Extraction of Milk Oligosaccharides from Lactose Mother Liquor

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1 SUMMARY

Oligosaccharides are a diverse group of bioactive sugars that are a critical part of a new-born's nutrition. In particular, sialylated oligosaccharides have been shown to be beneficial in stimulating brain development and providing resistance to infection in the first months of life, among many other benefits. Breast milk is naturally rich in these molecules and is the best possible diet for an infant, whereas infant formula based on cows' milk is much lower in oligosaccharides.

Because of the large throughput of the dairy industry, there is an opportunity to separate and enrich the small fraction of oligosaccharides to create an ingredient with a higher oligosaccharide content that could be added to infant formula to provide formula-fed babies with a level of nutrition closer to breast milk. Lactose mother liquor is a low value by-product of lactose manufacture and has been identified to have one of the highest oligosaccharide contents of any dairy stream, including a significant portion of sialylated oligosaccharides. The objective of this work was to develop a food grade, industrial scale process for enriching oligosaccharides from mother liquor to at least 4% on a dry basis.

While the laboratory characterisation and high purity isolation of milk oligosaccharide species has developed significantly in the past 20 years, there is comparatively little understanding of high volume separations from process sources. This presents an opportunity to research the behaviour of oligosaccharides and other components of lactose mother liquor in different separation systems.

Technologies were initially evaluated by mass balances and economic criteria, with reference to final oligosaccharide purity and yield, likely production cost, and food safety considerations. Nanofiltration and size exclusion chromatography had distinct advantages in these areas, while other technologies were likely to either give poor purity for their cost or product with non-food grade chemicals.

A process was selected that centres on simulated moving bed chromatography, a continuous separation of the smaller lactose, monosaccharide, and mineral components of ultra-filtered mother liquor which are more strongly retained on a size exclusion resin from the larger oligosaccharides. A four-column laboratory scale system was built and used in trials to determine its suitability, with results showing that a raffinate containing up to 35%

oligosaccharides on a dry basis could be obtained at a flow rate of 145 mL/hour (1.03 column volumes/hour).

Nanofiltration was also trialled for the separation of oligosaccharides from ultra-filtered mother liquor, but the size difference between oligosaccharides and lactose is too small for current membrane technology to separate completely and a maximum enrichment of 2.5% (dry basis) oligosaccharides was reached. This is lower than the 4% minimum oligosaccharide requirement which is needed to meet the minimum oligosaccharide to lactose ratio. Attempts to optimise nanofiltration using lactose crystallisation and multiple nanofiltration stages gave small improvements but still fell short of the 4% target.

A sequence consisting of decanting, ultrafiltration, simulated moving bed chromatography, and evaporation was chosen as the recommended process for oligosaccharide enrichment.

CONTENTS

2 INTRODUCTION

In addition to the disaccharide lactose, mammalian milk contains oligosaccharides. These are sugars with 3-10 units (most commonly 3-5) that are diverse in structure and function, varying between species and individuals, and represent an essential part of a new-born's diet (Goto et al., 2010). The past 10-20 years have seen a large number of studies and clinical trials which suggest oligosaccharides bring a surprisingly wide range of benefits to the human new-born, from a balanced initial colonisation of the lower intestine to assistance with brain development, protection from pathogens, improved nutrient absorption, and reduced risk of cancer and heart disease later in life (Gibson, Probert, Loo, Rastall, & Roberfroid, 2004; Macfarlane, Steed, & Macfarlane, 2008).

The link between oligosaccharide consumption (particularly sialic acid containing oligosaccharides) and brain development is perhaps the most crucial, as the effects on the brain during the first six months last for the individual's lifetime (Wang, 2009). Human milk has the highest oligosaccharide component of any mammal studied so far, with a large proportion of sialic acid containing molecules. Amongst other mammals there is a correlation between milk oligosaccharide content and relative intelligence. Cattle and sheep, for instance, with less developed brains and relatively simple behaviour, have the lowest concentration of oligosaccharides among studied mammals. Goats, elephants, dolphins, and other mammals with higher intelligence generally have higher levels of oligosaccharides but still fall short of the levels found in human milk (Kunz, Rudloff, Schad, & Braun, 1999; Traving & Schauer, 1998). This link with brain development is one of the many reasons why breast milk is the preferred nutrition for infants in the first six months after birth.

Infant formula is widely used where breast milk is not available or not preferred, and its formulation is tightly regulated in New Zealand and Australia (Australia New Zealand Food Authority, 2013) to provide the best possible composition of lactose, protein, lipids, minerals, vitamins, and other bioactive compounds for the formula fed infant, but generally does not include oligosaccharides. Recently, synthetic galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS, made by the hydrolysis of inulin) have been added to some formulae, but these are the simpler oligosaccharides (galactosyl-lactose and related species) which lack the sialylated and fucosylated compounds found in human milk. GOS is commonly produced

by enzymatic action on lactose solutions, whereas the complex mixture of natural oligosaccharides found in human milk cannot be manufactured in the same way. Both GOS and FOS provide indigestible carbohydrates for utilisation by *Bifidobacteria*, leading to the establishment of a population of healthy bacteria in the gut, which has associated benefits with regard to overall gut health and immunity (Gibson & Roberfroid, 1995), but does not provide the brain development aid that is associated with sialylated oligosaccharides. Methods have been developed to synthesise sialic acid and individual sialyl-oligosaccharide species (Pelletier, Barker, Hakes, & Zopf, 2004), but to obtain a complex mix as found in milk, an extraction from the milk itself must be made.

Since bovine milk is by far the most commonly processed of all milks, there is an opportunity to isolate oligosaccharides from high volume dairy streams that would otherwise be considered by-products or waste. Although the concentration of oligosaccharides in these streams is still very low, with highly selective and efficient separation methods and a high feed rate, a high value health ingredient could be produced. Oligosaccharides have been successfully extracted from the milks of different mammals in high purity at a laboratory scale for use in chemical analyses, but a process scale, economically feasible method of isolating a natural oligosaccharide stream from bovine milk has not yet been documented. Success in concentrating caprine milk oligosaccharides on a process scale using ultrafiltration and nanofiltration has been noted in the literature recently (Martinez-Ferez et al., 2009), due to the much higher relative concentration of oligosaccharides compared to bovine milk. With the current size of the global goat milk industry at just over 15 million tons per year, the majority being produced in India, Bangladesh, and Sudan (FAO Statistics Division, 2011) where milk processing is less developed, an economically viable volume of concentrated oligosaccharide mix would be difficult to support. This compares with a global bovine milk production of 602 million tons per year in 2012 with 20 million tons per year in New Zealand alone (FAO Statistics Division, 2013).

Lactose mother liquor (sometimes called de-lactose permeate), the liquid obtained from lactose crystal manufacturing, is a low value stream that has been identified as a possible starting material for oligosaccharide enrichment. Reasons for this include its relatively high production volume, low value, and its higher oligosaccharide concentration compared with raw milk and other process streams. This increase in concentration occurs as other milk components are removed during processing, including casein and fat (usually during cheese

manufacture), whey protein (through ultrafiltration) and most of the lactose and water (during evaporation and crystallisation). Mother liquor has 35-50% total solids, made up primarily of ash and lactose, with small concentrations of protein, organic acids, vitamins, and monosaccharides. Its oligosaccharide content is around 0.5% on a wet basis, compared to about 0.1% in raw milk.

Before commercial processes can be developed, an understanding of how lactose mother liquor behaves in different separation processes and how the oligosaccharide concentration is affected in each was required, so that the best separation process can be selected and optimised. The objectives of this work were:

- Use mass balances, economic predictions, and process constraints to predict the success of a number of different processes for the enrichment of oligosaccharides.

- Evaluate one or two of the best processes by laboratory experiments and pilot plant trials to confirm predictions.

- Understand the science behind the selected processes and the fundamentals which affect final oligosaccharide purity and yield.

- Explain the chemistry behind successful separation where molecules are close in molecular weight and electrostatic properties.

- Design a process that gives a significant increase in oligosaccharide concentration at minimal cost, whilst meeting regulatory and process constraints.

An enriched oligosaccharide ingredient with a mix of natural species including sialyloligosaccharides would represent a technological breakthrough, in the sense that it achieves a separation that has not yet been made, and would create value as an infant formula ingredient.

3 LITERATURE REVIEW

3.1 Sources of Milk Oligosaccharides

3.1.1 Natural Sources

Oligosaccharides occur naturally in all milk, and at especially high concentrations (around 8-10 g/L) in human milk (Geisser, Hendrich, Boehm, & Stahl, 2005). Many of the benefits in breast-feeding infants, including immune system and brain function development, have been linked to the large profile (up to 200 different species) and concentration of oligosaccharides present in human milk. Human milk is referred to as the "gold standard" (Kunz & Rudloff, 2006) of nutrition for infants, and good formulated infant milk seeks to mimic it as closely as possible.

In particular, sialic acid containing oligosaccharides are recognised as especially important for growth and development of infants because of the roles they play as anti-infection agents and in helping brain development. Other mammals with comparable oligosaccharide levels are elephants and goats (Kunz et al., 1999; Martinez-Ferez et al., 2009), whereas bovine milk (along with conventional infant formula) is low at 0.03 – 0.06 g/L (Barile et al., 2009; Sarney, Hale, Frankel, & Vulfson, 2000).

According to Macfarlane et al. (2008), human milk is made up of 7% carbohydrates (total basis), of which the majority is lactose. Neutral oligosaccharides make up roughly 1% of the total mass of the milk, and acidic oligosaccharides 0.1%. The proportion of oligosaccharides in human milk also varies over the lactation period. Oligosaccharides account for 24% of the total carbohydrates in human colostrum, compared with 15-19% in mature milk (Macfarlane et al., 2008). Studies reporting the concentration of oligosaccharides in breast milk give a range of values but are generally between 6 and 12 g/L (Geisser et al., 2005), with the higher of these concentrations found only in colostrum. These levels of oligosaccharides are similar to those found in goat's milk (Martinez-Ferez et al., 2009).

3.1.2 Process Sources

Barile et al. (2009) describe permeate from cheese whey ultrafiltration as a source of milk oligosaccharides, identifying 15 structures, most of which have been found in human milk, including sialyloligosaccharides. This study looks at purifying oligosaccharides from lactose

mother liquor, the liquid product obtained after lactose crystallisation and centrifugation of permeate. Mother liquor is similar in composition to whey permeate but has reduced lactose and water components and therefore an elevated oligosaccharide content.

The large volume processed by the dairy industry in New Zealand raises the possibility of the isolation of oligosaccharides from lactose mother liquor. This stream contains oligosaccharides, as well as a large proportion of residual lactose and dissolved salts. There are also small quantities of protein and fat. The oligosaccharide composition of Fonterra mother liquor is subject to intellectual property control; however, there are other literature sources that give a typical mother liquor analysis. Estimates of the oligosaccharide content of mother liquor vary from 0.1 – 0.6% on a wet basis (Pelletier et al., 2004).

Although the concentration of oligosaccharides in mother liquor is still very low, an economical way of separating them from such high volume streams would yield a significant amount of product which can be sold as an ingredient in infant formula. Prices for bovine milk oligosaccharides are not available, however galactooligosaccharides currently sell for about US$ 5 – 10 per kilogram. A natural mix of bovine oligosaccharides would command a premium over this, because of the sialyloligosaccharide content and increased functionality.

3.1.3 Oligosaccharide Synthesis

Galacto oligosaccharide (GOS) mixtures have been produced for many years is a relatively simple process using bacterial and fungal enzymes such as beta-galactosidase (Prenosil, Stuker, & Bourne, 1987b) and only contain neutral oligosaccharides (with no sialic acid) comprising galactose and glucose monomers. The chemistry and synthesis of these molecules is discussed below in Section 3.2. Although these oligosaccharides are not of the type that this project seeks to isolate, they are relevant to this research because the presence of enzymes from the cheese-making process in the whey permeate and lactose mother liquor means that some GOS will be synthesised (and some broken down) during further processing (Barile et al., 2009).

GOS have been widely used in infant formula for functional benefits, although there is scepticism as to how helpful this addition is for the health of the infant, given that human milk oligosaccharides (HMO) are composed of more diverse monosaccharides and only a small

portion of HMO are neutral oligosaccharides. There are few similarities in function between acidic and neutral oligosaccharides (Kunz & Rudloff, 2006).

It has also been reported that sialyloligosaccharides can be manufactured *in situ* in dairy processing streams, and a patent for this process has been issued to Pelletier et al (2004). α-(2-3) *trans*-Sialidase enzymes are used to synthesize sialyloligosaccharides from high lactose concentrations. The synthesis of both GOS and sialyloligosaccharides are outside the scope of this project.

3.2 Chemistry of Oligosaccharides

3.2.1 Structures Found in Mammal Milk

Because of the range of monomers available and the broad categorisation of oligosaccharides as chains of 3 – 10 monosaccharides linked by glycosidic bonds (Mehra & Kelly, 2006), the possibilities for different structures are almost limitless. Most species are derived from lactose and can be broadly categorised as acidic oligosaccharides (those with one or more sialic acid or phosphate groups) or neutral oligosaccharides, with non-ionic structures. Monomer units can be linked together with (1-6), (1-4), or (1-3) bonds. The main monosaccharide building blocks for oligosaccharides in milk are sialic acids (N-acetyl neuraminic acid and N-glycolyl neuraminic acid), N-acetylglucosamine, L-fucose, D-glucose, and D-galactose. The general structures and some examples of oligosaccharides are shown in Figure 3.1.

The number of oligosaccharides discovered in human milk is greater than 130 (Kunz, Rudloff, Baier, Klein, & Strobel, 2000; Macfarlane et al., 2008), and the exact makeup of the oligosaccharide fraction varies from woman to woman and is dependent on blood type and genetics (Kunz & Rudloff, 2006). The number of oligosaccharide structures in bovine milk is much lower, around 28, and it has been found that the oligosaccharides in bovine milk are almost all found in human milk (Barile et al., 2009). Many longer chain structures, as well as all fucosylated oligosaccharides, are absent in bovine milk (Mehra & Kelly, 2006). The most abundant oligosaccharides in bovine milk are the trisaccharides galactosyl-lactose and sialyllactose, with traces of tetraose and higher oligosaccharides also present (Kunz et al., 2000), as shown in Table 3.1.

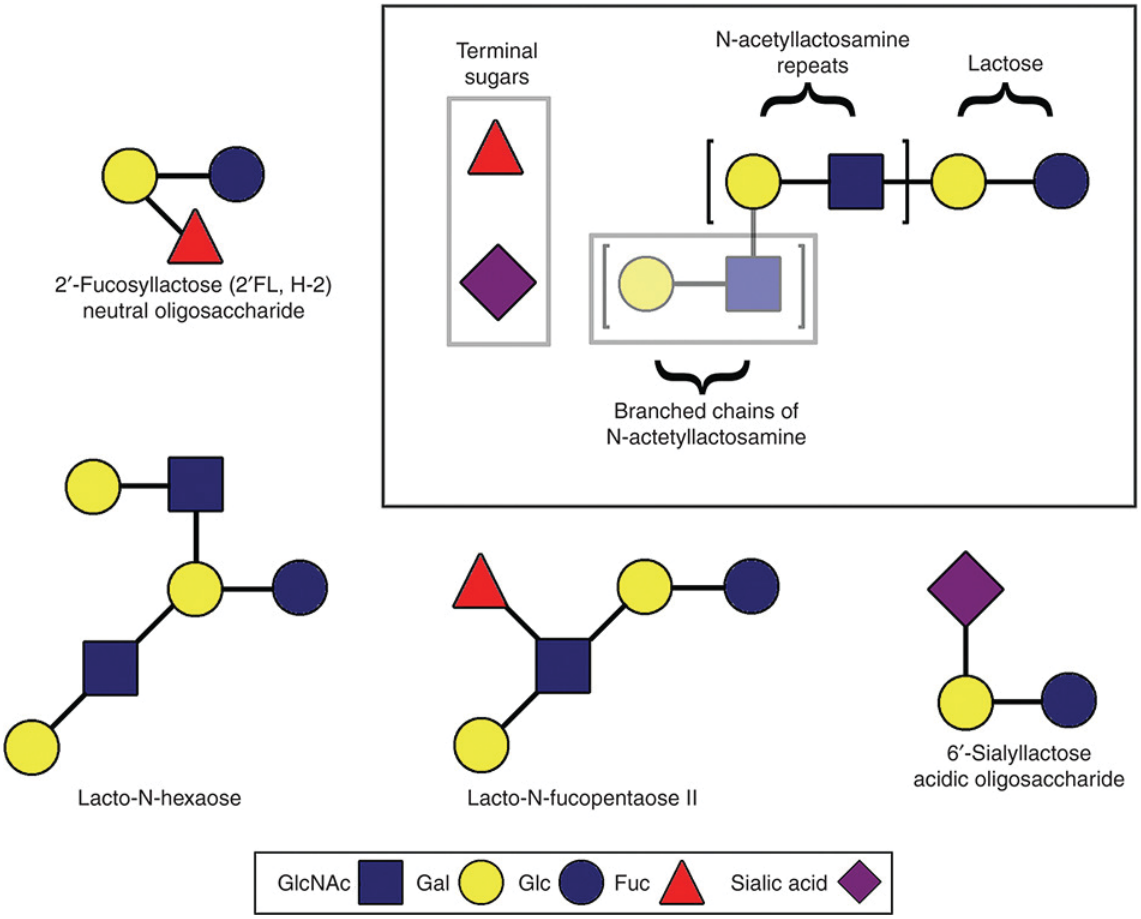

Figure 3.1: Structure and selected examples of milk oligosaccharides (from Newburg & Grave, 2014).

Porcine oligosaccharide compositions have also been investigated recently (Tao, Ochonicky, German, Donovan, & Lebrilla, 2010), with the discovery of predominantly sialylated compounds and low levels of fucosylated oligosaccharides. This composition makes the profile more similar to bovine milk than human milk.

Milk oligosaccharides (and other prebiotic oligosaccharides such as GOS) can be fermented by specific bacterial groups in the colon, e.g. *Bifidobacteria*. A by-product of this fermentation is short-chain fatty acids. These short chain fatty acids, in particular acetic, lactic, and formic, have antimicrobial effects with regard to certain strains of *Escherichia coli*, *Salmonella*, *Shigella*, and *Clostridia*. The endogenous human enzymes in the upper digestive tract are not able to breakdown most HMO or bovine milk oligosaccharides (BMO), leaving the metabolism to colonic bacteria in the lower tract (Tomomatsu, 1994).

Table 3.1: Oligosaccharides reported in bovine milks. From (Urashima et. al, 2011). NAc = Neuraminic acid, GL = Galactosyllactose, Gc = Glycolyl, SL = Sialyllactose, DSL = Disialyllactose, LNnH = Lacto-N-neohexaose, LNnT = Lacto-N-neotetraose, Le^x = Lewis X, Gal = Galacto, LNP = lectin nucleotide phoohydrolase, Lac = Lacto.

Neutral oligosaccharides		
1	*N*-Acetylgalactosaminylglucose	GalNAc(β1-4)Glc
2	LacNAc	Gal(β1-4)GlcNAc
3	Le^x trisaccharide	Gal(β1-4)GlcNAc Fuc(α1-3)
4	α3′-GalNAcL	GalNAc(α1-3)Gal(β1-4)Glc
5	Isoglobotriose	Gal(α1-3)Gal(β1-4)Glc
6	β3′-GL	Gal(β1-3)Gal(β1-4)Glc
7	β4′-GL	Gal(β1-4)Gal(β1-4)Glc
8	β6′-GL	Gal(β1-6)Gal(β1-4)Glc
9	*novo*-LNP I	Gal(β1-4)GlcNAc(β1-6) Gal(β1-3) > Gal(β1-4)Glc
10	β3′-GalNAcL	GalNAc(β1-3)Gal(β1-4)Glc
11	β6′-GlcNAcL	GlcNAc(β1-6)Gal(β1-4)Glc
12	LNnT	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
13	LNnH	Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)GlcNAc(β1-3) > Gal(β1-4)Glc
Acidic oligosaccharides		
14	3′-*O*-Lac phosphate	Gal(β1-4)Glc-3′-PO_4
15	3S-Gal	Neu5Ac(α2-3)Gal
16	3′-SL	Neu5Ac(α2-3)Gal(β1-4)Glc
17	6′-SL	Neu5Ac(α2-6)Gal(β1-4)Glc
18	3′-Neu5GcL	Neu5Gc(α2-3)Gal(β1-4)Glc
19	6′-Neu5GcL	Neu5Gc(α2-6)Gal(β1-4)Glc

20	6′-SLacNAc	Neu5Ac(α2-6)Gal(β1-4)GlcNAc
21	6′-Neu5GcLacNAc	Neu5Gc(α2-6)Gal(β1-4)GlcNAc
22	-	Neu5Ac(α2-3)Gal(1-3)Gal(1-4)Glc
23	DSL	Neu5Ac(α2-8)Neu5Ac(α2-3)Gal(β1-4)Glc
24	6′-SLacNAc-1-*O*-phosphate	Neu5Ac(α2-6)Gal(β1-4)GlcNAc-1-PO_4
25	6′-SLacNAc-6-*O*-phosphate	Neu5Ac(α2-6)Gal(β1-4)GlcNAc-6-PO_4
26	3′-SLacNAc	Neu5Ac(α2-3)Gal(β1-4)GlcNAc
27	3′-S- β6′-GL	Gal(β1-6)Gal(β1-4)Glc | Neu5Ac(α2-3)
28	6′-S- β3′-GL	Gal(β1-3)Gal(β1-4)Glc | Neu5Ac(α2-6)

3.2.2 Sialic Acid Structure and Function

Sialic acids are a diverse group of acylated derivatives of a 9-carbon monosaccharide. They are acidic monosaccharides that can be joined with lactose or other carbohydrates to form acidic oligosaccharides (Tang, Liang, Cai, & Mou, 2008). Sialyl oligosaccharides are present in high concentrations in breast milk and sialic acids have a number of important roles in the body, including acting as binding and transport agents for positively charged ions, repelling infectious cells and destructive enzymes, and inhibiting proteolysis in glycoproteins (Barile et al., 2009; Tang et al., 2008). More systemic effects include better brain function development and easier digestion of food. Specific sialic acids are also hypothesised to be potential risk factors in conditions such as tumours, cancers, diabetes, and heart disease (Tang et al., 2008).

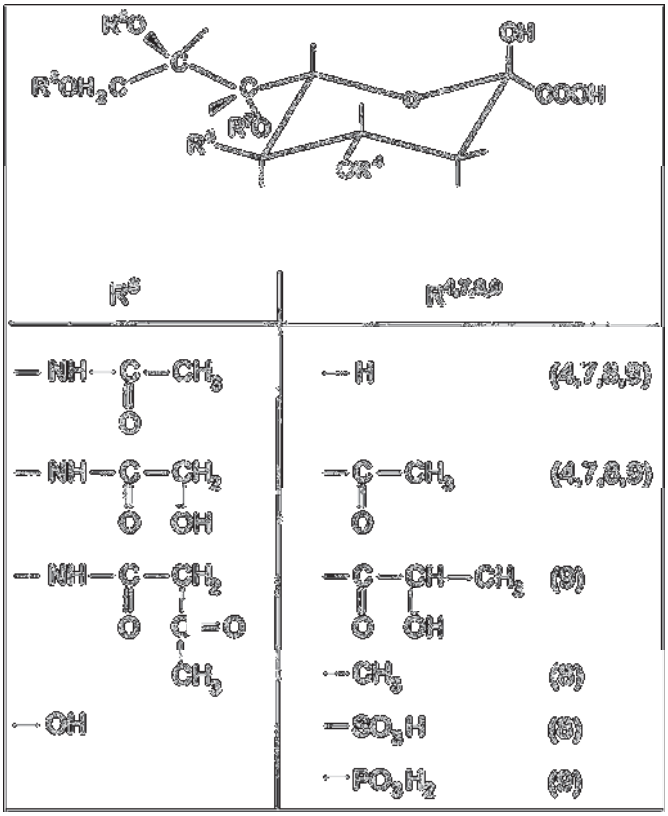

Figure 3.2: Chemical structure of the sialic acid molecule and a list of natural substituents, the positions of which are indicated in brackets (from Kunz et al, 1999).

The four most common sialic acids are differentiated by the substituents on the C-5 site: Neuraminic acid, *N*-acetylneuraminic acid (Neu-5-Ac), *N*-glycolylneuraminic acid (Neu-5-Gc), and deaminoneuraminic acid (KDN) (Tang et al., 2008), as shown in Figure 3.2. Neu-5-Ac is the most common sialic acid in human and bovine milk, while goats' milk is predominantly Neu-5-Gc (Wang, 2009).

3.2.3 Galacto-oligosaccharides (GOS)

GOS is produced industrially, primarily for the Chinese infant formula market, by the same reaction that hydrolyses lactose to glucose and galactose, as discovered in the 1950's (Macfarlane et al., 2008; Prenosil, Stuker, & Bourne, 1987a). Both α- and β-galactosidases are known to synthesize GOS, although β-galactosidase does so at a rate 60 times faster (Goulas, Tzortzis, & Gibson, 2007), so is the standard method in industry. The enzyme reaction uses a galactose transfer mechanism, and in the case of transfer onto a water molecule, the products are monomers (i.e. lactose hydrolysis). The recipient can, however, be another lactose

molecule, in which case the product is a trisaccharide of Gal-Gal-Glu, the most basic and abundant of the GOS group. For this reason, the yield of GOS is strongly dependent on the concentration of lactose being reacted – the higher the concentration of lactose, the more chance there is of the transfer occurring to another lactose molecule (Park & Oh, 2009).

When the lactase enzymes (e.g. *Kluyveromyces* β-galactosidase) are immobilised on a medium during synthesis, the yield of GOS drops by 14% for trisaccharides, and 28% for tetrasaccharides (Prenosil et al., 1987a). Media used for immobilisation have included ion exchange resins, chitosan, cellulose, agarose beads, and cotton cloths. The yield of GOS is typically in the range from 24 – 55%, depending on the reaction conditions (Goulas et al., 2007; Macfarlane et al., 2008). This GOS solution can then be purified by activated carbon adsorption or filtration, and concentrated (Macfarlane et al., 2008), or by fermentation by *Saccharomyces cerevisiae* to remove glucose (Goulas et al., 2007), although this technique does not remove any residual galactose.

Depending on the microbial source of β-galactosidase, different rates of oligosaccharide production can be obtained. *A. Oryzae* was found in one study to be the best source (Prenosil, Stuker, Hediger, & Bourne, 1984), although enzyme from *B. Bifidum* has been used in some cases as well (Goulas et al., 2007). Optimal conditions for β-galactosidase activity are typically around pH 6.8 and 40 – 45 °C (Goulas et al., 2007).

Although GOS are not the target for this research, the bacteria present in the cheese whey from which the bovine oligosaccharides are isolated may have significant transgalactosylase activity, leading to the production of small amounts of GOS (Barile et al., 2009). Such a process would give a mixture of GOS and naturally occurring oligosaccharides, which would raise the overall concentration for extraction.

3.3 Analytical Methods

Quantification and characterisation of the oligosaccharides present in a mixture using analytical methods is an important step before a process is designed to concentrate them. It is also necessary for determining the oligosaccharide content of the results of any experimental extraction. A number of techniques described in the literature will therefore be described here.

3.3.1 High pH Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

HPAEC-PAD is the most frequently used technique to determine the oligosaccharide content of milks, for instance by Tang et al. (2008) and Marinez-Ferez et al. (2009), as it can give clear peaks of individual species of oligosaccharides including both acidic and neutral molecules. Essentially, the alkaline eluents ionise the sugars, which are able to be separated on an anion exchange resin. The detection is then made by measuring the current on a gold electrode when the carbohydrates pass it. According to Tang et al. (2008), the method can detect sialic acid to ppm levels and is highly accurate, safe, and more straightforward than other methods.

3.3.2 Other Chromatographic Methods

High performance liquid chromatography (HPLC), gas chromatography (GC), and high performance thin-layer chromatography (HPTLC) have enabled oligosaccharide fractions to be characterised and quantified in many mammals including humans, cows, sheep, goats, and buffalo (Mehra & Kelly, 2006), although for sialic acid determination, these methods require derivatisation of the hydroxyl groups to give higher sensitivity. HPLC with a light scattering detector was used by Sarney et al. (2000) to quantify oligosaccharides purified by nanofiltration.

HPLC with size exclusion media was used by Geisser et al. (2005) to quantify the oligosaccharide contents of mixtures obtained by simulated moving bed chromatography. Size exclusion methods are only suitable for looking at the whole oligosaccharide fraction as opposed to individual species, since the resolution is based purely on size difference and many different oligosaccharides are very similar in molecular weight.

More simply, FPLC (fast protein liquid chromatography) has been used by Martinez-Ferez et al. (2009). This method runs at a lower pressure and, as with HPLC, allows only the quantification of the entire oligosaccharide fraction and not the individual oligosaccharide species. This means, however, that an alternative low cost oligosaccharide standard of increasing molecular weight (e.g. a malto-oligosaccharide mix) can be used, which does not require the often expensive specific oligosaccharide species that HPAEC-PAD does.

3.3.3 Mass Spectrometry

Mass spectrometry (MS) methods are expensive (Tang et al., 2008), although they can eliminate the need for oligosaccharide standards that are required for chromatography. Samples must be pure to achieve maximum ionisation (Barile et al., 2009), and for this reason MS is often linked to GC, although as with other chromatographic methods, GC requires prior derivatisation of hydroxyl groups (Fong, Ma, & McJarrow, 2011). MS does not guarantee consistent ionisation, and some researchers e.g. Barile et al. (2009) use relative quantitation rather than real quantitation.

Barile et al. (2009) used ultrafiltration and solid phase extraction to purify the oligosaccharides from protein, fat, and mineral contaminants, before performing both nanoelectrospray ionisation Fourier transform ion cyclotron resonance mass spectrometry (nESI FT-ICR MS) and matrix-assisted laser desorption/ionisation (MALDI) FTICR. Both methods were found to be accurate and reproducible, and did not require the use of a commercial standard. nESI FT-ICR gave a higher sensitivity for acidic oligosaccharides (e.g. sialyllactose), although MALDI-FTICR could identify neutral oligosaccharides which were not detected using nESI FT-ICR.

3.3.4 Other Methods

Other methods for identification and quantification of oligosaccharides include gel electrophoresis, used by Sarney et al. (2000) as part of the FACE (Fluorophore-assisted carbohydrate electrophoresis) to compare the oligosaccharide composition of milks of different sources, and capillary electrophoresis (Bao & Newburg, 2008).

3.4 Oligosaccharide Health Benefits

The benefits arising from consumption of oligosaccharides can be categorised into two broad groups – “prebiotic” effects related to all milk oligosaccharides (acidic and neutral), and those only related to acidic oligosaccharides.

3.5 Oligosaccharides as Prebiotics

Oligosaccharides are widely considered to be prebiotics – that is, they assist in maintaining a healthy gut micro flora through selective stimulation of bacterial growth, in particular *Bifidobacteria* and *Lactobacilli* species (Gibson & Fuller, 2000; Hawkins, 1993). For this reason, prebiotics have become a highly desirable ingredient in foods and medicine, and enjoyed immense popularity initially in Japan (Tomomatsu, 1994) and now in many other countries.

Such bacteria have been shown to deliver a host of benefits to the human body, including resistance to bacterial and viral infection through production of short chain fatty acids (SCFA)(Durand, Cordelet, Hannequart, Beaumatin, & Grivet, 1992; Macfarlane et al., 2008), increased immune system activity (Macfarlane et al., 2008), relief from constipation and diarrhoea, prevention of cancer (Tomomatsu, 1994), and synthesis of B-vitamins.

The term *prebiotic* should not be confused with *probiotic*, which is live bacteria delivered within a food or capsule. Since the beginning of the 20th century, it has been recognised that foods and medicines with "friendly" bacteria promote a healthy gut micro flora and boost longevity, as well as giving a number of benefits to the host (Gibson & Fuller, 2000; Hawkins, 1993). During the 1970's *Bifidobacteria* cultures were deliberately added to foods and termed "probiotics" for the first time, and these became very popular during the 1980's and 1990's as health-conscious people sought to boost their immune system and obtain other health benefits (Hawkins, 1993). There is, however, also a new class of foods, including both prebiotics and probiotics, called "synbiotics" (Macfarlane et al., 2008).

Prebiotics have, however, been demonstrated to be superior to probiotics for inducing a change in the gut microflora (Macfarlane et al., 2008). Practical reasons for the choice of prebiotics over probiotics are compelling for a few reasons: (i) For any noticeable probiotic effect, a large dose of bacteria (more than 10^6 CFU/g in food ingested) is needed, which can be difficult to attain (Hawkins, 1993). (ii) Probiotic inclusion demands a suitable food medium and storage conditions, given bacteria are sensitive to heat, shear, oxygen levels, and pH (Tomomatsu, 1994). This limits both the type of food that can be used as well as the processing that can take place subsequent to culture addition. Prebiotic ingredients, on the other hand, are mostly stable in high temperatures and acid, so can be added to a large range of food types (Macfarlane et al., 2008).

The potential use of prebiotics is even wider than that for normal dietary fibre, given it does not increase viscosity, requires a smaller daily dosage, has no objectionable flavours or textures, and is water soluble (Tomomatsu, 1994). For these reasons it is easy to include prebiotics in food. Both naturally occurring oligosaccharides (such as those found in milk) and those synthesised enzymatically (GOS and fructo-oligosaccharides) are legal food ingredients and not classed as novel foods (Macfarlane et al., 2008).

3.5.1 Dosage

Studies have shown (Macfarlane et al., 2008) that up to 10 g per day of fructo-oligosaccharide in the diet was beneficial, but 14 g per day led to abdominal discomfort and flatulence. Tomomatsu (1994) reported lower effective daily dosages of either 3 g FOS, 2 - 2.5 g GOS, 2 g Soy oligosaccharides, or 0.7 g xylo-oligosaccharides. In either case, there appears to be an upper limit to oligosaccharide consumption that is determined by abdominal discomfort and flatulence. The average intake of oligosaccharides is estimated (Tomomatsu, 1994) to be just 0.8 g per day from a normal western diet.

3.6 Criteria for Prebiotics

The exact definition of a prebiotic has not been easy to establish, and with many manufacturers seeking to label their food as prebiotic, this has been the subject of a great number of studies, which have covered a range of effects and criteria for prebiotic function. Gibson, Probert, Loo, Rastall, & Roberfroid (2004) provide a review on this topic and give the following criteria, all of which must be met for an ingredient to be considered a prebiotic:

1. Resistant to gastric acidity, mammalian enzymes, and absorption in the upper intestine.
2. Fermented by bacteria in the colon
3. Selectively stimulates growth or activity of bacterial species in the colon.

The first of these criteria is relatively easy to demonstrate, as resistance to human enzymes can be shown either *in vitro* or *in vivo* by a number of techniques. Almost all oligosaccharides are resistant to digestion by human enzymes, as is dietary fibre (Gibson et al., 2004).

Again, the second of these criteria is readily demonstrated by either *in vitro* (with batch or continuous culture systems inoculated with faecal slurry) or *in vivo* by adding the oligosaccharide in question to the food of human subjects or rats. In humans, the recovery of carbohydrate is ascertained by collecting hydrogen gas from exhaled air or by analysing the faeces, or in rats by anaesthetising and killing the rats for gastric analysis (Gibson et al., 2004).

The third criterion, however, requires measurement of the bacterial composition change, which is not easy to do. Many studies, for example Xiao et al. (2010) and Marcobal et al. (2010), have used pure cultures or a mix containing a limited range of bacteria *in vitro* which, whilst it shows that the bacteria studied can grow on a given carbohydrate, is not

representative of the colonic microbiota, and ignores the effects of the interactions between different bacteria, thus not showing that the carbohydrate stimulates some bacteria and not others. Some methods have been developed to overcome this, including *in vitro* testing using faecal inocula in gut models, for example that of Sharp, Fishbain, & Macfarlane (2001), although even this model fails to account for the variation among different regions in the colon (Gibson et al., 2004). More recent work has focussed on techniques such as denaturing gradient gel electrophoresis (DGGE) and reverse transcriptase polymer chain reaction (rtPCR) for assessing changes in gut microflora without the need for culturing samples (Reid et al., 2009; Tannock et al., 2004).

Furthermore, it is difficult to measure the resulting change in bacterial population, given that some bacteria cannot be cultured on agar plates and up to 50% of the species in the colon have yet to be identified. Some techniques, such as fluorescence *in situ* hybridisation (FISH), polymerase chain reaction, flow cytometry, and gel electrophoresis can be used, although each has significant limitations (Macfarlane et al., 2008).

3.6.1 Foods Currently Considered as Prebiotics

Currently there are only a few groups that fit all three criteria, namely fructo-oligosaccharides (FOS), Galacto-oligosaccharides (GOS, sometimes also referred to as transgalactosylated-oligosaccharides), inulin, and lactulose. Other candidate prebiotics, such as soybean oligosaccharides, xylo oligosaccharides, isomalto oligosaccharides, lactosucrose, and gluco-oligosaccharides have not been shown to fill all three criteria (usually criterion 3), although there is potential that further testing will allow a prebiotic classification (Gibson et al., 2004) and many are considered prebiotics despite the lack of thorough research, for example xylo-oligosaccharides (Vegas et al., 2008).

The fact that GOS (as produced from lactose) meets these criteria means that their use in foods has a sufficient body of evidence behind it to say that there are benefits of their consumption. *Bifidobacteria* prefer galactose, allowing them to compete for it well, and GOS and FOS have also been shown to promote *Bifidobacteria* growth, whereas xylooligosaccharides and pyrodextrins do not give the same effect (Macfarlane et al., 2008). Oligosaccharides from cows' milk contain a large proportion of neutral species, giving it a similar effect to GOS.

3.6.2 Other Potential Prebiotics

Lactulose was discovered to be bifidogenic in the 1950's, although it is classed as a drug and cannot be sold as a food (Harju, 1992). Lactulose, as well as Lactitol and Lactobionic acid, are derivatives of lactose and are all resistant to digestion (thus fulfilling the first criterion).

3.7 Benefits of prebiotics

3.7.1 Bacterial Profile Change

The bacteria stimulated by prebiotics are almost exclusively from the *Bifidobacterium* or *Lactobacillus* species (Macfarlane et al., 2008), and include *B. breve* and *B. infantis* (common in babies), *B. longum* and *B. adolescentis* (more common in adults), as well as *B. bifidum*. Studies have shown that in particular, GlcNAc, a major component of some human and bovine oligosaccharides, enhances the growth of *Bifidobacteria* (Kunz & Rudloff, 2006). Even within the B*ifidobacteria* genus, some species undergo much faster growth than others (Tao et al., 2010). *Lactobacillus acidophilus* is also stimulated, although their overall proportion in the gut is not high. Although *Bifidobacteria* and *Lactobacilli* have been shown to metabolise GOS and FOS, other bacteria such as *Clostridia* and *Bacteroides* can utilise these sources for energy too (Marcobal et al., 2010).

Ito et al. (1990) looked at correlations between oligosaccharide consumption and *Bifidobacteria* counts in human volunteers, by administering up to 10 g of Oligomate-50 (a mix with 52% GOS) daily. The result was a significant ($P<0.05$) linear relationship in which the ratio of *Bifidobacteria* to total bacteria increased from 0.26 ±0.12 to 0.48 ±0.19 with 10 g GOS ingestion. This study also looked at *Lactobacilli* numbers, but concluded there was only a slight increase after GOS ingestion. A high natural variation of *Lactobacilli* populations makes it difficult to establish the effect of oligosaccharides. The effect is similar in babies, where those fed with a mixture of 10% long-chain fructo-oligosaccharides and 90% GOS had higher *Lactobacillus* and *Bifidobacteria* counts and had faecal compositions similar to that of breast-fed babies (Macfarlane et al., 2008).

Infants are in particular need of prebiotic foods, because their immune systems are generally weak and the selective stimulation of *Bifidobacteria* helps to colonise a beneficial micro flora. Numerous studies have found that infants who are fed on breast-milk have higher faecal

Bifidobacteria counts than those fed on formula (Hawkins, 1993), due to the relatively high content (around 8 g/L) of oligosaccharides found in human milk. More importantly, however, is the finding that the counts of harmful bacteria such as *Clostridia* and *Enterococci* are much lower in breast-fed infants, even if the actual number of *Bifidobacteria* is unchanged (Kunz & Rudloff, 2006). In this sense, it is the relative bacterial ratio change that is important.

Although infants are in particular need of dietary oligosaccharides and this project is primarily concerned with applications for infant formula, there is benefit for people of any age. *Bifidobacteria* levels have been shown to naturally decrease with age and with other factors including mental stress (Tomomatsu, 1994), so a regular intake of oligosaccharide-containing foods is beneficial in countering this.

3.7.2 Short Chain Fatty Acid (SCFA) Production

The primary mechanism by which oligosaccharides deliver their benefit is selective metabolism by bacteria to produce SCFA such as acetate, lactate, and formate, all of which lower the pH of the gut and act as broad spectrum bacteriocins, inhibiting harmful bacteria such as *E. coli*, *Salmonella*, *Shigella*, *Staphylococcus*, and *Clostridia* (Hawkins, 1993; Macfarlane et al., 2008).

In summarising a number of studies, Rao, Srinivasjois, & Patole (2009) noted that the pH of stools in infants decreased by an average of 0.65 in subjects given infant formula supplemented with GOS prebiotics, due to SCFA production.

Durand et al. (1992) added 10 g of GOS to an *in vitro* intestinal setup with a continuous nutrient stream, and found an increase in SCFA production from 49.7 mmol per day (control) to 126.4 mmol per day (after 5 – 6 days of transgalactosylated oligosaccharides [TOS] addition). This change was seen most clearly in the acetate portion of the SCFA, which increased from 53.7% to 63.0% of the total SCFA present. Propionate and butyrate levels also increased after transgalactosylated oligosaccharide (TOS) addition.

Andrieux & Szylit (1991) studied the effect of TOS on bacterial activity and metabolite production in rats, finding that the total SCFA rose from 15.6 to 29.3 µmol/g when 5% TOS was introduced to the diet. There was minimal further increase in total SCFA when the TOS level was raised to 10%, although the SCFA composition changed dramatically to include a much

higher proportion of acetate, lactic acid, and succinic acid. Proportions of butyrate, valerate, and isoacids decreased.

Ito *et al* (1993) found that TOS intake increased the ratio of *Bifidobacteria* to total bacteria from 0.28 to 0.51 by lowering faecal pH. Given that 40 – 55% of colonic solids is bacterial mass (Tomomatsu, 1994), this shift in *Bifidobacteria* ratio is a significant result.

Other oligosaccharides such as lactulose, lactitol, and lactobionic acid are also fermented to SCFA (Harju, 1992).

3.7.3 Toxic Compound Reduction

It was stated by Tomomatsu (1994) that 3-6 g per day of oligosaccharide is effective in decreasing levels of toxic compounds by 40% or more. Ammonia and indole are two examples of such toxic compounds, and it is also reported by Ito *et al* (1993) that these were reduced by oligosaccharide ingestion. Fucosylated OS have been reported to inhibit diarrhoea caused by toxins of *E. coli*, *Campylobacter jejune*, and *Calicivirus* (Tao et al., 2010).

There is, however, great difficulty in establishing any conclusive evidence in the reduction of toxic compounds because of the length of the intestine and the great number of variables that cannot be controlled (Macfarlane et al., 2008). Further *in vivo* testing is needed to validate any effect.

3.7.4 Bowel Conditions

It is well documented that oligosaccharides help some aspects of bowel function, in particular stool consistency (Andrieux & Szylit, 1991; Chichlowski, German, Lebrilla, & Mills, 2011; Chow et al., 2014). On the other hand, most studies report little or no change in other aspects such as stool frequency or weight, e.g. Ito et al. (1990). Silk, Davis, Vulevic, Tzortzis, and Gibson (2009) studied the effect of TOS addition to the diet and found that measures such as stool consistency, flatulence, bloating, anxiety, and (most significantly) symptoms of irritable bowel syndrome, showed significant improvement.

3.7.5 Cancer Reduction

Some products of metabolism by harmful bacteria are pro-carcinogens, and by reducing the number of the bacteria mentioned above through beneficial bacteria growth, cancer risk can

be reduced (Macfarlane et al., 2008; Tomomatsu, 1994). Harju (1992) also reports that lactulose changes the bile metabolism, reducing the risk of colonic cancer.

3.7.6 Allergy Prevention

It has been found that infants with allergies generally have lower bifidobacteria counts than those without allergies (Macfarlane et al., 2008). Pirapatdit et al.(2008) studied the effect of dietary α-GOS (58% disaccharide, 28% trisaccharide, 14% OS more than three units long) in mice, finding that allergic peritonitis was suppressed in the mice that had been fed a diet including α-GOS.

Similarly, oligosaccharides from human milk have been understood to reduce inflammation in cells (Kunz et al., 2000) and reduce the incidence of inflammatory diseases (Kunz & Rudloff, 2006).

3.7.7 Mineral Absorption

Research into calcium absorption shows that increased *Bifidobacteria* counts also correlate with a higher calcium uptake, leading to a reduced risk of osteoporosis (Macfarlane et al., 2008). Bioavailability is also enhanced among infants (Geisser et al., 2005). Kruger, Brown, Collett, Layton, and Schollum (2003) found that FOS and inulin mixtures improved bone mineral density in rats.

3.7.8 Cholesterol and Satiety

A reduction in serum cholesterol has also been reported in some cases (Hawkins, 1993), as well as an increase in the ratio of HDL to LDL cholesterol in women (Tomomatsu, 1994). Larkin, Astheimer, & Price (2007) conducted a study which included either prebiotics (resistant starch) or probiotics (yoghurt) with a soy diet, finding that total cholesterol was significantly decreased in both cases.

It is also reported by Macfarlane et al. (2008) that FOS intakes of 8 g per day resulted in higher satiety and an associated decrease in food consumption.

3.7.9 Immunological Stimulation

Bifidobacteria have also been shown to increase the amount of IgA in the blood (Macfarlane et al., 2008), although there are few papers that support this. This is important for infants as it allows it to increase its resistance to infection.

3.8 Direct Roles of Oligosaccharides

Oligosaccharides, in particular those containing sialic acid residues, have been shown to effect changes in the body which are not related to the selective growth of *Bifidobacteria* in the colon. These are discussed here.

3.8.1 Epithelium Receptor Analogues

A recently described function of sialic acid containing oligosaccharides is that of mimicking the epithelium receptors in the intestine, and thus acting as soluble receptor analogues for viruses and pathogenic bacteria so that they are simply excreted from the body (Macfarlane et al., 2008; Sarney et al., 2000). It is known that attachment to the epithelium is generally required for infection, so by preventing this, oligosaccharides reduce the chances of a wide range of infectious agents such as *Influenza* virus, *V. cholerae*, and *E. coli* causing illness (Kunz et al., 2000). This is of course crucial for infants, whose immune system is still developing and is weak (Geisser et al., 2005).

3.8.2 Brain Development and Maintenance

Human milk oligosaccharides are also known to contribute greatly to brain development in the early stages of life, and Neu-5-Ac (a sialic acid) in particular has been reported to be essential in the myelination process in neonates (Wang, 2009).

The contribution to the development of brain function in infants by providing large amounts of galactose has also been covered by Kunz et al (2000). The monomers obtained when oligosaccharides are broken down by bacteria in the gut are absorbed into the blood stream and utilised to synthesize brain cells at a time when there is not enough galactose being absorbed from lactose consumption alone.

The galactose residues present in the oligosaccharides in breast milk have been traced using markers, showing that galactose molecules ingested by mothers are not metabolised by the liver, but incorporated directly into oligosaccharides in the mammary glands (Kunz et al., 2000).

3.9 Separation of Oligosaccharides

Oligosaccharide production can take a number of forms, including enzymatic synthesis of sialyloligosaccharides in dairy streams (Pelletier et al., 2004), using genetically engineered bacteria to ferment substrates, or expression using transgenic animals (Mehra & Kelly, 2006). Because the aim of this work was to enrich a natural oligosaccharide mix, with the full range of acidic and neutral oligosaccharides, isolation from genetically modified organisms was not studied in depth.

The difficulties encountered in the extraction of oligosaccharides from any sort of mixture are many and complex. Two general approaches may be taken in this regard: The first is to directly remove the oligosaccharides from the solution and retain the other components in a single waste stream. The other is to selectively remove the other components over a number of stages of treatment to eventually be left with a solution with a reasonably high oligosaccharide content that can be subsequently concentrated by evaporation or reverse osmosis.

Because the oligosaccharide fraction in bovine milk contains both neutral and acidic species in approximately equal proportions and there is a range of molecular sizes, a technique that targets the entire range of oligosaccharides is desirable as this more accurately represents the variance in HMO (Marx et al., 2014), and techniques for isolation of individual species are already available (Michalak et al., 2014). This means that the second approach, that of removing non-oligosaccharide components over a series of separations, is more likely to give a suitable product.

Of the components present in mother liquor, lactose is anticipated to be the most difficult to separate from the oligosaccharides. The reason for this is that the molecular weight of lactose (360 Da, in hydrated form) is close to that of the smallest oligosaccharide (Galactosyl-lactose, 504 Da), whereas other components are either smaller than lactose (i.e. monosaccharides and minerals) or much larger (i.e. proteins and fat). Additionally, the electrostatic properties are similar to that of neutral oligosaccharides, making charge based separation difficult. This has been noted by others (Geisser et al., 2005), and in some cases the lactose is not targeted for removal at all (Martinez-Ferez et al., 2009).

3.9.1 Lactose Crystallisation

A seemingly obvious method of removing the lactose (the component of mother liquor in highest concentration) would be to further crystallise it from the mother liquor. This step would take place as part of a series of steps (ultrafiltration, ion exchange) to remove components other than oligosaccharides from mother liquor.

This approach has not been successful, however, as other work has shown that dissolved lactose below a certain concentration in mother liquor cannot be crystallised (Wong & Hartel, 2014). This may be due to the effect of other components in the mother liquor such as minerals, organic acids, or even oligosaccharides themselves. The understanding of this phenomenon is outside the scope of the project, although if progress is made in this area then further lactose crystallisation may become a possibility.

3.9.2 Ultrafiltration

Ultrafiltration (UF) has a few major benefits over nanofiltration (NF) and other techniques, including its easy scalability, economic performance (mostly by running at lower pressures), and the minimal impact it has on product quality (Martinez-Ferez et al., 2009). This technique is usually reserved for compounds of greater molecular weight; in particular proteins, but some low molecular weight cut off (MWC) UF membranes have been studied and found to give appropriate separation (Vegas et al., 2008). In reality it is difficult to draw a line between UF and NF, and it is more important to test different membranes for performance, given a particular requirement.

The molecular weight cut-off provides minimal information about the actual product retention, since pore distribution can also have a major effect on the ability of molecules to pass through (van Reis & Zydney, 2007). Additionally, the electrostatic interactions resulting from charged molecules can change the effective size of a solute, as can the pH or ionic strength of the solution. This difference can be exploited in UF to allow separation of molecules with very small molecular weight differences. Although this has been applied to protein separation, the same cannot be said of oligosaccharides.

Martinez-Ferez et al.(2009) used a 50 kDa ceramic tube to isolate goat's milk oligosaccharides from protein. They found they were able to recover 95% of the oligosaccharides and remove

94% of the protein using three diavolumes, although these results are hardly comparable to cow's milk in terms of oligosaccharides because the initial concentration is much higher.

The most likely application of UF in this study will be in combination with nanofiltration or other low molecular weight separations so that the higher molecular weight material (residual protein, suspended solids, and fats) can be collected as UF retentate and the oligosaccharides (along with smaller molecules) can pass through the UF membrane. Further downstream processing would then separate the oligosaccharides from smaller species.

Any UF membrane used would need to be very high quality (free from defects) and replaced often, as the reduction in macromolecule concentration needs to be very high to ensure the oligosaccharides are in high concentration after nanofiltration, creating a higher value product.

3.9.3 High performance Tangential Flow Filtration (HPTFF)

This application of membrane technology allows separation of molecules very close in molecular size and has reportedly been used in extracting oligosaccharides from monomers (van Reis & Zydney, 2007). The main mechanism by which this is achieved is a co-flow operation, where a pressure is maintained on the permeate side to give a constant pressure drop across the length of the filter (van Reis & Zydney, 2007). Charged membranes can also be incorporated to enhance the separation of ionic groups. Although the technology appears capable, there are few examples of its use in the literature so any application would require thorough testing before commercialisation.

3.9.4 Nanofiltration

Nanofiltration has a lot of promise for the removal of oligosaccharides, and some studies have seen effective separations with purities of up to 97% and retentions of oligosaccharide up to 80%. There is a large variation between membrane types, with some suppliers performing much better in this particular separation than others. It was found that pressures of around 8 bar were required for the best performing membranes, though changes in the pressure could be made which resulted in different recoveries or purities, depending on the total solids content of the solution.

Sarney et al. (2000) successfully extracted oligosaccharides from human milk by using nanofiltration in combination with enzymatic pre-treatment to hydrolyse the lactose to monosaccharides. The loss of oligosaccharides during enzyme contact was found to be

minimal, and the hydrolysis was complete within 4-5 hours. From a sample containing 2.6% (dry weight) oligosaccharides, a retentate with more than 97% purity (dry weight) was obtained after four diafiltration cycles. This compares to just 50% oligosaccharides in the retentate achieved without enzymatic hydrolysis after the same filtration. The NF-CA-50 (Intersep) membrane proved best of the three that were selected after initial pretesting, and no difference in separation was found between 10 and 40 bar operating pressure.

Although this is a positive result, the authors do not give the result for when they used the same experimental method with bovine milk, only mentioning that the yield was very low.

Vegas et al (2008) evaluated several nanofiltration membranes for the recovery of xylo oligosaccharides from rice husk waste, finding that the 4 kDa polymeric tubular ESP04 and 1 kDa ceramic monolithic Kerasep Nano (both from Novasep) gave the best results. Other membranes were impermeable to monosaccharides or allowed too much of the oligosaccharide fraction through. There were some trade-offs, as expected, between fractionation (best with the ESP04) and recovery and flux (better with the Kerasep Nano). Yields were around 71%, whilst purity was generally over 91% for both membranes. Again, the initial concentration of oligosaccharides was high (56.5% on a dry basis).

Nanofiltration is an easily scalable technology (Sarney et al., 2000), and requires little in the way of other processes to obtain an enriched fraction. Some drawbacks related to nanofiltration are the propensity for fouling and concentration polarisation (Vegas et al., 2008). The former can be overcome by good design and frequent cleaning and membrane replacement, whilst the latter can be reduced by having a turbulent feed stream and thereby reducing the boundary layer at the membrane.

3.9.5 Membrane Chromatography

This separation uses microfiltration membranes that include functional ligands, allowing highly selective interactions to take place. The benefits over conventional chromatography are much higher flow rates, lower pressure drops, and shorter processing times (van Reis & Zydney, 2007). The technology has found application in the pharmaceutical industry for high value products. Different membrane materials can be modified to include ion exchange components.

Little was found in the literature about such techniques in the food industry because it is typically associated with high value extractions. Because the interactions are highly specific and there are many different species of oligosaccharide to extract, it is unlikely this method will relevant to this process.

3.9.6 Cation and Anion Exchange

These processes would be used together in series to remove components other than oligosaccharides, in particular dissolved salts and other polar molecules. The separation does not rely on a size exclusion principle, but rather discriminates on the basis of charge through adsorption onto media.

A distinct advantage of ion exchange processes is that they can be modified and adapted to meet the specific needs of a process. This can be done through selection of different resins and using different eluents to ensure only the components of interest are recovered from the process. The pH can also be adjusted to exploit the differences in charge, as described by Shimatani, Murakami, Idota, and Ido (1992). The patent describes a method of reducing the pH of whey to between 2 and 5, then passing the solution through a cation exchanger. Using lactose crystallisation to treat the exchanger-passed liquid, concentrations of 300 mg/L sialyllactose were obtained, and this increased to 3300 mg/L or even 4750 mg/L (dry basis) when used in combination with ultrafiltration and diafiltration for removal of salts.

In one type of setup, the oligosaccharides, with either neutral or relatively weak acidic charges, would be retained in the flow, whilst charged molecules such as minerals are segregated into either of the anion or cation columns and eluted later by altering the composition of the eluent. In this sense, the process would be a demineralisation step, requiring protein and lactose removal in other steps.

Alternatively, ion exchange can be used to target acidic oligosaccharides, in particular sialyllactose. This requires a selective resin and modification of the eluent to remove impurities whilst still retaining the acidic oligosaccharides. Elution is typically done using a large volume of salt (e.g. NaCl or NaOAc) and later desalted using reverse osmosis or a similar technique (Brian, Zopf, Lu, McCauley, & Partsch, 1998). This process would be largely standalone in nature as it could target oligosaccharides whilst leaving all other components in the waste stream. The main disadvantage of this method is that only the acidic oligosaccharides are collected from a fraction that contains a large proportion of neutral

oligosaccharides, and as stated earlier, both fractions are beneficial in the final product. Another problem is the addition of some eluents jeopardises the safety of the final ingredient, which is subject to very stringent regulations as an infant formula component.

Maischberger et al. (2008) used ion exchange to remove the impurities lactobionic acid and monosaccharides from a mixture of GOS, where the lactobionic acid was a product of lactose oxidation. They found that 99.7% of the lactobionic acid was removed from the GOS after three consecutive runs. Monosaccharides were then removed using a strong cation exchange resin, which effectively ionises the sugars to allow attachment to the column. Again, very high removal rates were obtained (99.7% for glucose, 99.2% for galactose). There was significant loss of GOS (60.4% recovery over both stages), although the purity was very high, at 99.1%.

Another application of anion and cation exchange involves the elution of oligosaccharides with lithium acetate, as described in the patent by Brian et al (1998). This can be achieved because lithium salts of sialic acid have low solubility in organic solvents, whereas other salts are more soluble. Although the oligosaccharide fraction is almost pure upon elution, the toxicity of lithium acetate means it would be difficult to render the product safe for consumption.

3.9.7 Simulated Moving Bed (SMB) Chromatography

Process Description

SMB, illustrated in Figure 3.3, is the most developed continuous chromatographic technology, utilising what is effectively a counter-current chromatographic separation between solid phase (resin) and liquid phase (eluent). As opposed to a hypothetical true moving bed, with a solid phase moving around a loop counter to the liquid phase flow (which would be almost impossible to create in practice), a simulated moving bed instead periodically rotates the inlet (feed and eluent) and outlet (raffinate and extract) points around the loop with a stationary solid phase to create a frame of reference by which, relative to each of the inlets and outlets, the solid phase is moving counter-current to the liquid phase (Amalgamated Research Inc., 2008). This allows a continuous process with a large number of columns to be developed, which is of much more useful for industrial separation than conventional (batch) chromatography. SMB chromatography uses much lower quantities of eluent and has a higher productivity than conventional chromatography (Buhlert, Lehr, & Jungbauer, 2009; Geisser et al., 2005).

Hernandez et al. (2009), in evaluating potential fractionation techniques for GOS, concluded that size exclusion chromatography gave the best purity and recovery, and allowed a range of oligosaccharides to be recovered. Other techniques compared were diafiltration, yeast treatment, and activated charcoal adsorption.

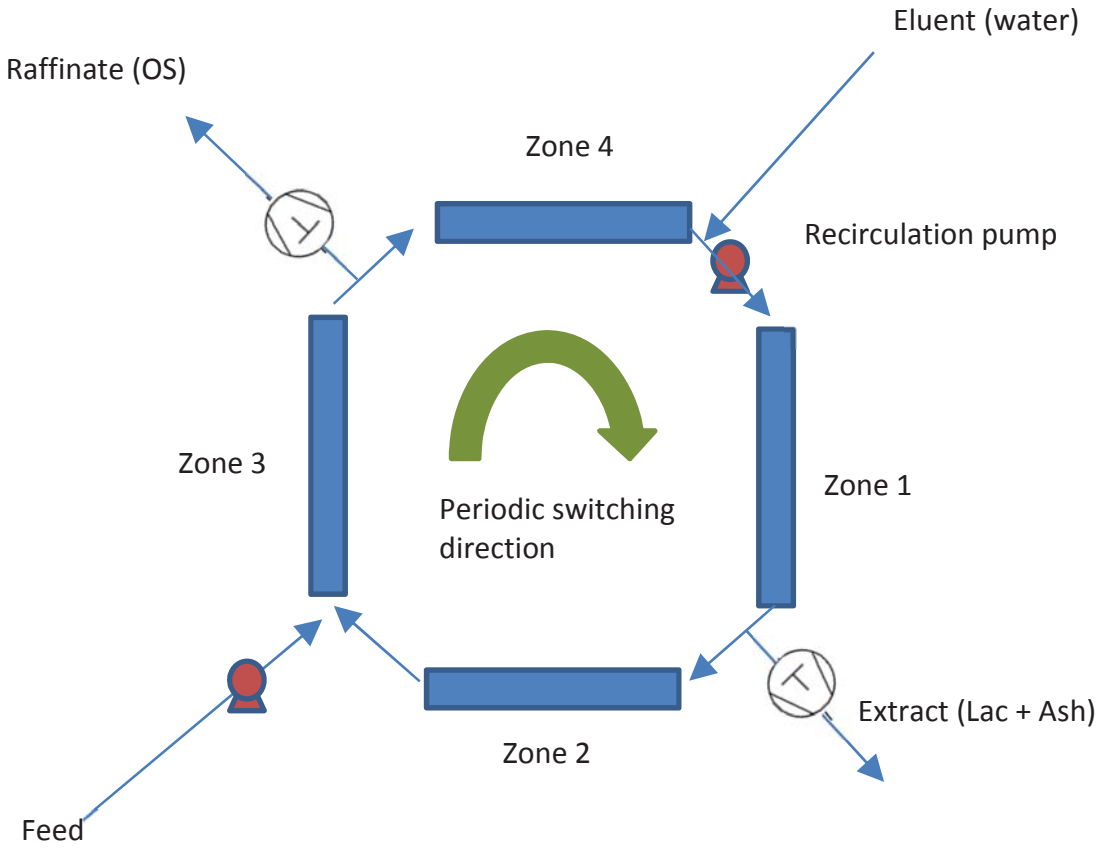

Figure 3.3: Typical SMB setup with 4 columns. The feed, extract, eluent, and raffinate streams rotate periodically in the direction of fluid flow to simulate counter-current flow (Futterer, 2010).

Common uses for SMB are mostly found in the sugar industry (although it was in the petro-chemical industry that the process was initially developed), particularly in glucose-fructose separations, using ion exchange resin with warm water as eluent. The high fructose corn syrup (HFCS) industry is particularly well developed and has used SMB to enrich the fructose fraction of a mix with glucose for around 25 years (da Silva, de Souza, de Souza, & Rodrigues, 2006), including reactive SMB technology, where the isomerisation reaction and separation take place within the same unit operation (Toumi & Engell, 2004; Zhang, Hidajat, & Ray, 2004). This process is well developed and literature is available on its analysis and optimisation (Azevedo

& Rodrigues, 2001, 2006; Toumi, Hanisch, & Engell, 2002).Most recently, SMB has been applied to chiral technology, separating enantiomers which are very difficult to separate by conventional means (Lee, 2009). Some other research has made separation between monosaccharides and disaccharides possible, whilst oligosaccharide separations are less common (Blehaut & Nicoud, 1998).

Laboratory Setups

Variations in the number of columns are common, as well as a single column form of SMB described by Buhlert et al. (2009). The extract, feed, raffinate, and eluent are introduced or removed *via* distributor plates positioned at a regular spacing up the column to give four distinct zones, as with multi-column SMB, and a recycle line provides flow from the bottom of the column to the top. By calculating the height equivalent to a theoretical plate (HETP), it was shown that the packing was equivalent in each of the four zones of the column. Plug flow was also demonstrated to be present by calculating dispersion numbers. The advantages are less complex connection tubing, more uniform packing, reduced total headspace, and simplified cleaning operations. These factors combine to reduce the complexity and cost of SMB chromatography. Purities obtained were up to 86.8% (from an initial purity of 3.4%) for vitamin B12, and 97.7% (from an initial purity of 96.6%) for dextran.

A range of pilot scale SMB systems is available for experimental work, including Varicol, which adds the ability to stagger the switching time between columns for more efficient separation (Aniceto & Silva, 2015), and systems designed to achieve ternary separation through “pseudo-simulated moving bed”, where the feed and eluent are introduced in an initial step equivalent to a series of preparative columns before being separated in a second step on a SMB without further eluent or feed addition (Jiang, Huang, & Wei, 2014; Mata & Rodrigues, 2001; Palacios, Kaspereit, Ziomek, Antos, & Seidel-Morgenstern, 2009).

Media Selection

SMB processing can utilise any form of resin, including size exclusion (most relevant for this project), ion exchange, or reverse phase resins. The requirement is that the eluent remains a constant composition, ruling out bind and elute methods (more similar to solid phase

extraction). Size exclusion gel seems to be preferred over other gel types for oligosaccharide separations (Geisser et al., 2005), although ion-exchange chromatography has also been effective, mainly in separation acidic oligosaccharides from neutral ones (Smith, Zorf, & Ginsburg, 1978).

Size exclusion gels are designed to encourage steric hindrance of large molecules and kinetic transport of small molecules within the pore structure, and are ideally free from any adsorptive activity, although in practice this is seldom achieved (Specht & Frimmel, 2000). The larger molecules are prevented from entering micro-pores within the beads and pass through the smaller volume of macro-pores around the beads to elute first, whilst the small molecules follow a more tortuous path through the micro-pores and are eluted later (Lee & Chang, 1996). From a thermodynamic perspective, the diffusion coefficient (K) into and out of the pores is governed by

$$K = \exp\left(\frac{-\Delta H^\circ}{RT}\right)\exp\left(\frac{\Delta S^\circ}{R}\right) \qquad \text{Equation 3.1}$$

where R is the gas constant, T is absolute temperature, ΔS° is the change in conformational entropy, and ΔH° is the change in enthalpy. For size exclusion chromatography, ΔH° is ideally equal to zero, since there should be no enthalpic reactions between the solutes and media. The equation then becomes

$$K = \exp\left(\frac{\Delta S^\circ}{R}\right) \qquad \text{Equation 3.2}$$

i.e. movement into and out of pores is governed purely by entropy differences (Mori & Barth, 1999).

Size exclusion media are manufactured from a variety of materials including silica, suited to low temperature, high pressure, protein separation, and methacrylate, which is less sensitive to high temperatures and pH and more suited to polymer separation. Other media are manufactured from agarose or cross-linked polyacrylamide.

For oligosaccharide enrichment, size exclusion media are preferred because they are generally not able to discriminate on the basis of charge, selecting instead on the basis of molecular weight, or, more precisely, hydrodynamic size. Among size exclusion gels available, a separation of oligosaccharides would generally require the smallest separation range – with an effective separation between 100 and 1000 Da. One such product is Superdex peptide (GE

Healthcare, Auckland), with a separation range of 100 – 7000 Da, aimed at peptide purification.

For a process scale SMB system, the pressure drop requirements dictate that a coarse particle size needs to be used in the columns, since they are much longer. This means for any experimental work or pilot scale trials, an appropriate resin and pressure drop needs to be chosen so that preparative scale conditions can be properly estimated.

A summary of the properties of an ideal medium for oligosaccharide isolation can now be given:

- Separation range of 200 – 1000 Da to exploit difference between oligosaccharides and lactose.
- Narrow pore size distribution, to give a high selectivity in this range.
- Non-adsorptive for hydroxyl groups and anionic species.
- Large bead size (>100μm diameter) to allow for a low pressure drop over preparative columns.
- High resistance to osmotic shock (needed because of the high concentration of ash in the feed).
- Long resin lifetime, to minimise replacement costs.

Application to Oligosaccharide Enrichment

SMB can only separate the feed into two streams – the process will isolate either the most retained (extract) or least retained fraction (raffinate), so there would need to be either upstream or downstream processing to remove either all of the larger molecules (proteins and lipids) or all of the smaller molecules (lactose, monosaccharides, and ash). Since lactose is most similar in molecular weight to oligosaccharides and therefore most difficult to remove, as discussed earlier, it would make sense to undertake this removal with SMB and use UF to remove proteins and lipids.

Geisser et al. (2005) performed SMB separations on human milk, with lipids and proteins removed by centrifugation and precipitation, to obtain oligosaccharides, and found that a size exclusion (SEC) gel gave more stability than a ligand exchange chromatography gel (MCI) for continuous use, although the latter gave higher flow rates. Analysis of the products by HPLC

gave less than 4% lactose in the raffinate and more than 96% lactose in the extract for the SEC gel, and 0-20% lactose in the raffinate and more than 96% lactose in the extract for MCI. Although this is high purity for human milk oligosaccharides, which has a relatively high initial concentration, this degree of separation would need to be replicated for the much lower oligosaccharide concentrations in bovine milk to allow any meaningful increase in oligosaccharide concentration from cow's milk. No literature has been found regarding use of SMB to extract the full fraction of oligosaccharides from bovine milk.

Operation

Determining the correct flow rates and switching time for a chromatographic separation can be difficult, and requires a model to be developed based on the retention times obtained on an experimental batch chromatography column with the same resin. The difficulty of these calculations increases when non-linear isotherms are encountered, such as Langmuir isotherms, although much literature is devoted to this type of separation (Futterer, 2010; Fütterer, 2009; Mazzotti, Storti, & Morbidelli, 1997). Fortunately, the very low concentration of oligosaccharides found in mother liquor means that the adsorption during chromatography can be modelled by a linear (Henry's) isotherm, the application of which is relatively simple and described by Rajendran (2008).

Chromatography is a promising technology because unlike filtration or electrodialysis, where there is likely to be some degree of leakage of contaminants into the product stream, it can theoretically deliver a pure extract if the correct parameters are used. This can be difficult to maintain at large scale, however, due to non-uniformities in the bed packing and larger solid phase particle sizes. Another major issue with chromatographic separations, whether SMB or batch operations, is contamination from proteins and fats which, if they are in high concentration, can clog pores and lead to irregularities and deterioration of the equipment over time (Prusisz & Pohl, 2008).

3.9.8 Preparative Continuous Annular Chromatography (P-CAC)

This is another recent development in the field of separation processing, and involves an annulus packed with resin rotating slowly as a fixed feed point introduces the material to be

separated (Finke et al., 2002). Eluent is distributed evenly across the top of the column. The difference in affinity for the resin gives different helices as the feed flows under gravity to the bottom of the cylinder, where stationary collection points can receive the separated fractions. The main benefit of this process is that multi-component separations can be made relatively simply, which is not the case for SMB chromatography.

Although the theory seems promising, there may be factors that prevent its use for the separation of oligosaccharides. Most importantly, as the flow rate of feed is increased, as with other forms of chromatography, the selectivity suffers as dispersion and irregular bead sizes become more significant, meaning large beds or expensive high grade beads. It is also a relatively inefficient use of the solid phase, given that only a small portion of it is being used at any time, increasing the initial cost of stationary phase.

3.9.9 Solid Phase Extraction with Graphitised Carbon

Also an adsorption process, solid phase extraction relies on the difference in affinity between oligosaccharides and other constituents, for graphitised carbon. Ward (2009) successfully extracted oligosaccharides from human milk using this technique, obtaining over 5 g of oligosaccharide from a litre of milk of unspecified initial concentration (usually 8 – 12 g/L). As with the nanofiltration carried out by Sarney et al. (2000), lactose was digested using β-galactosidase of microbial origin. It was found that the columns used for extraction could bind up to about 20% of their weight in oligosaccharides, although overloading the column with material washed the HMOs from the column and gave poor recoveries. For elution, increasing concentrations of butanol released monosaccharides and disaccharides first, before eluting pure oligosaccharides at more than 1% butanol (Ward, 2009). Acidic HMOs were more strongly adsorbed to the carbon than neutral HMOs and were eluted with 4% butanol and 0.1% Trifluoroacetic acid (TFA).

Earlier work was carried out by Packer, Lawson, Jardine, & Redmond (1998), demonstrating that solid phase extraction using standard activated carbon columns is an efficient method of removing contaminants from oligosaccharides, including proteins, salts, and detergents. Moreover, it is suggested that neutral and acidic oligosaccharides can be separated from each other. In this case, the eluent was acetonitrile for the recovery of neutral oligosaccharides and 0.05% TFA was added (see also Ward (2009)) for recovery of acidic oligosaccharides. Recoveries of up to 90-97% were achieved, although the purity of oligosaccharides was not

given and it is likely that some of the charged monosaccharide groups would elute in the same wash as the acidic oligosaccharides. According to the authors, salts and neutral monosaccharides elute with water (first wash), and proteins are said to remain in the column even after the final wash to remove acidic oligosaccharides.

A subsequent study (Redmond & Packer, 1999) again demonstrated that acidic and neutral oligosaccharides can be separated easily, along with the removal of contaminating salts (which anion-exchange chromatography cannot remove). Again, it is unclear from the paper what degree of purity was obtained for oligosaccharide fractions.

Although solid phase extraction of this form gives a reasonably pure sample of oligosaccharides, the process requires step-elution of the columns to obtain the desired fraction, and it is more difficult to achieve a product with oligosaccharides of the entire size range (Hernández et al., 2009). Furthermore, acidic oligosaccharides, probably containing sialic acid, require TFA as well as an organic modifier for elution, which would require subsequent treatment for removal before inclusion in a food product.

Instead of binding the oligosaccharides, Montane, Nabarlatz, Martorell, Torne-Fernandez, & Fierro (2006) used the activated carbon column to bind the lignin impurities from almond shell hydrolysis liquor. Although this is helpful for removing single components, it would be far less feasible for removing multiple components whilst still retaining both acidic and neutral oligosaccharides.

3.9.10 Electrodialysis

This process involves passing an electric current through a solution, with the aim of separating molecules on the basis of charge, and is commonly employed for solution demineralisation. Essentially, the positively and negatively charged molecules are attracted to the anode and the cathode respectively, and pass through ion exchange membranes and are retained within alternate channels containing diluate where they are blocked by ion exchange membranes of the opposite type. The feed streams, in alternate channels become demineralised over the length of the unit; see Figure 3.4 (Bazinet, Lamarche, & Ippersiel, 1998).

Advantages of electrodialysis are the low energy consumption and environmental friendliness, convenient design, and ease of operation (Bazinet et al., 1998), although highly charged

molecules are easier to separate, meaning oligosaccharides with only a slight charge would remain largely unaffected.

Electrodialysis with ultrafiltration (EDUF) was carried out by Aider, Brunet, & Bazinet (2009) on a pure solution of chitosan oligomers, based on the discovery that chitosan has a net positive charge at pH 4. They found that dimers migrated most easily, followed by trimers and tetramers, which is consistent with the principle of electrodialysis, that small charged molecules are most easily separated. Higher electric field strength, from 2.5 – 10 V/cm, was found to increase the migration of chitosan, although increased solution flow velocity did not give any significant effect. Times of up to 1.5 seconds were needed for the dimers to migrate to the membrane.

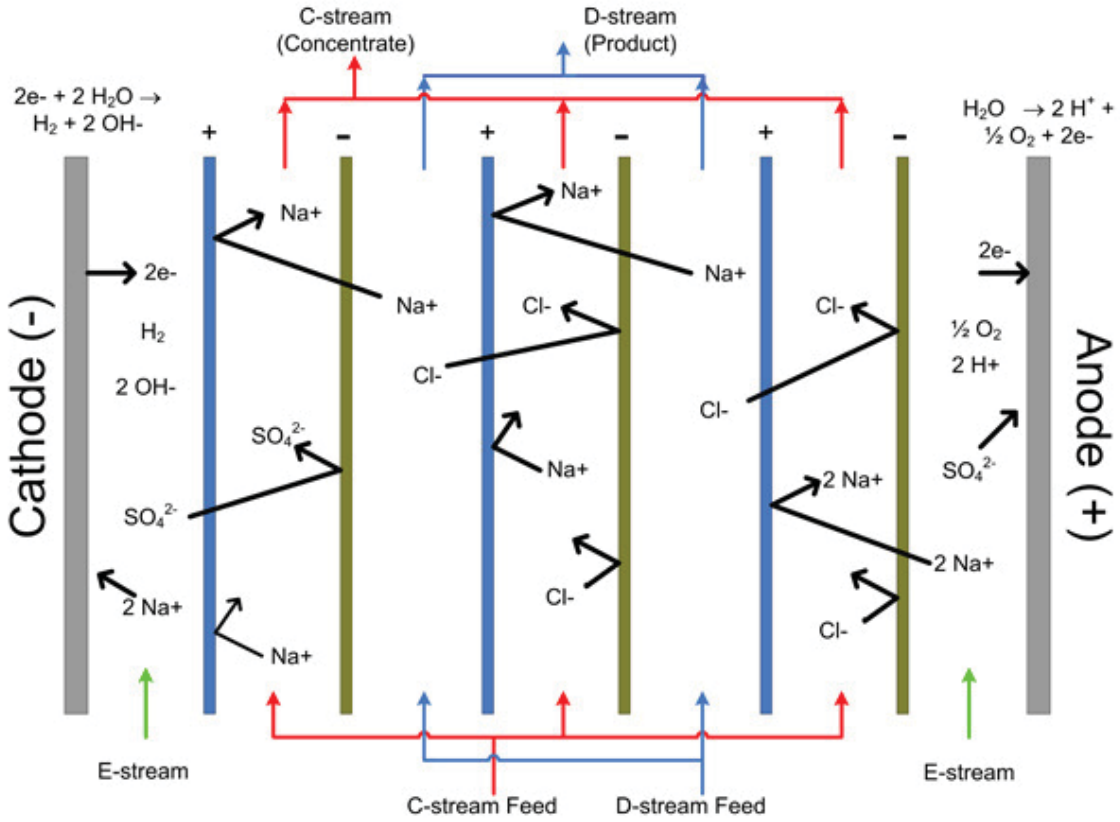

Figure 3.4: Schematic of electrodialysis unit showing anion exchange (blue) and cation exchange (green) membranes with diluent (D), concentrate (C), and electrolyte (E) streams. From EET Corporation (www.eetcorp.com).

In previous work on chitosan (Aider, Brunet, & Bazinet, 2008), it was found that the MWCO of the membrane used in EDUF determined the length of oligomer that was allowed to pass

through. A 500 Da membrane was completely impermeable, whereas the 5 kDa membrane initially only allowed dimers and trimers through, and the 20 kDa membrane allowed all oligomers through.

Perez, Andres, Alvarez, Coca, and C.G. Hill (1994) carried out experiments to de-ash whey permeate and whey retentate using electrodialysis. They found that ash removal rates of up to 0.3 kgh^{-1} per m^2 of membrane area were attainable, and above that, the dissociation of water at high electric currents and concentration polarisation at the membranes became limiting factors.

De-ashing only removes the mineral matter from a feed solution, so electrodialysis is unlikely to be a useful technique unless combined with lactose and protein removal. In a series of processes that remove different components from mother liquor to give a final product with a high concentration of oligosaccharides, electrodialysis is an easy, cheap way to remove ash.

The suitability of Electrodialysis is reviewed further by mass balances and economic considerations in Chapter 4.

3.10 Summary

Although a large amount of research has been carried out to demonstrate that bovine milk oligosaccharides are valuable as prebiotics and for brain development in infants, a large scale process for their isolation has not yet been developed. Successful attempts to isolate human and goat milk oligosaccharides, which are found in higher concentration in the respective milk, give some insight into the technologies likely to work, but the much lower concentration in bovine milk creates an opportunity to understand the mechanisms of separation and develop a process that can exploit the difference between oligosaccharides and other milk components.

4 MASS BALANCES & ECONOMICS

4.1 Introduction

After reviewing the literature against the objectives of the process (Section 3.9), it is clear that some separation processes are simply not suitable for enrichment of oligosaccharides from mother liquor. These include processes where the technology is not appropriate to the size of the oligosaccharide molecules, processes using non-food grade chemicals (e.g. some types of solid phase extraction), or where the separation basis is too selective to capture a range of oligosaccharides, such as ion exchange or affinity chromatography. There are, however, a number of processes that are documented in the literature that could theoretically be included in a process to enrich oligosaccharides. These are nanofiltration, size exclusion chromatography (using simulated moving bed or preparative continuous annular chromatography), lactose crystallisation, enzyme treatment, and electrodialysis. As well as this, clarification, evaporation, reverse osmosis, and spray drying are included for pre- and post- processing.

Before committing to laboratory and pilot plant trials for each of these, it was important to establish whether these process operations are (i) able to achieve a significant increase in oligosaccharide concentration, given the conditions and scale of the process to be developed and (ii) economically viable, since the aim of the project is to create a high value product from a low value dairy stream.

A calculated mass balance is the simplest way to establish the likely conditions for each scenario, and when extended to include cost and revenue predictions, can give a good idea of whether or not a setup is feasible. Inherent in such a mass balance is a range of assumptions, where values are not known exactly but are estimated using comparable examples from literature or existing processes. Although there is a lack of a precision in using assumptions like this, they can be used to quickly see and assess how likely a setup is to succeed. Once two or three of the best processes are selected, based on their initial performance in a mass balance, the assumptions can be confirmed or changed based on experimental work before a final process is decided on. A sensitivity analysis was used to identify which of the variables are most influential, where the assumed values were changed in the mass balance to their highest and lowest expected values to observe the effect on the final purity of product and profit margin.

This Chapter presents the methods and results of the mass balance, while the following Chapters in this thesis present the results of the experiments to confirm the values of the most influential assumptions.

4.2 Process Descriptions

The different unit operations described in Section 4.1 above can be placed in various arrangements to achieve an increase in oligosaccharide concentration; these arrangements are presented in Figure 4.1, and will be referred to in this Chapter and subsequent Chapters as they are evaluated for effectiveness and cost.

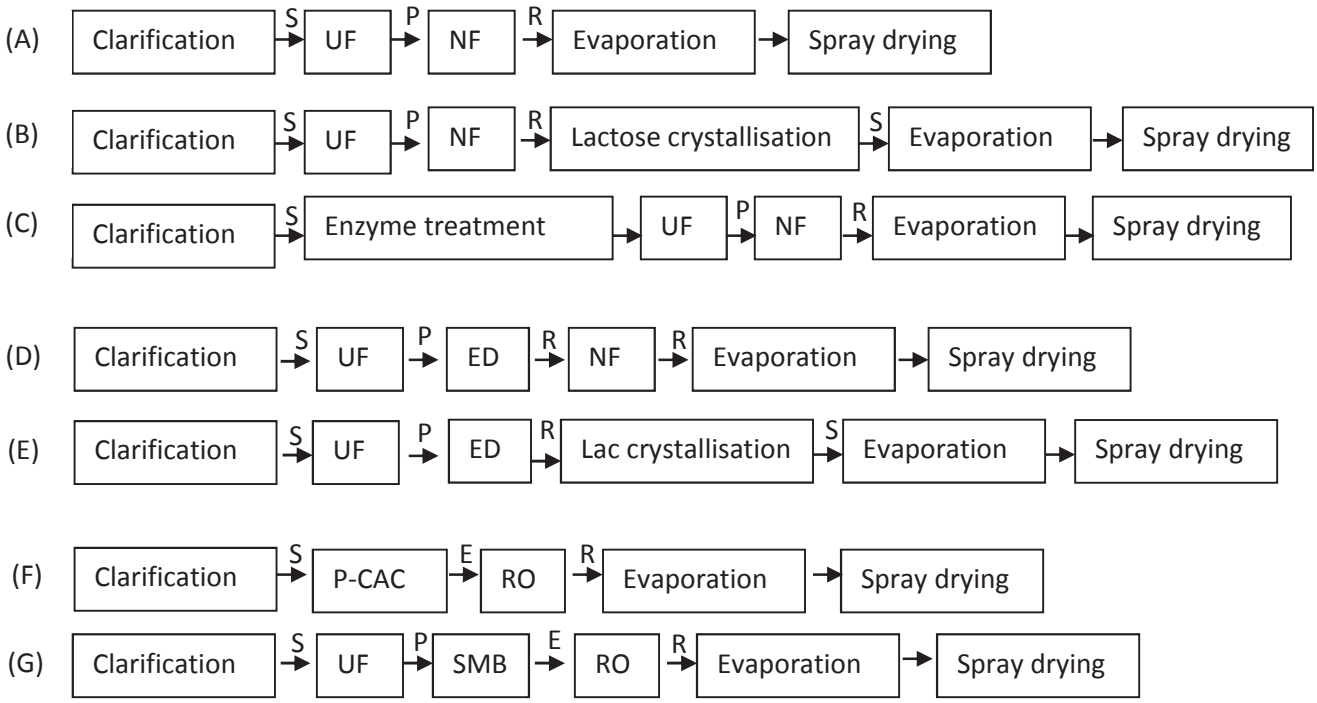

Figure 4.1: Process flow descriptions for 7 possible oligosaccharide enrichment arrangements. UF - Ultrafiltration, NF – Nanofiltration, ED – Electrodialysis, RO – Reverse Osmosis, P-CAC – Preparative Continuous Annular Chromatography, SMB – Simulated Moving Bed, S – Supernatant, P – Permeate, R – Retentate, E – Extract.

4.3 Methods

Excel spreadsheets (Microsoft Corporation, 2010) were developed to model the changes in flow rates and composition of process streams for the different options. A basis of 100 kg of mother liquor feed was used in each case, and simple mass balancing equations were used to

meet the conservation of mass requirement and calculate the flow and concentration at each stage.

Literature covering similar processes and information from within Fonterra was used to make assumptions and estimates for unknowns. New Zealand capital cost information was obtained from Bouman, Jesen, and Wake (2005) and adjusted for inflation and exchange rates to find the installed cost of each arrangement, and together with estimated operating expenses and revenue, the IRR was calculated. Other calculations of importance for designing the process were sizes of equipment (e.g. membrane area, chromatographic resin volume), estimation of daily production volume, and guidance in choosing between different processes for the same function (e.g. water removal by either evaporation or reverse osmosis).

An example using the NF process, arrangement (A) from Figure 4.1, is worked through here for reference. The mother liquor and filtration parameters are presented in Table 4.1. Note that all concentrations expressed as a percentage in this thesis are mass fractions unless otherwise specified.

The fraction of each mother liquor component ($X_{ML,i}$) was converted to a component flow ($Q_{ML,i}$) using:

$$Q_{ML,i} = X_{ML,i} \times Q_{ML,T} \qquad \text{(Equation 4.1)}$$

where $Q_{ML,T}$ is the total mass flow of mother liquor. The first stage, decanting, is preceded by a 1:1 water dilution, then the sludge ($Q_{S,i}$) and clarified mother liquor ($Q_{C,i}$) streams were calculated based on the component mass flows and their removal in the clarifier (R_C). For the crystals in the sludge:

$$Q_{S,crystals} = Q_i \times R_D R_C \qquad \text{(Equation 4.2)}$$
$$= Q_i \times 1$$

For all other components, the only fraction in the sludge is that held up with the crystals, where the concentration is the same as in the clarified mother liquor and the mass flow is the product of the mass fraction, the flow rate of crystals, and the hold-up fraction:

$$Q_{S,i} = X_{ML,i} \times Q_{S,crystals} \times 10\% \qquad \text{(Equation 4.3)}$$

Table 4.1: Starting parameters for NF process mass balance

Mother liquor characteristics	Assumed Value
Max daily flow rate	167 m^3/d
Yearly production (Hautapu)	20,000 m^3
Season length	250 days
Daily production length	20 hours
Oligosaccharide content	0.3%
Dissolved lactose content	21%
Crystallised lactose content	0.5%
Protein content	1.5%
Fat content	0.15%
Ash content	15%
Monosaccharides content	2%
Water content	59.55%
Filtration Parameters	Assumed Value
Volume concentration factor (UF)	6
Volume concentration factor (NF)	5
Protein and fat retained by UF	99%
Protein and fat retained by NF	100%
Oligosaccharides retained in NF	90%
Lactose retained in NF	30%
Ash, monosaccharides retained in NF	0%
Number of diavolumes (NF)	3
Other Process Variables	Assumed Value
Crystals removed in clarifier	100%
Liquid holdup with crystals	10%
Moisture content of spray dried powder	5%

The remaining flow in the clarified mother liquor is then:

$$Q_{C,i} = Q_i - Q_{S,i} \qquad \text{(Equation 4.4)}$$

The component fractions for the sludge and clarified mother liquor were then found:

$$X_{C,i} = \frac{Q_{C,i}}{Q_{C,T}} \qquad \text{(Equation 4.5)}$$

$$X_{S,i} = \frac{Q_{S,i}}{Q_{S,T}} \qquad \text{(Equation 4.6)}$$

A summary of the flows into and out of the clarifier is given in Table 4.2.

Table 4.2: Clarifier flows for NF process

Component	**Flow (kg/d)**			**Mass Fraction**		
	Feed	Sludge	Clarified mother liquor	Feed	Sludge	Clarified mother liquor
Fat	250	0.2	250	$8x10^{-4}$	$7x10^{-5}$	$8x10^{-4}$
Protein	2500	1.2	2499	0.0075	$7x10^{-4}$	0.0075
Dissolved lactose	34083	17	34067	0.10	0.009	0.10
Lactose crystals	833	833	0	0.0025	0.91	0
Oligosaccharides	500	0.2	500	0.0015	$1x10^{-4}$	0.0015
Ash	25000	12.6	24987	0.075	0.007	0.075
Monosaccharides	3333	2.0	3331	0.01	$9x10^{-4}$	0.01
Water	266833	123.4	266701	0.8	0.073	0.8

The next stage, ultrafiltration, can be analysed in a similar way. The mass flows of the components that are retained by the membrane (protein and fat) in the retentate are found by:

$$Q_{UF,R,i} = Q_{UF,F,i} \times R_{UF,i} \qquad \text{(Equation 4.7)}$$

where $Q_{UF,R,i}$ and $Q_{UF,F,i}$ are the flows in the retentate and feed, respectively, and $R_{UF,i}$ is the retention of component i in the UF membrane.

The volume concentration factor, defined as the initial feed volume divided by the final retentate volume, is then used to predict the total mass of the retentate from the feed flow, using:

$$Q_{UF,R} = VCF \times Q_{UF,F} \qquad \text{(Equation 4.8)}$$

Components that are not significantly retained by the membrane are assumed to be found in the retentate at the same concentration as in the feed. The component flows in the retentate can then be calculated by multiplying their concentration by the total retentate flow. The permeate component flows are then found by subtracting the retentate flow from the feed flow.

A summary of the component flows and concentrations is presented in Table 4.3.

Table 4.3: UF flows for NF process

Component	Flow (kg/d)			Mass Fraction		
	Feed	Retentate	Permeate	Feed	Retentate	Permeate
Fat	250	247	2	$8x10^{-4}$	0.004	$9x10^{-6}$
Protein	2499	2474	25	0.0075	0.043	$9x10^{-5}$
Dissolved lactose	34067	5679	28396	0.10	0.10	0.10
Oligosaccharides	500	83.3	417	0.0015	0.0015	0.0015
Ash	24987	4166	20828	0.075	0.075	0.075
Monosaccharides	3331	555	2777	0.01	0.01	0.01
Water	266701	44461	222306	0.80	0.73	0.81

Nanofiltration is more complex because of the addition of diafiltration water as well as the feed (UF permeate). One diavolume is defined as the volume of water equal to the total volume of the feed, i.e. the volume of feed would be doubled with the addition of one diavolume. Although the diafiltration volume is added gradually throughout the filtration process, for simplicity the assumed three diavolumes will be considered to be added along with the UF permeate at the beginning of the process. The density of water was assumed to be 1000kgm^{-3} in this work. The mass of each component in the feed is therefore the same as that of the UF permeate except for the water, which is given by:

$$Q_{NF,W,F} = Q_{UF,W,P} + N_D \times Q_{UF,P}$$ (Equation 4.9)

where N_D is the number of diavolumes.

The mass of each component (except water) in the retentate was then found based on its retention factor:

$$Q_{NF,R,i} = R_{NF,i} \times Q_{NF,F,i}$$ (Equation 4.10)

The total mass of the retentate was found by multiplying the feed mass by the volume concentration factor (VCF):

$$Q_{NF,R} = VCF \times Q_{NF,F}$$ (Equation 4.11)

And the difference between the sum of the dry component masses and the total mass is equal to the mass of water in the retentate:

$$Q_{NF,W,R} = Q_{NF,R} - \sum Q_{NF,R,i}$$ (Equation 4.12)

Table 4.4: Component flows for NF process

Component	Flow (kg/d)			Mass Fraction		
	Feed	Retentate	Permeate	Feed	Retentate	Permeate
Fat	2	2	0	$2x10^{-6}$	$1x10^{-5}$	0
Protein	25	25	0	$2x10^{-5}$	$1x10^{-4}$	0
Dissolved lactose	28396	12494	15902	0.026	0.055	0.018
Oligosaccharides	417	383	33	$4x10^{-4}$	0.0017	$4x10^{-5}$
Ash	20828	4166	16663	0.019	0.018	0.019
Monosaccharides	2777	555	2222	0.0025	0.0024	0.0025
Water	1046557	209311	837246	0.95	0.92	0.96

The mass of each component in the permeate was then calculated by subtracting the component mass in the retentate from that in the feed, and concentrations were calculated as fractions of the total mass for each stream. The filtration flows are summarised in Table 4.4.

For the final processes, evaporation and spray drying of the NF retentate, only water was assumed to be lost. The other final mass components are then equal to those in the NF retentate, and the mass of water was calculated iteratively as the total mass of product multiplied by the assumed moisture content of the final product (5%). The final product concentrations and mass flows are shown in Table 4.5.

Table 4.5: Final product components for NF process

Component	Final Product Stream	
	Flow (kg/d)	Mass Fraction
Fat	2	$3x10^{-4}$
Protein	25	0.0029
Lactose	12494	0.76
Oligosaccharides	383	0.042
Ash	4166	0.13
Monosaccharides	555	0.021
Water	855	0.05

For the economic analysis, the sizing of equipment was needed with delivered and installed costs calculated based on the throughput of the process and cost data from Bouman et al. (2005). The main components for the NF process are shown in Table 4.6

Table 4.6: Main capital costs for NF process

Equipment	Requirements	Delivered cost (NZD)
ML Holding tank	6 hours supply, 50 m^3	$60,000
Clarifier	120 L/s dry solids	$215,000
UF pump	17 m^3/h	$42,000
UF plant	17 m^3/h, 20 h/day	$310,000
NF holding tank	1 hr supply, 14 m^3	$28,000
NF pump	55 m^3/h	$80,000
NF plant	55 m^3/h, 20 h/day	$2,340,000
TOTAL		$3,075,000

A Lang factor of 3.78 was applied to the delivered equipment cost, which covers direct plant costs, contractors' fees, overheads, and contingency. Working capital was assumed to be 15% of the installed cost. The total capital cost was then:

$$Capital\ costs + Working\ capital$$

$$= \$11{,}623{,}500 + \$461{,}250$$

$$= \$12{,}084{,}750$$

Operating expenses were then calculated and are presented in Table 4.7:

Table 4.7: Main operating expenses for NF process.

Item	Usage	Cost per unit	Yearly cost (NZD)
Mother liquor	20,000 m^3/year	\$100/m^3	\$2M
Water	100,000 m^3/year	\$0.20/m^3	\$20,000
UF Membrane replacement	Once per year	US\$150/m^2	\$36,000
NF Membrane replacement	Once per year	US\$250/m^2	\$60,000
Labour	3 full time operators	\$100,000	\$300,000
Power	250 kW	\$0.10/kW	\$60,000
Packaging	17,100 kg/day	\$50/t	\$103,000
Lab testing	17,100 kg/day	\$400/t	\$821,000
Effluent	873,000 kg/day	\$40/t	\$82,000
TOTAL			\$3.48M

Revenue from sale of products was calculated based on a "cost in use", meaning that infant formula manufacturers will buy product at a certain rate per kilogram of finished infant formula product, assumed to be \$0.40/kg formula. Since oligosaccharides are to be added to achieve the same levels as found in human milk, the product addition rate can be calculated and the profit per kg found. It is important to note that manufacturers will generally only pay

for the oligosaccharides in a product, regardless of the accompanying lactose, minerals, protein, or other nutrients. This means for example that 1 kg of a product containing 50% oligosaccharides would sell for the same price as 2 kg of product containing 25% oligosaccharides, since the total oligosaccharide content is the same (500 g in each). This means that a process that achieves a lower concentration of oligosaccharides may in fact be more economical to produce and sell if its capital and operating costs are lower than a process giving higher oligosaccharide purity. This estimate assumes a linear relationship between oligosaccharide purity and purchase price, which is likely to be the case for purities < 90%, as expected in the studied processes.

A minimum oligosaccharide content for the finished product was also calculated to be approximately 4%, based on the ratio of oligosaccharides to lactose in mothers' milk. Oligosaccharide contents of lower than 4% (with the balance made up of lactose and/or minerals) would mean that the maximum levels for lactose or mineral content would be overshot in attempting to meet the oligosaccharide level requirement.

Economic feasibility was measured by the internal rate of return (IRR). The IRR essentially describes the return on investment for a particular project as a percentage over a specified number of years. Although any IRR of greater than zero is technically profitable, if investment funds are not readily available then a higher IRR is required. In the case of this work, the option that returns the highest IRR for seven years is the most desirable option, assuming the gross profit is similar in each case. Accordingly, only the options with an IRR of greater than 30% were selected for further analysis. An example for the NF process is shown in Table 4.8. The same method was applied to each of the seven options from above, and the full spreadsheets for the NF and SMB arrangements are presented in Appendix 11.1.

Once the IRR was found for each option and the poorer options eliminated, the numerical assumptions for each option were varied individually to their lowest and highest expected values and the effect on the final IRR noted. This gives a picture of which assumptions are linked to variables that have a large effect on the final IRR, and are therefore most important in finding a more certain value for through experiments or further research. As an example, the daily operating hours for a proposed process might be assumed to be 20 hours (allowing 4 hours for downtime and cleaning). The highest expected value for this variable might be 23 hours, and the lowest might be 16 hours. The spreadsheet is then modified accordingly, and

the new IRR noted for each modification. In this case, the resulting IRR might change by 1% from the lowest to the highest value, indicating that this variable is not significant. In the case of two variables being linked, the second variable was evaluated separately at two different levels of the first variable to determine how strong the linkage is. A significance factor (0-5) was assigned to each assumption based on the difference between the minimum and maximum IRR.

Table 4.8: NF IRR calculation

Production Rate	2050 t/year
Cost in use	$0.40/kg finished product
Production cost	$1.58/kg
Addition rate in infant formula	9.7%
Sale price	$4.13/kg
Sale margin	$2.55/kg
Revenue	$8.5 M/year
Operating cost	$3.48 M/year
Operating profit	$5.02 M/year
Capital cost	$12.1 M
IRR (7 years)	34%

4.4 Results

The IRR from the mass balance for each process option is presented in Table 4.9, along with an indication whether the minimum oligosaccharide (OS) content of 4% was met. As the table shows, all but option (F) gave a positive IRR. Continuous annular chromatography requires significantly more resin than simulated moving bed chromatography (as discussed in Section 3.9.8), and since resin is one of the major on-going costs for the process, the profitability is reduced. Processes with electrodialysis failed to meet the minimum oligosaccharide requirement of 4% (solids basis), even though the IRR was high when paired with crystallisation. This is because the acidic oligosaccharides are also affected by the electric current and some are removed from the stream with the ash component, reducing their concentration in the product.

Table 4.9: Internal rate of return for different process arrangements

Arrangement from Figure 4.1	Description	IRR (7 years)	Meets minimum OS content?
(A)	Nanofiltration (NF)	34%	Yes
(B)	NF with crystallisation	41%	Yes
(C)	NF with enzymatic treatment	27%	Yes
(D)	NF with electrodialysis (ED)	13%	No
(E)	ED with crystallisation	55%	No
(F)	Preparative continuous annular chromatography	-24%	Yes
(G)	Simulated moving bed chromatography	65%	Yes

Of the other processes involving nanofiltration, all were able to meet the minimum oligosaccharide requirement and gave good returns on investment, although the enzymatic treatment option returned an IRR of less than 30%. This is because the operating cost includes enzymes which are a significant cost.

The best performing process in terms of IRR was simulated moving bed chromatography. This process allows a continuous counter-current separation which can be tailored to give a high purity product (see Section 3.9.7 for a full explanation). Although chromatographic media are required, the volume is small compared with continuous annular chromatography. Media use is still a crucial factor in the process's economic viability, and is dealt with in the sensitivity analysis.

The most important variables for the three selected processes are summarised as a sensitivity analysis in Table 4.10 (refer to Figure 4.1 for the process description of each). The full results, including variables with lower significance, can be found in Appendix Section 11.1.

Table 4.10: Sensitivity analysis on three preferred processes with significance (0 = no significance, 5 = most significant).

Nanofiltration (A)						
Variable	Initial Value	Max Value	Min Value	IRR (max)	IRR (min)	Significance (0-5)
Peak mother liquor flow rate (million L/month)	5	6	2	23%	102%	4
Average mother liquor flow rate (million L/month)	1.7	3.3	0.83	77%	5%	4
OS content of mother liquor	0.3%	0.5%	0.1%	200%	-8%	5
Cost of mother liquor (NZD)	$0.09/L	$0.30/L	$0.05/L	<< 0%	89%	5
Volume concentration factor of UF	6	10	2	58%	31%	3
Heat induced loss of sialyl compounds	0%	20%	0%	10%	32%	3
Nanofiltration OS leakage	10%	30%	1%	20%	90%	4
Nanofiltration recycle ratio	50%	80%	0%	28%	68%	3
Number of diavolumes	3	6	1	23%	76%	4
Nanofiltration with Crystallisation (B)						
Variable	Initial Value	Max Value	Min Value	IRR (max)	IRR (min)	Significance (0-5)
Peak mother liquor flow rate (million L/month)	5	6	2	28%	115%	4
Average mother liquor flow rate (million L/month)	1.7	3.3	0.83	86%	8%	4
OS content of mother liquor	0.3%	0.5%	0.1%	319%	-8%	5
Cost of mother liquor (NZD)	$0.09/L	$0.30/L	$0.05/L	<<0%	102%	5
Heat induced loss of sialyl compounds	0%	20%	0%	20%	41%	3
Nanofiltration OS leakage	10%	30%	1%	18%	94%	4
Nanofiltration recycle ratio	50%	80%	0%	29%	68%	3
Number of diavolumes	3	6	1	33%	61%	3
Removal of lactose during crystallisation	80%	95%	70%	19%	82%	4

Simulated Moving Bed Chromatography (G)						
Variable	Initial Value	Max Value	Min Value	IRR (max)	IRR (min)	Significance (0-5)
Max mother liquor flow rate (million L/month)	5	6	2	55%	87%	3
Average mother liquor flow rate (million L/month)	1.7	3.3	0.83	79%	50%	3
OS content of mother liquor	0.3%	0.5%	0.1%	164%	<< 0%	5
Cost of mother liquor (NZD)	$0.09/L	$0.30/L	$0.05/L	-7%	83%	4
Volume concentration factor UF	6	10	2	80%	27%	4
Reverse osmosis membrane flux (L/m^2h)	20	50	5	68%	44%	3
Resin lifetime (years)	1	2	0.25	81%	-10%	4
Cost of resin (NZD/kg)	$400	$600	$150	20%	93%	4

Some things to note from the table above are:

(1) In each case, the oligosaccharide concentration of mother liquor is the most important factor, and has the potential to make the process unprofitable if below 0.1% (total basis). This is because the cost of processing is determined by the volume of feed, whereas the revenue from product sales is determined by the volume of oligosaccharides produced. If the volume of feed is high in relation to the volume of oligosaccharides, the costs outweigh the revenues and profit is negative.

Prior to this project, there were limited data for oligosaccharides in mother liquor (in contrast to data on oligosaccharides in raw milk), and assumptions had to be made based on individual analyses and anecdotal research. Because the success of any of the options depends on this concentration, it is important to find the long term average oligosaccharide concentration and variability for the supply being used. Research is presented in Chapter 5 to determine this.

(2) Some important variables cannot be determined experimentally. These include the mother liquor flow rate (i.e. feed availability), peak flow rate, and the cost of mother liquor. Instead, commercial data from the production sites needs to be obtained (where possible) to give an accurate estimate. There will still be considerable uncertainty in these estimates, since previous conditions cannot be guaranteed in the future, especially with a downstream product such as mother liquor.

(3) The cost of mother liquor is a significant factor, but is also subject to some variability. Mother liquor is currently processed with other ingredients into stock feed and sold on to farmers, so the cost described here is the loss in revenue from this stream that would need to be offset by the profit of processing the mother liquor for oligosaccharides. Alternatively, the waste products of oligosaccharide manufacture could be recombined to give a similar product with slightly reduced volume, meaning the stock feed revenues could be mostly retained, but this would require further capital and is not covered here.

(4) Resin lifetime is an important factor in simulated moving bed chromatography, though it is difficult to establish a value for this without moving to full scale production. Because there are no instances of this process being used on a production scale for dairy streams in the literature, a direct value cannot be found and instead an estimate has to be made based on single column chromatography data.

Although difficult to quantify, resin lifetime can be extended by keeping the feed clean of species that cause fouling (e.g. proteins, as targeted by UF pre-treatment), or strongly ionic species that could cause osmotic shock when injected onto the column. A sacrificial pre-treatment column with a low-cost ion exchange resin could be used here to protect the more costly SMB resin. The SMB resin should also be chosen to minimise interaction with ionic species, through a strongly hydrophobic, inert, resin structure, and cleaned with an appropriate solution on a regular basis.

(5) The uncertainties in some variables are best clarified by experimental analysis. These include the oligosaccharide content of mother liquor (as mentioned above), the leakage of target molecules through nanofiltration membranes, volume concentration

factors and recycle ratios in membrane processes, and the extent of lactose crystallisation. The following chapters are tasked with finding more certain values for these variables by rigorous laboratory and pilot plant experimentation.

(6) Some assumed values that are not listed in Table 4.10 are still important and need to be determined by experimental work. These include the purity and recovery of oligosaccharides in the simulated moving bed process, for which the first question needs to be whether the process is capable of the separation, before trying to determine what degree of separation is possible. Similarly for membrane separation, membrane life, flux, and rejection coefficients are important variables to study, although they have a lesser bearing on the IRR. The purpose of the sensitivity analysis is not to give an exhaustive and precise collection of the variables and their uncertainty, but rather to highlight any that might not have otherwise been thought of as important.

4.5 Conclusions

Based on a review of the literature, seven different arrangements of unit operations were developed for the enrichment of oligosaccharides, including ultra- and nanofiltration, two types of continuous size exclusion chromatography, lactose crystallisation, electrodialysis, and enzyme treatment. Comprehensive mass balances in Excel were used to predict flow rates, compositions and together with economic data, an IRR for each scenario.

Variables from the three scenarios that had the best IRR (nanofiltration, nanofiltration with lactose crystallisation, and simulated moving bed chromatography) were analysed for their effect on the overall IRR by changing the assumptions in the spreadsheet to the highest and lowest expected values for each and noting the change in IRR. Important factors in all scenarios were the flow rates and cost of mother liquor, and most importantly its oligosaccharide content. Other important factors were the retention of oligosaccharides in nanofiltration and the extent of lactose crystallisation, and the resin lifetime in simulated moving bed chromatography.

Variables which can be quantified by experimental work are investigated in the following chapters of this thesis, whilst other variables require commercial information or will still carry significant uncertainty until a full scale process is begun.

5 MOTHER LIQUOR SEASONAL ANALYSIS

5.1 Introduction

Based on the mass balance and economic analysis presented in Section 4.4, the concentration of oligosaccharides in mother liquor was found to have the most significant effect on the economic viability of the project. This is because the process is more efficient when the feed concentration is higher (i.e. similar volume of fluid to be processed with higher extract flow rate). The oligosaccharide content was assumed to be 0.3% (on a total basis; approximately 0.8% on a dry basis) but this was based on a small number of samples and had considerable uncertainty. The internal rate of return (IRR) for a nanofiltration process would vary between -8% and 200% depending on the actual oligosaccharide concentration. For SMB chromatography the difference was from well below 0% to 164%. It is therefore critical to understand what the actual oligosaccharide content is, and how much it varies within a season. Mother liquor composition is unique to each production site (Hautapu, Kapuni, and Clandeboye) and the starting material from which the lactose is produced (WPC, WPI, MPC etc.). Its composition also fluctuates across the production season making it difficult to predict the concentration from literature or mass balances. Instead, mother liquor samples over a full season were analysed for oligosaccharide content as well as protein, ash, total solids, and lactose, to establish the expected concentration and variability for the enrichment process. The concentrations of the main components were estimated *via* mass balances from Section 4.3 and are presented in Table 5.1 below.

Table 5.1: Mother liquor composition

Component	**wt%**
Oligosaccharides	0.3
Dissolved lactose	21
Crystallised lactose	0.5
Protein	1.5
Fat	0.15
Ash	15
Monosaccharides	2
Water	59.55

5.2 Methods

Fortnightly mother liquor samples were collected from a sampling tap at Fonterra Kapuni, immediately downstream from all lactose crystallisation and recovery stages, within 1 hour of manufacture and without dilution. Seventeen samples covered the entire production season (usually from November to May) with an overlap of four months from the previous year, and were kept frozen at -18 °C until analysed. Although -18 °C prevents microbial activity, a portion of the mother liquor remains liquid at this temperature.

In this work, trisaccharide content was used to represent total oligosaccharide content, as the target species are mostly this size. For total trisaccharide, lactose, and monosaccharide determination by HPLC, the samples were defrosted, diluted tenfold with milli-Q water, passed through cation exchange cartridges and 0.22 µm syringe filters (Sigma-Aldrich Ltd., Auckland NZ). Injections of 10 µL were made onto an Aminex 87C Ca^{2+} anion exchange column (300x7.8 mm, 9 µm particle size; BioRad Laboratories Ltd., Auckland NZ) with milli-Q water as eluent at a flow rate of 0.5 mL/min on an Agilent 1100 series system. Detection was by refractive index. Maltotriose, lactose, and galactose were used as standards to represent trisaccharides, disaccharides, and monosaccharides respectively. This use of standards means that the concentration of oligosaccharides reported encompasses different trisaccharides, including neutral and acidic species. HPLC chromatograms were integrated using Agilent Chemstation software. Resolution between peaks was good, giving reliable and consistent results and minimal ambiguity in peak areas.

Ash was measured by combustion in a muffle furnace at 600 °C for 5 hours, and total solids were found by thermogravimetric analysis at 102 °C for 2 hours. Both ash and total solids measurements were carried out at Fonterra Kapuni.

5.3 Results and Discussion

Figure 5.11 shows the change in trisaccharide concentration over the course of the 2011-2012 season at Fonterra Kapuni, as well as the latter half of the previous season, 2010-2011. It can be seen that the concentration is relatively stable and generally increases over the season from 0.84% to 1.16% (dry basis), with a similar trend to other data which show the trisaccharide content of bovine milk increasing across the season (Tao, DePeters, German, Grimm, & Lebrilla, 2009). This is slightly higher than the initial estimation of 0.8% on a dry basis, meaning the IRR of the process will be similar or higher than predicted in Section 4.4.

Although the change in concentration of mother liquor is similar to the trend of increasing concentration in bovine milk, it is not purely dependent on the composition of the milk, however, for two main reasons:

Firstly, the recycle of lactose-rich streams back to the crystallisation process over the course of a season leads to oligosaccharide build-up (as well as other non-lactose components such as ash, monosaccharides, and proteins), as generally only lactose and water are removed in the crystallisation process. An imbalance of other components is set up (assuming negligible mother liquor hold-up with the lactose crystals) since they are not removed from the system at the same rate they are added in the first few months of the season. The longer this recycle process is continued and the higher the proportion that is recycled, the higher the concentration of oligosaccharides and other components will become over the season. This inference is supported by data which show the ash concentration also increasing slightly over the course of the season (Figure 5.2), indicating that other non-lactose components are following the recycle path. Although the mineral content of bovine milk does generally increase over the season, the content relative to lactose (the other major component of mother liquor) remains similar across the season, so the mother liquor entering the bunkers is unlikely to cause the change in concentration.

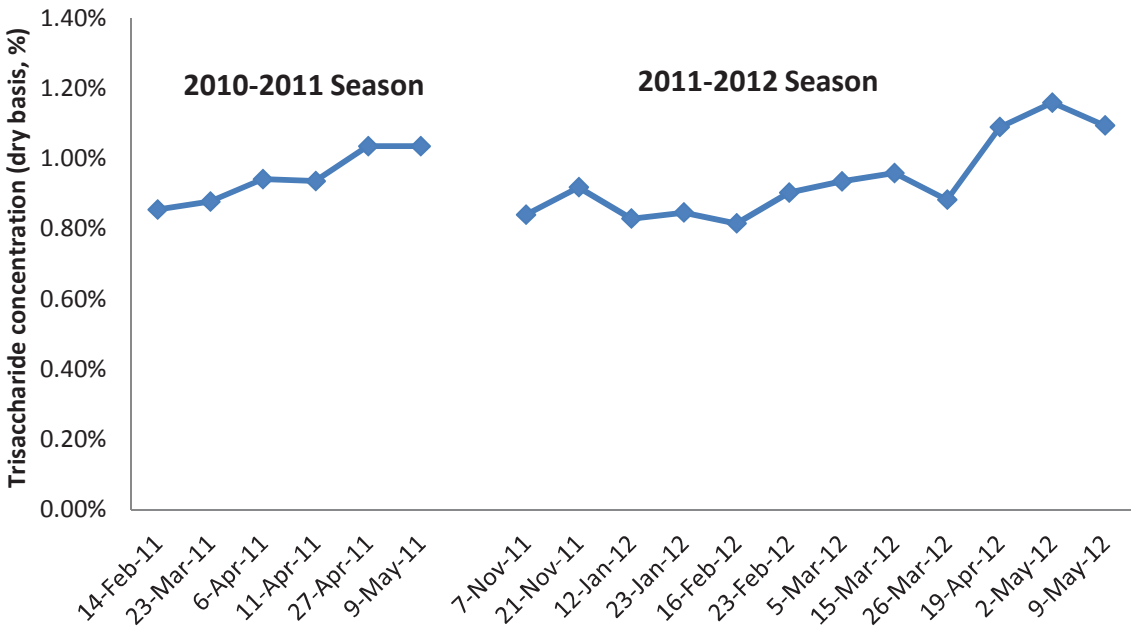

Figure 5.1: Trisaccharide concentration in Kapuni mother liquor over the 2010-2011 and 2011-2012 production season

Secondly, the oligosaccharide concentration can increase by enzymatic action on lactose. The permeate to be processed at Kapuni is concentrated to above 70% total solids and collected in large bunkers during the peak season for processing later, when supply is lower. The time in the bunkers can be as long as six months. According to research on Galacto-oligosaccharide (GOS) formation, high concentrations of lactose in the presence of β-galactosidase enzymes can lead to formation of GOS by addition of free galactose to a lactose molecule in preference to hydrolysis of lactose to form monosaccharides (Prenosil et al., 1987a). Although the majority of cultures and enzymes should have been removed in ultrafiltration of whey prior to arrival at Kapuni, any which remain or are subsequently introduced (e.g. surface moulds) would have favourable conditions to convert some of the lactose to oligosaccharides. This process has been noted (Wood, 1971) though the extent of this has not been measured. It should be noted that this is not necessarily a desirable process for the increase of oligosaccharides, because only GOS are being produced, and not the more beneficial acidic oligosaccharides as found in human milk.

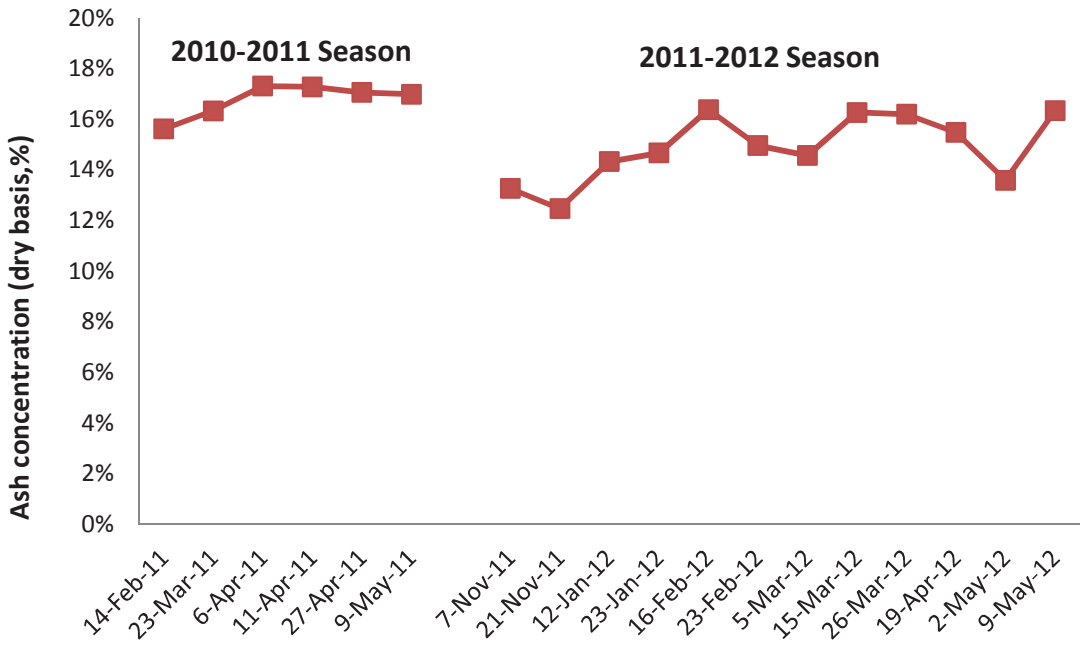

Figure 5.2: Ash content of mother liquor from Fonterra Kapuni over the 2010-2011 and 2011-2012 season

Although the project has targeted the full range of oligosaccharides for addition to infant formula which includes some Galacto-oligosaccharides, the proportion of acidic

oligosaccharides should remain as high as possible so that the formula ingredient is as close to human milk as possible.

Comparison of the ash concentration in same month across both years indicates that the variation from year to year is negligible. This is to be expected, since the bunkers are emptied at the end of each season and there is no variation carried over from the previous season. In general the variation from season to season from external factors (such as weather) are minor compared to the changes within each season, but if the processes used to produce mother liquor or the starting material for lactose crystallisation change, the concentration of oligosaccharides may change more significantly. In a commercial process it is important to measure oligosaccharide levels every year to monitor and track any changes and to reassess the feasibility of a process to isolate them.

5.4 Conclusions

The estimate of oligosaccharide content used in feasibility studies, based on previous analyses of mother liquor, was about 0.8% (dry basis), but there was considerable uncertainty as to its average value and variation across the season. These trials have shown that oligosaccharide content in Kapuni mother liquor varies between 0.8 and 1.0% (dry basis) during most of the season (from November to April) and can increase to 1.2% in the months prior to shutdown April – May). According to the mass balance and economic analysis, 0.8% oligosaccharides in lactose mother liquor would give an IRR of more than 60% for a nanofiltration process and more than 40% for a simulated moving bed process, and higher concentrations would increase these further. Provided the actual values of other process variables are close to their assumed figures (as will be examined in the following chapters), both a nanofiltration and simulated moving bed system will be viable options for enrichment of oligosaccharides from lactose mother liquor.

6 HEAT TREATMENT EXPERIMENTS

6.1 Introduction

In Chapter 4 there were a number of assumptions made about the processes for extracting oligosaccharides from mother liquor for the purpose of predicting which technologies were most appropriate, and to select parameters that need to be verified by experimental analysis. One of these was the heat sensitivity of the target oligosaccharides.

There is uncertainty as to whether sialyloligosaccharides (the acidic fraction of oligosaccharides) from mother liquor are hydrolysed at high temperatures with a corresponding decrease in concentration. It is important to establish whether this takes place, and to what extent it occurs, because lactose crystallisation, evaporation and spray drying processes have been considered for concentration and drying of an enriched oligosaccharide product and any heat induced losses of oligosaccharides in these processes means difficulty meeting targets and loss of revenue.

6.2 Experimental

Mother liquor samples were collected from a tap within the processing plant at the Hautapu lactose crystallisation plant in January 2011, and kept at -18 °C until analysed. Triplicate 5 mL samples were each diluted 40 times with milli-Q water at the treatment temperature before placement in a water bath, bringing the sample close to the target temperature immediately. Temperatures of 40, 50, 60, 70, and 80 °C and times of 5, 10, 15, 20, and 30 minutes were used. After heat treatment, the samples were cooled rapidly in an ice bath and kept refrigerated until analysed.

High performance anion exchange chromatography with pulsed Amperometric detection (HPAEC-PAD) was used for analysis of the acidic oligosaccharide fraction. Samples were prepared by being defrosted, diluted tenfold, and passed through a 0.22 µm syringe filter. Elution was made on a CarboPac PA1 column (250x2.5 mm; Thermo-Fischer Scientific Inc., Auckland NZ) at 0.25 mL/min using a Dionex chromatography system, starting with 100 mM NaOH as eluent and ending with 100 mM NaOH + 200 mM sodium acetate with a linear gradient. Standards used were 3’ and 6’ sialyllactose, 6’ sialyllactosamine, and sialic acid.

6.3 Data Analysis

HPAEC-PAD Chromatograms were interpreted using Dionex Chromeleon software by drawing a baseline and peak divisions where necessary. Peak areas were found by integration and converted to a percentage of the original oligosaccharide level. Since the standard curve was linear in this case (with R^2 values between 0.97 and 0.99), it was not necessary to convert the area to a concentration before calculating the percentage loss of oligosaccharides. Sample chromatograms were more difficult to analyse than standards because of sloped base lines and lack of base line resolution in all cases. Consistency was targeted, and because the results are in the form of a comparison relative to an original concentration, it is more important to have a consistent peak measurement technique than absolute accuracy.

A typical chromatogram is shown in Figure 6.1, with the relatively clear elution of standards at the bottom and the more complex elution of the sample above. A few difficulties in interpreting these chromatograms will be outlined here:

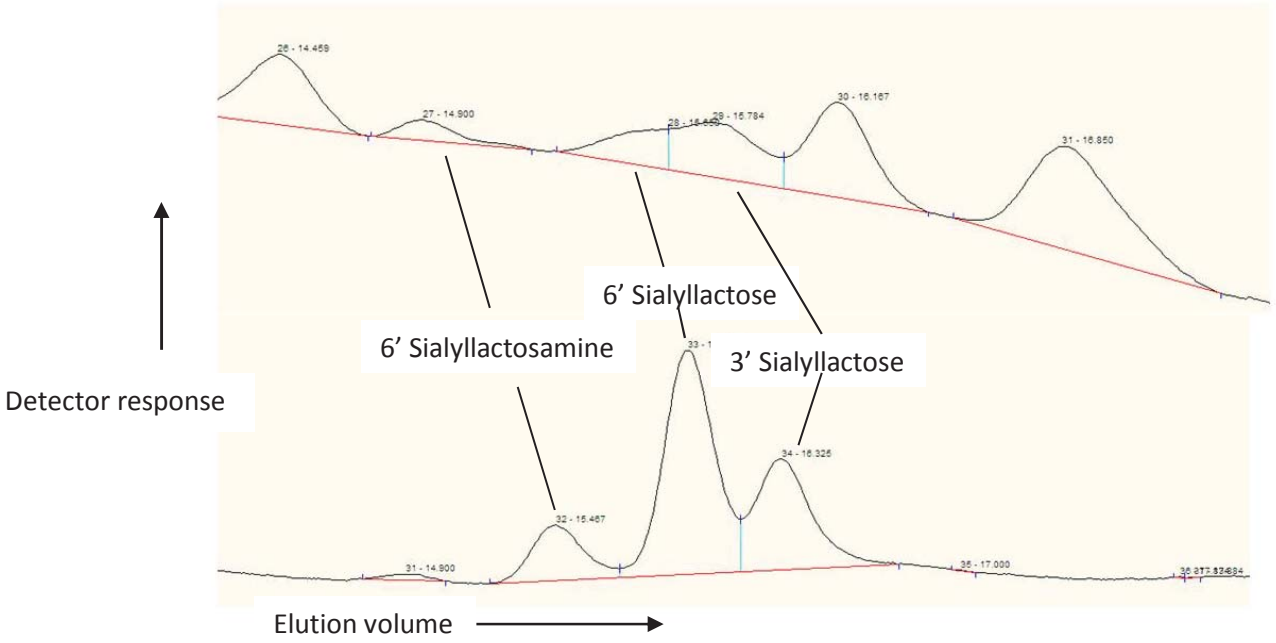

Figure 6.1: Typical chromatogram from HPAEC-PAD analysis of oligosaccharide standard (below) and mother liquor samples (top)

The two chromatograms are aligned laterally with regard to elution volume, although it is clear that the prominent peaks in the sample are not matched exactly to the standard peaks. This is problematic because it creates uncertainty as to what species the peaks in the sample represent. Tracking of gradual movement of the peaks between each elution can in some

cases reveal where the three main peaks in the standard elution correspond to on the sample chromatogram. In this case the sample elutes slightly earlier than the standards, as indicated in the figure, but sometimes it is impossible to know which peaks in the sample correspond to those in the standard. The peak movement observed may be due to small differences in pressure and compaction, or irreversible binding of solutes or impurities to the chromatographic media.

The second difficulty in analysing the HPAEC-PAD chromatograms is the lack of resolution between peaks. Because the different acidic oligosaccharides are very similar in size and charge (especially 3' and 6' Sialyllactose) it is difficult to separate them, even on the most specialised column, meaning their elution times are similar and the peaks close together. The measuring of peak size by integration of these peaks is not straightforward as one needs to determine a point to split the two peaks. If the split is made at the low point (or inflection point) on the chromatogram between the two peaks, this can lead to a false reading, as the peak overlap hidden on either side of the split will not be accurately reflected in the division, especially if the two peaks are different in shape and size. However for the sake of consistency, and since the low point is the only precise point to target, the split is made there, as is seen in the examples in Figure 6.1.

A third difficulty relates to the slope of the baseline. Here, one must decide when to draw a straight line between the bases of two distant peaks, between which the peaks of interest do not reach down to the baseline, and when to draw a shorter line between the bases of peaks close together. The approach taken here is to use a common baseline across neighbouring peaks where possible, but to ensure at least every 2-3 peaks that the trace touches the baseline. Sharp deviations in the baseline were also avoided. Figure 6.1 shows examples of groups of 2 or 3 peaks sharing a common baseline. Again, the most important approach is consistency, especially between treatment of the samples and standards.

6.4 Results

Results showed that for each of the three oligosaccharide compounds measured, there was only a very small loss (<5%) after heat treatment at 80 °C for 30 minutes, the most extreme treatment given, as shown in Figure 6.2. At lower temperatures there was no significant loss of oligosaccharides after 30 minutes.

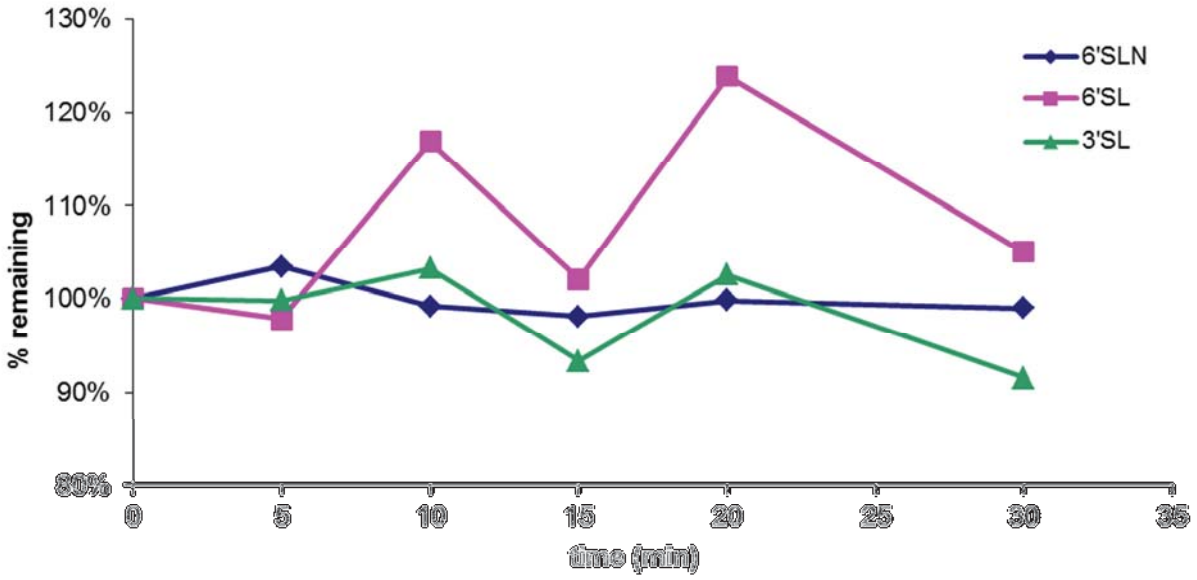

Figure 6.2: Percentage of sialyl oligosaccharides remaining after heat treatment of mother liquor at 80 °C. 6'SLN is 6' Sialyllactosamine; 6'SL and 3'SL are 6' and 3' Sialyllactose, respectively.

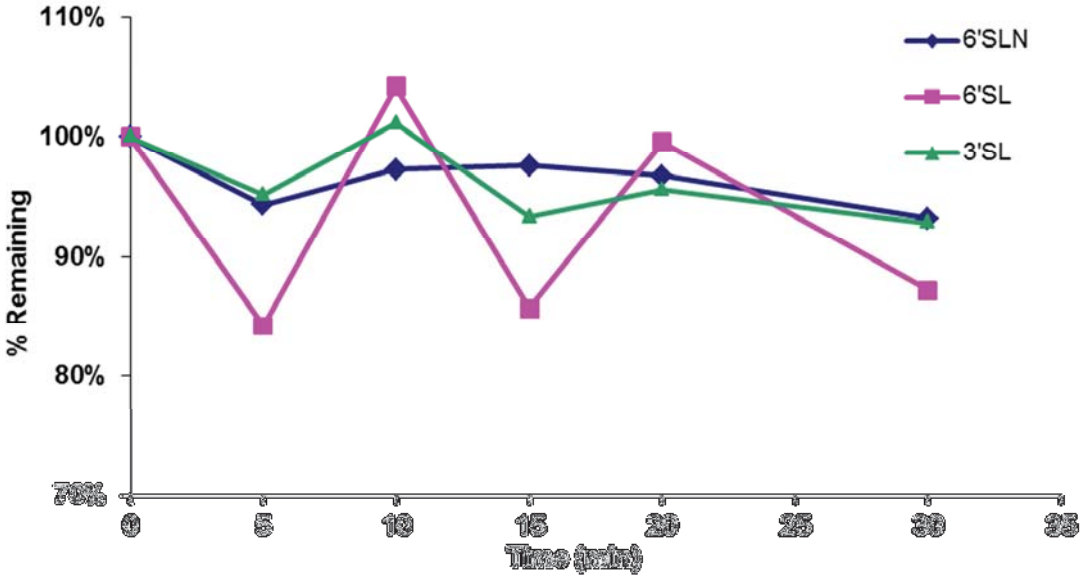

Figure 6.3: Percentage of sialyl oligosaccharides remaining in mother liquor after heat treatment at 60 °C.

Variability was high for 6'sialyllactose (calculated errors were higher than 20% in some cases), as seen in the undulating plot, whereas for 6'sialyllactosamine and 3'sialyllactose the results were more reliable and gave straighter lines. This difference in variability between species was seen at all temperatures studied, and is a result of the peak corresponding to 6'sialyllactose on

the chromatogram being comparatively small and flat, and therefore more difficult to measure consistently. The main source of error is the analysis of the samples and peak area measurement (as discussed above) rather than variation between different samples. Because 6'Sialyllactosamine is much lower in concentration than the other two species measured, the effect of its variability on the overall oligosaccharide concentration variability is small.

When the overall process is considered, this is an encouraging result, because it means that acidic oligosaccharides are less heat sensitive than previously thought and even in extended heat treatment processes up to 80 °C there is minimal loss of the target oligosaccharides in the timeframe tested. Evaporation processes typically operate at temperatures lower than this, assuming lower than ambient pressures are used. Spray drying can require higher particle temperatures (approaching 100 °C) but at much shorter residence times, meaning that the overall degradation is likely to be minimal. Both of these processes can be safely incorporated into an oligosaccharide enrichment process without worry of loss of the target compounds.

Results showing that there is minimal heat induced loss of sialyl oligosaccharides fit well with the observation that there is still a significant concentration of sialyl oligosaccharides in lactose mother liquor, despite being treated previously at temperatures higher than 60 °C for a number of hours in a crystallising evaporator. If high losses of oligosaccharides were noticed after a short time at 80 °C, then their presence in lactose mother liquor would be hard to explain.

Research into heat stability of sialyl-oligosaccharides is not reported in literature, meaning that this result is significant and may be applied in other studies. Further work is recommended here, looking at thermal stability of a wider range of species, at higher temperatures and longer duration.

6.5 Conclusion

Experimental results show that there is no significant loss of acidic oligosaccharides from mother liquor subjected to temperatures of up 80 °C for lengths of time up to 30 minutes. These are the most extreme conditions expected in downstream processing (evaporation and spray drying) of an enriched oligosaccharide product. This gives confidence that steps with elevated temperature can be used in the overall process.

Chapters 7, 8, and 9 cover filtration and simulated moving bed chromatography, and Chapter 10 summarises which process operations are most suited to enriching oligosaccharides (including high temperature processes) and how they are best arranged.

7 MEMBRANE FILTRATION EXPERIMENTS

7.1 Introduction

In Chapter 4 a number of different arrangements of unit operations were looked at for the enrichment of oligosaccharides and three options were found with good internal rates of return (greater than 30%) for further study. All three options included ultrafiltration, and two were based on using nanofiltration to separate the smaller components in mother liquor. The sensitivity analysis (Section 4.4) also revealed four significant variables related to filtration that needed to be confirmed by experiment, most importantly the volume concentration factor in ultrafiltration and the leakage of oligosaccharides through the nanofiltration membrane. Membrane filtration was therefore an important process to understand for this project.

Filtration is now a very common means of separation in many different industries and for a wide range of applications, from micro filtration (MF) for bacteria and particulates down to reverse osmosis for removal of salts and ions from water or concentration of solids. The reason for this is that it is relatively cheap compared to other separation technologies, can be adapted for a variety of applications, takes up little space, and there is usually no need for modifying chemicals. The main disadvantage is that unless there is a large difference in solute size (generally one order of magnitude or more), separation is only partial, and not complete. Furthermore, “size” is defined as molecular weight by filtration manufacturers, while in reality there can be large variances in the lengths of molecules depending on physico-chemical properties.

Its use is therefore mainly in food, chemical, and environmental processing where lower purities are acceptable, or in downstream processes for biological and pharmaceutical products where large differences in size exist, for instance in desalting or isolating proteins. As technology develops further, it may become possible to perform high purity separations of similarly sized solutes, but for now these applications are not commercially possible.

Filtration is commonly described as one of two types. Dead end filtration (sometimes called normal flow filtration) describes the process where all of the retentate is collected against the membrane as the process continues, with a build-up occurring over the course of the run. This is more suited to applications where the permeate is of interest and where the material to be

retained is low in concentration in the feed. Tangential flow filtration is a different process where the permeate is forced through the membrane at right angles to the feed flow across the length of the membrane, and the retentate continues in this flow to be collected or recycled after it has exited the membrane cartridge. In this sense there is continuous flow of both the permeate and retentate. A typical tangential flow filtration process is shown in Figure 7.1. Tangential flow filtration has clear advantages over dead end filtration, because it can be used continuously and can take feed streams with much higher concentrations of retentate, as the convection across the membrane surface is strong, and was used exclusively in this project.

Three streams generally comprise tangential flow filtration: A feed, containing the components to be separated, a permeate, containing the portion that has migrated through the membrane, and the retentate, the material that has not crossed the membrane. The feed velocity across the membrane surface is generally much higher than the permeate velocity, promoting strong convection and reducing solute build up and fouling. This is achieved by recycling the retentate back to the feed tank at a flow rate much higher than the permeate flow. A heat exchanger is often used to maintain the desired operating temperature in the feed, and pressure sensors before and after the membrane show the pressure drop along the length of the membrane.

Tangential flow filtration can be run in either batch or continuous mode. In batch processes, all of the retentate is recycled back to form the feed, and the process is stopped when the desired (or maximum) total solids in the feed is reached by permeate leaving the system. In continuous processes, the majority of the retentate is still recycled, but a small portion is removed, with fresh feed added to the feed tank to keep the volume and concentration of the feed constant (see Figure 7.1). To efficiently increase the total solids of the retentate in a full scale continuous process, it is common to use several stages of filtration, each stage increasing the feed concentration by a few percent.

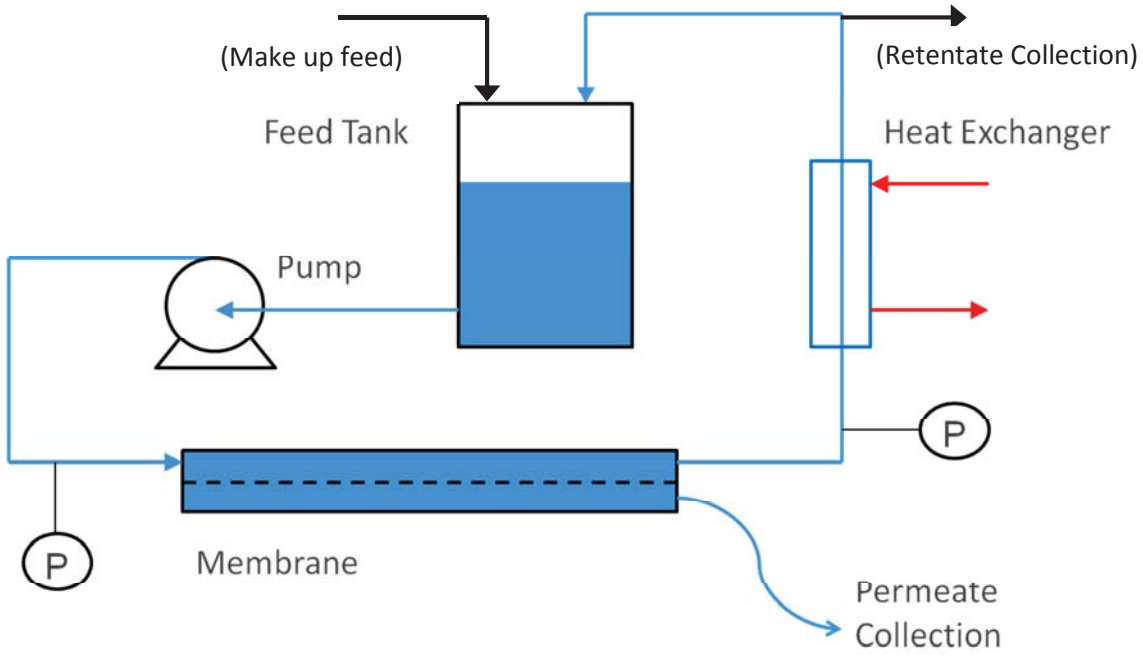

Figure 7.1: Diagram of tangential flow filtration in batch mode (without streams in parentheses) or continuous mode (including streams in parentheses)

Membranes are often described with a molecular weight cut off (MWCO), which is usually defined as the highest molecular weight species that will be allowed to pass through the membrane, but in practice there is no definite cut off for a given membrane, and a proportion of a given solute will be allowed through whilst a proportion is retained. Instead, rejection coefficients are used to describe the passage of different species across a particular membrane. Rejection coefficients, R_i, are defined as follows:

$$\boldsymbol{R_i = 1 - \frac{C_{P,i}}{C_{F,i}}} \qquad \text{(Equation 7.1)}$$

where $C_{P,i}$ and $C_{F,i}$ are the concentrations of component i in the permeate and feed, respectively.

For a membrane with a nominal MWCO of 10 kDa, the rejection coefficient for a 10 kDa protein would typically be higher than 80%, whilst a 5 kDa protein might have a rejection coefficient of 40-50% across the same membrane. In all cases these need to be confirmed experimentally, as it depends on the flow rate, pressure, fouling, and the chemical makeup of the feed.

Other important measures in filtration are the purity, defined in this work as the concentration of the target compound (oligosaccharides) divided by the total solids concentration, and the recovery, defined as the concentration of target compound in the product stream (the retentate in this case) divided by its concentration in the feed.

7.2 General Approach

Since the molecular weight of oligosaccharides (approx. 500 Da to 2000 Da) lies between both the smaller and larger species present in mother liquor, any single membrane separation stage will not be able to produce a pure oligosaccharide stream. If filtration is to be the primary means of separation, a two-stage approach (Figure 7.2) needs to be considered, one for the removal of larger species (proteins, fats, and remaining particulate matter) and one for the removal of smaller species (minerals, monosaccharides and small organic acids, and lactose).

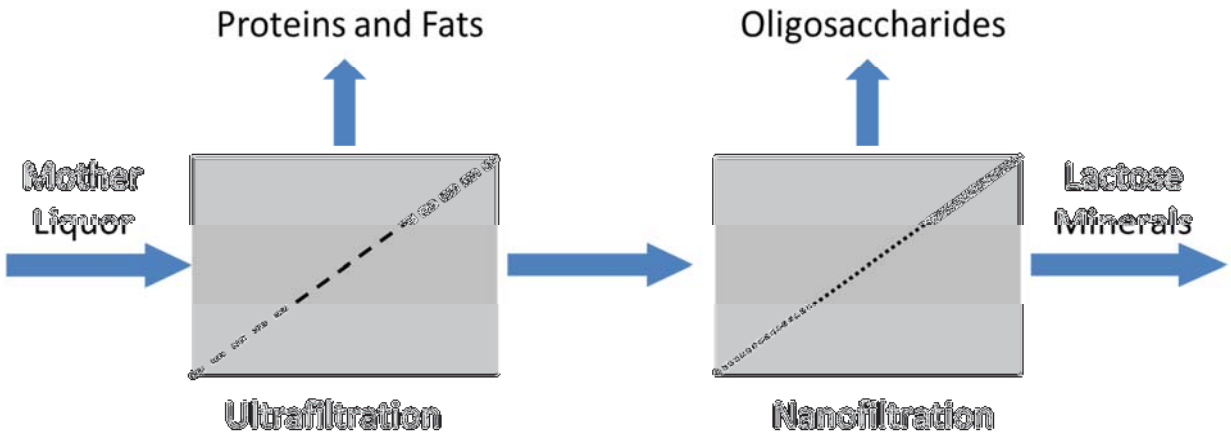

Figure 7.2: Two-stage approach to filtration of mother liquor

For the first stage, removal of proteins and larger molecules from the mother liquor, an ultrafiltration membrane is needed. This membrane needs to allow molecules smaller than 2 kD to pass through, the upper limit of oligosaccharide size (Mehra et al., 2014), and block anything larger. In this case, the smallest of the dairy protein sizes is more than an order of magnitude larger than this (14 kDa), so the separation is, in theory, straightforward. The permeate from the first stage forms the feed for the second stage.

For removal of lactose, minerals, and other small molecules, a nanofiltration or tight ultrafiltration membrane is needed, which retains oligosaccharides but allows smaller

molecules through. This is a much more difficult separation to make, since lactose, in particular, is similar in size to the smallest of the oligosaccharides (lactose = 342 Da, smallest oligosaccharides = 504.4 Da). The retentate of the second stage is the product stream, rich in oligosaccharides, and the permeate is the waste stream, with mainly lactose and minerals. The nanofiltration work presented below essentially assumes that lactose is the most difficult component to remove from the oligosaccharides and presents data detailing the differences between the two, given that the minerals, monosaccharides and organic acids are smaller in size than lactose and should therefore have lower rejection coefficients in the conditions being used.

For the simulated moving bed chromatography approach, dealt with in Chapter 9, ultrafiltration is required prior to introduction of the feed to the simulated moving bed system but nanofiltration is not.

7.3 Experimental Method

7.3.1 Setup

The Lab20 is the same as that shown in Figure 7.1 (above) and comprises a feed tank of approximately 50 L and a high pressure homogeniser pump through which the feed is forced into the membrane unit. The stainless steel membrane module holds any spiral membrane of the same dimensions (50 mm diameter, 300 mm length) so can be used for reverse osmosis, nanofiltration, or ultrafiltration. After passing through the membrane, the permeate is allowed to flow through a flexible hose for collection, waste, or recycle back to the feed tank. The retentate exits through a shell and tube heat exchanger and is returned either to the feed tank or to a collection point via a 3-way valve.

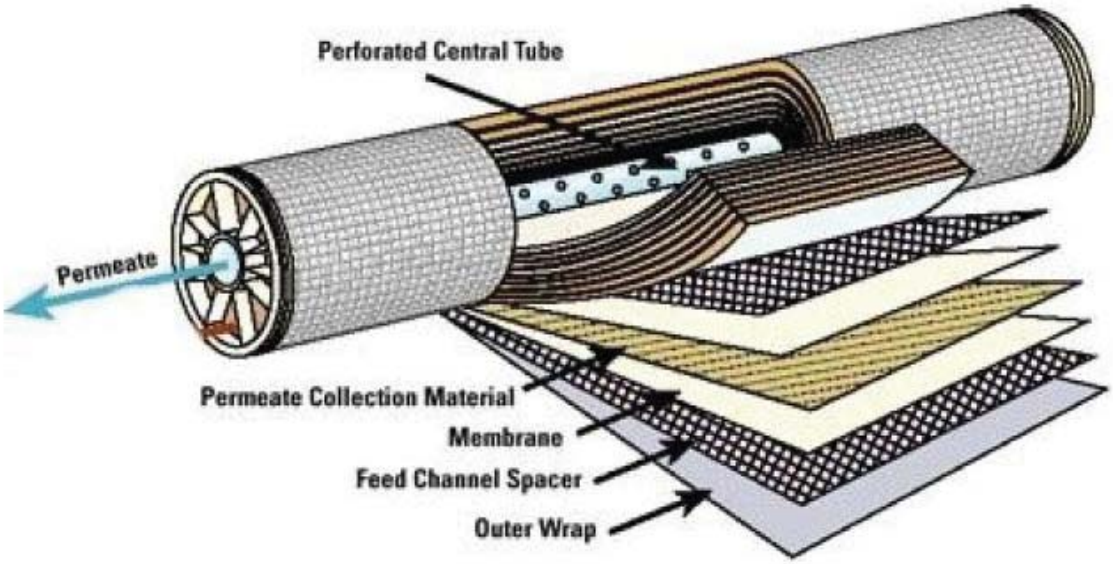

Figure 7.3: Diagram of spiral wound filter module (Lenntech BV, 2013)

The Lab20 operates under industrial conditions so that parameters including pressure, temperature, membrane module shape, and flow velocities are similar to commercial processes, though it requires relatively small quantities of feed and can be managed by one person. This means that process scale up is relatively straightforward and easily predicted from pilot plant trials, as opposed to laboratory scale trials where basic conditions of temperature, pressure, and fluid flow are different. The permeate and retentate return pipes also give flexibility for a variety of operating modes.

Membrane modules were all spiral wound membranes from the Osmonics Desal range (GE Healthcare, Auckland, NZ) with an area of 0.32 m^2, diameter of 50 mm, and length of 300 mm. Construction includes a permeate collection sheet and feed channel spacer wound into a spiral around a central perforated tube used for permeate collection, as shown in Figure 7.3. The membrane modules were fitted to the Lab20 filtration unit housing.

7.3.2 Ultrafiltration Trials

A 5 kDa Osmonics Desal PT1812C - 34D spiral wound membrane (GE Healthcare, Auckland, NZ) was trialled initially for the separation of proteins from lactose mother liquor. Results (Figure 7.4) showed that the 5 kDa membrane was retaining too much of the oligosaccharide fraction as well as the proteins and led to the choice of a 10 kDa membrane (Osmonics Desal PW1812C

- 34D) as a replacement. The 10 kDa membrane was used under the same operating conditions as the 5 kDa membrane.

150 L mother liquor was diluted 1:1 by weight with RO water, preheated to 50 °C in milk cans with hot water rings, mixed thoroughly, and separated in a disc-bowl clarifier with de-sludging every five minutes. Approximately 275 L of diluted, clarified mother liquor was obtained and frozen at -18 °C in 20 L pails until used.

For each UF run, 15-20 L clarified mother liquor was defrosted at 4 °C for 48 hours, diluted at a 1:1 ratio by weight with warm RO water into 50 L milk cans, weighed, and preheated to 50 °C using hot water rings while being stirred. The filtration unit and membrane were also preheated to 50 °C by circulating distilled water at the target temperature for 10 minutes. The diluted mother liquor was then added to the feed tank to begin the process.

Trials to determine the effect of feed concentration on separation efficiency were carried out at a constant pressure (7 bar) by manually adjusting the pump speed when required. Permeate was collected and continuously weighed, while retentate was recycled back to the feed tank. The collected permeate mass was used to determine the volume concentration factor (VCF), which is defined as:

$$\boldsymbol{VCF} = \frac{\boldsymbol{Initial\ mass\ of\ feed}}{\boldsymbol{Initial\ mass\ of\ feed - permeate\ collected}} \qquad \text{(Equation 7.2)}$$

The VCF gives an indication of the reduction in volume of retentate relative to the starting volume. Because of the removal of permeate from the system, the retained compounds increase in concentration over the course of the run which increases viscosity and reduces the flow rate across the membrane. Samples of retentate and permeate were taken at certain VCFs across the run. The process had to be stopped when the concentration of solids in the retentate reached a point (usually between 15-20% total solids, measured by a handheld refractometer) that made the flux impractically slow (below 15 $Lm^{-2}h^{-1}$). At such slow flow rates, the fouling and risk of damage to the membrane increases sharply.

Trials to determine the effect of pressure on separation efficiency were carried out by initially using the method above to attain a certain concentration of total solids, then redirecting the permeate (as well as the retentate) back into the feed tank, so that the system was in full

recycle. This meant that the volume and concentration in the feed tank were constant (neglecting leakage and fouling) and the system was in steady state. The pressure was then adjusted to 5, 6, 7, 8, 9, and 10 bar by increasing or decreasing the pump speed, and left for 30 minutes to stabilise at each step before collecting samples of permeate and retentate. Concentration was fixed at about 17% total solids. Changes in pressure must always be increases rather than decreases (without flushing with water in between changes), because the concentration build-up and gel formation at the membrane surface which occurs at high pressures is not easily reversed by a decrease in pressure. For this reason the trials could not be randomised, either in this case or for nanofiltration trials (see below, Section 7.3.3).

In both VCF and pressure studies, flow rate, retentate and permeate refractive index, temperature, accumulated permeate mass, and pressure were measured at 15 minute intervals. The target temperature of 50 °C was maintained using the shell and tube heat exchanger to warm the recycled retentate with hot water at approximately 60 °C prior to its return to the feed tank. The service water flow rate and temperature for the heat exchanger was adjusted manually based on temperature readings from the feed tank.

Cleaning was carried out after each trial by recirculation of NaOH and Reflux 7C (Orica NZ Ltd) at a pH of 10.9 for 20 minutes at 50 °C, before rinsing for 10 minutes, then addition and recirculation of phosphoric acid (pH 2.8) for 20 minutes at the same temperature. After subsequent rinsing for 10 minutes, sanitisation was done with approx. 14 mL NaOH in 50 L of RO water (pH 10.9) and sodium hypochlorite added (about 80-100 mL) to give enough free chlorine for sterility. Finally, the system was rinsed for 10 minutes with RO water and cooled. On occasions where the equipment would not be used for a week or longer, a sterilising storage solution was introduced into the system, otherwise RO water was used for storage.

7.3.3 Nanofiltration Trials

Initially an Osmonics Desal DK1812C - 34D nanofiltration membrane was used for separation of oligosaccharides from the smaller molecules in mother liquor. This membrane is specified as rejecting 98% $MgSO_4$ and does not have a nominal MWCO as ultrafiltration membranes do. After initial trials it was found that most of the lactose was being retained as well as the oligosaccharides (Figure 7.7), so it was replaced with an Osmonics Desal GE-1812C-34D membrane with a 1 kD nominal MWCO. This membrane is technically a tight UF membrane,

because the pore structure and function within the membrane more closely resembles that found in ultrafiltration. To differentiate this stage from the previous one (with 5 or 10 kDa ultrafiltration membranes), throughout the thesis it will be referred to as nanofiltration.

The feed material for nanofiltration trials was the permeate from the ultrafiltration runs that were carried out using the optimal conditions from the previous trials. The feed and membrane were preheated to the operating temperature of 45 °C and the feed added to the feed tank after being weighed. In these trials the feed was not diluted, because the total solids were already within a good range for processing (less than 10%).

Trials to investigate dependence on concentration and pressure were carried out in the same way as the ultrafiltration trials above, with a pressure of 17 bar used for VCF experiments and pressures of 17, 20, 23, 26, and 30 bar used in pressure experiments with a constant concentration of around 15% total solids. Samples were taken 30 minutes after each increase in pressure to allow the system to reach steady state.

Further trials involving multiple stages of nanofiltration and simulated continuous processing were also conducted and are presented in Chapter 8.

Cleaning of the nanofiltration membrane was done with 15-20 mL B615 (Orica NZ Ltd) in 50 L RO water at pH 10.9, recirculated at 45 °C for 20 minutes at 10 bar, then after rinsing with water, 15-20 mL R400 (Orica NZ Ltd) was added into 50 L water (target pH 2.9) and recirculated at the same pressure and temperature for 15 minutes. After the final rinse and cooling, the system was left in RO water or sanitising solution if not being used within the next week.

7.3.4 Sample Analysis

Samples that were taken during the trials were analysed for composition and the results used to determine rejection coefficients (Equation 7.1). Total solids were measured by oven drying at 105 °C until a constant weight was reached, and ash content measured in a thermo-gravimetric analyser at 550 °C. Total oligosaccharides, lactose and galactose were measured by HPLC using the method in Section 5.2, and acidic oligosaccharides were measured by HPAEC-PAD using the method also in Section 5.2. Total nitrogen and non-protein nitrogen were measured by the Kjeldahl method and the true protein level calculated from the difference between the two.

7.4 Results

7.4.1 Ultrafiltration

Rejection coefficients from the 5 kDa UF trials were calculated from the composition data using and plotted against total solids to show the dependency of rejection coefficients on concentration in Figure 7.4. Raw composition data can be found in Appendix Section 11.2.

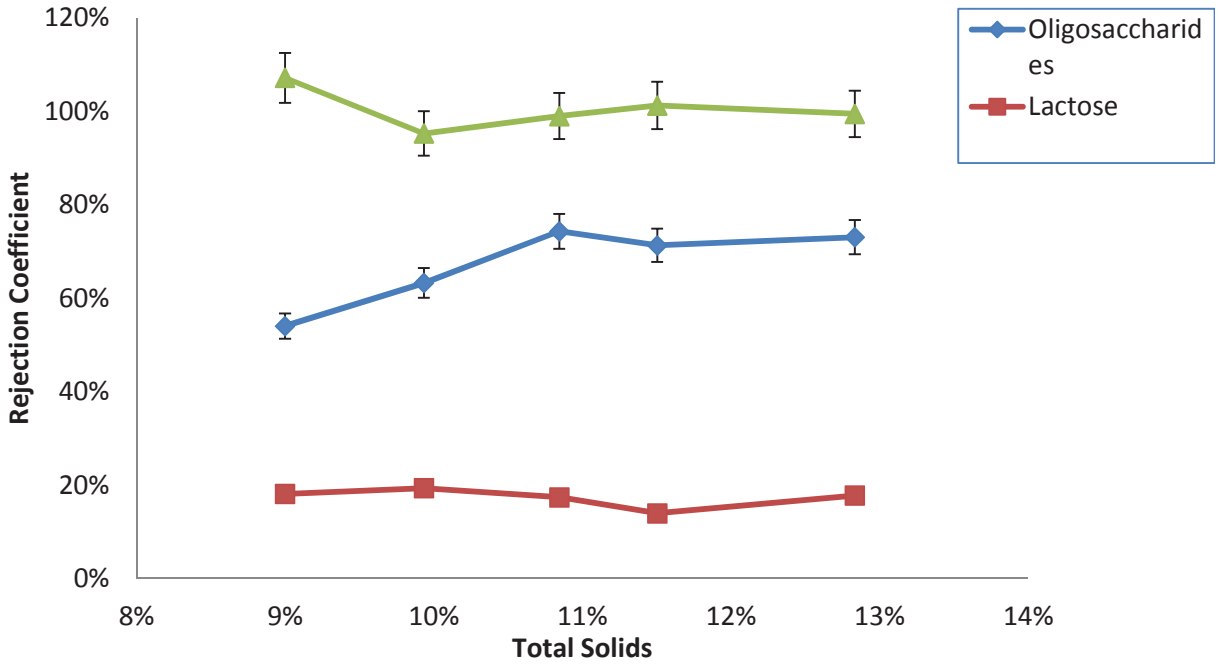

Figure 7.4: Rejection coefficients for three major components in lactose mother liquor in 5 kDa ultrafiltration across a range of total solids at 7 bar.

In Figure 7.4 the rejection of protein is complete (100%), which is the primary goal of the ultrafiltration step, but the rejection of oligosaccharides is also high (50 – 75%). This means a significant portion of the oligosaccharide fraction is being retained in the waste stream with the protein, when it should be allowed through the membrane at this stage. Because of this problem, a looser membrane (10 kDa) was selected and the experiment repeated. The results from this are presented in Figure 7.5. The difference from Figure 7.4 is clear: The protein is still fully retained at all concentration levels (although with some experimental error) whilst the oligosaccharide and lactose rejections are below 20%, where before the oligosaccharide rejection was more than 50%.

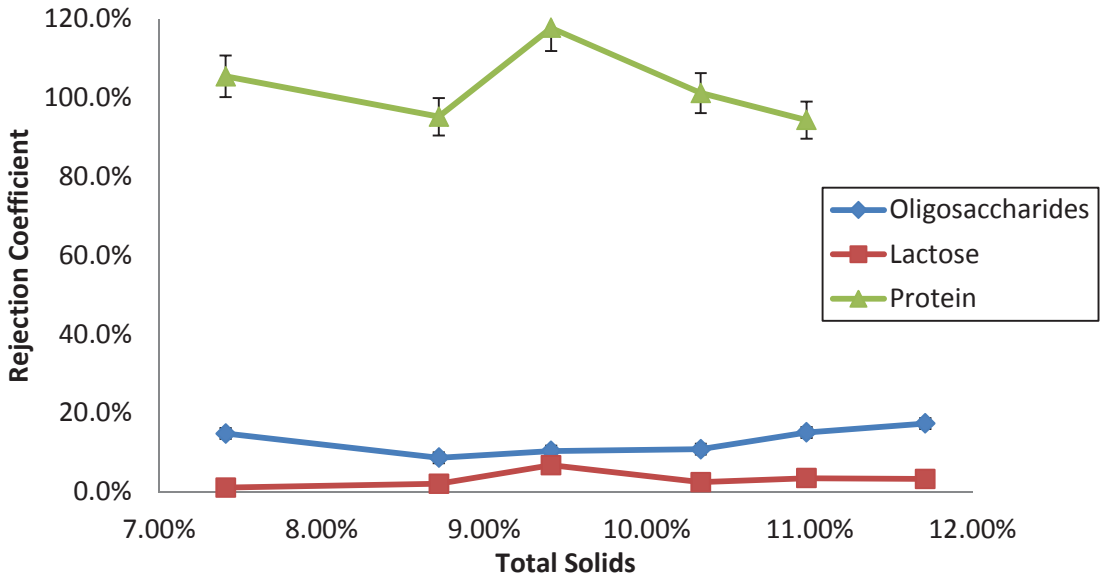

Figure 7.5: Rejection coefficients at different concentrations for 10 kDa ultrafiltration membrane at 7 bar.

The variability in protein rejection coefficient is because of the small initial protein content of mother liquor and the negligible content (if any) in the permeate. These concentrations are at the lower detection limits of the methods used, so the percentage variation can be large. In the cases of rejection being reported as over 100%, the non-protein nitrogen was reported higher than the total nitrogen. This is impossible in reality, and suggests minor experimental error in the determination. This leads to an apparent negative mass of true protein in the permeate. Because these calculated true protein levels were close to 0% and within the detection limits, it is assumed that no protein was present in the permeate.

Although it could be assumed that a 5 kDa membrane would not hinder the flow of molecules with a molecular weight of less than 1 kDa (i.e. oligosaccharides and lactose), the nature of membrane filtration means that in practice there are some factors which restrict their passage. One of these is that the conformation of the molecule is more important than its molecular weight. This means that although two species might have a similar molecular weight, their size can be very different because of ionic charges and pH, or the way the atoms are arranged in the molecule (i.e. compact and globular or linear and spread out). Oligosaccharides are known to behave as though they have a higher molecular weight than they actually do when at neutral pH, as with mother liquor, so in an ultrafiltration membrane they are more retained than is expected for their molecular weight (Frank & von der Lieth, 1997). As well as the molecular conformation, those species with electrostatic charges (including acidic sialyl

oligosaccharides) will have a hydration shell of attached water molecules at neutral pH, increasing the apparent size by up to 50% or more, especially with sialic acid.

Additionally, small blockages, gel layers, and fouling can occur during the course of the process which make pores smaller and less accessible to oligosaccharides and lactose. This is an unavoidable part of the process, especially when proteins are present, which are especially susceptible to fouling. By increasing the molecular weight cut off to 10 kDa, these effects are significantly reduced for oligosaccharides and lactose, and the small molecules are allowed through whilst the protein is retained. The smallest of the dairy proteins, α-lactalbumin, is 14 kDa, so by using a membrane with a higher molecular weight cut off there is a danger that some protein will be allowed through. The 10 kDa membrane was therefore selected as the best membrane to use for the removal of proteins (and any larger material present) prior to further processing.

It is also important to see what the effect of pressure is on the ultrafiltration process, because higher pressures can sometimes allow a greater flux through the membrane which improves productivity. A plot of pressure and rejection coefficients for protein, lactose, and oligosaccharides is presented in Figure 7.6. It can be seen that lactose and oligosaccharide rejection coefficients are significantly higher at pressures above 7 bar, whilst the protein continues to be rejected at or near 100%.

The increase in rejection coefficients is likely due to the formation of a thicker gel layer against the membrane under high pressure, as described by Wijmans, Nakao, and Smolders (1984). This hinders the transport of lactose and ash through the gel layer and membrane, whilst the water has a comparatively free path and is found in higher concentration in the permeate.

Because the goal is to allow free passage of the small molecules across the membrane, 7 bar is the highest pressure that can be considered for this 10 kDa ultrafiltration membrane. This aligns well with common practice in ultrafiltration, where pressures between 5-7 bar are most often used.

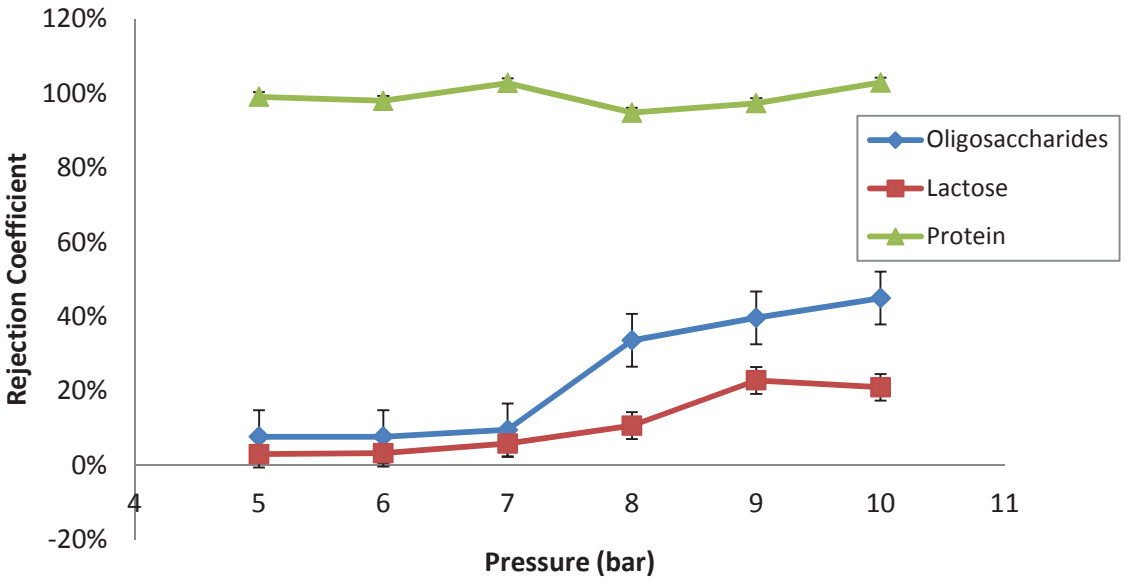

Figure 7.6: Rejection coefficients at different pressures for 10 kDa ultrafiltration

The relationship between pressure and membrane flux (presented in Appendix Section 11.2) showed that unlike in nanofiltration, higher pressures do not necessarily lead to a higher flux. In this case the flux stayed at the same level (6.6 $Lm^{-2}h^{-1}$) at each of the pressures studied, probably because the membrane is above the pressure-dependent region of pressure. This indicates there is no advantage in increasing pressure, and confirms that lower pressures (5-7 bar) are better because of the low rejection coefficients for small molecules.

7.4.2 Nanofiltration

The initial nanofiltration trials were carried out with a true nanofiltration membrane as described in Section 7.3.3. The specified rejection is 98% $MgSO_4$, with no other molecular weight indication, making it hard to predict results for oligosaccharides and lactose. The observed rejection coefficients (presented in Figure 7.7) are high for both species, and there is very little difference between them, especially at low solids concentrations.

Nanofiltration with this membrane is not a viable option for separating oligosaccharides from lactose, since there is no significant difference in the rejection coefficient and almost all of the lactose is retained with the oligosaccharides, as well as about half of the ash content. A

different membrane was selected, this time with a 1 kDa molecular weight cut off, with the intention of allowing more lactose through the membrane whilst still retaining the oligosaccharide fraction. A membrane of this type is often referred to as a "tight ultrafiltration" membrane. The initial results of testing with this membrane, at different solids concentrations, are shown in Figure 7.8.

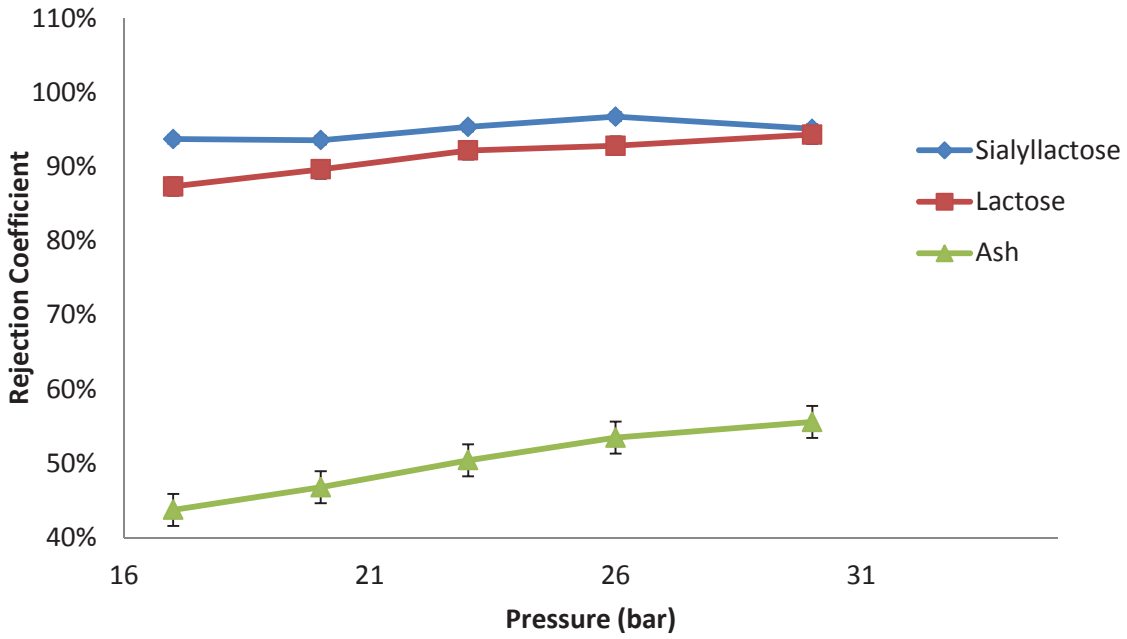

Figure 7.7: Rejection coefficients at different pressures for sialyllactose, lactose, and ash in "true" nanofiltration (98% MgSO4 rejection).

The difference between the two membranes in Figure 7.7 and Figure 7.8 is obvious, with the tight ultrafiltration membrane rejecting less than 50% of the lactose at total solids of 11% or higher, whilst still rejecting around 90% of the oligosaccharides. This degree of separation, although still not complete, was enough to give an increase of oligosaccharides from 1.0% to 2.5% on a dry basis – the increase in composition coming from the removal of lactose, minerals, monosaccharides, and organic acids in the permeate. This falls short of the requirement for at least 4% oligosaccharides from Chapter 4, but there is scope for improving this by modifying the conditions and adding further stages (dealt with in Chapter 8). The improved separation at higher total solids may be due to the effect of a thicker and more concentrated gel layer at the membrane surface hindering the transmission of oligosaccharides through the membrane more so than lactose.

The reason for the reversal in rejection at 8.5% total solids in Figure 7.8 is unclear, but likely to be due to issues reaching steady state early in the filtration process.

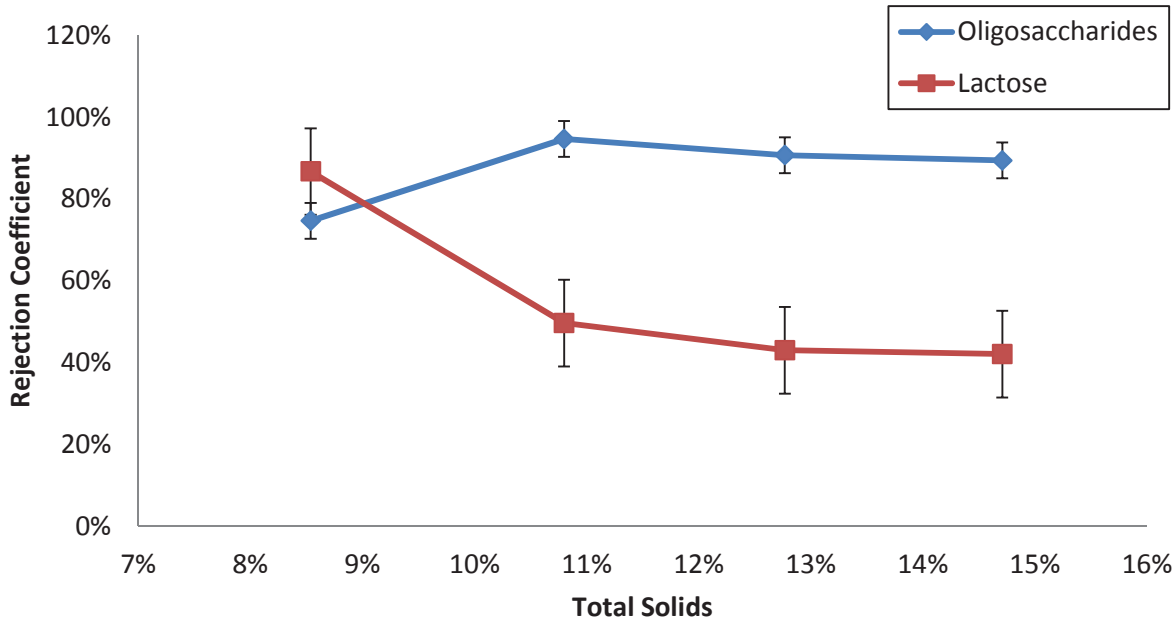

Figure 7.8: Rejection coefficient at different total solids for oligosaccharides and lactose in 1 kDa membrane at 17 bar.

The total solids of the feed in the nanofiltration process is not easily raised to the minimum of 10-11% which is shown in Figure 7.8 to encourage better separation. The reason for this is that even with a relatively high solids feed (i.e. 10-15%) for the ultrafiltration process, the permeate (which forms the feed to the nanofiltration stage) is always lower in total solids. The ultrafiltration feed cannot be increased past 15% solids because of fouling and significant flux decrease, meaning the maximum permeate solids is about 10%. This maximum occurs towards the end of the ultrafiltration run and most of the collected permeate lies in the range of 5-8% solids. Unfortunately this is a constraint of the system and cannot be avoided unless an intermediate concentration step is used. This adds significant cost and inefficiency in processing which is better to avoid if possible.

A simple option for increasing solids is to begin the nanofiltration process without collecting retentate until the total solids has reached a point where the rejection coefficients are favourable. This would not affect the yield, since the retentate stays in the system as the concentration is increasing. In a full scale continuous process, this could be achieved by having

a pre-concentration step where permeate is continuously removed to increase total solids before the retentate is sent to a second stage where the retentate is collected.

A further trial was done looking at the effect of pressure, the results for which are in Figure 7.9. The total solids concentration in the retentate was constant at 7.9% at all points in the trial, which was in the region of poorer separation in Figure 7.8. This total solids level was chosen because of the higher flux and ease of processing at the lower concentration. This explains why the overall separation is not as good as it was at the higher concentrations, and means that this information should not be looked at in isolation to provide maximum purity and recovery data but should be used to give an idea of which pressures best enhance separation. Because some oligosaccharides are so similar in molecular weight to lactose (as well as in charge), this is the most difficult of all the components to separate in the process and it is important to optimise ahead of other measures.

As can be seen from Figure 7.9, the effect of pressure is minimal. Apart from the outlier at 19 bar, the oligosaccharide rejection follows a gentle downward trend, whilst the lactose rejection is relatively constant. The distance between the rejection coefficients of the two components decreases very slightly as pressure is increased. This is a reflection of the way nanofiltration membranes are constructed. In contrast with ultrafiltration membranes, which are designed to cope with pressures of no more than 10 bar, they are designed to withstand pressures of up to 40 bar and are constructed with more rigidity and less flexibility in the membrane structure. This is why the pores do not shrink under higher pressures like they do with ultrafiltration membranes, and the performance is similar across the pressure range.

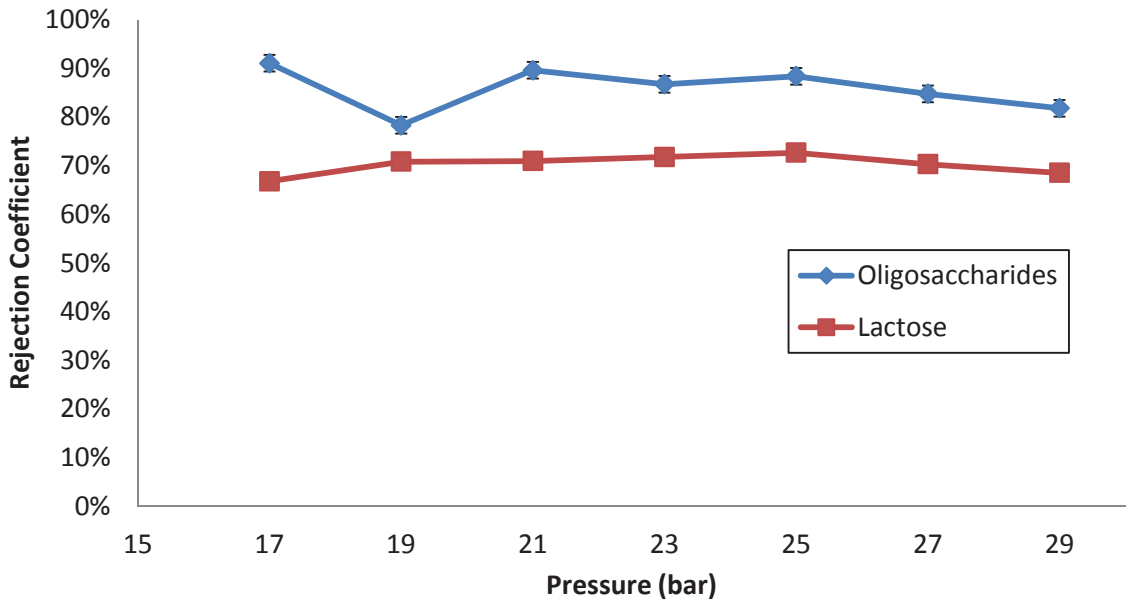

Figure 7.9: Rejection coefficients of oligosaccharides and lactose at increasing pressure in 1 kDa ultrafiltration. Total solids were 7.9%.

The rejection coefficients for both components are high (greater than 50%), leading to a system with a high recovery but low purity. This is because the oligosaccharide concentration in the permeate is very low, which means that almost all of it (94-96%) is recovered in the retentate, but the lactose is also mostly retained, meaning that the resulting purity is very low (2-2.5%). On the other hand if the rejection coefficients were low, the recovery of oligosaccharides would also be low because most would be lost in the permeate, but the purity would be higher because much less of the lactose fraction would be retained. Because the initial lactose concentration is so high compared to the oligosaccharide concentration, it needs to have a very low rejection coefficient to increase the purity of oligosaccharides being retained. Diafiltration and multiple stage nanofiltration can help exploit the differences in purity and recovery and are covered in Chapter 8.

Another factor that is important to consider is membrane flux. Unlike in ultrafiltration at the pressures studied (Section 7.4.1), flux here is in the pressure dependent region, as shown in Figure 7.10. In this case the relationship is linear (R^2 >0.99), which is to be expected for pressures up to 40 bar (the manufacturer's specified limit). This is a significant result, because by altering the pressure, the flux, and therefore productivity of a given membrane area, can be doubled. This means that only half the membrane area is required to achieve the same production rate if 30 bar is used instead of 15 bar. This leads to decreased capital and on-

going costs as membrane housings are installed with associated pipe work and services, and membrane cartridges are periodically replaced.

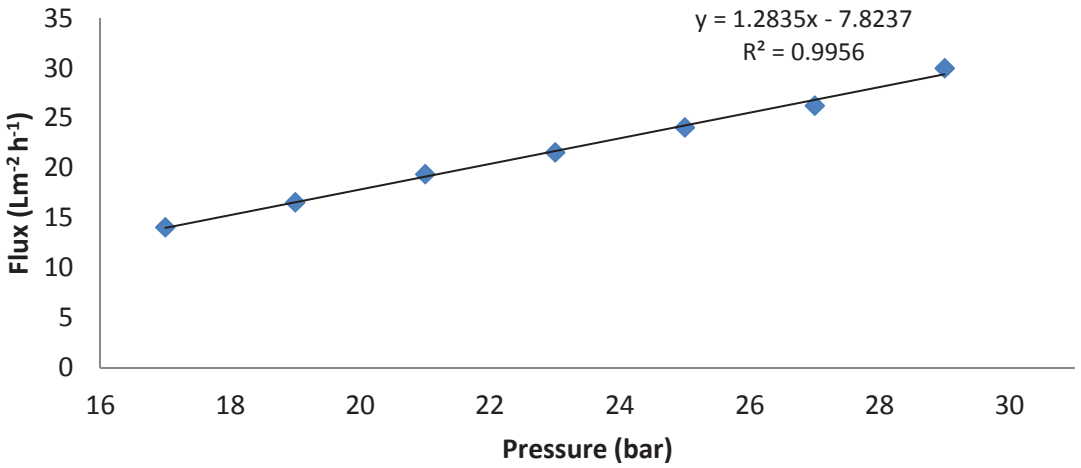

Figure 7.10: Flux dependence on pressure in nanofiltration

The fact that higher pressures give a higher flux whilst also slightly decreasing the separation between the oligosaccharides and lactose (Figure 7.9) leads to a trade-off between productivity and purity of product. At this stage, however, the separation at the best conditions studied still falls short of the minimum oligosaccharide content of 4% (Chapter 4) so the focus is to bring this up to specification before the economics of this process option are considered. This means selecting a pressure in the lower part of the range initially and seeking to optimise the separation (see Chapter 8), although the question of flux versus separation will be revisited if there is an indication that the minimum requirement will be met.

7.5 Discussion

Ultrafiltration was investigated as a process for the removal of protein and other large molecules from mother liquor prior to processing with either nanofiltration (with or without lactose crystallisation) or simulated moving bed chromatography. The 5 kDa membrane initially trialled was too tight to allow adequate flow of oligosaccharides across the membrane and was therefore not used for protein removal. A 10 kDa membrane corrected this problem and allowed rejection coefficients of less than 20% for oligosaccharides and lactose, whilst retaining all of the protein.

While the 10 kDa membrane is better when used in series with nanofiltration, this does raise the possibility of using a single stage of 5 kDa ultrafiltration without a nanofiltration step following. In this case, the protein and oligosaccharides would be largely retained, whilst the lactose and ash would be found predominantly in the permeate (waste stream). The retentate had a solids content of 10-12% and an approximate composition as shown in Table 7.1:

Table 7.1: Composition of 5 kDa UF retentate

Component	Dry basis percentage
Protein	5-7%
Lactose	52-57%
Oligosaccharides	1.8-2.1%
Ash	16-18%
Monosaccharides	5-7%

Note that the difference in rejection coefficients (Figure 7.4) is approximately 50%, which is very similar to the difference in rejection coefficients for the 1 kDa nanofiltration membrane. The retentate oligosaccharide content is slightly less, while the main difference is the inclusion of protein in the product. This is generally not an issue for addition to infant formula as many products currently add bovine proteins, although the more complex the product is, the less flexibility manufacturers have when formulating products. For this reason, and the slightly lower oligosaccharide purity, a process using both ultrafiltration and nanofiltration is preferred.

Ultrafiltration performance in terms of rejection and flux was not found to be dependent on feed concentration, but pressures above 7 bar across the membrane led to higher rejection coefficients for oligosaccharides and lactose, most likely due to an increase in gel layer thickness and concentration. This membrane is therefore suitable for removal of large molecules at pressures of 5-7 bar.

A true nanofiltration membrane with a stated rejection of 98% $MgSO_4$ was trialled for the separation of oligosaccharides from lactose, but proved too tight to allow lactose through the membrane, as the hydrodynamic diameter of lactose is 0.87 nm at the temperature studied (Ribeiro et al., 2006), larger than its molecular weight would suggest, and approaching the nominal pore size of the membrane (1 nm). This was replaced with a looser, 1 kDa, membrane, with the goal of allowing more lactose through and achieving a significant separation.

Results showed that at higher feed solids, a partial separation occurred with the 1 kDa membrane, with a final oligosaccharide purity (dry basis) of up to 2.5% from an initial purity of 0.8% and recovery of up to 96%, with oligosaccharides rejected at about 90% and lactose rejected at as low as 40%. The rejection coefficient of the ash component was approximately 10-15%, much lower than the 45-55% rejection using the tight nanofiltration (NF) membrane. Pressure, studied between 17 and 30 bar, was a less significant factor in the degree of separation, with a slight trend towards poorer separation at higher pressures. The membrane flux is strongly correlated with pressure, with a two-fold increase in flux observed across the pressure range.

Because the initial lactose concentration is so high in relation to the oligosaccharide concentration, even if the majority of the lactose is allowed through the membrane there can still be a significant concentration of it in the retentate with the target oligosaccharides. This makes it very hard to achieve a high purity, and it was found throughout the experimental work that the highest oligosaccharide purity obtained was just 2.5% on a dry basis, compared to the target of 4% for addition to infant formula. It can be concluded that nanofiltration is only capable of a partial enrichment in cases like this where the molecular weights are so similar, although this enrichment can be improved by optimising feed concentration and pressure.

In the mass balance in Section 4.4, it was assumed that the rejection coefficient of oligosaccharides was 90 ±10%, and that the rejection coefficient for lactose was 30% (with a sensitivity range from 0 to 50%). The rejection of oligosaccharides has minimal effect on the purity of the oligosaccharide stream (which is more dependent on removal of other compounds due to its low concentration) but determines the yield of the process and is therefore a strong factor in the economic feasibility. Both concentration dependent and

pressure dependent experiments in this chapter confirmed that the rejection coefficient in the 1 kDa membrane is close to the predicted 90%.

The rejection of lactose was close to the upper limit of the sensitivity analysis in the best case (at high feed concentration) and beyond this limit in the pressure dependence studies, indicating that the initial assumption was incorrect. Although the rejection of lactose is not an important factor regarding the final internal rate of return, it has a strong bearing on the final purity of the oligosaccharide stream, because it is an impurity in the final product.

7.6 Conclusions

A single stage of ultrafiltration followed by single stage of nanofiltration is incapable of producing an oligosaccharide stream with 4% purity. Nanofiltration can be incorporated into a broader process that combines it with other technology to achieve the 4% purity target, and options for this are covered in Chapter 8, where lactose crystallisation and multistage nanofiltration are considered.

8 MEMBRANE ARRANGEMENTS FOR ENHANCED SEPARATION OF OLIGOSACCHARIDES

8.1 Introduction

In Chapter 7 it was shown that by processing lactose mother liquor using a single stage of NF, a maximum oligosaccharide concentration is soon reached. That concentration depends on factors such as membrane selectivity, temperature, pressure, fouling, and upstream processing, and typically lies between 2.0-2.5% (dry basis). The dynamics for a single stage have been discussed in Chapter 7.

By adding further stages to the process - either identical NF stages, NF stages with different characteristics, or other unit operations altogether – an improvement to the single stage maximum was sought. This chapter explores a few selected arrangements by modelling and experimental validation where possible, and evaluates them with reference to the final oligosaccharide purity, recovery, and economic viability.

8.2 Single Stage NF with Lactose Crystallisation

8.2.1 Description

Since lactose is the most concentrated component in mother liquor and it is difficult to separate from oligosaccharides by size exclusion methods, removal of lactose by crystallisation is an alternative worth considering. Crystallisation is a well-researched and relatively cheap method for lactose separation.

In this process the mother liquor would first be ultra-filtered for protein and fat removal, before an NF stage which is targeted purely at ash removal (contrary to previous setups where the NF stage was designed to remove both ash and lactose). Lactose crystallisation would take place after the UF and NF stage using a crystallising evaporator, as is currently done in lactose production. The quality of the crystals that form is of little importance, since food grade and pharmaceutical grade lactose are produced by separate processes and are not required from mother liquor. The completeness of their removal is important, however, as well as the minimisation of oligosaccharide content in the associated slurry.

8.2.2 Modelling

The mass balance for the process was determined using Excel and is shown in Appendix Section 11.3. A final OS concentration of 2.56% (dry basis) and a recovery of 85% were calculated. Since this system has no recycle loops, no simultaneous equations or optimisation procedures are required. Some of the key assumptions made in this model were that the rejection coefficients for the NF membrane were constant at 0.9, 0.7, and 0.1 for oligosaccharides, lactose, and ash respectively (as determined by experiments in Chapter 7), which in turn assumes uniform pressure and concentration conditions and negligible fouling. Final total solids of 15% for the retentate and steady state conditions for the feed tank were also specified, with no accumulation or change in concentration assumed. For the crystallisation step it was assumed that the lactose crystallised from the mother liquor was only limited by reaching a high total solids that prevents normal liquid pumping (about 50% TS for mother liquor). This corresponds to a lactose removal of 80%, which is similar to the most efficient industrial lactose crystallisation processes (Paterson, 2009).

8.2.3 Confirmation of Model Results

A pilot plant trial was designed and carried out to confirm the results found in the above model. Although a continuous, steady state process is described above, this is very difficult to achieve on a small scale, given the lack of automation and relatively small feed tank compared to hold-up volume. Instead, a simulated continuous process was used where the initial feed was first concentrated to the desired total solids (15%) by removing permeate and recycling retentate, before switching to a semi-continuous mode where about 100 mL fresh feed was added every minute and 10-20 mL of retentate was removed in the same time period. The addition ratio of fresh feed to retentate is based on the calculated recovery of the process, and should ensure constant total solids in the feed tank, as well as a constant tank level. A summary diagram is shown to explain the steady state setup in Figure 8.1. A refractometer was used to track total solids in the feed tank, as well as visual monitoring of the tank level, so that the feed addition rate and retentate collection rate could be modified if needed.

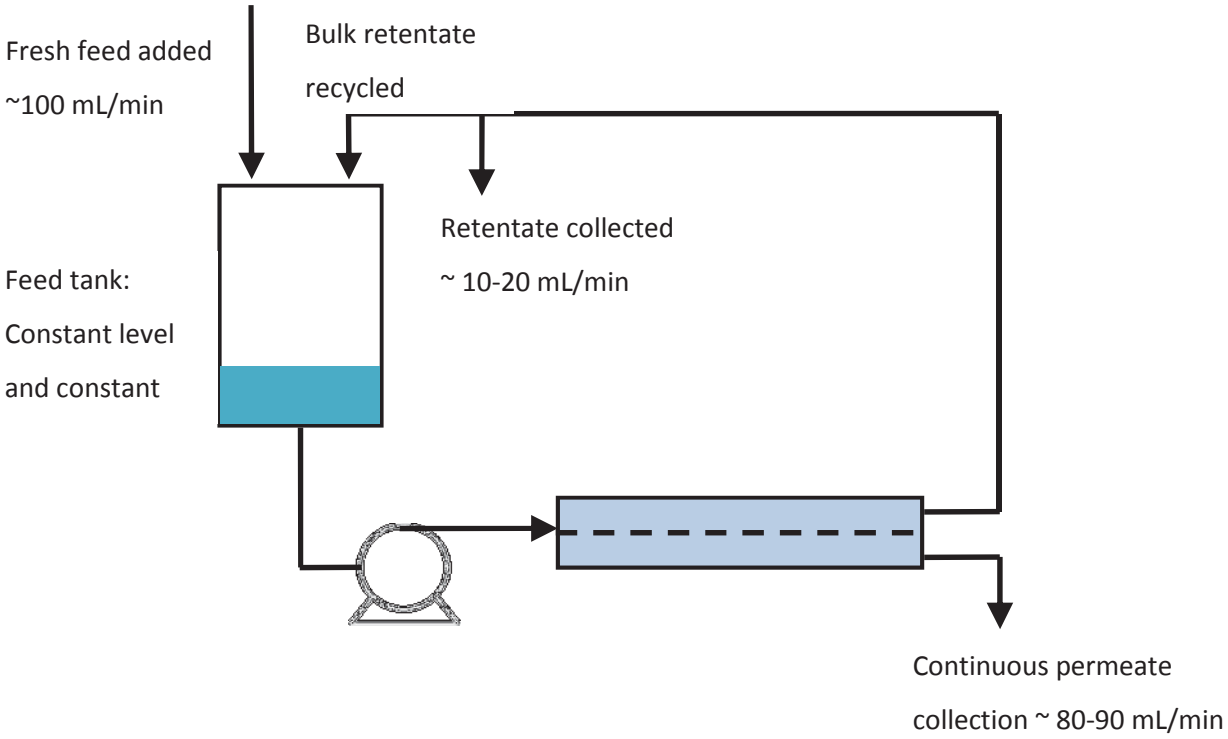

Figure 8.1: Diagram of Lab20 filtration plant set up to simulate continuous, steady state conditions

The mass of collected retentate was 327 g, which was then evaporated in an oven at 80 °C over 18 hours to increase the lactose concentration and begin crystallisation. This produced a large amount of sediment and 20.27 g of crystallisation mother liquor, which was separated by centrifugation (10 minutes at 600 *g*) and analysed by HPLC for total oligosaccharide content according to the method described in Section 5.2. The resulting oligosaccharide content, 2.4 ± 0.2% (dry basis), is a threefold improvement on the ultrafiltered mother liquor, but only matches the highest levels obtained using only nanofiltration.

The actual oligosaccharide content is slightly lower than that predicted in the model (2.56%), but still within the bounds of the experimental error. The assumptions made in order to simplify the model bring with them some uncertainty, including the assumption that 15% total solids in the retentate would be achieved, when in fact this varied between 13-15% over the course of the run.

There is also some uncertainty in the rejection coefficients used, which can fluctuate by around 10% from the average value due to fluctuating pressure and temperature. The small volume in the feed tank leads to pressure loss as air bubbles enter the pump, and temperature fluctuations in the service water for the heat exchanger are quickly transferred into the feed

tank, both of which significantly change the rejection coefficients for the components in the mother liquor.

8.2.4 Conclusion

Incorporating lactose crystallisation into the NF process gives a negligible improvement on the purity of oligosaccharides obtained by nanofiltration alone. This improvement is still not enough to be able to produce an oligosaccharide mix with high enough concentration to be used in infant formula (at least 4%).

8.3 Two Stage NF with Lactose Crystallisation

8.3.1 Description

Since there was a significant increase in oligosaccharide concentration by using lactose crystallisation, removing more of the mineral component by another stage of nanofiltration was investigated in the hope that oligosaccharide content could be increased further. Even with a low rejection coefficient (i.e. relatively unhindered flow through the membrane), the concentration of minerals in the retentate remains significant after one stage of NF because the liquid remaining in the retentate still has dissolved minerals in it. This is commonly overcome by what is called diafiltration, where fresh water is added to a retentate whose water content has been reduced by permeate removal to a level too low to enable effective filtration. The addition of fresh water allows filtration to continue, with further removal of small molecule impurities from the retentate. Although diafiltration can be carried out continuously within the same stage, it will be analysed here as two discrete stages.

Removing more mineral content means more lactose can be removed. The theory behind this (as discussed in Section 3.9.1) is that lactose will not crystallise in the presence of minerals above a critical concentration (about twice the concentration of lactose in solution), so by removing more mineral content, more lactose can be removed until that threshold is reached.

It is also important to note that because a tighter membrane can be used for ash removal, the recovery of the oligosaccharides is higher than in NF membrane processes designed to separate lactose. This means up to 4-5 stages could be incorporated with an overall oligosaccharide loss of less than 20%.

8.3.2 Modelling

As for the above case of a single stage NF followed by crystallisation, an Excel spreadsheet was set up to calculate the likely concentration of oligosaccharides in the final product (see Appendix 11.3). The conditions were the same as above for the first stage, and prior to the second stage of NF, water was added to the retentate to bring it back to the same moisture content as the initial feed (97%). The same assumptions as for the single stage model regarding rejection coefficients, recovery factors, lactose removed by crystallisation, and steady state, continuous processing conditions were also applied to the second stage.

As a result, a dry basis concentration of 2.96% oligosaccharides was predicted, with a recovery (yield) of 79%.

8.3.3 Confirmation of Model Results

The same Lab20 setup as that in Figure 8.1 above was used for producing a retentate for lactose crystallisation. In this case there was a second stage of NF processing, which was achieved by simply collecting the retentate from the first stage, adding water to bring to the required total solids level (3%), and starting a second run using this as feed on the next day (after CIP of the membrane according to the method in Section 7.3). This is representative of a typical industrial scale membrane filtration plant, where a series of membranes is used, the retentate from the first providing the feed to the second, and so on.

Retentate was crystallised at 80 °C in an oven for 18 hours, before being cooled to room temperature and centrifuged at 600 *g* for 10 minutes and the mother liquor analysed by HPLC, according to the method in Section 5.2. From 230.17 g of retentate, 11.39 g of mother liquor and 12.66 g of crystals were obtained (dry, unwashed), with the rest evaporated as water. The oligosaccharide content of the mother liquor by HPLC was 2.5 ±0.3% (dry basis).

The difference between this experimental value and that obtained through modelling (2.96%) is significant, so at this point the assumptions made were checked to see which are likely to be responsible for this. Firstly, as discussed with the single stage case above, the rejection coefficients are dependent on factors such as pressure, temperature, membrane fouling, and changing concentration, all of which are difficult to control in a pilot scale rig. Secondly, a constant retentate refractive index and tank level is difficult to maintain without automation

and this affects the recovery of each stage, in turn affecting the concentration of oligosaccharides in the retentate.

While the above may have a small to moderate effect on the final concentration, it is more likely that the lactose crystallisation stage is not removing as much lactose as possible. This is because there must be a point where the evaporation is stopped, the mixture is cooled, and the crystals are separated out, but it is very difficult to know whether that point is the optimal one. By evaporating further water, there may be more lactose crystallised, but there is also the danger of drying out the mixture too far when the proportion of moisture is already very low. In an industrial setting this would be easier to control as crystals are continuously removed and concentrations are always monitored, but in a lab experiment such as this, it is likely that more lactose remained in solution than necessary. The experimental results showed that only 12.66 g (61%) of the lactose was recovered as crystals, compared to the 70% assumed by the model. This suggests that the crystallisation was not as complete as it would be in a better controlled process.

8.3.4 Conclusion

Two stage NF with subsequent crystallisation improved the oligosaccharide concentration (dry basis) compared to single stage NF alone and a single stage NF with crystallisation. This may be able to be improved further using a more thorough crystallisation process.

8.4 Filter Series

8.4.1 Description

An alternative to crystallisation is to have a simpler series of filters in which the retentate from each stage is processed further, and the permeate from each stage is discarded. This avoids the complication of having recirculating flows, as is found with cascading systems (dealt with in Section 8.5). A clear disadvantage of this process is that the recovery of oligosaccharides is inherently lower, since in every stage there will be some fraction of oligosaccharides in the permeate which is not recovered. On the other hand, the purity of the final product is enhanced with each stage, and so a trade-off presents itself whereby for a lower recovery and higher operating cost, a higher purity of final product is attainable. The number of stages used depends on how important it is to attain high purity at the cost of higher losses. If there is an

abundance of feed compared to processing capacity, for instance, it is less important to have a high yield.

Since water is expressed in the permeate at each stage, it needs to be replaced prior to the next stage. This can be achieved either by using reverse osmosis to recover and reuse water from the outgoing permeate streams, or by fresh water addition.

8.4.2 Modelling

An Excel spreadsheet was set up to predict the resulting oligosaccharide purity of a series of membranes (see Appendix 11.3). Water was added into the feed for each stage except the first to bring the total solids content down to 3%. The recovery for each stage was set to 5%, which is close to the lowest possible recovery based on the requirement for retentate moisture being greater than 50%. Otherwise the same assumptions regarding rejection coefficients, recovery factors, and continuous steady state processing as above were applied. The purity and recovery of target oligosaccharides are presented in Table 8.1.

Table 8.1: Effect of multiple identical filtration stages on purity and recovery of oligosaccharides using 1 kDa NF.

Number of Stages	Oligosaccharide Purity (dry basis)	Oligosaccharide Recovery
0 (feed)	1.0%	100%
1	1.93%	90.5%
2	2.29%	81.9%
3	2.67%	74.1%
4	3.11%	67.1%
5	3.36%	60.7%

8.4.3 Discussion

As can be seen from Table 8.1, oligosaccharide purity increased steadily with the addition of each stage, with a corresponding decrease in recovery, although the fifth stage of filtration only increases the concentration by 0.25%. Together with the loss of up to 40% of the oligosaccharides in a process where recovery is important and the increased cost of pumping, membranes, and housings, this suggests that such an arrangement is not the most beneficial.

Model confirmation experiments were not carried out for this arrangement, since the initial predictions were poor. Experimental data from two stage filtration without lactose crystallisation shows that oligosaccharide concentrations are within 15% of the modelled second-stage level.

8.4.4 Conclusion

Multiple stages of membrane filtration without recovery or lactose crystallisation will theoretically result in too much loss of the target oligosaccharides to be considered for an arrangement for enrichment. Fewer stages of nanofiltration with a lactose crystallisation stage give a much more cost effective and efficient separation.

8.5 Counter-Current Membrane Cascade

8.5.1 Description

To overcome the problem of yield reduction over a number of stages, a membrane cascade has been incorporated into processes in some industries, as described by Vanneste et al. (2011). Originating in the petro-chemical industry, membrane cascades have been applied to sugar separations in more recent years. A membrane cascade uses membrane filtration to achieve a counter-current separation between solutes, as is common by other separation processes in distillation and gas-liquid contacting columns. The key difference between counter-current separations using membranes and those using columns is that with membranes, each stage is discrete and can be controlled independently, whereas conditions within a column stage are specified by the overall process.

Figure 8.2 shows the most basic membrane cascade, a three stage “one up, one down” arrangement. The feed enters the middle of the three membranes, and the permeate is directed to the lower stage while the retentate is directed to the higher stage. The lower stage is thus richer than the feed in more permeable compounds, in this case ash and lactose, while the higher stage is richer in the more retained compound (oligosaccharides). From the lower stage, the retentate is returned to the central stage and mixed with the feed, while the permeate forms a final waste stream (in this case). The permeate stream from the higher stage is returned to the central stage, while the retentate is the final enriched oligosaccharide stream.

This arrangement can be extended to more than three stages, depending on whether greater purity or recovery are needed. The effect of adding downstream stages is primarily to increase the *recovery* of oligosaccharides in the system. This happens because in the permeate from the feed stage, some oligosaccharides, although a depleted fraction, remain. Without further stages to recover these, they would be lost in the waste. In subsequent downstream stages, oligosaccharides will preferentially be retained and will be gradually carried upstream through the system. The final permeate is ideally free from oligosaccharides and contains water, lactose, and ash. The number of stages is limited only by cost and practicality, and the limited increase to purity and recovery from adding extra stages will render the process uneconomical at some point.

The number of upstream stages of a membrane cascade has a direct bearing on the *purity* of the final retentate, since lactose and ash that was retained in the first stage can be removed by subsequent stages to increase the proportion of oligosaccharides. The upstream stages may therefore be called *retentate enriching*, whilst the downstream stages are *permeate stripping*. The number of stages required of each will be the product of purity and recovery requirements and process economic calculations.

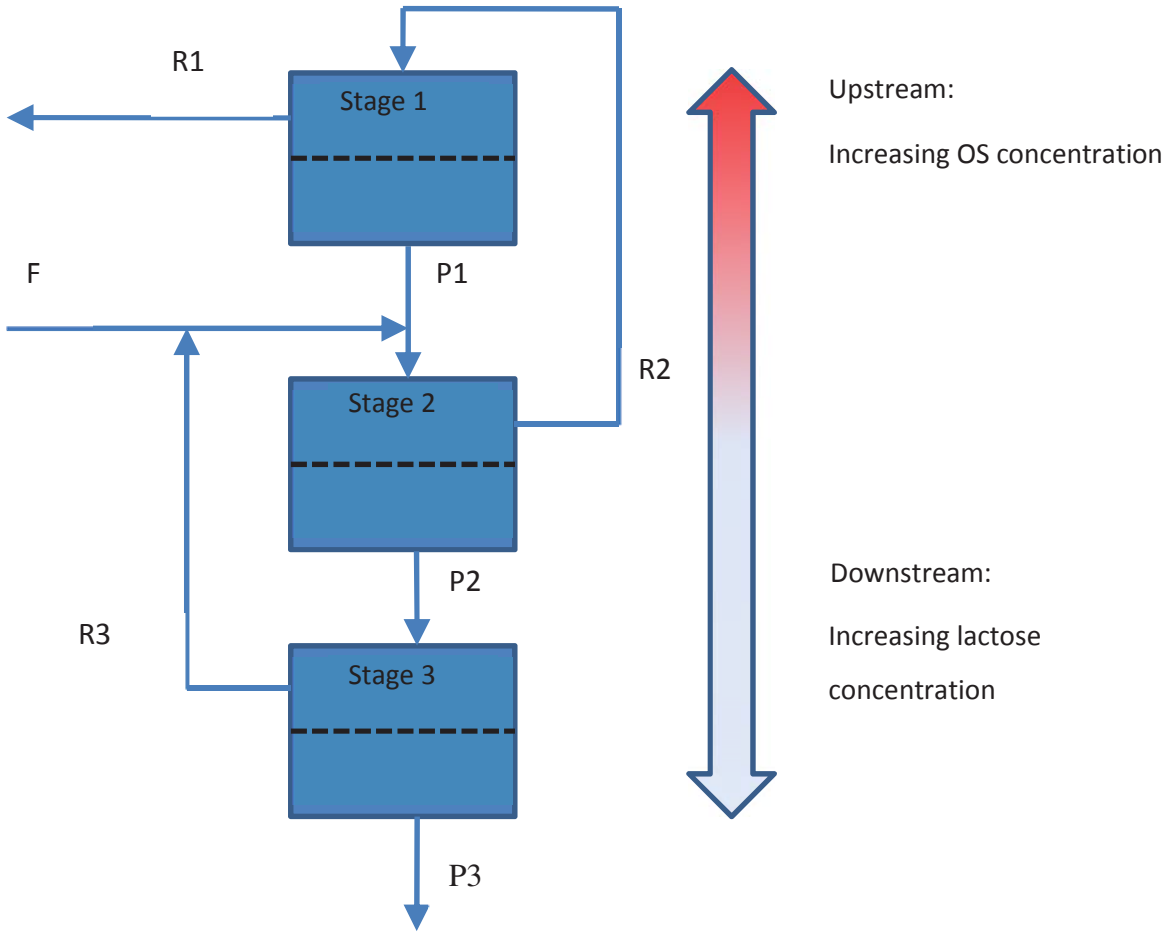

Figure 8.2: Basic membrane cascade setup with a "one-up, one-down" arrangement for oligosaccharide separation. F = Feed, R = Retentate, P = Permeate. Stages with lower numbers than the feed stage are referred to as upstream stages or retentate enriching stages, whilst those with higher numbers are downstream stages or permeate stripping stages for simplicity.

For the purpose of this work, final oligosaccharide purity is more important than recovery, since there is a target purity (4% dry basis) that needs to be attained. Once this purity is reached, recovery becomes more important in order to boost product output and reduce the per kilogram cost.

The rejection coefficients, as discussed regarding single stage NF are still important in each stage of a membrane cascade. Another important factor to understand in dealing with membrane cascades is that of *stage cut*. Stage cut is defined as the proportion of the feed flow rate which is taken as retentate, and can be set for each stage, n, separately:

$$SC_n = \frac{Q_{Rn}}{Q_{Fn}} \qquad \text{(Equation 8.1)}$$

where SC is the cut for stage *n*, Q_{Rn} is the retentate flow from stage *n*, and Q_{Fn} is the feed flow to the stage in kgs^{-1}

The effect of the stage cut is to determine the upstream and downstream flows between each stage: Small stage cuts indicate the bulk of mass moving downstream in the permeate streams, whilst large stage cuts indicate bulk upstream movement, towards the final retentate. Stage cuts are best determined by optimisation studies and are heavily dependent on the relative starting concentration of the more retained compounds to that of the less retained compounds. For this situation very small stage cuts are required, since the oligosaccharide concentration is around 1/100 of the combined ash and lactose concentration. The proportion of flow rate of final retentate to final permeate should be roughly the same as the proportion of oligosaccharides in the feed.

8.5.2 Modelling

To assess the suitability of using membrane cascades for the separation of oligosaccharides from mother liquor, a model of a simple three stage cascade was developed based on a mass balance around each stage. The feed for the central stage *n* is the sum of the retentate from the downstream stage, *n*+1, the permeate from the upstream stage, *n*-1, and the initial process feed. Similarly, the feed for the upstream stage is the retentate from the central stage, and the feed for the downstream stage is the permeate from the central stage. The concentrations in the feed are specified by component balances from the incoming streams.

The concentration in the permeate of any stage was found by rearranging the formula for the rejection coefficient, and substituting in the rejection coefficients found in single stage study (see Chapter 7):

$$C_{Pi} = (1 - R_i)C_{Fi} \qquad \text{(Equation 8.2)}$$

where C_{Pi} and C_{Fi} are the concentrations of component *i* in the permeate and feed in gL^{-1}, respectively, and R_i is its rejection coefficient.

With the concentrations of each component calculated for the feed and permeate, the last requirement is to determine the total flow rate in the permeate, which can be simply calculated as:

$$Q_P = Q_F \times SC \qquad \text{(Equation 8.3)}$$

The component flows in the permeate are then calculated as the product of the total permeate flow and the component concentration. With the feed and permeate conditions fully specified, the retentate flow is the difference between the two, assuming a steady state process.

Because the feed to each stage is at least partially specified by the outputs from a previous stage, there arises a complex system of simultaneous equations (see Appendix 11.4) which must be solved by an appropriate software package. The equations were first entered into an Excel spreadsheet, beginning with the central stage (initially assuming zero values for streams from other stages) before calculating the downstream and upstream stage conditions.

The rejection coefficients were fixed at 0.9, 0.7, and 0.1 for oligosaccharides, lactose, and ash, respectively, which, for simplicity, were set as the same for every stage. These values were ones commonly seen in filtration experiments using a single NF membrane (see Chapter 7). The stage cut of each stage was set initially to 0.5, although this was later decreased in optimisation studies.

Setting the Excel spreadsheet to enable iterative calculation (maximum of 100 calculations with a tolerance of 0.001), the spreadsheet was calculated and returned feasible answers. A final purity cell was added so that the oligosaccharide concentration could be calculated on a water-free basis, using the equation:

$$P_{OS} = \frac{X_{OS}}{1 - X_w} \qquad \text{(Equation 8.4)}$$

where X_i is the mass fraction of component i and P_{OS} is the purity of oligosaccharides (usually expressed as a percentage). This final purity is the important measure that needs to be optimised, but this optimisation relies on the stage cut for each stage – in this case three variables, which Excel does not have the functionality within the solver algorithm to handle. There is the possibility of optimising with regard to each factor individually, but this is time

consuming and assumes there is no interaction between these variables. Furthermore, when more stages are added, as would be expected when the system is well understood and the best arrangement is sought, the degrees of freedom increase accordingly and successful optimisation becomes complicated.

To find an optimised solution, Engineering Equation Solver (EES), which includes a multi-variable optimisation tool, was utilised. EES requires a series of equations to be listed in a file which are then solved simultaneously, and the order the equations are entered is not important. The equations used are listed in Appendix 11.4. For a specific solution, an equal number of equations and unknown variables must be provided, whereas for optimisation fewer equations than variables are required. Guesses can be entered for each variable and bounds set, which in this case include restricting the outputs to positive numbers and setting the minimum water content in process streams to 50%, which is a requirement for continued fluid flow.

The optimisation dialog box gives two options, either direct search method, or variable metric method. These options were used alternately, and the best optimisation taken, noting it was not always the same method giving the best result in each case.

It is important to remember that using an optimisation function is not guaranteed to actually find the optimum, especially when three or more factors are involved. To illustrate this, all the possible solutions to the model based on different input variables can be imagined as a response surface in 3-dimensional space, with various peaks, troughs, saddles, valleys, and degrees of gradients. The optimisation function will generally follow an “uphill” path as far as it can, moving along the various axes as needed, until it reaches a peak. This peak represents a local maximum of the optimised variable (in this case a maximum oligosaccharide concentration), but this may not be a global maximum; there may be other higher peaks that lie close to the current one, or other peaks that are much further away. It is impossible to know where all of these peaks are, and how many exist, without systematically calculating the output value for every possible combination of input variables.

With three stages, hence three different recovery values, and a system of linear equations, it can be reasonably assumed that there will be only one or two maxima and the plot will be mostly uniform. As further stages are added, however, this cannot be expected, and the best

strategy is to change the starting guess values for optimisation to determine whether other maxima are present.

8.5.3 Modelling Results

Table 8.2 shows the results of using EES with the above method to optimise a three stage counter current cascade. A final (dry) concentration of 1.867% was obtained. 1600 function calls with a relative tolerance of $1x10^{-4}$ were specified. Relative tolerance is a measure of the error allowable in the final calculated value relative to the value itself. This figure was checked for errors using a Microsoft Excel spreadsheet with the same values for the recovery of each stage, and found to be accurate.

Interestingly, the final value is actually lower than that obtained from a single stage of filtration as we saw in the previous chapter, which from experiments was around 2-2.5%. The model for a filter series, in Section 8.4, also gives a higher concentration than this. This implies that a three stage cascade in this case is not achieving any further separation. Some possible reasons for this are discussed here.

Table 8.2: Initial guesses and optimised values for membrane cascade modelling parameters

Parameter	Initial Guess / Value	Final Value
SC1	50%	23.3%
SC2	50%	15.6%
SC3	50%	1.3%
Final OS purity	0.61%	1.867%

A difficulty in working with membrane cascades, and a contributing reason for there being no improvement in oligosaccharide concentration, is that of solvent management. This arises because at each stage, water is the most permeable compound present within the solution and so the concentration of water is always higher in the permeate than in the retentate, and this is exacerbated for systems where the rejection coefficients of the other components are

relatively high, especially in the upstream stages of a cascade where the retentates from previous stages are used. High stage cuts at each stage also mean that the retentate has had most of its water removed prior to entering the higher stage. Evidence of this lack of water is that during the optimisation process, in all cases the water component (X_w) of the final retentate was constrained to the minimum possible value – either zero, if a non-negative constraint was in place, or in this case 0.5, to maintain fluid flow.

A few potential solutions to the lack of solvent can be seen, and results are presented in Table 8.3. One is to simply add fresh water into each upstream stage to replace what has been lost in the previous stage – in the case of a three stage cascade, adding water to the top stage, seeing as the middle and lower stages have sufficient water from the feed to be able to function properly. A simpler method would be to dilute the feed prior to introducing it to the cascade.

Table 8.3: Adjustments made to membrane cascade model and resulting changes

Change Made	Final Optimised Oligosaccharide Purity
Increase water component of feed to 99.9%	1.9517%
Use RO to return water from permeate to make up retentate stream to 97% water	1.9557%
Add fresh water to make upstream stage feed to 97% water	1.9469%
Single stage (for comparison)	1.9476%

Another solution is to use reverse osmosis (RO) membranes to dewater the permeate, and to use this water as make-up water for the upstream stage. The disadvantage here is that an RO process uses further operational and capital costs, but the advantage is a dewatered waste stream (permeate) which will be easier to deal with if further waste processing is required.

These approaches were each incorporated into the existing model and the optimisation procedure above was repeated to assess whether they would improve the final concentration to higher than what is achieved with a single stage. As can be seen from Table 8.3, the increase in oligosaccharide purity is minimal, and only marginally better than that achieved from a single stage. This might seem contradictory, since more stages are used and there is no improvement, but this indicates that a membrane cascade is simply not a useful method for oligosaccharide separation.

The reason for this is that the concentration of oligosaccharides is so low to begin with that the fraction of retentate compared to permeate at each stage has to be very small. With such a concentration of the solids in the retentate, the water content very quickly reaches its minimum constraint, and even with addition of fresh water in the upstream stages this is not significantly improved.

8.5.4 Conclusion

Although on paper, the recovery and purity of an integrated cascade of this type is impressive, modelling shows that it cannot achieve a separation any better than a single stage of NF with an oligosaccharide concentration so low in comparison to the other components. A membrane cascade is not a recommended process.

8.6 Overall Conclusion

With the current nanofiltration membrane technology it is very difficult to separate compounds of similar molecular weight, and single stage filtration trials gave modest increases in oligosaccharide concentration. Some selected arrangements have been investigated for overcoming this limitation, including multiple stages of nanofiltration and lactose crystallisation.

While multiple stages of filtration gave an increase in purity, it also led to significant losses in yield when recovery stages were not used. A membrane cascade, using recovery stages to increase yield, was constrained by low water contents and did not give an increase in purity.

A two-stage nanofiltration process with subsequent lactose removal by crystallisation gave the best increase in purity, up to 3% oligosaccharides (dry basis) from a starting concentration of 0.5%. This came with a yield of 79%, although this could be improved by using a tighter membrane which targets ash removal rather than lactose. Experimental validation of the model result gave a lower value (2.06%) which is most likely a result of lab based lactose crystallisation which was not as complete as the processes used in industrial scale crystallisation. Experimental error and batch simulation of continuous processing also contributed to this discrepancy.

Even this increase in oligosaccharide concentration is not enough to create an enriched stream which could be added to infant formula, which requires 4% oligosaccharides on a dry basis to reach the same ratio of oligosaccharides to lactose that is found in mothers' milk. Until membrane technology progresses to a point where such fine separations can be carried out, membrane processes are not suited to this process.

Simulated moving bed chromatography, another process using size-based separation, is covered in the following chapter.

9 SIMULATED MOVING BED CHROMATOGRAPHY

9.1 Introduction

Chapter 4 led to the selection of three processing options that gave a good projected internal rate of return and were expected to meet the minimum oligosaccharide content. Of these three, the best was simulated moving bed (SMB) chromatography. Nanofiltration, either in a standalone process or as part of a series including lactose crystallisation or other nanofiltration stages, was shown to give only a modest enrichment of oligosaccharides (final purity less than 4% in all cases) in Chapters 7 and 8. By contrast, according to literature covered in Section 3.9.7, the SMB process can give complete separation of components very similar in molecular weight. It is also highly adaptable to different species, is compact and can deliver high flow rates, and has already been successfully used in separation of oligosaccharides from human milk (Geisser et al., 2005). It is, however, more expensive than nanofiltration and lactose crystallisation because of the cost of chromatographic resin.

Although SMB is common in some industries, it has had limited application in the dairy industry, and the predictions made in Chapter 4 have considerably more uncertainty associated with the process than those for filtration and other operations. This chapter will give a full overview of the technology and its principles, then show the evaluation process for different resins and how the chosen resin was applied to a pilot scale process with results. Final oligosaccharide purity and yield are used to evaluate results as in previous chapters.

9.2 Size Exclusion Principles

Given that the process requires a separation based on size differences, size exclusion chromatography (SEC) was identified as the most suitable solid phase. Size based separation can be achieved using other types of resin (for example cation exchange in HPLC sugar separations (Geisser et al., 2005).

An important requirement for SMB chromatography is a net continuous movement of all solutes through the columns, without permanent binding. The velocity varies with each species but for each species it must be uniform. This is because the feed and solvent are introduced and extract and raffinate are drawn out at constant rates. This in turn requires that the interaction between the solutes, resin and solvent not be of a “bind and elute” type where

a second solvent type or change in concentration is needed for the elution of solutes that were bound under the first solvent (as is common in solid phase extraction). Instead, one solvent is used for the entire process. Continuous, cyclical steady state operation is critical for prediction and control of the SMB process.

Such a requirement does not necessarily rule out the use of certain resin types, but the interaction between the solutes and solid phase needs to be understood and an appropriate system chosen. An equilibrium function, or in most cases an adsorption isotherm is used to describe the concentration of bound solutes as a function of their concentration in the mobile phase, and can vary for each interaction type. The different chromatography media and the resulting interaction between solutes and solid phase will be outlined briefly here, and size exclusion media will be discussed in more detail.

1) Adsorption: Solutes or gas particles adhere to the surface of the solid phase. The equilibrium between the solid phase and liquid (mobile) phase can usually be described by one of the common adsorption isotherms (Henry's, Langmuir, Anti-Langmuir, Freundlich, GAB, BET, Kisliuk, or mixed), depending on the exact nature of the interaction, and accounts for the different retention times of each solute. The adsorption process can be either continuous, or of a bind and elute mechanism (Geankoplis, 1993).
2) Partition chromatography: Solutes are in equilibrium between the mobile phase and a thin film of liquid which coats the solid phase. This type is not commonly used in the separation of sugars (Geankoplis, 1993).
3) Ion exchange: Solutes are electro-statically attracted to the solid phase surfaces with the opposite charge. Commonly used in sugar analysis, differentiating on the number of OH groups and any acidic groups. The process can be either continuous, or bind and elute (Geankoplis, 1993).
4) Affinity chromatography: A very specific type of chromatography for proteins where a specific interaction between the target molecule and an immobilised receptor molecule takes place (e.g. antibodies and antigens). Not used in sugars separation (Geankoplis, 1993).

Size exclusion is different from all of the above because there is (theoretically) no interaction between the solid phase and the solutes. Factors other than size, such as molecular charge,

reactivity or affinity for certain groups are largely irrelevant and play no part in the separation. Manufacturers design the media to be inert and to offer no binding sites to solutes. Although in practice this is difficult to achieve completely, and some groups can attach to the resin, in general the effect of this is negligible. Examples of resin types include hydroxylated methacrylic polymers (Toyopearl®), cross-linked dextran with epichlorohydrin (Sephadex™), composites of cross-linked agarose and dextran (Superdex®), or hydrophilic copolymer with amide and hydroxymethyl groups (Trisacryl®).

Separation in a size exclusion resin occurs because the pores in the solid phase allow the mobile phase to flow through but exclude molecules too big to enter the pores (steric hindrance). Small molecules which can flow through the smallest pores in the resin have the greatest volume to flow through (i.e. inter-particle spaces + all internal pore spaces) and elute last. Large molecules, too large to flow through the pores within the solid phase, have the smallest volume to flow through (i.e. only inter-particle spaces) and elute first. The separation is thus determined by a particular species' available void fraction, which increases with decreasing solute size. Between the largest and smallest particles is a continuum of sizes and elution times which provides a separation across a certain size range. The extent of the separation range depends on the size of the largest and smallest pores in the solid phase. It is also important to note that the size of a species is not solely dependent on molecular weight, and the hydrodynamic size is partly determined by the pH conditions and ionic strength of the solution, meaning that molecules similar in molecular weight could have different hydrodynamic sizes and thus different elution times.

Figure 9.1 illustrates the size exclusion principle as a matrix of resin with pores within each gel particle. The large molecules are too large to pass through the small pores but freely pass through the gaps between particles. In these spaces between the particles, their concentration relative to the solvent is the same as that in the feed, since the solvent has no preference for either the small pores or large pores and all of the large molecules must travel through the large pores. Since the larger molecules cannot pass through the small pores, their concentration there is zero.

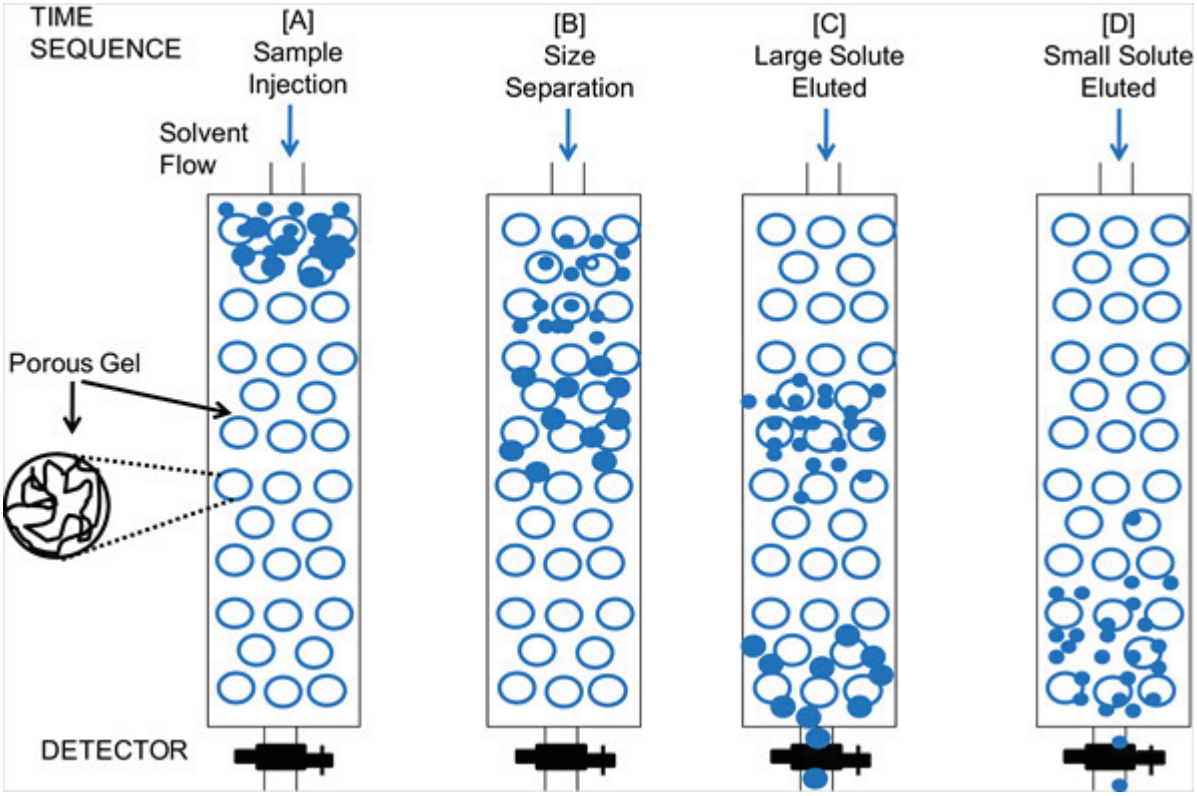

Figure 9.1: Sequence of separation by SEC showing smaller particles being retained in the pores and larger molecules passing through more quickly.

In the case of the small molecules, which are able to travel through any of the pores, their concentration in all pores is the same as that in the feed, relative to the solvent concentration.

Since the probability of a solute being able to enter a given pore is dependent purely on its size and not on the behaviour of the other solutes (as is usually the case with adsorption or affinity chromatography), the equilibrium between the concentration of a given solute in the mobile phase and that in the pores of the solid phase is linear. The same mobile phase is present in both cases and the solid phase does not restrict the passage of solutes which are small enough to fit through the pores, even at high concentrations.

In the case where the solute was very close in size to the pore it was trying to pass through, then the molecule's passage could be described by a probability of it making it through the pore, as it would depend on random factors such as its orientation at the time it reaches the pore or its spatial alignment with the pore. This is analogous to membrane filtration, where a "rejection coefficient" is given for a species that is not fully rejected or allowed through the membrane. In these cases, the concentration in the pore may not be equal to that in the

larger pores, but the relationship is still linear, assuming convection is strong enough to prevent concentration polarisation at the pore entries (Fütterer, 2009).

Using this model, it can be inferred that under uniform conditions, there is a linear relationship for each solute between the concentrations in each part of the column, which means that for a SMB process a linear model can be used. The assumptions in drawing this conclusion are that the solid phase does not interact with the solvent or solutes, and that entry into a pore is not determined by the concentration of solutes.

9.3 SMB Principles

SMB is an adaption of chromatography for continuous binary separation, and is finding new applications at both small and large scales. Instead of the feed solution passing through a single column for separation and collection or detection of the different components, a series of between 3 and 16 columns is connected in a loop with feed and eluent inputs and raffinate and extract outputs. The principle is best understood by considering a hypothetical "true moving bed" as shown in Figure 9.2. In a true moving bed, the chromatography medium is imagined as filling a continuous loop and moving in a counter-clockwise direction at a controlled flow rate.

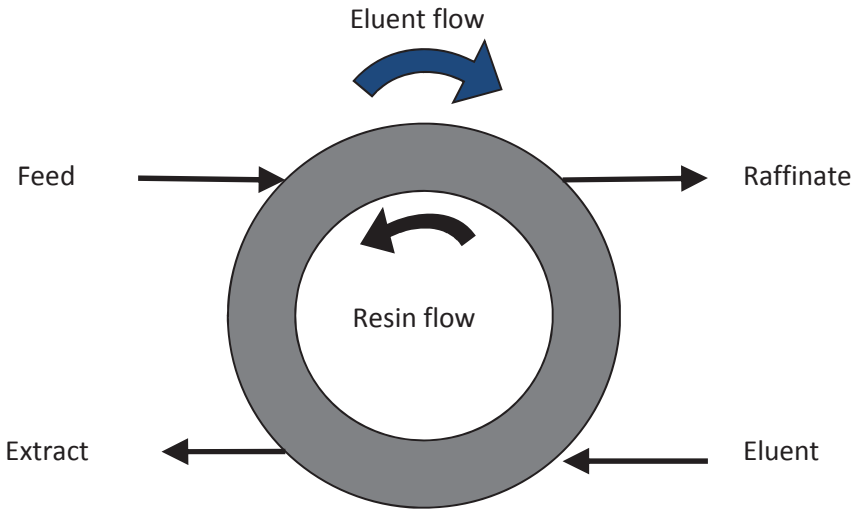

Figure 9.2: Hypothetical true moving bed setup. See text for full explanation.

Eluent is introduced at one of the ports and is pumped in a clockwise direction, against the

flow of the resin. When the feed, containing the components to be separated, enters the system, those components with a stronger affinity or adhesion to the resin (or in the case of size exclusion, a more tortuous path to follow through the resin) will move in a counter clockwise direction, whereas those with a weaker affinity for the resin will be more affected by the eluent flow and will move in the clockwise direction. The extract outlet then collects the more retained compounds whilst the raffinate outlet collects the less retained compounds. By altering the flow rates of the resin, eluent, and feed, in most cases the process can be tailored to give pure extract or raffinate, or both (Fütterer, 2009).

A true moving bed is a physical impossibility, since the increasing pressure drop created by the movement of resin against an eluent flow would be impossible to manage given the small pore sizes and slow permeation rates. Instead of having an actual "moving bed", a simulated moving bed is used. Figure 9.3 shows a four-column SMB setup. This is achieved by having a stationary solid phase within a number of columns, and a periodic switching of the inlet and outlet ports by one column length in the same direction as the eluent. This means that although the resin is not actually moved inside the system, from the point of view of any of the four ports, the resin periodically moves in the opposite direction from the eluent. This periodic switching is of course not exactly the same as a continuous movement, but by adding more columns and switching more frequently, the process becomes closer and closer to steady state. In practice, only 6-8 columns are needed to approach 100% purity and recovery for most applications (Amalgamated Research Inc., 2008).

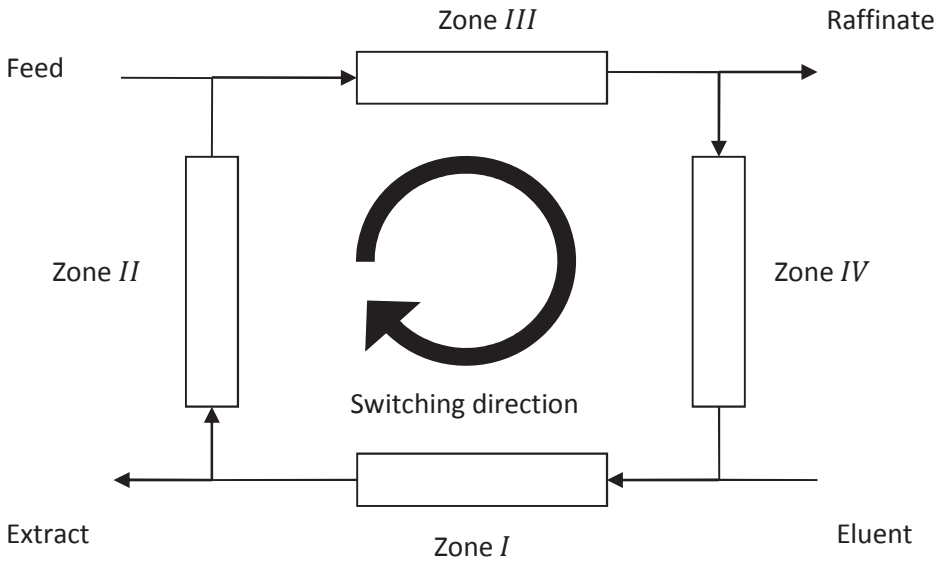

Figure 9.3: Simulated moving bed setup

The simulated moving bed system is divided into three or four zones, each containing at least one column, which moves with each switch according to the inlet and outlet ports. Each zone has a distinct purpose in the separation.

Zones II and III, immediately before and after the feed inlet, are tasked with the separation of oligosaccharides from lactose and other smaller molecules. Since the smaller molecules are more retained in size exclusion chromatography (see Section 9.2 above for a discussion on separation basis), they move through the columns more slowly and are eventually found in the extract. Oligosaccharides are less retained and move more quickly through the resin to be found in the raffinate. For both of these requirements to be met, the flow rates of zones II and III need to be such that the oligosaccharides travel a distance of more than one column length in the time between switches, but lactose and minerals travel less than a column length during the same time period. This means that, regardless of which time point during the switching period the components in the feed are released, they will always eventually be found in the correct outlet stream.

Furthermore, zone II should have a slightly lower flow rate than zone III (but still within the constraints above) so that the components within the two zones can move more quickly towards their respective outlets and more feed can be introduced to make up the difference. The greater the difference in flow rates between zone II and III, the higher the feed flow rate

and the higher the productivity, though this can lead to incomplete separation where the components of interest are close in retention time.

Zone I has the highest flow rate and has the task of flushing all remaining components into the extract stream and thereby regenerating the column. After the next switch, the column(s) in zone I become zone IV, which needs to be free from extract compounds. Zone IV has the lowest flow rate and collects all of the material that exits from zone III without being taken in the raffinate. Zone IV is often referred to as the solvent regeneration or solvent recycling step, since the end product is free from all solutes and is feed into zone I. Zone IV is the only zone whose inclusion is optional, since recovery of solvent is not always required, especially when water is used and the scale is small. In this work all four zones are used, with one column in each zone.

The switching time is another crucial parameter in the process and must be set and closely controlled to ensure that the components are found in the correct streams as described here and that the columns run reproducibly. To calculate the flow rate for each zone and the switching time, it is first necessary to decide which resin is the most appropriate for this application, then to obtain parameters from single column data using the chosen resin and column size so that the SMB set points can be determined.

9.4 Resin Screening

9.4.1 Introduction

The degree of separation and productivity of the SMB process is heavily dependent on the type of resin used. The differences between resin types can come from the different polymers used to construct the resin structure and the degree of cross-linking, as well as physical factors such as the gel's rigidity and bead size. The pores within the resin can be homogenous, as is the case with traditional style products, or can have a range of pore sizes, which is a more recent development and allows separation of a greater range of molecular weights and higher selectivity. The goal of resin screening is to test a range of resins in a simple single column elution to determine which is most suitable for the intended separation (Geankoplis, 1993).

Suitability is a product of a number of different factors. The most important of these is the peak resolution, which is the ability to separate the signal of two different species. The formula for resolution is:

$$R = \frac{(t_{r2} - t_{r1})}{1/2(w_1 + w_2)} \qquad \text{(Equation 9.1)}$$

where t_r and w are the time points and widths of two adjacent peaks. This effectively gives the quality of separation, so the higher the resolution between oligosaccharides and lactose (the closest species in molecular size), the more suitable the resin is for this separation (Azevedo & Rodrigues, 2006).

Other factors that need to be considered are the maximum flow rate, which is in turn determined by the bead size, compressibility, and pressure limit, and resin cost and lifetime. Maximum flow rate and resin cost can be found easily, though the lifetime of the resin cannot. In this case, anecdotal evidence from other studies will provide some guidance although uncertainty will still remain until an automated process is set up.

Hydrophobicity is also important in size exclusion gels, as any protein fragments that may be in the feed will bind to hydrophilic groups, reducing resin lifetime and increasing cleaning time and cost. Similarly, resistance to osmotic shock is desirable, as the feed is high in mineral content and ionic strength, which could alter the hydration of the resin beads.

It is important to note that the resolution and other measures achieved in a resin screening will not be directly transferrable to a full scale SMB setup, since the number of columns and their length can be different and operating conditions (flow rate, injection volume, and pressure) are difficult to predict at this stage and are unlikely to be the same. The goal is to qualitatively determine which of the resins is *most* appropriate, and to proceed with more rigorous, quantified testing with the chosen resin.

9.4.2 Experimental

Four different size exclusion resins were tested for their ability to resolve oligosaccharides from lactose. These resins were selected for testing based on having an appropriate separation range (between 200 and 1000 Da), a large particle size for process scale and low pressure operation (larger than 100 µm diameter hydrated bead size, except for Superdex 30),

and recommendations from literature (see Section 3.9.7). The resins chosen and their manufacturer specifications are shown in Table 9.1.

Table 9.1: Manufacturer data for size exclusion resins screened in FPLC

Resin	Hydrated Bead Size (µm)	Separation Range (Da)	Pressure drop (bar/m)
Trisacryl GF-05 M	80 – 160	200 - 2500	3
Sephadex G-25 Coarse	170 – 520	100 - 5000	Unspecified
Toyopearl HW40-C	50-100	100 - 7000	3
Superdex 30 (Prep grade)	24-44*	0 - 10 000	5

*Dry bead size; no hydrated size given.

An Akta Explorer FPLC (Fast Protein Liquid Chromatography) system was used to test each resin. Resins were ordered as dry beads or slurries and then packed using the slurry method at constant pressure into 70 cm x 16 mm I.D. columns (GE Healthcare, Auckland NZ) for connection to the FPLC system. These columns are much thinner than those likely to be used in an industrial application, which affects the through put (which is a related to surface area) and also introduces wall effects that could stagger elution times and lead to lower resolution and purity.

For each resin, the column was first equilibrated with 2-3 column volumes of demineralised water until a constant UV signal was obtained, then for the first trial a 2 mL injection of sugar standard (0.15 g/L maltotriose, 1 g/L lactose, and 0.5 g/L galactose) was made directly onto the column using the sample pump. Eluent flow was at the highest solvent flow that pressure restrictions allow (2- 4 mL/min, depending on resin type) with Milli-Q water as eluent. The injection volume and flow rate were both decreased for subsequent runs, and the difference (if any) in peak resolution using UV detection at both 197 and 214 nm was noted. If the chromatogram showed better resolution between peaks with a lower flow rate or injection volume (down to a minimum of 1 mL/min and 0.5 mL injection), this was considered optimal for the given resin. If there was no increase in resolution by reducing flow rate and injection volume, the initial run with larger injection and faster flow rate was considered best, for productivity reasons.

Samples of clarified and ultra-filtered (10 kDa) mother liquor, prepared as described in Section 7.3.2, were also run on each column to confirm the resolution of oligosaccharides from the

other sugars. These samples were injected at the same volume and flow rate that was used in the injection which gave the best resolution. The concentration of sugars in the mother liquor was approximately the same as that in the sugar standard (0.15 g/L maltotriose, 1 g/L lactose, and 0.5 g/L galactose) for easier comparison.

Fractions from mother liquor runs were collected in a repeat run of the best conditions for each resin using an automatic fraction collector. To keep the number of fractions manageable, 2 mL fractions were only taken for the 30-40 mL eluted either side of the oligosaccharide and lactose peaks, and 5 or 10 mL fractions were taken over the remainder of the elution. The fractions were analysed by HPLC, using the method in Section 5.2, although this time without dilution because of the dilution already inherent in chromatography. HPLC results were used to confirm the elution time of the peaks as recorded by the UV detector and give an indication of the concentrations in the samples.

9.4.3 Results

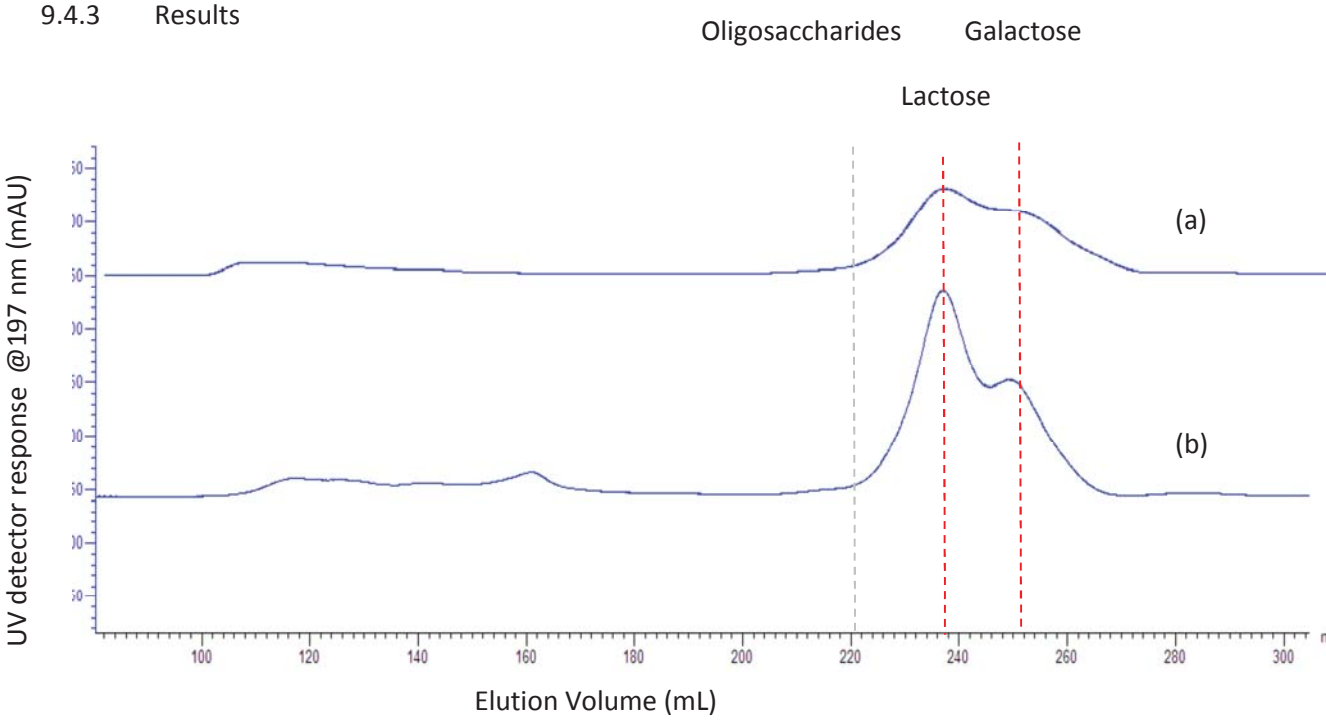

Figure 9.4: Chromatograms for Trisacryl GF05 with standard sugar solution at (a) 2 mL/min, 1 mL injection (with vertical offset) and (b) 1 mL injection, at 0.5 mL/min on a 700x16 mm column.

Trisacryl GF05

A chromatogram comparison is shown in Figure 9.4, where injections of 1 and 2 mL of standard sugar solution were made onto Trisacryl GF05. The eluted peaks are marked,

including the approximate location of the oligosaccharide peak if it were present. Note that the 2 mL injection has markedly poorer resolution between the lactose and galactose than the 1 mL injection. The reason for this is that the increased injection volume means that the sample fills a greater portion of the column length, and without the necessary degree of separation the components eluted last from the beginning of the injection will be eluted at the same time as the first components from the end of the injection. This can be overcome by using wider columns, giving more cross sectional area and therefore less sample height in the column, or by extending the length to allow a more complete separation.

The oligosaccharide peak is not visible in either chromatogram. Further trials with this resin, including injections of standards and mother liquor sample at lower eluent flow rates, showed that there was no resolution between the oligosaccharide and lactose peaks under any conditions, and that the resin was therefore not suitable. This is a surprising result, given the manufacturer's claim of effective separation between 200 and 2500 Da. A reason for this could be that the pore size distribution is too broad, tailoring this resin towards applications where solutes have a large difference in size rather than a separation like this.

Sephadex G-25

The relevant portion of the chromatogram of a 2 mL injection of standards (same mix as above) at 2 mL/min on Sephadex G-25 is shown in Figure 9.5. As with Trisacryl GF-05, there is no clear oligosaccharide peak under these conditions with Sephadex G-25. Resolution between lactose and galactose was also poor, suggesting that there would need to be a large improvement in resin performance for an oligosaccharide peak to be distinguished. The final peak (at about 260 mL elution volume) is most likely from a small molecule impurity, such as inorganic minerals, in the injection standard.

Injecting standards at smaller volumes and lower flow rates (minimum 0.5 mL injection at 1 mL/min) failed to improve the separation. Samples of ultra-filtered mother liquor gave similar results, with lactose, monosaccharides, and oligosaccharides not resolved into distinct peaks. As with Trisacryl GF05, the pore distribution may not be appropriate for a high resolution separation of molecules close in molecular size.

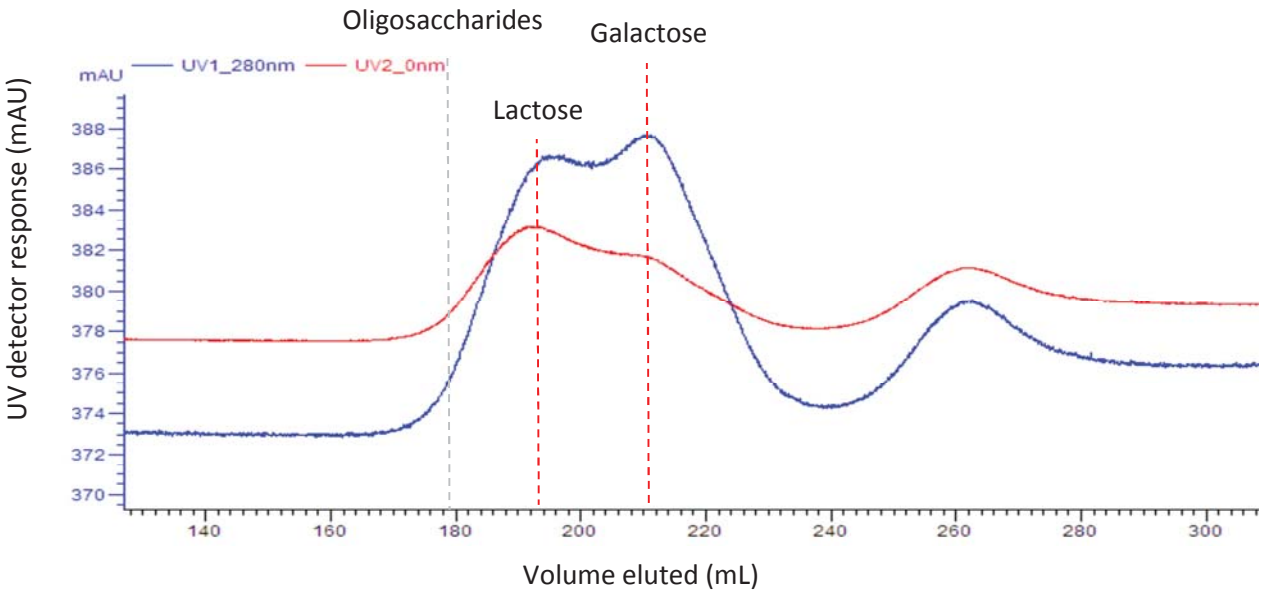

Figure 9.5: 2 mL injection of standards onto Sephadex G-25. Flow rate 2 mL/min, UV detection at 197 nm (blue) and 214 nm (red)

Toyopearl HW40-C

The result of a 2 mL injection of standards (same mix as above) onto Toyopearl HW40-C is shown in Figure 9.6. In contrast to the previous two resins trialled, the oligosaccharide peak is resolved along with the lactose and galactose peaks, as marked on the diagram. Although the resolution in this trial is still far from ideal, this shows that the resin performs better than the previous two and, with the multiple column arrangement of SMB chromatography, has potential for more effective and efficient separation.

The chromatogram from an injection of ultra-filtered mother liquor sample is shown in Figure 9.7. The locations of the peaks (confirmed by HPLC analysis of collected samples in a subsequent trial with fraction collection) are marked on the chromatogram.

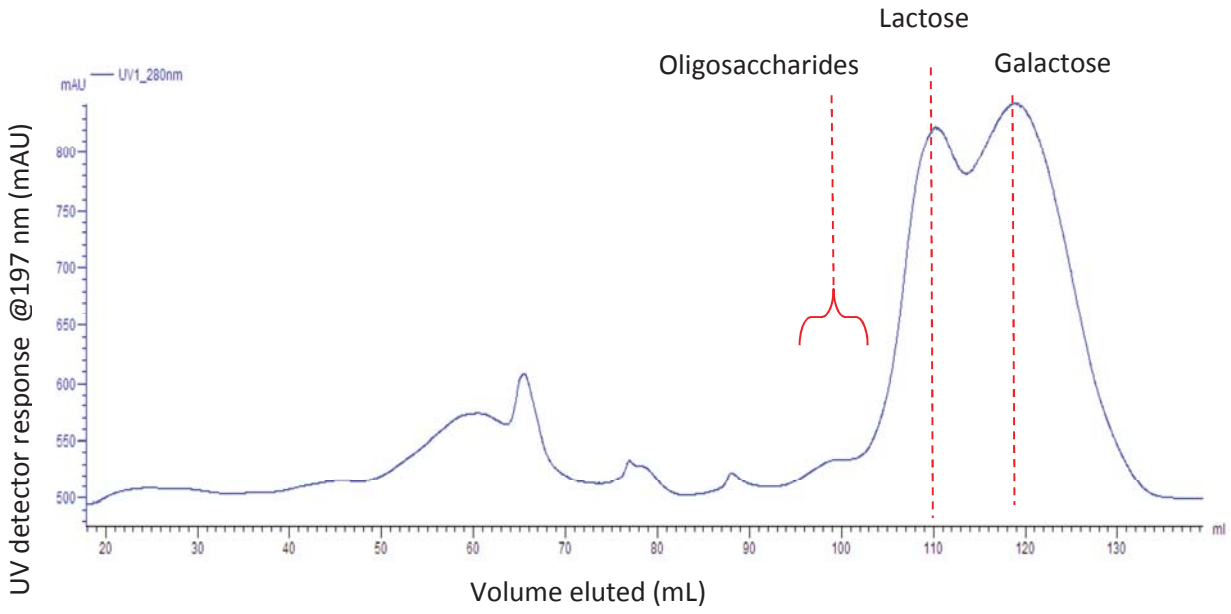

Figure 9.6: Elution of standards on Toyopearl HW40-C. Injection volume 2 mL, flow rate 4 mL/min, 700x16 mm column. UV detection at 197 nm.

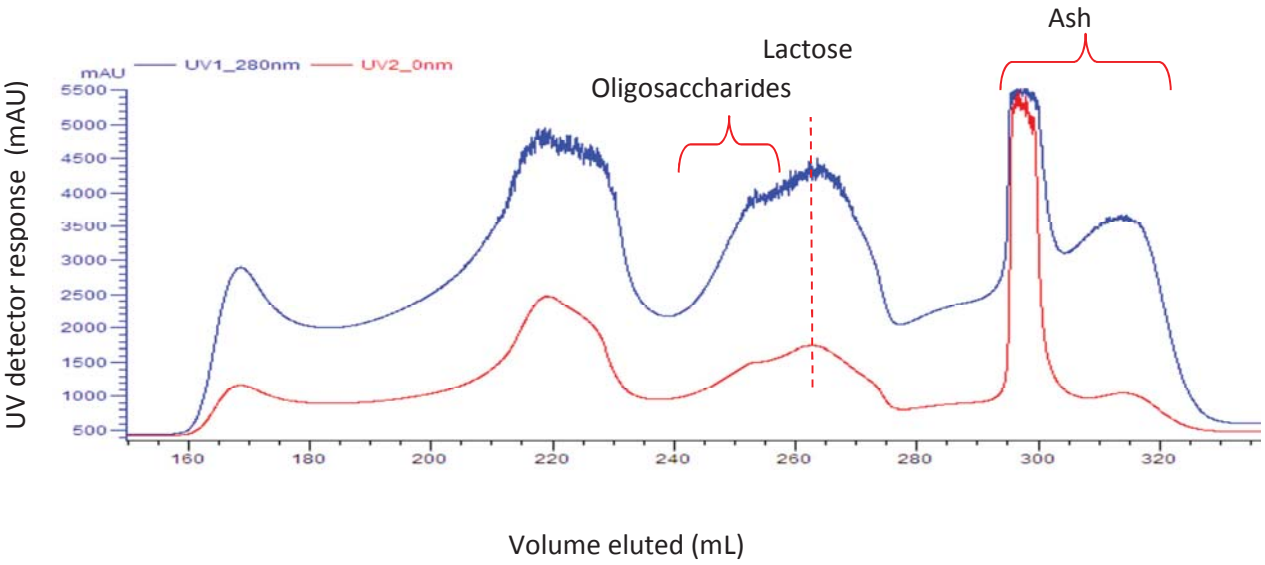

Figure 9.7: Elution of ultrafiltered mother liquor sample on Toyopearl HW40-C. 2 mL injection eluted at 2 mL/min on a 700x16 mm column. UV detection at 197 nm (blue) and 214 nm (red).

Again, the oligosaccharide peak can be discerned although the resolution from the lactose peak is limited. Note that the relative concentration of oligosaccharides, lactose and monosaccharides is different from that in the standards, and there are other components present such as ash (after the lactose peak), and polysaccharide, protein, and/or particulate impurities (prior to the sugar peaks) which are not present in the standard mixture.

Furthermore, the relative concentrations are not represented accurately in the UV detector trace, because the different compounds give different responses at each wavelength.

The composition of the mother liquor to be separated in the SMB process may not be exactly the same as the sample that was used here, because the pre-treatment was done with a pilot scale membrane and the samples were subject to different storage time and conditions. This means that the separation may have been slightly different, even though the degree of separation between oligosaccharides and lactose was very similar. It must be stressed again that the goal of resin screening was not to quantify the separation of each resin, but to compare the different resins and select the best one.

Superdex 30

Superdex 30 gave poor results with standards and similar results when injected with mother liquor sample (Figure 9.8). The sugars are all eluted in the same broad peak and the ash component is close in elution volume to the sugars.

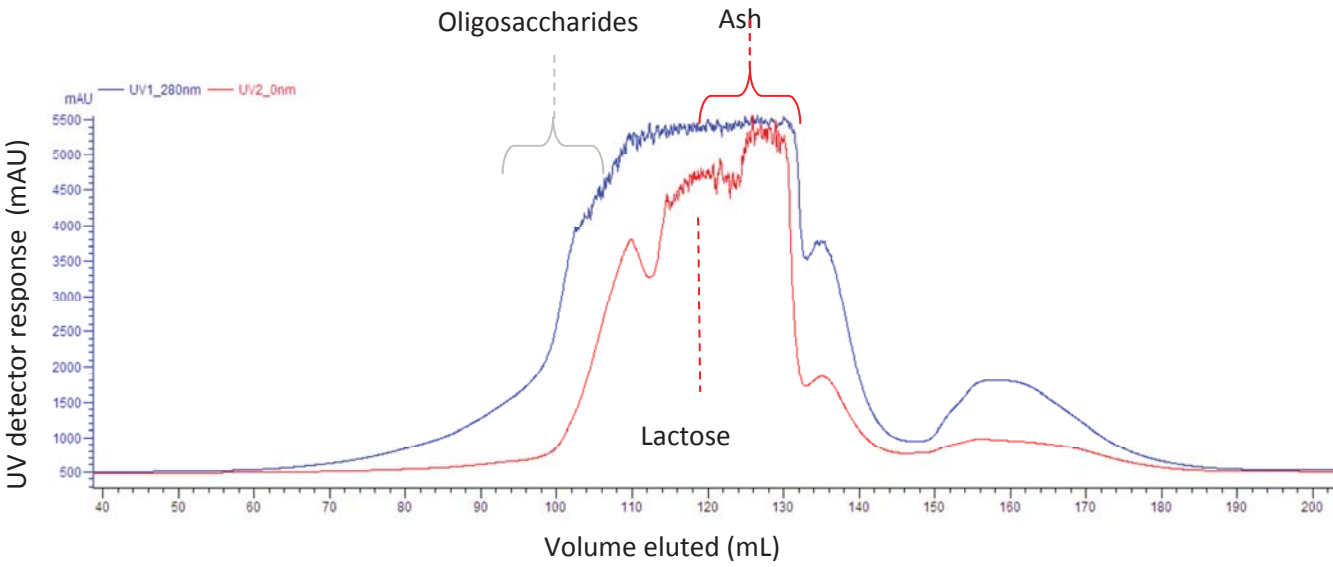

Figure 9.8: Ultra-filtered mother liquor sample (2 mL) eluted on Superdex 30 (prep grade) at 2 mL/min, 700x16 mm column. UV detection at 197 nm (blue) and 214 nm (red).

Superdex 30 is suited to low cost, efficient separation of components that have a large difference in molecular size (e.g. desalting proteins) but is not suited for separation of components that are close in size such as lactose and oligosaccharides.

9.4.4 Conclusions

Toyopearl HW40-C was the only one of the four resins to display an oligosaccharide peak under any conditions. Moreover, this resolution was maintained up to the maximum flow rate allowable under the 3 bar pressure limit with injections of up to 2 mL. This aligns well with the findings of others, e.g. Geisser et al. (2005). Based on this conclusion, Toyopearl HW40-C was selected for work on the SMB trials.

The superior performance of Toyopearl HW40-C over the other three resins trialled for oligosaccharide separation could be due to a number of reasons, including a narrower pore size distribution, larger number of pores on each particle, higher surface area to volume ratio, or improved resin particle structure. Because the chemistry of each resin is proprietary information, it is difficult to know which of these factors is most important in this case. These results reflect a lack of size exclusion media products suited to this type of separation. The HFCS industry uses cation exchange resins successfully, though these are not suited to separations where the feed is complex and needs to be separated based on size rather than charge.

9.5 SMB Experimental Setup

A simulated moving bed system was designed and built for the purpose of testing the effectiveness of SMB for the enrichment of oligosaccharides. Figure 9.9 shows the setup of the system. Four columns of 16 mm I.D and 700 mm length (GK 16/70, GE Healthcare) were interconnected with 1/16” chromatography tubing and Luer three-way valves (Cole-Parmer, Illinois, USA) for connection to the inlet and outlet lines.

Columns were filled with Toyopearl HW40-C resin using a slurry preparation of 10-20% ethanol in distilled water at a 1:1 ratio of resin to solvent and adjusted to a uniform packed height by removal or addition of further resin. To ensure the columns had the same volume of resin, test runs were made with 1 mL of sample and the elution time of the main peaks was

determined by UV detector and compared across columns. Resin was removed or added to give retention times within ±1%. Unused resin was stored in 20% ethanol at 4 °C until needed.

Four pumps were used in the setup: A recirculation pump was connected in-line between columns four and one, and feed, extract, and raffinate pumps controlled the flow rates for respective inlets and outlets. The extract and raffinate pumps were former FPLC piston pumps (Biopharma) with direct flow rate input, minimal pulsing, and high accuracy and precision. Because of the back pressure from the system, these pumps were tasked with simply restricting the flows of extract and raffinate to the required level (see Section 9.6 for discussion on pressure and flow). Raffinate and extract were collected at the pump outlets for analysis or disposal.

The feed and recirculation pumps were high precision, low volume micro annular gear pumps (model mzr-4605, Pump Systems Ltd, Christchurch NZ) with programmable speed via computer software (Faulhaber Motion Manager 5, www.faulhaber.com). These pumps, although positive displacement, had significant back leakage under the pressures in the system (up to 3 bar), and therefore required continual adjustment to achieve the correct flow rate in each case. Refer to the SMB trials (Section 9.5) for more detail on flow rate control.

Solvent intake was driven by the suction prior to the recirculation pump, provided by the deficit in flow rate given by:

$$Q_S = Q_E + Q_R - Q_F \qquad \text{(Equation 9.2)}$$

The recirculation pump was moved after each switch to be in the position after the solvent inlet.

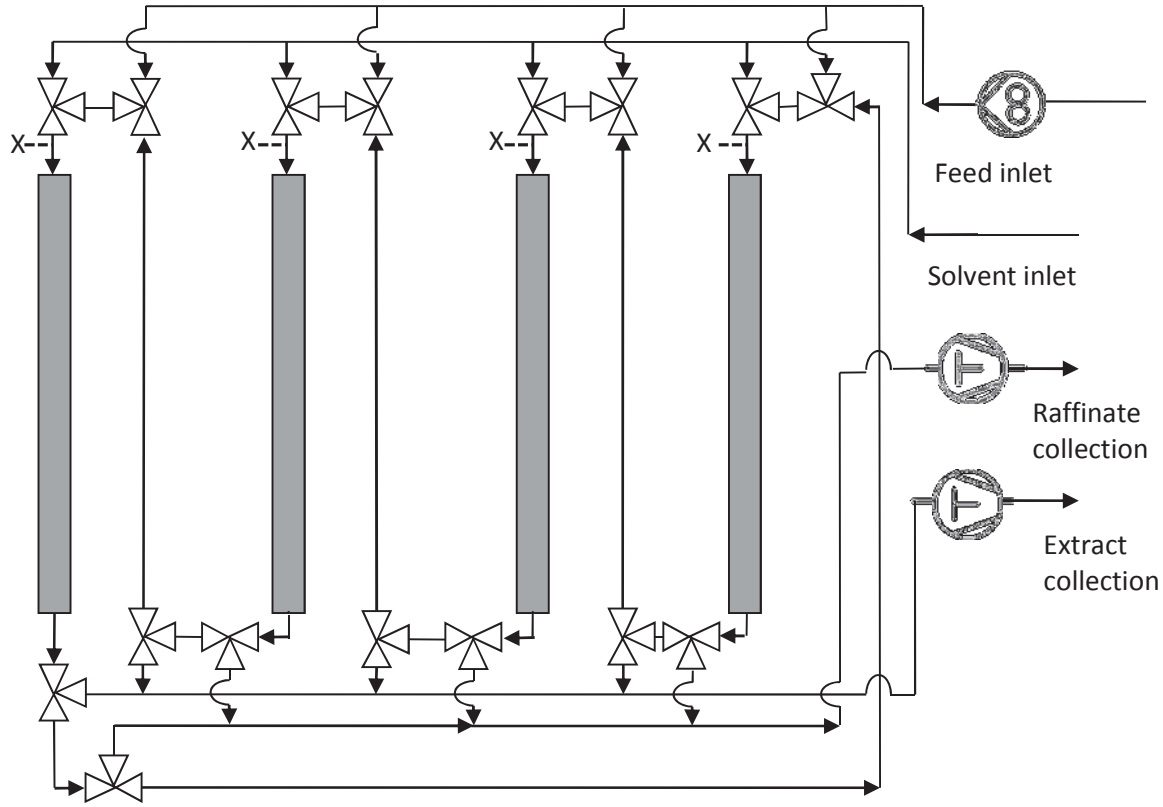

Figure 9.9: Diagram of SMB setup. Valves and pump speeds were manually controlled to meet the operating conditions calculated in Chapter 9.6. The recirculation pump was at one of the points marked X, depending on the switch position.

9.6 SMB Calculations

In order to find the parameters for the SMB process, a single injection of ultra-filtered mother liquor (2 mL) was made with the four columns in "open" configuration, where solvent was pumped (flow rate 5 mL/min) into column one, then flowed through all four columns with no other inlet or outlet valves open and exited via a UV detector with data logger measuring voltage signal at 0.5 s intervals. Fractions were collected in 2 mL samples for HPLC analysis for confirmation of the peaks observed from the UV detector, according to the method in Section 5.2 (but without sample dilution). The results from this exercise are shown in Figure 9.10.

The important points on this graph are as follows:

Dead time, t_0 = 36 min

Retention time of first peak, t_1 = 38 min

Retention time of oligosaccharides, t_{OS} = 62 min

Retention time of lactose, t_L = 68 min

Retention time of last peak, t_F = 72 min

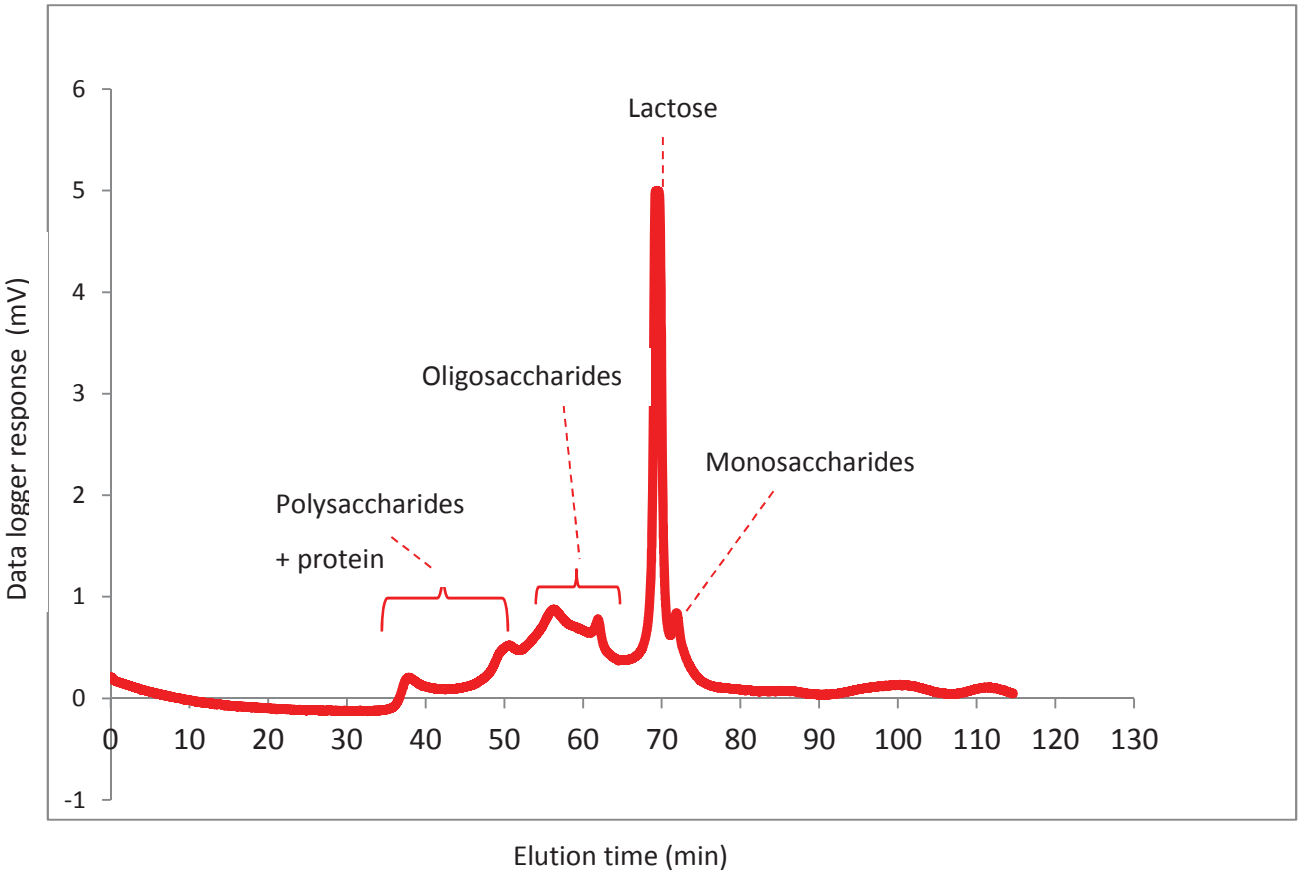

Figure 9.10: Pilot trial for SMB parameter calculation. Oligosaccharide, lactose and monosaccharide peaks labelled with confirmation from HPLC analysis (as described in Section 5.2).

Note that although the centre of the lactose peak is closer to 70 min, 68 min (the approximate beginning of the peak) was selected for use in the parameter calculations because this will lead to the selection of flow rates and a switch time which exclude more lactose than it would otherwise. This comes at the expense of productivity, as will be discussed later, and if optimisation studies are to be done, this can be adjusted.

Firstly, the porosity of the resin, ε, was calculated:

$$\varepsilon = \frac{t_0 \times Q}{V_C} = 0.32 \qquad \text{(Equation 9.3)}$$

where Q = eluent flow rate (5 mL/min) and V_c = total column volume (560 mL)

The retention factors, K_i, were found by:

$$K_i = \left(\frac{t_i}{t_0} - 1\right) \times \frac{\varepsilon}{1-\varepsilon} \qquad \text{(Equation 9.4)}$$

where t_i = the retention time of component *i*; $K_1 = 0.026$, $K_{OS} = 0.34$, $K_L = 0.42$, $K_F = 0.47$. The selectivity, α, between lactose and oligosaccharides is given by:

$$\alpha = \frac{K_L}{K_{OS}} = 1.24 \qquad \text{(Equation 9.5)}$$

The requirements for complete separation in the SMB process (as described in Section 9.3) are:

$$QI > s \times K_F \qquad \therefore \frac{QI}{s} > 0.47 \qquad \text{(Equation 9.6)}$$

$$QII > s \times K_{OS} \qquad \therefore \frac{QII}{s} > 0.34 \qquad \text{(Equation 9.7)}$$

$$QIII < s \times K_L \qquad \therefore \frac{QIII}{s} < 0.42 \qquad \text{(Equation 9.8)}$$

$$QIV < s \times K_1 \qquad \therefore \frac{QIV}{s} < 0.026 \qquad \text{(Equation 9.9)}$$

where Q is the flow rate in each zone (I, II, III, IV) in mL/min and *s* is the effective flow rate of the stationary phase (mL/min). Furthermore:

$$QIII > QII \qquad \text{(Equation 9.10)}$$

since the feed inlet is between zones II and III and requires a positive flow.

To determine the flow rates, a value for one of the flows needed to be chosen, from which the others could be calculated. Since the highest flow rate is in zone I, a value 20% lower than the upper pressure limit of the resin and column diameter could be used to define a nominal flow rate (QI_n), which for the chosen configuration is:

$$QI_n = 3 \text{ mL/min} \qquad \text{(Equation 9.11)}$$

This gives a value of $s = 6.38$, and flow rate criteria for the other zones:

$QII > 2.17$ mL/min (Equation 9.12)

$QIII < 2.68$ mL/min (Equation 9.13)

$QIV < 0.17$ mL/min (Equation 9.14)

The inequalities above specify the boundary of complete separation, i.e. if the flow rates are brought outside the specified range then the separation will be incomplete (leading to either impure raffinate or extract, or both). This may be desirable in some applications where high purity or yield is not important and throughput can be optimised, but for this work, where proof of the concept is the goal, maximum separation is most important.

Alternatively, if QII and $QIII$ are brought further within their respective limits, the system will remain in the zone of complete separation but the productivity will decrease due to the reduction in feed flow rate ($Q_F = QIII - QII$). Again, there may be instances where low throughput is acceptable in pursuit of complete separation. For this work, a feed flow rate of at least 0.25 mL/min was desirable for reducing process control issues relating to very small flow rates (see Section 9.7). With a very small feed flow rate the extract and raffinate streams also become more dilute, creating difficulties with HPLC analysis.

From the above inequalities, the difference between the bounds of QII and $QIII$ was 0.51 mL/min, so points were selected for QII and $QIII$ such that the difference between their flow rates was 0.27 mL/min, approximately halfway between the boundary of complete separation, 0.51, and zero. QII was therefore set as:

$QII = 2.17 + 0.12 = 2.29$ mL/min (Equation 9.15)

and $QIII$ was decreased by the same value to:

$QIII = 2.68 - 0.12 = 2.56$ mL/min, (Equation 9.16)

giving a difference of 0.27 mL/min. This gives a buffer from the boundary of complete separation to allow for inaccuracies in the control of flow rate and switch time, while also retaining a high enough feed flow rate.

QI and QIV were also set 10% away from the boundary of ideal operation and to allow for inaccuracies in flow rates and switch time. For QI and QIV the flow rates were therefore:

$QI = QI_n + 10\% = 3.3$ mL/min (Equation 9.17)

$QIV = 0.17 - 10\% = 0.15$ mL/min (Equation 9.18)

The flow rates of the pumps were then found:

$Q_F = QIII - QII = 0.27$ mL/min (Equation 9.19)

$Q_E = QI - QII = 1.01$ mL/min (Equation 9.20)

$Q_R = QIII - QIV = 2.41$ mL/min (Equation 9.21)

$Q_{Recirc} = QI = 3.30$ mL/min (Equation 9.22)

The flow rate of the solvent, drawn in by the deficit in flow rate between the inlets and outlets, is given by:

$Q_S = Q_E + Q_R - Q_F = 3.15$ mL/min (Equation 9.23)

The switch time, t_s, was chosen so that in zone III, where the feed is introduced, the oligosaccharides introduced at the beginning of the switch time would be recovered in the raffinate, whilst the lactose introduced at the beginning of the switch time would not reach the end of zone III before the next switch. From Figure 9.10, this time is 66 minutes across four columns at 5 mL/min, so for a single column at the calculated $QIII$ flow rate of 2.56 mL/min, the time is:

$t_s = \frac{t_p \times Q_p}{N_c \times QIII}$ (Equation 9.24)

= 32.233 min

where t_p is the elution time of the target point and Q_p is the flow rate using the pilot trial in Figure 9.10, and N_c is the number of columns.

It is also important to consider the differences in pressure across the system to ensure that fluid flows in the correct direction. Figure 9.11 shows the pressure (estimated from pressure drop provided from supplier) at each point (all in kPa.g). Note that these are not measured or calculated pressures, but they illustrate the gradients within the system.

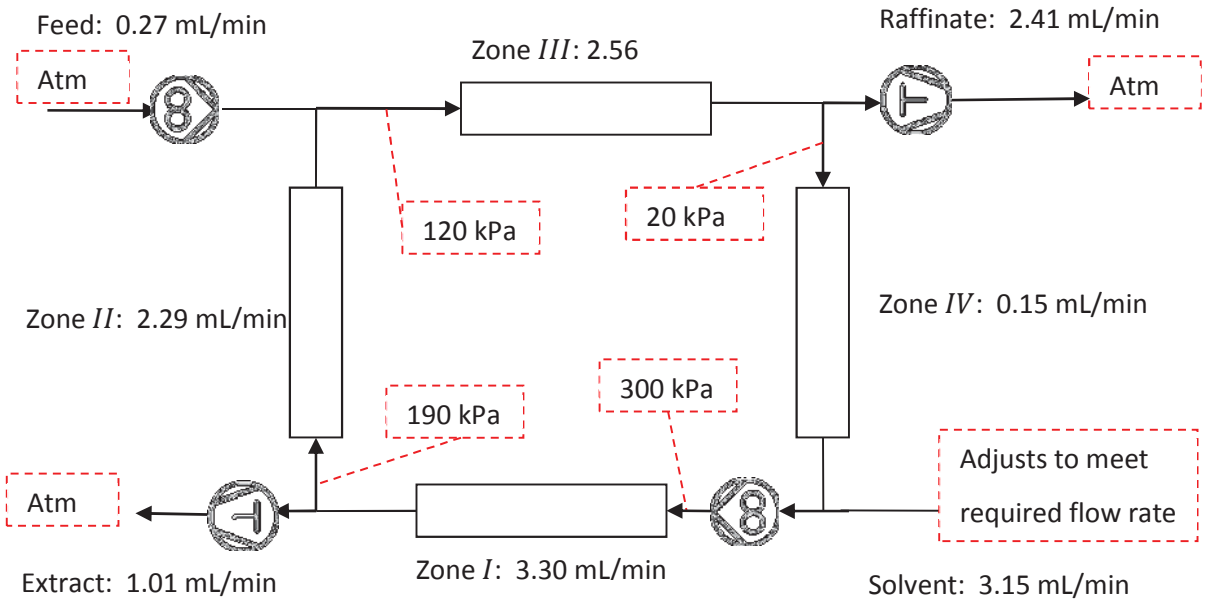

Figure 9.11: Estimated pressure at different points in the SMB process

The discharge side of the recirculation pump (between zones IV and I), is where the pressure is highest (approximately 300 kPa). The pressure drop in the first zone is the greatest because of the high flow rate, and then the extract is drawn off by a piston pump. Because the pressure is greater on the feed side of the pump, its job is to restrict the flow to a given rate rather than to increase the extract pressure. Because the piston pump is well sealed, it is capable of this, although an accurate flow restriction device would be equally effective.

Part of the flow from zone I enters zone II, where further pressure is lost, although due to the lower flow rate the pressure drop is smaller. At the end of zone II, the feed is added via a micro-annular gear pump. Given that the pump is a positive displacement type, the pressure on the discharge side will naturally increase to match the pressure in the system, provided the flow rate is less than the sum of the extract and raffinate streams.

In practice, the pump has some back-leakage and the flow rate cannot be set independently of the system pressure, leading to a requirement for calibration, as described in Section 9.7.

After further pressure is lost through zone III, the raffinate is drawn off using an identical pump and flow restriction mode to the extract collection point. The remaining flow is directed through zone IV at a very low flow rate, where the pressure drops further, prior to the solvent intake and entry into the recirculation pump. The solvent is drawn through the recirculation pump by suction at close to atmospheric pressure to make up the deficit in flow between the

outlets (extract and raffinate) and inlet (feed). The eluent intake is the only place in the system where the columns are linked to atmospheric pressure, and provides the degree of freedom required for equilibration of inevitable fluctuations in flow and pressure.

There will be no problems with pressure becoming too high or low provided the target flow rates are achieved, the maximum pressure of the columns is not exceeded (which is most likely in zone I), the valves are correctly set, and tubing is free of air bubbles and blockages. Section 9.7 describes the SMB operation procedures, designed to achieve these requirements.

9.7 SMB Trials

SMB trials were carried out using the initial parameters calculated above to assess the effectiveness of separating oligosaccharides from other lactose mother liquor components. The method employed was as follows:

1. The four columns were equilibrated with fresh RO water for at least two hours at 3 mL/min to remove ethanol storage solution and any impurities.
2. The columns were labelled A, B, C, D, and arranged with valves and tubing according to Figure 9.9. The solvent inlet and recirculation pump were connected between columns D and A, the extract (lactose rich) outlet between columns A and B, the feed inlet between columns B and C, and the raffinate (oligosaccharide rich) outlet between columns C and D. The first column of Table 9.2. summarises the initial arrangement. The system was checked for leaks or pockets of air, and valves replaced or air purged as necessary.
3. With the feed, raffinate, extract, and solvent valves open, the recirculation pump was set to 1000 rpm, before switching the extract and raffinate pumps on at the target flow rate (1.01 and 2.41 mL/min respectively). The feed pump was then switched on to 700 rpm, with water used in place of mother liquor for start-up.
4. The flow rate of the feed pump was measured by setting the feed vessel on electronic scales and measuring the mass decrease across a one-minute time period, with the assumption that 1 g = 1 mL of feed. To attain the required flow rate, the pump speed was increased or decreased using the control interface software. After waiting at least one minute for the system to reach equilibrium, the flow rate was checked again and adjusted if necessary.

5. To determine whether the flow rate of the recirculation pump was correct, the flow rate in zone IV was observed, since this is the only zone whose flow rate is determined solely by the speed of the recirculation pump. This was measured by opening the valve slightly to allow an air bubble into the line, then recording the time taken for the bubble to travel through a section of tubing with known volume (50 µL). The recirculation pump speed was increased or decreased to meet the required flow rate, and checked again after a minute or more.

Table 9.2: Column assignment for each switch in the SMB process

Zone	Initial/4th switch	1st switch	2nd switch	3rd switch
1	A	D	C	B
2	B	A	D	C
3	C	B	A	D
4	D	C	B	A

6. Because changing the feed pump or recirculation pump speed affects the pressure in the system and therefore the flow rate of both pumps, steps 4 and 5 were repeated until both flow rates were accurate.
7. The water in the feed vessel was replaced with ultra-filtered mother liquor to begin the SMB process. The timer was started and feed measurement scales reset.
8. Feed flow rate and the flow rate in zone IV were monitored continuously over the period of the switch time, with small adjustments made as necessary.
9. When the switch time was reached, all pumps were stopped and valves closed to prevent back flow. The tubing between the feed pump, extract and raffinate lines, and the four columns, as well as the recirculation pump, were shifted to the corresponding point on the next column in sequence, so that the zones were matched to the columns as described in the second column of Table 9.2.
10. The valves were reopened, the pumps restarted at the flow rates from the previous switch period, and the timer started. Flow rates were again monitored and pump speeds adjusted to keep close to the target.

11. The process was repeated five times in order to bring the system to steady state, with the arrangement of columns progressing through those described Table 9.2, returning to the initial arrangement after the 3rd switch.
12. After five switches, the process was continued in the same way with sample collection. Five 1.5 mL grab samples of both raffinate and extract were taken for each switch, at approximately 7 minute intervals. Composite samples were collected by accumulating the extract and raffinate over the full switch period and taking a 100 mL sample of each.
13. The SMB process was continued for at least 12 switches, given that it takes around 8-10 switches to attain steady state (depending on the adjustment of the system). To finish the process, the pumps were shut down and the system cleaned with RO water overnight. Ethanol in 20% solution was circulated through the system for longer term storage.
14. Samples were analysed by HPLC for oligosaccharide, lactose, and monosaccharide content according to the method in Section 5.2, and the results used to evaluate the effectiveness of separation.

9.8 Results

Figure 9.12 shows the ratio of oligosaccharides to other measured sugars (lactose and monosaccharides) in the initial SMB trial. The graph is arranged to show successive switches, labelled with the column (A,B,C or D) that occupies zone I, and shows the complete trial, including the initial period where the system is yet to reach steady state. It can be seen that the raffinate is completely devoid of the target oligosaccharides until the penultimate switch of the trial, at which point the ratio rises quickly to 2.64. Oligosaccharides are present in the extract, however, as early as the third switch. This indicates that the switch time was too short initially, since the oligosaccharides do not have time to reach the raffinate drain before the switch occurs.

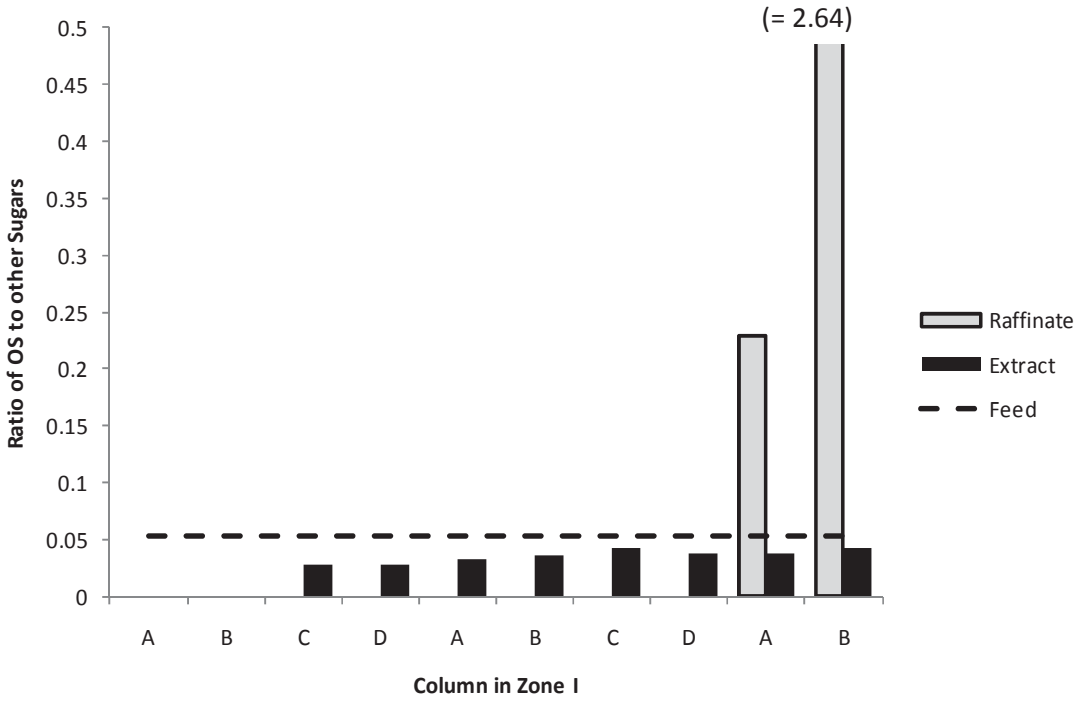

Figure 9.12: Ratio of oligosaccharides to other sugars in initial SMB trial

In the last two switches, a pulse of oligosaccharide-rich raffinate is produced, most likely because of a higher feed flow rate (and therefore higher flow rate in zone III) due to difficulty of pump speed control. Although the higher ratio of oligosaccharides in the raffinate is targeted, this result is not reliable because of its short duration and the fact that the oligosaccharide content in the extract did not decrease correspondingly, indicating unsteady state operation. If the system were perfectly calibrated and controlled, the ratio of oligosaccharides to other sugars in the raffinate would be consistent from the first switch, although the concentration would be low to begin with as the oligosaccharides are mixed with the water in the system. In the same way, the extract should be composed entirely of smaller molecules and not have any oligosaccharide present at any stage, although the solution will be dilute at first.

The first problem (switch time being too short) was corrected by giving an allowance for the volume of the valves and connectors between columns, which had previously been ignored in calculations. Because the flow rates are already in the optimal range for the pressure limitations, the switch time was instead changed. The total volume of valves, pumps, and tubing between columns was estimated at 60 mL, making the effective column volume

approximately 10% larger than estimated from column volume alone. The switch time was therefore increased by 10% to 35.45 min for the next trial.

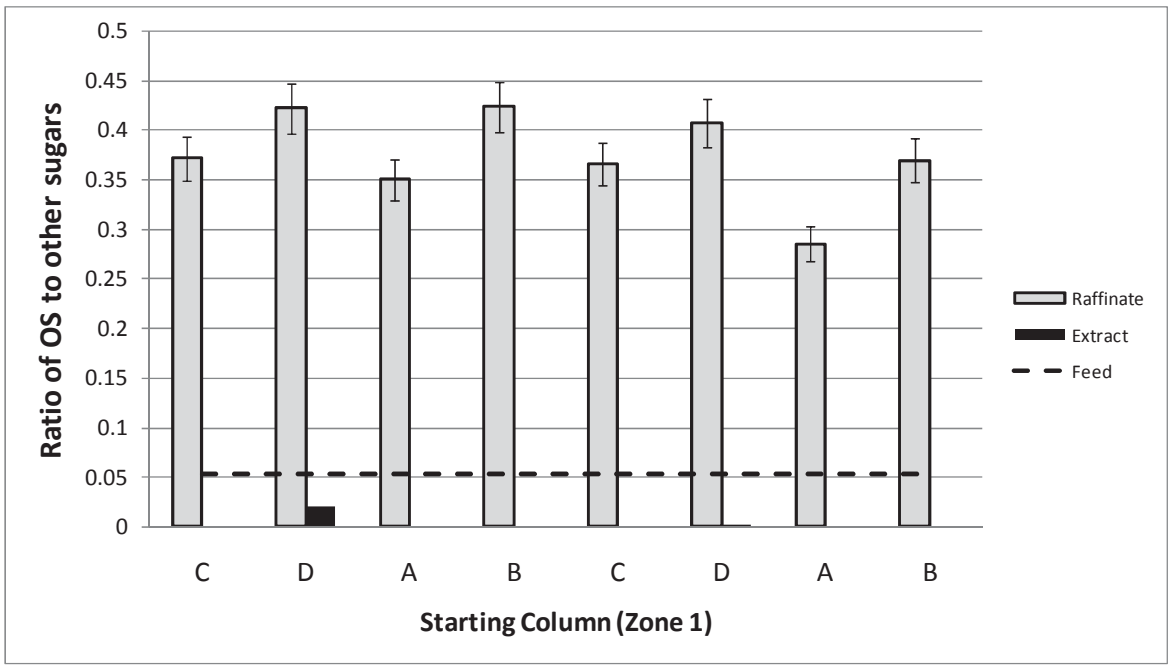

Figure 9.13: Ratio of Oligosaccharides to other sugars in adjusted SMB process

The second problem (that of lacking control of the pump flow rates) was addressed through becoming more familiar with the system and checking and adjusting the pump flow rates more frequently during the trial. The cumulative mass of feed across the switch period was also monitored, as well as instantaneous flow rate measurement, so that the correct amount was pumped into the system. The process was also run for longer, with 15 switches in total. Figure 9.13 shows the results from the adjusted process. The graph shows the final 8 switches of the process, after 7 initial switches to allow the system to stabilise and come to steady state. Error bars indicate the standard error from the HPLC analyses (based on triplicate trials). A number of observations can be made from the results in Figure 9.13:

1. In contrast to the first trial, oligosaccharides are present in a ratio of 0.35 or higher for seven of the eight samples, and the extract is free from oligosaccharides in all but two of the samples. This is much closer to what was expected for the trial based on calculations.
2. The system tends to favour recovery, rather than purity. Because the extract is mostly free of oligosaccharides, the oligosaccharides are almost exclusively found in the raffinate. This is a positive result if the goal is to recover the highest mass of

oligosaccharides, but the trade-off is a reduction in the purity of the raffinate, meaning a product with lower oligosaccharide content which would be less valuable.

3. Although there appears to be stability across the period of the trial, there is a cycling of the raffinate composition (higher when columns B or D are in position 1, lower when columns A or C are in position 1). It can also be noted that oligosaccharides only appear in the extract when column D is in position 1. This points to differences between the columns themselves, most likely a slight difference in resin volume or packing density, and therefore retention time. With mechanical column packing efficiency, as would be used in a process scale system, this is not likely to be an issue.
4. As well as the cyclical variation described above, there is also more random variation which can be seen by comparing the two times the first column was the same (i.e. every fourth switch). The cause of this is difficulty controlling flow rate for the feed and recirculation pumps and some back flow during the switching process. This random variation is smaller than that in the initial trial due to more experience in setting the correct pump speeds, more frequent adjustments, and speed of changing the valves between switches.

The ratio of oligosaccharides to other sugars, as presented in Figure 9.13, does not include the ratio of ash content, which was found almost exclusively in the extract. The composition of the raffinate on a dry basis was approximately 25-30% oligosaccharides, 60-65% lactose, 0-5% monosaccharides, and 0-5% ash, while the composition of the extract (dry basis) was approximately 0-2% oligosaccharides, 65-70% lactose, 5-10% monosaccharides, and 20-25% ash.

Overall, this is an encouraging result as it demonstrates that SMB chromatography is capable of separating BMO from lactose mother liquor where nanofiltration was unable to under the conditions trialled. Further trials with these parameters were carried out, though the ratio of oligosaccharides to other sugars could not be improved.

9.9 Conclusions

SMB chromatography is generally used in pharmaceutical and chemical engineering applications because of its high selectivity and relatively high cost, but has been increasingly used in tasks where high purity is not required and productivity can be increased. Bovine milk

oligosaccharides, a potentially high value ingredient, falls into this category as it does not need to be in a pure form to be effective as an ingredient, although a minimum concentration of about 4% is needed so that the proportion of lactose added is not too high (Chapter 4.3).

Toyopearl HW40-C, a size exclusion gel, gave a much better separation than other resins at the screening stage, and was used in a bench-top SMB setup to determine the likely separation performance in a larger scale operation. Initial results were poor, due to inaccurate switch time and flow rate selection, along with difficulty in flow rate control. By adjusting the switch time to allow for extra volume from tubing and valves, and tightening the control of the pump flow rates and lengthening the run time, good separation was obtained, with a raffinate containing 25-35% on a dry basis continually produced at a flow rate of 145 mL/hour across a period of more than five hours.

The concentration and reliability of oligosaccharide-rich raffinate production indicates that SMB is a process that is well suited to separation of oligosaccharides from mother liquor. Chapter 10 puts the SMB operation in the context of a full process including upstream processing (ultrafiltration) and revisits the economic viability of the process compared to other options.

Although this is an original result and can be seen as a significant contribution to science, this application of SMB to BMO enrichment requires further work with optimisation and scale up before being used in industry. In particular, wider columns with negligible wall effects should be investigated as a way to increase oligosaccharide purity, and variations on the number of columns can be adjusted to optimise the extract purity and minimise resin use.

10 CONCLUSIONS

Having looked at a number of approaches to the enrichment of oligosaccharides from lactose mother liquor, a preferred arrangement needs to be selected based on performance and cost. A process flow diagram showing the seven options was shown in Figure 4.1 and is repeated here for convenience (Figure 10.1):

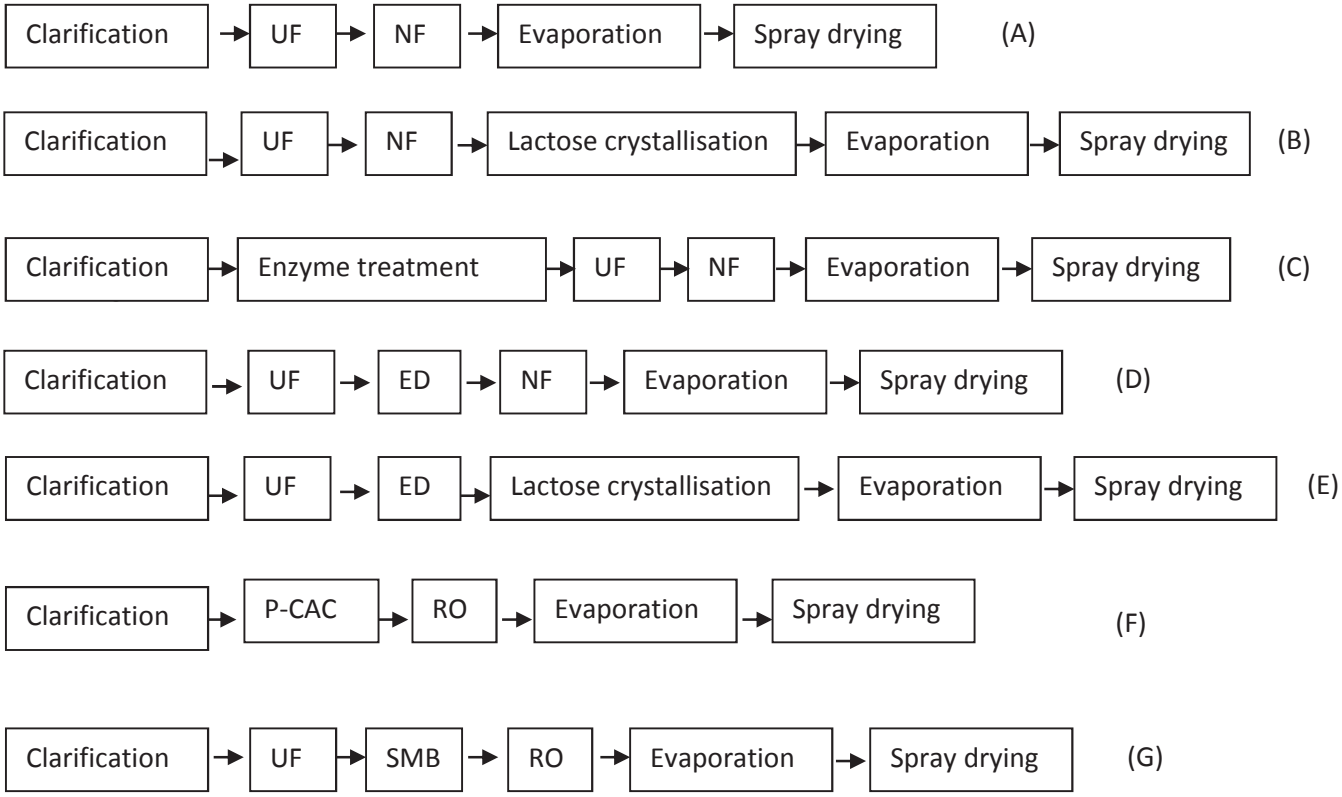

Figure 10.1: Process flow options for enrichment of oligosaccharides from Lactose Mother Liquor

Options (C), (D), (E), and (F) were rejected in Chapter 4 because mass balances showed that they would be either incapable of producing an oligosaccharide stream with the minimum required concentration or that the IRR would be too low to be economically viable.

Chapters 7 and 8 demonstrated that although membrane technology is relatively cheap and can be scaled easily for any application, the difference in molecular weight between lactose and the target oligosaccharides is not large enough for a differentiation to be made across a nanofiltration membrane. Adding further stages of filtration and coupling it with lactose crystallisation did not improve the separation to the 4% oligosaccharide purity requirement. These results lead to the rejection of options (A) and (B).

With all other options rejected, the process that centres on simulated moving bed (SMB) is the only one that is capable of producing an oligosaccharide stream with a concentration of over 4%. Although the process was presented in Chapter 4 as being economically viable, this was based on assumptions about the process that had not been verified by experiment. The key assumptions from Section 4.4 and the actual values as measured are presented in Table 10.1.

Table 10.1: Comparison of initial SMB assumptions with actual values

Parameter	Assumption	Experimental Value
Oligosaccharide content of mother liquor	0.3%	0.3-0.4%
Volume concentration factor (UF)	6	7-8
Purity of oligosaccharide stream	80%	25-35%
Yield of oligosaccharide stream	80%	90-100%
Internal rate of return	65%	60-65%

Although the purity of the oligosaccharide-rich raffinate was not as high as originally predicted, a high recovery was targeted and gave better results than predicted in Section 4.4 and thus the overall internal rate of return is almost as high as that predicted.

A process centred on the SMB process has been shown to be the most effective in enriching oligosaccharides, including sialyloligosaccharides, from lactose mother liquor and gave a final product with up to 35% purity and 100% yield. This is the recommended option for a commercial process.

The properties of the resin used in the SMB process is the most significant factor in attaining a high purity, as demonstrated in the resin screening process in Section 9.4 where it was found that Toyopearl HW40-C was the only one of four resins tested to give separation between oligosaccharides and lactose. Although Toyopearl HW40-C was able to give 35% purity in these trials, the resin is not optimised for this application, and a specific resin for oligosaccharide and lactose separation is not currently manufactured. It is recommended, therefore, that a new resin is developed for oligosaccharide separation with the following properties:

- High resolution between disaccharides and trisaccharides with very narrow pore size distribution, giving a binary separation.

- Strongly hydrophobic bead structure to prevent binding of protein fragments or other impurities.
- Resistance to osmotic shock from high mineral and ion concentrations.
- Large bead size (>100 µm hydrated diameter) to allow for a low pressure drop and high flow rate across large columns.

A resin with these properties would allow an efficient and high purity process to be scaled up for production of an oligosaccharide-enriched stream.

11 APPENDICES

11.1 Mass balance and Sensitivity Analysis (on attached CD)

11.2 Ultrafiltration Trial Data (on attached CD)

11.3 Mass Balance for Single and Double stage Nanofiltration with Crystallisation, and Filter Series (on attached CD)

11.4 Equations for Engineering Equation Solver program

The following equations were entered into Engineering Equation solver for solving simultaneous equations in Section 8.5. The mass fraction, mass flow, and rejection coefficient are calculated for each component for the feed and for the retentate and permeate of each stage.

```
F = 100
xF_OS = 0.00015          [Initial feed specifications]
xF_L = 0.01785  [x = mass fraction]
xF_A = 0.012
xF_W = 0.97
F_OS = xF_OS*F
F_L = xF_L*F
F_A = xF_A*F
F_W = xF_W*F

[Stage 0 - feed stage]
rj0_OS = 0.9      [Rejection coefficients]
rj0_L = 0.7
rj0_A = 0.1

F0 = F+Pm1+R1  [Feed flows]
F0_OS = F_OS+Pm1_OS+R1_OS
F0_L = F_L+Pm1_L+R1_L
F0_A = F_A+Pm1_A+R1_A
F0_W = F_W+Pm1_W+R1_W

xF0_OS = F0_OS/F0       [Feed composition]
xF0_L = F0_L/F0
xF0_A = F0_A/F0
xF0_W = F0_W/F0

P0 = (1-REC0)*F0          [Permeate flows]
P0_OS = xP0_OS*P0
P0_L = xP0_L*P0
P0_A = xP0_A*P0
```

```
P0_W = P0-P0_OS-P0_L-P0_A

xP0_OS = (1-rj0_OS)*xF0_OS      [Permeate composition]
xP0_L = (1-rj0_L)*xF0_L
xP0_A = (1-rj0_A)*xF0_A
xP0_W = P0_W/P0

R0 = F0*REC0    [Retentate flows]
R0_OS = F0_OS-P0_OS
R0_L = F0_L-P0_L
R0_A = F0_A-P0_A
R0_W = F0_W-P0_W

xR0_OS = R0_OS/R0       [Retentate composition]
xR0_L = R0_L/R0
xR0_A = R0_A/R0
xR0_W = R0_W/R0

[Stage 1 - raffinate stripping]
rj1_OS = 0.9      [Rejection coefficients]
rj1_L = 0.7
rj1_A = 0.1

F1 = P0  [Feed flows]
F1_OS = P0_OS
F1_L = P0_L
F1_A = P0_A
F1_W = P0_W

xF1_OS = F1_OS/F1       [Feed composition]
xF1_L = F1_L/F1
xF1_A = F1_A/F1
xF1_W = F1_W/F1

P1 = (1-REC1)*F1          [Permeate flows]
P1_OS = xP1_OS*P1
P1_L = xP1_L*P1
P1_A = xP1_A*P1
P1_W = P1-P1_OS-P1_L-P1_A

xP1_OS = (1-rj1_OS)*xF1_OS      [Permeate composition]
xP1_L = (1-rj1_L)*xF1_L
xP1_A = (1-rj1_A)*xF1_A
xP1_W = P1_W/P1

R1 = F1*REC1    [Retentate flows]
R1_OS = F1_OS-P1_OS
R1_L = F1_L-P1_L
R1_A = F1_A-P1_A
```

```
R1_W = F1_W-P1_W

xR1_OS = R1_OS/R1       [Retentate composition]
xR1_L = R1_L/R1
xR1_A = R1_A/R1
xR1_W = R1_W/R1


[Stage m1 - raffinate stripping]
rjm1_OS = 0.9    [Rejection coefficients]
rjm1_L = 0.7
rjm1_A = 0.1

Fm1 = Pm2+R0  [Feed flows]
Fm1_OS = Pm2_OS+R0_OS
Fm1_L = Pm2_L+R0_L
Fm1_A = Pm2_A+R0_A
Fm1_W = Pm2_W+R0_W

xFm1_OS = Fm1_OS/Fm1          [Feed composition]
xFm1_L = Fm1_L/Fm1
xFm1_A = Fm1_A/Fm1
xFm1_W = Fm1_W/Fm1

Pm1 = (1-RECm1)*Fm1  [Permeate flows]
Pm1_OS = xPm1_OS*Pm1
Pm1_L = xPm1_L*Pm1
Pm1_A = xPm1_A*Pm1
Pm1_W = Pm1-Pm1_OS-Pm1_L-Pm1_A

xPm1_OS = (1-rjm1_OS)*xFm1_OS        [Permeate composition]
xPm1_L = (1-rjm1_L)*xFm1_L
xPm1_A = (1-rjm1_A)*xFm1_A
xPm1_W = Pm1_W/Pm1

Rm1 = Fm1*RECm1       [Retentate flows]
Rm1_OS = Fm1_OS-Pm1_OS
Rm1_L = Fm1_L-Pm1_L
Rm1_A = Fm1_A-Pm1_A
Rm1_W = Fm1_W-Pm1_W

xRm1_OS = Rm1_OS/Rm1          [Retentate composition]
xRm1_L = Rm1_L/Rm1
xRm1_A = Rm1_A/Rm1
xRm1_W = Rm1_W/Rm1


[Stage m2 - raffinate stripping]
rjm2_OS = 0.9    [Rejection coefficients]
```

```
rjm2_L = 0.7
rjm2_A = 0.1

Fm2 = Pm3+Rm1           [Feed flows]
Fm2_OS = Pm3_OS+Rm1_OS
Fm2_L = Pm3_L+Rm1_L
Fm2_A = Pm3_A+Rm1_A
Fm2_W = Pm3_W+Rm1_W

xFm2_OS = Fm2_OS/Fm2          [Feed composition]
xFm2_L = Fm2_L/Fm2
xFm2_A = Fm2_A/Fm2
xFm2_W = Fm2_W/Fm2

Pm2 = (1-RECm2)*Fm2  [Permeate flows]
Pm2_OS = xPm2_OS*Pm2
Pm2_L = xPm2_L*Pm2
Pm2_A = xPm2_A*Pm2
Pm2_W = Pm2-Pm2_OS-Pm2_L-Pm2_A

xPm2_OS = (1-rjm2_OS)*xFm2_OS         [Permeate composition]
xPm2_L = (1-rjm2_L)*xFm2_L
xPm2_A = (1-rjm2_A)*xFm2_A
xPm2_W = Pm2_W/Pm2

Rm2 = Fm2*RECm2       [Retentate flows]
Rm2_OS = Fm2_OS-Pm2_OS
Rm2_L = Fm2_L-Pm2_L
Rm2_A = Fm2_A-Pm2_A
Rm2_W = Fm2_W-Pm2_W

xRm2_OS = Rm2_OS/Rm2          [Retentate composition]
xRm2_L = Rm2_L/Rm2
xRm2_A = Rm2_A/Rm2
xRm2_W = Rm2_W/Rm2


[Stage m3 - raffinate stripping]
rjm3_OS = 0.9   [Rejection coefficients]
rjm3_L = 0.7
rjm3_A = 0.1

Fm3 = Rm2+Pm4           [Feed flows]
Fm3_OS = Rm2_OS+Pm4_OS
Fm3_L = Rm2_L+Pm4_L
Fm3_A = Rm2_A+Pm4_A
Fm3_W = Rm2_W+Pm4_W

xFm3_OS = Fm3_OS/Fm3          [Feed composition]
```

```
xFm3_L = Fm3_L/Fm3
xFm3_A = Fm3_A/Fm3
xFm3_W = Fm3_W/Fm3

Pm3 = (1-RECm3)*Fm3  [Permeate flows]
Pm3_OS = xPm3_OS*Pm3
Pm3_L = xPm3_L*Pm3
Pm3_A = xPm3_A*Pm3
Pm3_W = Pm3-Pm3_OS-Pm3_L-Pm3_A

xPm3_OS = (1-rjm3_OS)*xFm3_OS        [Permeate composition]
xPm3_L = (1-rjm3_L)*xFm3_L
xPm3_A = (1-rjm3_A)*xFm3_A
xPm3_W = Pm3_W/Pm3

Rm3 = Fm3*RECm3       [Retentate flows]
Rm3_OS = Fm3_OS-Pm3_OS
Rm3_L = Fm3_L-Pm3_L
Rm3_A = Fm3_A-Pm3_A
Rm3_W = Fm3_W-Pm3_W

xRm3_OS = Rm3_OS/Rm3         [Retentate composition]
xRm3_L = Rm3_L/Rm3
xRm3_A = Rm3_A/Rm3
xRm3_W = Rm3_W/Rm3

[Stage m4 - raffinate stripping]
rjm4_OS = 0.9   [Rejection coefficients]
rjm4_L = 0.7
rjm4_A = 0.1

Fm4 = Rm3 + Pm5         [Feed flows]
Fm4_OS = Rm3_OS+Pm5_OS
Fm4_L = Rm3_L+Pm5_L
Fm4_A = Rm3_A+Pm5_A
Fm4_W = Rm3_W+Pm5_W

xFm4_OS = Fm4_OS/Fm4         [Feed composition]
xFm4_L = Fm4_L/Fm4
xFm4_A = Fm4_A/Fm4
xFm4_W = Fm4_W/Fm4

Pm4 = (1-RECm4)*Fm4  [Permeate flows]
Pm4_OS = xPm4_OS*Pm4
Pm4_L = xPm4_L*Pm4
Pm4_A = xPm4_A*Pm4
Pm4_W = Pm4-Pm4_OS-Pm4_L-Pm4_A

xPm4_OS = (1-rjm4_OS)*xFm4_OS        [Permeate composition]
```

```
xPm4_L = (1-rjm4_L)*xFm4_L
xPm4_A = (1-rjm4_A)*xFm4_A
xPm4_W = Pm4_W/Pm4

Rm4 = Fm4*RECm4       [Retentate flows]
Rm4_OS = Fm4_OS-Pm4_OS
Rm4_L = Fm4_L-Pm4_L
Rm4_A = Fm4_A-Pm4_A
Rm4_W = Fm4_W-Pm4_W

xRm4_OS = Rm4_OS/Rm4          [Retentate composition]
xRm4_L = Rm4_L/Rm4
xRm4_A = Rm4_A/Rm4
xRm4_W = Rm4_W/Rm4

[Stage m5 - raffinate stripping]
rjm5_OS = 0.9   [Rejection coefficients]
rjm5_L = 0.7
rjm5_A = 0.1

Fm5 = Rm4       [Feed flows]
Fm5_OS = Rm4_OS
Fm5_L = Rm4_L
Fm5_A = Rm4_A
Fm5_W = Rm4_W

xFm5_OS = Fm5_OS/Fm5          [Feed composition]
xFm5_L = Fm5_L/Fm5
xFm5_A = Fm5_A/Fm5
xFm5_W = Fm5_W/Fm5

Pm5 = (1-RECm5)*Fm5  [Permeate flows]
Pm5_OS = xPm5_OS*Pm5
Pm5_L = xPm5_L*Pm5
Pm5_A = xPm5_A*Pm5
Pm5_W = Pm5-Pm5_OS-Pm5_L-Pm5_A

xPm5_OS = (1-rjm5_OS)*xFm5_OS         [Permeate composition]
xPm5_L = (1-rjm5_L)*xFm5_L
xPm5_A = (1-rjm5_A)*xFm5_A
xPm5_W = Pm5_W/Pm5

Rm5 = Fm5*RECm5       [Retentate flows]
Rm5_OS = Fm5_OS-Pm5_OS
Rm5_L = Fm5_L-Pm5_L
Rm5_A = Fm5_A-Pm5_A
Rm5_W = Fm5_W-Pm5_W

xRm5_OS = Rm5_OS/Rm5          [Retentate composition]
```

xRm5_L = Rm5_L/Rm5
xRm5_A = Rm5_A/Rm5
xRm5_W = Rm5_W/Rm5

zOS_dryfraction = xRm5_OS/(1-xRm5_W) [Final OS conc dry]
zOS_yield = Rm5_OS/F_OS [Final OS yield dry]

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