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INVESTIGATION INTO THE ACIDIC PROTEIN FRACTION OF BOVINE WHEY AND ITS EFFECT ON BONE CELLS

A THESIS PRESENTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTERS OF SCIENCE IN CHEMISTRY

AT MASSEY UNIVERSITY,

NEW ZEALAND

BERNADETTE JANE MULLAN

JANUARY 2010

Abstract

Milk is provided to new borns as their first food source and it contains essential nutrients, vitamins and other beneficial components, such as enzymes and antibodies that are required for rapid growth and development of the new born and for sustained growth over time.

Milk contains two main types of proteins; casein proteins and whey proteins. Although casein proteins account for up to 80% of the proteins found in bovine milk, it is the whey protein that has become of high interest because of its bioactive content. Whey, a very watery mixture of lactose, proteins, minerals and trace amounts of fat, is formed from milk when the milk is coagulated and/or the casein proteins are removed from the milk.

Bovine whey protein, including both the acidic and basic fractions (low and high isoelectric point, respectively), has previously been studied *in vitro* (cell based) and *in vivo* (using rats) for its impact on bone to determine if it can help improve bone mineral density and help reduce the risk of developing bone diseases, such as osteoporosis.

Bone is constantly undergoing a remodelling process of being dissolved and reformed and the two main cell types responsible for this bone remodelling process are mature osteoclasts, which dissolve (resorb) bone, and osteoblasts, which reform the bone.

Prior work has shown that acidic protein fractions derived from different sources of whey protein concentrate (WPC) have both *in vivo* and *in vitro* activity on bone, particularly anti-resorptive properties. However, the component(s) which confer activity have not yet been identified. In this thesis, work was undertaken to better understand the analytical composition of three types of WPC (cheese, mineral acid and lactic acid) and their associated acidic protein fractions and relate this to bone activity in the hope of identifying where the activity lies. Bone activity was assessed using *in vitro* screening with osteoblast cells (MC3T3-E1) and osteoclast cells (RAW 264.7).

Comparison of the cell-based bone activity of the parent WPCs and corresponding acidic fractions indicated that the acidic fractions derived from both mineral acid and lactic WPC were superior in their ability to inhibit osteoclast development. Although compositional data was complex and definitive correlations with both bone bioactivities could not be made, it appeared that elements common to both the acidic fractions were a higher proportion of GLYCAM-1 and bone sialoprotein-1 (osteopontin). Further studies to more closely investigate the bone bioactivity of the acidic fractions are warranted.

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To my parents, Philip and Irene, thank you; the sacrifices you have made for me as parents, and the support and encouragement you have given me throughout my life, have allowed me to become the person I am today and for this I will be forever grateful.

Hamish, my husband, your support and patience throughout my study has been greatly appreciated. I know the responsibilities you have taken on to allow me the time to do the long hours needed to get this thesis finished and for that I thank you.

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Abbreviations

α-Lac	Alpha-lactalbumin
AF	Acid fraction
AOAC	Association of Official Analytical Chemists
ASE	Accelerated solvent extraction
ASG	Analytical Services Group
β-Lac	Beta-lactoglobulin
BMD	Bone mineral density
BME	Beta-mercaptoethanol
BSA	Bovine serum albumin
BT	Breakthrough (fraction)
CPPs	Casein phosphopeptides
CR05	Mineral acid WPC 80 manufactured October 2007
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
EPA	Eicosapentaenoic acid (C20:5N-3)
ESI	Electrospray ionization
FCS	Fetal calf serum
FPLC	Fast protein liquid chromatography system
FRC	Fonterra Research Centre, Palmerston North
FSOT	Fused silica open tubular
GLYCAM 1	Glycosylation-dependent cell adhesion molecule 1
GMP	Glycomacropeptide
GP	Gel permeation
GS19	Cheese WPC 80 manufactured February 2008
HBS-EP	10mM HEPES, 150mM NaCl, 3mM EDTA, 0.005% Surfactant P20
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HESI	Heated electrospray ionization
HPAEC	High performance anion exchange chromatography

HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HS15	Lactic acid WPC 80 manufactured March 2008
ICP-OES	Inductively coupled plasma optical emission spectrophotometry
ID	Internal diameter
IDF	International Dairy Federation
IgA	Immunoglobulin A
IGF-I	Insulin-like growth factor-I
IgG	Immunoglobulin G
IgM	Immunoglobulin M
ISO	International Organization for Standardization
KCl	Potassium chloride
LC	Liquid chromatography
LF	Lactoferrin
LOD	Limit of detection
LOQ	Limit of quantitation
L-PC	Lysophosphatidylcholine
L-PE	Lysophosphatidylethanolamine
LTQ	Linear trap quadrupole
MBP	Milk basic protein
MEMα	Minimal essential medium α
MFGM	Milk fat globule membrane
MS	Mass spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCBI	National Centre for Biotechnology Information
NMKL	Nordic Committee on Food Analysis
NPN	Non-protein nitrogen
NZDRI	New Zealand Dairy Research Institute
NZTM	New Zealand Testing Methods
OPN	Osteopontin
PAGE	Polyacrylamide gel electrophoresis
PC	Phosphatidylcholine
PE	Phosphoethanolamine
P*i*	Isoelectric point

PI	Phosphatidylinositol
PP3	Proteose peptone component 3
PP5	Proteose peptone component 5 (β-casein-5-phosphate 1-105/107)
PP8	Proteose peptone component 8
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
PVDF	hydrophilic polyvinylidene fluoride
RANK-L	Receptor activator for nuclear factor κ B ligand
RAW 264.7	Mouse leukaemic monocyte macrophage cell line
RP-HPLC	Reverse phase high performance liquid chromatography
RP-LC-MS/MS	Reverse phase-liquid chromatography-mass spectrometer
SDS	Sodium dodecylsulfate
SM	Sphingomyelin
SPP 1	Secreted phosphoprotein 1
SPR	Surface plasmon resonance
TEM	Transmission electron microscope
TFA	Trifluoroacetic acid
TGF-β1	Transforming growth factor-β1
TGF-β2	Transforming growth factor-β2
TMB	3,3,5,5-tetramethylbenzidine
TN	Total nitrogen
TRAP	Tartrate-resistant acid phosphatase
Tris-HCl	2-Amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride (tris(hydroxymethyl)aminomethane hydrochloride)
Trp Hyd	Trypsin hydrolysate
UF	Ultrafiltered
UV-Vis	Ultraviolet - visible
WPC	Whey protein concentrate
WPC 80	Whey protein concentrate 80
WPI	Whey protein isolate

1. Introduction

1.1 Bone Health and Bovine milk

It has long been known that milk is good for growing bones and has, compared to other food sources, more beneficial effects on bone health (*1-3*). The purpose of this section is to outline the composition of milk, the basic structure and metabolism of bone and the association between milk and bone with reference to the work that has previously been done to determine why milk is good for bones and with specific emphasis on milk whey proteins.

1.1.1 Milk Structure and Composition

Bovine milk is a white opaque liquid that is produced by bovines after giving birth, as with all mammals. Milk acts as the first food source for new born young and provides the nutrition required for rapid growth and development of the new born before they are able to digest other types of foods. Milk is also a method of being able to pass antibodies and other beneficial nutrients, such as vitamins and minerals, from a mother to a new born (*1, 4-6*). The proteins from milk contain all 9 essential amino acids required by humans.

Bovine milk composition consists, on average, of 87.5% water, 4.7% lactose, 3.7% fat, 3.4% protein and 0.7% inorganic minerals (ash) (such as calcium, iron, phosphate, magnesium, and many others) although this can vary for breed, animal feed, location and the stage of lactation of the animal from which the milk is sourced (*2, 7, 8*). Milk also contains enzymes and vitamins (vitamins A, C, thiamine, and many others) as well as dissolved gases, such as oxygen and nitrogen (*9*). Milk can be described as a colloidal suspension of milk fat globules, casein micelles and globular proteins in a watery solution (serum) with lactose, minerals, vitamins and soluble proteins (*10*).

Figure 1 below shows a Transmission Electron Microscope (TEM) image of milk fat globules and casein micelles as found in milk.

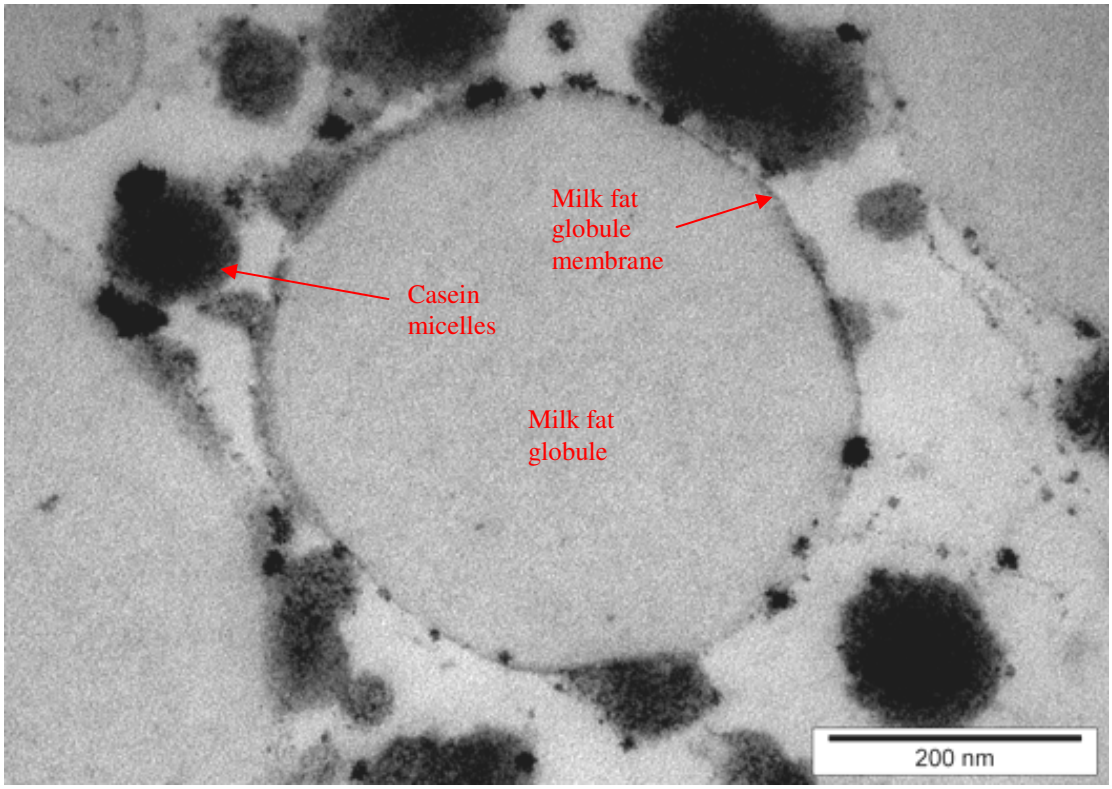

Figure 1: Image showing milk fat globules and casein micelles found in milk. Image, as supplied by Michael Loh, Fonterra Research Centre, Palmerston North, was taken with a Philips CM10 TEM at 130000x magnification.

The milk fat forms milk fat globules, surrounded by a milk fat globule membrane (MFGM), comprised of proteins, polypeptides and lipids. Milk fat globules can range in size from 1.0µm to 8.0 µm in diameter and are formed mainly from triglyceride, which makes up 98% of total milk fat (*11*). C16:0 fatty acid, palmitic acid, is the predominant fatty acid found in milk. Fatty acids are released during metabolism of triglycerides, giving rise to the free fatty acids (*12*).

Milk proteins consist of two main types of protein; casein proteins, the main protein found in bovine milk and constituting up to 80% of total milk protein, and whey proteins (*2*). The caseins occur in micellar form while the whey proteins are found principally in the serum (non-micellar) fraction of milk. Caseins are phosphate-

containing proteins that can be categorised into 4 main types; alpha-s1-casein (α-s1), alpha-s2-casein (αs-2), beta-casein (β), and kappa-casein (κ) (*13*). Casein micelles are formed by the different types of caseins (α-s1, α-s2 and β). A casein micelle is held together by internal calcium phosphate bridges with a surrounding layer of κ-casein that helps maintain the micelle in solution. Caseins are easily digestible and as such make a good source of amino acids for nursing young (*14*). Casein proteins leave the stomach as degraded peptides and are relatively more digestible than whey proteins, which leave the stomach as intact proteins (*14*). Whey proteins can be categorised into 4 major types; α-lactabumin (~20%), β-lactoglobulin (~60%), serum albumin (~7%) and immunoglobulins (IgG, IgA and IgM) (13%) (*15, 16*). Other minor proteins found in milk include lactoferrin, lactoperoxidase, lysozyme and β2 microglobulin. The different types of whey protein have a variety of nutritional and functional attributes that relate to the proteins structure and biological function. Whey proteins are more water soluble then casein proteins and are more susceptible to denaturing due to temperature or pH change (*17*).

Whey is formed from milk when the milk is coagulated and/or the casein proteins are removed from the milk, resulting in a very watery mixture. Whey is a liquid consisting of lactose, proteins, minerals and trace amounts of fat. Approximately 6% of whey is total solids, comprised of ~70% lactose and ~0.7% whey proteins (*18*).

1.1.2 Bone Structure

Bone makes up the body's endoskeleton and provides the structural framework of the body and protection for the body's vital organs (such as the brain or heart) (*19*). Muscles attach to bone and pull against bone to allow the body movement. Bone also functions in the body as a manufacturer of red and white blood cells and as a mineral depository (for calcium phosphate, hydroxyapatite, etc) and energy storage (lipids (fats) stored in the bone marrow). Bone is living tissue that is composed of both organic and inorganic (mineral) material and its structure is made up of other tissues such as endosteum, periosteum, marrow (red and yellow), cartilage, osseous tissue, nerves, collagen and blood vessels. The mineral phase of bone is composed primarily of

calcium phosphate, largely in the crystalline hydroxyapatite form, while the organic phase is constituted predominantly by collagen type I.

Bone mineral density (BMD), often referred to as bone density, relates to the amount of minerals found in bone and how strong the bone will be. Bone consists of two main tissue types; firstly a sponge-like tissue comprising of a lattice network of thin, irregular shaped plates giving rise to a 'honey-comb' structure (known as cancellous bone), and secondly a compact (hard) calcified tissue, that forms the outside surface of bone and gives bone its structure and strength.

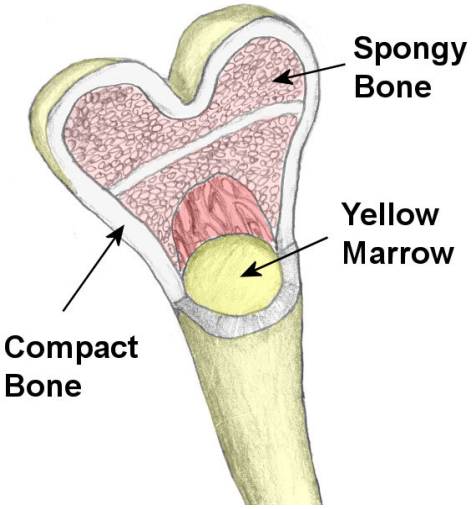

Figure 2: A simple image of a bone taken from http://www.teachpe.com/anatomy/bone_structure.php

Bone is constantly undergoing remodelling, being dissolved and reformed. The main two cell types responsible for this bone remodelling process are mature osteoclasts, which dissolve (resorb) bone, and osteoblasts, which reform the bone, both of which are found in the periosteum (*20-24*).

Osteopontin (OPN), a phosphorylated glycoprotein found in the extracellular matrices of mineralized tissues and at sites of inflammation plays an important role in bone remodelling, in particular in facilitating bone cell attachment to bone, acting as a "biological glue" between old and newly formed bone (*25-27*). OPN is produced by pre-osteoblast cells, when bone formation is initiated, and is imbedded in bones by

mature osteoblasts where pre-osteoclast cells bind to the bone before maturing into bone resorbing osteoclasts (*28, 29*).

There are biological triggers that stimulate either osteoclasts or osteoblasts to form when required, forming a cycle of remodelling such that bone formation is in equilibrium with bone resorption. When this cycle gets out of equilibrium diseases and conditions such as osteoporosis, which results from a loss of bone density, begin to develop (*20, 30*). This can cause major problems with everyday life situations and can even become life threatening, especially since it is normally the elderly who suffer the most from these diseases, although in rare cases the young can also be affected (*31, 32*). Osteoporosis causes the bone to become weak and increases the chance of bone breaking (*33*). This risk increases in the elderly especially as they are more prone to falls than the young. Older women are at a higher risk of developing osteoporosis than men for 2 main reasons. Firstly, at menopause the body stops production of the female hormone estrogen, which can lead to an estrogen deficiency. Estrogen plays an important part in the bone remodelling cycle, as it inhibits the growth of osteoclasts (*34-36*). Hence after menopause bone resorption can begin to exceed bone formation, resulting in rapid bone loss which can lead to osteoporosis (*37*). Secondly, because women tend to have 30% smaller bone structure and mass compared to men, there is an increase in percentage of bone loss (*38*). Between the ages of 20 and 80 an average woman loses one-third to half of her bone density, while for men this is much less (*39*). Women also tend to live longer then men, so not only do women lose bone density faster then men but they have a longer time period over which this occurs, causing more bone loss to occur over a lifetime (*40*).

Calcium is a critical factor for maintenance of bone health and strength. Lack of calcium in the blood stream due to dietary or other disorders can also contribute to the development of osteoporosis. Other triggers include immobilization (i.e. lack of use such as a bone in a cast or a bed ridden patient) (*41, 42*), insufficient intake of nutrients (including magnesium, potassium, vitamin D and vitamin C) (*33, 43-46*), patients suffering from certain diseases and genetic disorders (for example coeliac disease or Turner syndrome) (*40, 47-49*), smoking (*44, 50*) and reduced estrogen levels in both women, as discussed above, and in men (*34, 35, 51, 52*).

1.1.3 Milk and Bone

Bovine milk contains high levels of calcium, but it is not only the high levels of calcium that makes milk good for bones. Milk additionally contains other components, such as lactose and milk protein, that can improve the absorbance of calcium for the body, thus effectively improving the bioavailability of the milk calcium for bone (*53*). Milk also contains other nutrients, such as magnesium and vitamin D, that are needed for bone growth and development (*31, 54-57*). Bones are the body's depository of calcium and other such minerals. When the body requires more calcium than is available from the diet, it resorts to resorbing bone to release more calcium into the blood stream for the body's use (*23, 58, 59*). If the diet continues to lack calcium, or other nutrients that assist with calcium absorption (such as Vitamin D), this can lead to bone being resorbed more rapidly than it is being reformed (*31, 60, 61*). Therefore a diet sufficient in calcium can help prevent excessive bone being resorbed. In this respect, milk (and its products such as cheese, yoghurt, etc) is a good source of bioavailable calcium and other nutrients needed to support calcium absorption (*31, 53, 57*).

However, calcium and other minerals and vitamins are not the only components needed for good bone growth and maintenance. Recent research has indicated that lipids, in particular polyunsaturated fatty acids (PUFA), may have an impact on bone growth and development, and in particular may affect osteoclast maturation and stimulation of osteoblast cells (*24, 36, 62-66*). The studies also implicate that a low ratio of n-6 fatty acids to n-3 fatty acids in the diet could be an important factor in maintaining BMD (*24, 67, 68*). Fatty acids play an important role in maintaining signalling between cells and can be found as structural phospholipids in high concentrations in all membranes (*68*). Fatty acids help regulate physiological functions within the body and it is believed that this is why fatty acids, particularly n-3 fatty acids, play an important role in bone remodelling and calcium absorption (*62, 66, 68*).

1.1.4 Milk Proteins and Bone

The health-conferring benefits of whey protein have been widely recognised (*69-71*). There has been a considerable amount of research on the effect of whey proteins on bones and bone cells (osteoblasts and osteoclasts) and whey proteins have been shown

to have a positive impact, not only on bone density and increased energy and force required to break a bone, but also on the rate of bone reformation and osteoblast stimulation (*15, 16, 72-76*). A Japanese company, Snow Brand Milk Products Co., Ltd., one of the largest dairy companies in Japan, manufactures a product from milk called MBP® (milk basic protein) that is made from the basic (alkaline) proteins found in whey. MBP® is claimed to contain several bioactive ingredients that are good for bone health. These are proposed to act by regulating the activity of bone resorbing osteoclast cells and increasing the number of bone reforming osteoblast cells. This in turn helps make bone more receptive to calcium from the bloodstream, while also reducing excessive calcium loss from the bones thereby helping to reduce the risk of osteoporosis (*37, 54, 55*). Snow Brand believe their MBP® product should be used not only by older people, both men and women, but also by younger women to help build up bone density and increase peak bone mass. This can help prevent the development of osteoporosis as they get older and enter the postmenopausal stage of their lives (*3, 37*).

Additionally, individual whey proteins have come under increasing scrutiny in recent years for their bioactive properties on bone. In particular lactoferrin, a minor basic whey protein has been shown to have potent anabolic effects on bone along with anti-resorptive properties (*77-80*). Growth factors such as IGF-I, TGF-β and angiogenin, known to be anabolic to bone (*30, 81-86*), are also present in milk and angiogenin is also found in the whey basic protein fraction (*87*).

A fraction of milk, known to contain heat-stable acid-soluble glycoproteins, phosphoproteins and peptides, is known as the proteose peptone fraction, which accounts for ~10% or more of the total whey protein found in bovine milk. Its main constituents are known as proteose peptone components 3, 5, 8 fast and 8 slow (PP3, PP5, PP8F and PP8S respectively) (*88, 89*). PP5 has been determined to be derived from β-casein and belongs to a phosphoserine rich group of casein peptides known as casein phosphopeptides. Casein phosphopeptides (CPPs), mainly derived from α-s-caseins and β-caseins and normally formed *in vivo* after enzymatic cleavage of casein during digestion and/or commercially produced via trypsin digestion of casein, have

been shown to aid in the solubility, bioavailability and uptake of calcium from milk (*90-93*).

1.1.5 Whey Acidic Protein Fraction and Bone

The so-called whey acidic protein fraction of milk is isolated by anion exchange of either fresh whey, whey protein concentrate (WPC) or whey protein isolate (WPI)(*94-97*) and contains mainly phosphorylated and/or sialylated proteins and peptides, such as casein phosphopeptides and OPN, and those with low isoelectric point (P*i*). Phospholipids may also be present.

Previous work has investigated whether whey acid protein fractions can be used to inhibit osteoclast cell development and reduce bone mineral density loss using both *in vitro* (cell based) and *in vivo* (using rats) studies (*76, 96-99*). In these studies, various WPC substrates were used as starting sources for preparation of the acidic protein fractions or hydrolysates thereof. WPC is manufactured from fresh whey by the removal of non-protein ingredients, such as lactose, using methods such as membrane filtration (ultra-filtration and diafiltration) and is a spray dried product containing usually 80% or more protein by weight.

This work indicated that there may be components found in the acid fraction of WPC that could have positive effects on bone growth. Oral feeding studies with such fractions have shown reduced bone loss in rats which have been ovariectomised (had their ovaries removed) (*96, 98*). Rats are often used for these studies as they have a similar cancellous bone remodelling to humans as well as having a similar skeletal anatomy. Ovariectomised rats are used in studies to simulate post-menopausal conditions, as ovariectomy results in a decline in plasma estrogen. As discussed above, post-menopausal woman have reduced amounts of estrogen in their bodies, which can lead to a higher chance of osteoporosis developing due to bone mineral density losses (*55, 100-103*).

Although overall encouraging, the rat feeding studies have had mixed results. Some studies appear to have positive outcomes, where bone loss seems to have been reduced compared to the controls; other studies in which rats were fed a diet of increasing

amounts of whey protein acidic fraction or other milk components, such as casein, seem to show no effect on bone loss (*103, 104*). These mixed results could be due to several factors, such as the length of time for the studies. Studies shorter in time are unlikely to show as significant a change in BMD and calcium absorption as those of greater length due to the nature of the bone remodelling process, which occurs over a prolonged time. An additional reason for mixed results from the rat studies was the use of WPC substrates generated from different sources, resulting in different levels of casein and whey peptides and whey proteins in the acidic fractions used for oral feeding. As an example, whey (and hence WPC) generated from the manufacturing of cheese contains high amounts of glycomacropeptide (GMP) (also known as caseinmacropeptide, CMP (*105*)), a κ-casein macropeptide generated by the renneting process, whereas other sources of whey contain negligible amounts of this component (*97*).

1.2 Present Work

1.2.1 Present Work

The work outlined in this thesis was premised upon prior studies on the effect of the whey acidic protein fraction on bone (*76, 96-99, 104*). As discussed above, oral feeding studies using acidic protein fractions from various WPC substrates had shown some promise in ameliorating bone loss in ovariectomised rats. However, characterisation of the fractions was preliminary and no hard and fast conclusions could be drawn as to the component(s) contributing to the bioactivity. To better understand the bone activity of whey, an understanding of the composition of whey and its associated acidic protein fraction was needed, especially as to the content of nutrients and proteins/peptides known to influence the bone remodelling process (e.g. vitamins, growth factors, OPN). This compositional data could then be used to locate, if possible, the source of the bone activity of whey and potentially reveal novel bone agents.

To this end, the composition and bone cell activities of different types of WPC were assessed and compared to the whey acidic protein fractions and their hydrolysed derivatives to determine any differences and to indicate whether these differences could highlight a component of whey that induces activity on osteoclast and/or osteoblast cells.

Following on from previous research (*76, 96-99, 104*), three different types of WPC 80 powder were employed for this study to better enable differentiation of activity. Acidic protein fractions were prepared from all three WPC substrates and the fractions further hydrolysed with gastrointestinal tract enzymes. Bone activity was assessed using *in vitro* screening with osteoblast and osteoclast cells.

The activation or deactivation (loss) of bone activity due to digestion/hydrolysis by enzymes found in the gut (pepsin and trypsin) is of interest as it relates to what would occur naturally in the body when these proteins are ingested and could lead to a better understanding of how whey proteins help with bone growth and development of newborns.

2. Preparation of Whey Acidic Protein Fractions

2.1 Description and Source of WPC Substrates

WPC 80 powders were used in this study. The 80 in WPC 80 refers to the protein content of the dried product, which contains more than 80% protein by weight.

The WPC 80 products used were: mineral acid WPC 80 (CR05); cheese WPC 80 (GS19); and lactic acid WPC 80 (HS15). The type of WPC relates to how it is made and the source it comes from as described below.

Mineral acid WPC 80 is the by-product of mineral casein manufacturing and is generated using mineral acids such as hydrochloric and sulfuric acids. The milk is acidified (pH~4.6), then heated to 40-50^{o}C to coagulate the caseins. The recovered whey is clarified, chilled and then ultrafiltered (UF) and diafiltered. The resulting whey (UF) retentate is pH adjusted to ~6.7 with sodium hydroxide and then evaporated and spray-dried.

Cheese WPC 80 is generated as the by-product of cheese making, in which enzymes (specifically rennet) are used to coagulate the caseins. The whey is clarified, separated (to reduce fat) and thermalised to kill the starter and enzymes. Citric acid is used to acidify the whey slightly prior to ultrafiltration. Following ultrafiltration and diafiltration, the pH of the whey retentate is adjusted to neutral with sodium hydroxide and the whey is evaporated and spray-dried.

Lactic acid-WPC 80 is generated as the by-product of lactic casein manufacturing. Skim milk is fermented with lactic starter bacteria until the pH decreases to ~pH 4.6. The milk is then heated to 40-50^{o}C to coagulate the casein and the whey is recovered as for mineral acid whey above.

The three WPC 80 powders used in this project work were from different Fonterra manufacturing sites and were supplied in 25kg sealed bags and are described in Table 1 below.

Table 1: The details of the three WPC 80 powders used in this study.

Product type	**Production Factory**	**Group-Spec**	**Cypher (month year)**	**Material Number**
Cheese WPC 80	Hautapu WPC factory 1239	081-3921	GS19 (February 2008)	104578
Lactic Acid WPC 80	Whareroa Whey factory 4739	081-0094	HS15 (March 2008)	106134
Mineral Acid WPC 80	Edgecumbe WPC factory 4139	081-1643	CR05 (October 2007)	104556

The cypher refers to when the product was made. The first letter refers to the month and the second letter to the year. The numbers indicate the day the product was made. The cypher numbers are used as sample identification numbers throughout this thesis.

2.2 Chemicals and Materials in Fraction Preparation

Where possible all chemicals used to prepare these fractions were analytical grade or higher (Merck Limited, Palmerston North, New Zealand), unless otherwise specified.

FPLC Specifications:

AKTA explorer system, made by Amersham Biosciences:

P-900 Pump: Pumps solvents using Lines A and B.

UV-900 Monitor: Monitors the UV.

pH/C-900 Monitor: Monitors the pH (when a pH probe is inserted inline) and measures the conductivity.

P960 pump: Sample loading pump

Tubing size: 0.5 mm internal diameter (ID)

FR-902 Flow restrictor: FR-902 is pre-set to 0.2 MPa and acts as a back-pressure regulator.

Column description

XK50/100 technical specifications are as follows:

Internal Diameter = 50mm

Length = 100cm

Volume = 1274-1862mL

Bed Height = 65-95cm

Operating pressure = ≤ 3 bar (43 psi, 0.3 MPa)

Operating temperature = 4–60°C

pH stability = 1–14

Mesh size of net = 10 µm, nylon

Tefzel tubing = 1.2-mm ID

Packing = Q Sepharose Big Beads, anion exchange resin, 100 to 300µm in size

Dialysis Tubing description

The Dialysis Tubing used is as follows:

6 Spectra/Por Dialysis Membrane, MWCO 1000, Nominal Flat Width: 45mm, Diameter: 29mm, Vol/Length: 6.4ml/cm, Length: 10m/33ft, Mfg date: 08/12/08 from Spectrum laboratories, Inc. Order Number: 132640.

2.3 Summary of Fraction Generation

Fractions were prepared in accordance with previous methods used to generate bioactive acidic whey protein fractions (Kruger *et al.*, 2006) (*96*). In general the WPC 80 samples were weighed and water added to make a ~10% w/v sample solution. The sample solution was then left to dissolve with continuous magnetic stirring for an hour at room temperature. After an hour the sample solution was checked to ensure all powder had been dissolved and there were no clumps. The pH of the sample solution was then adjusted to pH 4.5 with 10% (v/v) hydrogen chloride (HCl). The sample

solution was then left over night in the cold room (4°C) with magnetic stirring at a gentle speed. The term 'sample' refers to the WPC80 powder whereas the term 'sample solution' refers to the mixture of sample and water.

The next morning the sample solution was removed from the cold room and stirred to room temperature. The sample solution was then centrifuged and filtered to remove particulates before being loaded onto the anion exchange column using a fast protein liquid chromatography system (FPLC). Figure 3 below shows a schematic diagram of the GE Healthcare ÄKTAexplorer 10 FPLC system used for this study (product code 18-1300-00).

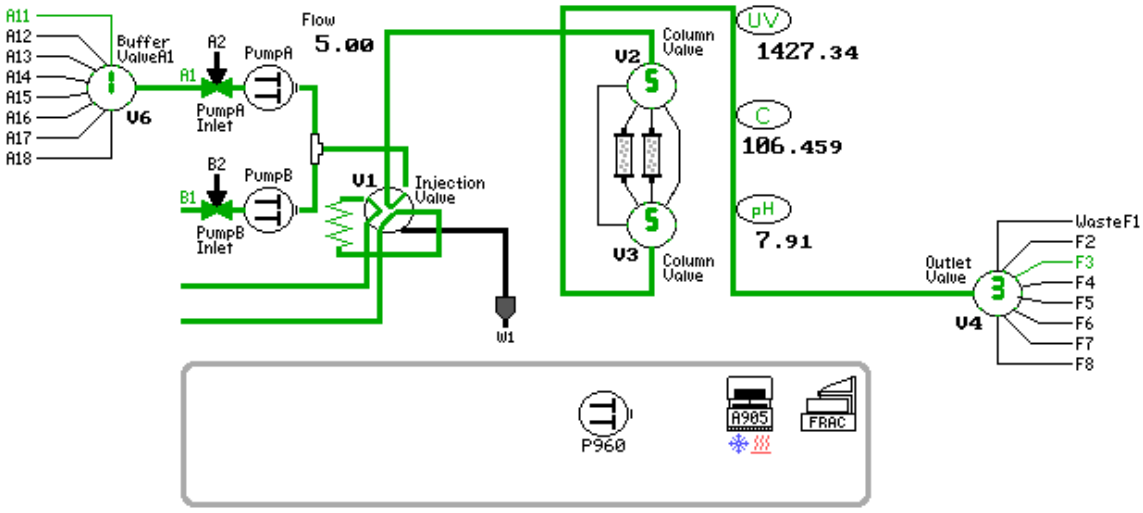

Figure 3: Schematic diagram of the ÄKTAexplorer 10 FPLC system.

The Figure 3 above shows a schematic layout of the FPLC system used to generate the fractions in this study. Pump A and Pump B relate to the main pump (P900). P960 is the sample pump used to load the sample solution onto the column via the injection valve. The A905 sample holder and the fraction collector, although interfaced with the system, were not used for this work. C refers to the in-line conductivity meter and pH refers to the pH probe. However, for this work, the probe was not in place and elution was monitored by UV absorbance.

Due to the large volume of sample solution being loaded on to the column, the time taken to load the sample solution was extensive (more then 20 hours) so the sample solution was kept in an ice bath with the temperature maintained between 7°C and 12°C. The temperature was not allowed to drop below this level as lower temperatures resulted in increased viscosities and caused the pressure to build rapidly in the system, which could have resulted in the column bursting or leaking at the joins in the system. The breakthrough (non-bound material) was collected into Schott glass bottles also in an ice bath.

Once all the sample solution had been loaded onto the column, the column was flushed through with water until the UV had decreased to near baseline. The flush-through was collected and combined with the breakthrough. Once the sample solution had been flushed through the system a salt gradient was applied to wash the bound sample from the column. This fraction was collected and designated the acid fraction and was the main fraction of interest. The acid fraction was collected until the UV absorbance had dropped and levelled out to near baseline, indicating that the bound material had been removed from the column. The column was washed with further bed volumes of salt and then put back into water over a fast gradient. Once the column had been flushed through with water, the system and column were put into 20% ethanol for storage. Unless otherwise stated all water used for this work was Milli-Q water.

2.4 Development of Fractionation Procedure

Protein purification protocols were developed using the ÄKTAexplorer 10 FPLC system. Cheese WPC 80 (GS19) was chosen to initially trial the FPLC due to the high content of glycomacropeptide (GMP) giving rise to a higher proportion of acidic protein/peptide available to bind to the column. Running conditions were more-or-less optimised using this substrate.

2.4.1 Small-Scale Trials

Small scale trials were conducted as a prelude to larger scale preparative chromatography, to gauge column performance, loading and elution conditions.

100g of GS19 powder was placed into a 2 litre Schott bottle. A litre of water was slowly added to the powder, with constant magnetic stirring. The sample solution was left to mix for 30 minutes. Once the sample solution was well mixed and checked to ensure there were no clumps of dry powder, the pH was measured. The pH of the sample solution was adjusted to 4.5 using 10% (v/v) HCl. The sample solution was left stirring for another 10 minutes before the pH was checked again to ensure it was still at 4.5. The sample solution was then placed in an ice bath with the temperature maintained between 4^{o}C and 5^{o}C.

The initial FPLC trials were set up with a small XK16 (~30mL) ion exchange column (from GE Healthcare/Life Sciences), packed with Q Sepharose Big Beads, a strong anion ion exchange resin with an ionic capacity of 180-250pmole/mL gel. The beads are spherical and range in size from 100 to 300 µm in size. The Q Sepharose Big Beads had been stored in 20% ethanol, thus the column was flushed with ~100mL of water (just over 3 column volumes) in preparation for use.

150mL of GS19 sample solution was loaded onto the column at a flow rate of 1.5mL/min using the pre-calibrated sample loading pump (P960). The system did not have a pH probe inline but the UV was monitored at 280nm, 254nm and 215nm, and conductivity was also recorded. The temperature of the sample solution at the start of the run was 4.8^{o}C.

Once the sample loading was complete, the column was flushed with 75mL (2.5 times the column volume) of water (Line A), at 1.5mL/min flow rate, to remove the unbound solution material from the column (total run time to this point of 150 minutes).

At ~157 minutes, a gradient was initiated from 100% water (Line A), to 100% 1M sodium chloride (NaCl) (Line B) over a time period of 10 minutes with a maintained flow rate of 1.5mL/min.

Elution of bound protein by the 1M NaCl eluent was monitored by UV absorbance. However, after more than 5 times the column volume (~158mL) of 1M NaCl had been passed through the column, the 215nm UV absorbance signal had still not returned to starting baseline. It was decided that since the other two UV absorbance signals (280nm and 254nm) had reached near baseline, sufficient proteinaceous material had been eluted from the column (See Figure 4). Therefore, at ~265 minutes into the run, a 10 minute gradient was applied to move the column back to 100% water (Line A).

The run was stopped at ~330 minutes. The entire FPLC system (including the column) was rinsed with water before being rinsed with and stored in 20% ethanol.

Figure 4 below shows the three UV absorbance signals and conductivity spectra for this small column trial run. It should be noted that the scale on the left side of the run (bottom of the page) is in UV absorbance units of mAU and the scale on the right side (top of the page) is in conductivity units of mS/cm. This figure shows the relationship between the UV absorbance signals and conductivity as material flows from the column.

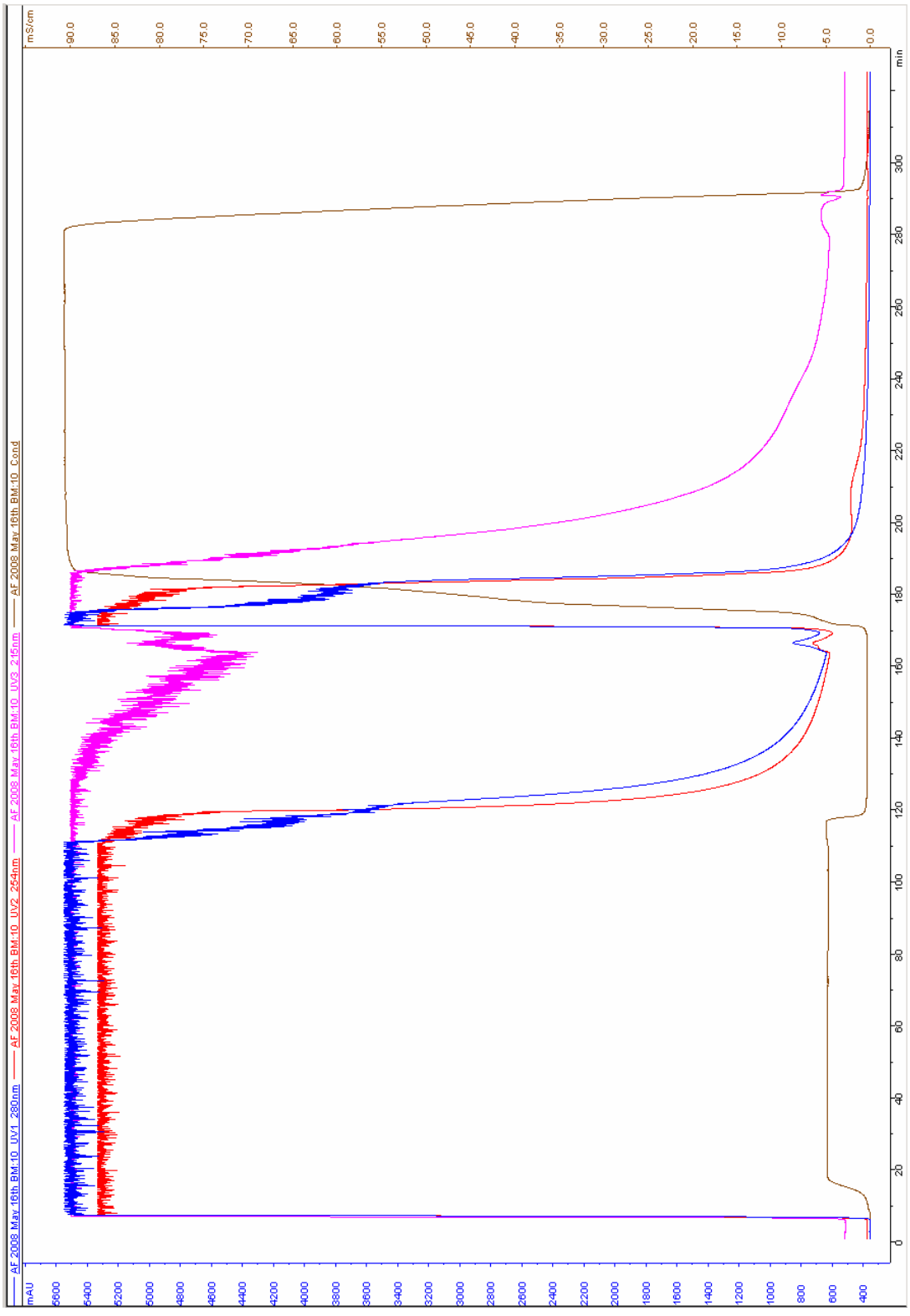

Figure 4: The FPLC UV absorbance (215nm pink, 254nm red and 280nm blue) and conductivity spectra (brown) show the loading of sample solution onto the column (0 to ~100min), the sample being washed through the column with water (~100 to ~157min) and the removal of bound material with salt (~157 to ~270min) before being put back into water (~270min to ~330min).

2.4.2 Larger-Scale Preparative Trials

Scale up of this procedure for preparative work employed a XK50/100 (~1L) column (from GE Healthcare/Life Sciences) packed with Q Sepharose Big Beads, to give an ion exchange column with a volume of ~900mL. The specifications for this column can be found in the Chemicals and Materials in Fraction Preparation section.

During the trial run the pressure rose almost to 1MPa, which is beyond the pressure limit of the XK50/100 column so investigations were undertaken to reduce this pressure. The tubing in the FPLC system was changed to 0.5mm I.D. from 0.25mm I.D. tubing. It was also thought that the 10µm column filter might be blocking and causing the pressure to build so it was decided that the sample solution should be centrifuged and filtered first before being loaded on to the column. It was also decided to keep the sample solution a little warmer to help reduce sample solution viscosity.

500g of GS19 powder was placed in a 10L plastic bucket. 5L of water was added slowly while the sample was magnetically stirred. A stirring rod was also used to help mix and break up the clumps of powder that formed. The sample was left to mix for 30 minutes, after which the pH was measured and adjusted to pH 4.5 with 10% HCl. The sample was left mixing for another 10 minutes and the pH checked to ensure it was still at pH 4.5.

Aliquots (~220mL) of the sample solution were placed into 6 x 250mL centrifuge bottles. The samples were centrifuged at 7,000g for 30 minutes at 10^{o}C using the Induction Drive Centrifuge, Beckman Model J2-2IM, and Rotor JA14 (L2 centrifuge). The supernatant was removed from the centrifuge bottles and collected in a 2L Schott bottle, resulting in ~900mL of solution. This step was repeated to give over 1500mL of centrifuged sample solution.

The centrifuged sample solution was then vacuum filtered using a 5.0µm hydrophilic polyvinylidene fluoride (PVDF) membrane filter (Durapore® Membrane Filter, Millipore, supplied by Biolab Limited, NZ). Approximately 200mL of sample solution went through the filter before it blocked and a new filter was needed. This procedure

was repeated until the filtrates pooled together gave over 1100mL of sample solution, collected in a 2L Schott bottle in an ice bath (maintained between 7 and 12^{o}C).

1000mL of the filtered GS19 sample solution was then loaded onto the column at a flow rate of 5mL/min using the pre-calibrated sample loading pump (P960). The temperature of the sample solution at the start of the run was 7.5^{o}C. The temperature of the sample solution was monitored throughout the entire loading to ensure it did not exceed the 12^{o}C upper limit. After 350mL of sample solution had been loaded onto the column (70 minutes), the UV absorbance signals started to rise (See Figure 5) and at this stage the collection line was put into a clean 2L Schott bottle in an ice bath for the breakthrough/flow-through to be collected.

Once the sample loading was complete, the column was flushed with water (Line A), using the P-900 pump at an initial flow rate of 1.0mL/min increasing to 5.0mL/min over a 5 minute period. The flow rate was maintained at 5.0mL/min for 30 minutes before it was reduced to 2.0mL/min to run overnight.

The breakthrough already collected was stored at 4^{o}C overnight. The collection of the breakthrough coming off the column was continued overnight in a new collection bottle; however, overnight the temperature of the ice bath was allowed to rise to room temperature.

Once 1700mL (~1.8 column volumes) of water had been loaded onto the column the system was paused and the column set to by-pass, so Line B could be prepared in 1M NaCl from its storage of 20% ethanol*.

The system was restarted at 1mL/min with 100% water (Line A) and the column brought back in-line. A 10 minute gradient to 100% 1M NaCl (Line B) was performed and the breakthrough collection was stopped. A new bottle collected the “waste” that came off the column between the breakthrough and the eluted acid fraction. The breakthrough fractions were pooled together and stored at 4^{o}C.

* Due to the lengthy load-time it was preferred not to have line B prepared and sitting in salt.

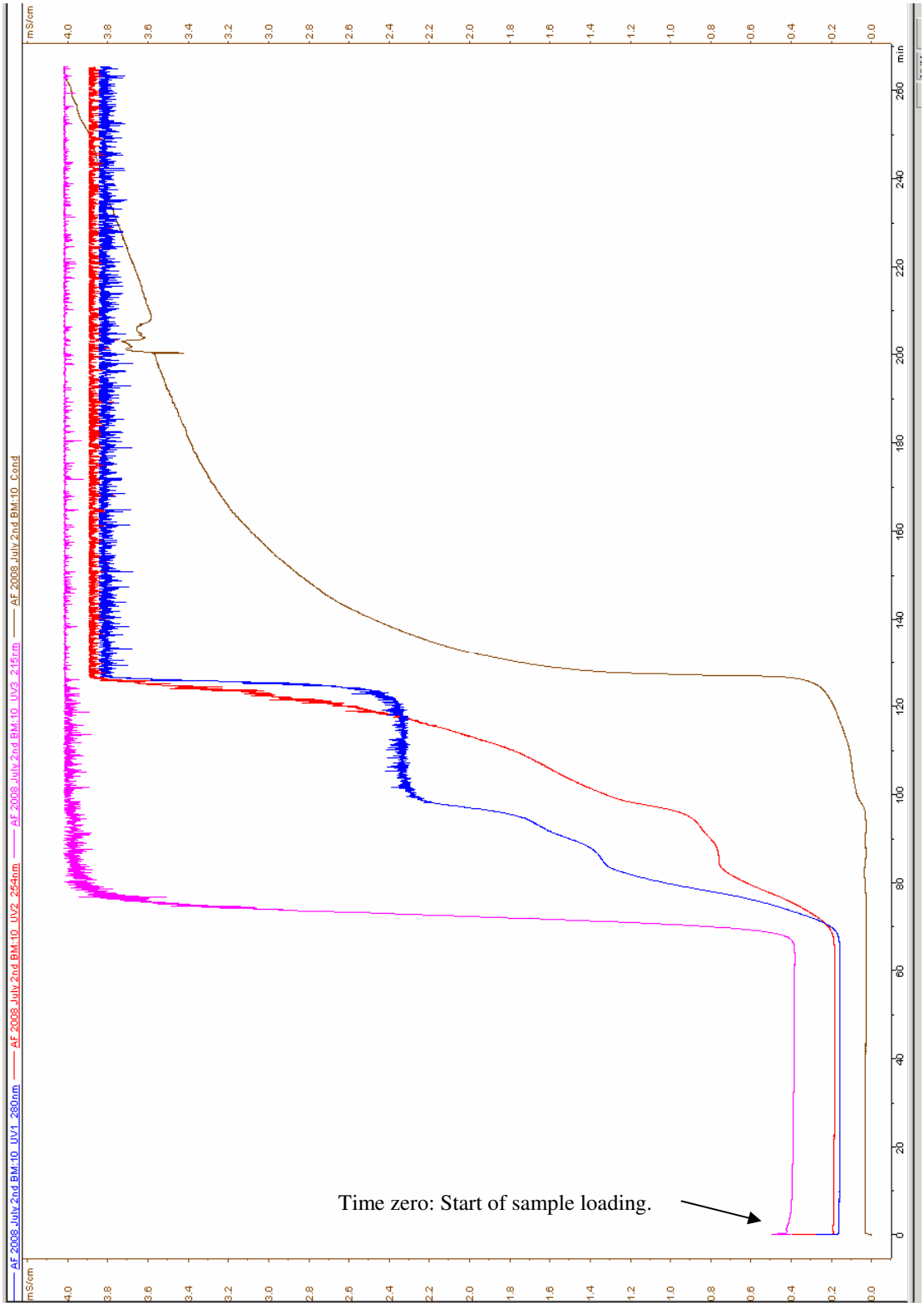

Figure 5: The FPLC UV absorbance (215nm pink, 254nm red and 280nm blue) and conductivity spectra (brown) of sample solution loading on the 1L Q Sepharose Big Beads column, showing that as the sample moved through the column not all of the material was bound, resulting in an increase in the UV absorbance and conductivity as the sample emerged from the column.

Note that this run is illustrated in two parts (Figure 5 and 6). When the sample had finished loading, the system (P-900 pump) was stopped to enable rinsing of the sample from the loading loop and P-960 pump. On resumption of flow through the P-900 pump the recording automatically defaulted to time zero (hence although the spectrum shown in Figure 6 is a continuation from Figure 5, the run time scale is reset to 0 minutes).

Once the gradient had finished, and there was 100% B (1M NaCl) flowing on to the column, the flow rate was increased in steps to 5.0mL/min and the pressure was monitored to ensure it did not rise too high. After 540mL of 1M NaCl had been loaded onto the column the UV absorbances began to rise and a new collection bottle was set in place to begin collection of the acid fraction.

After 1860mL of 1M NaCl (~2 times column volume) had been loaded on to the column and, even though the 215nm UV absorbance signal had not returned to starting baseline, it was decided that the other two UV absorbance signals (280nm and 254nm) had dropped significantly and sufficient proteinaceous material for this trial had been eluted from the column. The acid fraction that had been collected was placed in the cold room overnight. A 10 minute gradient was then applied to return the column to 100% water. Once the gradient had been completed, the system was paused, the column by-passed and line B flushed with water to remove the salt from the line. After line B had been flushed the pump was set to 50% Line A and 50% Line B with both lines in water, giving 100% water loading onto the column, at a flow rate of 1.7mL/min. The column was put back in-line and the collection bottle changed. Water was washed continuously through the system at a flow rate of 1.7mL/min overnight. Figure 6 shows the UV absorbance signals and conductivity spectra for this 1L column trial.

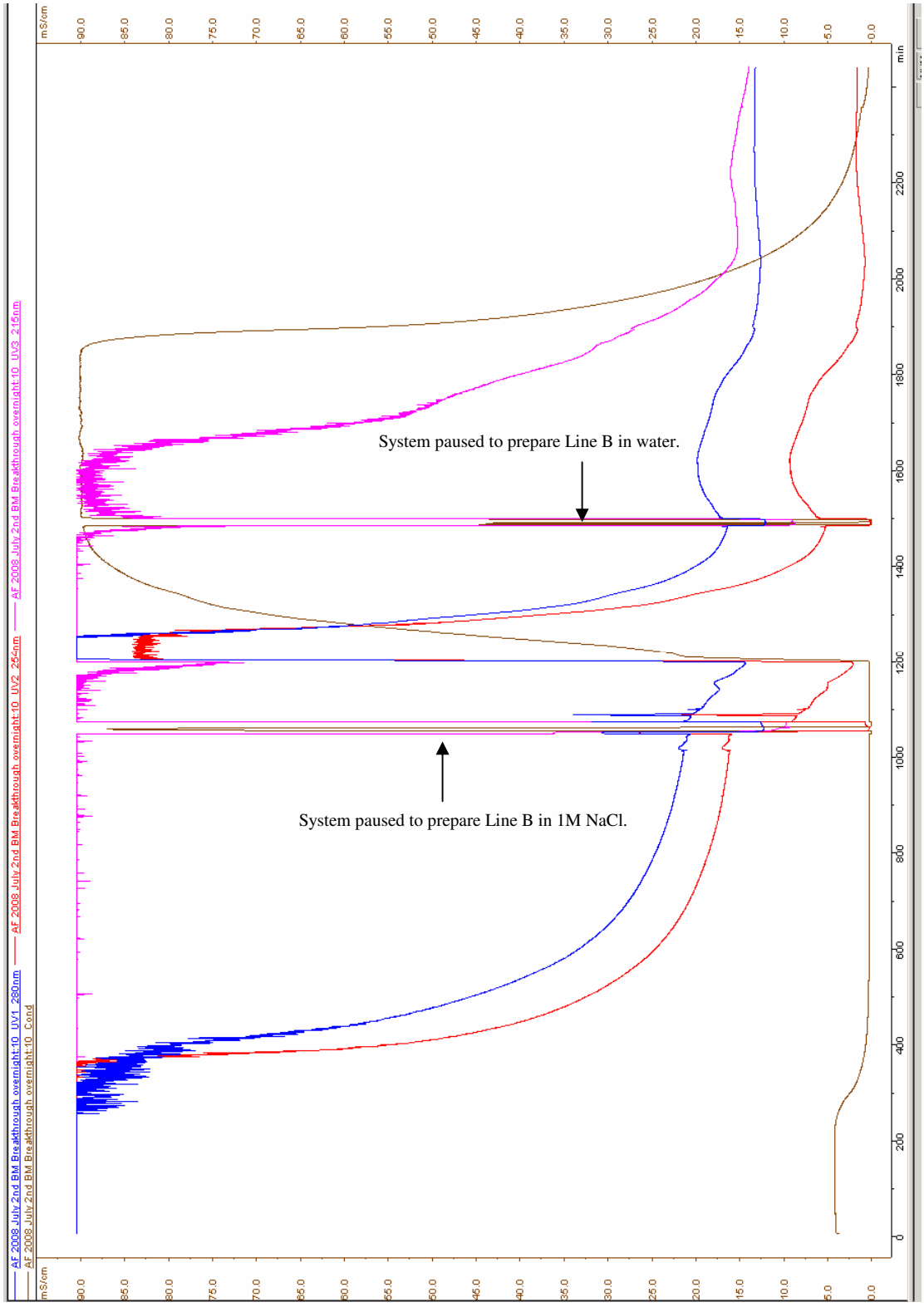

Figure 6: The FPLC UV absorbance (215nm pink, 254nm red and 280nm blue) and conductivity spectra (brown) showing the water flush-through of unbound sample and salt elution step with 1M NaCl on the 1L Q Sepharose Big Beads column. As indicated, salt loading was between ~1080 minutes and ~1490 minutes, with water being flushed through prior to and following the salt step.

2.4.3 Modifications to Procedure

In the procedure that had been used, 1M NaCl was loaded onto the column until the UV absorbance signals dropped to near baseline and levelled out. The column was then switched back to water.

Because of the difference in response of the UV absorbance signals in these trial runs, mainly the long tailing of the UV absorbance signals in the larger column trial, a modified procedure was implemented to improve this tailing by removing the bound material more quickly and more efficiently.

The modified procedure employed 2M NaCl in addition to 1M NaCl to remove bound material and employed 50:50 lines A (water) and B (2M NaCl). After sample loading and flushing through of sample with water, a succession of 2L of 1M NaCl, 300mL of 2M NaCl and 200mL of 1M NaCl were used to remove bound material from the column.

To remove more solid (undissolved) material from the sample solution making it easier to filter, the sample solution was centrifuged for 60 minutes instead of 30 minutes. Due to the high sample volume involved in the preparative fraction runs, two centrifuges were used to prepare the sample solution; the Sorvall® Evolution RC Centrifuge with the Fiberlite® Piramoon Technology Inc. F8- 6x 1000y Rotor (L1 centrifuge) and the Beckman centrifuge, as described above (L2 centrifuge). The sample was prepared the day before and left stirring in the cold room overnight.

As the sample solution was still hard to filter alternatives were investigated. A 114µm GAF Filter™ bag (manufactured by Eaton-Hayward, Cleveland, Ohio, USA) was trialled with 5L of 10% (w/v) sample solution. The GAF Filter™ bag was left hanging above a bucket overnight to find out how well the sample solution drained. By morning less then 200mL had filtered through overnight. The GAF Filter™ bags are designed to be used with a vacuum system. Since there was no system available for such a small volume of sample, this approach was thus abandoned.

2.5 Isolation of the WPC Fractions

The fractions were isolated using the general procedure described above. Below are the key points for the different WPC fraction types.

2.5.1 GS19 – Cheese WPC80:

- 500g of cheese WPC80 was weighed and 5000mL of water added.
- L1 centrifuge was spun at 10,000 g force and held 6 x 1L plastic centrifuge bottles with aliquots of ~650mL of sample solution in each. L2 centrifuge was spun at 7,000 g force and held 6 x 250mL plastic centrifuge bottles with ~220mL aliquots in each. In both cases centrifugation was 60 minutes at 10^{o}C.
- The pooled centrifuged sample solution was then vacuum filtered using a 5.0µm hydrophilic PVDF membrane filter.
- The P-960 flow rate was calibrated to 3.0mL/min on the sample solution.
- The temperature of the sample solution at the start of the run was 11.7^{o}C and the conductivity was 4.43mS/cm.
- The starting UV absorbance signals were: 215nm = 1246mAU; 254nm = 677mAU; 280nm = 593mAU.
- At 3mL/min the pressure was stable at 0.32MPa at the start of the run.
- 4000mL of the clarified sample solution was loaded on to the column at 3mL/min, (22 hours 37 minutes load time).
- 1540mL (column volume) of water was loaded onto the column before a 10 minute gradient to 1M NaCl was applied (50 % Line A/water and 50% Line B/2M NaCl) at 5mL/min.
- After 1950mL (column volume) of 1M NaCl had been loaded onto the column a gradient 2M NaCl (100% Line B) was applied over 10 minutes.
- After 300mL (column volume) of 2M NaCl had been loaded onto the column a gradient was applied to return to 1M NaCl (50% Line A and 50% Line B).
- After ~200mL of 1M NaCl had been loaded on the column a fast gradient to 0.3M NaCl (85% Line A and 15% Line B) was employed over 3 minutes before the system was paused and Line B put into water. The system was started again at

50% Line A and 50% Line B to flush the salt from Line B and to load water onto the column.

- 2500mL (column volume) of water was loaded onto the column before flushing the column with 20% ethanol for storage.
- ~1800mL of acid fraction was collected from the column.
- The acid fraction was placed in 7 lengths of dialysis tubing, holding ~260mL of fluid each, and then placed into two 10L plastic bucket filled with water and placed at 4^{o}C, with magnetic stirring to keep the water moving.
- The dialysis tubing was moved to 2 new buckets of water after ~6 hours and then again after another ~16 hours. After another ~30 hours the dialysis tubing was removed, pierced and the acid fraction collected in a 5L glass beaker.
- After dialysis there was 2360mL of acid fraction which gave a yield of 24.5g dry powder after freeze-drying. (See materials section for dialysis tubing specifications).
- The pH of the acid fraction after dialysis was pH 5.58; the conductivity was 626µS/cm.
- ~5000mL of breakthrough was collect; 900mL was freeze dried, giving 70.6g of powder. (The remaining breakthrough was put into 2L plastic milk bottles and stored at -85^{o}C in case it was required later.)
- The total solid of the breakthrough and the starting sample solution was measured using a SMART Trac™ microwave (manufactured by the CEM Corporation, Matthews, North Carolina, USA) with an internal balance. 3-5g of the sample was weighed onto sample pads (on which the SMART Trac™ microwave balance had been zeroed) that absorbed the liquid sample. The sample was then heated (max setting 102^{o}C) in the SMART Trac™ microwave until a constant weight was reached, for 10seconds, giving a total solid calculation.
- The starting sample solution had a duplicate average total solid of ~9% and the breakthrough had a duplicate average total solid of ~6%.
- The breakthrough and the acid fraction powders were placed in heat-sealed sachets and stored in a cold room at 4^{o}C until needed.

2.5.2 CR05 – Mineral WPC80:

This fraction was prepared in the same manner as the GS19 – Cheese WPC80 fraction with modifications as noted below:

- 600g of CR05 powder was weighed and 6000mL of water was added.
- 5000mL of the clarified sample solution was loaded on to the column at 4mL/min, over a time period of 20 hours, 50 minutes.
- ~1860mL (column volume) of water was loaded onto the column before a gradient to 1M NaCl was applied.
- ~1550mL (column volume) of 1M NaCl was loaded before applying a gradient to 2M NaCl over 5 minutes.
- 270mL of 2M NaCl was loaded before applying a gradient back to 1M NaCl over 5 minutes.
- 180mL of 1M NaCl was loaded before applying a gradient to 100% water. The column was flushed with 2.5L of water before being put back into 20% ethanol for storage.
- 1300mL of acid fraction was collected, giving 2350mL after dialysis, and a yield of 22.7g powder after freeze-drying.
- After dialysis the acid fraction had a pH of 5.56 and a conductivity of 369µS/cm.
- 7000mL of breakthrough was collected; 900mL was freeze dried, giving a yield of 70.3g powder.
- The 10% starting solution had a duplicate average total solid of ~9% and the breakthrough had a duplicate average total solid of ~7%.

2.5.3 HS15 – Lactic WPC80:

This fraction was prepared in the same manner as the GS19 – Cheese WPC80 with modifications as noted below:

- 700g of HS15 powder was weighed and 7000mL of water was added.
- 5000mL of the clarified sample solution was loaded on to the column at 3mL/min, over a time period of 27 hours, 47 minutes.
- ~2000mL (column volume) of water was loaded onto the column before a gradient to 1M NaCl was applied.

- ~2000mL (column volume) of 1M NaCl was loaded before applying a gradient to 2M NaCl over 10 minutes.
- 300mL of 2M NaCl was loaded before applying a gradient to1M NaCl over 5 minutes.
- 200mL of 1M NaCl was loaded before applying a gradient to 100% water. The column was flushed with 2.5L water before being put back into 20% ethanol for storage.
- 1900mL of acid fraction was collected, giving 2820mL after dialysis, which gave a yield of 21.1g powder after freeze-drying.
- After dialysis the acid fraction had a pH of 5.90 and a conductivity of 449µS/cm.
- 6500mL of breakthrough were collected; 900mL was freeze dried giving a yield of 57.81g powder.
- The 10% starting solution had a duplicate average total solid of ~9% and the breakthrough had a duplicate average total solid of ~5%.

Figures 7 and 8 below show the spectra generated by the FPLC system during the HS15 acid fraction collection run. Figure 7 shows the UV absorbance spectra and Figure 8 shows the conductivity spectrum. As the runs were approximately the same only the HS15 spectra have been included here, but they are representative of all three of the fraction collection runs.

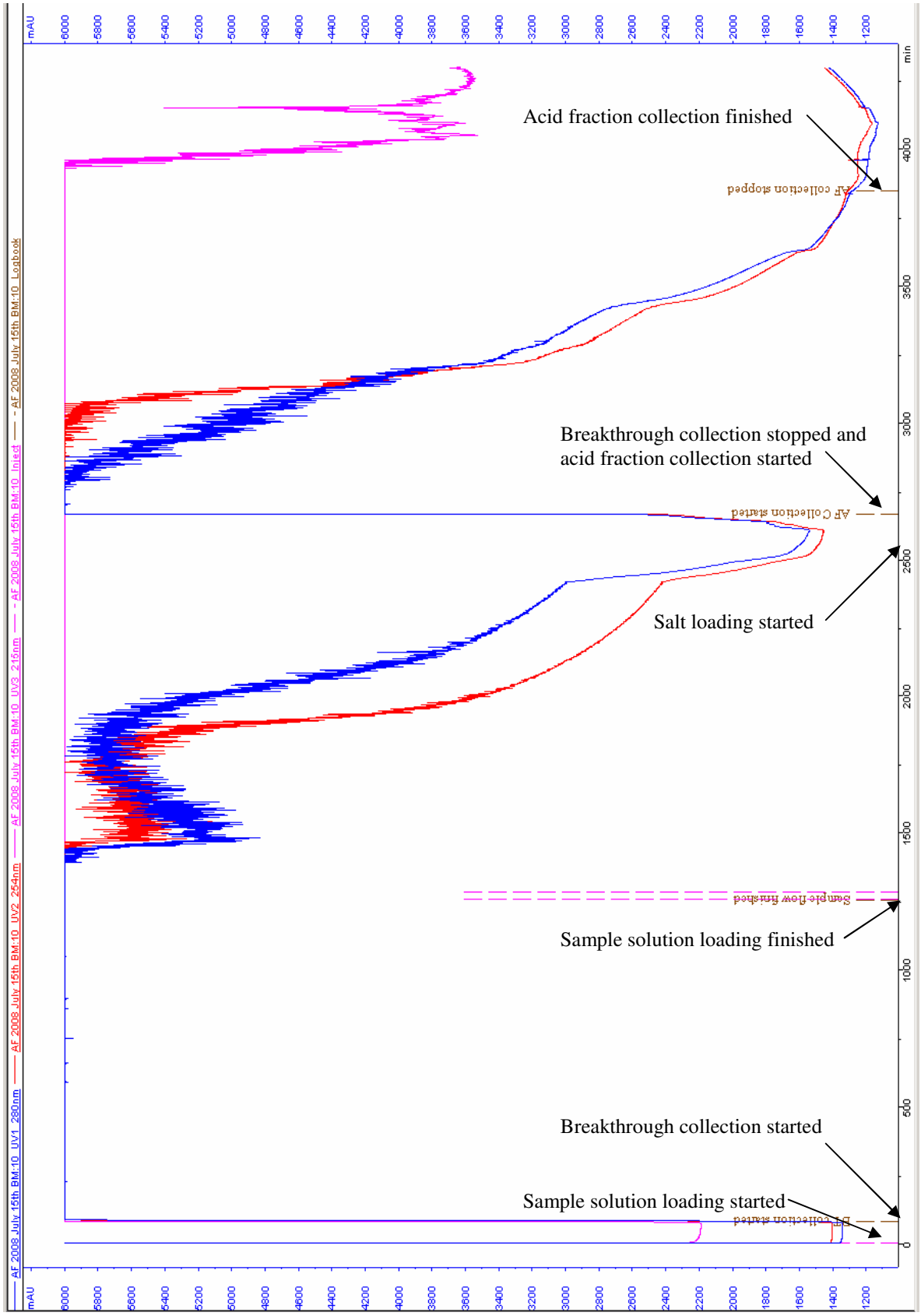

Figure 7: The FPLC UV absorbance spectrum (215nm pink, 254nm red and 280nm blue) for the HS15 fractionation showing the absorbance changing as material elutes from the column under different conditions.

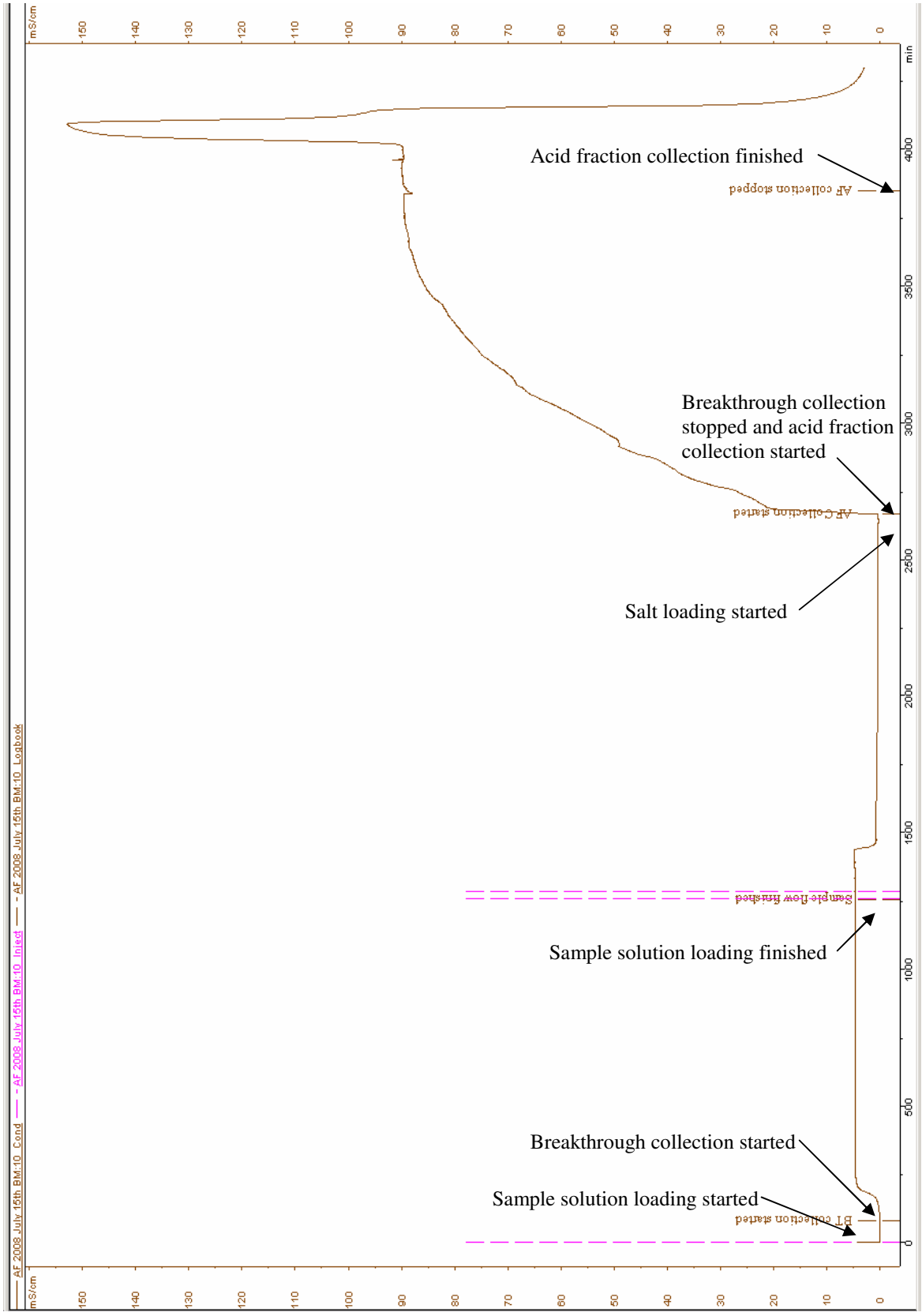

Figure 8: The FPLC conductivity spectrum (brown) for the HS15 fractionation showing the conductivity changing under different buffer conditions. The peak near 4100 min is due to the 2M NaCl loading on the column.

3. Compositional Analysis

3.1 Experimental

Note: A number of the procedures below were performed by the Analytical Services Group (ASG) at the Fonterra Research Centre, Palmerston North, with samples being incorporated into routine testing. These procedures are indicated by an asterisk. Procedures labelled NZTM (New Zealand Testing Methods) are confidential testing methods privy to Fonterra. The principles of each procedure are outlined in Appendix 1.

3.1.1 Chemicals and Materials

For the methods performed at the Fonterra Research Centre, by other technicians or myself, all chemicals used were analytical grade or higher (Merck Limited, Palmerston North, New Zealand), unless otherwise specified. The chemicals used are detailed in the appropriate places in the following procedures.

Materials and instruments used have been stated with the experimental methods descriptions.

3.1.2 Determination of Element Composition*

The powder samples were analysed for mineral composition using New Zealand Testing Method (NZTM) 3.9.21: Minerals by Acid Digestion and Inductively Coupled Plasma-Optical Emission Spectrophotometry (ICP-OES) for Dairy Products, based on Environmental Systems Monitoring Laboratory methods (*106, 107*). The elements tested for included calcium, iron, magnesium, manganese, phosphorus, potassium, sodium, zinc and copper.

3.1.3 Determination of Vitamin D3 Content*

Vitamin D3 was determined in the powder samples using NZTM 3.17.7: Vitamin D3 (Cholecalciferol) and Vitamin D2 (Ergocalciferol) by High Performance Liquid Chromatography, Nordic Committee on Food Analysis (NMKL) (*108*), Indyk, 1996 (*109*); Kurmann, 1994 (*110*); European Committee of Standardisation, 2000 (*111*); Indyk, 1985 (*112*).

3.1.4 Determination of Vitamin K Content

The vitamin K testing was contracted out to Asure Quality Chemistry Laboratory in Auckland for analysis. Vitamin K was determined using Association of Official Analytical Chemists (AOAC) Method 999.15 (*113*), Indyk, 2000 (*114*) .

3.1.5 Determination of Total Nitrogen and Protein Content*

The powder samples were analysed for total nitrogen (TN) content using NZTM 3.15.8: Total Nitrogen by the Kjeldahl Block Digestion (Macro) Method and Calculation of Protein for Cheeses, Liquid Milks, Milk Powders and Protein Products. This method is based on International Organization for Standardization (ISO) 8968-2/International Dairy Federation (IDF) 20-2: 2001: Milk-Determination of Nitrogen Content. Part 2: Block Digestion Method (Macro Method) (*115*) and AOAC Method 991.20: Nitrogen (total) in milk, 1995 (*116*), by Barbano, 1990 (*117*); Grappin, 1988 (*118*); McKenzie, 1994 (*119*); and Vogel, 1989 (*120*). The acid fraction powders that were hydrolysed with trypsin (see below under PAGE Gels) were analysed for total nitrogen by micro-TN at Otago University, due to a very limited amount of sample being generated for bone assay testing.

3.1.6 Determination of Non-Protein Nitrogen Content*

Non-protein nitrogen (NPN) was determined in the powder samples using NZTM 3.15.3: Non-Protein Nitrogen for Liquid Milks and Milk Powders, IDF Provisional Standard 20B, 1993 (*121*), Rowland, 1938 (*122*).

3.1.7 Determination of Fat Content

The fat content of the parent and fractionated powders was determined using NZTM 3.6.11: Fat Using Accelerated Solvent Extraction for Milk Powders, Protein Products and Cream Powders. This method is based on 2 reports: New Methods for Fat Extraction of Dairy Products Part I: Evaluation of the Dionex Accelerated Solvent Extractor. NZDRI Report 1997-098 (*123*); and New Methods for Fat Extraction of Dairy Products Part II: Validation of Accelerated Solvent Extraction for Fat in Dried Milk Products. NZDRI Report 1998-116 (*124*).

In principle this gravimetric fat determination test is based on extracting the fat from the sample using the Dionex ASE-200 (Accelerated Solvent Extraction) at 80^{o}C and high pressure to increase the efficiency of the extraction process. The solvent used to extract the fat from samples was 2:3:3 hexane:dichloromethane:methanol. The extract was collected in previously dried and weighed ASE collection vials. The collected extract was then completely dried under nitrogen flow in a heat block preheated to 60^{o}C with the temperature being increased at a rate of 2^{o}C per minute up to 102^{o}C. Once a temperature of 102^{o}C was reached the extract was left to dry for an hour. The vials were then left to cool to room temperature for 10 minutes before being weighed and the fat determined by weight difference. The fat was then used to determine the fatty acid profile of the fractions and parent samples.

3.1.8 Determination of Fatty Acid profile*

The fatty acid profile of the fat extracted from the samples using the Accelerated Solvent Extraction (ASE) method described above, was determined using the ASG routine laboratory testing method: Determination of Total Fatty Acids including phospholipids in Whey Protein Concentrate Powders and Complex Milk Lipids, based on the work of Morrison, 1964 (*125*); Bannon, 1986 (*126*); Patton, 1975 (*127*); and Eder, 1992 (*128*).

This test principle is based on the glyceride and glycerophospolipid fatty acids being transesterified with sodium methoxide in methanol. A boron trifluoride/methanol solution is added to the sample and heated to above 90^{o}C for 15 hours, generating

methyl esters of sphingomyelin fatty acids and free fatty acids. Hexane is then added to the samples, followed by addition of a small amount of boron trifluoride neutralising solution. The samples are then centrifuged, the upper layer is diluted with hexane and placed in an autosampler vial and capped. The derived methyl esters of the constituent fatty acids are then separated using gas chromatography. For these samples the Shimadzu GC-17A (capillary column) gas chromatographer was used with a 30m x 0.32mm, 0.25μm film thickness, column (β = 320) with a 0.6 – 1.0 m deactivated fused silica open tubular (FSOT) as pre-column. Initial temperature was 50°C increasing to 220°C over the course of the run.

3.1.9 Determination of Total Phosphate Content*

Total phosphate was determined as inorganic P (as PO_4^{3-}) in the powder samples using the ASG routine laboratory testing method: The Analysis of Chloride, Lactose and Phosphate by Autoanalyser, based on the AOAC method, 1965 (*129*) and Method Sheet CP2-075-10, 1979 (*130*).

3.1.10 Determination of Amino Acid Profile*

To determine the amino acid profile for the powder samples, the acid stable amino acids were measured using the NZTM 3.17.36: Acid stable amino acids by high performance liquid chromatography for dairy protein products, Hewavitharana, 1999 (*131*); Tsao, 1994 (*132*); Pearce, 1988 (*133*); and Richardson, 2005 (*134*).

The samples were analysed for tryptophan with the Dionex High Performance Anion Exchange Chromatography (HPAEC) system with a pulsed amperometric detector (PAD) using NZTM 3.17.35: Tryptophan by HPAEC for protein products and colostrum powder, based on the Dionex Application Note 142 (*135*) and the work done by Tsao, 1994 (*136*).

The sulfur amino acids, cystine and methionine, were analysed by AgResearch using performic acid oxidation.

Using all three tests it was possible to construct an amino acid profile of the parent and fractionated samples.

3.1.11 Determination of Growth Factors

Powder samples were analysed for 3 growth factors - Insulin-like Growth Factor-I (IGF-I), Transforming Growth Factor-β1 (TGF-β1) and Transforming Growth Factor-β2 (TGF-β2) using a sandwich enzyme-linked immunosorbant assay (ELISA). The reference method used to test for IGF-I was NZTM 3.11.15: Measurement of Insulin-like Growth Factor-I in milk, colostrum and milk and colostrum products by ELISA Using R&D Kit. The reference method used to test for TGF-β2 was NZTM 3.11.19: Measurement of Transforming Growth Factor-β2 in milk, colostrum and milk and colostrum products by ELISA Using R&D Kit. Analysis of TGF-β1 was based on work done by Clark, 1980 (*137*); Hornbeck, 1994 (*138*); and Daughaday, 1980 (*139*). All methods were also based on the DuoSet ELISA development kits supplied by R&D Systems: IGF-I Catalogue # DY291, TGF-β1 Catalogue # DY240 and TGF-β2 Catalogue # DY302. The 96-well microplates were scanned at 450nm using a Sunrise™ (Tecan, Switzerland) microplate reader with Magellan™ 6 software (Tecan, Switzerland).

3.1.12 Determination of Lactoferrin Content*

Lactoferrin was determined in the powder samples by the surface plasmon resonance (SPR) method NZTM 3.11.21: Bovine Lactoferrin by SPR-Biosensor Immunoassay. This method is based on the work of Indyk, 2005 (*140*) and Palmano, 2002 (*141*).

3.1.13 Determination of Molecular Weight Profile

The powder samples were analysed by Jean Garman at the Fonterra Research Centre in Palmerston North to determine their molecular weight profiles using size exclusion high performance liquid chromatography (HPLC). The pepsin and trypsin hydrolysates of the parent and fractionated powders were also analysed by this method. The method follows NZTM 3.24.7: Molecular Weight Profile by High Performance Liquid Chromatography for Protein Hydrolysates, based on Swergold, 1983 (*142*).

Briefly, the method employed a size exclusion HPLC-GP (gel permeation) column (30 cm TSK-GEL® G5000SW$_{XL}$ column with TSK-GEL® SW$_{XL}$ guard column) using a mobile phase of 0.1% trifluoroacetic acid, 36% acetonitrile and 63.5% water at a flow rate of 0.8mL/min and molecular weight standards over the range ~165 to ~36000 Daltons. The samples were weighed and then dissolved in the mobile phase at a concentration of 1mg/mL, before being filtered in preparation for the HPLC (Shimadzu, Kyoto, Japan). Sample injection volume was 20µL. The absorbance's of the eluting peptides are monitored at 205nm with a UV/Visible spectrophotometer detector (model SPD-6AV, Shimadzu, Kyoto, Japan) and compared against calibration standards (supplied by Sigma, Sigma-Aldrich New Zealand Ltd).

3.1.14 Determination of Proteins by Resource™ HPLC

The powder samples were analysed for the whey proteins α-lactalbumin, β-lactoglobulin, bovine serum albumin and glycomacropeptide (α-Lac, β-Lac, BSA, and GMP, respectively) and lactoferrin (LF) using the Resource™ HPLC method NZTM 3.15.10 Whey Proteins by Reverse Phase High Performance Liquid Chromatography. Whey proteins are separated according to their hydrophobicity using reversed-phase chromatography with UV detection (214 nm), Elgar, 2000 (*143*); Coolbear, 1993 (*144*); Palmano, 2002 (*141*); Kronman, 1964 (*145*); Tanford, 1952 (*146*); and Wetlaufer, 1974 (*147*). Briefly, samples reconstituted in water were centrifuged to remove any particulate matter and the soluble fraction analysed by HPLC (Waters, Milford, MA, USA) as above.

3.1.15 Determination of Phospholipids by Mass Spectrometry

Samples were separated into polar and non-polar phases using the Folch/Svennerholm separation method which is based on the work done by Svennerholm, 1980 (*148*) and Folch, 1957 (*149*). The samples were then analysed for phospholipid content by Carmen Norris using Mass Spectrometry Unit, Fonterra Research Centre in Palmerston North. The HPLC/MS methodology is that as found in Fong, 2008 (*150*).

3.1.16 Determination of Enzyme Hydrolysis using PAGE Gels

Samples were hydrolysed with pepsin and trypsin enzymes before being run on polyacrylamide gel electrophoresis (PAGE) gels, with controls, to determine the level of digestion.

Trypsin Digest:

The samples were weighed out and diluted with 0.2M ammonium bicarbonate, pH ~ 8, to make a 10mg/mL solution in 16mL Kilmax test tubes. The samples were placed in a 37°C waterbath for 10 minutes to allow the solution to reach 37°C. For the sample and time zero control, 100µL of solution was then removed and placed in a 600µL Eppendorf microcentrifuge tube and 100µL of SDS/BME (sodium dodecylsulfate/β-mercaptoethanol) sample buffer was added in preparation for SDS-PAGE. Trypsin (from Sigma: T1426-250MG 056K7775) was added to the remaining samples in the Kilmax tubes at a 1:100 (w/w) ratio and also at a 1:40 (w/w) ratio and incubation continued at 37°C. Time point aliquots (100µL) were taken at 5, 10, 30, 45 and 60 minute intervals and treated the same as for time zero. The SDS/BME buffer was also used to inactivate the enzyme and prevent further proteolysis in the samples. The sample aliquots were then stored at -30°C until required for gels. The remaining solution was frozen down until it was confirmed by PAGE gels that the hydrolysis had worked. The solution was then defrosted and heated to above 80°C for 10 minutes to inactivate the trypsin enzyme. The solution was then freeze dried to convert it to a powder.

Pepsin Digest:

Different ways of getting the pepsin to digest the sample were tried, including using different pepsins, but the following method gave the best results.

Samples were dissolved in water, the pH was adjusted to between pH 2.50 and 2.90 with 1M HCl, and then water was added to make 10mg/mL solutions. The pH was checked to ensure it was still between pH 2.50 and 2.90. As with the trypsin digest, the samples were equilibrated for 10 minutes at 37°C before time point zero was collected and pepsin was added to the samples in ratios of 1:100 (w/w) and 1:40 (w/w) (Pepsin: Sigma P-7000, Lot 129F0148). Time point aliquots of 100µL were taken at 5, 10, 30 and 60 minute intervals and the enzyme activity was stopped by adding 100µL of SDS

buffer. The sample aliquots were then stored at -30°C until required for gels. The remaining solution was adjusted to between pH 7.00 and 7.50 with 1M sodium hydroxide (NaOH) to inactivate the pepsin. The remaining solution was frozen and stored at -30°C until it was confirmed by SDS-PAGE gels that the hydrolysis had worked, then the solution was defrosted and freeze dried.

SDS-PAGE:

Laboratory made SDS-PAGE gels were initially used to test if the hydrolysis was working. After the best enzyme digestion conditions were determined, Criterion pre-cast gels (8-16% Tris-HCl, 1.0mm, Bio-Rad Laboratories, New Zealand) were used for the samples because they gave greater consistency between gels, were larger and able to give better separation of the bands.

5µL of BME was added to 100µL of each hydrolysed sample in 600µL Eppendorf microcentrifuge tubes, the contents were mixed by vortexing and heat treated in a beaker of boiling water on a hot plate for 4-5 minutes. The samples were allowed to cool for ~1 minute before 20µL of each sample was loaded across the gel. The BIO-RAD All Blue Precision Plus Protein™ standard (Catalogue #161-0373) was loaded on the gel at 5µL and was run at either end of the gels to give the molecular weight scale. The standard contained 10 protein bands ranging from 10kD up to 250kD. Samples of the trypsin and pepsin were also run on the gels for comparison.

The gel running conditions for the pre-cast gels, using Bio-Rad power supply (model 1000/500), were as follows:

Program: T/V-H

Volts: 210

Current: 80

Power: 6.5

Time: 2 hours

For the laboratory-made SDS-PAGE gels the conditions were the same except for the run time, which was set at 1.2 hours.

The gels were stained for 1-2 hours with Amido Black and destained with 10% acetic acid with regular changes of the destain buffer over the next 3 days. Once the gels had been destained sufficiently they were examined for band separation and the amount of hydrolysis each sample had undergone at each time point. The gels of interest were scanned using the ImageScanner™ III (from GE Healthcare/Life Sciences).

3.1.17 Determination of Proteins using RP-LC-MS/MS

The hydrolysed acid fraction powders were dissolved in loading buffer (0.1% formic acid/0.1% trifluoroacetic acid (TFA)) and centrifuged/filtered to remove any particulate material and placed in glass vials for analysis of their peptide content using reverse phase-liquid chromatography-Mass spectrometer (RP-LC-MS/MS).

Using a LC Packing U3000 LC System in the capillary flow mode the sample was first loaded onto a 300µm id x 1mm capillary PepMAP trap at 30µL/min, as a pre-concentration/desalting step, before the sample was loaded onto a 150mm x 300µm, 300A, 3µm particle size, capillary C18 PepMAP column at 3µL/min.

Solvent A was 0.2% formic acid and solvent B was 90% acetonitrile and 0.2% formic acid. The peptides were separated on and eluted off the column using a gradient of low acetonitrile (5% Solvent B) to high acetonitrile (90% Solvent B) over the course of the run (total time 35 minutes) before the column was re-equilibrated to starting conditions for the next sample.

A linear trap quadrupole (LTQ) OrbiTrap™ mass spectrometer, equipped with an electrospray ionisation source, was connected to the LC system. Information-dependent acquisition experiments were performed according to the following parameters: full scan carried out on the OrbiTrap™ with 30000 resolution, followed by MS/MS of the top six most intense peaks in the LTQ. Former target ions were excluded for 20 seconds.

<u>RP-LC/MS/MS analysis - Protein identification</u>

Protein identification was carried out using the MS/MS spectral data matching search engine SEQUEST (Version 2.0.0.4,) as described. The filtered MS/MS data (spectra

were rejected if less than 10 peaks) were searched against the National Centre for Biotechnology Information (NCBI) Bos Taurus genomic database (NCBI build 2.1, based on Btau_2.0, www.ncbi.nlm.nih.gov), a reversed NCBI Bos Taurus genomic database (where the amino acid sequence of each Bos Taurus entry was reversed), the NCBI Bos Taurus database (extracted from the NCBI nr database) and the Swiss-Prot database (other mammalia only, Version 25-07-06, www.expasy.org/sprot).

The following search parameters were used: MS error ± 0.1 Da; MS/MS error ± 0.1 Da; fixed modifications: carbamidomethyl cysteine; variable modifications: phosphorylation of serine and threonine residues, oxidation of methionine. A maximum of two missed tryptic cleavages was allowed. Protein matches were considered to be valid if there were at least two valid matched peptides with a p score less than or equal to 0.005. Beyond this score, additional unique peptides matching the protein were not necessarily validated. Valid peptides were required to have all of the major peaks in the spectra assigned.

3.1.18 Determination of Bone Activity

The parent, fraction and digested powder samples were submitted to Dr Wei-Hang Chua at Massey University, Institute of Food, Nutrition and Human Health for bone activity analysis. This testing was done to assess the impact the powders had on cell proliferation in osteoblasts and the effect they had on differentiation of osteoclast precursors into mature osteoclasts.

The methods used, as taken from a report by Dr Wei-Hang Chua (*151*), are as follows:

Osteoblast Proliferation

The testing to assess the impact the powders had on cell proliferation in osteoblasts was done in 2 stages: First the Murine preosteoblast (MC3T3-E1) cells were cultured, based on the work of Takada, 1996 (*16*); secondly the powders were tested for osteoblast cell proliferation, based on the work of Yamamura, 1999 (*75*). Murine preosteoblast (MC3T3-E1) cells were maintained at 37^{o}C in a 5% CO_2 and 95% Air environment in Minimal essential medium α (MEMα) medium with 10% (v/v) fetal calf serum (FCS) supplemented with penicillin-streptomycin-glutamine antibiotics. The cells were

seeded at 1x 10^5 cells/mL and grown to confluence (5-7 days) after which they were subcultured, the culture media removed and the cells rinsed with phosphate buffered saline (PBS, pH 7.4). The cells were then trypsinised with trypsin-ethylenediaminetetraacetic acid (EDTA), rinsed in MEMα with 10% FCS, centrifuged and resuspended in medium. The cells were then seeded into 96-well flat bottom plates at 0.75 x 10^5 cells/mL and incubated at 37°C for 24 hours before being incubated for a further 24 hours in medium with 0.1% BSA.

The powder samples, at concentrations of 100ng/mL, 1, 10, and 100 µg/mL, were solubilised in medium with 0.1% BSA, 0.20 µm filtered-sterilised and then added to the cells in the 96-well flat bottom plate. This was done in triplicate. The cells were incubated in medium with 0.1% BSA for 24 hours in 5% CO_2 at each of the different sample concentrations. Lactoferrin was included at 150µg/mL as a positive control for cell proliferation.

Quantification of the cell proliferation was done using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colourimetric assay, where the yellow MTT is reduced to purple formazan crystals by active cells. These formazan crystals are then solubilised using dimethyl sulfoxide (DMSO) and the formazan is quantified colourimetrically at 550nm.

Osteoclast Maturation (Osteoclastogenesis)

Testing of the effect the powders had on differentiation of osteoclast precursors into mature osteoclasts was based on the work of Yun *et al.*, 2007 (152) and Collin-Osdoby *et al.*, 2003 (153). The murine macrophage RAW 264.7 cell line can be used to generate osteoclasts. RAW 264.7 cells were plated in wells and treated with murine receptor activator of nuclear factor κ B ligand (RANK-L). Different concentrations of the powder samples, at 0.1, 1, 10 and 100µg/mL, were added to the cell culture media and incubated for 5 days, with a media change on day 3. At day 5 an aliquot of cell culture media was removed and quantified colourimetrically for tartrate-resistant acid phosphatase (TRAP). The TRAP enzyme is a primary indicator of osteoclast formation. The cells growing in the wells were fixed then stained for TRAP before being counterstained with hematoxylin, causing osteoclasts to appear as large stained purple

red multinucleated cells, and in some cases larger giant cells. All the samples were assayed in duplicate and all cell culture measurements of TRAP were conducted in triplicate. As a control the samples were assayed with RANK-L present and absent, as it is known that osteoclast formation requires the presence of RANK-L (*154*).

4. Results and Discussion Section

4.1 Results and Discussion

4.1.1 Composition of WPC Powders and Fractions

The following results give compositional details of the parent WPC substrates and resulting fractions. The aim was to gather sufficient information on both macro and micro components of the powders so as to be able to compare and contrast in terms of possible contributors to bone bioactivity, and derive an overall fingerprint for each for future comparisons. As WPC is a complex blend of many constituents, analyses above and beyond typical compositional analysis were based on those parameters previously reported to have effects on bone at the *in vitro* level and at the *in vivo* level (e.g. fatty acids (*64, 65, 155*), growth factors (*80, 85, 86*) and Vitamin D/K (*60, 156-160*)).

4.1.2 Elemental Composition and Total Phosphate

The elemental composition of the parent powders and the acid fraction and breakthrough fractions varied for all three WPCs and may be related to mode of manufacture. Table 2 shows the elemental composition of the parent powders and the acid fraction (AF) and breakthrough fraction (BT) powders.

Table 2: ICP-OES results for the parent WPC and fractionated powders.

Element	GS19 – Cheese WPC80			CR05 – Mineral WPC80			HS15 – Lactic Acid WPC80		
	Parent	AF	BT	Parent	AF	BT	Parent	AF	BT
Inorganic as PO4 (mmol/kg)	17.2	<5.2	7.1	12.3	<5.2	<5.2	35.5	<5.2	24.9
Calcium (mg/kg)	4345	97	5370	778	91	927	2255	<Q.L.[1]	2930
Copper (mg/kg)	0.6	1.8	1.7	0.7	1.8	1.4	1.0	4.2	1.3
Iron (mg/kg)	11.3	33.3	<Q.L.[2]	3.3	21.7	<D.L.[3]	3.2	12.1	<D.L.[4]
Potassium (mg/kg)	6510	<Q.L.[5]	7300	120	82	133	7510	<Q.L.[6]	9340
Magnesium (mg/kg)	451	<D.L.[7]	542	29	<D.L.[8]	35	180	<D.L.[9]	233
Manganese (mg/kg)	0.24	0.20	0.28	0.02	0.08	0.07	0.14	0.08	0.17
Sodium (mg/kg)	1835	21100	2070	9175	13800	10100	5160	22000	6830
Phosphorus (mg/kg)	2950	5720	2440	2295	10400	1550	3895	11400	2330
Zinc (mg/kg)	6.2	<Q.L.[10]	8.4	5.6	8.6	7.0	9.0	2.6	<D.L.[11]

Notes:

Limit of detection (LOD); Limit of quantitation (LOQ)

[1] Ca < LOQ, LOQ = 60.50 mg/kg

[2] Fe < LOQ, LOQ = 9.34 mg/kg

[3] Fe < LOD, LOD = 3.21 mg/kg

[4] Fe < LOD, LOD = 3.40 mg/kg

[5] K < LOQ, LOQ = 72.40 mg/kg

[6] K < LOQ, LOQ = 74.80 mg/kg

[7] Mg < LOD, LOD = 7.28 mg/kg

[8] Mg < LOD, LOD = 6.70 mg/kg

[9] Mg < LOD, LOD = 7.53 mg/kg

[10] Zn < LOQ, LOQ = 5.92 mg/kg

[11] Zn < LOD, LOD = 6.31 mg/kg

It is interesting, because of its importance to bone growth, to see the variation in calcium levels between the different types of WPCs and between the parent WPCs and their associated fractions. The data in the table above shows that the bulk of the calcium from the GS19 and HS15 parent WPC resides in the breakthrough fractions, taking into consideration that the total solid of the acid fraction is only ~6% of the total solid of the breakthrough collected (although only a proportion of the breakthrough fraction was freeze dried down, total breakthrough solids were estimated from final volume yield and partial dry weight yields). For CR05, the balance of calcium found in the two fractions is very similar, when the total solids amount is taken into consideration, but the overall amount of calcium found in this parent WPC is low compared to the other two parent WPCs. This situation is similarly true for potassium and magnesium.

The lower mineral content (with the exception of sodium) of the CR05 parent WPC, compared to GS19 and HS15, might be related to the differences in the whey manufacturing, especially the extent of ultra filtration.

Copper, iron and phosphorus appear to associate more in the acid fraction than the breakthrough fraction. The high concentration of phosphorus in the acid fraction may be attributed to the retention of organic phosphate, associated with proteins and lipids, on the anion exchange column, whereas the inorganic phosphate, most likely in the form of calcium phosphate, would most likely be found in the breakthrough fraction. Iron associates strongly with the acid fraction, with almost all in the parent WPCs being found in the acid fraction, this may be due to the binding of iron to whey proteins (*161*).

The parent WPCs, and related breakthroughs, contain relatively high concentrations of sodium, when compared to the other compositional elements tested; however, the acid fractions appear to contain extremely high concentrations of sodium. The high sodium results may be related to the elution buffer, NaCl, not being completely removed via dialysis, with the sodium and chloride ions being retained, and may also relate to the ~30% increase of the acid fraction volume, after dialysis, due to increased retention of water containing the charged ions. It may also be that the sodium has bound to the acidic proteins Although the conductivities of the acid fraction solutions after dialysis were low, due to the large volume, the freeze dried acid fraction powder had an

increased ratio of salt to solid material, 1-2% of the acid fraction total solid material being sodium.

4.1.3 Vitamin D3 and Vitamin K Content

The parent samples were tested and returned values of <2µg/100g and "*not detected*" for Vitamin D3 and Vitamin K respectively, therefore the fraction samples were not tested. At these negligible levels, vitamin D3 and K were assumed to have no contribution to bone bioactivity for the parent or fractionated WPCs.

4.1.4 Total Nitrogen, Protein and Non-Protein Nitrogen

On a weight percentage basis it appears the protein from the parent WPCs is relatively evenly distributed between the acid fractions and the breakthroughs of the respective WPCs. All fractions contained ~70-87% protein. However, when the dry weight yield of the fractions is considered, the bulk mass of the proteins passed through the column and was collected in the breakthrough fraction, i.e. the parent WPCs and fractions all contain ~70-87% protein by weight, but on a yield basis the majority of the protein was captured in the breakthrough and only ~4-6% of the original protein loaded was isolated in the acid fraction (see Experimental Section - Isolation of the Fractions). The protein concentration was calculated from the TN using the standard conversion factor of 6.38 for milk proteins (*115*). Table 3 shows the protein composition of the parent powders and the acid fraction and breakthrough fraction powders.

Table 3: TN, protein and NPN content of the parent WPC and fractionated powders.

Test	GS19 – Cheese WPC80			CR05 – Mineral WPC80			HS15 – Lactic Acid WPC80		
	Parent	AF	BT	Parent	AF	BT	Parent	AF	BT
Non Protein Nitrogen (% w/w)	0.71	8.26	1.10	0.11	2.36	0.26	0.65	4.34	0.21
Kjeldahl Total Nitrogen (% w/w)	12.42	11.16	12.16	12.99	12.54	13.52	12.15	11.19	12.46
Calculated Protein (TN x 6.38)	79.21	71.20	77.58	82.88	80.01	86.26	77.49	71.39	79.50

It is interesting to note that the acid fractions appear to have a higher percentage of NPN compared to the breakthrough fraction. In milk, NPN is normally contributed to by compounds such as urea, free amino acids, growth factors, ammonia and uric acid (*162, 163*). The high concentration of NPN found in the acid fractions of these WPCs may be contributed to by small peptides selectively captured in this fraction.

4.1.5 Fat Content

Table 4 shows the fat results obtained by the ASE method for the parent WPCs and the fractionated powders. With the exception of GS19, it appears that, on a weight percentage basis, the fat content from the parent WPCs was reasonably evenly distributed into the respective acid and breakthrough fractions. Thus both CR05 and HS15 acid fractions contained significant amounts of fat (5-6%). (It should be noted that although whey itself contains little fat, the UF membrane processes employed in the manufacture of WPC results in the retention and, hence, concentration of the fat components in the WPC).

Table 4: Fat content of the parent WPC and fractionated powders.

Test	GS19 – Cheese WPC80			CR05 – Mineral WPC80			HS15 – Lactic Acid WPC80		
(% w/w)	Parent	AF	BT	Parent	AF	BT	Parent	AF	BT
Fat by ASE	7.27	0.86	10.10	7.34	6.11	5.83	7.68	5.04	9.62

The milk fat is composed of triglycerides, phospholipids and small amounts of free fatty acids. The low level of fat found in the GS19 acid fraction may result from an overloading of the column with GMP, resulting in the displacement of the fat and other possible peptide/proteins. Another possible reason for the low level of fat found in the GS19 acid fraction could be due to associated with measurement error complications in the extraction of the fat using the ASE, where the sample required a second extraction due to a blockage occurring in the first extraction in the ASE extraction cell.

4.1.6 Fatty Acid Analysis

The levels of most fatty acids measured in the three parent WPCs and fractions were consistent with levels of fatty acids normally found in milk fat (*11*), although there

were minor variations in content no doubt associated with WPC origin e.g. C16 (palmitic acid). Table 5 contains the fatty acid results for the fats which were ASE-extracted from the parent and the fractionated powders and Figure 9 represents these results graphically.

There appeared to be no major differences in fatty acid profiles between the three acid fractions, although of note, C16 was lower in the CR05 acid fraction, and both C18:3N-3 and C20:5N-3 (EPA) were lower in the GS19 acid fraction. When compared to the parent WPC substrates, the fatty acid profiles of the respective acid fractions were generally similar. However, contents of C4-C14 fatty acids were somewhat lower in the acid fractions, while contents of C18:2N-6 and C18:1N-9 fatty acids were higher. This may reflect a higher proportion of phospholipid in the acid fractions as the phospholipid fraction is characterised by a low content of fatty acids below C14 with the major fatty acids being C18:1N-9, C18:2N-6 and C16:0/C18:0 (*11*).

It has been shown that fatty acids of 14 to 18 carbon atoms in length, particularly C16:0, strongly inhibit osteoclastogenesis in bone marrow culture assays and also osteoclast development in RAW264.7 cell assays, as well as stimulating osteoblast cell proliferation (*62*). The presence of fat in the WPC powders and acid fraction may well contribute to *in vivo* bone activity, although for *in vitro* assays, release of fatty acids from the triacylglycerol backbone would seem to be a pre-requisite for activity (*62*).

Table 5: Fatty acid composition of the parent WPC and fractionated powders.

Fatty Acid	GS19 – Cheese WPC80			CR05 – Mineral WPC80			HS15 – Lactic Acid WPC80		
(wt % in Fat)	Parent	AF	BT	Parent	AF	BT	Parent	AF	BT
C04:0	2.1	1.5	1.9	2.2	1.3	2.3	1.9	1.4	1.2
C06:0	1.4	1.0	1.2	1.6	1.0	1.4	1.3	1.0	0.9
C08:0	0.8	0.6	0.6	1.0	0.6	0.9	0.8	0.6	0.5
C10:0	2.0	1.3	1.8	2.6	1.4	2.4	2.0	1.4	1.6
C10:1	0.2	0.0	0.2	0.2	0.1	0.2	0.2	0.1	0.0
C12:0	3.7	3.1	3.5	3.4	2.2	3.3	3.9	2.8	3.8
C13:0 BR	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0
C12:1	0.1	0.0	0.3	0.1	0.1	0.1	0.1	0.2	0.0
C13:0	0.1	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.0
C14:0 BR	0.1	0.0	0.1	0.1	0.0	0.1	0.1	0.1	0.0
C14:0	10.2	7.6	9.8	9.9	6.6	9.6	9.9	7.5	8.5

C14:1	0.9	0.4	0.7	0.5	0.4	0.4	0.8	0.5	0.5
C15:0 ISO BR	0.4	0.2	0.3	0.3	0.2	0.2	0.3	0.2	0.2
C15:0 ANTE-ISO	0.5	0.4	0.5	0.5	0.4	0.4	0.4	0.4	0.4
C15:0	1.2	1.0	1.2	1.1	0.8	1.0	1.1	0.9	0.9
C16:0 BR	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.0
C16:0	31.9	28.6	32.0	25.0	19.6	25.0	29.9	24.9	28.3
C16:1	1.9	1.6	1.6	1.4	1.4	1.3	1.7	2.0	1.3
C17:0 ISO BR	0.4	0.2	0.3	0.4	0.4	0.4	0.4	0.3	0.3
C17:0 ANTE-ISO	0.5	0.4	0.5	0.4	0.4	0.4	0.4	0.3	0.3
C17:0	0.7	0.5	0.5	0.6	0.5	0.5	0.6	0.4	0.4
C17:1	0.4	0.2	0.4	0.3	0.4	0.3	0.3	0.4	0.3
C18:0	8.9	10.2	9.1	11.7	13.1	12.0	10.4	10.6	10.3
C18:1N-9	19.1	24.9	20.3	19.4	27.1	19.8	19.1	26.6	22.3
C18:1N-7	2.8	2.7	2.9	5.7	5.7	5.5	3.5	3.5	3.1
C18:2N-6	2.1	3.2	2.3	2.2	4.0	2.3	2.0	3.9	2.9
C18:3N-3	0.7	0.6	0.7	1.1	1.8	1.1	0.8	1.9	1.0
C18:2 CONJ.	0.2	0.0	0.2	0.3	0.3	0.2	0.2	0.2	0.2
C18:4N-3	0.1	0.0	0.0	0.1	0.1	0.0	0.1	0.0	0.0
C20:0	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.2
C20:1N-11	0.7	0.8	0.6	1.1	1.4	1.0	0.8	1.0	0.7
C20:1N-9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C20:2N-6	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.1	0.2
C20:3N-6	0.3	0.4	0.3	0.2	0.5	0.2	0.3	0.5	0.4
C20:4N-6 (AA)	0.2	0.2	0.2	0.2	0.4	0.2	0.2	0.4	0.2
C20:3N-3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C20:4N-3	0.1	0.0	0.1	0.1	0.3	0.1	0.1	0.2	0.2
C20:5N-3 (EPA)	0.2	0.1	0.1	0.2	0.5	0.2	0.2	0.4	0.3
C22:0	1.2	1.9	1.4	1.3	1.5	1.5	1.4	1.1	2.2
C22:1N-13,N-11	0.0	0.4	0.0	0.0	0.1	0.0	0.0	0.0	0.0
C22:1N-9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C23:0	1.7	2.6	1.9	1.9	2.0	2.1	2.0	1.4	3.1
C23:1	0.1	0.2	0.2	0.3	0.5	0.4	0.2	0.3	0.4
C24:0	1.4	1.6	1.1	1.4	1.2	1.3	1.6	0.8	2.0
C24:1	0.2	0.3	0.2	0.2	0.3	0.3	0.2	0.2	0.3

Note:

Fatty Acid	Common Name	Scientific Name	Molecular Formula
C04:0	Butyric acid	Butanoic acid	$C_4H_8O_2$
C06:0	Caproic acid	Hexanoic acid	$C_6H_{12}O_2$
C08:0	Caprylic acid	Octanoic acid	$C_8H_{16}O_2$
C10:0	Capric acid	Decanoic acid	$C_{10}H_{20}O_2$
C10:1	Caproleic acid	9-decenoic acid	$C_{10}H_{18}O_2$
C12:0	Lauric acid	Dodecanoic acid	$C_{12}H_{24}O_2$

C13:0 BR	Isoundecylic acid	11-methyl-dodecanoic acid	$C_{13}H_{26}O_2$
C12:1	Lauroleic acid	9-dodecenoic acid	$C_{12}H_{22}O_2$
C13:0	Tridecylic acid	Tridecanoic acid	$C_{13}H_{26}O_2$
C14:0 BR	Isomyristic acid	12-methyl-tridecanoic acid	$C_{14}H_{28}O_2$
C14:0	Myristic acid	Tetradecanoic acid	$C_{14}H_{28}O_2$
C14:1	Myristoleic acid	9-tetradecenoic acid	$C_{14}H_{26}O_2$
C15:0 ISO BR	Isopentadecylic acid	13-methyl-tetradecanoic acid	$C_{15}H_{30}O_2$
C15:0 ANTE-ISO	12-methyl myristic acid	12-methyl-tetradecanoic acid	$C_{15}H_{30}O_2$
C15:0	Pentadecyclic acid	Pentsdecanoic acid	$C_{15}H_{30}O_2$
C16:0 BR	Isopalmitic acid	14-methyl-pentadecanoic acid	$C_{16}H_{32}O_2$
C16:0	Palmitic acid	Hexadecanoic acid	$C_{16}H_{32}O_2$
C16:1	Palmitoleic acid	9-hexadecenoic acid	$C_{16}H_{30}O_2$
C17:0 ISO BR	15-methyl palmitic acid	15-methyl-hexadecanoic acid	$C_{17}H_{34}O_2$
C17:0 ANTE-ISO	14-methyl palmitic acid	14-methyl-hexadecanoic acid	$C_{17}H_{34}O_2$
C17:0	Margaric acid	Heptadecanoic acid	$C_{17}H_{34}O_2$
C17:1	9-heptadecylenic acid	9-heptadecenoic acid	$C_{17}H_{32}O_2$
C18:0	Stearic acid	Octadecanoic acid	$C_{18}H_{36}O_2$
C18:1N-9	Oleic acid	cis-9-octadecenoic acid	$C_{18}H_{34}O_2$
C18:1N-7	Vaccenic acid	cis-11-octadecenoic acid	$C_{18}H_{34}O_2$
C18:2N-6	Linoleic acid	cis-9,12-octadecadienoic acid	$C_{18}H_{32}O_2$
C18:3N-3	Alpha-linolenic acid (ALA)	cis-9,12,15-octadecatrienoic acid	$C_{18}H_{30}O_2$
C18:2 CONJ.	Conjugated linoleic acid	(9Z,11E)-octadecadienoic acid	$C_{18}H_{32}O_2$
C18:4N-3	Stearidonic acid (Moroctic acid)	cis-6,9,12,15-octadecatertraenoic acid	$C_{18}H_{28}O_2$
C20:0	Arachidic acid	Eicosanoic acid	$C_{20}H_{40}O_2$
C20:1N-11	Gadoleic acid	cis-9-icosenoic acid	$C_{20}H_{38}O_2$
C20:1N-9	Eicosenoic acid (Gondoic acid)	cis-11-eicosenoic acid	$C_{20}H_{38}O_2$
C20:2N-6	Eicosadienoic acid	cis-11,14-eicosadienoic acid	$C_{20}H_{36}O_2$
C20:3N-6	Dihomo-gamma-linolenic acid (DGLA)	cis-8,11,14-eicosatrienoic acid	$C_{20}H_{34}O_2$
C20:4N-6 (AA)	Arachidonic acid (AA)	cis-5,8,11,14-eicosatetraenoic acid	$C_{20}H_{32}O_2$
C20:3N-3	Eicosatrienoic acid (ETE)	cis-11,14,17-eicosatrienoic acid	$C_{20}H_{34}O_2$
C20:4N-3	Eicosatetraenoic acid (ETA)	cis-8,11,14,17-eicosatetraenoic acid	$C_{20}H_{32}O_2$
C20:5N-3 (EPA)	Eicosapentaenoic acid (EPA)	cis-5,8,11,14,17-eicosapentaenoic acid	$C_{20}H_{30}O_2$
C22:0	Behenic acid	Docosanoic acid	$C_{22}H_{44}O_2$
C22:1N-13,N-11	Cetoleic acid	cis-9/cis-11 docosenoic acid	$C_{22}H_{42}O_2$
C22:1N-9	Erucic acid	cis-13-docosenoic acid	$C_{22}H_{42}O_2$
C23:0	Tricosylic acid	Tricosanoic acid	$C_{23}H_{46}O_2$
C23:1	ω-tricosenoic acid	22-tricosenoic acid	$C_{23}H_{44}O_2$
C24:0	Lignoceric acid	Tetracosanoic acid	$C_{24}H_{48}O_2$
C24:1	Nervonic acid	cis-15-tetracosenoic acid	$C_{24}H_{46}O_2$

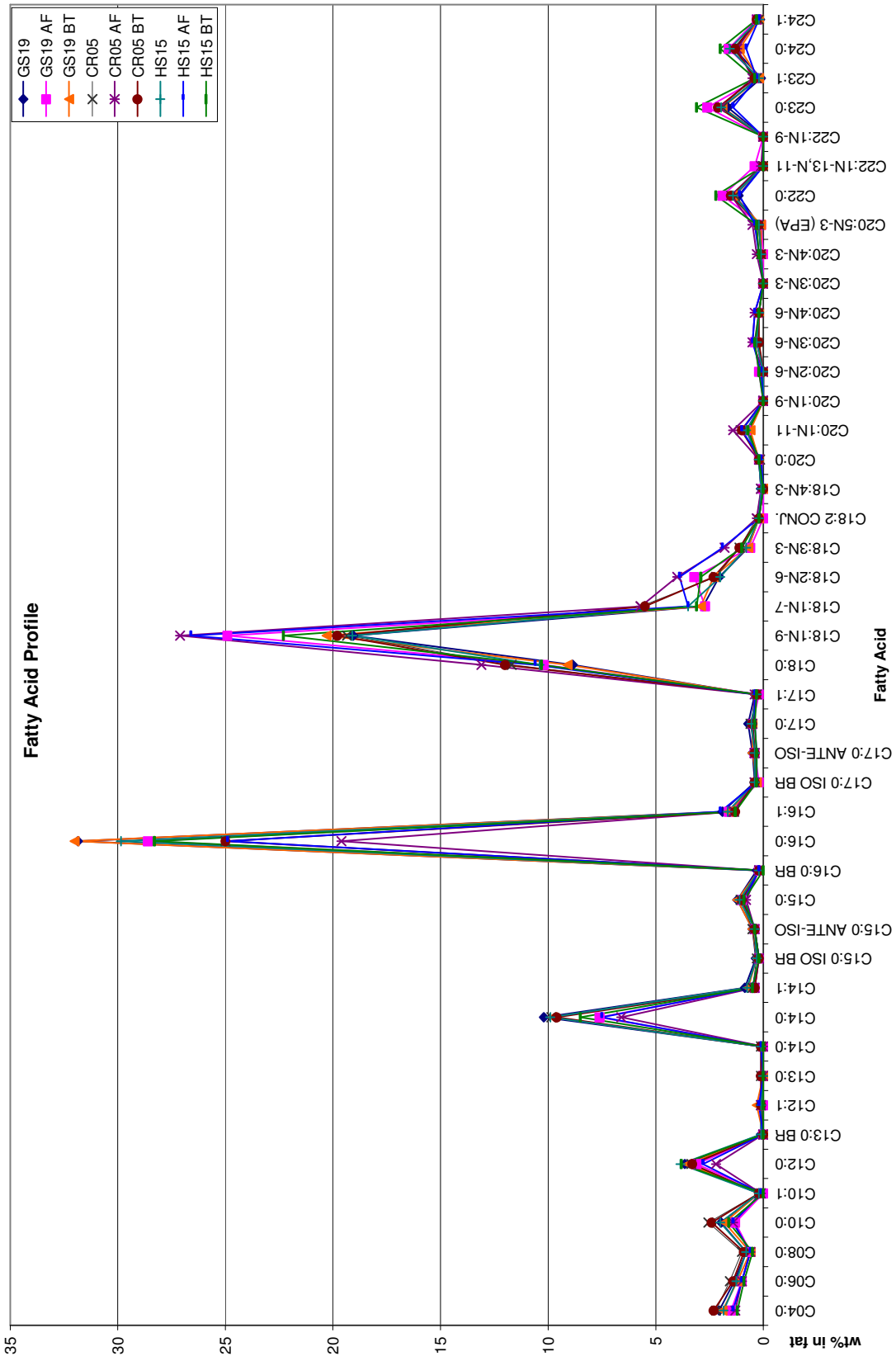

Figure 9: Fatty acid profile of the parent WPC and fractionated powders.

4.1.7 Phospholipids Analysis by Mass Spectrometry

The Mass Spectrometry (MS) results (Table 6 and 7) indicate that, based on peak area, phosphatidylcholine and sphingomyelin are the most dominant phospholipids found in the parent WPC and the fractionated powders, which is consistent with the general pattern of phospholipid representation in milk fat (*11*). Phosphatidylserine is found to be the next abundant phospholipid, especially in the acid fractions.

For the parent WPCs CR05 and GS19 appear to contained similar amounts of phospholipids, significantly more then HS15. This trend is also true for the breakthrough fractions. Unfortunately, due to an instrument breakdown during a previous analysis and a limited sample quantity, there was not enough GS19 acid fraction sample available for a repeat analysis by mass spectrometry.

The phospholipids peak areas for CR05 and HS15 acid fraction compare favourably with each other, with the exception of CR05 containing significantly higher level of sphingomyelin, as is the trend seen in the parent and breakthrough fractions also.

Table 6: Phospholipids distribution in the parent WPC powders (MS peak area).

Phospholipid (Peak Area)	GS19 - Parent Cheese WPC80	CR05 - Parent Mineral WPC80	HS15 - Parent Lactic Acid WPC80
PI	159006899	181272060	181194453
PE	474612468	705050915	482060447
PC	9728484153	9355935684	8637467285
L-PE	100328346	189774471	72156252
SM	8415896147	10057417924	4569715799
L-PC	554294841	883219809	386055899
PS	603194389	1191869204	510458807
Total	20035817243	22564540067	14839108942

Notes:

PI = Phosphatidylinositol

PE = Phosphoethanolamine

PC = Phosphatidylcholine

L-PE = Lysophosphatidylethanolamine

SM = Sphingomyelin

L-PC = Lysophosphatidylcholine

PS = Phosphatidylserine

Table 7: Phospholipids distribution in the acid and breakthrough fractions.

Phospholipid (Peak Area)	CR05 - AF	HS15 - AF	GS19 - BT	CR05 - BT	HS15 - BT
PI	382818144	288175774	65952629	95319854	80162426
PE	952396962	935651137	366062615	530453334	215829887
PC	10315606283	11172053227	5583189510	5654194000	5221016079
L-PE	188010733	223589790	56612636	48015420	12445595
SM	11175894694	6921471414	3516396658	6315051509	3459884254
L-PC	834204038	996274315	317299600	563725480	169031867
PS	1986931211	2637856763	302728139	467336245	229878728
Total	25835862065	23175072420	10208241787	13674095842	9388248836

It is noteworthy to mention that the level of phospholipid measured in the CR05 and HS15 acid fractions is about twice that measured in the corresponding breakthrough. This finding implies that there is selective capture of the phospholipids in the acid fraction by the anion exchange column. This data is shown graphically in Figure 10a and Figure 10b**.**

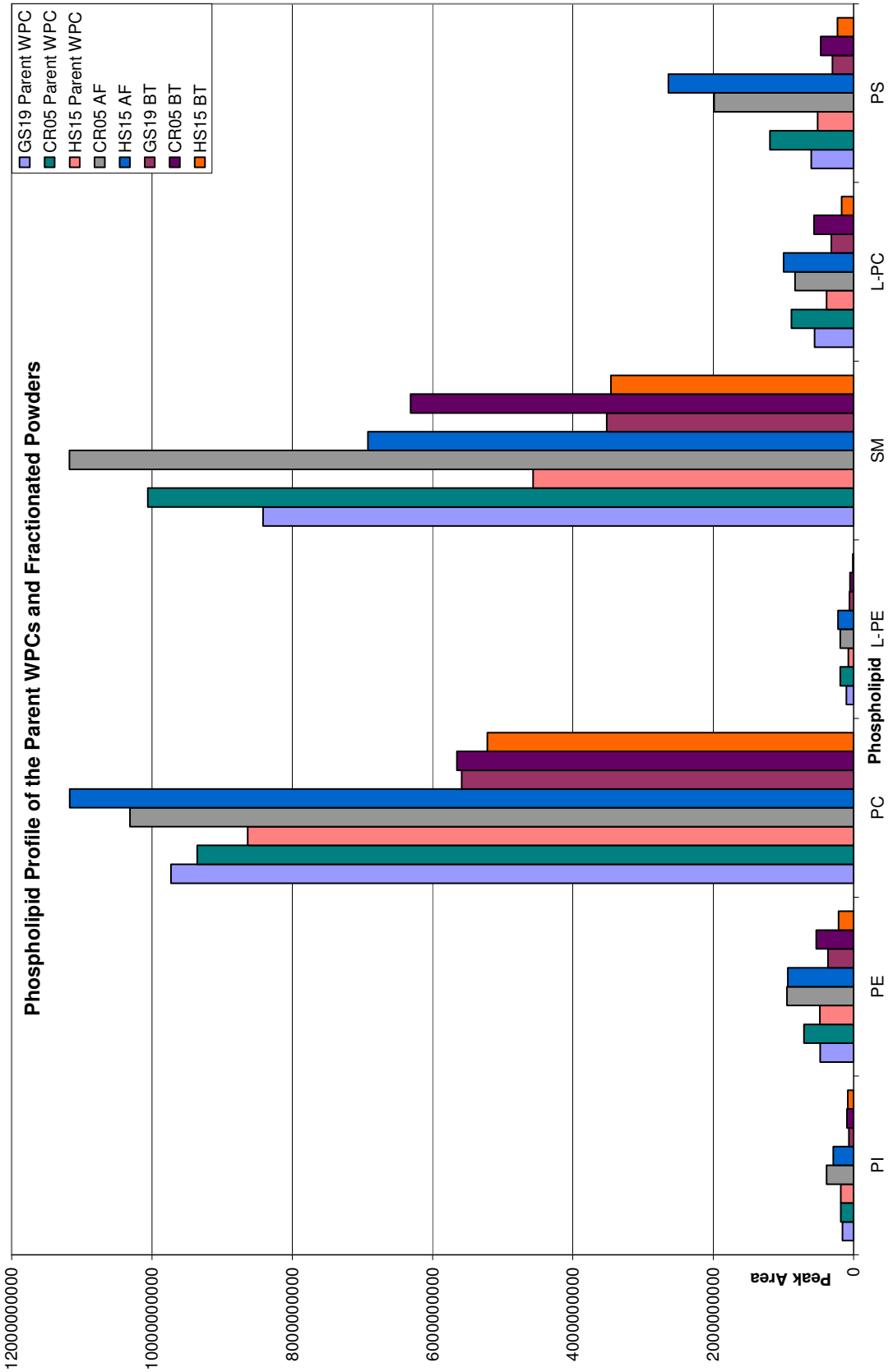

Figure 10a: Phospholipid profile of the parent WPCs and fractionated powders. (See Figure 10b for a smaller scale graph of the low concentration phospholipids).

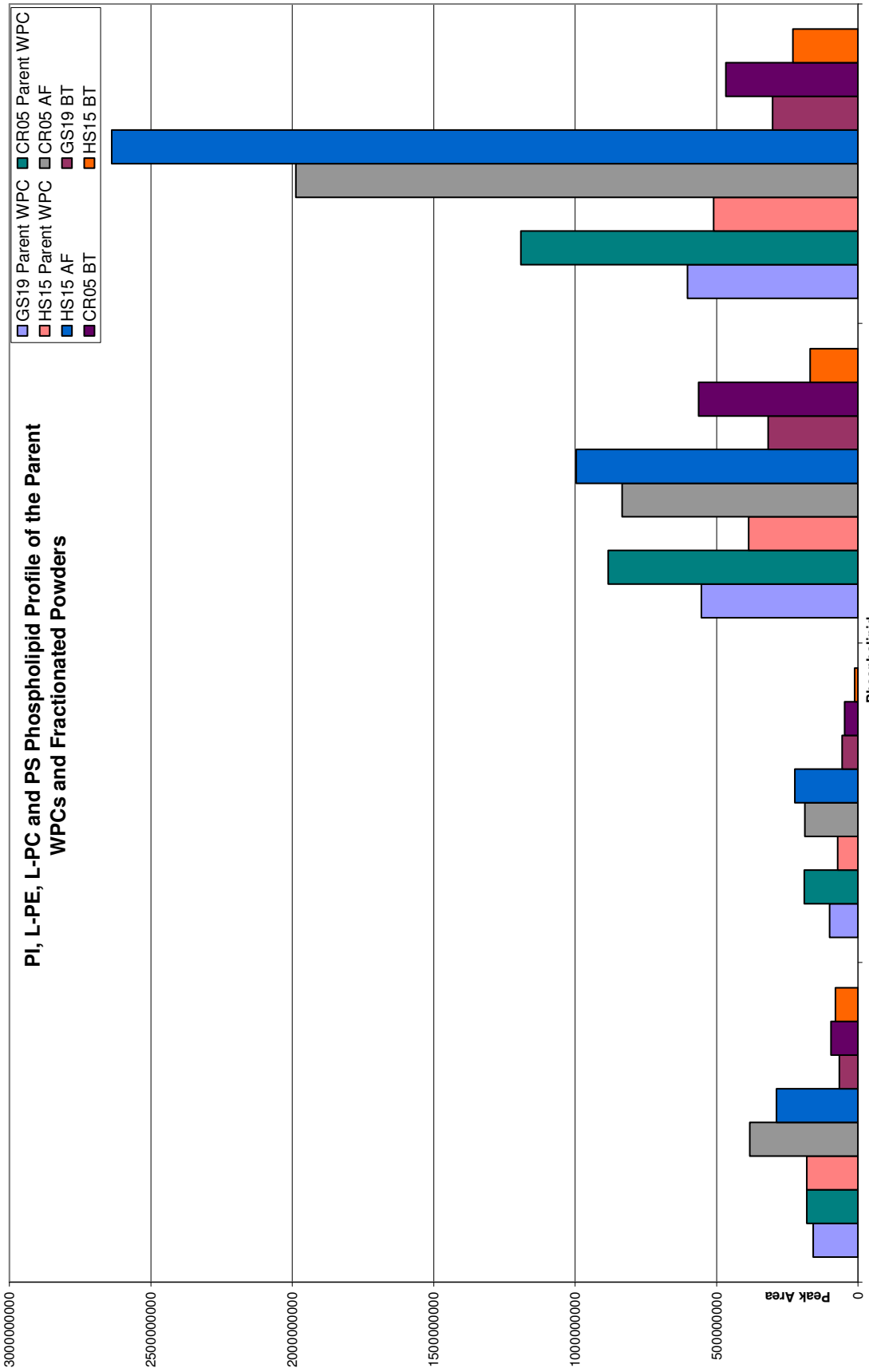

Figure 10b: PI, L-PE, L-PC and PS phospholipid profile of the parent WPCs and fractionated powders.

4.1.8 Analysis of Whey Proteins by Resource™ HPLC

Table 8 shows the results for analysis of whey proteins in the parent and fraction powders.

Table 8: Analysis of whey proteins in the parent WPC and fractionated powders.

Protein	GS19 - Cheese WPC80			CR05 - Mineral WPC80			HS15 - Lactic Acid WPC80		
(g/100g)	Parent	AF	BT	Parent	AF	BT	Parent	AF	BT
GMP +Phos	16.47	71.81	12.18	2.65	35.45	0.84	4.17	51.07	1.11
PP3 + PP5	4.88	9.45	3.66	4.19	35.21	2.18	3.12	7.45	3.24
α-lac	7.86	0.00	9.60	15.59	1.01	19.18	8.31	0.79	11.03
Lf	0.60	0.00	0.55	1.04	0.00	0.57	1.55	0.35	0.98
BSA	1.93	0.00	2.62	1.68	0.00	2.07	1.45	0.00	1.73
β-lac	32.96	0.21	39.30	43.13	5.97	53.29	40.77	6.63	54.63
IgG	3.83	0.00	3.47	3.91	0.00	3.70	2.98	0.00	1.50

Notes:

GMP + Phos = Glycomacropeptide plus other phosphorylated and/or sialylated proteins/peptides (e.g. α-s-casein peptides, osteopontin) (K. P. Palmano, personal communication)

PP3 = Proteose peptone component 3 (lactophorin)

PP5 = Proteose peptone component 5 (β-casein-5-phosphate 1-105/107)

α-lac = α-lactalbumin

Lf = lactoferrin

BSA = bovine serum albumin

β-lac = β-lactoglobulin

IgG = Immunoglobulin G

Although the parent powders contained relatively high amounts of β-lactoglobulin (β-lac), the major whey protein of milk, there appears to be only a small proportion of this in the acid fractions and the bulk can be found in the breakthrough fractions of the WPCs. This is a similar case for α-lactalbumin (α-lac). However, relative to overall protein content, the CR05 and HS15 acid fractions do contain significant amounts of β-lac (7-10%), while the GS19 acid fraction contains almost none at all, but this may be

related to the high concentration of GMP in the GS19 acid fraction displacing the β-lac on the column.

The major protein of the GS19 acid fraction appears to be GMP, which is what would be expected due to the high amount of GMP in the GS19 parent powder. GMP is an acidic phosphorylated heterogeneous peptide with a MW of ~8kDa, which contains no aromatic amino acids and, even at pH 3, has a net negative charge (*164*). The large GMP (+ Phos) peak found in the HS15 and CR05 acid fractions can mainly be attributed to other acidic (probably phosphorylated and/or sialylated) proteins and/or peptides, such as osteopontin, with very little being attributed to GMP.

The Resource™ HPLC profile for the parent WPCs and acid fractions are shown below in Figures 11 and 12 and illustrate inherent differences between the parent substrates and the acid fractions. The breakthrough HPLC profiles can be found in Appendix 2.

Worth noting is the significant amount of PP3/PP5 found in the acid fractions, especially in the CR05 powder. Although PP5 is generally more abundant than PP3 the RP-HPLC method does not resolve the two (*143*), hence the 'PP5' labelled peak on the chromatograms (Figures 11 and 12) are actually a sum of both. The phosphorylated glycoprotein PP3 is highly hydrophobic and has an apparent MW of ~28kDa (*88*). Recently the bovine PP3 gene has been shown to have homology with the murine gene for glycosylation-dependent cell adhesion molecule 1 (GLYCAM 1), which functions as a endothelial cell surface ligand for L-selectin, a leukocyte (white blood cells produced in the red bone marrow) adhesion molecule (*165-167*). PP3 is produced in the mammary gland, shows association with the MFGM and has no relation to casein, whereas PP5 is considered a minor casein protein as it consists of residues 1-105 and 1-107 of the β-casein sequence (*88, 165, 166, 168-170*). PP5 contains 5 phosphate moieties which confer acidic properties on the molecule and a MW of ~12-13kDa (*170*).

It is worth noting that only the more hydrophilic species of GMP have been captured into the GS19 acid fraction (Figure 12d): correspondingly, the breakthrough (Appendix 2, Figure A3a) contains the less hydrophilic species.

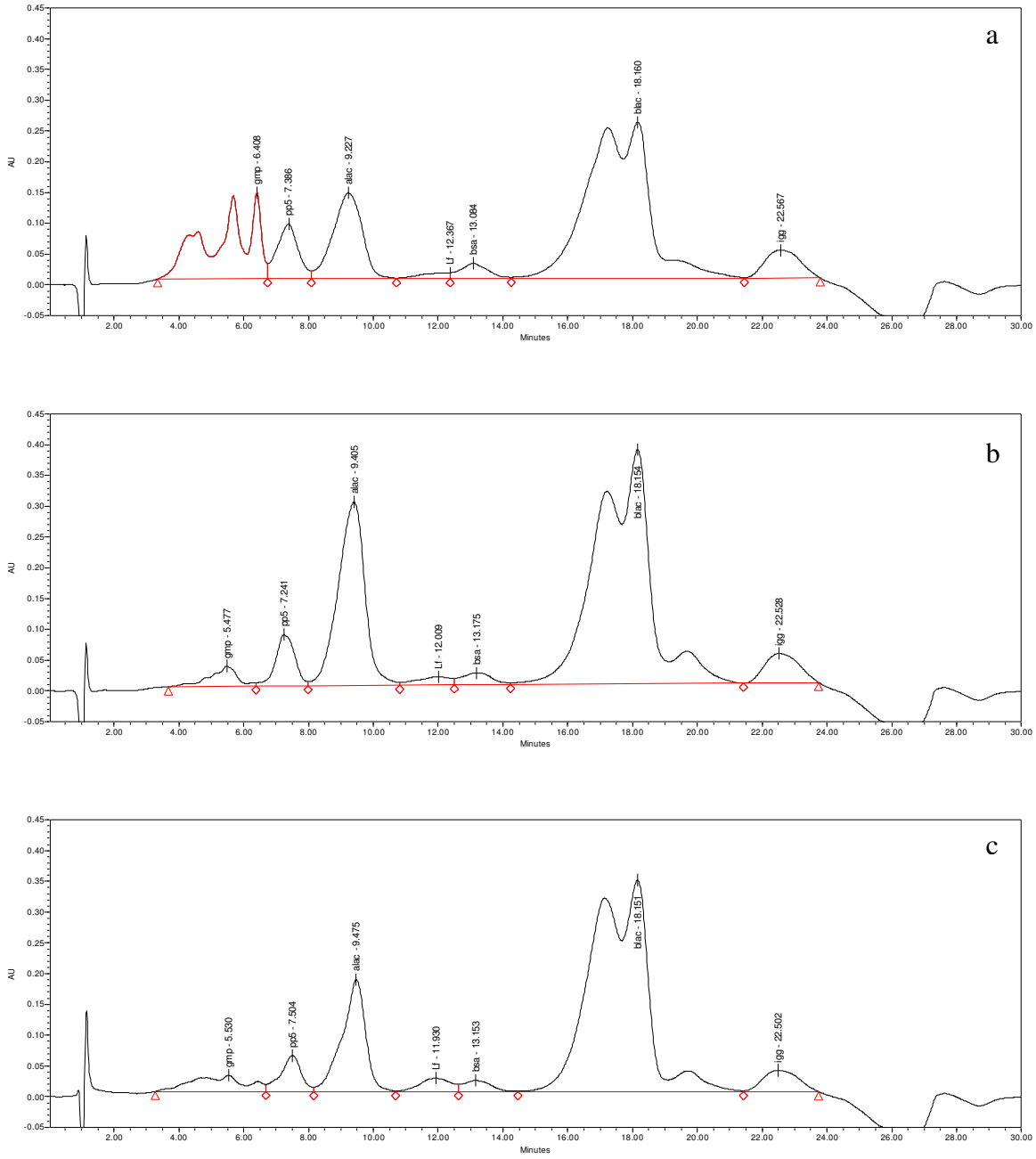

Figure 11: Resource™ HPLC Profiles of the parent WPCs. a) GS19 Parent WPC; b) CR05 Parent WPC; c) HS15 Parent WPC.

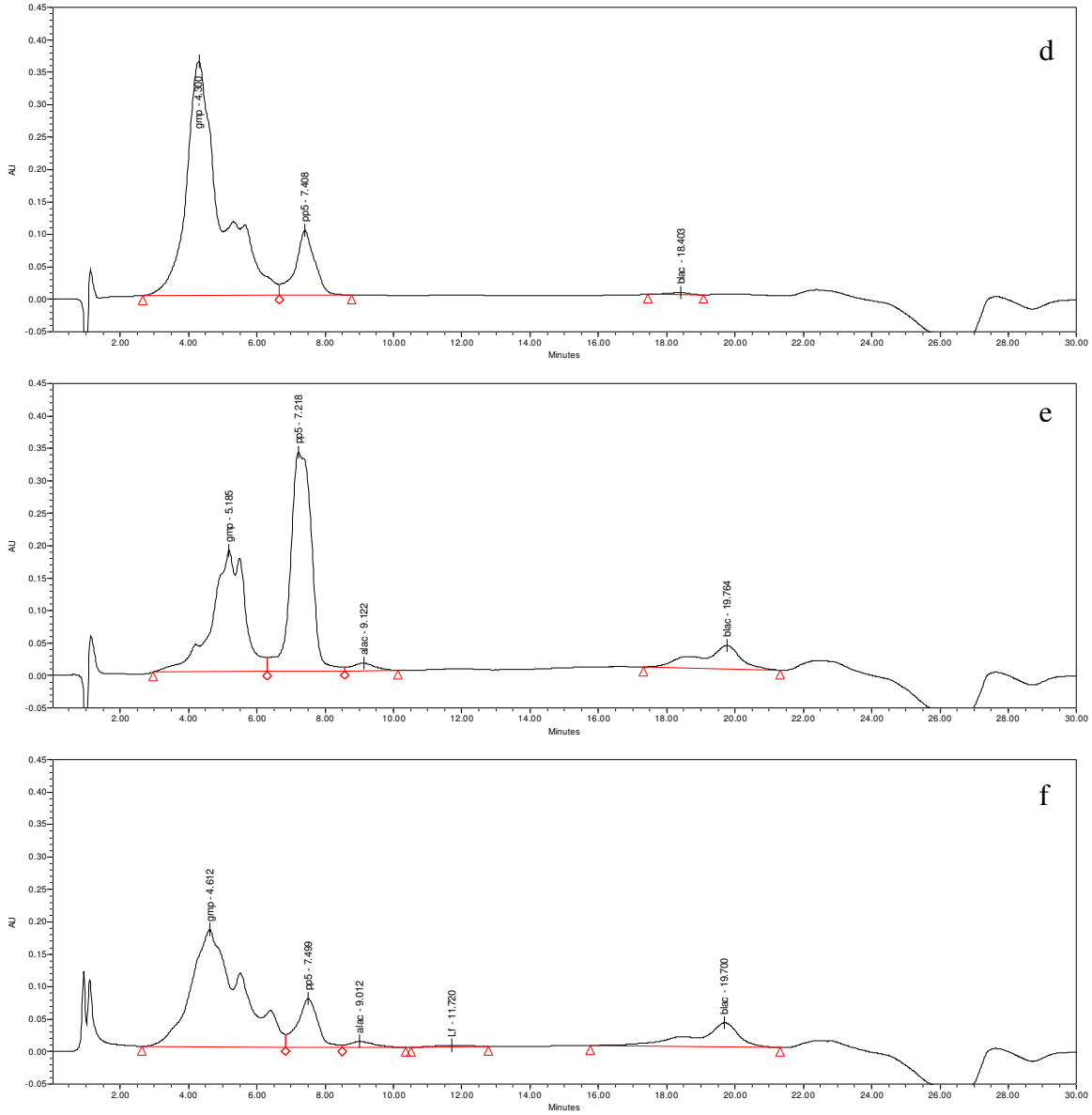

Figure 12: Resource™ HPLC Profiles of the acid fractions. d) GS19 Acid Fraction; e) CR05 Acid Fraction; f) HS15 Acid Fraction

4.1.9 Amino Acid Analysis

The amino acid results show that the three parent WPCs were similar to each other in their amino acid composition and were of the typical composition expected for WPC 80 (*171*), allowing for variation due to whey source. Table 9 contains the amino acid results for the parent powders and fractionated powders and Figure 13 represents these results graphically.

Table 9: Amino acid results for the parent WPC and fractionated powders.

Amino Acid	GS19 – Cheese WPC80			CR05 – Mineral WPC80			HS15 – Lactic Acid WPC80		
(% w/w)	Parent	AF	BT	Parent	AF	BT	Parent	AF	BT
Cystine	1.94	0.14	2.38	2.62	0.47	3.23	2.30	0.30	2.80
Methionine	1.88	1.26	1.66	1.90	1.03	1.96	1.87	0.99	1.87
Aspartic Acid	8.86	6.09	8.86	9.92	8.38	10.80	9.31	6.47	9.49
Serine	4.33	5.50	4.02	3.87	6.35	3.87	3.79	5.58	3.35
Glutamic Acid	15.20	14.50	14.50	14.10	17.30	15.00	14.00	13.30	14.50
Glycine	1.62	0.88	1.62	1.75	1.14	1.85	1.81	1.01	1.57
Histidine	1.55	0.42	1.57	1.86	2.19	1.94	1.75	1.38	1.72
Arginine	2.39	0.78	2.42	2.75	3.28	2.68	2.76	1.66	2.66
Threonine	5.77	9.08	5.27	4.54	4.54	4.61	4.52	6.63	4.23
Alanine	4.41	3.60	4.36	4.21	2.54	4.63	4.49	2.96	4.64
Proline	5.30	7.18	4.79	3.93	5.62	3.97	4.27	5.61	4.23
Tyrosine	2.82	0.30	2.92	3.42	1.62	3.80	3.17	0.65	3.29
Valine	5.25	5.40	4.99	4.61	3.82	4.98	4.66	3.63	4.61
Lysine	8.01	4.45	8.36	8.74	6.62	10.20	8.59	4.75	9.18
Isoleucine	5.27	6.25	4.97	4.79	5.22	5.15	4.56	4.90	4.72
Leucine	8.75	2.48	9.11	10.30	6.22	11.50	9.77	3.65	10.80
Phenylalanine	2.82	0.76	2.82	3.25	2.73	3.52	3.18	1.60	3.20
Tryptophan	1.56	0.09	1.46	2.09	0.32	2.22	2.07	0.18	2.00

The amino acid compositions of the three breakthrough fractions are almost mirror images of the parent WPCs with a few exceptions, such as a higher concentration of cystine and leucine in the breakthrough compared to the corresponding parent WPC.

Of interest is the higher concentration of proline and isoleucine found in the acid fractions when compared to the corresponding parent WPC and breakthrough fraction; this may be due to the retention of casein peptides on the column, such as PP5 and α-casein phosphopeptides, as caseins are high in proline (*171-173*).

The low levels of histidine, arginine and phenylalanine present in the GS19 acid fraction compared to the other two acid fractions could be due to the absence of these amino acids in GMP, which makes up a large proportion of the GS19 acid fraction (*164*). The high threonine content of GMP may also be the reason for the high threonine concentration in the GS19 acid fraction.

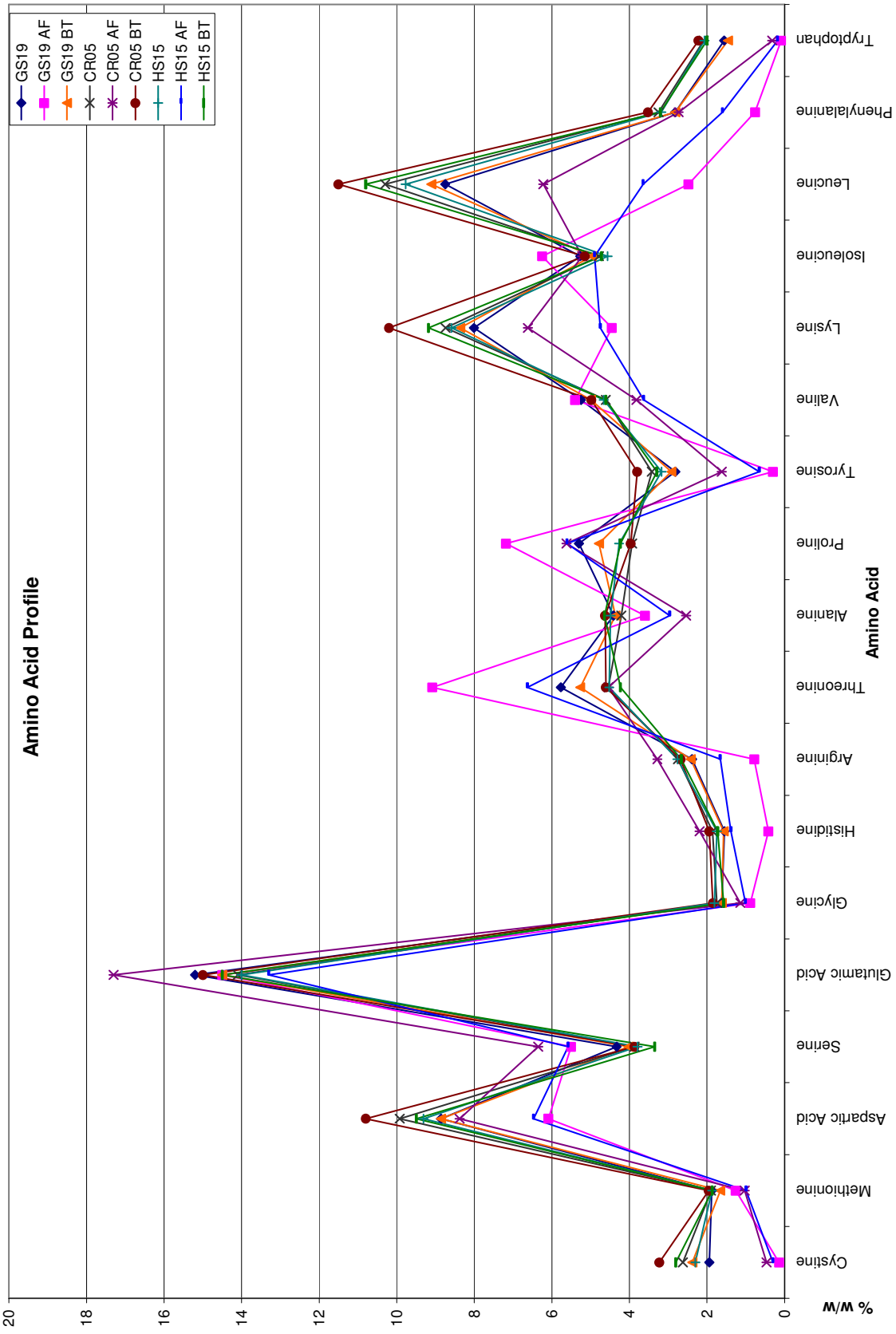

Figure 13: Amino acid profile of the parent WPC and fractionated powders.

4.1.10 IGF-1, TGF-β1 and TGF-β2 Growth Factors and Lactoferrin

Table 10 shows the IGF-1, TGF-β1, TGF-β2 growth factors (ELISA) and lactoferrin (SPR) results for the parent WPCs and fractionated powders.
The different WPC types varied greatly in the concentration of the different growth factors they contained. On a mass balance basis it appears the bulk of the growth factors, from the parent WPCs, are found in the corresponding breakthrough fractions.

Table 10: Growth factors and lactoferrin results for the parent WPC and fractionated powders.

Test	GS19 - Cheese WPC80			CR05 - Mineral WPC80			HS15 - Lactic Acid WPC80		
	Parent	AF	BT	Parent	AF	BT	Parent	AF	BT
IGF-1 (ng/g pwd)	76.91	9.18	108.33	29.34	<LOD	54.78	58.66	2.64	60.10
TGF-β1 (ng/g pwd)	14.03	10.75	17.84	43.14	61.78	44.35	113.42	15.56	31.84
TGF-β2 (ng/g pwd)	129.88	11.97	100.16	14.23	73.23	57.03	81.59	21.83	21.23
LF (mg/g)	6.64	0.28	6.27	8.22	1.06	7.51	16.00	1.29	19.00

Notes:

IGF-1 = Insulin-like Growth Factor-I

TGF-β1 = Transforming Growth Factor-β1

TGF-β2 = Transforming Growth Factor-β2

LF = Lactoferrin

GS19 parent WPC and breakthrough fraction appear to contain higher concentrations of IGF-I and TGF-β2 compared to CR05 and HS15. However, these levels are comparatively low when translated into solutions for dosing on to bone cells. As an example 1mg/mL (the highest concentration tested) of GS19 WPC powder would give ~77pg/mL of IGF-1. IGF-1 has been shown to have an effect on bone cells but at these low levels, especially in the acid fractions, it is unlikely to be the reason for the bone activity discussed below.

In terms of parent powders, HS15 contained the highest concentration of TGF-β1, with 113.42ng/g powder, but the CR05 fractions contained quite significant amounts, with

61.78ng/g powder in the acid fraction and 44.35ng/g powder in the breakthrough. It should be noted that the CR05 parent WPC TGF-β2 seems disproportionately low compared to its fractions. This may be due to the ELISA assay requirement for full activation/release of the growth factors, which in this case may not have gone to completion and therefore not all of the TGF-β2 being measured. Nevertheless, it appears that there has been some selective capture of TGF β1 and β2 into the CR05 acid fraction. Again, it is uncertain whether the levels of these growth factors in a 1mg/mL test sample would contribute significantly to bone activity. The effect of these growth factors would have to be tested independently.

Lactoferrin levels as assessed by ELISA were consistent with those from RP-HPLC (Resource™). The ELISA assay, however, has the benefit of measuring lower concentrations of lactoferrin more accurately and also is some measure of bioactivity, being based on lactoferrins ability to bind antibody. The low concentration of lactoferrin found in the acid fractions is to be expected as lactoferrin is a basic protein, with an isoelectric point of ~9, and would not be expected to bind to the anion exchange column. The residual concentrations of lactoferrin found in the acid fraction may be attributed to lactoferrin associating with other proteins that are found in the acid fraction. Lactoferrin of high purity is known to stimulate osteoblast proliferation at concentrations of 1-100µg/mL and decrease the number of newly developed osteoclast cells in bone assays at concentrations of 10µg/mL and greater (*78*). What is not known is the effect of the matrix on the availability of lactoferrin to bone cell receptors and thus, the ability of lactoferrin to affect bone cell growth. For example, the HS15 parent WPC and breakthrough contained levels of lactoferrin, which at 1mg/mL powder would give a higher concentration than the 10µg/mL concentration shown to prevent osteoclast development. However, although the corresponding acid fraction had a 10-fold lower lactoferrin concentration, both the parent WPC and acid fraction inhibited osteoclast development (see bone assay section below) while the breakthrough had no effect. These results lead to the interpretation that it is not lactoferrin, or lactoferrin alone, which gave rise to the bone activity observed in this study. It may also be that doses as low as 1-10µg/mL are not effective on the particular bone cell lines used for this study, so very low concentrations of lactoferrin, as occur in the parent WPCs and acid fractions, may indeed not contribute to bone activity.

4.1.11 Molecular Weight Profiles

Table 11 below shows the molecular weight profile for the parent WPC, acid fraction and breakthrough fraction powders. For each of the different WPCs, the parent WPCs and the corresponding breakthrough fractions had very similar molecular weight profiles, where approximately 50% of the molecular weight was above 20kD, 34-48% was between 5-20kD and between 0.3-1% of the molecular weight was below 1kD. The filtration of the samples before analysis most likely removed insoluble material from the sample, such as insoluble proteins or peptides; however, it is expected that this would not have greatly affected the results.

Table 11: Molecular weight profile for the parent WPC and fractionated powders.

Molecular	GS19 - Cheese WPC80			CR05 - Mineral WPC80			HS15 - Lactic Acid WPC80		
Weight (%)	Parent	AF	BT	Parent	AF	BT	Parent	AF	BT
>20 kD	48.93	4.06	52.00	53.10	27.28	51.94	54.98	17.55	51.89
5-20 kD	34.16	19.81	37.10	45.65	58.53	47.40	38.77	32.04	44.10
1-5 kD	16.33	74.48	10.50	0.92	12.44	0.37	5.31	43.68	3.30
<1 kD	0.57	1.65	0.40	0.33	1.75	0.29	0.94	6.74	0.71

The GS19 parent WPC and breakthrough fraction differed somewhat from the CR05 and HS19 parent and breakthrough fractions in the 1-5kD molecular weight range. Notably, the GS19 WPC and corresponding breakthrough fraction had more material in the 1-5kD range (16.3% and 10.5%, respectively) whereas, CR05 had 0.9% and 0.4%, respectively, for the parent powder and the breakthrough fraction and HS15 had 5.3% and 3.3%, respectively, for the parent powder and the breakthrough fraction.

The molecular weight profiles for the acid fractions for all three WPCs were different, with GS19 having only ~4% above 20kD, ~20% between 5-20kD, ~74% between 1-5kD and ~2% below 1kD; CR05 having ~27% above 20kD, ~59% between 5-20kD, ~12% between 1-5kD and ~2% below 1kD; HS15 having ~18% above 20kD, ~32% between 5-20kD, ~43% between 1-5kD and ~7% below 1kD. Figure 14 shows the molecular weight profile chromatograms for the acid fractions with the molecular weight cut-off points indicated. The parent WPC and fractionated powders molecular weight chromatograms can be viewed in the Appendix 3. Of note is that the majority of the material in the GS19 acid fraction eluted in the 1-5kDa range. The major

component of this fraction was shown to be GMP, which has a nominal molecular weight of ~8kDa (*164*). It is known, however, that the calibration in the lower molecular weight range is not as accurate as that for the higher molecular weight range.

The molecular weight profiles were able to show key differences between the various WPCs and their fractions, not otherwise apparent by other analytical means. In this sense they serve as blueprints against which to compare and contrast future fractions. These results can be viewed graphically in Figure 15**.**

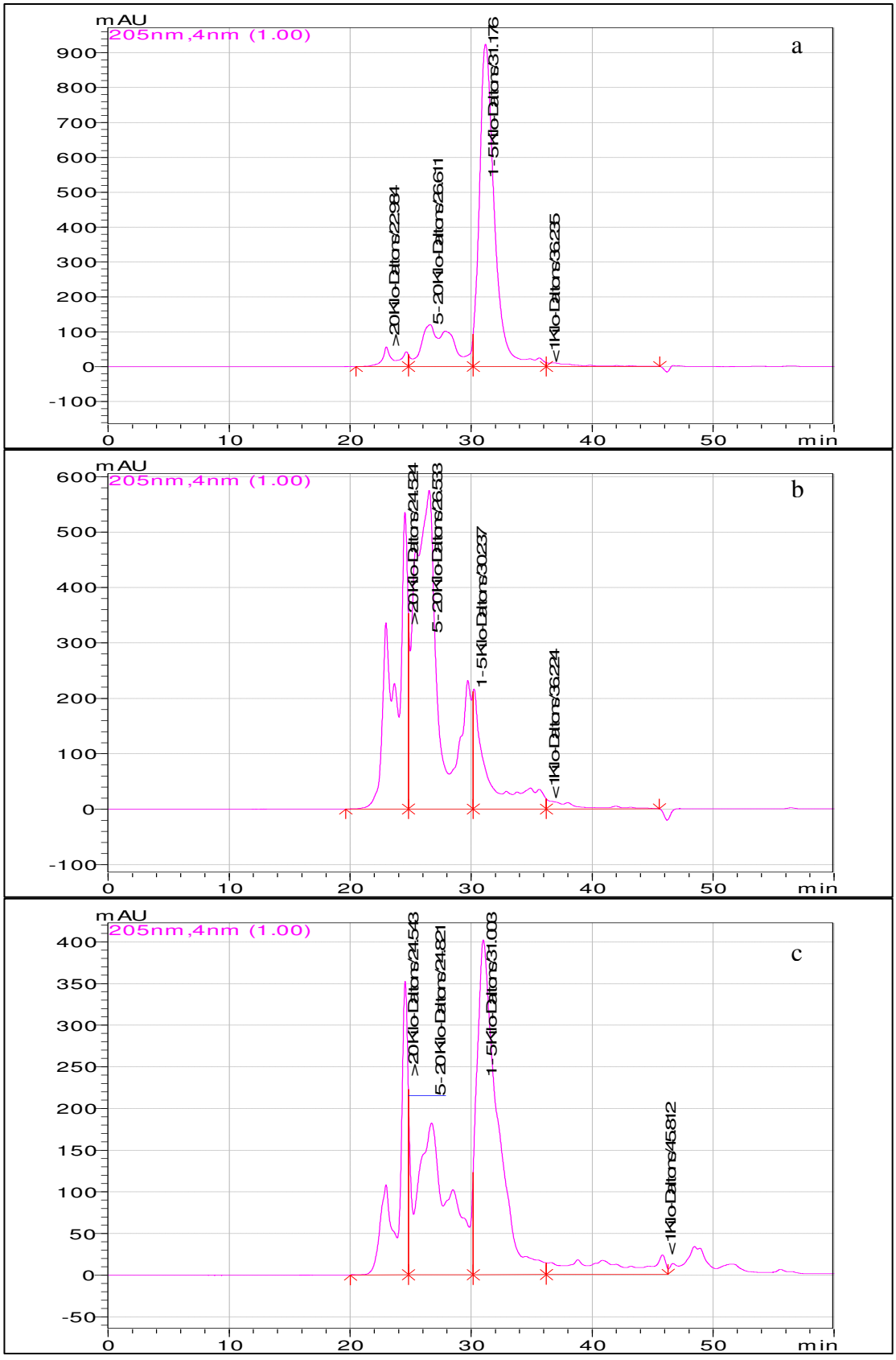

Figure 14: Molecular weight profile chromatograms for acid fractions: a) GS19 AF; b) CR05 AF; c) HS15 AF

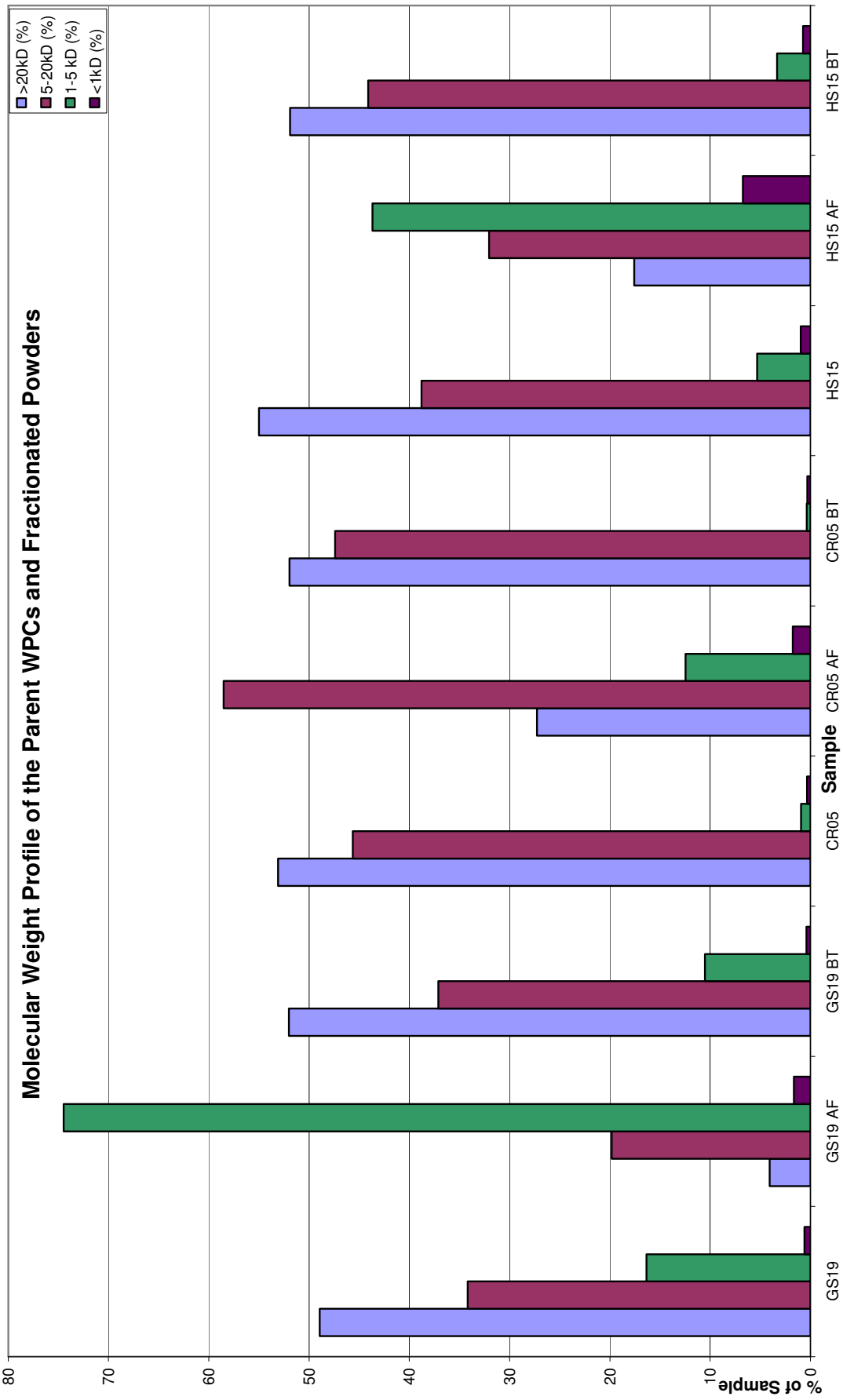

Figure 15: Molecular weight profile of the parent WPCs and fractionated powders.

4.1.12 Enzyme Hydrolysis of Acid Fraction Powders

It is well known that bioactivity can be encrypted within proteins, and that enzyme hydrolysis of proteins can release bioactive peptides. Prior research has shown that a pepsin hydrolysate of the acid fraction from mineral acid WPC was anti-osteoclastogenic, whereas the parent acid fraction was inactive (*99*). It is also possible that active peptides are formed in the gut after oral intake and contribute to the *in vivo* activity observed with previous acid fraction preparations (*96, 97*). Conversely, bioactivity can be destroyed by hydrolysis, as in the case of growth factors. Enzyme hydrolyses of the acid fractions were performed using both trypsin and pepsin (physiologically relevant enzymes) to not only confirm previous findings but also to extend them. Although bioactivity has not yet been definitively attributed to any specific protein or peptide component, it was hoped that hydrolysis might act as a diagnostic tool to reveal either active peptide (or peptides), or diminished the activity of the intact proteins.

The trypsin digests samples were also analysed for MS protein composition and molecular weight profiles (see below).

4.1.13 Determination of hydrolysis by SDS-PAGE

For the trypsin digestion, at 1:40 (w/w) and 1:100 (w/w) ratio, samples were taken at 0, 5, 10, 20, 30, 45 and 60 minute intervals for all three WPC types and run on SDS-PAGE gels, which are illustrated in Appendix 4.

For the pepsin digestion, which was done after the trypsin gels had been viewed, samples were taken at 0, 10, 30 and 60 minute intervals for all three WPC types and samples run on gels. SDS-PAGE gels of the acid fraction powders following digestion with pepsin at 1:40 (w/w) and 1:100 (w/w) ratio are illustrated in Figures 16 and 17 respectively. A Precision Plus Protein™ standard was applied to the tracks on either side of the gels to give an indication of molecular weight. The standard included markers of 10, 15, 20, 25, 37, 50, 75, 100, 150 and 250 kD in size.

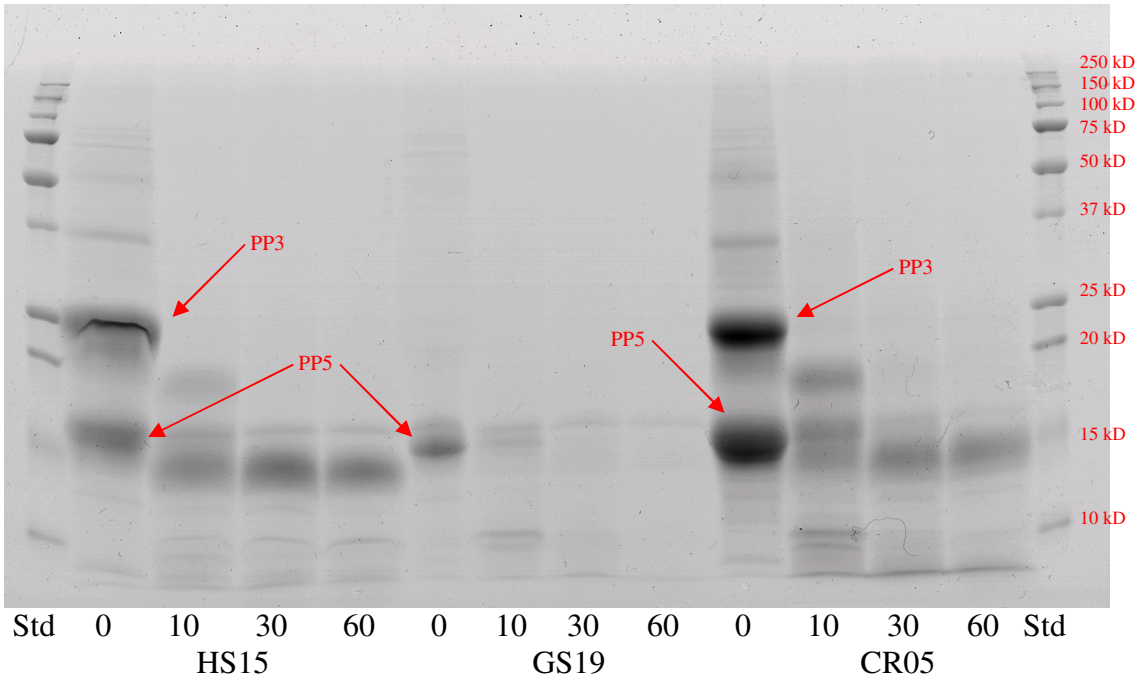

Figure 16: SDS-PAGE Gel of acid fractions incubated with pepsin at 1:40 enzyme to substrate (w/w) ratio.

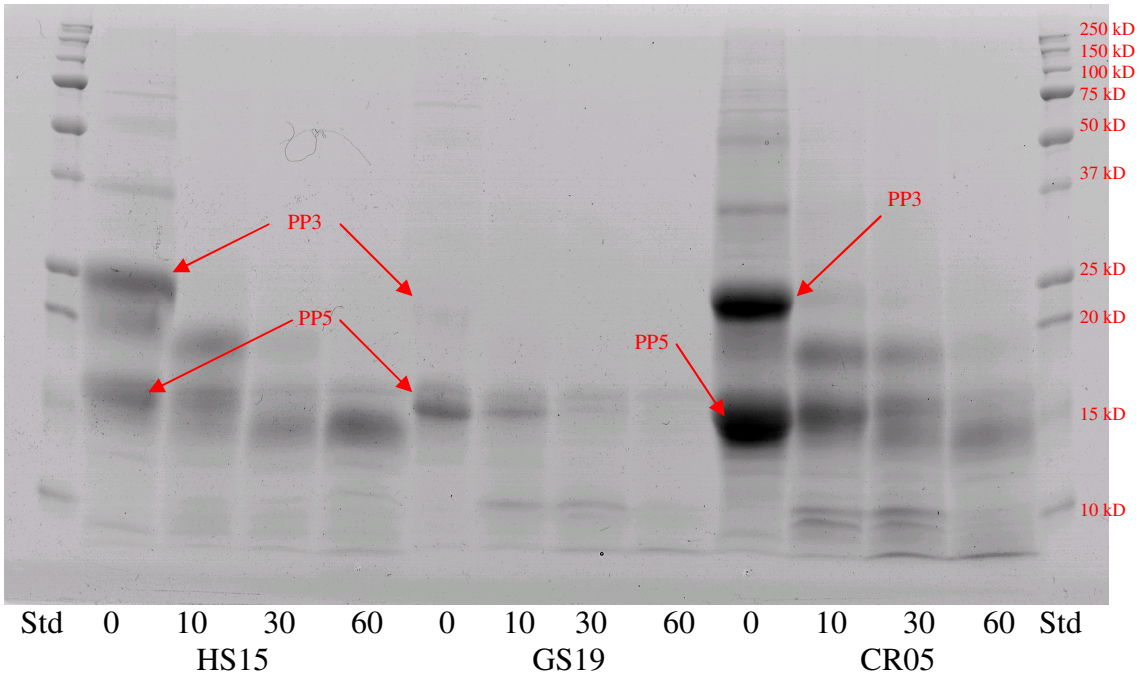

Figure 17: SDS-PAGE Gel of acid fractions incubated with pepsin at 1:100 enzyme to substrate (w/w) ratio.

Protein bands on gels were tentatively identified on the basis of in-house knowledge and also according to Sorensen and Petersen (*88*) and Andrews (*170*). Note that β-lac also runs in the region of PP5, although not annotated on the gels.

It can be seen from the gels that there was little apparent difference in degree of hydrolysis when enzyme to substrate ratio was either 1:100 (w/w) or 1:40 (w/w). The scanned gels show a marked difference in the acid fraction of the GS19 compared to those of HS15 and CR05. The GS19 acid fraction was predominantly GMP, as described above, but as the macropeptide is resistant to staining (K. P. Palmano, personal communication), little was apparent. Note also that, due to the negative charge, GMP does not run as the rest of the proteins on SDS-PAGE gels and usually runs at a higher apparent MW than its nominal MW of ~8kDa (*164*). Nevertheless, the sample appeared to be well digested by the pepsin. The HS15 and CR05 acid fractions were more similar and differences in end-point digestion were more subtle.

The trypsin digested samples showed similar degrees of hydrolysis to the pepsin and it was not surprising that, when the acid fractions were digested with trypsin, the percentage of molecular weight material above 20kD decreased significantly for all three WPC derivatives. Table 12 shows the molecular weight profile for the acid fraction powders that had undergone hydrolysis using trypsin at a 1:100 (w/w) ratio.

Table 12: Molecular weight profile for the trypsin digest acid fractions powders.

Molecular Weight (%)	GS19 - Cheese WPC80 AF 1:100 Trp Hyd	CR05 - Mineral WPC80 AF 1:100 Trp Hyd	HS15 - Lactic Acid WPC80 AF 1:100 Trp Hyd
>20 kD	0.66	7.36	1.47
5-20 kD	2.89	35.44	8.32
1-5 kD	73.95	38.37	57.82
<1 kD	22.5	18.82	32.39

For GS19 acid fraction digested with trypsin the bulk of the material, ~74%, was between 1-5kD, followed by ~23% being below 1kD, with very little in the 5-20kD (~3%) and >20kD (0.66%) molecular weight range. This was very different from CR05 where ~35% was between 5-20kD, ~38% was between 1-5kD and ~19% was below 1kD, leaving ~7% above 20kD. HS15 has a similar profile for its digested acid

fraction to that of GS19, although the numbers do vary. Only ~1.5% of HS15 digested acid fraction is above 20kD, ~8% between 5-20kD, the bulk being between 1-5kD (~58%) and ~33% being below 1kD.

Below in Figure 18 are the molecular weight profile chromatograms with the molecular weight cut-off points indicated for the acid fractions digested with trypsin at 1:100 w/w. These results can be viewed graphically in Figure 19.

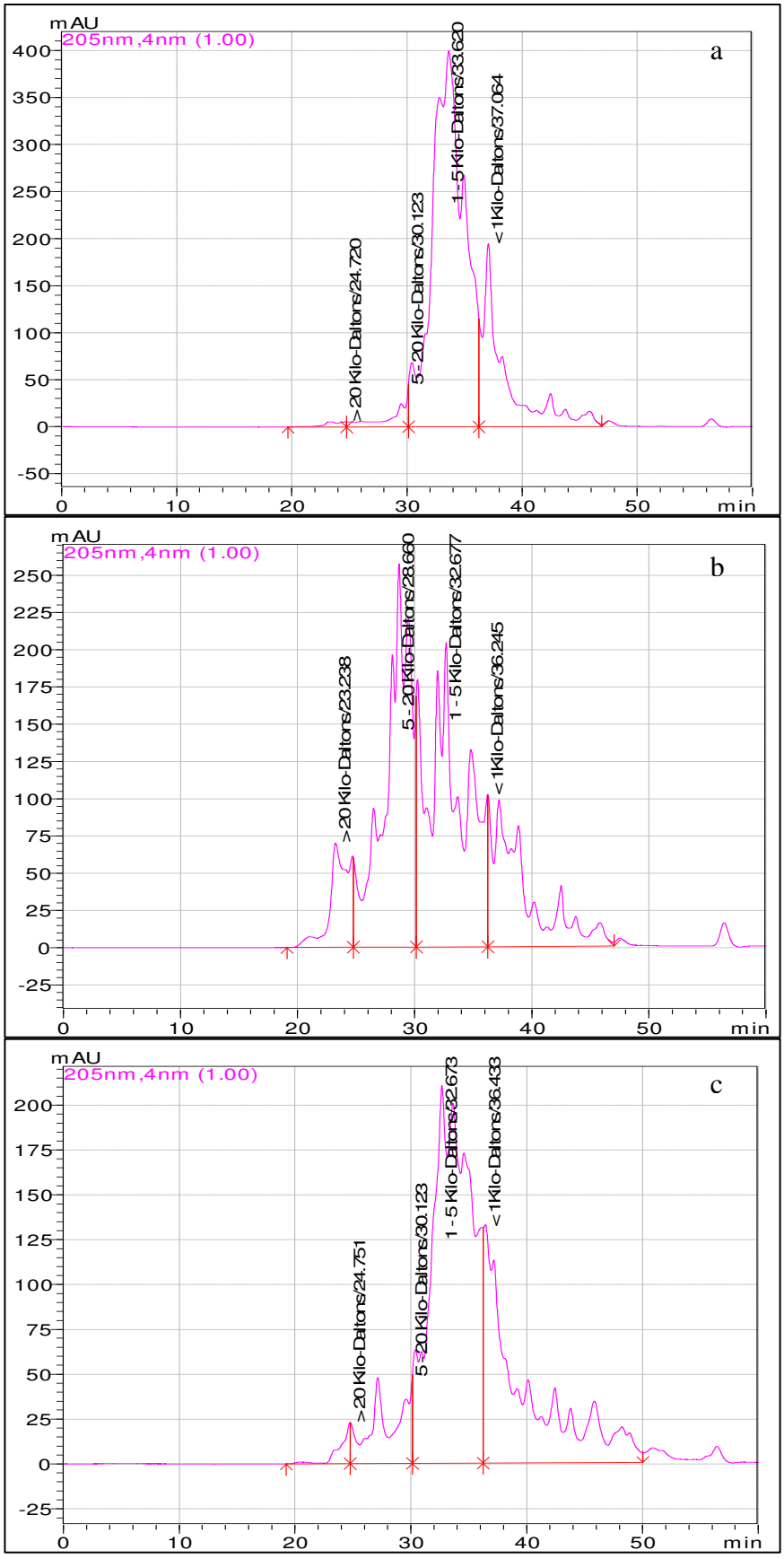

Figure 18: Molecular weight profile chromatograms for trypsin digested acid fractions: a) GS19 AF; b) CR05 AF; c) HS15 AF

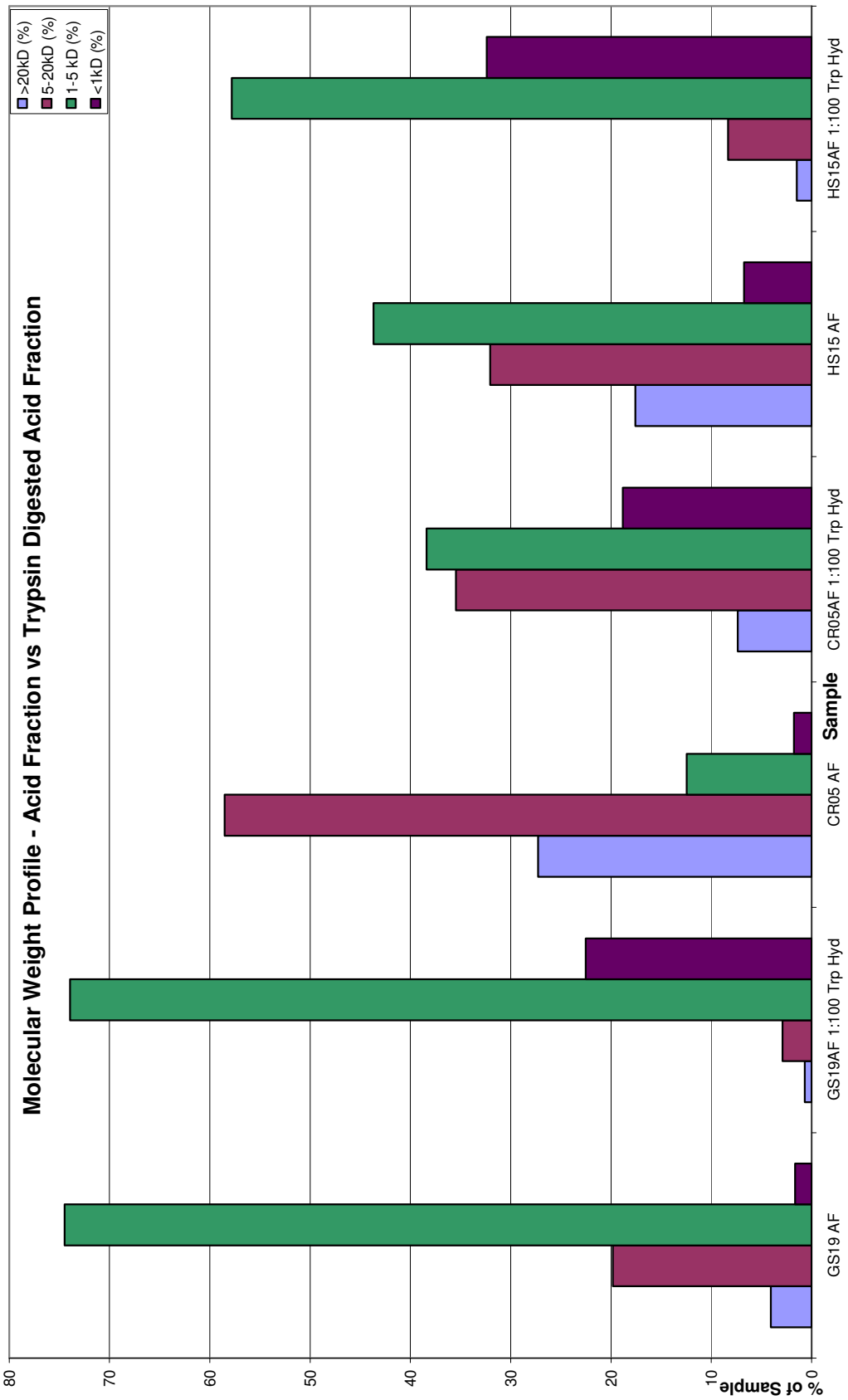

Figure 19: Molecular weight profile of the acid fraction and trypsin digested acid fraction powders.

4.1.14 Protein Analysis From Mass Spectrometry

The genomic database results for the acid fractions digested by trypsin at the different ratios of 1:40 and 1:100 can be found below in Table 13. These results show the relative proportions of peptides found in the samples based upon MS peak intensity data. The numbers are indicative only and are not exact quantitative values for the protein or peptide fragments found in the samples. Note that the pepsin hydrolysed material was not submitted for MS protein composition as it is more difficult to get a valid identification on peptides from pepsin hydrolysis since pepsin is less specific than trypsin in its cleavage sites.

Table 13: The % peak area of the proteins/peptides identified by in the genomic database for the 1:40 and 1:100 acid fraction trypsin digested powders.

	GS19 AF		CR05 AF		HS15 AF	
Protein/Peptide	1:40	1:100	1:40	1:100	1:40	1:100
albumin	0.50	1.46	0.17	0.06	1.36	0.56
alpha-1 acid glycoprotein	-	-	-	-	1.42	1.19
alpha-2-HS-glycoprotein	-	-	0.20	0.20	0.35	0.26
butyrophilin, subfamily 1, member A1	0.09	0.07	-	-	-	-
calmodulin 3	-	-	-	0.20	-	-
casein alpha-S1	21.44	28.23	9.01	16.00	5.86	4.03
casein alpha-S2	-	-	-	1.52	-	0.34
casein beta	51.64	49.68	37.33	30.06	21.50	16.37
casein kappa	3.69	3.79	8.24	23.14	9.17	15.77
glycosylation-dependent cell adhesion molecule 1	1.96	2.96	20.47	13.17	13.84	49.09
lactoglobulin, beta	8.39	9.36	6.70	6.00	10.62	8.71
lactoperoxidase	-	-	-	-	-	0.09
lactotransferrin	-	-	0.07	0.07	0.25	-
milk fat globule-EGF factor 8 protein	-	-	0.11	0.11	-	-
mucin 15	-	-	-	0.21	-	0.35
PREDICTED: calmodulin 2 (phosphorylase kinase, delta)	-	-	0.17	-	-	-
PREDICTED: cationic trypsin	5.64	2.23	5.74	1.12	13.78	1.01
PREDICTED: similar to calmodulin 2	-	-	-	0.17	-	-
PREDICTED: similar to TRB@ protein isoform 1	5.64	2.23	5.74	1.12	13.78	1.01
secreted phosphoprotein 1	1.00	-	6.22	6.67	8.07	1.09
serine proteinase inhibitor, clade A, member 1	-	-	-	-	-	0.12

It should be noted that as highly phosphorylated peptides do not bind to the capillary PepMAP trap column, used for desalting before the capillary LC-MS, they are exhausted with the waste and thus not all peptides were necessarily captured and recorded.

Although results should be interpreted with caution and numbers can not be used as exact values, the mass spectrometry analysis of the trypsin digested acid fractions indicated that fractions from all three types of WPC contained high levels of casein peptides (made up of the different types of casein - alpha-S1, alpha-S2, beta and kappa casein), accounting for over ~76% of the GS19 digested acid fraction peak area, over ~50% of the CR05 digested acid fraction peak area but just over ~36% of the HS15 digested acid fraction peak area. The beta-casein peptides were no doubt largely due to the presence of PP5 (see Resource™ analysis). It is also worth noting that trypsin does not digest GMP readily, with only two-three sites available in the N-terminal, which can cause issues with GMP recognition in the genomic database.

All three types of acid fraction also appeared to contain reasonably high amounts of beta lactoglobulin, making up ~8-9% of the GS19 acid fraction peak area, ~9-11% of the HS15 acid fraction peak area and ~6-7% of the CR05 acid fraction peak area. These results are in agreement with the Resource™ results for CR05 and HS15 but not for GS19, but this may be due to the lack of GMP signal distorting the results.

Important information to come from the MS protein results was the relative levels of glycosylation-dependent cell adhesion molecule 1 (GLYCAM 1), which shares its amino acid sequence with PP3 (*165-167*), and secreted phosphoprotein 1 (SPP1, also known as osteopontin). Based on relative peak intensities, both CR05 and HS15 appeared to contain relatively significant amounts of these proteins. GLYCAM 1 made up ~13-21% of the CR05 acid fraction peak area, ~13% of the HS15 1:40 acid fraction peak area and ~49% of the HS15 1:100 acid fraction peak area. GS19 appeared to contain very low amounts of both GLYCAM 1 and SPP1, which could be of interest in relation to the bone activity observed. This is one of the major differences between the powders and is in line with SDS-PAGE analysis. As previously discussed, osteopontin (OPN) is a well known acidic phosphorylated glycoprotein that plays an important role in the bone remodelling process and is associated with both casein micelles and whey

(*25, 26, 95, 174*). OPN is known to encourage the attachment of bone cells to the bone matrix and plays a role in cell signalling and migration. OPN, and its fragments have been shown to be superior to CPP in their ability to inhibit the formation of calcium phosphate precipitates and may have an influence on the efficiency of calcium absorption (*175*).

This data can be viewed graphically in Figure 20 and also in Figure 21where the higher intensity protein signals have been removed to focus in on the protein signals of lower intensity, such as albumin. Note that lactoferrin (noted in Table 13 as lactotransferrin) was also detected by this means, even though it is in fact a basic protein, low levels appear to co-isolate with the acidic fraction of WPC.

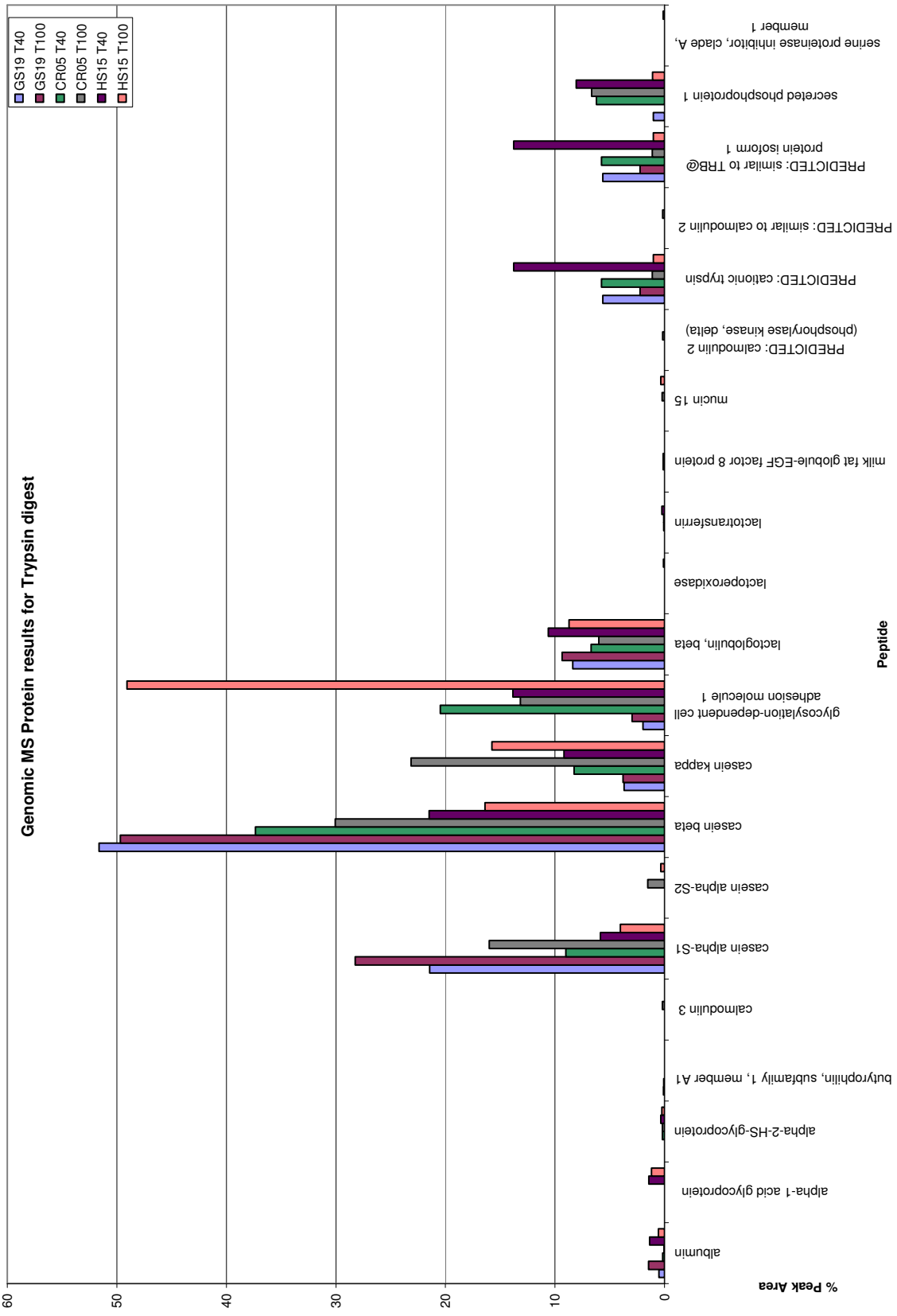

Figure 20: MS relative peak intensity areas for proteins and peptides found in the trypsin hydrolysed acid fractions.

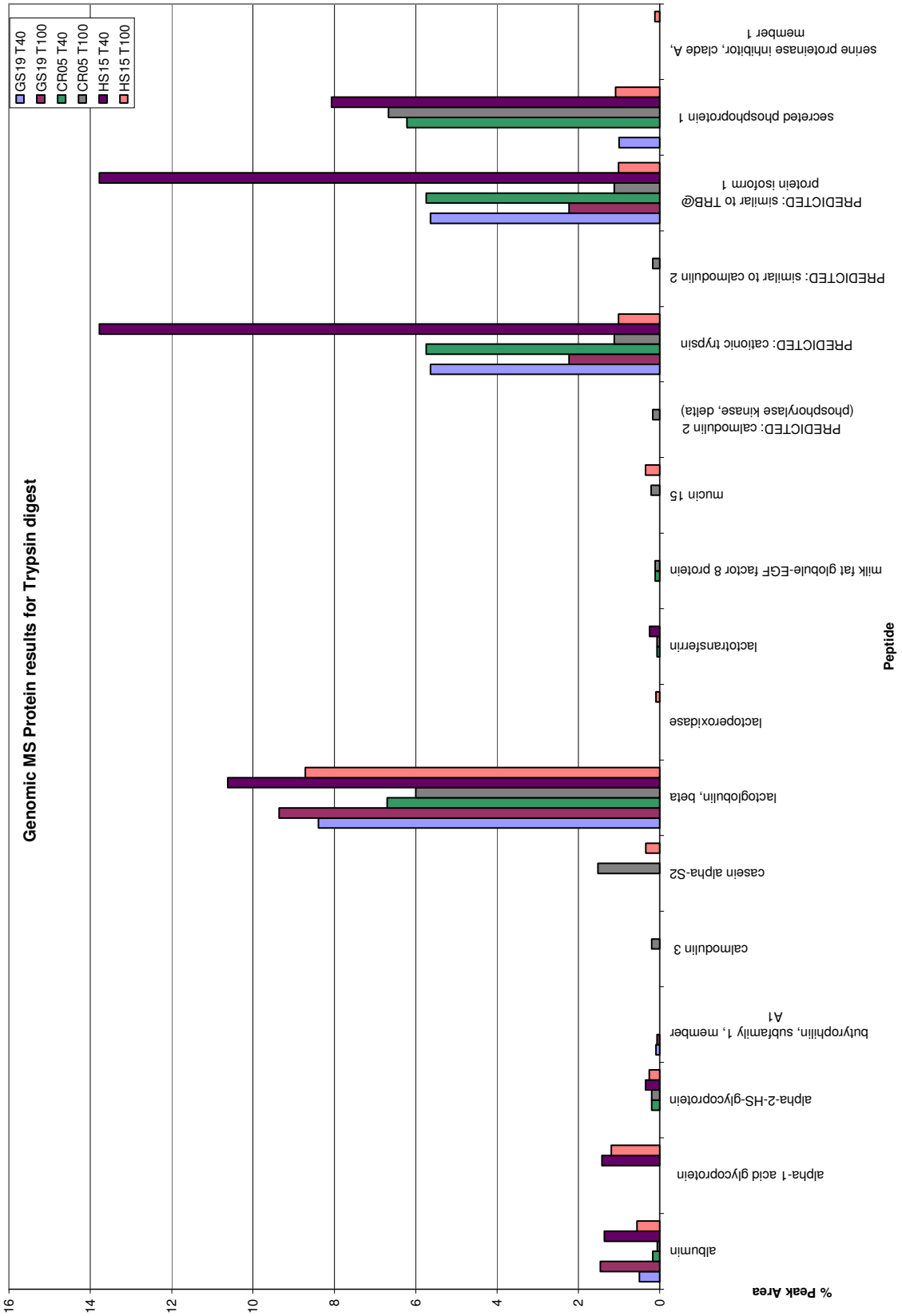

Figure 21: MS relative peak intensity areas for proteins and peptides found in the trypsin hydrolysed acid fractions; the higher intensity protein peaks have been removed to focus on protein peaks of lower intensities.

4.1.15 Bone Activity Results

The parent WPC, acid and breakthrough fractions, and the trypsin and pepsin digested acid fraction powders were submitted for bone activity analysis to determine level of activity on both cell proliferation in MC3T3-E1/Clone 4 osteoblasts and on osteoclast development (murine macrophage RAW 264.7 cell line).

These results were very important to the undertaking of this study and showed very clearly which powders had the desired characteristic and which should be further investigated to determine the active component or components.

Desired characteristics

This work was undertaken to identify a component, or components, showing bone activity, and therefore had the potential to reduce the risk, and development of, osteoporosis. For the purposes of this work, not all bone activity was desired; for example, increased osteoclast development was not a desired characteristic as it may increase the resorption rate of bone and thus increase the risk of developing osteoporosis. The desired characteristics were that osteoclast development was inhibited (for reasons discussed above) and that osteoblast cell proliferation was not inhibited, as this would prevent the reformation of bone, which could also lead to an increased risk of developing osteoporosis or other unwanted bone diseases or conditions.

GS19 Osteoblast Cell Proliferation

At concentrations of 1mg/mL, the GS19 parent WPC and breakthrough fraction stimulated osteoblast cell proliferation, with no (or very limited) activity at lower concentrations. The acid fraction showed no or very little activity in cell proliferation, with only one of the three replicates stimulating cell proliferation at 1µg/mL. The pepsin digested acid fractions (both 1:40 and 1:100) showed inhibition of cell proliferation at 1mg/mL and it was found that the level of inhibition was concentration dependent for the 1:100 fraction. The trypsin digested acid fraction did not appear to have as large an effect on cell proliferation, as the pepsin digest acid fraction did, but did show low levels of stimulation at a range of concentrations tested.

GS19 Osteoclast Development

At the different concentration levels (0.1, 1, 10 and 100µg/mL) and conditions (RANK-L present and absent) tested, the GS19 parent WPC, trypsin digested acid fraction (both 1:40 and 1:100) and the pepsin 1:40 digested acid fraction showed no effect on the development of osteoclasts from RAW 264.7 murine macrophage cells. However, the pepsin 1:100 digested acid fraction appeared to show some inhibition of osteoclast development at concentrations greater then 1µg/mL and the undigested acid fraction showed some inhibition at the lowest concentration of 1µg/mL, but not at higher concentrations. The breakthrough fraction was found to stimulate osteoclast development at concentrations of 0.1µg/mL and higher (as measured by an increase in TRAP levels).

CR05 Osteoblast Cell Proliferation

The CR05 parent WPC was found to stimulate osteoblast cell proliferation at a concentration of 1mg/mL, as too did the trypsin 1:100 digested acid fraction. The acid and breakthrough fractions both showed cell proliferation at concentrations of 100µg/mL and 1mg/mL. The trypsin 1:40 digested acid fraction and the pepsin 1:100 digested acid fractions appeared to have no effect on cell proliferation at any of the concentrations tested. At odds with these results, the pepsin 1:40 digested acid fraction appeared to cause inhibition of cell proliferation at 1mg/mL.

CR05 Osteoclast Development

The CR05 parent WPC and breakthrough fraction showed no effect on osteoclast development at any of the tested concentrations, however, there was an increase in TRAP levels at the 1µg/mL breakthrough fraction, indicating an increase in the number of osteoclast cells. Inhibition of osteoclast development was observed for the acid fraction and trypsin digested acid fractions (both the 1:40 and 1:100) at concentrations of 100µg/mL. Inhibition was also observed at concentrations of 10µg/mL and higher and at 1µg/mL and higher, respectively, for the 1:40 and 1:100 pepsin digested acid fractions.

HS15 Osteoblast Cell Proliferation

The HS15 parent WPC showed stimulation of osteoblast cell proliferation at concentrations of 100µg/mL and 1mg/mL. Neither the acid fraction nor the breakthrough fraction had any effect on cell proliferation at any concentrations tested. The trypsin and pepsin digested acid fractions also showed no effect on cell proliferation.

HS15 Osteoclast Development

The HS15 parent WPC inhibited osteoclast development at 100µg/mL concentration, whereas the acid fraction inhibited development at concentrations of 10 and 100µg/mL. The trypsin digested acid fractions also showed inhibition of osteoclast development at concentrations of 10 and 100µg/mL, however, the pepsin digested acid fraction only showed inhibition at 100µg/mL. The breakthrough fraction showed no effect on the development of osteoclasts at any of the concentrations tested.

Table 14 summarizes the effects of the parent WPCs and their fractions on osteoblast cell proliferation and osteoclast development as described above. Included in Appendix 5 are the summary graphs from Dr Wei-Hang Chua's bone activity results report (*151*)

From the results of the bone assay testing it is shown that although the GS19 parent powder had no effect on osteoclast development the GS19 breakthrough fraction stimulated osteoclast growth, the opposite of what was desired. The GS19 acid fraction had the desired characteristics of inhibiting osteoclast development with no effect on osteoblast cell proliferation. However these characteristics were generally lost when the powder was digested with either pepsin or trypsin, indicating a loss of protein and/or peptide integrity or integral sequences required for the activity observed in the original (undigested) acid fraction.

The effects of CR05 and HS15 powders on osteoclast development, on the whole, were similar to each other, with the exception of the parent WPCs, where CR05 showed no effect but HS15 inhibited development. While the CR05 and HS15 breakthrough

fractions had no effect on osteoclast development, the other fractions (including digests) inhibited osteoclast development.

Table 14: Bone activity summary of the powders and desired characteristics.

Sample	Cell Proliferation	Osteoclast Development
Desired Characteristics	No Effect	Inhibition
GS19 Parent WPC	Stimulation	No Effect
GS19 Acid Fraction	No Effect	Inhibition
GS19 Breakthrough	Stimulation	Stimulation
GS19 AF -Trypsin Digest 1:40	Stimulation	No Effect
GS19 AF -Trypsin Digest 1:100	Stimulation	No Effect
GS19 AF - Pepsin Digest 1:40	Inhibition	No Effect
GS19 AF - Pepsin Digest 1:100	Inhibition	Inhibition
CR05 Parent WPC	Stimulation	No Effect
CR05 Acid Fraction	Stimulation	Inhibition
CR05 Breakthrough	Stimulation	No Effect
CR05 AF -Trypsin Digest 1:40	No Effect	Inhibition
CR05 AF -Trypsin Digest 1:100	Stimulation	Inhibition
CR05 AF - Pepsin Digest 1:40	Inhibition	Inhibition
CR05 AF - Pepsin Digest 1:100	No Effect	Inhibition
HS15 Parent WPC	Stimulation	Inhibition
HS15 Acid Fraction	No Effect	Inhibition
HS15 Breakthrough	No Effect	No Effect
HS15 AF -Trypsin Digest 1:40	No Effect	Inhibition
HS15 AF -Trypsin Digest 1:100	No Effect	Inhibition
HS15 AF - Pepsin Digest 1:40	No Effect	Inhibition
HS15 AF - Pepsin Digest 1:100	No Effect	Inhibition

The major differences between the CR05 and HS15 powders occurred in the osteoblast cell proliferation assay. The HS15 parent powder stimulated cell proliferation, whereas the corresponding fractions had no effect. The CR05 parent WPC, acid and breakthrough fractions and the 1:100 trypsin digest fraction all stimulated osteoblast cell proliferation but the 1:40 trypsin digest and the 1:100 pepsin digest had no effect on the osteoblast cell proliferation. However, the CR05 1:40 pepsin digest fraction showed an interesting and in the context of this study, negative characteristic of inhibiting osteoblast cell proliferation.

5. Conclusion Section

5.1 Overview and Conclusions

Relatively comprehensive compositional analyses of WPC powders and acid fractions have highlighted commonalities between powders and also major and subtle differences which may be important for bone activity. The fractions are complex and there could be a number of potential contributors to bone activity that have tracked through from parent to acid fraction. Bone activity assays have shown that the best activity profiles are associated with the acid fractions of HS15 (lactic acid WPC 80) and CR05 (mineral acid WPC 80), but not the high GMP acid fraction of GS19 (cheese WPC 80). This information can be used for further investigations. It has been important to fingerprint the source WPC powders as these may be used as starting points for future commercial extractions, which may include the use of wider fractions as bone prophylactics rather than (expensive) isolated protein/peptides. However, if bone activity can be tracked to a single peptide then there is the possibility of using synthetic chemistry to generate the active molecule for commercial use.

Interpretation of the HPLC resource data, the molecular weight profiling data, the SDS-PAGE gels and the MS data, showed that the GS19 acid fraction is markedly different from the CR05 and HS15 acid fractions in protein composition, which can mainly be attributed to the high GMP content. This fraction did not exhibit strong bone activity.

It is interesting that small but perhaps physiologically significant amounts of components known to have bone remodelling activity have been found in the acid fractions. E.g. lactoferrin and TGF-β growth factors. This could be interpreted to mean that the bone activity of the acid fractions is possibly due to a combination of active components found in the acidic protein fraction of whey, rather than a single active component.

Of particular interest is the high level of the glycosylation-dependent cell adhesion molecule 1 (GLYCAM 1) and secreted phosphoprotein 1 (SPP1) found in the more active CR05 and HS15, but not in the less active GS19, trypsin digested acid fraction. GLYCAM 1, a mucin-like endothelial glycoprotein, is a milk protein synthesized in the mammary gland that has been shown to exhibit homology with proteose peptone component 3 (PP3), as previously discussed. Glycoproteins have been shown to have essential functions in bone and cartilage, and in the inflammatory process, but due to the vast complexity of carbohydrate structures their exact roles are still unclear. Secreted phosphoprotein 1 (SPP1), most commonly known as osteopontin (OPN) is a glycoprotein and has a role as a linking protein. It is an extracellular structural protein and, as an organic component of bone, it has a function in the formation, remodelling and maintenance of bones and teeth.

One of the most important outcomes of this study has been the confirmation that the acidic protein fractions of both mineral and lactic WPC, have bone bioactivity *in vitro* and that this bioactivity effect appears to be greater than that observed in the parent WPC.

Further and more specific studies need to be undertaken to determine what the active component(s) of the acidic protein fraction of the mineral and lactic WPCs are. The work detailed in this study will act as a major stepping stone for future research in this area.

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Appendices

Appendix 1: Principles of analytical procedures used in this study

Appendix 1.1: Determination of Element Composition

The principle of this method relies on the samples being digested with nitric and hydrochloric acids using commercial microwave systems or enclosed polycarbonate or polypropylene vials before analyses by Inductively Coupled Plasma Optical Emission Spectrophotometry (ICP-OES). ICP-OES takes advantage of elements emitting electromagnetic radiation at particular wavelengths when the atoms and ions are excited by inductively coupled plasma (ionised gas). The concentration of the element in the sample can be determined using the intensity of the emission with comparison to known standards.

Appendix 1.2: Determination of Vitamin D3 Content

The sample first undergoes alkaline saponification (hydrolysis of the ester under alkaline conditions) before the vitamin D3 is extracted and concentrated. This crude extract then undergoes a semi-preparative liquid chromatographic (LC) fractionation stage where the fraction of the eluting sample containing the D3 is collected as it comes through a 5µm silica column before being analysed using isocratic reversed-phase high performance liquid chromatography (HPLC), with dual wavelength detection at 265 nm and 280 nm, with 100 % methanol as the running buffer at 1.5-2.0mL/min. Quantitation of the Vitamin D3 is determined by use of internal standards and the peak ratio of Vitamin D2 and D3.

Appendix 1.3: Determination of Vitamin K Content

In principle this method uses lipase enzymatic digestion of the fat and the precipitation of the released fatty acids. The vitamin K is extracted from the sample using hexane. The sample mixture separates into phases; the upper hexane phase is siphoned off and dried to dryness under nitrogen flow. This sample residue is then re-dissolved in a small volume of methanol and is analysed by HPLC using a 5µm C18 column

(monomeric or polymeric) and fluorescence detector at λ_{ex} 243 nm, λ_{em} 430nm). Between the column and the fluorescence detector is a post-column Zn redactor which reduces the Vitamin K and allows it to be detected by the fluorescence detector. The vitamin K content of the sample is calculated using a 5 point standard curve and calculations are based on peak area (or height).

Appendix 1.4: Determination of Total Nitrogen and Protein Content

A solvent mixture of concentrated sulfuric acid, copper (II) sulfate and potassium sulfate is used to digest the samples, in a block-digestion apparatus, at temperatures of 410-430^{o}C for between 1.75 and 5.5 hours. The copper (II) sulfate acts as a catalyst for the conversion of the organic nitrogen in the sample to ammonium sulfate. The sample is then allowed to cool to room temperature before sodium hydroxide is added in excess to release the ammonia. The sample is then steam distilled, using a steam distillation unit, into a boric acid solution. The sample is then titrated with hydrochloric acid and the amount of nitrogen in the sample is calculated from the amount of ammonia produced.

The protein content of the sample is determined by calculations based on the TN content of the sample. The protein is calculated using a standard conversion factor of 6.38 x TN for milk proteins.

Appendix 1.5: Determination of Non-Protein Nitrogen Content

Non-protein nitrogen (NPN) was determined in the powder samples using NZTM 3.15.3: Non-Protein Nitrogen for Liquid Milks and Milk Powders. This method is based on the IDF Provisional Standard 20B, 1993 (*121*) and also on work done by Rowland, 1938 (*122*).

A 10% sample solution is diluted 1:4 with a trichloroacetic acid solution in a stepwise fashion with gentle mixing. The addition of trichloroacetic acid causes the proteins and fat in the sample to precipitate out of solution and settle on the bottom of the flask. The solution is then filtered through a dry pleated filter paper into a dry flask. The clear

filtrate is then analysed for the nitrogen content not associated with protein (NPN) using NZTM 3.15.8.

Appendix 1.6: Determination of Total Phosphate Content

The sample is dissolved in 0.5% tri-sodium citrate before being analysed using an autoanalyser. The orthophosphate in the sample reacts with the reagents used, ammonium molybdate and ammonium metavanadate, resulting in the formation of a colour complex whose absorption is measured at 405nm along with standards.

Appendix 1.7: Determination of Amino Acid Profile

For the acid stable amino acids the samples are first hydrolysed with HCl before being diluted with water and an internal standard added. AccQTag, from Waters Corporation, is then used to derivatise a filtered aliquot of the sample. The sample is then analysed using reverse phase HPLC equipped with a fluorimeter.

For tryptophan an internal standard is added to the samples before they are hydrolysed with NaOH in the presence of starch. The hydrolysate is then diluted, with water, and the amount of tryptophan is determined using the Dionex HPAEC system and the generation of a standard curve.

The sulfur amino acids, cystine and methionine, were analysed by AgResearch using performic acid oxidation. The method principle is described in Aitken and Learmonth, 2002 (*176*).

Appendix 1.8: Determination of Growth Factors

The tests all follow the same principle, although the methods are slightly different in terms of volumes and reagents used. The antibodies and standards are specific for each kit.

The basic principles of the test are as follows: the 96-well plate is coated with capture antibody (specific to each kit and able to bind the specific growth factor to the plate).

The plate is sealed and left to incubate overnight at room temperature. The following morning any capture antibody not bound to the plate is washed off. A blocking reagent is added to the wells and left to incubate to bind to any gaps in the wells where the capture antibody has not bound. The samples are made up in water and are allowed significant time to mix before use. The samples undergo pre-treatment/activation before use: this is done by adding 1N HCl to a small aliquot of sample, mixing and then leaving to react for 10 minutes before adding 1.2 N NaOH/0.5 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and reagent diluent (reagent diluent is specific for each kit). The blocking reagent is washed off and the sample and standards are plated and left to incubate. After 2 hours any unbound material is washed away and the detection antibody (a secondary biotinylated antibody) is added and incubated for 2 hours. The unbound material is then washed off and the plate is incubated with a Streptavidin/Hourseradish peroxidase (HRP) Conjugate. Following incubation, any unbound material is washed off and a colourless substrate solution of H_20_2/TMB (3,3,5,5-tetramethylbenzidine) is added. The intensity of the resulting yellow colour is proportional to the amount of growth factor antibodies bound. The reaction is allowed to occur for 10 minutes before being stopped with the addition of 2N H_2SO_4. The plates, also containing standards, are then scanned at 450nm with a microplate reader and the optical density of each well is determined. A standard curve is generated and the amount of growth factor in each sample is calculated with reference to the standard curve.

Appendix 1.9: Determination of Lactoferrin Content

The Biacore Q (an automated biosensor) was used for this determination of lactoferrin. Goat anti-bovine lactoferrin was immobilised on a flow cell of a CM 5 sensor chip (a sensor chip consist of carboxymethylated dextran covalently attached to a gold surface), see Figure A1.

The samples were dissolved and diluted in 0.5M NaCl HBS-EP (10 mM Hepes, 3.4 mM EDTA, 0.005% surfactant P20, pH 7.4) buffer before being loaded on to the chip using the Biacore Q. As the sample passes through the flow cell, with 0.15 M NaCl HBS-EP running buffer, the lactoferrin from the sample interacts with the antibodies bound to the flow cell. All unbound material is then washed away leaving only the

lactoferrin bound to the antibodies on the chip. As the injected sample interacts with the immobilized antibody, the refractive index of the polarized light at the interface between the sensor surface and the solution alters to a degree proportional to the change in mass at the surface due to the binding of lactoferrin to the antibodies. This change in refractive index is detected in real time and recorded as a "sensorgram" (SPR response plotted against time), see Figure A2.

The bound material is then removed from the antibodies by a process of regeneration using 10 mM glycine-HCl, pH 1.75.

Standards are analysed at the same time as the samples and the concentration of lactoferrin can be determined from the standard curve.

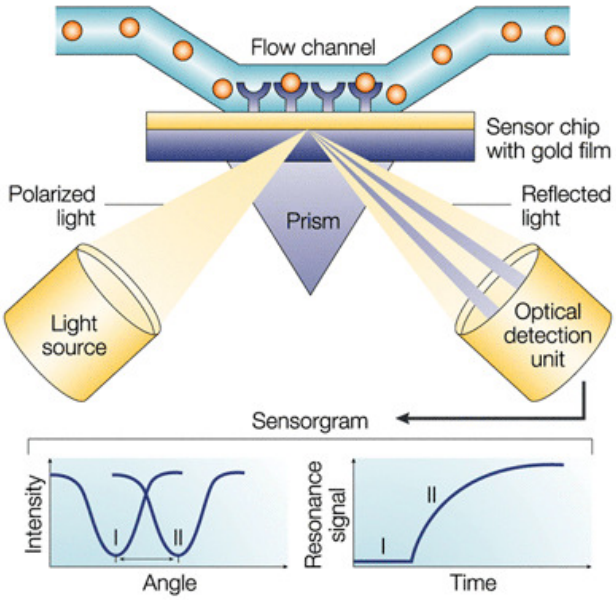

Figure A1: Surface plasmon resonance (SPR) method. (Taken from http://www.nature.com/nrd/journal/v1/n7/fig_tab/nrd838_F2.html)

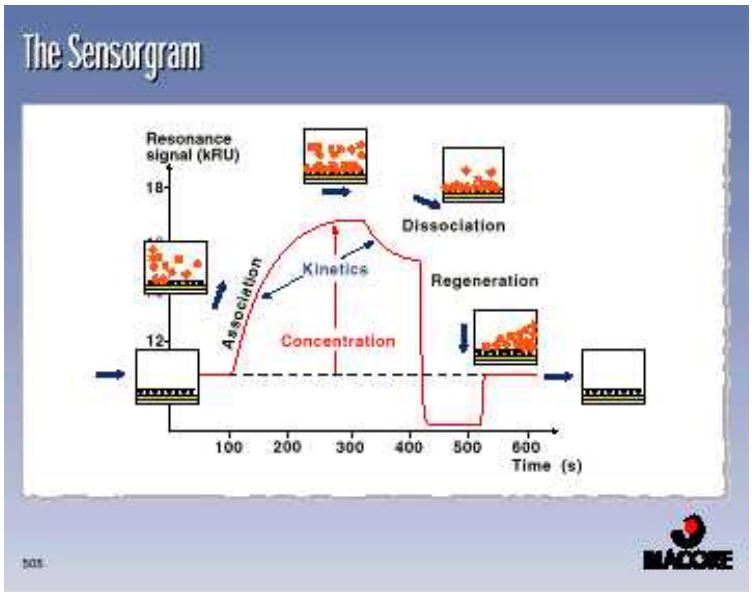

Figure A2: Biacore sensogram. (Taken from http://www.astbury.leeds.ac.uk/facil/SPR/spr_intro2004.htm)

Appendix 1.11: Determination of Proteins by Resource™ HPLC

The samples were first dissolved in water and made up to a concentration of 2mg/mL before being centrifuged and aliquoted in HPLC vials ready for analyses by HPLC (Waters, Milford, MA, USA). David Elgar (Fonterra Research Centre) completed the HPLC work on the prepared samples.

The HPLC method uses a gradient of 2 buffers to separate the whey proteins on a RPC 1-mL Resource™ column. Buffer A is water with 0.1% TFA and Buffer B is 90% acetonitrile with 0.1% TFA. The non-linear gradient runs from 80% to 0% Buffer A over a 23 minute time period at room temperature at a flow rate of 1 mL/min. Standards are also run alongside the samples and the concentration can be determined based on the standard curve generated.

Appendix 1.11: Determination of Phospholipids by Mass Spectrometry

The samples were dissolved in methanol before chloroform was added to give a ~1:2 chloroform:methanol mixture. The samples were allowed to mix with gentle rocking for 30 minutes before being centrifuged for 30 minutes to remove any solid material.

The supernatant was removed. Water and 1:2 chloroform:methanol were added to the pellet, mixed and centrifuged again. This supernatant was pooled with the first supernatant and water was added. After mixing by inversion and centrifuging, 2 phases formed, the upper phase contained non-polar material, and the lower phase contained the polar material (this is where the phospholipids were found). The upper phase was removed to a 5mL volumetric flask. Potassium chloride (KCl, 0.01M) and methanol were added to the lower phase, mixed and centrifuged again. The upper phase was removed and pooled to the first collection in the 5 mL volumetric flask and made up to the mark with 50% methanol. The lower phase was transferred to a 5 mL volumetric flask and made up to the mark with 1:2 chloroform:methanol. The lower phase was mixed and transferred to HPLC vials ready for analysis by HPLC/Mass Spectrometry.

The HPLC/MS methodology was from Fong, 2008 (*150*). HPLC details: Alliance HPLC, UV-Vis detector, refrigerated auto-sampler (set to 5°C), column Thermo Scientific, 150mm x 2.1mm, 3µm, APS-2 Hypersil hydrophilic column set at a column temperature of 50°C. The column was protected using a 10mm x 2.1mm guard column of the same packing material.

The sample was loaded onto the column and the phospholipids were separated with an acetonitrile/ammonium acetate buffer gradient at a flow rate of 0.5mL/min before being introduced into the Mass Spectrometer, a Thermo Scientific TSQ Quantum triple quadrupole mass spectrometer using a heated electrospray ionization (HESI) probe. The electrospray ionization (ESI) voltage, set at 3750 V, was used to generate the ions, which were focused with a capillary temperature of 300°C, a sheath gas flow of 30L/min and an auxiliary gas flow of 10L/min.

Appendix 2: Resource™ Breakthrough Fraction HPLC Profiles

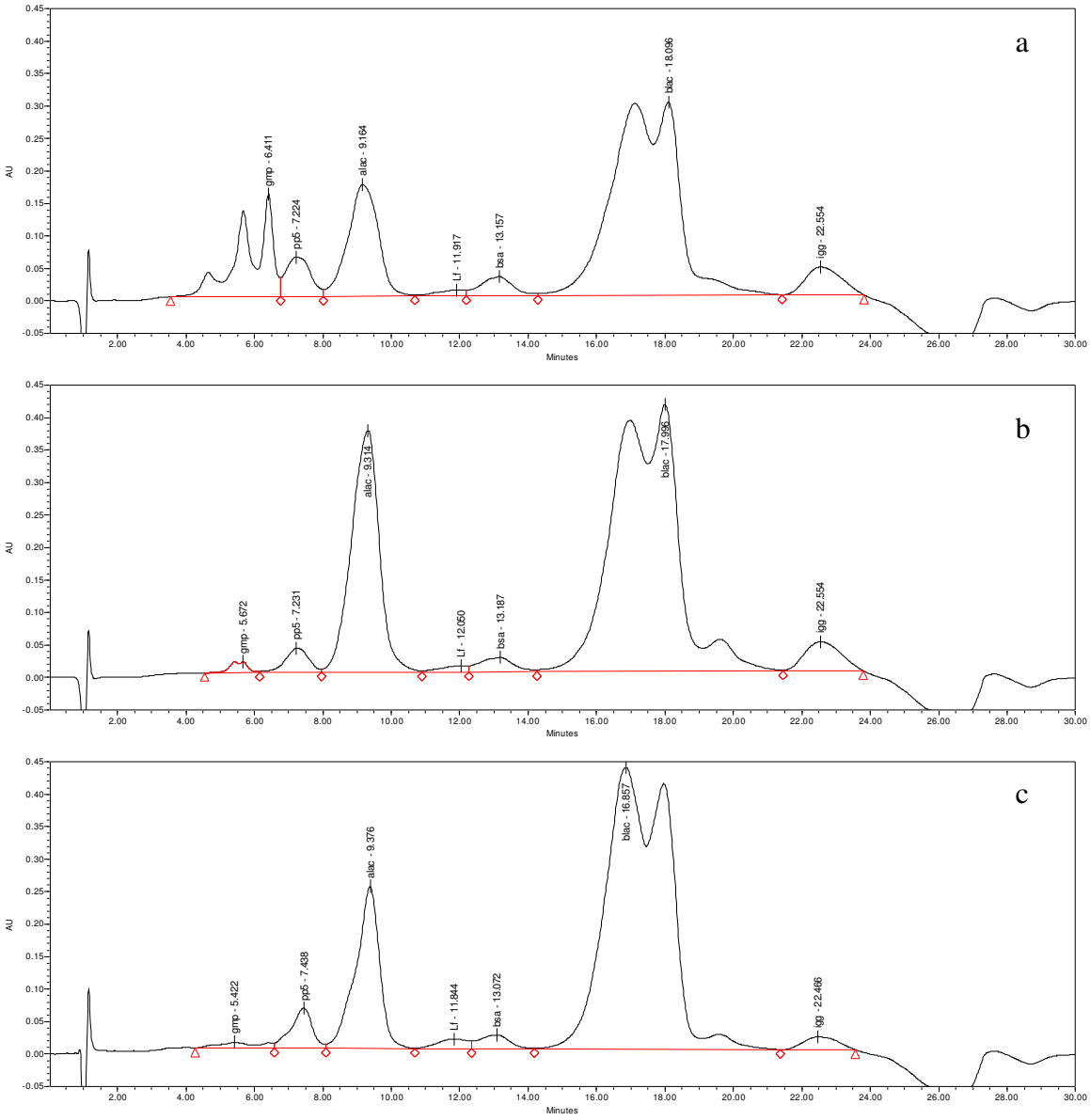

Figure A3: Resource™ Breakthrough Fraction HPLC Profiles for: a) GS19 Breakthrough Fraction; b) CR05 Breakthrough Fraction; c) HS15 Breakthrough Fraction

Appendix 3: Molecular Weight Profile

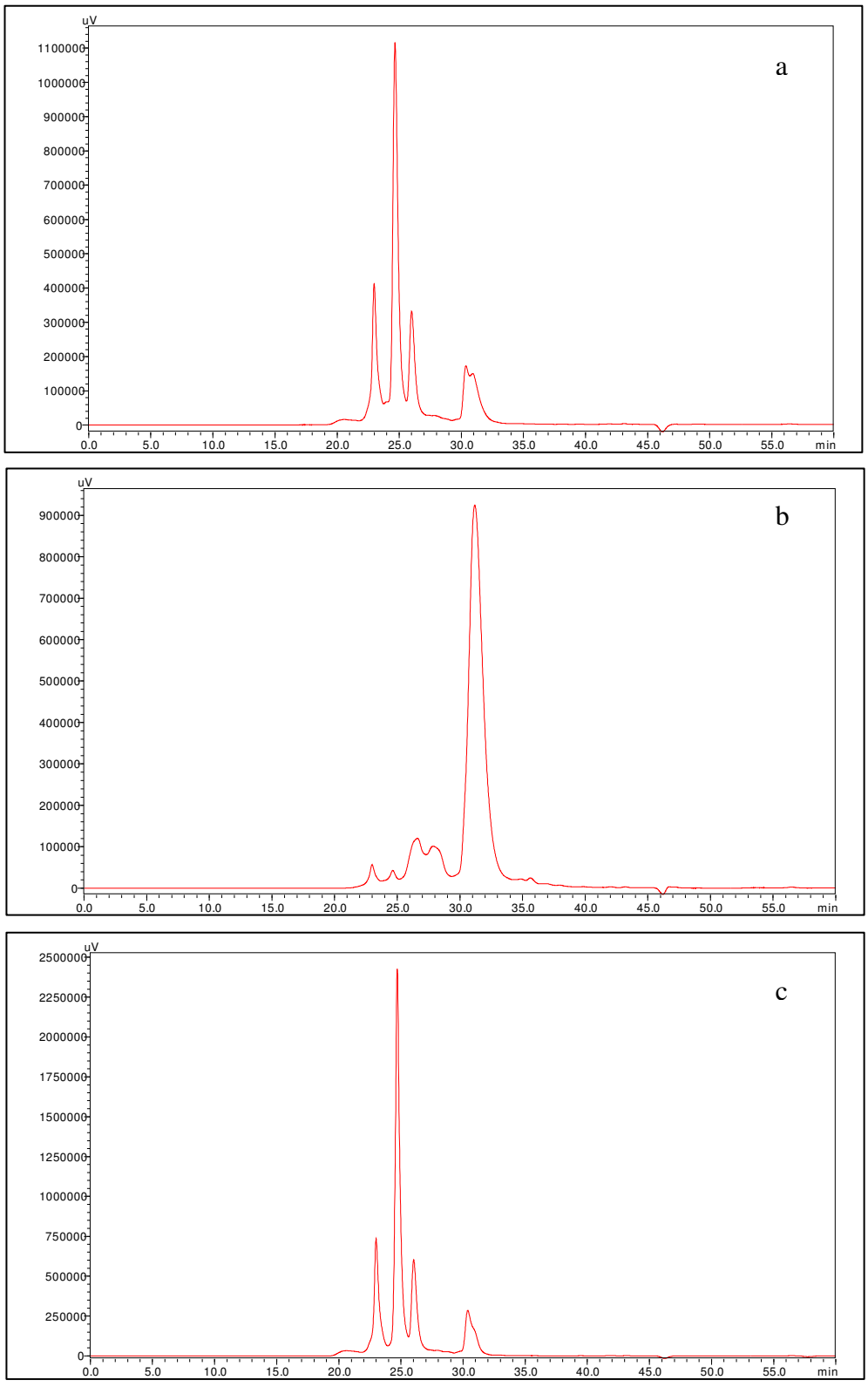

Figure A4: Molecular weight profiles for: a) GS19 Parent WPC; b) GS19 Acid Fraction; c) GS19 Breakthrough Fraction.

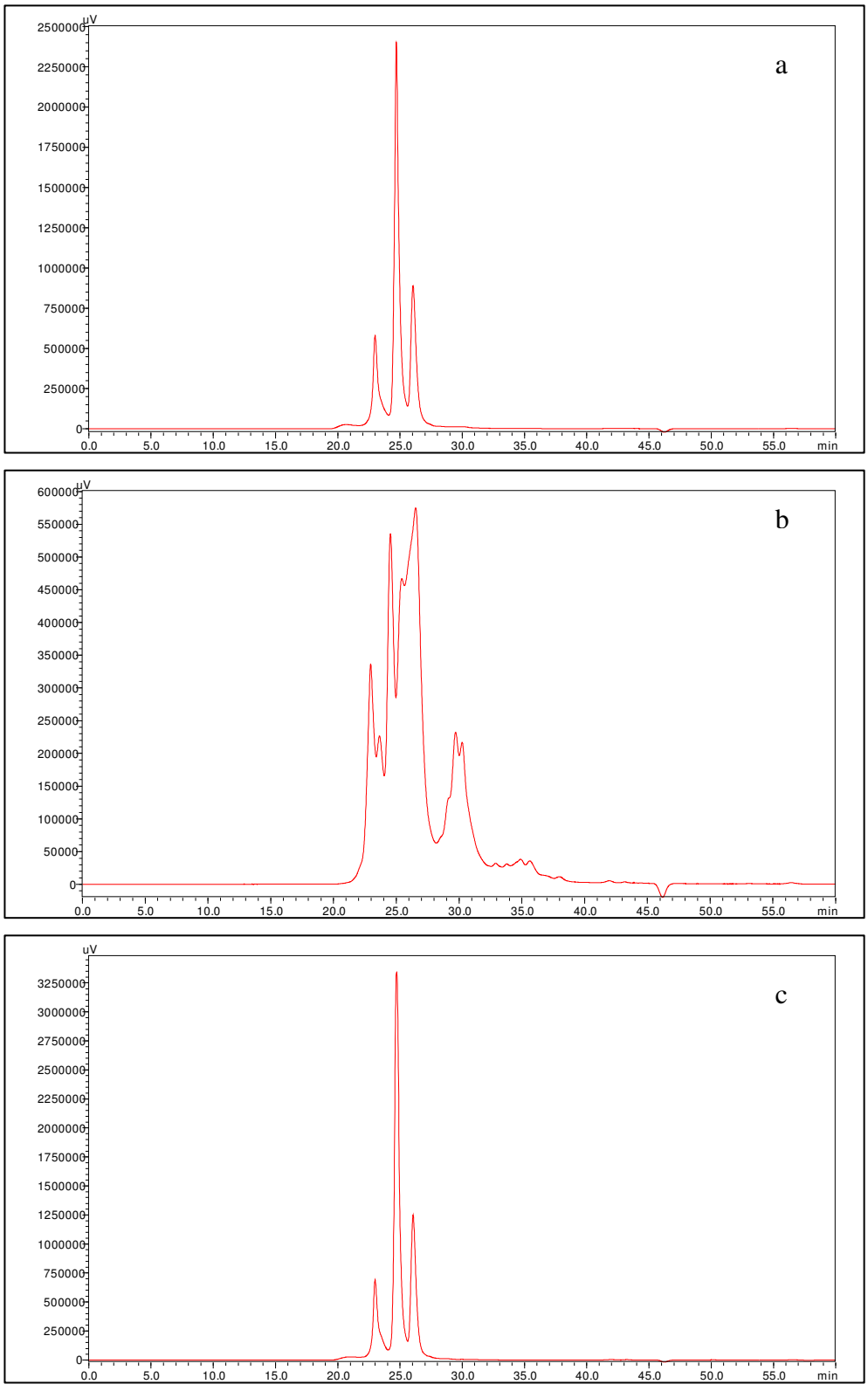

Figure A5: Molecular weight profiles for: a) CR05 Parent WPC; b) CR05 Acid Fraction; c) CR05 Breakthrough Fraction.

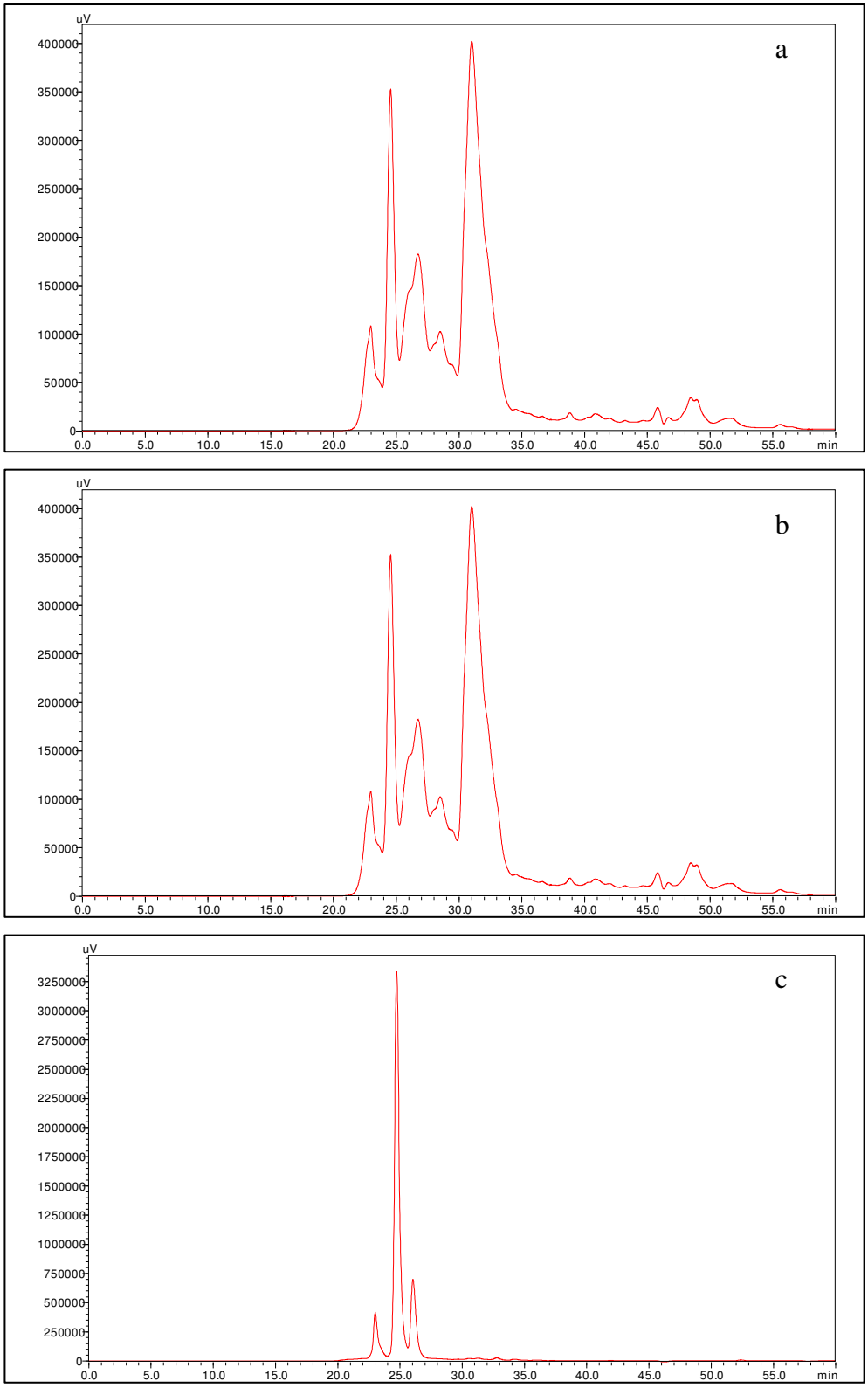

Figure A6: Molecular weight profiles for: a) HS15 Parent WPC; b) HS15 Acid Fraction; c) HS15 Breakthrough Fraction.

Appendix 4: Trypsin Digest SDS PAGE

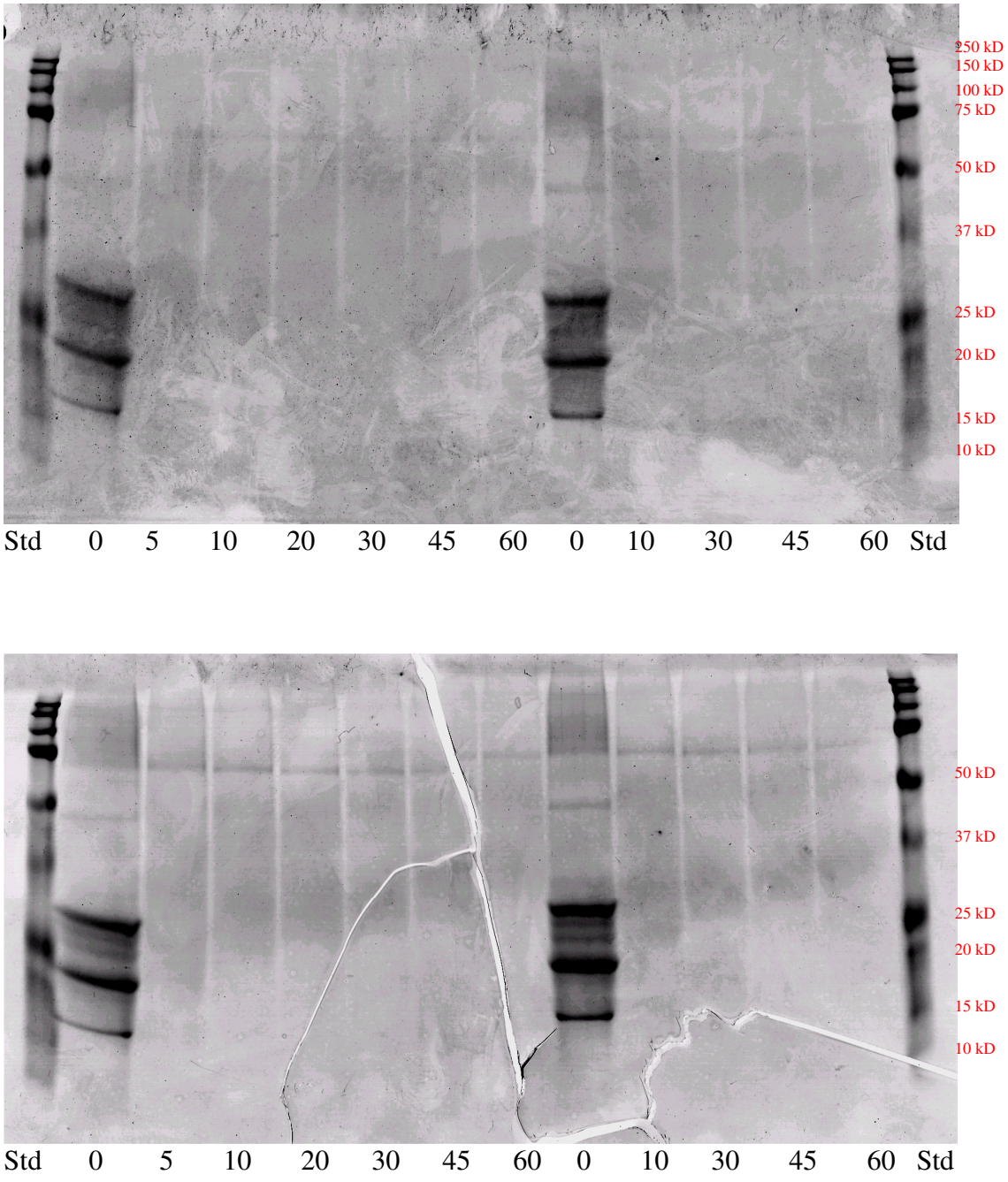

Figure A7: PAGE gels of the GS19 trypsin digested acid fractions at 1:40 (top) and 1:100 (bottom) enzyme to substrate (w/w) ratio.

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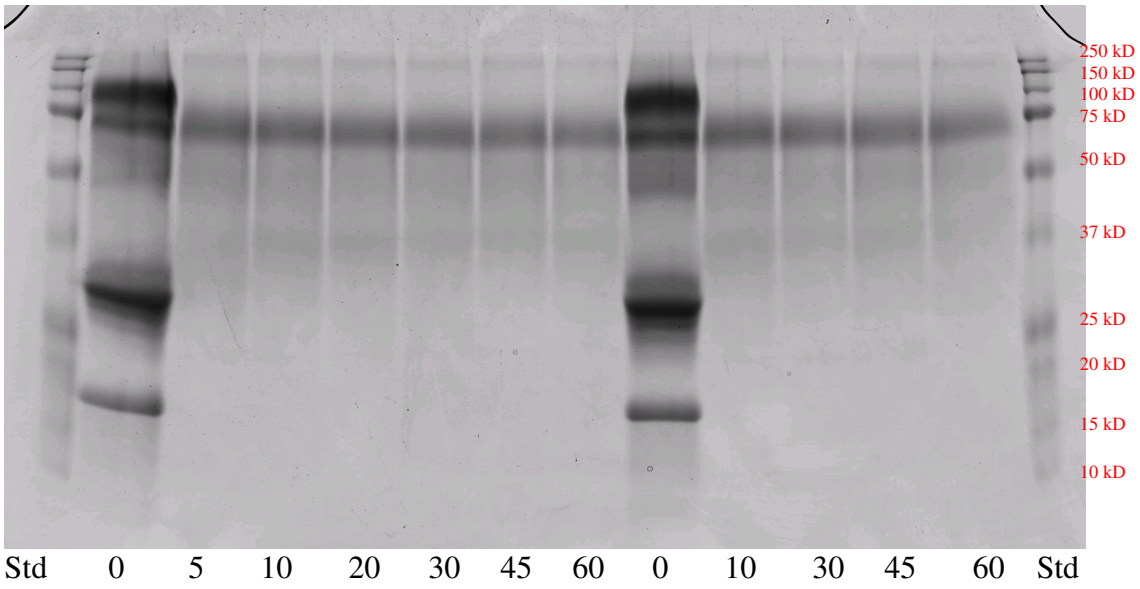

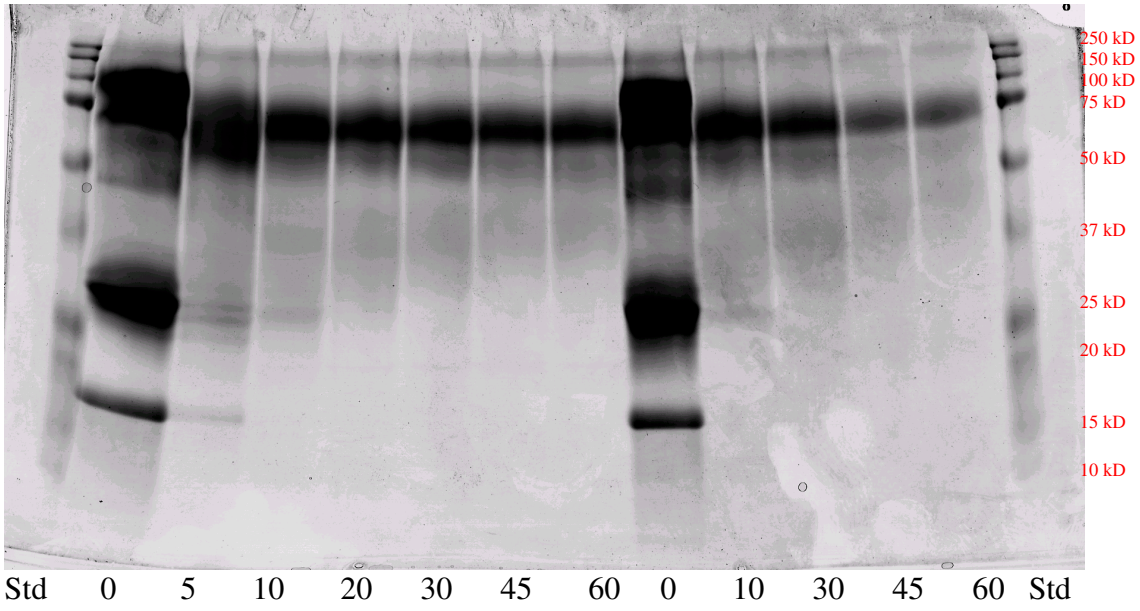

Figure A8: PAGE gels of the CR05 trypsin digested acid fractions at 1:40 (top) and 1:100 (bottom) enzyme to substrate (w/w) ratio.

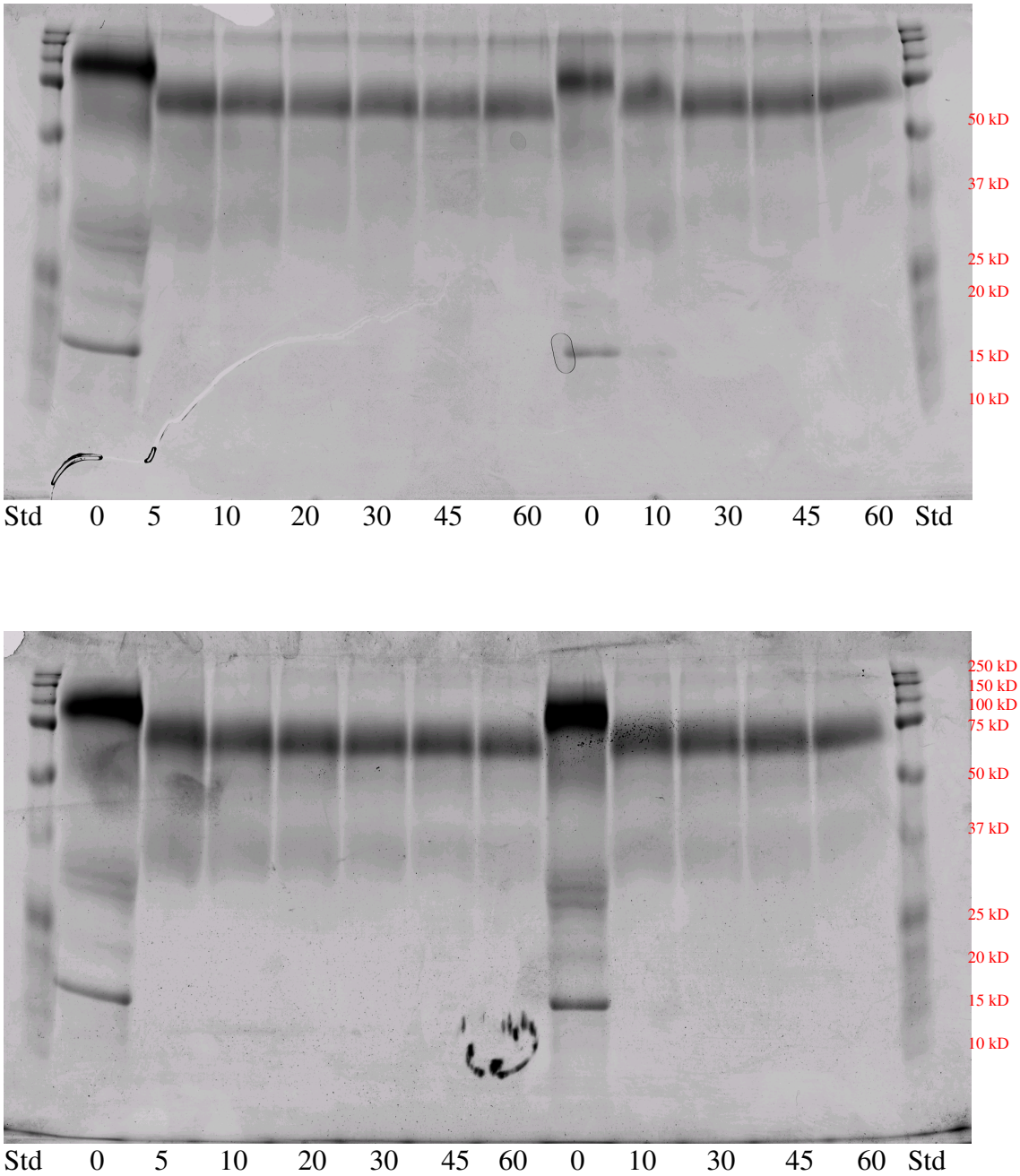

Figure A9: PAGE gels of the HS15 trypsin digested acid fractions at 1:40 (top) and 1:100 (bottom) enzyme to substrate (w/w) ratio.

NOTE: the samples did not run in the gels in the expected fashion, being retarded in their migration for reasons unknown. Hence the molecular weights of the components bear no relation to the MW markers. Nevertheless, the gels were indicative that sufficient hydrolysis had occurred. A more accurate reflection of the samples can be seen from the gels in the main body of the thesis (Results and Discussion: Determination of hydrolysis by SDS PAGE section).

Appendix 5: Bone Activity Results

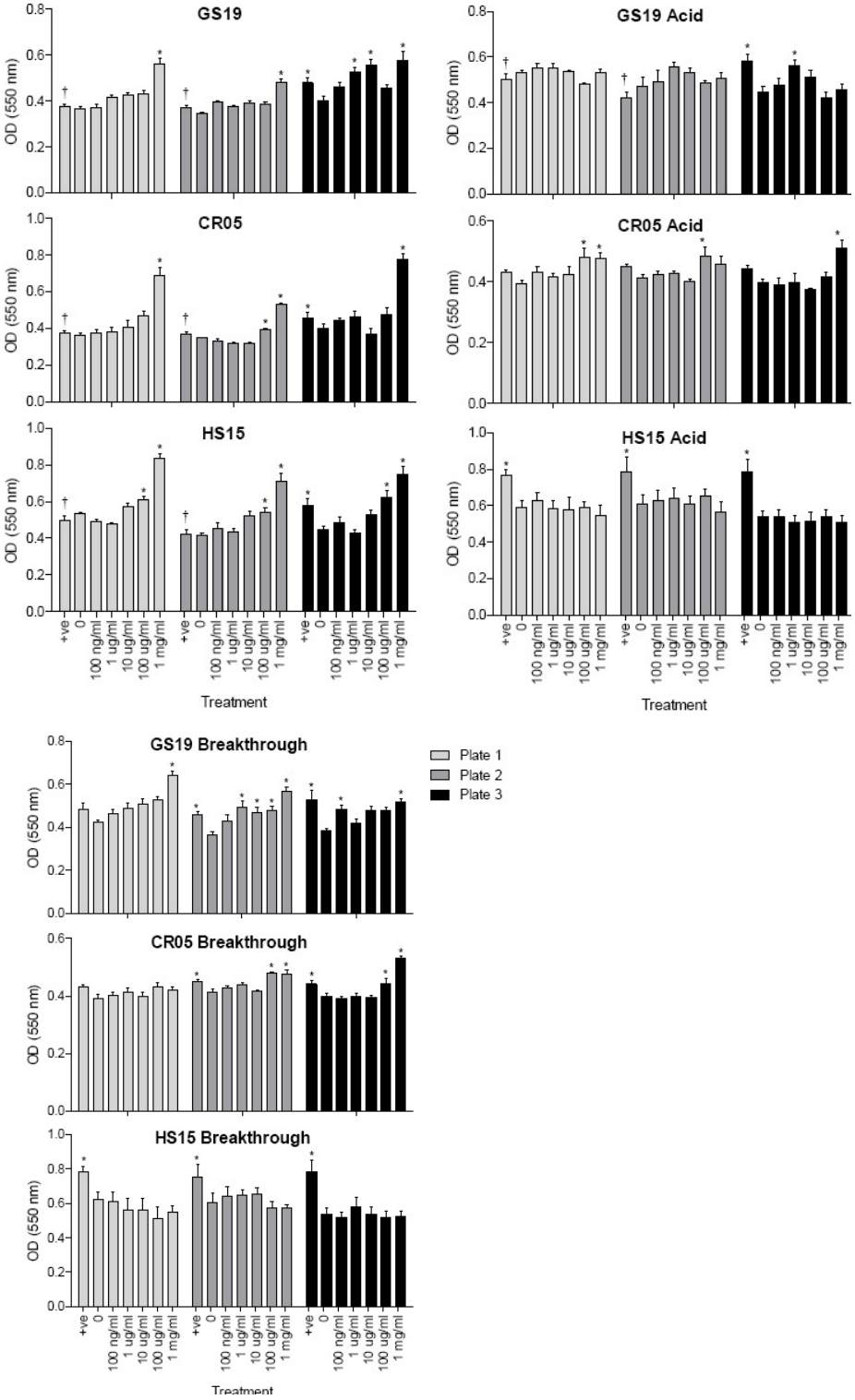

Figure A10: Effect of the three WPC parent powders, acid and breakthrough fractions on osteoblast cell proliferation. Statistical significance ($p < 0.05$) from the 0 control is indicated by an asterisk. Means ± SEM. With permission, these graphs have been included from the bone activity results report by Dr Wei-Hang Chua (*151*).

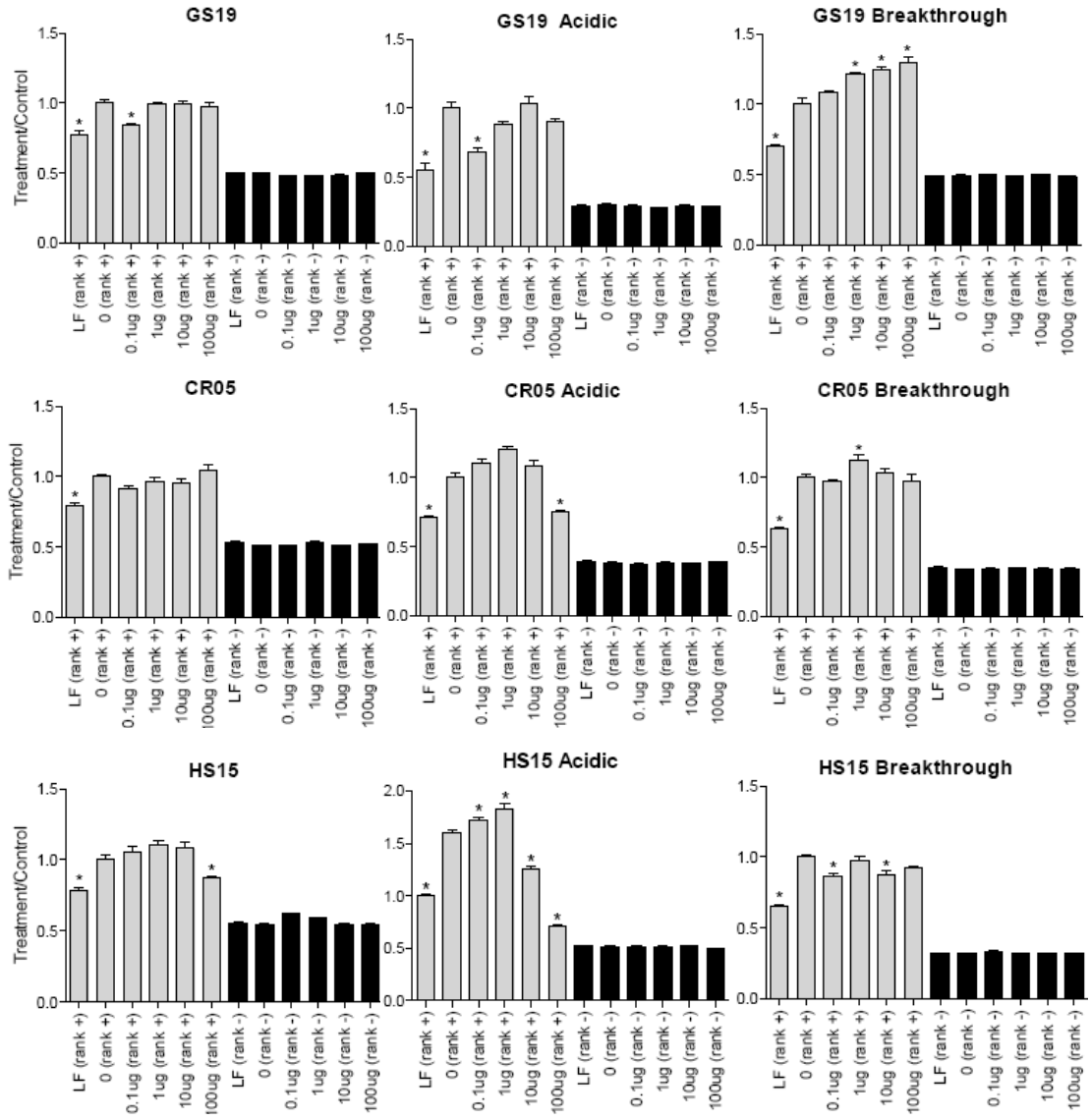

Figure A11: Effect of the three WPC parent powders, acid and breakthrough fractions on Raw 264.7 osteoclastogenesis. Significance ($p < 0.05$) from 0 control indicated by asterisk. Means ± SEM. With permission, these graphs have been included from the bone activity results report by Dr Wei-Hang Chua (*151*).

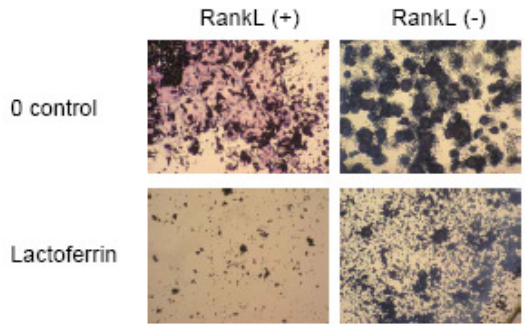

Figure A12: An image of the effect of the control and lactoferrin on osteoclatogenesis (osteoclast development) under the conditions of RANK-L present and absent as taken from, with permission, the bone activity results report by Dr Wei-Hang Chua (*151*).