# PHYSICOCHEMICAL, NUTRIENT AND MICROBIOLOGICAL ANALYSIS OF *OSHIKUNDU*; A CEREAL BASED FERMENTED BEVERAGE FROM NAMIBIA

A THESIS SUBMITTED IN FULFILMENT

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**ABSTRACT**

The aim of this study was to determine the physicochemical, nutrients, predominant microflora mainly looking at lactic acid bacteria and spoilage bacteria in *Oshikundu* samples collected from Omusati, Oshana, Ohangwena and Oshikoto regions. *Oshikundu* is brewed from pearl millet (*Pennisetum glaucum* L. R.Br.,) meal locally known as *Mahangu*, malted sorghum (*Sorghum bicolor L. Moench*), bran and water. *Oshikundu* pH ranged between 3.33 ± 0.127 in Oshana region and 3.60 ± 0.014 in Oshikoto region. Titratable acidity as lactic acid was found to be ranged between 1.20 % in Oshikoto and 1.68 % in Ohangwena region. Meanwhile acetic acid ranged between 0.10 % in Omusati and 0.30 % in Ohangwena region. The protein content ranged between 0.133 ± 0.007 and 0.178 ± 0.02 (mg/mL), insoluble fibre, 0.025 ± 0.01 % and 0.028 ± 0.004 %, energy, 46.8 ± 8.45 and 54.4 ± 4.17 (kJ/100 mL) on dry basis. Micro-nutrients including vitamin $B_1$ and $B_2$, were detected in *Oshikundu.* Minerals were analysed and revealed the presence of B, Ca, Cu, Fe, K, Mg, Mn, Na, S, Zn and P which was the highest (ranged between 3.914 ± 0.452 and 11.511 ± 7.264 ppm). The microflora of *Oshikundu* included 6 predominant Lactic acid bacteria (LAB): *Lactobacillus plantarum*, *Lactococcus lactis* ssp. *lactis*, *Lactobacillus delbrueckii* ssp. *delbrueckii*, *Lactobacillus fermentum*, *Lactobacillus pentosus* and *Lactobacillus curvatus* ssp. *curvatus*. Five bacterial species were identified that are likely to be responsible for *Oshikundu* spoilage; *Enterobacter cloacae*, *Enterobacter sakazakii*, *Pseudomonas luteola*, *Pseudomonas aeruginosa* and *Serratia ficaria*. Further studies on microflora, starch structure and characterization of flow behaviour are necessary in understanding the rheological properties of *Oshikundu* for process design and product quality evaluation.

**ACRONYMS**

| | |
|---|---|
| **ADF** | Apparent Degree of Fermentation |
| **ANOVA** | One Way Analysis of Variance |
| **AOAC** | Association of Official Analytical Chemists |
| **API** | Analytical Profile Index |
| **CFU** | Colony Forming Units |
| **DNA** | Deoxyribose Nucleic Acid |
| **EA** | Apparent Extract |
| **ER** | Real Extract |
| **GC-MS** | Gas Chromatography Mass Spectrometer |
| **HPLC** | High Performance Liquid Chromatography |
| **ICP-OES** | Inductively Coupled Plasma Atomic Emission Spectroscopy |
| **IK** | Indigenous Knowledge |
| **LAB** | Lactic acid bacteria |
| **mPas** | MilliPascal-second |
| **MRS** | de Man Rogosa Sharp |
| **PCA** | Plate Count Agar |

| | |
|---|---|
| **PCR** | Polymerase Chain Reaction |
| **PDA** | Potato Dextrose Agar |
| **ppm** | Parts Per million |
| **RDF** | Real Degree of Fermentation |
| **Rpm** | Rotation per minute |
| **SD** | Standard Deviation |
| **SPSS** | Statistical Package for Social Sciences |
| **TPC** | Total Plate Count |
| **UNESCO** | United Nation Educational, Scientific and Cultural Organization |
| **Nuffic** | Netherlands Organization for International Cooperation Protocol |
| **UV** | Ultraviolet |

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## PUBLICATIONS AND PRESENATIONS FROM THIS RESEARCH WORK

The following publications have been published from this thesis research work:

1. **Werner Embashu**, Ahmad Cheikhyoussef and Gladys Kahaka, **2012**. Survey on Indigenous Knowledge and Household processing methods of *Oshikundu;* a cereal-based fermented beverage from Oshana, Oshikoto, Ohangwena and Omusati Regions in Namibia. MRC, University of Namibia, Windhoek, Namibia.

2. Hina Mu Ashekele, **Werner Embashu** and Ahmad Cheikhyoussef, **2012**. Indigenous Knowledge System Best Practices from Namibia: The Case of *Oshikundu* Processing Methods. *Trends in Applied Sciences Research*, 7: 913-921.

3. **Werner Embashu**, Ahmad Cheikhyoussef and Gladys Kahaka. Physiochemical and Nutritional Analysis of *Oshikundu*: fermented Beverage from Namibia. Paper presented at the 2nd Indigenous Knowledge Systems Symposium, 8-9 October **2012** University of Namibia, Windhoek, Namibia.

4. **Werner Embashu**, Ahmad Cheikhyoussef, Gladys Kahaka and Selma Lendelvo (**2013**). Processing Methods of *Oshikundu,* a Traditional Beverage from Sub-tribes within Aawambo Culture in the Northern Namibia. *Journal of Studies in Humanities and Social Sciences*, 2 (1): 117-127.

5. **Werner Embashu**, Gladys Kahaka and Ahmad Cheikhyoussef, **2013**. Physicochemical and nutritional analysis of *Oshikundu*: traditional fermented beverage from Namibia. Paper presented at the EFFoST Annual Meeting: Bio-based Technologies in the Context of European Food Innovation Systems, 12-15th November 2013, Savoia Hotel Regency, Bologna, Italy.

6. **Werner Embashu**, Ahmad Cheikhyoussef and Gladys Kahaka (**2013**). Micro-nutrient analysis of *Oshikundu*: A traditional fermented beverage from Namibia. Paper presented at the 1st Annual Science Research Conference, 25-26 October 2013, University of Namibia, Windhoek, Namibia. pp: 15-16.

7. **Werner Embashu**, Ahmad Cheikhyoussef and Gladys Kahaka, 2013. *Oshikundu*: a Cereal-based, Fermented Namibian Beverage. In: Kazhila C Chinsembu, Ahmad Cheikhyoussef, Davis Mumbengegwi, Lawrence N Kazembe and Kasanda, C.D. (Eds.) Scientific Indigenous Knowledge of Namibia. Accepted book chapter to be published by UNAM Press, Windhoek, Namibia.

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## DEDICATION

I would like to dedicate this work to my mother Mrs Ally Embashu for all the love, support and guidance that helped mould me into who I am today.

**DECLARATION**

I, Werner Embashu, hereby declare that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.



…… Date …….

# CHAPTER 1: INTRODUCTION

## 1.1. General Introduction

Africa has a diverse range of fermented food and beverages. This is due to the diversity of fermented products derived from the heterogeneity of traditions found all over Africa, cultural preference, different geographical areas where they are produced: and the staple or by-products used in fermentation (Marshall & Mejia, 2011). In addition to forming part of traditional heritage, they also contribute to human nutrition and health (Musaiger, Ahmed & Rao, 2000). These fermented products became part of culture over time and a feature among different communities. These fermented beverages are not known only to quench thirst but for their biochemical contribution to human diet. This is brought about the complex micro and macronutrients required by human bodies as cereal food and beverages can be considered as great contributor of dietary protein, carbohydrates, minerals and fiber (Blandino, Al-Aseeri, Pandiella, Cantero & Webb, 2003; Kohajdová & Karovičová, 2007).

In many instances, it is likely that the methods of production of fermented products were unknown and came about by chance and then have been passed down by cultural and traditional values to subsequent generations (Marshall & Mejie, 2011). Some of the most popular fermented products derived from grains, fruits and vegetables are alcoholic based, and some fermented food products which are important in meeting nutritional requirements of a large proportion of the global population (Blandino et al., 2003), have a long history of production via “kitchen” fermentation, contributing to household nutrition and important social-cultural practices (Marshall & Mejie, 2011).

## 1.2. Statement of the Problem

The multiplicity of traditional food and beverages in Namibia is the manifestation of a diverse culture such as *Aawambo, Hereros, Tswanas, Himbas, Damaras, Namas, Caprivians* and *Akavango*. *Oshikundu* is one of the many traditional fermented beverages from the northern and north-eastern parts of Namibia that is popular among the rural inhabitants and over the years it has become a source of income to many rural women. Despite its daily consumptions especially by children, its nutrient content and microbial composition remain unknown. Although, *Oshikundu* is an important daily beverage, it has not gained popularity among urban dweller and affluent young people. This may be due to its short shelf life, long fermentation period and possibly inconsistent organoleptic properties. This might be due to variations in preparation methods and/ or ingredients. Organoleptic properties such as colour and taste are the main driving force in consumer's choice. *Oshikundu* is only localized to the north and north-east of Namibia among *Aawambo* culture and a part of Kavango region, which has been a source of food and culture for many generations. This however, can be dispersed to other ethnic groups around the country to increase consumption and add value to *Oshikundu* as an indigenous fermented beverage.

There is a gap in the scientific knowledge when it comes to the micro-flora, and their contribution to nutritional quality of *Oshikundu*. In addition, microbiota of traditional fermented cereal-based foods could help shape, maintain or restore gut microbiota (Guyot, 2012), and *Oshikundu* also can be one of them. *Oshikundu* is prepared in rural area where access to safe and clean water remains a challenge (Embashu, Cheikhyoussef, Kahaka, 2013). However, there are no known reported cases of food poisoning associated with *Oshikundu* and understanding the microbial community of the beverage will help better understand this. Food safety is an

important aspect and becomes crucial when it comes to children, who are the primary consumers. The traditional art of *Oshikundu* brewing is passed on verbally and experimentally from generation to generation, and no formal channels are followed in preservation and in brewing, hence there is a need to document this as Indigenous knowledge (IK).

### 1.3. Objectives of the Research

The overall objective of this study is to profile physicochemical, nutrients characteristics, the predominant microflora and the essential microorganisms responsible for fermentation and those for spoilage of *Oshikundu.* The research will focus on *Oshikundu* from four regions in the central-Northern part of Namibia, namely Omusati, Oshana, Ohangwena and Ohangwena. The physicochemical, nutrients content and microbiological properties will help better understand the kinetics and dynamics of *Oshikundu.* This will contribute towards the shelf life extension and new product development in the near future.

The specific objectives of this research included:

1. Collection of different representative samples of *Oshikundu* within sub-groups from *Aawambo* culture within the four regions: Oshikoto, Ohangwena, Oshana and Omusati.
2. Documentation of the traditional household processing method(s) of *Oshikundu.*
3. Conduct of different physicochemical tests (pH, titratable acidity (lactic and acetic acid), total organic acids, flavour (volatile compounds) and viscosity on *Oshikundu* samples.
4. Measurement of the sugar and alcohol contents.

5. Determination of the approximate contents (total ash, carbohydrate, energy, proteins and total moisture, total solid);
6. Determination of the micro-nutrients composition (minerals, amino acids, and vitamins);
7. Screening the micro-flora screening and starter cultures characterization by determining the:
    a) Essential microorganisms for *Oshikundu* fermentation.
    b) Microorganisms that cause *Oshikundu* spoilage.

### 1.4. Significance of the Study

The research looked at profiling the micro-flora of *Oshikundu* for future starter culture development in Namibia. This research also looked at determination of the types of micro-flora at the genus level, since if this micro-flora is known it will be easy to alter, modify the fermentation process to optimize the starter culture and to improve quality and shelf life of *Oshikundu* for the commercialization purpose in the future. Micro-flora profiling is significant since it will enable determine the diversity of different species in *Oshikundu*; to know the quantities (estimates) of the different species. The nutritional composition adds value to *Oshikundu* among the consumers in this four regions and perhaps other regions and tribes within Namibia. This will help in educating and understand that *Oshikundu* is not just a thirst quenching beverage, but also contain important nutrients. Consequently, *Oshikundu* can be a national beverage instead of its association to only one or two tribes in Namibia.

### 1.5. Limitation of the Study

1. Ideally, the study would include a large number sample within *Oshiwambo* sub-culture in all the four regions, but due to distribution in population and geographical location, this would not

be possible in this study. This would make the study extensive and costly to screen and collect a large number of *Oshikundu* samples.

2. Some form of reimbursement was demanded by interviewees for providing *Oshikundu* samples. This is a challenge during sample collection as the people reluctant to be interviewed and provide samples.

3. During the screening and strains identification phases; a number of isolates is expected to be generated. Screening bacteriocinogenic strains for their protective characteristics will be a very expensive.

4. Genetic identification will be instrumental to this research, but this will not be done since the budget will not allow procuring an isolation kit, primer, sequencing and delay in procurement process (finance department and vendor).

5. Further analysis (micro flora, ingredients starch structure, characterization of flow behaviour) may be of great addition to this study of which will not be done due to lack of human expertise in the field (food chemistry) within the University of Namibia.

# CHAPTER 2: LITERATURE REVIEW

## 2.1. Physicochemical Characterization

Titratable acidity and pH plays a role in food analysis; and these are interrelated concepts that are determined in different ways. Titratable acidity is the measure of total acid concentration (total acidity) contained in food, which is done through exhaustive titration of intrinsic acids with a standard base, which is a better predictor of acid impact than pH. Usually expressed as predominant acid and in some cases two acids may be present in large quantity and the predominant acid change with time (Sadler & Murphy, 2010). Meanwhile, pH is the measure of hydronium ion concentration or negative logarithm of the hydronium $H_3O^+$ concentration in an aqueous medium (Sadler, 2010; Chang, 2007). Inorganic acids such as phosphoric and carbonic (arising from carbon dioxide) acids plays an important and even predominant in food and beverage acidulation. The organic acid present in food and beverages, influence the flavour (i. e, tartness), colour (through impact on anthocyanin and other pH-influenced pigments), microbial stability (via inherent pH-sensitive characteristics of organism) and maintain quality (arising from varying chemical sensitivities of food components to pH) and the ability of microorganism to grow in food depends on pH more than on titratable acidity (Sadler & Murphy, 2010).

Acids that contributes to titratable acidity includes lactic, acetic, malic, citric, tartaric, oxalic, ascorbic and sulphuric acid; where phosphoric and carbonic plays important role in acidulation, tartaric acid influence flavour, colour and microbial stability (Sadler & Murphy, 2010).

## 2.2. Micro and Macro -Nutrients

### 2.2.1. Amino Acids

There are 20 different types of amino acids (Table 1). Some amino acids cannot be synthesized by humans and can be classified based on structure or nutritionally (Eastwood, 1999). Amino

acids have the same basic structure, an amino group ($-NH_3^+$) and a carboxylic acid group ($-COO^-$) attached to the same carbon atom (α-carbon) and the differences comes only to what is attached to the α-carbon (R) (figure 1). Some amino acids are termed essential (table 1) since humans cannot synthesize them; hence we need to take them through food intake. Apart from amino acids serving as nutrients, they are also involved in the synthesis of many metabolic products such as purine and pyrimidine, dopamine, noradrenaline, adrenaline and thyroid hormones (Geissler & Powers, 2009).

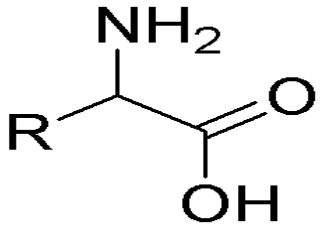


**Figure 1:** Amino acids skeleton and varying side chain denatured by R

**Table 1**: Amino acids (20) grouped into essential and non-essentials

| Essential amino acids | Non-essential amino acids |
|---|---|
| Arginine | Alanine |
| Histidine | Asparagine |
| Isoleucine | Aspartate |
| Leucine | Cysteine |
| Lysine | Glutamine |
| Methionine | Glycine |
| Phenylalanine | Proline |
| Threonine | Serine |
| Tryptophan | Tyrosine |
| Valine | Glutamate |

### 2.2.2. Minerals

Minerals are inorganic molecules that are essential dietary constituents. Minerals and trace elements play an important biochemical and physiological functions in human body which include Calcium, Phosphorus, Iron, Zinc, Iodine, Potassium, Selenium and Sodium (Geissler & Powers, 2009). Minerals and trace elements have to be taken in through food and each has a metabolic function and consequences of deficiency (Table 2).

**Table 2**: Minerals with their functions and deficiencies (Geissler & Powers, 2009;).

| **Elements** | **Function (s)** | **Deficiency** |
|---|---|---|
| Calcium | Formation of bones components, cell signalling and blood clotting | Muscle weakness, hypocalcaemia and metacarpophalangeal joints |
| Phosphorus | Formation of inorganic bones components, cell signalling | |
| Iron | Energy metabolism, antioxidant, immune function | Anaemia and Haemochromatosis |
| Zinc | Enzyme functions, DNA synthesis, cell division | Male hypogonadism, growth retardation, hepatosplenomegaly |
| Iodine | Production of thyroid hormones | Goitre |
| Potassium | Maintain fluid and electrolyte balance | Hypokalemia |
| Selenium | Transcriptional enhancers, enzyme functions | Keshan disease (cardiomyopathy) |
| Sodium | Blood pressure, Muscles and nerves system | Hyponatremia and Hypernatremia |

### 2.2.3. Vitamins

Vitamins are relatively low-molecular weight compounds which the body is incapable of synthesizing and required in small amounts (Eastwood, 1999). The human body depends on

them as a source of nutrients, required for normal metabolism and insufficient levels of vitamins could result in deficiency diseases (Pegg, Lander Jr & Eitenmiller, 2010). Vitamins are divided into fat and water soluble vitamins. Water soluble vitamins include Ascorbic acid (Vitamin C), Thiamine (Vitamin $B_1$), Riboflavin (Vitamin $B_2$), Niacin, Vitamin $B_6$ , Folic acid, Vitamin $B_{12}$ , Biotin and Pantothenic acid. Meanwhile fat soluble vitamins include Vitamins A, D, E and K (Eastwood, 1999).

### 2.2.4 Protein

A protein molecule is made of hundreds or thousands of amino acids units joined together (Lean, 2006). The arrangement and shape of amino acids determine the biological properties of the proteins (Eastwood, 1999). Primary structure of proteins is essential in living things with numerous functions in enzymes, structures, immunity and transport (Barasi, 2003), play a role in cell signalling (e.g., Calmodulin), act as a precursors for the synthesis of hormones (eg., steroid hormones) (Taylor et al, 1991; Geissler & Powers, 2009). In humans, proteins are needed to maintain normal functions of the body and can be taken through food and beverages or are synthesised by our bodies. Deviation from this can lead to diseases and conditions such as protein-energy malnutrition. Protein-energy malnutrition is a condition similar to starvation, this occurs when the body needs for protein, energy or both are not met and this is manifested in conditions such as Kwashiorkor and Marasmus (Ramakrishnan & Rao, 1995).

### 2.2.5. Carbohydrates

Carbohydrates are the major food source of metabolic energy the sugars, starch and fibre. Chemically, they are composed of carbon, hydrogen and oxygen in the ratio $C_n$:$H_{2n}$:$O_n$ (Geissler & Powers, 2009). Carbohydrates are important in food as a major source of energy; digestible carbohydrates are converted into monosaccharide (BeMiller, 2010). Carbohydrates are classified

based on their length of polysaccharide chain. Monosaccharides are single unit sugar which exists in one of two forms: aldose which contains an aldehyde group (e.g. glucose) and ketoses (figure 2) containing a ketone group such as fructose (Geissler & Powers, 2009).

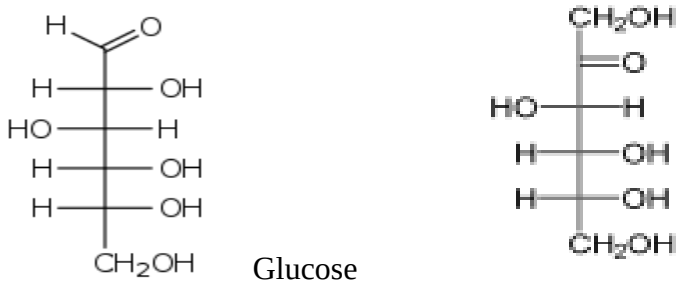


**Figure 2:** Glucose and fructose structures (Geissler & Powers, 2009).

### 2.2.6. Fibres

Dietary fibre is the edible part of plant that helps in digestion and absorption in the human small intestine with complete or partial fermentation in the large intestines (BeMiller, 2010). Fibre affects the rate of digestion of foods, the absorption of nutrients and the movement of waste products (stool) through the colon (Geissler & Powers, 2009; BeMiller, 2010). Fibre is important in inhibiting gastric emptying, lipid and carbohydrate metabolism, lowering plasma total and Lower density lipoprotein cholesterol, postprandial glucose and insulin response (Geissler & Powers, 2009).

## 2.3. Fermentation

Great emphasis has been recently laid on the role of traditional food and beverages in the health and nutritional status of the people, especially for those of low social classes; in addition, traditional food and beverages constitute an essential aspect of cultural heritage and they are highly regarded by local communities (Musaiger et al, 2000). Despite the lack of scientific knowledge, many local people understand the benefits of indigenous food in maintaining their culture and in health promotion. In addition, indigenous foods are being studied for scientific

identification, nutritional composition and cultural food uses however, there is still much to be done and learned (Mbhenyane, 2005) on cultural influence as well as traditional processing technology.

Many traditional fermented food and beverages produced in Africa, Asia and South American countries are mainly from raw materials (Schwan, Ramos, Almeida, Pereira, Cardoso, & Dias, 2010). The production of fermented foods and beverages is based on empirical knowledge transferred from generation to generation (Schwan et al, 2010). The preparation of many indigenous fermented beverages remains an indigenous science in homes and small-scale industries. Generally, spontaneous cereal-based fermentation are carried out by yeasts, lactic acid bacteria and Fungi, which sometimes cooperate together in a complex microbial consortium (Schwan et al, 2010).

Fermentation is one of the oldest, economical forms of food and beverage preservation in the world (Rhee, Lee, & Lee, 2011; Blandino et al., 2003; Chelule, Mbongwa, Carries, & Gqaleni, 2010). The word fermentation signifies the gentle bubbling or boiling condition in these processes. The definition of fermentation in biochemistry is the extraction of energy from carbohydrates and other organic substrates without using $O_2$ as an electron acceptor (Li, 2004; Blandino et al., 2003). A broad definition, describes fermentation as a process in which microorganism produces chemical changes in organic substrates through the action of enzymes produced by these microorganisms (Li, 2004). Meanwhile, fermented beverages are defined as those products that have been subjected to the effect of microorganisms or enzymes to cause desirable biochemical changes (Blandino et al., 2003). Fermented beverages contribute to human diet in many developing countries, since fermentation is an inexpensive technology which preserves food, improves its nutritional value and enhances its sensory properties (Nzigamasabo

& Nimpagaritse, 2009). Depending on the biological activity of microorganisms for production of a range of metabolites, these metabolites can suppress the growth and survival of undesirable microflora (Kohajdovă & Karovičovă, 2007).

Fermentation play an important role in ensuring the food security of millions of people around the world, particularly marginalised and vulnerable groups (Marshall & Mejie, 2011). Fermentation also leads to detoxification, destruction of undesirable factors present in raw food such as cyanide and phytates. Fermentation also contributes to reduction of some secondary metabolites such as tannins and polyphenols in addition to enhance taste, aroma, shelf life, texture, nutritional value and other attractive properties of food (Nzigamasabo & Nimpagaritse, 2009). Fermentation is classed as alcoholic, acidic and alkaline fermentation. Alcoholic fermentation is in which ethanol the main product from the sugar metabolism by yeast. Acid fermentation includes lactic fermentation (pH reduction) from Lactic acid bacteria (LAB) where lactic acid is the main product from sugar transformation. Meanwhile Alkaline fermentation involves ammonia production from amino acid deamination (pH increase) (Hui et al., 2012. Fermentation plays at least five roles in food and beverages (Steinkraus, 1994; Kohajdovă & Karovičovă, 2007). These are:

1. Enhancing of human dietary through development of a wide diversity of flavours, aromas and textures in food.
2. Preservation of substantial amounts of food through lactic acid, alcoholic, acetic acid and alkaline fermentations.
3. Enrichment of food substances biologically with protein essential amino acids, essential fatty acids and vitamins.
4. Detoxification during food and beverages fermentation processing and lastly,

5. A decrease in cooking times and fuel requirements.

Fermentation in beverages has a fundamental role in enhancing sensory characteristics such as flavour, texture or colour and also transformation of nutrients. Flavour is one of the sensory attribute that is transformed by fermentation. Carbohydrates, proteins, lipids, organic acids, amino acids, phenolic compounds and glycosides are transformed into non-volatile and volatile flavour that affects taste and aroma and thus beverage acceptability. Hence flavour diversification is an immerging trend because of consumer demand for novel and diverse flavour. These flavour compounds have a profound impact on a range of other sensory attributes such as; sweetness (e.g., mannitol), sourness (e.g., lactic acid), savouriness or umami (e.g., $_{L}$ –glutamic acid), bitterness (e.g., hydrophobic peptides), fruitiness (ester) and sulphurous notes (volatile sulphur compound) (Hui et al., 2012).

Flavour is not only derived from microbial fermentation fermentation of microbial, but raw materials also have an effect as well as manufacturing processes. Lactic acid fermentation is widely applied in tropical climates as a low-cost method of enhancing food quality, safety and shelf life (Nout & Sarkar, 1999).

One cannot predict what new industries will be developed from further study of the indigenous fermented food and beverages, but it is highly likely with the new emphasis on biotechnology and genetic engineering that detailed study of indigenous fermented food and beverages will offer many benefits for mankind, insuring the extensive supplies of food needed by millions of consumers (Steinkraus, 2004).

### 2.4. Lactic Acid Bacteria (LAB)

Lactic acid bacteria (LAB) is a large group of closely related bacteria that are gram-positive, catalase-negative (table 4), non-sporing rods, cocci that utilize carbohydrate (fermentable sugars)

and have similar properties of lactic acid production, which is one of their end products (Chelule et al., 2010; Blandino et al., 2003). Traditionally, LAB comprises four Genera *Lactobacillus, Leuconostoc*, *Pediococcus*, and *Streptococcus* (McKay & Baldwin, 1990; Oberman & Libudzisk, 1996; Suskovic, Kos, Matosic & Maric 1997; Blandino et al., 2003). However, several new genera have been suggested for inclusion in the group of LAB due to a recent taxonomic revision (Axelsson, 1998). Lactic acid bacteria have a long history of safe use in fermented food and beverages. In addition, they also have beneficial influence on nutritional and sensory characteristic as well as on the standardization of end product (Abd El Gawad, Abd El Fatah, & Al Rubayyi, 2010).

Lactic acid fermentation is a biological process by which sugars such as glucose, fructose, and sucrose, are converted into cellular energy and the metabolic product (Ray, 1996). Fermentation by LAB is characterized by the accumulation of organic acids and the accompanying reduction in pH, and the levels and types of organic acids produced during the fermentation process depend on the species of organisms, culture composition and growth conditions (Lindgren & Dobrogosz, 1990). These organic acids are also involved in conferring antimicrobial properties. The antimicrobial effect of organic acids lies in the reduction of pH, as well as the un-dissociated form of the molecules (Gould, Accolas, 1991; Podolak, Zayas, Kastner, Fung, 1996). Some of the main reasons for the fermentation practice using LAB are to increase food palatability and improve the quality of food by increasing the availability of proteins and vitamins; furthermore, LAB confers preservative and detoxifying effects on food (Chelule et al., 2010; Rhee et al., 2011). Anti-microbial effect contributes towards the safety, nutritional value, shelf life and acceptability of a wide range of cereal based foods (Blandino et al., 2003).

Lactic acid bacteria play an important role in food fermentation processes. LAB are resistant to low pH condition and high osmotic pressure, and this characteristics makes them useful in industrial application such as preservation and microbial safety, due to production of organic acids, carbon dioxide, ethanol, hydrogen peroxide, diacetyl, antifungal compounds (free fatty acid), bacteriocins and antibiotics (Hui et al., 2012). During cereal fermentation, several volatile compounds are formed, which contribute to a complex blend of flavours (table 3) in products and the presence of aromas such as those conferred by diacetyl, acetic acid and butyric acids makes fermented cereal-based products more appetizing (Kohajdovă & Karovičovă, 2007) in small quantity.

**Table 3**: Compounds formed during cereal fermentation, adopted from Kohajdovă & Karovičovă (2007).

| Organic acids | Alcohol | Aldehydes and Ketones | Carbonyl compounds |
|---|---|---|---|
| Butyric Heptanoic<br>Succinic Isovaleric<br>Formic Propionic<br>Valeric n-Butyric<br>Caproic Isoburyric<br>Lactic Caprylic<br>Acetic Isocaprilic<br>Capric Pleagronic<br>Pyruvic Mevulinic<br>Palmitic Myristic<br>Crotonic Hydrocinnamic<br>Itaconic Benzylic<br>Lauric | Ethanol<br>n-propanol<br>Isobutanol<br>Amyl alcohol<br>Isoamyl alcohol<br>2,3-Butandieol<br>β-phenylethyl alcohol | Acetaldehyde<br>Formaldehyde<br>Isovaleraldehyde<br>n-valderaldehyde<br>2-Methyl butanol<br>n-Hexaldehyde<br>Acetone<br>Propionaldehyde<br>Isobutyraldehyde<br>Methyl ethyl Ketone<br>Butanone<br>Diacetyl<br>Acetoin | Furfural<br>Methional<br>Glyoxal<br>3-Methyl butanal<br>2-Methyl butanal<br>Hydroxymethyl furfural |

### 2.5. Starter Culture

Bacteria and yeast are the commonly and widely used for food fermentation and flavour formation in pure, defined or undefined mixed culture. The specific type of culture and function vary with food substrate, intrinsic and extrinsic factors such as pH, temperature and water activity (Hui, et al., 2012). The main bacteria involved in food fermentation are mainly acetic

acid bacteria (e.g. vinegar), bacilli (e.g. some soya fermented products), lactic acid bacteria (e.g. dairy products), propionic acid bacteria (e.g. Swiss cheese), staphylococci and micrococci (fermented sausage) and *Zymomonas* sp. (e.g. some fermented beverages) (Hui et al., 2012).

Microorganisms involved in fermentation can be divided into monoculture: only one species of microorganisms is necessary to produce product, Multi-culture: more than one microorganism is required belonging taxonomically to different species, Uni-multiculture: two or more strains of the same species are used together and Poly-culture: many different microorganisms and the species specifically required to make the product are unknown (Hesseltine, 1983).

The starter culture may be a pure culture or mixture of known organisms, and each product requires its own special starter culture and these contains different mixtures of organisms. According to Cogan and Accolas (1996), starters usually consist of a cultivation medium, such as grains, seeds, or nutrient liquids that have been well colonized by the microorganisms used for the fermentation.

### 2.6. Yeast

Yeast has been reported to be involved in several types of indigenous African fermented foods and beverages (Greppi et al., 2013). Yeasts contribute to the organoleptic properties of the final fermented product, they are capable of upgrading the nutritional value of the foods and they are reported to have several probiotic effects that can contribute to the improvement of human health (Greppi et al., 2013).

### 2.7. Pearl Millet (*Pennisetum glaucum*)

Pearl millet, commonly known by Oshiwambo name as "O*mahangu*" in Namibia, is one of the most important staple cereals grown mostly by *Aawambo* in Omusati, Oshana, Oshikoto, Ohangwena and part of Kavango regions). Pearl millet (*Pennisetum glaucum* (L.) R. Br.)

classified as *P. americanum, P. spicatum* and *P. typhoides* is a cultivated, small-grain tropical cereal grass (Taylor, 2004). However, pearl millet should not be confused with the other millets, such as finger millet (*Eleusine coracana*), foxtail millet (*Setaria italica*), common or prose millet (*Panicum miliaceum*), and fonio (*Digitaria exilis*). Millet is a staple food for millions of people in Africa (Adebayo *et al*, 2010). Throughout Africa, indigenous tropical cereal grains sorghum, pearl millet and finger millet have been used since prehistory in making fermented beverages (Dewar & Taylor, 1999).

Pearl millet is a traditional staple food crop of the semi-arid tropical zones of Africa and Asia, it is cultivated in areas with a rainfall ranging from 150 mm to 800 mm per annum (Mallet & Du Plessis, 2011). It is important in food security in dry marginal agricultural areas are immense (Taylor, 2004). It has a nutritional value superior to other cereals like maize, wheat or rice. Pearl millet is digested more slowly and thus delays hunger and it has a higher content of proteins, lipids and amino acids (Mallet et al, 2011). Pearl millet is the most drought tolerant cereal and can grow in soils with low fertility, which are unsuitable to other cereals. Being particularly resistant to moisture stress and adapted to high temperatures, it has a low yield, which also explains its high nutritional value compared to other cereals (Hama, Icard-Verniere, Guyot, Picq, Diawara, & River, 2011). The pearl millet grain (figure 3) nutrients are distributed unequally in the different fractions of the grains (table 4). Endosperm, the largest fraction, which represents from 75% to more than 80% of the kernel weight, is the starch rich fraction whereas the pericarp contains mainly fibres; the germ contains most of the lipids, minerals, and phytates, the latter being the main chelating factors that impair absorption of minerals in the duodenum and hence micronutrient bioavailability (Hama et al., 2011).

**Table 4:** Compositions of different fractions of pearl millet (Hama et al., 2011).

| Grain structure | Bran/ Pericarp | Germ | Endosperm |
|---|---|---|---|
| % of kernel weight | 7.5 | 17.4 | 75.1 |
| Ash/minerals (%) | 13.9 | 72.2 | 13.9 |
| Lipids (%) | 5.9 | 87.8 | 6.3 |
| Phytates (%) | 25 | 67 | 8 |

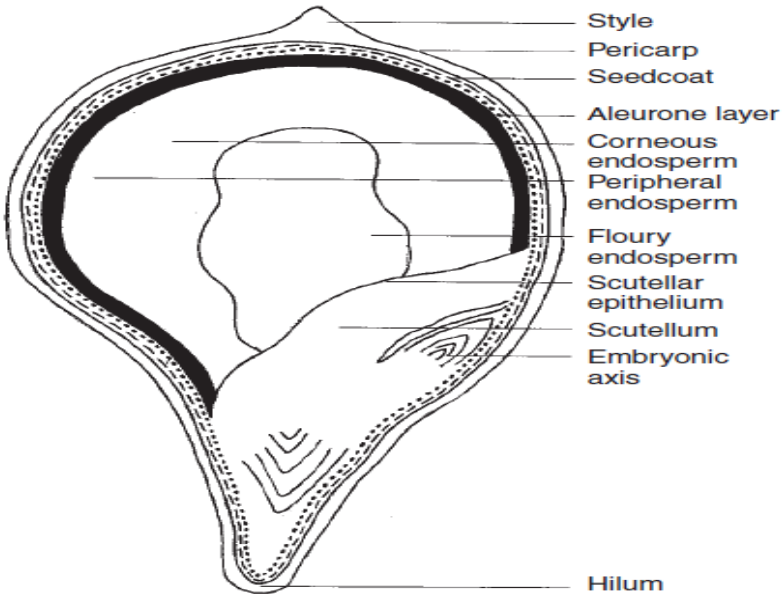


**Figure 3:** Diagrammatic longitudinal section through a pearl millet grain (Taylor, 2004).

Pear Millet grain is processed into *Mahangu* meal (figure 4): Decortication (separating the endosperm from the outer envelopes), then pounding into flour. *Mahangu* processing is mainly done manually using a traditional wooden mortar and pestle. Decortication separates the bran, consisting of the outside envelopes (pericarp, testa) and a portion of the germ, from the endosperm (mainly consisting of starch) (Mallet & Du Plessis, 2011**).** Pearl millet and sorghum contain nutritional inhibitors, the level of which is reduced before consumption, through Lactic acid steeping and fermentation (figure 4). The presence of polyphenols gives the grain its bitter

taste and reduces its digestibility, phytic acid is also found in pearl millet and sorghum grain, mainly in the aleurone layer (a thin layer rich in protein and fat found between the endosperm and the pericarp). Phytic acid has anti-nutritional effects on minerals and affects the digestibility of proteins and starch, by binding to minerals and proteins preventing absorption and adsorption in the body (Mallet et al., 2011**)**. In the crop field, the bitterness makes the grain less attractive to birds and reduces pre-harvest losses. To reduce the undesirable tastes due to the presence of polyphenols in sorghum and millet, housewife decorticates the grains and often allows the processed product to ferment by soaking in water for more than 24 hours.

These processing techniques (decortication and fermentation), lower the polyphenol levels in the grains. Phytic acid forms insoluble compounds (phytates) with mineral elements such as calcium, iron, magnesium, sodium, and zinc, making them unavailable for use by humans. Phytic acid can also combine strongly with proteins at certain pH levels (Mallet et al., 2011). Grain decortication reduces the fiber content and improves the taste, texture and nutritional value of the finished product, removes the bitterness (polyphenols) that is found mainly in the pericarp and the testa, the layer situated immediately below the pericarp.

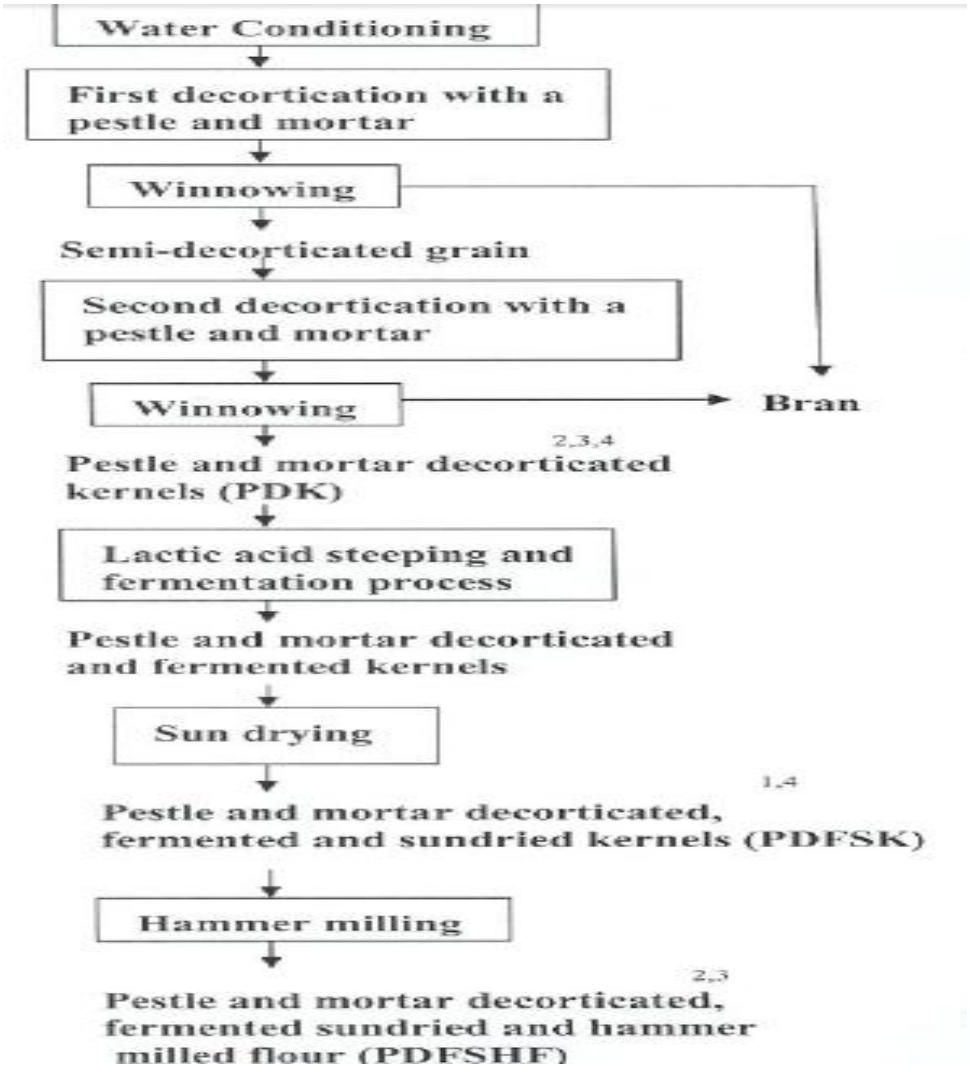


**Figure 4:** Flow diagram of the traditional Namibian milling process (Barrion, 2008).

### 2.8. Sorghum (*Sorghum bicolor*)

Sorghum (*Sorghum bicolor*) (L.) Moench is the major cereal crop produced in Africa. Sorghum has similar nutritional value to maize and is consumed as a major source of energy and protein by millions of people in the semi-arid area of Africa and Asia (Serna-Saldivar & Rooney, 1995). Sorghum is malted to brew opaque beer in most parts of Africa, including South Africa and European type beer (e.g., lager) and non-alcoholic malt beverages in several African countries (Taylor & Dewar, 2001). Malting is the limited germination of cereals in moist air, under

controlled conditions, while the objective of mobilizing the endogenous hydrolytic enzymes especially α-amylase of the grain which attacks α-(1-4) glucosidic bonds in starch molecules (Taylor & Belton, 2002). The nutrients are distributed unequally in the different fractions of the sorghum grains (table 5). Sorghum malting has many benefits according to Lefyedi, (2007). According to Lefyedi (2007), the general benefits of sorghum malting are:

1. Results in high levels of amylase
2. Reduce anti-nutritional factors
3. Enhances vitamin content
4. Improve mineral content
5. Improves in vitro digestibility of sorghum protein
6. Improves the composition and content of essential amino acids
7. Increase the vitro starch digestibility

**Table 5:** Structure and composition of different fractions sorghum grains (Hama et al., 2011).

| **Grain structure** | **Bran/ Pericarp** | **Germ** | **Endosperm** |
|---|---|---|---|
| % of kernel weight | 6 | 7 | 82 |
| Ash/minerals (%) | 13 | 71 | 2.9 |
| Lipids (%) | 4 | 80 | 10 |
| Crude fiber (%) | 53 | 16 | 30 |

### 2.9. *Oshikundu*

*Oshikundu* is a traditional cereal based fermented, very low alcoholic drink made from Pearl millet (*Mahangu* mill) and malted sorghum meal. However, both alcoholic and non-alcoholic varieties exist. The alcohol is mainly from yeast fermentation from malt sorghum. It is brewed by rural women for their daily household consumption and it is drunk the same day. *Oshikundu* is a sour-sweet drink and has quite a strong cereal taste. *Oshikundu* is not only brewed for

household consumption. Women sell it at open markets in many towns especially in the northern Namibia as a mean of earning a little income. It is a perishable beverage with a shelf life of less than six hours. Benchmarking *Oshikundu* traditional production method (s) to United Nation Educational, Scientific and Cultural Organization (UNESCO) and Netherlands organization for international cooperation protocol (Nuffic) indigenous knowledge system best practices, show applicable potential to incorporate *Oshikundu* brewing in Sub-Aawambo tribes development process (Mu Ashekele, Embashu & Cheikhyoussef, 2012). The UNESCO-Nuffic indigenous knowledge best practices criteria is a tool to identify indigenous knowledge practices and enhance their sustainability and development potential, through innovation and transformation. The traditional household production methods of *Oshikundu* was compared to UNESCO-Nuffic criteria by check listing elements of the traditional practices which should be looked at to ensure great impact on the indigenous knowledge practice.

*Oshikundu* processing methods have shown potential for commercialization (Mu Ashekele et al., 2012). In addition, the incorporation into school feeding program to address malnutrition. It can be given to school children as a daily beverage and the social economic implication. Such that children taking *Oshikundu* instead of exotic beverages at school are regarded as from poverty stricken households. This is in agreement with Mu Ashekele et al. (2012) and Embashu et al. (2013a), that commercialization of the production of *Oshikundu* would guarantee the utilization of local traditional resources, preservation of culture, job creation for subsistence farmers and the reduction of poverty.

*Oshikundu* is part of Aawambo culture knowledge transfer is passed on verbally. Traditional food and beverages constitute a vital body of indigenous knowledge (IK) (Aworh, 2008). Since IK is not documented, it is therefore on the risk of being lost to future generations (Dweba &

Mearn, 2011). In Namibia this is a problem when it comes to preservation of traditional knowledge of food and beverages, and this also includes *Oshikundu* processing knowledge. The preservation of traditional technology can serve as a template for transformation of the development of new products and consequently improved nutrition for this reason the art of traditional processing method needs to be documented in order to be transformed into a technology that incorporate objective methods of process control, optimization and standardise quality of the end product without losing the desired attributes (Gaden, Bokanga, Harlander, Hesseltine & Steinkraus, 1992). This can be applied to the traditional processing technology of *Oshikundu* for large scale production.

Despite *Oshikundu* being an important daily beverage, its social value is decreasing among the sub-tribes Aambandja (Omusati region) and *Aakwanyama* (Ohangwena region) valued the importance of the social values of *Oshikundu* and they mostly live in remote areas; *Aakwambi*, *Aandonga* and *Aakwanyam*a (Oshana region), *Aakwanyama*, *Aakwambi*, *Aangandjela*, *Aakwaaludhi*, *Aakolonkadhi*, *Aandongona* and *Aambalantu* (Omusati region) and *Aandong*a (Oshikoto region) have less value of *Oshikundu* comparatively (Embashu et al., 2013a).

# CHAPTER 3: MATERIALS AND METHODS

## 3.1. Research Design

A total of 22 *Oshikundu* samples were collected from different villages from Oshikoto, Ohangwena, Oshana and Omusati regions (figure 5) in 500 mL sterile plastic bottles. Sample providers were interviewed on the household processing method of *Oshikundu*, using questionnaire written in English and Oshiwambo. Sample providers were remunerated with a bag with basic needs such as sugar, tea, biscuits and sweets as a token of appreciation. Samples were stored and transported in a portable fridge below $4^{o}$ C and pH was taken at point of sampling. Interviews were conducted with local people on the traditional brewing method of *Oshikundu*.

## 3.2. Sample Collection and Storage

Samples of *Oshikundu* were collected from Ohangwena, Oshikoto, Omusati and Oshana regions in Namibia (Figure 5). This was done using 500 mL sterilized plastic bottles, which were kept and transported in the fridge at or just below $4^{o}$C, before taken to the University of Namibia in the Biotechnology laboratory and kept below $-1^{o}$C, until further analysis for physicochemical, nutritional and microbial analysis.

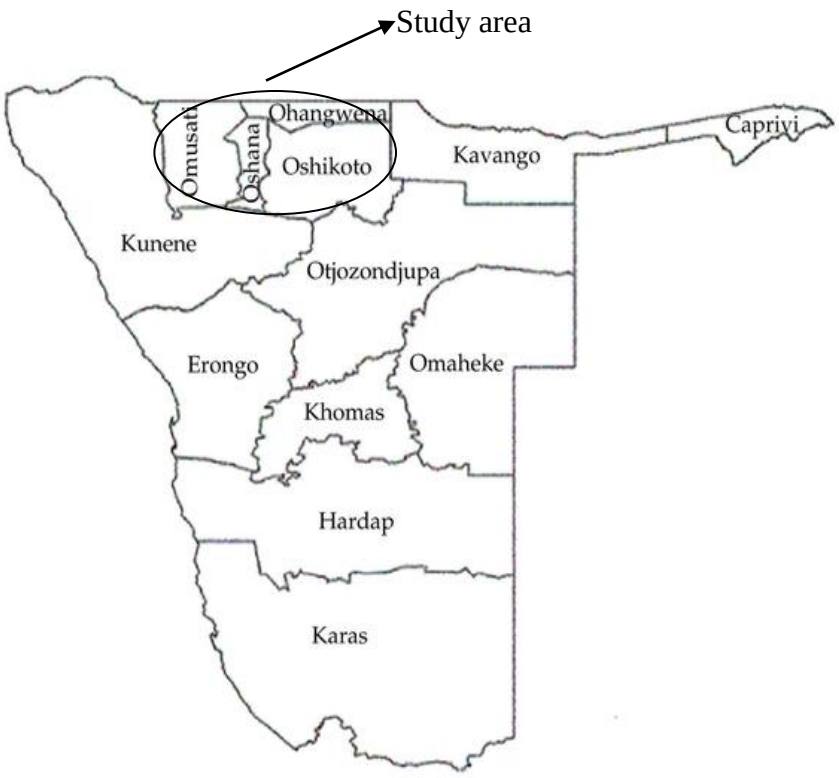


**Figure 5:** Study area Ohangwena, Omusati, Oshana and Oshikoto region

### 3.3. Research Instruments

- pH meter (Eurotech, Singapore)
- High Pressure Liquid Chromatography (HPLC) (PerkinElmer Flexar, USA)
- Gas Chromatography-Mass Spectrophotometer (GC-MS) (Thermo-Fisher, USA)
- Laminar flow
- Incubator
- Ultraviolet spectrophotometer (Gene sys 20, Thermo)
- Oven
- Centrifuge (Eppendorf 5810R, Germany)
- Viscosity meter

- Furnace
- Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-OES)

### 3.4. Physicochemical Characterization

#### 3.4.1. pH Measurement

The pH of *Oshikundu* was measured using a pH meter (Eurotech, Singapore) immediately as soon (on the spot) as samples were collected and this was done in triplicate. Additional measurements were also taken from freshly brewed *Oshikundu* in the laboratory.

#### 3.4.2. Titratable Acidity

The total acidity of *Oshikundu* was measured as lactic and acetic acid. Acetic acid was measured by procedure outlined by James (1995). 10.00 mL of *Oshikundu* was diluted into a 100 mL conical flask, and then double distilled $H_2O$ was added to the mark. Pipette 10 mL of the dilute in a conical flask and add 3 drops of Phenolphthalein. Titrate to faint pink endpoint with 0.1 M NaOH solution. The reaction is given by the equation below (James, 1995).

$$NaOH_{(aq)} + CH_3COOH_{(aq)} \rightarrow CH_3COONa_{(aq)} + H_2O_{(l)}$$

Percentage acetic acid was given by:

$$\% \text{ Acetic acid (M/V)} = T \times 0.6$$

T = Mean titre (in mL) of 0.1 M NaOH solution to neutralize acidity in 10 mL of diluted *Oshikundu*.

Lactic acid was measured by procedure outlined by James (1995). 10 mL *Oshikundu* was titrated of in conical flask. After that, 1 mL of 0.5 % Phenolphthalein solution was added and

titrate with M/9 NaOH solution until faint pink colour. The reaction is given by the equation below (Nielsen, 1998).

$$CH_3\,CH(OH)COOH_{(aq)} + NaOH_{(aq)} \rightarrow CH_3CH(OH)COONa_{(aq)} + H_2\,O_{(l)}$$

Percentage lactic acid is given by:

Titratable acidity (as % lactic acid) = (mL M/9 NaOH used/ 10 mL sample used).

### 3.4.3. Viscosity

*Oshikundu* viscosity was measured using a portable viscosity meter (Viscotech Hispanis S.L. Vendrell, Spain). About 50 mL of *Oshikundu* was measures by inserting the spindle (R 1) in a 100 mL beaker and viscosity was recorded as given by the viscosity meter.

### 3.4.4. Alcohol Content

About 25 mL aliquots of freshly brewed *Oshikundu* sample was centrifuged (Eppendorf 5810R, Germany) for 5 minutes at 1000 rpm before gravity filtration. Alcohol content was measured using alcohol analyser (Anton Paar, DMA 4500) using auto-sampler with frequent washing with water after every three samples and Heineken beer was used as a positive control.

### 3.4.5. Kinetics of Acid Evolution during *Oshikundu* Fermentation

Freshly brewed *Oshikundu* was prepared using Mahangu meal, malted sorghum meal and bran bought from Oshakati open market. Twenty two of the original samples were used for back-sloping and create a duplicate of each to be used in the analysis. *Oshikundu* was prepared with 100 g Mahangu flour, 40 g sorghum malt flour, 10 g bran and 1 L of water. *Oshikundu* pH, lactic acid and acetic acid were measured at different time interval: $t_0 = 0$, $t_1 = 2$, $t_2 = 4$, $t_3 = 6$, $t_4 = 8$, $t_5 = 24$, $t_6 = 30$, $t_7 = 48$ hours

### 3.4.6. Organic Acids

Organic acids in freshly *Oshikundu* were analysed following the method as outlined by Friedrich, (2001) with few modifications. About 15 mL of *Oshikundu* was filtered with Whatman no.4 filter paper before concentrated using a rotary evaporator to final volume of 7 mL. Concentrated *Oshikundu* samples were filtered through 0.45 µm syringe filter into vials. Organic acids standards (Merck, Germany): Maleic acid, D-Tartaric acid, (-)Shikimic acid, Succinic acid, Phytic acid dodecasodium salt hydrate, Oxalic acid and Malonic acid were prepared by weighing a 0.100 g to the nearest 0.1 mg in 10 mL volumetric flask and fill to the mark with HPLC grade water (Sigma, Germany). Both samples and standards were analysed using HPLC (PerkinElmer Flexar, USA) using column (Zorbax SB-C18, 4.6 × 150 mm, USA). The condition for the HPLC was adjusted (annex 1). Retention time for the organic acids standards was compared to samples retention time for peaks identification (annex 7).

### 3.4.7. Volatile Compounds Analysis

Freshly prepared *Oshikundu* was used for volatiles analysis. About 5 mL of *Oshikundu* was centrifuged (Eppendorf 5810R, Germany) for 5 minutes at 1000 rpm before gravity filtration. An amount of 1 mL of *Oshikundu* filtrate was extracted with 2 mL of dichloromethane ($CH_2CL_2$). About 1 µL of sample was injected in the GC-MS (Thermo scientific focus, ITQ 700) with a column 30 × 0.25 mm × 0.25 µm was used.

## 3.5. Proximate Analysis

### 3.5.1. Moisture and Total Solids

In a pre-weigh dry crucible with lid, 5 g aliquot of *Oshikundu* was weigh and dried in an oven at 100$^{o}$C. Crucibles were cooled to room temperature before placed in a desiccator after moisture

evaporated to dryness. Crucibles were re-weighed and the data was used to calculate moisture and total solids using this equation (James, 1995; Bradley, Jr, 2010).

$$\% \text{ Moisture} = \frac{W_2 - W_3}{W_2 - W_1} \times 100$$

Where $W_1$ =Initial weigh of crucible and lid

$W_2$ = Weigh of crucible, lid and food

$W_3$ = Weigh of crucible, lid and food after drying

$$\% \text{ Total solid} = 100 - \% \text{ Moisture}$$

**3.5.2. Ash**

Ash in *Oshikundu* was done by dry ashing. In a pre-weigh a dry crucible with lid, 5 g aliquot of *Oshikundu* was weighed and dried in an oven at 100 $^{o}$C. After moisture was evaporated to dryness, crucibles were placed in a furnace at 550 $^{o}$C and kept there for overnight. Crucibles were left to cool to room temperature before placed in the desiccator using tongs. Dry crucibles with ash were weighed and data was used to calculate percentage ash using this equation (Marshall, 2010).

$$\% \text{ Ash (dry matter)} = \frac{\text{Weight of crucible after ashing} - \text{Weigh of empty crucible}}{\text{Weight of sample} \times \text{dry matter coefficient}} \times 100$$

Where Dry matter Coefficient = % solids/ 100

**3.5.3. Protein**

Protein in *Oshikundu* was quantified using UV spectrophotometer (Gene sys 20, Thermo) according to Bradford (1976) and Kruger (2004). An amount of 0.1250 g Coomassie Blue R250

(Merck, Germany) was dissolve in 50 mL of 95 % ethanol ($C_2H_6O$) before mixing with 100 mL of 85 % phosphoric acid ($H_3PO_4$) and made up with distilled water in a 1 L volumetric flask. The solution was filtered through Whatman no. 1 filter paper. The filtrate was coved with foil and stored in the dark. An amount 0.0316 g of albumin bovine serum (Sigma-Aldrich, Germany) was dissolved in distilled water to make 25 mL in a volumetric flask to make 1.264 mg/mL concentration. The solution was frozen at -20$^{o}$C before used for analysis. A standard curve was prepared and absorbance was measured at 595 nm using UV spectroscopy (Gene sys 20, Thermo, USA).

### 3.5.4. Carbohydrates

About 25 mL aliquots of freshly *Oshikundu* sample was centrifuged (Eppendorf 5810R, Germany) for 5 minute at 1000 rpm before gravity filtration. Energy, reduced and unreduced sugars content of *Oshikundu* was measured using alcohol analyser (Anton Paar, DMA 4500).

### 3.5.5. Insoluble Fibers

Insoluble Fiber analysis in *Oshikundu* was done according to Nielsen (1998). About 1 g of *Oshikundu*, 40 mL phosphate buffer (pH 8.2) and heat stable α-amylase were added. The mixture was incubated for 15 minutes at 100$^{o}$ C before cooling to 60$^{o}$C. Protease was added and incubated for 30 minutes at 60$^{o}$C. The pH was adjusted with hydrochloric acid to 4.7 before the addition of 300 µL amyloglucosidase and incubates for 30 minutes at 60$^{o}$C. The digest was filtered and washed the residue with 10 mL of water two times. Insoluble fiber was collected on the filter paper, and this was washed with 10 mL of 95% ethanol then twice with 10 mL acetone. Insoluble fibre was calculated according to the equation below:

Insoluble fibre = mass of residue – (mass of ash + mass of protein)

### 3.6. Micronutrients Analysis

#### 3.6.1. Amino Acids

Amino acids in *Oshikundu* were analysed following method by Zhao et al., (2013). *Oshikundu* 10 mL was centrifuged (Eppendorf 5810R, Germany), gravity and filtered with a 4.5 µm nylon filter before analysis. Both samples and standards were analysed using High Performance Liquid Chromatography (HPLC) (PerkinElmer Flexar, USA) using column (Zorbax SB-C18, 4.6 × 150 mm, USA) and analysed the chromatogram. The conditions for the HPLC were adjusted (annex 2).

#### 3.6.2. Minerals

Mineral analysis for *Oshikundu* was done following method by Ayaz, Torun, Colak, Sesli, Millson, Glew (2011) with minor modifications. About 5 mL aliquots of *Oshikundu* samples from Oshana, Oshikoto, Ohangwena and Omusati region were digested in an Erlenmeyer flask with 20 mL of concentrated nitric acid ($HNO_3$) and 1 mL of perchloric acid ($HClO_4$) and covered with paraffin film. Samples were left to stand for 1 hour at room temperature before heating on a hot plate for 15 minutes at $50^0$ C, before left on a magnetic stiller for 24 hours. The digest, 5 mL was diluted with distilled water before analysis with Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-OES) (Thermo Scientific, iCAP 6000, UK). Different individual concentrations of 16 standards were prepared and a standard curve was used in quantifying elements in *Oshikundu.*

#### 3.6.3. Vitamins

Fat soluble vitamins in *Oshikundu* were determined using methods outlined by Prabhu, Goodman & Reuter, (2010) with few modifications. To 1 mL *Oshikundu*, 10 mL of water: acetonitrile (2:8) was added and mixed well by shaking several times. The mixture was

centrifuged (Eppendorf 5810R, Germany) for 5 minutes at 1000 rpm and remove the supernatant by decantation. 1 mL aliquots of *Oshikundu* supernatant was further diluted to 10 mL with water: acetonitrile (2:8). 500 µl sample aliquots were further diluted with 5 mL of water:acetonitrile (2:8) and filtered using 4.5 µm nylon filter prior to analysis. A standard curve was constructed by weighing 10 mg of vitamin A, vitamin E, vitamin $D_3$ to prepare a concentration of 1000 µg/mL (ppm) and 500 mg of vitamin $K_1$ for 500 µg/mL concentrations. The four standards were dissolved in 10 mL water:acetonitrile (2:8) and filtered using 4.5 µm nylon filter before analysis. Both samples and standards were analysed using High Performance Liquid Chromatography (HPLC) (PerkinElmer Flexar, USA) using column (Zorbax SB-C18, 4.6 × 150 mm, USA). The conditions for the HPLC column were adjusted (annex 3). Retention time for the standards was compared to sample chromatograms (annex 7).

Water soluble vitamins in *Oshikundu* were quantified (Shahnaz, Yasmin & Khan, 2009) with few modifications. About 10 mL of *Oshikundu* was transferred into a 250 mL round bottom flask. To each *Oshikundu* sample, 80 mL of distilled water was added and followed by 10 mL 0.1 N Hydrochloric acid (HCl) and reflux for 15 minutes. The mixture was cooled to room temperature, only 15 mL of *Oshikundu* sample was centrifuge (Eppendorf 5810R, Germany) at 1400 rpm for 5 minutes. The supernatant was filtered using 4.5 µm nylon filter before analysis. About 0.01 g of thiamine (vitamin $B_1$) and riboflavin (vitamin $B_2$) were dissolved in water to make 1mg/mL concentration. The two standards were filtered with 4.5 µm nylon filter before analysis. Both samples and standards were analysed using High Performance Liquid Chromatography (HPLC) (PerkinElmer Flexar, USA) using column (Zorbax SB-C18, 4.6 × 150 mm, USA) and analysed the chromatogram. The conditions for the HPLC were adjusted (annex 4). Retention time for the standards was compared to sample chromatograms (annex 7).

### 3.7. Microbiological Analysis

#### 3.7.1. Total Plate Count (TPC)

*Oshikundu* total plate count was done by aseptically transferring 1 mL into 9 mL of sterile Peptone water buffered (Biolab, Merck, Germany) and mixed thoroughly. Serial dilution ($10^{-1}$ to $10^{-9}$) was done for each of the 22 samples and incubated at 37 $^{o}$C for 24 hours. 100 µl dilute was inoculated on Plate Count Agar (PCA) and spread with the aid of spreader. The readings were taken after 24hrs of incubation at 35ºC. Results were expressed in Log CFU/mL (Colony Forming Units/mL).

100 µL aliquots of appropriate dilutions was inoculated in Nutrient broth and de Man Rogosa Sharp (MRS) broth for 24 hours at 37 $^{o}$C. 100 µL aliquots from the Nutrient and MRS broth were plated on the following media: Brilliant Green agar, Nutrient Agar, Bacillus cereus Agar Base, M17 agar, Rogosa agar and Man Rogosa Sharp (MRS) Agar, Malt extract Agar and Potato dextrose agar (PDA). This was done using a spreader before incubation at 37 $^{o}$C between 24-28 hours. The plates were stored in the fridge at 4$^{o}$C until further analysis for two days. The respective selective media were prepared as per manufactures instructions.

#### 3.7.2. Isolation of Lactic Acid Bacteria

Lactic acid bacteria strains were isolated from *Oshikundu* aseptically by transferring 1 mL into 9 mL of sterile Peptone water buffered (Biolab, Merck, Germany) and mixed thoroughly. Serial dilution ($10^{-1}$ to $10^{-9}$) was done for each of the 22 samples and incubated at 37 $^{o}$C for 24 hours.

a) 100 µL aliquots of appropriate dilutions was inoculated on MRS agar and incubated anaerobic at 37$^{o}$C for 48 hours for isolation of *Lactobacilli* ssp. (De Man, Rogosa, & Sharpe, 1960).

b) 100 µL aliquots of appropriate dilutions was inoculated on M17 agar incubated anaerobic at 37$^{o}$C for 48 hours for isolation of *Lactobacillus, Enterococci and Streptococcus thermophilus* (Therzaghi & Sandine, 1975).

c) 100 µL aliquots of appropriate dilutions were inoculated on Rogosa agar incubated anaerobic at 37$^{o}$C for 48 hours for isolation of *Lactobacillus* ssp.

After incubation, colonies were further purified by successive streaking on MRS, M17 and Rogosa agar. The pure isolates were preserved in sterile 25% glycerol and stored at -80$^{o}$C until further use.

### 3.7.3. Phenotypic Characterization of Lactic Acid Bacteria

Preliminary characterization of isolates through gram stain and catalase test was done. Pure isolates were recovered in MRS broth before strike on MRS, M17, Rogosa agar and incubated at 37$^{o}$C for 24 hours. Catalase test was done by placing a drop of 3 % hydrogen peroxide on a clean slide and spread a colony from each of the selective media. Gas bubble production was observed, indicating catalase positive and no gas bubble indicate catalase negative (Harrigan & McCance, 1976; Abd El Gawad et al., 2010). Cell morphology was observed using an optical microscope (Olympus BX51, Japan) (Gerhardt et al., 1981; Sneath, Mair, Sharpe & Holt, 1986; Abd El Gawad et al., 2010).

### 3.7.4. Preliminary Characterization of Yeast

Cell morphology and cellular arrangement were observed using an optical microscope (Olympus BX51, Japan). Growth on selective media was used by visual inspection and compare colour of different colony as a preliminary Characterization of yeast and moulds.

### 3.7.5. Preliminary Characterization of *Enterobacteriaceae*

Potassium hydroxide test was done by placing a drop of potassium hydroxide on a clean slide and mix a colony from nutrient agar. A slimy mixture indica's KOH positive (+) and no slimy KOH negative (-) (Harrigan & McCance, 1976; Abd El Gawad et al., 2010). Gram stain was performed to indicate gram reaction. Slides were viewed using optical microscope (Olympus BX51, Japan) for gram reaction as gram positive (+) indicated a purple colour or gram negative (-) indicated by a red colour (Bartholomew & Finkelstein, 1958). The KOH positive (+) and gram negative (+) isolates were selected for *Enterobacteriaceae* identification.

### 3.8. Biochemical Identification of LAB

A total of 20 isolates from MRS, M17 and Rogosa agar that are gram positive (+) and catalase negative (-) were selected for carbohydrate test using API 50 CH stripe/CHL medium for identification. The selection was also based on sub-groups within *Aawambo* in the four regions (Oshana, Oshikoto, Ohangwena and Omusati). API 50 CH strips and API 50 CHL medium according to manufacturer's instructions (BioMèrieux, France) was used to identify *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*. The API LAB PLUS software (BioMèrieux, France) was used according manufactures instruction in interpretation of results.

### 3.9. Biochemical Identification of Yeast

A total of 25 isolates from Malt extract agar and Potato dextrose agar were selected for API 20 C AUX for identification. The selection was also based on sub-groups within *Aawambo* in the four regions (Oshana, Oshikoto, Ohangwena and Omusati). API 20 C AUX was done according to manufacturer's instructions (BioMèrieux, France) was used to identify yeast and mould to genus and specie level. The API LAB PLUS software (BioMèrieux, France) was used according manufactures instruction in interpretation of results.

### 3.10. Biochemical Identification of Spoilage Bacteria

A total of 25 isolates from Nutrient agar were selected for API 20 E for identification of bacterial that are likely to cause spoilage in *Oshikundu*. The selection was also based on sub-groups within *Aawambo* in the four regions (Oshana, Oshikoto, Ohangwena and Omusati). *Enterobacteriaceae* API 20 E according to manufacturer's instructions (BioMèrieux, France) was used to identify *Enterobacteriaceae* to genus and specie level. The API LAB PLUS software (BioMèrieux, France) was used according manufactures instruction for identification of *Enterobacteriaceae*.

### 3.11. Genotypic Characterization

*Oshikundu* isolates of Lactic acid bacteria identified (8) with API LAB PLUS, DNA were extracted using Puregene yeast/bacteria kit (Qiagen, USA) following the kit instructions with few modifications (annex 5). Polymerase Chain Reaction (PCR) was done using a thermo cycler ( Eppendorf, Germany) with the following primers (Hesham, Khan, Liu, Zhang, Wang, Yang, 2006): *Pediococcus*-F (5'-AGA GTR TGA TCM TYG CTW AC-3'), *Pediococcus*-R (5'-CGY TAM CCT WTT ACG RCT-3'), *Lactococcus*-F (5'-AGA GTT TGA TCC TGG CTC AGG A-3'), *Lactococcus*-R (5'-GGA GGT GAT CCA GCC GC-3'), F-lac (5'-GCA GCA GTA GGG AAT CTT CCA-3'), R-lac (5'-GCA TTY CAC CGC TAC ACA TG-3'), 27F (5'-AGA GTT

TGA TCC TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3'). The un-purified PCR-products were sent for sequencing at Inqaba biotech, South Africa. Isolates of yeast identified with API LAB PLUS, DNA was extracted using Puregene yeast/bacteria kit (Qiagen, USA) following the kit instructions with few modifications (annex 6). Polymerase Chain Reaction (PCR) was done using a thermo cycler with primers NL1 (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCC ATA TCA ATA AGC GGA GGA AAA G-3') (GC clamp underlined) and reverse primer LS2 (5'-ATT CCC AAA CAA CTC GAC TC-3') (Hesham et al., 2006). The un-purified PCR-products were sent for sequencing to Inqaba biotech (Johannesburg, South Africa).

### 3.12. Data Analysis

Measurements were done in triplicates and average means were calculated with standard deviation (±SD) for each of the regions. Kolmogorov-Smirnov Test was done to check for normal distribution for each of the regions data, before one way analysis of variance (ANOVA). One way ANOVA was used to compare the means. For the significantly different mean, Post hoc test was used to identify where the difference is using SPSS.

### 3.13. Research Ethics

*Oshikundu* samples were collected with the permission from the local people and through their Councillors and Governors in the four regions. This was done so that the residents in the regions can be informed and explained the research objectives. *Oshikundu* brewed in the laboratory (not food grade) was not used for any other purpose apart from experimental analysis only.

# CHAPTER 4: RESULTS

## 4.1. *Oshikundu* Traditional Processing Method(s)

During sampling, it was found that *Oshikundu* is a popular daily beverage in many households among *Aawambo* within Ohangwena, Omusati, Oshana and Oshikoto regions. Respondents between the age of 18 and 70 years old, have indicated that *Oshikundu* has been a part of *Aawambo* culture for hundreds of years and the processing technology has been passed on verbally from generation to generation. This knowledge transfer is carried out by mothers to their daughters or grandmother to their granddaughters. They further added that men hardly play a role in this knowledge transfer and the actual brewing process. Women from the four regions brew *Oshikundu* with *Mahangu* meal (*Pennisetum glaucum* (L.) R. Br.), malted Sorghum meal (*Sorghum bicolor*) (L). Moench), *Mahangu* bran and water, and two procedures are followed independently illustrated in Figure 6.

Processing methods of raw materials, storage and traditional milling of malted sorghum and *Mahangu* to final product *Oshikundu* (figure 7). In the first procedure (figure 6), boiled water is added to *Mahangu* meal and the mixture is left to cool to room temperature with occasional stirring. Malted sorghum meal is then added to the mixture. Meanwhile, in the second procedure, warm water is added to *Mahangu* and malt sorghum meal is mixed by stirring (Embashu et al., 2013a). Malt pearl millet was also reported to be used in brewing, although none of the respondents have indicated to have used it. In both procedures bran is added at this step and this step is optional depending on the availability and preference of using bran in brewing. Back-slopping is done at this step whereby a previously fermented *Oshikundu* is also added. This mixture is diluted with water depending on the amount of starting material used and desired

volume of the final product. Some respondent have indicated to add porridge, which will be eaten by children. The mixture is then left to ferment at room temperature on average one and half hour after which *Oshikundu* will be ready to drink. *Oshikundu* however has a very short shelf life of less than six hours based on respondents on average.

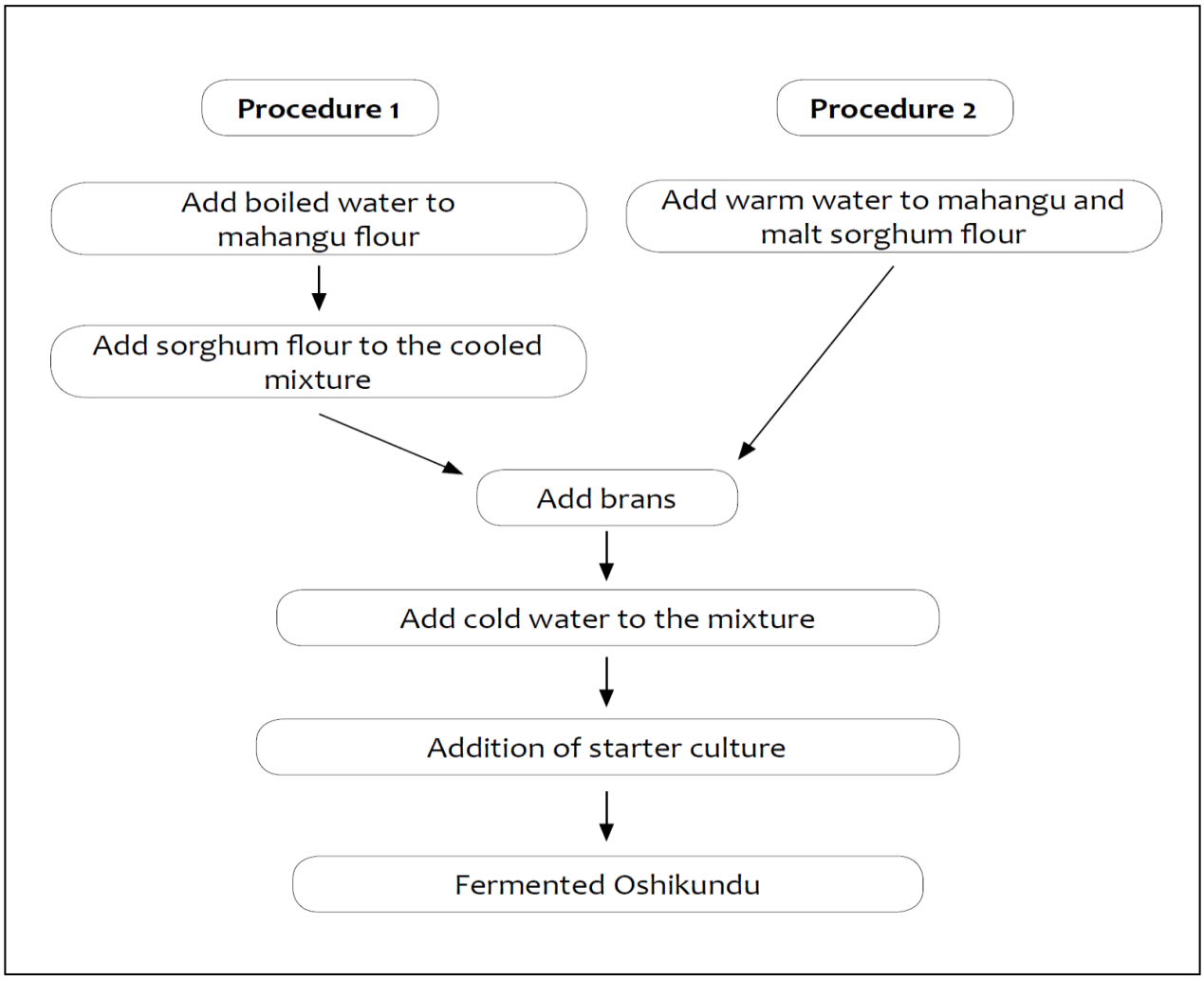


**Figure 6:** A flow chart outlining common traditional processing methods of *Oshikundu* in Oshana, Oshikoto, Ohangwena and Omusati regions (Embashu et al., 2013a).


**Figure 7:** The processing of *Oshikundu* from raw materials, storage, traditional milling, ingredients and final product A) Mahangu Field, B) storage (*Oshigadhi*), C) milling by using pestle and mortar, D) *Mahangu* meal, E) malted sorghum meal, F) *Oshikundu* (Embashu et al., 2013b).

### 4.2. Physicochemical Properties

#### 4.2.1. pH

*Oshikundu* pH ranged from 3.33 ± 0.127 in Oshana to 3.60 ± 0.014 in Oshikoto region as shown in table 6. *Oshikundu* pH is an acidic beverage and it is consistent within all the four regions. *Oshikundu* pH was recorded to be lowest in Oshana followed by Ohangwena, Omusati and the highest was in Oshikoto region. There was no significant difference ($P > 0.05$) in comparing *Oshikundu* pH mean within the four regions.

**Table 6:** *Oshikundu* average pH with standard deviation (±) from Ohangwena, Omusati, Oshana and Oshikoto region

| Regions | | | |
|---|---|---|---|
| Ohangwena | **Omusati** | **Oshana** | **Oshikoto** |
| 3.36 ±0.045 | 3.40 ±0.146 | 3.33±0.127 | 3.60±0.014 |

### 4.2.2. Titratable Acidity

Titratable acidity of *Oshikundu* as acetic acid was found to range from 0.10% to 0.30% in Omusati and Ohangwena regions respectively. Meanwhile lactic acid ranged from 1.20% and 1.68% in Oshikoto and Ohangwena region (figure 8). Lactic acid was relatively higher in Ohangwena followed by Omusati, Oshana and lower in Oshikoto. Acetic acid was observed to be high in Ohangwena followed by Oshana, Oshikoto and lower in Omusati region. There was no significant difference ($p > 0.05$) in comparing means for acetic acid of *Oshikundu* mean among Ohangwena, Omusati, Oshana and Oshikoto, and also comparing mean for lactic acid.

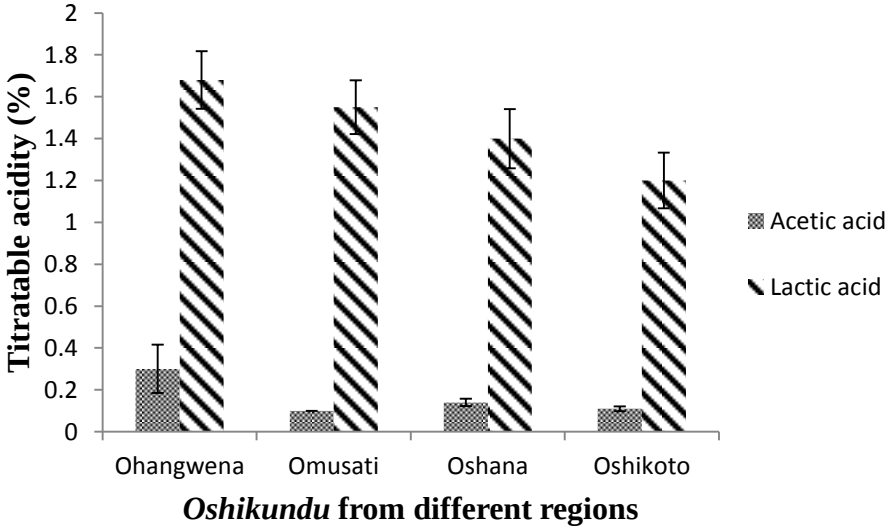


**Figure 8:** *Oshikundu* titratable acidity as acetic acid and lactic acid from Ohangwena, Omusati, Oshana and Oshikoto study regions.

### 4.2.3. Viscosity

*Oshikundu* viscosity ranged between 23.4 ± 1.154 mPas to 41.0 ± 6.082 mPas. While the Torque ranged between 14.4 ± 0.838% to 24.9 ± 3.464% (table 7). The viscosity was found to be high in Ohangwena region in comparison with other samples, followed by Omusati, Oshikoto and Oshana region the lowest. Torque also was found to be the highest in Ohangwena followed by Omusati, Oshikoto and Oshana with the lowest. There is a significant difference ($P < 0.05$) in comparing the mean viscosity for *Oshikundu* between the four regions and also in comparing the mean torque among the four regions.

**Table 7**: Viscosity and Torque of *Oshikundu*

| | **Regions** | | | |
|---|---|---|---|---|
| | Ohangwena | Omusati | Oshana | Oshikoto |
| **Viscosity (mPas)** | 41.0 ±6.082 | 40.0 ±10.785 | 23.3 ±1.154 | 24.3 ±4.932 |
| **Torque (%)** | 24.9 ±3.464 | 24.3 ±6.585 | 14.4 ±0.838 | 14.8 ±3.031 |

### 4.2.4. Alcohol

*Oshikundu* alcohol content was less than 2 % v/v from all the four regions (figure 9). *Oshikundu* alcohol content was found to be relatively higher in Omusati, and then followed by Ohangwena, Oshana and Oshikoto regions. There is no significant difference between Ohangwena and Omusati (A) regions, and between Oshana and Oshikoto regions (B). There is a significant difference level ($P > 0.05$) between the region A and B.

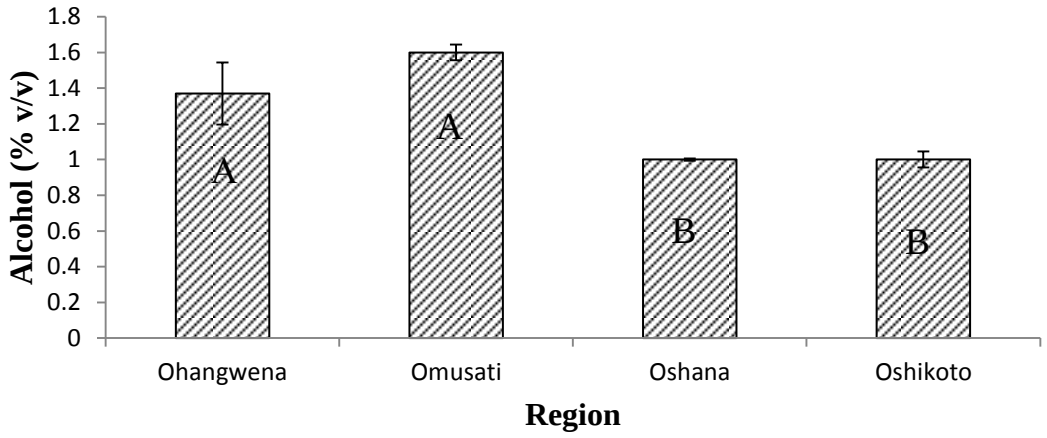


**Figure 9:** *Oshikundu* alcohol content

### 4.2.5. Acid Evolution Kinetics during Oshikundu Fermentation

*Oshikundu* pH, acetic and lactic acid varied with time within 48 hours (figure 10). *Oshikundu* pH decreases with time from 4.01 to 3.71 in 48 hours. The pH was monotonic between 24 - 48 hours, which showed little or no decrease. Acetic acid content did not increase within 48 hours of measurements. Meanwhile lactic acid increased from 1.1 % to 3.2 % within 48 hours. There was a sharp increase in the amount of lactic acid between 6-30 hours. Lactic acid and pH intersect at16 hours, as the pH level decrease and lactic acid increase.

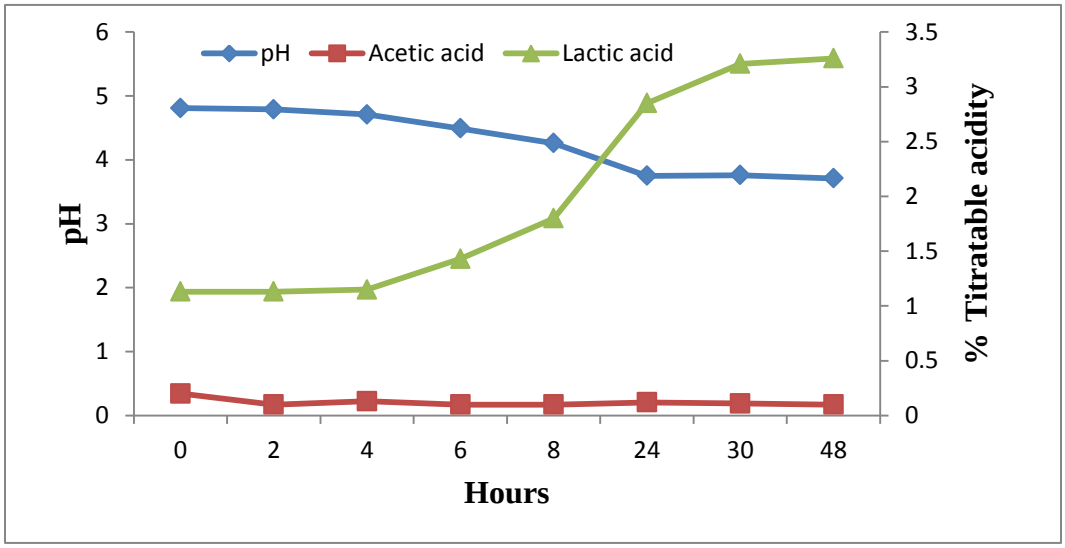


**Figure 10:** Changes in pH, lactic acid and acetic acid during fermentation of *Oshikundu*

### 4.2. 6.Organic Acids

*Oshikundu* was found to mainly contain six organic acids that were detected (figure 11) within the Ohangwena, Omusati, Oshana and Oshikoto region. Comparing the retention time for

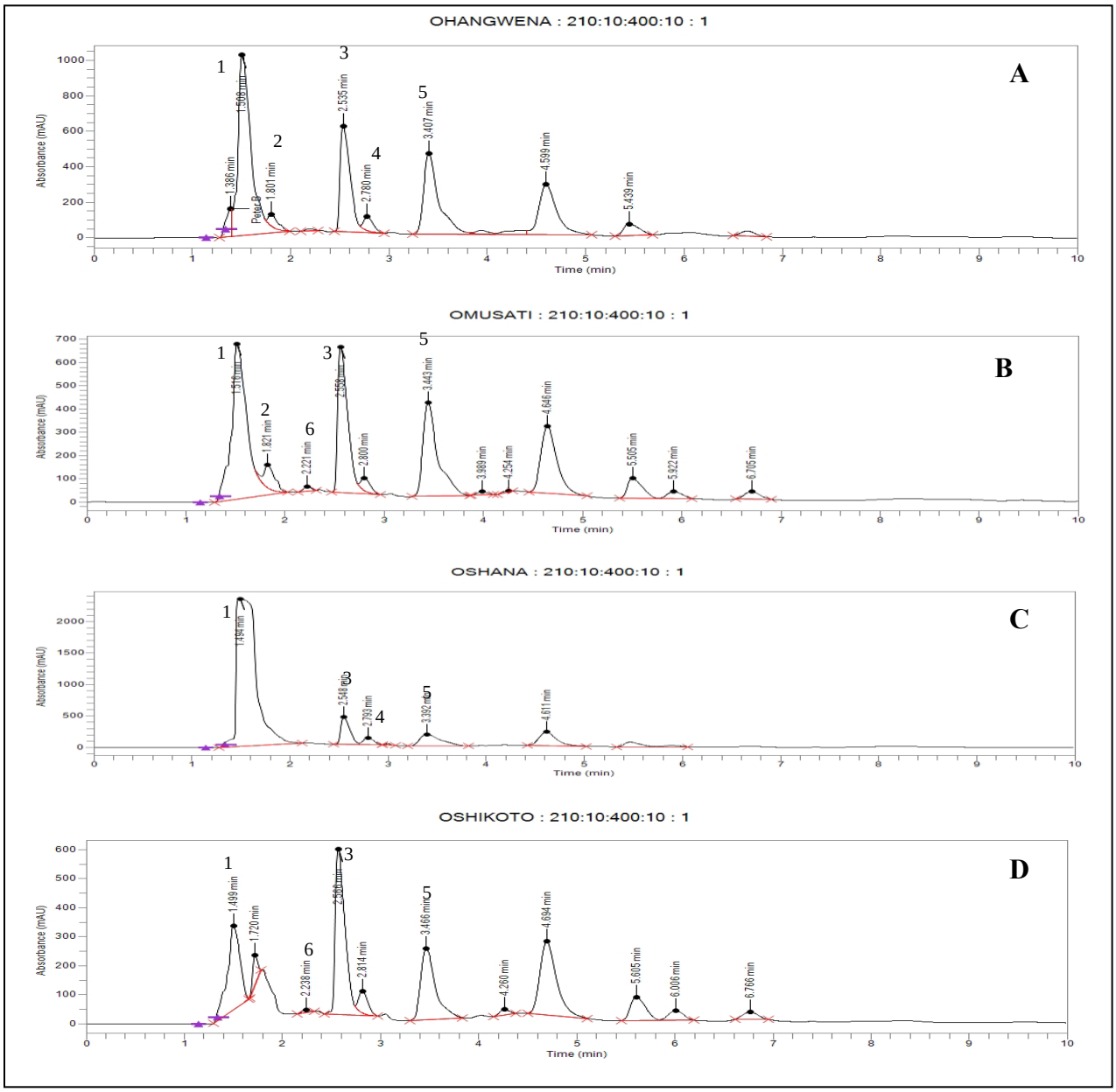


**Figure 11:** Chromatograms of organic acids in *Oshikundu* samples from A) Ohangwena, B) Omusati, C) Oshana and D) Oshikoto region analysed using HPLC: 1)lactic acid, 2) Acetic acid, 3) Shikimic acid, 4) Maleic acid, 5) Succinic acid and 6) Phytic acid.

organic acids standards to *Oshikundu* samples from the respective regions for identification, organic acid in *Oshikundu* from Ohangwena region (Fig. 11.A) are acetic acid, Lactic acid, Shikimic acid, Maleic acid and Succinic acid, with lactic acid being in more concentration compared to others. Omusati region's organic acids were identified as lactic acid, acetic acid, phytic acid, shikimic acid and succinic acid. Oshana region's organic acids were also identified as lactic acid, shikimic acid, maleic acid and succinic acid. Meanwhile, Oshikoto region organic acids were identified as lactic acid, shikimic acid, succinic acid and phytic acid only. Lactic acid, shikimic acid and succinic acid were all present in all the *Oshikundu* samples from all four regions. Lactic acid being the highest in concentration when compared to other organic acid identified in all tested samples.

### 4.2.7. Volatile Compounds

Six possible compounds were identified from the mass spectrum (figure 12) in all the four samples from Ohangwena, Omusati, Oshana and Oshikoto regions from *Oshikundu*. However, the Mass Spectrometer library used for identification was a demo version, it is limited to a hundred compounds and the result may not be accurate. Ohangwena region digitoxin, Vitamin A aldehyde, Pregn-4-ene-3,20-dione, 11-hydroxy and prednisolene were identified. In Omusati region, Digitoxin and vitamin A aldehyde were also identified. In Oshana region, vitamin A aldehyde, Pregn-4-ene-3,20-dione, 11-hydroxy, Retinal, 9-cis and digitoxin were identified. And in Oshikoto region, Gibberellic acid, Beclomethasone, Digitoxin and Vitamin A aldehyde were also identified. Digitoxin and Vitamin A aldehyde were identified in all the samples from all the four regions. Five of the identified compound has a steroidal skeleton (figure 13) in their structures except vitamin A aldehyde.

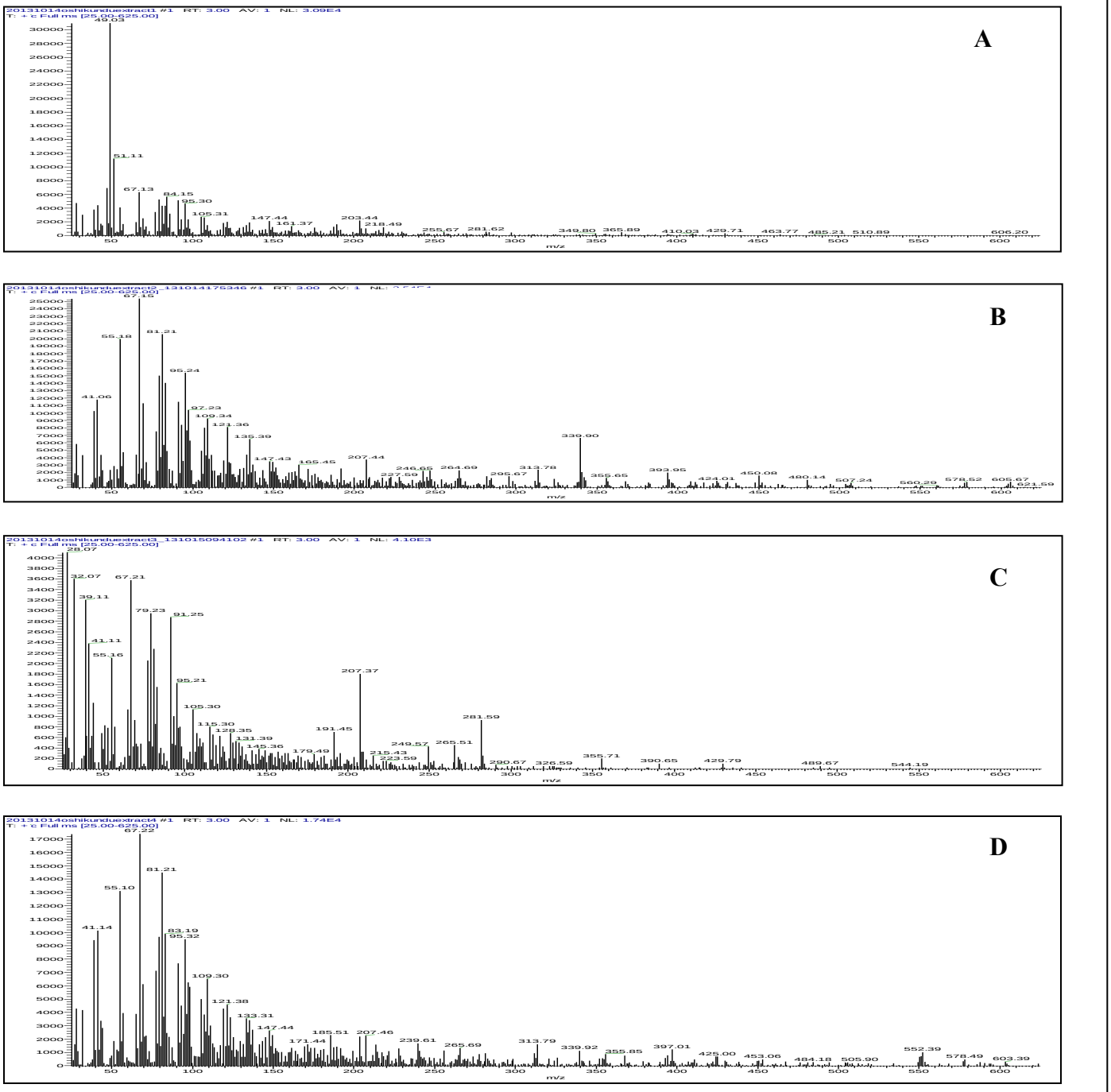


**Figure 12:** Mass spectra for *Oshikundu* samples from A) Ohangwena, B) Omusati, C) Oshana and D) Oshikoto region analysed using GC-MS.

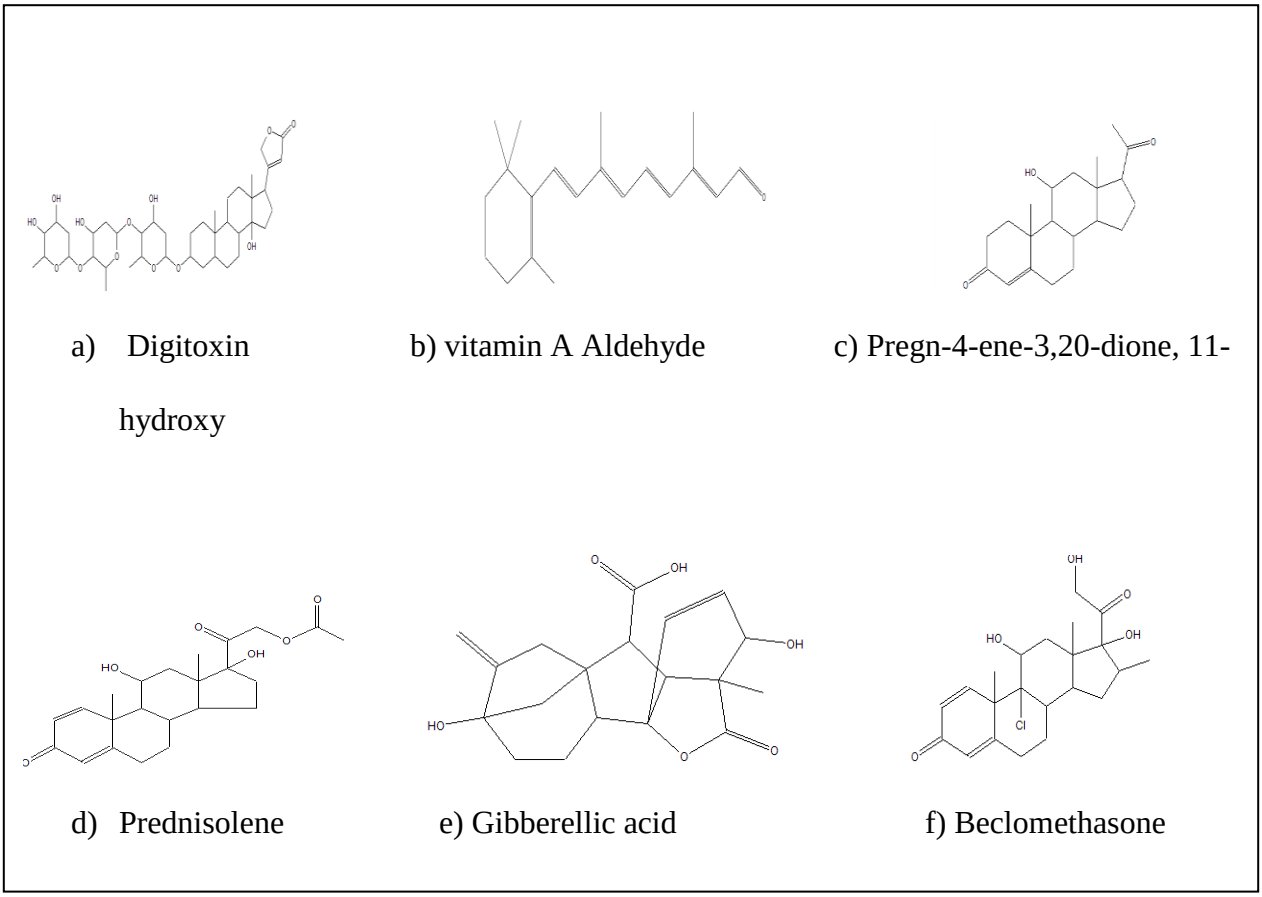


**Figure 13:** Structure of compounds identified in *Oshikundu*: a) Digitoxin, b) Vitamin A aldehyde, c) Pregn-4-ene-3,20-dione, 11-hydroxy, d) Prednisolene, e) Gibberellic acid, f) Beclomethasone.

### 4.3. Proximate Analysis

#### 4.3.1. Moisture, Total Solids and Ash

*Oshikundu* moisture content ranged between 94.9 ± 5.190% to 96.8 ± 0.464%, total solids ranged between 2.0 ± 0.926 % to 4.2 ± 0.951 % and ash ranged between 0.070 ± 0.050 % to 0.114 ± 0.007 % (table 8). Moisture in *Oshikundu* was high in Ohangwena region, in comparison to other regions, followed by Omusati, Oshana and lower in Oshikoto region. There was no significant difference ($P > 0.05$) in comparing the moisture Means between the regions. Total solids were found high in Oshana as compared to other regions followed by Omusati, Ohangwena then

Oshikoto region respectively. There is no significant difference (P> 0.05) in comparing Means between the four regions. Ash content was high in comparison to other regions in Ohangwena, Oshikoto, and Oshana and lower in Omusati region. There is a significant difference (P<0.05) in comparing the means between the regions. The significant difference was found when one compares Ohangwena to Omusati region and Ohangwena to Oshana regions (table 8).

**Table 8:** *Oshikundu* moisture, total solids and ash content from Ohangwena, Omusati, Oshana and Oshikoto regions, *the mean difference is significant at 0.05 level. ±: Standard deviation.

| | **Ohangwena** | **Omusati** | **Oshana** | **Oshikoto** |
|---|---|---|---|---|
| **Moisture (%)** | 96.8 ±0.464 | 96.2 ±1.123 | 95.7 ±0.951 | 94.9 ±5.190 |
| **Total Solids (%)** | 3.1 ±0.464 | 3.7 ±1.123 | 4.2 ±0.951 | 2.0 ±0.926 |
| **Ash (%)** | 0.114 ±0.007* | 0.070 ±0.050* | 0.079 ±0.031* | 0.88 0.044 |

### 4.6.2. Protein

*Oshikundu* protein content ranged between 0.131-0.175 mg/mL (figure 14). Protein in *Oshikundu* was high in Ohangwena and Omusati, followed by Oshana and lower in Oshikoto region.

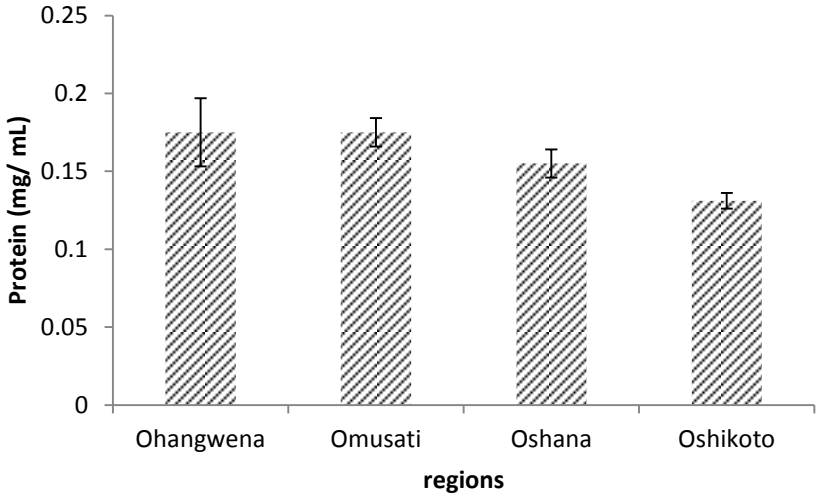


**Figure 14:** *Oshikundu* protein content.

There was no significant difference (P> 0.05) in comparing the mean between the four regions

### 4.6.3. Carbohydrates

*Oshikundu* real extract (Er) ranged between 0.95 ± 0.104 to 1.44 ± 0.075% m/m, Apparent extract (Ea) ranged between 0.79 ± 0.274 to 1.03 ± 0.291% m/m, Plato ranged between 1.95 ±1.102 to 3.55 ± 0.435 %, Real degree of fermentation (RDF) ranged between 41.67 ± 33.657 to 64.30 ± 0.718 %, Apparent degree of fermentation (ADF) ranged between 66.02 ± 2.0160 to 79.19 ± 0.8915 % m/m and energy 38.77 ± 2.5243 to 58.34 ± 2.8006 kJ/ 100 mL (table 9). *Oshikundu* from Omusati region has the highest Er, Ea, plato, RDF, ADF and energy compare to the other regions. Meanwhile Ohangwena region follows high in plato, RDF, ADF and energy but lower in Er and Ea compared to Oshana region. Oshikoto has the lowest Ea, Er, plato, RDF, ADF and energy in comparison with other regions. There is a significant difference ($P< 0.05$) in comparing means for Er and energy between the regions. However, there is a significant difference ($P< 0.05$) in comparing means for Ea, Plato, RDF and ADF among the four regions.

**Table 9:** *Oshikundu* estimates of Real extract (Er), Apparent extract (Ea), Plato, Real degree of fermentation (RDF), apparent degree of fermentation and energy from Ohangwena, Omusati, Oshana and Oshikoto regions. *There mean difference is significant at 0.05 levels. Standard deviation (±)

| Regions | Er (%m/m) | Ea (%m/m) | Plato (%) | RDF (%) | ADF (%m/m) | Calories (kJ/100 ml) |
|---|---|---|---|---|---|---|
| **Ohangwena** | 1.35 ±0.112* | 0.82 ±0.198 | 3.55 ±0.435 | 61.84 ±6.733 | 76.21 ±8.3061 | 51.90 ±6.2283* |
| **Omusati** | 1.44 ±0.075* | 1.03 ±0.291 | 4.00 ±0.187 | 64.30 ±0.718 | 79.19 ±0.8915 | 58.34 ±2.8006* |
| **Oshana** | 1.41 ±0.102* | 1.02 ±0.097 | 3.01 ±0.100 | 53.57 ±1.681 | 66.02 ±2.0160 | 44.11 ±1.5492* |
| **Oshikoto** | 0.95 ±0.104* | 0.79 ±0.274 | 1.95 ±1.102 | 41.67 ±3.657 | 70.68 ±8.3289 | 38.77 ±2.5243* |

### 4.6.4. Insoluble Fibres

*Oshikundu* insoluble fibre was found to range between 0.25 - 0.31 % on wet basis (figure 15). Oshikoto region was found to be the highest insoluble fibre followed by Omusati, Ohangwena and Oshana region. There is no significant difference (P> 0.05) in comparing means of fibre between the four regions.

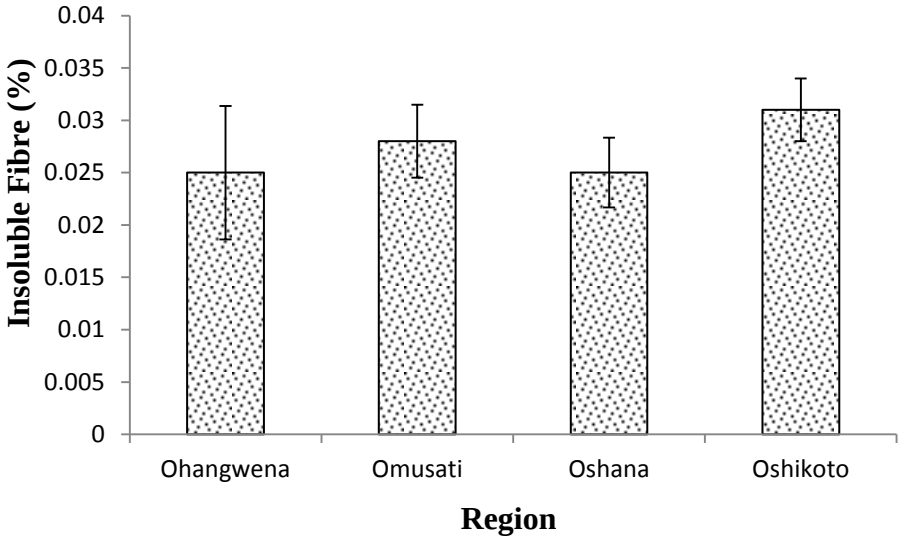


**Figure 15:** *Oshikundu* insoluble fibre

## 4.7. *Oshikundu* Micronutrients

### 4.7.1. Amino Acids

Despite the unavailability of derivatization reagents of amino acids in *Oshikundu* samples and their standards, the sample chromatogram gave less than five peaks (figure 16). These peaks could not be correctly identified however; an attempt was made to identify peaks according to Henderson, Ricker, Bidlingmeyer & Woodward (2000). Retention time of the sample peaks was compared to retention time of the standards used by Henderson et al, (2000). The possible amino acids are Aspartate and Glutamate at 1,2 and 3. Meanwhile at peak 4 possible amino acids are Glycine, Theomine, Citrulline and Arginine all from the four respective regions. Despite this, the

peaks could not be confirmed nor identified that indeed they are due to the presence of amino acids in *Oshikundu*.

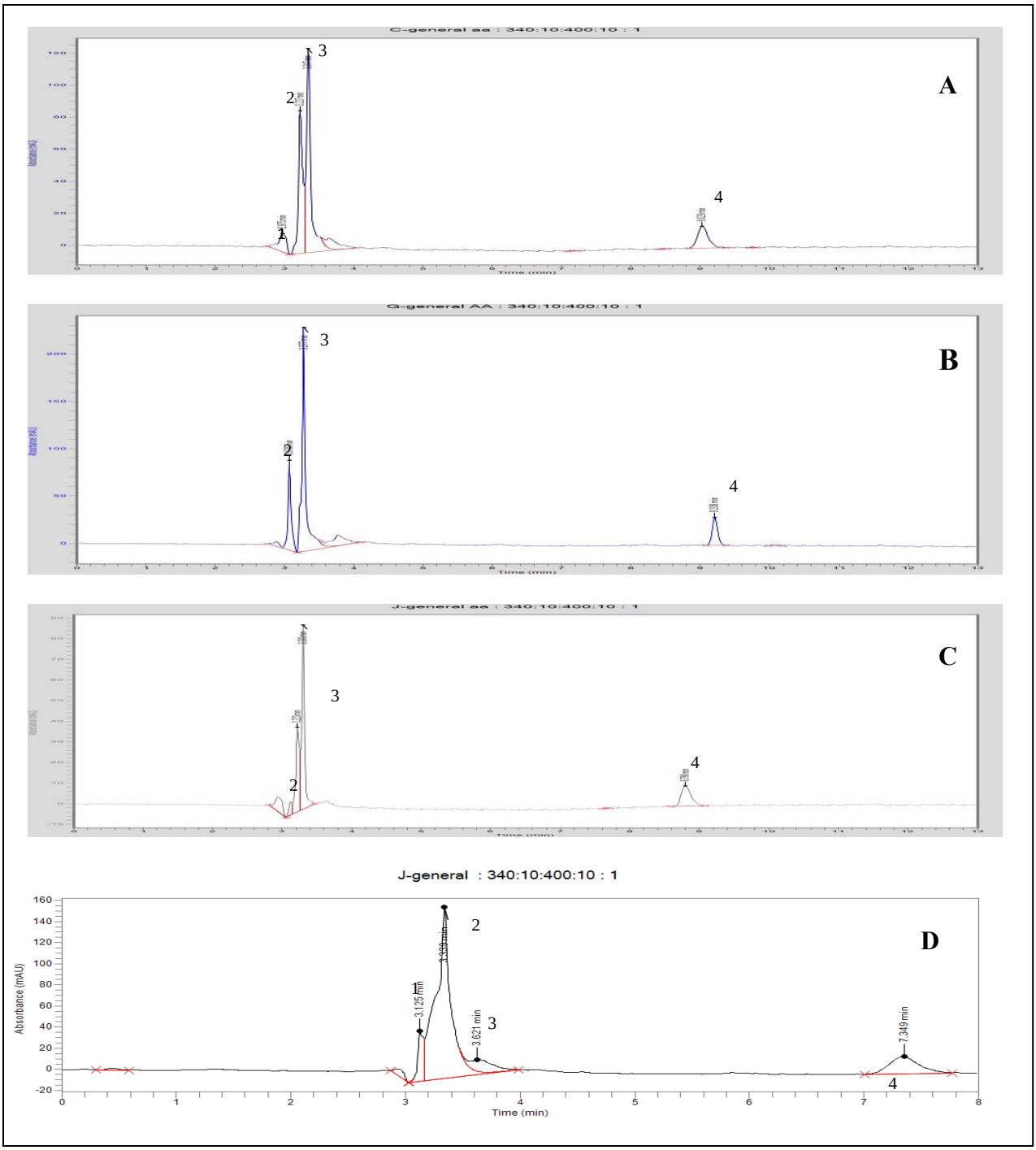


**Figure 16:** Chromatograms of amino acids in *Oshikundu* samples from A) Ohangwena, B) Omusati, C) Oshana and D) Oshikoto regions. Aspartate and Glutamate could be possibly at 1,2 and 3 meanwhile Glycine, Theomine, Citrulline and Arginine possibly at peak 4.

### 4.7.2. Minerals

Fifteen elements were detected in *Oshikundu* from Ohangwena, Omusati, Oshana and Oshikoto region respectively (table 10). *Oshikundu* is high in Phosphorus, followed by potassium, magnesium and calcium. Meanwhile it is low in cerium. Phosphorus was found to range between 3.914 ± 0.452 to 11.511 ± 7.264 ppm and potassium ranged between 4.333 ± 0.461 to 8.466 ± 5.170 ppm. Magnesium was found to range between 0.847 ± 0.138 to 2.503 ± 1.024 ppm and calcium ranged between 0.697 ± 0.281 to 2.593 ± 1.357 ppm. There is significant difference (P> 0.05) in comparing means for Ce, Fe, K, Ni, P, Zn and S between the four regions. There is a significant difference (P> 0.05) in comparing means of B, Ca, Cr, Cs, Cu, Mg, Mn and Na.

**Table 10:** *Oshikundu* elements from Ohangwena, Omusati, Oshana and Oshikoto regions. *The mean difference is significant at 0.05 level. ±: Standard deviation.

| Elements (ppm) | Ohangwena | Omusati | Oshana | Oshikoto |
|---|---|---|---|---|
| **B** | 0.645 ±0.307* | 1.100 ±0.107* | 1.804 ±0.303* | 1.842 ±0.088* |
| **Ca** | 2.593 ±1.357* | 1.223 ±0.239* | 0.697 ±0.281* | 0.856 ±0.376* |
| **Ce** | 0.018 ±0.048 | 0.018 ±0.800 | 0.080 ±0.049 | 0.049 ±0.017 |
| **Cr** | 0.032 ±0.016* | 0.017 ±0.002* | 0.008 ±0.003* | 0.011 ±0.001* |
| **Cs** | 0.313 ±0.232* | 0.481 ±0.107* | 0.206 ±0.157* | 0.160 ±0.201* |
| **Cu** | 0.052 ±0.028* | 0.004 ±0.004* | 0.007 ±0.019* | 0.034 ±0.007* |
| **Fe** | 0.230 ±0.183 | 0.220 ±0.030 | 0.032 ±0.075 | 0.050 ±0.016 |
| **K** | 8.466 ±5.170 | 6.705 ±0.411 | 4.512 ±0.482 | 4.333 ±0.461 |
| **Mg** | 2.503 ±1.024* | 1.637 ±0.143* | 0.923 ±0.159* | 0.847 ±0.138* |
| **Mn** | 0.025 ±0.130* | 0.020 ±0.004* | 0.009 ±0.002* | 0.005 ±0.002* |
| **Na** | 1.134 ±0.615* | 0.537 ±0.043* | 0.323 ±0.019* | 0.380 ±0.050* |
| **Nd** | 0.039 ±0.009 | 0.019 ±0.009 | 0.024 ±0.009 | 0.020 ±0.009 |
| **Ni** | 0.015 ±0.007 | 0.011 ±0.001 | 0.006 ±0.003 | 0.006 ±0.001 |
| **P** | 11.511 ±7.264 | 8.433 ±0.404 | 5.243 ±1.111 | 3.914 ±0.452 |
| **S** | 0.360 ±0.141 | 0.270 ±0.025 | 0.158 ±0.015 | 0.109±0.011 |
| **Zn** | 0.098±0.089 | 0.093±0.049 | 0.152±0.083 | 0.140±0.113 |

### 4.7.3. Vitamins

#### *4.7.3.1. Water Soluble Vitamins*

Two water soluble vitamin B-complexes were both identified in the four regions (figure 17).

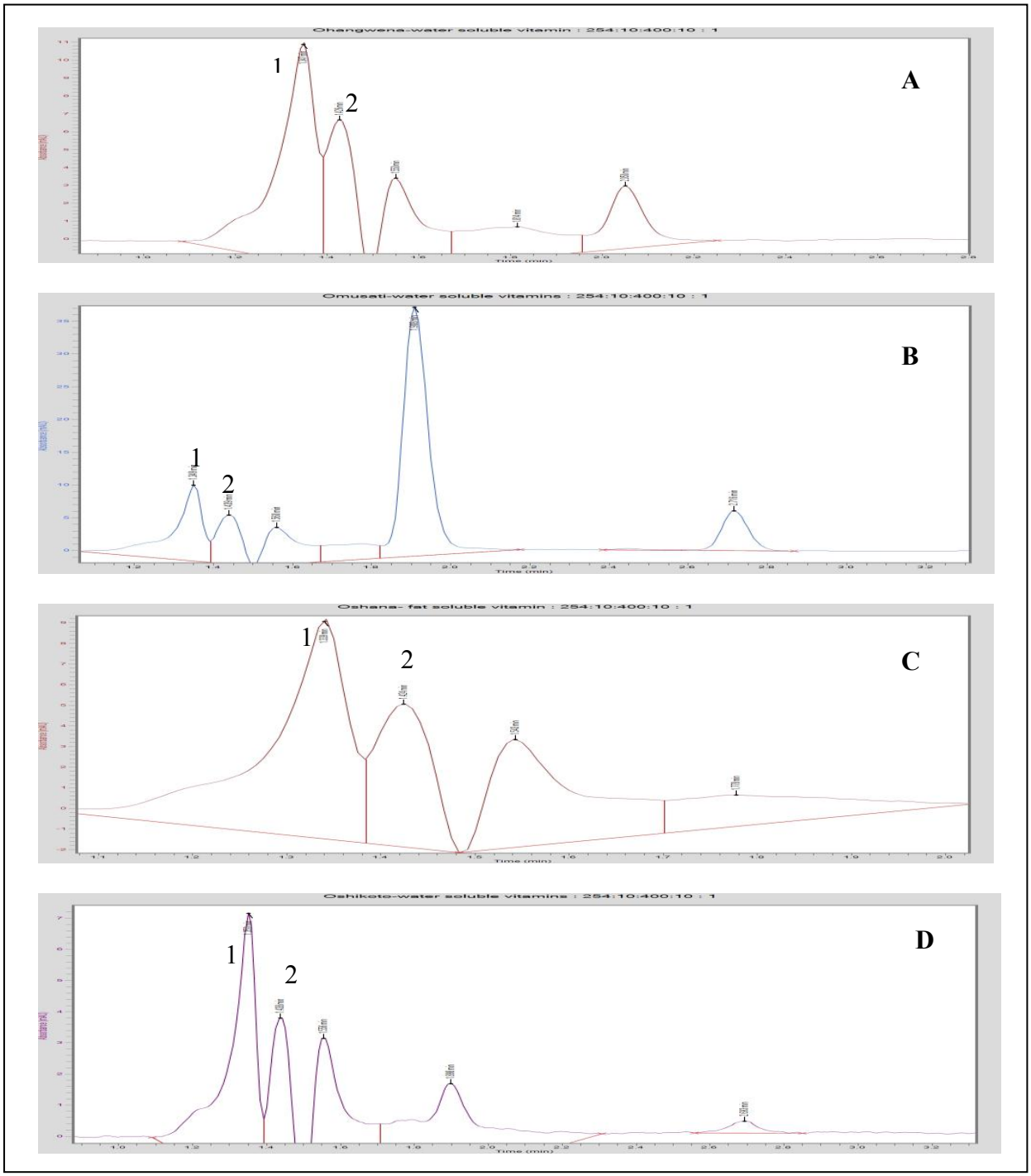


**Figure 17:** Chromatogram of water soluble vitamins in *Oshikundu* from A) Ohangwena, B) Omusati, C) Oshana and D) Oshikoto region. 1) Vitamin $B_2$ , 2) Vitamin $B_1$

### *4.7.3.2. Fat Soluble Vitamins*

In comparison of the retention time between fat soluble vitamins standards (annex 7) and

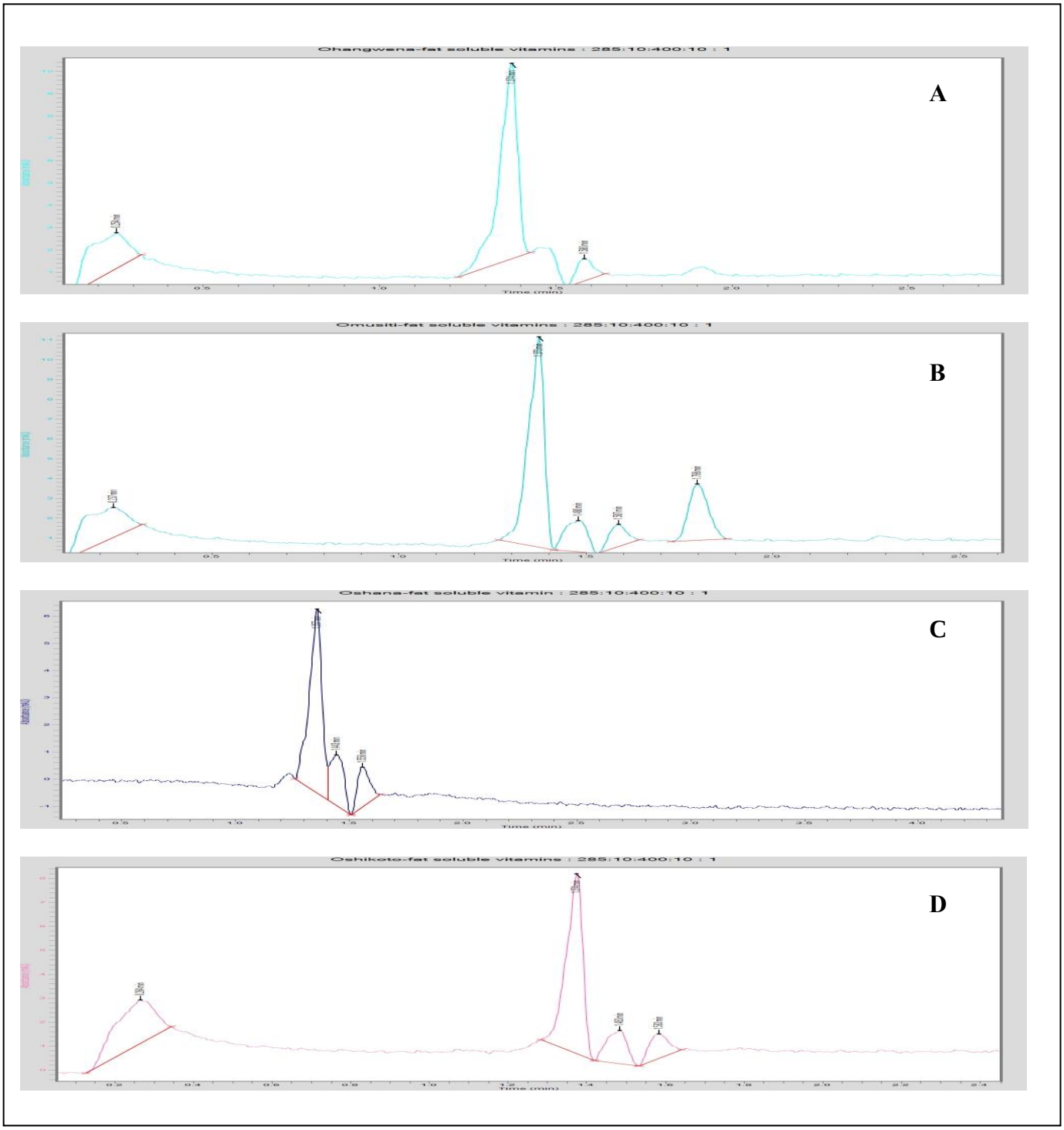


**Figure 18:** *Oshikundu* chromatogram for fat soluble vitamins, a) Ohangwena, b) Omusati, c) Oshana, d) Oshikoto region.

*Oshikundu* samples peaks (figure 18), none of the peaks could be identified. All the standards eluted between 4 and 20 minutes, meanwhile samples peaks eluted before two minutes (figure 18).

### 4.8. *Oshikundu* Microbiology

#### 4.8.1 Total Plate Count

Oshikoto region had the highest Log colony forming units per mL (8.20) of *Oshikundu* in comparison with other regions, followed by Oshana (7.46 Log CFU/mL), Ohangwena (6.78 Log CFU/mL) and the lowest is Omusati (6.36 Log CFU/mL) (table 11). However, there was no growth observed on green brilliant agar for isolation of pathogens and related microbes.

**Table 11:** Total Plate Count (TPC) after 24 hours at $10^6$ dilutions for *Oshikundu* from Oshana, Oshikoto, Omusati and Ohangwena regions.

| Regions (Log CFU/ mL) | | | |
|---|---|---|---|
| Ohangwena | **Omusati** | **Oshana** | **Oshikoto** |
| **6.78** | 6.36 | 7.46 | 8.20 |

#### 4.8.2. Lactic acid Bacteria

*Oshikundu* lactic acid bacteria were identified to genus level; consequently six were identified as dominant bacteria (figure 19). These are *Lactobacillus plantarum* 2 (23 %), *Lactococcus lactis* ssp. *lactis* 1 (23 %), *Lactobacillus delbrueckii* ssp. *delbrueckii* (15 %), *Lactobacillus plantarum* 1 (15 %), *Lactobacillus fermentum* 2 (8 %), *Lactobacillus pentosus* (8 %) and *Lactobacillus curvatus* ssp. *curvatus* (8 %). *Lactobacillus* species are the dominant in *Oshikundu* in addition to *Lactococcus*. *Lactobacillus plantarum* 2 and *Lactococcus lactis* ssp. *lactis* 1 are the most frequently identified in all the samples from the four regions.

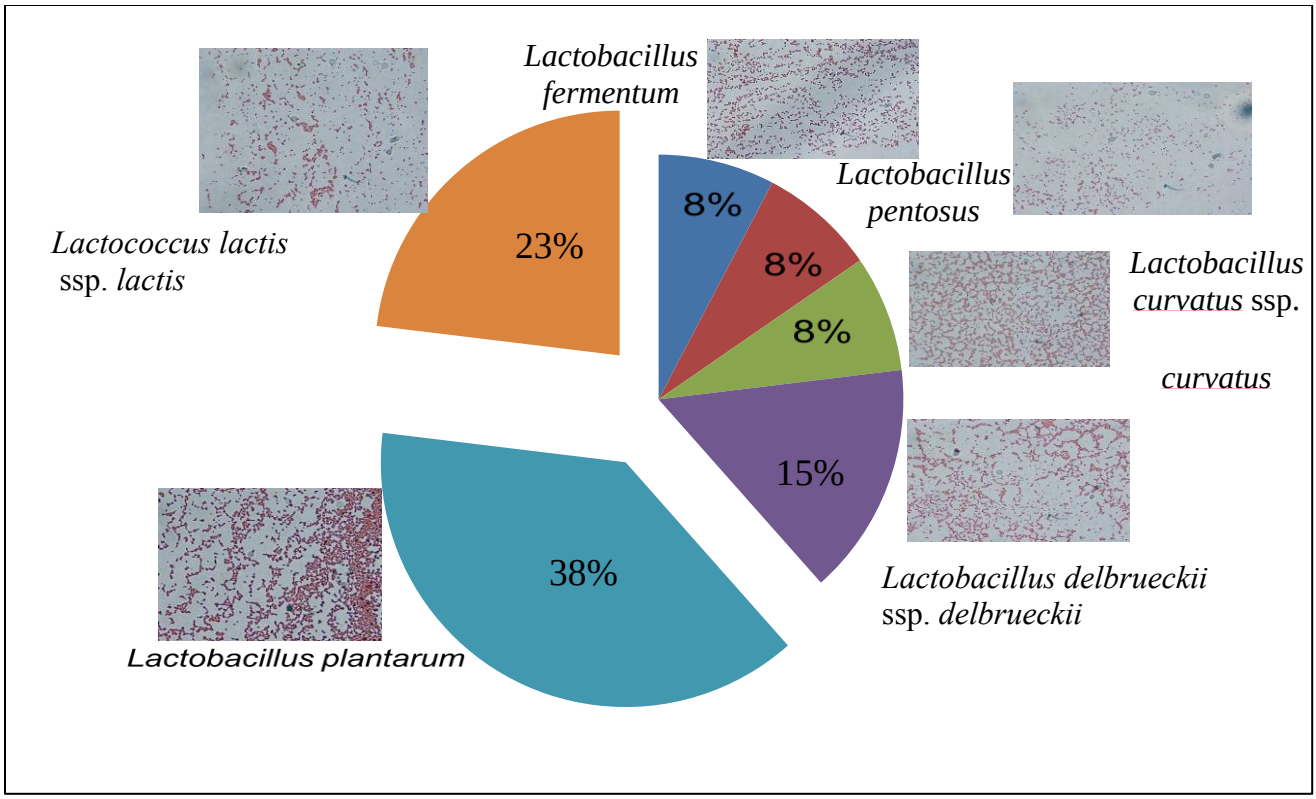


**Figure 19:** Dominant Lactic acid bacteria identified in *Oshikundu* using API 50 CH/CHL medium.

### 4.8.3. Yeast

The result from sequencing could not be included in this write up, since they are not yet available from South Africa and due to time constraints.

### 4.8.4. Spoilage Bacteria

Bacterial isolates were identified to genus levels (figure 20) that are likely to cause spoilage of *Oshikundu*. These are *Enterobacter cloacae* (56.25 %), *Enterobacter sakazakii* (25 %), *Pseudomonas luteola* (6.25 %), *Pseudomonas aeruginosa* (6.25 %) and *Serratia ficaria* (6.25 %). *Enterobacter cloacae* is the most frequently identified in all the samples, followed by *Enterobacter sakazakii*.

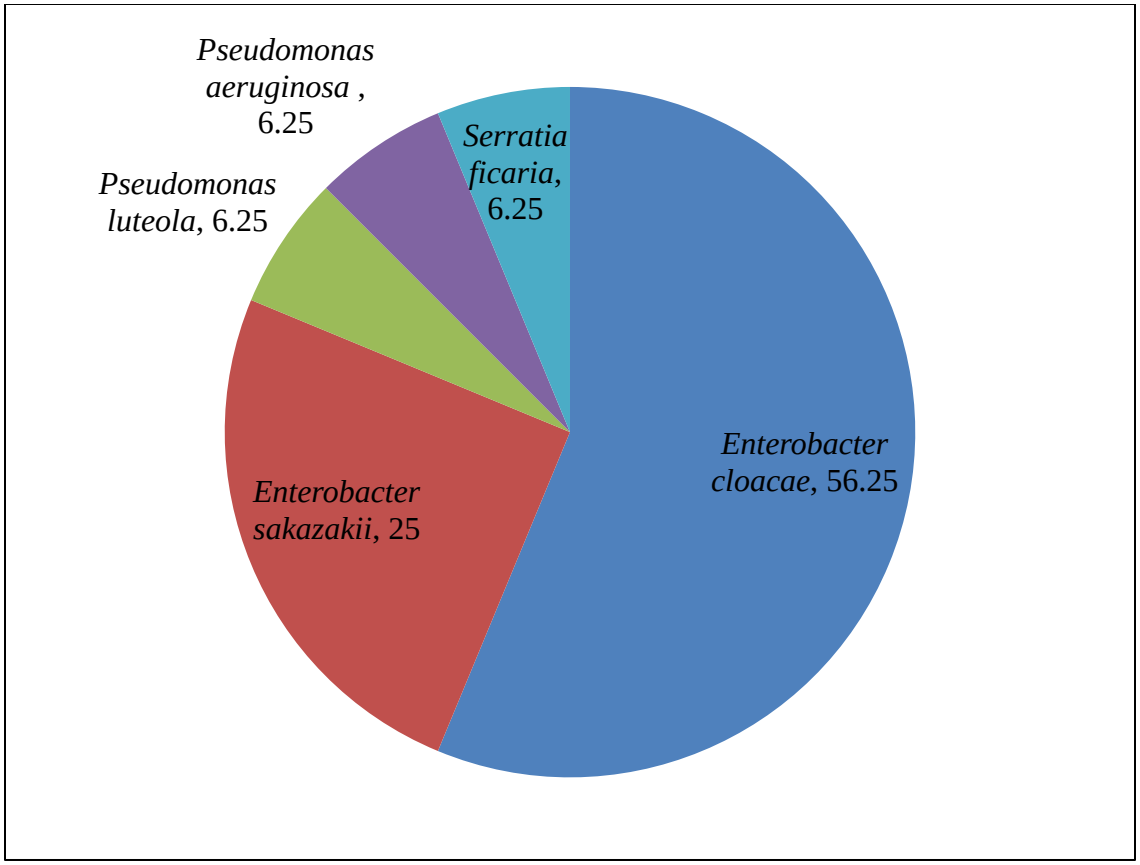


**Figure 20:** Likely spoilage bacterial species in *Oshikundu* identified using API 20 E.

# CHAPTER 5: DISCUSSION

## 5.1. *Oshikundu* Processing Method(s)

The traditional processing technology of *Oshikundu* is passed on from mothers to daughters. Women play a major role in knowledge transfer and preservation, not only in the case of *Oshikundu.* They are responsible for the perfection and consequently the evolution in the brewing process of *Oshikundu*. The art of traditional brewing of *Oshikundu* may seem to be simple, but the science behind it is intriguing. Women in Aawambo culture may not know the science behind the processing method of *Oshikundu*, but they understand the techniques and their relevancy within the processing steps.

The two procedures in *Oshikundu* brewing, procedure 1 (figure 3) is being commonly used even though some brewers have improvised and modified some steps. The addition of malted sorghum meal after a cooled mixture in procedure 1 (figure 3), brewers, believe that this has an effect on the taste of *Oshikundu*. However, some brewers have modified the procedure and add boiled water to malted sorghum and Mahangu meals. Sorghum and pearl millet grain undergoes malting before milling and is used in *Oshikundu* brewing. Malted sorghum plays an important role in the fermentation of *Oshikundu*. The malting process enables the production of enzymes such as amylases, which hydrolyze grain starch to sugar (glucose and maltose) (Taylor, 2003) and thus free sugar is available for lactic acid bacteria to ferment *Oshikundu*. The lactic acid bacteria especially lactobacillus ferments the sugar (glucose) into lactic acid and yeast into ethanol and carbon dioxide. Certain steps in the traditional art of brewing have been ingeniously optimised to result in non-alcoholic products (Dewar & Taylor, 1999). In *Oshikundu* brewing process, the use of boiled water limits the amount of enzymatic hydrolysis of starch to

fermentable sugar and hence this favours a lactic acid, rather than alcoholic fermentation (Taylor, 2003). Therefore, this could be the reason for low alcohol content in *Oshikundu,* even though sorghum malt would naturally contribute yeast. The high boiled water temperature (approx. $60^{o}$ C) enable the growth of thermophilic lactic acid bacteria but not yeast; later when the temperature falls towards the range suitable for alcoholic fermentation, mesophilic bacteria takes over (Taylor, 2003).

Under traditional brewing conditions, it is a challenge to reach and maintain an optimal temperature for all the steps: this leads to an overlap in the brewing process across different steps, consequently two or more steps takes place at the same time (Dewar & Taylor, 1999). The brewing steps of *Oshikundu* may take place simultaneously depending on the brewer, extraction of sugar from malt sorghum meal and fermentation take places in one step and temperature. Temperature is one of the crucial parameter in brewing and this directs the type of microorganism, sugar extract, enzyme activity that leads to the final product. Back slopping in *Oshikundu* has been in practice for many generations, but this has also its disadvantages. The inconsistency in the organoleptic properties of *Oshikundu* is considered to be one major drawback in the product characteristics and form a major challenge to develop *Oshikundu.*

**5.2. Physicochemical Characterization**

*Oshikundu* pH ranged between 3.33 ± 0.127 and 3.60 ± 0.014 (table 7) and titratable acidity, acetic acid and lactic acid ranged between 0.10% to 0.30% and 1.20% and 1.68%, respectively. The *Oshikundu* pH decreases with time as compares to lactic acid content (figure 10). The major organic acids in *Oshikundu* were lactic and acetic acid. *Oshikundu* lactic acid increases over time and as a result of accumulation of lactic acid and hence the acidity increases. The increase in acidity is the direct result of production of organic acids. Acetic acid in *Oshikundu* did not

increase within 48 hours. As the acidity increases, *Oshikundu* pH decreases. As the *Oshikundu* pH decreases over time, the taste becomes sour and gives off a bad odour. The acidic pH inhibits and favours certain microorganism in *Oshikundu*.

The presence of some organic acids plays a role on *Oshikundu* quality and shelf life. The presence of phytic acid can probably serve to our advantage as an antioxidant but can possibly be also an anti-nutrient. Minerals bind to phytic acid and become unavailable for absorption. Shikimic acid is a very useful organic acid in amino acid biosynthesis was detected in *Oshikundu* coming from plant tissues used in brewing. Maleic acid was also detected in *Oshikundu* which may contribute to the sour taste after six hours. The combination of these organic acids influences *Oshikundu* colour, flavour and microbial stability.

The volatile compounds detected in *Oshikundu* were digitoxin, vitamin A Aldehyde, Pregn-4-ene-3, 20-dione, 11-hydroxy, Prednisolene, Gibberellic acid and Beclomethasone. Some of the identified compounds are toxic, and they have a steroidal skeleton in their structure. The library used in identification is a demo version,which made it difficult to accurately identify the volatile compounds in *Oshikundu*. Another reason could be that the compounds in *Oshikundu* were highly volatile and degrade before analysis. *Oshikundu* alcohol content is below 2 %, and this maybe due to the lactic acid bacteria favoured fermentation. *Oshikundu* viscosity of samples used in this study was not high, since much of it is made up of water.

### 5.3. *Oshikundu* Nutrient Profile

*Oshikundu* is very high in moisture content, which is above 90 %. This is a disadvantage when it comes to microbial spoilage and quality. Spoilage microbes are more likely to be favoured by high water content and as a result *Oshikundu* shelf life is under six hours. However, high water content provides consumers with daily water intake through *Oshikundu* and this helps in keeping

them hydrated. Total solid in *Oshikundu* much of it is from malt sorghum and *Mahangu* meal. Ash is a good indicator of minerals in *Oshikundu*. *Oshikundu* has a considerable amount of protein. The protein could have been increased due to fermentation, as its one of its advantages.

*Oshikundu* is low in calories, which is below 60 kJ/ mL. This is consistent with the Plato of *Oshikundu*, which is the degree of fermentability of the malt sorghum mix which is very low. But the degree to which sugar in cold mix has been fermented into alcohol, Real degree of fermentation (RDF) is very high. The specific gravity of Oshikundu at the completion of fermentation; Apparent degree of fermentation (ADF) is also high. The amount of sugar that is fermented, Real extract (Er) is also low as well as the extract content and produced alcohol; Apparent extracts (Ea).

*Oshikundu* has insoluble fibre, which can be contributed from the ingredients as they come from plant materials. Amino acids were not correctly identified based on their standards chromatograms and retention time. Amino acid standards and *Oshikundu* samples need to be derivatized, which was not done due to lack of derivatization reagent. *Oshikundu* has many elements, P being the highest (11.511 ± 7.264 ppm) (table 11) followed by K, Mg and Ca are the most abundant. Other important elements also detected in *Oshikundu* are B, Cu, Fe and Zn. These elements in *Oshikundu* collectively serve important functions in the human body such as normal brain function, skeletal, immune system and calcium absorption. Water soluble vitamins were also detected in *Oshikundu*, vitamin $B_1$ (thiamine) and vitamin $B_2$ (Riboflavin). Vitamin B-complex detected in *Oshikundu* plays an important role in normal function of human body such as catabolism of sugar and amino acids and energy generation from protein.

In *Oshikundu*, no fat soluble vitamins were detected. Fat soluble vitamins could be present but they were not properly extracted to be available in the extracting solvent for detection. Another

reason could be since much of *Oshikundu* is mostly water, fat soluble vitamins will degrade and only water soluble vitamins are able to be available.

### 5.4. *Oshikundu* Microbiology

The total plate count was low and ranged between 6.36 and 8.20 Log CFU/ mL of *Oshikundu* which is below the limit to constitute not safe for drinking. There was no growth on brilliant green agar, for isolation of pathogens and related microbes. This means that *Oshikundu* can be considered safe to drink. This is very important, since within these four regions access to clean and safe water used in brewing *Oshikundu* remains a challenge.

Dominant lactic acid bacteria in *Oshikundu* are *Lactobacillus plantarum* 2, *Lactococcus lactis* ssp. *lactis* 1, *Lactobacillus delbrueckii* ssp. *delbrueckii*, *Lactobacillus plantarum* 1, *Lactobacillus fermentum* 2, *Lactobacillus pentosus* and *Lactobacillus curvatus* ssp. *curvatus*. *Oshikundu* is dominated by *lactobacillus* and they are able to survive high acidic environment (Blandino et al., 2003). Some of the lactic acid bacteria such as *Lactobacillus lactis, Lactobacillus Fermentum, Lactobacillus Plantarum* are used in probiotic (Blandino et al., 2003). Both of these lactic acid bacteria are found in *Oshikundu*. These bacteria are responsible for the production of lactic acid which is the main organic acid in *Oshikundu*. The organic acid produced by these bacteria may also have antimicrobial properties, which gives *Oshikundu* an advantage as a choice of beverage.

These bacteria are able to survive this low pH and pathogenic bacteria such as Salmonella typhimurium are destroyed, heat-resistant bacteria spores do not germinate and the rate of growth of spoilage bacteria is much reduced (Taylor, 2003). This could be the reason why there is a very low known reported cases, if any of *Oshikundu* related food poisoning in Namibia, due to

inhibition of pathogen microbes. Diarrhoea diseases are the common illnesses in infants and young children, which is the result of being ascribed to water supply and sanitation; food prepared under unhygienic conditions and heavy contamination with pathogens are the major contributor of child mortality through combination of diarrhoea diseases, nutrients malabsorption, and malnutrition (Sahlin, 1999).

LAB in food initially present in low number will outnumber other microorganism and inhibit their growth on the basis of extending shelf life and improving microbiological safety of lactic-fermented foods (Sahlin, 1999; Adams, 1990). The low temperature in *Oshikundu* brewing, favour the growth of thermophilic homofermentative *Lactobacillus* spp., notably *L. delbrueckii* which produce a laevorotatory isomer that can be nutritionally preferable (Dewar, & Taylor, 1999). Some of *Lactobacillus plantarum* strains (found in *Oshikundu*) have an important function in lowering blood serum cholesterol in Sprague-Dawley rats (Huang et al, 2013).

However, other bacterial species were also detected in *Oshikundu*, these include: *Enterobacter cloacae, Enterobacter sakazakii, Pseudomonas luteola, Pseudomonas aeruginosa* and *Serratia ficaria*. These microbes are likely to be responsible for the spoilage of *Oshikundu*. More especially the *Pseudomonas* species proliferate in high moisture environment, which *Oshikundu* is the case. Food and beverages are perishable within days or weeks in the natural course of events; this is brought by chemical reactions, action of microorganism or action of both (Lean, 2006).

## CHAPTER 6: RECOMMENDATIONS

Efficient transfer and adaptation of technologies is often limited by inadequate basic scientific knowledge of the processes involved, a lack of appropriate starter cultures, and process control for these technologies: more research and development geared toward a better understanding of the technologies applied in small-scale traditional fermentation is essential (Marshall & Mejie, 2011). There is a need to take up the traditional processing technology of *Oshikundu* to modern processing technology.

With the physicochemical, nutritional, Lactic acid bacteria and spoilage bacteria profile of *Oshikundu*, a starter culture can be developed from LAB and come up with a uniform product. This will address the draw backs of back-sloping in *Oshikundu* brewing. This will also ensure consistency in organoleptic properties in the final product such as aroma, taste and colour. A detailed study is needed on the physicochemical and microbial properties of *Oshikundu*. *Oshikundu* can be introduced in the school feeding program all over the country since the main ingredients are common in Namibia and create a job market for small farmers in the Ohangwena, Omusati, Oshana and Oshikoto region. *Oshikundu* can also be taken up to commercial level and be sold as bottled or in sachets. An inactive form of the starter culture can be introduced in a pre-mixed sachet and can be ready to drink in as little time as under one hour. Another alternative is to ferment *Oshikundu,* filter it, sterilize and completely stop fermentation before bottling.

To prolong the shelf life of *Oshikundu*, more research can be done by using plant or traditional fruits based tissues to increase the number of beneficial microorganism and in the process inhibit the growth of the harmful and spoilage bacteria.

## CHAPTER 7: CONCLUSION

*Oshikundu* is an important daily beverage in *Aawambo* households from Ohangwena, Omusati, Oshana and Oshikoto regions. The traditional art of *Oshikundu* brewing has been in existence for many generations and knowledge transfer is done by women to their daughters verbally. *Oshikundu's* combinations of physicochemical properties such as pH, lactic acid, alcohol and viscosity have a profound effect on the final product's chemical stability and shelf life. *Oshikundu* has a number of macro and micro nutrients such as carbohydrates, protein, vitamin $B_{1,}$ vitamin $B_{2,}$ amino acid and minerals such as P, K, Mg, Ca, B, Cu, Fe, S and Zn. The combination of all the nutrients provides a package with important nutrients. *Oshikundu* is characterized by lactic acid bacteria that have important function. The presence of these bacteria in *Oshikundu* makes it a safe beverage to drink. There are also spoilage microbes in *Oshikundu* that contribute to the short shelf life of the product.

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**ANNEX 1**

**Adjusted conditions of HPLC (PerkinElmer Flexar, USA) column for Organic acids analysis**

| | | |
|---|---|---|
| **Instrument** | PerkinElmer Flexar, USA | |
| **Auto sampler** | | |
| **Detector** | | |
| **Column** | Zorbax SB-C18, 4.6 × 150 mm, USA | |
| **Column temperature** | $40^{o}$ C | |
| **Detector wavelength** | **Time (min)** | **Wavelength (nm)** |
| | 10.0 | 210 |
| Injection volume | 10 µL fixed Loop | |
| Flow rate | 1.0 mL/min | |
| Mobile phase | $KH_2PO_4$, pH adjusted to 2.4 with o-Phosphoric acid. | |

**Gradient program**

| **Type** | **Time** | **Flow** (min) | **%A** (mL/min) | **Curve** |
|---|---|---|---|---|
| Equil | 0.5 | 1.0 | 100 | 0 |
| Run | 10.0 | 1.0 | 100 | 1 |

**ANNEX 2**

**Adjusted conditions of HPLC (PerkinElmer Flexar, USA) column for Amino acid analysis (Zhao et al., 2013)**

| | | |
|---|---|---|
| **Instrument** | PerkinElmer Flexar, USA | |
| **Auto sampler** | | |
| **Detector** | | |
| **Column** | Zorbax SB-C18, 4.6 × 150 mm, USA | |
| **Column temperature** | $40^{o}$ C | |
| **Detector wavelength** | **Time (min)** | **Wavelength (nm)** |
| | 0.00 | 340 |
| | 2.40 | 440 |
| Injection volume | 2 µL fixed Loop | |
| Flow rate | 0.7 mL/min | |
| Mobile phase | A: (20 µM NaAc, 0.08 % TEA, 0.3 % THF, 0.004 % EDTA, pH 7.2) | |
| | B: (20 µM NaAc, 40 % CAN, 40 % MeOH, pH 7.2) | |

**Gradient program**

0- 17 minutes solvent A reduced from 100 to 40 % and solvent B increase from 0 to 60 % (linear gradient).

17- 18 minutes solvent B was kept at 100 %

24- 25 minutes solvent A increased from 0 to 100 % (linear gradient)

**ANNEX 3**

**Adjusted conditions of HPLC (PerkinElmer Flexar, USA) column for fat soluble vitamin (A, E, $D_3$ , $K_1$) analysis**

| | | |
|---|---|---|
| **Instrument** | PerkinElmer Flexar, USA | |
| **Auto sampler** | | |
| **Detector** | | |
| **Column** | Zorbax SB-C18, 4.6 × 150 mm, USA | |
| **Column temperature** | $40^{o}$ C | |
| **Detector wavelength** | **Time (min)** | **Wavelength (nm)** |
| | 0.00 | 325 |
| | 2.40 | 248 |
| | 3.60 | 265 |
| | 4.50 | 285 |
| | 6.20 | 248 |
| | 7.50 | 325 |
| Injection volume | 2 µL fixed Loop | |
| Flow rate | 0.7 mL/min | |
| Mobile phase | A: water, pH adjusted to 3.0 w/acetic acid:Methanol (1:1) | |
| | B: Acetonitrile | |

**Gradient program**

| **Type** | **Time** | **Flow** (min) | **%A** (mL/min) | **%B** | **Curve** |
|---|---|---|---|---|---|
| Equil | 0.5 | 1.0 | 20 | 80 | 0 |
| Run | 3.5 | 1.0 | 20 | 80 | 1 |
| Run | 1.0 | 1.0 | 0 | 100 | 1 |
| Run | 2.0 | 1.0 | 20 | 80 | 1 |
| Run | 3.3 | 1.0 | 20 | 80 | 1 |

**ANNEX 4**

**Adjusted conditions of HPLC (PerkinElmer Flexar, USA) column for fat soluble vitamin (A, E, $D_3$ , $K_1$) analysis**

| | | |
|---|---|---|
| **Instrument** | PerkinElmer Flexar, USA | |
| **Auto sampler** | | |
| **Detector** | | |
| **Column** | Zorbax SB-C18, 4.6 × 150 mm, USA | |
| **Column temperature** | $40^{o}$ C | |
| **Detector wavelength** | **Time (min)** | **Wavelength (nm)** |
| | 0.00 | 254 |
| Injection volume | 20 µL fixed Loop | |
| Flow rate | 1 mL/min | |
| Mobile phase | A: Methanol | |
| | B: Potassium dihydrogen phosphate, pH adjusted to 4.2 using for formic acid | |

**Gradient program**

| **Type** | **Time** | **Flow** (min) | **%A** (mL/min) | **%B** | **Curve** |
|---|---|---|---|---|---|
| Equil | 8 | 1.0 | 20 | 80 | 0 |
| Run | 3.5 | 1.0 | 20 | 80 | 1 |
| Run | 1.0 | 1.0 | 0 | 100 | 1 |
| Run | 2.0 | 1.0 | 20 | 80 | 1 |
| Run | 3.3 | 1.0 | 20 | 80 | 1 |

**ANNEX 5**

**DNA extraction from Gram-positive bacteria using Gentra Puregene Yeast/bacteria kit**

1. Prepare overnight culture
2. Transfer 500 µL of cell culture to a 1.5 mL microcentrifuge tube on ice
3. Centrifuge for 5 seconds at 13000-16000 ×g to pellet cells.
4. Carefully discard the supernatant by pipetting or pouring.
5. Add 300 µL Cell suspension solution and pipet up and down.
6. Add 1.5 µL Lytic solution, and pipet up and down.
7. Sonicate for 5 minutes at 35 $^{o}$C.
8. A 1.5 µL RNase A solution, and mix by vortex at the lowest speed and incubate for 15-60 minutes at 37$^{o}$ C.
9. Incubate for 1 minute on ice to quickly cool the sample
10. Add 100 µL Protein Precipitation solution and vortex for 20 seconds at high speed
11. Centrifuge for 3 minutes at 13000- 16000 × g. (the precipitated protein should form a tight pellet. If the protein pellet in not tight, incubate in ice fir 5 minutes and repeat the centrifuge).
12. Pipet 300 µL 70 % ethanol into a clean 1.5 mL microcentrifuge tube and add the supernatant from the previous step by pouring carefully.
13. Mix by vortex at the minimum speed
14. Centrifuge for 1 minute at 13000- 16000 × g. (DNA will be visible as a white pellet).
15. Carefully discard the supernatant, and drain the tube by inverting on a clean piece of absorbent paper, taking care that the pellet remains in the tube
16. Add 50 µL DNA Hydration solution and vortex for 5 seconds at medium speed to mix

17. Incubate at 65$^{o}$ C for 1 hour to dissolve the DNA
18. Incubate at room temperature (15-25$^{o}$ C) overnight with gentle shaking. Ensure tube cap is tightly closed to avoid leakage. Sample can then be centrifuge briefly and transferred to a storage tube.

# ANNEX 6

**DNA extraction Yeast using Gentra Puregene Yeast/bacteria kit**

1. Prepare overnight culture containing 1-3 × $10^8$ cells
2. Transfer 1 mL of the cell suspension to a 1.5 mL microcentrifuge tube on ice.
3. Centrifuge for 5 seconds at 13000-16000 ×g to pellet cells.
4. Carefully discard the supernatant by pipetting or pouring.
5. Add 300 µL Cell suspension solution and pipet up and down.
6. Add 1.5 µL Lytic Enzyme solution, and mix by inverting 25 times. Incubate for 30 minutes at 37$^o$ C.
7. Centrifuge for 1 minute at 13000- 16000 × g to pellet cell.
8. Carefully discard the supernatant by pipetting or pouring
9. Add 300 µL Protein Precipitation solution and vortex vigorously for 20 seconds at high speed.
10. Centrifuge for 3 minute at 13000- 16000 × g.
11. Pipet 300 µL ethanol into a clean 1.5 mL micro-centrifuge tube and add the supernatant from the previous step by pouring carefully.
12. Mix by vortex at minimum speed.
13. Centrifuge for 3 minute at 13000- 16000×g (DNA may be visible as a small white pellet).
14. Carefully discard the supernatant, and drain the tube by inverting on a clean piece of absorbent paper, taking care that the pellet remains in the tube.
15. Add 300 µL absolute ethanol and invert several times to wash the DNA pellet.
16. Centrifuge for 1 minute at 13000- 16000 × g.

17. Carefully discard the supernatant. Drain the tube on a clean piece of absorbent paper, taking care that the pellet remains in the tube. Allow to air dry for 5 minutes.
18. Add 300 µL isopropanol and incubate at room temperature.
19. Centrifuge for 1 minute at 13000- 16000 × g.
20. Carefully discard the supernatant, and drain the tube by inverting on a clean piece of absorbent paper, taking care that the pellet remains in the tube. Allow to air dry for 5 minutes
21. Add 50 µL DNA Hydration solution and vortex for 5 seconds at medium speed to mix
22. A 1.5 µL RNase A solution, and mix by vortex at the lowest speed and incubate for 15-60 minutes at $37^{o}$ C.
23. Incubate at $65^{o}$ C for 1 hour to dissolve the DNA
24. Incubate at room temperature (15-$25^{o}$ C) overnight with gentle shaking. Ensure tube cap is tightly closed to avoid leakage. Sample can then be centrifuge briefly and transferred to a storage tube.

**ANNEX 7**

**Standards retention time for Vitamins and organic acids**

| **Compounds** | **Retention time (minutes)** |
|---|---|
| Vitamin A | 4.911 |
| Vitamin $B_1$ | 1.593 |
| Vitamin $B_2$ | 1.373 |
| Vitamin $D_3$ | 14.566 |
| Vitamin E | 16.052 |
| Vitamin $K_1$ | 19.988 |
| Lactic acid | 1.511 |
| Acetic acid | 1.803 |
| Shikimic acid | 2.557 |
| Maleic acid | 2.794 |
| Succinic acid | 3.402 |
| Phytic acid | 2.243 |