Suitable organ(s) in wild *Cucumis africanus* for the production of Nemafric-BL phytonematicide. *Acta Agriculturae Scandinavica, Section B – Plant Soil Science*. http://dx.doi.org/10.1080/09064710.2015.1122829.
5. Shadung, K.G., Mashela, P.W. and M.S. Mphosi. 2016. Response of cucurbitacin B concentration in Nemafric-BL phytonematicide to increasing storage period. Journal of Stored Products and Postharvest Research 7(3):32-36.
6. Shadung, K.G. and P.W. Mashela. 2016. Storage dynamics of cucurbitacin A in phyto-inventories of Nemarioc-AL phytonematicide. Transylvanian Review (In press).
7. Shadung, K.G., Mashela, P.W. and V.L. Mulaudzi. 2016. Responses of cucurbitacin A and cucurbitacin B concentrations from fruits of two *Cucumis* species to drying method. HortTech (Submitted).

8. Shadung, K.G., Mashela, P.W. and M.S. Mphosi. 2016. Suitable drying temperature for preserving cucurbitacin active ingredient in fruits of *Cucumis myriocarpus* and *Cucumis africanus.* HortTech (Submitted).
9. Shadung, K.G., Mashela, P.W. and A.R. Ndhlala. 2016. Influence of storage period on concentration of cucurbitacin B from mealed *Cucumis africanus* fruit. Journal of Agricultural Science and Technology (Submitted).
10. Shadung, K.G., Mashela, P.W., Mphosi, M.S. Mulaudzi, L.V. and I. Ncube. 2016. Cucurbitacin residues in tomato plants treated with Nemarioc-AL and Nemafric-BL phytonematicides. *Acta Agriculturae Scandinavica, Section B – Plant Soil Science* (In preparation).
11. Shadung, K.G., Mashela, P.W., Mphosi, M.S. Mulaudzi, L.V. and I. Ncube. 2016. Influence of storage period on active ingredient of Nemarioc-AL Phytonematicide. Transylvanian Review (In preparation).
12. Shadung, K.G., Mashela, P.W., Mphosi, M.S. Mulaudzi, L.V. and I. Ncube. 2016. Responses of foliar essential nutrient elements in tomato plant treated with Nemarioc-AL and Nemafric-BL phytonematicides. *Acta Agriculturae Scandinavica, Section B – Plant Soil Science* (In preparation).

B. Conference presentations

1. Shadung, K.G., Mashela, P.W., Mphosi, M.S. and L.V. Mulaudzi. 2016. Establishing harvest time of *Cucumis myriocarpus* fruit using active ingredient for nemarioc-AL phytonematicide production. 9th Annual International Symposium on Agricultural Research, July 11-14, Athens, Greece.

2. Shadung, K.G., Mashela, P.W., Mphosi, M.S. and Z.P. Dube. 2016. Effect of Nemrioc-AL and Nemafric-BL phytonematicides on essential mineral elements of tomato plants. Autumn International Scientific Conference on Food Safety and Security, May 16-18, University of Johannesburg, South Africa.
3. Shadung, K.G., Mashela, P.W., Mphosi, M.S., Mulaudzi, L.V. and I. Ncube. 2016. Nemarioc-AL and Nemafric-BL phytonematicides residues in tomato plants. Autumn International Scientific Conference on Food Safety and Security, May 16-18, University of Johannesburg, South Africa.
4. Shadung, K.G., Mashela, P.W., Mphosi, M.S., Mulaudzi, L.V., Dube, Z.P. and I. Ncube. 2016. Responses of cucurbitacins to drying temperatures from two *Cucumis* species. Combined Congress, January 18-21, University of the Free State, Bloemfontien, South Africa.
5. Shadung, K.G., Mashela, P.W., Mphosi, M.S., Mulaudzi, L.V., Ncube, I. and Z.P. Dube. 2016. Responses of cucurbitacin a to different harvest time of *Cucumis myriocarpus* fruit: Indigenous to South Africa. Combined Congress, January 18-21, University of the Free State, Bloemfontien, South Africa.
6. Shadung, K.G., Mamphiswana, N.D., Mashela, P.W., Mphosi, M.S., Mulaudzi, V.L. and I. Ncube. 2016. Optimising storage length of Nemafric-BL phytonematicide after processing. Combined Congress, January 18-21, University of the Free State, Bloemfontien, South Africa.
7. Dube, Z.P., Shadung, K.G. and P.W. Mashela. 2016. Residual effect of Nemarioc-AL and Nemafric-BL phytonematicides in tomatoes. Combined Congress, January 18-21, University of the Free State, Bloemfontien, South Africa.

8. Shadung, K.G., Mashela, P.W., Mphosi, M.S., Mulaudzi, V.L. and I. Ncube. Developing quality protocols for nemafric-BL phytonematicide. Post-Graduate Research Day, October 1-2, University of Limpopo, South Africa.
9. Shadung, K.G., Mashela, P.W., Mphosi, M.S., Mulaudzi, V.L. and I. Ncube. 2015. Using cucurbitacin b level in *Cucumis africanus* for establishing the harvesting time. Combined Congress. January 19-22, Tramonto, George, WC, South Africa.
10. Shadung, K.G., Mashela, P.W., Mphosi, M.S., Mulaudzi, V.L. and I. Ncube. 2015. Influence of storage time of ground crude extracts of *Cucumis africanus* fruit on concentration of cucurbitacin B. Combined Congress. January 19-22, Tramonto, George, WC, South Africa.
11. Shadung, K.G., Mashela, P.W. and M.S. Mphosi. 2014. Optimising fertiliser requirement for Cucumis africanus medicinal plant for low input production systems. 2nd International conference on Advances in Plant science. November 18-22, Four Points by Sheration, Kuching, Sarawak, Malaysia.
12. Shadung, K.G., Mashela, P.W., Pelinganga O.M. and M.S. Mphosi. 2013. Optimising NPK fertiliser requirement for *cucumis africanus* medicinal plant indigenous to South Africa. African Crop Science Society Conference. October 14-17, Entebbe, Uganda.
13. Shadung, K.G., Mashela, P.W. Mphosi, M.S and O.M. Pelinganga. 2013. Responses of *cucumis myriocarpus* to npk fertilisation in low-inputs food security vegetable programme. African Crop Science Society Conference. October 14-17, Entebbe, Uganda.

14. Shadung, K.G., Mashela, P.W. and M.S. Mphosi. 2013. Optimising NPK fertiliser requirement for *cucumis africanus* medicinal plant indigenous to South Africa. Post-Graduate Research Day, October 04, University of Limpopo, South Africa.

CHAPTER 1
RESEARCH PROBLEM

1.1 Introduction

Fruits of wild cucumber (*Cucumis myriocarpus* Naude.) and wild watermelon (*Cucumis africanus* LF.) are internationally used in medicinal systems, nutraceutical, pharmaceutical, cosmeceutical and pesticidal industries (Lee *et al.*, 2010; Mashela *et al.*, 2011; Mphahlele *et al.*, 2012; Thies *et al.*, 2010; Van Wyk *et al.*, 2002; Van Wyk and Wink, 2012). The plant species have since attained international distribution since cucurbitacin A and B have the economic potential for uses in various industries, including in all ten medicinal systems (Van Wyk and Wink, 2012), namely, traditional medicine, traditional Chinese medicine, traditional European medicine, traditional African medicine, traditional Indian medicine, traditional North American medicine, traditional Central/South American medicine, traditional Australian medicine, modern medicine and experimental medicine (Van Wyk and Wink, 2012). *Cucumis myriocarpus* and *C. africanus* fruits contain cucurbitacin A ($C_{32}H_{46}O_9$) and cucurbitacin B ($C_{32}H_{46}O_8$), respectively, as potent active ingredients (Chen *et al.*, 2005; Jeffrey, 1978). The two thermo-stable chemical compounds (Krieger, 2001) are the triterpenoids, which are chemicals with a base structure that contains at least C-30 atoms (Chen *et al.*, 2005; Van Wyk and Wink, 2012). Cucurbitacin A, a partial polar molecule, is slightly soluble in water (Jeffrey, 1978) and oxidises readily to cucumin ($C_{27}H_{40}O_9$) and leptodermin ($C_{27}H_{38}O_8$) chemical compounds, whereas the insoluble cucurbitacin B is non-polar and stable (Jeffrey, 1978).

Fruits from the two *Cucumis* species are seasonal and cannot be stored in fresh form due to the high incidents of post-harvest decays. Mphahlele *et al.* (2012) identified the

causal agent as the acid-loving fungus, *Penicillium simplicissimum* (Oudem.) Thom. Usually, decay promotes losses of active ingredients through microbial degradation and leaching. For instance, in the Kingdom Fungi, digestive enzymes are excreted in the substrate, with the externally digested nutrients being absorbed by the mycelia (Campbell, 1990), resulting in large quantities of active ingredients being lost. Preservation techniques had been developed to ameliorate losses of active ingredients in plant organs for various medicinal systems (Danso-Boateng, 2013; Diaz-Maroto *et al*., 2002; Mudau and Ngezimana, 2014; Rocha *et al*., 2011). Fermented crude extracts on such technologies (Pelinganga *et al*., 2012) had since received widespread acceptance in climate-smart agriculture (Mashela *et al*., 2011).

Fruits collected from the two *Cucumis* species are used to produce Nemarioc-AL and Nemafric-BL phytonematicides through the fermented crude extract technology (Pelinganga, 2013). The two phytonematicides consistently suppressed nematode population densities, including the notorious root-knot (*Meloidogyne* species) nematodes (Mashela *et al*., 2015; Pelinganga and Mashela, 2012; Pelinganga *et al*., 2012). Products developed from mature fruits are less variable in chemical composition (Mashela *et al*., 2015), whereas those from leaves have ingrained variabilities due to the inherent seasonal mobilities of secondary metabolites and mobile essential nutrient elements from leaves to other organs (Mudau *et al*., 2008). In *C. myriocarpus*, cucurbitacin A is compartmentalised mainly in roots and fruit, whereas in *C. africanus*, cucurbitacin B occurs in all plant organs (Jeffrey, 1978). However, studies on comparative distribution of this active ingredient in fruit, stems, leaves and roots of *C. africanus* had not been undertaken. Additionally, in both

Cucumis species the influence of the developmental phase of fruits on concentrations of cucurbitacins remains undocumented.

Information on quality protocols and effects of chemical residues on cultigens from the two phytonematicides could help to address the issues of 'inconsistent results' in phytonematicides – as expounded elsewhere (McSorley, 2011). Development of quality protocols and comprehensive investigation of chemical residue effects on cultigens are an integral part of the development of pesticides (Banks and Bristow, 1999). Quality protocols ensure that the product would retain its "true-to-type" efficacy characteristics whenever it is used (Besterfield *et al*., 2003). Products are characterised by their performance and when the latter surpasses expectation, the product is viewed as being of high quality (Parasuraman *et al*., 1990; Zeithaml, 1988). Quality (Q) is intangibly based on perception but could be depicted using the $Q = P/E$ relation, where Q = quality, P = performance and E = expectation (Banks and Bristow, 1999). In most cases, when $Q > 1.0$, the end-user has exceptional acceptance of the product, *vice versa* (Banks and Bristow, 1999). Although P and E are mostly based on perceptions, the inventor is expected to quantify both variables in line with the existing legislation. The latter is intended to provide end-users with specifications for protecting the consumer and the environment (Mashela *et al*., 2015). In development of phytonematicides, the environment may include non-target organisms like the cultigens that are being protected against the target nematode pests, including various soil microorganisms (Taurayi, 2011).

The International Organization for Standardisation (ISO 9000) defines quality as the degree to which a set of inherent characteristics fulfils prescribed quality requirements

(Anon., 2000). The ISO 9000 being critical for both quality control and quality assurance. In terms of ISO 9000, quality can be used with adjectives such as poor, good and/or excellent (Anon., 2000). The term ‘inherent’ as used in quality, is defined as an “embodiment”, which could be viewed as a permanent characteristic (Bank and Bristow, 1999). The characteristics could be quantitative or qualitative, whereas the requirement element could be viewed as an expectation that was stated or implied by the inventor, its end-users and other stakeholders (Anon., 2000). In reality, quality has nine different but interdependent dimensions, namely, (a) performance, (b) features, (c) conformance, (d) reliability, (e) durability, (f) service, (g) response, (h) aesthetics and (i) reputation (Banks and Bristow, 1999). A product could be excellent in one or more of the nine dimensions, whereas it could be average or poor in others. Few, if any products, excel in all nine dimensions (Banks and Bristow, 1999). In most cases, quality products are dependent on a few dimensions, with marketing agencies having the responsibility to identify the relative importance of each dimension and then magnifying them as being ideal to fulfil the identified needs for the end-users, with negligent effects on health and environmental risks (Sachdev and Verma, 2004).

Persistent health and environmental concerns from the end-users regarding synthetic chemical nematicides led governments, internationally to unilaterally and/or collectively withdrawing the products from the agrochemical markets (Govindasamy and Italia, 1998; Lambropoulou and Albanis, 2006; Thompson, 1998; WHO, 1984). In South Africa, the government established and enforced the Maximum Residue Limits (MRL) policy for pesticide residues in agricultural produce through an amendment to Act No. 36 of 1947. This Act outlines the criteria for the registration of inputs for use in agricultural systems. Similarly, ISO frameworks had been adopted to safe-guide health

and environmental concerns at international levels (Anon., 2000). Globalisation of produce and ISO 9000 product quality framework resulted in the withdrawal of most inputs from the agrochemical markets, particularly the synthetic chemical pesticides (Speth, 2004). Following the 2005 cut-off withdrawal date of ozone-depleting fumigant nematicides (Mashela *et al*., 2015), efforts to research and develop alternative strategies for managing nematode population densities in various cropping systems increased dramatically (McSorley, 2011). The Land Bank Chair of Agriculture – University of Limpopo, initiated the Green Technologies Research Programme (GTRP), which focused on research and development of botanicals as phytonematicides, phytoinsecticides and phytoherbicides (Mashela *et al*., 2011; 2015).

Using a multi-disciplinary approach, the GTRP had been involved in the development of Nemarioc-AL and Nemafric-BL phytonematicides (Mashela *et al*., 2015). Pelinganga *et al*. (2012) developed the liquid formulation of Nemafric-BL and Nemarioc-AL phytonematicides for use through irrigation in tomato (*Solanum lycorpersicum* L.) production systems. The application of botanicals through irrigation systems for managing nematodes had been referred to as botinemagation (Mashela *et al*., 2015). Pelinganga *et al.* (2013) demonstrated that the mean concentration stimulation point (MCSP) for each of the two products on tomato was approximately 3%, thus the code-names 3% Nemarioc-AL and 3% Nemafric-BL phytonematicides. The MCSP is the concentration of the phytonematicide that could hardly be phytotoxic to a given crop, but would consistently suppress nematodes (Mashela *et al*., 2015). Since the two products are produced from plant organs, their quality could invariably

be prone to changes in the active ingredients, which are inherently depended on various environmental factors.

1.2 Problem statement

The quality of Nemarioc-AL and Nemafric-BL phytonematicides, as products from plant organs is greatly influenced by the pre-harvest and post-harvest conditions that fruits are exposed to. The manufacturer has control over conditions under which the materials are prepared, stored as phyto-inventories and/or as finished products prior to distribution. In addition to efficacy information, the manufacturer, is expected to provide information on various quality protocols of the products in order to safe-guard the environmental and consumer health. Quality protocols of the two phytonematicides are required to enhance the registration of the two products to enable their uses under agricultural systems.

1.3 Rationale

Phytonematicides constitute an important part of climate-smart agriculture, due to their being environment-friendly (Mashela *et al.*, 2015). Nemarioc-AL and Nemafric-BL phytonematicides are being researched and developed for use in climate-smart agriculture. However, information is not readily available on the quality protocols and the environment-friendliness of these plant-based products. The establishment of quality protocols for Nemafric-BL and Nemarioc-AL phytonematicides, along with their potential chemical residue status in crops are intended to influence policy and to enhance the registration of the two products by the Department of Agriculture, Forestry and Fisheries in terms of Act No. 36 of 1947. Currently, there are no exact official quality protocols for phytonematicides (Anon., 2000). Therefore, the use of active

ingredients in quantifying quality protocols could serve as the basis for policy framework in registration of phytonematicides. Additionally, quality protocols would ensure that the end-users get 'true-to-type' products in relation to the efficacy and shelf-life of the products.

1.4 Aim and objectives

1.4.1 Aim

The development of quality protocols for Nemafric-BL and Nemarioc-AL phytonematicides with respect to fulfilling expectations with regards to consistency of the products and their related potential chemical residues in crops would be indispensable in the registration and up-scaling of the two products.

1.4.2 Objectives

The objectives of the study were to:

1. Determine (a) whether the content of cucurbitacin B in all organs of *C. africanus* was equally distributed to allow for the use of whole plants in the production of Nemafric-BL phytonematicide and (b) whether the accumulation of cucurbitacin A and B during different developmental fruit stages in *C. myriocarpus* and *C. africanus* could enhance the establishment of optimum harvest times, thereby ensuring improved quality of Nemarioc-AL and Nemafric-BL phytonematicides, respectively.
2. Investigate whether sun-, shade- and freeze-drying methods relative to the oven-drying method at 52°C would affect concentrations of cucurbitacins A and B in fruit pieces of *C. myriocarpus* and *C. africanus,* respectively.

3. Determine whether increasing the drying temperatures relative to 52°C compromise temperature would affect the concentrations of cucurbitacin A and B in fruit pieces of *C. myriocarpus* and *C. africanus*, respectively.
4. Investigate (a) whether the storage of phyto-inventories for Nemarioc-AL and Nemafric-BL phytonematicides would influence the concentration of cucurbitacin A and B in sealed and unsealed containers at room temperature and (b) whether the storage of manufactured Nemarioc-AL and Nemafric-BL phytonematicides would affect the concentrations of cucurbitacin A and B, respectively.
5. Determine whether the location where *C. myriocarpus* and *C. africanus* fruits were harvested would affect the concentration of cucurbitacin A and B, respectively.
6. Investigate whether tomato plants treated with Nemarioc-AL and Nemafric-BL phytonematicides under field conditions would contain residues of cucurbitacin A and B with improved foliar essential mineral nutrients.

1.5 Reliability, validity and objectivity

Reliability was ensured by using statistical levels of significance ($P \leq 0.05$ or R^2), validity was achieved through repeating the experiments in time, whereas the objectivity was attained by ensuring that the findings were discussed on the basis of empirical evidence, as shown in the statistical analyses, in order to eliminate all forms of subjectivity.

1.6 Bias

Bias was minimised by ensuring that the experimental error in each experiment was reduced through (a) using a high number of replications in each experiment, and (b) by assigning treatments at random within the selected research designs.

1.7 Ethical considerations

In this study, indigenous plant materials are being used to develop Nemarioc-AL and Nemafric-BL phytonematicides. Where the commercial use of the developed products is envisioned, the GTRP research team would ensure that the moral or legal rights of any potential claimants are respected through consultations in line with the University of Limpopo research policies and the appropriate legal framework in South Africa. Ethical considerations as outlined above would endure beyond the termination of the current study.

1.8 Significance

The developed quality protocols would ensure the availability of empirically-based information on the two phytonematicides. Findings would also expedite the registration of Nemarioc-AL and Nemafric-BL phytonematicides in terms of Act No. 36 of 1947. Additionally, the protocols would expand knowledge and influence policy dialogue in research and development of quality protocols for the developing phytonematicide industry.

1.9 Format of thesis

Following the description and detailed outlining of the research problem (Chapter 1), the review on the work done and not yet done on the research problem ensured

(Chapter 2). Then, each of the six objectives constituted separate chapters (Chapter 3-8). In the final chapter (Chapter 9), findings in all chapters were summarised and integrated to provide the significance of the findings and recommendations with respect to future research, culminating with conclusions that were intended to provide a take home message regarding the entire study. Literature citation and referencing followed the Harvard style using author-alphabets as prescribed by the relevant University of Limpopo Senate-approved policy framework.

CHAPTER 2
LITERATURE REVIEW

2.1 Contemporary pesticide issues

A number of contemporary issues related to global warming resulted in most countries legislating against the excessive use of pesticides. These issues, should phytonematicides not be properly used, could also affect the sustainable availability of these materials on agrochemical markets. Although this review is not intended to provide a detailed review on the contemporary issues of pesticides, few are outlined to introduce the problem statement in context.

2.1.1 Residues in produce and products

The presence of chemical residues in food and feed had been a serious concern during the pesticide era (Keikotlhaile and Spanoghe, 2011). The major concern had been their toxic effects such as those interfering with the reproductive system, foetal development and their carcinogenic capacity and triggering parkison disease and asthma in human beings (Gilden *et al*., 2010). The European Union had been rejecting foodstuff that contained pesticide chemical residues, ranging from tea, egg powder, chilies, green vegetables and cashew kernels (Rajendran, 2003; Vahab *et al*., 1991). In citrus trees treated with 18 g aldicarb/tree, chemical residues were found to be higher in leaves, with similar high residues in grape leaves and fruits and in sugar beets (National Research Council, 1986). Watermelon (*Citrullus lanatus* Thunb.) had also been shown to contain high chemical residues from fumigant nematicides used several years ago (Akan *et al*., 2015).

2.1.2 Environmental impact

Synthetic chemical pesticides could affect both biotic and abiotic components of the environment, with chemical residues found in soil, air, water, food and various ecosystems (Kumar, 2012; Rajendran, 2003). Pesticides get into ground water through leaching and into surface water through runoff (Rajendran, 2003). Rajendran (2003) also observed that some packaged water and soft drinks contain substantial amounts of pesticide chemical residues. The nontarget effects of nematicide applications were shown to alter some soil flora and fauna (Chitwood, 2003). Previously, the use of methyl bromide affected mycorrhizae which resulted in poor plant growth and accumulation of phosphorus in the soil (Klein *et al*., 1996).

2.1.3 Persistence and degradability

Under natural field conditions volatilisation, microbial degradation and leaching, all reduce pesticide concentrations (Celik *et al*., 1995). Photochemical degradation occurs when chemicals are on the soil surface where solar energy can be readily absorbed directly by the pesticide or indirectly by the soil components acting as photocatalyst (Földényi *et al*., 2011). Microbial degradation can also help to decrease considerable pesticide quantities, thereby negating their persistence in the environment (Johnson, 1998; Racke and Coats, 1988). In human beings, some of the previously used pesticides were persistent and remain in the body, thereby, causing long-term exposure (Keikotlhaile and Spanoghe, 2011). Generally, non-fumigants had shorter lifespans in the soil when compared with those of fumigants (Parsons and Witt, 1989). Fumigants with a half-life greater than 100 days in water and soil were categorised as being persistent, whereas those with a half-life less than 10 days were viewed as being nonpersistant (Vogue *et al*., 1994). However, certain fumigant

nematicides had a lifespan ranging from one to eight years, depending on the application depth and the applied concentration, whereas non-fumigant nematicides have a lifespan ranging from two weeks to two months, depending on whether they were applied on foliage or roots, as well as the applied concentrations (Van Gundy and McKenry, 1975).

2.1.4 Mobility in soil

The mobility of synthetic pesticides in the soil is influenced by the soil texture, pH and environmental conditions (Bowman, 1988; Jones and Norris, 1998). The distribution of the nematicide in the soil is the major factor affecting management of nematode population densities. Whiteheat *et al.* (1979) suggested that non-volatile nematicides exerted their effects on nematodes through contact in the soil rather than by systemic action through the plant. Garabedian and Hague (1982) conducted a study to evaluate the mobility of four different nematicides in the soil and noted that in sandy loam soil, aldicarb and oxamyl systemic nematicides were weakly adsorbed, and therefore, were highly mobile, whereas carbofuran and terbufos were strongly adsorbed and less mobile. Temperature also plays an important role on the efficacy of nematicides (Chitwood, 2003), with increase temperature stimulating or altering the efficacy of the nematicides. Consequently, when nematicides are applied in soils with low temperatures, efficacy could be affected (Grove and Haydock, 2000).

Nemarioc-AL (L = liquid formulation) and Nemafric-BL phytonematicides have the potential to serve as alternatives to the eco-unfriendly chemical synthetic nematicides (Mashela *et al.*, 2015). The latter had been implicated in ozone-depletion, which is one of the contributing factors to global warming (Speth, 2004). The two phytonematicides

had been researched and developed from wild cucumber (*Cucumis myriocarpus* Naude.) and wild watermelon (*Cucumis africanus* LF.) fruits, which are indigenous to Limpopo Province, South Africa (Kristkova *et al*., 2003). The objective of this review was to assess the work done on the problem statement, followed by the work not yet done on the problem statement.

2.2 Allelochemicals as active ingredients in phytonematicides

Plants produce a vast number of organic chemical compounds, which are responsible for various physiological functions (Mandal *et al*., 2007). The phytochemical compounds are categorised as primary or secondary metabolites. The primary metabolites are needed for plant growth and development, whereas the physiological roles of secondary metabolites in plants are not well-documented (Hartmann, 1991; Lai, 2008). The latter possibly play crucial roles in plant defences against herbivores (Palo and Robbins, 1991; Rosenthal and Berenbaum, 1991), insect pests (Adeyemi, 2010) and pathogens (Inderjit *et al*., 1999; Kosuge, 1969; Rice, 1984). Roles in defence range from serving as feeding deterrents/anti-feedants, repellents, allelopathic and also induce physical defence structures (Adeyemi and Mohammed, 2014; Bennett and Wallsgrove, 1994; Mashela *et al*., 2015; Nicholson and Hammerchmidt, 1992; Rattan, 2010). The secondary metabolites used by plants in interference with plants from different species are referred to as allelochemicals (Inderjit *et al*., 1999; Rice, 1984).

Allelochemicals are widely used in medicines, with more than 80% of the global population being depended on medicines that contain active ingredients derived from phytochemicals (Fabricant and Farnsworth, 2001; Wadood *et al*., 2013; Wink, 2012).

Globally, there are ten medicinal systems that are dependent upon allelochemicals for their efficacies (Van Wyk and Wink, 2004). Allelochemicals are heterogeneously distributed in plant organs (Macheix *et al.*, 1990). In some cases the materials are localised in root, bark and fruit organs or are temporarily stored in leaves (Cunningham, 1993). For instance, in wild watermelon (*Cucumis africanus* LF.), cucurbitacin B is stored in all organs of the plant, whereas cucurbitacin A in (*Cucumis myriocarpus* Naude.) is stored in seeds and roots (Jeffrey, 1978). Chemical compounds accumulate in leaves during photosynthetically active phase of plant growth are mobilised to other plant organs for overwintering storage (Mudau *et al.*, 2008). The latter could introduce high variability when these organs are used as resources for allelochemical compounds. The quality and quantity of active ingredients in plants are also affected by biotic and abiotic factors (Li and Wardle, 2001; Prance, 1994), the harvested organs (Pushpan *et al.*, 2012) and the post-harvest handling practices (Colegate and Molyneux, 1993; Houghton and Raman, 1998).

Traditionally, the majority of plants with medicinal properties are harvested from the wild (Fennell *et al.*, 2004). Due to limited information about the site where active ingredients are concentrated, whole plants are usually harvested and used. Due to increasing demands for toxic-free and eco-friendly products, product research and development using organs from indigenous plants with insecticidal, nematicidal and medicinal properties are increasing (Lee *et al.*, 2010; Mashela *et al.*, 2011; Thies *et al.*, 2010; Van Wyk and Wink, 2012; Van Wyk *et al.*, 2002). In research and development of Nemarioc-AL and Nemafric-BL phytonemacides, fruits of *C. myriocarpus* and *C. africanus* were used, respectively (Mashela *et al.*, 2011; Mashela *et al.*, 2015). In Neemate 10G, for instance, neem (*Azadirachta indica* A. Juss) seeds

were used (Hassan *et al.*, 2015). In cases where the active ingredients are compartmentalised, it would not be desirable to harvest the entire plant in the production of commercial products. However, since in most cases the organ with the highest concentration of the target allelochemical in not known, the whole plant is harvested.

In research and development of plant-based products, the variability of active ingredients in the harvested organ impacts negatively on the overall efficacy and quality of products (Agerbirk *et al.*, 2001; Azevedo *et al.*, 2001; Isman, 1997; Prakash and Rao, 1997). Commercial products from neem leaves have various degrees of efficacies, which negatively impact on product quality and therefore, the credibility of the products (Childs *et al.*, 2001; Weaver and Subramanyam, 2000). The same could be said for those that are from leaves of Lantana camara, which is being researched and developed for use as phytonematicides (Taurayi, 2011). In Nemarioc-AG (G = granular formulation) and Nemafric-BG phytonematicides, Maile (2013) demonstrated that the two products increased population densities of the citrus nematode (*Tylenchulus semipenetrans*). Subsequent studies (Mashela *et al.*, 2015), however, demonstrated that the nematode population densities under phytonematicide-treated conditions were dependent upon the application interval and/or sampling time. Some degree of standardisation throughout the value-chain, therefore, appears to be necessary if product quality is to be ensured. Although this approach entails standardisation in both pre- and post-harvest handling, the focus on this review was deliberately on the latter.

2.3 Phytonematicides from *Cucumis* species

Nemarioc-AL and Nemafric-BL phytonematicides are liquid (L) formulations, whereas Nemarioc-AG and Nemafric-BG are granular (G) formulations (Mashela *et al.*, 2015). Letters prior to the formulation represent active ingredients, with A being cucurbitacin A ($C_{32}H_{46}O_9$) (Figure 2.1). This active ingredient in *C. myriocarpus* fruit and roots, is not stable and rapidly disintegrates to cucumin ($C_{27}H_{40}O_9$) and lepdermin ($C_{27}H_{38}O_8$) (Jeffrey, 1978). In Nemafric-BL phytonematicide, B stands for cucurbitacin B ($C_{32}H_{48}O_8$) (Figure 2.1), and occurs in all organs of *C. africanus*. This cucurbitacin is insoluble in water and is also naturally stable (Chen *et al.*, 2005; Jeffrey, 1978).

Cucurbitacins are highly oxygenated tetracyclic triterpenoids that occur mainly in the Cucurbitaceae Family (Chen *et al.*, 2005). Plants that contain cucurbitacins are generally known for their analgesic, antimicrobial, anti-inflammatory, antipyretic and antitumor activities (Chen *et al.*, 2005; Geissman, 1964).

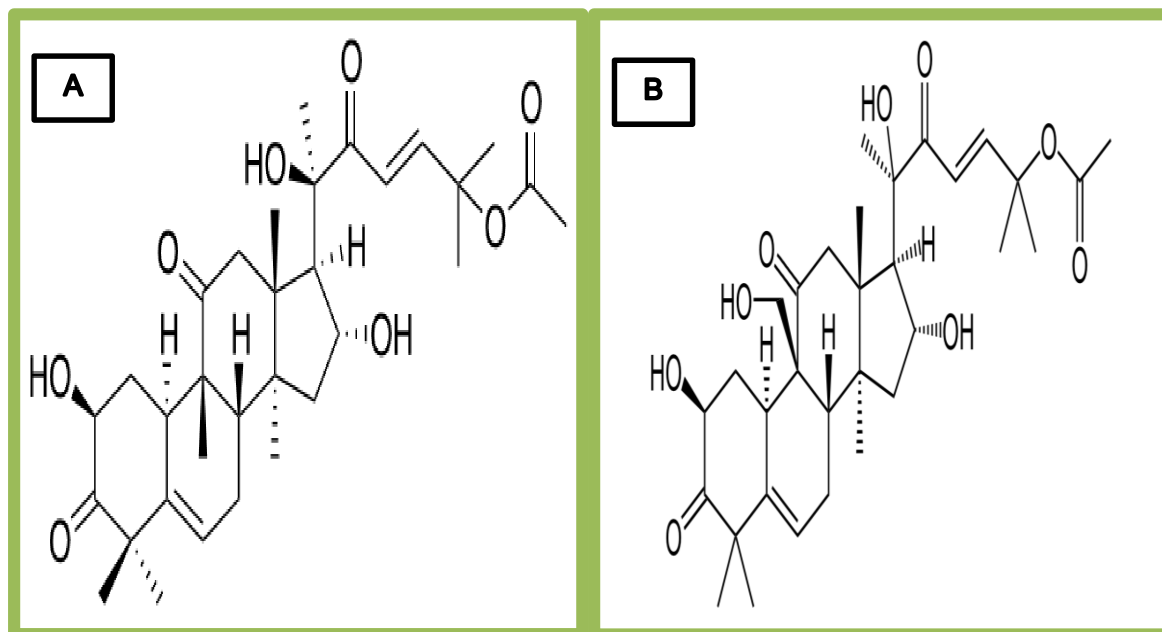


Figure 2.1 The chemical structure of cucurbitacin A ($C_{32}H_{46}O_9$) (A) and cucurbitacin B ($C_{32}H_{46}O_8$) (B) (Chen *et al.*, 2005).

Cucurbitacins and their derivatives are grouped into 12 categories (Chen *et al*., 2005), which are arbitrarily named cucurbitacin A to cucurbitacin T. Cucurbitacins have pharmacological, nematicidal and cosmetic properties as well as attractant or antifeedant activities towards insects (Attard and Scicluna-Spiteri, 2004; Dantas *et al*., 2006; Mashela *et al*., 2015). Cucurbitacins are also widely being researched for their cytotoxic, hepatoprotective, anti-inflammatory, anti-cancer and cardiovascular effects (Dhiman *et al.,* 2012; Lee *et al*., 2010; Miro, 1995).

Biosynthesis of cucurbitacins: Active compounds in plants are produced through numerous pathways, namely, (a) malonic acid pathway, (b) mevalonic acid pathway (MVA) and (c) shikimic acid pathway (Lai, 2008). The shikimic acid pathway synthesises aromatic compounds in plants, leading to the formation of tryptophan, tyrosine and phenylalanine, whereas malonic acid pathway plays a role in the biosynthesis of flavonoid compounds (Taiz and Zeiger, 1998; Van Wyk and Wink, 2004). The MVA pathway and MEP/DOXP pathway (Figure 2.2) play a role in the synthesis of triterpenoids which are mainly steroids and cucurbitacins (Chen *et al*., 2014; Van Wyk and Wink, 2004). From acetyl coA, when the MVA pathway was used, there were eight precursors and at least eight different enzymes prior to the formation of the stable cucurbitacin or steroids molecules (Figure 2.2) (Chen *et al*., 2014). However, when MEP/DOXP pathway was followed, there were eight precursors and six enzymes (Chen *et al*., 2014).

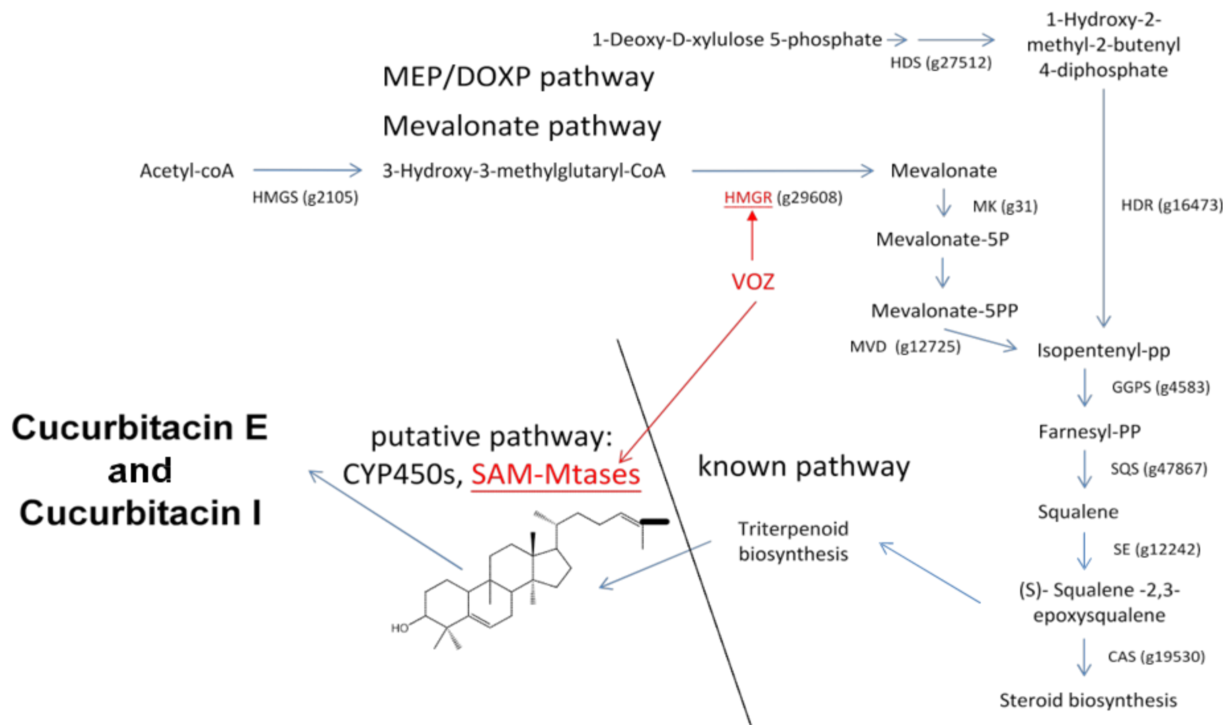


Figure 2.2 Biosynthesis pathways of cucurbitacin E and I in *Aquilaria agallocha* (Chen *et al.,* 2014).

2.4 Green technologies research programme

In Green Technologies Research Programme (GTRP), Nemarioc-AG (G = Granular formulation), Nemarioc-AL (L = Liquid formulation), Nemafric-BG and Nemafric-BL phytonematicides had been studied with the focus being on product performance. The GTRP comprises five technologies, *viz*. (1) ground leaching technology, (2) botinemagation technology, (3) intergeneric grafting technology, (4) agronomics technology and (5) chemical technologies (Mashela *et al*., 2011).

2.4.1 Ground leaching technology

The ground leaching technology (GLT) involved the use of Nemarioc-AG in granular formulation at approximately 2 g/plant, which translated to 8 kg for 4 000 tomato plants/ha (Mashela, 2002; Mashela and Nthangeni, 2002). Nemarioc-AG reduced

population densities of *Meloidogyne* species by 90%, 90% and 80% under greenhouse (Mashela, 2002), microplot (Mofokeng *et al.*, 2004; Shakwane *et al.*, 2004) and field conditions (Mashela, 2007), respectively. The performance of Nemarioc-AG in GLT system was comparable to that of aldicarb and fenamiphos in suppression of *Meloidogyne* species in tomato production (Mashela *et al.*, 2008).

2.4.2 Intergeneric grafting technology

Host-status and host-sensitivity demonstrated that the two *Cucumis* species were highly resistant to *M. incognita* races 2 and 4 and *M. javanica* (Pofu *et al.*, 2009). The two *Cucumis* species were further developed to serve as seedling rootstock for watermelon (*C. lanatus*) under greenhouse and microplot conditions (Pofu *et al.*, 2010a,b; 2009). Under field conditions the rootstocks retained their resistance to *Meloidogyne* species, but were tolerant to the spiral nematode (*Helicotylenchus dihystera*) and the ring nematode (*Criconema mutabile*) (Pofu *et al.*, 2011a).

2.4.3 Agronomic technology

Agronomic technology involved sexual propagation and development of *in vitro* propagation protocols for *C. myriocarpus* and *C. africanus* (Mafeo, 2005; Maila, 2015). Mafeo and Mashela (2009) observed that seeds of *C. myriocarpus* have autoallelopathy, which suggested that seeds needed to be leached in order to improve germination. In order to address autoallelopathy, in *vitro* studies were established (Maila, 2015), where it was observed that leaching alone in *C. africanus* resulted in germination of seeds, whereas in *C. myriocarpus* the treatment did not result in germination. However, combining leaching and scarification improved germination

(Maila and Mashela, 2013). Mass propagation of disease-free, uniform and true-to-type plantlet protocols was successful for in both *Cucumis* species (Maila, 2015).

2.4.4 Botinemagation technology

Botinemagation technology involved the application of Nemarioc-AL and Nemafric-BL phytonematicides through irrigation systems (Mashela *et al*., 2015). Nemarioc-AL phytonematicide reduced population density of *M. incognita* race 2 in roots and soil under greenhouse conditions by 97-99% and 47-90% respectively, under microplot conditions by 61% and 52%, respectively, and under field conditions by 79-85% and 79-85% respectively (Pelinganga *et al*., 2012; Pelinganga *et al*., 2013; Pelinganga, 2013). Also, under the three environments, Nemafric-BL phytonematicide, when applied through botinemagation, reduced *M. incognita* race 2 population densities in roots and soil by 85-97% and 45-96%, respectively, 72% and 77%, respectively, and 79-85 and 79-85%, respectively (Pelinganga *et al*., 2012; Pelinganga *et al*., 2013; Pelinganga, 2013).

2.4.5 Chemical technology

Fruits and roots of *C. myriocarpus* contain cucurbitacin A, which comprises cucumin ($C_{27}H_{40}O_9$) and leptodermin ($C_{27}H_{38}O_8$) (Jeffrey, 1978; Rimington, 1938; Van Wyk *et al*., 1997). Cucurbitacin A is the only cucurbitacin that is soluble in water (Chen *et al*., 2005). In contrast, cucurbitacin B ($C_{32}H_{48}O_8$), which occurs in all organs of *C. africanus*, is insoluble in water (Chen *et al*., 2005). Bioactivities of cucurbitacin A and B demonstrated that among other mechanisms, reduced nematode population densities by increasing egg hatch, thereby exposing the infective juvenile stages to lethal concentration of phytonematicides (Dube *et al*., 2016).

2.4.6 Drawbacks of phytonematicides

The major drawback of phytonematicides was their high level of phytotoxicity to the crops being protected. Nemarioc-AG phytonematicide was highly phytotoxic to eight monocotyledonous and ten dicotyledonous crops when applied as drenches at planting with most crops failing to emerge (Mafeo and Mashela, 2009; Mafeo and Mashela, 2010). Similarly, Nemarioc-AL and Nemafric-BL phytonematicides were both highly phytotoxic to tomato seedling when applied at transplanting in high concentrations (Pelinganga and Mashela, 2012; Pelinganga *et al.*, 2012). In order to quantify non-phytotoxicity concentrations, the Curve-fitting Allelochemical Response Dosage (CARD) computer-based model (Liu *et al.*, 2003) was adapted (Pelinganga, 2013). The success Nemafric-B and Nemarioc-A phytonematicides depended on striking a balance between avoiding phytotoxicity and improving nematode suppression.

Non-phytotoxic concentrations of Nemarioc-AL and Nemafric-BL phytonematicides were researched and developed using density-depended growth (DDG) patterns, which have three distinct growth responses: stimulation, saturation and inhibition growth (Liu *et al.*, 2003; Mafeo, 2012; Mashela *et al.*, 2015; Nicholson, 1933; Pelinganga, 2013; Salisbury and Ross, 1992). At saturation, the plant no longer responds to phytonematicides, but with continuous application the phytonematicide becomes inhibitory to plant growth. Pelinganga and Mashela (2012) developed the concept of “30-day-week-month”, which demonstrated that, at shorter application intervals, the two products inhibited growth of tomato plants and might also increase chemical residues in soil. In contrast, Mashela *et al*. (2013) recommended the use of empirically-based concentrations and application intervals, which had been

determined for both phytonematicides, *Meloidogyne* species and tomato plants (Pelinganga *et al*., 2013).

Due to the seasonality nature of the availability of fruits from *Cucumis* species, large quantities have to be harvested. Fruits of the two *Cucumis* species are highly perishable and the causal agent had been identified as the fungus *Penicillium simplicissimum* (Mphahlele *et al*., 2012). The feeding mode in fungi constitutes the release of hydrolytic enzymes onto the substrate and after digestion, the nutrients are absorbed (Campbell, 1990). This feeding mode, technically referred to as absorption (Campbell, 1990), results in large losses of active ingredients in produce and therefore, preservation after harvest is indispensable.

2.5 Post-harvest preservation of active ingredients

2.5.1 Drying methods

Medicinal plants used in various industries are normally dried to preserve the active ingredients, thereby prolonging their shelf-life and reducing the post-harvest decays (Mphahlele *et al*., 2012; Reynolds and Williams, 1993). Also, drying at low temperatures could slow down enzymatic reactions without eliminating them (Özcan *et al*., 2005). The choice of a drying method depends on the harvesting time of the plant produce, plant organ and the active ingredients to be preserved (de Freitas Araújo and Bauab, 2012). Plant produce can be dried through the sun-, oven-, shade- and freeze-drying methods. However, empirical work for the drying of fruits from the two *Cucumis* species had not been documented. The four methods briefly reviewed.

Sun-drying: Sun-drying is a method used to preserve most grains, vegetables, fruits and other agricultural produce in the developing countries (Szulmayer, 1971). The use of modern machinery for drying is scarce due to their unaffordability to most smallerholder farmers (Okoro and Madueme, 2004). However, the sun-drying method has not always been suitable to large scale farming, due to the inability of users to control temperature fluctuations, high labour cost, large area requirements, fungal growth and encroachment of insects, birds and rodents (Downs and Compton, 1955). The sun-drying method has other limitations such as irregular loss of moisture, which can result in patchy decays (Tanko *et al*., 2005). In general the rate of drying is low, with increasing risks of spoilage during the drying process (Anand *et al*., 2002). Also, the final moisture content of the dried product could rather be high due to low air temperatures. Generally, the concentration of malnutrition substances such as vitamins are greatly reduced in dried products (Anand *et al*., 2002). The sun-dried plant materials may lose various active ingredients because during drying most physical, chemical and nutritional changes occur, thereby affecting the product quality attributes (Di Scala and Crapiste, 2008). Intensive solar radiation could have adverse effects on quality, due to high losses of essential oils through volatilisation or colour changes through cyto-photolysis (Bouwmeester *et al*., 1995).

Shade-drying: Shade-drying requires full air circulation and it should, therefore, not be undertaken inside conventional buildings, but in an open-sided shed which was erected for such a purpose (Sulieman *et al*., 2013). Oztekin and Martinov (2007) demonstrated that shade-drying and sun-drying methods had numerous drawbacks due to their inability to handle large quantities produced by mechanical harvesters and

could hardly achieve the high quality standards required in the processing of medicinal phyto-inventories.

Oven-drying: The oven-drying method is the simplest and most effective way to dry plant materials and it is also faster than the traditional drying methods of sun- and shade-drying. Most of the medicinal plants are dried in the temperature range of 30–40°C in order to enhance the preservation of active ingredients (Müller and Heindl, 2006). Oven-drying can be used on a small and large production scale (Afolabi, 2013). This method is preferred over traditional methods because it can dry highly succulent fruits and vegetables (Reynolds and Williams, 1993). Generally, oven-drying has two phases, namely, (a) the initial fast rate moisture loss and (b) the slow rate moisture loss. During the first phase water evaporates rapidly from plant organs to form a thin boundary-layer of high humidity air, which eventually determines the rate of drying in this drying phase (Reynolds and Williams, 1993). Positive air movement over the produce surfaces reduces the thickness of the high-humidity layer, which increases the evaporation rate. The second phase begins when the rate of water movement to the surface of the fruit is less than the rate of evaporation from the surface, thereby speeding the drying process by increasing the rate through which water can move through the tissues.

Freeze-drying: During freeze-drying, the plant produce can be reduced to a very low water content and remain stable for long periods with limited risks to pathogen infection. The method prolongs shelf-life and longevity by preventing microbial growth and retarding lipid oxidation (Marques *et al*., 2009). In freeze-drying the produce is frozen, thereby reducing the surrounding pressure and adding enough heat to allow

the frozen water in the produce to sublime directly from the solid phase to the gaseous phase. Freeze-drying is an excellent drying method for quality preservation of active ingredients intended for pharmaceutical uses. However, its energy consumption is excessive when compared with those in other drying methods (Ratti, 2001). Freeze-dried plant produce may experience chemical breakdown of active ingredients within a few months of storage (Afolabi, 2013).

2.5.2 Drying temperature and exposure time

Effects of drying temperature: Drying temperature is the most important factor for preserving the active ingredients for both volatile and non-volatile chemical compounds (Saha *et al*., 1995). Essential oils in plant organs are the most sensitive to drying temperatures. The sensitivity of chemical compounds determines the temperature to be used during the drying process, with high temperatures promoting loss through volatilisation and/or degradation of active ingredients (Venskutonis, 1997). Enzymes are highly variable in their optimum temperature requirements and are generally known to undergo thermal inactivation in plant produce at temperatures above 60°C, but become completely denatured at 80°C (Saha *et al*., 1995). Generally, temperatures above 100°C completely destroy secondary metabolites and completely weaken the efficacy of the active compounds in plant produce (Lozano *et al.,* 2000). However, in purified forms, most active ingredients from plants have high boiling temperatures, with cucurbitacin A ($C_{32}H_{46}O_9$) boiling at 731°C at 760 mmHg (Krieger, 2001), where 760 mmHg implies at 1 atmosphere or at sea level. Similarly, cucurbitacin B ($C_{32}H_{48}O_8$) boils at 699°C at 760 mmHg (Krieger, 2001). In contrast, methyl bromide boils at 3.56°C at 760 mmHg (Windholz, 1983), whereas the boiling temperature for Nemacur is 49°C at 760 mmHg (Pesticide Manual, 1979).

Effects of drying time: An increase in drying time increases loses of active ingredients in plant produce (Idah *et al.*, 2010; Kissinger *et al.*, 2005; Mahanon *et al.*, 1997). During prolonged drying times, high moisture content is removed from the plant material, therefore, resulting in increased losses on secondary metabolites (Idah *et al.*, 2010). Empirically-based information suggested that losses in stability of phytochemicals were inversely proportional to increases in drying times (Mahanom *et al.*, 1997). Phytochemicals such as chlorophyll, ascorbic acid, niacin, riboflavin and carotenoid in leaves were lost in large quantities when produce were oven-dried at 50°C for 9 h than when at 70°C for 5 h (Mahanom *et al.*, 1997). Drying of plant organs for medicinal purpose is at comparatively low temperatures (30 - 40°C), but it takes much longer (Mahanom *et al.*, 1997). Higher drying temperatures are generally associated with relatively shorter drying periods (Kissinger *et al.*, 2005). When drying plant produce, ideal drying temperatures should be consistent with the ideal drying period. As a rule of thumb, at temperatures below 60°C plant produce should be dried for not more than 72 h, whereas at temperatures above 60°C produce should be dried for not more than 12 h (Kissinger *et al.*, 2005). High heat favours rapid removal of water (Kissinger *et al.*, 2005), with resultant faster changes in cellular structures and rapid enzyme denaturisation (Gamal *et al.*, 2012).

2.5.3 Denaturing of active ingredients

Denaturing of chemical compounds occurs due to exposing plant products to excessive drying temperatures or to optimum temperatures for extended periods. Therefore, drying temperatures might cause degradation of metabolites responsible for antioxidant capacity, such as vitamin C and A, along with phenolic chemical compounds and carotenoids, via chemical reactions such as the formation of

polymerization products or their structural rearrangements (Sujuta *et al*., 1992). Like most chemical reactions, the rate of an enzyme-catalysed reaction increases within the limits of density-dependent growth patterns as temperature increases (Cadwallader, 2002). Variations in one unit temperature below 10°C may introduce molecular changes of 10 to 20% in active ingredients (Ihl *et al*., 1998). Most enzymes are adversely affected by high temperatures since their reaction rates increase rapidly with temperature (stimulation phase) to an optimum level (neutral phase) and then decline (inhibition phase) with further increases in temperature (Cadwallader, 2002). Due to the high thermo-sensitivity of most phyto-enzymes, they are rapidly denatured at temperatures above 60°C, but could inadvertently be deactivated at low temperatures when exposure periods are long (Cadwallader, 2002).

2.6 Storage of phyto-inventories

Growth of plants is strongly associated with seasons (Mander, 1997). Plant produce are generally prone to rapid post-harvest decay (Mander, 1997; Mphahlele *et al*., 2012). The former dictates the need for harvesting large quantities, whereas the latter dictates the need to preserve active ingredients and then storing the phyto-inventories prior to use (FAO, 1989). The senescence stage in plant growth compromises the active ingredients in organs, thereby, affecting the final quality of products (Colegate and Molyneux, 1993). Since most plant organs are not available all year-round, it is imperative to harvest, preserve and store the produce as phyto-inventories. The chemical composition of phyto-inventories during storage can change due to any of the three, namely, chemical breakdown, volatilisation and microbial attack (Fennel *et al*., 2004). During storage of phyto-inventories, changes in active ingredients may occur, depending on factors such as enzymes, light, pH and temperature (Fennel *et*

al., 2004). In most cases, the occurrence of the following chemical changes are modulated by deteriorating enzymes; (a) oxidases – oxidise phenolics and unsaturated fatty acids, (b) peroxidases – oxidise terpenes and terpenens, (c) hydrolases – split esteric and glycosidic bonds, which break-up polysaccharides and (d) isomerases – catalyse the isomerisation of ergot alkaloids (Fennel *et al*., 2004). Both pre-storage and storage conditions play an important role in preventing rapid degradation of phyto-inventories during storage (Fennel *et al*., 2004).

2.7 Work not yet done on the research problem

The quality protocols and potential residues in tomato crops from the two phytonematicides constitute the perceived gaps in the research problem. The identified gaps in the research problem in relation to the development of quality protocols of Nemarioc-AL and Nemafric-BL phytonematicides included the selection of suitable (a) organs, (b) harvest-time, (c) drying method, (d) drying temperature, (e) storage period of phyto-inventories, (f) storage period of phytonematicides, (g) location for producing *Cucumis* species and (h) chemical residues in fruits where the crop was treated with phytonematicides.

CHAPTER 3
VARIATION OF CUCURBITACIN QUALITY AND QUANTITY EXTRACTED FROM DIVERSE PLANT PARTS OF *CUCUMIS* SPECIES

3.1 Introduction

Maturity tests are inherently used in various horticultural crop industries to establish the optimum harvest time (Lebrun *et al*., 2004; 2008; Lee *et al*., 1983; Saevels *et al*., 2003). This ensures consistent quality of the produce (Anon., 2000) and therefore, the quality of the products (Parasuraman *et al*., 1990). Generally, maturity tests could include the quantification of total soluble solids (TSS), total acidity, pH, acid/sugar ratio, tannins, volatile compounds, ascorbic acid and oil content (Abbasi *et al*., 2011; El-Buluk *et al*., 1995; Hulme, 1971; Kader, 2002). Most established maturity tests require some form of equipment and/or laboratory procedures (Wilson, 2013; Wilson and Baietto, 2009) and could not always be accessible to developing industries. Generally, maturity tests should not be confounded with maturity indices, which comprise the use of various shapes and colours during produce grading (Cristo, 1994).

Accumulation of allelochemicals over time is characterised by density-dependent growth (DDG) curves (Pelinganga *et al.,* 2012; Salisbury and Ross, 1992), which have three stages, *viz*., stimulation, neutral and inhibition phases (Liu *et al*., 2003; Pelinganga *et al.,* 2012; Salisbury and Ross 1992). In most cases, DDG patterns are quantified by quadratic relations (Mashela *et al*., 2015; Salisbury and Ross 1992), which allow for the computation of the optimum point in neutral phase (Mashela *et al*., 2015), referred to as the optimum independent variable (x-axis) for the highest level of the dependent variable (y-axis). Pelinganga *et al.* (2012) used DDG patterns to establish the application interval of 3% Nemarioc-AL and 3% Nemafric-BL phytonematicides as 16 and 18 days, respectively, for effective suppression of root-

knot (*Meloidogyne* species) nematode population densities in tomato (*Solanum lycopersicum* L.) production. Should the accumulation of cucurbitacins during the developmental stages of fruits follow DDG patterns, this could allow for the determination of the optimum harvest time when fruits have the highest concentrations of cucurbitacins. The objectives of this study was to determine (a) whether the content of cucurbitacin B in all organs of *C. africanus* was equally distributed to allow for the use of whole plants in the production of Nemafric-BL phytonematicide and (b) whether the accumulation of cucurbitacin A and cucurbitacin B during different developmental fruit stages in *C. myriocarpus* and *C. africanus* could enhance the establishment of optimum harvest times, thereby ensuring improved quality of Nemarioc-AL and Nemafric-BL phytonematicides, respectively.

3.2 Materials and methods

3.2.1 Study location

The field studies were conducted during October 2013 and April 2015 at the Green Technologies Research Centre (GTRC), University of Limpopo, Limpopo Province, South Africa (23°53'10"S, 29°44'15"E). The site had Hutton sandy loam (65% sand, 30% clay, 5% silt), with organic C at 1.6%, electrical conductivity 0.148 dS/m and pH(H_2O) 6.5. Hot and dry summers had maximum temperatures ranging from 28°C to 38°C, with summer rainfall being less than 500 mm.

3.2.2 Plant growth and cultural practices

Seeds of *C. myriocarpus* and *C. africanus* were prepared as previously described (Maila and Mashela, 2013) prior to sowing in seedling trays containing Hygromix-T (Hygrotech, Pretoria North, South Africa) growing medium. At two-leaf stage,

seedlings were hardened off for 5 days. Uniform 4-week old seedlings of each *Cucumis* species were transplanted on the field in parallel trials. Plot size was 1 m × 1 m (Figure 3.1), with each containing 4 seedlings. Three days after transplanting, each plant was fertilised with 3 g 2:3:2 (22) to provide a total of 186 mg N, 126 mg K and 156 mg P per ml water and 2 g 2:1:2 (43) – providing 0.35 mg N, 0.32 mg K and 0.32 mg P, 0.9 mg, 0.75 mg Fe, 0.075 mg Cu, 0.35 mg Zn, 1.0 mg B, 3.0 mg Mn and 0.07 mg Mo per ml water (Shadung and Mashela, 2013; Shadung *et al*., 2013). Plants were irrigated using overhead sprinklers every other day.

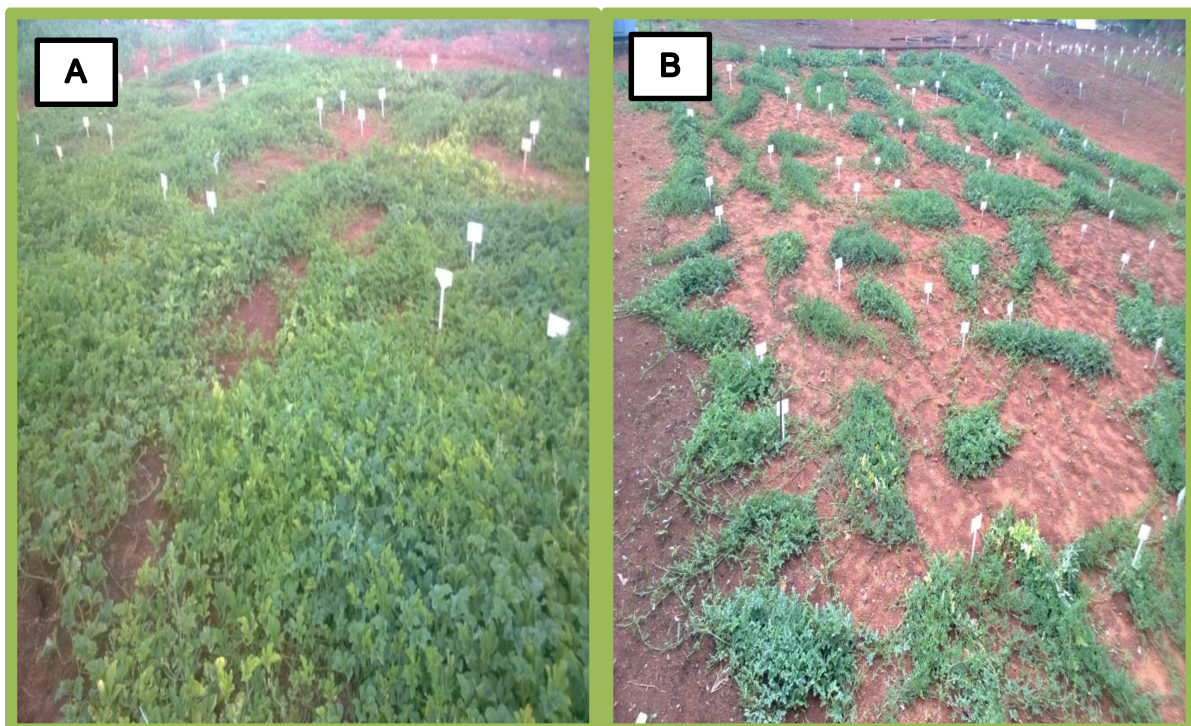


Figure 3.1 Plots for *Cucumis myriocarpus* (A) and *Cucumis africanus* (B) at eight weeks after planting.

3.2.3 Experimental design and treatments and procedures

<u>Suitable organs</u>: Fruit, leaves, stems and roots of *C. africanus* were collected at 110 days after transplanting, arranged in completely randomised design, with 5 replications

(n = 20), dried at 52°C for 72 h in air-forced ovens and ground in a Wiley mill to pass through a 1-mm sieve.

Optimum harvest time: Fruit samples were collected at 60, 67, 74, 81, 89, 96, 103 and 110 days after transplanting. This provided eight treatments for comparison. Eight fruits of *C. africanus* and *C. myriocarpus* were harvested weekly per replication (Figure 3.2), cut into pieces, dried at 52°C for 72 h in an air-forced oven and ground in a Wiley mill to pass through a 1-mm-pore sieve (Makkar, 1991).

3.2.4 Extraction of cucurbitacins

A representative subsample 4 g of dried crude extracts of each organ were extracted with 100 ml methanol and dichloromethane [1:1 (v/v)] solution on a rotary evaporator set at 60 rpm at 40°C for 4 h. After extraction, sub-samples were concentrated by reducing the volume to 30 ml under reduced pressure on a rotary evaporator and then 1 ml aliquots centrifuged at 4500 rpm for 10 minutes before filtering through 0.22 μm-pore filter (Miller, Sigma). Concentrations of cucurbitacin were quantified using the isocratic elution Shimadzu HPLC Prominence with detection using Shimadzu CTO-20A diode array detector. Quantification was performed in a wide pore reverse phase C18 (25 cm × 4.0 mm, 5 μm) discovery (Sigma-Aldrich) using 2:3 (v/v) methanol and deionised water as a mobile phase at a flow rate of 1.0 ml/min in an oven at 35°C, with wavelengths monitored at 230 nm for 43 minutes.

3.2.5 Data collection

Quantification of cucurbitacin A and B was accomplished by comparing the retention times and peak areas under that of the samples (Appendix 3.1; 3.3) to those of pure

(98%) cucurbitacin A (Appendix 3.2) and cucurbitacin B (Appendix 3.4) standards (Wuhan ChemFaces Biochemical Co. Ltd., Wuhan: China), which were dissolved in methanol and prepared in serial dilutions of 0.02, 0.04, 0.06, 0.08 and 1.0 μg.ml^{-1}.

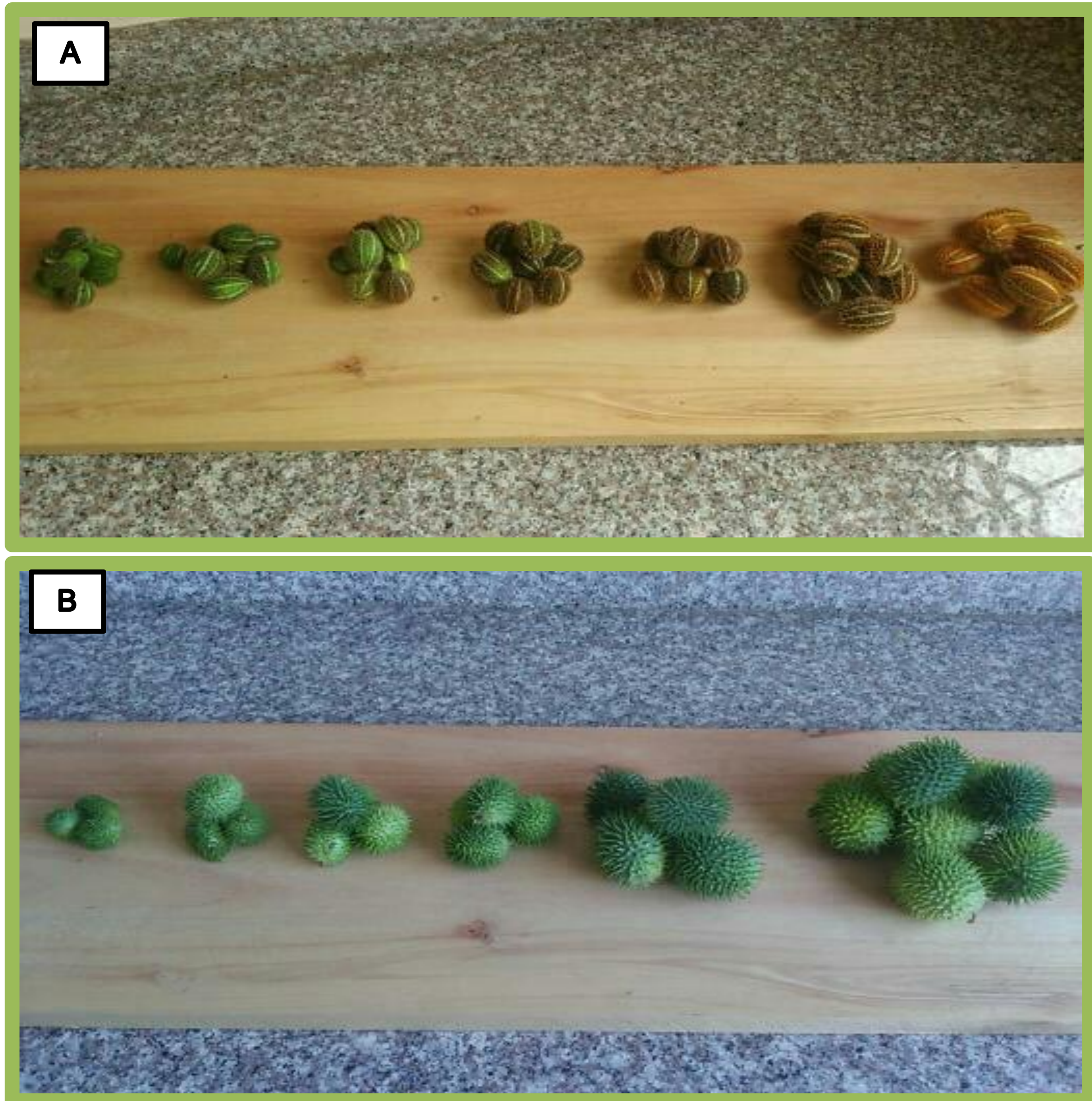


Figure 3.2 Different developmental stages of *Cucumis myriocarpus* fruit starting from 67 days (A) and *Cucumis africanus* fruit starting from 74 days (B) after transplanting.

3.2.6 Data analysis

Cucurbitacin A and B data were subjected to analysis of variance (ANOVA) procedure using SAS software (SAS Institute Inc, 2008). When the treatments were significant at the probability level of 5%, the degrees of freedom and their associated sum of squares were partitioned (Appendix 3.5–3.7) to determine the percentage contribution of sources of variation to the total treatment variation (TTV) among the treatment means (Johnson and Berger, 1982; Little, 1981; Steyn *et al.*, 2003). Mean separation was achieved using either Fisher's Least Significant Difference test or Waller-Duncan Multiple Range test. The variable with significant ($P \leq 0.05$) treatment means were further subjected to lines of the best fit using cucurbitacin A and B responses to different harvest time and modelled by the regression curve estimations resulting in a quadratic equation: $Y = b_2x^2 + b_1x + a$, where Y = Cucurbitacin A and B concentration and x = harvest time with $-b_1/2b_2 = x$ being the value for the optimum cucurbitacin A and B concentration. Unless otherwise stated, only treatment means significant at the probability level of 5% were discussed.

3.3 Results

3.3.1 Suitable organs

The effect of days to harvest on cucurbitacin concentration: Fruit, leaves, stems and roots had significant differences on concentration of cucurbitacin B (Appendix 3.5). Organs contributed 59% in TTV of cucurbitacin B from *C. africanus*.

Relative impact: Ground fruit had significantly higher concentrations of cucurbitacin B than the other organs, whereas the variables in other organs did not differ. Relative to

cucurbitacin B in fruits, leaves, roots and vines contained 94, 70 and 67% less cucurbitacin B concentrations (Table 3.1).

Table 3.1 Concentration of cucurbitacin B in four organs of *Cucumis africanus* (n = 20).

Variable	Y-value ($\mu g.ml^{-1}$)^y	Relative impact (%)^z
Fruit	$1.418^a \pm 0.487$	–
Leaf	$0.082^b \pm 0.011$	−94
Root	$0.426^b \pm 0.124$	−70
Vine	$0.470^b \pm 0.077$	−67

^{y} Column means ± SE (Standard error) followed by same letter were not different ($P \leq 0.05$) according to Fisher's Least Significant Difference test.

^{Z} Relative impact = [(treatment/control – 1) × 100].

3.3.2 Optimum harvest time

Cucumis myriocarpus: Similarly, harvest time had highly significant ($P \leq 0.01$) effects on concentration of cucurbitacin A in *C. myriocarpus* fruit (Appendix 3.6), contributing 51% in TTV of concentration cucurbitacin A. Cucurbitacin A (y-axis) and harvest time (x-axis) had quadratic relationships, where the model explained 87% of the observed variation in cucurbitacin A (Figure 3.3). The concentration of cucurbitacin A was optimised at 4.10 weeks starting from 60 days after transplanting four-week old seedlings (Table 3.2), which translated to harvest time of approximately 89 days after seeding. Relative to initial harvest time, all other harvest times had strong impact to

the accumulation of cucurbitacin A concentration as shown by relative increases ranging from 157 to 285% (Table 3.3).

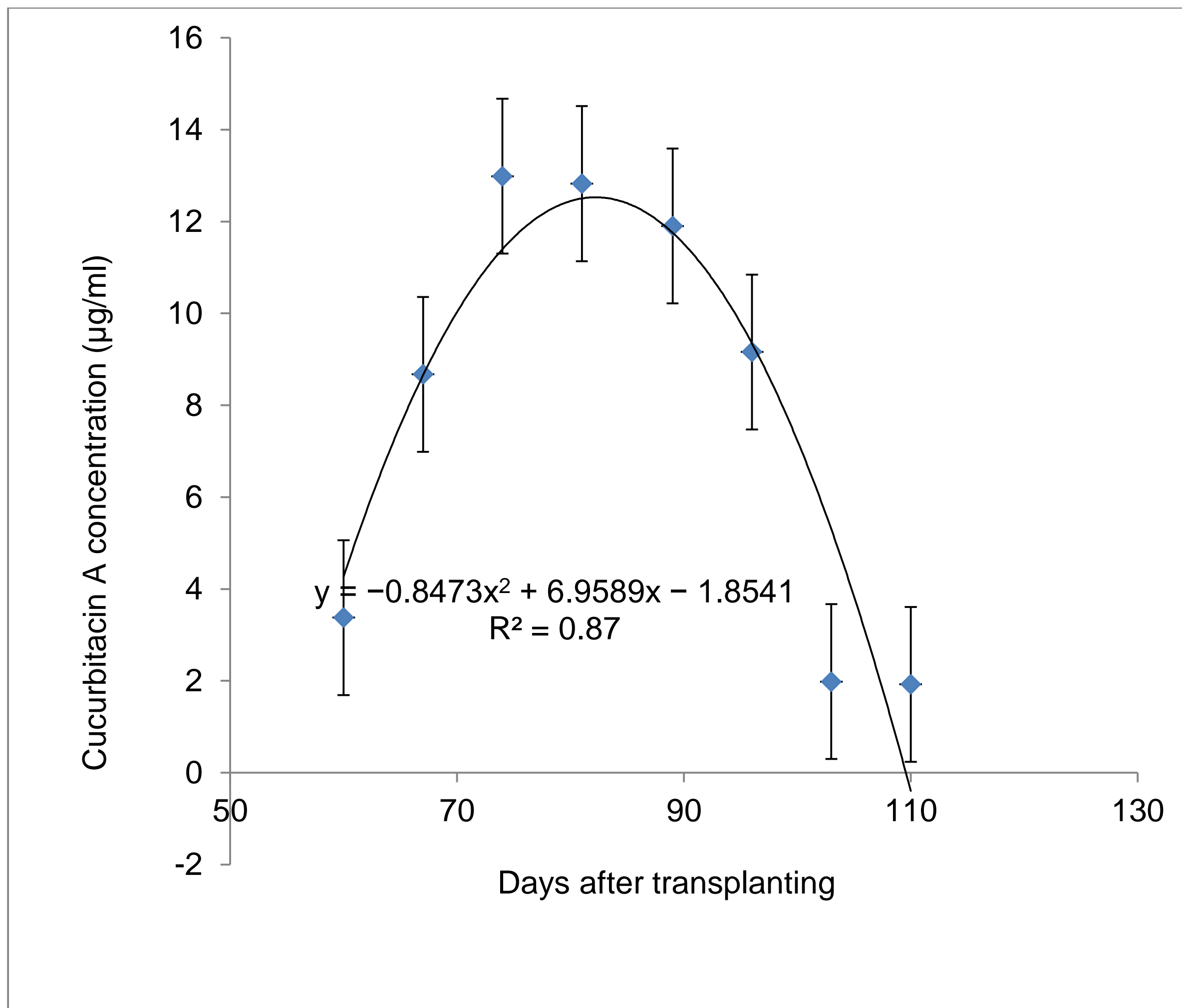


Figure 3.3 Responses of cucurbitacin A concentration from *Cucumis myriocarpus* overtime (T_0 = 60 DAT) (n = 96).

Cucumis africanus: Harvest time had highly significant ($P \leq 0.01$) effects on concentration of cucurbitacin B in *C. africanus* fruit (Appendix 3.3), contributing 73% in TTV. Cucurbitacin B (y-axis) and harvest time (x-axis) exhibited quadratic relationships, where the entire model was explained by 87% of the observed variation (Figure 3.4). The concentration of cucurbitacin B was optimised at 5.0 weeks starting from 60 days after transplanting the four-week old seedlings (Table 3.2), which

translated to harvest time of approximately 95 days after seeding. Relative to initial harvest time (60 days), all other harvest times had strong impact to the accumulation of cucurbitacin B concentration in fruits as shown by relative increases ranging from 22 to 898% (Table 3.3).

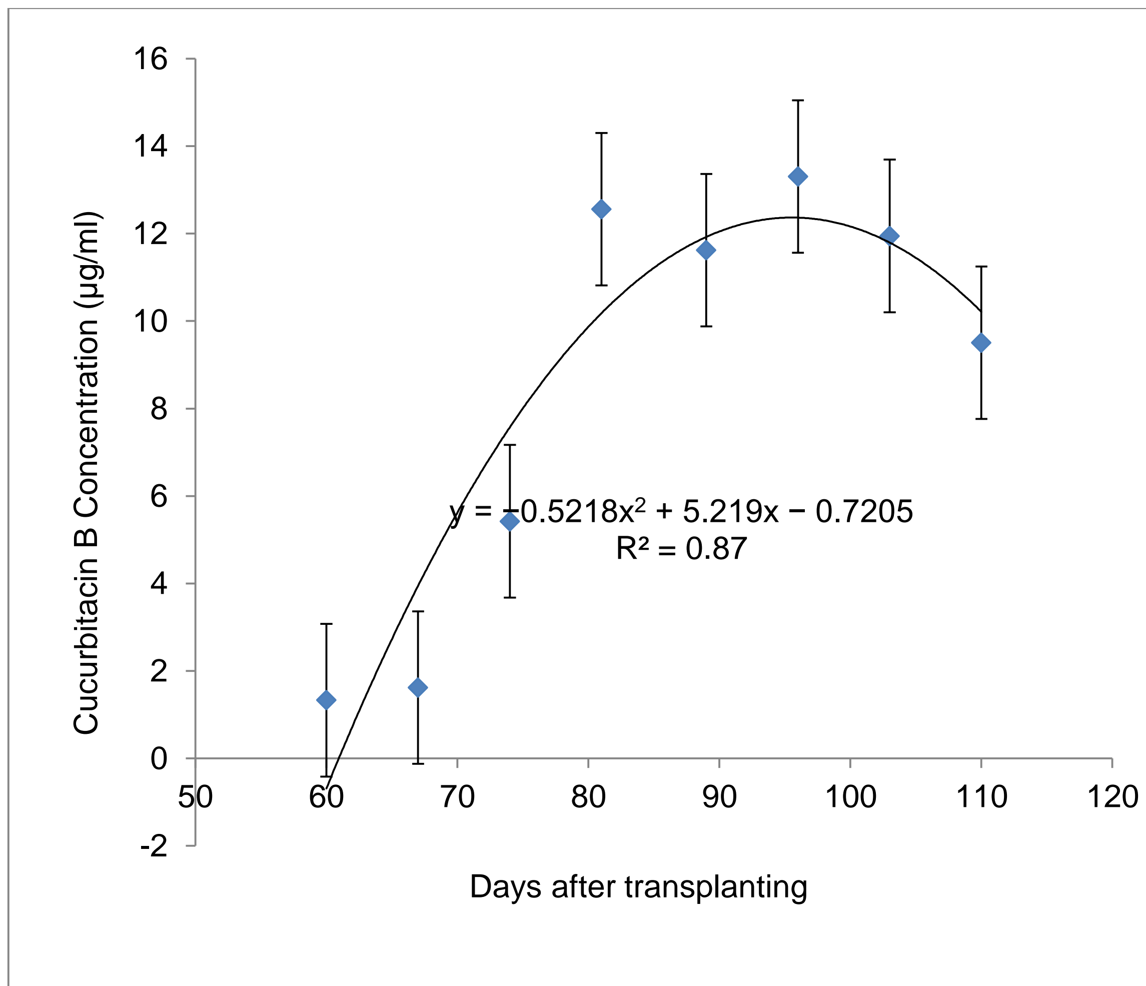


Figure 3.4 Responses of cucurbitacin B concentration from *Cucumis africanus* overtime (T_0 = 60 DAT) (n = 96).

Table 3.2 Optimum harvest times of *Cucumis myriocarpus* and *Cucumis africanus* fruits (n = 96).

Plant variables	Quadratic relationship	R^2	x^z	P ≤
Cucurbitacin A	$Y = -0.8473x^2 + 6.9589x - 1.8541$	0.87	4.10	0.01
	Optimum harvest time (weeks)		4.10	
Cucurbitacin B	$Y = -0.5218x^2 + 5.219x - 0.7205$	0.87	5.0	0.01
	Optimum harvest time (weeks)		5.0	

Calculated optimum harvesting time (x) = $-b_1/2b_2$.

Table 3.3 Relative accumulation of cucurbitacin A and B in fruits of *Cucumis myriocarpus* and *Cucumis africanus* over increasing developmental stages of fruits (n = 96).

	Cucumis myriocarpus		*Cucumis africanus*	
Treatment (Days)	Y-value ($\mu g.ml^{-1}$)[y]	Relative impact[z] (%)	Y-value ($\mu g.ml^{-1}$)	Relative impact (%)
60	3.376[c]	–	1.332[d]	–
67	8.673[b]	157	1.619[cd]	22
74	12.988[a]	285	5.423[bcd]	307
81	12.827[a]	280	12.558[ab]	843
89	11.903[a]	253	11.623[abc]	773
96	9.159[ab]	171	13.306[ab]	898
103	1.984[c]	−77	11.946[a]	796
110	1.922[c]	−85	9.505[abc]	613

[y] Column means followed by same letter were not different (P ≤ 0.05) according to Waller-Duncan Multiple Range test.

[z] Relative impact = [(treatment/control – 1) × 100].

3.4 Discussion

3.4.1 Suitable organs

Findings in this study supported the view that cucurbitacin B occurred in all organs of *C. africanus* (Jeffrey, 1978). However, the distribution is not equivalent in all organs. The highest concentration occurred in fruit, whereas those in leaves, roots and vines were less but equivalent among the three organs. In traditional African medicines, *C. africanus* roots, vines and leaves, in this order, are preferred when compared to fruit, since the latter is prone to post-harvest decay (Limpopo Provincial Traditional Organisation: Pers. Communication).

In comparison to other organs, concentrations of secondary metabolites accumulated in fruits tend to be more stable (Moco *et al.*, 2007), whereas those in other organs, particularly in leaves, fluctuate with seasonal changes (Alves *et al.*, 2007; Mudau *et al.*, 2008). Products produced from fruits tend to be more stable in terms of the desired quality, whereas those from leaves tend to fluctuate in quality (Alves *et al.*, 2007). In the production of Nemafric-BL phytonematicide, the targeted active ingredient in *C. africanus* is cucurbitacin B, which had been consistent in nematode suppression when fruit were used (Mashela *et al.*, 2011).

3.4.2 Optimum harvest time

Generally, there are five developmental stages during the formation of fruits in *Cucumis* species (Shaik *et al.,* 2011). Flowering is followed by fruit formation, which is characterised by rapid cell division of the ovary. Elongation stage, which proceeds for two to three weeks, ensues. Thereafter, fruit maturation stage occurs, characterised by fruit swelling without increases in fruit size. The colour of fruits changes from green

to slightly yellow to signify fruit ripening. Finally, senescence sets in, resulting in softening and thereafter, fruit shrivelling (Oh *et al.,* 2011; Shaik *et al.,* 2011). Consequently, results of the study suggested that the optimum concentrations of cucurbitacin A and B coincided with the maturity stages in *C. myriocarpus* and *C. africanus* fruits as observed in other maturity standards (Abbasi *et al.,* 2011; El-Buluk *et al.,* 1995; Hulme, 1971; Kader, 2002).

The response of cucurbitacin A and B levels to various developmental fruit stages demonstrated a DDG pattern (Liu *et al.,* 2003). Biological entities respond to extrinsic and intrinsic factors in the context of DDG pattern (Liu *et al*., 2003; Salisbury and Ross, 1992). Declining and increasing cucurbitacin A and B concentrations coincided with senescence and maturity stages, respectively. Results the current study agreed with those of cucurbitacin concentrations in *Ecballium elaterium* (l.) A. Rich. and *Trichosanthes cucumerina* L. var*. cucumerina,* where they reached the highest peak during the fruit maturation stages (Attard and Scicluna-Spiteri, 2004; Devendra *et al.,* 2011).

The production of allelochemicals is depended upon the physiological and developmental stage of plants. Developmental stages have an impact on the metabolic pathways, namely, shikimic-, malonic- and mevalonic-acid, which are responsible for accumulation of secondary metabolites (Achackzai *et al*., 2009; Devendra *et al.,* 2011; Ramakrishna and Ravishankar, 2011). The observed decline in the concentrations of cucurbitacin A and B during senescence in the current study was an important observation since this could also have a detrimental effect on quality of Nemarioc-AL and Nemafric-BL phytonematicides, respectively. However, the

mechanism involved in this decline is not yet clear, but could suggest the existence of degradation activities which are the properties of all chemical compounds as shown by the concepts of half life cycles (Mitchell *et al*., 2013).

Generally, secondary metabolites are released by plants for defense against various pathogens (Adeyemi, 2010; Inderjit *et al*., 1999; Kosuge, 1969; Palo and Robbins, 1991; Rice 1984; Rosenthal and Berenbaum, 1991). The concentration of secondary metabolites is variable and depends on the growing conditions, which have an impact on the metabolic pathways responsible for their accumulation. Due to various active ingredients from secondary metabolites being in demand because of various uses in industries such nutraceutical, pharmaceutical, cosmetical and pesticidal industries (Lee *et al*., 2010; Mashela *et al*., 2011; Thies *et al*., 2010; Van Wyk and Wink, 2012; Van Wyk *et al*., 2002), there is a need to optimise their production.

3.5 Conclusion

In conclusion, the inclusion of other organs in the production of Nemafric-BL phytonematicide would invariably introduce the dilution effect, which might potentially affect the quality of the product. Empirically-designed trials would be necessary to establish the dilution factor and the potential economic benefits of including whole organs in the production of Nemafric-BL phytonematicide using the fermentation process. Results also suggested that the concentrations of cucurbitacin A and B in *C. myriocarpus* and *C. africanus* fruits, respectively, were optimised at fruit maturation stages. After fruit maturation, the concentrations of cucurbitacins declined, confirming the importance of optimising harvest times. Using the concentrations of cucurbitacin,

the harvest times for *C. myriocarpus* and *C. africanus* fruits were optimised at 89 and 95 days, respectively, after transplanting the four-week old seedlings.

CHAPTER 4
INFLUENCE OF DRYING METHODS ON CUCURBITACINS

4.1 Introduction

Over many centuries, drying methods served as the initial process in preparation of botanicals and thereby, the preservation of the related active ingredients (Lusia *et al.*, 2015). Drying lowers moisture content and thereby prevents microbial degradation (Oztekin and Martinov, 2007). Drying methods can either be thermal (sun- and oven-drying) or non-thermal (shade- and freeze-drying) methods (Chan *et al.*, 2008; Lusia *et al.*, 2015). However, drying can result in unintended consequences by destroying potent chemicals through biochemical degradation and/or volatilisation of targeted active ingredients (Sharma and Prasad, 2003). The unintended consequences could result in the reduction of bioactive compounds and thereby affecting the quality of the products. The reduction of target active ingredients is undesirable because this may affect the doses and dosages, thereby compounding the unintended consequences. For example, at low concentrations, cucurbitacins consistently stimulate plant growth, whereas at high concentrations the materials consistently inhibit growth (Mashela *et al.*, 2015).

The choice of a drying method is particularly important when the target chemical compounds are not thermo-stable (Bravo, 1998; Hermann, 1995). Cucurbitacin A and B are believed to be thermo-stable (Chapter 6; Gry *et al.*, 2006). In most medicinal plants, low drying temperatures from 30 to 50°C are preferred in order to protect the active ingredients (Muller and Heindl, 2006). Temperature for quantification of tannins in plant materials through ovens was optimised at 52°C (Makkar, 1991). Although

cucurbitacin are not related to tannins, chopped fruits of the two *Cucumis* species had been dried at 52°C in air-forced ovens (Mashela, 2002). The efficacy of oven-drying *C. myriocarpus* and *C. africanus* fruits at 52°C in relation to the concentration of cucurbitacins had not been compared sun-, shade- and freeze-drying methods. The objective of this study was, therefore, to investigate whether other drying methods relative to the oven-drying method at 52°C would affect cucurbitacin A and B in fruit pieces of *C. myriocarpus* and *C. africanus*, respectively.

4.2 Materials and methods

4.2.1 Study location

Fruits from the two *Cucumis* species from cultivated fields in April 2015 were harvested at the Green Technologies Research Centre (GTRC), University of Limpopo, South Africa (23°53'10" S, 29°44'15" E) and cultural practices were as described previously (Chapter 3).

4.2.2 Raising of *Cucumis* species

The two *Cucumis* species were raised in parallel fields as described previously (Chapter 3). Fourty fruits from each plot were harvested, cut into pieces and divided into four equal portions, stacked in cooler boxes.

4.2.3 Experimental design and drying

Fourty fruits, from each of the four plots, were harvested at 110 days after planting, chopped into pieces and divided into four equal portions. Each portion per drying method was laid in a completely randomised design within each drying method, with five replications. Five plastic saucers were oven-dried in an air-forced oven at 52°C

(EcoTherm, Labotech) for 72 h. Under sun-drying, five separate portions of fruit were uniformly spread on plastic pot saucers, occasionally turned and left to dry under direct exposure to sunlight for 3 days. At night the materials were covered to ensure that they were not moistened by dew. The remaining five portions were placed in a table top freeze dryer (Ilshin Lab Co. Ltd, USA) and allowed to dry for 3 days at – 45°C. For shade-drying, the prepared materials were left in the shade for 5 days, but were also covered at night. After drying, the materials were ground in the Wiley mill to pass through 1-mm sieve.

4.2.4 Extraction of cucurbitacins and data collection

A representative subsample of 4 g dried crude extracts of fruits per treatment were extracted as described previously (Chapter 3). Briefly, after extraction, the materials were quantified for cucurbitacins using isocratic elution Shimadzu HPLC Prominence with detection using Shimadzu CTO-20A diode array detector and appropriate standards.

4.2.5 Data analysis

Cucurbitacin A and B data were subjected to analysis of variance procedure using SAS software (SAS Institute Inc., 2008). When treatments were significant ($P \leq 0.05$), the sum of squares were partitioned to determine the percentage contribution of sources of variation to total treatment variation (TTV) in concentrations of the two cucurbitacins (Johnson and Berger, 1982; Little, 1981; Steyn *et al.*, 2003). Mean separation was achieved using Fisher's Least Significant Difference test.

4.3 Results

4.3.1 Cucurbitacin A

The four drying methods had significant ($P \leq 0.05$) effects on cucurbitacin A, contributing 43% in total treatment variation of cucurbitacin A (Appendix 4.1). Oven-drying at 52°C resulted in significantly ($P \leq 0.05$) higher concentration of cucurbitacin A than that under the shade-drying method (Table 4.2). Relative to oven-drying method, shade-drying methods reduced concentrations of cucurbitacin A by 85%. However, the effects of oven-drying and shade-drying were each not different to those of sun- and freeze-drying methods.

4.3.2 Cucurbitacin B

The four drying methods also resulted in significant ($P \leq 0.05$) effect on cucurbitacin B, contributing 40% in total treatment variation concentration of this variable (Appendix 4.2). Similarly, relative to oven-drying, sun-, freeze- and shade-drying methods reduced cucurbitacin B by 81, 60 and 46%, respectively (Table 4.2). However, the effects of sun-, freeze- and shade-drying methods on cucurbitacin B were not different.

4.4 Discussion

Cucurbitacins in fruits of both *Cucumis* species resulted in the highest concentrations under the drying method, which was consistent with the view that cucurbitacins were thermo-stable (Chen *et al.*, 2014). This thermo-stability can also provide some explanation for the comparative reduced concentrations of both cucurbitacins under freeze- and shade-drying methods. The dynamics of drying under the four methods, as reviewed previously (Chapter 2), are device, and could account to some of the

observed variabilities. In this study, focus on the observed differences was deliberately directed on cucurbitacins as chemical compounds.

Table 4.1 Influence of four drying methods on cucurbitacin A and B from fruits of *Cucumis myriocarpus* and *Cucumis africanus*, respectively (n = 20).

Drying method	Cucurbitacin A		Cucurbitacin B	
	Y-value (μg.ml^{-1})^{y}	Relative impact (%)^{z}	Y-value (μg.ml^{-1})	Relative impact (%)
Oven	1.1780^{a}±0.103	–	3.1980^{a}±0.655	–
Sun	0.3200^{ab}±0.081	−73	0.5860^{b}±0.274	−81
Freeze	0.6560^{ab}±0.418	−44	1.2740^{b}±0.814	−60
Shade	0.1660^{b} ±0.096	−85	1.7400^{b}±0.465	−46

^{y} Column means ± SE (Standard error) followed by the same letter were not different ($P \leq 0.05$) according to Fisher's Least Significant Difference test.

^{z} Relative impact = [(treatment/oven-dried – 1) × 100].

Chen *et al*. (2014) observed that eight enzymes were involved in catalysing precursors of the cucurbitacins through mevalonic acid MVA pathway. Since the eight catalytic enzymes were thermo-stable when drying occurred at 52°C, the activities of MVA remained uninhibited so that the stable molecules, namely, the cucurbitacins, continued to be formed. The proposed hypothesis could also explain the observed reduced concentrations of cucurbitacin under freeze- and shade-drying methods, where the bioactivities of the enzymes were reduced due to increased inactiveness at low temperatures. In contrast, under the sun-drying method, volatilisation of the

constituent inputs required for biosynthesis of various precursors in the cucurbitacins might have interfered with bioactivities of MVA pathway.

Drying below 60°C has negligent effects in denaturing of enzymes responsible for catalysing acetyl coA into the Krebs cycle in the mitochondria (Sukrasno, 2014). Acetyl coA is an important input in the transformation process of cucurbitacins (Chen *et al.*, 2014). All three pathways used by secondary metabolites, namely, shikimic-, malonic- and mevalonic-acid pathways, are intended to modulate the quantities of acetyl coA that enter the Krebs cycle (Campbell, 1990). Temperatures between 30 and 50°C had been recommended for drying most medicinal plants (Muller and Heindl, 2006), with the drying time being inversely proportional to increasing temperatures (Sharma and Prasad, 2003). During early stages of exposure to low temperatures, cellular respiration increases (Bowsher and Tobin, 2001), thereby increasing precursors for secondary metabolites in all pathways. However, high temperatures denature enzymes required to drive the secondary metabolite processes for thermo-unstable chemical compounds and also increase the volatilisation of others, thereby reducing the concentrations of targeted active ingredients (Makkar, 1991). However, thermo-stable chemical compounds like the cucurbitacins behave differently.

Cucurbitacin A ($C_{32}H_{46}O_9$) from *C. myriocarpus* fruit and cucurbitacin B ($C_{32}H_{48}O_8$) from *C. africanus* fruit (Chen *et al.*, 2005), have boiling points at 731°C and 699°C at sea level (760 mmHg), respectively (Krieger, 2001). When fruits from *C. myriocarpus* and *C. africanus* were dried at 52°C for 72 h and stored at room temperature in air-tight sealed and unsealed containers, in support of the thermo-stability hypothesis, the concentrations of cucurbitacin A and B continued to increase quadratically over a six-

month-storage period (Chapter 6). Similar increases were observed in sun-dried aerial parts of *Thymus daenensis,* where thymol ($C_{10}H_{14}O$) and carvacrol ($C_{10}H_{14}O$) increased during a 3-month-storage period at room temperature (Rowshan *et al.*, 2013). The two active ingredients in *T. daenensis* are monoterpenes (Zarshenas and Krenn, 2015). In contrast, oven-drying at 45°C for 48 h decreased flavonoid contents in Centella (*Centella asiatica*) organs (Mohd *et al.*, 2009).

Quantitatively, regardless of the drying method, per unit mass *C. africanus* fruit appeared to contain larger quantities of cucurbitacins than *C. myriocarpus* fruit. In *C. africanus*, cucurbitacin B is accumulated in all organs of the plant, whereas cucurbitacin A in *C. myriocarpus* is accumulated in fruit and roots only (Jeffrey, 1978). The standards used in quantifying cucurbitacin A and B in the current study could not detect other chemical compounds in the subsamples. Cucurbitacin A is generally not stable and disintegrate into two bioactive chemical compounds, cucumin ($C_{27}H_{40}O_9$) and leptodermin ($C_{27}H_{38}O_8$) (Rimington, 1938), which could explain consistent low values of cucurbitacin A under various drying methods.

4.5 Conclusion

Oven-drying method at 52°C for 72 h for both *Cucumis* species resulted in the highest quantities of cucurbitacin B, whereas those of cucurbitacin A were significantly higher than those under shade-drying. Due to large quantities required for drying, oven-drying method could be viewed as the appropriate drying method for preparing fresh fruits of the two *Cucumis* species for the manufacturing of Nemarioc-AL and Nemafric-BL phytonematicides, as well as for use in other industries.

CHAPTER 5
SUITABLE DRYING TEMPERATURE FOR PRESERVING CUCURBITACINS

5.1 Introduction

The recommended drying temperature range for various organs in medicinal plants is 30-40°C (Müller and Heindl, 2006). However, when fruit pieces from wild cucumber (*Cucumis myriocarpus* Naude.) and wild watermelon (*Cucumis africanus* LF.) were dried within the recommended range, most of the materials were lost to decay, with a blue, bluish-green, or olive green colours, surrounded by white mycelium and a band of water-soaked tissues that characterise *Penicillium simplicissimum* Oudem. infection (Mphahlele *et al*., 2012). Preliminary optimum drying temperature to prevent growth of mycelia and therefore subsequent decay, was at 52°C (Mashela, 2002). This temperature could be viewed as a compromise temperature, since there was no information on the impact of the compromise and higher temperatures on the targeted active ingredients in fruits of the two *Cucumis* species. The objective of this study, therefore, was to determine whether increasing the drying temperatures relative to the 52°C compromise temperature would affect the concentrations of cucurbitacin A and B in fruit pieces of the *C. myriocarpus* and *C. africanus*, respectively.

5.2 Materials and methods

5.2.1 Study location

Fruits from *C. myriocarpus* and *C. africanus* were collected from cultivated fields in April 2015 at the Green Technologies Research Centre (GTRC), University of Limpopo, South Africa (23°53'10" S, 29°44'15" E). Preparation of *Cucumis* seedlings, raising of plants and cultural practices were as described previously (Chapter 3).

5.2.2 Experimental design and treatments

Sixty fruits, from each of the six plots, were harvested at 110 days after planting, chopped into pieces and divided into six equally portions. Each portion per plot was laid in a completely randomised design within six oven-drying temperatures, namely, 52, 60, 70, 80, 90 and 100°C, with five replications. Exposure period was 72 h, followed by grinding in a Wiley mill to pass through 1-mm-opening sieve. Prior to further processing, samples were stored in hermetically-sealed plastic bottles at room temperature.

5.2.3 Extraction of cucurbitacins

A representative subsample of 4 g dried crude extracts of fruits per treatment were extracted as described previously (Chapter 3). Briefly, after extraction, the materials were quantified for cucurbitacins using isocratic elution Shimadzu HPLC Prominence with detection using Shimadzu CTO-20A diode array detector and appropriate standards.

5.2.4 Data analysis

Cucurbitacin A and B data were subjected to analysis of variance (ANOVA) procedure using SAS software (SAS Institute Inc, 2008). When the treatments were significant at the probability level of 5%, the degrees of freedom and their associated sum of squares were partitioned (Appendix 5.1–5.2) to determine the percentage contribution of sources of variation in total treatment variation (TTV) among the treatment means (Johnson and Berger, 1982; Little, 1981, Steyn *et al.*, 2003). Mean separation was achieved using Waller-Duncan Multiple Range test. The variable with significant ($P \leq 0.05$) treatment means were further subjected to lines of the best fit using cucurbitacin

A and B responses versus drying temperatures. Relationships were modelled by the regression curve estimations from the quadratic equations to generate the ($Y = b_2x^2 + b_1x + a$), where Y = Cucurbitacin A and B concentration and x = oven-drying temperature with $-b_1/2b_2 = x$ being the value for the optimum cucurbitacin A and B concentration (Mamphiswana *et al*., 2010). Unless otherwise stated, only treatment means significant at the probability level of 5% were discussed.

5.3 Results

5.3.1 Treatment effects on cucurbitacins

Increasing oven-drying temperatures had highly significant ($P \leq 0.01$) effects on concentrations of cucurbitacin A and B (Appendix 5.1), contributing 65 and 71% in TTV of cucurbitacin A and B concentrations, respectively (Appendix 5.1).

5.3.2 Relative impact

Concentration of cucurbitacin A was the highest at 60°C (Table 5.1). Relative to 52°C, temperature at 60°C increased cucurbitacin A by 4%. The reductive effects (25%) at 70°C did not result in different effects from those at 52°C. Similarly, although the relative effects started to steep down from 70°C (53-92%), the actual temperature effects were not different up to 100°C. In contrast, in cucurbitacin B, the effects of the 52°C were significantly higher than those at higher temperatures, which were not different from one another from 60°C to 100°C (Table 5.1). Relative to 52°C, increasing temperature reduced cucurbitacin B by 28 to 85%.

5.3.3 Generated models

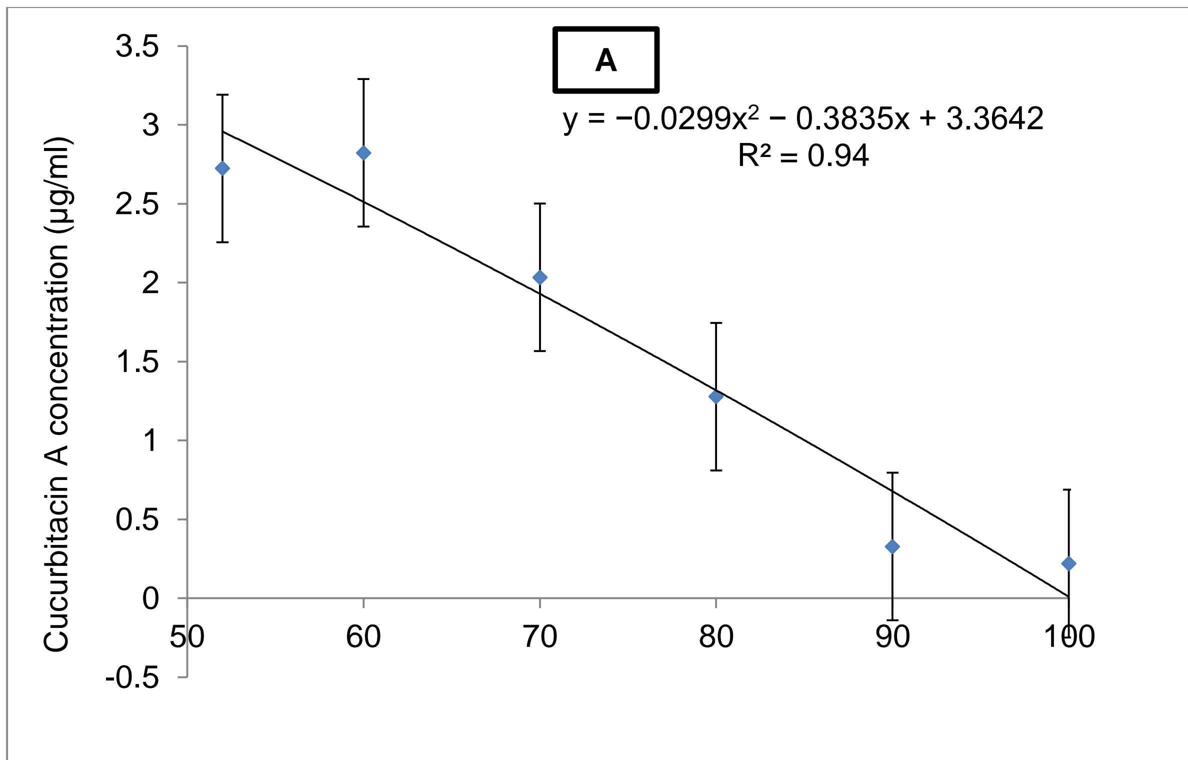
Cucurbitacin A and B concentrations over increasing temperatures exhibited quadratic relationships (Figure 5.1). The models explained 94 and 95% of the observed quadratic relations in cucurbitacin A and B, respectively.

Table 5.1 Responses of cucurbitacins to six levels of oven-drying fruits of *Cucumis myriocarpus* and *Cucumis africanus* from 52°C to 100°C (n = 30).

Oven-drying temperature (°C)	Cucurbitacin A		Cucurbitacin B	
	Y-value (μg.ml^{-1})^{Y}	Relative impact (%)^{z}	Y-value (μg.ml^{-1})	Relative impact (%)
52	2.724^{b}±1.139	–	6.706^{a}±1.433	–
60	4.840^{a}±0.658	4	3.568^{b}±0.506	−28
70	1.200^{bc}±0.174	−25	2.980^{b}±0.820	−49
80	0.930^{bc}±0.579	−53	3.600^{b}±0.923	−48
90	0.328^{c}±0.087	−88	2.940^{b}±1.409	−81
100	0.220^{c}±0.120	−92	1.100^{b}±0.376	−86

^{y} Column means ± SE followed by the same letter were not different ($P \leq 0.05$) according to Waller-Duncan Multiple Range test.

^{z} Relative impact = [(treatment/Oven-dried – 1) × 100].



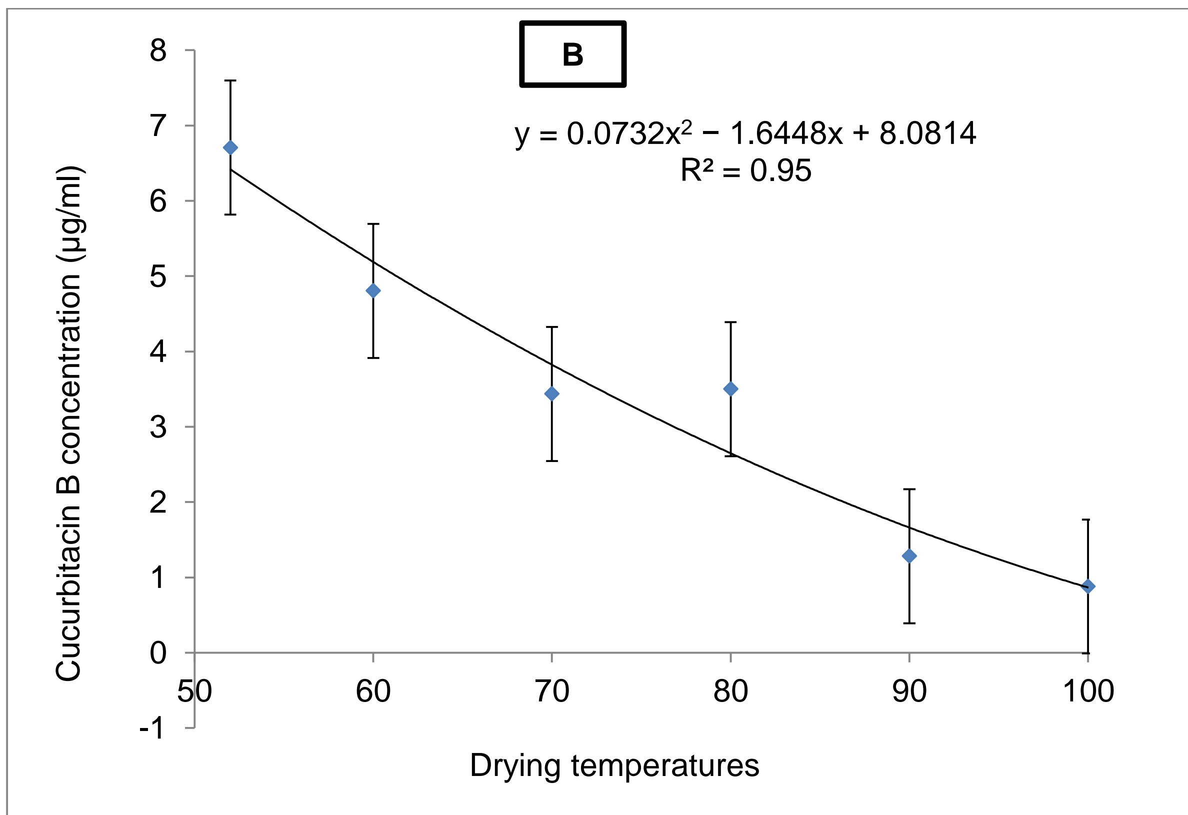


Figure 5.1 Relationship between cucurbitacin A and B concentrations from fruits of *Cucumis myriocarpus* (A) and *Cucumis africanus* (B), respectively, over increasing drying temperatures at 72 h-exposure time.

5.4 Discussion

Concentrations of cucurbitacin A and B triterpenoids (Chen *et al*., 2005) versus increasing temperatures were inversely proportional. Du *et al*. (2003) and Hwang *et al*. (2014) observed that concentrations of ginsenoside from ginseng roots, another triterpenoid, also decreased when dried at 40, 55 and 70°C. Similar findings were noted with the pyrethrins, which are the monoterpenoids (Morris *et al.,* 2006). Rosmarinic acid and sinenselin (phenolic compounds) from misai kucing (*Orthosiphon staminiues*), increased with increasing temperature from 40°C to 55°C, but decreased when dried at 70°C (Abdullah *et al.,* 2011). Drying bush tea (*Athrixia phylicoides*) between 45°C and 65°C reduced total phenolic content when compared with freeze- and shade-drying methods (Mudau and Ngezimana, 2014).

Mostly, the reduction of chemical compounds with increasing temperature had been attributed to the degradation of chemical compounds (Phillips *et al*., 1960). Chemical degradation with increasing temperature depends on the chemical bonds within the chemical compounds (Phillips *et al*., 1960). In essential oils with weaker bonds, for example, drying temperature for *Origanum vulgare* subsp. *hirtum* was optimised at 40°C for 72 h (Novák *et al.,* 2011), whereas at high temperatures most essential oils were volatilised (Faridah *et al*., 2010; Radünz *et al*., 2003). Cucurbitacins are thermo-stable, with boiling temperatures of cucurbitacin A and B being at 731°C and 699°C at 760 mmHg, respectively (Krieger, 2001). The decrease in cucurbitacin with increasing temperatures from 52°C agreed with observations in other chemical compounds (Abdullah *et al*., 2011; Du *et al*., 2003; Hwang *et al*., 2014; Morris *et al*., 2006).

Drying fruits from the two *Cucumis* species at 52°C should be viewed as a balance between preserving the material from *P. simplicissimum* induced post-harvest decay and retention of cucurbitacins from thermal degradation (Mashela, 2002). Optimising for temperature in cucurbitacin A and B would not be necessary since the optimum range would be falling below the 52°C compromise temperature. However, it could still be necessary to establish the appropriate exposure time at 52°C since the degree of drying at a given temperature is inversely proportional to exposure periods (Kissinger *et al*., 2005). For instance, since drying at 52°C resulted in exposure period of 72 h, it could imply that drying at 60°C for cucurbitacin A could require a relatively shorter period. This could reduce losses due to degradation and volatilisation (Barbieri *et al*., 2004; Gregory *et al.,* 2005), which could explain the lower retention of cucurbitacin A.

In both *Cucumis* species, cucurbitacin concentrations versus increasing drying temperatures, had quadratic relations which suggested that the relations subscribed to density-dependent growth (DDG) patterns (Liu *et al*., 2003). Most biological entities, when exposed to appropriate series of biotic and/or abiotic factors respond in DDG patterns (Salibury and Ross, 1992). The three stages of DDG patterns, namely, stimulation, neutral and inhibition (Liu *et al*., 2003), had been scantily reported in relation to chemical active ingredients and temperature. In most cases, due to the limited ranges used, one phase was observed. For instance, in the current study and those of others (Abdullah *et al*., 2011; Du *et al*., 2003; Morris *et al*., 2006), only the inhibition ranges were exhibited. At lower temperature ranges, however, Abdullah *et al.* (2011) observed that the two phenolic compounds (Rosmarinic acid and sinenselin) increased with increasing temperatures (40 − 55°C), which is a reflection of the stimulation range within the DDG patterns (Liu *et al*., 2003). Concentrations of the two

cucurbitacins could not be optimised since beyond the 52°C compromise temperature the chemical responses to temperature were already in the inhibition phase of the DDG patterns (Mashela *et al.*, 2015).

5.5 Conclusion

The 52°C compromise temperature was already beyond the optimisation temperatures for both cucurbitacin A and B. Above this temperature, the observed trend was generally that of temperature that would gradually reduce cucurbitacin concentrations. However, it is recommended that a trial be conducted with acceptable range for drying medicinal plants (30 − 40°C) to at least 70°C in order to allow for optimisation of cucurbitacins. Also, if 52°C is retained, the exact time frame that would optimise the concentrations of the cucurbitacins should be established.

CHAPTER 6
INFLUENCE OF STORAGE PERIOD ON CUCURBITACINS

6.1 Introduction

Poor quality plant material at pre- and post-harvests causes huge losses in products developed from plant materials (Isman, 1997; Niazi, 2009; Orwa *et al.*, 2004). Most, of the losses are incurred in plant produce during inventory prior to processing (Brecht, 1995; Kader, 2002; 2005; Salveit, 1997). Active ingredients in phyto-products are instrumental in product quality, with their degradation during inventory resulting in insufficient quantities of active ingredients (Lai, 2008). At pre- and post-harvests, both extrinsic and intrinsic factors contribute to the change in the stability of chemical compounds, inevitably altering the quality and quantity of active ingredients (Gobbo-Neto and Lopes, 2007; Isman, 1997). Development of quality protocols and comprehensive investigation of the effect of storing inputs prior to processing are integral to the development of phyto-products (Banks and Bristow, 1999). Quality protocols ensure that products retain their true-to-type performance characteristics when properly handled and applied by the end-users (Besterfield *et al.*, 2003). Product quality is distinguished through the performance of the product and when this surpasses expectation, the product is viewed as being of high quality (Parasuraman *et al.*, 1990). Effects of both intrinsic and extrinsic factors on inputs along the value-chain could be instrumental in phyto-products quality (FAO, 1997).

Storage period of fermented products plays an important role in product quality (Rogers, 2010). Product quality (Q) is a function of its performance (P) and expectation (E), conceptualised as Q = P/E (Besterfield *et al.*, 2003). In South Africa, Nemarioc-AG (G = granular formulation), Nemafric-BG, Nemarioc-AL (L = liquid formulation) and

Nemafric-BL phytonematicides are being developed for the management of population densities of root-knot (*Meloidogyne* species) nematodes in various cropping systems (Mashela *et al.*, 2011). Crude extracts from *Cucumis* fruits are fermented using effective microorganisms (EM), comprising lactic acid bacteria, yeast, smaller number of photosynthetic bacteria, actinomycetes and other negligent forms of microorganisms (Higa and Ke, 2001) to produce stock solutions for Nemarioc-AL and Nemafric-BL phytonematicides. Diluted Nemarioc-AL and Nemafric-BL phytonematicides are applied through irrigation, a process referred to as botinemagation (Pelinganga *et al.*, 2013). However, the stock solutions for Nemarioc-AL and Nemafric-BL phytonematicides are being retained for 90 days, with frequent preparations during the crop growing seasons necessitating the storage of dried powder. Azadirachtin, an active ingredient in neem (*Azadirachta indica*), one of the well-documented triterpenoid (Schmutterer, 2002), stores poorly in neem products, due to high degradation rates (Chandler *et al.*, 2011).

The influence of storing dried powder from *Cucumis* fruits in relation to quality of these phyto-inventories is not documented. Similarly, the influence of storing the manufactured Nemarioc-AL and Nemafric-BL phytonematicides on concentration of cucurbitacins is also not documented. The objectives of this study were, therefore, to investigate (a) whether the storage of phyto-inventories for Nemarioc-AL and Nemafric-BL phytonematicides would influence the concentrations of cucurbitacin A and B in sealed and unsealed containers at room temperature and (b) whether storage of Nemarioc-AL and Nemafric-BL phytonematicides would affect the concentrations of cucurbitacin A and B, respectively.

6.2 Materials and methods

6.2.1 Study location and raising of seedling plants

Plants of *C. myriocarpus* and *C. africanus* were raised under irrigated field conditions at the Green Technologies Research Centre, University of Limpopo, Limpopo Province, South Africa (23°53'10" S, 29°44'15" E) and seedling of *Cucumis*, raising of plants and cultural practices were as described previously (Chapter 3).

6.2.2 Preparation and experimental design

Phytonematicide inventories: Fruits of *C. myriocarpus* and *C. africanus* were harvested at maturity (110 days after transplanting) and prepared as previously described (Chapter 3). Storage treatments were initiated from April to September 2014. One set of milled material was stored hermetically in sealed 100 ml plastic containers, whereas the remainder was in unsealed containers, with each container having 10 g material (Figure 6.1). Treatments, *viz*., 0 (refers to no storage period), 1, 2, 3, 4 and 5 storage period (months), were arranged in completely randomised design, with 5 replications (n = 30). Containers were placed on a shelf in the storeroom and for each container type, five were removed for analysis/month.

Phytonematicide products: Fruits of *C. myriocarpus* and *C. africanus* were harvested at 110 days after transplanting and prepared as described previously (Chapter 3). Approximately 80 g and 40 g crude extract of *C. myriocarpus* and *C. africanus* fruits, were placed in 20-litre-plastic containers and 300 ml molasses, 100 g brown sugar, 300 ml effective microorganisms (EM) and 16 litre chlorine-free tapwater added and hermetically sealed (Nzanza and Mashela, 2012). The mixtures were fermented for 14 days at room temperature until pH declined to 3.7. Gases were allowed to escape from

the container through a 5 mm-diameter tube with the hermetically-glued end to a hole on the lid of the 20 l container, with an outlet end dangling in chlorine-free tapwater container in a litter bottle.

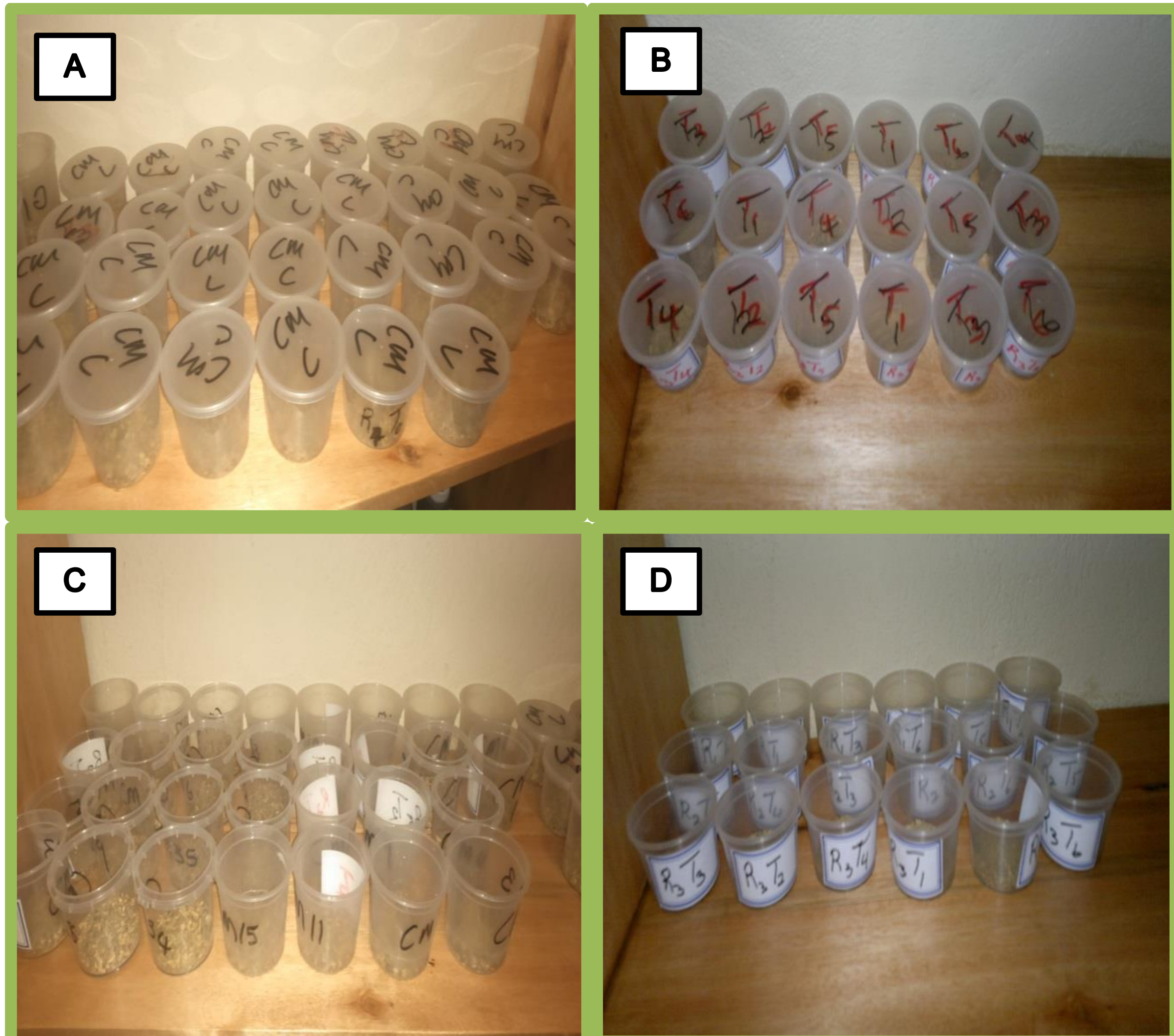


Figure 6.1 Stored dried crude extracts; sealed *Cucumis myriocarpus* (A), sealed *Cucumis africanus* (B), unsealed *Cucumis myriocarpus* (C) and unsealed *Cucumis africanus* (D).

After fermentation, 200 ml were pipetted into 300 ml plastic containers, which were hermetically sealed (Figure 6.2). Treatments, *viz.*, 0 (refers to no storage period), 1, 2, 3, 4 and 5 month storage time, were arranged in randomised complete block design,

with five replications. Samples were stored temperature in a dark room at room, with initial storage period (T_0) being the control.

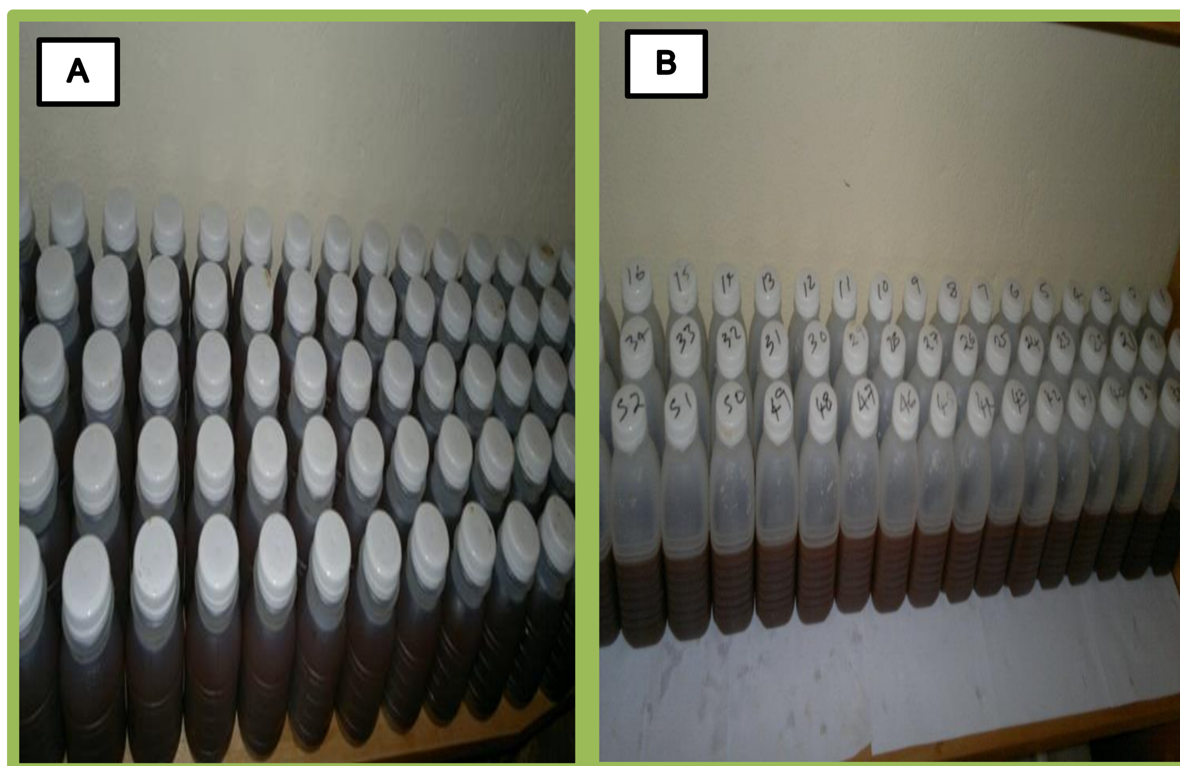


Figure 6.2 Nemarioc-AL (A) and Nemafric-BL (B) phytonematicides stored at room temperature in a dark room.

6.2.3 Extraction and quantifying cucurbitacins

Phytonematicide inventories: A representative subsample of 4 g dried crude extracts of fruits were extracted as described previously (Chapter 3). Briefly, after extraction, the materials were quantified for cucurbitacins using isocratic elution Shimadzu HPLC Prominence with detection using Shimadzu CTO-20A diode array detector and appropriate standards.

Phytonematicide products: Prior to storage (T_0) and then monthly, 1 ml subsamples were collected from 10 containers and centrifuged at 4500 rpm for 10 minutes before

filtering through 0.22 μm-pore filter (Miller, Sigma). Concentration of cucurbitacin A and B were quantified as previously described (Chapter 3).

6.2.4 Data collection

Quantification of cucurbitacin A and B were accomplished by comparing the retention times and peak areas under that of the samples to those of pure (98%) cucurbitacin A and B standards (Wuhan ChemFaces Biochemical Co. Ltd., Wuhan: China), which were dissolved in methanol and prepared in serial dilutions of 0.02, 0.04, 0.06, 0.08 and 1.0 $\mu g.ml^{-1}$.

6.2.5 Data analysis

Cucurbitacin A and B data were subjected to analysis of variance procedure using SAS software (SAS Institute Inc., 2008). When the treatments were significant ($P \leq 0.05$), the degrees of freedom and their associated sum of squares were partitioned (Appendix 6.1−6.6) to determine the percentage contribution of sources of variation to the total treatment variation (TTV) among the treatment means (Steyn *et al*., 2003). Mean separation was accomplished using Fisher's Least Significant Different test or Waller-Duncan Multiple Range test. Variables with significant ($P \leq 0.05$) treatments were further subjected to lines of the best fit using cucurbitacin A and B responses versus increasing storage times.

6.3 Results

6.3.1 Phytonematicide inventories

Treatment effect on cucurbitacins: Storage period had significant effects on concentration of cucurbitacin A, with storage contributing 73 and 94% in TTV of

cucurbitacin A in sealed and unsealed containers, respectively (Appendix 6.1−6.2). Similarly, storage time had significant effect on concentration of cucurbitacin B, contributing 71 and 86% to total treatment variation in cucurbitacin B when dried materials were stored in sealed and unsealed containers, respectively (Appendix 6.3−6.4).

<u>Relative impact</u>: In sealed containers, relative to storage time T_0, cucurbitacin A increased to a peak of 294% at the end of four months of storage and then declined to 239% by the end of six months (Table 6.1). Similar effects were observed in unsealed containers, however, with reduced magnitudes (Table 6.1). Similarly, concentration of cucurbitacin B increased with increasing storage times (Table 6.2). Relative to T_0, concentrations of cucurbitacin B increased by as high as five times, regardless of whether the containers were sealed or unsealed. In sealed and unsealed containers, cucurbitacin B increased by 9−705% and 45−1759%, respectively (Table 6.2).

<u>Generated Models</u>: Cucurbitacin A concentration over increasing storage time exhibited quadratic relations (Figure 6.3). Relationship of cucurbitacin A over increasing storage period were explained by 97 and 96% in sealed and unsealed containers, respectively. Optimum storage times (x), using $x = -b_1/2b_2$ relation from the quadratic equations for sealed and unsealed containers were 4.20 and 4.22 months, respectively (Table 6.3). Similarly, cucurbitacin B concentration and storage time exhibited a quadratic relationship (Figure 6.4). Relationship of cucurbitacin A over increasing storage period were explained by 98 and 95% in sealed and unsealed containers, respectively. However, optimum storage time (x) using $-b_1/2b_2$ could not

be achieved since the increase of cucurbitacin B was still within the stimulation range by storage time (Figure 6.4).

Table 6.1 Responses of cucurbitacin A concentration in phyto-inventories of Nemarioc-AL phytonematicide stored in sealed and unsealed containers over six months (n = 35).

Treatment (months)	Sealed		Unsealed	
	Y-value ($\mu g.ml^{-1}$)[y]	Relative impact (%)[z]	Y-value ($\mu g.ml^{-1}$)	Relative impact (%)
0[x]	0.290[c]	–	0.396[d]	–
1	0.486[bc]	68	0.658[cd]	66
2	0.976[ab]	236	0.874[bc]	120
3	1.108[a]	282	0.990[ab]	150
4	1.143[ab]	294	1.180[a]	198
5	1.140[a]	293	1.015[bc]	156
6	0.982[ab]	239	0.960[ab]	142

[x] 0 = Refers to no storage period

[y] Column means followed by the same letter were not different ($P \leq 0.05$) according to Waller-Duncan Multiple Range test.

[z] Relative impact = [(treatment/control – 1) × 100].

Table 6.2 Responses of cucurbitacin B concentration in phyto-inventories of Nemafric-BL phytonematicide stored in sealed and unsealed containers (n = 30) over five months.

Treatment	Sealed		Unsealed	
(months)	Y-value ($\mu g.ml^{-1}$)[y]	Relative impact (%)[z]	Y-value ($\mu g.ml^{-1}$)	Relative impact (%)
0[x]	3.49[c]	−	1.384[b]	−
1	1.92[c]	−45	2.002[b]	45
2	3.798[c]	9	4.07[b]	194
3	4.666[c]	34	4.46[b]	222
4	17.46[a]	400	18.78[a]	1959
5	28.108[b]	705	25.62[a]	1751

[x] 0 = Refers to no storage period

[y] Column means followed by the same letter were not different ($P \leq 0.05$) according to Waller-Duncan Multiple Range test.

[z] Relative impact = [(treatment/control – 1) × 100].

Table 6.3 Responses of cucurbitacin A concentration in phyto-inventories of Nemarioc-AL phytonematicide stored in sealed and unsealed containers (n = 35) over six months.

Variables	Quadratic relationship	R^2	x^z	$P \leq$
Sealed	$y = -0.0528x^2 + 0.4434x + 0.2307$	0.96	4.20	0.01
	Storage time		4.20 months	
Unsealed	$y = -0.0398x^2 + 0.3358x + 0.378$	0.97	4.22	0.01
	Storage time		4.22 months	

Optimum storage time (x) = $-b_1/2b_2$.

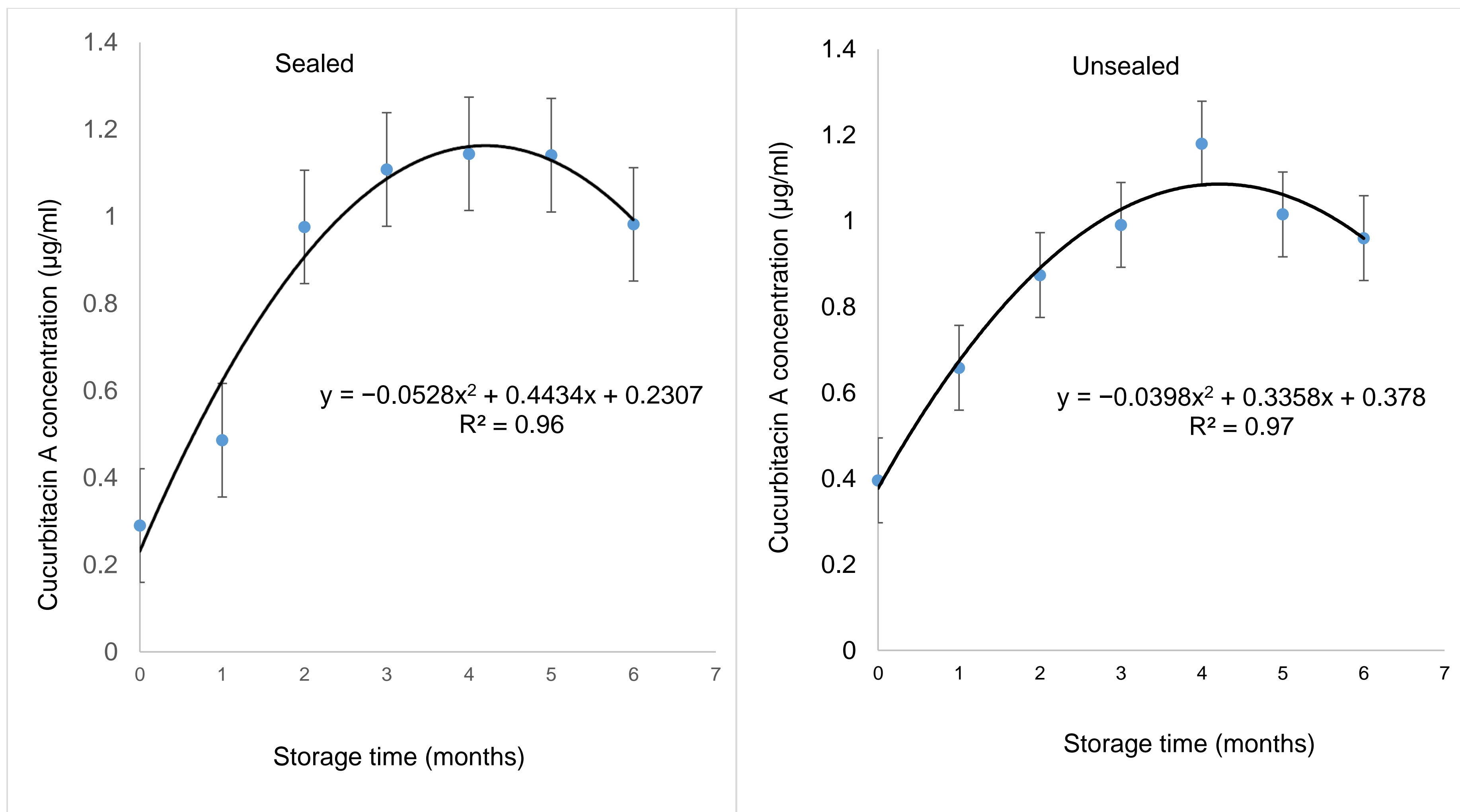


Figure 6.3 Quadratic relationships of cucurbitacin A concentration in phyto-inventories of Nemarioc-AL phytonematicide stored in sealed and unsealed containers over six months (n = 35).

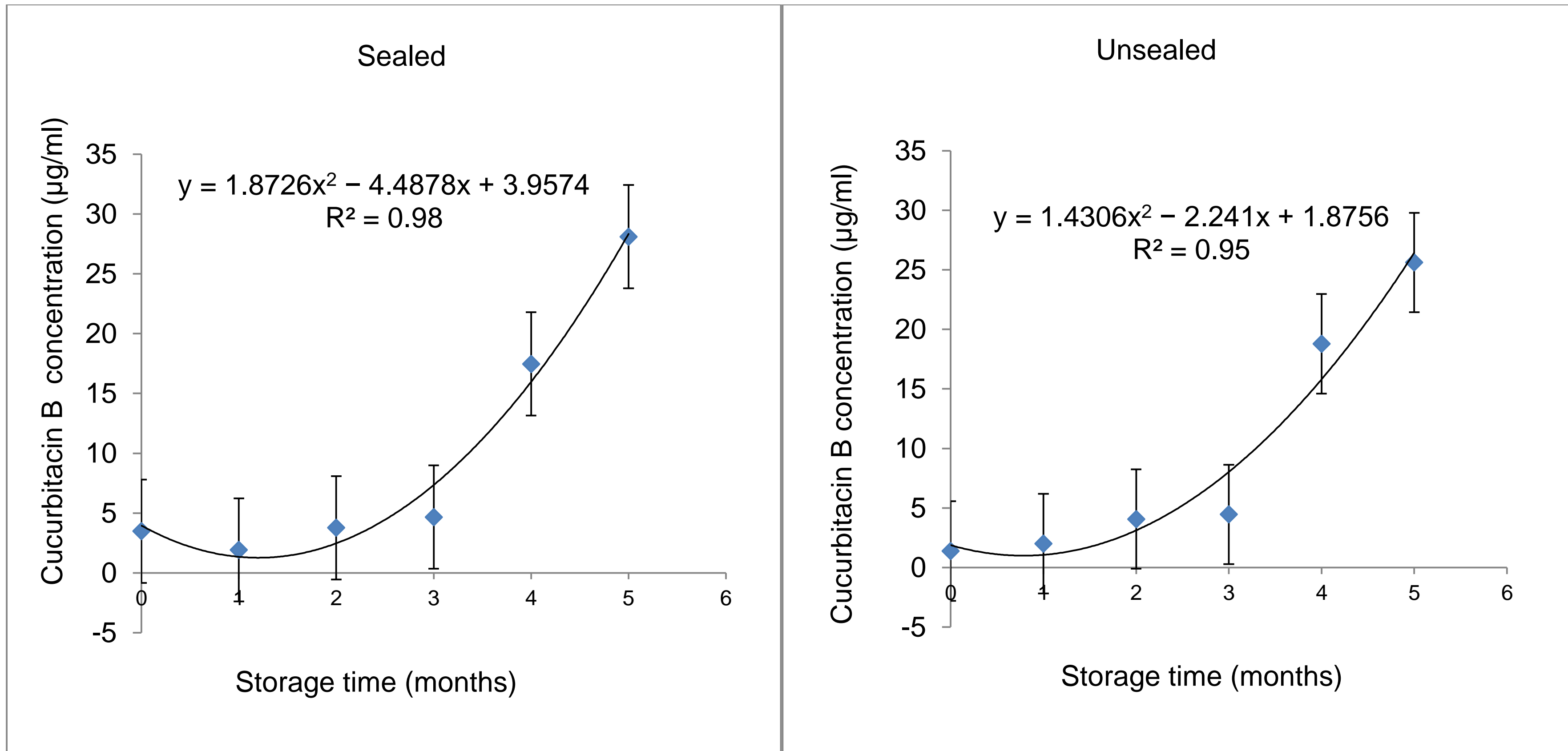


Figure 6.4 Quadratic relationships of cucurbitacin B concentration in phyto-inventories of Nemarioc-AL phytonematicide stored in sealed and unsealed storage containers over six months (n = 30).

6.3.2 Phytonematicide products

Total treatment variation: The interaction between the 2014 and 2015 growing seasons was not significant ($P > 0.05$) and therefore, data were pooled ($n = 60$) and re-analysed. Storage period had highly significant ($P \leq 0.01$) effects on concentration of cucurbitacin in both phytonematicides. Treatments contributed 62 and 68% in TTV of cucurbitacin A and B, respectively (Appendix 6.5−6.6).

Relative impact: Relative to T_0, storage increased concentration of cucurbitacin in both phytonematicides (Table 6.4). Cucurbitacin A was increased by 152 to 361% in the first three months and thereafter the rate of increase declined to 114% by the fifth month in Nemarioc-AL phytonematicide. Similarly, cucurbitacin B increased by 599 to1182% in the first three months and thereafter gradually decreased to 348% by the fifth month in Nemafric-BL phytonematicide.

Quadratic relations: Concentration of cucurbitacin A and B versus storage period exhibited quadratic relationship (Figure 6.5). The model explained the observed relationship for cucurbitacin A and B by 97 and 93%, respectively.

Optimum storage period: Using $x = -b_1/2b_2$ relation, concentrations of cucurbitacin A and B in Nemarioc-AL and Nemafric-BL phytonematicides were optimised at 3.90 and 2.71 months (Table 6.5).

Table 6.4 Responses of cucurbitacin A and B concentrations in Nemarioc-AL and Nemafric-BL phytonematicides during five months of storage (n = 60).

Storage period (month)	Nemarioc-AL phytonematicide		Nemafric-BL phytonematicide	
	Y-value ($\mu g.ml^{-1}$)[y]	Relative impact (%)[z]	Y-value ($\mu g.ml^{-1}$)	Relative impact (%)
0[x]	0.076[c]	–	1.233[b]	–
1	0.192[c]	152	8.619[b]	599
2	0.334[ab]	339	14.535[a]	1079
3	0.350[a]	361	15.807[a]	1182
4	0.307[ab]	304	9.446[ab]	666
5	0.163[bc]	114	6.53[ab]	348

[x] 0 = Refers to no storage period

[y] Column means followed by the same letter were not different ($P \geq 0.05$) according to Fisher's Least Siignificant test.

[z] Relative impact (%) = [(treatment/control − 1) × 100].

Table 6.5 Storage time for cucurbitacins in Nemarioc-AL and Nemafric-BL phytonematicides (n = 60).

Plant variable	Quadratic relationship	R^2	x^z	P ≤
Cucurbitacin A	$Y = -0.0364x^2 + 0.2779x - 0.1827$	0.97	3.90	0.01
	Optimum storage period		3.90 months	
Cucurbitacin B	$Y = -1.797x^2 + 9.8488x + 1.2124$	0.93	2.74	0.01
	Optimum storage period		2.74 months	

[z] Optimum storage time (x) = $-b_1/2b_2$.

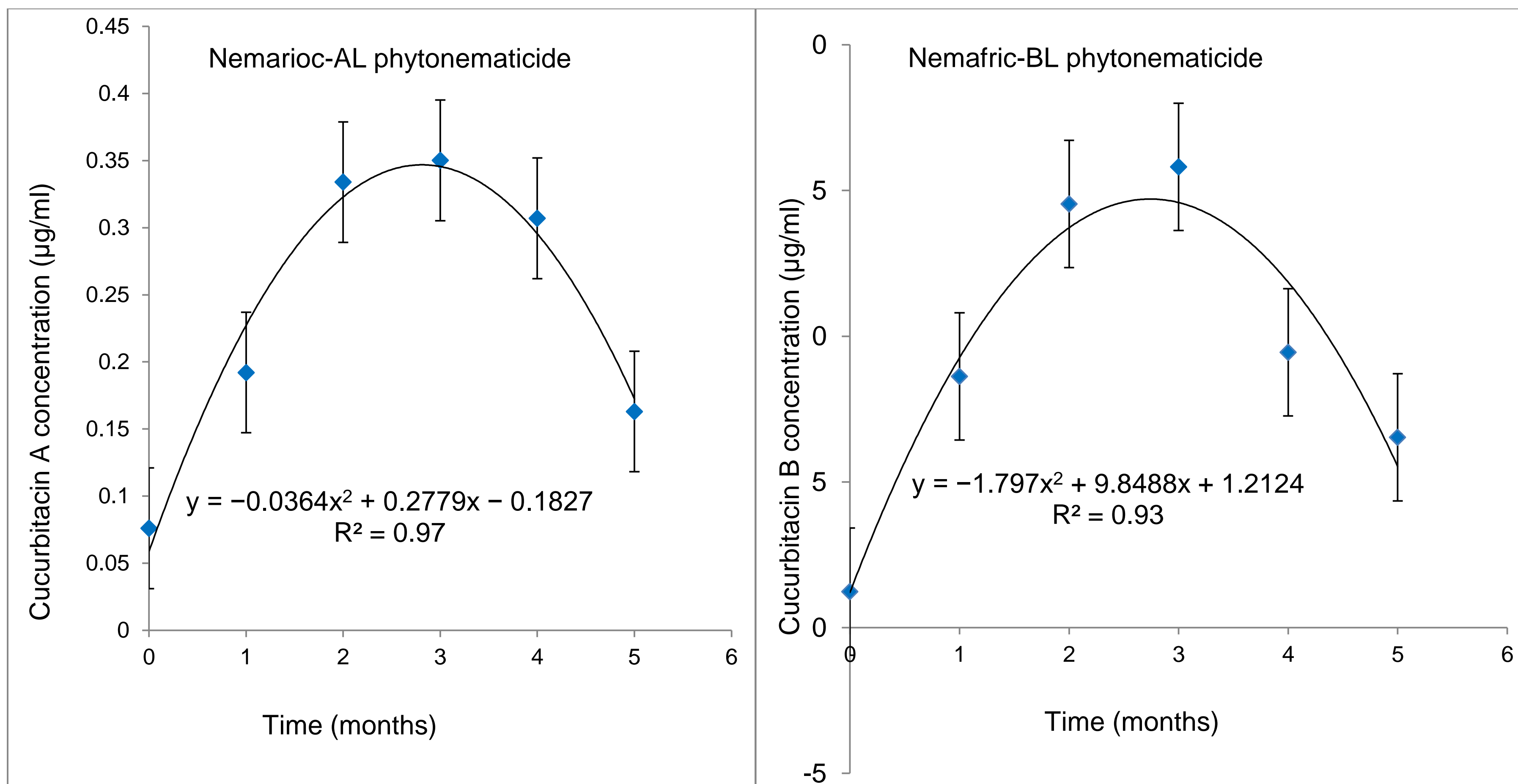


Figure 6.5 Quadratic curve of cucurbitacin A and B concentration in stored Nemarioc-AL and Nemafric-BL phytonematicides (n = 60).

6.4 Discussion

6.4.1 Phytonematicide inventories

Cucurbitacin A: Storage period had an effect on cucurbitacin A concentration, regardless of whether the container was hermetically sealed or exposed to atmospheric gases. Storage period and conditions are among the factors that affect quality of phyto-inventories used in product development (Li and Wardle, 2001). Sukrasno *et al.* (2012) reported that storage of dried crude *Curcuma xanthorhiza* rhizome increased bioactive compounds, namely, a-curcumene ($C_{15}H_{22}$), xanthorrhizol ($C_{15}H_{22}O$) and germacron ($C_{15}H_{22}O$) and also decreased diepi-a-cedren ($C_{15}H_{24}$), which are monoterpenes. Stafford *et al.* (2005) reported that bioactive ingredients namely, eucomnalin ($C_{17}H_{14}O_6$) and 3,9-dihydroeucomnalin ($C_{17}H_{16}O_6$) in *Eucomis autumnalis* maintained their stability when stored for 3 months in the dark at room temperature in dried form. McGaw (2001) also observed that in dried powdered leaves of *Schotia brachypetala,* fatty acids, which were responsible for antibacterial activities, remained stable.

Cucurbitacin A concentration and increasing storage periods exhibited quadratic relationships – confirming the existence of density-dependent growth (DDG) patterns (Liu *et al.*, 2003; Mashela *et al.*, 2015; Salisbury and Ross, 1992). DDG patterns are dominant when microorganisms are exposed to increasing allelochemical concentrations (Liu *et al.*, 2003), with most entities following such patterns when exposed to increasing units of either abiotic or biotic entities (Salisbury and Ross, 1992). DDG patterns are characterised by three phases, namely, stimulation, neutral and inhibitory phases (Liu *et al.*, 2003; Mashela *et al.*, 2015). The three phases had been consistently observed when various plant or nematode variables were regressed

on increasing concentrations of cucurbitacins (Mashela *et al.*, 2015). This is the first report to demonstrate that concentration of cucurbitacin A on increasing storage time exhibiting DDG patterns.

The initial increase in cucurbitacin A suggested that during drying at 52°C the enzymes for biosynthesis of cucurbitacin A from different precursors were not denatured (Chen *et al.*, 2014). During storage, the unstable precursor proceeded through the cucurbitacin-synthesis pathway (Chen *et al.*, 2014), thereby increasing the accumulation of the cucurbitacins in the stored materials. The decreasing concentration of cucurbitacin A could be due to breakdown of this active ingredient into cucumin ($C_{27}H_{40}O_9$) and leptodermin ($C_{27}H_{38}O_8$) (Jeffrey, 1978), which were not quantified in our study. It could be probable that as the concentrations of cucurbitacin A decline, those of cucumin and leptodermin gradually increase. The two chemical compounds have high insecticidal properties (Damalas, 2011; Gobinda *et al.*, 2014). Should the two also constituents have nematicidal properties, the decline in concentrations of cucurbitacin A would not have immediate effects on the efficacy of Nemarioc-AL phytonematicide on nematode suppression.

Generally, phyto-inventories undergo oxidative and degradative processes that could gradually reduce the quality of the phyto-inventories. Heat, oxidation and exposure to the high levels of oxygen and microbial activities accelerate the listed breakdown processes (Fennell *et al.*, 2004). Storage of dried *C. myriocarpus* fruit was optimised at 4.20 and 4.22 months after drying at 52°C from sealed and unsealed containers, respectively. The two optima should be interpreted with caution, (a), the storage time of the phyto-inventories should not be restricted to the optimum periods, since the

concentrations above the optima were still much higher than at T_0, (b), the storage time should be increased to determine the concentration where $C_0 = C_f$, with C_0 and C_f being the initial and final concentrations, respectively, (c) the storage of phyto-inventories could result in different performance of the products and, therefore, quality of the products.

Cucurbitacin B: Increases in cucurbitacin B over five months suggested that storage of powdered material would improve the quality of Nemafric-BL phytonematicide either in granular or liquid formulation. Similar increasing tendencies in active ingredients were observed during a three-month storage of aerial parts of *Thymus daenensis,* which were shade dried at room temperature prior to storage (Rowshan *et al*., 2013). Thymol ($C_{10}H_{14}O$) and carvacrol ($C_{10}H_{14}O$) in *T. daenensis* are monoterpene chemical compounds (Van Wyk and Mink, 2012). The two monoterpenes as secondary metabolites have their acetyl coA molecules exiting glycolysis prior to the Krebs cycle through the methylerythritol 4-phosphate (MEP) pathway (Chen *et al*., 2014; Novak *et al*., 2010), whereas the acetyl coA for cucurbitacins exit glycolysis using the mevalonate pathway, both pathways with a series of enzyme-mediated precursor (Chen *et al*., 2014).

Makkar (1991) demonstrated that different drying temperatures had distinguishable effects on the concentration of secondary metabolites in various plant organs. Generally, most secondary metabolites remain stable when dried at temperatures below 60°C, with thermo-degradation increasing above this temperature (Sukrasno, 2014). The latter could possibly be attributed to denaturing of enzymes which are required to reduce energies of activation for precursors to continue being transformed

to the stable chemical compounds, which are, in our case, cucurbitacin B. Also, it had been suggested that cucurbitacins are thermostable, thus, heat cannot break some bonds within cucurbitacin molecules (Gry *et al*., 2006).

Incidentally, after drying, triterpenoids precursors could have been trapped in tissues within the mevalonic acid pathway where at least eight different precursors with different enzymes are involved in biosynthesis of the final product (Chen *et al*., 2014). In the current study, *C. africanus* fruit were cut into pieces and dried at 52°C (Mashela, 2002). The observed increase in cucurbitacin B during storage suggested that the drying temperature and storage conditions allowed enzymatic activities to proceed after drying and during storage, with the precursors eventually forming cucurbitacin B, which is biologically a stable chemical compound in *C. africanus* fruit. Also, the observed quadratic curves are characteristics of density-dependent growth (DDG) patterns in variables exposed to increasing concentrations of secondary metabolites (Liu *et al*., 2003; Pelinganga and Mashela, 2012) or concentrations of secondary metabolites overtime (Pelinganga *et al*., 2013). Failure to optimising the cucurbitacin B concentration during storage, as is usually the case in DDG studies (Liu *et al*., 2003), suggested that our study was terminated during the characteristic stimulation phase of the DDG patterns (Liu *et al*., 2003). However, because after stimulation there is the neutral phase where optimum values are inherently achieved, the latter would possibly be followed by the inhibition phase in concentration of cucurbitacins. Consequently, it would be necessary to conduct further trials in order to derive the optimum storage time of the material to serve as a cut-off time for storing inputs for stock solutions of Nemafric-BL phytonematicide for both granular and liquid formulations.

6.4.2 Phytonematicide products

The quality of phytonematicides is dependent upon the concentration of active ingredients, which is directly associated with their performance. Active ingredient in phytonematicides, being secondary metabolites, is in a state of continuous change (Luckner, 1984) due to microbial degradation and/or auto-oxidation (Gunatilaka, 2006). Loss of product quality in commodities is a global concern (Drew and Myers, 1997; Straus, 2002), which had since necessitated the development of regulatory standards, collectively referred to as shelf-life (WHO, 2002). In our study, the active ingredient in Nemarioc-AL and Nemafric-BL phytonematicides over increasing storage period exhibited strong quadratic relation, which was characteristic of density-dependent growth (DDG) patterns (Mashela *et al.*, 2015). In the biofertiliser produced from EM-fermented plant materials, the chemical, physical and microbial characteristics over increasing storage period also exhibited DDG patterns (Ngampimol and Kunathigan, 2008).

Gradual stimulation followed by gradual inhibition in concentration of cucurbitacin A and B over increasing storage period of Nemarioc-AL and Nemafric-BL phytonematicides within DDG context could be attributed to a series of both extrinsic and intrinsic factors. EM bioactivities are depended upon the availability of energy and carbon from the substrates (Higa, 1991; Higa and Wididana, 1991). EM is widely used in fermenting plant materials to produce biofertilisers, biopesticides, phytonematicides and feeds (Ngampimol and Kunathigan, 2008; Pelinganga and Mashela, 2012; Pelinganga *et al.*, 2012). Commercially available South African EM comprises photosynthetic bacteria, lactic acid bacteria, yeast, actinomycetes and fermenting fungi (Higa, 1991; Higa and Parr, 1994; Higa and Wididana, 1991). Upon depletion of

readily available sources (sugar + molasses), EM degrade the plant crude extracts, thereby releasing active ingredients into solution (Margarita and Dengel, 2003), with various forces coming into play as the concentration of the active ingredients increases. For example, during the early stages, EM increased cucurbitacins A and cucurbitacin B in solution of the two phytonematicides, through degradation of fruit crude extracts as sources of readily available energy and carbon. However, with time, once fruit crude extracts were depleted, EM could have started to attack cucurbitacin A and B for the same sources, thereby reducing the concentration of cucurbitacins. Maatooq *et al*. (1995) noted that the observed decrease in cucurbitacin E-glycoside concentration of bitter Hawkesbury watermelon (*Citrullus vulgaris* Thunb.) was primarily ascribed to microbial activities. Additionally, auto-oxidation also contribute to the reduction of active ingredients in most active chemical compounds (Allen, 2013).

Reductions in cucurbitacins were also observed at low pH in bitter Hawkesbury watermelon solution extracts stored at varying temperatures over time (Martin *et al*., 2002). EM-produced products like the two phytonematicides are naturally acidic, with the release of active chemical compounds through fermentation directly reducing product pH (Merlin *et al*., 2013; Rizk *et al*., 2007). Miller and Blackwell (1986) reported that a persistent drop in pH could result in enzyme inactivation, which would stop the fermentation process. However, pH did not appear to have played a role in the DDG patterns of cucurbitacin concentrations. At the end of the fermentation process, pH of the mixture is invariably less than 3.7 (Mashela *et al*., 2015). One should appreciate that in addition to sources of energy and carbon for immobilisation by EM, the concentration of active chemical compounds from plant materials using the fermentation process could be affected by pH, temperature, light, presence and form

of precursors and the substrate components (Bhattacharyya and Jha, 2011; Gautam *et al*., 2011; Jain and Pundir, 2011; Kumara and Rawal, 2008; Sudarkodi *et al*., 2012; Zain *et al*., 2009).

6.5 Conclusion

Findings of this study suggested that phytonematicide input-inventories from dried *C. myriocarpus* and *C. africanus* improve the quality of cucurbitacin A and B, respectively. The optimum concentration in dried *C. myriocarpus* was achieved at 4.20 and 4.22 months of storage in sealed and unsealed containers, respectively, whereas, in dried *C. africanus* an increase in concentration of cucurbitacin B during storage suggested that the biological synthesis of cucurbitacins from various precursors continued in favour of the stable outputs – the cucurbitacins. Information on optimum storage would assist the manufacturer to decide on the shelf-life of cucurbitacin A and B in powdered materials from the two *Cucumis* species for the successful development and commercialisation of Nemarioc-AL and Nemafric-BL phytonematicides.

In Nemarioc-AL and Nemafric-BL phytonematicides the concentration of the active chemical ingredient was optimised at 3.90 and 2.74 months, respectively. Concentrations of cucurbitacin in Nemarioc-AL and Nemafric-BL phytonematicides were stimulated up to ca. 99 and 71 days, respectively. These should, however, not be viewed as being equivalent to shelf-life. In both phytonematicides, six months after $T_{0,}$ the concentration of cucurbitacins was still more than three-hundred time to that at $T_{0.}$ The latter suggested that the products were still suitable for use as phytonematicides. The derived quadratic equation for two phytonematicides were

important because at any storage period (months), the concentration of cucurbitacins could be estimated and/or future predictions could be made.

CHAPTER 7
THE EFFECT OF HARVEST LOCATION ON CUCURBITACIN QUALITY AND QUANTITY IN TWO *CUCUMIS* SPECIES

7.1 Introduction

The variabilities in active ingredients are informed by factors such as plant species, preferred organ for accumulation of active ingredients, environmental conditions, climatic conditions, latitude or longitude and the extent of use by the Kingdom Animalia (Cunningham, 1993; Li and Wardle, 2001). Active ingredients in fruits tend to be more stable than those in other organs (Alves *et al.*, 2007; Moco *et al.,* 2007; Mudau *et al.*, 2008). Cucurbitacin A and B in wild cucumber (*Cucumis myriocarpus* Naude.) and wild watermelon (*Cucumis africanus* LF.) fruits, respectively, are widely used in nutraceutical, pharmaceutical, cosmetical and pesticidal industries (Lee *et al.*, 2010; Mashela *et al.*, 2011; Thies *et al.*, 2010; Van Wyk and Wink, 2012; Van Wyk *et al.*, 2002). In most cases, fruits from the two *Cucumis* species are collected from the wild, but efforts were made to determine various agronomic performance (Mafeo, 2005; Maila *et al.*, 2016). Due to the heterogeneity of the environment, it is likely that the cucurbitacin content would vary with location, thereby resulting in quality issues when products are developed from such fruits. The objective of the study was to determine whether the location where *C. myriocarpus* and *C. africanus* fruits were harvested would affect the concentration of cucurbitacin A and B, respectively.

7.2 Materials and methods

7.2.1 Locations, collection and preparation of samples

Cucumis myriocarpus and *C. africanus* fruits were collected from five districts in Limpopo Province, South Africa, with different coordinates, rainfall and parent soil groups (Table 7.1). The samples were collected during two seasons in April 2015. At

each location, 10 fruit for each *Cucumis* species per field were collected from randomly selected 10 maize (*Zea mays* L.) fields to constitute 10 samples/location. The samples were put in paper bags and transported to the laboratory in a cooler box. Fruits were stored in a cold room at 5°C until all locations were serviced and cut into pieces to increase the exposure drying surface area at 52°C in air-forced ovens for 72 h.

Table 7.1 Coordinates, rainfall and soil group where *Cucumis myriocarpus* and *Cucumis africanus* fruits were collected as per districts of Limpopo Province.

District	Location	Rainfall [z]	Soil group
Sekhukhune	Makubu (24°25'32.99"S,30°13'9.19"E)	500	Melanic
Capricorn	Khureng (24°33'44.5"S, 29°23'14.64"E)	400	Duplex
Waterberg	Ga-Seleka (23°13'3.72"S, 27°54'19.94"E)	500	Anthropic
Mopani	Siyandhani (23°16'47.32"S, 30°40'11.78"E)	550	Vertic
Vhembe	Dzwerani (23°02'44.60"S, 30°24'52.61"E)	600	Vertic

[z] (mm/annum).

7.2.2 Extraction of cucurbitacins and data collection

A representative subsample of 4 g dried crude extracts of fruits per location were extracted as described previously (Chapter 3). Briefly, after extraction, the materials were quantified for cucurbitacins using isocratic elution Shimadzu HPLC Prominence with detection using Shimadzu CTO-20A diode array detector and appropriate standards.

7.2.3 Data analysis

Cucurbitacin A and B data were first subjected to analysis of variance (ANOVA) procedure using SAS software (SAS Institute Inc., 2008). When treatments were not significant ($P \leq 0.05$) (Appendix 7.1−7.2), data were subjected to Kruskal Wallis non-parametric one-way analysis of variance (Appendix 7.3−7.4). Mean separation was achieved using Dunn's All-Pairwise Comparison Test.

7.3 Results

Treatment effects on cucurbitacins

Different locations did not have significant ($P \leq 0.05$) effects on concentrations of cucurbitacin A and B when using parametric ANOVA. However, when the data were subjected to the Kruskal Wallis non-parametric test, location had highly significant effects on cucurbitacin concentrations (Table 7.2). Fruit of *C. myriocarpus* collected in Sekhukhune and Mopani had the highest concentration of cucurbitacin A than those from Vhembe (Table 7.2). However, the cucurbitacins in fruits from districts with the highest and lowest concentrations, each were not different to concentrations from Capricorn and Waterberg. Fruits of *C. africanus* collected in Capricorn contained the highest concentration of cucurbitacin B than those collected from Waterberg. However, cucurbitacins in fruit from either district were not different from those collected in Sekhukhune, Mopani and Vhembe (Table 7.2).

Table 7.2 Location effects on cucurbitacin A and B in fruits of *Cucumis myriocarpus* and *Cucumis africanus* (n = 50).

Location	Cucurbitacin A Y-value (µg.ml^{-1})^{z}	Cucurbitacin B Y-value (µg.ml^{-1})
Makubu	32.40^{a}(0.547±0.124)	26.15^{ab}(1.847±0.854)
Khureng	20.05^{ab}(0.483±0.139)	35.05^{a}(2.199±1.257)
Ga-Seleka	27.45^{ab}(0.260±0.040)	12.60^{b}(2.710±1.800)
Siyandhani	33.60^{a}(0.974±0.503)	25.30^{ab}(3.340±0.560)
Dzwerani	14.00^{b}(0.651±0.149)	28.40^{ab}(0.390±0.170)

^{z} Column means followed by the same letter were not different ($P \leq 0.05$) according to the group ranking of Dunn's All-Pairwise Comparison Test. Column means in brackets are cucurbitacins concentrations as generated from parametric ANOVA, which were not different.

7.4 Discussion

In this study, using parametric tests demonstrated that location had no significant effects on cucurbitacins, with coefficient of variation (CV) values being quite high. This observation suggested that other factors which were not considered were more important than location in the accumulation of cucurbitacins in fruits. Thereby suggesting that the fruits were not from the same populations as required for the performing parametric ANOVA (Gomez and Gomez, 1984).

Observations in this study did not support those reported elsewhere on botanicals used for the protection of stored products against insect pest (FAO, 1999).

Phytochemical variability in english tea bush (*Lippia multiflora* Moldenke), black grain (*Chamaecrista nigricans* Vahl.), lemongrass (*Cymbopogon schoenanthus* L.) and leaves, stems and roots of violet tree (*Securidaca longepedunculata* Fres.), were not affected by location, but by other soil variables (FAO, 1999). However, active ingredients in other plant organs, tended to be highly variable when compared to those in fruits (Moco *et al.*, 2007; Zubairi *et al.*, 2014). In flower heads of pyrethrum (*Tanacetum cinerariifolium* Trevir.), the pyrethrin ($C_{21}H_{28}O_3$), which is used in phyto-insecticides, greatly varied with location (Ambrozic *et al.*, 2007). Similarly, the active ingredient azadirachtin ($C_{35}H_{44}O_{15}$) in leaves and seeds of neem (*Azadirachta indica* A. Juss) were negatively affected when harvested at different regions in India (Devaranavadagi *et al.*, 2003; Tomar *et al.*, 2011).

However, when using non-parametric tests location had significant effects on cucurbitacins. Observations in this study agreed with those reported on podophyllotoxin ($C_{22}H_{22}O_8$), 4'-Demethylpodophyllotoxin ($C_{21}H_{20}O_8$), total lignan ($C_{27}H_{30}O_{13}$), quercetin ($C_{15}H_{10}O_7$) and kaempferol ($C_{15}H_{10}O_6$) of traditional Chinese herbal medicine, Himalayan mayapple (*Sinopodophyllum hezandrum* Royle T.S. Ying) roots and rhizomes showed to be affected by locations (Liu *et al.*, 2015).

Accumulation of active ingredients are either directly or indirectly affected by environmental factors, which may be characterised by climatic and soil factors (Bjerke *et al.*, 2005; Dong *et al.*, 2006; Liu *et al.*, 2015; Searles *et al.*, 2001; Zidorn and Stuppner, 2001). Factors such as altitude, light, temperature, humidity and soil type are mostly main contributing factors to varying active ingredients in plant intended

(Dong *et al*., 2011). In this study, concentrations of cucurbitacin varied as affected by location, implying that *C. myriocarpus* and *C. africanus* fruits should be cultivated.

Generally, the active ingredients in botanicals vary greatly in quality and quantity (Agerbirk *et al*., 2001; Azevedo *et al*., 2001; Isman, 1997) due to factors such as genetic make-up, ecological and environmental differences, harvested organs and harvest time (Bopana and Saxena, 2007; FAO, 1999). Commercial biopesticides researched and developed from neem have high variability in the active ingredient azadirachtin (Weaver and Subramanyam, 2000). The variability had been associated with climatic conditions, natural habitats and season variation (Devaranavadagi *et al.,* 2003*).*

7.5 Conclusion

In the current study, location had no significant effect on the accumulation of cucurbitacins in fruits of *C. myriocarpus* and *C. africanus*. However, due to high variability in concentrations of cucurbitacins, it is not recommended that fruits from different location be combined for the production of the two phytonematicides.

CHAPTER 8
CUCURBITACIN RESIDUES AND FOLIAR ESSENTIAL NUTRIENT ELEMENTS IN TOMATO PLANTS TREATED WITH PHYTONEMATICIDES

8.1 Introduction

Nemarioc-AL and Nemafric-BL phytonematicides are highly effective on suppression of nematode population densities (Pelinganga and Mashela, 2012; Pelinganga *et al*., 2012; Pelinganga *et al*., 2013), as shown against root-knot (*Meloidogyne* species) nematodes and the citrus nematode (*Tylenchulus semipenetrans* Cobb 1913) under diverse conditions (Mashela *et al*., 2011; 2015). The two products are produced from fruits of wild cucumber (*Cucumis myriocarpus* Naude.) and wild watermelon (*Cucumis africanus* LF.), indigenous to South Africa (Kristkova *et al*., 2003). In phytonematicides efficacy trials, care should be taken to ensure that (a) phytotoxicity on crops is avoided, (b) the products suppress nematodes consistently and (c) the products do not leave chemical residues in edible parts of the crops. Phytotoxicity, efficacy and consistency trials for Nemarioc-AL and Nemafric-BL phytonematicides had been completed under different conditions (Mashela *et al*., 2011), with scant information on the status of nutrient elements and chemical residues on cultigens (Mashela *et al*., 2015; Pelinganga *et al*., 2012). The two trials on the influence of the two phytonematicides on foliar nutrient elements had been under greenhouse and microplot conditions in pots (Mashela, 2002; Pelinganga, 2012). The objective of this study was to investigate whether tomato plants treated with Nemarioc-AL and Nemafric-BL phytonematicides under field conditions would contain residues of cucurbitacin A and B with improve foliar essential nutrient elements.

8.2 Materials and methods

8.2.1 Study location

Experiments for Nemarioc-AL and Nemafric-BL phytonematicides were conducted on an open field system at the Green Technologies Research Centre (GTRC), University of Limpopo, South Africa (23º53''10'S, 29º44''15'E). The location was characterised by summer rainfall with mean annual rainfall of less than 500 mm and maximum/minimum average temperatures of 28/19ºC. The experiments were conducted in summer (October - December) 2015. The site was characterised by Hutton soil (65% sand, 30% clay, 5% silt 1.6% organic C, ECe 0.148 $dS.m^{-1}$ and pH(H_2O) 6.5).

8.2.2 Plant materials and cultural practices

Seedlings of *C. myriocarpus* and *C. africanus* were raised under field conditions, fruits harvested at 110 days after transplanting and prepared as described previously (Chapter 3). Nemarioc-AL and Nemafric-BL phytonematicides were also prepared as described previously (Chapter 4). Uniform four week-old nematode-free tomato cv. 'Rodade' seedlings were transplanted at 0.5 m and 1 m inter-row and intra-row spacing, respectively. Three days after transplanting, each plant was fertilised with 3 g 2:3:2 (22) as described previously (Chapter 3). Irrigation was achieved through a drip irrigation system at 2 h every other day, which was increased by 2 h every month of until harvest. Plants were scouted weekly for insect pests which were managed using pesticides recommended for tomato production, whereas plants were sprayed weekly with alternations of Bravo, Dithane M-45 and copper oxychloride for disease management.

8.2.3 Experimental design and treatments

Treatments, untreated control, 3% Nemafric-BL and 3% Nemarioc-AL phytonematicides, were arranged in randomised complete block design, with 13 replications. Treatments were initiated at transplanting. Phytonematicides were drench-application of 1 000 ml solution in the irrigation basin around the stem at 16 and 18 days for Nemarioc-AL and Nemafric-BL phytonematicides, respectively (Pelinganga *et al*., 2012).

8.2.4 Data collection

At 110 days after initiating the treatments, with a withholding period of 14 and 16 days for Nemarioc-AL and Nemafric-BL phytonematicides, respectively, fruit were collected and plant height measured from the soil surface to the tip of the flag leaf (Figure 8.1). Stems were cut off at the soil surface and diameters measured at 5 cm above the severed ends using a digital vernier caliper. Mature leaves were collected from tomato plants, dried at 70° for 24 h and finely ground through a Wiley mill to pass through a 1-mm sieve. Fresh fruit were weighed, shoots and roots were oven-dried at 70°C for 72 h and weighed, whereas cut fruit were dried at 52°C. Approximately 0.10 g ground leaf materials were digested in 40 ml 4% nitric acid (HNO_3), followed by placing the container on a vortex to allow for complete wetting of the mixture. The materials were magnetically stirred, thereafter incubated in a 95˚C waterbath for 90 minutes, allowed to cool down at room temperature, filtered, decanted into 50 ml tubes which were covered with a foil and then Ca, Cu, Fe, K, Mg, Mn, Na, P and S analysed using the inductively coupled plasma optical emission spectrometry (ICPE-9000).

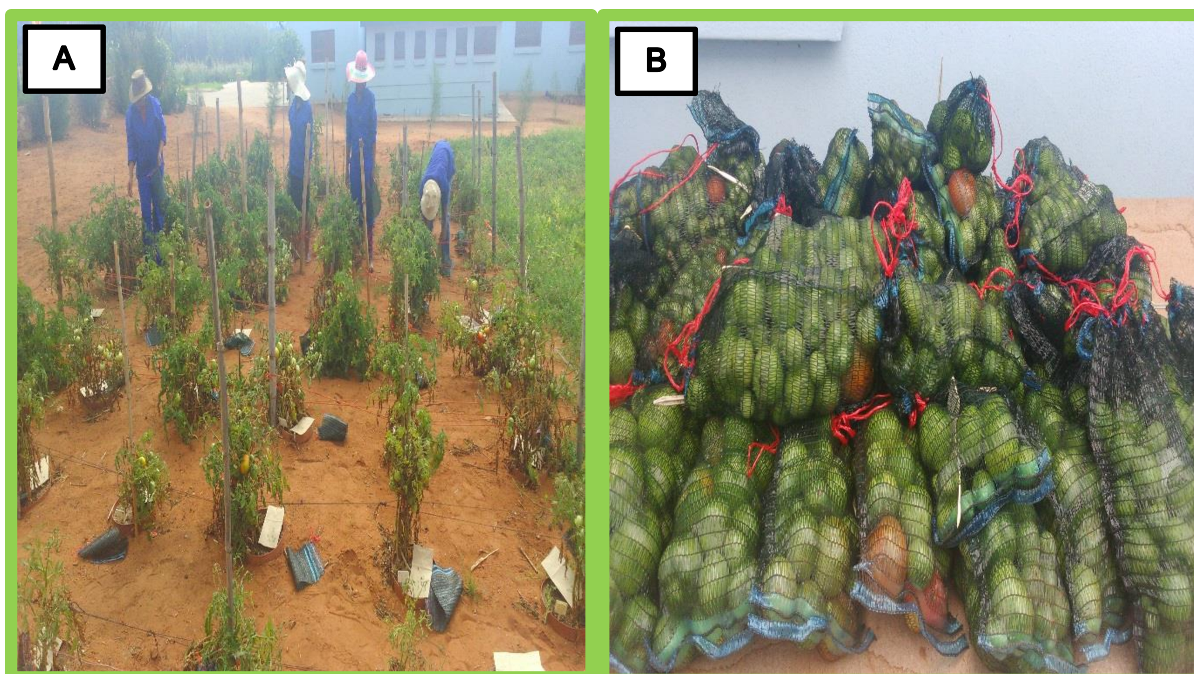


Figure 8.1 Tomato field (A) and fruit (B) at 110 days after initiation of treatments.

8.2.5 Extraction and quantification of cucurbitacins

A 4 g dried tomato fruit representative subsample was extracted as described previously (Chapter 3). Briefly, after extraction, the materials were quantified for cucurbitacins using isocratic elution Shimadzu HPLC Prominence with Shimadzu CTO-20A diode array detector and the appropriate cucurbitacin standards (Chapter 3).

8.2.6 Data analysis

Data were subjected to analysis of variance procedure using SAS software (SAS Institute Inc, 2008). When treatments were significant ($P \leq 0.05$), the sum of squares were partitioned to determine the percentage contribution of sources of variation to the total treatment variation (TTV) in variables measured (Johnson and Berger, 1982; Little, 1981). Mean separation was achieved using Fisher's Least Significant Different

test. Unless otherwise stated, treatment means significant at the probability level of 5% were discussed.

8.3 Results

8.3.1 Plant variables

Treatment effects were not significant ($P \geq 0.05$) for fruit number, plant height, stem diameter and dry shoot mass, except chlorophyll content (Appendix 8.1−8.5), contributing 1, 3, 3, 3 and 11% in TTV of the variables, respectively (Table 8.1). Treatment effects were significant for foliar Mg, Na, P, Ca and Fe (Appendix 8.6−8.8, 8.10−8.11), contributing approximately 17, 58, 19, 18 and 52% to the TTV, respectively (Table 8.2). Relative to untreated control, 3% Nemarioc-AL phytonematicide increased leaf Mg, Na, P and Ca by 28, 38, 27 and 25%, respectively, whereas leaf Fe decreased by 26% (Table 8.3). Similarly, 3% Nemafric-BL phytonematicide increased leaf Mg, Na, P and Ca by 18, 54, 22 and 25%, respectively, but decreased Fe by 62% (Table 8.3).

8.3.2 Phytonematicide residues

Using cucurbitacin A (Figure 8.2 B) and cucurbitacin B (Figure 8.3 B) standards versus the controls (Figure 8.2 A; 8.3 A) and tomato fruit samples treated with Nemarioc-AL phytonematicide (Figure 8.2 C), cucurbitacin A residues were not detected in tomato fruit samples. Similarly, in tomato plants treated with Nemafric-BL phytonematicide (Figure 8.3 C), cucurbitacin B residues were not detected in tomato fruit samples.

Table 8.1 Responses of sum of squares to Nemarioc-AL and Nemafric-BL phytonematicides for fruit number, plant height, stem diameter, chlorophyll and dry shoot mass of tomato 'Rodade' at 110 days after the treatment under field conditions (n = 39).

		Fruit No.		Plant height		Stem diameter		Chlorophyll content		Dry shoot mass	
Source	DF	SS	%	SS	%	SS	%	SS	%	SS	%
Replication	12	388.5	34^{ns}	4770	39^{ns}	2.2006	61^{ns}	607.6	49^{ns}	21469.2	41^{ns}
Treatment	2	1.76	1^{ns}	344	3^{ns}	0.1016	3^{ns}	126.4	11^{**}	1478.5	3^{ns}
Error	24	735.5	65	7089	58	1.3068	36	497.4	40	29184.8	56
Total	38	1125	100	12203	100	3.6091	100	1231	100	52123.5	100

^{ns} = Not significant at $P \leq 0.05$; ** = significant at $P \leq 0.05$.

Table 8.2 Responses of mean sum of squares of essential nutrient minerals to Nemarioc-AL and Nemafric-BL phytonematicides applied on tomato 'Rodade' at 110 days after the treatment under field conditions (n = 39).

		Mg		Na		P		K	
Source	DF	SS	%	SS	%	SS	%	SS	%
Replication	12	283.3	37^{ns}	17.222	24^{ns}	520.9	23^{ns}	1330.2	39^{ns}
Treatment	2	127.9	17^{**}	42.085	58^{**}	433.5	19^{**}	434.47	13^{ns}
Error	24	356.3	46	12.715	18	1300	58	1682.5	49
Total	38	767.5	100	72.023	100	2254	100	3447.3	100
		Ca		Fe		S		Cu	
		SS	%	SS	%	SS	%	SS	%
Replication	12	2438.4	39^{ns}	0.9987	20^{ns}	368.82	44^{ns}	1.9666	40^{ns}
Treatment	2	1149.8	18^{**}	2.5754	52^{**}	34.647	4^{ns}	0.2203	4^{ns}
Error	24	2702.4	43	1.3679	28	435.2	52	2.754	56
Total	38	6290.6	100	4.942	100	838.66	100	4.9409	100

^{ns} = Not significant at P ≤ 0.05; ** = significant at P ≤ 0.05.

Table 8.3 Relative accumulation of essential nutrient minerals to 3% Nemarioc-AL and 3% Nemafric-BL phytonematicides applied on tomato 'Rodade' at 110 days after the treatment under field conditions (n = 39).

Treatment	Mg		Na		P		Ca		Fe	
	Value[y]	%[z]	Value	%	Value	%	Value	%	Value	%
Control	$15.80^{b}\pm1.18$	−	$4.56^{c}\pm0.18$	−	$28.30^{b}\pm1.13$	−	$46.59^{b}\pm2.26$	−	$1.02^{a}\pm0.11$	−
Nemarioc-AL	$20.15^{a}\pm0.91$	28	$6.30^{b}\pm0.26$	38	$35.94^{a}\pm2.82$	27	$58.18^{a}\pm3.59$	25	$0.75^{b}\pm0.05$	−26
Nemafric-BL	$18.71^{ab}\pm1.36$	18	$7.04^{c}\pm0.29$	54	$34.61^{a}\pm1.55$	22	$58.05^{a}\pm3.86$	25	$0.39^{c}\pm0.04$	−62

[y] Column means ± SE followed by the same letter were not different ($P \leq 0.05$) according to Fisher's Least Significant Difference test.

[z] Relative impact (%) = [(treatment/control - 1) x 100].

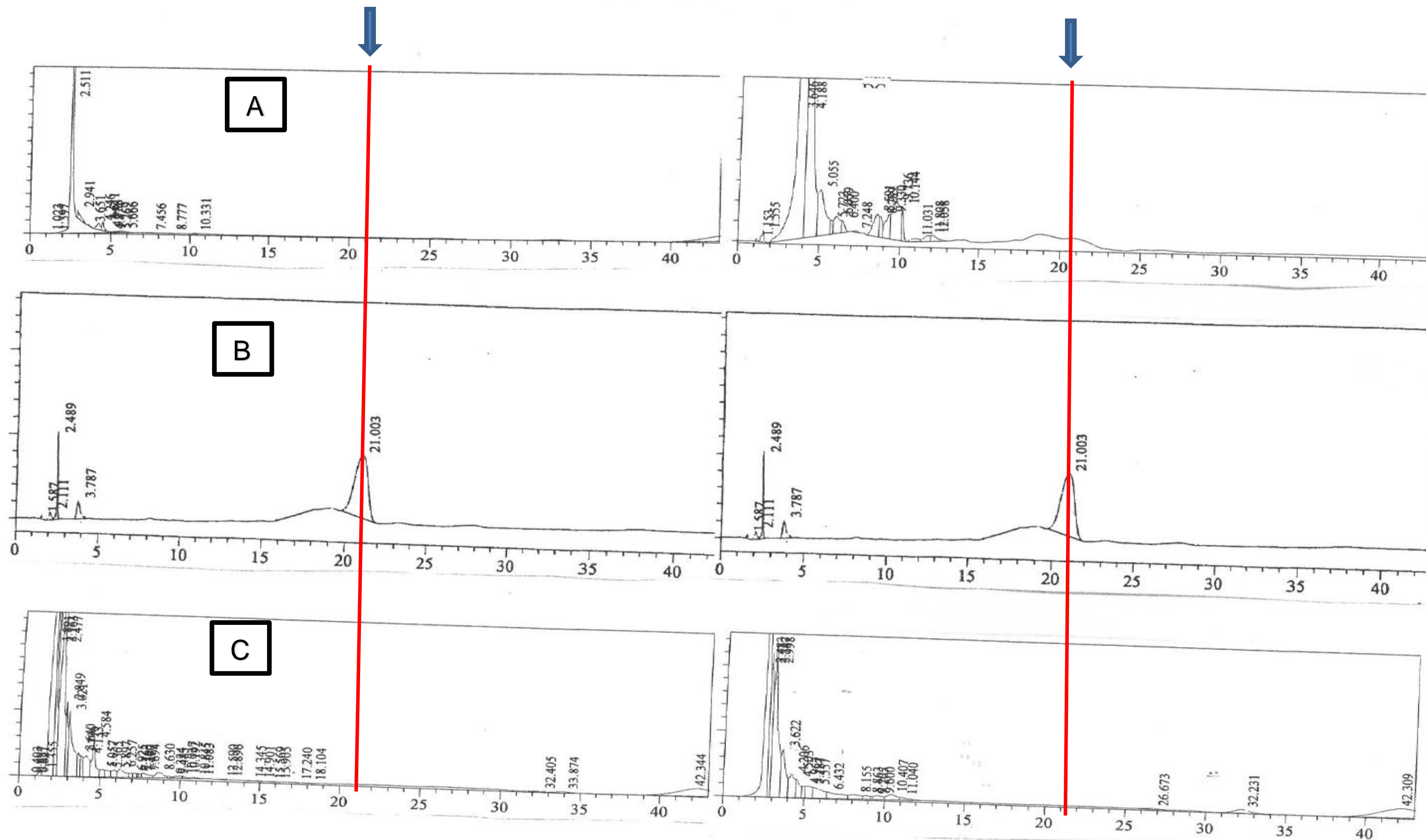


Figure 8.2 Chromatograms for control (A), cucurbitacin A standard (B) and tomato fruit samples from plants where nematodes were managed using Nemarioc-AL phytonematicide (C).

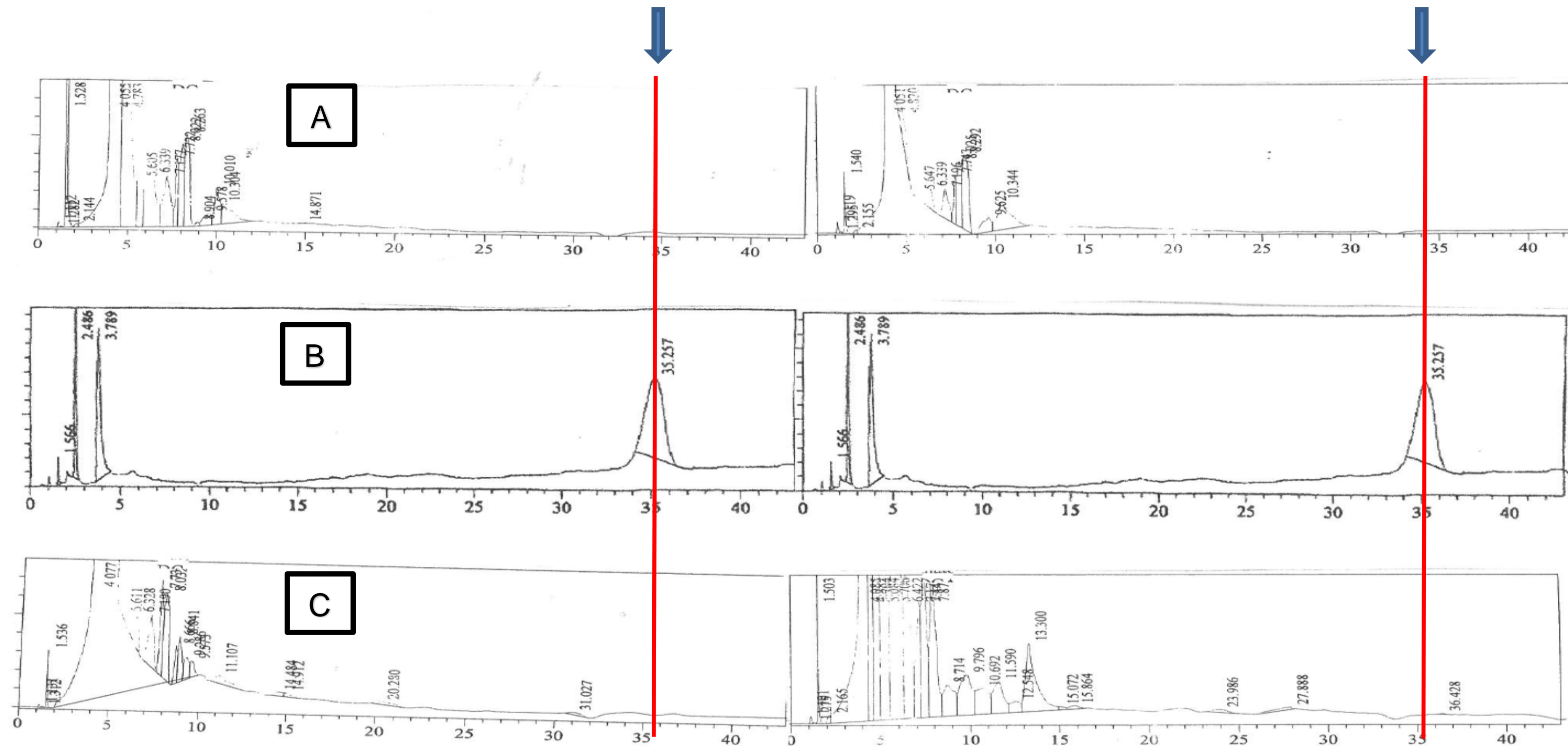


Figure 8.3 Chromatograms for control (A), cucurbitacin B standard (B) and tomato fruit samples from plants where nematodes were managed using Nemafric-BL phytonematicide (C).

8.4 Discussion

8.4.1 Plant variables

Nemarioc-AL and Nemafric-BL phytonematicides had no significant in all plant variables measured except for chlorophyll content. Pelinganga (2013) observed treatment effects on dry shoot mass, dry root mass and plant height for Nemarioc-AL phytonematicide, whereas for Nemafric-BL phytonematicide only plant height was affected by treatments under field conditions. Under field conditions, phytonematicides have unobstructed movement in the soil, which might be downstream or sidestream, depending on soil type, slope and irrigation intensity. Some of the listed factors would affect the concentration of the phytonematicide within the rhizosphere, unlike in pot trials where phytonematicide movements are restricted. Phytonematicide effects on chlorophyll content agreed with the findings of Mashela *et al.* (2013), where Nemafric-BL 3% and 6% resulted in increased chlorophyll content on tomato plants growing on soil where phytonematicides were applied in the preceding crop. The increased chlorophyll content in the current study could be explained by the improved Mg content in leaves as articulated by Mohammad and Rafiq (2011). Magnesium is one of the essential elements required in the synthesis of chlorophyll ($C_{55}H_{72}O_5N_4Mg$) (Bhosle *et al.*, 1975).

8.4.2 Leaf nutrient elements

Different responses in leaf nutrient elements in tomato plants to Nemarioc-AL and Nemafric-BL phytonematicides were consistent with previous observations on tomato plants under microplot conditions (Pelinganga, 2013). Generally, an increase in Ca in the soil increases Ca content in tomato fruit, fruit firmness and shelf-life extension (Cooper and Bangerth, 1976; Paiva *et al.*, 1998). Also, Ca ameliorates the blossom-

end rot in tomato fruit (Taylor and Locascio, 2004), whereas P is responsible for the number of blossoms in early plant growth and fruit set (Sainju *et al*., 2003; Zobel, 1966). Concentration of phytochemicals such as ascorbic acid, flavonoids and lycopene in tomato fruits may also increase due to increasing P content in leaves (Dorais *et al*., 2008). Insufficient essential nutrient elements in tomato plants, especially Ca, may induce physiological disorders that range from blotchy ripening, catface, fruit cracking and blossom-end rot (Franco *et al*., 1999; Nonami *et al*., 1995; Saure, 2001; Taylor and Locascio, 2004). Decrease in Fe content in leaves of tomato was observed in the current study, which might be due to Fe existing as Fe^{3+} in the soil bound with Fe hydroxides, suggesting that plants needed to mobilize Fe in the soil by first making it soluble before it could be absorbed and transported (Jeong and Guerinot, 2009).

8.4.3 Cucurbitacin residues in fruits

Active ingredients in Nemarioc-AL and Nemafric-BL phytonematicides are as in fruits of *C. myriocarpus* and *C. africanus* plants, namely, cucurbitacin A ($C_{32}H_{46}O_9$) and cucurbitacin B ($C_{32}H_{48}O_8$), respectively (Jeffrey, 1978). In the current study, residues from the two phytonematicides were not detected in all tomato fruit. The two cucurbitacin molecules are non-polar, with cucurbitacin A being slightly polar and soluble in water (Gry *et al*., 2006) and cucurbitacin B being insoluble (Jeffrey, 1978). Generally, non-polar molecules, including glucose, cannot be transported through the bipolar membranes of the symplastic pathway of the endodermis in roots into the vascular bundle (Campbell, 1990). From one to seven days after drench-application, azadirachtin ($C_{35}H_{44}O_{16}$) residues were, however, observed in olive, with rapid decline from 0.35 ppm in Day 1 to less than 0.02 ppm in Day 7 (Caboni *et al*., 2002). However,

when using neem products on strawberry, azadirachtin chemical residues in berries were not detected (Caboni *et al*., 2006). The azadirachtin findings along with the undetected concentrations of cucurbitacin A and B at with-holding periods of 14 and 16 days after application of Nemarioc-AL and Nemafric-BL phytonematicides, respectively, suggested that the two products each could be "viewed" as being "safe" for use in tomato production.

In context of density-dependent growth (DDG) patterns, small quantities of cucurbitacin in edible produce and products would be highly risky since they could stimulate cell division (Lee *et al*., 2010). The latter would eventually result in cancer. However, at high concentrations, where growth of cell would be inhibited, cucurbitacins could result in cytotoxicity (Lee *et al*., 2010). Therefore, observations in this study, where cucurbitacins were undetectable, were important for the tomato industry in respect to the commercialisation prospects of Nemarioc-AL and Nemafric-BL phytonematicides.

8.5 Conclusion

At 3% and their respective application intervals, Nemarioc-AL and Nemafric-BL phytonematicides, improved the nutrition elemental status of leaves in tomato plants, without leaving traces of cucurbitacin residues in fruit. Along with consistent efficacy reports on nematode suppression and non-phytotoxicity effects, the current observation strengthens the potential commercialisation of the two products for use in tomato crop protection against nematodes.

CHAPTER 9
SUMMARY, SIGNIFICANCE OF FINDINGS, RECOMMENDATIONS AND CONCLUSIONS

9.1 Summary

The study was primarily involved in research and development of quality protocols for Nemarioc-AL and Nemafric-BL phytonematicides using the active ingredients cucurbitacin A and B, respectively. Two phytonematicides are manufactured using fermented crude extracts of fruits from wild cucumber (*Cucumis myriocarpus* Naude.) and wild watermelon (*Cucumis africanus* LF.), respectively. Although cucurbitacin B is distributed in all organs of *C. africanus* plant, the highest was in fruit. The fruits of both *Cucumis* species should be harvested at maturity, beyond which senescence sets in, resulting in reduced concentrations of cucurbitacin A and B. The selection of (a) suitable organs, (b) optimum harvest period, (c) drying method, (d) optimum drying temperature, (e) storage period of phyto-inventories, (f) storage period of phytonematicides and location from which fruits were collected, were shown to influence concentrations of cucurbitacin A and B. Finally, chemical residues of cucurbitacin A and B were tested in tomato fruits where nematode population densities were managed using Nemarioc-AL and Nemafric-BL phytonematicides, respectively, without traceable residues of the two cucurbitacins.

9.2 Significance of findings

Development of quality protocols is required by legislation prior to the registration of pesticides for user, consumer and environmental protection. In most instances, quality protocols for botanicals have not been developed due to the belief that the products are short-lived and therefore, with limited risks. This view creates challenges since botanicals rely mostly on allelochemicals for their efficacy, which can be highly toxic

to various organisms. In South Africa, the Department of Agriculture, Fisheries and Forestry is the responsible authority for the registration of inputs used in agriculture, with a clear set of regulations that include efficacy, toxicology, quality protocols, chemical residues and bioactive mechanisms.

9.3 Recommendations

In all the studies, the concentration of cucurbitacin in fruit of *C. myriocarpus* appeared to be lower than those in *C. africanus*. Cucurbitacin A in fruit of *C. myriocarpus* oxidises rapidly into cucumin and leptodermin, therefore, it would be necessary that the efficacy of the two chemical compounds on nematode suppression be tested, to verify whether Nemarioc-AL phytonematicide has three complementary active ingredients on nematodes. Cucurbitacin B occurs in all organs of *C. africanus* plant; despite the fact that the highest concentration was in the fruit, further trials are necessary to establish if the use of whole plant would result in dilution effects that would negatively impact efficacy on nematode suppression. During storage, the concentration of cucurbitacins over increasing storage period, regardless of whether the material was as phyto-inventories or finished products, the relations were primarily within the DDG patterns. In the current study, at termination of the respective trials the concentration of cucurbitacins was already declining gradually, but it had not yet declined to concentrations that were below those at harvest. Studies are therefore necessary to establish the duration when the concentrations would be equivalent to those at initial storage time and perhaps below. Although cucurbitacin residues were not detected in tomato fruit, testing should extended to other crops, including leaf, root and stem vegetable crops. Additionally, since the phytonematicides are applied through the

drench method, it would be prudent to monitor the degradation of the cucurbitacins in soil solutions overtime under different cropping systems.

9.4 Conclusions

This study, focused exclusively on the development of quality protocols and chemical residues in produce, with a number of major findings, namely, (a) fruits were suitable organs for development of the two phytonematicides from the two *Cucumis* species, (b) fruits should be harvested within specific periods, those from *C. myriocarpus* and *C. africanus*, for instance, at 89 and 95 days after transplanting, (c) oven-drying method was viewed as the appropriate preservation method for cucurbitacins, with the suitable temperature being at 52ºC, (d) during storage of both phyto-inventories and finished phytonematicide products, cucurbitacins increased through density-dependent growth patterns, which is characterized by three phases, namely, stimulation, neutral and inhibition phases, (e) the study also showed that it was not advisable to randomly use fruits from different locations to manufacture phytonematicides due to inherent variability in cucurbitacin concentrations and (f) fruits from tomato plants that were treated with the two products to manage nematode population densities had no cucurbitacin residues. Nemarioc-AL and Nemafric-BL phytonematicides, with active ingredients cucurbitacin A and B, respectively, have the potential of serving as alternatives to synthetic nematicides in management of nematode population densities. Findings in the current study provided preliminary quality protocols in research and development of phytonematicides for registration in terms of Act No. 36 of 1947. The protocols were developed in an attempt to influence policy in relation to registration of application of phytonematicides for use in agricultural systems.

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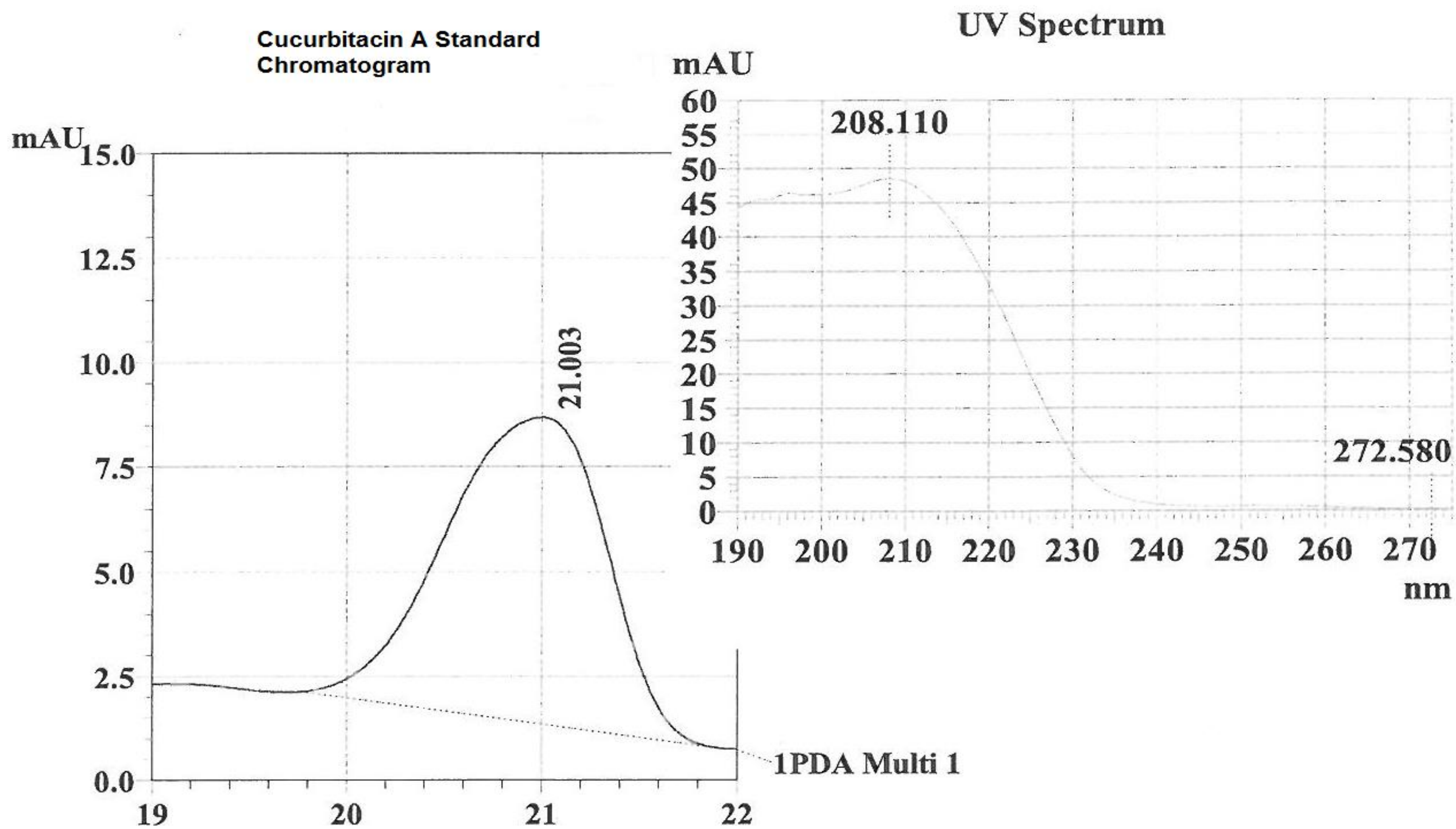
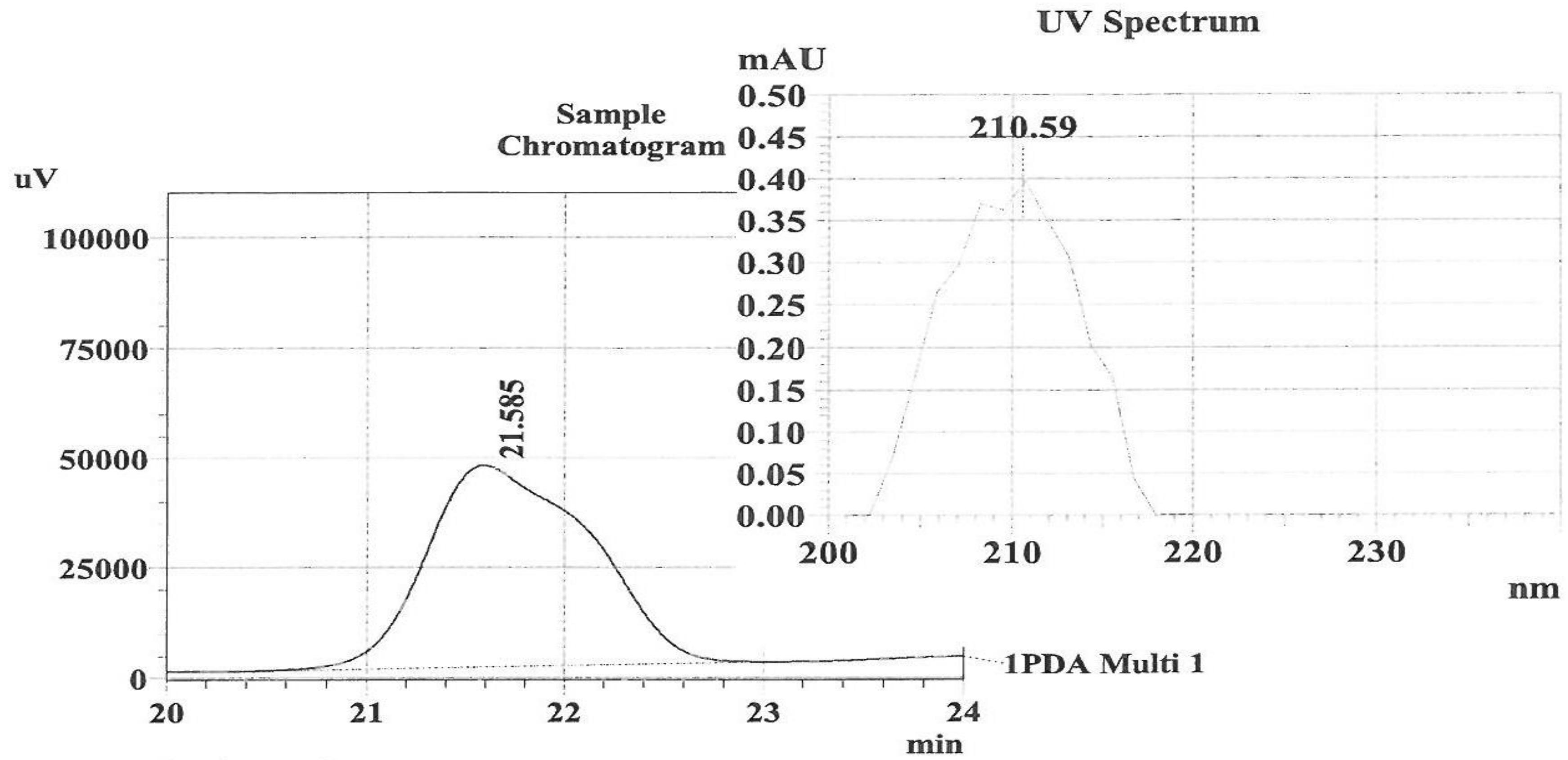
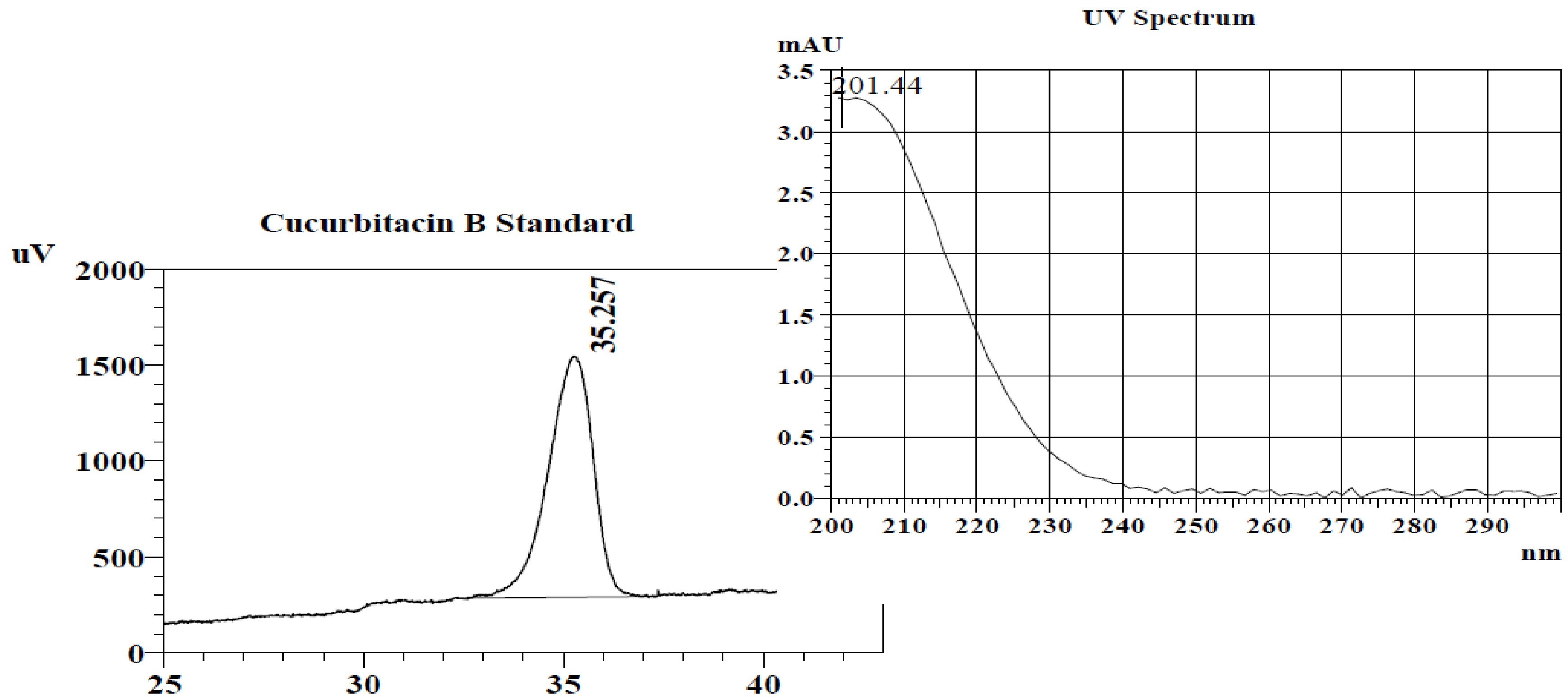


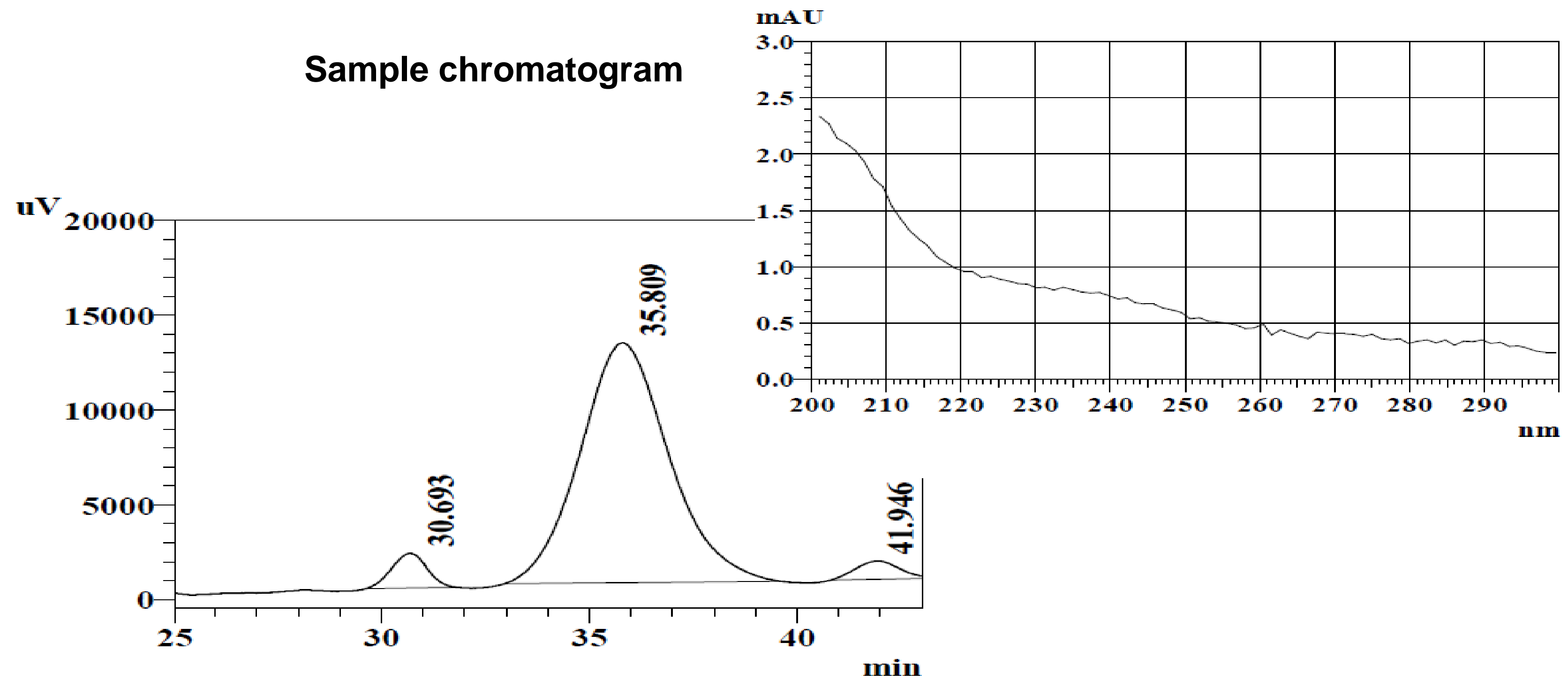
Appendix 3.1 Comparative cucurbitacin A HPLC chromatograms at 230 nm of *Cucumis myriocarpus* standard.



Appendix 3.2 Comparative cucurbitacin A HPLC chromatograms at 230 nm of *Cucumis myriocarpus* sample.



Appendix 3.3 Comparative cucurbitacin B HPLC chromatograms at 230 nm of *Cucumis africanus* standard.



Appendix 3.4 Comparative cucurbitacin B HPLC chromatograms at 230 nm of *Cucumis africanus* sample.

Appendix 3.5 Analysis of variance for cucurbitacin B concentration responses to varying organs of *Cucumis africanus.*

Source	DF	SS	%	F	P ≤
Treatment	3	5.9231	59**	5.09	0.01
Error	16	4.1599	41		
Total	19	10.0830	100		

ns = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 3.6 Analysis of variance for cucurbitacin A concentration responses to harvesting time of fruit from *Cucumis myriocarpus*.

Source	DF	SS	%	F	P ≤
Replication	11	434.33	6 ns	13.11	0.00
Treatment	7	3648.7	51**		
Error	77	3061.62	43		
Total	95	7144.65	100		

ns = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 3.7 Analysis of variance for cucurbitacin B concentration responses to harvest time of fruit from *Cucumis africanus.*

Source	DF	SS	%	F	P ≤
Replication	11	99.536	16 ^{ns}		
Treatment	7	427.33	73**	6.86	0.00
Error	77	62.325	11		
Total	95	589.19	100		

^{ns} = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 4.1 Analysis of variance for cucurbitacin A concentration responses to different drying methods of fruit from *Cucumis myriocarpus*.

Source	DF	SS	%	F	P ≤
Treatment	3	3.0118	43**	3.99	0.02
Error	16	4.0291	57		
Total	19	7.0410	100		

^{ns} = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 4.2 Analysis of variance for cucurbitacin B concentration responses to different drying methods of fruit from *Cucumis africanus.*

Source	DF	SS	%	F	P ≤
Treatment	3	18.3404	40**	3.54	0.03
Error	16	27.6141	60		
Total	19	45.9545	100		

ns = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 5.1 Analysis of variance for cucurbitacin A concentration responses to different oven-drying temperatures of fruit from *Cucumis myriocarpus*.

Source	DF	SS	%	F	P ≤
Treatment	3	79.118	65**	9.03	0.01
Error	16	42.039	35		
Total	19	121.157	100		

ns = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 5.2 Analysis of variance for cucurbitacin B concentration responses to different oven-drying temperatures of fruit from *Cucumis africanus.*

Source	DF	SS	%	F	P ≤
Treatment	5	141.362	71**	3.77	0.01
Error	24	58.368	29		
Total	29	198.729	100		

ns = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 6.1 Analysis of variance for cucurbitacin A concentration responses to different storage time of dried fruit from *Cucumis myriocarpus* in sealed containers.

Source	DF	SS	%	F	P ≤
Treatment	6	5.8957	73**	4.31	0.00
Error	28	2.2177	27		
Total	34	8.1135	100		

ns = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 6.2 Analysis of variance for cucurbitacin A concentration responses to different storage time of dried fruit from *Cucumis myriocarpus* in unsealed containers.

Source	DF	SS	%	F	P ≤
Treatment	6	2.8650	94**	7.28	0.00
Error	28	0.1947	6		
Total	34	3.0597	100		

ns = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 6.3 Analysis of variance for cucurbitacin B concentration responses to different storage time of dried fruit from *Cucumis africanus* in sealed containers.

Source	DF	SS	%	F	P ≤
Treatment	5	2792.85	71**	11.87	0.00
Error	24	1129.10	29		
Total	29	3921.95	100		

ns = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 6.4 Analysis of variance for cucurbitacin B concentration responses to different storage time of dried fruit from *Cucumis africanus* in unsealed containers.

Source	DF	SS	%	F	P ≤
Treatment	5	3921.11	86**	30.54	0.00
Error	24	616.88	14		
Total	29	4537.39	100		

ns = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 6.5 Analysis of variance for cucurbitacin A concentration responses to storage time for Nemarioc-AL phytonematicide.

Source	DF	SS	%	F	P ≤
Replication	9	0.19677	7 ns		
Treatment	5	1.68007	62**	9.33	0.00
Error	45	0.83423	31		
Total	59	2.71107	100		

ns = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 6.6 Analysis of variance for cucurbitacin B concentration responses to storage time for Nemafric-BL phytonematicide.

Source	DF	SS	%	F	P ≤
Replication	9	483.53	5^{ns}		
Treatment	5	6075.61	68^{**}	5.15	0.00
Error	45	2374.80	27		
Total	59	8933.95	100		

^{ns} = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 7.1 Analysis of variance for cucurbitacin A concentration responses to different harvesting locations of fruit from *Cucumis myriocarpus*.

Source	DF	SS	%	F	P ≤
Treatments	4	2.7313	8^{ns}	1.04	0.3
Error	45	29.4842	92		
Total	49	32.2155	100		

^{ns} = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 7.2 Analysis of variance for cucurbitacin B concentration responses to different harvesting locations of fruit from *Cucumis africanus.*

Source	DF	SS	%	F	P ≤
Treatments	4	49.076	7^{**}	0.94	0.4
Error	45	589.003	91		
Total	49	638.079	100		

^{ns} = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 7.3 Non-parametric analysis of variance for cucurbitacin A concentration responses to different harvesting locations of fruit from *Cucumis myriocarpus*.

Source	DF	SS	%	F	P ≤
Between	4	2789.8	27^{**}	4.12	0.00
Within	45	7612.3	73		
Total	49	10402	100		

^{ns} = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 7.4 Non-parametric analysis of variance for cucurbitacin B concentration responses to different harvesting locations of fruit from *Cucumis africanus.*

Source	DF	SS	%	F	P ≤
Between	4	2664.8	26^{**}	3.87	0.00
Within	45	7740.7	76		
Total	49	10405.5	100		

^{ns} = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 8.1 Analysis of variance for fruit number responses to 3% Nemarioc-AL and 3% Nemafric-BL phytonematicides.

Source	DF	SS	%	F	P ≤
Replication	12	388.57	34^{ns}		
Treatment	2	1.76	1^{ns}	0.03	0.96
Error	24	735.57	65		
Total	38	1125.90	100		

^{ns} = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 8.2 Analysis of variance for plant height responses to 3% Nemarioc-AL and 3% Nemafric-BL phytonematicides.

Source	DF	SS	%	F	P ≤
Replication	12	4770	39^{ns}		
Treatment	2	344	3^{ns}	0.63	0.53
Error	24	7089	58		
Total	38	12203	100		

^{ns} = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 8.3 Analysis of variance for stem diameter responses to 3% Nemarioc-AL and 3% Nemafric-BL phytonematicides.

Source	DF	SS	%	F	P ≤
Replication	12	2.2006	61^{ns}		
Treatment	2	0.1016	3^{ns}	1.01	0.37
Error	24	1.3068	36		
Total	38	3.6091	100		

^{ns} = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 8.4 Analysis of variance for chlorophyll responses to 3% Nemarioc-AL and 3% Nemafric-BL phytonematicides.

Source	DF	SS	%	F	P ≤
Replication	12	607.6	49^{ns}		
Treatment	2	126.4	11^{**}	3.31	0.05
Error	24	497.4	40		
Total	38	1231	100		

^{ns} = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 8.5 Analysis of variance for dry shoot mass responses to 3% Nemarioc-AL and 3% Nemafric-BL phytonematicides.

Source	DF	SS	%	F	P ≤
Replication	12	21469.2	41^{ns}		
Treatment	2	1478.5	3^{ns}	0.66	0.52
Error	24	29184.8	56		
Total	38	52123.5	100		

^{ns} = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 8.6 Analysis of variance for magnesium responses to 3% Nemarioc-AL and 3% Nemafric-BL phytonematicides.

Source	DF	SS	%	F	P ≤
Replication	12	283.3	37^{ns}		
Treatment	2	127.9	17^{**}	4.31	0.02
Error	24	356.3	46		
Total	38	767.5	100		

^{ns} = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 8.7 Analysis of variance for sodium responses to 3% Nemarioc-AL and 3% Nemafric-BL phytonematicides.

Source	DF	SS	%	F	P ≤
Replication	12	17.222	24^{ns}		
Treatment	2	42.085	58^{**}	39.72	0.00
Error	24	12.715	18		
Total	38	72.023	100		

^{ns} = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 8.8 Analysis of variance for phosphorus responses to 3% Nemarioc-AL and 3% Nemafric-BL phytonematicides.

Source	DF	SS	%	F	P ≤
Replication	12	520.9	23^{ns}		
Treatment	2	433.5	19^{**}	4.00	0.03
Error	24	1300	58		
Total	38	2254	100		

^{ns} = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 8.9 Analysis of variance for potassium responses to 3% Nemarioc-AL and 3% Nemafric-BL phytonematicides.

Source	DF	SS	%	F	P ≤
Replication	12	1330.2	39^{ns}		
Treatment	2	434.47	13^{ns}	3.10	0.06
Error	24	1682.5	49		
Total	38	3447.3	100		

^{ns} = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 8.10 Analysis of variance for calcium responses to 3% Nemarioc-AL and 3% Nemafric-BL phytonematicides.

Source	DF	SS	%	F	P ≤
Replication	12	2438.4	39^{ns}		
Treatment	2	1149.8	18^{**}	5.11	0.01
Error	24	2702.4	43		
Total	38	6290.6	100		

^{ns} = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 8.11 Analysis of variance for iron responses to 3% Nemarioc-AL and 3% Nemafric-BL phytonematicides.

Source	DF	SS	%	F	P ≤
Replication	12	0.9987	20^{ns}		
Treatment	2	2.5754	52^{**}	22.59	0.00
Error	24	1.3679	28		
Total	38	4.942	100		

^{ns} = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 8.12 Analysis of variance for sulfur responses to 3% Nemarioc-AL and 3% Nemafric-BL phytonematicides.

Source	DF	SS	%	F	P ≤
Replication	12	368.82	44^{ns}		
Treatment	2	34.647	4^{ns}	0.96	0.39
Error	24	435.2	52		
Total	38	838.66	100		

^{ns} = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05

Appendix 8.13 Analysis of variance for copper responses to 3% Nemarioc-AL and 3% Nemafric-BL phytonematicides.

Source	DF	SS	%	F	P ≤
Replication	12	1.9666	40^{ns}		
Treatment	2	0.2203	4^{ns}	0.96	0.39
Error	24	2.754	56		
Total	38	4.9409	100		

^{ns} = Not significant at P ≤ 0.05

** = Significant at P ≤ 0.05