EXTRACTION AND PURIFICATION OF SIALIC ACID FROM SUBMANDIBULAR MUCIN OF CATTLE AND GOAT

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AHMADU BELLO UNIVERSITY

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE IN BIOCHEMISTRY

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NIGERIA

JUNE, 2014

DECLARATION

I hereby declare that the work in this thesis entitled 'EXTRACTION AND PURIFICATION OF SIALIC ACID FROM SUBMANDIBULAR MUCIN OF CATTLE AND GOAT' was performed by me in the Department of Biochemistry under the supervision of Dr. A.B. Sallau and Prof. D.A. Ameh. The information herein derived from literature has been duly acknowledged in the text and a list of references provided. No part of this thesis has been previously presented for another degree or diploma at any university to the best of my knowledge.

Nkeonye, Ogechi Lynda

_____	_____	_____
Name	Signature	Date

CERTIFICATION

This thesis entitled 'EXTRACTION AND PURIFICATION OF SIALIC ACID FROM SUBMANDIBULAR MUCIN OF CATTLE AND GOAT' by Nkeonye, Ogechi Lynda meets the regulations governing the award of the degree of Master of Science in Biochemistry, of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to my family.

ACKNOWLEDGEMENT

I would like to extend my profound gratitude to God Almighty for giving me the grace and opportunity to write this thesis. May His name be praised forever more. My gratitude also goes to my dedicated supervisors, Dr. A.B. Sallau and Prof. D.A. Ameh for their untiring efforts and support that made this study a success; my parents, Prof and Mrs. Nkeonye for their endless love and support and to my siblings for always being there for me.

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ABSTRACT

In this study, Sialic acid (Neuraminic acid) was extracted, purified and partially characterized from submandibular mucin of cattle and goat. The extraction involved homogenization, treatment with enzymes (phospholipase and lipase) and acid hydrolysis. Ion exchange chromatography was used to purify the sialic acids. The partial characterization involved thin-layer chromatography and Fourier transform infrared spectroscopy (FTIR). Treatment of the submandibular mucin with phospholipase led to an increase in release of sialic acid with optimum release of sialic acids at 90 minutes while treatment of the homogenate with lipase led to a slight increase in sialic acids released. Acid hydrolysis of the homogenized submandibular glands led to a 15 fold increase in sialic acids released. The purification fold after carrying out ion exchange chromatography on the sialic acid extracts of the cattle and goat submandibular mucin was 16.59 and 12.11 respectively. Thin-layer chromatography revealed that both sialic acid extracts moved at the same pace with a standard sialic acid from Sigma. The FTIR spectra of the purified sialic acid and that of the standard sialic acid had wavenumbers that are indicative of alkyl halide, alkenes, allene, alkane and carboxylic acid which are the functional groups that are found in sialic acids. The results indicated that the sialic acid contained in goat submandibular gland is more and of similar quality with that from cattle submandibular gland. The results have indicated that sialic acids are extractible from the submandibular mucins with the aid of phospholipase and lipase.

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LIST OF ABBREVIATIONS

CMP - Cytidine Monophosphate

CTP - Cytidine Triphosphate

FTIR - Fourier-Transform Infrared Spectroscopy

GlcNAc - *N*-Acetylglucosamine

GNE - UDP- *N*-Acetylglucosamine-4-epimerase

GPI - Glycophosphatidylinositol

HIBM - Hereditary Inclusion Body Myopathy

HPAE-PAD - High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection

HPLC - High Performance Liquid Chromatography

IQ - Intelligence Quotient

ISSD - Infantile Sialic acid Storage Disease

KDN - 2 Keto-3-Deoxynonic Acid

LDL - Low Density Lipoprotein

LPS - Lipopolysaccharide

ManNAc - *N*-Acetylmannosamine

MS - Mass Spectroscopy

NANA - *N*-Acetylneuraminic Acid

Neu5Ac/NeuAc - *N*-Acetylneuraminic Acid

Neu5Gc/NeuGc - *N*-Glycolylneuraminic Acid

RO - Reverse Osmosis

SA - Sialic Acid

Sias - Sialic Acid

SSD - Sialic acid Storage Disease

TLC - Thin-Layer Chromatography

UDP - Uridine Diphosphate

CHAPTER ONE

1.0 INTRODUCTION

Sialic acid is a derivative of a nine-carbon monosaccharide. It comprises of a large family of *N*- and *O*-substituted neuraminic acids. The amino group of sialic acid is linked to either an *N*-acetyl or *N*-glycolyl group, which yields *N*-acetylneuraminic acid (Neu5Ac) or *N*-glycolylneuraminic acid (Neu5Gc) respectively. The hydroxyl groups of both sialic acids are often modified. It is a naturally widespread carbohydrate with numerous biological functions, including blood protein half-life regulation, variety of toxin neutralization, starting reagent of biochemical derivatives for the synthesis of pharmaceuticals, cellular adhesion and glycoprotein lytic protection (Varki *et al.*, 2009; Schauer, 2004a). Sialic acid is covalently bound to the side chains of mucin by a 2-6' glycosidic bond; however, the amount and types of sialic acids present in mucin vary by species of animal and system of isolation. For example, 22% of the dry weight of bovine submaxillary mucin is sialic acid, but the vast majority of the sialic acid is *N*-acetylneuraminic acid, with minor amounts of *N*-glycolylneuraminic acid (Mizan *et al.*, 2000).

Their first level of diversity results from the different alpha linkages that may be formed between the Carbon-2 (C-2) of sialic acid and underlying sugars by specific sialyltransferases, using cyclic monophosphate bound to sialic acid (CMP-Sias) as high-energy donors. The most common linkages are to the C-3 or C-6 positions of galactose residues or to the C-6 position of *N*-acetylgalactosamine residues (Varki and Schauer, 2009).

In “Primary” sialic acids: 2-keto-5-acetamido-3,5-dideoxy-D-glycero-D-galactononulosonic acid (Neu5Ac) and 2-keto-3-deoxy-D-glycero-D-galactonononic acid (KDN), the only difference is

the substitution at the 5-carbon position. The most common in vertebrate cell types is *N*-glycolylneuraminic acid. All other sialic acids are thought to be metabolically derived from these two (Varki and Schauer, 1999). Sialic acids rarely occur free in nature. They are commonly present as components of oligosaccharide chains of mucins, glycoproteins and glycolipids. Sialic acids are found in all vertebrates and within each organism they are found in virtually all tissues but not generally found in plants, prokaryotes or invertebrates. The mammalian central nervous system has the highest concentration of sialic acid. The majority (65%) is present in gangliosides and glycoproteins (32%) with the remaining 3% as free sialic acid (Brunngaber *et al.*, 1972). A lot of researches have shown that the concentration of sialic acid in the human body serves as a marker for certain pathological conditions such as cancer and artherosclerosis (Sobenin *et al.*, 1998). Sialic acids are found in high concentrations in submandibular mucin (saliva), human milk, pancreas, brain, nervous tissue, urine, gastric juice, serum, tear, adrenal glands and heart of vertebrates (Wang and Brand-Miller, 2003). However, the concentrations of sialic acid in these tissues vary with age, ethnicity and dietary sialic acid intake (Wang, 2012).

Several studies have proved that sialic acid has a lot of beneficial roles in the body (Varki and Gagneux, 2012). As a result of this discovery, scientists are using sialic acid in more research works and they are also incooperating the acid and its analogs into various pharmaceutical products and dietary supplements (Ghaderi *et al*., 2012). Sialic acid analogs are antiviral therapeutics as well as crucial tools in bacterial pathogenesis research, immunobiology and development of cancer diagnostic imaging. The scarce supply of sialic acid hinders production of these materials (Lundgren and Boddy, 2007). This has led to an increase in the demand for sialic acid. However, the production of sialic acid using contemporary methods does not meet the high

demand for the acid thereby leading to high cost of sialic acid. This high cost of sialic acid has driven scientists to research on cheaper methods of sialic acid extraction and production. So far, sialic acid has been isolated from different sources such as egg yolk, milk whey and edible bird nest, which requires a lengthy and cumbersome purification process. The overall yields are typically low (˜10-20%) and the purity is marginal. (Boddy and Lundgren, 2007).

1.1 Statement of Problem

There is increased need for sialic acid; and its production using contemporary methods is partly laborious. There are also challenges in the chemical synthesis of sialic acid because of the complex structure of the molecule. The cost of sialic acid is also relatively high. In developed countries today, a large proportion of tissues containing high amounts of sialic acid are thrown off as wastes in abattoirs.

1.2 Justification

- There is a need to come up with novel methods of sialic acid production that are cheaper and less laborious which is the main focus of this work.
- A large proportion of sialic acid (submandibular gland) is thrown off as waste in some abattoirs and 1gram of sialic acid costs about ₦80,000. It is therefore imperative to find a way of making a good use of this "waste".
- It is important to find a way of applying biochemical principles to improve on the already existing protocol of sialic acid extraction.

1.3 Objectives

1.3.1 General objective

- To purify sialic acids from submandibular mucin of goat and cattle.

1.3.2 Specific objectives

- To extract and purify sialic acids from submandibular mucin of cattle and goat.
- To quantify sialic acids from submandibular mucin of cattle and goat and establish which of the submandibular mucins (cattle and goat) is a better source of sialic acids.
- To characterize sialic acids from submandibular mucin of cattle and goat.

1.4 Hypothesis (null)

Sialic acids cannot be extracted, purified and characterized from submandibular mucin of cattle and goat.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Sialic acids

2.1.1 History

In 1936, a scientist called Gunnar Blix isolated and identified an acidic amino sugar which he named sialic acid because he extracted it from saliva. In 1941, another scientist called Ernst Klenk isolated the crystalized form of the same molecule from the brain and named it neuraminic acid. Subsequently, the structure for the compound was proposed. In 1957, it was discovered that the molecules were the same so the two names were retained. The name sialic acid was derived from the Greek word *sialos* meaning saliva. Sialic acids are sometimes referred to as Sias, lactaminic acid, NANA or *N*-Acetyl-D-neuraminic acid (Varki and Schauer, 2009).

2.1.2 Occurrence

Sialic acids rarely occur free in nature. They are more commonly present as components of oligosaccharide chains of mucins, glycoproteins, and glycolipids. They usually occupy terminal, non-reducing positions of oligosaccharide chains of complex carbohydrates on outer and inner membrane surfaces in various linkages, mainly to galactose, *N*-acetylgalactosamine, and other sialic acid moieties, where they are highly exposed and functionally important (Wang and Brand-Miller, 2003). Sialic acid is found in a wide variety of substances and tissues in humans and animals occurring most abundantly in glycoproteins and glycolipids. Sialic acids are critical in determining glycoprotein bioavailability, function, stability and metabolism.

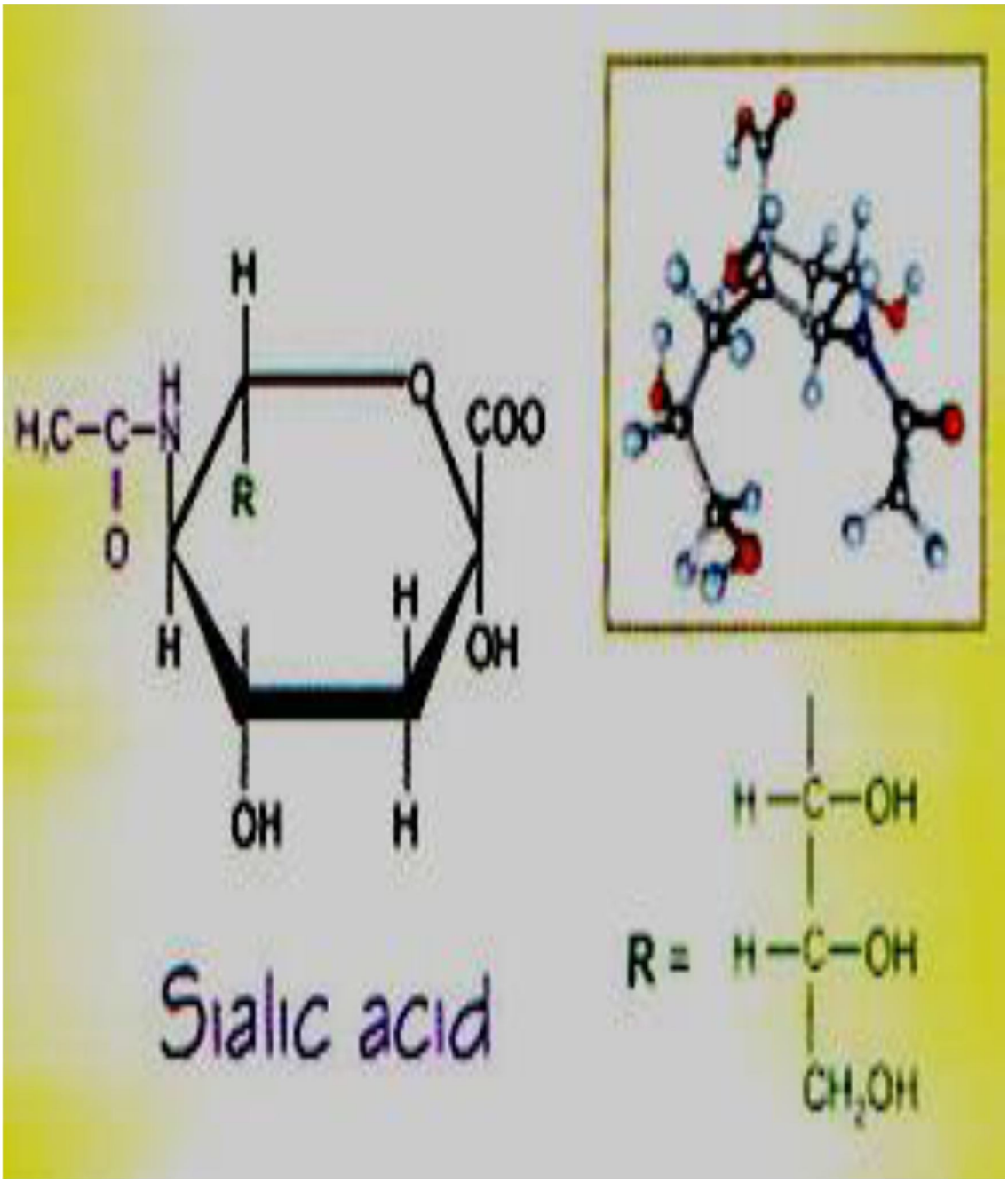


Figure 2.1: Structure of *N*-acetylneuraminic acid (Wainscot, 2004)

2.1.3 Diversity in Structure & Linkage

Sialic acids are a structurally unique molecule of 8 or 9-carbon monosaccharides and characteristically contain an anomeric carboxylate, a deoxygenated methylene C-3 ring carbon, an oligohydroxylated side chain at C-6 and are differentially functionalized at C-5. The most common sialic acid is *N*-acetylneuraminic acid (Ress and Linhardt, 2004). Their structural diversity is high and the mechanism for their biosynthesis is complex (Schauer, 2004b). Sialic acids comprise of a large family of *N*- and *O*-substituted neuraminic acids. It has the empirical formula $C_{11}H_{19}NO_9$ and a molecular weight of 309.27grams per mole. The amino group of neuraminic acid is linked to either an *N*-acetyl or *N*-glycolyl group, which yields *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) respectively. The hydroxyl groups of both sialic acids are often modified. All other sialic acids are apparently metabolically derived from these two with the exception of some bacterial molecules such as legionaminic acid (Varki *et al.*, 2009).

Sialic acids are typically found at the outermost ends of *N*-glycans, *O*-glycans, and glycosphingolipids (and occasionally capping the side chains of GPI anchors). They are subject to a wide variety of modifications. The carboxylate group at the 1-carbon position is typically ionized at physiological pH, but it can also be occasionally found in lactone ester with hydroxyl groups of adjacent saccharides. The 5-*N*-acetyl group can also be hydroxylated, giving *N*-glycolylneuraminic acid (Neu5Gc). Occasionally, the 5-*N*-acetyl group is de-*N*-acetylated, giving neuraminic acid (Neu). These four molecules (Neu5Ac, Neu5Gc, KDN, and Neu) have the potential for additional substitutions at the hydroxyl groups on the 4-, 7-, 8-, and 9-carbons (*O*-acetyl, *O*-methyl, *O*-sulfate, and phosphate groups). Additional complexity arises from the fact that the *O*-acetyl esters can migrate along the side chain (from the 7- to the 9-position) under

physiological conditions. Unsaturated and dehydro forms of sialic acids are also known to exist. (Varki *et al.*, 2009). Sialic acids usually occur α-ketosidically linked to glycoproteins and glycolipids and are often positioned as terminal monosaccharides in these glycoconjugates (Schauer, 2004a). The two main types of sialic acids: NeuAc and NeuGc which differ by one oxygen atom, usually occur as terminal structures attached to galactose residues at the non-reducing termini of *N*-glycans, *O*-linked glycans and glycosphingolipids. The way that NeuAc and NeuGc attach to other sugar residues is different from that of the hexoses found in therapeutic glycoproteins-the glycosidic linkage configurations for these sialyl groups are usually either α2,3 or α2,6 compared to α1,x or β1,x for hexoses (where x indicates the carbon atom of the adjacent monosaccharide). Glycosidically bound sialic acids in naturally occurring glycans are in the α-form and free sialic acids in solution are mainly in the β form (Varki *et al.*, 2009). The *O*-acetylation of sialic acids in various positions is a frequent modification of these residues in glycoproteins and glycolipids of higher animals and some bacteria. Sialic acid *O*-acetylation is involved in the regulation of many cell biological and pathophysiological events (Lrhorfi *et al.*, 2007). The *O*-acetylated sialic acids have been found frequently in the deuterostomate lineage from the echinoderms to man, in addition to a variety of bacterial species. Two types prevail, sialic acids *O*-acetylated at the pyranose ring, at C-4, or at the glycerol side chain, preferably at C-9 (Schauer and Kamerling, 1997; Tiralongo and Schauer, 2004). The *O*-acetylation of sialic acids can alter its sensitivity to neuraminidases (Corfield *et al.*, 1986).

Over 50 natural sialic acids have been discovered. There are over 50 different derivatives of sialic acid (Warwas *et al.*, 2007). Sialic acids are present in mammalian and avian tissues predominantly in the form of lipooligosaccharides, lipopolysaccharides and as the glycan

component of glycoproteins. They exist in a variety of glycosidic linkages, most typically α(2-3) and α(2-6) to galactose (or lactose), as well as α(2-8) and α(2-9) linkages in homopolymers of Neu5Ac. Since, humans do not generally produce Neu5Gc and have been shown to possess antibodies against Neu5Gc, the presence of this sialic acid in a therapeutic agent can potentially lead to an immune response. Consequently, glycoprotein sialylation and the identity of the sialic acids, play important roles in therapeutic protein efficacy, pharmacokinetics and potential immunogenicity (Hurum and Rohrer, 2012).

Diversity in sialic acid structure is generated through substitutions at the hydroxyl and amino groups located on carbons 4, 5, 7, 8, and 9 (Angata and Varki 2002; Schauer 2004a). The first level of diversity results from the different alpha linkages that may be formed between the C-2 of sialic acids and underlying sugars by specific sialyltransferases, using CMP-sialic acids as high energy donors. The most common linkages are C-3 or C-6 positions of galactose residues or to the C-6 positions of *N*-acetylgalactosamine residues. Sialic acids can also occupy internal positions within glycans, the most common being when one sialic acid residue is attached to another, often at C-8 position. The second level of diversity arises from a variety of natural modifications. The C-5 position of sialic acids can have an *N*-acetyl group or a hydroxyl group. The 5-*N*-acetyl group can also be hydroxylated, giving *N*-glycolylneuraminic acid. Less commonly, the 5-amino group is not *N*-acylated, giving neuraminic acid. These four core sialic acid molecules (Neu5Ac, Neu5Gc, Kdn and Neu) can carry one or more additional substitutions at the hydroxyl groups on C-4, C-7, C-8 and C-9. The carboxylate group at the C-1 is typically ionized at physiological pH, but can also be condensed into a lactone with hydroxyl groups of adjacent saccharides or into a lactam with a free amino group at C-5 (Varki *et al.*, 2009).

2.1.4 Functions of Sialic Acid in the Body

Sialic acids are structurally diverse molecules that have important roles in the physiological reactions and characteristics of prokaryotes and eukaryotes. These include the ability to mask epitopes on underlying glycan chains and to repulse negatively charged moieties (Byrne *et al.*, 2007). Other biological functions of sialic acids include glycoprotein lytic protection, cellular adhesion, a variety of toxin neutralization and blood protein half-life regulation. Sialic acids on human red blood cells serve as a cell receptor for certain viruses eg. Enterovirus 70, influenza A and influenza B virus (Alexander and Dimock, 2002). Sialic acid-rich glycoproteins (sialoglycoproteins) bind selectin in humans and other organisms. Sialic acid-rich oligosaccharides on the glycoconjugates (glycolipids, glycoproteins, proteoglycans) found on surface membranes help keep water at the surface of cells (Severi *et al.,* 2007). Sialic acids serve as an immune moderator that affects the flow resistance of mucus which in turn, repels bacteria, viruses and other harmful microbes. Due to their cell surface location, these acidic molecules shield macromolecules and cells from enzymatic and immunological attacks and thus contribute to innate immunity (Schauer, 2004b). Since high levels are found in the human brain and kidney, it is speculated that sialic acid may play a key role in brain development and learning and in lessening the risk of kidney stone formation. Animal studies indicate that this essential saccharide does appear to improve both memory and cognitive performance. Sialic acid is also found in such other tissues as the skin and testes leading to speculation that disruptions like skin diseases and reproductive problems could be reversed with supplementation of this essential sugar. Sialic acid also influences blood coagulation and cholesterol levels, lowering LDL (bad cholesterol) (Ricci , 2008).

Sialic acid levels are markedly reduced in those with rheumatoid arthritis, confirming that this saccharide plays an important part in the immune system (Ricci, 2008). The high expression of sialic acids on outer cell membranes on the interior of lysosomal membranes and on secreted glycol-proteins suggests that they have roles in the stabilization of molecules and membranes, as well as in modulating interactions with the environment. Some functions arise from the relatively strong electronegative charge of sialic acid for example, binding and transport of ions and drugs, stabilizing the conformation of proteins including enzymes and enhancing the viscosity of mucins. Sialic acids can also protect molecules and cells from attack by proteases or glycosidases, extending their life time and function. Furthermore, sialic acids can regulate the affinity of receptors and are reported to modulate processes involved in transmembrane signaling, fertilization, growth and differentiation (Varki and Schauer, 2009).

Another prominent role of sialic acid is dualistic; they act either as masks or recognition sites. They mask antigenic sites, receptors and most importantly, penultimate galactose residues (Varki *et al.*, 2009). Gangliosides, an important class of sialic acid containing antigenic glycotopes, are currently being evaluated as anti-cancer agents. Specific tumor types express gangliosides (GM **4** and GD series,) as well as specific glycopeptides (sTn) selectively on their cell surface. Thus, the potential for use of sialic acid based therapeutic agents is promising. The specific expression of these surface carbohydrate antigens by certain pathogens and tumor cell lines has resulted in a concerted effort to synthesize sialoconjugates as biological probes, small molecule inhibitors and as part of higher ordered, multivalent vaccines (Ress and Linhardt, 2004).

Some derivatives of sialic acids play some important biological roles that have made them to become targets for extensive research. Examples are 9-*O*-acetyl-*N*-acetylneuraminic acid which

is a specific ligand for the agglutinin of influenza virus C. Another one is 4-*O*-acetylated *N*-acetylneuraminic acid which is a specific ligand for the agglutinin of murine hepatitis S virus (Zanetta *et al.*, 2006). The human erythrocyte has a net negative charge and the bulk of this is because of the ionized sialic acid that surrounds the erythrocyte. The role of membrane sialic acid in erythrocyte survival is unclear, although there is evidence for a reduction in sialic acid and surface charge in older erythrocytes (Durocher *et al.*, 2011). Sialic acid level in blood plasma and circulating glycoproteins is considered to be a marker for a number of pathologic conditions, including atherosclerosis, cancer, etc. Sialic acid is crucial in determining serum half-life and immunogenicity of glycoproteins. Sialic acid analogs are antiviral therapeutics as well as crucial tools in bacterial pathogenesis research, immunobiology and development of cancer diagnostic imaging. The scarce supply of sialic acid hinders production of these materials (Lundgren and Boddy, 2007).

The number of therapeutic proteins has increased dramatically over the past years and most of the therapeutic proteins in the market today are glycoproteins. The terminal monosaccharide of *N*-linked complex glycans is typically occupied by sialic acid. The presence of this sialic acid affects absorption, serum half-life, and clearance from the serum; as well as the physical, chemical and immunogenic properties of the respective glycoprotein. From a manufacturing perspective, the degree of sialylation is crucial since sialylation varies the function of the product. In addition, insufficient or inconsistent sialylation is also a major problem for the process consistency. Sialylation of over-expressed glycoproteins in all mammalian cell lines commonly used in biotechnology for the production of therapeutic glycoproteins is incomplete

and there is a need for strategies leading to homogenous, naturally sialylated glycoproteins (Bork *et al.*, 2009).

Dietary sialic acids are important for infant development, serving both immune system and cognitive development roles. Although these functionalized neuraminic acids are present in all mammalian milk, the proportions vary significantly according to the species (Malacarne *et al.*, 2002). A large body of evidence shows that breastfeeding provides long-term cognitive advantages, particularly for infants born small or premature (Wang, 2012). Numerous studies have suggested that breastfeeding is associated with higher intelligence, but it is difficult to disentangle the effects of social and educational factors from nutritional ones (Horwood and Fergusson, 1998).

Sialic acids have also been found to play a huge role in brain development. Brain development is time dependent (Uauy *et al.*, 2001). The different developmental processes follow a highly ordered sequence of events that are under strict genetic control but can also be influenced by epigenetic factors. Once the sequence of growth events has occurred, brain growth cannot be restarted (Morgan, 1990). The blood of pregnant and lactating women has been found to contain a high concentration of sialic aids. Experimental studies in animals have shown that brain structure and function are permanently affected by early nutrition (Wang and Brand-Miller, 2003). In addition to that, the human breast milk has a high concentration of sialic acids. Several studies have shown that the IQ of children who were breastfed is higher than those of children who were bottle-fed. All these discoveries have made scientists to conclude that sialic acids are essential for complete growth and development of the brain. Even though children are born with

developed neurons, the synaptic connections between these neurons are not fully developed. Sialic acids and polysialic acids are the building blocks of brain gangliosides and in the early life of an infant; they play a huge role in formation of synaptic connections, neuronal growth and memory formation. Sialic acid, which is a key component of both human milk oligosaccharides and neural tissues, may be a conditional nutrient during periods of rapid brain growth (Wang, 2012). Brain growth and maturation are associated with an increase in gangliosides and sialoglycoproteins (Kracun *et al.*, 1992), whereas advancing age and congenital retardation syndromes are associated with a decrease (Svennerholm *et al., 1997*).

N-acetylneuraminic acid (NANA) binds to and neutralizes toxic hydrogen peroxide (H_2O_2) under physiological conditions (Lijima *et al.*, 2004).

2.1.5 Safety

There does not appear to be any data that indicate any adverse reactions to a supplementation of sialic acid. On the contrary, researchers who constantly used high doses in experimental animals, found that the learning abilities of these animals increased. Nor did it seem to matter whether the dose was given orally or intravenously, the result was the same, indicating that the sugar is just as potent using either route. However, if there is a metabolic disorder that prevents the proper absorption of the sugar, disorders can be aggravated, causing a reversal in the positive effects seen in others. Malabsorption could result in mental retardation and ataxia (muscle coordination failure), as well as enlarged livers, developmental delays, and an excess excretion of sialic acid in the urine. Therefore, establishing whether this or other sugars are properly assimilated is very important. This is just one reason why taking all eight essential sugars together is much better

than trying to take them individually and guessing the amounts. All work together to help each other, thereby drastically reducing the risk of problems when taking only one sugar. It is suggested that the maximum safe dosage would be 140mg for a healthy 150-pound adult (Durocher *et al.*, 2011).

2.1.6 Dietary Sources

Good sources of sialic acids are red meat, eggs, milk and whey protein isolate (Wang *et al.*, 2007).

2.1.7 Biosynthesis

Although the liver can synthesize sialic acid *de novo* from glucose, the activity of the limiting enzyme, UDP-*N*-acetylglucosamine-2-epimerase, is low during the neonatal period (Gal *et al.*, 1997). Two polysialyltransferases (α-2,8-sialyltransferase II and IV, abbreviated as *ST8SiaII* and *ST8SiaIV*, respectively) are the key enzymes involved in sialic acid metabolism and have been linked to learning behavior (Muhlenhorf *et al.*, 2001). All mammals, including human beings, have the capacity to synthesize sialic acid in all tissues from simple sugar precursors. Cells from higher animals and various microorganisms produce sialic acid in a long pathway starting from glucose (Wang and Brand-Miller, 2003). Neonates cannot synthesize sialic acids properly because of reduced activity of the enzyme UDP-*N*-acetylglucosamine-2-epimerase (Wang *et al.*, 2007).

In bacterial systems, sialic acids are biosynthesized by an aldolase enzyme. The enzyme uses a mannose derivative as a substrate, inserting three carbons from pyruvate into the resulting sialic

acid structure. These enzymes can be used for chemoenzymatic synthesis of sialic acid derivatives (Hai *et al.*, 2006). With the exception of the β-linked CMP donor sugar, naturally occurring sialic acid glycosides are found in the α-configuration, their synthesis being catalyzed by sialyltransferases. All of the sialyl transferases are thus inverting enzymes (Morley *et al.*, 2009). Sialic acid is synthesized in the cytoplasm of the cell and transported into the nucleus where it is activated with CTP to form the activated nucleotide donor of Sia, CMP-Sia. The CMP-Sia returns to the cytoplasm where it is transported into the Golgi compartment and serves as the donor substrate of Sia for the sialyltransferases, including ST8Sia IV and ST8Sia II. The CMP-Sia exerts negative feedback inhibition on GNE, the key bifunctional cytoplasmic enzyme that regulates the biosynthesis of Sia, thus limiting excess production of free Sia (Wang, 2012).

The main metabolic precursors of all known sialic acids are *N*-acetylneuraminic acid (Neu5Ac) and 2-keto-3-deoxynonic acid (Kdn). In vertebrate systems, they are derived by condensation of Man*N*Ac-6-P (for Neu5Ac) or Man-6-P (for Kdn) with phosphoenolpyruvate. The Man*N*Ac-6-P is produced by a bifunctional enzyme that converts UDP-Glc*N*Ac to Man*N*Ac-6-P and UDP in two steps. Missense mutations of this gene give rise to hereditary inclusion body myopathy (HIBM) in humans and inactivation causes embryonic lethality in the mouse. Condensation of the sugar phosphates with phosphoenolpyruvate yields the corresponding Sialate-9-phosphosphates, which must be dephosphorylated by a specific phosphatase, giving free Sias in the cytoplasm. In contrast, Neu5Ac biosynthesis in prokaryotes involves condensation of Man*N*Ac with phosphoenolpyuruvate, giving non-phosphorylated Neu5Ac (Varki *et al.*, 2009).

2.1.8 Catabolism of Sialic Acid

Once a sialic acid molecule is released into the lysosome of a vertebrate cell, it is delivered back to the cytoplasm by a specific exporter called sialin. This allows the sialic acid molecule to be either efficiently utilized or degraded. Some microorganisms can also directly scavenge sialic acid molecules from the extracellular space, using high-efficiency transporters. Free sialic acid molecules can be relatively efficiently taken up into mammalian cells via fluid-phase macropinocytosis, eventually arriving in the lysosomes, from which they are exported into the cytoplasm by Sialin (Varki *et al.*, 2009). If sialic acid molecules are not reused in eukaryotic cells, degradation can occur catalyzed by cytoplasmic Sia-specific pyruvate lyases that cleave the molecule into N-acetyl-mannosamine and pyruvate (Varki and Schauer, 2009).

2.1.9 Physiological and Pathological Role of Sialic Acids

The saliva of pregnant women increases in sialic acid concentration during the course of gestation (Salvolini *et al.*, 1998). A non-human sialic acid is *N*-glucolylneuraminic acid (Neu5Gc). Although humans are genetically deficient in producing Neu5Gc, small amounts are present in human cells *in vivo* (Bardor *et al*., 2005). It is present in red meat and is often used as an ingredient in some pharmaceutical products. The human body is known to produce antibodies in response to the presence of *N*-glucolylneuraminic acid in the body. Since our immune response vary, some people's immune response may initiate a chronic inflammatory process. In addition to that, the attached sugars are targets for infectious diseases such as cholera, malaria and influenza. Controlling the ratio of NeuAc to NeuGc is critical for manufacturers. The reason is that NeuAc is the normal type of sialylation found in humans while NeuGc is found in non-human glycoproteins and is considered undesired, aberrant form of sialylation for therapeutic glycoproteins. A widely used method for determining the NeuAc:NeuGc ratio is HPLC analysis

of sialic acids labeled with fluorescent DMB (1,2-diamino-4,5-methylenoxybenzene) (Fernandes, 2006).

The *N*-glycolyl neuraminic acid is produced by many vertebrates but not produced by humans. However, in humans, it is found in foetal tissues and metastatic cells (Higashi *et al.*, 1985). The *N*-glycolyl neuraminic acid in the human body comes from consumption of red meat and dairy products. Meanwhile, healthy humans have significant and differing levels of circulating anti-Neu5Gc antibodies (Nguyen *et al.*, 2005; Tangvoranuntakul *et al.*, 2003). Current hypothesis shows that chronic inflammation related to this antigen–antibody reaction could be facilitating both carcinogenesis and atherogenesis (Varki and Varki, 2007). When a glycoprotein loses sialic acid residues, it has a reduced serum half-life, and in some cases reduced activity. Therefore, it is important to know the sialic acid content of a glycoprotein when assaying its function or its efficacy as a pharmaceutical therapeutic (Tan *et al.,* 2012).

Abnormalities in sialic acid metabolism are seen in infants who fail to grow, who regress in development, who have enlarged livers and/or spleens, who show a coarsening of facial features, and who display a failure to produce pigmentation of the skin and hair. As a result of the fact that sialic acids are involved in so many cellular functions, disturbances of their biosynthesis and degradation can lead to medical problems. Sialic acids are vulnerable to the action of microbial esterases, sialidases and lyases because of their exposed position. The action of these enzymes can affect the amount of ligands present, masking of antigenic sites, stabilization of membranes and immunological and other functions of sialic acids. Metastatic cancer cells often express a high density of sialic acid-rich glycoproteins. This helps these late-stage cancer cells enter the blood stream. Changes in sialic acids have also been found to be involved in degenerative

diseases such as atherosclerosis and diabetes as well as neurological disorders such as Alzheimer's disease and alcoholism (Varki *et al*., 2009).

There are several human sialic acid disorders which are known such as Hereditary Inclusion Body Myopathy (HIBM), sialuria, Salla disease and galactosialidosis. Deficiencies in the human enzyme Neu1 leads to sialidosis, a rare lysosomal storage disease. Hereditary Inclusion Body Myopathy (HIBM) is a severe progressive metabolic myopathy caused by a defect in the biosynthetic pathway for sialic acid (SA), a critical component of many muscle proteins, resulting in a deficiency in SA in the muscles of HIBM patients (Eisenberg *et al.*, 2008).

HIBM is caused by missense mutations of UDP-GlcNAc-2-epimerase/ManNAc kinase gene. Sialuria is caused by mutations in the binding site of the feedback inhibitor CMP-Neu5Ac of the GNE (Bork *et al*., 2005). Galactosialidosis is caused by galactosi-dase-peptidase-sialidase complex deficiency. Sialic acid storage disease is an inherited disorder that primarily affects the nervous system. People with sialic acid storage disease have signs and symptoms that may vary widely in severity. Sialic acid storage diseases (SSDs) are rare autosomal recessive neurodegenerative disorders that may be present as a severe infantile form, infantile sialic acid storage disease (ISSD), or as a slowly progressive adult form that is prevalent in the Finnish population, called Salla disease. ISSD is clinically distinct and very serious; visceromegaly, coarse facial features, failure to thrive and early death are its main features. ISSD has been identified in only a few dozen infants worldwide. Salla disease occurs mainly in Finland and Sweden and has been reported in approximately 150 people. Salla disease is a less severe form of sialic acid storage disease. It is characterized by psychomotor developmental delay, hypotonia

and ataxia, usually appearing during the first year of life; phenotypic variation is wide. Only a few cases of various ethnic origins have been reported outside Finland, but there is a growing interest in considering free sialic acid disorders in infants with developmetal delays and growth retardation, regardless of their descent (Valianpour *et al.*, 2004).

A few individuals have been identified as having intermediate severe Salla disease. The known forms of SSD are primarily caused by a transport defect across the lysosomal membrane which leads to the accumulation of free sialic acid *N*-acetylneuraminic acid (NANA) in tissues, fibroblasts and urine. The origin of the transport defect lies in a mutation in the *SLC17A5* gene, which encodes for sialin protein (Valianpour *et al.*, 2004). Defective free sialic acid transport can be established by quantitative analysis of NANA in urine relative to the concentration of creatinine. Several types of analysis are available to quantify NANA, including colorimetric methods, thin-layer chromatography, gas chromatography-mass spectrometry, HPLC with fluorescence detection, HPLC with ultraviolet detection, enzymatic assays, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) and HPLC with mass spectrometry (HPLC-MS). The main disadvantages of these methods are a lack of sensitivity, a lack of speed or both (Valianpour *et al.*, 2004).

2.1.10 Disadvantages of Sialic acids

a. It serves as a receptor for some viruses eg. 2,3-*N*-linked sialic acids are necessary for adeno-associated virus (AAV2.5T) to enter into a human cell (Dickey *et al*., 2011).
b. Some bacteria feed/scavenge sialic acids leading to immune invasion. Some bacteria either scavenge sialic acids from their environment or synthesize it *de novo*. The bacteria

that synthesize sialic acid *de novo* includes *E. coli* K1, *Neisseria meningitidis* and *C. jejuni* (Vimr *et al.*, 2004). The metabolite UDP-GlcNAc is the precursor for sialic acid biosynthesis and is produced by most cells, as it is used in cell wall biosynthesis; the combined actions of the NeuC and NeuB proteins convert this to Neu5Ac via ManNAc (Severi *et al.*, 2007). Many pathogens secrete a sialidase that releases sialic acid from a diverse range of host sialoglycoconjugates. (Corfield, 1992); however, other sialic acid-utilizing bacteria, such as the respiratory pathogen *H. influenzae*, lack genes for a sialidase yet are reliant on host-derived sialic acid (Bouchet *et al.*, 2003). Presumably free sialic acid is made available to such pathogens by other, sialidase-expressing bacteria living in the same niche (Shakhnovich *et al.*, 2002), or, as hypothesized by Sohanpal *et al.* (2004, 2007), by host sialidases that are activated in the course of inflammation.

As sialoglycoconjugates can be predominant components of mammalian cell surfaces, the incorporation of sialic acid into bacterial cell-surface features (be those sialic acid-containing capsules or sialylated LPS) is hypothesized to allow bacterial pathogens to disguise themselves as host cells and thus circumvent and/or counteract the host's immune responses through a strategy of 'molecular mimicry' (Harvey *et al.*, 2001).

c. Sialic acid stimulates growth of *T. forsythia* biofilms. In order to be able to utilize sialic acid, *T. forsythia* must transport sialic acid into the cell across both the outer and inner membranes (Roy *et al.*, 2010).

2.1.11 Sialic acid Analysis

There are many methods which are currently being used for sialic acid analysis such as High Performance Liquid Chromatography (HPLC) with Flourescence detection and High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAE-PAD). There have been a number of chromatographic methods described for sialic acid analysis. Sialic acids are not volatile enough to be separated by gas chromatography. Ion-exchange chromatography is a good choice for separation of sialic acids from neutral sugars because it takes advantage of the ionic character of the carboxyl groups (Xia and Gilmer, 1994).

A new method for analyzing sialic acids in glycoproteins is high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD). It has high precision and linear detection for *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc). Using the Thermo Scientific Dionex CarboPac PA20 Fast Sialic Acid column, this rapid method separates Neu5Ac and Neu5Gc with a total analysis time of < 5 minutes, providing high-throughput sample analysis while reducing eluent consumption and waste generation (Hurum and Rohrer, 2012).

Typically, sialic acids are released from glycoproteins by acid hydrolysis or by enzymatic digestion before analysis. Once the sialic acids are liberated, there are many options for quantification. Numerous spectroscopic methods exist, although interferences in these methods can cause overestimation of the concentration of sialic acids in many samples. Therefore, chromatographic methods that separate the sialic acids from potentially interfering compounds are preferred. Sialic acids from biological sources have low concentration and high diversity (Xia and Gilmer, 1994).

2.2 Sialidase

The important role played by sialylated glycoconjugates in both homeostasis and disease states has led to the extensive study of the enzymes responsible for the addition and removal of sialic acid (Morley *et al.*, 2009). Sialidase also known as neuraminidase is the name of the hydrolytic enzyme present from virus to higher eukaryotes, which specifically cleaves glycosidic linkages between terminal sialic acid residues and mucopolysaccharides. Sialidases are commonly found in viruses, bacteria, fungi, protozoa, and vertebrates, but not in invertebrates. Sialidase can be used directly on intact glycoproteins as a gentle means of removing sialic acid groups for quantification, while leaving the glycan chain attached to the protein. Sialidases are commonly distributed in bacterial species. Sialidase activity varies in different strains of bacteria (Corfield, 1992).

2.4 Submandibular Mucin

Salivary mucins are viscous secretions of high molecular weight glycoproteins whose main function is the lubrication and protection of oral and tracheal epithelium (Herp *et al.*, 1979). The mucins are highly glycosylated, containing 60-80% carbohydrate, which is largely responsible for their physical and chemical properties (Slomiany and Slomiany, 1978). These molecules are highly glycosylated; the oligosaccharides (50–80% of the weight) are linked to serine and threonine residues clustered in mucin domains on the protein backbone (Thomsson *et al.*, 2002). Some mucins are linked to cell membranes due to the presence of a domain hydrophobic around the membrane that promotes retention in the plasma membrane , others are secreted on mucosal surfaces and, in mammals, in saliva. They have important functions in protecting epithelia of the airways and digestive tracts. The submandibular gland is located in the lower region of the

buccal cavity; beneath the floor of the mouth. In humans, the submandibular gland weighs about 15 grams. The secretory viscous cells of the submandibular gland have distinct functions. The mucous cells are the most active and therefore the major product of the submandibular gland is saliva. In particular, the serous cells produce salivary amylase, which aids in the breakdown of starches in the mouth. Mucous cells secrete mucin which aids in the lubrication of the food bolus as it travels through the esophagus (Koeppen and Bruce, 2010).

The submandibular gland's highly active acini account for approximately 70% of salivary volume. The parotid and sublingual glands account for the remaining 30%. Its secretions are regulated *directly* by the parasympathetic nervous system and *indirectly* by the sympathetic nervous system. The sympathetic nervous system regulates submandibular secretions through vasoconstriction of the arteries that supply it. Increased sympathetic activity reduces glandular blood-flow, thereby decreasing the volume of fluid in salivary secretions, producing an enzyme rich mucous saliva. Nevertheless, direct stimulation of sympathetic nerves will cause an increase in salivary enzymatic secretions. In summary, the volume decreases, but the secretions are increased by parasympathetic and sympathetic innervation (Koeppen and Bruce, 2010).



Plate I: Photograph of cattle submandibular gland (Mg x 1). Photographed by me in June, 2012.

2.5 Challenges in existing methods of sialic acid extraction and production

Although sialic acid occurs naturally in the non-reduced terminals of glycoproteins and glycolipids *in vivo*, it is difficult to produce sialic acid from a natural substance containing it both in a large amount and at a low cost. It is will also be difficult to purify it on an industrial scale with high purity (Koketsu *et al*., 1993). Synthetic routes in sialic acid chemistry are challenging, because of the target molecule's complexity. Enzymatic synthesis of sialic acid has a high yield (80%) and generates a pure product through a direct crystallization of the enzymatic mixture. The major drawbacks of enzymatic synthesis in sialic acid production are its cost and scalability at the industrial level (Boddy and Lundgren, 2007).

For sialic acid extracted from whey, the process includes ultrafiltration of casein whey, thermal flocculation of the proteins of the ultrafiltration retentate, phosphotungstic treatment of the supernatent obtained from the flocculation step, acid hydrolysis of the precipitate and treatment of the hydrolysis supernatent for the extraction of *N*-acetyl neuraminic acid (NANA) (Eustache, 1977).

In the case of sialic acid production from egg yolk, the cost is not favorable, since a large amount of lipid contained in egg yolk strongly emulsifies the egg yolk solution after hydrolysis in separating sialic acid from egg yolk, which in turn significantly hampers the purification process. Also, Japanese Patent Laid-Open No. 40491/1989 states that sialic acid can be purified by hydrolyzing a milk substance such as cow's milk, defatted milk, butter milk or whey, desalting the resulting hydrolyzate by electrodialysis and subsequently performing electrodialysis again. However, this method poses problems of decreased percent yield of sialic acid due to its loss

during desalting and decreased purity of sialic acid due to the presence of salt impurities during purification as a result of the incapability of complete desalting (Koketsu *et al.*, 1993).

A method of purifying sialic acid to high purity characterized in that the solution containing sialic acid obtained by hydrolyzing delipidated egg yolk is desalted, after which sialic acid is adsorbed to anion exchange resin and then eluted. Reagents such as methanol, diethylether or acetone can be used for dilipidating the egg yolk. To achieve hydrolysis, a process essential for making the sialic acid in delipidated egg yolk in a free form, both acid hydrolysis and enzyme hydrolysis are applicable with no limitation, but acid hydrolysis is preferred. In the case of enzyme hydrolysis, one or more enzymes such as neuraminidase, protease and sugar hydrolase are selected according to the purpose and deactivated by heating etc. after reaction under optimum conditions for respective enzymes. Accordingly, neuraminidase yields Neu5Ac, while protease yields various Neu5Ac-bound oligosaccharides. Any means of desalting can be used, as long as it is a traditionally known means, such as a reverse osmosis (RO) membrane filtration, electrodialysis or a dialytic membrane filtration. If the hydrolysate is not desalted, there will be a substantial decrease in the amount of sialic acid adsorbed to the anion-exchange resin. The eluates of sialic acid obtained after anion exchange chromatography is subjected to concentration under reduced pressure, membrane drying, spray drying or lyophilization to yield high purity sialic acid (Koketsu *et al.*, 1993).

Sialic acid is also being produced from genetically engineered microbes usually *Eschericha coli*. The microbe is modified by: introducing an inactivating mutation into a first DNA molecule encoding a sialic acid transporter in the microbe; introducing an inactivating mutation into a

second DNA molecule encoding a sialic acid aldolase in the microbe; and introducing a first enzyme-encoding recombinant DNA molecule in the microbe, wherein said first enzyme-encoding recombinant DNA molecule encodes an enzyme selected from the group consisting of a sialic acid synthase and a UDP-*N*-acetylglucosamine-2-epimerase (Boddy and Lundgren, 2007).

Another method of sialic acid production comprises the step of culturing a microorganism in a culture medium, wherein said microorganism comprises heterologous genes encoding a sialic acid synthase (NeuB), a UDP-GlcNAc epimerase (NeuC), said micro-organism being devoid of a gene encoding CMP-Neu5Ac synthase (NeuA) or wherein a gene encoding CMP-Neu5Ac synthase (NeuA) has been inactivated or deleted; and wherein endogenous genes coding for sialic acid aldolase (NanA), for ManNac kinase (NanK) and for sialic acid transporter (NanT) have been deleted or inactivated (Samain, 2011). In both animals and bacteria, the biosynthesis of Neu5Ac is initiated by UDP-GlcNAc 2-epimerase, which forms ManNAc from UDP-GlcNAc. In animals ManNAc is then phosphorylated at C-6 by a specific ManNAc kinase; ManNAc-6-P is metabolized further by Neu5Ac-9-phosphate synthase to Neu5Ac 9-phosphate which is then dephosphorylated into Neu5Ac. Many bacteria including E. coli K12 are able to catabolise Neu5Ac and use it as a carbon energy source. The catabolic pathway for Neu5Ac has been identified in E. coli: a specific permease encoded by nanT transports Neu5Ac into the cytoplasm, where it is cleaved into ManNAc and pyruvate by the aldolase encoded by nanA (Vimr & Troy, 1985). By disrupting the nanA and nanK genes, degradation of Neu5Ac and ManNAc is prevented. The sialic acid produced is purified by centrifuging the culture medium and direct crystallization of the supernatant (Samain, 2011). However, further improvement is

needed because even the most extensively engineered baculovirus/insect cell systems require media supplementation with *N*-acetylmannosamine, an expensive sialic acid precursor, for efficient recombinant glycoprotein sialylation (Geisler and Jarvis, 2012).

Chemical synthesis can be used to produce homogenous glycans in larger quantities than is available from most cellular production systems. Enzymes can be used together with chemical methods to further simplify the process of glycans synthesis. The fundamental challenge of glycans synthesis is the requirement for modifying one specific hydroxyl group in the presence of many others. In addition to that, glycans cannot yet be synthesized using a general commercial system. However, a number of methodologies are currently being developed by synthetic chemists to address the inherent challenges in glycans chemistry that have prohibited automation (Seeberger *et al.*, 2009).

2.6 Phospholipase

Phospholipase is an enzyme that catalyzes the hydrolysis of phospholipids from membranes (Singh and Ranganathan, 2014) into phospholipids and other lipophilic substances. The known types of phospholipase are phospholipase A_1 (PLA_1), phospholipase A_2 (PLA_2), phospholipase B, phospholipase C and phospholipase D. PLA_1 cleaves the SN-A acyl chain while PLA_2 the SN-2 acyl chain releasing arachidonic acid. Phospholipase B also known as lysophospholipase cleaves both the SN-1 and SN-2 acyl chains. Phospholipase C cleaves phopspahate leaving diacylglycerol and a phosphate-containing head group. It also plays a central role in signal transduction, releasing the second messenger inositol Triphosphate. Phospholipase D cleaves after the phosphate, releasing phosphatidic acid and an alcohol (Vasudevan and Sreekumari,

2007). PhospholipasePLA$_2$ is commonly found in mammalian tissues as well as insect and snake venom (Nicolas *et al.*, 1997).

2.7 Lipase

Lipases are a class of enzymes which catalyze the hydrolysis of long chain triglycerides to fatty acids and glycerol. They constitute the most important group of biocatalysts for biotechnological applications. Due to their low optimum temperature and high activity at very low temperatures, which are favorable properties for the production of relatively frail compounds (Babu *et al*., 2007). Lipase is produced by the pancreas, liver, intestine, tongue, stomach and other cells. They occur in plants, animals and microorganisms (Vakhlu and Kour, 2006). Lipolytic enzymes are currently attracting an enormous attention because of their biotechnological potential (Benjamin and Pandley, 1998). They constitute the most important group of biocatalysts for biotechnological applications. Furthermore, novel biotechnological applications have been successfully established using lipases for the synthesis of biopolymers and biodiesel, the production of enantiopure pharmaceuticals, agro chemicals and flavor compounds (Jaeger and Eggert, 2002).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and Reagents

N-butanol, Thiobarbituric acid: 0.1M solution of 2-thiobarbituric acid in distilled water adjusted to pH 9.0 with NaOH, Concentrated HCl, NaCl, Formic acid, Periodate reagent: 25mM-periodic acid in 0.125N-H_2SO_4 (pH 1.2), Sodium Arsenite: 2% of solution of sodium arsenite in 0.5N-HCl, Dowex-50, phospholipase from Sigma Chemical Company, lipase from Sigma Chemical Company, sodium phosphate and sodium hydrogen phosphate.

3.1.2 Equipment

Centrifuge, waterbath, spectrophotometer, pH meter, Fourier Transform Infra-red Spectroscope, hot plate, refrigerator and blender.

3.2 Methods

3.2.1 Collection of Submandibular Tissue

The submandibular glands were acquired from the slaughter immediately after the animals were slaughtered. The submandibular tissue was submerged in ice cold dextrose saline and brought to the laboratory for use. All subsequent experiments were carried out at room temperature. The unwanted fat and connective tissue were cut off with a knife then, the respective glands were weighed and their values were recorded.

3.2.2 Extraction of Sialic Acids

3.2.2.1 Homogenization of Submandibular Gland

The glands (13.4g) were chopped into small pieces then 100 ml of 50mM phosphate buffered saline, pH 6.8 was added to it and it was homogenized. Thereafter, the homogenate was centrifuged at 1500 x *g* for 5 minutes then the supernatant was assayed for sialic acids.

3.2.2.2 Assay for Sialic Acids

The free sialic acids were quantified using the method described by Aminoff (1961). The sample (500µl) was pipetted into a test tube. Distilled water (500µl) was pipetted into a different test tube as blank. Periodate reagent (250µl) was added to the test tubes. The tubes were then incubated at 37^0C for 30 minutes in a water bath. The excess periodate was reduced with 200µl of sodium arsenite reagent. As soon as the yellow colour of the liberated iodine disappeared, 2000µl of thiobarbituric acid reagent was added and the test tubes were covered with aluminum foil. The test tubes were incubated in a boiling water bath for 7.5 minutes in a boiling water bath. The colored solutions were then cooled and shaken with 5000µl of acid butanol reagent. The test tubes were then centrifuged at 3000 x *g* for 5 minutes to facilitate rapid separation of two phases. The colour intensity in the butanol layer was read at 549 nm after zeroing the spectrophotometer with the blank.

3.2.2.3 Treatment of Homogenized Submandibular Gland with Enzymes

Twenty milliliters of the homogenate was incubated with 0.15mls of phospholipase (0.1U) and 3mg of lipase respectively for 3 hours and at 30 minutes intervals. The resulting enzyme-treated

homogenate contained free and maybe bound sialic acids. After this, the enzyme-treated homogenate was assayed again for sialic acid concentration.

3.2.2.4 Acid hydrolysis

Acid hydrolysis was done using 0.1N HCl in a 1:1 ratio with the enzyme-treated homogenate. A fraction (10mls) of each of the samples was taken and an equal volume of 0.1N HCl was added. The pH was adjusted to 3.5 from 5.8 with concentrated formic acid. The resulting solution was heated at 80^0C in a water bath for 1hr. The resulting hydrolysates were assayed for the released sialic acids. The hydrolysate was centrifuged at 300 x *g* for 40 minutes. The supernatants were retained and the pellets were discarded. The supernatant was put into a dialysis bag and dialyzed against phosphate buffer (pH 7.2) for 48 hours respectively. Total sialic acid was assayed using the method described by Aminoff (1961).

3.2.3 Purification of Sialic Acids

The extracted sialic acids were purified using ion exchange chromatography. The dialysate, 20mls was applied unto an anion exchange resin (Dowex-50). The column was pre-equiliberated with 50mM phosphate buffer, (pH 7.2). The sialic acids were eluted with 10mls of step-wise gradient from 0.0-0.5M sodium chloride (NaCl). The fractions obtained were collected in 2ml capacity Eppendoff tubes. Fractions obtained were assayed for sialic acid concentration to determine the fractions containing the sialic acids. These fractions were pooled together, the sialic acid concentration requantified and used for partial characterization.

3.2.5 Thin Layer Chromatography (TLC)

A capillary tube was used to spot a few drops of the sialic acid extracts, the standard sialic acid and standard mucin on an aluminum thin layer plate coated with silica gel. The spots were kept to dry after which the aluminum plate was put into a tank containing 5ml of a mixture of *N*-butanol, acetic acid and water in the ratio 2:1:1 respectively then the tank was covered. After the plate was removed from the tank, it was sprayed with 20% H_2SO_4 and heated in an oven at 100^0C for 3 to 5 minutes (Kiyohara *et al*., 2011).

3.2.6 Fourier Transform Infrared Chromatography (FTIR)

An FTIR-8400S spectrometer equipped with a standard detector connected to an IR solution software was used for FTIR data collection. FTIR spectra were collected from 10 scans, at a resolution of 2 cm^{-1} and at mid infrared region (4700-340 cm^{-1}) against a polystyrene background.

CHAPTER FOUR

4.0 RESULTS

4.1 Purification profile for sialic acid extract from cattle and goat submandibular mucin.

The different stages of sialic acid purification from cattle submandibular mucin is presented in Table 4.1. The table shows the concentrations of sialic acid at each stage of purification. The stages shown on the table are homogenization, treatment with phospholipase and lipase, acid hydrolysis and ion exchange chromatography. The purification fold increased with each successive stage. The purification fold at the end was 16.59 and the percentage yield was 25.28%. Table 4.2 shows the different stages of sialic acid purification from goat submandibular mucin. The table shows the concentrations of sialic acid at each stage of purification. The stages shown on the table are homogenization, treatment with phospholipase and lipase, acid hydrolysis and ion exchange chromatography. The purification fold increased with each successive stage. The final purification fold was 12.11 while the percentage yield was 27.53%.

4.2 Treatment of Submandibular Mucin with Enzymes

Figures 4.1 and 4.2 are graphs showing sialic acid concentration during incubation of cattle and goat submandibular mucin with phospholipase and lipase respectively with time. In Figure 4.1, the two homogenized submandibular mucins were incubated with phospholipase and the sialic acid concentrations were taken at 30 minute intervals. As presented by the graph, it is seen that there is a linear increase in sialic acid concentration with time in the first 90 minutes of incubation with phospholipase. The acid hydrolyzed mucins also had a higher concentration of sialic acids compared to the submandibular mucins which was not acid hydrolyzed. In Figure 4.2, represents the sialic acid concentration of the submandibular mucins after their incubation

with phospholipase and lipase for 3 consecutive hours. The sialic acid concentration was also taken at 30 minute intervals. The sialic acid concentration of the acid hydrolyzed and non-acid hydrolyzed submandibular mucin of cattle and goat was almost constant during and after incubation with lipase.

Table 4.1: Purification table for sialic acid extract from cattle submandibular mucin.

Fractions	Volume (ml)	Sialic acid conc. (mg/ml)	Total sialic acid (mg)	Purification fold	% Yield
Homogenate	100	0.022	2.2	1.00	100
Phospholipase	25	0.082	2.05	3.72	93.18
Lipase	25	0.084	2.1	3.82	95.5
Acid hydrolysis	20	0.342	6.84	15.55	32.16
Ion exchange	22	0.365	8.03	16.59	25.28

Table 4.2: Purification table for sialic acid extract from goat submandibular mucin.

Fractions	**Volume (ml)**	**Sialic acid conc. (mg/ml)**	**Total sialic acid (mg)**	**Purification fold**	**% Yield**
Homogenate	100	0.046`	4.6	1.0	100
Phospholipase	25	0.125	3.13	2.72	68.04
Lipase	25	0.127	3.18	2.76	69.13
Acid hydrolysis	20	0.546	10.92	11.87	42.13
Ion exchange	30	0.557	16.71	12.11	27.53

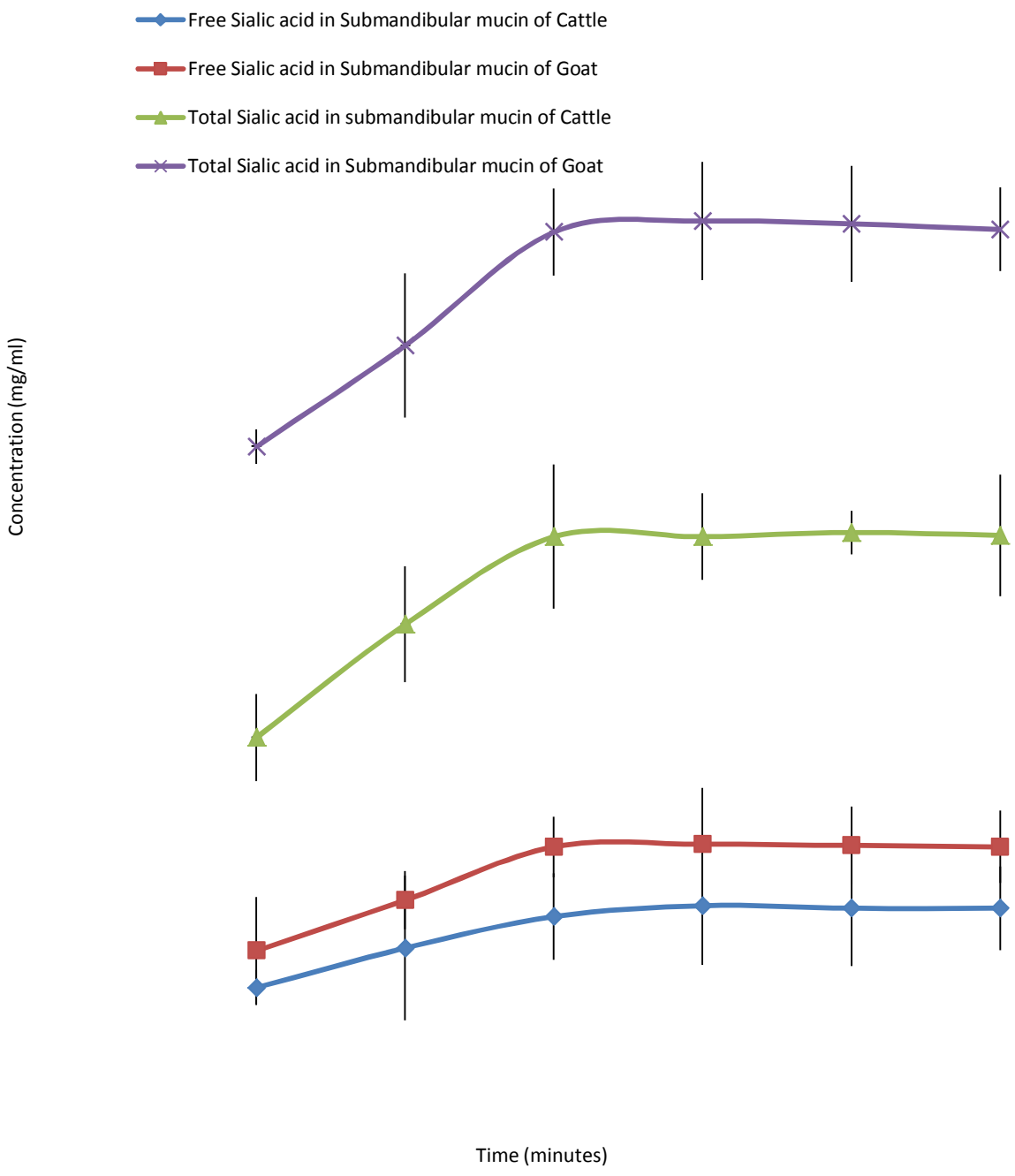


Figure 4.1: Effect of incubation of submandibular mucin with phospholipase at various time intervals

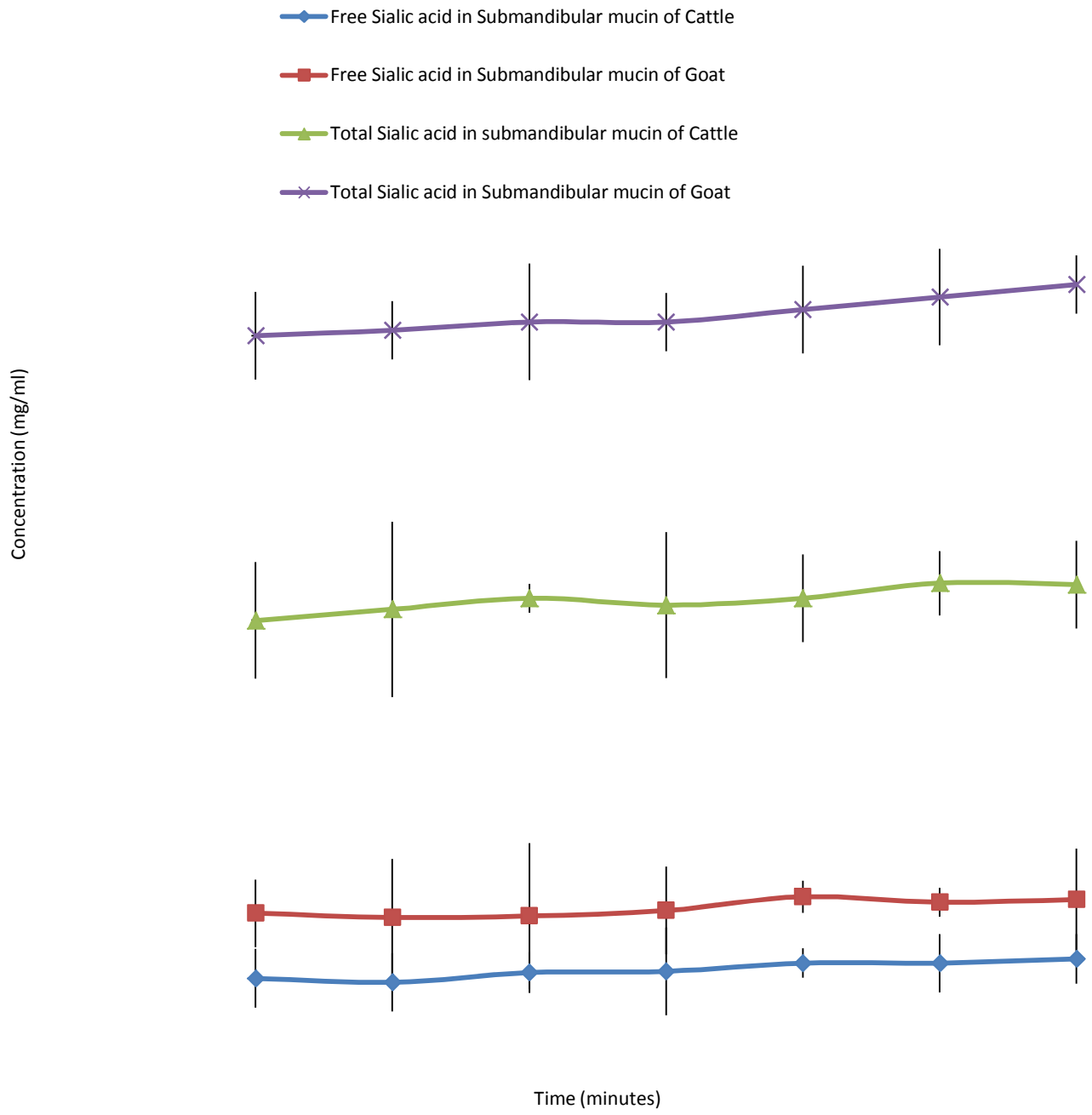


Figure 4.2: Effect of incubation of submandibular mucin with phospholipase and lipase at various time intervals

4.3 Ion exchange elution profile of sialic acid from cattle and goat submandibular mucin

The elution profile for sialic acid extract from submandibular mucin of cattle is presented in Figure 4.3. A total of 44 eluates were collected, 11 of which contained sialic acids. The 19th fraction had the highest sialic acid concentration, followed by the 20th, 21st and 22nd fractions.

The elution profile for sialic acid extract from submandibular mucin of goat is presented in Figure 4.4. A total of 45 eluates were collected, 15 of which contained sialic acids. The sialic acids were eluted with 0.0M to 0.5M NaCl. The 19th fraction had the highest concentration of sialic acid followed by the 20th, 22nd and 24th fractions.

4.4 Sialic acid proportion in cattle and goat submandibular gland

The proportion of sialic acid in cattle and goat submandibular gland is shown in Table 4.3. The percentage of sialic acid in one gram of cattle and goat submandibular gland is 1.23% and 2.56% respectively

Table 4.3: Sialic acid proportion in submandibular gland of cattle and goat

Submandibular gland	(mg of Sias/g of submandibular gland)	Mass of submandibular gland (grams)	% of Sias/gram of submandibular gland
Cattle	12.30	13.38	1.23
Goat	25.56	13.40	2.56

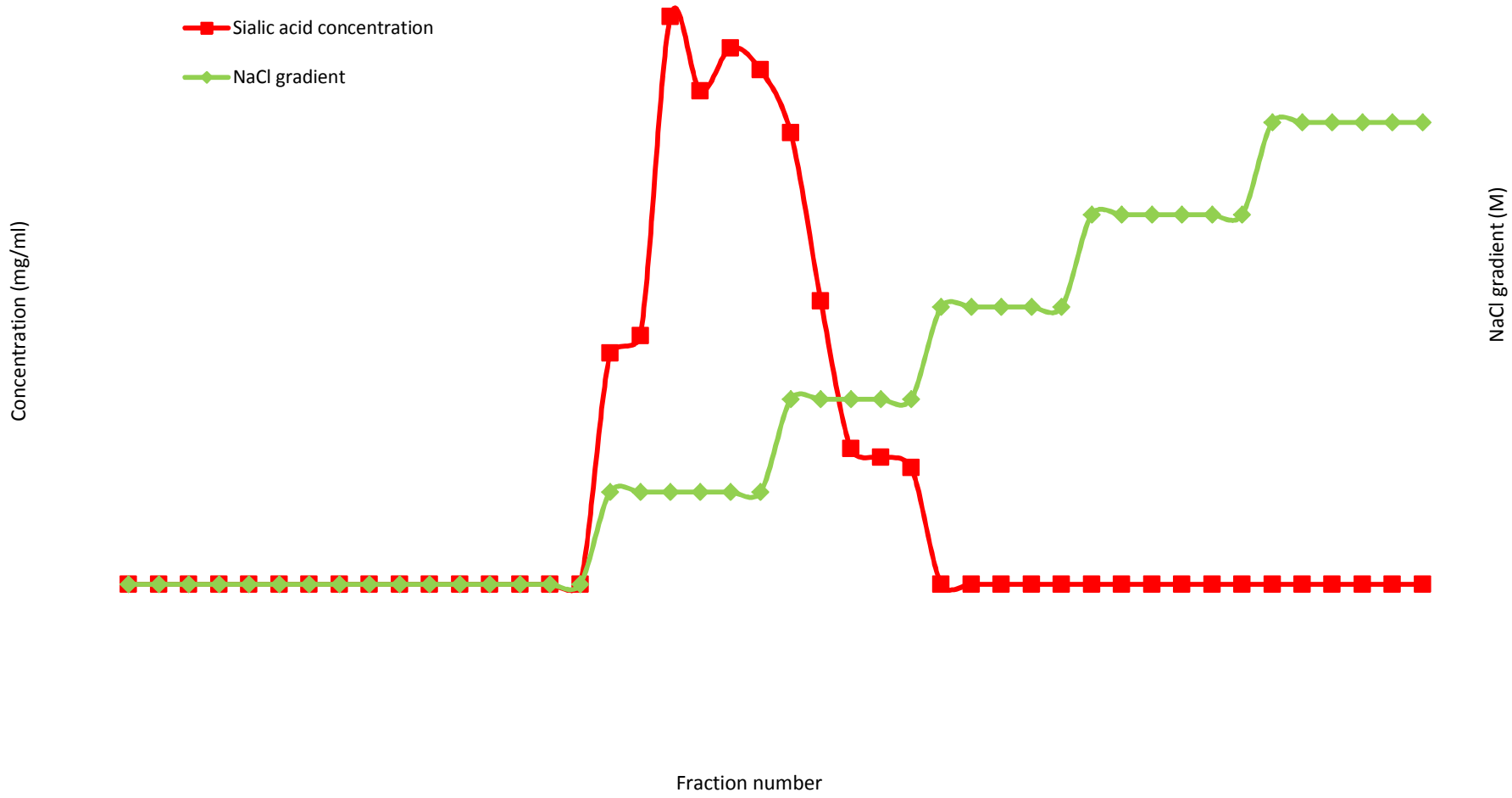


Figure 4.3: Ion exchange elution profile for sialic acid from submandibular mucin of cattle.

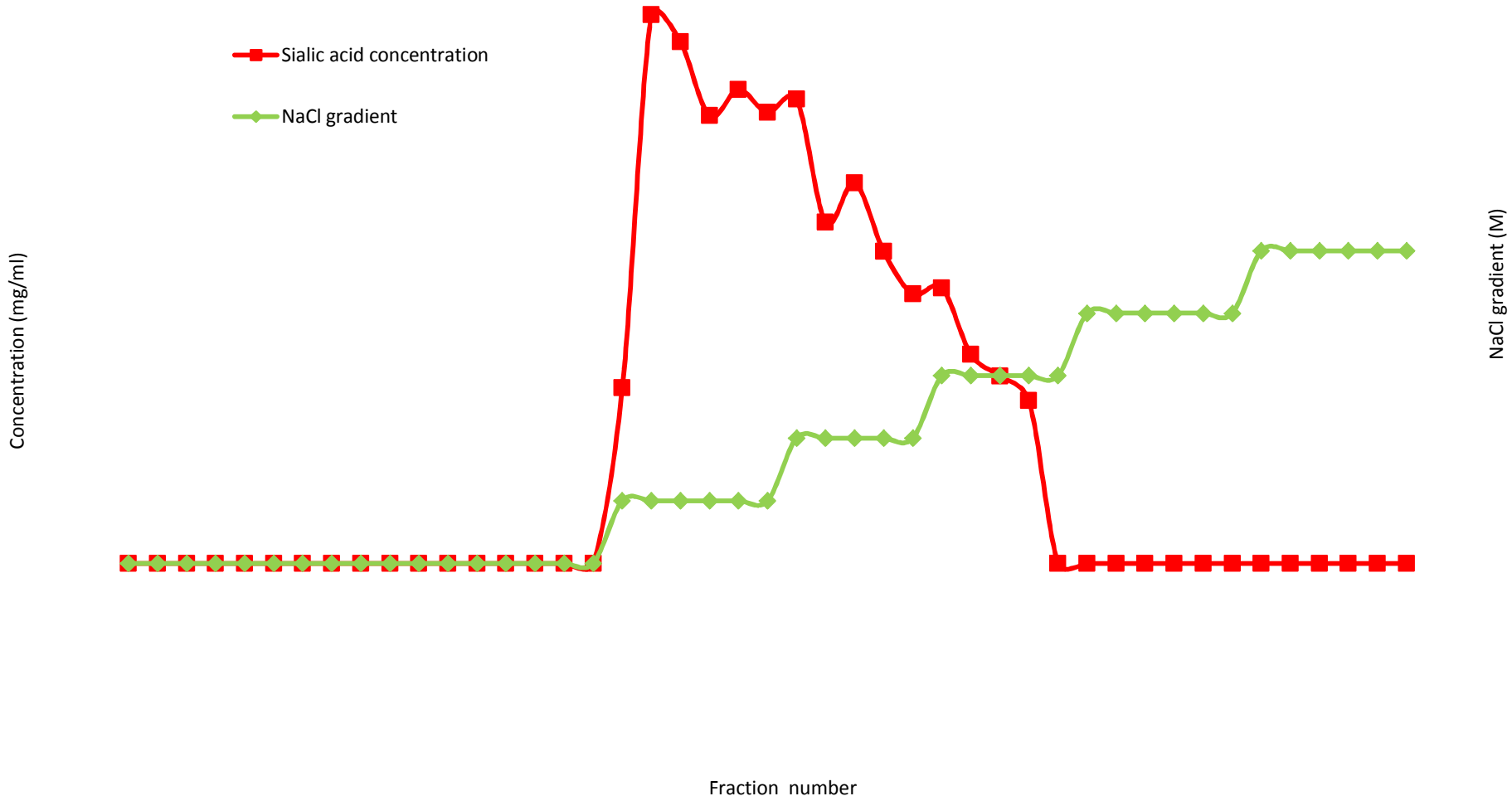


Figure 4.4: Ion exchange elution profile for sialic acid from submandibular mucin of goat.

4.5 Thin-Layer Chromatography of Sialic Acid Extract

Thin-layer chromatogram of the sialic acid extract is shown in Plate II. The spots of both the sialic acid extract from cattle and goat submandibular mucins moved together then merged at the point near the top of the thin-layer plate. The first spot on the thin-layer plate is from Standard mucin from Sigma, the second spot is from Standard sialic acid from Sigma, the third spot is from the sialic acid extract from goat submandibular mucin and the fourth spot is from the sialic acid extract from cattle submandibular mucin.

4.6 FTIR Spectra for Sialic Acid Extracts

Figure 4.5 shows the FTIR spectrum for the sialic acid extract gotten from cattle. It has 5 peaks corresponding to 5 different functional groups. The peaks are of wavenumbers 461.97 cm^{-1}, 1643.41 cm^{-1}, 2087.05 cm^{-1}, 2346.48 cm^{-1} and 3432.44 cm^{-1}. The FTIR spectrum for the sialic acid extract from goat is presented in Figure 4.6. The spectrum has 5 visible peaks. The peaks are of wavenumbers 459.07cm^{-1}, 1644.37 cm^{-1}, 2091.87 cm^{-1}, 2343.59 cm^{-1} and 3441.12 cm^{-1}.

The FTIR spectrum for powdered standard sialic acid is presented in Figure 4.7. The spectrum shows 13 peaks corresponding to 13 different functional groups. The peaks are of wavenumbers 593.13 cm^{-1}, 695.36 cm^{-1}, 920.08 cm^{-1}, 1053.17 cm^{-1}, 1404.22 cm^{-1}, 1609.65 cm^{-1}, 1754.32 cm^{-1}, 2199.89 cm^{-1}, 2639.67 cm^{-1}, 3393.86 cm^{-1}, 3546.25 cm^{-1}, 3915.63 cm^{-1} and 4161.56 cm^{-1}.

The FTIR spectrum for the powdered standard sialic acid in 0.2M NaCl solution is presented in figure 4.8. The spectrum gave 6 peaks. The peaks are of wavenumbers 462.93 cm^{-1}, 1122.61 cm^{-1}, 1643.41 cm^{-1}, 2090.91 cm^{-1}, 3434.37 cm^{-1} and 3940.7 cm^{-1} (Krishna *et al*., 2013).

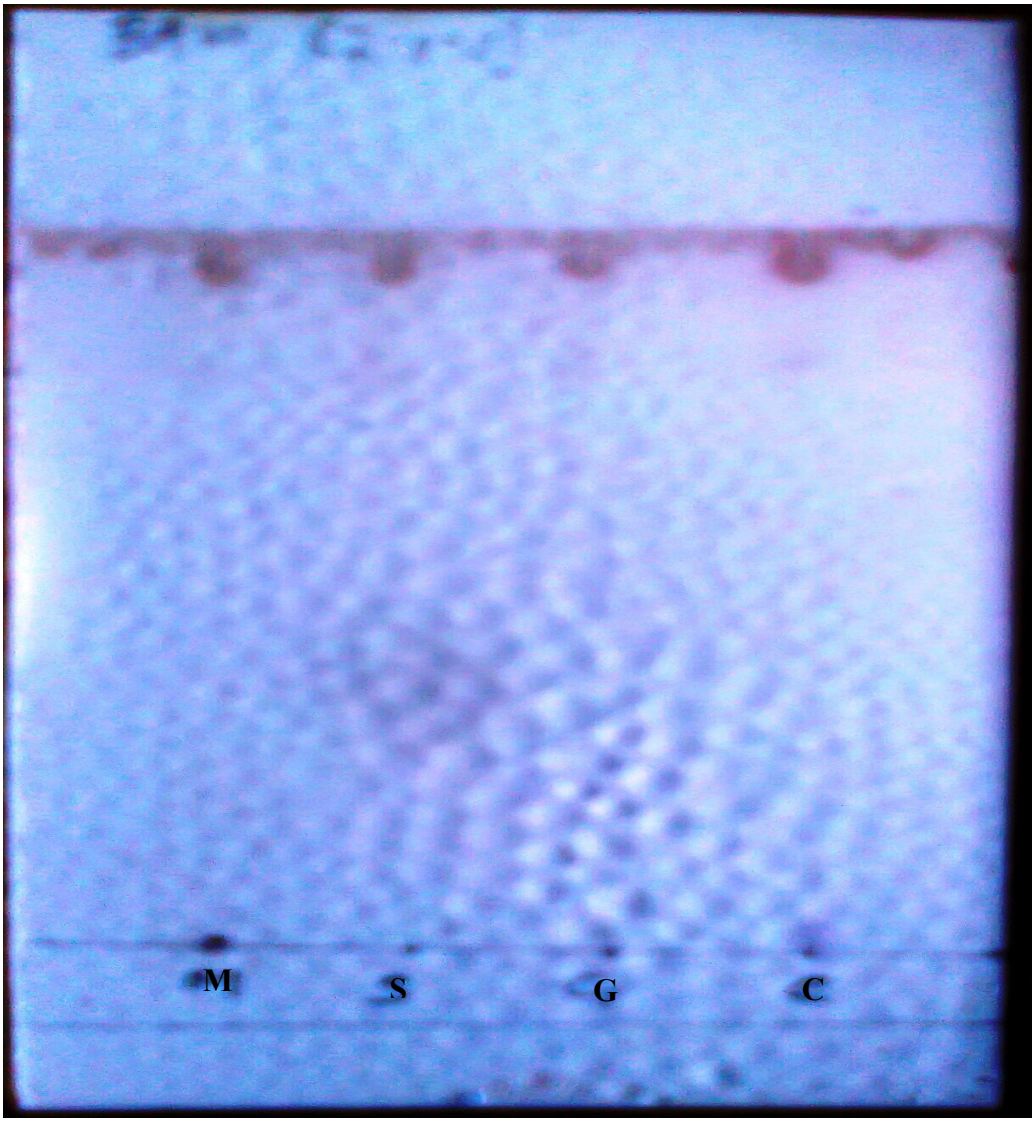


Plate II: Thin Layer Chromatography plate for standard sialic acid and sialic acid extract from cattle and goat.

Where M = Standard mucin from Sigma

S = Standard sialic acid from Sigma

G = Sialic acid extract from goat

C = Sialic acid extract from cow

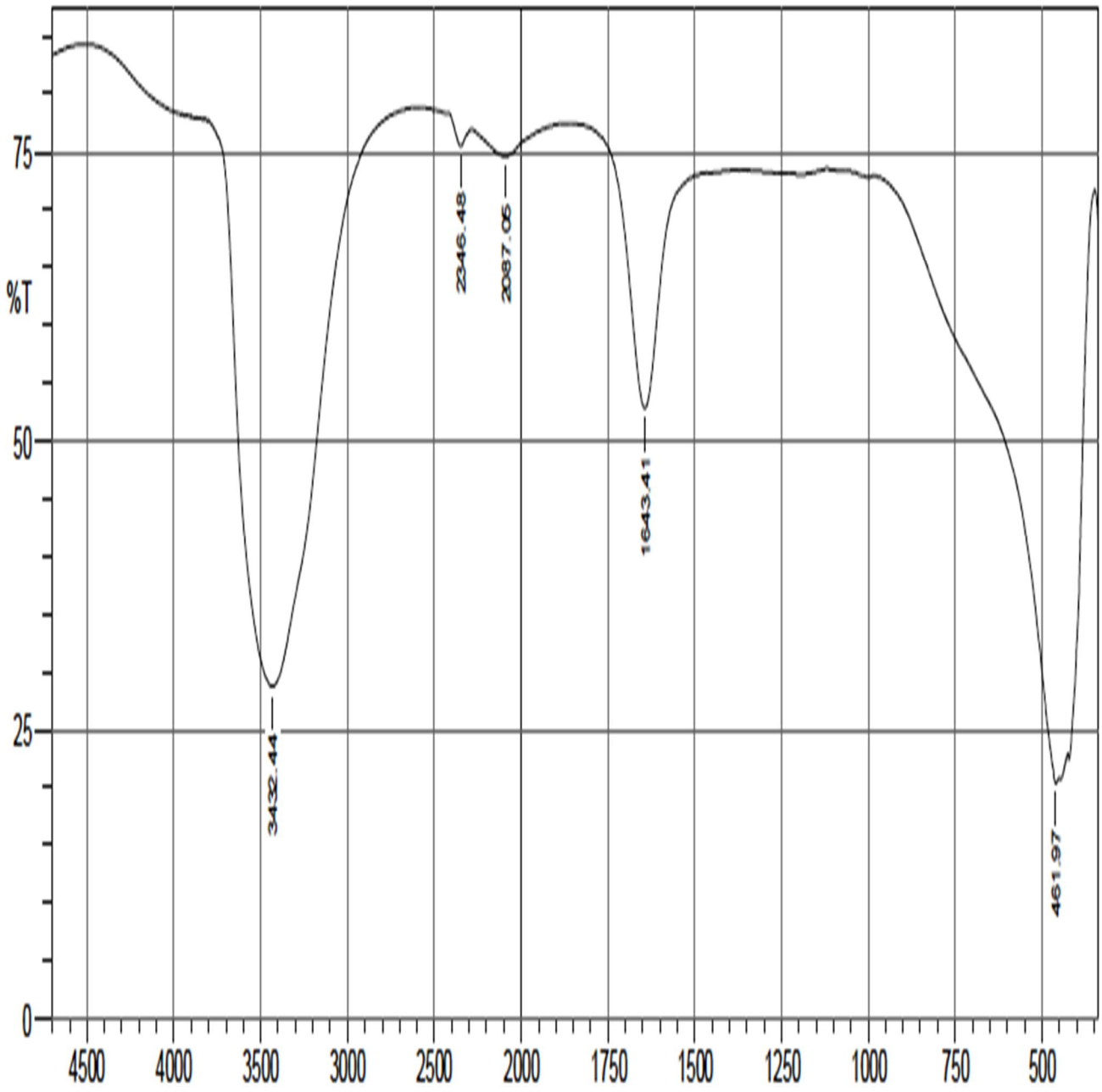


Figure 4.5: FTIR spectrum for sialic acid extract from cattle submandibular mucin

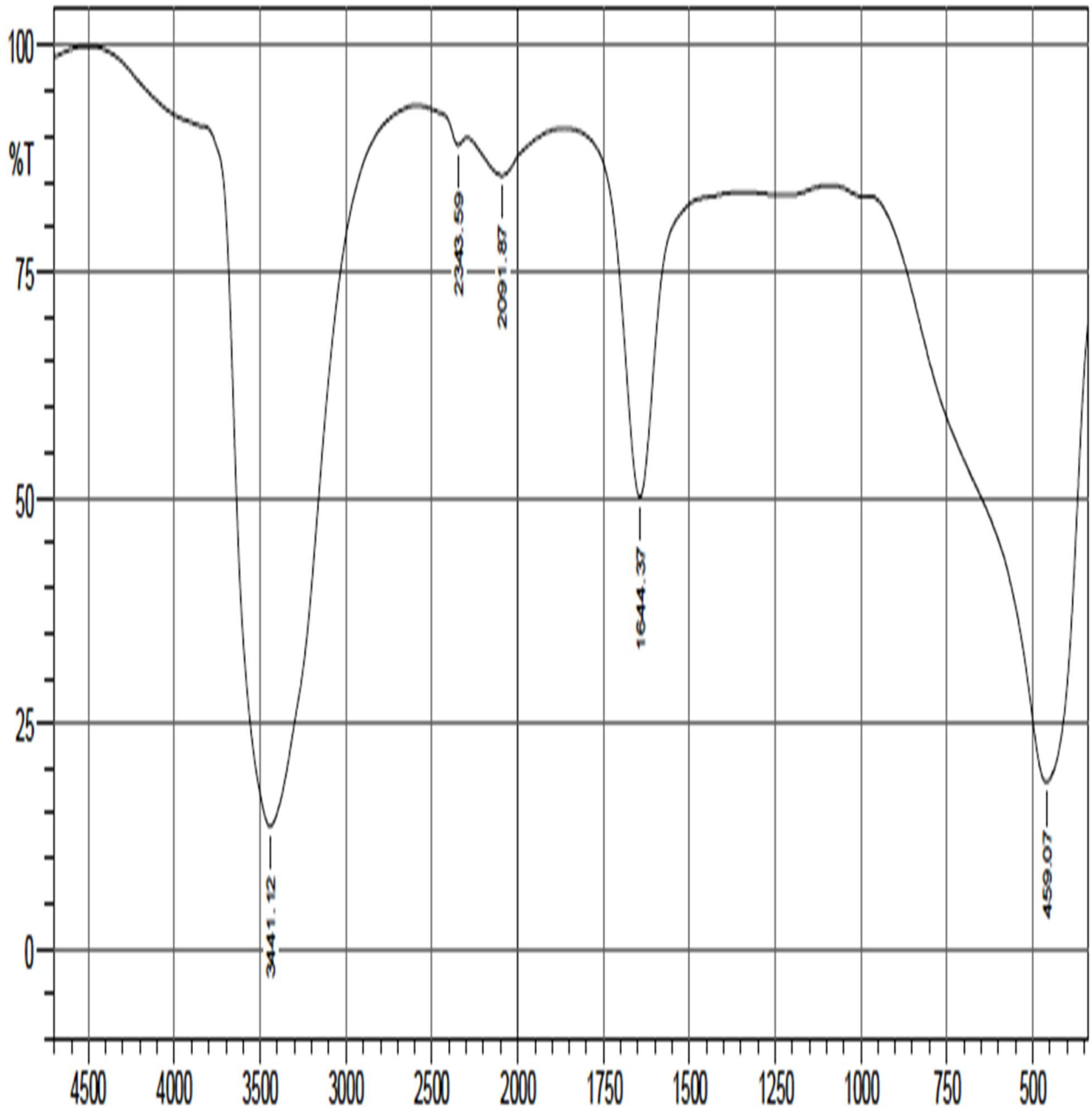


Figure 4.6: FTIR spectrum for sialic acid extract from goat submandibular mucin

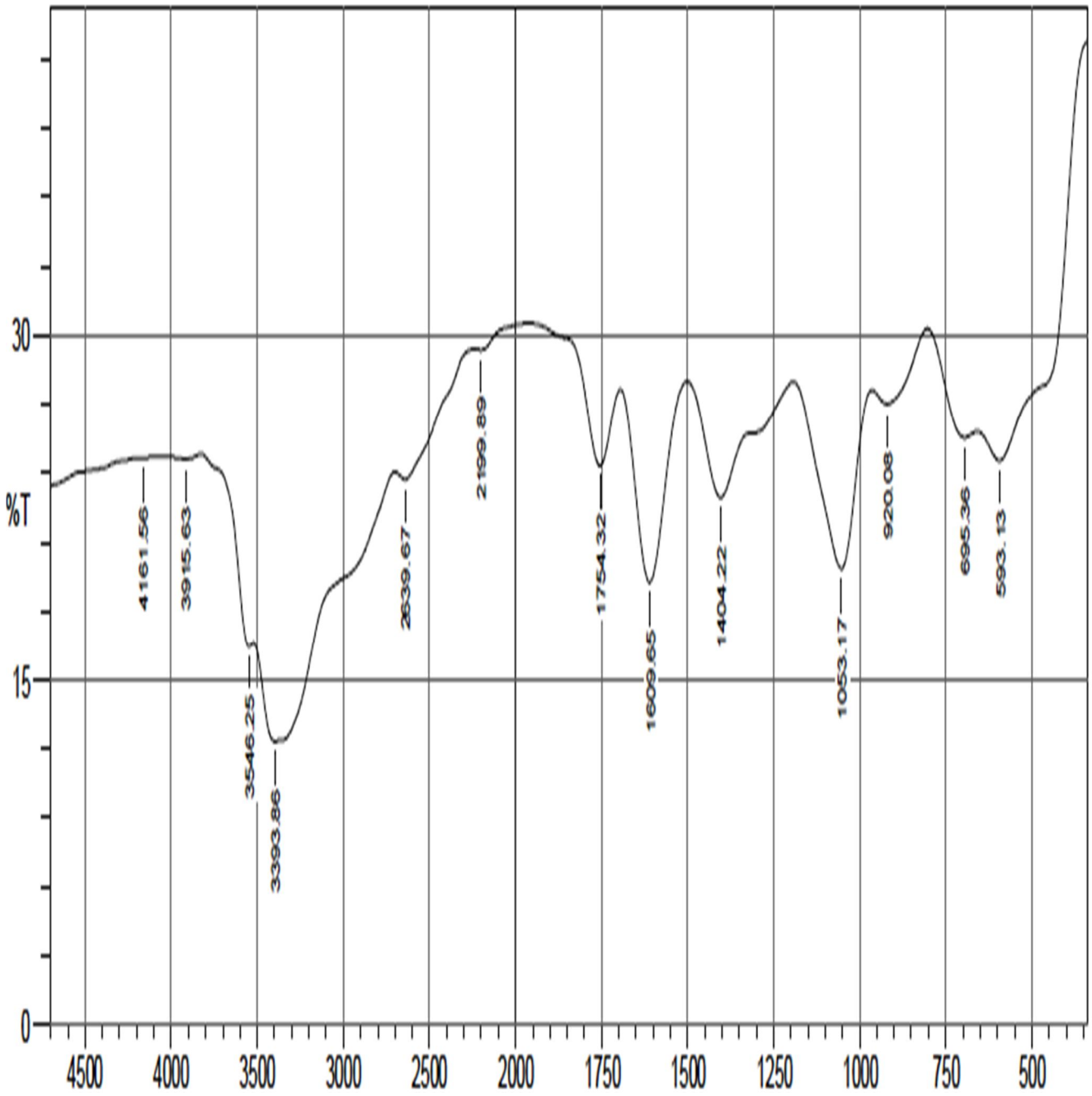


Figure 4.7: FTIR spectrum for standard sialic acid

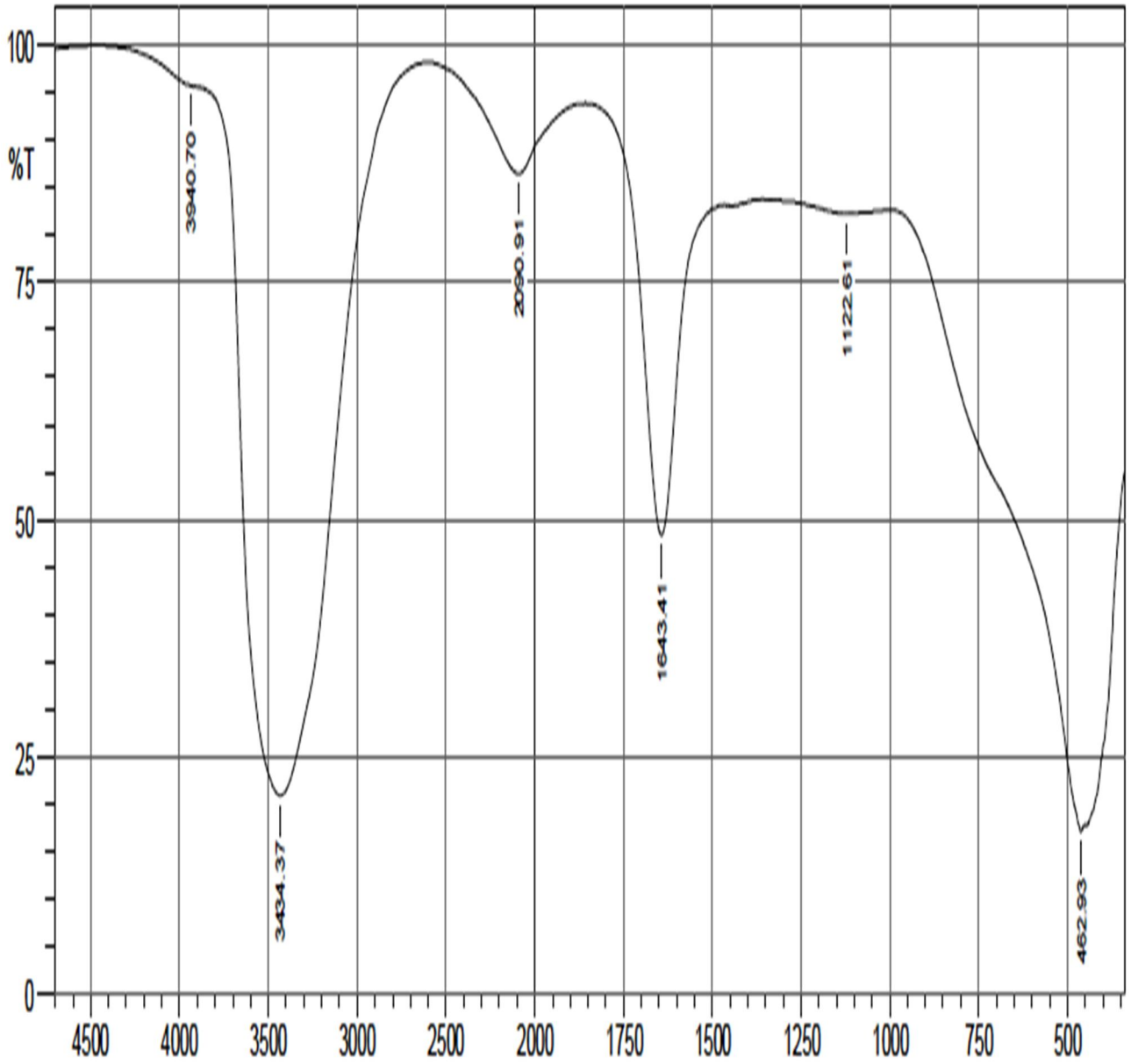


Figure 4.8: FTIR spectrum for standard sialic acid in 0.2M NaCl solution

CHAPTER FIVE

5.0 DISCUSSION

As a result of the high cost of sialic acids and its ever increasing demand, there are many studies which are currently focused on ways of getting a cheaper means of sialic acid production so that products containing sialic acids can be more affordable. This study is one of those and it is focused on isolating sialic acid from submandibular mucin.

In this report, purification of sialic acid from cattle and goat submandibular mucin was carried out. It was observed that phospholipase, lipase and mild acid hydrolysis led to an increase in free sialic acid concentration of the homogenized submandibular mucin. This is because acid hydrolysis helps to break down most of the bonds which are attached to the sialic acid molecules. Similarly, since sialic acid is linked to glycoconjugates through glycosylphosphatidylinositol (GPI) bonds, one would suggest that the action of phospholipase and lipase on this structure is linked to the release of sialic acid.

In Figure 4.1 a graph is shown representing the effect of incubating the homogenized submandibular mucin of cattle and goat with phospholipase. After the incubation of the homogenized goat and cattle submandibular mucin with phospholipase, the linear increase in the quantity of sialic acid hydrolyzed in the first 90 minutes may indicate that the hydrolytic action of the enzyme within that time is first order. Typical of enzyme catalyzed reactions, initial periods of interaction of enzymes and substrates is usually characterized by linear increase in the release of products to a certain level, after which the linearity diminishes. The loss of linearity after 90 minutes could be due to the fact that most of the phospholipids bound to sialic acid

molecules have been completely hydrolyzed or that there is a saturation of the active site of the phospholipase by the phospholipids available.

After incubation with phospholipase and lipase consecutively, the slight increase in sialic acid concentration during the incubation is an indication that either lipase has a lesser effect on sialic acid hydrolysis from submandibular mucin after treatment with phospholipase or the action that lipase would have carried out has already been carried out by phospholipase. The commonly used treatment for complete release of sialic acid by incubation with 1N H_2SO_4 for 1 hour at 80^0C usually results in complete destruction of *O*-acetyl groups and even in some destruction of the sialic acid molecule itself. Milder conditions have therefore been used (0.5N formic acid, pH 2.1, 80^0C, 1 hour). With this method less destruction of *O*-acetyl groups occurs but the release of sialic acid is incomplete (40-60%) (Varki and Diaz, 1984). However, for this research, HCl was used to substitute H_2SO_4. The submandibular mucin hydrolysis with 0.1N HCl and the eventual release of sialic acid is a demonstration of the hydrolytic role of HCl with regards to sialic acid hydrolysis from the glycoconjugate.

During ion exchange chromatography, sialic acids were eluted using 0.1M, 0.2M and 0.3M concentrations of NaCl because the sialic acid molecules were not tightly bound to the anionic exchanger. More eluates containing sialic acid were obtained from the goat sialic acid extract than the cattle sialic acid extract showing that goat submandibular mucin contained more sialic acid than that of cattle.

Although the analytical conditions do not routinely distinguish between the free and nucleotide sugar forms of sialic acid, it has been reported that the precursor pool is predominantly free sialic acid (Thomas *et al.*, 1985), a contention supported by thin layer chromatography analysis (Collins *et al*., 2000). The presence of sialic acid in the extracts was confirmed by a spot which moved at the same rate with a spot given by a solution of standard sialic acid on the same thin-layer plate.

The purification fold of the sialic acid extracted from cattle was more than that of the extract from goat while the percentage yield for the extracted sialic acid from goat was more than that extracted from cattle.

Unlike the previously listed methods of sialic acid extraction and production which require the purchase of raw materials such as eggs (Koketsu *et al*., 1993) or culture medium substrate (Geisler and Jarvis, 2012), the extraction of sialic acid from submandibular mucin of cattle and goat is cheaper because the major raw material being used which is the head of cattle and goat are considered as 'waste' in developed countries. Also, in the case of producing sialic acid from egg yolk on a commercial scale, separation of the egg shells or egg yolk from the egg white for many eggs will be tedious. In the case of sialic acid extraction from bovine submandibular mucine, enzymes have not been used and the results showed that incubation of submandibular mucine with phospholipase led to an increase in quantity of hydrolyzed sialic acids.

Sialic acid is also being extracted from bovine submandibular mucin. The extraction involves the homogenization of the submandibular gland, acid hydrolysis, dialysis with 0.1N HCl or H_2SO_4 and ion exchange chromatography

For FTIR, the analysis time was less than five minutes and required the use of a minute quantity of the sample. The FTIR spectra obtained from both the submandibular mucin of cattle and goat had 5 bands each in the same positions. The major bands in the two sialic acid extracts correspond with a broad O-H stretch absorption. This is partly due to the fact that the extracts are in aqueous form. The first FTIR absorption bands in the submandibular mucin of goat and cattle of wavenumbers 3441.12cm^{-1} and 3422.44cm^{-1} respectively are characteristic of O-H stretch and N-H stretches thereby confirming the presence of carboxylic acid, primary amides and secondary amines (Johny *et al.,* 2011). The second absorption bands in both the goat and cattle FTIR spectra corresponding to wavenumbers of 2343.59cm^{-1} and 2346.48cm^{-1} are characteristic of alkenes. The third absorption bands in the spectra for the sialic acid extracts from goat and cattle of wavenumbers 2091.87cm^{-1} and 2087.05cm^{-1} respectively are characteristic of allenes. The fourth absorption bands of wavenumbers 1644.37cm^{-1} and 1643.41cm^{-1} are characteristic of C-H stretch. The fifth band in the two spectra for submandibular mucin of goat and cattle identifies the presence of an alkyl halide in the two sialic acid extracts. Since NaCl was used to elute the sialic acid extract during ion exchange chromatography, it is suspected that the halide present in the sialic acid extract is chlorine. This shows that the quality of sialic acid from both cattle and goat submandibular mucin are almost identical. In the FTIR spectrum obtained for standard freeze dried sialic acid, 13 bands were seen. However, when the standard sialic acid was

dissolved in 0.2M NaCl, the FTIR spectrum obtained was similar to that obtained from submandibular mucin of cattle and goat. This is due to the fact that the sialic acid extract from the mucin was eluted with NaCl solutions during ion exchange chromatography. However, in the FTIR spectrum obtained for a solution of standard sialic acid in 0.2M NaCl, a sixth absorption band was observed. A faint band in a similar position was almost visible in the spectra obtained for the sialic acid extracts from goat and cattle submandibular mucin. The sixth band however is yet to be identified.

Therefore, the FTIR analyses of the two sialic acid extracts and the standard sialic acid solution in 0.2M NaCl reveal the presence of an alkyl halide, secondary amine group, primary amide, hydroxyl group, alkene, alkane and allenes. By visual recognition, there isn't much difference between the absorption bands obtained from the three spectra.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

- The percentage of sialic acid obtained per submandibular gland of cattle and goat were 1.23% and 2.56% respectively.
- The sialic acid extracted from the cattle and goat submandibular mucin moved at the same speed on the thin-layer plate with the standard sialic acid gotten from Sigma.
- The FTIR spectrum revealed that the components of the sialic acid extracts and those of the Standard sialic acid in 0.2M NaCl were almost identical.

6.2 Conclusion

Phospholipase cleaved off more bound sialic acid molecules than lipase during incubation with submandibular mucin. The enzymes had optimum activity after incubation with submandibular mucin for 120-150 minutes. This study shows that goats contain more sialic acid per gram of submandibular gland (2.56%) than cattle (1.23%). The average cattle have a submandibular gland which is about 5 times bigger than that of a goat. Most companies would rather sell pure products than impure products. Therefore, producing sialic acid from cattle will be a better option since the sialic acid extract from cattle had a higher purification fold than that from goat. This study was not time consuming and it led to the extraction and partial purification of sialic acids. Since, the heads of animals are not consumed in developed countries and many cattle are killed regularly, using the submandibular glands of the cattle and goat for the production of sialic acids will not be expensive. Also, the other reagents used in sialic acid extraction are relatively cheap.

The FTIR spectra for the sialic acid extracts obtained from the submandibular gland of cattle and goat revealed that cattle and goat have sialic acid of similar quality. The FTIR spectrum for a solution of standard sialic acid in 0.2M NaCl was almost identical with that obtained from the extracted sialic acid from cattle and goat submandibular mucin. The percentage of sialic acid extracted is higher than calculated because after the submandibular gland gotten from the cattle or goat is immersed in dextrose saline, the absorbed water will result in a higher weight of the submandibular gland.

6.3 Recommendation

When carrying out sialic acid analysis, there is no need to centrifuge the submandibular mucin before adding periodate reagent. This is because the tissue which will be discarded after centrifugation and decantation still contains sialic acids.

Since the FTIR spectrum of the sialic acid extracts from cattle and goat submandibular mucin is almost identical with that of a solution of standard sialic acid in 0.2M NaCl, it would be better to carry out dialysis after ion exchange chromatography so that Na and Cl ions won't be present in the purified extract and interfere with results.

In addition to that, High performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) is the best method of chromatography for characterizing charged amino sugars, it should be carried out of sialic acid extracts from cattle and goat so one can tell the ratio of NeuAc to NeuGC.

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APPENDIX I

Concentration of free and total sialic acid after incubation of goat and cattle submandibular mucins with phospholipase.

Time in minutes	FSC(mg/ml)	FSG(mg/ml)	TSC(mg/ml)	TSG(mg/ml)
30	0.026 ± 0.017	0.051 ± 0.037	0.199 ± 0.03	0.400 ± 0.012
60	0.053 ± 0.01	0.086 ± 0.02	0.277 ± 0.04	0.470 ± 0.05
90	0.075 ± 0.02	0.122 ± 0.021	0.338 ± 0.05	0.549 ± 0.03
120	0.082 ± 0.032	0.125 ± 0.039	0.338 ± 0.03	0.556 ± 0.041
150	0.080 ± 0.041	0.124 ± 0.027	0.341 ± 0.015	0.554 ± 0.04
180	0.080 ± 0.01	0.123 ± 0.025	0.339 ± 0.042	0.551 ± 0.029

Values are for three determinations ± SD

FSC = Concentration of free sialic acid from cattle submandibular mucin

FSG = Concentration of free sialic acid from goat submandibular mucin

TSC = Concentration of total sialic acid from cattle submandibular mucin

TSG = Concentration of total sialic acid from goat submandibular mucin

APPENDIX II

Concentration of free and total sialic acid after incubation of cattle and goat submandibular mucins with phospholipase and lipase.

Time in minutes	FSC(mg/ml)	FSG(mg/ml)	TSC(mg/ml)	TSG(mg/ml)
30	0.07095 ± 0.02	0.115 ± 0.023	0.316 ± 0.04	0.511 ± 0.03
60	0.0.068 ± 0.02	0.113 ± 0.04	0.324 ± 0.06	0.515 ± 0.02
90	0.075 ± 0.014	0.117 ± 0.05	0.331 ± 0.10	0.520 ± 0.04
120	0.076 ± 0.03	0.126 ± 0.03	0.326 ± 0.05	0.520 ± 0.02
150	0.081 ± 0.01	0.123 ± 0.011	0.331 ± 0.03	0.529 ± 0.03
180	0.081 ± 0.02	0.123 ± 0.01	0.342 ± 0.022	0.537 ± 0.033
210	0.084 ± 0.017	0.125 ± 0.35	0.341. ± 0.03	0.546 ± 0.02

Values are for three determinations ± SD

FSC = Concentration of free sialic acid from cattle submandibular mucin

FSG = Concentration of free sialic acid from goat submandibular mucin

TSC = Concentration of total sialic acid from cattle submandibular mucin

TSG = Concentration of total sialic acid from goat submandibular mucin

APPENDIX III

Evaluation of FTIR spectrum for sialic acid extract from submandibular mucin of cattle.

Peak number	**Absorption frequency(cm^{-1})**	**Functional group**	**Intensity**	**Assignment**
1	461.97	Alkyl halide	s	C-I stretch
2	1643.41	Alkene	vw-m	C=C stretch
3	2087.05	Allene, ketene	m-s	X=C=Y
4	2346.48	Alkane		C=H stretch/C=H deformation combination
5	3432.44	Carboxylic acids	s, broad	O-H stretch
		Heterocyclic 2^0	w	N-H stretch
		Amine	s	N-H stretch
		1^0 Amide		

Intensity abbreviations: vw = very weak, w = weak, m = medium, s = strong, vs = very strong

APPENDIX IV

Evaluation of FTIR spectrum for sialic acid extract from submandibular mucin of goat.

Peak number	Absorption frequency(cm^{-1})	Functional group	Intensity	Assignment
1	459.07	Alkyl halide	s	C-Cl stretch
2	1644.37	Alkene	vw-m	C=C stretch
3	2091.87	Allene, ketene	m-s	X=C=Y
4	2343.59	Alkane		C=H stretch/C=H deformation combination
5	3441.12	Carboxylic acids	s, broad	O-H stretch
		Heterocyclic 2^0	w	N-H stretch
		Amine	s	N-H stretch
		1^0 Amide		

Intensity abbreviations: vw = very weak, w = weak, m = medium, s = strong, vs = very strong

APPENDIX V

Evaluation of FTIR spectrum for standard sialic acid

Peak number	Absorption frequency(cm^{-1})	Functional group	Intensity	Assignment
1	593.13	Alkyl halide	s	C-X stretch
2	695.36	Aromatic	s	C-H stretch
3	920.08	Alkene	s	C=C stretch
4	1053.17	Alcohol, ether, esthers, Carboxylic acids or anhydrides	s	C=O
5	1404.22	Methyl group, alkane Nitro	s m m	C-H N=O
6	1609.65	1^0 and 2^0 amines and amides	m-s	N-H
7	1754.32	Acid anhydride Alkyl carbonate		
8	2199.89	Allene	m-s	X=C=Y
9	2639.67	Carboxylic acid	m	-OH
10	3393.86	Alcohol, amine	m-s	-OH
11	3546.25	Phenols, -OH stretch	broad, s	-OH -COOH

		Amine		-NH
		Amide	s	$-CONH_2$
12	3915.63			
13	4161.56			X-H

Intensity abbreviations: vw = very weak, w = weak, m = medium, s = strong, vs = very strong

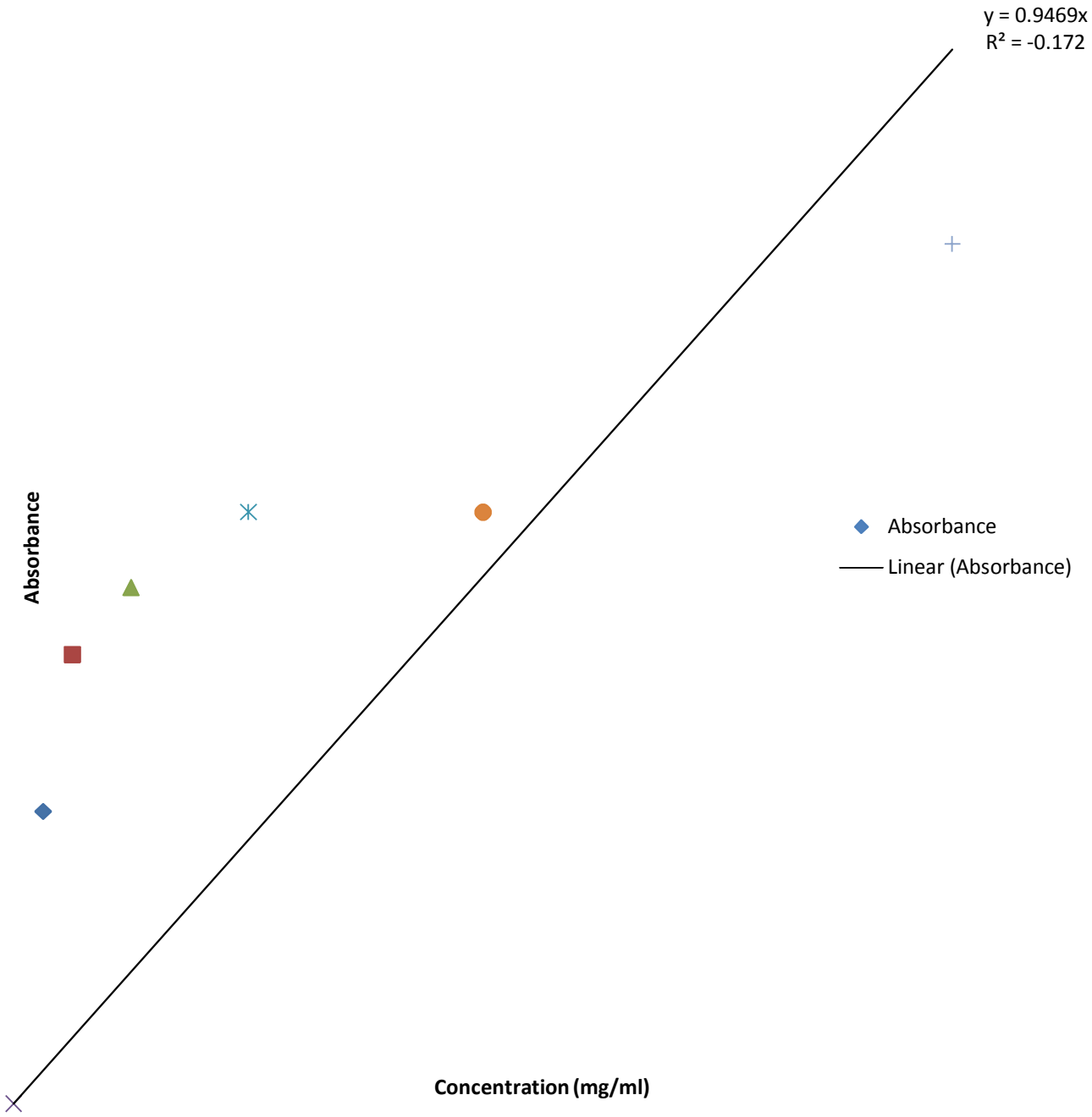
APPENDIX VI

Evaluation of FTIR spectrum for standard sialic acid in 0.2M NaCl.

Peak number	**Absorption frequency(cm^{-1})**	**Functional group**	**Intensity**	**Assignment**
1	462.93	Alkyl halide	s	C-X stretch
2	1122.61	Alcohol	m-s	C=O stretch
3	1643.41	Alkene	vw-m	C=C stretch
4	2090.91	Allene		X=C=Y combination
5	3434.37	Hydroxy group	broad,	Hydrogen bonded O-H
		1^0 Amide	s	stretch
		Carboxylic acid	s, broad	N-H stretch
		Heterocyclic 2^0	m	
		Amine		N-H stretch
6	3940.7			

Intensity abbreviations: vw = very weak, w = weak, m = medium, s = strong, vs = very strong

APPENDIX VII



Standard curve for sialic acid.