# ANTI-NUTRITIONAL CONSTITUENT OF *COLOCASIA ESCULENTA* (AMADUMBE) A TRADITIONAL CROP FOOD IN KWAZULU-NATAL

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# DEDICATION

**I can do all things through**
**Christ which strengthened me**
**Philippians 4:13**

# ABSTRACT

*Colocasia esculenta* L. Schott belongs to the family Aracea and is grown for its edible corms as a staple food throughout subtropical and tropical regions of the world. Amadumbe (the Zulu name for *Colocasia esculenta*) is consumed by and holds an important place in the diet of local rural people in Kwazulu-Natal, South Africa.

Three Amadumbe phenotypes were evaluated for their nutritional qualities. Like all known tubers, the locally grown Amadume contained high carbohydrate levels, adequate protein and low lipid content. Essential fatty acids (linoleic and linolenic) were identified as components of the Amadumbe lipids. Amadumbe was generally low in mineral content, apart from potassium and magnesium levels that were relatively high. Some anti-nutrients (protease inhibitors, lectin, phenolic compounds, alkaloids, oxalates, phytates, cyanogens and saponin) present in Amadumbe were also identified and quantified. The anti-nutrient levels were generally low and thus may not pose an immediate effect on the health of consumers. Reduction of the anti-nutrients through processing (cooking, frying, roasting) was observed to enhance the nutritional value of these tubers. However, their presence suggests that a steady consumption may lead to toxic levels.

Two proteins (A1 and B2) with α-amylase inhibitor activity, and a steroidal saponin (gamma-sitosterol) were extracted and partially characterised. The α-amylase inhibitors were extracted and partially purified through ammonium sulphate precipitation and chromatographic fractionation on diethylaminoethyl (DEAE)-Sephacel and Sephadex G-100. The molecular weights of the two inhibitors were estimated to be 17 000 and 19 000 dalton, respectively. The inhibitors were fairly heat-stable, with optimum activity at 40° C, pH 6.0. Both inhibitors showed activity against mammalian α-amylases, but were devoid of activity against fungal amylases. Inhibitor A also showed activity against plant amylases.

The steroidal saponin extracted from Amadumbe was characterized through TLC, HPLC, GC-MS, IR and NMR spectroscopic analysis and identified to be gamma-

sitosterol, an isomer of beta-sitosterol which is known to have a variety of high biological activity.

Studies of the effect of beta-sitosterol on absorptive and digestive enzymes in Sprague-Dawley rats revealed that oral administration of beta-sitosterol had no apparent gross or microscopic lesions in the liver, kidney or small intestine. The administered β-sitosterol significantly decreased serum aspartate aminotransferase (ALT) and alanine aminotransferase (AST) levels. $Na^+/K^+$-ATPase and intestinal disaccharidases activities were also significantly reduced in beta-sitosterol fed rats.

These results do suggest that even though Amadumbe is a neglected crop in South Africa, it is a highly nutritional crop; the consumption of it could be beneficial to diabetic and hypertensive patients.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ACAT | Acyl-coenzyme A cholesterol acyltransferase |
| ALT | alanine aminotransferase |
| AI | amylase inhibitor |
| AIA | amylase inhibitor activity |
| AOAC | Association of Official Analytical Chemists |
| apoB | apolipoprotein B |
| AST | aspartate aminotransferase |
| ATP | adenosine triphosphate |
| ATR | attenuated total reflectance |
| BAPNA | benzoyl-DL-arginine-*p*-nitroaniline |
| BP | Esikhawini boiled purple |
| BPH | benign prostatic hypertrophy |
| BW | Esikhawini boiled white |
| CCK | cholecystokinin |
| DM | dry matter |
| DNS | dinitrosaliccyclic acid |
| EI-MS | electron impact-mass spectrometry |
| EP | Esikhawini purple |
| ER | endoplasmic reticulum |
| EW | Esikhawini white |
| FAO | Food and Agriculture Organization |
| FP | Esikhawini fried purple |
| FW | Esikhawini fried white |
| GA | glycoalkaloids |
| GC-MS | gas chromatography-mass spectrometry |
| HCN | hydrogen cyanide |
| HPLC | high-performance liquid chromatography |
| HPLC-UV | High-Pressure Liquid Chromatography with Ultra-Violet Detector |
| IR | infra-red |
| KCN | potassium cyanide |
| KZN | Kwazulu-Natal |

| | |
|---|---|
| MakP | Makatini purple |
| MakW | Makatini white |
| MNU | methylnitrosourea |
| MtP | Mtubatuba purple |
| MtW | Mtubatuba white |
| MUFA | monounsaturated fatty acid |
| ND | no date |
| NMR | nuclear magnetic resonance |
| PI | proteinase inhibitor |
| PVP | polyvinylpolypyrrolidone |
| RP | Esikhawini roasted purple |
| RW | Esikhawini roasted white |
| TAN | tropical ataxic neuropathy |
| TG | triacylglycerol |
| $TH_1$ | T-helper-1 |
| TIA | trypsin inhibitor activity |
| TMS | tetramethylsilane |
| WHO | World Health Organisation |

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# CHAPTER 1: GENERAL INTRODUCTION

Nutritional value is the main concern when a plant is considered as food source. However, endogenous toxic factors characteristic of plant material can also affect the content of nutrients. These toxic factors act as anti-nutrients and adversely affect the organism. Anti-nutrients are chemicals which have been evolved by plants for their own defence, among other biological functions. Anti-nutrients reduce the maximum utilization of nutrients (especially proteins, vitamins and minerals), thus preventing optimal exploitation of the nutrients present in a food and decreasing the nutritive value (Ugwu and Oranye, 2006). Anti-nutrients vary in chemical structures, ranging from amino acids to proteins; from simple amines to alkaloids, glycosides and many phenolic compounds. The biological effects of all these chemicals are diverse and complex.

When man ingests plant foods to meet nutritional needs, a wide variety of these non-nutrient phytochemicals are ingested at the same time. Processing is expected to inactivate these anti-nutritional factors and increase the availability of bioactive compounds. However, the health risk to consumers of large quantities of residual anti-nutrients cannot be ruled out.

In sub-Saharan Africa, approximately three quarters of the population live in rural areas. Most of their dietary energy comes from staple cereals, such as maize. Given the limited resources and restricted access to different foods in rural societies, most African communities have developed diets that maximize the use of local foodstuffs. The advancements in grain production have not brought significant benefits to areas where tuber crops are the major staples. Therefore, emphasis should be placed on such tuber crops as Amadumbe (*Colocasia esculenta*), which is a staple food in many developing nations of West-Africa (as taro/cocoyam), Asia and the Pacific (as cocoyam). Amadumbe (*Colocasia esculenta*) is widely grown in the sub-tropical parts of South Africa as a traditional food

crop. Amadumbe is not extensively commercialized at present, but is mostly grown in rural areas or on small farms in Kwazulu-Natal, South Africa.

Literature abounds with research carried out on *Colocasia esculenta* species in other parts of the world, but little or no information is available regarding the composition, structural activity and biochemical mechanisms of anti-nutritional factors in local *Colocasia esculenta* (Amadumbe). Studies investigating these factors could help the government in formulating food and nutrition policies for South Africa.

The overall hypothesis of this study was, therefore, that if the constituents of various nutritional and anti-nutritional factors inherent in *Colocasia esculenta* (Amadumbe), a traditional crop food grown in Kwazulu-Natal, were identified, this tuber could be more efficiently utilized in food and nutrition policies for South Africa.

The project was divided into three specific aims to meet the overall objective:

- to determine the proximate composition, the mineral and the anti-nutrient content of Amadumbe from Kwazulu-Natal, South Africa, in order to compare the factors with those observed in *Colocasia esculenta* from other areas of the world. At the same time, the best processing method for maximum elimination of the anti-nutritional factors would be identified;

- to isolate and characterize some of the screened anti-nutritional factors to determine the structural activity and biochemical properties of these toxicants occurring naturally in Amadumbe tubers;

- to perform a nutritional evaluation using rats with a specific, identified anti-nutritional factor from *Colocasia esculenta* to determine the biological effects of the anti-nutritional factor.

# SECTION A

# PROXIMATE COMPOSITION AND SOME ANTI-NUTRIENTS IN PROCESSED AND UNPROCESSED *COLOCASIA ESCULENTA*

## SUMMARY

Amadumbe (*Colocasia esculenta*) is widely grown in the subtropical parts of South Africa as a subsistence crop. Like most root crops, Amadumbe is high in carbohydrate content with low levels of protein and lipids. Amadumbe contain some essential fatty acids. Preliminary screening of Amadumbe revealed the presence of some anti-nutrients (substances in food of plant origin that interfere directly with the absorption of nutrients). Processing (boiling, frying and roasting) of Amadumbe showed a reduction in the content of these anti-nutritional factors (amylase inhibitor, trypsin inhibitor, oxalate, alkaloids, saponin, phytate and total phenols). Of the three different treatments, boiling appeared to be the most effective in reducing levels of all the investigated anti-nutrients in the white variety of Amadumbe. Any of the domestic processes used in this research work could be employed detoxifying most of the investigated anti-nutritional factors in *Colocasia esculenta*.

# CHAPTER A-1

# LITERATURE REVIEW

## A.1.1 Introduction

A community will accept certain foods as suitable for their consumption and these foods, because of custom and the people's partiality towards them, become regarded as traditional food crops. There are many examples of plant foods consumed as traditional dietary staples that are indigenous to Africa – for example: cassava, yam, plantain, sweet potato, millets and sorghum (Food and Agriculture Organization of the United Nations, 1997).

Amadumbe (*Colocasia esculenta*) is a non-indigenous specie widely grown in the sub-tropical parts of South Africa as a subsistence crop. Locally developed cultivars are used as a dietary source of starch. As a tuber crop, Amadumbe makes a significant contribution to the diet of local people in Kwazulu-Natal, where it is easily available. Several authors have evaluated the chemical composition of whole corms and cormels of taro, also known as cocoyam [*Colocasia esculenta* var. Schott of the tropics and subtropics in Africa (Sefa-Dedeh and Agyir-Sackey, 2004; Oscarsson and Savage, 2007)], but there is little or no information available on the nutritional quality of Amadumbe (*Colocasia esculenta* var Schott), the variety that is traditionally used in Kwazulu-Natal, South Africa.

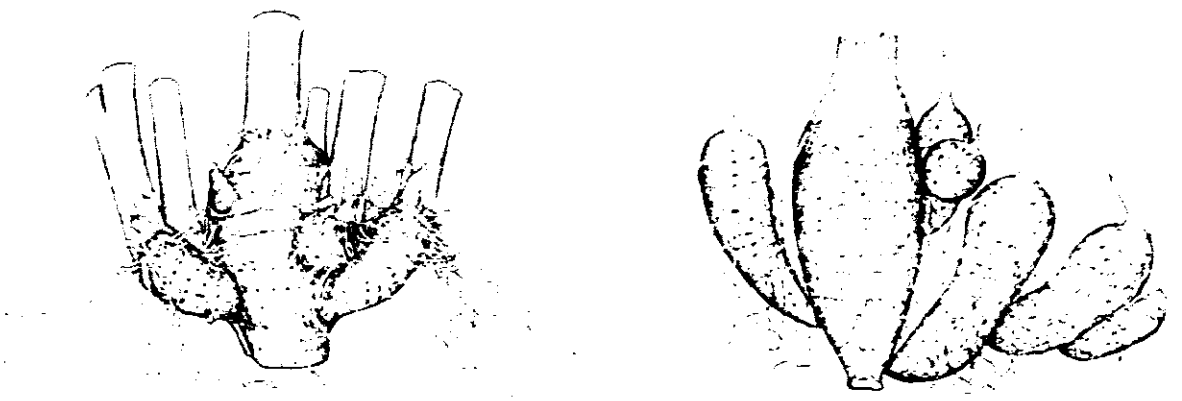
Anti-nutrients are compounds that limit the digestion and absorption of nutrients and result in reduced bioavailability of nutrients and flatulence production (Brune *et al.* 1989, Medoua *et al.*, 2007). Technological processes (for example, cooking) partially eliminate most anti-nutrients so that their acute toxicity is, as a rule, mild (Polomý, 1997; Adeparusi, 2001; West *et al.*, 2007). However, the possible health risk to those consuming food with high quantities of residual anti-nutrients cannot be ruled out. This

section of the study therefore investigates the nutritional and anti-nutritional status of processed and unprocessed Amadumbe.

### A.1.2 *Colocasia esculenta* (Amadumbe)

Amadumbe (Zulu name) belongs to the genus *Colocasia*, within the subfamily Colocasioideae of the monocotyledonous family Araceae. Taro or cocoyam is the common name for edible aroids which are important staple foods in many parts of this world (Chay-Prove and Goebel, 2004). Onwueme (1999) noted the problems arising from the taxonomy of the genus *Colocasia* owing to its complicated vegetative propagation. Purseglove (1972, cited in Onweume, 1999) identified two major botanical varieties:

i) *Colocasia esculenta* (L.) Schott var. *esculenta*;
ii) *Colocasia esculenta* (L.) Schott var. *antiquorum* (Schott) Hubbard & Rehder, which is synonymous with C. *esculenta* var. *globulifera* Engl. & Krause.



*(a) Colocasia esculenta var. esculenta* *(b) Colocasia esculenta var. antiquorum*

**Figure A1-1: Diagram showing the differences in corm structure between these two botanical varieties (a) Colocasia esculenta var. esculenta (a) and (b) Colocasia esculenta var. Antiquorum** (Hawaiian kalo, 2007)

Plants of the genus *Colocasia* are edible aroids with large leaves and one or more food-storing underground stems (corms). The corm is made up of starchy ground parenchyma with a thick brown skin consisting of central circular leaf scares and scales (Lee, 1999). Onwueme (1999) observed that the *Colocasia esculenta* var. *esculenta* is made up of a central corm, bulky and cylindrical in shape, with not many cormels (Figure A1-1a), whereas *Colocasia esculenta* var. *antiquorum* has a number of large cormels arising from a small, central, globe-shaped corm (Figure A1-1b). Amadumbe is classified as *Colocasia esculenta* var. Schott (Van Wyk and Gericke, 2000).

Taro (Amadumbe) has been described as a palatable, glabrate, annual herb. The leave blades (laminae) are large, 250 to 850 mm in length, 200 to 600 mm in width and 275 to 300 mm in thickness. The leaf laminae are carried on long, erect leafstalks (Figure A1-2). The leaf shape is described as being complete and lanceolate, with the apex tapering to a concave point (Lee, 1999).

*Colocasia esculenta* is best adapted to a warm, moist environment, ideally tropical or subtropical areas with long frost free periods. Amadumbe requires an average daily temperature above 21° C for normal production. High humidity with well-distributed summer rainfall or supplemental irrigation is ideal, partly because of the large, transpiring surfaces of Amadumbe laminae. Crops are normally grown in low-lying areas ranging from sea level to 1200 m elevation, only where moist conditions and stable temperatures generally prevail (Onwueme, 1999). Amadumbe is highly tolerant of saturated soil conditions, such as those found in wetlands, and artificial drainage of these soils is seldom required. Amadumbe cultivation does little harm to the wetland if it is restricted only to the less sensitive parts of the wetland. This presumes that artificial fertilizers and pesticides are not used and tillage is carried out by hand. Excessive drainage should be avoided and the cultivated area should be minimised. (Cultivation, food & health: amadumbe, n.d.).


**Figure A1-2: The aboveground portion of *Colocasia esculenta* (Amadumbe or Taro)**
(Missouri Botanical Garden, n.d.)

*Colocasia esculenta* (L.) Schott is an ancient crop grown throughout Africa for its edible corms and leaves, as well as for its traditional uses. Matthews (2004, cited in Oscarsson and Savage, 2007) surmised that *Colocasia esculenta* (L.) Schott had its origins in the tropics between India and Indonesia and the Food and Agricultural Organization [FAO] (1992, cited in Oscarsson and Savage, 2007) commented that this plant had been grown in the South Pacific for hundreds of years.

According to Bradbury and Nixon (1998), many *Colocasia esculenta* cultivars have a sharp, pungent taste and should not be eaten raw, because they can cause swelling of lips, mouth and throat. To enable comfortable ingestion, the tubers have to be thickly peeled and cooked over a long period (Saikai 1979; Crabtree and Baldry, 1982). Onayemi and Nwigwe (1987) identified the following constituents in *Colocasia esculenta*, once the causticity had been removed: digestible starch, high-quality protein, essential amino acids, vitamin C, thiamin, riboflavin and niacin.

In the rural areas of Kwazulu-Natal (SA), Amadumbe is the main starch source in cooked meals. Considered traditional African fare, the tubers are used in the same way as potatoes: fried as chips (Figure A1-3), mashed or barbequed whole (African affair, 2005). The young Amadumbe (cocoyam or taro) leaves can also be used as a vegetable (Aregheore and Perera, 2003): they are boiled for 15 minutes like spinach and are utilized as a supplement to maize. Leaves may be used in making salad and the Indian delicacy *puripatha*, often created in Durban, SA, is wrapped in Amadumbe leaves (Cultivation, food and health: amadumbe, n.d). Lee (1999) observed that taro (Amadumbe or cocoyam) is one of the only major staple foods where both the leaf and underground parts are used and have equal importance for human consumption. The excellent digestibility (98.8%) of the small starch grains of taro suggests efficient release of nutrients during digestion and absorption of this food (Lee, 1999).

Much work has been done on *Colocasia esculenta* (L.) var. Schott. As mentioned before, cocoyam and taro are the other common names used for these edible aroids. Cocoyams are grown for local consumption in West Africa and their corms and cormels are used in the same manner as yams or potatoes for local dishes (Onayemi and Nwigwe, 1987).

Taro is produced in the Pacific Islands and parts of Asia as a main staple food crop. In New Zealand, it is developed as a secondary food crop. These plants contain digestible starch, protein of good quality, vitamin C, thiamin, riboflavin, niacin and a high oxalate content (Onayemi and Nwigwe, 1987; Sefa-Dedeh and Agyir-Sackey, 2004; Pérez *et al.*, 2007; Catherwood *et al.*, 2007; Huang *et al.*, 2007; Oscarsson and Savage, 2007).

*Colocasia esculenta* has been broadly investigated for proximate composition and anti-nutrient screening, but the data were not comparable because of variations in genotypes, locations and experimental analysis. Plants are generally sensitive to environmental stress such as light intensity, rainfall, length of growing season, temperature and length of day, as well as agronomic factors such as soil fertility, weeds and plant density (McLean *et al.*, 1974; Robertson *et al.*, 1962; Singh *et al.*, 1972). Information is scanty or non-existent in literature on the presence of nutrients and anti-nutrients in Amadumbe. There are also no published studies on the effect of domestic cooking on the levels of anti-nutrients in Amadumbe tubers of Zululand, South Africa.

**Figure A1-3 Amadumbe chips with mango atchar** (www.woolworths.co.za)

Traditional crops are developed in rural areas because they have a high nutritional value, they are resistant to drought and cultivation is easy as long as standard agricultural practices are followed. By utiling indigenous knowledge about Amadumbe, not only has the community gained economic benefits directly, but indigenous African fare has also

acquired an increased status. The upmarket South African chain store Woolworths has used indigenous knowledge provided to sell organically grown Amadumbe (Makgoba, 2004).

### A.1.3 Fatty acids

#### A.1.3.1 Definition and structure

Fatty acids (carboxylic acids) often have a long, branchless tail, with carbon atoms linked in open chains (aliphatic). This chain can either be saturated or unsaturated. If the fatty acid does not contain any double bonds or other functional groups along the chain, it is termed saturated, whereas if it contains carbon-carbon double bonds, it is called unsaturated. If only one double bond is present, it is mono-unsaturated and with two or more double bonds, it is polyunsaturated. Linoleic acid and alpha-linoleic acid are essential fatty acids – that is, those which are needed by the body. These acids cannot be synthesised by humans, but are widely distributed in plant oils (Fatty acid, 2008).

#### A.1.3.2 Occurrence and food sources

Fatty acids can occur as either free or bound forms. In the bound form, they are attached to other molecules, such as in triglycerides or phospholipids (Fatty acid, 2008). Although fatty acids are found in plants, animals and bacteria, they tend to be more complex in plants and bacteria than in most animals (Fatty acids, 2008). Essential fatty acids provide practically all the polyunsaturated fat needed by man. Omega-3 and Omega-6 fatty acids are drawn from a diversity of sources, which include fish, leafy vegetables, seeds from plants such as pumpkins, chia and sunflowers, a variety of vegetable oils (for example: flaxseed/linseed hemp, soya, canola/rapeseed) and nuts (for example: peanuts, walnuts) (Essential fatty acid, 2008).

### A.1.4 Anti-nutrients

Foods are complex substances that contain many chemical compounds, more than 50 of which are required to nourish the body. These nutrients include water, proteins, lipids, carbohydrates, minerals and vitamins. Additionally, most plant foods also consist of natural compounds or anti-nutrients that appear to function generally in defense against herbivores and pathogens. Anti-nutrients are potentially harmful and give rise to a genuine concern for human health in that they prevent digestion and absorption of vitamins, minerals and other nutrients. They may not be toxic as such, but can reduce the nutritional value of a plant by causing a deficiency in an essential nutrient or preventing thorough digestion when consumed by humans or animals (Prathiba *et al.*, 1995). Several anti-nutritional factors are present in root and tuber crops and are partially neutralized during ordinary cooking. (Bhandari and Kawabata, 2004). The remaining anti-nutrients can, however, be responsible for the development of serious gastric distress and may interfere with digestion of nutrients, which inevitably results in chronic deficits in absorption of nutrients (Kelsay, 1985; Jood *et al.*, 1986; Brune *et al.*, 1989). Anti-nutritional factors include cyanogens, glycosides, saponins, phytate, enzyme inhibitors (trypsin and amylase inhibitors), lectins (haemagglutinins), oxalate and total polyphenols.

#### A.1.4.1 Proteinase inhibitors (PI) – Trypsin inhibitor

Numerous biochemical and physiological processes involve proteolytic enzymes. Kowalska *et al.* (2007) list the following activities for which these enzymes are responsible:

- "cellular protein digestion;
- intracellular protein turnover associated with defense mechanisms;
- elimination of misfolded proteins;
- activation of proenzyme, regulatory proteins and receptors;
- the release of hormones and biologically active peptide;
- assembling processes;
- cellular differentiation and ageing;

- seed development and mobilization of storage protein during seed germination or seedling growth;
- pathogen suppression; and
- pest proteinases."

Proteinase inhibitors which occur naturally play a crucial role in balancing body functions (Kowalska, 2007). Protease can be inactivated by being blocked by inhibitors and/or by proteins being broken down into simpler substances through hydrolysis (Troncoso *et al.*, 2007).

Proteinase inhibitors are proteins that bind to proteases, inhibiting proteolytic activity, and have been detected in animals, plants and microorganisms (Bhattacharyya *et al.*, 2007; Rawlings *et al.*, 2004). Zhang et al. (2004) observed the proteinase inhibitors accumulate at high levels in many plants because of attacks by bacteria and fungi, wounds and plant hormones. Bhattacharyya *et al.*, (2007) identified 59 individual families of proteinase inhibitors, which could be classified mainly as serine, cysteine, aspartic or metallo inhibitors. By the 1930s, the presence of proteolytic enzyme inhibitors had been detected in plants, although these had been identified in animals during the nineteenth century. Read and Haas (1938) reported that an aqueous extract of soybean flour inhibited the ability of trypsin to liquefy gelatin. The fraction of soybean protein responsible for this effect was partially purified by Bowman (1944) and Birk (1961) and subsequently isolated in crystalline form by Kunitz (1945).

Proteolytic enzyme inhibitors are widespread throughout all living entities (Bhattacharyya *et al.*, 2007). Proteinase inhibitors in plants could be a form of storage protein (Valueva and Mosolov, 1999) or may be to protect the plant against infections and disease. Generally, these proteins form part of a defense mechanism in plants to defend them against proteinases of pests and pathogens and discourage herbivores (Tiffin and Gaut, 2001; Tamhane *et al.*, 2005). Proteinase inhibitors cause amino acid deficiencies which affect the development and growth of an insect. When gut proteinases are inhibited or digestive enzymes are vastly over-produced, the insect may die because

essential amino acids not available for the synthesis of other proteins (Bolter and Jongsma, 1997; Pompermayer *et al.*, 2001).

Kowalska *et al* (2007) commented that an abundant source of protein proteinase inhibitors may be found in plant seeds and storage. Other substances, such as tannins (Price and Butler, 1980; Quesada *et al.*, 1996) and several indigestible polysaccharides (Price *et al.* 1988), may also inhibit digestive enzymes. However, the contribution of these compounds to the total trypsin inhibitor activity (TIA) is minimal in most foods (Ikeda and Kusano, 1983).

Trypsin is one of the serine-protease inhibitors identified and characterized in plants (Tremacoldi and Pascholati, 2002; Richardson, 1991). In general, trypsin inhibitors are low molecular weight proteins formed by association of identical peptide chains of smaller size. Normally these chains are devoid of carbohydrates, but have between one and eight disulphide bonds (Mueller and Weder, 1990). A common characteristic of plant protease inhibitors is their resistence to pH extremes, heat and hydrolysis by proteases as a result of the level of S-S bridges (Proteinase inhibitors, 1999).

Based on the molecular masses, cysteine content and number of reactive sites, these inhibitors have been categorized into two families, the Kunitz Type and the Bowman Birk Type. Bhattacharyya (2007) identified Bowman Birk Type inhibitors as "... usually 8-kDa proteins with seven disulfide bridges..." and Kunitz Type as "... 20-kDa proteins with just two disulfide linkages". Although active sites are much the same, it would appear that protease inhibitors from different sources have varied structures, molecular weights and chemical compositions (Richardson, 1977; Liener, 1994). These inhibitors inhibit trypsin and chymotrypsin at independent binding sites by reacting with some functional groups in the active site of the enzyme. This inhibition prevents the substrate from entering the active site or leaves the site catalytically inactive (Bender and Hézdy, 1965; Rittschof *et al.*, 1990).

A protein which inactivates trypsin is found in raw soybeans. This protein causes an increase in the size of the pancreas (hypertrophy), together with increased secretory pancreatic activity (Pimentel *et al.*, (1996). Lyman and co-workers (Green and Lyman, 1972; Lyman *et al.*, 1974) have shown that levels of the proteolytic enzymes trypsin and chymotrypsin in the small intestine determine the efficacy of the system regulating negative feedback inhibition, which, in turn, controls pancreatic secretion. Liener (1981) commented that the pancreas is stimulated to produce more enzymes when the level of trypsin is reduced: that is, when it is combined with the inhibitor. Cholecystokinin (CCK) is the major hormone responsible for activating pancreatic enzymes. If too little trypsin is available, CCK is released from the jejunal endocrine cells of the small intestine (Liddle, 2007). These relationships are illustrated in Figure A1-4.

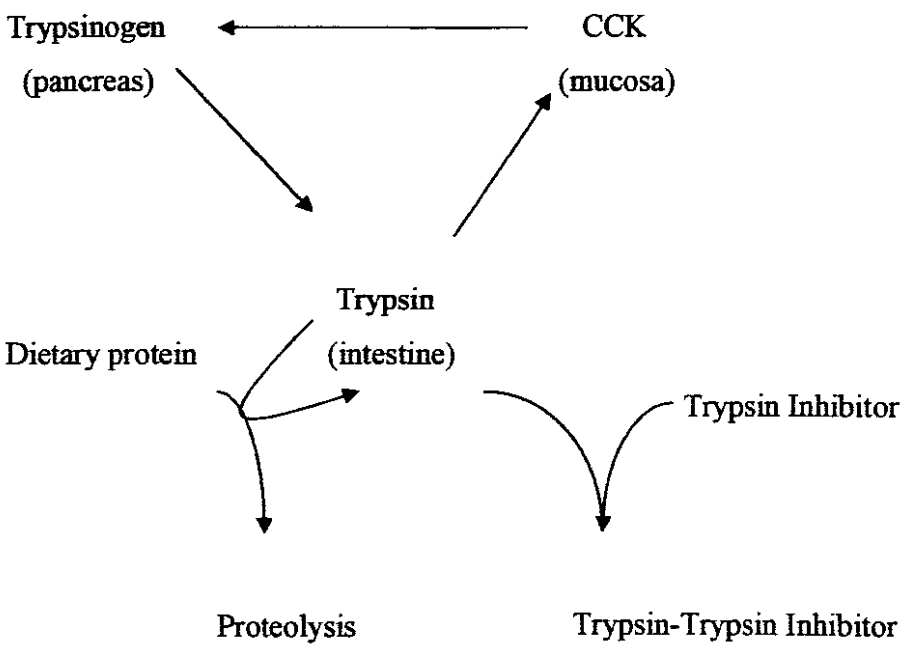



**Figure A1-4: Mode of action of soybean trypsin inhibitors on the pancreas**
(Anderson *et al.*,1979)

Proteinase inhibitors may add to their natural biological functions, the treatment of human pathologies such as blood clotting and haemorrhage, inflammation and cancer (Neuhof *et al.*, 2003; Fook *et al.* 2005; Mello *et al.*, 2006).

### A.1.4.2 Amylase inhibitors

In order to assimilate starches, which are complex storage carbohydrates, amylase (a digestive enzyme) and other enzymes have to break them down (Choudhury *et al.*, 1996). Through amylase [α-1,4-glucan-4-glucanhydrolase – Enzyme Code (EC) number 3.2.1.1], starch is broken down to maltose units, then to glucose monomer units. There are plants which have proteinaceous α-amylase inhibitors, as well as proteinase inhibitors (Franco *et al.*, 2000; Mello *et al.*, 2002; Payan, 2004).

Several types of plants, especially those in the legume family, have been used to extract amylase inhibitors (Marshall and Lauda, 2006). A large number of sweet potato genotypes have also been reported to contain α-amylase inhibitors (Sasikiran *et al.*, 1999; Rekha *et al.*, 1999). Pancreatic and salivary amylase action is affected by amylase inhibitors (Saunders, 1975; Pace *et al.*, 1978) and faeces reflect this by evidencing a greater proportion of undigested starch. Pusztai *et al.* (1995) observed that the nutritional quality of the food ingested consequently decreases, and Boivin (1987) surmised that undigested starch in the colon because of high level of amylase inhibitors may cause diarrhea.

Diabetes mellitus is a chronic disease, associated with abnormally high levels of glucose in the blood, caused by an insufficient insulin production or lack of responsiveness to insulin or both (WHO, 1999). A means of treating diabetes is through the reduction of postprandial hyperglycemia by activating decreased absorption of glucose in the digestive tract. The action of carbohydrate-hydrolyzing enzymes, α-amylase and α-glycosidase is inhibited to achieve this state, thus decreasing and delaying digestion of carbohydrates. This leads to a reduction in the rate of glucose absorption and a consequent decrease in plasma glucose after meals (Rhabasa-Lhoret and Chiasson, 2004; Ali *et al.*, 2006).

It has been claimed that α-amylase inhibitors assist weight loss, although initial research showed them ineffective in reducing carbohydrate absorption (Bo-Linn *et al.*, 1982;

Hollenbeck *et al.*, 1983; Carlson *et al.*, 1983). Interestingly, subsequent investigation indicated that highly concentrated types of amylase inhibitors could potentially reduce such absorption in humans (Udani and Singh, 2007). Franco *et al.* (2000) noted that many studies have been undertaken to determine the value of α-amylase inhibitors as a means of strengthening a diverse range of plants against insect and microbial pests.

### A.1.4.3 Lectins

Lectins or phytohemagglutinins may be defined as large protein or glycoprotein molecules (Liener, 2000) and have been identified in more than 800 plant species, including legume seeds, pulses and tubers (Noman *et al.*, 2007; Van Nevel *et al.*, 1998; Ignacimuthu *et al.*, 2000). Molecules which are found on the epithelial cells of the intestinal walls and contain carbohydrate can be bound by lectins. The toxic potential of lectins is determined by the degree of this binding (Liener, 2000). These proteins reflect their toxicity by inhibiting growth in experimental animals (Liener, 1986) and by causing diarrhea, nausea, bloating and vomiting in humans (Liener, 1982). Because the presence of lectins irritates the intestinal wall, over-secretion of mucus from this membrane may affect its enzymatic and absorptive efficacy. When lectins are together with other anti-nutrients, this harmful effect may be more pronounced (Francis *et al.*, 2001). Lectins are classified into several groups based on their sugar-binding specificity. Francesca *et al.* (2001) observed that apparently most lectins have relative masses of between 100 000 and 150 000 daltons and are composed of tetramers.

Lectins have the ability to recognize carbohydrates which bind and agglutinate red blood cells and can therefore be used for blood typing (Pusztia, 1992). Autoclaving and aqueous heat treatment – 100°C for 10 minutes – are two methods which eliminate lectins (Grant, 1991). Thus, there would appear to be little cause for concern regarding toxicity if food is cooked properly.

### A.1.4.4 Total polyphenols

Polyphenols are a large and varied class of metabolites widely spread throughout the plant kingdom: they are a complex but important group of naturally occurring compounds (Ryan *et al*, 1999). Phenolics are secondary metabolites: that is, they are not directly involved in any metabolic process (FAO, 1995). Plants produce them during natural development (Harborne, 1982) and as a result of stress conditions such as wounding, infection and ultraviolet radiation (Beckman, 2000).

These compounds, derived from phenylalanine and tyrosine, are an extremely diversified group of phytochemicals (Shahidi and Naczk, 2004). This class of plant metabolites contains more than 8000 known compounds, ranging form simple phenols – such as phenol itself – through to materials of complex and variable composition, such as tannins (Harborne, 1993; Bravo, 1998). One thing that all phenolic compounds have in common is that their molecular structure includes an aromatic hydrocarbon group to which a hydroxyl functional group (-OH) is attached.

Plant foods mostly contain phenolic acids in the bound form. The principal phenolic acids which may be found in plants are alternative derivatives of hydroxybenzoic and hydroxycinnamic acids, the most common of which are hydroxycinnamic acids (Mattila and Hellström, 2007). Examples of the latter are caffeic, *p*-coumaric and ferulic acids, which are often found in foods as simple esters with quinic acid or glucose. These derivatives differ in the patterns of the hydroxylations and methoxylations of their aromatic rings (Shahidi and Naczk, 1995; Hermann, 1989). The methoxylations of their pungent rings and the patterns of the hydroxylations differentiate these derivatives (Shahidi and Naczk, 1995; Hermann, 1989).

Phenolics may add to the smell, taste (bitterness, sharpness), colour and oxidative stability of food (Maga, 1978; Robbins, 2003). The dissociability of their –OH group renders phenols acidic. They are also easily oxidized and form polymers (dark aggregates). Phenolic compounds discolour and darken because of enzyme activity, as

polyphenoloxidase catalyzes into quinines. As phenolic compounds oxidize, for example, yam tubers which have been cut and left open to the air turn brown (Ozo *et al.*, 1984; Osagie and Opoku, 1984).

Plant phenolic compounds are a large and heterogenous group of secondary metabolites with low molecular weight. Examples of these are flavonoids, phenolic acids and lignans (Makoi and Ndakidemi, 2007). Phenolic compounds, especially tannins, inhibit digestive enzymes and interfere with the way in which vitamins and minerals are used. These compounds may also bind and precipitate proteins, thus causing a reduction in nutritional value (Chung *et al.*, 1998; Chavan *et al.*, 2001). Indeed, polyphenols have long been labelled anti-nutrients because of this ability.

The anti-nutritional tag may also be a result of the way in which polyphenols inhibit the rate of activity of digestive enzymatic processes. Many enzymes are thus inhibited: for example, hydrolases, protein phosphokinases, isomerases and oxygenases (Ferguson, 2001). Additionally, phenolic acids have strong antioxidant activities and exhibit anticarcinogenic, antiviral, anti-inflammatory, antibacterial and vasodilatory actions (Duthie *et al.*, 2000; Breinholt, 1999).

Flavonoids are polyphenolic secondary metabolites that are omnipresent in higher plants. Many are simply recognized as flower pigments in most angiosperm families (Harborne, 1994). However, their presence is not confined to flowers but include all parts of the plants. About 2/3 of the polyphenols we obtain in our diets are flavonoids. Armstrong (2007) defined flavonoids as: "... 3-ring phenolic compounds consisting of a double ring attached by a single bond to a third ring".

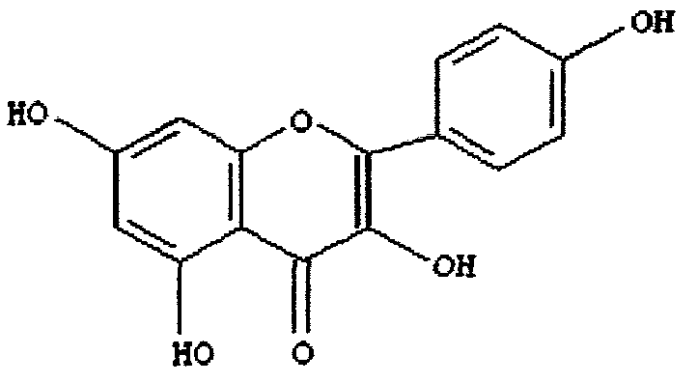



**Figure A1-5: Structure of a flavonoid (flavonol): Phenolic compound composed of the benzene rings with hydroxyl (OH) groups** (adapted from Armstrong, 2007).

Many of the flavonoids (Figure A1-5) that occur in plants are in the form of glycosides, where one or more simple sugar, such as glucose or galactose, is attached to them (De Rijke *et al.*, 2006). To sustain healthy tissues and achieve the correct balance of hormones and antioxidants in the body, flavonoids (bioflavonoids and isoflavones) are recommended by some nutritionists. (Brown *et al.*, 1998). However, because flavonoids may precipitate proteins (Jende-Strid, 1991), they demonstrate anti-nutritional properties.

Tannins form another group of phenolic compounds, usually divided into hydrolysable tannins and condensed tannins (proanthocyanidins), and are caustic and bitter-tasting. They bind and precipitate proteins, decreasing the digestibility of protein and carbohydrate. This results in growth depression, in all probablility owing to enzyme-resistant substrates formed by interaction between tannins and protein/starch. Digestibility of the substrates is compromised by interaction between tannin and the enzymes (Deshpande and Salunke, 1982; Griffiths, 1986).

Deshpande *et al.* (1986) reported that polyphenols react with proteins and enzymes, so that they can also act as trypsin and amylase inhibitors. Other anti-nutritional effects that have been attributed to tannins include damage to the intestinal mucosa (Mitjavila *et al.*,

1977), (Hagerman and Butler, 1991) and an interference with the absorption of iron (Garcia-Lopez *et al.*, 1990), glucose (Welch *et al.*, 1989) and vitamin B (Singh, 2004).

### A.1.4.5 Alkaloids

Alkaloids are one of the largest groups of chemical compounds synthesised by plants (Raffauf, 1996). They are generally found as salts of plant acids such as oxalic, malic, tartaric or citric acid (Watkins *et al.*, 2004) Alkaloids are small organic molecules, common to about 15 to 20 per cent of all vascular plants, usually comprising several carbon rings with side chains, one or more of the carbon atoms being replaced by a nitrogen. They are synthesized by plants from amino acids. Decarboxylation of amino acids produces amines which react with amine oxides to form aldehydes. The characteristic heterocyclic ring in alkaloids is formed from Mannich-type condensation from aldehyde and amine groups (Hendricks and Bailey, 1989).

The chemical type of their nitrogen ring offers the means by which alkaloids are subclassified: for example, glycoalkaloids (the aglycone portion) glycosylated with a carbohydrate moiety. They are formed as metabolic by-products. Insects and hervibores are usually repulsed by the potential toxicity and bitter taste of alkaloids (Levin, 1976; Robbers *et al* 1996).

Tubers of the common potato (*Solanum tuberosum*) have a natural content of the two toxic and bitter glycoalkaloids (GA) α-solanine and α-chaconine (Finotti *et al.*, 2006) [Figure A1-6]. The levels are normally low and without adverse affects on food safety and culinary quality. However, consumption of potato tubers with unusually high GA contents (300-800 mg $kg^{-1}$) has occasionally been associated with acute poisoning, including gastro-intestinal and neurological disturbances, in man (Van Gelder, 1991). Tuber GA levels are inheritable and can vary considerably between different species. Environmental factors experienced by tubers during germination, growth, harvest and storage may affect GA levels further (Jadhav *et al.*, 1981; de la Cuadra *et al.*, 1994).

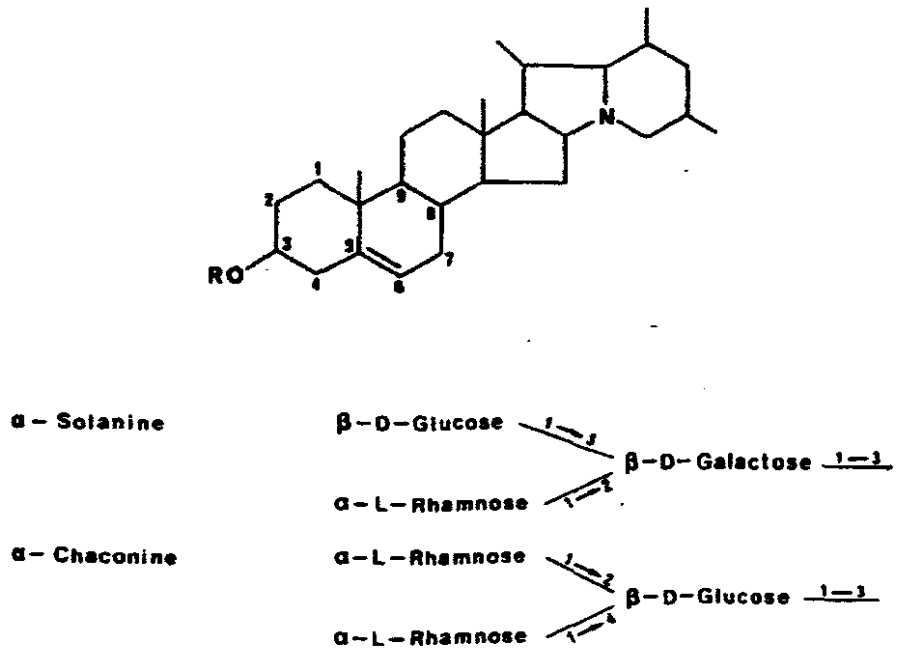



**Figure A1-6: Structures of two major glycoalkaloids found in potatoes: α-solanine and α-chaconine** (Wong, 1989)

Alkaloids are considered to be anti-nutrients because of their action on the nervous system (Cheeke and Kelly, 1989), disrupting or inappropriately augmenting electrochemical transmission. Indeed, the physiological effects of alkaloids have on humans are very evident. Cholinesterase is greatly inhibited by glycoalkaloids, which also cause symptoms of neurological disorder (Jackson, 1991). Other toxic action includes disruption of the cell membrane in the gastrointestinal tract (Friedman *et al.*, 2003).

Alkaline pH conditions generally enhance absorption of glycoalkaloids, where binding with sterols in cell membranes causes extra disruption (Roddick, 1979). Lethal doses for humans range between 3 and 6 mg/kg body weight, although susceptibility varies considerably among individuals. A dose of more than 2 mg/kg is usually considered toxic

(Wong, 1989). Korpan *et al.* (2004) identified poison symptoms as including vomiting, diarrhoea, abdominal pain, apathy, weakness and unconsciousness

Nicotine, caffeine, quinine and strychnine are well-known examples of alkaloids. Randolph (2008) claimed that alkaloids in food might well be at least partially responsible for the food-allergy effect of addition, where withdrawal from the food causes disagreeable symptoms.

### A.1.4.6 Oxalate

The plant *oxalis*, commonly known as wood sorrel, gave rise to oxalic acid (chemical formula HOOC-COOH), a strong, organic acid which has been found to be widely distributed in plants (Liebman, 2002). See Figure A1-7 for structure.

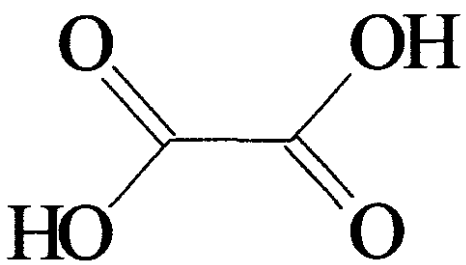



**Figure A1-7: Structure of oxalic acid**

Strong bonds are formed between oxalic acid and various other minerals, such as calcium, magnesium, sodium and potassium (Fink, 1991; Noonan and Savage, 1999). This chemical combination results in the formation of oxalate salts. A salt formed from oxalic acid is known as an oxalate: for example, calcium oxalate. Some oxalate salts, such as sodium and potassium, are soluble, whereas calcium oxalate salts are basically insoluble. The insoluble calcium oxalate has the tendency to precipitate (or solidify) in the kidneys or in the urinary tract, thus forming sharp-edged calcium oxalate crystals

when the levels are high enough (Bradbury and Nixon, 1998). These crystals play a role to the formation of kidney stones (Noonan and Savage, 1999).

When oxalic acid is consumed, it irritates the lining of the gut and can prove fatal in large doses. Hui (1992) stated that intake of 5g or more of oxalic acid could be fatal to humans while Munro and Bassir (1969) estimated the threshold of oxalate toxicity in man to be 2-5g/100g of the sample. Oxalic acid is a common and wide-spread component of most plant families. While the levels of this acid in these plants are generally low, it is the high concentrations in the leaves and corms of plants consumed daily that are of concern.

Oxalate is an anti-nutrient which under normal conditions is confined to separate compartments. However, when it is processed and/or digested, it comes into contact with the nutrients in the gastrointestinal tract (Kaushaiya *et al.*, 1988). When released, oxalic acid binds with nutrients, rendering them inaccessible to the body. If food with excessive amounts of oxalic acid is consumed regularly, nutritional deficiencies are likely to occur, as well as severe irritation to the lining of the gut.

Most taro cultivars have an astringent taste and can cause swelling of lips, mouth and throat if eaten unprocessed. This causticity is caused by closely-packed, needle-like, calcium oxalate crystals, which can penetrate soft skin (Bradbury and Nixon, 1998). Thereafter, an irritant, probably a protease, present on the sheathlike bundle of needles (raphides) can cause discomfort in the tissue (Bradbury and Nixon, 1998; Paul *et al.*, 1999). Both the tubers and the leaves can give this reaction (FAO, 1992) but this effect is reduced by cooking (Bradbury and Nixon, 1998).

### A.1.4.7 Phytate

Phytic acid, which is hexaphosphate of myo-inositol, is very common in the plant kingdom and is found mainly in mature seeds such as legumes, fruits, vegetables and cereal grains (Chan *et al.*, 2007). Phytic acid is the primary storage compound of phosphorus in plants, accounting for up to 80 per cent of the total phosphorus (Raboy, 2001; Steiner *et al.*, 2007). Josefsen *et al.*, (2007) reported that the negatively charged

phosphate in phytic acid strongly binds to metallic cations (e.g. Ca, Fe, K, Mg, Mn and Zn) and forms a mixed salt called phytin or phytate.

Phytate has been classified as an anti-nutritional factor in the diets of humans. The anti-nutritive effect of phytic acid is based on its molecular structure (Figure A1-8). Phytic acid's twelve negative charges from the six phosphate groups bind different di- and trivalent cations into a stable complex. Complete dissociation of phytic acid depends on the pH conditions (Pallauf *et al.*, 1998). Phytate forms insoluble complexes because they are negatively charged under physiological conditions. These complexes cannot be digested or absorbed in the gastrointestinal tract owing to the absence of the intestinal phytase enzyme (Iqbal *et al.*, 1994). Deficiencies of phosphorus and nutritionally important minerals in human populations can be a result of cations bound in the phytic acid salt and of low bioavailability of phosphorus (Gibson *et al.*, 2006).

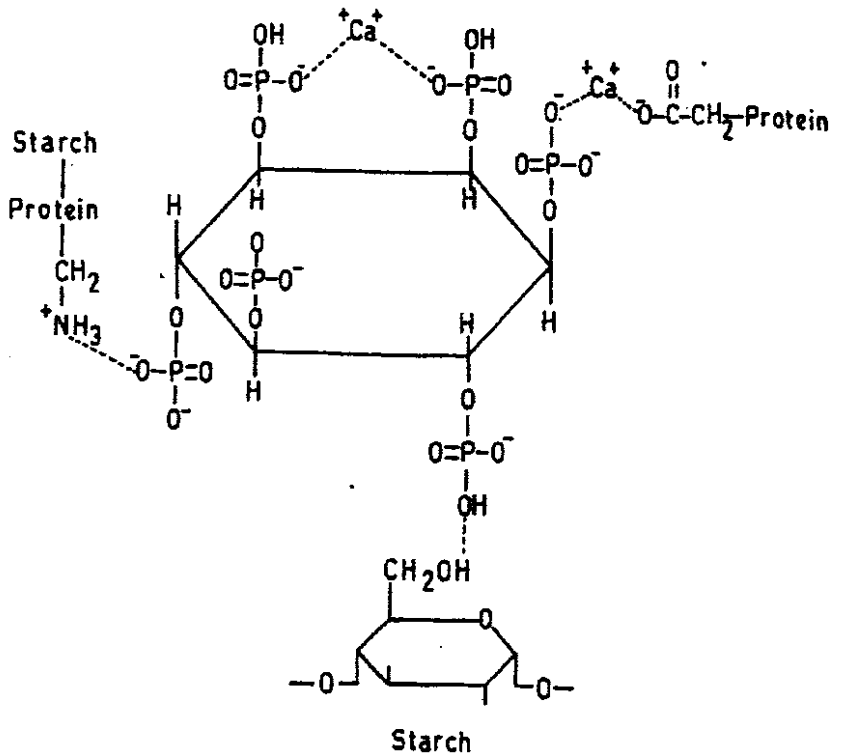



**Figure A1-8: Possible interactions of phytic acid with minerals, proteins and starch** (Thompson, 1988).

The ability of phytic acid to complex with proteins and particularly with minerals has been a subject of investigation for several reasons, predominantly from chemical and nutritional viewpoints. Phytic acid forms complexes with nutritionally important minerals such as calcium, copper and iron ($Fe^{2+}$ and $Fe^{3+}$). This reaction decreases the solubility of the metals, which are therefore not readily absorbed from the intestine (Brune *et al.*, 1989; Sandberg *et al.*, 1993; Weaver and Kannan, 2002).

There is strong concern that reduced mineral absorption, owing to consumption of food products rich in phytates, may lead to borderline malnutrition (Reddy and Pierson, 1994). It has also been shown that calcium ions interact with protein and phytate to decrease the solubility of proteins further (De Rham and Jost, 1979; Frokiaer *et al.*, 2001). As a consequence, phytate has been shown to inhibit the proteolysis of a number of enzymes (including pepsin, trypsin and amylases of the intestinal tract) important in digestion (Vaintraub and Bulmaga, 1991).

Certain functional properties of proteins, which are dependant on their solubility and hydration, can be negatively affected by the reduced solubility of proteins as a result of protein-phytate complex. Such hydrodynamic properties (viscosity, gelation) include emulsifying capacity, foaming and foam performance, and dispersibility in aqueous media (Urbano *et al.*, 2000). Phytic acids may also affect the digestibility of starch. Phytic acid and starches are structurally capable of combining via phosphate linkages (Sirkka, 1997). Thus, adverse effects on the digestion of starches and proteins, as well as reduced bioavailability of essential dietary minerals, are the result of high phytate contents in tubers.

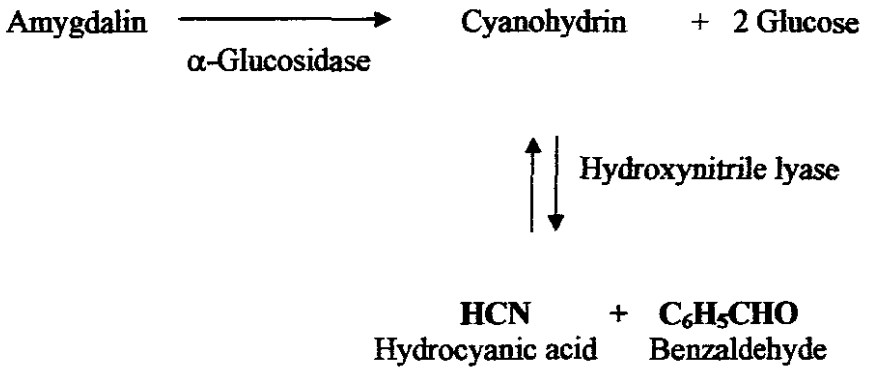
Investigators have shown that phytic acid may be beneficial with regard to human health, including as an anti-cancer agent (soft tissue, colon, metastatic lung cancer, mammary cancers) [Shamsuddin *et al.*, 1997; Febles *et al.*, 2002], as an inhibitor in renal stone development (Grases *et al.*, 2000) and as an anti-oxidant agent. Phytic acid has an anti-oxidative function because it chelates with iron by combining with all the available Fe

coordination sides to inhibit OH radical production (Obata, 2003; Muraoka and Miura, 2004).

Indeed, it is possible to use phytic acid as an uncommon, versatile food preservative because of its anti-oxidant or iron-chelating properties (Hix *et al.* 1997). Thompson (1988) suggested that the interaction among phytate, dietary starch and protein could be beneficially utilized in the treatment of diabetes and hyperglycemia. This apparent discrepancy between unhealthy and healthy properties of phytate clearly calls for a re-evaluation of this storage compound (Grases *et al.* 2001).

### A.1.4.8 Cyanogens

Cyanogen molecules consist of two CN groups that are bonded together at their carbon atoms: N≡C-N≡C. Cyanogens are glycosides of 2-hydroxynitriles and are widely distributed among plants (Rosenthal and Berenbaum, 1991; Bisby *et al.*, 1994). When plants are wounded by herbivores or other organisms, the cellular compartmentation breaks down and cyanogenic glycosides come into contact with an active β-glucosidase, which hydrolyses them to yield 2-hydroxynitrile (Vetter, 2000). This is further cleaved into the corresponding aldehyde or ketone and hydrogen cyanide (HCN) by hydroxynitrile lyase (eq. A1-1) (Conn, 1981). It is the low pH in the stomach which deactivates the β-glucosidase. Because the environment of the gut is alkaline, reactivation of part of the enzyme fraction is a possibility. The collective name given to glycosides, cyanohydrins and hydrogen cyanide is cyanogens (Møller and Seigler, 1999).




**Equation A1-1: Breakdown of cyanogenic glycosides to release hydrogen cyanide** (Wong, 1989)

The ability of plants to produce HCN, known as cyanogenesis, is exhibited by at least 1000 species representing approximately 90 families and at least 250 genera (Miller *et al.*, 2006). About 60 cyanogenic glycosides are known from higher plants (Nahrstedt, 1987; Seigler, 1991) and occur in at least 2000 plant species, of which a number of species are used as food in some areas of the world. Two examples of staple foods containing cyanogenic glycosides are cassava and sorghum (Okafor, 2004; Agbor-Egbe and Mbome, 2006). Figure A1.9 shows the structure of some of the best known cyanogenic glycosides.

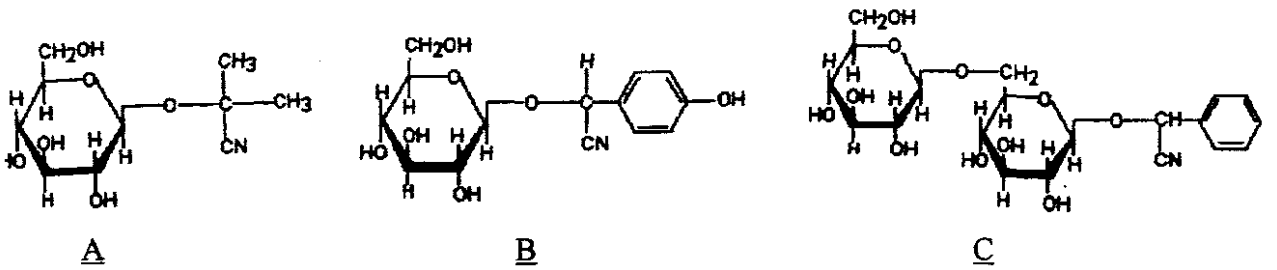



**Figure A1-9: Structures of some common cyanogenic glycosides** (A) Linamarin (B) Dhurrin (C) Amygdalin. (Simeonova and Fishbein, 2004)

Cytochrome oxidase, the terminal oxidase of aerobic organisms, is the primary site of action for ingested cyanide, an effective inhibitor of many metalloenzymes (Enneking and Wink, 2000). The enzyme cytochrome oxidase in the mitochondria of cells is inactivated by hydrogen cyanide binding to the $Fe^{2+}$/ $Fe^{3+}$ contained in the enzyme. This results in a reduction of oxygen usage in the tissues (Vetter, 2000) and oxygen starvation at cellular level, owing to the effects of cyanide poisoning, can result in death. Respiratory failure is therefore the cause of death since the respiratory centre nerve cells are extremely sensitive to hypoxia (Okolie and Osagie, 1999).

Other long-term diseases associated with dietary cyanide intake include (i) konzo (Cliff *et al.*, 1997), a paralytic disease; (ii) tropical ataxic neuropathy (TAN) [Onabolu *et al.*, 2001], a nerve-damaging disorder that renders a person unsteady and uncoordinated; (iii) goiter and cretinism (Delange *et al.*, 1994). More than 10 000 people in Mozambique, Tanzania and the Democratic Republic of the Congo have been paralysed by a devastating disease that developed from prolonged exposure to sublethal levels of dietary cyanide (Shorter, 1997).

Sheeba and Padkaja (1997) observed that the palatability and shelf-life of cassava products may be prolonged by processing. The levels of cyanogenic glycosides and hydrogen cyanide are also reduced to safer limits by processing (peeling, slicing, boiling) before consumption (Sheeba and Padmaja, 1997; Feng *et al.*, 2003).

Bourdoux *et al.*, (1982) and others concluded that, based on total root cyanide content, less than 50 ppm may be classified as innocuous; 50-100 ppm as moderately poisonous; and more than 100 ppm as dangerously poisonous. In 1991, the World Health Organisation (WHO) set the safe level of cyanogens in cassava flour at 10ppm (FAO/WHO, 1991). The acceptable limit in Indonesia has been set at 40 ppm (Damardjati *et al.*, 1993; Djazuli and Bradbury, 1999).

### A.1.4.9 Saponins

Saponins (Figure A1-10) are a group of varied, biologically active glycosides which derive their name from their ability to form stable, soaplike foams in aqueous solutions. They represent a complex and chemically diverse group of compounds. Saponins consist of a polycyclic aglycone that is either a triterpenoid ($C_{30}$) or steroid ($C_{27}$) sapogenin, attached via ester and ether linkages to a sugar side chain (Schwarz, 1993). Glucose, galactose or a pentose or methylpentose may make up the sugar moieties.

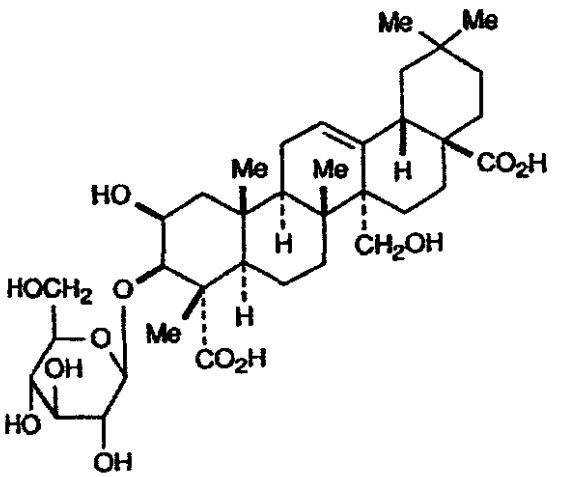



**Figure A1-10: Molecular structure of saikosaponins from bupleurum.**
(Dharmananda, 2000)

Saponins have purgative properties, forming oil-in-water emulsions and producing abundant amounts of foam when dissolved in water. The ability of a saponin to foam is caused by the combined hydrophobic sapogenin and the hydrophilic side chain. Saponins are non-volatile compounds that are known to have a bitter taste and reduce the palatability of livestock feeds (Curl *et al.* 1985; Bishnoi and Khetarpaul, 1994). It is this undesirable, bitter taste that creates a major problem in the utilization of saponin-containing plants and their products (Davies and Lightowler, 1998).

However, if saponins have a triterpenoid aglycone with glucuronic acid, they may taste of licorice, sweetened by the sugar moiety (Grenby, 1991). Saponins occur in a wide variety of plants such as peanuts, lentils, lupins, alfalfa, soybeans, peas, oats and spinach (Smartt, 1976; Oakenfull, 1981; Price *et al.* 1987; Cuadrado *et al.*, 1995; Huhman and Sumner, 2002).

Saponins are attracting considerable interest as a result of their diverse properties, both deleterious and beneficial (Cuadrado *et al.*, 1995). One of the well-known biological effects of saponins is their ability to split erythrocytes (Khalil and El-Adaway, 1994) and to make the intestinal mucous membrane permeable (Johnson *et al.*, 1986). Saponins are amphiphilous compounds which interact with biomembranes of animals, fungi and even bacteria. The hydrophobic part of the molecule forms a complex with cholesterol inside the membrane and their hydrophilic sugar side chain binds to external membrane proteins. Thus, the fluidity of biomembranes is disturbed, which leads to the formation of holes and pores. As a consequence, cells become leaky and die (Oakst and Knowles, 1977).

Clinical reports have also shown that the immune system is affected by saponins in ways that lower cholesterol levels and help to protect the human body against cancers (Pathirana *et al.*, 1980). This hypocholesterolemic effect has been ascribed to a complexation of the saponin with cholesterol and bile acids, which results in a decrease in their absorption from the intestines (Matsuura, 2001). A saponin-rich diet can be used for the treatment of hypercalciuria in humans, as an antidote against lead poisoning and in the inhibition of dental caries and platelet aggregation (Shi *et al.* 2004). Saponins also reduce blood lipids (Chunmei *et al.*, 2006), lower the risks of developing cancer (Messina and Bennik, 1998) and lower blood-glucose response (Sajadi Tabassi *et al.* 2007).

### A.1.5 Effect of processing on nutrients and anti-nutrients

Several factors influence the nutritional content of food. These include the genetic make-up of the plant, the soil in which it is grown, use of fertilizer, prevailing weather, maturity at

harvest, packaging, storage conditions and method utilized for processing (Morris *et al.*, 2004).

The primary purpose of processing is to render food palatable and develop its aroma, but it has inevitable consequences on the nutritional value of foods. Washing and peeling result in the loss of many water-soluble vitamins, since these are more concentrated in the peel and outer layers. With careful control of the processes, nutrient losses can be minimized without affecting palatability.

The term "food processing" covers an enormous field, from simple boiling to the use of irradiation. The types of cooking methods differ in countries around the world and also vary with the ethnic background of the family. Processing (cooking) can be both beneficial and detrimental to nutrient composition of foods. It is known that processing techniques may decrease the food value of some nutrients (Nestares *et al.*, 1996): for example, there is some inevitable leaching of nutrients into the cooking water during processing. The cooking water may or may not be discarded, depending upon cultural and personal preference.

On the other hand, processing may enhance the nutritional quality of food by reducing or destroying the anti-nutrients present in it, as well as increasing the digestibility of proteins and starches. Elimination or inactivation of anti-nutritional compounds is absolutely necessary to improve the nutritional quality and effectively utilize human foods to their full potential. Processing generally inactivates heat-sensitive factors such as enzyme inhibitors, lectins and volatile compounds such as HCN. A typical example is the protein in legumes, which is made more digestible by heating because of the inactivation of anti-nutrients such as trypsin inhibitors (Siddhuraju and Becker, 1992).

The use of some processing methods, such as boiling, baking, microwave- and pressure cooking are known to achieve reduction or elimination of anti-nutritional factors (Udensi *et al.*, 2005; Bhandari and Kawabata, 2006; Habiba, 2002; Khokhar and Chauhan, 1986). Radiation processing and extrusion have also been used as a means to inactivate anti-

nutritional factors naturally present (El-Niely, 2007; Alonso *et al.* 2000). Extrusion cooking was found to be a more versatile, quick and efficient method to reduce anti-nutrients when compared with other traditional processing methods (Alonso *et al.* 2000). Soaking, sprouting, fermentation and cooking methods have also been investigated. Combination of cooking and fermentation improved nutrient quality and drastically reduced the anti-nutiritional factors to safe levels much greater than any of the other processing methods tested (Obizoba, 1991). Excessive heat processing, however, should be avoided, since it adversely affects the protein quality of foods. It is therefore important that processing is done within the recommended guidelines e.g. for heat, pH, as over processing will further destroy not only nutrient content but also taste and appearance (Morris *et al.*, 2004).

### A.1.6 Aim and outline of Section A

Taro (Amadumbe) is a staple food extensively eaten in the tropics and subtropics of Africa (Oscarsson and Savage, 2007). The nutritional value of taro has been extensively studied, but the researcher is not aware of any such study pertaining to Amadumbe, the South-African cultivar: hence, the importance of investigating the nutritional quality of Amadumbe.

Different domestic processing methods are used to reduce the health risk associated with food consumption. It is therefore necessary to estimate the levels of nutrients and anti-nutrients in processed and unprocessed Amadumbe.

The objectives of Section A were:

- to evaluate the chemical composition of three Amadumbe phenotypes (proximate, mineral and anti-nutritional factors) among these phenotypes;
- to investigate the effect of domestic processing methods (boiling, frying, roasting) on anti- nutritional factors present in Amadumbe.

# CHAPTER A-2

# MATERIALS AND METHODS

## A.2.1 Introduction

This chapter gives a brief description of the materials and methods used to determine the nutritional and anti-nutritional composition of *Colocasia esculenta* grown in Zululand, South Africa. See Appendices A and B for details.

## A.2.2 Materials

### A.2.2.1 Food material

Two varieties of *Colocasia esculenta* L. Schott (Amadumbe) – white and purple – were obtained from local markets at Esikhawini and Mtubatuba and from Makatini Experimental Farm, Kwazulu-Natal (KZN), South Africa.

### A.2.2.2 Reagents

(See Appendix A for details of how reagents were prepared.)

| | |
|---|---|
| α-Amylase | Sigma |
| α-Chymotrypsin from human pancreas | Sigma |
| β-glycosidase | Sigma |
| Ammonium molybdate | Kleber chemicals |
| Anthrone reagent | Merck |
| Ascorbic acid | Merck |
| Benzoyl-DL-arginine-*p*-nitroaniline (BAPNA) | Sigma |
| Bispyrazolone | Fluka |

| | |
|---|---|
| Bromocresol green | Sigma-Aldrich |
| Calcium oxalate | Fluka |
| Dinitrosalicylic acid (DNS) | Merck |
| Ferric ammonium sulphate [$FeNH_4(SO_4)_2$] | Merck |
| Papain | Sigma |
| Phosphoric Acid ($H_3PO_4$) | Merck |
| Polyvinylpolypyrrolidone (PVP) | Fluka |
| Potassium antimonytartrase | Merck |
| Potassium cyanide (KCN) | Merck |
| Potassium ferricyanide [$K_3Fe(CN)_6$] | Merck |
| Potassium hydrogen phosphate ($K_2HPO_4 \cdot 3H_2O$) | Merck |
| Potassium permanganate ($KMnO_4$) | Merck |
| Protease: *Bacillus Licherniformis* | Sigma |
| Pyridine | Merck |
| Saponin | Sigma |
| Sodium phosphate ($Na_3PO_4$) | Merck |
| Trypsin | Sigma |
| Trypsin type 1 Bovine pancreas | Sigma |
| Tungstophosphoric acid | Merck |
| Type xiii Fungal protease: *Aspergillus saitoi* | Sigma |
| Type xiv Bacterial protease: *Streptomyces griseus* | Sigma |
| Type xviii Fungal protease:Rhizopus | Sigma |
| Vanillin | UNILAB |

### A.2.2.3 Special equipment utilized

Soxhlet Extractor (Soxtec HT-6, Tacater AB, Höganas)
PerkinElmer Atomic Absorption Spectrophotometer (Carl Zeiss) Model AAS-3
GPR centrifuge (Beckman)
Spectrophotometer (Pharmacia biotech-novaspec)
UV Spectrophotometer
Laborota 400 Rotary evaporator (Heidolph)

### A.2.3 Methods

(See Appendix B for details of methodology)

### A.2.3.1 Sample preparation

Healthy Amadumbe tubers were washed, hand-peeled and cut into small pieces. Only tubers with little or no skin wounding were selected. Samples were divided into two groups: one group was referred to as *processed* and the other group as *unprocessed.* The unprocessed samples were dried at 55° C for 24 hours and then were milled into a fine powder which was stored in brown bottles until used. The processed portion was subdivided into three different parts (those to be boiled, fried and roasted), processed as described in Section A.2.3.2 and screened for the presence of nutrients and anti-nutrients.

### A.2.3.2 Processing techniques

Approximately 500 g each of the processed portion of *Colocasia esculenta* samples were separately subjected to the following processing techniques:

i. Boiling: Amadumbe samples were boiled in distilled water on a stove for 30 minutes, after which they were drained.
ii. Frying: Amadumbe samples were deep-fried in domestic cooking oil (100 per cent sunflower oil) for 15 minutes.

iii. Roasting: Amadumbe samples were roasted in a baking pan in an oven for 30 minutes at 180° C.

The processed samples were sun-dried and then milled into a fine powder, which was stored in bottles until used.

### A.2.3.3 Proximate composition

Protein, moisture, carbohydrate, ash and crude fat contents were determined as described in AOAC methods (1990). The crude protein content was calculated by converting the nitrogen content determined by the micro-Kjeldahl method (*N* x 6.25). The moisture content was determined by oven-drying at 110° C to a constant weight. The total carbohydrate and starch contents were percolated with ethanol and perchloric acid respectively and then reacted with anthrone (Hansen and Møller, 1975). Ash content was determined by incineration of the samples, the weights of which were known, in a furnace oven. Fat content was determined gravimetrically by extraction, using petroleum ether on a Soxhlet extraction unit. The dried residue was quantified gravimetrically and expressed as percentage of lipid.

### A.2.3.4 Fatty acid composition in Amadumbe

#### A.2.3.4.1 Lipid extraction

Purple and white Amadumbe samples from Esikhawini local market were separately extracted with a methanol-chloroform (2:1, v/v) mixture over 24 hours in an oven shaker at room temperature. This was then filtered under suction and the residue re-extracted with a methanol-chloroform-water (2.5:1.3:1, v/v) mixture for another 24 hours. The filtrates were combined and then separated in the separating funnel into chloroform and water fractions. The chloroform layer was then mixed with 30 ml of benzene and dried under vacuum. The residual lipids were weighed and redissolved in a minimal volume of a methanol-chloroform mixture and stored in the fridge (Bligh and Dryer, 1959).

In another experiment, Amadumbe samples were extracted with hexane. The extract was filtered through rough filter paper and then finely filtered through a 0.45 μm pore filter paper. The hexane was evaporated *in vacuo* and the residue dissolved in an acetonitrile-2-propanol-hexane (2:2:1, v/v) mixture.

### A.2.3.4.2 Iodine value

A known amount (5 mg) of the methanol-chloroform extracted lipid was dissolved in chloroform and Dam's reagent was added. The mixture was then left at room temperature in a dark room. Potassium iodide was added as the source of iodine in the mixture with the starch indicator. The mixture was titrated with thiosulfate solution to determine the liberated iodine. The value was used to determine the number of double bonds present in the lipid extract. (Yasuda, 1931; AOAC, 1984).

### A.2.3.4.3 Column chromatography

About 20 g of silicic acid was preheated in the oven at 120° C. A slurry was then prepared with 35 ml of chloroform and poured into a column chromatography tube (48 mm length x 2.15 mm internal diameter). The air in the tube was gently disloged and the column was washed with 2 column volumes of chloroform. The sample was then runned and eluted with different solvents of chloroform (175 ml), acetone (700 ml) and methanol (175 ml).

### A2.3.4.4 High Pressure Liquid Chromatography-Mass Spectroscopy (HPLC-MS)

The hexane extracts of Amadumbe were redissolved in methanol and water (1:1, v/v). The lipid extracts were then injected into a LCMS machine to determine the fatty acid composition. Standard fatty acids were similarly treated. Their profiles were used to identify and quantify the fatty acids of Amadumbe (See Appendix C).

### A.2.3.5 Mineral analysis

The standard Association of Official Analytical Chemists (AOAC, 1990) method was used to digest the samples. The digests were diluted with HCl and the mineral composition and concentrations of Na, Ca, K, Zn, Fe and Mg were determined, using a PerkinElmer Atomic Absorption Spectrophotometer.

### A.2.3.6 Determination of anti-nutrients

#### A.2.3.6.1 Trypsin inhibitor

The method described by Smith et al. (1980) was used to determine the antitrypsin activity. Trypsin activity was measured by using BAPNA as substrate in the presence and absence of a sample extract. *p*-Nitroanilide released was measured using a spectrophotometer at 410 nm. Trypsin inhibitor activity (TIA) was therefore expressed as the decrease in trypsin activity per unit weight of sample, using the formula:

$$\text{TIA} = \frac{2.632 \cdot D \cdot At}{S} \text{ mg pure trypsin inhibited } g^{-1} \text{ sample}$$

D is the dilution factor, *At* is the change in absorbance and S is the amount of sample weighed out.

#### A.2.3.6.2 Amylase inhibitor

α-Amylase and α-amylase inhibitory activities were estimated according to the method utilized by Bernfeld (1955). One α-amylase unit (1UI) was defined as the amount of enzyme that will liberate 1μmol of maltose from the starch under the assay conditions (10 minutes, 37° C, pH 6.9). The amylase inhibitors activity (AIA) was determined as the percentage decrease in α-amylase activity (at the stated conditions) in the presence of Amadumbe extracts.

### A.2.3.6.3 Lectin

Amadumbe samples were homogenized with a sodium borate buffer in a shaker overnight. The samples were then filtered and serial dilutions were made.

#### A.2.3.6.3.1 Collection of platelets

The rats were anaesthetized with ether and blood was immediately collected from abdominal aorta into centrifuged tube containing ADA (anticoagulant) [1 ml ADA: 5 ml blood]. The blood was then centrifuged (15 minutes at 1200 rpm, 3 minutes at 2200 rpm and 15 minutes 3200 rpm) and resuspended in 5 ml of washing buffer [1 ml sediment: 20 ml resuspending buffer].

#### A.2.3.6.3.2 Measurement of platelet aggregation

The method of Hwang *et al.* (1974) as modified by Mekhfi *et al.* (2004) was adopted for the measurement of platelet aggregation. A 1:20 dilution of platelets was prepared in RB. A sample of washed platelets (0.4 ml) was mixed with $CaCl_2$ to a final concentration of 1.3 mM. Aggregation was initiated by adding 1 U/ml of thrombin. The development of platelet aggregation was recorded at 546 nm over five minutes.

Experiments with Amadumbe extracts were monitered by pre-incubating the washed platelets with extracts for one minute. The platelet aggregation was immediately initiated by adding thrombin.

Percentage of stimulation of clotting was calculated by using the formula:

$$\% \text{ stimulation} = \frac{\Delta A \text{ control} + \Delta A \text{ test} \times 100}{\Delta A \text{ control}}$$

$\Delta A$ is the change in absorbance at 546nm

### A.2.3.6.4 Total polyphenols

The Prussian Blue Method, utilized by Price and Butler (1977), was used to determine total phenol content. The phenols were extracted into 2 M HCl. Timed additions were performed on the extracted sample, using 0.10 M $FeNH_4(SO_4)_2$ and 0.008 M $K_3Fe(CN)_6$ to develop colour. Absorbance was measured spectrophotometrically at 720 nm. The total phenol concentration was calculated and expressed as a gallic-acid equivalent.

### A.2.3.6.5 Tannin

Tannins were determined by the method utilized by Van-Burden and Robinson (1981). The Amadumbe samples were weighed and extracted into distilled water. 0.1 M $FeCl_3$ in 0.1 N HCl and 0.008 M potassium ferrocyanide were added to the filtrate. The absorbance was measured at 120 nm within 10 minutes. Gallic acid was used as a standard to draw the standard curve, from which the tannin content was estimated.

### A.2.3.6.6 Flavonoid

Flavonoids were determined using the method of Boham and Kocipai-Abyazan (1994). Samples were repeatedly extracted into 80% aqueous methanol. The filtrate was evaporated into dryness over a water bath and weighed. The percentage yield of the flavonoid was calculated.

### A.2.3.6.7 Cyanogens

Cyanogens were assayed enzymatically, using the method described by O'Brien et al. (1991). Amadumbe samples were homogenized in a 0.1 M orthophosphoric/ethanol extraction medium. An aliquot of extract was added to a phosphate buffer (0.1M $H_3PO_4$ and $Na_3PO_4$; pH7) and β-glycosidase (5EU $ml^{-1}$). The liberated HCN was reacted with pyridine/pyralozone reagent to develop colour and the absorbance was measured spectrophotometrically at 620 nm. KCN was used as a standard to draw the standard curve, from which the cyanogen content was estimated.

### A.2.3.6.8 Phytate

Double acid extraction (HCl and $H_2SO_4$) was performed on sample materials for three hours. Samples were filtered under vacuum through Whatman no 1 filter paper. Colour was developed by adding ammonium molybdate, ascorbic acid and potassium antimonytartrase and absorbance was measured at 820 nm. A standard curve was prepared, expressing the results as potassium-hydrogen-phosphate equivalent. The concentration of phytate was calculated from its phosphorus content.

### A.2.3.6.9 Alkaloid

Amadumbe alkaloids were detected by the method utilized by Harborne (1973). Amadumbe samples were soaked in a 10 per cent solution of acetic acid in ethanol for four hours. This was filtered and the extract was concentrated on a water bath. Precipitation was executed by adding concentrated ammonium hydroxide. The precipitate thus obtained was washed with diluted ammonium hydroxide, dried and weighed. The percentage yield of the alkaloid was calculated.

### A.2.3.6.10 Oxalate

Oxalate was determined employing the method used by Munro and Bassir (1969). The oxalate was extracted with 0.15 per cent citric acid and treated with tungstophosphoric acid. Precipitated oxalate was solubilized with hot diluted $H_2SO_4$ and titrated against $KMnO_4$. Oxalate content was expressed as calcium oxalate equivalent.

### A.2.3.6.11 Saponin

Saponin content was determined utilizing the method employed by Fenwick and Oakenfull (1981). Saponin was extracted for 24 hours in a reflux condenser containing pure acetone. Re-extraction with methanol in the Soxhlet apparatus was carried out for another 24 hours. Colour development was achieved by using vanillin in ethanol and

sulphuric acid. Absorbance was measured spectrophotometrically at 500 nm. Saponin was used as a standard to draw the standard curve, from which the saponin content of sample was estimated.

*All analyses were done in duplicate and the results reported are the mean values.*

# CHAPTER A-3

# RESULTS

## A.3.1 Introduction

Nutritional value is the main concern when a crop is considered as a food source. Amadumbe is cultivated as a subsistence staple in parts of South Africa. Information on nutritional and anti-nutritional values of processed and unprocessed Amadumbe tubers grown in KZN, South Africa is given in this chapter.

## A.3.2. Proximate composition

The proximate composition of the two varieties of *Colocasia esculenta* tuber, from different locations, was determined by standard procedures. The data for the processed and the unprocessed samples are presented in Tables A3-1a – A3-1c. In general, the proximate composition of the two varieties of Amadumbe studied is similar to that of all known tubers. However, differences between the two varieties, as well as between the different locations, were observed in the proximate composition values obtained.

The moisture and ash contents of Amadumbe are presented in Table A3-1a. Water content was high in the investigated starchy staples, which, on average, ranged between 84 and 89 per cent. The unprocessed Esikhawini varieties showed the highest moisture content.

**Table A3-1a: The moisture and ash content (g/100g DM) of processed and unprocessed Amadumbe (*Colocasia esculenta*) tubers**

| Location | Moisture | Ash |
|---|---|---|
| | | |
| **Esikhawini white (EW)** | 89 | 4.4 |
| Boiled white (BW) | 88 | 4.4 |
| Roasted white (RW) | 84 | 3.6 |
| Fried white (FW) | 85 | 4 |
| | | |
| **Esikhawini purple (EP)** | 89 | 3.3 |
| Boiled purple (BP) | 88 | 3.2 |
| Roasted purple (RP) | 87 | 4 |
| Fried purple (FP) | 85 | 3.2 |
| | | |
| **Mtubatuba white (MtW)** | 87 | 5.4 |
| **Mtubatuba purple (MtP)** | 86 | 4.4 |
| | | |
| **Makatini white (MakW)** | 87 | 5.4 |
| **Makatini purple (MakP)** | 86 | 4.9 |

The ash content of Amadumbe tubers ranged between 3.2 and 5.4 per cent of the dry-weight material. The mean ash content for the unprocessed tubers was 4.6 per cent and that of the processed tubers, 3.7 per cent. The ash content of Amadumbe is significant in that it contains nutritionally important minerals.

The crude fat and protein content of the Zululand *Colocasia esculenta* species are shown in Table A3-1b.

**Table A3-1b: The crude fat and crude protein composition (g/ 100g DM) in unprocessed and processed Amadumbe**

| Location | Crude fat | Crude protein |
|---|---|---|
| | | |
| **Esikhawini white (EW)** | 0.8 | 5.04 |
| Boiled white (BW) | 0.78 | 5.04 |
| Roasted white (RW) | 1.58 | 6.87 |
| Fried white (FW) | 10.58 | 4.2 |
| | | |
| **Esikhawini purple (EP)** | 0.28 | 4.5 |
| Boiled purple (BP) | 0 | 4.56 |
| Roasted purple (RP) | 2.52 | 4.36 |
| Fried purple (FP) | 15.05 | 3.95 |
| | | |
| **Mtubatuba white (MtW)** | 1.54 | 5.39 |
| **Mtubatuba purple (MtP)** | 1.06 | 3.72 |
| | | |
| **Makatini white (MakW)** | 0.73 | 5.08 |
| **Makatini purple (MakP)** | 0.99 | 3.89 |

The lipid content for the unprocessed corms ranged between 0.73 and 1.54 per cent. The crude fat content for fried EW and EP Amadumbe tubers (10.58 and 15.05 per cent respectively) was higher than the mean crude fat value for the boiled and roasted samples, as well as higher than that of the unprocessed samples.

The crude protein content of the unprocessed *Colocasia esculenta* tubers was found to range between 3.72 and 5.39 per cent. MtP tubers presented the lowest crude protein content, whilst MtW showed the highest values. All the unprocessed tubers had a protein content of less than six per cent. No apparent increases or decreases were observed in the crude protein content for processed tubers.

**Table A3-1c Carbohydrate content (g% DM) of unprocessed and processed *Colocasia esculenta* tubers**

| Location | Carbohydrates | |
|---|---|---|
| | **Starch** | **Soluble sugar** |
| **Esikhawini white (EW)** | 28 | 4.0 |
| Boiled white (BW) | 25 | 3.0 |
| Roasted white (RW) | 27 | 2.0 |
| Fried white (FW) | 18 | 2.7 |
| | | |
| **Esikhawini purple (EP)** | 25 | 2.0 |
| Boiled purple (BP) | 22 | 3.1 |
| Roasted purple (RP) | 20 | 2.5 |
| Fried purple (FP) | 15 | 2.0 |
| | | |
| **Mtubatuba white (MtW)** | 16 | 1.1 |
| **Mtubatuba purple (MtP)** | 19 | 1.7 |
| | | |
| **Makatini white (MakW)** | 24 | 2.3 |
| **Makatini purple (MakP)** | 23 | 2.1 |

The unprocessed *Colocasia esculenta* tubers revealed high levels of starch (28-16 per cent), the predominant component of dry matter (Table A3-1c). The soluble sugar content of Amadumbe tubers ranged from 1.1 (MtW) to 4.0 per cent (EW) on a dry-weight basis. Processing decreased most of the total carbohydrate content of the Amadumbe tubers, possibly because of the leaching away of the component.

## A.3.3 Mineral analysis

The composition of mineral nutrients in the processed and unprocessed Amadumbe species studied are given in Tables A3-2a and A3-2b.

**Table A3-2a: Macronutrient profile of unprocessed and processed *Colocasia esculenta* (mg/100g DM)**

| Location | Na | Ca | K | Mg | P | S |
|---|---|---|---|---|---|---|
| **Esikhawini white** | 11.1 | 24.7 ± 0.26 | 408 | 79.6 ± 0.77 | • | • |
| Boiled white | 13.9 | 35.3 ± 0.27 | 312 | 102.6 ± 1.17 | • | • |
| Roasted white | 25.8 | 41 ± 0.80 | 362 | 101.5 ± 0.69 | • | • |
| Fried white | 23 | 32.5 ± 0.59 | 350 | 103.1 ± 1.37 | • | • |
| | | | | | | |
| **Esikhawini purple** | 26.4 | 40.3 ± 0.53 | 360 | 88.9 ± 1.10 | • | • |
| Boiled purple | 42 | 39.4 ± 0.62 | 250 | 99.6 ± 2.71 | • | • |
| Roasted purple | 17.2 | 45 ± 1.13 | 298 | 103.9 ± 1.22 | • | • |
| Fried purple | 27.02 | 33.5 ± 0.41 | 268 | 74.1 ± 0.72 | • | • |
| | | | | | | |
| **Mtubatuba white** | 13.4 | 41.3 ± 0.06 | 464 | 108.6 ± 0.91 | • | • |
| **Mtubatuba purple** | 20 | 47.8 ± 1.13 | 458 | 122.1 ± 0.52 | • | • |
| | | | | | | |
| **Makatini white** | 19.6 | 37.5 ± 0.58 | 740 | 93.45 ± 0.77 | • | • |
| **Makatini purple** | 14.3 | 41.4 ± 0.60 | 642 | 121.5 ± 1.18 | • | • |

• *Macro-element (not quantified) present in Amadumbe tubers*

In general, a variation in mineral distribution was noted between varieties. The presence of 15 minerals was investigated in the processed and unprocessed tubers; six were quantified (Tables A3-2a and A3-2b). The composition and concentration levels of these nutrients

varied significantly among cultivars. Potassium was the most abundant macromineral (740 mg 100 $g^{-1}$ DM) in the unprocessed tubers. Magnesium was the second most common mineral and varied between 79 and 122 mg 100 $g^{-1}$ DM Appreciable amounts of calcium (24.7- 47.8 $^{-1}$ DM) and sodium (11.1 - 42 mg $100^{-1}$ DM) were also noted.

**Table A3-2b: Micro-element contents of unprocessed and processed *Colocasia esculenta* (mg/100g DM)**

| Location | Zn | Fe | Cu | Ni | Al | Mn | Cr | Ba | As |
|---|---|---|---|---|---|---|---|---|---|
| **Esikhawini white** | 2.13 ± 0.03 | 2.33 ± 0.05 | • | • | • | • | • | • | • |
| Boiled white | 3.25 ± 0.05 | 2.62 ± 0.03 | • | • | • | • | • | • | • |
| Roasted white | 3.37 ± 0.10 | 4.34 ± 0.02 | • | • | • | • | • | • | • |
| Fried white | 3.09 ± 0.08 | 11.48 ± 0.19 | • | • | • | • | • | • | • |
| | | | | | | | | | |
| **Esikhawini purple** | 3.83 ± 0.06 | 2.12 ± 0.03 | • | • | • | • | • | • | • |
| Boiled purple | 2.99 ± 0.09 | 2.58 ±0.06 | • | • | • | • | • | • | • |
| Roasted purple | 2.18 ± 0.12 | 6.65 ± 0.20 | • | • | • | • | • | • | • |
| Fried purple | 2.45 ± 0.05 | 12.87 ± 0.02 | • | • | • | • | • | • | • |
| | | | | | | | | | |
| **Mtubatuba white** | 1.44 ± 0.02 | 4.13 ± 0.06 | • | • | • | • | • | • | • |
| **Mtubatuba purple** | 5.27 ± 0.15 | 2.07 ± 0.02 | • | • | • | • | • | • | • |
| | | | | | | | | | |
| **Makatini white** | 2.49 ± 0.06 | 3.12 ± 0.02 | • | • | • | • | • | • | • |
| **Makatini purple** | 3.17 ± 0.07 | 2.29 ± 0.002 | • | • | • | • | • | • | • |

• *Micro-element (not quantified) present in Amadumbe tubers*

With regard to micronutrients (Table A3-2b), Zn was the most abundant, with 3.05 mg 100 $g^{-1}$ DM as a mean value for the unprocessed tubers. Iron content ranged between 2.07

and 4.13 mg 100 $g^{-1}$ DM in the unprocessed tubers. The concentration of iron and zinc was the lowest of quantified minerals observed in the varieties studied.

### A.3.4 Fat and fatty-acids composition

The percentage yield of crude fat extract from Amadumbe with methanol-chloroform is presented in Table A3-3a.

**Table A3-3a: Yields of crude fat of Amadumbe extracted with methanol-chloroform mixture (2:1, v/v)**

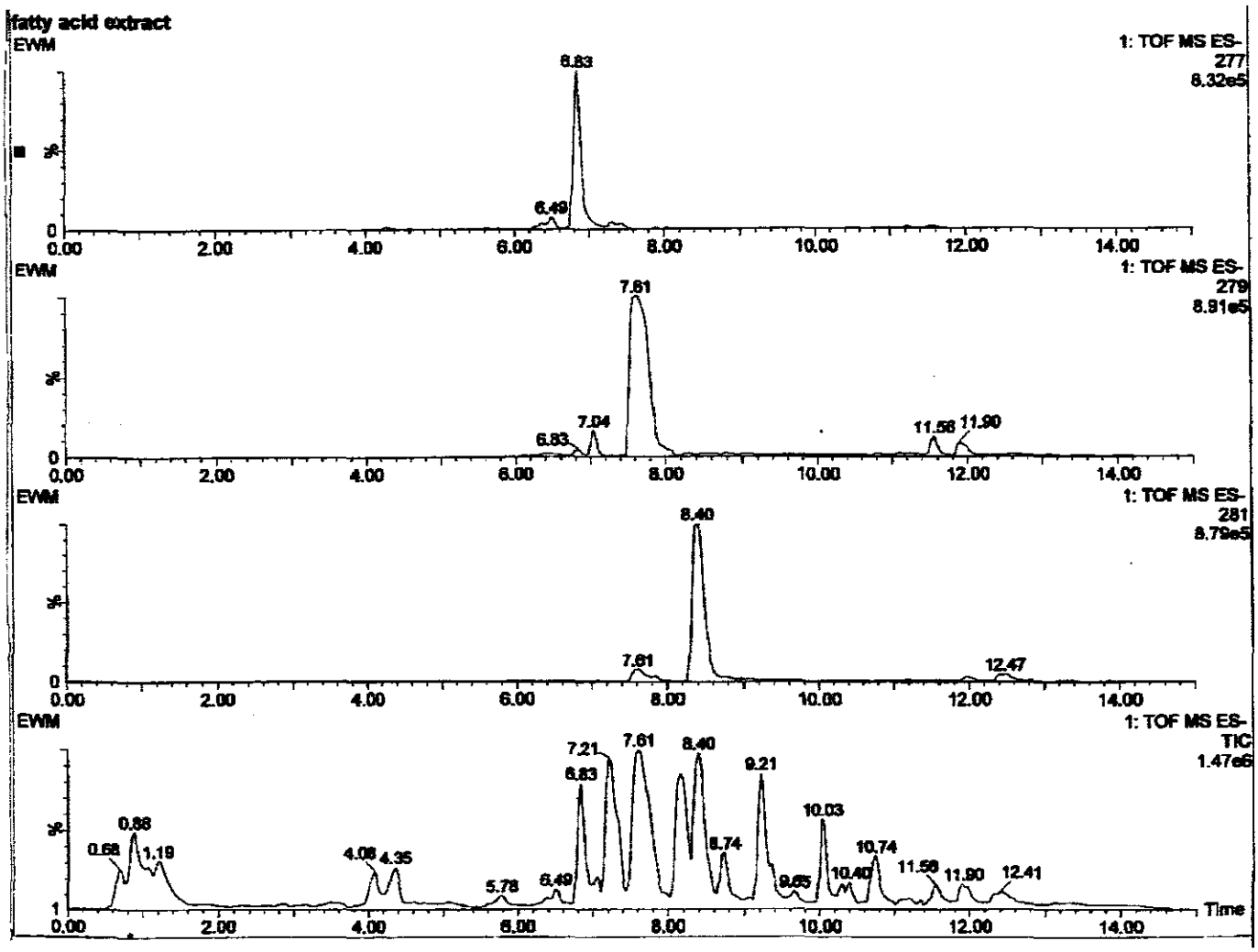
| Location | Crude fat (%) |
|---|---|
| Esikhawini white | 3.3 |
| Esikhawini purple | 3.6 |

The iodine numbers and the corresponding degree of unsaturation for the extracted lipids are presented in Table A3-3b.

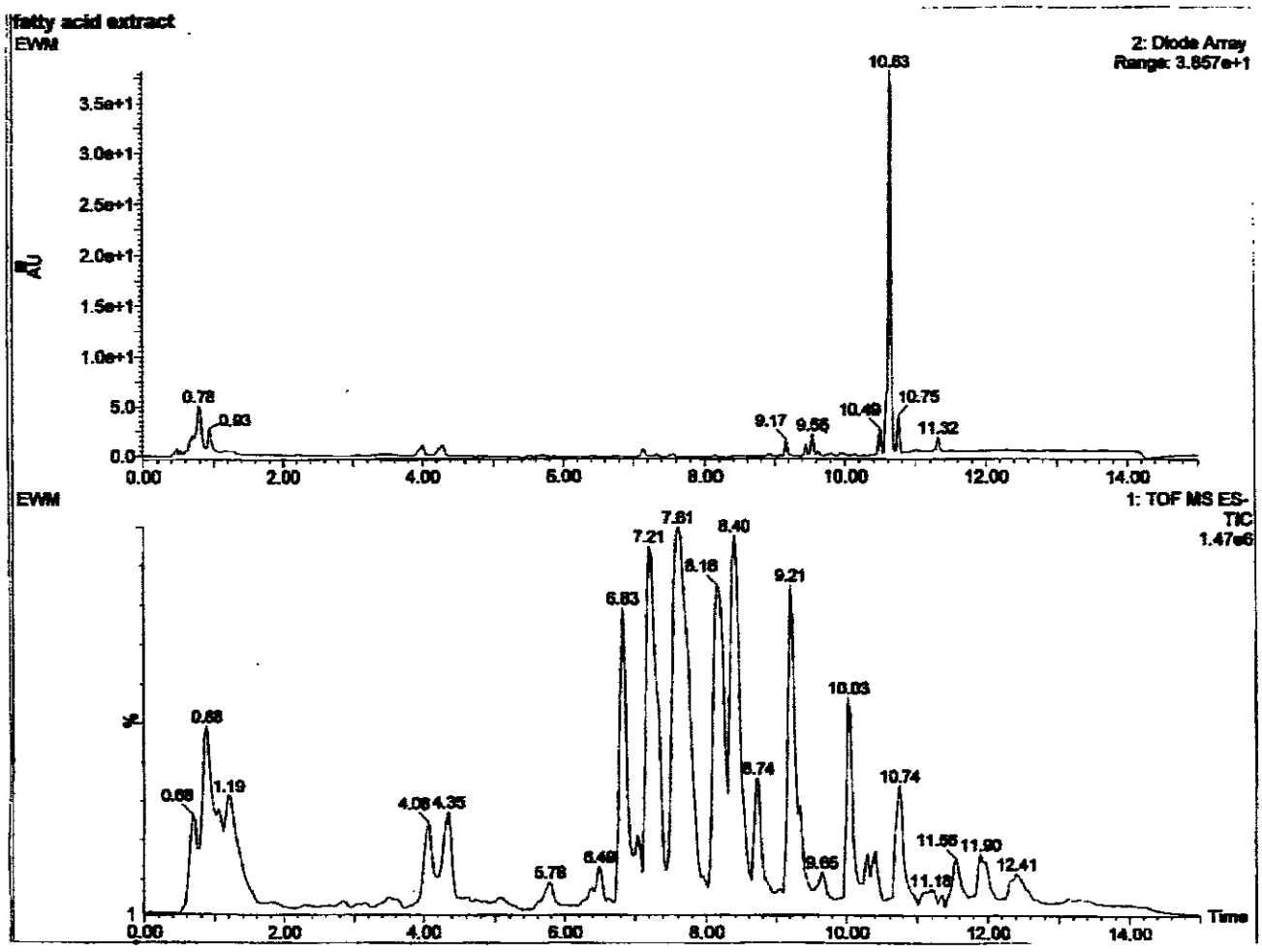
**Table A3-3b: Iodine value of Amadumbe lipid extract**

| Location | Iodine value (g/100g) | Degree of unsaturation (per mole) |
|---|---|---|
| Esikhawini white | 73 | 37 |
| Esikhawini purple | 29 | 15 |

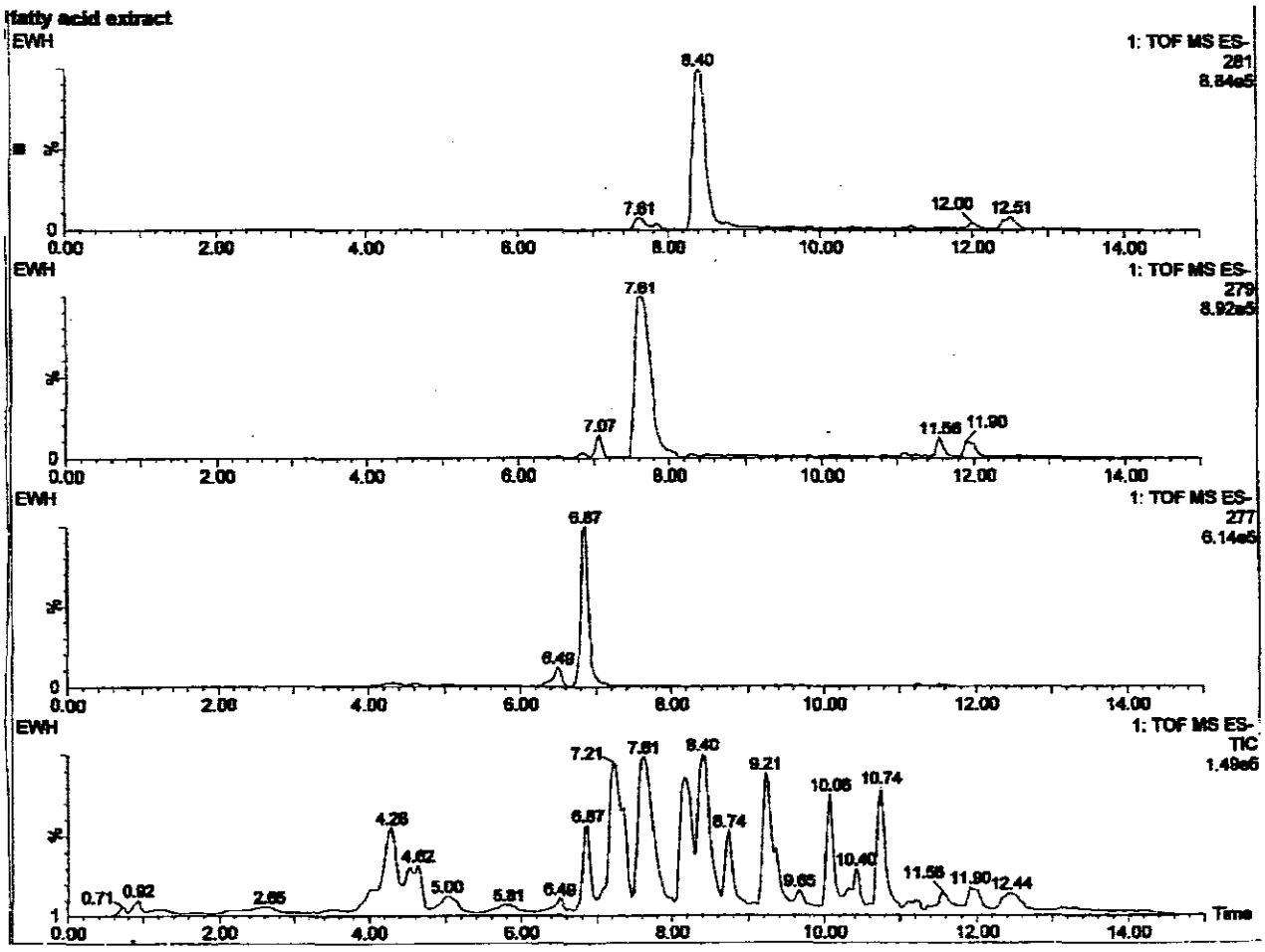
Figures A3-1 (a-d) shows the results of the investigation into the composition of fatty acids in Amadumbe, using HPLC-MS chromatographic techniques.



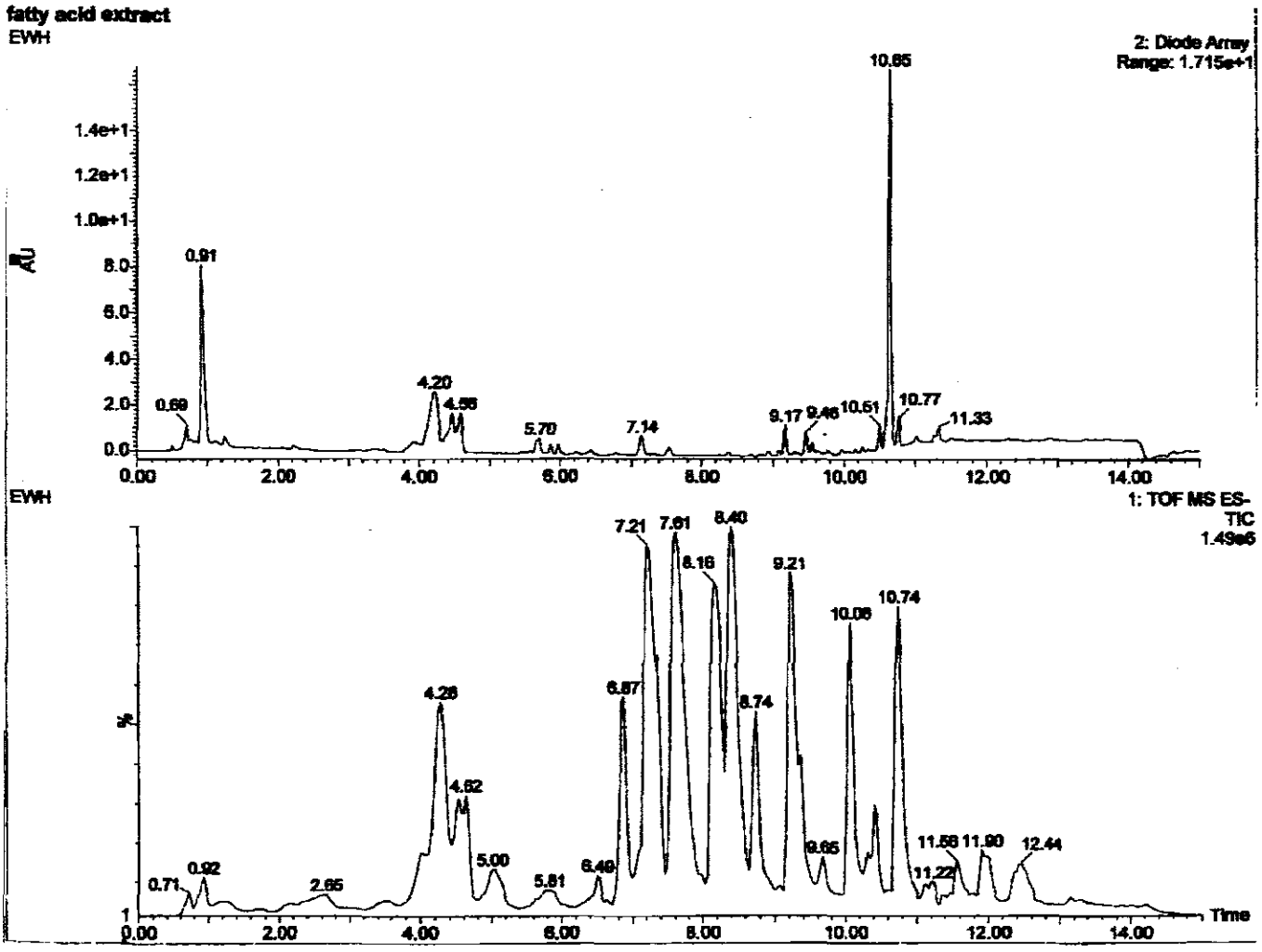

**Figures A3-1 a Esikhawini white Amadumbe extract (methanol-chloroform)**



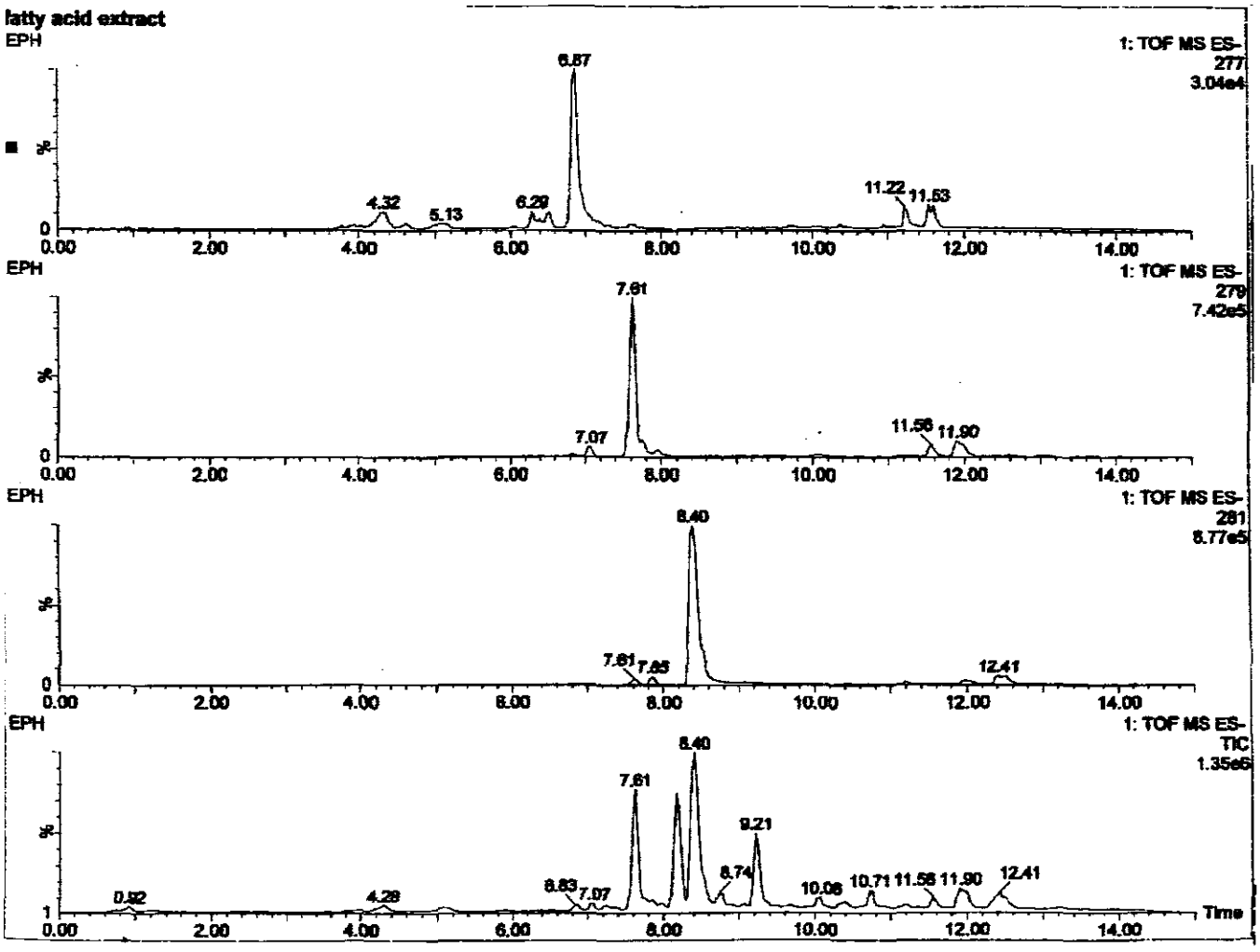

**Figures A3-1 a1 Esikhawini white Amadumbe extract extension (methanol-chloroform)**



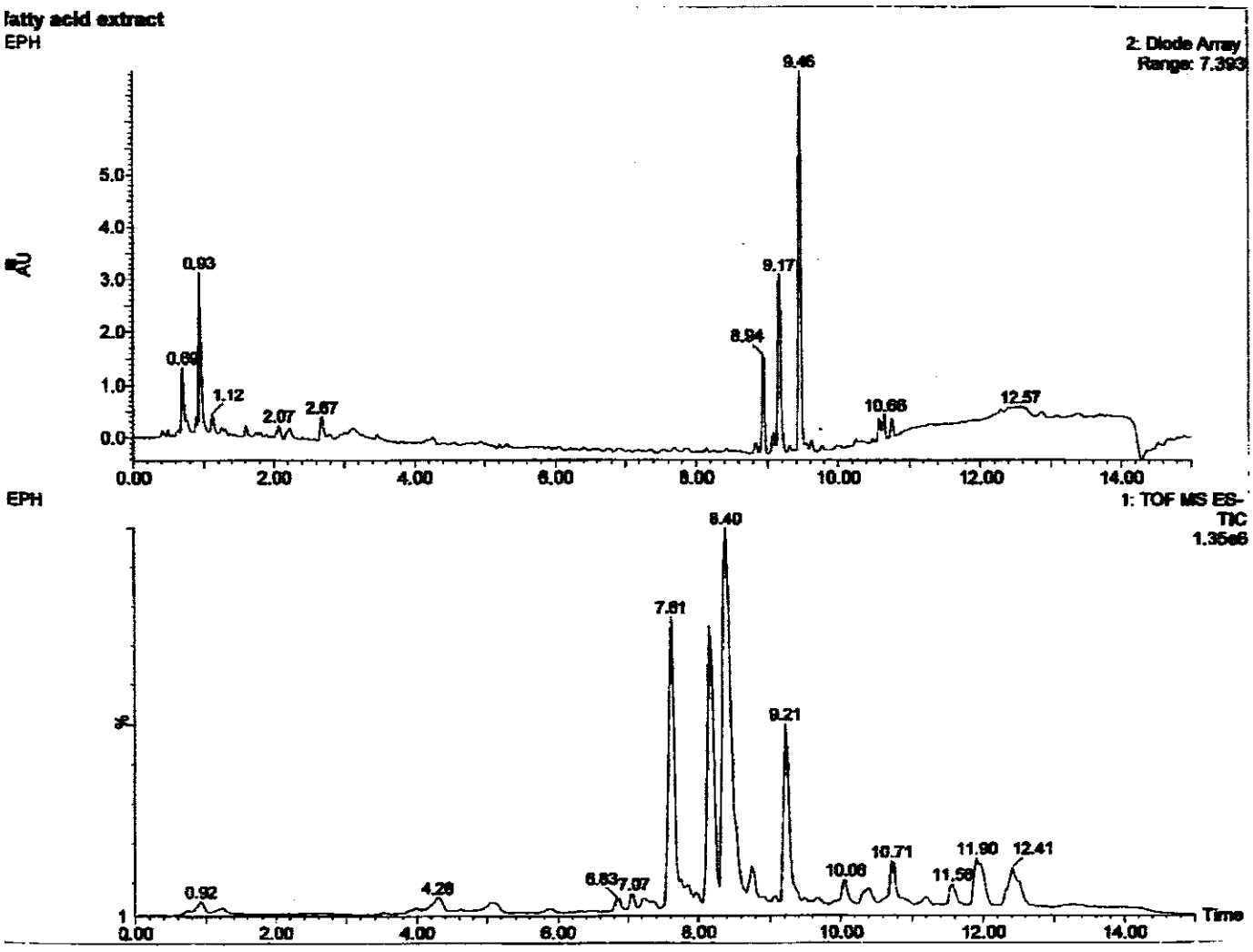

**Figures A3-1 b Esikhawini white Amadumbe extract (hexane)**



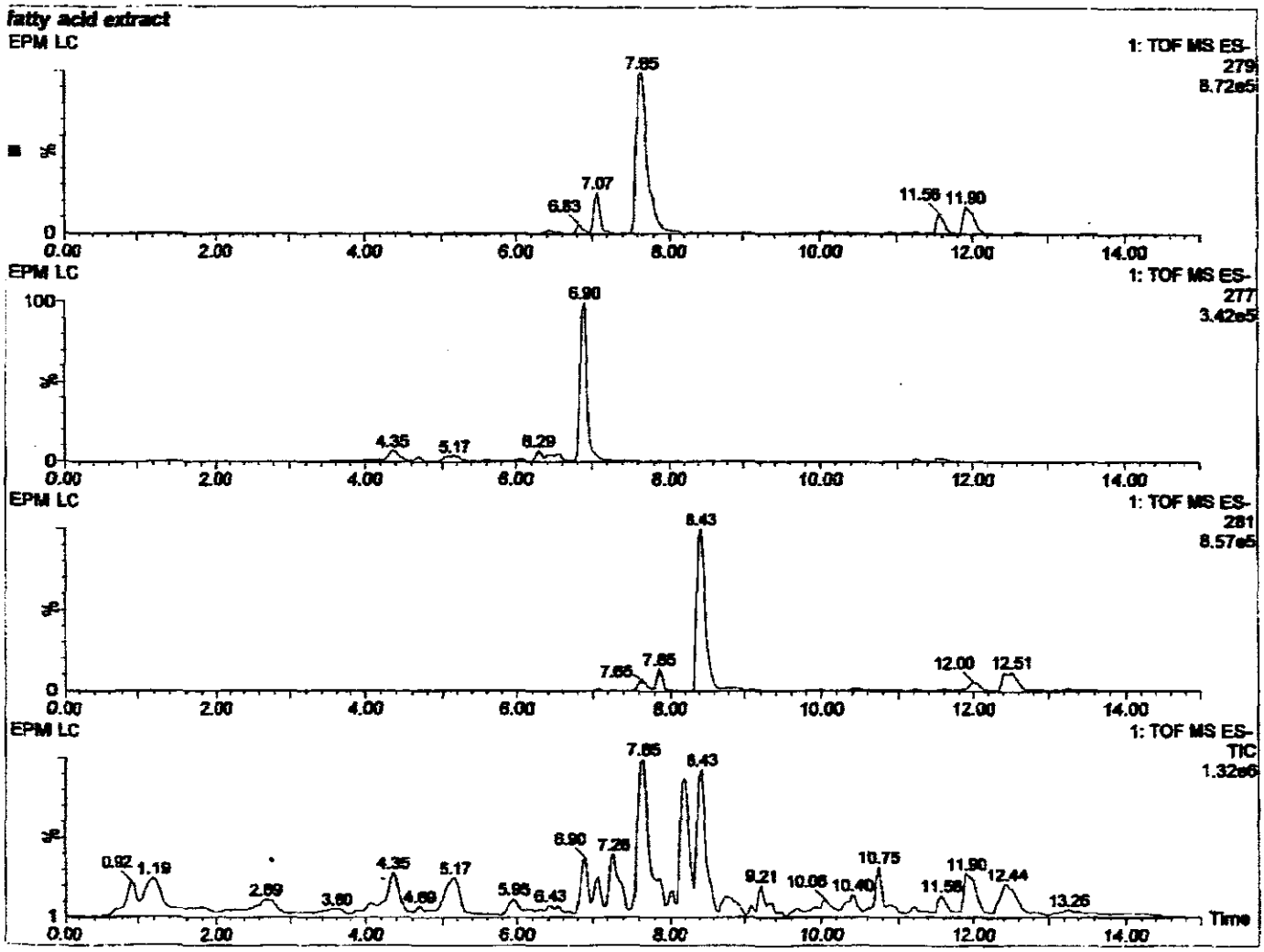

**Figures A3-1 b1 Esikhawini white Amadumbe extract extension (hexane)**



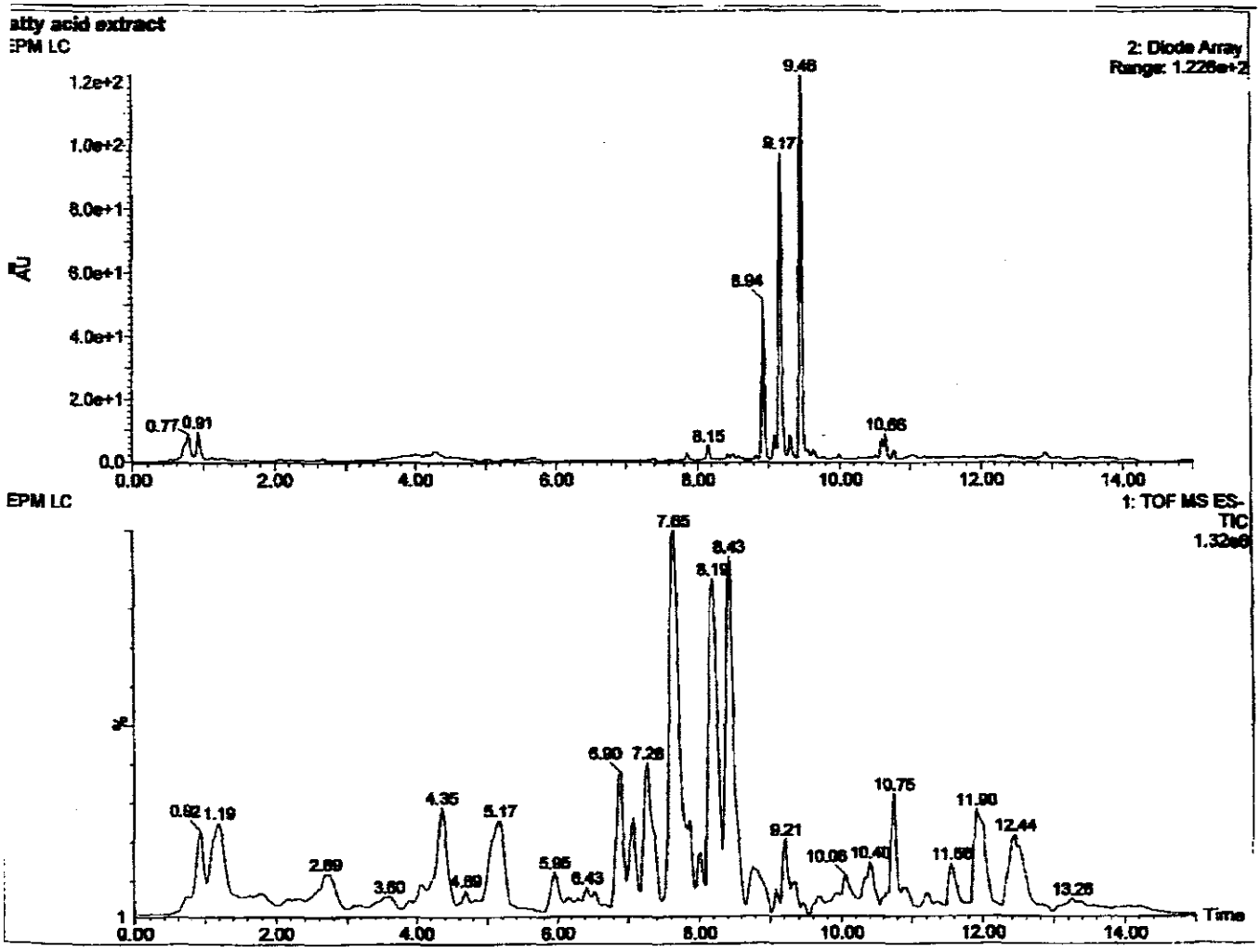

**Figures A3-1 c Esikhawini purple Amadumbe extract (methanol-chloroform)**




**Figures A3-1 c1 Esikhawini white Amadumbe extract extension (methanol-chloroform)**




**Figures A3-1 d Esikhawini purple Amadumbe extract (hexane)**




**Figures A3-1 d1 Esikhawini purple Amadumbe extract extension (hexane)**

**Figures A3-1 (a-d, and a1-d1) Chromatographic separation of Amadumbe (*Colocasia esculenta*) fatty acid**

Each peak on the separate chromatograms in Figure A3-1 (a-d) show the retention time with the relative molecular weights for the lipid extracts. Table A-3-3c shows the exact retention times for each sample extract.

**Table A3-3c: Fatty acids identified in Amadumbe *Colocasia esculenta***

| Fatty-acid extract | Retention times [$t_R$ (min)] | Molecular mass | $t_R$ of other peaks (min) |
|---|---|---|---|
| EWM | 6.83 | 277 | 10.63 |
| | 7.61 | 279 | 10.79 |
| | 8.40 | 281 | 10.49 |
| EPM | 7.65 | 279 | 9.46 |
| | 6.90 | 277 | 9.17 |
| | 8.43 | 281 | 8.94 |
| EWH | 8.40 | 281 | 10.65 |
| | 7.61 | 279 | 10.77 |
| | 6.87 | 277 | 10.51 |
| EPH | 6.87 | 277 | 9.46 |
| | 7.61 | 279 | 9.17 |
| | 8.40 | 281 | 8.94 |

### A.3.5 Anti-nutritional factors

The levels of anti-nutritional factors in the locally grown Amadumbe are important in the assessment of their nutritional status. Results obtained for the protease inhibitors on processed and unprocessed tubers are shown in Table A3-4a.

**Table A-3-4a: Trypsin inhibitor activity, amylase inhibitor activity and lectin activity of unprocessed and processed Amadumbe tubers**

| Location | Anti-nutrients (mg/g)[a] | | |
|---|---|---|---|
| | **Trypsin Inhibitor (TIA)** | **Amylase Inhibitor (AI)** | **Lectin Activity** |
| **Esikhawini white (EW)** | 19.7 | 21 | 246.47 |
| Boiled white (BW) | 0 (100) | 11 (48) | 316.8 (0) |
| Roasted white (RW) | 0 (100) | 5 (76) | 261.51 (0) |
| Fried white (FW) | 0 (100) | 6 (71) | 270.49 (0) |
| | | | |
| **Esikhawini purple (EP)** | 16.5 | 25 | 284.42 |
| Boiled purple (BP) | 0 (100) | 9 (64) | 284.56 (0) |
| Roasted purple (RP) | 0 (100) | 7 (72) | 329.48 (0) |
| Fried purple (FP) | 0 (100) | 6 (76) | 337.94 (0) |
| | | | |
| **Mtubatuba white (MtW)** | 20.6 | 26 | 570.72 |
| **Mtubatuba purple (MtP)** | 19.7 | 21 | 593.28 |
| | | | |
| **Makatini white (MakW)** | 17 | 31 | 651.09 |
| **Makatini purple (MakP)** | 19.1 | 33 | 650.19 |

[a] *Figures in parentheses indicate the percentage decrease over the values of the corresponding raw tuber*

The results show the presence of α-amylase inhibition in varying levels, ranging from 21 to 33 per cent. On processing, the α-amylase inhibitor activities were reduced by between 48 and 76 per cent.

Inhibition of trypsin obtained with the screening procedures ranged from 16.5 to 20.6 mg $g^{-1}$. The TIA content was found to be lowest in the raw tubers of the Esikhawini purple variety (16.5 mg pure trypsin inhibited/g DM) and highest (20.6/g DM) in Mtubatuba white. All three domestic processing methods completely inactivated the initial trypsin enzyme inhibitor level in the Amadumbe tubers.

The inhibitory effect of the crude extract on other proteases was also investigated and results are expressed in Table A3-5 Apart from the activity of papain, which was not inhibited, all other proteases were inhibited by crude Amadumbe extract. It is noted that the inhibitory activity on fungal proteases was minimal.

**Table A3-5: Inhibitory effect of crude extract on other proteases**

| Protease enzymes | Esikhawini | | Makatini | |
|---|---|---|---|---|
| | **white** | **purple** | **white** | **purple** |
| **Protease (*Bacillus Licheniformis)*** | 0.55 | 0.42 | 0.5 | Undetected |
| **Type xiii Fungal** | 1.5 | 0.15 | 0.25 | 0.15 |
| **Type xiv Bacterial** | 11.1 | 10.4 | 9.65 | 12.2 |
| **Type xviii Fungal: Rhizopus** | 0.45 | 0.5 | Undetected | 2.5 |
| **Papain** | Undetected | Undetected | Undetected | Undetected |
| **α-chymotrypsin** | 0.5 | 0.45 | Undetected | 0.55 |

Both varieties of Amadumbe contained lectins that stimulated platelet agglutinization by about 300-600%, as showed in Table A3-4a.

**Table A3-4b: The phenolic compounds in the processed and unprocessed Amadumbe tubers**

| Tubers | Antinutrients (mg/g)[a] | | |
|---|---|---|---|
| | **Total phenols** | **Tannins** | **Flavonoids** |
| **Esikhawini white (EW)** | 11.5 | $5.1 \times 10^{-5}$ | 0.03 |
| Boiled white (BW) | 7.1 (38) | $8.1 \times 10^{-6}$ (84) | 0.009 (70) |
| Roasted white (RW) | 10.0 (13) | $1.7 \times 10^{-5}$ (66) | 0.01 (67) |
| Fried white (FW) | 10.8 (6) | $1.9 \times 10^{-5}$ (63) | 0.014 (53) |
| | | | |
| **Esikhawini purple (EP)** | 13.0 | $1 \times 10^{-5}$ | 0.031 |
| Boiled purple (BP) | 9.8 (24) | $7.8 \times 10^{-6}$ (22) | 0.01 (68) |
| Roasted purple (RP) | 12.2 (6) | $7.3 \times 10^{-6}$ (27) | 0.013 (58) |
| Fried purple (FP) | 14.0 (0) | $5.5 \times 10^{-6}$ (45) | 0.01 (68) |
| | | | |
| **Mtubatuba white (MtW)** | 13.3 | $1 \times 10^{-5}$ | 0.034 |
| **Mtubatuba purple (MtP)** | 11.9 | $4.2 \times 10^{-5}$ | 0.039 |
| | | | |
| **Makatini white (MakW)** | 8.0 | $1.6 \times 10^{-5}$ | 0.049 |
| **Makatini purple (MakP)** | 14.0 | $6.5 \times 10^{-5}$ | 0.043 |

[a] *Figures in parentheses indicate the percentage decrease over the values of the corresponding raw tuber*

The total phenol, tannin and flavonoid contents obtained in processed and unprocessed Amadumbe tubers are given in Table A3-4b. Unprocessed tubers contained fairly low levels of free phenolics and low levels of tannin. The reduction for total phenols was highest during boiling, 38 per cent and 24 per cent for the white and purple Amadumbe respectively.

Phytochemical screening was carried out to determine the presence of flavonoids in the Amadumbe tubers. Yellow colouration was observed, indicating the presence of flavonoids (Figure A3-2) [Boham and Kocipai-Abyazan (1974)]. Flavonoid content decreased during processing, as can be seen in the difference in colouration observed between the screened processed and unprocessed samples (Figure A3-2).

**EW EP MtW MtP MakW MakP BW BP RW RP FW RP**

**Figure A3-2: Phytochemical screening for flavonoids in the unprocessed (left) and processed (right) tubers**

**Key**

| | | |
|---|---|---|
| EW – Esikhawini white | EP – Esikhawini Purple | MtW – Mtubatuba White |
| MtP – Mtubatuba Purple | MakW – Makatini White | MakP – Makatini Purple |
| BW – Boiled White | BP – Boiled Purple | RW – Roasted White |
| RP – Roasted Purple | FW – Fried White | FP – Fried Purple |

The total flavonoid content of the unprocessed and processed tubers was then quantified. Unprocessed flavonoid content ranged from 0.03 to 0.049 mg/g of dry matter and that of processed tubers, from 0.01 to 0.009 mg/g (Table A3-4b).

The results of the analysis of alkaloids, oxalates, phytates, cyanogens and saponins of Amadumbe obtained from different locations are shown in Table A3-4c.

**Table A3-4c: The levels of some anti-nutritional factors in processed and unprocessed tubers (*Colocasia esculenta*) from Zululand**

| Location | Anti-nutrients (mg/g)[a] | | | | |
|---|---|---|---|---|---|
| | **Alkaloids** | **Oxalates** | **Phytates** | **Cyanogens** | **Saponin** |
| **Esikhawini white (EW)** | 0.19 | 0.13 | 3.1 | 0.012 | 0.136 |
| Boiled white (BW) | 0.04 (79) | 0.06 (54) | 1.3 (58) | 0.001 (92) | 0.056 (59) |
| Roasted white (RW) | 0.04 (79) | 0.14 (0) | 1.1 (65) | 0.003 (75) | 0.079 (42) |
| Fried white (FW) | 0.13 (32) | 0.14 (0) | 0.8 (74) | 0.002 (83) | 0.123 (10) |
| | | | | | |
| **Esikhawini purple (EP)** | 0.18 | 0.10 | 2.8 | 0.025 | 0.145 |
| Boiled purple (BP) | 0.06 (67) | 0.06 (40) | 1.4 (50) | 0.008 (68) | 0.052 (64) |
| Roasted purple (RP) | 0.08 (56) | 0.21 (0) | 1.8 (36) | 0.003 (88) | 0.10 (31) |
| Fried purple (FP) | 0.04 (78) | 0.1 (0) | 0.8 (71) | 0.006 (76) | 0.095 (34) |
| | | | | | |
| **Mtubatuba white (MtW)** | 0.22 | 0.07 | 2.2 | 0.013 | 0.125 |
| **Mtubatuba purple (MtP)** | 0.17 | 0.09 | 3.2 | 0.018 | 0.119 |
| | | | | | |
| **Makatini white (MakW)** | 0.14 | 0.08 | 2.6 | 0.014 | 0.122 |
| **Makatini purple (MakP)** | 0.22 | 0.07 | 3.2 | 0.013 | 0.124 |

[a] *Figures in parentheses indicate the percentage decrease over the values of the corresponding raw tuber*

There was variation in the total oxalate content of the corms in this study between tubers from the different localities. The highest losses of oxalate (a decrease from 0.13 mg/g to 0.06 mg/g – 54 per cent) occurred when the white Amadumbe sample was boiled. When the samples were roasted and fried, there was an increase in oxalate content.

The phytate content of the Amadumbe tubers investigated is given in Table A3-4c. Phytate concentration ranged between 2.2 – 3.2 mg/g. Results indicated that phytate content was partially reduced by the cooking treatments. The phytate content of the processed tubers was reduced by up to 74 per cent.

The cyanogen content of Amadumbe tubers ranged from 1.2 – 2.5 mg HCN equivalent per 100 g dry weight of tubers. The results, presented in Table A3-4c, showed low variability of HCN content. The processing techniques employed were effective in substantially reducing the cyanogens to low levels (0.1 – 0.8 mg HCN equivalent/100g). Boiling for 30 minutes was found to be highly effective in reducing the HCN by up to 92 per cent in white and 68 per cent in purple Amadumbe. A loss of HCN content (75 per cent in white and 88 per cent in purple Amadumbe) was also observed when the tubers were roasted for 30 minutes or fried for 15 minutes.

Phytochemical screening revealed the presence of alkaloids at different concentration levels in all the unprocessed samples. A precipitate or flocculation in any of the samples was regarded as a positive test result for alkaloids (Figure A3-3).


**Figure A3-3: Phytochemical screening for alkaloids in the unprocessed tubers**

**Key**

| | | |
|---|---|---|
| MtW – *Mtubatuba White* | MtP – *Mtubatuba Purple* | EW – *Esikhawini White* |
| EP – Esikhawini Purple | MakW – Makatini White | MakP – Makatini Purple |

The alkaloid concentrations in the processed and unprocessed *Colocasia esculenta* tubers are shown in Table A3-4c. Total alkaloid concentration for Amadumbe corms ranged between 0.14 and 0.22 mg/g. Decrease in the alkaloid content during processing was reflected as ranging up to 79 per cent.

The saponin content, ranging from 0.119 to 0.145 mg/g in Amadumbe tubers, is given in Table A3-4c. Saponin reduction during processing was found to range between 10 and 64 per cent.

# CHAPTER A-4

# DISCUSSION

## A.4.1 Introduction

The nutritional and anti-nutritional values of processed and unprocessed Amadumbe tubers grown in KZN, South Africa, are discussed in this chapter.

## A.4.2.1 Proximate composition

Like all tubers, Amadumbe tubers have high moisture content. This is reflected in results reported for taro in the Pacifics, where moisture content ranges between 63 and 85 per cent (Bradbury and Holloway, 1988). These figures are higher than results reported by Huang et al. (2000) for taro corms in Hawaii and by Sefa-Dedeh and Agyir-Sackey (2004) for cocoyams in the eastern region of Ghana. The texture of food fluctuates with a change in moisture level; decreased water movement results in more structured endosperm (Hook *et al.* 1982).

The ash content of the tested Amadumbe tubers was between 3.2 and 5.4 per cent of the dry weight and reflected the mineral content. The ash contents in this study were higher than values reported for other cultivated taro species (Huang *et al.*, 2007). Differences in the ash contents of Amadumbe corms might be related to their species origin, fertility, geographical sources or planting periods (Bradbury and Holloway, 1988). Yam species are reported to contain relatively high level of minerals generally (Afoakwa and Sefa-Dedeh, 2001; Liu *et al.*, 1995).

Carbohydrates supply the most energy in a diet and for this reason make up a greater percentage than fats or proteins. The presence of starch contributes to the textural properties of many foods. Starch has many industrial functions, including gelling agent, colloidal stabilizer and thickener (Lii *et al*, 1996). Potato, rice, wheat and corn are the most important sources of starch, but differ noteably in structure and morphology (Singh *et al.*, 2007).

The starch and soluble sugar levels of the different Amadumbe corms indicated that Amadumbe tubers store a high level of starch, ranging between 15 and 28 per cent. For this reason, they can be considered carbohydrate foods (Swinkels, 1985). These starch levels are similar to those of between 19.2 and 26.1 per cent, recorded for taro by Huang *et al*,. (2000; 2007). Differences in starch content were observed among investigated Amadumbe corms from different locations. This finding corroborates the observations of Jane *et al.* (1992) that the carbohydrate content of taro cultivated in different locations showed variation.

Lee (1999) estimated the digestibility of taro starch to be 98.8 per cent and the size of the taro starch grain as one tenth the size of that of the potato. The good levels of starch in taro and cocoyams mean that these tubers can be used in the preparation of foods which prevent allergic diseases, in the manufacture of infant meals and for patients with chronic liver problems, peptic ulcers and inflammatory bowel disease (Sefa-Dedeh and Agyir-Sackey, 2004; Emmanuel-Ikpeme *et al.*, 2007).

The soluble sugar levels of Amadumbe tubers (1.1 – 4.0 per cent) were generally higher than those reported for taro corms (Huang *et al.*, 2007) and other roots, including yam, cassava, and potato (Bradbury and Holloway, 1988; Wanasundera and Ravindran, 1994). However, levels were similar to those reported for sweet potato (Zhang *et al.*, 2004). The higher soluble sugar content of Amadumbe tubers highlights their superior taste as a staple food (Huang *et al.*, 2007). Levels of soluble carbohydrates may indicate a degree of dormancy; however, Amadumbe samples were freshly collected.

The crude fat analysis of Zululand *Colocasia esculenta* species is shown in Table A3.1b. Crude fat content ranged from 0.73 to 1.54 per cent in the unprocessed tubers. The total fat content for the Amadumbe tubers is within the range of values that have been reported for cocoyam (Sefa-Dedeh and Agyir-Sackey, 2004). The proximate composition for fat was also found to be similar to or higher than that of potato and sweet potato (Noman *et al.*, 2007). These values are relatively higher than other root and tuber crops (Agbor-Egbe and Rickard, 1990; Rickard and Coursey, 1981). As was expected, the fried tubers had a higher level of fat (10.58 per cent to 15.05 per cent) than the raw tubers or those cooked using another method in this investigation.

The main functions of proteins are growth and replacement of lost tissues in the animal body. The variations in the mean values of the protein content of the different varieties were distinct. Environmental factors such as duration of growing season, length of day, rainfall, light intensity and temperature, as well as agronomic factors such as plant density, wild plants or soil fertility, can influence protein content (Robertson *et al.*, 1962; Singh *et al.*, 1972; McLean *et al.*, 1974). All the tubers had a protein content of less than six per cent, as is generally the case with most starchy root crops. Similar low protein levels have been reported for other cocoyam and taro varieties (Onayemi and Nwigwe, 1987; Agbor-Egbe and Rickard, 1990; Sefa-Dedeh and Agyir-Sackey, 2004). Amadumbe is normally consumed with other vegetables, a practice that undoubtedly complements the poor protein content of the tuber.

For each Amdumbe variety, processing methods showed no apparent or consistent effect on the moisture, ash and protein contents. Fat content only increased drastically with frying. The differences in processing may therefore be due to various differences in other components (Afoakwa and Sefa-Dedeh, 2001). Variations in the nutritional composition of roots and tubers from different places have been attributed to variations in the genetic background, as well as to varying climatic, seasonal and the agronomic factors (Onwueme, 1982).

### A.4.2.2 Mineral analysis

Diet is responsible for several existing problems relating to human health. Deficiency diseases could be prevented by sufficient intake of specific micronutrients that are involved in many biochemical processes. Vegetables and fruits are particularly important sources of minerals (Milton, 2003; Smolin and Grosvenor, 2000). Diets high in fruits and vegetables are also linked to decreased risk of diseases such as diabetes and cancer and daily consumption of these foods is being encouraged (Bernstein *et al.*, 2002; Leterme, 2002).

The mineral profile of Amadumbe species is comparable to that of taro, cocoyam, potato and sweet potato (Sefa-Dedeh and Agyir-Sackey, 2004 and Huang *et al.*, 2000; Noman *et al.* 2007). It is apparent that the Zululand variety of *Colocasia esculenta* is rich in mineral nutrients, especially in potassium and magnesium. It is thought that potassium might play an important role in the control of hypertension and in lowering the risk of strokes (NRC 1989). Magnesium plays a marked role in maintaining good health: for example, lowering of blood pressure, acting as an antacid, (Yamori, *et al.*, 1994) and counteracting asthma (Britton *et al.*, 1994).

Very few aspects with regard to the environmental and physiological processes that are responsible for the uptake of minerals in plants have been identified. This notwithstanding, substantial variations in mineral concentration in Amadumbe were generally observed. The discrepancies have been reported to be due to the influence of species, the age of the plant and environmental differences such as concentration of minerals in the soil, pH, water supply and climate variations (Underwood and Schuttle, 1999; Hofman *et al.*, 2002; Alfaia *et al.*, 2003).

Cooked samples of Amadumbe tubers were higher in sodium, calcium, magnesium, zinc and iron, while the potassium content was relatively lower than in the raw samples. The cooking process, which allows extraction of nutrients from the tissues, may have increased the mineral nutrient levels while reducing moisture content (Booth *et al.* 1992).

The relative proportion of Na, Ca, K, Mg, Zn and Fe in the Amadumbe tubers is shown in Figure A4-1. It is apparent that a daily diet of Amadumbe would offer a good measure of these minerals.

The data on the proximate analysis and mineral content of the Amadumbe tuber varieties also confirmed their potential as food resource. With regard to the supply of nutrients, Amadumbe tubers may be considered a good source of carbohydrates, potassium and magnesium.

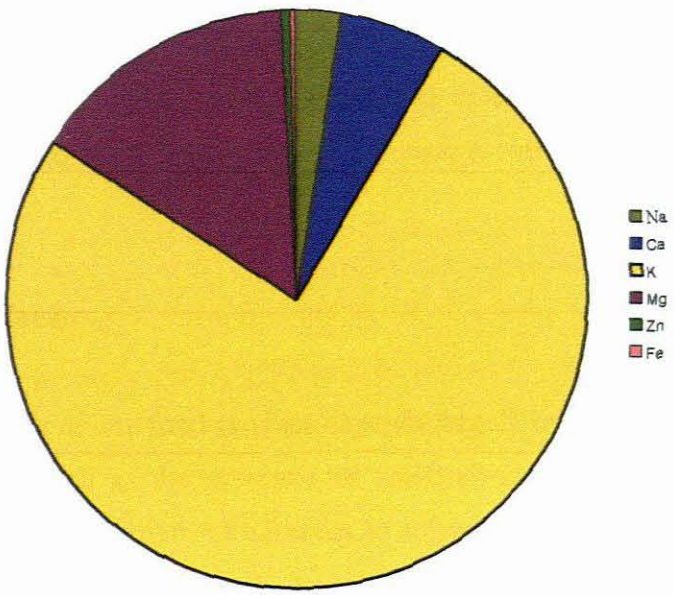



**Figure A4-1: The covering percentage for each mineral in 1 g of an Amadumbe tuber**

### A.4.3 Fatty acids composition

Different percentage yields were obtained for crude fat extraction during proximate composition and extraction undertaken to determine fatty acid composition. The difference in yield was due to the different solvents used. Petroleum ether was used for crude fat analysis (proximate composition) and methanol-chloroform was used to

determine fatty acid composition. Petroleum ether extracts only non-polar lipids, while methanol-chloroform extracts both non-polar and polar lipids.

Iodine values are used to measure the relative degree of unsaturation in fats. This value calculated from fatty acid composition may give a value closely related to the number of double bonds present in the fat.

The standard for oleic acid was used to determine fatty acid composition of Amadumbe by comparing the molecular weights given by each peak to relative retention times. The results showed that the investigated Amadumbe extract contained oleic acid, linolenic and linoleic acid, with linoleic acid being the most abundant. This is in agreement with studies undertaken by Hussain *et al.* (1984), who determined that palmitic, oleic and linoleic acids were the main fatty acids in *Colocasia esculenta* (L.) Schott, with linoleic acid predominating.

### A.4.4 Anti-nutritional factors

The nutritional value of any food product depends largely on the availability and quality of the nutrients as well as the presence of anti-nutritional factors. Anti-nutrients are known to reduce the availability of nutrients to animals and humans (Rackis *et al* 1986; Gupta, 1987). The higher the concentration of the metabolites, the greater the risk they pose to the health of the consumer. During domestic processing, anti-nutrients are partly removed and their toxicity is lowered. Nevertheless, consuming large quantities of food with anti-nutrients may still result in a health risk. The screening of the two varieties of Amadumbe tubers from the three geographical areas revealed no specific pattern in the anti-nutrient levels. It was therefore decided to process only the two varieties from the local Esikhawini areas because of ease of collection. This screening was conducted to determine the efficiency of the local processing methods (boiling, frying and roasting) employed in detoxifying the anti-nutrients present in the raw materials.

TIA indicated that the trypsin inhibitor in Amadumbe tubers could inhibit type I trypsin from bovine pancreas. The small variation in TIA may be due to different environmental factors. The handling conditions, degree of maturity and physiological conditions all play a major role in the production of anti-nutritional factors (Sotelo *et al.*, 1998). Apart from the activity of papain, which was not inhibited, all other proteases were inhibited (albeit to varying degrees) by the crude Amadumbe extract. Protease inhibitor reacts with some functional groups in the active site of the enzyme, forming an irreversible trypsin enzyme-trypsin inhibitor complex. This complex reduces trypsin in the intestines, which leads to less protein digestibility and ultimately causes slower animal growth (Siddhuraju and Becker, 2001). In this study, the initial enzyme inhibitor was completely inactivated during processing. It is well established that trypsin inhibitors are heat-labile (Bhandari and Kawabata, 2006; Khokhar and Chauhan, 1986). Reduction of TIA is expected to enhance the digestibility of proteins in processed Amadumbe tubers.

The AIA shows that α-amylase inhibitors were fairly heat-stable, compared to the trypsin inhibitors in these tubers. These observations were in close agreement with reports of Liener and Kakade (1980) who observed that, despite their susceptibility to heat inactivation, amylase inhibitors might persist through cooking temperatures. Similarly, Prathibha *et al.*, (1995) and Bhandari and Kawabata (2006) reported that α-amylase inhibitors from *Dioscorea* tubers did not show much reduction in activity, even at 100° C, and were heat stable.

Lectins were present in both Amadumbe varieties from all three localities. Lectins are heat-labile (Friedman and Brandon, 2001; Liener, 1989): however, the lectins present in Amadumbe were not destroyed during the different processing procedures. Armour *et al.*, (1998) reported that lectin activity was relatively heat-resistant for soya bean during aqueous heat treatment

Unprocessed Amadumbe tubers were shown to contain fairly low levels of phenolic compounds. The total free phenolic content in the present study was found to be higher than the values reported in sweet potato (Huang *et al.*, 2006) and taro in Cameroon

(Njintang *et al.*, 2006), whereas tannin content was similar to the values for potato and sweet potato (Noman *et al.*, 2007). The level of phenolic compounds in plant sources depends on factors such as cultivation techniques, cultivar, growing conditions and ripening process, among others (Naczk and Shahidi, 2006). A reduction in these compounds during processing may have been due to heat degradation of the tannin molecules or to the formation of water-soluble complexes (Uzogara *et al.*, 1990; Bakr and Gawish, 1991). The reduction during boiling was the highest (38 per cent and 24 per cent of the white and purple tubers respectively) and may be attributed to leaching out of the phenol into the cooking medium under the influence of the concentration gradient (Uzogara *et al.*, 1990; Vijayakumani *et al.*, 1997). The flavonoid content was also lower than that of potato (Chu *et al.*, 2000) and much lower than that of sweet potato (Huang *et al.*, 2006). Flavonoid content decreased considerably during processing. The tubers are partially hydrolyzed during boiling and these hydrolytic changes influence both their distribution between the lipidic and aqueous phases and their reaction with lipidic free radicals (Price *et al.*, 1998).

One very important factor reducing the consumption of cocoyam is the presence of oxalates which impart an acrid taste or cause irritation when foods containing them are eaten (Holloway *et al.*, 1989). Oxalates can have a harmful effect on human nutrition and health, especially by reducing calcium absorption and aiding the formation of kidney stones (Noonan and Savage, 1999). The levels of oxalates in the locally grown Amadumbe are therefore also important in the assessment of nutritional status. The tissue in this study was sampled after the removal of the Amadumbe skin as the skin is never cooked or eaten and is known to contain high levels of oxalates. Holloway *et al.*, (1989) reported that the presence of oxalate becomes less concentrated from the skin towards the centre of the taro corm. The total oxalate content of the corms in this study showed variation in plants from different localities. Oxalate values for the crude extract of the local *Colocasia* species were found to be much higher than the values ranging between 430 and 1560 μg/100 g obtained by using anion-exchange high performance liquid chromatography (Huang and Tanadjadja, 1992). The oxalate values obtained for the

samples in this study were also higher than the values of between 443 and 842 μg/100 g reported by Onayemi and Ngiwe (1987).

However, levels of oxalate obtained from the unprocessed Amadumbe samples investigated were lower than those recorded for three different local varieties of taro grown under irrigation at Kaohsiung, Taiwan. The latter oxalate levels ranged between 234 and 411 mg/100 g of dry matter (Huang *et al.*, 2007). Levels were also lower than the oxalate contents of some tropical foods investigated by Holloway et al. (1989), who reported on the oxalate content of the tubers of four different cultivars of taro grown in Fiji. In their study, the total oxalates ranged from 65 mg/100 g fresh weight (FW) for *Colocasia esculenta* to 319 mg/100 g for giant swamp taro. The oxalate content for the investigated samples was also lower than that reported for paddy- and upland-cultivated taro cultivars (Huang *et* al, 2007).

Cooking treatments were found to be effective in reducing the oxalate content of the tubers under investigation. The highest losses of oxalate (54 per cent) occurred when boiling the white Amadumbe sample. Boiling may cause considerable cell rupture and facilitate the leakage of soluble oxalate into cooking water (Albihn and Savage, 2001). When the samples were roasted and fried, there was an increase in oxalate content. This apparent increase could be related to the relative increase in dry matter as Amadumbe is roasted. Oxalate is broken down at 200° C, but this temperature is rarely reached when roasting or frying. Similar cooking studies on oca (*Oxalis tuberose*) showed that boiling considerably reduced the oxalate concentration in the whole tuber, while baking increased the concentration of soluble oxalates in the cooked tissue (Albihn and Savage, 2001). Sangketkit *et al.* (2001), Savage (2002) and Quinteros *et al.* (2003) all observed a similar trend regarding the decrease in soluble oxalate after boiling and the increase in oxalate with baking.

Hui (1992) reported that ingestion of 5 g or more of oxalic acid could be fatal to humans while Munro and Bassir (1969) determined the limit of oxalate toxicity in man to be 2–5 g/100 g of the sample. Care should be taken in high-quantity consumption of Amadumbe,

owing to the adverse effects of high oxalate intake. Therefore, the reduced oxalate content resulting from boiling Amadumbe tubers could have a positive impact on the health of consumers, particularly as the reduction of oxalate levels with boiling is expected to enhance the bioavailability of essential dietary minerals in these tubers. Hence, boiling the tuber would reduce the nutritional problems that the high levels of oxalates could cause.

Levels of phytate in Amadumbe were relatively higher compared to those found by Huang et al. (2007) in taro. However, these levels were lower than the values reported for cocoyam (Marfo *et al.*, 1990). Generally, the phytic-acid content in these samples is lower than those of other tropical root crops, including yam (Medoua *et al.*, 2007) and cassava (Marfo *et al.*, 1990). The phytate content of a plant could be determined by the availability of phosphorus in the environment (Raboy and Dickinson, 1993; Buerkert *et al.*, 1998). There are also other factors, such as environmental changes, genetics, locality, soil types, irrigation conditions, year and fertilizer treatment that can affect the phytate and phosphorus content (Dost and Tokul, 2006). The evident reduction in phytic acid during cooking may be caused by leaching into the cooking medium, degeneration by heat or the formation of insoluble complexes between phytate and other components, such as phytate-protein and phytate-protein-mineral complexes (Vijayakumari *et al.*, 1998; Siddhuraju and Becker, 2001).

Cooking has been reported to lower the phytate levels in yam (Bhandari and Kawabata, 2006) and several plant foodstuffs (Vijayakumari *et al.*, 1997; Saikia *et al.*, 1999; Badifu, 2001). The high content of phytate in the investigated *Colocasia esculenta* tubers is of nutritional importance because the phosphorus of the phytate molecule is unavailable to humans. Phytate also lowers the availability of many other necessary dietary minerals (Siddhuraju and Becker, 2001). Therefore, the reduction of phytate in the Amadumbe tubers is expected to enhance the bioavailability of proteins and dietary minerals. There is current support for the supposition that reduced levels of phytate may enhance health through antioxidant and anticarcinogenic activity (Harland and Morris, 1995; Sirkka, 1997).

The cyanogen levels for Amadumbe tubers from the three different localities are higher than HCN levels for potato and sweet potato (0.005 mg/100 g and 0.004 mg/100 g respectively), as well as for yam (0.06 mg/100 g) [Noman *et al.*, 2007; Bhandari and Kawabata, 2006]. Total cyanide levels in roots increase in a year of low rainfall owing to water stress on the plant (Bokanga *et al.*, 1994). The total cyanide content of plant parenchyma is dependent on the cultivar and location, as well as on a variety of other factors (Cardoso *et al.*, 2005). Processing Amadumbe was highly efficient in substantially reducing the cyanogens to low levels.

The reduction of hydrogen cyanide through boiling may be because free cyanide and bond cyanide are both water soluble and, hence, may be leached out during boiling. The heat treatment involved in roasting and frying may have caused the vaporization of the free cyanide (Udensi *et al.*, 2007). The results indicated that the cyanogen levels found in the processed and unprocessed tubers studied were satisfactorily below the safety level for cyanide poisoning. For humans, the lethal dose of HCN taken by mouth is estimated to be only 0.5 to 3.5 mg/kg body weight (Bradbury, 1991). However, smaller amount of cyanogens could have several long-term adverse effects on human health (Bhandari and Kawabata, 2004; Okolie and Osagie, 1999). It has been reported that higher intake of cyanogens could result in the development of neurological disease in humans (Montgomery, 1980). The results obtained showed that although the cyanogen content showed a relative decrease during processing, the amount remaining might be slightly toxic to people who consume high quantities of Amadumbe tubers.

The saponin content of the studied samples was found to be higher than reported saponin levels for *Jatropha curcas* L. (Martinez-Herrera *et al.*, 2006) and for citrus-fruit peel (Oluremi *et al.*, 2007). Gahlawat and Sehgal (1993) stated that loss of saponins during processing might signify their thermolabile nature, resulting in structural changes. Khokhar and Chauhan (1986) also mentioned that reduction of saponins may be due to the formation of a poorly extractable complex between them and sugar or amino acids.

Thus, it was observed that several anti-nutritional factors were present in Amadumbe. Their presence could impair the digestion of starch and protein, which could reduce the nutritional value of Amadumbe tubers and limit their utilization as food.

*Colocasia esculenta* is an underutilized crop in South Africa at present. Despite its importance nutritionally, as well as from the viewpoint of industry and health, Amadumbe has not drawn sufficient attention to encourage development of its potential. However, there has been collaboration among key role players in Assegai Organics, the Ezemvelo Farmers Organisation and the University of Kwazulu-Natal. This has resulted in Woolworths, a reputable supermarket chain, selling Amadumbe products commercially. The possibility of increasing the utilization of Amadumbe lies in developing suitable processing technology to decrease the anti-nutritional levels, securing consumer acceptance, marketable products and achieving economic feasibility. The health-promoting capacity of food is wholly dependent on processing history (Nicoli *et al.*, 1999). Processing methods used in this study decreased most of the anti-nutrients present in the tubers and would therefore increase digestibility of the starch and protein.

# CHAPTER A-5

# CONCLUSION

## A.5.1 Nutritional and anti-nutritional evaluation

The increased recognition of the health-protecting effects of non-nutrient, bio-active food components found in fruits and vegetables has directed immense attention toward vegetables as a vital part of daily diet. *Colocasia esculenta* L. is an important staple food for various populations (Huang *et al.*, 2006). Amadumbe tubers make a significant contribution to the diet of rural people in Zululand. However, their nutritional value has not been studied adequately.

The nutritional value of any food depends largely on the quality of ingredients used. This is measured in terms of the food's capacity to encourage growth and maintain the animals consuming it in good condition. Food content includes both the useful and the harmful components of food. Issues relating to food safety are causing more concern than ever: it has to be ensured that no food will be harmful to the consumer when it is processed and/or eaten according to its intended use (FAO/WHO, 1991). The study of the composition of food is not only related to the nutrients in the human diet, but also to the potentially dangerous anti-nutrient elements.

Nutritional composition of *Colocasia esculenta* is similar to that reported for most cultivated taro and cocoyam species in several parts of the world. The high moisture and carbohydrate content and the low amounts of fat and protein are typical of most roots crops (Onayemi and Nwigwe, 1987). The use of Amadumbe as food material for human consumption could be encouraged. It has been shown that Amadumbe tubers are generally low in mineral content apart from potassium and magnesium. This notwithstanding, if they are consumed together with ingredients high in micro- and macromineral elements, their health value, and hence utilization, would increase.

The study showed that Amadumbe tubers also contain essential fatty acids, linoleic (omega-6) and linolenic (omega-3) acid. Therefore, from a nutritional viewpoint, it may be concluded that, apart from the good starch content in Amadumbe, this food is a fine source of essential fatty acids.

It has been reported that several anti-nutritional factors are present in root and tuber crops (Bhandari and Kawabata, 2004). The levels of anti-nutritional factors in locally grown Amadumbe are important in the assessment of its nutritional status. In this investigation, the Amadumbe tuber was found to contain α-amylase and trypsin inhibitors, lectins, total phenols, alkaloids, oxalates, phytates, cyanogens and saponin. When the values recorded for Amadumbe were compared with those of taro, cocoyam, yam and other roots and tubers investigated in other studies, the oxalate (Holloway *et al.*, 1989; Huang *et al.*, 2007) and phytate (Marfo *et al.*, 1990) content were observed to be low, while total phenols (Njingtang *et al.*, 2006) and cyanogens (Bhandari and Kawabata, 2004) were considered high.

### A.5.2 Processing and anti-nutritional factors

The different cooking methods studied had diverse effects on reducing the levels of the α-amylase inhibitor, total phenols, tannin, flavonoids, alkaloids, oxalates, phytates, cyanogens and saponins.

Boiling appeared to be the most effective processing method to reduce most anti-nutrients in Amadumbe tubers. The reduction of anti-nutrient levels on processing this foodstuff enhances the nutritional value of Amadumbe tubers. Thus, it may be stated from the results of this study that the anti-nutritional factors, though present in raw tubers, should not pose a problem with regard to human consumption if the tubers are properly processed.

In conclusion, Section A of this study indicates the potential of the Amadumbe tuber as an unconventional dietary carbohydrate source for rural people living in Zululand. However, the principal problems that could undermine this potential are the presence of anti-nutrients typified by oxalate, cyanogens, tannin and phytate. Two of the anti-nutritients (α-amylase inhibitor, saponin) are further characterized in Section B. In order to reduce the effects of these anti-nutrients, which may be a potential health-hazard, proper processing before consumption is recommended. Supplementation from other sources, such as vegetables eaten with the cooked Amadumbe, could improve the nutritional value of these tubers.

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# SECTION B

## PURIFICATION AND CHARACTERIZATION OF α-AMYLASE INHIBITOR AND SAPONIN PRESENT IN *COLOCASIA ESCULENTA*


### SUMMARY

Two proteins with α-amylase inhibitors, A-1 and B-2, were partially purified from *Colocasia esculenta* tubers (extraction with 1% PVP, 80 per cent ammonium sulphate precipitation, ion-exchange chromatography on DEAE-Sephacel and chromatography on Sephadex G-100). Using gel chromatography on G-100, the molecular weight of A-1 and B-2 were found to be approximately 17 000 and 19 000 respectively. The inhibitors inactivated α-amylases of animal origin, but had no effect on fungal amylase. Inhibitor A-1 also exhibited activity towards plant amylases, while inhibitor B-2 evidenced no activity on plant amylases. Inhibitor A-1 was most active (with human-salivary amylase) at pH 6. Inhibitor A-1 was completely destroyed at temperatures above 50° C, while inhibitor B-2 was stable up to 70° C.

A steroidal saponin was also isolated from the tubers of *Colocasia esculenta*. The structure was elucidated mainly with TLC, IR (Infrared spectroscopy) and GC-MS (Gas Chromatography - Mass Spectroscopy) spectroscopic analysis. The compound was determined to be gamma-sitosterol.

# CHAPTER B1-1: α-AMYLASE INHIBITOR (AI)

# LITERATURE REVIEW

## B1.1.1 Introduction

Many edible plant species consist of compounds that inhibit enzymes, principally hydrolases. Most of these inhibitors are proteins by nature, which particularly inhibit enzymes by forming complexes that block the active site or modify enzyme conformation, ultimately reducing the catalytic function. In the previous section (Section A), α-amylase inhibitor activity was identified as one of the anti-nutrients present in Amadumbe. In this section, α-amylase inhibitors in *Colocasia esculenta* will be isolated from white Esikhawini tubers and partially characterized.

## B1.1.2 α-Amylase inhibitor

Starch is a storage carbohydrate present in seeds and tubers of numerous plants (Teotia *et al.* 2001). Starch consists of two components: amylose and amylopectin. Amylose is a linear glucose polymer, which contains α-1,4 linkages and is a non branched polymer. Amylopectin, on the other hand, is a highly branched polymer of glucose in which linear chains of α-1,4 glucose residues are interlinked by α-1,6 linkages [Buléon *et al.*, 1998]. Prior to starches being absorbed, they first need to be broken down into glucose and smaller oligosaccharides. Digestive enzymes (amylase and isomaltase) are responsible for this catalytic reaction (Marshall and Lauda, 1975; Choudbury *et al.*, 1996).

Amylases (α-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) are a group of glycoside hydrolases widely distributed in microorganisms, plants and animal tissues (Whitaker, 1988; Payan, 2004). α-Amylases hydrolyze long, complex, starch chain molecules to release maltotriose and maltose from amylose and maltose, glucose and "limit dextrin" from amylopectin (Moore *et al.*, 2005). Without calcium, the α-amylases which are

calcium metalloenzymes, are completely unable to function. MacGregor *et al.*, (2001) commented that these amylases are crucial to the carbohydrate metabolism of many autotrophic and heterotrophic organisms.

α-Amylase catalyzes the break down of α-(1,4) glycosidic linkages found in starch components and other related carbohydrates (Dolečková-Marešová *et al.*, 2005). In autotrophic organisms, sugars are gradually released from starch which has previously been stored, thus providing energy for proper growth. Amylases have established many valuable functions within society, some of which are disease testing, fruit ripening and malt production (Teotia *et al.*, 2001). α-Amylases also play a major part in breaking down starch in germinating seeds (Jones and Jacobsen, 1991). Usually, the major constituent of man's diet is carbohydrate and the main carbohydrate ingested is starch. α-Amylases are the most important means heterotrophic organisms use to digest starch (Silva *et al.*, 2000).

Several compounds found in nature are responsible for inhibiting enzymes, especially the hydrolases (Kokiladevi, 2005). Current investigations in the field of proteinase and amylase inhibitors and the growing interest in these phytochemicals in industry and pharmacy have led researchers to inquire directly into their exact biological function. Some of these inhibitors are proteins which decrease specific enzyme activities, such as the inhibitors of proteases and amylases (Whitaker and Feeney, 1973).

In 1934, Chrzaszcz and Janicki identified AI in wheat and since then, numerous studies have recognized the fact that α-amylase inhibitors occur naturally in a wide variety of plants. Richardson (1991) and Franco et al. (2002) proposed that α-Amylase inhibitors may simply be classified by their tertiary structure into six different classes, which researchers identify as lectin-like, knottin-like, cereal-type, Kunitz-like, γ-purothionin-like and thautamin-like. The molecular weight, disulfide bond content, three-dimensional structure and stability to heat and denaturing agent (Teles *et al.*, 2004) differentiate among these families. Each family of α-amylase inhibitors shows particular specificity features:

### B1.1.2.1 Lectin-like α-amylase inhibitor (AI)

Lectin-like α-amylase inhibitors have been purified and characterized from different varieties of common bean (*Phaseolus vulgaris*) [Le Berre-Anton *et al.*, 1997; Lee *et al.*, 2002]. These inhibitors have two variants with a high degree of sequence homology (Suzuki *et al.*, 1994). Three inhibitors are encoded by two different alleles. One of these inhibitors inhibits both mammalian and insect-amylases, whereas another inhibits different insect α-amylases (Franco *et al.*, 2002).

### B1.1.2.2 Knottin-like α-amylase inhibitor

The knottin-like family contains the AI from the Mexican crop plant, amaranth (*Amaranthus hypochondriacus*). This AI is the smallest known, natural, proteinaceous α-amylase inhibitor (Chagolla-Lopez *et al.*, 1994). The seeds of the amaranth inhibit insect α-amylases, but are inactive against mammalian enzymes (Pereira *et al.*, 1999). The tertiary protein structure shows knots particularly rich in disulphide bridges. This structure can exist both as individual miniproteins of around 32 amino-acid residues and as domains in larger molecules (Svensson *et al.*, 2004).

### B1.1.2.3 Cereal-type α-amylase inhibitor

This is a large protein family which includes α-amylase inhibitors from cereal seeds (Barber *et al.*, 1986; Garcia-Maroto *et al.*, 1991). Members of this family are known for their activity on α-amylases from mammals and insects, but have also shown inhibitory activity against α-amylases from birds and bacteria (Franco *et al.*, 2002).

### B1.1.2.4 Kunitz-like α-amylase inhibitor

Macedo *et al* (2007) defined plant Kunitz inhibitors as proteins (Mr ~ 18,000 – 24 000 Da) which have one or two polypeptide chains. They have a low cysteine content, generally with four cysteine residues organized into two disulphide bridges.

### B1.1.2.5 γ-Thionin-like α-amylase inhibitor

The major utilization of the γ-thionine α-amylase inhibitor family is in processes promoting plant-defense through several means: adaptation of membrane permeability (Castro *et al.*, 1996), blockage of protein synthesis (Mendez *et al.*, 1990) and digestive enzyme inhibition (Wijaya *et al.*, 2000). γ-Purothionin-like proteins have shown specificity to inhibit insect digestive α-amylase (Bloch and Richardson, 1991).

### B1.1.2.6 Thaumatin-like α-amylase inhibitor

Proteins from the thaumatin-like α-amylase inhibitor family have the ability to modify the properties of fungal cell walls. Schimoler-O'Rourke *et al.*, (2001) identified Zeamatin as a 22 kDa protein isolated from *Zea mays*, pointing out that it has noteworthy amino acid homology to taumatin 3-like proteins. It inhibits insects, but not mammalian α-amylases (Svensson *et al.*, 2004).

Amylase inhibitors prevent dietary starches from being absorbed and are therefore also known as starch blockers. Reports on purified AI have shown that intraluminal amylase activity is significantly inhibited when perfused into the duodenum (Layer *et al.*, 1985). When dietary starch is ingested with AI, it reduces the post-prandial increase in glucose significantly in both normal and diabetic patients (Layer *et al.*, 1986). Diarrhea may result from undigested starch in the colon, which is the cause of high amounts of amylase inhibitors (Boivin *et al.*, 1988).

α-Amylase inhibitors could function as part of the defense mechanism in plants. In leguminous plants (Giri and Kachole, 1998; Melo *et al.*, 1999) and cereals (Franco *et al.*, 2000; Yamagata *et al.*, 1998), the role of amylase inhibitors as plant-defense proteins is pronounced and has received much focus (Gatehouse *et al.*, 1986; Farmer and Ryan, 1990). α-Amylases and proteinases are inactivated by these inhibitors in the insect gut, thereby acting as insect anti-feedants. This interaction is believed to make plants less palatable. Indeed, it can even be lethal to insects. Thus, these inhibitors present the plants with some selective advantages.

Increasing natural defense mechanisms in plants can enhance agricultural activity and food safety by decreasing intensive use of pesticides (Huang *et al.*, 1997; Chen *et al.*, 1999). These inhibitors, however, often show confined specificities: a given inhibitor may inhibit the major digestive enzymes of one insect species, but not of another (Morton *et al.*, 2000). This specificity has been widely investigated, with some inhibitors capable of acting against insect α-amylases or against mammalian enzymes only (Franco *et al.*, 2000). As a result, α-amylase inhibitors show potential for utilization in several fields, including protection of crops, obesity and treatment of diabetes (Tormo *et al.*, 2006).

Diabetes mellitus is a metabolic disorder of multiple aetiology characterised by chronic hyperglycaemia with disturbance of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both (WHO, 1999). Glucose peaks occur after the intake of a meal in animals. α-Amylase inhibitors can reduce these peaks until the body is capable of processing the glucose. This is achieved by reducing the speed with which α-amylase can convert starch to simple sugars. Breuer (2003) held that this is especially important for diabetics who exhibit low insulin levels which hamper the prompt removal of extracellular glucose from the blood. Ali *et al* (2006) observed that reducing hyperglycaemia, using extracts of six selected Malaysian plants, after meals is one therapeutic approach which could be adopted in the treatement of diabetes.

Clinical use of inhibitors of intraluminal α-amylase activity has appeal because, in theory, controlled reduction of starch digestion could influence carbohydrate uptake in

diabetes mellitus or obesity. Octivio and Rigden (2000) referred to α-amylase and its inhibitors as 'drug-design targets' from which compounds for the treatment of diabetes, obesity and hyperlipaemia could be developed.

### B1.1.3 Aim and outline of Section B-1

Amylase inhibitors have been extracted from several types of plants, especially those in the cereal and legume family. In contrast to α-amylase inhibitors in cereal (Roy and Gupta, 2000; Heidari *et al.*, 2005; Muralikrishna and Nirmala, 2005) and legumes (Giri and Kachole, 1998; Melo *et al*, 1999), which have been extensively studied, little is known about the structural features and properties of tuber α-amylase inhibitors.

The aim of Section B-1 was therefore to extract, isolate and characterize α-amylase inhibitors from Amadumbe. The objectives were:

- the extraction, isolation and partial purification of α-amylase inhibitors through ion-exchange and gel chromatography;
- to undertake kinetic studies (pH and temperature optima); and
- to investigate the selectivity of inhibitor action on different α-amylases.

# CHAPTER B1-2

# MATERIALS AND METHODS

## B1.2.1 Introduction

This chapter gives a brief description of the materials and methods used to isolate, partially purify and characterize α-amylase inhibitors from white *Colocasia esculenta* grown in Esikhawini, Zululand, South Africa. (See Appendices A and B for details).

## B1.2.2 Materials

The white variety of Amadumbe (*Colocasia esculenta)* tubers was obtained from the local market in Esikhawini, Kwazulu-Natal, South-Africa. Commercially available amylases (human saliva, type IX-A; porcine pancreatic, type I-A; sweet potato, barley, *Bacillus* species, type II-A; *Aspergillus oryzae*) and all other reagents used were obtained from Sigma Chemical Company.

## B1.2.3 Methods

(See Appendix B for details of methodology.)

### B1.2.3.1 Extraction and purification of α-amylase inhibitor

The scheme for the extraction and purification is shown in Figure B-1.

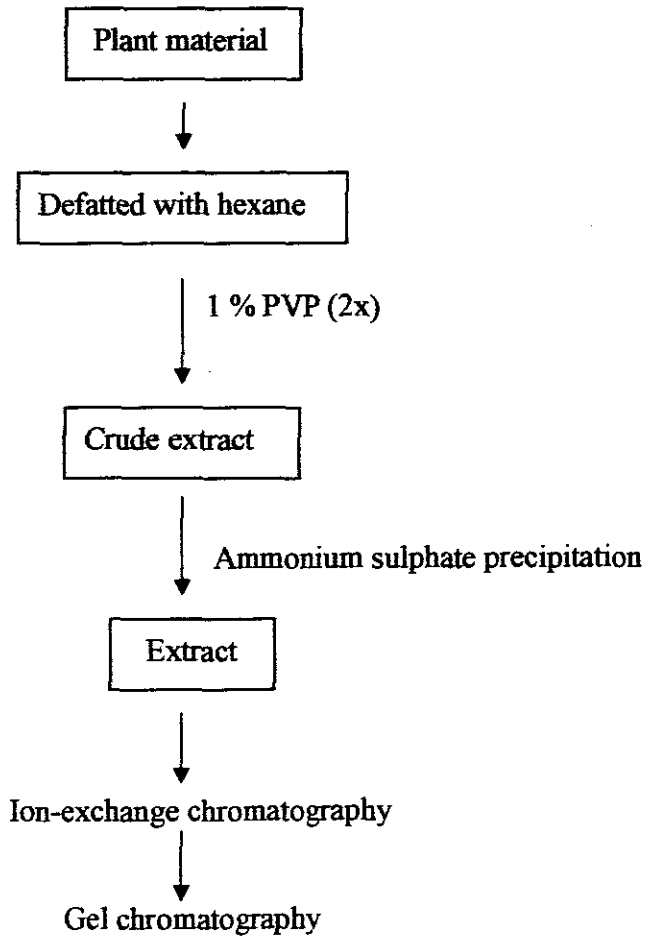



**Figure B1-2.1: Extraction protocol for isolating α-amykase inhibitor from Amadumbe tubers**

Tubers with no physical signs of infection were washed, peeled, cut into small pieces (2 cm x 3 cm) and dried at 40° C for 24 hours. The dried material was milled (68 mesh) and the flour defatted with hexane and air-dried. Twenty grams of the defatted flour were added to 100 ml of distilled water (containing one per cent PVP), stirred for two hours and filtered. The residue was re-extracted and the combined filtrate centrifuged at 12000 g for 20 minutes.

The supernatant (designated as the crude extract) was subjected to 80 per cent $(NH_4)_2SO_4$ saturation and left overnight at 4° C. Protein pellets obtained after centrifugation (12 000 g x 20 minutes) were redissolved in a minimum volume of phosphate buffer (0.02 M, pH6.9, containing 0.3 M NaCl), dialysed extensively against the buffer (designated the ammonium sulphate extract) and analysed for amylase inhibitor (AI) activity.

#### B1.2.3.1.1 Ion-exchange chromatography

The ammonium sulphate extract was further purified through ion-exchange chromatography (6 cm x 1.1 cm DEAE-Sephacel, equilibrated with 0.02 M phosphate buffer). The column was eluted with a linear NaCl gradient of 0-0.5 M at the flow rate of 20 ml/h and 5 ml fractions were collected). The absorbance of the effluent was monitored at 280 nm. Individual peaks were pooled and analysed for protein and AI activity.

#### B1.2.3.1.2 Gel chromatography

Two peaks (A and B, Figure B1-3.2) with AI activity were then separately chromatographed on a Sephadex G-100 column (35 cm x 1.1 cm, equilibrated with the phosphate buffer and eluted with the same buffer at a flow rate of 15 ml/hr. Five ml fractions were collected and the absorbance at 280 nm was determined). Pooled fractions were analysed for protein and AI activity. Fractions (A-1 and B-2, Figure B-4) with AI activities were collected, dialysed extensively, freeze-dried and dissolved in de-ionized water.

### B1.2.3.2 Enzyme/inhibitor assay

The method described by Bernfeld (1955) was used to assay for α-amylase and AI activities. One unit of amylase is defined as the amount of enzyme that will liberate 1 μmol of maltose from starch under the assay conditions (pH 6.9, 37° C, 5 min). Inhibitory activity is expressed as the percentage of inhibited enzyme activity out of the total enzyme

activity used in the assay. The incubation time of the enzymes with the inhibitor fractions was 20 minutes at 37° C.

#### B1.2.3.3 Molecular weight determination

Proteins were determined using the Sigma kit. Molecular weight was determined by gel chromatography on Sephadex G-100, under the conditions described by Sigma. Cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), alcohol dehydrogenase (150 kDa), and β-amylase (200 kDa) were used as molecular weight markers.

#### B1.2.3.4 Kinetic studies

##### B1.2.3.4.1 α-Amylase inhibitor specificity

α-Amylase inhibitory activities were estimated as in Section B.1.2.3.2, using α-amylases from human saliva, sweet potato, barley, porcine pancreas, *Bacillus* and *Aspergillus oryzae*. The AI with the corresponding amylase was pre-incubated for 30 minutes at 37° C in phosphate buffer pH 6.9 before adding starch to initiate the reaction.

##### B1.2.3.4.2 Optimum pH

The pH optimum of the inhibitor was determined by varying the pH of the test reaction mixture using the following buffers (0.1 M): sodium acetate (pH 5), sodium phosphate (pH 6–7), Tris–HCl (pH 8) and glycine–NaOH buffer (pH 9–10). To determine the pH of the α-amylase inhibitor, it was pre-incubated in different buffers (pH 1–10) for five minutes.

##### B1.2.3.4.3 Optimum temperature

The temperature optimum of the inhibitor was evaluated by measuring α-amylase activity at different temperatures (20 – 100° C) in phosphate buffer (pH 6.9).

# CHAPTER B1-3

# RESULTS

## B1.3.1 Introduction

In this chapter, the results for the isolation, partial purification and characterization of the two α-amylase inhibitors from Amadumbe are presented.

## B1.3.2.1 Extraction and purification of α-Amylase inhibitors

The overall results of the purification procedure of α-amylase inhibitors from *Colocasia esculenta* have been summarized in Table B1-3.1. Preliminary studies (Figure B-1) suggested that 80 per cent ammonium-sulphate saturation was best for salting out the inhibitor protein from the crude extract.

**Table B1-3.1: Purification of amylase inhibitors from Amadumbe**

| Fraction | Total protein (mg) | Inhibitor (units) | Specific activity (units/mg) | Yield (%) | Purification |
|---|---|---|---|---|---|
| Crude extract | 20.9 | 62 | 2.9 | 100 | |
| Ammonium sulphate | 18.5 | 56 | 3.02 | 88.5 | 1.04 |
| **DEAE sephacel:** | | | | | |
| Peak A | 7.2 | 54 | 7.5 | 33.44 | 2.59 |
| Peak B | 5.9 | 50.5 | 8.56 | 28.22 | 2.93 |
| **Sephadex G-100** | | | | | |
| Peak A1 | 3.7 | 41.8 | 11.29 | 17.70 | 3.89 |
| Peak B1 | 5.6 | 39.8 | 12.83 | 14.83 | 4.42 |

*Note: The inhibitor units were calculated using human-salivary amylase.*

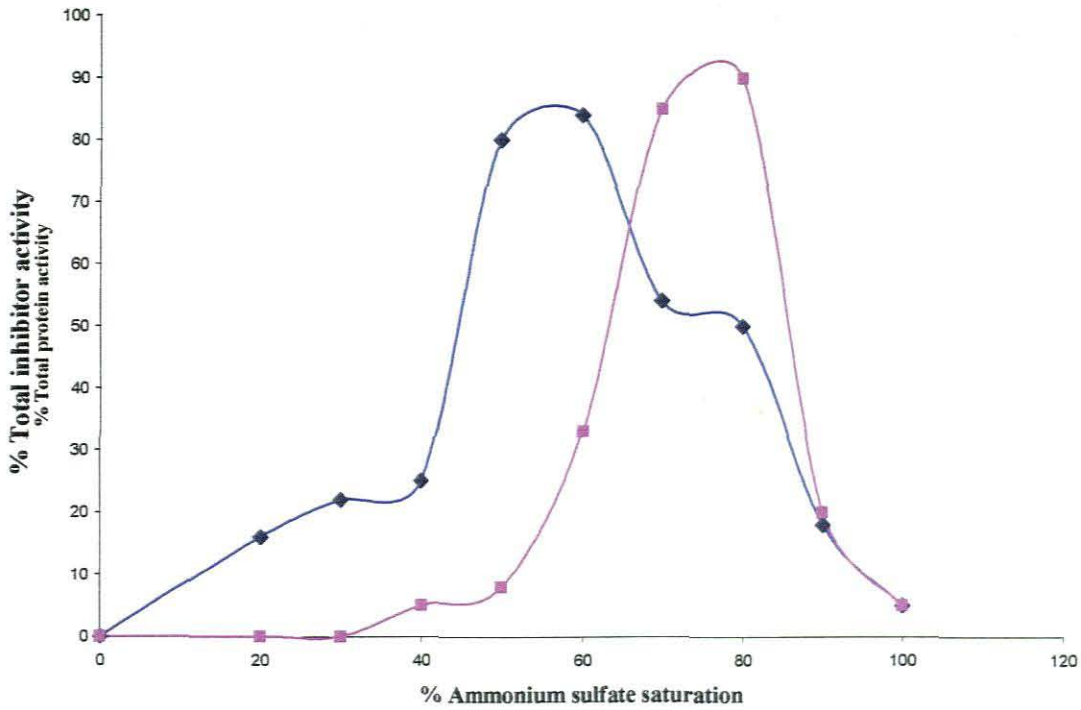



**Figure B1-3.1: Ammonium sulphate precipitation of the Amadumbe crude extract**

**Key**

inhibitor activity (■) total protein (♦)

The profile of the ion-exchange chromatography is shown in Figure B1-3.2. Only two (A and B) of the five protein peaks showed α-amylase inhibitory activity.

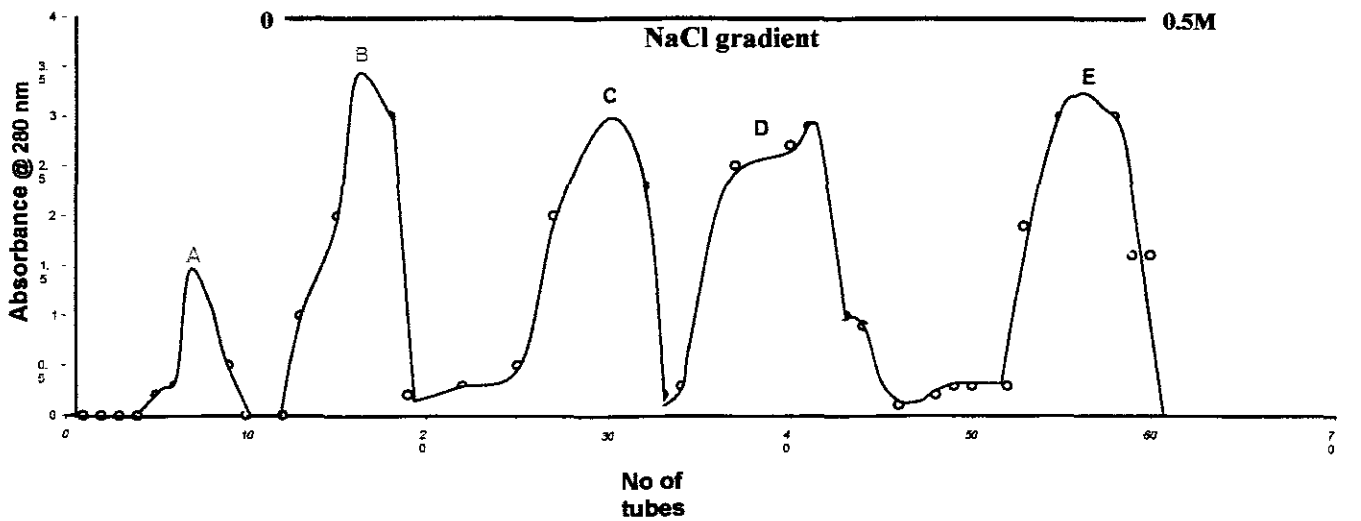



**Figure B1-3.2: Ion-exchange chromatography of extract on DEAE-Sephacel**

The inhibitor peaks (Figure B1-3.3) from the DEAE-Sephacel chromatography were subsequently separated by gel filtration. Only two (A1 and B2) of these proteins had inhibitory activity against human salivary α-amylase.

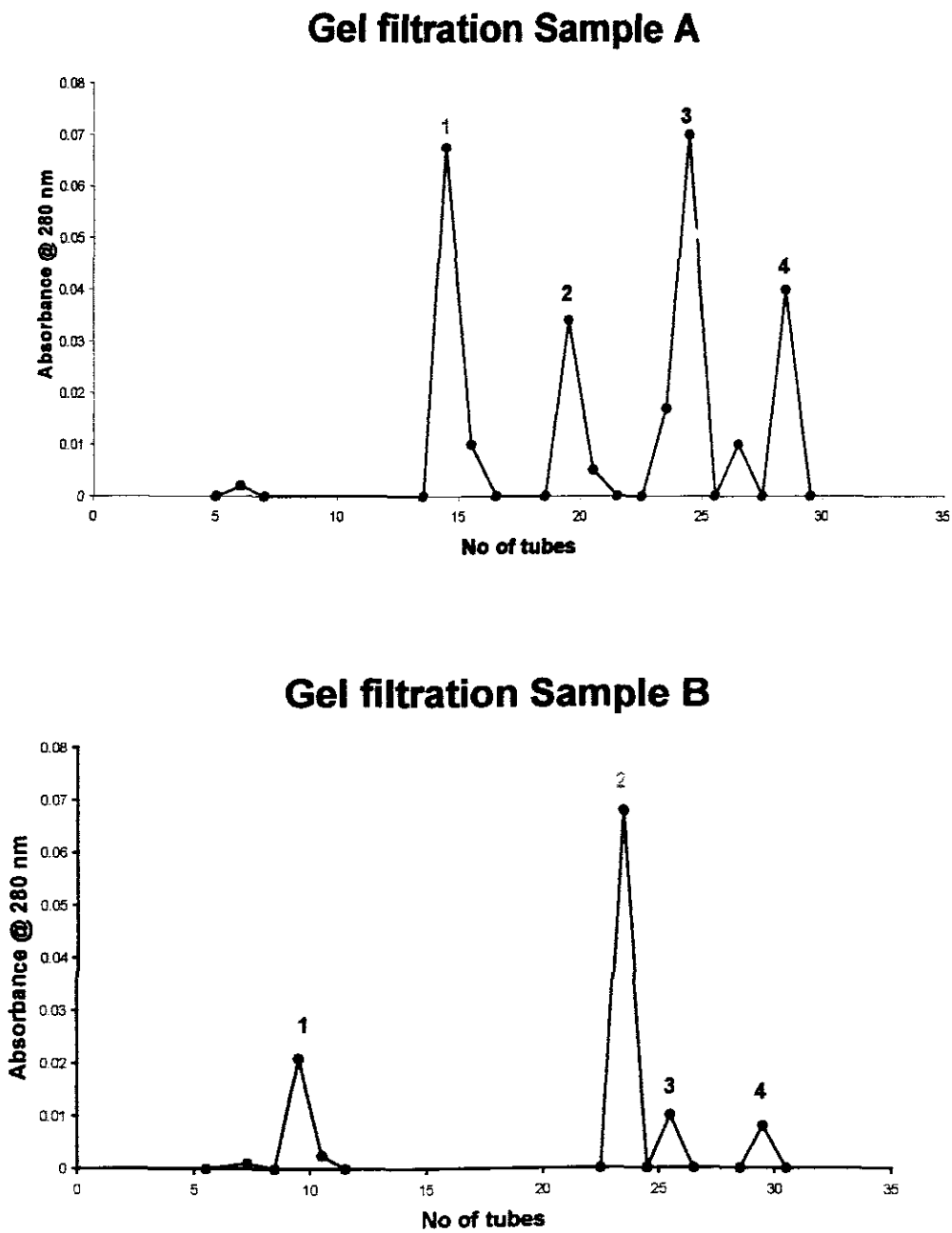



**Figure B1-3.3: Sephadex G-100 column chromatography of fraction A and B after ion-exchange chromatography on DEAE-Sephacel**

The molecular weight of AI-A1 and AI-B as determined by gel filtration on Sephadex G-100, was estimated to be about 17 kDa and 19 kDa respectively.

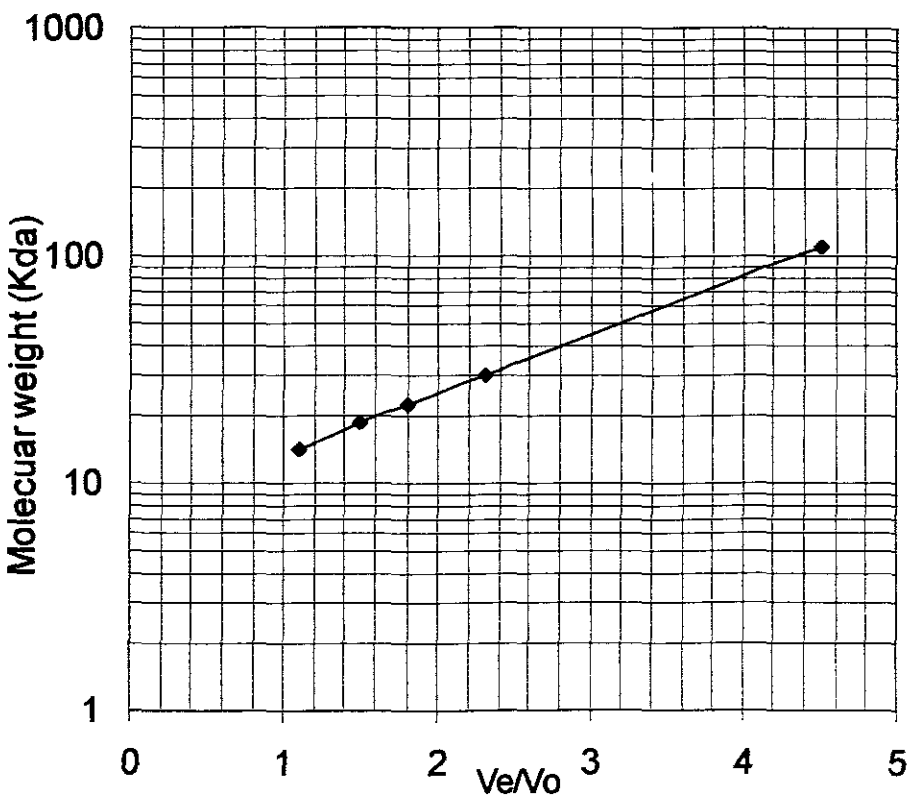



**Figure B1-3.4: Standard Log Molecular Weight graph using gel filtration**

**Standards**

| | |
|---|---|
| cytochrome C (12 400) | carbonic anhydrase (29 000) |
| alcohol dehydrogenase (150 000) | β-amylase (200 000) |

### B1.3.2.2 Kinetic studies

The inhibitory activity of the purified proteins on amylases from different sources is presented in Table B1-3.2. The two proteins were found to inhibit mammalian and bacterial amylases. While protein A1 inhibited amylase from plant sources, protein B2 showed no such effect. The two proteins could not inhibit fungal amylase.

**Table B1-3.2: Effect of α-amylase inhibitors present in Amadumbe against amylases from different sources**

| Action of inhibitors on different amylases | | |
|---|---|---|
| **Source of amylases** | **Percentage inhibition** | |
| | **A1** | **B2** |
| Human salivary | 62 | 56 |
| Barley | 56 | 0 |
| Sweet potato | 4.7 | 0 |
| *Bacillus* species | 10.2 | 23 |
| *Aspergillus* species | 0 | 0 |
| Porcine pancreas | 28.5 | 48.5 |

The effect of temperature on the two proteins is presented in Figure B1-3.5. The two proteins were most active at 40° C. They were completely inactivated at temperatures above 80° C.

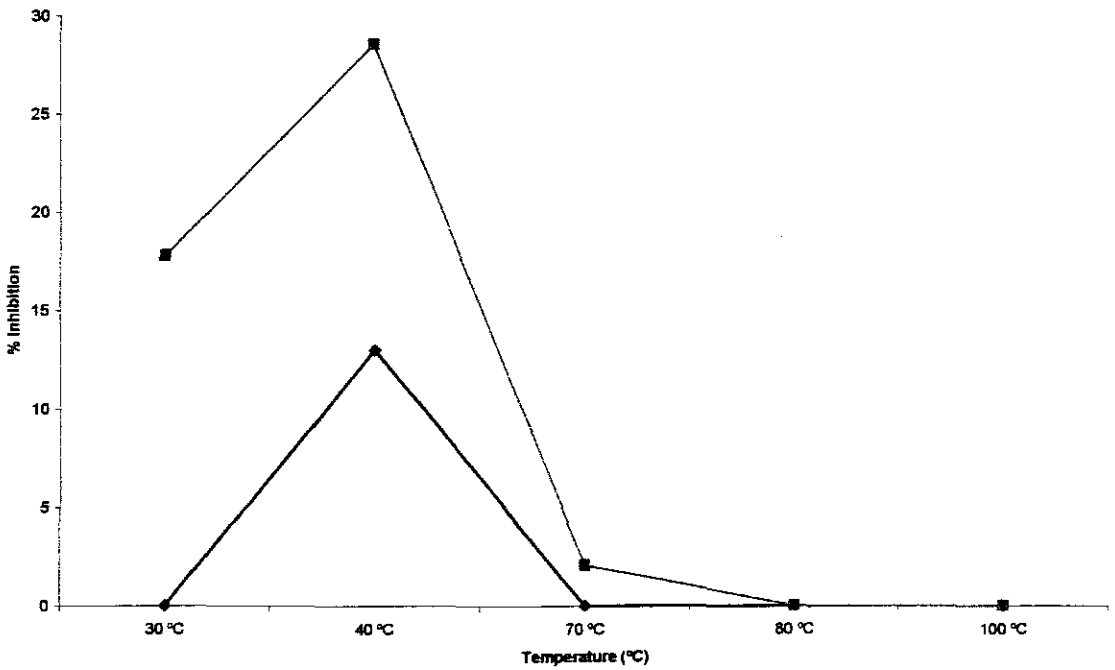



**Figure B1-3.5: Effect of temperature on the activity of *Colocasia esculenta* α-amylase inhibitors.**

**Key**

AI-A1 (♦) AI-B2, (■)

Optimum pH for amylase inhibitor activity in *Colocasia esculenta* is shown in Figure B1-3.6. A two-peak pattern was found, which showed an optimum at pH 4.0 and 6.0.

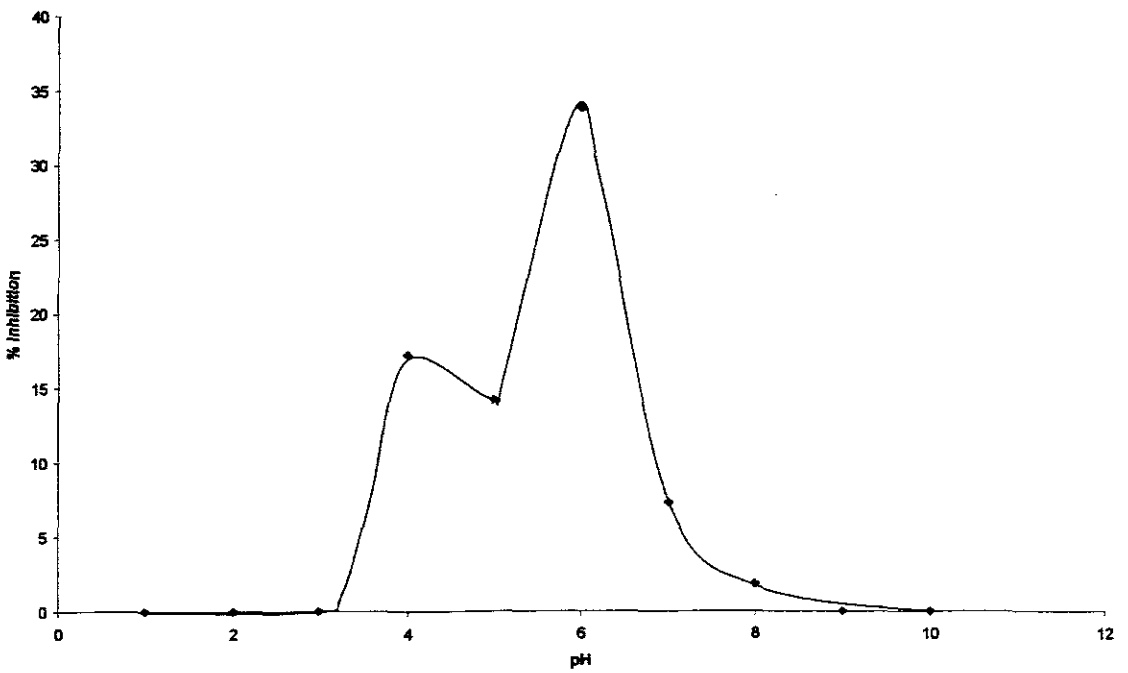



**Figure B1-3.6: Effect of pH on the stability of Amadumbe α-amylase inhibitor**

The enzyme solutions at various pH values were incubated at 25° C for 10 minutes and residual activity was measured as described in Appendix B.

# CHAPTER B1-4

# DISCUSSION

## B1.4.1 Introduction

In this chapter, the results of the isolation, partial purification and characterization of the two α-amylase inhibitors from Amadumbe are discussed.

## B1.4.2 Isolation of α-amylase inhibitor

A 3.89- and 4.42-fold purification of AI-A1 and AI-B2 respectively was obtained. The proteins had specific activity of 11.29 and 12.83 respectively.

The purified α-amylase inhibitors (AI-A1 and AI-B2) from *Colocasia esculenta* showed a molecular weight of approximately 17 kDa and 19 kDa respectively. The little available information on α-amylase inhibitors present in *Colocasia antiquorum* (Sharma and Pattabiraman, 1980), taro (Seltzer and Strumeyer, 1990) and sweet potato (Rekha *et al.*, 2004) seemed to indicate that most tubers had two proteins that showed inhibiting activity. The molecular weight of these proteins ranged between 11 and 25 kDa.

## B1.4.3 Kinetic studies

### B1.4.3.1 Action of inhibitors on different α-amylases

α-Amylase inhibitors show strict target enzyme specificity and recognize only one out of several closely related isoenzymes or enzymes from different species (Weselake *et al.*, 1983; Franco *et al.*, 2000). Payan (2004) observed that amylase-inhibitor complexes have general features of inhibition on different amylases: (i) the inhibitor inhibits primarily via interactions with the enzyme substrate active site (ii) side-chains originating from the

inhibitor molecule generally occupy the subsites of the enzyme (iii) structural elements involved in the inhibition action are likely to correspond to flexible components of the free structures of the molecules.

Literature (Sharma and Pattabiraman., 1980, 1982; Ida *et al.*, 1994) indicates that many AI present in tubers are active against mammalian amylases, but exhibit no activity on plant amylases. It is apparent that the two AI present in Amadumbe will complement each other, providing the Amadumbe tubers with a wider spectrum against intruders.

It is apparent that both inhibitors had no effect on fungal amylase. Amadumbe grow in moist, humid conditions and fungal infections are prevalent. It is possible that fungus has become resistant to the action of α-amylase inhibitors. Sharma and Pattabiraman (1982) have reported similar results for *Dioscorea alata*.

Bifunctional properties have been demonstrated by a number of inhibitors and have therefore received particular attention as appealing candidates for pest-control. This biological activitiy of inhibitors, inhibiting both serine proteinases and α-amylases, is very useful (Maskos *et al.*, 1996). α-Amylase inhibitors show great variety in their effectiveness against target enzymes. α-Amylase inhibitors found in wheat (Franco *et al.*, 2000), barley (Richardson, 1991) and Indian finger millet (Campos and Richardson, 1983) efficiently inhibit α-amylases from different insect sources. Thus, these α-amylase inhibitors can play a key role in plant defense against pests and pathogens.

### B1.4.3.2 Effect of temperature on inhibitor activity

Temperature is one of the most important parameters that affect the rate of enzyme hydrolysis. The optimum temperature displayed for both inhibitors was 40° C and, at extreme temperature, inhibitors became inactive. Optimum temperature for the navy-bean amylase inhibitor was 37° C, but activity was lost at 90° C (Hoover and Sosulski, 1984). A similar optimum temperature of 37° C was also observed for the α-amylase inhibitor from a *Pacchyrhizus erosus* tuber (Noman *et al.*, 2006). It can be concluded from these

results that amylase inhibitors are fairly heat-stable (Prathibha *et al.*, 1995) and that the residual activity in processed Amadumbe (Section A) could be attributed to this property.

### B1.4.3.3 Effect of pH on the interaction of amylase with the inhibitor

The AI protein was not active at acidic pH. The proteins isolated from *Colocasia antiquorum* (Sharma *et al.*, 2006) were basic proteins. Because of the two peaks of optimal pH points, it is possible that isoenzymes are present. A similar optimum pH 7.0 was observed for the α-amylase inhibitor from the *P. erosus* tuber (Noman *et al.*, 2006).

The partially purified and characterized α-amylase inhibitors from Amadumbe showed similar properties when compared with α-amylase inhibitors from other tubers. Although the Amadumbe α-amylase inhibitors showed activity against mammalian amylases, processing greatly inactivated the biological activity of the inhibitors.

# CHAPTER B2-1: SAPONIN

# LITERATURE REVIEW

## B2.1.1 Introduction

Many different secondary metabolites are synthesized collectively by plants. This can either be as a response to pathogen attacks and stress or part of the plant's normal growth. Although these secondary metabolites are not required for growth and reproduction, they are important in that they offer the plant selective advantages: for example, restraining the growth of neighboring plants or protecting the plant against pests, pathogens and stress (Wink, 1999; Morrissey and Osbourn, 1999).

### B2.1.2.1 Saponin - chemistry

Saponins are a pharmacodynamic group of secondary metabolites with a wide spectrum of biological activities. They are found in more than 90 plant families (Sondhia, 2005), such as peanuts, lentils, lupins, alfalfa, oats and spinach (Fenwick and Oakenfull, 1983; Huhman and Sumner, 2002; Woldemichael *et al.*, 2003).

Saponins are characterized by surfactant properties because they contain both hydrophobic and hydrophilic components and, in most cases, give stable, soap-like foams in aqueous solutions. Saponins are glycosidic compounds containing a carbohydrate and a non-carbohydrate unit in the same molecule. An acetal linkage joins the carbohydrate residue to a non-carbohydrate residue or aglycone at carbon atom position 1. The sugar component is called the glycone. Saponins are surface-active compounds because of a lipid-soluble aglycone and water-soluble sugar chain(s) in their structure. This amphiphilic characteristic gives saponins detergent, wetting, emulsifying and foaming properties (Ibanoglu and Ibanoglu, 2000; Sarnthein-Graf and La Mesa, 2004; Wang *et al.*, 2005)

The chemical structure of its sapogenin (aglycone) determines the type to which the saponin belongs – *steroidal* or *triterpenoid* (Figure B2-1.1). Saponins are glycosides because they contain one or more sugar chains attached to the aglycone backbone (Hostettmann and Marston, 1995).

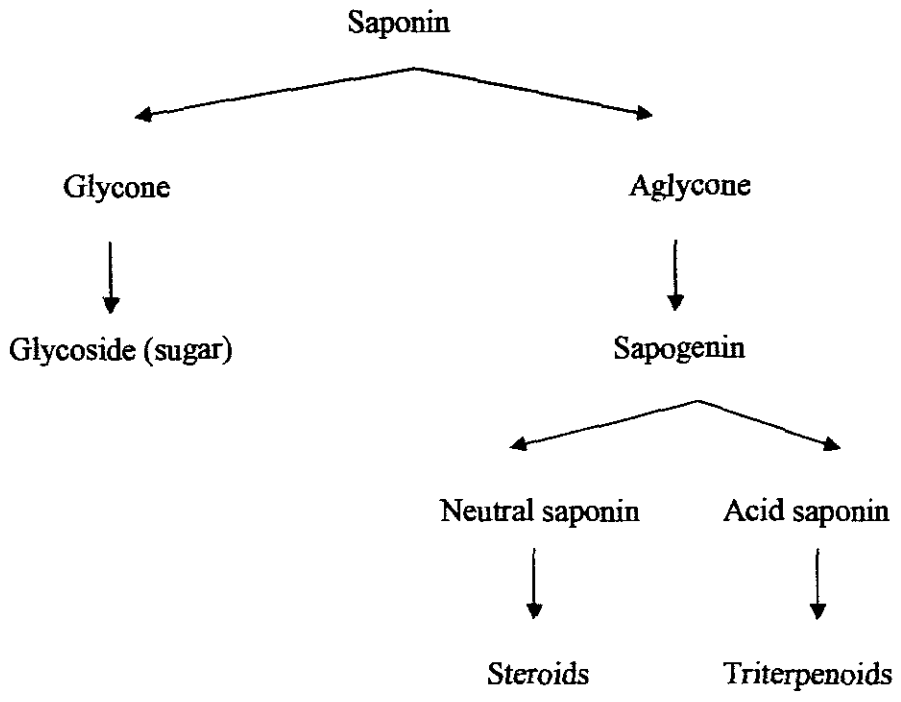



**Figure B2-1.1: Types of saponin** (Friedli, n.d)

Both types of sapogenins are synthesized from a similar pathway, involving the head-to-tail coupling of acetate units. However, after the formation of the triterpenoid hydrocarbon, squalene (Holstein and Hohl, 2004), there is a split in the pathway that leads to steroids in one direction and to cyclic triterpenes in the other (Figure B2-1.2)

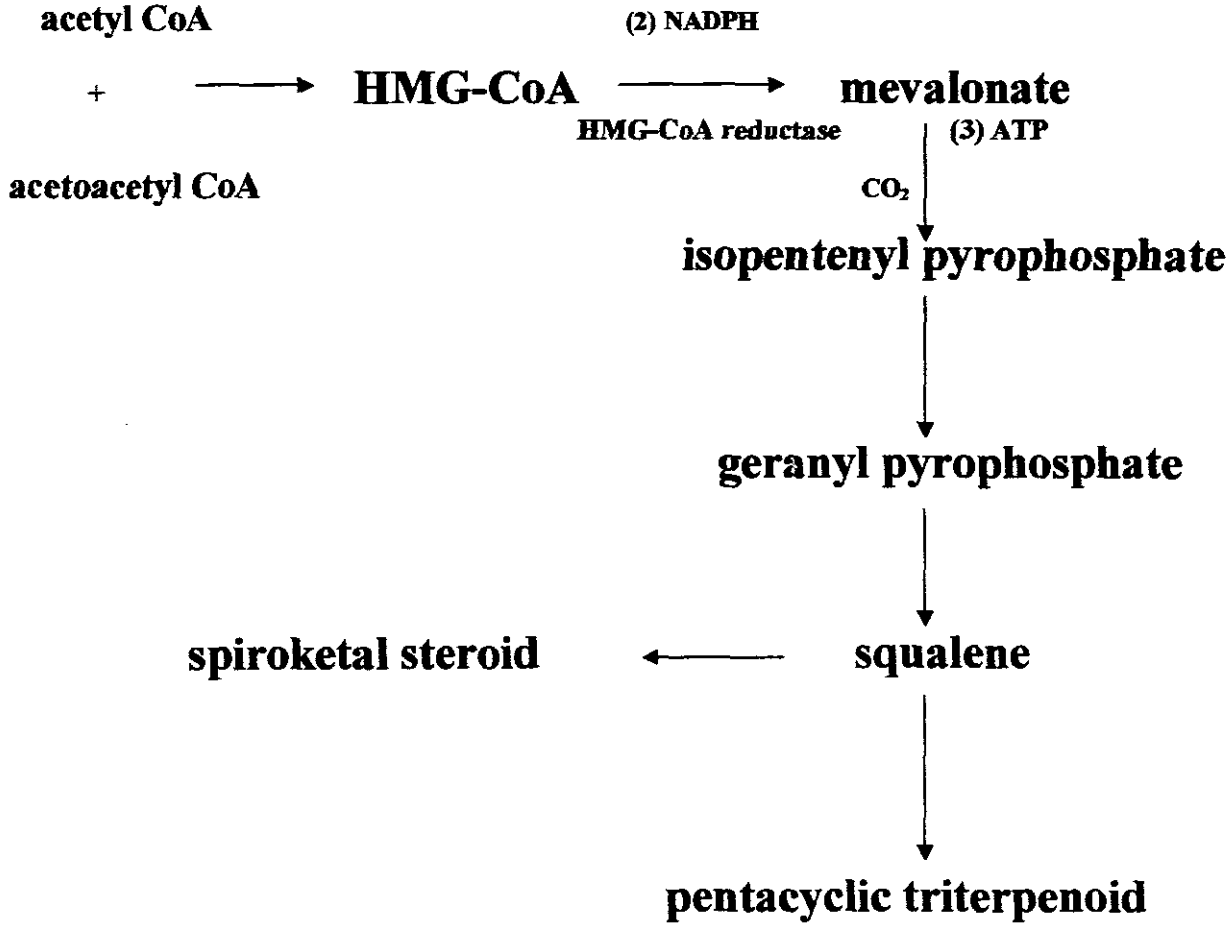



**Figure B2-1.2: Pathway to biosynthesis of triterpenes** (adapted from Focosi, 2005)

### B2.1.2.2 Triterpenoid saponin

Triterpenoid are a large group of compounds with 30 carbon atoms with four basic ring skeletal structures. The large group of compounds to which triterpenes belong are arranged in a four- or five-ring, planar-base molecule, containing 30 carbons with several oxygen attached. It is believed that squalene is the prescursor of all terpenoid compounds including triterpenoids. Initial steps in the synthesis of triterpenoid saponins in plants include the cyclization of 2,3-oxidosqualene via the isoprenoid pathway, giving rise to a number of different potential products (Hostettmann and Marston, 1995; Haralampidis *et al.*, 2002). The skeleton formed is then determined by the type of cyclase that is involved

in the cyclization reaction. There are about 20 groups of triterpenes, the specific structure of the triterpene determining to which group it belongs.

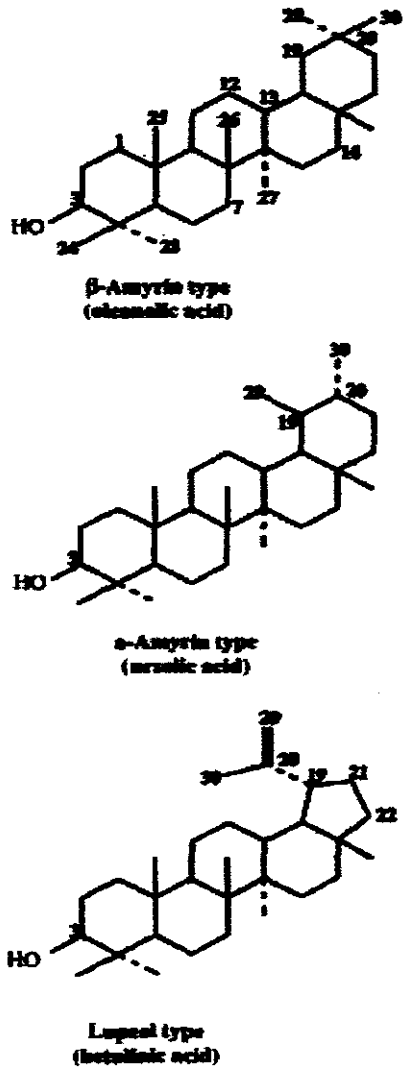



**Figure B2-1.3: Basic aglycone (sapogenin) skeletons –triterpene**

(Oleszek and Bialy, 2006)

### 32.1.2.3 Steroidal saponin

The triterpenes and the plant steroids are broadly described as saponins (Kelly, 2005). Sterols form an important group among the steroidal saponins. Like triterpenes, phytosterols are synthesized in the isoprenoid pathway. Triterpenes and plant sterols are differentiated by the structure of the carbon skeleton: triterpene saponins have a 30-carbon skeleton and sterols have a 27/29-carbon skeleton (Ukpabi and Ukpabi, 2003). Sterols were initially described as compounds having a structure similar to that of cholesterol, but this definition did not take their stereochemistry into account. Nes (1977) proposed a new definition, describing sterols as compounds developing from squalene or its oxide by a cyclization process. Therefore, the biosynthetic route to plant sterols also follows an isoprenoid biosynthetic pathway with isopentenyl pyrophosphate, derived from mevalonate, as the key building block (Piironen, 2000). However, in photosynthetic organisms (as opposed to yeast and fungi), it differs in that the important intermediate in the route from squalene is cycloartenol rather than lanosterol (Gibbons *et al.*, 1971). The basic sterol from which other sterol structures are defined is 5α-cholestan-3β-ol (Sterols, 2008). The phytosterols include campesterol, beta-sitosterol, and stigmasterol etc.

Steroidal saponins are comprised of two major classes: furostanol glycosides with an open side chain at C-22 and spirostanol glycosides with a closed spiroketal ring at C-22 (Figure B2-1.4). Furostanol glycosides are the foundation from which spirostanol glycosides are formed through hydrolysis of the C-26 sugar and spontaneous cyclisation of the side chain from the spiroketal (Inoue and Ebizuka, 1996).

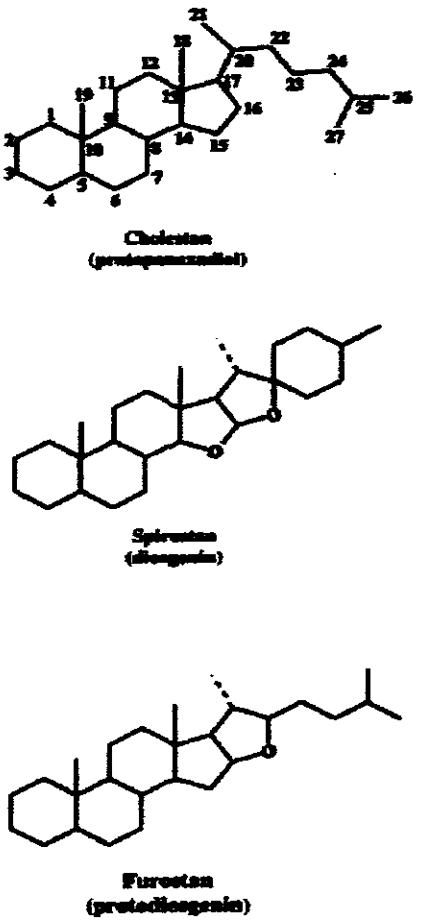


**Figure B2-1.4: Basic aglycone (sapogenin) skeletons – steroidal**

(Oleszek and Baily, 2006)

### B2.1.2.4 Biological activities

Because they have a number of both positive and negative properties, saponins have attracted a great deal of interest (Shi *et al.*, 2004). These properties include sweetness and bitterness (Kitagawa, 2002; Heng *et al.*, 2006), foaming and emulsifying characteristics (Price *et al.*, 1987), anti-nutritional effects (Fenwick *et al.*, 1992), pharmacological and medicinal properties (Attele *et al.*, 1999) as well as haemolytic properties (Oda *et al.* 2000; Sparg *et al.*, 2004). One of the most researched attributes of saponins is their ability to swell and rupture erythrocytes, causing a release of the pigment haemoglobin

(the *in vitro* haemolytic activity) (Oda *et al.*, 2000). Although the concept that they are harmful to human health has been questioned (Reddy and Pierson, 1994), saponins have been reported to retard growth in animals (Cheeke 1971; 1976).

The ability of saponins to lower cholesterol, demonstrated in animal (Matsuura, 2001) and human trials (Jones *et al.*, 1997) has been extensively researched. This trait of saponins has been attributed to their inhibiting cholestrol absorption from the small intestine or their inhibiting reabsorption of bile acids (Oakenfull and Sidhu, 1990). Clinical studies have suggested that the health-promoting components of saponins affect the immune system, impacting this system through adjuvant activity (Cheeke, 1999). The ability of saponins to operate as immunological adjuvants by improving the immune response to antigens has been recognized since the 1940s (Bomford *et al.*, 1992; Francis *et al.*, 2002). It is through the immunde response that the immune system helps to protect the human body against cancers. A number of triterpene and steroid saponins have shown anti-cancer activity (Berhow *et al.*, 2000; Kerwin, 2004). A saponin-rich diet offers several benefits, including a reduction in the occurrence of renal stones, the inhibition of dental carries, improved platelet aggregation and a positive response to treating hypercalciuria in humans (Shi *et al.*, 2004).

### 2.1.3 Aim and outline of Section B-2

Saponins can be found in a wide variety of plants including alfalfa (Cheeke *et al.*, 1977), soybeans (Berhow *et al.*, 2002; Kitagawa *et al.*, 1998) and legume seeds (Price *et al.*, 2006). Little is known about levels of saponins in *Colocasia esculenta* (Amadumbe).

The aim of Section B-2 was to extract, isolate and identify saponins from Amadumbe. The objectives were:

- to extract and isolate saponin using chromatographic methods (TLC and column chromatography);
- to elucidate saponin structure, using spectroscopy (IR and GC-MS).

# CHAPTER B2-2

# MATERIALS AND METHODS

## B2.2.1 Introduction

This chapter gives a brief description of the materials and methods used to isolate, partially purify and characterize a saponin from white *Colocasia esculenta* grown in Esikhawini, Zululand, South Africa. (See Appendix B for methodology).

## B2.2.2 Plant material

White tubers from *Colocasia esculenta* (Amadumbe) were collected from the local market at Esikhawini, Kwazulu-Natal, South Africa. The plant materials were collected between January and March 2006.

## B2.2.3 Methods

(See Appendix B for details of methodology)

### B2.2.3.1 Saponin extraction and isolation

The extraction procedure is illustrated below.

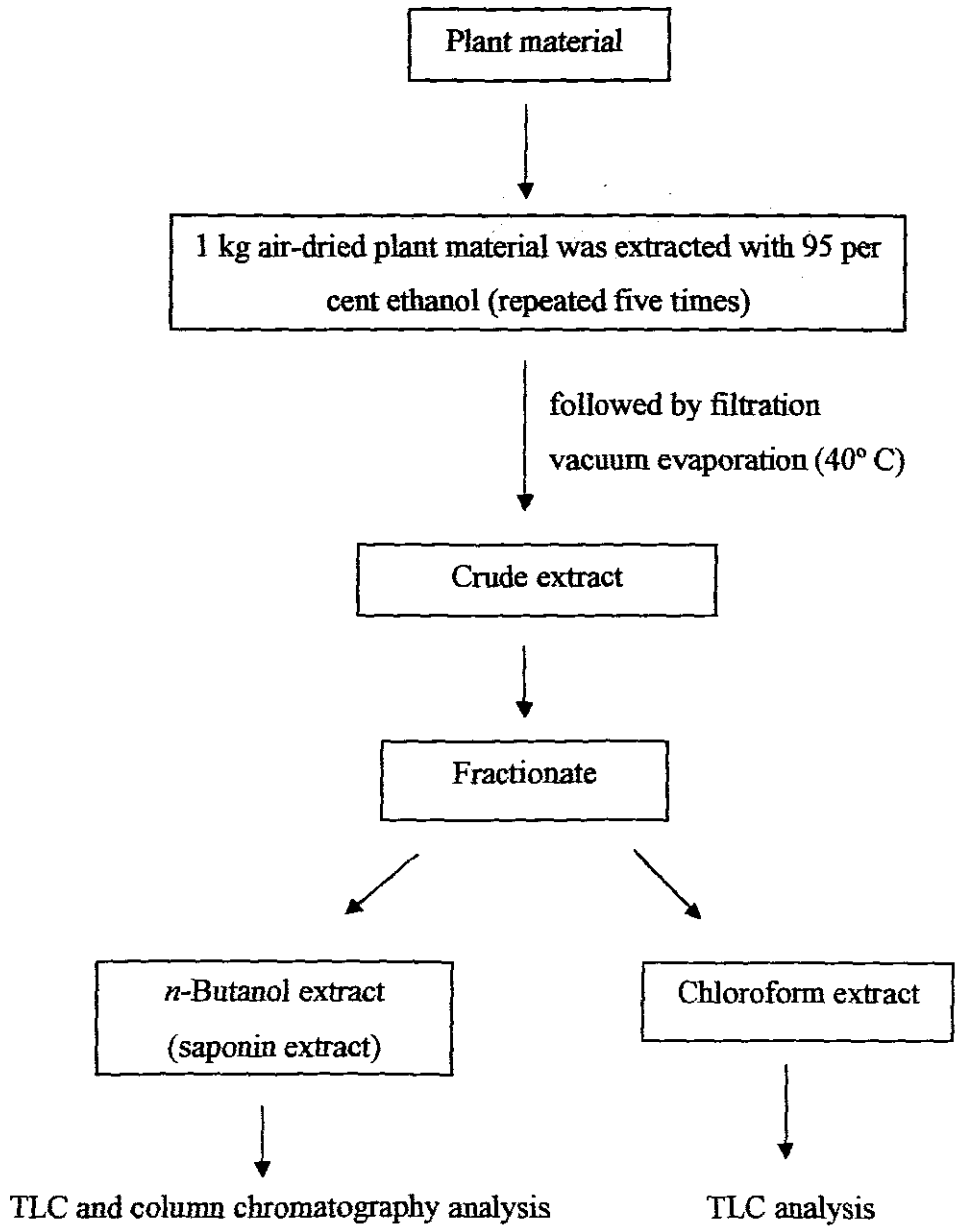



**Figure B2-2.1: Extraction protocol for obtaining native saponins from Amadumbe tubers**

Air-dried and powdered Amadumbe tubers were extracted with 95 per cent ethanol. The ethanolic extract was successively partitioned with chloroform and *n*-butanol respectively. The *n*-butanol-saponin fraction was then analysed by TLC. TLC procedure was optimized using the eluents chloroform:methanol:water in varying ratios. It was observed that there were many compounds present in the fraction, although at different $R_f$ values. The fraction obtained when the mobile phase composition of chloroform:methanol:water was 65:5:10 and 85:10:5 gave the best TLC separation. The TLC plates were evaluated and the $R_f$ values determined. Only one of the compounds was reproducible by comparison with the standard $R_f$ values of 0.64. Similar fractions from the TLC plates were combined by scraping off and were analysed by spectroscopic methods for identification.

### B2.2.3.2 Chromatographic methods

#### B2.2.3.2.1 Thin-layer chromatography

TLC was performed on Silica gel 60 $F_{254}$ aluminium baked sheets of 20 cm by 20 cm (Merck). Detection was undertaken, using iodine crystals and anisaldehyde/sulphuric acid/ acetic acid (90:5:5 v/v).

#### B2.2.3.2.2 Column chromatography

The *n*-butanol fraction was subjected to column chromatography, using Silica gel 70-240 mesh. Chloroform ($CHCl_3$) was initially used as the eluting solvent. Polarity of the solvent was increased by adding methanol until a ratio of 50:50 was reached. Fractions were pooled according to TLC analysis.

### B2.2.3.3 Spectroscopy

#### B2.2.3.3.1 Infrared spectroscopy (IR)

IR is a technique using the interaction of substances with infrared electromagnetic radiation to identify different functional groups in an unknown substance. IR spectra were measured using a PerkinElmer Spectrum 100 series and attenuated total reflectance (ATR) as the sampling technique. Liquid isolates were used.

#### B2.2.3.3.2 High-Pressure Liquid Chromatography with UV detector analysis (HPLC-UV)

Reversed-phase HPLC analysis, using a Shimadzu liquid chromatograph with a Teknokroma nucleosil 100 C18 column (5µm x 25 mm x 4 mm) was used to detect conjugation in the isolate. Detection was conducted by means of a Prominence Diode Array detector, at room temperature. The mobile phase used for HPLC experiments was 10 per cent acetonitrile ($CH_3CN$) and water. The samples were injected, using a Prominence autosampler, and were monitored for 15 minutes, at flow rate of 1.0 ml/min.

#### B2.2.3.3.3 Gas chromatography mass spectrometry (GC-MS)

GC-MS is an analytical technique used to separate the ions according to mass/charge (m/z). Ionization of the molecules by high energy electrons in an electric field is used to determine molecular weight. GC-MS was run on an Agilent 6890 GC system, including an HP 5973 MS instrument, using a J & W HP5-MS fused silica capillary column (30 m x 0.25 mm x 0.25 µm) under the following conditions:

- filament current: 4.2 A;
- column temperature: 180/260° C;
- programmed increase: 5° C/min;
- carrier gas: He;
- head pressure: 12 psi;
- EI-MS: 70 eV;

- ion source temperature: 250° C.

Silica gel (200-300 mesh and 10-40 μm), RP-18 (40-63 μm) and Sephadex LH-20 were used for column chromatography.

### B2.2.3.3.4 Nuclear magnetic resonance (NMR)

NMR is a method which has been developed to determine molecular structure by absorption of radio waves in the presence of a strong magnetic field. NMR spectra were run on a Bruker AM-400 (for $^{1}H$ and $^{13}C$-NMR) instrument, with tetramethylsilane (TMS) as internal standard.

# CHAPTER B2-3

# RESULTS

## B2.3.1 *Introduction*

In this chapter, the results for the isolation, partial purification and characterization of saponin B1 from Amadumbe are presented.

## B2.3.2 Extraction and purification of saponin

The crude ethanolic extract of Amadumbe was partitioned between chloroform and *n*-butanol. The *n*-butanol fraction was analysed by TLC. Figure B2-3.1 shows the separated spots.

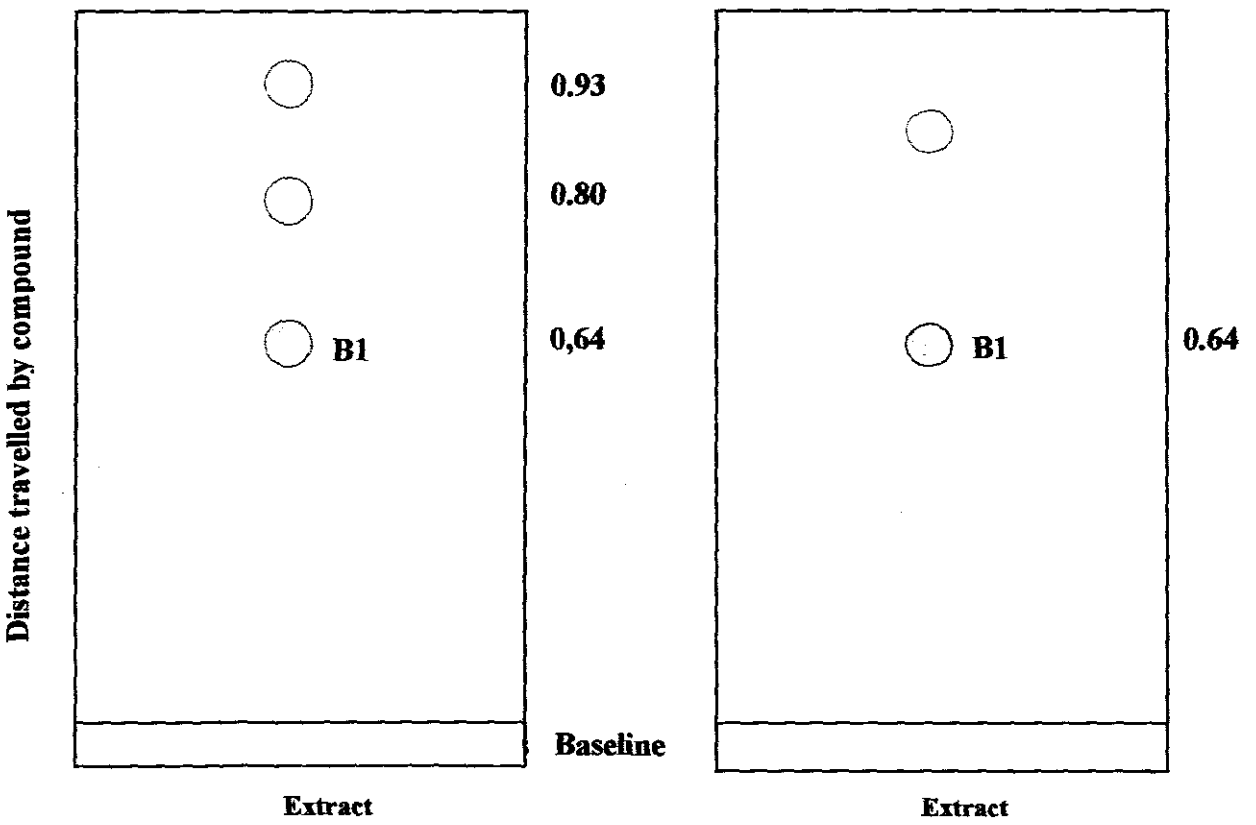



**Figure B2-3.1: Thin-layer chromatographic examination of saponin compounds from an Amadumbe (*Colocasia esculenta*) tuber**

Aliquots of 10 µl from crude extract were chromatographed on a gel 60 $F_{254}$ plate in the solvent system $CHCl_3$:MeOH:$H_2O$ [65:35:10]. The spots were located by means of the standard references. Comparison of $R_f$ value makes it possible to research complex mixtures qualitatively (Fernand 2003). The compounds with an $R_f$ value of 0.64 (saponin B1) were scraped off and dispersed in chloroform.

The combined saponin B1 was identified using IR and GC-MS. The spectra were compared with the spectra available in the library of the computerized GC-MS.

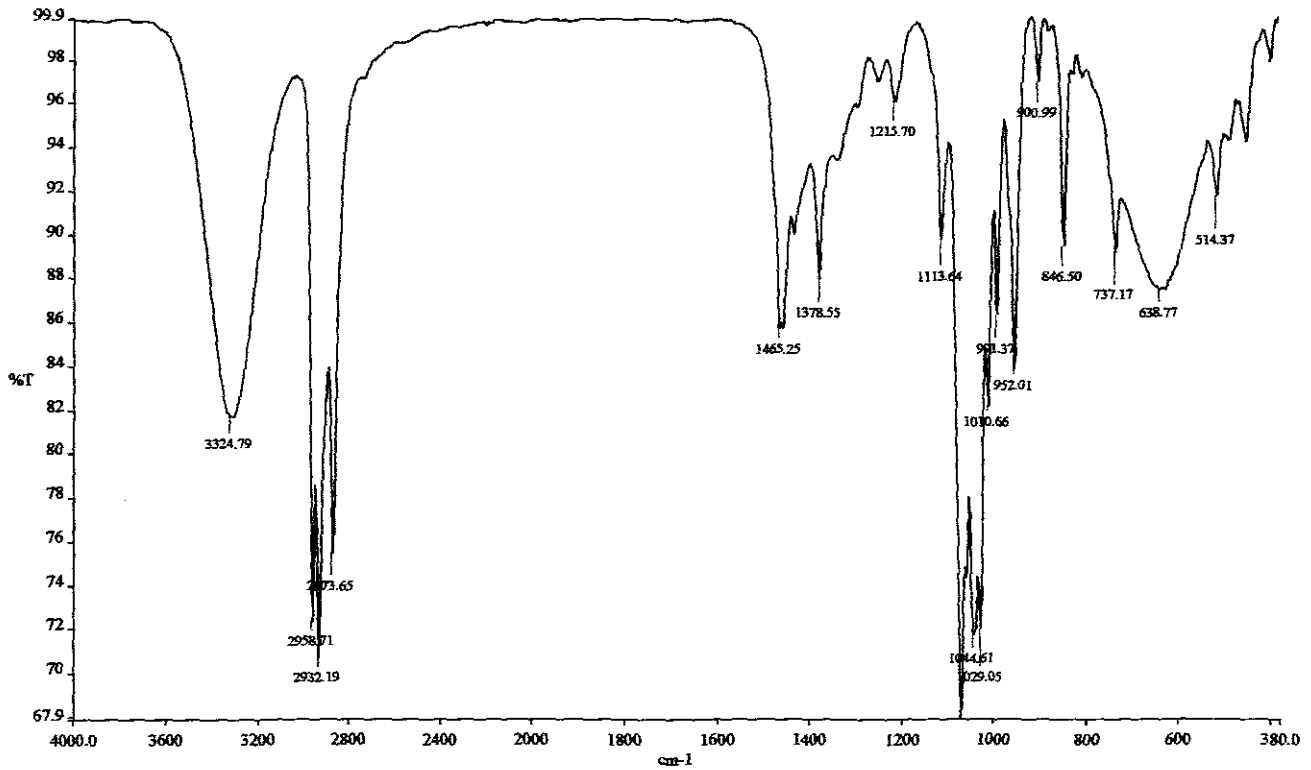



**Figure B2-3.2: Infrared spectrum of saponin B1 fraction of white *Colocasia esculenta*** *(Measurements were carried out at 27° C)*

The IR spectrum showed a peak at 3324 $cm^{-1}$ (OH), 2940 (C-H), an ether linkage 1000-1100 $cm^{-1}$, 2873.65 – 2932.19 $cm^{-1}$ (C=H stretching) and 1465.25 (C=C stretching). This spectrum suggests that there are functional groups present in the compound saponin B1.

The GC-MS of saponin B1 (Figure B2-3.3) showed a molecular-ion peak with a retention time of 22.54, which indicates the molecular formula $C_{29}H_{50}O$.

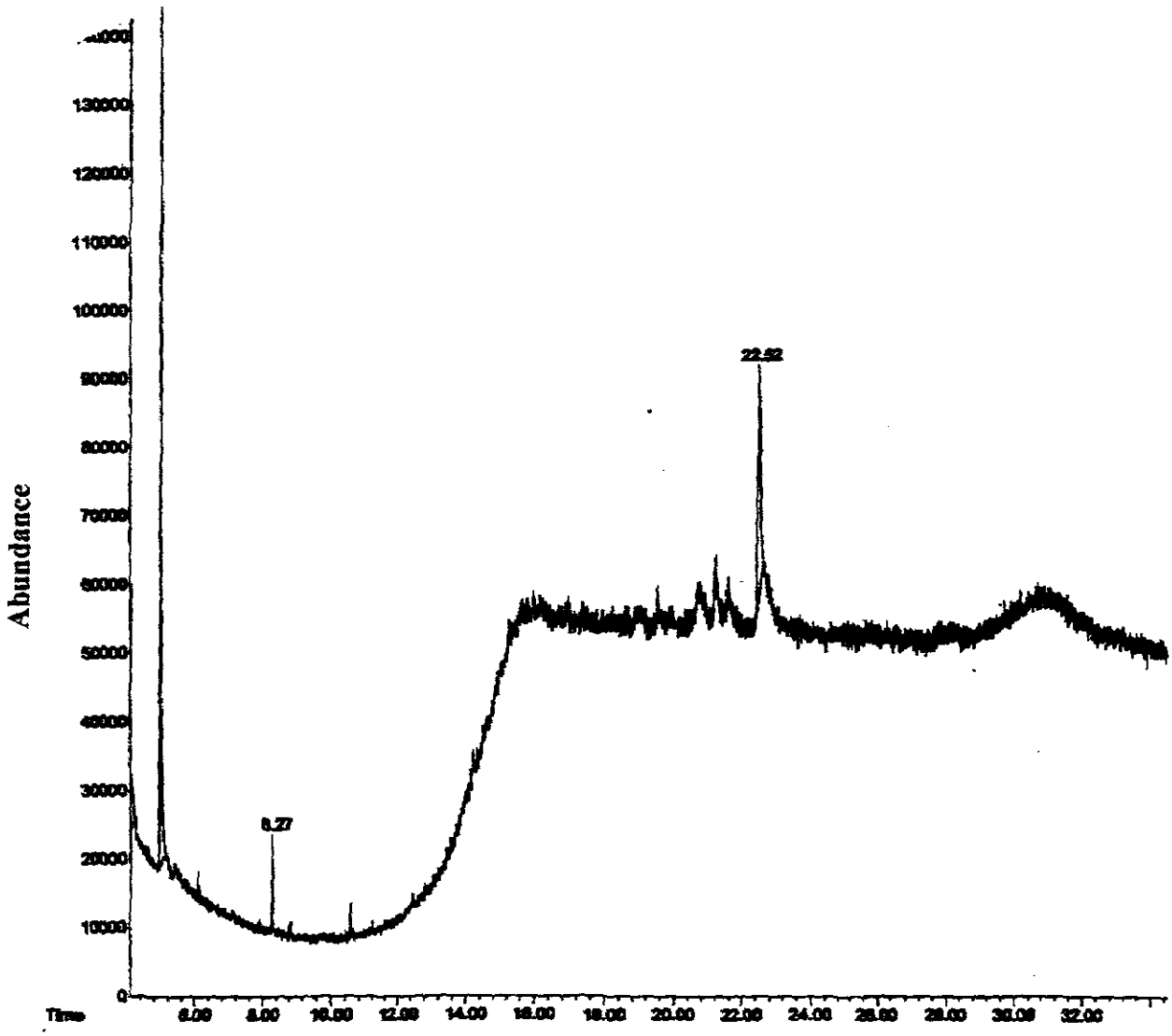



**Figure B2-3.3: Chromatogram of saponin B1 from Amadumbe anaylsed by GC-MS**

GC-MS was used to identify the isolated compound. The GC-MS spectrum showed a molecular-ion peak at 414 m/z, indicating a molecular weight of 414 dalton.

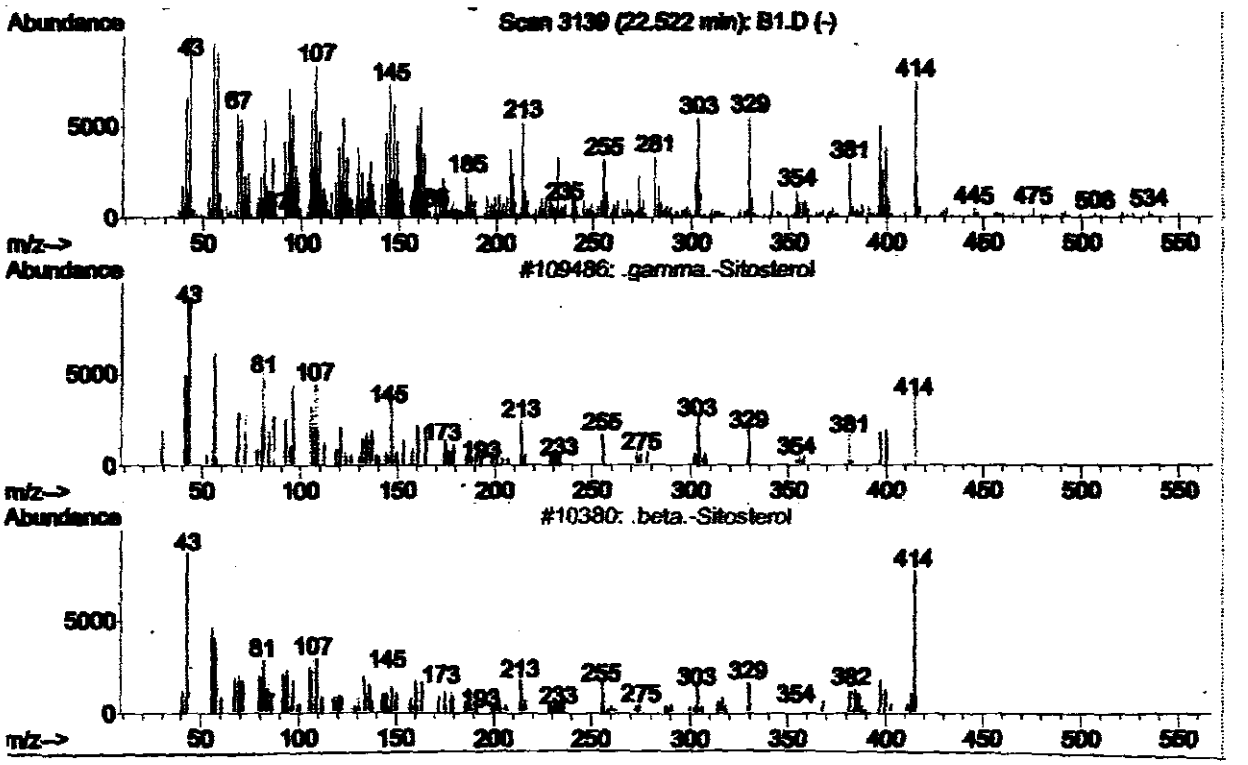



**Figure B2-3.4: Gas Chromatographic profile of saponin B1 analysed by GC-MS**

Identification of components was confirmed by comparison of collected mass spectra with those of authenticated standards and spectra from the National Institute for Standards and Technology (NIST) mass spectral library, Search Version 2.0.

The GC-MS spectra obtained for the isolated compound gives two suggestions as gamma-sitosterol and beta-sitosterol. The gamma-sitosterol gave a 98% match while beta-sitosterol gave 80% match. The fragmentation pattern (Figure B2-3.5) confirmed the presence of a sitosterol type skeleton. The IR spectrum also verifies all the functional group peaks identified in the spectra. Thus, gamma-sitosterol was assumed to be the compound.

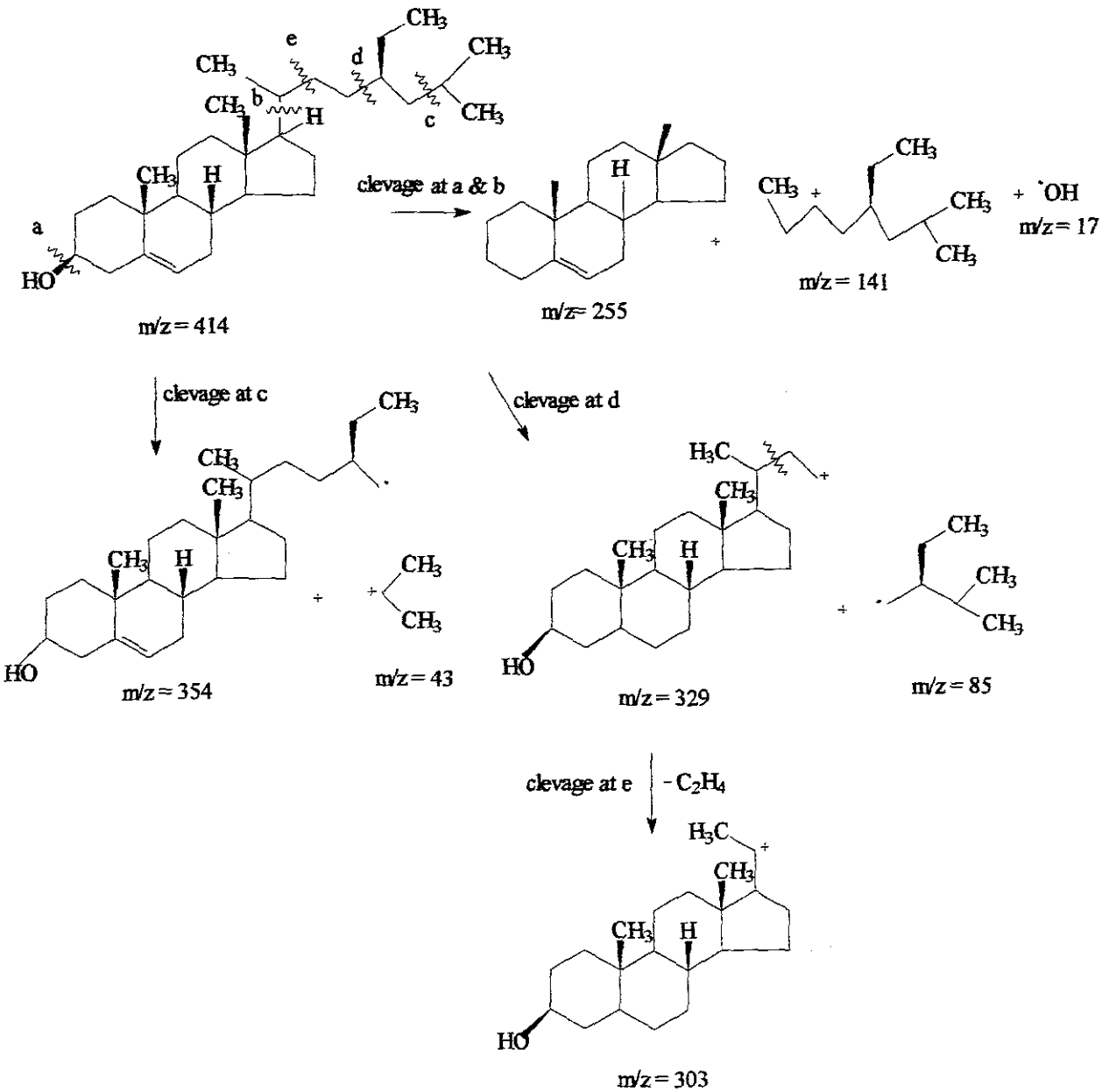



**Figure B2-3.5: Major fragmentation pattern of sitosterol irrespective of the isomeric form.**

# CHAPTER B2-4

# DISCUSSION

## B2.4.1 Introduction

In this chapter, the results for the isolation, partial purification and characterization of saponin B1 from Amadumbe are discussed.

## B2.4.2 Saponin

The isolated saponin B1 present in Amadumbe tubers was identified using TLC, IR and GC-MS techniques. The $R_f$ value was calculated (0.64) to give a tentative identification of a sterol. $R_f$ value of compound B1 was similar to $R_f$ value (0.65) of 5α-cholestan-3β-ol, the basic sterol from which other sterol structures are defined (Smith and Goad, 1975). The elucidation of this saponin was further identified by means of IR and GC-MS. Several researchers have investigated steroids in detail using GC-MS (Budzikiewicz *et al.*, 1964). Sterol fragmentation patterns are quite complex and it is possible that the typical fragmentation pattern of a specific structural unit appears as one set of sterol type and not another (Wretensjö, 2004). The molecular formula of compound B1 was $C_{29}H_{50}O$, based on the retention time of 22.54 (Tai *et al*,2004). The GC-MS spectrum showed a molecular-ion peak at 414 m/z, indicating a molecular weight of 414 (Phuruengrat and Phaisansuthichol, 2006).

Vibrations of functional groups of this compound were detected by infrared. The IR spectrum of compound B1 showed a band of $V_{max}$ 3324 $cm^{-1}$, which indicated the presence of –OH group without H-bonding. Furthermore, there were additional features in the spectra that identified the position and configuration of C-H and C=C stretching. Taking all the above-mentioned facts into consideration, the conclusion drawn was that

the phytosterol had a hydroxyl group at $C_3$ and a side chain originating at $C_{17}$. The numbering of carbon atoms in the sterol structure is shown in Figure B2-4.1.

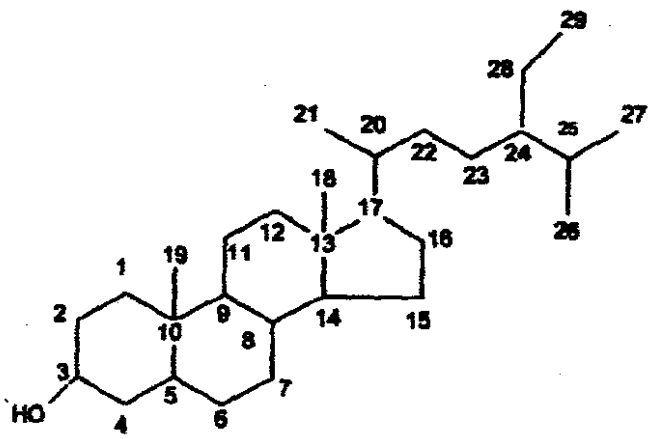
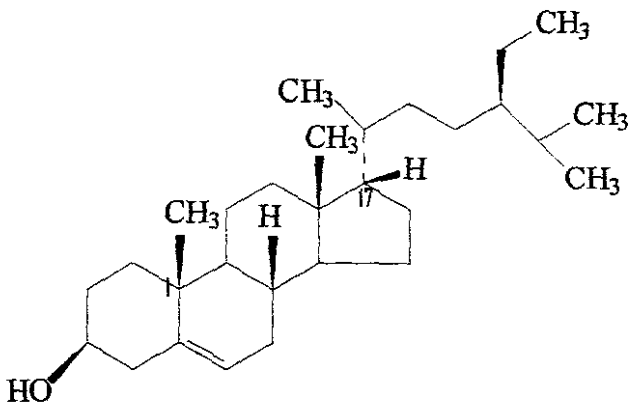



**Figure B2-4.1: Numbering of sterol carbons** (Wretensjö, 2004)

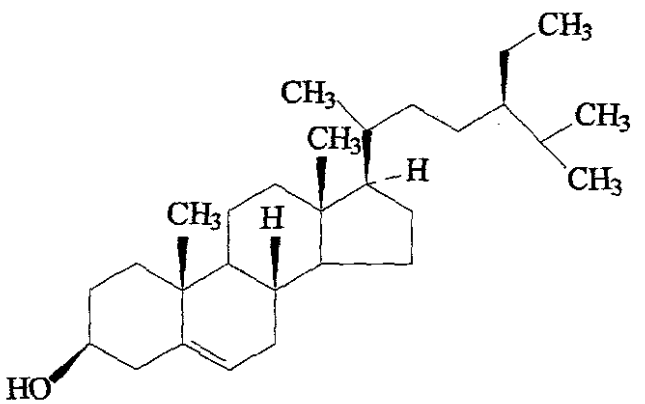
Thus, based on the above spectral data and chemical evidence, the structure of saponin B1 was recognized as gamma-sitosterol (Figure B2-4.2). It has four ring structures, a hydroxyl group at $C_3$ and a side chain originating at $C_{17}$ of the steroid skeleton. HPLC analysis was carried out, but did not show any conjugation.




γ - Sitosterol

**Figure B2-4.2: The structural formula of gamma-sitosterol**

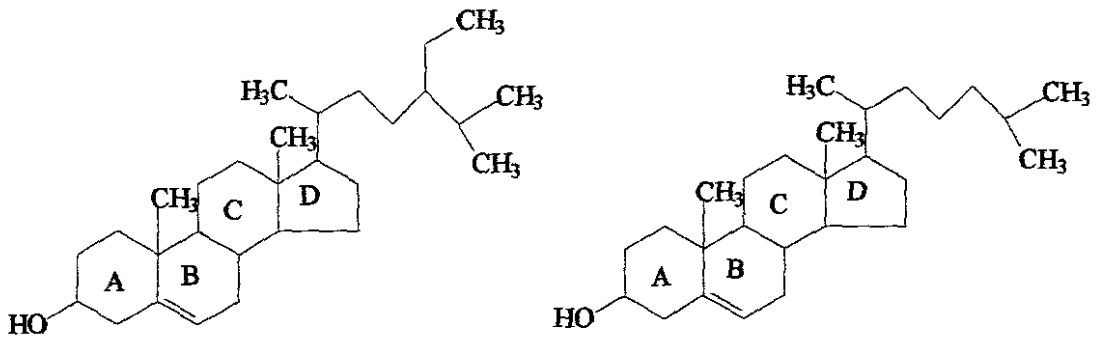
Sitosterols are the most extensively studied members of phytosterols. Sterols such as beta-sitosterol and stigmasterol are the predominant ones in taro (Durward *et al* 1997). Their role in taro is not yet known (Osagie 1977). Gamma-sitosterol is a stereoisomer of beta-sitosterol and differs only in the spatial configuration of the $C_{17}$ side chain (Figure B2-4.3) (Best *et al.*, 1954). Because gamma-sitosterol is an isomer of beta-sitosterol and beta-sitosterol is the predominant sterol in taro; it could be assumed that beta-sitosterol is present in Amadumbe.




β -Sitosterol

**Figure B2-4.3: The structural formula of beta-sitosterol**

Plant sitosterols are structurally related to cholesterol and differ in their chemical structure only because of an ethyl group present at the $C_{24}$ position of the side chain (Figure B2-4.4). Gamma- and beta-sitosterols differ from cholesterol in that they are not appreciably absorbed from the digestive tract (Best *et al.*, 1954).




Sitosterol ($C_{29}H_{49}OH$) Cholesterol ($C_{27}H_{45}OH$)

**Figure B2-4.4: The structural formula of gamma- and beta-sitosterol differs from that of cholesterol only in the presence of an ethyl group on carbon 24**

Various reports suggested that plasma total and LDL cholesterol levels in animals (Pollak and Kritchevsky, 1981; Moghadasian *et al.*, 1997) and humans (Pelletier *et al.*, 1995; Gylling *et al.*, 1995; Jones *et al.*, 1997) can be lowered by consumption of unsaturated phytosterol mixtures rich in beta-sitosterol. Circulating cholesterol concentrations may be lowered by phytosterols and stanols in a number of ways:

- by direct competitive blocking of intestinal cholesterol absorption (Miettinen and Vanhanen, 1994);
- by displacing cholesterol from bile salt micelles (Child and Kuksis, 1986);
- by increasing bile salt excretion (Salen *et al*, 1970); or
- by hindering cholesterol esterification rate in the intestinal mucosa (Ikeda and Sugano, 1983).

Investigations show that beta-sitosterol is one of those remarkable plant nutrients which are an important, non-toxic nutrient, essential for the maintenance of health and protection against several serious health disorders and diseases.

Gamma- and possibly beta-sitosterol present in Amadumbe tubers can increase the tubers' nutritional value because the sitosterols have many health-promoting biological activities. The phytosterol content of vegetables and fruits is not affected by intense processes such as boiling, neutralization, bleaching and deodorization because phytosterols are very stable (Normen *et al.*, 1999; Bortolomeazzi *et al.*, 2000). Therefore, processing will not decrease the nutritional value of the tuber.

# CHAPTER B-5

# GENERAL CONCLUSION

## B.5.1 α-Amylase inhibitor

Two active forms of the inhibitor, AI-A1 and AI-B2, were detected during the purification procedures. The apparent molecular weights, based on gel chromatography on Sephadex G-100, were approximately 17 kDa and 19 kDa respectively.

The optimum pH and temperature for the α-amylase inhibitors were pH 6 and 40° C respectively, but the enzyme activity was significantly destroyed at pH less than four and more than eight, as well as above a temperature of 70° C.

The results obtained for the studied α-amylase inhibitors present in Amadumbe are in agreement with the inhibitors studied for other tubers. The Amadumbe amylase inhibitors may not be of much nutritional relevance, since cooking was found to decrease their biological activity.

## B.5.2 Saponin

The isolated compound characterized by TLC, IR and GC-MS was found to be gamma-sitosterol.

The objective of the next section was to conduct a nutritional evaluation using one of the isolated anti-nutritional factors. Beta-sitosterol has many biological activities and is an isomer of gamma-sitosterol. Therefore, it was decided to conduct the nutritional evaluation using gamma-sitosterol. Gamma-sitosterol was not commercially available,

whereas beta-sitosterol was. It was then decided to substitute gamma-sitosterol for beta-sitosterol in the nutritional evaluation.

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# SECTION C

## EFFECTS OF INGESTED BETA-SITOSTEROL ON DIGESTIVE AND ABSORPTIVE ENZYMES IN RATS

## SUMMARY

The purpose of this study was to investigate the effect of beta-sitosterol on intestinal disaccharidases and $Na^+/K^+$-ATPase activities in Sprague-Dawley rats. During the test period, food and water consumption, body weights and clinical signs were examined. Haematological changes included elevated alanine aminotransaminase (ALT) and aspartate aminotransaminase (AST) levels. Histopathology revealed that the beta-sitosterol had no apparent effect on the liver, kidneys or small intestine. The supplemented diet reduced $Na^+/K^+$-ATPase and intestinal disaccharidases activities.

# CHAPTER C-1

# LITERATURE REVIEW

## C.1.1 Introduction

Plants are the only producers of phytosterols, which are cholesterol-like chemicals. The most common phytosterols are beta-sitosterol, campesterol and stigmasterol (Ling and Jones, 1995). Phytosterols are known for their ability to lower plasma cholesterol and there have been many studies reporting their hypocholesterolaemic effects (reviewed by Ling and Jones, 1995; Pollak and Kritchevsky, 1981). Epidemiological studies have also proposed that increased dietary phytosterol intake may decrease the risk of colon cancer in humans (Hirai *et al.*, 1986; Nair *et al.*, 1984).

In the previous sections, Amadumbe tubers were screened for the presence of anti-nutrients. From the screened anti-nutrients, α-amylase inhibitor and saponin were extracted, isolated and identified. The saponin was structurally elucidated as gamma-sitosterol. Beta-sitosterol is a structural isomer of gamma-sitosterol and is a compound with many biological activities. Therefore, gamma-sitosterol would be the perfect anti-nutrient compound for nutritional evaluation. Gamma-sitosterol was not commercially available and, because beta-sitosterol is the stereoisomer, it was decided to use beta-sitosterol for the nutritional evaluation. A large body of research on the cholesterol-lowering activity of beta-sitosterol has already been conducted; thus, the effects of beta-sitosterol on digestive and absorptive enzymes were investigated in this study.

## C.1.2.1 Stereochemical structure

Sterols are necessary cell membrane components and are produced by both animals and plants. Plant cells consist of a number of complex mixtures of sterols, whereas animals

and fungal cells contain one major sterol: cholesterol and ergosterol, respectively. More than 100 different types of phytosterols (plant sterols) have been identified (Moreau *et al.*, 2002). Seeds, nuts, fruits and vegetable oils are some of the different parts of a plant that contain significant amounts of plant sterols (Weihrauch and Gardner, 1978; Moreau *et al.*, 2001). The phytosterol content of these vegetables and fruits is not influenced by intense processes such as boiling, bleaching and deodorizing since phytosterols are very stable compounds (Normen *et al.*, 1999; Bortolomeazzi, 2000). The human body is unable to produce plant sterols: thus, all plant sterols are regulated by dietary intake. Beta -sitosterol, stigmasterol and campesterol are the most abundant in plants (Rosenblum *et al.*, 1993; Law 2000; Hicks and Moreau, 2001).

### C.1.2.2 Pharmacochemistry and pharmacokinetics

Researchers have been fascinated by the concept of a protein-mediated transport system responsible for intestinal uptake of cholesterol for more than a decade (Thurnhofer and Hauser, 1990). Identification of the molecular defects responsible for phytosterolemia (Berge *et al.*, 2000; Lee *et al.*, 2001) and possible confirmation of the existence of a specific transport protein located in the brush border membrane mediating intestinal sterol absorption (Detmers *et al.*, 2000; Hernandez *et al.*, 2000; Kramer *et al.*, 2000) has shed new light on the cellular transport of cholesterol and plant sterols. Plasma phytosterol levels are generally very low compared to cholesterol levels in mammalian tissues. This is due primarily to poor absorption from the intestine and faster excretion from the liver (Ling and Jones, 1995). Absorption of beta-sitosterol is also made difficult during its passage through the gut because these phytosterols are bound to the fibers of the plant (Bouic, 1998).

The plant sterol mixture has very low solubility (Moghadasian, 2000): it is not soluble in either water or oil (Kim *et al.*, 2002). About five per cent of an ingested dose of supplemental beta-sitosterol is absorbed from the gastrointestinal tract and is transported via the portal circulation to the liver (Salen *et al.*, 1970). Systemic circulation is

responsible for transport of beta-sitosterol to other tissues in the body. Some beta-sitosterol is glucoronidated in the liver and a portion is also metabolized to cholic acid and chenodeoxycholic acid (Marteau *et al.*, 1980). Excretion is mainly via the biliary route.

In an attempt to improve sterol solubility, plant sterols have been esterified with fatty acids in order to produce oil-soluble plant sterol esters (Mattson, 1964, U.S. Patent No. 5,502,045).

### C.1.2.3 Biological activities

Beta-sitosterol offers a remarkable display of scientifically recognized benefits for major areas of health. Health benefits include:

- lowering cholesterol (Farquhar *et al.*, 1956; Lees *et al*, 1977);
- anti-diabetic properties (Ivorra, 1988);
- anti-tumor activities (Romero and Lichtenberger, 1990; Janezic and Rao, 1992);
- anti-bacterial properties (Hess *et al.*, 1995; Padmaja *et al.*, 1993);
- anti-fungal abilities (Smania *et al.*, 2003);
- anti-inflammatory predilection (Bouic *et al.*, 2001);
- anti-atherogenecity activities (Moghadasian *et al.*, 1997);
- anti-ulcer properties(Jayaraj *et al.*, 2003); and
- preventing cardiovascular events (Miettinen *et al.*, 1990; Gylling and Miettinen, 1997).

Human research already includes the treatment of hypercholesterolemia, benign prostatic hypertrophy (BPH) and rheumatoid arthritis with beta-sitosterol (Pegel, 1997). Success in these areas, particularly with regard to cardiovascular diseases, may be attributed to the cholesterol-lowering effects of plant sterols (Drexel *et al.*, 1981; Miettinen *et al.*, 2000).

Beta-sitosterol has been successfully used to lower cholesterol levels in humans, with almost no change in diet or exercise. Many scientific studies have shown that, because beta-sitosterol and cholesterol are very similar in chemical composition, beta-sitosteriol interferes with cholesterol absorption by inhibiting its absorption in the gut (Ling and Jones, 1995; Westrate and Meijer, 1998; Hallikainen and Uusitupa, 1999). This lowering of cholesterol helps to prevent the surplus rise in serum cholesterol from building up on the walls of the heart arteries.

Bouic *et al.* (1996) reported on a series of *in vivo* and *in vitro* studies which clearly demonstrate that beta-sitosterol has immunomodulatory properties. Beta-sitosterol both improves an under-performing immune system to help fight viral and other infections and also corrects the underlying immune dysfunction of an overactive immune system as in autoimmune disorders such as arthritis. Rheumatoid arthritis, for example, is an inflammatory disease characterized by dysregulation of the immune system. B-lymphocytes become overactive and secrete antibodies that destroy synovial tissue of the joint. Beta-sitosterol and beta-sitosterol glucoside in combination have been shown to increase the levels of T-Helper-1 ($TH_1$) cells, down-regulating antibody production by B-lymphocytes. The phytosterol mixture also decreased sections of pro-inflammatory cytokines by macrophages, thereby decreasing inflammation (Calpe-Berdiel *et al.*, 2007).

Sterols have been found to stimulate the body's immune system to fight the infections which disrupt the lives of HIV-positive patients (Bouic *et al.* 2001). Scientific reports have shown that CD4 lymphocyte counts can be maintained by beta-sitosterol and its glucoside, thereby slowing disease progression (Bouic *et al.*, 1996). The studies also revealed an important decrease in IL-6 levels, possibly slowing viral replication rates in infected cells and thereby decreasing viral loads (Bouic, 1997).

Studies show that beta-sitosterol is among the most successful treatments available for prostate enlargement (Buck *et al.*, 1996; Klippel *et al.*, 1997). The growth of human prostate cancer cells may be inhibited by beta-sitosterol: this phytosterol apparently

replaces some of the cholesterol in the cell membrane, which ultimately triggers the relaying of an instruction to the cells not to divide (Awad *et al.*, 2000).

Thus, dietary plant sterols also have possible anti-cancer effects. Beta-sitosterol may offer protection against breast cancer by stimulating apoptosis and by inhibiting tumor growth in cancer tissue (Awad and Fink, 2000). Epidemiological studies have shown positive association between higher dietary phytosterol intake and populations at lower risk for colonic cancer (Hirai *et al.*, 1986). Experimental studies by Raicht *et al.*, (1980) have shown that rats fed a diet containing 0.3 percent beta-sitosterol had a significantly lower incidence of tumours when given methylnitrosourea (MNU), a chemical carcinogen, than those on diets without beta-sitosterol supplement.

The toxic potential of a test article may be considerably affected by several factors such as administration route, dosing form and the experimental conditions (Dain and Jaffe, 1988; Dethloff *et al.*, 1996; Kim *et al.*, 2001). With regard to toxicity, no obvious side effects of phytosterols have been observed in studies to date, except in individual with phytosterolemia, an inherited lipid disorder (Ling and Jones, 1995). Beta-sitosterol has been fed to rats in dosages of between 0.5 and 5 mg $kg^{-1}$ (Young *et al.*, 2004; Malini and Vanithakumari, 1990, 1992). Kim *et al* (2002) investigated subchronic toxicity of plant sterol ester by administering 1000, 3000 and 9000 mg $kg^{-1}day^{-1}$ to rats . Based on their results, 9000 mg $kg^{-1}day^{-1}$ was considered to be the toxic dose since it resulted in the suppression of body weight gain in both sexes and male cardiomyopathy.

### C.1.3 Digestive enzymes

#### C1.3.1 Disaccharidases

The nutritional building blocks derived from digestion are used by metabolic enzymes in every cell, tissue and organ of the body to restore damage and decay, to combat and overcome disease, to repair wounds and to maintain overall health and well-being.

Digestive enzymes do the all important work of hydrolyzing food into the nutritional components required to build and maintain health.

Disaccharides (sucrose, lactose and maltose) are important sources of metabolic energy (Dilworth *et al.* 2005). Disaccharidases are integral enzymes of the small intestinal brush border membrane responsible for the hydrolysis of carbohydrates which must occur before monosaccharides can be absorbed (Lorenzsonn *et al.*, 1987; He *et al.*, 2006). These enzymes are sucrase (EC 3.2.1.26), lactase (EC 3.2.1.23) and maltase (EC 3.2.1.20) respectively. The end-products (glucose and fructose, galactose) resulting from the activities of these enzymes are actively translocated from the intestine to the blood by ATPases (Dilworth *et al.*, 2005).

Although the factors controlling the development of intestinal disaccharidases are not known (Herzenberg and Herzenberg, 1959), it appears that the intestinal dissacharidases of mammals develop in a way that allows the utilization of the carbohydrates that the animal is likely to encounter under natural feeding conditions (Siddons, 1969). Thus, dissacharidase activities appear to be broadly correlated to dietary habits (Vonk and Western, 1985). Food influences the brush border enzyme activity in rats and is also necessary for the maintenance of normal mucosal stability (Yamada *et al.*, 1981; Holt and Yeh, 1992). Thus, the activities of specific enzymes are reduced when their substrates are sporadic or absent from an animal's diets (Zarling and Mobarhan, 1987; Rivera-Sagredo *et al.*, 1992).

On the other hand, studies suggest that carbohydrate intake increase dissacharidase activities (Shinohara *et al.*, 1986; Samulitis-Dos *et al.*, 1992). The overall digestive capacity of an animal is sensitive to changes in the concentrations of intestinal enzymes. These changes may affect the nutritional status of the animal, as well as the functioning of other systems, including neurological, renal, cardiac and pulmonary systems (Lee *et al.*, 2003).

Intestinal disease may be determined by the activities of disaccharidases. Greater enzyme activity does not have any known clinical significance, but decreased enzyme activity results in a digestive defect of disaccharide, which, clinically, may result in osmotic diarrhea, crampy abdominal pain or gaseousness (He *et al*, 2006).

### C.1.3.2 ATPase

ATPases are a group of enzymes that catalyze the hydrolysis of adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and a free phosphate ion (Figure C1-1). Energy is released from this dephosphorylation reaction and is used to drive other chemical reactions.

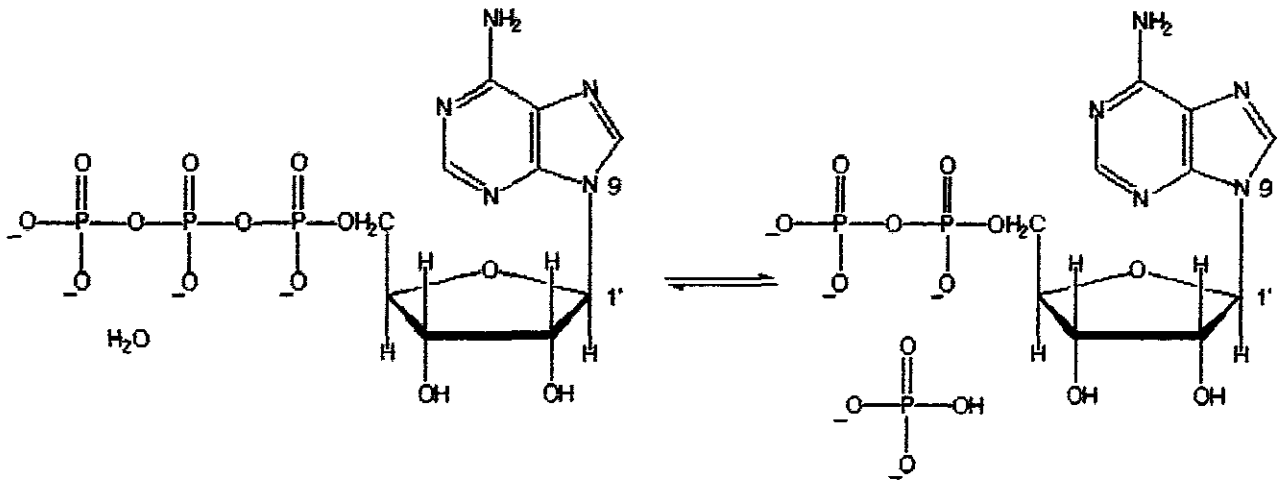

**Figure C1-1: Hydrolysis of ATP to ADP, the fundamental mode of energy exchange in biological systems** (Moyna, 1999)

Some ATPases are basic membrane proteins and transport solutes across the membrane and are therefore called transmembrane ATPases. Sodium-potassium exchanger is a primary example of a transmembrane ATPase which establishes the ionic concentration balance that maintains the cell potential (Figure C1-2) [Skou, 1957]. Sodium-potassium ATPase ($Na^+/K^+$- ATPase) is a wide-spread membrane enzyme which uses the hydrolysis of ATP to regulate cellular $Na^+$ and $K^+$ levels and fluid volume. Sodium-

potassium ATPase creates concentration gradients of sodium and potassium ions across the plasma membrane of animal cells. The gradients provide the source of energy for membrane transport of substances. Ion gradients play an essential part in the flow of metabolites: for example, the basic glucose transport in intestinal enterocytes. Prolonged flow will dissipate these gradients, making them less efficient. Thus, it is essential to rebuild these gradients.

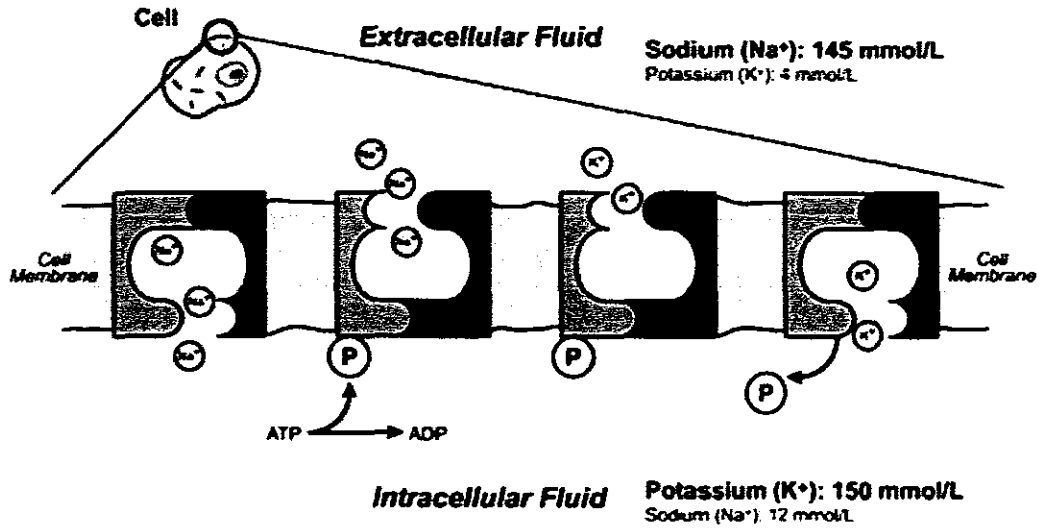


**Figure C1-2: The sodium-potassium exchanger, which establishes the ionic concentration balance that maintains the cell potential.** [(Oregon State University: Micronutirent Information Centre (2008)]

ATPase is responsible for generating and maintaining transmembrane ionic gradients that are of vital importance for cellular function and adjuvant activities such as pH maintenance, volume regulation and creation of action potentials and secondary active transport (Mobasheri *et al.*, 2004). Organs that transport a great deal of sodium and potassium can be used as sources for experimental work regarding this enzyme. The outer medulla of the mammalian kidney is an excellent source: more than 45 per cent of energy usage of kidney and brain is consumed by $Na^+$, $K^+$-ATPase (Cornelius, 1996).

## C.1.4 Aim and outline of Section C

The administration of a chemical compound may bring about significant changes in the structure, function, metabolic transformation and concentration of biomolecules, enzymes and metabolic pathways. These changes, be they rapid or slow, may lead to diverse biochemical mechanisms producing comparable pathological, clinical and laboratory findings (Murray *et al.*, 2000).

Extensive studies of the hypocholesterolaemic results (Lees *et al.*, 1977; Richter *et al.*, 1996; Vanstone *et al.*, 2001) of beta-sitosterol have been carried out, but the effect of this compound on digestive enzymes has, to the best of the author's knowledge, not been studied previously.

The objective of Section C was to determine the effect of dietary beta-sitosterol on the digestive and absorptive enzymes of rats. To achieve this:

- beta-sitosterol, esterified to oleic acid, would be fed to rats through stomach pumps;
- the rats would be sacrificed and the intestinal disaccharidases (sucrase, maltase and lactase) and ATPase activities monitored;
- organs (liver, kidneys and small intestines) would be histologically examined;
- the animals' blood would be tested for total glucose, total proteins, triacylglycerols, total cholesterol, liver enzymes and blood-cell counts.

# CHAPTER C-2

# MATERIALS AND METHODS

## C.2.1 Introduction

Two types of experiments were conducted for the nutritional evaluation. A six-day, short-term, preliminary toxicity test was used to determine the dietary concentration of beta-sitosterol for the two-week experimental test. This chapter explains the experimental design, with materials and methods for each of the parameters tested.

## C.2.2 Materials and methods

| | |
|---|---|
| Beta-sitosterol | Sigma |
| Oleic acid | Sigma |
| Sodium caseinate | Sigma |
| Glucose (GO) Assay Kit | Sigma |

### C.2.2.1 Ethical consideration

The experimental protocol was performed according to national and international guidelines. The animal experiment was approved by the Ethics Committee of the Faculty of Science and Agriculture of the University of Zululand, KZN, South Africa. (See Appendix C).

### C.2.2.2 Solubilisation of beta-sitosterol

Beta-sitosterol was solubilised by esterification in the presence of sodium caseinate solution (US Patent 6491952). Beta-sitosterol was administered as a suspension in oleic acid (Sjoberg, 2002).

### C.2.2.3 Preliminary toxicity test

#### C.2.2.3.1 Animals

Only male, inbred, Sprague-Dawley rats, weighing between 200 and 250 g were used in this section of the study. The animals were randomly assigned to five groups, two per cage. The animals were housed in solid-bottom plastic cages, with wood shavings for bedding. They were kept in a well-ventilated room, with the temperature ranging between 25 and 28° C and the humidity between 50 and 55 per cent.

#### C.2.2.3.2 Diet

All the animals were maintained on a stock, pelleted diet, with food and water available constantly. The preliminary toxicity test-group animals were fed by means of a stomach pump with 10, 5 and 2.5 mg $kg^{-1}day^{-1}$ of beta-sitosterol (Table C2-1). The two control groups received oleic acid and distilled water respectively. Because it is a clinically-intended route for the test article, oral administration was selected for the present study.

**Table C2-1: Composition of administered materials for preliminary toxicity test groups**

| | Group | Composition of administered materials[1] | | |
|---|---|---|---|---|
| | | **Oleic acid (w/v)*** | **Beta-sitosterol (w/v)** | **Distilled water** |
| **A** | Distilled water | - | - | 1 ml |
| **B** | Oleic acid | 0.03 mg/ml | - | - |
| **C** | Beta-sitosterol (10 mg $kg^{-1}day^{-1}$) | 0.03 mg/ml | 2.5 mg/ml | - |
| **D** | Beta-sitosterol (5 mg $kg^{-1}day^{-1}$) | 0.03 mg/ml | 1.25 mg/ml | - |
| **E** | Beta-sitosterol (2.5 mg $kg^{-1}day^{-1}$) | 0.03 mg/ml | 0.63 mg/ml | - |

[1] 1 ml

* *oleic acid was added to a three per cent sodium caseinate solution.*

### C.2.2.3.3 Experimental design

All animals were acclimatized for five days under standard laboratory conditions. There were two rats in each group for the short-term preliminary toxicity test (Groups A-E). The animals were given their respective diets for six days: Group A received 10 mg $kg^{-1}day^{-1}$ of beta-sitosterol; Group B received 5 mg $kg^{-1}day^{-1}$ of beta-sitosterol; and Group C received 2.5 mg $kg^{-1}day^{-1}$ of beta-sitosterol. Group D received the equivalent amount of oleic acid and Group E received distilled water, both groups performing as controls. The body weight, food and water consumption of each rat were monitored daily. All animals were observed for clinical signs of toxicity and mortality throughout the study period.

## C.2.2.4 Experimental test

### C.2.2.4.1 Experimental design

The animals were selected and housed as described in Section C2.2.3. They were fed a standard, balanced diet of pellets and water was made available *ad libitum*. Animals were divided into three groups and received their respective supplemented diets for two weeks: Group A received 10 mg $kg^{-1}day^{-1}$ of beta-sitosterol; Group B received distilled water as a control; and Group C received the equivalent amount of oleic acid as a control. Body weight, food and water consumption of each rat were measured daily. All animals were observed for clinical signs of toxicity throughout the study period. After two weeks, the animals were sacrificed by a quick blow to the back of the neck, after overnight food deprivation.

### C.2.2.4.2 Haematology

The animals were fasted overnight, prior to necropsy and blood collection. Blood samples were collected in ethylene diamine tetra acetic acid (EDTA) tubes by cardiac puncture, for haematological testing and in serum separator tubes (SST) for serum

biochemistry (samples were allowed to clot before centrifuging). Collected blood samples were sent to Ampath Pathology Laboratory in Richards Bay, South Africa.

Serum samples were obtained by centrifuging the clotted blood samples at 3500 rpm for five minutes (Ojiako and Nwanjo, 2006). The sera of each group were analysed for aspartate aminotransferase (AST), alanine aminotransaminase (ALT), alkaline phosphatase (ALP), glucose, triglyceride (TG), protein and cholesterol, using a Roche Modular chemistry analyser. White blood cell (WBC) and red blood cell (RBC) counts of the EDTA-treated blood were determined by the standard specified method for using an ADVIA 120/2120.

#### C.2.2.4.3 Histology

Kidneys, lungs and intestines were collected and placed in 10 per cent neutral buffered formalin for histopathological evaluation. Fixation of tissues was then routinely processed, embedded in paraffin wax and sectioned at 5 µm, according to standard histological procedures. Sections were stained with haematoxylin and eosin (HE) for routine light microscopical histological examinations.

### C.2.2.5 Measurement of enzyme activities

#### C.2.2.5.1 Disaccharidases

Brush border maltase, lactase and sucrase activities in the small intestine were determined, using the Dahlqvist method (1968). Disaccharidase activity was assayed by measuring glucose liberated through respective disaccharide hydrolysis, using a glucose oxidase-peroxide system. One unit of enzyme activity is defined as the amount of enzyme hydrolyzing 1 µmol of the disaccharide in one minute at 30° C.

### C.2.2.5.2 $Na^+/K^+$-ATPase

$Na^+/K^+$-ATPase activity was determined through ouabain-sensitive ATP hydrolysis in intestinal samples (Vásárhelyi *et al.*, 1986). $Na^+/K^+$-ATPase activity was measured by determination of the inorganic phosphate liberated through the hydrolysis of the substrate adenosine triphosphate at 37° C. One unit of activity was defined as the amount of enzyme that catalyses the reaction of 1 µmol of ATP per minute.

### C.2.2.6 Statistical analysis

Data was analysed using the statistical programme Genstat ® (Lane and Payne, 2006). A one-way analysis of variance (ANOVA) as well as two sample t-tests were conducted and least significant differences (LSD) at the 5% level, were used to determine significant differences between treatment means.

# CHAPTER C-3

# RESULTS

## C.3.1 Introduction

Beta-sitosterol is present in a wide variety of plants (and therefore in most diets) and possesses a wide spectrum of biological activities. This chapter presents the results, following administration of beta-sitosterol to Sprague-Dawley rats.

## C.3.2 Preliminary toxicity test

### C.3.2.1 Food and water consumption

Table C3-1 shows the amount of food and water taken in by the rats in the six-day period. Food and water consumption did not appear to differ markedly in any group treated with beta-sitosterol when it was compared with the control groups over the six-day period.

**Table C3-1: Average food and water consumption in rats over a six-day period in preliminary studies**

| Group | Diet | Average food intake[1] | Average water intake[2] |
|---|---|---|---|
| Control A | Distilled water | 13.8 g | 20.6 ml |
| Control B | Oleic acid | 17.1 g | 23.9 ml |
| C | β-sitosterol (10 mg $kg^{-1}day^{-1}$) | 13.8 g | 20.1 ml |
| D | β-sitosterol (5 mg $kg^{-1}day^{-1}$) | 12.2 g | 17.0 ml |
| E | β-sitosterol (2.5 mg $kg^{-1}day^{-1}$) | 11.9 g | 19.5 ml |

[1] g/day/rat

[2] ml/day/rat

## C.3.2.2 Body weight changes

The animals were weighed every day and the average weight for each group was plotted (Figure C3-1).

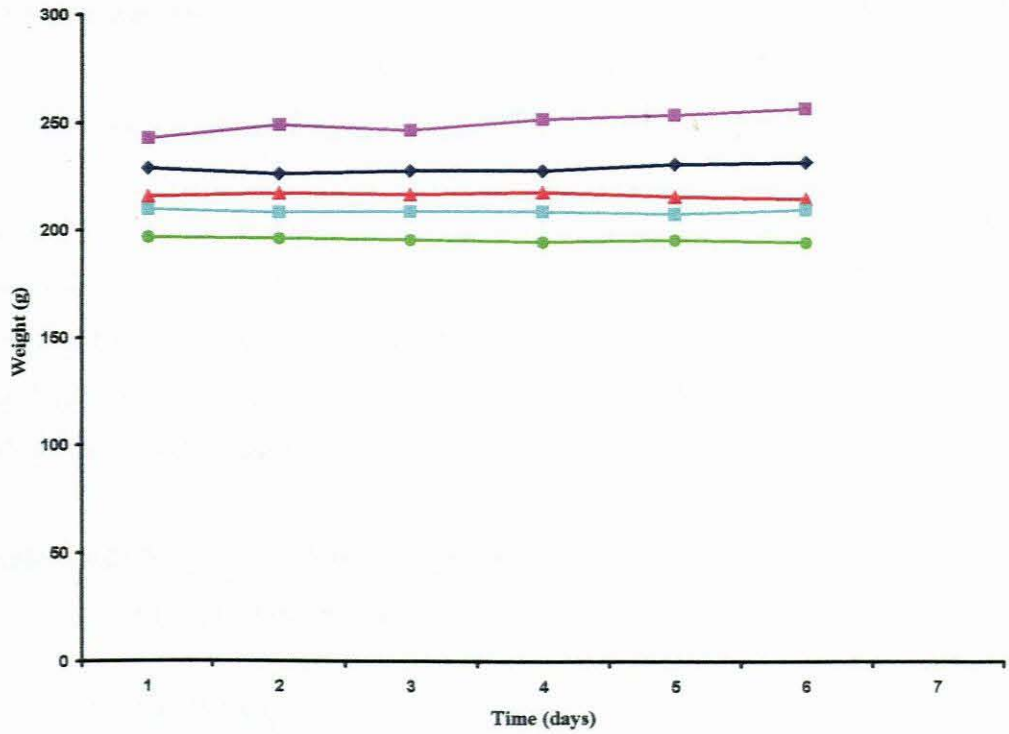


**Figure C3-1: Growth rates of rats fed diets containing beta-sitosterol**

**Key**

distilled water (♦); oleic acid (■); 10 mg $kg^{-1}day^{-1}$ beta-sitosterol (▲);
5 mg $kg^{-1}day^{-1}$ beta-sitosterol (■); 2.5 mg $kg^{-1}day^{-1}$ beta-sitosterol (•).

The body weight gain was comparable to the respective control groups and no apparent difference was noticed in mean body weights, recorded daily for each group, between the treated and the control groups during the treatment periods.

### C.3.2.3 Clinical signs

During the preliminary toxicity test, no diarrhea or other abnormalities in animal behaviour or appearance were observed. No mortality was recorded during the short-term, preliminary period.

## C.3.3 Experimental test

### C.3.3.1 Food and water consumption

Table C3-2 shows the amount of food (g/rat/day) and water (g/rat/day) the rats consumed during the 14-day period. There was no significant difference in the feed intake of the beta-sitosterol group compared with the two control groups. A small but significant ($p<0.05$) difference in water intake was observed between the beta-sitosterol and distilled water groups.

**Table C3-2: Average food and water consumption of rats during a 14-day period of experimental studies**

| Group | Diet | Average food intake[1] | Average water intake[2] |
|---|---|---|---|
| A | Distilled water | 16.0 g | 23.6 ml |
| B | Oleic acid | 16.1 g | 24.9 ml |
| C | β-sitosterol | 17.8 g | 25.1 ml[3] |

[1] g/rat/day

[2] ml/rat/day

[3] Significantly different from distilled water control group ($p<0.05$)

## C.3.3.2 Body weight changes

The animals were weighed daily and the change in average weight for every group was plotted (Figure C3-2). All the animals apparently gained weight over the study period. Animals fed with beta-sitosterol seemingly gained more weight than the other groups over the two-week period. The body weight/food consumed (turn over of food) is similar (Figure C3-3) for all the groups.

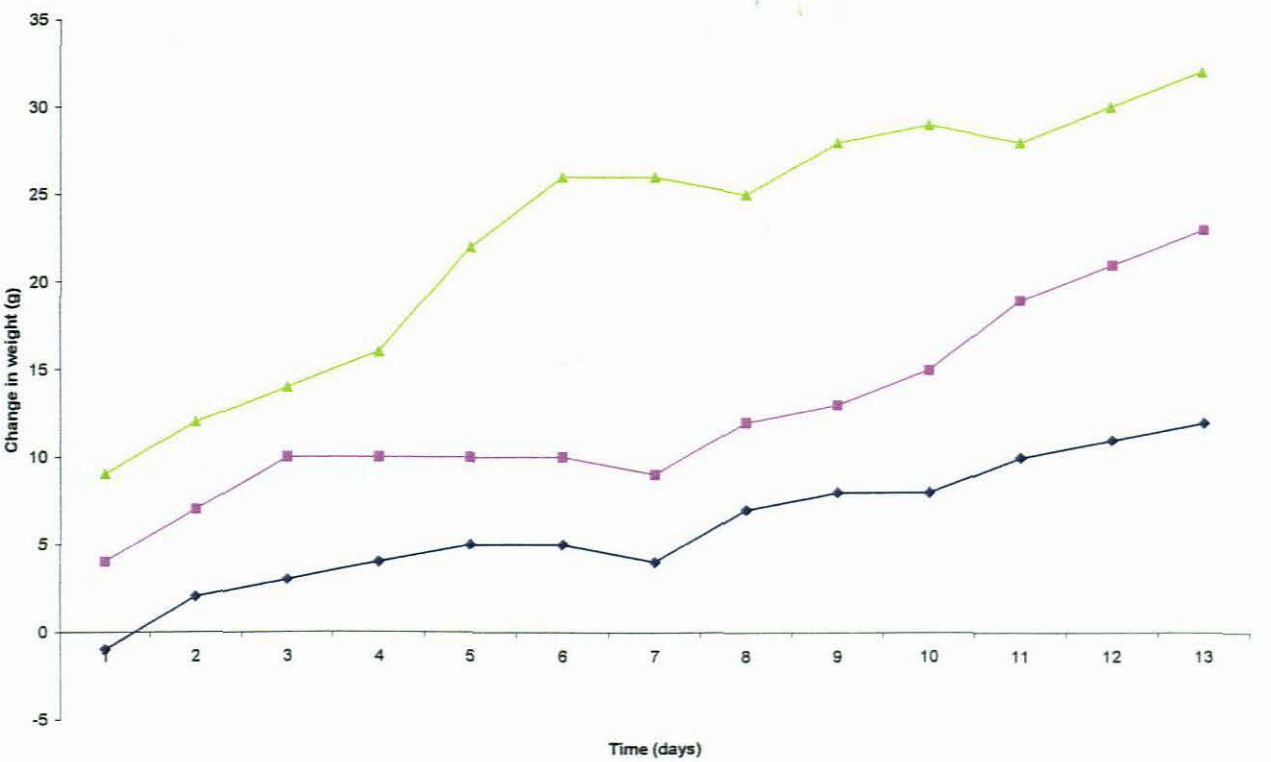


**Figure C3-2: Body weight gains of male Sprague-Dawley rats**

**Key**

distilled water (♦) oleic acid (■) β-sitosterol (▲)

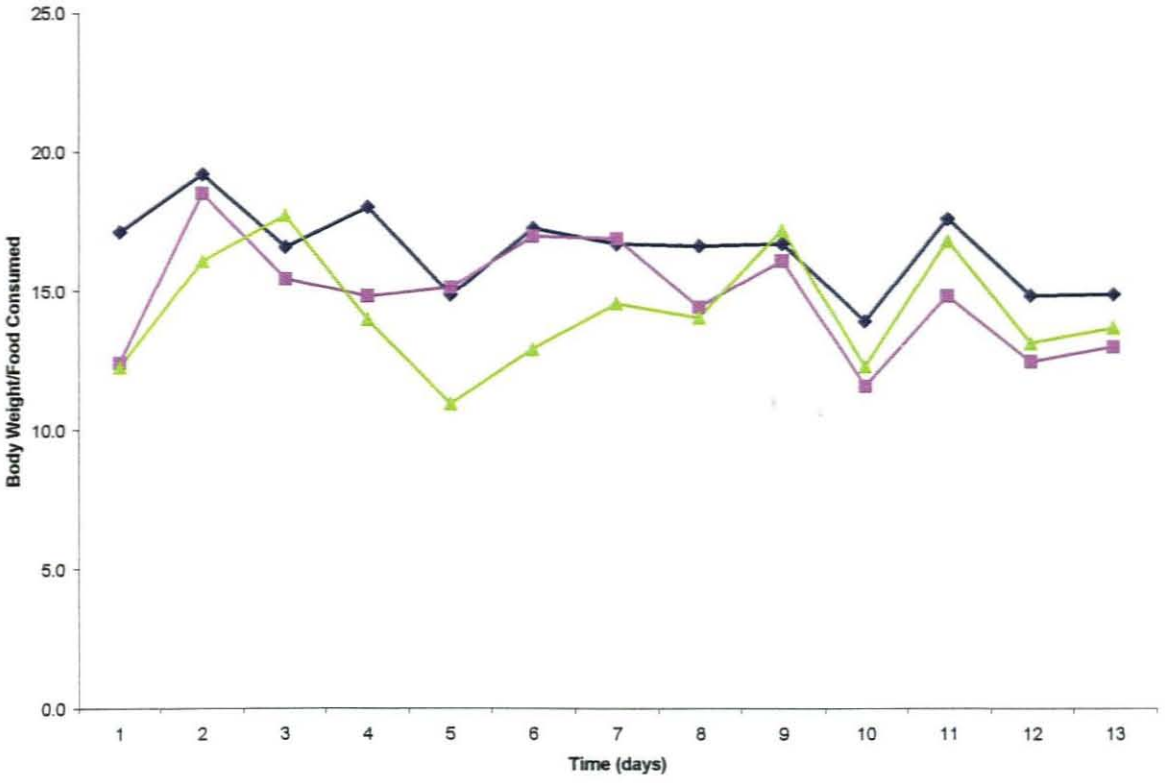


**Figure C3-3: Body weight/food consumed of male Sprague-Dawley rats**

**Key**

distilled water (■) oleic acid (▲) β-sitosterol (■)

### C.3.3.3 Clinical signs

The appearance of the test animals, compared with the control animals, was quite normal (Figure C3-4).

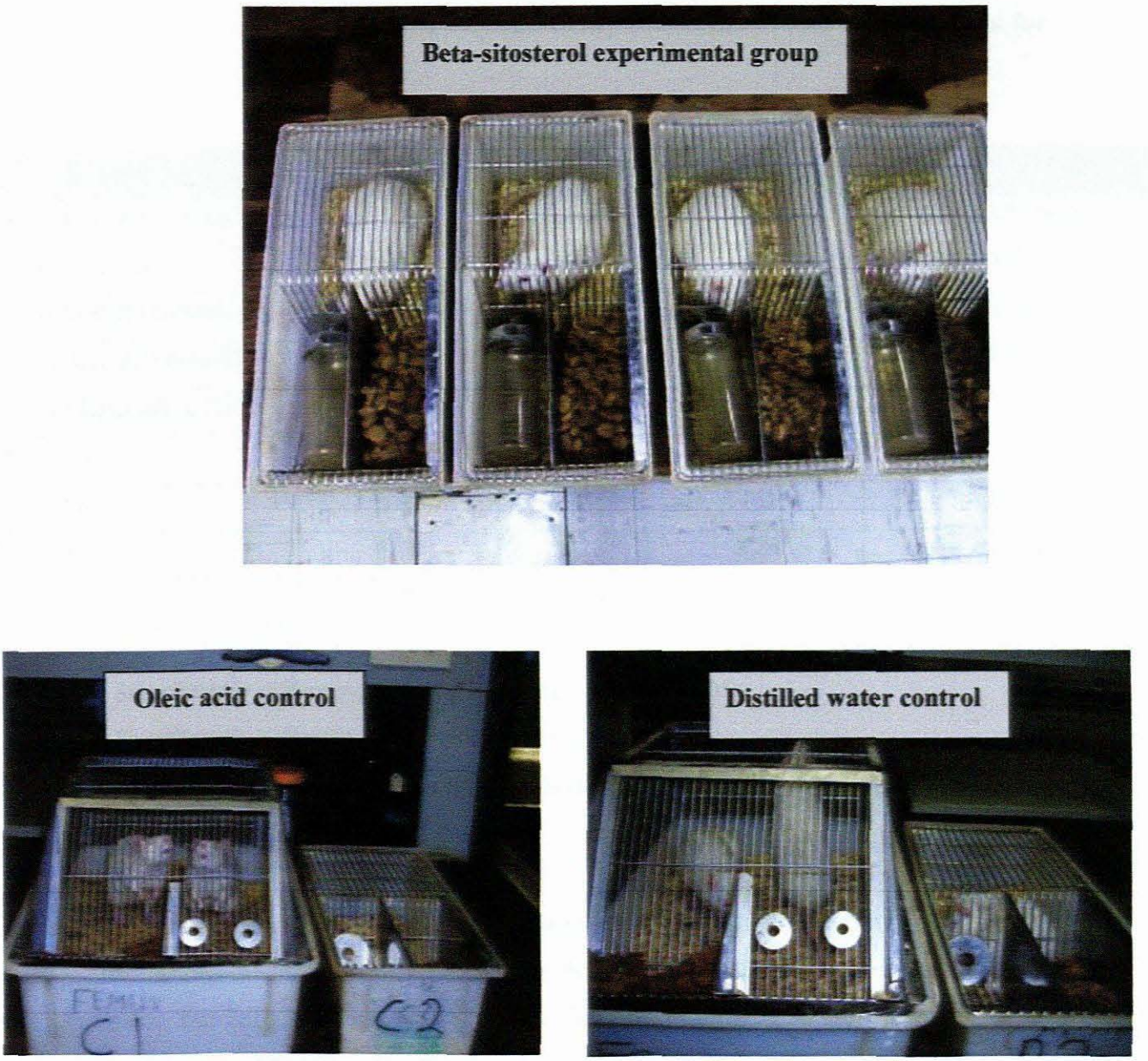


**Figure C3-4: Male Sprague-Dawley rats used for experimental tests**

### C.3.3.4 Haematology tests

Serum biochemistry and haematological profiles are summarized in Table C3-3.

**Table C3-3: Summary of serum biochemistry and haematological parameters of male Sprague-Dawley rats orally administered with beta-sitosterol for 14 days**

| Parameter | Distilled water | Oleic acid | Beta-sitosterol | Reference range |
|---|---|---|---|---|
| Random glucose (mmol/L) | 5.0 | 4.8 | 4.8 | 1.110 – 20.92 mmol/L |
| Total protein (g/L) | 71 | 77 | 69[2] | 75 – 107 g/L |
| Triacylglycerol (mmol/L) | 1.2 | 1.5 | 0.9[2] | 0.2147 - 3.040 mmol/L |
| Total cholesterol (mmol/L) | 2.50 | 2.55 | 2.20 | 1.088 – 14.89 mmol/L |
| Alkaline phosphatase (U/L) | 128 | 164 | 130[2] | 50 – 754 U/L |
| AST (U/L) | 1695 | 2197 | 852[3] | 154 – 369 U/L |
| ALT (U/L) | 375 | 500 | 161[3] | 7 – 28 U/L |
| RBC count | 7.89 | 8.22 | 8.24[1] | 4.25 – 9.31 x $10^6$ µl |
| WBC count | 2.55 | 3.18 | 3.71[3] | 3.35 – 17.35 x $10^3$ µl |

[1] Significantly different from distilled water group ($p<0.05$)

[2] Significantly different from oleic acid group ($p<0.05$)

[3] Significantly different from both controls ($p<0.05$)

Random glucose levels showed no significant difference among the groups. Total protein for the beta-sitosterol group differed significantly ($p<0.05$) from the oleic-acid group. A minor, but not significant, decrease was observed in total cholesterol levels in the rats treated with beta-sitosterol compared with the two controls. Triacylglycerol levels decreased significantly ($p<0.05$) for rats fed beta-sitosterol compared with rats fed oleic-acid. This is in line with the known hypocholestrolemic activity of beta-sitosterol.

ALT and AST values were significantly elevated in rats in the groups receiving distilled water and oleic acid in their diet ($p<0.05$). The group receiving beta-sitosterol exhibited the ability to lower the activity of the two enzymes significantly ($p<0.05$).

A significant increase in RBC count was observed between beta-sitosterol and distilled water groups ($p<0.05$). There was also a significant increase between the beta-sitosterol and two control groups with regard to total WBC counts ($p<0.05$)

### C.3.3.5 Pathology and organ weights

On the fifteenth day of the experiment, the animals were sacrificed and the livers, kidneys and small intestines were immediately prepared for analysis. No differences were observed on the relative weights of collected organs from the different groups of animals (*Table C3-4*).

**Table C3-4: Effect of beta-sitosterol on absolute and relative organ weights of male Sprague-Dawley rats**

| Group | Absolute organ weight | | Body weight | Liver | Kidney |
|---|---|---|---|---|---|
| | Liver | Kidney | | Relative organ weight (g organ weight/100 g) | |
| Distilled water | 6.10 g | 1.45 g | 231 g | 2.64 | 0.62 |
| Oleic acid | 7.04 g | 1.71 g | 252 g | 2.79 | 0.67 |
| Beta-sitosterol | 6.80 g | 1.60 g | 256 g | 2.65 | 0.63 |

Animals treated with beta-sitosterol showed no significant difference in the relative organ weights of the liver and kidneys when compared with the two control groups. Macroscopically, both livers and kidneys appeared normal in all the groups. Furthermore, no dramatic adverse histopathological changes were observed in the liver, kidney duodenum and ileum studies of these groups (Figure C3-5 – C3-7).

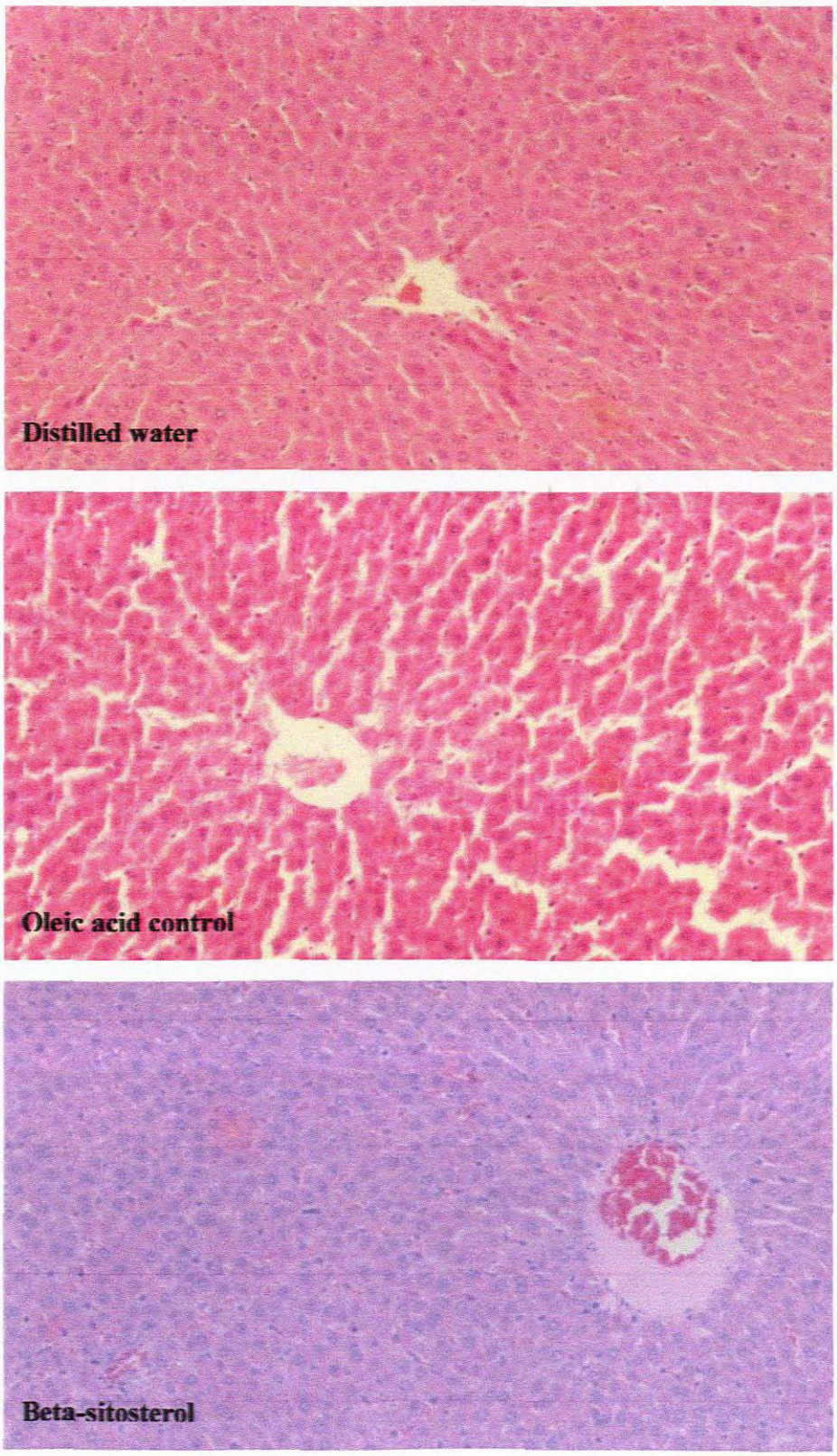


**Figure C3-5: Representative photomicrographs of histology of liver (X10)**

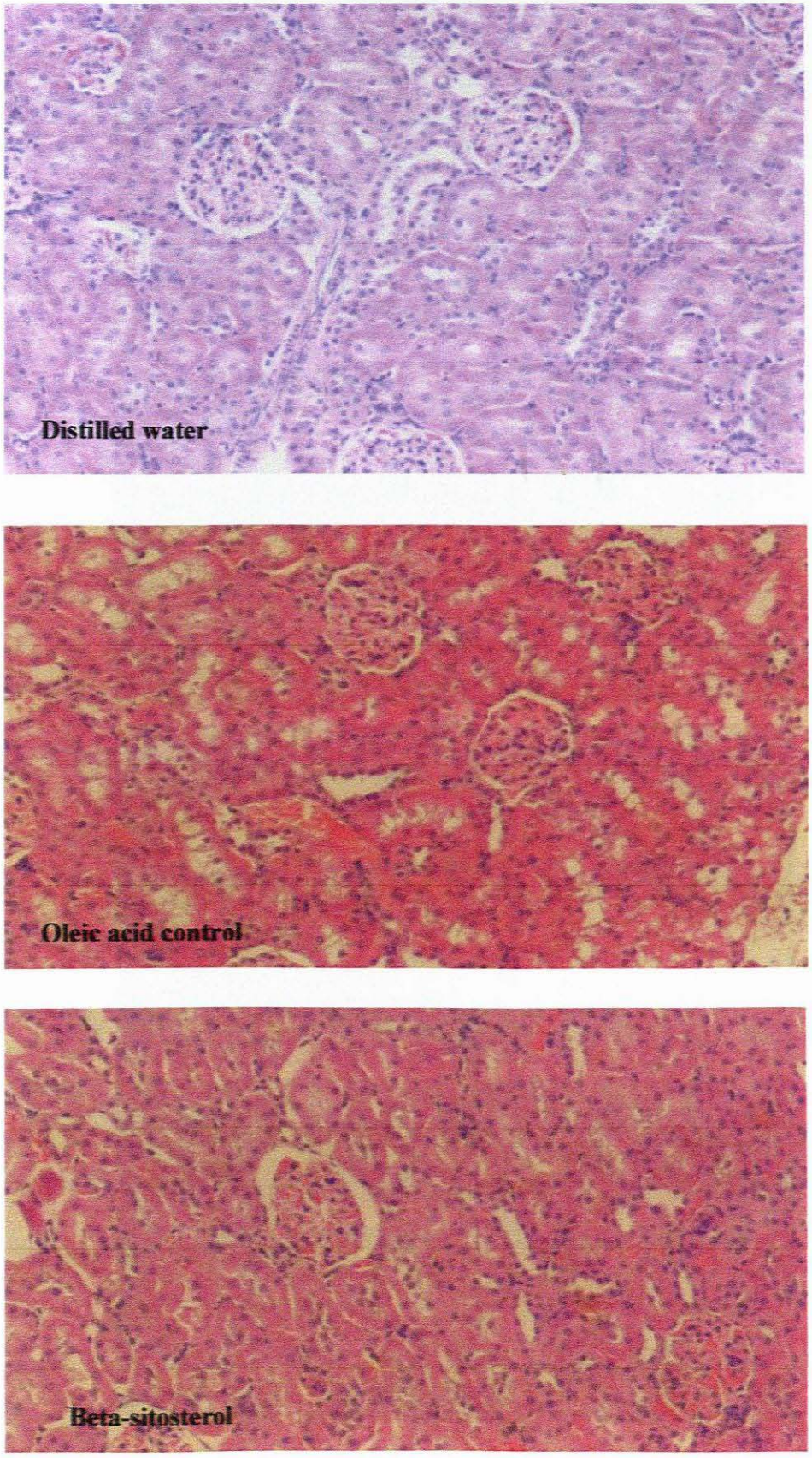


**Figure C3-6: Representative photomicrographs of histology of kidney (X10)**

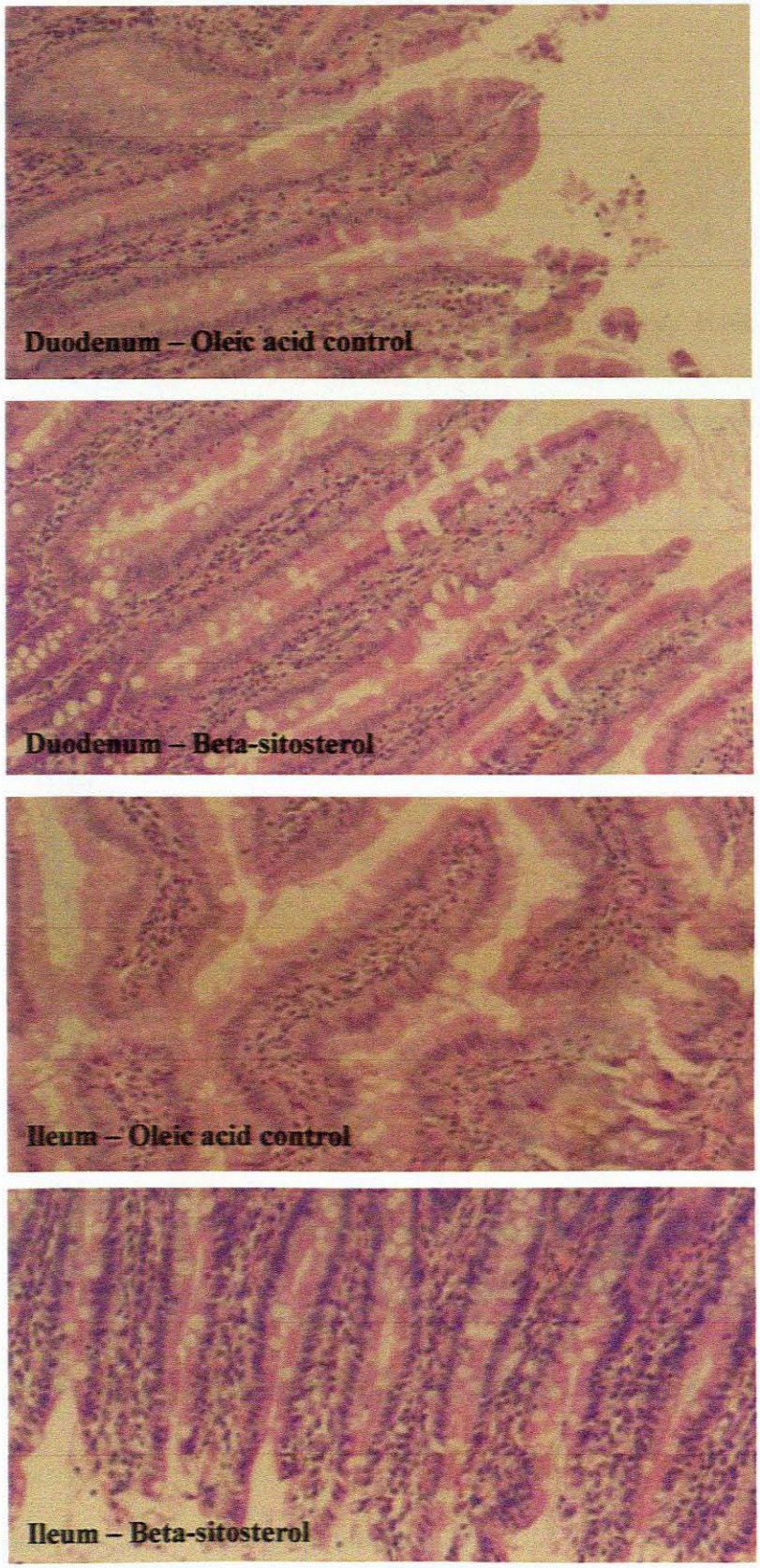


**Figure C3-7: Representative photomicrographs of histology of duodenum and ileum (X10)**

## C.3.3.6 Digestive enzyme activities in small intestine

### C.3.3.6.1 ATPase activity

Figure C3-8 shows the lower and upper intestinal $Na^+/K^+$-ATPase activity in the different rat groups. Oleic acid increased the $Na^+/K^+$-ATPase activity significantly ($p<0.05$) in both regions of the intestine. The beta-sitosterol significantly decreased the activity of the enzyme ($p<0.05$).

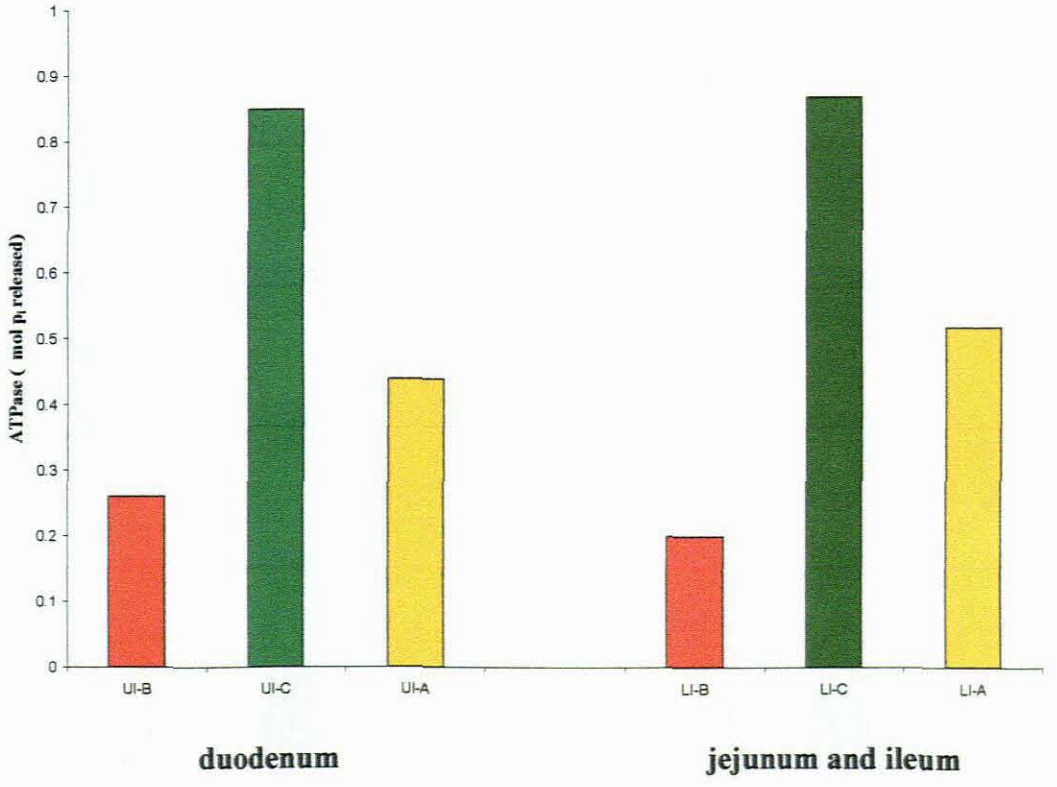


**Figure C3-8: Lower and upper intestinal $Na^+/K^+$-ATPase activity in rats**

**Key**

UI – upper intestine LI – lower intestine ■ A: beta-sitosterol group ■ B: distilled water group ■ C: oleic acid group

### C.3.3.6.2 Disaccharidase activity

Figures C3-9 a, b and c show the activity (U/ml homogenate) of small intestine disaccharidases of the rat groups.

**Key**

UI – upper intestine LI – lower intestine ■ A: beta-sitosterol group ■ B: distilled water group ■ C: oleic acid group.

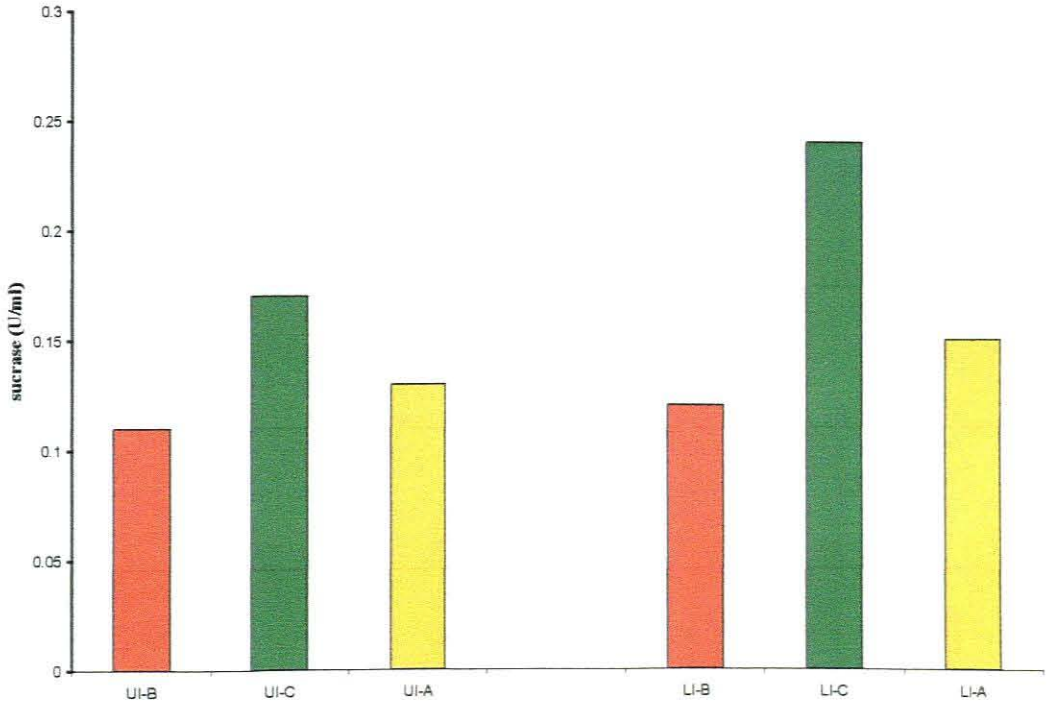


**Figure C3-9 a: Sucrase**

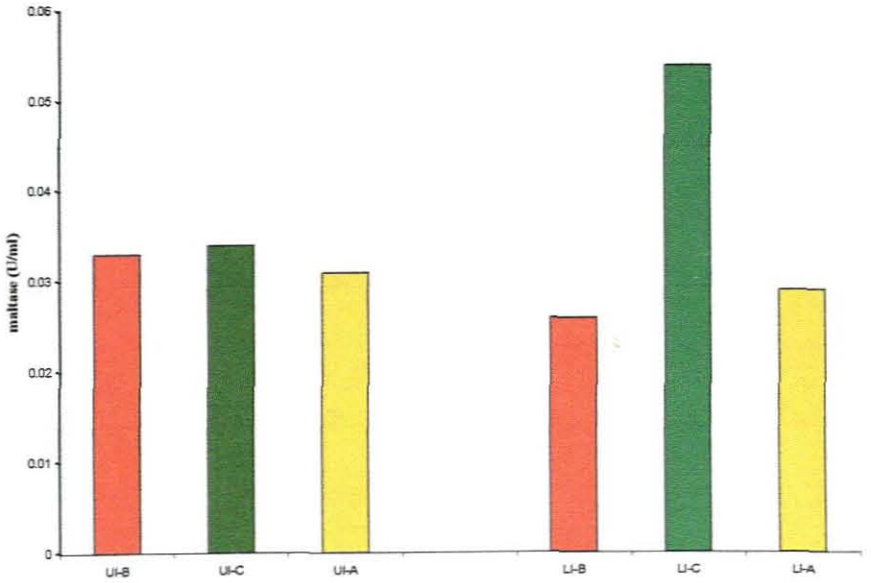


**Figure C3-9 b: Maltase**

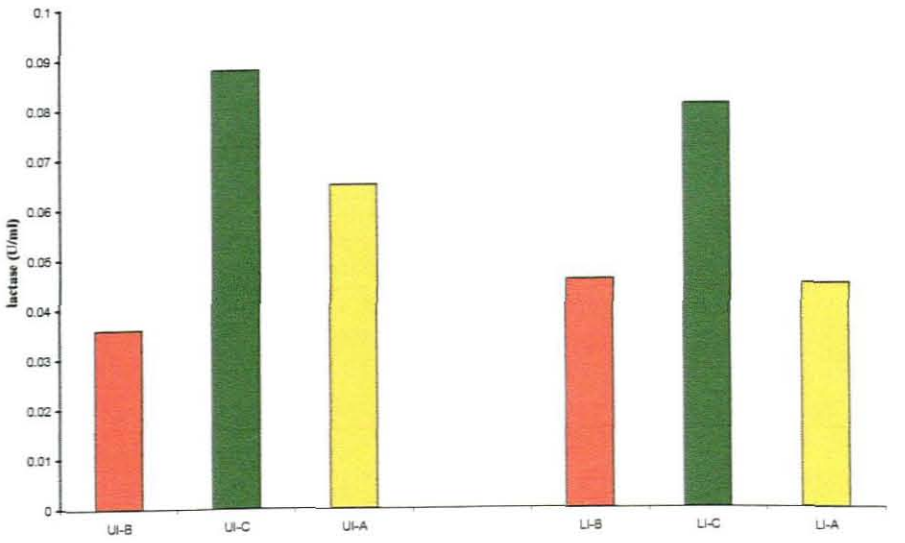


**Figure C3-9 c: Lactase**

# CHAPTER C-4

# DISCUSSION

## C.4.1 Introduction

This section of the study was conducted to investigate the effects of beta-sitosterol on the digestive and absorptive enzymes of Sprague-Dawley rats. Results obtained for the nutritional evaluation are discussed in this chapter.

## C.4.2 Experimental test

The rats were in good health throughout the study: no mortality was observed in any of the groups tested with beta-sitosterol. This is in agreement with studies conducted on phytosterols. Similar studies have consistently demonstrated a lack of toxicity in animals and humans fed beta-sitosterol, except for individuals with an extremely rare genetic condition, sitosterolaemia (Malini and Vanithakumari, 1990; Hicks and Moreau, 2001).

Plasma phytosterol levels are generally very low in mammalian tissues. Poor absorption from the intestine and quicker excretion from the liver, compared to that of cholesterol, are responsible for these low levels (Ling and Jones, 1995). Intestinal absorption of total dietary beta-sitosterol consumed is negligible in mammals. Animal studies have demonstrated that this absorption should possibly be as little as five percent (Borgstrom, 1968; Gould, 1955). In rats, the absorption of beta-sitosterol is about four percent (Sanders *et al.*, 2000). A reason for the low absorption of plant sterols might be that plant sterols are poorly esterified, possibly due to the low affinity of ACAT (acyl-coenzyme A cholesterol acyltransferase) for these components (Bhattacharyya, 1981; Ntanios *et al.*, 1998). Within the enterocyte, free cholesterol is is esterified by ACAT, incorporated into chylomicrons, which are subsequently excreted into the circulation and converted into a chylomicron-remnant by the action of lipoprotein lipase (Kwiterovich, 2000). Absorption

of free cholesterol depends on mixed micelles: mixtures of free cholesterol, mono- and diacylglycerols, fatty acids, phospholipids and bile salts (De Jong *et al.*, 2003). Evidence has been presented to support the view that cholesterol absorption would take place only in the presence of fat. It has been demonstrated that the absorption of cholesterol is made possible by fat. The fatty acid component of fat is the active factor for this absorption (Kim and Ivy, 1952; Swell *et al.*, 1956). The most efficient fatty acid promoting cholesterol absorption has been reported to be oleic acid (Swell *et al.*, 1956). Cholesterol is structurally similar to beta-sitosterol. Consequently, the solubility of beta-sitosterol was enhanced by esterifying it with oleic acid. The results in this study showed that beta-sitosterol became active in the rats when 0.01 mg $kg^{-1}$ $day^{-1}$ of oleic acid was added to the beta-sitosterol.

Changes in the physiological state of mammals can alter the concentration of a number of organic constituents in the blood serum: for example, glucose, protein, cholesterol and liver enzymes (Malini and Vanithakumari, 1990). Triacylglycerol levels, which are independent risk factors for cardiovascular problems (Wierzbicki and Mikhailidis, 2002), showed a significant decrease in levels for rats fed beta-sitosterol compared with rats fed oleic acid. Similar effects have been observed in cells incubated with oleic acid; oleic acid increased both the intracellular pool and biosynthesis of triacylglycerols (Pullinger *et al.*, 1989; Ellsworth *et al.*, 1986). The potential target for the influence of oleic acid is in the assembly of the apoB-containing (apolipoprotein B) lipoproteins. This occur in regions of the ER with high diacylglycerol:acyltransferase activity that has a high capacity for the formation of triacylglycerol (Borén *et al.*, 1990). Oleic acid could be used to manipulate important steps in the assembly process (Borén *et al.*, 1993). The decreased level observed in beta-sitosterol fed rats is in line with the known hypercholesterolemic activity of beta-sitosterol (Duivenvoorden *et al.*, 2006).

A slight decrease in serum protein level was observed in the group receiving beta-sitosterol in its diet. The slight change in the serum protein levels may have resulted from altered rates of anabolism and catabolism (Malinia and Vanithakumari, 1990). When compared to the two control groups, serum cholesterol was slightly, but not

significantly, decreased after beta-sitosterol treatment. Earlier reports have shown that beta-sitosterol is a potent inhibitor of dietary and serum cholesterol in rats (Gould, 1955), mice (Behar and Anthony, 1955), rabbits (Bhattacharyya and Lopéz, 1979) and dogs (Shipley *et al.*, 1958). Therefore, in the present investigation, the observed decrease in serum cholesterol concentration appears to be due to the inherent hypocholesterolemic effect of beta-sitosterol. An increased sample size would be necessary to increase the inhibitory effect on cholesterol. Owing to poor solubility and bioavailability of phytosterols, the lowering effect on serum cholesterol of phytosterols is not consistent. High dosages of between 25 and 50 g/d are required for efficacy (Moreau *et al.*, 2002).

Aminotransferase enzymes, ALT and AST, are largely used in the assessment of liver damage (Al-Habori *et al.*, 2002; Dobbs *et al.*, 2003). These enzymes can be measured in serum since membrane damage to the liver releases the enzymes into circulation. In the serum biochemical analysis, the most notable results were significantly elevated ALT and AST. Such a significant increase in enzymatic activity of serum ALT and AST reveals a very important pathological change in cell-membrane permeability or hepatic-cell rupture (Benjamin, 1978). This rise in liver enzyme activity is not necessarily an indication of the liver's ability to synthesize the enzymes. Instead, it signifies a loss of material from damaged hepatocytes (Woodman, 1980).

It is generally assumed that an increase of these enzyme activities reflects active inflammation and necrosis of hepatic cells. The levels of alkaline phosphatase remained fairly stable in this investigation and, therefore, do not appear to confirm the early stages of viral infection. However, it should be borne in mind that increases in alkaline phosphatase levels are not as sensitive an indicator of hepatic viral infection as are elevated ALT and AST (Gopal and Rosen, 2000).

The significantly elevated ALT and AST levels were not correlated to any observable clinical changes in the livers. Clinical observations included liver weights, as well as macroscopic and microscopic histological examinations of the livers and kidneys. A

significant increase was observed in the liver and kidney weights in the group receiving oleic acid, but, macroscopically and microscopically, all livers and kidneys appeared normal. With viral infections, ALT and AST levels are elevated even before the clinical signs and symptoms of disease occur. ALT values increased 13.4 and 17.9 times respectively for distilled water and oleic acid control groups, whereas ALT levels increased 4.6 and 6.0 times respectively for the two control groups. This is in agreement with Johnston (1999), who reported that in typical viral or toxic liver injury, the serum ALT levels rise more than the AST value. Observations, using an electron-microscope, might have revealed damage to hepatocytes which could have caused elevated ALT and AST levels. Oxidative stress at the sub-organelle (mitochondria and macrosome) level could have led to build up of free radicals that could have caused increased ALT and AST levels (Chen *et al.*, 1998; İçen *et al.*, 2005; Balkan *et al.*, 2002). Silva *et al.*, (2004) have observed and increase in AST and ALT activities in the liver of bullfrog oil-treated animals compared to the control. Bullfrog oil is a MUFA-rich (monounsaturated fatty acid) animal derived oil. MUFA account for 55% of total fatty acids, consisting of 38 % oleic acid. Their results indicate that the increase in oxidative stress (lipid peroxidation and catalase activity) in the liver of bullfrog oil-treated animals promoted damage to liver cells, which led to an increase of AST and ALT activities in the liver (Silva *et al.*, 2004). According to this study beta-sitosterol administration can improve the stress if obtained by oleic acid.

White blood cells (WBC) are generally crucial in fighting any infection (Schalm *et* al., 1975). In this study, there was a significant decrease in the WBC count for the two control groups, which might imply reduction in the ability to respond to early infection. However, the normal WBC levels for the group fed beta-sitosterol were significantly increased in comparison with the control rats fed distilled water and oleic acid. This might indicate a boost to the immune system. The group receiving beta-sitosterol also showed significantly lower AST and ALT levels relative to both control groups. Bouic et al. (1996) demonstrated that beta-sitosterol may have immunomodulatory properties and that even though this phytosterol is poorly absorbed and not synthesized in the human body, daily intake is needed to sustain an optimal immune response. Therefore, these results might

indicate that beta-sitosterol boosts the production of WBC and could also have a possible hepatoprotective function (Banskota *et al.*, 2000).

Gross inspection of the livers, kidneys and small intestines revealed hardly any visual lesions attributable to sterol treatment. There were no remarkable differences in variability of the histopathological parameters in the groups studied. All the groups showed normal cellular architecture, with distinct hepatic cells, sinusoidal spaces and periacinar veins. Glomeruli, Bowman's space, proximal convoluted and distal convoluted tubules appeared normal for all the kidneys.

The availability of the surface area for various aspects of digestion and absorption in the duodenum, jejunum and ileum is very important for proper functioning of the small intestine. Following the histopathological examination, no adverse changes in villus height or mucosal surface area were discovered in the duodenum or ileum and, therefore, no apparent effects on the functioning of the intestine were observed. None of the minor changes appeared to be related to the exposure to diets containing beta-sitosterol supplement. This suggests that beta-sitosterol is devoid of any toxic complications or side effects at the dose tested. These findings are in agreement with previous reports that the liver and kidneys did not show any adverse effects after long-term exposure to oral administration of beta-sitosterol in rat, rabbit and dog models (Swell *et al.*, 1956; Shipley *et* al., 1958; Malini and Vanithakumari, 1990; Hepburn *et al.*, 1999).

Intestinal ATPase activity was significantly increased in the control group receiving oleic acid in their diet, in both intestinal locations. The $Na^+/K^+$-ATPase showed less significantly elevated activities after beta-sitosterol intake, compared with rats in the control group receiving the oleic acid. Information on the effect of beta-sitosterol on ATPase activity is scanty in literature. Takahashi *et al* (2006) showed that ATPase activity of ABAC1 (ATP-binding cassette protein A1) was reduced in a dose-dependent manner by the addition of cholesterol, decreasing by 25 percent in the presence of 20 percent cholesterol. Beta-sitosterol, which does not have a double bond in the acyl chain as cholesterol, showed a similar inhibitory effect as cholesterol. ATPase activity may be

decreased by sitosterols as a result of their affect on membrane fluidity (Silva *et al.*, 2005). Increased $Na^+/K^+$-ATPase activity generates an inward sodium ion gradient that leads to increased glucose translocation across the cell membrane (Omoruyi, 1991). A reduction in ATPase activity can result in lowered glucose translocation from the intestine into blood. This could be useful in the treatment of diabetics (McAnuff *et al.*, 2005).

When rats fed with beta-sitosterol were compared with rats fed the oleic-acid and/or distilled water control diet, a significant inhibitory change was observed. Upper intestinal sucrase and maltase and both proximal and distal intestinal lactase activity showed a significant inhibitory effect ($p>0.05$). The dissemination of disaccharidase activities among mammals is extremely diverse (Martínez del Río and Stevens, 1988). The distribution pattern of the disaccharidases along the small intestine is also significantly influenced by the locus of disaccharide hydrolysis because the location of optimum activity presumably reflects the site of absorption. A number of mammals have showed this inconsistent distribution of disaccharidase activity along the small intestine (Dahlqvist, 1964; Malhotra and Philip, 1964, 1965).

This non-uniform distribution of disaccharidase activity along the small intestine was also observed in this study. The modified changes in enzyme activity of the small intestine depend on various exogenous factors (McCarthy *et al.*, 1980). These disaccharide mucosal enzymes are physiologically necessary in the digestion of food consumed for assimilation. They provide essential nutrients for maintenance and rapid growth (Lee *et al.*, 2003). Reduced disaccharidase activity is indicated by reduced blood glucose levels from less absorbable glucose formed from carbohydrate digestion (McAnuff *et al.*, 2005). There are other factors that can influence disaccharidase activities, such as obesity and age (Flores *et al.*, 1990), hormones (Raul *et al.*, 1984) and a decrease of luminal proteases (Zarling and Mobarhan, 1987). Thus, with a decrease in the blood-glucose levels of the rats treated with beta-sitosterol, the decreasing effect on the activities of the disaccharidase and ATPase could suggest the value of beta-sitosterol an as anti-diabetic (hypoglycemic) agent.

## CHAPTER C-5

# CONCLUSION

**C.5.1** Beta-sitosterol was administered to rats over a period of 14 days. No evidence of toxicity was observed in the rats given daily oral dietary supplements containing beta-sitosterol. No gross or microscopic alterations of any tissues were observed. There was no histological evidence of deposition of the plant sterol. Elevated ALT and AST levels could be due to possible oxidative stress whereas beta-sitosterol showed possible hepatoprotective function in lowering AST and ALT, an avenue which could be investigated further.

Secondly, the present study indicates that ingested beta-sitosterol decreases $Na^+/K^+$-ATPase and disaccharidases activity, resulting in the apparent decrease in blood glucose. The hypoglyceamic properties of beta–sitosterol could be further investigated

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# CHAPTER 2

# GENERAL CONCLUSION

## 2.1 Introduction

The mass production of highly refined foods has tended to overshadow traditional foods which in many ways are more nutritious. In search for solutions to problems of food insecurity in developing countries, there is need to re-examine the role of these traditional foods. Amadumbe is widely grown in the sub-tropical parts of South Africa. The locally developed cultivars are commonly used as staple foods in the Kwazulu-Natal province, South Africa. When any crop is being considered as a food source the nutritional value is of utmost importance. The nutritional and anti-nutritional evaluation of unprocessed and processed Amadumbe tubers revealed the following:

## 2.2 Nutritional and anti-nutritional evaluation

Proximate analysis showed Amadumbe to be highly nutritious with high carbohydrate, adequate protein and low lipid content. Amadumbe was also shown to contain essential fatty acids in the form of linoleic and linolenic acid. The tubers are generally low in mineral content except for potassium and magnesium.

Even though anti-nutritional substances were found in unprocessed Amadumbe, domestic processing (boiling, frying, roasting), were observed to effectively reduce the content of the anti-nutrients Boiling was the most effective method to decrease the levels of anti-nutrients. Zululand *Colocasia esculenta* can therefore be used as food material for humans but tubers should be processed properly to not pose a long-term health problem in humans.

## 2.3 Characterization and nutritional evaluation of selected anti-nutrients in Amadumbe

The characterization of two anti-nutients α-amylase inhibitors and gamma-sitosterol highlighted some of the benefits of the consumption of Amadumbe. The α-amylase inhibitors present in the Amadumbe tubers showed inhibitory activity against mammalian amylases and could therefore cause a marked decrease in the availability of digested starch. This may be beneficial in the management of diabetes mellitus and obesity.

Gamma-sitosterol was also isolated from Amadumbe tubers. It is an isomer of beta-sitosterol which is a highly active biological compound. The nutritional evaluation of beta-sitosterol has shown that by lowering the activity of disaccharidases, it could cause a decrease in glucose concentration in the blood of rats. Therefore the gamma-sitosterol present in these tubers could possibly be valuable by reducing blood sugar levels in diabetics and obesity.

The death rate among adults in South Africa is increasing. AIDS-related illnesses are not likely to be the entire reason for the increase in death rates. With reported increase in urbanization and the associated changes in life-style, incidences of obesity and diabetes could be additional factors.

The results of this study do suggest that even though Amadumbe is a neglected crop in South Africa, it is a highly nutritional crop, the consumption of which could be beneficial to diabetic and hypertensive patients.

## 2.4 Suggestions for future work

Further studies on Amadumbe are suggested on these lines

1. To extract and partially characterize other anti-nutritional factors in Amadumbe tubers.

2. Studies on the properties of these anti-nutrients and their effect on the human health.

3. Investigate the extent to which α-amylase inhibitors are capable of interfering with the digestive process of humans on consumption of Amadumbe.

4. Extract beta-sitosterol and/or gamma-sitosterol from Amadumbe to feed rats and do full investigation on effect of the beta-sitosterol on glucose absorption which could be beneficial in diabetes and obesity.

# Appendix A

## PREPARATION OF REAGENTS

### A.A.1 Anthrone reagent

1 g of Anthrone was dissolved in 500 ml of 72% $H_2SO_4$.

### A.A.8 Indicator

0.0172 g bromocresol green and 0.0078 g of methyl red were added to 25 ml of ethanol.

### A.A.3 Dam's reagent

Preparation of pyridine dibromide solution.

This was prepared by the addition of 2.06 ml pyridine in 5 ml glacial acetic acid to another solution of 1. 85 ml concentrated sulphuric acid in 5 ml glacial acetic acid and was cooled To this mixture of 0.63 ml of bromide diluted with 500 ml glacial acetic acid.

Potassium iodide solution (10%) 10 g of KI dissolved in 100 ml of distilled water. Starch indicator solution of 1 g soluble starch dissolved in potassium chloride solution which was allowed to boil and cool.

### A.A.2 0.05 M Tris-Hydrochloric Acid buffer pH 8.6

6.05 g Tris (hydroxyl methyl) amino methane was dissolved in a litre of distilled water and labelled 'A'.4.3 ml of HCl was diluted to 1 litre with distilled water and labelled solution 'B'. 50 ml of A was mixed with 5 ml of B and made up to 200 ml with distilled water. The pH was adjusted to 8.6 with 4.0 M NaOH solution or dilute HCl solution.

### A.A.3 Enzyme trypsin

10 mg bovine-pancreas trypsin type I was dissolved in 0.001 M HCl. The enzyme was stable in a refrigerator for two weeks.

100 mg protease was dissolved in 1 litre of tris-HCl buffer, pH 8.0, (i.e. α-chymotrypsin type vii from bovine pancreas, protease type xiv from *Streptomyces Griseus*, proteinase type xviii from *Rhizopus* species and protease type xiii fungal from *Aspergillus Saitoi*).

### A.A.4 0.001 M BAPNA (Benzoyl-DL-arginine-*p*-nitroaniline)

43.5 mg of benzoyl-DL-arginine-*p*-nitroaniline (BAPNA) was dissolved in 1 ml of dimethyl sulfoxide (DMSO) solution. This was diluted to 100 ml with 0.05 M tris-HCl buffer pH 8.6, containing 0.02 M calcium chloride. Care was taken to dissolve all BAPNA in the DMSO, as the presence of any crystals causes precipitation to occur on standing. A constant temperature was maintained throughout preparation.

### A.A.5 Phosphate buffer pH 6.9

18.73 g of sodium dihydrogen orthophosphate and 8.15 g of disodium hydrogen orthophosphate were mixed, dissolved together in a litre of distilled water and the pH was adjusted to 6.9.

### A.A.6 Soluble starch

1 g of soluble starch was dissolved in 100 ml of phosphate buffer.

### A.A.7 Dinitrosalicylic acid (DNS)

1 g of 3.5 dinitrosalicylic acid was dissolved in 20 ml of 2 M NaOH and 50 ml of distilled water. 30 g of Rochelle salt was added and made up to 100 ml with distilled water.

**A.A.8 0.04 M Sodium borate buffer pH 8**

2.473 g of boric acid was dissolved in 1000 ml distilled water. 2.012 g of sodium borate dissolved in 250 ml of distilled water. Then 166.67 ml of sodium borate was mixed together with 1000 ml of boric acid.

**A.A.9 ADA**

100 g of dextrose; 68.296 g of 0.065 M citric acid and 124.95 g of 0.085 ml trisodium citrate were dissolved in 5000 ml of distilled water.

**A.A.10 Washing buffer (pH 6.5)**

32.77 g of 0.113 M NaCl; 3.741 g of 4.3 mM $K_2HPO_4$; 14.64 g of 24.4 mM $NaH_2PO_4$; 5.45 g of 5.5 mM glucose and 1.86 g of 1 mM EDTA were dissolved in 5000 ml of distilled water.

**A.A.11 Resuspending buffer (pH 7.4)**

(i) 41 ml 0.03 M HCl was added into 50 ml of 0.03 M tris amminomethane and the solution was made up to a 100 ml with distilled water.

(ii) 8.12 g of 0.14 M of NaCl and 0.99 g of 0.005 M of glucose was dissolved in (i).

**A.A.9 0.1 M Ferric ammonium sulphate [$FeNH_4(SO_4)_2$] in 0.1 M HCl**

8.3 ml of concentrated HCl was diluted to 0.1 M by bringing the concentrated acid to 1 L with distilled water. Ferric ammonium sulphate was made by dissolving 4.8 g of the dodecahydrate salt $FeNH_4(SO_4)_2$ .12 $H_2O$ in 100 ml of the 0.1 M HCl. The resulting solution was pale yellow.

**A.A.9 0.008 M Potassium ferricyanide [$K_3Fe(CN)_6$]**

0.26 g of potassium ferricyanide was dissolved in 100 ml of distilled water. The resulting solution was yellow.

**A.A.10 Vanillin solution**

800 mg of vanillin was dissolved in 10 ml of 99.5 per cent ethanol.

**A.A.11 0.1 M Orthophosphoric/ethanol medium**

6.78 ml of orthophosphoric acid was dissolved in 1 litre of distilled water and 250 ml of ethanol was added to the orthophosphoric acid.

**A.B.1 Phosphate buffer**

18.74 g of sodium dihydrogen orthophosphate ( ) and 8.16 g of disodium hydrogen orthophosphate ( ) were mixed and dissolved together in a litre of distilled water. pH was adjusted to 6.9.

**A.B.2 Soluble starch**

1 g of soluble starch was dissolved in 100 ml of phosphate buffer.

**A.B.3 Dinitrosalicylic acid**

1 g of 3.5 dinitrosalicylic acid was dissolved in 20 ml of 2 M NaOH and 50 ml of distilled water. 30 g of Rochelle salt was added and the resulting mixture was made up to 100 ml with distilled water.

**A.B.4 Tris-HCl**

0.72 ml HCl was added to 10 ml of distilled water. 6.06 g of Tris was dissolved in 200 ml of distilled water and 9.75 ml of HCl. The resulting mixture was made up to 250 ml, then up to 1000 ml with distilled water and the pH was adjusted to 7.5.

**A.B.5 0.4 M NaCl phosphate buffer**

23.38 g of NaCl was dissolved in 500 ml of phosphate buffer.

**A.B.6 G-100 Sephadex**

10 g of Sephadex was dissolved in 500 ml of phosphate buffer.

## A.C.1 10 per cent buffered formalin

1.75 g of sodium hydrogen orthophosphate ($Na_2HP_42H_2O$) and 3.25g of di-sodium hydrogen orthophosphate ($Na_2HPO_4$) was dissolved in 25 ml of boiling water. 50 ml of 40 per cent formalin was added and the resulting mixture was made up to 400 ml with distilled water.

# Appendix B

## DETAILS OF METHODOLOGY

### B.A.1 Proximate analysis (AOAC, 1990)

#### B.A.1.1 Ash determination

Volatile organic matter was driven off when 2.5 g of the sample was ignited and kept at 600° C for six hours in an electric furnace. The residue was quantitated and the mass of the inorganic matter over the mass of the organic matter was noted as percentage ash.

#### B.A.1.2 Moisture determination

50 g of the sample was transferred to previously weighed and dried crucibles, then dried in a thermostatically controlled oven at 110° C for 24 hours. Samples were then removed, cooled in desiccators and weighed. The final weight over the initial weight was recorded as the percentage of moisture.

#### B.A.1.3 Crude fat determination

0.5 g of dry samples was pre-extracted with 25 ml of petroleum ether for 50 minutes with an automatic soxhlet system (Soxtec HT-6, Tacater AB, Höganas). The solvent in the extraction cups was evaporated overnight in an oven (70° C) and weighed to calculate the fat percentage.

#### B.A.1.4 Soluble carbohydrate determination [Hansen and Møller (1975)]

Extraction and determination of soluble carbohydrates were performed according to the methods of Hansen and Møller (1975). One gram of each sample was quantitatively transferred into burettes stuffed with glass wool. These burettes had been previously set up as shown in Figure B-1. The samples were wet with 2 ml of 80 per cent ethanol each and stirred to remove air bubbles in order to avoid channel formation.

Liquid surfaces were formed with ethanol. The air spaces were created by inserting a stopper of glass wool as shown in Figure B-1. The air space was to prevent diffusion of the carbohydrate into the solvent. The soluble carbohydrates were percolated from the sample.

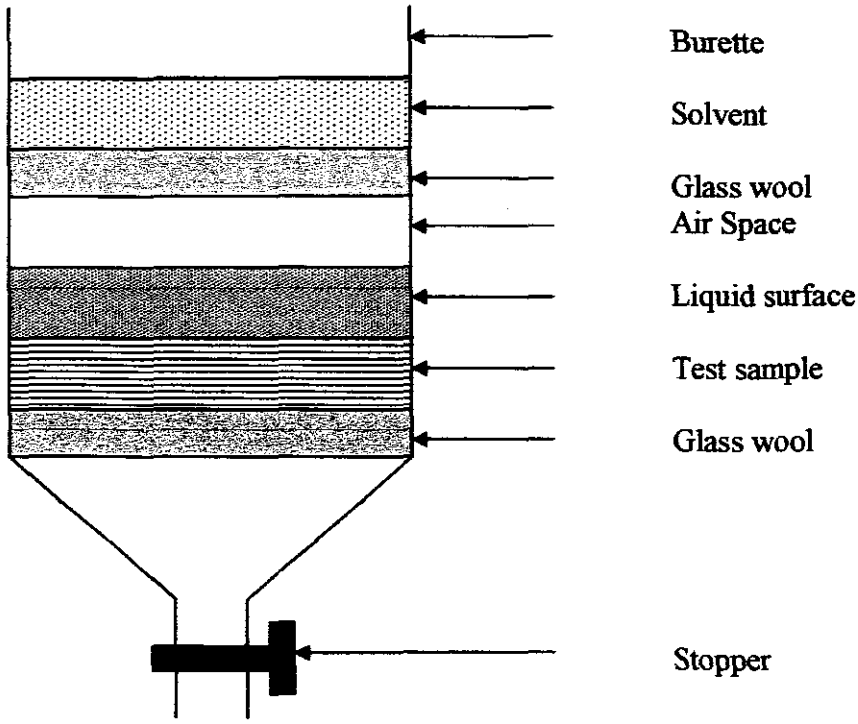



**Figure B-1: Burette packaging for starch percolation**

After ethanol percolation, the residues containing starch were further percolated with 25 ml of 35 per cent perchloric acid each. The residues were thoroughly mixed with 2 ml perchloric acid first, which resulted in their swelling. This was also percolated at the rate of 1.5 ml $hour^{-1}$. 2 ml of the test solution containing starch and glucose was pipetted into test tubes and kept at 0° C. 10 ml of anthrone reagent, which had been cooled to 0° C, was added to the 2 ml test solution. The reaction was thoroughly shaken and heated for exactly 10 minutes in a 100° C water bath. Thereafter, the test tubes were cooled to 0° C and the absorbance was read at 630 nm against 2 ml of 30 per cent perchloric acid, 10 ml of anthrone reagent for starch, 2 ml of 80 per cent ethanol and 10 ml of anthrone reagent for soluble sugars. The amounts of starch and soluble

sugars were estimated from standard graphs, prepared using potato starch (starch) and glucose (soluble sugar).

### B.A.1.5 Protein determination (Kjeldahl method of nitrogen determination)

#### B.A.1.5.1 Digestion

1 g of the dried and ground sample was transferred into a digestion flask. One Kjeldahl tablet and 10 ml of concentrated $H_2SO_4$ were added to each flask. The blank was prepared using the tablet and concentrated $H_2SO_4$. The mixture was cautiously heated on the digestion stand in a fume cupboard until the acid became clear. When the digestion was completed, samples were removed to cool, 2 ml of distilled water was added and the samples were cooled again.

#### B.A.1.5.2 Distillation

5 ml of the sample and 20 ml of NaOH solution were added to the digestion flask. A conical flask was prepared by adding 10 ml of saturated boric acid and 6 drops of mixed indicator (0.0172 g bromocresol green and 0.0078 g methyl red) so that the condenser tip was immersed. Distillation was completed and 30 ml of the distillate was collected in the conical flask. The tip of the condenser was removed from the conical flask.

#### B.A.1.5.3 Titration

The distillate was titrated with 0.05 M HCl to a pink endpoint. The crude protein content of the Amadumbe tubers was calculated from the results, using a factor of 6.25 to convert the amount of nitrogen to crude protein.

#### B.A.1.5.4 Fatty acid determination

#### 3.A.1.5.4.1 Lipid extraction [Bligh and Dyer (1959)]

50 g of Amadumbe were extracted with 150 ml of methanol-chloroform (2:1, v/v) mixture overnight in the oven shaker. The homogenate was filtered and the filter residue was re-extracted overnight with a mixture of methanol-chloroform (2:1, v/v) and 40 ml of distilled

water. The homogenate was filtered and the filter residue was then washed with 75 ml of methanol-chloroform (2:1, v/v). The filtrates were combined in the separating funnel with 125 ml of chloroform and 145 ml of water and phases allowed to separate. The chloroform layer was then collected and diluted with benzene and concentrated in vacuo. The residual lipids were immediately dissolved in 25 ml of chloroform-methanol (1:1, v/v) mixture.

#### B.A.1.5.4.2 Hexane extraction

15 g of dried Amadumbe was dissolved in 90 ml of hexane. The mixture was stirred occasionally for 15 minutes. Solid particles were filtered using rough filter paper and the extract was filtered again using fine filter paper with 0.45 µm pores. Hexane was evaporated at room temperature to yield the plant oil.

#### B.A.1.5.4.3 Iodine value

5 mg lipid was dissolved in 5 ml chloroform and to this solution 5 ml of Dam's reagent was added in a 50 ml Erlenmeyer flask. The solution was mixed and left at room temperature in the dark for 15 minutes. 0.5 ml of potassium iodide was added to this mixture with 0.5 ml of water and few drops of starch indicator. A standard thiosulfate solution was used to titrate the liberated iodine to the endpoint colour of milky. A blank was titrated containing 5 ml of chloroform only.

#### B.A.1.5.4.4 Column Chromatography

20 g of silicic acid was preheated in the oven at 120 °C. A slurry of the gel was then prepared with 35 ml chloroform in a beaker and poured into a 48x2.15 cm chromatography tube,the stopcock was openend slowly to dislodge the air bubbles which result in settling of the column. The solvent level was allowed to drop to the top of silicic acid; the bed was washedwith 2 column volumes of 35 ml chloroform. The Amadumbe exract was added in the column at the top of the solvent and the elution of the column was done at the flow rate of 3 ml/min with

175 ml chloroform, 10 column volumes, 40 column volumes with 700 ml acetone and 10 column volumes with 175 ml methanol.

### B.A.1.5.4.5 Liquid Chromatography Mass-Spectroscopy

The extracts of Amadumbe extracted with hexane and methanol-chloroform mixture were dissolved in methanol and the dichloromethane. 200 µl of this solution was then made up to 2 ml using the mixture of methanol and water. The analysis of the fatty acids was conducted under the following conditions by LCMS whereby the mass spectrometer conditions were kept in the capillary voltage of 3500 kV, cone voltage, 150 kV, desolvation temperature 250 °C and desolvation gas flow rate 300 °C. The solvents used was methanol and water at the flow rate of 0.035 ml $min^{-1}$. The dissolved extracts were then injected in *LCMS at an injection volume of 5 µl.*

## B.A.2 Determination of anti-nutrients

### B.A.2.1 Trypsin inhibitor [Smith *et al.* (1980)]

1g Amadumbe powder was extracted by homogenizing it in 20 g/litre of NaCl at the ratio 1:10 (w/v). The homogenate was stirred for 24 hours at room temperature, passed through a layer of cheese cloth and centrifuged at 9000 rpm for 15 minutes. The supernatant was passed through glass wool to remove any floating lipid materials.

#### B.A.2.1.1 Enzyme inhibitor assay

The following additions were pipetted into a series of 10 ml test tubes containing:

- reagent blank: 2 ml of distilled water (test tube a);
- standard trypsin: 2 ml of standard trypsin solution, 2 ml of water distilled water (test tube b);
- sample blanks: 1 ml of diluted sample extract, 1 ml of distilled water (test tube c);
- test samples: 1 ml of diluted sample extracts, 1 ml of distilled water, 2 ml of standard trypsin solution (test tube d).

After mixing the solutions and heating them to 37° C for 10 minutes, 5 ml of BAPNA solution (previously warmed to 37° C) was pipetted into each test tube and the resulting solution was mixed. After an incubation of exactly 10 minutes at 37° C, the reaction was stopped by adding 1 ml of 39 per cent (v/v) acetic acid. The absorbance was measured at

410 nm and the colour remained stable for several hours. Solutions of trypsin and BAPNA were prepared as described by Kakade et al. (1974). This BAPNA is an artificial substrate that becomes yellow when it reacts with the trypsin, thus revealing the non-inhibited trypsin (Kakade *et al.*, 1974).

**B.A.2.1.2 Calculation**

The change in absorbance (*At*), owing to trypsin inhibition $ml^{-1}$ diluted sample extract is ($A_b$ – $A_a$) – ($A_d$ – $A_c$), where the subscripts refer to tubes (a) – (d) referred to above. The percentage inhibition in each sample tube is given by 100 *At*/($A_b$ – $A_a$). If this is less than 40 per cent or more than 60 per cent, the assay must be repeated to provide a more suitable dilution (*D*) of the sample suspension. The trypsin inhibitor activity (TIA) was calculated in terms of mg pure trypsin $g^{-1}$ sample as weighed (mg $g^{-1}$):

$$TIA = \frac{2.632 \cdot D \cdot At}{S} \quad \text{mg pure trypsin inhibited } g^{-1} \text{ sample}$$

**B.A.2.2 Amylase inhibitor [Bernfeld (1955)]**

50 g of dried Amadumbe powder was homogenized and defatted with hexane. The samples were extracted with distilled water containing 1 per cent polyvinylpolypyrrolidone (PVP). The resulting crude extract was centrifuged at 10 000 x *g* for 20 minutes. The precipitate was discarded and supernatant was utilized for enzyme and inhibitory-enzyme assay. Enzyme and inhibitors, buffered with phosphate buffer, pH 6.9 containing 7 mM NaCl, were pre-incubated for 10 minutes at 37° C. Two per cent starch was utilized as substrate. After the addition of 10 ml of dinitrosalicylic acid (DNS), reaction was stopped at 100° C and absorbance was measured at 530 nm. One α-amylase unit (1UI) was defined as the amount of enzyme that would liberate 1 µmol of maltose from the starch under the assay conditions (10 minutes, 37 °C, pH 6.9). The amylase inhibitor's activity (AIA) was calculated from the equation, through maltose generated:

$$AIA = \frac{A_{530\,nm}\,\text{amylase} - A_{530\,nm}\,\text{plant extract}}{A_{530\,nm}\,\text{amylase}} \times 100$$

### B.A.2.3 Lectins

#### B.A.2.3.1 Collection of blood

The rats were anaesthetized with ether and blood was immediately collected from abdominal aorta into centrifuged tube containing ADA (anticoagulant) [1 ml ADA: 5 ml blood]. The blood was then centrifuged for 15 minutes at 1200 rpm and then further centrifuged for 3 min. at 2200 rpm. Supernatant was centrifuged after 15 min. at 3200 rpm and the sediment was resuspended in 5 ml of washing buffer which was then centrifuged for 15 min. at 3000 rpm. The sediment was suspended in resuspending buffer [1 ml sediment: 20 ml resuspending buffer].

#### B.A.2.3.2 Estimation of lectins

10 g of Amadumbe samples were homogenized with 200 ml of sodium borate buffer in shaker overnight. Samples were filtered with cheesecloth and serial dilutions were made as follows: 1:0, 1:1, 1:2, 1:4, 1:8, 1:10 and 1:20.

Test tubes were prepared in duplicates and 1 ml of diluted extract, 0.5 ml of platelets obtained from rats were added together with thrombin. For the control, 1 ml of sodium borate buffer was added instead of the extract and for the blank only the buffer was used. Absorbance was measured at 546 nm at an interval of 0 and 1 minute.

### B.A.2.4 Total polyphenols [Prussian Blue Assay – Price and Butler (1977)]

10 g of each sample was homogenized in 100 ml of 2 M HCl and heated in a water bath for 95° C for 60 minutes. This was then cooled to room temperature (28° C ±2° C) and filtered. The filtrate was made up to 500 ml with distilled water. 1 ml of this extract sample was diluted with 50 ml of distilled water. Timed additions of 3 ml of 0.10 M $FeNH_4(SO_4)_2$ were conducted and, exactly 20 minutes later, timed additions of 3 ml of 0.008 M $K_3Fe(CN)_6$ were started. Solutions were swirled and, after 20 minutes, absorbance was measured spectrophotometrically at 720 nm. A standard curve was prepared, expressing the result as gallic-acid equivalent.

**B.A.2.4 Tannin [Van-Burden and Robinson (1981)]**

Weigh out 500 mg of the sample into a 50 ml plastic bottle. Add 50 ml of distilled water and shake for 1 hour in a mechanical shaker. Filter this into a 50 ml volumetric flask and made up to the mark. Pipette 5 ml of the filtered out into a test tube. Mix this with 0.008 M potassium ferrocyanide and 2 ml of 0.1 N HCl. Measure the absorbance at 120 nm within 10 minutes.

**B.A.2.5 Flavonoid [Bohm and Kocipai-Abyazan (1994)]**

100 ml 80% aqueous methanol at room temperature was used to extract 10 g of the plant sample repeatedly. The entire solution was filtered through No 42 (125 mm) whatman filter paper. After transferring the filtrate into a crucible, it was evaporated into dryness over a waterbath. It was weighed to a constant weight.

**B.A.2.6 Saponin [Fenwick and Oakenfull (1981)]**

20 g of sample material was weighed and placed in the Soxhlet extractor with acetone for 24 hours. Constituent parts, such as lipids and pigments, were thus removed. The solvent was changed to methanol and extraction was continued for another 24 hours. At this point, the method used so far (Hai *et al.*, 1976) was modified. Instead of bringing the sample up to 250 ml as suggested in the original method, it was concentrated. The methanolic extract was then transferred to a rotary evaporator and evaporated to dryness. Dry extracts were suspended in approximately 10 ml of methanol. The colour was developed with 0.5 ml of 8 per cent vanillin solution in ethanol (freshly prepared for each determination) and 5 ml of 72 per cent (v/v) sulfuric acid was added to 0.5 ml of the methanol solution of the sample. The mixture was blended well, warmed in a water bath at 60° C for 10 minutes and then cooled in ice-cold water. The methanol blank with the reagents resulted in a strong yellow colour. Absorbance was recorded at 500 nm. Saponin was used as a standard to draw the standard curve.

### B.A.2.7 Cyanogenic determination [O'Brien *et al.* (1991)]

Amadumbe samples were homogenized with 160 ml of orthophosphoric/ethanol extraction medium for two minutes. The homogenate was washed on glass fibre filter.

*Total cyanogens*: an aliquot of extract (0.1 ml) was added to 0.4 ml pH 7.0 buffer A (prepared from 0.1 M $H_3PO_4$ and 0.1 M $Na_3PO_4$) in a stoppered Quickfit test tube, then β-glucosidase preparation (0.1 ml), with the activity of 5 EU $ml^{-1}$, was added. After a 15-minute incubation at 30° C, 0.2 M NaOH (0.6 ml) was added, followed by buffer A (2.8 ml; pH 6.0). Aliquots were assayed as in the colorimetric procedure described below.

*Colorimetric procedure*: Chloramine T reagent (0.2 ml: 0.5 per cent w/v) was added to 4 ml buffered extract in a stoppered Quickfit test tube and mixed well. Tubes were placed in ice/a water bath for 5 minutes, then pyridine-pyrazolone reagent (0.8 ml) was added in a fume cupboard. After 90 minutes, the absorbance at 260 nm was determined. The concentration of the samples was extrapolated from the standard curve, using KCN as a standard. Duplicate analyses were undertaken and blanks with the extraction medium were performed for each analysis.

### B.A.2.8 Oxalate [Munro and Bassir, (1969)]

In this method, 2.0 g of each sample powder was extracted, with 0.15 per cent citric acid, for up to six hours. The solutions were filtered under vacuum. Prior to determination, the heavy metals in the acidified extracts were precipitated with 5 ml tungstophosphoric acid reagent and centrifuged at 1500 revolutions per minute for five minutes. The supernatant was discarded and precipitates solubilized with hot, diluted $H_2SO_4$. The content of each test tube was then titrated against 0.01 M $KMnO_4$. Titration with potassium permanganate can reveal the presence of oxalic acid. The acid is a weak reductant and needs an oxidant as strong as permanganate in order to react. Calcium oxalate was used as the standard and oxalate contents were expressed as an amount equivalent to 0.3 g/100 ml of calcium oxalate.

**B.A.2.9 Alkaloid [Harborne (1973)]**

5 g of the sample was weighed into a 250 ml beaker and 200 ml of 10 per cent acetic acid in ethanol was added, covered and allowed to stand for four hours. The mixture was filtered and the extract was concentrated, using a water bath, to one-quarter of the original volume. Concentrated ammonium hydroxide was added dropwise to the extract until precipitation was complete. The whole solution was allowed to settle and the precipitate was collected, washed with dilute ammonium hydroxide and then filtered. The alkaloid was the residue, which was dried and weighed.

**B.A.2.10 Phytate determination [Mehlich (1953)]**

2 g of sample material was extracted in 30 ml of double acid (0.05 M HCl and 0.025 M $H_2SO_4$) for three hours. Samples were filtered under vacuum through Whatman no 1 filter paper. The following solutions were added to 5 ml of the extract:

- 50 ml 0.05 M sulfuric acid;
- 20 ml ammonium molybdate;
- 20 ml ascorbic acid;
- 10 ml potassium antimonytartrase (the ratio 5:2:2:1).

The mixture was allowed to stand for 30 minutes at room temperature to allow colour to develop. The absorbance of the samples was measured spectrophotometrically at 820 nm. A standard curve was prepared, expressing the results as a potassium hydrogen phosphate equivalent. The concentration of phytate was calculated from its phosphorus content.

**B.B1 Isolation of amylase inhibitor**

**B.B1.1 Extraction of *Colocasia esculenta* α-amylase inhibitor from tubers**

Amadumbe tubers were obtained from the local market at Esikhawini in Kwazulu-Natal, South Africa. Tubers were washed, peeled, cut into small pieces (2 cm x 3 cm) and dried overnight at 40° C. The dried tubers were ground into flour, homogenized and defatted with hexane, then filtered and air-dried. 20 g of the defatted flour was extracted for α-amylase inhibitor with 100 ml of distilled water containing 1 per cent polyvinylpolypyrrolidone (PVP).

The mixture was stirred continuously for two hours. The residue was re-extracted and the combined filtrate centrifuged at 12 000 g for 20 minutes. The precipitate was discarded and the crude extract supernatant was subjected to 80% $(NH_4)_2SO_4$ saturation and left overnight at 4 °C. Precipitated protein was recovered after centrifugation (12 000 g x 20 min) and the protein pellet redissolved in a minimum volume of phosphate buffer (0.02 M, pH6.9, containing 0.3 M NaCl). The protein suspension was transferred to dialysis tubing and dialysed against the buffer (designated the ammonium sulphate extract). The dialysed sample was centrifuged and the fraction was analysed for amylase inhibitor activity.

### B.B1.2 Ion-exchange chromatography

The dialysed material, dissolved in 0.02 M phosphate buffer (pH6.9), was loaded on a column (6 x 1.1 cm) of DEAE-Sephacel, equilibrated with the same buffer. The column was eluted with a gradient of 0–0.5 M NaCl at a flow rate of 20 ml $h^{-1}$. Four inhibitors, containing fractions eluted from the column, were pooled and analysed for protein and AI activity. The absorbance was measured at 280 nm and the graph plotted against tube numbers.

### B.B1.3 Gel chromatography

The ultimate purification procedure was performed by chromatography on Sephadex G-100. During chromatography, the major peaks retained from ion-exchange dissolved in phosphate buffer were loaded on to a Sephadex G-100 column (35 x 1.1 cm), equilibrated with the same buffer. The elution was performed at a flow rate of 15 ml $h^{-1}$, fractions were pooled and analysed for protein and AI activity. Fractions (A-1 and B-2) with AI activities were collected, dialysed extensively, freeze-dried and dissolved in deionized water.

### B.B1.4 Molecular weight determination by gel filtration chromatography

The relative molecular weight ($M_r$) of the native enzyme was determined by using a Sephadex G-100 column. Elution was carried out at the flow rate of 15 ml $h^{-1}$, with an elution buffer comprising 50 mM tris-HCl pH 7.5. The calibration curve was constructed using protein markers: 2 mg $ml^{-1}$ cytochrome c (12 400), 3 mg $ml^{-1}$ carbonic anhydrase (29 000), 5 mg $ml^{-1}$ alcohol dehydrogenase (150 000) and 4 mg $ml^{-1}$ β-amylase (200 000). 2 mg $ml^{-1}$ of

Dextran blue was used to determine the void volume (Vo). A calibration curve between log-log molecular weights of protein markers and the partition coefficient values ($K_{AV}$) was constructed.

### B.B1.5 Enzyme assay

The α-amylase and α-amylase inhibitor activity were measured using Bernfeld's method (Bernfeld, 1955). α-Amylase inhibitor extracts were added to a one-per-cent (w/v) starch solution in a 20 mM phosphate buffer, containing 0.4 mM NaCl (pH 6.9) and α-amylase. The mixtures were incubated at 37° C for 30 minutes. Reaction was stopped by the addition of 10 ml of dinitrosalicylic acid reagent (DNS). This solution was boiled for five minutes, then cooled and the absorbance was read at 530 nm. One amylase unit is defined as the amount of enzyme that will liberate 1 μmol of maltose from starch under the assay conditions (pH 6.9; 37° C; five minutes). Inhibitory activity is expressed as the percentage of inhibited enzyme activity out of the total enzyme activity used in the assay.

### B.B1.2 Kinetic studies

For all kinetic studies other than pH, both enzymes obtained after gel-filtration on Sephadex G-100 were used.

### B.B1.2.1 pH

The effect of pH was checked using the following buffers (0.1 M): sodium acetate (pH 5), sodium phosphate (pH 6–7), Tris–HCl (pH 8) and glycine–NaOH buffer (pH 9–10).

The AI activity was assayed at different pH values (pH 1 – pH 10) for five minutes at 37° C and the remaining amylase activity was determined.

### B.B1.2.2 Temperature

The optimum temperature for inhibition of the inhibitor was determined by assaying the amylase activity at temperatures ranging from 20° C to 100 °C at pH 6.9. Constant amounts (1 ml) of AI were preincubated with phosphate buffer (pH 6.9) for five minutes at 2-100° C. Remaining amylase activity was determined.

### B.B1.2.3 Assay of α-amylase inhibitor activity

The specificity of AI-A1 and AI-B2 was evaluated against amylases from different sources: human saliva, porcine pancreas, sweet potato, barley, *Bacillus* species, *Aspergillus oryzae* α-amylases, dissolved in phosphate buffer pH 6.9 For inhibitory assays, α-amylase inhibitors were pre-incubated with α-amylase enzymes in phosphate buffer for 30 minutes at 37 ° C.

## B.B2 Isolation of saponin

### B.B2.1 Saponin extraction and isolation

Saponins were extracted from white Amadumbe tubers (*Colocasia esculenta*) obtained from the local market at Esikhawini, KZN, South Africa. One kilogram of the tubers were air-dried, powered and extracted five times at room temperature with 95 per cent ethanol. The ethanolic extract was concentrated under vacuum on a rotary evaporator at 40° C. The resulting extract (15.53 g) was suspended in 10 ml of water and partitioned into a $CH_3Cl$-$H_2O$ (1:1, v/v) mixture to furnish the 30 ml $CH_3Cl$-soluble fraction and an aqueous layer. The aqueous layer was extracted with 30 ml *n*-butanol (*n*-BuOH) to give 40 ml *n*-butanol and $H_2O$-soluble fraction.

### B.B2.2 Thin-layer chromatography

Aliquots of 10 µl of the methanol extract were manually spotted on to Silica gel 60 $F_{254}$ Al plates (20 x 20 cm), using a glass capillary. The solvent extracts (10 µl per plate) were applied as separate spots to a TLC plate about 2 cm from the edge (spotting line). After sample application, the plates were placed vertically into a solvent-vapor-saturated TLC chamber. Plates were developed in system: chloroform/methanol/water (65:35:10 v/v) and developed for a distance of 12 cm, air-dried and sprayed with anisaldehyde, sulphuric acid, acetic acid (95:5:5). The TLC plate was dried for between five and ten minutes in an oven at 100°. Spots were visualized by gentle heating with hot air (90° C) for ten minutes. Detection was also done with iodine crystals.

### B.B2.3 Column chromatography

*n*-Butanol extract was further separated by column chromatography using glass columns with sintered glass filters. Silica gel (Merck) was loaded as slurry prepared in the respective solvents, eluting with $CH_3Cl$ and methanol (until a ratio of 50:50 was reached). Fractions were collected under gravitational flow. Fractions were pooled according to TLC analysis.

### B.B2.4 HPLC-UV analysis

Analysis was performed using a Shimadzu liquid chromatograph system (Palo Alto, CA, USA), equipped with autosampler and Prominence Diode Array detector. A Teknokroma nucleosil 100 $C_{18}$ column (5 µm x 25 mm x 4 mm) was used. A binary gradient elution system, consisting of water (A) and 10 per cent acetonitrile (B), was utilized and separation was achieved using the following gradient program: 0-15 min, 90 per cent. The flow-rate was 0.1 ml/min and the system operated at room temperature (23 ± 1° C).

### B.B2.5 Gas chromatography mass spectrometry

Gas chromatography/electron ionization mass spectrometry (GC-MS) was performed on a Agilent 6890 GC system, including a HP 5973 mass spectrometer equipped with an electron impact (EI) source on an HP 5 MS capillary column (30 m x 0.25 mm x 0.25 µm). GC parameters were as follows: injector split 1:40, helium carrier gas flow rate 30 cm/min. Initial temperature was set at 50° C (hold 2 minutes) and the temperature ramp was 20° C/min to 300 °C (10 minutes hold). Spectra were obtained over *m/z* 35-550. Injection volume was 1 µl. System control and data evaluation were realized using the Enhanced ChemStation software package, G1701BA Rev. 01.00, incorporating the NIST 98 MS spectral library for sample identification.

### B.B2.6 NMR

NMR experiments were done on a Bruker AM-400 spectrometer (Bruker, Rheinstetten, Germany) equipped with an HX inverse probe ($^1$H:500 MHz; $C^{13}$:125 MHz; solvent: pyrimidine-$d_5$). System control and data evaluation were realized with the XWINNMR software package, version 2.6 (Bruker, Rheinstetten, Germany).

## B.C.1 Esterification of beta-sitosterol

200 mg of beta-sitosterol was dissolved in 200 mg of oleic acid at 80° C. The solution was added to 80 ml of a three per cent solution of sodium caseinate at 60-80° C under strong stirring.

## B.C.2 Analyses of digestive enzyme activities

### B.C.2.1 Preparation of tissues

The intestine of each rat was divided into two portions: the proximal segment (duodenum) representing the upper intestine, and the mid and distal segments (jejunum and ileum) representing the lower intestine. The rat intestine was free of food and rinsed in 0.9 per cent NaCl. A fraction was prepared from the two parts of the intestine by homogenizing the parts in 00 M phosphate buffer. The homogenate was centrifuged at 10 000 x *g* for 10 minutes and the supernatant was frozen until required for enzymatic assays.

### B.C.2.2 Disaccharidases [Dahlqvist (1968)]

The principle of this method is as follows: an intestinal homogenate is incubated with the appropriate disaccharide. The disaccharidase activity is then interrupted by boiling the solution and the glucose liberated is measured with a glucose-oxidase reagent. Five hundred microlitres of substrate at 56 mM was added to 200 µl of homogenate (100 µl for sucrase and maltase) and incubated for 60 minutes at 37° C (15 minutes for maltase). The reactions were terminated by incubating at 100° C for five minutes. The liberated glucose was measured, using a Glucose (GO) Assay kit. The absorbance of each tube was measured spectrophotometrically at 540 nm.

The activity was calculated as follows:

$$\text{Units/ml of homogenate} = \frac{\mu\text{g glucose} \times F}{180 \times 60 \times N}$$

**Key**

$F$ = dilution factor of the homogenate (1.6 for the sucrase and maltase; 1.4 for the lactase);

180 = molecular weight of the glucose;

60 = incubation time in minutes;

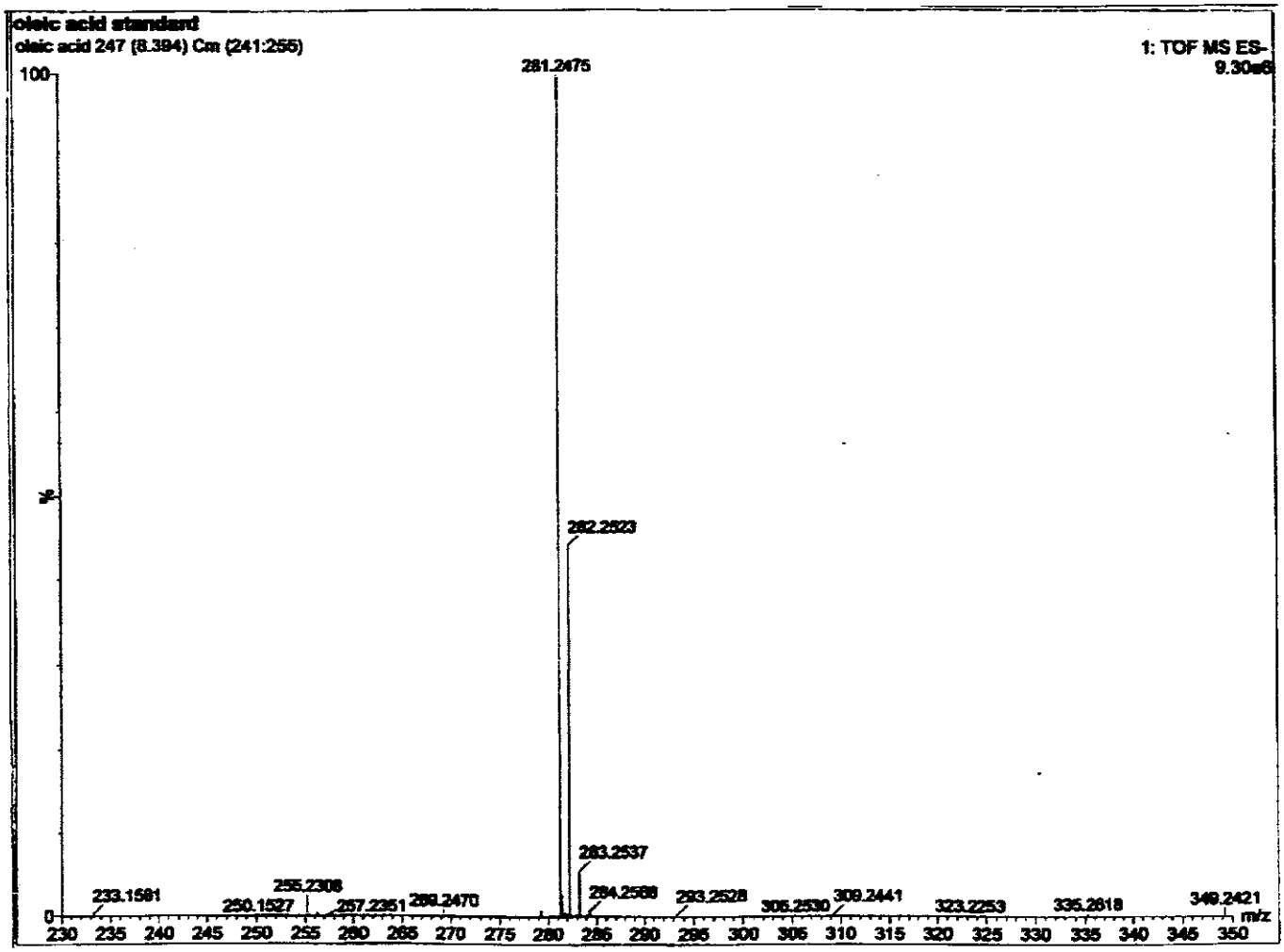
$N$ = number of molecules of glucose liberated by hydrolysis in each sugar ($n$ = 1 for sucrose and lactose, $n$ = 2 for maltose) [Rodriguez-Castilla *et al.*, 1996].

### B.C.2.3 ATPase [Vásárhelyi *et al.* (1986)]

The $Na^+/K^+$-ATPase activity of freeze-thawed samples was determined by measuring the release of inorganic phosphate (Pi) associated with the hydrolysis of ATP. The samples (250 µl) were added to 4750 µl of Reagent 1 [final concentration per litre: 100 mM of NaCl, 20 mM of KCl, 2.5 mM of $MgCl_2$, 0.5 mM of ethylene glycol tetraacetic acid (EGTA), 50 mM of tris-HCl (pH 7.4), 1 mM of ATP, 1 mM of phosphoenolpyruvate, 0.16 mM of nicotineamide adenine dinucleotide (NADH), 5 kU of pyruvate kinase, 12 kU of lactate dehydrogenase]. After 300 s, 65 µl of 10 mM ouabain was added to inhibit ouabain-sensitive ATPase activity. The $Na^+/K^+$-ATPase is composed of the stoichiometric of two obligatory major polypeptides, the α-subunit (~ 112 kDa) and the β-subunit (~ 45 kDa). The binding sites for ATP, cations and ouabain are localized in the α-subunit, which is responsible for the atalytic activity of the enzyme. The change in absorbance was monitored at 340 nm. The $Na^+/K^+$-ATPase activity was calculated from the difference in $A_{340}$.

# Appendix C

## OLEIC ACID STANDARD



# Appendix D

## CERTIFICATE FROM ETHICS COMMITTEE

University of Zululand Ethics Committee
C/O Dr Brent Newman
Department of Zoology
University of Zululand
Private Bag 1001
Kwadlangezwa
3886

27 July 2005

To whom it may concern

### ETHICS EVALUATION OF RESEARCH PROJECT PROPOSAL

This letter serves to confirm that Ms Ronalda McEwan (Student No 056257), registered for a Doctoral Degree in the Department of Biochemistry and Microbiology at the University of Zululand, in accordance with appropriate rules submitted a research project proposal to the Ethics Committee of the University of Zululand. The research project will investigate the Antinutritional constituent of *Colocasia esculenta* (Amadumbe), a traditional food crop in Kwazulu-Natal.

Based on the research protocol stipulated the above-said Ethics Committee could find no reason to reject the proposed research provided that relevant internationally accepted procedures pertinent to the maintenance and experimental treatment of laboratory held rats are adhered to. The students Supervisor and Co-supervisor, Prof AR Opoku and Prof T Djarova respectively, have indicated that appropriate procedures will be followed in this regard. The candidate is however advised to ensure that these procedures are correctly implemented, and that all facilities are available for inspection by members of the Ethics Committee from time to time. Should these facilities and procedures be found to be deficient in terms of internationally acceptable standards, the Ethics Committee, on behalf of the University of Zululand, reserves the right to call for the termination of experiments, which then may or may not be permitted to continue following rectification of relevant problems.

The candidate is also advised to consult relevant Medical Research Council of South Africa rules and regulations with regard to the use of animals for experimental purposes, particularly Guidelines on Ethics for Medical Research: Use of Animals in Research and Training, ISBN 1-919809-53-8, 2004. A copy of this document will be made available in electronic format by the Chairman of the Ethics Committee should this be required.

Yours sincerely
Brent Newman (PhD)
Chairman
Ethics Committee
University of Zululand

# Appendix E – Posters presented at conferences

## Antinutritional constituent of *Colocasia esculenta* (Amadumbe) a traditional crop food in Kwazulu-Natal

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### Objective

**To evaluate the natural toxic substances(the available literature abounds to nutritive values with little or no information as to Amadumbe's antinutrient constituent) and increase the knowledge of the antinutritional factors in Amadumbe which may provide one of the practical and most natural means for improving both the nutritional value and safety of the local Zululand diet.**

### Introduction

- Antinutrients are chemicals that evolved in plants to protect them from insects, plant pathogens and other organisms.
- Antinutritional factors include e.g., cyanogens, glycosides, saponins, phytate, enzyme inhibitors (trypsin and amylase inhibitors), lectins (haemagglutinins), oxalate and tannin, etc.
- Antinutrients are partly removed by technological processes and thus their acute toxicity is as a rule mild - however the health risk of the consumers of large quantities of food cannot be ruled out.
- Extensive studies of various antinutrients have revealed that although not necessarily toxic *per se*, are plant compounds which decrease the nutritional value of a plant food, usually by making an essential nutrient unavailable or indigestible when consumed by humans or animals. (Liener and Hasdai, 1986).
- These factors also have been linked to the aetiology of goiter, tropical ataxic neuropathy and endemic spastic paraparesis, nausea, bloating and vomiting (Liener, 1982).

- *Amadumbe* (*Colocasia esculenta*) is widely grown in the sub-tropical parts of South Africa as a traditional food crop. It is commonly used in rural communities in Zululand.
- The locally developed cultivars are a very good source of starch
  - λ the tuber are processed by baking, roasting or boiling it
  - λ the leaves are processed like spinach by boiling them for 15 minutes and are also used in making salad

### Methods

Amadumbe tubers collected from various locations in Zululand were screened for the presence of antinutrients:
Total phenol - Prussian blue method (Price and Butler, 1977)
Oxalate - Method Munro and Basir (1969)
Alkaloids - Method Cuadra *et al* (1994)
Trypsin inhibitor - Method of Kakade *et al* (1974)
Amylase inhibitor - Method of Bernfeld (1955)

### Results

| | Eshowe white | Eshowe purple | Mtubatuba white | Makuzini white | Makuzini purple |
|---|---|---|---|---|---|
| Total phenol | 11.5 mg/g | 13.0 mg/g | 13.3 mg/g | 8.0 mg/g | 14.0 mg/g |
| Oxalate | 0.13 mg/g | 0.10 mg/g | 0.07 mg/g | 0.08 mg/g | 0.07 mg/g |
| Alkaloids | 0.19 mg/g | 0.18 mg/g | 0.22 mg/g | 0.14 mg/g | 0.22 mg/g |
| Trypsin inhibitor | 19.7 mg/g | 16.5 mg/g | 20.6 mg/g | 17 mg/g | 19.3 mg/g |
| Amylase inhibitor | 21 mg/g | 25 mg/g | 26 mg/g | 31 mg/g | 33 mg/g |

### Conclusion

- At every level of government, food and nutrition are important issues to the health of the nation.
- Nutrition security is influenced by wide range of factors that may lead to inadequate or excessive nutrient intake or may impair utilization.
- From this screening it is concluded that Amadumbe like many other staple foods of the human diet such as grains, tubers and legumes contain antinutritional factors.
- The antinutrient levels are low and thus may not have an immediate effect on the human health - however their presence suggest that a steady consumption of them may lead to toxic levels
- Food safety and quality is secured through effective food quality control at all stages of production, processing and handling.

### Further work

- A selected group of toxicants in the amadumbe variety with the most antinutrients that is present with the preliminary screening, will be extracted and partially purified through various chromatographic methods [High Performance Liquid Chromatography (HPLC) and Thin layer chromatography (TLC)].
- The purified toxicants will then be further analysed to evaluate their molecular weight,composition and concentrations by using Mass Spectrophotometry.
- Nutritional evaluation of the selected antinutritional factors from *Colocasia esculenta* on rats.

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# α-Amylase Inhibitor of Amadumbe (*Colocasia esculenta*): Isolation, purification and selectivity toward amylases from various sources

University of Zululand

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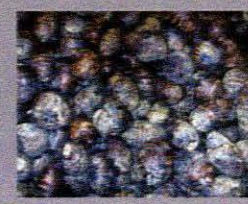
Department Microbiology and Biochemistry, University of Zululand, South Africa

## Introduction

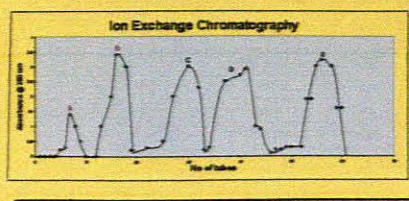
- Amadumbe (*Colocasia esculenta*) is widely grown in the sub-tropical parts of South Africa as a traditional food crop. It is commonly used in rural communities in Zululand.
- The locally developed cultivars are a very good source of starch - the tuber are processed by baking, roasting or boiling it.
- Like most plant foods *Phaseolus vulgaris* (kidney beans), cereals, rye, wheat, mangoes and Amadumbe contain antinutrients.
- Antinutrients are partly removed by technological processes and thus their acute toxicity is as a rule mild – howeverthe health risk of the consumers of large quantities of food cannot be ruled out.
- Amylase inhibitors are antinutrients that inhibit α-amylase activity in digesting starch.
- Highly concentrated versions of amylase inhibitors did show potential for reducing carbohydrate absorption in humans.
- High amounts of amylase inhibitors may cause diarrhea due to the effects of undigested starch in the colon (Whitney & Rolfes, 1994).
- Purified starch blocker extracts have also been shown to reduce the subsequent rise in blood sugar levels of diabetics.
- This paper describes the characteristics of the amylase inhibitor present and active in Amadumbe.

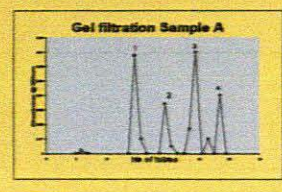
## Methods

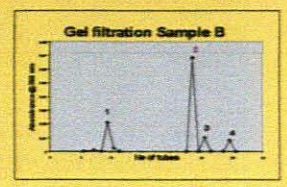
- The defatted flour was extracted with distilled water containing 1% polyvinylpolypyrolidone (PVP)
- The crude extract was subjected to ammonium sulphate precipitation and chromatographic (Diethyl amino ethyl (DEAE)- sephacel, G-100 Sephadex) purification.
- 0 – 0.5 NaCl gradient was introduced in the ion-exchange chromatography after tube 20
- Amylase activity was measured according to method described by Bernfeld (1955)
- Amylase Inhibitor is expressed as % decrease in amylase activity in the presence of the extract
- Amylase Inhibitor was characterised:
  - Action of inhibitors on different amylases
  - Action of inhibitors at different temperatures
  - Action of inhibitor using different pH with human salivary amylase
  - Molecular weight was determined by gel chromatography on Sephadex G-100



## Results and Discussion




α-Amylase Inhibitor Activity

| Action of inhibitors on different amylases | | | Action of inhibitors in different temperatures | | | Action of inhibitors using different pH with human salivary amylase | |
|---|---|---|---|---|---|---|---|
| Sources of amylase | % inhibition | | Temperature | % inhibition | | pH | % inhibition |
| | A | B | | A | B | | A |
| Human salivary | 82 | 56 | 20°C | 15 | 35.7 | 1 | 0 |
| Barley | 56 | 0 | 30°C | 0 | 17.9 | 2 | 0 |
| Sweet potato | 4.7 | 0 | 40°C | 13 | 28.5 | 3 | 0 |
| Bacillus species | 10.2 | 23 | 70°C | 0 | 2.87 | 4 | 17.2 |
| Aspergillus oryzae | 0 | 0 | 80°C | 0 | 0 | 5 | 14.2 |
| Porcine pancreas | 28.5 | 48.5 | 100°C | 0 | 0 | 6 | 33.8 |
| | | | | | | 7 | 7.3 |
| | | | | | | 8 | 1.8 |
| | | | | | | 9 | 0 |
| | | | | | | 10 | 0 |

### Discussion

- Two proteins (A, B) with α-Amylase inhibitor activity were obtained.
- Inhibitor A has a molecular mass of 17kDa; inhibitor B has a molecular mass of 19 kDa.
- Both proteins inhibited α-amylase of animal origin, but had no effect on fungal amylase.
- While inhibitor A exhibited activity towards plant amylases, inhibitor B has no activity on plant amylases.
- The inhibitors were most active (with human salivary amylase) at pH6.
- Inhibitor A was completely destroyed at temperatures above 50 °C, while inhibitor B was stable up to 70 °C.

## Conclusion

- The two α-amylase inhibitors isolated from Amadumbe are similar to well characterised plant amylase inhibitors(Marshall and Lauda, 1975)
- Their high activity exhibited on mammalian amylase emphasises the need to study the role of these inhibitors in human nutrition.

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University of Zululand

# Effect of Processing on Antinutritional Constituent of *Colocasia esculenta* (Amadumbe)

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*Colocasia esculenta*

## Introduction

- Amadumbe (*Colocasia esculenta*) is widely grown in the sub-tropical parts of South Africa as a traditional food crop. It is commonly used in rural communities in Zululand.
- The locally developed cultivars are a very good source of starch.
  the tuber are processed by baking, roasting or boiling it and the leaves are processed like spinach by boiling them for 15 minutes and are also used in making salad
- Like most plant foods *Phaseolus vulgaris* (kidney beans), cereals, rye, wheat, mangoes and Amadumbe contain antinutrients.
- Antinutrients are chemicals that evolved in plants to protect them from insects, plant pathogens and other organisms.
- Extensive studies of various antinutrients have revealed that although not necessarily toxic *per se*, are plant compounds which decrease the nutritional value of a plant food, usually by making an essential nutrient unavailable or indigestible when consumed by humans or animals. (Liener and Hasdai, 1986).
- These factors also have been linked to the aetiology of goiter, tropical ataxic neuropathy and endemic spastic paraparesis, nausea, bloating and vomiting (Liener, 1982).
- Antinutrients are partly removed by technological processes.

## Methods

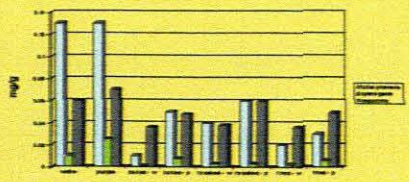
Amadumbe tubers collected from Eskhawini local market and processed in three different ways (boiled, roasted, fried) were screened for the presence of antinutrients:

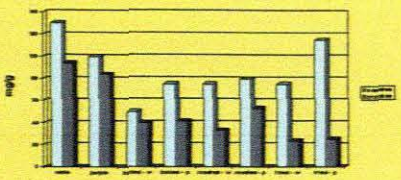
- Total phenol – Prussian blue method (Price and Butler, 1977)
- Oxalate – Method Munro and Bestr (1969)
- Alkaloids – Method Cuadra *et al* (1994)
- Cyanogens – Method Cooke (1978)
- Saponins – Method Fenwick and Oakenfull (1981)
- Phytate – Method Latta and Eskin (1980)

## Results

Processed Amadumbe chips

| Antinutrients | Unprocessed | | Boiled | | Roasted | | Fried | |
|---|---|---|---|---|---|---|---|---|
| | white | purple | white | purple | white | purple | White | purple |
| Total phenols | 11.5 mg/g | 13.0 mg/g | 7.1 mg/g | 9.8 mg/g | 10.0 mg/g | 12.2 mg/g | 10.8 mg/g | 14.0 mg/g |
| Alkaloids | 0.19 mg/g | 0.18 mg/g | 0.04 mg/g | 0.08 mg/g | 0.04 mg/g | 0.08 mg/g | 0.13 mg/g | 0.04 mg/g |
| Oxalates | 0.13 mg/g | 0.10 mg/g | 0.06 mg/g | 0.06 mg/g | 0.14 mg/g | 0.21 mg/g | 0.14 mg/g | 0.10 mg/g |
| Phytates | 3.1 mg/g | 2.8 mg/g | 1.3 mg/g | 1.4 mg/g | 1.1 mg/g | 1.9 mg/g | 0.9 mg/g | 0.9 mg/g |
| Cyanogens | 0.012 mg/g | 0.025 mg/g | 0.001 mg/g | 0.009 mg/g | 0.003 mg/g | 0.003 mg/g | 0.002 mg/g | 0.008 mg/g |
| Saponin | 0,138 mg/g | 0.145 mg/g | 0.058 mg/g | 0.052 mg/g | 0.079 mg/g | 0.10 mg/g | 0.123 mg/g | 0,095 mg/g |





## Discussion and Conclusion

- From the results obtained boiling is the best way of processing – antinutrients are washed away with water during boiling.
- Antinutrients evaporates during frying.
- From this screening it is concluded that Amadumbe like many other staple foods of the human diet such as grains, tubers and legumes contain antinutritional factors.
- The antinutrient levels are low and thus may not have an immediate effect on the human health – however their presence suggest that a steady consumption of them may lead to toxic level
- Processed Amadumbe shows a decrease in antinutritional factors

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