Cyanogenic Glycosides in Plant Foods

Islamiyat Folashade Bolarinwa

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 2013

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

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisors Professor Mike Morgan and Dr Caroline Orfila for their guidance, constructive comment, patience and support during the course of this project.

I would like to thank Dr Lisa Marshall for allowing me to use the hplc in her laboratory.

I am very grateful to Education Trust Fund (ETF) of Nigeria and to Ladoke Akintola University of Technology (LAUTECH) for funding this research.

I would like to thank my colleagues, Nasser Al-Shabib, Swen Langer, Merfat Al-Maghrabi, Heba Sindi, Hanis Yahyah, Ebun Oladele, Wessal Almabruk, Sarah Hamli, Maha Thaiban, Abdul-rahman Sweiden and Nicola Kraut for their friendship, good humour and helpful nature that help to create an excellent working environment.

I am grateful to all members of staff of the School of Food Science and Nutrition. Especially Dr Mahmood Akhtar, Dr Melvin Holmes, Dr Tristan Dew, Mr Ian Hardy, Mr Mattew Taylor, Mr Paul Kajda and Mr Miles Ratcliffe for their help and assistance.

Special thanks to my sister, Dr S.T.Taiwo for her spiritual and financial support, and to Dr A. Salaudeen and family for their precious help and support.

Many thanks to my lovely kids, Abeedah, Abubakr and Aisha and my husband, Mr A. Bolarinwa for their endurance, patience, support and love during the course of this project.

ABSTRACT

Consumption of cyanogenic plants can cause serious health problems for humans. The ability to detect and quantify cyanogenic glycosides, capable of generating cyanide, could contribute to prevention of cyanide poisoning from the consumption of improperly processed cyanogenic plants. In the first part of the present study, an efficient extraction method for amygdalin from foods was developed by comparing the extraction efficiency of water and ethanol at room and boiling temperature for various extraction times. Efficiencies of the extraction methods were evaluated by using a modified HPLC method. In general, boiling ethanol was more efficient for amygdalin extraction from foods. Amygdalin contents of a wide range of commercially-available food products were determined for the first time. In the second part, the effect of processing on amygdalin levels in apple juice was investigated. Apple juices produced from different apple varieties were subjected to various processing conditions such as freezing, pasteurization and holding for various times. The results obtained showed that processing can result in reduction of amygdalin level in apple juice and that the reduction of amygdalin by enzymatic degradation probably depends on the level of the enzyme in the processed foods and the utilisation of optimum conditions for the enzyme activities. High-temperature treated commercially-available apple juice had lower amygdalin levels compared to low-temperature treated juices, indicating the degradation of amygdalin at high processing temperatures. In the last part, the development of a sensitive ELISA method for amygdalin was investigated. Antibody produced by using an immunogen that was synthesized using the cyanuric chloride method for coupling of hapten to carrier protein was highly immunoreactive and very specific for amygdalin. The ELISA developed using this antibody was very sensitive (LOD = 0.2ng/ml). The results obtained from the determination of amygdalin in foods using the ELISA method showed excellent correlation with those obtained from HPLC method.

The work described is the first comprehensive study on detection of a particular cyanogenic glycoside and use of an antibody-based method for determination of amygdalin in processed foods. The results obtained suggested that while the consumption of products available in the UK with the potential for containing amygdalin are unlikely to present a health risk to consumers, monitoring of new processing protocols would be wise.

Publications Arising From Research in the Thesis

Islamiyat F. Bolarinwa, Caroline Orfila, Michael R.A. Morgan (2013) *Amygdalin Content of Seeds, Kernels and Food Products Commercially-available in the UK*. Submitted manuscript.

Conference presentations

Islamiyat F. Bolarinwa, Caroline Orfila, Michael R.A. Morgan. Poster presentation: *Improving Detection Methods for Cyanogenic Glycosides in Foods.* First Africa College International Conference on Food security, Health and Impact Knowledge Brokering, Leeds, England, UK, June, 2011.

Islamiyat F. Bolarinwa, Caroline Orfila, Michael R.A. Morgan. Poster presentation: *An Enzyme-linked Immunosorbent Assay (ELISA) for the Determination of Amygdalin, a Cyanogenic Glycoside, in Foods*. 245th American Chemical Society National Meeting and Exposition on Chemistry of Energy and Food, New Orleans, Louisiana, USA, April, 2013.

CONTENTS

List of figures

List of tables

Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
B-cell	Bone marrow lymphocytes
Bp	Boiling point
BSA	Bovine serum albumin
CC	Cyanuric chloride
DNA	Deoxyribonucleic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
ELSD	Evaporative light scattering detector
Fe	Iron
GCMS	Gas chromatography-mass spectrometry
HCN	Hydrogen cyanide
IgG	Immunoglobulin G
IRMA	Immunoradiometric assay
KLH	Keyhole limpet hemocyanin
LOD	Limit of detection
LOQ	Limit of quantification
M	Moles per litre
MHC	Major histocompatibility complex
mRNA	Messenger Ribonucleic acid
OVA	Ovalbumin
PAD	Pulsed amperometric detector
PBST	Phosphate buffered saline with tween 20
PCR	Polymerase chain reaction
RIA	Radioimmunoassay
RP-HPLC	Reversed-phase high performance liquid chromatography
ScFv	Single-chain variable fragment
TMB	3, 3', 5, 5'- Tetramethyl benzidine

TNBS	Trinitrobenzene sulfonic acid
UHT	Ultra-heat treated
UV	Ultraviolet
VH	Heavy chain variable region
VL	Light chain variable region
v/v	Volume to volume ratio
w/v	Weight to volume ratio

Chapter 1

Introduction

1.1 Plant toxins

Plant toxins are naturally-occurring secondary metabolites that may be poisonous to pathogens, herbivores and humans. They protect plants from predators by acting as chemical defense compounds against herbivores, insects and pathogens (Table 1-1). They usually behave by altering specific mechanisms involving enzymes, receptors and genetic targets in particular cells and tissues (Wink, 1988). They may be present in the whole plant or in seeds, fruits, leaves or stalks. Depending on plant species, the poisonous constituent may be concentrated in one or more parts of the plants or it may be present throughout the plant (Chandra *et al*., 2012). According to a review by Wittstock and Gershenzon (2002), plant parts that are of high risk of attack by herbivores or other predators tend to accumulate high levels of toxic compounds compared to the parts that are rarely attacked. Although not all plant secondary metabolites are toxins, this study focused on the secondary metabolites that are toxins. The toxins are toxic to humans, animals and insects when they come in contact with them either internally or externally. Plant toxins such as hypericin are also used as signals to control physiological responses to attack (Kliebenstein, 2012).

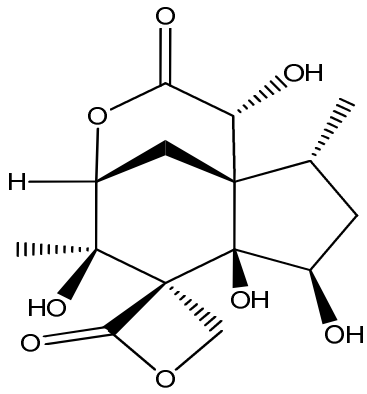
Plant toxins are classified based on their structural and chemical constituents: alkaloids, glycosides, proteins, oxalates, anti-vitamins, tannins, volatile ethers, phytoestrogens, terpenes, glucosinolates and

phenylpropanoids (Wink, 1988; Chandra *et al*., 2012). Structures of examples of plant toxins are shown in Figure 1-1.

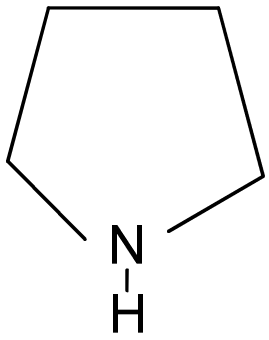
Table 1-1: Chemical protection strategies of plant secondary metabolites

Compound	**Biological activity**
Alkaloids	Toxic for vertebrates, arthropods, bacteria
Cyanogenic glucosides	Toxic for animals
Flavonoids/anthocyanins	Antimicrobial, insectistatic
Glucosinolates	Repellant for animals, antimicrobial
Non-protein amino acids	Toxic for animals, antimicrobial
Phenylpropanoids	Antimicrobial
Terpenes	Repellent for animals, antimicrobial

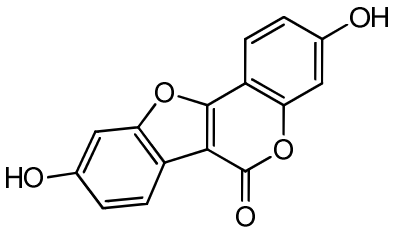
(Adapted from Wink, 1988)



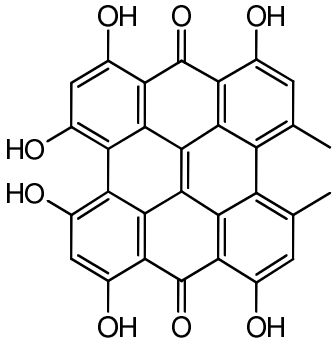
Anisatin (found in Japanese anise plant)



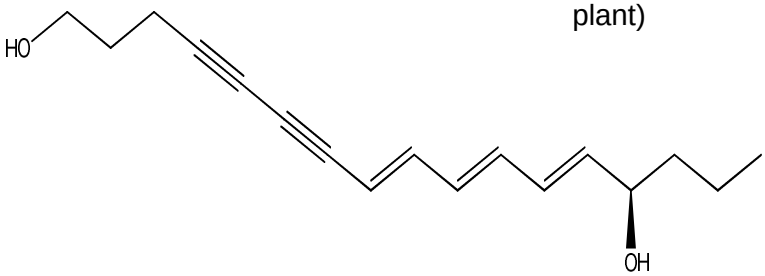
Pyrrolizidine (found in the leaves of tobacco and carrot)



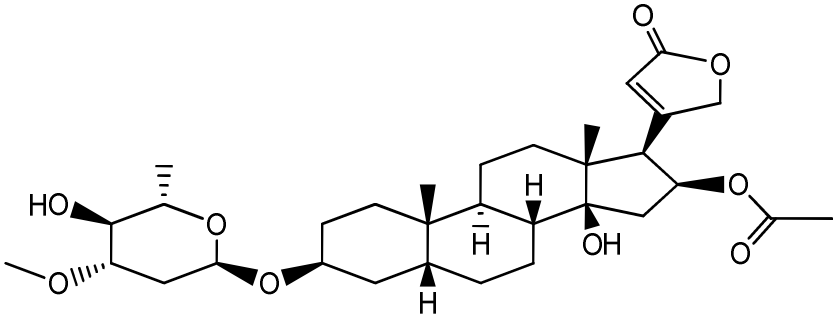
Coumestrol (found in alfalfa, red clover and soybeans)



Hypericin (found in St John's wort plant)



Cicutoxin (found in western water-hemlock)



Oleandrin (found in nerium oleander and Laurier rose plant)

Figure 1-1: Structures of example plant toxins

Examples of specific natural plant toxins from each class of plant toxin are listed below;

(i) Plant toxins categorised based on the chemistry of the compounds;

- Nitrile containing compounds;
  - Alkanenitriles e.g. cyanaogenic glycosides.
  - α, β-unsaturated nitriles e.g. samentosin.
  - Aromatic nitriles e.g. Cytotoxin (Fleming, 1999).
- Sulfur containing compounds e.g glucosinolate (Halkier & Gershenzon, 2006).
- C17 conjugated polyacetylene compounds e.g. cicutoxin, oenanthotoxin (Schep *et al*., 2009).
- Glucoside of methylazoxymethanol e.g. cycasin (Wogan & Busby, 1980).
- Ester derivatives of 1-hydroxymethyl-1, 2-dehydropyrrolizidine e.g. pyrrolizidine alkaloids (Wogan & Busby, 1980).

(ii) Plant toxins categorized based on their toxic effect;

- Carcinogens e.g. pyrrolizidine alkaloids, cycasin (Wogan & Bussy, 1980).
- Those that inhibit cellular respiration e.g. cyanogenic glycosides (Wajant & Mundry, 1994; Hamel, 2011).
- Those that discrupt cellular membranes e.g. saponin (Osbourn , 1996).

- Central nervous system stimulatory effect (including seizures) e.g. cicutoxin, oenanthotoxin (Schep *et al*., 2009).
- Growth inhibitor e.g. glucosinolate (Ulmer *et al*., 2001).

Several plant species are cultivated for food and many plants or plant parts (seeds, leaves, fruits) are consumed as food. Seeds are important food 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. Table 1-2 shows examples of plant toxins and their harmful effects.

Table 1-2: Source of some plant toxins and their harmful effects

Plant name	Plant part	Toxin	Harmful effect
Black locust	Bark, seeds	Glycoprotein-abrin, ricin, lectin	Constipation, diarrhea, muscle weakness, ataxia
Buckwheat	Whole plant except ripe seeds	Fagopyrin, dianthroquinones	Photosensitization, sloughing of skin
Castor bean	Seeds	Ricin, lectin	Diarrhea, depression, anorexia, bloat
Choke cherry	Wilted leaves	Cyanogenic glycosides-prunasin, amygdalin	Increased salivation, nervousness
Crotolaria	Whole plant	Pyrrolizidine alkaloids	Weight loss, depression
Lupin	Whole plant	Quinazolidine alkaloids, anagyrine	Teratogenic, respiratory failure
Potato	Foliage	Glycolalkaloids-solanine	Diarrhea, abdominal cramps, headache
Rhubarb	Whole plant	Oxalic acid	Nephrotoxic
Rosary pea	Whole plant	Abrin	Photosensitivity, vomiting, convulsive seizures, pulmonary edema
Sweet pea	Whole plant	Glycosides	Diarrhea, abdominal cramps, headache
Tobacco	Whole plant	Nicotine, anabasine	Teratogenic

(Adapted from Chandra *et al.,* 2012)

1.1.1 Cyanogenic glycosides

Cyanogenic glycosides are the most important defense-related secondary metabolites and class of natural plant toxin in plant foods. Cyanogenic glycosides are plant toxins that consist of α-hydroxinitrile aglycones and a sugar moiety (Vetter, 2000). They are widely distributed in the plant kingdom, being present in more than 2,500 species (Ganjewala *et al*., 2010). The structure of some cyanogenic glycosides and their sugar moiety is shown in Table 1-3. Cyanogenic glycosides are common in certain families such as the Fabaceae, Rosaceae, Leguminoseae, Linaceae and Compositae and identification of their constituents' is a useful tool for informative taxonomic markers (Vetter, 2000). Many studies have shown the efficiency of cyanogenic glycosides as defence agents in nature. For example, feeding experiments conducted by Ballhorn *et al*. (2009) showed that when two natural insect herbivores of lima bean (*Gynandrobrotica guerreroensis and Cerotoma ruficornis*) were fed with high cyanide-containing leaves and low cyanide leaves, the total leaf consumption was low (15%) when the insects were provided with high cyanide leaves compared to when low cyanide leaves (total leaf consumption was 85%) were available for their consumption, thus indicating the high repellent activity of high cyanogenic potential to herbivores. In another study by Tattersall *et al*. (2001), when the pathway for the biosynthesis of the cyanogenic glycoside dhurrin was transferred from sorghum into an acyanogenic plant (*Arabidopsis thaliana*), the transgenic *Arabidopsis thaliana* had resistance to flea beetle conferred upon it.

In addition to their defence function in plants, cyanogenic glycosides have been reported to serve important functions in primary metabolism. According to Moller (2010), a high concentration of cyanogenic glycosides in seeds during germination may serve to provide aspartate or asparagines for transmination reactions required to balance amino acid supply to the developing seedling. Cyanogenic glycosides have been reported to serve as storage forms for reduced nitrogen (Poulton, 1990).

Several thousand plant species, including economically important food plants, synthesize cyanogenic glycosides; linamarin in cassava and butter bean, dhurrin in sorghum and macadamia nut, amygdalins in almond, peach, sweet cherry and sour cherry (Jones, 1998; Vetter, 2000; Donald, 2008). The level of cyanogenic glycosides in plants depends on the age and variety of the plant as well as environmental factors. For instance, cassava crops grown in low altitude areas have been reported to contain high levels of cyanogenic glycosides while those grown in high altitude areas contain low levels of cyanogenic glycosides (Oluwole *et al*., 2007). Although most plants produce small amounts of cyanide, more than 300,000 plants produce different secondary metabolites including cyanogenic glycosides (Zagrobelny *et al*., 2008). Levels of cyanogenic glycosides in different parts of the plants vary and also vary between the same parts of different individual plants of the same species. While high concentrations of cyanogenic glycosides are usually found in plant leaves, cyanogenic

glycosides may also be concentrated in plant roots, seeds or other plant tissues (Seigler, 1975). Table 1-4 lists the major cyanogenic glycosides of food plants and their cyanogenic potential.

The occurrence of cyanogenic glycosides in food and fodder can be a significant social and economic problem in many parts of the world. In Africa, dietary use of cassava (*Manihot esculenta* Crantz) has been associated with cyanide poisoning, tropical neuropathy disease and konzo (Tylleskär *et al*., 1992; Mlingi *et al*., 1992; Teles, 2002; Ernesto *et al*., 2002). In 1992, cyanide from cyanogenic glycosides of cassava was responsible for the death of three people in Nigeria (Alade and Tunwashe, 1992). Recently, five Nigerians apparently died of cyanide poison after eating a meal prepared with cassava flour (Makinde, 2010).

Despite the toxicity of cyanogenic glycosides, farmers continue to grow crops with high levels of cyanogenic glycosides, because they act as natural pesticides to protect crops against animal pests.

Table 1-3: The chemical structure of some cyanogenic glycosides and their sugar moiety

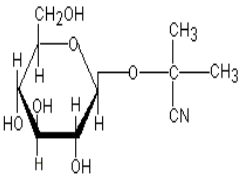
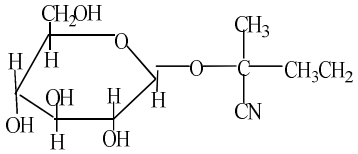
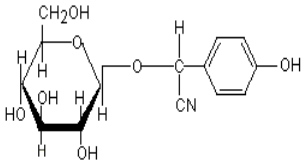
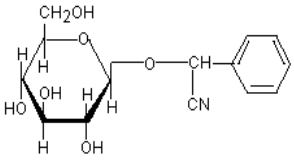
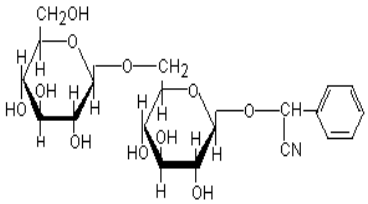
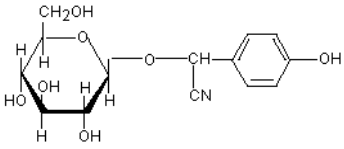
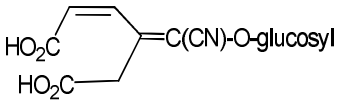
Configuration	Glycoside	Sugar
	Linamarin	D-glucose
	Lotaustralin	D-glucose
	Dhurrin	D-glucose
	Prunasin	D-glucose
	Amygdalin	Gentiobiose
	Taxiphyllin	D-glucose
	Triglochinin	D-glucose

Table 1-4: Major cyanogenic glycosides of food plants and their cyanogenic potential

Food	Major cyanogenic glycosides present	Potential for Cyanide generation (mg HCN/kg)
Cassava (*Manihot esculenta*) – root	Linamarin	15-1000
Sorghum (*Sorghum vulgare)* – leaves	Dhurrin	750-790
Flax (*Linum usitatissimum*) - seed meal	Linamarin, linustatin, neolinustatin	360-390
Lima beans (*Phaseolus lunatus*)	Linamarin, lotaustralin	2000-3000
Giant taro (*Alocasia macrorrhizos*) - leaves	Triglochinin	29-32
Bamboo (*Bambusa arundinacea)* - young shoots	Taxiphyllin	100-8000

(Haque & Bradbury, 2002; Simeonova & Fishbein, 2004).

1.1.1.1 Biosynthesis of cyanogenic glycosides

Cyanogenic glycosides are derived from five amino acids; valine, isoleucine, leucine, phenylalanine and tyrosine, and from the non-proteinogenic amino acid, cyclopentenyl glycine. For example, linamarin and lotaustralin are derived from valine, isoleucine and leucine, dhurrin is derived from tyrosine, and amygdalin and prunasin are derived from phenylalanine (Seigler, 1975; Conn, 1979). Several authors have described the biosynthesis of cyanogenic glycosides in different plants. For instance the biosynthesis of

the tyrosine-derived cyanogenic glycosides dhurrin has been described extensively in sorghum and other plants such as seaside arrow grass, cassava and barley (as reviewed by Ganjewala *et al.,* 2010).

The biosynthetic pathway of cyanogenic glycosides is illustrated in Figure 1-2. The first and the second steps of cyanogenic glycoside biosynthesis are catalysed by a cytochrome P450 through two successive N-hydroxylations of the amino group of the parent amino acid, followed by decarboxylation and dehydration; α-hydroxynitrile is produced from the action of a second cytochrome P450 on the aldoxime (Kahn *et al*., 1997; Bak *et al*., 1998). Hydroxynitrile (also known as cyanohydrin) is generated following an initial dehydration reaction to form a nitrile through the hydroxylation of the alpha carbon and glycosylation of the cyanohydrin moiety (the final step in cyanogenic glycoside synthesis) is catalysed by UDPG-glycosyltransferase (Jones *et al*., 1999).

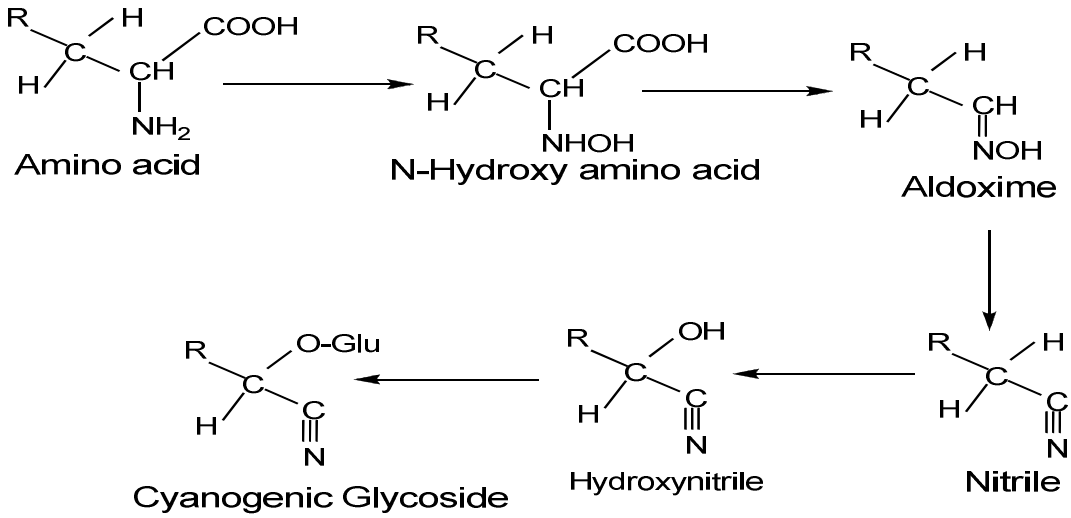


Figure 1-2: The biosynthetic pathway for cyanogenic glycosides from its precursor amino acid.

1.1.1.2 Cyanogenesis

Cyanogenesis is the ability of plants and other living organisms to release hydrogen cyanide (Poulton, 1990; White *et al*., 1998; Zagrobelny *et al*., 2008; Ballhorn *et al*., 2009). Although cyanogenic glycosides are not toxic when intact, enzymic action on cyanogenic plants causes release of hydrogen cyanide causing potential toxicity issues for animals including humans. While cyanogenic glycosides are stored in vacuoles within the cell, the enzymes are stored in the cytoplasm. When the plant tissues are disrupted by herbivores or during processing, toxic hydrogen cyanide could be released from the hydrolysis of cyanogenic glycosides by plant endogeneous enzymes; β-glucosidase and α-hydroxynitrile lyases (Zagrobelny *et al*., 2004).

In plants, cyanogenic glycosides serve as important chemical defense against herbivores due to their ability to generate hydrogen cyanide (Zagrobelny *et al*., 2004; Ganjewala *et al*., 2010). In humans, consumption of cyanogenic plants could cause acute cyanide poisoning with associated symptoms including; anxiety, headache, dizziness, confusion, decreased consciousness, hypotension, paralysis and coma. Cases of cyanide poisoning are common from the consumption of plants of *Rosaceae* (peach, apricot, plum, apple seed), *Leguminosae* (Lima beans, broad beans), *Euphorbiaceae* (cassava) or members of the genus *Sorghum* (Jones, 1998; Francisco & Pinotti, 2000; Vetter, 2000). Indeed, incidents of acute cyanide poisoning have been reported from Literature. For instance, a woman was

affected with cyanide poisoning after ingesting apricot kertnels (Suchard *et al*., 1998), thirteen people were also reported to be intoxicated with cyanide after eaten 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), three people were also affected with cyanide posoining after consuming a cassava based meal (Akintonwa & Tunwashe, 1992).

1.1.1.3 Enzymatic hydrolysis of cyanogenic glycosides in food plants

Enzyme activity results in cleavage of the carbohydrate moiety of the cyanogenic glycoside to yield corresponding cyanohydrins which further decompose to release hydrogen cyanide and an aldehyde or ketone (Poulton, 1990).

In cassava, the cyanogenic glycosides linamarin (α-hydroxybutyronitrile-β-D-glucopyranoside) and lotaustraulin (ethyl linamarin) are located in the plant vacuoles and the enzyme is located in the cell wall (Gruhnert *et al*., 1994). Hydrolysis of linamarin from cassava starts from the disintegration of the root tissue during processing or chewing to release the endogenous enzyme (linamarase) that hydrolyses linamarin to glucose and acetone cyanohydrins (which are unstable in neutral/alkaline solution). During processing, reduced

moisture and/or increased temperature facilitate the spontaneous breakdown of cyanohydrins to toxic hydrogen cyanide (McMahon, 1995).

In sorghum, the cyanogenic glycoside dhurrin (4-hydroxymandelonitrile-β-D-glucopyranoside) is stored in the plant vacuoles (separated from the enzymes). When the plant tissue is crushed, the enzymes and dhurrin are brought in contact and hydrolysis is initiated by β-glucosidase (dhurrinase) which hydrolyzes the cyanogenic glycoside to form hydroxymandelonitrile and glucose. Hydroxymandelonitrile further decomposes spontaneously or enzymatically by the action of hydroxynitrile lyase to form hydrogen cyanide and hydroxybenzaldehyde (Wajant *&* Mundry, 1994). Enzymatic hydrolysis of linamarin and dhurrin is illustrated in Figure 1-3.

Amygdalin (D-mandelonitrile-β-D-gentiobioside) is one of the most common cyanogenic glycosides. It can be found in plant families of the Caprifoliaceae, Mimosaceaw, Oleaceae and Rosaceae (Vetter, 2000) and is present in kernels and seeds of fruits such as apples, apricots, almonds, cherries, plums and peaches (Donald, 2009). Amygdalin and prunasin (D-mandelonitrile-β-D-glucopyranoside) are degraded by endogenous enzymes (β-glucosidases) to form non-glycosidic cyanogen molecules; mandelonitrile and free (CN^-) cyanide ion (Tunçel *et al*., 1998). Enzymatic degradation of amygdalin is divided into three parts. The first part involves the splitting of amygdalin to prunasin and glucose by the enzyme amygdalin lyase. The second part is the hydrolysis of prunasin to mandelonitrile and glucose by

the enzyme prunasin lyase and the final stage of the hydrolysis is the breaking down of mandelonitrile to benzaldehyde and hydrogen cyanide (HCN) by hydroxynitrile lyase (Haisman & Knight, 1967). Enzymatic hydrolysis of amygdalin is illustrated in Figure 1-4.

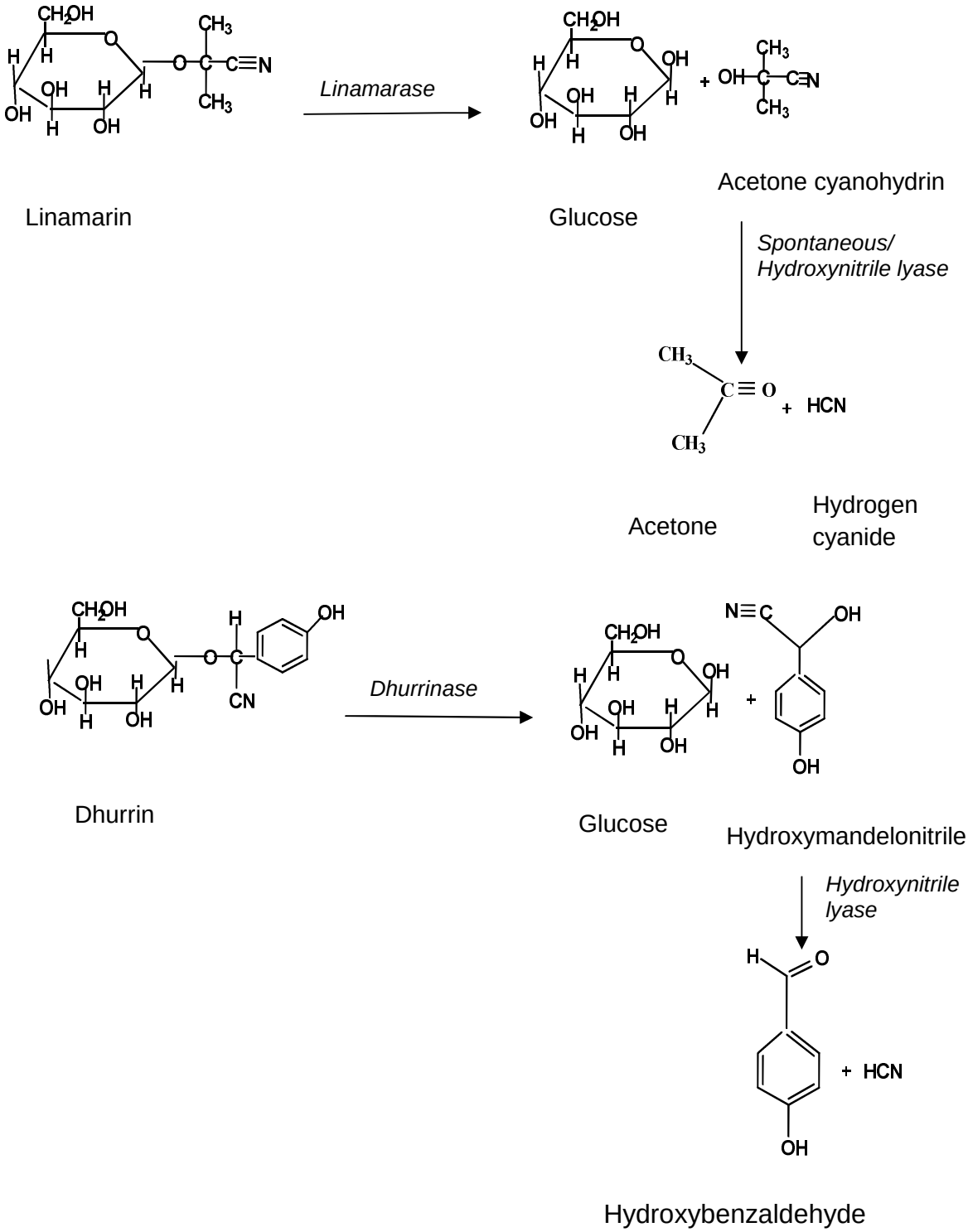


Figure 1-3: The structures and hydrolytic products of linamarin and dhurrin.

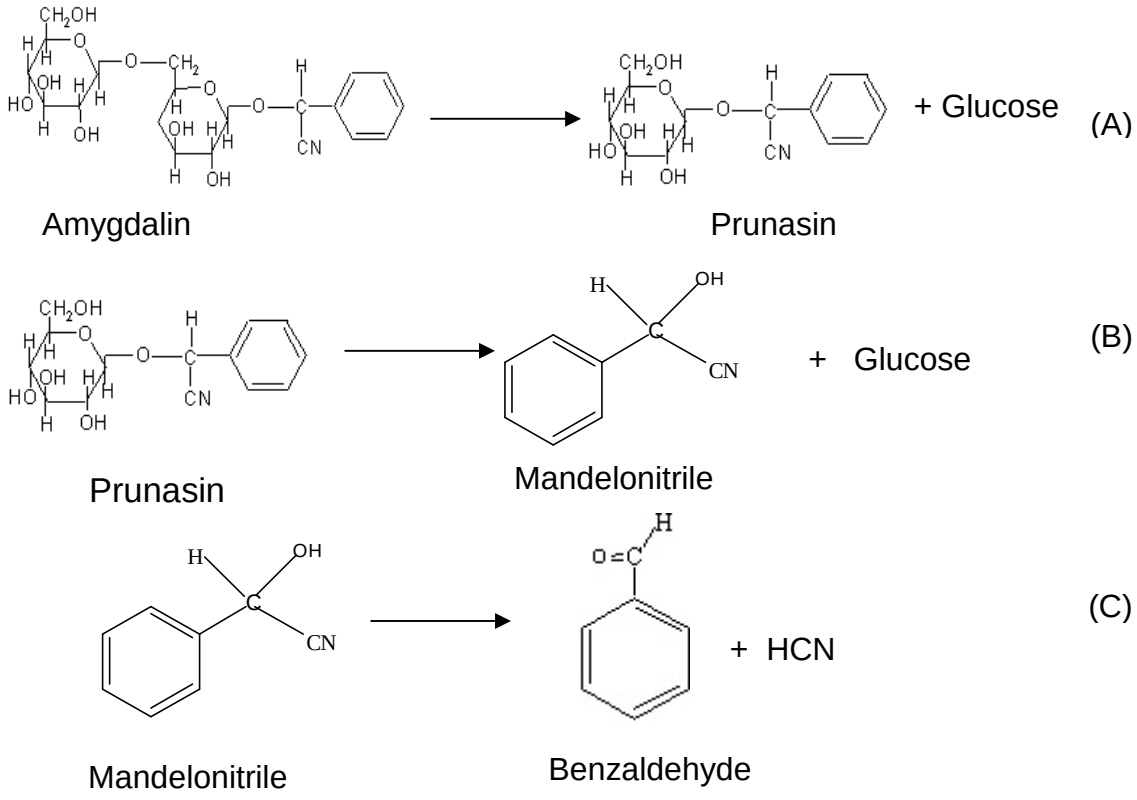


Figure 1-4: Enzymatic hydrolysis of amygdalin and prunasin to form hydrogen cyanide. (A) Splitting of amygdalin to prunasin and glucose by the enzyme amygdalin lyase, (B) The breakingdown of prunasin to mandelonitrile and glucose by the enzyme prunasin lyase, (C) the hydrolysis of mandelonitrile to benzaldehyde and hydrogen cyanide.

1.1.1.4 Mechanism of cyanide poisoning

Cyanide is a highly fatal and rapidly acting poison. The toxicity of cyanide is largely attributed to cessation of aerobic cell metabolism. Cyanide causes intracellular hypoxia by reversibly binding to the cytochrome oxidase a_3 (which is necessary for the reduction of oxygen to water in oxidative phosphorylation) within the mitochondria. Binding of cyanide to ferric ion in

cytochrome oxidase a_3 inhibits the terminal enzyme in the respiratory chain and halts electron transport and oxidative phosphorylation (which is essential to the synthesis of adenosine triphosphate (ATP) and the continuation of cellular respiration (Beasley & Glass 1998; Hamel, 2011).

The exposure of humans or animals to hydrogen cyanide either through the skin, eye or oral ingestion results in absorption and distribution of cyanide in the blood, and subsequent binding of hydrogen cyanide with Fe^{2+} of the hemoglobin to form cyanohemoglobin. Cyanide also binds to Fe^{3+}/Fe^{2+} of cytochrome oxidase enzyme (present in the mitochondria cell), inactivating the enzyme and causing a reduction in oxygen utilization by the body tissues. This may result in cytotoxic hypoxia, a shift from aerobic metabolism to anaerobic metabolism (because of decrease in the ATP/ADP ratio) and an increase in the levels of glucose and lactic acid in the blood. 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 after exposure, exposure to high concentrations of cyanide can cause instant death.

1.1.1.5 Clinical manifestation of cyanide poisoning

The acute lethal dose for cyanide after oral ingestion in humans is in the range of 0.5 to 3.5mg/kg body weight (Speijers, 1993). The onset of signs and symptoms of cyanide poisoning is usually less than 1 minute after inhalation and within a few minutes after ingestion (Hamel, 2011). The clinical features of acute cyanide poisoning are variable (Table 1-5). 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 tried (Yen *et al*., 1995).

1.1.1.6 Treatment of cyanide poisoning

Cyanide poisoning is treatable when quickly recognised and immediately countered with an antidote. An ideal cyanide antidote would act rapidly and effectively with little to no treatment-limiting adverse effect. Currently, treatment of cyanide poisoning involves the use of a cyanide antidote kit (amyl nitrite + sodium nitrite + thiosulfate) or hydroxocobalamin (Borron, 2006). Cyanide antidote kit provides an effective treatment when combined with supportive treatment such as a 100% O_2 ventilator, vasopressors and sodium bicarbonate (Yen *et al*., 1995). Hydroxocobalamin (Vitamin B12a) detoxifies cyanide by binding with it to form the renally excreted, non-toxic cyanocobalamin or Vitamin B12. Because it binds without forming methemoglobin, hydroxocobalamin can be used to treat patients without compromising the oxygen carrying capacity of haemoglobin. The antidotal effect is enhanced by thiosulphate. Hydroxocobalamin antidote is safer in

patients who have pre-existing hypotention or are pregnant (Beasley & Glass 1998; Hamel, 2011).

Table 1-5: Clinical manifestation of the toxic effects of cyanide

System	Manifestation
Central nervous	Anxiety Headache Giddiness Dizziness Confusion Mydriasis Bright retinal veins Decreased consciousness Seizures Paralysis Coma
Respiratory	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) Hypoventilation Apnea (cells cannot take up oxygen)
Cardiovascular	Trachycardia Hypotension Supraventricular tachycardia Artrioventricular blocks Ventricular fibrillation Asystole

(Adapted from Hamel, 2011).

1.1.1.7 Human health effect of cyanogenic glycosides

The potential toxicity of food produced from cyanogenic plants depends on the likelihood that consumption could result in concentrations of hydrogen cyanide (HCN) that are toxic to exposed humans or animals. The lethal dose of orally ingested hydrogen cyanide for a 60kg adult man ranges from 30-210 mg equivalent HCN (Nhassico *et al*., 2008). Chronic cyanide exposure associated with the consumption of insufficiently processed bitter cassava (in regions of the world where cassava is the major source of dietary energy), almond kernels and apricot seeds have been associated with a number of cyanide-induced disorders including:

i. Cyanide poisoning: occurs as a result of consumption of bitter cassava, almond kernels or apricot kernels and their products without proper processing. The clinical symptoms are vomiting, nausea, dizziness, stomach pains, weakness, headache, diarrhea and occasionally death (Lasch & Shawa, 1981; Mlingi *et al*., 1992; Kwok, 2008; Akyildiz *et al*., 2010; Sanchez-Verlaan *et al*., 2011).
ii. Tropical ataxic neuropathy (TAN): occurs in older people due to a monotonous consumption of cyanogenic glycosides from a bitter cassava diet for many years, resulting in chronic thiamine deficiency from inactivation of thiamine by the cyanogenic glycosides (Adamolekun, 2010a). The disease is characterized by unsteady walking, loss of sensation in the hands and feet, blindness, deafness and weakness (CCDN, 2008).

iii. Konzo: an upper motor neurone disease of acute onset due to continuous large intake of cyanogenic glycosides 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 of the lower limbs in children and women of child-bearing age (Tylleskär *et al*., 1992; Ernesto *et al*., 2002; Adamolekun, 2010b).

iv. Goitre: 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 (Gbadebo & Oyesanya, 2005). Table 1-6 shows the different parts of the world where cyanide-induced disease are prominent.

Table 1-6: Cyanide-induced disease observed in particular Countries

Country	Food	Disorder
Jamaica	Cassava, Lima beans	Peripheral neuritis
Nigeria	Cassava	Goitre, tropical ataxic neuropathy, cyanide poisoning
Mozambique	Cassava	Severe paralyzing illness (konzo)
Zaire	Cassava	Goitre
Turkey	Apricot kernels	Cyanide poisoning
Israel	Apricot kernels	Cyanide poisoning
France	Almond seeds	Cyanide poisoning

(Lasch & Shawa, 1981; Jones, 1998; Akyildiz *et al*., 2010; Sanchez-Verlaan *et al*., 2011).

1.1.1.8 Mechanism of cyanide detoxication in human

Cyanide is detoxified by the enzyme rhodanase (a sulfur transferase enzyme present in the liver) with the help of sulfur donors, such as sulfur-containing amino acids (cysteine and methionine) or their products of metabolism, to thiocyanate which is excreted in the urine with very little in the faeces (Oke, 1973). When hydrogen cyanide is converted to thiocyanic acid, there is a 200-fold reduction in toxicity. However, the detoxication mechanism in the

body will only cope with minutes amount of cyanide generated from the consumption of small amounts of cyanogenic plants, but not with a toxic amount or a large dose of cyanide introduced artificially to the body.

Another important independent pathway for cyanide detoxication is cyanocobalamin (vitamin B_{12}). Cyanocobalamin occurs in the liver to some extent as the hyroxocobalamin (vitamin B_{12a}), which is capable of reacting with cyanide to form cyanocobalamin. Cyanide detoxication also occurs to a lesser extent by mercaptopyruvate (cyanide sulfur transferase) (Oke, 1973). The major substrates for this conversion are thiosulfate and 3-mercaptopyruvate. 3-mercaptopyruvic acid can arise from cysteine via transamination or deamination and this compound can provide sulfur as rapidly as thiosulfate for cyanide detoxification (Figure 1-5).

Although the human body is protected against cyanide toxicity through detoxification of ingested cyanogenic glycosides by rhodanase, the amount of cysteine and methionine in the diet is a contributing factor to cyanide-induced disease as sulfur-containing amino acids are required for the detoxification process. The high consumption of cyanogenic plants and their products can therefore result in the depletion of essential amino acids and in thiamine deficiency when the diet is short of protein and thiamine (Rosling, 1994; Padmaja, 1996; Ngudi *et al*., 2002; Ademolekun, 2010b).

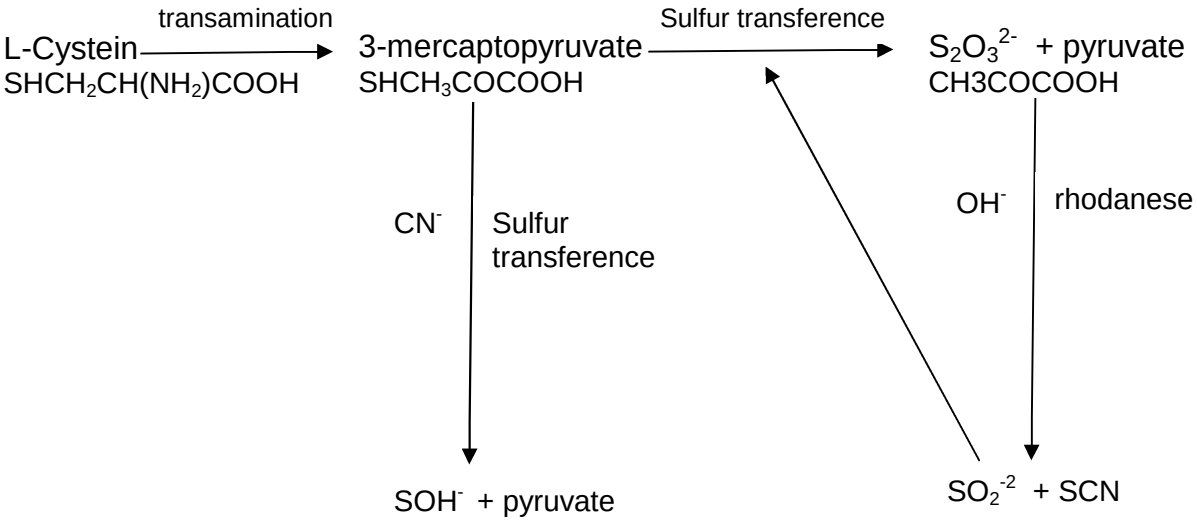


Figure 1-5: The reaction of cyanide with 3-mercaptopyruvate to form sulfur and thiosulfate.

1.1.1.9 Toxicity of cyanogenic glycosides

Toxicity of amygdalin and linamarin are associated with free cyanide, toxic levels of cyanogenic glycosides are therefore estimated in terms of the amount of hydrogen cyanide liberated. Data on the concentration of cyanides in the total diet are lacking (WHO, 2004), and as a result there is inconsistencies in the toxic levels of amygdalin and linamarin reported in literatures (Table 1-7).

Safe level of intake of cyanogenic glycosides could not be estimated because of lack of quantitative toxicological and epidermiological information. However, world health organisation (FAO/WHO, 1991)

concluded that a level of up to 10mg/kg HCN in cassava flour is not associated with acute toxicity.

Table 1-7: Toxic level of cyanogenic glycosides in food

Cyanogenic compound	**Toxic level (mg HCN/kg)**	**Reference**
Amygdalin		
	65	Humbert *et al*., 1977
	340	Sadoff *et al*., 1978
	9 – 59	Solomonson, 1981
	74	Shragg *et al*., 1983
	50	Holzbecher *et al*., 1984
Linamarin		
	100 – 2000	Oke, 1980
	50	Osuntokun, 1981
	14 – 30	Ministry of health Mozambique, 1984
	50 – 60	Speijers, 1993

The increased in belief of complementary or alternative treatment for cancer has led to the use of amygdalin or laetrile for cancer treatment. However, according to the study conducted by national cancer institute in 1982 which involve 175 cancer patients. The trial found out that tumor size had increased in all patients that were treated with amygdalin/laetrile and that minimal side effects were observed except in two patients who consumed bitter almonds instead of amygdalin/laetrile and suffered from cyanide

poisoning (NCI, 2013). Nevertheless, the consumption of sweet almonds seeds have been reported to prevent cancer development in healthy people (Edgar Cayce's, 2013).

1.1.1.10 Prevention of cyanide-induced disease

Cyanide-induced disease can be prevented by effective removal of cyanogenic compounds in food plants prior to consumption. Cyanogenic plants are subjected to various processing methods such as cooking; steaming, boiling, roasting, baking and other processes such as peeling, soaking, grating, fermentation and sun drying, to reduce their cyanide content to safe levels (Kemdirim *et al*., 1995; Tunçel *et al*., 1995; Obilie *et al*., 2004; Cardoso *et al*., 2005; Perera, 2010). In eastern and southern Africa, cassava is 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 59ppm of HCN equivalents, compared to the WHO safe level of 10ppm (Montagnac *et al*., 2009).

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 left to ferment for several days, the water-soluble cyanogens squeezed out and the residual HCN gas is removed by roasting. This process reduced the

cyanogen content of the product (gari or farinha) significantly (Montagnac *et al*., 2009).

Recently, a new processing method that reduced the total cyanide content of cassava by three to six-fold of its former value (i.e from 43ppm to 7ppm) was developed by Bradbury & Denton (2007). The 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 the cassava flour) to allow the catalytic breakdown of linamarin to hydrogen cyanide.

Adequately processed cyanogenic plants, effective detection methods for residual cyanogens 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; Adamolekun, 2010b).

1.1.1.11 Analysis of cyanogenic glycosides

Traditionally, the cyanogenic glycosides in food plants are quantified in terms of total cyanide released after acid or enzymatic hydrolysis of cyanogenic plant rather than the glycoside content itself. Although this quantification 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 hydrogen cyanide (boiling point of 26^{o}C). In addition, discrepancies occur in acid hydrolysis due to the formation of amides and ammonia while in spontaneous autolysis by enzymes, the activity of enzymes is influenced by the conditions prevailing in the heterogeneous plant substrate, the accompany secondary reactions and the accumulation of products of hydrolysis (Zitnak, 1973). Nevertheless, the method has been used for the determination of the cyanogen potential of different plants such as cassava roots and cassava products (Cooke, 1978; Ikediobi *et al*., 1980; Bradbury *et al*., 1991; Yeoh & Tan, 1994; Brimer *et al*., 1998; Djazuli & Bradbury, 1999), lima beans, sorghum, wheat, millet, barley (Okafor, 2005) peach stone, apricot stone, apple seed, giant taro, bamboo shoot (Haque & Bradbury, 2002) and flaxseeds (Kobaisy *et al*., 1996).

Other numerous indirect quantitative methods have also been employed for determination of cyanogenic glycosides in plants. These methods include amperometric measurement with cyanide-sensitive electrode (Dalgaard & Brimer, 1984), spectrophotometric estimation with alkaline picrate (Williams, 1979) or methemoglobin complex (Ajaelu *et al*., 2008), thin-layer chromatography for a densitometric direct assay of cyanogenic glycosides (Brimer *et al*., 1983), colorimetric methods (Tatsuma *et al*., 2000) and alkaline picrate methods (Egan *et al*., 1998; Yeoh & Sun, 2001; Adindu *et al*., 2003; Ngudi & Lambein, 2003). A resorcinol method which is based on enzymatic hydrolysis of cyanogenic plants and trapping of hydrogen cyanide

in alkaline solution of resorcinol reagent followed by measuring the absorbance at 488nm has also been developed (Drochioiu *et al*., 2008). This method has been reported to be more sensitive than the picrate methods (Drochioiu *et al*., 2008).

Recent research efforts for direct quantification of cyanogenic glycosides are based on instrumental methods. Passion fruit cyanogenic glycosides (amygdalin, prunasin and sabunigrin) were quantified by GC-MS (Chassagne, 1996). A reversed phase chromatography with UV and pulsed amperometric detection was developed by Wasserkrug & Rassi (1997) for the detection of linamarin, amygdalin and prunasin and their degradation products (mandelonitrile and benzaldehyde). An HPLC method with a refractive index detector was developed for the determination of linamarin in cassava root cortex (Sornyotha *et al*., 2007). Although, these methods are sensitive, accurate and reproducible, they are expensive and time consuming in terms of sample purification.

Despite the inherent sensitivity, simplicity, rapidity and cost effectiveness of immunoassay methods, the use of immunoassay for quantitative determination of cyanogenic glycosides in plant foods has not been reported. Until now, there are only two reports on immunoassay development for the qualitative analysis of amygdalin in food (Cho *et al*., 2006; Cho *et al*., 2008).

1.2 Immunoassay

1.2.1 Definition

The term “immunoassay” generally refers to quantitative and characterizing methods for analyzing the properties of the targets (sometimes analytes) of antibody binding. Immunoassay has been one of the fastest-growing and most widely used analytical techniques, and involves techniques for the detection and quantification of antigens or antibodies (Edwards, 1996; Price & Newman, 1997). Several variations exist in the design of particular immunoassays. Generally, all assay names contain the word “immuno” combined with another word indicating the type of label used, along with the word “assay”. For example, in isotopic immunoassays, radioimmunoassays (RIAs) described an assay system in which the detection label is a radioisotope (Miles & Hales, 1968). RIA was first described in 1960 for measurement of endogeneous plasma insulin in man by Yalow and Berson (Yalow & Berson, 1960). In non-isotopic immunoassays, a variety of markers or labels, individually or in combination, are used to follow and measure the reactions. For example, enzyme immunoassays (EIAs), fluorescent immunoassay, and chemiluminescent immunoassay (Voller *et al*., 1978; Quan *et al*., 2006).

The word “immuno” and “metric” are used when the immunoassay involves the use of reagents in stoichiometric excess. Thus, “immunometric assay” is used to describe reagent excess assays (Deshpande, 1996). However, depending on the label used, such an assay may be denoted as an

immunoradiometric assay (IRMA), immunoenzymometric assay or immunofluorometric assay (Edwards, 1985; Hemmilä, 1985).

1.2.2 Enzyme-linked immunosorbent assay

The term “enzyme-linked immunosorbent assay” (ELISA), was first coined and developed by Engvall & Perlmann (1971) and Van Weemen & Schuurs (1971). ELISA is a term generally used for reagent-excess enzyme immunoassays of specific antibodies or antigens. Enzyme immunoassay (EIA) was developed as an alternative to radioimmunoassay. This is because radioisotope-labeled reagents are unstable, hazardous to health, and disposal of radioactive wastes is problematic. In contrast, enzymes used in EIA are very stable and cause no health or waste disposal problems. In addition, EIA can be as sensitive as radioimmunoassay, if not more so (Engvall & Perlmann, 1972). The enzymes commonly used as labels in EIA are described in Table 1-7.

There are two types of ELISA, namely; two-site ELISA (non-competitive ELISA) and competitive ELISA. The two-site ELISA or non-competitive ELISA is a sensitive and specific technique for quantifying molecules in solution. The technique is based on the use of two antibodies. The two antibodies recognize separate epitopes of the antigen to be measured and thus bind to the antigen simultaneously. The immobilised antibody specific to the substance to be measured is called the “capture” antibody. The capture antibody is most commonly coated onto a high-capacity protein binding

microtitration plate wells. To prevent unspecific binding, any vacant binding sites on the plate are sometimes blocked with the use of a protein such as bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). The sample or standards are then incubated on the plate to allow the antigen to bind to the capture antibody. The bound sample or standard can be detected using a secondary antibody (which recognizes a different epitope on the antigen) known as the detector antibody. The secondary antibody is usually linked to an enzyme, and the addition of suitable substrate results in a colorimetric reaction. The colour can be measured using a spectrophotometer. The resulting optical density is proportional to the amount of antigen present in the sample (Kemeny & Chantler, 1988; Jordan, 2005). A typical representation of the non-competitive ELISA technique is shown in Figure 1-6.

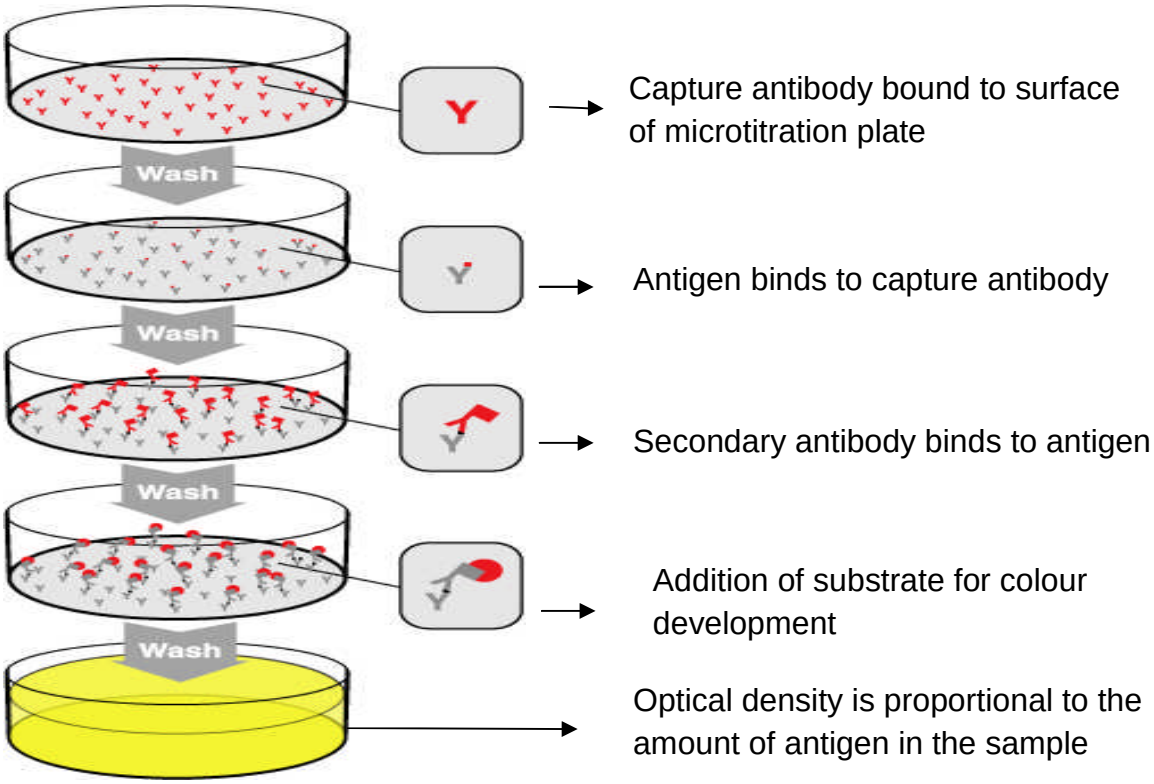


Figure 1-6: Non-competitive ELISA assay showing the capture antibodies and the detector antibodies (modified from Mabtech, 2010).

Competitive ELISA involves the use of two forms of the antigen; one the sample antigen to be assayed and the other, a constant level of “tracer” or specific antigen immobilized in the microtitration plate well. Plates are coated with the specific antigen and these are then incubated with a mixture of antibody and the test sample. If there is no antigen in the test sample the antibody becomes fixed to the antigen sensitized surface. If there is antigen in the test solution this combines with the antibody, which cannot then react with the sensitized solid phase. The amount of antibody attached is then indicated by an enzyme labeled antibody 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 labeled antibody (Porstmann & Kiessig, 1992) and may be of greater specificity compared to the two-site ELISA (Makarananda *et al.*, 1995).

Table 1-8: Enzymes commonly used as labels for EIA

Enzyme	Source	pH (optimum)	Spectrum activity (U/mg at 37°C)	Molecular weight	Chromogenic substrates and measurement
Alkaline phosphatase	Calf (intestine)	9-10	1,000	100,000	*p*-nitrophenyl-phosphate λ = 405nm (*p*NP)
β-galac -tosidase	*E. coli*	6-8	600	540,000	*o*-nitrophenyl-β-D-gala-ctopyranoside (*o*NPG) λ = 420nm Chlorophenolic red-β-D-Galactopyranoside (CPRG) λ = 574nm
Peroxidase	Horseradish	5-7	4,500	40,000	H_2O_2/2,2'-azino-di(3-ethylbenzthiazoline sulfonic acid-6)(ABTS) λ = 415nm H_2O_2/3,3',5,5'-tetramethylbenzidine (TMB) λ = 450nm H_2O_2/ *o*-phenylenediamine (*o*PD) λ = 492nm
Glucose oxidase/peroxid ease	*Aspergillus niger*	4-7	200	186,000	Coupled enzyme reaction Glucose + chromogen for HRP
Urease peroxidise	Jack bean niger				Glucose + chromogen for HRP
Urease	Jack bean	6.5-7.5	10,000	483,000	Urea/bromcresol yellow λ = 588nm

(Adapted from Porstmann & Kiessig, 1992)

1.2.3 Advantages of enzyme-immunoassay methods over radioimmunoassay (Wisdom, 1976)

- They provide specific and sensitive assays of wide applicability.
- Equipment required is relatively cheap and is widely available.
- Reagents are relatively cheap and have a long shelf-life.
- A separation step or complicated extraction procedure may not be required.
- The variety of labels available may allow multiple, simultaneous assays to be performed.
- There is potential for automation.
- No problem of radiation hazards.

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 makes 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 antibodies with appropriate affinities and specificities.

1.2.4 Immunoassay applied to food

Immunoassays are highly quantitative analytical methods that at the highest level comply with strict regulatory requirements mandated by government agencies, and are mostly used in the clinical diagnostics industry. In addition to the sensitivity and specificity of immunoassay methods, they are also simple; they do not require sophisticated instrumentation and toxic organic solvents, and are relatively cheap compared to chromatographic, colorimetric, HPLC or other standard procedures (Mickova *et al*., 2003). The success of immunoassay in clinical chemistry has encouraged their use in food analysis. 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. Quan *et al*. (2006) developed a sensitive enhanced chemiluminescence enzyme linked immunosorbent assay (ECL-ELISA) for the detection of fumonisin B_1 (FB_1) in food and animal feeds. An enzyme-linked immunosorbent assay (ELISA) developed by Pastor-Navarro

et al. (2007), was able to detect 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 are very useful because of their suitability for mass production, high sensitivity, large number of labels and nanotechnology applications (Hervás *et al*., 2009). Extensive research has been carried out on the development and use of immunoassay for the detection of mycotoxins (such as ochratoxin A, and aflatoxin B_1) (Morgan *et al*., 1983a; Ward *et al*., 1990; Yu *et al*., 2005), residual insecticides and herbicides in several food commodities (Krikunova *et al*., 2002; Bo *et al*., 2008). Immunoassay has also been used for the determination of traces of peanut protein contamination in complex food matrices (Morgan *et al*., 1986; Mills *et al*., 1997; Holzhauser & Vieths, 1999).

Mycotoxin toxicity represents a considerable hazard in the nutrition of man and farm animals. Classical methods (such as HPLC) for mycotoxin determination are time consuming in terms of sample preparation. High sensitivity and specificity of immunoassay make it possible to minimize the size of samples required and the volume of extracting solvents and to omit the time-consuming purification procedures. This is advantageous for routine analysis and for screening large sets of samples. For instance, an indirect

competitive ELISA was recently used to assess aflatoxin B_1 and *Aspergillus spp*. infection in 1,200 rice samples in India (Reddy *et al*., 2009).

1.2.4.1 Determination of pesticide residue

The current analytical methods used for the determination of residual pesticides are high-performance liquid chromatography (HPLC) and gas chromatography connected with mass spectrometry (GCMS). Although these methods are sensitive, a lot of time is required for the purification of the sample extracts. Highly sensitive and specific immunoassay has now been developed for the determination a number of pesticides and fungicides and their metabolites. For instance, the immunoassay method developed for parathion-methyl (a non-systemic organophosphorus) pesticide was very sensitive (0.2ng/ml) (Kim *et al*., 2003). In another study, the immunoassay method developed for fungicide diniconazole determination showed that the fungicide hexaconazole and tebuconazole do not give a cross reaction with the antiserum against diniconazole, which is structurally very similar (Jiang *et al*., 2011).

Immunoassays are now utilized for the determination of substances occurring naturally in plant raw-materials, for example glycoalkaloids (which gives rise to the bitter taste of potatoes) (Morgan *et al*., 1983; Stanker *et al*., 1996).

1.3 Antibodies

Antibodies belong to the family of macromolecules known as immunoglobulins. Immunoglobulins are glycoproteins which are present at 12-15mg/ml in the blood serum. The five immunoglobulin classes in mammals are IgG, IgM, IgA, IgD and IgE. IgG is one of the most abundant immunoglobulin classes in terms of concentration, with a molecular mass of 170kDa. It represents 70 to 75% of serum immunoglobulins and constitutes the majority of the secondary immune response to most antigens (Turner, 1981). IgG antibodies are secreted by plasma cells produced by the B lymphocytes of the adaptive immune system. Antibody genes are assembled from pieces of DNA scattered widely throughout the genetic material (Deshpande, 1996). As the B-cell that produces antibody matures, it rearranges these gene components such that the new gene and the antibody it encodes become unique. As the B-cell containing this gene arrangement proliferates, all its descendants will make this unique antibody (Paraf & Peltre, 1991).

The basic structure of IgG antibody resembles the letter Y (Figure 1-7). Each molecule of IgG antibody has two identical heavy polypeptide chains (H) and two identical light chains (L), which are stabilized and linked by intrachain and interchain disulfide bonds. The Fc (fragment crystallisable) portion of an antibody is the stem of the Y shaped molecule that is a dimer of the two last heavy chain domains while the Fab (fragment antigen binding) is the remaining upper part. The two upper domains of IgG antibody (Fab), one

from an H and one from an L chain facing each other in a tip of the IgG antibody Y's arm form the antibody determinant or paratope, and are uniquely shaped to fit a specific portion of an antigen called "epitope" (Paraf & Peltre, 1991). The specificity of antigen-antibody reaction makes antibodies very useful and highly selective reagents in immunoassays.

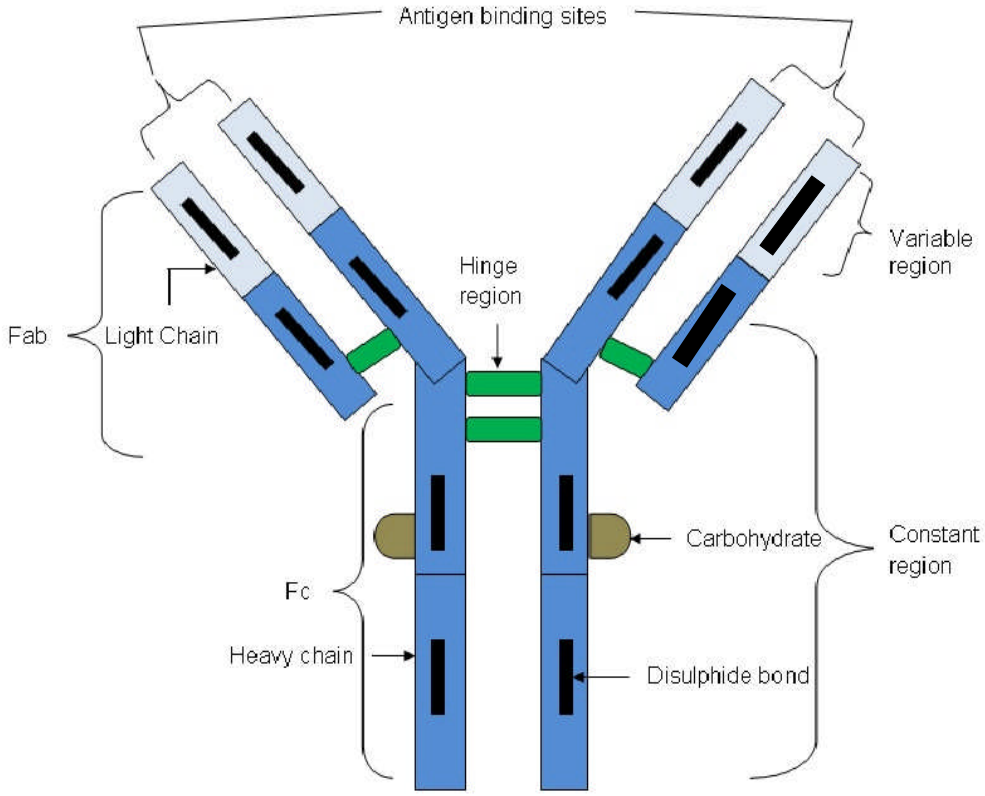


Figure 1-7: The general structure of antibody (IgG molecule) showing the heavy and the light chains and the antibody binding site (modified from Paraf & Peltre, 1991).

1.3.1 Antigen-antibody reaction

Antigen and immunogen are terms often used interchangeably. The word "antigen" is used for a substance that is recognized by antibody *in vitro* while "immunogen" defines the antigen which induces antibody production *in vivo* and binds specifically to antibody *in vitro.* Antigens or immunogens can 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, antibodies able to bind to small molecules can also be produced under specific conditions.

Generally, antibodies do not bind to the whole of an antigen, rather they recognize one of several characteristic surface features of an antigen called the "epitopes" or "antigenic determinants". A particular antigen can have several different epitopes or may have several identical epitopes. Each antibody is specific for a particular epitope of the antigen. Thus antibodies are specific for the epitopes rather than the whole antigen molecule. Antigens are linked to antibody by hydrogen bonds, electrostatic forces, Van der Waals forces and hydrophobic bonds (Figure 1-8). Because antigens are not covalently linked to antibody, there is usually a thermodynamic reversibility of the antigen-antibody reaction (Coico *et al*., 2003).

Specificity of an antibody for its antigen is related to the antibody's affinity. A close fit of an epitope into a paratope will result in high binding forces with a

slow dissociation rate of the complex, whereas a loose contact between epitopes and paratope (which could be due to a poor structural complementary) results in a low mutual affinity and a great dissociation rate of the immune complex (Kemeny, 1991).

The antigen-antibody affinity is expressed by an affinity constant K_A resulting from the application of the mass action law to the following equation at equilibrium:

Free Ag + free Ab $\rightleftharpoons$ Ag-Ab complex

$$K_A = \frac{\text{(Ag-Ab) (bound)}}{\text{(Ag) (Ab) (free)}}$$

Ag is antigen, Ab is antibody.

K_A is obtained by the formula whose terms are concentrations measured at equilibrium (Benjamini *et al*., 2000).

.

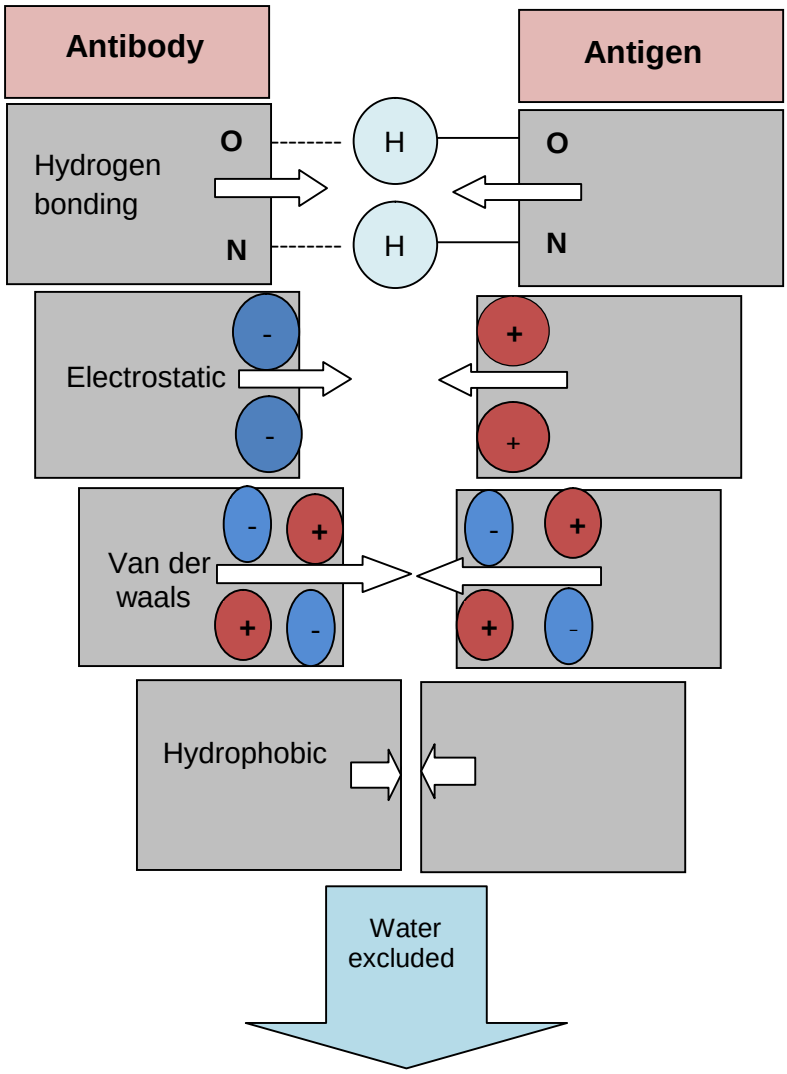


Figure 1-8: Schematic representation of antigen-antibody linkage showing the intermolecular forces involved in antibody-antigen reactions (adapted from Steward, 1984).

1.3.2 Antibody production

The immune systems of animals are capable of producing antibodies in response to the presence of foreign proteins or other molecules within the tissues of the animal. The intensity of the response is determined by the size of the immunogen molecule, its chemical characteristics and how different it is from the animal's own proteins. 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 antibody molecules in blood and some body secretion (Burns, 2005). Although, the natural purpose of antibody production is to protect against invading pathogens, antibodies can be produced experimentally by the injection of an animal with an analyte (antigen) alone or bound to a protein carrier to induce the formation of specific antibodies in the body of the animal. Generally animals are capable of producing different types of antibodies which can bind at specific sites (epitope) of the antigen. There are three types of antibody preparations; polyclonal antibodies (pAb), monoclonal antibodies (mAb) and recombinant antibodies.

1.3.2.1 Polyclonal antibodies

Polyclonal antibodies are antibodies synthesized by several cell types of immunized animal lymphocytes. Conventionally, polyclonal antibodies are produced by immunizing animals with the antigen of interest. Immunization of animals results in the activation of B-lymphocytes and subsequent

antibody production, each different antibody recognizing one epitope; all antibodies together recognizing hundreds or thousands of epitopes. This collection of antibodies produced by many different B-cells is termed "polyclonal" for the various B clones it is derived from. Thus, polyclonal antibodies are mixtures of different immunoglobulins binding to multiple sites on the antigen used for immunization. Polyclonal antibodies have been raised in numerous species including mice, hen, rabbits, goats and sheep (Kascsak *et al*., 1987; Clarke *et al*., 1993; Bean, 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, rabbit is the most common species of choice for polyclonal antibodies intended for routine work (Bean, 2001). In addition, polyclonal antibody raised in rabbits offers the advantage of ready availability and general high quality of a wide variety of anti-rabbit antibody secondary detection reagents (Burns, 2005). Polyclonal antibodies are reagents of choice for general purpose analytical applications in immunoassay systems. Polyclonal antibodies have been used extensively in food immunoassay (Ramesh *et al*., 2007; Burkin *et al*., 2010; Suárez-Pantaleón *et al*., 2010; Jiang *et al*., 2011; Kondo *et al*., 2011).

1.3.2.2 Monoclonal antibodies

The production of specific homogeneous antibodies (monoclonal antibodies) with predefined binding characteristics that can be produced in large

amounts from immortal cell lines has been made possible through hybridoma technology. Monoclonal technology involves the fusing of an antibody-producing B cell with a myeloma cell line (lacking the DNA salvage pathway) to generate a continuous growing hyridoma producing single antibody specificity. Thus, the genes coding for the specific heavy and light chains are captured from the B cell and expressed by the hybridoma. A single B cell from the polyclonal mixture which is isolated and immortalized is termed “monoclonal”. Each mammalian B cell contains the capacity to produce an antibody that recognizes a single epitope, generally consisting of 6 to 12 amino acids. Although hybridomas are capable of producing large amounts of antibodies, the antibodies will all be identical and have identical epitope binding properties (Köhler & Milstein, 1975; Stewart, 2001). Monoclonal antibodies are generated using either immune rodent spleen cells or lymphoid cells from other immunized species (Dean & Shepherd, 2000). For example, monoclonal antibodies are produced when antigen-producing lymphocytes (e.g. splenocytes of immunized mice) are fused with myeloma cells and the hybrid cells are selected in a particular culture medium and screened. The antibodies generated may be highly specific with identical physical, biochemical and immunological properties in addition to their immortality (Goding, 1996; Fremy & Usleber, 2003). Monoclonal antibodies are commonly used in immunoassay techniques for pesticides, herbicides and mycotoxin contamination in agricultural products (Ward *et al*., 1990; Schneider & Hammock, 1992; Abad *et al*., 1997; Moreno *et al*., 2001; Qiant *et al*., 2009; Ackermann *et al*., 2011; Fang *et al*., 2011).

1.3.2.3 Recombinant antibodies

Advances in specific antibody production have led to recombinant technology using phage display technology. Recombinant technology has opened a new route for the generation of antibodies. Instead of immortalizing B cells for production of monoclonal antibodies, the antibody heavy (H) and light (L) chain variable region (V) genes are immortalized by gene technology (Léger & Saldanha, 2000). Recombinant antibody technology is based on the isolation and sequencing of the genes that encode antibodies. By altering the sequence of the antibody genes, the binding sites can be manipulated to give more desirable characteristics which include increased or broadened specificity and affinity (Choudary *et al*., 1995). The DNA coding for specific antibodies and fragments of antibodies has been introduced into bacteria, yeast and mammalian cell lines by recombinant molecular genetics techniques. Antibody genes from hybridomas or lymphocytes from immunized animals are used to construct libraries of antibody genes. For example, the IgG mRNA of B cells of immunized animals can be used as a source of variable region (V) gene libraries. Murine VH and VL genes are amplified using PCR and used to construct libraries of millions of recombinant single-chain variable fragment (scFv). ScFv antibodies consist of the variable regions of the light and heavy chains of antibodies linked via a short peptide spacer. Repertoires of scFv are cloned into the gene encoding the minor coat protein (gene III) of filamentous bacteriophage, thus creating a large library of phage, each displaying an individual heavy and light chain combination. The fusion