7-31-2013 12:00 AM

Identification of the Salivary Proteome in Children Throughout the Course of Dental Eruption

Nada Tabbara
The University of Western Ontario

Supervisor
Dr. Michael Rieder
The University of Western Ontario Joint Supervisor
Dr. Walter Siqueira
The University of Western Ontario

Graduate Program in Biochemistry
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

IDENTIFICATION OF THE SALIVARY PROTEOME IN CHILDREN THROUGHOUT THE COURSE OF DENTAL ERUPTION

(Thesis format: Monograph)

by

Nada Tabbara

Graduate Program in Biochemistry

A thesis submitted in partial fulfillment

of the requirements for the degree of

Master of Science

The School of Graduate and Postdoctoral Studies

The University of Western Ontario

London, Ontario, Canada

ABSTRACT

The salivary proteome is recognized as a valuable source of potential oral and systemic disease biomarkers. Major efforts in salivary research have been dedicated to identify and characterize salivary proteins present in saliva using both classical biochemical methods and proteomics approaches in adults. Despite considerable research on the salivary proteome, little attention has been given to the changes in the salivary proteome occurring in children, specifically from 0-3 years of age. Through the use of anionic PAGE, SDS PAGE, HPLC and MS/MS, salivary protein profiles in children before, during and after dental eruption were compared with edentulous adult controls. We identified substantive qualitative and quantitative differences in the salivary proteome between children and adults, suggesting a greater emphasis is warranted in the study of the changes in the salivary proteome as a function of age and dental status.

KEY WORDS

Saliva, dental eruption, edentulous adults, salivary proteins, proteomics, protein quantification, anionic PAGE, SDS-PAGE, HPLC, mass spectrometry.

When you sense a thirst for knowledge,

why not

perform mouthwatering research?

When you feel the answer on the tip of your tongue,

why not

start by looking there?

DEDICATION

For the reader, I hope you find all that you are looking for.

&

For Ma, Pa, S, and E, you are the very best.

ACKNOWLEDGMENTS

A great number of people have made my graduate school experience what it is and I am deeply grateful to all those who have supported me throughout my years of research at Western.

Thank you to my supervisors and advisory committee for teaching me through this project.

To my friends and family, near and far, thank you for being the best I could ever dream of. E, thank you for your uplifting love and support. I am very blessed to have you in my life.

To my colleagues, past and present, classmates, labmates, floormates and schoolmates, I am so happy to have met you, shared special moments and countless hours brushing shoulders during our many adventures and successes. Thank you for your unsurpassed companionship. Good luck friends, now and in the future!

Thank you to Dr. Gilles Lajoie, Cindy Xiao, Paula Pittock and Jamie Seabrook for sharing their knowledge.

To my first research supervisor, Dr. Gregory Dekaban, thank you for welcoming me as a first year undergraduate student onto your team. Thank you for introducing me to the research process I continue to explore.

To my fourth year honors project supervisors, Dr. John McCormick and Dr. Joaquín Madrenas, thank you for inviting me into your outstanding labs. Thank you for teaching, supporting, and inspiring me to continue in research.

To Dr. Michael Rieder, thank you for all of the years of mentorship throughout my undergraduate and graduate degrees. I have learned so much from you, as a scientist and a person, and am profoundly grateful for the experience you have shared. I am honored to be one of your ‘loyal troops’.

Thank you with all my heart.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

°	degrees
µg	microgram
µL	microliter
µm	micron
1D	one dimensional
2D	two dimensional
Å	Angstrom
AU	Absorbance Unit
AUC	Area under the curve
BCA	bicinchoninic acid
C	Celsius
CA	carbonic anhydrase
cm	centimeter
Da	Dalton
dd H2O	double distilled water
DTT	dithiothreitol
F	female
FA	formic acid
GCF	gingival crevicular fluid
h	hour
HAS	human serum albumin
HPLC	high performance liquid chromatography
kDa	kilo Dalton
M	male
MG1	mucin glycoprotein 1
MG2	mucin glycoprotein 2
min	minutes
mL	milliliter
mm	millimeters
mM	millimolar
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MW	molecular weight
NCBInr	NCBI non-redundant database
NCBI	National Center for Biotechnology Information
nL	nanoliter
nm	nanometer
No.	number
PAGE	polyacrylamide gel electrophoresis

pH	potential of hydrogen
PN	Part Number
PRP	proline-rich protein
rpm	rotations per minute
SDS	sodium dodecyl sulfate
SLPI	secretory protease inhibitor
SPSS	software package for statistical analysis
TFA	trifluroacetic acid
V	volts
VEGh	Von Ebner's gland protein
w/w	weight per weight
wks	weeks

Chapter 1 – Literature Review

1.1 Introduction to saliva

Saliva is indispensible in the maintenance of health and homeostasis in the body. The critical importance of saliva is strikingly evident in individuals with reduced salivary flow who experience: tooth decay/loss, acute irritation of oral mucosa, and severe difficulties with airflow, speaking, swallowing, food clearance and taste. Saliva's utility extends still further, far beyond the oral cavity, with the discovery of oral and systemic disease biomarkers in saliva. The use of salivary biomarkers as diagnostic tools must be preceded by a clear understanding of salivary biochemistry in different conditions and throughout the stages of life.

Saliva is defined as the mixture of fluid, organic, and inorganic components derived in large part from salivary gland secretions, the gingival fold, oral mucosa, desquamated epithelial cells, blood cells, food, and microorganisms (1-7). The complex composition of saliva reflects the dynamic equilibrium that exists between host, external forces and microbial flora present in the oral cavity (8). The large inter- and intra-individual variation present in the salivary biochemical composition throughout the life stages poses both a challenge to understanding saliva's biochemical properties, but also a tremendous opportunity to uncover stage-specific biological data potentially relevant in the clinical setting (9-12).

From a biochemical perspective, it is recognized that proteins in saliva represent a rich source of relevant biological information (6). Great effort has been dedicated to identifying the proteins present in adult saliva (4, 7, 13-25). Despite the magnitude of research devoted to salivary proteomic research, few have studied the salivary proteome in children (26-29)**.** In order to address this deficit and develop a deeper understanding of the salivary proteome at different life stages, this thesis is focused on the protein profile of saliva in children as their primary dentition erupts, from the ages of 0 to 3 years.

1.2 Overview of the salivary glands

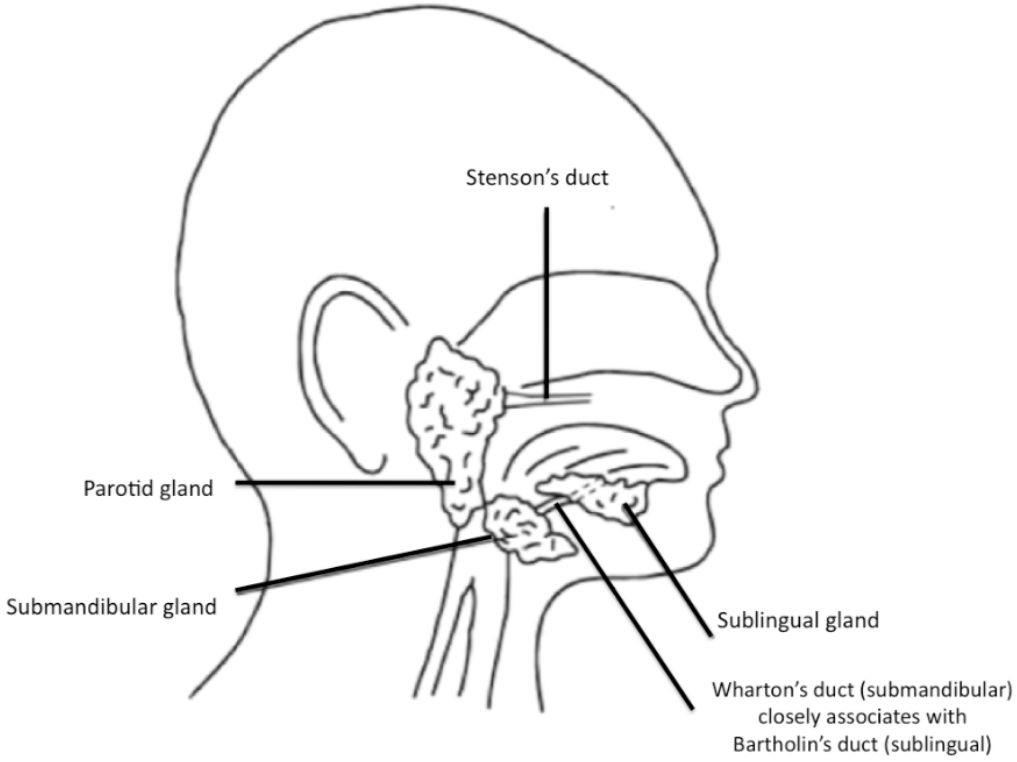
Salivary glands are responsible for producing and actively secreting protein-containing fluid into the oral cavity. These secretions represent an important contribution to whole saliva (30, 31). Salivary glands are classified into two categories: major and minor (30).

The major salivary glands contribute approximately 90% of the fluid present in whole saliva. The three paired major salivary glands are: the parotid, submandibular, and sublingual glands (30). The minor salivary glands supply approximately 10% of the fluid present in whole saliva. There are an estimated 400-600 minor salivary glands in the oral cavity. They are located in the mucosa of most of the soft tissue surfaces in the mouth, including the cheeks, lips, palate and tongue (lingual/Von Ebner glands) (6, 31, 32).

1.2.1 Salivary gland anatomy

The parotid gland, the largest of the major salivary glands, is located near the mandibular ramus and provides mainly serous fluids through the Stenson's duct that opens into the oral environment near the second upper molars. The submandibular gland is located near the lower jaw bone and provides mostly serous fluids through the Wharton's duct that opens to the oral environment near the junction of the tongue and the floor of the mouth. The sublingual gland is located near the submandibular gland and provides mainly mucous fluids through the Bartholin's duct that opens to the oral environment near the junction of the tongue and the floor of the mouth in close association with the Wharton's duct. A schematic illustrating the anatomy of the major salivary glands and the associated ducts is found in Figure 1.1.

Figure 1.1 – Anatomy of the major salivary gland and associated ducts. This schematic depicts the location and form of the major salivary glands (parotid, submandibular and sublingual) and the corresponding ducts (Stenson's, Wharton's, and Bartholin's) through which glandular saliva is introduced into the oral cavity.


Stenson's duct
Parotid gland
Submandibular gland
Sublingual gland
Wharton's duct (submandibular)
closely associates with
Bartholin's duct (sublingual)

1.2.2 Salivary gland histology

Salivary glands contain two types of epithelial cells: Ductal and Acinar. Ductal cells are primarily involved in establishing the ionic composition of the glandular secretions. Ductal cells alter the electrolyte content of the fluid originating from the acinar cells primarily by reabsorbing sodium chloride (33, 34). Ductal cells also secrete proteins, although far fewer than acinar cells (34). Acinar cells make up the acini, the secretory endpiece of the salivary gland ductal trees. Acinar cells function by synthesizing and secreting the majority of the functionally significant host-derived salivary proteins, as well as actively transporting water and electrolytes (31).

Acinar cells are classified into two categories: Serous and mucous. The type of secretion produced by a gland is determined by the ratio of serous to mucous acinar cells (6, 31). Serous acinar cells secrete a proteinaceous, watery fluid, largely lacking mucus. They are present in the parotid, submandibular, palatal, and lingual glands. Mucous acinar cells secrete a mucous-rich substance, with high viscosity and elasticity. They are present in the submandibular, sublingual, labial, palatal, and lingual glands (6).

1.3 Regulation of salivary secretion

Health and homeostasis of the oral environment depends greatly on the presence of saliva and its protein composition. In order to maintain this vital role, salivary flow is under the control of both the parasympathetic and sympathetic branches of the autonomic nervous system (31). Both branches positively regulate flow from salivary glands (35). The type of stimulation determines the ratio of activation between

the two branches of autonomic control. In healthy individuals, resting or 'unstimulated' salivary flow rate is approximately 0.4 mL/min with a standard deviation of 0.21 mL/min. In contrast, 'stimulated' salivary flow rate is approximately 1.6 mL/min with a standard deviation of 2.1 mL/min. It is important to note the high standard deviations in both the 'unstimulated' and 'stimulated' saliva, as this reflects the wide range of normal healthy values in salivary secretion (3).

Parasympathetic control of salivary secretion

Parasympathetic innervation is responsible for initiating salivary secretion and maintaining high secretion rates (35). Cholinergic parasympathetic nerves innervate all salivary glands. Parasympathetic stimulation of muscarinic cholinergic and alpha-adrenergic receptors on the parotid salivary glands leads to a high flow rate of a fluid containing low protein and high ion concentrations. Parasympathetic stimulation can be sustained over long periods and accounts for the majority of salivation control. Baseline amounts of salivary fluid and protein secretion are maintained at a 'resting' or 'unstimulated' rate in the absence of appropriate stimuli. (31).

Sympathetic control of salivary secretion

Sympathetic nerves are unable to initiate or maintain secretions independently. Rather, these nerves potentiate parasympathetic effects through the release of noradrenaline targeted at stimulating alpha- and beta-adrenergic receptors on acinar and ductal cells to induce the release of stored proteins (31, 35). The presence of stimuli (i.e. mastication) leads to a drastic increase in salivary flow, up to ten fold,

signaled by sympathetic stimulation. Sympathetic stimulation is short-lived, releasing large amounts of digestive enzymes and macromolecules such as mucin, which help lubricate and protect soft tissues in the oral cavity. Sympathetic stimulation of submandibular and sublingual glands results in a low flow rate of fluid containing a high protein concentration (34).

Salivary protein secretion

Protein secretion is initiated by the binding of neurotransmitters to the appropriate receptors on the basolateral membrane of acini secretory cells. The chief neurotransmitter released by parasympathetic nerves is acetylcholine. Noradrenaline is the primary neurotransmitter released by sympathetic nerves. Once bound, the neurotransmitters signal the induction of downstream intracellular mechanisms, beginning with second messengers and terminating in the release of salivary proteins out of the acinar secretory vesicles and into the acinar lumen. The majority of salivary gland protein excretion results from the exocytosis of protein storage granules in acinar cells (31). The type of stimuli, including environmental and physiological factors, such as: chemical or mechanical stimulation, psychological stress, pathological conditions and pharmacological stimuli, alters the protein profile of the secretions (36). It is for this reason that great attention is given to the types of stimuli enlisted for the collection of saliva samples destined for analysis. In this study, to account for the variation in the protein profile of the secretions caused by the factors listed above, samples were carefully collected from individauls in the absense of chemical or mechanical stimulation (unstimulated whole saliva). All individuals were maintained in a restful state before and

during collection. Individuals must have met the inclusion criteria of being in a state of good health and free of medications. These careful considerations helped to enable appropriate comparisons between different age groups.

1.4 Composition of whole saliva

Since the beginning of salivary research in the early 20th century, saliva has been recognized as a complex fluid. It has been stated that saliva is more appropriately regarded as a fluid tissue than a solution (37).

Whole saliva originates from major (parotid, submandibular, sublingual) and minor salivary gland secretions, as well as serum filtrate components (gingival crevicular fluid), host cells derived from tissues throughout the oral cavity (desquamated cells from the oral epithelium), microorganisms and microbial products (2, 5), blood and serum products from wounds, nasal and bronchial secretions (19, 38-40), and food debris (30). Plasma proteins are also present in saliva and are introduced through several avenues. Passive diffusion, ultrafiltration (evident at tight cellular junctions) and the contributions of serum transudate originating at the gingival sulcus referred to as gingival crevicular fluid (GCF), are the most common modes of entry for plasma proteins into saliva.

Water constitutes approximately 99.5% of the total volume of whole saliva (6, 7). Proteins account for an estimated 0.3% of the total volume of whole saliva (6). Inorganic and trace substances (i.e. electrolytes, sugars, lipids, hormones and nitrogenous products) comprise the remaining 0.2% of whole saliva's total volume. Inorganic and

trace substances, while relatively low in concentrations, are critical to saliva's function in the maintenance of oral health. Unstimulated saliva has an average pH range of 5.7-7.1, while stimulated saliva is known to have a pH of up to 7.8 (3).

Electrolytes present in saliva include: sodium, potassium, calcium, chloride, magnesium and bicarbonate. These common electrolytes are present in final concentrations that deem saliva hypotonic to other body fluids. Nitrogenous products, such as urea and ammonia, are also present in saliva (1, 6, 7, 41).

Saliva contains an estimated 700 species of microorganisms (42). Microorganisms contribute an assortment of enzymes to saliva's composition (43). Proteolytic enzymes, from multiple sources (i.e. bacterial, burst leucocytes), cleave a great number of salivary proteins into peptides. The extensive proteolysis and deglycosylation evident in saliva is another important consideration for salivary research. Some proteins may be granted protection from proteolytic cleavage if bound to hydroxyapatite, the primary constituent of tooth enamel. Histatin 1 is a good example of a protein that avoids proteolytic cleavage by binding to the tooth surface (44).

Host-derived proteins present in saliva are commonly divided into structurally related groups, referred to as salivary protein families. The major salivary protein families are described in the next section.

1.5 Salivary protein families

The eight major salivary protein families are: Amylases, Histatins, Mucins, Statherins, Cystatins, Carbonic Anhydrases, Peroxidases, and Proline-rich proteins.

Table 1.1 summarizes the primary protein source, chief known function, molecular weight, modifications/isoforms if present, and concentration measured in whole saliva. It is important to note that concentrations of salivary proteins vary, and the numbers provided are mean values or ranges measured in resting whole saliva in adults.

Table 1.1 - Summary of Protein Families found in Saliva. The source, function, molecular weight, modification/isoforms and concentration in whole saliva for the eight major protein families (Amylase, Histatins, Mucins, Statherins, Cystatins, Carbonic Anhydrases, Peroxidases, and Proline-rich Proteins) are highlighted in this table.

Protein	Source	Function	MW	Modifications/Isoforms	Conc. in whole saliva
Amylase (17, 22, 45-47)	Chiefly the parotid gland	Digestion – Hydrolyzes starches (i.e. amylose, amylopectin, maltose, glucose) by catalyzing hydrolysis of alpha 1-4 glycosidic linkages in starch. Protection – Selective binding of oral microorganisms (i.e. *S. gordonii, S. minits, S. oralis*), preventing bacterial attachment and aiding bacterial clearance.	53-57 kDa	Glycosylated and unglycosylated isoforms present	380-500 µg/mL
Histatins (17, 48-50)	Major salivary glands (parotid, submandibular and sublingual) and minor salivary glands (sublingual)	Short distinct functional domains determine histains' biological functions. Anti-fungal: Kills *C. albicans* (in both yeast and mycelial form) through the proposed mechanism of taking on a helical structure that disturbs cell membranes. Histatins are also potent *C. albicans* growth inhibitors Anti-bacterial: Inhibits the trypsin-like activity of *P. gingivalis* (gram negative bacteria associated with forms of periodontal disease). Does not inhibit host trypsin or chymotrypsin activity. Inhibits bacterial-induced hemagglutination, and bacterial colonization. Histatins also bind hydroxyapatite, complex with metal ions, inhibit crystal growth of calcium phosphate salts and stimulate wound-closure Histatin 1 - protects tooth enamel and pellicle formation (typical of phosphorylated salivary proteins) Histain 5 – most potent candidacidal property amongst histatins	3-6 kDa	Histatins exist in 3 major isoforms (Histatin 1, 3, and 5) Histain 1, the only phosphorylated isoform, is phosphorylated on serine 2 Major forms of histatin undergo proteolytic cleavage to form minor forms of histatin	2-8 µg/mL
Mucins (1, 7, 17, 51-56)	Submandibular, sublingual glands Primarily sublingual and minor mucous glands	Provides salivary viscoelasticity and lubrication. Protection – physical barrier from bacterial protease activity, helps regulate bacterial and fungal colonization by selectively modulating adhesion of microorganisms to oral tissue surface, lubrication, preventing desiccation Concentrates anti-microbial salivary components to mucosal interface Helps form acquired enamel pellicle	120-1000 kDa (Glycosylaton account for 40-80% of mass)	Two structurally distinct species of mucins secreted by salivary glands – MG1 (oligomeric) and MG2 (monomeric) Glycosylation –high carbohydrate content largely on serine and threonine residues	10-500 µg/mL
Statherins (17, 55)	Produced by acinar cells	Inhibits crystal growth of calcium phosphate salts Inhibit the spontaneous precipitation of calcium phosphate salts from the supersaturated concentrations present in saliva. Binds bacteria Binds with high selectivity and great affinity to hydroxyapatite	5380 Da	Phosphoproteins rich in tyrosine, glutamine, and proline	2-12 µg/mL

Cystatins (17, 55)	Isolated from submandibular secretions	Bind hydroxyapatite (3 times weaker than statherin) Inhibit crystal growth of calcium phosphate salts (10 times weaker than statherin)	14 kDa	SN, S, and S1 isoforms. Exist in phosphorylated and unphosphorylated forms.	240-280 µg/mL
Carbonic Anhydrases (17, 57)	Submandibular and parotid glands	Protection – involved in salivary pH regulation Low salivary concentrations of CA-VI are associated with increased prevalence of caries	42 kDa	7 isozymes and several homologous carbonic anhydrase-related proteins Can be glycosylated	4.6 µg/mL
Peroxidases (58-61)	GCF and neutrophil granulocytes (Myeloperoxi-dase), Salivary glands (Lactoperoxi-dase)	Anti-bacterial action –Involved in the intracellular metabolism of H_2O_2 leading to production of hypothiocyanite (OSCN-) an even more effective bactericidal and fungicidal agent. Helps prevent decalcification of enamel caused by bacterial acid production resulting from carbohydrate fermentation.	78-280 kDa	2 major forms found in saliva - Myeloperoxidase and Lactoperoxidase	1-5 µg/mL
Proline-rich protieins (43, 55, 62)		Solubilize calcium phosphate to inhibit crystal growth. Remineralization – in the early and late acquired enamel pellicle through the binding of hydroxyapatite (acidic PRPs). Caries prevention – a subset of basic PRPs differs in individuals with resistance to caries formation.		PRPs are classified as acidic, basic and glycosylated	

1.6 Functions of saliva

Saliva plays important roles in the mouth and upper portion of the gastrointestinal tract that are critical for preserving health and homeostasis in the body. Saliva's functions, related to its fluid characteristics and composition, can be classified in the following 5 broad categories: Protection/maintenance of teeth and oral mucosa, digestion, swallowing/clearance, airflow/speech, and taste.

1.6.1 Protection and maintenance of teeth and oral mucosa

Protection through lubrication

Lubrication, defined as the ability of a substance to decrease friction between moving surfaces, is regarded as one of the most vital functions of saliva. Saliva lubricates the mucosa and helps protect against irritation (i.e. mechanical, thermal, and chemical) (7, 30, 31). The importance of appropriate lubrication is readily observed in the effects of abrasive wear of epithelial surfaces and the destruction of tooth tissue when sufficient lubrication is not present (6). Lubrication has been associated with several salivary proteins, including: mucins (53, 63), statherin (63, 64), amylase (65), proline-rich glycoproteins (3, 66), and acidic proline-rich proteins (63).

Protection through immunological defense

Saliva is the first line of oral immune defense (67). The immunological defense provided by saliva is of great importance, as the oral cavity serves as the entry point of a wide range of substances into the alimentary track. This first line of defense protects through anti-bacterial, anti-fungal and anti-viral functions. Anti-bacterial functions include specific (i.e. secretory immunoglobulin A) and non-specific mechanisms (i.e. lysozyme, lactoferrin, myeloperoxidase, cystatins, histatins, Von Ebner's gland protein (VEGh), secretory leukocyte protease inhibitor (SLPI), calprotectin, lactoperoxidase, chromogranin A). Anti-fungal and anti-viral properties are similarly achieved with a combination of specific (i.e. secretory immunoglobulin) and non-specific components (anti-fungal: histatins, chromogranin A; antiviral: cystatins, mucins, SLPI). Mechanical cleansing of bacteria and the dilution of detrius add to the immunological protective functions of saliva.

Protection through buffering

Buffering and acid neutralization from oral and gastric sources is also conferred by saliva's composition, namely: bicarbonate, phosphate, and the negatively charged residues in salivary proteins. The neutralization of acids produced by acidogenic microorganisms offers teeth protection by preventing enamel demineralization (30). The salivary peptide, sialin, is a good example of saliva's buffering activity. Sialin increases the pH on the tooth surface and releases ammonia and carbon dioxide after undergoing hydrolysis by bacterial ureases (3, 41). The carbonic acid-bicarbonate system serves as an excellent example of an important pH buffer in stimulated saliva and phosphate

buffer in unstimulated saliva (3). In saliva, hydronium and bicaronate ions combine to form carbonic acid. Carbonic anhydrase functions to regulate pH by facilitating the conversion of carbonic acid into water and carbon dioxide. Through the actions of bicarbonate ions working in concert with carbonic anhydrase, the pH is effectively increased through the net eilmation of hydronium and bicarbonate ions and production of water and carbon dioxide.

Maintenance of teeth and oral mucosa

Saliva occupies a vital role in maintaining the physical-chemical integrity of tooth enamel. In addition to lubrication of the hard and soft tissues and buffering capacity of saliva, the maintenance of the integrity of teeth and oral mucosa is achieved through saliva's ability to protect against demineralization (mucins, Ca^{2+}, phosphate), and recuperate mineral loss through remineralization (PRPs, statherin, Ca^{2+}, phosphate) (31).

The stability of tooth enamel's hydroxyapatite composition is controlled by salivary pH and concentrations of free calcium, phosphate and fluoride. The supersaturated calcium phosphate concentration, with respect to hydroxyapatite, leads to the formation and maintenance of the protective pellicle present on the enamel surface. Maintaining the equilibrium between calcium phosphate demineralization and remineraliztion is a critical function of salivary proteins (55).

1.6.2 Digestion

Saliva is also responsible for the initiation of starch and lipid digestion. Digestive enzymes, such as alpha-amylase, cleave starches into maltose, maltotriose, and dextrins, contributing to the digestive process (30).

1.6.3 Swallowing/Clearance

Saliva dilutes and mechanically cleanses non-adherent particles (i.e. bacteria, cellular, and food detritus), aiding in their clearance from the oral cavity. As an added benefit, the clearance of food detritus, in particular excess carbohydrates, results in a reduction of the availability of sugar for microorganism metabolism (7). Food bolus formation aiding swallowing is also helped by the presence of saliva (30). By extension, the clearing capabilities of saliva are not limited to the oral cavity, as it also aids esophageal clearance.

1.6.4 Airflow/Speech

The fluid properties of saliva are critical to facilitate airflow and enhance speech quality. The importance of this function is most evident in instances with reduced salivary flow.

1.6.5 Taste

By serving as a solvent, saliva also facilitates taste by aiding in dissolving taste compounds and enhancing interaction of food products with taste buds (30). While the salivary fluid in acini starts isotonic to plasma, as it travels through the duct network it

becomes hypotonic in glucose, sodium, chloride and urea concentrations (3, 34). This aids in the perception of dissolved substances by gustatory buds.

1.6.6 Conclusion to functions of saliva

With such vital functions, saliva is of monumental importance in the maintenance of teeth, oral mucosa and overall oral health (3). While the functions of a great number of proteins and peptides contained in saliva are not yet well understood, it is clear there is a great deal of redundancy in salivary composition for it to be able to accomplish these functions and uphold the integrity of the teeth and oral mucosa with the required ecological balance (3, 31).

1.7 Current Knowledge of the salivary proteome

Proteomic studies centered on identifying the salivary proteome have increased dramatically in the past decade. The investigation of proteins on a large scale draws upon an ever-improving toolbox of techniques (13, 24, 28, 68-77).

During the past decade, more than 3000 different proteins have been identified in saliva (15, 18, 24, 25, 75, 78, 79). In 2010, Loo and colleagues from the University of California-Los Angeles compiled recent data from multiple laboratories to form a list of 2290 proteins present in whole saliva (72). Most recently, the same laboratory identified 1,166 proteins as the core salivary proteome (80). This compiled data set was consulted during the analysis of proteins in this study. The ongoing fluctuation in the size of the salivary proteome catalogue is closely related to the challenges present in the analysis and categorization of salivary proteins. One of those challenges is the great number of structurally related proteins found in saliva. The abundance of structurally related

proteins is commonly shared with other proteomes, most notably in blood, and has important implications with the application of mass spectrometric-based methods for the identification of proteins. Protein identification with MS/MS relies on sequencing peptides unique to a protein. If a sequenced peptide is common between a number of proteins, the accurate identification of the parent protein becomes more difficult. To address this, a variety of proteomic tools are often enlisted to accurately distinguish unique protein species.

The critical stage of any study examining proteins in a complex mixture is protein separation. A wide variety of protein separation techniques have been applied to the study of salivary proteins. Gel electrophoresis and liquid chromatography are two of the most powerful protein separation techniques applied in salivary studies.

Both 1- and 2-dimensional PAGE (1D- and 2D-PAGE) are effective methods of protein separation and have been widely used to separate salivary proteins (4, 6, 14, 29, 81). Both methods provide visuals of profiles of protein mixtures that can be used to assess variability between samples, people, and groups. 2D-PAGE is a powerful technique of protein separation but harbors significant limitations that remain highly relevant in salivary protein research. 2D-PAGE is ill suited for the detection of small MW proteins, highly acidic or basic proteins, highly hydrophobic proteins, as well as proteins in low abundance (6). The presence of many small MW proteins and peptides, as well as a significant number of highly acidic, basic, hydrophobic and proteins present in very low concentrations in saliva, invites alternative methods of protein separation, such as liquid chromatography.

1.8 Purpose of thesis

With a great impetus to realize the tremendous translational potential of saliva as a diagnostic resource, the focus must now be concentrated on developing and understanding the basic biochemistry of saliva at all stages of life. The successful and meaningful analysis of salivary proteins necessitates optimized methods of collection, processing and storage conditions (82).

Until now, the majority of proteomic studies have focused on adult populations, with very little attention given to characterizing the salivary proteome in children. Clinically, it is known that children and adults differ in an array of biological parameters used to assess health status via monitoring and diagnostic tools. Saliva, as well as blood, have both been shown to harbour clinically relevant age-dependent differences. While the focus of this study is not centered around gender, race, or environtmentally-related differences, these are also factors that warrant continued attention as they may provide further valuable biological insights. It is imperative to understand the standard baseline of the salivary protein profile, and how it changes with age, among other factors, in order to establish appropriate comparisons between individuals at different states of health.

With this knowledge, the purpose of this thesis is centered on the following two central aims:

1. To establish and optimize techniques for the analysis of the salivary proteome in children, as well as adults. Establishing techniques and optimizing methods is a crucial step in the successful and meaningful analysis of salivary samples.

2. To identify qualitative and quantitative changes in salivary protein profiles as primary dental eruption events unfold in children from the ages of 0 to 3 years.

1.9 Hypothesis

We hypothesize that there are observable qualitative and quantitative changes in the salivary protein profile throughout the course of dental eruption, in children from age 0 to 3 years.

Chapter 2 – Changes in the salivary proteome during the course of dental eruption

2.1 Experimental Design Overview

2.1.1 Sample Collection

The eligibility of individuals to donate unstimulated whole saliva for this study was based on the following inclusion criteria. The individual must be deemed healthy according to their medical history, and therefore free of any acute or chronic medical conditions (i.e. Asthma, diabetes, renal or cardiac conditions). At the time of sampling, the individual must be included in one of the following four categories:

Children with no teeth – Absence of primary dentition
Children with 1-19 primary teeth – Partial primary dentition
Children with 20 primary teeth – Complete primary dentition
Adults with 0 permanent teeth – Complete denture patients

Samples were rejected from this study if any of the “Exclusion Criteria” in Table 2.2 were met. Individuals must not be taking any medication to avoid potential drug-related effects on salivary flow and/or composition. Collection of unstimulated whole saliva from individuals meeting the “Inclusion Criteria” listed in Table 2.2 was sampled in the described manner.

Table 2.1 - Summary of group categories. Groups A, B, and C represent children participants pre-, during, and post-dental eruption, respectively. The age ranges described in the table for Groups A, B, and C, serve only as guidelines. Samples from children were grouped according to the number of teeth present at the time of collection. Group D represents the edentulous adult controls.

	Group		*No. of Teeth*	*Description*
Children	A	Pre-dental eruption	0	Children approx. 0-6 months old*
	B	During dental eruption	1-19	Children approx. 7-24 months old*
	C	Post-dental eruption	20	Children approx. 25-36 months old*
Adults	D	No dentition (edentulous)	0	Adults <65 years of age with complete dentures

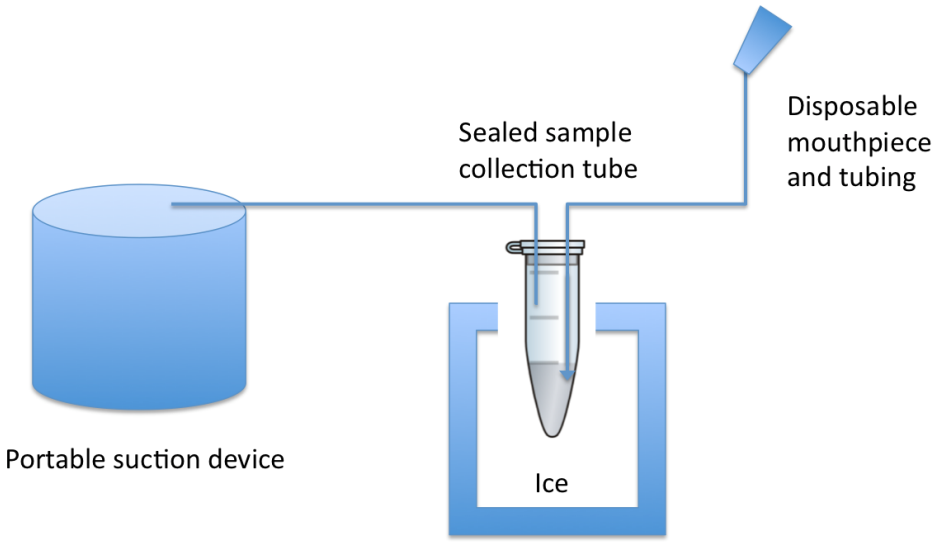
*Age categories serve only as guidelines indicating the stage of dental eruption. Samples were classified strictly based on the number of teeth full and partially erupted at time of collection.

Table 2.2 - Criteria used in the selection of individuals to donate saliva. All individuals included in this study were required to meet the inclusion criteria. Salivary samples were excluded from the analysis if any of the exclusion criteria were met.

Inclusion Criteria	Exclusion Criteria
Healthy – including their medical history	Presence of any chronic or acute medical conditions
Children approx. 36 months or younger	Consumption of any food or water within 1 hour prior to collection
OR	If individual became stressed during collection
Edentulous adults 65 years or younger	Any medications (as they may interfere with salivary secretion and composition)

All saliva samples were collected between the hours of 10:00 AM and 12:00 PM, to minimize any inter-individual variation of saliva composition associated with circadian rhythms (83). Unstimulated whole saliva was aspirated using a portable suction device and disposable mouthpiece attached to a 1.5 mL eppendorf snap cap tube, as depicted in the schematic in Figure 2.1. To prevent proteolytic degradation of salivary proteins, collection tubes remained on ice at all times during collection. To achieve an accurate assessment of resting, unstimulated whole saliva, great care was put forth during collection to ensure the individual was seated comfortably while collection took place. Sample was discarded if subject became stressed or if child began to cry. Upon completion of sample collection, all samples were stored at – 40°C.

Figure 2.1 - Schematic of suction device adapted for collection of unstimulated whole saliva samples. The portable suction device, shown on the left of the schematic, produced a gently powered suction that was connected to the eppendorf snap cap lid with a needle adaptor. A second needle adaptor attached to a disposable mouthpiece and tubing was inserted to the same eppendorf snap cap tube, as depicted in the figure. This set up resulted in a disposable mouthpiece terminal with gentle but effective suction, well suited to collect whole saliva from children and adults. All collection tubes remained on ice during the collection process.


Disposable
mouthpiece
and tubing
Sealed sample
collection tube
Portable suction device
Ice

2.1.2 Patient Demographics

Table 2.3- A summary of the unstimulated whole saliva samples collected for this study. The following patient demographic parameters from all four groups, Group A (Pre-dental eruption), Group B (During dental eruption), Group C (Post-dental eruption), and Group D (Adult controls) are summarized: Age, sex, volume of unstimulated whole saliva collected, and number of teeth present.

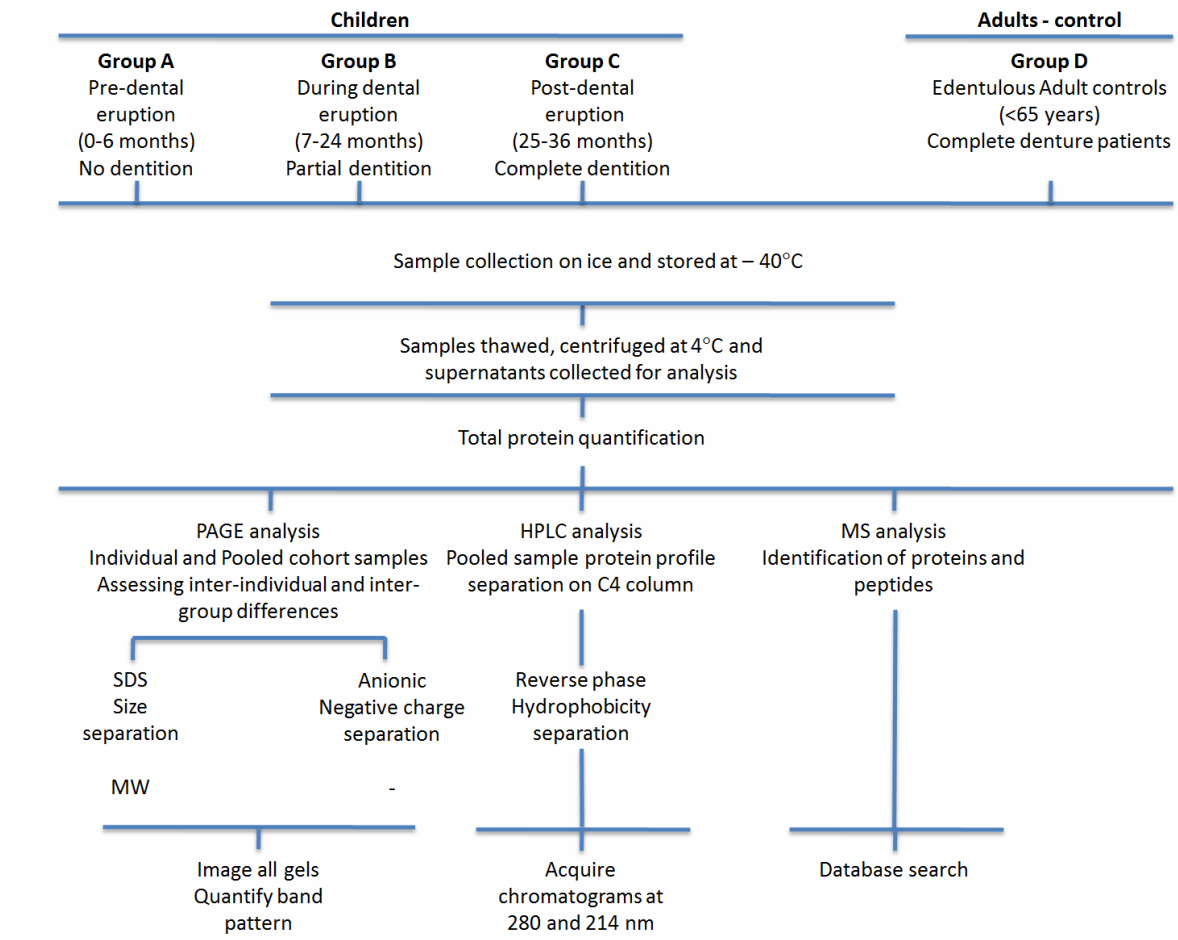
Group	Description	Sample ID	Age (months)	Sex	Volume collected (µL)	No. of Teeth visible
		A001	7	F	200	0
A	**Pre-dental eruption** (n = 12)	A002	2.75	M	200	0
		A003	0.75	M	50	0
		A004	5.75	M	500	0
		A005	6.5	M	800	0
		A006	6.75	F	500	0
		A007	6.5	M	400	0
		A008	2.75	M	500	0
		A009	4.25	F.	500	0
		A010	5.5	F	400	0
		A011	4.75	F	300	0
		A012	2.75	F	500	0
		B001	6.5	F	300	4
B	**During dental eruption** (n = 10)	B002	6.25	M	200	1
		B003	12.25	M	100	7
		B004	23	M	200	16
		B005	16	M	400	2
		B006	15.25	F	500	6
		B007	19.25	M	500	14
		B008	9.75	F	600	6
		B009	19.75	M	100	16
		B010	14	M	100	8
		C001	35.5	F	200	20
C	**Post-dental eruption** (n = 12)	C002	39.25	F	300	20
		C003	42	M	600	20
		C004	23.5	F	150	20
		C005	41.75	M	200	20
		C006	38	M	100	20
		C007	31.5	M	500	20
		C008	37.75	M	500	20
		C009	29	M	400	20
		C010	31	F	500	20
		C011	31.75	M	500	20
		C012	29	M	500	20
			Age (years)			
		D004	58.833	F	2000	0
D	**Adult controls** (n = 10)	D005	44.5	F	2500	0
		D006	47.083	F	1000	0
		D008	49.75	M	2200	0
		D009	61.75	F	200	0
		D010	37.58	F	3000	0
		D011	35	M	500	0
		D012	55.083	M	200	0
		D013	52.33	M	200	0
		D014	56	M	1000	0

2.1.3 Project Experimental Design

The goal of this study was to examine and compare the salivary proteome in children during the course of dental eruption in children and edentulous adult controls. Hence, children were divided into three cohorts (pre-, during, and post-dental eruption) and adult controls grouped into a fourth cohort. Samples were analysed individually, as well as pooled in each cohort, to assess both inter-individual and inter-group differences. The overall design of the experimental approach used in the analysis is shown in Figure 2.2. The scheme describes the analysis flow for all samples.

Whole saliva samples were centrifuged and the supernatants collected. The supernatants were then quantified for total protein concentration. The supernatants were then used to complete the three major avenues of analysis seen in Figure 2.2, PAGE, HPLC and MS. Separation of proteins based on molecular weight, negative and positive charge was achieved using PAGE. The minimal protein requirement for PAGE analysis permitted the analysis of pooled cohort samples in both gel types (SDS and anionic) and individual samples to be examined with both SDS and anionic PAGE. Separation as a function of the degree of protein hydrophobicity was realized through the use of reverse phase HPLC. Protein identification was attained through mass spectrometric analysis. Processing and analysis of all whole saliva samples used in this study was completed according to the following methodologies.

Figure 2.2 - The overall experimental design for the analysis of unstimulated whole saliva samples. The first stage of the experimental design involved recruiting children pre-, during, and post-dental eruption (Groups A, B, and C, respectively), as well as edentulous adult controls (Group D) to participate in the study. Saliva sample collection was followed by total protein quantification. The samples were then analyzed first with PAGE, followed by HPLC, and then LC-MS, as described in the methods.


Children
Adults - control
Group A
Pre-dental eruption
(0-6 months)
No dentition
Group B
During dental eruption
(7-24 months)
Partial dentition
Group C
Post-dental eruption
(25-36 months)
Complete dentition
Group D
Edentulous Adult controls
(<65 years)
Complete denture patients
Sample collection on ice and stored at – 40°C
Samples thawed, centrifuged at 4°C and supernatants collected for analysis
Total protein quantification
PAGE analysis
Individual and Pooled cohort samples
Assessing inter-individual and inter-group differences
HPLC analysis
Pooled sample protein profile separation on C4 column
MS analysis
Identification of proteins and peptides
SDS
Size separation
Anionic
Negative charge separation
Reverse phase
Hydrophobicity separation
MW
-
Image all gels
Quantify band pattern
Acquire chromatograms at 280 and 214 nm
Database search

2.2 Materials and method

Due to the mixture of components present in whole saliva, in particular proteolytic enzymes and mucins, precautions are taken and all procedures adhered to with respect to sample collection, processing prior to analysis, sample storage and treatment, to ensure the successful preservation and analysis of all samples.

2.2.1 Unstimulated whole saliva collection

All samples were collected from study participants with full consent and approval (See APPENDIX 2 for General Letter of Information and Consent). The collection protocol was approved by the Health Sciences Research Ethics Board (HSREB) at Western. Parents or Legal Guardians were consulted for sample collection from child participants. All sampling was conducted between a two-hour period (10:00 AM and 12:00 PM) to minimize inter-individual variation of saliva composition associated with circadian rhythms (83). To ensure true resting and unstimulated saliva samples, participants did not consume any food or water for a full hour prior to collection. All participants were relaxed and seated upright throughout collection. At the first signs of distress, most relevant for the youngest volunteers, collection was stopped and samples discarded. This procedure ensured all samples reflected resting unstimulated whole saliva. Samples were collected with a sterile disposable mouthpiece connected to a 1.5 mL polypropylene microtube. A portable suction device was used to create a gentle suction in the tube to facilitate saliva collection. See Figure 2.1 for an illustration of the set-up successfully adapted for the collection of unstimulated whole saliva. Throughout

sampling, collection tubes were stored on ice to inhibit proteolytic degradation of salivary proteins outside the oral cavity.

2.2.2 Pre-analysis sample processing

Immediately after collection saliva samples were centrifuged at 14 000 rpm for 20 min at 4°C. The supernatants were gently aspirated with a 200 µL pipette to avoid disturbing the pellet. The supernatants were aliquoted into microfuge tubes and frozen at -40°C. Prior to analysis, aliquots were thawed on ice.

2.2.3 Total protein quantification

The most suitable method for quantifying total protein concentration in whole saliva samples was determined by comparing the Bradford Protein Assay Kit (Thermo Scientific, Rockford, IL) and the Pierce BCA Protein Assay kit (Thermo Scientific, Rockford, IL) for sensitivity and reproducibility. The Pierce BCA Protein Assay proved to be the most appropriate and was used to analyze all saliva samples.

2.2.4 Protein separation via polyacrylamide gel electrophoresis

Polyacrylamide gels were used in two modes (SDS and Anionic native) to separate proteins based on size and negative charge, respectively. Each sample was run individually to assess inter-individual variability in protein pattern. In addition, samples were pooled and run according to their group to identify inter-group variability. Polyacrylamide gels (8.3 X 7.3 cm X 0.1 cm, 10% acrylamide) were cast in a MiniPROTEAN® IV system (Bio-Rad, Hercules, California). The gels were pre-run at 30 V for 30 minutes immediately prior to sample loading. SDS gels were loaded with 20 µg

of total protein, while Anionic gels required 100 µg of total protein to allow for sufficient protein separation and band visualization. The Anionic gels required a larger quantity of total protein to be loaded because this gel type separates only negatively charged species, unlike SDS gels which are able to separate proteins regardless of their charge. Negatively charged proteins constitute a fraction of the total proteins present in saliva, thus requiring more total protein loading in Anionic gels to provide sufficient quantities for visualization. Samples run on SDS-PAGE were placed in boiling water for 5 min and allowed to cool to room temperature prior to loading. While SDS is an effective denaturing agent, heating the samples further enhances the action of SDS by further disrupting protein structure. The gels were stacked and run at a constant voltage of 100 V in a MiniPROTEAN® Tetra Cell (Bio-Rad, Hercules, California) for 1.5-2 h, or until the dye front had migrated to approximately 0.5 cm from the bottom edge of the gel.

2.2.5 Gel imaging, image analysis and band quantification

Coomassie Brilliant Blue and silver staining were tested for their suitability as a staining option for both SDS and Anionic PAGE gels. Coomassie and silver have detection limits of approximately 100 ng, and 1 ng, respectively (84). While silver staining has a much lower limit of detection, the narrow linear dynamic range, relative to Coomassie makes this staining method less suitable for quantification (84-86). Due to the importance of quantifying the protein profile in the gels, Coomassie was selected for use in this study over silver staining. All gels were stained with the colorimetric stain Coomassie Brilliant Blue (Thermo Scientific, Rockland, Illinois). To minimize background staining, destaining was achieved by incubating gels for 2 hours with destaining solution (40% Methanol, 10% Acetic Acid in milliQ dd H_2O) under gentle

agitation, followed by 3 washes with milliQ ddH_2O. Gel image acquisition was achieved using an Epson Perfection 4990 Photo scanner at the Best Scanning Quality, with 24-bit colour and 400 dpi. The scanned gels were analysed using TotalLab Quant v12.5. Band intensity was quantified and the gel-specific background subtracted to normalize intensity data.

2.2.6 Protein separation via high performance liquid chromatography

A reversed phase C4 column was chosen (XBridge™ BEH300 C4 3.5 µm, 4.6 mm X 150 mm, Waters) to separate the complex mixture of whole proteins based on degree of hydrophobicity. To equally represent every individual from each group, equivalent amounts of protein were pooled for each run. Pooled protein samples containing 100 µg of total protein from each group were diluted with 0.1% TFA to reach a total volume of 1 mL. Pooled samples were syringe filter sterilized using a 0.2 µm Supor® Membrane (PN 4602, Pall Corporation, Ville St. Laurent Quebec) with a 1mL syringe (Reference number: 329650, Franklin Lakes, New Jersey). Immediately after filtration, samples were injected onto the equilibrated C4 column following a full blank method run to confirm the absence of carryover between samples. The method was initialized with 100% Buffer A (0.1% TFA in milliQ dd H_2O) and gradually increased Buffer B (ACN + 0.1% TFA in milliQ dd H_2O) concentrations until a final concentration of 55% acetonitrile was reached with a 110 min method. A dual UV/Visible detector (Model: 2489, Waters) allowed for the simultaneous measurement of absorbance at 214 nm and 280 nm to visualize both peptide and protein profile patterns across all groups.

2.2.7 Sample preparation for mass spectrometric analysis

Due to the vast range of total protein concentrations determined in the samples, utilizing a protocol with a standardized volume would have resulted in a drastic over-representation of samples with a high protein concentration, and an under-representation of samples with a low protein concentration. Therefore, standardized weight was selected, rather than volume of saliva supernatant, to provide each individual with equal protein representation in the MS analysis. A total of 5 µg of total protein from each individual was combined in each pooled group sample. Low-protein binding 0.5 ml polypropylene microtube and low-protein binding tips were enlisted in every step of MS sample preparation to minimize protein loss prior to analysis. Pooled samples were aliquoted and immediately processed, or frozen at – 40°C. Aliquots containing 15 µg of pooled total protein from each group were prepared as follows for LC MS/MS analysis. The final volume of each tube was brought to 50 µL with 4 M Urea, 10 mM DTT, 50 mM NH_4HCO_3, pH 8.0) and incubated for 1 hour at room temperature (~25°C) to denature and reduce samples. Samples were then diluted 4-fold with 50 mM NH_4HCO_3, pH 8.0. Mass Spectrometry grade Trypsin was added 5% w/w (Promega) followed by incubation at 37°C for 16 h to complete proteolytic digestion. Samples were dried with an Eppendorf Vacufuge™.

2.2.8 Mass spectrometric analysis

Prior to analysis, samples were cleaned with a C18-ZipTip (Millipore, Watford, United Kingdom) to eliminate accumulated salts and denaturants (i.e. Urea) and optimize signal-to-noise ratio on spectra. Analyses were performed using liquid

chromatography with an Agilent 1100 Capillary LC system (Palo Alto, California) in-line with a linear ion trap quadrupole tandem mass spectrometer (Thermo Electron, San Jose, California). Digested proteins were separated with a C18 pre-column consisting of polyimide-coated fused silica capillary column (100 µm internal diameter X 5.0 cm length) (InnovaQuartz, Phoenix, Arizona) and a micro-liquid chromatography analytical column (75 µm X 10 cm) (Proxeon Biosystems, Odense, Denmark) with C18 resin (5 µm diameter bead, 200 Å pore) (Varian, Palo Alto, California) that also functioned as a microelectrospray emitter. The reverse phase chromatography was achieved with an 80 minute gradient elution from optima grade water to acetonitrile (Fisher Scientific, Ottawa, Ontario) each containing 0.1% formic acid (FA) (VWR, Mississauga, Ontario) and 0.2% protein sequencing grade trifluoroacetic acid (TFA) (Sigma-Aldrich, Oakville, Ontario) with an injection volume of 5 µL and a flow of 200 nL/min. The mass spectrometer was operated in data-dependent acquisition mode cycling automatically through acquisition of full-scan mass spectrum and three MS/MS spectra sequentially on the three most abundant ions present in the initial MS scan. All samples were run in duplicate with identical experimental parameters.

2.2.9 Searching the data base

All MS/MS samples were analyzed using Mascot (version 2.2, Matrix Science, London, United Kingdom) and X! Tandem (version 2007.01.01.1). The samples were searched against the NCBInr database assuming trypsin digestion. Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.05 Da and a parent ion tolerance of 3.0 Da. In Mascot, variable modifications were specified as follows: Oxidation of methionine, deamination of asparagine, deamination of unknown, acetylation and carbamylation of the n-terminus. No fixed modifications were specified.

Scaffold (version Scaffold_2_06_02, Proteome Software Inc., Portland, Oregon) was enlisted to validate MS/MS based peptide and protein identifications. Peptide identifications with a greater than 95.0% probability were accepted (87). Protein identifications with a greater than 95.0% probability, as well as a minimum of 1 identified peptide, were assigned by the Protein Prophet algorithm (88).

2.3 Results

2.3.1 Comparison of total protein quantification methods

Two protein quantification methods, Bradford and bicinchoninic acid (BCA) assay, were compared to determine the most suitable technique for total protein quantification of unstimulated whole saliva samples. Both methods are widely used to quantify protein concentrations in proteomic studies.

The BCA assay is similar to the Lowry method, with the added advantage of increased sensitivity. It relies on protein forming complexes with Cu^{2+} under alkaline conditions followed by the release of Cu^{+} from the reduction of the copper-protein complexes, leading to a colorimetric change. The amount of protein present determines the amount of reduction and resulting quantifiable colour modification. This method measures the amount of cysteine, cystine**,** tryptophan, tyrosine and peptide bonds, all of which are capable of reducing Cu^{2+} to Cu^{+}. When measured at 592 nm, the linear working range of the BCA assay reaches 2,000 μg/ml, well above the average total protein concentration of whole saliva of approximately 1,000 μg/ml.

The Bradford assay has the advantage of working very quickly and requiring small amount of sample. Minimizing the amount of limited biological sample required is of great importance. However, the assay sensitivity is poor, relative to the BCA assay. The Bradford method uses Coomassie Brilliant Blue dye to bind protein through hydrophobic and ionic interactions. The anionic form of the dye is stabilized through this protein binding, resulting in a colour change detected at 595 nm. The linear working range of the Bradford assay is limited to at or below 1,000 μg/ml.

The most suitable protein quantification assays are those in which the properties being measured are equally distributed within all samples. The BCA assay's detection of peptides bonds and four amino acids in salivary samples is much more representative of the total protein concentration than the less uniformly distributed hydrophobic and ionic interactions detected by the Bradford method.

Ultimately, due to the requirement of a larger linear working range, heightened sensitivity, and a reliable total protein concentration measurement for each sample, the BCA assay was selected, a stable, reliable, reproducible method, appropriate for protein concentrations from 20-2,000 μg/ml. Bovine serum albumin, a common protein standard used in salivary proteomic studies, was selected as protein standard for the total protein quantification assays.

2.3.2 Total protein quantification

All saliva samples collected from 44 individuals from 4 groups (Group A: Children pre-dental eruption (no teeth), Group B: Children during dental eruption (partial primary dentition), Group C: Children post-dental eruption (complete primary dentition), Group D: Adults edentulous (complete denture patients)) were subject to total protein concentration quantification using the BCA assay. The results are summarized in Table 2.4.

Table 2.4 Summary of total protein concentration in collected saliva samples from all four groups, Group A (pre-dental eruption), B (during dental eruption), C (post-dental eruption), and D (adult controls) as measured with the BCA assay. The individuals ranged in age from 2 wks – 61.75 years of age.

Clinical Parameters with Total Protein Concentration

GROUP A –Children –pre-dental eruption

	Age (# of months)	Sex	Protein concentration [µg/mL]	Teeth erupted (#)
A001	7.00	F	**508.64**	0
A002	2.75	M	**585.00**	0
A003	0.75	M	**1205.63**	0
A004	5.75	M	**886.18**	0
A005	6.50	M	**235.45**	0
A006	6.75	F	**379.27**	0
A007	6.50	M	**506.36**	0
A008	2.75	M	**1679.27**	0
A009	4.25	F.	**745.81**	0
A010	5.50	F	**522.00**	0
A011	4.75	F	**854.18**	0
A012	2.75	F	**284.73**	0

GROUP B –Children –during dental eruption (Partial Primary Dentition)

	Age (# of months)	Sex	Protein concentration [µg/mL]	Teeth erupted (#)
B001	6.50	F	**580.27**	4
B002	6.25	M	**346.73**	1
B003	12.25	M	**972.82**	7
B004	23.00	M	**704.55**	16
B005	16.00	M	**448.55**	2
B006	15.25	F	**3207.27**	6
B007	19.25	M	**967.27**	14
B008	9.75	F	**463.09**	6
B009	19.75	M	**689.64**	16
B010	14.00	M	**833.45**	8

GROUP C –Children – post dental eruption (Complete Primary Dentition)

	Age (# of months)	Sex	Protein concentration [µg/mL]	Teeth erupted (#)
C001	35.50	F	**1084.18**	20
C002	39.25	F	**821.09**	20
C003	42.00	M	**957.18**	20
C004	23.50	F	**797.64**	20
C005	41.75	M	**1362.73**	20
C006	38.00	M	**932.49**	20
C007	31.50	M	**751.55**	20
C008	37.75	M	**1280.36**	20
C009	29.00	M	**1484.18**	20
C010	31.00	F	**1003.09**	20
C011	31.75	M	**495.64**	20
C012	29.00	M	**1260.36**	20

GROUP D –Adults – edentulous (Complete Denture Patients)

		Age (# yrs)	Sex	Protein concentration [µg/mL]	Teeth present (#)
D004	(1)	58.83	F	**1421.94**	0
D005	(2)	44.50	F	**826.90**	0
D006	(3)	47.08	F	**1583.16**	0
D008	(4)	49.75	M	**852.26**	0
D009	(5)	61.75	F	**848.64**	0
D010	(6)	37.58	F	**969.09**	0
D011	(7)	35.00	M	**2074.05**	0
D012	(8)	55.08	M	**1393.87**	0
D013	(9)	52.33	M	**5398.86**	0
D014	(10)	56.00	M	**1181.09**	0

2.3.3 Statistical analyses of clinical data

In order to identify any significant trends or differences in the clinical data, a statistical analysis was enlisted. The clinical parameters of age, sex, number of teeth present, and protein concentration in collected saliva samples were subjected to analysis. The nonparametric nature of the collected data was examined with the Kruskal-Wallis, the Mann-Whitney U Test, and Spearman's analysis. SPSS statistics was the software chosen to complete the statistical analysis of the clinical data. Statisticians regard SPSS as the gold standard software for statistical analyses.

The Kruskal-Wallis hypothesis test is a nonparametric one-way analysis of variance. It is useful in the comparison of more than two independent samples. A significant result from a Kruskal-Wallis test states that a minimum of one of the samples is different from the other samples. The Kruskal-Wallis hypothesis test determined that the distribution of the protein concentration was the same across all four groups. The Kruskal-Wallis test also determined that the distribution of the volume of saliva collected was the same across all four groups.

The second nonparametric analysis enlisted was the Mann-Whitney U hypothesis test. This test analyzes for significant differences between specific sample pairs. The Mann-Whitney U hypothesis test works from a null hypothesis describing two groups as the same opposed to an alternative hypothesis.

In accordance with the Kruskal-Wallis test, The Mann-Whitney U hypothesis test also determined that the distribution of the volume of saliva collected was the same

across all four groups. Both the Kruskal-Wallis test and the Mann-Whitney U test level of significance were set at 0.05.

Spearman's analysis, often referred to as Spearman's rho, is the third nonparametric test applied to this clinical data set. This analysis measures two variables' statistical dependence upon each other.

Spearman's rho determined there was a significant correlation between age and protein concentration in the collected samples, with a correlation coefficient of 0.48 (n=44) at the level of 0.01. A significant correlation was still seen when the males and females were tested separately for correlations between age and protein concentration. Males (n=26) and Females (n=18) had correlation coefficients of 0.635 at the level of 0.01 and 0.42 at the level of 0.05, respectively. Spearman's analysis determined no significant correlation between volume of saliva and protein concentration, volume of saliva and age, and finally, protein concentration and teeth erupted.

Appendix 3 includes the tabulated results of the clinical data and statistical analyses of the Kruskal-Wallis hypothesis test, the Mann-Whitney U hypothesis test, and the Spearman's analysis.

Making use of the measured total protein concentrations in this study, a future sample size calculation was completed to inform the determination of sample sizes in future studies. The calculated mean values of the youngest (Group A) and oldest children (Group C), and their associated standard deviations were used, as they represented the pre- and post-dental eruption populations, a primary focus of this research. With clinical consultation determined a clinically relevant difference limit of

200 µg/mL of total protein concentration between groups. A power of 0.80, and an alpha of 0.05 were the standard values assigned to this calculation, representing an 80% chance of detecting a clinically relevant difference when one is present, and a 5% chance of detecting a clinically relevant difference when there is none. Using these parameters, a future sample size is recommended to be 48 per group (n = 48), bringing a study with four groups to a total study size of 192 (n = 192).

2.3.4 Protein separation via polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was used to separate proteins based on negative charge (Anionic-PAGE) and size (SDS-PAGE). Loading each lane of a gel with a sample from a single individual allowed the assessment of intra-group variability, a visual representation of differences between individuals of a single group. Inter-group variability, the differences between groups, was assessed by pooling samples from Group A and running the resultant pooled samples in a single lane alongside individual lanes of pooled samples from Group B, C and D.

Protein profiles were quantified first using Photoshop followed by TotalLab Quant v12.5. Photoshop allowed for pixel counts of individual bands, but was unable to take into account the intensity of the stained protein. Band density can vary even within the same gel. Therefore, using only a count of pixels and not intensity, large diffuse protein bands were often overestimated, while small densely packed bands were largely underestimated for the amount of protein they contain. In order to take band intensity into proper account, to achieve a truly accurate measurement of protein quantity in all cases, gels were re-analyzed and bands re-quantified using TotalLab Quant v12.5. With the ability to consider band intensity as well as band surface area, TotalLab Quant

v12.5 allowed for the quantification and comparison of protein profiles from all individual and group samples successfully.

2.3.5 Anionic PAGE

The following section displays the anionic PAGE comparing pooled protein from each of the four groups run alongside purified human serum albumin. The stacking gel was maintained atop the separating gel to confirm the large amount of protein that was unable to enter the separating gel to be separated and visualized in this manner. The resultant band intensity quantification as measured by TotalLab Quant v12.5 is also displayed in this section.

Figure 2.3 - Anionic-PAGE Inter-group variability. Each lane represents protein pooled from all the individuals in a single group (100 μg loaded in each lane). Gel was stained with Coomassie Brilliant Blue. Lane indicated with an 'X' was loaded with 4 μg of human salivary protein standard - human serum albumin (HSA). Lanes A, B, C, and D represent pooled samples from Groups A, B, C, and D, respectively.

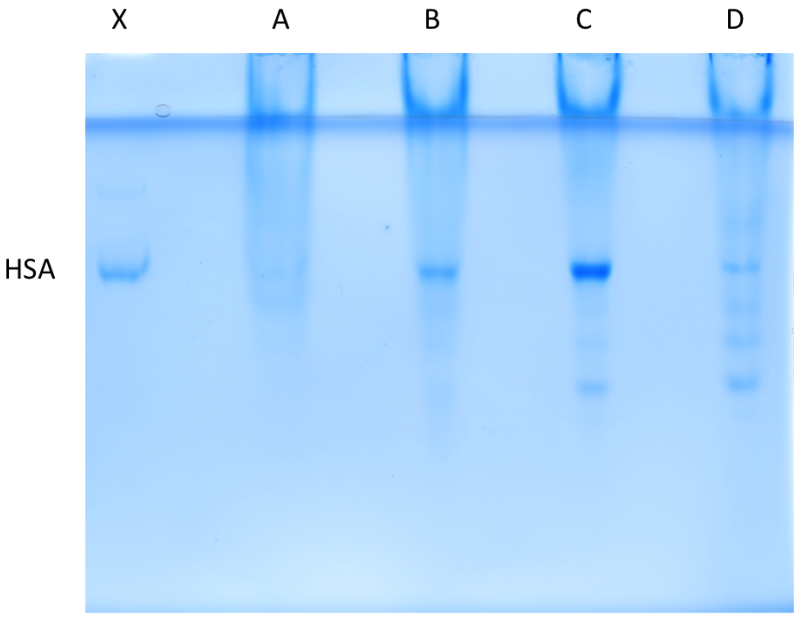
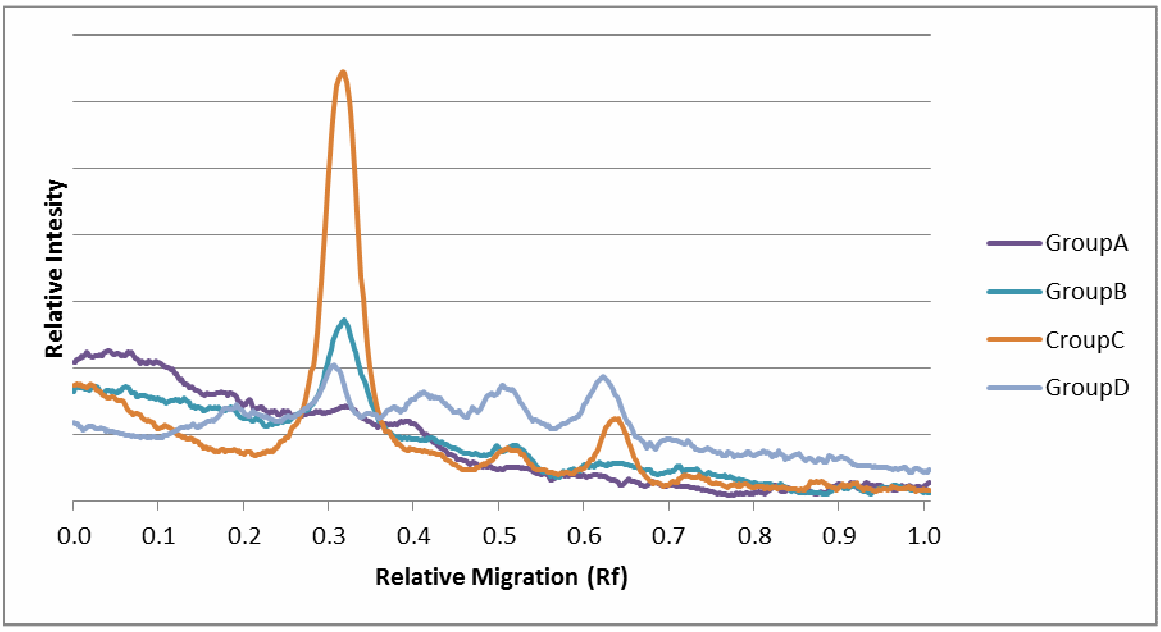

X
A
B
C
D
HSA

Figure 2.4 - Inter-group variability visualization through band intensity quantification of anionic-PAGE. Each line represents the relative intensity of stained salivary protein from each lane consisting of pooled samples from all the individuals in a single group.


Relative Intesity
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
Relative Migration (Rf)
GroupA
GroupB
CroupC
GroupD

2.3.6 SDS PAGE

The following section displays the SDS PAGE comparing individual saliva samples run along a molecular weight standard ranging from 10-250 kDa. The stacking gel was maintained atop the separating gel to confirm that the vast majority of proteins were not retained at the interface between the stacking gel and the separating gel, unlike the large amount of protein that was unable to enter the separating gel to be separated and visualized in the anionic PAGE seen earlier. The resultant band intensity quantification as measured by TotalLab Quant v12.5 is also displayed in this section.

Through the quantification of band intensities in the gel run with pooled saliva samples from each group (Inter-group gel, Fig 2.10), and the summation of intensities all the individual profiles seen in the intra-group gels (Fig. 2.5) a comparison of trends can be drawn between the two methods (non-pooled intra-group gels, and pooled inter-group gels). A summary of this comparison is displayed in Figures 2.11 and 2.12.

Figure 2.5 - SDS-PAGE Intra-group variability. A), B), C), and D) display results from Groups A, B, C, and D respectively. Gels are seen here stained with Coomassie Brilliant Blue. Furthermost left lane in each gel was loaded with a molecular weight marker, numbers indicating kilo Daltons (kDa). Each lane to the right of the molecular weight marker represents 20 μg of total protein from a single individual, therefore each lane represents a different individual.

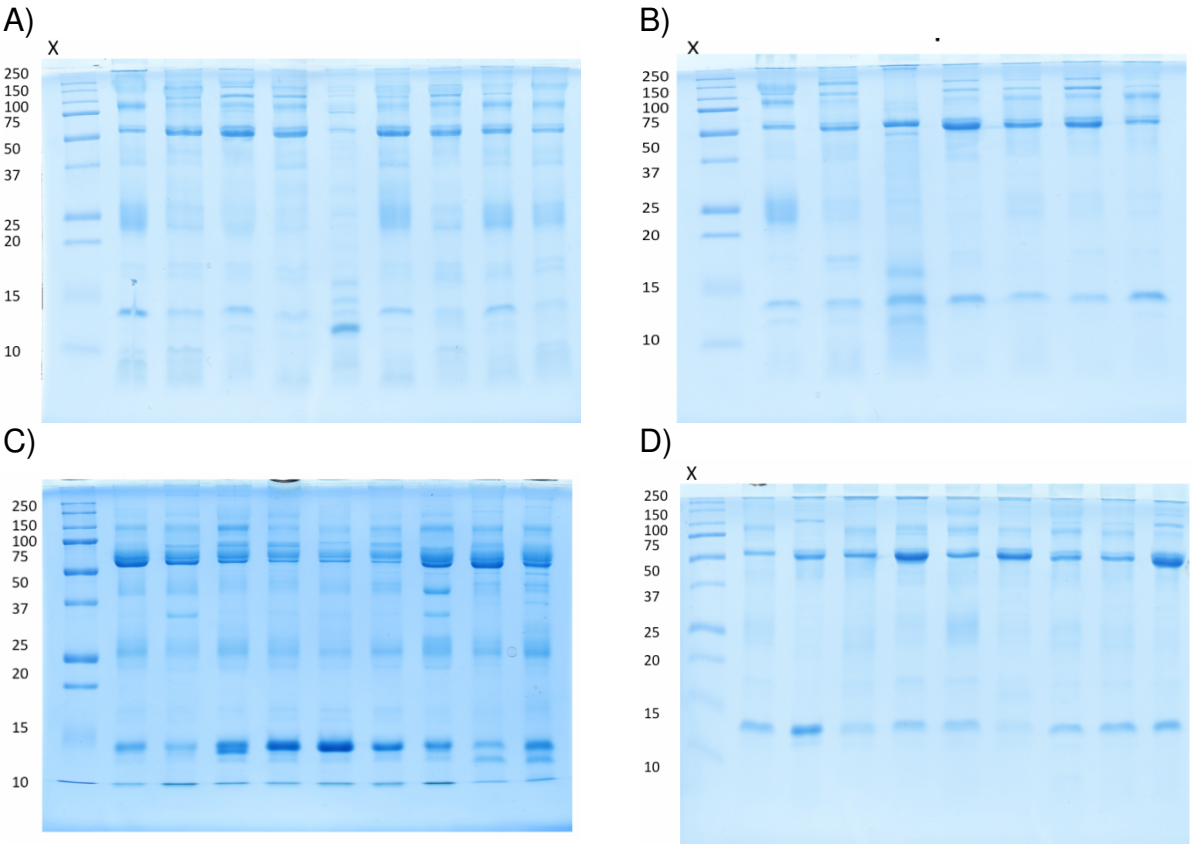

A)
X
250
150
100
75
50
37
25
20
15
10
B)
X
250
150
100
75
50
37
25
20
15
10
C)
250
150
100
75
50
37
25
20
15
10
D)
X
250
150
100
75
50
37
25
20
15
10

Figure 2.6 - Group A - Intra-group variability visualization through band intensity quantification of SDS-PAGE. Each line represents the relative intensity of stained protein from each lane consisting of salivary proteins from a single individual in Group A (Children pre-dental eruption) from Figure 2.5 A.

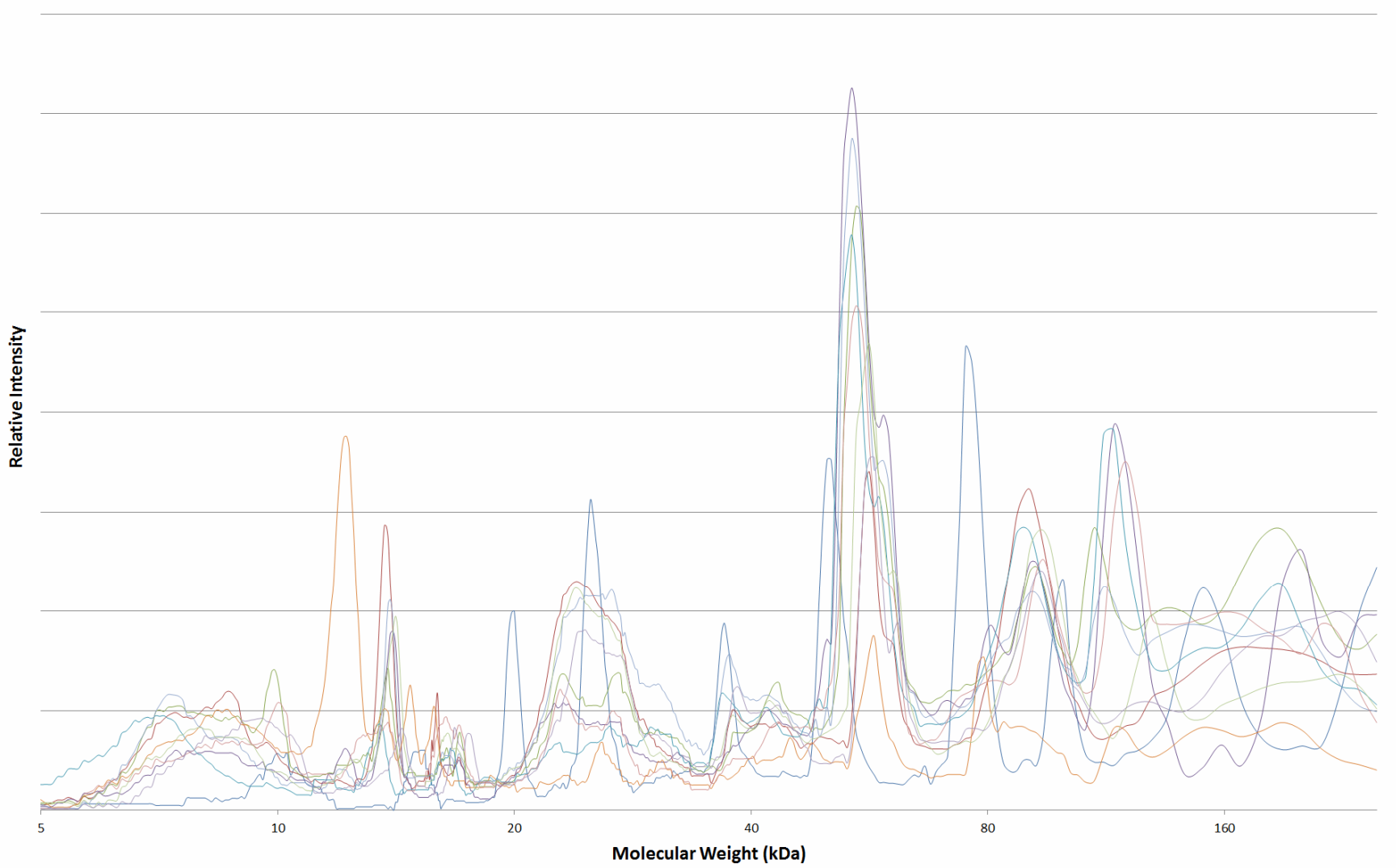

Relative Intensity
5
10
20
40
80
160
Molecular Weight (kDa)

Figure 2.7 - Group B - Intra-group variability visualization through band intensity quantification of SDS-PAGE. Each line represents the relative intensity of stained protein from each lane consisting of salivary proteins from a single individual in Group B (Children during dental eruption) from Figure 2.5 B.

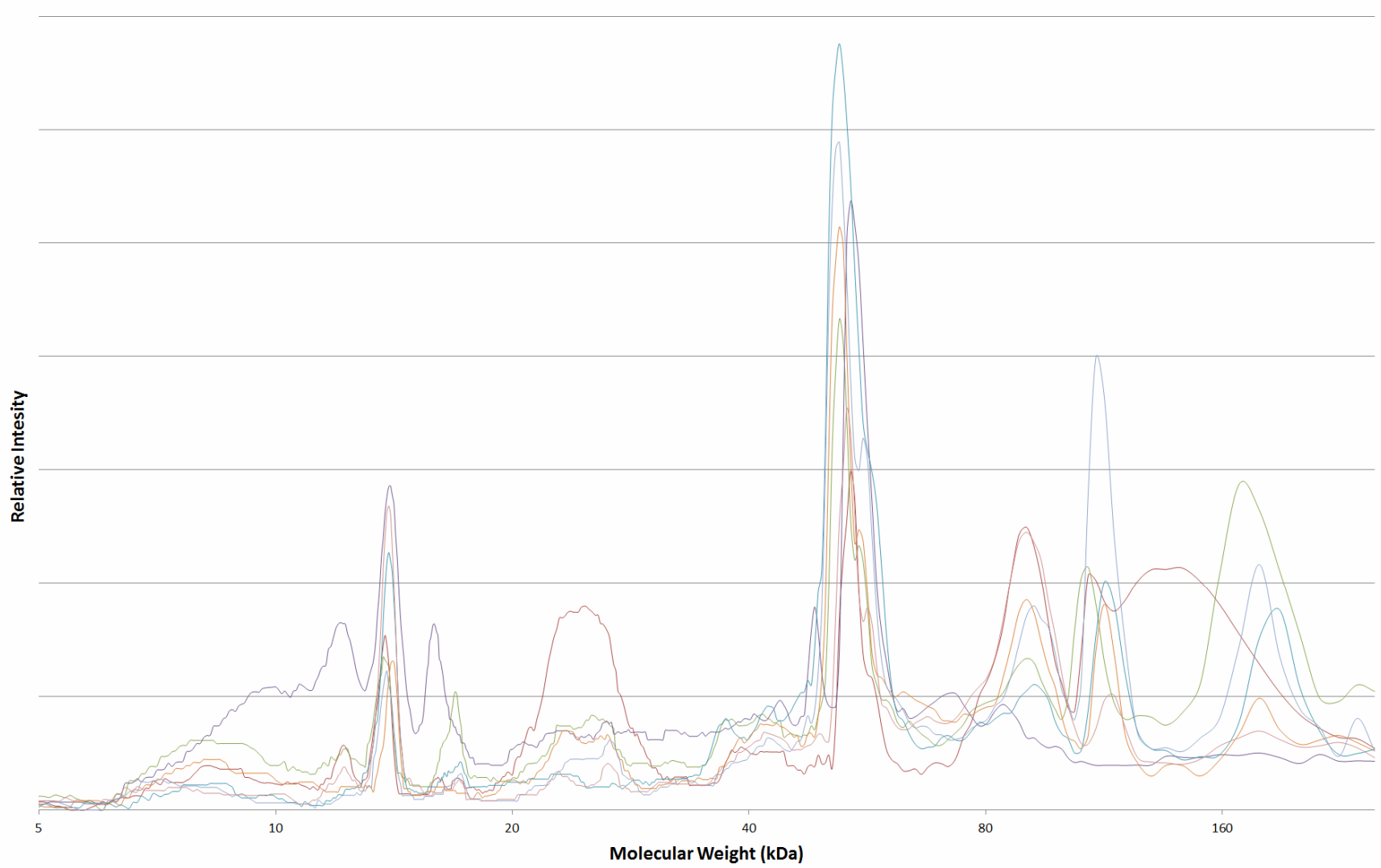

Relative Intesity
5
10
20
40
80
160
Molecular Weight (kDa)

Figure 2.8 - Group C - Intra-group variability visualization through band intensity quantification of SDS-PAGE. Each line represents the relative intensity of stained protein from each lane consisting of salivary proteins from a single individual in Group C (Children post-dental eruption) from Figure 2.5 C.

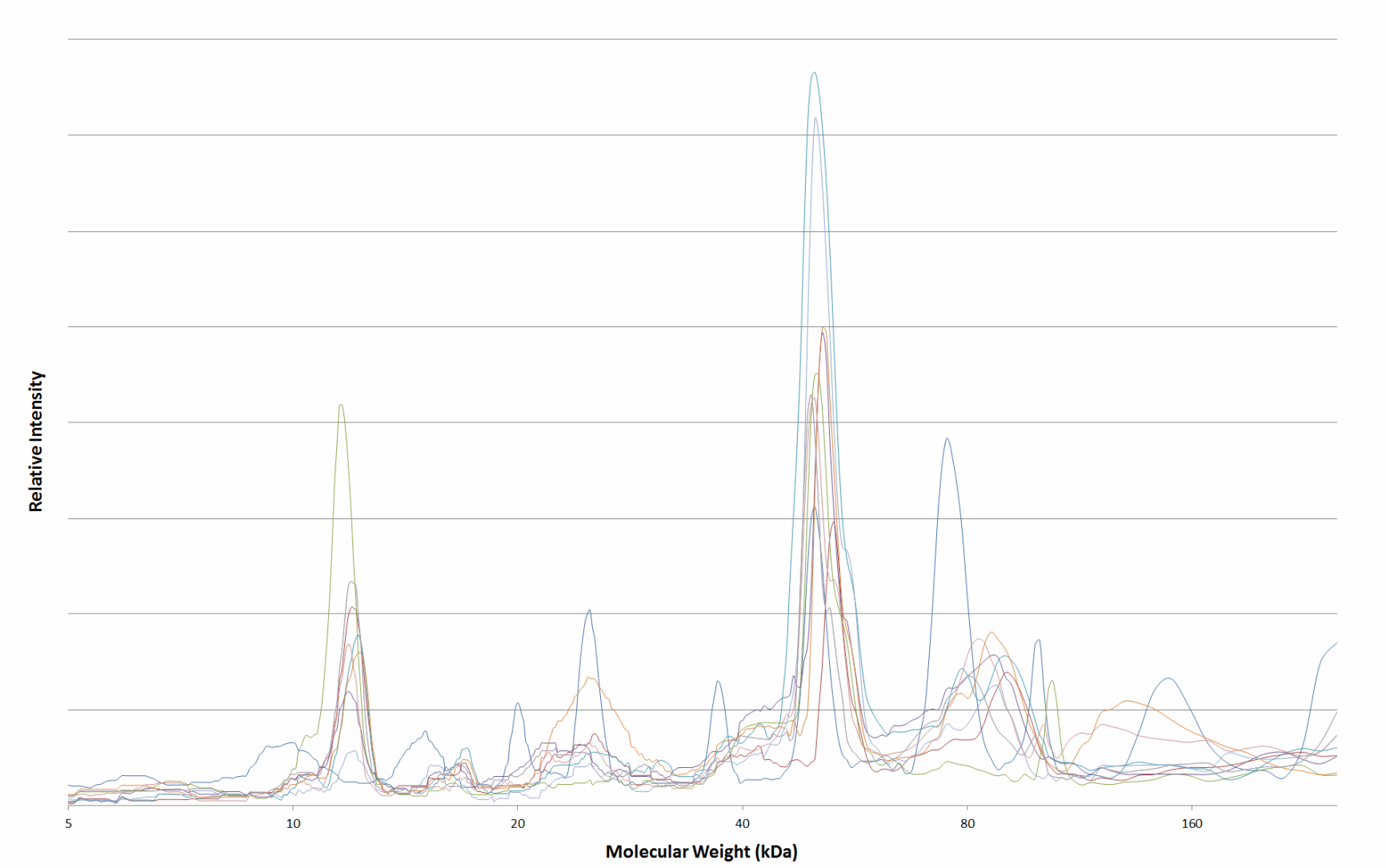

Relative Intensity
5
10
20
40
80
160
Molecular Weight (kDa)

Figure 2.9 - Group D - Intra-group variability visualization through band intensity quantification of SDS-PAGE. Each line represents the relative intensity of stained protein from each lane consisting of salivary proteins from a single individual in Group D (Adult controls) from Figure 2.5 D.

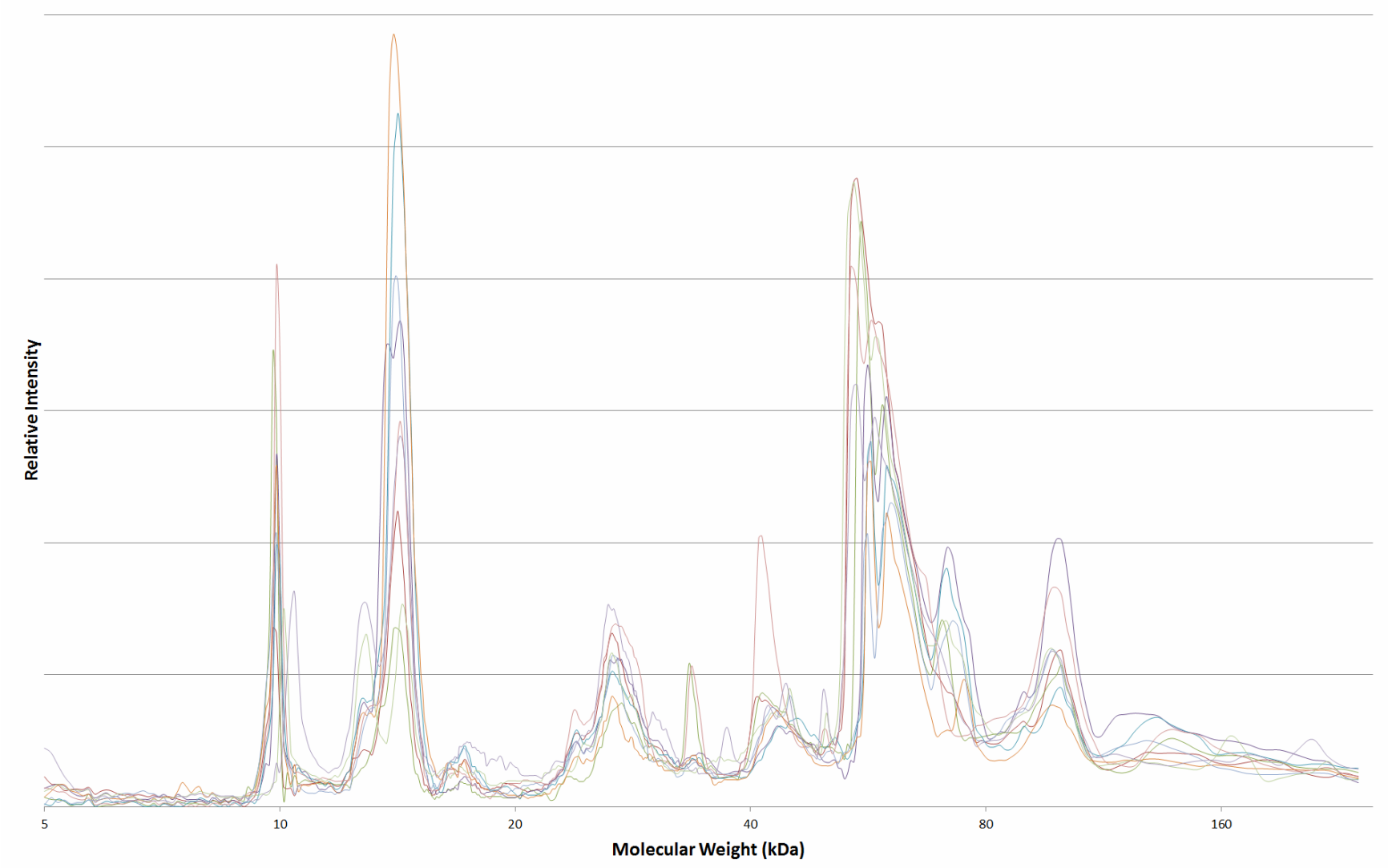

Relative Intensity
5
10
20
40
80
160
Molecular Weight (kDa)

Figure 2.10 - SDS-PAGE Inter-group variability. Each lane represents protein pooled from all the individuals in a single group (20 μg loaded in each lane). Lanes A, B, C, and D represent all the pooled samples from Group A (Children pre-dental eruptions), Group B (Children during dental eruption), Group C (Children post dental eruption), and Group D (Adult controls), respectively. Gel is seen here stained with Coomassie Brilliant Blue. Furthermost left lane was loaded with a molecular weight marker, numbers indicating kilo Daltons (kDa).

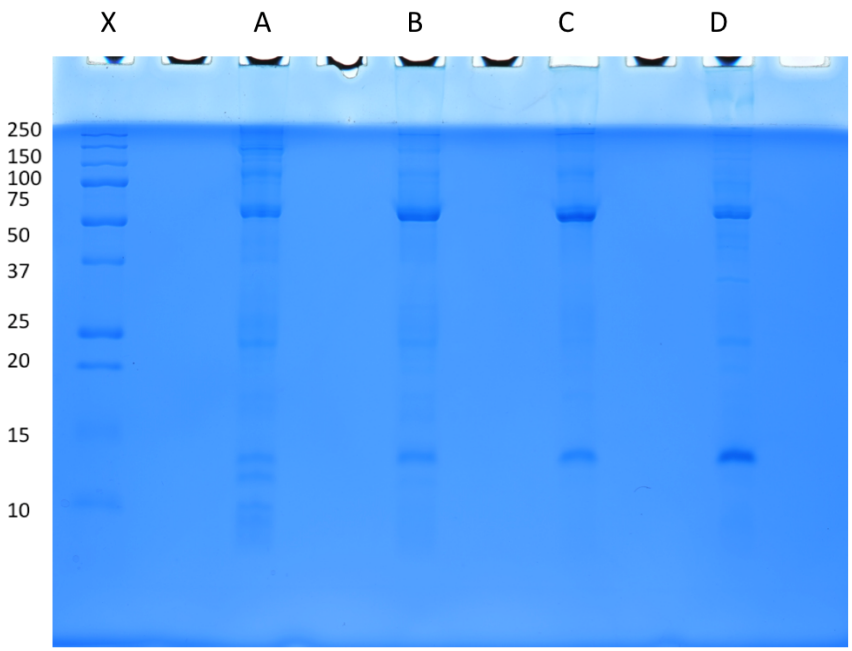

X
A
B
C
D
250
150
100
75
50
37
25
20
15
10

Figure 2.11 - SDS-PAGE Inter-group variability. Each line represents the quantified intensities of a single lane visualized on the single gel displayed in Figure 2.10. Each lane of the gel represents protein pooled from all the individuals in a single group. Lines A, B, C, and D represent all the quantified band intensities of pooled samples from Group A (Children pre-dental eruptions), Group B (Children during dental eruption), Group C (Children post dental eruption), and Group D (Adult controls), respectively.

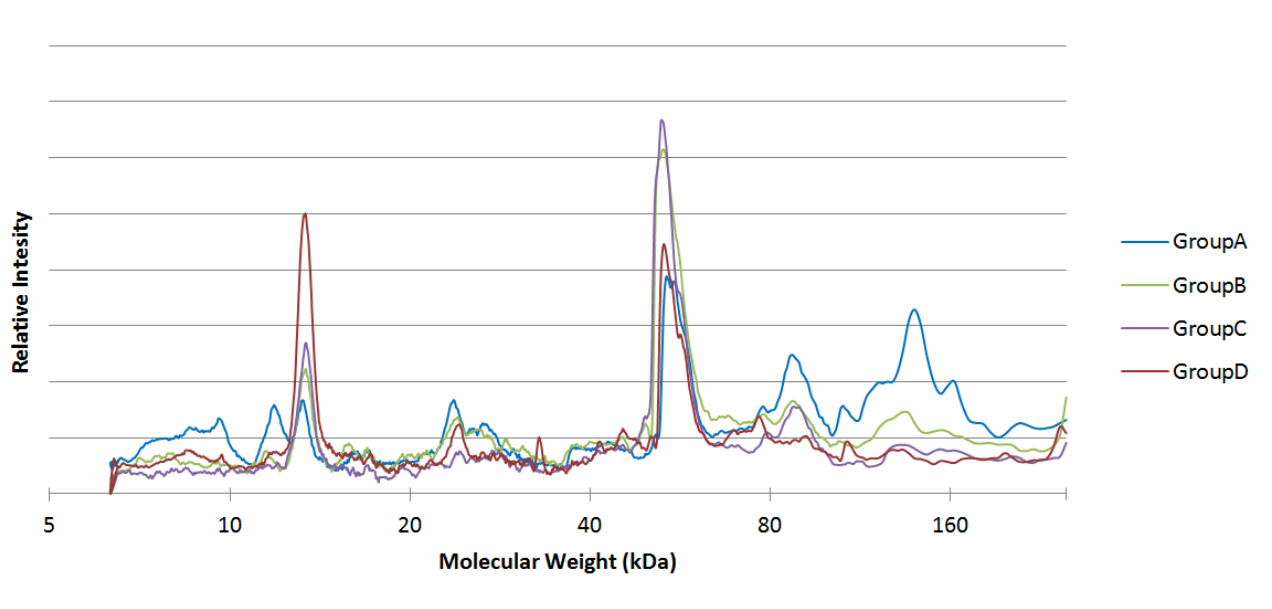
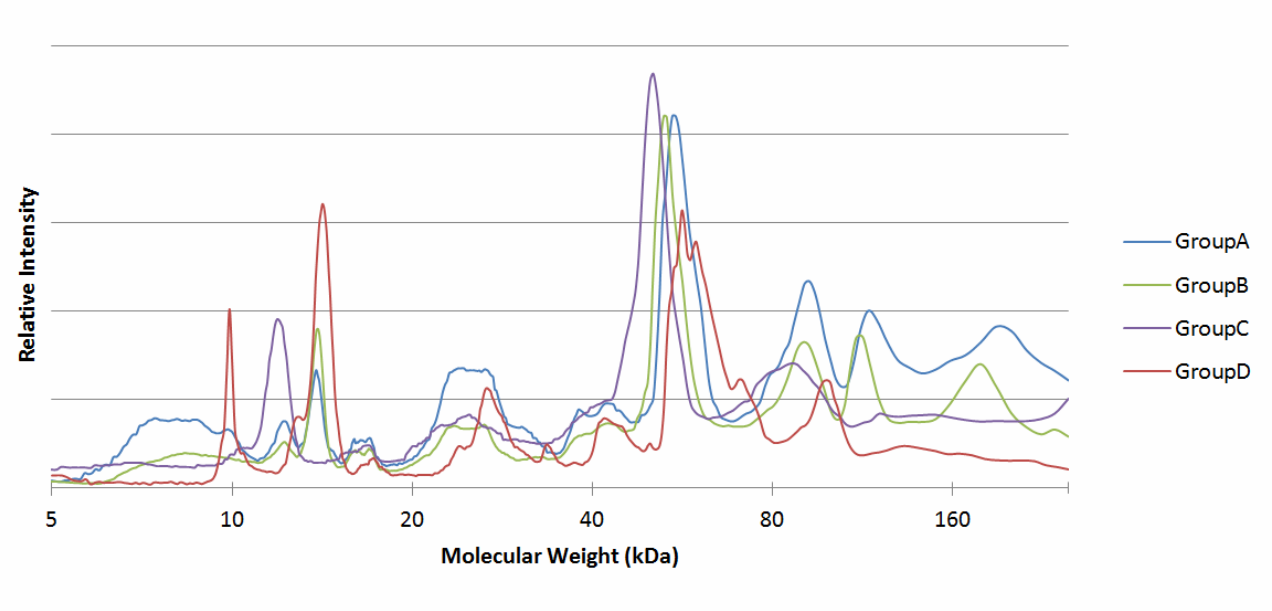

Relative Intesity
5
10
20
40
80
160
Molecular Weight (kDa)
GroupA
GroupB
GroupC
GroupD

Figure 2.12 -. SDS-PAGE Inter-group variability measured on the four separate gels displayed in Figure 2.5 A, B, C, D, representing Group A (Children pre-dental eruptions), Group B (Children during dental eruption), Group C (Children post dental eruption), and Group D (Adult controls). Each line in this figure represents the quantified intensities summed across one of the four separate gels seen in Figure 2.5. Lines A, B, C, and D represent all the quantified band intensities across all the lanes in Figure 2.5A, B, C, and D, respectively.


Relative Intensity
5
10
20
40
80
160
Molecular Weight (kDa)
GroupA
GroupB
GroupC
GroupD

2.3.7 High performance liquid chromatography

High performance liquid chromatography (HPLC) was used in two capacities in this study. The first application provided another avenue of protein separation and independent visualization of the whole protein profiles present in saliva in all four groups. The second application of HPLC was in-line with the mass spectrometer, serving to separate trypsin digestion protein fragments immediately prior to mass spectrometric analysis.

HPLC provides an excellent method of visualizing profiles of protein mixtures, separating proteins based on a range of properties (i.e. hydrophobicity, net charge, size/shape, metal binding etc.). In order to gain information about the whole protein profile of each group, the HPLC was used to analyze whole protein (avoiding tryptic-digestion) samples pooled from all individuals in each group. To add a unique dimension of separation not previously achieved with the PAGE analysis (Anionic – separated proteins based on their negative charge, SDS – separated proteins based on their molecular weight), a reverse phase column has been selected separate based on protein hydrophobicity. The column selected for sample analysis was a C4 XBridge^{TM} column. After extensive method optimization, the column proved its ability to reproducibly separate the complex protein mixture found in whole saliva, as well as parotid saliva. Parotid saliva served as a less-complex protein mixture but with much in common with whole saliva. Being far less complex in nature, the use of parotid saliva reduced unnecessary loading on the column during the comprehensive optimization process.

Chromatograms indicating successful and reproducible protein profile acquisition are displayed in Figures 2.13-2.16.

Once appropriate gradient range was established, flow rate and amount of protein loaded was optimized for consistent visualization and separation of peaks while minimizing the required amount of limited biological sample. Figures 2.13 and 2.14 displays chromatograms of protein profiles with loading of either 50 µg or 100 µg of total protein from parotid saliva. Figures 2.15 and 2.16 displays the same process applied to unstimulated whole saliva.

The detection sensitivity of the HPLC with the optimized method for C4 RP-HPLC column was determined through the use of isolated protein (albumin) loaded in known concentrations. The resulting single peak in each chromatogram was integrated to relate the area under the curve (AUC) with the amount of protein loaded. This forms the basis for quantitative analysis of visualized peaks in sample-derived chromatograms by relating peak size with a previously quantified value. The results of the relationship between the area under the curve and amount of isolated albumin protein over a range of concentrations (1.25μg–25μg) are displayed in Figure 2.17, with the raw chromatograms included in Appendix 4.

Figure 2.13 - Triplicate chromatograms produced by loading a total of 50 µg of parotid saliva protein on the C4 XBridge™ column. The chromatograms display the pattern of isolated parotid saliva separated on the reverse-phase C4 column run with a method of increasing organic solvent to a final concentration of 55% acetonitrile and 0.1% TFA.

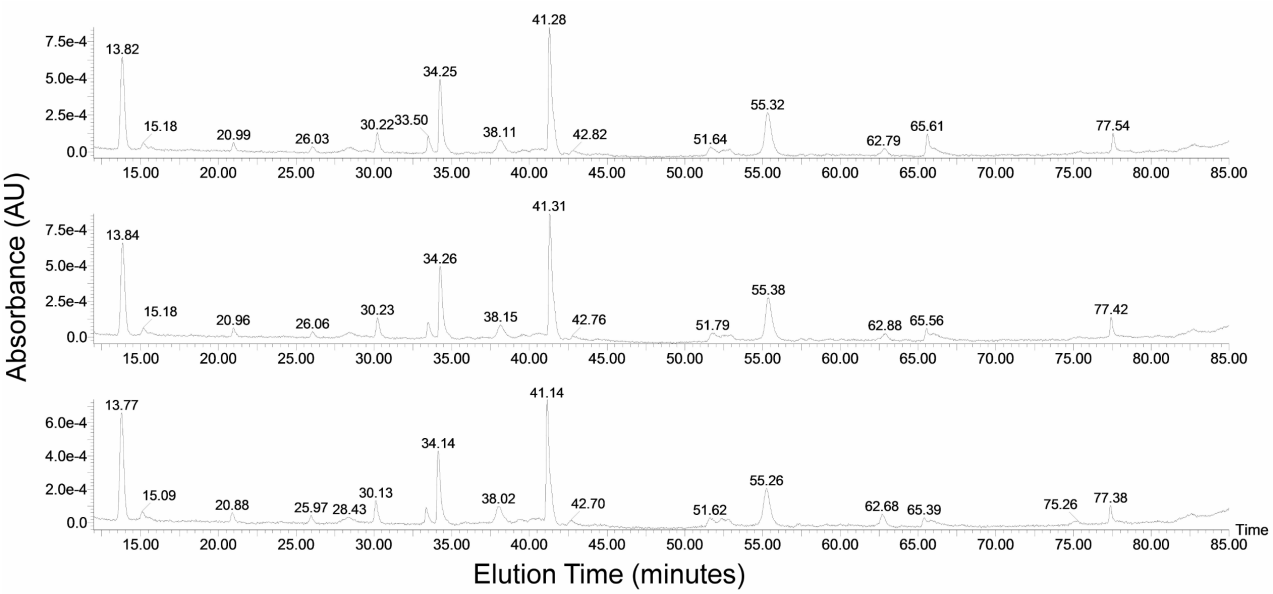

Absorbance (AU)
7.5e-4
5.0e-4
2.5e-4
0.0
13.82
15.18
20.99
26.03
30.22
33.50
34.25
38.11
41.28
42.82
51.64
55.32
62.79
65.61
77.54
13.84
15.18
20.96
26.06
30.23
34.26
38.15
41.31
42.76
51.79
55.38
62.88
65.56
77.42
6.0e-4
4.0e-4
2.0e-4
0.0
13.77
15.09
20.88
25.97
28.43
30.13
34.14
38.02
41.14
42.70
51.62
55.26
62.68
65.39
75.26
77.38
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00
55.00
60.00
65.00
70.00
75.00
80.00
85.00
Time
Elution Time (minutes)

Figure 2.14 - Triplicate chromatograms produced by loading a total of 100 µg of parotid saliva protein on the C4 XBridge™ column. The chromatograms display the pattern of isolated parotid saliva separated on the reverse-phase C4 column run with a method of increasing organic solvent to a final concentration of 55% acetonitrile and 0.1% TFA.

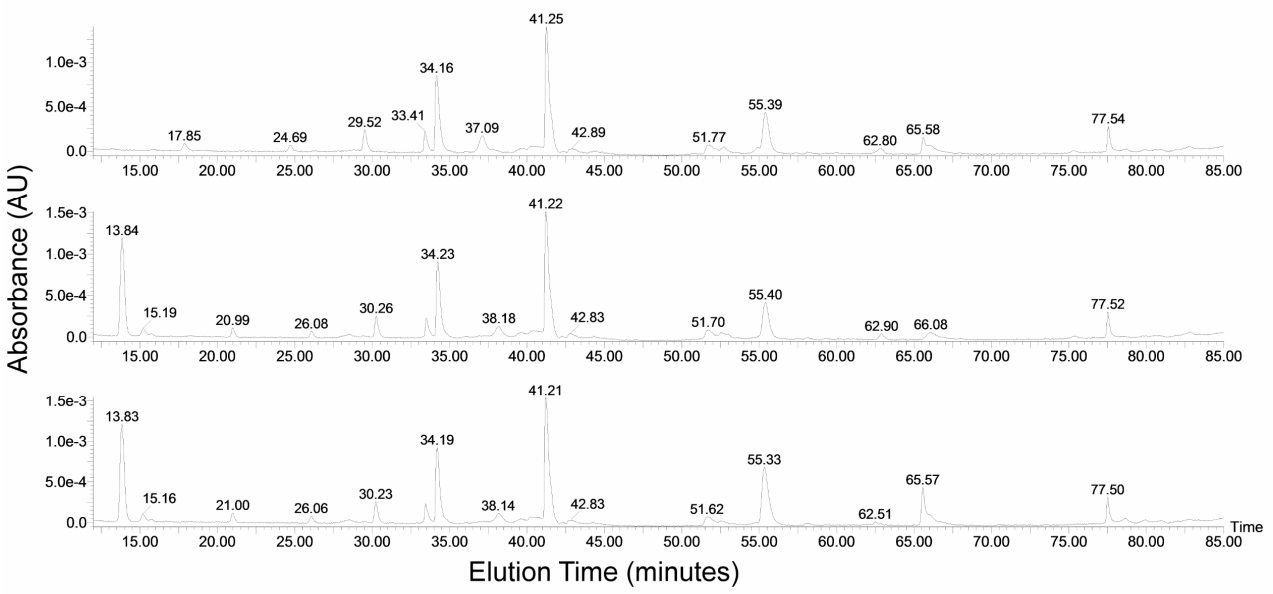

Absorbance (AU)
1.0e-3
5.0e-4
0.0
41.25
34.16
55.39
17.85
24.69
29.52
33.41
37.09
42.89
51.77
62.80
65.58
77.54
1.5e-3
1.0e-3
5.0e-4
0.0
41.22
13.84
34.23
55.40
15.19
20.99
26.08
30.26
38.18
42.83
51.70
62.90
66.08
77.52
1.5e-3
1.0e-3
5.0e-4
0.0
41.21
13.83
34.19
55.33
15.16
21.00
26.06
30.23
38.14
42.83
51.62
62.51
65.57
77.50
Time
15.00 20.00 25.00 30.00 35.00 40.00 45.00 50.00 55.00 60.00 65.00 70.00 75.00 80.00 85.00
Elution Time (minutes)

Figure 2.15 - Triplicate chromatograms produced by loading a total of 50 µg of unstimulated whole saliva protein on the C4 XBridge™ column. The chromatograms display the pattern of unstimulated whole saliva separated on the reverse-phase C4 column run with a method of increasing organic solvent to a final concentration of 55% acetonitrile and 0.1% TFA.

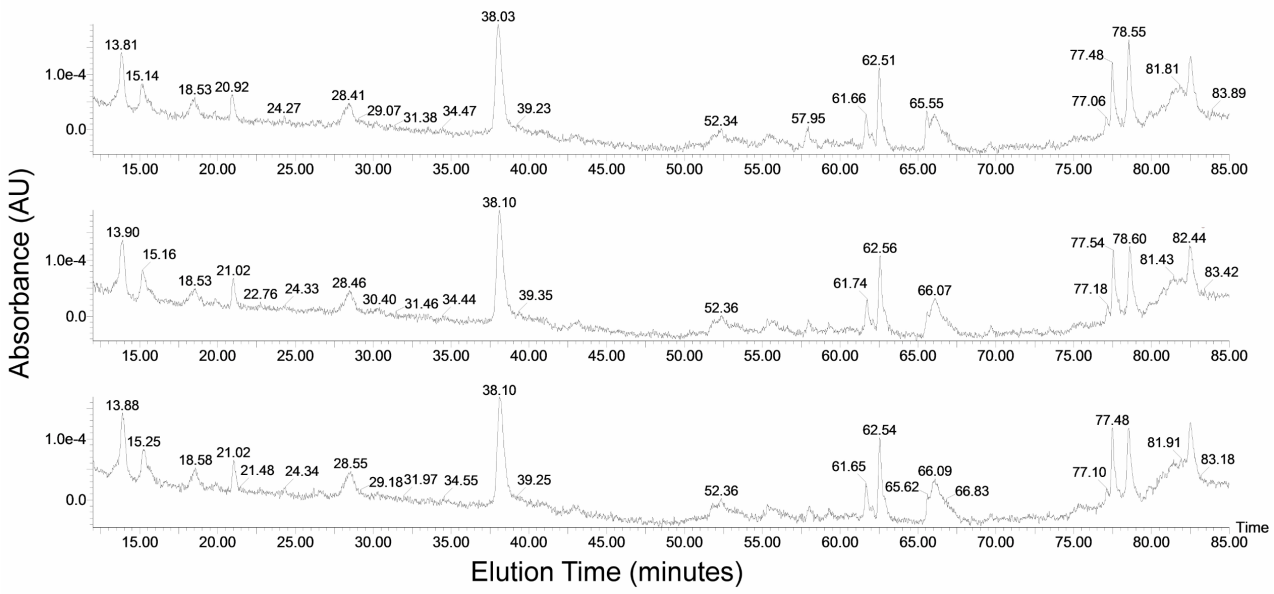

Absorbance (AU)
1.0e-4
0.0
13.81
15.14
18.53
20.92
24.27
28.41
29.07
31.38
34.47
38.03
39.23
52.34
57.95
61.66
62.51
65.55
77.06
77.48
78.55
81.81
83.89
13.90
15.16
18.53
21.02
22.76
24.33
28.46
30.40
31.46
34.44
38.10
39.35
52.36
61.74
62.56
66.07
77.18
77.54
78.60
81.43
82.44
83.42
13.88
15.25
18.58
21.02
21.48
24.34
28.55
29.18
31.97
34.55
38.10
39.25
52.36
61.65
62.54
65.62
66.09
66.83
77.10
77.48
81.91
83.18
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00
55.00
60.00
65.00
70.00
75.00
80.00
85.00
Time
Elution Time (minutes)

Figure 2.16 – Triplicate chromatograms produced by loading a total of 100 µg of unstimulated whole saliva protein on the C4 XBridge™ column. The chromatograms display the pattern of unstimulated whole saliva separated on the reverse-phase C4 column run with a method of increasing organic solvent to a final concentration of 55% acetonitrile and 0.1% TFA.

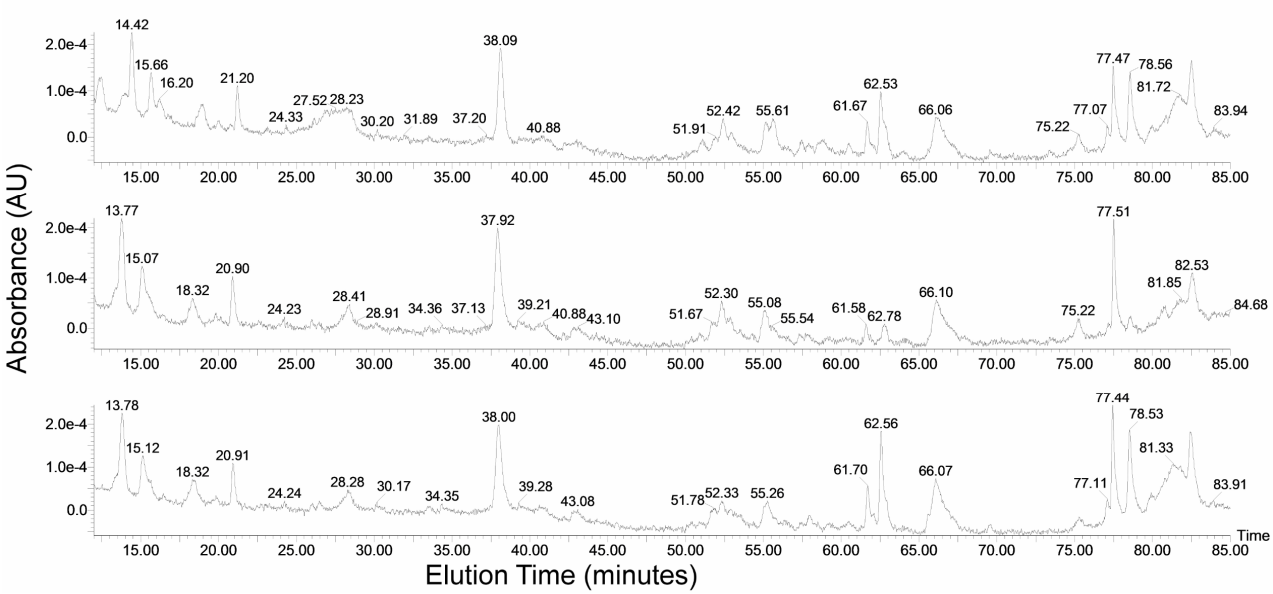

Absorbance (AU)
2.0e-4
1.0e-4
0.0
14.42
15.66
16.20
21.20
24.33
27.52
28.23
30.20
31.89
37.20
38.09
40.88
51.91
52.42
55.61
61.67
62.53
66.06
75.22
77.07
77.47
78.56
81.72
83.94
13.77
15.07
18.32
20.90
24.23
28.41
28.91
34.36
37.13
37.92
39.21
40.88
43.10
51.67
52.30
55.08
55.54
61.58
62.78
66.10
75.22
77.51
81.85
82.53
84.68
13.78
15.12
18.32
20.91
24.24
28.28
30.17
34.35
38.00
39.28
43.08
51.78
52.33
55.26
61.70
62.56
66.07
77.11
77.44
78.53
81.33
83.91
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00
55.00
60.00
65.00
70.00
75.00
80.00
85.00
Time
Elution Time (minutes)

Figure 2.17 - Relationship between area under the curve and amount of HSA loaded onto the C4 XBridge™ column. The area under the curve (AU*time) was measured for quantities of HSA ranging from 5 – 60 µg.

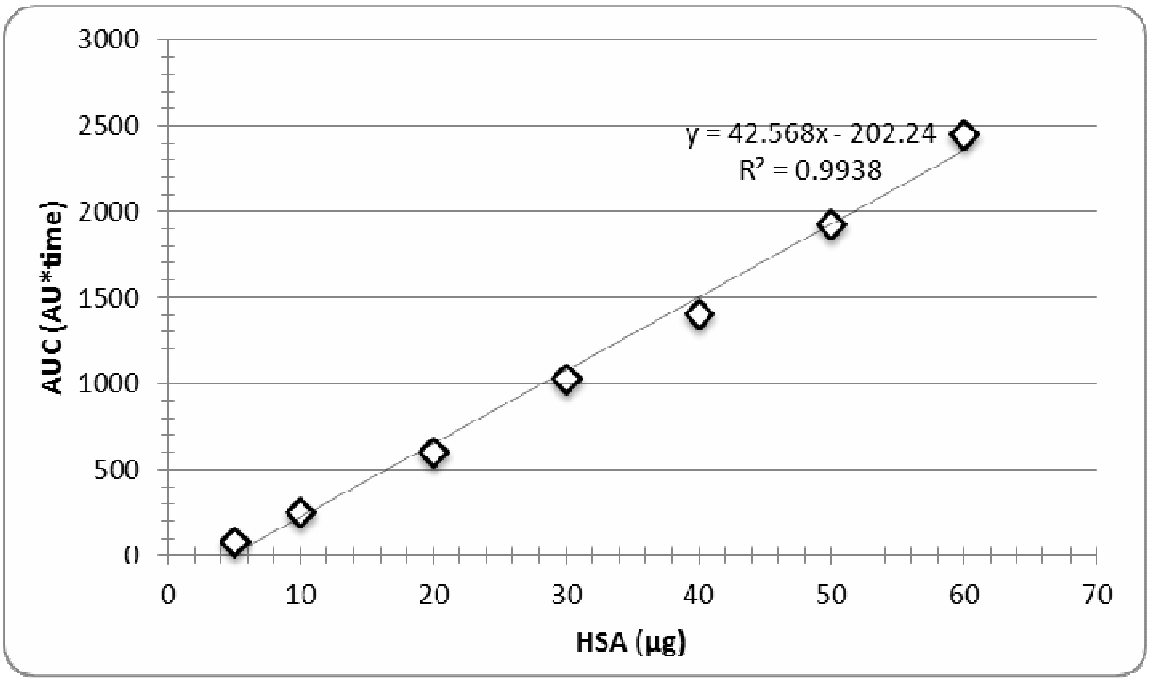
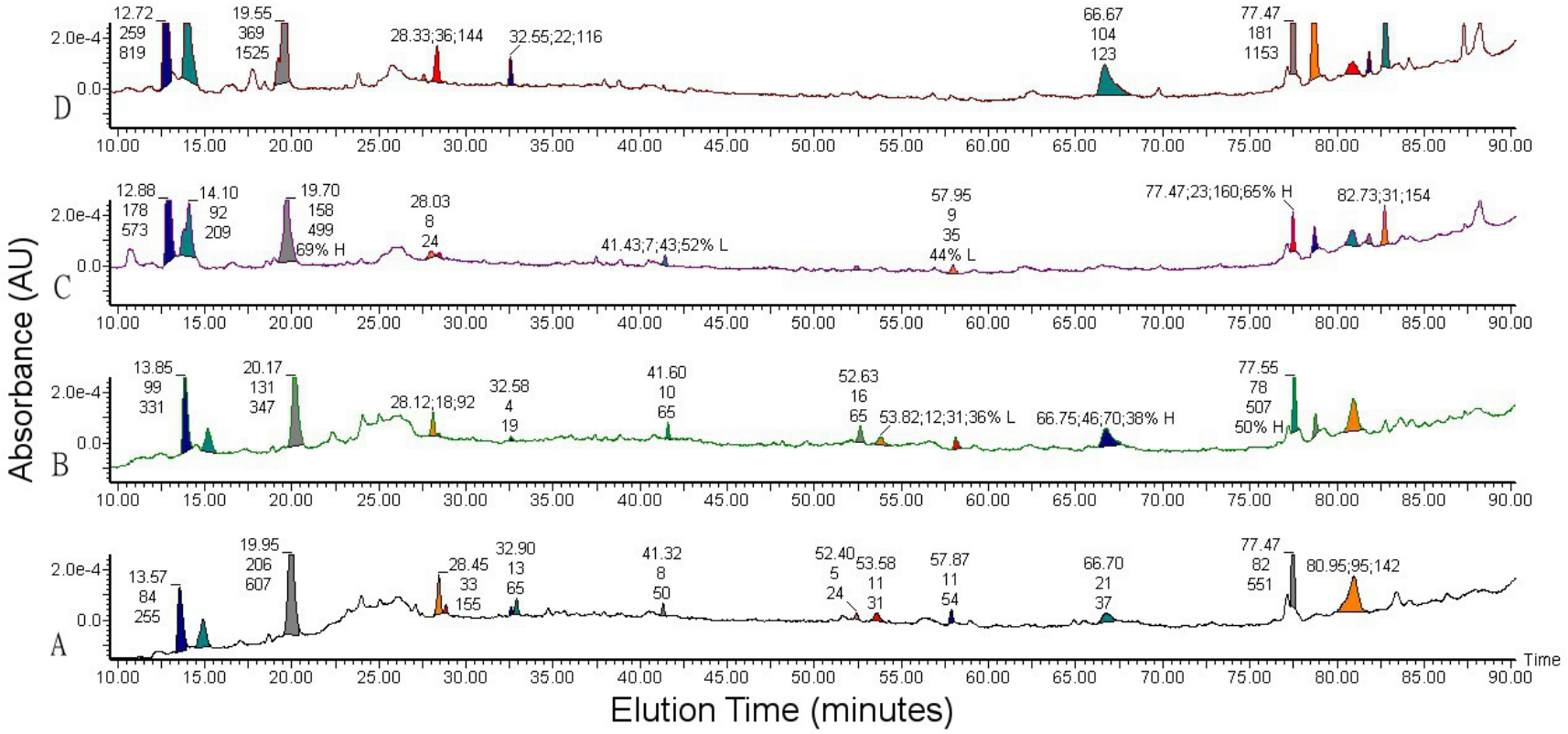

3000
2500
2000
1500
1000
500
0
AUC (AU*time)
y = 42.568x - 202.24
R² = 0.9938
0
10
20
30
40
50
60
70
HSA (µg)

Figure 2.18 – Chromatograms A, B, C, and D, represent 100 µg of pooled unstimulated whole saliva from Groups A (Children pre-dental eruption), B (Children during dental eruption), C (Children post dental eruption), and D (Adult controls), respectively, on a C4 X-Bridge reverse-phase column. The chromatograms display the pattern of unstimulated whole saliva separated on the reverse-phase C4 column run with a method of increasing organic solvent to a final concentration of 55% acetonitrile and 0.1% TFA. A minimum of ten of the most prominent and shared peaks from each chromatogram were highlight for comparison between groups.


Absorbance (AU)
D
C
B
A
2.0e-4
0.0
12.72
259
819
19.55
369
1525
28.33;36;144
32.55;22;116
66.67
104
123
77.47
181
1153
12.88
178
573
14.10
92
209
19.70
158
499
69% H
28.03
8
24
41.43;7;43;52% L
57.95
9
35
44% L
77.47;23;160;65% H
82.73;31;154
13.85
99
331
20.17
131
347
28.12;18;92
32.58
4
19
41.60
10
65
52.63
16
65
53.82;12;31;36% L
66.75;46;70;38% H
77.55
78
507
50% H
13.57
84
255
19.95
206
607
28.45
33
155
32.90
13
65
41.32
8
50
52.40
5
24
53.58
11
31
57.87
11
54
66.70
21
37
77.47
82
551
80.95;95;142
10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00
55.00
60.00
65.00
70.00
75.00
80.00
85.00
90.00
Time
Elution Time (minutes)

Once the method was proven to be consistent and able to successfully separate the proteins found in unstimulated whole saliva, the pooled unstimulated whole saliva samples from each of the four groups were loaded and chromatograms compared. Figure 2.18 displays an overview of the entire 90-minute method for all four groups. Appendix 5 includes more detailed views of the chromatographic patterns throughout the length of the chromatographic run.

2.3.8 Mass spectrometric analysis

Mass spectrometric results are summarized in Table 2.5. A total of 79 proteins were successfully identified in one or more of the four pooled sample groups. A total of 48, 48, 58, and 50 proteins were successfully identified in pooled samples from Groups A, B, C, and D, respectively.

Table 2.6 summarizes the quantification of relative abundance of the top 15 identified proteins by Scaffold with a minimum cut-off of 2 unique peptide counts, if a peptide was detected at all. In this study, the count of unique peptides represents the number of unique parent ions, identified for a protein, that meet the default minimum intensity limit for MS/MS analysis. The term spectrum counts represents the total number of MS/MS fragmentation spectra that map to peptides of a given protein. Unique spectrum counts is the sum of nonrepeated MS/MS fragmentation spectra, represented as a subset of the total spectrum counts.

Table 2.5 –Mass spectrometric protein identifications for children pre-, during, post-eruption, and edentulous adults, Groups A, B, C, D, respectively. This table summarizes the name, accession number, molecular weight, and number of unique peptide counts, for each of the 79 identified proteins, across Groups A, B, C, and D. The numbers corresponding to each protein identified in each group represents the number of unique peptide counts. The MS/MS results were analyzed using Mascot (version 2.2) and X! Tandem (version 2007.01.01.1), and searched against the NCBInr database. Scaffold (version Scaffold_2_06_02) was used to validate the search results, with an acceptable peptide probability of greater than 95.0%. The number zero (0) indicates no peptides were detected from the specified protein.

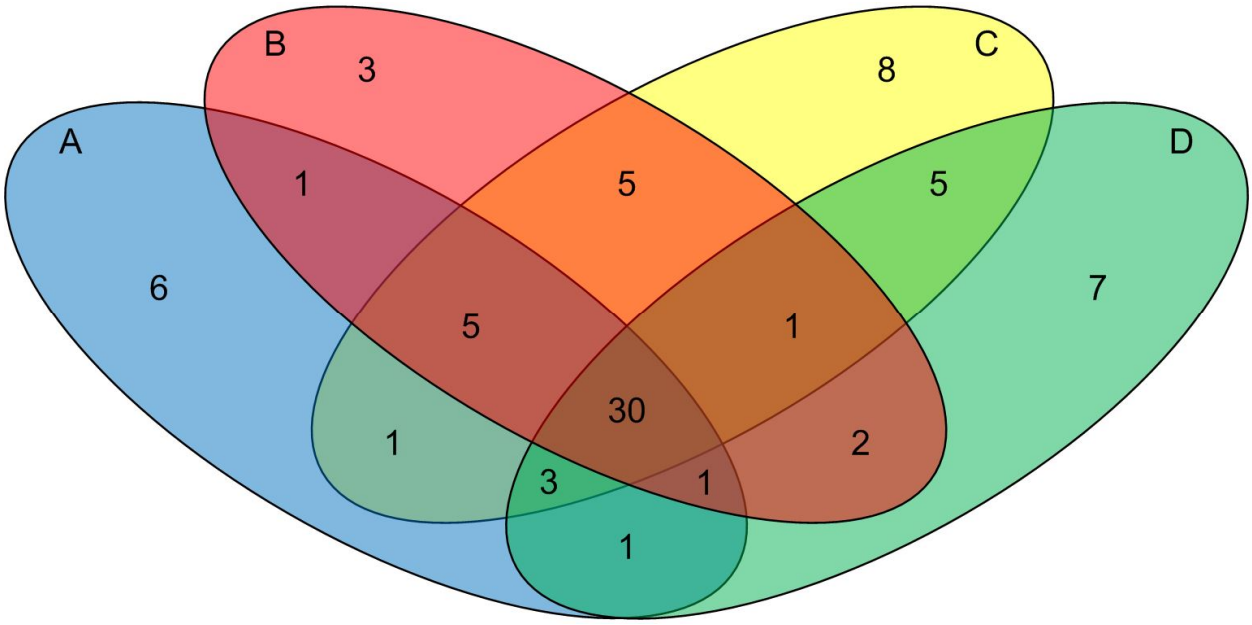
#	Identified Proteins (79)	Accession Number	Molecular Weight	Group A	Group B	Group C	Group D
1	Chain A, Structure Solution And Refinement Of The Recombinant Human Salivary Amylase	gi|14719766 (+1)	56 kDa	37	34	37	23
2	albumin, isoform CRA_h [Homo sapiens]	gi|119626071 (+11)	69 kDa	12	20	20	12
3	peptide PB,saliva	gi|350218	6 kDa	7	10	14	14
4	mucin-5B precursor [Homo sapiens]	gi|301172750	596 kDa	16	11	11	7
5	unnamed protein product [Homo sapiens]	gi|189053131 (+2)	19 kDa	10	8	12	10
6	Polymeric immunoglobulin receptor [Homo sapiens], transmembrane secretory component	gi|238236 (+2)	83 kDa	11	11	11	6
7	parotid secretory protein [Homo sapiens]	gi|16755850 (+1)	27 kDa	12	7	8	7
8	unnamed protein product [Homo sapiens]	gi|14042015	53 kDa	7	9	10	7
9	hypothetical protein LOC352999 precursor [Homo sapiens]	gi|58219024	38 kDa	8	6	10	6
10	carbonic anhydrase VI [Homo sapiens]	gi|119592012	35 kDa	7	7	6	6
11	Chain B, Mhc-Like Zinc Alpha2-Glycoprotein And Prolactin Inducible Protein	gi|145579641	14 kDa	5	6	6	6
12	Mucin 7, secreted [Homo sapiens]	gi|19343619 (+1)	39 kDa	9	7	4	3
13	Chain A, Zn-Alpha-2-Glycoprotein	gi|58176763	32 kDa	5	7	7	4
14	lactoperoxidase isoform 1 preproprotein [Homo sapiens]	gi|40549418	80 kDa	12	2	4	5
15	lipocalin-1 precursor [Homo sapiens], von Ebner's gland protein	gi|4504963	19 kDa	8	10	5	0
16	cystatin SA-III=potential precursor of acquired enamel pellicle	gi|235948 (+1)	14 kDa	1	7	7	7
17	actin, beta [Homo sapiens]	gi|14250401 (+9)	41 kDa	2	5	8	2
18	bactericidal/permeability-increasing protein-like 1 precursor [Homo sapiens]	gi|15055535 (+1)	49 kDa	4	3	4	5
19	small proline-rich protein 3 [Homo sapiens]	gi|4885607 (+3)	18 kDa	3	0	6	7
20	deleted in malignant brain tumors 1, isoform CRA_b [Homo sapiens]	gi|119569694 (+14)	178 kDa	6	3	5	2
21	cystatin-SA precursor [Homo sapiens]	gi|4503105	16 kDa	1	4	6	4
22	cystatin-B [Homo sapiens]	gi|4503117	11 kDa	4	4	3	4
23	cystatin-SN precursor [Homo sapiens]	gi|19882251 (+1)	16 kDa	1	3	4	5
24	unnamed protein product [Homo sapiens]	gi|158256038 (+2)	54 kDa	2	2	3	5
25	immunoglobulin light chain [Homo sapiens]	gi|149673889	23 kDa	1	3	4	3
26	suprabasin isoform 1 precursor [Homo sapiens]	gi|260436922	61 kDa	4	1	4	2
27	Ig L-chain V-region [Homo sapiens]	gi|27552515 (+1)	23 kDa	1	3	5	2
28	unnamed protein product [Homo sapiens]	gi|158261509 (+3)	52 kDa	3	7	1	0
29	Chain A, Human Cystatin C	gi|14278690 (+1)	13 kDa	2	1	5	1
30	transcobalamin-1 precursor [Homo sapiens]	gi|21071008	48 kDa	4	1	1	3
31	beta-casein [Homo sapiens]	gi|288098 (+1)	25 kDa	8	0	0	0
32	beta-2-microglobulin precursor [Homo sapiens]	gi|114319049 (+23)	12 kDa	3	3	2	0
33	cystatin D [Homo sapiens]	gi|398711 (+1)	16 kDa	0	2	3	2
34	Chain B, T-To-T(High) Quaternary Transitions In Human Hemoglobin	gi|60594354 (+30)	16 kDa	0	0	7	0
35	Chain A, Role Of Amino Acid Residues At Turns In The Conformational Stability And Folding Of Human Lysozyme	gi|6730358	15 kDa	2	2	1	1
36	keratin 1 [Homo sapiens]	gi|11935049 (+3)	66 kDa	1	1	2	2
37	monoclonal IgM antibody heavy chain [Homo sapiens]	gi|41388180	64 kDa	0	3	3	0
38	histatin-1 precursor [Homo sapiens]	gi|4504529	7 kDa	3	1	0	1

Table 2.5 (Continued):

#	Identified Proteins (79)	Accession Number	Molecular Weight	Group A	Group B	Group C	Group D
39	salivary proline-rich protein precursor [Homo sapiens]	gi|190510	25 kDa	0	2	0	3
40	hCG2006898 [Homo sapiens]	gi|119571628	16 kDa	1	0	1	3
41	hypothetical protein [Homo sapiens]	gi|34365139	52 kDa	2	1	1	1
42	immunoglobulin J chain [Homo sapiens]	gi|114319027 (+2)	20 kDa	1	2	2	0
43	PREDICTED: nucleobindin 2 isoform 2 [Pan troglodytes]	gi|114636384 (+5)	50 kDa	2	0	2	1
44	peptide,salivary low MW	gi|223364	1 kDa	1	1	2	0
45	hemoglobin alpha-1 globin chain [Homo sapiens]	gi|13650074 (+16)	15 kDa	0	0	4	0
46	Chain A, Crystal Structure Of Pi Class Glutathione Transferase	gi|11514448 (+22)	23 kDa	2	0	0	2
47	kallikrein 1, renal/pancreas/salivary, isoform CRA_b [Homo sapiens]	gi|119592319 (+10)	24 kDa	0	0	2	2
48	RecName: Full=Ig kappa chain C region [Homo sapiens]	gi|125145 (+2)	12 kDa	0	0	2	2
49	Chain A, Crystal Structure Of Human Enolase 1	gi|203282367 (+5)	47 kDa	0	0	3	1
50	lactotransferrin [Homo sapiens]	gi|119585171 (+28)	78 kDa	3	1	0	0
51	unnamed protein product [Homo sapiens]	gi|189053201 (+3)	13 kDa	0	0	1	3
52	beta-casein [Homo sapiens]	gi|29674	7 kDa	3	0	0	0
53	RecName: Full=Vitamin D-binding protein [Homo sapiens]	gi|139641 (+9)	53 kDa	0	2	1	0
54	hypothetical LOC389429, isoform CRA_a [Homo sapiens]	gi|119568478	31 kDa	0	2	1	0
55	alpha-2-macroglobulin-like 1, isoform CRA_b [Homo sapiens]	gi|119609009 (+4)	161 kDa	0	1	0	2
56	albumin, isoform CRA_p [Homo sapiens]	gi|119626079 (+2)	23 kDa	1	0	2	0
57	cystatin-A [Homo sapiens]	gi|4885165	11 kDa	0	0	0	3
58	unnamed protein product [Homo sapiens]	gi|194387966 (+3)	17 kDa	0	0	1	2
59	Chain A, Apo-Human Serum Transferrin (Non-Glycosylated)	gi|110590597	75 kDa	0	0	3	0
60	unnamed protein product [Homo sapiens]	gi|34526199	53 kDa	0	1	1	0
61	Chain A, Alpha-Lactalbumin [Homo sapiens]	gi|157829683 (+2)	14 kDa	2	0	0	0
62	immunoglobulin variable region [Homo sapiens]	gi|323432327	15 kDa	0	1	1	0
63	desmoglein-3 preproprotein [Homo sapiens]	gi|119964718 (+2)	108 kDa	0	0	2	0
64	Chain A, High Resolution Crystal Structure Of The Unliganded Human Acbp	gi|118137768 (+1)	10 kDa	0	0	0	2
65	extracellular glycoprotein lacritin precursor [Homo sapiens]	gi|15187164	14 kDa	0	2	0	0
66	mammaglobin-B precursor [Homo sapiens]	gi|4505171	11 kDa	0	2	0	0
67	cysteine-rich secretory protein 3 [Homo sapiens]	gi|119624753 (+6)	26 kDa	2	0	0	0
68	basic salivary proline-rich protein 3 precursor [Homo sapiens]	gi|117306167 (+4)	31 kDa	0	0	0	2
69	alpha-amylase [Homo sapiens]	gi|178585	58 kDa	2	0	0	0
70	interleukin-1 receptor antagonist protein isoform 3 [Homo sapiens]	gi|10835147 (+5)	18 kDa	0	0	0	2
71	unnamed protein product [Homo sapiens]	gi|158256510	68 kDa	0	0	0	2
72	Chain A, Mixed Disulfide Intermediate Between Mutant Human Thioredoxin And A 13 Residue Peptide	gi|1065111 (+15)	12 kDa	0	0	0	2
73	hypothetical protein LOC644054 [Homo sapiens]	gi|212276011 (+1)	9 kDa	0	0	0	2
74	glyceraldehyde-3-phosphate dehydrogenase, isoform CRA_a [Homo sapiens]	gi|119609192 (+7)	35 kDa	0	0	2	0
75	complement C3 precursor [Homo sapiens]	gi|115298678 (+2)	187 kDa	0	0	1	0
76	unnamed protein product [Homo sapiens]	gi|194373909 (+5)	51 kDa	0	0	1	0
77	secretoglobin family 1D member 1 precursor [Homo sapiens]	gi|5729907	10 kDa	0	1	0	0
78	Casein alpha s1 [Homo sapiens]	gi|118764211 (+6)	22 kDa	1	0	0	0
79	lectin, galactoside-binding, soluble, 3 binding protein, isoform CRA_a [Homo sapiens]	gi|119609949 (+7)	65 kDa	0	0	1	0

Figure 2.19 – Venn Diagrams describing the number of proteins identified in Group A (Children pre-dental eruption), B (Children during dental eruption), C (Children post dental eruption), and D (Adult controls). a) TOP Venn Diagram represents the number of proteins identified in the four groups with a minimum of 1 or more unique peptides identified with MS/MS. b) BOTTOM Venn Diagram represents the number of proteins identified in the four groups with a minimum of 3 or more unique peptides identified with MS/MS.

a)



b)

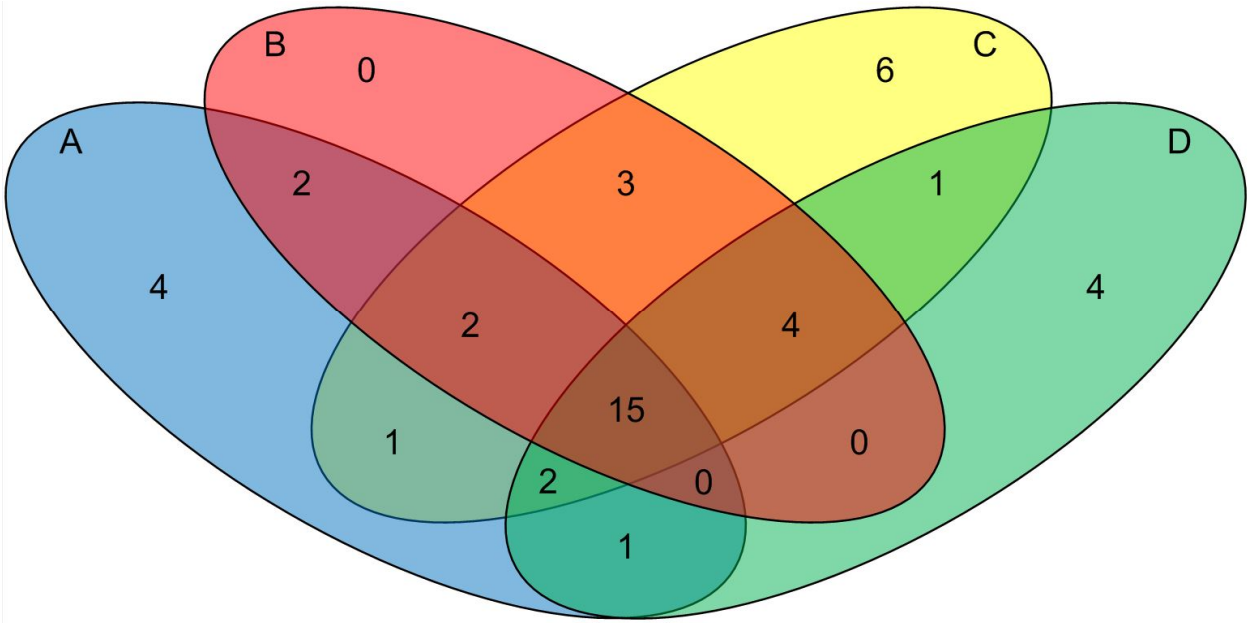


Table 2.6 – Summary of 15 most abundant proteins from mass spectrometric protein identifications for children pre-, during, post-eruption, and edentulous adults, Groups A, B, C, D, respectively. This table summarizes the name, molecular weight, number of unique peptide counts, spectrum counts, and unique spectrum counts for the top 15 most abundant proteins across Groups A, B, C, and D, identified in Table 2.5. Scaffold (version Scaffold_2_06_02) was used to validate the search results, with an acceptable peptide probability of greater than 95.0%. The number zero (0) indicates no peptides and therefore no spectra were detected from the specified protein.

#	Identified Proteins (79)	Molecular Weight	Group A			Group B			Group C			Group D		
			Unique Peptide Counts	Spectrum Counts	Unique Spectrum Counts	Unique Peptide Counts	Spectrum Counts	Unique Spectrum Counts	Unique Peptide Counts	Spectrum Counts	Unique Spectrum Counts	Unique Peptide Counts	Spectrum Counts	Unique Spectrum Counts
1	Chain A, Recombinant Human Salivary Amylase	56 kDa	37	134	45	34	208	47	37	234	52	23	134	35
2	albumin, isoform CRA_h [Homo sapiens]	69 kDa	12	21	17	20	35	27	20	62	30	12	29	17
3	peptide PB,saliva	6 kDa	7	29	10	10	32	12	14	39	18	14	53	17
4	mucin-5B precursor [Homo sapiens]	596 kDa	16	22	16	11	11	11	11	12	11	7	7	7
5	unnamed protein product [Homo sapiens]	19 kDa	10	72	13	8	24	9	12	52	15	10	24	12
6	Polymeric immunoglobulin receptor, transmembrane secretory component	83 kDa	11	18	12	11	16	12	11	30	14	6	20	9
7	parotid secretory protein [Homo sapiens]	27 kDa	12	18	15	7	8	8	8	10	8	7	8	7
8	unnamed protein product [Homo sapiens]	53 kDa	7	16	9	9	13	11	10	22	13	7	23	8
9	hypothetical protein LOC352999 precursor [Homo sapiens]	38 kDa	8	9	8	6	12	7	10	22	12	6	10	7
10	carbonic anhydrase VI [Homo sapiens]	35 kDa	7	13	8	7	15	7	6	19	7	6	15	7
11	Chain B, Mhc-Like Zinc Alpha2-Glycoprotein And Prolactin Inducible Protein	14 kDa	5	23	6	6	23	10	6	19	8	6	11	7
12	Mucin 7, secreted [Homo sapiens]	39 kDa	9	22	12	7	18	10	4	10	5	3	7	3
13	Chain A, Zn-Alpha-2-Glycoprotein	32 kDa	5	9	5	7	10	7	7	10	8	4	9	6
14	lactoperoxidase isoform 1 preproprotein [Homo sapiens]	80 kDa	12	14	12	2	2	2	4	5	4	5	6	6
15	lipocalin-1 precursor [Homo sapiens], von Ebner's gland protein	19 kDa	8	10	9	10	14	12	5	6	5	0	0	0

2.4 Discussion

2.4.1 Importance of Sample Preparation and Experimental Design

Importance of Sample Preparation

Sample preparation, including collection, storage and processing is of fundamental importance when working with whole saliva. During the analysis of whole saliva, one must keep in mind the multiple sources that contribute to saliva's composition, including host-derived and exogenously introduced substances. Ordinary activities, such as brushing one's teeth, may be sufficient activity to cause minor injury and introduce serum components into whole saliva. Salivary composition is influenced by enzymatic activity of both host and bacterially derived proteins. This influence can be mitigated through careful collection, storage and processing techniques. Such techniques include collecting and storing samples at temperatures that inhibit metabolism, centrifuging saliva samples immediately after collection to eliminate bacterial and cellular debris. Adding enzyme inhibitors to collection tubes to prevent protein cleavage post-collection, as well as during storage and processing is a potentially useful measure to be taken to protect the biochemical properties of saliva samples in future studies. The practical aspects of sample collection and analysis are of fundamental importance to the completion and interpretation of this thesis and salivary research as a whole.

The effects of quality handling of samples on the biochemical properties and composition of saliva should not be underestimated. This is especially the case when dealing with studies in children. As was the case in this research, the precisely

controlled and monitored collection of unstimulated whole saliva samples from healthy children presented more challenges in some respects than the collection of unstimulated whole saliva from adult controls. It was imperative to maintain a consistent collection protocol in all sample collections, including the requirement of all collections to be performed: during the same limited window of time during the day, from individuals in good health who had not had anything to eat or drink for a full hour before the time of collection, for all individuals to be at rest before collection starts and to maintain the state of rest until sample collection is completed, and for all individuals to be free of medications. The significance of ensuring all individuals were free of medications is outlined later in this discussion. Ensuring all of these criteria were fully met for each collection was a source of difficulty in collecting saliva from children. Special mention is given to the challenge of the maintenance of a restful state in very young children before and during saliva collection.

Being aware of, and controlling as many variables as possible in the collection process reduces the number of potential confounders that may affect the composition of saliva unrelated to the hypothesis being tested, such as the variation in the salivary proteome with age/developmental stage, at the focus of this work.

Once collected, the processing of samples became of primary importance. Salivary samples were never left at room temperature during processing or storage. Unless the samples were actively being processed, they were kept frozen. The handling of saliva requires mindful planning of the number of times a single saliva sample requires freezing and thawing. During the freeze-thaw cycle, some salivary proteins (namely the large glycoproteins of the mucin family) come out of solution. The

precipitation of proteins out of solution can be greatly prevented through limiting the number of freezes and thaws experienced by a sample. Samples can generally be safely frozen twice before any noticeable changes in the consistency of the saliva appears.

A consideration to keep in mind is the importance of standardizing methods of sample collection and processing between studies to enable wide-scale comparison of findings. A standard method of processing whole saliva samples described throughout the literature includes the centrifugation of samples followed by the collection and analysis of the supernatant. The whitish pellet that remains at the bottom of the centrifuged tube is rarely discussed. The composition of the pellet may also prove to be a useful source of biological information, as proteins and other molecules of potential interest, may be trapped in the debris.

Importance of Experimental Design

The selection of techniques in this study of the changes in the salivary proteome during the course of dental eruption was designed to offer as much useful information as possible with the amount of samples collected. As previously mentioned, both 1- and 2-dimensional PAGE (1D- and 2D-PAGE) are effective methods of protein separation and have been widely used to separate salivary proteins (4, 6, 14, 29, 81). Both methods provide visuals of profiles of protein mixtures that can be used to assess variability between samples, people, and groups. 2D-PAGE is a powerful technique of protein separation but harbors significant limitations that remain highly relevant in salivary protein research. 2D-PAGE is ill suited for the detection of small MW proteins,

highly acidic or basic proteins, highly hydrophobic proteins, as well as proteins in low abundance (6). The presence of many small MW proteins and peptides, as well as a significant number of highly acidic, basic, hydrophobic and proteins present in very low concentrations in saliva, makes it clear that 2D-PAGE is not of great use to study the changes in the complete salivary proteome. Instead, two varieties of 1D-PAGE were employed to separate and visualize proteins in their native state (anionic PAGE) and in a denatured state (SDS-PAGE). With the use of both the negative charge (anionic) and molecular weight (SDS) as differentiating characteristics, a third mode of separation and visualization was then employed. Reverse-phase HPLC was used to separate whole proteins based on their degree of hydrophobicity. The fourth technique for protein analysis involved digesting the salivary proteins with trypsin prior to separation via reverse-phase HPLC inline with the mass spectrometer. This variety of techniques worked in concert together to maximize the quality and quantity of useful information and cross-technique validations.

2.4.2 Protein Quantification

Quantification of Total Protein Concentration

In addition to the discussion of quality handling of the saliva samples during collection, storage and processing, the quantification of protein concentration must be highlighted. The very first quantification of salivary protein concentration in any sample in this study was the measurement of total protein concentration. While numerous methods are available to achieve the quantification of total protein in a sample, saliva's varied and complex composition, as well as the large normal range of total protein

concentration, narrows the selection of appropriate protein concentration assays. This work compared the results of two well-established protein quantification methods, the Bradford assay and the BCA assay. The most suitable protein quantification assays are those in which the properties being measured are equally distributed within all samples. The BCA assay's detection of peptides bonds and four amino acids in salivary samples is much more representative of the total protein concentration than the less uniformly distributed hydrophobic and ionic interactions detected by the Bradford method. For this reason, the BCA assay was selected for this study. Ultimately, the basic salivary composition greatly guides the selection of the techniques and technologies applicable to its study. Once the total protein concentration of all the samples were achieved, the focus shifted to the accurate quantification of individual or subsets of proteins within saliva, such as those achieved with the quantification of protein profile bands in both SDS and anionic PAGE experiments.

Quantification of PAGE Band Intensities

The quantification of bands from both types of PAGE (SDS and anionic) demonstrated clear visual relative quantitative representations of protein profiles for individuals in all four groups. Anionic PAGE revealed a striking difference in the protein profile of children with complete dental eruption (Group C), compared to the children with no dentition (Group A), children with partial dentition (Group B), and adults with no dentition (Group D) (Figures 2.3 and 2.4). The greatest difference is seen in the section of the gel with the same relative migration as the human serum albumin control. Albumin is a major salivary protein derived from serum exudate that enters the oral cavity at the interface between the teeth and gums through a source known as gingival

crevicular fluid (GCF). An increase in the number of teeth erupted produces a larger interface surface between the teeth and gums and therefore a larger the potential contribution of serum exudate components harbored in GCF.

As expected, the group with the highest number of erupted teeth (Group C) has the highest concentration of potential HSA. The group with a fewer number of erupted teeth (Group B) has a notably lower concentration of potential albumin. Lastly, the two groups with no teeth (Groups A and D) have an even lower concentration of potential albumin. The relative protein quantification provided by the MS analyses (seen in Table 2.6) are perfectly aligned with this trend of albumin concentration increasing as the number of teeth increase. These findings serve to highlight the importance of studying children throughout the course of dental eruption, as clear quantitative changes are detectable at different stages of development. These changes must be taken into account prior to development of standard baseline measurements of salivary protein profiles.

The SDS PAGE also revealed a striking difference in the protein profile of children at different stages of dental eruption as well as the adult edentulous controls. Figures 2.6-2.9 suggest the degree of variation between individuals of the same group (intra-group variability) decreases with age. The greatest differences in the protein profiles are seen in the youngest children (Group A- approximate age 0-6 months), with increasingly more conformity to a single protein profile trend as the average age of the subjects increases (Groups B, C, and finally adults in Group D).

The inter-group variation summaries displayed in Figures 2.11 and 2.12 illuminate the prominent differences in the average protein profiles of each of the four groups. While both figures summarize the same information, the data was acquired in two ways. Figure 2.11 summarizes data of an independent gel loaded with each lane representing a single group, while Figure 2.12 displays data of summed protein profiles from different gels (Figure 2.12). While similar conclusions can be drawn from both summaries, attention is drawn to Figure 2.11, as it provides a more direct comparison of the protein profiles run simultaneously. As seen in Figure 2.11, Group A (the youngest children in this study, representing the salivary proteome pre-dental eruption) appears to have a far greater abundance of large MW proteins (80 kDa or greater) relative to all the other groups. The relative protein quantification provided by the MS analyses (seen in Table 2.6) are also perfectly aligned with this observation. The relative abundance of mucin-5B precursor (596 kDa) is seen in much greater abundance in the youngest cohort (Group A), than in the other 3 groups. This may indicate a lower amount of proteolytic cleavage experienced in children with no dentition. In the absence of teeth, as is the case in Group A, the proteins that are known to adhere to the surface of teeth are not yet established in a stable or permanent way in the oral environment. With a decrease in the number of surfaces available for the adherence of proteolytic enzymes, it is expected to observe a lower concentration of these enzymes and their associated proteolytic activity. Related to the anionic PAGE results, the SDS PAGE results in Figure 2.11 displayed the greater abundance of protein at approximately 60 kDa in Group C relative to all the other groups. HSA, the most abundant protein in blood, has a known molecular weight of 67 kDa. As underscored previously in the discussion of the

anionic PAGE results, this striking difference of Group C relative to the other groups may be an indication a protein derived from GCF, as Group C is the only group with fully present dentition and therefore a maximum interface of teeth and gums (the entry point of GCF into saliva).

While these results across PAGE platforms are promising, quantifications of protein profiles will be more useful once protein identities can be confidently assigned. To enhance the quality of comparison, and help control for variability between migrations of samples on different gels, an internal control of a single individual can be run consistently on all gels.

2.4.3 Protein Separation

As the resolution of separation is increased, an even greater wealth of information may be derived from the analysis. We were able to separate proteins in whole saliva based on molecular, negative charge and hydrophobicity. Additional separation may also be achieved using techniques such as positive charge or protein binding affinities to further develop the complete picture of the components of the salivary proteome at different ages.

While often very time-consuming, method optimization with liquid chromatography provides very high-resolution separation on a multitude of dimensions, dependent on column and gradient selection. Chromatograms also allow for the potential of peak quantification if a panel of isolated proteins may be acquired to correlate the area under the curve with absorbance for each protein species.

2.4.4 Proteins Identified

The MS analysis completed in this study resulted in the identification of fewer than 100 proteins (79 proteins) across all groups. The rather low number of protein identifications across the four groups could be expanded when greater sample volumes are collected and made available to repeat the MS runs more than the duplicates completed in this study. A greater number of runs would further increase the likelihood of identifying more lower abundance proteins. It is important to note that proteins present in lower abundance are often of greatest interest in diagnostic testing. A longer liquid chromatography separation method, from the 80 minutes described in this study to 2-4 hours, would enhance protein separation, and may result in an increase in the number of successful protein identification.

Alternative pre-processing of the samples prior to loading on the inline HPLC may serve to further enhance the number and confidence of protein identifications. Due to the presence of high-abundance proteins (i.e. albumin, mucin, complement component proteins), immunodepletion may be used to help reveal proteins in lower abundance. Mucin is a good candidate to target with immunodepletion because of its great abundance in whole saliva, its large size (>500 kDa), and extensive glycosylation making it highly susceptible to aggregation. Immunodepletion, much like any purification or simplifying measure, may be associated with undesired effects, such as the elimination of other protein species through non-specific binding, or their close association with the targeted and eliminated protein. The detection sensitivity and quantitation technologies that continue to advance in the MS field have the potential to

provide a more detailed assessment of differentially expressed proteins in saliva, especially if the complexity of the samples can be successfully managed.

Even with the limited number of protein identifications, a number of interesting stories have come to light. For example, beta-casein, a member of a phosphoprotein family present in mammalian milk, was detected only in the youngest cohort (Group A – children from approximately 0-6 months of age). This finding helps to validate the techniques of sample collection and analysis, as children 0-6 months of age have a diet primarily composed of mammalian milk.

The relative protein quantification trends provided by the MS analyses (seen in Table 2.6) serve as rough estimates of trends present in the salivary proteome. More replicates and greater number of identified peptides and spectra are required to draw definitive and absolute conclusions. This study describes relative abundance trends in specific proteins between groups. The relative abundance trends are extracted from Table 2.6 by comparing the total number of unqiue spectra counts for each protein in the different groups. As previously defined, unique spectrum counts is the term used to represent the number of one of a kind MS/MS fragmentation spectra that map to peptides of a given protein. The number of unqiue spectrum counts may be greater than the number of unique peptide counts due to the presence of variable modifications, such as oxidation of methionine. The same unique peptide may be counted as multiple unique spectra if the variable modifications are present in one spectra, but not in another. Spectral counting offers a means of comparing protein abundance across groups, if the same preparation and isolation techniques were applied for each experiment.

There are critical considerations to keep in mind when using abundance comparisons obtained through spectral counting. The first major consideration to highlight is the appropriate use of inter-group comparisons of a given protein, rather than inter-protein comparisons even within the same group, which are not reliable. Abundance comparisons through spectral counting does not allow for a proper comparison of different proteins and their relative abundance. This is due to technical considerations of MS-based approaches that depend on the efficiency of protein digestion (related to size, and number of lysine and arginine residues, etc.), the ionization efficiency, and the quality of HPLC separation (i.e. presence of co-elution). It is for this reason that the rank order of the top 15 quantified proteins is not emphasized significantly, but rather the focus is given to the relative abundance of a single protein between different groups. Comparing the relative abundance of one protein with another even within the same group requires careful attention and validation, due to the points mentioned above. The quality of HPLC separation, and presence of co-elutioning proteins is also a point of consideration for valid inter-group comparisons of a given protein, as the variable composition of samples between groups can serve to mask a protein in some groups but not equally in others.

Despite the issues associated with reliability of protein abundance comparison, spectral counting provides a reasonable estimation method for ball-park global quantification of abundant proteins. The results that were achieved in this study serve as a good reference point from which to continue exploring. The following paragraphs highlight the most significant findings in abundance trends, as determined by the quantification of unique peptide and spectrum counts. The use of unqiue ion counts is

another very valid route to describe these findings, and is preferred by some researchers. For the purpose of this analysis and as is often the case, both measurements result in the same relative rank order of the top most abundant identified proteins, and resultant abundance trends. The cut-off determined for categorizing the top 15 proteins (as displayed in Figure 2.6) was determined by the minimum unique peptide count set at 2, if a peptide was detected at all.

Amylase (56 kDa) was found to be in lowest abundance in the adult controls (Group D), relative to all the child cohorts (Groups A, B, and C). The highest abundance of amylase was observed in children post dental eruption (Group C). This could reflect the requirement of higher amylase concentrations in individuals with complete dentition, as amylase functions to prevent bacterial attachment to the tooth surface and assist in bacterial clearance.

Albumin's (69 kDa) relative abundance trend serves as an excellent example of cross-technique validation in this study. The relative MS quantification of albumin validates the findings from the anionic gels, as the relative abundance of albumin increases as the number of erupted teeth increases. Albumin's abundance is nearly 2-fold greater in children with all of their primary dentition (Group C), relative to children pre-dental eruption (Group A). The relative abundance of albumin in Group B (children during dental eruption) is between that of Group A and Group C, as to be expected if the albumin concentration is related to the potential GCF contribution in children with teeth versus children without any teeth.

Lactoperoxidase isoform 1 preproprotein (80 kDa) was detected in the youngest group (Group A) with approximately a two-fold greater abundance than in all the other groups (Groups B, C, and D). The presence of this lactoperoxidase precursor molecule in saliva is not a surprise, as lactoperoxidase is a well-known endogenous salivary protein produced by the salivary glands. Lactoperoxidase is one of the two major peroxidases in whole saliva, along with myeloperoxidase, functioning primarily as an anti-bacterial agent. The heighten abundance of this innate immune molecule in the youngest group (Group A) is again well inline with the large innate immune response in infants, that compensates for their weaker adaptive immune system which is not fully developed in the first few months of life.

As previously mentioned, the relative abundance of mucin (mucin-5B precursor, and mucin 7) is highest in children pre-dental eruption (Group A), relative to all the other groups, as suggested and described in the SDS-PAGE results.

Von Ebner's gland protein (19 kDa) was detected in all the children groups (Group A, B, and C), but not in the edentulous adult controls (Group D). This finding has not previously been documented.

The following proteins were not seen to change dramatically between the four groups: Carbonic anhydrase VI (35 kDa), Prolactin Inducible Protein, unnamed protein products with a MW of 53 kDa and 19 kDa, and a hypothetical protein of 38 kDa. The unnamed protein products and hypothetical protein are good candidates for further interaction studies, as very little is known about them and their functions in whole saliva.

To enable the further relative and absolute quantitative measurements of salivary proteins in different groups, proteins may be labeled prior to separation and mass spectrometry. Protein labeling (iTRAQ) was attempted during the course of this work but required more biological samples than were presently available from the collection from all individuals to return meaningful results. Through the combination of immunodepletion to increase the probability of low abundance protein identification, as well as protein labeling, more meaningful quantitative results of a panel of salivary proteins may be achieved.

2.4.5 Future Work

The motivation for identifying and understanding the changes in the salivary proteome for all ages in health or pathology is to lay the groundwork for the development of diagnostic tools to assess the physiological state of individuals through non-invasive salivary testing. Understanding the differences in salivary proteins and the salivary proteome present throughout life in health and disease is a necessity to accurately identify salivary protein-drug interactions, to accurately measure salivary drug concentrations and identify disease markers in saliva.

The advancement of useful clinical tools must be firmly planted in an understanding of the biochemistry of saliva and the ways in which composition is altered with age, states of health and presence or absence of drugs. The work with unstimulated saliva from healthy individuals, such as that of this research, is truly the foundation that must be set before the study of the changes in composition of the salivary proteome can be expanded to include diseased states and drug-induced

changes in salivary biochemistry. The recognition of a drugs ability to alter the salivary proteome is of tremendous importance, especially in the development of drug-specific assays for the monitoring of drug concentrations in saliva.

Some of the most helpful advances in salivary research are those that bring to light the often unmentioned considerations that are critical to producing reproducible and meaningful results. This study encourages future work to be mindful of the careful monitoring of salivary sample collection, storage and processing techniques. Future work must recognize the importance of careful selection of individuals free of medications, as this is imperative at this stage of analysis. Many drugs are able to interfere with the process of salivary secretion, as well as binding salivary proteins. Much attention is needed to elucidate the drug-associated changes in the salivary proteome, once a healthy baseline is confidently identified at different ages and states.

To achieve this aim, future work is needed with a focus on the determination of protein identities separated with PAGE and liquid chromatography. The use of mass spectrometric identification of proteins can allow us to better elucidate differences, specifically in young children who have been seen here to have notably unique salivary protein profiles as compared not only with adult controls, but also between developmental stages in children as dental eruption events unfold.

We must remain conscious of the developmental stage of an individual as we work towards future diagnostic test development. As seen in this study, age plays a noticeable role in the salivary protein profile. This age-specific focus on protein profiling needs to be continued to provide a higher resolution image of the dynamic changes in

the salivary proteome. In future studies, the close monitoring of the age and developmental stage (i.e. pre- or post-dental eruption) of an individual will be very useful to account for and limit the variation of the protein profile related to age. More replicates are needed to draw definitive conclusions on the relative and absolute quantified changes in the salivary proteome. A greater sample size ($n > 48$ per group), as determined by this study, and larger sample volumes will serve to increase the amount of testing possible, and as a result, increasing the quantity of findings and confidence in future studies.

Once a more comprehensive description of the changing salivary proteome in healthy children is achieved, protein profiles of children with specific pathologies may be compared to identify presence of any distinguishing salivary protein markers. The greater degree of protein profile variability observed in this study in children 0-6 months of age, relative to children 6 months-3 years of age, as well as adult controls, will hopefully stir further excitement and enthusiasm in the efforts focused on uncovering of the complexities of the dynamic nature of the salivary proteome in the youngest of children. It is this youngest cohort that may benefit the most from repeated non-invasive and pain-free salivary diagnostics to inform clinical decisions. With a better understanding, salivary protein markers may then be further explored for relevance in diagnostic, prognostic and/or condition/treatment monitoring tests. This will provide insights into the potential use of salivary drug concentrations for therapeutic drug monitoring in children.

By addressing the limitations of this present research, future efforts can build on this knowledge and continue to move the field of salivary biochemistry, and ultimately salivary diagnostics in children, forward and into the medical and dental clinics.

Chapter 3 – References

1. Humphrey SP, Williamson RT. A review of saliva: Normal composition, flow, and function. J Prosthet Dent. 2001 Feb;85(2):162-9.

2. Macarthur DJ, Jacques NA. Proteome analysis of oral pathogens. J Dent Res. 2003 Nov;82(11):870-6.

3. Edgar WM, Dawes C, O'Mullane DM. Saliva and oral health. 3rd ed. London: British Dental Association; 2004.

4. Huang CM. Comparative proteomic analysis of human whole saliva. Arch Oral Biol. 2004 Dec;49(12):951-62.

5. Guo T, Rudnick PA, Wang W, Lee CS, Devoe DL, Balgley BM. Characterization of the human salivary proteome by capillary isoelectric focusing/nanoreversed-phase liquid chromatography coupled with ESI-tandem MS. J Proteome Res. 2006 Jun;5(6):1469-78.

6. Schipper RG, Silletti E, Vingerhoeds MH. Saliva as research material: Biochemical, physicochemical and practical aspects. Arch Oral Biol. 2007 Dec;52(12):1114-35.

7. de Almeida Pdel V, Gregio AM, Machado MA, de Lima AA, Azevedo LR. Saliva composition and functions: A comprehensive review. J Contemp Dent Pract. 2008 Mar 1;9(3):72-80.

8. Rosa N, Correia MJ, Arrais JP, Lopes P, Melo J, Oliveira JL, et al. From the salivary proteome to the OralOme: Comprehensive molecular oral biology. Arch Oral Biol. 2012 Jul;57(7):853-64.

9. Pfaffe T, Cooper-White J, Beyerlein P, Kostner K, Punyadeera C. Diagnostic potential of saliva: Current state and future applications. Clin Chem. 2011 May;57(5):675-87.

10. Kaufman E, Lamster IB. Analysis of saliva for periodontal diagnosis--a review. J Clin Periodontol. 2000 Jul;27(7):453-65.

11. Miller CS, King CP,Jr, Langub MC, Kryscio RJ, Thomas MV. Salivary biomarkers of existing periodontal disease: A cross-sectional study. J Am Dent Assoc. 2006 Mar;137(3):322-9.

12. Kalk WW, Vissink A, Stegenga B, Bootsma H, Nieuw Amerongen AV, Kallenberg CG. Sialometry and sialochemistry: A non-invasive approach for diagnosing sjogren's syndrome. Ann Rheum Dis. 2002 Feb;61(2):137-44.

13. Goncalves Lda R, Soares MR, Nogueira FC, Garcia C, Camisasca DR, Domont G, et al. Comparative proteomic analysis of whole saliva from chronic periodontitis patients. J Proteomics. 2010 May 7;73(7):1334-41.

14. Siqueira WL, Salih E, Wan DL, Helmerhorst EJ, Oppenheim FG. Proteome of human minor salivary gland secretion. J Dent Res. 2008 May;87(5):445-50.

15. Denny P, Hagen FK, Hardt M, Liao L, Yan W, Arellanno M, et al. The proteomes of human parotid and submandibular/sublingual gland salivas collected as the ductal secretions. J Proteome Res. 2008 May;7(5):1994-2006.

16. Huq NL, Cross KJ, Ung M, Myroforidis H, Veith PD, Chen D, et al. A review of the salivary proteome and peptidome and saliva-derived peptide therapeutics. Int J Pept Res Ther. 2007;13(4):547-64.

17. Oppenheim FG, Salih E, Siqueira WL, Zhang W, Helmerhorst EJ. Salivary proteome and its genetic polymorphisms. Ann N Y Acad Sci. 2007 Mar;1098:22-50.

18. Hu S, Xie Y, Ramachandran P, Ogorzalek Loo RR, Li Y, Loo JA, et al. Large-scale identification of proteins in human salivary proteome by liquid chromatography/mass spectrometry and two-dimensional gel electrophoresis-mass spectrometry. Proteomics. 2005 Apr;5(6):1714-28.

19. Vitorino R, Lobo MJ, Ferrer-Correira AJ, Dubin JR, Tomer KB, Domingues PM, et al. Identification of human whole saliva protein components using proteomics. Proteomics. 2004 Apr;4(4):1109-15.

20. Yao Y, Berg EA, Costello CE, Troxler RF, Oppenheim FG. Identification of protein components in human acquired enamel pellicle and whole saliva using novel proteomics approaches. J Biol Chem. 2003 Feb 14;278(7):5300-8.

21. Fang X, Yang L, Wang W, Song T, Lee CS, DeVoe DL, et al. Comparison of electrokinetics-based multidimensional separations coupled with electrospray ionization-tandem mass spectrometry for characterization of human salivary proteins. Anal Chem. 2007 Aug 1;79(15):5785-92.

22. Hardt M, Thomas LR, Dixon SE, Newport G, Agabian N, Prakobphol A, et al. Toward defining the human parotid gland salivary proteome and peptidome: Identification and characterization using 2D SDS-PAGE, ultrafiltration, HPLC, and mass spectrometry. Biochemistry. 2005 Mar 1;44(8):2885-99.

23. Walz A, Stuhler K, Wattenberg A, Hawranke E, Meyer HE, Schmalz G, et al. Proteome analysis of glandular parotid and submandibular-sublingual saliva in comparison to whole human saliva by two-dimensional gel electrophoresis. Proteomics. 2006 Mar;6(5):1631-9.

24. Wilmarth PA, Riviere MA, Rustvold DL, Lauten JD, Madden TE, David LL. Two-dimensional liquid chromatography study of the human whole saliva proteome. J Proteome Res. 2004 Sep-Oct;3(5):1017-23.

25. Xie H, Rhodus NL, Griffin RJ, Carlis JV, Griffin TJ. A catalogue of human saliva proteins identified by free flow electrophoresis-based peptide separation and tandem mass spectrometry. Mol Cell Proteomics. 2005 Nov;4(11):1826-30.

26. Cabras T, Pisano E, Boi R, Olianas A, Manconi B, Inzitari R, et al. Age-dependent modifications of the human salivary secretory protein complex. J Proteome Res. 2009 Aug;8(8):4126-34.

27. Castagnola M, Inzitari R, Fanali C, Iavarone F, Vitali A, Desiderio C, et al. The surprising composition of the salivary proteome of preterm human newborn. Mol Cell Proteomics. 2011 Jan;10(1):M110.003467.

28. Amado FM, Vitorino RM, Domingues PM, Lobo MJ, Duarte JA. Analysis of the human saliva proteome. Expert Rev Proteomics. 2005 Aug;2(4):521-39.

29. Ruhl S, Rayment SA, Schmalz G, Hiller KA, Troxler RF. Proteins in whole saliva during the first year of infancy. J Dent Res. 2005 Jan;84(1):29-34.

30. Pedersen AM, Bardow A, Jensen SB, Nauntofte B. Saliva and gastrointestinal functions of taste, mastication, swallowing and digestion. Oral Dis. 2002 May;8(3):117-29.

31. Proctor GB, Carpenter GH. Regulation of salivary gland function by autonomic nerves. Auton Neurosci. 2007 Apr 30;133(1):3-18.

32. Silvers AR, Som PM. Salivary glands. Radiol Clin North Am. 1998 Sep;36(5):941,66, vi.

33. Melvin JE, Yule D, Shuttleworth T, Begenisich T. Regulation of fluid and electrolyte secretion in salivary gland acinar cells. Annu Rev Physiol. 2005;67:445-69.

34. Turner RJ, Sugiya H. Understanding salivary fluid and protein secretion. Oral Dis. 2002 Jan;8(1):3-11.

35. Barrett KE. Gastrointestinal physiology. New York: Lange Medical Books/McGraw-Hill, Medical Pub. Division; 2006.

36. Sivakumar T, Hand AR, Mednieks M. Secretory proteins in the saliva of children. J Oral Sci. 2009 Dec;51(4):573-80.

37. Glantz PO, Friberg SE, Wirth SM, Baier RE. Thin-section transmission electron microscopy of human saliva. Acta Odontol Scand. 1989 Apr;47(2):111-6.

38. Hardt M, Witkowska HE, Webb S, Thomas LR, Dixon SE, Hall SC, et al. Assessing the effects of diurnal variation on the composition of human parotid saliva: Quantitative analysis of native peptides using iTRAQ reagents. Anal Chem. 2005 Aug 1;77(15):4947-54.

39. Castagnola M, Cabras T, Vitali A, Sanna MT, Messana I. Biotechnological implications of the salivary proteome. Trends Biotechnol. 2011 Aug;29(8):409-18.

40. Kaufman E, Lamster IB. The diagnostic applications of saliva--a review. Crit Rev Oral Biol Med. 2002;13(2):197-212.

41. Edgar WM. Saliva: Its secretion, composition and functions. Br Dent J. 1992 Apr 25;172(8):305-12.

42. Chen T, Yu WH, Izard J, Baranova OV, Lakshmanan A, Dewhirst FE. The human oral microbiome database: A web accessible resource for investigating oral microbe taxonomic and genomic information. Database (Oxford). 2010 Jul 6;2010:baq013.

43. Helmerhorst EJ, Oppenheim FG. Saliva: A dynamic proteome. J Dent Res. 2007 Aug;86(8):680-93.

44. McDonald EE, Goldberg HA, Tabbara N, Mendes FM, Siqueira WL. Histatin 1 resists proteolytic degradation when adsorbed to hydroxyapatite. J Dent Res. 2011 Feb;90(2):268-72.

45. Koyama I, Komine S, Yakushijin M, Hokari S, Komoda T. Glycosylated salivary alpha-amylases are capable of maltotriose hydrolysis and glucose formation. Comp Biochem Physiol B Biochem Mol Biol. 2000 Aug;126(4):553-60.

46. Granger DA, Kivlighan KT, el-Sheikh M, Gordis EB, Stroud LR. Salivary alpha-amylase in biobehavioral research: Recent developments and applications. Ann N Y Acad Sci. 2007 Mar;1098:122-44.

47. Hirtz C, Chevalier F, Centeno D, Rofidal V, Egea JC, Rossignol M, et al. MS characterization of multiple forms of alpha-amylase in human saliva. Proteomics. 2005 Nov;5(17):4597-60.

48. Oudhoff MJ, Bolscher JG, Nazmi K, Kalay H, van 't Hof W, Amerongen AV, et al. Histatins are the major wound-closure stimulating factors in human saliva as identified in a cell culture assay. FASEB J. 2008 Nov;22(11):3805-12.

49. Oppenheim FG, Yang YC, Diamond RD, Hyslop D, Offner GD, Troxler RF. The primary structure and functional characterization of the neutral histidine-rich polypeptide from human parotid secretion. J Biol Chem. 1986 Jan 25;261(3):1177-82.

50. Tsai H, Bobek LA. Human salivary histatins: Promising anti-fungal therapeutic agents. Crit Rev Oral Biol Med. 1998;9(4):480-97.

51. Prakobphol A, Levine MJ, Tabak LA, Reddy MS. Purification of a low-molecular-weight, mucin-type glycoprotein from human submandibular-sublingual saliva. Carbohydr Res. 1982 Oct 1;108(1):111-22.

52. Veerman EC, van den Keybus PA, Vissink A, Nieuw Amerongen AV. Human glandular salivas: Their separate collection and analysis. Eur J Oral Sci. 1996 Aug;104(4 (Pt 1)):346-52.

53. Tabak LA, Levine MJ, Mandel ID, Ellison SA. Role of salivary mucins in the protection of the oral cavity. J Oral Pathol. 1982 Feb;11(1):1-17.

54. Schenkels LC, Veerman EC, Nieuw Amerongen AV. Biochemical composition of human saliva in relation to other mucosal fluids. Crit Rev Oral Biol Med. 1995;6(2):161-75.

55. Johnsson M, Levine MJ, Nancollas GH. Hydroxyapatite binding domains in salivary proteins. Crit Rev Oral Biol Med. 1993;4(3-4):371-8.

56. Gerken TA. Biophysical approaches to salivary mucin structure, conformation and dynamics. Crit Rev Oral Biol Med. 1993;4(3-4):261-70.

57. Kivela J, Parkkila S, Parkkila AK, Rajaniemi H. A low concentration of carbonic anhydrase isoenzyme VI in whole saliva is associated with caries prevalence. Caries Res. 1999 May-Jun;33(3):178-84.

58. Mansson-Rahemtulla B, Rahemtulla F, Baldone DC, Pruitt KM, Hjerpe A. Purification and characterization of human salivary peroxidase. Biochemistry. 1988 Jan 12;27(1):233-9.

59. Ihalin R, Loimaranta V, Tenovuo J. Origin, structure, and biological activities of peroxidases in human saliva. Arch Biochem Biophys. 2006 Jan 15;445(2):261-8.

60. Welk A, Meller C, Schubert R, Schwahn C, Kramer A, Below H. Effect of lactoperoxidase on the antimicrobial effectiveness of the thiocyanate hydrogen peroxide combination in a quantitative suspension test. BMC Microbiol. 2009 Jul 9;9:134,2180-9-134.

61. Ashby MT. Inorganic chemistry of defensive peroxidases in the human oral cavity. J Dent Res. 2008 Oct;87(10):900-14.

62. Ayad M, Van Wuyckhuyse BC, Minaguchi K, Raubertas RF, Bedi GS, Billings RJ, et al. The association of basic proline-rich peptides from human parotid gland secretions with caries experience. J Dent Res. 2000 Apr;79(4):976-82.

63. Hahn Berg IC, Lindh L, Arnebrant T. Intraoral lubrication of PRP-1, statherin and mucin as studied by AFM. Biofouling. 2004 Feb;20(1):65-70.

64. Proctor GB, Hamdan S, Carpenter GH, Wilde P. A statherin and calcium enriched layer at the air interface of human parotid saliva. Biochem J. 2005 Jul 1;389(Pt 1):111-6.

65. Aguirre A, Mendoza B, Reddy MS, Scannapieco FA, Levine MJ, Hatton MN. Lubrication of selected salivary molecules and artificial salivas. Dysphagia. 1989;4(2):95-100.

66. Hatton MN, Loomis RE, Levine MJ, Tabak LA. Masticatory lubrication. the role of carbohydrate in the lubricating property of a salivary glycoprotein-albumin complex. Biochem J. 1985 Sep 15;230(3):817-20.

67. Amerongen AV, Veerman EC. Saliva--the defender of the oral cavity. Oral Dis. 2002 Jan;8(1):12-2.

68. Jehmlich N, Dinh KH, Gesell-Salazar M, Hammer E, Steil L, Dhople VM, et al. Quantitative analysis of the intra- and inter-subject variability of the whole salivary proteome. J Periodontal Res. 2013 Jun;48(3):392-403.

69. Al Kawas S, Rahim ZH, Ferguson DB. Potential uses of human salivary protein and peptide analysis in the diagnosis of disease. Arch Oral Biol. 2012 Jan;57(1):1-9.

70. Goncalves Lda R, Soares MR, Nogueira FC, Garcia CH, Camisasca DR, Domont G, et al. Analysis of the salivary proteome in gingivitis patients. J Periodontal Res. 2011 Oct;46(5):599-606.

71. Fleissig Y, Reichenberg E, Redlich M, Zaks B, Deutsch O, Aframian DJ, et al. Comparative proteomic analysis of human oral fluids according to gender and age. Oral Dis. 2010 Nov;16(8):831-8.

72. Loo JA, Yan W, Ramachandran P, Wong DT. Comparative human salivary and plasma proteomes. J Dent Res. 2010 Oct;89(10):1016-23.

73. Zhang L, Xiao H, Karlan S, Zhou H, Gross J, Elashoff D, et al. Discovery and preclinical validation of salivary transcriptomic and proteomic biomarkers for the non-invasive detection of breast cancer. PLoS One. 2010 Dec 31;5(12):e15573.

74. Dowling P, Wormald R, Meleady P, Henry M, Curran A, Clynes M. Analysis of the saliva proteome from patients with head and neck squamous cell carcinoma reveals differences in abundance levels of proteins associated with tumour progression and metastasis. J Proteomics. 2008 Jul 21;71(2):168-75.

75. Hu S, Loo JA, Wong DT. Human saliva proteome analysis. Ann N Y Acad Sci. 2007 Mar;1098:323-9.

76. Hu S, Li Y, Wang J, Xie Y, Tjon K, Wolinsky L, et al. Human saliva proteome and transcriptome. J Dent Res. 2006 Dec;85(12):1129-33.

77. Hirtz C, Chevalier F, Centeno D, Egea JC, Rossignol M, Sommerer N, et al. Complexity of the human whole saliva proteome. J Physiol Biochem. 2005 Sep;61(3):469-80.

78. Yan W, Apweiler R, Balgley BM, Boontheung P, Bundy JL, Cargile BJ, et al. Systematic comparison of the human saliva and plasma proteomes. Proteomics Clin Appl. 2009 Jan 1;3(1):116-34.

79. Bandhakavi S, Stone MD, Onsongo G, Van Riper SK, Griffin TJ. A dynamic range compression and three-dimensional peptide fractionation analysis platform expands proteome coverage and the diagnostic potential of whole saliva. J Proteome Res. 2009 Dec;8(12):5590-60.

80. Wong DT. Salivaomics. J Am Dent Assoc. 2012 Oct;143(10 Suppl):19S-24.

81. Hu S, Denny P, Denny P, Xie Y, Loo JA, Wolinsky LE, et al. Differentially expressed protein markers in human submandibular and sublingual secretions. Int J Oncol. 2004 Nov;25(5):1423-30.

82. Rehak NN, Cecco SA, Csako G. Biochemical composition and electrolyte balance of "unstimulated" whole human saliva. Clin Chem Lab Med. 2000 Apr;38(4):335-43.

83. Dawes C. Circadian rhythms in human salivary flow rate and composition. J Physiol. 1972 Feb;220(3):529-45.

84. Weiss W, Weiland F, Gorg A. Protein detection and quantitation technologies for gel-based proteome analysis. Methods Mol Biol. 2009;564:59-82.

85. Chevallet M, Luche S, Rabilloud T. Silver staining of proteins in polyacrylamide gels. Nat Protoc. 2006;1(4):1852-8.

86. Dyballa N, Metzger S. Fast and sensitive coomassie staining in quantitative proteomics. Methods Mol Biol. 2012;893:47-59.

87. Keller A, Nesvizhskii AI, Kolker E, Aebersold R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. Anal Chem. 2002 Oct 15;74(20):5383-92.

88. Nesvizhskii AI, Keller A, Kolker E, Aebersold R. A statistical model for identifying proteins by tandem mass spectrometry. Anal Chem. 2003 Sep 1;75(17):4646-58.

Appendices

Appendix 1

Ethics Approval for Use of Human Subjects

Office of Research Ethics
The University of Western Ontario

Western

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. W.L. Siqueira
Review Level: Expedited
Review Number: 16181E
Revision Number: 1
Review Date: March 24, 2010
Approved Local # of Participants: 100
Protocol Title: Composition, Structure and Function of Salivary Proteins
Department and Institution: Dentistry, University of Western Ontario
Sponsor: NSERC-NATURAL SCIENCES ENGINEERING RSRCH COU
Ethics Approval Date: April 20, 2010
Expiry Date: June 30, 2016
Documents Reviewed and Approved: Revised Letter of Information and Consent. Assent Letter.
Documents Received for Information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly also report to the HSREB:

a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) all adverse and unexpected experiences or events that are both serious and unexpected;
c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

Chair of HSREB: Dr. Joseph Gilbert
FDA Ref. #: IRB 00000940

This is an official document. Please retain the original in your files.

cc: ORE File

Appendix 2

Letter of Information for Research Participants

LETTER OF INFORMATION

Project Title

Composition, Structure and Function of Salivary Proteins

Researcher(s)

Dr. Walter L. Siqueira DDS; PhD
Nada Tabbara

The pronouns 'you' and 'your' should be read as referring to the participant rather than the parent/guardian/next of kin who is signing the consent form for the participant.

Background

Human saliva is important for the protection of teeth and gums and therefore long-term oral health. A better understanding of which substance(s) in saliva help in this protection is needed so that clinicians can utilize such substances or similar substances in the prevention and treatment of oral disease conditions.

Purpose

The purpose of this research study is to determine how proteins and other molecules found in saliva are important for the protection of teeth and to what extent these proteins participate in the development of oral diseases. Saliva is a liquid continuously made by the salivary glands that coats the teeth and soft tissues of the mouth. Saliva is important because we know that people with dry mouth (xerostomia) and certain other conditions which affect the saliva suffer from mouth ulcers (sores), and more mouth infections such as caries (cavities), yeast infections (thrush or Candida), and possibly bleeding gums and loosening teeth (periodontal disease). In addition, there is a protein film on tooth surfaces known as the acquired enamel pellicle (AEP). This structure forms on teeth within seconds upon exposure to saliva. It affects the mineral of the tooth surface as well as the kinds of bacteria, which collect on its surface. These features make knowledge of AEP extremely important. Our studies with AEP have the ultimate goal of interfering with oral disease processes such as caries, periodontal disease and other oral infectious and inflammatory conditions. Thus, we are inviting you to donate saliva and/or tooth pellicle so that we can undertake analysis of this material.

What Happens in This Research Study

You will be one of approximately 100 subjects to be asked to participate in this study.

All or part of the research in this study will take place at the following location(s): Dental Clinic of Schulich school of Medicine & Dentistry, The University of Western Ontario.

Western

Schulich
MEDICINE & DENTISTRY

I. Initial Screening

Before you participate in the study we will take a medical and dental history. For both medical and dental history, general questions such as "do you have diabetes? or do you have periodontal disease?" will be asked. We also will perform a visual oral examination to assess your oral health status. These medical and dental histories as well as the dental examination are necessary in order for us to determine whether you are eligible for this study.

II. Collection of Saliva and/or Enamel Pellicle

Because there are several different kinds of saliva glands that make different types of saliva, we have to collect saliva in slightly different ways. Whole saliva is a mixture made up of combined secretions from all of the salivary glands. To collect whole saliva, we will ask you to spit or drool into a test tube several times per minute. We will collect this over a number of minutes depending upon how much saliva you produce. We may stimulate saliva flow by asking you to chew on plain wax or to suck on some sour candy, or we may use a Q-tip swab to put a few drops of lemon juice (citric acid) on your tongue. The collection of saliva from the individual parotid glands (in upper jaw) and the individual submandibular glands (in lower jaw) involves the use of small, plastic devices that are held in place on the openings of the glands by slight suction (negative pressure). The device used in the lower jaw is further held in place with the use of routine dental impression (model) material. The plastic devices are connected to tubing that empty into test tubes. This may also take several minutes. Again, we may stimulate this flow by having you chew or use the sour candy or sour drops that we will provide for you. For the collection of saliva from subjects 1 month old to 6 years old will be utilized a suction device that will provide a slight suction through a sterile soft plastic butterfly catheter. This catheter will be placed in the oral cavity of the subject for a time frame of 1 to 3 minutes.

If you choose to participate in this study, you may be a donor of saliva for one or more times at your convenience. Although multiple donations are usually not made more than once per week you can donate saliva more frequently, if you wish. There is no known medical reason to limit the number of times you donate saliva. The volunteers will receive a visual examination as well as a dental cleaning each time a donation is made.

The total time for this part of the study is estimated to be 10-20 minutes

The collection of enamel pellicle requires a polishing of your tooth surfaces similar to a routine dental cleaning treatment in a dental office carried out by a dentist or a hygienist. After this you are allowed to proceed with your daily activities but refrain from any drinking or eating for a period of 1-2 hours. Your are then asked to return 1 or 2 hours after the dental cleaning treatment to the Dental Clinic at Schulich Dentistry where the pellicle formed over this time period will be collected. Pellicle collection involves swabbing the tooth surface with a wetted absorbent filter paper to remove the pellicle. We will collect this pellicle from most or all of your teeth. This generally takes about 10 minutes. The pellicle material is then placed into test tubes for later analysis. If you choose to participate in this study you may be a donor of pellicle material one or more times at your convenience. Although multiple donations are usually not more than once per week you can donate more frequently. We allow you to donate pellicle 4 times within a 3-month period if you wish to do so. There is no known medical reason to limit the number of times you donate salivary pellicle.

III. Post-collection processing and analysis

Immediately after collection, saliva and pellicle samples that we collect from you will be processed for biochemical analysis. It will not be possible to return samples to you once we begin processing, and you will not be given the results of the tests.
The AEP and saliva will be analyses to determine the physical characteristics of the proteins such as their molecular size, charge and three-dimensional shape, their chemical characteristics such as arrangement of amino acids within the proteins, and their biological characteristics such as how these molecules may actually work.

Risks and Discomforts

There are no reports of pain or discomfort during the saliva and/or pellicle collection procedure. None of the collection procedures are experimental. The tooth polishing procedures are the same as used in regular cleanings and should not cause any untoward effects. In very rare cases minor and very transient tooth sensitivity has been observed. If this should occur we will treat the affected sites with topical fluoride applications and/or suggest the use of desensitizing toothpastes.

Potential Benefits

The results from this study will help us to understand better the role of salivary components in oral health and may lead to new tools to prevent or combat oral disease.

Alternatives

Your alternative is to not participate in the study.

Subject's Rights

By consenting to participate in this study you do not waive any of your legal rights. Giving consent means that you have heard or read the information about this study and that you agree to participate. You will be given a copy of this form to keep.

Right to Refuse or Withdraw

Taking part in this study is voluntary. You have the right to refuse to take part in this study. If you decide to be in the study and then change your mind, you can withdraw from the research. Your participation is completely up to you. Your decision will not affect your being able to get health care at this institution. It will not affect your enrollment in any health plan or benefits you can get. If you choose to take part, you have the right to stop at any time. If there are any new findings during the study that may affect whether you want to continue to take part, you will be told about them as soon as possible.

Confidentiality

All scientific data and information obtained for the purpose of this study may be used for publications, and/or teaching. Your name will not appear in any publications or document generate from this study. All private information will be kept confidential and will not be shared by other institutions or resources. In addition,

private Information in hard copies will be kept in locked file cabinet and information in electronic versions will be kept in computer under controlled access with password.

Contact Person(s) for Participants

The investigator(s) will try to answer all of your questions. If you have questions or concerns at any time, or if you need to report an injury while participating in this research, concerns or problems related to your participation in this study, including any research-related injury, you are asked to contact Dr. Walter Siqueira, principal investigator a
can reach Dr. Walter Siqueira at

Project Title

Composition, Structure and Function of Salivary Proteins

Researcher(s)

Dr. Walter L. Siqueira DDS; PhD
Nada Tabbara

Signing this document indicates that you have read this consent form (or have had it read to you), that your questions have been answered to your satisfaction, and that you voluntarily agree to participate in this research study. You will receive a copy of this signed consent form.

If you have any questions about rights as a research participant or the conduct of the study you may contact the Office of Research Ethics

Subject or Subject's Parent or Guardian (Signature and Printed Name) Date

Investigator (Signature and Printed Name) Date

Appendix 3

Results

Table A3.1 - Clinical data summary – Inter-group Age Comparison

Clinical Data Inter-group Age Comparison
AGE

	Group A		Group B		Group C		Group D	
	Age (months)	Standard Error	Age (months)	Standard Error	Age (months)	Standard Error	Age (months)	Standard Error
N	12		10		12		10	
Mean	4.667	0.583	14.200	1.777	34.167	1.661	597.491	33.490
95% CI for Mean								
Lower Bound	3.383		10.180		37.822		521.731	
Upper Bound	5.951		18.220		30.511		673.251	
5% Trimmed Mean	4.755		14.153		37.822		599.379	
Median	5.125		14.625		34.324		612.480	
Variance	4.083		31.581		33.625		11215.852	
Standard Deviation	2.021		5.620		5.754		105.905	
Minimum	0.750		6.250		23.500		420.000	
Maximum	7.000		23.000		42.000		741.000	
Range	6.250		16.750		18.500		321.000	
Interquartile Range	3.750		10.440		9.440		167.260	
Skewness	-0.600	0.637	-0.052		-0.230	0.637	-0.479	0.687
Kurtosis	-0.737	1.232	0.942		-0.773	1.232	-0.728	1.334

Table A3.2 - Clinical data summary – Inter-group Total Protein Comparison

Clinical Data Inter-group Total Protein Comparison
TOTAL PROTEIN CONCENTRATION

	Group A		Group B		Group C		Group D	
	Total protein (µg/mL)	Standard Error	Total protein (µg/mL)	Standard Error	Total protein (µg/mL)	Standard Error	Total protein (µg/mL)	Standard Error
N	12.0		10.0		12.0		10.0	
Mean	699.4	119.5	921.4	262.9	1019.2	83.1	1655.0	454.6
95% CI for Mean								
Lower Bound	436.3		326.7		836.4		672.0	
Upper Bound	962.4		1516.0		1202.0		2638.0	
5% Trimmed Mean	670.7		826.3		1022.5		1493.0	
Median	553.5		697.1		980.1		1287.5	
Standard Deviation	414.0		831.2		287.7		1374.2	
Minimum	235.5		346.7		495.6		826.9	
Maximum	1679.3		3207.3		1484.2		5398.9	
Range	1443.8		2860.6		988.6		4572.0	
Interquartile Range	467.1		509.2		471.9		854.5	
Skewness	1.3	0.6	2.8	0.7	0.0	0.6	2.7	0.7
Kurtosis	1.8	1.2	8.2	1.3	-0.5	1.2	7.8	1.3

Table A3.3 - Clinical data summary – Inter-group Volume of Saliva Comparison

Clinical Data Inter-group Volume of Saliva Comparison VOLUME OF SALIVA								
	Group A		Group B		Group C		Group D	
	Volume of Saliva (mL)	Standard Error	Volume of Saliva (mL)	Standard Error	Volume of Saliva (mL)	Standard Error	Volume of Saliva (mL)	Standard Error
N	12		10		12		10	
Mean	0.404	0.057	0.300	0.060	0.371	0.049	1.280	0.335
95% CI for Mean								
Lower Bound	0.280		0.165		0.262		0.523	
Upper Bound	0.529		0.435		0.480		2.037	
5% Trimmed Mean	0.402		0.294		0.373		1.244	
Median	0.450		0.250		0.450		1.000	
Variance	0.038		0.036		0.029		1.120	
Standard Deviation	0.196		0.189		0.171		1.058	
Minimum	0.050		0.100		0.100		0.200	
Maximum	0.800		0.600		0.600		3.000	
Range	0.750		0.500		0.500		2.800	
Interquartile Range	0.280		0.400		0.300		2.080	
Skewness	0.056	0.637	0.373	0.687	-0.394	0.637	0.441	0.687
Kurtosis	0.703	1.232	-1.508	1.334	-1.530	1.232	-1.481	1.334

Table A3.4 - Clinical data summary – Inter-group Number of Teeth Erupted Comparison

Clinical Data Number of Teeth Erupted TEETH ERUPTED					
	Group A	Group B		Group C	Group D
	Teeth Erupted (#)	Teeth Erupted (#)	Standard Error	Teeth Erupted (#)	Teeth Erupted (#)
N	12	10		12	10
Mean	0	8	1.74483	20	0
95% CI for Mean					
Lower Bound	N/A	4.0529		N/A	N/A
Upper Bound	N/A	11.9471		N/A	N/A
5% Trimmed Mean	N/A	7.9444		N/A	N/A
Median	N/A	6.5		N/A	N/A
Variance	N/A	30.444		N/A	N/A
Standard Deviation	N/A	5.51765		N/A	N/A
Minimum	N/A	1		N/A	N/A
Maximum	N/A	16		N/A	N/A
Range	N/A	15		N/A	N/A
Interquartile Range	N/A	11		N/A	N/A
Skewness	N/A	0.496	0.687	N/A	N/A
Kurtosis	N/A	-1.166	1.334	N/A	N/A

Table A3.5 - Nonparametric Correlations - Age and Protein Concentration

Spearman's rho

		Age	Protein Concentration
Age	Correlation Coefficient	1	0.48**
	Significance (2-tailed)		0.001
	N	44	44
Protein Concentration	Correlation Coefficient	0.48**	1
	Significance (2-tailed)	0.001	
	N	44	44

** Correlation is significant at the level of 0.01 (2-tailed)

Table A3.6 - Nonparametric Correlations – Protein Concentration and Age in Females

Spearman's rho

		Protein Concentration	Age
Protein Concentration	Correlation Coefficient	1	0.635**
	Significance (2-tailed)		0.005
	N	18	18
Age	Correlation Coefficient	0.635**	1
	Significance (2-tailed)	0.005	
	N	18	18

** Correlation is significant at the level of 0.01 (2-tailed)

Table A3.7 - Nonparametric Correlations - Protein Concentration and Age in Males

Spearman's rho

		Protein Concentration	Age
Protein Concentration	Correlation Coefficient	1	0.42**
	Significance (2-tailed)		0.033
	N	26	26
Age	Correlation Coefficient	0.42**	1
	Significance (2-tailed)	0.033	
	N	26	26

** Correlation is significant at the level of 0.05 (2-tailed)

Table A3.8 - Nonparametric Correlations – Volume of Saliva and Protein Concentration

Spearman's rho

		Volume of Saliva	Protein Concentration
Volume of Saliva	Correlation Coefficient	1	0.066
	Significance (2-tailed)		0.672
	N	44	44
Protein Concentration	Correlation Coefficient	0.066	1
	Significance (2-tailed)	0.672	
	N	44	44

Table A3.9 - Nonparametric Correlations – Age and Volume of Saliva

Spearman's rho

		Age	Volume of Saliva
Age	Correlation Coefficient	1	0.252
	Significance (2-tailed)		0.098
	N	44	44
Volume of Saliva	Correlation Coefficient	0.252	1
	Significance (2-tailed)	0.098	
	N	44	44

Table A3.10 - Nonparametric Correlations – Protein Concentration and Teeth Erupted

Spearman's rho

		Protein Concentration	Teeth Erupted
Protein Concentration	Correlation Coefficient	1	0.067
	Significance (2-tailed)		0.668
	N	44	44
Teeth Erupted	Correlation Coefficient	0.067	1
	Significance (2-tailed)	0.668	
	N	44	44

Table A3.11 - Nonparametric Correlations – Protein Concentration and Teeth Erupted in Females

Spearman's rho

		Protein Concentration	Teeth Erupted
Protein Concentration	Correlation Coefficient	1	0.184
	Significance (2-tailed)		0.464
	N	18	18
Teeth Erupted	Correlation Coefficient	0.184	1
	Significance (2-tailed)	0.464	
	N	18	18

Table A3.12 - Nonparametric Correlations – Protein Concentration and Teeth Erupted in Males

Spearman's rho

		Protein Concentration	Teeth Erupted
Protein Concentration	Correlation Coefficient	1	-0.021
	Significance (2-tailed)		0.918
	N	26	26
Teeth Erupted	Correlation Coefficient	-0.021	1
	Significance (2-tailed)	0.918	
	N	26	26

Table A3.13 - Nonparametric Tests – Hypothesis Test Summary – Kruskal-Wallis

Hypothesis Test Summary				
	Null Hypothesis	**Test**	**Significance**	**Decision**
1	The distribution of Age is the same across all Groups	Independent-Samples Kruskal-Wallis Test	0	Reject the null hypothesis
2	The distribution of Protein Concentration is the same across all groups	Independent-Samples Kruskal-Wallis Test	0.598	Retain the null hypothesis
3	The distribution of Volume of Saliva is the same across all Groups	Independent-Samples Kruskal-Wallis Test	0.266	Retain the null hypothesis
4	The distribution of Teeth Erupted is the same across all Groups	Independent-Samples Kruskal-Wallis Test	0	Reject the null hypothesis

Asymptotic significances are displayed. The significant level is 0.05.

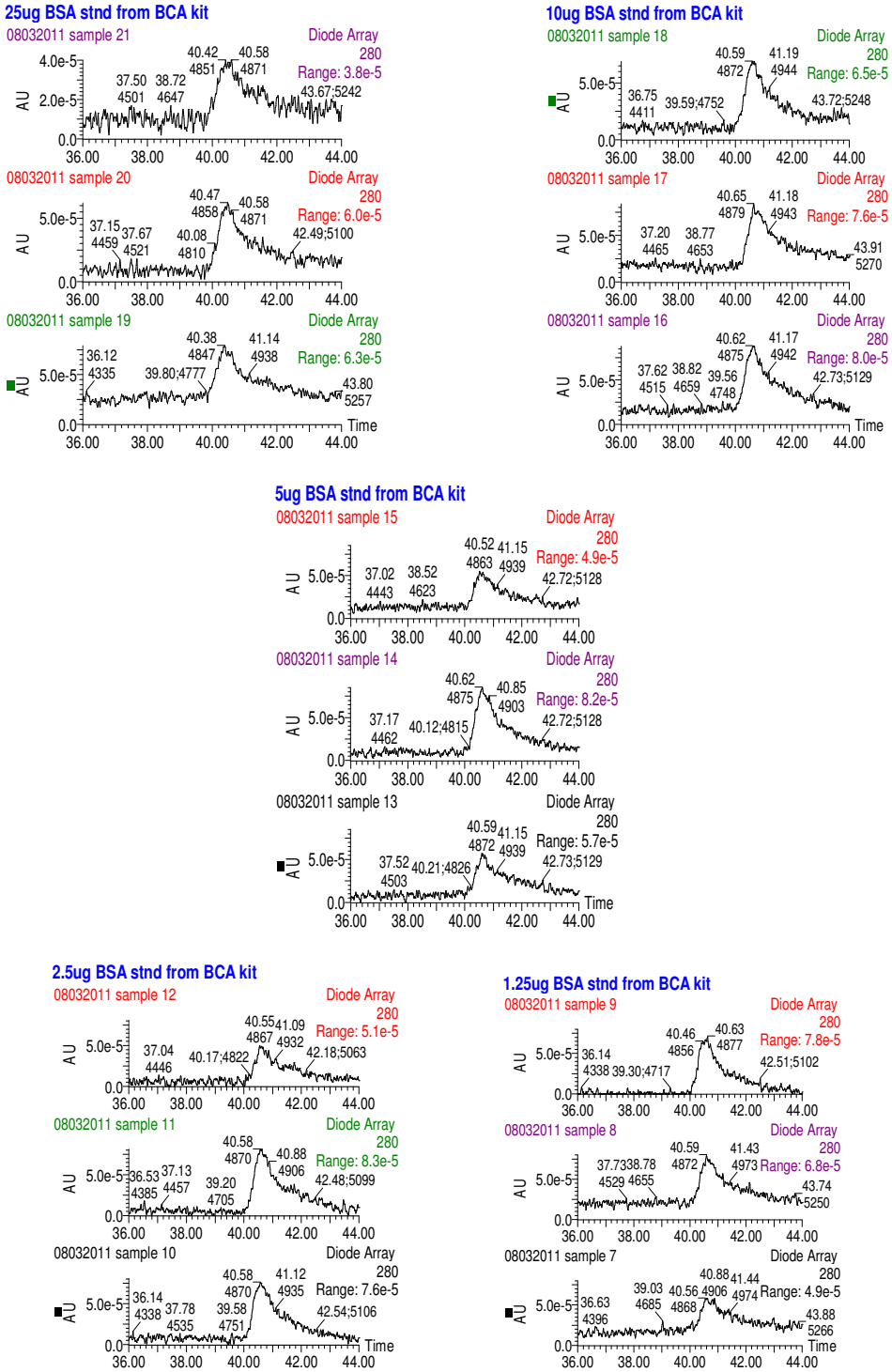
Table A3.14 - Nonparametric Tests – Hypothesis Test Summary – Mann-Whitney U

Hypothesis Test Summary				
	Null Hypothesis	**Test**	**Significance**	**Decision**
1	The distribution of Age is the same across all Groups	Independent-Samples Mann-Whitney U Test	0	Reject the null hypothesis
2	The distribution of Protein Concentration is the same across all groups	Independent-Samples Mann-Whitney U Test	0.024	Reject the null hypothesis
3	The distribution of Volume of Saliva is the same across all Groups	Independent-Samples Mann-Whitney U Test	0.81	Retain the null hypothesis
4	The distribution of Teeth Erupted is the same across all Groups	Independent-Samples Mann-Whitney U Test	0	Reject the null hypothesis

Asymptotic significances are displayed. The significant level is 0.05.

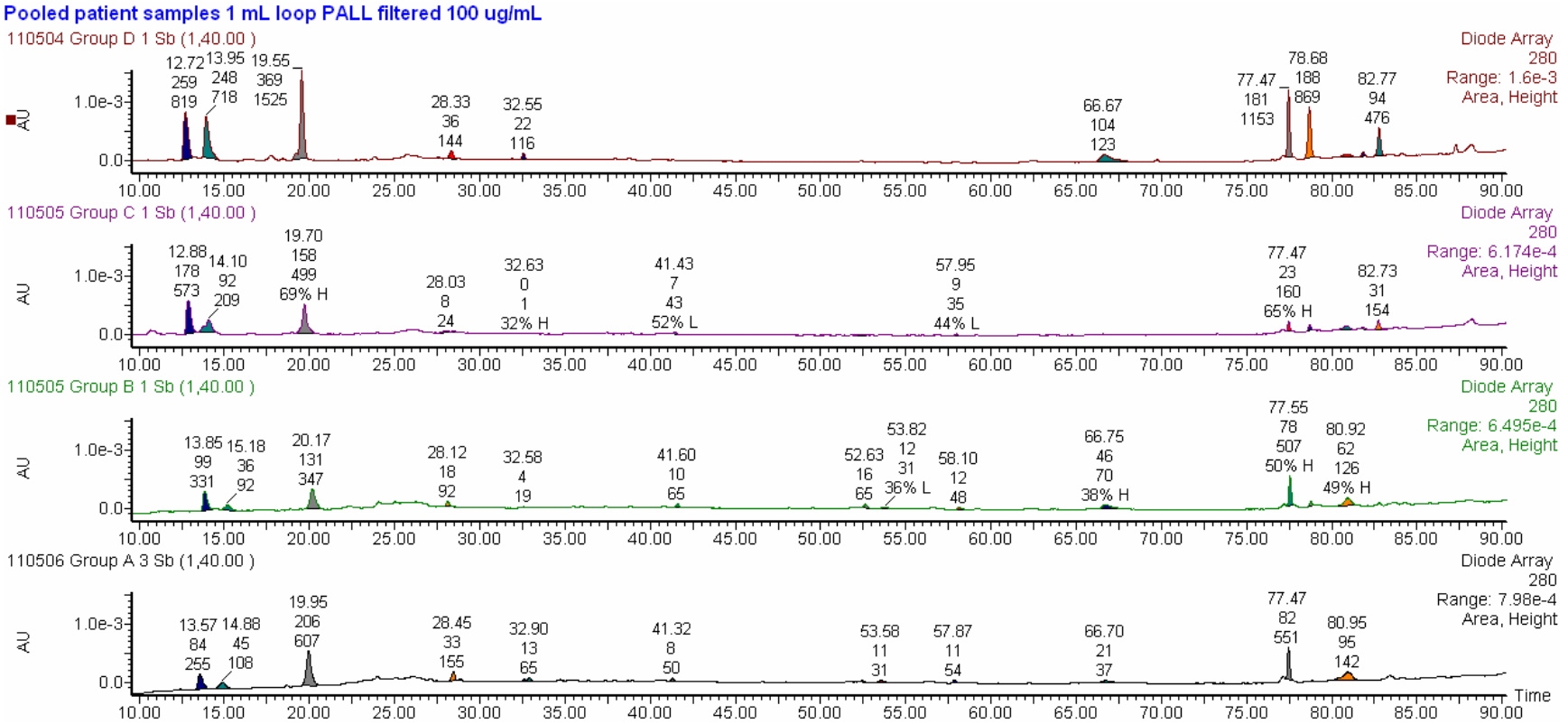
Appendix 4

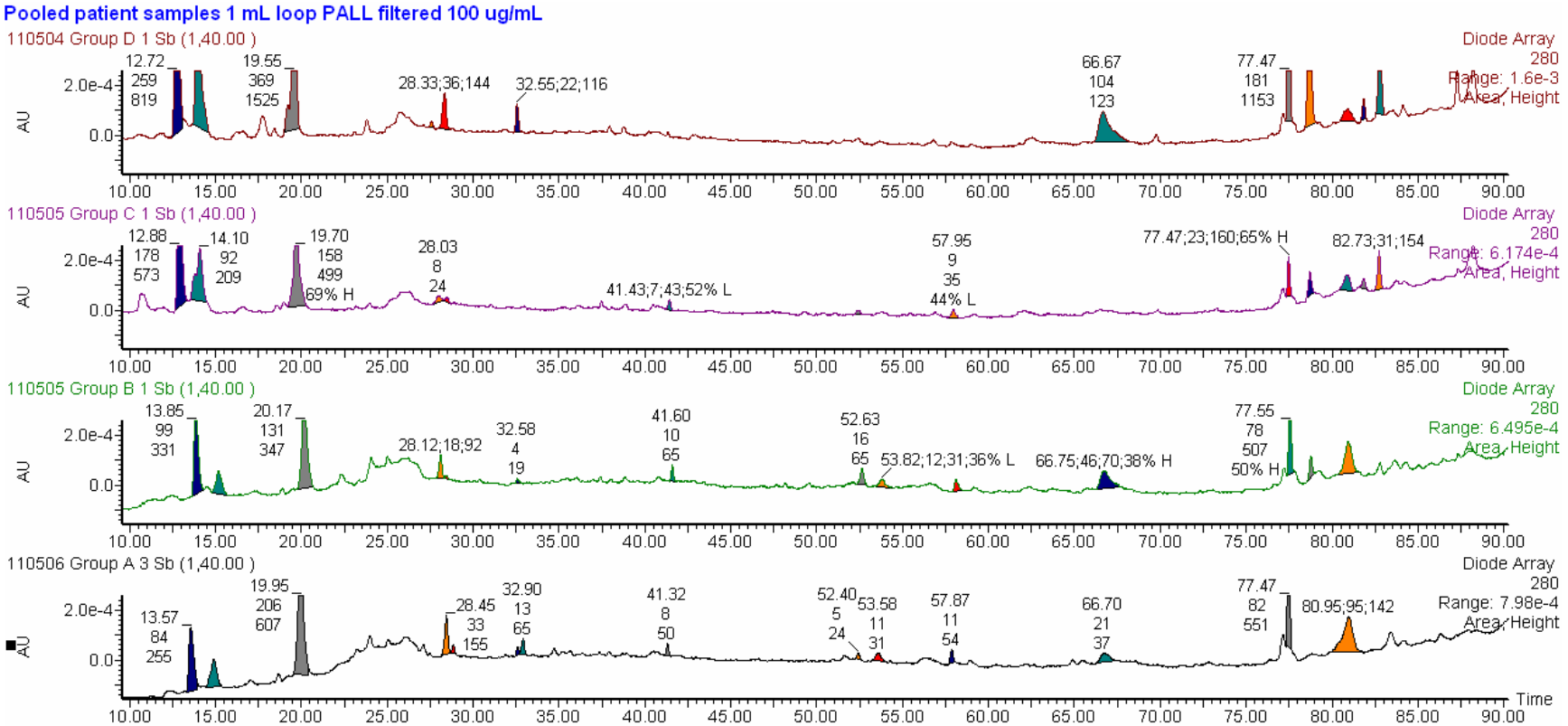
Results – HSA Area under the curve of chromatograms

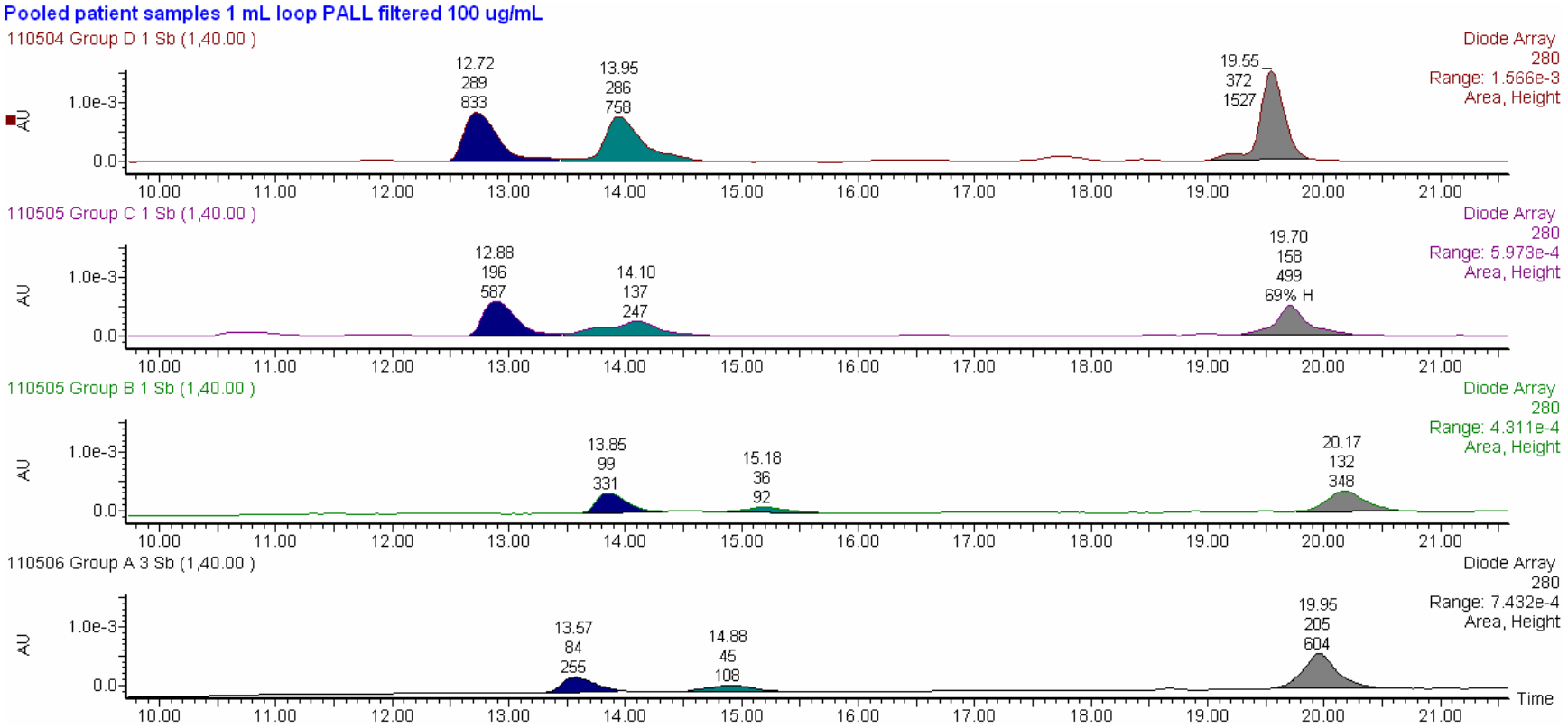


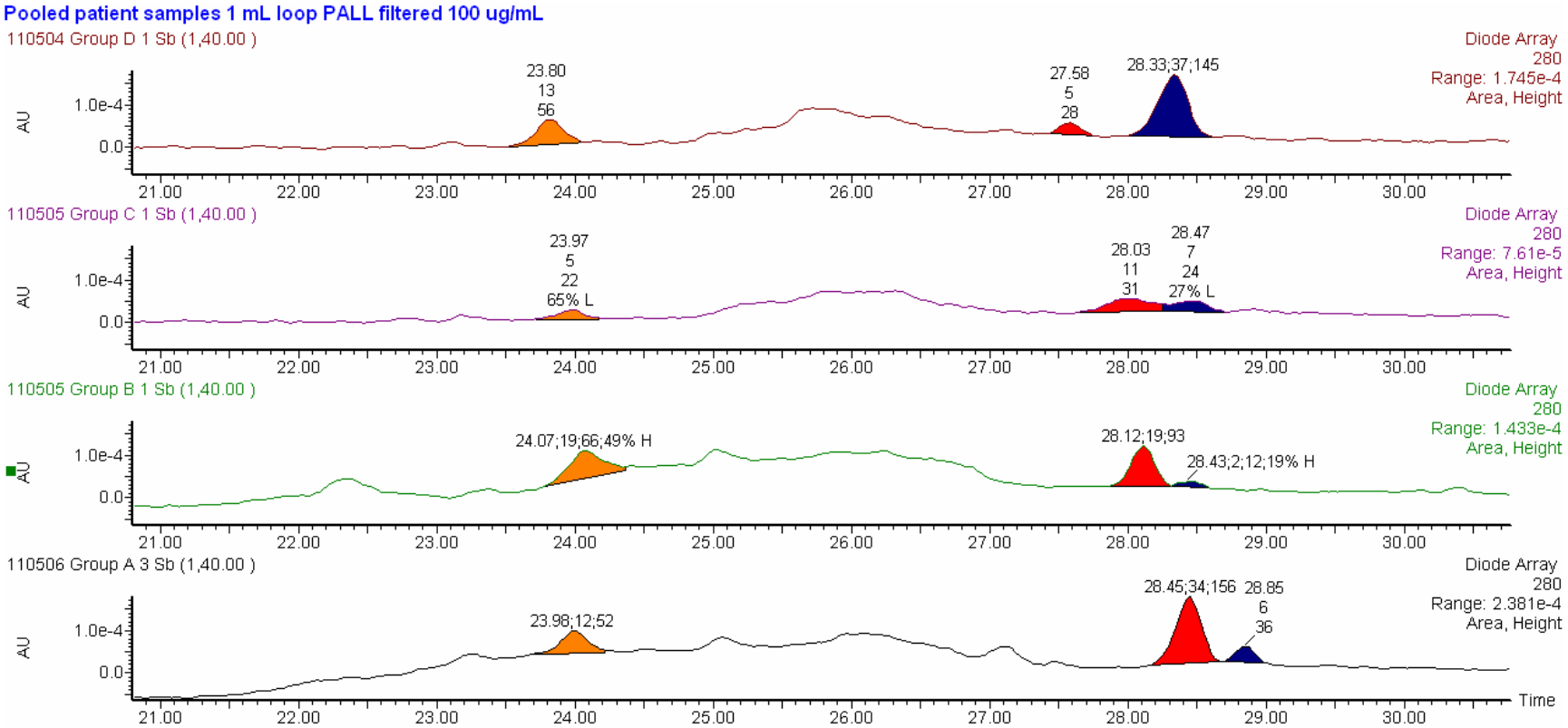
Appendix 5

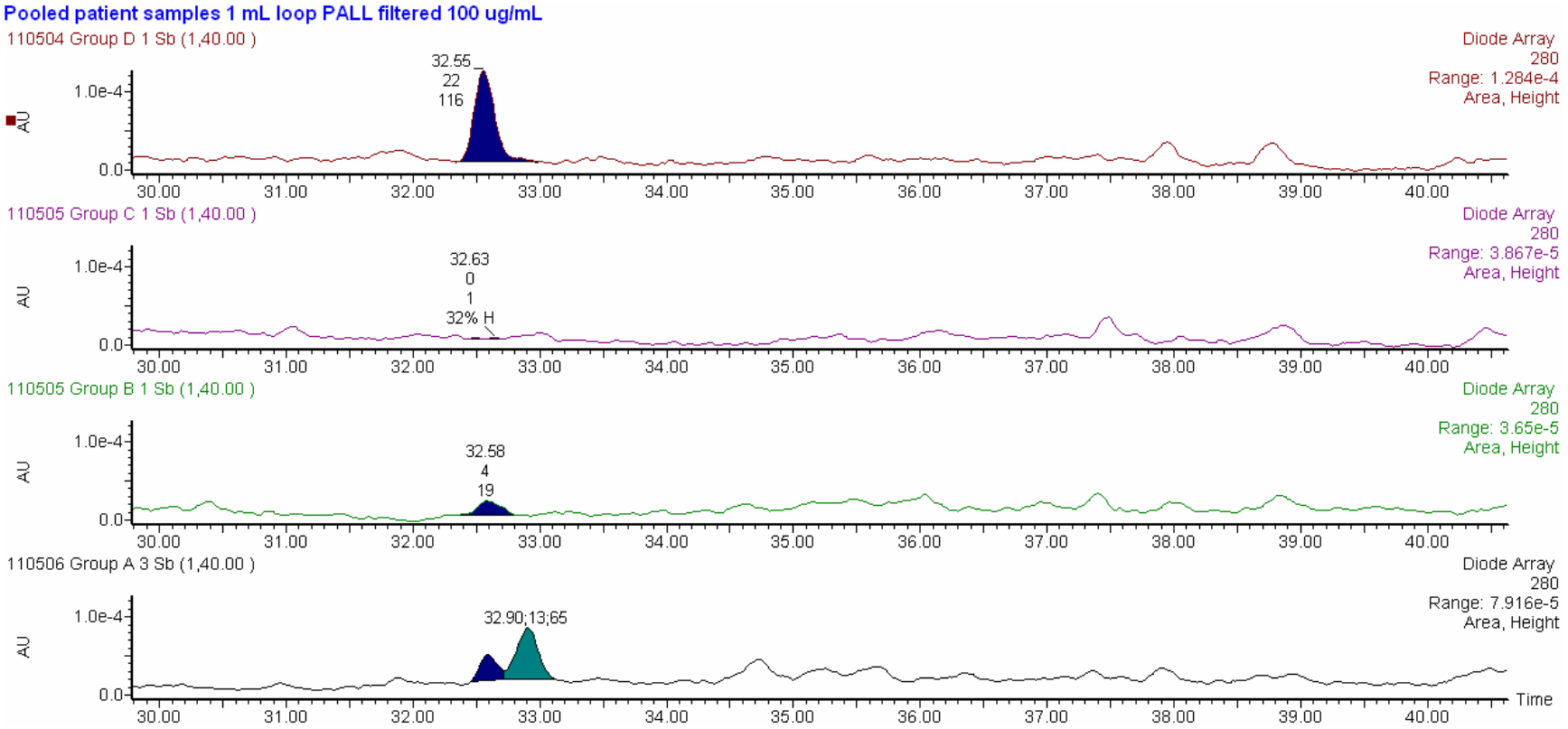
Results – Unstimulated whole saliva chromatograms


Pooled patient samples 1 mL loop PALL filtered 100 ug/mL
110504 Group D 1 Sb (1,40.00)
Diode Array
280
Range: 1.6e-3
Area, Height
AU
1.0e-3
0.0
12.72 259 819
13.95 248 718
19.55 369 1525
28.33 36 144
32.55 22 116
66.67 104 123
77.47 181 1153
78.68 188 869
82.77 94 476
10.00 15.00 20.00 25.00 30.00 35.00 40.00 45.00 50.00 55.00 60.00 65.00 70.00 75.00 80.00 85.00 90.00
110505 Group C 1 Sb (1,40.00)
Diode Array
280
Range: 6.174e-4
Area, Height
12.88 178 573
14.10 92 209
19.70 158 499 69% H
28.03 8 24
32.63 0 1 32% H
41.43 7 43 52% L
57.95 9 35 44% L
77.47 23 160 65% H
82.73 31 154
110505 Group B 1 Sb (1,40.00)
Diode Array
280
Range: 6.495e-4
Area, Height
13.85 99 331
15.18 36 92
20.17 131 347
28.12 18 92
32.58 4 19
41.60 10 65
52.63 16 65
53.82 12 31 36% L
58.10 12 48
66.75 46 70 38% H
77.55 78 507 50% H
80.92 62 126 49% H
110506 Group A 3 Sb (1,40.00)
Diode Array
280
Range: 7.98e-4
Area, Height
13.57 84 255
14.88 45 108
19.95 206 607
28.45 33 155
32.90 13 65
41.32 8 50
53.58 11 31
57.87 11 54
66.70 21 37
77.47 82 551
80.95 95 142
Time

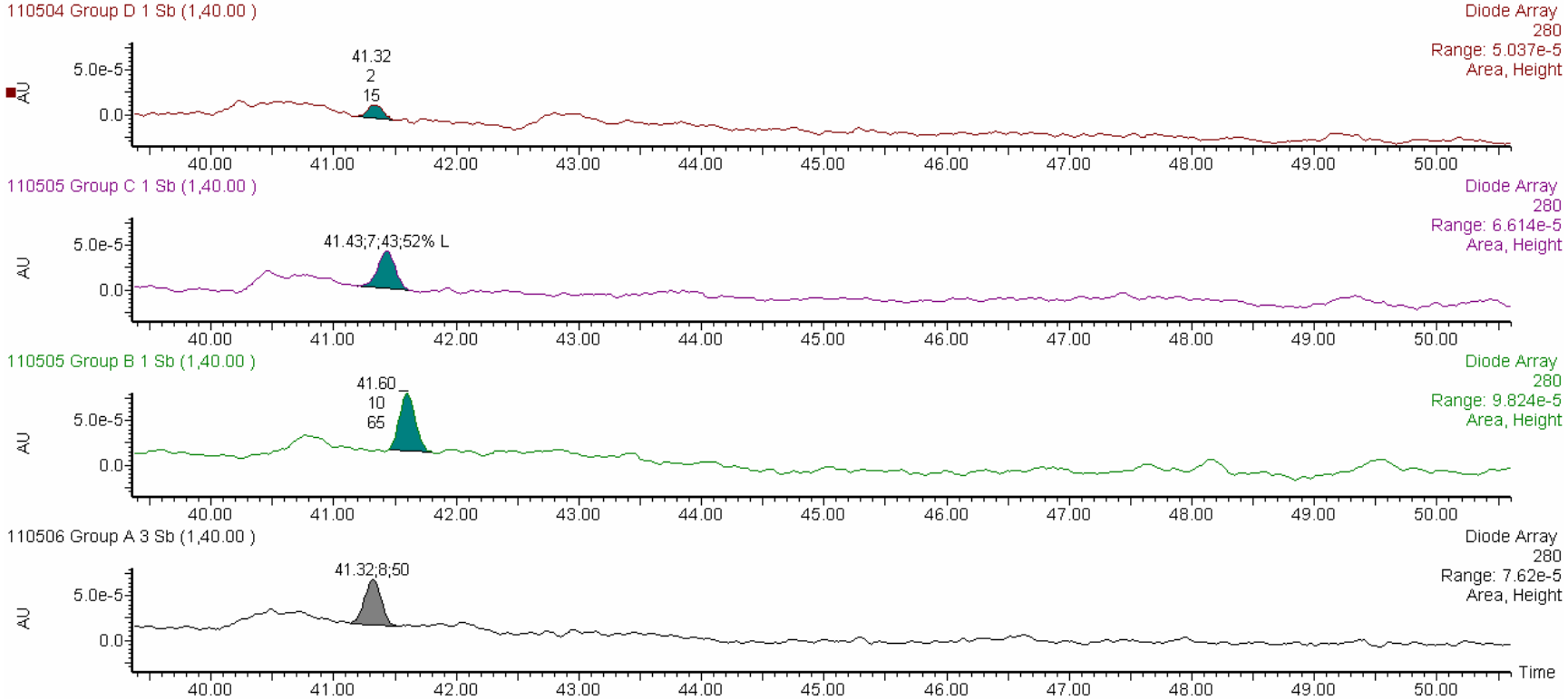

Pooled patient samples 1 mL loop PALL filtered 100 ug/mL
110504 Group D 1 Sb (1,40.00)
Diode Array
280
Range: 1.6e-3
Area, Height
12.72
259
819
19.55
369
1525
28.33;36;144
32.55;22;116
66.67
104
123
77.47
181
1153
110505 Group C 1 Sb (1,40.00)
Diode Array
280
Range: 6.174e-4
Area, Height
12.88
178
573
14.10
92
209
19.70
158
499
69% H
28.03
8
24
41.43;7;43;52% L
57.95
9
35
44% L
77.47;23;160;65% H
82.73;31;154
110505 Group B 1 Sb (1,40.00)
Diode Array
280
Range: 6.495e-4
Area, Height
13.85
99
331
20.17
131
347
28.12;18;92
32.58
4
19
41.60
10
65
52.63
16
65
53.82;12;31;36% L
66.75;46;70;38% H
77.55
78
507
50% H
110506 Group A 3 Sb (1,40.00)
Diode Array
280
Range: 7.98e-4
Area, Height
13.57
84
255
19.95
206
607
28.45
33
155
32.90
13
65
41.32
8
50
52.40
5
24
53.58
11
31
57.87
11
54
66.70
21
37
77.47
82
551
80.95;95;142
AU
2.0e-4
0.0
Time
10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00
55.00
60.00
65.00
70.00
75.00
80.00
85.00
90.00

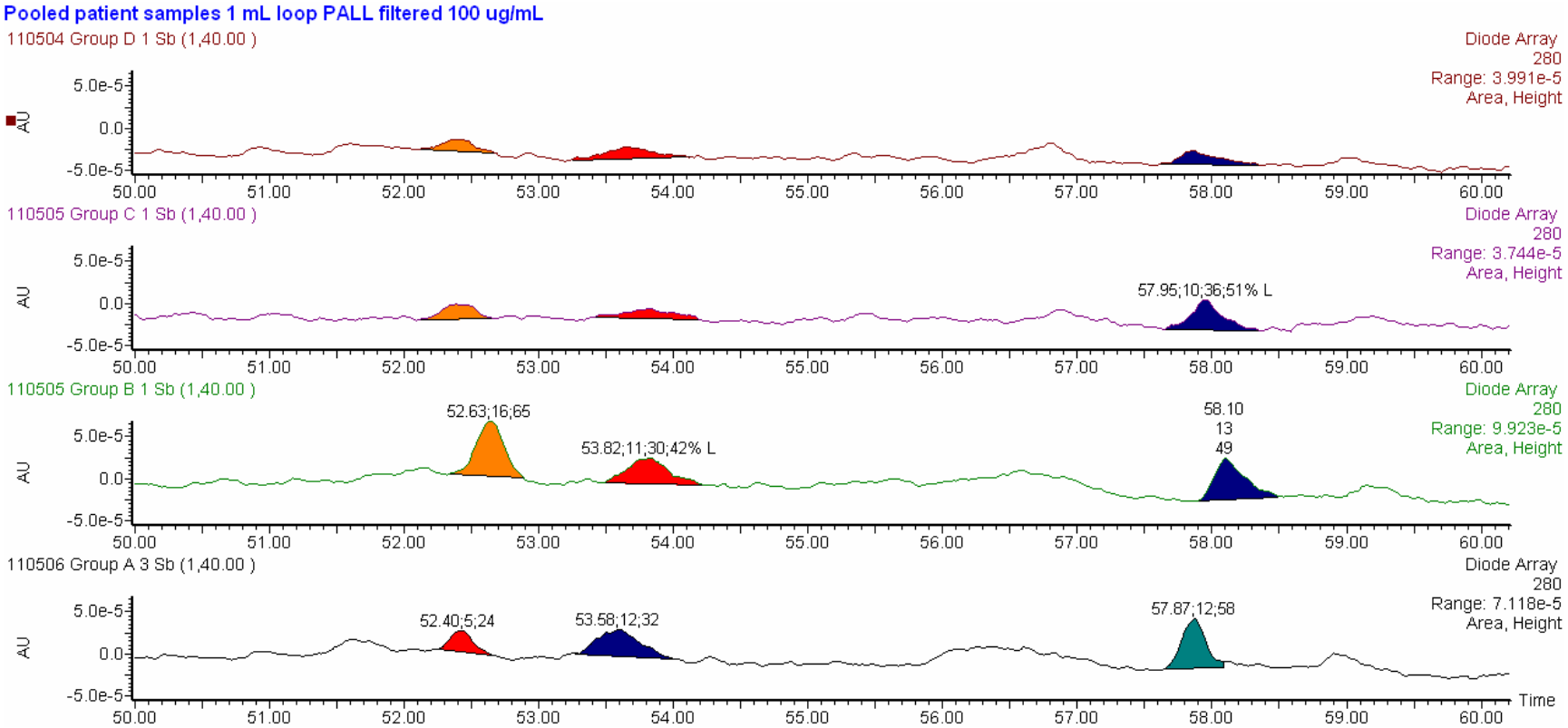

Pooled patient samples 1 mL loop PALL filtered 100 ug/mL
110504 Group D 1 Sb (1,40.00)
Diode Array
280
Range: 1.566e-3
Area, Height
12.72
289
833
13.95
286
758
19.55
372
1527
110505 Group C 1 Sb (1,40.00)
Diode Array
280
Range: 5.973e-4
Area, Height
12.88
196
587
14.10
137
247
19.70
158
499
69% H
110505 Group B 1 Sb (1,40.00)
Diode Array
280
Range: 4.311e-4
Area, Height
13.85
99
331
15.18
36
92
20.17
132
348
110506 Group A 3 Sb (1,40.00)
Diode Array
280
Range: 7.432e-4
Area, Height
13.57
84
255
14.88
45
108
19.95
205
604
AU
1.0e-3
0.0
10.00
11.00
12.00
13.00
14.00
15.00
16.00
17.00
18.00
19.00
20.00
21.00
Time

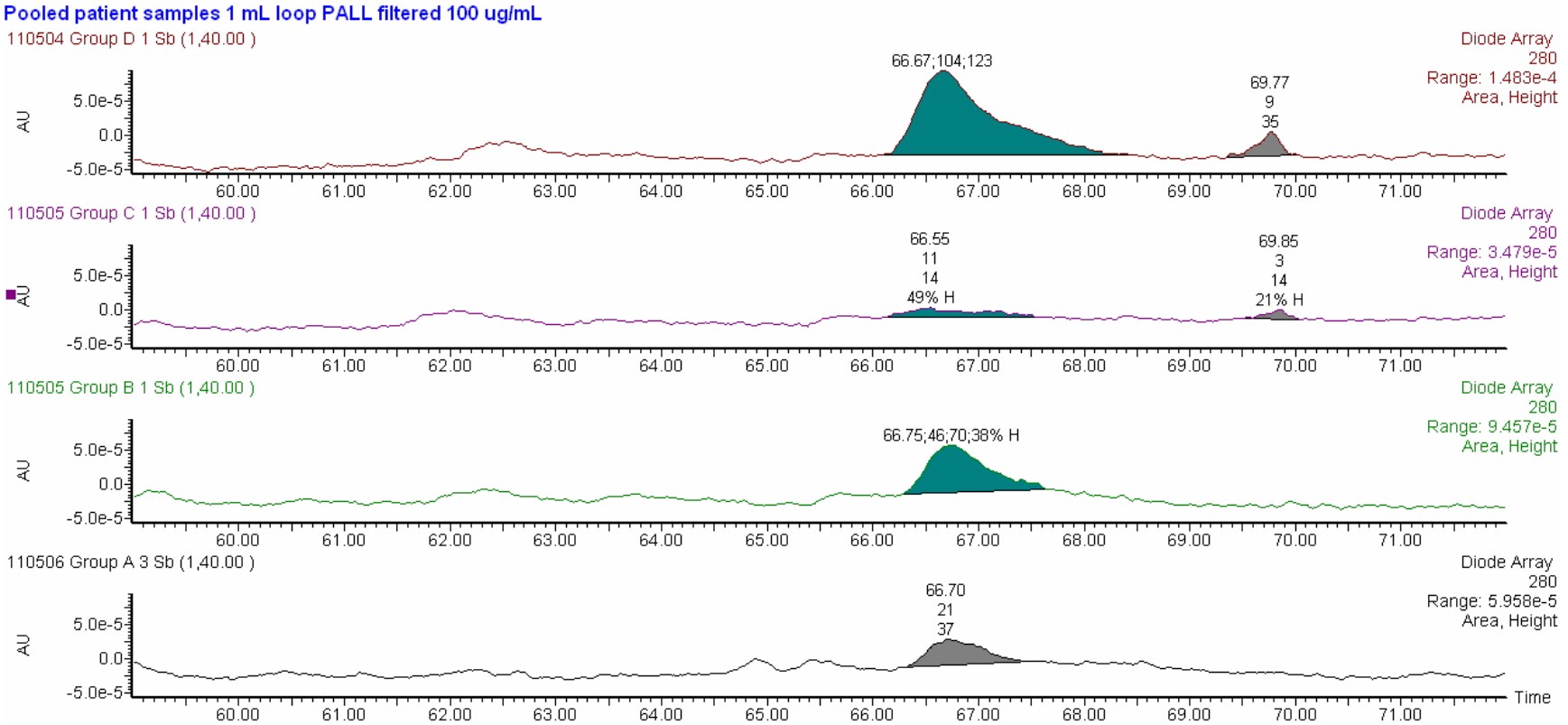

Pooled patient samples 1 mL loop PALL filtered 100 ug/mL
110504 Group D 1 Sb (1,40.00)
Diode Array
280
Range: 1.745e-4
Area, Height
23.80
13
56
27.58
5
28
28.33;37;145
AU
1.0e-4
0.0
21.00
22.00
23.00
24.00
25.00
26.00
27.00
28.00
29.00
30.00
110505 Group C 1 Sb (1,40.00)
Diode Array
280
Range: 7.61e-5
Area, Height
23.97
5
22
65% L
28.03
11
31
28.47
7
24
27% L
110505 Group B 1 Sb (1,40.00)
Diode Array
280
Range: 1.433e-4
Area, Height
24.07;19;66;49% H
28.12;19;93
28.43;2;12;19% H
110506 Group A 3 Sb (1,40.00)
Diode Array
280
Range: 2.381e-4
Area, Height
23.98;12;52
28.45;34;156
28.85
6
36
Time


Pooled patient samples 1 mL loop PALL filtered 100 ug/mL
110504 Group D 1 Sb (1,40.00)
Diode Array
280
Range: 1.284e-4
Area, Height
32.55
22
116
AU
1.0e-4
0.0
30.00
31.00
32.00
33.00
34.00
35.00
36.00
37.00
38.00
39.00
40.00
110505 Group C 1 Sb (1,40.00)
Diode Array
280
Range: 3.867e-5
Area, Height
32.63
0
1
32% H
110505 Group B 1 Sb (1,40.00)
Diode Array
280
Range: 3.65e-5
Area, Height
32.58
4
19
110506 Group A 3 Sb (1,40.00)
Diode Array
280
Range: 7.916e-5
Area, Height
32.90;13;65
Time

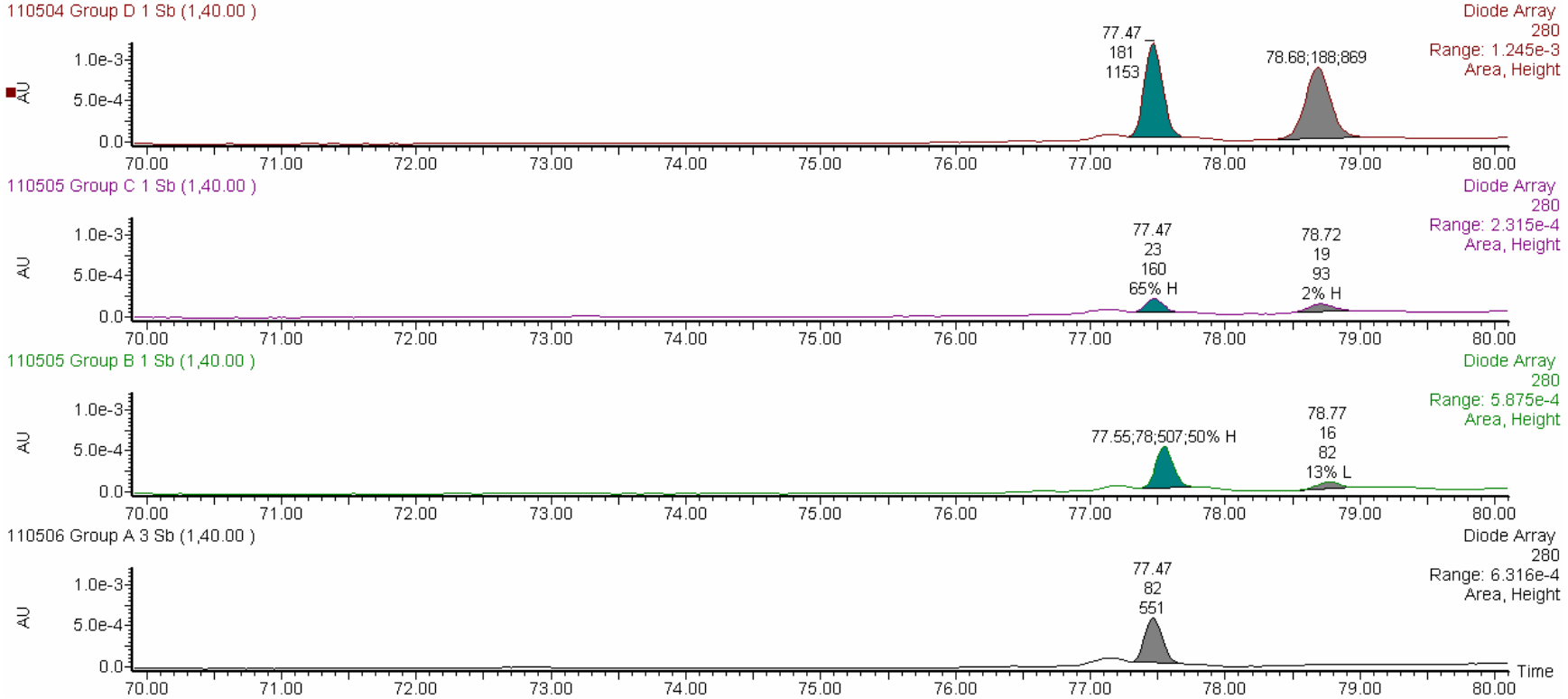
Pooled patient samples 1 mL loop PALL filtered 100 ug/mL

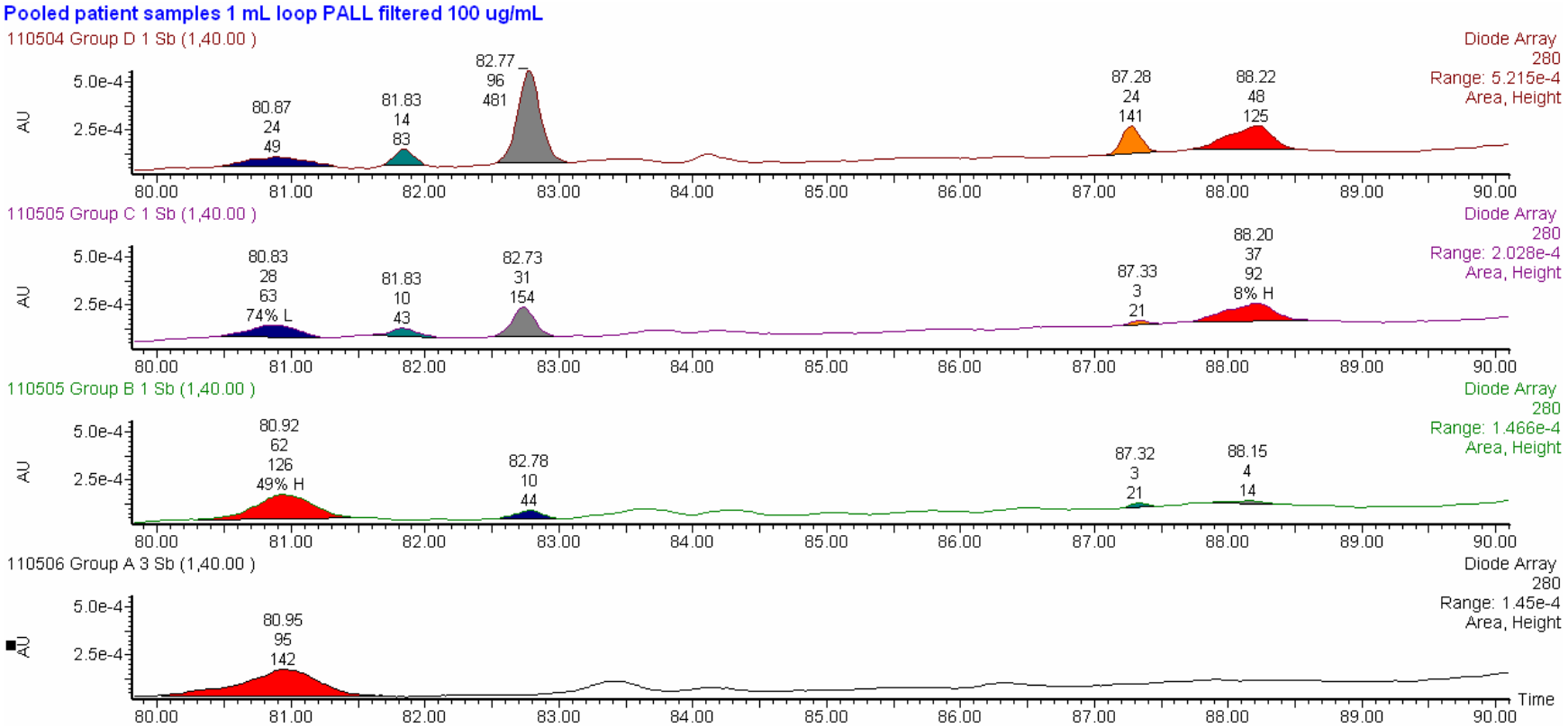



Pooled patient samples 1 mL loop PALL filtered 100 ug/mL
110504 Group D 1 Sb (1,40.00)
Diode Array
280
Range: 3.991e-5
Area, Height
AU
5.0e-5
0.0
-5.0e-5
50.00
51.00
52.00
53.00
54.00
55.00
56.00
57.00
58.00
59.00
60.00
110505 Group C 1 Sb (1,40.00)
Diode Array
280
Range: 3.744e-5
Area, Height
57.95;10;36;51% L
110505 Group B 1 Sb (1,40.00)
Diode Array
280
Range: 9.923e-5
Area, Height
52.63;16;65
53.82;11;30;42% L
58.10
13
49
110506 Group A 3 Sb (1,40.00)
Diode Array
280
Range: 7.118e-5
Area, Height
52.40;5;24
53.58;12;32
57.87;12;58
Time


Pooled patient samples 1 mL loop PALL filtered 100 ug/mL
110504 Group D 1 Sb (1,40.00)
Diode Array
280
Range: 1.483e-4
Area, Height
66.67;104;123
69.77
9
35
110505 Group C 1 Sb (1,40.00)
Diode Array
280
Range: 3.479e-5
Area, Height
66.55
11
14
49% H
69.85
3
14
21% H
110505 Group B 1 Sb (1,40.00)
Diode Array
280
Range: 9.457e-5
Area, Height
66.75;46;70;38% H
110506 Group A 3 Sb (1,40.00)
Diode Array
280
Range: 5.958e-5
Area, Height
66.70
21
37
AU
5.0e-5
0.0
-5.0e-5
60.00
61.00
62.00
63.00
64.00
65.00
66.00
67.00
68.00
69.00
70.00
71.00
Time

Pooled patient samples 1 mL loop PALL filtered 100 ug/mL




Pooled patient samples 1 mL loop PALL filtered 100 ug/mL
110504 Group D 1 Sb (1,40.00)
Diode Array
280
Range: 5.215e-4
Area, Height
AU
5.0e-4
2.5e-4
80.87
24
49
81.83
14
83
82.77
96
481
87.28
24
141
88.22
48
125
110505 Group C 1 Sb (1,40.00)
Diode Array
280
Range: 2.028e-4
Area, Height
80.83
28
63
74% L
81.83
10
43
82.73
31
154
87.33
3
21
88.20
37
92
8% H
110505 Group B 1 Sb (1,40.00)
Diode Array
280
Range: 1.466e-4
Area, Height
80.92
62
126
49% H
82.78
10
44
87.32
3
21
88.15
4
14
110506 Group A 3 Sb (1,40.00)
Diode Array
280
Range: 1.45e-4
Area, Height
80.95
95
142
Time
80.00
81.00
82.00
83.00
84.00
85.00
86.00
87.00
88.00
89.00
90.00

Appendix 6

Details of calculation for future sample size recommendation for comparison using two independent means as mentioned in Section 2.2.3 and described by Altman DG, Machin D, Bryant TN, Gardner MJ. Statistics with confidence, 2nd edition. BMJ Books, 2000.

$n = 2 * (Z_{1-\alpha/2} + Z_{1-\beta})^2 * \sigma^2 / \Delta^2$

$n = 2 * (1.96 + 0.84)^2 * 350^2 / 200^2$

$n = 2 * 7.84 * 122{,}500 / 40{,}000$

$n = 48.02$ or 48 per group

Conclusion: 48 subjects are needed in each group to have an 80% chance of detecting a clinically meaningful difference in total protein concentration of 200 ug/mL between groups, assuming an alpha of 0.05, and a standard deviation of 350 ug/mL.

Curriculum Vitae

Nada Tabbara

EDUCATION

M.Sc. candidate, Biochemistry
University of Western Ontario

Honors BMSc. Specialization, Microbiology and Immunology 2003-2007
Honors thesis title: Identification of a Novel T Cell
Receptor for Bacterial Superantigens
Co-Supervisors: Dr. Joaquín Madrenas and Dr. John K. McCormick
University of Western Ontario

SCHOLARSHIPS & AWARDS

Dental Research Day Certificate of Merit 2010

WRF Award 2nd Place Poster Presentation 2010
23rd Annual Western Research Forum

Department of Paediatrics Graduate Student Scholarship 2009
Schulich School of Medicine & Dentistry

Rising Researcher Travel Award for Poster Presentation 2009
Canadian Child Health Clinician Scientist Program
7th Annual National Symposium, Halifax NS

POSTER AND ORAL PRESENTATIONS

24th Annual Western Research Forum 2011
"Evidence of changes in the salivary proteome in children
during dental eruption"
University of Western Ontario

Departmental Seminar, Biochemistry 2011
"Changes in the salivary proteome during the course
of dental eruption"
Schulich School of Medicine & Dentistry

23rd Annual Western Research Forum 2010
"Identification of the Salivary Proteome in Children
Pre- and Post-dental eruption"
University of Western Ontario

Department of Biochemistry Research Day 2010

Schulich Dental Research Day 2010
"Evidence of changes in the salivary proteome in children during dental eruption"
Schulich School of Medicine & Dentistry

CCHCSP 7th Annual National Symposium, Halifax NS 2009
"Identification of the salivary proteome in children throughout the circadian rhythm"
Canadian Child Health Clinician Scientist Program
Halifax, Nova Scotia. Poster.

PUBLICATION

McDonald EE, Goldberg HA, **Tabbara N**, Mendes FM, Siqueira WL. Histatin 1 resists proteolytic degradation when adsorbed to hydroxyapatite (2011). *J Dent Res.* 90(2):268-72.

LEADERSHIP INVOLVEMENTS

Margaret Moffat Research Day 2011
Executive Committee Member
Career Team Leader
Expert Panel Moderator

Schulich Strategic Planning Process Focus Group 2011
Graduate Student representative

Western Humanitarian Award Senate Selection Sub-committee 2010

Western International Student Centre – EC Group Leader 2009-2010

Operation Smile Students' Association 2006-2007
President – Western Chapter

RELATED RESEARCH EXERIENCE

Dr. Michael Rieder's Clinical Pharmacology Laboratory
Robarts Research Institute, Research Associate 2006-2009

Dr. Grant McFadden's Microbiology and Immunology Laboratory
Siebens-Drake Research Institute, Research Assistant 2005-2006

Dr. Gregory E. Dekaban's Biotherapeutics Laboratory
Robarts Research Institute, Research Assistant 2004-2005