Detection of Human Salivary α-Defensins by LC-ESI-MS and Evaluation of Levels in Athletes after Physical Stress

by

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Declaration

I certify that this work has not been accepted in substance for any degree, and is not concurrently being submitted for any degree other than that of Doctor of Philosophy being studied at the University of Greenwich. I also declare that the work is the result of my own investigations except where otherwise identified by references and that I have not plagiarised the work of others.

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Conference Presentations

The following published abstracts/conference presentations have arisen from the material detailed in this thesis:

Poster Presentations

British Society for Proteome Research Meeting, University of Reading, UK (20-22nd July, 2015): Evaluation of salivary antimicrobial peptide (α-defensins) in relation to physical stress.

The 29th Protein Society Meeting, Barcelona, Spain (23rd-25th July, 2015):

1) Evaluation of salivary antimicrobial peptide (α-defensins) in relation to physical stress.

2) Investigation of potential correlation between salivary antimicrobial peptides (α-defensins) and cortisol after physical stress.

3) Validation of an LC-ESI-MS assay for the detection of human salivary neutrophil α-defensins in human saliva.

The 5th International Antimicrobial Peptide Meeting, Burlington House, London (7-8th September, 2015): Evaluation of salivary antimicrobial peptide (α-defensins) in relation to physical stress.

The 36th British Mass Spectrometry Society Meeting, University of Birmingham, UK (15-17th September, 2015): Evaluation of salivary antimicrobial peptide (α-defensins) in relation to physical stress.

These posters are presented in Appendix E.

Oral Presentation

The 36th Biological Mass Spectrometry Meeting, Imperial College, London (24th September, 2015): Evaluation of antimicrobial peptide (HNP 1-4) in human saliva by LC-ESI-MS.

Research publication

1. Fernando Naclerio, Eneko Larumbe‐Zabala, Nadia Ashrafi, Marco Seijo, Birthe Nielsen, Judith Allgrove, Conrad P. Earnest (2016). Effects of Protein-Carbohydrate Supplementation on Immunity and Resistance Training Outcomes: A double blind, randomized, controlled clinical trial. Eur J Appl Physioly. DOI 10.1007/s00421-016-3520-x (presented in Appendix F).

Abstract

The aim of this study was to optimise and validate an LC-ESI-MS method to determine the level of individual human neutrophil alpha defensins (HNP1-4) after physical stress. A method for the analysis of individual (HNP1-4) in a saliva matrix by LC-ESI-MS has been established. An Agilent 1200 series HPLC, coupled with a Synapt G1 (Q-TOF-ESI-MS), was used for the separation and detection of HNP1-4. The need for solid phase extraction (SPE) was established. An LC-MS optimisation, focusing on mobile phases, column performances, and ion intensities, was carried out in order to enhance the detection of individual HNPs in human saliva. During LC-MS optimisation, two different mobile phase compositions (methanol with water; acetonitrile with water) and three different additives (formic acid, acetic acid, ammonium format with formic acid) were compared in response to electrospray ionisation (ESI) ion intensity of salivary HNP1-4. Kinetex® column separation efficiency was evaluated using two different column dimensions (50 x 2.1 mm and 50 x 3 mm) and two different stationary phases (C18 and C8). The performance of the core shell Kinetex® column (homogenous porous shell) was also compared to a new ultra ACE® (encapsulated bonded phase) column. The results showed that a SPE method prior to the analysis resulted in cleaner mass spectra compared to a method without SPE; whereby co-elution of proline rich proteins, peptides and other unknown peptides were observed to cause ion suppression. The peak area reduction between the HNPs in the SPE and without SPE treated samples ranged from 17-65%. HNPs were extracted by SPE with a recovery of 80-91%. Aqueous 0.1% acetic acid: methanol (50:50) provided enhanced ion intensities for individual HNPs. The Kinetex® C8 (50 x 3.0 mm, 2.6 µm) column facilitated a better separation of individual HNPs compared to the Ultra Core Super C18 ACE® (50 x 3.0 mm, 25 µm), Kinetex® C18 (50 x 3.0 mm, 2.6 µm) and Kinetex® C18 (50 x 3.0 mm, 5 µm) columns. The LC-MS method was linear for concentrations of HNP2 between 0.05 and 1 ng/µL with a detection limit of 0.05 ng/µL and quantification limit of 0.1 ng/µL ($R^2 = 0.99$). Inter-intra assay precision was 0.3–15%, respectively. As part of the method validation, HNP1-3 salivary levels were determined by ELISA and the data compared with that obtained by LC-MS. No significant correlation was found between the two methods ($R^2= 0.96$) for the detection of these potential antimicrobial peptides. The optimised, validated LC-MS method was applied to examine the relative levels of individual HNP1-4 in participants undertaking a rigorous physical activity intervention. The levels of individual HNP1-4 were found to be higher after exercise (at both 30 and 60 min intervals) compared to baseline assessments, where participants consumed either placebo (PL) or carbohydrate (CHO) supplements. In addition, the level of

individual HNPs at 30 mins post exercise increased by around 80-134% and 103-132% for participants who consumed either PL or CHO supplements. A further increase in the levels of individual HNPs was observed around 185-415% and 90-264% at 60 mins post exercise. These levels differed significantly (HNP1, p=0.00; HNP2, p=0.05; HNP3, p=0.03; HNP4, p=0.01) in athletes that consumed either PL or CHO supplements. Furthermore, the levels of individual HNPs were investigated after supplementing with a commercially available multi-ingredient (MTN) and compared with a CHO supplement. A similar trend was observed for individual HNPs at both 30 and 60 min intervals but they did not significantly differ at any time at the intervals (post 30 mins HNP1, p=0.85; HNP2, p=0.16; HNP3, p=0.07; HNP4, p=0.40 and post 60 mins HNP1, p=0.17; HNP2, p=0.20; HNP3, p=0.09; HNP4, p=0.30). The above findings provide evidence that the level of individual HNPs varies and is sensitive to physical stress. Physical stress may induce air-inflammation and damage to the airway epithelial cells, which could be part of a normal stress response. A possible correlation between the average response of HNP1-3 and cortisol was investigated by ELISA. The findings suggest that there is no correlation between the changes in the levels of salivary HNP1-3 and cortisol. In conclusion, the optimised and validated LC-MS method revealed that individual levels of HNPs can be determined accurately and precisely, which makes a novel contribution to sports and clinical research.

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List of Abbreviations

AMPs	Antimicrobial Peptides
ANS	Autonomic Nervous System
AUC	Area Under the Curve
C18	Carbon 18
C8	Carbon 8
CF	Cross fit
CHO	Carbohydrate
CMJ	Countermovement Vertical Jump
CV	Coefficient of Variation
Da	Daltons
EIC	Extracted Ion Chromatogram
ELISA	Enzyme-Linked Immunosorbent Assay
ESI	Electrospray Ionisation
F	Flow rate
FA	Formic Acid
HBD	Human β Defensin
HMB	β-Hydroxy β-Methylbutyric acid
HNP	1-4 Human Neutrophil α-Defensin
HPAA	Hypothalamic–Pituitary–Adrenal-Axis
HPLC	High Performance Liquid Chromatography
HQC	High concentration
ID	Internal Diameter
IgA	Immunoglobulin A
kDa	Kilo Daltons
kV	Kilovolt
L	Length
LC	Liquid Chromatography
LL-37	Cathelicidin
LOD	Limit of Detection
LOQ	Limit of Quantitation
LQC	Low concentration
m/z	Mass to Charge Ratio
MALDI	Matrix-Assisted Laser Desorption Ionisation
MQC	Medium concentration
MS	Mass Spectrometry
MTN	Multi-Nutritional
N	Column efficiency
p	Probability
PA	Peak Area

PL	Placebo
PRP3	Proline Rich Proteins
Q-TOF	Quadruple Time of Flight
RIA	Radio Immunoassay
RIL	Rayleigh instability limit
RMBP	RM Bench Press
RP	Reverse Phase
RPE	Rate of Perceived Exertion
S/N	Signal to noise ratio
SPE	Solid Phase Extraction
STD	Standard deviation
TFA	Trifluoroacetic Acid
tG	Gradient time
TIC	Total Ion Current
u	Linear Velocity
UBPT	Upper Body Power Test
UV	Ultraviolet radiation
V	Volts
v/v	Volume Concentration
$VO_{2\ max}$	Maximal Oxygen Uptake
WSPE	Without Solid Phase Extraction

Key Terms

Antimicrobial peptides: these are small (<100 amino acids) cationic peptides that demonstrate antimicrobial activity. AMPs are known as multifunctional molecules, and have a central role in infection and inflammation.

Cortisol: a steroid hormone with many functions in the body. It represents a chemical response of the body to stress and plays a major role in the mobilisation of glucose into the blood stream.

Crossfit: a training performance with additional resistance training elements (external overweight). Crossfit training is the most efficient way to enhance and muscle endurance.

Charge deconvolution: the process of obtaining the molecular weight from the number of charge states of a multiply charged ion from individual isotopes.

Extracted ion chromatogram (EIC): this is created by plotting the intensity of the signal observed at a chosen *m/z* value or set of values as a function of the retention time.

Electrospray ionisation (ESI): this is a method by which ions can be transferred or produced in the gas phase. It is especially useful in producing ions from macromolecules because it overcomes the propensity of these molecules to fragment when ionised.

Gradient: a method of changing the mobile phase composition over time to affect a separation, this typically enables the analysis of a mixture of analytes which have a broad range of polarities and retention characteristics in a single run, and for many applications this offers significant benefits over isocratic methodology.

High performance liquid chromatography electrospray ionisation mass spectrometry (HPLC-ESI-MS): this is an analytical technique that can be used for the separation, detection and quantitation of proteins and peptides present within a complex matrix.

Human neutrophil α-defensins: small, cationic antimicrobial peptides that are part of the mucosal immune system, possessing a broad spectrum of antibacterial and antifungal activity.

Internal standard: bioanalytical techniques, such as LC-ESI-MS, can employ various methods to quantify an unknown analyte; however, the main methods used are either standard addition or adding an internal standard. An internal standard can also be used to ensure the recovery of an analyte and instrument sensitivity.

Ion suppression: matrix effects are the alternation of ionisation efficiency in the presence of co-eluting substances. Ion suppression is one form of matrix effect that negatively affect detection capability, precision and accuracy.

Isobaric ions: two or more analytes of the same nominal molecular weight; co-elution is known as an isobaric effect. The presence of co-eluting isobaric ions can lead to ion suppression.

Label free quantitation: a method that aims to determine the amount of analytes present within a sample without chemically modifying the analytes.

Mobile phase: liquid chromatography separation is based on the selective distribution of analytes between a liquid mobile phase and a stationary phase. The mobile phase in reverse phase high performance liquid chromatography usually consists of an aqueous/organic solution and commonly an organic modifier.

Number of theoretical plates (N): columns with high plate numbers are considered to be more efficient, that is they have a higher column efficiency than columns with a lower plate count. A column with a high number of theoretical plates will have a narrower peak at a given retention time than a column with a lower N number.

Particle size: the mean diameter of the spherical supports used to pack a column, is a physical dimension that has a significant impact on the performance of an HPLC column.

Placebo: an inactive substance that is visually identical in appearance to the liquid supplements that are being tested in this study. A placebo is used to compare the effects of an inactive substance with those of the experimental supplements

Solid phase extraction: one of the most popular sample preparation techniques involving the use of chromatographic sorbents in a column format. A sample is passed through the column bed and analytes are retained on the sorbent while the sample matrix liquid passes through. The sorbent bed is then washed to remove undesired interferences, and the purified analytes are subsequently eleuted from the column.

Supplements: a product taken orally that contains one or more ingredients (proteins, carbohydrates) that are known as supplements. Supplements (also called ergogenic aids) are used to enhance athletic performance and may include vitamins, minerals, carbohydrates, proteins or a combination of these.

Signal to noise ratio (S/N): this is a measure that is used to compare the level of a desired signal to the level of background noise.

Total ion current (TIC): this results from the detection of ions over the period of the MS scan. In general, a TIC chromatogram is created by totalling the intensities of all the mass spectral peaks belonging to the same scan over time

Ultra-core shell: uses a solid silica core and a porous outer silica shell. The manufacturing of an ultra-core shell utilises an encapsulated bonding technique, which helps to minimise the influence of non-derivatised silanol groups. The end capping reagent is generally a smaller silane (SiH_4), which is later used for derivatisation. This process reduces unwanted interactions between polar or charged analytes, because the number of available silanol groups has been reduced. High efficiency separations can be achieved, comparable with UPLC, but potentially without the high back pressures.

van Deemter curve: this describes the relationship between the height equivalent of a theoretical plate (HETP) and the linear velocity; and is partly dependent on the diameter of the particles packed into the analytical column. A smaller particle diameter can significantly reduce the HETP which results in higher efficiency.

VO_2 max: The maximum or optimum rate at which the heart, lungs, and muscles can effectively use oxygen during exercise. It is used as a way of measuring a person's individual aerobic capacity.

Overview

Human saliva is increasingly being used for proteomic and biomarker-discovery studies due to the ease of collection and simpler work flow compared to when using plasma and blood samples (Hu *et al.,* 2006). Recently, the combination of emerging biotechnologies and salivary diagnostics has extended the range of saliva-based diagnostics from the oral cavity to the entire physiologic system, as most compounds found in the blood are also present in saliva (Hu *et al.,* 2006; Mori *et al.,* 2006). Saliva contains a variety of electrolytes, enzymes, antibodies (immunoglobulins) and antimicrobial peptides (AMPs), such as defensins, and cathelicidins. Antimicrobial peptides and proteins are important components against invading pathogens (De Smet *et al.,* 2005; Bals *et al.,* 1998). Due to advances in mass spectrometry technologies, liquid chromatography-electrospray ionisation-mass spectrometry (LC-ESI-MS) is now considered one of the most convenient analytical techniques for proteomic studies (Zhao *et al.,* 2014; Li *et al.,* 2012; Wasinger *et al.,* 2013). The advantage of using LC-ESI-MS lies in the fact that it is an extremely sensitive technique that enables the rapid separation and identification of peptides (Vitrino *et al.,* 2000; Gardner *et al.,* 2008).

Mucosal Immunity and AMPs

The mucosal surfaces of the body are thin, permeable barriers and their physiological activities are involved in gas exchange (the lungs), food absorption (the gut), sensory activities (eyes, nose, mouth, and throat), and reproduction (uterus and vagina). These mucosal surfaces are directly exposed to the environment, which makes them particularly vulnerable to infection (Gorr *et al.,* 2009; Boman *et al.,* 2003; Gorr *et al.,* 2011); however, mucosal secretions contribute to innate immunity as the first line of defence, and the salivary glands are considered to be part of the common mucosal immune system (Brandtzaeg *et al.,* 2004). AMPs are relatively small (<10 kDa), cationic and amphipathic peptides of variable chain length, sequence and structure. Over the past two decades, several AMPs have been isolated from diverse animals, both vertebrates and invertebrates, plants, and also from bacteria and fungi. These peptides exhibit a broad-spectrum of activity against a wide range of microorganisms, including Gram-positive and Gram-negative bacteria, protozoa, yeast, fungi and viruses (Wilson *et al.,* 2013; Mori *et al.,* 2006; Ganz *et al.,* 2003; Lai *et al.,* 2009).

A diverse range of antimicrobial peptides (defensins, histatins and cathelicidins) and proteins (immunoglobulins) are found within saliva, and similar to other mucosal surfaces, many of

these AMPs form a constitutive barrier to prevent foreign micorbes from entering the oral cavity. Each mucosal location has a unique profile of AMPs, and salivary AMPs are produced from different cell sources, e.g. macrophages, neutrophils, and epithelial cells.

Role of AMPs in Exercise and Mucosal Immunity

The immune system provides a unique opportunity to evaluate the role of stress (physiological or physical) and numerous studies have suggested that exercise induces considerable physiological change within the immune system (West *et al.,* 2006; Gleeson *et al.,* 2007; Walsh *et al.,* 2011; Allgrove *et al.,* 2008; Beaven *et al.,* 2008; Moreira *et al.,* 2009; Cadore *et al.,* 2009; Thomas *et al.,* 2009; Usui *et al.,* 2011; Davison *et al.,* 2009). The effect of exercise on mucosal immunity has mainly been focused on salivary immunoglobulins, especially different forms of immunoglobulin and hormones (Gleeson *et al.,* 2000; Usui *et al.,* 2011; Talebi *et al.,* 2013; Laing *et al.,* 2003; Rosa *et al.,* 2014). This is not surprising given that IgA and IgG are the predominant immunoglobulins within saliva and there is a relationship between mucosal immunity and upper respiratory illness symptoms (Neville *et al.,* 2008; Woof *et al.,* 2006). It is well-known that physical and mental stress provokes the release of cortisol from the hypothalamic-pituitary-adrenal axis, and thus stress can modulate various immune responses (Gleeson *et al.,* 2000).

However, the effects of exercise on other factors within mucosal secretions that contribute to innate immunity have been unexplored. To date, no studies have systematically investigated the effect of acute (minutes to hours) and chronic (days to weeks) changes in AMP concentrations (α-defensins) in saliva before and after exercise or training. Few studies have measured the salivary concentration of human neutrophil α-defensin (HNP1-3) following prolonged training, and in this study HNP1-3 were measured using an enzyme-linked immunosorbent assay (ELISA) which does not discriminate between individual HNP peptides due to structural similarities (Davison *et al.,* 2009; Gillum *et al.,* 2015; Kunz *et al.,* 2015). Consequently, there is a need to develop an analytical method that will discriminate between defensins. Currently, there is no report within the scientific literature of any investigation that has assessed the relative level of each HNP1-4 by mass spectrometry (MS) in relation to physical stress (exercise), although a few studies have quantified HNP1 by Liquid chromatography coupled with ultraviolet (LC-UV) (Tanida *et al.,* 2003; Mizukawa *et al.,* 1999) or HNP1-3 by Liquid chromatography coupled with electrospray ionisation (LC-ESI-MS) (Cabras *et al.,* 2010; Goebel *et al.,* 2000; Van den Broek *et al.,* 2010; Peulso *et al.,* 2007; Pisano

et al., 2005; Vitorino *et al.*, 2004). These studies have some limitations, such as insufficient sample clean-up. Davidson (2009) demonstrated that the level of salivary HNP1-3 significantly increased following an aerobic exercise. Another study which focused on AMPs (ß-defensin-2 and cathelicidins LL-37 levels), revealed that an aerobic exercise caused an increase in the saliva concentration of these analytes during and after exercise, whereas salivary IgA concentration and secretion rates decreased after exercise. Usui (2011) suggests that this type of exercise might also modify antimicrobial peptide profiles within oral secretions. The authors concluded that it is possible that aerobic endurance exercise could partly enhance oral innate immunity, although the precise mechanism(s) of the exercise induced an increase in antimicrobial peptides remain unclear.

Research Question

Is high performance liquid chromatography (HPLC) ESI-MS a more selective and sensitive technique than ELISA to detect changes of individual HNP1-4 levels in athletes undertaking rigorous exercise intervention?

Aims and objectives

The aims and objectives of this study were to:

1. Develop, optimise and validate an LC-ESI-MS method for the detection and relative quantification of salivary α-defensins. This extends to sample preparation.
2. Evaluate the physiological responses of HNP1-4 in athletes over a 2.5 h exercise intervention and during recovery (30 mins and 60 mins).
3. Determine the salivary α-defensin levels by ELISA.
4. Compare the data obtained by HPLC-MS and ELISA with respect to sensitivity, selectivity and reproducibility, and determine if LC-ESI-MS is a viable method for the detection of human α-defensins.
5. Determine the levels of stress hormone (cortisol) by ELISA during the exercise intervention and consider if there could be a correlation between changes in α-defensins and stress hormone levels.

Chapter 1 - Introduction

Over the last seventy-five years' interest in salivary research has increased steadily due to the development and advancement of techniques for investigating the biochemical and physicochemical properties of components in saliva. The multifunctional role of saliva in speech, lubrication, digestion, oral and general health, alongside the finding that saliva contains chemical markers which can aid in the detection of systemic disease, has increased interest in this bio-fluid for diagnostic and clinical research (Garett *et al.,* 1975; Mandel *et al.,* 1993; Zhang *et al.,* 2013; Schipper *et al.,* 2007). Interest in salivary research increased from the mid-1950s to mid-1960s, when for the first time, the Burgen and Ellison Group investigated the composition and complexity of saliva (Burgen *et al.,* 1956; Ellison *et al.,* 1960). This chapter introduces the biochemical composition of human saliva, its function and production, together with an introduction to α-defensins. The aim of this chapter is to provide a literature review of the existing analytical work on the detection and quantitation of salivary α-defensins (HNP1-4); therefore, in this chapter, qualitative and quantitative methods for the analysis of HNP peptides are reviewed. Furthermore, this chapter also presents a discussion on how physical stress induces the activation of neutrophils, and the effects of exercise on α-defensins and hormone levels (cortisol) are also highlighted. Finally, the function and production of a commonly used biomarker of physical stress, cortisol, is described, and the reasons for comparing the levels of this stress hormone against levels of antimicrobial peptides.

1.1 Saliva and Diagnostic Perspectives

The liquid chromatography (LC) mass spectrometry (MS) approach has aided the diagnostic potential of plasma and human saliva, especially through the identification and quantification of proteins; however, this is challenged by the large dynamic range of protein abundance within these fluids. Human plasma/serum is dominated by immunoglobulins and albumins that constitute 60-80% of the total weight (Bjorhall *et al*., 2005), with the most abundant plasma proteins representing 99% of the total protein content of this matrix. The remaining fraction is composed of proteins with a much lower abundance, including proteolytically cleaved protein fragments. However, in whole saliva, the most abundant proteins represent approximately 40% of the saliva protein content (Whiteaker *et al*., 2007). The salivary proteome contains a larger proportion (14%) of low-molecular-weight proteins (< 20 kDa), in contrast to only 7% for the plasma proteome. The highest fraction of proteins found in whole saliva range in size between

20 and 40 kDa (26%), whereas the 40-60 kDa range is the largest fraction for plasma (18%); in total, 65% of saliva proteins have a molecular weight less than 60 kDa, compared to 36% of plasma proteins. For the proteins that are common between saliva and plasma, the molecular weight distributions are similar to the distributions of the salivary proteome, with a tendency toward the low-molecular-weight end, except for the highest molecular weight range (≥ 200 kDa). Approximately 27% of whole saliva proteins are also found in plasma, and such a significant overlap in protein content between saliva and plasma has led to the proposal that saliva could be used as a diagnostic alternative to blood tests (Loo *et al.,* 2009).

1.2 Saliva

Saliva is an oral liquid secreted by the salivary glands, and human saliva is mainly composed of water (99.55%), with the remaining 0.45% consisting of electrolytes (sodium, potassium, calcium, chloride, magnesium, bicarbonate, and phosphate), enzymes (α-amylase, lipase, ribonuclease, kallikrien, peroxidase, and lysozyme), antibodies (immunoglobulins), proteins (proline rich proteins, mucins), antimicrobial compounds (histatins, cystatins, defensins, and cathelicidins) and hormones (cortisol, testosterone) (Humphrey *et al.,* 2001).The most abundant proteins and peptides within human saliva are proline-rich proteins (PRPs), mucins and amylase (Scarano *et al.,* 2010; Pfaffe *et al.,* 2011; Lamkin *et al.,* 1993). Salivary PRPs are usually divided into acidic (MW 16 kDa), basic (MW 6-9 kDa) and glycosylated (MW 36 kDa) groups (Schenkels *et al.,* 1995). Human saliva contains two types of mucins, termed low and high molecular weight mucin glycoproteins: MG1 (>100 kDa) and MG2 (150-200 kDa) (Schenkels *et al.,* 1995). Amylase is a protein enzyme (40-60 kDa) and is one of the most abundant components present in saliva (Pfaffe *et al.,* 2011). Proline rich proteins are constitutively expressed in humans and 60-70% are secreted from the parotid gland (Kauffman *et al.,* 1979). Pfaffe (2011) and Schenkels (1995) described the major proteins found in unstimulated human saliva, and proline rich proteins were found to account for 37%, in comparison to α-amylase 20%, mucins 20%, cystatins 8%, albumin 8%, IgA 2%, statherins 1%, histatins <1 %, IgM <1 %, IgG <1 %, lysozyme <1 %, lactoferrin <1 %, and cathelicidins and defensins (percentages are not available).

In general, human saliva contains a large array of proteins and peptides that have important biological functions. Protein components that have been identified in all of the major glandular secretions are proline-rich proteins, amylase, statherin, histatins, lysozyme, and secretory IgA; whereas cystatins and mucins have been identified in submandibular/ sublingual secretions

(Yao *et al.,* 2003). However, some proteins are specific to saliva and have no common equivalent with serum proteins; these are synthesised by the acinar cells, for example amalyse and lactoferrin and their secretion is a response to mastication. Interestingly, HNP1-4 were found to be undetectable in the parotid, submandibular, and sublingual glands. As a large number of neutrophils continuously migrate from the blood through the gingival crevice into the oral cavity, salivary HNPs could theoretically be derived from these cells (Abiko *et al.,* 2003; Pisano *et al.,* 2005). Blood components enter the watery fluid of the salivary duct via two processes; active transport or passive diffusion. Within the salivary glands, transfer mechanisms include intracellular and extracellular routes, e.g. movement across membranes via active and passive transport (Figure 1.1), with passive transport being the most common intracellular route.

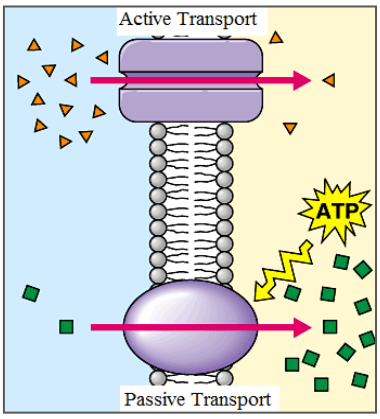


Figure 1.1 Transport of molecules from blood to salivary glands (Dawn *et al.,* 2008).

Passive transport is the movement of molecular substances across a cell membrane, and unlike active transport, it does not require an input of chemical energy. The movement of molecules depends upon their concentration inside and outside a cell, and molecules move neutrally from an area of high concentration to a lower concentration, known as passive diffusion (Kufman *et al.*, 2002; Wang *et al.*, 2015). Antimicrobial peptides (HNP1-4) are actively secreted into saliva by an energy dependent process (active transport).

1.2.1 Production of Saliva

Saliva is produced by three major glands, the parotid, submandibular and sublingual glands, and by various minor glands. The parotids are the largest glands, located in front of and just below each ear, while the submandibular glands can be found under the tongue, and the sublingual glands are located below the jawbone (Figure 1.2). Minor glands are found in the

lower lip, tongue, palate, cheeks, and pharynx. The terms ‘major’ and ‘minor’ refer to the anatomical size of the glands (Edgar *et al.,* 2014) and the major glands produce more saliva (90% of the total volume of saliva) compared to the minor glands. The parotid glands consist of serous acinar cells, which secrete thin, watery, amylase rich saliva, and this is the largest salivary gland in humans.

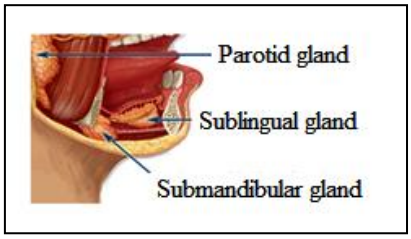


Figure 1.2 Anatomy of the salivary glands (Larian *et al.,* 2014).

Despite its large size, it only produces approximately 25% of saliva (Edgar *et al.,* 2014). The submandibular gland consists of secretory serous and mucous acinar cells, which produce viscous and mucins-rich saliva. The major function of this mucus is to protect against infectious agents, such as fungi, bacteria and viruses. This gland produces approximately 60% of saliva (Edgar *et al.,* 2014). The sublingual is the smallest of the major salivary glands, and numerous ducts are found under the sublingual folds of the mucous membrane on the floor of the mouth, beneath the tongue near the midline. This gland consists of mucous acinar cells, which secrete fluid that is predominantly mucous in character. Approximately 7-8% of saliva is produced by this gland (Edgar *et al.,* 2014). The structure of the salivary glands consists of a secretory end piece (acini) and a branched ductal system. There are three types of duct present in the salivary glands: intercalated ducts which have a low cuboidal epithelium and a narrow lumen; striated ducts which are lined with columnar cells; and excretory ducts which have cuboid cell types until the terminal section, which is lined with stratified squamous epithelium. In addition, the acini are surrounded by myoepithelial cells, the function of which is to assist in propelling the secretions into the ductal system. The fluid formation within salivary glands occurs in the end pieces (acini), where serous cells produce a watery seromucous secretion and mucous cells produce a viscous mucin-rich secretion. These secretions result from interstitial fluid from blood in capillaries, which is then modified by the end piece cells and secreted into the lumen. From the lumen it passes through the ductal system, where it is further modified, mostly within the striated ducts, where ion exchange occurs and secretions are changed from an isotonic solution to a hypotonic one. The composition of saliva is further modified in the excretory ducts before it is finally secreted into the mouth (Edgar *et al.,* 2014). Each salivary gland

produces and secretes either serous or mucous fluid; serous fluid is enriched with proteins and water, whereas mucous fluid is enriched with glycoproteins and water. The salivary glands have a high blood flow and the salivary secretion is exclusively controlled by the sympathetic and parasympathetic autonomic nervous system.

1.2.2 Function of Saliva

Salivary function can be organised into five major categories that serve to maintain oral health and create an appropriate ecologic balance (Table 1.1): (1) buffering action and clearance; (2) antibacterial activity; (3) maintenance of tooth integrity; (4) taste and digestion; and (5) lubrication and protection (Moss *et al.*, 1995; Mandel *et al.*, 1985).

Table 1.1 The function, components and mechanisms of saliva (Nielsen *et al.*, 2008).

Function	Components	Mechanisms
Buffering action and clearance	Sodium, potassium, calcium, bicarbonate, phosphate, urea, proteins	Saliva buffers neutralises the acids produced by microorganisms and maintain salivary pH.
Antibacterial action	Immunoglobulins (IgA, IgG, IgM), mucins, lysozymes, peroxidase, proteins, peptides	The immunoglobulins neutralise viruses, serve as antibodies for bacterial antigens, and work to aggregate bacteria; thus inhibiting bacterial attachment to dental tissue.
Maintenance of tooth integrity	Calcium, phosphate, proteins	Help to maintain the integrity of teeth by modulating remineralisation and demineralisation.
Taste and digestion	Amylase, lipase	The hypotonicity of saliva enhances the tasting capacity of salty food and nutrient sources depending on the presence of zinc. Taste and digestion of carbohydrates are achieved through α-amylase which breaks up the carbohydrate into sugars while saliva lipase initiates fat digestion.
Lubrication	Mucins, statherins, proline rich proteins	Mucins are responsible for lubrication (to protect against dehydration) and also maintenance of salivary viscosity. Mucins also protect oral tissues against proteolytic attack by microorganisms. Lubricant effects help mastication, speech and deglutition.

1.3 Defensins: Antimicrobial peptide

Antimicrobial peptides are small (<100 amino acids) cationic peptides that demonstrate antimicrobial activity. Defensins constitute a major family of antimicrobial peptides that play a central role in infection and inflammation, where they act as part of the host defences against infectious agents, including bacteria, viruses, fungi and parasites (Lai *et al.*, 2009). Based primarily on the spacing between the cysteine residues and the topology of the disulphide bridges, defensins are organised into three classes; α, β and θ (Hazlett *et al.*, 2011). Human α-

defensins are small (3500 - 4000 Da) peptides, whereas, β-defensins are larger (4000 - 7000 Da). A third novel class of defensins has been identified in rhesus macaque leukocytes, which is only 18 amino acids in length and is referred to as θ-defensins (Tang *et al.,* 1999; Nguyen *et al.,* 2003; Selsted *et al.,* 2005; Wang *et al.*, 2014). Nguyen (2003) reported that θ-defensins are structurally dissimilar to α and β defensin family and are only found in non-human primates. Defensins have also been identified in many multicellular organisms, including plants, such as *Arabidopsis thaliana* (Thomma *et al.,* 2002), invertebrates, such as *Mytilus edulis* and *Mytilus galloprovincialis* (Charlet *et al.,* 1996; Hubert *et al.,* 1996; Thomma *et al.,* 2002), and other vertebrate animals, such as rabits (Selsted *et al.,* 1984), rats, guinea pigs, hamsters, mice (Lehrer *et al.,* 2002).

1.3.1 α-Defensins

In 1985 the Lehrer Group isolated a family of α-defensins from human blood (Selsted *et al.,* 1985), and based on the source, property and size, these peptides were named the human neutrophil peptides (HNP1, HNP2, and HNP3). These three defensins have nearly identical amino acid sequences. It is believed that HNP2, which is 29 amino acids in length, could arise following post-translational proteolytic cleavage of the N-terminal amino acid of HNP1 or HNP3 (Wang *et al.,* 2014). A fourth human neutrophil defensin (HNP4) was isolated and characterised form an azurophil granule (Wilde *et al.,* 1989). HNP4 has a distinct peptide sequence of 33 amino acids (Table 1.2). HNP1, HNP2 and HNP3, which differ only by the first amino acid, account for 5-7% of the total neutrophil proteins; whereas, HNP4 compromises less than 2% of the total defensins found within neutrophils (Klotman *et al.,* 2006; Wilde *et al.,* 1989; Selsted *et al.,* 1985). In 1992 and 1993, Jones and colleagues identified human defensins 5 and 6 (HD5 and HD6), a family of α-defensins from human paneth cells. HD5 and HD6 also have a distinct peptide sequence of 32 amino acids (Table 1.2). In total, six α-defensins have been found in humans (Klotman *et al.,* 2006). Human α-defensins 1-4 are designated as human neutrophil peptides (HNP1, HNP2, HNP3, and HNP4), as they are mainly expressed by neutrophils (Ganz *et al.,* 1985). However, HD5 and HD6 peptides are tissue specific, as they are only expressed in the paneth cells of human intestines and the female reproductive system (Wang *et al.,* 2014).

1.3.2 β-Defensins

β-defensins are found primarily in association with epithelial surfaces in humans, in particular, the skin, gut, trachea, and oral epithelia, particularly the gingiva (Table 1.3). Members of the human β-defensin family were identified in 1990s. The first ß-defensin (HBD1) was reported

in the plasma and epithelial cells of the urinary and respiratory tract (Bensch *et al.*, 1995; Valore *et al.*, 1998). Human β-defensin 2 (HBD2) was isolated from psoriatic skin using an affinity chromatography procedure utilising columns coated with microbial components (Harder *et al.*, 1997), and is expressed on skin and epithelia of the respiratory and gastrointestinal tract (Bals *et al.*, 1998; Haynes *et al.*,1999). In addition, HBD3 was discovered through bioinformatics and functional genomic analyses of the Human Genome Project (Gracia *et al.*, 2001). However, in order to prove the existence of this peptide, the tissue distribution of HBD3 expression was evaluated via real-time quantitative (RT-PCR) and it was detected in the uterus, lungs, kidney, prostate, neutrophils, placenta, testis, heart and skeletal muscle (Gracia *et al.*, 2001). In the same year, Harder (2001) reported the detection of HBD3 in skin tissues by high performance liquid chromatography (RP-HPLC). Later, Dunsche (2002) reported the detection of HBD3 in oral tissue through real-time quantitative RT-PCR. Likewise, HBD4 was also predicted based on the screenings of genomic sequences, and HBD4 expression was evaluated by real-time quantitative RT-PCR and detected in the uterus, neutrophils, lung, kidney, thyroid gland, and testis (Gracia *et al.*, 2001). Recently, Li (2016) has also determined the levels of HBD1, HBD2, HBD3 and HBD4 by RT-PCR in gingival epithelia tissue. For human β-defensins, more than 28 transcriptionally active β-defensin genes have been found in the human genome, based on a computational search strategy (Schutte *et al.*, 2002; Pazgier *et al.*, 2006). Pazgier (2006) compiled a list of β-defensin genes, but despite the significant progress made in the identification of these peptides in recent years, the structure-function relationship of β-defensins remains unexplored. To date, only a few genes have been cloned, isolated and fully characterised (HBD1-4) at the protein/peptide level (Suarez *et al.*, 2015). In humans the most studied β-defensins are HBD1-4, therefore these are highlighted in Table 1.3. The secondary structure of the defensin family depends upon the disulphide bridges between the six cysteine residues. The disulphide bridges between the cysteine residues in the six α-defensins are between Cys^1-Cys^6, Cys^2-Cys^4 and Cys^3-Cys^5, whereas in the four β-defensins the bridges are between Cys^1-Cys^5, Cys^2-Cys^4, and Cys^3-Cys^6 (Tang *et al.*, 1999).

1.3.3 Defensins and their Multifunctional Role

To date, both α and β-defensins have demonstrated broad antibacterial activity. The antibacterial properties of the six human α-defensins against the Gram-positive bacteria *Staphylococcus aureus* and the Gram-negative bacteria *Enterobacter aerogenes* and *Escherichia coli* were investigated by Harder (1999), and the results indicate that their potential antibacterial activities against *S. aureus* were HNP2>HNP1>HNP3>HNP4, whereas the

potential microbicidal effects against gram negative bacteria (*E. coli* and *E. aerogenes*) were HNP4>HNP2>HNP1=HNP3. HBD1-4 has also been shown to possess microbicidal activities *in vitro* against a variety of bacteria (Harder *et al.,* 2001; Gracia *et al.,* 2001), and it was reported that HBD2 preferentially kills Gram-negative bacteria, such as *Pseudomonas aeruginosa*, rather than Gram-positive bacteria, such as *S. aureus* (Harder *et al.*,1999). Compared to HNP2, HNP1 and HNP3 contain an additional amino acid residue at the N-terminus: alanine in HNP1 and aspartate in HNP3, and this additional acidic residue in HNP3 may make it less active than HNP1 or HNP2 in killing *S. aureus, S. aeruginosa*, and *E. coli*. HD5 has been shown to be as effective as HNP2 against *S. aureus* and also as effective as HNP4 against Gram-negative bacteria, whereas HD6 shows little or no antibacterial activity. Although the structure and multifunctional role of HNP1-3 has been extensively studied due to their structural similarity, HNP4, HD5 and HD6 have been less well studied because of the absence of an efficient method (Wu *et al.,* 2004). The action of HNP1 was studied in relation to the tuberculosis pathogen *Mycobacterium tuberculosis,* and it was found to lead to significant clearance of the tuberculosis pathogen from the lung, liver and spleen of infected mice (Sharma *et al.,* 2001). To confirm this finding, HNP1-3 levels were measured by radioimmunoassay and were found to be significantly higher in the plasma fluid of patients with tuberculosis than that of healthy subjects. This suggests the involvement of HNPs in the pathogenesis of tuberculosis, and the assessment of their plasma levels could be a useful marker of disease severity (Ashitani *et al.,* 2002). HBDs have also been found to be capable of adherence to the tuberculosis pathogen (Hazlet *et al.,* 2011; Santiago *et al.,* 2005, 2006; Fattorini *et al.,* 2004). In terms of their antifungal activities, HNP1-3 has been shown to be active against *Candida albicans* and *Candida neoformans (*De Smet *et al.,* 2005), and *in vitro,* purified HNP4 has been demonstrated to kill *C. albicans* (Wilde *et al.,* 1989). Studies have suggested that AMPs can interact with a variety of molecular targets, present either on the cell surface (including membranes) or within cells. The most common proposal for this action is that AMPs interact with the lipid bilayers of a microorganism. The mechanism of action depends on the electrostatic interactions between the cationic peptide characteristics of the defensins and the negatively charged phospholipid components present in the microbial membranes. This proposal is supported by the fact that several members of each defensin class are effective microbicides against Gram-positive and Gram-negative bacteria, and fungi *in vitro* (Ganz *et al.,* 2003; Lehrer *et al.,* 1985, 1989; Ericksen *et al.,* 2005; Selested *et al.,* 2005; Wilson *et al.,* 2013).

Table 1.2 Structural, biological source and antimicrobial activity of α-defensins family (Wang *et al.*, 2014; Klotman *et al.*, 2006).

Year	Name	Sequence	Length (AA)	Mw* (Da)	Cell sources	Biological fluid	Activity
1985	HNP1	ACYCRIPACIAGERRYGTCIYQGRLWAFCC	30	3442.08	Neutrophils, monocytes, macrophages, natural killer cells, B cells and T cells	Saliva, Plasma, Breast milk, BALF, Serum, Tears	G, V, F
1985	HNP2	CYCRIPACIAGERRYGTCIYQGRLWAFCC	29	3371.00	Neutrophils, monocytes, macrophages, natural killer cells, B cells and T cells	Saliva, Plasma, Breast milk, BALF, Serum, Tears	G, V, F
1985	HNP3	DCYCRIPACIAGERRYGTCIYQGRLWAFCC	30	3486.09	Neutrophils, monocytes, macrophages, natural killer cells, B cells and T cells	Saliva, Plasma, Breast milk, BALF, Serum, Tears	G, V, F
1989	HNP4	VCSCRLVFCRRTELRVGNCLIGGVSFTYCCTRV	33	3709.45	Neutrophils	Saliva, Plasma, Breast milk, BALF, Serum, Tears	G, V, F
1992	HD5	ATCYCRTGRCATRESLSGVCEISGRLYRLCCR	32	3582.18	Intestinal paneth cells and vaginal epithelial cells (HD5 only)	ND	G, V, F
1993	HD6	AFTCHCRRSCYSTEYSYGTCTVMGINHRFCCL	32	3708.27	Intestinal paneth cells	ND	V, F

*Disulphide connectivity Cys^{2}-Cys^{30}, Cys^{4}-Cys^{19}, Cys^{9}-Cys^{29} (HNP1, HNP3, HNP4), Cys^{1}-Cys^{29}, Cys^{3}-Cys^{18}, Cys^{8}-Cys^{28} (HNP2), Cys^{3}-Cys^{31}, Cys^{5}-Cys^{20}, Cys^{10}-Cys^{30} (HD5), Cys^{4}-Cys^{31}, Cys^{6}-Cys^{20}, Cys^{10}-Cys^{30} (HD6). G = Gram positive and Gram negative bacteria; V= viruses; F= fungi; AA = Amino acid. Peptide: HD = human α-defensin; HNP= human neutrophil peptide. Biological fluid: BALF= Bronchoalveolar lavage fluid; ND= not detected.

Table 1.3 Structural, biological source and antimicrobial activity of β-defensins family (Wang *et al.*, 2014; Klotman *et al.*, 2006; Fang *et al.*, 2003).

Years	Name	Sequence	Length (AA)	Mw* (Da)	Cell sources	Biological fluid	Activity
1995	HBD1	DHYNCVSSGGQCLYSACPIFTKI QGTCYRGKAKCCK	36	3928.56	Epithelial cells, monocytes, macrophages, monocyte-derived dendritic cells, keratinocytes, pancreas, kidney, lung, prostate, placenta, thymus, and testis	Saliva, Plasma	G, F
1997	HBD2	GIGDPVTCLKSGAICHPVFCPRR YKQIGTCGLPGTKCCKKP	40	4328.22	Epithelial cells, monocytes, macrophages, monocyte-derived dendritic cells and keratinocytes	Saliva, Plasma	G, V, F
2001	HBD3	GIINTLQKYYCRVRGGRCAVLSC LPKEEQIGKCSTRGRKCCRRKK	44	5155.19	Epithelial cells, monocytes, macrophages, monocyte-derived dendritic cells and keratinocytes	Saliva Plasma (ND)	G, V, F
2001	HBD4	FELDRICGYGTARCRKKCRSQE YRIGRCPNTYACCLRKWDESLL NRTKP	48	4366.11	Epithelial cells	Saliva Plasma (ND)	G

*Disulphide connectivity Cys^{5}-Cys^{34}, Cys^{12}-Cys^{27}, Cys^{17}-Cys^{35} (HBD1), Cys^{8}-Cys^{37}, Cys^{15}-Cys^{30}, Cys^{20}-Cys^{38} (HBD2), Cys^{11}-Cys^{40}, Cys^{18}-Cys^{33}, Cys^{23}-Cys^{41} (HBD3), Cys^{6}-Cys^{33}, Cys^{13}-Cys^{27}, Cys^{17}-Cys^{34} (HBD4). G = Gram positive and Gram negative bacteria; V= viruses; F= fungi; AA = Amino acid. Peptide: HBD = human β-defensin; ND= not detected.

Both α and β-defensins are active against human immunodeficiency virus (HIV) (Mehlotra *et al.,* 2016), and De Leeuw (2007) has compiled the modes of action of defensins against HIV. The inhibition of HIV replication was first reported for α-defensins (Nakashima *et al.,* 1993; De Leeuw *et al.,* 2007), and more recently, anti-HIV activities have been described for human α and β-defensins (Wang *et al.,* 2003; Zhang *et al.,* 2002). A number of mechanisms for anti-HIV activity by defensins have been proposed, including the suggestion of a direct interaction of defensins with the virus itself. Gracia *et al.* (2010) reported that HNP1-3 levels, as assessed by ELISA, in monocyte-derived dendritic cells were significantly higher in HIV-infected patients compared to healthy controls. The study also suggested that HIV-infected patients with greater secretions of HNP1–3 had a significantly lower risk of disease progression (Gracia *et al.,* 2010). However, a few recent studies have reported that higher α-defensin levels are associated with increased HIV acquisition, and thus these peptides can be regarded as a risk factor (Pellett Madan *et al.,* 2015; Hirbod *et al.,* 2014). Nevertheless, the role of defensins in HIV infection and diseases progression requires further investigation (Melthora *et al.,* 2016).

In addition to their antimicrobial activity, increasing evidence suggests that defensins may also play a significant role in infectious diseases and cancer. Increased levels of HNP1-3, as measured by ELISA, were reported in the serum of colorectal cancer patients (Melle *et al.,* 2005; Albrethsen *et al.,* 2005; Albrethsen *et al.,* 2006) and were significantly higher compared to healthy subjects. HNP1 and average response of HNP1-3 were found at significantly higher levels in oral squamous cell carcinoma (Mizukhawa *et al.,* 1999), breasts cancers (Li *et al.,* 2005), bladder cancer (Gunes *et al.,* 2013), pulmonary disease (Paone *et al.,* 2011) and sepsis (Panyutich *et al.,* 1993). The upregulation of HNP1-3 originates from tumour infiltrating immune cells, including neutrophils (Droin *et al.,* 2010). An immunochemistry based study reported that tumour cells could produce HNP1-3 peptides by themselves (Muller *et al.,* 2002; Melle *et al.,* 2005). However, the exact mechanism awaits further investigation. Therefore, in the near future it may be possible to use α-defensins to monitor advanced diseases. Taken together, both α and β defensins play a multiple role in innate immunity. In this study, α-defensins (HNP1-4) were investigated and in the following subsection the focus will be on this family.

1.4 Published Work on α-Defensins

Within the last fifteen years, the number of publications relating to human neutrophil α-defensins (HNP1-4) has increased significantly (Figure 1.3). The keywords: human neutrophil α-defensins were used following US National Liberary of Medicine and National Institues of Health (NCBI) Pubmed search engine in order to determine the number of manuscripts published concerning this family of peptides (Siqueira *et al.,* 2011). Defensins initially attracted interest due to their potent multifunctional role against pathogens.

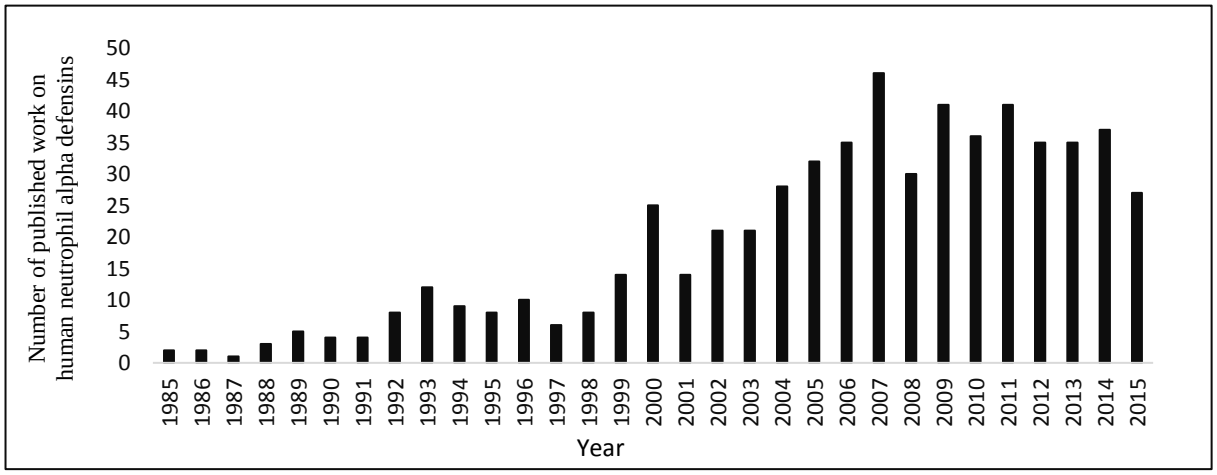


Figure 1.3 Numbers of published manuscripts on α-defensins from the period of 1986 to 2015. The bar chart derived from US National Liberary of Medicine and National Institues of Health (NCBI) Pubmed search engine (https://www.ncbi.nlm.nih.gov/pubmed/?term=human+neutrophil+alpha+defensins).

The role of antimicrobial peptides is particularly important in the oral cavity where there is constant challenge by microorganisms. In periodontal health and disease, neutrophils play a critical role in defending the host against infection by ingesting and killing invading microorganisms. Therefore, the aims of the existing publication were various and included investigation of the structural aspects of these biomarkers, how neutrophil is active against microbes, identification of these peptides, and evaluation of their levels in various oral and systemic diseases. Overall, the bar chart (Figure 1.3) represents an insight into the interest that has been growing throughout the years on human neutrophil α- defensins. These were analysed from the period of 1986 to 2015 via the PubMed search engine. With regard to the number of existing works on human neutrophil α-defensin in various biological fluid (plasma, serum, saliva) and the number analysed by ELISA and radioimmunoassay (RIA) were higher compared to analysis using MS. Interestingly, only a few studies have reported the detection levels of human neutrophil α-defensins in saliva by mass spectrometer (Figure 1.3). Therefore, the next subsection reviewed the analytical techniques that have been used to separate and

detect the α-defensins family in a saliva matrix, as well as a comparison of the analytical techniques in relation to sensitivity and specificity.

1.5 Existing Analytical Techniques for the Detection and Quantitation of α-Defensins

In the past decade the development of mass spectrometry has led to a new era in biological biomarker discovery that potentially will have a huge impact on future disease diagnosis (Wasinger *et al.,* 2013; Scherl *et al.,* 2015; Grebe *et al.,* 2016). To date, analyses of α-defensins (HNP1-4) present within plasma and serum has mostly been performed via immunoassays. Proteome analysis by mass spectrometry is a relatively new technique compared to immunoassays, but it is believed that in the near future mass spectrometry will replace immunoassays for routine analysis (Scherl *et al.,* 2015; Grebe *et al.,* 2016). The focus of the subsection will be on the detection of salivary α-defensins (HNPs) by antibody-based techniques, LC-ESI-MS, LC-UV, and other mass spectrometry techniques.

1.5.1 Antibody Based Techniques

An enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) are simple, convenient and can achieve high throughput when automated (Rosner *et al.,* 2007). The RIA developed by Yalow and Berson (1959) involves radioactively labelling a known quantity of an antigen before mixing with a known amount of antibodies specific for the antigen and allowing the two to chemically bind. A sample containing an unknown amount of antigen is added and this competes with the radioactively labelled antigen for binding sites. Increasing the amount of non-radioactively labelled compound results in a concomitant decrease in the amount of radioactive compound bound to the antibody, and the amount of bound antigen is measured using a gamma counter. The readings obtained can be used to create a calibration curve, and subsequently, concentrations of unknown samples can be extrapolated from this (Hawker *et al.,* 1973); a RIA can be accurate and highly reliable (Furuyama *et al.,* 1970; Dufau *et al.,* 1972). Alternatively, a 96-well plate is coated with antibodies specific to the compound of interest and an enzyme-linked compound competes with known/unknown and unlinked amounts of the compound for antibody binding sites. Unbound compound is washed off the plate and bound compound is measured by its reaction with a substrate, as illustrated by a colour change. Absorbance values are read using a plate reader and a calibration curve is constructed for quantification (Lequin *et al.,* 2005). The main difference between an ELISA and a RIA is the manner in which the ‘tracer’ antigen is labelled (Booth *et al.,* 1982).

An ELISA measures the average response of HNP1-3 and has been widely used since 1993 to assess various oral and systemic diseases. For example, the average concentration of defensins (HNP1-3) present in plasma was measured and was found to reach micro molar concentrations in septic patients (Panyutich *et al.*, 1993). Mukae (2007) examined the levels of HNP1-3 (average response) in the serum of patients with various lung diseases, and in another study, HNP1–3 (average response) concentrations in serum were found to be elevated in colon cancer patients (Melle *et al.*, 2005), while more recently, the concentrations of HNP1-3 were measured in the serum of patients with acute exacerbation of fibrotic interstitial pneumonia (Sakamoto *et al.*, 2015). The evaluation of salivary HNP1-3 levels (average) were examined in patients with oral disease (Mumcu *et al.*, 2012), and a few studies have also determined salivary HNP1-3 levels after physiological stress (Davison *et al.*, 2009; Gillum *et al.*, 2015; Kunz *et al.*, 2015). Many other studies also have reported average responses for HNP1–3 concentrations in plasma and serum matrices for various systemic diseases by ELISA (Gunes *et al.*, 2013; Milewski *et al.*, 2011; Paone *et al.*, 2011; Vordenbaumen *et al.*, 2010; Albrethsen *et al.*, 2006; Albrethsen *et al.*, 2005). In addition, average concentrations of HNP1-3 have also been measured from the plasma of patients with various systemic disease by RIA (Yamaguchi *et al.*, 2009; Hoover *et al.*, 1997; Mukae *et al.*, 2002; Ashtani *et al.*, 2002; Ashthani *et al.*, 1998). Antibody based assays provide high sensitivity and the detection of a particular analyte is relatively straightforward. However, for the detection of defensins, an antibody based approach is limited by the lack of availability of antibodies with high enough specificity to distinguish between HNP1, 2 and 3. In general, antibody-based assays do not discriminate between closely related isoforms. Most readily available assays measure the average responses for the three defensins, with a limit of detection in human plasma and serum of 0.00015 ng/µL (ELISA) and for plasma analysis 0.022 ng/µL (RIA) (Table 1.4). Recently (2016), My BioSource has introduced an ELISA kit for the analysis of individual HNP1, HNP2 and HNP3, which reports a limit of detection of 0.0001 ng/µL for HNP1, 0.0001 ng/µL for HNP2, (0.00187 ng/µL for HNP3, and $3x10^{-5}$ ng/µL for HNP4. However, this kit has not yet been validated for the detection of defensins present in a saliva matrix. In addition, the cost of the commercially available ELISA kits for individual HNP1-3 is £499.99 (1 x 30 sample runs corresponding to 5 participants). Other biomedical companies, such as Kamiya (Seattle, USA) and Cell Sciences (Massachusetts, USA), previously offered individual kits, however, these have now been discontinued.

1.5.2 Liquid Chromatography based Techniques

1.5.2.1 LC-UV

To date, high performance liquid chromatography (HPLC) is one of the most popular analytical techniques, and it has the ability to separate, identify, and quantitate compounds that are present in any sample that can be dissolved in a liquid (Kupiec *et al.*, 2004). HPLC uses a liquid mobile phase to transport analytes through a packed stationary phase column. In addition, HPLC is equipped with a detector which is used to respond to a physiochemical property of an analyte (Lloyd *et al.*, 2010). Few studies have been conducted on the detection and quantitation of individual α-defensins by HPLC-UV (Table 1.4). In 1999 the levels of human defensin (HNP1) were determined in the human saliva of patients with oral inflammation (Mizukhawa *et al.*, 1999), and higher levels were found in patients with oral and maxillofacial inflammation compared to control samples. Whole saliva samples were subjected to centrifugation and a 10 µL aliquot of the supernatant was separated on a reverse phase column. Defensin (HNP1) was quantified by comparing the peak height of the eluted defensin from the saliva sample with a HNP1 standard. Similar to the findings of Mizukhawa *et al.*, the absolute concentration of HNP1 was also elevated in saliva of patients with oral candidiasis (Tanida *et al.*, 2003; Kuchukkolbasi *et al.*, 2011). However, concerns exist with regards to the separation of HNPs (co-elution due to their structural similarities) and sample preparation (HNPs retention time interferes with that of other salivary glycoproteins). The limit of detection for the analysis of salivary HNP1 by LC-UV was reported as 100 ng/µL. Nevertheless, a good correlation (R^2 = 0.982) was observed for the EIC peak areas of HNP1-3 using LC-UV and LC-MS when comparing gingival crevicular fluid (Cabras *et al.*, 2005). In addition, gingival crevicular fluid does not contain proline rich proteins and peptides.

1.5.2.2 LC-MS

The electrospray ionisation (ESI) mechanism was investigated forty-seven years ago, when Dole and his group studied the ionisation mechanism of ESI (Dole *et al.*, 1968), although the real breakthrough took place in 1988, when Fenn and co-workers demonstrated that ESI is suitable for larger biomolecules, and the Nobel Prize in Chemistry was awarded for this work in 2002. ESI-MS allows large, non-volatile molecules to be directly analysed from a liquid phase and is normally coupled with a separation technique, such as HPLC (Gelpi *et al.*, 1995; Niessen *et al.*, 1995). HPLC-ESI-MS has become one of the most widely-used analytical techniques for the analysis and sequencing of peptides (Fenn *et al.*, 1989; Cao *et al.*, 1998; Owens *et al.*, 1998), and is also used for analysing carbohydrates, oligonucleotides, natural

products, pesticides, and small molecules (Herderich *et al.*, 1997; Strege *et al.*, 1999; Apffel *et al.*, 1998). The process of mass analysis in LC-MS involves the separation of analyte ions by a mass analyser and is central to this technology.

1.5.2.2.1 Mass analysers

There are three basic types of mass analysers currently used in proteomics research; the ion trap, time-of-flight (TOF), and quadrupole or mass filter. The quadrupole mass analyser was first described in the 1950s by the Nobel Prize-winner Wolfgang Paul. A quadrupole (Q) mass analyser has four parallel electrical rods typically with circular cross sections; two rods carry a positive charge and the other two rods carry a negative charge. In general, a higher radio frequency (RF) is applied to two rods and the other two are linked with a direct current (DC). Ions, formed in the ionisation chamber, are pulsed towards a quadrupole by an electrical field in the range of 5 kV. Consequently, positively charged ions travel towards negatively charged rods and negatively charged ions travel towards positively charged rods. Once the polarity has been changed, the ions switch their movement pathway before striking the rods and thus are transmitted through. The ions enter through a small orifice present at the centre of the rods and undergo specific trajectories based upon their *m/z* values. Only ions with very short/narrow intervals for their *m/z* values have stable trajectories and successfully pass through the quadrupole rods to the detector. All other ions with lower or higher *m/z* values have unstable trajectories and are filtered out (Pasa *et al.*, 2004). The major advantages of quadrupole analysers are low cost, relatively small size, robustness, and ease of maintenance.

A single quadrupole system contains only one mass filtering quadrupole, while a triple quadrupole system consists of three quadrupoles. In a triple quadrupole, Q1 and Q3 act as mass filters, while Q2 is acts as a collisional cell. A quadrupole possesses limited capability in terms of mass range (usually <4000 *m/z*), resolving power, and the ability to perform MS/MS analysis. This final disadvantage can be overcome by attaching addtional quadrupoles, such as in a triple quadrupole instrument (QQQ) or linking to a time-of-flight analyser (QToF). The time-of-flight ion separation methodology is one of the simplest and most popular analysers for mass spectrometers. It relies on the free flight of the ionised molecules in a drift tube before reaching the detector (Aneed *et al.*, 2009). The time required for an ion to travel a set distance and strike a detector enables the *m/z* ratio to be calculated.

Table 1.4 Quantitation of HNPs using ELISA, LC-UV and LC-MS.

Technique	Peptide	Sample Matrix	Limit of Detection (ng/µL)	Advantages	Disadvantages	Reference
ELISA	HNP1-3	Plasma	0.00015 [1]	High sensitivity	Less specific (not possible to discriminate between individual HNPs due to their structural similarities)	1. Sakamoto *et al.*, 2015 2. This work (ELISA)
	HNP1-3	Saliva	0.00015 [2]			
LC-UV	HNP1	Saliva	100 [3]	Fast analysis	Low sensitivity and less specific. Quantitation is based on retention time	3. Mizukhawa *et al.*, 1999 4. Kucukkolbasi *et al.*, 2011 5.Tanida *et al.*, 2003
	HNP1	Saliva	100 [4]			
	HNP1	Saliva	100 [5]			
LC-MS	HNP1 HNP2	Saliva	100 [6]	High mass resolution, high sensitivity and specificity for HNPs	Ion source can be difficult to clean and has a tendency to become contaminated with residues from previous experiments.	6. Goebel *et al.*, 2000 7. Van den Broek *et al.*, 2010 8. This work
	HNP1 HNP2 HNP3	Plasma	0.003 [7]			
	HNP1 HNP2 HNP3	Serum	0.003 [7]			
	HNP2	Saliva	0.05 [8]			

When an electric field is applied to a free ion, it will give the ion a kinetic energy of zV, where z is the ion charge and V is the applied voltage. The flight time (t) is determined by the energy (E) to which an ion is accelerated, the distance (d) it has to travel, and its mass to the charge ratio. There are two well know formulae that apply to time-of-flight analysis. One is the formula for kinetic energy:

$$E = 1/2mv^2$$

where,

E= kinetic energy
m = mass
v = velocity

The above equation explains that for a given kinetic energy, E, smaller masses will have larger velocities, and larger masses will have smaller velocities. Instead of measuring velocity, it is much easier to measure the time it takes an ion to reach the detector. The second equation is the familiar velocity (v) equals distance (d) divide by time (t): v = d/t. This gives the basic time-of-flight relationship. For a given energy (E) and distance (d), the mass is proportional to the square of the flight time of the ion.

In ion trap analysers the ions are first captured or trapped for a specific interval (Aebersold *et al.,* 2003). The ion trap analyser employs similar principles as the quadrupole analyser, as it uses an electric field for the separation of ions via mass to charge ratios and then traps ions in a controlled manner. A quadrupole ion trap analyser has a ring electrode and an end cap electrode. The ion trap is operated by a fixed RF frequency supplied to the ring electrode whilst the endcap electrode has a constant DC current (usually = 0). An ion trap operates by storing ions in a trap and manipulating those using DC and RF electric fields in a series of carefully timed events (Chromacademy 2010). The main difference between an ion trap and quadrupole is that an ion trap is capable of trapping ions for long periods of time (milliseconds to *days*), providing plenty of time for ions to fall apart spontaneously (unimolecular decomposition), to experience undesirable interactions with other ions (space charge effects), and neutral molecules (ion-molecule reactions). This provides some unique capabilities, such as extended length MS/MS experiments and high sensitivity.

1.5.2.2.2 Mode of acquiring LC-MS data

The most common modes of acquiring LC-MS data are full scan (resulting the total ion count), selected ion monitoring (SIM) and multiple reaction ion monitoring (MRM). The most

selective mode to use for single quadrupole MS is SIM, where a fixed set of DC and RF voltages is applied to the quadrupole and thus only a single *m/z* can pass, while ions with different *m/z* ratios are filtered out. MRM is the common mode when using a triple quadrupole MS/MS for quantitative analysis, allowing enhanced sensitivity and selectivity. The first quadrupole filters a specific precursor ion of interest, and the collision cell is optimised to produce a characteristic product ion by collision of the precursor ion with a neutral collision gas, such as argon, in a process termed collision induced dissociation (CID). Generated product ions are transferred into the third quadrupole where a number of specific *m/z* ions are allowed to pass, and all other product ions are filtered out (Schreiber *et al.,* 2010; Schulze *et al.,*2010). Mass spectrometry profiling of the proteome and peptidome is a relatively new concept in clinical diagnostics. Since its introduction at the beginning of 2000, peptide analysis has offered a new promising approach for the quantitative-qualitative analysis of endogenous peptides present in biological samples (Grebe *et al.,* 2015; Terraciano *et al.,* 2011). Quantitation of HNPs in various biological fluids has been acquired through either the full scan range or MS/MS mode and the next subsection highlights some of these studies. Of the many LC-ESI-MS-based studies involving biological fluids (serum, plasma, saliva, bronchoalveolar lavage fluid and tears), only one report validated an LC-ESI-MS method for the detection of plasma and serum α-defensins (Van den Broek *et al.,* 2010).

1.5.2.2.3 Full scan and MS/MS

The levels of HNP1-3 in human saliva were quantified following an external standard (HNP1, HNP2) calibration curve (absolute quantitation) method using the extracted ion chromatogram (EIC) peak area (Goebel *et al.,* 2000). Unstimulated saliva was collected and solid phase extraction was used for the clean-up of the saliva samples prior to LC-MS analysis. The purpose of the study by Goebel *et al.* (2000) was to identify HNP1-3 present in human saliva and therefore both full scan (400 – 1600 m /z) and MS/MS data acquisition was utilised. HNP1 and HNP2 were identified in saliva $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$; however, HNP3 was only tentatively identified. In order to confirm the presence of HNP3 the authors compared the MS/MS product ion pattern of HNP1 and HNP2 with the tentatively identified $[M + 5H]^{5+}$ ion of HNP3.The authors highlighted that defensins are complicated compounds to sequence by LC/MS/MS due to the presence of the three disulphide bridges; hence, the assignment of the exact sequence sections and charge state of the peaks was not carried out. The limit of detection of HNP1 and HNP2 by a triple stage quadrupole detector was determined to be 0.1 ng/µL. Defensins were separated via a C18 column (150 x 2.1 mm) with 5-µm particle size (Grace

Alltech Alltima), eluting the peptides over a 60-minute gradient. Gardner (2009) highlighted that the disulphide linkages present in these peptides are not amenable to fragmentation via standard low-energy collisional activation, and must be reduced in order to produce appropriate product ions. Unstimulated saliva samples were cleaned by solid phase extraction analysis and the levels of HNP1-3 were quantified using an internal standard (bradykinin) calibration curve (absolute quantitation) method using an EIC peak area. Their study confirms that individual HNP1-3 profiles are different from each other. Tandem MS analysis was performed using a Thermo LTQ ion trap mass spectrometer and exploratory tandem MS experiments were also carried out for all defensin standards using AB Sciex API 4000 triple quadrupole mass spectrometer. The purpose of Gardner's study was to optimise a tandem method for the analysis of salivary HNP1-3 and therefore, no other effects (stability, precision and matrix) were reported.

An LC-ESI-MS method for the analysis of individual α-defensins (HNP1-3) present in plasma and serum was validated by Van den Broek *et al.* (2010). Peptides were extracted from plasma and serum by protein precipitation with 1% TFA (v/v) in acetonitrile and the samples were subjected to centrifugation (5 min; 14,000×g) at room temperature. The supernatant was collected and evaporated under nitrogen air, and the lyophilised powder was reconstituted with acetonitrile/water/formic acid (25/75/0.25, v/v) before LC-MS analysis. Individual HNP1-3 levels were quantified following the addition of an internal standard (human defensins 5, HD5) using the EIC peak area for absolute quantitation. However, Van den Broek et *al.* (2010) observed examples of ion suppression by the internal standard (HD5) and recommended the use of isotope-labelled internal standards for the absolute quantification of levels of individual HNP1-3. The limit of detection by triple stage quadrupole detector was 0.003 ng/µL. Furthermore, individual levels of HNP1-3 in intensive care patients (n= 11) were measured and HNP2 levels were found to be significantly higher compared to HNP1 and HNP3. Cabras (2010) analysed the salivary secretory peptidome profile in children affected by type 1 diabetes, and HNP1, HNP2 and HNP4 were quantified (semi-quantitative) using the EIC peak area. Saliva samples were acidified with 0.2% TFA and subjected to centrifugation. The acidic supernatant was separated from the precipitate before LC-MS analysis. The main aim of the study by Cabras was to detect as many as possible proteins and peptides present in saliva. Both full scan and tandem MS/MS were utilised to confirm the structure of salivary proteins and peptides fragments. The aim of the work of Vento (2013) was to evaluate the relative levels of α-defensins as markers of prenatal infection/inflammation, in samples of bronchoalveolar

lavage fluid (BALF) from premature newborns, in relation to the detection of the *Candida* MN antigen. The extracted ion current procedure was based on the extraction of the current associated with three multiply charged ions specific for each α-defensin from the total ion current chromatographic profile. The levels of individual HNPs were also investigated in the bronchoalveolar lavage fluid of patients with pneumonia, and relatively quantified using the EIC peak area using an ion trap mass spectrometry detector (Tirone *et al.,* 2010). The relative abundance of the HNPs was approximated using the EIC peak area in the saliva of patients with Sjogren's syndrome using an ion trap detector (Peulso *et al.,* 2007).

It is clear that the mode for acquiring LC-MS data depends upon the main purpose of a study, and both full scan and tandem mass method can be utilised for the detection of defensins. ESI is the most commonly used ionisation method for the analysis of peptides and proteins. Peptides and proteins can carry multiple charges, and each charge state has its own isotopic envelope and the ion intensity profile varies depending on the mobile phase solvent pH; therefore, it is important to carefully choose the data acquiring mode. Abaye *et al.,* (2011) demonstrated that increasing the percentage of organic solvent (decreasing the polarity of the mobile phase) decreased the ion intensity response of 3.2–3.7 kDa peptides, such as calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP), glucagon-like peptide 1 (GLP1) and HNP2 (see details in chapter 2). However, it is not difficult to imagine that during gradient method development, where the mobile phase ratio is changing, and any slight pipetting error during mobile phase solvent preparation may change the ion intensity profile and thus the charge state distribution of salivary proteins and peptides. Therefore, it is vital to select the appropriate data acquiring mode so that more than one indication ion can be monitored. Most current instruments have limited *(m/z)* ranges and this has an impact upon their ability to accurately identify and quantitate proteins and peptides with good sensitivity. However, sample cleaning is essential before MS analysis. The upper mass range limit is typically 10,000- 20,000 *m/z* for Q-TOF and QQQ (Grebe *et al.,* 2016), although the *m/z* range limit depends on the model and configuration.

1.5.2.2.4 Other Mass spectrometry approaches

In addition to ESI, other soft ionisation mass spectrometric methods have been utilised to detect defensins present in bio fluids. Both ESI and matrix-assisted laser desorption ionisation (MALDI) are very sensitive analytical techniques utilising analyte concentrations that are as low as a picomolar (Aneed *et al.,* 2009). One of the main differences between MALDI and ESI

is the state in which a sample is introduced to the ion source; ESI uses a solvated sample that is infused into the instrument, whereas the solid state is typically used in MALDI. Therefore, when interfaced with a fast and rapid analytical technique, such as HPLC, ESI is possibly more efficient for quantitative measurements (Cohen *et al.*, 2006). In 1993, as an extension of MALDI, surface enhanced laser desorption (SELDI) was first introduced (Hutchens *et al.*, 1993) and later commercialised by Ciphergen Biosystems Inc. (California, USA) in 1997. MALDI and SELDI coupled with ToF are widely used in proteomic screening to search for potential new biomarkers, and both techniques operate via similar principles. In general, a sample of interest is applied to a plate or chip, left to air dry or placed under a stream of nitrogen gas, and the sample is subsequently co-crystallised with a matrix. The components of the mixture are brought into the gas phase via a laser beam, usually a nitrogen laser or pulsed UV laser beam, which hits the sample matrix crystal, leading to absorption of the laser energy by the matrix, and subsequent desorption and ionisation of the analytes present in the sample (Liu *et al.*, 2011). The main difference between MALDI and SELDI are in the construction of the sample targets. In SELDI, sample analysis is by protein chip arrays, which use various chromatographic surfaces or biological surfaces to capture proteins from complex biological mixtures according to their physicochemical properties. Chromatographic surfaces are hydrophobic, hydrophilic, ion exchange, immobilised metal, or other chemistries. These surfaces are often used for profiling proteins from biological mixtures for biomarker discovery. Unbound proteins and mass spectrometric interfering compounds are removed by subsequent on-spot washing with appropriate buffer solutions (Reddy *et al.*, 2003). The analytes of interests that are retained on the array surface are analysed and detected by SELDI-TOF-MS using a protein chip reader. One of the key features of SELDI-TOF is on-spot sample cleaning, which helps to improve the sensitivity. However, for MALDI, analysis typically benefits from sample purification before mixing with the matrix (Meuleman *et al.*, 2008; Vorderwuelbecke *et al.*, 2005). Few studies have determined the levels of defensins using a MALDI-ToF instrument, although the relative abundance (ion intensity) of HNP1, HNP2 and HNP3 in gingival crevicular fluid has been evaluated by MALDI-TOF (Lundy *et al.*, 2005). This is one of the earliest published studies and the main purpose was to evaluate the ion intensity profiles of HNP1-3. The authors note that HNP1 was identified as the most abundant ion and HNP3 as the least abundant. Lundy (2005) also compared the relative abundance of HNP1-3 between healthy control and periodontal patients, and a decreased relative abundance was observed in the periodontist patients. The overexpression of HNP1-3 was found in squamous cell carcinomas of the human tongue and was compared to autogenous non-tumour tissues. Peptides

were extracted from tongue tissue, separated by HPLC, and fractions were also analysed by MALDI-ToF, where relative quantitation was based on ion intensity (Lundy *et al.*, 2005). Ion intensity profiles were also evaluated in the sputum of patients with lung diseases (Terraciano *et al.*, 2010), and in the tears of dry eye patients (Lo *et al.*, 2010). SELDI-TOF has also been successfully employed for the analysis of defensins, and up-regulated levels of HNP1-3 were found in serum and colon cancer tumours when compared. HNP1-3 were separated by HPLC and fractions analysed by SELDI-ToF. Serum samples were added to various protein chip arrays to monitor for the presence of HNP1-3 in serum, where the immobilised metal affinity capture (IMAC30) chip was used for the analysis of HNP1-3. Pilot studies have also been performed using different chips, and the NP20 (Normal Phase) (Ciphergen) chip was chosen for tissue screening (Albrethsen *et al.*, 2004). Albrethsen (2006) used SELDI-TOF and confirmed the plasma levels of HNP1-3 in patients with colorectal cancer (CRC). The aim of the study was to confirm whether the amount of HNP1–3 present in tumours and plasma from CRC patients correlates with the Dukes' stages using an immunoassay (ELISA) and SELDI-TOF. The Dukes' staging system is divided into 4 groups - A, B, C and D, where Dukes' stage A is an early stage and Dukes' D is advanced. The peak intensity of HNP1–3 was found to be low in plasma and was not detected in any sample from a healthy individual or CRC patient. However, levels of HNP1–3 determined by ELISA increased in Dukes' stages C and D, but not in A and B.

This subsection presents a review of LC-ESI-MS, MALDI, SELLDI, LC-UV and ELISA analysis for the detection and quantification of HNP1-4. ESI tends to produce multiple ions for proteins and peptides, whereas MALDI typically produces a less complex isotope profile which makes data analysis much easier but there is a smaller degree of certainty (Aneed *et al.*, 2009). In contrast, SELDI combines the power of MALDI with the selectivity of a protein chip technique (Hutchens *et al.*, 2000). In addition, SELDI is mostly optimised for cancer diagnosis and biomarker discovery. Nevertheless, the choice of mass spectrometry based instrument depends on availability in the laboratory, and all these techniques can be used for the analysis of α-defensins. LC-UV can also be used for the analysis of individual HNP1-4, specifically in gingival crevicular fluid, where minimal sample cleaning is required. In addition, it is clear that regardless of which mass spectrometry approach is used, the most crucial step is sample cleaning. Careful sample handling at the protein and peptide level is key for successful analysis at the MS level. Consequently, it is necessary to take great care, and in some instances to optimise each step involved, in order to obtain better efficiency and sensitivity. LC-MS is

currently an established method for the analysis of proteomic studies; however, ELISA remains the primary method used for peptide analysis in exercise research. The present review suggests that LC-MS offers an alternative method for analysis of HNP1-4, simultaneously providing quantification of individual HNPs together with high sensitivity and specificity.

1.6 Challenges and Perspective: The Salivary Proteome Analysis by Electrospray Ionisation

Electrospray ionisation mass spectrometry allows the salivary proteome to be examined in detail, such as for the presence or absence of proteins, and levels of expression (Caporossi *et al.,* 2010; Good *et al.,* 2007; Hu *et al.,* 2007; Lee *et al.,* 2009; Tarawneh *et al.,* 2011). Out of the thousands of proteomic studies published to date, only a small minority has attempted to provide a comprehensive quantitative description of the salivary proteome (Bantscheff *et al.,* 2007; Wasinger *et al.,* 2013). The focus of this subsection is to present the challenges of analysing saliva by electrospray ionisation, highlighting sample handling, and the complexity of the analysis of the salivary proteome. Despite the phenomenal impact of electrospray ionisation (ESI) and peptide separation (liquid chromatography) techniques within proteomics, the identification and quantification of all of the proteins present in a salivary sample remains an unmet technical challenge (Wasinger *et al.,* 2013). Analysis of human saliva is inherently challenging because the salivary proteome contains a large number of proteins within an extremely wide range of physicochemical properties, such as size, charge, and hydrophobicity, which lead to large differences in ESI responses (Tarawneh *et al.,* 2011; Bantscheff *et al.,* 2007). Therefore, proteomics analysis of saliva by mass spectrometry is not inherently quantitative, and the amount of analyte compared to MS signal intensity does not always demonstrate a linear relationship (Wasinger *et al.,* 2013). Although there are some challenges in quantitative analysis by mass spectrometry, the further development of a series of specific stable-isotope labelling strategies for an analyte of interest to obtain absolute quantitative analysis will be possible.

During bioanalysis, matrix components present within biological samples can affect the response of the analyte of interest. These phenomena, termed generally as matrix effects, can lead to inaccurate quantitation, and it is therefore important that they are addressed during bioanalytical method development and validation. Matrix effects are complex and biological fluid specific, with each biological matrix presenting different management challenges, and each type of analytical method being affected by matrix components differently (Chiu *et al.,*

2010). Similar to plasma and serum, many proteins (mucins) present in human saliva are glycosylated (Hu *et al.*, 2007).

Ion suppression is one of the most common problems in MS, and matrix effects associated with a LC-MS method include ion suppression or ion enhancement caused by co-eluting matrix components. Matrix effects are often assessed via post column infusion and the post-extraction spike method; consequently, sample preparation is often required. If sample complexity has not been significantly reduced, then low signal-to-noise ratios may result, which will limit the overall sensitivity (Taylor *et al.*, 2005; Bonfiglio *et al.*, 1999; Muller *et al.*, 2002; Sterner *et al.*, 2000). Therefore, sample preparation helps to remove and pre-concentrate the analytes of interest from the target matrices (Annesely *et al.*, 2003; Tang and Kebarle *et al.*, 1993; Buhrman *et al.*, 1996; Bonfiglio *et al.*, 1999; Boyd *et al.*, 2008; Wieling *et al.*, 2002). Furthermore, the ESI source interface also influences its susceptibility to matrix effects. In addition to sample cleaning, there are other challenges which need to be addressed for the successful validation and quantitation of salivary analytes. Mallet (2004) studied the influence of several additives and their concentrations on the ESI responses of acidic and basic drugs. The results showed a clear decrease in the response when the concentration of the additive (formic acid, acetic acid, trifluoroacetic acid, ammonium formate, ammonium bicarbonate) was increased from 0.05% to 1%, and the suppression effect was also reported by Benijts and co-workers (Mallet *et al.,* 2004; Benijts *et al.,* 2004). Improved chromatography efficiency can also help to separate matrix components from the analytes. Providing a longer or shorter chromatographic retention of the analyte, matrix components can be separated, thereby eliminating ion suppression (Matuszewski *et al.*, 1998). Complex matrices and tryptic digests of a protein often require very long gradient run times, and method development and optimisation take months to establish depending on the requirement of the chromatographic separation. However, for a complex biological mixture, sample cleaning can be an alternative procedure which plays an important role in enhancing the dynamic range of a mass spectrometer.

Saliva is susceptible to many physiological and biochemical processes, both locally and systemically, and the saliva matrix is challenging to analyse (Helmerhorst *et al.*, 2007). Salivary content can be altered as a result of physiological processes occurring at different points during the day, as well as in response to oral stimuli (eating, drinking and oral hygiene) (Caporossi *et al.*, 2010). In subjects, these circumstances cannot be controlled and therefore

sample standardisation, gender, age, diet, circadian rhythm and sample storage, all need to be carefully considered as aspects that may influence the final findings (Battino *et al.*, 2002). The challenge of using saliva for diagnostic purposes is the low concentration (100-1000 fold) of analytes in comparison to blood concentrations (Pfaffe *et al.*, 2010). However, with multistep sample preparation (in particular analyte concentration) and a sensitive method (MS/MS), salivary biomarkers can be detected. In general, internal standards (IS) are used to determine the concentration of the analytes of interest by calculating the response factor, and the general rule is these must not interfere with sample components. Wang (2007) demonstrated a difference in retention time between an analyte and a stable isotope IS, caused by the deuterium isotope effect, resulted in a differing degree of ion suppression. Furthermore, the co-elution of an IS and an analyte could also have mutual effects (ion enhancement or ion suppression) on the responses of each (Wang *et al.,* 2007; Sojo *et al.,* 2003; Liang *et al.,* 2003). Finding an appropriate IS is the first difficulty, as an endogenous analyte at a high concentration in a biological fluid will also supress other endogenous analytes which are present in lower concentrations (Chapter 4). Therefore, careful assessment is vital during method development. In recent years, the number of publications related to the saliva proteome and mass spectrometry, and consequently the number of identified proteins/peptides present within saliva, have increased significantly (Siqueira *et al.,* 2011). There are several key advantages to analysing saliva including: (1) low invasiveness; (2) minimal cost; (3) easy sample collection; and (4) ease of obtaining of multiple samples. Saliva can also be used in clinically challenging situations, such as obtaining samples from children, disabled or anxious patients, where blood sampling could be difficult to perform (Javaid *et al.,* 2016). Rather than provide a compressive list of the advantages of using saliva, the next subsection highlights the other technical challenges encountered with saliva analysis. Saliva is a heterogeneous complex fluid composed of proteins, glycoproteins, electrolytes and small organic compounds, as well as compounds transported from the blood (Battino *et al.,* 2002). The importance of a comprehensive list of all major proteins (details in subsection 1.2) present in saliva is that they can cause challenges in the analysis of small peptides present in the saliva matrix. The presence of amylase, glycoproteins and histatins within saliva increases the complexity of the analysis of less abundant small and medium size proteins and peptides due to the high abundance of these proteins. The presence of salivary proteases also constitutes a challenge and requires sample treatment. A few studies have suggested that histatin and proline rich proteins and peptides in particular, display a high level of susceptibility to whole saliva protease (Castangola *et al.,* 2004; Campese *et al.,* 2009; Helmerhorst *et al.,* 2010). Campese (2009) investigated the

concentration of whole saliva and glandular secretions on the analysis of PRPs and histatins, and the resulting degradation curve illustrated the rapid proteolysis of histatin and proline rich proteins in whole saliva compared to glandular secretions. It is not difficult to imagine that all this proteolysis and deglycosylation will have significant effects on the structure and function of salivary proteins and peptides (Siqueira *et al.*, 2011). Another major challenge is to collect and store saliva under conditions whereby minimal proteolysis occurs (Ferreiro *et al.*, 2002). Surprisingly little research has been carried out on ways of minimising the degradation process, and the most common method is the addition of 0.1-0.2% additives (trifluoroacetic acid, formic acid or acetic acid) to saliva (Castangola *et al.*, 2011). When considering the storage conditions, itis preferable to store saliva specimens at −80°C rather than at −20°C, where the preservation of the protein content cannot be guaranteed for more than 1 month (Scripper *et al.*, 2007). In order to avoid biased results, it is also important to perform a cross validation study for the analysis of the salivary proteome. Another important factor which needs to be considered in order to decrease the risk of variation, bias, and errors, is communication between researchers and the collaborative group. One generally considers that 70% of errors are due to human intervention (mostly due to communication problems), while only 30% appear to be instrumental related errors (Plebani *et al.*, 1999; De Bock *et al.*, 2009). Therefore, these factors need to be carefully considered prior to planning for quantitative analysis. The ultimate goal of analysing proteins and peptides found in biological fluids is often to use the information for health screening and disease detection. In total, 27% of plasma proteins are also found in saliva, and plasma proteins and peptides have proved their value as clinical analytes, as these bio-fluids provide a bigger picture. Nevertheless, saliva has attracted increased attention because it provides advantages over other body fluids due to its non-invasive collection and cost-effectiveness.

1.7 Stress

Stress is defined as the feeling of being under too much mental or emotional pressure and arises when individuals perceive that they cannot adequately cope with the demands being made on them or with demands being made on them with threats to their wellbeing (Lazarus *et al.*, 1966). Stress can be psychological or physical, and both forms can be acute or chronic. Psychological stress is referred to as events or situations that challenge a person's psychological and/or physiological homeostasis (Cannon *et al.*, 1935). Three categories of stress have been identified: a) cataclysmic events, which include natural disasters and war; b) personal stressors, which are negative life events, including death, divorce, loss of job, or

absence of positive life events; and c) daily hassles which are chronic background stressors, e.g. work environment (Lazarus *et al.*, 1977). Physical stress includes environmental stressors, such as extreme cold, heat and exercise stress. Exercise can encompass a short burst, such as less than an hour of cardiovascular exercise or weight training, or a longer endurance effort that lasts for more than an hour, such as a marathon. The time it takes for the body to return to 'normal' and reach homeostasis depends on the severity of the stress (Tanner *et al.*, 2011). Physical stress (exercise) is the most widely used method in research, as the variability of the study can be controlled by the given parameters of the exercise. Salivary measures of biomarkers, such as the steroid hormone cortisol have over the past two decades become acceptable in human endocrinology (Carter *et al.*, 2007; Helhammer *et al.*, 2008; Dabbs *et al.*, 1993), and most studies consider salivary cortisol levels to be a reliable measure of the hypothalamic–pituitary–adrenal (HPA) axis adaptation to stress (Hellhammer *et al.*, 2008) and the hypothalamic-pituitary-gonodal axis (Cook *et al.*, 1996; Escribano *et al.*, 2012; Merlot *et al.*, 2011). Stress biomarkers are commonly measured in blood plasma and serum, and also in urine and saliva. In this project, the response of cortisol in saliva and its levels were compared with to those for salivary α-defensins (HNP1-4). Therefore, the next subsection will focus on the function and production of cortisol and will highlight the importance of comparing hormone levels with antimicrobial peptides (α-defensins).

1.8 Cortisol

Cortisol is a steroid hormone (glucocorticoid class of hormone) which is produced by the cells of the adrenal cortex within the adrenal glands (Figure 1.4) in response to signals from the pituitary gland and hypothalamus of the brain (Scott *et al.*, 2011).

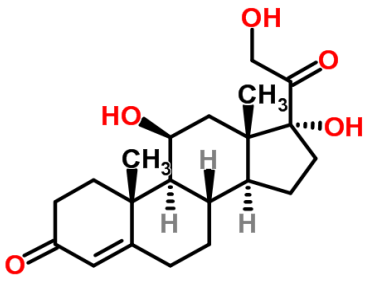


Figure 1.4 Chemical structure of cortisol (Chemspider 2006).

1.8.1 Function of Cortisol

Cortisol is a stress hormone that has many functions and is critical for regulation, as it helps the metabolism of fat, protein, and carbohydrate to maintain blood glucose (glycogenesis), and in the regulation of blood pressure and cardiovascular function. This is accomplished through insulin and its signalling central and peripheral mechanisms, and by the breakdown of stored protein into glucose (Juhan *et al.,* 1998). Insulin functions not only as a peripheral regulator of nutrient storage and the release of circulating substrates, but as a key afferent signal to the central nervous system for the control of energy balance (Porte *et al.,* 2005). Cortisol stimulates gluconeogenesis and the mobilisation of free fatty acids to make more glucose available (Salway *et al.,* 2006).

1.8.2 Production of Cortisol

The production of cortisol by cells of the adrenal cortex is dependent upon two messengers in a chain called the HPA axis. When experiencing a stress, the hypothalamus releases the messenger corticotrophin releasing hormone which is transported to the anterior pituitary and signals specialised cells to release adrenocorticotropic hormone into the bloodstream. This is transported to the adrenal glands where it signals cells of the zona fasciculate and reticularis layers of the adrenal cortex to increase production of cortisol and androgens (Brook *et al.,* 2001).

1.9 Reason for Comparing Cortisol and α-Defensins Levels

The central nervous system (CNS), the endocrine system and the immune system, are the most complex systems that interact with each other. Almost all immune cells, such as neutrophils, T and B cells, monocytes and macrophages, have receptors for the stress hormone that are associated with hypothalamic pituitary adrenal (HPA) axis (Glaser *et al.,* 2005), with the glucocorticoid receptor (GR) being the one to which cortisol bind. The endogenous glucocorticoid hormone cortisol diffuses through the cell membrane into the cytoplasm and binds to the GR. Neutrophils are formed in the bone marrow and secrete neutrophil α-defensins (HNP 1-3). They are the most abundant white blood cell type, constituting 70% of the total population. In response to stress, the endocrine system, governed by the HPA axis, releases cortisol, and this binds GRs present on white blood cells to mediate the stress response (Baschant *et al.,* 2010; Strickland *et al.,* 2001; Buttgereit *et al.,* 2002). The mechanism underlying these interactions is complex and it will probably take many years to fully understand how these three systems interact. However, it is beyond the scope of this thesis to understand whether potential physiological changes associated with stress hormones could also

be involved in the action of antimicrobial peptides, and this is an important new area that deserves exploration. Furthermore, this study was not designed to investigate the underlying mechanism between stress hormones and neutrophil (HNP 1-4) activation. Less is known about the correlation between immune peptides (HNP1-4) and stress hormone (cortisol), and therefore, this is one of the earliest studies to demonstrate the relationship between the levels of cortisol and HNP1-4 before and after physical stress. In addition, it will help to confirm the association as to whether the stress hormone is associated with local levels of these antimicrobial peptides.

1.10 Physical Stress Induces the Activation of Neutrophils

Physical stress (exercise) results in the initial activation of neutrophils, although their responses will vary depending on their phagocytic activity. Degranulation is a cellular process that releases antimicrobial cytotoxic molecules from secretory vesicles, called granules, which are present inside cells. Degranulation is used by several different immune cells, including granulocytes (neutrophils, basophils, and eosinophils). Studies measuring degranulation have reported an increased level of granular enzymes following exercise depending on the intensity. This indicates that physical stress in general will elicit the activation of neutrophils (Pyne *et al.,* 1994; Peake *et al.,* 2004; Khanfer *et al.,* 2010). Although neutrophil degranulation and phagocytic activity increase after exercise, only a few studies have suggested that reduced degranulation depends upon individual innate immunity in response to bacterial stimulation that can last for many hours (Peake *et al.,* 2004; Pedersen *et al.,* 2009). Heavy or prolonged exercise for more than ninety minutes could cause a weakened immune response for up to 72 hours after an exercise session (Niemen *et al.,* 2005). Therefore, it is important to consider supplementation after physical stress, which may reduce the risk of major illnesses. In this study, resistance exercise (2.5 hours) with two different types of supplementation was examined in athlete participants (Chapter 7). The next subsection highlights an introduction of resistance vs aerobic endurance training and discuss the existing manuscripts in relation to aerobic and resistance training for the analysis of α-defensins and cortisol.

1.11 Resistance versus Aerobic

Regular aerobic exercise, also known as “cardio”, improves cardiovascular endurance by increasing the stroke volume, cardiac output, and arteriovenous oxygen difference (Takeshima *et al.,* 2004). Aerobic endurance activities, such as running, cycling and swimming, require high levels of oxygen to be delivered to the muscles. The use of oxygen is known as oxygen uptake or VO_{2max}, and in sports science, oxygen uptake is the most basic measure of aerobic

fitness. After exercise the immune system generally returns to normal function within a few hours, but consistent regular exercise seems to make these changes a slightly more long-lasting. When moderate aerobic exercise is repeated on a near-daily basis, there is a cumulative effect that leads to a long-term immune response. Research has shown that those who walk at 70-75% $VO_{2\ max}$ for forty minutes per day had half as many sick days due to colds or sore throats compared to those who did not exercise (Niemen *et al.,* 2005). In general, resistance training is performed at a higher intensity than can ever be achieved by aerobic endurance exercises alone. Resistance exercise is related to the upper body, lower body and total body. Aerobic exercise is a physical activity that meets the energy provision via the oxidative energy system during the exercise (Stroth *et al.,* 2009). Resistance training improves muscular strength and muscular endurance by changing neural adaptation, muscle hypertrophy, and hyperplasia; it also has an effect on body composition changes by increasing the resting metabolic rate (Wilmore *et al.,* 1978; Shelmadine *et al.,* 2009).

1.12 α-Defensins and Cortisol Responses to Physical Exercise

Physiological stress can elicit changes in hormone and AMPs, particularly in response to exercise, and prolonged or moderate exercise can result in a temporary depression of certain aspects of the immune function (Gleeson *et al.,* 2007). Multiple studies have reported the measurement of cortisol in relation to the exercise response (Table 1.5, Table 1.6 and Table 1.7). As noted, few studies have reported the level of salivary antimicrobial peptides (HNP 1-3) in response to an exercise intervention (Davison *et al.,* 2009; Gillum *et al.,* 2015; Kunz *et al.,* 2015). The cortisol and AMPs response to exercise is dependent upon several factors, including intensity, duration, mode of exercise (aerobic endurance versus resistance), and training status of the subject. Therefore, the focus of this subsection is on the responses of α-defensins and cortisol after exercise.

1.12.1 Cortisol and Physical Exercise

Cortisol level increases in proportion to exercise intensity but the secretory limit is also dependant on exercise duration. It is well established that cortisol level increases after exercise, with the majority of studies reporting this finding (Table 1.5 and Table 1.6). Most of the studies investigating aerobic endurance exercise have reported that exercise at or above 60% VO_{2max} intensity generally produces a significant increase in cortisol. Allgrove (2008) reported a higher level of cortisol at 75% VO_{2max} compared with 50% VO_{2max} was observed immediately after exercise. Further research by Allgrove (2009) confirmed that levels of salivary cortisol increased by 68% at 65% VO_{2max} after 2 hours of cycling. Hill (2008) examined 12 active men

performing 30 minutes of cycle ergometer at 40, 60 and 80% VO_{2max} and reported that only the moderate (60%) and high (80%) intensity trials elicited a significant increase in serum cortisol. A recent study by Alghadir (2015) investigated aerobic endurance training on four consecutive weeks following cycle ergometer at 65-75% VO_{2max} also confirmed an increase in the levels of salivary cortisol. However, exercise duration can also affect levels of cortisol. Tremblay (2005) examined 8 males performing 40 minutes, 80 minutes and 120 minutes of treadmill running at 55% VO_{2max}, and found cortisol levels increased in response to the 120 minutes run in the second hour of running and showed a decline across time during the other shorter sessions. It is suggested that when exercise is performed under 60% VO2max, hormonal responses will only occur when a certain amount of work is done, i.e. running for an adequate period of time (Viru et al., 1992), and Tremblay et al. (2005) demonstrated that this was 80 minutes of running at low intensity (55% VO_{2max}). Existing research on marathon running also supports that long duration exercise will increase cortisol levels (Leers et al., 2006; Petraglia et al., 1988; Franca et al., 2006; Marinelli et al., 1994; Karkoulias et al., 2008). Petraglia (1988) measured cortisol before and after an athletic competition, and found that plasma cortisol levels significantly rose in all athletes, with a higher increase in 10,000m (n=8) and 1500m (n=7) competitors compared to 100m (n=7) competitors. Leers (2006) found an increase of more than two-fold in cortisol levels after a marathon race, and this was supported by Karkoulias (2008), who also found a significant increase in cortisol after completing a marathon. Similarly, Stuempfle (2010) found an increase in cortisol after a 161 km race (28.3 ± 7.4 hours). These results suggest that longer duration exercise (>20 minutes) leads to a higher increase in cortisol, although the effects of intermittent exercise on cortisol are less clear. Eliakim (2009) studied the response of a one-hour volleyball practice on serum cortisol levels and found no change. Similarly, Moreira (2009) reported no significant change in salivary cortisol after a competitive football match, a finding supported by Sari-Sarraf (2007), who studied a football training session and also found no significant increase in salivary cortisol. However, Protzner (2015) reported an increase in salivary cortisol after soccer and a kickboxing match. This variation in the results could be due to athletes becoming accustomed to performing a certain type and intensity of exercise, and therefore adapting (Vuorimaa *et al.*, 2008) and requiring extra stress to elicit a hormone response. In addition, the results may have been affected by diurnal variation, as the samples were collected in the morning but exercise did not commence immediately; therefore, the decrease may have represented the natural sharp decline in cortisol concentrations.

Table 1.5 Cortisol level after aerobic endurance.

Compound	Sample matrix	Exercise type	Participants	Effects on the level	References
Cortisol	Saliva	Treadmill running	16 students	↑ increased	Alghadir *et al.*, 2015
Cortisol	Blood	Cycle ergometer	10 males	↑ increased at 85% VO_{2max}	Jin *et al.*, 2015
Cortisol	Saliva	Cycle ergometer	8 males and 8 females	↑ increased	Allgrove *et al.*, 2009
Cortisol	Saliva	Cycling	17 schoolchildren	↑ increase	Thomas *et al.*, 2009
Cortisol	Blood	Cycle ergometry	12 men	↑ significant at 70-85% VO_{2max}	Hill *et al.*, 2008
Cortisol	Saliva	Cycle ergometer	10 males	↑ significant	Allgrove *et al.*, 2008
Cortisol	Saliva	Cycle ergometer	12 volleyball players	↑ significant	Cordova *et al.*, 2010
Cortisol	Saliva	Cycle ergometer	8 male athletes	↑ post exercise	Davison *et al.*, 2007
Cortisol	Plasma	Treadmill running	8 males	↑ increased	Tremblay *et al.*, 2005
Cortisol	Serum	Cycle ergometry	40 young males	↑ no change	Hiruntrakul *et al.*, 2010
Cortisol	Plasma	Running	17 males	↑ increased at 65% VO_{2max}	Hackney *et al.*, 1999
Cortisol	Blood	Cycle ergometry	82 males	↑ increased at 60% VO_{2max}	Viru *et al.*, 1992
Cortisol	Saliva	Cycle ergometry	10 active males	↑ increased at 75% VO_{2max}	Jacks *et al.*, 2002
Cortisol	Saliva	Treadmill Running	60 students	↑ increased at 70-85% VO_{2max}	Budde *et al.*, 2010
Cortisol	Blood	Running and cycling	5 males	Running: ↑ increased at 70-75% VO_{2max} Cycling: : ↑ increased at 65% VO_{2max}	Brownlee *et al.*, 2005
Cortisol	Blood	Cycling	12 endurance athletes	↑ increased at 70% VO_{2max}	Viru *et al.*, 2001
Cortisol	Serum	Treadmill Running	20 trained runners	↑ increased at 80% VO_{2max}	Vuorimaa *et al.*, 2008
Cortisol	Plasma	Marathon	25 males and 2 females	↑ post exercise	Stuempfle *et al.*, 2010
Cortisol	Serum	Marathon race	11 marathon runners	↑ post exercise	Karkoulias *et al.*, 2008

Table 1.6 Cortisol level after resistance training.

Hormone	Sample matrix	Exercise type	Participants	Effects on the level	References
Cortisol	Blood	Resistance	10 males	↑ post exercise	Jin *et al.*, 2015
Cortisol	Blood	Resistance	8 males	↑ post exercise	Conceicao *et al.*, 2014
Cortisol	Saliva	Resistance	23 rugby players	↓ significant	Beaven *et al.*, 2008
Cortisol	Blood	Resistance	50 active men	↑ post exercise	Brownlee *et al.*, 2005
Cortisol	Serum	Resistance	20 males	No change	Willoughby *et al.*, 2014
Cortisol	Blood	Resistance	10 males	↑ post exercise	Leite *et al.*, 2011
Cortisol	Blood	Resistance	35 participants	No change	Shikorski *et al.*, 2013
Cortisol	Plasma	Resistance	Eight males	↑ after post exercise	Kraemer *et al*., 1993
Cortisol	Plasma	Resistance	4 males	No change	Fry *et al.*, 2010
Cortisol	Blood	Resistance	21 trained and untrained men	↑ untrained	McMillan *et al*., 1993
Cortisol	Serum and saliva	Resistance	21 middle aged men	↑ post exercise	Cadore *et al.*, 2008
Cortisol	Plasma and saliva	Resistance	10 males	↑ post exercise	Hough *et al.*, 2011
Cortisol	Plasma	Resistance	10 males	↑ post exercise	McCaulley *et al.*, 2009
Cortisol	Saliva	Resistance	13 male and 13 female athletes	↑ post exercise	Le Panse *et al.*, 2010
Cortisol	Serum	Volleyball	14 males and 14 female	No change	Eliakim *et al.*, 2009
Cortisol	Saliva	Football match	22 males	No change	Moreira *et al.*, 2009
Cortisol	Saliva	Soccer	10 males	No change	Sari-Sarraf *et al.*, 2007
Cortisol	Saliva	Rugby	20 rugby players	↑ post exercise	Elloumi *et al.*, 2003
Cortisol	Saliva	Soccer	8 athletes	↑ post exercise	Protzner *et al.*, 2015

Further studies are required to establish an overall trend for the cortisol response to resistance exercise. One of the drawbacks of resistance exercise is in recruiting participants. There is no clear trend regarding cortisol levels and crossfit exercise, and the majority of studies show an increase in levels of cortisol after resistance training. Cadore (2008) found that serum and salivary cortisol were both significantly increased after a resistance training bout consisting of two exercises at 75% 1RM (8 repetitions each) and two exercised at 75% and 65% 1 RM (8 and 12 repetitions, respectively). Similarly, Hough (2011) conducted a resistance test in 10 males consisting of 8 sets of a 10 repetition max squat test, and plasma cortisol levels increased from pre-exercise to post exercise, a finding supported by many other studies (McCaulley *et al.,* 2009; McMillan *et al*., 1993; Kraemer *et al*., 1993; Jin *et al.,* 2015). Le Panse (2010) revealed a significant increase in salivary cortisol after three bench press attempts in an international power lifting competition ($p<0.01$), although other researchers, such as Fry (2010), have shown no change in cortisol levels for a protocol involving 10 speed squats at 70% 1RM, and this result is supported by findings from Hakkinen *et al.* (1987) and Beaven *et al,* (2008). Therefore, further studies are required to establish an overall trend for the cortisol response to resistance exercise.

1.12.2 α-Defensins and Physical Exercise

It is well known that physical stress may result in changes (IgA levels) in the immune system of athletes (Trochimial *et al.,* 2012). To date, few studies have investigated the levels of α-defensins (average response) following physical stress. The aim of this subsection is to discuss the published studies on the analysis of α-defensins in response to physical stress, and other antimicrobial peptides (β-defensin and cathelicidin) and their levels during and after exercise are also highlighted. This is relevant because it supports the notion that levels of other antimicrobial peptides (β-defensin and cathelicidin) also change during and after physical stress. Table 1.7 presents the findings of studies which compare levels of antimicrobial peptides (HNP1-3 β-defensin and cathelicidin) after exercise. Cathelicidins (LL-37) are a family of antimicrobial peptides and in humans, only one cathelicidin, LL-37 (4.4 kDa) has been characterised. This peptide was first isolated in 1989 from bovine neutrophils (Generao *et al.,* 1989; Zanetti *et al.,* 1990; Frank *et al.,* 1990; Durr *et al.,* 2006), but can also be found in neutrophils, monocytes, natural killer cells, T lymphocyte cells, B lymphocytes cells, epithelial cell, skin, gastrointestinal and respiratory tracts (Gudmundsson *et al.,* 1996; Frohm *et al.,* 1997; Bals *et al.,* 1998; Agerberth *et al.,* 2000). To date, all the studies have reported a significant increase in salivary antimicrobial peptides after physical stress.

Table 1.7 Antimicrobial peptides level after aerobic endurance.

Protein and peptide	Sample matrix	Exercise type	Participants	Effects on the level	References
HNP1-3	Saliva	Cycle ergometers	5 males and 4 females	↑ significant at 75% VO_{2max}	Gillum *et al.*, 2015
HNP1-3	Saliva	Cycle ergometers	12 male athletes	↑ significant at 60% VO_{2max}	Davison *et al.*, 2009
HNP1-3	Saliva	Cycle ergometers	17 males	↑ significant	Kunz *et al.*, 2015
HBD2	Saliva	Cycle ergometers	10 males	↑ significant at 75% VO_{2max}	Usui *et al.*, 2011
LL-37	Saliva	Cycle ergometers	10 males	↑ significant at 75% VO_{2max}	Usui *et al.*, 2011
LL-37	Saliva	Cycle ergometers	12 males	↑ significant at 60% VO_{2max}	Davison *et al.*, 2009
LL-37	Saliva	Cycle ergometers	17 males	↑ significant	Kunz *et al.*, 2015
LL-37	Saliva	Cycle ergometers	4 males and 4 females	↑ significant at 75% VO_{2max}	Gillum *et al.*, 2015

Peptide name: HBD2 = human β-defensin 2, LL-37= cathelicidins, HNP1-3= human neutrophil α-defensins

Further studies are required to explore whether various intensities and different exercise types (resistance training) also affect the levels of α-defensins (HNP1-3) alongside other AMPs and hormones.

1.13 Summary

Current proteome research utilises LC-ESI-MS as a technique with reliable sensitivity, although ELISA remains the primary proteomic analytical method. Proteome analysis of α-defensins by ELISA has been commonly used in the area of sports science, gastroenterology, cancer biology or oral biology but this chapter has suggested that LC-MS has the ability to provide simultaneous high-throughput quantification, with high specificity and sensitivity. Minimal research has been conducted into the innate immunity of humans in relation to the consequences of exercise or physical stress on mucosal secretions. This chapter has highlighted the response of stress hormone and AMPs to physical stress and this study aims to bridge some of the gaps identified regarding responses to exercise.

Chapter 2 - Practical Considerations of Electrospray Ionisation

To date, mass spectrometry (MS) has become one of the most popular analytical techniques for both quantitative and qualitative applications (Karas *et al.,* 1988; Yamashita *et al.,* 1984; Fenn *et al.,* 1989; Cohen *et al.,* 2006; Aneed *et al.,* 2009; Cole *et al.,* 2010). Over the past fifty-five years MS has been repeatedly transformed, and its applications have been extended through the use of new methods of ionisation. The advent of new methods of ion generation, novel mass analysers, and new tools for data processing, have made it possible to analyse small organic compounds through to large biological molecules. The aim of this project is to optimise and validate a LC-MS method for the detection and relative quantification of individual salivary HNPs. Chapter 1 reviewed the existing literature on analytical techniques and identified LC-MS as a suitable choice for the measurement of structural homologous antimicrobial peptides (HNP 1-4). To accomplish this, the first and foremost requirement is to understand how electrospray ionisation (ESI) works and the parameters that can affect the detection limits of ESI, which include: (a) choice of solvents (pH) and analyte concentration; (b) choice of additives and their concentration; (c) contaminants (electrolytes) present in complex biological matrix; and (d) choice of flow rates of the solution through the spray capillary. In addition, the following aspects should be considered for the detection and relative quantitation of salivary HNPs by LC-ESI-MS: (1) minimisation of the matrix effects and analyte loss during sample preparation (Chapter 4); (2) development and optimisation of the LC-MS parameters - solvents, additives, columns performance (Chapter 5) and (3) evaluation of the validation parameters such as: detection limit, quantitation limit, intra-interday precisions, stability (Chapter 6). This chapter provides an opportunity to understand how the analyte characteristics, concentration, solvent pH and contaminates of analytes could affect the ESI signals. It highlights examples of the practical considerations of ESI which include: analyte characteristics, concentration and its effects on the ESI response, analytes pKa and solvent pH on the ESI response, and detection limits (electrolyte effects on the ESI response). This chapter also describes how ESI functions through the separation of ions, and the interpretation of ESI mass spectra. Overall, this chapter has been written to explain ‘how ESI works’ and it focuses on the practical considerations of ESI that were relevant to the experimentation work presented in Chapters 4-6.

2.1 Mass Spectrometry

In mass spectrometry, chemical compounds are converted into gas phase molecules and their mass-to-charge (*m/z*) ratio are measured (Figure 2.1). The sample is introduced via the inlet system either as a solid, liquid or a gas and the choice of ionisation mode depends on the nature of the sample. The ionisation chamber also depends on either a vacuum or atmospheric pressure system. In the ion source, analytes are converted into gas phase ions, which are separated by the mass analyser according to their mass-to-charge ratio.

$$M^+ \text{ (Ion)} + H^+ \longrightarrow MH^+ \quad \textbf{(}\text{Mode of ionisation for ESI)}$$

The ion MH^+ is known as the pseudo molecular ion due to the change in overall mass by 1 Da. Two of the most important properties of an ion are its charge (*z*) and its mass (m). Ions carry charges and can therefore, be handled or their path controlled through the use of electric and magnetic fields.

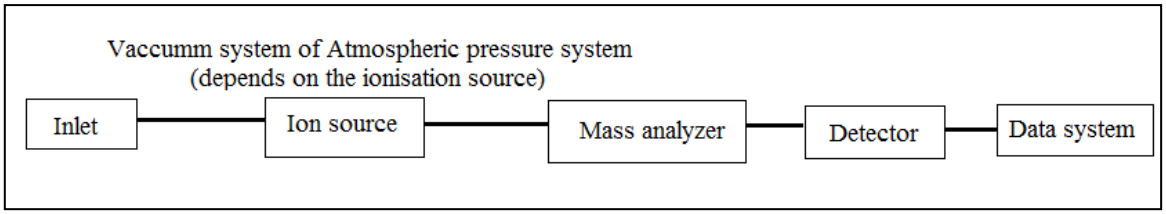


Figure 2.1 Principal components of a mass spectrometer.

2.2 Mechanism of Electrospray Ionisation

The eluent is introduced by HPLC at a suitable flowrate in ESI-MS, and based on the nature of the analyte both negative and positive modes can be selected. In the positive ion mode, the capillary tip is the positive electrode and the sampling cone (plate) is the negative electrode, which is set to a higher voltage (typically 2-5 kV). Ions within the eluent solution are repelled from the inner walls of the sprayer needle and moved towards the droplet formed at the capillary tip. This mode causes positive ions to predominately populate the spray droplet where the analyte forms cations in solution. When the charged density at the liquid meniscus is raised due to the repulsion of cations, the columbic forces are also increased. The point at which the surface charge repulsion matches the surface tension of the eluent is termed the Rayleigh instability limit. When the number of ions and columb explosion overcome this limit, the shape of the meniscus is changed to a conic, in order to relieve the charge repulsion and the cone meniscus is referred to as the Taylor Cone.

Upon formation of the cone, a stream of droplets containing a vast excess of cations emerges from its surface and this process is repeated until the size of the droplets is small enough to emit gas phase ions. This process is termed an electrospray (Figure 2.2).

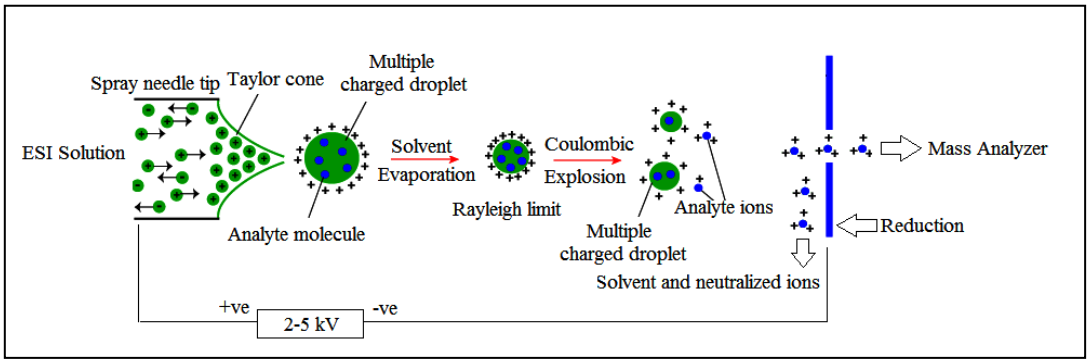


Figure 2.2 Schematic representation of the formation of ions during ESI (Gates *et al.*, 2002).

When the solution forms the Taylor Cone, the droplets that contain an excess of positive or negative charge detach from its tip. In the ion evaporation model theory, droplets are driven electrically and are vaporised with the aid of warm nitrogen gas, the droplets then break down, reducing their size and shifting inside the sources. Thus, repulsive forces among the ions on the surface of the shrinking droplets become very high and help the ions to desorb into the gas phase by exceeding the surface tension of the solvent (Iribarne *et al.*, 1976; Kebarla *et al.*, 2000). An alternative theory of ESI ion formation, known as the charge residue model, involves continuous evaporation of the solvent accompanied by droplet fragmentation, so that a single ion (multiply charged) is formed at the end of this process (Fenn *et al.*, 1989). Both these ESI ion formation mechanisms have an evidence base, and it has been suggested that the ion evaporation model is more suitable for small ions and the charge residue model is more applicable to macromolecular ions (Tang and Kebarle *et al.*, 1993; Kelly *et al.*, 1992).

The main difference between the two proposed mechanisms is that in the first, the columb explosion will break the highly charged droplets into smaller ones and eventually lead to ions being desorbed into the gas phase, whereas in the charge residue model, continuous evaporation of the solvent is accompanied by droplet fragmentation, so that a single ion (multiply charged) is formed at the end of this process.

2.3 Interpretation of Electrospray Ionisation Mass Spectra

Understanding ESI mass spectra is usually complex. However, advantage of multiple charging makes it possible to observe large proteins at lower *m/z* values, and the subsection below explains the interpretation of ESI mass spectra in detail.

2.3.1 Multiple Charge Ion Formation

Single, double and multiple charged ions can be determined by assessing the isotopic pattern of the ions in a mass spectrum.

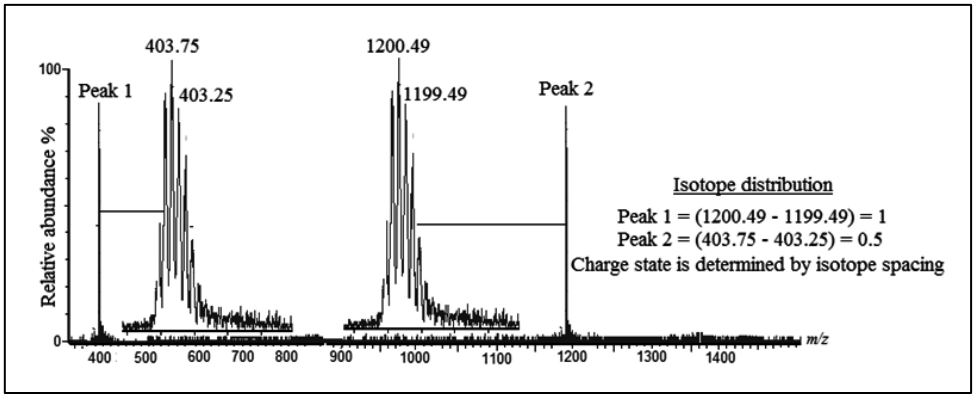


Figure 2.3 Schematic diagram represents how the isotope distribution can be calculated from an ESI mass spectrum. The charge state is determined by the isotope spacing. As an example, unknown single charged peptides from human saliva samples have been used.

In the ESI spectrum shown in Figure 2.3, an ion is seen at *m/z* 1200.49 and another at *m/z* 403.75, thereby demonstrating that the 2+ charge means that the isotopes are separated by 1/2 Da, while the 3+ charge indicates that the isotopes are separated by 1/3 Da, and for the 4+ charge state the isotopes are separated by 1/4 Da.

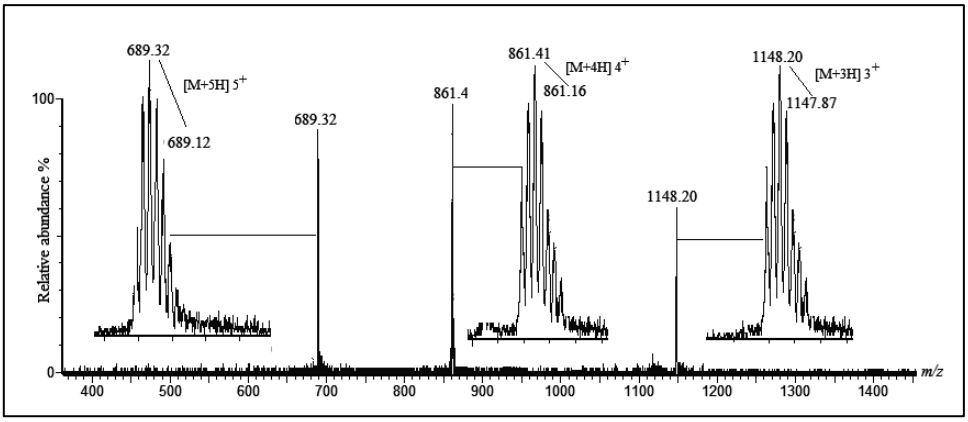


Figure 2.4 An ESI mass spectrum representing the isotope distribution of a multiple charged peptide (HNP1) present in human saliva.

The isotopic distribution is also confirmed if the peaks correspond to the same compound. The isotopic pattern of these ions indicates a single charge state (*m/z* 1200.49, isotope ions separated by 1 mass unit) and a double charge state (*m/z* 403.75, isotope ions separated by 1/2 a mass unit). Another example of charge state isotope spacing of a known peptide (HNP1) is shown in Figure 2.4.

The arrows indicate that for the 3+ charge state *m/z* of 1148.68, isotopes are separated by 1/3 Da; for the 4+ charge state *m/z* of 861.2734, isotopes are separated by 1/4 Da, and for the 5+ charge state *m/z* of 689.2214, isotopes are separated by 1/5 Da.

2.3.2 Charge Deconvolution

The molecular weight of an intact protein can be calculated by the number of charge states of proteins, corresponding to individual peaks.

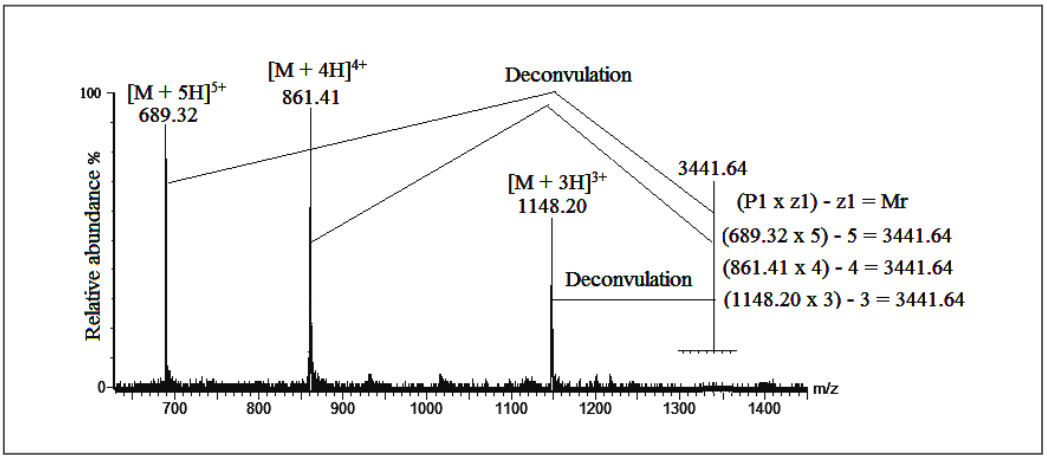


Figure 2.5 Calculation of deconvolation from an ESI spectrum. Deconvolation is determined by ions and charge state, and it also helps to confirm the molecular weight of an analyte. As an example, a multiple charged peptide (HNP1) from a human saliva sample has been utilised.

The molecular weight can be determined by: (P1 x z1) – z1 = Mr where,

Mr = Molecular weight of the sample;
P1 = *m/z* value for peak 1in the spectrum;
Z1 = charge of peak 1.

An ESI spectrum of human salivary HNP1 (positive ionisation mode) shows how to deconvolate the molecular weight (Figure 2.5). The above procedure may be time-consuming but works well for pure compounds and simple mixtures. When complex mixtures are analysed, correct manual assignment of all the peaks becomes increasingly difficult. The ESI spectrum

creates a large number of peaks for molecular weights above 20 kDa, such as for bovine albumin serum (mw 66.5 kDa), and this requires the use of specialist software (Magtran, MaxEnt) in order to enable reliable mass assignments of unknowns.

2.4 Practical Considerations of Electrospray Ionisation

For successful application of ESI, understanding the analyte characteristics is of key importance in determining the ESI response. The suitability of ESI analysis depends upon the particular analyte and the next subsection highlights the practical considerations that may affect the ESI response during peptide analysis.

2.4.1 Effects of Analyte Characteristics on the Electrospray Ionisation Response

The ESI response varies significantly depending on the different characteristics (amino acid sequence) of analytes. To enhance the ESI response it is essential to understand the characteristics of the analytes (Tang and Kebarle *et al.,* 1993; Cech *et al.,* 2001).

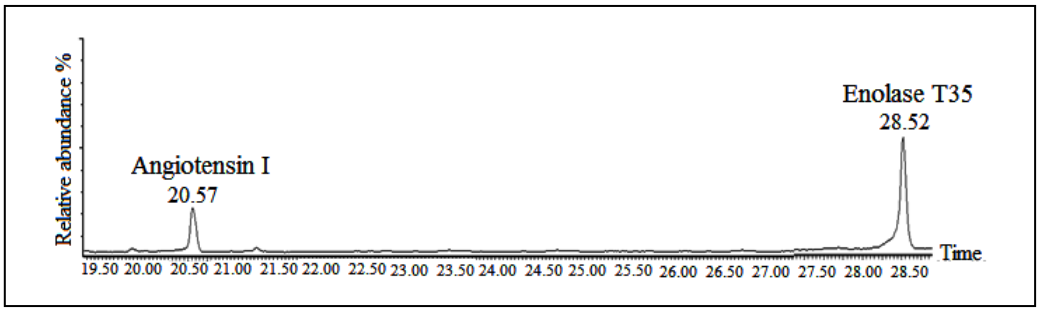


Figure 2.6 Total ion current chromatogram of an equimolar mixture of angiotensin I and enolase T3ESI chromatogram of the two peptides responses varies significantly, with the angiotensin I peptide response being less than that of enolase T35. The peptides were separated using a linear MeCN: H_2O: AC (0.1%) gradient on a Kinetex® C18 column (50 x 3.0 mm, 2.6 µm), at a flow rate of 0.6 mL/min.

ESI is typically performed in polar solvents, such as water, acetonitrile (MeCN) and methanol (MeOH), and analytes with significant non-polar regions should favour the air-solvent interface at the surface of the electrospray droplets, where the polar region can be desolvate (Irebarne *et al.,* 1976; Tang and Kebarle *et al.,* 1993; Cech *et al.,* 2001). The example provided in this subsection is relevant to the practical work described in Chapter 4 for the analysis of HNPs in a saliva matrix and an example of a stable peptide mixture is presented in order to understand analyte characteristics, concentration and its effects on the ESI response. Figure 2.6 and Figure 2.7 show a chromatogram and a spectrum of an equimolar peptide mixture of one mole of two different peptides (angiotensin I and enolase T35), which were separated using a linear MeCN: H_2O: acetic acid (0.1%) gradient on a Kinetex® C18 column (50 x 3.0 mm, 2.6 µm), at a flow rate of 0.6

mL/min. Angiotensin I and enolase T35 peptides are positively charged species with an equimolar concentration, yet their ESI responses are very different. Enolase contains eight hydrophobic (non-polar) amino acids, whereas angiotensin I contains five non-polar amino acids. The EIC obtained for angiotensin I and enolase T35 ions corresponds to $[M + 2H]^{2+}$ and $[M + 3H]^{3+}$ and this finding seems in line with that of Iribarne (1976), where an increased ESI response for non-polar analytes was reported.

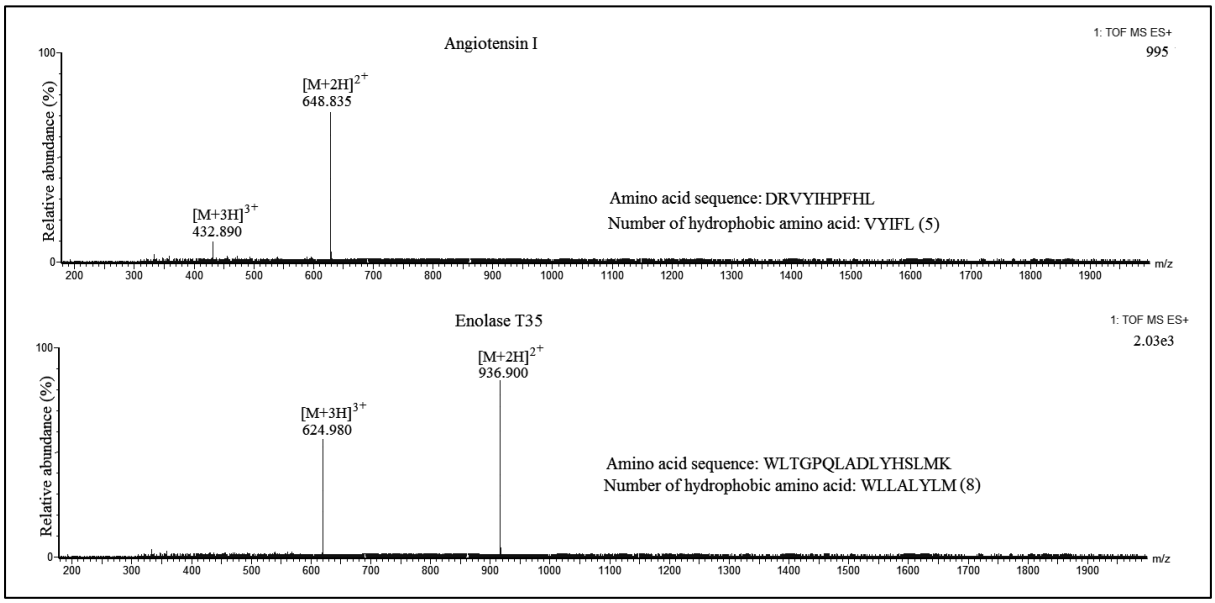


Figure 2.7 ESI mass spectrum of an equimolar mixture of angiotensin I and enolase T35. The observed variation in the ESI response for the charged species could be due to differences in analyte structure.

The existing literature also suggests that nonpolar ions would enter the gas phase more readily (Tang and Kebarle *et al.,* 1993; Apffel *et al.,* 1995), and this could be one of the reasons that nonpolar ions are less attracted to water. Ions are therefore easily attached to the non-polar analytes in the gas phase, which results in a higher ion signal intensity. Poor ion intensity cannot only be defined by the polarity of an analyte, as it also relies on the complex structure of an analyte and the solvent pH. If a non-polar analyte contains disulphide bonds, then it will not easily be ionised; thus non-polar analytes also lead to poor ionisation. The next subsection explores the importance of the role of analyte pKa and solvent pH.

2.4.2 Effects of Analyte pKa and Solvent pH on the Electrospray Ionisation Response

This subsection highlights the importance of analyte characteristics and solvent pH effects on the ESI response and the example presented is relevant to the practical work described in Chapter 5 for the analysis of HNPs within a saliva matrix. The ionisation mode of ESI involves protonation $[M + H]^+$ or deprotonation $[M - H]^-$, and for an analyte to become protonated it

should be basic in solution and in the gas phase. The same principal applies for acidic analytes and deprotonation. Basic analytes generally ionise best at a low pH, but acidic analytes perform best at a higher pH (Wang *et al.*, 1997). It is well established that protonated ions of basic analytes can be observed when ESI-MS analysis is performed with basic solutions (the usual rule-of-thumb is 2 pH units higher or lower than the analyte pKa), whereas deprotonated ions can be observed in the ESI analysis of acidic solutions (Kelly *et al.*, 1992; Wang *et al.*, 1997; Zhou *et al.*, 1995).

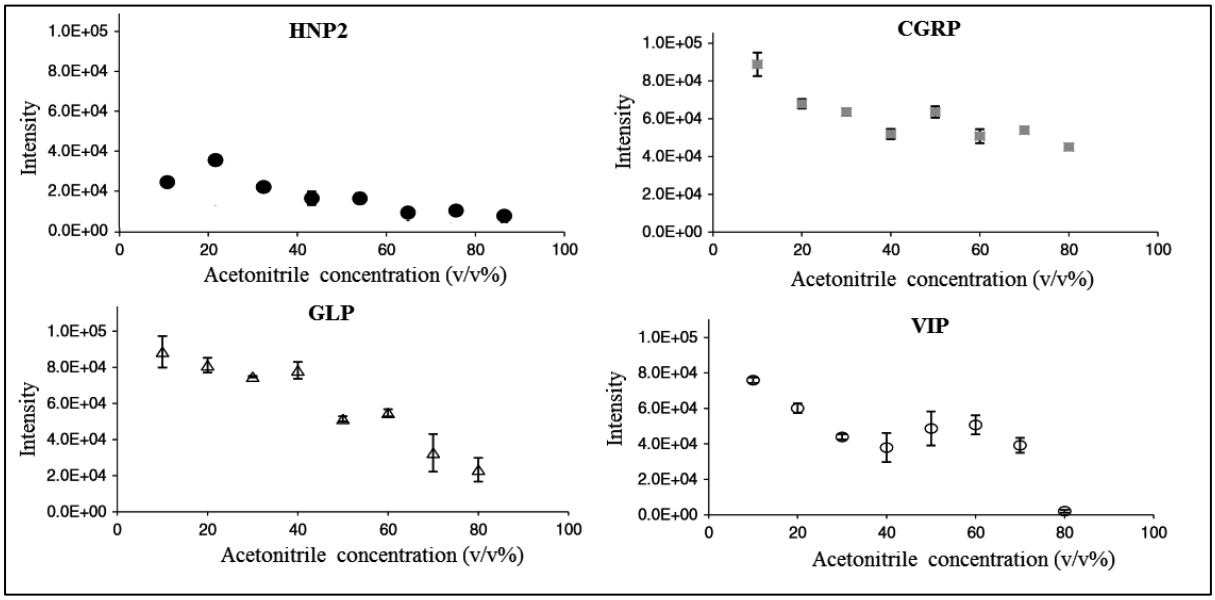


Figure 2.8 Effects of decreasing the mobile phase polarity on the ion intensity profile of HNP2, CGRP, GLP and VIP peptides. Increasing MeCN concentration (10- 80% v/v) on the ESI response for individual peptides for the ions (a) $[M + 5H]^{5+}$ for HNP2, (b) $[M + 4H]^{4+}$ for CGRP, (c) $[M + 4H]^{4+}$ for GLP and (d) $[M + 4H]^{4+}$ for VIP (Abaye *et al.*, 2011).

Abaye (2011) investigated analyte responses when decreasing the polarity of the mobile phase (acetonitrile concentration from 10-80% v/v) on four medium sized (3125-3370 Da) peptides, including calcitonin gene-related peptide (CGRP); glucagon-like peptide (GLP); vasoactive intestinal peptide (VIP), and HNP2. Abaye (2011) demonstrated that increasing the percentage of organic solvent (decreasing the polarity of the mobile phase) decreased the ion intensity response of individual peptides (Figure 2.8). The data revealed that the analyte response changes through with the change of organic solvent pH. Changes in pH, which occur when increasing the percentage of organic solvent, lead to poor ionisation. The data also shows that polarity does not always explain the poor ionisation that has been observed for HNP2, which is the most non-polar peptide. One reason for this could be the presence of disulphide bonds that produce more structurally restricted peptides, and thus lead to poor ion intensity compared

to CGRP, GLP and VIP. Other studies have reported similar findings, whereby more non-polar compounds (in this case peptides) ionise better than polar ones (Cech *et al.,* 2001; Hendrickson *et al.,* 2006; Ehrmann *et al.,* 2008).

2.4.3 Effects of Electrolytes on the Electrospray Ionisation Response

This subsection highlights the effects of electrolytes on the ESI response, and the example provided is relevant to the practical work described in Chapter 4 and Chapter 6 for the analysis of HNPs. The ESI ion source is sensitive to electrolytes and saliva contains a variety of electrolytes: sodium, potassium, calcium, chloride, magnesium, bicarbonate, and phosphate. Samples entering the MS should be of the highest possible purity and deprived of compounds that compete with the analytes for ionisation or cause signal suppression, such as inorganic salts. Therefore, the ESI response for a salivary matrix is dependent upon the quality of the input and on earlier sample preparation/processing steps. The presence of electrolytes is often a limiting factor in determining the lower limit of detection with ESI.

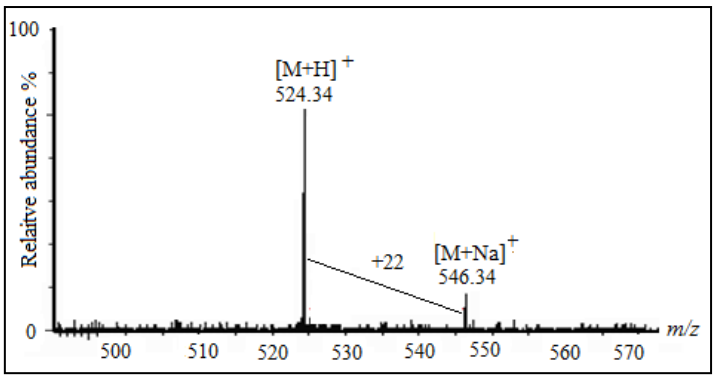


Figure 2.9 ESI mass spectrum of protonated molecule and a related sodium adduct in saliva matrix.

In addition, ESI is typically performed in polar solvents with additives and Mallet (2004) studied the influence of several additives and their concentrations on the ESI responses of acidic and basic drugs. Mallet's findings showed a decrease in the response when the concentration of an additive (FA, AC, TFA, ammonium format, ammonium bicarbonate) was increased from 0.05% to 1%. Additives at higher concentrations will compete with the analytes for the excess charge, which results in signal suppression. The natural presence of electrolytes within a complex matrix or any electrolytes (additives) added to the ESI solution mean that some common interactions may take place between a protein and dissociated salts. Such interactions result in charge transfer reactions, neutralisation reactions, and/or ion-paring reactions. As an example, sodium adducts in a saliva matrix were evaluated as these have been

observed at a retention time of 5.33 minutes and ions at *m/z* 524.34. A saliva sample was treated with 0.1% AC 1:1 (v/v), subjected to centrifugation at 12,000 *g* for ten minutes at 4°C and analysed by LC-MS. Sodium adducts which is present in the sample matrix and often causes a limiting factor to determine the lower limit of detection for analyte of interest with ESI (Figure 2.9). Chemical noise is another type of interference, and can be manifested as a fixed pattern of noise revealed at specific *m/z* units. This chemical noise is generally caused by the mass analysis of charged species other than the analyte. Thus, interferences are often either adducts in the electrospray solution, species generated electrochemically, or neutral species present in the atmosphere around the ESI spray that are charged in the gas phase by proton transfer (Ramsey *et al.,* 1993; Purves *et al.,* 1998; Guevremont *et al.,* 2000). To avoid discharge and a poor ESI response, sample optimisation (clean up) and the use of mobile phase additives at the lowest possible concentration must be considered.

2.5 Summary

This chapter presents an account of 'how ESI works' and has highlighted the relevant practical considerations of ESI for peptide analytes. However, it represents only a limited account of ESI and extensive details can be found in reviews (e.g. Cole *et al.,* 2010).

Chapter 3 - Sample Optimisation: Separation and Detection of α-Defensins

3.1 Background

Fields that utilise saliva now include metabolomics, genomics, proteomics and bioinformatics (Javaid *et al.,* 2015). However, the composition of the saliva matrix, its biochemical properties and inherent variability and instability, may be problematic. The sensitivity and specificity of the LC-ESI-MS method often allows for the detection of low-abundance proteins and peptides within biological studies (Zhang *et al.,* 2010). Saliva is a complex matrix and in order to enhance the ESI response, sample optimisation may be important.

This chapter outlines the issues related to the methods of sample preparation used in previous studies to analyse salivary HNPs. Few studies have reported the detection and analysis of human neutrophil α-defensins within the saliva matrix (Cabras *et al.,* 2005; Vitorino *et al.,* 2004; Peulso *et al.,* 2000). These studies have some limitations, such as insufficient sample clean-up, which could result that the peptides were co-eluted during the analysis leading to the possibility of ion suppression or enhancement thus potentially compromising quantitation. Ion suppression and enhancement is one form of matrix effect that negatively affects detection capability, precision and accuracy (Mallet *et al.,* 2004).

Goebel (2000) and Gardner (2009) reported the separation of individual α-defensins using solid phase extraction (SPE) sample preparation method. However, in both studies there was insufficient information regarding the selection of the solvent composition to conduct SPE. Therefore, a pilot study is necessary to analyse salivary HNPs so that a single method can be finalised for the main study. This pilot study assesses the impact of different sample treatments, such as with SPE and acidification without SPE for the analysis of individual HNPs (Cabras *et al.,* 2005; Vitorino *et al.,* 2004; Peulso *et al.,* 2005; Goebel *et al.,* 2000; Mizukhawa *et al,* 1998). Sample optimisation parameters are also described in this chapter, including analyte recovery, sample drying efficiency, sample treatment, and matrix effects. Overall, the focus of this chapter is on the relevant conceptual features of sample preparation methods with respect to practical experimentation for the analysis of HNPs.

3.1.1 Sample Preparation Techniques

It is well established that both the sensitivity and accuracy of peptide identification and quantification can be affected by many factors, including sample purity and LC-MS analysis. The presence of electrolytes within body fluids (e.g. plasma, saliva etc.) is normal. The

sensitivity of ESI towards some inorganic compounds has already been discussed in Chapter 2. To date, without-SPE (acidification) and with-SPE have been the preferred methods of sample preparation for the analysis of salivary HNPs. Thus, the fundamental characteristics of without-SPE and SPE for analysing salivary HNPs are discussed in this sub-section.

3.1.2 Acidification

Generally, acidifying saliva samples is performed using formic acid, acetic acid or trifluoroacetic acid, and the benefit of this technique is it takes less time. The acidification technique was used by Lupi *et al.,* (2003) to analyse salivary cystatins, and the application of the acidification technique by Lupi *et al.,* was modified by Vitorino (2004) by adding a subsequent filtration stage for separating salivary peptides from all the mucins and proteins present in the saliva to maximise the number of peptides identified. HNP3, along with other AMPs (such as histatins and statherin), were detected and identified by Vitorino (2004), while Peulso (2005) and Cabras (2010) executed the acidification technique (as per the work of Lupi) for the relative quantification of salivary HNPs, as well as other peptides and proteins.

3.1.3 Solid Phase Extraction

Solid phase extraction is one of the most popular sample preparation techniques and involves the use of chromatographic sorbents within a column format. A sample is passed through a column bed and analytes are retained on the sorbent while the sample matrix liquid passes through. The stationary phase of the column then washed to remove undesired interferences, and the purified analytes are subsequently eluted from the column (Figure 4.1) (Chromacademy 2010). The advantage of SPE is that a wide variety of stationary phases are available for the selective removal of analytes from desired analytes, including those with polar, hydrophobic and ionic interactions. The challenge, however, is to choose the washing and elution solvents and any additives. Analyte recovery is typically achievable at around 85-95% for a particular analyte (Matuszewski *et al.,* 2003). However, in some cases, interfering components may not be selectively separated from the analyte. Goebel (2000) reported the use of SPE for the analysis of salivary HNPs, where SPE was carried out for eledoisin, kassinin, substance P, and physalaemin peptides. The solvent composition used by Goebel (2000) was 50% MeCN in 1% aqueous AC, and this solvent composition was also used in the experiments of Gardner (2008).

3.2 Pilot Study

LC-ESI-MS is a current analytical method of choice for the quantitation of analytes within biological matrices (Scherl *et al.,* 2015; Grebe *et al.,* 2016; Taylor *et al.,* 2005); however,

matrix effects may lead to significant analytical errors (Xu *et al.*, 2005). Guidance on bioanalytical method validation, asserts that the matrix effects require monitoring to ensure that the sensitivity, precision, and selectivity remain uncompromised, particularly when procedures are based on LC–MS (Huber *et al*., 2010). Matrix effects are the alternation of the ionisation efficiency in the presence of co-eluting substances (Tang and Kebarle *et al.*, 1993), and can affect the development of MS methods, as they may lead to ion suppression, inhibiting ionisation and sensitivity during ESI, with LOQ adversely affected (Buhrman *et al.*, 1996). Thus, the minimisation of matrix effects to maintain sensitivity is essential when developing a method. It is not always possible to separate analyte from interferences within a chromatogram in order to avoid ion suppression (Chromacademy 2010). Hence, the optimisation of various methods for sample preparation to obtain improved separation efficiency is necessary for specific analytes. The general method described in Chapter 3 described the sample preparation, storage and collection of saliva. The purpose of the sample preparation used in this project is to clean a sample before analysis and concentrate it. Goebel (2000) and Lupi (2003) assessed two methods of sample preparation: SPE and acidification, and these two preparation methods have also been investigated in this study. This pilot study serves the purpose of comparing the two sample preparation techniques for analysing individual HNPs within the human saliva matrix. Valuable insights were obtained from both methods for individual HNPs and the drying efficiency, extraction recovery, mobile phase solvents and matrix effects are also discussed in the context of LC-MS separation efficiency of SPE.

3.3 Materials and Methods

3.3.1 Chemicals and Reagents

LC-MS grade water was obtained from Fisher Scientific (Dartford, UK), LC-MS grade methanol and acetonitrile (Hipersolv Chromanorm) was obtained from VWR (Leicestershire, UK) and acetic acid (≥ 98% purity) and formic acid (≥ 95% purity) from Sigma-Aldrich (Gillingham, UK).

3.3.2 Instruments and LC-MS Experimental Conditions

Saliva samples were analysed by LC-MS using an Agilent 1200 series HPLC from Agilent Technologies (Santa-Clara, USA) coupled to a Quadrupole Time of Flight Mass Spectrometer from Waters Synapt G1 (Manchester, UK). The saliva was separated on a Kinetex® Core Shell C8 column (2.6 µm, 50 x 3.0 mm) (Phenomenex, USA). The mobile phase compositions were A: 0.1% (v/v) acetic acid in H_2O and B: 0.1% (v/v) acetic acid in MeOH, and were delivered

at a flow rate of 0.6 mL/min with a gradient of B 2-95% over a 45 minutes: In general, Eluent B was increased from 2 to 50% over 19 minutes, followed by an increase to 95% over 2 minutes. Eluent B was maintained at 95% for 3 minutes, followed by a rapid return to the initial condition of B (2%), which was maintained for 20 minutes for re-equilibration of the column. The injection volume was 10 µL. An EIC was used for the analysis of salivary HNP1-3. Individual HNP1-4 levels were calculated from the EIC peak areas of HNP1-4 ions corresponding to $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$.

3.3.3 Sample Collection

3.3.3.1 Circadian Rhythm

A circadian rhythm is a biological process that displays an oscillation over 24 hours. Circadian (daily) low flow occurs during sleep (Dawes *et al.,* 1974) and circadian flow variations affect the concentration levels of salivary components. In the 1970s, studies reported that cortisol exhibited a circadian variation and may be secreted episodically (Hellman *et al.,* 1970; Krieger *et al.,* 1971). Circadian variations affect the concentration level of hormone (cortisol), and Harris *et al.,* (2010) reported that levels of cortisol increase on awakening (0 mins) and peak after 45 minutes, followed by a steady decrease throughout the day. Interestingly, Davison (2009) reported the circadian variation of HNP 1-3 over a 2.5-hour period in the morning as detected by ELISA, although no significant variation was found for the average response of salivary HNP 1-3. Therefore, when measuring cortisol or salivary HNPs it is important to control for these variables. Consequently, in the study presented in this thesis all the samples were collected at approximately the same time of the day (3-5 pm) to avoid circadian variation.

3.3.3.2 Stimulated versus Unstimulated saliva

The concentration of salivary components including α-defensins and cortisol secretion may be different under stimulated and unstimulated conditions (Von Zastow *et al.,* 1987). It is also well documented that glandular contribution by volume changes following stimulation (Mandell *et al.,* 1972, Shannon *et al.,* 1974, Edgar *et al.,* 1990). Unstimulated whole saliva is expected to yield higher levels of serum derived proteins and peptides, while stimulated whole saliva is expected to have a greater concentration of glandular derived proteins and peptides (Mandell *et al.,* 1993, Shannon *et al.,* 1974, Edgar *et al.,* 1990; Von Zastow *et al.,* 1987; Edgar *et al.,* 1992). However, α-defensins are serum derived peptides and cortisol is a serum derived hormone and therefore, in this study unstimulated saliva was collected from the participants.

Participants were advised to refrain from drinking for ten minutes before producing saliva sample, and from eating for a minimum of two hours before collection. There are several ways of collecting saliva, such as draining, drooling, suction and swabbing. Spitting and draining are the most reliable and reproducible methods for the collection of unstimulated whole saliva, and both methods provide similar information (Navazesh *et al.,* 1993). The suction and swab methods provide some degree of stimulation and variability and are therefore not recommended for unstimulated whole saliva collection. Consequently, in this study unstimulated whole saliva samples were collected using the spitting method (Navazesh *et al.,* 1993).

3.3.3.3 Saliva Storage

The correct storage of saliva is important to minimise degradation and the reduction of salivary components, which can affect the accuracy and precision of the results. In this study, the concentration levels of salivary hormone (cortisol) were compared with the α-defensins (HNP1-4) family. Therefore, saliva storage stability was considered for the cortisol hormone, as studies have suggested that salivary hormone is more stable at -80°C (Whembolua *et al.,* 2006; Kirschbaum *et al.,* 2000). No storage stability study has been optimised for HNPs in the human saliva matrix; however, HNP analytes were considered stable when 85–115% of the initial concentration was found in human plasma and serum after storage at -80°C (Van den broek *et al.,* 2010). These results support the storage of saliva samples at -80°C for measuring cortisol and HNPs in human saliva.

3.3.4 Sample Preparation

3.3.4.1 Method A using A SPE

Prior to LC-MS analysis, unstimulated saliva was diluted (by adding 1 mL, 1% aqueous acetic acid) and saliva was subjected to centrifugation at 12,000 *g* for 10 minutes at 4°C. Discovery® DSC-18 500 mg (3 mL tubes) SPE cartridges from Supleco (Bellefonte, PA, USA) were used to clean up the saliva samples. The SPE cartridges were conditioned in 15 mL of 50% MeOH in 1% aqueous acetic acid, then washed with 5 mL of HPLC grade water and the SPE cartridges were equilibrated (5 mL of 1% aqueous AC). The previously diluted 1 mL saliva sample was loaded onto the cartridges at a flow rate of 1 mL/min. The cartridge was washed with 10 mL of HPLC grade water in order to remove any electrolytes and salts and the peptides were eluted with 1 mL of 50% MeOH in 1% aqueous acetic acid (Figure 3.1). The eluent from the SPE was

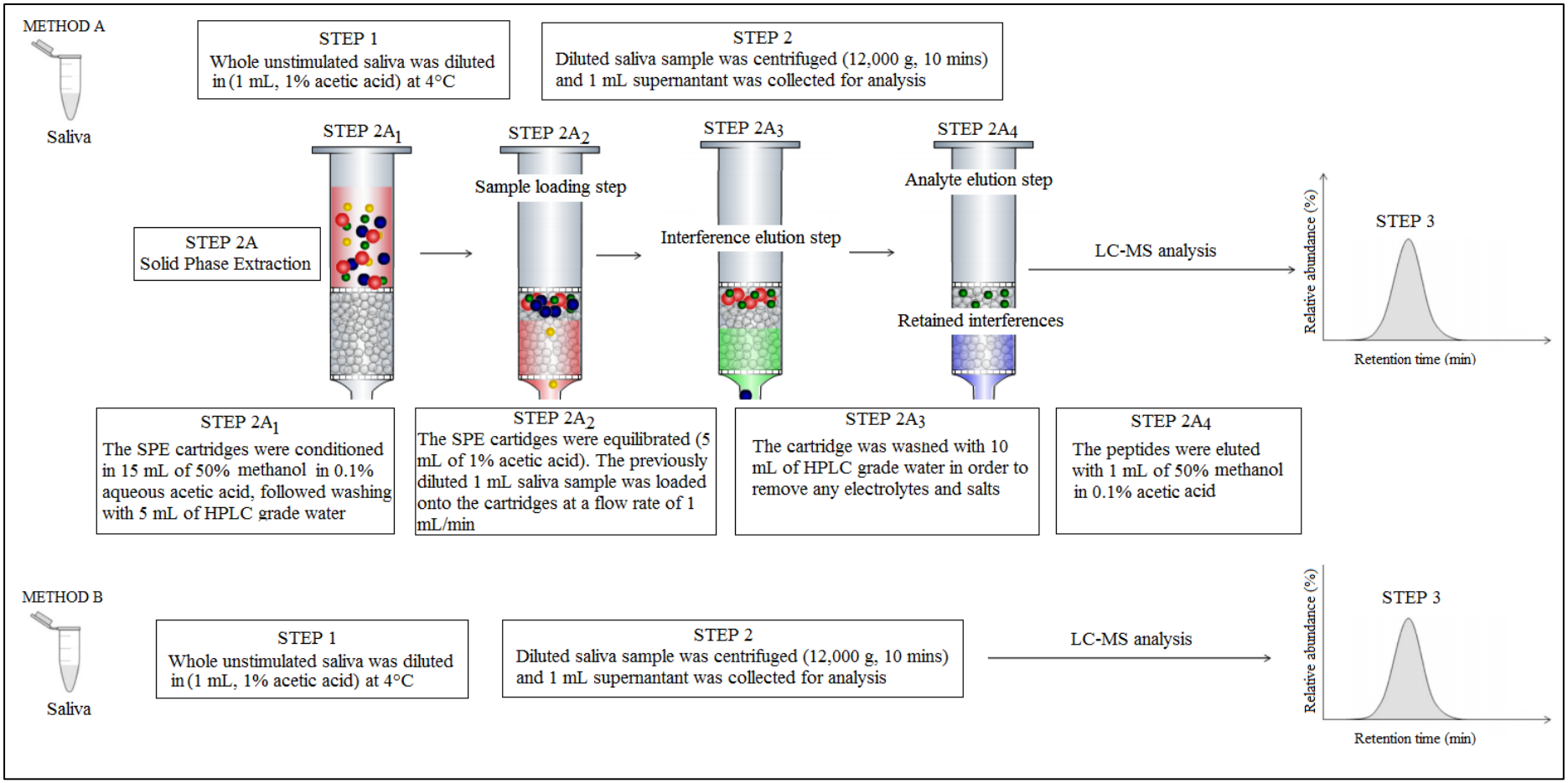


Figure 3.1 Schematic diagram of saliva sample preparation for the analysis of individual HNP1-4 prior to LC-MS analysis. Two different saliva sample treatments were investigated: Method A (without SPE) and Method B (with SPE). Both sample treatment methods utilised the same STEP 1-2, process for the analysis of salivary HNPs.

dried using an Thermo Savant SPD 1010-130 integrated speedvac concentrator (Thermo Scientific, USA) and the saliva samples were reconstituted with the desired mobile phase. To determine whether the method had introduced any contamination that might interfere with the sample, a blank run was extracted under the same conditions.

3.3.4.2 Method B using acidification

In total, 0.1% AC 1:1 (v/v) was added to whole, unstimulated saliva and the saliva was subjected to centrifugation at 12,000 *g* for 10 minutes at 4°C and analysed by LC-MS (Figure 3.2).

3.3.5 Mobile Phase Extraction Buffer

To compare the effects on the LC-ESI response, eight different SPE solvent buffers were investigated are in Table 3.1

Table 3.1 Composition ratios for eight different SPE solvent buffers.

Mobile phase solvents	Mobile phase solvents composition ratio
H_2O+ MeOH + FA	H_2O+ MeOH (50:50) + 1% FA
H_2O+ MeOH + AC	H_2O+ MeOH (50:50) + 1% AC
H_2O+ MeOH + AC	H_2O+ MeOH (50:50) + 0.1% AC
H_2O+ MeOH + FA	H_2O+ MeOH (50:50) + 0.1% FA
H_2O+ MeCN + FA	H_2O+ MeCN (50:50) + 1% FA
H_2O+ MeCN + AC	H_2O+ MeCN (50:50) + 1% AC
H_2O+ MeCN + AC	H_2O+ MeCN (50:50) + 0.1% AC
H_2O+ MeOH + FA	H_2O+ MeCN (50:50) + 0.1% FA

3.3.6 Internal Standard and Matrix Effects

The saliva matrix effects were assessed by comparing the peak areas (EIC) of Leu-enkaphelin, which was spiked into saliva samples before and after SPE. Three different concentrations of Leu-enkaphelin (0.3, 0.6 and 0.8 ng/µL) were added into saliva to compare the matrix effects. Matrix effects were calculated using the equation below:

$$\frac{\text{EIC peak area of spiked Leu-enkapheline into saliva matrix}}{\text{EIC peak area of spiked Leu-enkaphelin into mobile phase}} \text{ X } 100$$

3.3.7 Extraction Recovery

To account for losses, Leu-enkaphelin (0.3 ng/µL) was spiked into each saliva sample prior to SPE and Leu-enkaphelin levels were calculated from the EIC peak area of Leu-enkaphelin ions corresponding to $[M + H]^{1+}$.

Extraction recovery (%) was calculated using the equation below:

$$\text{Leu-enkaphelin} = \frac{\text{EIC peak area of spiked Leu-enkapheline into saliva before SPE}}{\text{EIC peak area of spiked Leu-enkaphelin into saliva after SPE}} \text{ X } 100$$

3.4 Statistical Analysis

Statistical analysis was performed using Microsoft Excel 2013 with Excel Add-Ins and analysis tool pack ANALYS32.XLL. The presented data were analysed using a one-way analysis of variance (ANOVA) and independent t-tests.

3.5 Results and Discussion

3.5.1 Sample Optimisation

3.5.1.1 Effects of SPE

A better separation and increased peak area (EIC) was observed in the SPE treated sample at a retention time of between 19-20 minutes for individual HNPs, whereas without SPE, two broad/tailing peaks were observed for this time interval (Figure 3.2, Figure 3.4, Figure 3.6 and Figure 3.8). The individual HNP 1-4 eluted at the retention time of 19.13, 19.41, 19.48 and 20.67 mins without SPE treated and retention time of 19.20, 19.43, 19.56 and 20.72 with SPE treated samples. In the SPE treated samples, peaks are well separated as interference from a protein and peptide has been removed. As indicated in the EICs, interferences have been eliminated and a cleaner spectrum and chromatogram are obtained using the SPE technique for individual HNPs in comparison to without SPE (Figure 3.2- Figure 3.10). A wide range of molecules can lead to ion suppression, especially when present in high concentrations within an extract and are eluted in the same retention window as the analytes of interest. The possible origins of ion suppression are multiple (Annesely *et al.*, 2003). Ion suppressor agents include plastic and polymer residues, proton exchange promoting agents such as organic acids, calibration products and buffers, or material released by the SPE phase (Mallet *et al.*, 2004). The SPE treated sample revealed more intense ion peaks $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$ for each individual HNP, whereas the same ions are more difficult to observe in samples that did not undergo SPE treatment (Figure 3.3, Figure 3.5, Figure 3.7 and Figure 3.9) and the

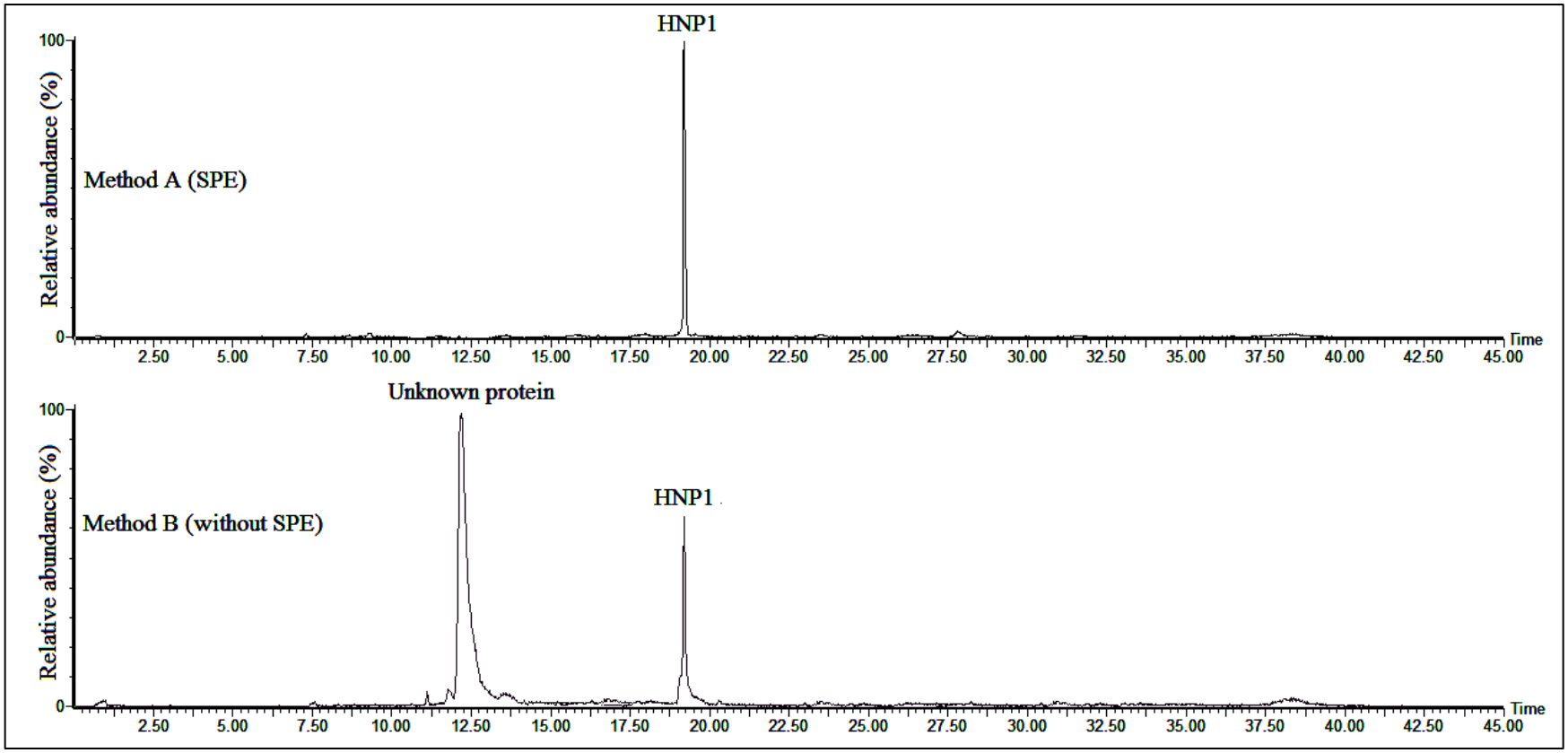


Figure 3.2 Extracted ion chromatogram of salivary HNP1. The EIC obtained for HNP1 ions corresponding to $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$. Two different sample treatments were investigated: Method A (SPE sample treatment) and Method B (without SPE sample treatment). Both methods show an interesting insight for the analysis of salivary HNP1. Method A shows a cleaner EIC compared to Method B; furthermore, an increased S/N (approximately 1 fold) was observed for HNP1 with the SPE treated sample compared to the without SPE treated sample.

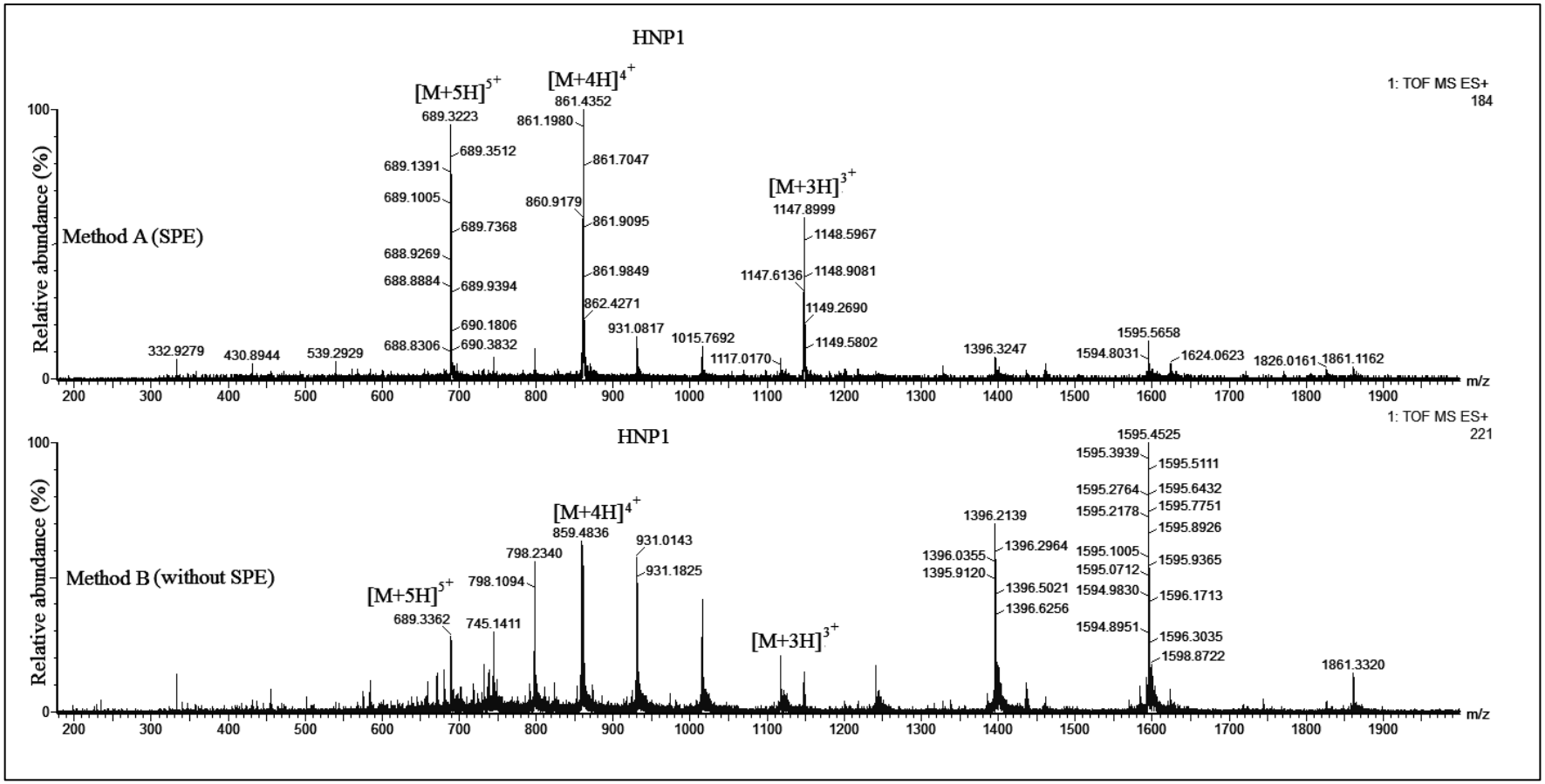


Figure 3.3 ESI-MS spectrum of salivary HNP1. ESI-MS spectrum obtained for HNP1 ions corresponding to $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$. Method A shows a cleaner ESI-MS spectrum compared to Method B. The co-elution of protein was deconvulated by Magtran and the identity of the interference was confirmed to be a proline rich protein (PRP3; MW= 11,161).

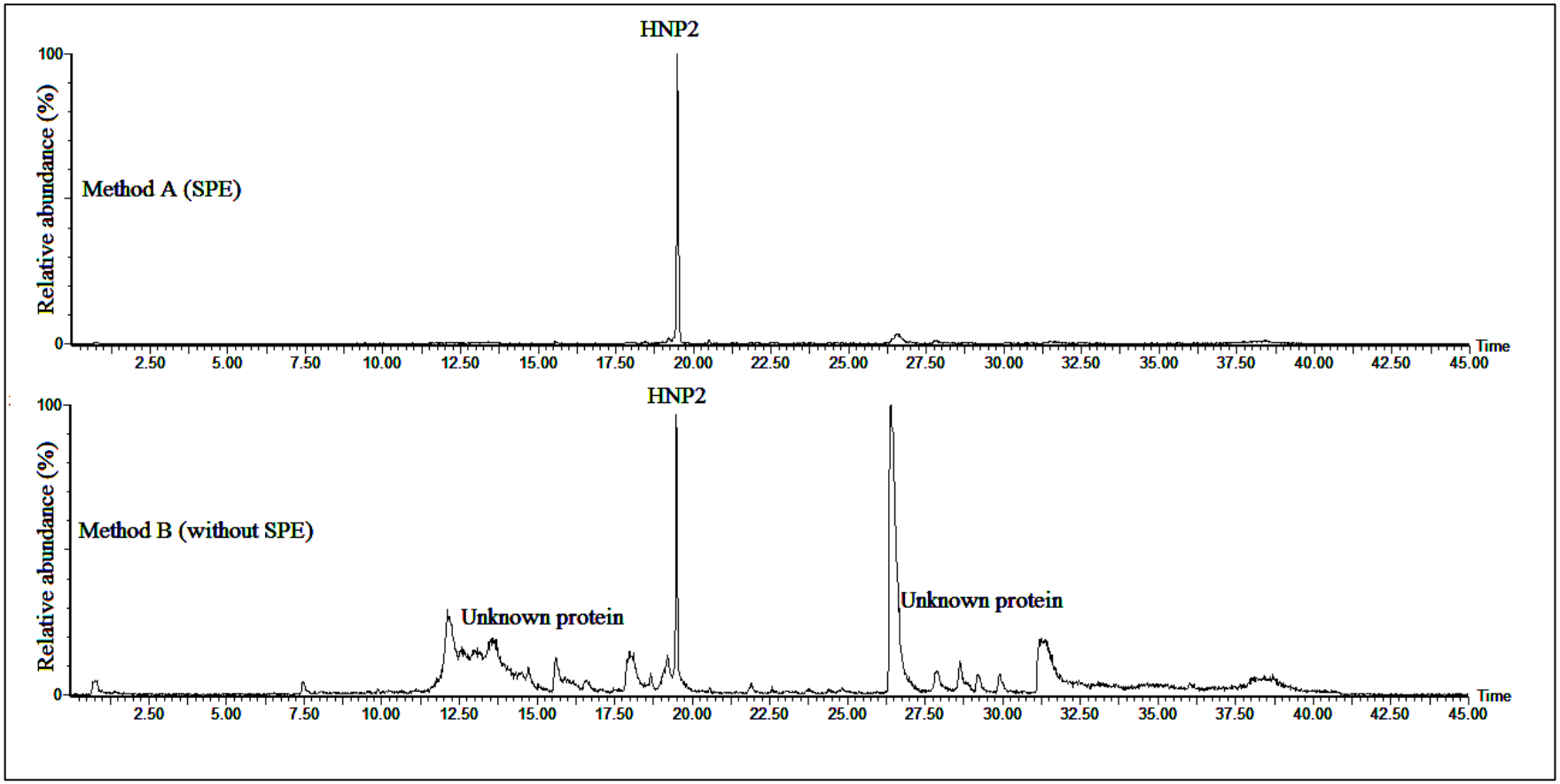


Figure 3.4 Extracted ion chromatogram of salivary HNP2. The EIC obtained for HNP 2 ions corresponding to $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$. Two different sample treatments were investigated: Method A (SPE sample treatment) and Method B (without SPE sample treatment). Both methods show an interesting insight for the analysis of salivary HNP2. Method A shows a cleaner EIC compared to Method B, furthermore, an increased S/N (approximately 9 fold) was observed for HNP2 with the SPE treated sample compared to the without SPE treated sample.

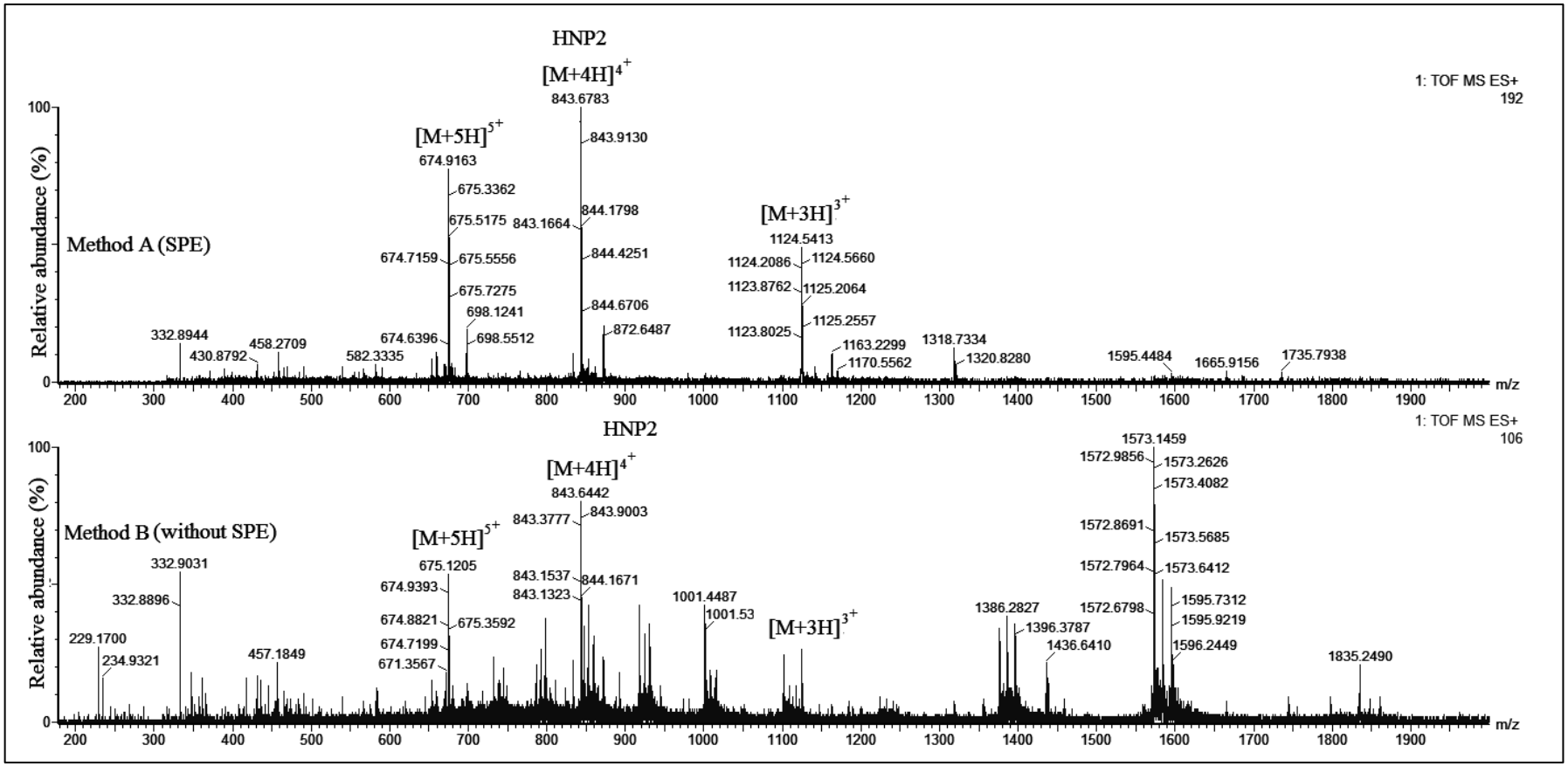


Figure 3.5 ESI-MS spectrum of salivary HNP2. ESI-MS spectrum obtained for HNP2 ions corresponding to $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$. Method A shows a cleaner ESI-MS spectrum compared to Method B. The co-elution of peptide was deconvulated by Magtran and the identity of the interference was confirmed to be a proline rich peptide (PRP3; MW= 5792).

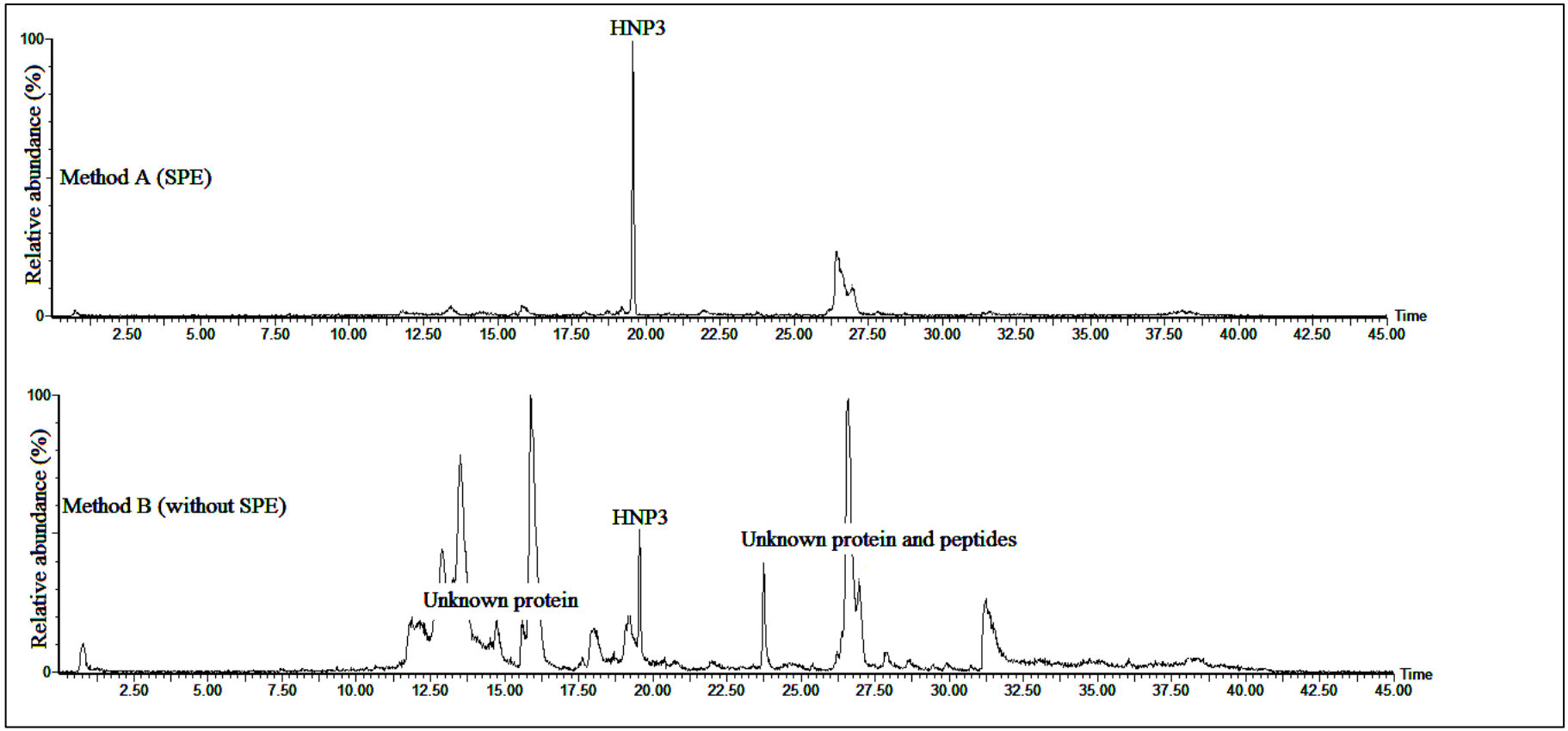


Figure 3.6 Extracted ion chromatogram of salivary HNP3. The EIC obtained for HNP3 ions corresponding to $[[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$. Two different sample treatments were investigated: Method A (SPE sample treatment) and Method B (without SPE sample treatment). Both methods show an interesting insight for the analysis of salivary HNP3. Method A shows a cleaner EIC compared to Method B; furthermore, an increased S/N (approximately 9 fold) was observed for HNP3 with the SPE treated sample compared to the without SPE treated sample.

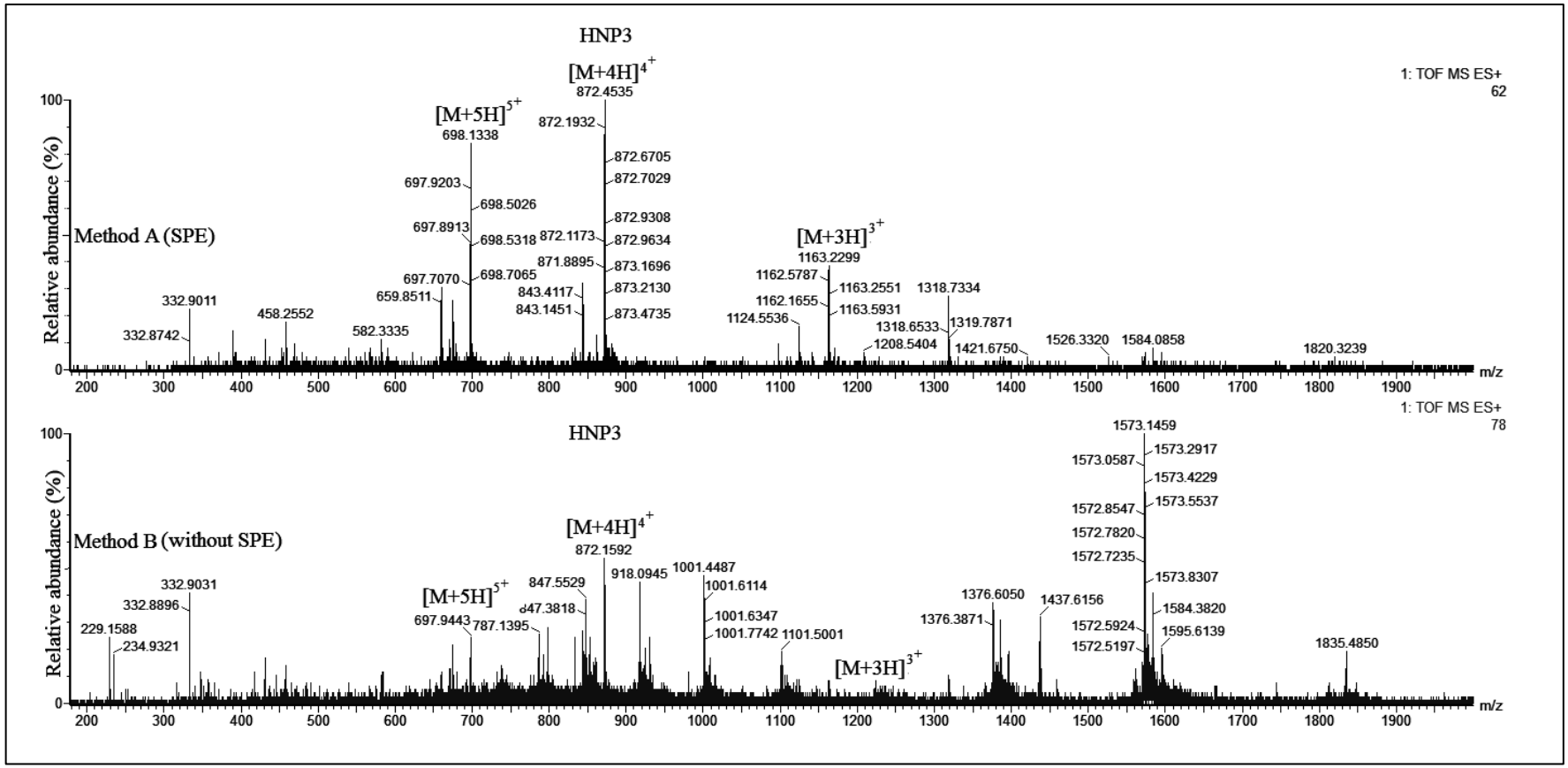


Figure 3.7 ESI-MS spectrum of salivary HNP3. ESI-MS spectrum obtained for HNP3 ions corresponding to $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$. Method A shows a cleaner ESI-Ms spectrum compared to Method B. The co-elution of peptide was deconvulated by Magtran and the identity of the interference was confirmed to be a proline rich peptide (PRP3; MW= 5792).

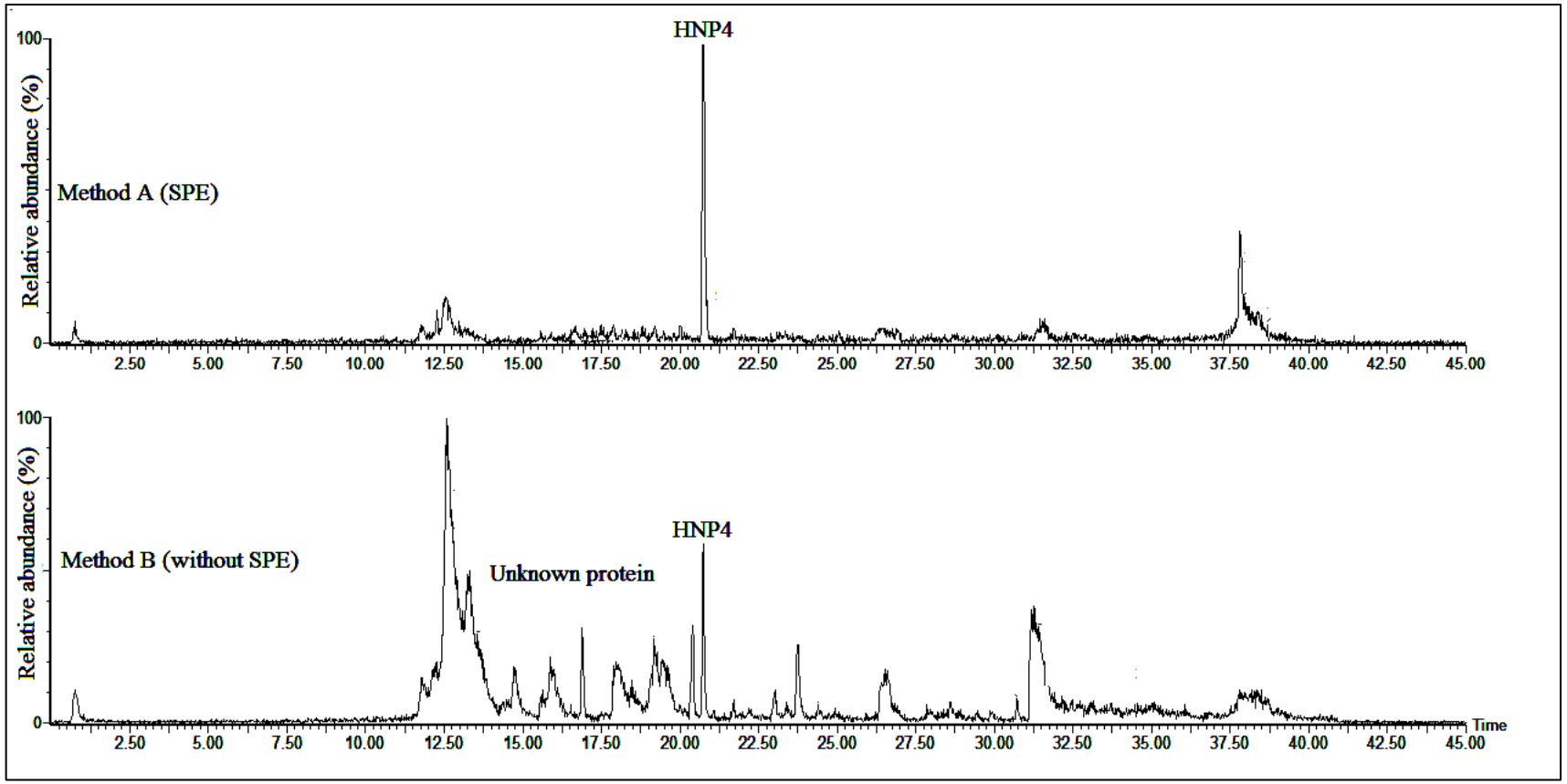


Figure 3.8 Extracted ion chromatogram of salivary HNP4. The EIC obtained for HNP4 ions corresponding to $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$. Two different sample treatments were investigated as follows: Method A (SPE sample treatment) and Method B (without SPE sample treatment). Both methods show an interesting insight for the analysis of salivary HNP4. Method A shows a cleaner EIC compared to Method B; furthermore, an increased S/N (approximately 2 fold) was observed for HNP4 with the SPE treated sample compared to the without SPE treated sample.

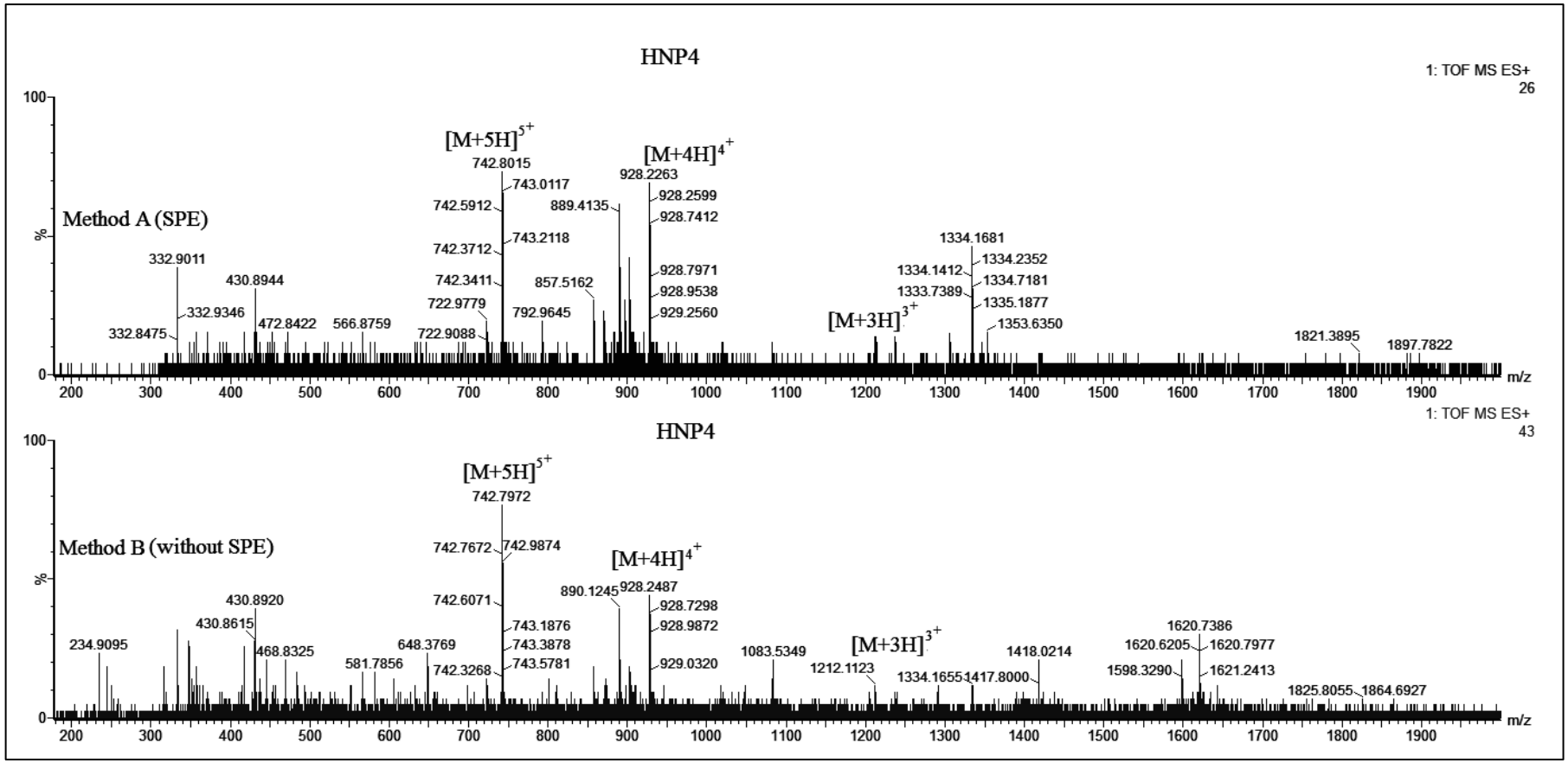


Figure 3.9 ESI-MS spectrum of salivary HNP4. ESI-MS spectrum obtained for HNP4 ions corresponding to $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$. Method A shows a cleaner ESI-MS spectrum compared to Method B. The co-elution of peptide was deconvulated by Magtran and the identity of the interference was confirmed to be an unknown peptide.

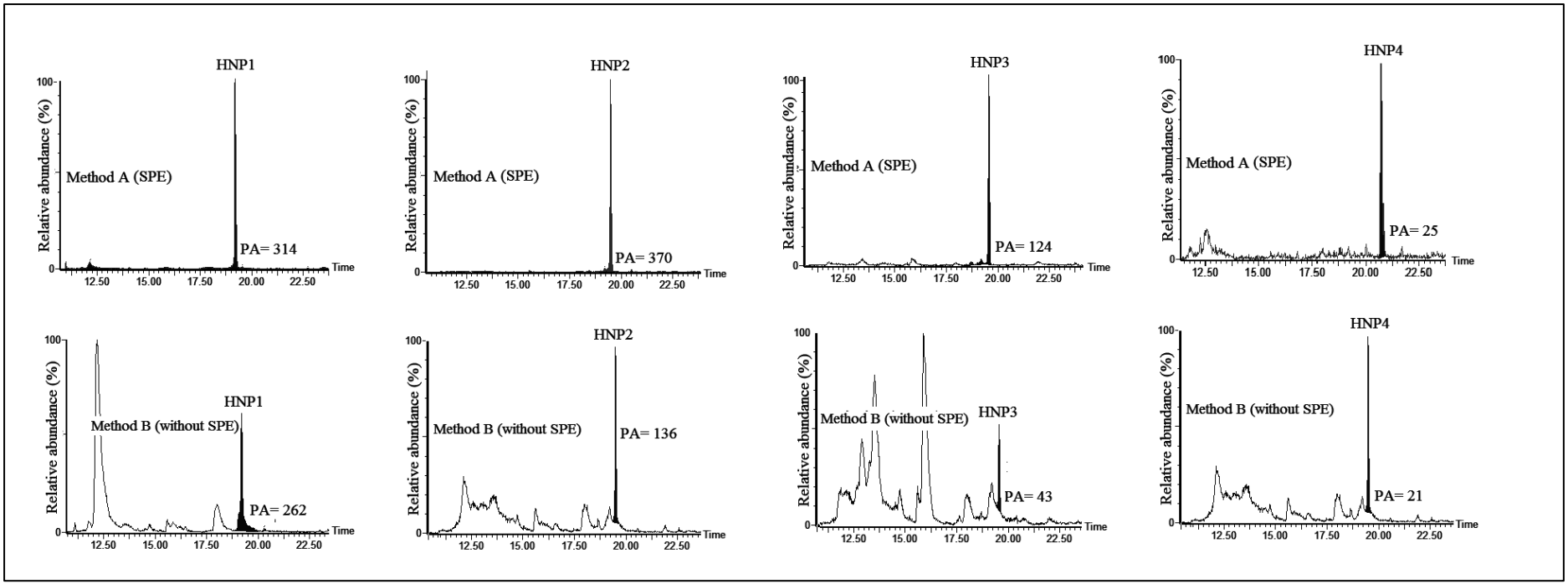


Figure 3.10 Extracted ion chromatograms obtained of individual HNP1-4 ions corresponding to $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$. Two different sample treatments were investigated: Method A (SPE sample treatment) and Method B (without SPE sample treatment). The peak area reduction between the HNPs in the SPE and without SPE treated samples ranged from 17 – 65%, which indicates the SPE clean-up was effective.

reason for this is the presence of co-eluting components. The peptide list was deconvoluted by Magtran and the identity of the interference was tentatively confirmed to be proline rich proteins (MW 11,161). The ions at m/z 1861, 1595, 1396, 1241,1117, 1015, 931, 859, 798, 745, correspond to the multiple charged ions $[M + 6H]^{6+}$, $[M + 7H]^{7+}$ and $[M + 8H]^{8+}$, $[M + 9H]^{9+}$, $[M + 10H]^{10+}$ and $[M + 11H]^{11+}$, $[M + 12H]^{12+}$, $[M + 13H]^{13+}$ and $[M + 14H]^{14+}$, $[M + 15H]^{15+}$ of PRP3; Peulso (2007) reported that α-defensins 1-3 and proline rich proteins (PRPs) co-elute. Multiple charged ions $[M + 6H]^{6+}$, $[M + 7H]^{7+}$ and $[M + 8H]^{8+}$, $[M + 9H]^{9+}$, $[M + 10H]^{10+}$ and $[M + 11H]^{11+}$, $[M + 12H]^{12+}$, $[M + 13H]^{13+}$ and $[M + 14H]^{14+}$, $[M + 15H]^{15+}$ of PRP3 are still visible in the mass spectrum, although at a much lower intensity (Figure 3.3). The structural variation between the HNPs and PRPs could cause a reduction in the recovery of PRPs from the SPE phase. The co-eluting/interfering proteins present in the HNP2-3 mass spectra (Figure 3.5 and Figure 3.7) are likely to be a proline rich proteins (P-B, MW 5792), as confirmed by Magtran, while the co-eluting species within the HNP4 spectrum (Figure 3.9) could not be identified. The peak area reduction between the HNPs in the SPE and without SPE treated samples ranged from 17-65% (Figure 3.10). This demonstrates the effectiveness of washing off the samples (SPE).

3.5.1.2 Internal Standard and Matrix Effects

The peak areas of Leu-enkephalin between a human saliva matrix and non-saliva matrix were compared with one another in order to evaluate the saliva matrix effects (Figure 3.11).

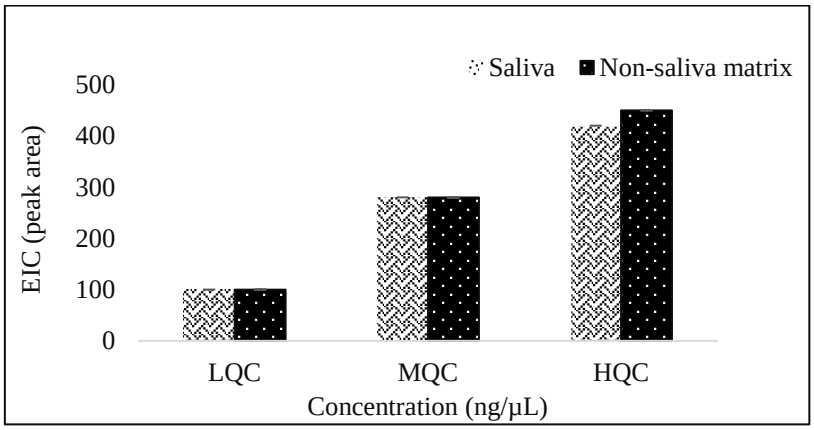


Figure 3.11 Matrix effects of Leu-enkephalin were compared with human saliva and non-saliva matrix. Three different concentrations such as low concentration quality control (LQC= 0.3 ng/uL), medium concentration quality control (MQC=0.6 ng/uL) and high concentration quality control (HQC=0.8 ng/uL) standards of Leu-enkephalin were spiked into the saliva samples prior to SPE and then analysed by LC-MS. The peak area was calculated from EIC for single charged ions corresponding to $[M + 1H]^{1+}$ for Leu-enkephalin. Bars represent ± mean std (n=3).

Three different concentrations, LQC = 0.3, MQC = 0.6 and HQC = 0.8 ng/uL, were spiked into the saliva samples prior to SPE and then analysed by LC-MS. Leu-enkephalin was chosen as the internal standard due to it being a well-known stable peptide often used for in mass spectrometry and typically having a single charged ion corresponding to the $[M + 1H]^{1+}$ charge state (Polfer *et al.*, 2007; Schnier *et al.*, 1997, Sztaray *et al.*, 2011). The endogenous internal standard, HNP2, was also investigated but due to stability concerns, further analysis of matrix effects could not proceed. The internal standard showed a different retention times (~15 min) compared to that of the HNPs. However, no matrix effects on the internal standard were observed in the saliva and non-saliva matrix samples. A paired t-test revealed that there were no significant differences in the peak area of Leu-enkephalin between the saliva matrix and non-matrix saliva samples (Figure 3.11) for LQC, MQC and HQC ($p = 0.01$).

3.5.1.3 Extraction Recovery

The sample extraction recovery of Leu-enkephalin was tested at three different concentrations (LQC: 0.3 ng/µL; MQC: 0.6 ng/µL; and HQC: 0.8 ng/µL) within the saliva matrix and the recovery data for Leu-enkephalin in the spiked saliva were 88 – 94% for LQC, MQC and HQC (Table 3.2).

Table 3.2 Recovery of Leu-enkephalin following SPE sample treatment.

Sample (n=3)	Concentration (ng/µL)	Peak area (mean ± std)	% Recovery
Spiked Leu-enkephalin in human saliva (Pre-extraction)	LQC	74 ± 4.0	-
	MQC	111 ± 0.8	-
	HQC	127 ± 0.7	-
Spiked Leu-enkephalin in human saliva (Post extraction)	LQC	82 ± 1.6	88
	MQC	118 ± 0.2	94
	HQC	136 ± 1.5	93

Three different concentrations such as low concentration quality control (LQC= 0.3 ng/µL), medium concentration quality control (MQC=0.6 ng/µL) and (high concentration quality control HQC=0.8 ng/µL) standards of Leu-enkephalin were spiked into the saliva samples prior to and after SPE and analysed by LC-MS.

There is no recommended threshold for sample recovery, and although an ideal recovery would be 100%, anything above 80% for complex biological fluids is acceptable (U.S. Department of Health *et al.*, 2001). However, the extent of recovery of the analytes and internal standard/quality control should be consistent (U.S. Department of Health *et al.*, 2001).

3.5.1.4 Sample Drying Efficiency

Sample drying and concentrating efficiency affects method reliability and reproducibility. Sample drying efficiency was investigated for individual HNPs using two methods: a vacuum centrifuge SpeedVac SPD 1010-130 (ThermoFisher, Colorado, USA) and a manual nitrogen dryer. Saliva drying efficiency was assessed by comparing the peak areas of individual HNPs (Figure 3.12). A significant difference ($p < 0.05$) was observed between the efficiency of the vacuum centrifuge SpeedVac SPD 1010-130 (ThermoFisher, Colorado, USA) and the manual nitrogen dryer.

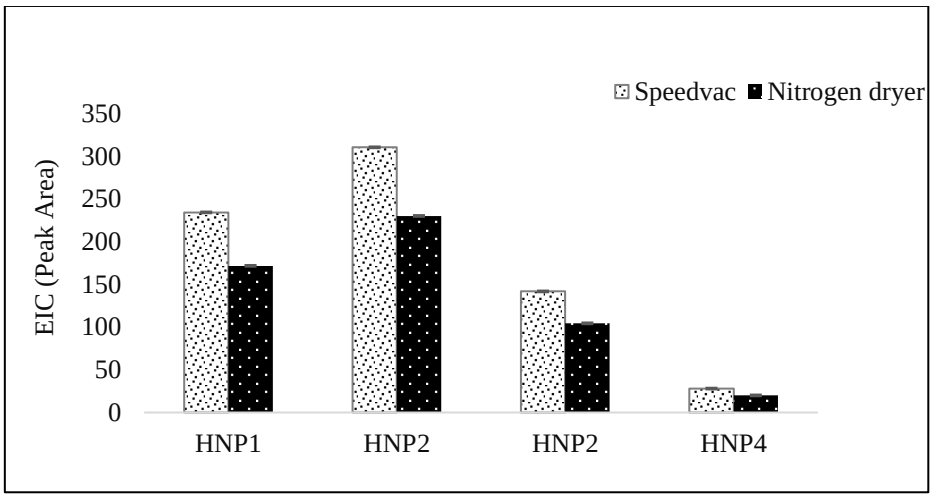


Figure 3.12 Peak area after sample drying for individual HNPs. The peak area was calculated from the EIC for individual HNP1-4 ions corresponding to $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$. Bar chart represents mean ± std (n=3).

Nitrogen evaporation is a manual procedure and thus needs to be consistently monitored. Usually, the method tends to lead to poor final dryness, as it leaves more space for human error. However, with respect to volatile solvents, relative rapidity can be observed during nitrogen evaporation.

3.5.1.5 ESI Response

SPE was conducted to separating the HNPs within the saliva matrix from other components. Different mean peak areas were obtained using eight different SPE buffer extraction solvents and ($p = 0.05$, Table 3.3).

Table 3.3 ESI response using various mobile phase solvents and additives.

Mobile phase extraction buffer	pH	HNP1 (mean ± std)	HNP2 (mean ± std)	HNP3 (mean± std)	HNP4 (mean± std)
MeOH + H_2O (50:50) + 1% FA	2.59	170 ± 5.3	132 ± 5.0	139 ± 13	62 ± 0.4
MeOH + H_2O (50:50) + 1% AC	2.88	354 ± 10	256 ± 8.0	153 ± 0.3	84 ± 3.6
MeOH + H_2O (50:50) + 0.1% AC	3.11	106 ± 4.0	107 ± 1.3	63 ± 1.4	57 ± 1.0
MeOH + H_2O (50:50) + 0.1% FA	3.01	82 ± 4.5	58 ± 1.0	24 ± 1.6	36 ± 2.5
MeCN + H_2O (50:50) + 1% FA	2.48	201 ± 1.0	173 ± 3.1	127 ± 2.4	47 ± 1.9
MeCN + H_2O (50:50) + 1% AC	2.91	302 ± 7.3	191 ± 9.8	165 ± 1.6	90 ± 3.6
MeCN + H_2O (50:50) + 0.1% AC	3.91	113 ± 1.1	94 ± 1.6	53 ± 0.1	36 ± 0.2
MeCN + H_2O (50:50) + 0.1% FA	3.56	58 ± 1.8	31 ± 2.1	21 ± 1.3	13 ± 0.6

The peak area was calculated from EIC for individual HNP 1-4 ions corresponding to $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$. The table represents mean ± std (n=3).

ESI responses can also be affected by the recovery of the analyte; thus Leu-enkephalin (0.6 ng/uL) as an internal standard was spiked into the saliva prior to and after SPE. The recovery data for Leu-enkephalin in spiked saliva showed 90–94% recovery. The peak area was noted to increase when the pH range was 2.48 to 2.91 in the mobile phase condition [MeOH + H_2O (50:50) + 1% AC; MeOH + H_2O (50:50) + 1% AC; MeCN + H_2O (50:50) + 1% FA and MeCN + H_2O (50:50) + 1% AC]. The data obtained from the investigation suggests that the pH ranges (2.48 to 2.91 and 3.01 to 3.91) differ ($p > 0.05$). The cationic characteristics of the HNP peptides could drive such differences, since they may ionise more efficiently in the range pH 2.48 to 2.91 compared to the range pH 3.01 to 3.91.

3.6 Summary

Saliva contains thousands of proteins and peptides which can cause ion suppression of HNPs during ESI analysis. Thus, optimisation of common separation methods and a comparison of the matrix effects at the time of LC-MS analysis was necessary. A cleaner mass spectrum was confirmed through comparing without SPE and with SPE. SPE appears to have removed the matrix compounds in individual HNPs and minimised ion suppression. The ESI response of individual HNPs may be affected by the mobile phase of SPE extraction, hence the final analysis used aqueous 0.1% acetic acid: methanol (50:50). The significance of washing the saliva samples through SPE before analysing individual HNPs was proven in this pilot study. This chapter demonstrates how HNP recovery can be influenced by sample drying efficiency, and this is the reason that vacuum centrifugation was implemented in the final study.

Chapter 4 - LC-MS Method Optimisation

4.1 Background

In proteomic research, ESI has become one of the most popular analytical technique (Ong *et al.,* 2005; Lill *et al.,* 2004). The selectivity and sensitivity of LC-ESI-MS analysis depends not only on the ionisation technique but also on the LC method (Snyder *et al.,* 2011; Lee *et al.,* 2005; Beck *et al.,* 2012). Reverse-phase LC is one of the most commonly used analytical techniques for biomolecules separations (Boyson *et al.,* 2001). The aim of this project was to optimise an LC-MS method for the analysis of individual HNPs present in human saliva. Chapter 1 highlighted the importance of using LC-MS compared to previous approaches using ELISA. Considering the biological importance of HNPs and the future clinical implications, it is clear that an optimised LC-MS method is needed. To accomplish this, it is important to optimise various LC and MS parameters; therefore, the focus of this chapter is on LC parameters, such as solvent composition (various mobile phase solvents with different additives and pH), gradient length, column choice and ion intensity. Furthermore, the theoretical background for LC-MS parameters (stationary phase of column, mobile phase solvents and the van Deemter curve) is discussed. In general, this chapter focuses on the most important theoretical aspects of LC-MS that are relevant to the practical experimental work.

4.2 Quantitative Approaches

Different analyte responses in MS vary due to ionisation efficiency and analyte characteristics, making quantification difficult. However, over time many quantitative proteomic approaches have been established, and the most common are known as labelling and label-free quantitation. The next subsection will explore the basic principles of the label-free and isotope labelled approaches.

4.2.1 Labelling Based Approach

The common workflow is to tag peptide containing samples with equivalent reagents, one of which includes a heavy mass tag and the other a light mass or no tag. The labelled samples are analysed by mass spectrometry after being mixed and fractionated using LC. The peaks in the mass spectra reveal the ratio of the two-different isotopic or mass tag variants. The ratio is then used to identify protein or peptide relative abundances (Figure 4.1). Over the past decade several MS labelling based quantitative proteomic strategies have been published (Thompson *et al.,* 2003; Schmidt *et al.,* 2005; Dayon *et al.,* 2008; Boersema *et al.,* 2009), with most relying

on the labelling of samples from different sources with stable isotopes (^{2}H, ^{13}C and ^{15}N) followed by quantitative analysis using a mass spectrometer. In general, stable isotope labels need to be carefully designed in order to prevent the introduction of systematic errors caused by the dissimilar behaviour of compounds with different labels. In addition, it is difficult to use the labelling based approach for proteins because of multiple charging.

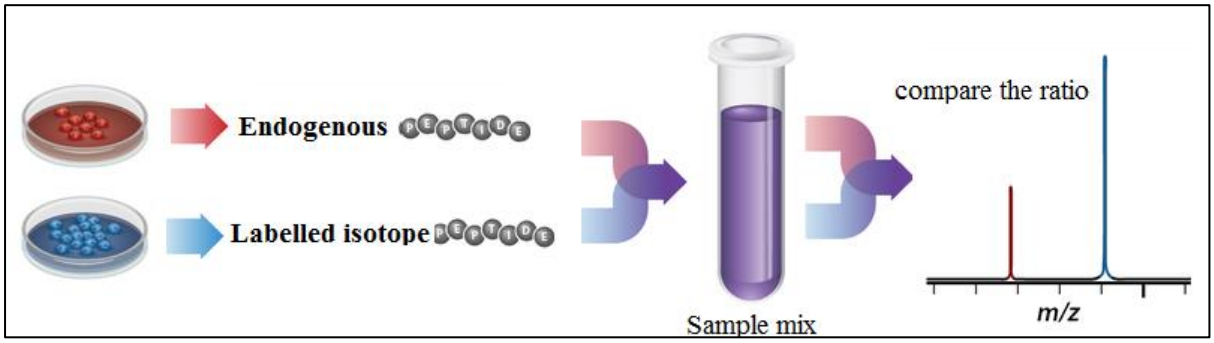


Figure 4.1 Schematic diagram of labelling based quantitation. A fixed amount of an internal standard (Labelled isotope) is added to unknown samples and the ratio of the unknown and isotope labelled samples compared (modified from Megger *et al.*, 2013).

4.2.2 Label-Free Approach

The standard addition (label-free quantitation) method involves spiking a series of unknown samples with known amounts of an analytical standard in increasing quantities. Each spiked aliquot must be extracted and processed to give final extract solutions with a fixed final volume (Figure 4.2).

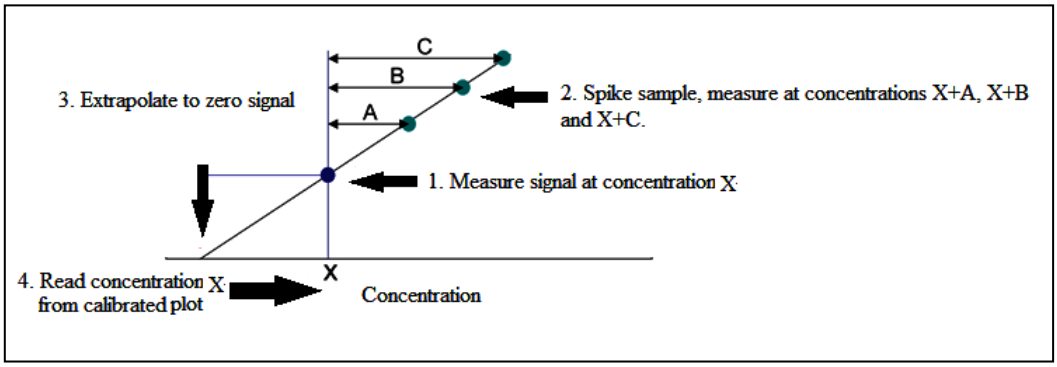


Figure 4.2 Example of standard addition quantification method. The response for each spiked sample (y-axis) is plotted against the amount of standard added to each sample (x-axis) and a best-fit line determined; the intercept should correspond to where the added amount of the standard is zero. The desired quantity (unknown) is determined from the intercept on the axis representing the amount of added sample (x-axis) (Tanner *et al.*, 2012).

The total amount of analyte measured is a combination of the spiked amount and an unknown amount, and measurement is followed by extrapolation of the calibration line to the zero response. The major limitation of this method is the large amount of sample required and the time involved (Wieling *et al.,* 2002; Boyd *et al.,* 2008). The label-free quantitative proteomics approach involves the measurement of chromatographic peak areas (also termed mass spectrometric signal intensities) of peptide precursor ions. Depending on the chromatographic method (e.g. reversed-phase LC), peptides are separated according to their particular physical properties (e.g. hydrophobicity, charge) and finally detected in a mass spectrometer.

In the acquired mass spectrum, each peptide with a particular charge and mass generates one mono-isotopic mass peak, and the intensity of this peak as a function of the retention time can be visualised in an in an extracted ion chromatogram. The area of a chromatographic peak has been shown to correlate linearly over a wide range with the protein/peptide abundance, which makes measurement feasible for quantitative studies (Bondarenko *et al.,* 2002; Megger *et al.,* 2013).

4.2.3 Semi-Quantitative Approach for HNPs

Different approaches have particular advantages and limitations; the advantage of using labelling based over label-free methods is the simultaneous measurement of different labelled samples within a single experiment, and also it provides an absolute concentration. Label-free analysis via peptide ion intensities requires a higher mass resolution mass spectrometer, as the precursor ion needs to be measured accurately. The Association of Bimolecular Resources Facilities has conducted a comprehensive study where eight known proteins were quantified in different sample mixtures. Their study demonstrated gel based approaches, as well as MS based techniques (label-free and labelling based quantitation approaches), and monitored protein ratios were close to the expected values, especially for proteins with the lowest abundance using the label-free approach (Turck *et al.,* 2007; Megger *et al.,* 2013).

In agreement with earlier studies, the label-free approach was also found to yield a higher proteome coverage, and the authors were able to show that among these techniques the label-free approach has the largest dynamic range compared to the labelled based approach (Bondarenko *et al.,* 2002; Turck *et al.,* 2007; Patel *et al.,* 2009; Tian *et al.,* 2009; Griffin *et al.,* 2010; Lundgren *et al.,* 2010; Neilson *et al.,* 2011; Collier *et al.,* 2011; Merl *et al.,* 2012). Therefore, in this study a label-free (standard addition) and data analysis (chromatographic peak area) method was chosen to compare the relative levels of individual HNPs.

4.3 van Deemter Curve

Band broadening is a phenomenon that reduces the efficiency of the separation, and leads to poor resolution and can lead to poor chromatographic performance (accuracy of quantitation). The degree of band broadening (loss of efficiency) increases depending on the type of column used and in 1956 J. J. van Deemter derived an equation that included the main factors contributing to column band broadening. van Deemter described the individual terms (Eddy diffusions = A, longitudinal diffusions = B, mass transfer = C) and also the curve, which related the plate height to the linear velocity of the mobile phase flowing through a column (van Deemter *et al.*, 1956).

4.3.1.1 Eddy Diffusion

Eddy diffusions (A term) describe the variations in the mobile phase flow or analyte flow path within a chromatography column. Multiple paths (variation in the mobile phase flow or analyte flow) arise due to inhomogeneity in column packing (Figure 4.3). Eddy diffusion can be minimised by selecting well-packed columns (smaller particle size); therefore, in this project three different particle size columns (2.5, 2.6 and 5 µm) were investigated.

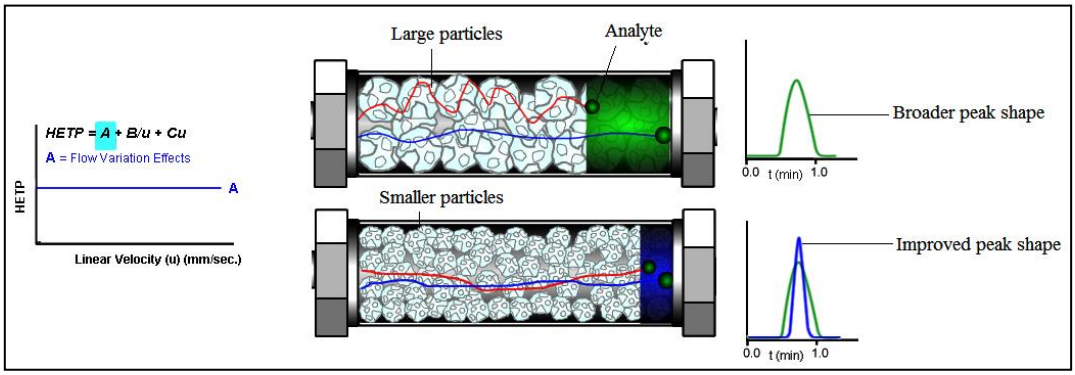


Figure 4.3 Illustration of eddy diffusion. Eddy diffusion (A term) describes the multiple path variation which arises due to inhomogeneity in column packing. A smaller particle size column provides improved peak shape compared to a larger particle size column (modified from Chromacademy 2010)

4.3.1.2 Longitudinal Diffusion

Longitudinal diffusion (B term) usually occurs when a HPLC system contains internal volumes that are larger than necessary (tubing length is too long, tubing is too wide, tubing is joined by unions using the wrong column nuts and ferrules). Usually, the longitudinal effect has a much larger effect at a low mobile phase flow rate (Figure 4.4).

Therefore, minimising the longitudinal diffusion (optimum or higher mobile phase flow rate with narrow columns) reduces the effects of the longitudinal diffusion factor.

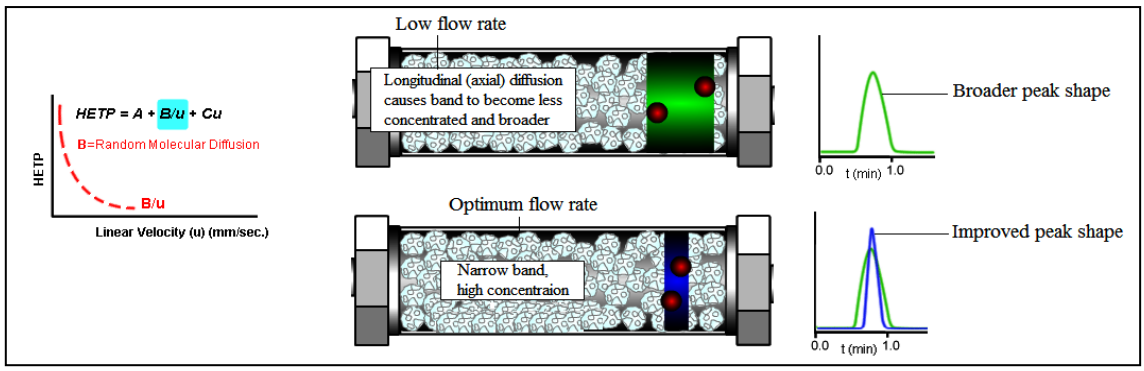


Figure 4.4 Illustration of longitudinal diffusion. Longitudinal diffusion (B term) usually occurs when a HPLC system contains internal volumes that are larger than necessary. The peak shape can be improved by using an optimal flow rate (modified from Chromacademy 2010)

4.3.1.3 Mass Transfer

Analyte residence time in the stationary phase of a column is variable and usually causes band broadening. The effects of mass transfer (C term) can be minimised by reducing the size (diameter) of the packing material. Mass transfer is also affected by the linear velocity of the mobile phase (Figure 4.5). The analysis of peptide separations are of great importance in biological research. However, the sample complexity is enormous with typically hundreds of species encountered in proteomics samples. Evidently, the chromatographer is con-fronted with an enormous separation challenge (Vanhoenacker *et al.*, 2009).

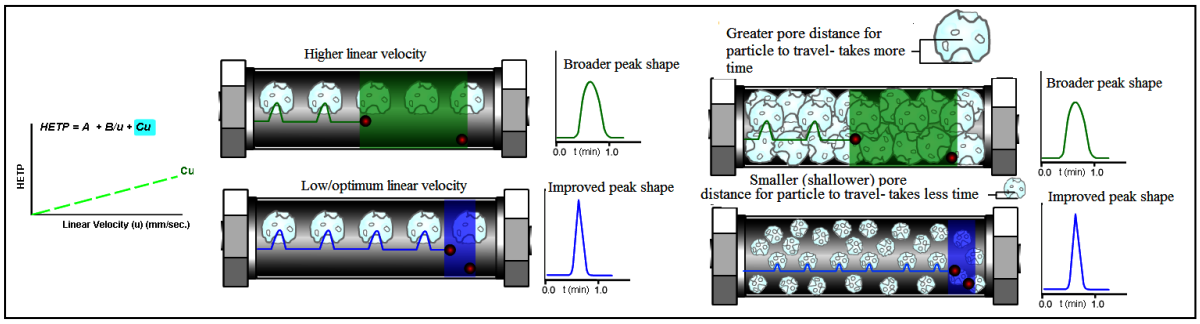


Figure 4.5 Illustration of mass transfer. Mass transfer (C Term) band broadening processes in the pore structure of the stationary phase particles. Peak shape can be improved by using a smaller particle size column and optimum flow rate (modified from Chromacademy 2010).

The separation efficiency for complex biological fluids are dependent on stationary phase and internal diameter of the column. However, for a successful operation of LC, understanding the Van-Deemter curve is of key importance to determine the LC response (Swartz *et al.*, 2005; Guillarme *et al.*, 2010). A longer column with a smaller particle size and smaller internal diameter often requires long run times and the pressure of such a column increases dramatically

(Joseph *et al.,* 2010; Petersson *et al.,* 2008). Alternatively, a short column packed with a smaller particle size and smaller internal diameter is also capable of maintaining the quality (separation efficiency) of the data (Joseph *et al.,* 2010). Based on van Deemter's theory, a smaller particle size column (e.g. 2.6 µm) was used for the optimisation of the separation efficiency of individual salivary HNPs. As a part of the optimisation process, a larger particle size (5 µm) column was also compared with the smaller (2.6 µm) column.

4.4 Chromatographic Parameters

4.4.1 Column

Reversed-phase (C18), a non-polar stationary phase has become one of the most popular columns for peptide separation (Lee *et al.,* 2005). The most common solid support for a reversed-phase column is silica endcapped with C18 alkyl chains, as it has a highly robust hydrophobic phase that produces good retention with hydrophobic (non-polar) analyte molecules.

A reversed-phase column is also suitable for polar compounds when the mobile phase contains additives. A C18 packed column is a good starting point for most chromatographic separations, since it maximises retention of moderately polar to non-polar compounds (Agilent Technologies 2012; Lee *et al.,* 2005). A core shell Phenomenex Kinetex C18 column was compared with an ACE UltraCore shell C18 column.

The manufacturing process utilises an encapsulated bonding technique and this process helps to minimise the influence of non-derivatised silanol groups. The endcapping reagent is generally a smaller silane (SiH_4), which is later used for derivatisation, and this reduces unwanted interactions between polar or charged analytes, because the number of available silanol groups has been reduced (Hichrom 2014). A core shell column utilises a sol-gel processing technique that incorporates nano-structuring technology, whereby a homogeneous porous shell is grown on a solid silica core to create a core-shell particle (Chromacademy 2010). This study is the first to investigate the separation efficiency of individual HNPs in human saliva using this type of column.

4.4.2 Flow Rate

Flow rate is a key component in van Deemter's band broadening theory. It is important to note that there is an optimum flow rate for individual analyte separation, which is the minimum point on the curve corresponding to the lowest plate height. Flow rate plays an important role

in improving the efficiency of the separation; however, some mobile phase solvents, such as MeOH, are more viscous and result in higher back-pressures, e.g. compared to MeCN.

While optimising the flow rate, the pressure limit of a LC system should be considered; in general, a column with an internal diameter (ID) of 2.0-4.6 mm, the typical flow rate is around 0.2-3 mL/min (depends on the length and particle size of the column). In this study, columns with 2.1 mm and 3.0 mm ID were investigated, where the optimum flow rate for the 2.1 mm ID column is around 0.3-0.7 mL/min and for the 3.0 mm ID column is around 0.6-1.3 mL/min (Petersson *et al.,* 2007; Joesph *et al.,* 2010; Phenomenex 2013). In addition, 0.6 mL/min was used for the 3.0 mm ID column and for method transfer 0.3mL/min was used for the 2.1 mm internal diameter column.

4.4.3 Mobile Phase Solvents and pH

The mobile phases in reversed phase HPLC usually consists of aqueous and organic solutions and commonly an organic modifier, with additives usually helping to control retention and resolution. The most common LC-MS mobile phase solvents are water, MeOH and MeCN. Chromatographically, during reversed phase HPLC water is the 'weakest' eluotropic solvent, as it is very polar and is repelled by the stationary phase more than any of the other solvents utilised. Adding organic solvents (MeOH or MeCN) and organic additives (FA, AC) helps a hydrophobic analyte to elute earlier; thus solvent modifiers and additives speed up elution and reduce retention time.

If the desired resolution cannot be achieved, or in order to improve optimisation further, it can be useful to investigate different mobile phase solvents and additives for particular analytes. In general, for peptide separation TFA as an additive is far superior to FA and AC; however, TFA is not compatible with ESI because of signal suppression from ion pairing and spray instability from surface-tension effects (Eshraghi *et al.,* 1993; Mirza *et al.,* 1994; Apffel *et al.,* 1995; Gustavsson *et al.,* 2001; McCalley *et al.,* 2004).

Consequently, a strategy to improve peptide separation would be the use of a mobile-phase modifier with a higher ionic strength at a specific pH. When a sample contains an ionisable analyte, the mobile phase pH can be the most important variable for LC-ESI-MS separation, and if the pH of the mobile phase is not properly controlled it can be a source of many problems. As highlighted in Chapter 2, basic analytes usually ionise well at a low pH where pH<pKa, while acidic analytes at ionise better at a higher pH where pH>pKa.

Therefore, ionisable compounds such as peptides are very sensitive to the mobile phase pH. Another factor that needs to be considered when optimising the mobile phase pH is the stability of the column. As a general rule, silica based columns should be operated at 2<pH<8.

Some investigations have shown that mobile phase modifiers composed of ammonium formate with FA (AFFA) provide similar separation characteristics to those obtained using mobile phases modified with TFA (McCalley *et al.,* 2003; Johnson *et al.,* 2013). It has also been suggested that AF and FA additives can improve load tolerance. FA has consistently shown high sensitivity to column overloading, especially for basic analytes (McCalley *et al.,* 2003, 2011).

The resemblance of AFFA to TFA in terms of separation characteristics makes it a potential candidate for use as a mobile-phase modifier in LC-MS, but only one investigation has been conducted to date that has evaluated the ability of AFFA to permit the efficient ionisation of peptide analytes (Johnson *et al.,* 2013).

Therefore, this study has investigated two different mobile phase solvents (MeOH, MeCN) and three different additives (FA, AC, AFFA) for the separation of individual HNPs using four columns with different stationary phases. The efficiency of the separation was assessed based on the ion intensity progile.

4.5 Materials and Methods

4.5.1 Chemicals and Reagents

The chemicals and reagents used in this study are listed in detail in Chapter 3.

4.5.2 Columns

A Kinetex® Core shell C18 and C8 column packed with 2.6 µm particles (50 x 3.0 mm) and a Kinetex® Core shell C18 column packed with 5 µm particles (50 x 2.10 mm) were obtained from Phenomenex (Torrance, CA, USA). An Ultra Core Super C18 ACE® column packed with 2.5 µm particles (50 x 3.0 mm) was obtained from Hichrom Ltd (Reading, Berkshire, UK).

4.5.3 Flow Rate

Mobile phase composition was delivered at a flow rate of 0.6 mL/min for 3 mm ID columns and 0.3 mL/min for 2.10 mm ID columns. The saliva samples were run in quadruplicate.

4.5.4 LC Mobile Phase

The composition ratios for the six different mobile phase solvents used are listed in Table 4.1

Table 4.1 Composition ratios for six different mobile phase solvents.

Mobile phase	Mobile phase solvents composition ratio	pH
H_2O+ MeCN + FA	A: 0.1% (v/v) FA in H_2O and B: 0.1% (v/v) FA in MeCN	3.56
H_2O+ MeOH + FA	A: 0.1% (v/v) FA in H_2O and B: 0.1% (v/v) FA in MeOH	3.01
H_2O+ MeCN + AC	A: 0.1% (v/v) AC in H_2O and B: 0.1% (v/v) AC in MeCN	3.91
H_2O+ MeOH + AC	A: 0.1% (v/v) AC in H_2O and B: 0.1% (v/v) AC in MeOH	3.11
H_2O+ MeCN + AFFA	A: 0.1% FA+ 10 mM AF in H_2O and B: 0.1% FA+ 10 mM AF in 80% MeCN	4.15
H_2O+ MeOH + AFFA	A: 0.1% FA+ 10 mM AF in H_2O and B: 0.1% FA+ 10 mM AF in 80% MeOH	4.39

4.5.5 Gradient

Gradient solutions were adjusted based on the use of different mobile phase solvents and additives. In general, A: aqueous solvents contain 0.1% additives (v/v) and B: organic solvents contain 0.1% additives (v/v). Eluent B was increased from 2 to 50% over 19 minutes, followed by an increase to 95% over 2 minutes. Eluent B was maintained at 95% for 3 minutes, followed by a rapid return to the initial condition of B (2%), which was maintained for 20 minutes for re-equilibration of the column. The total gradient length was 45 minutes.

4.5.6 Sample preparation

SPE cartridges from Supleco (Bellefonte, PA, USA) were used to clean up the saliva samples (details in Chapter 3).

4.5.7 Equations

Column efficiency (theoretical plates) was calculated from the EIC for individual HNPs:

$$N=5.545\left(\frac{t_R}{W\frac{1}{2}}\right)^2$$

where,

t_R = retention time of the retained compound (individual HNPs),

$W_{1/2}$ = peak width at half height.

4.6 Statistical Analysis

Statistical analysis was performed using Microsoft Excel 2013 with Excel Add-Ins, analysis tool pack, ANALYS32.XLL. The presented data were analysed using a one-way ANOVA and independent t-tests.

4.7 Results and Discussion

4.7.1 LC-MS Optimisation

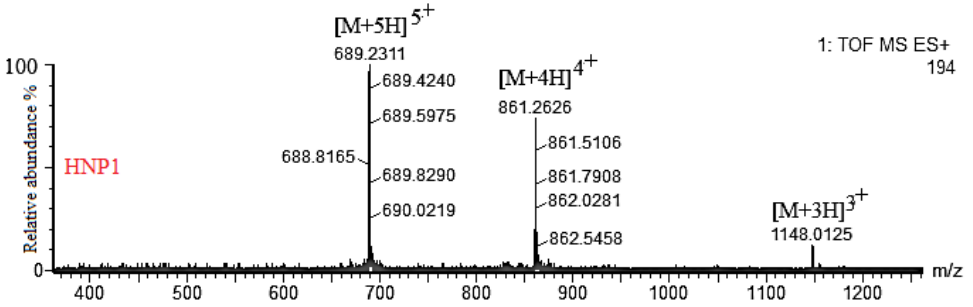
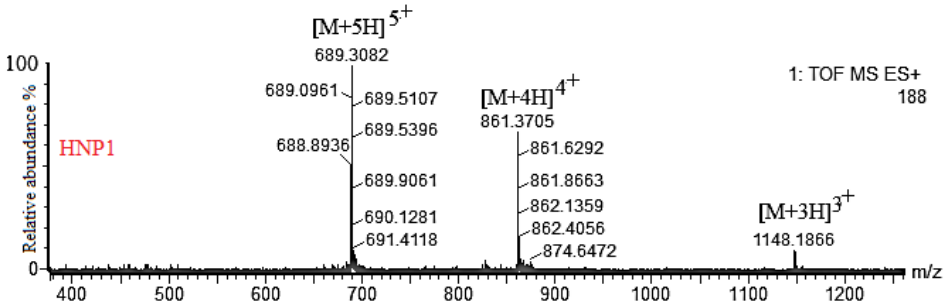
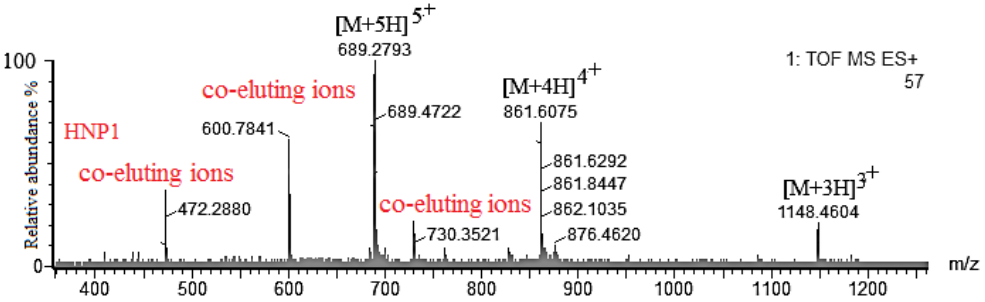
4.7.1.1 Gradient

Reversed-phase gradient HPLC enables the analysis of a mixture of analytes possessing a broad range of polarities and retention characteristics in a single run, and for many applications this offers significant benefits over isocratic methodology. A linear gradient is commonly used to separate proteins and peptides that have similar affinities for the stationary phase. A longer (70 mins), medium (45 mins) and shorter (30 mins) gradient length was investigated.

The EIC was obtained for multiple charged ions corresponding to $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$ for the analysis of HNP1. The salivary HNP1 eluted at 9.87 min with the shorter gradient, 16.13 min and 36.96 with the medium and longer gradients, respectively (data not shown). The ions of HNP1 at 1148, 861 and 689 *m/z* correspond to $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$ and isobaric ions of an unknown peptide (MW 3298.59) show an ion at *m/z* 472.23, corresponding to $[M + 7H]^{7+}$, and an unknown peptide (MW 1199.5) shows an ion at *m/z* 600.78, corresponding to $[M + 2H]^{2+}$ (Table 4.2: ESI spectrum).

There is evidence of co-eluting ions from what appears to be an unknown peptide causing interference when analysing the HNP1 with the short gradient (25 mins). However, the 45 minutes and 90 minutes' gradients, respectively, provided sufficient chromatographic resolution to separate out this interference (Table 4.2: ESI spectrum). The longer gradient was time consuming with no measurable benefit, therefore a medium gradient length was chosen as the final gradient method.

Table 4.2 Optimisation of the gradient length for the separation of HNP1. A similar trend was observed for HNP2, HNP3 and HNP4.

Gradient length	Solvent compositions		ESI-MS Profile (ion intensity)
Long (70 mins)	Time (mins)	% B	
	0	2	
	2	2	
	40	50	
	42	95	
	44	95	
	46	2	
	70	2	
Medium (45 mins)	Time (mins)	% B	
	0	2	
	2	2	
	19	50	
	21	95	
	23	95	
	25	2	
	45	2	
Short (30 mins)	Time (mins)	% B	
	0	2	
	2	2	
	16	50	
	17	95	
	18	95	
	20	2	
	30	2	

ESI mass spectrum of HNP1 in saliva separated using a long, medium and short gradient length The EIC was obtained for multiple charged ions corresponding to $[M+3H]^{3+}$, $[M+4H]^{4+}$, $[M+5H]^{5+}$ of HNP1.

4.7.1.2 Column Performance

The stationary phase and particle size can influence the performance of a column (Snyder *et al.*, 2011; Lee *et al.*, 2005; Beck *et al.*, 2012). Therefore, column performance was investigated based on different particle sizes (2.6, 5 µm) and stationary phases (C18, C8). The band-broadening model describes the relationship between the height equivalent of theoretical plate (HETP) and linear velocity, which is partly dependent on the diameter of the particle packed into the analytical column. Smaller particle diameters can significantly reduce the HETP, which results in higher efficiency and a flatter profile of the van Deemter curve (van Deemter *et al.*, 1956). The particle size of the stationary phase plays an important role when selecting a stationary phase. Therefore, the Phenomenex Kinetex® C18 column with smaller particles (2.6 µm) and large particles (5µm), and different stationary phases (Phenomenex Kinetex® C18 versus Kinetex® C8) were compared for individual salivary HNPs (Figure 4.6).

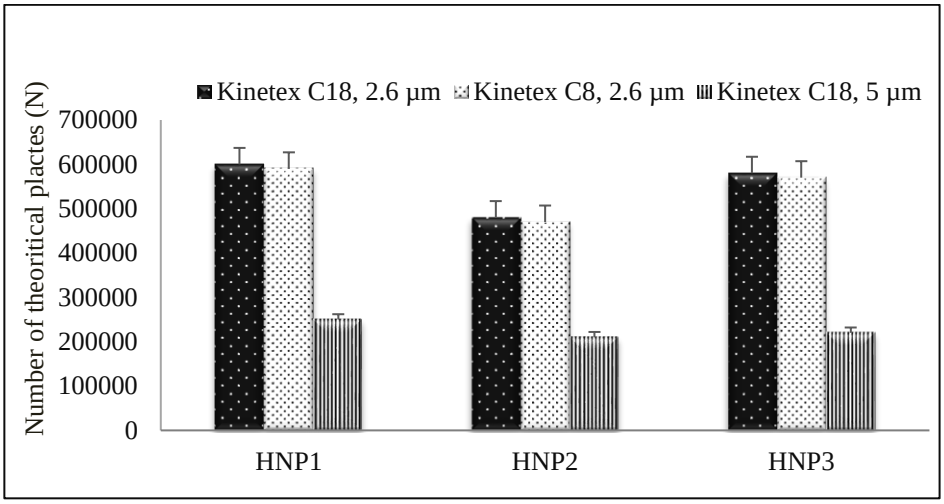


Figure 4.6 Bar chart represents the response of HNPs using different particle sizes (2.6 versus 5 µm) and stationary phases (C18 versus C8) column. The initial mobile phase solvents (A: 0.1% (v/v) FA in H_2O and B: 0.1% (v/v) FA in MeCN) were used. (i) At a constant flow rate (0.6 mL/min), Kinetex® 2.6 µm column efficiency (theoretical plates) was higher than the Kinetex 5 µm column. At a flow rate (0.6 mL/min), the Kinetex® C18 showed similar efficiencies (theoretical plates) compared to C8. The theoretical plates were calculated from EICs for multiple charged ions corresponding to $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$ for the analysis of salivary HNPs.

Columns with higher theoretical plate numbers are considered to be more efficient; therefore, column separation efficiency (number of theoretical plates) was calculated from EIC for salivary HNPs. EICs were obtained for multiple charged ions corresponding to $[M+3H]^{3+}$, $[M+4H]^{4+}$, $[M+5H]^{5+}$ for the analysis of salivary HNPs. A significance difference was observed ($p=0.05$) between the Kinetex® C18 2.6 µm and 5 µm columns. The Kinetex® 2.6 µm column resulted

in a higher separation efficiency (number of theoretical plates) compared to the Kinetex® 5 µm column (Figure 4.6). However, no significant difference was observed (p=0.10) between the stationary phases C18 versus C8. The column performance for particle size (2.6 vs 5 µm) and stationary phase (C18 vs C8) was investigated using mobile phase solvents and additives (A: 0.1% (v/v) FA in H_2O and B: 0.1% (v/v) FA in MeCN).

However, further experiments were required using various mobile phase solvents and additives to compare column performance and the column performance investigation was expanded. The column performance for three different core shell Kinetex® C18 (50 x 3mm, 2.6 µm), Kinetex® C18 (50 x 3mm, 5 µm), and Kinetex® C8 (50 x 3mm, 2.6 µm) and an ultra-core shell ACE® C18 (50 x 3mm, 2.5 µm), using six different mobile solvents with three different additives, was assessed. This was the first study to investigate column performance between the Kinetex® core shell and the ACE Ultra-core shell using six different mobile phase solvents and three different additives for the analysis of individual salivary HNPs. The core shell and ultra-core shell column performance using six different mobile phases and three different additives is discussed in the following sections.

4.7.1.3 Core Shell versus Ultra-Core Shell

When developing a method for the separation of complex mixtures (saliva, blood, serum), it is best to try several different hydrophobic phases to determine which has the best selectivity for that particular mixture of peptides. C18 columns are generally preferred for peptides and small proteins less than about 5,000 Da, and the smallest and most hydrophilic peptides are often best separated on small pore C18 columns. Proteins larger than 5,000 Da or small polypeptides that are particularly hydrophobic are best separated on C4 or C8 columns.

C8 columns are similar to C18 columns in their application but sometimes offer a different selectivity or ability to separate particular peptides (Pearson *et al.,* 1982; Garnick *et al.,* 1988). Core shells composed of Kinetex® C18 (50 x 3mm, 2.6 µm), Kinetex® C18 (50 x 3mm, 5 µm), Kinetex® C8 (50 x 3mm, 2.6 µm) and ultra-core shell ACE® C18 (50 x 3mm, 2.5 µm) were investigated for the separation of individual HNPs. The residence time of HNP1 was 0.81 min longer for Kinetex® C18 (50 x 3mm, 5 µm) and 0.2 min longer in Kinetex® C18 (50 x 3mm, 2.6 µm) column compared to the Kinetex® C8 column and as a result peak broadening was observed for the mobile phase [A: 0.1% (v/v) FA in H_2O and B: 0.1% (v/v) FA in MeCN] (Figure 4.7).

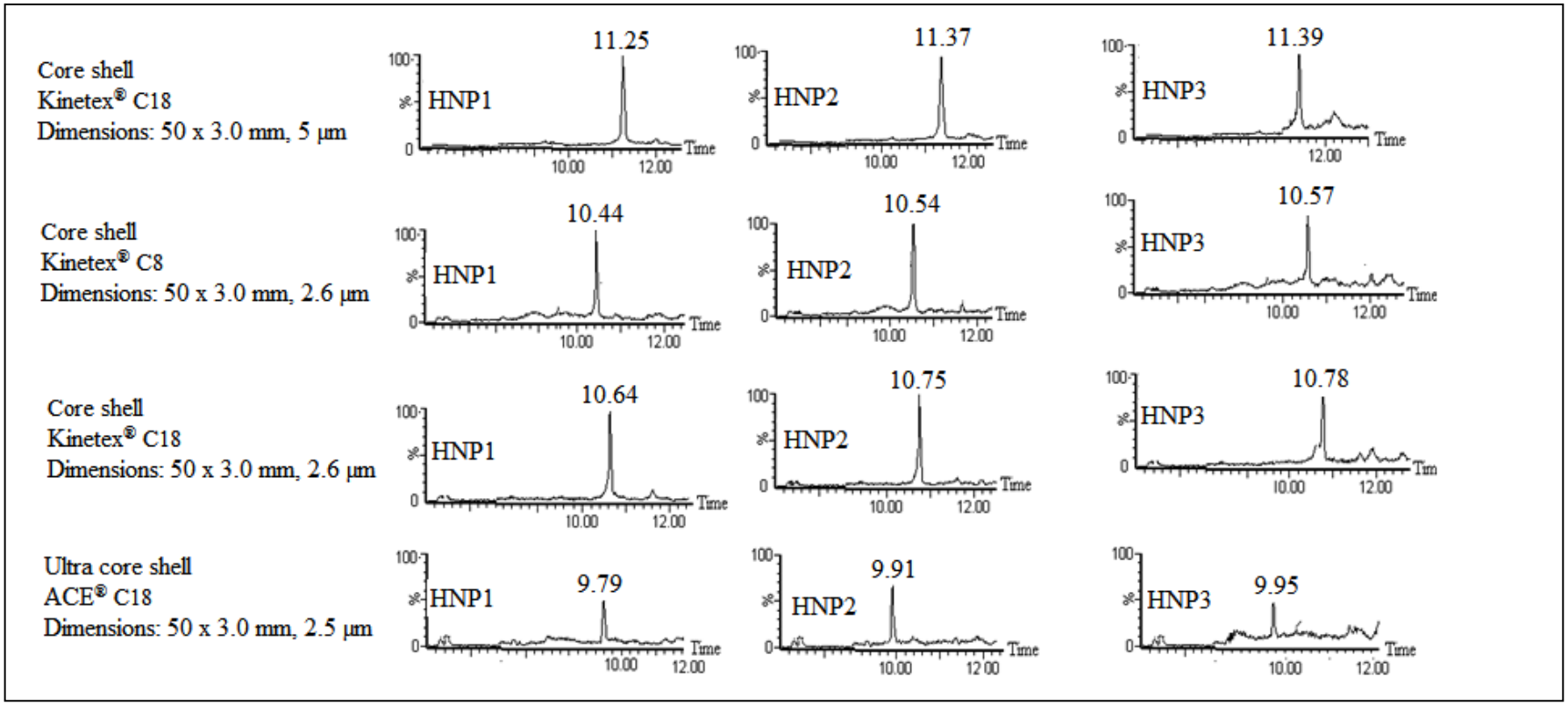


Figure 4.7 The separation of individual HNPs in saliva using different stationary phase columns. Extracted ion current chromatogram of HNPs was obtained by multiply protonated charge state of $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$.

However, the residence time of HNP1 was 0.65 min longer for Kinetex® C8 (50 x 3mm, 2.6 µm) compared to the ACE® C18 (50 x 3mm, 2.5 µm) column, but the peak height was half than that for HNP1 for the ACE® C18 column compared to the Kinetex C8 column. The findings suggested that the ACE® C18 column provides poor separation compared to the Kinetex® C8 column. A similar trend was also observed for HNP2 and HNP3 and for six different mobile phase compositions.

4.7.1.4 ESI-MS Response

Human saliva is a complex mixture due to the number of proteins and peptides present in the saliva, making this sample more challenging. Longer run times are often required for 5 µm particle size columns whereas a 2.6 µm particle size column improves resolution and sensitivity. Kinetex® core shell columns have an advantage over porous silica columns in that the column back pressure is lower, allowing higher flow rates and enabling faster column equilibration and hence faster analysis times (Preti *et al.,* 2016).

To further test the column performance with various mobile solvents, the ESI-MS response (ion intensity) was measured. ESI-MS signal intensities depend on multiple factors, such as mobile phase pH and organic percentage, LC separation, ESI source parameters, and type and concentration of the mobile phase. The solution and gas phase ions are completely different environments, and the ions observed in the gas phase may not directly correspond to those present in the solution. For practicing mass septrometrists, understanding the gas phase ions represents an important and interesting challenge. Sometimes, a combination of solution and gas phase ion chemistry provides vital information leading to an understanding of the observed mass spectra. As the gas phase is a very different environment compared to the liquid phase provided by a polar solvent, many ions can become modified when entering the gas phase, and this is particularly the case for protonated bases (peptides). Since the basicity order in solution is often different from that in the gas phase, proton transfer to the stronger gas phase base can occur on formation of the gas phase ions (Tang and Kebrala *et al.,* 1993; Wilm *et al.,* 2011; Litlle *et al.,* 2006). The ESI-MS responses (ion intensity) were calculated for individual HNPs corresponding to $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$ from the EICs (Figure 4.8, Figure 4.9 and Figure 4.10). An increased ion intensity ($p = 0.05$) was observed for HNP1 for the Kinetex® C8, 2.6 µm column using a mobile phase of 0.1% (v/v) AC in H_2O and B: 0.1% (v/v) AC in MeOH, out of six different mobile phases (Figure 4.8). A similar trend was also observed for the salivary HNP2 and HNP3 (Figure 4.9 and Figure 4.10).

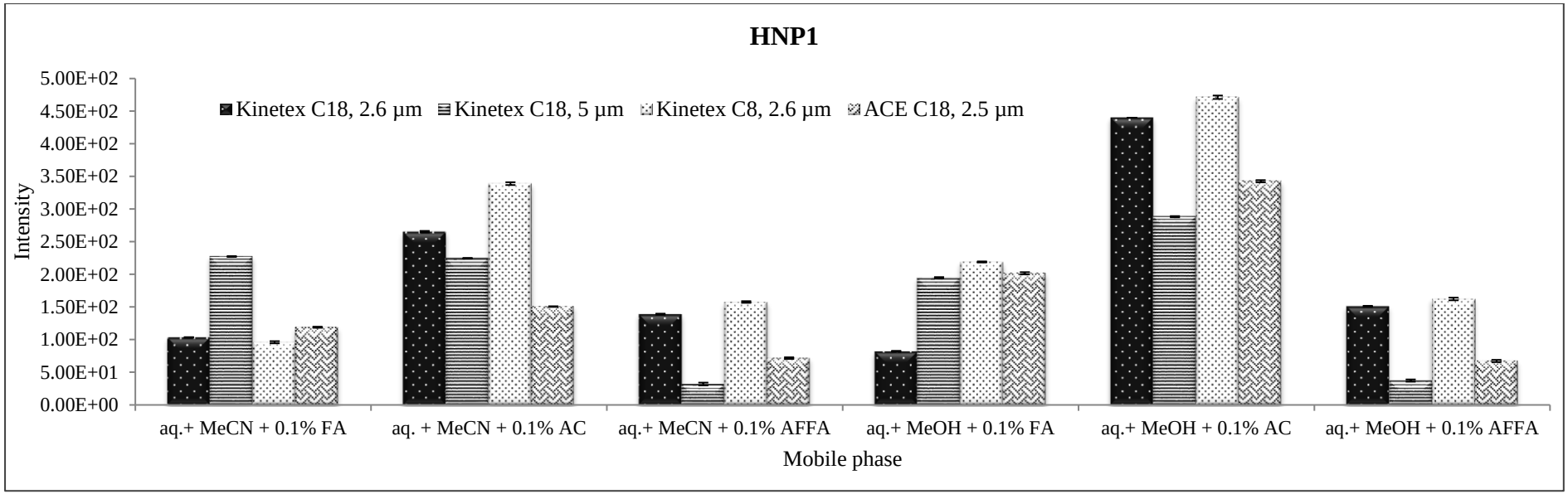


Figure 4.8 ESI response of HNP1 was investigated using six different LC mobile phase solvents. The six different mobile phase solvents contained three different additives (0.1% v/v) with three different particle sizes (2.6, 2.5 and 5 µm) and two different stationary phase (C18 versus C8) columns. The bars represent mean ± std (n= 3).

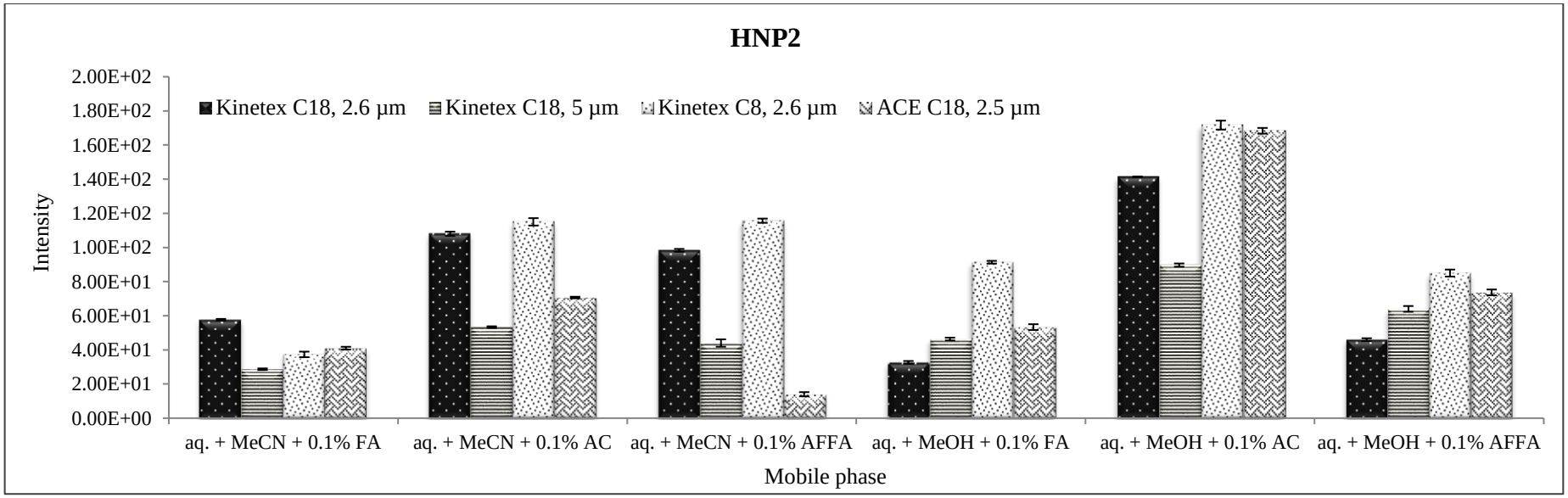


Figure 4.9 ESI response of HNP2 was investigated using six different LC mobile phase solvents. The six different mobile phase solvents contained three different additives (0.1% v/v) with three different particle sizes (2.6, 2.5 and 5 µm) and two different stationary phase (C18 versus C8) columns. The bars represent mean ± std (n= 3).

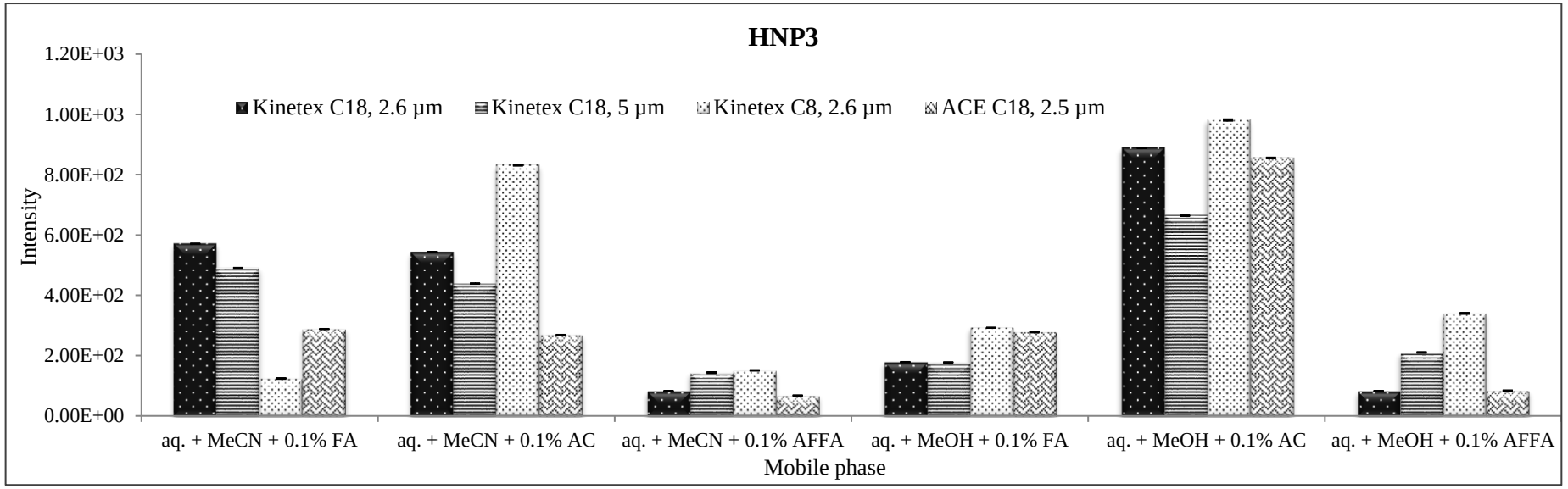


Figure 4.10 ESI response of HNP3 was investigated using six different LC mobile phase solvents. The six different mobile phase solvents contained three different additives (0.1% v/v) with three different particle sizes (2.6, 2.5 and 5 µm) and two different stationary phase (C18 versus C8) columns. The bars represent mean ± std (n= 3).

The mobile phase MeOH resulted in higher ESI ion intensities compared to ionisation in MeCN, which is likely attributable to a more rapid desolvation of the ESI droplets (MeCN bp. ≈ 82 °C, MeOH bp. ≈ 65 °C). The impact of AC, FA and AFFA on the evaluation of signal intensity was also examined for individual HNPs in the saliva matrix. The addition of AFFA and FA increases the ionic strength of the mobile phases, which should increase ion pairing, and thus may lead to ESI signal suppression. It has been suggested that an increase in ionic strength of the AFFA mobile phase has the potential to increase ion pairing, which in turn, could reduce the peptide-charge state and lead to a decrease in signal intensity (Johnson *et al.*, 2013). For basic analytes, FA has traditionally been reported as a poor mobile phase modifier compared to AC (Apffel *et al.*, 1995; Gustavsson *et al.*, 2001). Regardless, for any dimensions of the column, the mobile phase composition of 0.1% (v/v) AC in H_2O and B: 0.1% (v/v) AC in MeOH, provided the highest ion intensity for individual HNP 1-3 (Figure 4.8, Figure 4.9 and Figure 4.10). One of the other possible reasons for the increase in ion intensity could be related to pH. In general, if the pH is near the pKa, smaller changes in pH could make large changes in the retention, which could affect the chromatographic resolution. For proteins and peptides, the pKa value is normally predicted from the charge state of the ion, consequently it is difficult to know the exact pKa of a peptide. The change in pH (4.15 - 3.01 = 1.38 units) affected the ion intensity of the Kinetex® C18, 2.6 µm column for six different mobile phases for HNP1, and similar trends were observed for HNP2 and HNP3 (Figure 4.8, Figure 4.9 and Figure 4.10). The reason for an increase in the ion intensity could also be related to the cationic characteristics of the HNP peptides, in that they separate more efficiently in the pH range of 3.00 - 3.56, compared to the pH range of 3.60 - 4.39.

4.8 Summary

This chapter has highlighted the importance of the mobile phase solvent and choice of column for chromatographic separation and ESI response. A medium gradient length was chosen rather than a shorter gradient to ensure better separation. A Phenomenex Kinetex C8 column with smaller particle size packing was the final choice of column, as it provides higher efficiency (theoretical plates) compared to the performance of the other columns. The mobile phase solvent MeOH with AC resulted in higher ESI ion intensities compared to MeCN with AC..

Chapter 5 - Method Validation

5.1 Background

Antimicrobial peptides (AMPs) present in saliva are key components of the innate immune system that functions as a primary defence against infection. Defensins (HNP1-4) are part of the mucosal immune system and have a broad spectrum of antibacterial and antifungal activity (Chapter 1). Several studies have reported increased levels of HNPs in biological fluids (saliva, plasma, and serum) during colorectal cancer patients (Melle *et al.,* 2005; Albrethsen *et al.,* 2005; Albrethsen *et al.,* 2006), breasts cancers (Li *et al.,* 2005), bladder cancer (Gunes *et al.,* 2013), pulmonary disease (Paone *et al.,* 2011) and sepsis (Panyutich *et al.,* 1993) measured by ELISA. However, the relationship between exercise and the salivary levels of defensins have not yet been explored. Therefore, the quantitation of individual HNP levels during exercise could deepen our understanding of mucosal immunity. A reliable analytical method which is capable of detecting α-defensins (HNP1-4) within a single assay would be a valuable diagnostic and prognostic tool for the assessment of exercise induced inflammation. Here, the validation of an LC-MS method that allows simultaneous semi-quantification of four individual HNPs (HNP1, HNP2, HNP3 and HNP4) in human saliva is presented. This validated method has provided an opportunity to assess individual HNP levels in athletes' saliva, in order to understand their response to exercise. A cross-correlation of the data with the commonly used ELISA is also included.

The main objective of any analytical technique is to obtain consistent, reliable and accurate data, and method validation is used to evaluate the quality, reliability and consistency of the analytical results. Goebel (2000) presented a LC-ESI-MS method for the detection of salivary HNP1 and HNP2 and the limit of detection (LOD) was estimated to be around 100 ng/µL but no other validation parameters (stability, precision, and extraction recovery and matrix effects) were reported, while Van den Broek *et al.* (2010) presented the validation of a LC-ESI-MS method for the analysis of individual α-defensins (HNP1-3) in plasma and serum. This chapter describes the method validation guidelines, performance characteristics (linearity, precision, accuracy, recovery, stability), and the validation of the LC-MS method for the detection of individual HNPs in human saliva.

Table 5.1 Method validation guidelines (ICH 1996).

Validation criteria	Validation requirement	Acceptable range
Specificity	Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. These might typically include impurities, degradants, and matrix effects. Besides chromatographic separation, the sample preparation step can also be optimised for best specificity.	For qualitative/qualitative analysis.
Linearity	Linearity is the ability of a method to obtain results which are within a given range, to obtain results which are directly proportional to the amount of analyte. This is established by calculating the regression line of the results and comparing it with analyte concentration.	Regression line R2 value >0.95 is expected for a method to be classed as linear.
LOD	The limit of detection of an individual analytical procedure as the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated as an exact value.	S/N 3:1.
LOQ	The lowest amount of analyte in a sample which can be quantitatively determined with accuracy.	S/N 10:1.
Precision Intraday Interday	The precision of an analytical procedure is usually expressed as the variance standard deviation or coefficient of variation of a series of measurements. Intraday expresses the precision under the same operating conditions over a short interval of time. Interday within-laboratories variations: different days, different analysts, different equipment, etc.	The coefficient of variation should be better than 20% at lower concentration and better than 15% at higher concentration.
Accuracy	Expresses the closeness of agreement between the test results and the value which is accepted as the true value. It indicates the deviation between the mean value and the true value. It should be presented as percent recovery of a known added amount of analyte in the sample.	All results should fall within ±20% of the expected value at the lower concentrations and within ±15% at higher concentrations.

5.2 Method Validation Guidelines

The validation characteristics (Table 5.1), according to the ICH, are accuracy, precision (interday and interday), specificity, LOD, limit of quantitation (LOQ), linearity and stability. The validated method has been applied in the pilot study.

5.3 Materials and Methods

5.3.1 Participants

Five male and five female student athletes (age: 25 ± 2.7 years; height: 1.88 ± 0.7 m; body weight: 86 ± 13 kg) volunteered to participate in the pilot study, and nineteen males and four female athletes (age: 23 ± 2.39 years; height: 1.7 ± 0.1 m; body weight: 76 ±11 kg) participated in the cross-validation study. Participants were recruited from the sports science group at the University of Greenwich, Medway. Ethical approval was obtained from the University of Greenwich Ethics Committee.

5.3.2 Instrumentation

The experimental instrument conditions for MS and LC (mobile phase and column) were listed in Chapter 3.

5.3.3 Sample Preparation

5.3.3.1 Analysis of individual HNPs by LC-MS

SPE cartridges from Supleco (Bellefonte, PA, USA) were used to clean up the saliva samples (details in Chapter 3).

5.3.3.2 Analysis of HNP1-3 by ELISA

The saliva samples were centrifuged (12,000 *g*, 10 mins; 4°C) and the supernatant diluted (1000×) in dilution buffer. Each sample was analysed in duplicate by ELISA (Hycult Biotech Inc, Netherlands) following to the manufacturer's instructions. A calibration curve was constructed consisting of eight prepared standards; 0.00015 to 0.01 ng/µL HNP1-3. Absorbance (450 nm) values for the saliva samples were interpolated from the calibration standards using a four-parameter logistic curve (My Assays, Version 2015).

5.3.4 LC-MS Validation

5.3.4.1 Linearity

The HNP2 standards (0.05, 0.1, 0.3, 0.6, 0.8 and 1 ng/µL) were prepared in HPLC grade water and reconstituted with the mobile phase [(MeOH + H_2O (50:50), both with 0.1% acetic acid

v/v]. The standards were analysed with LC-MS in triplicates and the signal-to-noise ratio (S/N) was plotted against concentration.

5.3.4.2 Precision

The intermediate precision (intra and interday) was investigated by spiking HNP2 into saliva post-SPE. The HNP2 standard was added at three different concentrations (0.3 ng/µL, 0.6 ng/µL and 0.8 ng/µL) and the level analysed in triplicate (n=3). The endogenous concentrations of HNP1, HNP3 and HNP4 were also assessed in these saliva samples. The samples were left at room temperature and re-analysed after 24 hours. The reproducibility was calculated from the EIC (peak area) and expressed as a coefficient of variation (CV%).

5.3.4.3 Recovery

Extraction recovery was determined by spiking HNP2 and leu-enkaphalin into saliva at three different concentrations (HNP2: LQC: 0.3 ng/µL, MQC: 0.6 ng/µL, and HQC: 0.8 ng/µL; leu-enkaphalin: LQC: 0.3 ng/µL, MQC: 0.6 ng/µL, and HQC: 0.8 ng/µL). Both peptides were dissolved in [(MeOH + H_2O (50:50)]. The endogenous concentrations of HNP1, HNP3 and HNP4 were assessed by LC-MS (n=4), and the sample recovery was calculated from the EIC (peak area) and expressed as a CV%. Extraction recovery calculation for Leu-enkephalin can be found in chapter 3 and the extraction recovery was calculated for HNP2 using the equation below:

$$\text{HNP2} = \frac{\text{(EIC peak area of endogenous HNP2)} - \text{(EIC peak area of spiked HNP2 into saliva before SPE)}}{\text{(EIC peak area of spiked HNP2 into saliva after SPE)}} \text{ X } 100$$

5.3.4.4 Stability

The stability of the standard (HNP2) in saliva was investigated after one month of storage. Each saliva sample was split into two, and HNP2 was spiked into the saliva (post-SPE) at three different concentrations (0.3 ng/µL, 0.6 ng/µL and: 0.8 ng/µL). The relative levels of HNP1, HNP3 and HNP4 were also assessed in these saliva samples. Following SPE, one of the sample were reconstituted in mobile phase and analysed by LC-MS (n=3) and one stored as a lyophilised powder for one month (-80°C). The stability was calculated from EIC (peak area) and expressed as a CV%. The stability of the standard leu-enkaphalin in saliva was also assessed and compared with similar levels in a freshly prepared non-saliva matrix.

5.4 Statistical Analysis

Statistical analysis was performed using Microsoft Excel 2013 with Excel Add-Ins, analysis tool pack, ANALYS32.XLL. The data is presented as the mean % increase of HNP1-3 for post-

thirty and post-sixty mins after exercise. The percentage increases were plotted against each other to assess the correlation between the ELISA and LC-MS methods.

5.5 Results and Discussion

5.5.1 LC-MS Validation

5.5.1.1 Linearity

The standard (HNP2) gave a linear response (EIC peak area vs concentration) in the range of 0.05 to 1 ng/µL (R^2 = 0.99). The mean (n=3) were 10 ± 6.7; 20 ± 0.1; 34 ± 0.1; 80 ± 1.6; 108± 3.1; 140 ± 3.2 (0.05, 0.1, 0.3, 0.6, 0.8, 1 ng/µL, respectively). The S/N ratio was used to establish the LOD and LOQ for HNP2 (Figure 5.1). The LOQ was 0.1 ng/µL and the LOD was determined to be 0.05 ng/µL.

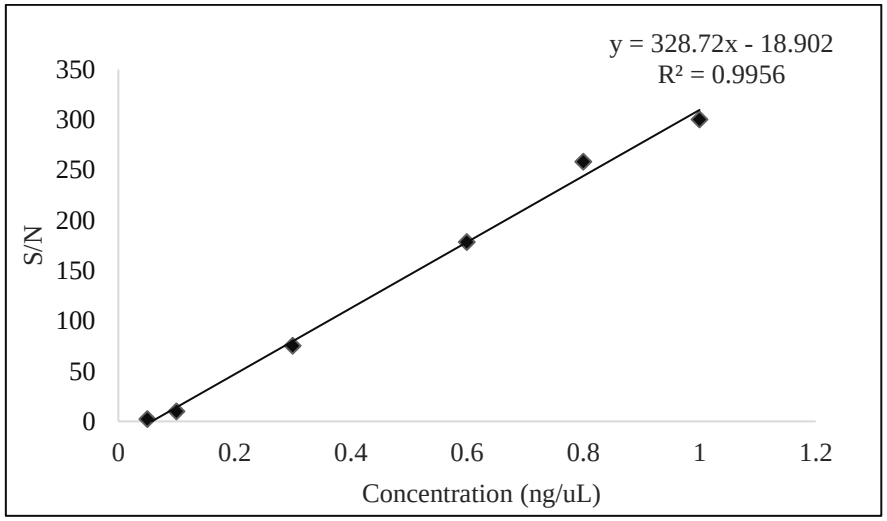


Figure 5.1 Calibration graph illustrating the signal to noise ratio of HNP2 against concentration.

5.5.1.2 Precision

The intra-interday precision of HNPs is presented in Table 5.2. Interday precision was established from reanalysis of the same sample over two successive days. The intraday CV% of the EIC response of spiked HNP2 at three different concentrations (LQC: 0.3 ng/µL; MQC: 0.6 ng/µL; HQC: 0.8 ng/µL) was in the range of 0.6 - 3.1%, while the interday CV% of the response was 10-12%. The HNP2 at three different concentrations in the saliva matrix was found to be higher at the lower reference range of quantification than the upper range for intraday precision. The intraday CV% for HNP 1-4 (EIC peak area response) was in the range of 0-5% and the interday CV% was 6-15%. The accepted validation criteria for repeatability is a CV of <20% (ICH *et al.*, 2005; Huber *et al.*, 2010); therefore, the HNP1-4 intraday CV%

were all within the acceptable range. The intraday CV % for HNP 1-3 (EIC peak area response) after SPE in the saliva matrix was lower compared to the findings of Van den broek (2010) who reported an intraday CV% for HNP1-3 (ng/µL) of 9.8-14.0% in plasma and 5.6-6.7% in serum. However, the interday CV % for HNP 1-3 (EIC peak area response) after SPE in the saliva matrix seems comparable to the findings of Van den broek (2010) who reported an intraday CV% for HNP1-3 (ng/µL) of 10-19% in plasma and 7-10% in serum.

5.5.1.3 Stability

The stability of the endogenous peptides HNP1, HNP3, HNP4 and HNP2 spiked into the saliva matrix at three different concentrations was assessed up to twenty-four hours at room temperature (interday precision), and after one month in storage at -80°C (as a lyophilised powder following SPE). The CV % was calculated by comparing the EIC peak area compared to freshly prepared samples. The CV% of endogenous analytes (HNP1, HNP3 and HNP4) after one month at -80°C were in the range of 9-13%, 15-19%, and ,7-18%, respectively, while the CV% of spiked HNP2 at three different concentrations (LQC: 0.3 ng/µL; MQC: 0.6 ng/µL; and HQC: 0.8 ng/µL) after one month was 15-17%. The CV% of the stability study of the spiked HNP2 in saliva at three different concentrations (LQC: 0.3 ng/µL; MQC: 0.6 ng/µL; HQC: 0.8 ng/µL) was in the range of (0-17%) at room temperature (12-24h) and at -80°C for one month. Van den broek (2010) found that spiked HNP2 in serum at three different concentrations (LQC: 50 ng/µL; MQC: 250 ng/µL; HQC: 800 ng/µL) was in the range of 4-7% for 2.5 hours at room temperature and at -80°C. The stability of the spiked HNP2 at three different concentrations in the saliva matrix was found to be higher at the lower reference range of quantification than the upper range (Table 5.2). The accepted validation criteria for stability is a CV of <20% (ICH *et al*., 1996; Huber *et al*., 2010); therefore, HNP 1-4 stability CV% scores were all within the acceptable range. The saliva samples were freshly prepared and samples were analysed by LC-ESI-MS within twenty-four hours. The observed fluctuations in the CV% at -80°C remains unclear.

5.5.1.4 Recovery

The recovery of the standard (HNP2 and leu-enkaphalin) was investigated by comparing the EIC peak areas in the saliva matrix before and after SPE.The standard (HNP2 and leu-enkaphalin peptide) was spiked at three different concentrations (HNP2: LQC: 0.3 ng/µL, MQC: 0.6 ng/µL, and HQC: 0.8 ng/µL, and Leu-enkephalin: LQC: 0.3 ng/µL, MQC: 0.6 ng/µL, and HQC: 0.8 ng/µL).

Table 5.2 Intra-and interday precision, and stability data for HNPs 1-4 in human saliva.

		Intraday (12 hours)		Interday (24 hours)		Stability (-80◦C, one month)	
Defensins	**Concentration (ng/uL)**	**Peak area (mean ± std)**	**Peak area (CV%)**	**Peak area (mean ± std)**	**Peak area (CV%)**	**Peak area (mean ± std)**	**Peak area (CV %)**
HNP1	-	81 ± 0.2	0.31	85 ± 3.8	2.53	100 ± 12.6	13.92
	-	82 ± 1.0	1.22	82 ± 2.5	1.70	89 ±10.1	11.36
	-	79 ± 0.8	1.03	83 ± 3.5	2.38	84 ±7.7	9.20
HNP2	LQC	53 ± 1.6	3.13	50 ± 2.8	4.02	60 ± 10.4	17.23
	MQC	90 ± 0.5	0.64	91 ±1.8	2.35	100 ±13.9	13.89
	HQC	116 ± 2.6	2.26	116 ± 2.5	2.22	130 ±20.2	15.52
HNP3	-	43 ± 1.6	3.67	43 ± 1.4	3.03	50 ± 10.0	19.73
	-	41 ± 0.9	2.38	47 ± 2.0	4.38	46 ±7.0	15.29
	-	42 ± 0.8	1.94	47 ±1.5	3.21	47 ±7.7	16.38
HNP4	-	19 ± 0.9	4.69	19 ± 1.3	3.81	22 ± 4.1	18.67
	-	18 ± 0.5	3.00	19 ±1.3	4.38	19 ± 1.8	9.45
	-	17 ± 0.0	0.36	19 ± 0.5	1.69	18 ± 1.4	7.56

HNP2 were spiked into the saliva samples at three different concentrations (0.3, 0.6 and 0.8 ng/ µL) post SPE and analysed by LC-MS (n = 3). The intermediate precision was calculated from the EIC (peak area) and expressed as a %CV. The endogenous relative levels of HNP1, HNP3 and HNP4 were also assessed in these samples. Samples were stored as a lyophilised powder (-80◦C) after SPE and reassessed after one month.

Table 5.3 Recovery data of HNP2 using SPE sample treatment (n= 5).

Sample	Concentration (ng/uL)	Peak area (mean ± std)	% Recovery
Saliva	-	33 ± 1.7	-
Spiked HNP2 in saliva (Pre extraction)	LQC	53 ± 1.6	-
	MQC	90 ± 0.5	-
	HQC	116 ± 2.6	-
Spiked HNP2 in saliva (Post extraction)	LQC	58 ± 1.6	80
	MQC	101 ± 1.5	83
	HQC	124 ± 0.8	91

Three different concentrations such as low concentration quality control (LQC= 0.3 ng/uL), medium concentration quality control (MQC=0.6 ng/uL) and (high concentration quality control HQC=0.8 ng/uL) standards of HNP2 were spiked into the saliva samples prior to and after SPE and analysed by LC-MS. The intermediate precision was calculated from the EIC (peak area) and expressed as a CV%.

Table 5.4 Recovery data of leu-enkaphalin using SPE sample treatment (n= 5).

Sample	Concentration (ng/uL)	Peak area (mean ± std)	% Recovery
Spiked Leu-enkephalin in saliva (Pre extraction)	LQC	74 ± 4.2	-
	MQC	111 ± 0.8	-
	HQC	127 ± 0.7	-
Spiked Leu-enkephalin in saliva (Post extraction)	LQC	82 ± 1.6	88
	MQC	118 ± 0.2	94
	HQC	136 ± 1.5	93

Three different concentrations such as low concentration quality control (LQC= 0.1 ng/uL), medium concentration quality control (MQC=0.3 ng/uL) and (high concentration quality control HQC=0.5 ng/uL) standards of Leu-enkephalin were spiked into the saliva samples prior to and after SPE and analysed by LC-MS. The intermediate precision was calculated from the EIC (peak area) and expressed as a CV%.

The recovery data revealed an acceptable recovery range of 80-91% for HNP2 and 89-93% for Leu-enkephalin in the saliva matrix (Table 5.3 and Table 5.4). The percentage recovery of HNP2 after SPE in the saliva matrix was lower compared to the findings of Van den broek (2010), where recovery of standard HNP2 was 86-121% in plasma and 90-128% in serum. Compounds co-eluting with the chosen analyte can affect analyte recovery in an LC-MS assay. The HNP2 standard was not stable in solution (approximately six hours) and therefore HNP2 could not be used as an internal standard (Van den broek *et al.,* 2010; Hills *et al.,* 1999). Van den broek (2010) showed examples of ion suppression by the internal standard (HNPs) and recommended use of isotope-labelled internal standards for the absolute quantification of individual HNP1-3. Over recent decades, Leu-enkephalin has become a commonly used reference compound in MS. It is a small molecule (MH+ = 556.27 Da), which is easy to interpret and ionises well (Polfer *et al.,* 2007; Schnier *et al.,* 1997; Sztaray *et al.,* 2011). Therefore, Leu-enkephalin as the standard was utilised in the main study. The primary reason for using an internal standard (Leu-enkephalin) in this study in a complex mixture of saliva was to ensure the recovery of the analyte and instrument sensitivity.

5.5.2 Pilot Study

To establish if the developed LC-MS method is sufficiently sensitive to detect individual HNP 1-4 in male and female participants' saliva samples a pilot study was conducted. HNP4 could not be detected in some participants' saliva samples, as the levels of HNP4 is 100 fold less compared to HNP1-3 (Fu *et al.,* 2003). In this study, HNP1 (EIC peak area) was the most abundant peptide compared to HNP2, HNP3 and HNP4. Cabras (2010), Peulso (2007) and Goebel (2000) have also noted a similar trend between HNP 1-4. In addition, HNP2 was found to be 1.3 fold lower compared to HNP1, whereas HNP3 was found to be 2.3 fold lower and HNP4 5.8 fold lower compared to HNP1.

Goebel (2000) and colleagues determined the levels of HNP1 and HNP2 in twenty healthy controls and reported ranges of 0.7-23 ng/µL and 0.5-17 ng/µL, respectively, and noted that in healthy controls the levels of HNP1 and HNP2 varies significantly. Variation in the levels of individual HNPs was also observed in the plasma and serum matrix. Van den broek (2010) and colleagues reported the levels of HNP1, HNP2 and HNP3 as 0.004-0.042 ng/µL, 0.033-0.127 ng/µL, and 0.005-0.019 ng/µL, respectively in plasma and 0.016-0.108 ng/µL, 0.113-0.267 ng/µL, and 0-0.149 ng/µL, respectively in serum. The measurement (EIC peak area) of

individual HNPs in saliva compared with LC-MS-based work in different matrices (serum and plasma) and the commonly used immunoassays for the quantitation of HNP1-3, including RIA and ELISA, are summarised in (Table 5.5). The HNP1-3 concentrations measured by RIA were around 0.102-0.402 ng/µL in plasma for controls (Hoover *et al.*, 1997), while other studies have reported the concentration of HNP1-3 in plasma to be around 0.2-0.8 ng/µL, 0.1-0.23 ng/µL, 0.01-0.09 ng/µL, and 0.25-0.5 ng/µL, when measured via RIA (Yamaguchi *et al.*, 2009; Hoover *et al.*, 1997; Ashthani *et al.*, 2002; Ashtani *et al.*, 1998).

Davison (2009) reported the concentration of HNP1-3 was 0.1-0.5 ng/µL for saliva, while in this study the concentration of HNP1-3 was in the range of 0.0018-0.0023 ng/µL by ELISA. A recent report by Gillum (2015) and Kunz (2015) the concentration of HNP1-3 was in the range of 1000-3500 ng/µL and 0.757-0.827 ng/µL for saliva by ELISA. Salivary levels can vary about 100 fold between subjects, and it is difficult to define normal ranges for individual antimicrobial peptides within saliva (Gorr *et al.*, 2009; Tao *et al.*, 2005). The observed variation within these results and in other published work may be the due to differences between the health statuses of individual subjects and sample handling. In addition, saliva contains low concentrations (100-1000 fold) of analytes in comparison to blood, and the range of HNP1-3 concentrations in this study is comparable with plasma levels concentrations (Yamaguchi *et al.*, 2009; Hoover *et al.*, 1997; Ashthani *et al.*, 2002; Ashtani *et al.*, 1998).

The approach presented in this thesis (sample preparation, quantitation and validation) are specific compared to previously published methods. Combined with its applicability, as shown in saliva samples from male and female athletes for the analysis of individual HNPs, this method provides an opportunity to measure large sets of data collected from various fields (sports, biomedical and clinical science) in order to assess the diagnostic values of these antimicrobial peptides.

Table 5.5 Comparison of individual HNPs using LC-MS in different matrices.

Techniques	Matrix	Participants	Comparison with other LC-MS studies		References
			Peptide	Mean (EIC peak area)	
		10 participants [1]	HNP1	3.2	1. This work
			HNP2	2.9	
			HNP3	1.3	
			HNP4	1.3	
	Saliva	31 participants [2]	HNP1	25	2. Cabras *et al.*, 2010
			HNP2	22	
			HNP3	ND	
LC-MS			HNP4	4	
		9 participants [3]	HNP1	1.6	3. Peulso *et al.*, 2007
			HNP2	1.3	
			HNP3	0.7	
			HNP4	0.3	
			Peptide	Concentration (ng/µL)	
	Saliva	20 participants [4]	HNP1	0.7-23	4. Goebel *et al.*, 2000
			HNP2	0.5-17	
	Plasma	11 participants [5]	HNP1	0.004-0.042	5. Van den broek *et al.*, 2010
			HNP2	0.033-0.127	
			HNP3	0.005-0.019	
	Serum	11 participants [5]	HNP1	0.016-0.108	
			HNP2	0.113-0.267	
			HNP3	0-0.149	
		23 participants [1]	HNP1-3	0.0018-0.0023	6. Davison *et al.*, 2009
		23 participants [6]	HNP1-3	0.1-0.5	7. Gillum *et al.*, 2015
		8 participants [7]	HNP1-3	1000-3500	8. Kunz *et al.*, 2015
		17 participants [8]	HNP1-3	0.757-0.827	
	Plasma	44 participants [9]	HNP1-3	0.027-0.079	9. Sakamoto *et al.*, 2015
	Plasma	57 participants [10]	HNP1-3	0.25-0.5	10. Yamaguchi *et al.*, 2009
RIA	Plasma	86 participants [11]	HNP1-3	0.102-0.402	11. Hoover *et al.*, 1997
	Plasma	30 participants [12]	HNP1-3	0.2-0.8	12. Mukae *et al.*, 2002
	Plasma	37 participants [13]	HNP1-3	0.1-0.23	13. Ashthani *et al.*, 2007

5.5.3 Cross Validation

One of the objectives of this study was to compare the data obtained by LC-MS with that obtained by ELISA for the analysis of salivary HNPs. The data reveals that there is a good correlation (Figure 5.2) between the LC-MS and ELISA data for the measured levels of salivary HNP1-3 (n= 210; R^2 = 0.96). Therefore, the developed LC-MS method can be considered an accurate analytical technique to measure individual HNP1-3 levels in a biological matrix such as saliva. Method validation parameters (linearity, accuracy and recovery) for ELISA and LC-MS are presented in Table 5.6

Table 5.6 Method validation parameters for ELISA and LC-MS.

Technique	Peptide	Linearity (HNP2 Standard)	Precision (%) (Saliva Matrix)	Recovery (%) (Saliva Matrix)
ELISA	Averaged response from HNP1-3	0.00015-0.01 ng/µL (R^2 = 0.99)	Interday < 2% Intraday < 5%	90-101%
LC-MS	Individual HNP1-3	0.05-1 ng/µL (R^2 = 0.99)	Interday 0-5% Intraday 11-14.95%	80-91%

The LC-MS method showed lower intermediate precision (inter < 5% and intraday <14.95%) compared to ELISA (inter < 2% and intraday < 5%). A significant difference in analyte recovery was observed between the ELISA and LC-MS methods.

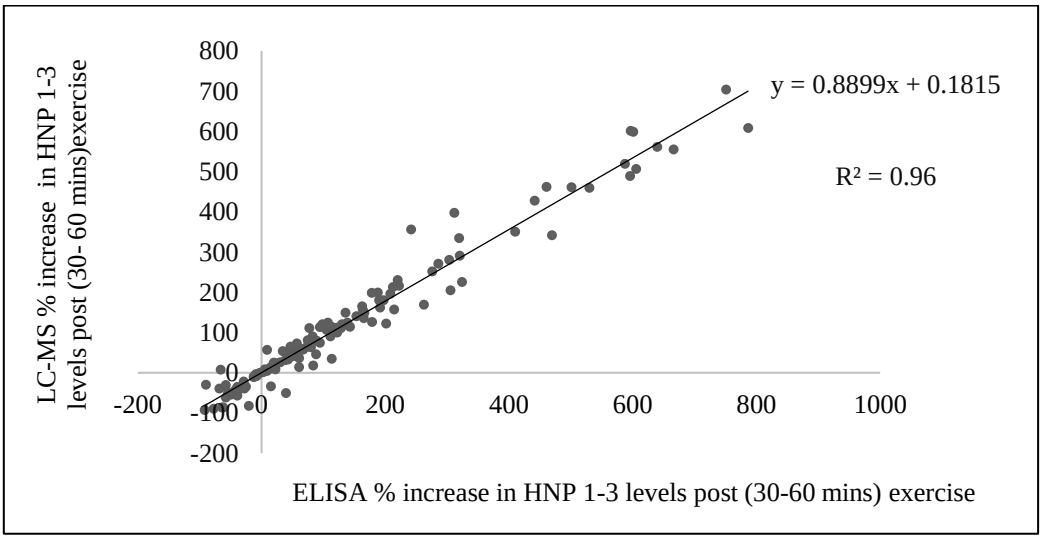


Figure 5.2 Correlation between LC-MS and ELISA data (n= 210). The data is calculated as a mean (% increase) of HNP1-3 for post 30 and post 60 minutes after exercise. The percentages increases were plotted against each other to assess the correlation between the (ELISA and LC-MS) methods.

ELISA provided higher recovery (90-101%) compared to LC-MS (80-91%), and while both methods showed similar linearity, ELISA provided better (0.00015-0.01 ng/µL) sensitivity compared to LC-MS (0.05 ng/µL). However, it is worth noting that the LC-MS data (combined increases in HNP1-3 levels post exercise intervention) regarding average response tended to be higher than the levels determined by ELISA (Figure 5.2), although this could be due to the fact that the saliva samples were stored at -80ºC for longer between collection and analysis.

5.6 Summary

ELISA is an extremely sensitive method. However, ELISA is both relatively expensive and time consuming. A correlation was observed between the LC-MS and ELISA data for the analysis of salivary HNPs of 0.96. The developed LC-MS method can be considered an accurate analytical technique to measure individual HNP1-3 levels in saliva. This study has confirmed that LC-MS can be used to determine the salivary levels of HNP1-3 with lower sensitivity, but with higher specificity compared to ELISA because ELISA is unable to distinguish between HNPs 1-3 and measures the sum total whereas LC-MS was able to quantify the abundance of individual HNPs. The measurement using LC-MS in this study measured the relative increases in HNPs 1-3 whereas ELISA was able to give absolute values for the sum total of HNPs 1-3.

Chapter 6 - Evaluation of α–Defensins and Cortisol levels in Response to Physical Stress

6.1 Background

AMPs are major components of the innate immune system (Bal *et al.,* 2000; De Smet *et al.,* 2005), and Bowdish (2005) reported that AMPs are crucial for both prevention and clearance of infection. There is limited research regarding the mechanism by which exercise alters salivary AMP concentrations (Davison *et al.,* 2009; Gillum *et al.,* 2015; Kunz *et al.,* 2015), although Usui (2011) reported an increase in salivary levels of β-defensins (HBD2) and cathelicidins (LL-37) following exercise, while Davison (2009) reported an increase in levels HNP1-3. Currently, the quantification of α-defensins is predominantly accomplished via an ELISA; however, LC-MS is emerging as an alternative method, and such studies offer an insight into individual α-defensin responses following physical stress. The primary aim of this chapter is to investigate the levels of HNPs after a crossfit intervention exercise and to confirm whether the results obtained agree with the existing literature (Davison *et al.,* 2009; Gillum *et al.,* 2015; Kunz *et al.,* 2015).The effects of exercise on immunomodulatory components (α-defensins and cortisol) and the response of the innate immune system have been found to be suppressed or altered depending on exercise intensity (Chapter 1).

In addition, prolonged exercise of more than ninety minutes may weaken an athlete's immune system for up to seventy-two hours afterwards (Niemen *et al.,* 2005). Therefore, two different studies were conducted to investigate supplement consumption: study 1, placebo versus carbohydrate supplement, and study 2, carbohydrate versus multinutritional supplements on salivary levels of individual α-defensins and cortisol before and after exercise. Hormones play an important role in innate immunity and it is well known that physical and mental stress elicit the release of cortisol from the hypothalamic pituitary adrenal axis (HPA), and this results in the modulation of various immune responses (Hellhammer *et al.,* 2009). It is surprising that to date, only one study has investigated the correlation between salivary cortisol and AMPs (β-defensins, cathelicidins) (Usui *et al.,* 2011). Furthermore, no studies have been undertaken to compare the individual levels of α-defensins and cortisol. Therefore, the relationship between α-defensins (HNP1-3) versus cortisol responses after exercise was investigated for both studies conducted in this chapter. Furthermore, the ELISA results for HNP1-3 levels presented in chapter 6 was used for the correlation comparison with cortisol.

6.2 Materials and Methods

6.2.1 Participants

Study 1: Five male athletes (age 22 ± 1.5 years, body weight 79 ±10 kg and height 181 ± 0.07 cm) and five female athletes (age 26 ± 1.9 years, body weight 65 ± 9 kg and height 175 ± 0.09 cm) volunteered to participate in this study.

Study 2: Twelve male athletes (age 24 ± 2.5 years, body weight 85 ± 11 kg, and height 191 ± 0.01 cm) volunteered to participate in this study.

Prior to commencing the studies, participants received written and verbal instructions via an information sheet (Appendix C) detailing what the study involved. They also completed a health questionnaire (Appendix B) and gave their informed written consent (Appendix A).

6.2.2 Familiarisation Sessions

Each participant undertook three sessions prior to the actual study session to familiarise themselves with the prolonged crossfit exercise training. In total, the participants were required to attend the laboratory on seven different occasions. Participants were also asked to produce unstimulated saliva in order to familiarise themselves with the collection procedure, and instructions were provided about the crossfit exercises to be completed prior to the familiarisation trial, with time allocated to practice the exercises with guidance. Following the familiarisation sessions, participants attended for the main testing session for each study.

6.2.3 Crossfit Exercise

The crossfit exercise protocol is illustrated in Figure 6.1.

6.2.3.1 Pre Interval Saliva Sample Collection

For visits made between 3-5pm, participants' saliva samples were collected before the warm-up (pre) interval based on the method described in Chapter 3. Participants could drink water during the trial but were asked to stop drinking ten minutes before each saliva sample to avoid dilution. All samples were stored at -80°C immediately after each collection.

6.2.3.2 Warm up

Participants performed a warm-up session for about 10 minutes (Table 6.1) after collection of the pre-interval saliva sample.

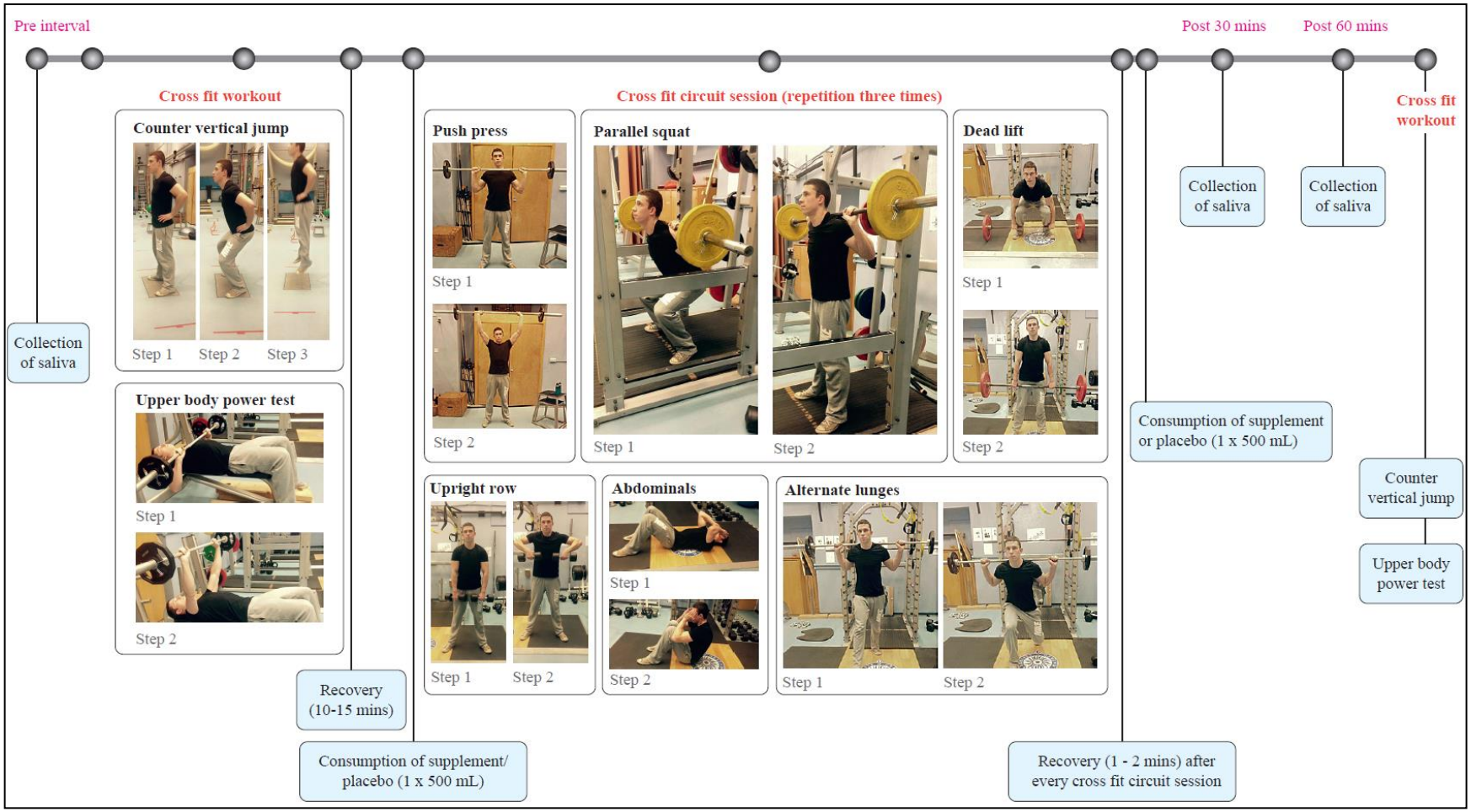


Figure 6.1 Schematic diagram of resistance training performance. A supplement or placebo was given to each participant. Salivary samples for two different interventions (post 30 and post 60 mins) were compared to the pre interval sample for level of HNPs and cortisol. Each participant performed a crossfit workout and crossfit circuit session.

Table 6.1 Warm up exercise performances.

Exercise	%1 RM	Sets	Repetition
Light Jogging, high knees and side steps	N/A	N/A	N/A
Dynamic stretching (leg raises and hip rotations)	N/A	N/A	N/A
Bodyweight squat	50	1	10
Push-ups	N/A	1	10
Lunges	50	1	10

%1RM = maximal weight

6.2.3.3 Crossfit Workout Measurement

In this study muscular power performance was utilised during crossfit workout measurements. Muscular power is the ability to exert a maximal force in as short a time as possible, such as jumping or weight lifting. In addition, muscular power is one of the main common fitness components and plays an important role in success in sports (Cooper *et al.,* 2014; Kodesh *et al.,* 2015). After the warm up session, each participant performed the crossfit workout for about fifteen minutes (Figure 6.1).

6.2.3.4 Countermovement Movement Jump

In a countermovement movement jump (CMJ) a jumper starts from an upright standing position, makes a preliminary downward movement by flexing at the knees and hips, then immediately extends the knees and hips to jump vertically up off the ground. Three consecutive CMJs without increased force generation by the arms were performed onto a force plate (kistler force platform).

6.2.3.5 Upper Body Power Test

An upper body power test (UBPT) was determined from a 1 RM bench press (1RMBP) (Baechle *et al.,* 2008). In general, a 1RMBP is used for evaluation of muscle strength and 1RM value was calculated as follows (Brzycki *et al.,* 1997):

$$1\ RM = 100 \times \text{maximal weight} / (102.78 - 2.78 \times \text{repetition})$$

Participants performed weight lifting: eight repetitions at approximately 50%; three repetitions at 75%; one repetition at 85%; 1 repetition at 95% and 1 repetitions at 100% of the perceived 1-RM. A maximum of five speed repetition tests was assessed for the bench press exercise. Maximum peak and average accelerative power were selected from the best of the five repetitions with 50% of the previously determined 1 RM. This test was used as an index of

lower body power and the neuromuscular fatigue index (Bosco *et al.*, 1982). An optical rotary encoder (Winlaborat) was used for measuring the power applied during each repetition during the UBPT.

6.2.3.6 Recovery

After the crossfit workout session (CMJ and UBPT), participants rested for 10-15 minutes.

6.2.3.7 Supplementation Protocols

Study one investigated a placebo (total energy ~0.005 kcal) versus a CHO supplement (total energy ~ 230 kcal) over two consecutive weeks: CHO supplement one week and placebo next or vice versa. Study two investigated a CHO supplement (total energy ~ 230 kcal) versus a MTN supplement (total energy ~ 230 kcal) for four consecutive weeks (CHO supplement one week and MTN supplement the next or vice versa). The first 500 mL of intake was ingested by participants immediately after completing their crossfit workouts (CMJ and UBPT), and the second 500ml was consumed immediately after completing the crossfit circuit sessions (Figure 6.1).

6.2.3.8 Nutritional Composition of Supplements

The nutritional composition of the supplements is listed in Table 6.2.

Table 6.2 Nutritional composition of supplements.

MTN Supplement		**CHO Supplement**		**Placebo**	
Total energy: 230 kcal		Total energy: 230 kcal		Total energy: 0.005 kcal	
Ingredients	Amount (g)	Ingredients	Amount (g)	Ingredients	Amount (g)
Protein	30	Protein	0	Protein	0.5 g
Carbohydrate	21	Carbohydrate	56	Carbohydrate	0.9 g
Total Fat	4.6	Total Fat	0	Fat	0.5 g
Creatine Monohydrate	5.1				
Glutamine	5.1				
β-Hydroxy β-methylbutyric acid (HMB)	1.5				
Potassium Bicarbonate	0.5				
Sodium Bicarbonate	0.5				
Bioperine	0.005				
Chromium Picolinate	0.000241				

6.2.3.9 Crossfit Circuit Session

This session aimed to simulate an endurance circuit session designed to improve core strength. After the first dose (1 x 500 mL) of supplementation or placebo, participants performed three circuits involving one set of each exercise without rest, aside from the time needed to move from one exercise to the next (Figure 6.1 and Table 6.3).

Table 6.3 Characteristics of the familiarising performance of the resistance training.

Exercise	%1RM	repetition	RPE scale
Parallel-squat	70 to 75%	12	5-6 to 8-10
Upright row	70 to 75%	12	5-6 to 8-10
Alternate lunges	70 to 75%	12	5-6 to 8-10
Dead lift	70 to 75%	12	5-6 to 8-10
Push-press	70 to 75%	12	5-6 to 8-10
Abdominals	Body Weight	20	N/A

%1RM = maximal weight

6.2.3.10 Rate of perceived exertion

The ratings of perceived exertion (RPE) (Borg's RPE scale) were used as an indication of impending fatigue (Bosco *et al.*, 1982). RPE reported to be a valid global indicator of internal stress during prolonged, high-intensity intermittent exercise (Figure 6.2) (Foster *et al.*, 2001; Backhouse *et al.*, 2007).

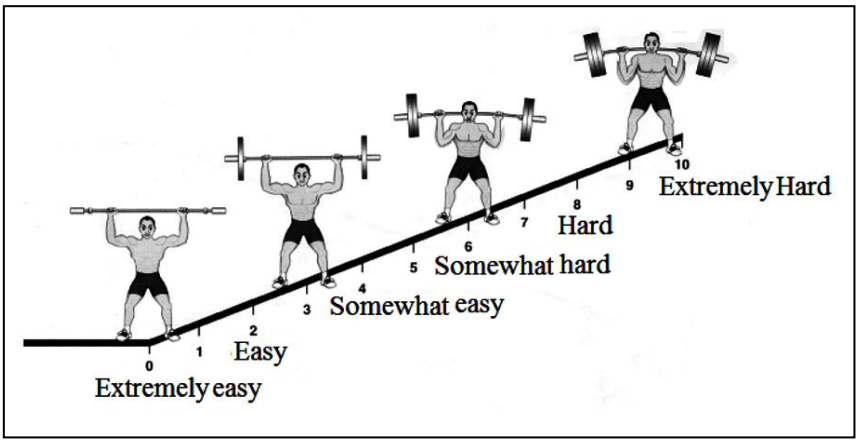


Figure 6.2 Illustration of rate of perceived exertion scale. RPE scale was used to assess the crossfit circuit session (Morishita *et al.*, 2014).

A recovery session of 1-2 minutes was provided between each set of the crossfit circuit exercise and participants RPE was assessed following the RPE scale (Figure 6.2). A second dose (1 x 500 mL) of supplementation or placebo was ingested after completion of the circuit session.

6.2.3.11 Post Interval Saliva Sample

Participants' post interval (post 30 mins and post 60 mins) saliva samples were collected after the completion of the circuit session and ingestion of the second dose (1 x 500 mL) of supplementation.

6.2.4 Sample Analysis

6.2.4.1 Analysis of HNP1-3 and Cortisol by ELISA

ELISA was performed for assessment of salivary HNP1-3 discussed in Chapter 5. Saliva (1 mL) was centrifuged (1500 *g*, 5 mins) and 25 µL was analysed in duplicate by a cortisol ELISA (Salimetrics, UK) according to the manufacturer's instructions. A calibration curve was constructed consisting of five prepared standards: 0.012 to 3.0 µg/dL cortisol and a zero sample. Absorbance (450 nm) values for the saliva samples were interpolated from the calibration standards using a four parameter logistic curve (My Assays, Version 2015).

6.2.5 Equations

The area under the curve (AUC) value in a time–response (time–concentration) curve is commonly used for the assessment of the total amount of substances secreted into a biological fluid during an observation period. This is useful for comparing with assessment using direct concentrations of peptides/ hormone at each time point.

$$AUC = trap_{\text{HNP1-3 (t= 30)}} + trap_{\text{HNP1-3 (t= 60)}}$$

$$AUC = trap_{\text{cortisol (t= 30)}} + trap_{\text{cortisol (t= 60)}}$$

where,

trap = trapezoidal rule, t= time interval.

The AUC was calculated using the trapezoidal rule (trap) to assess total change in the direct concentration of salivary HNP1-3 and cortisol levels during the exercise session (t = 0 to t = 30 and t = 60).

6.3 Statistical Analysis

Statistical analysis was performed using the statistical computer software package SPSS (v16.00; SPSS Inc., Chicago, IL, USA). A one-way ANOVA was used to examine the data for salivary cortisol and HNPs and if significant differences were observed, bonferoni post hoc adjustments for multiple comparisons were assessed. Additionally, correlations were analysed using Pearson product-moment correlation coefficients.

6.4 Results and Discussion

6.4.1 Pilot study

The main findings of the pilot study were that salivary levels of individual HNPs increased after resistance training (2.5 h).

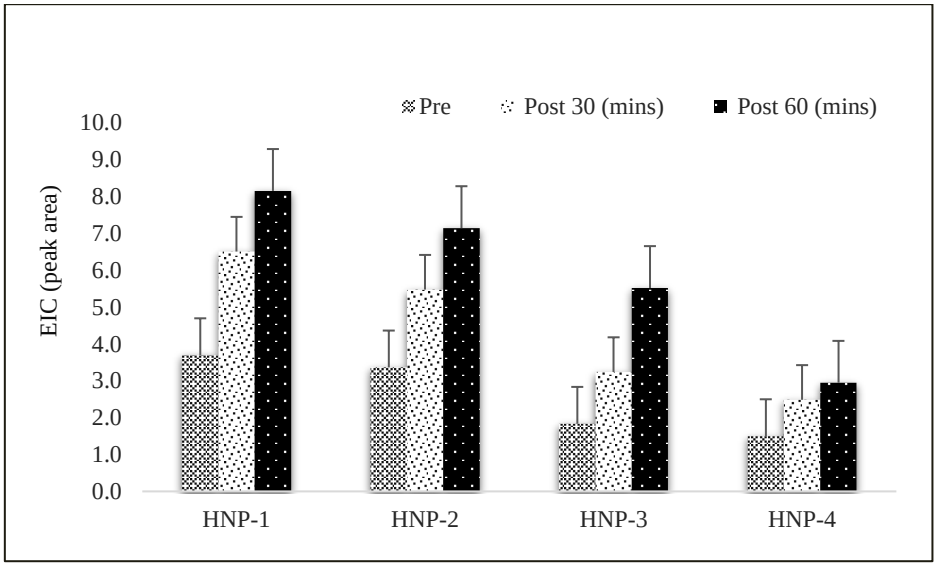


Figure 6.3 Peak area of individual HNP1-4 before (pre) and after (post 30 and post 60 mins) exercise by LC-ESI-MS, where athletes consumed placebo. The peak area was calculated from EIC (ions corresponding to $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$) for individual HNPs.

The levels of individual HNPs for participants who consumed the placebo before and after crossfit exercise between pre and post (30 and 60 mins) intervals are shown in Figure 6.3. An increase in the levels of individual HNPs were observed: a 2.2-fold increase between pre and post exercise at 30mins; a 1.3-fold increase between pre and post exercise at 60 mins; and a 1.7-fold increase between post exercise 30 and post exercise 60 mins for salivary levels of individual HNP1-4. The post-exercise increases in salivary HNP1–3 are in line with the findings from previous studies that have employed shorter duration acute exercise and examined other salivary AMPs and proteins (Davison *et al.*, 2009; Gillum *et al.*, 2015; Kunz *et al.*, 2015).

6.4.2 Exercise Performance

6.4.2.1 Resistance Workout

The characteristics in terms of the crossfit workout such as: counter vertical jump (CMJ) and upper body power (UBPT) tests are presented in Figure 6.4. The crossfit workout performances during (pre exercise) and after (post exercise) were investigated for CMJ and UPBT.

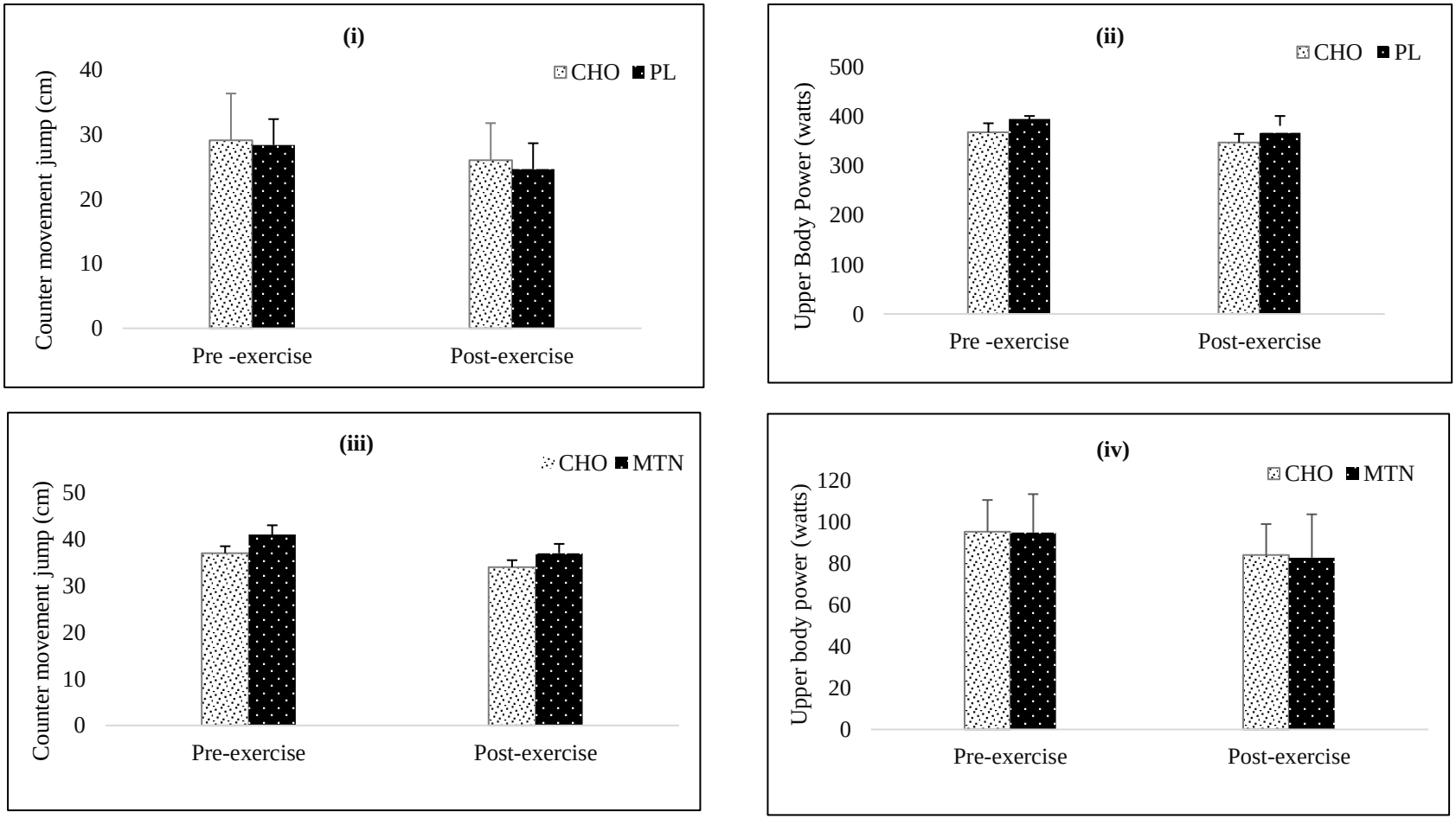


Figure 6.4 Crossfit workout performance which include: (i and iii) counter movement jump test and (ii and iv) upper body power test. No significant differences were found in athletes consuming either placebo (PL) or carbohydrate (CHO) supplements. A similar trend were observed in athletes consuming either CHO or multinutrional (MTN) supplements.

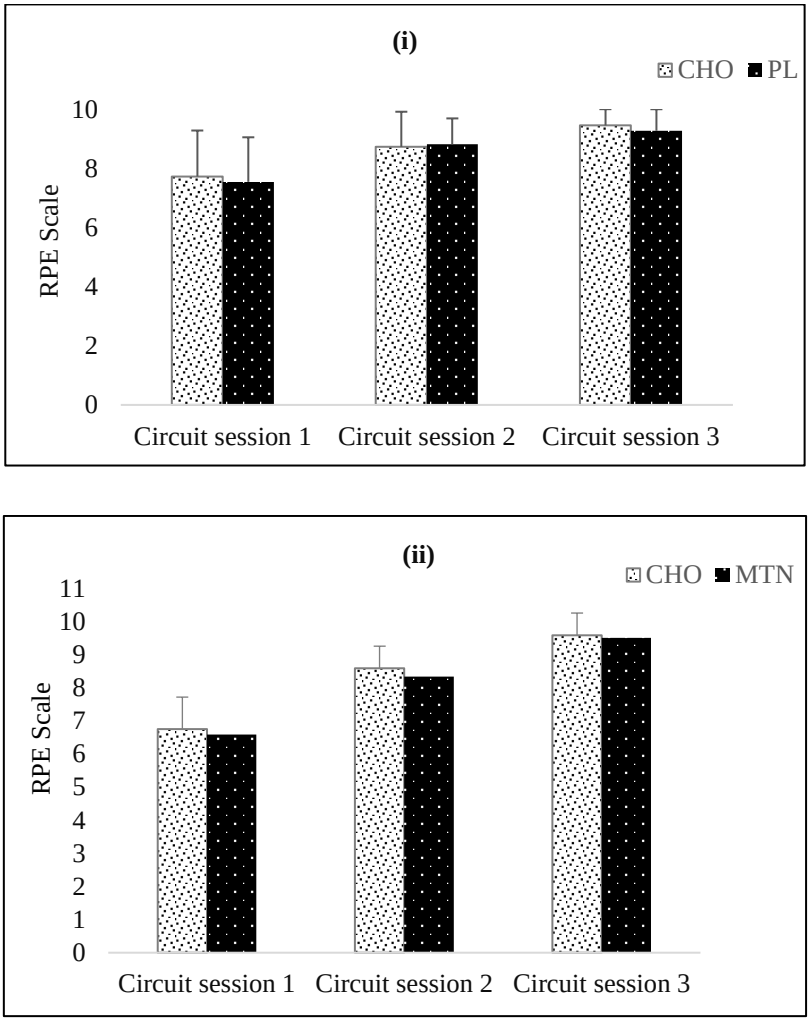


Figure 6.5 Bar charts represents data for the crossfit circuit session performance where participants consumed either placebo (PL) or carbohydrate (CHO) supplements. No significant differences were found between (i) RPE scores (0-10) for placebo versus CHO supplement and (ii) RPE scores (0-10) for CHO versus MTN supplements.

No significant ($p = 0.56$) differences between the PL versus CHO supplement and ($p = 0.69$) or between the CHO versus MTN supplements for the CMJ and UBPT were found Figure 6.4. Jump height was higher pre exercise for both supplement conditions but did not significantly differ (placebo versus CHO supplement, $p = 0.36$; CHO versus MTN supplements, $p = 0.89$) between pre and post exercise. Upper body power was higher at pre exercise for both supplement conditions but again did not significantly differ (placebo versus CHO supplement, $p= 0.23$; CHO versus MTN supplements, $p = 0.89$) between pre and post exercise. The existing literature has reported an improvement on exercise performance based on several weeks' performances with different intensity exercises (aerobic or moderate exercise) (Willems *et al.,* 2012; Schmitz *et al.,* 2010; Willoughby *et al.,* 2007; 2014; Tipton *et al.,* 2001; Darvishi *et al.,*

2013; Ivy *et al.,* 2002; Highton *et al.,* 2013). Therefore, further research involving the assessment of performance over several weeks is needed.

6.4.2.2 Resistance Circuit Session

The crossfit circuit session tests are presented in Figure 6.5. The crossfit circuit session performances during (pre exercise) and after (post exercise) were investigated using the RPE scale. The RPE scale in pre exercise for PL versus CHO supplement was lower and significantly different ($p = 0.86$) compared to post exercise, but there was no significant impact of CHO supplementation on the RPE session. A similar trend was observed for CHO versus MTN, supplements between pre and post exercise for the RPE session but again there was no significant impact of CHO and MTN supplementation on the RPE session. This finding suggests that the crossfit circuit sessions resulted in internal stress in the participants. Few studies have reported a reduction in pre exercise and an enhancement to post exercise (Welsh *et al.,* 2002; Backhouse *et al.,* 2007; Hough *et al.,* 2011), therefore, the findings of this study seem comparable with previous findings.

6.4.3 Level of Individual HNPs and Cortisol

The levels of individual HNPs and The levels of individual HNPs and cortisol were investigated in two different studies. The objective of the first study was to investigate levels of individual HNPs and cortisol in saliva in athletes that had consumed either a PL or a CHO supplement during the resistance training. cortisol before and after resistance training (pre to post 30 mins and pre to post 60 mins) for participants receiving either the placebo or a CHO supplement during the resistance training. The objective of the second study was to investigate levels of individual HNPs and cortisol in saliva in athletes that had consumed a commercially available multi-ingredient (MTN) supplement (composed of protein, creatine monohydrates, and carbohydrate) versus a CHO supplement. In both studies, the change in individual HNP levels (Figure 6.6 and 6.8) and cortisol (Figure 6.7 and 6.9) was determined pre, post 30 and post 60 mins after the resistance training. In study one, the EIC data shows that resistant training (physical stress) resulted in an increase in the level each individual HNP1-4 in athletes consuming either a placebo or CHO supplement (Figure 6.6); this increase was observed at both 30 and 60 min post exercise. In addition, at 30 mins post exercise the level of HNP1 was increased to 134%, HNP2 143%, HNP3 122% and HNP4 80% in athletes consuming the placebo and level of HNP1 was increased to 132%, HNP2 103%, HNP3 125%, and HNP4 117% in athletes consuming the CHO supplement.

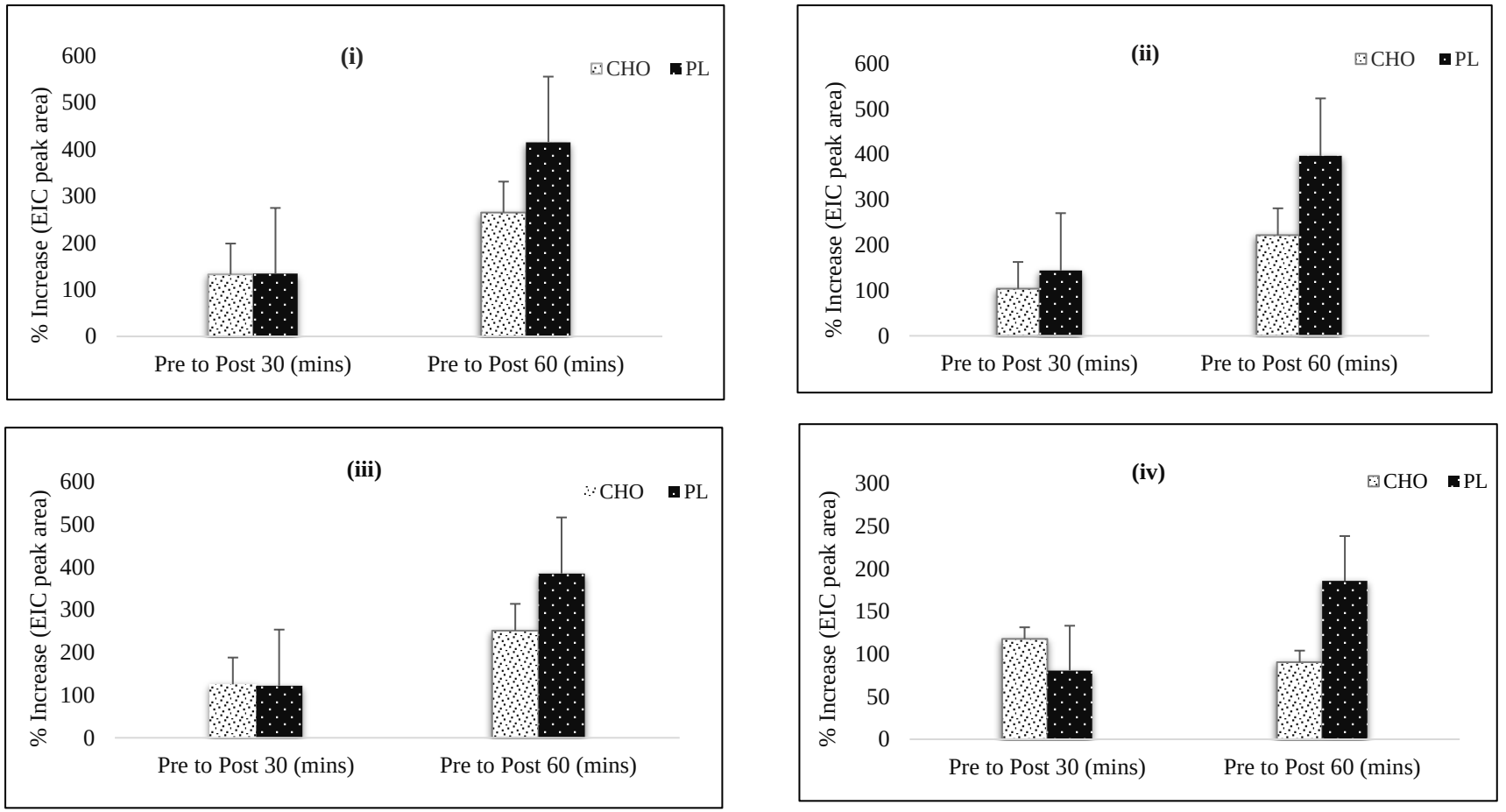


Figure 6.6 Peak area of individual HNP1-4 before (pre) and after (post 30 and post 60 mins) exercise by LC-ESI-MS, where athletes consumed either placebo (PL) or carbohydrate (CHO) supplements. The peak area was calculated from EIC (ions corresponding to $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$) for individual HNPs. A significant difference was found between the pre and post exercise (60 mins) for (i) salivary HNP1; (ii) salivary HNP2; (iii) salivary HNP3; and (iv) salivary HNP4. However, no significant differences were observed between pre exercise and post exercise (30 mins) for PL versus CHO supplement.

At 60 mins post exercise the level of HNP1 had further increased to 415.9%, HNP2 396.7%, HNP3 by 384.8%, and HNP4 by 185.8% in athletes consuming the placebo and levels of HNP1 to 264.9%, HNP2 221.8%, HNP3 250.8%, and HNP4 90.3% in athletes consuming the CHO supplement. In the second study, an increase in HNP levels (EIC) was also observed at 30 and 60 min post exercise in athletes consuming either the CHO or MTN supplement (Figure 6.8). At 30 mins post exercise the HNP1 level had increased to 104%, HNP2 366%, HNP3 225% and HNP4 104% in athletes consuming the CHO supplement, and the level of HNP1 was 35%, HNP2 82%; HNP3 126% and HNP4 5% in athletes consuming the MTN supplement. At 60 mins post exercise levels of HNP1 increased to 127%, HNP2 60%, HNP3 235%, and HNP4 51% in athletes consuming the CHO supplement and the level of HNP1 was 63%, HNP2 76%, HNP3 108% and HNP4 4% in athletes consuming the MTN supplement.

The post exercise increases in levels of HNP1-3 are in line with those of previous studies (combined response of HNP1-3). For example, Davison (2009) reported that the level of HNP1–3 (combined response) increased 266% and other antimicrobial peptides (such as cathelicidins) increased up to 200% after exercise at an intensity corresponding to 55–65% of VO_{2max}. This is one of the earliest studies that compared the combined levels of HNP1-3 using ELISA and evaluated levels at 2.5 hours after exercise. In another study, the levels of HNP1-3 (average response) were evaluated after 30 mins of cycling at 5 to 15% $VO_{2max,}$ and an increase of 80-99% was observed in HNP1-3 levels, and 80-122% for cathelicidins (LL-37) (Kunz *et al.,* 2015). Additional studies examining the relationship between exercise and salivary antimicrobial peptides (beta defensins 2, LL-37, average response of HNP1-3) have yielded similar results, demonstrating significant increases in their concentration (Gillum *et al.,* 2014; Usui *et al.,* 2011).

Compared with the data obtained in this study, the type of exercise is different, aerobic versus resistance, and this could result in the higher increases observed in HNP levels here. The increased level after 2.5 hours observed by Davidson (2009) could indicate that the defensin response continues to increase in the hours post exercise. It is yet to be determined if this increase in transient, however, the literature suggests that this is the case.

In both studies the percentage increases in HNP1, HNP2 and HNP3 levels 30 and 60 mins post exercise were not significantly different. However, a large standard deviation was observed between the levels (% increase) of HNP1-3 in individual athletes' saliva samples. Therefore, combined data, as provided by ELISA, could have been sufficient to study the individual

responses of HNP1-3. Nevertheless, a further study is required to confirm individual changes with a higher number of participants. Interestingly, the increases in HNP4 level at 30 and 60 minutes post exercise is on average smaller compared to that for HNP1-3 in both studies.

Overall, there was no significant difference between the increase in HNP levels at post 30 min in athletes who had consumed either the placebo or CHO supplement (HNP1, p=0.35; HNP2, p=0.15; HNP3, p=0.17; HNP4, p=0.06); however, the increases are significantly different at 60 min post exercise (HNP1, p=0.00; HNP2, p=0.05; HNP3, p=0.03; HNP4, p=0.01) (Figure 7.6). This indicates that supplementing with CHO significantly reduces HNP levels compared to the placebo. In the second study where participants consumed either a CHO or MTN supplement, the differences in HNP levels were on average lower in participants consuming the MTN supplement (Figure 6.8), although not significantly different at any time interval (post 30 mins HNP1, p=0.85; HNP2, p=0.16; HNP3, p=0.07; HNP4, p=0.40 and post 60 mins HNP1, p=0.17; HNP2, p=0.20; HNP3, p=0.09; HNP4, p=0.30).

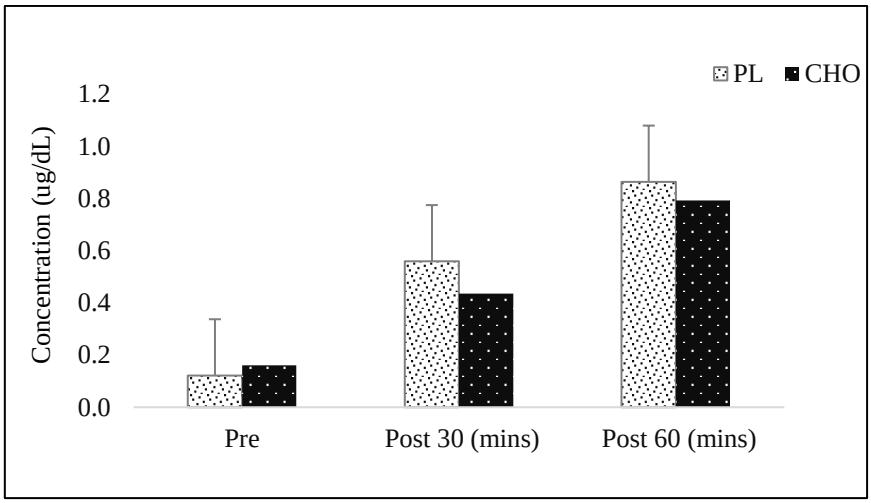


Figure 6.7 Salivary response of cortisol before (pre) and after (post 30 and post 60 mins) exercise by ELISA, where athletes consumed either placebo (PL) or carbohydrate (CHO) supplements. A significant difference was found between pre exercise and post exercise (30 and 60 mins) for PL versus CHO supplements.

Salivary cortisol levels (ELISA) increased in athletes consuming either the placebo or CHO supplement at 30 mins post exercise (500% vs. 400%). Cortisol levels increased in both groups at 60 mins post exercise (700% vs. 600%). However, due to the larger standard deviation variation, no significant difference (p= 0.06) (Figure 6.7) were obsereved. Likewise, in the second study, salivary cortisol levels (ELISA) were found to be increased for both CHO and MTN supplementation at 30 mins post exercise (250% vs. 66%), and levels increased in both

groups at 60 mins post exercise (100% vs 33%). A trend was observed whereby salivary cortisol was found to be higher between the pre exercise to post exercise 30 mins and 60 mins but it did not differ significantly (p=0.09, p=0.08 respectively) for both the CHO and MTN supplements (Figure 6.9). Resistance training may induce air-inflammation and damage to airway epithelial cells, which could be a part of the normal stress response. It is well established that exercise is associated with changes within a number of immune cells (Bishop *et al.,* 2002; Nehlsen *et al.,* 1997; Pyne *et al.,* 1994). These cell functions have been largely assessed *in vitro* and do not necessarily reflect the whole body response to a bacterial or viral challenge; however, this temporary change in immune cell function has been suggested to lead to an 'open window' for infection, which may account for the apparent increased susceptibility of athletes to infection (Pedersen *et al.,* 2000). However, further study is required to confirm this association. It will be interesting to assess microbial activity in future studies and upper respiratory risk in athletes. The consumption of a CHO supplement during and after prolonged exercise has been shown to attenuate immunosuppression by better maintaining glycaemia and reducing the magnitude of the release of stress hormone (Nieman *et al.,* 2001; Pedersen *et al.,* 1997; Gomes *et al.,* 2014). In addition, the consumption of a CHO supplement has been shown to limit the reduction in immune cell function (Bishop *et al.,* 2002). In this study, ingesting a CHO supplement reduced the elevation levels of individual salivary HNPs. A few studies have suggested that prolonged exercise is associated with changes in a number of aspects of circulating neutrophil functions, including degranulation and oxidative burst (Pyne *et al.,* 1994). However, the magnitude of these responses appears to be reduced by regular carbohydrate beverage ingestion during exercise (Bishop *et al.,* 2002; Nieman *et al.,* 1997, 1998; Scharhag *et al.,* 2002; Bishop *et al.,* 2000). The existing literature concerning the interaction between carbohydrate ingestion and neutrophil count after exercise is inconsistent. Some studies have reported that the neutrophil count is significantly higher after exercise (Niemen *et al.,* 1997; Nehlsen *et al.,* 1997; Niemen *et al.,* 2001; Bishop *et al.,* 2002, 2003), while others have reported that carbohydrates reduce neutrophil counts following exercise (Bishop *et al.,* 2002, 2003); however, the underlying explanation for this effect of carbohydrate is as yet undetermined (Gomes *et al.,* 2014). Ingesting the CHO supplement also reduced the salivary cortisol concentration, and several factors have been

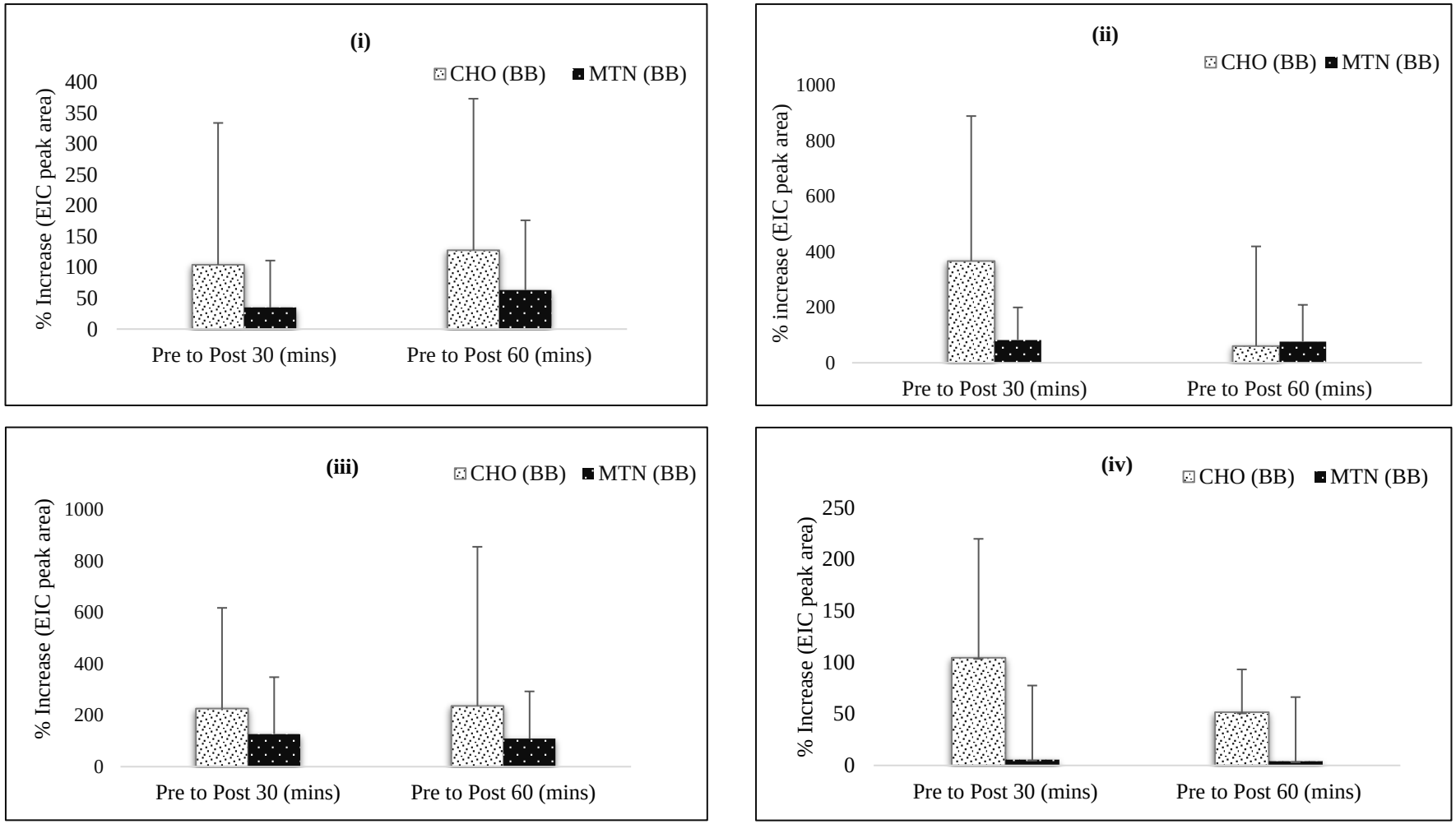


Figure 6.8 Peak of individual HNP1-4 before (pre) and after (post 30 and post 60 mins) exercise by LC-ESI-MS, where athletes consumed either carbohydrate (CHO) or multinutrional (MTN) supplements. The peak area was calculated from EIC (ions corresponding to $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$) for individual HNPs. No significant differences were found between pre exercise and post exercise 30 and 60 mins (i) salivary HNP1; (ii) salivary HNP2; (iii) salivary HNP3; (iv) salivary HNP4 for CHO versus MTN supplement.

shown to influence this, such as perceived stress, adrenal sensitivity, adrenal capacity, and glycaemia (Hellhammer *et al.,* 2009; Gomes *et al.,* 2015). The salivary cortisol levels in this study are comparable to the findings of previous studies (McAnulty *et al.,* 2003, 2005; Gomes *et al.,* 2015), which all investigated resistance exercise. However, a small number of studies using resistance training have also reported no effect of CHO supplementation on salivary cortisol responses (Beaven *et al.,* 2008; Cadore *et al.,* 2008). An investigation of the glycaemic index in this study would have been beneficial, and is one of the limitations of this study.

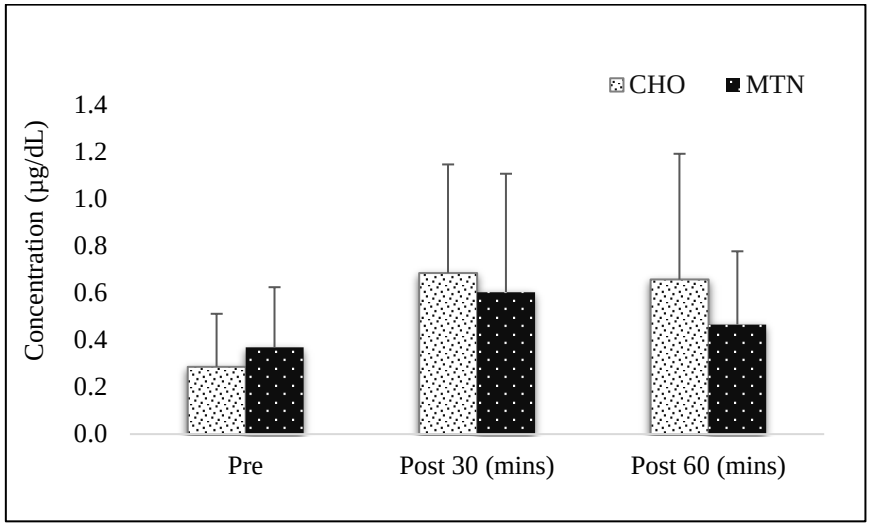


Figure 6.9 Salivary response of cortisol before (pre) and after (post 30 and post 60 mins) exercise by ELISA, where athletes consumed either carbohydrate (CHO) or multinutrional (MTN) supplements. No significant differences were found between pre exercise and post exercise (30 and 60 mins) for CHO versus MTN supplement.

A multi-ingredient product that could potentially increase muscle strength and mass while concomitantly improving metabolic function, oxidative stress, and body composition, may prove to be superior to a single-ingredient product. Many multi-ingredient products on the market contain whey protein, amino acids, carbohydrate, and creatine, and a number have been shown to augment physiological responses to resistance training (Candow *et al.,* 2007; Shelmadine *et al.,*2009; Spillane *et al.,* 2011; Willoughby *et al.,* 2007; Tipton *et al.,* 2004; Darvishi *et al.,* 2013; Ivy *et al.,* 2002).

Therefore, there is a need for additional research to confirm the effects of CHO or MTN ingestion on cortisol and HNP responses, including comparing different exercise modes, intensities, and a longer time interval. Overall, the consumption of a placebo vs. CHO or CHO vs. MTN supplement during and after resistance-training workout resulted in no impact on performance. Nevertheless, the consumption of the CHO or MTN supplement resulted in

decreased levels of HNPs compared to the placebo, which indicates that there could be a reduction in exercise-induced airway inflammation (Davison *et al.*, 2009). The ingestion of the CHO and MTN supplement also demonstrated a reduced response for cortisol (30 min and 60 min post exercise) compared to pre exercise.

Therefore, both studies support the hypothesis that both CHO and MTN supplementation influence a reduction of the immune response, although further research is required to confirm this association with a large number of participants. Therefore, there is a need for additional research to confirm the effects of CHO or MTN ingestion on cortisol and HNP responses, including comparing different exercise modes, intensities, and a longer time interval. Overall, the consumption of a placebo vs. CHO or CHO vs. MTN supplement during and after resistance-training workout resulted in no impact on performance. Nevertheless, the consumption of the CHO or MTN supplement resulted in decreased levels of HNPs compared to the placebo, which indicates that there could be a reduction in exercise-induced airway inflammation (Davison *et al.*, 2009). The ingestion of the CHO and MTN supplement also demonstrated a reduced response for cortisol (30 min and 60 min post exercise) compared to pre exercise. Therefore, both studies support the hypothesis that both CHO and MTN supplementation influence a reduction of the immune response, although further research is required to confirm this association with a large number of participants.

6.4.4 Possible Correlation between the Changes in Salivary HNPs and Cortisol Levels

AMPs are known to serve as the 'first line of defence' at mucosal surfaces and to contribute to innate immunity. Many studies have investigated the defence mechanism against pathogens in the oral cavity and have shown that AMPs play an important role at mucosal surfaces (Hancock *et al.*, 2000; Lai *et al.*, 2009; Doss *et al.*, 2010). Hormone also play an important role in innate immunity and it is well known that physical and mental stress can elicit the release of cortisol from the HPA axis. The ELISA results for HNP1-3 in Chapter 5 was used to compare the correlation with the cortisol. Stress, therefore, can modulate various immune responses (Gleeson *et al.*, 2000; Neville *et al.*, 2008) and so it is surprising that only one study has investigated the correlation between salivary cortisol and AMPs (ß-defensins, cathelicidins) (Usui *et al.*, 2011). Therefore, a correlation between the changes in salivary HNP1-3 and cortisol levels after exercise was investigated in both the first and second studies.

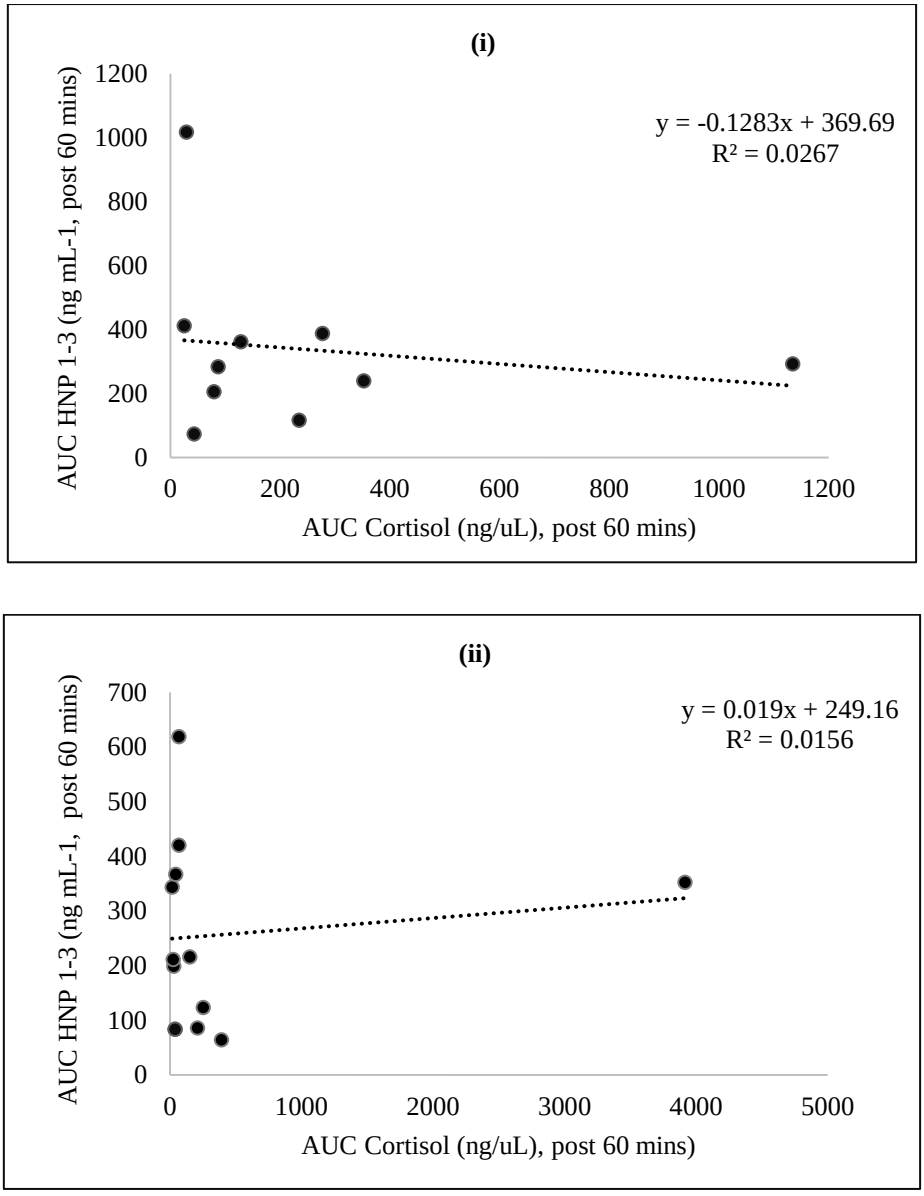


Figure 6.10 Correlation between the changes in salivary (HNP1-3) and cortisol levels by ELISA, where athletes consumed either (i) placebo or (ii) carbohydrate supplements. The AUC was calculated using the trapezoidal rule (trap) to assess the total change in the direct concentration of salivary HNP 1-3 and cortisol levels during the exercise session (t = 0 to t = 30 and t = 60).

The correlation was assessed for both first and second study (Figure 6.10 and Figure 6.11) between salivary cortisol and the combined response of HNP1-3 The findings indicate that there is no correlation between salivary HNP1-3 and cortisol (R^2 = 0.02; R^2 = 0.01) for the placebo and CHO supplement. The changes in salivary AMP (HNP1-3) concentrations (ng/µL) were not associated with cortisol levels (ng/µL). Likewise, there is no correlation between salivary HNP1-3 and cortisol following CHO supplementation (R^2 = 0.02) and MTN (R^2 = 0.08). To date, this study represents the first of its kind to investigate the correlation between

the HNPs and cortisol (stress hormone) after a crossfit training exercise (high resistance training). No significant correlation was observed between the response of cortisol and HNPs.

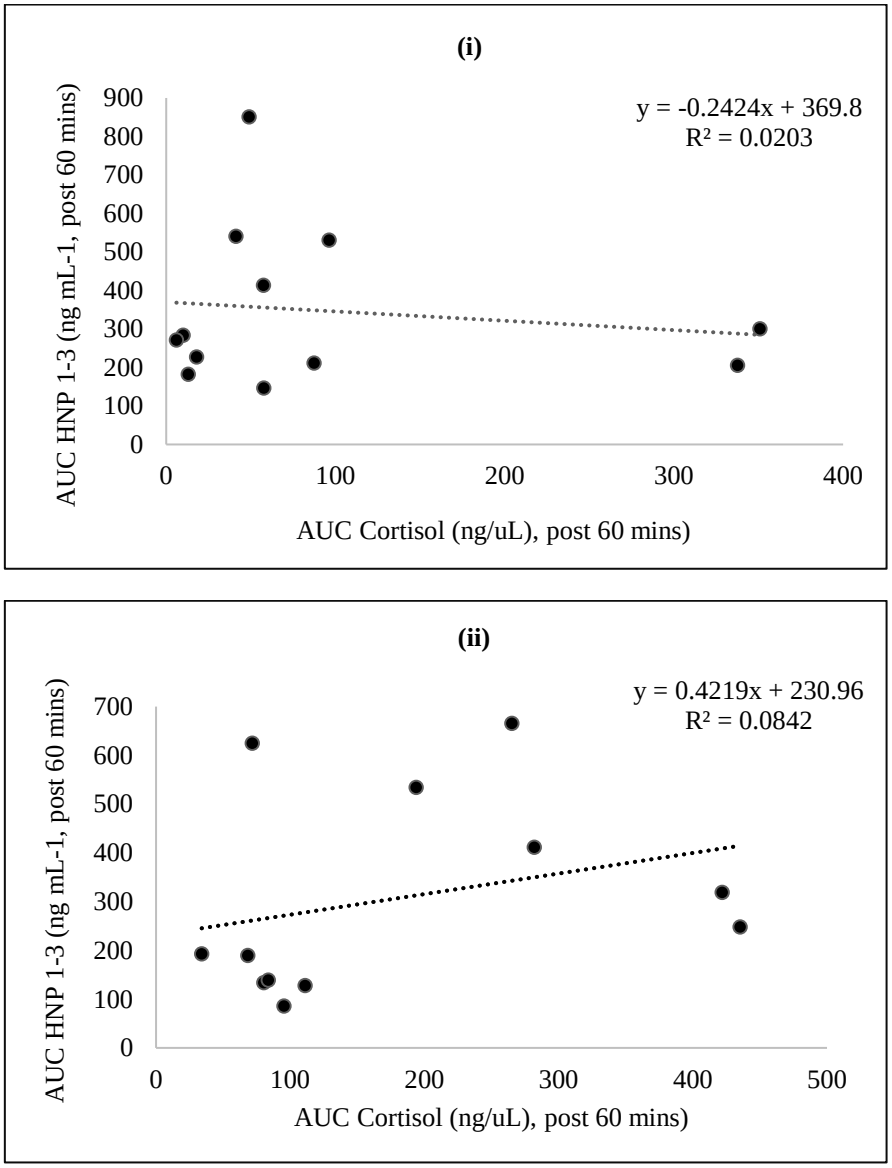


Figure 6.11 Correlation between the changes in salivary (HNP1-3) and cortisol levels by ELISA, where athletes consumed either (i) carbohydrate or (ii) multinutrional supplements The AUC was calculated using the trapezoidal rule (trap) to assess the total change in the direct concentration of salivary HNP 1-3 and cortisol levels during the exercise session (t = 0 to t= 30 and t = 60).

The present findings highlight the variability of cortisol and levels of HNPs 1-3 in response to resistance exercise, and it is not clear if the correlation between hormone and HNP1-3 levels has been affected by the time interval of the exercise. Both cortisol and levels of HNPs 1-3 demonstrated an increase after exercise but it is surprising that they do not correlate with each other. Further investigation is therefore required to determine the synergism between these molecules. Singh (2000) demonstrated that compensation may occur in mucosal fluids due to

synergism between substances, although an alternative explanation is that there is variation between individuals. In both studies, a few participants appeared to have greater salivary levels of individual HNPs and cortisol. Therefore, future investigations should utilise a larger cohort of participants (Singh *et al.*, 2000).

6.5 Summary

In conclusion, resistance intervention exercise resulted in an increase in individual HNPs and cortisol within human saliva, although the increased levels of HNP and cortisol could be part of the normal stress response. Regardless of the mechanism, it is certain that from the results of previous studies and those of this study that HNPs do play a role in the 'first line of defence' at mucosal surfaces. The aim of this study was to determine whether resistance training alters the salivary levels of HNPs and this has been established in both studies.

Chapter 7 - General Discussion

This work has shown that LC-MS can be used to relatively quantify the structural homologues HNP1-4. Physiological stress can elicit changes in hormones and AMPs (individual HNPs), particularly in response to exercise. The developed and validated LC-MS method used to detect individual HNPs makes a novel contribution to sports and clinical research. The studies presented in this thesis are the first to show optimisation of the LC-MS technique for individual salivary HNPs, revealing the impact of physiological changes to AMPs after exercise, and also comparing the relationship between salivary HNPs and cortisol. There were two main reasons for pursuing this research; firstly, ELISA is frequently used in research studies to determine levels of salivary peptides (sum response of HNP1-3), whilst a validated and specific LC-MS method to study individual salivary HNPs has not been developed. Secondly, investigations into changes resulting from physical stress have mostly been conducted using the cortisol and IgA.

The literature review in Chapter 1 stated that RIA and ELISA have both been successfully used in various fields (biomedical or clinical studies) for analysing levels of AMPs (HNP1-3) present in various biological fluids (mainly plasma and serum). In comparison to LC-MS, almost similar sensitivity is provided by ELISA. To date, no controlled study has optimised sample preparation, LC- MS conditions (column performance, phases of mobile and ion intensity), and validated a method for individual salivary HNP detection. The main objective of this research was to assess levels of individual HNP1-4 by LC-MS following physiological stress. To achieve this, sample preparation (Chapter 3) and the LC-MS method (Chapter 4) were optimised. Furthermore, an LC-MS method was validated and cross validated with ELISA (Chapter 5). The key findings from each chapter are discussed below.

7.1 Thesis Findings

A selective and sensitive LC-MS method for the detection of four individual peptides (HNP1-4) has been developed and validated, and the need for sample clean-up when detecting HNPs in a complex biological matrix as saliva was highlighted in Chapter 3. Matrix effects are the alternation of ionisation efficiencies in the presence of co-eluting substances. Without SPE treatment, the matrix effects contributed to a reduction in the detection of salivary HNP1-4 by 17-65%, as interfering compounds compete for charge and suppress the signal of the analyte of interest. Ion suppression or enhancement represents a major challenge in MS quantitative

analysis (Trufelli *et al.,* 2010). A significant difference was observed which was related to sample preparation (SPE against without SPE); therefore, it was concluded that appropriate cleaning (with SPE) should be untaken prior to analysis. No ion suppression was caused through the use of an internal standard peptide (Leu-Enkephalin), and the extraction recovery data falls within the > 80% range following (U.S. Department of Health 2001).

In addition, eight different SPE extraction solvent mixtures were investigated. These solvent mixtures effected the extraction of the peptides differently depending on the pH of the buffers. The effect of the extraction (recovery) was determined by assessing the peak area (EIC), and an increased peak area for individual HNPs with a pH range between 2.48 to 2.91, was observed for MeOH: H_2O (50:50) + 1% FA; MeOH + H_2O (50:50) + 1% AC; MeCN + H_2O (50:50) + 1% FA and MeCN + H_2O (50:50) + 1% AC. According to the data, a significant difference ($p > 0.05$) between pH ranges of 2.48 to 2.91 and 3.01 to 3.91 was noted.

Indeed, the overall charge state $[M + 3H]^{3+}$, $[M + 4H]^{4+}$ and $[M + 5H]^{5+}$ of the HNPs changed by one unit between pH 2-4. The pKa of the C-termimus (approximately 2.0) and glutamic acid (4.15), and for example changes to the pH, affected the overall charge state of the defensins. The extent of the ionisation/charge state affects the polarity of defensins, as well as their ability to interact with the stationary phase.

To develop an LC-MS method that determines the relative level of individual salivary HNPs it was necessary to optimise the separation efficiency and the ion intensities of the defensins. During the LC-MS optimisation, mobile phase solvent composition was shown to influence the analyte ion intensity profile. Six different mobile phase compositions were investigated (Chapter 4), and the data (ion intensity profile) suggests that a MeOH: H_2O solvent is the preferred solvent compared to MeCN: H_2O. During electrospray ionisation, the protic nature of MeOH promotes ionisation by providing a hydrogen ion for binding, which improves the ionisation of less ionisable compounds, and increases the analyte signal (Smith *et al.,* 2002).

The pH of the mobile phase solvent additives is a further factor which influences chromatographic performance and ionisation. Decreasing the mobile phase pH had a negative effect on the ion intensity profile (Chapter 4; Figure 4.8-4.9). It is also important to choose the most appropriate stationary phase and particle size. Higher separation efficiency was produced by smaller particle columns, suggesting that a Kinetex C8 column (2.6 µm) should be selected (Shen *et al.,* 2002). HNP4 was not detected in these samples; however, a similar performance

to HNP1-3 is expected due to the 33% sequence similarity compared to HNP1. The level of salivary HNP4 is 100 fold lower compared to HNP1-3. During column optimisation the sample could not be concentrated enough to detect HNP4, as a large sample volume was needed for repeat experiments (n= 4). However, for validation purposes, the sample was concentrated and all four defensins were detected. Optimisation of the gradient for the elution of HNPs was also evaluated in this study. When analysing HNPs with a shorter gradient (30 minutes), an unidentified peptide causing isobaric effects was noted due to the complexity of the saliva matrix. Hence, for separating individual HNPs a medium length (45 minutes) gradient was found to be appropriate, as the interfering ion was separated from the defensins. A longer gradient profile (70 mins) did not significantly improve ion intensity profile further.

The optimised LC-MS method was validated and the LOD for HNP2 was 0.05 ng/µL. This is sufficient for the purpose of this study and the sensitivity was comparable to ELISA. However, an increase in LOD may be achievable by performing MS/MS or using a different mass analyser, for example an ion trap or triple quadrupole. Other validation parameters were determined and these all fell in the acceptable range within the ICH Q2B guidelines for complex biological fluids.

An internal standard (Leu-Enkephalin) was spiked into the saliva to control for variation in injection, sample preparation, instrument parameters and matrix effects (Magni *et al.*, 2001). To date this is the first validated method for the analysis of individual HNP1-4 in saliva matrix. A pilot study was conducted and significant variation was observed in the levels of individual HNP1-4 from both female and male participants. Variation was also observed in the levels of individual HNPs in saliva among individuals in other studies utilising the LC-MS method (Goebel *et al.*, 2000; Cabras *et al.*, 2010). Furthermore, as the number of participants was low, an extension of this study would be helpful in determining if a larger population would still reflect the observed trends. When the data obtained from LC-MS versus ELISA were taken into account, both methods showed equal sensitivity while detecting the average response of HNP1-3 (Chapter 6).

The validated LC-MS method was used to study the change in the physiological levels of individual HNP1-4 following physical stress. Immunity has been reported to be weakened up to 72 hours after intensive exercise, i.e. more than 90 minutes (Niemen *et al.*, 2005). Two studies were developed and in the first exercise study the level of individual HNPs were evaluated in athletes given a placebo or CHO supplement, while in the second study levels of

individual HNPs were measured in the participants who received CHO or MTN supplementation.

According to the post interval (30 and 60 min) data, when compared to the pre interval data following ingestion of the placebo, a significant increase in the levels of HNPs was observed after 60 min of exercise. The results indicated that the HNP response occurs after only 30 mins and increases at least up to 60 mins after exercise. It will be necessary to repeat this study with longer post intervals (up to 24 hours) in order to assess whether the increase is transient and to assess when defensin levels returns to base level.

For example, in the case of IgA, levels were found to return to baseline following 18 hours of rest (Fedricks *et al.,* 2012); however, this will depend on the type and intensity of exercise performed. Interestingly, when supplementing with CHO, the HNP levels only increased significantly up to 30 mins post exercise, but this increase was significantly lower compared to the increase observed in athletes receiving the placebo. This indicates that a CHO supplement is beneficial for reducing inflammation induced by stress/exercise. The standard error is rather large and an increase in participant numbers is recommended for future studies. In addition, salivary cortisol levels (measured by ELISA) were significantly increased in athletes consuming either the placebo or CHO supplement at both 30 and 60 mins post exercise.

Following on from the findings from the first study, the second study was designed to explore the effects of acute stress on levels of individual HNPs in athletes supplemented with CHO versus MTN. Although the standard deviation is very large, a similar trend was observed as in the first study, where participant consumed CHO and the levels of HNPs seemed to increase 30 min post exercise but not at 60 mins post exercise. Likewise, supplementing with MTN did not significantly change HNP levels; however, a clear trend was observed whereby HNP1-3 levels seem to increase but not to the same extent as when supplementing with CHO even though the differences between CHO and MTN are not significant.

This could indicate that supplementing with MTN also has an enhanced effect on the immune response after intense exercise compared to supplementing with CHO alone. The carbohydrate source was same in the two formulations. The underlying biological mechanisms for the suppression of HNP levels when supplementing with either CHO or MTN is unclear; however, it is known that supplementing with carbohydrates during exercise has been shown to attenuate immunosuppression by better maintaining glycaemia and reducing the magnitude of stress

hormone release (Nieman *et al.,* 2001; Pedersen *et al.,* 1997; Gomes *et al.,* 2014). In addition, excessive neutrophil activation, for example via acute or prolonged physical stress, is known to result in superoxide production, which can lead to local tissue inflammation/tissue damage (oxidative stress) (Khanfer *et al.,* 2010; Mayadas *et al.,* 2009). This is an important line of investigation for future research into acute stress and the individual function of α-defensins, as longer term neutrophil activation are known to be detrimental to health.

The above findings are novel and provide clear evidence that individual HNP functions are sensitive to psychological stress. Indeed, these two studies have shown that human neutrophil functions are affected by CHO and MTN supplementation during exercise. Nevertheless, the lack of an association between the reduction in response of individual HNPs and responses to CHO and MTN supplementation during acute stress make it difficult to further elucidate the mechanisms underlying this. It is also difficult to determine which ingredient(s) contained within the products may be inducing the ergogenic outcome.

Given that limited research has focused on psychological stress effects on innate immunity, the replication of this research with a greater number of participants is necessary to confirm the current findings. There has been speculation concerning the role of the neuroendocrine system; specifically, the HPA axis and the sympathetic-adrenal-medullary axis, and the function of neutrophils (Khanfer *et al.,* 2012). Therefore, one objective of this study was to evaluate a possible correlation between the salivary hormone cortisol and HNPs following physiological stress.

Khanfer (2011) reported that stress, as expected, induced significant increases in cardiovascular activity. Despite examining potential neuroendocrine mediators, such as cortisol activation with stress, the observed changes were not associated with neutrophil function changes. Transient acute stressors might both enhance and decrease these neutrophil functions, thus the specific underlying mechanisms driving the changes in neutrophil function in response to psychological stress remain unknown. Further research is needed to replicate these findings; specifically, with a greater focus on stress hormone and the functions of individual HNPs. This would confirm the involvement of the HPA axis, and hormone and HNP functions during psychological stress exposure.

7.2 Limitations of the Study

In both studies the size of the sample was comparatively modest, as it was challenging to recruit athlete participants. Consequently, the findings need to be considered in light of this limitation. Further study and the duplication of this research is essential, particularly when considering that to date, only a few studies have studied HNP physiological levels during physical stress (Davison *et al.,* 2009; Gillum *et al.,* 2015; Kunz *et al.,* 2015). Undoubtedly, the level of the stress hormone cortisol and salivary peptides (HNPs) showed changes induced as a result of physical stress; however, it remains unclear how the levels of HNPs and hormones are altered by CHO and MTN supplementation. There is a need to explore the specific underlying mechanisms that supress levels of HNPs and hormones after supplementation compared to placebo levels. The present findings could be enhanced by studying the effect on HNP levels after longer post exercise periods (up to 24 hours) and analysing if salivary cortisol responses can be delayed.

Chapter 8 - Conclusions

The significance of the analysis of salivary AMPs after physical stress, and the need to establish a LC-MS approach was determined in this study. The examination of levels of salivary HNPs in different fields of research, including biomedical or sports science, has until now been ELISA based. Hence, the development of an LC-MS method offers the ability to investigate the response of individual salivary HNPs. The method optimisation consisted of sample preparation optimisation, LC-MS method (column performance, mobile phase solvents and additives) optimisation, and LC-MS method validation. HNPs were detected via LC-MS, as implemented by other researchers, and where there was a lack of a standardised approach (for instance, SPE separation effectiveness, mobile phase solvent choice, additives, column performance, inter-intraday exactness, and retrieval), then constancy and cross- validation has been established and authenticated via ELISA for the examination of individual salivary HNP1-4 levels. Some gaps in the existing research were identified, including the examination of individual salivary HNP levels after physical stress with CHO and MTN supplementation. The level of cortisol was also compared with HNP levels.

The established LC-MS method was specific and sensitive for salivary HNP analysis. A list of the conclusions obtained from the findings is presented below:

1. Optimising the sample preparation resulted in an improvement in the peak area (EIC) for individual HNPs. The SPE washing phase and the use of organic solvents and additives can affect the ESI response. A significant difference ($p = 0.05$) in the ESI response for the pH range of 2.48 to 2.91 was observed when using various washing phase organic solvents and additives. Aqueous 1% AC: MeOH (50:50) at pH 2.8 was found to yield the largest extracted ion chromatogram peak area for individual HNPs.

2. Speed vac drying of SPE fractions prior to sample analysis resulted in a significant increase in the EIC peak area for all HNPs ($p < 0.05$) compared to drying the fraction manually under a flow of nitrogen gas.

3. The separation of individual HNPs can be enhanced by modifying the LC mobile phase gradient. A shorter gradient resulted in the co-elution of peptides causing isobaric interference during MS analysis. Hence, a 45 min gradient is needed for the separation of HNPs.

4. Mobile phase solvents, additives, and column performance are important factors to consider when optimising the ESI response. Three mobile phase solvents (with three differing additives) and two different stationary phase columns (with two differing dimensions) were tested and the optimal column (in terms of increased ion intensity) was selected. A significant increase in ion intensity was observed for individual HNPs with the Kinetex® C8, 2.6 µm column using a mobile phase of 0.1% (v/v) AC in H_2O and B: 0.1% (v/v) AC in MeOH, after assessing six different mobile phases.

5. The LC-MS method was validated following ICH guidelines. The LOD was determined to be 0.05 ng/µL and extraction recovery was 80-91%. In addition, a good correlation between ELISA and LC-MS (R^2=0.963) supports that individual salivary HNPs are being measured accurately and precisely via LC-MS.

6. The level of individual HNP1-4 were found to be increased for up to 60 mins after exercise. The increase, i.e. the activation of neutrophils and therefore the release of defensins, was minimised in athlete supplemented with CHO or MTN compared to athletes who received a placebo; this could indicate reduced airway inflammation. A correlation between salivary HNPs and cortisol levels was not detected, but this could be due to a delayed cortisol response.

8.1 Future Research

An ultra-performance liquid chromatography (UPLC) based method could accelerate the analysis, and future work could investigate method transfer compatibility from HPLC to UPLC. The importance of sample cleaning was briefly discussed in Chapter 4, but despite analyte recovery and sample cleaning exhibited by SPE, this method was time consuming. Future work could investigate the compatibility of automated SPE in reducing sample preparation time. In addition, a full scan range (100-2000) *m/z* was used in this study and therefore other analytes within this *m/z* range could be investigated to assess their changes after physical stress. Other innate immunity modulators (AMPs), such as β-defensins and cathelicidins, could be investigated in future work in order to understand them in more depth. The exercise time interval was seen to affect the association between hormone and AMPs, although this has not yet been proven. An increase in levels after exercise was illustrated by both cortisol and AMPs but no correlation could be identified. Consequently, additional research with longer exercise time intervals would be beneficial.

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Appendices

Appendix A – Informed consent form

To be completed by the participant	
I have read the information sheet about this study	YES/NO
I have had an opportunity to ask questions and discuss this study	
I have received satisfactory answers to all my questions	YES/NO
I have received enough information about this study	
I understand that I am free to withdraw from this study:	YES/NO
• At any time	YES/NO
• Without giving a reason for withdrawing	
• (If I am, or intend to become, a student at the University of Greenwich) without affecting my future with the University	YES/NO
• Without affecting any medical or nursing care I may be receiving.	YES/NO
I agree to take part in this study	YES/NO
Signed (participant)	Date
Name in block letters	
Signature of investigator Nadia Ashrafi	Date

This project is supervised by: Dr Fernando Naclerio
Tel – 020 8331 9800 **Email –**f.naclerio@gre.ac.uk

Appendix B - Health Questionnaire

CONFIDENTIAL

Pre-test Health & Physical Activity Questionnaire

Date:	Sex: .....
Name:	Address: .....
D.O.B.....	.....
Tel. No:	.....
Fax No:	.....
E. Mail:	Postcode: .....

Please circle when appropriate

1. Do you, or have you ever smoked? Yes/No

   If yes please state the number/day..... Or when stopped.....

2. Do you drink alcohol regularly? Yes/No

   If yes how many units/week? (1/2 pint = 1 units).....

3. Have you consulted your general practitioner with the last 3 months? Yes/No

   If yes please give details.....

4. Are you on any medication at present? Yes/No

If yes please state which and for what.....

.....

5. When was the last time you had a medical check-up?

6. Have you ever suffered from?

Any heart condition Yes/No

If yes please specify.....

High blood-pressures (>140/90) Yes/No Fainting Yes/No

Heart or chest pains Yes/No Anaemia Yes/No

Family history of heart of vascular disease Yes/No

If yes please specify.....

High blood cholesterol (>5.2mmol/L) if known Yes/No

Any blood condition Yes/No

If yes please specify.....

HIV, Hepatitis A, B or C, Venereal Disease, Haemophilia, Any other

Respiratory problems (asthma, bronchitis, etc.) Yes/No

If yes please specify.....

Diabetes - NIDDM or IDDM (please circle) Yes/No

Epilepsy Yes/No

Cancer Yes/No

If yes please specify.....

7. Are you currently injured Yes/No

If yes please specify.....

8. Have you been ill within the last 3 weeks? Yes/No

If yes please specify.....

9. Have you ever "over-reached", had overtraining syndrome or chronic fatigue Syndrome? Yes/No

If yes please specify.....

10. To your knowledge are there any health related reasons for not Undergoing the tests that have been explained to you? Yes/No

If yes please specify.....

11. How many times do you exercise every week.....?

12. Do you weight train? (Frequency –number of times per week?)
……..

Play games? (Frequency –number of times per week &which).....

Swim, run or cycle? (Frequency, which & how long for each time?) .....

13. Are you out of breath during exercise; (always?) 10----------5----------0 (never)

14. Are there any relevant factors? .....

.....

15. Height (metres)..... Weight (kg).....

Signature of participant: **Date:**

Signature of researcher: **Date:**

RISK ASSESSMENT (ACSM Guidelines)

No. of Cardiopulmonary signs / symptoms ……

No of Risk Factors ……

Recommendation ……

Assessor's signature

Appendix C - Participant information sheet exercise study

Research Information Sheet for Participants Involved in the University of Greenwich Nutritional supplement Study – The effect on acute recovery from a single bout of whole body resistance exercise due to supplementation

Firstly thank you for agreeing to take part in this research project. I hope you find the information given here useful and adequately informative for your participation in the project. The research aims to monitor differences in performance due to supplementation with a commercially available product.

Procedures

You will initially attend the laboratory/sports hall at the University of Greenwich at Medway campus for an explanation of the study. This will take approximately 40 minutes and will involve:

- Assessing your suitability to the study and to complete the health questionnaire and informed consent form.
- This is also an opportunity for you to ask any questions that you may have.

The following meeting, should you agree to take part in the study, will see you return to the laboratory to perform a 2 familiarisation session (on separate days) on the squat, bench press, pull, abdominals and other common resistance exercises. You will then attend the laboratory again for another 4 occasions here you will have to perform 1 crossfit training session involving 3 circuits of 7 exercises.

Before and after this session you will be assessed in jump, maximal strength and muscular endurance capacity using two exercises: bench press and squat. Additionally salivary samples will be taken before, 30 and 60 minutes after crossfit or aerobic endurance exercises

Measurements

- Jump test on an force plate
- 1 repetition maximum on Squat, and bench press exercises
- Power at 50% of 1 repetition maximum on Squat, and bench press exercises
- Rate of perceived exertion
- Salivary markers antimicrobial peptide and hormone (using ELISA technique and LC-MS)

Requirements

On the day of testing a standardised meal should be consumed 3 hours prior to the exercise test and nothing else, except from 500ml of H_2O which is requested to be consumed 1 h prior to testing. Ergogenic aids are to be stopped at least 6 weeks prior to the study and abstained from during the course of the study except for those provided by the researchers. Caffeine is to be avoided 4 days prior to testing. Participants are instructed to abstain from any vigorous and unaccustomed physical activity 72 hours before testing with no exercise 24 hours before testing and arrive in the laboratory in a rested state.

Possible Risks/Discomforts

The risks involved in the study are minimal. Prior to the study you will have filled out a health questionnaire to assess your suitability for participation in the study. This study does involve strenuous exercise, but providing you perform in regular physical activity the sensation you experience will not be unfamiliar. Further, you will be instructed to exercise until volitional fatigue. All supplements contained in the commercially available multi nutrient formula 'Cyclone' are dosed within recommended amounts.

You are free to withdraw from the study at any time.

Benefits

You will increase your knowledge of nutrition in relation to exercise,

Confidentiality

Your contact information and testing results (held anonymously) will be stored on a USB memory stick which will be stored in a secure location. This information will be deleted at the end of the study.

I look forward to seeing you, if there are any problems please do not hesitate to call me on the email or telephone number below:

Supervisor: Dr Fernando Naclerio

Email: f.j.naclerio@gre.ac.uk

Appendix D - Participant Food Record Diary

CONFIDENTIAL

Name and Address

_____
_____
_____
_____
_____
_____

Food Record Diary

Please record everything that you eat and drink including supplements over a 3 day period in accordance with the dietary guideline provided.

Instruction for using the food diary

Everything that you eat and drink over a 3 day period should be recorded below (two week days and one weekend day), giving portion sizes, weights and volumes. Many food packaging has the weight on the labelling so if you have half a pack, then record half the weight, etc. and if possible collect clean food labels and bring them to the researcher. This will enable the researcher to analyse your diet. Please record the cooking method (boiled/grilled/fried etc.) and whether the food is fresh, canned, frozen or dried.

Time	Description of Food Eaten	Cooking method	Weight/portion size/volume
18.00	Pasta	Boiled	100g
	Chicken breast	Baked	125g
	Mixed frozen veg	boiled	80g
	Pasta sauce (tomato)	Jar	200g
18.00	Pasta	Boiled	Medium
	Chicken breast	Baked	Large
	Mixed frozen veg	Boiled	Medium
	Pasta sauce (tomato)	Jar	Half a jar

Appendix E - Poster Presentations

INVESTIGATION OF A POTENTIAL CORRELATION BETWEEN SALIVARY ANTIMICROBIAL PEPTIDES (α-DEFENSINS) AND CORTISOL AFTER PHYSICAL STRESS

Nadia Ashrafi

Fernando Naclario, Marcos Seijo, Chris Lapthorn, Patricia Wright, Frank Pullen, Birthe Nielsen,
Faculty of Engineering and Science, University of Greenwich, Central Avenue, Chatham, Maritime, Kent ME4 4TB. U.K
Email: an35@gre.ac.uk

Poster Number 073

AIMS AND OBJECTIVES: Numerous studies have investigated the effect of exercise on mucosal immunity but the focus has mainly been on salivary immunoglobulins (especially different forms of immunoglobulin A), lysozymes, cytokines, hormones (Gleeson *et al.,* 2000; Mackinnon *et al.,* 1993). It is well known that physical and mental stress provoke the release of cortisol from hypothalamic pituitary adrenal axis, by which stress can modulate various immune responses. The effects of physical stress (exercise) in mucosal secretions that contribute to innate immunity are virtually unexplored. Here we investigate a potential correlation between the stress hormone cortisol with antimicrobial peptides (α- Defensins) after physical stress. See (poster 069) for role of α-Defensins in innate immunity, physical stress and (poster 071) for the method development.

BACKGROUND

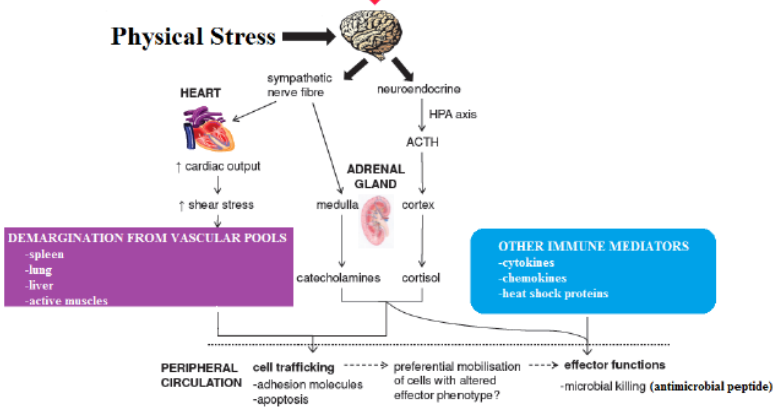
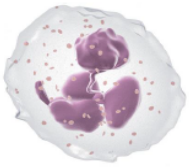


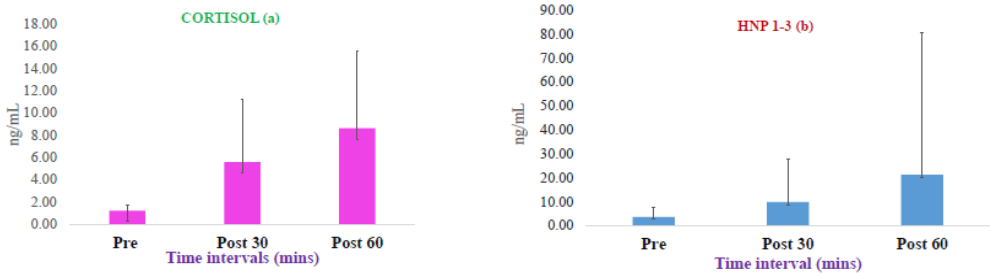
Fig.1. Schematic diagram of how physical stress affects the various parameters of human body and innate immunity.

WHY COMPARE STRESS HORMONES WITH ANTIMICROBIAL (α- DEFENSINS) PEPTIDE LEVELS?



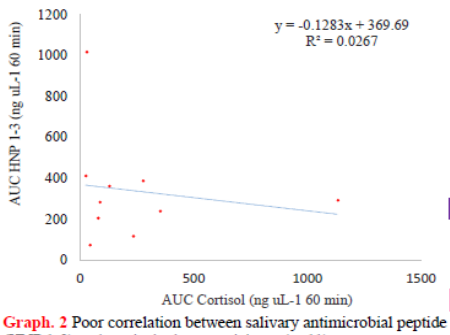
Neutrophils are the most abundant (70%) white blood cells, which secretes neutrophil α- Defensins (HNP1-3). Neutrophils are formed from bone marrow. In response to stress, the endocrine system, governed by the hypothalamus-pituitary-adrenal (HPA) axis, releases cortisol which binds glucocorticoid receptors in white blood cells to mediate the stress response.

CORTISOL AND ANTIMICROBIAL PEPTIDES (HNP 1-3) RESPONSE AFTER PHYSICAL STRESS



Graph. 1 (a). The bar chart represents a transient increase of cortisol and (b) a transient increase of HNP1-3 after exercise where, participant (n=11). Levels determined in saliva by ELISA.

CORRELATION BETWEEN CORTISOL AND HNPS



Graph. 2 Poor correlation between salivary antimicrobial peptide (HNP 1-3) and cortisol where participant (n=11).

DISCUSSIONS

Both cortisol and antimicrobial peptide showed a transient increase after exercise however no significant correlation were observed between cortisol and the HNPs 30 or 60 mins after exercise. To investigate a potential time delay in the responses further studies must be carried out. Singh (2000) demonstrated that a compensation may occur in mucosal fluids due to synergism between substances. Testosterone response was also investigated however, no correlation with the HNPs were observed (results not shown) post 30 or 60 mins after exercise.

CONCLUSION

The transient increase of HNPs could be affected by cortisol (synergism) or the exercise-induced airway inflammation. The increase levels of HNP could also be the part of the normal stress response.

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ACKNOWLEDGEMENTS

I would like to thank the British Mass Spectrometry (BSMM) for their fund and special thanks to my mass spectrometry and sports group.

EVALUATION OF SALIVARY ANTIMICROBIAL PEPTIDE (α- DEFENSINS) IN RESPONSE TO PHYSICAL STRESS BY LC-ESI-MS

Poster Number 069

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ROLE DO DEFENSINS IN INNATE IMMUNITY

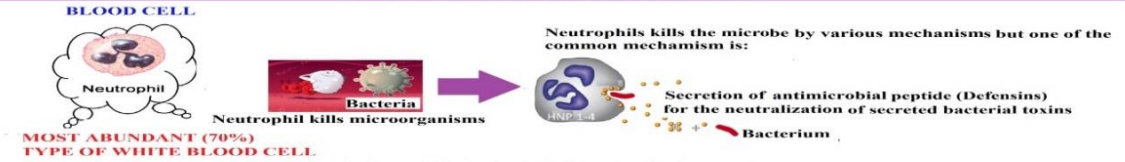


Fig.1. Defensins are multifunctional molecules that are part of the immune system.

RESPONSE TO STRESS

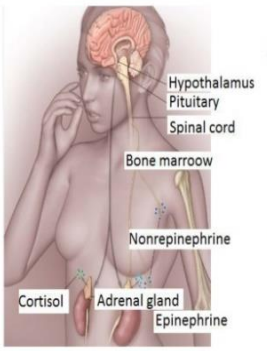


Fig.2. Correlation of stress and innate immunity modulator (α- Defensins).

In response to stress, the endocrine system, governed by the hypothalamus-pituitary-adrenal (HPA) axis, releases of cortisol which binds glucocorticoid receptors in white blood cells to mediate the stress response.

Neutrophils are formed from bone marrow. The most abundant white blood cells which secretes neutrophil α- Defensins (HNP 1-4).

AIMS AND OBJECTIVES

Considering the biological importance of antimicrobial activity of human neutrophil α-defensins (HNP 1-4), an optimised LC-MS assay **(See poster 071)** for the measurement of individual HNPs in saliva will aid in exploring whether HNPs biomarker has an impact on physical stress during a circuit training intervention exercise. To date, no studies have systematically investigated the effect of acute (min to hours) and chronic (days to weeks) change in salivary α-defensins family before and after circuit training exercise. Four salivary defensins were detected by Liquid Chromatography-Mass Spectrometry (LC-ESI-MS) and the effect on their levels after a circuit training exercise was investigated.

RESEARCH QUESTION: DOES PHYSICAL STRESS AFFECTS THE LEVEL OF DEFENSINS?

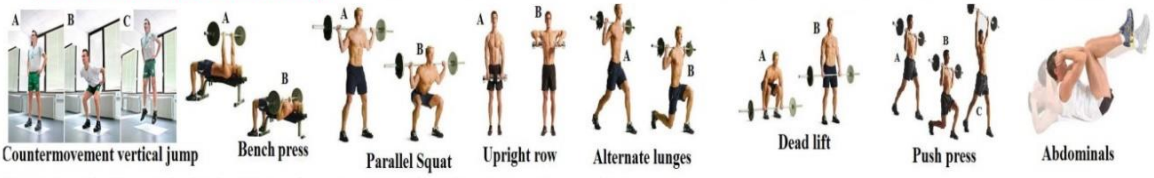
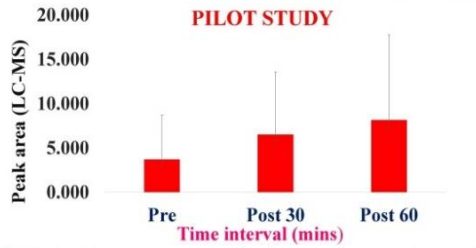
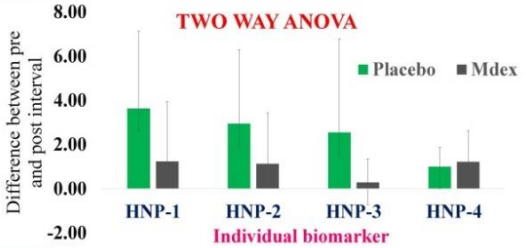


Fig.3. Schematic diagram of a circuit training performance exercise with resistance (external overweight).

EXPERIMENTAL RESULTS



Graph. 1. The bar chart represents a transient increase of HNP 1 where, participant (n=11) has consumed placebo drink. Similar trend has been observed for HNP-2, HNP-3 and HNP-4



Graph. 2. The bar chart represents the mixed effect model (two way ANOVA) data of HNP 1-4 for two different weeks where, participant (n=11) has consumed placebo and multidextrose (mdex) drink. .

DISCUSSION

The data reveals that the difference between pre and post exercise intervals is significant; $p < 0.03$ of HNP 1-4 for both drinks. Placebo and multidextrose (mdex) data shows that increases in salivary HNP levels may be a part of the normal stress response. However, mdex data shows decreased level of HNPs compared to the placebo data which could possibly be a result of multidextrose reducing air inflammation by providing a source of energy and reducing the stress level.

CONCLUSION

LC-MS assay demonstrates that HNP 1-4 could be used as potential stress biomarkers and the LC-MS assay is potentially more specific than ELISA assays, thus providing a more accurate readout of stress levels. HNP levels for athletes drinking multidextrose were reduced post-exercise (relative to placebo) indicating a reduction of the immune response.

CLINICAL IMPLICATIONS

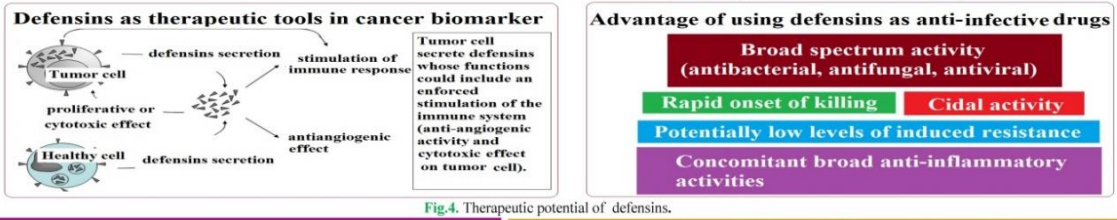


Fig.4. Therapeutic potential of defensins.

REFERENCES

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ACKNOWLEDGEMENTS

I would like to thank the British Mass Spectrometry (BSMM) for their fund and special thanks to my mass spectrometry and sports group.

VALIDATION OF AN LC-ESI-MS METHOD FOR THE DETECTION OF HUMAN SALIVARY α- DEFENSINS

Nadia Ashrafi
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Poster Number 071

AIMS AND OBJECTIVES

Antimicrobial peptides (α- Defensins) are commonly measured by ELISA, however, this technique does not discriminate between HNP1-3 due to their structural similarities (Fig.1). With advances in mass spectrometry, Liquid Chromatography-Mass Spectrometry (LC-ESI-MS) is often considered as a first choice for analysis of salivary peptides. The main objectives of the study were to optimise and validate an LC-ESI-MS method for the separation of individual HNPs in human saliva by LC- ESI-MS which includes sample preparation. HNP-1, HNP-2 and HNP-3 have identical amino acid sequences with the exception of an additional amino acid in the N-terminus of HNP1 (A) and HNP 3 (D). The α-defensins have three β strands and two loops created by the disulphide bridges between cysteine 2 and 4 and cysteine 3 and 5. A third disulphide bridge exists between cysteine 1 and cysteine 6. **See (poster 069) for the role of α- Defensins in innate immunity and in physical stress.**

HNP-1 ACYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP-2 CYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP-3 DCYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP-4 VCSCRLVFCRRTELRVGNCLIGGVSFTYCCTRV

Fig. 1. α- defensins (HNP1-4) sequence and 3D structure of α- defensins.

METHOD AND EXPERIMENTAL CONDITIONS

SAMPLE PREPARATION

Unstimulated whole saliva was collected and solid phase extraction was used to clean up the sample before LC-ESI-MS analysis.

LC CONDITIONS

Column: Kinetex C8 (3 x 50 mm, 2.6 µm)
Mobile phase:
A: H_2O + 0.1% CH3COOH (v/v)
B: MeOH + 0.1% CH3COOH (v/v)
Flow rate: 0.6 mL/min
Injection volume: 10 µL
Column temp: 35ºC

LC CONDITIONS

Gradient:

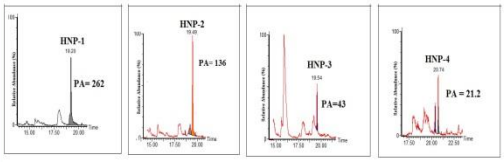
Time	% B
0	2
2	2
19	50
21	95
23	95
25	2
45	2

MS CONDITIONS

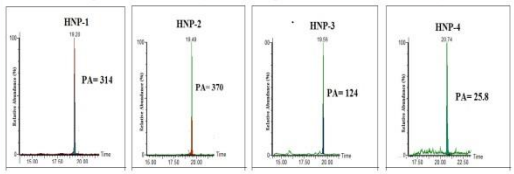
Ionisation mode: ESI +
Capillary Voltage: 3.8 kV
Sampling Cone: 40.0 V
Extraction cone: 4.1 V
Source temp: 150ºC
Desolvation temp: 500ºC
Cone gas flow: 100 L/hr
Mass range: 100-2000 *m/z*
Software: Mass Lynx and Magtran

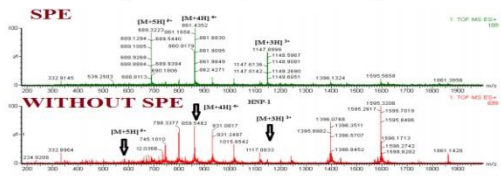
SAMPLE PREPARATION OPTIMISATION

Without solid phase extraction (Chromatogram for individual HNPs)



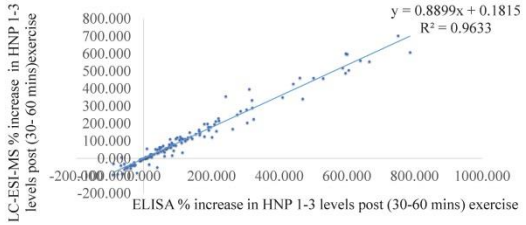
With solid phase extraction (Chromatogram for individual HNPS)





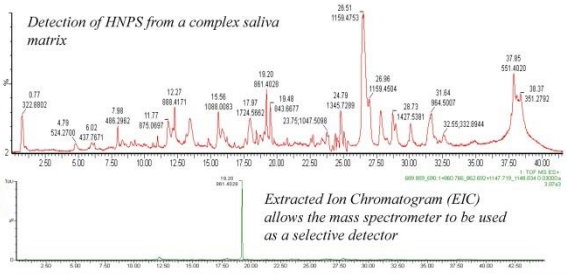
A comparison of the effect of SPE and without SPE treated saliva on the multiple charge states for HNP-1 where m/z 1148, 861 and 689 in the ESI mass spectra corresponding to[M+3H] 3+, [M+4H] 4+, [M+5H] 5+. In the SPE treated sample the ions at m/z 861 and 689 and 1147.89 are the most intense ions whereas these ions are more difficult to observe in the (without SPE) treated sample due to the presence of co-eluting components. Similar cleaner mass spectrum has been observed for HNP-2, HNP-3 and HNP-4.

LC-MS VS ELISA (CROSS-VALIDATION)

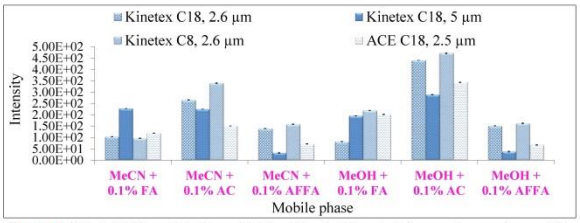


Graph 2: Correlation between LC-MS and ELISA data (n= 210). The slope is not equal to 1, which demonstrate that although there is a correlation but the two measurements do not agree at all. It is worth noticing that the LC-ESI-MS data (combined increases in HNP1-3 levels post resistance exercise intervention) on average tended to be higher than the levels determined by ELISA. This observation could be due to the fact that the saliva samples were stored (-80ºC) for longer between collection and analysis.

IDENTIFICATION OF HNPS



METHOD OPTIMISATION (LC-MS SEPARATION)



Graph 1. Effect of LC mobile phases (0.1% v/v) and different stationary phase columns (C8, C18) on ESI response of HNP-1. *N*= 4, bars represent ± std. Similar trend has been observed for HNP-2, HNP-3 and HNP-4. The results indicated that the ESI response for HNPs in saliva matrix is affected by the mobile phase solvent and additives. Methanol with acetic acid provides an enhancement of ion intensities signal was observed for individual HNPs. The Kinetex® C8 column, provides better separation compared to Ultra core super C18 ACE® and Kinetex® C18. This method can easily be adopted for similar molecular weight of peptides as HNPs and also for any other biological matrix.

LC-MS METHOD VALIDATION

Table 1: Method validation parameters by LC- ESI-MS (n= 3).

Technique	Peptide	Linearity (HNP-2 Standard)	Precision (%) (Saliva Matrix)	Recovery (%) (Saliva Matrix)
LC-MS	Individual HNP 1-4	0.05-1 ng/mL. (R^2 ~ 0.99)	Interday 0-5% Intraday 11- 14.95%	80-91%

Table 2: Intra- interday precision of HNP 1-4 in human saliva (n = 5) by LC-MS.

		Intraday		Interday	
Defensins	Concentration (ng/mL)	Peak area (mean ± sd)	Peak area (CV%)	Peak area (mean ± sd)	Peak area (CV%)
HNP-1	-	82.08 ± 4.06	4.95	86.88 ± 6.78	7.81
	-	82.00±1.00	1.22	87.84±8.25	9.39
	-	79.00 ±0.81	1.03	85.50 ±9.91	10.75
HNP-2	LQC	53.23 ± 1.67	3.13	58.45 ± 7.38	12.63
	MQC	90.33±0.58	0.64	97.67±10.37	10.62
	HQC	116.33 ± 2.62	2.26	125.67 ± 13.20	10.51
HNP-3	-	43.80 ± 1.61	3.67	48.74± 6.98	14.32
	-	41.05±0.98	2.38	45.69±6.56	14.36
	-	42.00 ±0.81	1.94	46.50 ±6.36	13.69
HNP-4	-	19.17 ± 0.90	4.69	20.09 ± 1.29	6.44
	-	18.37±0.55	3.00	19.85± 2.09	10.54
	-	17.97 ±0.06	0.36	20.04 ± 2.93	14.95

DISADVANTAGE OF USING ELISA

This study confirmed that LC-MS can be used to determine salivary levels of HNP 1-3 with similar sensitivity but higher specificity as the traditional ELISA. The disadvantage of using immunoassay for salivary HNP 1-3 measurement is that the kit is expensive, time-consuming and does not discriminate between the HNP 1-3.

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Appendix F – Publication

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ORIGINAL ARTICLE

Effects of protein–carbohydrate supplementation on immunity and resistance training outcomes: a double-blind, randomized, controlled clinical trial

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Abstract

Purpose To examine the impact of ingesting hydrolyzed beef protein, whey protein, and carbohydrate on resistance training outcomes, body composition, muscle thickness, blood indices of health and salivary human neutrophil peptides (HNP1-3), as reference of humoral immunity followed an 8-week resistance training program in college athletes.

Methods Twenty-seven recreationally physically active males and females ($n = 9$ per treatment) were randomly assigned to one of the three groups: hydrolyzed beef protein, whey protein, or non-protein isoenergetic carbohydrate. Treatment consisted of ingesting 20 g of supplement, mixed with orange juice, once a day immediately post-workout or before breakfast on non-training days. Measurements were performed pre- and post-intervention on total load (kg) lifted at the first and last workout, body composition (via plethysmography) vastus medialis thickness (mm) (via ultrasonography), and blood indices of health. Salivary HNP1-3 were determined before and after performing the first and last workout.

Results Salivary concentration and secretion rates of the HNP1-3 decreased in the beef condition only from pre-first-workout (1.90 ± 0.83 µg/mL; 2.95 ± 2.83 µg/min, respectively) to pre-last-workout (0.92 ± 0.63 µg/mL, $p = 0.025$, $d = 1.03$; 0.76 ± 0.74 µg/min, $p = 0.049$, $d = 0.95$), and post-last-workout (0.95 ± 0.60 µg/mL, $p = 0.032$, $d = 1.00$; 0.59 ± 0.52 µg/min, $p = 0.027$, $d = 1.02$). No other significant differences between groups were observed.

Conclusions Supplementation with a carbohydrate–protein beverage may support resistance training outcomes in a comparable way as the ingestion of only carbohydrate. Furthermore, the ingestion of 20 g of hydrolyzed beef protein resulted in a decreased level and secretion rates of the HNP1-3 from baseline with no negative effect on blood indices of health.

Keywords Immune status · Strength performance · Body composition · Muscle thickness · Blood indices of health

Abbreviations

AST/GOT	Alanine transaminase
AMP	Antimicrobial peptides
ALT/GPT	Aspartate transaminase
BM	Body mass
CHO	Carbohydrate
CK	Creatine kinase
DHEA	Dehydroepiandrosterone
η^2_G	Generalized eta squared
HDL	High density lipoprotein
HNP1-3	Human neutrophil peptides
LDL	Low density lipoprotein
ANOVA	One-way analysis of variance

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