Cyanogenic Glycosides in Cassava

Ahmad Farid Mohd Azmi

Submitted in accordance with the requirements for the degree of Doctor of Philosophy

To

The University of Leeds

School of Food Science and Nutrition

May 2019

Declarations

The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

Publishable materials from this thesis. The list of manuscripts below are under preparation.

Azmi, A. F. M., Orfila, C. and Morgan, M. R. A. Synthesis and characterization of hapten-protein conjugates for polyclonal antibody production against linamarin.

Azmi, A. F. M., Orfila, C. and Morgan, M. R. A. Monoclonal antibody development against linamarin.

Azmi, A. F. M., Orfila, C. and Morgan, M. R. A. Development and application of an enzyme-linked immunosorbent assay (ELISA) for the quantification of linamarin, a cyanogenic glycoside in food.

Azmi, A. F. M., Orfila, C. and Morgan, M. R. A. Linamarin content in fresh cassava and processed products commercially available in the UK.

Conferences:

Azmi, A. F. **M.** and Morgan, M. R. A. Raising polyclonal antibodies against linamarin – a cyanogenic glycoside in cassava. *The 2nd Annual Food Science & Nutrition PhD Conference* – University of Leeds, Leeds, UK, November 16, 2015. (Poster presentation, 1st prize award)

Azmi, A. F. M. and Morgan, M. R. A. Raising polyclonal and monoclonal antibodies against linamarin – a cyanogenic glycoside in cassava .*The 3rd Annual Food Science & Nutrition PhD Conference* – University of Leeds, Leeds, UK, November 17, 2016.

Azmi, A. F. M. and Morgan, M. R. A. Cyanogenic glycoside content in fresh cassava and processed products commercially available in the UK. *The 4th Annual Food Science & Nutrition PhD Conference* – University of Leeds, Leeds, UK, November 22, 2017.

Azmi, A. F. M. and Morgan, M. R. A. Cyanogenic glycoside content in fresh cassava and processed products commercially available in the UK. *4th International Conference on Humanities, Social Sciences & Technologies*, University of Leeds, Leeds, UK, 25-26 November 2017.

Acknowledgement

First and foremost, I would like to thank Allah the Almighty for giving me the willpower to complete my thesis and for the continuous blessings I had despite many difficult situations I endured throughout this journey.

A very special thanks to my family especially my wife, Hajar for all the sacrifices she had to make, and our 4 lovely children, Aiman, Nuha, Aidin and Hana for their patience, support and love during the course of my study. My parents, Mohd Azmi Razak and Asmah Ahmad who never stopped supporting and inspired me to achieve success in whatever path I embarked. They have been a constant source of love, support and strength. Without their unconditional love this whole journey would be a huge mountain to overcome.

I would like to express my sincere gratitude to my supervisors Professor Mike Morgan and Dr Caroline Orfilla for their guidance, constructive comments and feedbacks, patience and support throughout the course of this research endeavour. Despite his busy schedule, Mike still managed to allocate 30-45 minutes of his valuable time twice a month for us to have a discussion on my work, sharing his broad knowledge and enthusiasm for science and love for cricket, rugby and football.

I would like to thank my past and present colleagues, Merfat Al-Maghrabi, Ukpai Anayochukwu Eze, Nur Liyana Sulaiman, Bridget Dooshima, Lucia Perez Hernandez, Marjorie Ladd Prada, Denise Li, Sam Hutchinson, Michael Houghton, Jose Villa, Ayyoub, Idolo Ifie, Liam Morris for their friendship, football sessions, good humour and helpful nature that not only helped creating a productive and excellent working environment, there are also plenty of room for

laughter and shoulders to lean on whenever any of us facing hardships and challenges.

I am grateful to all members of staff of the School of Food Science and Nutrition especially Angela Morrison and Rhiannon Reese for their endless assistance whenever I faced issues and problems, Catherine Roberts, Matthew Taylor, Miles Ratcliffe, Ian Hardy, Jo Burke, Sara Viney for their continuous help and assistance throughout my studies.

Abstract

Cassava is a well-known cyanogenic plant, which contains the cyanogenic glycosides linamarin and lotaustralin, the former being the principal cyanogenic glycoside. The ability to detect and quantify cyanogenic glycosides, capable of generating cyanide, could contribute to prevention of acute and subacute cyanide poisoning from the consumption of improperly processed plants. Enzyme-linked immunosorbent assay (ELISA) can be used to detect and measure the amount of linamarin directly within the fresh cassava or cassava-related products with minimal sample preparation using antibodies as the key detector. However, there has been no antibody and ELISA developed for linamarin detection in foods, hence the need. The aim of the project was to generate polyclonal antibodies (pAbs) obtained by immunizing two New Zealand white rabbits with linamarin (hapten) conjugated to a carrier protein, bovine serum albumin (BSA) using cyanuric chloride as chemical linker to be used with the ELISA. Monoclonal antibodies (mAbs) development was attempted by immunizing four BALB/c mice with linamarin-CC-KLH and 3 BALB/c mice with linamarin-CC-BSA but failed to produce the desired antibodies. PAbs generated by both immunised rabbits were highly immunoreactive with antiserum titre of 1:100,000. The pAbs were able to establish positive inhibition assay towards free linamarin with high specificity and sensitivity (limit of detection of 0.0015 µg/ml and IC_{50} of 2.1 µg/ml). The optimised ELISA was able to determine the amount of linamarin in fresh cassava and processed products available in the UK market, ranging from 0.003 mg/kg to 43.08 mg/kg fresh and dry weight depending on the products. Levels of linamarin of some fresh cassava products are way beyond the safe limit of 10 mg/kg cyanide allowed in food, a potential health risk to

consumers. In contrast, highly processed cassava products contained less than 1.0 mg/kg of linamarin, indicating the effectiveness of proper food processing and preparation in reducing the linamarin content in foods.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Ab/s	Antibody/antibodies
Ag/s	Antigen/s
Ag-Ab	Antigen-antibody
ATP	Adenosine triphosphate
ARfD	Acute reference dose
BSA	Bovine serum albumin
CC	Cyanuric chloride
CDR/s	Complementary-determining regions
CFA	Complete Freund's adjuvant
CIA/s	Chemiluminescent immunoassays
CIMS	Chemical ionization mass spectrophotometry
CG/s	Cyanogenic glycoside/s
CN-	Cyanide ions
DMSO	Dimethyl sulfoxide
EIA/s	Enzyme immunoassays
EIMS	Electron impact mass spectrophotometry
ELISA	Enzyme-linked immunosorbent assay
FABMS	Fast atom bombardment mass spectrophotometry
Fab	Fragment antigen binding
Fc	Fragment crystallisable
FIA/s	Fluorescent immunoassays
Fv	Antigen-binding site
GCMS	Gas chromatography mass spectrophotometry
H	Heavy polypeptide chains
HAT	Hypoxthine-aminoterin-thymine
HC1/2/3	Heavy-constant 1/2/3
HCN	Hydrogen cyanide
HGPRT	Hypoxantine-guanine-phosphoribosyltransferase
HNMR	Hydrogen nuclear magnetic resonance
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
HV	Heavy-variable
IcELISA	Indirect competitive enzyme-linked immunosorbent assay
IEMA	Immunoenzymatic assay
IFMA	Immunofluorometric assay
IncELISA	Indirect non-competitive enzyme-linked immunosorbent
IFA	Incomplete Freund's adjuvant
IgG	Immunoglobulin G (gamma)
IgM	Immunoglobulin M (mu)
IgA	Immunoglobulin A (alpha)
IgD	Immunoglobulin D (delta)
IgE	Immunoglobulin E (epsilon)
IRMA	Immunoradiometric assay
KLH	Keyhole limpet haemocyanin
L	Light polypeptide chains
LC	Light-constant
LCMS	Liquid chromatography mass spectrophotometry
LoD	Limit of detection

LoQ	Limit of quantification
LSIMS	Liquid secondary ion mass spectrophotometry
LV	Light-variable
mAb/s	Monoclonal antibody/antibodies
MHC	Major histocompatibility complex
MST	Mercaptopyruvate sulphurtransferase
NIRS	Near infrared spectroscopy
OD	Optical density
OVA	Ovalbumin
OVO	Ovomucoid
pAb/s	Polyclonal antibody/antibodies
PBS/T	Phosphate buffer saline / Tween
PPD	Post-harvest physiological deterioration
RIA/s	Radioimmunoassays
SAA	Sulphur-containing amino acid
TAN	Tropical ataxic neuropathy
TDI	Total daily intake
TLC	Thin layer chromatography
TMB	Tetramethylbenzidine
TNBS	Trinitrobenzene sulfonic acid
UV	Ultraviolet
v/v	Volume to volume ratio
w/v	Weigh to volume ratio

Chapter 1 : Introduction

1.1 Cassava (*Manihot esculenta* Crantz)

1.1.1 Importance of cassava

Cassava (*Manihot esculenta* Crantz), also commonly known as tapioca, manioc or yuca, is an important economical and nutritional perennial woody tall shrub (Figure 1-1) cultivated and domesticated from one or more species of genus *Manihot*, belonging to the Euphorbiaceae family (Allem, 2002; McKey *et al*. 2010), native to South America. The genus Manihot comprises 98 species of which *M. esculenta* is the most widely cultivated member (Rogers and Appan, 1973; Nassar *et al*., 2008). It ranks fourth as a major food crop after rice, wheat and maize and the productivity of cassava in terms of calories per unit land area per unit of time is significantly higher than other staple food crops (Nambisan, 2011; Latif and Müller, 2014).

Figure 1-1: Cassava tree and roots. a) Cassava tree, b) cassava root (whole), c) cross section and d) peel and cortex.

The drought tolerance of cassava, combined with high yield on poor soils and the possibility to leave the starch-rich tubers in the soil for extended time periods thus enabling harvest on demand, renders the increasing use of cassava as a food source where in the past thirty years, the area under cultivation has doubled and is expected to increase further (Nweke *et al*., 2002; Chauynarong *et al.* 2009; Burns *et al*., 2012). Besides high yields, the plant has enormous potential for accumulation of starch, ability to produce moderate yields in marginal lands (semi-arid tropic: < 600 mm, sub-humid and humid-tropics: > 10,000 mm of rain per year) and is tolerant to diseases and pests (De Tafur *et al*., 1997; Pellet and El-Sharkawy, 1997). In terms of yield it is the most productive crop per unit land area especially when cultivated under tropical conditions (warm climate, mean day temperature of > 20°C, optimum leaf temperature between 25-35°C) (El-Sharkawy *et al*., 1992a), with a yield of between 25 and 60 tonnes/ha (L/ha = 10,000 m^2) (Garcia and Dale, 1999).

These attributes make cassava a favoured crop for both, small scale farmers and large scale plantation as it requires low input of time, labour and money hence often regarded as a valuable food security crop and an insurance against famine (Nambisan, 2011, Latif and Müller, 2014). The estimated total of world cassava production was 228 million tonnes in 2007 (FAO, 2008) and in 2012 it reached 276.7 million tonnes, an approximate of 10 million tonnes (1 tonne = 1000 kg) annual production, contributing to almost 40% production increase since 2000 according to the FAO (2013) and FAO (2014). By 2020 the production of cassava is predicted to reach an estimate of 291 million tonnes (Scott *et al*., 2000, FAO, 2014). However, in recent report by FAO, 2018, the production of cassava have

been sluggish due to changes in policies and incentives favouring cultivation of other crops in Africa and uncertainty of cassava sectors in Asia as they are strongly susceptible to developments in China, the principal destination for internationally traded cassava products. In 2008/2009 about 51% of cassava in the world was produced in Africa, 35% in Asia and only 14% in South America and the Caribbean (Howeler, 2017) and from that almost 70% of world's cassava production alone comes from five countries, namely Nigeria, Democratic Republic of Congo (Africa), Brazil (South America), Thailand and Indonesia (South East Asia) (Chauynarong *et al*., 2009, Burns *et al*., 2010).). It provides an important source of carbohydrates for more than 800 million people worldwide being extensively cultivated in nearly 105 countries mostly in tropical and subtropical regions of sub-Saharan Africa, South East Asia and South America, with an estimated total cultivated area greater than 13 million hectares and partly due to the amount of energy it yields per hectare is evidently more than other staple food crops. (El-Sharkawy, 2003; Montagnac *et al*., 2009a).

1.1.2 Nutritional value of cassava roots and leaves

About 70% of cassava tubers/roots and leaves (to a lesser extent) are edible, mainly used for human consumption either directly after cooking or in processed forms/products (Westby, 2002); the remaining 30% is used for animal feed and other industrial products such as starch, glucose and alcohol (El-Sharkawy, 2004). Tapioca, a commercially important starch product common in the United States and in South East Asia, mainly used in drinks and desserts is one of the products of cassava roots whereby the leaves are usually cooked as green vegetables (Dahniya, 1994; Moyo *et al*., 1998) normally served as part of a

sauce, eaten with starchy dishes like chikwange, cassava fufu, boiled cassava roots, rice and pounded yam (Latif and Muller, 2015). The nutritional values of cassava root and leaves have been extensively studied by several authors and recently reviewed by Montagnac *et al*. (2009a), Morgan and Choct (2016) on cassava as a whole and Latif and Muller (2015) mainly on cassava leaves.

Table 1-1: Proximate, vitamin and mineral composition of cassava roots and leaves.

	Raw cassava (Edible portion only)	Cassava roots	Cassava leaves
Proximate composition (g per 100g)			
Food energy (kcal)	160	110 to 149	91
Food energy (KJ)	667	526 to 611	209 to 251
Moisture (g)	59.68	45.9 to 85.3	64.8 to 88.6
Dry weight (g)	40.32	29.8 to 39.3	19 to 28.3
Protein (g)	1.36	0.3 to 3.5	1.0 to 10.0
Lipid (g)	0.28	0.03 to 0.5	0.2 to 2.9
Carbohydrate, total (g)	38.06	25.3 to 35.7	7 to 18.3
Dietary fiber (g)	1.8	0.1 to 3.7	0.5 to 10.0
Ash	0.62	0.4 to 1.7	0.7 to 4.5
Vitamins			
Thiamin (mg)	0.087	0.03 to 0.28	0.06 to 0.31
Riboflavin (mg)	0.048	0.03 to 0.06	0.21 to 0.74
Niacin (mg)	0.854	0.6 to 1.09	1.3 to 2.8
Ascorbic acid (mg)	20.6	14.9 to 50	60 to 370
Vitamin (mg)	-	5.0 to 35.0	8300 to 11800
Minerals			
Calcium (mg)	16	19 to 176	34 to 708
Phosphorus, total (mg)	27	6 to 152	27 to 211
Ca/P	0.6	1.6 to 5.48	2.5
Iron (mg)	0.27	0.3 to 14.0	0.4 to 8.3
Potassium (%)	-	0.25 (0.72)	0.35 (1.23)
Magnesium (%)	-	0.03 (0.08)	0.12 (o.42)
Cooper (ppm)	-	2.00 (6.00)	3.00 (12.0)
Zinc (ppm)	-	14.00 (41.00)	71.0 (249.0)
Sodium (ppm)	-	76.00 (213.00)	51.0 (177.0)
Manganese (ppm)	-	3.00 (10.00)	72.0 (252.0)

Adapted from Montagnac *et al*. 2009a

The roots are twice as rich as the leaves in carbohydrates, but the leaves contain more protein, lipid, minerals, vitamins and fibre. The total protein content in cassava leaves is 5 to 10 times higher than in roots and is comparable with the protein content of an egg based on grams of nitrogen (Latif and Muller, 2015). The lipid content is 10 times higher in leaves than in roots. Although the lipids and lipid-soluble components such as photosynthetic pigments are much more concentrated in leaves, some of them, such as volatile fatty acids, do not bring significant energy to the diet. Therefore, the energy density of the lipid is lower in leaves than in roots. The mineral content of cassava leaves is 2 to 5 times higher than of the roots. The roots typically have more phosphorus, but the leaves have a greater concentration of calcium (Gil and Buitrago, 2002). The calcium content in the leaves is 100 times higher than in roots and the phosphorus content is 2 to 3 times higher in roots than in the leaves. Cassava leaves are more concentrated than the roots in vitamins, and the minerals iron, potassium, magnesium, copper, zinc and manganese. Overall, their nutritional value is, consequently, lower than those of cereals, legumes and some other root and tuber crops in terms of mineral, vitamin, lipid and protein contents but the leaves are well provided in these, and should be added to diet consisting mainly of roots (Latif and Muller, 2015) or perhaps from other food plants or sources (El-Sharkawy, 2004), which sometimes hard to come by in certain areas plague with famine and drought.

1.1.3 Antinutritional aspects of cassava roots and leaves

Given the comprehensive reports on the nutritional value of cassava, it appears that cassava roots are a good energy dense source of carbohydrate meanwhile cassava leaves provide good sources of minerals, vitamins and fibre for human and animal consumption. Despite the positive attributes, it has a number of serious limitations as reported by Burns *et al*. (2009). Firstly, cassava roots are not tubers and therefore cannot be used for reproductive purpose (Ceballos *et al*., 2004). As a major consequences of this, Beeching *et al*. (1998) reported the problem of rapid post-harvest physiological deterioration (PPD) of the roots within one or two days following removal from the soil, which limits its marketability unless they are immediately processed or consumed (van Oirschot *et al*., 2000; El-Sharkawy, 2004; Lebot, 2009). Secondly, the roots are low in protein and some essential micro-nutrients which would results in unbalanced diet, thus, additional requirements for protein and other essential nutrients are commonly fulfilled by other food sources (El-Sharkawy, 2004), for instance, the cassava leaves (Latif and Muller, 2015). Thirdly, cassava contains a number of bioactive products that are harmful to human health. Antinutritional factors such as tannins, polyphenols, phytic acid and high fibre content could reduce nutrient bioavailability, nutrient uptake and digestibility and the presence of cyanogenic glycosides (CGs), may eventually lead to toxic exposure depending on the processing method and amount consumed (Montagnac *et al*., 2009b; Wobeto *et al*., 2007). The level of antinutrients also varies depending on the maturity status, climate condition and variety of cassava as reported by Wobeto *et al*. (2007).

In contrast, depending on the ingested amount, some of these compounds can either act as antioxidants and to some extent, anticarcinogens (Wobeto *et al*., 2007). For example, polyphenols found in the leaves are usually considered as antioxidants but they also bind with essential minerals and make them unavailable for absorption (Latif and Muller, 2015). In term of protein digestibility in young and old leaves, it was found to be 80% and 67% respectively (Bokanga, 1994). This reduction may be attributed to the condensed tannin in cassava leaves due to the indigestible tannin-protein complexes or negative effect on the enzyme activity (Lancaster and Brooks, 1983). Massey (2007) reported antinutritional property of oxalate, a simple dicarboxylic acid [$(COO)2^{2-}$] found in cassava leaves, which negatively affects the bioavailability of magnesium and calcium as the oxalates bind to the calcium and excreted through urine or formed crystals which might cause kidney stones. However, the negative effect of oxalates on human health depends on the oxalate and calcium levels as Wobeto *et al*. (2007) reported the calcium-to-oxalate ratio in five cassava cultivars was lower than the critical limit of calcium uptake. Therefore, the oxalate levels in cassava leaf may not negatively affect the calcium uptake present in cassava leaves.

Above all, the major concern among these antinutrients are the presence of CG, one of the plant secondary metabolites, which could release toxic hydrogen cyanide (HCN) in a process known as cyanogenesis. The levels of CGs content in cassava have been long associated with the bitter taste by several studies (Sundaresan *et al*., 1987; King and Bradbury, 1985; Bokanga, 1994). The bitter taste is often used by cassava farmers to predict toxicity of single raw roots by tasting the tip of the root parenchyma (Chiwona-Karltun *et al*. 2004), enabling

them to classify cultivars that are toxic and require extensive processing and the less toxic cultivars, mostly referred to as ‘cool’, ‘sweet’ or ‘non-bitter’ cassava. However, this relationship was still debatable as some studies failed to prove the association (Sinha and Nair, 1968; Pereira *et al*., 1981) but a recent comprehensive study by Chiwona-Karltun *et al*. (2004) found a strong correlation between bitterness and glycoside level which is consistent with the majority of the earlier studies. Studies indicated that roots from bitter cassava cultivars are preferred for making flour (Nweke, 1994) and it produces products that have superior taste and texture qualities (Dufour, 1993; 1994). In many parts of Africa, farmers who are prone to food insecurity preferentially grow ‘bitter’ cassava cultivars that yield toxic roots as it improves food security by conferring protection from theft and animal destruction (Kapinga, 1997). The methods for processing cassava that have evolved in Africa have been adapted to remove the toxicity and suit the local preferences of the desired food products (Lancaster *et al*., 1982). However, in other parts of the world where consumption of cassava occur occasionally, lack of knowledge in cassava toxicity and preparation could become a serious food safety issue. Cassava consumption can lead, therefore, to chronic health problems and acute cyanide poisoning unless the food products are appropriately processed.

1.2 Plant secondary metabolites

A characteristic feature of vascular plants is their capacity to synthesize an enormous variety of low molecular weight chemical compounds, the so-called secondary metabolites. Secondary metabolites are plant-produced organic compounds that play an important role in the defence of plants against herbivorous animals including insects and vertebrates, invading pathogens (bacteria or viruses), fungi growth as well as in their adaptation to the environment as such competing for better light source, water uptake and nutrients, although they are not directly involved in the growth and development of organisms (Bennett and Wallsgrove, 1994; Fürstenberg-Hägg *et al*., 2013; Sakamoto *et al*., 2017). More often, they serve as key components of active and potent chemical compounds and depending on plant species, they may be present in the whole plant or compartmentalized in seeds, fruits, leaves or stalks. According to a review by Wittstock and Gershenzon (2002), parts of the plant that are highly exposed and within reach of insects, herbivores or other predators have the tendency to synthesise and accumulate high levels of toxic compounds (mostly attributed to plant secondary metabolites) compared to parts that are well or partly hidden and out of reach. Tens of thousands of these secondary metabolites have already been isolated and their structures determined (Table 1-2) by various analytical methods such as mass spectrophotometry (electron impact, EIMS, chemical ionization/gas CI/GCMS, fast atom bombardment/liquid secondary ion, FAB/LSIMS), nuclear magnetic resonance (H-NMR, 13C-NMR), X-ray diffraction (Harbone, 1993), and high performance liquid chromatography (HPLC) (Sornyotha *et al*, 2007) to name a few.

Table 1-2: Number of known secondary metabolites of higher plants

Compounds	No. of structures
Cyanogenic glycosides	50
Sesquiterpenes	1,500
Tetraterpenes	350
Triterpenes/steroids	800
Diterpenes	1,000
Glucosinolates	100
Polyacetylenes	750
Amines	1,00
Phenylpropanoids	500
Flavonoids	1,200
Alkaloids	7,000
Nonprotein amino acids	400
Monoterpenes	1,000
Polyketides	700

(Adapted from Wink, 1988)

The specific odours, tastes and colours of plants are mostly contributed by these secondary metabolites which are distinct from the intermediate and products of primary metabolism that could be distinguished according to their family and species. Such restricted distribution of compounds enable them to be used as taxonomic markers (Bennett and Wallsgrove, 1994). They usually behave by altering specific mechanisms involving enzymes, receptors and genetic targets in particular cells and tissues and their formation is usually organ-, tissue-, cell- and often development-specific regulated by their respective genes (Wink, 1988, 1999). These secondary metabolites are therefore ultimately important for the fitness and survival of the plant producing them (Wink, 1999). Although not all plant secondary metabolites are toxins, this study focused on the secondary

metabolites that are, particularly CGs present in cassava. The toxins proved to be toxic to humans, animals and insects when they come into contact with them either internally or externally.

1.2.1 Plant toxins in plants

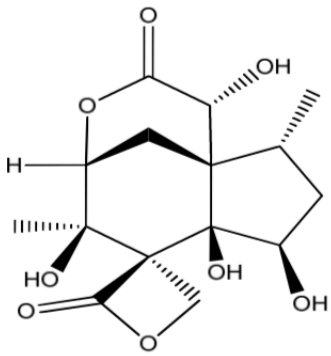
Most plants possess multiple lines of defence to avoid being eaten; these include physical methods such as hairs and spines to impale potential herbivores and chemical methods targeting the nervous, reproductive or digestive systems of potential herbivores as reviewed by Petersen (2011). Plant toxins are naturally-occurring chemical of secondary metabolites that may be poisonous to pathogens, herbivores and humans. They provide protection for plants from predators by acting as deterrent against herbivores, insects and pathogens as represented in Table 1-3.

Table 1-3: Chemical defence strategies of plant secondary metabolites

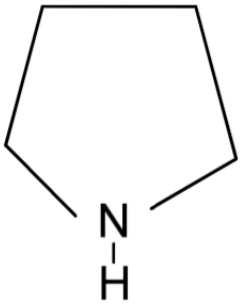
Compound	Defence Mechanism
Alkaloids	Toxic for vertebrates, arthropods, bacteria
Cyanogenic glycosides	Toxic for animals
Flavonoids/anthocyanins	Antimicrobial, insectistatic
Glucosinolates	Repellent for animals, antimicrobial
Non-protein amino acids	Toxic for animals, antimicrobial
Phenylpropanoids	Antimicrobial
Terpenes	Repellent for animals, antimicrobial

(Adapted from Wink, 1988 and Bolarinwa, 2013)

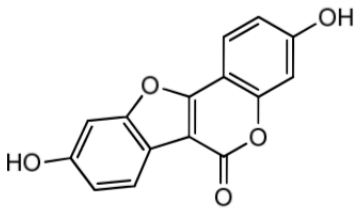
They may be present in the whole plant or concentrated in vital regions that are usually prone to animal disturbance such as seeds, fruits, leaves and stalks. According to Chandra (2012), the poisonous constituent may be concentrated in one or more parts of the plant depending on plant species. These toxins, if consumed or touched, are toxic to humans, animals and insects. Plant toxins are classified based on their structural and chemical constituents; alkaloids, glycosides, proteins, oxalates, anti-vitamins, tannins, volatile ethers, phytoestrogens, terpenes, glucosinolates and phenylpropanoids (Wink, 1988; Chandra *et al*., 2012). Structures of examples of plant toxins are shown in Figure 1-2.



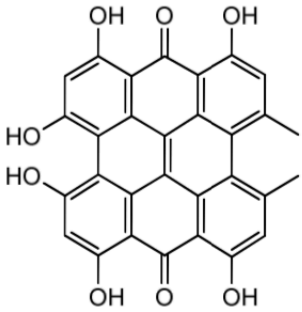
Anisatin (found in Japanese anise plant)



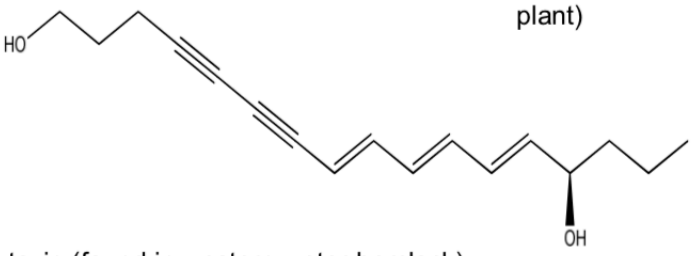
Pyrrolizidine (found in the leaves of tobacco and carrot)



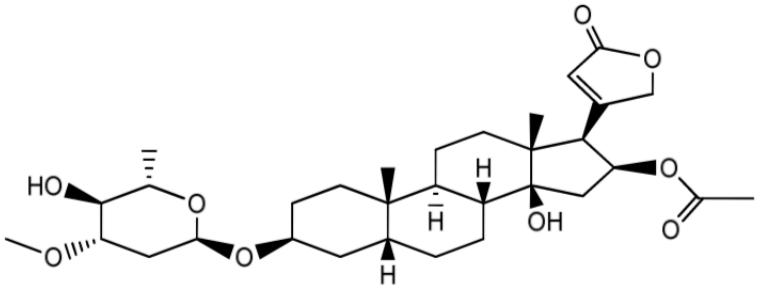
Coumestrol (found in alfalfa, red clover and soybeans)



Hypericin (found in St John's wort plant)



Cicutoxin (found in western water-hemlock)



Oleandrin (found in nerium oleander and Laurier rose plant)

Figure 1-2: Chemical structure of some of the plant toxins.

Examples of specific natural plant toxins from each class of plant toxin are listed in Table 1-4:

Table 1-4: Plant toxins categories based on their chemical constituents and toxic effect

Chemical constituents	Chemical compounds	Toxin name	References
Nitrile	Alkanenitriles	Cyanogenic glycosides	
	α, β-unsaturated nitriles	Samentosin	Flemming, 1999
	Aromatic nitriles	Cytotoxin	
Sulphur		Glucosinolate	Halkier & Gershenzon, 2006
C17 conjugated polyacetylene		Cucitoxin	Schep *et al*., 2009
		Oenanthotoxin	
Glucosides	Methylazoxymethanol	Cycasin	Wogan & Busby, 1980)
Ester derivatives	1-hydroxymethyl-1 2-dehydropyrrolizidine	Pyrrolizidine alkaloids	Wogan & Busby, 1980)
Toxic effects			
Carcinogens	Pyrrolizidine alkaloids	Cycasin	
Oxidative phosphorylation inhibition	Cyanogenic glycosides	Hydrogen cyanide	Wajant & Mundry, 1994; Hamel, 2011)
Cellular membrane disruption		Saponin	Osbourn, 1996
Stimulatory effect of central nervous system		Cicutoxin	Schep *et al*., 20009
		Oenanthotoxin	
Growth inhibitor		Glucosinolate	Ulmer *et al*., 2001

(adapted from Bolarinwa *et al*., 2013)

Several plant species are cultivated from food and many plants or plant parts (seeds, leaves, fruits) are consumed as food. Seeds are important sources for animals including humans because of their high nutrient contents (starch, protein, lipids, fibre, vitamins and minerals). Leaves and fruits are good sources of vitamins and minerals for humans. Although animals including humans depend on plants for sustenance, plant foods contain quite a large numbers of naturally-occurring toxins that are harmful to both animals and humans.

1.2.2 Cyanogenic glycosides

Cyanogenic glycosides (CGs), classified as phytoanticipins, are the most important defence-related secondary metabolites and a class of natural plant toxin mainly found in plant foods. Phytoanticipins are of low molecular weight, antimicrobial compounds that are present in plants before challenge by microorganisms or are produced after infection solely from pre-existing constituents (VanEtten *et al*., 1994). There are approximately 25 known CGs and these are generally found in edible parts of the plants, particularly in the seed of such fruits (Bolarinwa *et al*., 2016; Cressey and Reeve, 2019) and they are widely distributed in the plant kingdom, being present in more than 2,650 species (Conn, 1980; Poulton., 1990; Møller, 1999; Bak *et al*., 2006, Ganjewala *et al.*, 2010; Zagrobelny *et al*., 2018), commonly found in certain families belong to the Angyospermatophyta and remaining to the Dicotyledonopsida and Monocotyledonopsida. This includes economically important crop and edible plants as listed in Table 1-5.

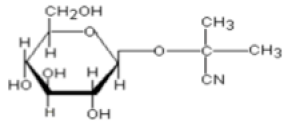
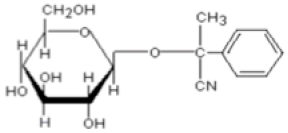
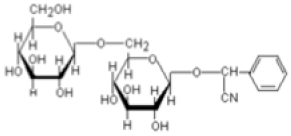
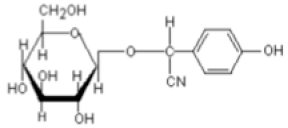
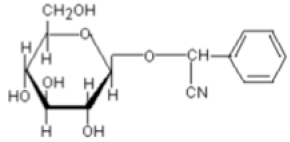
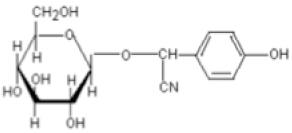
Table 1-5: Cyanogenic glycosides and its occurence in major food plants

Cyanoglucosides	Sugar moiety	Food plants
Linamarin	D-glucose	Cassava (Manihot esculenta) Lima beans (Phaseolus lunatus) Flaxseed (Linum usitatissimum) Kidney beans (Phaseolus vulgaris) Oats (Avena sativa)
Dhurrin	D-glucose	Wheat (Triticum aestivum) Rye (Secale cereale) Sorghum (Sorghum bicolor) Macademia nut (Macademia ternifolia)
Prunasin	D-glucose	Apple (Malus pumila) Quince (Cydonia oblonga) Passion fruit (Passiflora edulis)
Taxiphyllin	D-glucose	Bamboo shoots (Bambusa vulgaris)
Amygdalin	Gentiobiose	Almond (Prunus dulcis) Apricot (Prunus armeniace) Peaches (Prunus persica) Plums (Prunus domestica) Sweet cherry (Prunus avium) Sour cherry (Prunus cerasus)
Triglochinin	D-glucose	Millets (Eluesine coracana) Taro (Colocasia esculenta) Giant taro (Alocasia macrorrhizos)
Lucumin	Primeverose	Sapote (Pouteria sapota)

(Adapted from Jones, 1999; Vetter , 2000; Haque and Bradbury, 2002); Codex Committee in Foods (CCCF), 2008; Donald, 2008; Bolarinwa *et al*., 2016)

CGs are β-glucosides of α-hydroxynitrile (the aglycones) (Bjarnholt and Møller, 2008) biosynthesised from mainly five amino acids; the aliphatic protein amino acids L-valine, L-isoleucine and L-leucine and the aromatic amino acids L-phenylalanine and L-tyrosine, and from the cyclopentanoid non-proteinogenic amino acid, 2-(2'-cyclopentenyl)-glycine (Zagrobelny *et al*., 2004; Zagrobelny *et al*., 2018), and consist of a sugar moiety, mostly D-glucose, stored in the vacuoles (Flemming, 1999; Vetter, 2000). The structure arrangement includes a core carbon attached to a -CN moiety and two substitute groups (R1 and R2) and attached to a sugar, either a monosaccharide (glucose) or a disaccharide (gentiobiose) by a glycosidic bond. R1 may be a methyl group of a phenyl or p-hydroxyphenyl group. R2 is most commonly hydrogen but may also be a methyl or ethyl group (Cressey and Reeve, 2019). Accordingly, CGs are defined as aliphatic, aromatic or cyclopentenoid, based on their parent amino acids (Vetter, 2000; Zagrobelny *et al*., 2018). For instance, the two aliphatic CGs, linamarin and lotaustralin are derived from valine and isoleucine; the three aromatic CGs, dhurrin is derived from tyrosine, amygdalin and prunasin are derived from phenylalanine (Seigler, 1975; Conn, 1979), while cyclopentenoid CGs are derived from cylopentenyl glycine (Bjarnholt and Møller, 2008). Therefore, it is immediately apparent that there is a high degree of structural relatedness within this group of compounds. The structure of some CGs and their sugar moiety is shown in Table 1-6.

Table 1-6: The chemical structure of cyanogenic glycosides in major edible plants and their sugar moiety

Configuration	Glycoside	Sugar
	Linamarin	D-glucose
	Lotaustralin	D-glucose
	Amygdalin	Gentiobiose
	Dhurrin	D-glucose
	Prunasin	D-glucose
	Taxiphyllin	D-glucose

1.2.2.1 Functions of cyanogenic glycosides in plants

Significant numbers of CGs are produced in plants to mediate both general and specialised functions. Their primary function in plants is dependent on activation by β-glucosidase enzyme to break down and release toxic volatile HCN as well as a ketones or aldehydes to fend off herbivore and pathogen attack (Møller, 2010), hence dictating the plant-insect interactions and co-evolution as comprehensively reviewed by Zagrobelny *et al*., (2004). CGs have also been described as nitrogen storage compounds (Forslund and Jonsson, 1997; Busk

and Møller, 2002), nitrogen metabolism and transport (Lieberei *et al*., 1985; Selmar, 1993; Gleadow *et al*., 1998) and the control of germination (Esashi *et al*., 1996). They also serve as phagostimulants towards herbivores that specializes on them (Gleadow and Woodrow, 2002). Ressler *et al*. (1969) reported the degradation products of CGs such as β-cyanoalanine, possess potent neurotoxin property that serve to deter predators and is a catabolic product in some plants.

Furthermore, identification of these constituents in plants may serve as a useful tool for informative taxonomic markers (Vetter, 2000). In general, the functions and level of CGs in plants depends on the age and variety of the plant as well as the ecosystem, biotic and abiotic challenges in their surroundings. For instance, Oluwole *et al*. (2007) reported that cassava crops grown in low altitude areas contain high levels of CGs while those grown in high altitude area contain low levels of CGs. Levels of CGs in different parts of the plants vary and also vary between the same parts of different individual plants of the same species (Oluwole *et al,* 2007). While high concentrations of CGs are usually found in plant leaves, CGs may also be concentrated in plant roots, seeds or other plant tissues (Seigler, 1975).

Table 1-7 lists the major CGs of food plants and their cyanogenic potential where they usually act as natural pesticides to protect crops against animal pests.

Table 1-7 Major cyanogenic glycosides of food plants and their cyanogenic potential

Food plants	Scientific name	Plants part	Major cyanogenic glycosides present	Cyanogenic content (mg/kg fresh weight)
Cassava	*Manihot esculenta*	Leaves, roots	Linamarin	200-1300
Flax	*Linum usitatissimum*	Seed meal	Linamarin, linustatin, neolinustatin	360-390
Lima beans	*Phaseolus lunatus*	Seed	Linamarin	2000-3000
Sorghum	*Sorghum vulgare*	Leaves	Dhurrin	750-790
Apple	*Malus spp.*	Seeds	Amygdalin	690-790
Peach	*Prunus persica*	Kernels	Amygdalin	196-209
Apricot	*Prunus armeniace*	Kernels	Amygdalin	400-4000
Bitter almond	*Prunus dulcis*	Pits	Amygdalin	2900
Giant taro	*Alocasia macrorhizzos*	Leaves	Triglochinin	29-32
Bamboo	*Bambusa arundinacea*	Young shoots	Taxiphyllin	100-8000

(Adapted from Haque & Bradbury, 2002; Simeonova & Fishbein, 2004; Zhang *et al*., 2014)

However, the effectiveness of CGs as herbivore deterrent is not universal as described by Gleadow and Woodrow (2002). From the findings, Møller (2010) has outlined five important reasons that need to be considered whenever effectiveness of CGs as plant's defence mechanism is concerned:

i. The importance of CG concentration and its large variability between individual plants in a population.
ii. The importance of avoidance of CG containing plants when alternative feed sources are available.

iii. Importance of feeding style to avoid release of toxic HCN.

iv. Plant material containing CGs may not be cyanogenic due to lack of β-glucosidase activity required for HCN release.

v. Specialized herbivores and pathogens have coevolved to circumvent cyanogenesis based plant strategies.

1.3 Linamarin – a cyanogenic glycoside in cassava

Cassava, is a well-known staple plant which provides major source of calories in the tropics where its roots could be processed into varieties of other delicacies (Agbor-Egbe and Mbome, 2006; Aloys, 2006). It contains both linamarin (2-β-D-glucopyranosyloxy-2-2methylpropiononitrile) and lotausralin ([(2R)-2-β-D-glucopyranosyloxy-2-methylbutyronitrile]) at a ratio of 93:7, the former being the principal cyanogenic glycoside (CG) (Nartey, 1968; Cock, 1984). The linamarin and lotaustralin, its methyl variant, have a relatively broad distribution in the plant kingdom, having been demonstrated in the following plant families: Compositae, Euphorbiaceae, Linaceae, Papaveraceae and Fabaceae (Leguminosae) (Vetter, 2000). Linamarin (95~97% of total cyanogen content) contributes to the bitter taste of fresh cassava roots (Sundaresan *et al*., 1987), which may function as a feeding deterrent (Poulton, 1990) and is found in all parts of cassava except the seeds (McMahon *et al*., 1995). Both linamarin and lotaustralin are synthesised in the leaves of cassava plants and transported to the tuberous roots, probably in the phloem and some additional synthesis in the periderm of the tubers (Koch *et al.*, 1992; Jørgensen *et al*., 2005, Burns *et al.*, 2010). Their levels vary considerably between tissues within individual plants, among cultivars and with environmental conditions (Bruijin, 1971; Ramanujam *et al*., 1984; Nambisan and Sundaresan, 1994). The highest concentrations are in the young leaves, newly germinated seedlings and the outer layer of the tuber (Jørgensen *et al*., 2005), consistent with its primary function in herbivore defence (Belloti and Riis, 1994, Kakes, 1994, Gleadow and Woodrow, 2002).

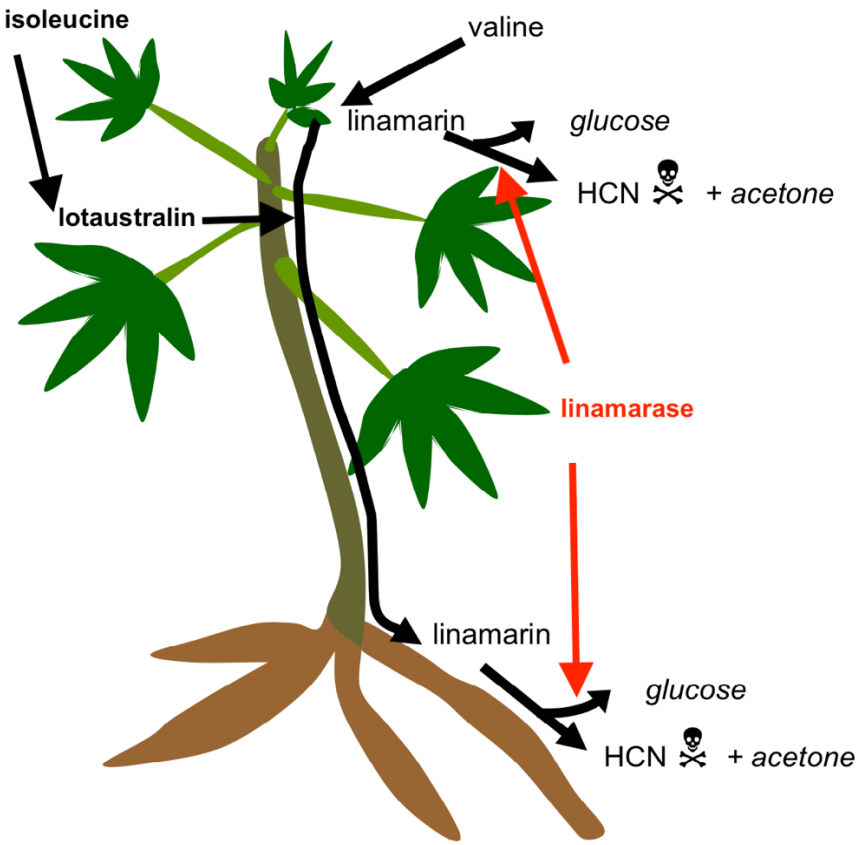


Figure 1-3: Linamarin (and lotaustralin) synthesis, distribution and degradation in cassava plant.

(Adapted from Burns *et al*. 2010)

Cyanide in cassava is not detected under normal physiological conditions but Haque & Bradbury (2004) reported that the linamarin (and lotaustralin), separately contained in the vacuoles within the cellular structure of the plant and the β-glucosidase enzyme, also known as linamarase, from either the latex or the region outside of the cell known as the apoplast, can be released and mixed due to mechanical damage caused by chewing, processing or preparation prior to consumption. Upon contact with the linamarase enzyme, linamarin will be hydrolysed to glucose and acetone cyanohydrins. The acetone cyanohydrin then breaks down spontaneously under certain conditions to form acetone and HCN (Conn, 1994). A simplified linamarin synthesis, distribution and degradation in

cassava plant is represented diagrammatically in Figure 1-3. This is the likely route of cyanide exposure in humans and animals and may present significant safety and health issues.

1.3.1 Biosynthesis of cyanogenic glycosides in cassava

CGs biosynthesis have been broadly studied and described extensively in many important plants, for instance the tyrosine-derived CGs dhurrin in sorghum (*Sorghum bicolor*), cassava (*Manihot esculenta*), seaside arrow grass (*Triglochin maritimum*) barley (*Hordeum vulgare*) as reviewed by Ganjewala *et al*. (2010). In plants, CGs biosynthetic pathway could be generally described in three steps as described below and depicted in Figure 1-4.

Step 1: Conversion of a precursor amino acid into aldoxime through two successive N-hydroxylation of amino group of parent amino acid by an enzyme of cytochrome-P450 family.

Step 2: Conversion of aldoxime into cyanohydrin catalysed by another cytochrome P-450 enzyme.

Step 3: Glycosylation of the cyanohydrins by a soluble enzyme uridine 5'-diphosphoglucose-glucosyl transferase (UGT).

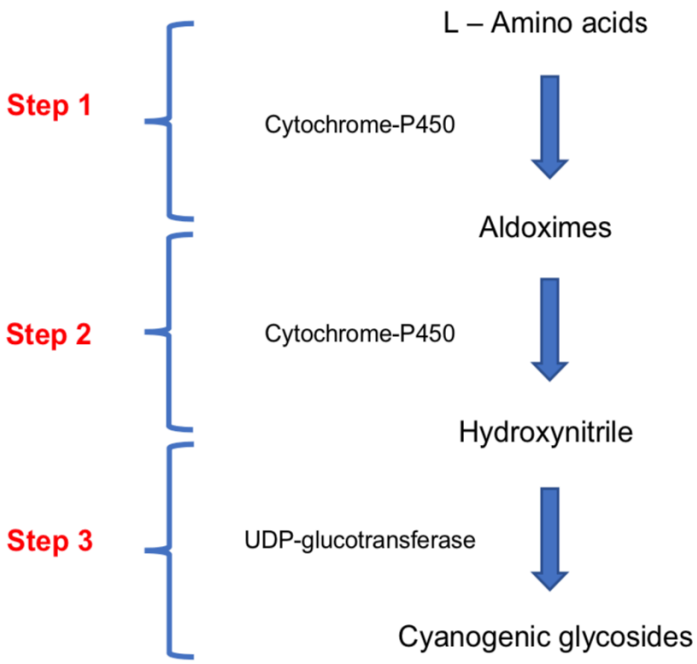


Figure 1-4: General scheme of the biosynthesis of cyanogenic glycosides in plants

(Adapted from Ganjewala *et al*. 2010)

The primary precursors for the biosynthesis of linamarin and lotaustralin in cassava are derived from L-valine and L-isoleucine respectively (Du *et al*. 1995). The general pathway involves the sequential conversion of the amino acids to the N-hydroxyaminoacid, an N, N-dihydroxyamino acid, an E- and Z-oxime, and an α-hydroxynitrile (cyanohydrin) by a multi-microsomal enzyme complex of cytochrome-P450-I (CYP79D1, CYP79D2) and cytochrome-P450-II (CYP71E7) (Nartey, 1969; Du *et al*. 1995; Andersen *et al*. 2000; Zhang *et al*. 2003). The first and the second steps of CG biosynthesis are catalysed by cytochrome CYP79D1 and CYP79D2 through two successive N-hydroxylations of the amino group of the parent amino acid, followed by decarboxylation and dehydration to produce the corresponding E-aldoxime which will subsequently converted to Z-aldoxime

(Zagrobelny *et al*. 2018). The cyanohydrin is produced from the action of a CYP71E7 on the Z-aldoxime via a dehydration reaction and C-hydroxylation (Kahn *et al*., 1997; Bak *et al*., 1998 and Jørgensen *et al*., 2011). Finally, the glycosylation of the cyanohydrin moiety (the final step in CG synthesis) by the UGT85B (a soluble enzyme) will form the CGs, linamarin and lotaustralin respectively (Jensen *et al*., 2011). The involvement of the microsomal systems catalysing the synthesis of the aglycone of linamarin and lotaustralin have been described by Koch *et al*. (1992) through the isolation from young etiolated cassava seedlings and root tissue, suggesting that both leaves and roots, to a certain extent or partly, are capable of linamarin synthesis (White *et al*., 1994; McMahon *et al*., 1995; Du *et al*., 1995). The biosynthetic pathway of linamarin and lotaustralin in cassava is illustrated in Figure 1-5.

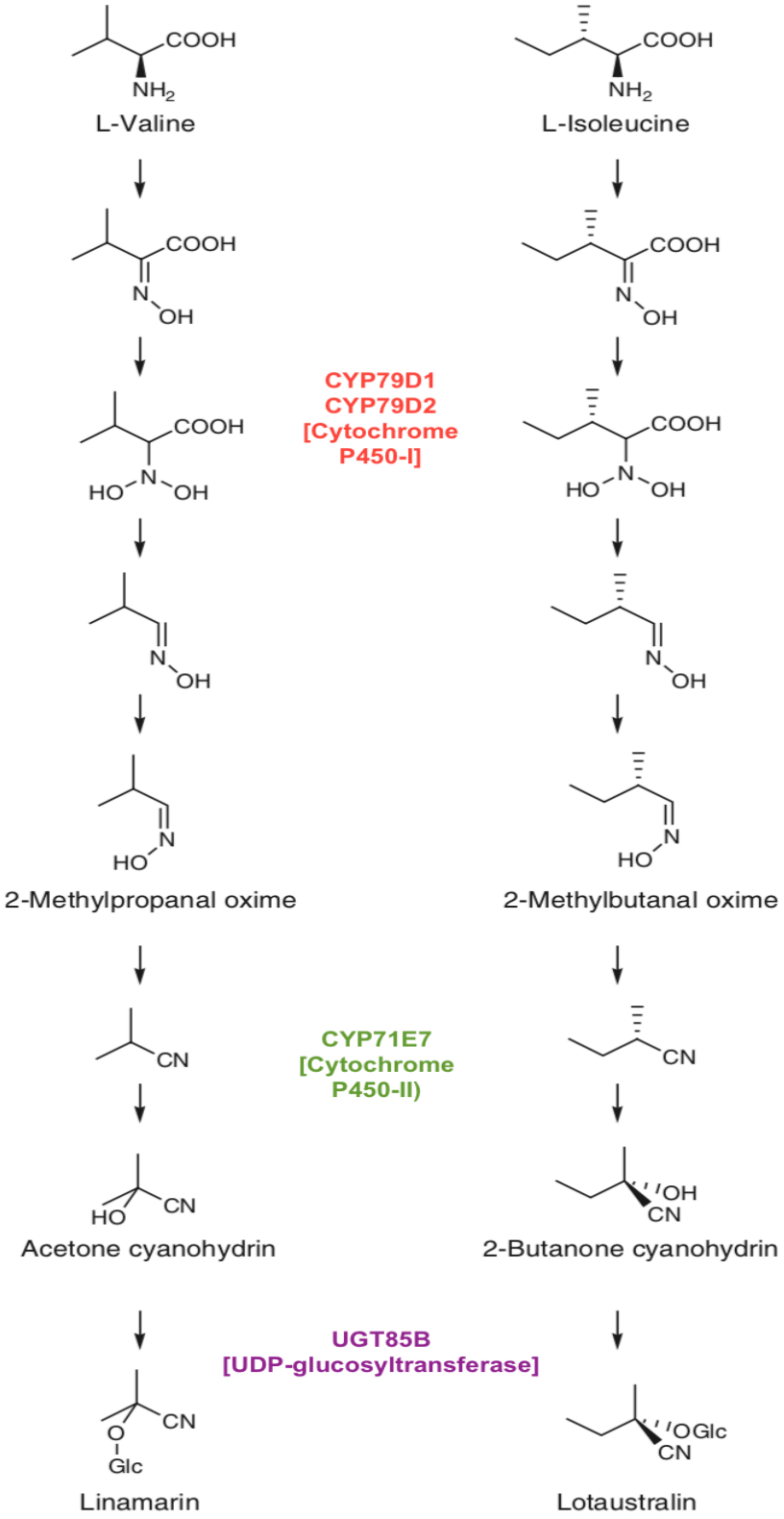


Figure 1-5: The biosynthetic pathway for cyanogenic glycosides in cassava.

(Adapted from Andersen *et al*., 2000; Burns *et al*, 2010 and Jørgensen *et al*., 2011)

1.3.2 Cyanogenesis, catabolism and detoxification of cyanogenic glycosides in cassava

CGs are not toxic when intact but have to be activated to release HCN for defence, as well as to recycle nitrogen and glucose into other metabolic processes (Zagrobelny *et al*., 2018). Cyanogenesis is the ability of plants and other living organisms to release HCN upon breakdown of CGs (Poulton, 1990; White *et al*., 1999; Zagrobelny *et al*., 2008; Balhorn *et al*., 2009) causing potential toxicity issues for animals including humans. In cassava, autotoxicity is prevented by spatial separation of the CGs (linamarin and lotaustralin), located in the plant vacuoles, meanwhile the enzymes are located in the chloroplast or apoplastic spaces in plants (Conn, 1980; Poulton, 1990; Gruhnert *et al*., 1994). Cyanogenesis in cassava starts from the disintegration of the tissues during chewing action of the herbivores, processing or meal preparation that releases both CGs and the endogenous enzymes (linamarase and α-hydroxynitrile lyase). The linamarase enzyme cleaves the glucose residue stabilising the linamarin structure, converting it into the corresponding α-hydroxynitrile, which at pH condition of more than 6 will spontaneously dissociates into a sugar, a keto compound (acetone), and liberates HCN, which is toxic (Zagrobelny *et al*., 2004). At pH lower than 6, α-hydroxynitrile does not dissociate spontaneously but an enzyme α-hydroxynitrile lyase may catalyse its dissociation (Hughes *et al*., 1994; Ganjewala *et al*., 2010). During processing, reduced moisture and/or increased temperature facilitate the spontaneous breakdown of α-hydroxynitrile to toxic HCN (McMahon, 1995). A simplified cyanogenesis reaction of linamarin in cassava is depicted in Figure 1-6.

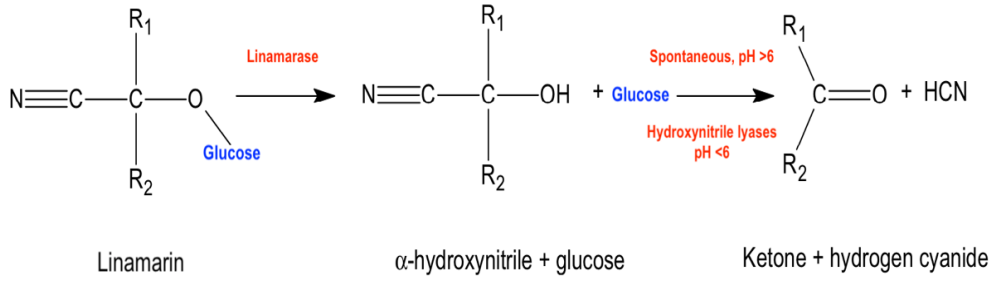


Figure 1-6: Structure and cyanogenesis pathway of linamarin in cassava. (Adapted from Bjarnholt and Møller, 2008)

HCN, the toxic, final product of dissociation of α-hydroxynitrile then detoxified via two pathways (Møller and Poulton, 1993). The endogenous detoxification of cyanide in plants and animals uses sulphur. The first detoxification pathway involves the binding of HCN to cysteine forming β-cyanoalanine and this reaction is catalysed by an enzyme of mitochondrial origin, β-cyanoalanine synthase (Zagrobelny *et al*, 2004). β-cyanoalanine synthase detoxifies HCN in pyridoxal phosphate (PLP) dependent reaction and produce β-cyanoalanine. The process is beneficial both ways as the detoxification of HCN prevents the mitochondrial degradation from the vulnerable attack of HCN and the resulted detoxification product β-cyanoalanine (a neurotoxin) serve to deter predators (Ressler *et al*, 1997). β-cyanoalanine is subsequently converted into asparagine by the enzyme β-cyanoalanine hydrolase (Miller and Conn, 1980). This route seems to be most common in plants and possibly in insects too (Ganjewala *et al*., 2010). The second pathway proceeds with binding of HCN into thiosulfate (an oxyanion of sulfur) forming thiocyanate catalysed by sulphurtransferase enzyme also of mitochondrial origin known as rhodanese (Bordo and Bork, 2002). Vertebrates predominantly utilizes thiocyanate route, however some plants and insect are

also reported to follow the same path. The whole metabolism, catabolism and detoxification processes of CGs is summarized diagrammatically in Figure 1-7.

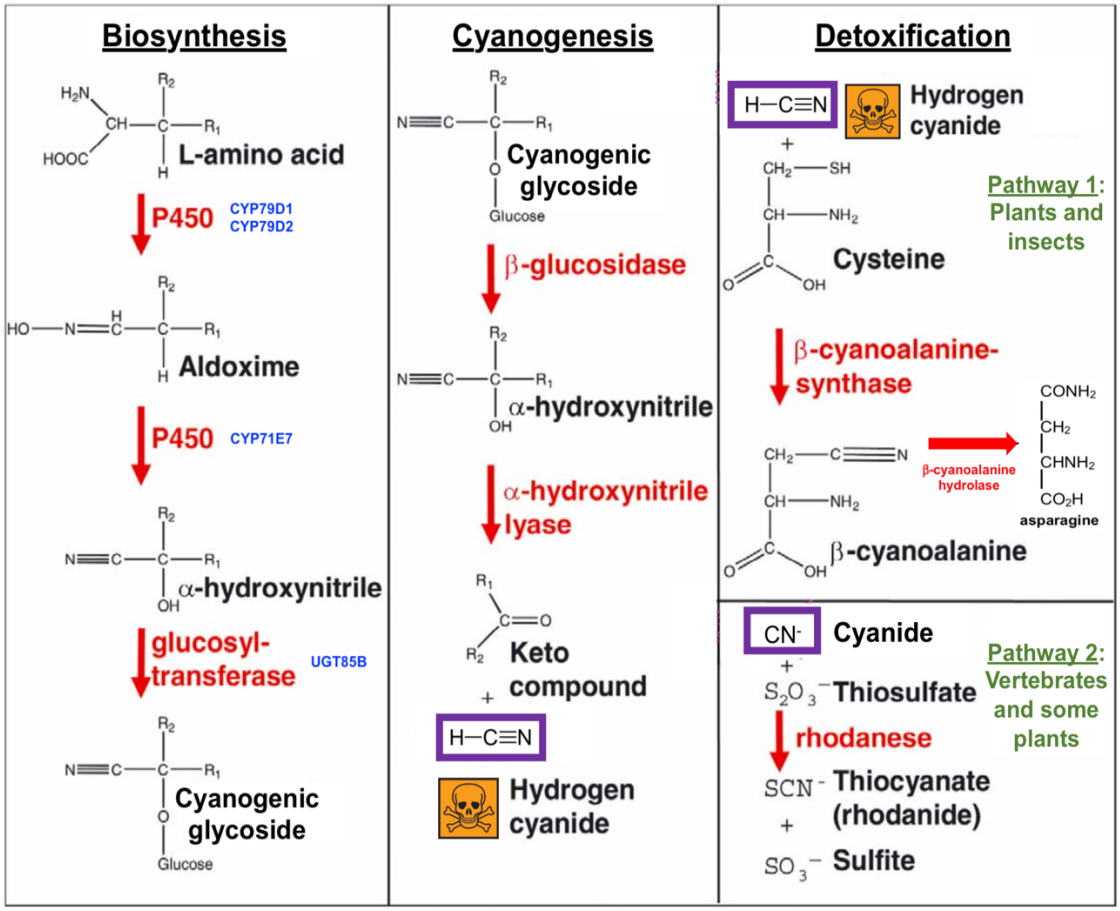


Figure 1-7: Metabolism, catabolism and detoxification of cyanogenic glycosides in plants, insects and higher animals. Enzymes involved are highlighted in red, HCN in purple.

(Adapted from Zagrobelny *et al*, 2004, Ganjewala *et al*, 2010 and Burns *et al* 2010)

1.3.3 Mechanism of cyanide poisoning

Cyanide released from the degradation of CGs is extremely toxic to all aerobic organisms (including human), could be fatal depending upon dose and is a rapidly acting poison (Piantadosi and Sylvia, 1984). Cyanide poisoning can occur as a result of inhalation, ingestion or injection of cyanide, dermal absorption,

parenteral administration by one of its salts or derivatives (Leybell *et al*., 2018). The toxicity of cyanide is largely attributed to the impairment of mitochondrial oxygen utilization due to cessation of aerobic cell metabolism (Hamel, 2011; Reade *et al*., 2012). This was achieved by reversibly binding to the cytochrome c oxidase (also known as cytochrome a,a_3) which is the terminal oxidase complex (Complex IV) necessary for the reduction of oxygen to water in the oxidative phosphorylation chain within the mitochondria (Hamel, 2011). Cyanide has high affinity towards metalloenzymes such as ferric iron (Fe^{3+}) and cobalt, consequently, able to bind to numerous critical enzyme systems in the body (Hall *et al*., 2007). Binding of cyanide to ferric ion (Fe^{3+}) in cytochrome c oxidase inactivates the terminal enzyme complex and halts the electron transfer through the oxidative phosphorylation preventing synthesis of adenosine triphosphate (ATP) causing reduction in oxygen utilization by the body tissues (Beasley and Glass, 1998; Hamel, 2011). This may result in cytotoxic hypoxia, a shift from aerobic metabolism to anaerobic metabolism (due to reduction in the ATP/ADP) and an increase in the levels of glucose and lactic acid in the blood which leads to high-anion-gap metabolic acidosis and central nervous system injury (Hall *et al*., 2007; Hammer *et el*., 2013). Cyanide also inhibits the tricarboxylic acid cycle by decreasing the rate of glycolysis and activating glycogenolysis, thus causing a reduction in energy available to the respiratory system, the heart and other cells (Speijers, 1993). Although toxicity of cyanide can develop over minutes or hours depending upon route of exposure, exposure to high doses of cyanide could certainly be lethal.

1.3.4 Clinical manifestation of cyanide poisoning from food plants

The clinical manifestation of cyanide poisoning are largely a reflection of cascading effects of intracellular hypoxia (Hamel, 2011). Consumption of cassava products with high concentrations of cyanogens can lead to illness or even death. Cases of cyanide poisoning from cassava consumption are most common among people who subsist on a monotonous cassava-based diet (Tylleskar *et al*., 1992; Cliff and Nicala, 1997; Banea-Mayambu *et al*., 2000). Cases of cyanide poisoning can also occur from the consumption of other food plants such as Rosacceae (peach, apricot, plum, apple seed), Leguminosae (lima beans, broad beans), or members of the genus Sorghum (Jones, 1998; Francisco and Pinotti, 2000; Vetter, 2000). The onset of signs and symptoms of cyanide poisoning usually could be seen in less than 1 minute after inhalation and within a few minutes after ingestion (Hall *et al*, 2007). Acute intoxication symptoms, which occur within hours of consumption of insufficiently processed cassava reflect reflexive attempts of the respiratory, neurologic and cardiovascular system to overcome tissue hypoxia. Early signs and symptoms include dizziness, fatigue, headache, palpitations, shortness of breath, hyperventilation and nausea. Stupor, coma, seizures, haemodynamic shock and cardiorespiratory arrest are common signs of late or severe poisoning (DesLauriers *et al*, 2006; Nelson, 2006). The clinical features of acute cyanide poisoning are listed in Table 1-8. The major determinants of severity and mortality are the source of exposure, the route and the magnitude of exposure and the effects and the time taken for any treatments that may have been attempted (Yen *et al*., 1995).

Table 1-8: Clinical manifestation of the toxic effects of cyanide

Human body systems	Manifestations
Central nervous system	**Early symptoms**
	Anxiety
	Headache
	Giddiness
	Dizziness
	Confusion
	Mydriasis
	Bright retinal veins
	Late symptoms
	Decreased consciousness
	Seizures
	Paralysis
	Coma
Respiratory system	**Early symptoms**
	Hyperventilation and tachypnea (due to hypoxic stimulation of peripheral and central chemoreceptor)
	Absence of cyanosis (caused by an increased in oxygen content in venous blood)
	Hypoventialtion
	Apnea (cells cannot take up oxygen)
Cardiovascular system	**Early symptoms**
	Tachycardia
	Late symptoms
	Hypotension
	Supraventricular tachycardia
	Atrioventricular blocks
	Ventricular fibrillation
	Asystole

(Adapted from Hamel, 2011)

1.3.5 Cyanogenic glycosides in cassava: Impacts on human health

The occurrence of CGs in food and fodder can be a significant social and economic problem in many parts of the world. Consumption of inadequately processed cassava can lead to dietary cyanide exposure. The toxicity produced by cassava consumption would depend on the cyanogen level in the consumed product, the type of cyanogen present, quantity of cassava consumed, nutritional status of the subject, quantity of protein consumed and the amount of detoxified product formed in the body (Nambisan, 2011). While many food crops are cyanogenic, unlike cassava, the CGs are generally either in parts of the plant that are not consumed (e.g. apple seeds, wheat leaves) or are eaten in small amounts (e.g., almonds). The risk of cyanide toxicity from cassava is high because not only is the part that is consumed highly cyanogenic but it also frequently forms a large proportion of the overall diet (Burns *et al*, 2010). The acute onset and chronic conditions of prolong exposure to cyanide include:

i. Acute cyanide poisoning: occurs as a result if consumption of bitter cassava and their products without proper processing. The clinical symptoms are vomiting, nausea, dizziness, abdominal pains, weakness, headache, diarrhea and occasionally death (Lasch and Shawa, 1981; Mlingi *et al*., 1992; Kwok, 2008; Akylidiz *et al*., 2010; Sanchez-Verlaan *et al*., 2011).

ii. Tropical ataxic neuropathy (TAN): occurs in older people due to a monotonous consumption of CGs from a bitter cassava diet for many years, resulting in chronic thiamine deficiency from inactivation of

thiamine by the CGs (Osuntokun 1994, Adamolekun, 2010a). The disease is characterized by unsteady walking, loss of sensation in the hands and feet, blindness, deafness, reduced visual perception, ataxic gait and weakness (Onabolua *et al*., 2001; CCDN, 2008). The mechanism of thiamine deficiency however vary in the different communities from where TAN has been reported. In the African communities where cassava is the staple diet, chronic thiamine deficiency resulted from poor concomitant thiamine intake and the inactivation of thiamine by CGs in cassava. The major pathway of the detoxification of cyanide is its reaction with cysteine to form iminothiozidine compound that is excreted in the saliva and urine. Hence, effectively, detoxification is dependent on the nutritional status, protein malnutrition and levels of sulfur containing amino acids. This is based largely on studies which showed high intakes of cassava foods and high levels of thiocyanate in saliva and urine of subjects in the endemic area of Nigeria. It is difficult to estimate the exposure to cyanide from the frequency of intake of cassava foods because of the wide variation in levels of CGs in foods that have been processed using different methods.

iii. Konzo: an upper motor neurone disease of acute onset due to continuous large intake of CGs from insufficiently processed bitter cassava, also resulting in thiamine deficiency from inactivation of thiamine when the sulfur in thiamine is utilized for detoxification of cyanide in the human body, thereby causing an irreversible paralysis (spastic paraperisis) of the lower limbs mainly in children (greater than 2 years of age) and women (less than 45 years of age) (Rosling, 1988; Tylleskar *et al.*, 1992; Ernesto et al., 2002; Adamolekun, 2010b; Nzwalo and Cliff, 2011; Cliff *et al*.,

2011). This is due to the high nutritional demands of pregnant and lactating women, and children, who have been weaned but have only limited access to alternative foods (Burns *et al*., 2010). Symptoms at onset often occur after a long walk and include sudden trembling in the legs (paresthesia), sensations of electrical charges in the spine and legs and loss of visual acuity (Tshala-Katumbay *et a*l., 2016)

iv. Goitre: exacerbation of goitre and cretinism results from interference by thiocyanate (the end product of cyanide detoxification in the human body) in iodine metabolism leading to dietary iodine deficiency and associated disorders (Delange *et al*., 1994, Gbadebo and Oyesanya, 2005, Nhassico *et al*., 2008).

In Africa, although cassava is a major staple, toxic effects due to its consumption are restricted to only few regions, and accounts for <1% of the cassava eating population (Tylleskar, 1994). TAN and konzo are prevalent only in cassava consuming populations and associated with high cyanogen intake due to the consumption of improperly processed bitter cassava, deficiency of sulphur and low protein intake (Cliff *et al*., 1985; Tylleskar et al., 1992; Osuntokun, 1981). Most poisoning cases reported have been from the Democratic Republic of Congo, Nigeria, Tanzania and Mozambique (Nhassico *et al*., 2008, Mlingi *et al*., 2011, Ciglenecki *et al*., 2011).

1.3.6 Toxicity of cyanogenic glycosides in cassava

Since toxicity of linamarin is associated with free cyanide, toxic levels of CGs are therefore estimated in terms of the amount of HCN liberated following disruption

and incubation period (Bradbury *et al*., 2010). The acute lethal dose of cyanide for human is 0.5-3.5 mg/kg body weight (Jones, 1998). The lethal dose of orally ingested HCN for a 60kg adult man ranges from 30 – 210 mg equivalent HCN (Nhassico *et al*., 2008) and a list of minimum lethal dose of HCN per body weight is shown in Table 1-9. World Health Organization (WHO) has set the safe level of total cyanogens in cassava flour at 10 mg/kg dry weight (10 ppm). Food Standards Australia New Zealand (FSANZ) amended Standard 1.4.1 of the Australia New Zealand Food Standards Code by including a maximum limit of 10 mg/kg for cassava chips (FSANZ, 2009, Cressey *et al.* 2013). In Indonesia, the acceptable limit of total cyanide in cassava products is set at 40 mg/kg fresh weight (40 ppm) (Hidayat *et al*., 2000, Cardoso *et al*., 2005). Since the toxic effect of cyanide depends on body weight, a child of approximately 20 kg could safely eat up to 1 kg of flour with a concentration of 10 mg/kg total cyanide, in a meal; and adult of 60 kg, up to 3 kg of flour with 10 mg/kg total cyanide. Considering that the daily per capita consumption of cassava products is up to 700-800 g in parts of Africa (Nhassico *et al*., 2008), a child and an average adult (60-80 kg) would start to reach their minimum lethal dose of cyanide if the cassava products contained 40 mg/kg or more of total cyanide. Moreover, on top of the body weight, the lethal dose that might affect each individuals would have to rely on other factors such as the other nutrients availability in diet (sulphur-containing amino acids, vitamin B_{12}) and the ability to detoxify the cyanide (conversion to thiocyanate, methemoglobin) in which these two factors often interrelates. The rate of absorption also plays an important role in cyanide toxicity, and even an acute lethal dose is tolerated without symptoms if it is split into even parts ingested, for example, hourly over the day. As the delay in absorption would allow time to detoxify the cyanide, this would therefore contribute to lower peak

levels. These mechanisms influencing the peak levels of cyanide in body tissues can be expected to be different for different foods, depending on the degree of plant tissue destruction, the type of the cyanogenic glycosides, the effectiveness of the accompanying β-glucosidase, the pH value of the stomach, the bacterial flora of the gut, an/or possible influences related to the plant matrix.

Table 1-9: Minimum lethal dose ranges of HCN in human per body weight, and the amount of cassava product, containing 10 mg/kg and 40 mg/kg HCN, required to reach these lethal doses (based on lethal dose of 0.5-3.5 mg/kg body weight).

Body weight (kg)	Lethal dose range of HCN (mg/kg)	Lethal amount of cassava product (kg)	
		10 mg/kg HCN	40 mg/kg HCN
10	5-35	0.5-3.5	0.13-0.88
20	10-70	1-7	0.25 – 1.75
40	20-140	2-14	0.50 – 3.50
60	30-210	3-21	0.75 – 5.25
80	40-280	4-28	1.00 – 7.00
100	50-350	5-35	1.25 - 8.75

(Adapted from Jones 1998, Burns et al., 2010)

With regard to the complex issue described, several incidents of acute cyanide poisoning from cassava meal or products have been reported from several case reports and literatures, mainly affecting those in tropical countries, although not their staple food but consume cassava occasionally as desserts and snacks. Cheok (1978), reported the first case of acute cyanide poisoning involving 2 siblings in East Malaysia (Sarawak) after eating a plate of ‘tapioca’ cake, made from wildtype cassava obtained from the forest. In 1992, cyanide from a meal

called *'gari'* was responsible for the death of three people in Nigeria (Akintowa and Tunwashe, 1992) and eight children were in bad condition after eating bitter cassava in Venezuela (Espinoza *et al*., 1992). In the same year, three sisters were admitted with cyanide poisoning in West Malaysia after eating 3-5 chunks of boiled cassava (Ariffin *et al*., 1992). In March 2005, more than 100 children were admitted to the hospital and left 27 children at San Jose Elementary School in Magini, Bohol, Philippines dead after eating caramelized cassava roots sold by a street vendor outside of the school as reported by The Seattle Times and China Daily. Five Nigerians apparently died of cyanide poisoning after eating a meal prepared with cassava flour (Makinde, 2010). In September 2017, an outbreak of 98 cases of suspected cyanide poisoning from consumption of a cassava dish made by combining hot water with cassava flour left two people dead in Kasese District, Uganda (Alitubeera *et al*., 2019).

Cases of cyanide poisoning from other cyanogenic food plants have also been highlighted in the literatures. For instance, a woman was affected with cyanide poisoning after ingesting apricot kernels (Suchard *et al*., 1998). A similar case of self-medication using apricot kernel extract of a 67-year- old man with cyanide level 25 times above acceptable limit was reported by Konstantatos *et al*. (2017). Thirteen people were also reported to be intoxicated with cyanide after eating apricot seeds (Akyildiz, 2010). A case of a girl who became unconscious after eaten apricot kernels was reported by Sahin (2011). In another report, an elderly woman was affected with cyanide poisoning after ingesting bitter almonds (Sanchez-Verlaan et al., 2011), and a man from Lancashire suffered cyanide poisoning after eating cherry seeds in July 2017 as reported by BBC News. Despite all the reported cases of cyanide poisoning and studies on the toxicity of

CGs, the awareness of consumers on this safety issue is still poor especially in regions where cassava is not the staple crop. People living in regions with higher cassava consumption are mostly aware of this issue although not necessarily understand the science behind it, but more often continuing the rituals that have been performed years ago and there is always a case of ignorance where farmers continue to cultivate crops with high levels of CGs due to taste preference, reduction in herbivory and protection against infections and theft (Cock, 1985, Nweke *et al*., 2002, Siritunga and Sayre, 2004).

1.3.7 Mechanism of cyanide detoxification in human

In human, intravenous and inhaled exposures to cyanide produce rapid onset of signs and symptoms than does oral ingestion due to the two former routes provide faster diffusion and direct distribution to target organs via the bloodstream (Hamel, 2011). Following an acute exposure, cyanide is detoxified by the enzyme rhodanese (a sulphur transferase enzyme present in the liver) with the help of rate limiting cofactor known as sulphane sulphur (Tor-Agbidye *et al*., 1999), forming a goiterogenic compound thiocyanate (SCN^-) which is rapidly excreted via the kidney through the urine with very little in the faeces (Oke, 1973, Dhas *et al*., 2011). The concentration of sulphane sulphur is dependent on the availability of sulphur-containing amino acids (SAA) such as cysteine and methionine from dietary diets (Cliff *et al*., 1985). However, this mechanism will only cope with minute amount of cyanide generated from the consumption of small amounts of cyanogenic plants. Exposure above the threshold will overwhelm the mechanism as seen in patients with acute poisoning or in patients with decreased kidney function (Benowitz, 2007). Nevertheless, thiocyanate still

remains the most useful biomarker for dietary cyanide exposure (Rosling, 1994) and according to Foss & Lund-Larsen, (1986), thiocyanate levels in urine have been used in several studies to measure exposure to cyanide quantitively. However, in much more recent report by EFSA, (2018), serial measurements of cyanide in whole-blood after ingestion was reported to be a far more reliable biomarker to assess acute cyanide exposure. Although the determination of linamarin or other partially absorbed CGs as well as their metabolite thiocyanate in urine is useful for comparing different chronic exposure levels, it cannot provide information on the absolute exposure, because the degree if absorption and the proportion of the CGs degraded to cyanide in the intestine or colon are not known and because urinary thiocyanate might be strongly confounded by other factors including smoking. Apart from that Onabolu *et al*. (2000) suggested both frequency of intake of cassava foods and the cyanogen content of the cassava foods contributed to dietary cyanide load.

Cyanide can also be temporarily trapped (reversible reaction) by the methemoglobin fraction in red blood cell corpuscle in the form of cyanomethemoglobin (Schutlz, 1984, Lunquist *et al*., 1985). This is a rapid buffer system that comes into play only after the CN^-/SCN^- conversion pathway has been saturated (Lunquist *et al*., 1985). Cyanide detoxification also occurs to a lesser extent by mercaptopyruvate sulphurtransferase (MST) (Oke, 1973, Moeller *et al*., 2017). MST catalyses the transfer of sulphur from 3-mercaptopyruvate to the active enzyme site, which then reacts with cyanide, forming the much less toxic thiocyanate (Moeller *et al*., 2017). 3-mercaptopyruvate can arise from cysteine via transamination or deamination and

this compound can provide sulphur rapidly for cyanide detoxification (Figure 1-8).

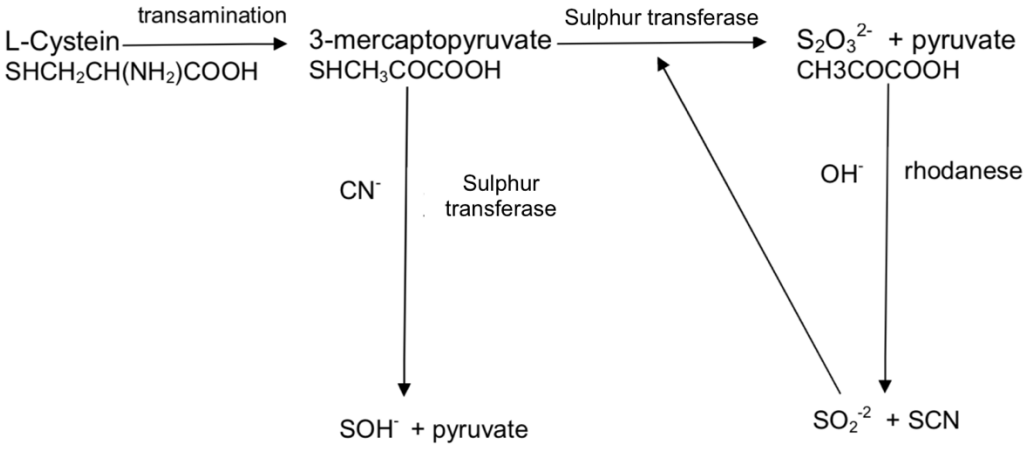


Figure 1-8: The reaction of cyanide with 3-mercaptopyruvate to form sulphur and thiosulfate.

Although the human body is protected to some extent against cyanide toxicity via the pathways describe previously, the amount of cysteine and methionine in the diet is a contributing factor to cyanide induced disease as SAA are required for the detoxification process. Diet consisting of food plants and products with high cyanogenic content without complementing it with adequate source of SAA will result in the depletion of SAA in the body and could consequently leads to the chronic conditions of prolong cyanide exposure.

1.3.8 Prevention of cyanide induced disease and intoxication

Cassava roots and leaves are consumed both, fresh and cooked in different parts of the world according to the taste preferences, economic status and availability of other food plants (Latif and Muller, 2015). Cyanide induced disease can be prevented by effective removal of cyanogenic compounds in food plants prior to consumption which could be achieved via proper and efficient processing technique. A wide diversity of processing methods are used in cassava consuming communities, and cassava processing have been extensively studied and documented where they were subjected to various processing methods such as peeling, slicing, soaking, grating and grinding followed by cooking, steaming, boiling, roasting, baking, deep frying, fermenting and sun drying to reduce their cyanide content to safe levels (Westby, 1991, Nambisan, 1994; Oke, 1994, Essers, 1994; Kemdirim *et al*., 1995; Dufour, 1988; Obilie *et al*., 2004; Cardoso *et al*., 2005; Bradbury, 2006; Montagnac *et al*., 2009a, Perera, 2010, Bradbury and Denton, 2014). Most of the methods reviewed in all of the literatures were able to reduce up to 99% of total cyanide content (with significant loses of vitamins and minerals) and usually involved combinations of processing techniques. Depending on the nature of the process, they either lead to hydrolysis of CGs to liberate HCN, which are volatilized and subsequently lost or the highly soluble CGs and its hydrolytic products are leached out in water (Nambisan, 2011). A simple slicing and baking, steaming and frying of the roots or leaves of high-cyanogenic cultivar results in very little loss of linamarin. This is due to inactivation of linamarase preventing further decomposition of the cyanogenic glycoside and stability of linamarin at high temperature (Burns *et al*., 2010). Cyanide is later released, during digestion, due to hydrolysis of the intact linamarin by the gut microflora enzymes (Jones, 1998).

In eastern and southern Africa, cassava is preferably processed into flour, which is produced by sun drying of the peeled root followed by pounding and sieving or heap fermentation, however, because this process does not allow enough contact between linamarase and linamarin, the product may contain up to 59 ppm of HCN equivalents, as compared to the WHO safe level of 10 ppm (Montagnac *et al*., 2009a). In western Africa and southern America, cassava parenchyma is ground, grated or crushed into small pieces to disrupt many plant cells and allow good contact between linamarin and linamarase. The moist mash is then soaked and left to ferment for several days, the water-soluble cyanogens squeezed out by dewatering and the residual HCN gas is removed by roasting. The percentage of cyanogens retained in these fermented foods can vary from 0% to 20%, higher retention can occur with inadequate processing (Westby and Choo, 1994; Essers and Nout, 1989). Recently, new processing method that reduced the total cyanide content of cassava by three to six-fold of its former value (i.e from 43 ppm to 7 ppm, over 80% reduction) was developed by Bradbury & Denton (2010). The ‘wetting’ method involved mixing dry cassava flour with water and spreading it on a thin layer in the shade for five hours or in the sun for two hours (prior to cooking) which allows the enzymatic breakdown of linamarin to HCN and volatilised into the warm environment.

Both the initial content of CGs and the method of processing influence the level and nature of cyanogens in the final processed products. Hence, adequately processed cyanogenic plants, effective detection methods for residual cyanogen in processed products and use of plant cultivars with low level of cyanogen content will reduce and help to maintain safe level of cyanogenic glycoside contents of cassava-based foods and other cyanogenic plants to 10ppm; the

safe level of cyanide recommended by World Health Organization (McMahon *et al.*, 1995; Ademolekun, 2010b, Nambisan *et al*., 2011).

1.3.9 Analysis of cyanogenic glycosides in cassava

Conventionally, the cyanogenic glucosides in food plants are quantified indirectly in terms of total cyanide released after acid or enzymatic hydrolysis of cyanogenic glycoside rather than the glycoside itself. Efficient extraction and complete hydrolysis is the key for accurate determination of plant cyanogens using these indirect methods. This quantification however does not give reliable and reproducible data because of the difficulty involved in the isolation and recovery of cyanide from digested samples (which arises as a result of the volatility of HCN boiling point of 26^{o}C). Various methods were used to determine linamarin in cassava which have been extensively studied and modified for improved precision, sensitivity and ease of use. Probably the most common analysis involves three main steps: (i) extraction of linamarin from cassava, (ii) hydrolysis of linamarin by linamarase to liberate HCN and (iii) analysis of the latter (Cooke, 1978; Borges *et al*., 1993; Bradbury *et al*., 1994). Extraction of linamarin from plant material is normally carried out using dilute acid such as 0.1M phosphoric acid in order to stop endogenous linamarase activity and to stabilise the cyanohydrins (Cooke, 1978; Essers *et al*., 1993). Few strategies have been adapted to hydrolyse cyanogens to cyanide which Bradbury *et al*. (1991) reported by acid hydrolysis, by autolysis (AOAC, 1990), or by enzymatic hydrolysis (Cooke, 1978; O'Brien *et al*., 1991; Essers *et al*., 1993). The acid hydrolysis requires use of strong acid which is laborious and time-consuming. In

addition, discrepancies might occur in acid hydrolysis due to formation of amides and ammonia.

Meanwhile, in spontaneous autolysis by enzymes, the activity of enzymes is influenced by the conditions prevailing in the heterogenous plant substrate, the accompany secondary reactions and the accumulation of products of hydrolysis (Zitnak, 1973). The hydrolysis of linamarin by the endogenous enzyme linamarase reaction time could take up to 24 hours for certain sample (Bradbury and Bradbury, 1994). This autolysis method however, is more suitable for fresh cassava material and it cannot be applied to cooked or roasted products since the endogenous enzyme is permanently inactivated. In enzymatic hydrolysis, exogenous linamarase is added to the acid-extracted sample after pH adjustments (pH 5-6). Enzymatic breakdown of linamarin is rapid at around 30^{o}C and takes less than 15 minutes depending on enzymatic activity (Cooke, 1978; Essers *et al*., 1993; Haque and Bradbury, 1999; Nambisan, 1999). Linamarase can be easily isolated from cassava latex (Haque and Bradbury, 1999; Nambisan, 1999). Both acid and enzymatic hydrolysis methods convert linamarin to cyanohydrins, which further decompose to HCN and acetone at alkaline pH levels (Bradbury and Bradbury, 1994).

Various techniques have been developed to determine endogenous HCN liberated from CGs by all the hydrolysis methods described previously. AOAC (1990) described a titration method whereby the endogenous cyanide is precipitated with $AgNO_3$ after steam distillation of HCN from autolysed cassava samples. This method however suffers from loss of cyanide during the distillation

process (Borges *et al*., 1993). Perhaps, the most notably used method since the early 1990s with various modifications and improvements throughout the years would be the use of alkaline picrate in which, cyanide reacts with alkaline picrate paper, and the change of colour is matched against a colour chart. The resulting chromophore can also be dissolved from the picrate paper for more accurate quantitative determination using a spectrophotometer (Bradbury *et al*., 1999; Bradbury, 2009). The picrate method was used in a micro diffusion method, where the change in colour of picrate pre-coated in an ion-exchange sheet is read in reflectometer (Saka *et al*., 1998). Although the picrate method is easy to use, it has certain disadvantages. The reaction can be really slow (~16-24 hours), the chemical needs special handling and storage and the response sometimes is imprecise due to the limitations mentioned previously. Nevertheless, the method has been used and later on extensively modified for the determination of the cyanogen potential of cassava roots and cassava products; picrate paper assay (Bradbury *et al*., 1991; Egan *et al*., 1998; Haque and Bradbury, 1999; Djazuli and Bradbury,1999, Bradbury and Denton, 2011, Burns *et al*., 2012); picrate based solid state detection (Brimer *et al*., 1998; Haque and Bradbury, 2002; Abban *et al*., 2011); Feigl-Anger paper based semiquantitative detection (Olsen *et al*., 2007; Takos *et al*., 2008). The picrate method was also implemented and incorporated into a paper strip indicator (Yeoh, 1993; Yeoh and Truong, 1993) and later on into an enzyme-based dipstick for semi-quantitative estimation of cyanogenic potential in cassava (Yeoh and Tan, 1994; Yeoh *et al*., 1996; Yeoh and Egan, 1997; Yeoh and Sun, 2001). This semi-quantitative method could be made quantitative by eluting the colour formed on the picrate paper in water and measuring its absorbance at 510nm (Yeoh and Egan, 1997).

Other indirect quantitative methods have also been employed for determination of CGs in plants. These methods include amperometric measurement with a cyanide-sensitive electrode (Dalgaard and Brimer, 1984; Tatsuma *et al*., 1996); use of methemoglobin complex (Ajaelu *et al*., 2008); colorimetric methods (Tatsuma *et al*., 2000); chemiluminescence assay (Ruengprapavut and Chulavatnatol, 1999); chloramine T/barbituric acid/isonicotinic acid method (Saka and Nyirenda, 2012); barbituric acid/pyridine method (Spectroquant® cyanide test from Merck) (Ballhorn *et al*., 2005; Takos *et al*., 2010); ninhydrin method (Drochioiu, 2002; Nagaraja *et al*., 2002; Drochioiu *et al*., 2004). A resorcinol method which is based on enzymatic hydrolysis of cyanogenic plants and trapping of HCN in alkaline solution of resorcinol reagent followed by measuring the absorbance at 488nm has also been developed (Drochioiu *et al*., 2008) and it was claimed that this method has been reported to be more sensitive than the picrate methods. Reaction of ninhydrin and cyanide was proposed for the spectrophotometric determination of free cyanide in environmental samples (Drochioiu *et al*., 2007); under flow injection conditions (Santelli *et al*., 2006; Themelis *et al*., 2009), in cuvetteless microspectrophotometry (Jain *et al*., 2010) and ninhydrin-based micromethod (Surleva and Drochioiu, 2013).

Direct quantification of CGs are at the moment based on various instrumental chromatographic methods which include cyanogens content in passion fruit using Gas Chromatography-Mass Spectrometry (GC-MS) (Chassagne, 1996; Bacala and Barthet, 2007); CGs in gooseberry using LC-MS (Bjarnholt *et al*., 2008); detection of CGs in grapevine using LC-MS/MS (Franks *et al*., 2005); methanol extraction and HPLC analysis of CGs in sorghum (De Nicola *et al*., 2011); GC and Near Infrared Spectroscopy (NIRS) (Golf *et al*., 2011); a reversed

phase chromatography with UV and pulsed amperometric detection developed by Wasserkrug and Rassi (1997) for the detection of linamarin, amygdalin and prunasin and their degradation products (mandelonitrile and benzaldehyde). Sornyotha *et al*. (2007) developed an HPLC method with a refractive index detector to quantify linamarin in cassava root cortex. Recently, Tivana *et al*. (2014) developed a direct quantification of total cyanide in various cassava products using a aquacyanocobyrinic (ACCA) chemosensor where it was firstly described by Zelder (2008) and Männel-Croise *et al*. (2009). An advantage of the chromatographic method is the quantification of cyanogens in their native form. These methods are summarized in Table 1-10. Although these methods are reliably accurate and sensitive in determining CGs, its wide application is limited as they often require expensive solvents and separation columns, complicated, time consuming sample pre-treatment, lack of CGs standards and are not easily adapted to rapid quality control monitoring purpose. There is therefore the need for a method that is simple, rapid, sensitive and cost effective.

Table 1-10: Various indirect and direct methods of detecting and quantifying cyanogenic glycosides in plants.

	Analysis	References
Indirect method	Titration method	AOAC, 1990
	- AgNO3 precipitation after steam distillation of HCN	
	Alkaline picrate method	Saka *et al*., 1998
	- Microdiffusion and ion-exchange sheet	
	Picrate paper assay	Bradbury *et al*., 1991; Egan *et al*., 1998; Haque and Bradbury, 1999; Djazuli and Bradbury, 1999; Bradbury and Denton, 2011; Burns *et al*., 2012.
	Picrate based solid state detection	Brimer *et al*., 1998; Haque and Bradbury, 2002; Abban *et al*., 2011
	Feigl-Anger paper based semiquantitative detection	Olsen *et al*., 2007; Takos *et al*., 2008
	Picrate paper strip indicator	Yeoh, 1993; Yeoh and Truong, 1993
	Enzyme-based dipstick for semi-quantitative estimation	Yeoh and Tan, 1994; Yeoh *et al*., 1996; Yeoh and Egan, 1997; Yeoh and Sun, 2001
	Amperometric measurement with cyanide-sensitive electrode	Dalgaard and Brime, 1984; Tatsuma *et al*., 1996
	Methemoglobin complex	Ajaelu *et al*., 2008
	Colorimetric methods	Tatsuma *et al*., 2000
	Chemiluminescence assay	Ruengprapavut and Chulvatnatol, 1999
	Chloramine T/barbituric acid/isonicotinic acid	Saka and Nyirenda, 2012
	Barbituric acid/pyridine method (Spectroquant® cyanide test from Merck)	Ballhorn *et al*., 2005; Takos *et al*., 2010
	Resorcinol method	Drochioiu *et al*., 2008
	Ninhydrin method – spectrophotometric determination	Drochioiu *et al*., 2007
	Ninhydrin method – under flow injection conditions	Santelli *et al*., 2006; Themelis *et al.,* 2009;
	Ninhydrin method – cuvetteless microspectrophotometry	Jain *et a*l., 2010
	Ninhydrin based micromethod	Surleva and Drochioiu, 2013

	Analysis	References
Direct method	Gas chromatography mass spectrometry (GC-MS) (passion fruit)	Chassagne, 1996; Bacala and Barthet, 2007
	Liquid chromatography mass spectrometry (LC-MS) (gooseberry)	Bjarnholt *et al*., 2008
	High performance liquid chromatrography (HPLC) (sorghum)	De Nicola *et al*., 2011
	Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) (grapevine)	Franks *et al*., 2005
	Gas Chromatography and Near Infrared Spectroscopy (NIRS)	Golf *et al*., 2011
	Reversed phase chromatography with UV and pulsed amperometric detection (linamarin, amygdalin and prunasin)	Wasserkrug and Rassi
	High performance liquid chromatography with refractive index detector (linamarin in cassava)	Sornyotha *et al.,* 2007
	Aquacyanocobyrinic (ACCA) chemosensor	Tivana *et al*., 2014

1.4 Antibodies

1.4.1 Definition

Antibodies (Abs), a category of macromolecules which are known as immunoglobulins (Ig). Ig are glycoproteins, which are present at 12-15 mg/ml in the blood serum (carbohydrate 2%-14%), synthesised by the white blood cells (lymphocytes) in response to invading foreign substances (Wild, 2013). The function of these highly specialised macromolecules is for immune recognition of foreign proteins (antigens) that will activate specific response reactions aimed at protecting the host (Kemeny, 1991, Liddell, 2013). Abs are the most crucial component in all types of immunological methods, as it will pretty much determine the assay simplicity, specificity and sensitivity of the assay (Hage, 1999). The five Ig classes in mammals are IgG, IgM, IgA, IgD and IgE, that differ on the basis of size, charge, amino acid composition and carbohydrate content, with IgG being the most abundant (molecular mass of 150 kDa) (Liddell, 2013). IgG represents 70 – 75% of total serum Ig and constitutes the majority of the secondary immune response to most Ags (Turner, 1981).

The basic structure of IgG antibody resembles the letter Y made up of two identical heavy polypeptide chains (H) and two identical light chains (L), which are stabilized and linked by intrachain and interchain disulphide bonds (Hsieh, 2010). The heavy chains are each made up of three constant domains (HC1, HC2, HC3) and one variable domain (HV), whereas the light chains are each made up of one constant domain and one variable domain (LC and LV) as depicted in Figure 1-9. IgG can be fractioned into two important fragments. The Fc (fragment crystallisable) portion of an Ab is the part below the flexible hinge

region or the ‘tail’ of the Y shaped molecule that is a dimer of the two last heavy chain domains (HC2 + HC3) while the Fab (fragment antigen binding) is the remaining upper part, combination of light and heavy chains (LC + LV and HC1 + HV). This fractionation method can be useful in assays where the Fc region is causing interference in assays.

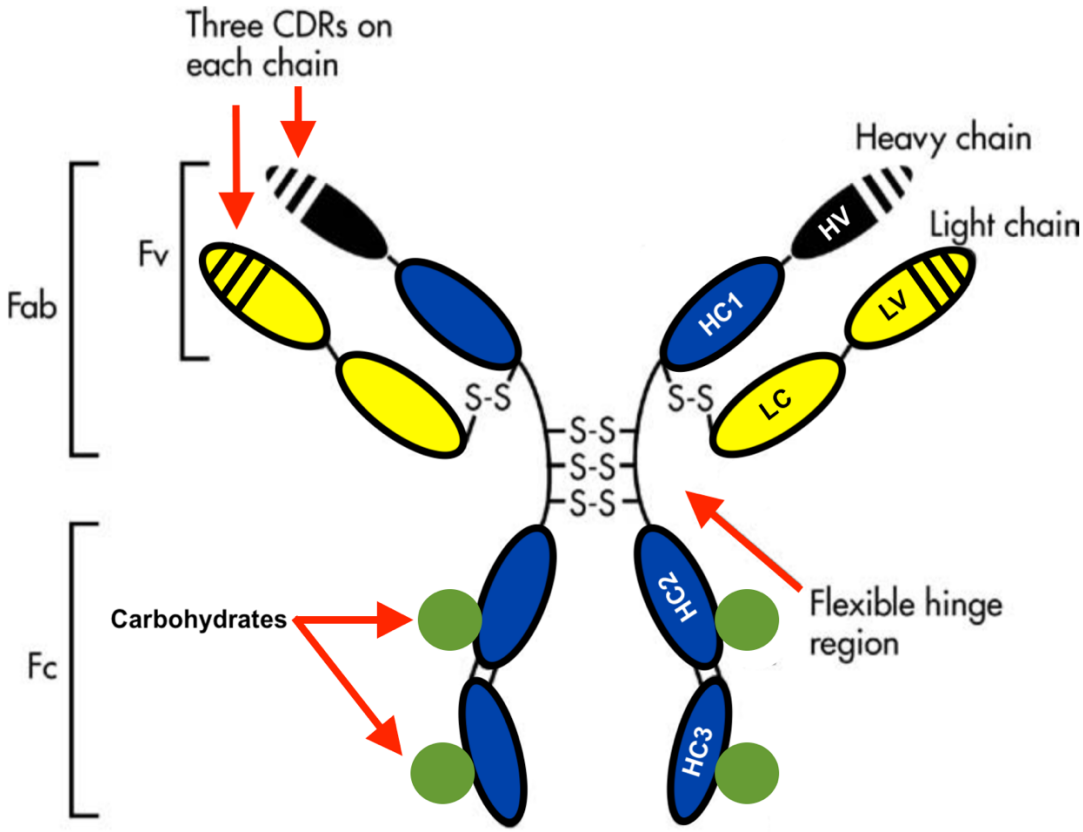


Figure 1-9: Basic antibody structure of human IgG illustrating a pair of identical heavy chain and light chains.

(Adapted and modified from Paraf and Peltre, 2012; Liddell, 2013)

Constant domains (HC and LC) as the name suggest are relatively constant in sequence with varying degree of glycosylation and are responsible mostly for the binding properties and interactions towards other effector cells involved in the immune response such as mast cells and phagocytes. The variable domains (HV and LV) form the antibody determinant or ‘paratope’, and are unique shaped to

fit a specific portion of an antigen called ‘epitope’, that leads to tremendous diversity of binding sites for different Abs. This can be achieved by the presence of three areas of hypervariable sequence each known as the complementarity-determining regions (CDRs) (Paraf and Peltre, 2012; Liddell, 2013). HV and LV (total of six CDRs) together form the antigen-binding site (Fv). Structural information about these sequences has been an important basis on emergence of variable immunoassays techniques, development and advancement throughout the years.

IgG antibodies are secreted by plasma cells produced by the B-lymphocytes of the adaptive immune system. Ab genes are assembled from pieces of DNA scattered widely throughout the genetic material (Deshpande, 1996). As the B-cell that produces Ab matures, it rearranges these gene components such that the new gene and the antibody it encodes become unique. Once the B-cell containing this gene arrangement proliferates, all its descendants will make this unique antibody (Paraf and Peltre, 1991). IgG is used almost exclusively in immunoassays. It is produced in the highest yield in response to immunization, binds with higher affinity to its epitope and is stable during isolation and purification process and has several functional sites that can be used for chemical coupling with minimal loss of antibody binding (Liddell, 2013). Furthermore, since immunoglobulins are proteins, therefore they can undergo *in-vitro* reactions developed specifically for proteins, e.g. iodination, conjugation, separation and all possible techniques which are routinely available for biochemist to make use of its advantageous features. The specificity of antigen-antibody reaction makes Abs very useful and highly selective reagents in immunoassays.

1.4.2 Antigen-antibody interaction

The fundamental feature of all immunological methods is the recognition of an analyte, the antigen (Ag), by a reagent, the antibody (Ab) which has specificity directed against the antigen. This interaction will form an antibody-antigen (Ag-Ab) complex that will lead to its elimination from circulation through the mononuclear phagocytic system. The attractive forces that stabilise this Ag-Ab interaction complex include a combination of hydrogen bonds, hydrophobic bonds, coulombic interactions and van der Waals forces (Steward ,1984). In general, these forces are weak individually in comparison to covalent bonds. However, combination of these forces altogether are able to form a strong bond (termed the affinity of the Ab) (Harlow and Lane, 1999) which leads to stabilization and they are of fundamental importance to the specificity of the Ag-Ab complex (Deshpande, 1996).

1.4.3 Immune response and antibody production

The immune system of animals are capable of recognising and responding in a protective manner towards invasion of foreign materials when they break through the physical barriers such as the skin or mucous membrane which form the animal's 1st line of defence. The immune response can be divided into two general systems, cell-mediated immunity and humoral immunity, where the former is dependent upon direct interaction of T-type lymphocytes and phagocytic cells with the foreign material, whereby the latter refers to the protection provided by a group of inducible globular proteins (immunoglobulins) produced by B-type lymphocytes (Rittenburg, 1990). In other words, the presence of the foreign protein initiates a sequence of events, mediated by the

cells of the immune system, that lead to the release of Ab molecules in blood and other body secretions (Burns, 2005). Two important properties are necessary for a complete Ag immunogenicity, i.e. the capacity to stimulate formation of Abs; and the ability to react specifically with these Abs. Ag and immunogen are terms often used interchangeably where Ag refers to any substance with which an Ab will bind, and the term immunogen refers to those substances capable of inducing an immune response, but both able to stimulate the immune system and are able to induce humoral response when introduced into an animal. Macromolecules such as proteins (glycoprotein, lipoprotein or nucleoprotein) and polysaccharides are mostly good immunogens (Catty, 1988). However, Abs able to bind to a small molecules can also be produced under specific conditions.

Although the natural purpose of Abs production is to protect against invading pathogens, Abs can be produced experimentally *in vitro* by the injection of an animal with an analyte (antigen) alone or bound to a protein carrier to induce the formation of specific Abs in the body of the animal. Generally, animals are capable of producing different types of Abs which can bind at specific sites (epitopes) of the Ag. The ideal Ab can be defined as possessing a high titre and high specificity as well as high affinity towards the Ag of interest. The affinity between an Ab and Ag represents the main factor for achieving a low detection limit in an immunoassay (Deshpande, 1996). There are three types of Abs usually used in immunoassays; polyclonal antibodies (pAbs), monoclonal antibodies (mAbs) and recombinant antibodies (rAbs), but in this study, only pAbs and mAbs will be discussed.

1.4.3.1 Polyclonal antibodies

PAbs, are normally produced by immunizing animals with the Ag of interest. The B-lymphocytes have specific antigen binding receptors on the outer surface of the cell membrane that reflect the specificity of the Ab that it will produce. Many immunogens contain a variety of different antigenic determinants and thus bind to and induce different B-lymphocytes to proliferate, producing variety of Abs which bind specifically to different epitopes on a given substance respectively. Thus, pAbs are a heterogenous mixture of Abs of varying binding affinities, different specificities and isotype able to recognize epitopes both on the immunogen and any impurities injected with it (Liddell, 2013). PAbs have been raised in numerous species including mice, hen, rabbits, goats and sheep (Kascsak *et al*., 1987; Clarke *et al*., 1993; Beans, 2001; Matsumoto *et al*., 2005). The choice of host depends on the intended use of the resulting antibody. Although goats and sheep are the species of choice for large-scale antiserum production, rabbits are the most common species of choice for pAb intended for routine work, which balances the low maintenance costs with decent volumes of serum (Beans, 2001, Liddell, 2013). In addition, pAb raised in rabbits offers the advantage of ready availability and general high quality of a wide variety of anti-rabbit Ab secondary detection reagents (Burns, 2005). PAbs are reagents of choice for general purpose analytical applications in immunoassay systems and have been used extensively in food immunoassay (Ramesh *et al*., 2007; Burkin *et al*., 2010; Suarez-Pantaleon *et al*., 2010; Jiang *et al*., 2011; Kondo *et al*., 2011).

1.4.3.2 Monoclonal antibodies

These are Abs produced by the clone of a single B-lymphocyte, often identified and isolated from pool of B-lymphocytes that synthesize pAbs, and therefore have identical specificities. As normal lymphocytes cannot normally survive *in vitro*, monoclonal technology involves fusing of an antibody-producing B–lymphocytes with immortal myeloma cell line (lacking the DNA salvage pathway) to produce hybrid cells that can be grown indefinitely in tissue culture, producing single Ab specificity, a Nobel pioneering work done by Köhler and Milstein in 1975. These hybridomas inherit both the ability to survive *in vitro* from the myeloma cell and the capacity to produce Ab from the lymphocyte (Rittenburg, 1990). Through a series of selection procedures, single-antibody secreting hybridoma cells can be isolated in individual tissue culture wells from which large colonies (monoclones) can develop through mitotic division, each secreting identical Abs, given rise to reagents of superb specificity able to distinguish very slight differences between epitopes of different immunogens (Liddell, 2013). These Abs can be made in limitless quantities since the hybridoma cells can be grown in tissue culture almost indefinitely at industrial scale, can be frozen for storage and recovered when required without the need for recharacterization as would be necessary for a new batch of pAbs (Liddell, 2013). The initial Abs or B-lymphocytes are mostly derived from a successful immunisation of target Ags in mice before the fusion with immortal myeloma cells from murine cell line. Other cell lines are currently emerging such as rats and more rarely human-derived Abs. Rabbit mAbs can also now be made which have certain advantages over mice. Ten of thousands of mAbs are now available commercially, in purified form or as native culture supernatant or ascitic fluid, and can be supplied with a variety of enzyme or fluorescent label attached that being extensively used for various

diagnostic tests, analytic and chemical use, therapeutic and clinical treatments (Drewe and Powell, 2002; Liddell, 2013).

Monoclonal antibodies (mAbs) are important reagents used extensively in biomedical research, diagnostic tools in food safety and toxicity, diagnosis of diseases and treatment of illnesses such as infections and cancer (CMPMA, 1999). It represents a novel way in which to target specific mutations and defects in protein structure and expression in a wide range of diseases and conditions (Liu, 2014). MAbs, as briefly described previously, are monovalent Abs which bind to the same epitope and are produced from a single B-lymphocyte clone. These Abs are produced by cell lines or clones obtained from animals that have been immunized with the substance (immunogen) that is the subject of study. Köhler and Milstein (1975) developed a technique that allows the growth of clonal populations of cell secreting homogenous Abs with a defined specificity. The generation of hybridomas involves immunising certain species against a specific epitope on an antigen and obtaining the B-lymphocytes from the spleen of the animal. The B-lymphocytes are then fused (by chemical or virus induced methods) with an immortal myeloma cell line (a type of B-cell tumour preferably from the same species that can divide indefinitely) lacking the hypoxantine-guanine-phosphoribosyltransferase (HGPRT) gene and not containing any other immunoglobulin-producing cells. This conferring of immortality to that single plasma cell by fusion to myeloma cell results in a hybrid B-cell line (hybridomas). The fusion is then cultured *in vitro* in selective medium (hypoxanthine-aminopterin-thymidine, HAT) where only hybridoma cells survive as they have inherited immortality from the myeloma cells and selective resistance (HGPRT gene) from the primary B-lymphocytes. Unfused B-lymphocytes will eventually

die due to their short lifespan and for the myeloma cells, as they lack HGPRT enzyme, they cannot synthesise nucleotides (purine and pyrimidine) using the salvage pathway as the *de novo* pathway is already inhibited by aminopterin (an antibiotic) in the selective medium, hence they will not survive (Little, 2000).

The initial culture of hybridomas contains mixture of Abs derived from many different primary B-lymphocyte clones, each secreting its own individual specific Ab into the culture medium (the Abs are still polyclonal at this stage). The cell culture medium can then be screened (ideally using ELISA) from many hundreds of different wells for the specific Ab activity required. Each individual clone can be separated by limiting dilution into different culture wells. The desired B-lymphocytes (positive and monoclonal) are grown larger culture wells and then recloned and retested for activity until one can be reasonably sure that the cell line is monoclonal (Li *et al*., 2010). The positive hybridomas and monoclonal Abs generated should be stored in several vials of approximately 10^7 cells in several different -70°C or liquid nitrogen freezers and the freezing medium should contain 10% dimethyl sulfoxide (DMSO) to prevent crystallization of water within the cells (Liddell, 2013). The cells can be propagated by tissue culture conventionally in large flasks, roller bottles, stirred flasks or bioreactors such as the hollow fiber reactor and air lift fermenter depending on the amount of mAbs needed. Ascitic mice is the easiest way to obtain a very concentrated Ab (5-10 mg/mL) from the ascitic fluid but this has been prohibited in certain countries due to the peritoneal tumour induced effect it had on the mice.

1.4.3.3 Antibody production against haptens

Small molecules (hapten) of less than 5,000 – 10,000 Da which, if present by itself, is unable to stimulate the immune system for antibody production. To generate an antibody against haptenic molecules requires the covalent linkage of the hapten to a larger, immunogenic, protein carrier, usually 20,000 to 40,000 Da or even higher molecular mass. Immunodominant structures usually project distally from the surface of the immunogen and are well exposed for inducing highly specific Abs. These sites are also less likely to be sterically hindered from closely approaching the antibody binding site. It is for these reasons that when preparing hapten-protein conjugates for immunisation, the coupling is usually done by the intermediary of a spacer arm (Catty, 1988; Rittenburg, 1990; Roucairol *et al*., 2007). A schematic representation of hapten on a carrier molecule is illustrated in Figure 1-10. The carrier protein, usually bovine serum albumin (BSA), keyhole limpet haemocyanin (KLH), or ovalbumin (OVA) contains many epitopes to which T-cells can respond in order to assist antibody-producing B-lymphocytes after immunization with a hapten-carrier conjugate. The B-lymphocyte receptor binds to the hapten and incorporates the hapten-carrier complex, thus the protein component yields primarily carrier related peptides that bind to MHC (major-histocompatability complex) class II molecules which are presented on the surface of the B-cells. T-cells with specificity for the MHC-presented carrier-derived peptides then activate the B-cells for the production of hapten-carrier specific Abs (Pedersen *et al.*, 2006).

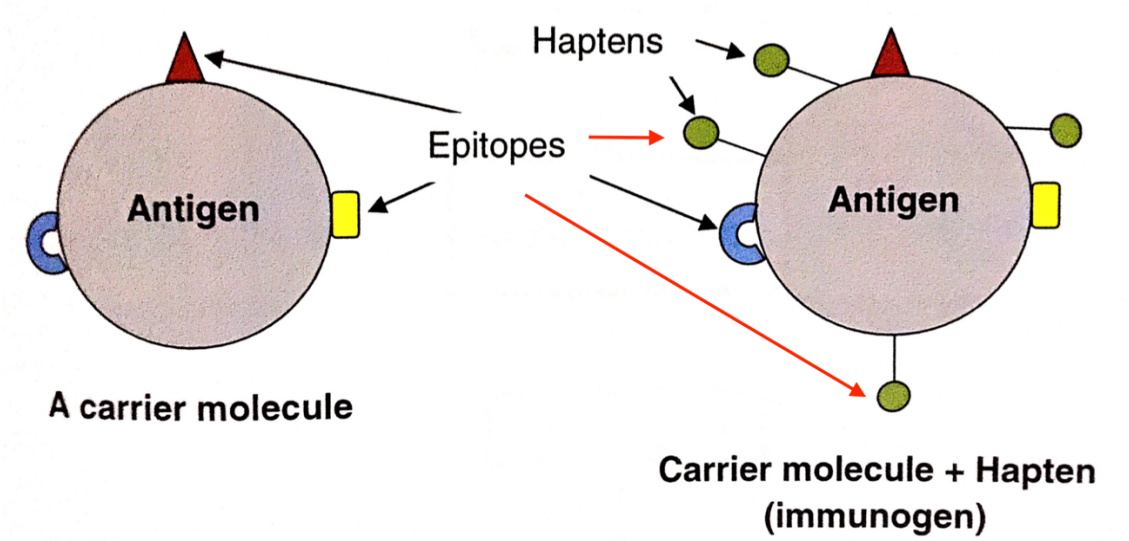


Figure 1-10: A schematic representation of hapten on a carrier molecule, showing the number of hapten bound to one molecule of carrier (adapted with modification from Paraf and Peltre, 1991)

There are four important criteria that have been well defined by Goodrow *et al*. (1998), about the design of an ideal hapten derivative: i) the stereo structure, electronic distribution and hydrophobicity on the hapten derivative should be similar to those on the original hapten; ii) a spacer arm with several-carbon-chain length to couple with carrier is preferred, but the spacer arm should not induce the immunized animal to produce 'arm antibodies' or neodeterminants; iii) the presence of active group at the end of the spacer should not affect the electronic distribution of hapten molecule; and iv) after coupling with carrier, the basic molecular structure of the hapten should be retained. Hence, the most basic requirement of hapten design is to maintain the original molecular structure of the hapten and to exposed it on the surface of the carrier as much as possible, which in turn, will maximise the recognition of the hapten by the immune cells of the immunized animal, stimulating the animal to produce a specific immune

response and generate high affinity and high specificity Abs against the hapten (Juan *et al.,* 2010). In general, antigenic structures or epitopes are mostly located on the hydrophilic parts of the immunogen, each of them being recognized by a specific antibody. The procedure for the production of antibody against hapten is illustrated in Figure 1-11.

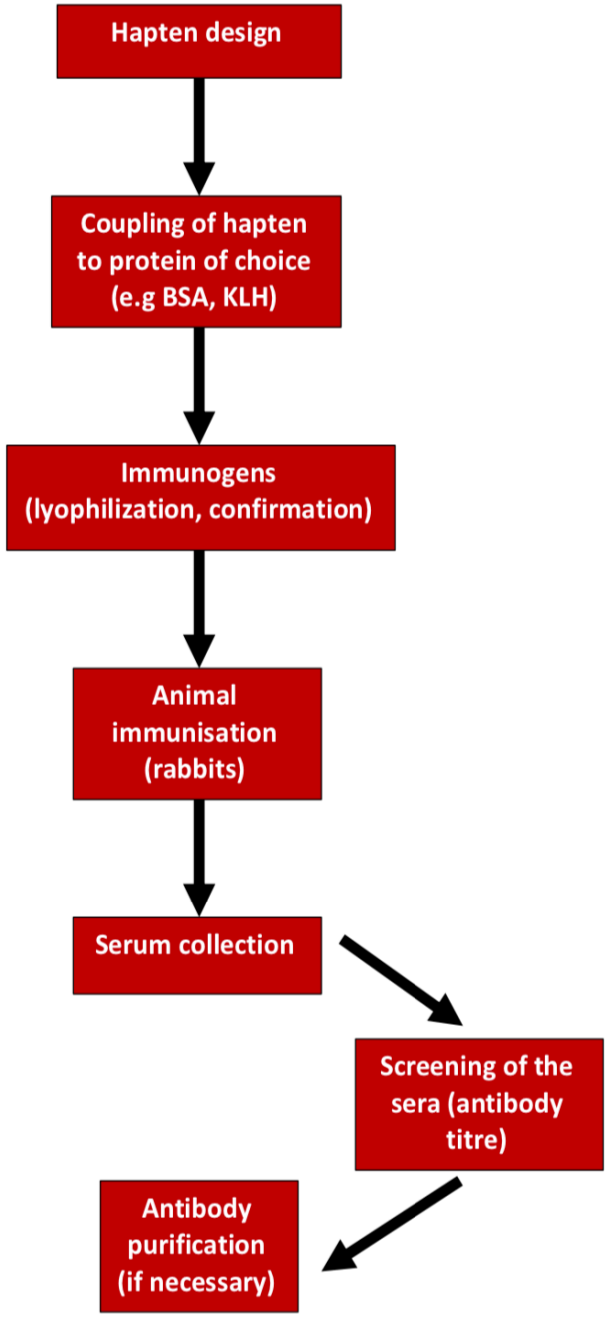
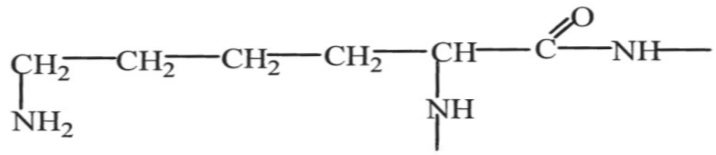


Figure 1-11:Procedure of antibody production against hapten.

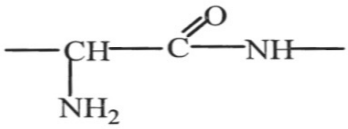
Linamarin is a very small molecule (hapten) of less than 1,000 daltons, 247 daltons to be exact (PubChem, 2005) in which, if present by itself, is unable to stimulate the immune system for antibody production. To generate an antibody against haptenic molecules requires the covalent linkage of the hapten to a larger, immunogenic, protein carrier, usually at least 20,000-40,000Da or even higher molecular mass. Immunodominant structures usually project distally from the surface of the immunogen and are well exposed for inducing highly specific Abs. These sites are also less likely to be sterically hindered from closely approaching the antibody binding site. It is for these reasons that when preparing hapten-protein conjugates for immunisation, the coupling is usually done by the intermediary of a spacer arm (Catty, 1988; Rittenburg, 1990; Roucairol *et al.*, 2007). Typically, haptens are conjugated to carrier proteins such as bovine serum albumin (BSA), keyhole limpet haemocyanin (KLH), ovalbumin (OVA), ovomucoid (OVO), thyroglobumin or fibrinogen. These proteins contain many epitopes to which T-cells can respond in order to assist antibody-producing B-lymphocytes after immunization with a hapten-carrier conjugate (Hancock and O'Reilly, 2005).

The B-lymphocyte receptor binds to the hapten and incorporates the hapten-carrier complex; thus, the protein component yields primarily carrier-related peptides that bind to MHC (major-histocompatability complex) class II molecules which are presented on the surface of the B-cells. T-cells with specificity for the MHC-presented carrier-derived peptides then activate the B-cells for the production of hapten-carrier specific Abs (Pedersen *et al.*, 2006). Among the carrier proteins used for hapten-protein conjugates, BSA is the most frequently

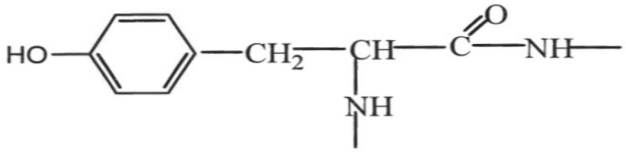
used protein because of its stability in some organic solvents allowing a wide range of chemical reactions to be attempted, the abundance of Ɛ-amino groups of the lysine residues available for conjugation (Figure 1-12), in addition to its wide availability with low cost and ability to yield soluble conjugates (Erlanger, 1980). KLH is also used by many but it is relatively expensive, and its high molecular weight might be limiting (Hsieh, 2010, Juan *et al*., 2010).



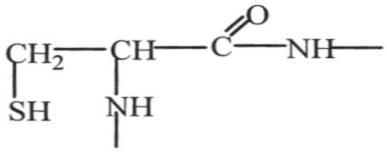
ε-Amino groups of lysine residues (59 available)



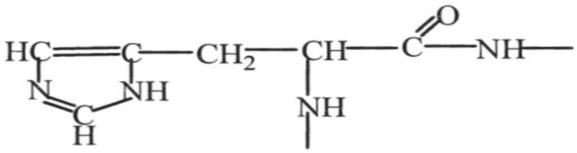
α-Amino groups (1 available)



Phenolic hydroxyl groups of tyrosine residues (19 available)



Sulfhydryl groups of cysteine residues (1 available)



Imidazole groups of histidine residues (17 available)

Figure 1-12: Functional groups (with number of availability) in bovine serum albumin (BSA), (adapted from Erlanger, 1980)

The preparation of immunogenic hapten-protein conjugates is governed by the nature of the reactive functional groups of hapten. That is, functional groups of the hapten govern the selection method to be used to conjugate the hapten to the functional groups of the carrier. Haptens are covalently conjugated to carrier proteins using various coupling methods. According to Juan *et al*. (2010) hapten-protein conjugates can be prepared by i) modifying the existing hapten, ii) using relevant intermediate or metabolite of the hapten and iii) resynthesizing the hapten-protein conjugate from raw materials.

A review conducted by Erlanger (1980) on the preparation of antigenic hapten-protein conjugates reports that conjugation methods such as carbodiimide and mixed anhydride procedures can be used for haptens with amino groups, succinic anhydride procedures can be used for haptens with carboxyl groups, while haptens such as alcohols, phenols, sugars, polysaccharides and nucleotides with available hydroxyl groups can be conjugated to protein with procedures using hemisuccinates (used for coupling alcohols to protein), aminophenyl derivatives (used for coupling of phenols and sugars to proteins) and oxidation to dialdehydes, a procedure which makes use of the reaction of vicinal hydroxyl groups with periodate to yield dialdehydes. The latter procedure is used for coupling of compounds such as glycols, glycerol derivatives and glycosides to protein. In another study, cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) has been reported to be an efficient coupling reagent for labelling Abs with enzymes (Abuknesha *et al*., 2005).

1.4.3.4 Primary antibody

Primary antibody refers to the Ab obtained from active immunization of laboratory animals with an immunogen of interest that will form antibody-antigen complex to the same antigen adsorbed onto a solid phase. The Abs are made in response to any immunogenic epitope in the injected material, includes any impurities in the preparation and any carrier protein or linker attaching them which have been described in section 1.4.3.1.

1.4.3.5 Secondary antibody

Secondary Abs are anti-immunoglobulin Abs. Because Abs are proteins and exhibit distinct structural difference when obtained from different species, they themselves can be used as immunogens. Thus, anti-antibodies can be raised for example, when rabbit immunoglobulin is injected into a goat. The secondary antibody is usually directed against species-characteristic determinants on the constant regions of the primary of the immunizing Ab to which it binds irrespective of the binding or antigenic specificity of the primary Ab (Deshpande, 1996). The secondary Ab used in immunoassay does not have the specificity to bind the Ag but only recognizes the primary Ab; thus it makes a link of the enzyme label to the bound immunomolecules without interfering with the primary Ag-Ab binding (Hsieh, 2010).

1.4.3.6 Antibody titre

In immunoassay, titre is the measurement of the amount and affinity of Ab generated from an immunised animal that recognises the Ag epitope. Ab titre can also be defined as the dilution factor which gives 2- times higher optical density values than the pre-immune control serum or the dilution that binds 50% of a certain amount of labelled antigen (Price & Newman, 1991). In hapten conjugation, different coupling methods may result in different Ab titres.

1.4.3.7 Antibody specificity

Specificity is described as the ability of one Ab to bind to one and not to another member of a family of chemically-related substances (Langman, 2000). Specificity is an important characteristics that determines Ab quality. The specificity of an immunoassay method is determined by the ability of the Ab to form a complex with only one kind of molecule (analyte) in the presence of different molecules (Catty, 1988). The structure of the Ag and the homogeneity of the Abs are important factors in determining specificity (Zhang *et al*., 2007). Specificity may be established by assaying a number of structurally related analytes and determining their cross-reactivity. Cross-reactivity can be defined as a situation in which at least two different Ags compete for the same antibody binding site (Benjamini, 2000). Abs with cross-reactivity ≤ 10% are very specific, and can be used for quantitative determinations, while Abs with cross-reactivity ranging from >10 to 100% can be used for qualitative tests, though Abs with cross-reactivity ranging from ≥ 50 to 150% can be used for semi-quantitative, group-specific tests (Fremy & Usleber, 2003).

1.5 Immunoassay

1.5.1 Definition

Immunoassays are analytical techniques based on the specific and high affinity binding characteristics of a group of inducible, often, animal-derived proteins called Abs with particular target molecule known as Ag (Hsieh, 2010). Higher vertebrates are remarkably proficient in synthesizing Ab with precise specificity to its target Ag that could come in variety of organic molecules and macromolecules provided, they are able to induce the humoral immune response. Both Ags and Abs are interchangeably used either as target molecules or capture molecules making immunoassay a versatile technique that has been one of the fastest growing and most widely used analytical techniques (Edwards, 1996; Price and Newman, 1997). The primary binding between the Ab and the target Ag forms the basis of the immunoassay, generating a signal that could be measured allowing quantification of the target and a wide variety of immunoassay formats have been developed to allow either visual or instrumental measurement of this primary binding reaction. The potential of rapidly measuring minute quantities of a specific analyte from within a complex sample matrix, often with little or no sample clean-up, is one of the attractive features that has led to the widespread application of immunoassay. In comparison to other analytical methods such as gas chromatography (GC), thin-layer chromatography (TLC), HPLC, and electrophoresis, immunoassay provides a rapid, cost effective, highly sensitive and specific analysis that is relatively simple to perform and interpret (Rittenburg, 1990).

Several variations exist in the design of particular immunoassays. Generally, all assay names contain the word “immune” combined with another word indicating the type of label used, along with the word “assay”, generally means test. Signal generation mentioned previously usually involves conjugation of either the antibody or the target to ta detectable label such as a radioisotope or an enzyme. Therefore, in isotopic immunoassays, a radioimmunoassay (RIAs) described an assay system in which the detection label is radioisotope and in non-isotopic immunoassays, enzyme immunoassays (EIAs), fluorescent immunoassay (FIAs), and chemiluminescent immunoassay (CIAs) described a variety of markers or labels, individually or in combination, used to in the respective assay system (Voller *et al*., 1978; Quan *et al*., 2006). RIA was first described in 1959 by Yalow and Berson for the analysis of the endogenous plasma insulin in human with a high degree of sensitivity and specificity. On the other hand, “immunometric assay” is used when the assay involves the use of reagents in stoichiometric excess (Deshpande, 1996). Such an assay may also be denoted as an immunoradiometric assay (IRMA), immunoenzymometric assay (IEMA) or immunofluorometric assay (IFMA), depending on the label used (Edwards, 1985; Hemmila, 1985). Immunoassays are popular techniques of choice because of their simplicities as well as the possibilities they offer in terms of automation to meet the numerous challenges faced in the analytical laboratories.

1.5.2 Enzyme-linked immunosorbent assay (ELISA)

Enzyme immunoassay (EIA) was developed as an alternative to some of the disadvantages of using the isotopic assay because radioisotope-labeled reagents are usually radioactive in nature, unstable, hazardous and disposal of

radioactive wastes is problematic. In addition, EIA can be as sensitive as radioimmunoassay, if not more so (Engvall & Perlmann, 1972). The work of Engvall and Perlmann (1971) together with Van Weemen and Schuurs (1971), have changed the world of immunoassay immensely. They invented and developed the “enzyme-linked immunosorbent assay” (ELISA), a term generally used for reagent excess enzyme immunoassays for specific Abs or antigens. In their work, they were able to prove the achievability of an enzyme immunoassay technique, showing that a sensitive signal could be generated by an enzyme-catalysed reaction, an idea that was opposed by others at that time who questioned how such large molecule like an enzyme could be linked to either the antigen or the antibody without affecting the reaction. Since then, the enzyme immunoassay has been the foundation of development of all immunoassays. Enzyme-linked immunosorbent assays (ELISAs) have been successfully developed not only for detection of macromolecular compounds (proteins, nucleic acids and bacteria, etc.) but also for analysis of low molecular weight compounds (hormones, toxins and drugs, etc.). The key ingredient in an immunoassay development is the antibody and the key process is its production, allowing a very specific detection associated with high sensitivity and high affinity. Attaining appropriate Abs is always a challenge even if the immune system can be exquisitely selective in producing Abs, particularly if the protein-hapten conjugate has been carefully designed. Therefore, one of the most vital procedures for generating Abs with highly sensitive and specific determination of small, low molecular weight molecules is the design of the hapten and the way the hapten is being linked to the protein carrier. The orientation of attachment should be such that the relevant structural determinants of the analyte are free to interact with the immune system so that the resulting Abs have a chance to

bind selectively to the desired analyte without cross-reaction with analogues or metabolites possibly present in the same samples (Roucairol *et al*., 2007). All ELISA protocols include the following five steps: i) coating of antibody or antigen on a solid phase (usually a microtitration plate); ii) blocking the remaining uncoated surface on the solid phase with blocking buffer containing non-specific protein such as bovine serum albumin or gelatine (this is to minimise the nonspecific reactions and also protect the adsorbed antigen or antibody from surface denaturation); iii) incubating with different immunoassay reagents at specified temperature and time; iv) washing the coated surface to separate free unbound molecules from bound molecules; and v) detecting the colour developed from the assay visually or spectrophotometrically (Hsieh, 2010). Specific procedures may vary with different variations of ELISA depending on the target analytes. There are several types of ELISA format that took advantage of the use of either immobilized antigen or Abs. Indirect non-competitive ELISA (incELISA) and indirect competitive ELISA (icELISA) will be discussed in section 1.5.2.1 since these were the ELISA formats used throughout the study.

1.5.2.1 Indirect and direct ELISA

All immunoassay signals can be detected directly or indirectly. In the direct detection method, the detecting molecules are purified and linked to the label to directly measure the amount of the Ag-Ab complex. In contrast, the indirect detection method uses a commercially available intermediate reagent to link the capture molecule. Most often a labelled anti species Ab is used for an antibody-captured assay to indirectly measure the amount of antibody-complex formed (Hsieh, 2010). Although an additional step is involved, indirect assays require

less immunoreagents and in many cases could be more sensitive because more labelling molecules can be linked to the detection Ag or antibody for enhanced signal production and the interaction between the enzyme and food sample matrices could be avoided due to absence of the enzyme label during incubation (Morgan, 1989). While the direct detection method is essential when an accurate quantification is required for the assay, the indirect detection method is used in most solid-phase immunoassays.

1.5.2.2 Indirect non-competitive ELISA

This is the simple form of indirect antibody-captured ELISA which is a sensitive technique for quantifying molecules in solution. It is often used in the early stage of immunoassay development to detect primary Abs in antisera or screen hybridoma supernatants for searching desired Abs. The soluble antigen is adsorbed (coating) onto the surface of the microtitration wells and incubated. To prevent nonspecific binding, any vacant binding sites in the plate are sometimes blocked with the use of a protein such as bovine serum albumin (BSA) or gelatine. After blocking the diluted samples of antisera or hybridoma supernatants are then added to the wells and incubated to allow the Abs to bind specifically to the immobilized antigen. After washing the unbound molecules, those bound Abs can be detected by adding the secondary antibody. The secondary antibody is usually linked to an enzyme, and the addition of suitable substrate results in a colorimetric reaction. The colour can then be measured using a spectrophotometer. The resulting optical density (OD) is proportional to the amount of antigen present in the sample (Kemeny and Chantler, 1988; Jordan, 2005).

1.5.2.3 Indirect Competitive ELISA

Competitive ELISA involves the use of two forms of the Ag, one is the sample Ag to be assayed and the other, a constant level of “tracer” or specific antigen immobilized on the microtitration plate well. Plates are coated with the specific Ag and these are then incubated with a mixture of Ab and the test sample. If there is no Ag in the test sample the antibody becomes fixed to the antigen-sensitized surface. If there is Ag in the test solution, it binds with the Ab, which cannot then react with sensitized solid phase. The amount of Ab attached is then indicated by an enzyme-labelled Ab conjugate and enzyme substrate. The amount of inhibition of substrate degradation in the test sample (as compared with the reference system) is proportional to the amount of antigen in the test (Voller *et al*., 1978). Competitive ELISA, unlike two-site ELISA, permits both simultaneous and sequential incubation of sample and labelled antibody (Porstmann & Kiessig, 1992) and may be of greater specificity compared to the two-site ELISA (Makarananda *et al*., 1995). The direct ELISA and both indirect non-competitive and competitive ELISA are depicted in Figure 1-13.

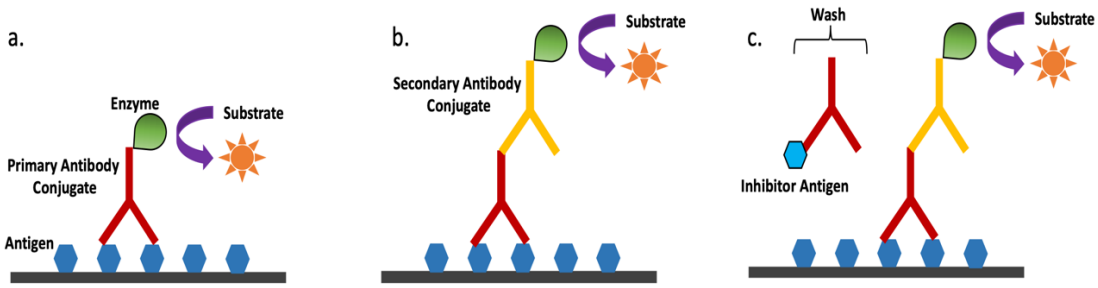


Figure 1-13: Different formats of enzyme-linked immunosorbent assay (ELISA). a. Direct ELISA; b. Indirect non-competitive ELISA; c. Indirect competitive ELISA.

Generally, immunoassay techniques can be a cost effective detection and quantification method for trace contaminants or toxins in a wide variety of foods. Most modern immunoassays for environmental and food contaminants are based on the use of enzyme tracers to provide quantification signals (Meulenberg, 1997). This is because enzyme-based assays have appropriate sensitivity for most purposes and have been developed in a variety of configurations. The long shelf-lives and ease of distribution of enzyme-based immunoassays also make them suitable for field use. Thus, immunoassay is a very useful analytical technique that can be used for the development of specific and sensitive assays that would permit the determination of trace residues of contaminants such as mycotoxins or phytotoxins in small volume of samples which could be otherwise difficult to analyse by other more expensive analytical methods. Successful development of an immunoassay technique would however depend on the availability of Abs with appropriate affinities and specificities.

1.5.3 Immunoassay application in food

Immunoassays are now widely utilized in food analysis for determination of food contaminants and in the agrochemical industry for detection of chemical residues in plants, water and soil (Meulenberg, 1997; Ramesh *et al*., 2007; Sathe *et al*., 2012). Different types of immunoassay techniques have been developed for the detection and quantification of hazardous compounds in food. Extensive research has been carried out on the development and use of various immunoassay techniques for monitoring occurrence and detection of mycotoxins (such as T-2, HT-2, deoxynivalenol, ochratoxin A and aflatoxin B_1) in wheat, barley, rice and corn (Morgan *et al*., 1983a; Ward *et al*., 1990; Yu *et al*., 2005;

Liu *et al*., 2012; Li *et al*., 2016). ELISA is the most commonly used method (Li *et al*, 2009b). ELISA has also been used in monitoring residual insecticides and herbicides in several food commodities (Krikunova *et al*., 2002; Bo *et al*., 2008). Quan (2006) developed a sensitive enhanced chemiluminescene enzyme-linked immunosorbent assay (ECL-ELISA) for the detection of fumonisin B_1 (FB_1) in food and animal feeds. Pastor-Navarro *et al*. (2007) was able to develop an ELISA for detection of different tetracycline residues in honey. In another study, Chen *et al*., 2007 developed a sensitive ELISA method for monitoring neomycin (an aminoglycoside antibiotic) residue levels in milk and other biological matrices. Recently, electrochemical immunoassays have been developed for detection of zearalenone mycotoxin in foods and very useful because of their suitability for mass production, high sensitivity, large number of labels and nanotechnology applications (Hervás *et al*., 2009). Immunoassay has also been used for the determination of tracers of peanut protein contamination in complex food matrices (Morgan *et al*., 1986; Mills *et al*., 1997; Holzhauser and Veiths, 1999). Despite numerous and elaborate studies done on detection and quantification of CGs in plant foods, surprisingly the use of immunoassay for its quantitative determination is still lacking, despite its inherent sensitivity, simplicity, rapidity and cost effectiveness.

1.6 Aim of the project

The aim of the study is to develop a rapid and sensitive enzyme-linked immunosorbent assay (ELISA) test for detection and quantification of linamarin, a cyanogenic glycoside in cassava and to determine its content in commercially available cassava-products in the UK.

The whole thesis describes the experimental approach and results towards the following area:

i) Linamarin (hapten) conjugation to a suitable carrier proteins and the production of the polyclonal antibody against linamarin.

ii) Development of monoclonal antibody against linamarin.

iii) Sample preparation to extract linamarin from plant tissues and food products and the development of an ELISA for the detection and quantification of linamarin in foods.

The use of immunoassay and development of antibodies against linamarin for its direct determination and quantification in fresh cassava and processed products have not yet been reported although total cyanogenic contents in cassava have been extensively studied and investigated through various other methods as discussed previously in section 1.3.9. Successful conjugation of the immunogen followed by immunisation would provide good antibodies to be used in the ELISA for the detection and quantification of linamarin in foods.

Chapter 2 : Materials and Methods

2.1 Chemicals, Consumables and Solvents

The following chemicals, consumables and solvents were obtained from:

Sigma-Aldrich Chemical Co. Ltd, Poole, Dorset, UK

- Naringin >=90%, from citrus fruit (N1376, 100 g)
- Cyanuric chloride, 99% (C95501, 250g)
- Acetone (31062, 2.5 L)
- Methanol amd chromasolv (34898, 2.5 L)
- Diethyl ether (296082, 1 L)
- Amygdalin, from apricot kernels (A6005, 100 mg)
- Sodium carbonate (S2127, 1 kg)
- Sodium bicarbonate, ReagentPlus®, ≥99.5%, crystalline (S8875,1 kg)
- Tween® 20 (P1379, 500 ml)
- 3,3', 5,5' Tetramethylbenzidine (TMB) liquid substrate system for ELISA (T0440, 1 L)
- 3,3', 5,5' Tetramethylbenzidine (TMB) liquid substrate, Supersensitive, for ELISA, ready to use solution (T4444, 100 ml)
- Gelatin from cold water fish skin (G7765, 250 ml)
- Gelatin from cold water fish skin, powder (G7041, 100 g)
- Sodium phosphate dibasic, anhydrous, ≥ 98.0% (71642, 1 kg)
- Phosphoric acid >=85wt% solution in water (438081, 2.5 L)
- Eppendorf® epT.I.P.S standard, capacity 0.5-20 µl (Z640085-1000EA)

- Eppendorf® epT.I.P.S standard, capacity 20-300 µl (Z640107-1000EA)
- Eppendorf® epT.I.P.S standard, capacity 50-1000 µl (Z640115-1000EA)

Fisher Scientific Ltd., Loughborough, Leicestershire, UK

- Disodium hydrogen orthophosphate dihydrate (S/4450/60,1 kg)
- Potassium chloride, laboratory reagent grade (P/4240/53, 500 g)
- Sodium chloride, for analysis, certified AR 99.5% (S/3161/53, 500 g)
- Sulphuric acid, 95-97% (07208, 2.5 L)
- Bovine serum albumin, free fatty acid (BP9704-100, 100 g)
- Sodium hydroxide (S/4800/60,1 kg)
- Hydrochloric acid, analytical reagent grade (~37%, H/1200/PB17, 2.5l)
- Potassium carbonate anhydrous, analytical reagent grade (P/4120/53, 500 g)
- Sodium phosphate monobasic for analysis, 99% (Acros Organics, 7558-80-7, 1 kg)
- BioDesignDialysis Tubing™ D304, 21.3 mm wet diameter – 3.57 ml/cm volume, 3500 MWCO (BID-050-020C, 20.5 m roll)
- Eppendorf® epT.I.P.S standard 165 mm, 1-10 mL (10333682)
- X1000 Microcentrifuge tube 2.0 ml, natural, snap cap (11558252)

Scientific Laboratory Supplies Ltd., Nottingham, UK.

- Whatman No. 1 150 mm filter paper circles (Fil2034)
- Whatman No. 1 70 mm filter paper circles (Fil2029)

Calbiochem, Merck Chemicals Ltd., Nottingham, UK

- Keyhole limpet hemocyanin (KLH), from *Megathura crenulata*, protein content 73% (374805, 250 mg)

Eppendorf UK Ltd.

- Eppendorf Research® Plus Pipette 0.5–1.0 µL
- Eppendorf Research® Plus Pipette 1.0–20 µL
- Eppendorf Research® Plus Pipette 10–100 µL
- Eppendorf Research® Plus Pipette 10–200 µL
- Eppendorf Research® Plus Pipette 100–1000 µL
- Eppendorf Research® Plus Pipette 1000–10,000 µL

Insight Biotechnology Ltd., Wembley, UK

- Linamarin, (sc-203439, 50 mg)

Invitrogen Life Technologies Ltd, Paisley, UK

- Goat anti-rabbit IgG (H+L) secondary antibody, HRP conjugate, 1 mg (G-21234). The species-specific enzyme-labelled secondary antibody was used as secondary antibody for the immunoassays using rabbit antisera.

Morrisons Supermarket, Leeds, UK

- Dried skimmed milk powder (Marvel, 1 kg)

CovalAb UK Ltd., Cambridge, UK

- Goat anti-mouse IgG (H+L) secondary antibody, HRP conjugate, 1 ml (LAB0252).
- Goat anti-rabbit IgG (H+L) secondary antibody, HRP conjugate, 1 ml (LAB0273).

Gilson Scientific Ltd., Bedfordshire, UK

- Pipetman L Multichannel 20-300 µl 12 channel (FA10016)

Greiner Bio-One Ltd., Gloucestershire, UK.

- 96 Well ELISA plate, flat bottom, high bind (655061)
- Pipette tip, to fit Gilson P200, yellow, 5000 per case (739295)
- Eppendorf tube with attached cap, 1.5 ml, natural, 4000 per case (616201)
- EASYseal plate sealer, transparent, 100 per case (676001)

VWR International Ltd., Leicestershire, UK

- Absolute ethanol, AnalaR NORMAPUR, ACS Reagent (20821.330, 2.5 L)
- 2,4,6-Trinitrobenzene sulfonic acid, TNBS, 1% in methanol (BC86)
- Potassium hydrogen orthophosphate (26923.298, 1 kg)
- Parafilm sealing film, length-75 m, width-100 mm (291-1212)

All chemicals and solvents were stored and handled according to manufacturer's specifications.

2.2 Equipment

The general laboratory equipment used in this study are as follows:

- Washing of polystyrene microplates was carried out using Tecan Hydroflex plate washer (Tecan Group Ltd., Männedorf, Switzerland).
- A high resolution (0.001 Abs) photodetector Multiskan FC® plate reader (Thermo Scientific, Vantaa, Finland) and Tecan Spark 10M reader (Tecan Group Ltd., Männedorf, Switzerland) were used to determine the ELISA microplate well optical densities at 405nm.
- Water (unless otherwise stated) was purified using a Millipore Direct-Q 5 (Millipore, Watford, UK) which is a tap-fed purification system that purifies potable water to ultrapure (Type 1) water with resistivity of 18.2 MΩ cm (25°C) and a TOC value below 5 ppb at temperatures between 20 – 25^{o}C.
- Incubation of samples, substrate and antibody was carried out using a Sanyo incubator, model MIR-162 (Sanyo Electric Co. Ltd., Moriguchi, Japan).
- Cassava samples were homogenised using a domestic blender, Tefal Mastermix BL800140, 850 watt (Tefal UK & Ireland, Berkshire, UK).
- Centrifugation of extracts were performed using a refrigerated centrifuge, Thermo Scientific Heraeus Megafuge 16R (Life Technologies Ltd, Paisley, UK).
- Solvent evaporation was performed using a rotary evaporator (Heidolph Hei-VAP Value Digital, Heidolph UK, Essex, UK).

- The pH of solutions were measured with a Hanna HI2211 pH Meter (Hanna Instruments INC., Woonsocket, USA), calibrated with standard buffer solutions of pH 4, 7 or 10 each time before use.
- Lyophilization of hapten-protein conjugates was carried out using a Labconco FreeZone 2.5L Freeze Dryer (Labconco, Kansas, MO, USA).
- UV absorbance of samples were measured using a Jenway 6700 UV-Vis spectrophotometer (Bibby Scientific Ltd., Staffordshire, UK) with quartz cuvettes, 1 ml volume (Spectronic Analytical Instruments, Leeds, UK).
- Mixing of solutions was carried out either by using IKA magnetic stirrer (IKA-Werke, Staufen, Germany) or by using an electric vortex mixer - Fisherbrand Whirlimixer (Fisher Scientific Ltd., Leicestershire, UK).
- Chemicals were weighed using an OHAUS Pioneer precision balance (OHAUS Europe GmBH, Switzerland) or AND precision balance (A&D Instruments, Oxfordshire, UK).
- Dispensing of small volumes of reagents and solutions was carried out using Eppendorf Research® Plus pipettes ranging from 0.5-10,000 µl, pre-calibrated before use.

2.3 Buffers, Solvents and Solutions

2.3.1 Antigen coating buffer

(0.05 M buffer solution of sodium carbonate bicarbonate, pH 9.6)

Sodium carbonate ($NaCO_3$, 2.65 g) and sodium hydrogen carbonate ($NaHCO_3$, 2.10 g) were each dissolved in water (500 ml) to obtain 0.05 M solutions. Sodium hydrogen carbonate (0.05M) was gradually added to sodium carbonate (0.05 M). 50% Hydrochloric acid (HCl) or 4 M sodium hydroxide (NaOH) were used to adjust the pH to 9.4. The buffer was prepared fresh for daily use and any excess solution was discarded.

2.3.2 Phosphate-buffered saline (PBS)

Sodium chloride (NaCl,16.0 g), disodium hydrogen orthophosphate ($Na_2HPO_4.12H_2O$, 3.56 g), potassium dihydrogen phosphate (KH_2PO_4, 0.4 g), potassium chloride (KCl, 0.4 g) were dissolved in water (1800 ml). The pH of the solution was adjusted to 7.4 using HCl (50%) or sodium hydroxide (4 M), as required. The solution was made up to 2L with water. The buffer can be stored at room temperature and was used within one week or kept at 4ºC and used within 2 weeks.

2.3.3 Phosphate-buffered saline plus Tween 20 (PBST)

PBST buffer was prepared as 2.4.1 except that Tween 20 (1.0 ml, 0.05%) was added after the pH adjustment to 7.4.

2.3.4 Blocking buffers

- **Gelatine-PBST (1%, v/v).** Gelatine (1.0 ml) was dissolved in PBST (99.0 ml) as prepared in 2.4.3. This blocking buffer was prepared fresh for daily use and any excess solution was discarded.
- **Milk-PBST (1%, w/v).** Skimmed-milk powder (1.0 g) was dissolved in PBST (100 ml) as prepared in 2.4.3. This blocking buffer was prepared fresh for daily use and any excess solution was discarded.
- **BSA-PBST (1%, w/v).** BSA (1.0 g) was dissolved in PBST (100 ml) as prepared in 2.4.3. This blocking buffer was prepared fresh for daily use and any excess solution was discarded.
- **Soy-PBST** (1%, w/v). Soy powder (1.0 g) was dissolved in PBST (100 ml) as prepared in 2.4.3. This blocking buffer was prepared fresh for daily use and any excess solution was discarded.

2.3.5 Enzyme substrate

3,3', 5,5' Tetramethylbenzidine (TMB) was used directly from stock solution as substrate for the horse radish peroxidase (HRP) attached to the secondary antibody. The TMB was kept in refrigerator at 4°C when not it use.

2.3.6 TMB-HRP reaction stop solution (2 M sulphuric acid)

Concentrated sulphuric acid (11.22 ml) was diluted in water (88.78 ml) with care, to make a 2 M solution, and used to stop enzyme reactions in ELISA.

2.3.7 Rabbit primary antibody serial dilutions (Log 10 dilution)

A series of primary antibody dilutions were prepared using the stock primary antibody obtained from the rabbit. Primary antibody (100 μl) was diluted with PBST (900 μl) in a 1.5ml eppendorf tube to give 1:10 dilution. An aliquot (100 μl) from the 1:10 dilution was diluted with PBST (900 μl) in another 1.5 ml eppendorf tube to make 1:100 dilution. These steps were repeated sequentially to get a series of dilutions up to $1:10^6$ (v:v).

2.3.8 Mouse/hybridoma primary antibody serial dilutions

2 sets of primary antibody dilutions were prepared using the stock primary antibody obtained from the mice and hybridoma (Log 10 and 2-fold dilutions). Due to very small amount of antisera (150 μl, 1/5 dilution, v/v) being delivered by CovalAb UK Ltd., some adjustment need to be made when preparing the dilutions.

2.3.8.1 Log 10 dilution

Since the volume of mice antisera provided were small (~ 150 μl), and pre-diluted to 1:5 dilution, (15 μl) was diluted with PBST (1485 μl) in a 2.0 ml eppendorf tube to give an initial dilution of 1:500 dilution. An aliquot (500 μl) from the 1:500 dilution was diluted with PBST (500 μl) in a 1.5 ml eppendorf tube to make a 1:1,000 dilution. An aliquot (100 μl) from the 1:1,000 was diluted with PBST (900 μl) to make a 1:10,000 dilution. The last steps were repeated sequentially to get a series of dilutions up to $1:10^6$ (v:v).

2.3.8.2 2-fold dilution

From the initial antiserum provided (1:5 dilution), (15 μl) was diluted with PBST (1485 μl) in a 1.5ml eppendorf tube to give 1:500 dilution. An aliquot (500 μl) from the 1:500 dilution was diluted with PBST (500 μl) into another 1.5 ml eppendorf tube to make the subsequent dilution. These steps were repeated sequentially to get a series of dilutions up to 1:64,000.

2.3.9 Secondary antibody preparation (1/1,000, v/v)

Secondary antibody (anti-rabbit IgG from goat with HRP, 10 μl) was diluted with PBST (990 μl) in a 15 ml conical tube to give a 1:1,000 dilution. If more than 10 ml was required, 20 μl of secondary antibody was pipetted instead and diluted with PBST (980 μl), in a 20 ml conical tube.

2.3.10 Antigen coating for microtitration plates (1μg/ml)

Coating antigen (linamarin-CC-KLH, 1.0 mg) was dissolved in coating buffer (1 L) to give a 1.0 μg/ml concentration. The solution was stirred for 1 hour and filtered using Whatman No. 1 filter paper to remove undissolved materials. The coating antigen solution was prepared fresh for daily use and any excess solution was discarded. Aliquots (300 μl) were pipetted into each well of the microtitration plate. The microtitration plate was then sealed using parafilm and incubated overnight at 4^{o}C. The microtitration plate was washed 5-times with PBST the following day and dried for immediate use or stored in dry place and away from sunlight for future use.

2.3.11 Standard antigen dilution

Standard antigen (linamarin, 1.0 mg) was dissolved in PBST (1.0 ml) buffer in a 1.5 ml eppendorf tube to make a 1.0 mg/ml dilution. An aliquot (100 μl) from the 1.0 mg/ml solution was diluted with PBST (900 μl) in another 1.5 ml eppendorf tube to make a 1:10 dilution. This process were repeated sequentially to get a series of dilutions up to $1:10^6$ (v:v).

2.3.12 Buffer preparation for hapten-protein conjugation

2.3.12.1 Potassium carbonate (5%, w/v)

Potassium carbonate (50.0 g) was dissolved in water (1 L).

2.3.12.2 Sodium carbonate/bicarbonate buffer, 0.05 M, pH 9.4

The carbonate-bicarbonate buffer is prepared as described in 2.4.1. The buffer was prepared fresh for daily use and any excess solution was discarded.

2.3.12.3 Sodium bicarbonate (4%, w/v), pH 9.4

Sodium bicarbonate (4.0 g) was dissolved in water (100 ml). The pH was adjusted to 9.4 using 4 M sodium hydroxide.

2.3.12.4 Phosphate buffer, 0.05 M, pH 5.5

Sodium phosphate monobasic (NaH_2PO_4, 5.751 g) and sodium phosphate dibasic (Na_2HPO_4, 0.294g) were each dissolved in water (1 L). Both solutions were then mixed to give a buffer of pH 5.5.

2.3.12.5 Preparation of dialysis membrane

Visking dialysis membrane was cut 15cm in length and soaked in water for approximately 1 hour and was used immediately.

Chapter 3 : Production of Polyclonal Antibodies against Linamarin

Summary

- For the first time, polyclonal antibodies against linamarin have been produced in rabbits.

- Linamarin was successfully conjugated to BSA and KLH using the cyanuric chloride method, the former used as immunogen for immunization and the latter used as solid phase antigen.

- Anti-linamarin antibody titre curves were obtained from both rabbits using an indirect non-competitive ELISA (incELISA). High antibody titre was obtained throughout all the test bleeds from both rabbits even at dilutions of 1 in 100,000.

- The highest antibody titre and activity was obtained from Day-74 test bleed in R074 and Day-88 test bleed in R076. Day-88 test bleed from R076 (R076D88) was selected and used in subsequent experiments in order to preserve identical conditions and consistency.

- A standard curve was successfully developed using an indirect competitive ELISA (icELISA) after few optimization procedures. This is the first immunoassay for the detection of linamarin to be reported.

3.1 Introduction

PAbs are generated by injecting an immunogen into an animal and collecting the blood fraction containing the Abs after an appropriate time. In order to produce good antisera, the hapten-protein conjugate (immunogen) is often prepared in adjuvants. The immunological adjuvants non-specifically enhance or modify the immune response to co-administered antigens (Baldridge and Lacy, 2000), which vigorously affect antibody production compared to when antigen is administered alone. First developed by Jules Freund in the 1940s (Freund *et al*., 1937, Freund, 1951), Freund's adjuvant is designed to provide slow release and presentation of the immunogen to the immune system over an extended period of time providing a strong, persistent immune response of the animal (Green and Manson, 1998). The antigen-adjuvant emulsions optimized the immune stimulus by focusing a general immunostimulant and Ag in the same microenvironment where they can interact with antigen-presenting cells and lymphocytes simultaneously, hence providing a method of stimulating, by a short course of injections, the development of Abs of high titre (Baldridge and Lacy, 2000). The adjuvants are used as a water-in-oil emulsion, often prepared from non-metabolizable oils (paraffin oil and mannide monooleate). During the emulsification process, the immunogen-containing water droplets become entrapped in the oil, forming particulates in a very viscous emulsion in which, upon administration into the host, acts as a depot of immunogen. The potency of this water-in-oil emulsion can be increased by incorporating immunostimulants.

There are two types of Freund's adjuvant used which are the complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA). The CFA, which

consists of the aqueous immunogen solution, mineral oil, an emulsifying agent, and heat-killed *Mycobacterium tuberculosis,* a very potent immunomodifier that attracts macrophages and other cells to the injection site which enhanced the host's immune response. Meanwhile, IFA is identical to CFA except that the *Mycobacterium tuberculosis* is omitted (Baldridge and Lacy, 2000). However, the main disadvantage of Freund's adjuvant is that it can cause granulomas, inflammation at the inoculation site and lesions. For this reason, the CFA is usually used only for the initial injections, and to minimize side-effects, IFA is used for the subsequent booster injections.

Although large volumes of antiserum are produced from immunizing goats or sheep, polyclonal antisera raised in rabbits are often of high quality (Hancock and O'Reilly, 2005). Two female New Zealand rabbits were the animal of choice used in this study as they produce adequate amount of sera, have ready availability and are easy to maintain. In addition, the anti-rabbit IgG (detection antibody) are of a high quality and are readily and commercially available. The use of more than one individual animal is recommended, because the immune response could vary significantly between individual animals (Hancock and O'Reilly, 2005). Since the carrier is immunogenic, immunization with a hapten-carrier conjugate elicits Abs to both carrier and hapten (Lewis, 2001).

Injecting the immunogen into the animals allows the hapten-carrier molecule to interact with both T and B-cells. The carrier portion of the conjugate activates T-cells, while Abs to both hapten and carrier are elicited by B-cells. The carrier proteins are responsible for the provision of necessary immunocompatibility

complex class II or T-cell receptor epitopes and the haptens can then serve as B-cell determinants (Hancock and O'Reilly, 2005). B-lymphocytes which are present in mammalian spleen, marrow and gut-associated lymphoid tissue are responsible for the production of Abs to individual epitopes on a specific Ag (target molecule). The epitope is the specific site on the Ag and bound by single Ab. There are two types of epitopes; a linear epitope which consist of a continuous amino acids sequence, and a conformational epitope which consists of a non-continuous sequence of amino acid that can be folded or overlapping peptide chains on the surface of the target (Hsieh, 2010). The presentation of these epitopes to the cells of the immune system which occurs during the immunization process induces B-lymphocyte priming. The primed B-cells undergo clonal expansion and secrete antibody until the antigen has been destroyed or removed. The pool of antibody molecules produced by these cells is called polyclonal because it is derived from multiple clones of lymphocytes, each clone generating Abs with unique specificity to single epitopes (Burns, 2005). PAbs are characterized based on their ability to distinguish between different antigens (specificity), their binding strength (avidity) and the optimal dilution of the antibody in an assay (antibody titre) (Green and Manson, 1998).

3.1.1 Aim of the chapter

The aim of the work described in this chapter was to design and conjugate linamarin, a hapten to a suitable carrier proteins in order to generate a good immunogen to be used for the production of the polyclonal antibody against linamarin, and another conjugate to be used as the solid phase antigen. Selected pAb will be used to develop the ELISA for the detection and quantification of linamarin.

This chapter describes the experimental approach and results towards the following areas:

i) Design and synthesis of linamarin protein conjugates for antibody production and for use in an assay.

ii) Immunization of rabbits in order to generate polyclonal antisera capable of recognizing linamarin.

iii) The setting up, optimization and preliminary characterization of an ELISA standard curve for the detection of linamarin.

3.2 Methods

3.2.1 Synthesis of hapten-protein conjugates

Linamarin was conjugated to BSA and KLH using a cyanuric chloride conjugation method based on the successful attempt by Bolarinwa (2013) with amygdalin and suitability of the method as mentioned previously in section 1.4.3.3. The chemical reaction is depicted in Figure 3-2. The linamarin-BSA conjugate was used as immunogen for the production of anti-linamarin antiserum while the linamarin-KLH conjugate was used as a solid phase antigen.

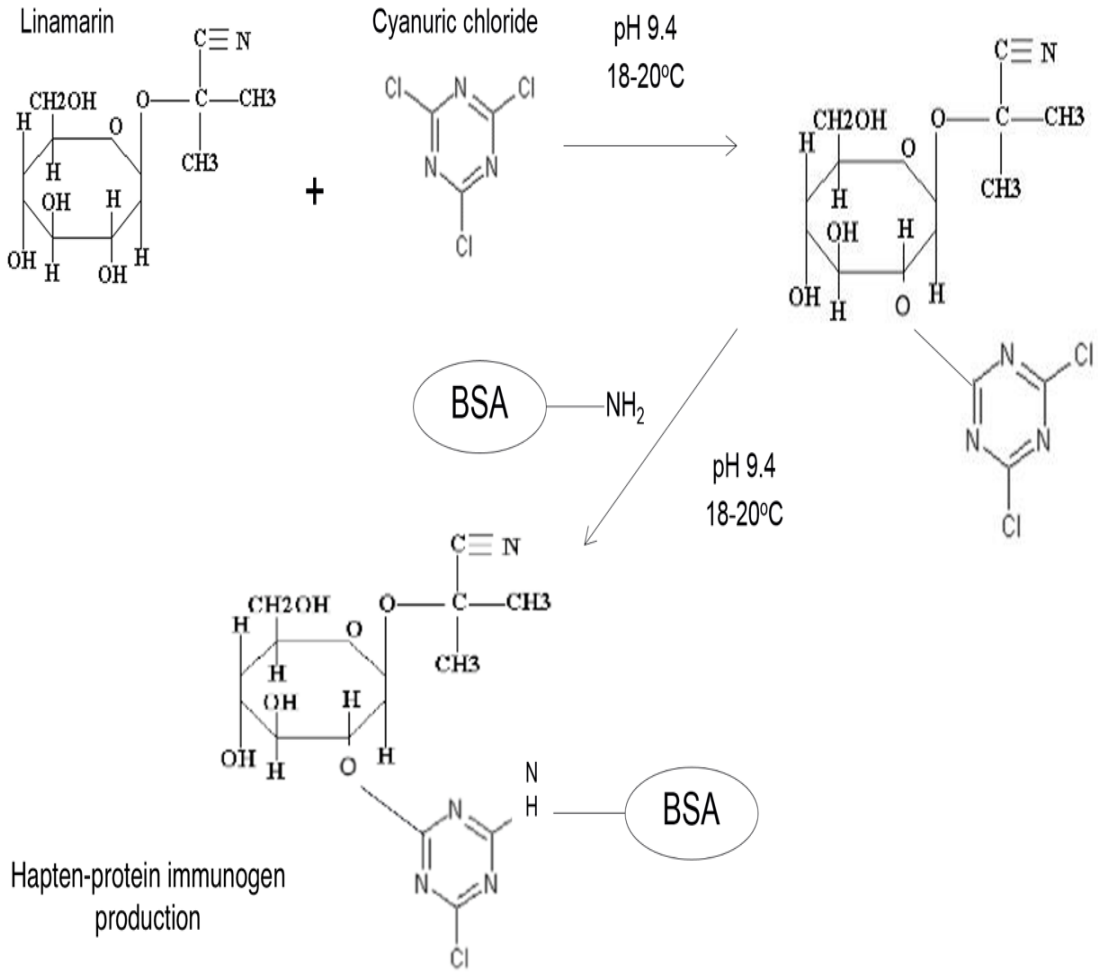


Figure 3-1: A schematic diagram showing the route for linamarin-CC (hapten) synthesis. Free linamarin standard was activated with cyanuric chloride by stirring a solution of linamarin with cyanuric chloride residue for 6 hours at room temperature.

3.2.1.1 Linamarin-BSA Conjugate

Linamarin was first activated with cyanuric chloride (CC) by dissolving CC (1.0 mg) in acetone (1.0 ml). An aliquot of the solution (0.3 ml) was then transferred into a glass vial and the contents of the glass vial was allowed to evaporate under the fume hood. Linamarin (2.3 mg) was dissolved in 0.5 ml sodium carbonate-bicarbonate buffer, pH 9.4 (5 µmol). Once the acetone had completely evaporated, the linamarin solution was added to the CC residue in the glass vial. The mixture was then stirred for 6 hours at room temperature (20 ± 2^{o}C) to generate the activated hapten (linamarin-CC). For the immunogen carrier protein, BSA (6.6 mg) was dissolved in 0.05 M sodium carbonate-bicarbonate buffer (pH 9.4, 0.5 ml) to give a 0.1 µmol concentration. The BSA solution was then added to the activated hapten mixture and incubated overnight at 37^{o}C. The protein:hapten mixture was then dialysed using 0.05 M phosphate buffer, (pH 5.5), for 6 hours the following day. The protein:hapten mixture was transferred into a 50 ml conical tube and lyophilised using a freeze-dryer to obtain a white compound (the linamarin-CC-BSA conjugate). The conjugate was stored at -20^{o}C until required. The linamarin-CC-BSA conjugate was used as immunogen to generate anti-linamarin Abs in rabbits.

3.2.1.2 Linamarin-KLH-conjugate

Similar procedures as described in 2.5.1 were followed. Linamarin (2.3 mg) of and KLH (10 mg) were used instead for the conjugation in order to provide the solid phase (coating) antigen to be used in ELISA.

3.2.2 Assessment of hapten-protein conjugates

3.2.2.1 UV-spectrophotometric method

The verification of hapten-protein conjugate and estimation of the number of moles of hapten conjugated to protein was estimated by UV spectrometry. Standard BSA, linamarin-CC-BSA or linamarin-CC-KLH conjugates (1.0 mg) were weighed in a 2.0 ml Eppendorf tube and dissolved in sodium carbonate-bicarbonate buffer (pH 9.4, 1.0 ml) respectively. Each of the solutions was vortexed for 1 minute before being transferred into a quartz cuvette. Absorption spectrum analysis was done using wavelengths between 200-400 nm for each sample. The number of moles of hapten per mole of protein in the conjugate was determined using the maximum absorbance of amygdalin at 260 nm to calculate the molar extinction coefficient. The number of moles for conjugated hapten was calculated using Beer Lambert's Law ($A = \varepsilon cl$), where:

A = Absorbance, ε = Molar extinction coefficient, c = concentration, l = pathlength.

3.2.2.2 Chemical method

2,4,5-trinitrobenzene sulfonic acid assay (TNBS) was used to estimate the molar conjugation ratio of linamarin-BSA and linamarin-KLH conjugates. The method is based on the determination of freely available amino acid groups present in the carrier protein before and after hapten conjugation following its reaction with TNBS. Reaction of TNBS with primary amines generated trinitrophenyl, a highly chromogenic derivatives that can be readily measured spectrophotometricaly. Carrier protein (BSA or KLH, 2.0 mg) and both conjugates (linamarin-BSA and linamarin-KLH) were separately dissolved in sodium bicarbonate buffer (4%, w/v, pH 9.4, 2.0 ml). Aliquots of the above solutions (40, 80, 120, 160, 200 µl) were dispensed in triplicate into the wells of a microtitration plate to generate the standard curve. The volume in each well was made up to 200 µl using sodium bicarbonate buffer (4%, w/v, pH 9.4). TNBS (50µl, 0.1%, v/v) in sodium bicarbonate buffer (4%, w/v, pH 9.4) was added into each well and incubated for 2 hours at 37^{o}C for colour development. The absorbance reading for the whole microtitration plate was measured using the plate reader at 405 nm. The percentage substitution of amino acid in the carrier protein was used to estimate the conjugation ratio of hapten to carrier protein.

$$\% \text{ Substitution} = \frac{\text{OD of carrier protein} - \text{OD of hapten-protein conjugate} \times 100}{\text{OD of carrier protein}}$$

*OD = Optical density value

3.2.3 Production of polyclonal antibody against linamarin

Anti-linamarin polyclonal antisera were raised in two rabbits by CovalAb UK Ltd., Cambridge, UK (CUK-1680). The immunization protocol employed was fairly standard and as follows. The immunogen (linamarin-CC-BSA) was used to immunize two female New Zealand White rabbits (weighing between 2 – 2.5 kg, coded 1529074 & 1529076) to generate pAbs. Blood was first collected from the ears of each rabbit for the pre-immune serum prior to the immunization process. The first injection, consisting of the immunogen (50 μg in 0.5 ml PBS) emulsified with Freund's complete adjuvant (0.5 ml), was injected intradermally on multiple sites on the back of the rabbits. The rabbits were further boosted by injecting intradermally with the immunogen (50 μg) dissolved in Freund's incomplete adjuvant (0.5 ml) three times at three-week intervals, with the final booster injection administered subcutaneously. Serum was taken via ear veins on days 53 (test bleed 1, 4-5 ml) and 74 (test bleed 2, 4-5 ml), and from the heart on day 88 (final bleed, 60-80 ml). The serum was separated from cells by centrifugation (3,000 rpm / 1,693 x g, 10 min at 4^{o}C) and the polyclonal antibody (serum) was stored at -20^{o}C until required. Table 3-1 shows a summary of the intradermal immunization protocol for the production of the anti-linamarin antibody.

Table 3-1: Summary of the immunization protocol for the production of anti-linamarin antibody

Day	Protocol
0	Pre-immune bleed (4-5ml) and starting immunisation with 1st intradermal injections of 0.5ml antigen + 0.5ml complete Freund/s adjuvant)
21	Second intradermal injections of 0.5ml antigen + 0.5ml incomplete Freund's adjuvant
42	Third intradermal injections of 0.5ml antigen + 0.5ml incomplete Freund's adjuvant
53	First test bleed (4-5ml)
63	Fourth subcutaneous injections 0.5ml antigen + 0.5ml incomplete Freund's adjuvant
74	Second test bleed (12-15ml)
88	Final bleed (60-80ml)

3.2.4 Determination of anti-linamarin titre

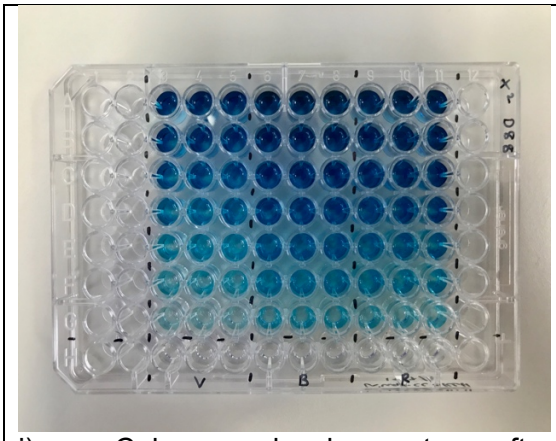
The activity of the pAbs obtained from the antisera was first investigated by constructing a titre curve. The primary antibody dilution was prepared as described in 2.3.7 and the microtitration plate was prepared as described in 2.3.10. Serial dilutions of the antiserum (bleeds 53, 74 and 88-days along with the pre-immune bleed as control) were dispensed into coated microtitration plate wells (200 μl/well) in triplicate. The microtitration plate was sealed using parafilm and incubated overnight at 4°C. The microtitration plate was washed 5-times with PBST the following day and left to dry. Secondary antibody (goat anti-rabbit IgG coupled with horseradish peroxidase enzyme, prepared as described in 2.3.11, 200 μl) was dispensed into each well and incubated for another 2 hours at 37°C. The microtitration plate was washed 5-times with PBST after the 2 hours incubation and left to dry. TMB substrate (200 μl per well) was dispensed into

each well and the plate left for 30 mins at room temperature to allow colour development. Noticeable changes in colour were observed. 2 M sulphuric acid (50 μl) was added into each well to stop the enzymic reaction. Finally the OD reading of each well was taken using a plate reader at 405 nm.

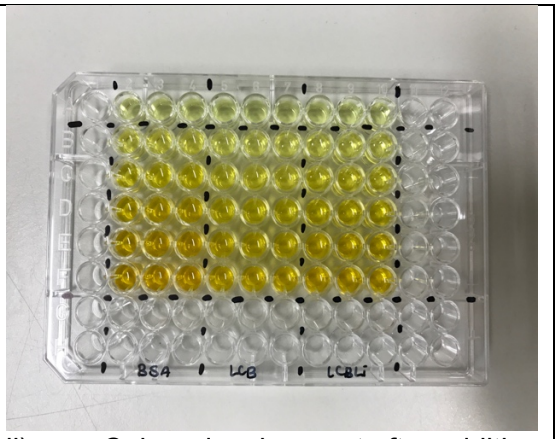
3.2.5 Development of an ELISA standard curve for linamarin

An icELISA was used to develop a linamarin standard curve. Linamarin-KLH conjugate was used as the solid phase antigen. The microtitration plate was prepared as described in 2.3.10. The blocking buffer of choice (prepared as described in 2.3.4, 300 μl) was dispensed into each well of the microtitration plate and incubated for 30 minutes at 37°C. Standard Ag (linamarin) serial dilutions were prepared as described in 2.3.11. Aliquots of each standard Ag dilution (100 μl) was then dispensed into each well of the microtitration plate in triplicate along with anti-linamarin Ab (100 μl; v:v in PBST). The microtitration plate was sealed using parafilm and incubated for 2 hours at 37°C. The microtitration was washed 5-times with PBST after the incubation and left to dry. The secondary Ab (prepared as described in 2.3.9, 200 μl per well) was dispensed into each well and incubated for another 2 hours at 37°C. The microtitration plate was washed 5-times with PBST after the incubation and left to dry. TMB substrate (200 μl) was dispensed into each well and left for 30 mins at room temperature. Noticeable changes in colour were observed. Finally 2M sulphuric acid (50 μl) was added into each well to stop the reaction. The OD reading of each well was then recorded using a plate reader at 405 nm. Subsequently, the average optical densities for each linamarin concentration was plotted against the log of

linamarin standard (ranging from 1.0 mg/ml to 1.0 x 10^{-6} mg/ml) to form a calibration curve. A calibration curve was run with each plate to minimise variation from plate to plate and the equation of the line was used to calculate the amount of linamarin in the analysed samples.



i) Colour development after substrate addition (TMB)



ii) Colour development after addition of stop solution (2M H_2SO_4)

iii) Plate washer and plate reader used throughout the study

Figure 3-2: Images showing colour development in ELISA and current laboratory setup including the Tecan Hydroflex Plate Washer and Multiskan FC ® Plate Reader. i) Blue colour development after addition of subtrate, ii) change of colour from blue to yellow after the addition of stop solution (2M sulphuric acid).

3.3 Results

3.3.1 Immunogen synthesis

Cyanuric chloride (2,4,6-trichloro-1,3,5-triazine, CC) was used as a bridging molecule in the synthesis of immunogen (linamarin-CC-BSA). The molar ratio between hapten and protein (linamarin:BSA) used was based on a chemical optimization study performed between naringin and BSA (Bolarinwa, 2013). The optimum initial molar ratio of 50:1, hapten:protein, was chosen based in terms of resulting yields and practicality. A similar approach was used in the present study to conjugate linamarin to BSA. The final product ratio after the conjugation was estimated to be between 15:1 and 25:1, on assumption that the carrier protein completely reacted with hapten in the conjugate. The lyophilised linamarin-BSA conjugate is shown in Figure 3-3, which will be further assessed to confirm successful conjugation.

Figure 3-3: Lyophilised linamarin-BSA conjugate. Freeze-dried for 36-48 hours and stored at -20°C.

3.3.2 Assessment of the hapten-protein conjugates

3.3.2.1 Spectrophotometric assessment

Verification of conjugate synthesis and estimation of the number of moles of hapten conjugated per mole of protein was estimated by using UV-visible spectrometry. The results of UV absorption spectra of linamarin, BSA and linamarin-CC-BSA conjugate from the CC conjugation method are shown in Figure 3-4. The absorption spectra revealed a single peak of unconjugated BSA at 280nm and a significantly lower peak for the linamarin-BSA conjugate. An absorbance peak at 280nm for unconjugated linamarin was totally absent.

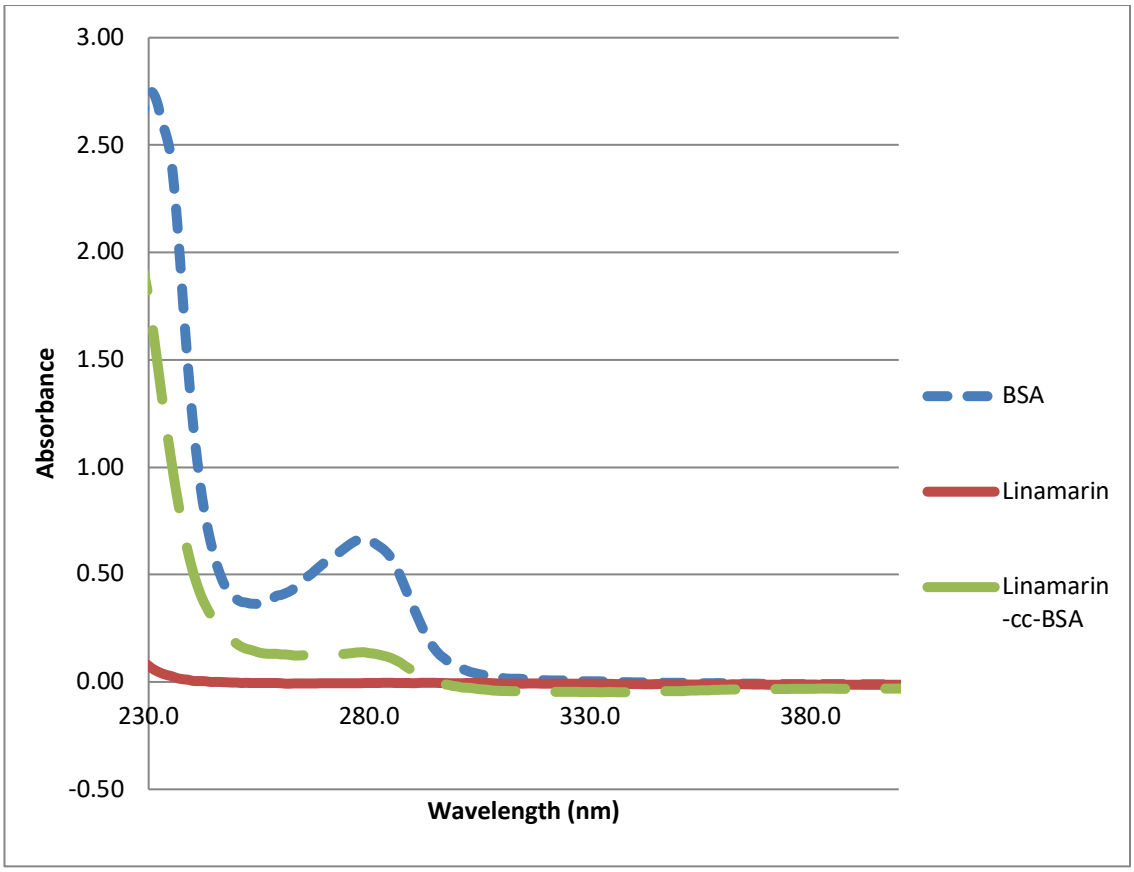


Figure 3-4: UV absorption spectra of BSA, linamarin and linamarin-CC-BSA conjugate obtained from the cyanuric chloride method, showing the absorption peaks of unconjugated linamarin, conjugated linamarin and BSA at different wavelength.

3.3.2.2 Chemical assessment

A chemical method, utilizing the 2,4,6-trinitrobenzene sulfonate (TNBS) assay, was used to reinforce the verification of linamarin-BSA conjugate using the estimation of its percentage conjugation. The method is based on the sulphuric acid digestion of the protein with subsequent conversion of the nitrogen to ammonium sulphate. Reaction of TNBS with primary amines generated trinitrophenyl protein-sulphite complexes, highly chromogenic derivatives that can be readily measured spectrophotometrically. The standard curves obtained from this assay are shown in Figure 3-5. Both standard curves show excellent linearity (R^2 values of 0.9955 for BSA and 0.9911 for linamarin-BSA conjugate). The standard curve of linamarin-BSA conjugate is clearly shifted much lower than the BSA standard curve.

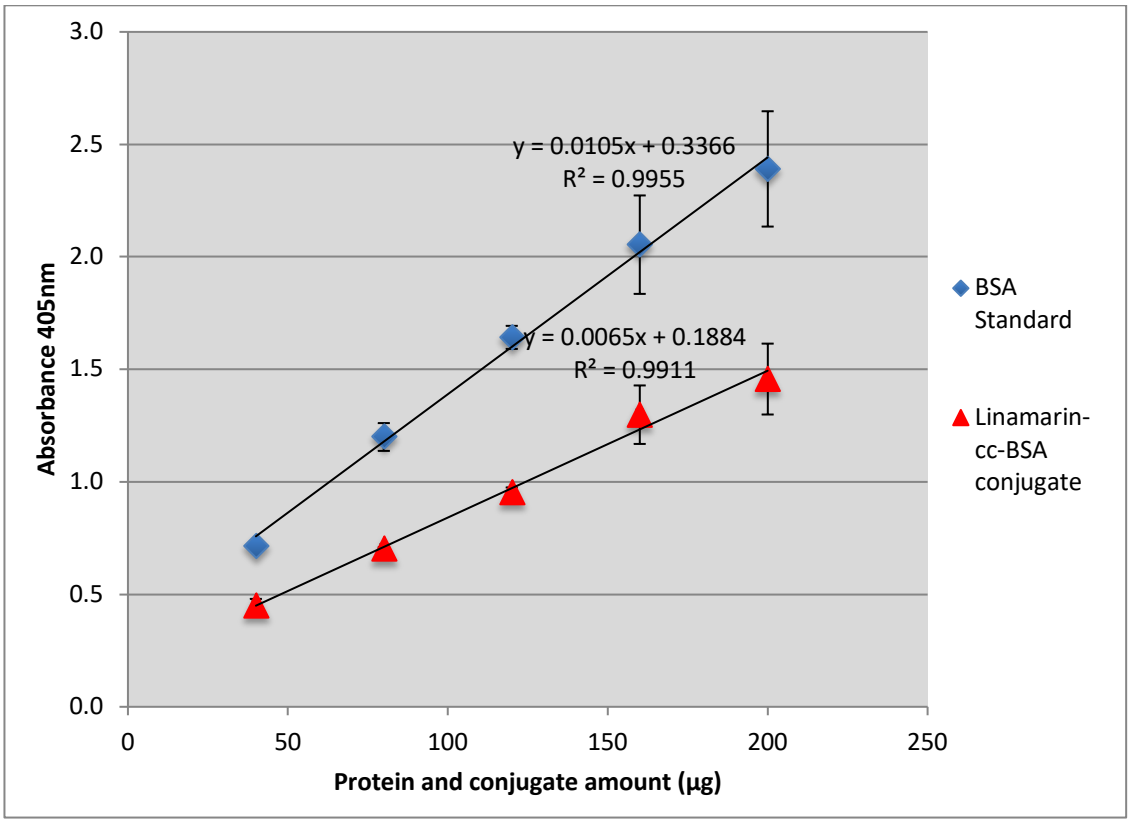


Figure 3-5: TNBS standard curves obtained with BSA and linamarin-CC-BSA conjugate obtained from the cyanuric chloride method, showing the reduction in free amino acids of BSA after conjugation to linamarin. Error bars represent the standard deviation of triplicate analyses.

3.3.3 Anti-linamarin antibody titre

An incELISA was applied to investigate the activity of antisera generated against linamarin from both rabbits, coded 1529074 – R074 (rabbit 1) and 1529076 – R076 (rabbit 2), which were immunised with linamarin-CC-BSA. Each antiserum was prepared in a serial dilution, starting from highly concentrated (row A, 1/10, v/v) and gradually diluted (row B, 1/100, v/v – G, $1/10^7$, v/v) in PBST buffer, together with blank samples (row H). This is to determine whether there are any anti-linamarin Abs present and if so, the lowest dilution of the antiserum that could bind to immobilised immunogen (linamarin-CC-KLH) with the pre-immune bleed as a control. Figure 3-6 shows the construction of a titre curve in a microtitration plate.

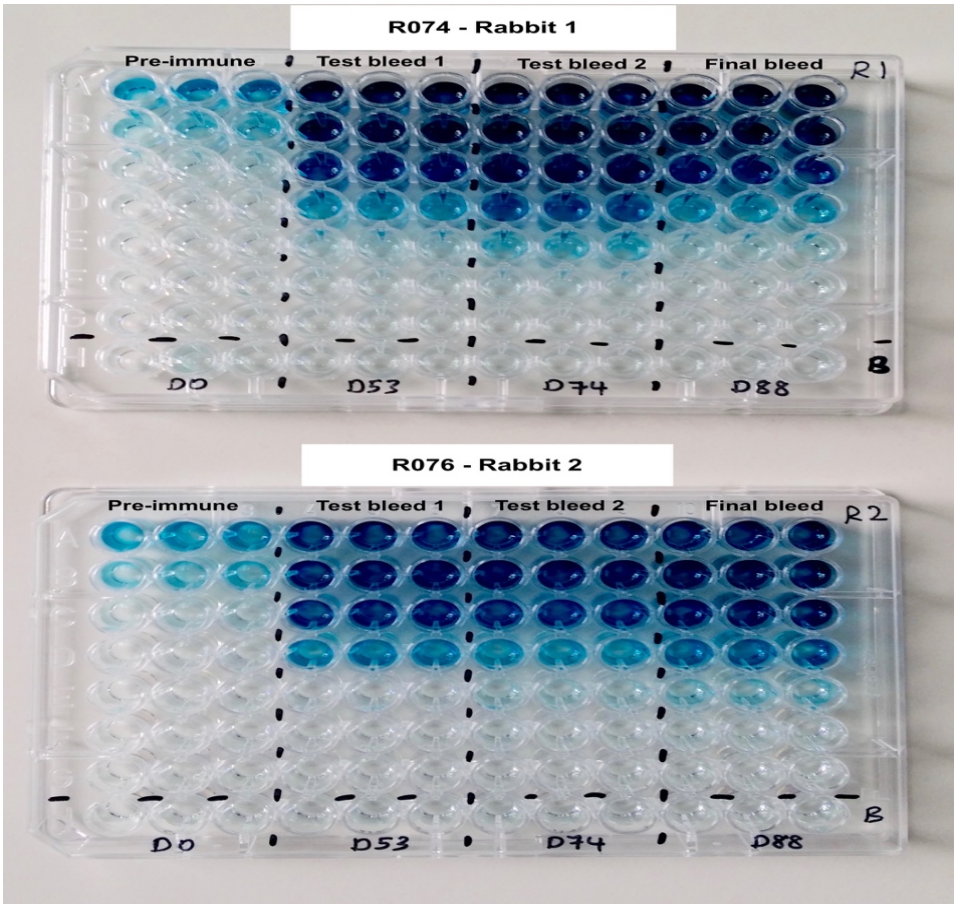


Figure 3-6 Indirect non-competitive ELISA for polyclonal anti-linamarin antibody titre. The activities of the two test bleeds and the final bleed together with the pre-immune serum for both rabbits (R074 and R076). The results represent triplicate determinations.

The blue colour in each of the wells indicates the primary Ab binding towards the immobilised linamarin-KLH conjugate, while the colourless reaction indicates zero binding. Darker colours indicate higher primary Ab binding to the linamarin-KLH conjugate meanwhile lighter colours indicate lower binding. For rabbit 1 (R074), the pre-immune serum shows some binding of the primary Ab towards the immobilised linamarin-KLH conjugate at 1/100 dilution. Test bleed 1 (Day 53 bleed) and the final bleed (Day 88) both have antiserum activity at 1/10,000 dilution. Test bleed 2 (Day 74) however, shows antiserum activity even at 1/100,000 dilution. For rabbit 2 (R076), the pre-immune serum activity was quite similar to rabbit 1, although test bleed 1 (Day 53) and test bleed 2 (Day 74) indicated antiserum activity at more than 1/10,000 dilution. The final bleed (Day 88) indicated antiserum activity at 1/100,000 dilution.

The colour generated and displayed in Figure 3-6 was in concordance to the curves shown in both figures 3-7 and 3-8. Polyclonal antisera produced from both rabbit 1 (R074) and 2 (R076) generated high optical density values, indicating significantly high antibody binding towards linamarin-CC-KLH even at dilutions of more than 1:100,000 with no significant difference between any of the test bleeds (Figure 3-7 and Figure 3-8). It can be clearly seen that in R074, the test bleed 2 (Day 74) shows the highest activity of all the bleeds meanwhile in R076, it was the final bleed (Day 88).

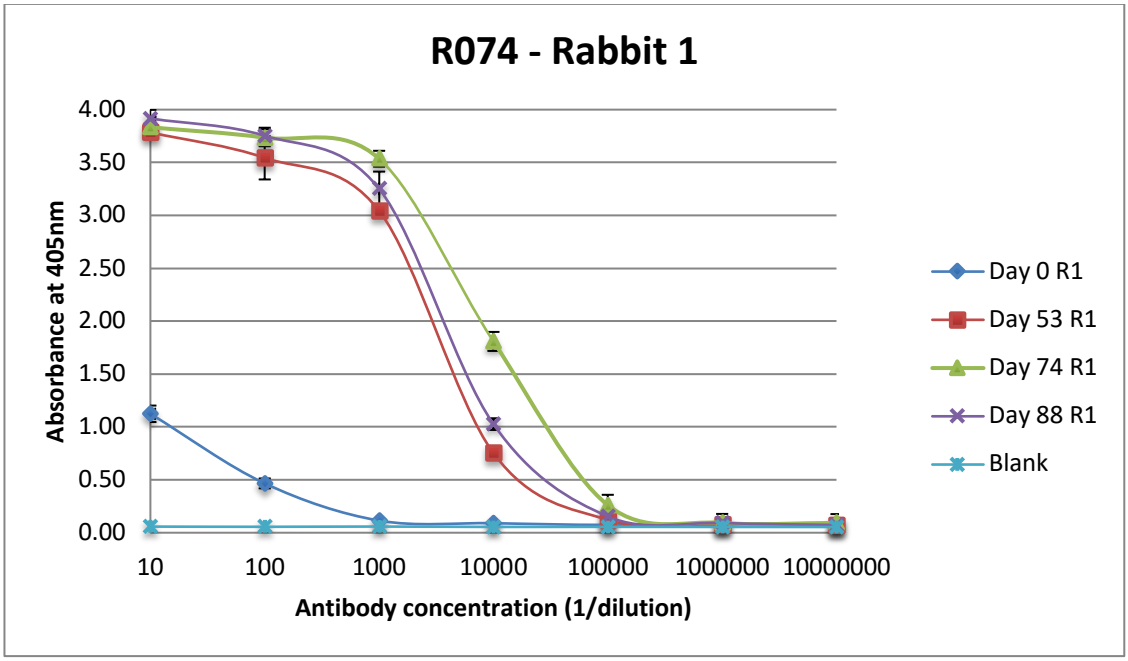


Figure 3-7: Titre curves of anti-linamarin antibody obtained from rabbit 1 (R074) immunized with linamarin – BSA conjugate (immunogen) from the cyanuric chloride method, showing the titre curves of 3 sera against solid phase antigen linamarin-KLH conjugate (1µg/ml) from the cyanuric chloride method, together with the pre-immune serum. Error bars represent standard deviation of triplicate analyses.

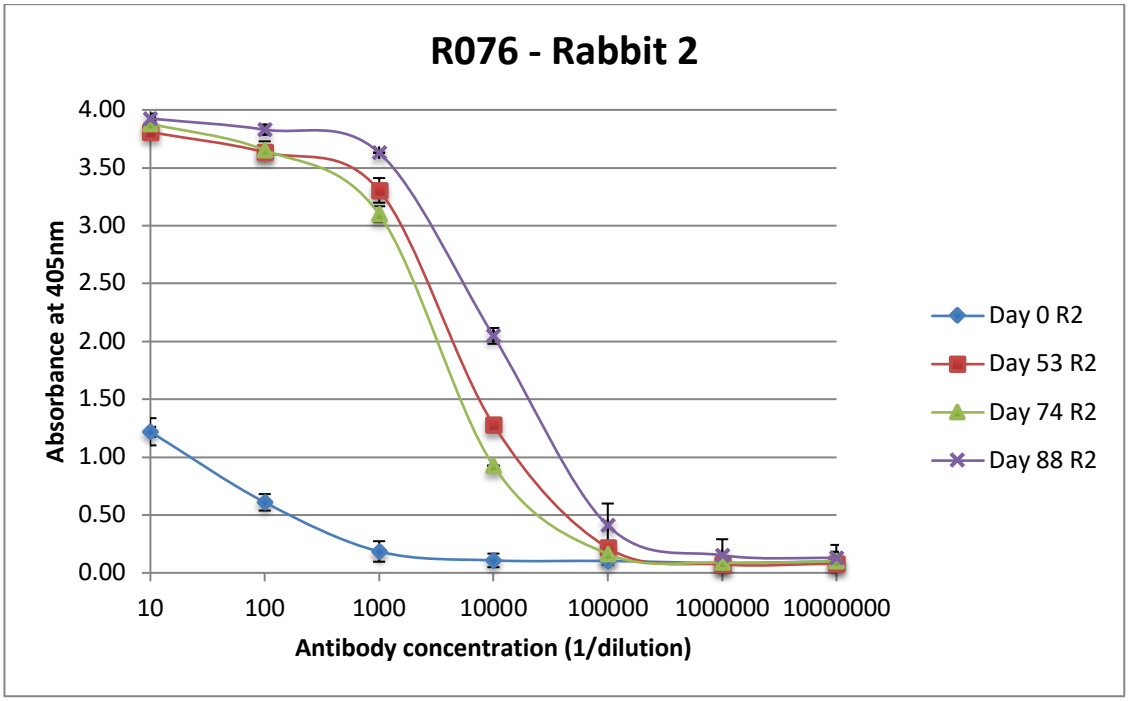


Figure 3-8: Titre curves of anti-linamarin antibody obtained from rabbit 2 (R076) immunized with linamarin – BSA conjugate (immunogen) from the cyanuric chloride method, showing the titre curves of 3 sera against solid phase antigen linamarin-KLH conjugate (1µg/ml) from the cyanuric chloride method, together with the pre-immune serum. Error bars represent standard deviation of triplicate analyses.

3.3.4 Development of an ELISA standard curve for linamarin

The antisera obtained from rabbits immunised with linamarin-CC-KLH immunogen were used to establish the standard curves. The assay was based on an icELISA using free linamarin standard at concentrations ranging from 1 to 100,000 ng/ml and linamarin-KLH conjugate for the immobilised phase. The procedure for the icELISA has been described previously in section 2.6.1. The curve was obtained by plotting the absorbance (405nm) on the y-axis and the logarithm of the concentration of standard linamarin on the x-axis as shown in Figure 3-9. The standard curve showed that as the concentration of free linamarin increased, the optical density decreased.

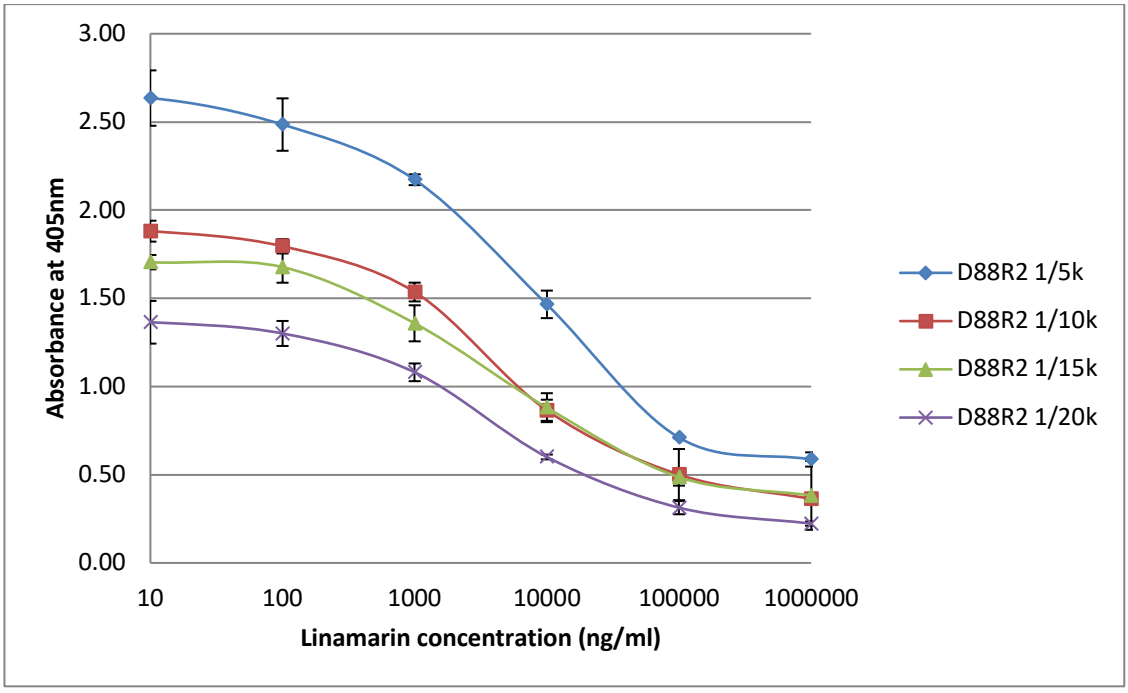


Figure 3-9: icELISA standard curve of linamarin obtained from antibody generated from the cyanuric chloride conjugate. The primary antibody dilution was done at 4 different dilutions, 1:5,000, 1:10,000, 1:15,000 and 1:20,000 and the solid phase antigen was linamarin-KLH. Overnight primary reaction incubation was at 4°C, secondary reaction was for 2 hours at 37°C and there was a 30min enzyme-substrate reaction time before stopping the reaction. Error bars represent standard deviation of triplicate analyses.

3.3.5 Optimization of an indirect competitive ELISA (icELISA) for linamarin

Ab dilution, incubation time, solid phase Ag concentration, and blocking buffers are factors that could be optimized to influence the sensitivity of the ELISA assay. However, it is imperative to determine which pool of antiserum to use and its optimum concentration to produce reliable and good standard curve. From the Ab titre results, test bleed 2 of R074 (R074D74) showed the highest affinity to linamarin in rabbit 1 meanwhile the final bleed of R076 (R076D88) from rabbit 2 showed similar affinity to linamarin. Although there is no significant difference between test bleed 2 of R074 (R074D74) and final bleed of R076 (R076D88), R076D88 was chosen and used throughout the subsequent experiments in order to preserve identical condition.

In order to achieve higher assay sensitivity with a better signal:noise ratio, a detection Ab (goat anti-rabbit IgG H+L HRP-conjugated) produced and supplied by CovalAb UK Ltd. was used for comparison with the detection antibody used previously from Invitrogen, Thermo Scientific. In addition, two different TMB substrates were investigated to improve the assay sensitivity. The TMB substrates used were TMB 1 (3,3',5,5'-tetramethylbenzidine liquid substrate system for ELISA, peroxidase substrate, Sigma Aldrich, UK) and TMB 2 (3,3',5,5'-tetramethylbenzidine liquid substrate, Supersensitive, for ELISA, Sigma Aldrich, UK). Both were slightly different in term of cost with the former being more expensive per 100 ml solution. The results of the investigation are shown in Figure 3-10. From the results, TMB 1 provided better signal amplification compared to TMB 2 although the difference between both is not

much. However, there was a huge leap in signal amplification between the two Abs used, suggesting that the detection Ab produced and supplied by CovalAb UK Ltd. had higher affinity towards the primary Ab compared to the one produced and supplied by Invitrogen, Thermo Scientific. Based on these results, TMB 1 and detection Ab from CovalAb UK Ltd. were used in subsequent experiments.

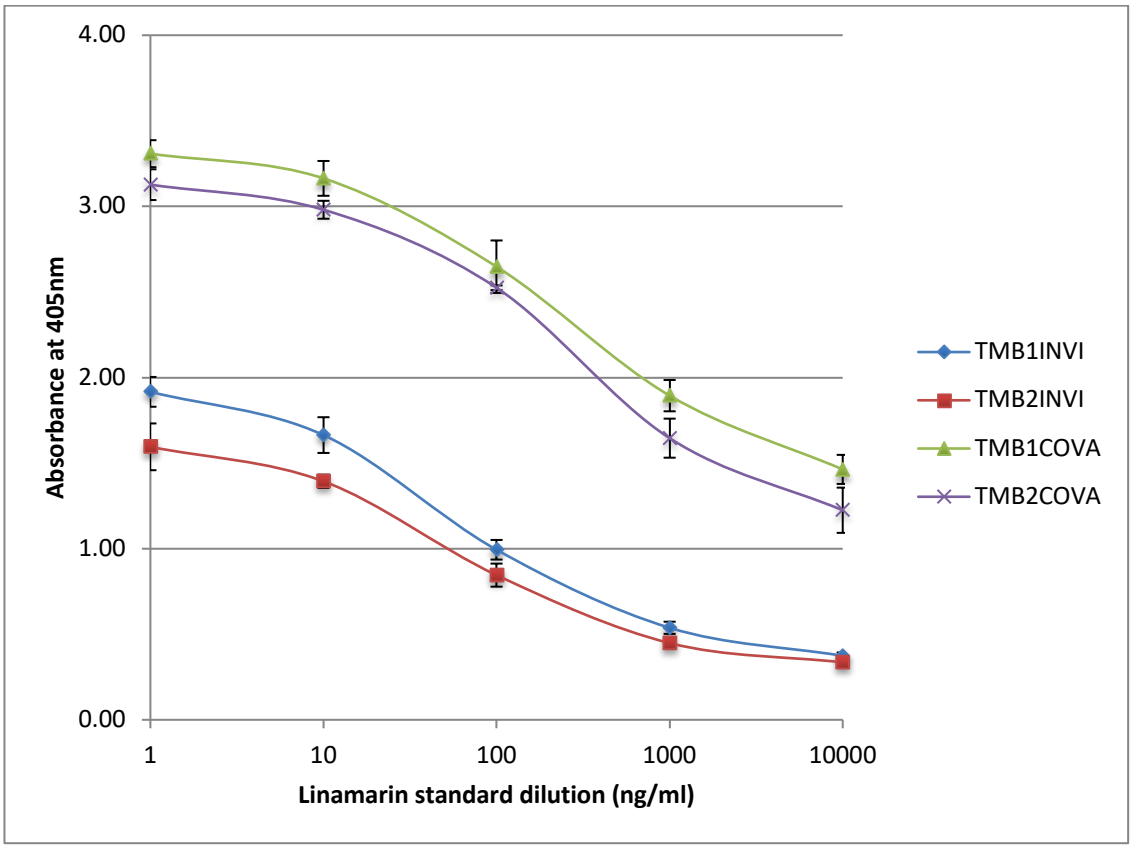


Figure 3-10: icELISA standard curve of linamarin obtained from antibody generated from the cyanuric chloride conjugate (R076D88), using two different detection antibodies and two different TMB substrates for signal generation. The antibody dilution was done at 1:15,000 and the solid phase antigen was linamarin-KLH. Overnight primary reaction incubation was at 4°C, secondary reaction was for 2 hours at 37°C and there was a 30min enzyme-substrate reaction time before stopping the reaction. Error bars represent standard deviation of triplicate analyses.

For the optimum primary Ab concentration determination, based on the results from Ab titre, several primary Ab dilutions were investigated as shown in Figure 3-11. Other conditions were kept constant for the time being; a solid phase Ag concentration of 1.0 µg/ml was used throughout the assay development, 1% skimmed-milk (w/v) was used as blocking buffer, primary incubation time was done overnight at 4°C, secondary reaction incubation time was 2 hours at 37°C and 30 minutes enzyme-substrate reaction time allowed before the reaction was stopped and absorbance measured.

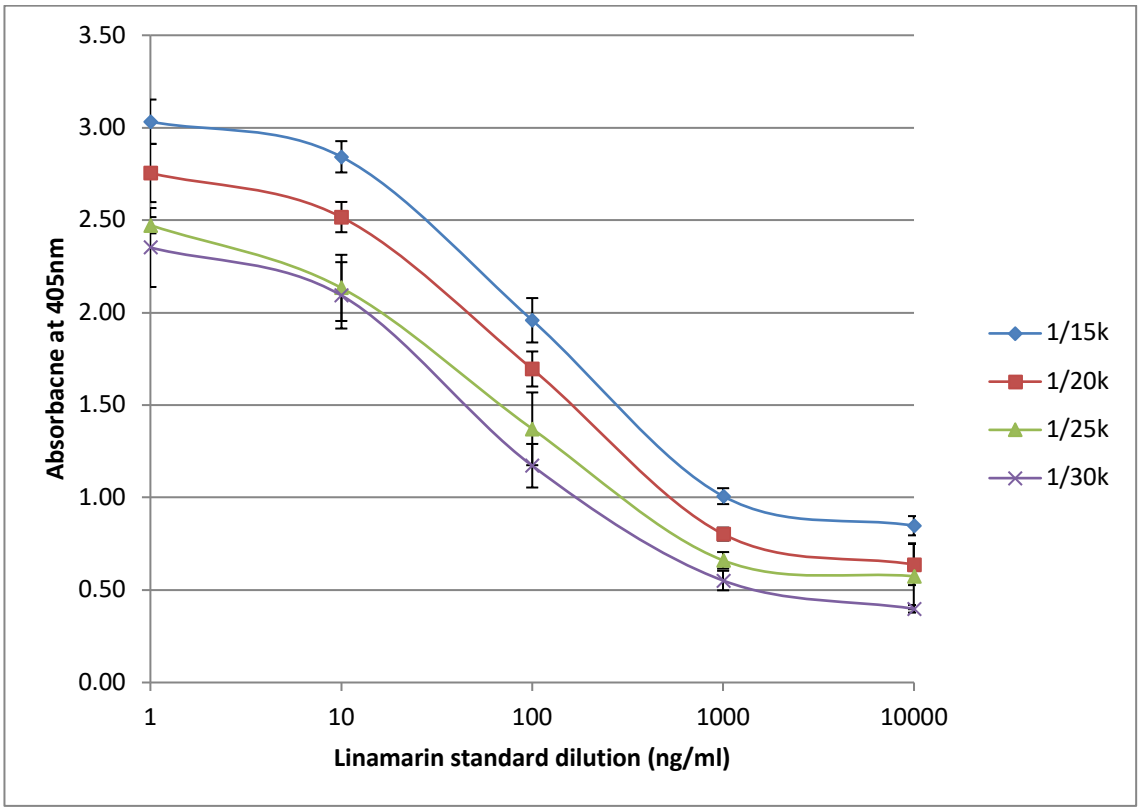


Figure 3-11 icELISA standard curve of linamarin obtained from antibody generated from the cyanuric chloride conjugate, using 1% dried skimmed milk as blocking buffer. The primary antibody dilution was done at 4 different dilutions, 1:15,000, 1:20,000, 1:25,000 and 1:30,000 and the solid phase antigen was linamarin-KLH. Overnight primary reaction incubation was at 4°C, secondary reaction was for 2 hours at 37°C and there was a 30min enzyme-substrate reaction time before stopping the reaction. Error bars represent standard deviation of triplicate analyses.

The icELISA was repeated several times using other dilutions up to 1/70,000 (v/v) as shown in Figure 3-12.

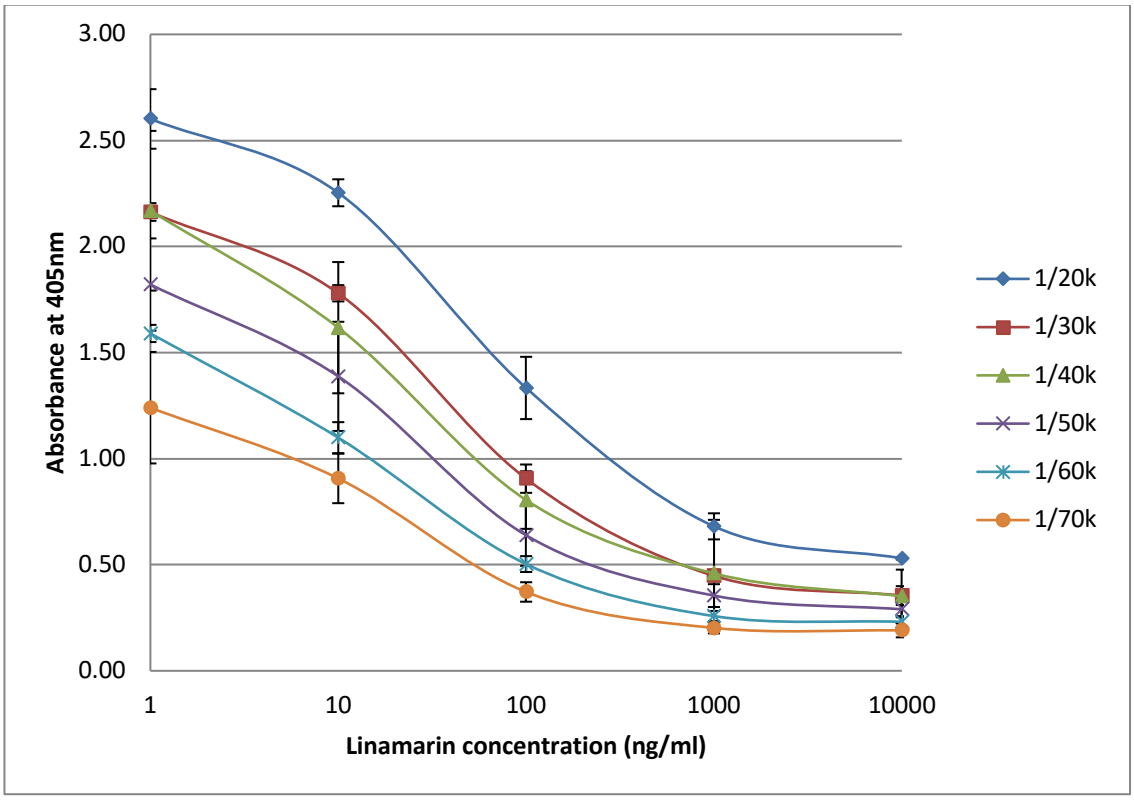


Figure 3-12: icELISA standard curve of linamarin obtained from antibody generated from the cyanuric chloride conjugate (R076D88), showing the binding of the antibody to the free linamarin standard. The antibody dilution was done at 8 different dilutions, ranging from 1:20,000 to 1:70,000 and the and the solid phase antigen was linamarin-KLH. Overnight primary reaction incubation was at 4°C, secondary reaction was for 2 hours at 37°C and there was a 30min enzyme-substrate reaction time before stopping the reaction. Error bars represent standard deviation of triplicate analyses.

The ELISA absorbance and sensitivity was greatly enhanced as shown in Figure 3-12 where even at dilution of 1/70,000, the primary Abs were still able to recognize and bind with the unbound linamarin standard. The dilution of 1/50,000 was chosen as the optimum dilution to be used for subsequent experiments.

The efficiency of different blocking agents used to block any non-specific reaction with the surface to improve the signal-to-noise ratio was investigated. The most common blocking buffers used in ELISA are BSA, gelatine from fish, non-fat (skimmed) milk and whole normal serum to name a few. BSA, dried skimmed milk and gelatine from fish were chosen as the blocking agents and were prepared as described in section 2.3.4. The standard curves indicating their blocking efficiency are shown in Figure 3-13. Apparently, BSA and gelatine seem to shift the curve to the right as compared to curve without any blocking (NB), meanwhile the use of dried skimmed milk powder shifted the curve to the left.

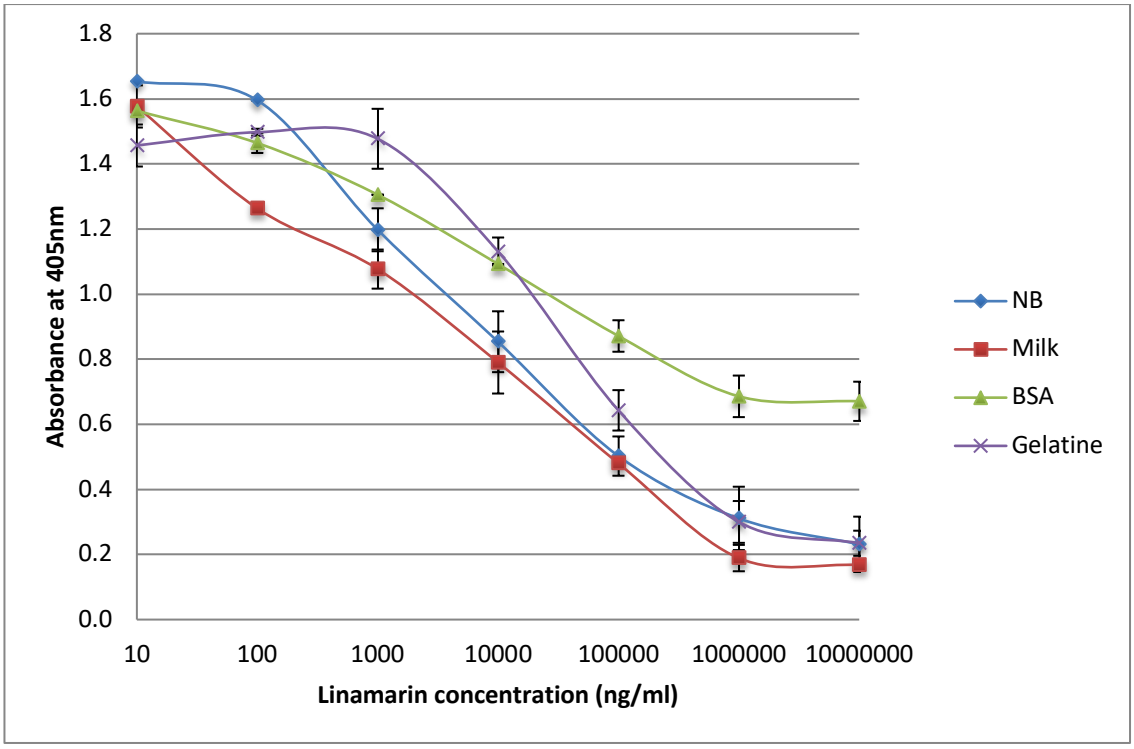


Figure 3-13: icELISA standard curve of linamarin obtained from antibody generated from the cyanuric chloride conjugate (R076D88), with different blocking buffers. The antibody dilution was done at 1/50,000 and the and the solid phase antigen was linamarin-KLH. Overnight primary reaction incubation was at 4°C, secondary reaction was for 2 hours at 37°C and there was a 30min enzyme-substrate reaction time before stopping the reaction. Error bars represent standard deviation of triplicate analyses.

Figure 3-14 shows the optimized icELISA standard curve for linamarin. The antibody dilution used was 1/50,000 and the solid phase antigen was linamarin-KLH (1.0 µg/ml). Dried skimmed milk (1%, w/v) was used as blocking buffer and incubated overnight at 4°C. Anti-rabbit IgG from CovalAb UK Ltd. was used as detection Ab and TMB 1 from Sigma Aldrich, UK was used as substrate. Overnight primary reaction incubation was at 4°C, secondary reaction was for 2 hours at 37°C and there was a 30 min enzyme-substrate reaction time before the reaction was stopped.

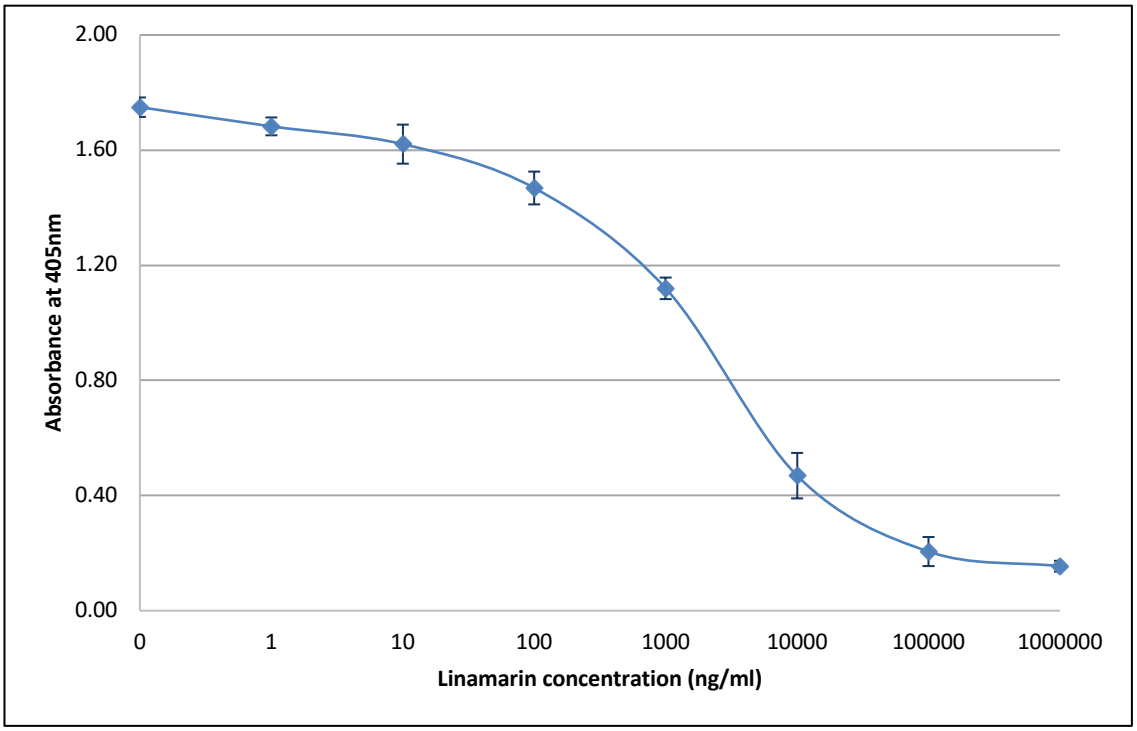


Figure 3-14: Optimized icELISA standard curve of antibody generated from the linamarin-CC-BSA conjugate (R076D88). The antibody dilution used was 1/50,000 and the solid phase antigen was linamarin-KLH. Each point represents the mean of 12 determination Error bars indicate standard deviations of the mean.

3.4 Discussion

There are no previous reports of Ab production against linamarin. The only cyanogenic glycoside for which Ab has been developed was against amygdalin (Bolarinwa *et al*., 2014). The production of good Ab against low molecular weight compounds is rather difficult to achieve because of their inability to directly stimulate the immune system for Ab production. The important requirement is that the molecular structure of hapten should have a certain degree of complexity or rigidity, such as containing a benzene ring, heterocyclic group or branch structure and so on (Juan *et al*., 2010). Otherwise, it would be difficult to produce Ab against the hapten or even if the Ab is produced, the Ab titre would probably be very low. Some studies have shown that the rate of successful production of Ab for haptens containing benzene ring is 1/3, while for hapten without a benzene ring the success rate is 1/11 (Szurdoki *et al*., 1992). Perhaps due to the difficulties in choosing an appropriate conjugation technique to synthesise a suitable immunogen for immunization, to this day there are only three reports on immunoassay development for the analysis of CGs in plant foods. There are two reports on the qualitative analysis of amygdalin (Cho *et al*., 2006; Cho *et al*., 2008), and one for the quantitative analysis of amygdalin as reported by Bolarinwa *et al*. (2014). The purpose of this study was to synthesize hapten-protein conjugates for the production of polyclonal anti-linamarin antibody for the first time and to develop a competitive ELISA standard curve for linamarin.

There are four important criteria that have been well defined by Goodrow *et al*. (1998), about the design of an ideal hapten derivative: i) the stereo structure, electronic distribution and hydrophobicity on the hapten derivative should be

similar to those on the original hapten; ii) a spacer arm with several-carbon-chain length to couple with carrier is preferred, but the spacer arm should not induce the immunized animal to produce ‘arm antibodies’ or neodeterminants; iii) the presence of active group at the end of the spacer should not affect the electronic distribution of hapten molecule; and iv) after coupling with carrier, the basic molecular structure of the hapten should be retained. Hence, the most basic requirement of hapten design is to maintain the original molecular structure of the hapten and to exposed it on the surface of the carrier as much as possible, which in turn, will maximise the recognition of the hapten by the immune cells of the immunized animal, stimulating the animal to produce a specific immune response and generate high affinity and high specificity Abs against the hapten (Juan *et al.,* 2010). Linamarin was conjugated to two carrier proteins (BSA and KLH) using the same conjugation method (cyanuric chloride, CC) to produce two different immunogens, the former was used as the immunogen for immunization and the latter was used as the coating Ag for the end-point assay. The CC conjugation method was chosen to conjugate linamarin to both BSA and KLH because of its simplicity and reactivity towards hydroxyl groups under mild conditions, which enabled coupling without affecting the structure of the target analyte (linamarin). In this technique, an alternative reactive group (i.e chloride group) was introduced into the linamarin structure prior to conjugation with the proteins. The idea was in line with earlier suggestions of an ideal hapten derivative that the coupling of alternative reactive groups away from antigenic determinants (characteristic structure and functional groups) of low molecular weight Ag prior to conjugation with carrier protein reduces the shielding effect and could increase the specificity of Abs produced against the Ag (Deshpande, 1996, Goodrow *et al*., 1998, Juan *et al.,* 2010). Linamarin was first treated with

CC to introduce dichlorotriazine by adding a solution of linamarin to dried CC residue in the activation process. Dried CC residue was used in the activation procedure in order to allow gradual solubilisation of the compound into the aqueous medium, preventing rapid breakdown and to avoid exposure of linamarin to the organic solvent (acetone) used to solubilise the CC. The first chlorine atom in the CC molecule reacted with hydroxyl groups of linamarin molecule to form stable linkages at low temperature and in alkaline pH to produce the conjugate (Abuknesha *et al.*, 2005). The chemical reaction is clearly depicted in Figure 3-2. The optimum molar ratio of protein to hapten was based on the study of chemical optimization experiments done by Bolarinwa (2013) using naringin (a flavanone glycoside) and amygdalin which were 1:15 and 1:20. The protein to hapten ratio obtained in this study was in concordance with the recommended ratio to be used for good antibody production. According to Erlanger (1980), a good antibody titre can usually be obtained with a hapten to protein ratio of between 8 to 25. Kemp and Morgan (1986) reported that a conjugate with a high protein to hapten ratio may be good as an immunogen, while one with lower ratio maybe desirable as a coating antigen. A hapten density of 15 to 30 molecules per carrier protein was also reported to be good for the production of high antibody titres with moderate antibody affinities (Singh *et al*., 2004).

Linamarin-BSA and linamarin-KLH were tested for successful conjugation by both spectrophotometric and chemical methods Spectrophotometric results revealed that the unconjugated BSA and linamarin-BSA conjugate both had an absorbance peak at 280nm while the unconjugated linamarin had none. The absence of absorbance peak for unconjugated linamarin could be explained by

the lack of chromophore in the structure that makes it impossible to absorb UV or visible lights and hence linamarin could not be detected by the UV scan at the selected wavelengths (200 – 400nm) or even at lower wavelengths. However, a reduced maximum peak of BSA in the linamarin-BSA conjugate suggested that a successful conjugation had occurred since the coupling of linamarin to BSA might have altered its maximum peak absorbance capacity, hence the reduction in the absorbance peak. The absence of an absorbance peak for linamarin means that the number of moles of conjugated hapten could not be calculated using the Beer-Lambert law. In order to reinforce the verification of a successful conjugation, a chemical assay method using TNBS assay was implemented.

This assay with its many variations is based on the sulphuric acid digestion of the protein with subsequent conversion of the nitrogen to ammonium sulphate (Goodwin and Choi, 1970). The assay protocol used in this study was as modified by Kemp and Morgan (1986) and Bolarinwa (2014), based on the determination of free amino acid groups present in the carrier protein before and after hapten conjugation following its reaction with TNBS. There are approximately 59 amino groups from lysine residues of BSA and of these, approximately 30-35 are accessible for use in coupling to hapten according to Erlanger (1980) and Singh *et al*. (2004). The standard curves obtained from BSA and linamarin-CC-BSA conjugate from each of the proteins when subjected to analysis with TNBS were significantly different (Figure 3-5). The results indicated there was a reduction in the number of available amino groups in the linamarin-BSA conjugate as compared to unconjugated BSA. The percentage substitution of linamarin calculated for linamarin-BSA from the cyanuric chloride conjugation method was 39%. Thus, the hapten-protein ratio of the immunogen was

calculated to be of 23:1, in concordance to other reports (Erlanger, 1980, Singh *et al*., 2004, Bolarinwa *et al*., 2014) suggesting a potentially good immunogen for antibody production. The CC method was initially devised for protein-protein conjugation and has only once before been used for hapten-protein conjugation. This is the first time linamarin was successfully conjugated to BSA using the CC method.

The immunogen (linamarin-CC-BSA) was used to immunised two New Zealand female rabbits weighing between 2.0 ~ 2.5 kg. IcELISA was used to determine the titre of the antisera. The titre of an antisera refers to the amount of specific, functional Ab present in the serum. Functionally, titre is generally indicated by the amount a serum can be diluted and still give good signal in a particular assay. Thus, the titre depends on the assay used and it measures both the affinity and amount of an Ab directed against the Ag of interest. An Ab with high titre is valuable not only because it means the serum can be used in more experiments, but, more importantly, that the non-specific components present in any serum will be less of a problem since they can be diluted out.

The colour generated by this indirect ELISA assay is due to the amount of secondary Ab (detection antibody – goat anti-rabbit IgG, H+L, HRP conjugated) bound to the primary Ab (rabbit anti-linamarin antibodies). The use of secondary Ab was very important at this stage to determine the presence of the desired primary Ab able to bind with linamarin-CC-KLH. This secondary Ab binds to the primary Ab because it recognizes the IgG Ab from a different species. HRP enzyme was used as the enzyme label and upon contact with the appropriate

substrate (TMB), it generated a signal colour that could be measured using a microtitration plate reader. The intensity of the signal produced is directly proportional to the amount of Ag captured in the plate and bound by the detection reagents. The quality and intensity of signal production and its detection in developing a new ELISA assay are imperative to achieve optimum sensitivity, specificity, precision and batch-to-batch consistency.

Polyclonal anti-linamarin titres were determined on microtitration plates coated with linamarin-CC-KLH conjugate. The pre-immune serum (serum taken just before the first immunization injection) for each rabbit shows slight but very minimal activity, which was expected due to non-specific binding of the sera towards the immobilised antigen and perhaps even towards the uncoated spaces on the wall of the microtitration wells. Both rabbits responded well to the immunogen challenge by producing Ab of a very high titre within 53 days (test bleed 1) of immunization showing antibody activity at a dilution of 1:10,000. Polyclonal antisera produced from both rabbit 1 (R074) and 2 (R076) indicated high Ab affinity towards immobilised linamarin-CC-KLH with no significant difference between any of the test bleeds as compared to the pre-immune serum. The antibody affinity of test bleed 2 (Day 74) of R074 and final bleed (Day 88) of R076 were exceptionally high as indicated by antibody activity detected even at dilution of 1/100,000, more so due to the fact that the antibody was derived from conjugated hapten. The final bleed from R076 (R076D88) was selected and used in subsequent experiments in order to preserve identical conditions and consistency.

The R076D88 bleed was chosen from the Ab titre results and investigated for use in standard curve at four different dilutions (v/v) 1/5,000, 1/10,000, 1/15,000 and 1:/20,000. The assay involves a competition between free standard linamarin with conjugated immobilised linamarin-KLH conjugate for binding to the Ab. In other words, the higher the amount of free linamarin present in the sample will reduce the number of primary Abs able to bind with the immobilised linamarin. Consequently, less enzyme-labelled secondary Ab binds to the primary Ab resulting in less enzyme activity and lower OD. The inverse relationship between the concentration of linamarin and the observed OD is clearly shown in Figure 3-8, which indicates that the anti-linamarin Abs were able to recognize and bind to the free linamarin, inhibiting the Abs from binding to the immobilised linamarin. The standard curves achieved indicated that even without subsequent optimization, the antibody was able to bind to free linamarin standard at a dilution of 1:20,000 with acceptable background noise.

The standard curve as shown in Figure 3-8 however was generated using detection Ab (goat anti-rabbit IgG H+L, HRP-conjugated) produced and supplied by Invitrogen, Thermo Scientific. As secondary Abs are mainly used for the indirect detection of a target to which a specific primary Ab is first bound, and it requires more steps than using a detectable primary Ab, indirect detection of the target Ag has the advantage of increased sensitivity due to the signal amplification from multiple secondary Abs binding to a single primary Ab. Moreover, a given secondary Ab can be used with any primary Ab of the same type and host species, making it an infinitely more versatile reagent than individual labelled primary Abs. Secondary Abs with specificity for the primary Abs of common species are commercially available pre-conjugated with many of

the common labels, making these detection reagents useful commodities. In order to achieve higher assay sensitivity with better signal:noise ratio, a detection Ab (goat anti-rabbit IgG H+L HRP-conjugated) produced and supplied by CovalAb UK Ltd. was used for comparison with the specific aims in mind. In addition, two different TMB substrates were investigated to determine one which produced better signal amplification. From the results, TMB 1 provided better signal amplification compared to TMB 2 although there was no significant difference between them. However, there was a huge leap in signal amplification between the two Abs used, suggesting that the detection Ab produced and supplied by CovalAb UK Ltd. has higher affinity towards the primary Ab compared to the one produced and supplied by Invitrogen, Thermo Scientific, which means that the assay sensitivity can be further enhanced quite significantly.

In an ELISA, it is important to block the unoccupied sites on the surface of the well of the microtitration plate to reduce the amount of non-specific binding of proteins during subsequent steps in the assay. The blocking decreases the opportunity especially for signal-generating Abs to bind non-specifically to the well hence reducing background signal/noise. A variety of blocking proteins ranging from non-fat milk to highly purified proteins have been used to block any remaining ‘sticky’ spots on the well (Gibbs, 2001). The blocking buffer should improve the sensitivity of the assay by reducing the background interference and an optimal blocking buffer maximizes the signal-to-noise ratio, does not react with Abs and alter or obscure the epitopes of the immobilized protein. An individual blocking buffer will not be compatible with every system; therefore, when developing any new ELISA, it is imperative to test several different blockers

for the highest signal-to-noise ratio in the assay due to each antibody-antigen pair having their own unique characteristics. The most common blocking buffers used in ELISA are BSA, gelatine from fish, non-fat (skimmed) milk and whole normal serum to name a few. Previous studies that worked on antibody development against hapten suggested that the use of 1% dried skimmed milk as blocking buffer was better compared to other buffers. The use of non-ionic detergent such as Tween 20 (0.05%, v/v) when used in conjunction with a protein blocker, provide added convenient and inexpensive blocking ability during wash steps by blocking areas on the surface that may become exposed due to protein/biomolecule desorption. In the current study, the use of dried skimmed milk (1%, w/v) was investigated and compared against other commonly used blocking agents, gelatine from fish (1%, w/v) and BSA (1%, w/v). From the results shown in Figure 3-12, the use of gelatine and BSA have actually increased the signal-to-noise ratio by shifting the standard curves to the right as compared to standard curve without any blocking. The use of 1% dried skimmed milk however, has shifted the curve to the left, which indicated an improved signal-to-noise ratio and hence further enhanced the sensitivity of the assay.

The present study demonstrated the use of CC conjugation procedure which involves activating the linamarin prior to conjugation to protein which enabled chloride bridging between the hydroxyl groups of linamarin and amine group of BSA could also be a useful alternative for other small molecular weight compounds which do not contain active groups such as -COOH, -NH2 or -OH or when the active groups on the hapten are very important to maintain the hapten immune characteristics and structural features to be used for coupling (Juan *et al*., 2010). The results indicated that anti-linamarin polyclonal antibody produced

from linamarin-BSA conjugate synthesized using the CC conjugation method performed exceptionally well in the icELISA.

3.5 Conclusion

A polyclonal anti-linamarin Ab was successfully raised against linamarin using a hapten linked to a carrier protein through a cyanuric chloride conjugation method. The linamarin antisera displayed an excellent Ab titre indicated by presence of binding activity even at 1:100,000 dilution. The Ab had desirable specificity towards linamarin as shown in the icELISA standard curve results which further optimized to improve the sensitivity of the assay. An Ab dilution of 1:50,000 was chosen for subsequent assay development and further characterization would be able to produce an ELISA with higher specificity and sensitivity.

Chapter 4 : Production of Monoclonal Antibodies against Linamarin

Summary

- Production of monoclonal antibodies against linamarin was attempted. There have been no previous reports of this activity.

- Seven female BALB/c mice were immunized into two separate procedures using linamarin-CC-KLH (4 mice) and linamarin-CC-BSA (3 mice) to generate polyclonal antibodies against linamarin.

- High antibody titre was obtained using non-competitive ELISA for all the test bleeds from both set of immunization.
    - Anti linamarin-KLH – 1/1000 ~ 1/100,000
    - Anti linamarin-BSA – 1/1000 ~ 1/70,000

- Inhibition assay (competitive ELISA) against free linamarin could not be established:
    - for selected hybridoma supernatants from antisera produced by immunization using linamarin-CC-KLH conjugate.
    - for antisera produced by immunization using linamarin-CC-BSA conjugate.

4.1 Introduction

The previous non-existence of specific Abs, both pAbs or mAbs is not surprising, since linamarin is a very difficult hapten to raise specific Ab response against. The lack of functional groups on linamarin and low molecular weight (147.2) makes it a difficult target for the immune system to recognize and elicit an appropriate immune response. Abs can only be raised against derivatives including the hapten and a chemical entity resembling an immunogenic conjugate. With help from chemical modifications, the Ab binding site encompasses the hapten residue with the inclusion of the chemical linker and sometimes even amino acid residues on the carrier protein (Tuomola *et al*., 2000). Chappey *et al.* (1994) reported that the influence of the spacer arm becomes crucial and high affinity against un-derivatized compounds are much more difficult to obtain when the molecular weight of the hapten is well below 300 Da. Despite the limitations, polyclonal Abs against linamarin have been successfully developed and described in the chapter 3 using linamarin conjugated to BSA via a cyanuric chloride method, and since the Ab titre of the pAbs produced against linamarin was exceptionally high (working range of 1:50,000 - 1:100,000, v/v), an attempt on mAbs production is highly probable. However, in immunoassays especially ELISA, both pAbs and mAbs have their advantages and disadvantages and the decision to use either of them depends on a number of factors, the most important of which are its intended use and whether the Ab is readily available from commercial suppliers or researchers (Lipman *et al*., 2005).

MAbs are often considered superior to pAbs simply because of their exquisite specificity of binding, homogeneity and consistency. The monospecificity provided by mAbs is useful in evaluating changes in molecular conformation, protein-protein interactions, phosphorylation states and in identifying single members of protein families. It also allows for the potential of structural analysis (e.g, x-ray crystallography or gene sequencing) to be determined for the Ab on a molecular level. In the context of immunoassay, however, the monospecificity of mAbs is not always an advantage. For example, when the Ab is required to be bound to a solid support or be labelled and still retain activity, the loss through denaturation of a sensitive Ab species leaves many others in a polyclonal preparation; not so for a monoclonal (Peppard, 2000). Lipman *et al*. (2005) also reported that small changes in the structure of an epitope (e.g., as a consequence of genetic polymorphism, glycosylation and denaturation due to chemical modifications, coupling procedures) can markedly affect the function of mAb. Monoclonal preparations may also be less avid in binding than polyclonal, leading to lower performance in immunoassay (Stewart and Lew, 1985). In contrast, because pAbs are heterogenous and recognize a host of antigenic epitopes, the effect of change on a single or small number of epitopes is less likely to be significant. PAbs are also more stable over a broad pH and salt concentrations, whereas mAbs may be highly susceptible to small changes in both. That being said, successful assays are often set up with monoclonal, in competitive binding or by using their high specificity to ‘capture’ the antigen, followed by a second monoclonal or polyclonal Ab for detection purposes (Peppard, 2000). Mixtures of mAbs for either capture or detection can also be used effectively. Furthermore, these combinations of Abs and use of accurate data treatment permit the development of immunoassays applied to the detection

of a group of closely related antigens (Glass *et al*., 2006), couples of cross-reacting analytes (Mercader and Montoya, 2007), (Wittmann and Hock, 1991), or degradation products (Mercader *et al*., 2008).

For the first time, pAbs have been successfully developed against linamarin. Although the pAbs were successfully generated within several months of initiating immunization, the same cannot be said for production of mAbs. One can reasonably expect a period of up to a year or longer in some cases, from immunization to generation of hybridomas, cloning and subsequent production of mAbs, often requiring considerable amount of expense and time. One major problem with pAbs is that the amount of Ab supply is limited to the size of the immunized animals, in this case the two New Zealand female rabbits. Once depleted, the next batch of pAbs generated from the same animal species would not be the same as the previous batch, leading to problems such as inconsistency and changes in avidity as they are harvested over time. In contrast to mAbs, once the desired hybridoma has been generated, mAbs can be generated as a constant and renewable resource, maintaining their consistency and homogeneity. To keep advancing the technique there is a need for new Abs and conjugates to novel analytes, because it is the availability for such immunoreagents that is the rate limiting step (Mercader *et al*., 2008). As far as linamarin is concerned, to our knowledge, no reports, whether using pAbs or mAbs, for analysis of linamarin in food have been published. The essential steps in the production of mAbs by the hybridoma method are illustrated in Figure 4-1.

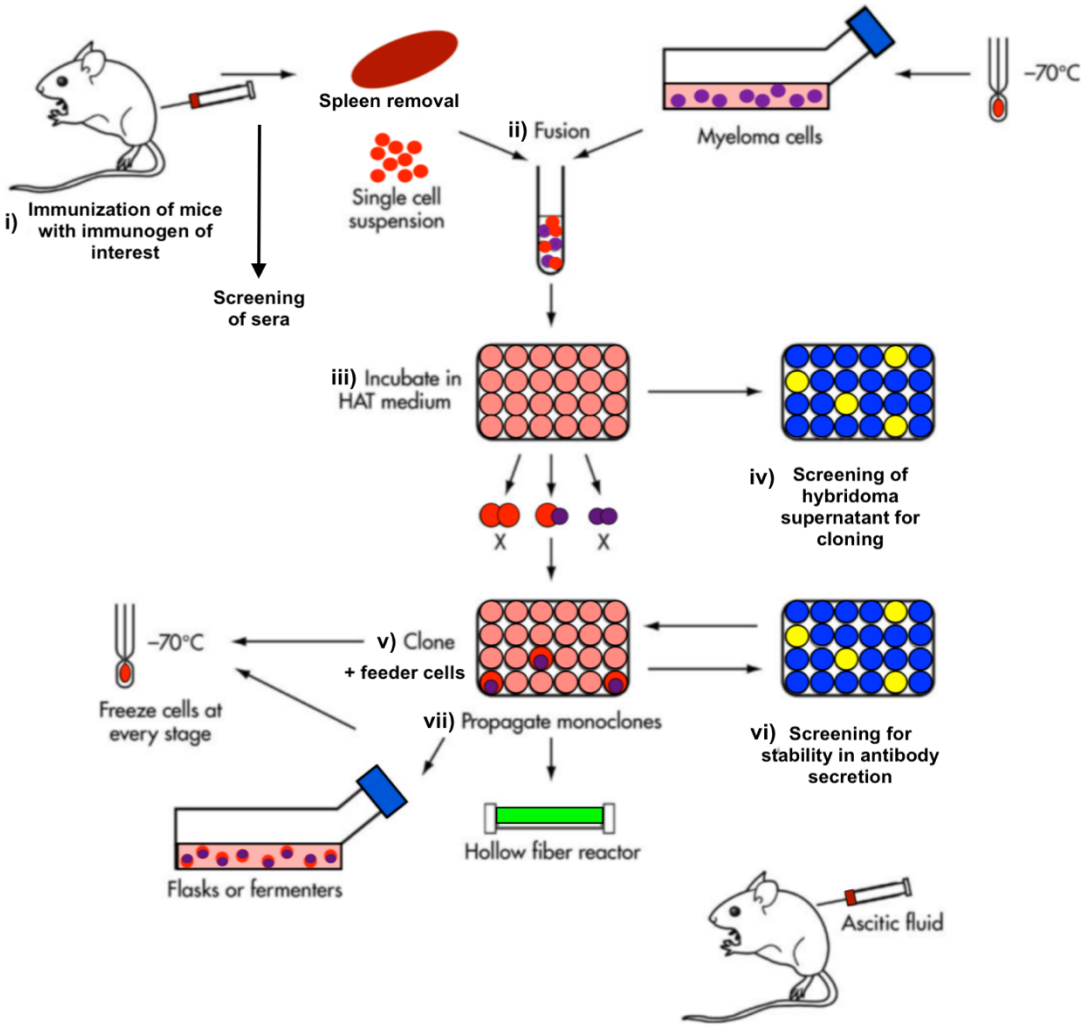


Figure 4-1: Essential steps in mAb production. A flowchart showing the main sequence of events in mAb production.

(Adapted from CMPMA, 1999 and Liddell, 2013)

4.1.1 Aim of the chapter

In continuation of the cyanuric chloride conjugated hapten-protein (linamarin-BSA conjugate) ability to elicit an immune response in rabbits that lead to production of an exceptionally good pAbs as described in chapter 3, the aim of the work described in this chapter was to use the same immunogen to immunise several BALB/c mice. The production of similarly good antisera could further be used to develop mAb against linamarin.

This chapter describes the experimental approach and results towards the following area:

i) Immunization of mice in order to generate polyclonal antisera capable of recognizing linamarin suitable for monoclonal antibody production.

ii) Production attempt, for the first time, of mAbs against linamarin using linamarin-KLH conjugate and linamarin-BSA, both conjugated using the cyanuric chloride method.

iii) Production of hybridoma cells through fusion, screening, cloning and culture of desired cells to produce mAbs.

iv) The setting up of Ab titre and ELISA standard curve for the detection of linamarin.

4.2 Methods: Monoclonal Antibody Production

Immunization with linamarin-KLH conjugate

KLH is well known as having higher immunogenicity compared to BSA mainly because of its sheer molecular weight and it has been used in various hapten-protein conjugation works, producing good immune response and Abs against various haptens. A set of different BALB/c mice (4) were injected with the immunogen (linamarin-KLH) to assess the immune response and explore possibilities of obtaining good quality Abs as opposed to the linamarin-BSA conjugate. Table 4-1 shows a summary of the intradermally immunization protocol for the production of the anti-linamarin Ab in mice provided by CovalAb UK Ltd.

Immunization with linamarin-BSA conjugate

Since the immunization of 2 New Zealand rabbits using the linamarin-BSA conjugate to generate pAbs were successful as described in previous chapter, the same conjugate was used to generate pAbs in 4 BALB/c female mice (between 6-8 weeks old) for mAbs production. Linamarin-CC-BSA and linamarin-CC-KLH conjugates were sent to CovalAb UK Ltd. for the immunization process, to be used as immunogen and a coating antigen respectively. Table 4-1 shows a summary of the intradermally immunization protocol for the production of the anti-linamarin Ab in mice provided by CovalAb UK Ltd.

Table 4-1: Summary of the immunization protocol for the production of the anti-linamarin antibody in mice.

Day	Protocol
0	Sampling of test serums (=30µl) and storage at -20°C
0 to 14	Multisite injections According to the protocol defined by Covalab Antigen and adjuvant (incomplete Freunds)
24	Sampling of test serums (= 30µl) and storage at -20°C – ELISA test(1)
27	IP injection Antigen + Adjuvant (incomplete Freunds)
38	Sampling of test serums (= 30µl) and storage at -20°C – ELISA test(1)
48	Subcutaneous injection Antigen + Adjuvant (incomplete Freunds)
69	Subcutaneous injection Antigen + Adjuvant (incomplete Freunds)
80	Sampling of test serums (= 30µl) and storage at -20°C – ELISA test(1)
97	Subcutaneous injection Antigen + Adjuvant (incomplete Freunds)
111	Subcutaneous injection Antigen + Adjuvant (incomplete Freunds)
122	Sampling of test sera (= 30µl) and storage at -20°C – ELISA test(1)
151	Boost BALB/c R
153	Fusion BALB/c R

Determination of anti-linamarin titre (screening)

Ab capture assays are often the easiest and most convenient of the screening methods. The non-competitive indirect ELISA similarly used for the determination of anti-linamarin titre in rabbits polyclonal antisera was implemented. The primary Ab dilutions were prepared as described in 2.3.8.1

and the microtitration plate was prepared as described in 2.4.10. Both sets of dilution (consisting of bleed 38, 80, 122, 153 or 255 days along with the pre-immune bleed as control) were dispensed into coated microtitration plate wells (200 μl/well) in duplicate. The microtitration plate was sealed using parafilm and incubated overnight at 4°C. The microtitration plate was washed 5-times with PBST the following day and left to dry. Secondary Ab (goat anti-rabbit IgG coupled with HRP enzyme, prepared as described in 2.3.9, 200 μl) was dispensed into each well and incubated for another 2 hours at 37°C. The microtitration plate was washed 5-times with PBST after the 2 hours incubation and left to dry. TMB substrate (200 μl per well) was dispensed into each well and the plate left for 30 mins at room temperature to allow colour development. Noticeable changes in colour were observed. 2 M sulphuric acid (50 μl) was added into each well to stop the enzymic reaction. Finally the optical density reading of each well was taken using a plate reader at 405 nm. The mouse that produced the highest Ab titre consistency throughout the immunization period was selected for the fusion stage.

Hybridisation and fusion

During the fusion stage, the spleen from the selected mouse was removed and the antibody-producing B-lymphocytes were isolated from the spleen cells and were fused with murine myeloma cell line (SP20Ag/14). The splenocytes were distributed and fused with the myeloma cells inside 12, 96 well-microtitration plates, which were then cultured in HAT selective medium for 2 weeks. An ELISA test was performed on the hybridoma supernatants to screen for hybridomas showing the desired Ab binding activity. The hybridisation and fusion were

performed by mixing the antibody-secreting cells with myeloma cells, centrifuged to generate good cell-to-cell contacts and fused with the aid of a fusing agent (polyethylene glycol, PEG). Three to five days before the fusion, the selected mouse was given a final boost on day 151 of the immunization. The boost was performed at least 3 weeks after the previous injection which allowed most of the circulating Abs to be cleared from the bloodstream by the mouse. The antibody-producing B-lymphocytes were isolated from the spleen of selected mouse and fused with murine myeloma cell line (SP20Ag/14). This hybridisation procedure was done by CovalAb UK Ltd. using their own protocol. Details of the hybridization and cell culture are shown in Table 4-2.

Table 4-2: Fusion and hybridisation details of mouse R.

Mouse	BALB/c R
Date of the last boost	01/11/2016
Number of spleen cells extracted	42 x 10^6
Seeding date	03/11/2016
Distribution	11 plates
Number of seeded wells	992
Density	0.2 x 10^6
Volume of culture medium per well	250µl
Culture medium	RPMI 1640*, 20% SVF*, HAT*, HCS*

*Royal Parkwell Memorial Institute (RPMI) 1640 growth media

*Stromal Vascular Fraction (SVF)

*Hypoxanthine Aminopterine Thymidine (HAT)

*Hybridoma Cloning Supplement (HCS)

Culture and propagation

After the fusion process, the hybridomas were distributed inside 11, 96 well-microtitration plates, which were then cultured in culture medium containing Royal Parkwell Memorial Institute (RPMI) 1640, 20% Stromal Vascular Fraction (SVF), Hypoxanthine Aminopterine Thymidine (HAT) and Hybridoma Cloning Supplement (HCS) for 2 weeks as described in Table 4-1. An ELISA test was performed on the hybridomas supernatants to screen for those secreting the desired Ab binding activity. Selected hybridomas was transferred into 24-wells plate and ELISA test was repeated 7-10 days after the transfer. Hybridomas (10 cell lines) were selected for freezing and this was done in 25cm^3 flasks where 3 vials (1 vial; 5-6 x 10^6 cells) are cryo-stored per hybridoma prior to the cloning stage. Partial Ab isotyping (IgG and IgM) were done at this stage as well.

Cloning and cryopreservation

Cloning of the selected hybridomas was done on a 96-well plate using the limiting dilution procedure. After 10-15 days, culture supernatants in each well were screened using ELISAs to determine positive wells. The 8 best cell lines were selected and transferred to a 24-well plate for another 7-10 days culture and were tested again with ELISA to select the best 3 clones. The selected clones were transferred to 25 cm^3 flasks for another 2 weeks of culture. Full isotyping of clones and determination of the Ig subclass (IgM, IgG1, IgG2a, IgG2b, IgG3 and light chains) was completed throughout the culture period. 3 vials (1 vial; 5-6 x 10^6 cells), each containing one clone per hybridoma was cryo-frozen and stored in liquid nitrogen until required.

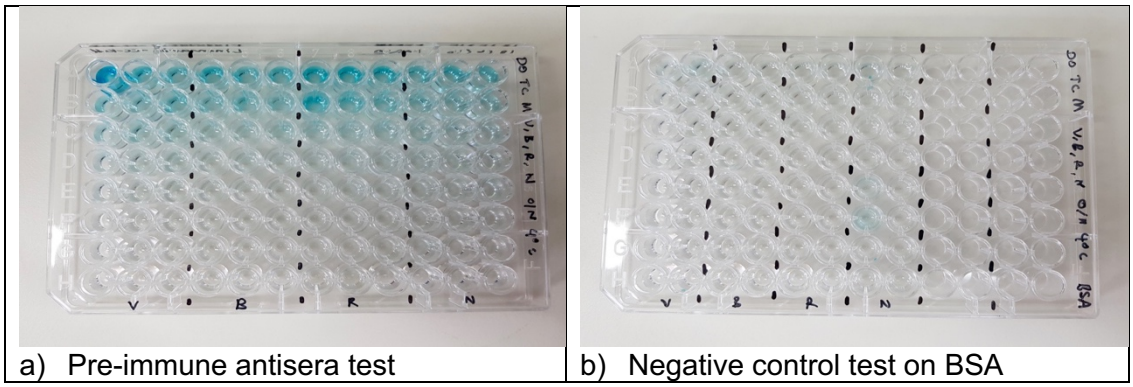
4.3 Results

4.3.1 Immunization with linamarin-KLH conjugate

Initially, the immunization of the mice was supposed to be carried out using linamarin-BSA conjugate (the same conjugate used for immunization of rabbits in Chapter 3) as agreed before the start of the project, but towards the end of the immunization period, CovalAb UK Ltd. informed that they had immunized the mice using linamarin-KLH instead which was supposed to be the solid phase antigen. After deliberate discussion with the scientific director of CovalAb, we have agreed to carry on with the project, based on the main aim of generating mAbs, in which, if successful, would produce clone of cells that secrete highly specific Abs against linamarin, regardless of the carrier used during the immunization period.

Four female BALB/c mice were injected with immunogen (linamarin-CC-KLH) for the immunization to take place. The immunoreactivity of the antisera collected from day 0 (pre-immune bleed), day 38, day 80 and day 122 generated from all mice, coded 1902V, 1902B, 1902R, 1902N (V, B, R and N) was evaluated using a non-competitive indirect ELISA against fixed amount (1.0 µg/ml) of solid phase antigen (linamarin-CC-BSA). Two sets of dilutions were used to test the antisera (2-fold and log 10 dilutions). This is to determine the lowest dilution of the antisera that could bind to the immobilized antigen above the background determined by the pre-immune bleed as a control. The antisera samples were arranged in the microtitration plate as shown is Figure 4-2 and Figure 4-3. Different shades of blue colour in each well represent the different amount of Ab

able to bind with the immobilised antigen based on their dilutions. The darker the colour, the higher amount of antibody-antigen complexes.



a) Pre-immune antisera test | b) Negative control test on BSA

Figure 4-2: Non-competitive indirect ELISA for polyclonal anti-linamarin antibody titre derived from mice. The activities of the a) pre-immune bleed (Day 0) for all mice (V, B, R, and N) and b) negative control test (on BSA). The results represent triplicate determinations for a), duplicate determinations for b).

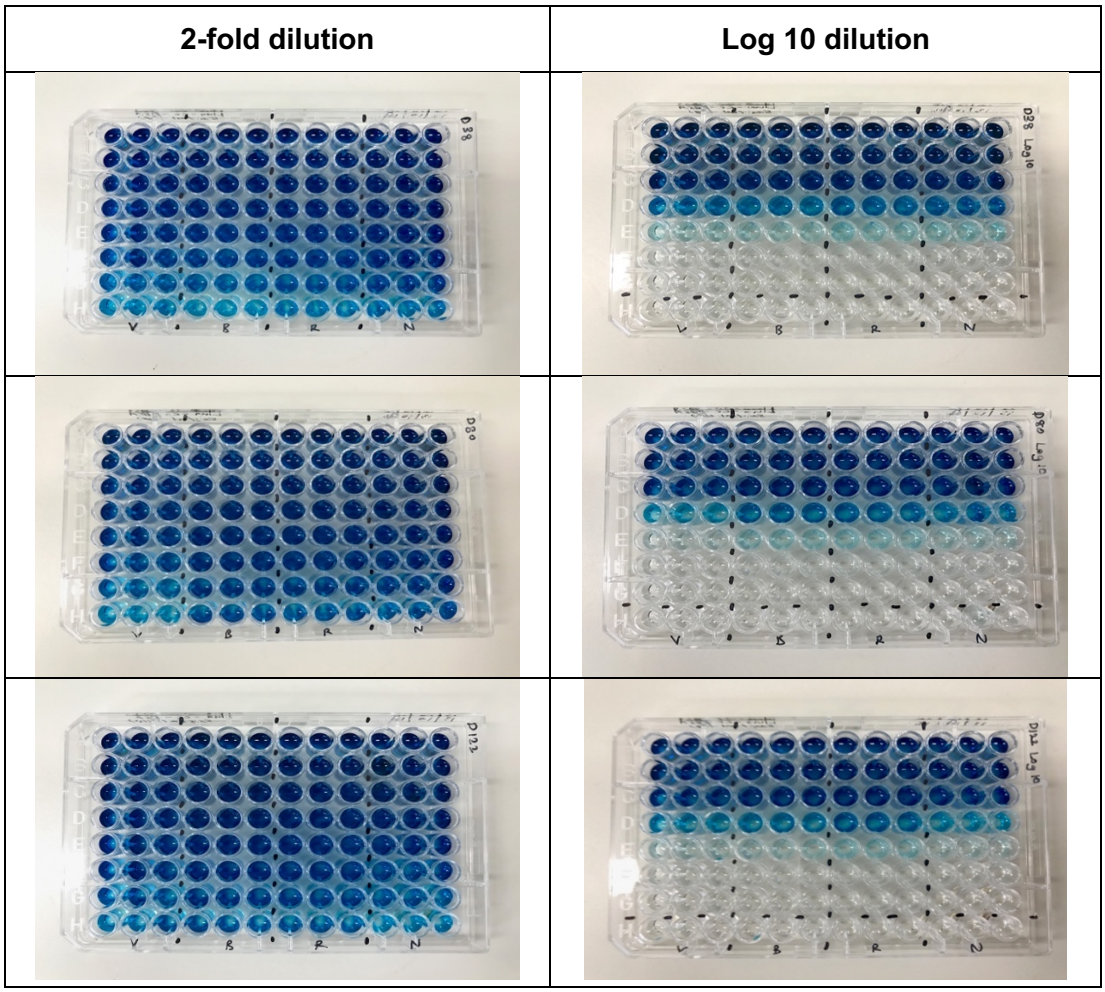


Figure 4-3: Non-competitive indirect ELISA for polyclonal anti-linamarin antibody titre derived from mice. The activities of all bleeds (Day 38, Day 80 and Day 122) for all 4 mice (V, B, R, and N). Each section represents triplicate determinations for each mouse down the antibody concentration as labelled.

The assessment of the immunoreactivity is based on the range of the optical density where; i) Titre < 0.20: no immunoreactivity, ii) 0.20 < titre < 0.80: low immunoreactivity, iii) 0.80 < titre < 3.20: good immunoreactivity and iv) Titre > 3.20: excellent immunoreactivity. From colorimetric observation, all test bleeds from the mice indicated positive reaction with the immobilised antigen compared to the background determined by the pre-immune bleed. The ELISA test using the 2-fold dilutions did not show much different in terms of colour intensity between the bleeds and dilutions. However, for the log10 dilutions, the shades of blue can be seen gradually turning from darker to lighter shade going down the Ab concentration. The optical density values were used to construct the Ab titre curves as shown in Figure 4-4 to 4-7 and were concurrent with the colorimetric observations. Remarkably, all four mice responded really well to the immunization in which the antisera that they produced were closely similar and able to recognize immobilised linamarin even at dilutions beyond 1/100,000. 2 booster injections were administered in between each test bleed resulting in the titre values to increase dramatically between the pre-immune bleed and the 1st test bleed (day 38). The subsequent booster injections seemed unable to improve the immunoreactivity of the antisera that much as shown in the 2nd test bleed (day 80) and the 3rd test bleed (day 120) results. Interestingly, similar results were exhibited by all four mice; i) all mice sera displayed high levels of pAbs with good immunoreactivity; the titre values were between 0.50 – 1.80 at 1/100,000 dilution, ii) the 1st test bleed (day 38) has the highest immunoreactivity. From the test, Mouse R antisera exhibited good consistency throughout the bleeds and hence were chosen for the fusion stage as described in section 4.2. Mouse V, B and N were scheduled for another booster injection to investigate the effect of further immunization on the Ab immunoreactivity.

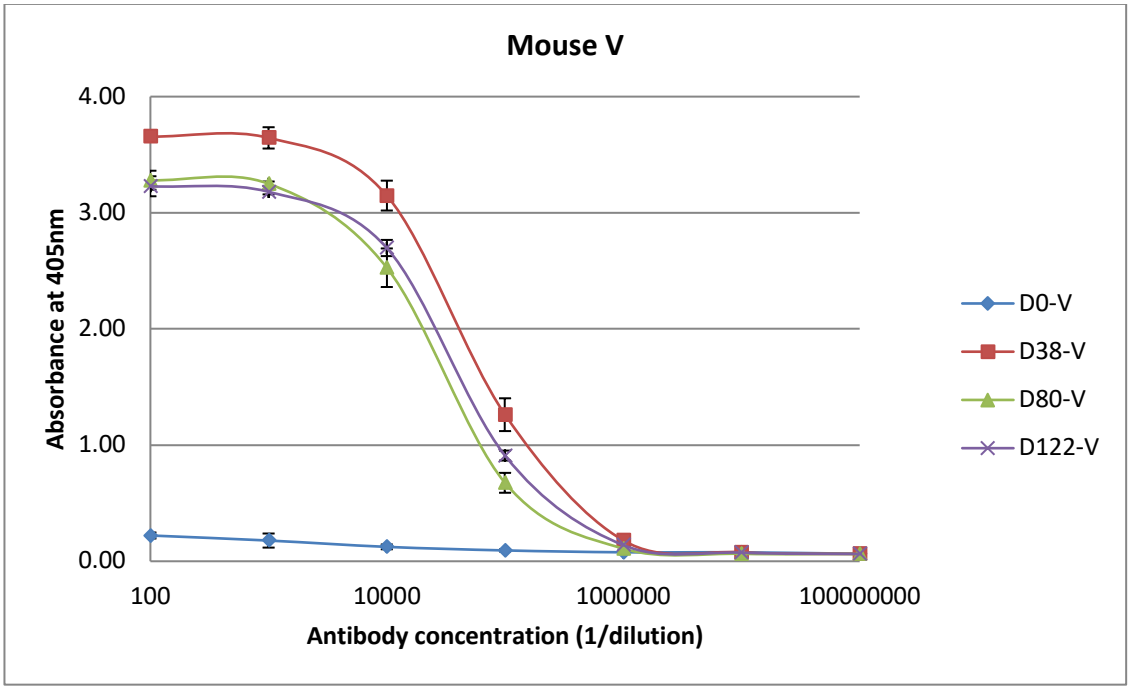


Figure 4-4: Titre curves of anti-linamarin antibody obtained from BALB/c mouse V immunized with linamarin – KLH conjugate (immunogen) from the cyanuric chloride method, each showing the titre curves for three different bleeds (Day 38, Day 88 and D122) along with the pre-immune bleed. Error bars indicate the standard deviations of triplicate analyses.

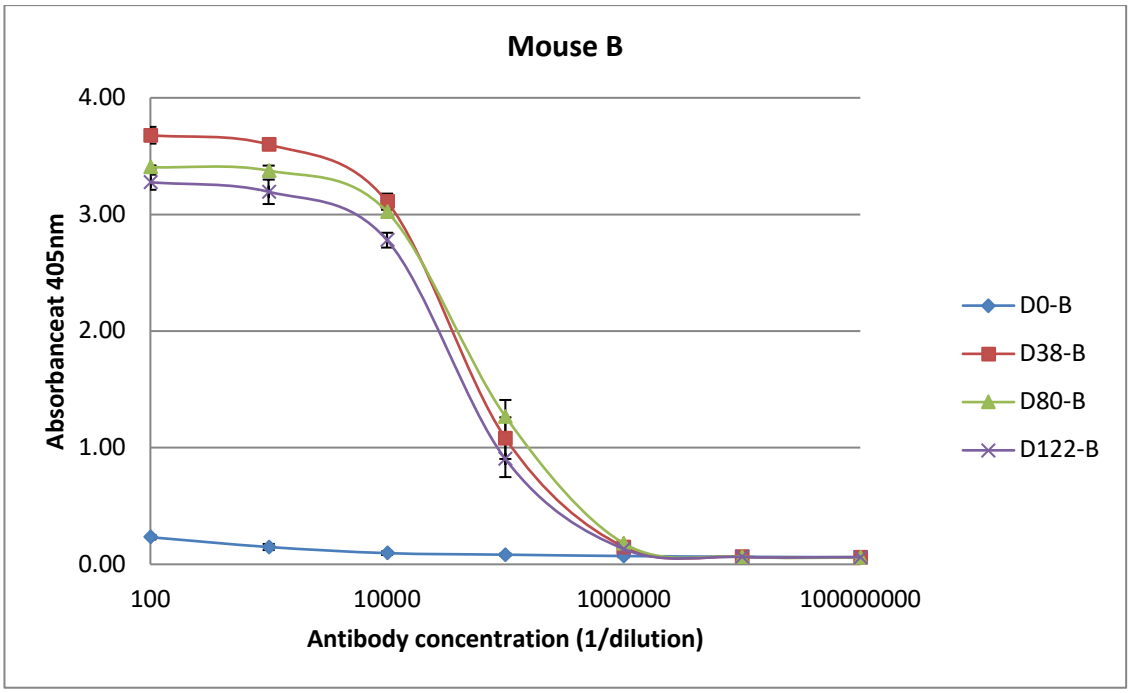


Figure 4-5 Titre curves of anti-linamarin antibody obtained from BALB/c mouse B immunized with linamarin – KLH conjugate (immunogen) from the cyanuric chloride method, each showing the titre curves for three different bleeds (Day 38, Day 88 and D122) along with the pre-immune bleed. Error bars indicate the standard deviations of triplicate analyses.

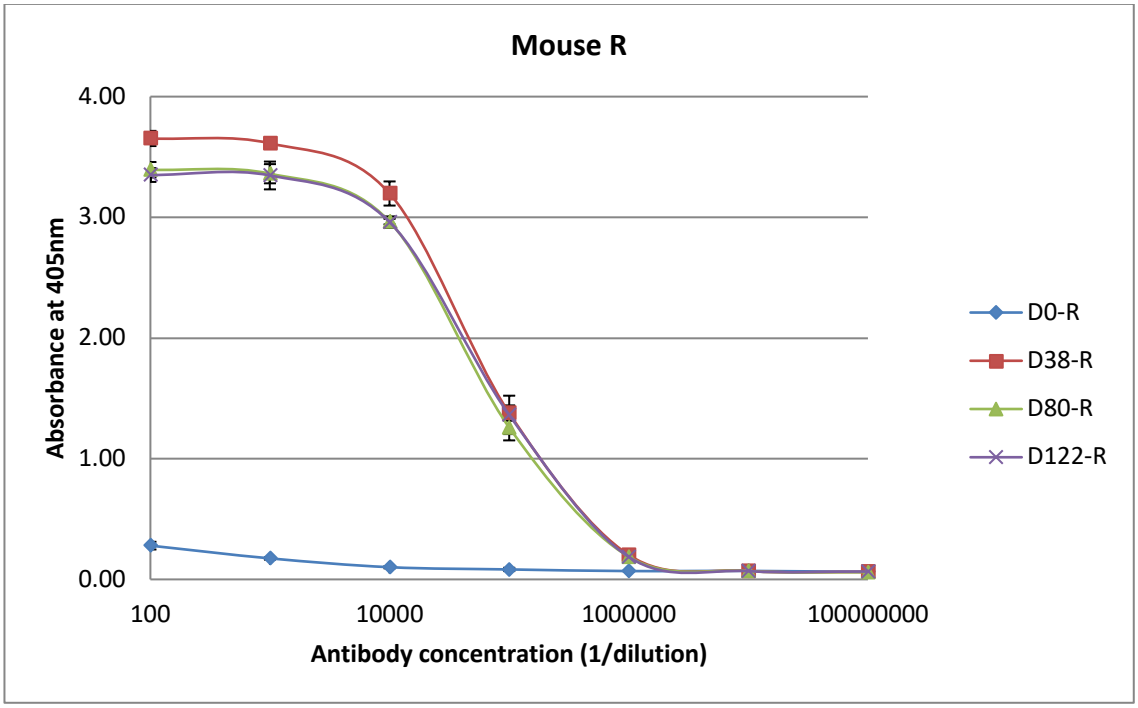


Figure 4-6 Titre curves of anti-linamarin antibody obtained from BALB/c mouse R immunized with linamarin – KLH conjugate (immunogen) from the cyanuric chloride method, each showing the titre curves for three different bleeds (Day 38, Day 88 and D122) along with the pre-immune bleed. Error bars indicate the standard deviations of triplicate analyses.

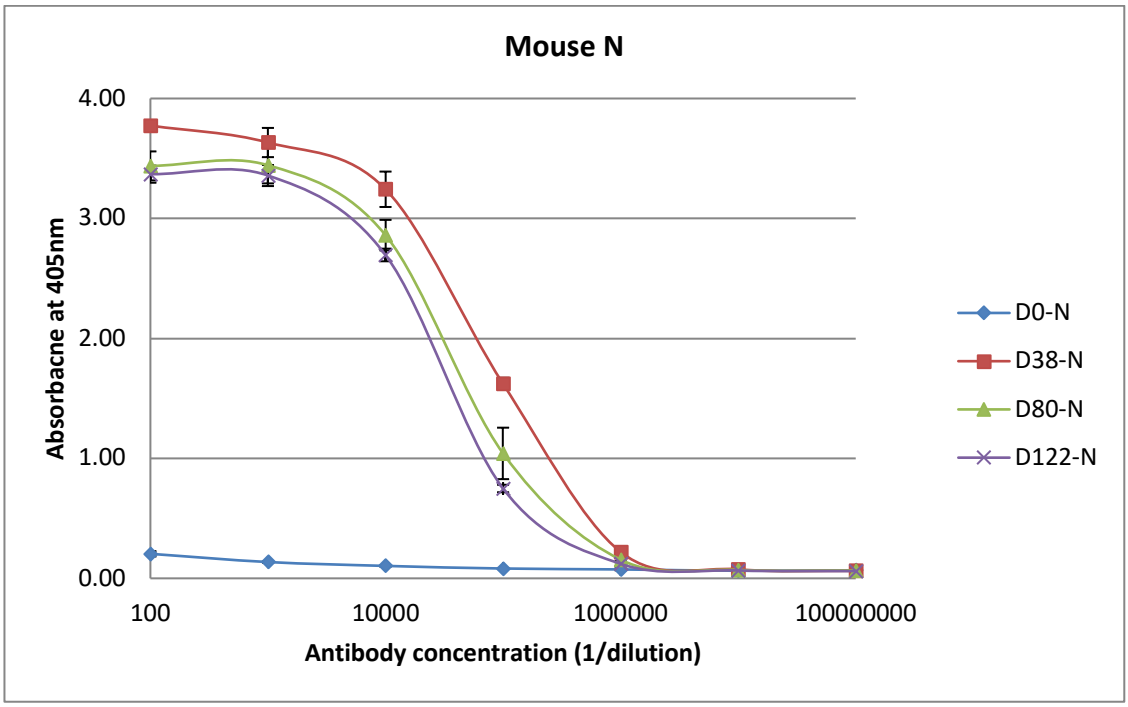


Figure 4-7: Titre curves of anti-linamarin antibody obtained from BALB/c mouse N immunized with linamarin – KLH conjugate (immunogen) from the cyanuric chloride method, each showing the titre curves for three different bleeds (Day 38, Day 88 and D122) along with the pre-immune bleed. Error bars indicate the standard deviations of triplicate analyses.

Following the hybridisation step and post culture period of two weeks, screening was performed on surviving clusters of hybridoma cells to determine those that were producing Abs against the antigen. The assessment of the immunoreactivity was based on the range of the OD where, 0.30 < OD < 0.50: low immunoreactivity; 1.00 > OD > 0.50: good immunoreactivity and OD > 1.00: very good immunoreactivity. There were 37 positive clusters of hybridoma cells identified from the screening as shown in Table 4-3. Clusters 1A3, 1G5, 1D9, 4D1, 4B2, 5B9, 6H6, 6G11, 6B12, 7A3 and 11H4 showed promising immunoreactivities with OD well over 2.000.

Table 4-3: Screening results for hybridomas culture supernatants by CovalAb UK Ltd.

Hybridomas	On antigen	Hybridomas	On antigen
1F2	1.895	**6A1**	1.932
1A3	2.537	**5B11**	1.015
1G5	2.732	**6H6**	2.554
1B9	0.435	**6H8**	0.415
1D9	2.551	**6F9**	0.625
1D10	0.426	**6H9**	0.350
2G8	0.328	**6G11**	2.690
2H12	0.458	**6H11**	0.377
3B7	1.378	**6B12**	2.531
3E12	0.821	**7G2**	0.379
4D1	2.537	**7A3**	2.569
4A2	1.357	**7F8**	0.330
4B2	2.590	**8D6**	0.831
4G8	1.155	**9H8**	0.502
5G5	1.159	**9H12**	0.916
5D8	1.315	**10F1**	0.316
5B9	2.488	**10D3**	0.497
5C10	1.451	**11H4**	2.605
2C7	1.631		

*Results from CovalAb UK Ltd.

Screening method: ELISA, Solid phase antigen: Linamarin-CC-BSA, Supernatant dilution: ½

indicates OD value over 2.00.

All 37 positive hybridomas were transferred into 24-well plates and grown for another 7-10 days to confirm the stability and secretion properties. The results are shown in Table 4-4.

Table 4-4: Hybridoma culture supernatants after 7-10 days growth. Negative control on BSA was also performed.

Hybridomas	On antigen	On BSA	Hybridomas	On antigen	On BSA
1F2	1.701	0.118	**6A1**	0.850	0.107
1A3	1.999	0.277	**5B11**	0.121	0.102
1G5	2.013	0.223	**6H6**	2.046	0.142
1B9	0.097	0.054	**6H8**	0.199	0.104
1D9	2.100	0.068	**6F9**	0.612	0.433
1D10	0.153	0.083	**6H9**	0.069	0.070
2G8	0.127	0.108	**6G11**	2.167	0.098
2H12	0.140	0.121	**6H11**	0.152	0.176
3B7	0.154	0.051	**6B12**	2.110	0.097
3E12	0.120	0.057	**7G2**	0.119	0.091
4D1	1.998	0.098	**7A3**	2.119	0.093
4A2	0.244	0.163	**7F8**	0.509	0.305
4B2	2.337	0.193	**8D6**	0.172	0.900
4G8	1.613	0.199	**9H8**	0.203	0.211
5G5	0.302	0.082	**9H12**	0.999	0.797
5D8	1.298	0.238	**10F1**	0.127	0.800
5B9	2.029	0.240	**10D3**	0.598	0.301
5C10	0.476	0.359	**11H4**	1.244	0.097
2C7	0.442	0.164			

*Results from CovalAb UK Ltd.

Screening method: ELISA, Solid phase antigen: Linamarin-CC-BSA, Supernatant dilution: ½

indicates OD value over 1.00.

The immunoreactivity of the Abs produced by all the hybridomas decreased as compared to the first screening results in Table 4-3. Most of the Abs produced by the hybridomas did not react with the BSA as indicated by the negative control results.

Table 4-5: Isotype of the antibodies produced by the culture supernatants from CovalAb. Only hybridomas that produced IgG Ab isotype were selected for further screening.

Hybridomas	Isotype	Hybridomas	Isotype
1F2	IgG	**6A1**	IgG
1A3	IgG	**5B11**	-
1G5	IgG	**6H6**	IgG
1B9	-	**6H8**	-
1D9	IgG	**6F9**	IgM
1D10	-	**6H9**	-
2G8	-	**6G11**	IgG
2H12	-	**6H11**	-
3B7	-	**6B12**	IgG
3E12	-	**7G2**	-
4D1	IgG	**7A3**	IgG
4A2	-	**7F8**	IgM
4B2	IgG	**8D6**	-
4G8	IgM	**9H8**	-
5G5	-	**9H12**	IgM
5D8	IgM	**10F1**	-
5B9	IgG	**10D3**	IgM
5C10	-	**11H4**	IgG
2C7	-		

*Results from CovalAb UK Ltd.

indicates IgG isotype only.

Out of the 37 hybridomas, only 13 were selected based on their immunoreactivity, stability of the Ab secretions and isotype they produced (Table 4-5). Hybridoma 1F2, 1A3, 1G5, 1D9, 4D1, 4B2, 5B9, 6A1, 6H6, 6G11, 6B12,

7A3 and 11H4 were selected for further testing. CovalAb UK Ltd. sent out the Abs in 0.5 ml eppendorf tubes pre-diluted in PBS (1:5; v/v) as shown in Figure 4-8.

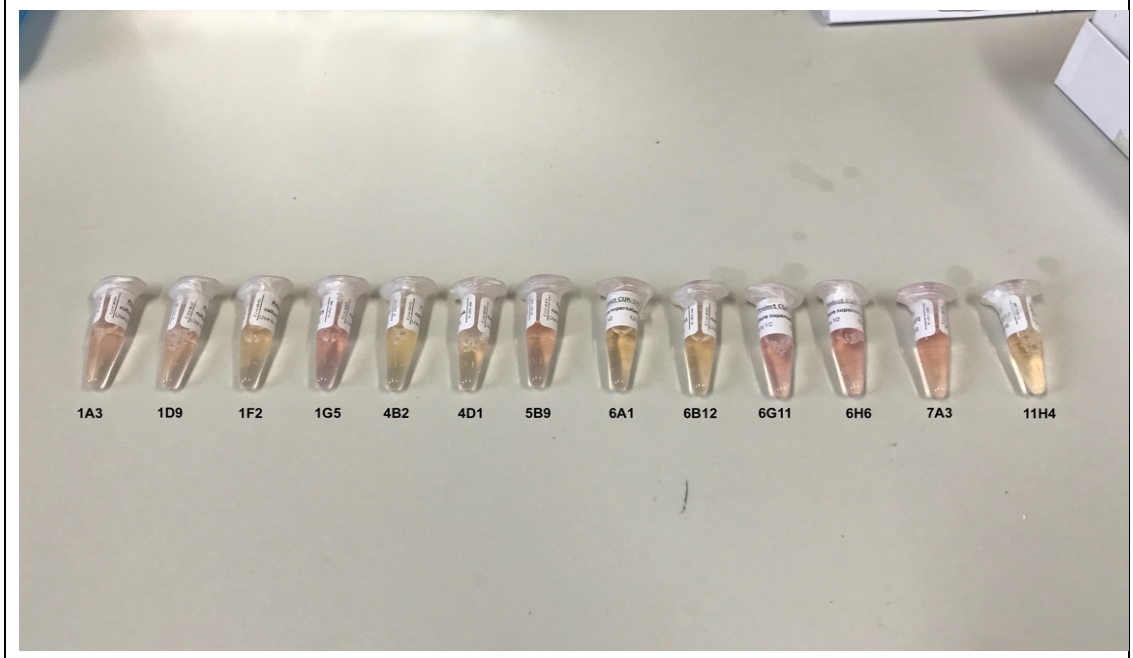


Figure 4-8: Antisera from culture supernatants of 13 selected hybridomas with IgG isotype sent by CovalAb Ltd. All antisera came pre-diluted in PBS (1:5).

Direct non-competitive ELISA was again used to determine the titre of the antiserum from each culture supernatant. Dilutions made were 1/5, 1/10, 1/16, 1/32, 1/64, 1/100, 1/1,000 and 1/10,000 and arranged as shown in Figure 4.9. The OD readings were listed in Table 4-5. Most of the culture supernatants showed gradual decreased in colour intensity and OD readings as the dilution ratio increased. All cultures have shown good immunoreactivity towards the solid phase antigen (OD: >1.00) except for culture 11H4 which OD values indicated poor recognition and binding properties. Major differences in immunoreactivity could be seen between 1/1,000 and 1/10,000 dilutions where there was a

significant change in colour intensity and OD readings. Culture 1D9, 4B2 and 6B12 still possessed good immunoreactivity at 1/10,000 (OD values >1.00) whereas others have already showed poor immunoreactivity and some to non-existence (1A3, 1F2, 4D1, 6A1 and 6H6).

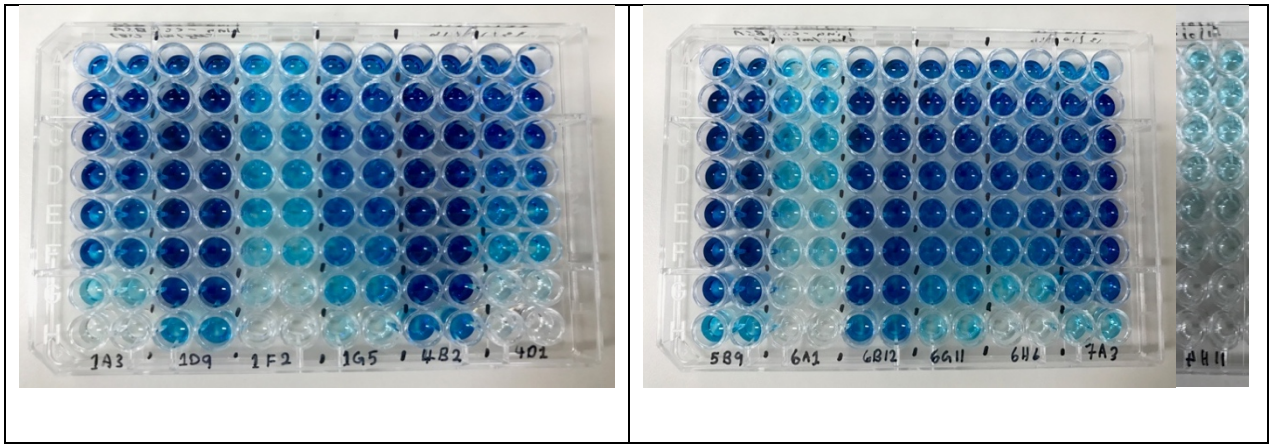


Figure 4-9: Non-competitive indirect ELISA for polyclonal anti-linamarin antibody titre derived from 13 selected hybridomas culture supernatants. Each section represents duplicate determinations.

Table 4-6: OD readings of the indirect non-competitive ELISA test in Figure 4-9

	1A3	1D9	1F2	1G5	4B2	4D1	5B9	6A1	6B12	6G11	6H6	7A3	11H4
1/5	2.81	3.49	1.74	2.50	3.72	3.49	3.35	1.45	3.42	3.51	3.08	2.86	0.56
1/10	2.68	3.46	1.12	2.24	3.54	2.44	3.28	0.83	3.44	3.15	2.65	2.70	0.23
1/16	2.66	3.58	1.06	2.43	3.72	2.77	3.27	0.59	3.29	3.16	2.70	2.80	0.21
1/32	2.36	3.53	0.73	1.98	3.65	1.72	3.35	0.47	3.24	2.69	2.55	2.67	0.16
1/64	1.67	3.51	0.60	2.00	3.75	1.24	3.24	0.34	3.18	2.34	1.95	2.73	0.12
1/100	1.64	3.36	0.37	1.25	3.53	0.82	3.20	0.30	2.97	1.81	1.50	2.25	0.09
1/1k	0.32	3.04	0.15	0.77	3.33	0.16	2.53	0.13	2.77	1.05	0.39	1.72	0.07
1/10k	0.08	0.89	0.07	0.18	1.35	0.09	0.76	0.07	1.32	0.32	0.10	0.33	0.06

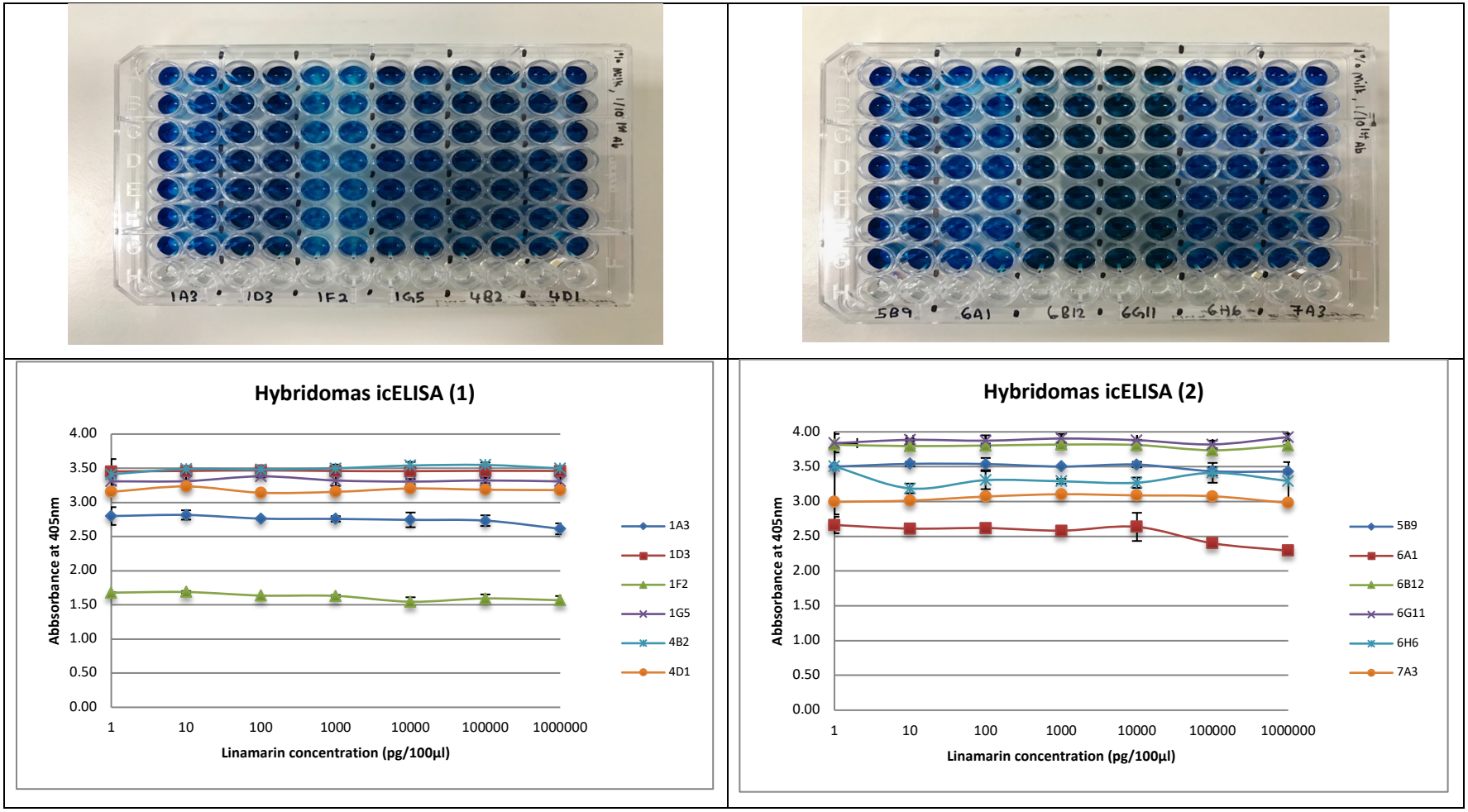


Figure 4-10: Indirect competitive ELISA for monoclonal anti-linamarin antibody titre derived from 12 selected hybridomas culture supernatants. Hybridomas icELISA (1): 1A3, 1D3, 1F2, 1G5, 4B2 and 4D1; Hybridomas icELISA (2): 5B9, 6A1, 6B12, 6G11, 6H6, 7A3. Each section represents duplicate determinations.

Hybridoma 11H4 was discarded due to very poor immunoreactivity (Figure 4-9 and Table 4-6). Twelve of the remaining culture supernatants were subjected to indirect competitive ELISA to investigate the ability of the Abs to bind with the standard linamarin (unbound linamarin). The results are shown in Figure 4-10. The competition between the unbound linamarin standard and immobilised antigen for binding with the Abs were poor. Thus, the inhibition of the Ab by unbound standard was not promising as most of the Abs tend to bind to the immobilised antigen rather than the unbound linamarin standard. However, some of the Abs in culture supernatants (1A3, 5B9, 6H6, 6A1 and 7A3) still demonstrated slight inhibition of the Abs binding to immobilised antigen between unbound linamarin concentration of 0.1 to 1.0 μg/100μl (Figure 4-10). This indicated that there might be a slightest chance to get a clone that would be able to secrete mAb, specific and has binding properties towards the standard linamarin after cloning by limiting dilution step.

Table 4-7: 10 selected culture supernatants based on the ELISA assessment for cloning.

	Antigen (Linamarin-cc-BSA, 5μg/ml)		Negative control (BSA, 5μg/ml)	
Hybridoma	**FSN**	**CovalAb**	**FSN**	**CovalAb**
1A3	2.814	2.787	0.050	0.081
1D9	3.489	2.872	0.056	0.100
1G5	2.499	2.719	0.050	0.081
4B2	3.722	2.743	0.058	0.147
4D1	3.487	2.639	0.059	0.081
5B9	3.349	2.953	0.078	0.075
6B12	3.424	2.686	0.063	0.098
6G11	3.506	3.189	0.064	0.141
6H6	3.080	2.818	0.059	0.097
7A3	2.858	2.728	0.057	0.153

*Results comparison from FSN and CovalAb UK Ltd.

OD readings from dilution 1/5 were used to compare the results from CovalAb and ten culture supernatants that displayed excellent immunoreactivity towards the solid phase antigen were selected for cloning (Table 4-7). The cloning was performed as described in section 4.2 by CovalAb UK Ltd. Following a 10-15 days of culture after the limiting dilution procedure, two of the hybridomas (1A3 and 4D1) were discarded due to instability and loss of ELISA activity. Eight remaining hybridomas were transferred to a 24-well plate for another 7-10 days culture and were tested again with ELISA to select the best three clones. The cultures were frozen accordingly at different steps of the protocol (Table 4-7). Inhibition assay (icELISA) was performed to investigate the ability of the mAbs to bind with the unbound linamarin standard. The test came out negative as the three selected clones (5B9, 6H6 and 7A3) were not able to show inhibition and binding property towards the unbound linamarin even after limiting dilutions. All the tests were done by CovalAb UK Ltd. However, the three other mice (V, B and N) were still subjected to further boost of immunogen injections just before the fusion stage of lymphocytes gathered from mouse R. The bleeds were taken from the mice on day 255 and were tested for their immunoreactivity and compared with mouse R bleed that was taken on day 153. Results are shown in Figure 4-11. No significant difference between the day 255 bleeds of mice V, B and N after prolong exposure to the immunogen as compared to mouse R bleed on day 153. Hence, from the reported results together with the negative inhibition assay that we did, and after lengthy discussion with CovalAb UK Ltd. we concluded that we were not able to generate mAb against linamarin using lymphocytes from mouse R and immunogen (linamarin-CC-KLH) and therefore had to put the project to halt. We initiated a new project, this time using linamarin-CC-BSA as the immunogen that will be explained in section 4.2.

Table 4-8: Number of vials frozen at different steps of protocol. Only positive hybridomas were frozen.

Hybridomas	Number of cells/vial	Date of frozen	Number of vials
7A3	6.5 x 10^6	23/01/2017	1
	6.9 x 10^6	27/01/2017	1
	9.9 x 10^6	31/01/2017	1
6H6	3.5 x 10^6	23/01/2017	1
	3.6 x 10^6	27/01/2017	1
	5.3 x 10^6	31/01/2017	1
5B9	4.1 x 10^6	23/01/2017	1
	6.4 x 10^6	27/01/2017	1
6B12	7.5 x 10^6	27/01/2017	1
	5.5 x 10^6	31/01/2017	1
	3.5 x 10^6	06/02/2017	1
1G5	5.8 x 10^6	27/01/2017	1
	4.2 x 10^6	31/01/2017	2
1D9	7.8 x 10^6	06/02/2017	1
	5.1 x 10^6	10/02/2017	2
4B2	4.8 x 10^6	06/02/2017	1
	4.9 x 10^6	10/02/2017	2
6G11	6.3 x 10^6	06/02/2017	1
	4.2 x 10^6	10/02/2017	2

*Results from CovalAb UK Ltd

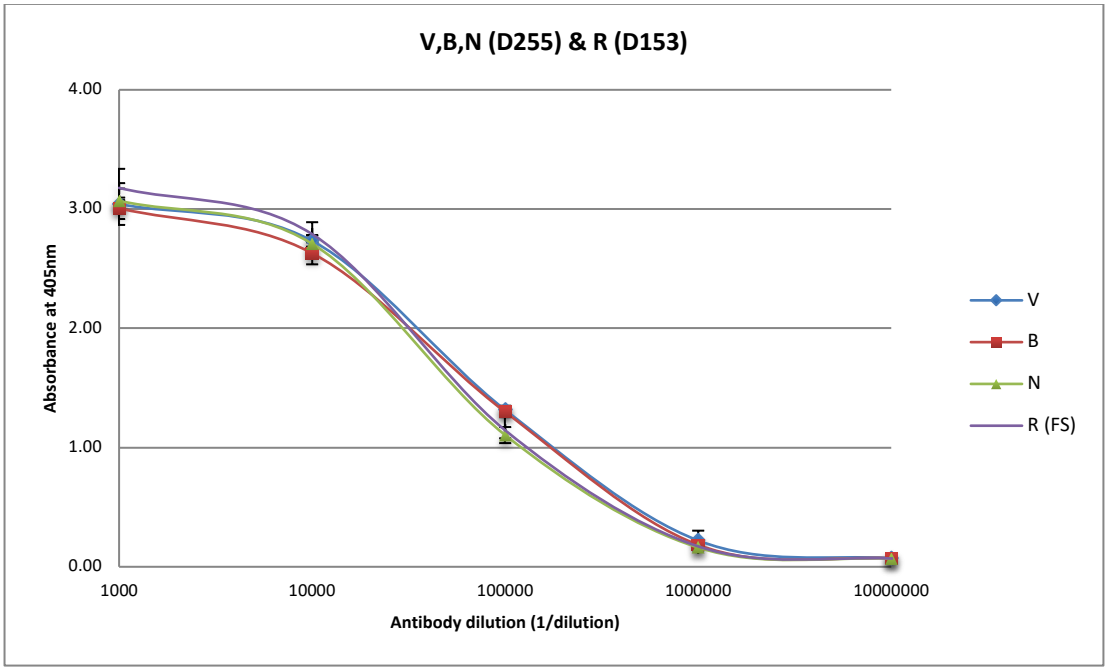


Figure 4-11: Non-competitive indirect ELISA for polyclonal anti-linamarin antibody titre derived from mice after prolong exposure to immunogen. The activities of all bleeds from mice V, B and N that went through booster injections and bleed taken on day 255 compared to mouse R bleed taken on day 153. Each section represents triplicate determinations for each mouse down the antibody concentration as labelled.

4.3.2 Immunization with linamarin-BSA conjugate

As we were unable to generate monoclonal antibody against linamarin using linamarin-CC-KLH as the immunogen, we decided to use the immunogen that had successfully generated excellent pAbs against linamarin in rabbit as described in Chapter 3, linamarin-CC-BSA. Four female BALB/c mice were injected with immunogen (linamarin-BSA) for the immunization to take place. However, one of the BALB/c mice (mouse N) died during the early stages of the immunization hence only bleeds from 3 surviving mice were gathered. The immunoreactivity of the antisera collected from day 0 (pre-immune bleed), day 38, day 88 and day 130 generated from all mice, coded MP-00050V, MP-00050B and MP-00050R (V, B & R) was evaluated by a non-competitive indirect ELISA using linamarin-KLH as the solid phase antigen. The titre curves of all the test bleeds from the mice are shown in Figure 4-12 to 4-14. The assessment of the immunoreactivity was similarly conducted as described in section 4.3.1 (i) Titre < 0.20: no immunoreactivity, ii) 0.20 < titre < 0.80: low immunoreactivity, iii) 0.80 < titre < 3.20: good immunoreactivity and iv) Titre > 3.20: excellent immunoreactivity). As expected, all mice responded well to the immunization in which the antisera that they produced exhibited high levels of pAbs showing good immunoreactivity towards bound linamarin even at dilutions beyond 1:30,000. 2 booster injections were administered in between each test bleed and as a result, the titre values increased significantly between i) the pre-immune bleed and the 1st test bleed (day 38), ii) the 1st test bleed (day 38) and the 2nd test bleed (day 88). However, the titre values between the 2nd test bleed (day 38) and 3rd test bleed (day 130) only indicated slight improvement after the booster injections.

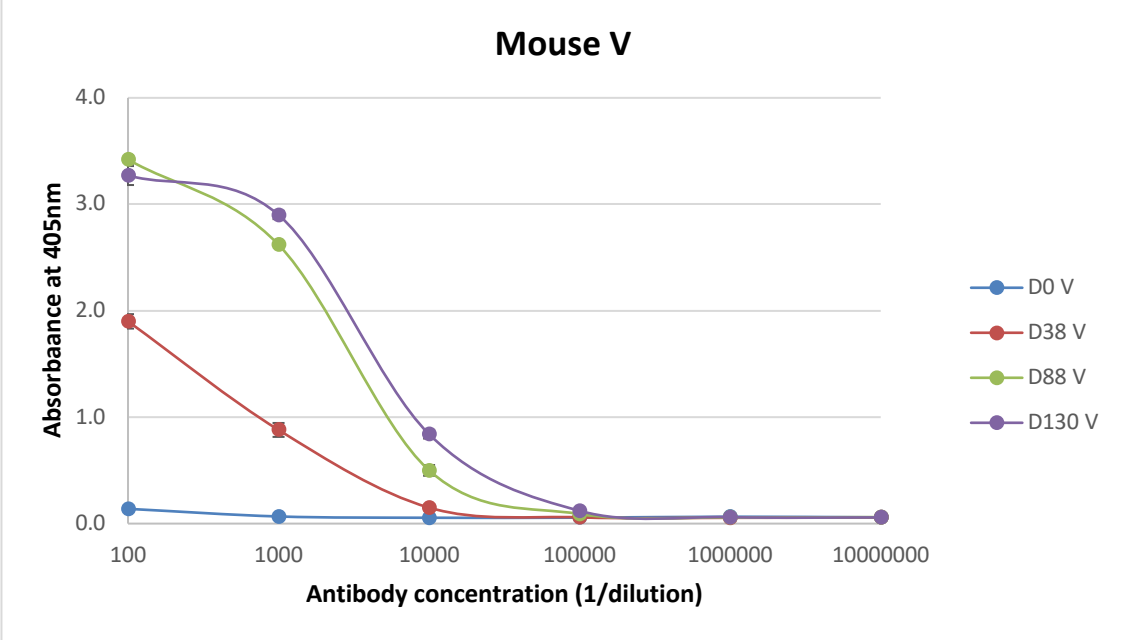


Figure 4-12: Titre curves of anti-linamarin antibody obtained from BALB/c mouse V immunized with linamarin-CC-BSA conjugate (immunogen), each showing the titre curves for three different bleeds (Day 38, Day 88 and D130) along with the pre-immune bleed. Error bars indicate the standard deviations of triplicate analyses.

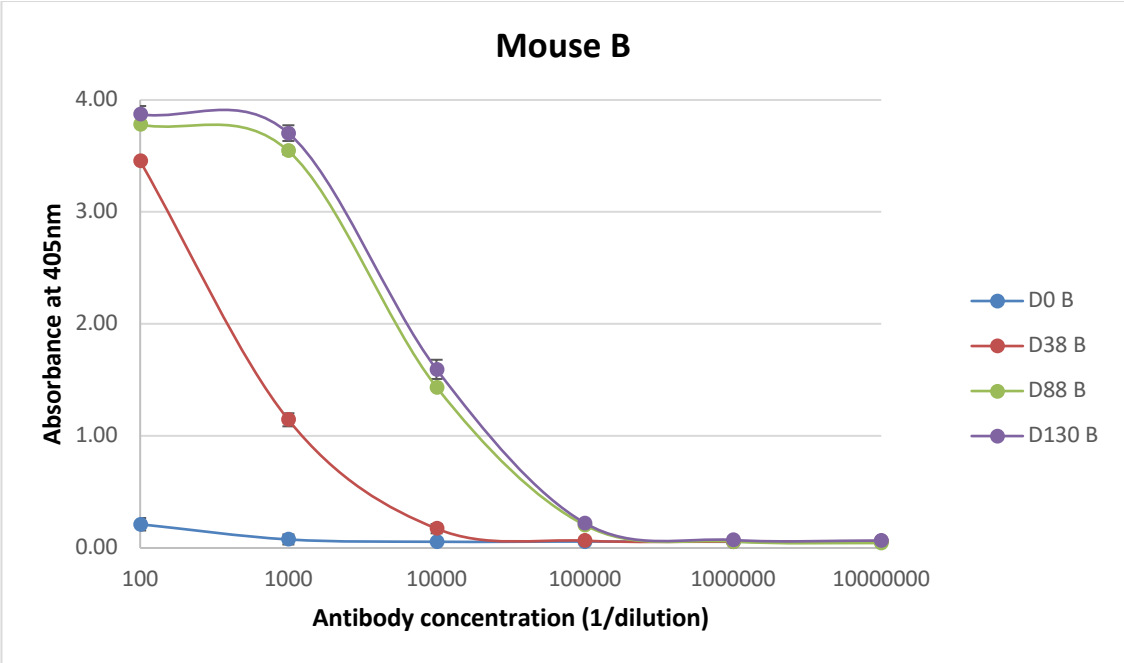


Figure 4-13: Titre curves of anti-linamarin antibody obtained from BALB/c mouse B immunized with linamarin-CC-BSA conjugate (immunogen), each showing the titre curves for three different bleeds (Day 38, Day 88 and D130) along with the pre-immune bleed. Error bars indicate the standard deviations of triplicate analyses.

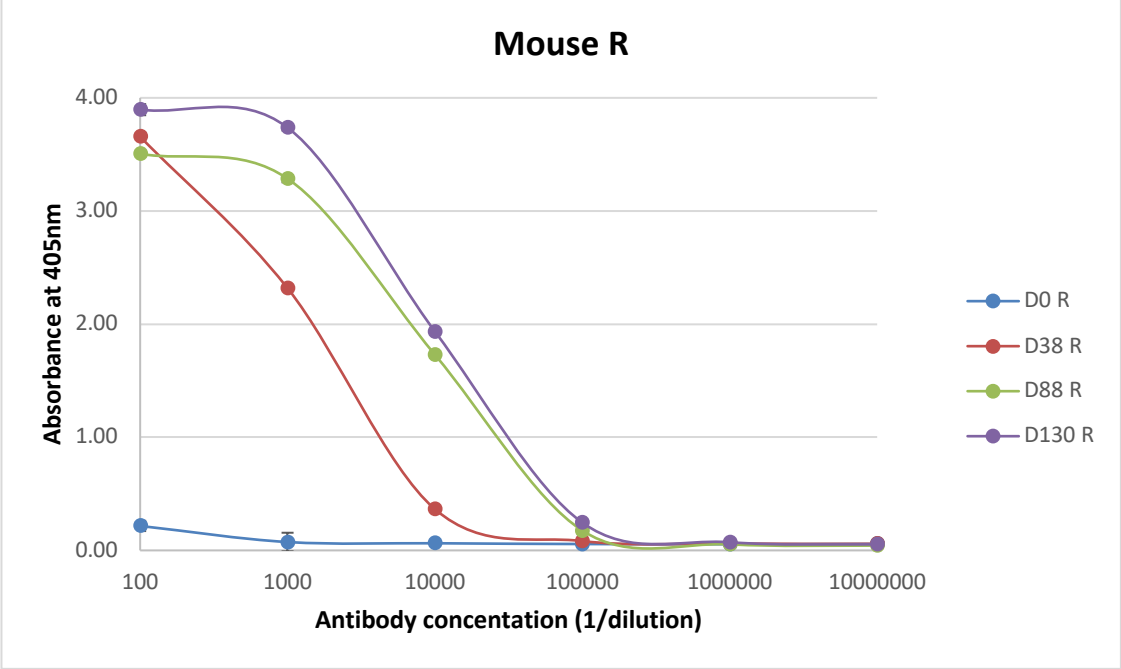


Figure 4-14: Titre curves of anti-linamarin antibody obtained from BALB/c mouse R immunized with linamarin-CC-BSA conjugate (immunogen), each showing the titre curves for three different bleeds (Day 38, Day 88 and D130) along with the pre-immune bleed. Error bars indicate the standard deviations of triplicate analyses.

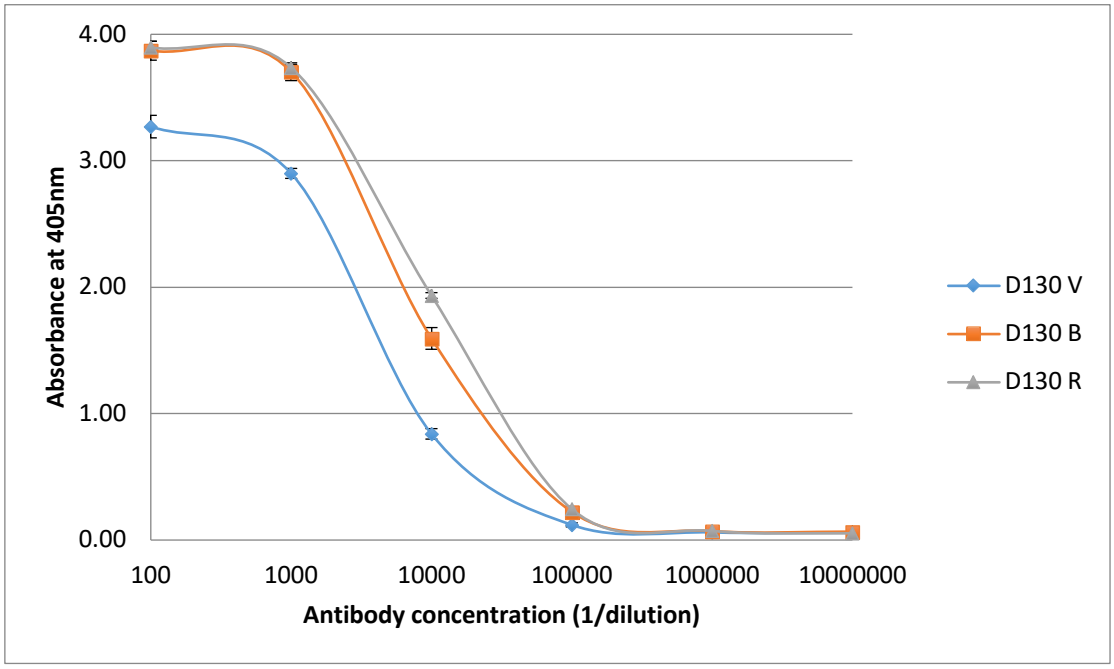


Figure 4-15: Day 130 antibody titres comparison between mouse V, B and R. Error bars indicate the standard deviations of triplicate analyses.

The Ab titre tests were performed using 2 sets of dilutions (2-fold dilution and log10 dilution). Figure 4-15 shows the Ab titre results for the day-130 test bleed of all mice using log10 dilution and the OD readings of 2-fold dilution test are shown in Table 4-8. Mouse B and Mouse R exhibited similarly close Ab titre with the latter being slightly better as the Ab concentration became more diluted. However, the Ab titres for all the mice this time around were not as good as the previous immunisation using linamarin-CC-KLH as the immunogen. In previous antisera titres, the immunoreactivity was still present at 1/100,000 dilution for all mice but in current titres, immunoreactivity was poor beyond 1/70,000 dilution and nearly absent passed the 1/100,000 mark.

Table 4-9: Day 130 antibody titres comparison between mouse V, B and R using 2-fold dilutions.

	D130 (Absorbance values at 405nm)		
1/dilution	**V**	**B**	**R**
2000	3.25	3.61	3.58
4000	3.14	3.29	3.37
8000	2.26	2.51	2.77
16000	1.20	1.66	1.90
32000	0.64	0.99	1.17
64000	0.34	0.51	0.60
128000	0.19	0.28	0.31
Blank	0.05	0.08	0.06
	Linamarin-CC-KLH 1µg/ml		

Based on the Ab titre results, both mouse B and R were selected for the indirect competitive ELISA test in order to check their binding ability towards unbound linamarin standard (Figure 4-16 and 4-17). Two bleeds from day 88 and day 130 were used although bleed from day 130 has the highest titre compared to other bleeds. Unfortunately, the results showed that the Ab did not bind with the unbound linamarin, therefore no significant inhibition occur even at higher linamarin concentration. Two different Ab dilutions were used in icELISA for day

88 bleed (1/5,000 and 1/10,000), meanwhile for day 130 bleed, 1/ 2,000 and 1/5,000 were used. For day 130 test, the Ab concentration used was higher because in day 88 test, 1/10,000 dilution resulted in very poor OD readings.

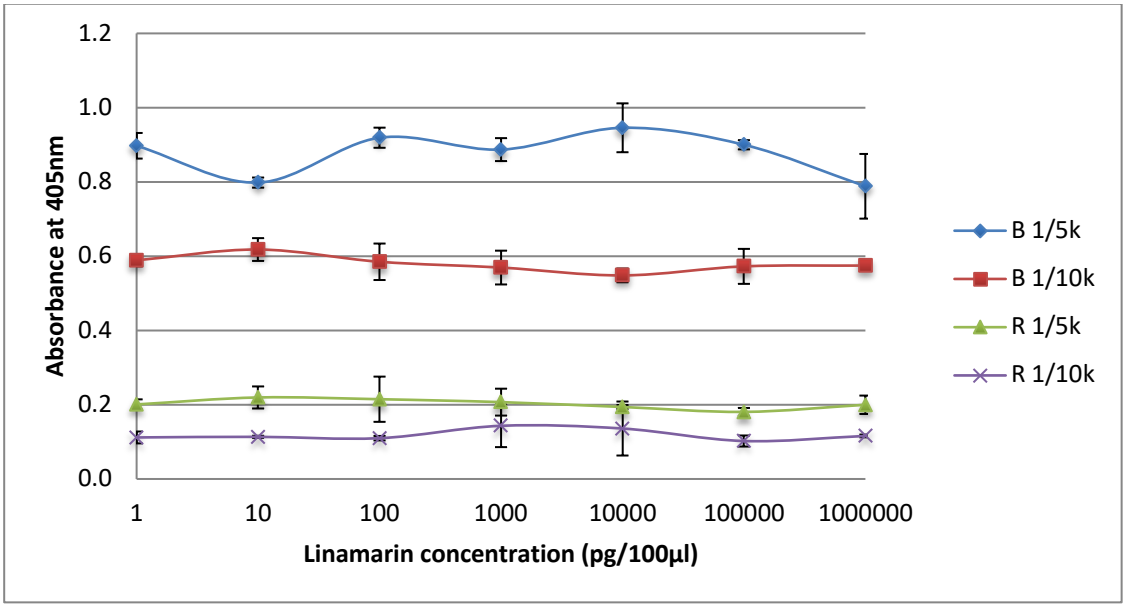


Figure 4-16: Indirect competitive ELISA results for day 88 test bleeds from Mouse B and R. Error bars indicate standard deviation of triplicate analyses.

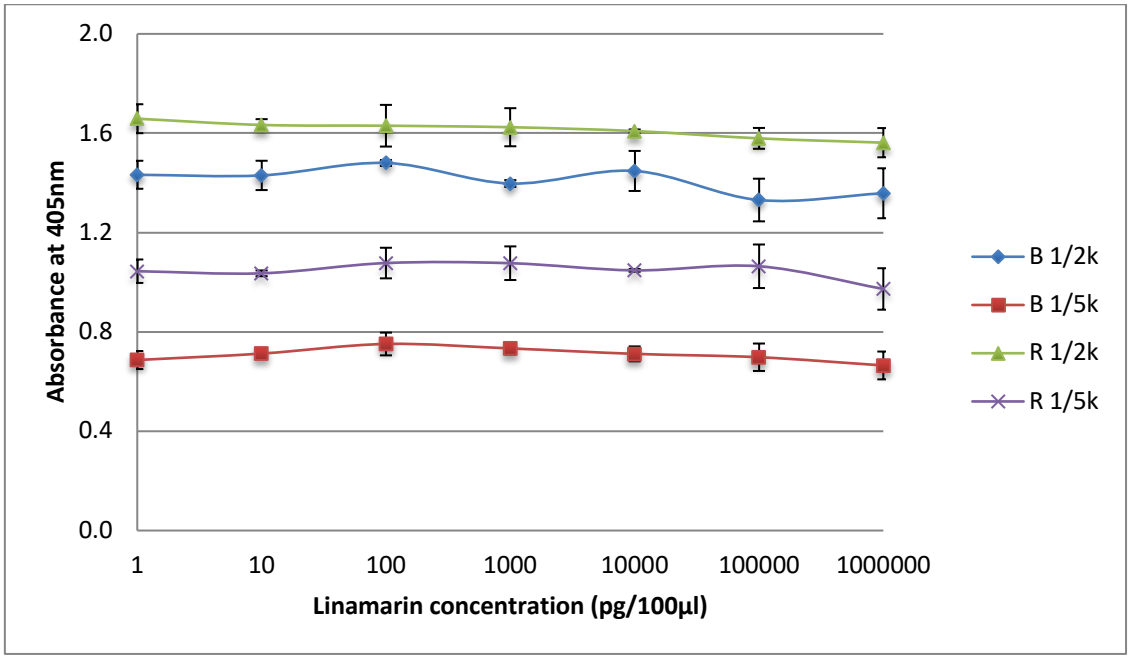


Figure 4-17: Indirect competitive ELISA results for day 130 test bleeds from Mouse B and R. Error bars indicate standard deviation of triplicate analyses.

The results in this section were quite similar to those obtained in previous section, where, both immunogens were able to elicit immune response in mice and produced Abs with excellent antiserum titre, showing immunoreactivity even at Ab dilution of 1:100,000 (linamarin-CC-KLH) and 1:70,000 (linamarin-CC-BSA), however they both failed to produce antihapten antibodies as indicated by the icELISA test. This approach was not pursued any further.

4.4 Discussion

The successful development of pAb of high immunoreactivity against linamarin previously discussed in Chapter 3 prompted the idea of synthesizing monoclonal antibody using the same immunogen (hapten-conjugate) synthesised using cyanuric chloride method. MAb production against linamarin has never been attempted yet at the time this study was conducted. The availability of mAbs would open up huge possibilities in all areas of Ab use because reagents could be created with specificity to a single domain (epitope) on the target substance. Additionally, Abs could be generated to compounds that had previously been regarded as impossible when using conventional serum production which explains the lack of literature on the matter, the CGs, currently being the main focus of this study. In previous chapter, the discussions were mainly focusing on the success of the conjugation method using cyanuric chloride bridging method producing an immunogen capable of eliciting the rabbit's immune response and eventually synthesised pAb that has high antibody titre and very low limit of detection and quantification towards linamarin. In this chapter however, the attempts made to produce mAb against linamarin were not giving the anticipated results whereby although both linamarin-CC-KLH and linamarin-CC-BSA conjugates were both able to elicit good immune response in mice, for some reason they both failed to recognize free linamarin. Despite the negative results, plenty of findings from both attempts could be used to improve the understanding on ways hapten and large immunogenic proteins interact with each other in terms of their hydrophobicity and hydrophilic properties, epitope orientations and electronic configurations on different carrier proteins, variable levels of immune response from different or even same hosts towards the hapten-conjugates.

These factors underlined the basis of designing optimum yet efficient conjugation method that will eventually lead to secretion of desired Abs with high specificity towards the free hapten instead of the hapten derivatives. This will be the main discussion in this section and perhaps it will provide the basis of mAb production against linamarin, covering most of the important aspects at the same time addressing problems and challenges for the betterment and future work.

It is well known that generation of specific and sensitive Abs against small molecules is greatly dependent upon characteristics of the hapten-protein conjugates (Rajesh *et al*., 2013). Ideally, conjugation should yield 100% conjugate of well-defined chemical structure, possess stable linkage and practical yet simple (Fasciglione *et al*., 1996). However these criteria are extremely hard to achieve and most of the conjugation procedures have large differences in terms of their conjugation efficiencies. The coupling of hapten to protein conjugate with optimal epitopes density without altering the basic molecular structure of the former is the most critical step required for the generation of good immunogen. Another important factor to be aware of is the introduction of new immunodominant epitopes (called neodeterminants) that are usually introduced by the most coupling agents which significantly reduces the specificity of humoral immune response (Fasciglione *et al*., 1996). Therefore, conjugation methods should be considered on the basis of that the carrier and coupling agents with low immunoreactivity should be selected to reduce the immune response against neodeterminants and at the same time able to increase antibody specificity against hapten.

In present study, BSA and KLH were both used as carrier proteins, the former being the carrier of choice to be used as immunogen initially and the latter as solid phase antigen to the hapten, linamarin. This was decided based on the high immune response obtained in rabbit, exhibiting excellent antiserum titre and working dilution of more than 1:100,000 (see Chapter 3). However, CovalAb UK Ltd. was somehow adamant in using linamarin-KLH conjugate although the initial request was to use linamarin-BSA conjugate. This mixed up was only brought to attention after the fusion procedure and since it was almost halfway through the mAbs development process and with extra assurances provided by the technical director from CovalAb UK Ltd. on their past experiences and successes in dealing with mAbs induced by hapten-KLH conjugates, the project was allowed to run. Similar to previous work in pAbs development, the immunisation responses and subsequent screenings were monitored with a non-competitive assay using homologous hapten labels, commonly done using hapten-conjugates having different carriers from that of the immunogen. The pre-fusion antiserum titre of the mice were excellent (1:100,000), as good as the rabbit's antiserum titre. Screening of the hybridoma supernatants revealed that most of the antiserum produced by the hybridomas failed to show significant binding to the free linamarin although some displayed slight inhibition at the highest linamarin concentration (Figure 4-10). While the use of carrier heterogeneity ensures that irrelevant carrier-specific antibodies are not measured, it still detects hapten-specific antibodies that include carrier protein structures in their epitopes which explains this type of behaviour. In the current work, the use of cyanuric chloride as the coupling agent (crosslinker, bridging molecule) between hydroxyl group in the glycoside and amino group in carrier protein was supposed to not have this issue, however it was suspected that the immune response in

mice was directed largely towards the bridging molecules producing antibodies specific for the linamarin-derivatives, and not for the free hapten, underlining the fact that the bridge effect is emphasised with small haptens (Chappey *et al*., 1994), although this was not the case with Abs produced by the rabbits. Despite the results, the slight inhibition by the free linamarin was enough to warrant continuation towards the cloning stage and perhaps after the limiting dilution and successful cloning, some of the hybridoma clones would have been able to produce mAb specific to the free linamarin. Final tests by CovalAb UK Ltd. on the supernatants of the best three selected hybridoma clones were negative. Perhaps, the immune response in mice was also largely directed against either the neodeterminants (the ε-lysine aminic bond) or those regions of the haptens involved in the coupling reaction. Due to the small size of the hapten, the bridging molecule will always recognised to some degree as well. In addition, most of the bridging molecules including spacer arms contained functional groups that were found to be important antigenic determinants (Tuomola *et al*., 2000). It is possible that the hapten, when linked to the carrier by a short linker group, was masked by or lost within the protein tertiary structure (Paxton *et al*., 1976). It was concluded that linamarin-CC-KLH conjugate was unable to generate mAbs specific to free linamarin perhaps due to factors mentioned and this approach was not pursued any further and prompted another attempt of mAb production; this time using the linamarin-CC-BSA conjugate.

The immunisation with linamarin-CC-BSA conjugate was expected to produce good immune response in mice, similarly as good as their rabbit counterparts or more so, those elicited by the KLH-conjugated hapten. Unfortunately, it was not

the case. The antiserum titre was slightly lower than those obtained from previous attempt (1:70,000), which is expected due to smaller molecular size of BSA as compared to KLH, though still considered an excellent titre nonetheless. As there was a high risk of choosing mAb with a high affinity for the hapten derivative but low affinity for the free hapten, a competitive assay step was included in the screening at an early stage (pre-fusion stage) on top of the non-competitive assay. The competitive assay result was not as expected as very little inhibition by the unbound linamarin occur and it seemed like the Abs had higher avidity towards the solid phase antigen. For some reason, the immunisation with linamarin-CC-BSA failed to produce antihapten antibodies in mice, as opposed to rabbit's immune response towards the same immunogen described in Chapter 3. Since the Abs from the immunization using linamarin-CC-BSA had also failed to establish a good inhibition assay and binding property towards free linamarin, due to budget and time constraint, this project was terminated at the end of the immunization stage.

Dealing with interspecies immunization, various immunogenic carriers have been successfully used; among them, BSA was the most widely assayed for many reasons. BSA is inexpensive and readily. In addition, this protein proved to be heterologous enough to provoke adequate mice immune response in most cases (Naar *et al.*, 1999) but not necessarily to haptens. Most of monoclonal antibodies approved for clinical application are of mouse origin. However, the mouse system is limited by a small spleen and mice used are usually inbred, thus offering less diversity of its immune responses. In contrast, as the original and still reliable model system to produce antibodies for laboratory use, the rabbit has a robust immune system able to generate antibodies with high affinity and

specificity. The rabbit immune system on the other hand generates antibody diversity and optimizes affinity by mechanisms different from those mice and other rodents (Zhang *et al*., 2017). Rabbits belong to the order Lagomorpha, which is evolutionary distinct from the order Rodentia, to which, for examples, mice and rats belong. Rabbit antibodies are able to recognize epitopes on antigens that are not immunogenic in rodents (Rief *et al*., 1998), increasing the total number of targetable epitopes and facilitating the generation antibodies to enhance the immunoreactivity (Yu *et al*., 2015). It has been observed that rabbits elicit strong immune response against small molecules and haptens, which is uncommon in rodents (Feng *et al*., 2011). Furthermore, rabbits use different mechanisms to genetically generate and diversify their primary and secondary antibody repertoires compared to mice, effectively creating a complementary set of binders (Weber *et al*., 2017). As of most strategies to generate mAb are based in the recovery of B cells from the spleen, bone marrow or blood, they are present in higher quantities in rabbits than in mice due to their overall larger body size (average body weight of a 3-month old laboratory rabbit is 2.5 kg compared to 25 g for a 6-week old laboratory mouse). That's 50 times more splenocytes containing B cells that could be recovered from rabbits as opposed to mice (Feng *et al*., 2011).

However, the main challenge of using rabbit antiserum to generate mAb is the absence of stable rabbit myeloma lines to generate viable hybridomas (Raybould and Takahashi, 1988). Raybould and Takahashi generated the first stable rabbit-mouse stable hetero-hybridoma by polyethylene glycol mediated fusion of rabbit spleen B cells with the mouse myeloma cell line SP2/0-Ag14. However, after several months they observed genetic instability and concomitant decrease of

mAb secretion by the hetero-hybridoma. The first rabbit homo-hybridoma was developed in 1995 in the laboratory of Katherine Knight (Spieker *et al*., 1995) and their findings subsequently led to the discovery of the first stable rabbit plasmacytoma cell line, 240E-1 which could be used as an efficient fusion partner to generate rabbit homo-hybridomas. However, the stability of the obtained homo-hybridomas was still a major concern and IgG secretion decreased over time. For this reason Zhu and Pytela (2009) attempted to further improve the initial 240E-1 cell line by iterative subcloning to screen clones with higher fusion efficiency, yielding hybridomas with higher genetic stability and more stable rabbit IgG secretion. The obtained fusion cell line of 240E-W and its successors 240E-W2 and 240-W3, which are characterised by higher fusion efficiency and the absence of endogenous rabbit heavy- and light-chain secretion (US Patent 7,429,487) are part of the proprietary RabMab platform of Epitomic, Inc (now Abcam, Inc.) and available commercially. The availability of stable rabbit fusion cell line provides an alternative to mAb generation through the murine cell line and perhaps this approach is something that could be considered in order to generate mAb against linamarin or a very small hapten.

Several authors have outlined the importance of the physicochemical interactions between haptens, carrier proteins and bridging molecules when designing hapten-conjugate to ascertain success in mAb production (Fasciglione *et al*. 1996; Harrison *et al*., 1991; Goodrow *et al*., 1995).

i) Carrier choice is critical in obtaining a high immune response measured as Ab titre, fusion efficiency and mean affinity constant.

ii) Hydrophobic haptens, modifying the tertiary structure of carrier proteins, are hidden in the core of the proteins thus avoiding or limiting any productive interaction with the immune system.

iii) Specific carriers should be used to obtain high specific immune response against hydrophobic haptens.

iv) Synthesis of a number of hapten derivatives with different bridge structures and attachment sites is generally recommended to explore maximum number of possibilities for obtaining good quality antibodies as well as for further assay improvement.

v) One should resist the temptation to use the existing functional groups of the hapten for the conjugation reaction when working with smaller molecules because the smaller it gets, the more important is the retention of the group's identity.

4.5 Conclusion

In general, the results of this study underline the importance of hapten design in the development of antibodies against compounds with simple structures and very low molecular weights. The hapten structure developed in this study successfully highlighted the choice of host animal for immunisation is something that should be considered especially when mAb generation is concerned. MAb should, however, be seen as complementary to those derived from animal serum because each has its place in immunochemistry. The unique specificity, defined affinity, and avidity of the mAb are very desirable when looking at cell surface markers or single epitopes on a viral protein. In contrast, the broad specificity of pAbs is a characteristic that may be desirable when screening for multiple strains

of a virus or in techniques such as immunoaffinity purification. MAbs may perform perfectly well in one assay format but may not for one reason or another convert to another.

Chapter 5 : Sample Preparation and Determination of Linamarin in Cassava and Food Products

Summary

- The indirect competitive ELISA for linamarin developed in chapter 3 was optimised and a sensitive standard curve was constructed using four parameter logistic regression analysis to achieve limit of detection (LoD) for linamarin of 1.5 ng/ml and working range of 0.215 μg/ml and 24.5 μg/ml.

- The cross-reactivity of the Ab with other structurally-related CGs was low amygdalin (4.80%), prunasin (6.48%) and mandelonitrile (6.40%).

- No apparent matrix effects were seen with the ELISA when linamarin standards were dissolved in assay buffer or cassava extract and analysed.

- The linamarin content of fresh cassava and processed products was successfully detected and quantified with the ELISA. The level of linamarin (HCN^- mg/kg, fresh or dry weight basis) equivalent were ranging from as low as 0.003 to 43.08 mg/kg. The results obtained were of satisfactory in concordance to other previous findings.

- Summary of cyanogen levels is shown in table below.

1.	Acute lethal dose of cyanide	0.5 – 3.5 mg/kg body weight
2.	Regulatory safe limit of total cyanide in food (WHO, FSANZ)	10 mg/kg
3.	Regulatory safe limit of total cyanide in food (Ministry of Industry and Trade of Indonesia)	40 mg/kg
4.	Highest concentration of linamarin found in a product (cassava root)	43.08 mg/kg fw
5.	Lowest concentration of linamarin found in a product (tapioca flour)	0.003 mg/kg dw

5.1 Introduction

One direct method that have not been studied as much in terms of CGs detection and quantification is the immunoassay. The inherent characteristics of immunoassay in terms of rapidity, reliability, high sensitivity and specificity make it an excellent analytical technique in the food and agrochemical industry. Immunoassays are now widely utilized in food analysis for determination of food contaminants, mycotoxins, pesticide residues, detection and quantification of proteins, enzymes, vitamins and other naturally occurring substances (Schneider & Hammock, 1992; Goodrow & Hammock, 1998; Spinks, 2000; Burkin & Galvidis, 2010; Ackermann *et al*., 2011, Lei *et al*., 2011). Despite its success, until now, there are only three reports on immunoassay development for the qualitative analysis of CGs in plant foods, amygdalin (Cho *et al*., 2006; Cho *et al*., 2008) and one for its quantitative analysis as reported by Bolarinwa *et al*., (2014). This trend can be rationalised by the unavailability of suitable Abs to perform the immunoassay, largely due to the difficulty in designing and producing a stable hapten-conjugate (immunogen). Enzyme-linked immunosorbent assay (ELISA) would fulfil this entire requirement in addition to its high throughput and ease of use. The successful production of pAbs in chapter 3 would allow the ELISA to be optimised and characterized for the detection and quantification of linamarin in food samples.

Direct and indirect analysis of CGs in general and linamarin in particular have been discussed in section 1.3.9. From the literature, there have been few studies conducted utilising the acid hydrolysis of the CGs and indirect semiquantitative picrate paper method detection of HCN developed by Egan *et al*., (1998) and

Bradbury *et al*. (1999) for total cyanide content analysis in fresh cassava and food products. Cardoso *et al*. (1998) conducted the first field trial of the picrate paper kit on cassava flour in Mozambique followed by Ernesto *et al*. (1999) where they analysed the cyanogen content in the roots and also the thiocyanate content in human urine. Djazuli and Bradbury (1999) conducted cyanogen content analysis of cassava roots and flour in Indonesia using both acid hydrolysis and picrate kit method. These indirect methods measured the total amount of HCN in samples that require samples to be processed Cyanogen content of cassava root of 179 cultivars grown in Indonesia were investigated and they found out only 40% of 179 cultivars grown in Indonesia is safe for consumption using the safe regulatory limit of 40 mg/kg body weight is used (set by Ministry of Industry and Trade of Indonesia). Adindu *et al.* (2003) investigated the cyanogen contents in ready-to-eat food products from cassava such as gari (20~30 mg/kg fresh weight), fufu (10~30 mg/kg fresh weight) and tapioca (10~20 mg/kg fresh weight) and found out the mean total cyanogen contents were all higher than 10 mg/kg fresh weight). Burns *et al*. (2012) conducted the analysis in Australia and found an alarming level of total cyanide concentration (262 mg/kg dry weight) in one of the cassava chips product. It will only take about 40-270 g (approximately a packet of crisps) of these crisps to reach the lethal dose for 20 kg child according to the 10 mg/kg body weight regulatory limit introduced by FSANZ in 2009. In New Zealand, Cressey *et al*. (2013) conducted analysis on all plant-based foods that contain CGs and they detected total hydrocyanic acid in all samples of cassava root (whole or grated) but not in any further processed products (flour, tapioca). Yeoh and Sun (2001) introduced an enzyme-dipstick technique based on the picrate method, and assessed 35-cassava based foods available in Singapore for residual cyanogen content. They ranged from 2 – 88 mg HCN

equivalent/kg. In the UK, studies on cyanogen contents (amygdalin) in food (fruit kernels ie apricot, cherry, peach, apple, apple juice, almonds) were conducted by Bolarinwa *et al*. (2014) using immunoassay and HPLC and traces of cyanogens were detected but unlikely to pose health problems to consumers. There is no report on cyanogen contents of fresh cassava and food products available in the UK market at the moment. UK only imported about 2,500 tonnes cassava and cassava products per year according to FAO, mainly to cater niche population of Black British people of African and Carribean descent and British Asian communities which consume cassava occasionally as part of special dishes or delicacies. These group of ethnicities would be the most at risk of getting intoxicated if certain imported batches of fresh cassava were of the bitter variant and the cassava products, post processing, still contain high traces of cyanogens. All cassava products that were bought in this study have no warning regarding possible risk of cyanogen traces and let alone handling and preparatory instructions. The availability of such data would be helpful in assessing the need for better monitoring, labelling and public awareness of the potential health issue this residual cyanogens in foods could pose, not only in those containing linamarin in particular but all plant-based foods containing CGs.

One of the challenges in using ELISA for detection of analyte in tissue sample is the sample preparation. Sample preparation for fresh cassava products requires careful planning due to the presence of the endogenous linamarase enzyme, which could be released due to mechanical damage to the tissue caused by processing or preparation prompting rapid degradation of linamarin to HCN. Sample preparation is also important to reduce the matrix effect in an ELISA. The sample preparation and extraction of linamarin from fresh cassava has been

studied previously. For example, extraction of linamarin using ethanol or methanol with the inactivation of endogenous linamarase enzyme by boiling or enzyme precipitation was investigated by Zitnak *et al*. (1977). The use of orthophosphoric acid during diced (1cm cubes) cassava parenchymal tissue (30-100g) homogenization was reported by Cooke (1978). The use of boiling 80% ethanol together with solvent:sample ratio of 10 to 1 v/w treated with blender for 2 mins, filtered and concentrated seems pretty much straight forward and simple (Brimer *et al*., 1983). King and Bradbury (1995) investigated the use of methanol during extraction for preparative HPLC and a mixture of hydrochloric acid (HCl) and methanol during extraction for analytical HPLC. Saka *et al*. (1978) and Linley *et al*. (2004) homogenized and extracted the linamarin using orthophosphoric acid. Sornyotha *et al*. (2007) investigated the use of different acids (HCl, H_2SO_4, H_3PO_4 or CH_3COOH), using a modified version of the method of Cooke (1978). Tivana *et al*. (2014) reported the use of phosphoric acid and water for extraction of linamarin in fresh and processed cassava products. Most of the extraction methods mentioned were necessary to i) inactivate the endogenous linamarase enzyme, ii) maintain the acidic pH of 4~6 to prevent dissociation of cyanohydrins to acetone cyanohydrin and HCN, iii) use the extract for total cyanogens assessment (free cyanide HCN/CN^-, acetone cyanohydrin and the glucosides, linamarin and lotaustralin). Considering the exhaustiveness of methods to extract and analyse cyanogens content in cassava from the literature, it was really surprising that ELISA was not one of them. Hence, methodology for sample preparation to extract linamarin from plant tissue to be assessed using ELISA was also lacking. This will be further discussed in the chapter.

5.1.1 Aim of the chapter

The main aim of this chapter is to use the pAbs developed described previously in chapter 3 to detect and quantify the amount of residual linamarin present in fresh cassava and food products available in the UK. In order to do so, the most optimal way of sample preparation from food sample is imperative and this has been compared to various extraction and purification methods for cyanide quantification in various food products that have been developed and improvised throughout the years.

This chapter describes the experimental approach and results towards the following area:

i) Optimisation of the indirect competitive ELISA for the determination of linamarin in fresh and processed cassava products.

ii) Optimal sample preparation for linamarin assessment using ELISA.

iii) Determination of linamarin in fresh and processed cassava products available in the UK.

5.2 Methods

5.2.1 Linamarin extraction from fresh cassava and food products

5.2.1.1 Extraction of linamarin using ethanol

The ethanol extraction method was adapted and modified from Brimer *et al*. (1983), Ruengpravut and Chulavatnol (1999) and Bolarinwa *et al*. (2014). Diced (1 cm x 1 cm) raw frozen cassava root (20 g) were immediately homogenized with chilled ethanol (kept in -20°C freezer overnight, 80 ml) for 15 s at low speed, followed by 1 min (2 times) at highest speed in a food processing blender. The homogenate was filtered through Whatman No. 1 filter paper into a small sized beaker (250 ml), placed in bucket of ice to maintain low working temperature and any residues remaining in the blender jar were rinsed off using more chilled ethanol (20 ml). The filtrate was then transferred into two (50 ml) conical tubes and placed in the ice bucket while waiting for other extraction to be completed. The filtrates were then centrifuged at 5,000 rpm / 4,696 x g rcf for 20 min at 4^{o}C. The supernatants were then transferred with care into a new conical tube avoiding agitation of the pellet. Ethanol was completely evaporated from the supernatants using a rotary evaporator (40 minutes, 80 mbar, 50°C, 75 rpm). Once the solvent has completely evaporated, linamarin was dissolved in water (100 ml). The sample was then transferred into smaller tubes for storage in -20°C until required. A similar method was used for linamarin extraction from cassava products.

5.2.1.2 Extraction of commercially available cassava products

Cassava products are not readily available in supermarkets or chain stores within the UK such as Morrisons, Tesco, Asda, Sainsbury or Aldi to name a few. However it could be purchased from local groceries stores specialized in international foods to serve certain ethnic or cultural backgrounds. Fresh and processed food samples used in this study were purchased from local supermarkets in Leeds, UK as mentioned previously and online during the period of June 2017 to December 2018 as listed in Table 5-1. A total of 5 local supermarkets (Morrisons, Asda, Tesco, Sainsbury, Iceland) and 2 local groceries shops (Continental and Sing Kee) were visited. Of those, only the 2 local groceries (Continental and Sing Kee) sold cassava products. Only 1 online retailer (Tropical Sun Foods) was found to deliver cassava products directly to consumers. All available cassava products from these three retailers were purchased. Items were mostly bought only once to have a snap-shot picture (overview) of the presence of linamarin in products sold in Leeds. All the fresh cassava parts and wet products were stored at -20°C prior to extraction and analysis.

Table 5-1: Commercially available raw cassava and processed cassava products in the UK market. These products were bought from Sing Kee Chinese Supermarket, Leeds, UK and CC Continental Supermarket, Leeds, UK.

No	Product	Product description	Place of purchase	Brand / Origin	Weight (g)
1.		Fresh cassava root	Continental Supermarket, Brudenell Grove, Leeds, UK	Class 2, Costa Rica	1,000
2.		Cassava leaves (grounded/frozen)	Continental Supermarket, Brudenell Grove, Leeds, UK	Ades, Cameroon	500
3.		Cassava chunks (frozen)	Continental Supermarket, Brudenell Grove, Leeds, UK	Daily Delight, India	900
4.		Cassava slices/diced (frozen)	Continental Supermarket, Brudenell Grove, Leeds, UK	Daily Delight, India	900
5.		Cassava dough (fermented)	Continental Supermarket, Brudenell Grove, Leeds, UK	Ghana	2,000
6.		Pure tapioca flour	Sing Kee Supermarket, Cross Stamford Street, Leeds, UK	UP, Thailand	455
7.		Black tapioca pearls	Sing Kee Supermarket, Cross Stamford Street, Leeds, UK	WuFuYuan, China	250

8.		Green tapioca pearls	Sing Kee Supermarket, Cross Stamford Street, Leeds, UK	AAAAA, Thailand	250
9.		White tapioca pearls	Sing Kee Supermarket, Cross Stamford Street, Leeds, UK	AAAAA, Thailand	250
10.		Cassava flour	Tropical Sun Foods (https://tropicalsunfoods.com/)	Tropical Sun, Jamaica	1,000
11.		Cassava chips (barbeque)	Tropical Sun Foods (https://tropicalsunfoods.com/)	Tropical Sun, Jamaica	80
12.		Cassava chips (lightly salted)	Tropical Sun Foods (https://tropicalsunfoods.com/)	Tropical Sun, Jamaica	80
13.		Gari Ijebu	Tropical Sun Foods (https://tropicalsunfoods.com/)	Tropical Sun, Jamaica	500
14.		Gari white	Tropical Sun Foods (https://tropicalsunfoods.com/)	Tropical Sun, Jamaica	500
15.		Gari yellow	Tropical Sun Foods (https://tropicalsunfoods.com/)	Tropical Sun, Jamaica	500

5.2.2 Optimization of the indirect competitive ELISA (icELISA) method

Ab dilution, incubation time, coating Ag concentration, and blocking buffers are factors that could be optimized to influence the sensitivity of the assay. Evaluation of the optimisation process was based on the IC_{50} value and the coefficient of the linear equation (R^2). From previous studies with amygdalin, an overnight incubation at 4°C of the primary Ab and Ag standard gave the lowest IC_{50} and higher R^2 values reflecting a higher sensitivity assay (Bolarinwa *et al.,* 2014). Lower IC_{50} values indicate better ELISA sensitivity. The same incubation time and temperature were implemented for subsequent assays. Coating Ag concentration of 1.0 µg/ml was used throughout the assay development. Previous studies that worked on Ab development against hapten suggested that the use of 1% dried skimmed milk as blocking buffer was better compared to other buffers. However, for this investigation, these studies were repeated using 1% BSA, 1% gelatine, 1% skimmed-milk and 1% soy as the blocking buffers. The use of a non-ionic detergent such as Tween 20 (0.05%, v/v), when used in conjunction with a protein blocker, provides added convenient and inexpensive blocking ability during wash steps by blocking areas on the surface that may become exposed due to protein/biomolecule desorption.

5.2.2.1 Cross-reaction determination

Cross-reaction determinations were carried out by incubating a range of concentrations of closely related CGs with the polyclonal anti-linamarin Ab (1:50,000 dilution) and comparing the response with that of linamarin. The cross reactivity of the Ab with some structurally-related CGs such as prunasin, dhurrin,

mandelonitrile and amygdalin was determined by icELISA. Cross-reactions of the pAb were determined using:

%Cross-reactivity (CR) = (IC_{50} of linamarin/IC_{50} of analogue) x 100

5.2.2.2 Determination of matrix effect

Non-specific interactions between Ab and the microtitration plate, proteins and other components in the sample, often known as matrix effects, can result in erroneous readings. The effect that other substances in the sample might have on the ability to detect the specific target material, are most commonly observed when using plasma and serum samples. Matrix components can affect the binding of antibody to protein or alter the signal-to-noise ratio hence it is important to investigate this effect prior to conducting assay on unknown samples. The determination of matrix effects was conducted by plotting normal standard curves (standards dissolved in assay buffer and standards dissolved in cassava extracts). The cassava extracts (containing non-detectable linamarin) were diluted in assay buffer (1:5 – 1:100, v:v, PBST) and used for the preparation of linamarin standard.

5.2.2.3 Determination of linamarin in food products

The extracted samples analysed by ELISA were diluted in assay buffer (1:10, v/v). Standard curves were performed with every analysis. Every ELISA plate had triplicate of negative control (secondary antibody control i.e primary antibody + analyte + no secondary antibody), triplicate of positive control (primary antibody + buffer (minus the analyte) + secondary antibody) and triplicate of

blank (no primary antibody + buffer (minus the analyte) + no secondary antibody). Unknown concentrations were determined by using the equation from the four parameter logistic regression (4PL) curve. The similar pre-treated samples were also tested using MColortest^{TM} Cyanide Test Kit from Merck for comparison purposes.

5.3 Results

5.3.1 Linamarin extraction from fresh cassava and food products

Three key areas were addressed prior to linamarin extraction from fresh cassava products: i) pre-processing storage condition, ii) suitability of the sample to be processed and iii) condition of the processing area. Fresh cassava tuber that had been bought fresh from the grocery shop was wrapped in a film and kept under separate storage conditions i) -86°C, ii) -20°C and iii) 4°C for a period of two weeks. The cassava tuber stored at -86°C had become rock solid and it was quite impossible to cut using a knife. The tuber had to be cut using a metal saw instead and the cross section of the tuber can be seen in Figure 5-1. The time taken to dice the sample into (1 cm x 1 cm) size was well over 5 mins and giving the sample ample time to thaw.

Figure 5-1: Fresh cassava tuber stored at -86°C. The tuber is frozen solid and difficult to cut normally using knife. Metal saw had to be used instead.

The cassava tuber stored in -20°C however, was much softer than the one stored in -86°C (Figure 5-2). Cutting it with a knife needed only a little bit of effort and

the metal saw easily sliced through the tuber. The time taken to dice this sample into (1 cm x 1 cm) size took about 2 mins.

Figure 5-2: Fresh cassava tuber stored in -20°C freezer.

The cassava tuber stored in 4°C refrigerator was not doing so well compared to the other two storage conditions (Figure 4-3). Although the tuber could be cut and dice easily but black spots were spotted within the root parenchyma indicating signs of PPD due to presence of hydroxycoumarins release due to stress conditions.

Figure 5-3: Fresh cassava tuber stored at 4°C refrigerator.

The processing area was initially to be conducted in a cold room in order to minimise enzyme activation but due to some technical difficulties, the processing was conducted on normal food preparation bench at room temperature.

Extraction of the fresh cassava samples were conducted using chilled ethanol stored in -20°C. After homogenization, sample filtration was done as depicted in Figure 5-4. All filtrates were collected and stored temporarily in a bucket of ice to maintain low working temperature in order to minimise linamarin degradation by the linamarase enzyme.

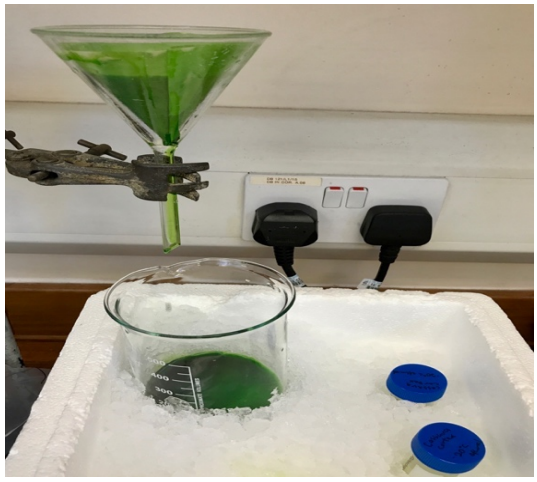
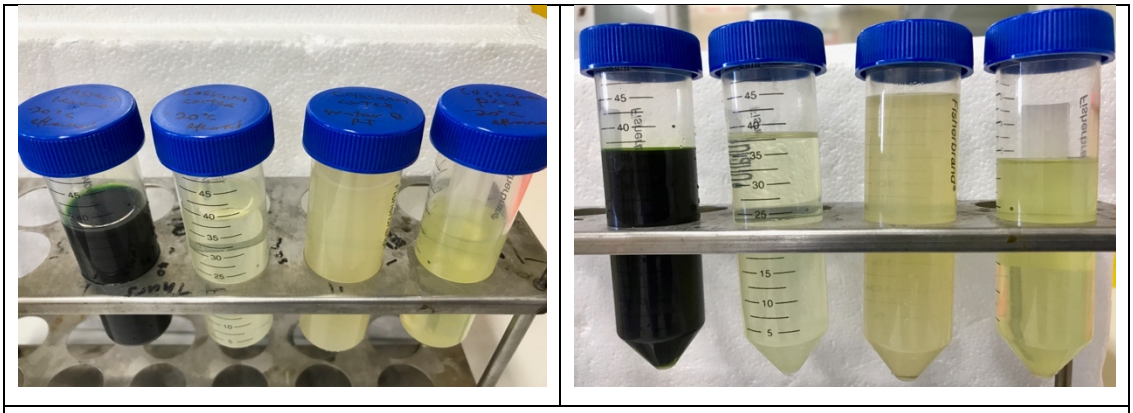


Figure 5-4: Extraction setup for linamarin from fresh cassava tuber and leaves. A bucket of ice was used to maintain low working temperature.

Homogenates residue and supernatants from ethanol extraction are shown in Figure 5-5 and 5-6. Cassava cortex residue which contain only the root parenchyma appeared to be whitish, peel residue was brownish due to the colour of the peel and cassava leaves was greenish due to the presence of chlorophyll.

a. Cassava cortex	b. Cassava peel	c. Cassava leaves

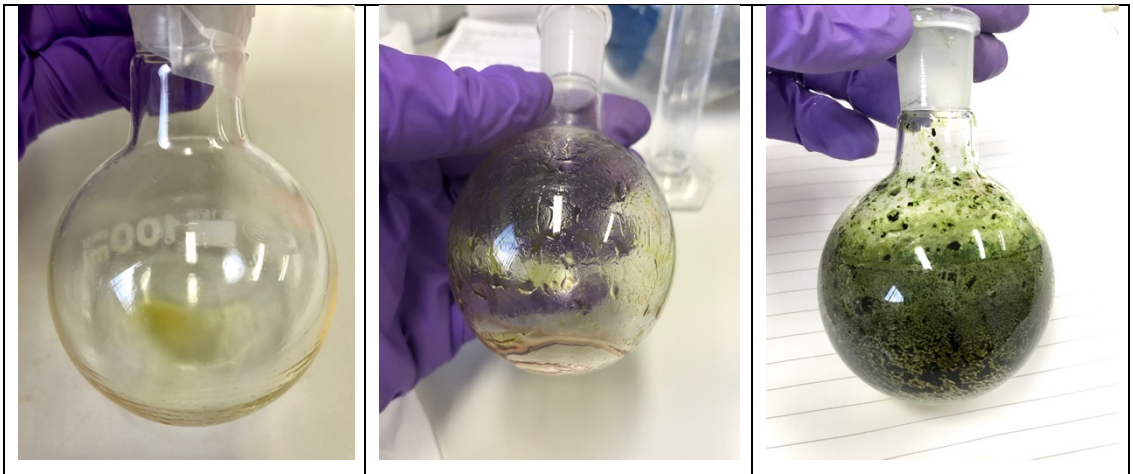
Figure 5-5: Sample homogenates residue left after filtration. a) cassava cortex, b) cassava peel and c) cassava leaves homogenised in chilled ethanol.



Leaves, tuber, tuber and peel, peel

Figure 5-6: Supernatants collected after centrifugation. From left a) cassava leaves, b) cassava tuber, c) cassava tuber and peel and d) cassava peel homogenised in chilled ethanol.

Ethanol from the filtrates were removed using the rotary evaporator and resulted in dried residue that can be seen adhered to the glass surface of the flask (Figure 5-7). The residue was dissolved in water (100ml). The sample was then transferred into smaller tubes for storage in -20°C until required. The same procedure was used for sample preparation on processed cassava products both in wet and dry forms.



a. Cassava cortex	b. Cassava peel	c. Cassava leaves

Figure 5-7: Sample filtrates after ethanol had been completely evaporated. Dried residue can be seen adhered to the glass surface of the flask.

5.3.2 ELISA optimisation

Most of the ELISA optimisation have been carried out in chapter 3. The optimised conditions include: i) primary Ab dilution (1 in 50,000 dilution), ii) secondary Ab manufacturer (CovalAb UK Ltd.), iii) type of TMB substrate (TMB1, refer section 3.3.5) and iv) blocking buffers (dried skimmed-milk) were chosen for subsequent icELISA assessment. Other conditions such as i) incubation time of antigen coating buffer (overnight at 4°C), primary Ab (overnight at 4°C for non-competitive ELISA, 2 hours at 37°C for competitive ELISA) and secondary antibody (2 hours at 37°C), ii) substrate incubation (30 mins) and iii) concentration of coating antigen (1.0 μg/ml) were based on work done by Bolarinwa and Al-Maghrabi (personal communication) where both have assessed and evaluated the conditions and suggested accordingly. All the conditions investigated for optimization were used to generate an icELISA standard curve against linamarin using polyclonal anti-linamarin Ab produced from CC conjugate at dilution of 1:50,000 (v/v). Mean values from 12 different standard curves were used to construct a standard curve using four parameter logistic analysis (4PL) provided by AAT Bioquest (2019) (Figure 5-8) where the value of the curve parameters is shown in the form of 4PL equation as follows:

$$y=\text{Bottom}+\frac{(\text{Top}-\text{Bottom})}{1+10^{(\text{LogEC}_{50}-x)*\text{Hillslope}}}$$

Where y is the absorbance obtained (nm), x is the concentration of the linamarin (log unit), (top) and (bottom) are the maximum and minimum responses (nm), respectively, Log EC50 is the value that produces a 50% signal response and Hillslope is the slope-like parameter (Hill coefficient).

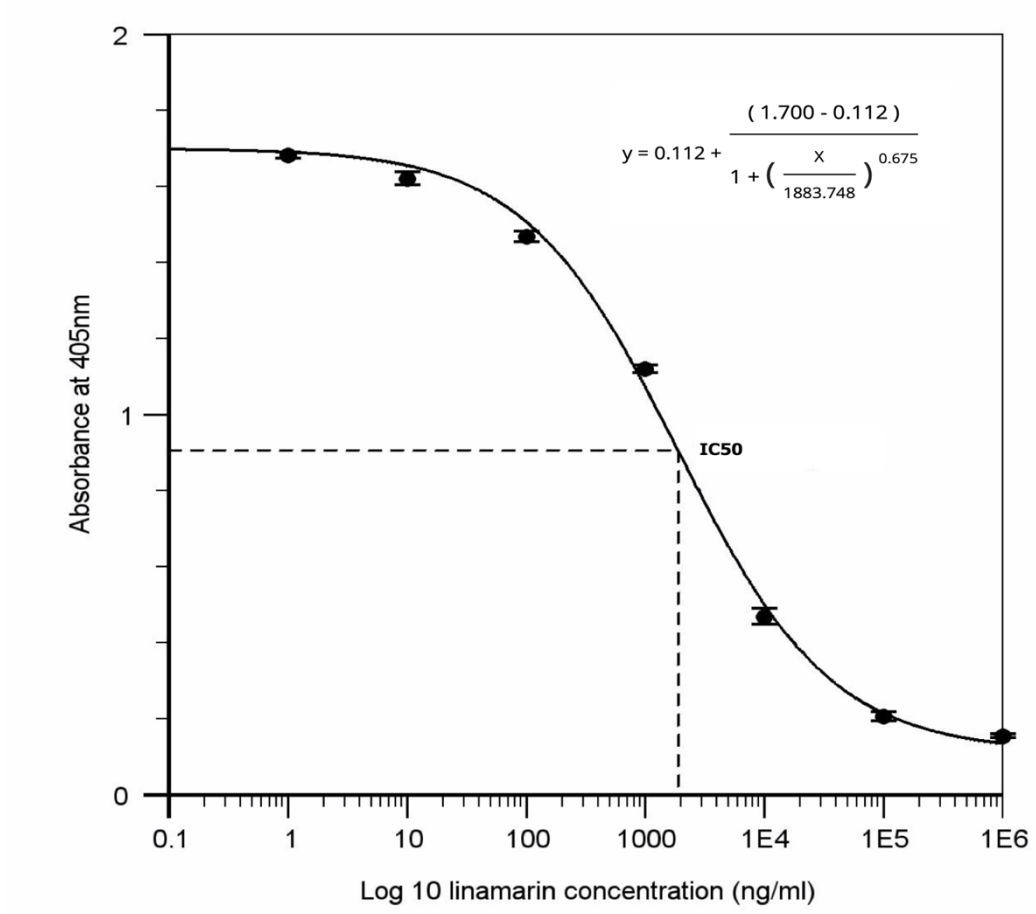


Figure 5-8: Optimised standard curve for the indirect competitive ELISA for determination of linamarin. The equation of the line is for concentrations which are higher than 1.5 ng/ml. Error bars indicate the standard deviations of 12 different curves.

The optimized icELISA standard curve obtained was characterised based on IC_{50}, limit of detection (LoD) and working range of the assay. The 50% inhibition (IC_{50}) of the standard curve was estimated to be 2,100 ng/ml (2.1 μg/ml) The LoD represent the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The LoD was calculated based on the value of three times the standard deviation of the blank value or the lowest concentration of linamarin utilized. The limit of quantification (LoQ) was calculated based on the value of ten times the standard deviation of the blank value. These values were then interpolated from the sigmoidal dose-response 4PL equation, and the corresponding concentration of linamarin value was then

calculated including the confidence interval. The LoD, was estimated to be 1.5 ng/ml (0.0015 μg/ml) and the working concentration range of the assay, which was defined as the concentration that corresponds to 20% inhibition (IC_{20}) and 80% inhibition (IC_{80}) was determined to be 215 ng/ml (0.215 μg/ml) and 24,500 ng/ml (24.5 μg/ml) respectively.

5.3.3 Cross-reaction of the polyclonal anti-linamarin antibody

The reactivity of the obtained antiserum towards other structurally related cyanogenic glycosides to linamarin was investigated to determine the specificity of the antibody. Figure 5-9 shows the icELISA results of linamarin and the other analogues. Table 5-2 shows the data for linamarin and the analogues including their chemical structures with the IC_{50} values and percentage of cross-reactivity (CR) against linamarin.

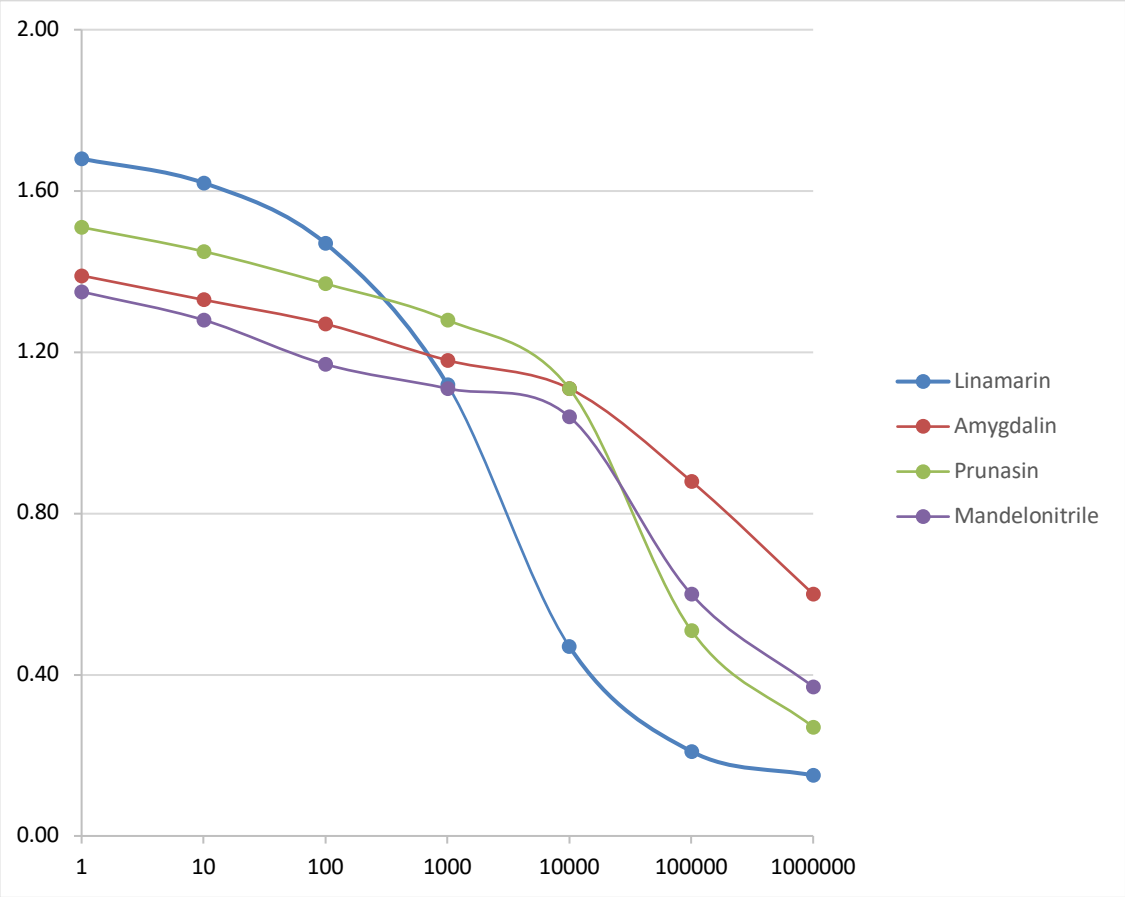
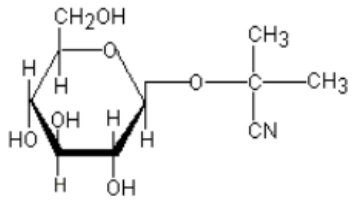
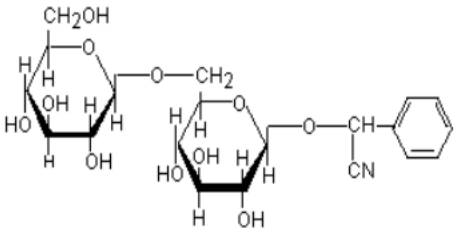
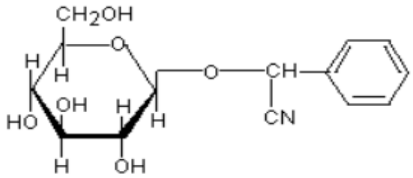
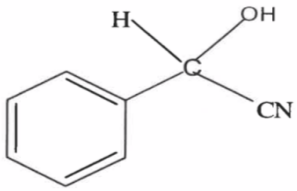


Figure 5-9: Competitive ELISA standard curve of linamarin obtained from antibody generated from the cyanuric chloride conjugate, using 1% dried skimmed milk as blocking buffer.

†The antibody dilution was 1:50,000 and the solid phase antigen was linamarin-KLH (1µg/ml) from the cyanuric chloride method with milk blocking, overnight primary reaction incubation at 4°C, secondary reaction for 2 hours at 37°C and 30 min enzyme-substrate reaction. Error bars represent standard deviation of triplicate analyses.

Table 5-2: Cross-reaction data for linamarin and three other structurally related cyanogenic glycosides.

Compound	Chemical structure	IC_{50} (μg/ml)	CR (%)
Linamarin		2.05	100
Amygdalin		42.74	4.80
Prunasin		31.67	6.48
Mandelonitrile		32.06	6.40

IC50 = Concentration of the analyte required for 50% inhibition of antibody binding to the coating antigen.

From the results, it can be seen that the antiserum (R076D88) had poor CR with all of the three cyanogenic glycosides tested as the percentage of CR is well below 10%. There is close similarities of CR values between amygdalin, prunasin and mandelonitrile (4.80%, 6.48% and 6.40%).

5.3.4 Matrix effect

Cassava flour extract containing undetectable levels of linamarin was diluted in assay buffer (1:5, 1:10 and 1:15, v/v) and was then used for preparation of linamarin standard. As can be seen from Figure 5-10, the linamarin standard dissolved in the cassava flour extract was superimposed with linamarin standard dissolved in assay buffer, which indicates the absence of matrix effect in the ELISA assay. Dilution factor of 5 prior to ELISA is adequate to give similar results to standard assay buffer and dilution factor of 10 and 15.

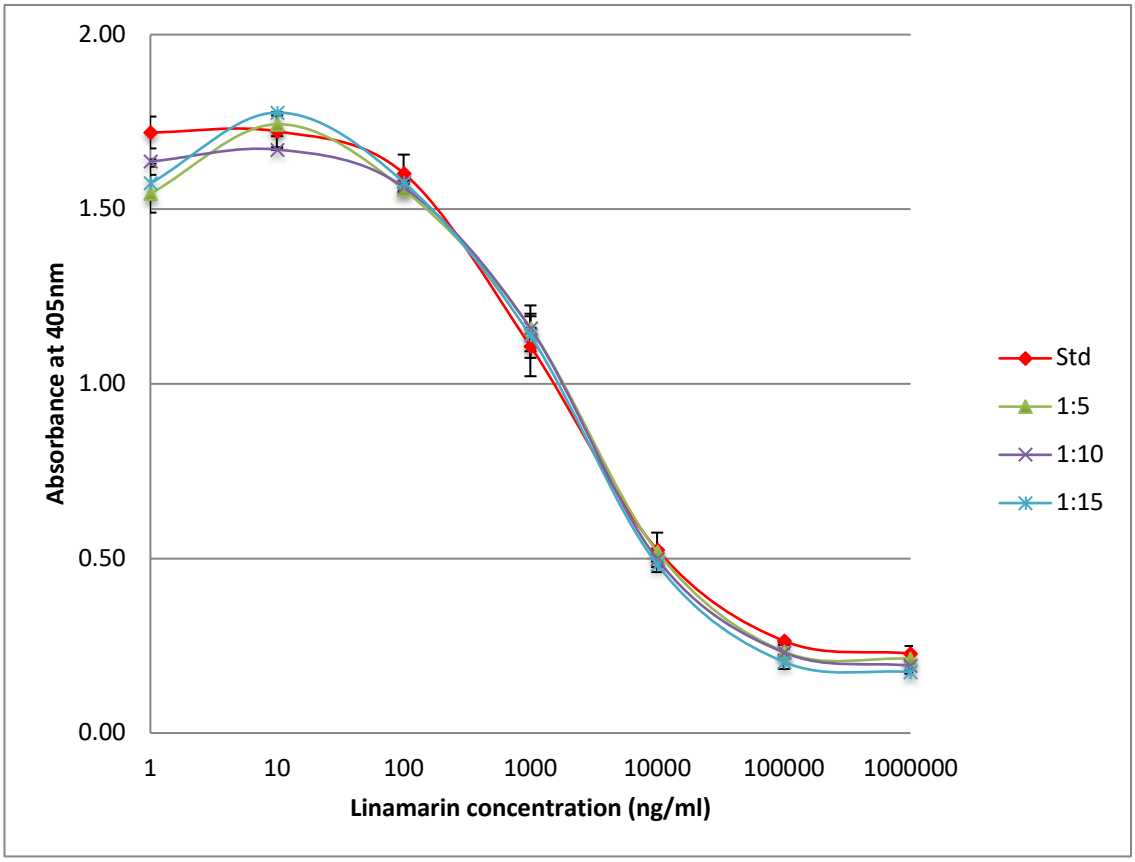


Figure 5-10: ELISA standard curve obtained by preparing linamarin in assay buffer and cassava flour extract diluted in assay buffer, i) Std – standard dissolved in assay buffer, ii) 1:5 – standard dissolved in cassava flour extract at 1:5 dilution, v/v, iii) 1:10 - standard dissolved in cassava flour extract at 1:10 dilution, v/v, iv) standard dissolved in cassava flour extract at 1:15 dilution, v/v. Error bars indicate the standard deviations of triplicate analyses.

The linearity (R2 = 0.9719) of the ELISA assay response with tapioca flour extract also confirms the absence of matrix effect in the assay (Figure 5-11).

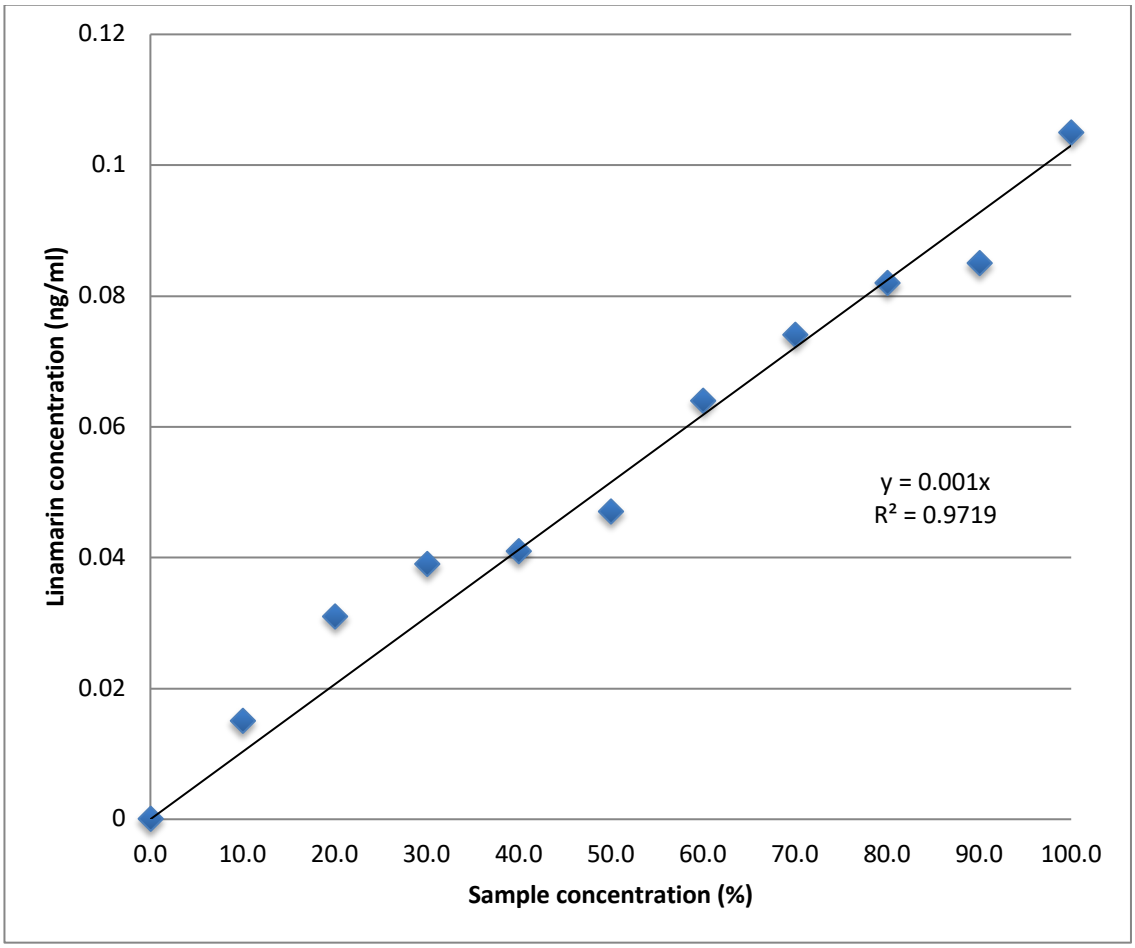


Figure 5-11: Linearity of the ELISA assay response with cassava flour extract. Error bars indicate standard deviations of triplicate analyses.

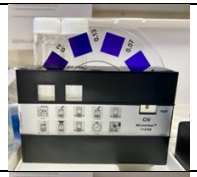
5.3.5 Determination of linamarin in food products

The results obtained from the analysis of a variety of food products using the optimised ELISA method are shown in Table 5-3. As a comparison, a cyanide test kit that measures the amount of free cyanide ions in the sample were also performed.

Table 5-3: Analysis of linamarin on fresh cassava and processed products (mg/kg fresh weight)

No	Sample		ELISA	MColortest™ Cyanide Test from Merck	
	Fresh cassava parts		Linamarin (mg/kg) fresh weight (wet product) dry weight (for dry product)		Free cyanide ions (mg/l CN^-)
1.		Fresh cassava root (stored in -20 °C)	43.08 ± 0.04		0.03
2.		Cassava leaves (grounded/frozen)	30.78 ± 0.04		0.3
3.		Cassava cortex (stored in -86°C)	14.89 ± 0.06		0.03
4.		Cassava peel	13.17 ± 0.02		0

	Processed cassava products				
5.		Cassava chunks (frozen)	14.51 ± 0.11		0
6.		Cassava slices/diced (frozen)	9.22 ± 0.13		0
7.		Cassava dough (fermented)	2.03 ± 0.22		0
8.		Pure tapioca flour	0.003 ± 0.001	<0.1	-
9.		Black tapioca pearls	0.036 ± 0.020	<0.1	-
10.		Green tapioca pearls	0.008 ± 0.003	<0.1	-
11.		White tapioca pearls	0.023 ± 0.015	<0.1	-

12.		Cassava flour	0.033 ± 0.014	<0.1	-
13.		Cassava chips (barbeque)	0.455 ± 0.18		0
14.		Cassava chips (lightly salted)	0.449 ± 0.20		0
15.		Gari Ijebu	0.270 ± 0.11		0
16.		Gari white	0.425 ± 0.6		0
17.		Gari yellow	0.282 ± 0.10		0

Each value is expressed as mean ± standard deviation (n = 6 determinants).
Samples with linamarin content of lower than 0.1 mg/kg were not tested.

From the results, the amount of linamarin detected in the cassava cortex (stored in -20°C) is the highest followed by cassava leaves, cassava cortex (stored in -86°C) and cassava peel. For the processed cassava products, the frozen cassava chunks and pieces still contain residual linamarin and for the rest of the dried products, the amount detected were well below 1.0 mg/kg dry weight. The results obtained from the cyanide test kit were negative for all the dried products and some free cyanide ions were detected in most of the fresh cassava samples.

5.4 Discussion

Successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Properties of a good solvent in plant extraction includes, low toxicity, ease of evaporation at low heat, promotion of rapid physiological absorption of the extract, preservative action, inability to cause the extract to complex or dissociate. The factors affecting the choice of solvent are quantity of target analytes to be extracted, rate of extraction, diversity of different compounds extracted, diversity of inhibitory compounds extracted, ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process and potential health hazard of the extractants, not to mention what is attended with the extract (Eloff, 1998). It is also important to take into account of the fact that the end product of the sample preparation will contain traces of residual solvent, hence the solvent should be non-toxic and should not interfere with the bioassay.

Linamarin is a hydrophilic compound and therefore suitable to be extracted using a non-polar solvent. Acetone, ethanol and methanol are all excellent solvents widely used for extraction of many hydrophilic and lipophilic components from plants especially tannins, phenolic compounds and other antioxidant compounds (Tiwari *et al*., 2011). Apart from that they are also more efficient in cell walls and seeds degradation which have non-polar character and facilitate the release of more compounds from cells and more importantly is the inactivation of the endogenous linamarase enzyme preventing the degradation of linamarin compound. Additionally, ethanol was found easier to penetrate the cellular membrane to extract the intracellular ingredients from the plant material (Wang,

2010). Polarity of ethanol can be easily manipulated by adding water v/v (10% water to 90% ethanol) to improve its extractant capability on certain compounds such as flavonoids. Methanol on the other hand is much more polar than ethanol but due to its cytotoxic nature, it is unsuitable for extraction in certain kind of studies as it may lead to incorrect readings and results (Cowan, 1999). With all the above in mind, supported by the findings of Brimer *et al*. (1983), Ruengpravut and Chulavatnol (1999) and Bolarinwa *et al*. (2014), chilled ethanol was opted as the solvent to extract linamarin from the cassava and food products. The amount of solvent to sample ratio used in this study was in agreement with previous reports that suggested ideal solvent to weight ratio of 10:1 (v/w) (Das *et al*., 2010).

In terms of recovery, analytical recovery refers to the amount of a target analysed relative to the 'true' amount present, usually expressed as a percentage. It is often difficult to recover 100% of a target analyte, particularly for a solid sample. Firstly, of course, there are losses during extraction; no process will be 100% efficient. Secondly, in real samples, low molecular weight analytes are often intimately embedded in the matrix. Thus, such analytes may be bound to insoluble structures or entrapped in networks of, for example, plant cell wall material. The usual way of calculating recovery is to 'spike' the sample with a known amount of the analyte dissolved in a volatile solvent. The solvent is allowed to evaporate, and the sample extracted and analysed. The amount of spike recovered relative to the amount added gives the recovery.

Of course, such experiments can never provide the recovery of the unknown, real analyte present, for the reasons described above. ‘Spiking’ will always give an over-estimate of true recovery, often a considerable over-estimate and therefore false re-assurance. Consistency of recovery (backed by assay validation) is a far more important measure. In the present example, the assay has been validated and consistency has been shown in several ways, but in particular by the data provided by the superimposability and linearity studies (see Figures 5-10 and 5-11).

Based on the biological nature of cyanogenic glycosides, recovery is unlikely to be a problem due to its solubility and lack of binding to matrix (since plants use glycosides as the means of solubilising and transporting materials around the organism. The biggest problem is that as soon as the plant material (solid, of course) is prepared for extraction by cutting, chopping, homogenising, etc, then the target analyte will start to degrade by enzymic action. This is, of course, why there has been a lack of quantitative methods of analysis for cyanogenic glycosides in the literature especially for linamarin (see section 1.3.9). Consequently, considerable efforts have been made in the present study to investigate sample extraction methods in order to maximise recovery and minimise losses. The results (see Figures 5-2 and 5-4) show that the optimal extraction procedure is as follows.

(i) Freeze the sample at -20ºC.

(ii) As soon as practically possible, homogenise the sample at 0ºC and extract with ethanol, also at 0ºC.

(iii) Any work prior to evaporation of the extractant requires working within the ice container to maintain low surrounding temperature.

Improvement of the ELISA method in this study was based on investigating the effect of varying some factors that were not usual in immunoassay optimisation (choice of substrate and detection antibody) to improve the signal to noise ratio (chapter 3). The results obtained from this study showed that the best assay sensitivity was observed with these sets of conditions: i) solid phase antigen concentration, 1.0 μg/ml; ii) blocking buffer, 1% dried skimmed milk-PBST; iii) detection antibody manufacturer and dilution, CovalAb UK Ltd. and 1:1,000; iv) incubation time of antigen coating, overnight at 4°C; v) incubation time of primary reaction, overnight at 4°C; vi) incubation time of secondary reaction, 2 hours at 37°C; vii) substrate reaction time, 30 mins. The standard curve for linamarin detection and quantification in cassava products was constructed using four parameter logistic (4PL) regression analysis software to provide more accurate assessment of the assay. This is true for any bioassays that depends on dose response or receptor-ligand binding assays that often follow a sigmoidal, or “s” shaped curve. This type of curve is particularly useful for characterizing ELISA because the results tend to be linear across a specific concentration magnitudes and beyond this linear range, the response quickly plateau and approach the minimum and maximum, one of the reason linear regression model is not preferable when dealing with this type of assay.

As this is the first time a pAb had been successfully generated against linamarin, reports of similar study were not available for comparison. Previous studies on

the use of antibodies and ELISA for detection and quantification of cyanogenic glycosides are also limited. The first study on successful production of pAbs to cyanogenic glycosides was reported by Cho *et al*. (2006) on amygdalin, raised against hapten-conjugate made using succinic anhydride to amygdalin in pyridine solution. Subsequent work by the same author and co-workers produced a recombinant antibody for the same analyte. Bolarinwa *et al*. (2014) reported a much simpler conjugation method utilising cyanuric chloride as the bridging molecule between the hapten and protein carrier and was able to produce highly sensitive working ELISA. Therefore, results from these three reports were used for comparison with the results from this study specifically on the limit of detection (LoD) and limit of quantification (LoQ). LoD is the lowest analyte concentration likely to be reliably distinguished from the blank and at which detection is feasible. LoQ is the lowest concetration at which the analyte can not only be reliably detected but at which some predefined goals for bias and imprecision are met. In other words, LoD values should be higher than the blank and LoQ values maybe equivalent to the LoD or it could be at a much higher concentration. These values are important for tests used to discriminate between the presence or absence of an analyte and to reliably measure low levels of analyte. LoD however is not to be confused with the assay sensitivity which is represented by the slope of the calibration curve. Cho *et al*. (2006) reported a detection limit of 0.1 μl/ml for the amygdalin ELISA method that employed pAb and detection limit of 0.5 ng/ml obtained from recombinant antibody. The LOD from this study (1.5 ng/ml) was more sensitive than the pAb amygdalin assay (0.1 μl/ml) (Cho et al., 2006) and not far off as compared to the recombinant Ab amygdalin assay (0.5 ng/ml) (Cho et al., 2008). Bolarinwa *et al*. (2014) reported the LoD of the pAb amygdalin ELISA standard curve of 0.2 ng/ml and IC_{50} of 50

ng/ml, with working concentration range between 10 ng/ml (IC_{20}) and 10,000 ng/ml (IC_{80}), after further optimization and characterization. The anti-amygdalin antibody was generated from the amygdalin conjugated to BSA using the cyanuric chloride method, similarly used in this study to conjugate linamarin to BSA. Both the detection limit (1.5 ng/ml) and IC_{50} value (2,100 ng/ml) of the competitive ELISA standard curve for linamarin developed in this study was slightly higher than the LoD determined in the competitive ELISA standard curve for amygdalin developed by Bolarinwa *et al.,* 2014. The Ab dilution used in this study (1:50,000) however is much higher compared to the anti-amygdalin study (1:30,000). Comparison between these findings are shown in Table 5-4.

Table 5-4: Working assay conditions of icELISA between linamarin and amygdalin.

	LOD (μg/ml)	IC_{20} (μg/ml)	IC_{50} (μg/ml)	IC_{80} (μg/ml)	Antibody dilution (1:dilution)
Linamarin	0.0015	0.215	2.10	24.5	1:50,000
Amygdalin	0.0002	0.01	0.05	10.0	1:30,000

IC_{50} = concentration of the analyte required for 50% inhibition of the antibody binding to the coating antigen. (IC_{20} = 20% inhibition, IC_{80} = 80% inhibition)
LOD = Limit of detection is concentration that corresponded to the optical density of zero binding (i.e direct binding of the antibody to the solid phase antigen) reduced by 3 times the standard deviation of the mean zero binding.

The lower LoD reported for the anti-amygdalin Ab was perhaps due to the presence of benzene ring in the amygdalin structure and its higher molecular weight (457.431) compared to linamarin (247.247), enabling it to elicit a better immune response and to generate pAb with higher affinity and specificity. The finding corresponds to the report of Szurdoki *et al*. (1992), which showed that the rate of successful production of Abs for a hapten containing benzene ring is 1/3

while for hapten without benzene ring the success rate is 1/11. In general, polyclonal antisera are complex mixtures of a considerable number of clonal products, and as a result they are capable of binding to multiple antigenic determinants (Diano *et al*., 1998). The above examples suggest that different hapten-conjugated proteins (immunogens), with different reactive groups, modified at different sites with different steric structure exposed with the introduction of spacer arm leading to different Abs in titre, affinity and specificity as reported by Kim *et al*. (2003). Nonetheless, both results from Bolarinwa *et al*. (2014) and presented in this study further strengthened the suitability and efficiency of cyanuric chloride to be used as bridging compound for conjugation involving cyanogenic glycosides or other very small hapten.

The cross-reactions of the antibody for a group of similar compounds were investigated to determine the specificity of the antibody. The results obtained (Figure 5-9) showed that the antibody was specific for linamarin, with cross reactions of 4.80% for amygdalin, 6.48% for prunasin and 6.40% for mandelonitrile respectively. The result indicates that although these compounds share close structural similarity to linamarin, the antibody exhibited direct recognition to part of the hapten distal from the bridge attachment to the protein carrier. The distal site recognition was part of specific antibody characteristic described by Szurdoki *et al*., (1995). Cross reactivity may be attributed to absence of specific antibody binding to the structural feature common to the hapten of interest and related compounds, whether they are of close proximity or distal to the hapten site of attachment to the protein for immunogen synthesis.

The matrix effect in the ELISA assay was investigated by comparing the linamarin standard prepared in cassava flour extract to those prepared in normal assay buffer. The results obtained showed that the standard curves obtained were quite similar without any significant difference between all of them. The absence of non-specific interference was also confirmed with the linearity of the ELISA assay response when tapioca flour extract was analysed (Figure 5-10).

Application of the ELISA assay to determination of linamarin and processed food products followed the matrix effect validation procedure. The results obtained from the determination of linamarin and food products using the optimised ELISA showed an acceptable results when compared to previous findings. Study by FSANZ suggested that there is sufficient enzymatic capacity in the microflora of the coecum to completely hydrolyze large amounts of linamarin as no unchanged linamarin was excreted in the faeces following oral ingestion. As total HCN levels are more readily determined than linamarin levels, the linamarin ARfD was converted to an ARfD for total HCN measured in cassava, for analytical convenience. One mole of linamarin can release one mole of hydrocyanic acid if hydrolysis is complete and on this basis linamarin ARfD equates to an ARfD for HCN in cassava of 0.08 mg/kg body weight, the ARfD for hydrocyanic acid provided a margin of exposure of seven which, given the steep dose-response curve for HCN toxicity, was considered to be appropriate. Linamarin content is expected to be high in the root parenchyma but that is not always the case as it can varies due to changes in the environment. The linamarin levels (HCN^- mg/kg equivalent) detected in the fresh cassava parts (root cortex, 43.08, peel, 13.17 and leaves, 30.78 mg/kg fresh weight, Class 2, Costa Rica) bought in the UK exceeded the regulatory limit set by WHO and FSANZ of 10 mg/kg. Since

linamarin content in cassava is usually measured as a whole tuber, the linamarin content of unprocessed whole root (root + peel) is 56.25 mg/kg, more or less similar to value reported by Burns *et al*. (2012). The commercially processed cassava (chunks and diced, Daily Delight, India), were subjected to minimal processing (peeling, washing, cut into chunks or diced into smaller pieces, packing and freezing). These processing methods have indeed reduced the amount of linamarin considerably (chunks, 14.51, diced, 9.22 mg/kg) as compared to the unprocessed root. Most of the linamarin, having good solubility in water perhaps had been leached out from the chunks and diced cassava, notably in higher amount from the diced variant due to increase in total surface area over volume. Cassava dough contains 2.03 mg/kg amount of linamarin. This was expected due to the amount of processing involved in preparing the cassava dough and the fermentation process that might occur during storage. The rest of the highly processed products (cassava and tapioca flour) contains almost undetectable amount of linamarin, same goes to all of the tapioca pearl and gari variants. Apparently both cassava crisps (lightly salted and BBQ flavoured) have linamarin residual of less than 1.0 mg/kg dry weight. As the commercial picrate kit method used by Bradbury to measure total cyanogens in food samples was not available to be purchased, another test kit was purchased and used to measure the amount of cyanide ions present in the samples for comparison purposes. This method however lacks the enzymatic break down of linamarin to HCN/CN^- and uses the König reaction instead for colorimetric determination of cyanogens in the sample. Since most of the linamarase enzymes have been inactivated and discarded during the extraction of linamarin, there should be very little or zero amount of cyanide ions present in the samples which clearly depicted by the results in Table 5-3. Very small presence of

HCN/CN^- in wet samples were expected due to the waiting time between chopping and homogenization in the extraction steps as linamarase enzyme might have not yet been fully inactivated and removed, provided there are still residual linamarin left in the product, HCN/CN^- liberation can still occur albeit at lower rate therefore giving positive readings. No HCN/CN^- were detected in all of the dried samples whereby all of the enzymes have been inactivated and leached out during the processing. The test kit was not the best kit to validate the results of the ELISA as it measures the amount of HCN/CN^- in sample meanwhile the latter measures the amount of the linamarin. Furthermore, the amount of HCN/CN^- detected in fresh leaves sample might have been falsely represented by the colorimetric indicator. This was due to the fact that the leaves extract was the only sample that had slightly greenish colour prior to the test and it caused the colour formation to be much darker than it probably should (Figure 5-5). This comparison however, able to highlight some of the limitation of semi-quantitative measurement using colorimetric method as opposed to the direct quantification of the CGs using ELISA.

In order to fully understand the results, it is imperative to note that the toxic effect of cyanide on human depends on body size, health status, the dose of cyanide ingested and the time duration over which it is ingested. Table 5-5 shows the amount of each product required to be consumed in order to reach the lethal doses according to different body weights based on the acute lethal dose of hydrogen cyanide for human which is 0.5-3.5 mg/kg body weight based on the results in Table 5-3. These calculations, however, do not take into account the general health and nutritional status of consumer, which may alter the effective lethal limit.

Table 5-5: Minimum lethal doses of cyanide and amount of products containing cyanogen required to reach the lethal doses.

	Total linamarin content (mg/kg)	Product consumption to reach the lethal amount (kg) based on body weight					
Body weight (kg)		10	20	40	60	80	100
Lethal range of cyanide (mg/kg)	0.5-3.5	5 - 35	10 - 70	21 - 140	30 - 210	40 - 280	50 - 350
Product							
Cassava roots	43.08	0.1 - 0.8	0.2 - 1.6	0.5 - 3.2	0.7 - 4.9	0.9 - 6.5	1.2 - 8.1
Cassava peel	13.17	0.4 - 2.7	0.8 - 5.3	1.5 - 10.6	2.3 - 15.9	3.0 - 21.3	3.8 - 26.6
Cassava leaves	37.08	0.1 - 1.9	0.3 - 1.9	0.5 - 3.8	0.8 - 5.7	1.1 - 7.6	1.3 - 9.4
Frozen cassava (chunks)	14.51	0.3 - 2.4	0.7 - 4.8	1.4 - 9.6	2.1 - 14.5	2.8 - 19.3	3.4 - 24.1
Frozen cassava (diced)	9.22	0.5 - 3.8	1.1 - 7.6	2.2 - 15.2	3.3 - 22.8	4.3 - 30.4	5.4 - 38
Cassava dough	2.03	2.5 - 17.2	4.9 - 34.5	9.9 - 69	14.8 - 103.4	19.7 - 137.9	24.6 - 172.4
Cassava flour	0.033	-	-	-	-	-	-
Tapioca flour	0.003	-	-	-	-	-	-
Black tapioca pearl	0.036	-	-	-	-	-	-
Green tapioca pearl	0.008	-	-	-	-	-	-
White tapioca pearl	0.023	-	-	-	-	-	-
Gari	0.425	-	-	-	-	-	-
Ijebu gari	0.282	-	-	-	-	-	-
Yellow gari	0.270	-	-	-	-	-	-
Cassava crisps (lightly salted)	0.449	-	-	-	-	-	-
Cassava crisps (BBQ)	0.455	-	-	-	-	-	-

*Products containing less than 1.0 mg/kg cyanogen were not calculated as they are negligible.

A teenager weighing 60 kg could eat 0.9 kg of cassava root before reaching the lethal limit but a child of 20 kg would only need to eat as little as 0.2 kg of the same cassava root to reach the lethal dose. However the food samples were

only sampled once and may not be representative of the linamarin content of foods available in the UK. Another limitation was that some of the foods (e.g fresh cassava roots or products) were not cooked. Various methods are employed in different parts of the world where cassava is consumed. These methods consist of different combinations of peeling, chopping, grating, slicing, soaking, drying, frying, boiling and fermenting. In Africa, where cassava flour is a major food product, wetting is an effective method of removing cyanide (Bradbury, 2006; Cumbana *et al*., 2007). Frozen or fresh roots that are usually boiled, baked or fried before consumption, can reduce the concentration of total cyanide in these products by 10-75% (Nambisan and Sundaresan, 1985; Montagnac *et al*., 2009b), but may not reduce the concentration below the safe limit. Based on the results presented here, it is strongly advisable that the maximum 10 mg/kg safe level of cyanogens in food products to be monitored to ensure that they are safe for consumption.

5.5 Conclusion

The developed ELISA method was considerably sensitive with LoD of 1.5 ng/ml and IC_{50} of 2.1 μg/ml. Cross-reactivity of the antibody with other related cyanogenic glycosides was low (4.80-6.40%). The antibody was therefore highly specific for linamarin. No matrix interference was found in the ELISA. The amount of residual linamarin retained in fresh unprocessed cassava root and leaves as well as those frozen ready-to-use products are the biggest concern according to the results of this study as the levels had exceeded the regulatory limit of cyanide allowed in food set by WHO, FSANZ (10 mg/kg). Based on these limited results, the acute public health implications will vary according to the level of total cyanogens in the specific cassava products and the amount of the product consumed by the consumer in the UK. Committee of Toxicity UK reported that there are limited data on chronic intake of cyanogenic glycosides available in the UK to make it a concern. Perhaps, with further survey and assessment using larger samples would give a clearer view of current cyanide occurrence in cassava based products to help authority set the ARfD, regulatory and maximum limit allowed in all the cyanogenic food plants available in the UK. Consumption of such cyanide-containing products, therefore, poses a health risk, especially to consumers who are unaware of the need for detoxification of cassava.

Chapter 6 : General Discussion, Future Work and Conclusions

6.1 General Discussion

Linamarin is one of the major cyanogenic glycoside, being present in the cassava, staple food to some 800 million people worldwide. The cassava are largely consumed raw or processed into wide varieties of products either for personal or commercial purposed. Consumption of improperly processed food products could result in serious health problems for consumers due to exposure to cyanide derived from linamarin. Children, more commonly then adults, are usually the victim of cyanide poisoning because of their low bodyweight. Multiple cases of cyanide poisoning in schools from consumption of small quantities of poorly cooked cassava from 'bitter' cultivars have been reported (section 1.3.6). Unintentional poisoning can also occurs in adults due to consumption of improperly processed food products (Akintowa & Tunwashe, 1992; Suchard *et al*., 1998; Sanchez-Verlaan *et al*., 2011). This is because most of the processing methods employed for cassava processing in industry or by the individuals do not necessarily involve complete removal of linamarin, thus resulting in the presence of residual linamarin in the final product. Therefore, the need for a sensitive detection method for monitoring linamarin in processed foods becomes important. There are exhaustive list of published information on the detection of linamarin using standard analytical method (which involved the use of laborious, expensive and highly specialised equipment). Furthermore, most reports as reviewed in section 1.3.9 measure total hydrogen cyanide-generation potential, not the individual compound. The quantitative analysis of linamarin in processed

food products has not been investigated and there is apparently no report on the use of immunochemical methods for linamarin detection. This present study therefore covers most of the experimental works performed in order to raise good pAb against linamarin and to establish a sensitive immunoassay capable of detecting and quantifying linamarin in cassava food products within the UK, data that is also have not been previously investigated and reported.

Since conventional methods for direct linamarin detection involved the use of expensive equipment, there is need for a rapid, simple, more sensitive and cost-effective method that can be applied away from the laboratory. An enzyme-linked immunosorbent assay (ELISA) method would fulfil these requirements. An ELISA method can provide visual results which are useful for field work as a form of qualitative analysis in addition to its quantitative analysis. The three published studies on the detection of amygdalin using an ELISA method were based on the quantitative and qualitative analysis of amygdalin in raw foods (Cho *et al*., 2006 & 2008, Bolarinwa *et al*., 2014). In the present study, pAb was produced against linamarin and the generated antibody was characterized and used to quantify the amount of linamarin in raw and processed food products. Establishing an ELISA technique for linamarin detection is not an easy task. The use of pAb and perhaps the ability of generating mAb against the analyte of interest has always been the long-term goal in immunochemical research. The rabbit's pAb produced in this study was highly immunoreactive and sensitive towards linamarin therefore enables a very sensitive and specific ELISA technique to be developed after several optimisation studies. This has prompted the mAb production against linamarin using the common animal host, mouse. Surprisingly, after two attempts of immunisation using two different conjugates

(linamarin-CC-KLH and linamarin-CC-BSA), both antisera, although has high titre, failed to bind with free linamarin in the inhibition assay. The results of this study underline the importance of hapten design in the development of antibodies against compounds with simple structures and very low molecular weights. Although the increased understanding of antibody–antigen interaction resulting in a more rational design of haptens, there is still a practical difficulty in predicting which chemical structure is required for the production of specific and sensitive antibodies against a particular analyte. The inability of mouse immune system to produce specific antibody against free linamarin successfully highlighted the choice of animal host for immunisation is something that should be considered carefully especially when mAb generation is concerned.

The results of this study also showed that specific antibody can be produced against non-chromophore, low molecular weight cyanogenic glycosides such as linamarin. The developed ELISA was more sensitive than the polyclonal ELISA reported by Cho *et al*. (2006) and was not that far off from those reported by Bolarinwa *et al*. (2014). The ELISA described in the present study has been internally validated in a number of ways, looking at sensitivity (Table 5-4, Figure 5-8), specificity (Figure 5-9), robustness (section 5.3.2), superimposability (Figure 5-10), linearity (Figure 5-11) and by ‘sense checking’ results from investigations into extraction and with the results from analysis of samples (section 5.3.6). However, correlating quantitative analysis of linamarin has proved extremely difficult. There is little published information on the detection of linamarin in food samples, even HPLC has not been utilised as might have been expected, this is because of the difficulties of working with glycosides in chromatographic procedures and because linamarin, as a non-chromophoric

compound, does not have a characteristic uv spectrum, which makes it even more difficult when it comes to detection. However, in the work of Bolarinwa *et al.* (2013), determination of amygdalin in food product using the optimised ELISA assay for amygdalin showed an excellent correlation with that obtained with the HPLC method with R^2 value of 0.983. As described in section 1.3.9, methods for estimation of linamarin have been described and most reports were based on colorimetric detection of derivatives formed after reaction with hydrogen cyanide released upon hydrolysis of the analyte, measuring the total cyanide-generating potential instead of individual compound. There are kits available commercially based on this principle but only the kit measuring the free cyanide ions present in a sample was available at the time of study. The picrate paper method kit used extensively and mainly by Bradbury was not available to be purchased from any of the scientific suppliers to make better comparison and correlation with the ELISA described in present work. Since there are currently no reported studies on linamarin quantification in food samples using ELISA therefore comparison was made with previous studies on cyanogenic potential of cassava products using picrate paper methods as depicted in Table 6-1. The reported measurement of total cyanide potential is understandably higher since it represents the total of cyanogenic potential in the sample consisting of HCN/CN^- liberated by the linamarin, acetonecyanohydrin and free cyanide ions meanwhile ELISA only measures the amount of linamarin present in the sample. Different cultivars and origin of the cassava might also contributed to the differences in HCN amount in each respective studies. The results of the quantitative analysis of linamarin in foods using the ELISA in this study exhibited close similarities to the result from previous studies indicating that the ELISA method could be a good alternative method for linamarin detection and quantification in foods.

Table 6-1: Methods of quantifying cyanogenic potential in cassava tuber.

Method	Plant parts/Origin/ Cultivar	Total cyanide (mg HCN equivalent/kg fresh weight)	Linamarin (mg/kg fresh weight)	Reference
icELISA	Cassava tuber, peeled (Costa Rica)		43.08	-
Picrate paper	Cassava tuber (TMS 50395, TMS63397)	41, 47		Bradbury *et al.* (1999)
Picrate paper	Cassava tuber (Indonesia)	57		Hidayat *et al.* (2000)
Picrate based dipstick	Cassava tuber (Singapore, Green Twig variety)	46		Yeoh and Sun (2001)
Picrate paper	Cassava tuber, peeled (Vietnam)	52		Burns *et al.* (2012)
Alkaline picrate solution	Cassava tuber (Nigeria)	36.65		Ezeigbo *et al*. (2015)

Nevertheless, the free cyanide ion test kit was purchased and used to further validate the assay. The results from the kit showed negative results for dried cassava products but for most wet products (Table 5-3), there were slight change of colour indicating the presence of cyanide ions within the samples. Although it did not measure the amount of linamarin in the samples, the kit however, able to point out the small presence of free cyanide ions in wet cassava samples potentially liberated during the extraction process, despite all the steps taken to minimise the hydrolysis of linamarin by the endogenous linamarase enzyme. Whilst correlation is often, rightly or wrongly, seen as the ultimate procedure in assay validation, in reality the ultimate procedure in validation should be inter-laboratory trials where a limited number of laboratories all analyse the same samples using the same method of analysis. Such studies are expensive and time-consuming to carry out and are therefore reserved for those analytes where

there is significant commercial interest, or where there are significant regulatory factors involved. Thus if it was decided that there should be a regulatory level governing the maximum amount of cyanogen glycoside present if human food in, say, the EU, then there would have to be an internationally-accepted method of analysis in place to support the regulations.

Finally, the amount of residual linamarin retained in fresh unprocessed cassava root and leaves as well as those frozen ready-to-use products are the biggest concern according to the results of this study as the levels had exceeded the safe limit of cyanide intake set by WHO, FSANZ (10 mg/kg). The estimated lethal dose would be around 0.5 ~ 3.5 mg/kg body weight. The UK Committee of Toxicity has set a total daily intake (TDI) of cyanide at 20 µg/kg body weight but none on the regulatory safe limit. This method could also be used to assess the effect of residual linamarin on chronic low dose dietary intake. The set limits however do not take into account the general health and nutritional status of the consumer, which may alter the effective lethal limit. Current labelling on cassava products imported into the UK are lacking hazardous warning related to presence of CGs in the product and not to mention any preparation or cooking instructions hence, the poor awareness of the public on this matter since cassava is not the main source of diet in the UK. These findings suggest that appropriate warning label and preparation instructions are necessary for any cassava products commercially selling in the UK.

6.2 Suggestion for future work

Based on the findings of this study, further works could be carried out in the future as follows:

i) Since this was the first time the cyanuric chloride conjugation method was used for immunogen production, studies on the use of this method for the synthesis of hapten-protein conjugate for antibody production against other CGs or other very small hapten could be investigated.

ii) The unsuccessful attempts made in producing mAb against linamarin in this study warrants another look into the alternative of using rabbit's generated monoclonal antibodies as opposed to mouse. The hapten-conjugate synthesis also could differ from batch to batch hence conjugates of multiple batches are advisable so that only those with optimum hapten-protein ratio is selected for immunization.

iii) The developed ELISA should be used for linamarin detection and quantification in fresh cassava and food products available in other countries that consume cassava as staple and in considerable amount compared to countries in this part of the world.

iv) HPLC analysis or other standard direct quantification methods will need to be performed concurrently in order to validate and compare the results obtain from the ELISA.

v) Further investigation of the development of a more rapid ELISA test in form of a lateral assay (dipstick test) for linamarin and other CGs would provide a rapid and easy point of care testing (POCT) method of analysis of CGs within the industry or on the field. Other use of the pAb or successfully generated mAb would be the incorporation onto self-assemble layers of graphene and gold nanoparticle on screen-printed electrode for highly sensitive analyte detection using a portable potentiometric or voltammetric device that could provide exact electrical or concentration reading within seconds.

6.3 Conclusion

The objectives of the present research which were, to generate antibodies against linamarin and use it to develop a highly sensitive enzyme-linked immunosorbent assay (ELISA) for the determination of linamarin content in varieties of fresh and processed cassava products available in the UK were all achieved. The unsuccessful attempts in producing monoclonal antibodies against linamarin have given valuable insights of the complexity involved when it comes to planning and producing new hapten-conjugate to raise the antibody against. This study also revealed a wide range of cyanide concentration in commercially available cassava based products in UK. As the negative impacts of excess cyanide consumption are well known, it is clear that careful regulation of the importation of cassava products may be necessary to monitor and control the amount of total cyanogen in both fresh and some of the ready-to-eat products. The use of immunoassays in determination of CGs in foods requires more research and therefore a reliable and sensitive ELISA method demonstrated in this study would be very important for the development of other immunoassay methods that was deemed impossible for very small haptens and perhaps, could further improve the awareness and prevention of consumption of hazardous foods.

References

Abban, S., Thorsen, L. and Brimer, L. (2011). A high-throughput microtiter plate based method for the quantitative measurement of cyanogenesis (rate of formation of HCN). *Nature and Science*, **9**, 64-68.

Abuknesha, R. A., Luk, C. Y., Griffith, H. H., Maragkou, A., and Iakovaki, D. (2005). Efficient labeling of antibodies with horseradish peroxidase using cyanuric chloride. *Journal of Immunological Methods*, **306**, 211-217.

Adamolekun, B. (2010a). Thiamine deficiency and the etiology of tropical ataxic neuropathy-Review. *International Health*, **2**, 17-21.

Adamolekun, B. (2010b). Etiology of konzo, epidemic spastic paraparesis assoc-lated with cyanogenic glycosides in cassava: Role of thiamine deficiency? Review. *Journal of the Neurological Science*, **296**, 30-33.

Adetunji, A. I., Duodu, K. G., Taylor, J. R. N. (2015). Inactivation of tannins in Milled sorghum grain through steeping in dilute NaOH solution. *Food Chemistry*, **175**, 225-232.

Adindu, M. N., Olayemi, F. F., and Nze-Dike, O. U. (2003). Cyanogenic potential of some cassava products in Port Harcourt markets in Nigeria. *Journal of Food Composition and Analysis*, **16**, p. 21-24.

Agbor-Egbe, T., Lape Mbome, I., 2006. The effects of processing techniques in reducing cyanogen levels during the production of some Cameroonian cassava foods*. Journal of Food Composition and Analysis*, **19**, 354-363.

Ajaelu, J. C., Bamgbose, J. T., Atolaiye, B. O., and Adetoye, A. A. (2008). The use of methemoglobin complex in estimating cyanogens potential of cassava and cassava products. *African Journal of Biotechnology*, **7**, 1585-1587.

Akintowa A., Tunwashe O. & Onifade A. (1994). Fatal and non-fatal acute poisoning attributed to cassava-based meal. *Acta Horticulturae*, **375**, 285-288.

Akyildiz, B. N., Kurtoğlu, S., Kondolot, M. and Tunç, A. (2010). Cyanide poisoning caused by ingestion of apricot seeds. *Annals of Tropical Paediatrics*, **30**, 39-43.

Alitubeera, P. H., Eyu, P., Kwesiga, B., Ario, A. R., Zhu, B-P. (2019). Outbreak of cyanide poisoning caused by consumption of cassava flour – Kasese district, Uganda, September 2017. *Morbidity and Mortality Weekly Report (MMWR) CDC*, **68(13)**, 308-311.

Aloys, N., (2006). Traditional cassava foods in Burundi – A review. *Food Reviews International*, **22**, 1-27.

Allem, A. C. (2002). The origins and taxonomy of cassava. In *Cassava: Biology, Production and Utilization*; Hillocks, R. J., Thresh, J. M., Belloti, A. C.,

Eds.; CABI Publishing: Oxon, UK, pp. 1-16.

Andersen, M. D., Busk, P. K., Svendsen, I., Moller, B. L. (2000). Cytochromes P-450 from cassava (*Manihot esculenta Crantz*) catalyzing the first steps in the biosynthesis of the cyanogenic glucosides linamarin and lotaustralin. Cloning, functional expression in *Pichia pastoris*. *Journal of Biological Chemistry*, **275**, 1966-1975.

AOAC. (1990). Hydrocyanic acid in beans, alkaline titration method*. Official methods of analysis* (15th edition), p.1213, Arlington, Va, USA.

Ariffin, W. A., Choo, K. E. and Karnaneedi, S. (1992). Cassava (ubi kayu) poisoning in children. *Medical Journal of Malaysia*, **47**, 231-234.

Bacala, R. and Barthet, V. (2007). Development of extraction and gas chromatography analytical methodology for cyanogenic glycosides in flaxseed (Linum usitatissimum). *Journal of AOAC International*, **90**, 153-161.

Bak, S., Kahn, R. A., Nielsen, H.L., Møller, B. L., and Halkier, B. A. (1998). Cloning of three A type cytochromes P450, CYP71E1, CYP98 and CYP99 from *Sorghum bicolour* (L) Moench by a PCR approach multifunctional Cytochrome P450 in the biosynthesis of the cyanogenic glucoside dhurrin. *Plant Molecular Bology*, **36**, 393-405.

Bak, S., Paquette, S. M., Morant, M.m Rasmussen, A. V., Saito, S., Bjarnholt, N., Zagrobelny, M., Jørgensen, K., Hamann, T., Osmani, S. (2006). Cyanogenic glycosides: A case study for evolution and application of Cytochromes P450. *Phytochemistry Reviews*, **5**, 309-329.

Balhorn D. H., Kautz S., Heil M., and Hegeman A. D. (2009). Cyanogenesis of wild lima beans (Phaseolus lunatus L) is an efficient direct defence in Nature. *PLoS ONE*, **4**,1-7.

Baldridge J. R. and Lacy M. J. (2000). Adjuvants. In Howard, G. C., and Bethell, D. R. (Eds), *Basic Methods in Antibody Production and Characterization,* CRC Press LLC, Boca Raton, Florida, US, **4**, pp. 19-28.

Banea-Mayambu, J. P., Tylleskar, K., Gebre-Medhin, M., Rosling, H. (2000). Dietary cyanide from insufficiently processed cassava and growth retardation in children in the Democratic Republic of Congo (formerly Zaire). *Annals of Tropical Paediatrics*, **20**, 34-40.

Beans, E. S. (2001). Polyclonal antibodies. In Howard, G. C., and Bethell, D. R. (Eds), *Basic Methods in Antibody Production and Characterization,* CRC Press LLC, Boca Raton, Florida, US, **5**, pp. 31-50.

Beasley, D. M. G., and Glass, W. L. (1998). Cyanide poisoning pathophysiology and treatment recommendations. *Occupational Medicine*, **48**, 427-431.

Belloti, A. C., Riis, L. (1994). Cassava cyanogenic potential and resistance to Pests and diseases. *Acta Horticulturae*, **375**, 140-150.

Benjamini, E., Coico, R., and Sunshine, G. (2000). Antigen-antibody interactions, immune assays and experimental systems. *In Immunology-A Short Course*, Benjamini, E., Coico, R., and Sunshine, G. (Eds). Wiley-Liss, New York, USA. pp. 87-114.

Bennett, R. N., Wallsgrove, R. M. (1994). Secondary metabolites in plant defence

mechanism. *New Phytologist*, **127**, 617-633.

Benowitz, N. L. (2007). Antihypertensive agents. In: Katzung BG, ed. Basic and Clinical Pharmacology. 10th ed. New York, NY: McGraw-Hill Co. Inc; 173-174.

Bjarnholt, N. and Møller, B. L. (2008). Hydroxinitrile glucosides. *Phytochemistry*, **69**, 1947-1961.

Bo, L., Hai-Yan, S., and Ming-Hua, W. (2008). An enzyme-linked immunoasorbent assay for the detection of bifenthrin. *Chinese Journal of Analytical Chemistry*, **36**, 34-38.

Bokanga, M. (1994). Processing of cassava leaves for human consumption. *Acta Horticulturae*, **375**, 203-207.

Bolarinwa I. F., (2013). Cyanogenic glycosides in plant foods*. PhD Thesis*, University of Leeds.

Bolarinwa, I. F., Orfila C. and Morgan M. R. A. (2014). Development and application of an enzyme-linked immunosorbent assay (ELISA) for the quantification of amygdalin, a cyanogenic glycoside, in food. *Journal of Agriculture and Food Chemistry*, **62**, 6299-6305.

Bolarinwa, I. F., Orfila C. and Morgan M. R. A. (2014). Amygdalin content of seeds, kernels and food products commercially-available in the UK. *Food Chemistry*, **152**, 133-139.

Bolarinwa, I. F., Orfila C. and Morgan M. R. A. (2015). Determination of amygdalin in apple seeds, fresh apples and processed apple juices. *Food Chemistry*, **170**, 437-442.

Bolarinwa, I. F., Oke, M. O, Olaniyan, S. A and Ajala, A. S. (2016). A review of cyanogenic glycosides in edible plants. In Toxicology New Aspects to This Scientific Conundrum. Larramendy, M (Ed.), IntechOpen, pp. 179-191.

Bordo, D. and Bork, P. (2002). The rhodanese/Cdc25 phosphatase super-family-Sequence-structure-function relations. *EMBO Reports*, **3**, 741-746.

Borges, M. F., Fukuda, W. M. G., and Caldas, R. C. (1993). Avaliação de três métodos para determinação de cianeto em mandioca. *Revista Brasileira de mandioca*, **12**, 75-80.

Bradbury, J. H., Egan, S. V., and Lynch, M. J. (1991). Analysis of cyanide in cassava using acid hydrolysis of cyanogenic glycosides. *Journal of Science of Food and Agriculture*, **55**, 277-290.

Bradbury, J. H., Bradbury, M. G., and Egan, S. V. (1994). Comparison of methods of analysis cyanogens in cassava. In M. Bokanga, A. J. A., Essers, N. Poulter, H., Rosling, and O. Tewe (Eds). *Proceedings of the International Workshop on Cassava Safety* (vol. **375**, pp. 87-96). Ibadan, Nigeria: Acta Horticulturae.

Bradbury, M. G., Egan, S. V., and Bradbury, J. H. (1999). Picrate paper kits for determination of total cyanogens in cassava roots and all forms of cyanogens in cassava products. *Journal of the Science of Food and Agriculture*, **55**, 277-290.

Bradbury, J. H. (2006). Simple wetting method to reduce cyanogen content of cassava. *Journal of Food Composition and Analysis*, **19**, 388-393.

Bradbury, J. H. (2009). Development of a sensitive picrate method to determine total cyanide and acetone cyanohydrin contents of gari from cassava. *Food Chemistry*, **113(4)**, 1329-1333.

Bradbury, J. H., and Denton, I. C. (2010). Rapid wetting method to reduce cyanogen content of cassava flour. *Food Chemistry*, **121**, 591-594.

Bradbury, J. H. and Denton, I. C. (2014). Mild method for removal of cyanogens from cassava leaves with retention of vitamins and protein. *Food Chemistry*, **158**, 417-420.

Brimer, L., Abrahamsson, M., Mlingi, N., and Rosling, H. (1998). A modified microdiffusion assay with solid-state detection for the determination of total cyanogens (CNp) in cassava flour. Comparison to the method of O'Brien et al. (1991). *Food Chemistry*, **62**, 239-242.

Bruijn, De G. H. (1971). A study of the cyanogenic character of cassava. *Meded-Landbouwhogeschool Wageningen*, **71**, 1-40.

Burkin, M. A., and Galvidis, I. A. (2010). Development of a competitive indirect ELISA for the determination of lincomycin in milk, eggs and honey. *Journal of Agricultural and Food Chemistry*, **58**, 9893-9898.

Burns, A. E., Gleadow, R. M., Cliff, J. Zacarias, A. M, Cavagnaro, T. (2009). Cassava: The drought, war and famine crop in changing world. *Sustainability*, **2**, 3572-3607.

Burns, A. E., Bradbury, J. H., Cavagnaro, T. R. and Gleadow, R. M. (2012). Total cyanide content of cassava food products in Australia. *Journal of Food Composition and Analysis*, **25**, 79-82.

Burns, A. E., Gleadow, R. M., Zacarias, A. M., Cuambe, C.E., and Miller, R. E. (2012). Variations in the chemical composition of cassava (Manihot esculenta Crantz) leaves and roots as affected by genotypic and environmental variation. *Journal of Agricultural and Food Chemistry,* **60**, 4946-4956.

Burns, R. (2005). Immunization strategies for antibody production. In Burns, R. (Ed), Methods in Molecular Biology, Totowa, New Jersey, USA, **295**, pp. 1-12.

Busk, P. K. and Møller, B. L. (2002). Dhurrin synthesis in sorghum is regulated at The transcriptional level and induced by nitrogen fertilization in older Plants. *Plant Physiology*, **129**, 1222-1231.

Catty, D. (1988). Properties of antibodies and antigens. In Antibodies-a practical approach. Catty, D. (Ed). IRL Press, Oxford, UK. pp. 7-18.

Cardoso, A. P., Mirione, E., Ernesto, M., Massaza, F., Cliff, J., Haque, M. R., Bradbury, J. H. (2005). Processing of cassava roots to remove cyanogens *Journal of Food Composition and Analysis*, **18**, 451-460.

CCDN. Cassava Cyanide Disease Network. (2008). News. In: Konzo, TAN and cyanogens from cassava flour and gari. *Issue number* **11**, June, pp. 1-4.

Chandra, S. J., Sandhya, S., Vinod K. R., David B., Sudhakar K., and Chaitanya R. (2012). Plant toxins-useful and harmful effects-a review. *Hygeia Journal of Drugs and Medicines*, **4**, 79-90.

Chappey, O., Debray, M., Niel, E., Schermann, J. M. (1994). Association

constants of monoclonal antibodies for hapten: heterogeneity of frequency distribution and possible relationship with hapten molecular weight. *Journal of Immunological Methods*, **172**, 219.

Chassagne, D., Crouzet, J. C., Bayonove, C. L. and Baumes, R. L. (1996). Identification and quantification of passion fruit cyanogenic glycosides. *Journal of Agricultural and Food Chemistry*, **44**, 3817-3820.

Chauynarong, N., Elangovan, A. V., Iji, P. A. (2009). The potential of cassava products in diets for poultry. *World Poultry Science Journal*, **65**, 23-26.

Chen, Y-Q., Shang, Y-H., Wu, X-P., Qi, Y-T., and Xiao, X-L. (2007). Enzyme-linked immunosorbent assay for the detection of neomycin in milk: effect of hapten heterology on assay sensitivity. *Food and Agricultural Immunology*, **18,** 117-128.

Cheok, S. S. (1978). Acute cassava poisoning in children in Sarawak. *Tropical Doctor*, **8**, 99-101.

Chiwona-Karltun, L., Brimer, L., Saka, J. D. K., Mhone, A. R., Mkumbria, J., Johansson, L., Bokanga, M., Mahungu, N. M. and Rosling, H. (2004). Bitter taste in cassava roots correlates with cyanogenic glucoside levels. *Journal of the Science of Food and Agriculture,* **84**, 581-590.

Cho, A-Y., Yi, K. S., Rhim, J-H., Kim, K-I., Park, J-Y., Keum, E-H., Chung, J., and Oh, S. (2006). Detection of abnormally high amygdalin content in food by an enzyme immunoassay. *Molecules and Cells*, **21**, 308-313.

Cho, A-Y., Shin, K-J., Chung, J., and Oh, S. (2008). A sensitive enzyme immunoassay for amygdalin in food extracts using a recombinant antibody. *Journal of Food Protection*, **71**, 2048-2052.

Cho, H-J., Do, B-Y., Shim, S-M., Kwon, H., Lee, D-H., Nah, A-H., Choi, Y-J and Lee, S-Y. (2013). Determination of cyanogenic compounds in edible plants by ion chromatography. *Toxicological Research Official Journal of Korean Society of Toxicology,* **29 (2)**, pp. 143-147.

Ciglenecki I., Eyema R., Kabanda C., Taafo F., Mekaoui H. and Urbaniak V. (2011). Konzo outbreak among refugees from Central African Republic in Eastern region, Cameroon. *Food and Chemical Toxicology*, **49**, 579-582.

Clapp R. C., Bisset F. H., Coburn R.A. and Long L. (1966). *Journal of Phytochemistry*, **5**,1323.

Clarke, J. R., Marquadt, R. R., Oosterveld, A., Frohlich, A. A., Madrid, F. J., M and Dawood, M. (1993). Development of a quantitative and sensitive enzyme-linked immunosorbent assay for ochratoxin A using antibodies from the yolk of laying hen. *Journal of Agricultural and Food Chemistry*, **41**, 1784-1789.

Cliff, J., Nicala, D. (1997). Long term follow-up of konzo patients. *Transactions of The Royal Society of Tropical Medicine and Hygiene*, **91**, 447-449.

Cliff, J., Muquinge, H., Nhassico, D., Nzwalo, H. and Bradbury J. H. (2011). Konzo and continuing cyanide intoxification from cassava in Mozambique. *Food and Chemical Toxicology*, **49**, 631-635.

Cock J. H. (1984). Cassava. In: P. R. Goldsworthy and N. M Fisher (Eds.) The

Physiology of Tropical Field Crops, Wiley, New York, pp. 529-549.
Codex Committee on Contaminants in Foods. (2008). Discussion paper on cyanogenic glycosides. FAO.WHO Rome.
Conn E. E. (1979). Biosynthesis of cyanogenic glycosides. *Naturwissenschaften* **66**, 28-34.
Conn, E. E. (1980). Cyanogenic compounds. *Annual Review of Plant Physiology*, **31,** 433-451.
Conn E. E., (1994). Cyanogenesis – a personal perspective. In M. Bokanga, A. J. A. Essers, N. Poulter, H. Rosling & O. Tewe (eds.). Proceedings of the international workshop on cassava safety Ibadan, Nigeria: *Acta Horticulturae*, **375**, 31-43.
Cooke R. D., (1978). An enzymatic assay for the total cyanide content of cassava (*Manihot esculenta* Crantz). *Journal of the Science and Food Agriculture*, **29**, 345-352.
Corkill L., (1942). *New Zealand Journal of Science and Technology B*, **23**, 178.
Cowan, M. M. (1999). Plant products as antimicrobial agents. *Clinical Microbiology Review*, **12(4)**, 564-582.
Cressey, P., Saunders, D., Goodman, J. (2013). Cyanogenic glycosides in plant-based foods available in New Zealand. *Food Additives & Contaminants: Part A*, **30:11**, 1946-1953
Cumbana, A., Mirione, E., Cliff, J. and Bradbury, H. (2007). Reduction of cyanide content of cassava flour in Mozambique by the wetting method. *Food Chemistry*, **101**, 894-897.
Dahniya, M. T. (1994). An overview of cassava in Africa. *African Crop Science Journal*, **2**, 337-343.
Dalgaard, L., and Brimer, L. (1984). Electrochemical detection of cyanogenic glycosides after enzymatic post-column cleavage. *Journal of Chromatography*, **303**, 67-76.
Das, K., Tiwari, R. K. S. and Shrivastava, D .K. (2010). Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. *Journal of Medicinal Plants Research***, 4(2)**, 104-111.
Delange F., Ekpechi L. & Rosling H. (1994). Cassava cyanogenesis and iodine deficiency disorders. *Acta Horticulturae*, **375**, 289-293.
De Nicola, G. R., Leoni, O., Malagutti, L., Bernardi, R. and Lazzeri, L. (2011). A simple analytical method for dhurrin content evaluation in cyanogenic plants for their utilization in fodder and biofumigation. *Journal o Agricultural and Food Chemistry*, **59**, 8065-8069.
De Tafur, S. M., El-Sharkawy, M. A and Calle, F. (1997). Photosynthesis and yield performance of cassava in seansonally dry and semiarid conditions. *Photosynthetica,* **33,** 229-257.
Deshpande, S. S. (1996). Enzyme immunoassays-from concept to product development. Chapman & Hall, New York, USA. pp. 24-274.
DesLauriers, C. A., Burda, A. M., Wahl, M. (2006). Hydroxycobalamin as a cyanide antidote. American Journal of Therapeutics, **13(2)**, 161-165.
Dhas, P. K., Chitra, P., Jayakumar, S. and Mary, A. R. (2011). Study of effect of

Hydrogen cyanide exposure in cassava workers. *Indian Journal of Occupational and Environmental Medicine*, **15(3)**, 133-136.

Diano, M., Bivic, A. L., and Hirn, M. (1998). Raising polyclonal antibodies using nitrocellulose-bound antigen. In Pound, J.D. (Ed.), *Methods in Molecular Biology: Immunochemical Protocols, 2nd ed.,* Humana Press Inc., Totowa, New Jersey, **80**, pp. 5-13.

Djazuli, M., and Bradbury, J. H. (1999). Cyanogen content of cassava roots and flour in Indonesia*. Food Chemistry*, **65**, 523-525.

Donald, G. B. (2009). Cyanogenic foods (assava, fruit kernels and cycad seeds). *Medical Toxicology of Natural Substances*, **55**, 336-352.

Drew, E., Powell, R. J. (2002). Clinically useful monoclonal antibodies in treatment. *Journal of Clinical Pathology*, **55**, 81-85.

Drochioui, G. (2002). Highly selective and sensitive reaction of cyanide with 2,2-dihydroxy-1,3-indanedione. *Analytical and Bioanalytical Chemistry*, **372**, 744-747.

Drochioiu, G., Mangalagiu, I., Avram, E., Popa, K., Dirtu, A. C. and Druta, I. (2004) Cyanide reaction with ninhydrin: elucidation of reaction and interference Mechanisms. *Analytical Sciences*, **20**, 1443-1447.

Drochioiu, G., Popa, K., Humelnicu, D., Murariu, M., Sandu, I. and Cecal, A. (2007). Comparison of various sensitive and selective spectrophotometric Assays of environmental cyanide. *Toxicological and Environment Chemistry*, **90**, 221-235.

Drochioiu, G., Arsene, C., Murariu, M., and Oniscu, C. (2008). Analysis of cyanogens with resorcinol and picrate. *Food and Chemical Toxicology*, **46**, 3540-3545

Du, L., Bokanga, M., Møller, B. L., Halkier, B. (1995). The biosynthesis of cyanoglucosides in roots of cassava. *Phytochemistry*, **39**, 323-326.

Dufour, D. L. (1988). Effectiveness of cassava detoxification techniques used by indigenous people in nortwest Amazonia. *Intercencia*, **14**, 88-91.

Dufour D. (1993). The bitter is sweet: a case study of bitter cassava (Manihot esculenta) use in Amazonia, in Tropical Forests, People and Food: Biocultural Interactions and Applications to Development, Ed by Hladik, O Linares, O, Pagezy, H, Semple, A and Hadley, M. UNESCO/Parthenon, Paris, pp. 575-588.

Dufour, D. (1994). Cassava in Amazonia: lessons in utilization and safety from native peoples. *Acta Horticulturae*, **375**, 175-182.

Edwards, R. (1985). Immunoassay-An introduction. In William Heinemann, London, UK. pp. 1-11.

Edwards, R. (1996). Immunoassays-Essential data. In BIOS Scientific, Oxford, UK. pp. 1-6.

Egan, S. V., Yeoh, H. H., and Bradbury, J. H. (1998). Simple picrate paper kit for determination of the cyanogenic potential of cassava flour. *Journal of the Science of Food and Agriculture*, **76**, 39-48.

Eloff, J. N. (1998). Which extractant should be used for the screening and isolation of antimicrobial components from plants. *Journal of Ethnopharmacology*,

60, 1-8.

El-Sharkawy, M. A., De Tafur, S. M. and Cadavid, L. F. (1992a). Potential photosynthesis of cassava as affected by growth conditions. *Crop Science*, **32**, 1336-1342.

El-Sharkawy, M. D. (2004). Cassava biology and physiology. *Plant Molecular Biology*, **56**, 481-501.

Engvall, E., and Perlmann, P. (1971). Enzyme-linked immunosorbent assay (ELISA). Quantitative assay for immunoglobulin G. *Immunochemistry*, **8**, 871-874.

Engvall, E., and Perlmann, P. (1972). Enzyme-linked immunosorbent assay, ELISA: III. Quantitation of specific antibodies by enzyme-labeled anti-Immunoglobulin in antigen coated tubes. *Journal of Immunology*, **109**, 129-135.

Erlanger, B. F. (1980). The preparation of antigenic hapten-carrier conjugates [4]: A survey. In Immunochemical Techniques Part A. Helen Van Vunakis, J. J. L. (Ed), *Methods in Enzymology*, **70**, 85-104.

Ernesto M., Cardoso A. P., Nicala D., Mirione E., Massaza F., Cliff J., Haque M. R. & Bradbury J.H. (2002). Persistent konzo and cyanide toxicity from cassava in Northern Mozambique*. Acta Tropica*, **82**, 357-362.

Esashi, Y., Maruyama, A., Sasaki, S., Tani, A. and Yoshiyama, M. (1996). Involvement of cyanogens in the promotion of germination of cocklebur seeds in response to various nitrogenous compounds, inhibitors of respiratory and ethylene. *Plant Cell Physiology*, **10**, 1-40.

Espinoza, O. B., Perez, M. and Ramirez, M. S. (1992). Bitter cassava poisoning in eight children: a case report. *Veterinary and Human Toxicology*, **34**, 65.

Essers, A. J. A. and Nout, M. J. R (1989). The safety of dark moulded cassava Flour compared with white. A comparison of traditionally dried cassava Pieces in North East Mozambique. *Tropical Science*, **29**, 261-268.

Essers, A. J. A. (1994). Making safe cassava flour from bitter cassava by indigeneous solid substrate fermentation. *Acta Horticulturae*, **375**, 217-224.

Ezeigbo, O. R., Ekaiko, M. U. and Ibegbulem, Z. O. (2015). Effect of cooking time on starch and cyanide contents of freshly harvested cassava tubers used for tapioca production. *British Biotechnology Journal*, **8(4)**, 1-6.

FAO (Food and Agriculture Organization of the United Nations). (2013). Agricultural statistics, Rome: *Food Agricultural Organization of the United Nations* [http://www.faostat.org, 03 January 2014]

FAO (Food and Agriculture Organization of the United Nations). (2014) Food Outlook. Biannual report on global food markets.

Fasciglione, F. G., Marini, S., Bannister., V. J. and Giardina, B. (1996). Hapten-carrier interactions and their role in the production of monoclonal anti-Bodies against hydrophobic haptens. In *Hybridoma*, ed. Mary Ann Libert, Inc., **15(1)**, pp.1-9.

Feng, L., Wang, X. and Jin, H. (2011). Rabbit monoclonal antibody: potential application in cancer therapy. *American Journal of Translational Research* **3**, 115-121.

Forslund, K. and Jonsson, L. (1997). Cyanogenic glycosides and their metabolic enzymes in barley, in relation to nitrogen levels. *Physiologia Plantarum*, **101**, 367-372.

Francisco, I. A. and Pinotti, M. H. P. (2000). Cyanogenic glycosides in plants. *Brazillian Archives of Biology and Technology*, **43**, 487-492.

Franks, T. K., Hayasaka, Y., Choimes, S. and Van Heeswijck, R. (2005). Cyanogenic glycosides in grapevie: polymorphism, identification and developmental patterns. *Phytochemistry*, **66**, 165-173.

Fremy, J. M., and Usleber, E. (2003). Technical communication: Policy on characterization of antibodies used in immunochemical methods of analysis for mycotoxins and phytotoxins. *J. AOAC International*, **86**, 868-871.

Freund, J., Casals, J., and Hosmer, E. P. (1937). Sensitization and antibody formation after injection of tubercle bacilli and paraffin oil. *Proceedings of the Society of Experimental Biology and Medicine*, **37**, 509.

Freund, J. (1951). The effect of paraffin oil and mycobacteria on antibody formation and sensitization. *American Journal of Clinical Pathology*, **21**, 645.

Furstenberg-Hagg, J., Zagrobelny, M. and Bak, S. (2013). Plant defence against insect herbivores. *International Journal of Molecular Science*, **14**, 10249 - 10297.

FSANZ. (2009). Final assessment report proposal P1002. Hydrocyanic acid in ready-to-eat cassava chips.First review report. Canberra, Report Number 2-09.

Ganjewala D., Kumar S., Asha D. S., and Ambika K. (2010). Advances in cyanogenic glycosides biosynthesis and analyses in plants: A review. *Acta Biologica Szegediensis*, **54**, 1-14.

Garcia, M., Dale, N. (1999). Cassava root meal for poultry. *Journal of Applied Poultry Science,* **8**, 132-137.

Gbadebo, A. M. and Oyesanya, T. M. (2005). Assessment of iodine deficiency And goiter incidence in parts of Yewa area of Ogun state southwestern Nigeria. *Enviromental Geochemistry and Health*, **27**, 491-449.

Gibbs, J. (2001). Effective Blocking Procedures. In *ELISA Technical Bulletin No. 3*, Corning Incorporation Life Sciences, Kennbunk, ME, US.

Gleadow, R. M., Foley, W., and Woodrow, I. E. (1998). Enahnced CO2 alters the relationship between photosynthesis and defence in cyanogenic *Eucalyptus cladocalyx* F. Muell*. Plant, Cell Environment,* **21**, 12-22*.*

Gleadow, R. M. and Woodrow, I. E. (2002). Constraints on effectiveness of cyanogenic glycosides in herbivore defense. *Journal of Chemical Ecology*, **28**, 1301-1313.

Golf, B. M., Moore, K. J., Fales, S. L & Pedersen, J. F. (2011). Comparison of gas chromatography, spectrophotometry, and near infrared spectroscopy to quantify prussic acid potential in forage. *Journal of the Science of Food and Agriculture*, **91**, 1523-1526.

Goodrow, M. H., Harrison, R. O. and Hammock, B. D. (1990). Hapten synthesis,

antibody development and competitive inhibition enzyme immunoassay for s-triazine herbicides. *Journal of Agricultural and Food Chemistry*, **38**, 990-996.

Goodwin, J. F. and Choi, S. Y. (1970). Quantification of protein solutions with trinitrobenzenesulfonic acid. *Clinical Chemistry*, **16(1)**, pp. 24-31

Greenfield, E. A. (2014). Generating Monoclonal Antibodies. In *Antibodies: A Laboratory Manual 2nd Edition.* Cold Spring Harbor Laboratory Press, **7**, pp. 201-221.

Green, J. A. and Manson, M. M. (1998). Production of polyclonal antisera. In Pound, J.D. (Ed.), *Methods in Molecular Biology: Immunochemical Protocols, 2nd ed.,* Humana Press Inc., Totowa, New Jersey, **80**, pp. 1-4.

Gruhnert, C., Biehl, B., and Selmar, D. (1994). Compartmentation of cyanogenic Glycosides and their degrading enzymes. *Planta*, **195**, 36-42.

Hadacek, F., Bachmann, G. (2015). Low molecular weight metabolite systems chemistry. *Frontiers in Environmental Science*, **3(12)**, 1-21.

Hage, D. S. (1999). Immunoassays. *Analytical Chemistry*, **71**, 294-304.

Hall, A. H., Dart, R., Bogdan, G. (2007). Sodium thiosulfate of hydroxycobalamin For the empiric treatment of cyanide poisoning? *Annual Emergency Medicine*, **46(6)**, 806-813.

Hamel, J. (2011). A review of acute cyanide poisoning with a treatment update. *CriticalCareNurse*, **31**, 72-82.

Hammer, G. B., Connolly, S. G., Schulman, S. R., Lewandowski, A., Cohane, C., Reece, T. L., Anand, R, Mitchell, J. and Drover, D. R. (2013). Sodium Nitroprusside is not associated with metabolic acidosis during intra-Operative infusion in children. *BMC Anesthesiology*, **13**, 1-9.

Hancock, D. C., and O'Reilly N. J. 2005. Production of polyclonal antibodies in rabbits. In Burns, R. (ed), *Methods in Molecular Biology: Immunochemical Protocols, 3rd ed.*, Humana Press Inc., Totowa, New Jersey, **295,** pp. 13-39.

Haque, M. R., and Bradbury, J. H., (2002). Total cyanide determination of plants and foods using the picrate and acid hydrolysis methods. *Food Chemistry*, **77**, 107-114.

Harbone, J.B. (1999). Classes and functions of secondary products. In: Walton N.J and Brown D. E (eds) *Chemicals from plants, perspectives secondary plant products.* Imperial College Press, London, pp. 1-25.

Harbone, J.B. and Turner, B.L. (1984). *Plant Chemosystematics*. Academic Press. London.

Harrison, R. O., Goodrow, M. H. and Hammock, B. D. (1991). Competitive inhibition ELISA for the s-triazine herbicides: assay optimization and antibody characterization. *Journal of Agricultural and Food Chemistry*, **39**, 122-128.

Hemmilä, I. (1985). Fluoroimmunoassays and immunofluorometric assays - Review. *Clinical Chemistry*, **31**, 359-370.

Hervás, M., Angel, López, M. A., and Escarpa, A. (2009). Electro-immunoassay using magenetic beads for the determination of zearalenone in baby

food: An anticipated analytical tool for food safety. *Journal of Analytica Chimica Acta*, **653**, 167-172.

Hidayat, A., Zuaraida, N., Hanarida, I. and Damardjati, D. S. (2000). Cyanogenic content of cassava root of 179 cultivars grown in Indonesia. *Journal of Food Composition and Analysis*, **13**, 71-82.

Holzhauser, T., and Vieths, S. (1999). Indirect competitive ELISA for determination of traces of peanut (*Arachis hypogaea* L.) protein in complex food matrices. *Journal of Agricultural and Food Chemistry*, **47**, 603-611.

Howeler, R.H. (2012). Recent trends in production and utilization of cassava in Asia. In: Howeler, Reinhardt H. (ed.). The cassava handbook: A reference Manual based on the Asian regional cassava training course, held in Thailand. Centro Internacional de Agricultura Tropical (CIAT), Bangkok, TH, pp. 1-22.

Hsieh, Y. P. (2010). Immunoassays, In Nielsen S. S., *Food Analysis 4th Edition*, New York, US, Springer, pp. 301-316.

Hughes, J., Carvalho, F., Hughes, M. (1994). Purification, characterization and cloning of of a hydroxynitrilelyase from cassava. *Archive of Biochemistry and Biophysics*, **311**, 496-502.

Ikediobi, C. O., Onyia, G. O. C., and Eluwah, C. E. (1980). A rapid and inexpensive enzymatic assay for total cyanide in cassava (*Manihot esculenta* Crantz) and cassava products. *Agricultural and Biological Chemistry*, **44**, 2803-2809.

Jensen, N. B, Zagrobelny, M., Hjerno, K., Olsen, C. E., Houghton-Larsen, J., Borch, J., Møller B. L., Bak, S. (2011). Covergent evolution in biosynthe-Sis of cyanogenic defence compounds in plants and insects. Nature Communications, 2.

Jiang, X-X., Shi, H-Y., Wu, N., and Wang, M-H. (2011). Development of an enzyme-linked immunosorbent assay for diniconazole in agricultural samples. *Food Chemistry*, **125**, 1385-1389.

Jørgensen, K., Bak, S., Busk, P. K., Sørensen, C., Olsen, C. E., Puonti-Kaerlas, J., Møller B. L. (2005). Cassava plants with a depleted cyanogenic gluco-side content in leaves and tubers. Distribution of cyanogenic glucosides, their site of synthesis and transport, and blockage of the biosynthesis by RNA interference technology. *Plant Physiology*, **139**, 363-374.

Jones, D. A. (1998). Why are so many food plants cyanogenic? *Phytochemistry*, **47**, 155-162.

Jones P. R., Møller B. L., and Høj P. B. (1999). The UDP-glucose: p-hydroxynitrile-o-glucosyltransferase that catalyzes the last step in synthesis of the cyanogenic glycoside dhurrin in Sorghum bicolor-isolation, cloning, heterologous expression and substrate specificity. *Journal of Biological Chemistry*, **274**, 35483-35491.

Jordan, W. (2005). Dual-antibody sandwich enzyme-linked immunosorbent assay. In Burns, R. (ed), *Methods in Molecular Biology* : *Immunochemical protocols 3rd ed.*, Humana Press Inc., Totowa, New Jersey, **295,**

pp. 207-214.

Juan, S., Rong-Mei, W., Yue-Qiu, W., Yu-Rong, T. & An-Ping, D. (2010). Hapten design, modification and preparation of artificial antigens. *Chinese Journal of Analytical Chemistry*, **38**, pp. 1211-1218.

Kahn R. A., Bak S., Svendsen I., Halkier B. A., and Møller B. L. (1997). Isolation and reconstitution of cytochrome P450ox and in vitro reconstitution of the entire biosynthetic pathway of the cyanogenic glycoside dhurrin from sorghum*. Plant Physiology*, **115**, 1661-1670.

Kakes, P. (1994). The function of cyanogenesis in cassava. *Acta Horticulturae*, **375**, 79-85.

Kapinga, R., Mlingi, N. and Rosling, H. (1997). Reasons for use of bitter cassava in southern Tanzania. *African Journal of Tropical Root Crops*, **2**, 81-84.

Kascsak, R. J., Rubenstein, R., Merz, P. A., TOnna-DeMAsi, M., Fersko, R., Carp, R. I., Wisniewski, H. M., and Diringer, H. (1987). Mouse polyclonal and monoclonal antibody to scrapie-associated fibril proteins. *Journal of Virology*, **61**, 3688-3693.

Kemdirim, O. C., Chukwu, O. A. and Achinewhu, S. C. (1995). Effect of traditional of cassava on the cyanide content of gari and cassava flour. *Plant Foods for Human Nutrition*, **48**, 335-339.

Kemeny, D. M., and Chantler, S. (1988). An introduction to ELISA. In *ELISA and other solid phase immunoassay*. Kemeny, D. M., and Challacombe, S. J. (Eds). John Wiley & Sons, New York, USA. pp. 1-29.

Kemeny, D. M. (1991). Immune cells and antibodies. *In A Practical Guide to ELISA*. Kemeny, D. M. (Ed), Pergamon Press, Oxford, UK. pp. 1-5.

Kemp, H. A., Morgan, M. R. A., (1986). Studies on the detrimental effects of bivalent binding in a microtitration plate ELISA and possible remedies. *Journal of Immunological Methods*, **94**, 65-72.

Kim, Y. J., Cho, Y. A., Lee, H. S. (2003). Investigation of the effect of hapten heterology on immunoassay sensitivity and development of an enzyme-linked immunosorbent assay for the organophosphorus insecticide Fenthion. *Analytica Chimica Acta*, **494**, pp. 29-40.

King N. L. R. and Bradbury J. H., (1985). Bitterness of cassava: Identification of a new apiosyl glucoside and other compounds that affect its bitter taste. *Journal of the Science of Food and Agriculture*, **68**, 223-230.

Koch, B., Nielsen, V. S., Halkier, B. A., Olsen, C. E., Møller, B. L. (1992). The biosynthesis of cyanogenic glucosides in seedling of cassava (*Manihot esculenta Crantz*). *Archive of Biochemistry and Biophysics*, **292**, 141-150.

Köhler, G., and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, **256**, 495-497.

Kondo, M., Tsuzuki, K., Hamada, H., Yamaguchi, Y., Uchigashima, M., Saka, M., Watanabe, E., Iwasa, S., Narita, H., and Miyaka, S. (2011). Development of an enzyme-linked immunosorbent assay (ELISA) for residue analysis of the fungicide azoxystrobin in agricultural products. *Journal of Agricultural and Food Chemistry*, **60**, 904-911.

Konstantatos, A., Kumar, M. S., Burrell, A., Smith, J. (2017). An unusual

presentation of chronic cyanide toxicity from self-prescribed apricot kernel extract. *BMJ Case Reports*, bcr-2017-220814.

Kramer, P. M. (1996). Biosensors for measuring pesticides residues in the environ ment: Past, present and future. *Journal AOAC*, **19**, 1245.

Krikunova, V., Hegedus, G., Le, H. M., Jouravleva, L., Eremin, S., Natangelo, M., Benfenati, E., and Székács, A. (2002). Development of an enzyme-linked immunosorbent assay (ELISA) for the herbicide propanil. *International Journal of Environmental Analytical Chemistry*, **82**, 865-878.

Kwok, J. (2008). Cyanide poisoning and cassava. Food Safety Focus. (19th issue February, 2008) – Incident in focus. (http://www.cfs.gov.hk/english/multi-media)

Lancaster, P. A., Ingram, J. S., Lim, M. Y. and Coursey, D. G. (1982). Traditional Cassava-based foods: surveu of processing techniques. *Economic Botany*, **36**, 12-45.

Langman, R. R. (2000). The specificity of immunological reactions. *Molecular Immunology*, **37**, 555-581.

Lasch, E. E. and Shawa, R. R. (1981). Multiple cases of cyanide poisoning by apricot kernels in children from Gaza. *Pediatrics*, **68**, 5-7

Latif, S., and Müller, J. (2014). Cassava – how to explore the "all-sufficient". Rural, **21**, S., 30-31.

Latif, S., and Müller, J. (2015). Potential of cassava leaves in human nutrition: A review. *Trends in Food Science and Technology*, **44**, 147-158.

Lee, G. U., Ahn, K. C., Park, O. S., Kang, S. Y. and Hammock, B. D. (2001). Development of an ELISA for the detection of the residues of the insect-Sides imadocloprid in agriculture and environment samples. Journal of Agricultura and Food Chemistry. **50**, 29-40.

Lewis, D. H. (2001). Antigens. In Howard, G. C. and Bethell, D. R. (Eds), *Basic Methods in Antibody Production and Characterization,* CRC Press LLC, Boca Raton, Florida, US, **3**, pp. 11-18.

Leybell, I., Borron S.W., Roldan, C. J., Rivers, C. M. (2018). Cyanide toxicity. *eMedicine*. Retrieved 21/4/2019 from (https://emedicine.medscape.com/article/814287-overview#a1)

Li, P. W., Zhang, Q., Zhang, W. (2009a). Immunoassays for aflatoxins. *TrAC Trends in Analytical Chemistry*, **28**, 1115-1126.

Li, P. W., Zhang, Q., Zhang, W., Zhang, J. Y., Chen, X. M., Jiang, J., Xie, L. H. and Zhang, D. H. (2009b). Development of a class-specific monoclona antibody-based ELISA for aflatoxins in peanut. Food Chemistry, 115, 313-317.

Li, Y., Zhang, J., Mao, X., Wu, Y., Liu, G., Song, L., Li, Y., Yang, J., You, Y. and Cao, X. (2016). High-sensitivity chemiluminescent immunoassay investi-Gation and application for the detection of T-2 toxin and major metabolite HT-2 toxin. Journal of the Science of Food and Agriculture, **97(3)**.

Liddell, E. (2013). Antibodies. In Wild. D, *The Immunoassay Handbook: Theory and applications of ligand binding, ELSA and related techniques*, 4th edt.,

Elsevier, UK, pp. 243-265.

Lieberei, R., Selmar, D. and Biehl, B. (1985). Metabolization of cyanogenic glucosides in *Hevea brasiliensis*. *Plant Systematics Evolution*, **150**, 49-63.

Linley C. K., Brimer L., Saka J. D. K., Mhone A. R., Mkumbira J., Johansson L., Bokanga M., Mahungu N. M., Rosling H., (2004). Bitter Taste in Cassava Roots Correlates with Cyanogenic Glycosides Levels, *Journal of the Science of Food and Agriculture,* **84**, 581-590.

Love B. and Snader K. M., (1965). Chemistry & Industry, **1**, 15.

Lunquist, P., Rosling, H. and Sorbo, B. (1985). Determination of cyanide in whole blood, erythrocytes and plasma. *Clinical Chemistry*, **31**, 591-595.

Ma, J., Dasgupta, P. K. (2010). Recent developments in cyanide detection: A review. *Analytica Chimica Acta,* **673**, 117–125.

Makarananda, K., Weir, L. R. and Neal, G. E. (1995). Competitive ELISa. In Pound, J. D. (Ed.), Methods in Molecular Biology 80: Immunochemical protocols, 2nd ed., Humana Press Inc., Totowa, New Jersey, USA. pp. 155-160.

Männel-Croisé C., Zelder F. (2009) Side chains of cobalt corrinoids control the sensitivity and selectivity in the colorimetric detection of cyanide. *Inorganic Chemistry*, **48**, 1272-1274.

Männel-Croisé, C., Probst, B., and Zelder, F. (2009). A straightforward method for the colorimetric detection of endogenous biological cyanide. *Analytical Chemistry*, **81**, 9493-9498.

Mao C. H., Blocher J. P., Anderson L. and Smith D. C., (1965). Phytochemistry, **4**, 297.

Matsumoto, K., Torimaru, A., Ishitobi, S., Sakai, T., Ishikawa, H., Toko, K., Miura, N., and Imato, T. (2005). Preparation and characterization of a polyclonal antibody from rabbit for detection of trinitrotoluene by a surface plasmon resonance biosensor. *Talanta*, **68**, 305-311.

Massey, L. K. (2007). Food oxalate: factors affecting measurement, biological variation and bioavailability. *Journal of the American Dietetic Association*, **107**, 1191-1194.

McMahon, J. M., White, W. L. B., Sayre, R. T. (1995). Cyanogenesis in cassava (Manihot esculenta Crantz). *Journal of Experimental Botany*, **46**, 731 – 741.

Medical Device and Diagnostic Industry. Rabbit monoclonal antibody: a new Diagnostics technology. Available at http://www/mddionline.com/article/ Rabbit-monoclonal-antibody-new-diagnostics-technology (posted on 26 June 2013 by Weimin Zhu).

Melville, J., and Doak, B.W. (1940). *New Zealand Journal of Science and Technology B*, **22**, 67.

Melville, J., and Fraser, J. G. (1951). *New Zealand Journal of Science and Technology* A, **33**, 56.

Meulenberg, E. P. (1997). Immunochemical detection of environmental and food contaminants: Development, validation and application-Review. *Food Technology and Biotechnology,* **35**, 153-163.

Mills, E. N., Diplock, A. T., Plumb, G. W., Lambert, N., and Morgan, M. R. A. (1997). Development of a rapid dipstick immunoassay for the detection of peanut contamination of food. *Food and Agricultural Immunology*, **9**, 37-50.

Mlingi, N. L. V., Nkya S., Tatala S. R., Rashid S. and Bradbury J. H. (2011). Recurrence of konzo in southern Tanzania: Rehabilitation and prevention using the wetting method. *Food and Chemical Toxicology*, **49**, 673-677.

Moeller, B. M., Crankshaw, D. L., Briggs, J, Nagasawa, H. and Patterson, S. E. (2017). In-vitro mercaptopyruvate sulfotransferase species comparison in humans and common laboratory animals. *Toxicology Letters*, **274**, 64-68.

Møller, B. L. (2010). Functional diversifications of cyanogenic glycosides. *Current Opinion in Plant Biology*, **13**, 338-347.

Møller, B. L. and Poulton, J. E. (1993). Cyanogenic glucosides. In: Lea, P. J. (Ed) Methods in Plant Biochemistry, vol. 9. Academic Press, San Diego, pp. 183-207.

Møller, B. L., Seigler, D. S. (1999). Biosynthesis of cyanogenic glycosides, cyano-Lipids and related compounds. In *Plant Amino Acids*; Singh, B. K., Ed,; Marcel Dekker: New York, NY USA, pp. 563-609

Montagnac, J. A., Davis, C. R. and Tanumihardjo, S. A. (2009a). Nutritional value of cassava for use as staple food and recent advances for improvement. *Comprehensive Reviews in Food Science and Food Safety*, **8**, 181-194.

Montagnac, J. A., Davis, C. R. and Tanumihardjo, S. A. (2009b). Processing techniques to reduce toxicity and antinutrients of cassava for use as a staple food. *Comprehensive Reviews in Food Science and Food Safety*, **8**, 17-27.

Morgan, N. and Choct, M. (2016). Cassava: Nutrient composition and nutritive value in poultry diets. Animal Nutrition, **2**, 253-261.

Morgan, M. R. A., McNerney, R., and Chan, H. W. S. (1983a). The enzyme-linked immunosorbent assay of ochratoxin A in barley. *Journal of the Association of Official Analytical Chemists*, **66**, 1481-1484.

Morgan, M. R. A., Kang, A. S., and Chan, H. W. S. (1986). Aflatoxin determination in peanut butter by enzyme-linked immunosorbent assay. *Journal of the Science of Food and Agriculture*, **37**, 908-914.

Moyo, C. C., Bebesi, I. R. M., Sandifolo, V. S. and Teri, J. M. (1998). Current status of cassava and sweet potato production and utilization in Malawi. In J. M. T. M. O. Akoroda (Ed.), *Southern African Root Crops Research Network (SARRNET) (Zambia),* pp. 51-67.

Naar, J., Branaa, P., Chinain, M. and Pauillac, S. (1999). An improved method for the microscale preparation and characterization of hapten-protein conjugates: The use of cholesterol as a model for nonchromophore hydroxylated haptens. *Bioconjugate Chemistry*, **10**, 1143-1149.

Nagaraja, P., Kumar, M., Yathirajan, H. and Prakash, J. (2002). Novel sensitive spectrophotometric method for the trace determination of cyanide in industrial effluent. *Analytical Science*, **18**, 1027-1030.

Nambisan, B. (1999). Cassava latex as a source of linamarase for determination of linamarin. *Journal of Agriculture and Food Chemistry*, **47**, 372-373.

Nambisan, B. (2011). Strategies for elimination of cyanogens from cassava for for reducing toxicity and improving food safety. *Food and Chemical Toxicology*, **49**, 690-693.

Nambisan, B. and Sundaresan, S. (1994). Distribution of linamarin and its metabolizing enzymes in cassava tissue. Journal of the Science of Food and Agriculture, **66**, 503-507.

Nartey, F. (1968). Studies on cassava, Manihot utilissima Pohl-I. Cyanogenesis: The biosynthesis of linamarin and lotaustralin in etiolated seedlings. *Phytochemistry*, **7**, 1307-12.

Nassar, N. M. A., Hashimoto, D. Y. C, Fernandes, S. D. C., (2008). Wild Manihot species: botanical aspects, geographic distribution and economic value. *Genetic and Molecular Research*, **7**, 16-28.

Nelson, L. (2006). Acute cyanide toxicity: mechanisms and manifestations. *Journal of Emergency Nursing*, **32(4)**, 8-11.

Ngudi, D. D., Kuo, Y-H., and Lambein, F. (2003). Cassava cyanogens and free amino acids in raw and cooked leaves. *Food and Chemical Toxicology*, **41**, 1194-1197.

Nhassico, D., Muquinge, H., Cliff, J., Cumbana, A. & Bradbury, J. H. (2008). Rising African cassava production, diseases due to high cyanide intake and control measures. *Journal of the Science of Food and Agriculture*, **88**, 2043-2049.

Nweke, F. (1994). Cassava processing in sub-Saharan Africa: implications for Expancing cassava production. *Outlook on Agriculture*, **23**,197-205.

Nweke, N. I., Spencer, D. S. C., Lynam, J. K. (2002). The cassava transformation : Africa's best-kept secret. Michigan State University Press , East Lansing, M. I., 273.

Nzwalo, H. and Cliff, J. (2011). Konzo: from poverty, cassava, and cyanogen Intake to toxico-nutritional neurological disease. *PLoS Neglected Tropical Diseases*, **5**, e1051.

Obilie, E. M., Tano-Debrah, K. and Amao-Awua, W.K. (2004). Souring and breakdown of cyanogenic glucosides during the processing of cassava into akyeke. *International Journal of Food Microbiology*, 93, 115-121.

Oke, O. L. (1973). The mode of cyanide detoxification-Review. In Chronic Cassava toxicity. Proceedings of an interdisciplinary workshop, London, England, 29-30 January 1973. International Development Research Centre. Monogram IDRC-010e. pp. 97-104.

Oke, O. L. (1994). Eliminating cyanogens from cassava through processing: technology and tradition. *Acta Horticulturae*, **375**, 163-174.

Oluwale, O. S. A., Onabolu, A. O., Mtunda, K. and Mlingi, N. (2007). Characterization of cassava (*Manihot esculenta Crantz*) varieties in Nigeria and Tanzania, and farmers' perception of toxicity of cassava. *Journal of Food Composition and Analysis*, **20**, 559-567.

Olsen, K. M., Sutherland, B. L. and Small, L. L. (2007). Molecular evolution of the Li/li chemical defense polymorphism in white clover (*Trifolium repens* L.). *Molecular Ecology*, **16**, 4180-4193.

Onabolua, A. O., Oluwolea, O. S. A., Bokanga, M. and Rosling, H. (2001). Ecological variation of intake of cassava food and dietary cyanide load in Nigerian communities. *Public Health Nutrition*, **4**, 871-876.

Osuntokun B. O. (1994). Chronic cyanide intoxification of dietary origin and a degenerative neuropathy in Nigerians. *Acta Horticulturae*, **375**, 311-321.

O'Brien, G. M. O., Taylor, A. J., and Poulter, N. H. (1991). Improved enzymic assay for cyanogens in fresh and processed cassava. *Journal of the Science of food and Agriculture*, **56**, 277-289.

Pastor-Navarro, N., Morais, S., Maquieira, A., and Puchades, R. (2007). Synthesis of haptens and development of a sensitive immunoassay for tetracycline residues application to honey samples. *Journal of Analytica Chimica Acta*, **594**, 211-218.

Paraf, A., and Peltre, G. (1991). Immunoassays in food and agriculture. Kluwer Academy, Lancaster, UK. pp. 9-35.

Paraf, A., and Peltre, G. (2012). Immunoassays in food and agriculture. Netherlands, Springer Science & Business Media.

Paxton, J. W., Rowell, F. J. and Ratcliffe, J. G. (1976). Production and characteri-Sation of antisera to diphenylhydantoin suitable for radioimmunoassay. *Journal of Immunological Methods*, **10**, 317.

Pedersen, M. K., Sorensen, N. S., Heegard, P. M. H., Beyer, N. H., and Bruun, L. (2006). Effect of different hapten-carrier conjugation ratios and molecular orientations of antibody affinity against a peptide antigen. *Journal of Immunological Methods*, **150**, 5-21.

Pellet, D. and El-Sharkawy, M.A. (1997) Cassava varietal response to fertilization Growth dynamics and implication for cropping sustainability. Experimental Agriculture, **33**,13-20.

Pereira, J., Seigler, D. and Splittstoesser, W. (1981). Cyanogenesis in sweet and bitter cultivars of cassava. *Horticultural Science*, **16**, 776-777.

Perera, C. O. (2010). Removal of cyanogenic glycosides from cassava during controlled drying. *Drying Technology*, **28,** 68-72.

Price, C. P., and Newman, D. J. (1991). Principles and practice of immunoassay. Macmillan Publishers, New York, USA. pp. 19-52.

Portsmann, T. and Kiessig, S. T. (1992). Enzyme immunoassay techniques. An overview. *Journal of Immunological Methods*, *150*, 5-21.

Poulton J. E. (1990). Cyanogenesis in plants-Review. *Plant Physiology*, **94**, 401-405.

Quan, Y., Zhang, Y., Wang, S., Lee, N., and Kennedy, I. R. (2006). A rapid and sensitive chemiluminescence enzyme-linked immunosorbent assay for the determination of fumonisin B_1 in food samples. *Journal of Analytica Chimica Acta*, **580**, 1-8.

Rajesh, K., Rana, V. K., Suri, R. C. (2013). Characterization of hapten-protein conjugates: antibody generation and immunoassay development for

pesticides monitoring. *BioNanoScience*, **3**, 137-144.

Ramanujam, T., Indira, P., Rajendran, P. G. (1984). Distribution of HCN in cassava and its relationship with anthocyanin pigments. *South Indian Horticulture*, **32**, 132-137.

Ramesh, A., Thirugnanam, P. E., and Balakrishnamurthy, P. (2007). Hapten synthesis, generation of polyclonal antibodies and development of ELISA for determination of thiamethoxam residues in food and environment samples. *Indian Journal of Biotechnology*, **6**, 365-371.

Raybould, T. J. and Takahashi, M. (1988). Production of stable rabbit-mouse hybridomas that secrete rabbit mAb of define specificity. *Science*, **240**, 1788-1790.

Reade, M. C., Davies, S. R., Morley, P. T., Dennet, J., Jacobs, I. C. (2012). Review article: Management of cyanide poisoning. Emergency Medicine Australasia, **24**, 225-238.

Ressler, C., Nigam, S. and Giza, Y. (1969). Toxic principle in vetch: isolation and identification of γ-L-glutamyl-1-β-cyanoalanine from common vetch seeds: distribution in some legumes. *Journal of the American Chemical Society*, **91**, 2758-2765

Ressler, C., Nigam, S. and Giza, Y. (1997). Neurotoxins in a vetch food: Stability to cooking and removal of γ-glutamyl-β-cyanoalanine and β-cyanoalanine and acute toxicity from common vetch (*Vicia sativa* L.) legumes. Journal of Agriculture and Food Chemistry, **45**, 189-194.

Rief, N., Wascho, C., Natainczyk, W., Montenarh, M and Gotz, C. (1998). Production and characterization of a rabbit monoclonal antibody against human CDC25C phosphatase. *Hybridoma*, **17**, 389-394.

Rittenburg, J. H. (1990). Fundamentals of immunoassay. *Development and application of immunoassay for food analysis*, Elsevier Science Publishers Ltd, pp. 29-57.

Rogers, D., Appan, S. (1973). Flora Neotropica Monograph, Hafner Press, New York.

Rosling H. (1988). Cassava toxicity and food security – A review of health effects of cyanide exposure from cassava and of ways to prevent such effects. Uppsala: International Child Health Unit, Uppsala University.

Roucairol, C., Azoulay, S., Nevers, M-C, Creminon, C., Grassi, J., Burger, A., and Duval, D. (2007). Development of a competitive immunoassay for efavirenz: Hapten design and validation studies. *Journal of Analytica Chimica Acta*, **589**, 142-149.

Ruengprapavut, S., and Chulavatnatol, M. (1999). New chemiluminescence assay for linamarin. *Life*, **48**, 219-223.

Saka, J. D. K., Mhone, A. R. K. and Brimer, L. (1998). An improved microdiffusion method with solid-phase detection for the determination of total cyanogens in fresh cassava. comparison to the method of Cooke (1978). *Journal of the Science of Food and Agriculture*, **76**, 334-340.

Saka, J. D. K. and Nyrenda, K. K. (2012). Effect of two ethnic processing technologies on reduction and composition of total non-glucosidic

cyanogens in cassava. *Food Chemistry*, **130**, 605-609.

Sakamoto, S., Putalun, W., Vimolmangkang, S., Phoolcharoen, W., Shoyama, Y., Tanaka, H. and Morimoto, S. (2018). Enzyme-linked immunosorbent assay for the quantitative/qualitative analysis of plant secondary Metabolites. *Journal of Natural Medicines*, **72**, 32-42.

Sanchez-Verlaan, P., Geeraerts, T., Buys, S., Riu-Poulenc, B., Cabot, C., Fourcade, O., Megarbane, B. and Genestal, M. (2011). An unusual cause cause of severe lactic acidosis: cyanide poisoning after bitter almond ingestion. *Intensive Care Medicine*, **37**, 406-413.

Santelli, R. R. Micelli, A. S. and de Carvalho, M. F. B. (2006) Automated flow injection method for monitoring total cyanide concentration in petroleum refinery effluents using ninhydrin as color reagent. Spectroscopy Letter, 39, 605-618.

Sathe, M., Ghorpade, R., Merwyn, S., Agarwal, G. S., and Kaushik, M. P. (2012). Direct hapten-linked competitive inhibition enzyme-linked immunosorbent assay (CIELISA) for the detection of O-pinacolyl methylphosphonic acid. *Analyst*, **137**, 406-413.

Schultz, V. (1984). Clinical pharmacokinetics of nitroprusside, cyanide, thiosulfate and thiocyanate. *Clinical Pharmacokinetics*, **9**, 239-251.

Scott, G. J., Rosegrant, M. W., and Ringler, C. (2000). Roots and tubers for the 21st century trends, projections and policy options, pp.1-64.

Seigler, D. S., (1975). Isolation and characterization of naturally occurring cyanogenic compounds-Review. *Phytochemistry*, **14**, 9-29.

Selmar, D. (1993). Transport of cyanogenic glucosides: Linustatin uptake by Hevea cotyledons. *Planta*, **191**, 191-199.

Singh, K. V., Kaur, J., Varshney, G. C., Raje, M., and Suri, C. R. (2004). Synthesis and characterization of hapten-protein conjugates for antibody production against small molecules. *Bioconjugate Chemistry*, **15**, 168-173.

Sinha, S. and Nair, T. (1968). Studies on the variability of cyanogenic glucoside content in cassava roots. *Indian Journal of Agricultural Sciences*, **38**, 958-963.

Siritunga, D., Sayre, R. (2004). Engineering cyanogen synthesis and turnover in cassava (Manihot esculenta). *Plant Molecular Biology*, **56**, 661-669.

Sornyotha S., Kyu K. L., Ratanakhanokchai K., (2007). Purification and detection of linamarin from cassava root cortex by High Performance Liquid Chromatography. *Food Chemistry*, **104**, 1750 – 1754.

Spieker-Polet, H., Sethupathi, P., Yam, P.C., Knight, K. L. (1995). Rabbit Monoclonal antibodies: generating a fusion partner to produce rabbit-Rabbit hybridomas. *Proceedings of the National Academy of Science of the United States of America*, **92**, 9348-9352.

Stewart, S. J. (2001). Monoclonal antibody production. In Howard, G. C., & Bethell, D. R. (Eds), Basic Methods in Antibody Production and Characterization. CRC, Boca Raton, London, UK. pp. 51-67.

Suarez-Pantaleon, C., Mercader, J. V., Agulio, C., Abad-Somovilla, A. and

Abad-Fuentes, A. (2010). Hapten synthesis and polyclonal antibody-. based immunoassay development for the analysis of forchlorfenuron in kiwifruit. *Journal of Agricultural and Food Chemistry*, **58**, 8402-8511.

Sundaresan S., Amma C. S. E. & Nambisan B. (1987). Bitterness in cassava in relation to cyanoglucoside content. *Indian Journal of Agricultural Sciences*, **57**, 37-40.

Surleva, A. and Drochioiu, G. (2013). A modified ninhydrin micro-assay for determination of total cyanogens in plants. *Food Chemistry*, **141**, 2788-2794.

Szurdoki, F., Bekheit, H. K. M., Marco, M. P. (1992). Synthesis of haptens and Conjugates for an enzyme imunassay for analysis of the herbicide Bromacil. *Journal of Agricultural and Food Chemistry*, **40**, pp. 1459-1465.

Szurdoki, F., Szekacs, A., Le, H. M. and Hammock, B. D. (2002). Synthesis of haptens and protein conjugates for the development of immunoassay for the insect growth regulator fenoxycard. *Journal of Agricultural and Food Chemistry*. **50**, 29-40.

Takos, A., Lai, D., Mikkelsen, L., Hacem, M. A, Shelton, D., Motawia, M S. (2010) Genetic screening identifies cyanogenesis-deficient mutants of lotus japonicus and revelas enzymatic specificity in hyroxynitrile glucoside metabolism. *The Plant Cell*, **22**, 1605-1619.

Tatsuma T., Tani. K., Oyama N. and Yeoh H. H., (1996). Linamarin sensors: Amperometric sensing of linamarin using linamarase and glucose oxidase. *Journal of Electroanalytical Chemistry*, **407**, 155-159.

Tattersall D. B., Bak S., Jones P. R., Olsen C. E., Nielsen J. K., Hansen M. l., Hoj P. B., and Moller B. L. (2001). Resistance to an herbivore through engineered cyanogenic glucoside synthesis. *Science*, **293**, 1826-1828.

Themelis, D. G., Karastogianni, S. C. and Tzanavaras, P. D. (2009). Selective determination of cyanides by gas diffusion-stopped flow sequential injection analysis and an on-line standard addition approach. *Analytica Chemica Acta*, **632**, 93-100.

Tivana L. D., Francisco J. D. C., Zelder F., Bergenstahl B. and Dejmek P., (2014). Straightforward rapid spectrophotometric quantification of total cyanogenic glycosides in fresh and processed cassava products, *Food Chemistry*, **158**, 20-27.

Tiwari, P., Kumar, B., Kaur, M., Kaur, G. and Kaur, H. (2011). Phytochemical screening and extraction: A Review. Internationale Pharmaceutica Scienca, **1(1),** 98-106.

Trim A.R., (1955) Modern Methods of Plant Analysis (edited by K. Paech and M V. Tracey), *Springer-Verlag, Berlin*, **2**, 295.

Tshala-Katumbay, D. D., Ngombe, N. N., Okitundu, D., David, L., Westaway, S. K., Boivin, M. J., Mumba, N. D., Banea, J-P. (2016). Cyanide and the Human brain: perspectives from a model of food (cassava) poisoning. *Annals of New York Academy of Sciences*, **1378**, 50-57.

Tuomola, M., Harpio, R., Mikola, H., Knuuttila, P., Linström, M., Mukala, V. M.,

Matikainen, M-T. and Lövgren, T. (2000). Production and characterisation of monoclonal antibodies against a very small hapten, 3-methylindole. *Journal of Immunological Methods*, **240**, 111-124.

Turner, M. W. (1981). Structure and function of immunoglobulins. *In Structure and Function of Antibodies*. Glynn, L. E., and Stewart, M. W. (Eds). John Wiley and Sons, New York, USA. pp. 1-57.

Tylleskar, T., Banea-Mayambu, M., Bikangi, N., Cooke, R. D., Poulter, N. H., Rosling, H. (1992). Cassava cyanogens and konzo, an upper motoneuron disease found in Africa. *Lancet*, **229**, 208-211.

Van Weemen, B. K., and Schuurs, A. H. W. M. (1971). Immunoassay using antigen-enzyme conjugates. *FEBS Letters*, **15**, 232-236.

Vetter J., (2000). Plant cyanogenic glycosides. *Toxicon*. **38**, 11-36.

Voller, A., Bartlett, A., and Bidwell, D. E. (1978). Enzyme immunoassays with special reference to ELISA techniques-Review. *Journal of Clinical Pathology*, **31**, 507-520.

Ward, C. M., Wilkinson, A. P., Bramham, S., Lee, H. A., Chan, H. W. S., Butcher, G. W., Hutchings, A., and Morgan, M. R. A. (1990). Production and characterization of polyclonal and monoclonal antibodies against aflatoxin B1 oxime-BSA in an enzyme-linked immunosorbent assay. *Mycotoxin Research*, **6**, 73-83.

Wasserkrug, K. and Rassi, Z. E. (1997). High performance liquid phase separation of glycosides. I. Reversed phase chromatography of cyanogenic glycoside with UV and pulsed amperometric detection. *Journal of Liquid Chromatography and Related Technology*, 20, 335-349.

Weller, M. G., Diemer, M., Wersching, C., Niessner, R., Sochor, H. (2003). Development of antibodies for the detection of N-acetyl-glufosinate, *Journal of Agriculture and Food Chemistry*, **51(23)**, pp. 6668-6675.

Westby, A., Choo, B. K. (1994). Cyanogen reduction during lactic acid fermentation of cassava. *Acta Horticulturae*, **375**, 209-215.

Westby A., (2002). Cassava utilization, storage and small scale processing. In R. J. Hillock, J. M. Thresh & A. C. Belotti (eds.), Cassava biology, production and utilization (pp. 281-300). Wallingford, UK: CABI Publishing.

Wild, D. (2013). The immunoassay handbook: theory and applications of ligand binding, ELISA and related techniques, 4th Edition In Wild. D. (eds.), John, R., Sheehan, C, Binder, S. and He, J., Elsevier, UK.

Williams, H. J. (1979). Estimation of hydrogen cyanide released from cassava by organic solvents. *Experimental Agriculture*, **15**, 393-399.

Wink, M. (1988). Plant breeding: importance of plant secondary metabolites for protection against pathogens and herbivores – review. *Theoretical and Applied Genetics* **75**, 225-233.

Wittstock, U., and Gershenzon. J., (2002). Constitutive plant toxins and their role in defense against herbivores and pathogens-review. *Current Opinion in Plant Biology*, **5**, 300-307.

Wobeto, C., Correa, A. D., Abreu, C. M. P., Santos, C. D. and Pereira, H. V.

(2007). Antinutrients in the cassava (Manihot esculenta Crantz) leaf powder at three ages of the plant. *Food Science and Technology (Campinas)*, **27**, 108-112.

Yallow, R. S. and Berson, S. A. (1959). Assay of plasma insulin in human Subjects by immunological methods. *Nature*, **184**, 1648-1649.

Wood, T., (1966). *Journal of the Science of Food and Agriculture*, **17**, 85.

Yen, D., Tsai, J., Wang, L-M., Kao, W. F., Hu, S-C., Lee, C-H., and Den, J-F. (1995). The clinical experience of acute cyanide poisoning. *American Journal of Emergency Medicine* ,**13**, 524-528.

Yeoh, H. H. (1993). Nylon linamarase electrode for rapid determination of linamarin. *Biotechnology Technique*, **7**, 761-764.

Yeoh, H. H., and Truong, V. D. (1993). Quantitative analysis of linamarin in cassava using a cassava β-glucosidase electrode. *Food Chemistry*, **47**, 295-298.

Yeoh, H. H., Tan, C. K. C. (1994). An enzyme-immobilized microplate for determination of linamarin for large number of samples. *Biotechnology Technique*, **8**, 337-338.

Yeoh, H. H., Lim, L. S., and Woo, H. C. (1996). An enzyme-bound linamarin indicator paper strip for the semi-quantitative estimation of linamarin. *Biotechnology Technique*, **10**, 319-322.

Yeoh, H. H., and Egan, S. V. (1997). An enzyme-based dip-stick for the estimation of cyanogenic potential of cassava flour. *Food Chemistry*, **60**, 119-122.

Yeoh, H. H., Sun, F. (2001). Assessing cyanogen content in cassava-based food using the enzyme-dipstick method. *Food and Chemical Toxicology*, **39**, 649-653.

Yu, F-Y., Chi, T-F., Liu, B-H., and Su, C-C. (2005). Development of a sensitive enzyme-linked immunosorbent assay for the determination of ochratoxin A. *Journal of Agricultural and Food Chemistry*, **53**, 6947-6953.

Yu, Y., Chen, Y., Ding, G., Wang M., Wu, H., Xu L. (2015). A novel rabbit Anti-hepatocyte growth factor monoclonal neutralizing antibody inhibits Tumor growth in prostate cancer cells and mouse xenografts. Biochemical Biophysics Research Communicators, **464**, 154-160.

Zagrobelny, M., Bak, S., Rasmussen, A. N., Jørgensen, B., Naumann, C. M. and Møller, B.L. (2004). Cyanogenic glycosides and plant-insect interactions. *Phytochemistry*, **65**, 293-306.

Zagrobelny, M., Bak, S., Møller, B.L. (2008). Cyanogenesis in plants and arthropods. *Phytochemistry*, **69**, 1457-1468.

Zagrobelny, M., Pinheiro de Castro, E. C., Møller, B.L. and Bak, S. (2018). Cyanogenesis in arthropods: From chemical warfare to nuptial gifts. Review, *Insects*, **9**, 1-51.

Zelder, F. H. (2008). Specific colorimetric detection of cyanide triggered by a conformational switch in vitamin B12. *Inorganic Chemsitry*, **47**, 1264-1266.

Zhang, P., Bohl-Zenger, S., Puonti-Kaerlas, J., Potrykus, I., and Gruissem, W.

(2003). Two cassava promoters related to vascular expression and storage root formation. *Planta*, **218**, 192-203.

Zhang Qi, Li, T-J., Zhu, X-X., Xu, L-N., Liu, F-Q., Hu, B-S., Jiang, Y-H., and Cao, B. (2007). Production and characterization of polyclonal antibody for the N-methylcarbamate insecticide metolcarb. *Journal of Agricultural Science China*, **6**, 175-179.

Zhu, W. and Yu, G. L. (2009). Rabbit hybridoma. In : An, Z (ed.) *Therapeutic Monoclonal Antibodies: From Bench to Clinic*. John Wiley & Sons: Hoboken, NJ, USA, pp 151-168.

Zitnak, A. (1973). Assay methods for hyrocyanic acid in plant tissues and their application in studies of cyanogenic glycosides in Manihot esculenta. Proceedings of an interdisciplinary workshop, London, England, 29 - 30 January 1973. International Development Research Centre. Monogram IDRC-010e, 89-96.

Zitnak, A., Hill D. C., Alexander J. C., (1977). Determination of Linamarin in Biological Tissues. *Analytical Biochemistry*, **77**, 1, 310-314.

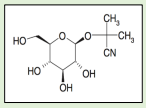
Appendices

Raising Polyclonal Antibodies Against Linamarin – a Cyanogenic Glycoside in Cassava

Ahmad F. M. Azmi[1,2], Michael R. A. Morgan[1]
[1]School of Food Science & Nutrition, University of Leeds, Leeds, LS2 9JT
[2]Centre for Defence Foundation Studies, National Defence University of Malaysia, 57000 Kuala Lumpur, Malaysia

1 The aim of this study is to develop a specific, sensitive immunoassay for determination of linamarin in cassava and cassava products.

Cassava (*Manihot esculenta* Crantz) also known as tapioca, manioc



Linamarin – a cyanogenic glycoside in cassava

Hydrogen cyanide – inhibits cellular respiration

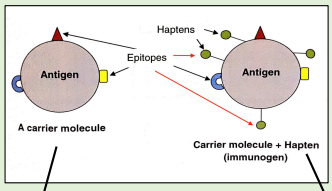
Cyanogenic glycosides release hydrogen cyanide (HCN) under certain conditions leading to toxic effects:

- Cyanide poisoning, tropical ataxic neuropathy, konzo – upper motor neurone disease, goitre, in extreme cases, death.

Immunoassay, using antibody specificity can be used in rapid, simple and cost effective methods of analysis to detect linamarin in food.

2 How we designed the immunogen?

Linamarin is of low molecular weight (<1000 daltons) – it can't elicit immune response to produce antibody unless it is conjugated to a bigger protein.

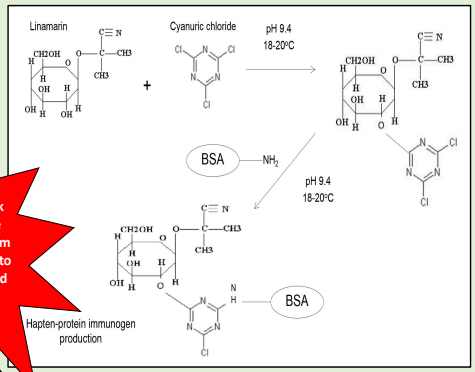


Carrier molecule = Bovine serum albumin (BSA)

Hapten = Linamarin

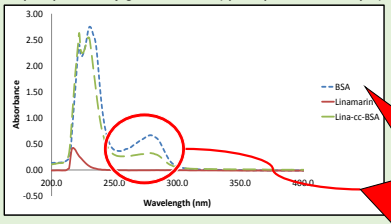
3 How did it occur chemically?

Linamarin-cyanuric chloride-BSA conjugation steps



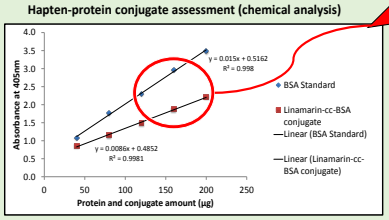
4 How we assessed the conjugate?

Hapten-protein conjugate assessment (spectrophotometric analysis)



UV absorption spectra of BSA (carrier protein), linamarin (hapten) and linamarin-cc-BSA (conjugate) from the cyanuric chloride method

Hapten-protein conjugate assessment (chemical analysis)

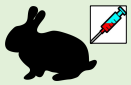


TNBS standard curves obtained with BSA and linamarin-BSA conjugate from the cyanuric chloride method, showing the reduction in free amino acids of BSA after conjugation to linamarin

Lower conjugate peak and reduction in free amino acids in BSA from TNBS assay compared to standard BSA indicated that conjugation had occurred!!

5 How we raised the antibody?

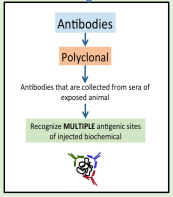
Immunization to raise the polyclonal antibodies



Immunization plan

Day	Procedure
0	Pre-immune bleeds (4-5 ml)
0	Injection (0.5 ml antigen + 0.5 ml complete Freund's adjuvant)
21	Injection (0.5 ml antigen + 0.5 ml incomplete Freund's adjuvant)
42	Injection (0.5 ml antigen + 0.5 ml incomplete Freund's adjuvant)
53	Test bleed (4-5 ml)
63	Injection (0.5 ml antigen + 0.5ml incomplete Freund's adjuvant)
74	Test bleed (12-15 ml)
88	FINAL BLEED (60-80 ml)

* Immunization using 2 New Zealand's white rabbit



6 What are we going to do with it?

Characterization of the immunoassay (ELISA) method to determine linamarin in food samples:

- Optimisation of the indirect competitive ELISA
- Cross-reactivity study
- Determination of matrix effect

And finally:

- Application of optimised ELISA method to quantify linamarin amount in fresh and cassava products.

7 Take home message

- ✓ The amount of linamarin retained in fresh and processed cassava are not negligible based on previous studies.
- ✓ The availability of an appropriate antibody could lead to the development of a simple dip-stick procedure that could be used away from specialised resources.

References:
1. Bolarinwa I. F., Orfila C. and Morgan M. R. A. (2014). Development and application of an enzyme-linked immunosorbent assay (ELISA) for the quantification of amygdalin, a cyanogenic glycosides, in food. J. Agric *Food Chem.* 62: 6299-6305.
2. Paraf A., and Peltre G. (1991). Immunoassays in food and agriculture. Kluwer Academy, Lancaster, UK. Pp. 9-35.

Cyanogenic glycoside content in fresh cassava and processed products commercially available in the UK

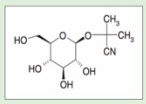
Ahmad F. M. Azmi[1,2], Michael R. A. Morgan[1]

[1]School of Food Science & Nutrition, University of Leeds, Leeds, LS2 9JT

[2]Centre for Defence Foundation Studies, National Defence University of Malaysia, 57000 Kuala Lumpur, Malaysia

1 The aim of this study is to develop a specific, sensitive immunoassay for determination of linamarin in fresh cassava and cassava products.

Cassava (*Manihot esculenta* Crantz) also known as tapioca, manioc



Linamarin – a cyanogenic glycoside in cassava

Hydrogen cyanide – inhibits cellular respiration

Cyanogenic glycosides release hydrogen cyanide (HCN) under certain conditions leading to chronic/acute toxic effects:

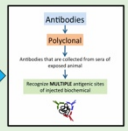
- Cyanide poisoning, tropical ataxic neuropathy, konzo – upper motor neurone disease, goitre, in extreme cases, death.

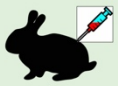
Immunoassay, using antibody specificity can be used in rapid, simple and cost effective methods of analysis to detect linamarin in food.

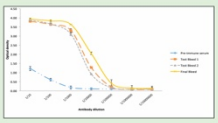
2 Why are we concerned about this?

3 How we detect and quantify linamarin?

The antibody against linamarin was obtained by immunizing 2 New Zealand white rabbit with conjugated linamarin.

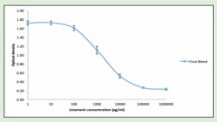






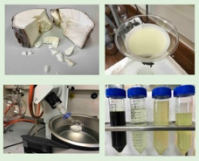
Antibody titre obtained from rabbit CUK1680R2 showing antibody activity even at dilution as low as 1/100,000.

Indirect competitive ELISA obtained against standard linamarin showing significant binding of the antibody towards the free linamarin at dilution of 1/50,000.



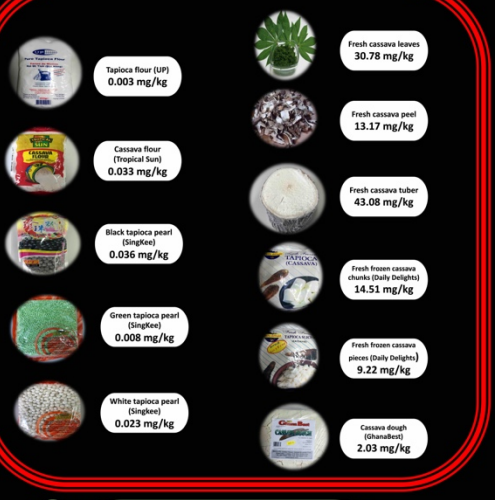
Linamarin content!!

ELISA



Sample extraction

4 Linamarin content in some of the products available in the UK



5 Results and Discussion

- Polyclonal antibody has been successfully developed against hapten (linamarin).
- The antibody has high specificity and sensitivity towards linamarin achieving antibody titre as low as 1/100,000 and working antibody concentration at 1/50,000.
- WHO/FAO has set the safe level of cyanide in cassava at 10 mg/kg and estimated the lethal dose would be around 0.5 ~ 3.5mg/kg body weight. The UK Committee of Toxicity set a total daily intake of cyanide at 20µg per kg body weight.
- Traces of linamarin have been detected in fresh cassava and processed cassava products commercially available in UK market which most of them were imported from other countries.

6 Take home message

- ✓ The amount of linamarin retained in fresh cassava is not negligible as they exceeded the safe level set by WHO/FAO, and there were minute trace of linamarin detected in highly processed cassava products.
- ✓ Current labelling on cassava products imported into the UK are lacking hazardous warning and cooking instructions which is a major concern.
- ✓ These findings suggest that appropriate warning label and cooking instructions are required on any cassava products commercially selling in the UK.

References:

1. Bolarinwa I. F., Orfila C. and Morgan M. R. A. (2014). Development and application of an enzyme-linked immunosorbent assay (ELISA) for the quantification of amygdalin, a cyanogenic glycosides, in food. *J. Agric Food Chem.* 62: 6299-6305.
2. Lawley R., Curtis L., Davis J. (2012). The Food Safety Hazard Guidebook 2nd Edition, Chapter 2.1: 266-269.
3. Makkar P. H., Siddharaju P., Becker K. (2007), Cyanogenic glycosides/cyanogens Methods in Molecular Biology, 393: 61-69.