Production, Fractionation, and Evaluation of Antioxidant Potential of Peptides Derived from Soy Protein Digests

by

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Author's Declaration

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Abstract

Oxidation plays an important role in the basic processes of life, such as the production of energy and phagocytosis employed by the immune system. However, when an imbalance between oxidants and antioxidants exists *in vivo*, oxidation can become uncontrolled and result in diseases such as arthritis, cancer, artherosclerosis, and Alzheimer's Disease. Dietary antioxidants including polyphenolic compounds, proteins, and peptides have been identified as being physiologically functional foods capable of contributing to the restoration of this oxidant-antioxidant balance.

The objective of this study was to explore the production of antioxidant soy peptides from a commercially available soy protein isolate (SPI) by enzymatic hydrolysis in a process similar to that occurring in the human digestive tract. In this study, Archer-Daniels Midland SPI PRO-FAM 974 was used as a raw material for the production of antioxidant soy peptides.

The digestion consisted of enzymatic digestion of the SPI (3.12 wt %) with pepsin (37ºC, pH 1.5) and/or pancreatin (40ºC, pH 7.8) either individually or sequentially. The enzyme concentration and digestion time for each enzyme was optimized using a 2^4 factorial experimental design to produce the greatest concentration of peptides quantified in PheGly equivalents by the OPA assay. A maximum peptide concentration of approximately 65 mM PheGly equivalents was achieved in the follow-up digests resulting from this factorial design model, using pepsin (0.15 g/L, 15 minutes) and pancreatin (4.5 g/L, 120 minutes) sequentially to digest the SPI.

Fractionation of the peptides by sequential dead-end membrane ultrafiltration with molecular weight cut-offs (MWCO) of 3 kDa and 1 kDa was performed to produce peptide fractions with increased antioxidant capacity. The permeate flux as a function of time was fit to empirical models, revealing that the membrane fouling resulting in the permeate flux decline is largely reversible and most likely the result of cake filtration.

Antioxidant capacity was quantified by the DPPH, FCR, and ORAC assays to determine the electron-donating and proton-donating capacities of the soy peptides. The electron-donating DPPH assay was not suitable to quantify the antioxidant capacity of the soy peptides due to poor peptide solubility in the assay media and sensitivity. The electron-donating FCR assay and the proton-donating ORAC assay were used to distinguish between the ultrafiltration and digestion conditions

employed to produce the soy peptides and the antioxidant capacity was quantified in equivalence to the standard antioxidant Trolox.

The soy peptide fraction with the greatest antioxidant capacity was produced by enzymatic digestion with pancreatin (4.5 g/L, 120 minutes) alone and had a molecular weight cut-off of between 3 kDa and 1 kDa. This fraction had an equivalent antioxidant capacity of approximately 190 mg Trolox/g sample in the ORAC assay and approximately 180 mg Trolox/g sample in the FCR assay.

A preliminary linear model for the optimum digestion and ultrafiltration conditions for the production of antioxidant peptides with the greatest ORAC antioxidant capacity was also developed. The model includes a positive pancreatin digestion time term and a negative pepsin digestion time term. No ultrafiltration terms were found to be significant in this preliminary model, but a large constant term persisted.

In conclusion, the enzymatic digestion of commercially available SPI with pancreatin and fractionated by ultrafiltration successfully produced a soy peptide fraction with increased antioxidant capacity.

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AAPH = 2,2-azobis-(2-amidinopropane) dihydrochloride

ACE = Angiotensin I-converting enzyme

ADM = Archer Daniels Midland

Da = Daltons

DH = Degree of hydrolysis

DPPH = 2,2-diphenyl-1-picrylhydrazyl

ET = Electron transfer

FCR = Folin-Ciocalteu reagent

HAT = Hydrogen atom transfer

MALDI-TOF = Matrix-assisted laser desorption/ionization-time of flight mass spectrometry

MWCO = Molecular weight cut-off

OPA = o-phthaldialdehyde

ORAC = Oxygen radical absorbance capacity

SEC = Size exclusion chromatography

SPI = Soy protein isolate

TNBS = 2,4,6-trinitrobenzenesulfonic acid

1   Introduction

Antioxidants are defined as "any substance which significantly delays or inhibits oxidation of a substrate when present at low concentrations compared to that of an oxidizable substrate" (Jun, Park, Jung, & Kim, 2004). Antioxidants are commonly used in the food industry to prevent lipid oxidation which can lead to product degradation and production of off-flavours, but they also have applications in other fields where oxidation will degrade the product quality (Gao, Miller, & Han, 2004).

Compounds such as polyphenols and carotenoids found in brightly coloured fruits and vegetables have been shown to possess beneficial health properties such as preventing cancer, diabetes, arthritis, atherosclerosis, and other age-related diseases (Kaur & Kapoor, 2001). The primary focus has been on the antioxidant properties of molecules such as flavonoids, polyphenols, and carotenoids derived from fruit and vegetable sources (Fukumoto & Mazza, 2000, Pulido, Bravo, & Saura-Calixto, 2000, Kaur *et al.,* 2001, Sanchez-Moreno, 2002) but some groups are also investigating the antioxidant properties of proteins and peptides derived from both animal and non-animal sources (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998, Jao & Do, 2002, Saito *et al.,* 2003). Wayner and co-workers recognized that 10-50% of the antioxidant properties found in human blood plasma could not be explained by known antioxidants such as vitamin E, urate, or ascorbate and theorized that a number of essential amino acids possessing labile hydrogen atoms could allow amino acids and peptides to behave as antioxidants (Wayner, Burton, Ingold, Barclay, & Locke, 1987). Research currently underway covers a wide range of potential antioxidant peptide sources such as rice, soybean, fish, skeletal muscle tissue, chicken eggs, and bovine milk.

The measurement of antioxidant capacity is a challenge because antioxidants can act by many different mechanisms, such as hydrogen-donating, electron-donating, free radical scavenging, and chelating metal ions which can initiate free-radical reactions. Therefore a single test for antioxidant capacity does not exist (Prior, Wu, & Schaich, 2005). In 2004, the First International Congress on Antioxidant Methods recommended the standardization of the testing of food, botanical, and nutraceutical antioxidants by two methods: the Oxygen Radical Absorbance Capacity (ORAC) fluorescence assay to measure the hydrogen-donating capacity of an antioxidant to protect a fluorescing target from attack by the free-radical AAPH, and the Folin-Ciocalteu Reagent (FCR) method to quantify the electron-donating capacity of an antioxidant to protect the FCR from degradation (Prior *et al.,* 2005).

Soybeans are a significant food crop with world-wide soybean consumption estimated at about 150 million tons in 2000 (De Meester, Kempener, & Mollee, 2000). While proteins make up approximately 40% of the total dry matter in soybeans, soy proteins are largely used as animal feed while the oil is reserved for human consumption, although interest is resurfacing for the use of soy proteins in other applications such as adhesives and polymers (Liu 1997, Kumar, Choudhary, Mishra, Varma, & Mattiason, 2002). In 1999, the US Food and Drug Administration approved the "use of health claims about the role of soy protein in reducing the risk of coronary heart disease" (Food and Drug Administration (FDA), 1999), which has lead to further interest in the soybean.

Enzymatic modification of proteins is a mild and targeted method to modify the functional properties of the proteins with high specificity under relatively mild conditions with limited effects on the nutritional properties (Kunst, 2000, Kumar *et al.*, 2002). The enzymatic modification of soy proteins is a well-documented process, including the production of traditional Asian foods such as tempeh and miso (Liu, 1997). More recently, enzymatic digestion by pepsin and pancreatin in a digestion model similar to that found in the human digestive tract has been employed to produce bioactive peptides, such as ACE inhibitor peptides (Lo & Li-Chan, 2005). The degree of hydrolysis is monitored by the o-phthaldialdehyde (OPA) assay, where an absorbing adduct between the OPA, β-mercaptoethanol, and the α-amino group of the peptide is measured by spectrometry and related to the concentration of peptides in the solution (Church, Swaisgood, Porter, & Catignani, 1983).

Once the enzymatic modification of the protein has been completed, a post-treatment method such as membrane ultrafiltration can be used to fractionate the peptides and proteins in the digestion media (Filipe & Ghosh, 2005, Kunst, 2000). In the current study, ultrafiltration was selected because it is a gentle, cost-effective method that can perform this separation without the introduction of potentially hazardous solvents while also reducing the processing which may be perceived as "unnatural" by the consumer (Chen, Chen, & Juang, 2007). An understanding of the size distribution of the peptides in each ultrafiltration fraction was determined through the application of MALDI-TOF and/or SEC-HPLC, similar to the methods employed by others in this field (Chen, Muramoto, Yamauchi,1995).

The production of antioxidant peptides between three and 16 amino acid residues from soy proteins has been demonstrated (Chen *et al.,* 1995, Gao *et al.,* 2004) although an understanding of the

factors and their interactions that contribute to the production of these peptides and their concentration is not well understood.

1.1   Objectives of this Study

The purpose of this study was to produce antioxidant soy peptides, by:

1. producing peptides from commercially-available soy protein isolate (SPI) by enzymatic hydrolysis with porcine pepsin and/or pancreatin under conditions similar to those in the human digestive tract,

2. fractionating the peptides according to size by dead-end membrane ultrafiltration,

3. determining the electron-donating and proton-donating antioxidant capacities of the peptide fractions, and

4. building an empirical model of the effects of the digestion and fractionation conditions on the production of antioxidant peptides.

2   Literature Review

2.1 Soybeans

The typical composition of soybeans is 40% proteins, 20% oil, 30% carbohydrates, 5% fiber, and 5% ash (Vereijken, 2000). The majority of the proteins found in soybeans are storage proteins, which are comprised of albumins (water-soluble proteins) and globulins (salt-solution soluble proteins). The two types of globulins found in soy proteins, which are the majority of the proteins, are often referred to as glycinin (11S) and conglycinin (one component of the 7S fraction), the names of which are derived from the genus name of the soybean plant, Glycine (Liu, 1997). Other proteins found in soybeans include hemaglutinin, trypsin inhibitors, beta-amylases and lipoxygenases (Kumar *et al.*, 2002). The 11S protein contains three to four times more methionine and cysteine per unit than the 7S protein although soy proteins are generally lacking in the sulphur-containing amino acids (Liu, 1997). The isoelectric point is the pH at which a molecule has a net charge of zero; for soy proteins, the isoelectric point is approximately pH 4.5 (Copeland, 1994, De Meester *et al.*, 2000).

Soy protein isolates (SPI) are a refined form of soybean proteins containing a minimum of 90% proteins. The proteins found in SPI are typically at least partially denatured due to the hexane extraction of soybean oil, exposure to temperatures of 60-100ºC to remove the remaining solvent and/or deactivate the trypsin inhibitors, and the alkali treatment, acid precipitation and/or aqueous alcohol washing of the proteins that appear in typical processing (Liu, 1997, De Meester *et al*., 2000, Kumar *et al*., 2002). The exact processing conditions employed in the production of the SPI used in this study were not disclosed by Archer Daniels Midland (ADM), the SPI manufacturer, but some level of denaturation due to the processing conditions employed is expected.

In recent years, soybeans have been used largely as an oilseed crop with the majority of the non-oil product being used as animal feed. However, this was not always the case as indicated by the traditional soy-based foods found in Asian culture and the recent popularity of soy ingredients in processed foods (Wang, Saito, Tatsumi, & Li, 2003). Components of the soybean are also being reincorporated into other fields such as adhesives, polymers, and construction materials (Kumar *et al.,* 2002).

2.2   Peptide Production

In biological systems, antioxidant ability has been associated with enzymes such as catalase, glutathione peroxidase, and superoxide dismutase that act on reactive oxygen species present *in vivo* (MacDonald-Wicks, Wood, & Garg, 2006, Chan & Decker, 1994). Proteins such as those found in soy protein isolates (SPI) have also credited with having antioxidant properties (Pena-Ramos & Xiong, 2002). However, one should note that some proteins such as heme proteins and lipoxygenases have been shown to be pro-oxidants (Elias, Kellerby, & Decker, 2008), therefore not all proteins should be assumed to have antioxidant properties.

A review of the recent literature reveals the prevalence of antioxidant peptides produced by several mechanisms. Fermentation with a bacterial culture has been employed to produce a change in the antioxidant capacity of the source material, such as the fermentation of milk using 25 lactic acid bacterial starter cultures by Virtanen and co-workers. This group found that improved antioxidant capacity was associated with fermentation with one of *Lactobacillus jensenii*, *Lactobacillus acidophilus*, or *Leconostoc mesenteroides* ssp. *cremoris* strains and that the nature of the antioxidant properties was associated with the stage of the fermentation (Virtanen, Pihlanto, Akkanen, & Korhonen, 2007).

Zhang and co-workers studied the effects of the proteases Alcalase, Chymotrypsin, Neutrase, Papain, and Flavorase on defatted rice endosperm protein and found that the digest with Neutrase produced peptides with the greatest antioxidant capacity (Zhang, Zhang, Wang, Guo, Wang, & Yao, 2009). Jao and Ko used Protease XXIII from *Aspergillus oryzae* to hydrolyze tuna cooking juices to produce peptides with improved antioxidant capacity (Jao & Ko, 2002). Techniques for the chemical modification of proteins are commonly employed but from the standpoint of safety of food for human consumption, enzymatic modification is the preferred method (Nakai & Modler 1996).

The enzymatic modification readily accomplished in the digestive tract of many organisms, where water is used to split the amino acid bond holding a peptide chain together as demonstrated in Figure 1 (Blanch & Clark, 1997) is also frequently employed in literature to produce antioxidant peptides.

$$R_1-\underset{\underset{H}{|}}{\overset{\overset{O}{||}}{C-N}}-R_2 + E + H_2O \rightarrow E + R_1-\overset{\overset{O}{||}}{C}-OH + \underset{H}{\overset{H}{\diagdown}}N-R_2$$
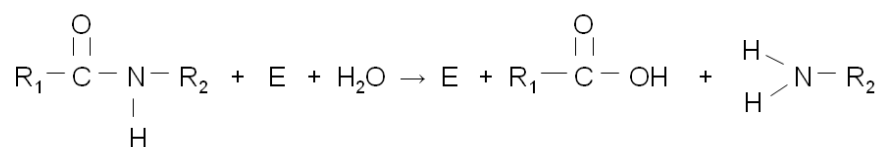
Figure 1:   Enzymatic hydrolysis of protein

Davalos and co-workers used pepsin to treat crude egg white to produce antioxidant peptides (Davalos, Miguel, Bartolome, & Lopez-Fandino, 2004a). Hernandez-Ledesma and co-workers have used pepsin and pancreatin sequentially to digest milk and infant formula by an *in vitro* method designed to mimic the human digestive tract, resulting in peptides with increased antioxidant capacity (Hernandez-Ledesma, Quiros, Amigo, & Recio, 2007). Similarly, Vilela and co-workers employed the enzymes pepsin and pancreatin to digest whey protein isolates with improved bioactive properties (Vilela, Lands, Chan, Azadi, & Kubow, 2006).

Certain naturally-occurring peptides have been isolated and shown to have antioxidant properties. Skeletal muscle tissues have been studied and the dipeptides carnosine, ophinine, and anserine have been identified as being powerful antioxidants (Chan *et al.,* 1994). Therefore, the peptide bond itself may play a role in the antioxidant capacity of peptides (Murase, Nagao, & Terao,1993). Further examples of peptides with demonstrated antioxidant capacity are summarized in Table 1, including the source of the protein or peptide and the processing performed.

While one may assume that a higher degree of hydrolysis would be best, short chain peptides have been demonstrated to be better absorbed by the digestive system and exhibit higher antioxidant capacities when compared to a mixture of amino acids in the same ratio (Wayner *et al.,* 1987, Turgeon, Bard, & Gauthier, 1991).

2.2.1 Soy Peptides

The health claim approved by the FDA that the consumption of soy proteins may lead to a reduction in the risk of coronary heart disease (FDA, 1999) indicates that the digestion of soy proteins by the human gastrointestinal system may have beneficial health effects. The partial hydrolysis of soy proteins has been shown to increase certain functional properties, including the presence of functional peptides in fermented soy foods (Liu, 1997, Wang *et al.,* 2003). Chen and co-workers digested the soy protein β-conglycinin with Protease M from *Aspergillus oryzae*,

Protease N from *Bacillus subtilus*, Protease P from *Aspergillus melleus*, Protease S from *Bacillus* sp., and pepsin; from the digest with Protease S, six antioxidant peptides with improved antioxidant capacity when compared to the source protein were isolated (Chen *et al.,* 1995).

One complicating factor is the bitter taste associated with soy peptides that may make them difficult to incorporate into mild-tasting foods. In the work done by Cho and co-workers, the 5-10 kDa fraction of soy peptides with the highest level of glutamic acid but the lowest levels of leucine, phenylalanine, and tryptophan were the most bitter while peptides with a molecular mass less than 1 kDa were much less bitter (Cho, Unklesbay, Hsieh, & Clarke, 2004). Therefore the amino acid residues present in the peptide may both be important factors in the antioxidant capacity of the peptide but also its flavour and therefore its ability to be incorporated into food products.

2.2.2 Pepsin

Pepsin (EC 3.4.23.1) is an enzyme from the family of carboxyl endoproteinases with an optimum pH of 2 found in the gastric juices of vertebrates (Copeland 1994). Endoproteinases act by hydrolyzing bonds within the polypeptide chains between specific amino acids thus producing smaller peptide chains (Nakai *et al.,* 1996). Pepsin cleaves at the carboxyl end of the hydrophobic and aromatic amino acids tyrosine, phenylalanine, tryptophan, and leucine (Lo, Farnworth, & Li-Chan, 2006), but will not cleave at valine, alanine or glycine, nor will it hydrolyze non-peptide amide or ester linkages (Sigma-Aldrich, 2008).

2.2.3 Pancreatin

Pancreatin is a mixture of many enzymes, including amylase, trypsin, lipase, ribonuclease, and protease which belong to the EC 3.4.21 family of serine endoproteinases (Sigma-Aldrich, 2009). Similar to pepsin, the pancreatin enzymes are also endoproteinases and therefore will hydrolyze the bonds between specific amino acids to produce smaller peptide chains (Nakai *et al.,* 1996). However, because pancreatin is composed of a mixture of enzymes, the exact behaviour of those enzymes will differ.

One component of pancreatin is the enzyme trypsin (EC 3.4.21.4), which has an optimum pH between 7 and 9 and is reversibly denatured below pH 4 or deactivated by raising the pH to 10.5 and the temperature to 65°C for 20 minutes (Mutilangi, Panyam, & Kilara, 1995). Trypsin preferentially cleaves at the carboxyl end of arginine and lysine residues and produces positively

charged residues at the C-terminal position (Lo *et al.,* 2006). Trypsin is also autolytic so solutions of trypsin should be prepared immediately before use; the enzymatic reaction is stopped by freezing the sample (Copeland, 1994).

Another component of pancreatin is chymotrypsin (EC 3.4.21.1) that cleaves at the carboxyl end of hydrophobic and aromatic amino acids tyrosine, tryptophan, phenylalanine, and leucine (Lo *et al*., 2006). Chymotrypsin, like trypsin, has an optimum pH between 7 and 9 and is reversibly denatured below pH 4 (Copeland, 1994).

Protease is a general term referring to an enzyme that cleaves peptide bonds in proteins, but without any further information on the nature of the pancreatin protease, additional information on the likely results of enzymatic digestion with this enzyme cannot be gathered. Furthermore, ribonucleases that cleave RNA, amylases that cleave sugars and starches, and lipases that cleave lipids, are of little interest in the production of soy peptides and therefore will not be discussed further.

2.3   Peptide Characterization

Degree of hydrolysis (DH), defined as the percentage of peptide bonds cleaved, is used to monitor enzymatic hydrolysis processes and to characterize protein hydrolysates. The degree of hydrolysis can be monitored on a continuous basis using the simple and rapid pH-stat technique, where the change in pH of the digestion media is monitored on a continuous basis and can be adjusted through the addition of an acid or a base. However, the pH-stat must be calibrated using other techniques such as the Bradford, OPA, ninhydrin, or TNBS assays (Turgeon *et al.,* 1991, Lemieux, Puchades, & Simard, 1990, Panasiuk, Amarowicz, Kostyra, & Sijtsma, 1998, Nielsen, Petersen, & Dambmann, 2001).

Once the peptides produced and fractionated, several researchers further characterized the peptides, some determining the molecular mass of the antioxidant peptides produced while others determined the amino acid sequence. Jun and co-workers used consecutive chromatographic methods to determine the molecular mass and sequence of the antioxidant *Limanda aspera* frame peptide (Jun *et al.,* 2004). Hernandez-Ledesma and co-workers identified the amino acid sequence of the antioxidant peptides derived from whey proteins by HPLC-MS/MS (Hernandez-Ledesma, Davalos, Bartolome, & Amigo, 2005). Chen and co-workers first isolated antioxidant soy peptides using a combination of size exclusion chromatography and reversed-phase HPLC (Chen *et al.,*

1995), then later used synthetic peptides to test the antioxidant behaviour of the earlier peptides identified in soy protein digests (Chen *et al.,* 1998). Livney and Dalgleish used MALDI-TOF MS to characterize the disulphide bonds in milk peptides produced by enzymatic hydrolysis (Livney & Dalgleish, 2004).

2.3.1 Bradford Assay

The Bradford assay is based on the change in absorbance at 595 nm of the dye Coomassie Blue G250 as a result of bonding the anionic form of the dye with the arginyl residues in a peptide chain in acidic media. The absorbance of the dye remains at 575 nm when in the unbound, cationic form and the dye does not bind to free amino acids (Walker, 1994, Bradford, 1976). The peptide-dye interactions have been reported to show some variability based on the peptide chains under investigation and interference from strong alkaline agents such as detergents has also been reported (Copeland 1994, Bradford 1976).

The Bradford method was employed by Vilela and co-workers to measure the reduction of protein concentration in the whey protein isolate digestion media as a result of enzymatic hydrolysis. The loss of protein measured by the Bradford assay was correlated to an increase in α -amino concentration measured by the OPA assay resulting from the enzymatic digestion (Vilela *et al.,* 2006).

2.3.2 OPA Assay

The basis of the o-phthaldialdehyde (OPA) assay is the reaction of the α-amino group of the peptide with the reagents OPA and a reducing agent, such as the β-mercaptoethanol, in an alkaline environment. The thio-substituted isoindole adduct produced by this reaction absorbs strongly at 340 nm (Church *et al.,* 1983).

The OPA method is widely employed in monitoring food protein hydrolysis reactions (Nollet, 2004). The assay is a simple and fast spectrometric method at 340 nm that can be performed at ambient temperature, and has good resolution down to a 7 μM concentration of α-amino groups in aqueous media (Lemieux *et al.,* 1990). The reaction of OPA with the primary amines is complete within 0.1 to one minute at room temperature (Shively, 1986) and the peptide content can be linearly related to a peptide or amino acid solution of known concentration; Nielsen and co-workers recommend serine as the standard peptide (Nielsen *et al.,* 2001). One disadvantage of the

OPA assay is the low response of cysteine, lysine, and hydroxylysine and the lack of reaction between OPA with proline and hydroxyproline (Zumwalt & Gehrke, 1998). Furthermore, the absorptivities of α-amino and ε-amino groups are similar, leading to potentially false high readings for peptide solutions rich in lysine, arginine, and glutamine (Church *et al.,* 1983).

Vilela and co-workers also measured the increase in α-amino content in the whey protein isolate digestion media as a result of enzymatic hydrolysis using the OPA method (Vilela *et al.,* 2006). Panasiuk and co-workers monitored the production of hydrolysates from pea proteins using the OPA assay (Panasiuk *et al.,* 1998), while Nielsen and co-workers monitored the production of peptides from sodium-caseinate or soy protein isolate (Nielsen *et al.,* 2001).

2.3.3 Ninhydrin Assay

The ninhydrin assay, like the OPA assay, is based on the production of a spectrometric compound by the reaction between the ninhydrin with the α-amino group, measured at 570 nm, in alkaline media (Lemieux *et al.,* 1990, Panasiuk *et al.,* 1998). Ninhydrin will also react with the ε-amino groups present in the solution, but this adduct absorbs at 440 nm and therefore does not give a false-positive spectrometric reading (Panasiuk *et al.,* 1998). The ninhydrin method is sensitive to a free amino acid concentration of 2 μM, but has the disadvantages of requiring heating and cooling steps and that the ninhydrin reagent will also react with ammonia and environmental oxygen (Turgeon *et al.,* 1991).

Panasiuk and co-workers also measured the concentration of pea protein hydrolysates with the ninhydrin method (Panasiuk et al., 1998), while Lemieux and co-workers determined the α-amino group concentration in cheddar cheese by this method (Lemieux *et al.,* 1990).

2.3.4 TNBS Assay

The 2,4,6-trinitrobenzenesulfonic acid (TNBS) method is performed in a slightly alkaline near pH 8, where the compound produced between TNBS and the α-amino groups is measured spectrometrically near 340 nm after 60 minutes (Lemieux *et al.,* 1990, Panasiuk *et al.,* 1998). The TNBS reagent is light sensitive and does not measure the presence of proline and hydroxyproline (Lemieux *et al.,* 1990). Furthermore, false-positive readings with ammonia and hydroxy ions present in the media (Lemieux *et al.,* 1990, Panasiuk *et al.,* 1998). Finally, TNBS is unstable,

toxic, and the solid TNBS must be handled carefully due to the risk of explosion (Nielsen *et al.,* 2001).

Lemieux and co-workers used the TNBS assay to determine the free amino acid content in cheddar cheese (Lemieux *et al,* 1990). Panasiuk and co-workers monitored the increase in pea protein hydrolysates, although high error was observed by this method when compared to the OPA and ninhydrin methods (Panasiuk *et al.,* 1998). The TNBS assay has lost popularity in recent years due to the increased speed and convenience of the OPA assay (Lemieux *et al.,* 1990).

2.3.5 MALDI-TOF Assay

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry is a separation method based on converting the samples into a mixture of ions, then analyzing these ions based on their mass to charge (m/z) ratio. MALDI describes the method of ionization while TOF describes the mass analyzer employed (Liebler, 2002).

Sample preparation involves mixing the sample with a chemical matrix to help transfer the excited protons from the laser to the proteins or peptides in the sample. The charged molecules are then ejected from the matrix into the gas phase. The ionization process produces positive and negative ions but when working with peptides and proteins, the singly charged positive ions produced when the peptide acquires a single proton are most often the samples of interest. The ions formed in the MALDI source are then directed to the TOF analyzer. The TOF analyzer measures the time elapsed for the ion to travel from entering the analyzer to making contact with the detector; the greater the mass to charge ratio, the faster the ions reach the detector (Liebler, 2002).

MALDI-TOF equipment is capable of producing MS data on very small samples (10^{-15} to 10^{-18} mol), covering a range of molecular masses from a few Daltons up to 20-30 kDa, and is also capable of distinguishing between peptide ions with mass to charge ratio differences of 0.001 amu (Horneffer, Foster, & Velikov, 2007). However, the presence of contaminants such as detergents, buffer salts, metals and organic modifiers can greatly inhibit the ionization of the sample at the MALDI source so removal of the these contaminants is important (Liebler, 2002).

Horneffer and co-workers characterized the sub-units of soy proteins using MALDI-TOF (Horneffer *et al.,* 2007) while Livney and Dalgleish were able to use the method to determine

disulphide bonds were formed during the thermal aggregation of milk proteins (Livney *et al.*, 2004).

2.3.6 HPLC

High-performance liquid chromatography (HPLC) is widely employed in food research to determine the composition of a particular food sample. The basis of the procedure is to fractionate the food sample based on a number of possible properties, such as molecular size, solubility, or charge, using a liquid stream following through a stationary medium (Nollet, 2004).

Size exclusion chromatography high pressure liquid chromatography (SEC-HPLC), also known as gel permeation chromatography, is a technique in which separation according to the hydrodynamic volume of the molecules occurs in a porous, non-adsorbing material with pores of approximately the same size as the target molecules in the solution to be separated (McNaught & Wilkinson, 1997). In SEC, the stationary phase often consists of hydrophilic carbohydrates and acrylamides in an open network formed by the cross-linking of the polymer chains. The swelling of this stationary phase causes openings in the structure while the degree of cross-linking between the polymers determines the size of the holes. Proteins or peptides larger than the swollen gel pores cannot penetrate the gel and therefore elute quickly from the column while smaller molecules will penetrate the open network to varying degrees and elute more slowly (Deyl, 1998).

Reverse phase (RP-HPLC) is used to determine the amino acid composition of a particular sample that has undergone derivatization of the amino acids. The column, typically packed with a silica compound, must be calibrated to the particular detector and eluting liquid to ensure that the various peaks representing the different amino acids can be clearly delineated (Nollet, 2004).

Chen and co-workers used size exclusion chromatography to fractionate soy peptides before purifying the peptides by reverse-phase (RP) chromatography, and finally processing the peptides using a gas-phase sequencer to determine the amino acid sequence (Chen *et al.,* 1995). Vilela and co-workers used ion exchange HPLC to determine the free amino acid content of the whey protein hydrolysates generated by enzymatic digestion (Vilela *et al.,* 2006) while Hernandez-Ledesma used RP-HPLC-MS to determine the amino acid sequence of the 3 kDa fraction of the Corolase digest of bovine milk proteins (Hernandez-Ledesma *et al.,* 2005). More examples of the peptide characterization techniques cited in literature are included in Table 1.

2.4 Membrane Ultrafiltration

Membrane ultrafiltration is a frequently cited technique for pre-processing of hydrolysates with antioxidant properties. Jun and co-workers produced a *Limanda aspera* frame peptide and fractionated it by ultrafiltration using 30, 10, 5, 3, and 1 kDa MWCO membranes (Jun *et al.,* 2004). Hernandez-Ledesma and co-workers fractionated milk hydrolysates by 3 kDa MWCO membrane ultrafiltration (Hernandez-Ledesma *et al.,* 2005) while Vilela and co-workers were studying small peptides and therefore used a 1 kDa MWCO membrane in their ultrafiltration set-up (Vilela et al., 2006). More examples can be found in Table 1.

Ultrafiltration is a membrane separation process where a membrane of a known molecular weight cut-off (MWCO) in the range of 1-500 kDa is used to fractionate a mixture based on difference in molecular mass when a trans-membrane pressure of 0-500 kPa is applied (Blanch *et al.,* 1997, Ghosh, 2003). Transport of the solute through the pores of the membrane is largely due to bulk convection with the solvent while 90% of the molecules larger than the MWCO are rejected by the membrane and remain in the retentate (Ghosh, 2003). Membrane ultrafiltration does not introduce any potentially hazardous chemicals (Chen *et al.,* 2007), is more cost-effective than chromatographic methods (Kinekawa & Kitabatake, 1996), is relatively simple to perform, and can fractionate the peptides into the desired molecular weight cut-off fractions based on the antioxidant peptides identified in literature (Ghosh, 2003, Chen *et al.,* 1995, Gao *et al.,* 2004).

Dead-end ultrafiltration requires a small working volume and when operated with stirring can also be used to ease the scale up to a cross-flow system if desired. The use of pressure as a driving force makes ultrafiltration a relatively gentle separation method, eliminating the higher temperatures that may exist in other separation methods (Belfort, Davis, & Zydney, 1994, Ghosh, 2003).

The general model used in dead-end ultrafiltration is the membrane hydraulic resistance model, also known as Darcy's Law (Belfort *et al.,* 1994, Ghosh 2003):

Equation 1:   Darcy's Law for membrane ultrafiltration

$$J_v = \frac{\Delta P}{\eta_0 R_m}$$

where J_v is permeate flux (permeate flow/unit area), ΔP is the trans-membrane pressure, η_0 is the viscosity of the permeate, and R_m is membrane resistance.

When filtering a solution other than pure solvent, the plot of permeate flux versus time can be divided into two domains. The first domain is attributed to the fouling of the membrane associated with a rapid permeate flux decline; the second domain is the remainder of the curve where the permeate flux slowly declines as a result of concentration polarization and cake formation. This second domain is also sometimes referred to as the "steady-state" domain because the permeate flux appears to have achieved a constant value although it slowly declines to a permeate flux of zero (Das, Saha, & Pugazhenthi, 2009).

One disadvantage of dead-end ultrafiltration performed without stirring is the significant fouling that can result as a result of the accumulation of solute at the membrane surface due to an initially higher rate of convective flux of solute to the membrane than can actually pass through the membrane (Blanch *et al.*, 1997). The accumulation of solute at the membrane surface results in a decrease in the permeate flux; a linear decline in permeate flux is observed in systems with a low concentration of fouling components while exponential decline in the permeate flux is observed in systems with a high concentration of fouling components present in the media to be filtered (Tansel, Bao, & Tansel, 2000, Belfort *et al.,* 1994). Equation 1 can be modified to include a resistance due to the fouling layer, often referred to as the cake (R_c), in series with the resistance due to the membrane (R_m) in the resistance in series model (Belfort *et al.,* 1994, Tansel *et al.*, 2000), as shown in equation 2:

Equation 2:   Resistance in series model for membrane ultrafiltration

$$J_v = \frac{\Delta P}{\eta_0\left(R_m + R_c\right)}$$

Other types of solute deposition or fouling, such as pore constriction and long-term adsorption, may reduce the permeate flux possible by dead-end ultrafiltration (Bowen, Calvo, & Hernandez, 1995). Four empirical models for membrane fouling have been developed by Bowen and co-workers to explain the flux decline experienced in the ultrafiltration of complex mixtures, such as BSA in aqueous saline solutions. These models are summarized in Equations 3-6, where J_P is the permeate flux (m/s) at some time t, J_0 is the permeate flux at time = 0, and k is a constant (Bowen *et al.,* 1995).

Equation 3:   Fouling model for standard blocking of membrane pores (Bowen *et al.,* 1995)

$$J_P = \frac{J_0}{(1+kt)^2}$$

Equation 4:   Fouling model for complete blocking of membrane pores (Bowen *et al.,* 1995)

$$J_P = J_0 \exp(-kt)$$

Equation 5:   Fouling model for intermediate blocking of membrane pores (Bowen *et al.,* 1995)

$$J_P = \frac{J_0}{(1+kt)}$$

Equation 6:   Fouling model for cake filtration (Bowen *et al.,* 1995)

$$J_P = \frac{J_0}{\sqrt{1+kt}}$$

The membrane material has an important role in the filtration process. Organic polymer-based materials are more popular than inorganic polymer materials because they tend to be less expensive, light, flexible and therefore easily shaped, and finally provide a variety of membrane chemistries. The more common organic polymer materials include polysulfone (PS), polyethersulfone (PES), cellulose acetate (CA), regenerated cellulose, polyamides (PA), polyvinylidedefluoride (PVDF), polyacrylonitrile (PAN) (Ghosh, 2003).

The organic membranes used in the ultrafiltration of protein solutions may contribute to fouling because proteins have a tendency to adsorb on different types of surfaces, typically in an irreversible manner (Bowen *et al.,* 1995, Ghosh, 2003). Furthermore, fractionation of protein-protein solutions with an ultrafiltration membrane is known to be difficult and is greatly affected by factors such as solution pH, salt concentration, protein concentration, permeate flux, and membrane properties (Filipe *et al.,* 2005).

2.5 Antioxidant Capacity

Compounds such as polyphenols and carotenoids have been shown to possess beneficial health properties (Kaur *et al.,* 2001), but proteins and peptides also have also been demonstrated to have antioxidant properties as well.

Antioxidants can be classified into two mechanistic groups: the primary antioxidants that reduce the rate of production of new free radicals by sequestering metal ions such as copper or iron and reducing hydroperoxides and the secondary or chain breaking antioxidants that trap free radicals directly thus interrupting the propagation of chain reactions (Wayner, Burton, Ingold, & Locke, 1985, Ou, Hampsch-Woodill, & Prior, 2001). Many assays are based this second mechanistic group, on the hydrogen atom transfer (HAT) reaction mechanism which is most relevant to human biology, while others are based on electron transfer (ET) mechanism which is also sometimes referred to as reducing potential, to trap or neutralize the synthetic free radicals. Still other antioxidant assays measure the ability of the antioxidant to suppress one particular reactive oxygen or nitrogen species such as peroxyl, superoxide, or peroxynitrite, or ability to act in a specific medium such as to prevent the peroxidation of linoleic acid (Huang, Ou, & Prior, 2005).

Because antioxidants act by so many different mechanisms, there is no single, definitive assay for measuring antioxidant capacity. Therefore, the First International Congress on Antioxidant Methods was held in 2004 and standardized testing procedures to measure the antioxidant capacity of food, nutraceutical, and dietary supplements were discussed. As reported by Prior and co-workers, the two standard methods are the oxygen radical absorbance capacity (ORAC) and the Folin-Ciocalteu reagent (FCR) assays. The ORAC assay was selected because it represents the hydrogen atom transfer (HAT) reaction mechanism and the FCR assay was selected as the electron transfer (ET) or reducing capacity assay (Prior *et al.,* 2005).

In addition to the ORAC assay, there are several other HAT mechanism assays that appear in literature, such as the total radical-trapping antioxidant parameter (TRAP) and β-carotene bleaching assays. Similarly for the ET mechanism assays, other options beyond the FCR assay, such as the Trolox equivalence antioxidant capacity (TEAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and copper(II) reducing capacity assays, can be found in the literature. Some of these other assays are included in Table 1.

2.5.1 FCR

The FCR method was originally developed by Folin and Ciocalteu as a method to quantify the amounts of tyrosine and tryptophan present in a protein sample (Folin & Ciocalteu, 1927). This method is commonly known as the total phenols assay due to its application in measuring the phenolic content of wines. However, this assay actually measures the reducing ability of a hydrophilic sample because the FCR is non-specific to phenolic compounds (Huang *et al.,* 2005).

The exact mechanism is unclear, but the current understanding is that the yellow Mo(IV) present in the solution is reduced to blue Mo(V), which can be measured through changes in absorbance near 750 nm (Huang *et al.,* 2005, Ainsworth & Gillespie, 2007). Under the basic test conditions (pH 10) of the assay, both phenolic and non-phenolic components can be measured; sodium carbonate is preferred to sodium hydroxide because the latter has been observed to form a haze in certain situations (MacDonald-Wicks *et al.,* 2006, Box, 1983). Colour development will fully occur between 1 and 6 hours at 20ºC although warmer temperatures can be used to speed the reaction to completion (Singleton, Orthofer, & Lamuela-Raventos, 1999). Gallic acid and α-catechin are typical standard antioxidants used in this assay, (Singleton *et al.,* 1999, Stevanato, Fabris, & Momo, 2004, Ainsworth *et al.*, 2007) although more recent versions have used Trolox as the standard antioxidant (Zielinska, Frias, Piskula, Kozlowska, Zielinski, & Vidal-Valverde, 2008).

2.5.2 ORAC

The ORAC (oxygen radical absorbance capacity) assay was originally designed by Cao and co-workers to measure the hydrogen-donating properties of serum. The assay employs the fluorescent properties of either phycoerythrin, a water-soluble pigment-containing protein with an affinity for reactive oxygen species, or the synthetic non-protein fluorescein (3',6'-dihydroxyspiro[isobenzofuran-1[3H],9'[9H]-xanthen]-3-one), both of which have distinct excitation and emission wavelengths and high fluorescence yield as the probe (Cao, Alessio, & Cutler, 1993, Ou *et al.,* 2001).

The free radical reaction is initiated with hydrophilic AAPH (2,2'-azobis[2-amidinopropane] dihydrochloride) as the peroxyl radical generator and loss of fluorescence is used to measure oxidative damage to the probe. The antioxidant capacity is determined from the difference in the area beneath the plot of time versus the fluorescent decay curve both with and without the antioxidant, also known as the area under the curve (AUC) method (Cao *et al.,* 1993, Ou *et al.,* 2001). The delay in the fluorescence decline is a direct result of the hydrogen-donating ability of the antioxidant (Gillespie, Chae, & Ainsworth, 2007).

The change in the area under the curve resulting from the addition of the test antioxidant is compared to that produced by Trolox, the standard antioxidant typically employed in this assay (Gillespie *et al.,* 2007). This assay combines both the inhibition time and inhibition percentage or degree of free radical action by antioxidants, because this reaction is driven to completion and not terminated after a set amount of time, unlike many other antioxidant assays such as the FCR (Kaur

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et al., 2001, Davalos, Gomez-Cordoves, & Bartolome, 2004b). A lipophilic version of the ORAC assay has also been developed (Prior *et al.,* 2003).

2.5.3 Antioxidant Peptides

Based on the superior antioxidant capacities and compositions of certain peptides described in literature, the behaviour of certain amino acid residues can be hypothesized. The peptides which have a good antioxidant capacity are typically between three and 20 amino acid residues in length and contain at least one non-polar residue. The disruption of the tertiary structure and cleaving of the protein chain to reveal the antioxidant amino acid sequence must also be considered. The amine bond appears to have a role in the antioxidant properties of the peptides because a mixture of amino acids in the same ratio as the peptide have been repeatedly demonstrated to have a much lower antioxidant capacity when compared to the peptide (Wu, Shiau, Chen, & Chiou, 2003).

The presence of a ring structure in the peptide, such as the phenolic ring in tyrosine or the imidazole ring in histidine, is also quite common and has been theorized to help stabilize the structure of the peptide once the peptide has neutralized a free radical (Elias *et al.,* 2008, Saito *et al.,* 2003, Chan *et al.,* 1994). Hernandez-Ledesma and co-workers credit the phenolic and indolic groups present tryptophan and tyrosine residues with the radical scavenging activity observed in milk hydrolysates (Hernandez-Ledesma *et al.,* 2005, Hernandez-Ledesma *et al.,* 2007). The phenolic ring in the tyrosine amino acid residue is a potent hydrogen donor and has been credited with contributing to the antioxidant potential of peptides derived from tuna cooking juices and other animal sources (Jao *et al.,* 2002, Jun *et al.,* 2004, Wayner *et al.,* 1987).

The imidazole ring in histidine has been credited with metal chelation properties exhibited by some peptides (Wu *et al.,* 2003). This ring structure has also been credited with contributing to the antioxidant ability of soy peptides due to the presence of a hydrophobic group at the end of a peptide chain (Chen *et al.*, 1995).

The presence of sulphur in the cysteine and methionine residues has also been proposed to be significant in the ability of a peptide to act as an antioxidant (Elias *et al.,* 2008).

More antioxidant proteins and peptides found in literature are presented in Table 1.

Table 1: Selection of Antioxidant Peptides from Literature

Protein Source	Processing	Peptide Sequence
Yellowfin Sole frame protein *(Limanda aspera)* (Jun *et al.*, 2004)	• Enzymatic digestion with MICE (Mackerel Intestine Crude Enzyme) followed by pepsin (conditions not provided) • 5 kDa UF fraction, HPLC gel permeation chromatography • Linoleic acid autoxidation	• Arg-Pro-Asp-Phe-Asp-Leu-Glu-Pro-Pro-Tyr
Bovine whey proteins α-lactabumin and β-lactoglobulin (Hernandez-Ledesma *et al.*, 2005)	• Enzymatic digestion with Corolase PP • 3 kDa UF permeate, then RP-HPLC-MS • ORAC (fluorescein)	• Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile • Met-His-Ile-Arg-Leu • Tyr-Val-Glu-Glu-Leu
Tuna cooking juices (Jao *et al.*, 2002)	• Digestion with protease XXIII from *A. oryzae* (37°C, 0-6h, 0.5% protease solution) • Gel filtration chromatography, HPLC • DPPH	• Pro-His-His-Ala-Asp-Ser • Val-Asp-His-Asp-His-Pro-Glu • Pro-Ala-Gly-Tyr • Val-Asp-Tyr-Pro • Pro-Lys-Ala-Val-His-Glu • Ser-His-Asp-Ala-His-Pro-Glu • Pro-Ser-His-Asp-Ala-His-Pro-Glu
Chicken egg white (Davalos *et al.*, 2004a)	• Digestion with pepsin (3h) • 3 kDa UF permeate, RP-HPLC • ORAC (fluorescein), induced oxidation of human LDL	• Tyr-Ala-Glu-Glu-Arg-Tyr-Pro-Ile-Leu
SPI from Protein Technology International (Gao *et al.*, 2004)	• Digestion with Alcalase or Corolase (50ºC, 3h) • Peptides appear to be from the G1, G2, or G3 subunit of glycinin, G4 or G5 precursor of glycinin • Aqueous hydrolysate is extracted with organic such as acetone • ABTS, β-carotene bleaching, linoleic acid peroxidation, and superoxide ion quenching	• Gly-Val-Ala-Trp, • Gly-Val-Ala-Trp-Trp, • Gly-Val-Ala-Trp-Trp-Met, • Gly-Val-Ala-Trp-Trp-Met-Tyr, • Trp-Trp-Met-Tyr, • Ser-Trp-Trp-Pro-Phe, • Gly-Val-Ala-Trp-Trp-Met*, • Gly-Val-Ala-Trp-Trp-Met*-Tyr, • Trp-Thr-Tyr where Met* refers to methionine sulfoxide (N-terminus is on the left)

Protein Source	Processing	Peptide Sequence
Rice bran protein (Adebiyi, Adebiyi, Yamashita, Ogawa, & Muramoto, 2009)	• Enzymatic digestion with pepsin (2h, other conditions not provided) • HPLC purification • ABTS antioxidant assay • Sequenced by MALDI-TOF	• Tyr-Leu-Ala-Gly-Met-Asn
Rice endosperm protein (Zhang *et al.,* 2009)	• Enzymatic digestion with Neutrase (protein 5% (w/v), 35ºC, 4 h, pH 7) • HPLC purification • DPPH activity • Sequenced by MALDI-TOF	• Lys-His-Asn-Arg-Gly-Asp-Glu-Phe
Soybean (Lv, Liu, Bao, Tang, Yang, & Guo, 2009)	• Enzymatic digestion with protease M (protein at 20 g/L, E:S = 1:100, 50ºC, pH 3, 60 min), centrifuged, then with deamidase (supernatant, E:S = 1:50, 50ºC, pH 7, 180 min) • UF fraction 10-30 kDa • Iron-chelating • Sequenced by MALDI-TOF	• Leu-Met-Asn-Leu-Ala-Ile-Arg-Cys-Arg-Leu-Gly-Pro-Met-Ile-Gly-Cys-Asp-Leu-Ser-Ser-Asp-Asp • Asp-Asn-Gln-Ser-Glu-Gln-Leu-Glu-Gly-Lys-Glu-Lys-Lys
Skeletal muscle (Chan *et al.,* 1994, Wu *et al.,* 2003)	• Comparison of muscle tissue extracts from beef, chicken, trout, pork, turkey, buffalo, cat, crab, dolphin, dog, donkey, frog, giant oyster, sheep, Siberian salmon, snake, sturgeon, squid, wallaby and blue whale	• Anserine (β -alanyl-L-1-methylhistidine) • Carnosine (β -alanyl-L-histidine) • Ophinine (β -alanyl-L-3-methylhistidine) • Taurine (2-aminoethanesulfonic acid) • Antioxidant enzymes (catalase, glutathione peroxidase, superoxide dismutase)

3 Materials and Methods

3.1 Equipment

The equipment used in this study is summarized in Table 2.

Table 2: Equipment

Equipment Name	Supplier	Product Code
Water bath	New Brunswick Scientific, USA	G-76
Timer	VWR, Canada	62344-585
pH meter	Cole Parmer, USA	Chemcadet, 5652-00
Thermometer (-20 – 100ºC)	VWR, Canada	61066-104
Freezer (-20ºC)	Wood's	Model E25P, type PA6AG1
Stopwatch	VWR, Canada	62344-898
Spectrophotometer (340 nm)	Varian, USA	Cary 1 Bio UV-Visible Spectrophotometer EL97043216
Ultrafiltration Cell (200mL)	Millipore, USA	Amicon 8200, prod# 5123
1kDa MWCO UF membrane (regenerated cellulose)	Millipore, USA	1000 NMWL YM1, cat# 13332
3kDa MWCO UF membrane (regenerated cellulose)	Millipore, USA	3000 NMWL PLBC, cat# PLBC 06210
Magnetic stir plate	VWR, Canada	Dyla-Dual 12620-970
Regulator	Prostar, USA	Platinum (0-60 psi)
Pressure gauge (0-60 psi)	Solfrunt, USA	33003
Scale	AE Adam, USA	AEP 2500g ± 10mg
Desktop PC	JKL Micro Distribution Inc, Canada	Intel Celeron Processor 768 MHz, 250 MB ram Windows XP v2002
Data collection program	National Instruments, USA	Labview 2004 "Adam Scale.vi"
Petri dishes, disposable	VWR, Canada	Polystyrene, 25384-302
Refrigerator (4ºC)	White Westinghouse, USA	Model R139SRW-1
Centrifuge tube (50mL)	VWR, Canada	21008-240
Centrifuge tube (15mL)	VWR, Canada	21008-214
1.5 mL acrylic (PMMA) disposable cuvettes	VWR, Canada	97000-590
4.5 mL (OPS) disposable cuvettes	VWR, Canada	58017-880
Cuvette caps	VWR, Canada	89000-628
Gyrotory Shaker	New Brunswick Scientific, USA	G2
Freeze-dryer (biology)	Thermo Savant, USA	Super Modulyo
Freeze-dryer (chemistry)	Labconco, USA	Freezezone 4.5

3.2 Chemicals and Materials

The chemicals and materials used in this study are summarized in Table 3.

Table 3: Chemicals and Materials

Material / Chemical Name	Supplier	Product Code
Soy Protein Isolate (SPI)	Archer Daniels Midland Company (ADM), USA	PRO-FAM 974 Product Code: 066974 Batch: 07021911
Pepsin, from porcine stomach mucosa	Sigma-Aldrich, Canada	P7012-5g
Hydrochloric acid	Fisher Scientific, Canada	A144P212-2.5L
Sodium hydroxide pellets	EMD Serono, Canada	SX0600-3 (2.5 kg)
Pancreatin, from porcine pancreas	Sigma-Aldrich, Canada	P1625-100g
Sodium phosphate, dibasic	Sigma-Aldrich, Canada	S5136-100g
Sodium carbonate, monohydrate	Sigma-Aldrich, Canada	S4132-500g
MilliQ water (>17MΩ)	Barnstead / Thermolyne, USA	NANOpure ultrapure water system D4754
o-phthaldialdehyde, 99% HPLC	Sigma-Aldrich, Canada	P0657-5g
2-mercaptoethanol	Sigma-Aldrich, Canada	M7154-500mL
Ethanol (99.9%)	UW Chem Stores	Ethanol Absolute
Sodium borate decahydrate, ACS Reagent	Sigma-Aldrich, Canada	S9640-500g
Sodium dodecyl sulphate	Sigma-Aldrich, Canada	L4509-100g
L-(+)-alpha-phenylglycine	MP Biomedicals, USA (obtained through VWR)	151834 (5g)
Tergazyme	Alconox, USA	Tergazyme Enzyme-Active Powdered Detergent
Nitrogen	Praxair, Canada	Nitrogen, compressed, 1066
(Trolox) 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, 97%	Sigma-Aldrich, Canada	238813-1g
MES Solution (1M)	Sigma-Aldrich, Canada	M1317-500 mL
(DPPH) 2,2-Diphenyl-1-picryl-hydrazyl	Sigma-Aldrich, Canada	D9132-1g
L-ascorbic acid, 99%	Sigma-Aldrich, Canada	A92902-100g
(FCR) Folin Ciocalteu's Phenol Reagent 2N	Sigma-Aldrich, Canada	F9252-100mL

3.3 Methods

3.3.1 Stock Solutions

Any stock solutions requiring special preparation or storage are detailed here. In the case of aqueous stock solutions not listed here, all were prepared with MilliQ water and stored in a closed glass bottle at room temperature.

0.1M Na_2HPO_4 buffer solution:

2.215 g Na_2HPO_4 was dissolved in 500 mL MilliQ water and mixed until fully dissolved. The pH of the buffer solution was adjusted to 7.8 with 1 M HCl. The buffer was stored in a capped bottle at room temperature.

1M PheGly calibration solution:

0.015 g PheGly (L-(+)-alpha-phenylglycine) was fully dissolved in 100 mL MilliQ water. The solution was then decanted into 1.5 mL centrifuge tubes and stored at -20ºC until needed.

Trolox solution

A 5 mM Trolox solution was prepared by dissolving 0.0250 g Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) in 20 mL ethanol. The solution was decanted into 1.5 mL centrifuge tubes and kept frozen at -20ºC until used, up to 30 days.

3.3.2 Enzymatic Digestion

The enzymatic digestion procedures detailed here were developed from the work of Vilela and co-workers (Vilela *et al.*, 2006). The conditions used for each set of experiments are summarized in Table 4.

Table 4:   Experimental Digestion Conditions

Experiment		Pepsin Digestion	Pancreatin Digestion
Preliminary Digestions	Pepsin	0.0375 g pepsin in 7.5 mL 0.01 M HCl Digestion time: 30 min (E:S = 1:125 w/w)	N/A
	Pancreatin	N/A	0.1500 g pancreatin in 30 mL 0.1 M pH 7.8 phosphate buffer Digestion time: 60 min (E:S = 1:30 w/w)
	Pepsin & Pancreatin	0.0375 g pepsin in 7.5 mL 0.01 M HCl Digestion time: 30 min	0.1500 g pancreatin in 30 mL 0.1 M pH 7.8 phosphate buffer Digestion time: 60 min
Factorial Design *	High	0.0468 g pepsin in 7.5 mL 0.01 M HCl (E:S = 1:100 w/w) Digestion time: 45 min	0.4680 g pancreatin in 30 mL 0.1 M pH 7.8 phosphate buffer (E:S = 1:10 w/w) Digestion time: 90 min
	Centre	0.0351 g pepsin in 7.5 mL 0.01 M HCl (E:S = 1:133 w/w) Digestion time: 30 min	0.2808 g pancreatin in 30 mL 0.1 M pH 7.8 phosphate buffer (E:S = 1:17 w/w) Digestion time: 60 min
	Low	0.0234 g pepsin in 7.5 mL 0.01 M HCl (E:S = 1:200 w/w) Digestion time: 15 min	0.0936 g pancreatin in 30 mL 0.1 M pH 7.8 phosphate buffer (E:S = 1:50 w/w) Digestion time: 30 min
Extensions	Extra Pancreatin	N/A	0.8450 g pancreatin in 30 mL 0.1 M pH 7.8 phosphate buffer (E:S = 1:5 w/w) Digestion time: 90 min
	Midpoint	0.0312 g pepsin in 7.5 mL 0.01 M HCl (E:S = 1:150 w/w) Digestion time: 30 min	0.1560 g pancreatin in 30 mL 0.1 M pH 7.8 phosphate buffer (E:S = 1:30 w/w) Digestion time: 60 min

* Factorial design digestion conditions described by Bissegger (Bissegger, 2008)

<u>Pepsin Digestion</u>

1. A pepsin solution of desired concentration was prepared by combining dried, frozen pepsin with room temperature 0.01 M HCl. The solution was stored at 4°C until needed.

2. A 3.12 wt% SPI solution in MilliQ water was prepared (4.68 g of SPI in 150 mL MilliQ water). Approximately 10 mL of the same solution was placed in a 15 mL centrifuge tube to be used as a control sample.

3. The pH of the SPI solution was adjusted to 1.5 with 10 M HCl. 150 mL of this SPI solution was then poured into a 500 mL Erlenmeyer flask.

4. Both the SPI sample and the control solution were placed in the water bath to warm to the desired temperature of 37°C.

5. The pepsin solution was removed from refrigerator and allowed to warm to room temperature while being stirred on the magnetic stir plate with a stir bar.

6. 10 μL samples were removed from the digestion vessel(s) and the control for use in the OPA assay.

7. The pepsin solution was added to the digestion vessel(s) according to the recipe, the timer was started, and the water bath shaker was started at shaker speed 4.

8. The OPA assay was performed on the samples at pepsin time = 0.

9. At the next sampling time, the shaker was stopped and the digestion vessel(s) were removed from the water bath. The digestion solution was mixed well using a magnetic stir plate and the 10 μL sample(s) for the OPA assay were removed. The control solution was also mixed and sampled. The digestion vessel(s) were returned to the water bath and the shaker was restarted. The OPA assay was then performed on the samples. This step was repeated as many times as desired throughout the digestion.

10. At the completion of the pepsin digest time, the digestion vessel(s) and control were removed from the water bath, placed on the magnetic stirrer and the 10 μL sample(s) for the OPA assay was removed.

   - If the digestion was complete at this point, 10 M NaOH was added to the digestion vessel to raise the solution pH to 7. The digest solution was decanted into centrifuge tubes and placed in the freezer (-20ºC) as soon as possible.

   - If the pepsin digestion was being followed with a pancreatin digestion, the process began at step 2 of the pancreatin digestion method.

Pancreatin Digestion

1. A 3.12 wt% SPI solution in MilliQ water was prepared (4.68 g of SPI in 150 mL MilliQ water) and placed in a 500 mL Erlenmeyer flask. Approximately 10 mL of the same solution was placed in a 15 mL centrifuge tube to be used as a control sample.

2. A pancreatin solution of desired concentration was prepared by combining dried, frozen pancreatin with room temperature 0.1 M Na_2HPO_4 buffer solution and mixing well. The pancreatin solution was stored at 4°C until needed.

3. The pH of the SPI solution was adjusted to 7.8 with 10 M NaOH.

4. Both the SPI sample and the control solution were placed in the water bath to warm to the desired temperature of 40°C.

5. The pancreatin was removed from the refrigerator and allowed to warm to room temperature while stirring.

6. 10 μL samples were removed from the digestion vessel(s) and the control for use in the OPA assay.

7. Pancreatin was added to the digestion vessel(s) according to the recipe, the timer was started and the water bath shaker was started at speed 4.

8. The OPA assay was performed on samples at pancreatin time = 0.

9. At the next sampling time, the shaker was turned off and the digestion vessel(s) and control were removed from the water bath. The solutions were well mixed using a magnetic stir plate and the 10 μL samples for the OPA assay were removed. The procedure was repeated for the control solution. The digestion vessel(s) were returned to the water bath and the shaker was restarted. The OPA assay was then performed on the samples. This step was repeated as many times as desired throughout the digestion.

10. At the completion of the pancreatin digest time, the digestion vessel(s) and control were removed from the water bath. The digestion solution was mixed well using a magnetic stir plate and the 10 μL samples for the OPA assay were removed.

11. 7 μL of 150 mM sodium carbonate solution for every 1 mL of starting SPI solution was added to each digestion vessel and the solutions were mixed well using a magnetic stir plate. The digest solution was decanted into centrifuge tubes and placed in the freezer (-20ºC) as soon as possible.

3.3.3 Bradford Assay

The Bradford assay to measure protein concentration in the digest samples was performed at the University of McGill in February 2007 according to the procedure described by Vilela and co-workers (Vilela *et al.,* 2006). The Bradford assay was also used by Bissegger to estimate the SPI solubility (Bissegger, 2008).

3.3.4 OPA Assay

The o-phthaldialdehyde (OPA) assay was developed from that described by Vilela and co-workers and Church and co-workers (Vilela *et al.,* 2006, Church *et al.,* 1983).

Method – 100 mL OPA solution:

1. 20 mL of an aqueous 10% w/w SDS solution and 50 mL of a 100 mM sodium borate solution were combined in a 100 mL volumetric flask.

2. 0.08 g of OPA was dissolved in 2 mL ethanol to make a 300 mM solution. Once dissolved, this solution was added to the volumetric flask.

3. 200 μL β-mercaptoethanol was added to the 100 mL volumetric flask.

4. The volumetric flask was then filled with MilliQ water to the 100 mL line.

5. The solution was transferred to a brown glass jar and the solution pH was adjusted to 9.0 with 1 M NaOH.

Method - OPA calibration curve:

1. 3 mL (two 1.5 mL centrifuge tubes) of the 1 M PheGly solution were thawed at room temperature.

2. A dilution series spanning 0 to 4000 µM PheGly was prepared.

3. 100 µL of each PheGly dilution was aliquoted into a 1.5 mL PMMA cuvette, to which 1 mL of OPA solution was added.

4. The solution was left to sit at room temperature for two minutes then transferred to the spectrophotometer to measure the absorbance at 340 nm.

5. The calibration curve was prepared to ensure linearity and good functioning of the equipment. A sample calibration curve is located in Appendix A.

Method – OPA sample testing:

1. A 10 µL sample was removed from the digestion vessel or control and transferred to the cuvette.

2. 1 mL of OPA reagent was added to the sample, mixed, and transferred to the spectrophotometer to be read after two minutes at 340 nm.

3. The results were inspected to ensure that they fell within the calibration range. If the absorbance for a particular sample fell outside of the calibration range, the solution was diluted up to ten times with MilliQ water and the OPA assay was performed again.

3.3.5 MALDI-TOF

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis to determine the molecular mass distribution of the resulting peptides with a spectrum of 500-4000 Da and 5-20 kDa was performed at the University of Guelph. The samples were desalted by the University of Guelph staff to obtain better peak resolution (Charcoglycan, 2008, Bissegger, 2008).

3.3.6 SEC-HPLC

The molecular weight distribution of selected protein hydrolysates was performed at Université Laval by gel permeation chromatography (GPC) on a Superdex Peptide HR 10/300 GL column (GE Healthcare, Baie d'Urfe, QC, Canada) with an exclusion limit of $1x10^5$ Da (13 μm, 10 x 300-310 mm) using a high-pressure liquid chromatography system (HPLC Waters, Mississauga, ON, Canada) (Beaulieu, Thibodeau, Bryl, & Charbonneau, 2008).

3.3.7 Membrane Ultrafiltration

Sequential ultrafiltration was performed in a dead-end system, first with a 3 kDa membrane and followed by a 1 kDa membrane, where both membranes were comprised of regenerated cellulose. The experimental conditions used in each set of experiments are summarized in Table 5; all ultrafiltrations were performed at 25˚C.

New Membrane Preparation Method

1. The new membrane was removed from its package and placed glossy-side down in a beaker containing 100-200 mL of MilliQ water at room temperature, according to the manufacturer's instructions (Millipore, no date).
2. The water was changed a minimum of three times during a one-hour period to remove the glycerin and sodium azide applied to the membrane surface by the manufacturer.
3. The membrane was removed from the beaker and rinsed with MilliQ water before being used.

General Ultrafiltration Method

1. The Amicon 8200 Ultrafiltration cell was assembled according to the manufacturer's instructions (Millipore, no date), either with or without the stirring arm, with the prepared membrane. The permeate line was clamped shut and directed into a covered collection vessel on the balance. The balance was connected to the personal computer running the Labview data acquisition software (2004, "Adam scale.vi"), as illustrated in Figure 2 and shown in Figure 3.
2. The solution to be filtered was poured into the ultrafiltration cell, the mass of the feed solution was recorded, and the lid with the nitrogen line attached was installed on the cell. The solution to be filtered was the digest solution, without any change in the pH, ionic strength, or water content. The ultrafiltration cell was then placed in the cell holder.

3. If a stirred cell were used, the ultrafiltration cell and holder were placed atop the magnetic stir plate and the stirrer was set to the desired speed. If no stirring were used, this step was skipped.
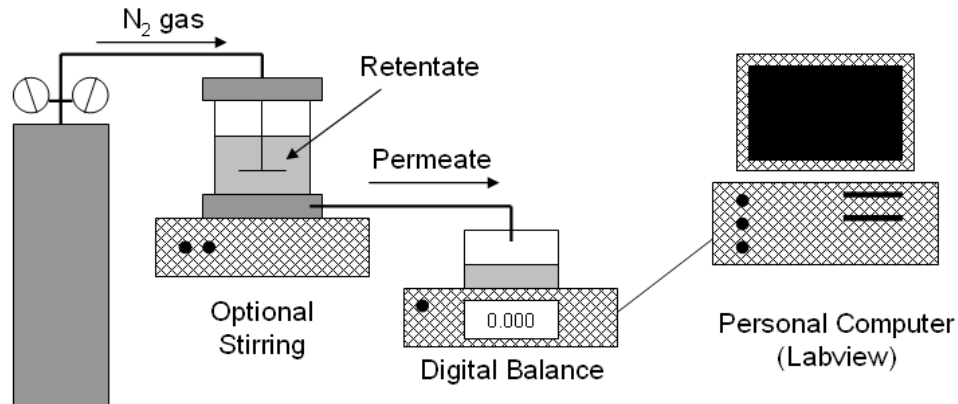
Figure 2: Experimental set-up for ultrafiltration experiments
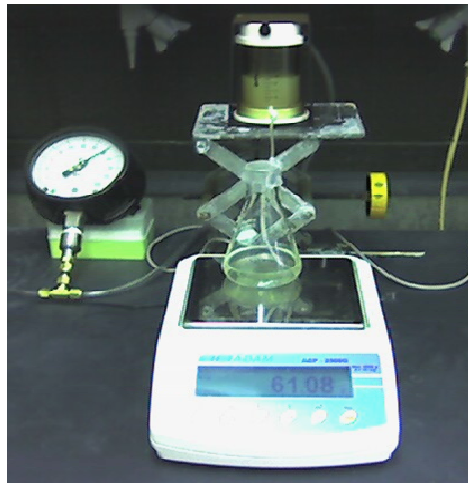
Figure 3: A portion of the actual ultrafiltration set-up, without stirring

4. The nitrogen cylinder was opened and the regulator was adjusted to deliver the desired pressure to the ultrafiltration cell.
5. The Labview data acquisition software was started to acquire the mass of permeate data over the duration of the experiment and the clamp on the permeate line was released. Samples of the permeate were not removed at different times throughout the process. The ultrafiltration process occurred at 25ºC.

6. Once the experiment was completed, the permeate line was clamped shut, the Labview program was stopped, and the final mass of permeate was recorded. The nitrogen cylinder was shut and the system was depressurized.

7. The ultrafiltration cell was removed from its holder and the lid with the attached nitrogen hose was removed from the cell.

8. The retentate in the ultrafiltration cell was weighed, transferred to capped centrifuge tubes, and placed in the freezer at -20ºC. Similarly, the permeate was either transferred to centrifuge tubes and placed in the freezer at -20ºC or it was saved for the next stage of ultrafiltration to be performed later the same day and stored in the refrigerator at 4ºC. The mass of the contents of the centrifuge tube was recorded.

9. The ultrafiltration cell was disassembled. The used ultrafiltration membrane was rinsed with MilliQ water to remove any visible residue, placed in disposable Petri dish in a 10% ethanol solution at room temperature, and the Petri dish was placed in the refrigerator at 4ºC, according to the manufacturer's instructions (Millipore, no date).

10. The remaining components of the ultrafiltration system were washed in a 1% Tergazyme solution, were rinsed by a series of tap water, deionized water, and finally MilliQ water before air drying.

Membrane Cleaning Method

1. The membranes to be cleaned were removed from the refrigerator and allowed to warm gradually to 25ºC.

2. According to the manufacturer's instructions, each membrane was rinsed with MilliQ water and was placed in a beaker with a 0.5% Tergazyme solution at 25ºC (Millipore, no date).

3. The beakers containing the membranes were covered and placed on the gyratory mixer for one hour.

4. At the end of the hour, the membranes were rinsed a minimum of three times with MilliQ water and either used immediately in an ultrafiltration experiment or placed in 10% ethanol solution in a clean Petri dish until used.

Water Flux

Prior to performing any experiment, the water flux of the membrane to be used was tested. Simply, the procedure described above was followed except that the solution to be filtered was MilliQ water. The water flux was calculated over a range of pressures between 20 psi and 50 psi to ensure a linear relationship, as illustrated in Appendix A. If the water flux was not linear, the membrane

was discarded and a new membrane was prepared. The experimental ultrafiltration conditions employed in this work are summarized in Table 5.

Table 5:   Experimental Ultrafiltration Conditions

Experiment	Ultrafiltration Conditions	
	3 kDa MWCO	**1 kDa MWCO**

Preliminary	Pressure: 40 psi
	With stirring
	Feed solution: 150 mL
	Target permeate mass:
	    Pepsin = 100 g
	    Pancreatin = 120 g
	    Pepsin & Pancreatin = 80 g
		Pressure: 40 psi
		With stirring
		Feed solution: 70 mL
		Permeate mass: 40 g

Factorial Design and Extensions	Pressure: 40 psi No stirring Feed solution: 150 mL Minimum run time: 800 min* Minimum permeate mass: 60 g*	Pressure: 40 psi No stirring Feed solution: 40 mL Minimum run time: 90 min* Minimum permeate mass: 20 g*
	* both minimum conditions needed to be met to stop the filtration. Due to the variation in permeate flux, the filtration runs had different run times	
	High = 1000 min Centre = 1000 min Low = 1200 min No Pepsin = 1200 min Extra Pancreatin = 800 min Midpoint = 1000 min	High = 180 min Centre = 90 min Low = 150 min No Pepsin = 150 min Extra Pancreatin = 150 min Midpoint = 90 min

3.3.8 Freeze-drying of Samples and Determination of Mass Fractions

1. Samples to be freeze-dried were placed in the -20ºC freezer for a minimum of 12 hours to ensure that they were frozen solid.

2. The "wet" mass of the samples was taken.

3. The samples were covered and further chilled with liquid nitrogen.

4. The chilled samples were placed in the glass pressure vessels and connected to the freeze-dryer until all moisture had been removed, approximately 72 hours. The freeze-dryer used was dependent on the equipment availability – either the Labconco or the Thermo-Savant unit.

5. The freeze-dryer was shut off and the samples were removed. The "dry" mass of the samples was recorded.

6. The dried samples were weighed, capped, and stored at room temperature.

7. The "dry" masses of the peptide samples were used to estimate the mass fraction of the peptides that permeated, were retained by the membrane ultrafiltration, or were lost. The feed to each stage of the fractionation process was considered as 100%.

3.3.9 DPPH Antioxidant Assay

The following solutions were prepared on the day of the experiment, according to the procedure described by Wang and co-workers (Wang *et al.,* 2003).

400 μM DPPH Solution Method

1. 0.0079 g DPPH was dissolved in 25 mL ethanol in a brown glass jar wrapped in tinfoil. The solution was mixed for approximately five minutes, until all of the DPPH had dissolved.

2. 20 mL MilliQ water and 5 mL 1 M MES buffer were added to the brown glass jar and the solution was mixed for approximately ten minutes.

3. The pH was adjusted to 6.0 with 10 M NaOH.

DPPH Assay Method

1. The following samples were prepared:

    Blank solution: in a 4.5 mL OPS cuvette, 1200 μL 0.2 M MES buffer and 1200 μL ethanol were combined. A cap was applied to the cuvette.

    Sample solution: in a 4.5 mL OPS cuvette, 300 μL 0.2 mM MES solution, 300 μL sample dissolved in MilliQ water, and 600 μL ethanol were combined.

    Control solution: 1200 μL ethanol was placed in a 4.5 mL OPS cuvette.

2. 1200 μL DPPH solution was added to the Sample and Control solutions. The cuvettes were capped and shaken lightly to mix the contents.

3. The cuvettes were transferred to the spectrometer to read the absorbance at 520 nm every minute for the desired period of time.

Calibration samples were run at the start and at the end of each day's experiments, as a minimum. Known antioxidants ascorbic acid and/or Trolox were used as calibration antioxidants and were substituted for the "sample" in the above "Sample solution" procedure. For ascorbic acid, calibration samples of 0.1, 0.2, 0.3, and 0.4 mM ascorbic acid in MilliQ water were prepared. For Trolox, calibration samples of 0.1, 0.2, 0.25, and 0.3 mM Trolox were prepared from the stock Trolox solution and MilliQ water. A sample calibration curve can be found in Appendix A.

3.3.10 FCR Antioxidant Assay

This assay was produced from the publications by Stevanato and co-workers, Singleton and co-workers, and Box. The Folin-Ciocalteu Reagent (FCR) assay is used to measure the electron-donating or reducing capacity of the hydrophilic antioxidant samples under investigation (Box, 1983, Stevanato *et al.,* 2004, Singleton *et al.,*1999).

FCR Assay Method

1. 20 μL sample was placed in a 4.5 mL cuvette and 150 μL Folin-Ciocalteu Reagent was added to the cuvette.

2. Five minutes was allowed to elapse.

3. 600 μL of a 15 wt% Na_2CO_3 solution was added to the cuvette.

4. 2230 μL of MilliQ water was added to the cuvette, bringing the total volume in the cuvette to 3 mL. The cuvette was capped and shaken lightly to mix the contents.

5. The cuvette was let sit for approximately two hours in a covered container, at room temperature.

6. After 120 minutes had elapsed from the addition of the FCR to the sample in step 1, the absorbance was read at 750 nm.

FCR Calibration Curve Method

A calibration curve was prepared by replacing the 20 μL sample with 20 μL of the appropriate dilution of Trolox; 1, 1.5, 2, 2.5 or 3 mM Trolox was prepared from the stock Trolox solution and MilliQ water. A sample calibration curve can be found in Appendix A.

3.3.11 ORAC Antioxidant Assay

The hydrophilic ORAC assay with AAPH as the peroxyl radical generator, as developed from publications by Prior and co-workers, Ou and co-workers, and Huang and co-workers was performed at the Agriculture and Agri-Food research facility in Summerland, BC (Prior *et al.,* 2003, Ou *et al.,* 2001, Huang, Ou, Hampsch-Woodill, Flanagan, & Deemer, 2002). The antioxidant capacity was expressed in terms of mg Trolox/g sample.

3.3.12 Statistical Analysis

A factorial design at two levels was performed and analyzed according to the methods described in Box, Hunter, and Hunter (Box, Hunter, & Hunter, 1978). Additional tests performed were added to the factorial design data to supplement the model, as described in Montgomery (Montgomery, 2005). Significance testing was performed using analysis of variance (ANOVA) and Tukey's paired comparison testing (Walpole, Myers, & Myers, 1998, Montgomery, 2005). Calculations were performed using Excel 2003 (Microsoft, US) and the statistical analysis was verified with Statistica (StatSoft, US).

4   Preliminary Investigations

4.1 SPI Characterization

ADM PRO-FAM SPIs were selected as the raw material for this research because they are commercially-available soy protein isolates (SPI) that have been designed and approved for use as a food additive. Therefore, they are both readily available and should be of a consistent quality when compared to a protein isolate that could be prepared in the laboratory. PRO-FAM 974 was used as the raw ingredient in all digests performed here, with the exception of PRO-FAM 892 that was used when determining whether to monitor the digestion progress using the Bradford or the OPA assay, as described in Section 4.2.1.

To gain an understanding of the properties of the raw SPI, data was gathered from a number of sources. The first source for information was the supplier, ADM, who provided a standard analysis for the raw material, ADM PRO-FAM 974. The solubility of this SPI was also determined experimentally over a range of pH.

4.1.1 ADM PRO-FAM

The PRO-FAM 974 soy protein isolate contains a minimum of 90% protein (Archer Daniels Midland, 2007). As part of the processing, the SPI is alcohol washed and contains minimal isoflavones at 0.4 mg total isoflavones/g protein (Chang, 2007). Isoflavones have been shown to be potent antioxidants (Shih, Yang, & Kuo, 2002) and therefore a low concentration of isoflavones is desirable in the SPI to be used in this study. The amino acid profile of the SPI as provided by ADM is summarized in Table 6. As expected for soy proteins, the SPI contains little of the essential sulphur-containing amino acid methionine and cystine/cysteine (Sathe, 2002).

Table 6: Typical Amino Acid Composition of PRO-FAM 974 (ADM, 2007)

Amino Acid	Typical g/100 g SPI
Aspartic Acid	11.5
Threonine	3.7
Serine	5.5
Glutamic Acid	19.2
Proline	5.2
Glycine	4.1
Alanine	4.3
Cystine	1.2
Valine	4.8
Methionine	1.4
Isoleucine	4.8
Leucine	8.0
Tyrosine	3.8
Phenylalanine	5.2
Histidine	2.7
Lysine	6.3
Arginine	7.5
Tryptophan	1.1

4.1.2 Solubility

Bissegger determined the solubility of the SPI PRO-FAM 974 at pH 1.5, 3, 5, 7, 9 and 11 in an aqueous solution by measuring the protein concentration of the solution using the Bradford assay (Bissegger, 2008). 100% solubility was defined as 31.2 mg SPI/1 mL MilliQ water corresponding to the initial amount of SPI added to the solution. The solubility data summarized in Table 7 indicates that the isoelectric point, defined as the point where the protein has a neutral charge and is therefore least soluble in water occurred near a pH of 5 (Sathe, 2002). According to the conditions tested, the maximum solubility is achieved near pH 11 at a solubility of 47.0%, equivalent to 14.7 mg SPI/mL MilliQ water (Bissegger, 2008).

Table 7: PRO-FAM 974 SPI solubility in MilliQ water between pH 1.5 and 11 (mean ± SD, n=3) (Bissegger, 2008)

Solution pH	% Solubility
1.5	37.0 ± 3.3
3	16.0 ± 2.1
5	0.0 ± 0.2
7	39.5 ± 1.4
9	45.7 ± 2.5
11	47.0 ± 1.6

The solubility of the SPI in sodium phosphate buffer and sodium chloride salt solutions was also tested. The addition of salts was found to decrease the solubility of the SPI when compared to water alone, with the use of a phosphate buffer and sodium chloride resulting in the lowest solubility for the SPI (Bissegger, 2008).

4.2   Preliminary Digestions

When performing enzymatic digestion, several factors including the enzymes to be employed and the conditions of pH, temperature, digestion time, and concentration of both the enzyme and the substrate to produce peptides must be considered (Kunst, 2000). Initial digestions of the SPI with pepsin and pancreatin were performed according to the procedure of Vilela and co-workers described in Chapter 3 (Vilela *et al.,* 2006).

4.2.1   Preliminary Digestion

As described by Vilela and co-workers, the SPI was combined with MilliQ water at 3 mg SPI/mL water. The pepsin solution was prepared at 5 mg pepsin/mL in 0.01 M HCl giving an enzyme to substrate ratio of 1:125 w/w and the digestion was performed for 30 minutes at 37ºC in a solution initially at pH 1.5. Similarly, the pancreatin solution was prepared at 5 mg/mL in sodium phosphate buffer adjusted to pH 7.8, resulting in an enzyme to substrate ratio of 1:30 w/w and the digestion proceeded at 40ºC for 60 minutes. To stop the digestion, 150 mM sodium carbonate solution was added and the digests were rapidly chilled on ice and stored at -20ºC.

The first digestion was performed at McGill University and compared the use of the Bradford assay with the OPA assay as the method to monitor the progression of a digestion with ADM PRO-FAM 892, which is very similar to the PRO-FAM 974 which was unavailable at the time (ADM, 2007). Briefly, the OPA assay measures the increase in peptide concentration in equivalents of phenyl-glycine dipeptide concentration while the Bradford assay monitors the decrease in protein concentration equivalent to BSA concentration. The results are summarized in Table 8 and indicate that the Bradford assay cannot distinguish between the peptide concentrations at any time during the digestion. Therefore the OPA assay was selected for monitoring the progression of future enzymatic digestions.

Table 8: Pepsin and Pancreatin Digestion of ADM PRO-FAM 892 SPI Monitored by the Bradford and OPA Assays (mean ± SD, n=3 Bradford, n=5 OPA)

Digestion Time (minutes)	OPA Assay (equivalent mM PheGly)	Bradford Assay (equivalent mg/mL BSA)
0 (add pepsin)	0.227 ± 0.006	0.018 ± 0.008
15	0.299 ± 0.017	0.000 ± 0.005
30 (pH to 7.8, add pancreatin)	0.309 ± 0.039	-0.003 ± 0.003
45	0.494 ± 0.065	0.029 ± 0.005
60	0.549 ± 0.015	0.019 ± 0.007
90	0.591 ± 0.030	0.019 ± 0.007

This initial testing was performed on digestions in 10 mL volumes in 15 mL capped centrifuge tubes. The digestion procedure was then scaled-up to be performed with 150 mL of SPI solution in a 300 mL Erlenmeyer flask. All digestion conditions other than the volume were kept constant with no measurable impact on the digestion results.

4.2.2 Individual Enzyme Effects

Next, the SPI (PRO-FAM 974) was digested with pepsin or pancreatin to study the effects of each enzyme to produce peptides. All digestions were performed in triplicate with solution volumes of 150 mL using either pepsin or pancreatin, as reported by Bissegger (Bissegger, 2008). The change in peptide concentration as a function of time is presented in Table 9. While all digestions were performed, a control solution was also placed in the water bath to measure the change in peptide concentration as a function of temperature; the peptide concentration throughout the experiments remained constant, measured as 14 mM PheGly equivalents.

Table 9: Peptide Concentration as a Function of Time as Monitored by the OPA Assay for ADM PRO-FAM 974 Digested with Pepsin and/or Pancreatin (mean ± SD, n=3, ND = not determined)

Digestion Time (minutes)	Peptide Concentration in equivalent mM PheGly		
	Pepsin	Pancreatin	Pepsin & Pancreatin
0	14.41 ± 1.48	15.30 ± 0.54	14.22 ± 0.31
15	21.40 ± 0.81	16.59 ± 1.05	20.91 ± 0.43
30	22.85 ± 0.87	18.73 ± 0.24	* 22.20 ± 0.77
45	ND	ND	24.76 ± 0.41
60	ND	23.18 ± 0.77	27.32 ± 1.10
90	ND	ND	31.93 ± 0.73

* pH adjusted to 7.8 and pancreatin added

A rapid initial increase in peptide concentration followed by a leveling off near 30 minutes can be seen in the Pepsin digest. The digestion with pancreatin, both with and without the pepsin, shows a

linear increase in the resulting peptide concentration and does not show any signs of decrease by the end of the 60 minute digestion time. Interestingly, after 30 minutes for the Pepsin digest and 60 minutes for the Pancreatin digest, a similar peptide concentration quantified as 23 mM PheGly was achieved. However, when the enzymes were applied sequentially in the Pepsin & Pancreatin digest, a peptide concentration of approximately 32 mM PheGly was achieved.

4.3   Fractionation by Ultrafiltration

The three preliminary digestions (Pepsin, Pancreatin, and Pepsin & Pancreatin) were fractionated by dead-end ultrafiltration with a 3 kDa MWCO membrane according to the procedure described in Section 3.3.7. The permeate was collected in one vessel for the duration of the 3 kDa filtration. The permeate collected was then further fractionated by dead-end ultrafiltration with a 1 kDa MWCO membrane. Both filtrations were performed under a constant, nitrogen-induced pressure of approximately 210 kPa (40 psi) and with stirring. The magnetic stir-plate was adjusted manually to agitate the feed solution without creating a significant vortex; no measurements were taken to quantify the stirring speed in RPM. The permeate flux for each filtration is presented in Figure 4 and summarized in Table 10.

Table 10:   Steady-state Flux for the 3 kDa and 1 kDa MWCO Ultrafiltration of the Pepsin, Pancreatin, and Pepsin & Pancreatin Digests under 210 kPa (40 psi) Nitrogen Pressure with Stirring (n=1)

3 kDa MWCO ultrafiltration		1 kDa MWCO ultrafiltration	
Feed solution	**Flux at 210 minutes (L/m^{2}h)**	**Feed solution**	**Flux at 50 minutes (L/m^{2}h)**
Pepsin	4.40	Pepsin Only	15.51
Pancreatin	10.35	Pancreatin Only	11.80
Pepsin & Pancreatin	6.56	Pepsin & Pancreatin	13.88

The flux versus time profiles in Figure 4 resemble the expected profile for dead-end ultrafiltration experiencing cake filtration, the permeate flux measured is initially high and declines at a decreasing rate until it reaches the lower steady-state flux value (Das *et al.,* 2009). Some of the variability in the data presented in Figure 4 can be attributed to adjustment of the stirrer speed to avoid vortex formation.
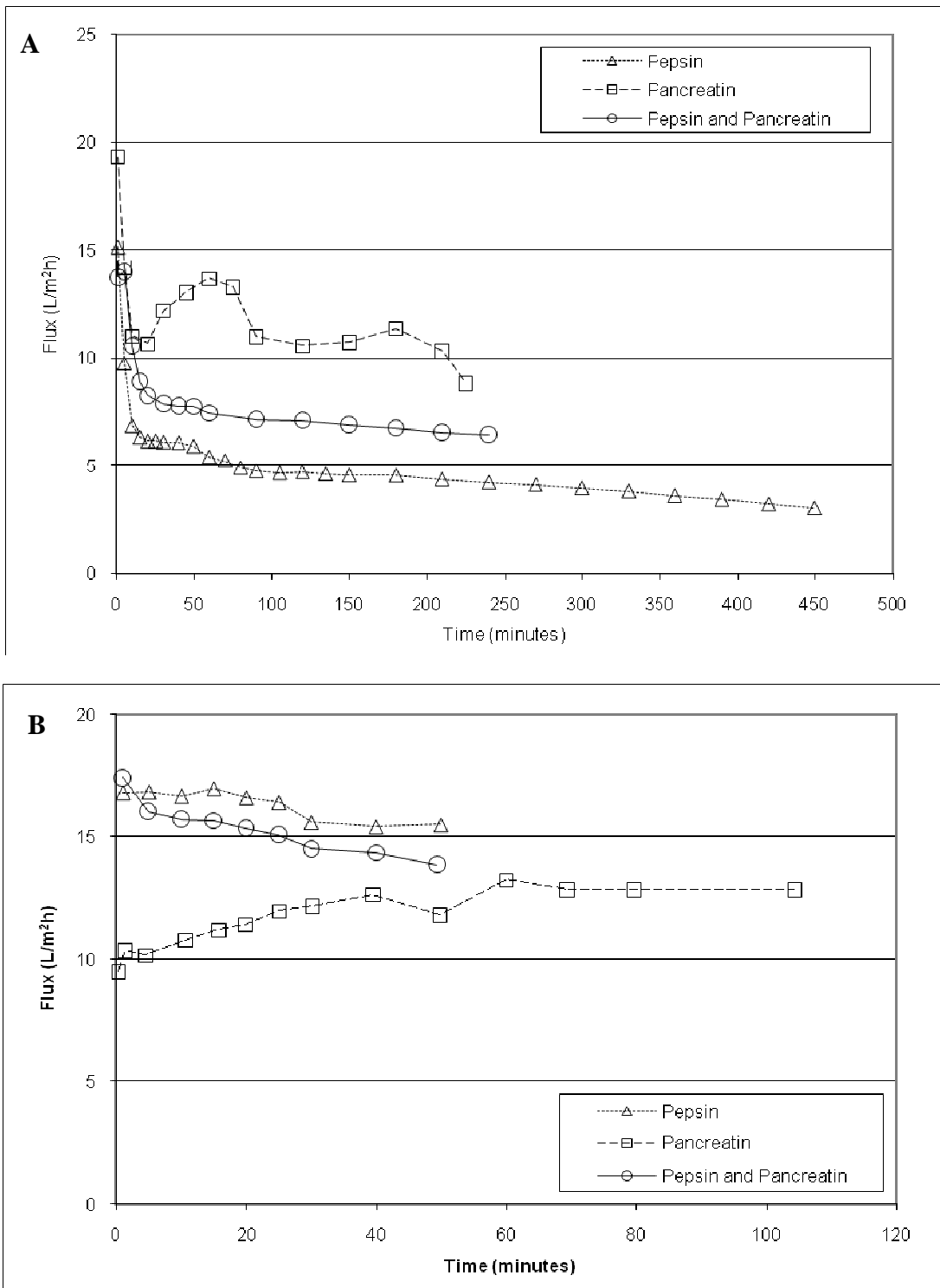
Figure 4: Time-flux Relationships for the 3 kDa (A) and 1 kDa (B) MWCO Ultrafiltration of the Pepsin, Pancreatin, and Pepsin & Pancreatin Digests under 210 kPa (40 psi) Nitrogen Pressure with Stirring (n=1)

The 3 kDa ultrafiltration data gathered suggests that the Pancreatin digest has the most desirable permeate flux at the relatively high value of 10 L/m^{2}h but the flux profile over time suggests very unusual behaviour which should be investigated further. The two remaining digests have lower permeate fluxes between 4 and 7 L/m^{2}h. The 1 kDa ultrafiltration data shows that all three digests appear to reach a constant permeate flux between 12 and 16 L/m^{2}h after approximately one hour, although the Pancreatin digest again shows unexpected behaviour with increasing permeate flux over time.

Samples of the permeate and retentate taken at the end of the filtration procedure for all digests were freeze-dried and stored at room temperature until antioxidant tests were performed.

4.4   Peptide Characterization

Four samples of freeze-dried digests were analyzed by MALDI-TOF at the University of Guelph to gain information about the molecular weights of the samples. The four samples tested were the raw ADM PRO-FAM 974 SPI, the Pepsin digest, the Pancreatin digest, and Pepsin & Pancreatin digest.

The MALDI-TOF analysis was performed over two different molecular mass spectra, 500 to 4000 Da and 5 to 20 kDa, to determine the size of peptides produced by digestion with each enzyme. Unfortunately, the range studied does not provide information on the make-up of the soy protein isolate because the majority of the glycinin and conglycinin subunits have molecular masses greater than 20 kDa (Horneffer *et al.,* 2007).

Figure 5 is the MALDI-TOF spectrum for the undigested PRO-FAM 974 SPI. Figure 5A has a significant peak at a molecular mass of 650 Da that does not correspond to known soy protein subunit but may instead be a peptide present in the SPI resulting from the processing conditions employed. Another significant peak is observed at a molecular mass near 9000 Da in Figure 5B, which may represent the acidic A5 subunit of glycinin (Horneffer *et al.,* 2007).

A comparison of Figure 5B, the 5-20 kDa spectrum for the undigested SPI, with Figure 6B, the 5-20 kDa spectrum for the Pepsin digest reveals a significant increase in the number of polypeptides in the range of 4-10 kDa. This suggests that the enzyme pepsin has cleaved a number of bonds and increased the number of peptides in the sample. Horneffer and co-workers indicated the presence of a fraction near 8900 Da similar to that observed in Figure 5B, which became more visible under

reducing conditions employed in their study, is likely the A5 acidic subunit of glycinin (Horneffer *et al.,* 2007). The peak near 650 Da is present in both Figures 5A and 6A, suggesting that this molecule is unaffected by the digestion with pepsin.
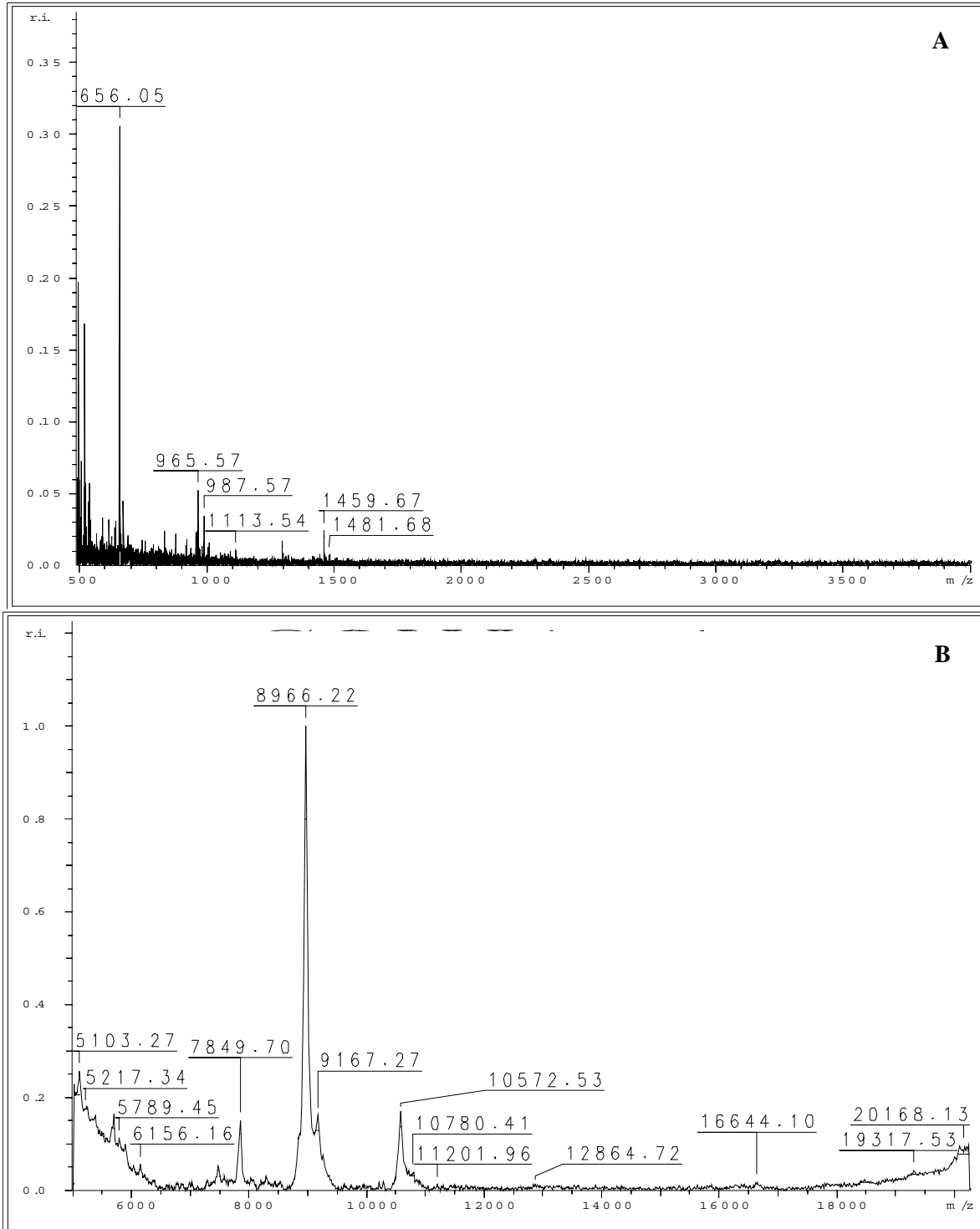
Figure 5:   MALDI-TOF for the PRO-FAM 974 SPI; A: 500-4000 Da, B: 5-20 kDa
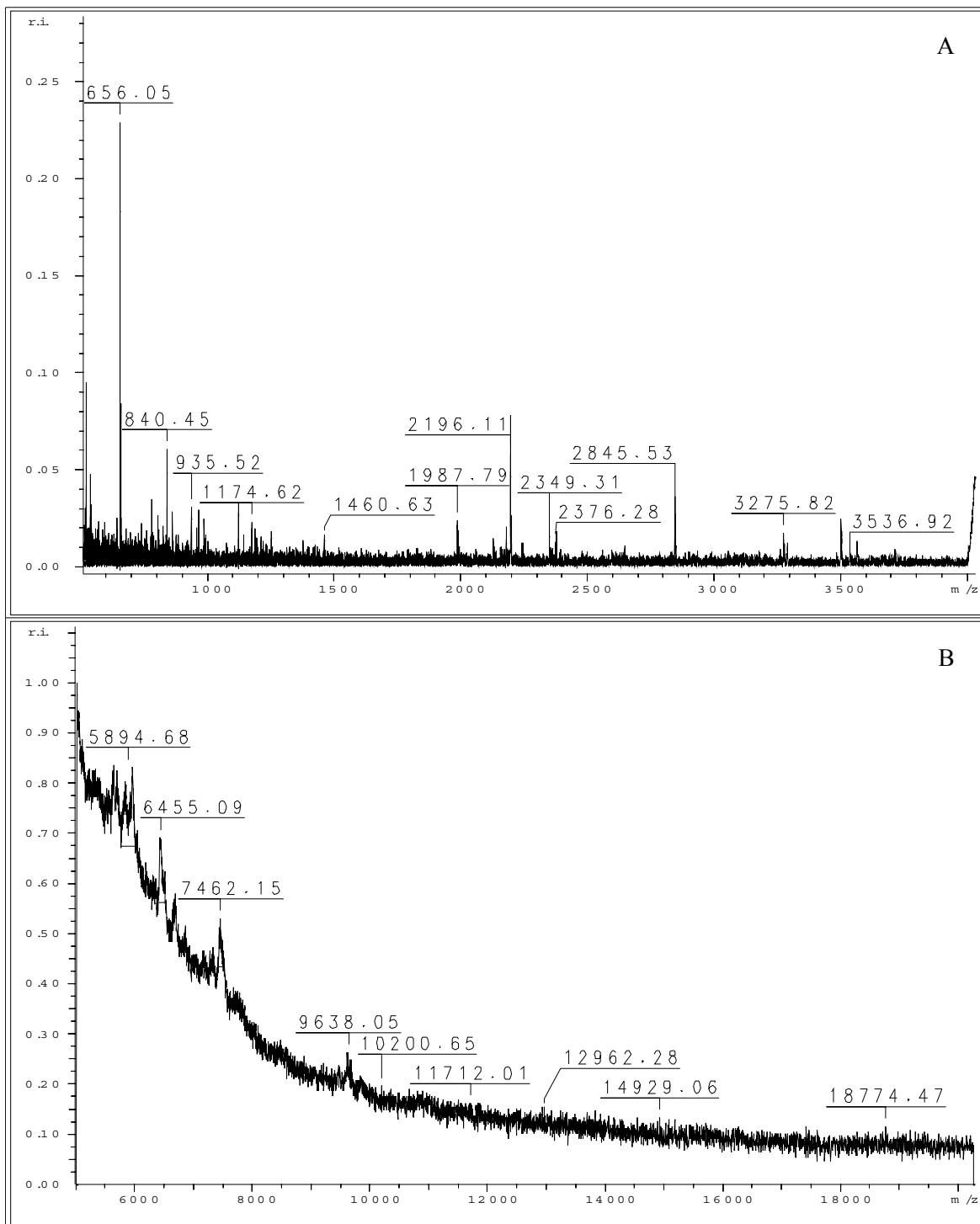
Figure 6: MALDI-TOF for the Pepsin Digest; A: 500-4000 Da, B: 5-20 kDa

Similarly, Figure 7 for the Pancreatin digest shows an increase in the amount of peptides between 4 and 10 kDa when compared to the MALDI-TOF spectrum for the undigested SPI. However, the profile between the two enzymatic digestions is different with the highest peak at 5600 Da and a secondary peak near 8100 Da for the Pancreatin digest, while the Pepsin digest shows a peak at 5900 Da. Figure 7A also has a peak near 650 Da, similar to Figures 5A and 6A.

Figure 8, the MALDI-TOF spectrum for the Pepsin & Pancreatin digest, resembles a smoothed pancreatin digest with the peaks near 5600 and 8100 Da dominating in Figure 8B. Furthermore, the peak near 650 Da which has been present in all other spectra is overshadowed by peaks near 520, 1230, and 1620 Da, as illustrated by Figure 8A. Therefore, a higher concentration of low molecular weight peptides and polypeptides were produced from the combination of enzymes in the Pepsin & Pancreatin digest when compared to the peptides resulting from the digests where pepsin and pancreatin were applied individually.
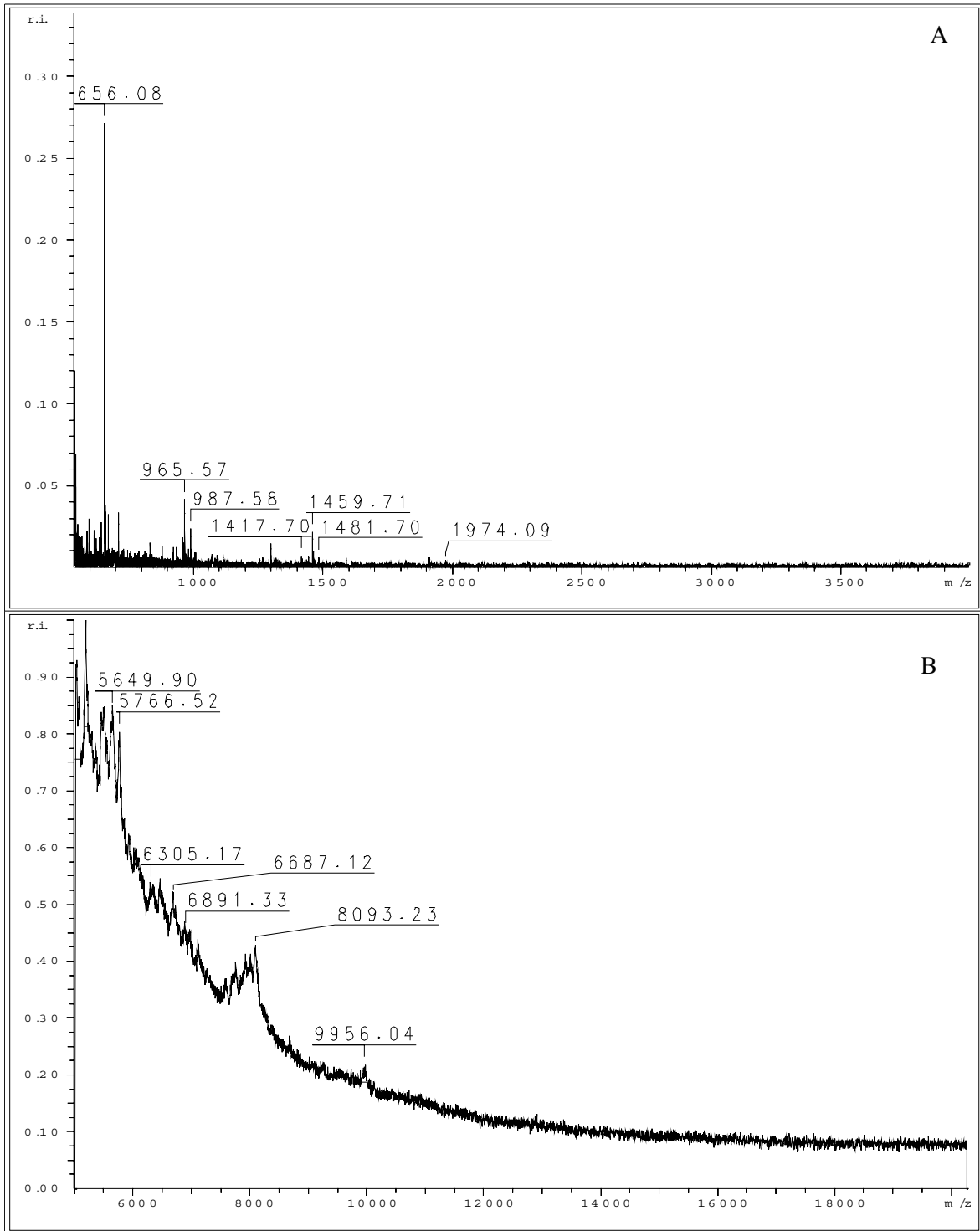
Figure 7: MALDI-TOF for the Pancreatin Digest; A: 500-4000 Da, B: 5-20 kDa
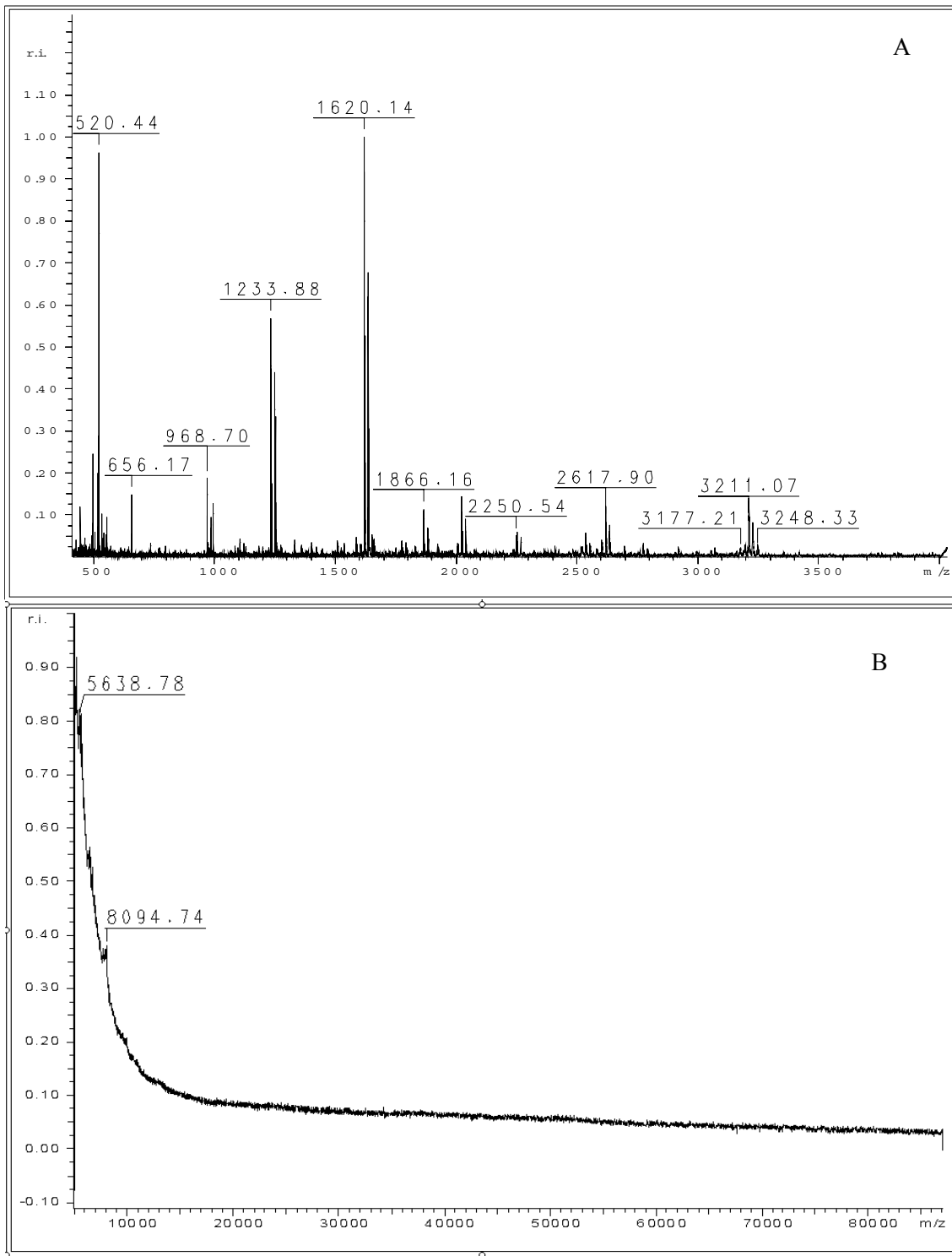
Figure 8: MALDI-TOF for the Pepsin & Pancreatin Digest; A: 500-4000 Da, B: 10-80 kDa

4.5 Antioxidant Capacity

The antioxidant capacity of the fractions generated by membrane ultrafiltration of the Pepsin, Pancreatin, and Pepsin & Pancreatin digests were tested in the DPPH, FCR, and ORAC assays. The data generated is quantified in terms of standard antioxidants for each assay.

4.5.1 DPPH Assay

The DPPH (α,α-diphenyl-β-picrylhydrazyl) assay was used to measure the antioxidant capacity of the fractions generated by membrane ultrafiltration of the Pepsin, Pancreatin, and Pepsin & Pancreatin digests to donate an electron to neutralize the purple-coloured free radical. The DPPH antioxidant capacities are summarized in Table 11 and are expressed in terms of g ascorbic acid/g sample.

Table 11: DPPH Antioxidant Capacity for the Pepsin, Pancreatin, and Pepsin & Pancreatin Digests, Expressed as Equivalents of Ascorbic Acid (n=2* and n=3, mean ± SD)

Digest	Ultrafiltration Fraction	mg ascorbic acid/g sample
Pepsin	3kDa Permeate	0.53 ± 0.25 * [a]
	1kDa Retentate	0.67 ± 0.15 [a]
	1kDa Permeate	0.71 ± 0.36 [a]
Pancreatin	3kDa Permeate	0.77 ± 0.13 [a]
	1kDa Permeate	0.11 ± 0.58 * [a]
Pepsin & Pancreatin	3kDa Permeate	0.56 ± 0.26 * [a]
	1kDa Retentate	0.34 ± 0.40 [a]
	1kDa Permeate	0.23 ± 0.98 [a]

Values with the same letter are not significantly different (P = 0.05)

Unfortunately, this assay was not performed on the 1 kDa retentate of the Pancreatin digest due to insufficient sample and therefore the results do not appear here. Furthermore, the turbidity that resulted between the DPPH assay solution and the aqueous digests made certain samples, such as the 3 kDa retentate solutions and undigested SPI, prohibited testing by the DPPH assay. The antioxidant capacities of the 3 kDa permeate, 1 kDa retentate, and 1 kDa permeate fractions of the three digests were not found to be significantly different from one another when compared by ANOVA at a 95% confidence level.

4.5.2 FCR Assay

The ability of the fractions generated by membrane ultrafiltration of the Pepsin, Pancreatin, and Pepsin & Pancreatin digests to donate an electron to reduce the Folin-Ciocalteu Reagent (FCR) in

an aqueous solution at pH 10 assay was measured. This antioxidant capacity was quantified in terms of equivalent mg of Trolox and summarized in Table 12.

Table 12: FCR Antioxidant Capacity for the Pepsin, Pancreatin, and Pepsin & Pancreatin Digests, Expressed as Equivalents of Trolox (n=3, mean ± SD; ND = not determined)

Digest	Ultrafiltration Fraction	mg Trolox/g Fraction	mg Trolox/ Fraction
Pepsin	3kDa Retentate	65.0 ± 5.2 [c]	151.6 ± 13.4
	3kDa Permeate	54.3 ± 3.0 [b]	86.3 ± 4.8
	1kDa Retentate	71.5 ± 3.6 [c]	32.9 ± 1.7
	1kDa Permeate	25.3 ± 2.1 [a]	11.4 ± 0.9
Pancreatin	3kDa Retentate	134.3 ± 4.2 [f]	471.4 ± 14.7
	3kDa Permeate	141.3 ± 33.9 [f,g,h]	185.1 ± 44.4
	1kDa Retentate	186.7 ± 12.8 [g]	ND
	1kDa Permeate	154.0 ± 5.9 [h]	160.2 ± 6.1
Pepsin & Pancreatin	3kDa Retentate	63.9 ± 0.8 [c]	ND
	3kDa Permeate	93.8 ± 1.3 [e]	ND
	1kDa Retentate	81.1 ± 3.9 [d]	ND
	1kDa Permeate	90.7 ± 2.7 [e]	ND

Values with the same letter are not significantly different (P = 0.05)

Unlike the DPPH assay, the sensitivity of the FCR assay was much greater and differences between both the enzymatic digestion conditions and ultrafiltration fractions were observed. The Pancreatin digest fractions consistently had a higher electron donating antioxidant capacity than both the Pepsin and the Pepsin & Pancreatin digests. Analysis of variance performed at a 95% confidence level revealed that the antioxidant capacities of certain fractions within a given digest were distinguishable, with the retentate fractions typically having better electron donating capacities compared to the permeate fractions of the same digests. The 1 kDa retentate fraction of the Pancreatin digest was significantly better at reducing the FCR when compared to all other digests and most other ultrafiltration fractions.

4.5.3 ORAC Assay

The ability of the fractions generated by membrane ultrafiltration of the Pepsin, Pancreatin, and Pepsin & Pancreatin digests to donate a proton to neutralize the peroxyl radical generator AAPH in the ORAC (oxygen radical absorbance capacity) in a hydrophilic solution was measured at the Agriculture and Agri-Food research facility. This antioxidant capacity of the digest fractions was quantified in terms of equivalent mg of Trolox in Table 13.

Table 13: ORAC Antioxidant Capacity for the Pepsin, Pancreatin, and Pepsin & Pancreatin Digests, Expressed as Equivalents of Trolox (n=3, mean ± SD, ND = not determined, N/A = not applicable)

Digest	Ultrafiltration Fraction	mg Trolox/g Fraction	mg Trolox/Fraction
PRO-FAM 974 (SPI)	N/A	14.1 ± 2.2 [a]	N/A
Pepsin (enzyme)	N/A	76.0 ± 2.4 [a]	N/A
Pancreatin (enzyme)	N/A	55.7 ± 2.7 [a]	N/A
Pepsin	1kDa Permeate	38.4 ± 3.8 [a]	17.3± 1.7
	1kDa Retentate	87.8 ± 3.8 [a, b]	40.4 ± 1.7
	3kDa Permeate	65.1 ± 4.9 [a]	103.5 ± 7.8
	3kDa Retentate	47.4 ± 0.4 [a]	111.4 ± 0.9
Pancreatin	1kDa Permeate	182.9 ± 20.2 [b]	190.2 ± 21.0
	1kDa Retentate	145.7 ± 4.5 [b]	ND
	3kDa Permeate	138.1 ± 13.0 [b]	180.9 ± 17.0
	3kDa Retentate	67.1 ± 2.0 [a]	235.5 ± 7.0
Pepsin & Pancreatin	1kDa Permeate	104.0 ± 7.9 [a, b]	ND
	1kDa Retentate	108.9 ± 3.9 [a, b]	ND
	3kDa Permeate	101.7 ± 5.6 [a, b]	ND
	3kDa Retentate	99.5 ± 12.1 [a, b]	ND

Values with the same letter are not significantly different (P = 0.05)

Analysis of variance was performed on the data at the 95% confidence level with Tukey's paired comparison testing to determine the significant differences in the antioxidant capacity in mg Trolox/g sample. The PRO-FAM 974 SPI was found to have a significantly lower antioxidant capacity when compared to the 3 kDa retentate, 3 kDa permeate, and 1 kDa retentate of the Pancreatin digest. The 1 kDa retentate of the Pancreatin digest had a statistically significantly higher antioxidant capacity compared to the 1 kDa permeate of the Pepsin digest. The 1 kDa permeate of the Pancreatin digest had a significantly higher antioxidant capacity when compared to the 1 kDa permeate, 3 kDa retentate, and 3 kDa permeate fractions of the Pepsin digest. The 3 kDa retentate of the Pancreatin digest has a significantly higher antioxidant capacity when compared to the 1 kDa permeate fraction of the same digest. The ORAC antioxidant capacity of all fractions of the Pepsin & Pancreatin digest were found to be statistically similar and could not be distinguished from any other ultrafiltration fraction or digest condition. These antioxidant capacity values suggest that not only the digest but also the ultrafiltration fraction of that digest contribute to the antioxidant capacity.

5   Digestion Condition Factorial Design

5.1 Digestions

As indicated in Chapter 4, the pepsin and pancreatin each appear to have a specific role in the production of antioxidant peptides, both in terms of the concentration of peptides produced and also the antioxidant capacity of those peptides. To study the effect of the enzymes, a 2^4 factorial design experiment was performed by Bissegger (Bissegger, 2008), varying the pepsin concentration (A), pancreatin concentration (B), pepsin digestion time (C), and pancreatin digestion time (D). Digestion conditions are summarized in Table 14.

Table 14:   High, Low, and Centre Point Conditions Employed in the Factorial Design Digests; Natural Values to the Left With the Coded Values to the Right (adapted from Bissegger, 2008)

Condition	Factor A: Pepsin Concentration		Factor B: Pancreatin Concentration		Factor C: Pepsin Time		Factor D: Pancreatin Time	
Low	0.149 g/L	-1	0.5 g/L	-1	15 minutes	-1	60 minutes	-1
Centre	0.223 g/L	0	1.5 g/L	0	30 minutes	0	90 minutes	0
High	0.297 g/L	+1	2.5 g/L	+1	45 minutes	+1	120 minutes	+1

The values for the high and low levels were selected by Bissegger; the low and high levels for the pancreatin to SPI ratio were 1:50 and 1:10 w/w respectively while the low and high levels for the pepsin to SPI ratio were 1:200 and 1:100 w/w respectively (Bissegger, 2008). The centre point enzyme concentrations were determined using the method described in Montgomery given by equation 7:

Equation 7:   Centre point calculation for factorial design (Montgomery, 2005)

$$x = \frac{value - (value_{low} + value_{high})/2}{(value_{high} - value_{low})/2}$$

A starting solution of 4.68 g SPI in 150 mL MilliQ water was used for all digests while pepsin and pancreatin were added according to the digestion methodology described in Chapter 3. Peptide concentration was measured at various points during the digestion while the peptide concentration measured at the end of the digestion was recorded as the final peptide concentration. The final peptide concentrations are summarized in Table 15, where the digestion levels are expressed in terms of the coded variables.

Table 15: Final Peptide Concentration for the Factorial Design Digests in Coded Variables (adapted from Bissegger, 2008)

Run Number	Factor A (Pepsin Concentration)	Factor B (Pancreatin Concentration)	Factor C (Pepsin Time)	Factor D (Pancreatin Time)	Peptide Concentration (mM PheGly equivalents)
1	+1	+1	+1	+1	48.14
2	-1	+1	+1	-1	47.00
3	+1	+1	-1	+1	47.28
4	-1	+1	-1	-1	45.50
5	-1	+1	+1	+1	48.47
6	-1	+1	-1	+1	46.76
7	-1	-1	+1	+1	36.75
8	-1	-1	-1	-1	27.49
9	0	0	0	0	41.62
10	0	0	0	0	38.00
11	0	0	0	0	42.60
12	+1	-1	+1	+1	33.61
13	+1	-1	+1	-1	30.41
14	+1	+1	+1	-1	45.85
15	-1	-1	+1	-1	32.08
16	+1	-1	-1	-1	25.91
17	-1	-1	-1	+1	30.97
18	+1	-1	-1	+1	34.74
19	+1	+1	-1	-1	44.37

The Centre digest was run in triplicate to give a measure of the standard error present in the digestion protocol (Walpole *et al*., 1998). The standard error for the digestion protocol was calculated to be 1.40 mM PheGly equivalents about a mean Centre digest peptide concentration of 40.74 mM PheGly equivalents. Therefore the error in this procedure is less than 5% and any factors found to contribute to the production of peptides in this empirical model can be considered significant.

The 16 digests were produced under conditions that were orthogonal to one another and therefore simplified analysis using the coded variables was performed. The relative significance of each term is tested with a Normal Plot; the terms which deviate from the linear behaviour have a significant impact on the production of peptides (Montgomery, 2005). Figure 9 is a Normal Plot for the factorial design in terms of the coded variables excluding the three centre point replicates to identify the factors that are significant in the production of peptides.
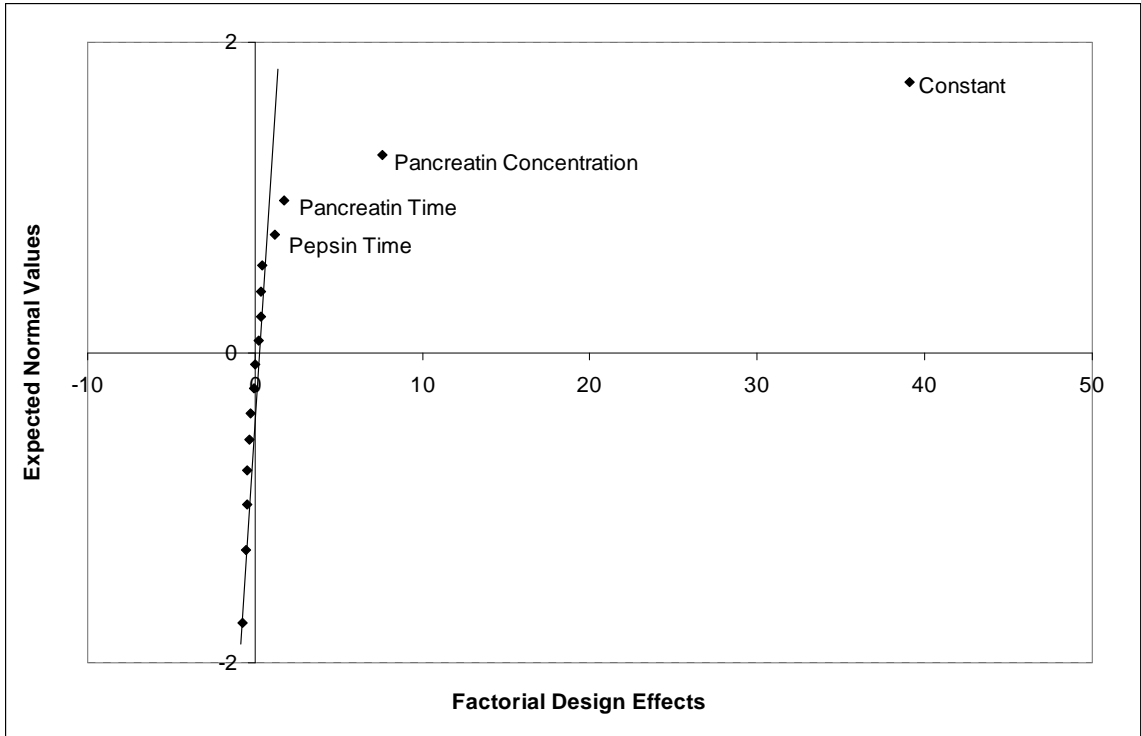
Figure 9: Normal plot for the factorial design in terms of the coded variables, revealing the factors that significantly impact the production of peptides.

The linear regression model to describe the factors affecting the production of peptides when expressed in terms of the coded variables is:

Equation 8: Empirical model for the production of peptides (coded variable form)

peptide concentration [mM PheGly] = 39.08 + 7.59 [coded pancreatin concentration] + 1.21 [coded pepsin time] + 1.76 [coded pancreatin time]

The model can also be expressed in terms of the natural variables, such as enzyme concentration (g/L) and time (minutes), giving:

Equation 9: Empirical model for the production of peptides (natural variable form)

peptide concentration [mM PheGly] = 20.02 mM + 7.59 [g/L pancreatin] + 0.08 [min pepsin]+ 0.06 [min pancreatin]

Furthermore, the data was inspected for any unexpected behaviour that cannot be explained by the linear model described in Equation 8. This was accomplished by plotting the residuals not explained by the model versus the values predicted by the model; any point which falls outside the expected range or if the data points show a non-random pattern indicates that the model does not

adequately explain the data (Walpole *et al.,* 1998). The plot of the residuals versus the values predicted by the model is given in Figure 10.
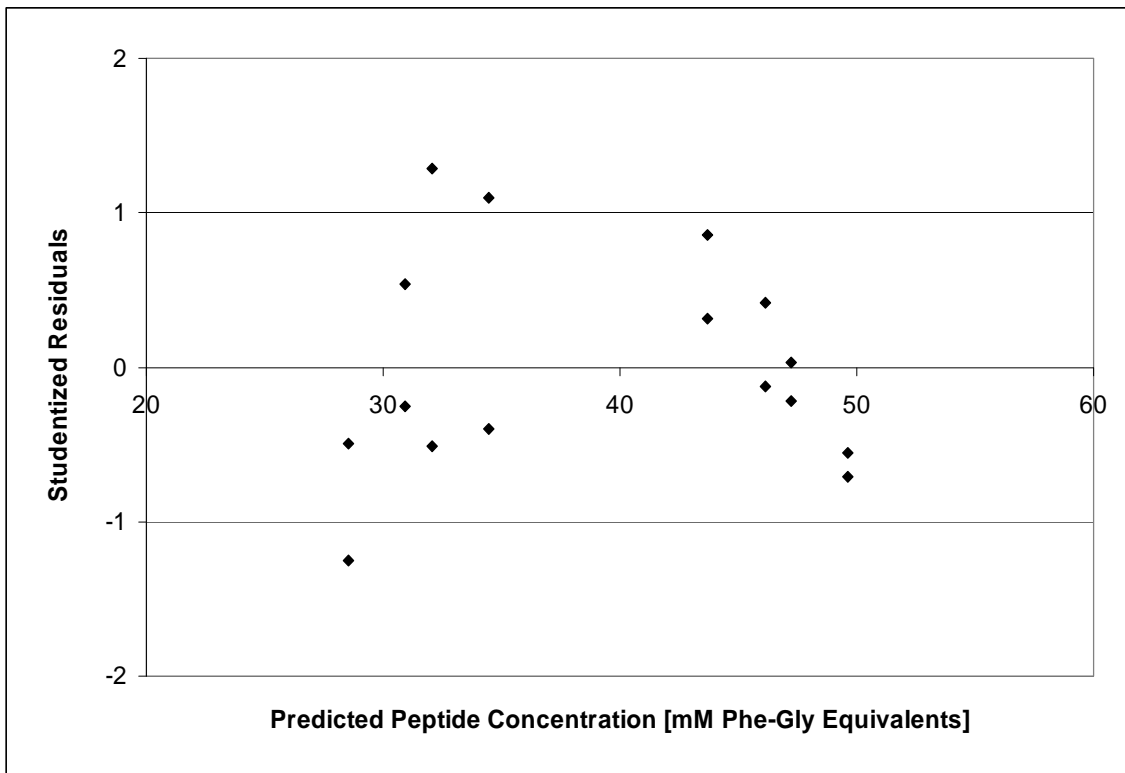

Figure 10:   Residuals versus the predicted values for the coded factorial design digests.

Figure 10 shows a random pattern, suggesting that no terms are missing from the digestion model. Furthermore, no point in the figure has a significantly high (+3) or low (-3) Studentized residual, which would otherwise indicate an outlier and require further investigation (Walpole *et al.*, 1998).

A review of the terms in the digestion model in terms of the natural variables in Equation 9 revealed a large constant term which is independent of the conditions tested, suggesting that any digests should have a minimum peptide concentration of approximately 20 mM PheGly equivalents. By comparison, undigested ADM PRO-FAM 974 SPI has a peptide concentration of approximately 13 mM PheGly equivalents. The term of next greatest importance is the pancreatin concentration, followed by the two time terms. All three of these factors have a positive influence on the concentration of peptides produced by enzymatic digestion. No interaction terms, such as pancreatin concentration x pancreatin digestion time, were found to have a significant effect on peptide concentration.

5.1.1 Additional Factorial Design Digests

The High (Run 1), Centre (Run 10), and Low (Run 8) digests were saved for fractionation by ultrafiltration and measuring the antioxidant capacity by the FCR and ORAC assays. Replicates of the High and Low digests were prepared so that additional testing could be performed in triplicate. The final peptide concentrations for each of these digests are summarized in Table 16.

Table 16: Final Peptide Concentrations for the Replicates of Selected Factorial Design Digests, in Coded Variables, Expressed as mM PheGly Equivalents

Run Number	Factor A	Factor B	Factor C	Factor D	Peptide Concentration (mM PheGly equivalents)
1	+1	+1	+1	+1	48.14
8	-1	-1	-1	-1	27.49
10	0	0	0	0	38.00
21	-1	-1	-1	-1	28.79
22	-1	-1	-1	-1	28.44
24	+1	+1	+1	+1	54.51
25	+1	+1	+1	+1	62.31

5.2 Fractionation by Ultrafiltration

The High Digests (Runs 1, 24, 25), the Centre Digest (Run 10), and the Low Digests (Runs 8, 21, 22) were frozen and saved for fractionation by ultrafiltration to study the effect of the digestion conditions on the filtration behaviour. The hydrolysate solutions were thawed without any change in pH or dilution. Approximately 25 mL of each hydrolysate solution was saved as a sample of the raw hydrolysate mixture; the remaining hydrolysate solution was filtered sequentially through the 3 kDa and 1 kDa MWCO membranes as described in Chapter 3 at 25ºC. The 3 kDa fractionation was run for a minimum of 600 minutes to produce a minimum of 60 g of permeate from approximately 160 mL of feed solution; the 1 kDa fractionation was run until approximately 50% fractionation between permeate and retentate by mass was achieved. Samples of permeate and retentate collected at the end of each ultrafiltration were freeze-dried and stored at room temperature until antioxidant tests were performed.

Figure 11 summarizes the time-flux relationship for the 3 kDa and 1 kDa ultrafiltration of the three types of digest while Table 17 summarizes the flux data after approximately 960 minutes for the 3 kDa MWCO filtration and after approximately 90 minutes for the 1kDa MWCO filtration.

Table 17: Steady-State Flux for 3 kDa and 1 kDa Ultrafiltration of the Factorial Design Digests under 210 kPa (40 psi) Nitrogen Pressure, Without Stirring (Low: n=3, mean ± SD; Centre: n=1, ND = not determined; High: n=2, mean ± SD) (includes some data from Bissegger, 2008)

Feed Solutions	3 kDa MWCO		1 kDa MWCO
	Flux at 180 minutes (L/m²h)	Flux at 960 minutes (L/m²h)	Flux at 90 minutes (L/m²h)
High Digest	2.23 ± 0.16	1.02 ± 0.10	2.89 ± 0.16
Centre Digest	1.71	ND	2.19
Low Digest	1.41 ± 0.16	0.75 ± 0.06	2.37 ± 0.05

The permeate flux VS time profiles in Figure 11 generally resemble the expected profiles for dead-end ultrafiltration with an initial high permeate flux followed by a decline to a lower, constant permeate flux (Das *et al*., 2009). The 3 kDa ultrafiltration of the High Digests have the highest permeate flux of the three digests at 1.7 L/m²h after 360 minutes. The Low and Centre digests have significantly lower permeate fluxes at a 95% confidence level after 360 minutes, although not significantly different from each other. When the 3 kDa permeate fluxes after 180 minutes are compared to those of the preliminary digestions, the permeate fluxes here are considerably lower than the values obtained for the Pancreatin Only digest of approximately 10 L/m²h after 210 minutes in Chapter 4.

The 1 kDa ultrafiltration of all three digests appear to reach a constant permeate flux between 3.1 and 3.7 L/m²h after approximately 30 minutes. The Run 1 High Digest 3 kDa ultrafiltration had an unusually high initial permeate flux of 57 L/m²h approximately three minutes into the filtration and the filtration was complete within seven minutes, which suggests a possible tear in the membrane. The two replicates had a considerably lower permeate flux of 6.0 L/m²h after 3 minutes of filtration and therefore, only the two replicates were used to produce Figure 11 and the data for Table 17. Neither the Centre nor Low filtrations behaved in a similar, anomalous manner. Also, none of the permeate fluxes after 30 minutes were found to be significantly different at the 95% confidence level.

A mass balance was performed on the 3 kDa and the 1 kDa ultrafiltration fractions by two different methods: total solids (results expressed as mass) and peptide content (results expressed as equivalent mM PheGly). The filtration mass balances given in Table 18 were calculated from the freeze-dried samples of the permeate and the retentate that were collected and freeze-dried.
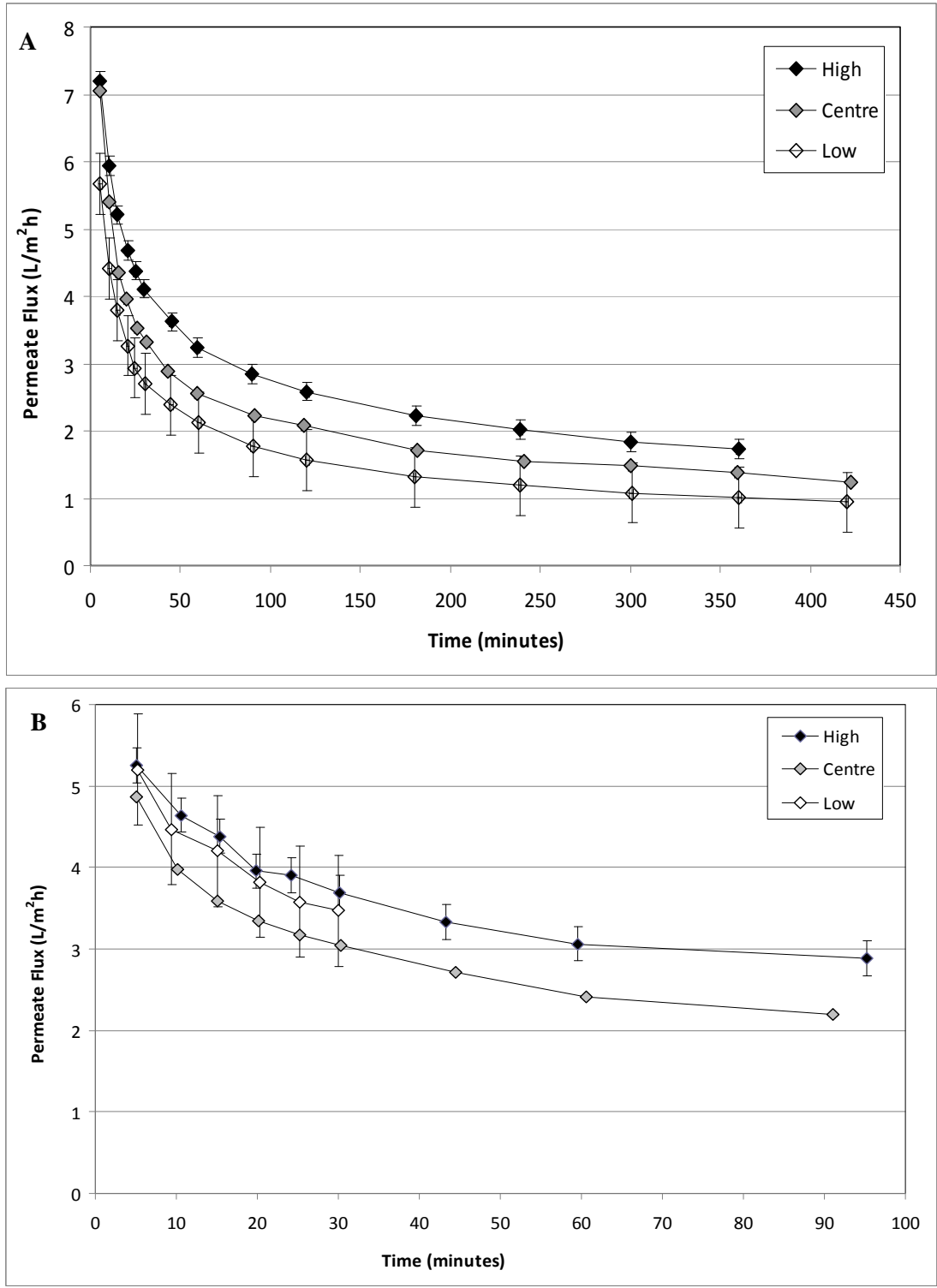
Figure 11: Time-permeate flux relationships obtained for the 3 kDa (A) and 1 kDa (B) MWCO ultrafiltration of the factorial design digests under constant 210 kPa (40 psi) nitrogen pressure with stirring (High and Low: n=3, mean ± SD. Centre: n=1) (includes some data from Bissegger, 2008)

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Table 18: Mass Balance on Filtration in Total Solids Expressed as Percent Mass (High and Low: n=3, mean ± SD; Centre: n=1) (includes some data from Bissegger, 2008)

Feed Solution	3kDa Fractionation (Mass %)			1kDa Fractionation (Mass %)		
	Retentate	Permeate	% Loss	Retentate	Permeate	% Loss
High Digest	50 ± 12 [a]	37 ± 14 [a]	13 ± 8 [b]	48 ± 5 [a]	35 ± 7 [a]	17 ± 13 [c]
Centre Digest	78 [a]	19 [a]	3 [b]	54 [a]	22 [a]	24 [c]
Low Digest	69 ± 16 [a]	26 ± 13 [a]	4 ± 9 [b]	62 ± 22 [a]	32 ± 16 [a]	6 ± 38 [c]

Values with the same letter are not significantly different (P = 0.05)

As summarized in Table 18, the separation of the feed solution quantified in terms of mass fractions at each stage indicates relatively low losses of 5-15% with the exception being the high variability in the losses documented for the Low Digest 1 kDa filtration. Half or more of the mass of the feed solution is retained by the 3 kDa membrane for all digests, with approximately 15% of the initial feed solution passing through the 1 kDa membrane. Similarly for the 1 kDa ultrafiltration, half or more of the feed mass is retained by the membrane. At a 95% confidence level, no significant difference between the recovery at each stage of the ultrafiltration was observed. The peptide content of the permeate and the retentate solutions, measured using the OPA assay on the freeze-dried samples, is summarized in Table 19.

Table 19: Peptide Balance on Filtration in mM PheGly Equivalents/g Fraction Collected, as Determined by the OPA Assay (High and Low: n=3, mean ± SD; Centre: n=1) (includes some data from Bissegger, 2008)

Feed Solution	3kDa Fractionation (mM PheGly/g Fraction)			1kDa Fractionation (mM PheGly/g Fraction)	
	Feed	Retentate	Permeate	Retentate	Permeate
High Digest	15.5 ± 2.0 [a]	14.8 ± 1.3 [a]	13.8 ± 0.8 [a]	15.1 ± 1.3 [a]	14.0 ± 1.2 [a]
Centre Digest	12.3 [a]	13.1 [a]	11.7 [a]	13.0 [a]	6.6 [a]
Low Digest	9.8 ± 1.6 [a]	10.8 ± 1.6 [a]	11.3 ± 1.7 [a]	10.6 ± 0.2 [a]	13.2 ± 0.9 [a]

Values with the same letter are not significantly different (P = 0.05)

The peptide content per gram of hydrolysates fraction, as shown in Table 19, is approximately equally split between all fractions from the ultrafiltration process, which is also independent of the nature of the feed digest solution on a mmol peptide/g sample basis. At a 95% confidence level, no difference between the peptide content of the ultrafiltration fractions could be measured. The total mmol peptides/hydrolysates sample, as shown in Table 20, was calculated to determine the effect of the size of each fraction collected on the peptide content.

Table 20: Total Peptide Content on on Filtration in mM PheGly Equivalents/Fraction Collected, as Determined by the OPA Assay (High and Low: n=3, mean ± SD; Centre: n=1) (includes some data from Bissegger, 2008)

Feed Solution	3 kDa Fractionation (mM PheGly/Fraction)			1 kDa Fractionation (mM PheGly/Fraction)	
	Feed	Retentate	Permeate	Retentate	Permeate
High Digest	118 ± 48	53 ± 16	41 ± 23	19 ± 10	13 ± 8
Centre Digest	54	47	6	3	1
Low Digest	59 ± 23	43 ± 14	21 ± 14	10 ± 6	7 ± 5

The peptide content of ultrafiltration of the Centre and High digests indicate that 50% of the feed solution was retained by the 3 kDa membrane while the permeate from the 3 kDa membrane was approximately equally split into the retentate and permeate of the 1 kDa filtration, as was observed in the mass fractions in Table 18. The Low digests follow a slightly different trend with approximately two thirds of the feed solution being retained by the 3 kDa membrane, then another two thirds of the retentate from the 3 kDa membrane being retained by the 1 kDa membrane. This difference in filtration behaviour in terms of peptide content may be attributed to the reduced cleavage of the proteins and the resulting longer peptide chains which could not pass through the respective membranes, although no significant difference was observed between in the quantity of peptides produced for each of the three digests at the same ultrafiltration conditions at a 95% confidence level.

5.2.1 Fouling Behaviour

A significant fouling layer was observed to form on the 3 kDa membrane during the ultrafiltration experiments but no significant fouling layer was observed to form during the 1 kDa membrane filtrations, as illustrated in Figure 12 for a selection of the filtrations performed.

To gain a better understanding of the fouling mechanisms present in this dead-end filtration process, the permeate flux data gathered for the ultrafiltrations was studied to give information on the fouling mechanisms present. Using the linearized form of the equations developed by Bowen and co-workers (Bowen *et al.*, 1995), the manipulation of the permeate fluxes for the 3 kDa and 1 kDa ultrafiltrations performed here both show evidence of cake fouling. The figures used in this determination can be found in Appendix B. This type of fouling is desirable because process scale-up to cross-flow ultrafiltration is expected to overcome this resistance simply by the design of the process (Ghosh, 2003).
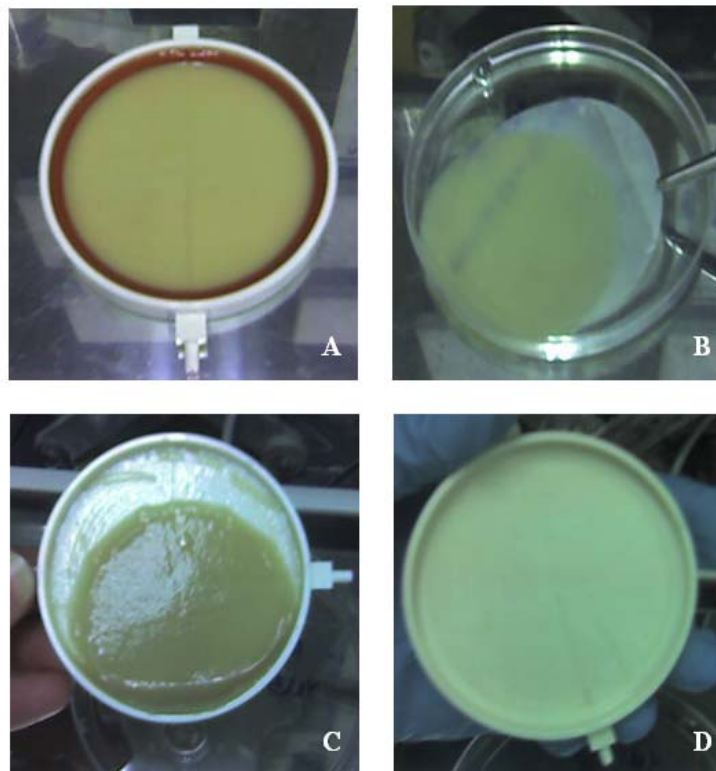
Figure 12: A) Visible fouling layer on 3 kDa membrane after Low digest (Run 22) filtration; B) same as A, with the fouling layer sliding off the wet membrane; C) fouling layer on 3 kDa membrane after High digest (Run 24) sliding off membrane and breaking apart; D) no visible fouling layer on 1 kDa membrane after filtration of High digest (Run 24)

To compliment this work, the 3 kDa and 1 kDa membranes used in the fractionation of one of each of the High and Low Digests (Runs 20 and 24 respectively) were lightly rinsed with MilliQ water to remove any visible fouling layer. The water flux for each membrane was measured before the enzymatic cleaning step to quantify the irreversible fouling effect. The water fluxes are summarized in Table 21 with typical water flux values obtained for the clean membranes. Before enzymatic cleaning, the water flux was slightly lower than that expected for a clean membrane, especially for the 3 kDa membrane. This suggests that the large majority of the fouling present in this system is reversible fouling although some foulant was not removed by the water rinse.

Table 21: Water Flux for Clean and Unwashed 3 kDa and 1 kDa Membranes under 210 kPa (40 psi) of Nitrogen (clean membrane: n=3, mean ± SD)

Identifier	Water flux ($L/m^2 h$)	
	3kDa membrane	**1kDa membrane**
Clean membrane	22.17 ± 0.61	15.57 ± 0.37
High Digest (Run 24)	21.18	13.52
Low Digest (Run 22)	21.16	13.44

5.3   Characterizing the Digests and Fractions

A better understanding of the molecular weight distribution of the peptides produced at the High (Run 24) and Low (Run 22) digestion conditions was obtained through the use of SEC-HPLC. SEC-HPLC was performed at Université Laval to further understand the molecular weight distribution resulting in a shift of the peptide peak at 215 nm, as illustrated in Figure 13.

Université Laval reported that the undigested SPI was difficult to dissolve in the buffer solution and therefore was run at the relatively low concentration of 0.5 mg sample/mL buffer solution, which is somewhat unexpected considering the solubility data gathered on the undigested SPI in Table 7. The undigested PRO-FAM 974 SPI has a small peak (approximately 5 mAu) at 840 Da. However, a more defined peak (greater than 25 mAu) can be seen at the molecular mass of 1.59 kDa for the High digest. A similarly significant peak is found at 1.95 kDa for the Low digest, along with a significant peak near 13 kDa potentially indicating an undigested fraction of the SPI, possibly the acidic fraction subunit A5 (approximately 10 kDa) or the basic fraction (approximately 20 kDa) (Horneffer *et al.*, 2007).
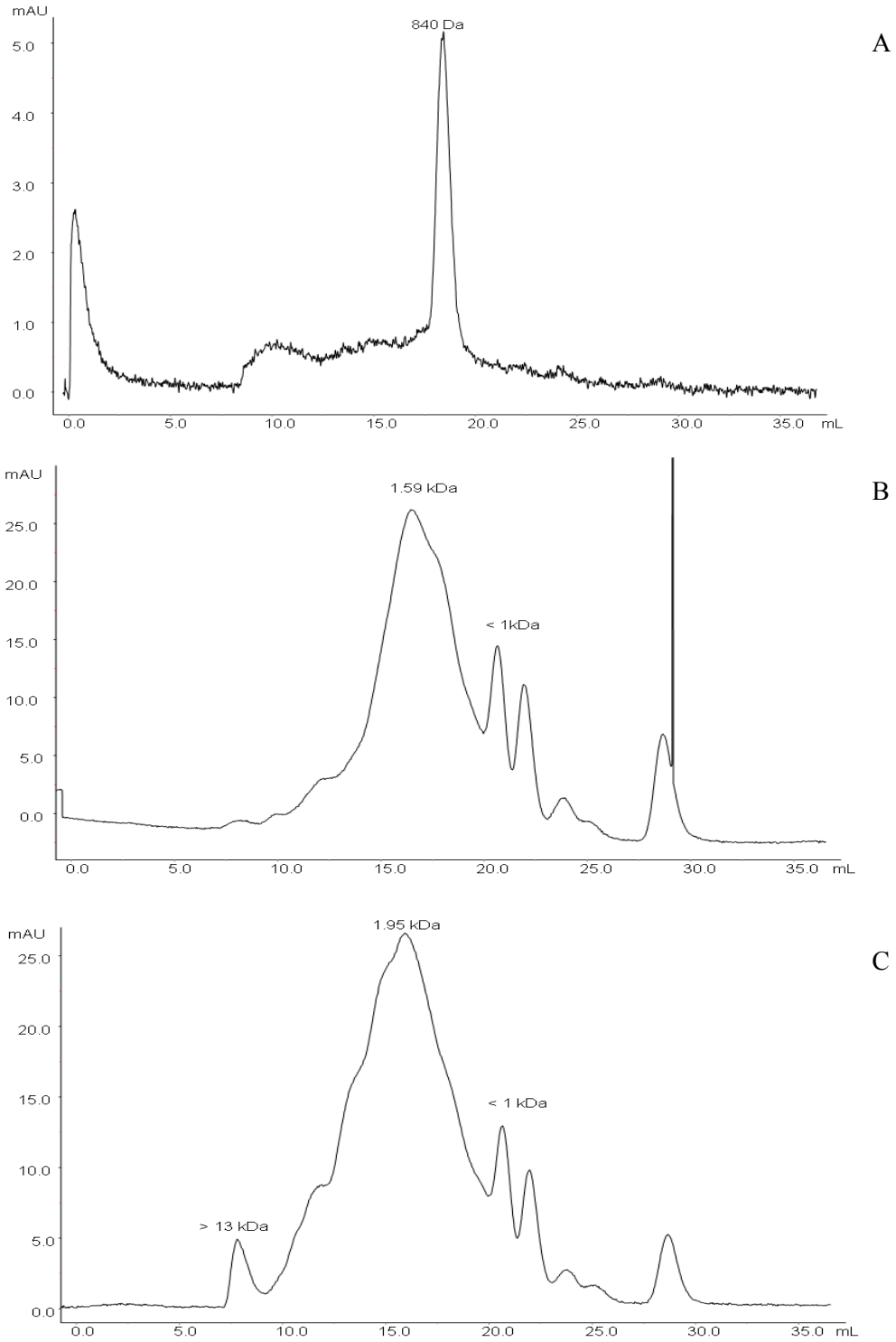
Figure 13: SEC-HPLC at 215 nm, sample dissolved in 50 mM phosphate buffer and 150 mM NaCl solution at pH 7. A) Undigested ADM PRO FAM 974 SPI at 0.5 mg/mL; B) High digest (Run 24) at 1.5 mg/mL; C) Low digest (Run 22) at 1.8 mg/mL.

5.4 Antioxidant Properties

Once fractionated by ultrafiltration, the High, Centre, and Low digests were freeze dried, and the antioxidant capacity of each fraction was determined by the DPPH, FCR, and ORAC assays.

5.4.1 DPPH Assay

The antioxidant capacities determined for the factorial design digests, expressed in terms of equivalent antioxidant capacity of mg Trolox/g sample in the DPPH assay, are summarized in Table 22. The antioxidant capacity of the feed and 3 kDa retentate fractions could not be determined due to the poor solubility of the samples in the ethanol-based DPPH assay medium. Furthermore, insufficient freeze-dried sample was available to determine the DPPH antioxidant capacity of the 3 kDa permeate and 1kDa permeate fractions of the Low (Run 8) digest.

Table 22: DPPH Antioxidant Capacity for the High, Centre, and Low Digests, Expressed as Equivalents of Trolox (Centre: n=1; Low and High: n=2* and n=3; mean ± SD)

Ultrafiltration Fraction	mg Trolox/g sample		
	High Digest	**Centre Digest**	**Low Digest**
3kDa Permeate	0.271 ± 0.059 [a,b]	0.209 ± 0.087 [a,b]	0.292 ± 0.092 * [a,b]
1kDa Retentate	0.267 ± 0.067 [a,b]	0.184 ± 0.069 [a,b]	0.267 ± 0.087 [a,b]
1kDa Permeate	0.294 ± 0.057 [a]	0.260 ± 0.013 [a,b]	0.392 ± 0.115 * [b]

Values with the same letter are not significantly different (P = 0.05)

Analysis of variance was performed at a 95% confidence level on the antioxidant capacities. The only significant difference was that the antioxidant capacity of the 1 kDa permeate fraction of the Low digest was slightly higher than that of the 1 kDa permeate of the High digest. Otherwise, the differences between replicates of the same digestion and filtration conditions were found to be as different from one another as other digestion or filtration conditions by the DPPH assay. Two conclusions may be drawn from this finding: first, that the samples measured have equivalent antioxidant abilities or, second, that the DPPH assay is unable to provide the sensitivity needed to distinguish between samples.

5.4.2 FCR Assay

The electron donating antioxidant capacities determined for the factorial design digests, expressed in terms of equivalent antioxidant capacity of mg Trolox/g sample for the FCR assay, are summarized in Table 23. Samples of the feed solutions for the High Run 1, Centre Run 10, and Low Run 8 digests were not available for antioxidant testing.

Table 23: FCR Antioxidant Capacity for the High, Centre, and Low Digests, Expressed as Equivalents of Trolox (Centre: n=1; Low and High: n=2* and n=3, mean ± SD, ND = not determined)

Ultrafiltration Fraction	mg Trolox/g sample		
	High Digest	**Centre Digest**	**Low Digest**
Feed	101.8 ± 13.4 * [a,b,c,d]	ND	78.9 ± 3.6 * [b,d]
3kDa Retentate	105.7 ± 13.8 [a,b,c,d]	111.1± 8.2 [b,c]	89.9 ± 10.9 [b,d]
3kDa Permeate	92.4 ± 9.5 [a,b]	89.6± 8.2 [b]	103.9 ± 22.7 [b,c,d]
1kDa Retentate	104.7 ± 28.3 [a,b,c,d]	83.9 ± 12.3 [b,d]	86.0 ± 16.7 [b,d]
1kDa Permeate	115.3 ± 11.1 [a,c,d]	117.9 ± 8.4 [c]	108.0 ± 5.5 [b,c,d]

Values with the same letter are not significantly different (P = 0.05)

Analysis of variance was performed at a 95% confidence level on the antioxidant capacities determined by the FCR assay. As was determined for the antioxidant capacities measured by the DPPH assay, the antioxidant capacities between replicates in the same digest group were found to be significantly different. Unlike the data gathered from the DPPH assay, some fractions for a given digest were found to be significantly different, suggesting that ultrafiltration can indeed be used to enrich antioxidant peptides or remove pro-oxidant peptides from a feed solution. Centre digests Run 10 and Low digests Run 8 and 21 revealed that a significant difference in the antioxidant capacity of the fractions tested for a given digest can be measured. High digest Run 25, which was removed from the digestion factorial design due to its anomalous behaviour, showed the only significant difference between fractions for the High digest condition. Furthermore, the standard deviation in replicated antioxidant capacity using the FCR assay is less than that observed in the DPPH assay, suggesting that this aqueous assay is better suited to measuring the antioxidant capacity of these peptides.

5.4.3 ORAC Assay

The antioxidant capacities determined for the factorial design digests in the ORAC assay, expressed in terms of equivalent antioxidant capacity of mg Trolox/g sample, are summarized in Table 24. Samples of the feed solutions for the High Run 1, Centre Run 10, and Low Run 8 digests were not available for antioxidant testing.

Table 24: ORAC Antioxidant Capacity for the High, Centre, and Low Digests, Expressed as Equivalents of Trolox (Centre: n=1; Low and High: n=2* and n=3, mean ± SD, ND = not determined)

Ultrafiltration Fraction	mg Trolox/g sample		
	High Digest	**Centre Digest**	**Low Digest**
Feed	125.5 ± 4.8 * [a,b]	ND	102.1 ± 6.6 * [a]
3kDa Retentate	117.4 ± 7.7 [a,b]	124.3 ± 5.6 [b]	109.3 ± 7.9 [a]
3kDa Permeate	125.3 ± 8.3 [a,b]	116.9 ± 4.8 [a,b]	110.3 ± 5.6 [a]
1kDa Retentate	113.1 ± 4.9 [a]	101.6 ± 6.0 [a]	110.3 ± 6.4 [a]
1kDa Permeate	134.4 ± 7.1 [a,b]	144.3 ± 6.9 [c]	125.9 ± 4.9 [b]

Values with the same letter are not significantly different (P = 0.05)

Tukey's paired comparison testing was performed on the ORAC antioxidant capacities at a 95% confidence level to identify and significant differences between digestion and fractionation conditions. The fractionation of the High digest had no significant effect on the antioxidant capacity of the peptides produced. At a 95% confidence level, the 1 kDa permeate fraction of both the Low and the Centre digests were shown to have a greater antioxidant capacity than the 1 kDa retentate, 3 kDa permeate, and 3 kDa retentate fractions. This suggests that not only the digestion conditions but also the ultrafiltration fraction have a significant effect on the antioxidant capacity measured by the ORAC assay.

6 Extensions from Factorial Design

6.1 Digestions

As described in Chapter 5, a factorial design model for the enzymatic digestion of SPI was produced (equations 8 and 9), describing the roles of the pepsin concentration (A), pancreatin concentration (B), pepsin digestion time (C), and pancreatin digestion time (D) on the production of peptides. This empirical model can be used to understand the factors involved in producing peptides. To expand the understanding of the role of enzymatic digestion on antioxidant peptide production, additional sets of digests were performed to explore observations made from the digestion factorial design analysis:

1. Pancreatin concentration had a significant, positive effect on the production of peptides over the range selected, and

2. Pepsin concentration had no significant effect on the production of peptides within the concentration range selected.

No significant connection between the digestion conditions and/or the ultrafiltration conditions employed and the antioxidant capacity of the peptides has been identified in Chapters 4 and 5. However, based on the work by Chen, Muramoto, and Yamauchi showing that peptides composed of five to 16 amino acid residues, the investigation of peptides of 3000 Da and smaller continued based on the optimum peptide producing conditions identified in equation 9 (Chen *et al*., 1995).

6.1.1 Effect of Increasing Pancreatin Concentration

Based on the results obtained from the factorial design experiments indicating that the concentration of pancreatin had the greatest positive impact on the resulting concentration of peptides from the enzymatic hydrolysis experiments, a series of experiments were performed with a pancreatin concentration greater than that used for the High condition in the factorial design work. A higher pancreatin concentration of 4.5 g pancreatin/L solution was selected and was applied with the conditions described earlier for the factorial design work. The peptide concentration obtained at the end of each digestion for each of the higher pancreatin concentrations is summarized in Table 25.

Table 25: Final Peptide Concentrations for the Higher Pancreatin Digests; Natural Values are to the Left with Coded Values to the Right

Run Number	Factor A (Pepsin Concentration)		Factor B (Pancreatin Concentration)		Factor C (Pepsin Time)		Factor D (Pancreatin Time)		Peptide Concentration (mM PheGly equivalents)
28	0.297 g/L	+1	4.5 g/L	+3	45 min	+1	120 min	+1	65.49
29	0.149 g/L	-1	4.5 g/L	+3	15 min	-1	120 min	+1	65.64
30	0.297 g/L	+1	4.5 g/L	+3	45 min	+1	60 min	-1	59.51
31	0.149 g/L	-1	4.5 g/L	+3	15 min	-1	60 min	-1	61.11

As suggested by the digestion model developed in equation 9, increasing the pancreatin concentration above the High level of 2.5 g/L was observed to increase the resulting peptide concentration of the digests. However, a maximum level for the peptide concentration was observed, as illustrated in Figure 14, where each digest appeared to achieve a maximum peptide concentration between 60 and 65 mM PheGly equivalents. This range of maximum peptide concentration coincides with the High digest Run 25 peptide concentration of 62.3 mM PheGly, although the average of the three High digests falls below 60 mM PheGly.
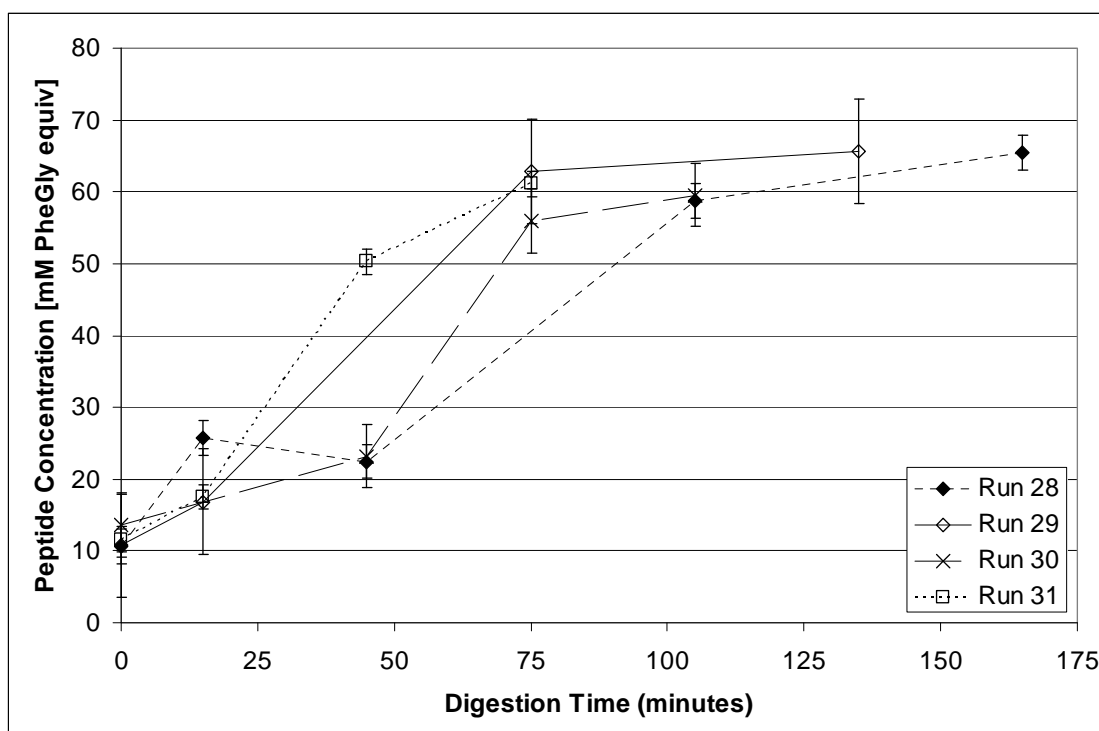
Figure 14: Digestion progression for the higher pancreatin concentration digests. Experimental conditions in Table 25.

The High digest conditions with the Higher pancreatin concentration of 4.5 g pancreatin/L solution (Run 28), was saved for fractionation by ultrafiltration and antioxidant testing to study whether an increase in the peptide concentration also increased the antioxidant properties of the resulting hydrolysates.

6.1.2 Effect of Eliminating Pepsin

Another extension of the model developed from the factorial design experiments was performed, based on the determination that the pepsin concentration was too high to show a significant effect in the production of peptides. Therefore, the pepsin concentration and subsequently the pepsin digestion time were set to zero and only the pancreatin concentration and time were adjusted. Based on the results obtained from the previous extension of the factorial design on increasing the pancreatin concentration, the concentration of pancreatin used was the High level and the new higher pancreatin level, as described in Table 26.

Table 26: Final Peptide Concentration for the No Pepsin Digests; Natural Values are to the Left with the Coded Values to the Right

Run Number	Factor A (Pepsin Concentration)		Factor B (Pancreatin Concentration)		Factor C (Pepsin Time)		Factor D (Pancreatin Time)		Peptide Concentration (mM PheGly equivalents)
26	0 g/L	-3	2.5 g/L	+1	0 min	-2	120 min	+1	35.37
27	0 g/L	-3	4.5 g/L	+3	0 min	-2	120 min	+1	58.22

The final peptide concentration for the digest performed with the High pancreatin condition of 2.5 g/L and no pepsin (Run 26) of approximately 35 mM PheGly is greater than that obtained for the Pancreatin digest with a pancreatin concentration of 0.8 g/L and no pepsin of approximately 23 mM PheGly. This suggests that pancreatin continues to cleave proteins into peptides until the maximum peptide concentration is achieved if given sufficient time. The High pancreatin digest performed without pepsin (Run 26), was saved for fractionation by ultrafiltration and antioxidant testing to study whether an increase in the peptide concentration also increased the antioxidant capacity of the resulting fractions.

6.1.3 Additional Midpoint Digest

In addition to the higher pancreatin concentration and removing pepsin, another digest with enzyme concentrations and digestion times between the Pepsin & Pancreatin and Centre digest conditions was performed. The concentration and time conditions employed and resulting peptide

concentration are summarized in Table 27. Both digests were fractionated by ultrafiltration and the antioxidant capacities of the resulting fractions were determined.

Table 27:   Final Peptide Concentration for the Midpoint Digests; Natural Values are to the Left with the Coded Values to the Right

Run Number	Factor A (Pepsin Concentration)		Factor B (Pancreatin Concentration)		Factor C (Pepsin Time)		Factor D (Pancreatin Time)		Peptide Concentration (mM PheGly equivalents)
20	0.198 g/L	-0.34	0.832 g/L	-0.67	30 min	0	90 min	0	38.10
23	0.198 g/L	-0.34	0.832 g/L	-0.67	30 min	0	90 min	0	39.73

6.1.4 Interpretation of Digestion Results

The factorial design model for the four factors that influence peptide production was evaluated for the eight additional digestions; the four factorial design replicates for the High and Low digests described in Chapter 5 were also included. The relative importance of each term in this expanded factorial design model was determined using the Normal Plot in Figure 15.
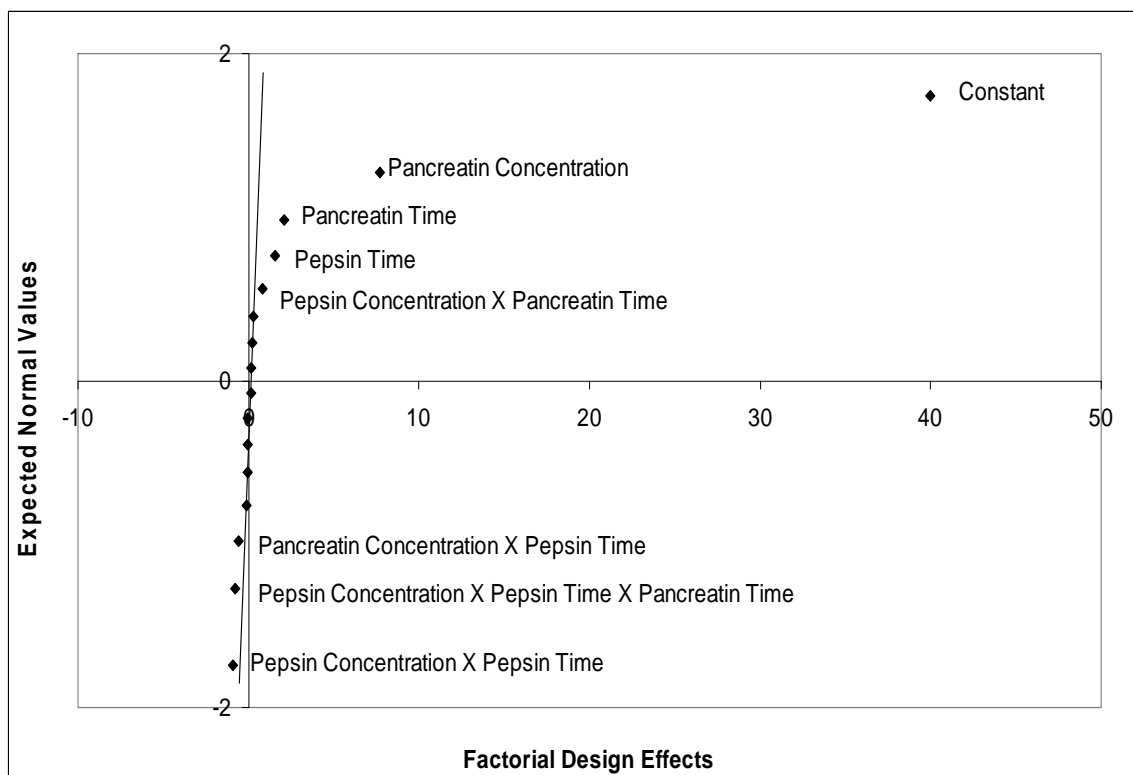
Figure 15:   Normal Plot for factorial design model with replicates and extensions.

As expected from the initial factorial design model described by equations 8 and 9, the constant, pancreatin concentration, pancreatin time, and pepsin time terms continued to contribute significantly to the production of peptides. However, several interaction terms have appeared and some or all may significantly contribute to the production of peptides for this expanded factorial model. Therefore, a series of residual plots were prepared to examine which of the interaction terms, if any, had a significant contribution.

All residual plots prepared showed a random distribution of the residuals, indicating the adequacy of a linear model for the production of peptides (Montgomery, 2005). However, a number of points were found to be outliers, having Studentized residuals greater than 3, which could be expected because the data points added to the original factorial design were not part of a designed set of data (Montgomery, 2005). These outlier points included one High digest (Run 25, 62.3 mM PheGly) and sometimes the No Pepsin digest (Run 26, 35.4 mM PheGly) depending on the model under consideration. All variations on the linear regression model starting with the basic form given by equation 8 up to the model containing all significant factors from the Normal Plot were used, but the outliers persisted to varying degrees depending on the model under consideration.

Therefore, the outliers were removed one at a time, starting with the High Digest (Run 25), and a satisfactory model was revealed. The empirical model that was found to best explain the data obtained, that is to reduce the magnitude of the residuals, is given by equations 10 and 11, in coded variable and natural variable forms respectively.

Equation 10: Extended empirical model for the production of peptides (coded variable form)

peptide concentration [mM PheGly] = 39.95 + 7.70 [coded pancreatin concentration]
+ 2.09 [coded pancreatin time] + 1.53 [coded pepsin time]
+ 0.79 [coded pepsin concentration x coded pancreatin time]
- 0.90 [coded pepsin concentration x coded pepsin time]

Equation 11: Extended empirical model for the production of peptides (natural variable form)

peptide concentration [mM PheGly] = 19.46 mM + 7.91 [g/L pancreatin] + 0.293 [min pepsin]
+ 0.005 [min pancreatin] + 0.283 [g/L pepsin x min pancreatin]
- 0.931 [g/L pepsin x min pepsin]

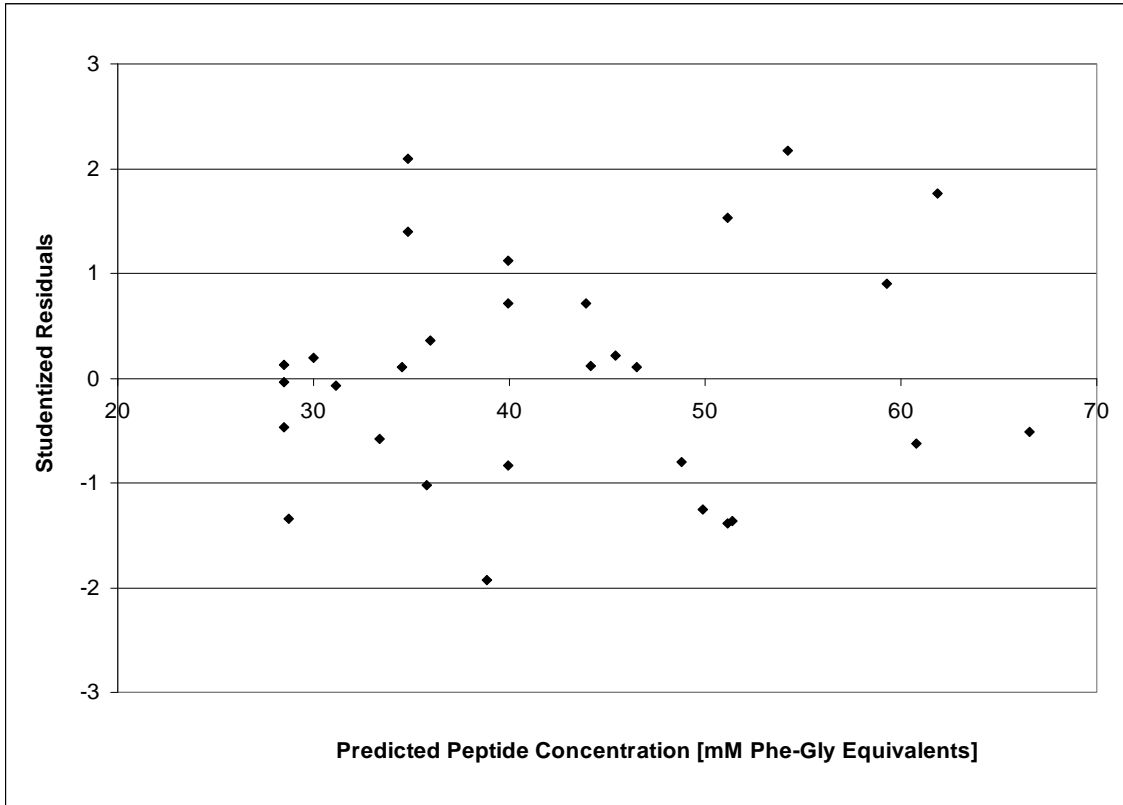The residual plot for model equation 10 is given in Figure 16.

Figure 16: Residual plot for the expanded factorial design model in coded variables with the addition of replicates and extensions, excluding High digest Run 25

The residual plot appears random which suggests that all terms needed to explain the data have been included in the model. Furthermore, all residual values fall between -3 and +3, indicating that no more outliers exist in the data (Montgomery, 2005). The general form of the model produced in equations 8 and 9 remains although two interaction terms between the pepsin time and the pepsin concentration or the pancreatin concentration has appeared. Furthermore, one of the interaction terms is negative, suggesting that the addition of pepsin to the digest may actually reduce the peptide concentration possible with a pancreatin only digest.

6.2   Fractionation by Ultrafiltration

Two extension digests were selected for fractionation by ultrafiltration, the High digest with No
Pepsin (Run 26) and the High digest with Higher Pancreatin (Run 28), in addition to the two
Midpoint digests (Runs 20 and 23), to study the effects of the digestion conditions on the
ultrafiltration behaviour. Approximately 25 mL of each hydrolysate solution was saved; the
remaining solutions of approximately 150 mL were filtered sequentially through the 3 kDa and 1
kDa MWCO membranes at 25ºC under 210 kPa (40 psi) nitrogen pressure. Samples of the
permeate and the retentate solutions were collected and freeze-dried at the end of each stage of the
filtration and stored at room temperature until antioxidant tests were performed. The permeate flux
data generated from this fractionation process is presented in Figure 17 and summarized in Table
28, after approximately 720 minutes for the 3 kDa MWCO filtration and 90 minutes for the 1 kDa
MWCO filtration.

Table 28:   Steady State Permeate Flux for the 3 kDa and 1 kDa MWCO Ultrafiltration of the
Extension Digests under 210 kPa (40 psi) Nitrogen Pressure, Without Stirring (n=1, n=2 for
Midpoint digest)

Feed Solutions	3 kDa membrane		1 kDa membrane
	Flux at 180 minutes (L/m^2h)	Flux at 720 minutes (L/m^2h)	Flux at 90 minutes (L/m^2h)
No Pepsin (Run 26)	1.31	0.84	2.51
Higher Pancreatin (Run 28)	2.37	1.29	2.76
Midpoint (Runs 20 and 23)	1.55 ± 0.08	0.93 ± 0.03	2.56 ± 0.33

The permeate flux VS time profiles in Figure 17 generally resemble the profiles expected for dead-
end ultrafiltration with an initial high permeate flux followed by a decline to a lower, constant
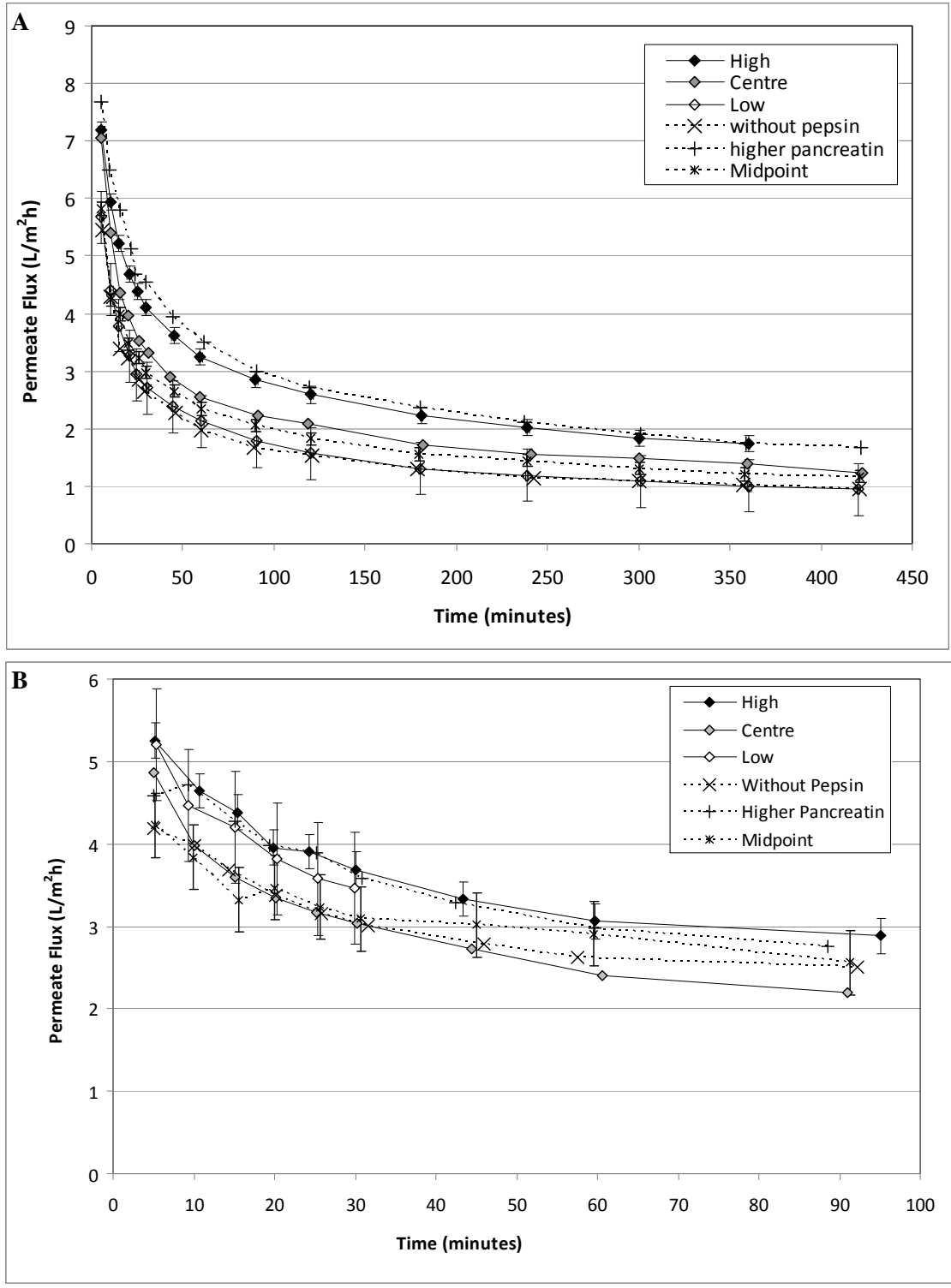permeate flux (Das *et al.,* 2009).

Figure 17: Time-permeate flux relationships for the 3 kDa (A) and 1 kDa (B) MWCO ultrafiltration of the Without Pepsin and Higher Pancreatin digests superimposed on the factorial design digests under constant 210 kPa (40 psi) nitrogen pressure, without stirring. (mean with SD error bars for replicates; Midpoint: n=2; High and Low: n=3; else n=1)

The 3 kDa ultrafiltration permeate fluxes revealed that the Higher Pancreatin digest closely follows the dead-end ultrafiltration behaviour of the High digest conditions from the factorial design digests while the No Pepsin digest closely modeled the Low digest behaviour. The Higher Pancreatin digest had a permeate flux of 2.4 L/m^2h after 180 minutes. However, this value is still less than the values obtained for the preliminary digestions; the permeate fluxes here are considerably lower than the values obtained for the Pancreatin digest of approximately 10 L/m^2h after 210 minutes. The No Pepsin digest had a permeate flux of 1.3 L/m^2h after 180 minutes which compared well to the Low digest flux of 1.41 ± 0.16 L/m^2h after 180 minutes. The Midpoint and Centre digests also compared well with permeate fluxes of 1.55 ± 0.08 and 1.71 L/m^2h after 180 minutes respectively.

The 1 kDa ultrafiltration permeate flux was observed to have the same pattern as the 3 kDa ultrafiltration process, where the flux decline for the Higher Pancreatin digest was similar to that for the High digest while the No Pepsin digest closely resembled the Low digest. The Midpoint digest initially followed a permeate flux decline comparable to the Low and Centre digests, but towards 45 minutes did not experience the same permeate flux decline and instead more closely resembled the permeate flux of the High digests after 90 minutes.

A mass balance was performed on the 3 kDa and 1 kDa ultrafiltration fractions using two different methods: total solids (results expressed as mass) and peptide content (results expressed as equivalent mM PheGly). The ultrafiltration total solids mass balances given in Table 29 were calculated from the permeate and retentate solutions that were collected and freeze-dried.

Table 29: Mass Balance on Ultrafiltration in Percent Mass Using Total Solids (n=1, Midpoint: n=2, mean ± SD)

Feed Solution	3 kDa Fractionation (mass %)			1 kDa Fractionation (mass %)		
	Retentate	Permeate	% loss	Retentate	Permeate	% loss
No Pepsin (Run 26)	61	27	12	61	40	-1
Higher Pancreatin (Run 28)	50	40	10	52	38	10
Midpoint (Runs 20 and 23)	54 ± 7	30 ± 0	17 ± 7	68 ± 8	27 ± 3	5 ± 5

From Table 29, the separation of the feed solution quantified in terms of mass fractions at each stage indicates relatively low losses, between a 1% gain in mass to a maximum 12% loss. From

the mass fractions, the same observation can be made as for the initial factorial conditions that half or more of the feed solution is retained by the 3 kDa membrane, with approximately 10 mass % of the initial feed solution collected in the permeate of the 1 kDa membrane. The peptide content of the permeate and retentate solutions, measured using the OPA assay for the freeze-dried samples, is summarized in Table 30.

Table 30: Peptide Balance on Ultrafiltration in mM PheGly Equivalents/g Fraction by OPA Assay (n=3, mean ± SD)

Feed Solution	3kDa Fractionation (Mass %)			1kDa Fractionation (Mass %)	
	Feed	Retentate	Permeate	Retentate	Permeate
No Pepsin (Run 26)	19.5 ± 1.7	23.6 ± 1.6	27.5 ± 2.9	27.1 ± 3.8	32.9 ± 2.1
Higher Pancreatin (Run 28)	22.4 ± 1.2	34.8 ± 22.4	21.5 ± 2.0	20.2 ± 2.3	19.6 ± 0.9
Midpoint (Runs 20 and 23)	15.1 ± 0.8	18.5 ± 1.1	19.7 ± 1.7	16.9 ± 1.7	20.3 ± 2.2

As stated in Table 30, the peptides are approximately equally split between all fractions as a result of the ultrafiltration, and this split is independent of the nature of the feed digest solution. This suggests that the enzymatic digestion is effective at producing peptides with a range of molecular weights and also that the MWCO of the membranes used in this study, 1 kDa and 3 kDa, has limited capacity to concentrate the peptides in any one fraction. To determine the effect of the size of each fraction collected on the peptide content, the total mmol peptides/hydrolysates sample collected were determined and tabulated in Table 31.

Table 31: Total Peptide Content on Ultrafiltration in mM PheGly Equivalents/Fraction Collected by OPA Assay (n=3, mean ± SD)

Feed Solution	3kDa Fractionation (mM PheGly/Fraction)			1kDa Fractionation (mM PheGly/Fraction)	
	Feed	Retentate	Permeate	Retentate	Permeate
No Pepsin (Run 26)	95.8 ± 8.2	70.9 ± 4.7	36.5 ± 3.9	15.4 ± 2.2	12.2 ± 0.8
Higher Pancreatin (Run 28)	142.0 ± 7.8	111.7 ± 71.8	54.0 ± 5.0	19.0 ± 2.1	13.7 ± 0.6
Midpoint (Runs 20 and 23)	86.7 ± 4.5	56.6 ± 3.4	33.5 ± 3.0	12.8 ± 1.3	6.2 ± 0.6

Samples of the permeate and retentate solutions were freeze-dried and stored at room temperature until antioxidant tests were performed.

6.2.1 Fouling Behaviour

Similar to the initial factorial design ultrafiltration work, a significant fouling layer was observed to form on the 3 kDa membrane but no significant fouling layer was observed to form on the 1 kDa membrane during the filtrations. To gain a better understanding of the fouling mechanisms present in this dead-end filtration process, the permeate flux data gathered for these two extension ultrafiltrations was studied to give information on the fouling mechanisms present.

Using the linearized form of the permeate flux relationships described by Bowen and co-workers in Equations 3-6 (Bowen *et al.,* 1995), these filtrations like the factorial design filtrations show evidence of cake fouling at the ultrafiltration membrane. The figures used in this determination can be found in Appendix B.

6.3 Antioxidant Properties

Once fractionated by ultrafiltration, the No Pepsin, Higher Pancreatin, and Midpoint digests were freeze dried, and the antioxidant capacity of each fraction was determined using the FCR and ORAC assays.

6.3.1 FCR Assay

The electron donating antioxidant capacities determined using the FCR assay for the extension digests, expressed in terms of equivalent antioxidant capacity of mg Trolox/g fraction and mg Trolox/fraction, are summarized in Table 32.

Table 32: FCR Antioxidant Capacity Expressed in Equivalents of Trolox (n=3, mean ± SD)

Filtration Fraction	mg Trolox/g Fraction			mg Trolox/Fraction		
	No Pepsin (Run 26)	Higher Pancreatin (Run 28)	Midpoint (Runs 20 and 23)	No Pepsin (Run 26)	Higher Pancreatin (Run 28)	Midpoint (Runs 20 and 23)
Feed	87.8 ± 16.1 [a,b]	82.5 ± 15.3 [a,b]	79.9 ± 16.1 [a]	430.4 ± 79.1	523.4 ± 96.7	458.4 ± 92.5
3 kDa Retentate	97.8 ± 25.3 [a,b]	72.0 ± 1.3 [b]	97.2 ± 23.2 [a]	293.4 ± 75.8	231.0 ± 4.3	297.8 ± 71.3
3 kDa Permeate	62.1 ± 24.1 [b]	76.4 ± 6.5 [b]	102.1 ± 12.7 [a]	82.6 ± 32.0	191.8 ± 16.3	173.5 ± 21.6
1 kDa Retentate	122.0 ± 67.1 [c]	70.5 ± 6.7 [b]	89.9 ± 13.5 [a]	69.5 ± 38.2	66.3 ± 6.3	67.8 ± 10.2
1 kDa Permeate	119.6 ± 4.4 [c]	68.6 ± 12.4 [b]	95.0 ± 9.2 [a]	44.3 ± 1.6	48.0 ± 8.7	29.0 ± 2.8

Values with the same letter are not significantly different (P = 0.05)

The antioxidant capacity of both the No Pepsin and the Higher Pancreatin digests prior to ultrafiltration are not significantly different from the antioxidant capacities obtained for the Low, Centre, and High digests performed as part of the initial factorial design. However, the 1 kDa retentate and permeate fractions of the No Pepsin digest exhibits higher antioxidant properties in the FCR assay. Tukey's paired comparison was performed at the 95% confidence level to indicate which fractions were significantly different from each other. Only the 1 kDa retentate and 1 kDa permeate of the No Pepsin digest were significantly higher at a 95% confidence level in antioxidant capacity than the other fractions tested here.

6.3.2 ORAC Assay

The ORAC hydrogen donating antioxidant capacities determined for the extension digests, expressed in terms of equivalent antioxidant capacity of mg Trolox/g fraction and mg Trolox/fraction, are summarized in Table 33.

Table 33: ORAC Antioxidant Capacity Expressed in Equivalents of Trolox (n=3, mean ± SD)

Filtration Fraction	mg Trolox/g Fraction			mg Trolox/Fraction		
	No Pepsin (Run 26)	Higher Pancreatin (Run 28)	Midpoint (Runs 20 and 23)	No Pepsin (Run 26)	Higher Pancreatin (Run 28)	Midpoint (Runs 20 and 23)
Feed	142.5 ± 5.5 [a]	118.3 ± 3.3 [c]	116.0 ± 6.7 [c]	698.5 ± 27.0	750.2 ± 20.8	662.1 ± 38.3
3kDa Retentate	139.2 ± 8.2 [a]	101.8 ± 0.9 [d]	128.9 ± 3.7 [a]	417.6 ± 24.7	326.9 ± 2.8	394.5 ± 11.3
3kDa Permeate	144.0 ± 8.8 [a]	127.0 ± 11.0 [a,c]	128.4 ± 5.0 [a]	191.5 ± 11.7	318.7 ± 27.6	219.0 ± 8.6
1kDa Retentate	151.2 ± 8.1 [a]	113.0 ± 6.2 [c]	114.3 ± 3.4 [c]	86.2 ± 4.6	106.2 ± 5.8	86.7 ± 2.6
1kDa Permeate	194.4 ± 8.5 [b]	129.5 ± 12.3 [a]	139.2 ± 7.9 [a]	71.9 ± 3.2	90.6 ± 8.6	42.6 ± 2.5

Values with the same letter are not significantly different (P = 0.05)

Similar to the antioxidant capacity measured by the FCR assay, the 1 kDa permeate of the No Pepsin digest exhibited a greater antioxidant capacity compared to the other fractions and digests studied here. Tukey's paired comparison testing was performed at the 95% confidence level and indicated that the digest performed without pepsin consistently had a higher antioxidant capacity than the corresponding fraction of the Higher Pancreatin digest. Because the extension digests were only performed once, the results show a promising trend but additional replicates are needed to confirm these findings. The ORAC antioxidant capacity of Midpoint digests Runs 20 and 23 in

mg Trolox/g fraction was found to be not significantly different from the Centre digest at a 95% confidence level.

6.4   Antioxidant Model

The effects of the digestion conditions and ultrafiltration fractionation on the antioxidant capacity of the peptides were modeled. The antioxidant capacity of the peptides to donate protons quantified using the ORAC assay was used to build this empirical model. The relative importance of each term in these antioxidant capacity models is summarized in equation 12. The validity of the model was tested by Normal and Residual Plots in Figures 18 and 19 respectively.

Equation 12:   Empirical model for the production of proton-donating antioxidant peptides
   (natural variable form)

ORAC antioxidant capacity [mg Trolox/g sample] = 160 mg/g + 0.68 [min pancreatin]
                                                                                                 − 0.07 [min pepsin]

From the limited ORAC antioxidant capacity information available, some preliminary figures were generated to help identify the terms that would likely have a significant effect on the resulting antioxidant capacity. These figures revealed that all of the individual parameters likely had a role to play in the antioxidant capacity and therefore no interaction terms could be included due to the limited number of independent experimental conditions used to produce the antioxidant capacity data. These figures are included in Appendix C.

Due to the undesigned nature of the ORAC antioxidant capacities generated, the model was generated using natural variables only. The empirical model that best explains the factors that contribute to the ORAC antioxidant capacity of the resulting peptides is very closely related to equation 9, the model developed in Chapter 5 using the factorial design method to determine which digestion conditions are best suited to maximizing peptide production. One should note that the antioxidant capacity constant term of 160 mg Trolox/g sample is considerably higher than the undigested SPI ORAC antioxidant capacity of approximately 14 mg Trolox/g sample, further indicating that at least one term is missing from this model.
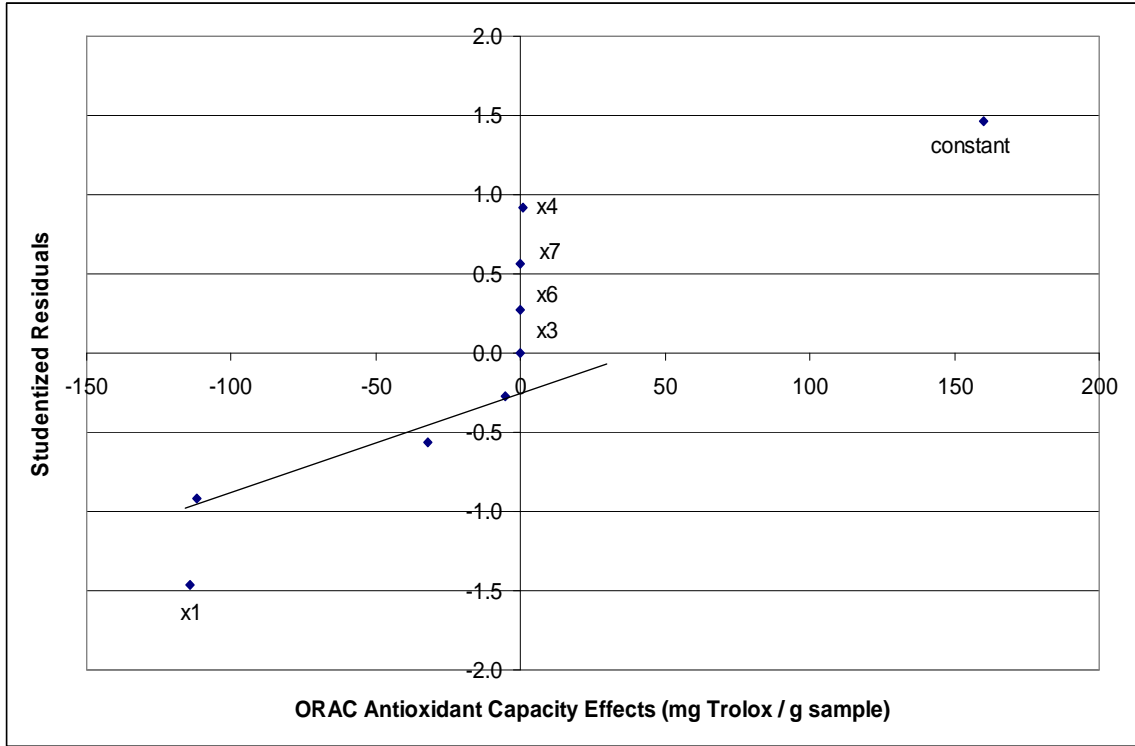
Figure 18: Normal plot in natural variables, revealing the factors that significantly impact the production of antioxidant peptides

From the Normal Plot, two possible options for antioxidant models exist: one case including two ultrafiltration fraction terms, which was found to be missing terms when the residual plot was considered. Therefore, the model given by equation 12 was selected. While the Studentized residual plot for this model does not indicate that an underlying function has not been addressed in the model, the Studentized residuals fall well outside the desired range of 3 to -3 (Montgomery, 2005). This finding indicates that equation 12 does not fully explain the factors that contribute to antioxidant peptide production.
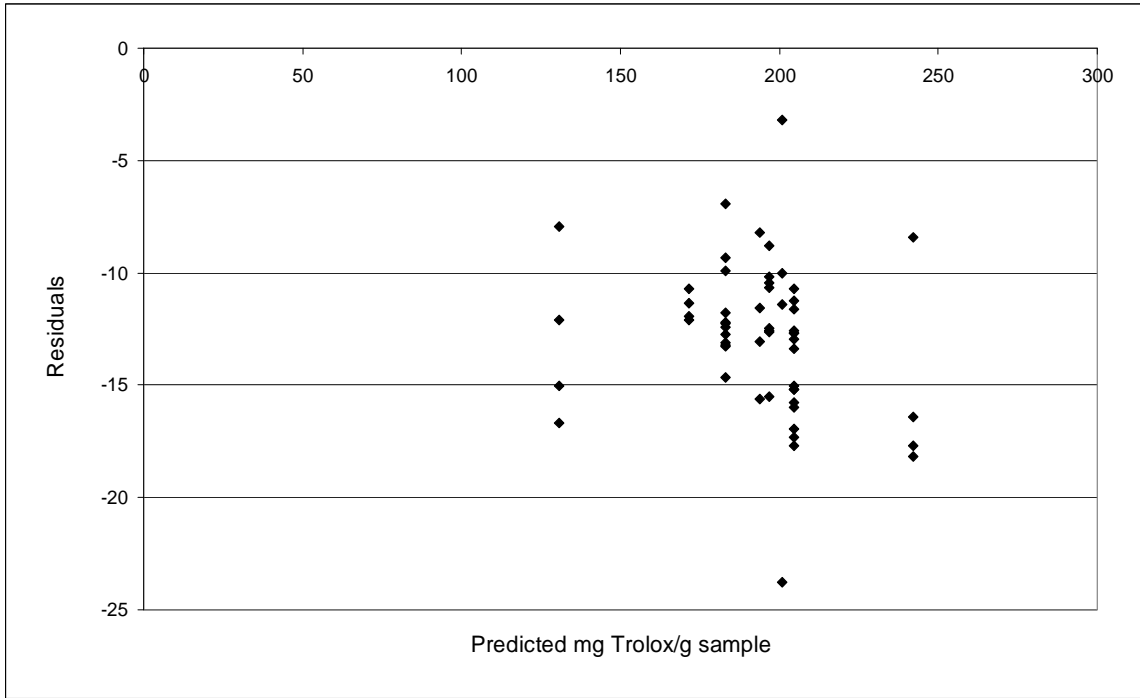
Figure 19: Studentized residuals versus the predicted values for the ORAC antioxidant capacities

7   Discussion

7.1 Soy Protein Source

A commercially available SPI was used as the raw ingredient in this study for several reasons, including its consistent composition, low cost of production, and larger production volumes when compared to a SPI produced in the laboratory. The SPI selected, ADM PRO-FAM 974, containing a minimum of 90% protein (ADM, 2007), is alcohol washed and therefore relatively low in isoflavones at 0.4 mg isoflavones/g protein (Chang, 2007).

Soy isoflavones, consisting primarily of genistein, diadzein, and glycetein, are bound to carbohydrates and have been linked to health benefits such as acting as potent antioxidants (Shih *et al.,* 2002), including the reduction of oxidative stress in rats (Khan & Sultana, 2004). Therefore, the low isoflavone content of the ADM PRO-FAM 974 SPI was desirable when studying the antioxidant properties of the peptides without the interference of other antioxidants.

7.2 Enzymatic Hydrolysis

Peptides have been demonstrated, in the large majority of cases, to have substantially higher antioxidant capacity than the intact proteins (Elias *et al.,* 2008) and therefore enzymatic hydrolysis was employed in this study to produce SPI peptides. Before considering the enzymatic hydrolysis of the SPI, the solubility of the SPI in aqueous solutions was investigated because the ability of the enzyme to access the desired amino acids will improve the degree of enzymatic hydrolysis. The digestion of the SPI with the enzymes pepsin and pancreatin from porcine sources over the range of conditions employed is discussed, starting with the experimental data, then the solubility of the SPI, the enzymes employed, and the peptide concentration monitoring technique.

The pepsin and pancreatin enzyme to substrate ratios, pH, temperature, and digestion times employed in this work are based on the conditions used by Vilela and co-workers (Vilela *et al.,* 2006), which dictated the conditions used in the preliminary digests described in Chapter 4. These digestion conditions provided a good preliminary understanding of the behaviour of the pepsin and pancreatin with the SPI. Each enzyme was able to increase the peptide concentration in the time allotted and under the digestion conditions employed individually (from approximately 13 mM PheGly prior to digestion to 23 mM PheGly after one enzyme digestion) and had an additive effect

on the peptide concentration when the two enzymes were applied sequentially (final peptide concentration near 32 mM PheGly).

Enzyme concentrations and digestion times for the factorial design work described in Chapter 5 were selected by Bissegger (Bissegger, 2008). The extension digestion conditions were selected based on the peptide concentrations and antioxidant capacities measured from the preliminary and factorial design digests.

7.2.1 Quantifying Total Peptide Concentration (OPA Assay)

The preliminary digestions of the SPI performed in Chapter 4 quickly indicated that the Bradford method could not be used to adequately follow the progression of the enzymatic digestion. Other methods for monitoring the progress of the digestion were considered, which lead to the adoption of the OPA method to monitor the digestion progression.

As presented in Section 2.3.2, the basis of the OPA assay is to measure the increase in absorbance at 340 nm as a result of the reaction of the α-amino group of the peptide(s) with OPA and β-mercaptoethanol in an alkaline environment (Church et al., 1983). The reaction of OPA with the primary amino groups is complete within 0.1 to 1.0 minutes at room temperature but these derivatives are unstable due to a sulphur-oxygen rearrangement and may lose the ability to be detected within as little as 10 minutes (Shively, 1986). A two minute delay between the combining of the peptide solution and the reading time is employed to obtain maximum colour development but the solution is read after exactly two minutes in all cases to avoid issues with absorbance decay. The good resolution in aqueous solutions with an α-amino concentration of as little as 7 μM and measuring the presence of peptides having a molecular weight less than 6 kDa (Lemieux et al., 1990) makes this method a good choice for monitoring the enzymatic digestion of the SPI. Acrylic (PMMA) cuvettes were selected for use with this assay due to the useable range of 280-800 nm, unlike the interference that could have resulted from using polystyrene (OPS) which has a useable range of 340-800 nm (VWR, 2006).

The low response of cysteine, lysine, and hydroxylysine, and the lack of reaction between OPA and proline and hydroxyproline (Zumwalt et al., 1998) were not expected to be a significant problem because proline makes up approximately 5% of the amino acids in the SPI, with the low response amino acids making up less than 10% of the amino acids (ADM, 2007). Furthermore, this poor

response would only be an issue if one of these two residues appears at the N-terminal of the peptides produced by the enzymatic digestion.

The absorbance values of α-amino and ε-amino groups in the OPA assay are similar (Church *et al*., 1983), leading to potentially false high readings for peptide solutions rich in lysine, arginine, and glutamine. This false positive result is not likely a significant source of error here since the amount of each amino acid present in solution is not expected to change as a result of the enzymatic digestion. However, the false positive of the ε-amino groups may have become an important consideration when measuring the peptide concentration in each of the ultrafiltration fractions.

The decision to use the dipeptide PheGly as the standard peptide is a reasonable choice, as these amino acids comprise approximately 10% of the amino acid residues present in the SPI (ADM, 2007) and do not contain any of the amino acids known to be problematic in this assay. Church and co-workers successfully used the dipeptide LeuGly as their standard when studying the proteolytic hydrolysis of β-lactoglobulin and β-lactalbumin (Church *et al.,* 1983); Vilela and co-workers successfully used PheGly as the standard for the digestion of whey protein isolates (Vilela *et al.,* 2006). Before every digestion was started, a calibration curve covering the range of peptide concentrations was performed and a linear response was obtained and required before the digestion was allowed to proceed. The OPA solution is known to age and the absorbance increases slightly with time so the calibration curve was also tested at the end of each digestion to obtain the most representative calibration curve.

The OPA assay pH of 9 is not problematic since it is far from the isoelectric point for soy proteins near pH 5, so poor SPI solubility in the assay media should not be an issue. The solubility of the peptides is likely different from the SPI due to the enzymatic digestion process, although the formation of a precipitate in the OPA assay media that would indicate a lack of solubility was not observed. Furthermore, the presence of the SDS and β-mercaptoethanol in the OPA solution ensures the denaturation of the peptide or protein and good access to the α-amino group (Church *et al*., 1983). A small amount (10 μL) of the digest media is added to the 1 mL of OPA solution for analysis; the pH of the digestion media of 1.5 for the pepsin portion of the digest does not likely play a role in the effectiveness of the peptide reading when compared to the pH 7.8 media for the pancreatin digests.

7.2.2 SPI Solubility

As presented in Section 2.1, soy proteins are a mixture of proteins largely comprised of the salt soluble globulins associated with the oil bodies (Liu, 1997). According to literature, the isoelectric point of soy proteins is approximately pH 5 (Sathe, 2002), which agrees well with the solubility data in Section 4.1.2 where the ADM PRO-FAM 974 SPI has a minimum solubility near pH 5.

The denaturation of globular proteins, which is expected as a result of the extensive processing the ADM PRO-FAM 974 SPI has likely undergone, leads to modification of the quaternary structure of some of the proteins and the unfolding of the polypeptide chains of the various soy proteins (Kumar *et al.,* 2002). As a result, amino acids with residues typically located near the interior of the protein are more accessible to water in an aqueous medium such as the digestion media used in this study, which may contribute to the decreased solubility, presented in Section 4.1.2. The maximum solubility of the SPI was approximately 15 mg SPI/mL MilliQ water near pH 11. However, poor solubility cannot always be correlated with a high degree of soy protein denaturation and may also result from protein aggregation (Arrese, Sorgentini, Wagner, & Anon, 1991).

For this study, the digestion conditions employed of pH 1.5 for the pepsin digestion and pH 7.8 for the pancreatin digestion are at pH above or below the SPI isoelectric point so reasonable substrate-enzyme interaction is expected, although the solubility of the SPI in the MilliQ water is still quite low at the pH employed. The solubility at the two digestion pH are comparable at approximately 12 mg SPI/mL MilliQ water so neither enzymatic hydrolysis reaction should have advantageous access to the substrate SPI.

7.2.3 Pepsin

The enzymatic hydrolysis with pepsin was performed near the optimum pH conditions for porcine pepsin of pH 2 to 4 and well below pH 6 where the enzyme is irreversibly denatured due to the destruction of the tertiary structure (Sigma-Aldrich, 2008, Qiao, Gumpertz, & van Kempen, 2002). Pepsin is an endoproteinase that preferentially cleaves at the C-terminus to the amino acid residues phenylalanine and leucine, and glutamic acid to a lesser degree (Sigma-Aldrich, 2008), resulting in peptides with hydrophobic and aromatic amino acids at the C-terminal (Lo *et al.,* 2005). Good digestion of the SPI proteins was expected because the three amino acids targeted by pepsin make up nearly one third of the amino acids found in the ADM PRO-FAM 974 SPI on a mass basis

(ADM, 2007). The significant increase in peptide concentration in the first 30 to 45 minutes of the enzymatic hydrolysis supports this finding, but the leveling off near a peptide concentration of 23 mM PheGly equivalents does not.

Qiao and co-workers have observed that pepsin is autolytic, that is the enzyme will hydrolyze the peptide bonds of the pepsin molecules, and that the loss of pepsin activity appears to follow first order kinetics. This group specifically considered the effects of porcine pepsin concentrations of 0.05, 0.10, and 0.20 mg/mL at pH 2 and 4 in citrate buffer (Qiao *et al*., 2002). The porcine pepsin used by Qiao and co-workers was obtained from Sigma-Aldrich as well but had a different reported activity of 3200-4500 units/mg protein compared to the porcine pepsin P7012 used in this study with an activity of 2500-3500 units/mg protein (Sigma-Aldrich, 2008). While there is some overlap in the range of enzyme activity, the activity of the enzyme used by Qiao and co-workers is expected to be greater than that used in this study. Therefore, at the same enzyme to substrate (w/w) ratio but the different enzyme activity ranges, it is expected that Qiao and co-workers will have produced more peptides and experienced increased hydrolytic behaviour from the pepsin.

Qiao and co-workers also observed that an increase in solution pH and pepsin concentration have been shown to increase the pepsin's half-life (Qiao *et al*., 2002). Therefore the digestion solution of pH 1.5 employed in this current study may have increased the autolytic effects of the pepsin employed.

The instructions provided by Sigma-Aldrich, the pepsin supplier, call for the use of cold 0.01 M HCl at levels of 0.01 to 0.05 mg pepsin/mL 0.01 M HCl (Sigma-Aldrich, 2008). The conditions employed in the factorial design work described in Table 4 are outside of this concentration range, with the Low condition having an enzyme concentration of 3.1 mg pepsin/mL 0.01 M HCl. Once added to the digestion media, the enzyme concentration falls to between 0.15 mg pepsin/mL digestion media for the Low pepsin concentration condition and 0.30 mg pepsin/mL digestion media for the High condition, both of which are still above the recommended pepsin concentration.

The enzyme to substrate ratio is another way of measuring the enzyme concentration and takes into account the amount of substrate available for the enzyme to act upon, ensuring that sufficient substrate is present. Other researchers have used pepsin to produce peptides at a variety of conditions. Kinekawa and co-workers used a porcine pepsin (Sigma-Aldrich P6687) with an activity of 3200-4500 units/mg to which they added an aqueous whey protein solution of 70

mg/mL at 1:200 enzyme to substrate w/w ratio, and performed the digestion at pH 2 and 37ºC for 1 hour (Kinekawa *et al*., 1996). Pena-Ramos and Xiong digested SPI with pepsin (Sigma-Aldrich, unknown type) at a 1:100 pepsin to substrate w/w ratio, 37ºC, pH 2, and 1 hour (Pena-Ramos *et al*., 2002). Lo, Farnworth, and Li-Chan performed a batch digestion of SPI with Sigma-Aldrich pepsin P7012, the same type employed here, in an aqueous solution with 5 g SPI, 100 mL water, and 200 mg pepsin yielding a 1:20 enzyme to substrate w/w ratio (Lo *et al*., 2006). Therefore the conditions in this study do not appear to be significantly different from those employed by other researchers claiming to mimic the conditions found in the human digestive tract, although pepsin with different enzyme activities were used in some cases. One should also note that mechanical factors involved in the *in vivo* digestion process are not considered here (Kong & Singh, 2008).

The pepsin enzyme to substrate ratios used in this study was varied between 1:200 and 1:100 w/w for the Low and High conditions respectively. Therefore, the combination of higher enzyme concentration and lower pH likely explains the observed decrease in hydrolysis between 30 and 45 minutes, most likely due to autolysis of the pepsin.

7.2.4 Pancreatin

The pancreatin used in this study is a mixture of enzymes obtained from the porcine pancreas. According to the supplier, the mixture in this study is a mixture of amylase, trypsin, lipase, ribonuclease, and protease that belong to the EC 3.4.21 family of serine endoproteinases with an optimum pH near 9 (Sigma-Aldrich, 2009). Endoproteinases cleave within the peptide chain, unlike exoproteinases that cleave from the ends of the peptide chains, producing a selection of smaller peptides.

Mutilangi and co-workers studied the roles of trypsin and chymotrypsin on milk proteins and observed a rapid reaction rate up to 20 minutes and a slower reaction rate afterward. The authors suggest that this decrease in reaction rate may be related to loss of enzyme activity, substrate exhaustion, and/or end-product inhibition, but when the reaction conditions were considered, only end-product inhibition would explain the decrease in reaction rate. Mutilangi and co-workers also indicated that the degree of hydrolysis obtained for each enzyme increased with an increase in the enzyme to substrate ratio (Mutilangi *et al*., 1995).

Also important to consider is that soybeans contain two trypsin inhibitors, Kunitz and Bowman-Birk, which should be denatured and therefore deactivated by the heat treatments employed in the

oil extraction and SPI production processes (Liu, 1997, Vereijken, 2000). However, heat treatment alone is not always sufficient; Wang and co-workers observed that 20% of Kunitz trypsin inhibitor activity remained in the soy flour they were studying after heating at 120ºC for 30 minutes although all of the Bowman-Birk trypsin inhibitor was inactivated (Wang, Faris, Wang, Spurlock, & Gabler, 2009). Therefore the soybean trypsin inhibitors may also be playing a role in the digestion profiles observed here.

Trypsin is known to be autolytic and therefore solutions of trypsin should be prepared immediately before use (Copeland, 1994). Digestion by trypsin and chymotrypsin is stopped by freezing the sample (Copeland, 1994), which was the method employed in this study to stop the digestion.

As with pepsin, other researchers have studied the effects of pancreatic enzymes in the hydrolysis of proteins. Pena-Ramos and Xiong studied the hydrolysis of SPI by chymotrypsin (Sigma-Aldrich, unknown type), at 37ºC, pH 8, and an enzyme to substrate ratio of 1:100 w/w for one hour (Pena-Ramos *et al*., 2002). Lo and co-workers studied the effects of a 4 wt% solution of trypsin (Sigma-Aldrich P7545) on SPI at pH 7.5 and 37ºC for 2 hours and found that the hydrolysis slowed considerably in the second hour and produced positively charged C-terminal peptides (Lo *et al*., 2006, Lo *et al*., 2005). Henn and Netto studied the digestion of 13 SPI with pancreatin (unknown type) at a 1:15 enzyme to substrate w/w ratio at pH 8 and 40ºC for a range of times from 48 to 252 minutes to obtain the desired degree of hydrolysis (Henn & Netto, 1998).

Gauthier and co-workers also studied the pepsin and pancreatin digestion of SPI and found that end-product inhibition, especially in the pancreatin digestion portion, resulted in decreased peptide production (Gauthier, Vachon, Jones, & Savoie, 1982). Garrett and co-workers employed similar enzyme concentration, temperature, pH, and time conditions to those used here to model digestion *in vitro* with both pepsin and pancreatin (Garrett, Failla, & Sarama, 1999).

7.2.5 Hydrolysis Conditions

A range of peptide concentrations resulted from the factorial design digestion study, revealing the impact of each of the four digestion factors: pepsin concentration, pancreatin concentration, pepsin digestion time, and pancreatin digestion time. The triplicate centre points showed good reproducibility with less than 5% variability and therefore the applicability of a factorial design to understand the relationship between these factors. Equations 8 and 9 express the linear relationship of these four factors on the resulting peptide concentration. The pepsin concentration was found to

have no significant effect on the final peptide concentration under the conditions employed in the factorial design work given by equations 8 and 9, likely because the pepsin to substrate w/w ratios are high and therefore that the enzyme's effect on producing soy peptides is essentially constant.

Based on the factorial design work performed in Chapter 5, additional digests were performed to test the suggestion from equation 8 that a higher peptide concentration could be achieved by using a higher pancreatin concentration. The peptide concentration reached a maximum near 65 mM PheGly when the pancreatin concentration was set to 4.5 g/L, although this enzyme concentration of nearly double the High pancreatin concentration may be too high since a peptide concentration of 62.3 mM was obtained for the High digest Run 25. This finding was not revealed in the initial factorial design work and equations 8 and 9 indicating no upper limit for the peptide concentration within the range of the enzyme concentrations and times selected. This upper peptide concentration may be due to substrate limitation as a result of either insufficient protein substrate or too high a pancreatin concentration. A second possibility may be the inactivation or autolysis of the pancreatin enzymes at this high peptide concentration.

pH was not actively monitored during the digestion process although a change in pH resulting from the hydrolysis reaction is expected. Furthermore, the ionic strength of the digestion media will also affect the enzyme activity (Whitaker, 1994). While the ionic strength of the digestion media employed here was initially set using 0.01 M HCl for the pepsin digestions and sodium phosphate buffer for the pancreatin digestions, the ionic strength will also be changing as the digestion progresses although it too was not actively measured in this work.

Finally, depending on the enzyme and its purity, some denaturation is expected as a result of the temperature employed. The temperatures used in this study for the pepsin and pancreatin digestions, 37°C and 40°C respectively, are not expected to significantly reduce the enzymes' activity over the duration of the digestion times employed (Whitaker, 1994).

When the additional digests with higher pancreatin concentration and without pepsin were included in the factorial design model, a different linear digestion model began to emerge, as represented by equations 10 and 11. Equations 10 and 11 indicate that pepsin concentration and pepsin digestion time are important factors in the production of peptides, represented by a negative interaction term in the digestion model. This suggests that the pepsin concentration may be significant, but may have been lumped in with the constant term in equations 8 and 9 due to the High and Low

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conditions selected for the pepsin concentration factor (Montgomery, 2005). Consider the constant term in equation 9, the non-coded form of the peptide production model based on the factorial design work, which has a value of 20 mM PheGly. One would expect this constant value to be much closer to the actual initial peptide concentration of approximately 13 mM PheGly observed for the raw SPI if all digestion terms were set to zero. This further supports the conclusion that the pepsin concentration factor term is lumped into the constant term resulting from the high pepsin concentration employed in the factorial design work. A full understanding of these implications is not known because a full factorial design digestion study was not performed at each of these new conditions but is an area for possible future work.

Temperature is known to play a role in the denaturation of proteins (Mutilangi *et al.,* 1995) and therefore a control sample of SPI in MilliQ water at the levels used in the digestion was run with each of the digests at the time and temperature conditions employed but without enzyme addition. The peptide concentration in this control sample was measured at the same time intervals as the digests and revealed that no significant change in peptide concentration indicative of digestion was occurring as a result of the temperature.

The difference in peptide concentration resulting from digestion with pepsin and pancreatin and also a difference in the type of peptides produced are expected due to the different enzymes employed. However, both pepsin and the pancreatin are endoproteinases so it is expected that both enzymes will be effective at cleaving proteins and longer peptides into small peptides and thus increasing the antioxidant capacity of the SPI (Vilela *et al.,* 2006). Due to the different cleavage sites possible, each enzyme is expected to produce different peptides from the same protein chain, although the majority of the peptides are expected to be somewhat hydrophobic. The hydrophobic peptides will be produced by pepsin and the pancreatic enzymes trypsin and chymotrypsin, all of which are endoproteinases known to cleave at hydrophobic residues. Wang and co-workers observed an improved digestibility of soy proteins with trypsin when the soy was pre-treated to break disulphide bonds (Wang *et al.*, 2009), which unfortunately pepsin will not do. Without knowing more about the exact make-up of the pancreatin enzyme and composition of the SPI, little more can be said about the peptides that are likely produced, except that both pepsin and pancreatin are effective at cleaving the PRO-FAM 974 SPI into smaller peptides and amino acids.

The production of peptides with hydrophobic ends by pepsin and pancreatin supports the polar paradox theory, which states that lipophilic antioxidants are more active in polar mediums while

polar antioxidants are more active in bulk oils (Brand-Williams, Cuvelier, & Berset, 1995). Virtanen and co-workers confirmed this finding, by demonstrating that fractions of milk fermentates containing more hydrophobic peptides had higher antioxidant capacities (Virtanen *et al.*, 2007). Therefore, both pepsin and pancreatin may be effective at producing peptides with good antioxidant capacities in an aqueous environment.

7.3   Membrane Ultrafiltration

The objective of the ultrafiltration was to fractionate a selection of hydrolysates according to the size of the peptides and obtain fractions with improved antioxidant characteristics. A selection of the enzymatic digestions were fractionated by two-stage sequential dead-end ultrafiltration using a 3 kDa followed by a 1 kDa MWCO regenerated cellulose membrane driven by 210 kPa (40 psi) nitrogen at 25ºC. One of each of the preliminary digests (Pepsin, Pancreatin, and Pepsin & Pancreatin) described in Chapter 4, the High, Centre, and Low digests prepared in Chapter 5, one of each of the Higher Pancreatin and No Pepsin digests from Chapter 6, and the two Midpoint digests were fractionated. Stirring was used in the ultrafiltration of the preliminary digests; no stirring was used in the remainder of the filtrations to study the type and severity of membrane fouling. One should note that while concentration of the antioxidant peptides might be considered the primary objective of the membrane ultrafiltration process, a second process where the removal of pro-oxidant peptides or contaminants may be occurring in the retentate. Furthermore, the change in composition resulting from the loss or accumulation of non-peptide components may also play a significant role in the antioxidant properties of the fractions.

The ultrafiltration of the soy digests was performed at 25˚C. At this temperature, the enzymes employed in the production of the peptides may be re-activated. The digestion protocol for pepsin called for the pH to be raised to at least pH 7; pepsin is irreversibly deactivated due to the destruction of its tertiary structure above pH 6 (Qiao *et al.*, 2002). Therefore pepsin is not expected to have been reactivated during the ultrafiltration process. However, pancreatin likely was not irreversibly denatured and this enzyme may have continued to hydrolyze the SPI during ultrafiltration. This possibility was not considered in this study; ultrafiltrations of pancreatin hydrolysates could be performed at 4ºC or have a heat treatment near 100ºC at the end of the digestion to denature the pancreatin to limit this possibility in future work.

7.3.1 Membrane Maintenance

All membranes were handled according to the manufacturer's instructions. All new membranes were washed for the minimum amount of time specified, were cleaned with Tergazyme after each filtration run, and stored in a 10% ethanol solution at 4ºC when not in use (Millipore, no date).

The water flux of each membrane was measured immediately before the digest fractionation was performed. The water flux at the desired pressure was not only required to be approximately 22 L/m^{2}h for the 3 kDa membranes and 16 L/m^{2}h for the 1 kDa membranes at 210 kPa (40 psi) but also needed to show a linear water flux associated with the trans-membrane pressures selected, as indicated in Appendix A. This linear behaviour is indicative of the hydraulic permeability of the membrane and a straight line indicates that the membrane is not prone to pressure-induced deformation (Ghosh, 2003). Any membrane that showed unexpected water flux behaviour, such as a significantly higher water flux indicative of a damaged membrane, was discarded and a new membrane was installed.

7.3.2 Peptide Recovery

Mass balances were performed on the fractions of the High, Centre, Low, Higher Pancreatin, and No Pepsin digests to determine the amount of feed material appearing in each of the 3 kDa retentate, 3 kDa permeate, 1 kDa retentate, 1 kDa permeate fractions. Some loss at each filtration step was expected for material that will have adsorbed onto or deposited within the pores of the ultrafiltration membrane or otherwise become attached to the ultrafiltration equipment and cannot be recovered.

From Table 18, greater losses appear to have occurred with the fractionation of the higher peptide concentration fractions, although the losses are not significantly different when the standard deviation associated with each fraction is considered. From Table 29, the ultrafiltrations of the Higher Pancreatin, Midpoint, and No Pepsin digests reveal that a loss of 10% at each filtration stage is obtained, which agrees with the average loss calculated for both the 3 kDa and 1 kDa fractions of the factorial design digests. Given the size and nature of the ultrafiltration system, up to 10% loss was expected.

The mass fraction data indicates that approximately 50% of the feed material is retained by the 3 kDa membrane while 40% is allowed to permeate through the membrane. For the 1 kDa membrane, a similar trend is observed where 50-60% of the mass is retained by the membrane and

40-30% is allowed to permeate through. This trend holds relatively well for the extension digests with 60% of the feed mass being retained by the 3 kDa membrane, and of that material allowed to permeate through, approximately 60% of it is retained by the 1 kDa membrane. There is a slight although not significant increase in the mass that permeates through the membrane with an increase in the peptide concentration of the feed solution. This follows logically that with a higher peptide concentration one would expect to have more short peptides present in the feed solution and therefore more of these shorter peptides would permeate through the membrane.

Peptide balances were performed on the fractionation of the High, Centre, Low, (Tables 19 and 20) Higher Pancreatin, No Pepsin, and Midpoint (Tables 30 and 31) digests using the OPA assay to quantify the peptides in mM PheGly equivalents. The recovery of peptides from the High, Centre, and Low digests follows the same trend as the total mass recovery, with approximately 50% being retained and 50% permeating through both the 3 kDa and 1 kDa membrane. Similarly, approximately half of the feed solution was retained while half permeated in the recovery of peptides from the Higher Pancreatin, No Pepsin, and Midpoint digests.

The peptide content of each fraction (mM PheGly/g sample) suggests that the lower the peptide concentration in the feed solution, the smaller the peptide concentration in the 1 kDa retentate fraction. This is especially true for the Low and Centre factorial design digests and supports the earlier suggestion that a higher peptide concentration in the digest corresponds to a greater amount of shorter peptides.

7.3.3 Fouling

The permeate flux was measured throughout the ultrafiltration process and plots of the flux decline over time are provided in Figure 4 for the Preliminary SPI digests, Figure 11 for the factorial design High, Centre, and Low digests, and Figure 17 for the extension digestion conditions. The expected permeate flux behaviour is an initially high permeate flux which declines rapidly due to the formation of a fouling layer and appears to reach a lower, "steady-state" permeate flux value which declines and eventually reach a permeate flux of zero (Das *et al.,* 2009, Ghosh, 2003). All figures for the 3 kDa and 1 kDa membrane ultrafiltration show the same general trend expected for dead-end membrane ultrafiltration where a fouling layer rapidly develops and thus decreases the permeate flux. The exception to this observed trend is the Pancreatin digest 3 kDa and 1 kDa ultrafiltration figures (Figure 4), largely due to adjustments made to the nitrogen pressure and stirring speed during the filtration.

Fouling is attributed to the adsorption and deposition of molecules in the feed solution, the SPI hydrolysates here, on the ultrafiltration membrane or within the pores, resulting in a decline in the permeate flux and a change in solute transmission behaviour. This fouling can be reversible or irreversible (Filipe *et al.,* 2005).

The permeate flux for the factorial design samples without stirring dropped to between 1.4 and 2.4 L/m^{2}h for the 3 kDa membrane ultrafiltrations after 180 minutes and the permeate flux for the 1 kDa membrane ultrafiltrations fell to less than 3 L/m^{2}h after 90 minutes. The permeate flux values after this time and before rinsing represent a loss of up to 80% compared to the initial permeate flux, which is expected for high retention situations and dead-end configuration far from desirable for production of large amounts of peptides. According to Tansel and co-workers, membranes are considered fouled when the permeate flux has been reduced by 60% of initial levels (Tansel *et al.,* 2000).

The permeate flux measured for the ultrafiltrations is positively related to a higher peptide concentration in the feed solution, thus the greater the degree of hydrolysis the greater the permeate flux for both the 3 kDa and 1 kDa membrane ultrafiltrations. One possible explanation is that the cellulose membranes used in this study are hydrophilic (Belfort *et al.,* 1994) and a lack of attraction between the membrane and the peptides with hydrophobic ends may also result in less fouling. The lack of strong bonds between the fouling layer and the membranes can also be observed in the photos in Figure 12 where the higher the peptide concentration, the less the fouling layer formed on the dead-end ultrafiltration membrane adheres to itself and the membrane.

Bowen and co-workers have described the fouling of membranes in four categories which can be determined by plotting a function of permeate flux over time and fitting it to a linear function (Bowen *et al.,* 1995). The permeate flux data generated by the ultrafiltration of the SPI digests was plotted and each of these linearized models was considered; these plots can be found in Appendix B. The model that best explains the fouling behaviour, especially for the extension digests presented in Chapter 6 which have been shown to have the highest antioxidant capacity per gram of ultrafiltration fraction, is that of cake filtration, given by Equation 6.

The linearized expression fits the experimental data generated for both the 3 kDa and 1 kDa membrane ultrafiltrations with an R^2 = 0.90 or greater and higher than the R^2 values for the other

three fouling models. However, Tansel and co-workers suggest that measuring the extent of fouling caused by different mechanisms is practically impossible (Tansel *et al.,* 2000), which is also supported by the plots in Appendix B. All models suggested by Bowen and co-workers explain the fouling observed over certain time ranges in terms of R^2 values over some portion of the ultrafiltration process, but not the entire filtration time.

The relationship between higher peptide concentration and increased permeate flux also matches the irreversible fouling data presented in Table 21 where the dead-end ultrafiltration membranes used in the filtration of the factorial design samples was rinsed with water and the water flux was tested. The water flux of the High and Low membranes increased considerably, although it was not equal to the water flux expected for a clean membrane; enzyme cleaning was required to restore the original water flux for the membrane. The water flux for the 3 kDa membranes was approximately 21 L/m^2h compared to the clean membrane water flux of 22 L/m^2h; the water flux for the 1 kDa membranes were approximately 13 L/m^2h compared to the clean membrane at 15 L/m^2h.

The filtration of the Pepsin, Pancreatin, and Pepsin & Pancreatin digests with stirring indicates that the fouling is largely of a reversible nature because the permeate flux declined to between 4 and 10 L/m^2h for the 3 kDa membrane and between 12 and 15 L/m^2h for the 1 kDa membrane. This data supports the scale-up to cross-flow filtration for larger volume production of antioxidant peptides as the portion of the fouling associated with the reversible fouling that would be removed by the cross-flow configuration accounts for the majority of the fouling present in this system (Blanch *et al.,* 1997).

For scale-up purposes, a low cost membrane material such as an organic cellulose-based membrane similar to that used here is recommended due to its non-adsorptive properties, low irreversible fouling, and low protein interaction (Darnon, Lafitte, Belleville, & Rios, 2002, Perry & Green, 1997). However, cellulose-based materials are only recommended for the ultrafiltration of dilute solutions (Millipore, no date) and short-time use, so another membrane material may be better for the ultrafiltration of more concentrated solutions (Perry *et al.,* 1997). Other factors which could be considered to improve the permeate flux in future filtrations include adjustments to the solution pH and salt concentration or trans-membrane pressure pulsations or changes in the feed flow rate to disturb the formation of the fouling layer (Ghosh, 2003). However, adjustments to the feed pH have conflicting benefits: Ghosh reports that filtration of an uncharged feed solution increases

fouling (Ghosh, 2003) while Burns and Zydney report that transmission of a protein is maximized near its isoelectric point (Burns & Zydney, 1999). The feed solutions used in this work had a pH near 7, which is far from the isoelectric point of the raw SPI, but is not known how it relates to the isoelectric point of the target antioxidant peptides.

7.3.4 Peptide Characterization

The analysis of the fractions by MALDI-TOF and SEC-HPLC support the findings from the OPA assay, that the peptide concentration is increasing as a result of the enzymatic digestion and that the digestion conditions do have a significant impact on the resulting peptides. The data gathered from these test methods also suggests that the use of pepsin and pancreatin is an effective way to produce peptides with a molecular mass of between 500 Da and 3 kDa.

The MALDI-TOF analysis was performed over the two molecular mass ranges of 500-4000 Da and 5-20 kDa, to determine the size profile of the SPI and the Pepsin, Pancreatin, and Pepsin & Pancreatin digests (Figures 5 to 9). Unfortunately, the range studied does not provide the full molecular mass profile of the SPI because the majority of the glycinin and conglycinin subunits have molecular masses greater than 20 kDa (Horneffer *et al*., 2007, Liu, 1997), but this method does provide some insight into the peptides produced by the enzymatic digestion.

The removal of contaminants such as the salts present in the digestion media is necessary to obtain good resolution using the MALDI-TOF method (Liebler, 2002) and so the digests were desalted prior to determining the MALDI-TOF profile. However, no reducing agent such as dithiothreitol or β-mercaptoethanol was used to prepare the samples so the molecular masses obtained in this analysis may represent free or molecules cross-linked by disulphide bonds (Vilela *et al*., 2006, Horneffer *et al*., 2007). Therefore, the OPA assay may indicate a higher peptide concentration than that indicated by the MALDI-TOF method since soy proteins do contain some disulphide bonds (Wang *et al.,* 2009).

Figure 5 shows the MALDI-TOF profile for the SPI, followed by Figures 6-8 for the Pepsin, Pancreatin, and Pepsin & Pancreatin digests. A peak appears near 650 Da on all digest profiles but post-source decay analysis was not performed so no additional information on the amino acid sequence of this peptide was gathered (Liebler, 2002). All three digestions show an increase in the number and concentration of peptides produced, with peaks corresponding to peptides at a variety of molecular masses. The Pepsin digest gave an increase in peptides in the range 4-10 kDa with a

significant peak near 5900 Da while Pancreatin gave a similar increase in 4-10 kDa peptides and a peak near 8100 Da. When the enzymes were used sequentially, the composition of the digest shows an increase in smaller peptides compared to the single enzyme digests, with peaks near 520, 1230, and 1620 Da. This finding confirms that pepsin and pancreatin cleave the SPI at different sites and effectively produce low molecular mass peptides.

Figure 13 gives the molecular mass distribution obtained for the SPI, High, and Low digests using size exclusion chromatography. This analysis was performed on a Superdex Peptide HR 10/300 GL column with a spherical composite of cross-linked agarose and dextran specially designed for separating peptides with a molecular mass of 100-7000 Da (GE Healthcare, 2009).

The molecular mass data provided on these three protein and peptide samples indicate that the undigested SPI contained a significant quantity of a peptide with a molecular mass of 840 Da. This peptide becomes less significant in the High and Low digests where other peptides dominate. In the Low digest, a peptide near 1.95 kDa (with a shoulder on the left) is the major component in the digestion mixture with a small peak near 13 kDa that may be a partially digested fraction of the SPI, while the High digest contains a peptide near 1.59 kDa as the major component. Other peptides are present in the High and Low digests such as two below 1 kDa, one of which may be the 840 Da present in the undigested SPI. One final peak appears in both digestion media near the end of the HPLC run, indicating a very small molecule that may be free amino acids or another component from the digestion media (Deyl, 1998).

7.4 Antioxidant Capacities

The next step was to quantify the antioxidant capacity of the peptides produced and to study whether the digestion conditions effective at producing large concentrations of peptides were also effective at producing peptides with significant antioxidant capacity. As indicated in Chapter 2, antioxidants may exhibit a variety of functional mechanisms and a variety of tests exist to measure antioxidant properties. Therefore, several test methods should be used to quantify the antioxidant properties of a given substance. In this work, the electron-donating assay first selected was the DPPH assay in a hydrophobic solution, followed by the FCR assay to measure the electron-donating capacity in a hydrophilic medium (Huang *et al.,* 2005). The hydrophilic ORAC assay with fluorescein was selected as the hydrogen-donating antioxidant assay.

7.4.1 DPPH Antioxidant Capacity

Blois suggested the use of DPPH (2,2-diphenyl-1-picrylhydrazyl) to mimic the stabilization of other free radicals by antioxidants (Blois, 1958). The basis of the DPPH assay is the scavenging of the stable, hydrophobic DPPH radical by antioxidants capable of donating an electron, thus reducing the deep purple radical to colourless hydrazine (Huang *et al.,* 2005). The scavenging reaction is monitored through the loss of absorbance near 517 nm when the antioxidant is added to the DPPH radical at concentrations up to $5x10^{-4}$ M in methanol or ethanol solutions (Blois, 1958). This absorption is independent of pH in the range of pH 5 to 6.5 so a buffer solution is combined with the ethanol in a 50:50 (v/v) mixture (Wang *et al.,* 2003).

Standard antioxidants used in the DPPH assay include ascorbic acid, which is known to neutralize the DPPH radical at a ratio of up to 2:1 respectively (Blois, 1958, Brand-Williams *et al.,* 1995). The rate of the decolourization reaction is based on the reaction kinetics of the antioxidant being studied with the DPPH radical in the medium selected and therefore reaction time can vary from less than 1 minute to more than 30 minutes (Brand-Williams *et al.,* 1995).

The DPPH assay was the first assay used to quantify the antioxidant capacity of the peptides produced in terms of the electron-donating or reducing capacity and quantified in relative units of the known antioxidants ascorbic acid and Trolox. A linear calibration curve with one of the standard antioxidants was generated before testing each set of fractions, as described in Chapter 4. Initially ascorbic acid was selected as the standard antioxidant but was replaced with Trolox, a water-soluble analog of tocopherol, in future assays due to the latter chemicals improved stability.

The electron-donating antioxidant capacity of selected fractions of the preliminary digests Pepsin, Pancreatin, and Pepsin & Pancreatin were determined, as summarized in Table 11, and the factorial design High, Centre, and Low digests Table 22. The antioxidant capacity of certain fractions such as the 3 kDa retentate were not quantified due to the high turbidity that resulted in the assay media; the peptides were immiscible with the 50/50 v/v ethanol and water assay media. Therefore, the DPPH assay was only run on the 3 kDa permeate, 1 kDa retentate, and 1 kDa permeate samples of the various digests performed.

Unfortunately, the DPPH assay was not sensitive enough to allow the fractions or digests tested to be distinguished from one another. More variability was observed in the antioxidant capacity values between replicates than between the different digests or ultrafiltration fractions for the High,

Centre, and Low digests. This leads to a few possible conclusions: the antioxidant capacities of the samples tested are equivalent, the DPPH assay is not sufficiently sensitive to distinguish between samples, or that the peptides produced have insignificant antioxidant capacity when donating electrons in a hydrophobic medium. This latter explanation may be the best, especially when the relatively low antioxidant capacity of approximately 0.3 mg Trolox/g sample for the factorial design digests is considered.

The antioxidant capacities observed for the SPI hydrolysates produced here were compared to other known peptides. Wang and co-workers studied the antioxidant ability of the fermented soy products and found that these peptide-containing samples had antioxidant capacities of between 0.75 and 1.9μg α-tocopherol/mg sample in the 50% ethanol DPPH assay (Wang *et al.,* 2003). Similarly, Chen and co-workers studied the antioxidant properties of soy peptides and measured the antioxidant properties in the 40% aqueous ethanol DPPH assay; the peptides were found to have negligible antioxidant ability at concentrations between 50 and 200μM while the standard antioxidant δ-tocopherol showed a significant dose-dependent response over the same concentration range (Chen *et al.,* 1998). The DPPH results expressed for the peptides produced by Wang and co-workers and Chen and co-workers are purified peptides; the SPI hydrolysates studied here were not purified to the same extent, and therefore interactions from impurities in the digestive solution may have affected the antioxidant capacity determination.

Issues also exist with the DPPH assay itself. The absorbance of the DPPH free radical degrades over time, thus the DPPH solution storage bottle was wrapped in aluminum foil to reduce the degree of UV degradation. This degradation also influenced the selection of a 10 minute assay time so as to minimize the effects of this colour loss. However, not all antioxidants may have fully reduced the solution in that time, thus giving a potentially false low antioxidant capacity for certain peptide fractions. For example, Brand-Williams and co-workers found that ascorbic acid is a very fast acting antioxidant with a reaction time of less than one minute, while δ-tocopherol took between 5 and 30 minutes to produce a sufficient colour change and BHA and BHT needed one to six hours (Brand-Williams *et al.,* 1995). Finally, the data generated by the DPPH assay were taken as the ratio of the final absorbance over the initial absorbance to minimize the impact of run-to-run variability resulting from this degradation, including the calibration data.

An additional drawback of the DPPH assay is that the DPPH radical is a relatively stable, organic, nitrogen radical which is significantly different from the more reactive peroxyl radicals often found

in lipid peroxidation reactions (MacDonald-Wicks *et al.,* 2006). The interaction of a potential antioxidant with the DPPH is dependent on its structural conformation, so the comparatively poor antioxidant capacity exhibited by the soy peptides in this study may be the result of a structural incompatibility (Brand-Williams *et al.,* 1995). Furthermore, the stoichiometry between the stable, synthetic DPPH radical and representative standard antioxidants is different; for example, Trolox scavenges two DPPH, ascorbic acid reduces nearly two DPPH, rosmarinic acid reduces nearly four DPPH, δ-tocopherol reduces nearly two DPPH, and BHA and BHT will each scavenge two or more DPPH free radicals (Sanchez-Moreno, 2002, Brand-Williams *et al.,* 1995). Finally, the DPPH radical may even react with other radicals and components of the system in unrelated reactions (Prior *et al.,* 2005).

Another possible reason for the poor results obtained from this assay is the solvent. Based on literature, the majority of researchers prepare their antioxidant samples (often polyphenolic extracts) and the DPPH reagent solution in very concentrated methanol or ethanol solutions (Brand-Williams *et al.,* 1995, Velioglu, Mazza, Gao, & Oomah, 1998). As indicated by Blois, several proteins are insoluble in the DPPH reagent and although attempts were made to overcome this issue with the use of a 50% ethanol solution, the sample solubility was an issue (Blois 1958, Wang *et al.,* 2003). A "polar paradox" exists in the field of antioxidant study, where lipophilic antioxidants are more active in polar mediums while polar antioxidants are more active in lipophilic mediums (Brand-Williams *et al.,* 1995), but that was not the case for the SPI peptides studied here.

Based on the poor sensitivity of the DPPH assay, the antioxidant capacities of the various digests and ultrafiltration fractions produced in this study could not be distinguished from one another. Therefore, the Folin-Ciocalteu Reagent (FCR) assay was identified as an alternate reduction-based assay.

7.4.2 FCR

Huang and co-workers recommend the Folin-Ciocalteu Reagent assay as the standard method to measure the reducing or electron-donating ability of a sample due to the large body of information available to compare antioxidants and relative ease of performing the assay (Huang *et al.,* 2005). Concerns have been raised by researchers about the interfering substances present in the sample, such as sugars, organic acids, some inorganic substances such as Fe(II), proteins, certain amino acids, ascorbates, citrates, and sulfites (Singleton *et al.,* 1999, Prior *et al.,* 2005, Stevanato *et al.,*

2004, MacDonald-Wicks *et al.,* 2006). While data exists for the antioxidant capacity of a variety of antioxidants quantified using the FCR assay, no references that used the FCR assay to quantify the antioxidant capacity of proteins and/or peptides using Trolox as the standard antioxidant were located.

In this study, the standard FCR assay described by Box and co-workers was used to measure the antioxidant capacity of all ultrafiltration fractions for the preliminary (Pepsin, Pancreatin, and Pepsin & Pancreatin), the factorial design (High, Centre, and Low), and the extension (No Pepsin, Higher Pancreatin, and Midpoint) digests. A linear calibration curve was prepared with Trolox over the concentration range described in Chapter 4 before any hydrolysate samples were tested. The FCR antioxidant capacities generated for these digests are summarized in Tables 12, 23, and 32.

Unlike the DPPH assay, the FCR assay was sensitive enough to reveal some significant differences in the electron-donating antioxidant capacities between both the digestion conditions and the ultrafiltration fractions. To start, the standard deviation in the FCR antioxidant capacity of the replicates of the factorial design digests was smaller than that observed for the DPPH antioxidant capacity values, indicating that the FCR assay is more robust than the DPPH assay.

The antioxidant capacity of the 1 kDa retentate fraction of the Pancreatin digest was found to be significantly higher than that 1 kDa permeate fraction of the Pepsin digest, further supporting the conclusion that different enzymes produce different antioxidant peptides. Differences were observed between some of the ultrafiltration fractions of selected digestion replicates for the Low and Centre digests, although no overall trends were observed. While the antioxidant capacity of the raw digests for the No Pepsin and Higher Pancreatin digests are not significantly different from the raw digest for the Low, Centre, or High digests, the antioxidant capacity of the 1 kDa retentate and 1 kDa permeate fractions (No Pepsin) were found to be significantly higher than all other fractions tested although not significantly different from each other. The antioxidant capacity for the 1 kDa retentate and permeate of the No Pepsin digest were estimated at 122.0 ± 67.1 mg Trolox/g sample (n=3) and 119.6 ± 4.4 mg Trolox/g sample (n=3) respectively.

Some concerns still remain with the adaptation of the FCR assay for use in quantifying antioxidants, especially antioxidant peptides or proteins. Box and co-workers indicated that below pH 9, the Folin-Ciocalteu reagent shows evidence of some blue colour formation but solution

remains yellow while absorbance decreases sharply above pH 11 (Box, 1983). The pH 10 employed for the FCR reaction media may denature some proteins and lead to false antioxidant capacity readings for antioxidants that would be used at a different pH. Box also observed that at 20°C, the FCR is stable for four hours, with slower colour development at 10°C and faster colour development at 30°C (Box, 1983). This suggests that the temperature should be carefully monitored when performing this assay. Finally, caution should be exercised when working with raw solutions such as the digest media tested here, where inorganic iron, molybdenum and sulfur containing compounds may interfere with the reduction of the Folin-Ciocalteu reagent and affect the antioxidant capacity estimation (Box, 1983).

7.4.3 ORAC

The oxygen radical absorbance capacity or ORAC assay is considered the standard method for determining the proton-donating antioxidant capacity of a sample due to its good reproducibility and its wide use in the published antioxidant research (Huang *et al.,* 2005). Prior and co-workers observed that the ORAC assay represented the hydrogen atom transfer mechanism, which is most relevant to human biology (Prior *et al.,* 2005). Another strength of this assay is the use of the area under the curve method to quantify the antioxidant capacity, which considers both the relative inhibition and the inhibition time, so slower acting antioxidants are not penalized as may have been the case in the DPPH assay (Davalos *et al.,* 2004a).

The ORAC method employed here is based on the loss of fluorescence of the synthetic, non-protein fluorescein due to free radical attack generated by AAPH in a hydrophilic medium. An antioxidant will prevent the degradation of fluorescein by donating hydrogen and neutralizing the peroxyl radicals generated by the hydrophilic radical generator AAPH. Other types of ORAC assays exist, with different fluorescent probes such as β-phycoerythrin or occur in hydrophobic media (Prior *et al.,* 2003, Ou *et al.,* 2001, Cao *et al.,* 1993, Huang, Ou, Hampsch-Woodill, Flanagan, & Deemer, 2002).

ORAC antioxidant capacity was expressed in terms of mg Trolox/g sample. The ORAC antioxidant capacity of the preliminary digests can be found in Table 13 and reveal that the undigested SPI, pepsin, and pancreatin all have significantly lower antioxidant capacities when compared to the SPI digests. Once digested with one or both of the enzymes, an increase in the antioxidant capacity of the SPI is observed with the Pepsin digest showing the smallest increase

and the Pancreatin digest showing the greatest increase of nearly ten times the ORAC antioxidant capacity of the SPI. Some difference between the ultrafiltration fractions can also be observed, with the greatest antioxidant capacity belonging to the 3 kDa retentate fraction of the Pancreatin digest, which was significantly higher than all other fractions of the preliminary and factorial design digests.

The observation that digestion improves ORAC antioxidant capacity continues through to the ORAC data for the factorial design digests where the application of enzymatic hydrolysis increased the antioxidant capacity by six to eight times. No significant differences were observed between the ORAC capacity of the different digestion conditions or their ultrafiltration fractions. Significant differences could be observed between the 1 kDa permeate fraction of the No Pepsin digest with an antioxidant capacity of 194.4 ± 8.5 mg Trolox/g sample (n=3) and all other digests and fractions (Tables 13, 24, 33).

From literature, Hernandez-Ledesma and co-workers identified the Corolase PP digest of whey protein produced a peptide (isolated by HPLC-MS/MS) with an antioxidant capacity of 2.6 µmol Trolox/µmol peptide in the ORAC assay (Hernandez-Ledesma *et al.,* 2005). Davalos and co-workers studied the hydrolysates from crude egg white digested with pepsin and identified a peptide (isolated by RP-HPLC) with a high radical scavenging ability in the ORAC assay of 3.8 µmol Trolox/µmol peptide (Davalos *et al.,* 2004b). Unfortunately this data from literature is difficult to correlate to the SPI antioxidants generated here since the purity of this digest sample is unknown.

One concern with the ORAC assay is the temperature sensitivity of the assay, resulting in the need for precise temperature control while the assay is performed, and therefore some difficulty reproducing experimental data between experimenters (Prior *et al.,* 2005). Also, the equipment demands may be another limiting condition since the assay calls for a temperature-controlled, 96-well, fluorescent plate reader (Gillespie *et al.,* 2007).

7.5   Antioxidant Model

The antioxidant capacity data generated in this work under the different digestion and ultrafiltration conditions were analyzed to indicate any trends that can be used to guide future work. An empirical model built from the antioxidant capacities was developed, although it was created from the digestions selected for further analysis due to limited antioxidant capacity testing possible. The ORAC hydrogen-donating antioxidant capacity was selected for this analysis since the peptides produced here appear to be most potent at donating hydrogen atoms.

A pancreatin concentration of 0 g/L and a pancreatin digest time of 0 minutes produce a lower ORAC antioxidant capacity, as indicated by the plots of the dependent variables (digestion and filtration conditions) versus the output variable (ORAC antioxidant capacity) in Appendix C. The fractionation variables do not reveal a clear trend in increasing or decreasing antioxidant capacity, although the 3 kDa permeate fraction for all the digests appear to have a narrower range of possible proton-donating antioxidant capacity values when compared to the antioxidant capacity of the other fractions, based on the antioxidant capacity measured for these fractions. Therefore, one can conclude that the enzyme pancreatin should be involved in the hydrolysis of the PRO-FAM 974 SPI at a time greater than zero minutes and must be included in the digestion protocol to produce a hydrolysate with a maximum antioxidant capacity. While both pepsin and pancreatin have been shown to be effective at hydrolyzing the SPI, one must recognize that both pepsin and pancreatin are autolytic and may be contributing antioxidant or pro-oxidant peptides to the digestion media studied here (Qiao *et al.,* 2002, Copeland, 1994).

8　　Conclusions and Recommendations

8.1　Conclusions

The purpose of this study was:

1. to produce peptides from commercially-available soy protein isolate (SPI) by enzymatic hydrolysis with porcine pepsin and/or pancreatin,

The enzymatic hydrolysis of a 3 wt% SPI solution with pepsin and pancreatin was found to increase the peptide concentration of the resulting solution from an initial peptide concentration of 13 mM PheGly equivalents to approximately 23 mM PheGly with each enzyme alone (pepsin 30 min, 37ºC, pH 1.5, 1:125 E:S w/w; pancreatin 60 min, 40ºC, pH 7.8, 1:30 w/w), to 32 mM PheGly when the two enzymes were used sequentially. Based on conditions produced from a full factorial model, a maximum peptide concentration near 65 mM PheGly could be achieved.

2. to fractionate the hydrolysates by dead-end membrane ultrafiltration,

The fractionation of the SPI hydrolysates by sequential membrane ultrafiltration with 3 kDa and 1 kDa MWCO membranes was demonstrated. 3 kDa fractionation conditions combining a minimum filtration time of 800 minutes and minimum permeate of 60 g for the factorial and extension digests resulted in a retention of approximately 50% of the feed solution for the High and Higher Pancreatin digests up to a retention of 70% for the Low digest. The 1 kDa fractionation with a minimum filtration time of 90 minutes and a minimum permeate of 20 g resulted in a retention of between 50% and 62% of the mass of the feed solution for the High and Higher Pancreatin digests compared to the Low digest. The peptide retention followed the same trend, with more peptides retained for the feed solutions having lower initial peptide concentrations.

Modeling of the permeate decline over time in the dead-end unstirred ultrafiltrations indicated that the fouling appears to follow the cake filtration model. Experimentally, the fouling was found to be largely reversible, with up to 86% of the fouling of the 3 kDa membrane and 95% of the 1 kDa membrane removed by a simple water rinse, for both the High and Low digests tested.

3. to determine the electron-donating and proton-donating antioxidant capacities of the
    peptide fractions, and

The antioxidant capacities of the peptides produced was quantified in terms of the electron-donating DPPH and FCR assays and the proton-donating ORAC assay. The DPPH assay not suitable to allow the digests and ultrafiltration fractions to be distinguished from one another at a 95% confidence level and is therefore not recommended for future testing. The FCR assay was sufficiently sensitive to distinguish the antioxidant capacity of some digests and fractions, with the lowest antioxidant capacity attributed to the 1 kDa permeate of the Pepsin digest at 25 mg Trolox/g sample and the highest to the 1 kDa retentate of the Pancreatin digest at nearly 190 mg Trolox/g sample. The ORAC assay was sensitive enough to distinguish between the peptide fractions as well, with the lowest antioxidant capacity of 47 mg Trolox/g sample found for the 1 kDa permeate of the Pepsin digest and the highest antioxidant capacity of approximately 195 mg Trolox/g sample to the 1 kDa retentate of the No Pepsin digest.

4. to model the effects of the digestion and fractionation conditions on the production of
    proton-donating antioxidant peptides.

An empirical model of the factors that contribute to the production of proton-donating antioxidant peptides as determined by the ORAC assay was developed. Due to the limited number of data points, only single factors and no interaction terms could be considered. The model developed (equation 12) indicates that pancreatin digestion time has a positive effect while pepsin digestion time has a negative effect on the antioxidant capacity of the peptides produced, but does not fully explain all of the conditions required to produce proton-donating antioxidant peptides.

Overall, the enzymatic cleavage of the ADM PRO-FAM 974 SPI with pancreatin and fractionation by membrane ultrafiltration is capable of producing enriched antioxidant peptide fractions.

8.2   Recommendations

1. Add more digestion conditions to the ORAC empirical antioxidant model such that a fractional factorial model can be built, especially de-coupled pepsin and pancreatin conditions. This will give a better understanding of the significant factors, such as the role of pepsin and pancreatin in producing antioxidant peptides. This includes studying lower pepsin concentrations than those considered here which appear to have an indistinguishable effect on the ORAC antioxidant capacity.

2. Investigate the autolytic properties of pepsin and pancreatin and the antioxidant capacity of the peptides resulting from this autolysis, both for pepsin and pancreatin individually and together.

3. Identify the peptide sequence with the greatest antioxidant capacity to optimize the process; perhaps a different starting material such as specific protein instead of SPI. This could be accomplished by running MALDI-TOF with a reducing agent such as β–mercaptoethanol on the ultrafiltration fraction that has the greatest antioxidant capacity.

4. Study the 3 kDa cross-flow ultrafiltration behaviour for the optimum enzymatic digestion condition(s) such as the No Pepsin digest. Scale up of the ultrafiltration procedure appears to be feasible based on the low irreversible fouling observed in this work.

5. The use of hypothetical test systems for measuring antioxidant capacity, such as the use of the synthetic fluorescence probe fluorescein, does not necessarily reflect the actual antioxidant capacity of the peptides. Instead, quantify the antioxidant properties in a medium comparable to the end use of the peptides, be it *in vivo*, incorporation into food products, or in other applications.

Permissions

May 11, 2010

To Whom It May Concern:

Author: Sonja Bissegger
Title: Production of Bioactive Soy Peptides
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Date: 2008

I, Sonja Bissegger, the author of Production of Bioactive Soy Peptides, give Mary Robinson permission to use the data in her University of Waterloo MASc thesis.

Sonja Bissegger

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Appendix A

Bradford Calibration

$$y = 1.0105x - 0.0001$$
$$R^2 = 0.999$$
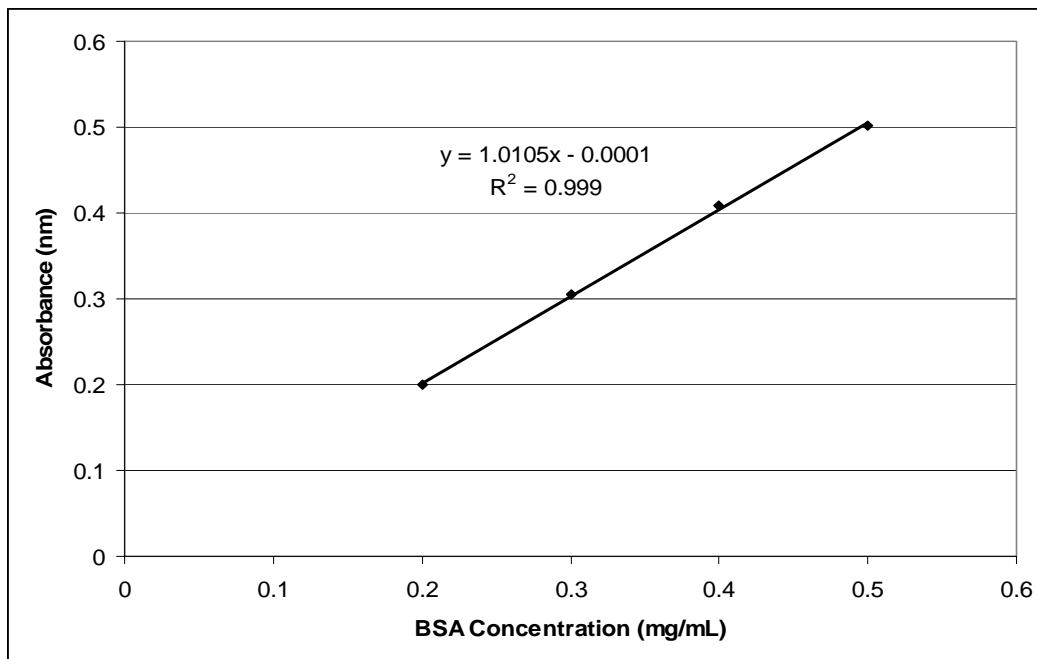
Calibration curve for the Bradford Assay, as performed at the University of McGill**.**

OPA Assay

$$y = 0.5736x + 0.0166$$
$$R^2 = 0.9984$$
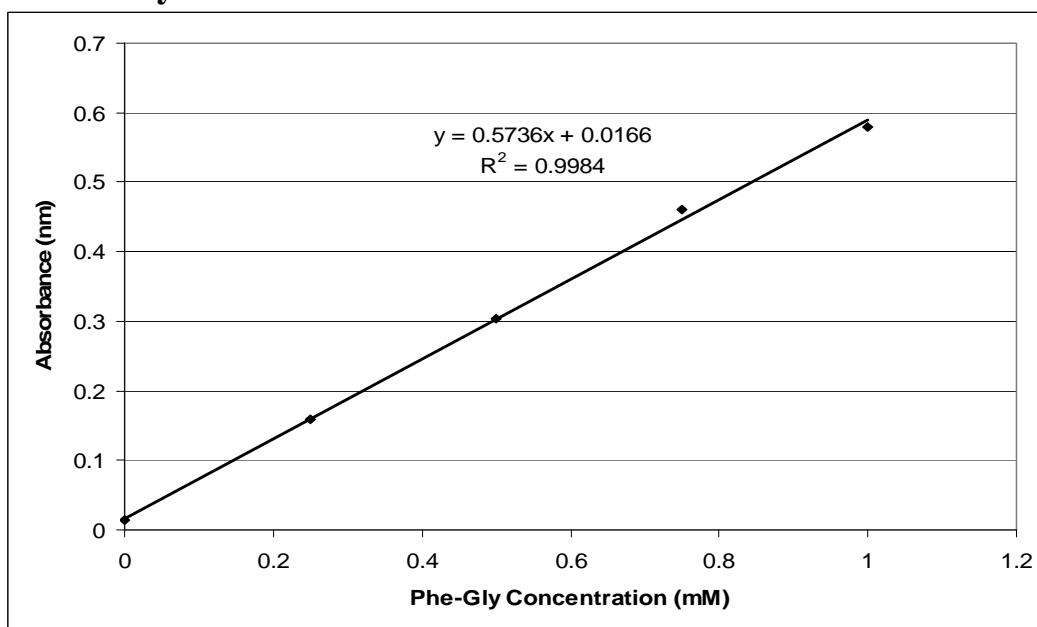
Typical calibration curve for the OPA Assay using PheGly 0-1000 μM as the standard peptide.

Ultrafiltration Water Flux

The figure below gives some typical results of a water flux experiment to check the integrity of the ultrafiltration membrane before any experiments were performed.

For the 3 kDa membranes, the pressure is increased stepwise from 70 kPa (10 psi) to 340 kPa (50 psi) in approximately 70 kPa (10 psi) steps, then decreased back to 210 kPa (40 psi), ensuring a linear relationship between the obtained flux and the pressure applied.

For the 1 kDa membranes, the pressure is increased stepwise from 210 kPa (40 psi) to 340 kPa (50 psi) in approximately 70 kPa (10 psi) steps, then decreased back to 70 kPa (10 psi) and raised in approximately 70 kPa (10 psi) increments to a final pressure of 210 kPa (40 psi). The reason for the higher starting pressure for the 1 kDa membranes is that a larger pressure differential needed to be used to start the filtration process and to purge any air bubbles from the system. The lower pressures were tested once the air bubbles had been purged.

The values displayed are average flux calculated over a time period of no less than three minutes during which time the pressure was held constant. Conversion factors and material properties of water were obtained from Perry's Chemical Engineers' Handbook (Perry *et al.,* 1997).

3kDa Water Flux
y = 0.1162x + 0.2309

1kDa Water Flux
y = 0.058x - 0.3596
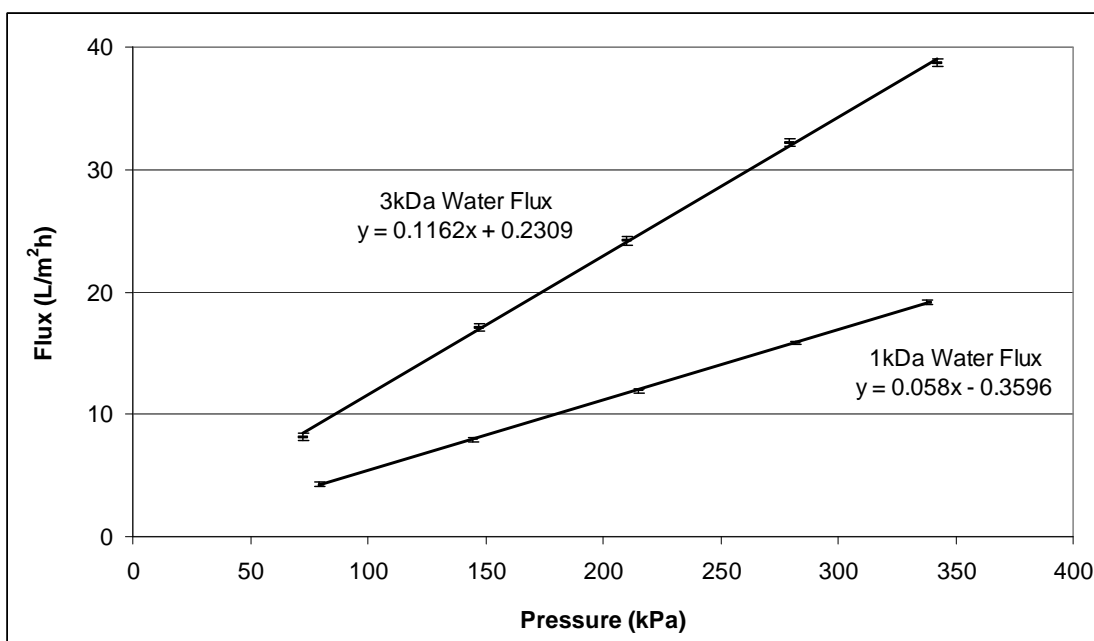
Pressure-flux relationship obtained with MilliQ water for the 3 kDa and 1 kDa ultrafiltration membranes. Values expressed are mean ± SD, n=3.

DPPH Assay

Trolox samples combined with DPPH to produce the calibration curve. From left to right: blank, control, 0.1 mM Trolox, 0.2 mM Trolox, 0.25 mM Trolox, and 0.3 mM Trolox. The colour change observed occurred nearly immediately after the Trolox was added and remains unchanged over the 15 minutes studied**.**

Calibration Curve
$y = -1.6107x + 0.9808$
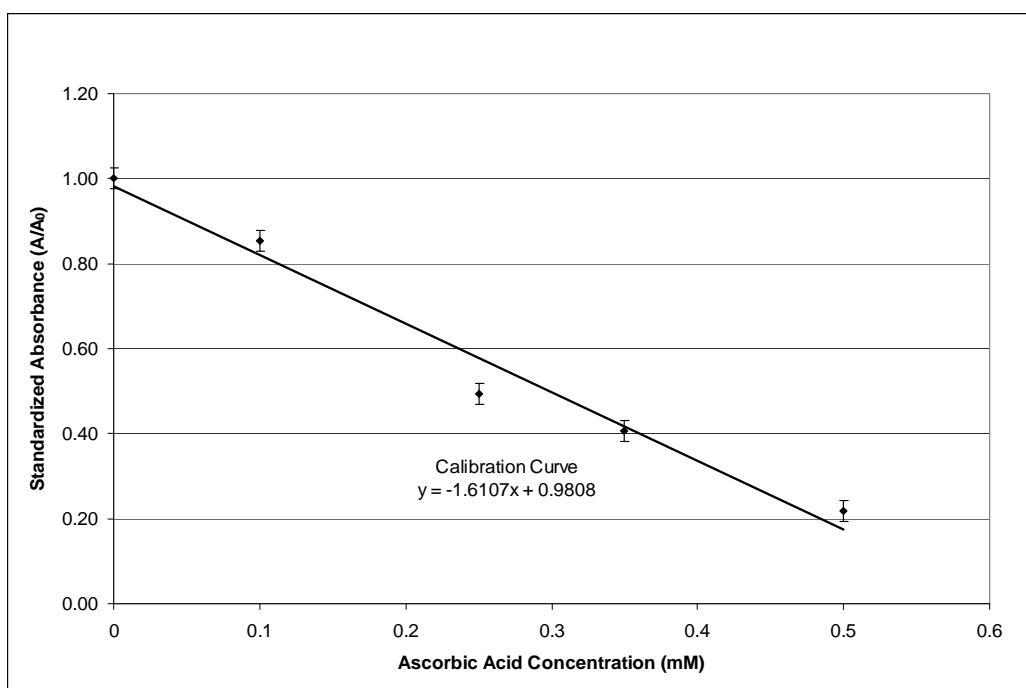
Ascorbic acid calibration curve for the DPPH assay. Values expressed are mean ± SD, n=2.
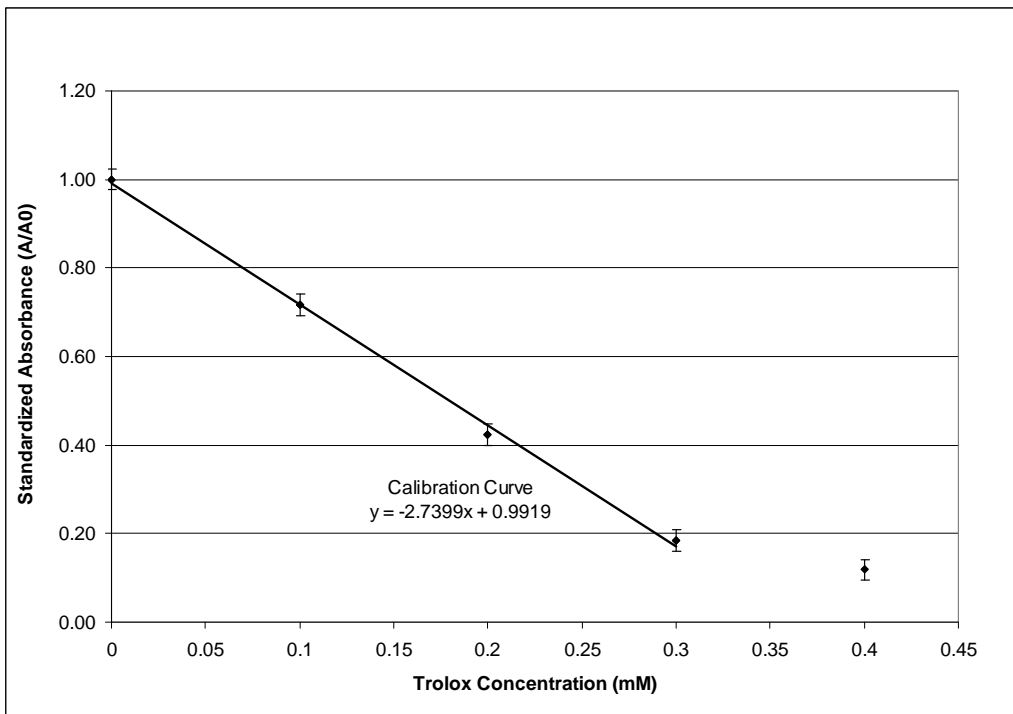
DPPH and Trolox calibration curve; note that the linear section is only up to 0.3 mM Trolox. Values expressed are mean ± SD, n=3.

As can be seen in these representative calibration curves developed with ascorbic acid and Trolox as the standard antioxidant, the calibration curves developed with Trolox were more reproducible than those developed with ascorbic acid. However, ascorbic acid is a very reactive reducing agent and known to degrade quickly (Frankel, 1998). Therefore, in the experiments performed from the factorial design and onwards, Trolox was used exclusively as the standard antioxidant. Note that the calibration curve becomes non-linear at ascorbic acid concentrations above 0.5 mM and at 0.4 mM Trolox and above.

FCR Assay

The FCR assay was developed from the publication by Singleton et al with a 15% Na_2CO_3 solution to maintain the desired final solution at pH 10 (Singleton *et al.,* 1999). Trolox was used as the standard antioxidant. The calibration curve was run at the start and end of every series of experiments to confirm minimal experimental drift. A sample calibration curve is given in the figure below, with a photograph of the samples used to prepare the calibration curve.
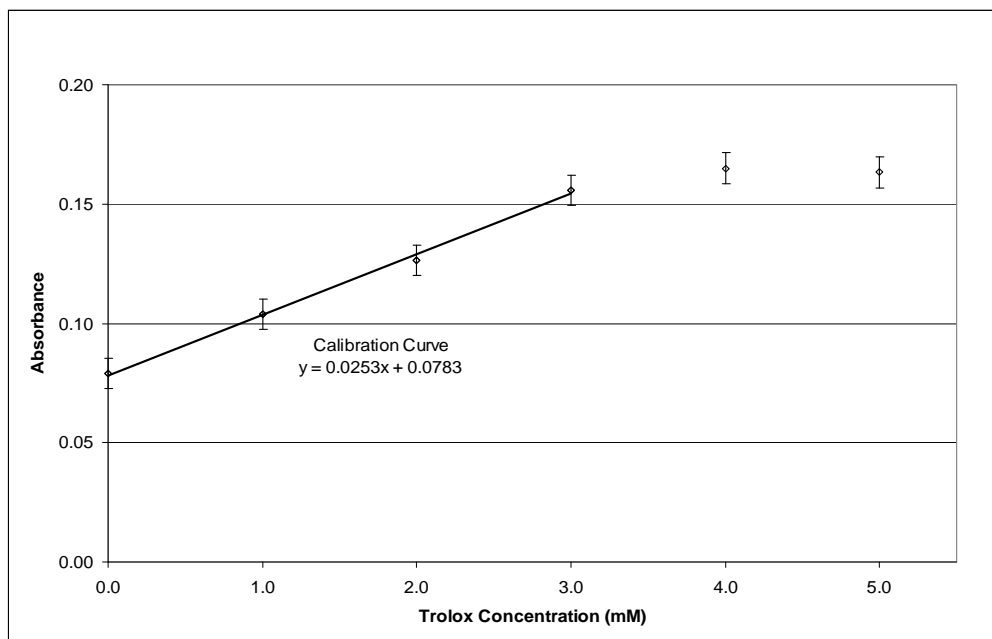
Trolox calibration relationship for the FCR assay; note that the linear section is up to approximately 3.0 mM Trolox. Values expressed are mean ± SD, n=3.

Trolox samples used to obtain the FCR calibration curve. From left to right: control, 1.0 mM Trolox, 1.5 mM Trolox, 2.0 mM Trolox, 2.5 mM Trolox, and 3.0 mM Trolox. Notice the increasing darkness of samples as the Trolox concentration increases from left to right.

Appendix B

As described in Chapter 3, selected digests were fractionated by sequential membrane ultrafiltration at 25°C under nitrogen at a constant pressure of approximately 210 kPa (40 psi). The time and permeate flux for the 3 kDa and 1 kDa MWCO membrane ultrafiltrations were recorded. The figures included in Appendix B are the product of the linearization of the four equations developed by Bowen and co-workers to model the flux decline over time resulting from membrane fouling as a result of standard blocking, complete blocking, intermediate blocking, or cake filtration for this filtrations.

The four models are, given in order of presentation in the graphs in the following pages of Appendix B, as developed by Bowen and co-workers: (Bowen *et al.*, 1995)

Mechanism	Fouling Model	Linearized Form	Plot
Cake Filtration	$J_P = \dfrac{J_0}{\sqrt{1+kt}}$	$\dfrac{1}{J_P^2} = \dfrac{k}{J_0^2}t + \dfrac{1}{J_0^2}$	$\dfrac{1}{J_P^2}$ vs t
Intermediate Blocking	$J_P = \dfrac{J_0}{(1+kt)}$	$\dfrac{1}{J_P} = \dfrac{k}{J_0}t + \dfrac{1}{J_0}$	$\dfrac{1}{J_P}$ vs t
Standard Blocking	$J_P = \dfrac{J_0}{(1+kt)^2}$	$\dfrac{1}{\sqrt{J_P}} = \dfrac{k}{\sqrt{J_0}}t + \dfrac{1}{\sqrt{J_0}}$	$\dfrac{1}{\sqrt{J_P}}$ vs t
Complete Blocking	$J_P = J_0 \exp(-kt)$	$\ln\left(\dfrac{1}{J_P}\right) = kt + \ln\left(\dfrac{1}{J_0}\right)$	$\ln\left(\dfrac{1}{J_P}\right)$ vs t

Where J_P = time variable permeate flux (m/s)
  J_0 = the permeate flux at time zero (m/s)
  t = elapsed ultrafiltration time (s)
  k = constants

3 kDa Ultrafiltration
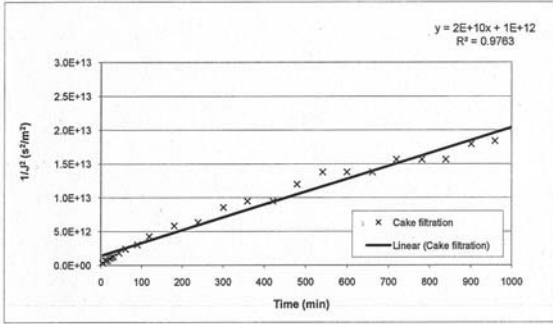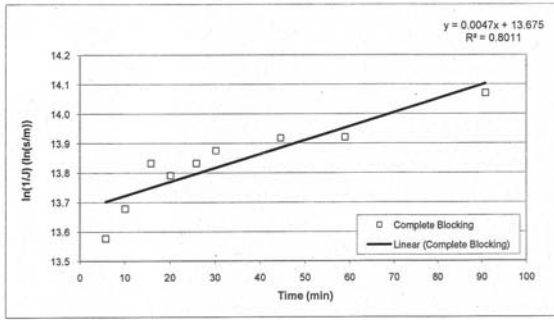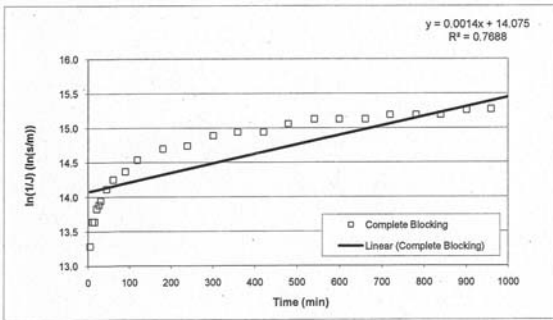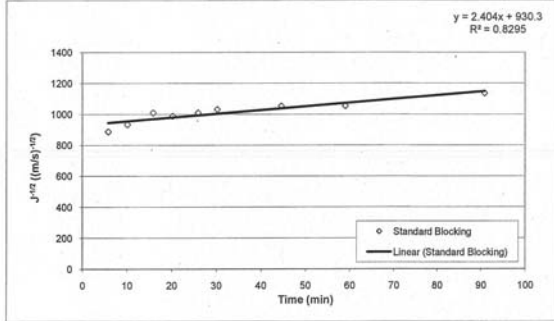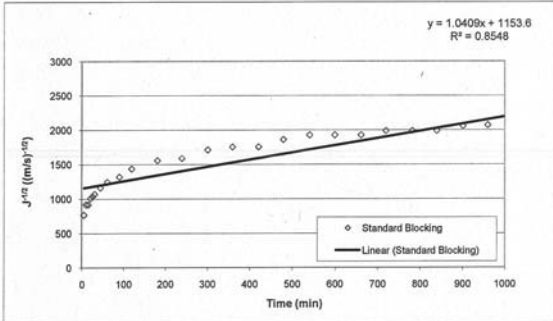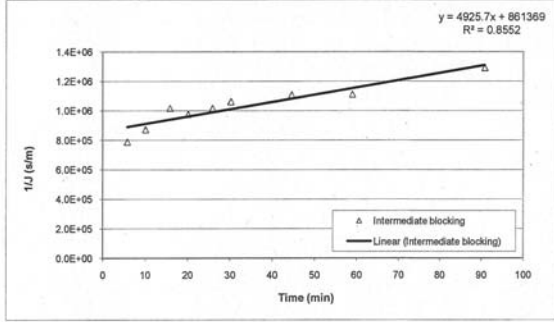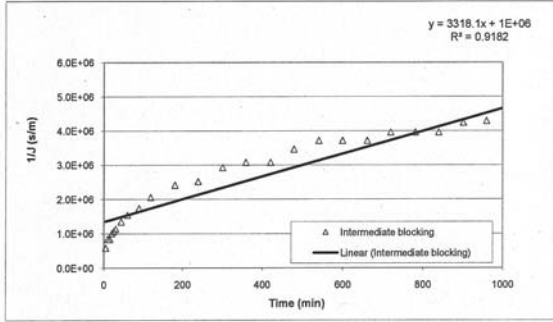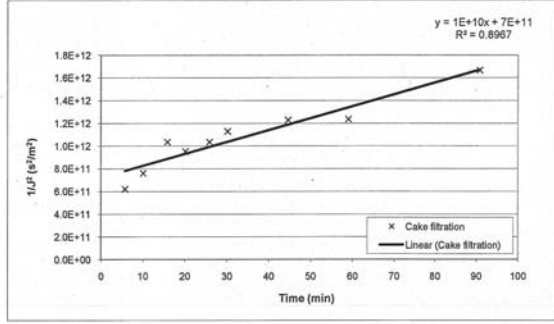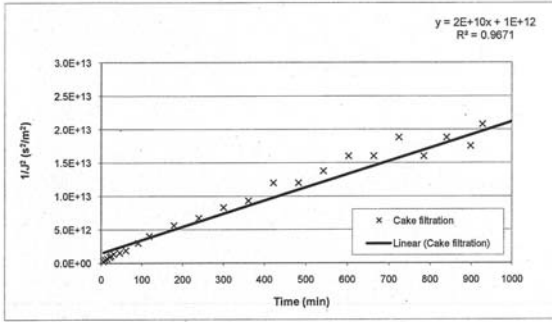
1 kDa Ultrafiltration

$y = 2E+10x + 1E+12$
$R^2 = 0.9783$

$y = 1E+10x + 7E+11$
$R^2 = 0.8967$

$y = 3318.1x + 1E+06$
$R^2 = 0.9182$

$y = 4925.7x + 861369$
$R^2 = 0.8552$

$y = 1.0409x + 1153.6$
$R^2 = 0.8548$

$y = 2.404x + 930.3$
$R^2 = 0.8295$

$y = 0.0014x + 14.075$
$R^2 = 0.7688$

$y = 0.0047x + 13.675$
$R^2 = 0.8011$

3 kDa Ultrafiltration

1 kDa Ultrafiltration

$y = 2E+10x + 1E+12$
$R^2 = 0.9671$

$y = 2E+10x + 6E+11$
$R^2 = 0.9647$

$y = 3467.8x + 1E+06$
$R^2 = 0.9003$

$y = 6750.5x + 821945$
$R^2 = 0.935$

$y = 1.0969x + 1131.2$
$R^2 = 0.8361$

$y = 3.0453x + 914.64$
$R^2 = 0.9107$

$y = 0.0015x + 14.022$
$R^2 = 0.7482$

$y = 0.0056x + 13.649$
$R^2 = 0.8798$

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3 kDa Ultrafiltration

1 kDa Ultrafiltration

3 kDa Ultrafiltration

1 kDa Ultrafiltration

$y = 2E+10x + 1E+12$
$R^2 = 0.9844$

$y = 2E+10x + 1E+12$
$R^2 = 0.9650$

$y = 3455.1x + 1E+06$
$R^2 = 0.9285$

$y = 6018.3x + 1E+06$
$R^2 = 0.9329$

$y = 1.1365x + 1125.6$
$R^2 = 0.8743$

$y = 2.6174x + 1010.3$
$R^2 = 0.9097$

$y = 0.0016x + 14.041$
$R^2 = 0.8001$

$y = 0.0046x + 13.842$
$R^2 = 0.8820$

3 kDa Ultrafiltration

1 kDa Ultrafiltration

3 kDa Ultrafiltration

1 kDa Ultrafiltration

3 kDa Ultrafiltration

1 kDa Ultrafiltration

3 kDa Ultrafiltration

1 kDa Ultrafiltration

$y = 1E+10x + 3E+11$
$R^2 = 0.9969$

$y = 1E+10x + 6E+11$
$R^2 = 0.9751$

$y = 3087.6x + 761310$
$R^2 = 0.9616$

$y = 5067.1x + 804367$
$R^2 = 0.9445$

$y = 1.2341x + 884.5$
$R^2 = 0.9127$

$y = 2.3923x + 902.34$
$R^2 = 0.9233$

$y = 0.0021x + 13.573$
$R^2 = 0.8420$

$y = 0.0045x + 13.619$
$R^2 = 0.8987$
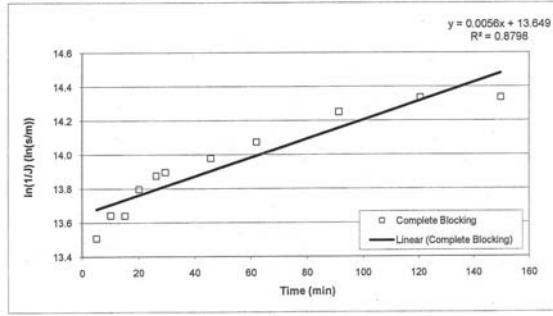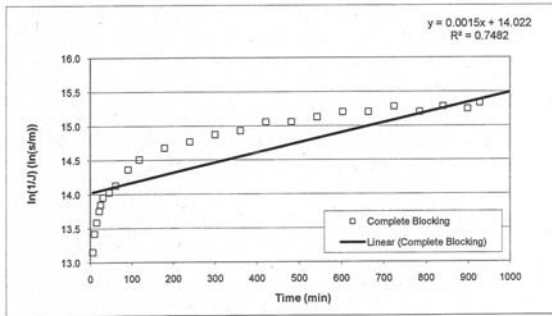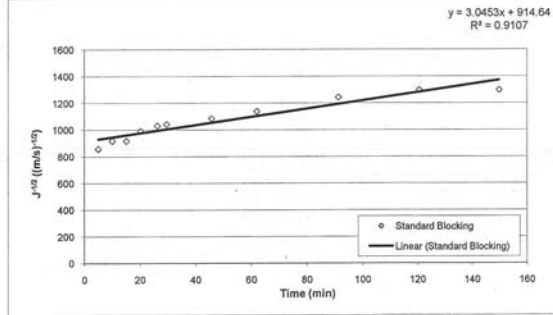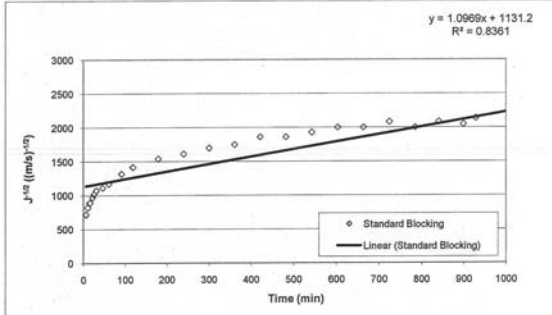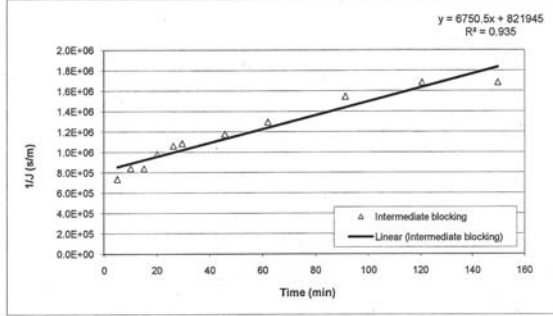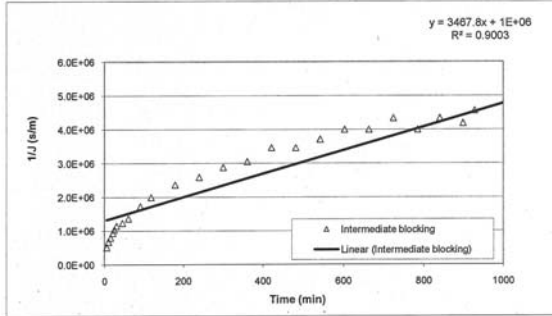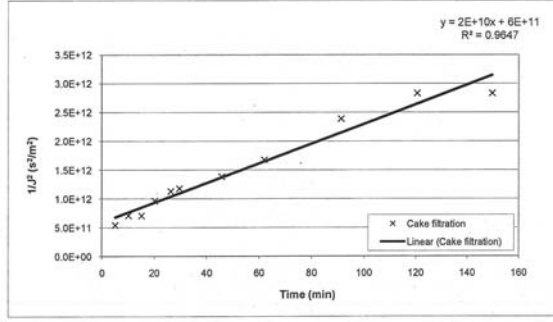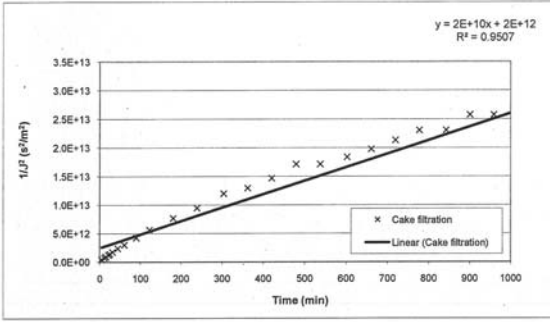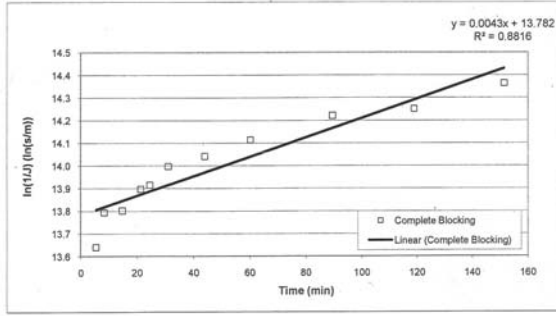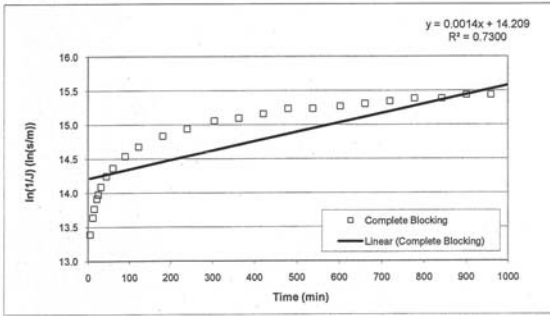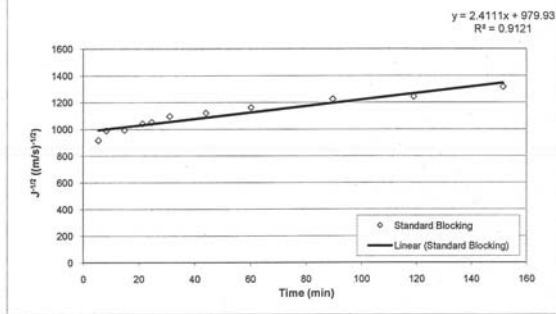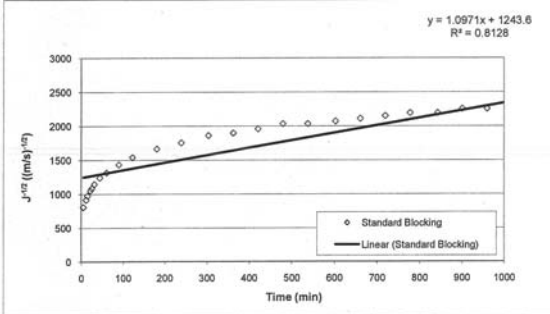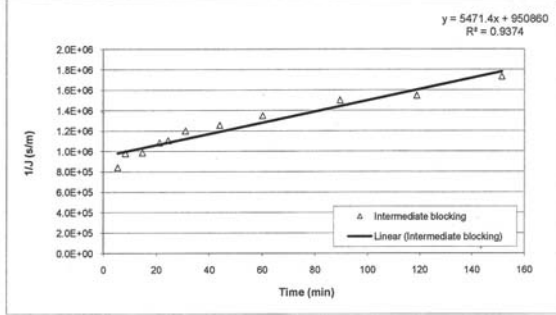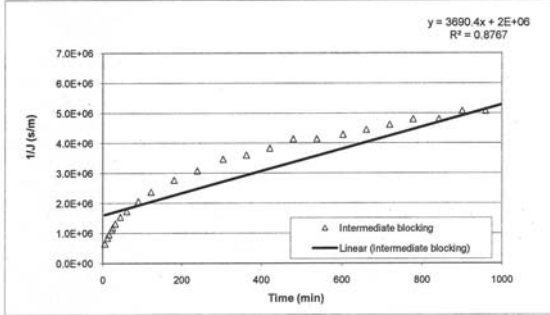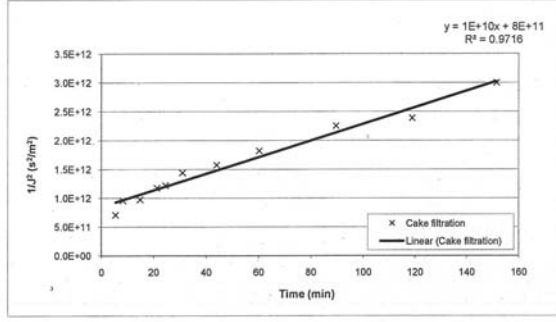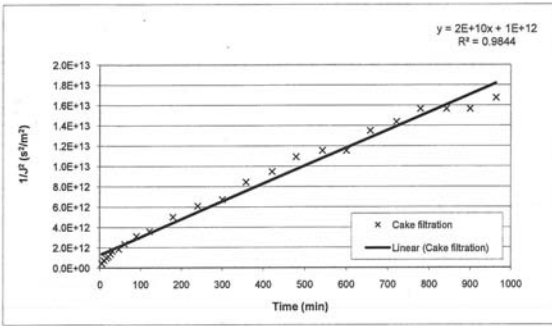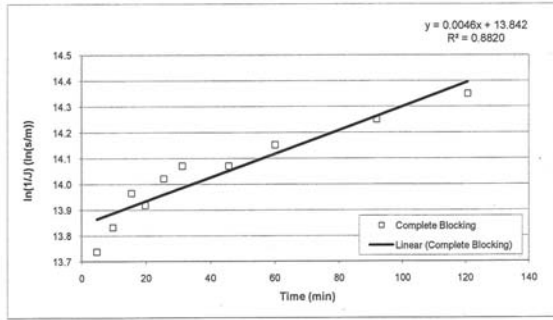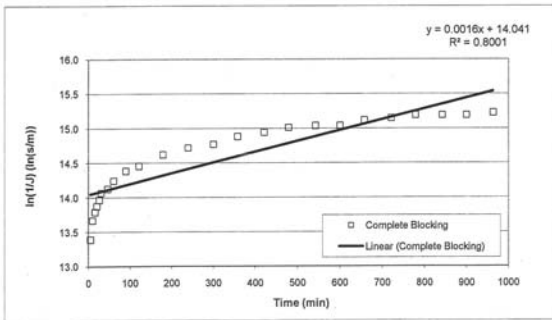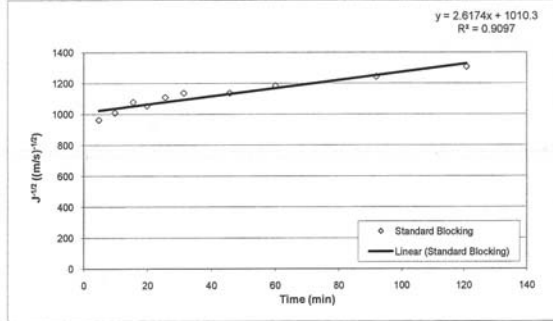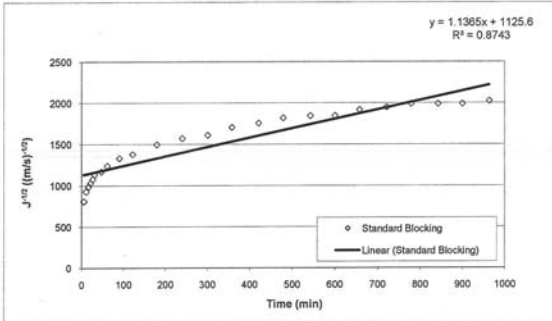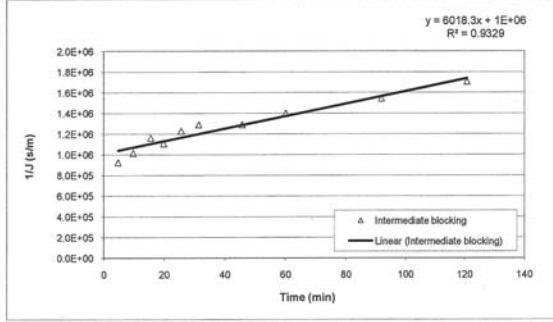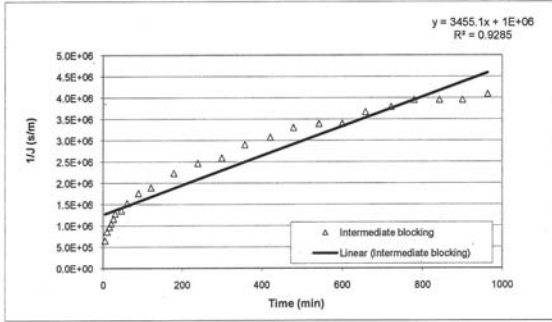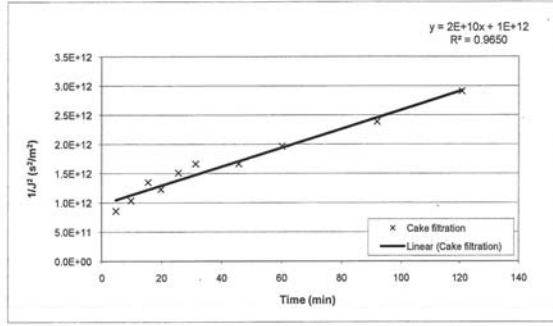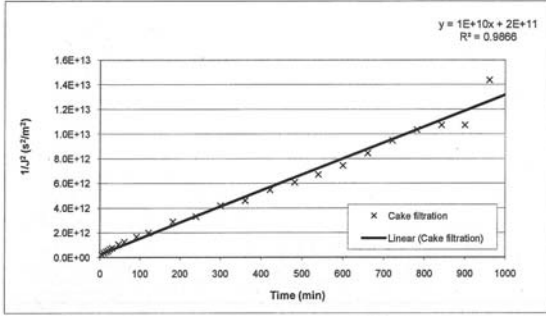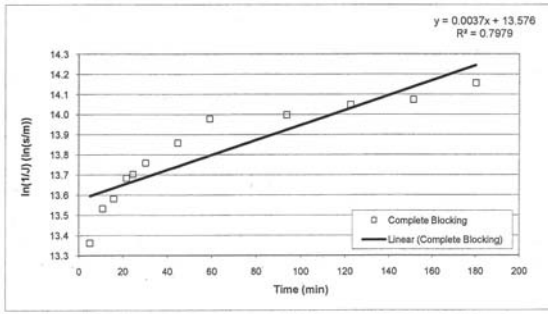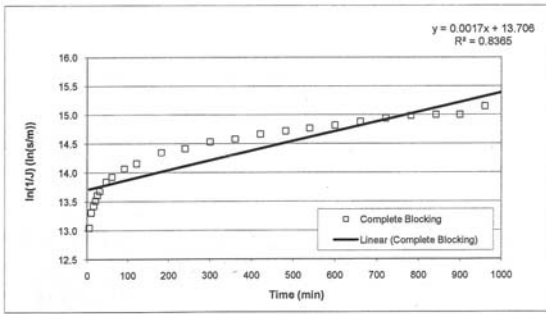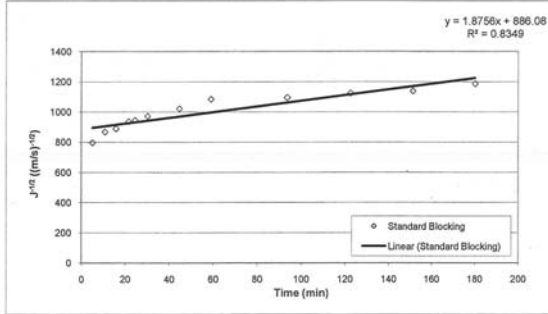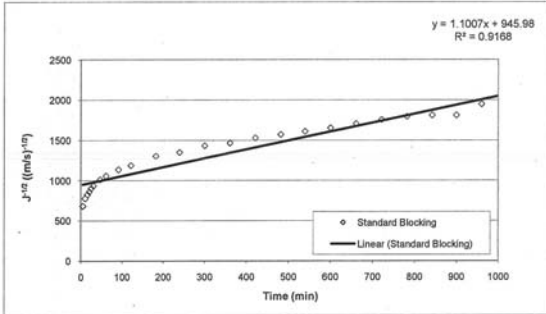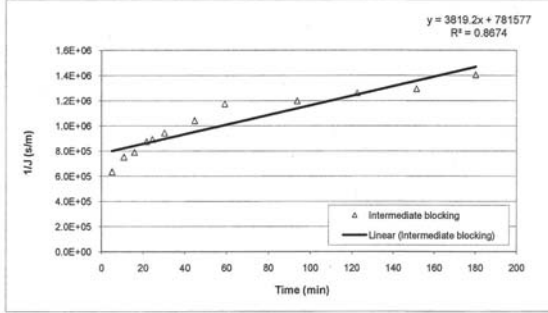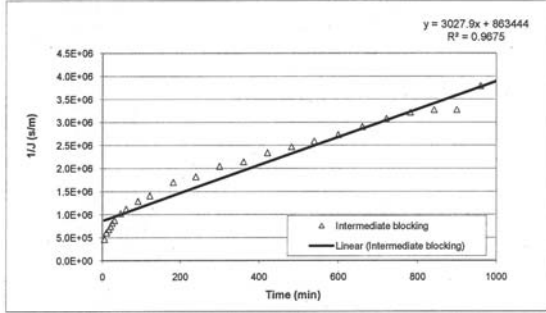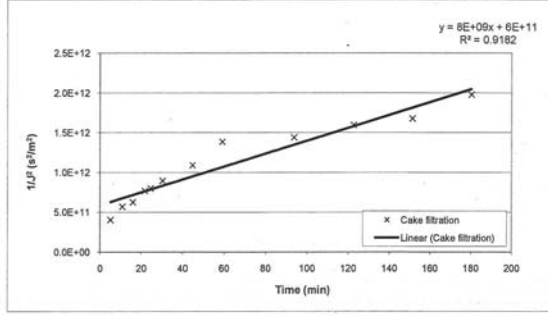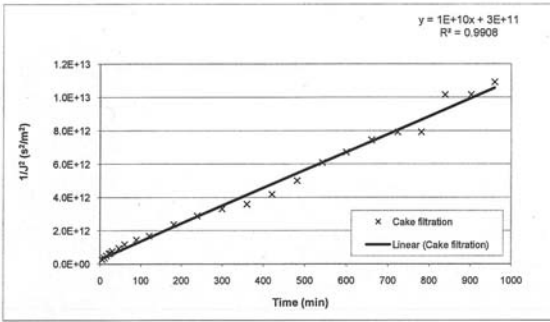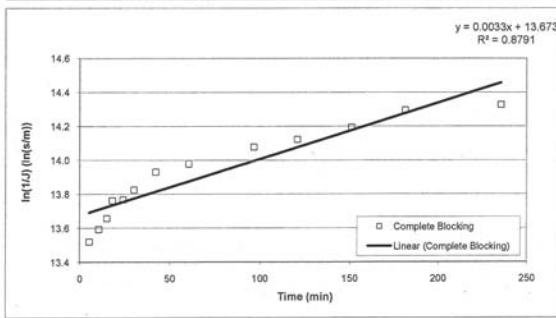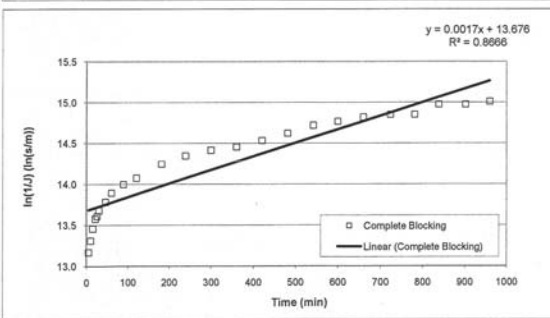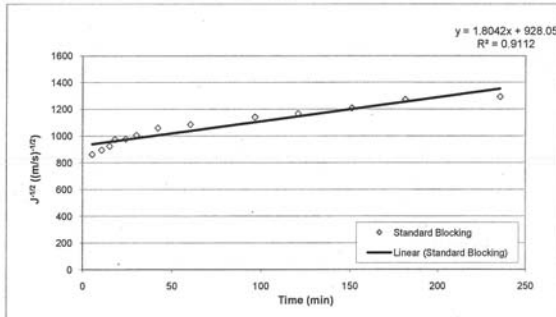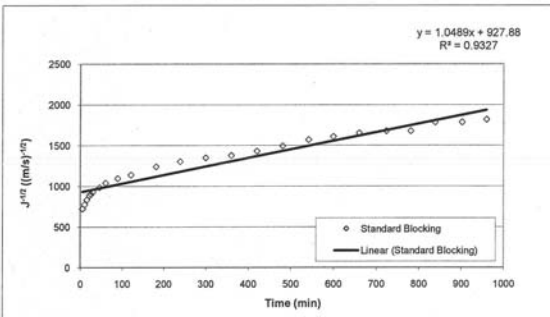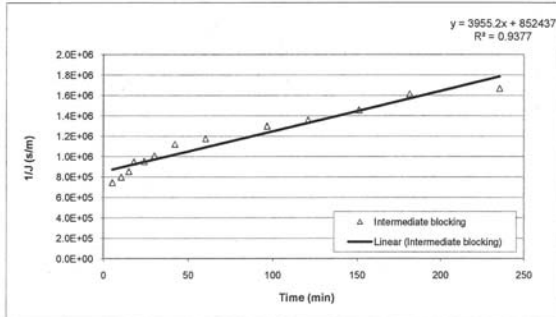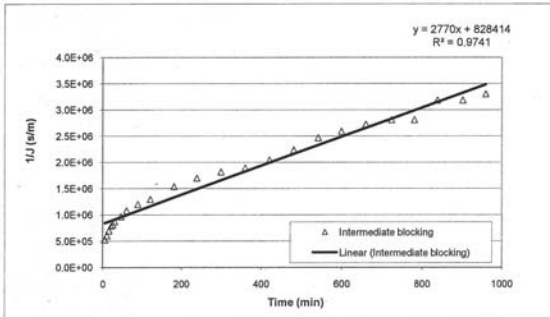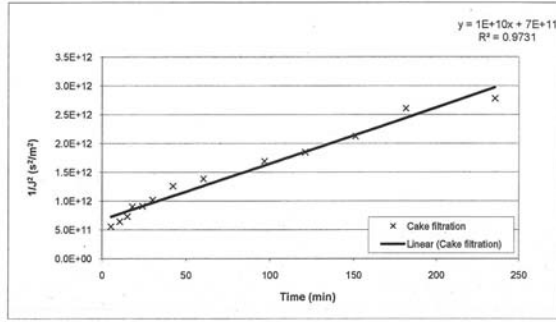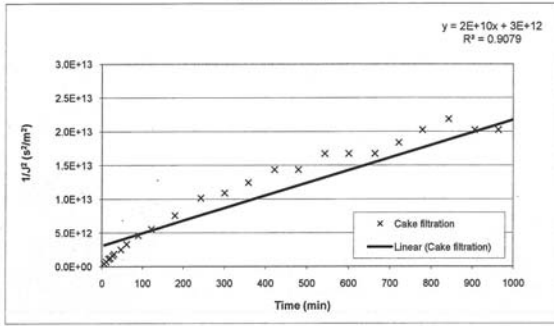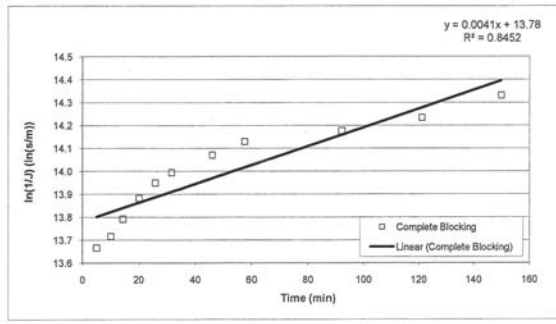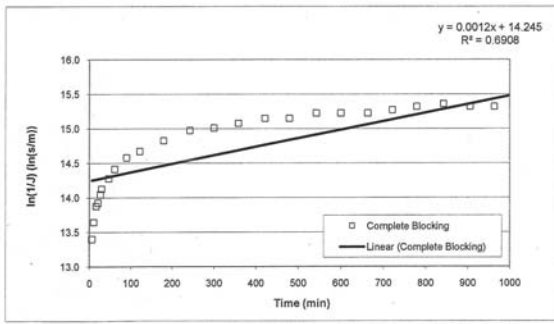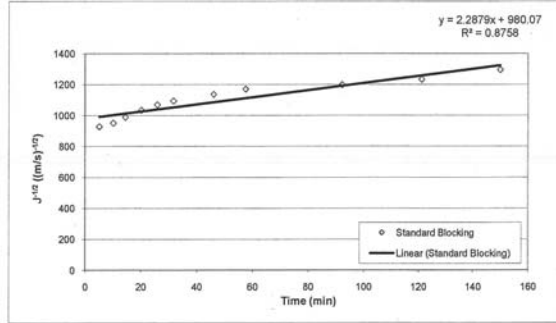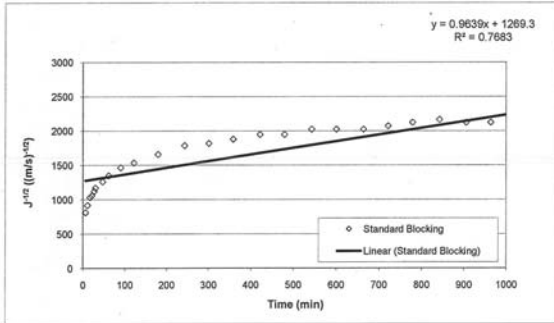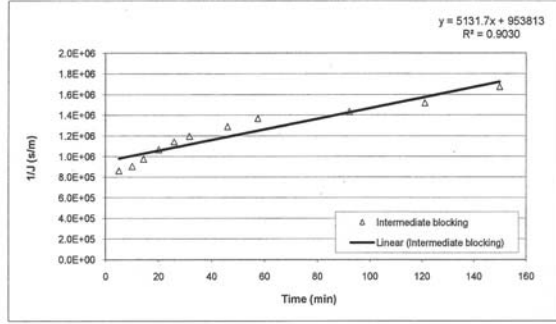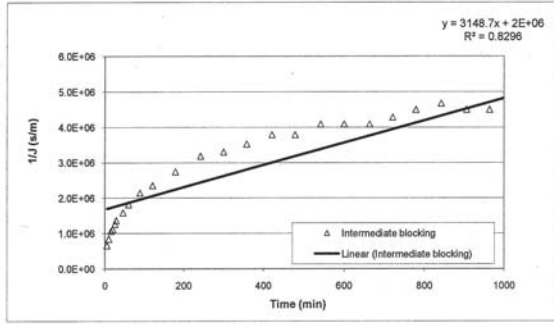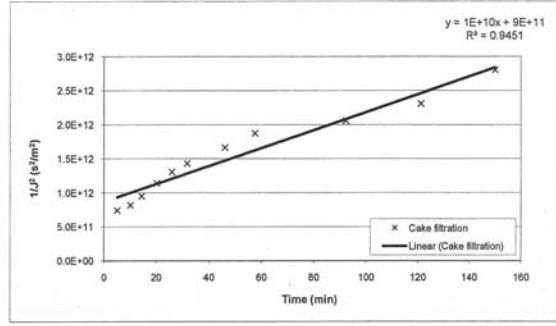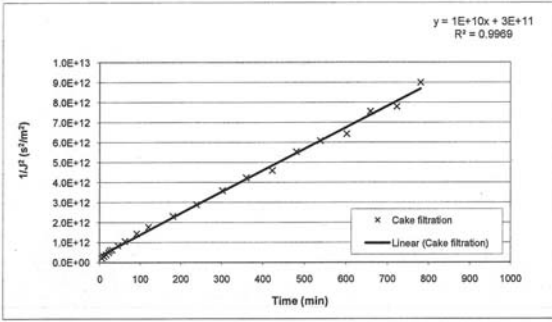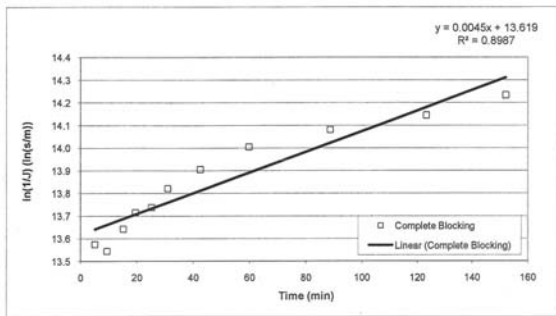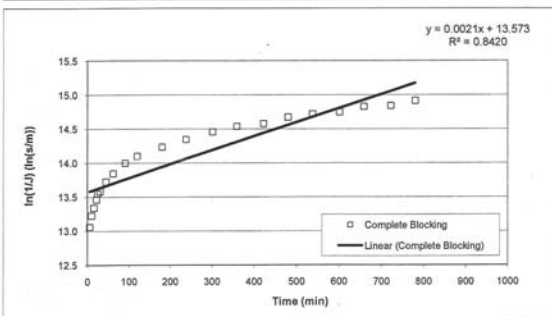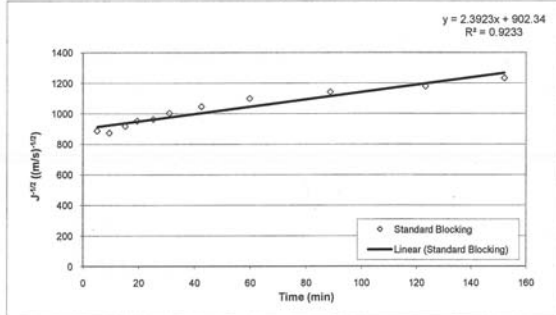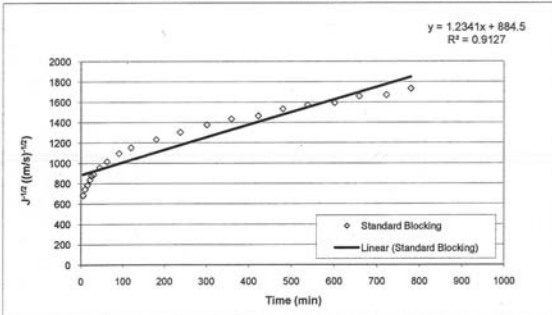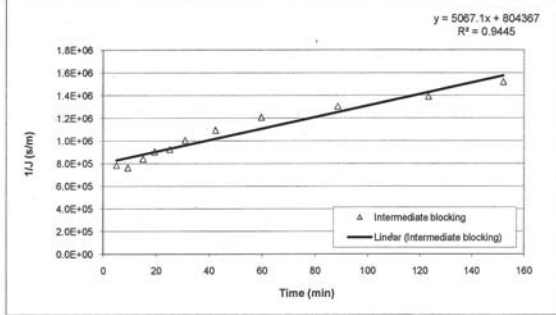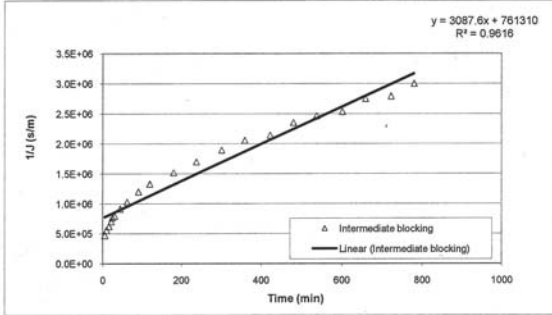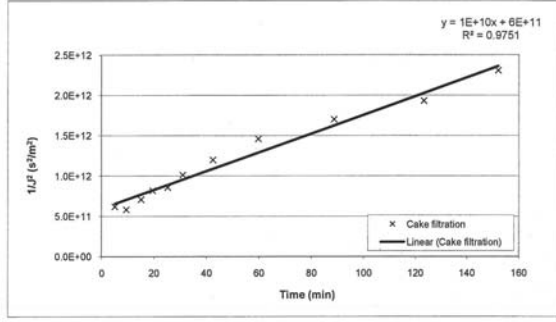
Appendix C

Due to the limited nature of the ORAC antioxidant capacities for the digests and ultrafiltration fractions, a full factorial model could not be produced. To help uncover which terms should be included in the limited model, a series of figures relating the independent variables (enzyme concentration, ultrafiltration fractions) to the output variable (ORAC antioxidant capacity) were produced and are summarized here.

Enzyme Concentrations VS ORAC Antioxidant Capacity
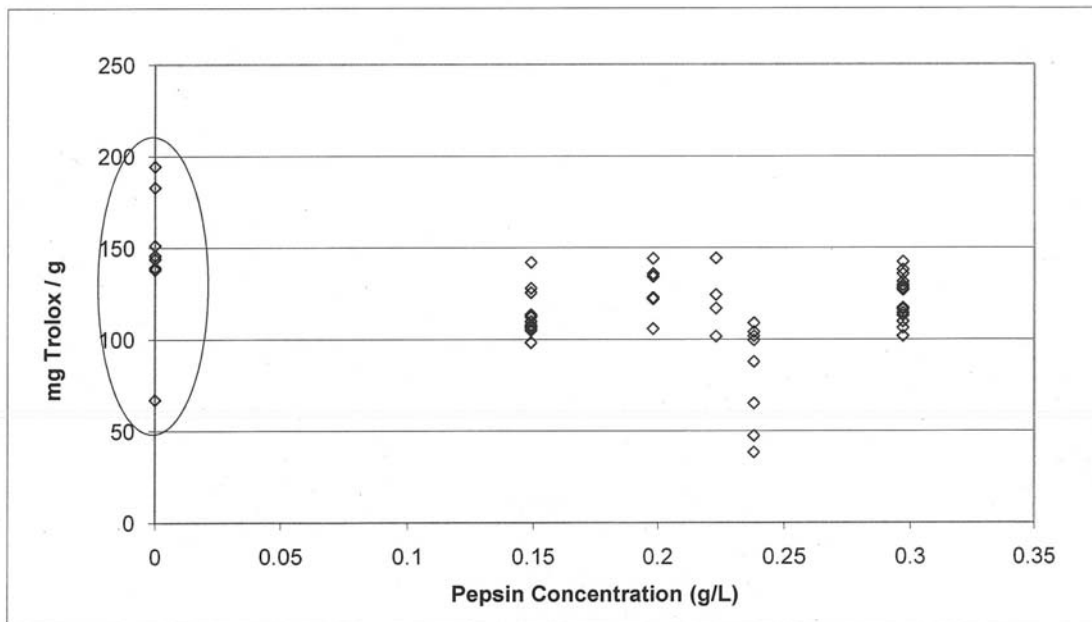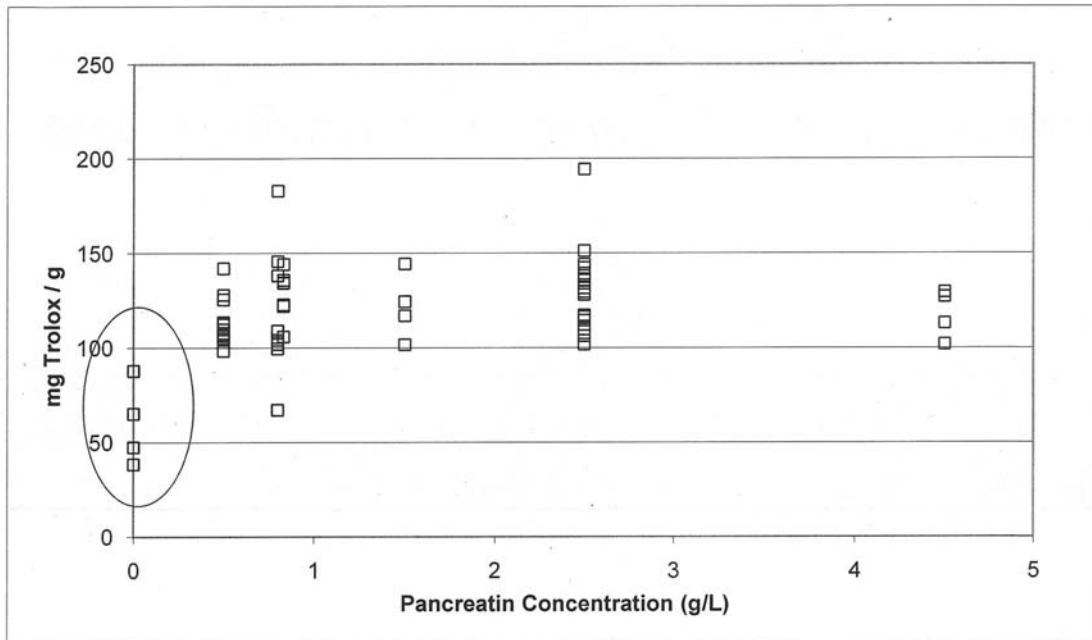
Observations:

Pepsin concentration (x1)   declining antiox with increasing pepsin conc
would be interesting to study in region 0-0.15 g/L
outlier at 0 g/L pepsin - associated with 1kDa permeate of Pancreatin digest
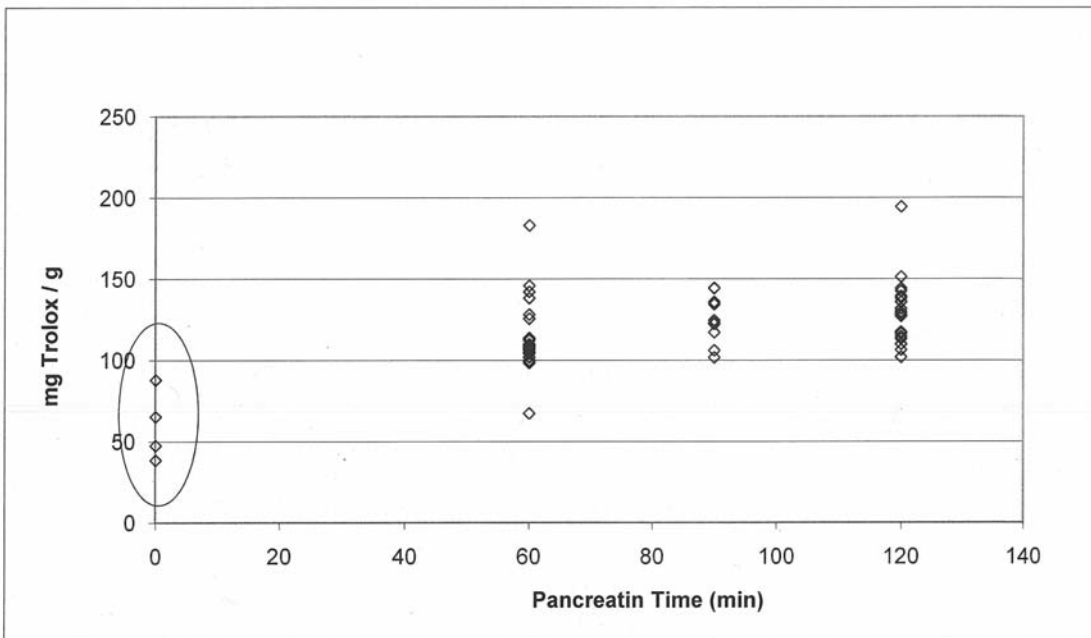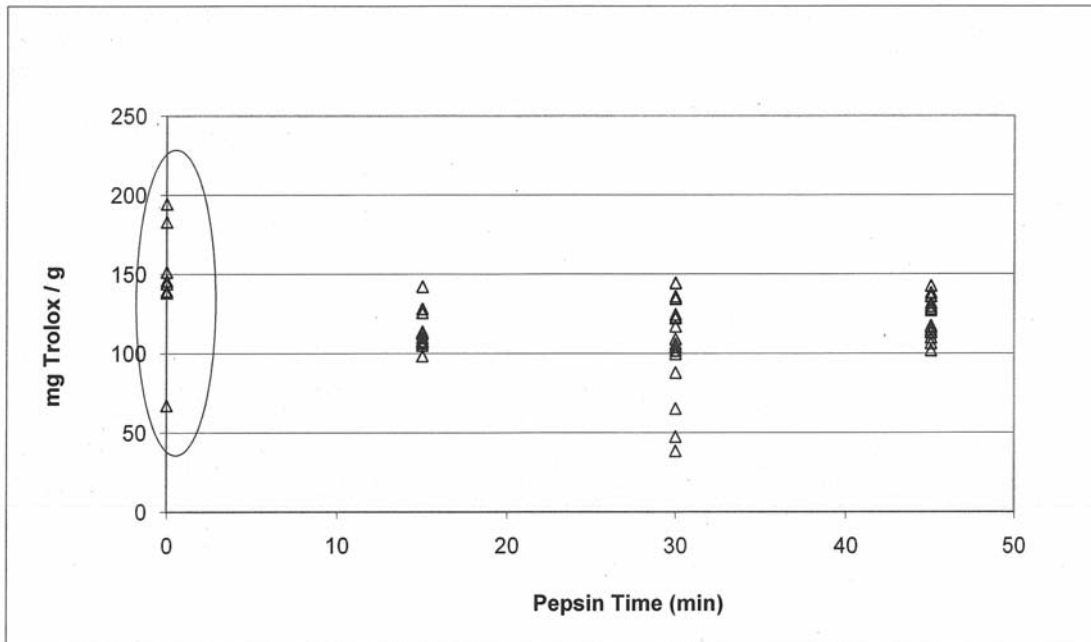
Pancreatin concentration (x2)   increasing antiox up to approx 0.5 g/L then levels out
couple of outliers of extremely high antiox at 1 and 2.5 g/L

Conclusions
keep x1 and x2 terms in model

Digestion Times VS ORAC Antioxidant Capacity

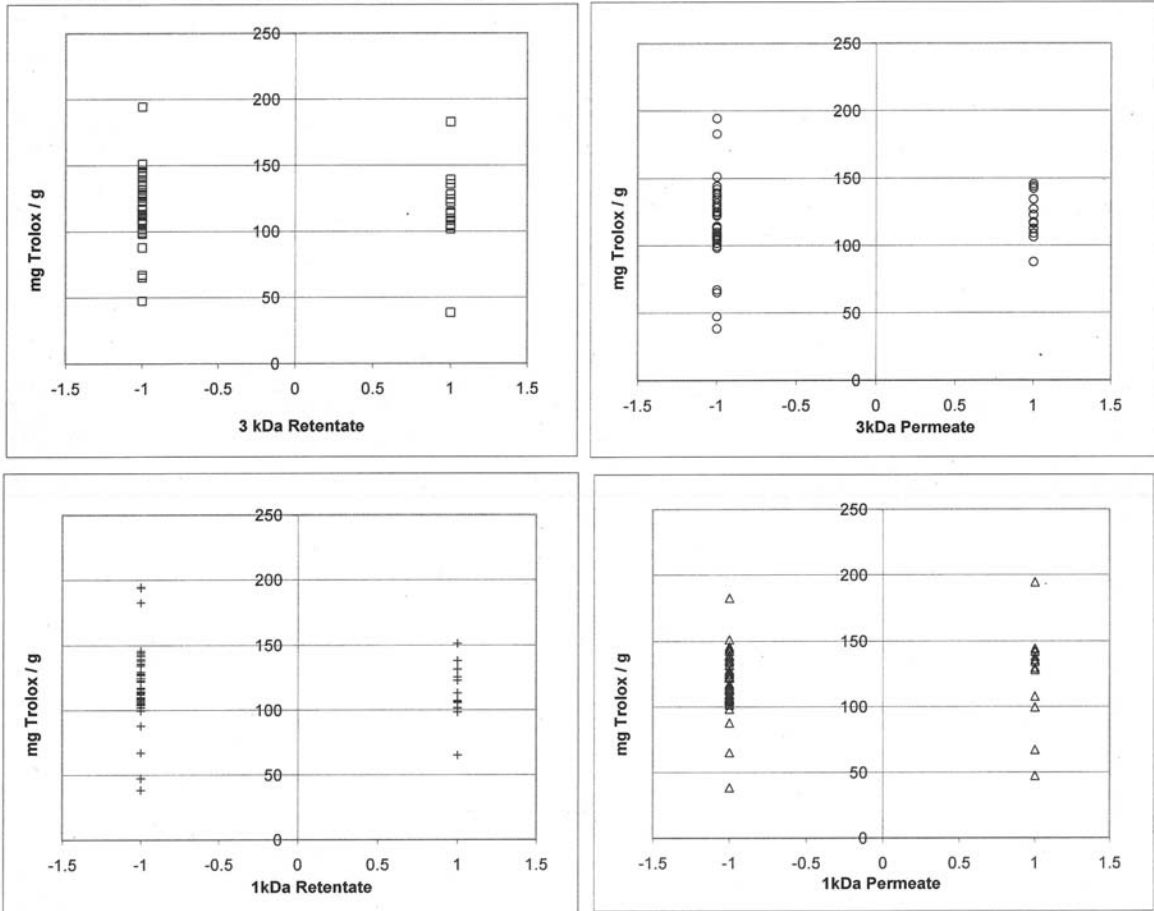
Observations:

Pepsin time (x3)   declining antiox with time above 0 minutes
                              outlier is 0 pepsin time associated with 1kDa permeate of Pancreatin digest

Pancreatin time (x4)   non-zero pancreatin time yields increased antioxidant capacity in ORAC

Conclusions
keep x3 and x4 in overall model

Ultrafiltration Fractions VS ORAC Antioxidant Capacity

Observations:

3kDa Retentate — no difference between (-1) not true and (1) true, although values appear more tightly grouped in the latter case

3kDa Permeate — very similar pattern expressed for these two ultrafiltration fractions - more variability in (-1) not true than true (1)
1kDa Retentate

1 kDa Permeate — no discernable effect of 1kDa permeate fraction on antioxidant capacity

Conclusions

fractionilation may or may not have an effect... keep filtration fraction terms in model (x5, x6, x7, x8)
likely filtration fractions are confounded with other terms, but insufficient data points exist here to study the interactions

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