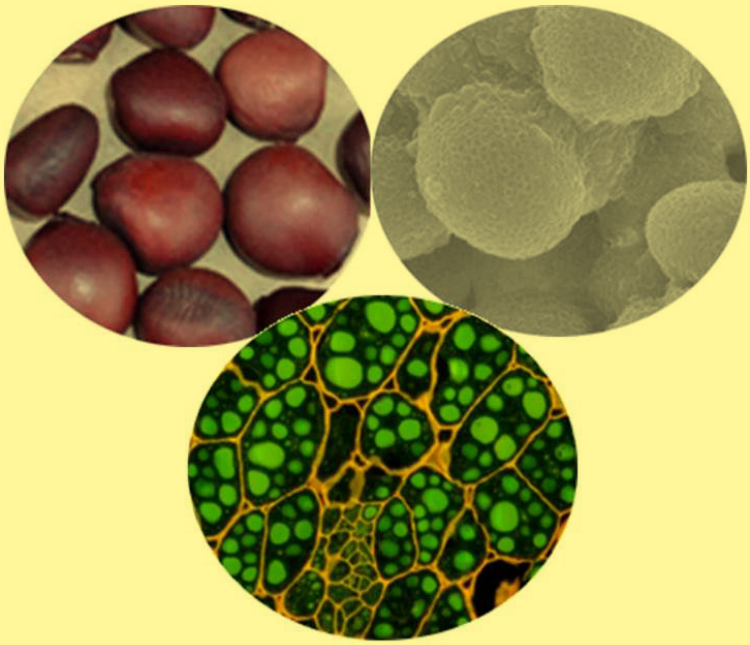
FACULTY OF SCIENCES
UNIVERSITY OF COPENHAGEN

Chemical and Structural Characterisation of Marama Bean *(Tylosema esculentum)* Carbohydrates



PhD thesis by Minah Mmoni Mosele

2012

Chemical and Structural Characterisation of Marama Bean *(Tylosema esculentum)* Carbohydrates

PhD thesis

Minah Mmoni Mosele

2012

Department of Food Science
Faculty of Science
University of Copenhagen

Title
Chemical and Structural Characterisation of Marama Bean *(Tylosema esculentum)* Carbohydrates

Submission
11 April[th] 2012

Defense
25 June[th] 2012

Supervisors
Associate Professor Åse Solvej Hansen
Professor Søren Balling Engelsen

Both from the Department of Food Science
Faculty of Science, University of Copenhagen, Denmark

Opponents
Associate Professor Thomas Skov (Chairperson)
Department of Food Science, Faculty of Science
University of Copenhagen, Denmark

Professor Marena Manley
Department of Food Science, Faculty of AgriSciences
University of Stellenbosch, South Africa

Professor Knud Erik Bach Knudsen
Department of Animal Health and Bioscience
University of Aarhus, Denmark

Illustrations
Cover illustration: Marama beans (brown picture), scanning electron microscopy of marama bean cotyledon showing protein bodies decorated with lipid bodies (grey picture), fluorescent microscopy of marama bean showing protein bodies (green fluorescence) and pectin (gold fluorescence) (Paper I). Pictures on pages between publications and on back cover by Minah Mmoni Mosele, (except picture for Paper II by Åse S. Hansen) depicting immature marama bean (Paper I), mature marama bean on a branch (Paper II) and marama beans mapping Botswana (back cover).

PhD Thesis • 2012 © Minah Mmoni Mosele
ISBN 978-87-7611-508-1
Printed by SL Grafik, Frederiksberg C, Denmark (www.slgrafik.dk)

PREFACE

This PhD has been accomplished at the Quality and Technology Section, Department of Food Science, Faculty of Science at the University of Copenhagen in partial fulfilment of the requirement for the PhD degree in Food Science. The financial support of The Government of the Republic of Botswana, Ministry of Infrastructure, Science and Technology is acknowledged for sponsoring this PhD project.

My heartfelt gratitude goes to my supervisors Associate Professor Åse Solvej Hansen and Professor Søren Balling Engelsen for their support and inspiration. To Åse, you were more than just a supervisor to me. I am indebted to Assistant Professor Jesper Harholt for availing himself for the most part of my practical work and every time I needed a brain to "pick on"; Associate Professor Helle Juel Martens, Associate Professor Flemming Hofmann Larsen and Associate Professor José Manuel Amigo for their support.

My appreciation to the ACABS Research Group, Aalborg University at Esbjerg Campus, especially Michael Madsen, for a fruitful external placement. I also wish to thank Lisbeth, Maria, Cemile and Abdel for the assistance with the laboratory work. My gratitude to my office mates Michael, Niels and Maja. My appreciation also to Oskar Jonsson, Umbio in Umeå (Sweden) and Paul Geladi at Umeå University in Sweden for the use of their facilities for hyperspectral imaging.

Thanks to all my co-authors, the Quality and Technology research group, especially Mette Holse for a wonderful working relationship and atmosphere. To my "Danish" family and friends: Manu and Gilit; Gilda; Stephen; Emma and Femi; Jens and Marina-Helena; Donahue and Judy; Kung and Tin; International Christian Community and Pinse Kirken. To my family, you are simply great; Setobele, Rati and Tlotla, I love you guys! Finally, to the Almighty God, all things are possible with You.

Minah Mmoni Mosele
April, 2012

ABSTRACT

The aim of this thesis was to characterise the chemical and structural composition of carbohydrates in marama bean (*Tylosema esculentum*) at two developmental stages of immature and mature. Marama bean is a wild growing legume indigenous to the Kalahari Desert and neighbouring sandy semi-arid regions of Namibia and South Africa. Despite its potential as a nutritional food, little is known about the carbohydrate composition. Most of the results reported herein focus on mature seeds, though immature seeds were used in some of the earlier work of this project because they were unavailable for further analyses.

In this study, various methods were used to elucidate the nature of the marama bean carbohydrates. Physicochemical (proximate composition) and histochemical (microscopy) methods gave a general overview (**Paper I**), where the carbohydrate content was calculated at 19-24% (mature seeds), constituted by cell wall polysaccharides, mainly pectin and cellulose. There seemed to be a positive relationship between carbohydrates increase and cell wall thickening, in relation to maturation, i.e. during maturation, carbohydrates increased with cell wall thickening as noticed under observation with fluorescent microscopy.

To measure the actual amounts of marama bean carbohydrates, high performance anion exchange-chromatography (HPAEC) with pulsed amperometric detection (PAD) was used. Immature and mature seeds had mannose and arabinose as the most abundant cell wall monomers, respectively, followed by galactose and glucose (**Paper II**). The arabinose was recalcitrant to extraction with strong alkali and was highly branched. It was also characterised by arabinan-like linkages recognised by the arabinan antibody LM6 and LM13 indicating pectic arabinan. The pectin amount was 4.2% in mature seeds, with a low degree of esterification, constituted mainly by homogalacturonan. The mannose was possibly from mannosylated proteins, because the linkages observed are typical of protein mannosylation. Starch was negligible at 0.2% in both immature and mature seeds (**Paper II**), which explains why the iodine stain test in light microscopy was negative (**Paper I**).

Fourier transform Raman spectroscopy (FT-Raman) revealed α-anomeric carbohydrates, observed mainly as pectin and galacturonic acid by nuclear magnetic resonance (NMR) (**unpublished**). The

extracted carbohydrates used for spectroscopy analyses showed residual proteins, suggesting a strong linkage of carbohydrates and proteins, already assumed as protein mannosylation in **Paper II**. This also suggests the existence of glycoproteins in marama bean. However this assumption needs to be further investigated. Since only about 5% of the 24% total carbohydrate content of marama bean could be quantified, the rest of the carbohydrate is considered to be non-starch polysaccharides and glycoproteins.

The chosen combination of methods has gone a long way in the characterisation of marama bean carbohydrates, with some limitations. Therefore this study forms a strong basis for further research in marama bean with great lessons for the next torchbearer.

LIST OF PUBLICATIONS

Paper I

Mosele, M. M., Hansen, Å. S., Hansen, M., Schulz, A., & Martens, H. J. (2011). Proximate composition, histochemical analysis and microstructural localisation of nutrients in immature and mature seeds of marama bean (*Tylosema esculentum*) - an underutilised legume. *Food Chemistry*, *127*, 1555-1561.

Paper II

Mosele, M. M., Hansen, Å. S., Engelsen, S. B., Diaz, J., Sørensen, I., Ulvskov, P., Willats, W. G. T., Blennow, A., & Harholt, J. (2011). Characterisation of the arabinose-rich carbohydrate composition of immature and mature marama beans (*Tylosema esculentum*). *Phytochemistry*, *72*, 1466-1472.

LIST OF ABBREVIATIONS

AIR	Alcohol insoluble residue
CBMs	Carbohydrate-binding modules
CDTA	1, 2-Diaminocyclohexane-N,N,N',N'-tetraacetic acid
CLSM	Confocal laser scanning microscopy
CoMPP	Comprehensive microarray polymer profiling
CP/MAS	Cross polarisation and magic angle spinning
DM	Dry matter
DP	Degree of polymerisation
ER	Endoplasmic reticulum
FT-IR	Fourier transform infrared
FT-Raman	Fourier transform Raman
GC-MS	Gas chromatography – mass spectrometry
GC-EI-MS	Gas chromatograph-electron impact-mass spectrometer
HG	Homogalacturonan
HPAEC-PAD	High performance anion exchange chromatography with pulsed amperometric detection
LB	Lipid bodies
LBG	Locust bean gum
M/G ratio	Mannose / galactose ratio
mAbs	Monoclonal antibodies
NIR-CI	Near infrared chemical imaging
NMR	Nuclear magnetic resonance
PAW	Phenol: acetic acid: water
PB	Protein bodies
RFOs	Raffinose family oligosaccharides
RGI	Rhamnogalacturonan I
RGII	Rhamnogalacturonan II
SEM	Scanning electron microscopy
SP/MAS	Single pulse and magic angle spinning
TEM	Transmission electron microscopy
TFA	Trifluoro acetic acid

TABLE OF CONTENTS

1 INTRODUCTION

This PhD study was set out to study the detailed chemical and structural composition of marama bean with emphasis on the carbohydrate fraction. Such information will benefit the food industry in Botswana in the use of marama bean as a food ingredient with regards to its functionality.

Therefore the aim of this thesis is to characterise the chemical and structural composition of carbohydrates in marama bean at two developmental stages, of immature and mature, corresponding to the stages utilised for consumption, in order to optimise the use of marama bean in food products. The immature seeds were not used in all the analyses because of their limited availability. The investigations were aimed at the chemical quantification and structural elucidation of simple to complex carbohydrates in marama beans by different techniques in microscopy, chromatography, spectroscopy and chemical imaging. The results of this study have been published in peer-reviewed journals, under the headings Paper I – II.

The marama bean (*Tylosema esculentum* Burchell A. Schreiber) is a wild perennial legume species and a prospective cultivated legume because of its exceptionally high nutritional value (Hartley *et al*., 2002). It is native to the Kalahari Desert and neighbouring sandy semi-arid regions of Southern Africa, in particular Namibia and South Africa (Castro *et al*., 2005; Hartley *et al*., 2002). The seeds, commonly called marama or morama bean, tsin or gemsbok bean, are an important component of the diet among the nomadic "hunters-gatherers" in these remote settlements, where few conventional crops can survive (National Research Council, 1979). The bean is commonly roasted and consumed as a snack.

It is known that mature marama bean seeds store large quantities of lipids and proteins in the cotyledons, both well above 30% (Bower *et al*., 1988; Amarteifio & Moholo, 1998; Holse *et al*., 2010; Mosele *et al*., 2011a). The protein content equals that of soya bean (*Glycine max*), and the lipid amount is almost twice that of soya bean (Vaidehi and Kadam, 1989; Nkama and Filli, 2006). The protein content is higher than that of peanuts (*Arachis hypogaea*) while the lipid content is similar (Adsule *et al*., 1989; Nkama and Filli, 2006). This shows that marama bean is equally, if not more nutritious than well known high nutritional value legumes. It has

been shown that mature marama beans do not contain potent allergens found in peanut and lupin (Holse *et al*., 2010).

The total carbohydrate content is approximately 24% (Bower *et al*., 1988; Amarteifio & Moholo, 1998; Mosele *et al*., 2011a). It has been shown that mature marama beans have a high content of dietary fibre with variation between 19 to 27% (Holse *et al*., 2010). The large variation is a result of the fact that marama bean is a non cultivated plant, and external factors such as soil composition and climate. There exists no previous study on the detailed analysis of carbohydrates in marama beans.

Carbohydrates, especially polysaccharides are important in the food industry because they can be used as thickeners, stabilisers, texturisers and gelling agents (Viñarta *et al*., 2006; Khurana & Kanawjia, 2007) and some are considered dietary fibres. In soya bean, the carbohydrate components include 5.3% oligosaccharides, 2.4 – 5.5% dietary fibre and 0.2 – 0.9% starch (Reddy *et al*., 1984; Karr-Lilienthal *et al*., 2005; Saldivar *et al*., 2011).

Structure elucidation of carbohydrates can be challenging due to their inherent heterogeneity and differences in extractability. Therefore detailed characterisation of the carbohydrates is not possible without their fractionation or extraction. The fractionation of marama bean carbohydrates was achieved by the removal of lipids and proteins, and they were studied with different analytical methods at two developmental stages, namely immature and mature stages of consumption.

The analytical methods used include an overall proximate composition of major constituents including total carbohydrates. The distribution and localisation of the various food components in biological materials are best understood by means of histochemistry, which involves the use of different microscopy techniques. In this study we used for example fluorescent microscopy with secondary staining for the detection of starch, pectin and cellulose. The limitation with histochemistry is that it is only able to detect the localisation of chemical components, without any quantification. Therefore in order to quantify the carbohydrates, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was employed to measure simple sugars, starch and cell wall polysaccharides. However, since their

quantification was not possible without fractionation or extraction, this posed a limitation, since some of these components could be lost during these procedures.

Fast and non-destructive spectroscopic techniques were also employed to further study the carbohydrates in pure marama bean flour and extracts. Vibrational spectroscopy and nuclear magnetic resonance (NMR) were used for analysing the composition and structure of the carbohydrates. In addition hyperspectral imaging in the form of near infrared chemical imaging (NIR-CI) was used to study intact marama bean tissue and flour, and compared them with pure commercial sugar samples.

The subsequent chapters of this thesis will be as follows.

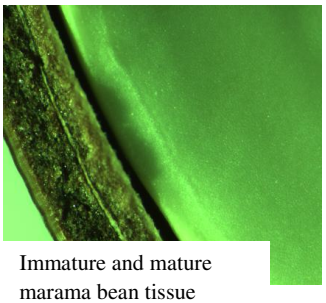
Chapter 2 provides background information of the marama bean, from its distribution, uses, to nutritional value.

Chapter 3 describes the carbohydrates in legumes based on their polymeric nature, i.e. monosaccharides, oligosaccharides and polysaccharides.

Chapter 4 describes the experimental work for the analyses of marama bean carbohydrates, for example, microscopy and spectroscopy and the results obtained.

Chapter 5 and 6 summarises the conclusions, recommendations and perspectives for further analysis of marama bean carbohydrates.

The overall experimental work is summarised in Figure 1. Firstly, the different food components were measured by proximate composition and studied with different microscopy techniques. Then the different carbohydrates components were measured by HPAEC-PAD and comprehensive microarray polymer profiling (CoMPP). Finally, different spectroscopy techniques were employed to elucidate the composition and structure of the carbohydrates.



Immature and mature marama bean tissue

Physicochemical composition

1. Proximate composition
- Moisture
- Lipid
- Protein
- Ash
- Carbohydrate (by difference)

Immature and mature marama bean tissue

Chemical compartmentalisation

2. Microscopy
- Lipid
- Protein
- Starch
- Cellulose
- Pectin
- Lignin

3. NIR-CI

Extracted immature and mature marama bean flour

Chemistry and structure of marama bean carbohydrates

4. HPAEC-PAD
- **Simple sugars**
- **Starch**
- **Alcohol insoluble fraction (cell wall)**

5. Linkage analysis by methylation

6. CoMPP

7. Spectroscopy
- **Fourier transform infrared (FT-IR)**
- **Fourier transform Raman (FT-Raman)**
- **NMR**

Figure 1: Overview of the experimental work performed during this thesis work.

2 THE MARAMA BEAN

2.1 Introduction

Grain legumes belong to the *Leguminosae* (*Fabaceae*) family, the third largest of flowering plants, with three subfamilies, *Caesalpinioideae*, *Mimosoideae* and *Palpilionoideae*. *Leguminosae* family have a characteristic flower structure bearing fruit pods which are consumed with the immature seeds, however, most of the seeds are consumed after maturation when they are dry (Chibbar *et al*., 2010). Marama bean is a wild perennial legume belonging to the tribe *Cercideae* in the subfamily *Caesalpinioideae*, and is thus related to *Cercis* and *Bauhinia* (Wunderlin *et al*., 1981). Before its establishment as a separate genus, *Tylosema* was included in *Bauhinia* (Castro *et al*., 2005). There are four other known species within the genus, namely, *T. fassoglense, T. argenteum, T. humifusum and T. angolense* (Castro *et al*., 2005). Other names of marama bean include morama bean, tsin bean or gemsbok bean (National Research Council, 1979).

2.2 Agronomy

The marama bean plant is a creeper with stems that lie prostrate on the ground in several directions (National Research Council, 1979), up to 6 metres long (Castro *et al*., 2005, Brink, 2006). The plant also has a big tuber (Figure 2) which can weigh up to 300 kg (Brink, 2006). This implies that a single plant would require far more space for cultivation than most legumes. The tuber serves as a water reserve for the plant during drought seasons (Mitchell *et al*., 2005). Big tubers are not edible as they become bitter and fibrous (Brink, 2006).

Figure 2: Picture of the marama bean plant showing stems with leaves and the underground tuber on sandy and grassland where it grows (Ngwako and Mogotsi, 2010).

The species of *Tylosema* are unique within the family *Fabaceae* in the fact that their flowers exhibit heterostyly, meaning that they are self incompatible because of spatial separation of the stigma and anthers (Hartley *et al*., 2002). This may reduce propagation potential, and obviously reduce seed production rates for cropping. In a field experiment carried out in Texas (USA), the marama bean plant took 4.5 years to produce edible seeds. The onset of flowering was at 2 years, development of fruits or seeds at 3.5 years, and final harvesting at full maturation a year later (Powell, 1987). Flowering of marama bean plant has also been seen in the third or fourth year after planting. Successful cultivation was also done in Australia, Kenya, Israel and South Africa (Van der Maesen, 2006). Other cultivation trials were also carried out in Namibia by the University of Namibia (Chimwamurombe, 2010) and Botswana (Botswana College of Agriculture) (unpublished data). Information on yield is not available, however it is assumed to be low since each pod contains 1-2 seeds in comparison to more seeds per pod in other legumes. However, we should bear in mind that this crop can grow where no other food crops can survive.

Marama bean mainly grows in the wild in the semi-arid regions of Southern Africa, in particular Botswana, Namibia and South Africa (Hartley *et al*., 2002; Castro *et al*., 2005). The regions where it grows are grasslands characterised by high temperatures (approximately 37 °C), low rainfall, in sandy and limestone soils (Van der Maesen, 2006).

2.3 Seed Structure

Mature legume seeds have three major components comprising seed coat, endosperm and embryo (Kadam *et al*., 1989; Weber *et al*., 2005; Chibbar *et al*., 2010). The seed coat and endosperm are initially formed followed by the embryo (Weber *et al*., 2005). The embryo includes the cotyledons, which are the major part of the seeds. In general, the seed coat is a transient storage organ for the growing tissue, accumulating starch and protein, before storage activity starts in the embryo. It modifies and controls nutrient supply to the embryo. The embryo grows by cell division (Figure 3). This is followed by cell expansion and accumulation of storage products, i.e. maturation. The endosperm disappears with maturation as observed in marama bean (Mosele *et al*., 2011a) (Figure 4). Marama beans store energy as lipid, which is not common for legumes as they mostly store energy in the form of starch. This exception is also seen in soya bean and peanut. Marama bean proteins exhibit themselves as electron-dense spherical bodies surrounded by lipid bodies (Amonsou *et al*., 2011; Mosele *et al*. 2011a), see Figure 4 and 5. The protein bodies are already in globular form during early deposition (Mosele *et al*., 2011a).

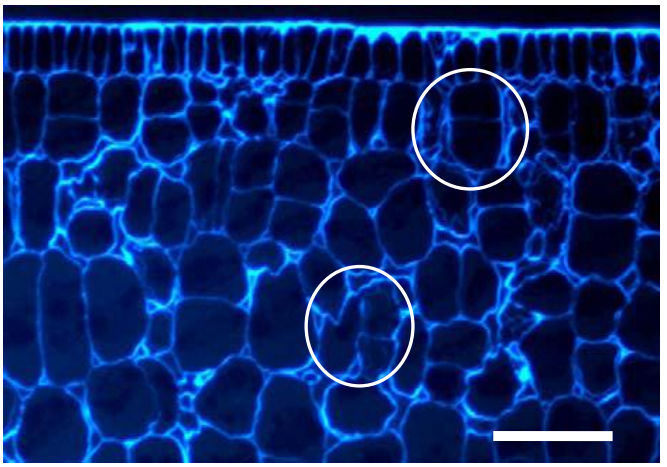


Figure 3: Fluorescence microscopy image of immature marama bean cotyledon showing cell division (white circles). The blue colour is cellulose after staining with Calcofluor white. Bar = 100 µm (Paper I).

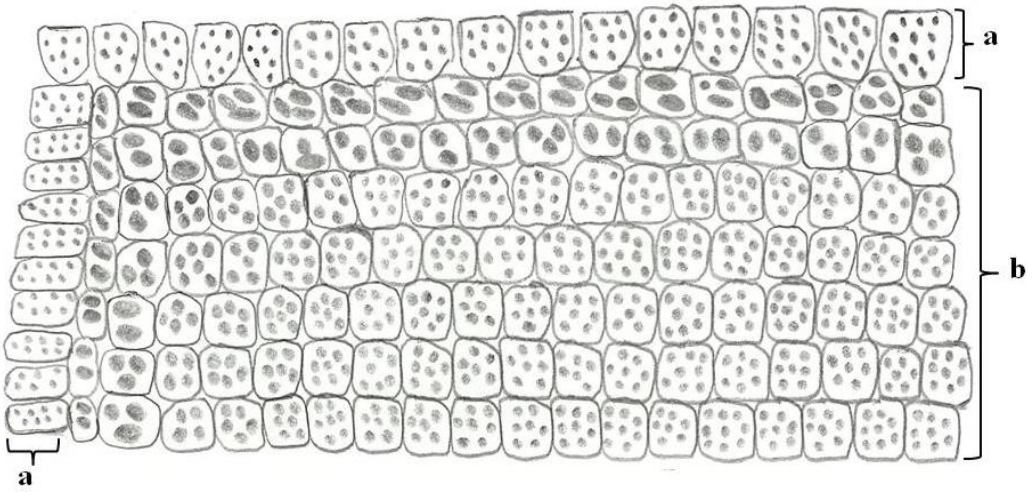


Figure 4: A sketch of the microstructure of mature marama bean cotyledon showing protein bodies (grey spherical bodies) embedded in a lipid matrix; in the a) Epidermis b) Storage parenchyma cells. Note the change in the size and number of the protein bodies from the epidermis through to the centre of the parenchyma cells (Mosele, unpublished).

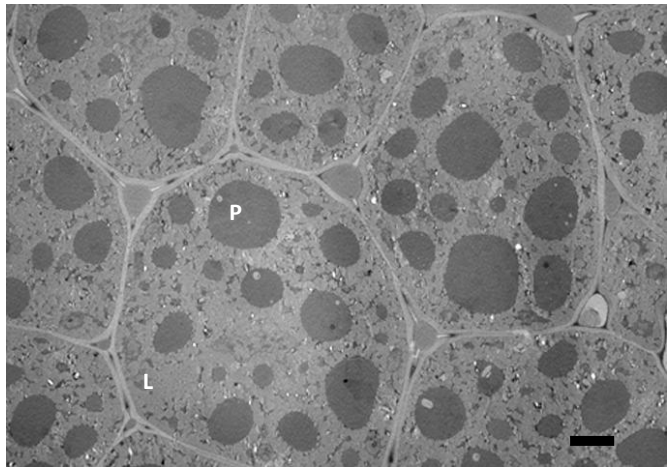


Figure 5: Transmission electron microscopy (TEM) image of mature marama bean cotyledon showing large protein bodies (dark grey, P) encircled by lipid bodies (light grey surrounding, L). Bar = 5 µm (Mosele, unpublished).

Immature marama bean cotyledon are white in colour with a tint of green, encapsulated in green pods containing one or two seeds (Mosele *et al*., 2011a), see Figure 6. Mature marama bean cotyledons are white to cream in colour (Figure 6), encapsulated in hard, woody seed coats, reddish brown in colour (Van der Maesen, 2006). Their decortication requires more mechanical effort than most beans. Mature marama bean seeds are on average 19.9 (± 1.6) mm in length, 17.6 (± 2.5) mm in width and 12.0 – 15.0 mm in thickness (Jideani *et al*., 2009; Mosele *et al*., 2011a).

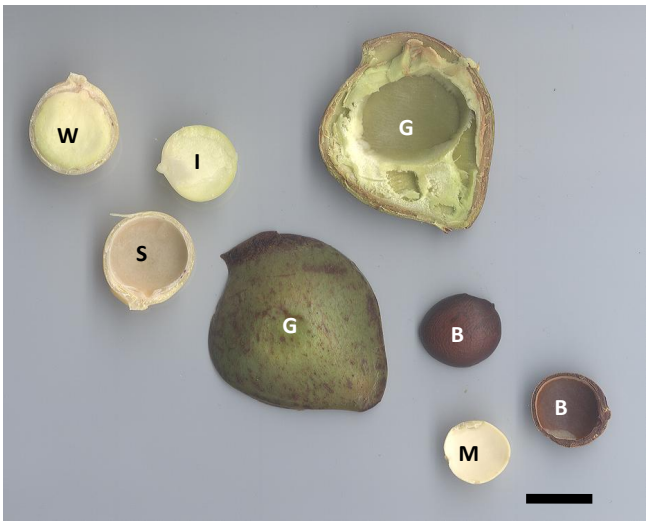


Figure 6: Picture of opened pod of immature marama bean seeds (G), immature cotyledon with seed coat (W); immature bean seed coat (S), immature bean cotyledon (I), mature bean seed coat (B), mature bean cotyledon (M). Bar = 20 mm (Paper I).

2.4 Traditional Use of the Marama Bean

Immature marama bean seeds, inclusive of the seed coat, are used as vegetables. Mature seeds are either boiled or roasted (Van der Maesen, 2006). They are normally eaten as a nutty snack after roasting in hot sand. The roasted mature seeds can be used to make butter like peanut butter (Van der Maesen, 2006; Mitei *et al*., 2009) and the flour can be added to cereals to increase their nutritive value (Maruatona *et al*., 2010). The prepared flour can also be used to make coffee or cocoa-like beverages. Oil can be pressed from mature seeds, used for food purposes in Botswana, Namibia and South Africa (Ketshajwang *et al*., 1998; Mitei *et al*., 2008). The National Food Technology Research Centre in Botswana has been engaged in marama bean product development and has made products such as marama milk and marama yoghurt, which could possibly be alternatives to already commercialised soya bean milk and yoghurt. The raw, dry mature seeds store well and remain edible for years under dry storage conditions (Van der Maesen, 2006).

2.5 The Chemical Composition of the Marama Bean

2.5.1 Moisture

The moisture content (Table 1) for immature seeds is approximately 67% (Mosele *et al*., 2011a) and in the range of 1.3 – 6.6% in mature seeds (Bower *et al*., 1988; Holse *et al*., 2010; Mosele *et al*. 2011a).

Table 1: Proximate composition (%) of immature and mature marama bean cotyledon (as is) (Paper I).

Sample	Moisture	Ash	Protein	Lipid	Carbohydrate[a]
Immature	66.8 (0.4)	2.2 (0.1)	20.8 (0.4)	1.5 (0.1)	8.6
Mature	5.3 (0.2)	3.0 (0.0)	32.3 (0.8)	40.0 (0.7)	19.4

Values in parentheses indicate standard deviations.

[a] Carbohydrate by difference.

2.5.2 Proteins

Marama bean has a significant amount of proteins at 21% (Table 1) in immature seeds (Mosele *et al*. 2011a) and 29 – 40% in mature seeds (Bower *et al*., 1988; Amarteifio and Moholo, 1998; Van der Maesen, 2006; Holse *et al*., 2010; Mosele *et al*. 2011a). The protein content equals that of soya beans (*Glycine max*) (Vaidehi and Kadam, 1989; Nkama and Filli, 2006) and is higher than that of peanuts (*Arachis hypogaea*) (Adsule *et al*., 1989; Nkama and Filli, 2006). Legumes' proteins are of great importance because they are complementary to those in cereals, and in their role especially in disease prevention (Guillon and Champ, 2002; Chibbar *et al*., 2010). The amino acids present are glutamic acid, being the highest at approximately 15% followed by arginine at approximately 11% of marama bean protein in defatted flour (Bower *et al*., 1988). Maruatona *et al*. (2010) also reported glutamic acid at highest amino acid.

2.5.3 Lipids

Mature marama bean seeds store large quantities of lipid (Table 1) at 40% (Bower *et al*., 1988; Amarteifio & Moholo, 1998; Holse *et al*., 2010; Mosele *et al*., 2011a). The lipid amount is almost twice that of soya beans (Vaidehi and Kadam, 1989; Nkama and Filli, 2006) and similar to that of peanuts (Adsule *et al*., 1989; Nkama and Filli, 2006). The oil yield is similar to that of peanut and it is fairly unsaturated (Ketshajwang *et al*., 1998). The highest fatty acid is oleic acid at 46 - 49%, followed by linoleic acid at 23% (Ketshajwang *et al*., 1998; Van der Maesen, 2006; Mitei *et al*., 2008).

2.5.4 Carbohydrates

The total carbohydrate content in mature marama bean (Table 1) is in the range of 18.9 - 24.1% (Bower *et al*., 1988; Amarteifio and Moholo, 1998; Mosele *et al*., 2011a). Surprisingly, the starch content is negligible in comparison to that in most legumes, at approximately 0.2% (Mosele *et al*., 2011b). Total dietary fibre constitutes up to 27% of the dry matter in mature seeds, (Holse *et al*., 2010), comprising the major part of carbohydrates in marama bean. Cellulose and pectin were observed by Mosele *et al.* (2011a, b) through fluorescence microscopy and CoMPP analysis.

2.5.5 Minerals

The ash content for both immature and mature seeds is approximately 3% as seen in Table 1 (Bower *et al*., 1988; Holse *et al*., 2010; Mosele *et al*., 2011), with potassium, phosphorus, magnesium, sulphur and calcium being the macroelements (Holse *et al*., 2010). The trace elements include zinc, manganese, iron and copper. The ash content is similar to that of cowpeas and is approaching that of soya beans (United States Department of Agriculture, 2011).

2.5.6 Vitamins

Vitamins reported for marama bean include vitamin A at 0.22 mg, essential for proper eyesight; folic acid (vitamin B_9) at 0.14 mg, vital during pregnancy; total vitamin B content at 11.50 mg, which was calculated by adding up the values of B_1, B_2, B_3, B_6, B_9 and B_{12} (Müseler and Schönfeldt, 2006). Vitamin B supports and increases metabolism, and maintains healthy skin, hair, muscle tone and nervous system. Vitamin E, regarded as an antioxidant, was at the level of 6.27 mg. The vitamin E content was dominated by γ-tocopherols in marama bean oil (Mitei *et al*., 2009) and marama bean seeds (Holse *et al*., 2010). Its level is similar to that found in lima beans and lower than the amount found in peas (Bramley *et al*., 2000).

2.5.7 Antinutritional Factors

Marama bean contains trypsin inhibitor at an average of 20% of the total protein. However, its activity can be destroyed by dry heat eliminating 70%, and by boiling (moist heat) removing 80% (Bower *et al*., 1988). Therefore the proteins in marama bean are bioavailable for use in different body functions.

2.5.8 Allergens and Cyanogenic Glycosides

Marama bean was found to be free from allergens found in lupine and peanut (Holse *et al*., 2010), making it a possible alternative snack for those allergic to peanut. There were also no cyanogenic glycosides found in marama bean (Holse *et al*., 2010).

2.5.9 Polyphenols

Marama bean ethanolic extracts from the seed coat and polyphenolic fractions from the cotyledon were found to have antibacterial and anticandidal effect, suggesting their possible use in antimicrobial drugs (Chingwaru *et al*., 2011).

2.6 The Functionality of the Marama Bean

Plant polysaccharides are important in the food industry because they can be used as thickeners, stabilisers, texturisers and gelling agents (Viñarta *et al*., 2006; Khurana and Kanawjia, 2007). Marama bean was found to contain cellulose and pectin, mostly in the form of homogalacturonan (Mosele *et al*., 2011b). Jideani *et al*. (2009) and Maruatona *et al*. (2010) studied the functional characteristics of marama bean flour and found that with roasting, the water and oil absorption capacities were increased beyond 100%, with or without defatting. Maruatona *et al*. (2010) also found that the protein solubility and emulsifying capacity were reduced with heating, and the foaming capacity was not affected.

3 LEGUME CARBOHYDRATES

Carbohydrates are building blocks for plants as well as sources of energy and storage material (Van Soest, 2004). They are made up of carbon, oxygen and hydrogen atoms. They can also be defined according to their cyclic form of a five-member (furanose) or six-member ring (pyranose) (IUPAC, 1996). Food carbohydrates play an important part in controlling functional properties in food systems such as texture, flavour and structure, based on their molecular weight (Lai and Lii, 2004). They are classified on the basis of the degree of polymerisation into mono-, di-, oligo-, or polysaccharides (Voragen, 1998; Quigley *et al*., 1999; Chibbar *et al*., 2010). They can also be classified on the basis of their digestibility, i.e. whether human digestive enzymes can hydrolyse them and be absorbed in the gastrointestinal tract (Khanna *et al*., 2006; Chibbar *et al*., 2010). The term "sugar" is normally used to describe mono-, di- and trisaccharides (Stick and Williams, 2009).

Apart from providing plant proteins, legume seeds are good sources of carbohydrates. The total carbohydrate content in legumes ranges from 22 – 45% (Aguilera *et al*., 2009b). Reddy *et al*. (1984) and Chibbar *et al*. (2010) reported the carbohydrate content in the range of 24 - 68%. Belitz *et al*. (2009) reported that the major carbohydrate was starch amounting to 75 – 80%. The total carbohydrate content (by difference) in mature marama bean has been reported before (Bower *et al*., 1988; Amarteifio and Moholo, 1998; Mosele *et al*., 2011a), but this fraction has not been studied in detail.

3.1 The Monosaccharides

Monosaccharides are carbohydrate molecules that cannot be broken down to simpler molecules, with a molecular formula of $C_n(H_2O)_n$ (BeMiller and Whistler, 1996; Coultate, 2002; Jouppila, 2006). Therefore a monosaccharide denotes a single sugar unit without glycosidic connection to other such units (IUPAC & IUBMB, 1996), e.g. glucose and arabinose. They have are made up of 3 to 8 carbon atoms, dominated by those with 5 or 6 carbon atoms (Coultate, 2002). The most common monosaccharides are hexoses, such as D-glucose and D-fructose; and pentoses, such as D-arabinose (Jouppila, 2006). The most abundant monosaccharide in nature is D-glucose (Damager *et al.*, 2010). It is found primarily found as a pyranose ring, called D-

glucopyranose in two anomeric forms of α-D-glucopyranose and β-D-glucopyranose (Damager *et al.*, 2010). D-glucose is the sole building block of such diverse polysaccharides such as amylose, amylopectin and cellulose (see earlier). In marama bean, the monosaccharides are glucose and fructose in immature seeds, and mainly arabinose in mature seeds (Mosele *et al*., 2011b). Other monosaccharides involved in the building of legume polysaccharides are α-D-galactose in galactans, α-D-arabinose in arabinans, α-D-mannose in glucomannans, α-L-rhamnose and α-D-galacturonic acid in pectins, β-D-mannuronic acid and α-L-guluronic acid in alginates.

3.2 The Glycosidic Linkage

Glycosidic linkage, the covalent bond between sugars, is of great importance in the structure of carbohydrates (Nikonenko *et al*., 2005). It is formed from a glycosyl donor and a glycosyl acceptor to form a glycosidic linkage of either α- or β- configuration (Stick and Williams, 2009). The glycosyl donor is normally a precursor of a molecule that has a significant positive charge at the anomeric carbon atom. It can be of either α or β configuration linked to the desired hydroxyl group of the glycosyl acceptor. For example, in starch, there are two different kinds of linkages, namely, a glucose moiety linked via its 1-position to either the 4- or 6-position of another glucose moiety, creating an α-(1→4) or α-(1→6) linkage (Damager *et al*., 2010). The glycosidic linkage is of particular importance in bigger molecules such as oligosaccharides and polysaccharides because its conformation defines the relative orientation of neighbouring sugar rings and hence the three dimensional (3D) structure of the molecules (Ravindranathan *et al*., 2001).

3.3 The Disaccharides

A disaccharide consists of two monosaccharide units bound by a glycosidic linkage formed by the loss of water, i.e. the loss of a hydrogen atom from one monosaccharide and a hydroxyl group from the other (Jouppila, 2006), e.g. sucrose and maltose. When a glycosidic linkage is established only between the lactol groups of two monosaccharides, then a nonreducing disaccharide is formed, and when one lactol group and one alcoholic HO group are involved, a reducing disaccharide results (Belitz *et al*., 2009). In terms of maltose a reducing sugar, two

glucose units are joined by an α-(1→4) bond, and is thus written O-α-D-Glcp(1→4)D-Glcp (Belitz *et al*., 2009).

Table 2 shows some of the low molecular weight carbohydrates (monosaccharides and disaccharides) or simple sugars found in selected legumes. Sucrose is the most abundant disaccharide in legumes ranging between 1 – 5% (Bravo *et al*., 1999; Martín-Cabrejas *et al*., 2008). It was also reported as the main soluble sugar in soya bean (Hou *et al*., 2009). The main disaccharides in marama bean also include sucrose and maltose (Mosele *et al*., 2011b).

Table 2: Soluble sugars of different legumes in % dry matter (Martín-Cabrejas *et al*. (2008).

Legume	Latin name	Arabinose	Glucose	Fructose	Ribose	Sucrose	Maltose	Raffinose
Cowpea	*Vigna unguiculata*	0.02 (0.1)	nd	0.01 (0.1)	0.02 (0.1)	1.39 (0.8)	0.06 (0.1)	0.40 (0.3)
Jack bean	*Canavalia ensiformis*	nd	0.16 (0.2)	nd	nd	0.94 (0.7)	0.12 (0.6)	1.18 (0.9)
Soya bean	*Glycine max*	nd	0.20 (0.6)	nd	nd	2.21 (1.8)	0.20 (0.3)	0.670.2)

nd = not detected

Values in parentheses indicate standard deviations.

Martín-Cabrejas *et al*. (2008) reported the total amount of soluble sugars in the range of 3.8 to 6.4% (dry matter) of five legumes studied, similar to the results observed by Abdel-Gawad (1993) at 5 - 6%. However, simple sugars were below 1% in marama bean (Mosele *et al*., 2011b). Mono- and disaccharides are added to food products to increase sweetness; to give colour and flavour, as a result of non enzymatic browning such as caramelisation reactions; and to increase storage stability by lowering the water activity of food products. Although their molecular weights are similar, their physicochemical and functional characteristics are quite different due partly to their various anomeric forms (Jouppila, 2006).

3.4 The Oligosaccharides

An oligosaccharide is a compound in which 2 – 10 monosaccharide units are joined by glycosidic bonds or linkages (Belitz *et al*., 2009). They derive their names from the number of monosaccharide units, and these include trisaccharides (e.g. raffinose), tetrasaccharides (e.g. stachyose), pentasaccharides (e.g. verbascose) and higher molecular weight oligosaccharides such as dextrin (IUPAC & IUBMB, 1996; Belitz *et al*., 2009). Oligosaccharides in legumes are present in higher concentration than in cereals. Predominant oligosaccharides are stachyose and verbascose (Belitz *et al*., 2009). These are the flatulence-causing, raffinose family oligosaccharides (RFOs) (Abdel-Gawad, 1993), at 2 – 5% of dry matter in legumes (Bravo *et al*., 1999), with soya bean having 3.5% (Martín-Cabrejas *et al*., 2008). RFOs consist of galactose units linked to sucrose via α-(1→6) glycosidic linkages (Peterbauer *et al*., 2001). The main oligosaccharide in marama bean is raffinose (Mosele *et al*., 2011b) and was also reported by Holse *et al*. (2011) among the water soluble carbohydrates in ^{1}H HR-MAS NMR.

Table 3 shows the amount of RFOs found in selected legumes, with lupin having an exceptionally higher amount. They form about 34 – 70% of the total soluble carbohydrates in legumes (Abdel-Gawad, 1993; Martín-Cabrejas *et al*., 2008).

Table 3: Total raffinose family oligosaccharides (FROs) of different legumes in % of dry matter (DM).

Legume	Latin name	RFOs	Authors
Soya bean	*Glycine max*	3.5[a]	Martín-Cabrejas *et al*., 2008
Faba bean	*Vicia faba*	3.6[b]	Dini *et al*., 1989
		3.8[b]	Abdel-Gawad, 1993
Lentil	*Lens culinaris*	2.7[b]	Abdel-Gawad, 1993
Common bean	*Phaseolus vulgaris*	2.5[b]	Abdel-Gawad, 1993
Cowpea	*Vigna sinensis*	4.1[b]	Abdel-Gawad, 1993
	Vigna Unguiculata	3.7[a]	Martín-Cabrejas *et al*., 2008
Jack bean	*Canavalia ensiformis*	2.9[a]	Martín-Cabrejas *et al*., 2008
Lupin	*Lupinus albus*	7.1[b]	Martínez-Villaluenga *et al*., 2005
	Lupinus luteus	11.0[b]	Martínez-Villaluenga *et al*., 2005
	Lupinus augustifolius	7.1[b]	Martínez-Villaluenga *et al*., 2005

[a]RFOs = raffinose family oligosaccharides (raffinose + stachyose + ciceritol)

[b]RFOs = raffinose family oligosaccharides (raffinose + stachyose + verbascose)

Oligosaccharides include prebiotic carbohydrates, which are defined as sugar-like compounds that are soluble in 80% ethanol and largely resist digestion by pancreatic and brush board enzymes (Cummings *et al*., 2004). They selectively stimulate the growth of either bifidobacteria or lactobacilli in the human colon.

3.5 The Polysaccharides

Polysaccharides are carbohydrates which are obtained from monosaccharide units greater than 20, where the monosaccharides units are also bound to each other by glycosidic linkages (BeMiller and Whistler, 1996; Belitz *et al*., 2009). The number of monosaccharide units is called “degree of polymerisation” (DP), with a few polysaccharides having a DP of less than 100 (BeMiller and Whistler, 1996). Well known polysaccharides include starch, cellulose and pectin. The monosaccharides may be joined in a linear pattern as in cellulose and amylose or in a branched fashion as in amylopectin. Amylose and amylopectin are the building blocks of starch (see below). When the polysaccharide is composed of the same sugar type, it is called a homoglycan; or heteroglycan if two or more of the monosaccharides are different. They are

often considerably less soluble in water than mono- and disaccharides. They are classified into storage polysaccharides such as starch; and structural polysaccharides such as cellulose (Chibbar *et al*., 2010). Polysaccharides constitute the major part of the cell wall classified into cellulose, hemicelluloses, and pectin, and these are represented in almost all cell walls in varying proportions (Harholt *et al*., 2010). Hemicelluloses and pectins constitute the matrix in which cellulose microfibrils are embedded. The interactions between the different polysaccharides ensure the strong, dynamic and flexible properties of the plant cell wall (Harholt *et al*., 2010).

The functional properties of polysaccharides are affected by their type of linkages. Perfectly linear polysaccharides (cellulose or amylose) are usually insoluble in water and can be solubilised only under drastic conditions, e. g. at high temperature, or by cleaving H-bonds with alkalis or other suitable reagents; while branched polysaccharides (amylopectin, pectin) are more soluble in water than their perfectly linear counterparts since the chain–chain interaction is less pronounced and there is a greater extent of solvation of the molecules (Belitz *et al*., 2009).

3.5.1 Starch

Starch consists of amylose and amylopectin (Chung and Liu, 2009). It is composed of a mixture of 20 - 30% amylose and 70 - 80% amylopectin (Scott *et al*., 1999; Chung and Liu, 2009). Table 4 shows the amylose content of different legumes as reported by Sandhu and Lim (2008). Amylose is made of long, linear α-D-(l→4) glucan chains with few (0.1%) α-D-(l→6) linkages (Hizukuri *et al*., 1981; Scott *et al*., 1999; Majzoobi *et al*., 2003; Belitz *et al.*, 2009). Amylopectin is also composed of linear α-D-(l→4) glucan chains but with more (4%) branched α- D-(l→6) linkages, giving it its branched structure (Hizukuri, 1986; Scott *et al*., 1999; Majzoobi *et al*., 2003). The molecular weight of amylose and amylopectin is negatively correlated to starch digestibility, while the molecular weight of amylopectin is negatively correlated to the amylose content (Sandhu and Lim, 2008). The main portion of starch granule's crystalline structure is apparently derived from amylopectin and it also depends on the water content of the plant material (Sandhu and Lim, 2008; Belitz *et al.*, 2009).

Table 4: Mean amylose content of starches from different legumes (Sandhu and Lim, 2008).

Legume types	Latin name	Amylose content (%)
Black gram	*Vigna mungo*	32.8 (0.5)
Chickpea	*Cicer arietinum*	30.4 (0.4)
Field pea	*Pisum sativum*	33.1 (0.5)
Lentil	*Lens culinaris*	31.6 (0.6)
Mung bean	*Vigna radiata*	31.6 (0.7)
Pigeon pea	*Cajanus cajan*	28.4 (0.5)

Values in parentheses indicate standard deviations.

Starch is the major source of carbohydrates or energy in the human diet (Reddy *et al*., 1984; Wang *et al*., 1998; Aguilera *et al*., 2009a) and varies in composition among legumes (Table 5). Soya bean has the lowest amount of starch, similar to that found in marama bean at 0.2% (Mosele *et al*., 2011b). Starch is found in granules. Legume starch granules, especially from beans, were found to be diverse in size (1 - 100µm) and their shape being greater in length than in width (Reddy *et al*., 1984; Coultate, 2002).

Table 5: Total starch of different legumes in % dry matter.

Legume	Latin name	Total starch	Authors
Chickpea	*Cicer arietinum*	31.8 (0.08)	Bravo *et al*., 1999
		51.9 (5.2)	Aguilera *et al*., 2009b
White bean	*Phaseolus vulgaris*	52.9 (1.9)	Aguilera *et al*., 2009b
Pink-mottled cream bean	*Phaseolus vulgaris*	51.8 (12.0)	Aguilera *et al*., 2009b
Soya bean	*Glycine max*	0.2 – 0.9	Reddy *et al*., 1984
		0.2 – 1.0	Saldivar *et al*., 2011

Values in parentheses indicate standard deviations.

3.5.2 Dietary Fibre

Dietary fibre is described as carbohydrate polymers with three or more monomeric units and the sum of indigestible carbohydrates, which includes non-starch polysaccharides (NSP) such as cellulose, hemicelluloses, gums and pectins found in plant food (Mehta and Kaur, 1992; BeMiller and Whistler, 1996; Khan *et al*., 2007; EC, 2008; Belitz *et al*., 2009). NSP also include xylans and mannans (Sinha *et al*., 2011). The EC (2008) further states that "the carbohydrate polymers of plant origin that meet the definition of fibre may be closely associated in the plant with lignin or other non-carbohydrate components such as phenolic compounds, waxes, saponins, phytates, cutin, phytosterols. These substances when closely associated with carbohydrate polymers of plant origin and extracted with the carbohydrate polymers for analysis of fibre may be considered as fibre. However, when separated from the carbohydrate polymers and added to a food these substances should not be considered as fibre". They contain long polymeric carbohydrate chains of several thousand monomer units, and make up to 90% of the cell wall in plants (Sinha *et al*., 2011).

Dietary fibre can be divided into soluble (pectin, galactomannan and some hemicellulose) and insoluble dietary fibre (cellulose and some hemicellulose). It has a number of health benefits such as prevention of cardiovascular diseases, decrease in blood cholesterol and glucose level, prevention of digestive system carcinogenic diseases and constipation (Vasić *et al*., 2009). The total dietary fibre in marama bean is 19 – 27% (Holse *et al*., 2010). It is a major part of carbohydrate in marama bean. Generally, legumes have a high amount of insoluble dietary fibre (Table 6), which was also observed in fourteen samples of marama bean analysed by Holse *et al*. (2010) where 96% of the total dietary fibre was insoluble.

Table 6: Total dietary fibre of different legumes in % of dry matter.

Legume	Latin name	SDF	IDF	TDF	Authors
Pea	*Pisum sativum*	2.4 (0.8)	22.8 (1.3)	25.2	De Almeida Costa *et al*., 2006
Common bean	*Phaseolus vulgaris*	2.6 (0.6)	22.6 (0.1)	25.2	De Almeida Costa *et al*., 2006
Lentil	*Lens culinaris*	1.4 (0.5)	21.4 (2.1)	22.8	De Almeida Costa *et al*., 2006
Cowpea	*Vigna unguiculata*	0.9 (2.0)	30.3 (16.7)	31.2	Martín-Cabrejas *et al*., 2008
Jack bean	*Canavalia ensiformis*	1.5 (3.2)	31.7 (4.4)	33.3	Martín-Cabrejas *et al*., 2008
Chickpea	*Cicer arietinum*	2.1 (0.1)	24.0 (0.9)	26.0	Bravo *et al*., 1999
		1.0 (0.1)	20.5 (2.7)	21.4	Aguilera *et al*., 2009b
White bean	*Phaseolus vulgaris*	5.8 (1.2)	21.1 (2.8)	27.0	Aguilera *et al*., 2009b
Pink-mottled cream bean	*Phaseolus vulgaris*	5.4 (0.9)	16.4 (2.4)	21.8	Aguilera *et al*., 2009b
Soya bean	*Glycine max*	2.7 (0.8)	52.1 (5.3)	54.8	Martín-Cabrejas *et al*., 2008
Marama bean	*Tylosema esculentum*	0.9 (0.4)	22.7 (2.2)	23.6	Holse *et al*., 2010

SDF = Soluble dietary fibre

IDF = Insoluble dietary fibre

TDF = Total dietary fibre

Values in parentheses indicate standard deviations.

3.5.2.1 Cellulose

Cellulose makes up the principal structural material in plant cell walls where it usually occurs together with hemicellulose, pectin and lignin (a non-carbohydrate) (Mehta and Kaur, 1992; BeMiller and Whistler, 1996; Belitz *et al*., 2009). It constitutes part of the indigestible carbohydrate of plant food and consists of β-(1→4) linear glucopyranosyl residues. Cellulose has no coiling or branching, and the molecule adopts an extended and rather stiff rod-like conformation, aided by the equatorial conformation of the glucose residues (Sinha *et al*., 2011). The multiple hydroxyl groups on the glucose residues from one chain form hydrogen bonds with oxygen molecules on the same or on a neighbouring chain, holding the chains firmly together side-by-side. Cellulose has a variable degree of polymerisation (DP), which is the number of glucose residues per chain depending on its origin. Cellulose never occurs as a single

chain, but exists as a crystalline array of many parallel, oriented chains (microfibrils), which are its fundamental structural units (Brown *et al.*, 1996).

Because of its high molecular weight and crystalline structure, cellulose is insoluble in water and has poor swelling power or ability to absorb water (Belitz *et al*., 2009). The amount of cellulose was reported to be in the range of 2 – 13% in seven legumes, with guar bean (*Cyamopsis tertagonoloba*) having the highest amount at 12.5 % (Khan *et al*., 2007). Cellulose was semi-quantified in marama bean using the CoMPP technique, where the cellulose was extractable with 4 M NaOH and Cadoxen. The presence of cellulose was recognised by the specific antibody CBM3a for semi-crystalline cellulose.

3.5.2.2 Galactomannan

Galactomannans are water soluble storage polysaccharides isolated from the endosperm of leguminous seeds such as locust and guar bean (Dea &Morrison, 1975; Parvathy *et al*., 2007). They consist of a main chain (backbone) of (1→4) linked β-D-mannopyranosyl residues with a single unit of α-D-galactopyranosyl side-chain residue (Dea & Morrison, 1975; Tapie *et al*., 2008; Pollard *et al*., 2010). Locust bean gum (LBG) is a galactomannan from the endosperm of locust bean composed of α-(1→4) linked β-D-mannan backbone with 1,6-linked α-D-galactose side groups (Dea and Morrison, 1975). Guar galactomannan is from the seed of guar bean composed of a backbone of β-1,4-D mannopyranosyl residues attached alternatively with a single unit of α-1,6-D-galactopyranosyl residue (Dea & Morrison, 1975). The mannose to galactose (M/G) ratio depends on both the plant source and the extraction method used (Tapie *et al*., 2008). For example, guar gum presents M/G ratio of approximately 1.5 whereas locust bean gum exhibits M/G ratio of approximately 3.5 (BeMiller and Whistler, 1996; Tapie *et al*., 2008). Other differences in galactomannans include solubility in water and ability to form gels. Guar gum is more soluble than locust bean gum, and is a better emulsifier as it has more galactose branch points (Naresh and Shailaja, 2006). Locust bean gum requires heat to reach full hydration and maximum viscosity. Galactomannans are extensively applied in the food industry as thickening and stabilising agents (Bourbon *et al*., 2010). They also have influence on textural attributes and mouthfeel (Yassen *et al*., 2005). Marama bean has a high amount of

mannose and galactose in the cell wall fraction (Mosele *et al*., 2011b), whether these are linked to galactomannan content is still to be established.

3.5.2.3 Pectin

Pectin refers to water soluble galacturonoglycans of varying methyl ester contents capable of forming gels (BeMiller and Whistler, 1996). These are mainly homogalacturonan (HG), rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII), with apiogalacturonan and xylogalacturonan as minor constituents (Øbro *et al*., 2004; Harholt *et al*., 2006; Scheller *et al.,* 2007; Harholt *et al*., 2010). The pectin constituents do not exist as separate entities but are covalently linked to each other (Harholt *et al*., 2010). HG is the most abundant pectin polysaccharide (approximately 70%) and it is a linear homopolymer (homoglycan) of α-1,4-linked galacturonic acid (Mohnen, 2008). The degree of esterification and distribution of methyl groups on the HG backbone are important in determining the application of pectin (Vincken *et al*., 2003).

RGI is the second most abundant pectin polysaccharide at 20 – 35% (Mohnen, 2008), a complex branched polymer with a backbone of disaccharide repeats (α-1,4-D-GalA-α-1,2-L-Rha), and the only type of pectin not built upon pure galacturonan backbones (Harholt *et al*., 2010). The rhamnose residues in its backbone can be constituted by β-1,4-galactan, branched arabinan and/or arabinogalactan side chains (Harholt *et al*., 2010). About ≤ 20% of the sugars in pectin could be neutral sugars such as rhamnose, glucose, galactose, arabinose and xylose (Coultate, 2002).

RGII is made up of a short stretch of homogalacturonic acid substituted with four different side chains. The residues in RGII include monosaccharides that are rarely found in other polysaccharides such as: D-apiose, 3-C-carboxy 5-deoxy-L-xylose (L-aceric acid), 2-O-methyl L-fucose, 2-O-methyl D-xylose, L-galactose, 3-deoxy-D-lyxo-2-heptulosaric acid (Dha) and 2-keto-3-deoxy-D-manno-octulosonic acid (Kdo) (Scheller *et al*., 2007). RGII consists of at least 12 different monosaccharides in more than 20 different linkages.

Pectin is abundant in cell walls that surround growing and dividing cells, in the middle lamella and tricellular junctions (Mohnen, 2008; Caffall *et al*., 2009; Mosele *et al*., 2011a), see Figure 7. The amount of pectin in legumes is 1.5 – 3%, with guar (cluster) bean having one of the highest amounts (Khan *et al*., 2007; Vasić *et al*., 2009). Marama bean was found to contain pectin estimated at 4.2% of mature bean flour, mostly in the form of homogalacturonan (Mosele *et al*., 2011b) and a major part of the pectin had α-linkages (Holse *et al*., 2011). However, the amount of pectin is underestimated as some of the sugars were lost during trifluoro acetic acid (TFA) hydrolysis. The main cell wall component in marama bean was arabinan, found in many cell walls and is generally considered to be part of the pectic network due to its association with RGI, where, along with arabinogalactan, form extensive side chains (Willats *et al*., 2001). However there are a number of instances in which arabinans appear to exist as independent polymers (Gomez *et al*., 2009).

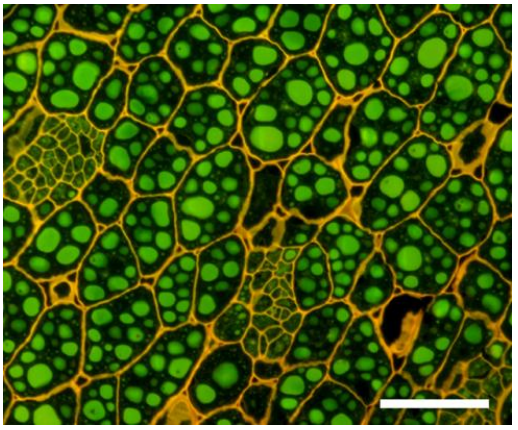


Figure 7: Coriphosphine O staining of mature marama bean showing yellow fluorescence from pectin in middle lamella and tricellular junctions. Bar = 100 µm (Paper I).

4 EXPERIMENTAL METHODS AND RESULTS

Identification and quantification of constituents in plant food materials is necessary for the development of new food products and industrial applications. In the present study, a detailed characterisation of the carbohydrates in marama bean was performed through different analytical techniques such as microscopy, chromatography, spectroscopy and hyperspectral imaging. Samples were prepared with and without extraction of lipids and proteins to enhance signals from carbohydrates especially in spectroscopic analyses. There are generally no standard methods for extraction of carbohydrates because of the large diversity of bio-macromolecular structures across the plant kingdom. Some of the preparations are important, for instance, the removal of water by freeze-drying to limit enzyme activity, because enzyme activity can cause degradation of the carbohydrates during storage. High drying temperatures (> 40° C) are avoided to prevent hydrolytic degradation and Maillard reactions (Andersson *et al*., 2006). For marama bean analyses, lipids were removed because they block the chromatographic columns, while the proteins were bound to the carbohydrates, therefore these were removed before chromatographic and spectroscopic analyses. The objective of this chapter is to present the principles of the methods of analyses used in this study as well as the primary results thereof.

4.1 The Chemical Compartmentalisation of the Marama Bean

The resolving power of the human eye is approximately 1 arc minute, which precludes direct observation of objects smaller than 73 μm at a typical viewing distance of 25 cm. With this kind of limitation, microscopic and imaging techniques are used to view objects with much smaller dimensions such as plant cell components. The techniques used depend on the type of radiation and the lenses used.

The distribution of various food components in plant material is best understood by histochemistry enabling the analysis of structural variations and changes during growth. Legumes store lipids, proteins and various types of polysaccharides as major reserves in their seeds. Although the basic microstructure of mature legume seeds is well-known, especially from commercial crops such as soybean (Webster and Leopold 1977) and peanut (Lott and

Buttrose 1977), less is known about the early deposition of seed reserves. With mature marama bean seeds, protein bodies of *Tylosema esculentum* and *Tylosema fassoglense* were studied by Amonsou *et al*. (2011). Even though their study did not present the complete microstructure of the seeds it is however complementary to this project. The current study then presents the first thorough elucidation of the marama seed microstructure. The methods and results of all the microscopy techniques are discussed in **Paper I**.

4.1.1 Transmission Electron Microscopy (TEM)

TEM is a technique that produces atomic-resolution images and generates signals which give information about the specimen chemistry and crystallography (Williams and Carter, 2009). In TEM, a thin specimen typically of the order of 5–100 nm is irradiated with an electron beam of uniform current density and the scattered electrons are collected and focused by the objective lens onto the image plane (Engel and Colliex, 1993; Reimer and Kohl, 2008). Electrons interact strongly with atoms by elastic and inelastic scattering. The distribution of the electron intensity behind the specimen is imaged onto a fluorescent screen with a lens system composed of three to eight lenses. A three- or four-stage condenser lens system permits variation of the illumination aperture and the area of the specimen illuminated (Reimer & Kohl, 2008). Therefore a phase-contrast image is produced by the interference of elastically scattered electrons with the unscattered electrons (Engel and Colliex, 1993). The image can be recorded by direct exposure of a photographic emulsion or an image plate inside the vacuum, or digitally via a fluorescent screen coupled by a fiber-optic plate to a CCD camera. The specimen needs to be specially prepared by different techniques such as electropolishing and ion-beam etching (Reimer and Kohl, 2008), as well as classical preparation methods such as embedding and ultrathin sectioning. A major limitation of TEM is the requirement to have thinner specimens. The thinning or cutting processes used affect the specimen's structures, therefore one has to be able to recognise the artifacts introduced by standard preparation methods (Williams & Carter, 2009). The samples in this study were stained and embedded in Spurr resin (a thermosetting polymer) before cutting into thin slices by an ultramicrotome, an instrument which allows the slicing of ultrathin sections.

Results (Paper I): The TEM results reveal an early protein deposition in the immature marama bean seeds (Figure 8). The amorphous protein bodies show cytosolic inclusions and seem to fuse with each other. At this developmental stage the protein bodies were closely associated with long strands of rough endoplasmic reticulum (ER) and sometimes small protein deposits were encircled by the ER (Figure 8). After biosynthesis at the ER, seed intracellular storage proteins bud off as separate organelles (protein bodies), which either accumulate in the cytoplasm or are sequestered into vacuoles (protein storage vacuole) by autophagy (Herman & Larkin 1999). In consistency with the chemical data, the lipid bodies (at approximately 2%) are not present at the immature stage. They are abundant at the mature stage of the seeds, numerous and electron-transparent encircling the protein bodies and lining the cytoplasmic side of the plasma membrane (Figure 9). The lipid bodies do not fuse and appear to be bound by a thin membrane, presumably a half unit membrane. We hypothesize that the marama bean protein bodies are of the cytosolic type forming large aggregates and not the protein storage vacuole type, which we would expect to be smooth-surfaced vesicles.

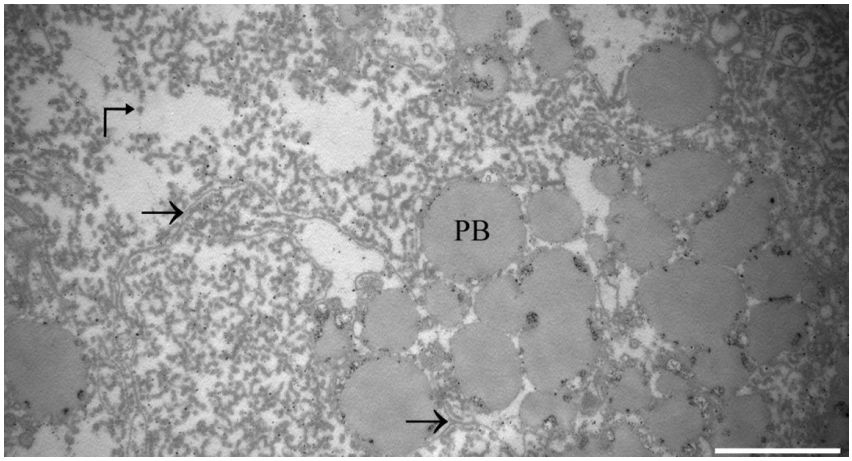


Figure 8: Transmission electron microscope (TEM) image of immature marama bean seed showing the amorphous appearance of spherical protein bodies (PB) and their aggregation. Their close association with ER (straight arrows) and ribosomes (bent arrow) can be seen. Bar = 10 µm (Paper I).

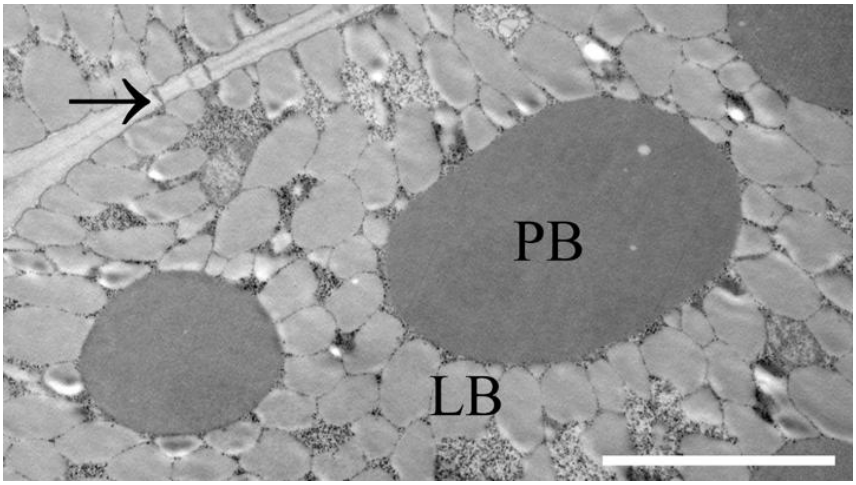


Figure 9: Transmission electron microscope (TEM) image of mature marama bean seed in the subepidermal cells showing protein bodies (PB) encircles by lipid bodies (LB) and lining the cytoplasmic side of the plasma membrane. Plasmodesmata through cell walls can be seen (arrow). Bar = 5 µm (Paper I).

4.1.2 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) allows for viewing of surfaces at cellular and sub-cellular levels, enabling visualisation of wide areas and irregular shaped specimens (Hoppert, 2003). The electron beam is produced by an electron gun and is then condensed by the first condenser lens, then focused by a second condenser lens into a thin coherent beam (Hoppert, 2003). A set of electromagnetic coils then scan the beam in a grid-like fashion. One set of coils move the beam in the *X* direction, and the other in the *Y* direction. The objective lens then focuses the scanning beam onto the specimen surface. This produces a number of signals, e.g. secondary electrons, back scattered electrons, etc., all of which may be collected using suitable detectors (Hoppert, 2003). The specimen needs to be stable after exposure and stable under vacuum because SEM instruments generally operate in a high vacuum and a very dry environment in order to produce the high energy beam of electrons needed for imaging and analysis (Echlin, 2009). Because of this, some biological materials have to be fixed, dehydrated and covered with a conductive layer, such as gold coating (Hoppert, 2003). After the fixation procedure, the specimens in this study were covered with a layer of gold/palladium to increase conductivity.

The difference between TEM and SEM is that in a TEM electrons are projected through a very thin slice of specimen to produce a 2D image on a phosphorescent screen and the brightness of a particular area of the image is proportional to the number of electrons that are transmitted through the specimen; while an SEM uses 2 to 3 nm spot of electrons that scans the surface of the specimen that are then detected by a sensor to produce a 3D image (Bozzola and Russell, 1999).

Results (Paper I): In the developing storage tissue the SEM results reveal a number of tiny globules, presumably protein bodies (Figure 10). In the mature seed, similar to the TEM results the protein bodies are decorated with lipid bodies (Figure 11). Cell wall material is also seen (Figure 9).

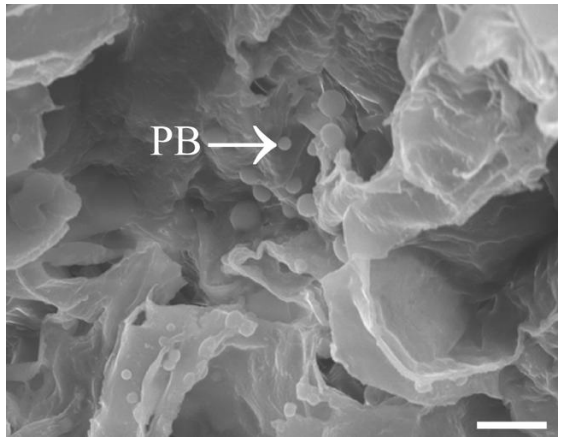


Figure 10: Scanning electron microscope (SEM) image of immature marama bean seed showing small spherical protein bodies (PB) (arrow). Bar = 10 µm (Paper I).

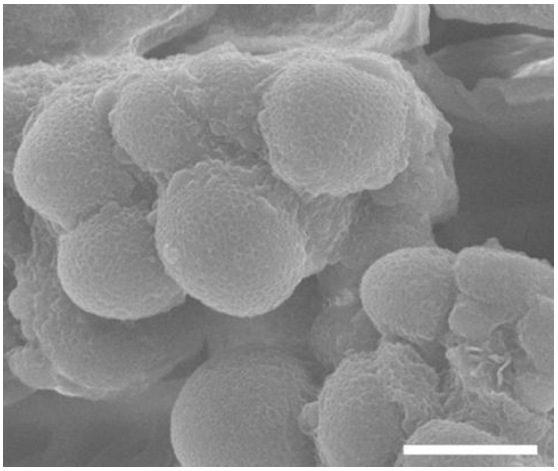


Figure 11: Scanning electron microscope (SEM) image of mature marama bean seed showing large proteins bodies covered with lipid bodies. Bar = 10 µm (Paper I).

4.1.3 Fluorescence Microscopy

Fluorescence is the property of atoms and molecules to emit light following excitation by an outside source of energy (Ghiran, 2011). Fluorescence microscopy can be successfully applied to locate components in a mixture when the component(s) of interest contain or have an affinity for a specific fluorophore (Palatini *et al*., 2002). A *fluorophore* is a molecule capable of fluorescence. Fluorescence is caused by conjugated, alternate double bonds that allow electrons to be displaced through the rigid and planar molecule (Ghiran, 2011). Therefore, the benzene ring (C_6H_6) is a very common group among fluorescent molecules. Certain organic molecules fluoresce whereas others do not. This can be referred to as autofluorescence. The fluorophores become observable when they absorb light, e.g. in chloroplasts and vacuoles. Fluorescent proteins can also be applied to living cells to investigate a wide range of intracellular processes in living organisms, as they have a wider response to a variety of biological events and signals (Claxton *et al*., 2007). For example, green fluorescent protein (GFP) isolated from jelly fish (*Aequorea victoria*), which has a high degree of fluorescence without the assistance of substrates or coenzymes. However, in some cases objects of interest are dyed with fluorescing stains that can be absorbed by the component of interest.

Some of the limitations in fluorescence microscopy include photobleaching, a process due to electrons transitioning from single to triplet state and interacting with other molecular species with triplet ground states such as oxygen, resulting in an irreversible change in the molecular structure of the fluorochrome and a permanent loss of fluorescence (Ghiran, 2011). Autofluorescence can also be problematic and is common in plant tissues where it arises from a variety of endogenous biomolecules e.g. chlorophyll and carotene, that absorb light in many regions of the near-ultraviolet and visible light spectrum interfering with the specific structure to be observed. It also arises from fixatives such as glutaraldehyde (Lichtman and Conchello, 2005). Another limitation is the degradation of the fluorophore, especially if slides are not stored properly.

Results (Paper I): In this study, fluorescence microscopy was very effective in the identification of proteins, lipids, pectin and cellulose. With SEM, it was necessary to assume that the spherical bodies present were proteins covered by lipid bodies, but this assumption was confirmed with light microscopy. Intracellular protein depositions as well as proteins within the cell walls were seen after staining with Aniline Blue Black under light microscopy (Figure 12). The pale blue substances within the parenchyma cell were also seen in the immature seeds indicating the early onset of protein deposition. The lipid bodies that surround the protein bodies were confirmed by Sudan Black staining (Figure 13) where the protein bodies are clearly unlabelled.

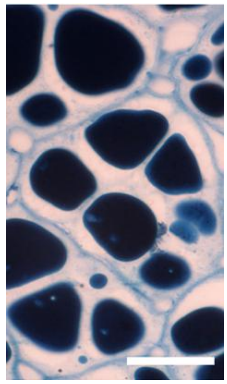


Figure 12: Aniline Blue Black staining of protein bodies and cell wall proteins without post fixation in mature marama bean seeds, and with lipids visible after post fixation (D). Bar = 25 µm (Paper I).

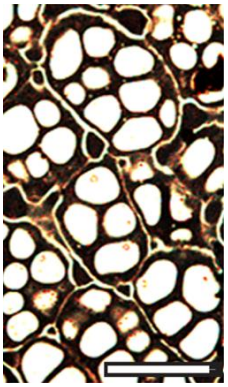


Figure 13: Sudan Black staining of lipid material surrounding unstained protein bodies after post fixation of mature marama bean seed. Bar = 25 µm (Paper I).

The absence of starch was also revealed under light microscopy (Figure 14). The iodine stain, an indicator for starch, showed the absence of a dark blue colour from starch structures in neither immature nor mature seeds. This was surprising as most legumes (with the exception of soya bean) contain considerable amounts of starch, as already discussed in the previous chapter.

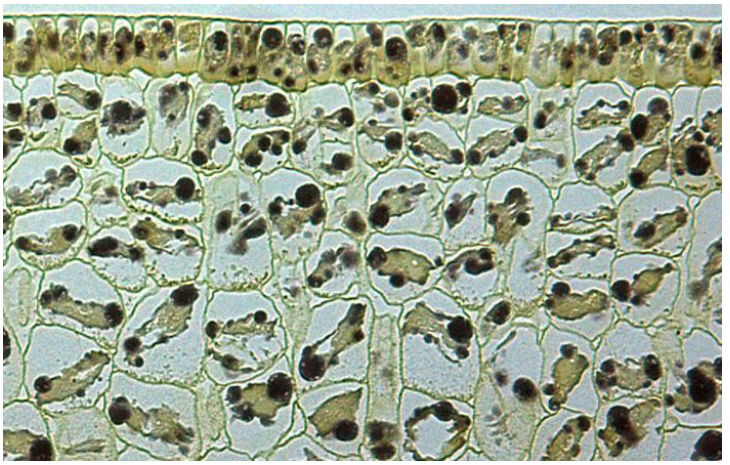


Figure 14: Immature marama bean cotyledon after iodine staining showing proteins (yellow brown areas) and lipophilic substances (black areas). There is no starch because of the absence of the dark blue colour. Bar = 420 µm. (Mosele, unpublished).

Both immature and mature seeds were positively stained for pectin and cellulose. Pectin was seen as yellow fluorescence from Coriphosphine O staining and cellulose was observed as a blue colour with Calcofluor White staining (Figure 3 and 6, respectively).

4.1.4 Confocal Laser Scanning Microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) has become an important tool in biology and medicine (Mauko *et al*., 2009), and in materials science (Claxton *et al*., 2007). CLSM is used for imaging thin optical sections in living and fixed specimen ranging in thickness up to 100 micrometers (Claxton *et al*., 2007). Coupled with photomultipliers that have high quantum efficiency in the near-ultraviolet, visible and near-infrared spectral regions, these microscopes are capable of examining fluorescence emission ranging from 400 – 750 nanometers, with or without the addition of a fluorophore. It has the ability to control depth of field, reducing or eliminating background information (that leads to image degradation) from the focal plane, and the capability to collect serial optical sections from thick specimens.

The basic principle of CLSM is the detection of excited light (from a laser excitation source) in the specimen, which is confocal with two pinhole apertures of the laser system (detector pinhole aperture) and detector (photomultiplier detector). Out-of-focus light is eliminated giving high quality images. Emitted light is projected by scanning mirrors through the lens and the objective into the specimen. Secondary fluorescence emitted from the specimen passes back to a beam splitter (dichromatic mirror) and is focused as a confocal point at the detector pinhole aperture. The same aperture discards rays that are out-of-focus. Refocusing of light shifts scanned points on the specimen to a new plane that becomes confocal. This forms contrasted images that are sequentially deeper optical slices of the sample. As a result, a 3D representation of specimen can be assembled (Claxton *et al*., 2007).

Results (Paper I): CLSM revealed that the lipids in marama bean are mostly neutral lipids that become positive with Nile Red staining (Figure 15). Neutral lipids include triaglycerols and cholesteryl esters (Fowler & Greenspan, 1985; Greenspan *et al*., 1985).

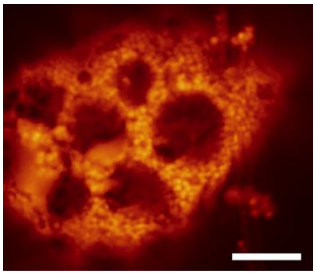


Figure 15: Confocal recording from top view of mature marama bean seed with Nile Red staining showed by the gold colour surrounding the dark spots. Bar = 10 µm (Paper I).

All the four microscopy techniques were able to elucidate the localisation and nutrient changes from the immature to the mature stage of seed development in marama bean from different complementary focal points. SEM showed the surface of the molecules in relation to one another, with a much higher resolution compared to fluorescence microscopy. However fluorescence microscopy revealed the chemistry of the molecules present in the specimen with the help of the different stains. It was also possible to observe that the molecules present such as pectin and cellulose increase with maturation (although not quantified), parallel with the general increase of cell wall thickness.

4.1.5 Near Infrared Chemical Imaging (NIR-CI)

Spectroscopy combined with conventional imaging i.e. hyperspectral imaging, is an emerging technique that measures spectral and spatial information of any sample within a short time and can be used to estimate a number of features/traits of a sample (Gowen *et al*., 2007). One such technique is NIR hyperspectral or chemical imaging (NIR-CI) which takes spectral images in the NIR region and collects the data in a three way data matrix called a hypercube (Williams *et al*., 2009). NIR vibrational spectroscopy is a technique based on the overlapping absorption of electromagnetic radiation, transmitted or reflected by the sample at wavelengths in the range of 780– 2500 nm resulting in spectra comprising of broad bands of overtones and combination tones of molecular vibrations involving C–H, O–H, S-H and N–H bonds (Huang *et al*., 2008; Labbé *et al*., 2008). The hyperspectral image is made up of many bands, where each spatial position and each pixel will contain the spectrum of that specific position, allowing biochemical constituents of a sample to be visualised in the different areas of the image (Gowen *et al*.,

2007). The 3D data matrix consists of the first two axes *x* and *y*, which are the vertical and horizontal pixels (spatial dimension), while the third, *λ*, is the spectral/wavelength dimension (Gowen *et al*., 2007; Williams *et al*., 2009) (Figure 16). Typical hypercubes have dimensions of 256 × 320 (pixels) × 118 (single channel images) (Williams *et al*., 2009). The translation of the spectral signature from each pixel into chemical concentrations will generate a set of chemical images, shown by the different colours (Figure 16), which show the distribution of each constituent within the sample matrix (Gendrin *et al*., 2008; Ravn *et al*., 2008). This allows visualisation of the internal structure and elucidation of the cluster size of each constituent in the sample.

In this study NIR-CI was used to investigate the gross pattern of the carbohydrates present in pure marama bean samples, i.e. mature marama bean cotyledon and flour, by selecting target compounds based on the carbohydrates identified in **Paper II**.

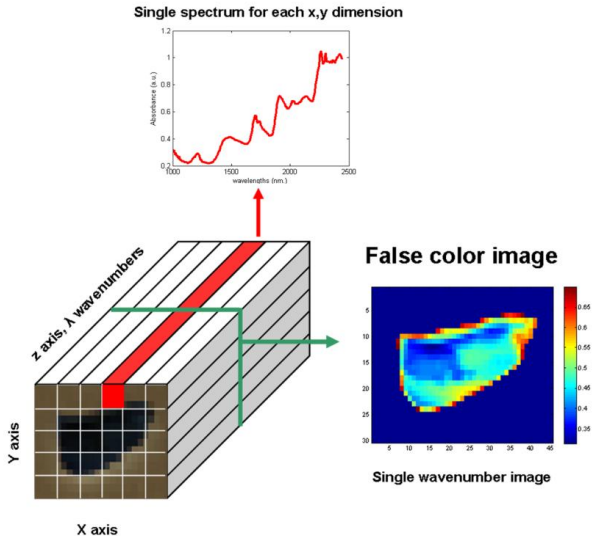


Figure 16: The structure of the final data cube dimensions (X × Y × λ) in near infrared chemical imaging (Mosele, unpublished).

With NIR-CI it was difficult to determine the specific carbohydrate composition in marama bean, even with the assistance of data from the different pure sugars and polymers used as references, probably because of poor spatial resolution. However the results show that the chemical components present are distributed evenly. They confirmed the presence of pectin, in

the form polygalacturonic acid, as well as cellulose. The results also showed that some of the arabinan present in marama bean is similar to that of the purified debranched arabinan standard used, indicating that the arabinan is not exclusively branched (**Paper II**).

4.2 The Composition and Structure of the Marama Bean Carbohydrates

4.2.1 The Extraction Process

Since plants are complex systems containing a multitude of different biomolecules, complete assignment or identification of the carbohydrates present especially in spectroscopy is difficult. To assist this process, extraction or separation of biopolymers may be necessary. In this study it was necessary to remove lipids and proteins for the adequate elucidation of carbohydrates. The following figure (Figure 17) shows the extraction process used before analysis of marama bean by FT-IR (Fourier transform infrared), FT-Raman (Fourier transform Raman) and NMR spectroscopy as well as analysis by HPAEC-PAD. However, the immature seeds had limited availability i.e. were not enough quantitatively, and therefore were only analysed by HPAEC-PAD. Defatting procedure 1 removes lipids from the seeds and was used before starch and simple sugars analyses. Procedure 2 also removes lipids, as well as soluble sugars and the remaining material is called alcohol insoluble residue (AIR). Deproteinising procedure 1 with $Ba(OH)_2$ hydrolyses the peptide bonds in proteins bringing the free amino acids into solution. In procedure 2 (NaOH) the amino acids are removed by precipitating them out of solution at their isoelectric point, while procedure 3 (phenol: acetic acid: water) unfolds the proteins and solubilises them. The effectiveness of the extraction procedures is discussed under the spectroscopy section.

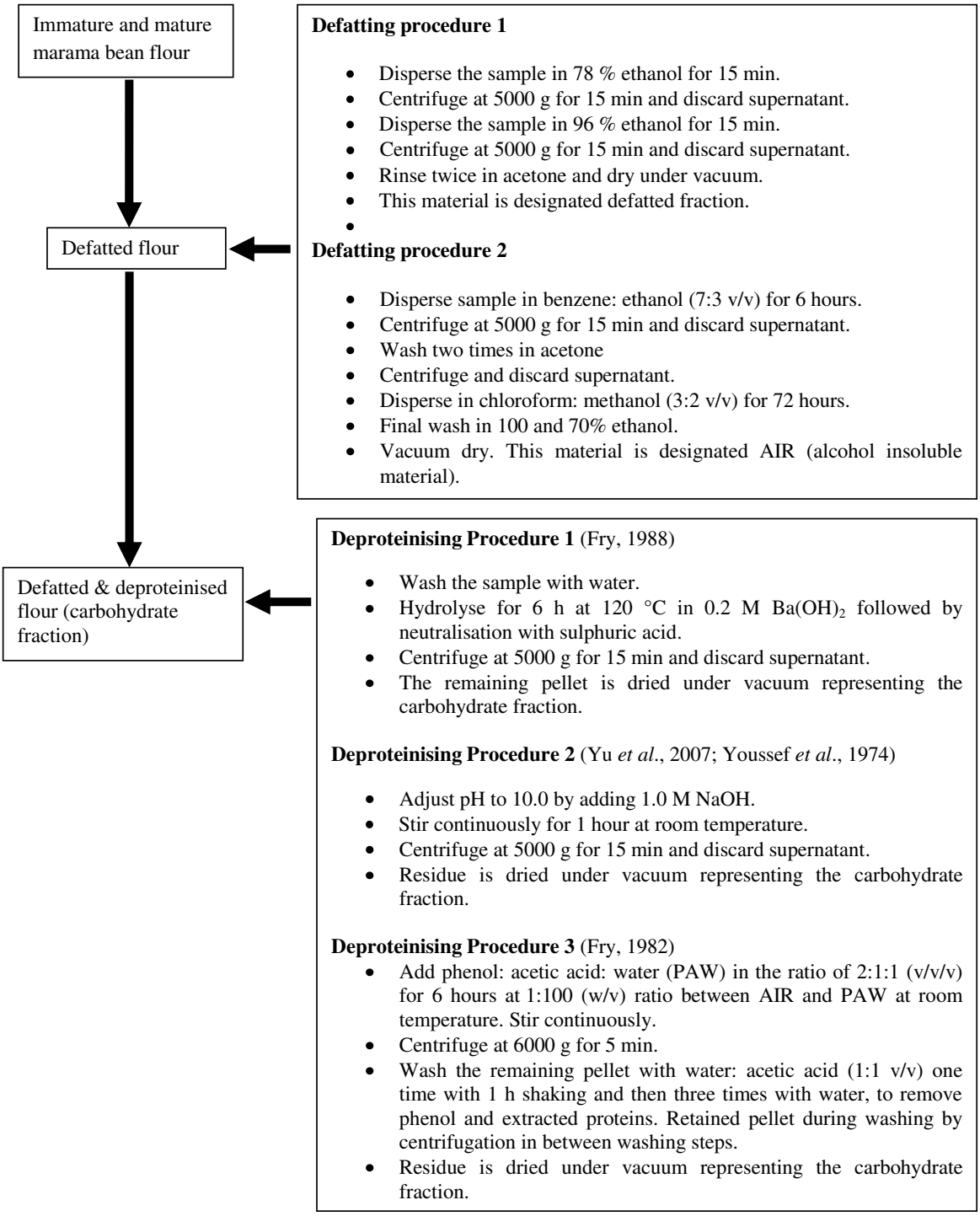


Figure 17: Defatting and deproteinising procedure for immature and mature marama bean flour for extracting the carbohydrates for chromatography and spectroscopy analyses.

4.2.2 High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

Chromatography refers to methods used for separating the components of a mixture by differential distribution between a stationary phase and a mobile phase, and used for purifying any soluble or volatile substance if the right adsorbent material, carrier fluid, and operating conditions are employed (Gambhir, 2008). Apart from being separation techniques, they are used to isolate individual components in a mixture and used for quantitative chemical analysis (Scott, 2003). The types of chromatographic techniques include liquid, gas and thin layer chromatography (Scott, 2003; Gambhir, 2008).

For this study, ion-exchange chromatography was used, a technique where ions of positive charges (cations) or negative charges (anions) in a liquid solution replace dissimilar and displaceable ions called counterions, having the same charge contained in a solid ion exchanger, which also contains immobile, insoluble and permanently bound co-ions of the opposite charge (Figure 18) (Henley *et al*., 2011). To be more specific, high-performance anion-exchange chromatography (HPAEC) was employed, which uses ion exchange resins as the stationary phase to separate monosaccharides (Scott, 2003). This method takes advantage of the weak acidic nature of carbohydrates to give highly selective separations at high pH. The technique coupled with pulsed amperometric detection (PAD) gives excellent signal-to-noise ratios without necessarily requiring derivatisation of the carbohydrates and is therefore highly specific and has high sensitivity to carbohydrates (Dionex; Folkes and Jordan, 2006). PAD detects only those compounds that contain functional groups that are oxidisable at the detection voltage employed. It also provides an easier method of analysis because nonsugar coextracts do not interfere with the separation. HPAEC-PAD has been successfully used for the analysis of neutral sugars and oligosaccharides in food (Corradini *et al*., 1997; Cordella *et al*., 2003). For this study the technique was also successfully used to quantify soluble sugars and starch from the defatted fraction, and sugars from AIR, i.e. cell wall material (Figure 17). The procedures of the analyses and the results are reported in **Paper II**.

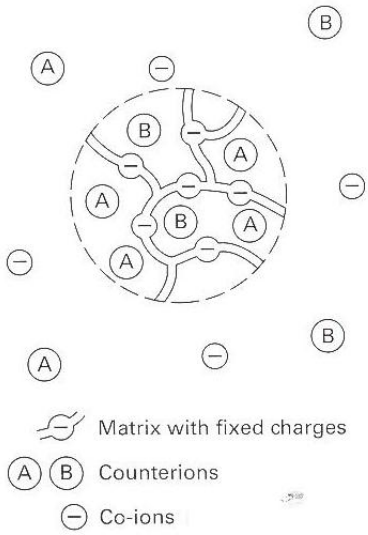


Figure 18: Adsorption operation in an ion exchange process (Henley *et al*., 2011, used with permission).

4.2.3 Methylation Analysis

Glycosyl linkages of AIR were also studied after methylation (per-O-methylation) by gas chromatograph-electron impact-mass spectrometer (GC-EI-MS). Permethylation involves converting hydroxyl groups to methyl ethers by methyl iodide in alkaline solution. These are then released by acid, reduced, acetylated and analysed. The linkages are deduced from the pattern of MS fragmentation (Andersson *et al*., 2006). The basic operating principle of GC involves volatilization of the sample in a heated inlet or injector of a gas chromatograph, followed by separation of the components of the mixture in a column. Only those compounds that can be vaporised without decomposition are suitable for GC analysis. Since carbohydrates are nonvolatile, they are derivatised before analysis to increase their volatility. A carrier gas, sometimes referred to as the mobile phase, usually hydrogen or helium, is used to transfer the sample from the injector through the column, and into a detector or mass spectrometer (Sparkman *et al*., 2011). A mass spectrometer can be used with or without prior separation by a chromatographic instrument such as the gas chromatograph to determine the size, quantity and chemical structure of inorganic and organic compounds via the determination of molecular

weight and the study of fragmentation patterns (Fay and Kussman, 2010). See **Paper II** for more details.

Results (Paper II): Quantification with HPAEC-PAD showed that the soluble sugars in immature marama bean seeds were dominated by glucose followed by myoinositol and fructose (Table 7); and dominated by sucrose in mature seeds, followed by raffinose and myoinositol (Mosele *et al*., 2011b). However the overall amount was less than 1 %. The amount of starch in both immature and mature seeds was also negligible (< 1%).

Table 7: Soluble sugar content of marama bean flour from immature and mature seeds cotyledons in dry matter (Paper II)

Soluble sugar	Immature (ng / mg)	Mature (ng / mg)
Myoinositol	11.5 (0.1)	8.9 (0.4)
Trehalose	0.6 (0.1)	n/d
Arabinose	2.7 (0.3)	4.5 (0.1)
Glucose	49.5 (7.4)	n/d
Fructose	10.1 (2.7)	n/d
Ribose	1.9 (0.2)	0.3 (0.3)
Sucrose	3.8 (0.8)	42.3 (0.7)
Raffinose	1.0 (0.4)	13.6 (1.9)
Maltose	0.8 (0.2)	5.3 (0.2)

n/d not detected

( ) values in parentheses indicate standard deviations

The sugars from AIR were dominated by a high amount of mannose in the immature seeds; and a high amount of arabinose in mature seeds, followed by mannose (Mosele *et al*., 2011b). The arabinose was highly recalcitrant as approximately 60% of the arabinose could only be extracted, even with harsh alkaline (6 M NaOH) extraction protocols before analysis with HPAEC-PAD. The mannose in both samples showed linkages characteristic for protein mannosylation indicating that the mannose in both samples originates from N-linked protein glycosylation also seen in other plants such as soya bean (Lis and Sharon, 1978). The linkage analysis also showed that the majority of the arabinose was linked via O-5, characteristic for

pectic arabinan and with attributes of a highly branched arabinan as indicated by the presence of 3,5- and 2,3,5-linked arabinose (Table 8). The occurrence of t-glucose and 4-glucose linkages possibly originate from cellulose.

Table 8: Linkage analysis of mature marama bean AIR after hydrolysis of proteins with $Ba(OH)_2$ (Paper II).

Sugar Linkage	% Area	std. Dev
t-arabinose	9.16	2.54
5-arabinose	10.13	2.05
2,3,5-arabinose	8.32	4.68
3,5-arabinose	5.18	3.54
sub-total	32.79	5.08
t-mannose	17.65	0.85
2-mannose	11.82	1.28
6-mannose	2.08	0.50
3,6-mannose	13.67	0.09
sub-total	45.22	2.55
t-galactose	3.02	0.86
4-galactose	3.34	0.03
2,4-galactose	1.81	0.22
sub-total	8.17	1.10
t-glucose	2.42	0.84
4-glucose	9.88	0.34
sub-total	12.30	1.18
t-fucose	<1	N/A
sub-total	<1	N/A
t-xylose	~1	N/A
sub-total	~1	N/A
2-Rhamnose	<1	N/A
2,4-Rhamnose	<1	N/A
sub-total	~1	N/A
TOTAL	100.00	

N/A not applicable

4.2.4 Comprehensive Microarray Polymer Profiling (CoMPP)

Comprehensive Microarray Polymer Profiling (CoMPP) is a method that has been used in the analysis of cell wall polysaccharides such as cellulose and pectin (Moller *et al*., 2007; Sørensen *et al*., 2008). It involves the sequential extraction of the major classes of cell wall polysaccharides with the generation of microarrays that are probed with monoclonal antibodies (mAbs) or carbohydrate-binding modules (CBMs) (Moller *et al*., 2007). The microarrays are ideally suited for the high throughput analysis of antibody specificities and are particularly useful for assigning the specificities to a large panel of mAbs with partially defined specificities, such as cell wall polysaccharides (Sørensen *et al*., 2009). Carbohydrate microarrays are challenging because of the chemical and structural diversity of polysaccharides, therefore quite laborious to elucidate the chemistry of all the structures present in a given sample. The detailed procedure and results are discussed in **Paper II**.

CoMPP analysis revealed that marama bean had cellulose and pectin, confirming the results observed in fluorescent microscopy and NIR-CI. The pectin observed was homogalacturonan (Figure 19), recognised by the specific mAb and extractable mostly with CDTA (1, 2-Diaminocyclohexane-N,N,N',N'-tetraacetic acid) (Mosele *et al*., 2011b). The pectin had a low degree of esterification. Xyloglucan a hemicellulose extractable with 4 M NaOH, was also observed at much higher values than cellulose, and together with the observed arabinan and galactan, indicate that marama bean has more hemicellulose than cellulose.

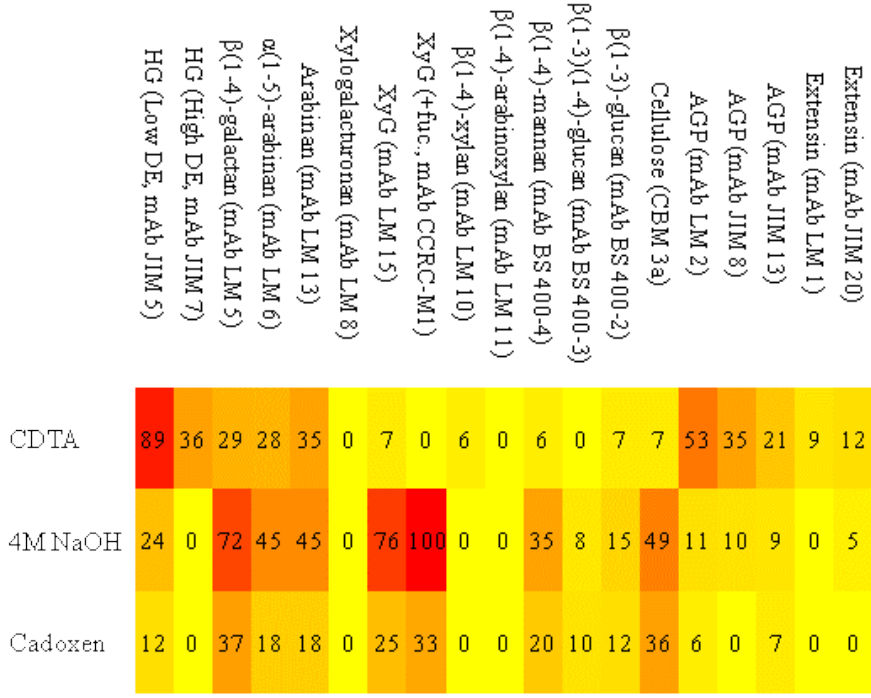


Figure 19: Relative quantification of alcohol insoluble residue from mature marama bean seeds as observed through CoMPP analysis displayed as a heat map. (HG = Homogalacturonan, Xyg = Xyloglucan, AGP = Arabinogalactan protein) (Paper II).

4.2.5 Spectroscopy Analyses

Spectroscopy is defined as the study of the interaction between radiation and matter as a function of wavelength (λ) or frequency and involves the interaction of electromagnetic radiation or some form of energy with molecules (Ghosh and Jayas, 2009). The molecules absorb the electromagnetic radiation and produce a spectrum either during the absorption process or as the excited molecules return to their ground-state. For this study, we used FT-IR, FT-Raman and NMR spectroscopy, and these techniques are described in the following sections. NMR provides information on the chemical structure, molecular mobility and ordering of molecules of solution- and solid-state samples (Van Soest, 2004). IR and Raman spectroscopy provide complementary information on molecular vibrations and structure of molecules (Thygesen *et al*., 2003; Van Soest, 2004). IR requires a change in the intrinsic dipole

moment of bonds as a function of time during the vibration and Raman a change in polarisability of functional groups (Li-Chan, 1996; Pavia *et al*., 2001; Van Soest, 2004). IR is more sensitive to functional groups (hydroxyls and carbonyls) while Raman is more sensitive to glycosidic bonds and pyranoid rings (Synytsya *et al*., 2003).

4.2.5.1 Fourier Transform (FT) –Infrared (IR) Spectroscopy

Infrared spectroscopy is based on molecular excitation to a higher energy state due to absorption of infrared radiation, which corresponds to the range encompassing the stretching and bending vibration frequencies of the bonds in most covalent molecules. Absorption occurs where the frequency of the incident radiation matches that of a vibrational mode so that the molecule is promoted to a vibrational excited state. The attenuation of this frequency of radiation from the beam after it passes through the sample is then detected (Smith and Dent, 2005). The radiation is referred to in wavenumbers, expressed as reciprocal centimetres (cm^{-1}) (Pavia *et al*., 2001). Infrared energy covering a range of frequencies passes through an interferometer (Figure 20) with a beam splitter, a fixed mirror, then onto a moving mirror and is directed onto the sample. The interferometer uses interference patterns to make accurate measurements of the wavelength of light. When the IR radiation is passed through a sample, some radiation is absorbed and the rest is transmitted to the detector and the detector measures the total interferogram from all the different IR wavelengths. A mathematical function called Fourier transform (FT) converts the interferogram (intensity versus time spectrum) to an IR spectrum (intensity versus frequency spectrum) (Davis and Mauer, 2010).

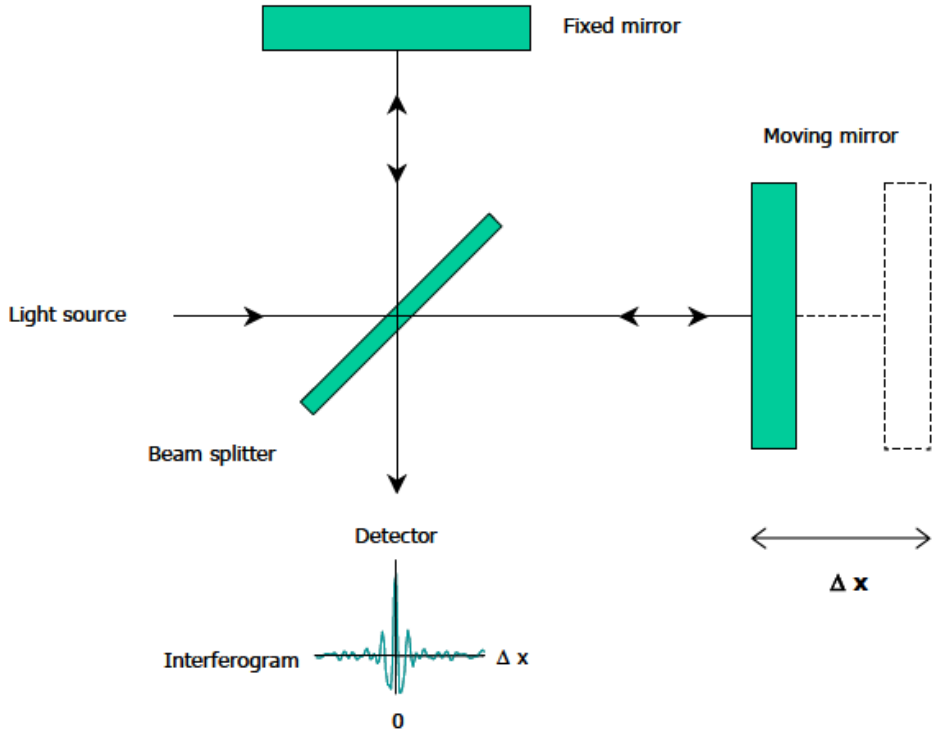


Figure 20: Diagram of an interferometer (Pedersen, 2002, used with permission).

As already mentioned, the information about the sample is obtained in the form of a spectrum where firstly, an interferogram (plot of intensity versus time) of the "background" (e.g. infrared-active atmospheric gases, carbondioxide and water vapour) is obtained before the sample can be analysed. The spectrum of the background is then automatically subtracted by the computer software from the rest of the results, giving information of the sample analysed (Pavia *et al*., 2001). FT-IR applications in food systems mostly use ATR (attenuated total reflectance) technology, which is less impeded by the high IR absorptivity of water commonly seen in conventional transmission IR spectroscopy (Dokken *et al*., 2005). The ease and rapid sampling has made it an attractive technique for liquids, solutions, viscous materials and flexible solids as well as studying plants and plant materials *in situ*. The use of different ATR crystals (e.g. ZnSe, Ge, or diamond) gives different internal reflection geometries.

Results (unpublished): In the IR spectra of the carbohydrate fractions ((NaOH, "PAW" and $Ba(OH)_2$)) a general reduction in lipids at approximately 2950 cm^{-1} and at approximately 1750 cm^{-1}(Figure 21 and 22) is observed (Dokken and Davis, 2007), in comparison to the raw fraction. The amide I and amide II peak at 1650 cm^{-1} and 1550 cm^{-1}, respectively are shown in Figure 21 (Dokken and Davis, 2007; Ahmed *et al*., 2010). This presence of proteins seen in the carbohydrate fractions suggests that the protein extraction processes were not effective, or that there is a close association of carbohydrates and proteins in marama bean, probably glycoproteins. This close association was also observed when marama bean was deproteinised with $Ba(OH)_2$, where approximately 50% of mannose and arabinose was removed with extensins (glycoproteins), measured by HPAEC-PAD. The carbohydrate fingerprint region (1200 – 700 cm^{-1}) in the carbohydrate fraction $Ba(OH)_2$ showed a broad signal between 1200 – 1000 cm^{-1} (Figure 23), indicating the presence of mixed polysaccharides with glycosidic linkages at 1100 cm^{-1} (Kačuráková *et al*., 2000), in particular pectin (Séné *et al*., 1994; Kačuráková *et al*., 2000; Coimbra *et al*., 2002).

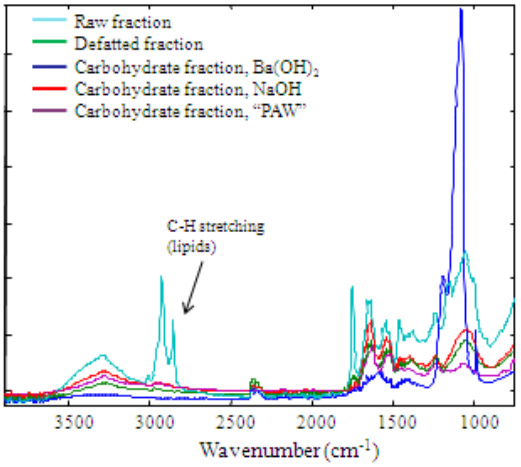


Figure 21: Infrared (IR) spectra (3500 – 1000 cm^{-1}) of raw, defatted and carbohydrate fractions ((NaOH, "PAW" and $Ba(OH)_2$)) of marama bean. Assignment is given in the figure.

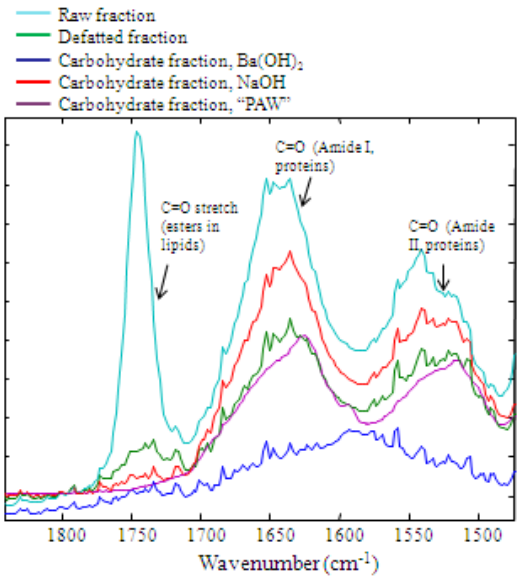


Figure 22: Infrared (IR) spectra (1800 – 1500 cm^{-1}) of raw, defatted and carbohydrate fractions ((NaOH, "PAW" and $Ba(OH)_2$))of marama bean. Assignments are given in the figure.

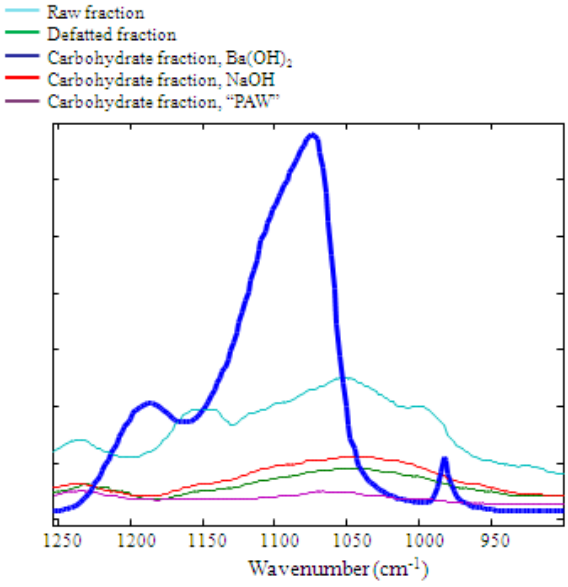


Figure 23: Infrared (IR) spectra (1250 – 950 cm^{-1}) of raw, defatted and carbohydrate fractions (NaOH, "PAW" and $Ba(OH)_2$) of marama bean, indicating pectic polysaccharides between 1200–1000 cm^{-1}, especially in the $Ba(OH)_2$ carbohydrate fraction.

4.2.5.2 Fourier Transform (FT) - Raman Spectroscopy

Raman spectroscopy is based on the shifts in the wavelength or frequency of an excited incident radiation beam that results from inelastic scattering on interaction between photons and the sample molecules (Li-Chan, 1996). It uses a single frequency of radiation to irradiate the sample and it is the radiation scattered from the molecule, one vibrational unit of energy different from the incident beam, which is detected (Smith and Dent, 2005). Continuous radiation, usually from an Nd:YAG (neodymium-doped yttrium aluminium garnet) laser is directed with mirrors onto the sample. The incident photons interact with the sample and the scattered light is collected by optical devices and directed to the interferometer. The modulated beam reaches the detector and the Raman spectrum is obtained by the application of Fourier transformation on the signal (interferogram) measured at the detector (Pappas *et al.*, 2000; Herrero, 2008). The advantage of FT-Raman is that it is not sensitive to water, which only gives a weak signal at 1640 cm^{-1} (H-O-H bending) and therefore ideal for analysing aqueous food products (Van Soest, 2004). However, it has some setbacks such as interference from fluorescence and photodecomposition of samples.

Results (unpublished): FT-Raman also revealed a reduction in the amount of lipids from C-H stretching in the fatty acid chains at approximately 2930 cm^{-1} (Figure 24) and at approximately 1745 cm^{-1} from C=O stretch in lipids, seen in the comparison between the raw and carbohydrate fraction from NaOH. The small shoulder just above 3000 cm^{-1} is also due to =C-H stretch from unsaturated fatty acids and is only found in the raw flour.

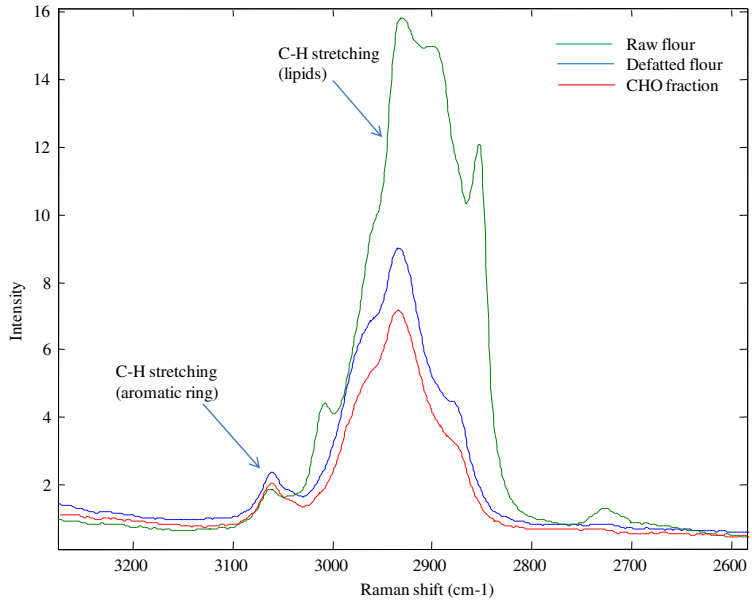


Figure 24: Raman spectra (3200 – 2600 cm^{-1}) of raw, defatted and carbohydrate fraction (NaOH extracted) of marama bean. Assignment is given in the figure Mosele, unpublished).

FT-Raman also revealed C-H stretching vibrations from aromatic compounds at approximately 3050 cm^{-1} seen in the NaOH carbohydrate fraction (Figure 24), which could also be from carbohydrates, revealing an overlap of proteins and carbohydrates, and implying that the deproteinisation procedure was not effective. However, the NaOH deproteinisation procedure has been successfully used to isolate proteins from soya bean and peanut. The amide I band at approx. 1650 cm^{-1} was also observed in all the samples, indicative of a high α-helical structure in proteins. Despite this, there was a prominent carbohydrate signal in the Raman spectra at the peak between 825 and 860 cm^{-1} (Figure 25, see curly brace) which shows a strong predominance of alpha-anomeric carbohydrates. The peak has previously been assigned to pectin at 855 cm^{-1} by Séné *et al*. (1994); Engelsen and Nørgaard (1996); Synytsya *et al*. (2003). The FT-Raman results again suggest a strong linkage of proteins and carbohydrates in marama

bean. Pectin in the form of homogalacturonan has recently been reported as one of the polysaccharides present in marama bean by CoMPP (Mosele *et al*., 2010b).

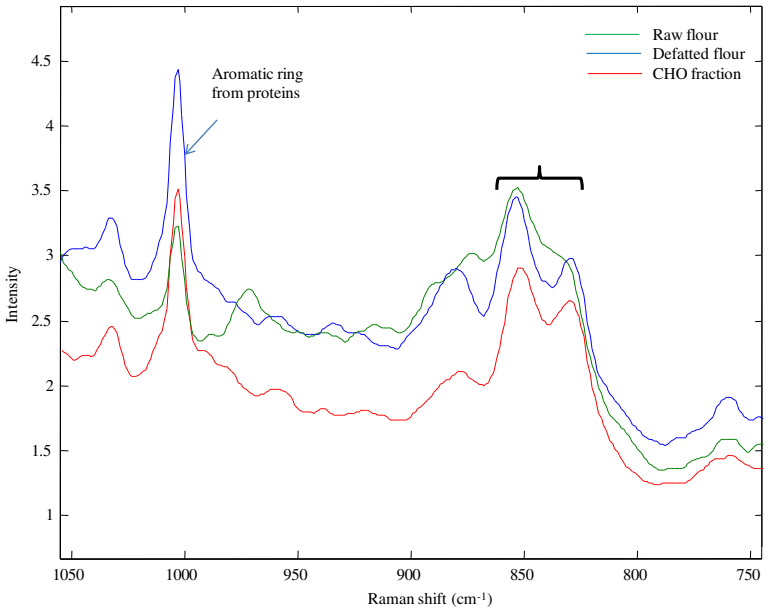


Figure 25: Raman spectra (1050 – 750 cm^{-1}) of raw, defatted and carbohydrate (CHO) fraction (NaOH extracted) of marama bean showing area for alpha-anomeric carbohydrates (curly brace) (Mosele, unpublished).

4.2.5.3 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy is another tool useful for the study of carbohydrates. The technique is based on nuclei having non-vanishing spin (e.g. ^{1}H and ^{13}C) and relies on the ability of nuclei to behave like small magnets and align with an external magnetic field. When the nucleus in a molecule is irradiated with a radio frequency signal it can change from being aligned with the magnetic field to being opposed to it. Its electrons also produce currents, which will produce an alternative magnetic field opposed to the external magnetic field. The total effective magnetic field that acts on the nuclei magnetic moment will therefore be reduced depending on the strength of the locally induced magnetic field (Poulsen, 2002). The protons in the nucleus of the

molecule do not experience the same magnetic field due to an effect called "shielding" or more commonly known as the chemical shift (Poulsen, 2002; Viereck *et al*., 2005). The chemical shift is normally measured in Hz, shifted relative to a reference signal and it is a very small number, divided by a much larger number of the spectrometer frequency also in Hz. The resulting number is multiplied by one million and gives the unit of parts per million used in NMR (Poulsen, 2002).

In ^{1}H NMR, carbohydrates are often dissolved in D_2O to reduce the intensity of the water/HDO resonance located in the same region as the resonances from the carbohydrates. Other common solvents include organic solvents such as deuterated chloroform and methanol. One dimensional experiments are made to estimate the monosaccharides present with anomeric proton resonances found in the shift range between 4.4–5.5 ppm and the ring protons between 3.0–4.2 ppm (Van Soest, 2004). Two dimensional experiments are also carried out to give more information on the chemical structure, molecular order and mobility, although they are time consuming.

Solid-state NMR (^{13}C NMR) is well-suited for studying marama beans, since most of the contained carbohydrates are insoluble. In ^{13}C NMR cross polarisation and magic angle spinning (CP/MAS), and single pulse and magic angle spinning (SP/MAS) detect immobile (rigid) and mobile segments of a sample, respectively (Gidley *et al*., 1991; Bardet *et al*., 2001). By using CP/MAS experiments resonances located in the immobile regions of the sample are enhanced, whereas in the SP/MAS experiments resonances of all sites are observed quantitatively correct. Other important information can be obtained by recording the spin lattice relaxation times, T_1, and the spin–spin relaxation times, T_2 of ^{1}H and ^{13}C atoms (Van Soest, 2004). The values obtained for the chemical shift make it possible to assign anomeric configurations of both native and derivatised polysaccharides and the number of anomeric signals shows the linkages of the sugars (Andersson *et al*., 2006). By combining the information gained, the structure of the parent polysaccharide can be obtained.

Results (unpublished): In NMR, CP/MAS spectra of different marama bean fractions showed that the deproteinisation procedure using PAW was more effective than using $Ba(OH)_2$ and NaOH. This can be seen in Figure 26 where the "PAW" sample contained less protein because

of no resonance around 130 ppm which are due to aromatic side chains in amino acids. The "PAW" fraction also showed a reduction in the resonances from aliphatic side chains in proteins at 0 – 50 ppm. However, it is not possible to exclude the presence of proteins or amino acids in the "PAW" sample because a protein molecule may contain a very low amount of amino acids with undetectable aromatic side chains. The signal of carbohydrates improved with PAW extraction as the resonances for carbohydrates were easily identified at approximately 21.0 ppm for O-$COCH_3$; 53.0 ppm for $COOCH_3$; and another broad peak at 172.0 ppm for carbonyl carbons in esters and acids. The resonances in the region 90 – 110 ppm originate from anomeric carbons in carbohydrates. The CP/MAS assignment suggests that the carbohydrates in marama bean are pectic polysaccharides. Holse *et al*. (2011) also mentions that the large part of the polysaccharides present in marama bean is pectin or galacturonic acid.

In general, when comparing the spectra of SP/MAS and CP/MAS of the different carbohydrate fractions, it is evident that the carbohydrate fractions have rigid or immobile molecules (Figure 26), an indication of insolubility, and a characteristic already covered in previous discussions.

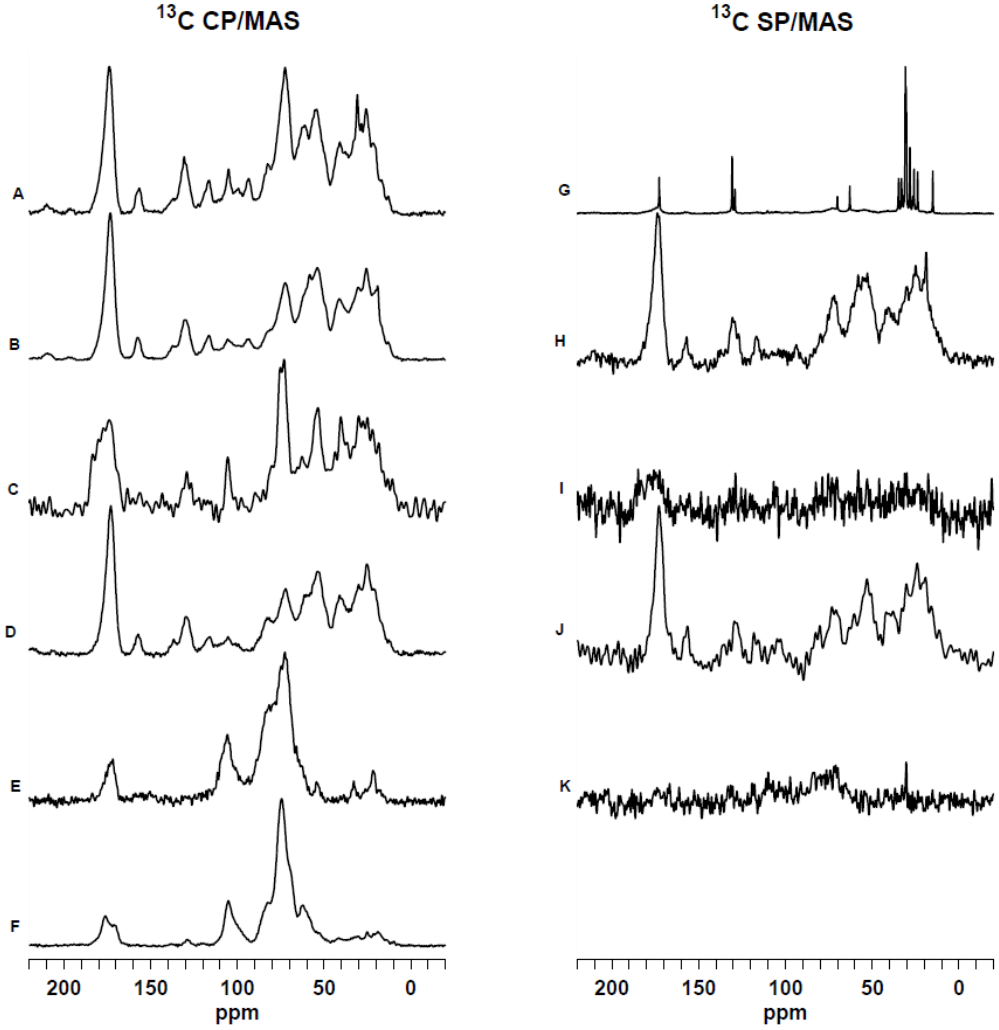


Figure 26: ^{13}C nuclear magnetic resonance cross polarisation and magic angle spinning (NMR CP/MAS) and ^{13}C nuclear magnetic resonance single pulse and magic angle spinning (NMR SP/MAS) spectra of different marama bean fractions (20-200 ppm) (Mosele *et al*., unpublished). A = CP/MAS, Raw; B = CP/MAS, Defatted; C = CP/MAS, CHO fraction, $Ba(OH)_2$, D = CP/MAS, CHO fraction, NaOH; E = CP/MAS, PAW; F = CP/MAS, Pure pellet, NaOH; G = SP/MAS, Raw; H = SP/MAS, Defatted; I = SP/MAS, CHO fraction, $Ba(OH)_2$, J = SP/MAS, CHO fraction, NaOH; K = SP/MAS, PAW.

4.3 Summary

Table 9 gives a summary of the method of analyses used in the study. These methods reveal the main carbohydrate as pectin, and negligible amount of simple sugars and starch. The pectin is made up of polygalacturonic acid and highly branched arabinan. The carbohydrates were strongly attached to proteins in a glycoprotein matrix, probably as a result of protein glycosylation. Spectroscopy analyses reveal that α-anomeric carbohydrates (which include pectin) dominated the carbohydrate fraction, from carbonyl carbons in esters and acids.

Table 9: A summary of methods of analyses used in the study

Techniques	Chemical/Physical Phenomena	Resolution	Advantages	Disadvantages
TEM	Electron transmission	<100nm	Information on surface features	Ultrathin sectioning
SEM	Electron transmission	>1nm	Visualisation of wide areas and irregular shaped specimens	Introduction of artifacts during sample preparation
Fluorescent microscopy	Fluorescence emission	<500nm	Visualisation of living organisms	Photobleaching
CLSM	Fluorescence emission and optical sectioning	<100μm	3D visualisation of organisms	Photobleaching
NIR-CI	Molecular vibrations and spatial resolution	<2500nm	Spatially resolved chemical analysis	Huge data sets
FT-IR	Molecular vibrations	>1μm	Sensitive, information on structure of molecules	Aqueous samples difficult to analyse
FR-Raman	Molecular vibrations	>50μm	Structure of molecules	Photodecomposition of samples
NMR	Nuclei spin	None	High sensitivity to structure of molecules	Complex data
HPAEC-PAD	Molecular distribution	None	High sensitivity to carbohydrates	Laborious sample preparation
CoMPP	Molecular mapping	None	High throughput analysis	Complex analysis
Methylation analysis	Molecular linkages	None	Information on molecular branching	Laborious sample preparation

5 GENERAL DISCUSSION

This thesis was set out to define or characterise the chemical and structural composition of carbohydrates in marama bean. The work has laid a strong foundation for further research in this area.

To commence the work, an understanding of the marama bean physico-chemical structure was in order, hence the use of microscopy techniques and proximate analysis. The microscopy techniques showed protein bodies (PBs) encircled by neutral lipid bodies (LBs), which are in droplet form (Mosele *et al*., 2011a). There was a trend of small PBs in the epidermal layer, which increase in size but decrease in number in the subepidermis, and finally increase in number but decrease in size at the centre of the cotyledon in mature seeds (Figure 4). The proteins and lipids were more in mature seeds than in immature seeds.

Through proximate composition, the total carbohydrate content of mature marama bean was calculated at 19 – 24% (Mosele *et al*., 2011a), the majority of it being dietary fibre at 23.4% (w/w) (Holse *et al*., 2011). What this suggests is that almost all of the carbohydrates in marama bean are dietary fibre, with 96% of it being insoluble. Out of the 24% (w/w), only about 5% (w/w) is accounted for by starch, simple sugars and pectin (Mosele *et al*., 2011a). The rest of the amount is assumed to be other non-starch polysaccharides, as well as N-linked glycoproteins based on the linkages observed (2- and 3,6-mannose) (Lis and Sharon, 1978). Protein glycosylation if present will inflate the carbohydrate content especially in the context of marama bean where the protein content is even higher than the carbohydrate content at 32% (w/w). It is also important to bear in mind that the calculated pectin amount is an underestimation because TFA hydrolysis used in the analysis destroys some of the sugars such as galacturonic acid (Wicks *et al*., 1991). The expectation is also that TFA will underestimate the amount of cellulose because it does not hydrolyse cellulose thereby releasing low amounts of glucose (Wicks et al., 1991; Chen *et al*., 1998; Zheng *et al*., 2008). A pre-treatment with sulphuric acid can be done before cellulose hydrolysation, however TFA alone is preferable because of its high volatility and high yields of monosaccharides from non-cellulosic polysaccharides (Wicks *et al*., 1991).

Light microscopy test for starch through iodine staining was negative. The reason is that its amount is low, measured by HPAEC-PAD at 0.2% (w/w) for both immature and mature seeds (Mosele *et al*., 2011b). This was surprising as most legumes (with the exception of soya bean) contain considerable amounts of starch, as already discussed in Chapter 3. The amount of simple sugars was also negligible at less than 1% (w/w) (Mosele *et al*., 2011b). Fluorescent microscopy tests in both immature and mature seeds were positive for pectin and cellulose (Mosele *et al*., 2011a). NIR-CI and CoMPP also revealed the presence of cellulose. All the observed carbohydrates were in the cell wall of the seeds. The main cell wall monomer was arabinose, which was highly branched as indicated by the presence of 3,5- and 2,3,5-linked arabinose (Mosele *et al*., 2011b). It was also linked via O-5, characteristic for pectic arabinan. The arabinose was recalcitrant to extraction.

Further studies with NIR-CI revealed that the pectin observed is made up of polygalacturonic acid, while in CoMPP the pectin components observed were homogalacturonan and arabinan (Mosele *et al*., 2011b). Galacturonic acid is the main backbone of homogalacturonan, therefore the results from the two analyses were exact. Arabinans are found in many cell walls and are generally considered to be part of the pectic network as they are associated with RGI (Willats *et al*., 2001; Harholt *et al*., 2010). The pectin had a low degree of esterification. Holse *et al*. (2011) also mentions that the large part of the polysaccharides in marama bean is present as pectins or galacturonic acids. The amount of pectin was calculated to be 4.2 % (w/w) by adding up the monosaccharides arabinose, galactose, rhamnose and galacturonic acid, which are well known as monomers for pectic substances.

FT-Raman spectra at the peak between 825 and 860 cm^{-1} also indicated a strong predominance of α-anomeric carbohydrates, mainly pectin (Séné *et al*., 1994; Engelsen *et al.,* 1996; Synytsya *et al*., 2003). The assignment of ^{13}C CP/MAS NMR resonances for carbohydrates also suggests that the carbohydrates in marama bean are pectic polysaccharides, especially the signals at approximately 21.8, 54.6 and 171.7 ppm. Pectin is an important food ingredient because of its function as a gelling and thickening agent.

FT-Raman, FT-IR and NMR results suggest a strong association between the proteins and carbohydrates in marama bean, which is observed from the different carbohydrate fractions extracted by the 3 deproteinisation procedures $Ba(OH)_2$, NaOH and PAW, where resonances from amino acids were observed in the carbohydrate fractions.

6 CONCLUSIONS

The study has given an insight into the carbohydrate composition of marama bean. The strength of this study is the use of diverse analytical techniques which have produced similar and complementary results, with different emphasises.

The main carbohydrate present in marama bean is pectin. The pectin is mainly galacturonic acid (homogalacturonan), with a low degree of esterification. The pectin is dominated by arabinan sidechains. The pectin was calculated to be 4.2%. The presence of rhamnogalacturonan was also noted, but at low amounts because the rhamnose observed in HPAEC-PAD was low and the rhamnose signal was weak from ^{13}C NMR CP/MAS. Since strong signals from the carbohydrates could only be seen with ^{13}C NMR CP/MAS (i.e. SP/MAS gave very weak, noisy signals), this supports the suggestion that marama bean carbohydrates are mainly insoluble.

Soluble (simple) sugars were negligible at less than 1%. These were dominated by glucose in immature seeds and sucrose in mature seeds. Starch was also found in negligible amounts (0.2%). In fact iodine staining test in light microscopy tested negative. Therefore, marama bean stores carbohydrates in the form of cell wall components instead of starch like other beans. The non cellulosic fraction of the cell wall had as much as 60% (w/w) arabinose, practically all from a highly branched arabinan. This arabinan forms part of the pectin network in the bean. It is also recalcitrant to extraction.

It was not possible to make specific assignments from the spectroscopy data, primarily because of the overlapping signals of proteins and carbohydrates, even after deproteinisation. This suggests the existence of glycoproteins and/or that carbohydrates and proteins are entangled in a tight network. Some form of glycosylation was also noted in FT-IR. Despite this, the presence of anomeric carbohydrates was confirmed by FT-Raman and ^{13}C CP/MAS, together with carbonyl carbons in acids and esters, galacturonic acid, i.e. pectin.

This thesis has laid a foundation for the profiling of carbohydrates in marama bean. Nevertheless, work still needs to be done where the polymers such as cellulose and pectin can be isolated and measured directly. This study has quantified only about 5% of the carbohydrates in marama bean,

accounted for by starch, simple sugars and pectin. The rest of the carbohydrate fraction can be accounted for by non-starch polysaccharides and carbohydrate-protein interactions such as glycoproteins. This study has showed that the marama bean carbohydrates are mainly pectin and thus it may offer an alternative gelling and thickening agent resource.

7 PERSPECTIVES

It is important that every local food industry seek food ingredients in their own backyard to minimise the cost of production and increase profit, with making affordable food products in mind. The identified pectin in marama bean, if well understood, could be an alternative to well known hydrocolloids from other legumes such as guar and locust bean. Future work should then focus on isolating pectin and cellulose, along with the elucidation of their functional properties to fully appreciate the uses of marama bean. This will be important especially for pectin since the current work is not fully conclusive on the type of pectin present in marama bean.

The observed close association of carbohydrates and proteins in marama bean also needs further investigation. With an improved separation of carbohydrates and proteins, fast and non-destructive spectroscopy techniques can be further employed to probe this carbohydrate-protein interaction or the postulated glycosylation.

Further studies into the characterisation of marama bean carbohydrates can be enhanced by using well-characterised legumes as references. Locust bean is especially well-suited for the purpose, since it comes from the same subfamily with marama bean and is extensively studied. A well chosen reference species can reduce the time-consumption and complexity of experimental data analysis.

In this study it turned out that seed variation within samples was considerable. This was especially seen in the microscopy results. Therefore further research should be carried out on seeds from the existing cultivation trials instead of seeds harvested from the wild.

Marama bean is a promising crop worth domestication and cultivation, firstly for its good nutritional value of naturally occurring high protein, lipid and dietary fibre content which rival that in soya bean and peanut. It also has a potential to shift from its current novel status to a revolution since it does not have known allergens. Marama bean has the potential for diverse food applications in the human diet (e.g. as a fortificant) and the food industry.

REFERENCES

Abdel-Gawad, A. S. (1993). Effect of domestic processing on oligosaccharide content of some dry legume seeds. *Food Chemistry*, *46*, 25–31.

Adsule, R. N., Kadam, S. S., & Salunkhe, D. K. (1989). Peanut. In D. K. Salunkhe, & S. S. Kadam, *CRC Handbook of World Legumes: Nutritional Chemistry, Processing Technology, and Utilization, vol. II*, 1-18. Florida: CRC Press.

Aguilera, Y., Esteban, R. M., Benitez, V., Molla, E., & Martin-Cabrejas, M. A. (2009a). Starch, functional properties, and microstructural characteristics in chickpea and lentil as affected by thermal processing. *Journal of Agricultural and Food Chemistry*, *57*, 10682-10688.

Aguilera, Y., Martín-Cabrejas, M. A., Díaz, M. F., Aguilera, Y., Benítez, V., Mollá, E., López-Andréu, F. J., Esteban, R. M. (2009b). Changes in carbohydrate fraction during dehydration process of common legumes. *Journal of Food Composition and Analysis*, *22*, 678-683.

Ahmed, E. G., Al-Muslet, N. A., Ahmed, M. M., Moharam, M. O., & Musaad, W. (2010). The use of fourier infrared spectroscopy and laser-Raman spectroscopy in bladder malignancy diagnosis, a comparative study. *Applied Physics Research*, *2*, 108–117.

Amarteifio, J. O., & Moholo, D. (1998). The chemical composition of four legumes consumed in Botswana. *Journal of Food Composition and Analysis*, *11*, 329–332.

Amigo, J. M. (2010). Practical issues of hyperspectral imaging analysis of solid dosage forms. *Analytical and Bioanalytical Chemistry*, *398*, 93–109.

Amigo, J. M., Cruz, J., Bautista, M., Maspoch, S., Coello, J., Blanco, M. (2008). Study of pharmaceutical samples by NIR chemical-image and multivariate analysis. *Trends in Analytical Chemistry*, *27*, 696–713.

Amonsou, E., Taylor, J., & Minnaar, A. (2011). Microstructure of protein bodies in marama bean species. *Food Science and Technology*, *44*, 42–47.

Andersson, R., Westerlund, E., & Åman, P. (2006). Polysaccharides: Structural, chemical and analytical aspects. In A. C. Eliasson, *Carbohydrates in food*, 2nd ed, 129-166. Florida: CRC Press.

Avallone, R., Plessi, M., Baraldi, M., Monzani, A. (1997). Determination of chemical composition of carob (*Ceratonia siliqua*): Protein, fat, carbohydrates and tannins. *Journal of Food Composition and Analysis*, *10*, 166-172.

Baldan, B., Bonaldo, A., Rascio, N., Vitale, A., Meggio, F., Profumo, P., & Mariani, P. (1995). *Cercis siliquastrum* L.: A comparative study of endosperm and embryo development and reserve accumulation. *International Journal of Plant Sciences*, *156*, 181–187.

Bardet, M., Foray, M. F., Bourguignon, J., & Krajewski, P. (2001). Investigation of seeds with high-resolution solid-state ^{13}C NMR. *Magnetic Resonance in Chemistry*, *39*, 733–738.

Belitz, H. D., Grosch, W., & Schieberle, P. (2009). *Food Chemistry, 4th ed*. Germany: Springer.

BeMiller, J. N., & Whistler, R. L. (1996). Carbohydrates. In O. R. Fennema, *Food Chemistry*, 3rd ed, 157-223. New York: Marcel Dekker.

Bower, N., Hertel, K., Oh, J., & Storey, R. (1988). Nutritional evaluation of marama bean (*Tylosema* esculentum, Fabaceae): Analysis of the seed. *Economic Botany*, *42*, 533–540.

Bozzola, J. J., & Rusell, L. D. (1999). *Electron Microscopy 2nd ed*. UK: Jones and Bartlett Publishers, Inc.

Bramley, P. M., Elmadfa, I., Kafatos, A., Kelly, F. J., Manios, Y., Roxborough, H. E., Schuch, W., Sheehy, P. J. A., & Wagner, K. H. (2000). Vitamin E. *Journal of the Science of Food and Agriculture 80*, 913–938.

Bravo, L., Siddhuraju, P., & Saura-Calixto, F. (1999). Composition of underexploited Indian pulses. Comparison with common legumes. *Food Chemistry*, *64*, 185-192.

Brink, M. (2006). *Tylosema*. In M.Brink, & G. Belay, *Plant resources of Tropical Africa 1. Cereals and pulses*, pp 190–191. Wageningen: PROTA (Plant Resources of Tropical Africa / Ressources végétales de l'Afrique tropicale) Foundation.

Brown Jr., R. M., Saxena, I. M., & Kudlicka, K. (1996). Cellulose biosynthesis in higher plants. *Trends in Plant Science*, *1*, 149-156.

Bourbon, A. I., Pinheiro, A. C., Ribeiro, C., Miranda, C., Maia, J. M., Teixeira, J. A., & Vicente, A. A. (2010). Characterisation of galactomannans extracted from seeds of *Gleditsia triacanthos* and *Sophora japonica* through shear and extensional rheology: Comparison with guar gum and locust bean gum. *Food Hydrocolloids*, *24*, 184 – 192.

Bubb, W. A. (2003). NMR spectroscopy in the study of carbohydrates: Characterising the structural complexity. *Concepts in Magnetic Resonance Part A*, *19A*, 1-19.

Cael, J. J., Koenig, J. L., & Blackwell, J. (1973). Infrared and Raman spectroscopy of carbohydrates. Part III: Raman spectra of the polymorphic forms of amylase. *Carbohydrate Research*, *29*, 123–134.

Caffall, K. H., Pattathil, S., Phillips, S. E., Hahn, M. G., & Mohnen, D. (2009). Arabidopsis thaliana T-DNA mutants implicate GAUT genes in the biosynthesis of pectin and xylan in cell walls and seed testa. *Molecular Plant*, *2*, 1000-1014.

Castro, S., Silveira, P., Coutinho, A. P., & Figueiredo, E. (2005). Systematic studies in *Tylosema* (Leguminosae). *Botanical Journal of the Linnean Society*, *147*, 99–115.

Chen, F. A., Dobashi, T. S., & Evangelista, R. A. (1998). Quantitative analysis of sugar constituents of glycoproteins by capillary electrophoresis. *Glycobiology*, *8*, 1045-1052.

Chibbar, R. N., Ambigaipalan, P., & Hoover, R. (2010). Molecular diversity in pulse seed starch and complex carbohydrates and its role in human nutrition and health. *Cereal Chemistry*, *87*, 342-352.

Chimwamurombe, P. M. (2010). Domestication of *Tylosema esculentum* (marama bean) as a crop for Southern Africa: Genetic diversity of the Omitara marama subpopulation of Namibia. *Aspects of Applied Biology*. 96, 37-43.

Chingwaru, W., Duodu, G., Van Zyl, Y., Schoeman, C.J., Majinda, R.T., Yeboah, S.O., Jackson, J., Kapewangolo, P.T., Kandawa-Schulz, M., Minnaar, A., & Cencic, A. (2011)**.** Antibacterial and anticandidal activity of *Tylosema esculentum* (marama) extracts. *South African Journal of Science*, *107*, 1-11.

Chung, H. J., & Liu, Q. (2009).Impact of molecular structure of amylopectin and amylose on amylose chain association during cooling. *Carbohydrate Polymers*, *77*, 807-815.

Claxton, N. S., Fellers, T. J., & Davidson, M. W. (2007). *Laser Scanning Confocal Microscopy*, Florida State University, USA.

Coimbra, M. A., Gonçalves, F., Barros, A. S., & Delgadillo, I. (2002). Fourier transform infrared spectroscopy and chemometric analysis of white wine polysaccharide extracts. *Journal of Agricultural and Food Chemistry*, *50*, 3405–3411.

Colquhoun, I. J., Ralet, M. C., Thibault, J. F., Faulds, C. B., & Williamson, G. (1994). Structure identification of feruloylated oligosaccharides from sugar-beet pulp by NMR spectroscopy. *Carbohydrate Research*, *263*, 243-256.

Cordella, C. B. Y., Mitão, J. S. L. T., Clément, M. C., Cabrol-Bass, D. (2003). Honey characterization and adulteration detection by pattern recognition applied on HPAEC-PAD profiles. 1. Honey floral species characterisation. *Journal of Agricultural and Food Chemistry*, *51*, 3234–3242.

Corradini, C., Canali, G., & Nicoletti, I. (1997). Application of HPAEC-PAD to carbohydrate analysis in food products and fruit juices. *Seminars in Food Analysis*, *2*, 99-111.

Coultate, T. P. (2002). *Food*, *the Chemistry of its Components*, 4th ed. Cambridge: Royal Society of Chemistry.

Cummings, J. H., Edmond, L. M., & Magee, E. A. (2004). Dietary carbohydrates and health: Do we still need the fibre concept? *Clinical Nutrition Supplements*, *1*, 5-17.

Dalgetty, D. D., & Baik, B. K. (2003). Isolation and characterization of cotyledon fibers from peas, lentils, and chickpeas. *Cereal Chemistry*, *80*, 310-315.

Dakia, P. A., Blecker, C., Robert, C., Wathelet, B., & Paquot, M. (2008). Composition and physicochemical properties of locust bean gum extracted from whole seeds by acid or water dehulling pre-treatment. *Food Hydrocolloids*, *22*, 807-818.

Damager, I., Engelsen, S. B., Blennow, A., Møller, B. L., & Motawia, M. S. (2010). First principles insight into the α-glucan structures of starch: Their synthesis, conformation and hydration. *Chemical Reviews*, *110*, 2049 – 2080.

Davis, R., & Mauer, L. J. (2010). Fourier transform infrared (FT-IR) spectroscopy: A rapid tool for detection and analysis of foodborne pathogenic bacteria. In A. Méndes-Vilas, *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology 2*, 1582-1594. Spain: FORMATEX.

De Almeida Costa, G. E., Queiroz-Monici, K., Reis, S. M. P. M., & De Oliveira, A. C. (2006). Chemical composition, dietary fibre and resistant starch contents of raw and cooked pea, common bean, chickpea and lentil legumes. *Food Chemistry*, *94*, 327-330.

Dea, I. C. M., & Morrison, A. (1975). Chemistry and interactions of seed galactomannans. *Advanced Carbohydrate Chemistry and Biochemistry*, *31*, 241–312.

Dini, A., De Simone, F., Ramundo, E., & Senatore, F. (1989). Oligosaccharides in five different *Vicia faba* L. cultivars. *Biochemical Systematics and Ecology*, *17*, 559-561.

Dionex (2010). *Technical note 20: Analysis of carbohydrates by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)*. http://www.dionex.com/en-us/webdocs/5023-TN20.pdf.

Dokken, K. M., & Davis, L. C. (2007). Infrared imaging of sunflower and maize root anatomy. *Journal of Agricultural and Food Chemistry*, *55*, 10517–10530.

Dokken, K. M., Davis, L. C., & Marinkovic, N. S. (2005). Use of infrared microspectroscopy in plant growth and development. *Applied Spectroscopy Reviews*, *40*, 301-326.

Dubois, M., Lognay, G., Baudart, E., Marlier, M., Severin, M., & Dardenne, G. (1995). Chemical characterisation of *Tylosema–Fassoglensis* (Kotschy) Torre and Hillc Oilseed. *Journal of the Science of Food and Agriculture*, *67*, 163−167.

Echlin, P. (2009). *Handbook of Sample Preparation for Scanning Electron Microscopy and X-Ray Microanalysis*. New York: Springer & Business Media.

Engel, A., & Colliex, C. (1993). Application of scanning transmission electron microscopy to the study of biological structure. *Current Opinion in Biotechnology*, *4*, 403-411.

Engelsen, S. B., & Nørgaard, L. (1996). Comparative vibrational spectroscopy for determination of quality parameters in amidated pectins as evaluated by chemometrics. *Carbohydrate Polymers*, *30*, 9–24.

European Commission (2008). Commission Directive 2008/100/EC. *Official Journal of the European Union*, *L285*, 9-11.

Fay, L. B., & Kussman, M. (2010). Mass spectrometry technologies. *Mass Spectrometry and Nutrition Research, Food Analysis Monographs No. 9.* Royal Society of Chemistry.

Folkes, D. J., & Jordan, M. A. (2006). Mono- and disaccharides: Analytical aspects. In A. C. Eliasson, *Carbohydrates in food*, 2nd ed, 1-40. Taylor & Francis Group.

Fowler, S. D., & Greenspan, P. (1985). Application of Nile Red, a fluorescent hydrophobic probe, for the detection of neutral lipid deposits in tissue sections: Comparison with oil Red O. *Journal of Histochemistry and Cytochemistry*, *33,* 833–836.

Fry, S. C. (1982). Phenolic components of the primary cell wall: Feruloylated disaccharides of D-galactose and L-arabinose from spinach polysaccharide. *Biochemical Journal*, *203*, 493-504.

Fry, S. C. (1988). *The Growing Plant Cell Wall: Chemical and Metabolic Analysis*. The Blackburn Press, Caldwell, NJ.

Gambhir, G. (2008). *Chromatography (a refresher course CPDHE)*. Acharya Narendra Dev. College.

Gendrin, C., Roggo, Y., & Collet, C. (2008). Pharmaceutical applications of vibrational chemical imaging and chemometrics: A review. *Journal of Pharmaceutical and Biomedical Analysis*, *48*, 533-553.

Ghiran, I. C. (2011). Introduction to fluorescence microscopy. In H. Chiarini & R. C. N. Melo, Light Microscopy, Methods and Protocols. *Methods in Molecular Biology Volume 689*, 93-136.

Ghosh, P. K., & Jayas, D. S. (2009). Use of spectroscopic data for automation in food processing industry. *Sensing and Instrumentation for Food Quality and Safety*, *3*, 3–11.

Gidley, M. J., McArthur, A. J., & Underwood, D. R. (1991). ^{13}C NMR characterisation of molecular structures in powders, hydrates and gels of galactomannans and glucomannans. *Food Hydrocolloids*, *5*, 129–140.

Gowen, A. A., O'Donnell, C. P., Cullen, P. J., Downey, G., Frias, J. M. (2007). Hyperspectral imaging – an emerging process analytical tool for food quality and safety control. *Trends in Food Science and Technology*, *18*, 590–598.

Greenspan, P., Mayer, E. P., & Fowler, S. D. (1985). Nile Red: A selective fluorescent stain for intracellular lipid droplets. *Journal of Cell Biology*, *100*, 965–973.

Guillon, F., & Champ, M. M. J. (2002). Carbohydrate fraction of legumes: Uses in human nutrition and potential for health. *British Journal of Nutrition*, *88*, S293-S306.

Harholt, J., Jensen, J. K., Sørensen, S. O., Orfila, C., Pauly, M., & Scheller, H. V. (2006). ARABINAN DEFICIENT 1 is a putative arabinosyltransferase involved in biosynthesis of pectin arabinan in arabidopsis. *Plant Physiology*, *140*, 49–58.

Harholt, J., Suttangkakul, A., & Scheller, H. V. (2010). Biosynthesis of pectin. *Plant Physiology*, *153*, 384 - 395.

Hartley, M. L., Tshamekeng, E., & Thomas, S. M. (2002). Functional heterostyly in *Tylosema esculentum* (Caesalpinioideae). *Annals of Botany*, *89*, 67 – 76.

Henley, E. J., Seader, J. D., & Roper, D. K. (2011). *Separation Process Principles* 3rd ed. John Wiley & Sons, Pte Ltd.

Herman, E. M., & Larkins, B. A. (1999). Protein storage bodies and vacuoles. *The Plant Cell*, *11*, 601–613.

Herrero, A. M. (2008). Raman spectroscopy for monitoring protein structure in muscle food systems. *Critical Reviews in Food Science and Nutrition*, *48*, 512-523.

Hizukuri, S. (1986). Polymodal distribution of the chain lengths of amylopectins and its significance. *Carbohydrate Research*, *147*, 342–347.

Hizukuri, S., Takeda, Y., Yasuda, M., & Suzuki, A. (1981). Multi-branched nature of amylose and action of debranching enzymes. *Carbohydrate Research*, *94*, 205–213.

Holse, M., Husted, S., & Hansen, Å. (2010). Chemical composition of marama bean (Tylosema esculentum) – a wild African bean with unexploited potential. *Journal of Food Composition and Analysis*, *23*, 648 – 657.

Holse, M., Larsen, F.H., Hansen, Å., & Engelsen, S.B. (2011). Characterization of marama bean (*Tylosema esculentum*) by comparative spectroscopy: NMR, FT-Raman, FT-IR and NIR. *Food Research International*, *44*, 373-384.

Hoppert, M. (2003). *Microscopic Techniques in Biotechnology*. Germany: Wiley – VCH.

Hou, A., Chen, P., Shi, A., Zhang, B., & Wang, J. (2009). Sugar variation in soybean seed assessed with a rapid extraction and quantification method. *International Journal of Agronomy*, *2009*, article ID 484571.

Huang, H., Yu, H., Xu, H., & Ying, Y. (2008). Near infrared spectroscopy for on/in-line monitoring of quality in foods and beverages: A Review. *Journal of Food Engineering*, *87*, 303–313.

Huisman, M. M. H., Schols, H. A., & Voragen, A. G. J. (1998). Cell wall polysaccharides from soybean (*Glycine max.*) meal. Isolation and characterisation. *Carbohydrate Polymers*, *37*, 87-95.

IUPAC & IUBMB (1996). Nomenclature of carbohydrates. IUPAC joint commission on biochemical nomenclature (JCBN). *Pure and Applied Chemistry*, *68*, 1919-2008.

Jakobsen, H. B., Martens, H., & Lyshede, O. B. (1994). Accumulation of metabolites during seed development in *Trifolium repens* L. *Annals of Botany*, *74*, 409–415.

Jensen, J. K., Sorensen, S. O., Harholt, J., Geshi, N., Sakuragi, Y., Moller, I., Zandleven, J., Bernal, A. J., Jensen, N. B., Sorensen, C., Pauly, M., Beldman, G., Willats, W. G. T., & Scheller, H. V. (2008). Identification of a xylogalacturonan xylosyltransferase involved in pectin biosynthesis in arabidopsis. *The Plant Cell*, *20*, 1289-1302.

Jideani, V. A., Van Wyk, J., & Cruywagen, M. H. (2009). Physical properties of *Tylosemia esculentum* and the effect of roasting on the functional properties of its flour. *African Journal of Agricultural Research*, *4*, 1208–1219.

Jouppila, K. (2006). Mono- and disaccharides: Selected physicochemical and functional aspects. In A. C. Eliasson, *Carbohydrates in food*, 2nd ed, 41-88. Taylor & Francis Group.

Kačuráková, M., Capek, P., Sasinková, V., Wellner, N., & Ebringerová, A. (2000). FT-IR study of plant cell wall model compounds: pectic polysaccharides and hemicelluloses. *Carbohydrate Polymers*, *43*, 195–203.

Kačuráková, M., Wellner, N., Ebringerová, A., Hromádková, Z., Wilson, R. H., & Belton, P. S. (1999). Characterisation of xylan-type polysaccharides and associated cell wall components by FT-IR and FT-Raman spectroscopies. *Food Hydrocolloids*, *13*, 34–41.

Kadam, S. S., Deshpande, S. S., & Jambhale, N. D. (1989). Peanut. In D. K. Salunkhe, & S. S. Kadam, *CRC Handbook of World Legumes: Nutritional Chemistry, Processing Technology, and Utilization, vol. II*, 23-50. Florida: CRC Press.

Karr-Lilienthal, L. K., Kadzere, C. T., Grieshop, C. M. & Fahey Jr., G. C. (2005). Chemical and nutritional properties of soybean carbohydrates as related to non-ruminants: a review. *Livestock Production Science, 97*, 1 – 12.

Ketshajwang, K.K., Holmback, J., & Yeboah, S.O. (1998). Quality and compositional studies of some edible leguminosae seed oils in Botswana. *Journal of the American Oil Chemists' Society*, *75*, 741-743.

Khan, A. R., Sahib Alam, S., Ali, S., Saiqa Bibi, S., & Iqtidar A. Khalil, I A. (2007). Dietary fibre profile of food legumes. *Sarhad Journal of Agriculture*, *23*, 763-766.

Khanna, S., Parrett, A., & Edwards, C. A. (2006). Nondigestible carbohydrates: Nutritional aspects. In A. C. Eliasson, *Carbohydrates in food*, 2nd ed, 273-303. Taylor & Francis Group.

Khurana, H. K., & Kanawjia, S. K. (2007). Recent trends in development of fermented milks. *Current Nutrition and Food Science*, *3*, 91–108.

Kök, M. S., Hill, S. E., & Mitchell, J. R. (1999). A comparison of the rheological behaviour of crude and refined locust bean gum preparations during thermal processing, *Carbohydrate Polymers*, *38*, 261-265.

Labbé, N., Lee, S. H., Cho, H. W., Jeong, M. K., & André, N. (2008). Enhanced discrimination and calibration of biomass NIR spectral data using non-linear kernel methods. *Bioresource Technology*, *99*, 8445-8452.

Lai, V. M. F., & Lii, C. Y. (2004). Roles of saccharides in texturisation and functional properties of foodstuffs. In P. Tomasik, *Chemical and Functional Properties of Food Saccharides*. Florida: CRC Press.

Li-Chan, E. C. Y. (1996). The application of Raman spectroscopy in food science. *Trends in Food Science and Technology*, *7*, 361-370.

Lichtman, J. W., & Conchello, J. (2005). Fluorescence microscopy. *Nature Methods*, *2*, 910–919.

Lis, H., & Sharon, N. (1978). Soybean agglutinin-a plant glycoprotein. Structure of the carboxydrate unit. *Journal of Biological Chemistry*, *253*, 3468-3476.

Lott, J. N. A., & Buttrose, M. S. (1977). Globoids in protein bodies of legume seed cotyledons. *Australian Journal of Plant Physiology*, *5*, 89–111.

Lott, J. N. A., Ockenden, I., Raboy, V., & Batten, G. D. (2000). Phytic acid and phosphorus in crop seeds and fruits: a global estimate. *Seed Science Research*, *10*, 11–33.

Majzoobi, M., Rowe, A. J., Connock, M., Hill, S. E., & Harding, S. E. (2003). Partial fractionation of wheat starch amylose and amylopectin using zonal ultracentrifugation. *Carbohydrate Polymers*, *52*, 269-274.

Martín-Cabrejas, M. A., Díaz, M. F., Aguilera, Y., Benítez, V., Mollá, E., & Esteban, R. M. (2008). Influence of germination on the soluble carbohydrates and dietary fibre fractions in non-conventional legumes. *Food Chemistry*, *107*, 1045-1052.

Martínez-Villaluenga, C., Frías, J., & Vidal-Valverde, C. (2005). Raffinose family oligosaccharides and sucrose contents in 13 Spanish lupin cultivars. *Food Chemistry*, *91*, 645-649.

Maruatona, G. N., Duodu, K. G., & Minnaar, A. (2010). Physicochemical, nutritional and functional properties of marama bean flour. *Food Chemistry*, *121*, 200 – 405.

Mauko, A., Muck, T., Mirtic, B., Mladenovic, A., & Kreft, M. (2009). Use of confocal laser scanning microscopy (CLSM) for the characterisation of porosity in marble. *Material Characterization*, *60*, 603–609.

Mehta, K., & Kaur, A. (1992). Reviews: Dietary fibre. *International Journal of Diabetes in Developing Countries*, *12*, 12-18.

Mitchell, R. A. C., Keys, A. J., Madgwick, P. J., Parry, M. A. J., & Lawlor, D. W. (2005). Adaptation of photosynthesis in marama bean Tylosema esculentum (Burchell A. Schreiber) to a high temperature, high radiation, drought-prone environment. *Plant Physiology and Biochemistry*, *43*, 969-976.

Mitei, Y.C., Ngila, J.C., Yeboah, S.O., Wessjohann, L., & Schmidt, J., (2008). NMR, GC-MS and ESI-FTICR-MS profiling of fatty acids and triacylglycerols in some Botswana seed oils. *Journal of the American Oil Chemists' Society*, *85*, 1021-1032.

Mitei, Y.C., Ngila, J.C., Yeboah, S.O., Wessjohann, L., & Schmidt, J. (2009). Profiling of Phytosterols, Tocopherols and Tocotrienols in Selected Seed Oils from Botswana by GC-MS and HPLC. *Journal of the American Oil Chemists' Society*, *86*, 617-625.

Mohnen, D. (2008). Pectin structure and biosynthesis. *Current Opinion in Plant Biology*, *11*, 266-277.

Moïse, J. A., Han, S., Gudynaite-Savitch, L., Johnson, D. A., & Miki, B. L. A. (2005). Seed coats: Structure, development, composition, and biotechnology. *In Vitro Cellular and Developmental Biology – Plant*, *41*, 620–644.

Moller, I., Sørensen, I., Bernal, A.J., Blaukopf, C., Lee, K., Obro, J., Pettolino, F., Roberts, A., Mikkelsen, J.D., Knox, J.P., Bacic, A., & Willats, W.G.T. (2007). High-throughput mapping of cell-wall polymers within and between plants using novel microarrays. *The Plant Journal*, *50*, 1118-1128.

Mosele, M. M., Hansen, Å. S., Hansen, M., Schulz, A., & Martens, H. J. (2011a). Proximate composition, histochemical analysis and microstructural localisation of nutrients in immature and mature seeds of marama bean (*Tylosema esculentum*) - an underutilised legume. *Food Chemistry*, *127*, 1555-1561.

Mosele, M. M., Hansen, Å. S., Engelsen, S. B., Diaz, J., Sørensen, I., Ulvskov, P., Willats, W. G. T., Blennow, A., & Harholt, J. (2011b). Characterisation of the arabinose-rich carbohydrate composition of immature and mature marama beans (*Tylosema esculentum*). *Phytochemistry*, *72*, 1466-1472.

Mpotokwane, S. M., Gaditlhatlhelwe, E., Sebaka, A., & Jideani, V. A. (2008). Physical properties of bambara groundnuts from Botswana. *Journal of Food Engineering*, *89*, 93–98.

Mullin, W. J., & Xu, W. (2000). A study of the intervarietal differences of cotyledon and seed coat carbohydrates in soybeans. *Food Research International*, *33*, 883-891.

Müseler, D. L., & Schönfeldt, H. C. (2006). The nutrient content of the marama bean (Tylosema esculentum), an underulised legume from Southern Africa. *AGRICOLA*.

Nakamura, A., Furuta, H., Maeda, H., Takao, T., & Nagamatsu, Y. (2002). Analysis of the molecular construction of xylogalacturonan isolated from soluble soybean polysaccharides. *Bioscience, Biotechnology, and Biochemistry*, *66*, 1155-1158.

Naresh, L., & Shailaja, U. M. (2006). Stabiliser blends and their importance in ice cream industry – a review. New Zealand Food Magazine.

National Research Council. (1979). Marama bean. *Tropical dry pulses: resources for the future*, 68 – 74. Washington DC: National Academy of Sciences.

Ngwako, S., & Mogotsi, K. (2010). *Commercialization of Morama (Tylosema esculentum (Burch.) A. Schreib)*, Powepoint presentation. Botswana: National Food Technology Research Centre.

Nikonenko, N A., Buslov, D. K., Sushko, N. I., & R.G. Zhbankov, R. G. (2005). Spectroscopic manifestation of stretching vibrations of glycosidic linkage in polysaccharides. *Journal of Molecular Structure*, *752*, 20–24.

Nkama, I., & Filli, K. B. (2006). Development and characterization of extruded fura from mixtures of pearl millet and grain legume flours. *International Journal of Food Properties*, *9*, 157 – 165.

Øbro, J., Harholt, J., Scheller, H.V., & Orfila, C. (2004). Rhamnogalacturonan I in Solanum tuberosum tubers contains complex arabinogalactan structures. *Phytochemistry*, *65*, 1429-1438.

Palatini, D. J., Zhang, S. L., Aral, B. & Crookham, H. (2002). Applying correlative brightfield, fluorescence, and Raman microscopy to determine and track component locations in formulations. *Microscopy and Microanalysis 8 (Supplement 2)*.

Pankajakshan, P., Blanc-Féraud, L., Zhang, B., Kam, Z., Olivo-Marin, J. C., & Zerubia, J. (2008). Parametric Blind Deconvolution for Confocal Laser Scanning Microscopy (CLSM)-Proof of Concept, *INRIA Research Report No 6493*. France: Institut National de Recherche en Informatique et en Automatique (INRIA).

Pappas, D., Smith, B. W., & Winefordner, J. D. (2000). Raman spectroscopy in bioanalysis. *Talanta*, *51*, 131-144.

Parvathy, K. S., Susheelammab, N. S., & Tharanathana, R. N. (2007). Hydration characteristics of guar gum samples and their fractions. *Food Hydrocolloids*, *21*, 630–637.

Pavia, D. L., Lampman, G. M., & Kriz, G. S. (2001). *Introduction to spectroscopy, a guide to students of organic chemistry*, 13-101.

Pedersen, D. K. (2002). Spectroscopic and chemometric exploration of food quality – Early prediction of meat quality. *PhD Thesis*. The Royal Veterinary and Agricultural University, Denmark.

Peterbauer, T., Lahuta, L. B., Blöchl, A., Mucha, J., Jones, D. A., Hedley, C. L., Górecki, R. J., & Richter, A. (2001). Analysis of the raffinose family oligosaccharide pathway in pea seeds with contrasting carbohydrate composition[1]. *Plant Physiology*, *127*, 1764–1772.

Pollard, M. A., Eder, B., Fischer, P., & Windhab, E. J. (2010). Characterisation of galactomannans isolated from legume endosperms of Caesalpinioideae and Faboideae subfamilies by multi detection aqueous SEC. *Carbohydrate Polymers*, *79*, 70 – 84.

Poulsen, F. M. (2002). *A brief introduction to NMR spectroscopy of proteins*. http://www.cs.duke.edu/brd/Teaching/Bio/asmb/current/2papers/Intro-reviews/flemming.pdf.

Powell, A. M. (1987). Marama bean (Tylosema esculentum, Fabaceae) seed crop in Texas. *Economic Botany*, *41*, 216 – 220.

Quigley, M. E., Hudson, G. J., & Englyst, H. N. (1999). Determination of resistant short-chain carbohydrates (non-digestible oligosaccharides) using gas-liquid chromatography. *Food Chemistry, 65*, 381–390.

Ravindranathan, S., Karlsson, T., Lycknert, K., Widmalm, G., & Levitt, M. H. (2001). Conformation of the glycosidic linkage in a disaccharide investigated by double-quantum solid-state NMR. *Journal of Magnetic Resonance*, *151*, 136-141.

Ravn, C., Skibsted, E., & Bro, R. (2008). Near-infrared chemical imaging (NIR-CI) on pharmaceutical solid dosage forms- Comparing common calibration approaches. *Journal of Pharmaceutical and Biomedical Analysis*, *48*, 554-561.

Reddy, N. R., Pierson, M. D., Sathe, S. K., & Salunkhe, D. K. (1984). Chemical, nutritional and physiological aspects of dry bean carbohydrates-A review. *Food Chemistry*, *13*, 25-68.

Redondo-Cuenca, A., Villanueva-Suárez, M. J., Rodríguez-Sevilla, M. D., & Mateos-Asparicio, I. (2006). Chemical composition and dietary fibre of yellow and green commercial soybeans (Glycine max). *Food Chemistry*, *101*, 1216–1222.

Reimer, L. & Kohl, H. (2008). *Transmission Electron Microscopy, Physics of Image Formation, 5th ed*. New York: Springer Science & Business Media.

Rombouts, F. M., & Thibault, J. F. (1986). Feruloylated pectic substances from sugar-beet pulp. *Carbohydrate Research*, *154*, 177-187.

Saldivar, X., Wang, Y., Chen, P., & Hou, A. (2011). Changes in chemical composition during soyben seed development. *Food Chemistry, 124*, 1369 – 1375.

Sandhu, K. S., & Lim, S. T. (2008). Digestibility of legume starches as influenced by their physical and structural properties. *Carbohydrate Polymers*, *71*, 245-252.

Schadel, W. E., Walter Jr., W. M., & Young, C. T. (1983). Cotyledon structure of resting peanut (*Arachis Hypogaea* L. cv. Florunner) seed before and after hydraulic pressing. *Journal of Food Science*, *48*, 1405–1407.

Scheller, H. V., Jensen, J. K., Sørensen, S. O., Harholt, J., & Geshi, N. (2007). Biosynthesis of pectin. *Physiologia Plantarum*, *129*, 283 - 295.

Scott, M. P., Jane, J. L., & Soundararajan, M. (1999). Carbon isotope ratios of amylose, amylopectin and mutant starches. *Phytochemistry*, *52*, 555-559.

Scott, R. P. W. (2003). *Book 1: Principles and Practice of Chromatography*, 1-104. Libraryforscience (LLC), Chrom-Ed Book Series.

Séné, C. F. B., McCann, M. C., Wilson, R. H., & Grinter, R. (1994). Fourier-transform Raman and fourier-transform infrared spectroscopy[1]: An investigation of five higher plant cell walls and their components. *Plant Physiology*, *106*, 1623 – 1631.

Sheppard, N. (2002). The historical development of experimental techniques in vibrational spectroscopy. In J. M. Chalmers & P. R. Griffiths, *Hanbook of Vibrational Spectroscpy, Theory and Instrumentation, Vol. 1*. UK: Jihn Wiley & Sons Ltd.

Shiga, T. M., & Lajolo, F. M. (2006). Cell wall polysaccharides of common beans (*Phaseolus vulgaris* L.) - composition and structure. *Carbohydrate Polymers*, *63*, 1-12.

Shiga, T. M., Lajolo, F. M., & Filisetti, T. M. C. C. (2003). Cell wall polysaccharides of common beans (*Phaseolus vulgaris* L.). *Ciência e Tecnologia Alimentos*, *23*, 141-148.

Shiga, T. M., Lajolo, F. M., & Filisetti, T. M. C .C. (2004). Changes in the cell wall polysaccharides during storage and hardening of beans. *Food Chemistry*, *84*, 53-64.

Sinha, A. K., Kumar, V., Makkar, H. P. S., De Boeck, G., & Becker, K. (2011). Non-starch polysaccharides and theri role in fish nutrition – A review. *Food Chemistry*, *127*, 1409-1426.

Smith, E., & Dent, G. (2005). *Modern Raman Spectroscopy - A Practical Approach*. England: John Wiley and Sons.

Sørensen, I., Pedersen, H. L., & A., Willats, W.G.T. (2009). An array of possibilities for pectin. *Carbohydrate Research*, *344*, 1872-1878.

Sørensen, I., Pettolino, F. A., Wilson, S. A., Doblin, M. S., Johansen, B., Bacic, A., & Willats, W.G.T. (2008). Mixed-linkage (1→3), (1→→4)-β-D-glucan is not unique to the Poales and is an abundant component of *Equisetum arvense* cell walls.*The Plant Journal*, *54*, 510-521.

Sparkman, D., Penton, Z. E., & Kitson, F. G. (2011). Gas chromatography. In D. Sparkman, Z. E. Penton & F. G. Kitson, *Gas Chromatography and Mass Spectrometry: A Practical Guide, 2nd ed.*, 15-83. UK: Academic Press.

Stick, R. V., & Williams, S. J. (2009). Carbohydrates: *The Essentials Molecules of Life, 2nd Ed.* UK: Elsevier Ltd.

Synytsya, A., Copikova, J., Matejka, P., & Machovic, V. (2003). Fourier transform Raman and infrared spectroscopy of pectins. *Carbohydrate Polymers*, *54*, 97-106.

Tapie, N., Malhiac, C., Hucher, N., & Grisel, M. (2008). Determination of galactose and mannose residues in natural galactomannans using a fast and efficient high-performance liquid chromatography/UV detection. *Journal of Chromatography A*, *1181*, 45–50.

Thygesen, L.G., Løkke, M. M., Micklander, E., & Engelsen, S. B. (2003). Vibrational microspectroscopy of food. Raman vs. FT-IR. *Trends in Food Science and Technology*, *14*, 50-57.

UmBio. (2011). UmBio Inspector. http://www.umbio.com.

United States Department of Agriculture (2011). USDA National Nutrient Database for Standard Reference, Release 24: Legumes and Legume Products. Http://www.ars.usda.gov/nutrientdata.

Vaidehi, M. P., & Kadam, S. S. (1989). Soybean. In D. K. Salunkhe, & S. S. Kadam, *CRC handbook of world legumes: nutritional chemistry, processing technology, and utilization, Vol. III*, 1-31. Florida: CRC Press.

Van der Maesen, L. J. G. (2006). Tylosema esculentum (Burch.) A.Schreib. In M. Brink, & G. Belay, *Plant resources of Tropical Africa 1. Cereals and pulses*, 187 – 190. Wageningen: PROTA (Plant Resources of Tropical Africa / Ressources végétales de l'Afrique tropicale) Foundation.

Van Soest, J. J. G. (2004). Spectroscopy of polysaccharides. In P. Tomasik, *Chemical and Functional Properties of Food Saccharides*, 371-385. Florida: CRC Press.

Vasić, M. A., Vujičić, B. L., Tepić, A. N., Gvozdanović-Varga, J. M., & Šumić, Z. M. (2009). Dietary fibre content in some dry legumes. *APTEFF (Acta Periodica Technologica)*, *40*, 113-110.

Viereck, N., Dyrby, M., & Engelsen, S. B. (2005). Monitoring thermal processes by NMR technology. In D. Sun, *Emerging Technologies for Food Processing*, 553-575. London: Elsevier Academic Press.

Viñarta, S. C., Molina, O. E., Figueroa, L. I. C., & Fariña, J.I. (2006). A further insight into the applications of exopolysaccharides from *Sclerotium rolfsii*. *Food Hydrocolloids*, *20*, 619-629.

Vincken, J. P., Schols, H. A., Oomen, R. J. F. J., McCann, M. C., Ulvskov, P., Voragen, A. G. J., & Visser, R. G. F. (2003). If Homogalacturonan Were a Side Chain of Rhamnogalacturonan I. Implications for Cell Wall Architecture. *Plant Physiology*, *132*, 1781-1789.

Voragen, A. G. J. (1998). Technological aspects of functional food-related carbohydrates. *Trends in Food Science and Technology*, *9*, 328–335.

Wang, T. L., Bogracheva, T. Y., & Hedley, C. L. (1998). Starch: As simple as A, B, C? *Journal of Experimental Botany*, *49*, 481-502.

Weber, H., Borisjuk, L., & Wobus, U. (2005). Molecular physiology of legume seed development. *Annual Review of Plant Biology*, *56*, 253-279.

Webster, B. D., & Leopold, A. C. (1977). The ultrastructure of dry and imbibed cotyledons of soybean. *American Journal of Botany*, *64*, 1286–1293.

Wicks, R. J., Moran, M. A., Pittman, L. J., & Hodson, R. E. (1991). Carbohydrate signatures of aquatic macrophytes and their dissolved degradation products as determined by a sensitive high-performance ion chromatography method. *Applied and Environmental Microbiology*, *57*, 3135-3143.

Williams, D. B., & Carter, C. B. (2009). *Transmission Electron Microscopy, a Textbook for Materials Science*. New York: Springer Science & Business Media.

Williams, P., Geladi, P., Fox, G., & Manley, M. (2009). Maize kernel hardness classification by near infrared (NIR) hyperspectral imaging and multivariate data analysis. *Analytica Chimica Acta*, *653*, 121–131.

Williats, G. T. W., McCartney, L., Mackie, W., & Knox, J. P. (2001). Pectin: cell biology and prospects for functional analysis. *Plant Molecular Biology*, *47*, 9 – 27.

Wunderlin, R., Larsen, K., & Larsen. S. S. (1981). Cercideae. In R. M. Polhill, & P. H. Raven, *Advances in legume systematics, part 1*, 107–116. Kew: Royal Botanic Gardens.

Yaseen, E. I., T.J. Herald, T. J., Aramouni, F. M., & Alavi, S. (2005). Rheological properties of selected gum solutions. *Food Research International*, *38*, 111–119.

Young, C. T., Pattee, H. E., Schadel, W. E., & Sanders, T. H. (2004). Microstructure of peanut (*Arachis Hypogaea* L. cv. 'NC 7') cotyledons during development. *Food Science and Technology*, *37*, 439–445.

Youssef, A. M., El-Fouly, M. M., & El-Baz, F. K. (1974). Isolation and chemical composition of protein concentrates from soya bean, rice bran and protelan. *Quality Plant-Plant Foods for Human Nutrition*, *XXIV*, 71–84.

Yu, J., Ahmedna, M., & Goktepe, I. (2007). Peanut protein concentrate: Production and functional properties as affected by processing. *Food Chemistry*, *103*, 121–129.

Zheng, Y., Pan, Z., Zhang, R., Wang, D., Labavitch, J., & Jenkins, B. M. (2006). Dilute acid pre-treatment and enzymatic hydrolysis of saline biomass for sugar production. American Society of Agricultural and Biological Engineers (ASABE), *An ASABE Meeting Presentation*, *Paper Number 067003*.

Paper I

Proximate composition, histochemical analysis and microstructural localisation of nutrients in immature and mature seeds of marama bean *(Tylosema esculentum)* - an underutilised legume

Minah M. Mosele, Åse S. Hansen, Michael Hansen, Alexander Schulz and Helle J. Martens

Food Chemistry

Food Chemistry 127 (2011) 1555–1561

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Proximate composition, histochemical analysis and microstructural localisation of nutrients in immature and mature seeds of marama bean (*Tylosema esculentum*) – An underutilised food legume

Minah Mmoni Mosele [a,b], Åse Solvej Hansen [a,*], Michael Hansen [c], Alexander Schulz [c], Helle Juel Martens [c]

[a] Department of Food Science, Faculty of Life Sciences, University of Copenhagen, Rolighedsvej 30, 1958 Frederiksberg C, Denmark
[b] Department of Extension and Training, National Food Technology Research Centre, Mpuutsane Industrial Area, Private Bag 008, Kanye, Botswana
[c] Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark

ARTICLE INFO

Article history:
Received 31 May 2010
Received in revised form 12 January 2011
Accepted 3 February 2011
Available online 26 February 2011

Keywords:
Marama bean
Morama bean
Tylosema esculentum
Proximate composition
Histochemistry
CLSM
Seed microstructure

ABSTRACT

Marama bean (*Tylosema esculentum*) is a wild-growing legume adapted to semi-arid conditions in southern Africa. Both immature and mature seeds are used as food by locals and marama bean has potential as a crop plant. Physicochemical and histochemical methods were used to study the accumulation of nutrients and their localisation in immature and mature seeds. The immature seeds had a high content of moisture (67%) and protein (21%), and a low content of lipid (1.5%). At maturity, proteins formed spherical bodies that were embedded in a droplet lipid matrix. The mature seeds are exceptional as they have a high content of protein (32%) and lipid (40%) and no starch. Staining of polysaccharides indicated increases of pectin and cellulose during maturation, parallel with the general increase of cell wall thickness; however, lignin was absent. The content and distribution of protein, lipid and carbohydrates in immature and mature marama beans make this underutilised nutritive legume a prospective crop plant and interesting for food processing applications.

1. Introduction

Marama bean (*Tylosema esculentum* Burchell A. Schreiber) is a wild perennial legume species and a prospective new crop in Southern Africa because of its exceptionally high nutritional value (Hartley, Tshamekeng, & Thomas, 2002). The species is native to the Kalahari Desert and neighbouring sandy semi-arid regions of Southern Africa, in particular Botswana, Namibia and the northern part of South Africa (Castro, Silveira, Coutinho, & Figueiredo, 2005; Hartley et al., 2002). Botanically, *Tylosema esculentum* belongs to the tribe Cercideae in the subfamily Caesalpinioideae within Fabaceae, and is thus related to *Cercis* and *Bauhinia* (Wunderlin, Larsen, & Larsen, 1981). Species of the genus *Tylosema* were previously included in *Bauhinia*, but were later established as a separate genus (Castro et al., 2005).

Apart from *T. esculentum*, there are four other known species within the genus, namely, *T. fassoglense, T. argenteum, T. humifusum and T. angolense* (Castro et al., 2005); among these, *T. fassoglense* is also edible (Brink, 2006). The species of *Tylosema* are unique within the family Fabaceae in the fact that their flowers exhibit heterostyly, meaning that they are self incompatible because of spatial separation of the stigma and anthers (Hartley et al., 2002). This may reduce propagation potential, and obviously reduces seed production rates for cropping. In a field experiment carried out in Texas (USA) the marama bean plant took 4.5 years to produce edible seeds, with flowering at 2 years, development of fruits or seeds at 3.5 years, and final harvesting at full maturation one year later (Powell, 1987).

The mature seeds of marama bean are encapsulated in hard, woody seed coats, reddish to brownish-black in colour (Van der Maesen, 2006). The seeds, commonly called marama or morama bean, tsin bean or gemsbok bean, are an important component of the diet among the nomadic "hunter-gatherers" in remote settlements, where few conventional crops can survive (National Research Council, 1979). Raw mature seeds of marama beans store well and remain edible for years under dry storage conditions (Van der Maesen, 2006).

The immature seeds of marama bean, inclusive of seed coat, are used as vegetables and the mature seeds are normally eaten boiled or roasted, and have a sweet flavour, with some bitterness in some varieties (Van der Maesen, 2006). The roasted mature seeds can be used to make butter, similar to peanut butter. Flour prepared from mature marama seeds can be used to prepare beverages and can also be added to cereals to increase the nutritive value (Van der Maesen, 2006).

* Corresponding author. Tel.: +45 35333241; fax: +45 35333245.
E-mail address: aah@life.ku.dk (Å.S. Hansen).

0308-8146/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2011.02.017

It is known that mature marama seeds store large quantities of lipids and proteins in the storage cotyledons, both well above 30% (Amarteifio & Moholo, 1998; Bower, Hertel, Oh, & Storey, 1988; Holse, Husted, & Hansen, 2010), making them comparable to soybeans (*Glycine max*) and peanuts (*Arachis hypogaea*). The marama beans are a source of edible oil used for cooking, especially in Botswana. Both, the composition of fatty acids in marama oil and the content of phytosterols and vitamin E have been investigated (Ketshajwang, Holmback, & Yeboah 1998; Mitei, Ngila, Yeboah, Wessjohann, & Schmidt, 2009). The essential amino acids present are tyrosine, being the highest, followed by arginine, leucine and lysine (Bower et al., 1988). Recently it has been shown that marama bean has a very high content of dietary fibre with variation between 19% and 27%, a high content of lignans, and no content of cyanogenic glycosides, as well as the potent allergens found in peanut and lupin (Holse, Husted, & Hansen, 2010).

The effects of food processing have only been studied on the functional properties of marama flour (Jideani, Van Wyk, & Cruywagen, 2009; Maruatona, Duodu, and Minnaar (2010). The domestication of marama bean should be encouraged because of its nutritional value and also due to its potential use as a food crop in arid areas (Bower et al., 1988) and regions with erratic rainfall (National Research Council, 1979).

Despite the potential of marama bean as a healthy nutritive crop for developing countries, not much is known about *in situ* localisation of nutrients. The nutritional value of immature seeds has not been investigated at all. Any developmental studies have been limited to the flowering parts of marama bean plant (Castro et al., 2005; De Frey, Coetzer, & Robbertse, 1992; Hartley et al., 2002).

Legumes store lipids, proteins and various types of polysaccharides as major reserves in their seeds. Although the basic microstructure of mature legume seeds is well-known, especially from commercial crops such as soybean (Webster & Leopold 1977) and peanut (Lott & Buttrose 1977), less is known about the early deposition of seed reserves. To our knowledge, there is one study on mature marama bean protein bodies (Amonsou, Taylor, & Minnaar, 2011), and that study did not present the complete microstructure of the seeds. Marama bean has commercial potential for agriculture in the areas where it grows, because of its high oil and protein contents and it is therefore important to study the nutritional value of the immature pods and the mature marama seed since the bean is consumed at both stages. The distributions of the various food components are best understood by way of histochemistry and this is important information in its processing and industrial applications. The aim of our study is to characterise the development and localisation of chemical components in marama bean seeds at two developmental stages, termed "immature" and "mature" stages of consumption, by means of physicochemical and histochemical analyses and electron microscopy.

2. Materials and methods

2.1. Plant material

Seeds of marama bean (*T. esculentum*) were collected in 2008, from multiple plants growing in their natural habitat in the southern region of Botswana. Whole pods with immature seeds, at the stage where they are normally consumed, were stored at −20 °C. Mature seeds were kept at 4 °C.

2.2. Linear dimensions

Linear measurements were taken according to the method of Mpotokwane, Gaditlhatlhelwe, Sebaka, and Jideani (2008), with slight modifications, as reported for marama bean by Jideani et al. (2009). Twenty seeds were randomly selected by collecting a handful from a random location in a bag for containing the seeds. The length (L), width (W) and thickness (T) were measured to an accuracy of 0.001 mm, using a vernier calliper.

2.3. Proximate composition

The samples were decorticated with a hammer and knife, and the cotyledons were milled into flour in a laboratory mill (IKA A10, Labortechnik, Staufen, Germany) for 15 s. Marama flour was passed through a 1 mm diameter mesh sieve, except for the flour from immature seeds because it formed a paste and was used in that state. All samples were analysed in triplicate. Proximate composition was determined by approved standard methods of analysis with a few modifications, as reported by Holse, Husted, and Hansen (2010). The methods used were AOAC (2000) for protein, moisture, lipid and ash. Results were expressed as a percentage in wet basis (as is).

2.4. Transmission electron microscopy (TEM)

Storage cotyledons were isolated from thawed immature seeds and dry mature seeds. Small 3 × 3 × 5 mm pieces of seed coat (in immature seeds only), and outer and central parts of cotyledons were fixed for 4 h in Karnovsky's fixative (5% glutaraldehyde, 4% paraformaldehyde, 0.1 M sodium cacodylate buffer), including a vacuum treatment, washed in cacodylate buffer at pH 7.3 and post-fixed in 1% osmium tetroxide (with 0.1 M cacodylate buffer) for 8 h at 4 °C. After washing in buffer and water, the samples were dehydrated in a graded acetone series, infiltrated with three different ratios of Spurr resin to acetone and embedded in Spurr resin within flat moulds. The resin was polymerised in an oven at 60 °C for 8 h. Ultra-thin sections were cut with a diamond knife, using a Reichert-Jung/LKB Supernova ultramicrotome and collected on pioloform-coated copper grids. Sections were contrasted with 1% uranyl acetate and lead citrate (2.7% in 3.5% sodium citrate) and examined in a Philips CM 100 TEM at 60 kV.

2.5. Light microscopy (LM)

Samples for LM were collected from the pool of samples prepared for TEM, as described above, except samples of endosperm which were collected in fresh tissue of immature and mature seeds. Semi-thin sections of 2 μm were cut with glass knives on the ultramicrotome and stained with 1% Aniline Blue Black in 7% acetic acid for proteins, periodic acid Schiffs (PAS) for insoluble carbohydrates containing 1,2-glycol groups, Sudan Black for lipids (saturated solution in 70% ethanol), 2% iodine solution (I_2 KI) for starch, phloroglucinol (mixture of 0.1 g phloroglucinol, 16 ml concentrated HCl and 84 ml 95% ethanol) for lignin and 0.01% Calcofluor White M2R for β-1, 4 linked glucans (cellulose), following the methods of O'Brien and McCully (1981). Coriphosphine O (0.03%) was used for staining pectin (Ueda & Yoshioka, 1976). Sections were viewed in immersion oil in a Nikon Eclipse 80i light and fluorescence microscope.

2.6. Scanning electron microscopy (SEM)

For SEM, specimens were cut with a razor blade into small wedges (3 × 5 × 5 mm) and fixed overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7, at 4 °C. After washing overnight in 0.1 M cacodylate buffer with 5% sucrose, pH 7, samples were washed for 5 min in the same solution and post-fixed for 1 h in 2% osmium tetroxide, in 0.1 M cacodylate buffer with 5% sucrose, pH 7, at room temperature. Following washing steps in buffer and water, samples were dehydrated in a graded ethanol series.

Further dehydration was done in hexamethyldisilazane (HDMS) for 30 min. Samples were mounted onto metal stubs with double-sided carbon tape and sputter-coated with a thin layer of gold/palladium under vacuum, using an automated sputter coater SC7640 (Quorum Technologies, Newhaven, UK). The samples were viewed in a Quanta 200 Microscope (FEI Company™).

2.7. Confocal laser scanning microscopy (CLSM)

Samples were cut from fresh samples with a razor blade into thick hand sections. They were specifically stained for neutral lipids with Nile Red (0.1 μg/ml) according to the method by Greenspan, Mayer, and Fowler (1985). After rinsing in water, images from intact cells were recorded in a Leica SPII confocal laser scanning microscope, using the 543 nm excitation line and 570–595 nm emission.

2.8. Statistical analysis

The quantitative data was presented as means of three replicates, using Microsoft Excel. Images were processed with Adobe Photoshop CS2.

3. Results and discussion

3.1. Linear dimensions

Mature marama bean seeds, on average, were 19.9 (±1.6) mm in lengths in a range of 17.0–23.0 mm, 17.6 (±2.5) mm in widths in a range of 11.5–20.5 mm and 13.1 (±0.9) mm in thicknesses in a range of 12.0–15.0 mm. Our results are similar to those reported by Jideani et al. (2009) for the same species.

3.2. Proximate composition

The protein content of mature seeds was above 30%, but it was already ca. 20% at the immature stage (Table 1) which means that the protein is stored in the seed from the early stage of development. The content of lipids was as high as 40% in the mature seeds but was negligible at the immature stage. Accordingly, lipid storage occurs in late stages of seed maturation. The carbohydrate content of mature seeds was twice that of immature seeds. The results of major chemical components in mature seeds are within the amounts reported by Bower et al. (1988), Amarteifio and Moholo (1998) and Holse, Husted, and Hansen (2010). The content of lipids in the immature seeds was similar to that reported by Redondo-Cuenca, Villanueva-Suárez, Rodríguez-Sevilla, and Mateos-Asparicio (2006) for green (immature) soybeans at 0.93 g/100 g. The ash and moisture contents for mature seeds were below ca. 5%. Immature seeds had a moisture content of ca. 67%.

The results confirm that mature marama beans have high contents of protein and lipid, comparable to those of other oil seeds such as soybean and peanut (Adsule, Kadam, & Salunkhe, 1989; Nkama & Filli, 2006; Redondo-Cuenca et al., 2006; Vaidehi & Kadam, 1989). However, marama bean protein content was slightly above that of peanut, and the content of lipids was almost twice that of soybean. Thus, marama bean would be a good crop for supplementation or fortification of diets of the local communities. From the chemical results we can conclude that lipid, protein and carbohydrate accumulate as the seeds mature and, like soybean and peanut, these major intracellular food reserves are stored in the thick cotyledons during seed development.

Table 1
Proximate composition (%) of immature and mature marama bean cotyledon (as is).

Sample	Moisture	Ash	Protein	Lipid	Carbohydrate[a]
Immature	66.8 (0.4)	2.2 (0.1)	20.8 (0.4)	1.5 (0.1)	8.6
Mature	5.3 (0.2)	3.0 (0.0)	32.3 (0.8)	40.0 (0.7)	19.4

Values in parentheses indicate standard deviations.
[a] Carbohydrate by difference.

3.3. Histochemistry of seed coat and endosperm

Immature and mature marama seeds are seen in Fig. 1A. The colour of the seed coat is cream in the immature seeds and brown in the mature seeds. The seed coat consists of a double layer of lignified macrosclereids with a callose-rich light line, underlying spongy parenchyma cells (Fig. 1B) and an inner epidermis. In contrast to e.g. soybean, a subepidermal layer of so-called hourglass cells storing proteins (Moïse, Han, Gudynaite-Savitch, Johnson, & Miki, 2005) does not exist in marama bean. The seed coat could potentially be a source of phenolic compounds for application as natural antioxidants (Oomah, Cardador-Martínez, & Loarca-Piña, 2005; Zadernowski, Borowska, Naczk, & Nowak-Polakowska, 2001). Direct microscopic localisation of phenolics is, however, restricted to anthocyanin-containing compartments, while other phenolics are auto-fluorescent and can be detected by fluorescence microscopy (Hutzler et al., 1998), as also noted in our study (not shown). At the immature stage, also, a nuclear endosperm is present (Fig.1D) which provides a temporal source of nutrition for the growing embryo. Later, cell walls develop in the endosperm (Fig. 1E), consisting of peripheral cells with dense proteins and lipids, but no starch (not shown). As in other Fabaceae species, e.g. *Trifolium* (Jakobsen, Martens, & Lyshede, 1994), the endosperm is used up during seed development and the nutrients are translocated into the thick cotyledons (Fig. 1F). This contrasts with the closely related species *Cercis siliquastrum,* where the endosperm does persist in the mature seeds (Baldan et al., 1995).

3.4. Histochemistry of cell walls in storage cotyledons

Coriphosphine O, Calcofluor White, and PAS were used as histochemical tests, specific for pectin, cellulose and 1–4 bound polyglucans (e.g. starch, cellulose and hemicelluloses), respectively (Luza, van Gorsel, Polito, & Kader, 1992; Marcus et al., 2008).

Parenchyma cell walls in the storage cotyledons are rich in pectin, especially within the middle lamella, as seen by Coriphosphine O fluorescent dye (Fig.1G–I). In the immature tissue the new cell walls are pectin-positive, visible as a thin straight line clearly depicting newly divided cells (Fig.1G). In mature seeds, pectin is a prominent constituent of epidermis cell walls and around intercellular spaces of the storage parenchyma cells (Fig.1I). These cells have primary cell walls which increase in thickness during maturation, consisting mainly of cellulose as indicated by Calcofluor White staining under UV excitation (Fig.1J,K). Provascular strands were noted (Fig. 3B), but lignification of prospective xylem elements had not started yet, as tested by phloroglucinol staining (not shown). PAS-staining (Fig. 1L–N), as well as iodine-staining (not shown), revealed that marama bean, in contrast to other legumes, e.g. peanut (Schadel, Walter, & Young, 1983; Young, Pattee, Schadel, & Sanders, 2004), does not store detectable amounts of starch. Insoluble polysaccharides, strongly positive with PAS, were deposited in the cell walls in both immature and mature seeds (Fig.1L–N). In these images, post fixation contrasting is seen as grey and black areas labelling lipophilic and proteinaceous substances. Compatible with the changes in proximate composition, it can be concluded that the thickening of cell walls during maturation is accompanied by an increase of insoluble carbohydrates, namely cellulose and pectin. It should further be noted that, within the outermost cells in immature cotyledons, the cytoplasm is somewhat shrunken, possibly due to some extent

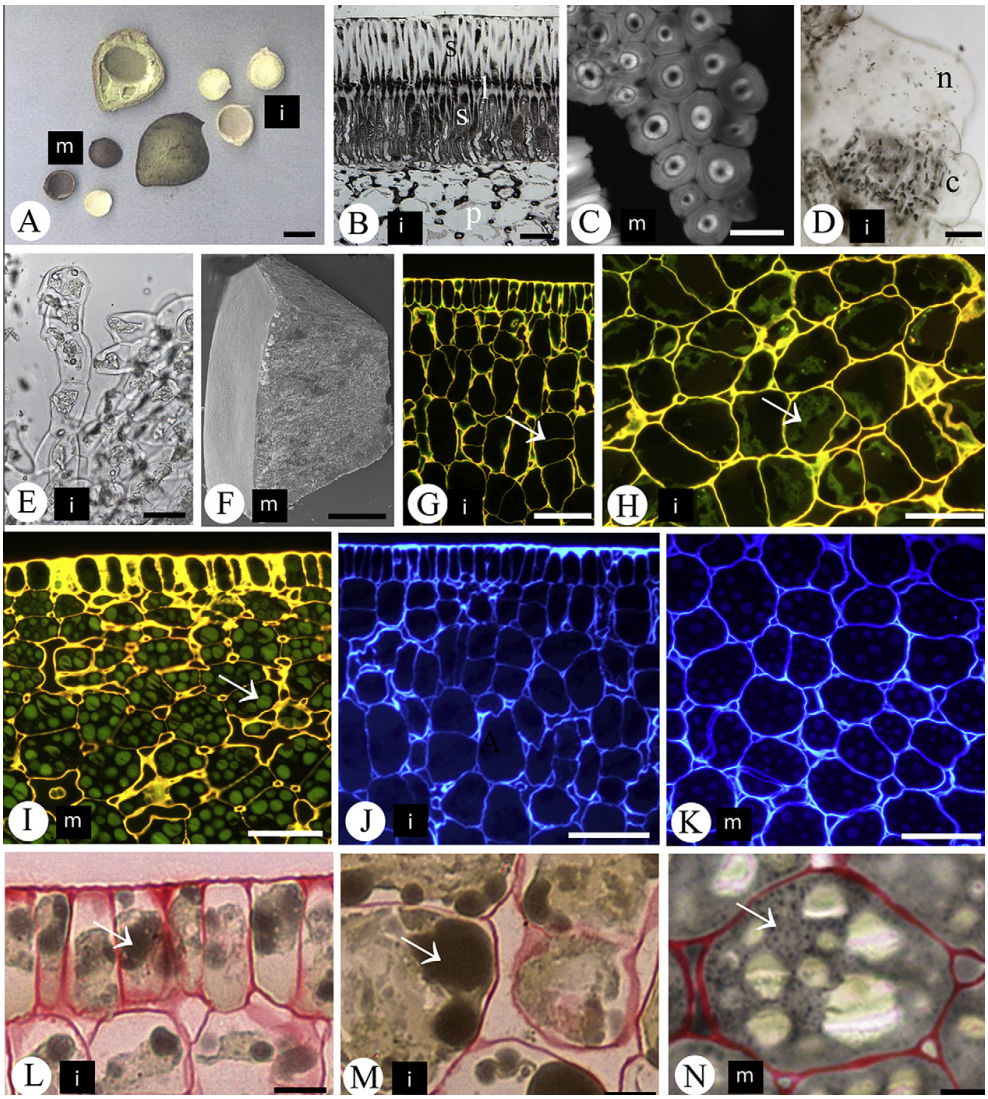


Fig. 1. Histochemistry of the cell wall of immature (i) and mature (m) marama seeds. Presence of pectin is indicated by Coriphosphine O, cellulose by Calcofluor White and 1,4 bound polyglucans by the PAS reaction. Opened pod of immature (i) and mature (m) seeds (A). Bar = 20 mm. Cross section of the seed coat showing the palisade layer of macrosclereids (s) with the light line of callose (l) and parenchyma tissue (p) (B). Bar = 25 μm. Cross section through a group of macrosclereids in the seed coat (C). Bar = 25 μm. Cellular (c) and nuclear (n) endosperm (D). Bar = 100 μm. Cells from the cellular endosperm (E). Bar = 100 μm. Block of storage cotyledon tissue, as trimmed for SEM (F). Bar = 1 mm. Pectin in the cell walls of developing storage cotyledon is seen as yellow fluorescence after Coriphosphine O staining (G). Newly formed cell walls (arrow). Bar = 100 μm. Pectin in central part of developing storage cotyledon (H). Note appearance of a green fluorescence from proteinaceous substances (arrow). Bar = 100 μm. Pectin and protein bodies (arrow) in mature tissue (I). Bar = 100 μm. Cellulose (blue) in immature (J) and mature cotyledons (K). Bar = 100 μm. Insoluble carbohydrates (red) detected by PAS reaction in immature cotyledons (L, M) and mature seed (N). Some cytoplasmic compounds are osmiophilic and stained grey to black due to OsO_4 fixation (arrows). Bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of dehydration during the collection period or non-optimal freezing conditions during transport.

3.5. Lipids and proteins in immature storage cotyledons

After biosynthesis at the endoplasmic reticulum (ER), seed intracellular storage proteins bud off as separate organelles (protein bodies, PBs), which either accumulate in the cytoplasm or are sequestered into vacuoles (protein storage vacuole, PSV) by autophagy (Herman and Larkins 1999). The terminology is, however, not clear, and we will (as many authors) refer to these storage vacuoles as protein bodies (PBs). In the SEM, many tiny globules, presumably PBs, are present in the developing storage tissue (Fig. 2A). Intracellular protein depositions, as well as

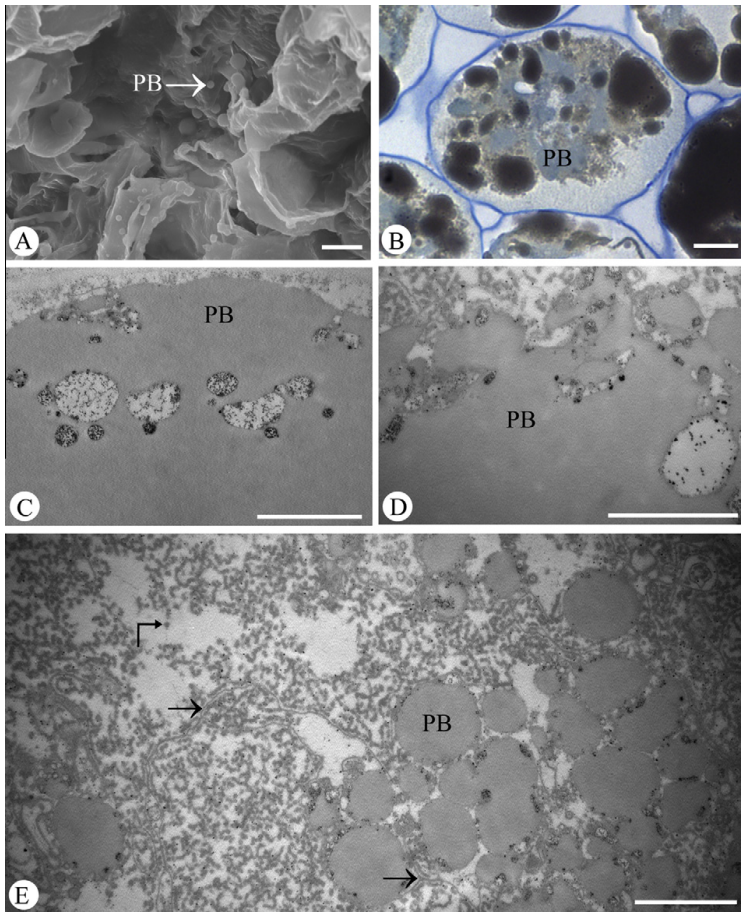


Fig. 2. The early deposition of protein and lipid in seeds of immature marama bean. SEM of developing storage parenchyma showing small spherical protein bodies (PB) (arrow) (A). Light microscopy labelling of cytoplasmic and cell wall proteins with Aniline Blue Black (blue). Also note black labelling of osmiophilic substances (B). TEM of the amorphous appearance of a PB (C). Aggregation of PBs (D). Spherical PBs in close association with ER (straight arrows) and ribosomes (bent arrow) (E). Bars = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proteins within the cell walls, are seen in the light microscope after staining with Aniline Blue Black (Fig. 2B). The pale blue substances within the parenchyma cell indicate the early onset of protein deposition, also recognised in TEM (Fig. 2C–E). The amorphous PBs show cytosolic inclusions (Fig. 2C) and seem to fuse with each other (Fig. 2D). At this developmental stage the PBs were closely associated with long strands of rough ER and sometimes small protein deposits were encircled by ER (Fig. 2E). From our electron microscopy data we hypothesise that the marama bean PBs are actually of the cytosolic type, forming large aggregates and not the PSV type, which we would expect to be smooth-surfaced vesicles. In consistency with the chemical data, lipid bodies (LBs) are not present at the early stage of maturity.

3.6. Lipids and proteins in mature storage cotyledons

At the mature stage of seed development, the voluminous cotyledons almost completely occupy the embryo sac cavity. Cells of the central part of the cotyledon are 30 ± 5 μm in length and 22 ± 5 μm in width. Protein bodies (13 ± 3 μm diameter) and lipid bodies (0.87 ± 0.17 μm diameter) are the dominant features of the cytoplasm (Figs. 3 and 4). PBs decorated with LBs can be seen in mature storage parenchyma, using SEM (Fig. 3A). Large PBs (up to 40 μm diameter) are located within the subepidermal cell layers (Fig. 3G and H). We observed a trend of small PBs in the epidermal layer, which increase in size but decrease in number in the sub-epidermis, and finally increase in number but decrease in size at the centre of the cotyledon (Fig. 3G–I). Interestingly, protein bodies are labelled green with Coriphosphine O (Fig. 3B, see Fig. 1H–I, for comparison). The finding that this pectin-specific dye (yellow emission) also specifically labels cytosolic proteins (green emission) was confirmed by control-staining of other protein-rich materials, e.g. sunflower seeds and *Phaseolus* beans (not shown). The PBs stained blue with Aniline Blue Black (Fig. 3C). In post-fixed samples PBs are surrounded by a greyish substance indicative of osmiophilic LBs (Fig. 3D). This was proved by Sudan Black staining (Fig. 3E) in which PBs and cell walls are clearly unlabelled. PBs consist of an electron-dense proteinaceous matrix, often containing spherical cytosolic inclusions (Fig. 3F and H) called globoids (Lott & Buttrose, 1977; Lott, Ockenden,

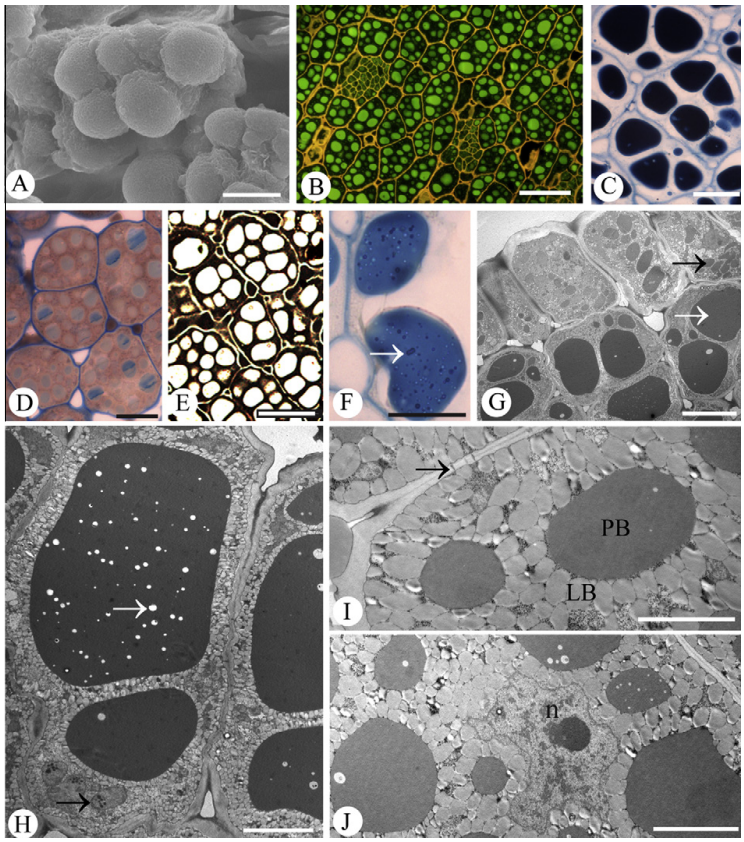


Fig. 3. Storage protein and lipid in seeds of mature marama bean. SEM of parenchyma cells with large proteins bodies (PBs) covered with lipid bodies (LBs) (A). Bar = 10 μm. Coriphosphine O-stained section, showing green fluorescence from PBs and yellow fluorescence from pectin in middle lamella (B). Bar = 100 μm. Aniline Blue Black staining of PBs and cell wall proteins without postfixation (C), and with lipids visible after postfixation (D). Bar = 25 μm. Sudan Black labels lipid material surrounding unstained PBs (E). Bar = 25 μm. Mineral globoids (arrow) in PBs (F). Bar = 25 μm. TEM of epidermis and subepidermal layers. Note that small size PBs in epidermis (black arrow) and large PBs in subepidermal cells (white arrow) (G). Bar = 10 μm. Subepidermal cells with LBs, proplastids (black arrow) and large PBs with numerous globoids (white arrow) (H). Bar = 5 μm. LBs encircling PBs. Plasmodesmata through cell walls (arrow) (I). Bar = 5 μm. Section showing large-lobed nucleus (n) (J). Bar = 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Raboy, & Batten, 2000). Studies of the elemental composition and distribution of mineral reserves within globoids in legumes revealed P, K, Mg and Ca salts of phytic acid (Lott & Buttrose, 1977). Cereal grains and oil seeds are particularly rich sources of phytate.

Lipids are abundant at the mature stage of the seeds. Numerous electron-transparent lipid bodies (LBs) encircle the PBs and line the cytoplasmic side of the plasma membrane (Fig. 3I). LBs do not fuse and appear to be bound by a thin membrane, presumably a half unit membrane. LBs, like PBs, probably originate from the ER. The distribution of LBs within individual parenchyma cells was judged by the application of Nile Red and confocal imaging in the *xyz*-plane. The fat is mostly neutral lipids, in droplet form, as Nile Red staining is specific for neutral lipids (Fig. 4A–C), usually triaglycerols or cholesteryl esters (Fowler & Greenspan, 1985; Greenspan et al., 1985).

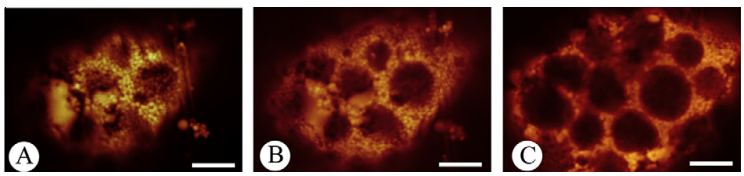


Fig. 4. Three-dimensional distribution of proteins and lipids in a parenchyma cell from seed of mature marama bean. Confocal recordings of Nile Red stained lipid bodies in top view (A) and sections obtained at two other focal planes (B and C) revealing the unlabelled PBs. Bars = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions and perspectives

Despite its exceptional nutritional quality, marama bean has not been domesticated and is thus not listed with other legume oilseeds, such as peanut and soybean. The present study employed physicochemical and histochemical methods and electron microscopy to elucidate the changes from the edible immature stage of seed development to the mature stage. It revealed a high protein content (21%) and moderate content of insoluble carbohydrates (9%) at the immature stage of the storage cotyledons, and a high protein (32%) and lipid (40%) content accompanied by increased carbohydrate content (19%) at the mature stage. The major carbohydrates in marama bean are insoluble polysaccharides (pectin and cellulose) stored as cell wall components. In the present study, it turned out that variation in seed development was considerably high between seeds collected at the same stage. Therefore seed variation should be kept in mind if selecting individual seeds for plant breeding work. Marama bean, being a wild legume, is a promising crop, worth domestication and cultivation for diverse food applications in the diets of communities and the food industry. Marama bean may, furthermore, have potential as a substitute for genetically modified legumes due to its naturally occurring high protein and lipid contents.

Acknowledgements

We are grateful to the National Food Technology Research Centre (NFTRC) for providing marama beans, to Piotr Binczycki for skilled preparation of the material for LM and TEM and to Professor Søren Balling Engelsen, Faculty of Life Sciences, University of Copenhagen for valuable comments on the manuscript. This work is part of a PhD project (M.M. Mosele) with the financial support of The Government of Botswana, Ministry of Infrastructure, Science and Technology.

References

Adsule, R. N., Kadam, S. S., & Salunkhe, D. K. (1989). Peanut. In D. K. Salunkhe & S. S. Kadam (Eds.), *CRC handbook of world legumes: Nutritional chemistry, processing technology, and utilisation, vol. II (pp 1–18)*. Florida: CRC Press.

Amarteifio, J. O., & Moholo, D. (1998). The chemical composition of four legumes consumed in Botswana. *Journal of Food Composition and Analysis, 11*, 329–332.

Amonsou, E., Taylor, J., & Minnaar, A. (2011). Microstructure of protein bodies in marama bean species. *Food Science and Technology, 44*, 42–47.

AOAC International – Association of Official Analytical Chemists. (2000). Official Methods of Analysis of AOAC. Washington DC: AOAC International.

Baldan, B., Bonaldo, A., Rascio, N., Vitale, A., Meggio, F., Profumo, P., et al. (1995). *Cercis siliquastrum* L.: A comparative study of endosperm and embryo development and reserve accumulation. *International Journal of Plant Sciences, 156*(2), 181–187.

Bower, N., Hertel, K., Oh, J., & Storey, R. (1988). Nutritional evaluation of marama bean (*Tylosema* esculentum, Fabaceae): Analysis of the seed. *Economic Botany, 42*(4), 533–540.

Brink, M. (2006). Tylosema fassoglense (Schweinf.) Torre & Hillc. In M. Brink & G. Belay (Eds.), *Plant resources of Tropical Africa 1. Cereals and pulses, pp 190–191*. Wageningen: PROTA Foundation (Plant Resources of Tropical Africa/Ressources végétales de l'Afrique tropicale).

Castro, S., Silveira, P., Coutinho, A. P., & Figueiredo, E. (2005). Systematic studies in *Tylosema* (Leguminosae). *Botanical Journal of the Linnean Society, 147*, 99–115.

De Frey, H. M., Coetzer, L. A., & Robbertse, P. J. (1992). A unique anther-mucilage in the pollination biology of *Tylosema esculentum*. *Sexual Plant Reproduction, 5*, 298–303.

Fowler, S. D., & Greenspan, P. (1985). Application of Nile red, a fluorescent hydrophobic probe, for the detection of neutral lipid deposits in tissue sections: Comparison with oil red O. *Journal of Histochemistry and Cytochemistry, 33*(8), 833–836.

Greenspan, P., Mayer, E. P., & Fowler, S. D. (1985). Nile red: A selective fluorescent stain for intracellular lipid droplets. *Journal of Cell Biology, 100*, 965–973.

Hartley, M. L., Tshamekeng, E., & Thomas, S. M. (2002). Functional heterostyly in *Tylosema esculentum* (Caesalpinioideae). *Annals of Botany, 89*, 67–76.

Herman, E. M., & Larkins, B. A. (1999). Protein storage bodies and vacuoles. *The Plant Cell, 11*, 601–613.

Holse, M., Husted, S., & Hansen, Å. (2010). Chemical composition of marama bean (Tylosema esculentum) – a wild African bean with unexploited potential. *Journal of Food Composition and Analysis, 23*, 648–657.

Hutzler, P., Fischbach, R., Heller, W., Jungblut, T. P., Reuber, S., Schmitz, R., et al. (1998). Tissue localisation of phenolic compounds in plants by confocal laser scanning microscopy. *Journal of Experimental Botany, 49*(323), 953–965.

Jakobsen, H. B., Martens, H., & Lyshede, O. B. (1994). Accumulation of metabolites during seed development in *Trifolium repens* L. *Annals of Botany, 74*, 409–415.

Jideani, V. A., Van Wyk, J., & Cruywagen, M. H. (2009). Physical properties of *Tylosemia esculentum* and the effect of roasting on the functional properties of its flour. *African Journal of Agricultural Research, 4*(11), 1208–1219.

Ketshajwang, K. K., Holmback, J., & Yeboah, S. O. (1998). Quality and compositional studies of some edible leguminosae seed oils in Botswana. *Journal of the American Oil Chemists' Society, 75*(6), 741–743.

Lott, J. N. A., & Buttrose, M. S. (1977). Globoids in protein bodies of legume seed cotyledons. *Australian Journal of Plant Physiology, 5*, 89–111.

Lott, J. N. A., Ockenden, I., Raboy, V., & Batten, G. D. (2000). Phytic acid and phosphorus in crop seeds and fruits: a global estimate. *Seed Science Research, 10*, 11–33.

Luza, J. G., van Gorsel, R., Polito, V. S., & Kader, A. A. (1992). Chilling injury in peaches: A cytochemical and ultrastructural cell wall study. *Journal of the American Society for Horticultural Science, 117*, 114–118.

Marcus, S., Verhertbruggen, Y., Herve, C., Ordaz-Ortiz, J., Farkas, V., Pedersen, H., et al. (2008). Pectic homogalacturonan masks abundant sets of xyloglucan epitopes in plant cell walls. *BMC Plant Biology, 8*, 1471–2229.

Maruatona, G. N., Duodu, K. G., & Minnaar, A. (2010). Physicochemical, nutritional and functional properties of marama bean flour. *Food Chemistry, 121*, 200–405.

Mitei, Y. C., Ngila, J. C., Yeboah, S. O., Wessjohann, L., & Schmidt, J. (2009). Profiling of phytosterols, tocopherols and tocotrienols in selected seed oils from Botswana by GC–MS and HPLC. *Journal of the American Oil Chemists' Society, 86*, 617–625.

Moïse, J. A., Han, S., Gudynaite-Savitch, L., Johnson, D. A., & Miki, B. L. A. (2005). Seed coats: Structure, development, composition, and biotechnology. In vitro cellular and developmental biology. *Plant, 41*, 620–644.

Mpotokwane, S. M., Gaditlhatlhelwe, E., Sebaka, A., & Jideani, V. A. (2008). Physical properties of bambara groundnuts from Botswana. *Journal of Food Engineering, 89*, 93–98.

National Research Council. (1979). Marama bean. Tropical dry pulses: resources for the future (pp 68–74). Washington DC: National Academy of Sciences.

Nkama, I., & Filli, K. B. (2006). Development and characterisation of extruded fura from mixtures of pearl millet and grain legume flours. *International Journal of Food Properties, 9*(2), 157–165.

O'Brien, T. P., & McCully, M. E. (1981). *The study of plant structure: Principles and selected methods*. Melbourne: Termarcaphi.

Oomah, B. D., Cardador-Martínez, A., & Loarca-Piña, G. (2005). Phenolics and antioxidative activities in common beans (*Phaseolus vulgaris* L). *Journal of the Science of Food and Agriculture, 85*, 935–942.

Powell, A. M. (1987). Marama bean (Tylosema esculentum, Fabaceae) seed crop in Texas. *Economic Botany, 41*(2), 216–220.

Redondo-Cuenca, A., Villanueva-Suárez, M. J., Rodríguez-Sevilla, M. D., & Mateos-Asparicio, I. (2006). Chemical composition and dietary fibre of yellow and green commercial soybeans (Glycine max). *Food Chemistry, 101*, 1216–1222.

Schadel, W. E., Walter, W. M., Jr., & Young, C. T. (1983). Cotyledon structure of resting peanut (*Arachis Hypogaea* L Cv. Florunner) seed before and after hydraulic pressing. *Journal of Food Science, 48*, 1405–1407.

Ueda, K., & Yoshioka, S. (1976). Cell wall development of *Micrasterias americana*, especially in isotonic and hypertonic solutions. *Journal of Cell Science, 21*, 617–631.

Vaidehi, M. P., & Kadam, S. S. (1989). Soybean. In D. K. Salunkhe & S. S. Kadam (Eds.), *CRC handbook of world legumes: Nutritional chemistry, processing technology, and utilisation, vol. III (pp 1–31)*. Florida: CRC Press.

Van der Maesen, L. J. G. (2006). Tylosema esculentum (Burch.) A.Schreib. In M. Brink & G. Belay (Eds.), *Plant resources of Tropical Africa 1. Cereals and pulses, pp 187–190*. Wageningen: PROTA Foundation (Plant Resources of Tropical Africa/Ressources végétales de l'Afrique tropicale).

Webster, B. D., & Leopold, A. C. (1977). The ultrastructure of dry and imbibed cotyledons of soybean. *American Journal of Botany, 64*(10), 1286–1293.

Wunderlin, R., Larsen, K., & Larsen, S. S. (1981). Cercideae. In R. M. Polhill & P. H. Raven (Eds.), *Advances in legume systematics, part 1* (pp. 107–116). Kew: Royal Botanic Gardens.

Young, C. T., Pattee, H. E., Schadel, W. E., & Sanders, T. H. (2004). Microstructure of peanut (*Arachis Hypogaea* L. cv. 'NC 7') cotyledons during development. *Food Science and Technology, 37*, 439–445.

Zadernowski, R., Borowska, J., Naczk, M., & Nowak-Polakowska, H. (2001). Effect of broad bean and pea phenolics on the activity of lipase and lipoxygenase. *Journal of Food Lipids, 8*, 263–270.

Paper II

Characterisation of the arabinose-rich carbohydrate composition of immature and mature marama beans *(Tylosema esculentum)*

Minah M. Mosele, Åse S. Hansen, Søren B. Engelsen, Iben Sørensen, Peter Ulvskov, William G. T. Willats, Andreas Blennow and Jesper Harholt

Phytochemistry

Phytochemistry 72 (2011) 1466–1472

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Characterisation of the arabinose-rich carbohydrate composition of immature and mature marama beans (*Tylosema esculentum*)

Minah M. Mosele [a,b], Åse S. Hansen [a], Søren B. Engelsen [a], Jerome Diaz [c], Iben Sørensen [c], Peter Ulvskov [c], William G.T. Willats [c], Andreas Blennow [c], Jesper Harholt [c,*]

[a] Quality and Technology, Department of Food Science, Faculty of Life Sciences, University of Copenhagen, Rolighedsvej 30, 1958 Frederiksberg C, Denmark
[b] Department of Extension and Training, National Food Technology Research Center, Mpuutsane Industrial Area, Private Bag 008, Kanye, Botswana
[c] Section for Plant Glycobiology, VKR Research Centre Pro-Active Plants, Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark

ARTICLE INFO

Article history:
Received 28 October 2010
Received in revised form 1 February 2011
Available online 3 May 2011

Keywords:
Marama bean
Morama bean
Tylosema esculentum
Legume
Starch
Soluble sugars
Cell wall composition
Pectin
CoMPP
Linkage analysis
HPAEC-PAD

ABSTRACT

Marama bean (*Tylosema esculentum*) is an important component of the diet around the Kalahari Desert in Southern Africa where this drought resistant plant can grow. The marama bean contains roughly 1/3 proteins, 1/3 lipids and 1/3 carbohydrates, but despite its potential as dietary supplement little is known about the carbohydrate fraction. In this study the carbohydrate fraction of "immature" and "mature" marama seeds are characterised. The study shows that the marama bean contains negligible amounts of starch and soluble sugars, both far less than 1%. The cell wall is characterised by a high arabinose content and a high resistance to extraction as even a 6 M NaOH extraction was insufficient to extract considerable amounts of the arabinose. The arabinose fraction was characterised by arabinan-like linkages and recognised by the arabinan antibody LM6 and LM13 indicating that it is pectic arabinan. Two pools of pectin could be detected; a regular CDTA (1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid) or enzymatically extractable pectin fraction and a recalcitrant pectin fraction containing the majority of the arabinans, of which about 40% was unextractable using 6 M NaOH. Additionally, a high content of mannose was observed, possibly from mannosylated storage proteins.

1. Introduction

Marama bean (*Tylosema esculentum* Burchell A. Schreiber) commonly called morama bean, tsin bean or gemsbok bean is an important component of the diet in settlements around the Kalahari Desert (National Research Council, 1979). It is a desiccant-tolerant plant with an ability to grow in high temperatures and dry environments such as the Kalahari area. Raw mature seeds of marama beans store well and remain edible for years (National Research Council, 1979). Leguminous seeds are an important part of the diet of rural communities in developing countries as they provide proteins, lipids and carbohydrates (Ketshajwang et al., 1998).

The mature seeds of *T. esculentum* are encapsulated in woody pods with 1–2 seeds and the pods open at maturity. Their seed coats, which are removed before consumption, are reddish to brownish black in colour (National Research Council, 1979). Previous work of mature marama seeds (Bower et al., 1988; Amarteifio and Moholo, 1998; Holse et al., 2010; Mosele et al., 2011) has indicated that they are a rich source of proteins and lipids, both above 30%, making them comparable to soya bean and peanut. They also have a considerable content of dietary fibre (19–27%), and mineral content is similar to that of peanut and approaching that of soya bean (Holse et al., 2010). The immature seeds are rich in proteins containing around 21% (w/w) (Mosele et al., 2011). Other studies have focused on the quality of marama bean oil (Mitei et al., 2008) and characterisation of the fatty acids, phytosterols and vitamin E compounds in the oil (Mitei et al., 2009).

Despite the potential of marama bean as a healthy nutritive crop for developing countries none of its carbohydrates have been thoroughly studied. Potential use of the carbohydrate fractions in food applications could yield valuable income for rural communities gathering marama beans. Studies on the use of pressed marama oil have been initialised and the defatted press rest might be readily available for potential extraction of useful carbohydrates.

Carbohydrates, especially polysaccharides are important in the food industry because they can be used as thickeners, stabilisers, texturisers and gelling agents (Viñarta et al., 2006; Khurana and Kanawjia, 2007). A number of studies have investigated the

* Corresponding author. Tel.: +45 35333306; fax: +45 35333300.
E-mail address: jesh@life.ku.dk (J. Harholt).

0031-9422/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2011.03.027

composition of carbohydrates in legume seeds, with special emphasis on starch and soluble sugars. Lentils and chickpeas contain around 50% (w/w) starch and approximately 20% (w/w) dietary fibre (Aguilera et al., 2009a). In soya bean, which compares to marama bean in nutritional quality, the carbohydrate components include 5.3% oligosaccharides, 2.4–5.5% polysaccharides (crude fibre) and 0.2–0.9% starch (Reddy et al., 1984). The main soluble sugar in soya bean is sucrose, followed by stachyose (Hou et al., 2009; Saldivar et al., 2011). The total carbohydrate content (by difference) of marama bean has been reported to constitute between 18.9% and 24.1% but has never been studied in detail (Bower et al., 1988; Amarteifio and Moholo, 1998; Mosele et al., 2011).

Structure elucidation of complex polysaccharides can be challenging due to the inherent heterogeneity and due to the differences in extractability. Analysis of legume seed cell wall is no exception and high protein content in the cell wall fraction does not make its structure elucidation any easier. Holse et al. (2011) characterised the bulk carbohydrate content of intact mature marama bean using different spectroscopic techniques. However a detailed characterisation of the cell wall polysaccharides was not possible without fractionation.

Dicot cell walls are generally considered to consist of three fractions, namely pectin, hemicellulose and cellulose. Pectin can be fractionated into homogalacturonan (HG), rhamnogalacturonan I (RG I), rhamnogalacturonan II (RG II) and xylogalacturonan (XGA). Apparently, these polymers are covalently linked to each other but it has proven very difficult to obtain unambiguous information on how the different pectic polysaccharides are connected wherefore several models exist (Vincken et al., 2003). Recent reviews have tried to elucidate the relationship of these pectic polysaccharides (Caffall et al., 2009; Scheller et al., 2007). RG I is built up of a backbone containing the disaccharide (α-1→4-GalA-α-1→2-Rha) as the basic repeating unit. The rhamnosyl residues can be substituted with galactan, arabinan or arabinogalactan side chains. The galacturonic acid (GalA) residues can furthermore be acetylated as in homogalacturonan. In some species the arabinose and galactose residues in RG I side chains can be substituted with ferulic and coumaric acid esters (Fry, 1982).

The aim of this study is to characterise the carbohydrate composition of marama bean seeds at the two developmental stages: immature and mature, corresponding to the stages utilised for consumption.

2. Results and discussion

2.1. Starch

Marama bean is virtually devoid of starch in both immature and mature seeds (Table 1). On average, the starch content is 0.2% dry mass, similar to that of mature soya bean as reported by Reddy et al. (1984) at 0.2–0.9%; Karr-Lilienthal et al. (2005) at 0.5%, and Saldivar et al. (2011) at 0.2–1.0%. Holse et al. (2011) also found starch signals to be absent in the infra red and NMR spectra of mature marama seeds. Carob seeds (*Ceratonia siliqua*), which is in the same family as marama bean also has a low starch content of 0.1% (Avallone et al., 1997). Other legumes such as pea, lentil and chickpea have a starch content of more than 40% (Dalgetty and Baik, 2003). Aguilera et al. (2009a) showed that total starch is 53.4% in chickpea (*Cicer arietinum* L.) and 46.3% in lentil (*Lens culinaris* L.).

The negligible amount of starch made detailed characterisation of starch, e.g. amylose/amylopectin ratio, unwarranted.

Table 1
Starch content of marama bean cotyledon from immature and mature seeds (% dry mass).

Sample	Starch content %
Immature seeds	0.19 (0.003)
Mature seeds	0.17 (0.003)

Values in parentheses indicate standard deviations, $n = 3$.

2.2. Soluble sugars

In general the content of soluble sugars is very negligible (less than 1% dry mass) in both immature and mature seeds. The main soluble sugar in immature seeds is glucose, followed by myoinositol and fructose (Table 2). Mature seeds had a higher amount of sucrose, followed by myoinositol and raffinose. The presence of raffinose in the mature seeds confirm the observations by Holse et al. (2011) using ^{1}H HR-MAS NMR.

In a study conducted by Aguilera et al. (2009b) the main soluble carbohydrate found in white beans and pink-mottled cream beans was also sucrose. However, other legumes contain raffinose family oligosaccharides (RFOs) as the main soluble sugars. The main soluble carbohydrate in soya bean (*Glycine max*), dolichos (*Lablab purpureus*) and cowpea (*Vigna unguiculata*) is RFOs (mainly stachyose), followed by sucrose, while jack bean (*Canavalia ensiformis*) has RFOs raffinose and ciceritol, followed by sucrose (Martín-Cabrejas et al., 2008).

A pattern of accumulation characterised by glucose, myoinositol, fructose, trehalose and ribose was observed at the immature stage, which disappeared at the mature stage as these sugars further decreased or were no longer detectable. The content of arabinose, raffinose, sucrose and maltose increased with maturation. Overall, a marked difference was observed between the two samples, with immature seeds having a higher amount of soluble sugars. However, soluble sugars represent a negligible percentage of total carbohydrates, as also observed for other legumes (Reddy et al., 1984).

2.3. Alcohol insoluble residue (AIR)

The composition of the non-cellulosic monosaccharides in AIR of mature marama seeds was characterised by a high content of arabinose, and with lower levels of mannose and galactose (Fig. 1). The immature seeds had a high content of mannose, followed by intermediate amounts of glucose and galactose. The mannose in the linkage analysis described below only show linkages characteristic for protein mannosylation indicating that the mannose in both samples originates from protein glycosylation (Lis and Sharon, 1978). As an abundance of cytoplasmic protein bodies have been visualised in marama seeds, possibly containing the majority of the protein associated mannose, the mannose detected in the sugar composition analysis of the non-cellulosic monosaccharides in AIR should not be considered part of the cell wall (Mosele et al., 2011). High mannose content has previously been reported in legume seeds with no evidence for its origin (Mullin and Xu, 2000).

Generally, legume seed cell wall contains high levels of galactose and arabinose, supposedly from long side chains of RG I (Huisman et al., 1998; Dalgetty and Baik, 2003). The presence of xyloglucan, mannan and callose (β-1,-3 glucan) was confirmed in CoMPP analysis (Fig. 2). However, based on the composition analysis of AIR the quantities of these polymers are minor compared to pectin. Both arabinogalactan protein (AGP) and extensin were also detected in the CoMMP analysis and with similar extraction profile as seen in Arabidopsis (Fig. 2) (Moller et al., 2007). Xylan could not be detected in this analysis and xylogalacturonan as recognised by the LM8 antibody, known to recognise an epitope present in a subpool of xylogalacturonans (Jensen et al., 2008; Nakamura et al., 2002) was not detected using CoMPP.

Table 2
Soluble sugar content of marama bean cotyledon from immature and mature seeds (% dry mass).

Soluble sugar	Immature (nmol/g)	Mature (nmol/g)	Immature (ng/mg)	Mature (ng/mg)
Myoinositol	64.1 (0.7)	49.3 (2.1)	11.5 (0.1)	8.9 (0.4)
Trehalose	1.6 (0.2)	n/d	0.6 (0.1)	n/d
Arabinose	18.2 (1.8)	30.0 (1.0)	2.7 (0.3)	4.5 (0.1)
Glucose	274.8 (41.0)	n/d	49.5 (7.4)	n/d
Fructose	55.8 (15.0)	n/d	10.1 (2.7)	n/d
Ribose	13.0 (1.2)	2.1 (1.8)	1.9 (0.2)	0.3 (0.3)
Sucrose	11.0 (2.4)	123.5 (2.1)	3.8 (0.8)	42.3 (0.7)
Raffinose	1.9 (0.8)	27.0 (3.7)	1.0 (0.4)	13.6 (1.9)
Maltose	2.2 (0.6)	15.4 (0.6)	0.8 (0.2)	5.3 (0.2)

n/d, not detected.
Values in parentheses indicate standard deviations, $n = 3$.

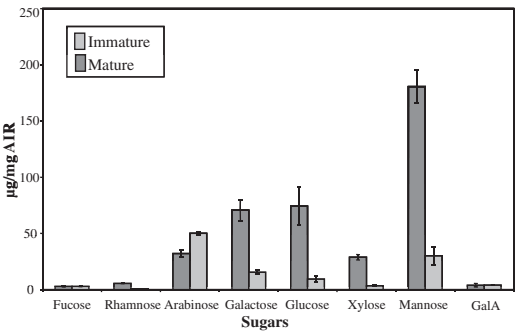


Fig. 1. Sugars (µg/mg) of the alcohol insoluble residue (AIR) of immature (unripe) and mature (fully ripe) marama seeds hydrolysed by TFA (GalA = galacturonic acid).

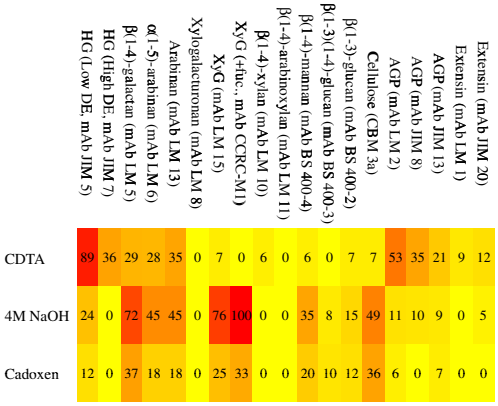


Fig. 2. Relative abundance of AIR cell wall components from mature marama seeds as judged by CoMMP analysis displayed as a heat map (HG = homogalacturonan, Xyg = xyloglucan, AGP = arabinogalactan protein).

To complement the sugar composition and CoMPP analysis, linkage analysis by permethylation was conducted. Linkage analysis of raw AIR generated unsatisfactory results with multiple interferring non-sugar peaks and hence $Ba(OH)_2$ hydrolysed AIR was used. $Ba(OH)_2$ removes proteins by hydrolysis and as protein was the single largest constituent of AIR this eliminated the interferring peaks. It should be noted that this technique also solubilises polysaccharides and approximately 50% (w/w) of the arabinose and mannose was removed by this method (results not shown) as measured by HPAEC-PAD. Furthermore peeling or solubilisation was observed as indicated by the occurrence of t-glucose and 4-glucose possibly originating from cellulose.

The linkage analysis (Table 3) showed that the majority of the arabinose was linked via O-5, characteristic for pectic arabinan and with attributes of a highly branched arabinan as indicated by the presence of 3,5- and 2,3,5-linked arabinose. The linkages observed for mannose corresponds well with the mannose originating from high mannose type N-linked protein glycosylation (Borisjuk et al., 2004; Lis and Sharon, 1978). The core GlcNAC (*N*-acetylglucosamine) was not detected but $Ba(OH)_2$ induced deacylation cannot be excluded. The presence of the 4-galactose and t-xylose linkages corresponds well with the presence of galactans and xylogalacturonan (Huisman et al., 2003). 2,4-Rhamnose were detected in trace quantities, but due to the low quantity the ratio between the two could not be accurately determined. Side reactions of the $Ba(OH)_2$ treatment prevents firm conclusions on the cell wall data but it is evident that the majority of the arabinose linkages resemble pectic arabinan linkages and the

Table 3
Linkage analysis of mature marama bean AIR after hydrolysis of proteins with $Ba(OH)_2$.

Sugar linkage	% area	Std. dev.
t-Arabinose	9.16	2.54
5-Arabinose	10.13	2.05
2,3,5-Arabinose	8.32	4.68
3,5-Arabinose	5.18	3.54
Sub-total	32.79	5.08
t-Mannose	17.65	0.85
2-Mannose	11.82	1.28
6-Mannose	2.08	0.50
3,6-Mannose	13.67	0.09
Sub-total	45.23	2.55
t-Galactose	3.02	0.86
4-Galactose	3.34	0.03
2,4-Galactose	1.81	0.22
Sub-total	8.16	1.10
t-Glucose	2.42	0.84
4-Glucose	9.88	0.34
Sub-total	12.30	1.18
t-Fucose	<1	N/A
Sub-total	<1	N/A
t-Xylose	~1	N/A
Sub-total	~1	N/A
2-Rhamnose	<1	N/A
2,4-Rhamnose	<1	N/A
Sub-total	~1	N/A
Total	100.00	

N/A, not applicable.

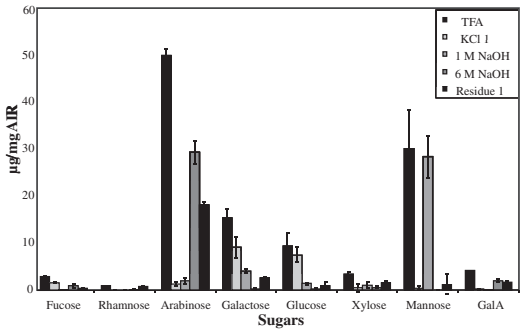


Fig. 3. AIR sugars (µg/mg) of mature marama seeds with sequential extraction (GalA = galacturonic acid).

majority of the mannose linkages resemble those present in protein glycosylation.

During maturation a decrease in content of non-cellulosic cell wall components was observed. Of all the single sugars, only arabinose increased in abundance. The reason for the relative decrease in non-cellulosic sugars from the AIR may be due to the increased abundance of protein in the mature sample. The changes observed in the composition of the cell wall sugars are probably due to cell wall adaptation to the maturation of the seed. In particular, the decrease in mannose content could be due to the crystallisation of protein in protein bodies (Mosele et al., 2011) making it impregnable to the TFA used for sugar composition analysis similar to cellulose microfibrils resistance to TFA hydrolysis.

To further characterise the cell wall, RG I was extracted from mature samples using an enzyme extraction protocol according to Harholt et al. (2006). Only low levels of RG I could be extracted and the relative arabinose to rhamnose ratio of the extracted RG I was lower than in the intact cell wall (results not shown). CoMPP analysis of the CDTA extract gave a similar result (Fig. 2). The data indicate the presence of two types of pectin: (1) a high HG pectin readily extractable with CDTA and (2) a high arabinan pectin requiring alkaline extraction procedures for solubilisation (Fig. 3).

In order to precisely quantify the extracted sugars and to complement the CoMMP analysis a modified sequential extraction was carried out using the AIR fraction from mature seeds (Fig. 4). The extraction protocol was designed to be different from the standardised CoMMP protocol in order to ensure extraction of specific polymers in the different extraction steps. KCl in high concentrations can extract ionically bound cell wall proteins. As already mentioned, both AGP and extensin were detected in the CoMPP analysis and since both of them contain arabinose in their glycan structures they could contribute to the high arabinose content of the cell wall (Figs. 1 and 3). However, the quantity of arabinose extracted by KCl was low. The KCl fraction contained the majority of the galactose found in the cell wall indicating its origin from AGP.

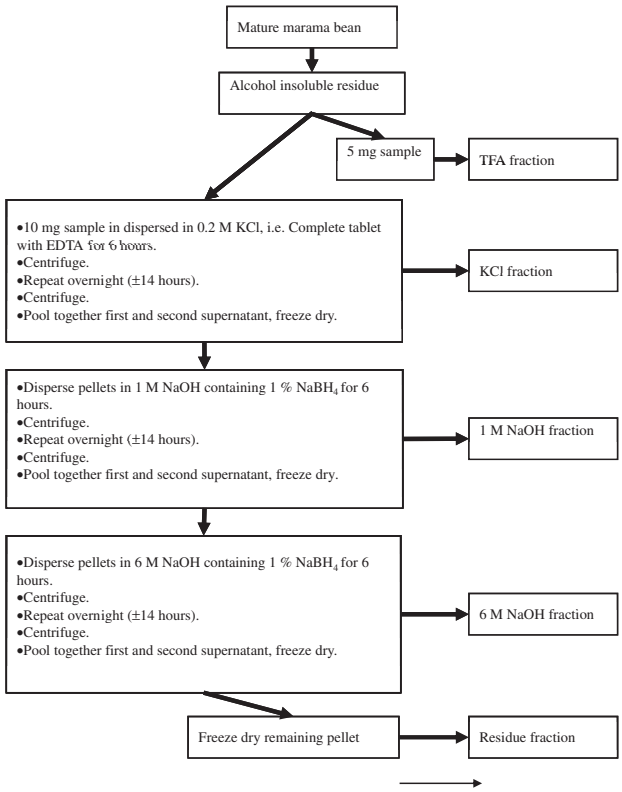


Fig. 4. Sequential extraction of sugars from alcohol insoluble residue (AIR) of mature seeds.

Since only low levels of xylose were extracted, the glucose cannot originate from xyloglucan. However, callose was observed in the CoMPP analysis and could be the origin of the glucose (Fig. 2). The next extraction was 1 M NaOH, and similar to both CDTA and enzyme based extraction it proved unsuccessful in extracting a high proportion of arabinose. The 1 M NaOH fraction contained nearly 100% of the mannose but only low levels of other sugars. Since legume seed proteins are extracted using alkaline extraction procedures, the mannose may originate from extracted proteins supporting the hypothesis that the mannose in the AIR fraction is from protein mannosylation. A harsher extraction of 6 M NaOH was also carried out which could release approximately 60% of the arabinose. Some of the galacturonic acid also needed 6 M NaOH for extraction, suggesting that this fraction of galacturonic acid is linked together with the arabinans providing evidence that the arabinans observed are pectic arabinan. The low amounts of rhamnose in the latter extractions indicate that the arabinans observed here are long chained. Similar extraction protocols have been used on other legume seeds in which the arabinose was also recalcitrant to extraction (Shiga and Lajolo, 2006; Shiga et al., 2003, 2004), but not to a degree as observed in marama beans. Using up to 4 M NaOH extractions Shiga and Lajolo (2006) could extract more than 96% of the arabinose from the cell wall of common bean. Less than 4% remained in the residue as unextractable arabinose, while in marama bean around 40% arabinose remained in the residue. Furthermore, there was a larger proportion of pectin in common bean that was extractable with water or CDTA, than observed when using enzyme mediated, 1 M NaOH or CDTA extraction of marama bean. The data suggest the presence of more ramified or branched arabinans in marama bean than in common bean.

Marama bean is normally not utilised and cooked like other beans. They are instead roasted and consumed like peanuts. In common bean a hard-to-cook phenomenon can be observed which relates to the cell wall polymers extractability (Shiga et al., 2004). Storage temperatures around or higher than room temperature seem to induce a hardening of the bean that is correlated with increasing recalcitrance of the cell wall polymers to extraction. Since marama beans are stored at elevated temperatures in their natural habitat the high recalcitrance could be an effect of acclimatisation to high temperatures.

Other legume seeds contain large amounts of arabinan but not to the same level as that observed in marama bean. The increased arabinan content of marama beans compared to common bean could be the result of an adaption to the decrease in moisture level of the seed. The moisture level of marama bean is low (ca. 5%) (Holse et al., 2010; Mosele et al., 2011) compared to what is observed in for example, soya bean and other legumes (>8%) (Guo et al., 2010; Khattab et al., 2009). It is hypothesised that arabinan may play a role in plant tissues that undergo desiccation and the plasticity of arabinan in the cell wall is fitting with this hypothesis (Gomez et al., 2009; Harholt et al., 2010).

The structure of marama bean arabinans is not fully elucidated and the reason for their resistance to extraction remains elusive. However, a tight association with cellulose could be anticipated as 40% of arabinose was not extracted using 6 M NaOH. Normally, 6 M NaOH will readily solubilise almost all cell wall polysaccharides except cellulose. It is already known that galactan and arabinan can interact with cellulose microfibrils (Zykwinska et al., 2005). The interaction is strongest for debranched arabinan unlike in the highly branched arabinan as observed in marama beans. The specific degree of hydration in the marama bean cell wall is not known. Since the water content is extremely low, it is likely that regions of the cell wall are (semi) crystalline increasing its recalcitrance. In Chenopodiaceae covalent cross-linking is possible through pectic side-chains terminated by feruloyl esters that can dimerise (Fry, 1986; Rombouts and Thibault, 1986; Colquhoun et al., 1994). This type or other types of cross-linking could also be present in marama bean further increasing recalcitrance to extraction. Further investigations are required in order to confirm the presence of such cross-linking.

3. Conclusions

Investigation of the carbohydrate fraction of marama bean supports the presence of a seed composition similar to that of other leguminous seeds, but with some atypical or unique characteristics. High levels of arabinose from arabinan and mannose from protein glycosylation were observed in the cell wall material. The arabinose level observed was higher than that of other leguminous species. The high arabinan content was not solubilised using standard pectin extraction methods, but was recalcitrant to extraction as 60% of the arabinose was extractable. Even after 6 M NaOH extraction a notable amount of arabinose could be detected in the residue. Starch and soluble sugars were less than 1% (w/w).

4. Materials and methods

4.1. Sample preparation

Two different samples of marama bean were analysed constituting two different developmental stages; immature (unripe) and the mature (fully ripe) stage of consumption. These samples were harvested in 2008 from their natural habitat in the southern region of Botswana. The samples were decorticated and the cotyledons were milled into flour in a laboratory mill (IKA A10, Labortechnik, Staufen, Germany) for 15 s. Three samples of each flour were aliquoted and analysed in parallel.

4.2. Analysis of starch

Starch in the immature and mature bean flour was quantified by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as released glucose after enzymatic hydrolysis of the starch. Defatted flour (100 mg) was extracted with 80% ethanol for 30 min at 80 °C and the supernatants were discarded. Additional washing two times with 15 ml 0.1% sodium dodecyl sulphate (SDS) and thereafter with two times 15 ml water, with centrifugation between each step were done. Thermostable α-amylase (Termamyl, Novozymes, DK) (300 U) in 50 mM (pH 7.0) MOPS buffer (4-morpholinepropanesulfonic acid sodium salt, 5 mM, calcium chloride and 0.02% sodium azide) was added to the samples, which were then incubated at 100 °C for 6 min. The temperature was then adjusted to 50 °C, and sodium acetate buffer (2.5 mM, pH 4.0) and 20 U of amyloglucosidase (Sigma, DK) were added. The samples were incubated for 30 min and centrifuged and the supernatants were analysed for glucose content according to the method by Blennow et al. (1998). The starch content was expressed as percentage of dry matter.

4.3. Analysis of soluble sugars

Soluble sugars in the immature and mature bean flour were quantified using HPAED-PAD. Defatted flour (100 mg) was placed in 15 ml conical tubes. The sugars were extracted with 80% ethanol for 30 min at 80 °C. The samples were centrifuged, and the supernatants recovered and dried overnight in a vacuum drier. The dry residue of the supernatants were dissolved in 100 µl of water and kept at −20 °C prior to analysis. Sugar analysis was performed as described by Lunde et al. (2008). The sugars were expressed in nmol/g and ng/mg of dry matter.

4.4. Isolation and identification of sugars from alcohol insoluble residue (AIR)

A weight of 50 mg of immature and mature bean flour was placed in micro tubes. Extraction with benzene:ethanol (7:3 v/v, 1 ml) was carried out for 6 h. After centrifugation the pellets were washed twice in acetone. They were further extracted with chloroform: methanol (3:2 v/v, 1 ml) for 72 h. After centrifugation, this was followed by washing in 100% and 70% ethanol, and vacuum drying. The residual material was designated as AIR. The samples were added trifluoro acetic acid (TFA) and hydrolysed for 1 h at 121 °C, dried under vacuum and re-suspended in 1 ml water. They were mixed, centrifuged and analysed by HPAEC-PAD according to the method by Øbro et al. (2004). The sugars were expressed in μg/mg of dry matter.

4.5. Comprehensive microarray polymer profiling (CoMPP) analysis of AIR

CoMPP was carried out as described by Moller et al. (2007) on the mature bean flour. Ten milligrams of AIR was sequentially extracted with 50 mM CDTA (1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, pH 7.5, 4 M NaOH with 0.1% v/v $NaBH_4$ and cadoxen (31% (v/v) 1,2-diaminoethane with 0.78 M CdO). The extracts were printed in three dilutions and three replicates giving a total of nine spots per sample. Primary antibodies were obtained from PlantProbes, Leeds, UK or BioSupplies, Melbourne, Australia. Secondary alkaline phosphatase-conjugated anti-rat or -mouse antibodies were obtained from Sigma–Aldrich (Cat. Nos. A8438, A3562 and A5588, respectively). Development of blots was performed using a BCIP/NBTC (5-bromo-4-chloro-3′-indolyphosphate/nitro-blue tetrazolium chloride)-based substrate. Arrays were scanned, converted to 16 bit grey-scale TIFF format images and spot signals quantified using the ImaGene 6.0 microarray analysis software (BioDiscovery, El Segundo, CA, USA). The extractions were performed three times and the data presented as a heat map is an average of these. The data was converted into the heat map format using the online BAR heatmapper tool (http://bar.utoronto.ca/ntools/cgi-bin/ntools_heatmapper.cgi).

4.6. $Ba(OH)_2$ hydrolysis of AIR

To prepare for linkage analysis (see Section 4.7) extensin (protein) was removed from mature bean flour with $Ba(OH)_2$. Without the protein hydrolysis, imprecise results were obtained.

The samples were washed with water. They were then hydrolysed for 6 h at 120 °C in 0.2 M $Ba(OH)_2$ followed by neutralisation with sulphuric acid to remove barium as precipitated $BaCO_3$, as described by Fry (1988). The supernatants were removed and the remaining pellets were analysed for linkages by permethylation.

4.7. Linkage analysis by permethylation

Freeze dried samples of $Ba(OH)_2$ treated AIR were permethylated using the Hakomori procedure (Hakomori, 1964) as modified by Huisman et al. (1998) and Verhoef et al. (2002). The permethylated samples were hydrolysed in 2 M TFA containing 1 μmol myoinositol as internal standard, at 121 °C for 1 h. The hydrolysed samples were air-dried and subsequently reduced using sodium borohydride. The reduced, permethylated samples were converted into alditol acetates and analysed using a Shimadzu gas chromatograph–electron impact-mass spectrometer (GC–EI-MS). The partly methylated alditol acetates were separated on a 0.25 × 30 mm vitreous silica capillary column of SP-2330 (Supelco). The temperature was held at 80 °C for 1 min upon injection, then programmed from 80 to 170 °C at 25 °C per min, then to 210 °C at 2 °C per min, then to 240 °C at 5 °C per min, with a 10 min hold at the upper temperature. Linkage composition was deduced from both the relative retention times and the electron impact-mass spectrum (EI-MS) (Carpita and Shea, 1989), and expressed as a percentage of dry matter.

4.8. Isolation and identification of sugars from AIR after sequential extraction

Sequential extraction of AIR from mature bean flour was done in order to follow the extraction pattern of the different sugar fractions from AIR (Fig. 4). All fractions, except TFA fraction, were washed several times in 70% ethanol, followed by centrifugation, until the pH of each fraction was below 10. All fractions were dried under vacuum, TFA hydrolysed and analysed for sugar composition as described in Section 4.4, according to the method by Øbro et al. (2004).

Acknowledgements

The authors wish to thank the National Food Technology Research Center (NFTRC) for providing marama beans. This work was supported by the Government of Botswana, Ministry of Infrastructure, Science and Technology and the Danish Villum Kann Rasmussen Foundation.

References

Aguilera, Y., Esteban, R.M., Benitez, V., Molla, E., Martin-Cabrejas, M.A., 2009a. Starch, functional properties, and microstructural characteristics in chickpea and lentil as affected by thermal processing. J. Agric. Food Chem. 57, 10682–10688.

Aguilera, Y., Martín-Cabrejas, M.A., Díaz, M.F., Aguilera, Y., Benítez, V., Mollá, E., López-Andréu, F.J., Esteban, R.M., 2009b. Changes in carbohydrate fraction during dehydration process of common legumes. J. Food Compos. Anal. 22, 678–683.

Amarteifio, J.O., Moholo, D., 1998. The chemical composition of four legumes consumed in Botswana. J. Food Compos. Anal. 11, 329–332.

Avallone, R., Plessi, M., Baraldi, M., Monzani, A., 1997. Determination of chemical composition of carob (*Ceratonia siliqua*): protein, fat, carbohydrates and tannins. J. Food Compos. Anal. 10, 166–172.

Blennow, A., Bay-Smidt, A.M., Olsen, C.E., Moller, B.L., 1998. Analysis of starch-bound glucose 3-phosphate and glucose 6-phosphate using controlled acid treatment combined with high-performance anion-exchange chromatography. J. Chromatogr. A 829, 385–391.

Borisjuk, L., Rolletschek, H., Radchuk, R., Weschke, W., Wobus, U., Weber, H., 2004. Seed development and differentiation: a role for metabolic regulation. Plant Biol. (Stuttg.) 6 (04), 375–386.

Bower, N., Hertel, K., Oh, J., Storey, R., 1988. Nutritional evaluation of marama bean (*Tylosema esculentum*, fabaceae): analysis of the seed. Econ. Bot. 42, 533–540.

Caffall, K.H., Pattathil, S., Phillips, S.E., Hahn, M.G., Mohnen, D., 2009. Arabidopsis thaliana T-DNA mutants implicate GAUT genes in the biosynthesis of pectin and xylan in cell walls and seed testa. Mol. Plant 2, 1000–1014.

Carpita, N.C., Shea, E.M., 1989. Linkage structure of carbohydrates by gas-chromatography–mass spectrometry (GC–MS) of partially methylated alditol acetates. In: Biermann, C.J., McGinnis, G.D. (Eds.), Analysis of Carbohydrates by GLC and MS. CRC Press, Inc., Boca Raton, pp. 157–216.

Colquhoun, I.J., Ralet, M.C., Thibault, J.F., Faulds, C.B., Williamson, G., 1994. Structure identification of feruloylated oligosaccharides from sugar-beet pulp by NMR spectroscopy. Carbohydr. Res. 263, 243–256.

Dalgetty, D.D., Baik, B.K., 2003. Isolation and characterization of cotyledon fibers from peas, lentils, and chickpeas. Cereal Chem. 80, 310–315.

Fry, S.C., 1982. Phenolic components of the primary cell wall. Feruloylated disaccharides of D-galactose and L-arabinose from spinach polysaccharide. Biochem. J. 203, 493–504.

Fry, S.C., 1986. Cross-linking of matrix polymers in the growing cell walls of angiosperms. Annu. Rev. Plant Physiol. 37, 165–186.

Fry, S.C., 1988. The Growing Plant Cell Wall: Chemical and Metabolic Analysis. The Blackburn Press, Caldwell, NJ.

Gomez, L.D., Steele-King, C.G., Jones, L., Foster, J.M., Vuttipongchaikij, S., McQueen-Mason, S.J., 2009. Arabinan metabolism during seed development and germination in arabidopsis. Mol. Plant 2, 966–976.

Guo, W., Wang, S., Tiwari, G., Johnson, J.A., Tang, J., 2010. Temperature and moisture dependent dielectric properties of legume flour associated with dielectric heating. LWT – Food Sci. Technol. 43, 193–201.

Hakomori, S., 1964. A rapid permethylation of glycolipid and polysaccharide catalyzed by methylsulfinyl carbanion in dimethylsulfoxide. J. Biochem. 55, 209–212.
Harholt, J., Jensen, J.K., Sørensen, S.O., Orfila, C., Pauly, M., Scheller, H.V., 2006. ARABINAN DEFICIENT 1 is a putative arabinosyltransferase involved in biosynthesis of pectin arabinan in arabidopsis. Plant Physiol. 140, 49–58.
Harholt, J., Suttangkakul, A., Scheller, H.V., 2010. Biosynthesis of pectin. Plant Physiol. 153, 384–395.
Holse, M., Husted, S., Hansen, Å., 2010. Chemical composition of marama bean (*Tylosema esculentum*) – a wild African bean with unexploited potential. J. Food Compos. Anal. 23 (6), 648–657.
Holse, M., Larsen, F.H., Hansen, Å., Engelsen, S.B., 2011. Characterization of marama bean (*Tylosema esculentum*) by comparative spectroscopy: NMR, FT-Raman, FT-IR and NIR. Food Res. Int. 44, 373–384.
Hou, A., Chen, P., Shi, A., Zhang, B., Wang, J., 2009. Sugar variation in soybean seed assessed with a rapid extraction and quantification method. Int. J. Agron. 2009, article ID 484571.
Huisman, M.M.H., Schols, H.A., Voragen, A.G.J., 1998. Cell wall polysaccharides from soybean (*Glycine max*) meal. Isolation and characterisation. Carbohydr. Polym. 37, 87–95.
Huisman, M.M.H., Schols, H.A., Voragen, A.G.J., 2003. Pectic substances from soybean cell walls distinguish themselves from other plant cell wall pectins. In: Voragen, F., Schols, H., Visser, R. (Eds.), Advances in Pectin and Pectinase Research. Kluwer Academic Publishers, Netherlands, pp. 159–168.
Jensen, J.K., Sorensen, S.O., Harholt, J., Geshi, N., Sakuragi, Y., Moller, I., Zandleven, J., Bernal, A.J., Jensen, N.B., Sorensen, C., Pauly, M., Beldman, G., Willats, W.G.T., Scheller, H.V., 2008. Identification of a xylogalacturonan xylosyltransferase involved in pectin biosynthesis in arabidopsis. Plant Cell 20, 1289–1302.
Karr-Lilienthal, L.K., Kadzere, C.T., Grieshop, C.M., Fahey Jr., G.C., 2005. Chemical and nutritional properties of soybean carbohydrates as related to non-ruminants: a review. Livest. Prod. Sci. 97, 1–12.
Ketshajwang, K.K., Holmback, J., Yeboah, S.O., 1998. Quality and compositional studies of some edible Leguminosae seed oils in Botswana. J. Am. Oil Chem. Soc. 75, 741–743.
Khattab, R.Y., Arntfield, S.D., Nyachoti, C.M., 2009. Nutritional quality of legume seeds as affected by some physical treatments: Part 1. Protein quality evaluation. LWT – Food Sci. Technol. 42, 1107–1112.
Khurana, H.K., Kanawjia, S.K., 2007. Recent trends in development of fermented milks. Curr. Nutr. Food Sci. 3, 91–108.
Lis, H., Sharon, N., 1978. Soybean agglutinin – a plant glycoprotein. Structure of the carbohydrate unit. J. Biol. Chem. 253, 3468–3476.
Lunde, C., Zygadlo, A., Simonsen, H.T., Nielsen, P.L., Blennow, A., Haldrup, A., 2008. Sulfur starvation in rice: the effect on photosynthesis, carbohydrate metabolism, and oxidative stress protective pathways. Physiol. Plant. 134, 508–521.
Martín-Cabrejas, M.A., Díaz, M.F., Aguilera, Y., Benítez, V., Mollá, E., Esteban, R.M., 2008. Influence of germination on the soluble carbohydrates and dietary fibre fractions in non-conventional legumes. Food Chem. 107, 1045–1052.
Mitei, Y.C., Ngila, J.C., Yeboah, S.O., Wessjohann, L., Schmidt, J., 2008. NMR, GC–MS and ESI-FTICR-MS profiling of fatty acids and triacylglycerols in some Botswana seed oils. J. Am. Oil Chem. Soc. 85, 1021–1032.
Mitei, Y.C., Ngila, J.C., Yeboah, S.O., Wessjohann, L., Schmidt, J., 2009. Profiling of phytosterols, tocopherols and tocotrienols in selected seed oils from Botswana by GC–MS and HPLC. J. Am. Oil Chem. Soc. 86, 617–625.
Moller, I., Sorensen, I., Bernal, A.J., Blaukopf, C., Lee, K., Obro, J., Pettolino, F., Roberts, A., Mikkelsen, J.D., Knox, J.P., Bacic, A., Willats, W.G.T., 2007. High-throughput mapping of cell-wall polymers within and between plants using novel microarrays. Plant J. 50, 1118–1128.
Mosele, M.M., Hansen, Å.S., Hansen, M., Schulz, A., Martens, H.J., 2011. Proximate composition, histochemical analysis and ultrastructural localisation of nutrients in immature and mature seeds of marama bean (*Tylosema esculentum*) – an underutilised legume. Food Chem. 127, 1555–1561.
Mullin, W.J., Xu, W., 2000. A study of the intervarietal differences of cotyledon and seed coat carbohydrates in soybeans. Food Res. Int. 33, 883–891.
Nakamura, A., Furuta, H., Maeda, H., Takao, T., Nagamatsu, Y., 2002. Analysis of the molecular construction of xylogalacturonan isolated from soluble soybean polysaccharides. Biosci. Biotechnol. Biochem. 66, 1155–1158.
National Research Council, 1979. Marama Bean, in Tropical Dry Pulses: Resources for the Future. National Academy of Sciences, Washington, DC, pp. 68–74.
Øbro, J., Harholt, J., Scheller, H.V., Orfila, C., 2004. Rhamnogalacturonan I in *Solanum tuberosum* tubers contains complex arabinogalactan structures. Phytochemistry 65, 1429–1438.
Reddy, N.R., Pierson, M.D., Sathe, S.K., Salunkhe, D.K., 1984. Chemical, nutritional and physiological aspects of dry bean carbohydrates – a review. Food Chem. 13, 25–68.
Rombouts, F.M., Thibault, J.F., 1986. Feruloylated pectic substances from sugar-beet pulp. Carbohydr. Res. 154, 177–187.
Saldivar, X., Wang, Y., Chen, P., Hou, A., 2011. Changes in chemical composition during soybean seed development. Food Chem. 124, 1369–1375.
Scheller, H.V., Jensen, J.K., Sørensen, S.O., Harholt, J., Geshi, N., 2007. Biosynthesis of pectin. Physiol. Plant. 129, 283–295.
Shiga, T.M., Lajolo, F.M., 2006. Cell wall polysaccharides of common beans (*Phaseolus vulgaris* L.) – composition and structure. Carbohydr. Polym. 63, 1–12.
Shiga, T.M., Lajolo, F.M., Filisetti, T.M.C.C., 2003. Cell wall polysaccharides of common beans (*Phaseolus vulgaris* L.). Ciênc. Tecnol. Aliment. 23, 141–148.
Shiga, T.M., Lajolo, F.M., Filisetti, T.M.C.C., 2004. Changes in the cell wall polysaccharides during storage and hardening of beans. Food Chem. 84, 53–64.
Verhoef, R., de Waard, P., Schols, H.A., Rättö, M., Siika-aho, M., Voragen, A.G.J., 2002. Structural elucidation of the EPS of slime producing *Brevundimonas vesicularis* sp. isolated from a paper machine. Carbohydr. Res. 337, 1821–1831.
Viñarta, S.C., Molina, O.E., Figueroa, L.I.C., Fariña, J.I., 2006. A further insight into the applications of exopolysaccharides from *Sclerotium rolfsii*. Food Hydrocolloids 20, 619–629.
Vincken, J.P., Schols, H.A., Oomen, R.J.F.J., McCann, M.C., Ulvskov, P., Voragen, A.G.J., Visser, R.G.F., 2003. If homogalacturonan were a side chain of rhamnogalacturonan: I. Implications for cell wall architecture. Plant Physiol. 132, 1781–1789.
Zykwinska, A.W., Ralet, M.C.J., Garnier, C.D., Thibault, J.F.J., 2005. Evidence for in vitro binding of pectin side chains to cellulose. Plant Physiol. 139, 397–407.

DEPARTMENT OF FOOD SCIENCE
PHD THESIS 2012 · ISBN 978-87-7611-508-1

MINAH MMONI MOSELE

Chemical and Structural Characterisation of Marama Bean *(Tylosema esculentum)* Carbohydrates

SL grafik