On-farm Fractionation of Milk Components

A thesis submitted in fulfillment

of the requirements for the degree of

Doctor of Philosophy

in Materials and Process Engineering

by

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WAIKATO
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Hamilton, New Zealand

April 2006

Abstract

Methods for on-farm extraction of low-concentration (minor) proteins from raw whole bovine milk directly after milking were explored. These minor proteins have high commercial value. Lactoferrin (LF) and lactoperoxidase (LP) were used as model proteins for extraction using cation exchange chromatography.

Laboratory fractionations showed that milk could be processed by conventional column chromatography without excessive column backpressures if resin with large particles sizes were used and the temperature was high enough so fat in the milk was malleable; ideally the milk should be near the secretion temperature of 37^{o}C. Processing parameters such as equilibrium and dynamic capacities were determined for SP Sepharose™ (GE Healthcare Technologies) and Bio Rex 70 (BioRad Laboratories) resins. SP Sepharose Big Beads (SP BB) were found to be more suitable than BR 70, for raw whole milk processing due to the larger size (200 μm).

Design considerations showed that column chromatography was not the most practical method for on-farm processing of fresh, raw whole milk. Trials with a single-stage stirred tank showed that SP BB resin could extract up to 65% of LF (initial LF concentration of 0.5 mg/mL) with a 10-minute adsorption time. The composite non-linear (CNL) model of Rowe *et al*. (1999) was used to describe LF uptake by SP BB resin in raw whole milk with initial LF concentrations of 0 to 1.0 mg/mL and resin:milk volume ratios of 0.010, 0.012, 0.017 and 0.024 over 45-minute contact times. The CNL model could be used to predict LF yields if initial feed concentration, milk and resin volumes, and contact times were known. Laboratory extractions showed that processing did not significantly affect bulk milk composition (fat, protein, lactose and total solids), indicating that the milk could be used for conventional processing after the minor proteins had been extracted.

Resin cleaning and regeneration studies, using a procedure similar to that recommended by the resin supplier, showed that the Sepharose resin had not degraded and there was no significant decrease in binding capacity after 50 extraction cycles.

A Protein Fractionation Robot (PFR) prototype based on a single-stage stirred tank and the operating parameters obtained from the laboratory trials was designed, assembled and

coupled to an Automated Milking System (AMS) to process fresh, raw whole milk from individual cows immediately after milking. The LF and LP extracted from the milk from 16 individual cows were 19.7 - 55.2% (35.6 ± 10.2%) and 21.2 - 99.5% (87.1 ± 12.0%) respectively. Generally, higher extraction levels were obtained at higher resin:milk ratios. The amount of LF extracted on-farm agreed within 14.1 ± 9.8% of those predicted by the CNL model, with predicted values generally being higher. The experimental on-farm adsorption values were calculated using data of LF recovered after elution, so differences between actual and predicted values may be due to losses during post-adsorption processing.

Economic feasibility studies, based on experimental data from the PFR and realistic wholesale prices for LF and LP ($400 and $150/kg respectively) showed that PFR-based processing is economically viable if the farmer is paid for the LF and LP produced as well as the bulk milk. This system would have a payback period of approximately five years and an internal rate of return of 14.5%. Further case studies determined the sensitivity of the economics to various operating parameters and value/cost assumptions, including producing recombinant human protein from transgenic bovine milk. These studies showed that the higher the value of the processed raw milk, the higher the absorptive capacity of the resin, and the higher the value of the extracted protein, the more favourable the economics. In the extreme case of producing a very high value therapeutic protein (e.g. $20 000), the payback period could be as low as 0.3 years, with an internal rate of return of 818%.

Acknowledgements

There are so many people who made this project possible. I am really grateful for that.

- Dexcel, especially Dr Norm Thomson (my mentor for years), the research manager and the senior management for allowing me to embark on this journey and their support. To the Greenfield site team for being ever so supportive. I am humbled to be affiliated with such an organization and its people and to Barabara Dow for statistical help.
- The team at Sensortec, especially Dr Rod Claycomb for providing the encouragement and motivation to see the project through and bringing a commercial focus to the project. To Johann for being affiliated to fractionation project and being called upon (almost always in urgency to help out). And thanks to Dr Tony Grabski (Wisconsin, U.S.A) for his help at the beginning of the project
- Enterprise Scholarship (Technology, New Zealand) for their support
- AGMARDT for funding the PFR prototype development and testing
- The Department of Materials and Process Engineering at The University of Waikato, for providing an awesome learning and supportive environment. A special tribute to my chief supervisor, Professor Conan Fee, who leads by example, sets high standards and works extremely hard (hence we ought to try and live up to his expectations!!). To Professor Janis Swan for the encouragement and support in Conan's absence and for help with editing the manuscript. Special thanks to the department's staff and technical team (Lisa, Brett & Brett and Paul) for their help and support through the years and whom I have got to know very well. It really does make for an awesome place to come to on a daily basis knowing there is such a friendly team of people around you. Thanks to our helpful friendly librarian, Cheryl Ward and department administrator, Mary Dalbeth.
- To the students in the department, thanks for the stimulating times. To my project team, Florian, Nathan and Andrew thanks for the hard work and truly fun times. Also students and staff from other departments that I have befriended over the years, thanks for your friendship
- To all those names I have not mentioned, actually far too many to add …. "a big thank you" for your patience
- To my family, Justin, Nikita and Pramesh, thanks so much for your love and support

Preface

The development of Automated Milking Systems (AMS) and associated technology as a future for milk harvesting in New Zealand has generated considerable commercial interest. It has the potential to address a lot of labour and productivity issues facing the dairying industry in New Zealand today. However, the uptake of the technology is low with the costs and economics of switching to such a high technology option still a concern for the farmers. The AMS is a shift in the way milk has been traditionally harvested. The concept for on-farm fractionation was looked at by DEC International Limited in mid 1990s. However, a feasibility study based on the dynamics of New Zealand dairy and market for bioactives in 1996 showed that it was not a viable technology for the market. Since then, the functional food market and the perception of dairy specialty foods have changed dramatically. Also with the introduction of the first AMS to New Zealand in 2001, an opportunity was presented to explore the practicality of this on-farm technology so this project was initiated in 2003 through this PhD project. The on-farm project's overall aim was to develop a platform technology to extract high-values proteins on-farm, in-line from freshly harvested, raw whole milk.

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Symbols and Abbreviations

α-La	α-lactalbumin
β-Lg	β-lactoglobulin
a	time constant, min^{-1}
AMS	Automated Milking System
BSA	bovine serum albumin
C	solution-phase protein concentration, mg mL^{-1}
c, m, x	constants
C_{LF}*	solution-phase protein concentration at equilibrium with solid-phase concentration, Q_{LF}*, mg mL^{-1}
CNL	composite non-linear model
Co	initial feed solution-phase protein concentration, mg mL^{-1}
IgG	immunoglobulin G
k	rate constant, min^{-1}
LF	lactoferrin
LP	lactoperoxidase
PFR	Protein Fractionation Prototype
PLC	programmable logic controller
q	solid-phase protein concentration, mg mL^{-1} of media
q_k	kinetic constant used in the CNL model, mg mL^{-1}
Q_{LF}*	solid-phase protein concentration at equilibrium with solution-phase concentration, C_{LF}*, mg mL^{-1}
SP BB	SP Sepharose Big Beads
SP FF	SP Sepharose Fast Flow
SPR	surface plasmon resonance
t	time, min
y(0)	zero-intercept for straight line fit of equation (1)

Greek

Φ	ratio of chromatography media volume to milk volume

1 Introduction

1.1 Background

Bovine milk has been used as a food source for many centuries. More recently, the minor milk components have been identified as important for human health and nutrition, with both nutraceutical and therapeutic applications. Examples include lactoferrin (LF) used in infant formulae, lactoperoxidase (LP) used as a natural preservative, and whey protein isolates used in sports and nutritional supplements.

Minor milk proteins are typically produced from dairy waste steams, particularly whey. Processing whey has evolved mainly to improve revenue while reducing the amount of waste produced (Smithers *et al*. 1996). High-value milk proteins such as LF and LP have also been extracted from skim (non-fat) milk.

Economy of scale drives production of commodities such as milk powder, casein, cheese and butterfat, leading to centralised processing in large factories (Figure 1-1). Currently, all milk fractionation is performed on bulk milk streams at the dairy processing plant, so farms that produce milk with particularly high concentrations of high-value minor components do not directly benefit. This lack of return on investment to the farmer has restricted the development of processes that can capture such benefits at the farm.

There are large variations in milk composition, both between cows and between milkings of individual cows. Farm management practices such as selective breeding, supplementary feeding, etc, can produce milk with different fat, proteins and lactose content. These differences, however, are minimised when milk from different suppliers is amalgamated into large silos before centralised processing.

Industrial milk processing involves many unit operations before the separation processes to extract valuable functional proteins (Figure 1-1). Such extensive processing may affect proteins yield and activity.

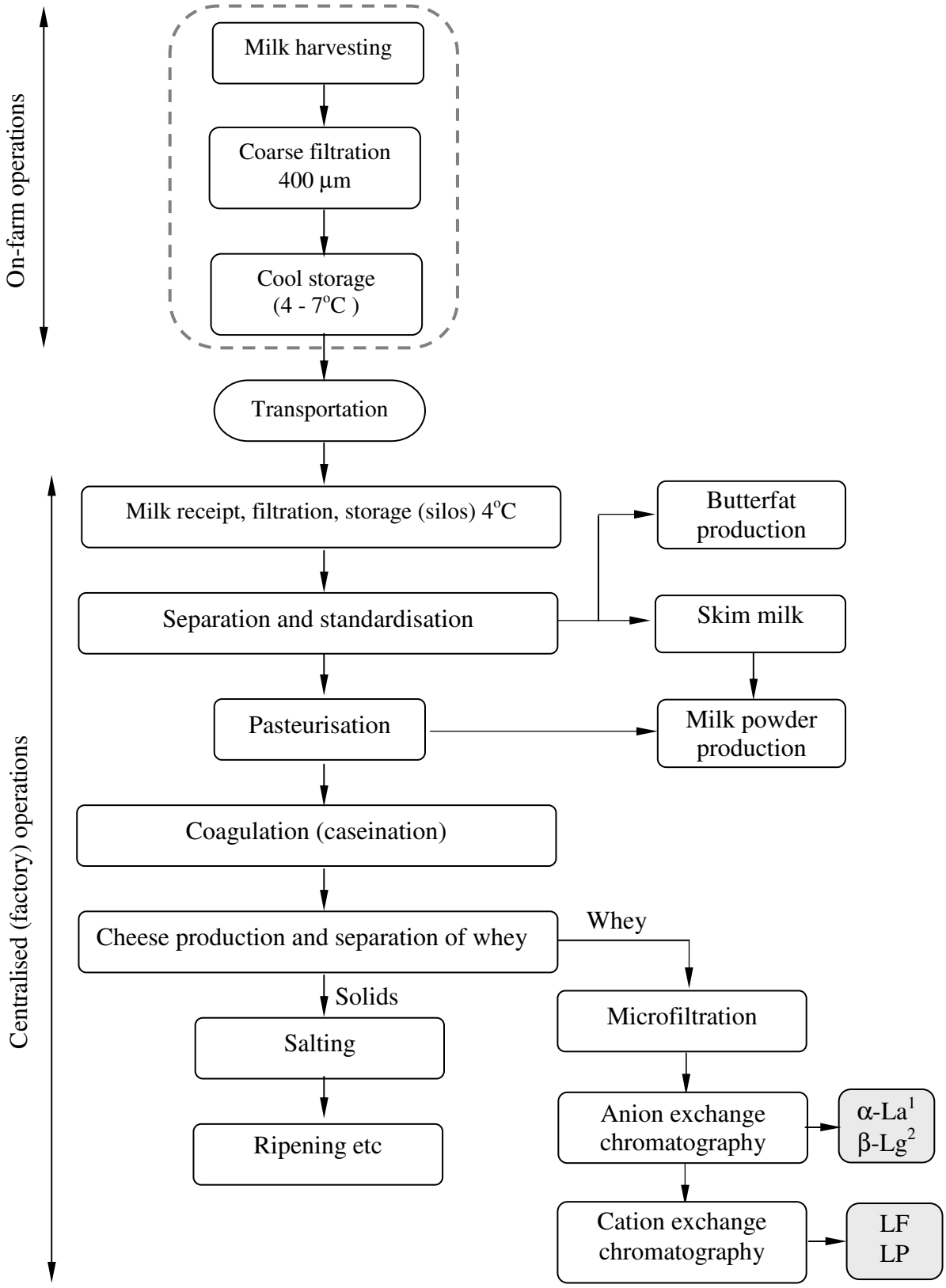


Figure 1-1: Commercial milk processing in a centralised facility.

[1] α-lactalbumin; [2] β-lactoglobulin.

Proteins are delicate molecules where structure and function are intertwined. It is a truism in the biotechnology industry that proteins should be processed as quickly as possible and in as few steps as possible because yields (Figure 1-2) and bioactivity are directly related to efficient and effective processing (Ladisch 2001; Harrison *et al*. 2003).

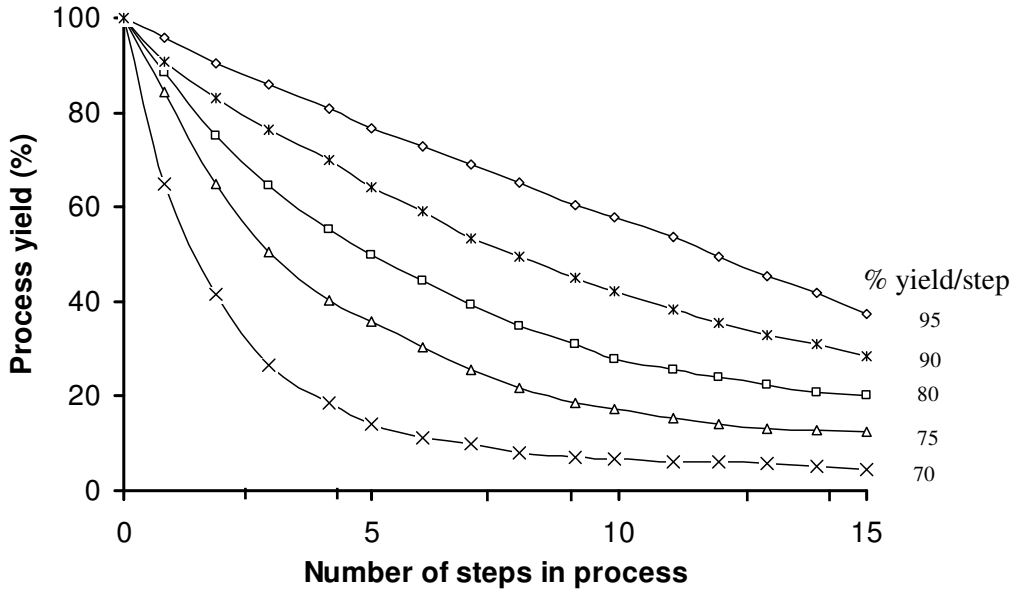


Figure 1-2: The effect of number of processing steps on yields.

Pre-treating milk before adsorption processes to capture proteins is universal (Humphrey and Newsome 1984, Andrews *et al*. 1985, Yoshida 1988). The current processes for functional dairy proteins use pharmaceutical protein separation and purification techniques (mainly ion exchange chromatography together with ultrafiltration) to produce proteins, whey proteins, whey protein isolates, immunoglobulins etc from filtered skim milk and whey.

An Automated Milking System (AMS) with a high degree of automation, herd management capability, and control and traceability has been demonstrated on a commercial farm (Dexcel and Sensortec Limited, Hamilton, New Zealand) (Woolford *et al*. 2003). This presents an opportunity to couple the AMS and protein fractionation for extracting thermally labile and unstable proteins from raw whole milk on-farm at the point of secretion. This process has the potential to increase product yield and activity.

The LF, LP, and other minor components of milk have high value. On-farm extraction creates the opportunity for individual farmers to improve their returns if they use practices that increase the concentrations of these components in their animals' milk. The concept of on-farm fractionation from fresh raw whole milk had not been previously attempted. It is generally accepted that fat fouls adsorbents and therefore must be removed before milk is further processed. Little or no information is available on processes about on-farm milk processing and/or from processing fresh, raw whole milk. This project was designed to explore the technical challenges of processing raw whole milk and develop a functional system suitable for on-farm operation.

1.2 Objectives

The primary goal of this thesis was to demonstrate the feasibility of on-farm fractionation of milk proteins from fresh, raw whole milk. A further goal was to couple a robotic fractionation system to the AMS.

The specific objectives for the project were to:

- investigate milk processing technologies for extracting high-value, specialty minor components
- explore chromatographic extraction of proteins from raw whole milk and determine adsorption capacities, kinetics and pressure-flow relationships of suitable adsorbents
- evaluate techniques and materials for extracting components from milk for their suitability for on-farm use
- develop an automatic fractionation platform that can be coupled to an AMS to extract components of interest

1.3 Thesis organisation

A general review on milk, its components, their extraction and separation technologies is presented in Chapter 2. In particular, LF and LP purification is reviewed with the aim of developing a process to extract them from raw whole milk. Laboratory methods and small scale fractionation experiments are described in Chapter 3 and the results and discussion are presented in Chapter 4. In Chapter 5, batch chromatography adsorption kinetics are explored using the new composite non-linear (CNL) model of Rowe *et al*. (1999). Data from both laboratory and on-farm adsorption experiments are fitted with the CNL model and used to explore the effects of operating parameters on yields. Chapter 6 describes the design of an extraction robot and the methodology used for on-farm experiments. Results from on-farm experiments are presented in Chapter 7. Chapter 8 contains an economic feasibility study based on the data for LF and LP extraction obtained with the robot and in the laboratory. Conclusions and recommendations for future work are presented in Chapter 9.

2 Literature Review

2.1 Introduction

Extracting proteins from dairy fluids using ion exchange chromatography has been extensively studied for many years. It is almost exclusively carried out on 'skim' (defatted) milk and/or whey (de-caseinated or cheese milk). The basis for on-farm extraction ('harvesting') of minor proteins from milk is protein purification chromatography (that is, selective adsorption of target proteins). This chapter reviews preparative chromatography techniques for extracting proteins from milk, although most analytical separations are generally not relevant to large-scale extraction of individual proteins. Before reviewing literature on industrial protein chromatography in general and milk protein purification in particular, a description of milk properties and its components is given, followed by a description of unit operations commonly used in dairy protein processing. The extraction/purification techniques of the two minor milk proteins LF and LP are described in detail because these were the model proteins used for on-farm capture. Chromatographic fractionation of raw whole milk is not described in the literature so this review discusses purification techniques for pre-treated milk such as skim and whey. The most suitable techniques for extracting proteins from raw whole milk are then discussed.

The composition of raw milk from individual cows can vary widely but milk processed in factories has a much more uniform composition and particle size. Since the protein fractionating system to be developed has to function with an AMS, some aspects of farming and harvesting milk are described.

2.2 Milk and its components

Milk is the first food of the neonate. Milk from domestic animals has been used for food for hundreds of years and it has been studied scientifically for well over 100 years. Hence it possibly is the best characterized of our major foods.

Total annual world production of milk is ~560 x 10^6 tonnes (85% cattle, 11% buffalo, 2% each sheep and goats). Other milking species such as camel, yak, horse and reindeer, are insignificant, although important in some regions (Fox 2001).

Milk is a complex colloidal dispersion of fat globules and various proteins in an aqueous solution of lactose, minerals and other minor components. Normal milk is approximately 87.4% water and 12.6% milk solids (3.7% fat, 8.9% milk solids non-fat; consisting of 3.4% protein, 4.8% lactose and 0.7% minerals and other minor components). Bovine, buffalo, ovine and caprine milk contain 3.4, 4.5, 5.5 and 3.0% weight percent protein respectively (Table 2-1) so ~20 x 10^6 tonnes of milk proteins are produced annually (Fox, 2001).

Table 2-1: Approximate composition of milk (g/L) (Playne *et al.* 2003).

Component	Cow	Human	Buffalo	Goat	Sheep
Total solids	125	129	171	130	163
Proteins	34	10	38	29	55
Casein proteins	28	4	--	23	--
Whey proteins	6	5.5	--	6	--
LF (mg/L)	10-50	2000	--	--	--
Fat	31	38	75	45	53
Lactose	48	71	49	41	46
Oligosaccharides	0.03-0.06	3.8	--	--	--
Riboflavin (mg/L)	1.57	0.43	1.02	1.14	4.36
Minerals					
Ash	7	2	8	8	9
Calcium	1.14	0.34	1.85	1.30	1.93
Phosphorous	0.93	0.14	1.25	1.06	0.99

Milk proteins can be broadly classified as caseins or whey proteins. The five bovine casein proteins (α-s_1, α-s_2, β, γ, and κ) are very well-characterized and have substantially different properties. Their molecular weights are between 20-25 kDa, they are phosphorylated, and display polymorphism (Fox 2001). The whey protein fraction of bovine milk contains four main proteins: β-lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA) and immunoglobulins (mainly IgG_1, with lesser amounts of IgG_2, IgA and IgM) (Table 2-2). Human milk contains no β-Lg and the principal immunoglobulin is IgA.

2.2.1 Factors affecting milk composition

On-farm practices such as breed, genetic selection, diet, and health can influence functionality of bovine milk (Auldist *et al.* 2000; Yang *et al.* 2000). For example, milk with softer fat (soft milk fat) can be obtained by grain feeding; and selective breeding can increase concentrations of special proteins such as α-casein and other minor milk components that have bioactive properties.

Table 2-2: Bovine milk proteins and their molecular weights (Varman and Sutherland 1994; Bylund and TetraPak 2003; Etzel 2004).

	Concentration (g/kg)	% of total protein	M_r of monomer (kDa)	pI
Casein				
α-s_1-casein	10.0	30.6	23	4.9/5.3
α-s_2-casein	2.6	8.0	25	4.9/5.3
β-casein	10.1	30.8	24	5.2
κ-casein	3.3	10.1	2	5.8
Total casein	26.0	79.5		
Whey proteins				
α-La	1.2	3.7	14	4.4
β-Lg	3.2	9.8	18.3	5.4
BSA	0.4	1.2	67	5.1
Immunoglobulins	0.7	2.1	up to 1000	5-8
Miscellaneous (including proteose-peptone)	0.8	2.4		
LF	0.1		77	7.9
LP	0.03		78	9.6
Total whey proteins	6.3	19.3		
Fat globule membrane proteins	0.4	1.2		
Total protein	32.7	100		
Glycomacropeptide	1.5		8.6	<3.8

M_r molecular weight

Supplementary feeding, breed effects, stage of lactation and seasonal changes influence milk composition (Coulon *et al.* 1994; 1998; Mackle *et al.* 1999; 2000; Nicholas *et al.* 2002; Turner *et al.* 2003a; 2003b; Auldist *et al.* 2004). Milking frequency also affects milk composition. For example, LF concentrations in cows milked once daily were higher than those milked twice daily (Farr *et al.* 2002). The LF levels in colostrum of Holstein-Friesian and Jersey (dairy breeds) is higher than in Japanese Black and Brown (beef breeds), being 2 mg/mL and 0.5 mg/mL respectively (Tsuji *et al.* 1990).

Functionality of milk components is also affected by post farm-gate practices such as storage conditions, pre-treatment, processing steps and product storage. Harvesting (milking) and post- harvest storage can affect milk quality and also enhance post-harvest deterioration during storage such as lipolysis, creaming, rancidity (off-flavours), denaturation and hydrolysis, which affect the factory processing characteristics of milk (Roupas 2001).

2.2.2 Milk harvesting

In New Zealand, milk is generally harvested twice daily (morning and afternoon). Effective farm layouts and dairy parlours minimize worker effort. Rotary machines on large revolving platforms can accommodate 60 or more cows, with automatic positioning and exit of cows. Labour-saving devices such as automatic gate openers, in-line milk metering and sampling, teat washing and cup removers have also been developed. Dairy farm staffing levels are between one person per 100 to 200 cows milked according to time spent milking and dairy parlour design. Hogeveen and Ouweltjes (2003) note two trends in developing high-tech milking equipment that may break the labour barriers to expansion: high-capacity milking parlours with high throughput of cows per person per hour and automatic milking systems where manual labour is replaced by a milking robot.

AMSs are generally used for housed cows (Fisher *et al.* 2004). Adoption of the AMS was slow initially, but by 2002 over 1000 herds (Figure 2-1) were being milked by AMS, with at least seven equipment suppliers offering such systems (Hillerton 1997; de Koning and van de Vorst 2002). The New Zealand dairying industry is predominantly pasture based; housed herds are limited, mainly due to the high cost of supplementary (grain) feeding. The AMS has been adapted to pasture-based dairying and systems exist in Ireland, Australia and New Zealand. Reported benefits of AMS are labour savings (Hayashi and Kawamura 2002; Fisher *et al.* 2004) and improved yields (Fisher *et al.* 2004).

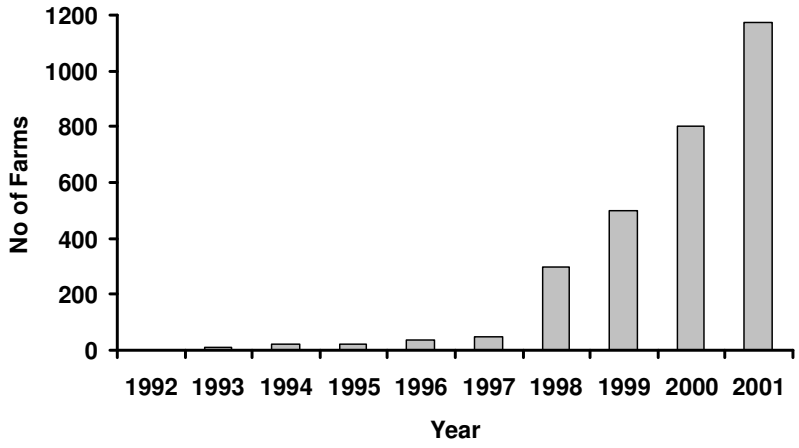


Figure 2-1: Number of farms with automatic milking systems in Europe (de Koning and van de Vorst 2002).

Currently the AMS cannot keep pace with high throughput dairies (e.g. 200-1000 cows being milked twice per day) because it is based on distributed milking with continuous cow traffic over 24 hours. Cost is also a deterrent being $250,000-$300,000 per robotic station that can handle 160 milkings in 24 hours (Dexcel 2006).

2.2.3 Milk as a source for transgenic proteins

Transgenic animals with foreign genes able to express specific proteins in their milk are used as sources of therapeutic proteins. Milk from transgenic dairy animals can be a viable, ample and specific pathogen-free source of recombinant and natural proteins to augment therapeutics derived from sources such as human plasma. Transgenic manufacture using animals or plants can be up to 20 g/L (Werner 1999) compared with production in bioreactors of 500-800 mg/L, making transgenic cattle an attractive vehicle for large-scale production of biopharmaceuticals (Owen and Chase 1997; van Berkel *et al.* 2002). It has been used for producing recombinant human LF (rhLF) and other examples using milk as a source of therapeutics have been reported (Denman *et al.* 1991; Ebert *et al.* 1991; Wright *et al.* 1991; Wolf *et al.* 1997; Degener *et al.* 1998; Pollock *et al.* 1999; Wright and Noble 1999; Andrews *et al.* 2000; Konrad *et al.* 2000; Fulton 2001; Baruah *et al.* 2003; Lindsay *et al.* 2004; Nikolov and Woodard 2004; Parker *et al.* 2004; Capezio *et al.* 2005). These natural and recombinant sources of therapeutic proteins generate the need for economic, high-resolution purification technologies.

2.2.4 Nutraceutical and functional foods

Functional foods (also referred to as physiologically functional foods, nutraceuticals, designer foods or pharmafoods) provide the consumer with an identified health benefit over basic nutritional value. More refined methods of fractionation and analysis of proteins has renewed interest in minor milk proteins. The new tools of biotechnology make it possible to take different approaches to studying components of milk. The current focus on milk protein components is based mostly on the physiological effects and hence their commercial value. It is now widely accepted that milk proteins demonstrate multiple functions such as bioactivity (Table 2-3) as well as being a high-grade source of dietary nitrogen in human nutrition (Tome and Debabbi 1998; Parodi 1999; Tsuda *et al.* 2000; Korhonen *et al.* 2001; Korhonen and

Pihlanto-Leppälä 2001; Meisel 2001; Parodi 2001; Steijns 2001; Kilara and Panyam 2003; Ohashi *et al.* 2003; Seifu *et al.* 2005; Severin and Xia 2005; Walsh and FitzGerald 2005).

The global functional food and nutraceutical market was worth US$50-63bn in 2004 (AROQ Limited 2004) and is expected to grow to US$167bn by 2010 (14% annually). Dairy foods represent a significant proportion of this market (Tome and Debabbi 1998; AROQ Limited 2004). Dairy constituents, notably the proteins and peptides, with multi-functional components, provide desirable features such as physical functional traits, nutritional qualities and an increasing array of substantiated bioactivities (Playne *et al.* 2003).

Table 2-3: Milk-derived components as possible ingredients of functional foods.

Components	Source
Antimicrobial and anti-inflammatory substances	Cheese, whey, colostral whey, fat globule membranes
Bioactive peptides	Caseins, whey proteins, fermented cheeses, fermented milks
Antibodies (immunoglobulins)	Colostral and cheese whey, fat free milk
Whey proteins and their hydrolylates	Cheese whey, acid whey, whey powder
Lactose and its derivatives	Milk, cheese whey
Growth factors, cytokines	Colostral whey
Conjugated linoleic acid (CLA)	Milk fat
Minerals (calcium salts)	Acid whey, fermented milks, cheeses
Organic acids and their salts	Cheese whey

The worldwide market for both consumer products and ingredients from milk will continue to grow, with technology often preceding market demand (Huffman and Harper 1999). The challenge for science and technology is to isolate these ingredients in a cost-effective manner while maintaining their inherent functional and nutritional traits. The approach to functional foods needs to be considered not only from a processing point of view but as a business model. Aspects such as market, consumer perceptions, food safety issues, value chain and regulatory issues need to be considered. Niche markets are valuable but usually difficult and costly to establish. Furthermore, palatable foods such as high-protein beverages must be developed to provide attractive routes for consumption (Etzel *et al.* 2006).

Technologies needed to harness the health and well-being potential of milk and milk products include analytical capability to determine the levels of components such as

peptides and surface proteins present in milk, bioactivity determinations and isolation/concentration processes (Marshall and Fenwick 1998). A review of chromatographic phases for process development by Bensch *et al.* (2005) highlighted the need to be "first to market" and to cut production costs to obtain fast process development while optimising processing parameters.

2.3 Protein purification

Laboratory-scale separation techniques for isolating proteins by salt or solvent precipitation, ion exchange chromatography and/or crystallization have been available for a long time. The processes available for commercial-scale production especially milk proteins, fall into three main categories: selective adsorption; membrane filtration based on different size and charge; and selective precipitation by adjusting physical properties of the solution.

These processes are used either individually or in combination followed by recovery using selective elution, filtration, etc. Various modes of purification exist. Column chromatography is widely used for selective adsorption and elution (Chisti and Moo-Young 1990; Strange *et al.* 1992), membrane filtrations using discs, filter cartridges and hollow fibres (Zydney 1998; Demmer and Nussbaumer 1999; Gosh 2002) and stirred tank systems in continuous and batch modes.

The highly specific and mild conditions needed to separate proteins and peptides from complex mixtures make separation difficult. Most separation schemes are scaled-up laboratory procedures (Korhonen *et al.* 1998) usually involving scaling chromatographic methods by adapting large-scale equipment.

It is essential to use or develop technologies that retain or enhance bioactivity of the desired component (Korhonen *et al.* 1998). Technologies that target protein functionality are already emerging. These include ultra pressure processing (DeSilva *et al.* 2003), membrane adsorbers (Splitt *et al.* 1996; Zydney 1998; Gosh 2002), crossflow filtration (Ulber *et al.* 2001), pulsed electric field processing (Bendicho *et al.* 2002), super-paramagnetic anion-exchangers in high-gradient magnetic fishing (Heebøll-Nielsen *et al.* 2004), colloidal gas aphrons generated for selective adsorption using surfactants (Fuda *et al.* 2004), and supercritical fluid or high pressure carbon dioxide extractions (Tomasula *et al.* 1997).

2.4 Chromatography

Chromatography can be operated in batch or continuous modes in packed columns, expanded (or fluidised) beds and stirred (suspended) tanks. The versatility of chromatography is due to the affinity of components for a sorbent phase based on size, charge, or hydrophobicity. This can often be modulated by adding solvents (reverse phase chromatography) or salts (ion exchange or hydrophobic interaction chromatography). Specialist sorbents (resins) are available for particular biomolecules (Hedhammer and Hober 2005).

2.4.1 Affinity chromatography

Affinity chromatography uses protein-protein interactions for selective extraction (Przybycien 1998). Specialist affinity media that target and capture a particular species in milk whilst leaving the rest of the milk for further processing can be formulated. However, toxic materials leaching from the media into the milk could be an issue and requires further work.

2.4.2 Ion exchange chromatography

Ion exchange chromatography is the most common type of adsorption chromatography. Ion exchange involves selectivity for cationic or anionic interaction with the target species. Anion exchange with diethlyaminoethyl (DEAE) moieties covalently attached to the adsorption matrix is more commonly used at pH 6-8 whilst cationic exchange is more commonly used at pH 4.5-6.0. Several factors can influence adsorption but two most important are pH and salt (ionic concentration). Once the target protein is adsorbed to the matrix, varying the salt concentration elutes both the target protein and background proteins. Typical protein binding is about 20-40 mg total protein per ml of matrix.

2.4.3 Column-based chromatography

Packed bed column chromatography offers high surface areas, giving high capacities and hence yields or recoveries if operated at optimum linear velocities (Chisti and Moo-Young 1990). This process is used extensively for biopharmaceuticals and on a very large industrial scale for whey fractionation and cation exchange capture of LF and LP from whey and skim milk feeds.

The media (resin) is packed into a column and solution passes though the bed. Columns are usually wide and short to minimize pressure drop and maximize flow rate. Industrial columns usually have beds 20 cm high and diameters up to 2.0 m, (total volume of 628 L) and operate at flow rates of 21 L/s. Media such as SP BB resin can minimize column clogging. On-line monitoring can be done using conductivity, flow rate, temperature, pH and UV (usually at 280 nm) detectors.

2.4.4 Batch (stirred tank) chromatography

Although batch procedures are less efficient than column techniques, they offer advantages, especially where very large sample volumes with a low protein concentration have to be processed. General practice is to allow an hour for adsorption and at least 30 minutes for desorption. Batch chromatography is very useful when concentrating dilute solutions or separating the target protein from gross contaminants during initial stages of a purification scheme.

Batch stirred-tank chromatography can be used to extract protein from complex mixtures and may be useful for on-farm separations because of the variable and, at times, high (up to 10% ^{w}/v) fat content in an individual cow's milk

Batch stirred-tank separation can be a rapid technique with minimal technical difficulties due to resin swelling or shrinking. Stirrer speed is important as fines are generated if the resin is stirred too vigorously. Fines can increase filtration time. Stirring speeds of 150 rpm (Rowe *et al.* 1999) and 200 rpm (Morison and Joyce 2005) have been used without damaging the resin.

2.4.5 Fluidised or expanded bed chromatography

In fluidised or expanded bed (EBA) adsorption, resin is suspended (fluidised) and the desired species are adsorbed from the feed stream flowing by. Column washing and elutions are done while the resin is suspended. A fluidised bed reduces problems associated with column clogging but adsorption and desorption times are longer than in column chromatography.

2.5 Selective precipitation

Selective precipitation involves adjusting physical properties of solution by adding organic solvents, enzymes or salts, pH changes and/or heating to promote selective insolubility. For example selective precipitaton is used to obtain caseins from milk

for making cheese (Pearce 1983; Uchida *et al.* 1996; Gesan-Guiziou *et al.* 1999). Crystallization can be used to recover milk components. For example, Harju and Heikkil (1989) recovered lactose by concentrating cheese whey to crystallize the lactose.

2.6 Membrane processing technologies

The dairy industry uses membrane processes such as cross-flow microfiltration (CFM), ultrafiltration (UF), microfiltration (MF), reverse osmosis (RO), dynamic membrane filtration (DMF), electro ultrafiltration (EU), and nano-filtration (NF) extensively for standardization of many products and protein concentration (Brans *et al.* 2004). Each process operates over a different particle size range (Figure 2-2). Ultrafiltration with polymeric or ceramic membranes completely retains whey proteins and produces retentate with lactose and minerals that can be further processed by evaporation and spray drying (Zydney 1998). Diafiltration can be used to further reduce lactose and minerals content in whey. There are many reviews on membrane filtration in the dairy industry (Marshall *et al.* 1993; Rosenberg 1995; Zydney 1998; Demmer and Nussbaumer 1999; Ghosh 2002).

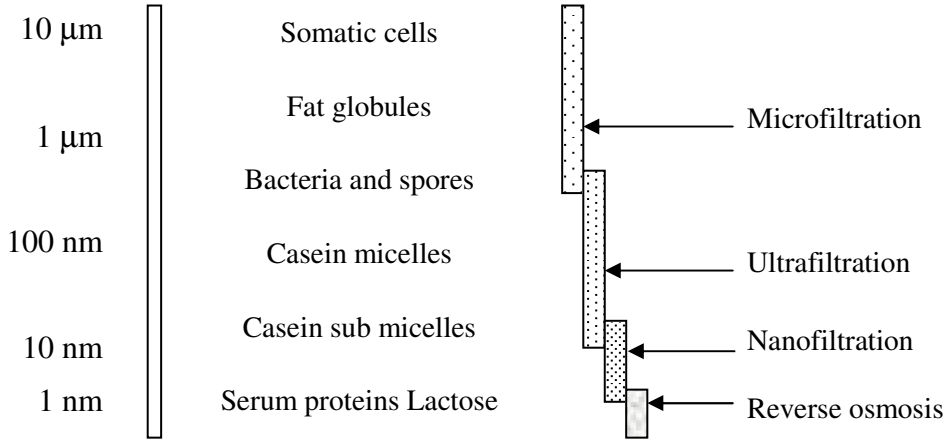


Figure 2-2. Membrane processes for separating milk components (Brans *et al.* 2004).

2.7 Overview of dairy protein fractionation

There are two main classes of dairy proteins. Traditionally, caseins have been extracted as cheese or dried as casein powder, leaving whey as a by-product. Whilst

butterfat and anhydrous butter fat have been traded as bulk products alongside milk powder, whey has been a waste product, often creating disposal issues. Membrane and gel filtration techniques developed in the 1970s allowed large-scale concentration of whey and manufacture of whey protein concentrates and isolates (Jelen 1983; Maubois *et al.* 1987; Jelen 1991; Hobman 1992). Large quantities of whey are produced. For example, Durham *et al.* (2003) reported that the Australian cheese industry produced 3.4 million tonnes of whey protein in 2001, which contained 160 000 tonnes of whey proteins but only 50 000 tonnes of whey was recovered. The low recovery, together with the associated effluent problems for disposing of the whey poses environmental risks. Other cheese producing countries have had similar issues. For example Germany produced 10 million tonnes of whey in 1996 as a by-product of cheese making (Ulber *et al.* 2001).

Issues with waste disposal led to innovations to recover and use the components in whey, firstly as whey protein concentrates and later as individual proteins and lactose fractions.

2.8 Properties of whey proteins and their extractions

The protein product, whey protein isolate (WPI), produced by single-stage batch anion exchange of waste from cheese production, is a crude mixture of acidic whey proteins and contains mainly α-La, β-Lg, BSA and immunoglobulins (mainly immunoglobulin G or IgG). Two whey proteins not captured by anion exchange chromatography because of their high isoelectric points are LF and LP. These basic proteins are extracted from whey or skim milk by cation exchange chromatography and sold as specialty ingredients.

The low total solids in whey (63 g/L) are mainly lactose (50 g/L) and proteins (6 g/L) (Doultani *et al.* 2004) of various molecular weights and isoelectric points (Table 2-2).

The LF and LP may be extracted from skim (non fat) milk or, more commonly, from whey produced as permeate during membrane concentration of milk or after precipitating out the caseins in cheese production (Zydney 1998; Tomita *et al.* 2002).

The literature reports many efficient protocols to fractionate milk (Mirabel 1978; Henricus 1983; Smithers *et al.* 1996) and for isolating natural milk components such as LF and LP (Kawakami *et al.* 1988; Burling 1989; Yoshida and Xiuyun 1991b; Kussendrager *et al.* 1994). Fat and caseins are normally removed before extracting recombinant proteins from the milk of transgenic animals (van Berkel *et al.* 2002).

Many authors have examined capture and analysis of whey proteins by chromatography (Lonnerdal and Carlsson 1977; Humphrey and Newsome 1984; Andrews *et al.* 1985; Chaplin 1986; Al-Mashikhi and Nakai 1987; Al-Mashikhi *et al.* 1988; Yoshida and Xiuyun 1988; Donnelly 1991; Visser *et al.* 1991; de Frutos *et al.* 1992; Morr and Ha 1993; Konecny *et al.* 1994; Francis *et al.* 1995; Torre and Cohen 1996; Felipe and Law 1997; Geberding and Byers 1998; Hahn *et al.* 1998; Noppe *et al.* 1999; Elgar *et al.* 2000; Xu *et al.* 2000; Ye *et al.* 2000c; Doultani *et al.* 2004). The milk and whey is usually extensively pre-treated before ion exchange capture of proteins in the laboratory. The following describes some recent examples of typical pre-treatments in laboratory studies.

- Hahn *et al.* (1998) examined the performance of several commercially available pharmaceutical grade cation exchangers (S-HyperD-F, Fractogel EMD 650(S), SP FF, Macro Prep High S) to extract protein from acid whey. Milk was first centrifuged at 4420×*g* for 30 min to remove fat, then acidified to precipitate casein and centrifuged at 17,700×*g* for 30 min, diluted with distilled water and then filtered through a 0.45-μm filter before being applied to the column.
- Bounous (1990) produced biologically active whey proteins by cooling milk to 4°C immediately after milking then precipitating the caseins by reducing pH to 4.6 with lactic acid. Rennet was added and the temperature raised to 30.5°C with low speed agitation. Whey was ultrafiltered with 10 000 and 17 000 molecular weight cut-off membranes, retaining α-La and whey protein concentrate.
- Doultani *et al.* (2004) used cation exchange chromatography to produce several protein products from mozzarella cheese whey. The pH was adjusted with H_2SO_4 and then whey was filtered through Whatman No.5 filter paper before being applied to a column packed with SP BB.
- Ye *et al.* (2000b) used both anion and cation exchange chromatography to isolate α-La, β-Lg, LF and LP from rennet whey produced from skim milk that had first

been defatted by centrifugation. After 1-hr incubation with rennet, the caseins were separated by filtration and the whey was then centrifuged at 10,000×*g* for 25 min before being applied to the column.

- Lyndsay *et al.* (2004) purified recombinant DNA-derived factor IX from milk of transgenic pigs. They removed fat by centrifugation and used EDTA to dissolve the casein micelles. The resultant fluid was diluted 5-fold in heparin loading buffer before being loaded onto a heparin-Sepharose Fast Flow column.

There is, therefore, a wide difference between the normal practices of dairy production processes and the usual requirement of protein purification processes to minimise the number of steps and to process quickly. The resulting loss in yield can be significant. Korhonen *et al.* (1998, 1991) lists deleterious effects of various process operations such as heat treatment, pH modification and storage on proteins and amino acids.

2.9 Properties and applications of LF and LP

2.9.1 LF

LF is a single-chain glycoprotein. The non-glycosylated form has a molecular weight of 80 kDa and pI 8 to 9 (Naidu 2002). It is a versatile, bioactive milk protein that plays an important role in immune response and helps protect the body against infections. Scientific studies show that LF also prevents growth of pathogens, has antibacterial and antiviral properties, controls cell and tissue damage caused by oxidation, and facilitates iron transport (Brock 2002).

Human milk has the highest LF concentrations (1.4 to 2.0 mg/mL) of the mammalian milks (Playne *et al.* 2003) compared with the average LF concentration in bovine milk of 0.1 mg/mL (Reiter 1985; Barth and Schlimme 1988; Renner 1989; Kawakami *et al.* 1990; IDF Bulletins 1991; Yamauchi 1991; Satue´-Gracia *et al.* 2000). There are large variations in LF concentrations (0.06 to 1.0 mg/mL) of milk from individual cows (Indyk and Filonzi (2005), which are similar to those observed in this study (0.08-1.13 mg/mL, section 7.3). Whey or skim milk (Table 2-4) is the normal source for LF used in product formulations. Highly purified bovine LF is used in nutritional formulations (especially infant formula), adult health products, medical nutritionals, cosmetics, health foods, beverages and animal feeds, shelf-life

extension of natural products such as meat (Taylor *et al.* 2004); its potential applications are extensive.

If LF could be captured from the milk of high LF-producing cows immediately after the cow is milked, both LF yields and bioactivity could be maximised. This also creates the opportunity to selectively extract LF from high-LF producing animals.

Table 2-4: The LF in various bovine milk fractions measured by reverse phase-high pressure liquid chromatography (Palmano and Elgar 2002).

Type of feed	Concentration (mg/mL)
Cheese (dried whey protein concentrate)	0.30 (% powder mass)
Acid (liquid whey protein concentrate)	0.99
Lactic (whey protein concentrate)	0.78
Basic fraction	0.40
Skim milk whey	0.15

2.9.2 LP

LP is present in most mammalian milks (FSANZ 2002; Naidu 2002) and has a molecular weight of 78 kDa and pI of 9.6 (Sievers 1981; Watanbe *et al.* 2000). It is one of the most abundant and active enzymes in bovine milk and catalyses the inactivation of a wide range of micro-organisms (Claeys *et al.* 2002). Hence, it acts as a natural preservative in milk. The LP-systems, which are harmless to mammalian cells, have been identified in the natural anti-microbial systems of human secretions such as saliva, tear-fluid and milk (Ekstrand and Bjorck 1986; Ekstrand 1989; Elgar *et al.* 2000; Palmano and Elgar 2002).

Concentrations of LP in raw whole milk are influenced by factors such as breed, stage of lactation, time of year, health status of animals etc. Values of 38 mg/L (Seifu *et al.* 2005) and 30 mg/L (Seifu *et al.* 2005) have been reported. LP is used in healthcare, animal feeds and food applications. A commercial application of LP is as coatings, especially in meat. LP is used to increase shelf life of milk by activation of LP-system in milk where refrigeration is unavailable (Seifu *et al.* 2005).

2.10 Unit operations used in dairy processing

Liquid milk products contain proteins, fats and sugars. Many processes are used to make commercial products such as milk powders, cheese, fats, and protein products.

Fat products include anhydrous milk fat (AMF or ghee) or butter. Powder products include whole milk powder, skim (no fat) milk powder and protein powders (whey protein isolates, caseinates) and individual protein powders. These products are used in many dairy products and other food formulations. Numerous unit operations are involved in processing them to a stable form.

Milk processing starts on farm with milk harvesting (using semi-automated processes involving pulsators that generate vacuum to aid milking). The harvested milk is immediately cooled and stored at 4-8^{o}C under gentle stirring before collection (1-2 day interval) by tankers (ambient temperatures) and transported to a centralized facility (factory) for processing. At the factory, milk is pumped into large silos and stored at 4-8^{o}C. Processing begins with centrifugation (removal of fat as cream) followed by pasteurization (variable heat treatment e.g. 72^{o}C for 15 seconds). Subsequent processing depends on the end use of the product, but can involve many filtration steps such as reverse osmosis and UF for concentration. Most liquid milk in New Zealand is either processed into various milk powders or used for cheese-making. The main unit operations involved (including fractionation of proteins) are evaporation and drying, centrifugation, crystallization, chromatography, ion exchange, electrodialysis, membrane processes and absorption (e.g. activated carbon or colour removal).

Many unit operations occur before LF and LP are captured using ion exchange chromatography (Figure 1-1). This scheme does not show all the chemical balancing operations, desalting, pumping cycles, mixing, screening, washing, etc.

2.11 LF and LP purification techniques

Johanson (1960) was one of the first to isolate LF from human milk using a calcium phosphate matrix. The process was based on a physico-chemical interaction between protein and the solid matrix. Other solid supports such as cellulose phosphate, silica, diatomaceous earths, or bentonite have also been used for LF adsorption (Spring and Peyrouset 1982; Foley and Bates 1987).

Fractionating LF and LP from cheese whey, skim milk and colostral whey has been demonstrated on a laboratory scale (Spring and Peyrouset 1982; Foley and Bates 1987; Burling 1989; Uchida *et al.* 1996) and on an industrial scale from whey, whey

protein concentrate, or skim milk using cation exchange resin. Ion exchange membranes have also been used to fractionate LF and LP (Chiu and Etzel 1997; Zydney 1998; Ghosh and Cui 2000; Ghosh 2002).

High purity LF, LP and secretory compounds have been separated from milk and whey using cross-linked polysaccharide, cellulose or acrylamide cation exchange resins with carboxyl, sulphonic acid or phosphoric acid functional groups (Uchida *et al.* 2003). The LP was eluted in buffer (0.2-0.5 ionic strength), secretory component (0.1-0.5 ionic strength) and LF (≥ 0.5 ionic strength). Uchida and coworkers used SP-Toyopearl, SP FF, sulfonated Chitopearl, and CM-Cellulofine with cheese whey that was desalted using UF before ion exchange from skim milk. Contacting was done in a stirred tank and the resin was retrieved and packed in column for washing and eluting the LF, LP and secretary components with NaCl. Because whey protein concentrates and individual whey proteins have commercial value, processing technologies have been patented for purifying LF and LP (Uchida *et al.* 1996) and whey proteins (Jensen and Larsen 1995).

Pre-treating milk prior to chromatographic capture of proteins is virtually universal (Lonnerdal and Carlsson 1977; Humphrey and Newsome 1984; Andrews *et al.* 1985; Chaplin 1986; Al-Mashikhi and Nakai 1987; Al-Mashikhi *et al.* 1988; Yoshida and Xiuyun 1988; Donnelly 1991; Visser *et al.* 1991; Yoshida and Xiuyun 1991a; de Frutos *et al.* 1992; Morr and Ha 1993; Konecny *et al.* 1994; Francis *et al.* 1995; Torre and Cohen 1996; Felipe and Law 1997; Etzel *et al.* 1998; Geberding and Byers 1998; Hahn *et al.* 1998; Noppe *et al.* 1999; Elgar *et al.* 2000; Xu *et al.* 2000; Ye *et al.* 2000a; Ye *et al.* 2000c; Ye and Ng 2000; Doultani *et al.* 2004). A review of downstream processing of recombinant proteins from transgenic feedstock identifies removal of fat globules and casein micelles from milk by centrifugation, acid precipitation, and/or membrane filtration as critical for reducing fouling in subsequent purification steps (Nikolov and Woodard 2004).

Pre-treatment by centrifugation, precipitation, Ca^{2+} chelation and/or filtration methods are done to remove fat and caseins before LF and LP are extracted. Chromatography especially ion exchange but to a lesser extent affinity, chelation, gel filtration and hydrophobic interaction based chromatography is used exclusively for extracting LF and LP from whey or defatted milk (Table 2-5). Batch foaming and

reverse micellar techniques are emerging for extracting LF and LP (Saleh and Hossain 2001; Fuda *et al.* 2004; Noh *et al.* 2005).

Table 2-5: LF and LP extractions.

Separation/purification technique	Target protein/compound	Reference
IEX chromatography (CM-Sepahdex-C50)	LF from human milk	(Johanson (1960)
IEX chromatography (DEAE cellulose)	LF from milk	(Goves (1965)
Metal-chelate affinity chromatography	LF from human milk	(Lonnerdal & Carlsson 1977)
Single chromatographic step affinity chromatography (Heparin Sepharose)	LF from human whey	(Blackberg & Hernell (1979)
Affinity chromatography	LF from human whey	(Bezwooda & Mansoor (1986)
IEX (Fast protein liquid chromatography)	LP, LF and lysozyme	(Ekstrand and Bjorck 1986)
Gel filtration chromatography	Immunoglobulins, LF from whey	(Al-Mashikhi and Nakai 1987)
Batch extraction (cellulose phosphate) (80% yield, 96% purity)	LF from human whey	(Foley and Bates 1987)
Immobilised monoclononal antibodies (specialised affinity chromatography)	LF	(Kawakami *et al.* 1987)
Gel filtration chromatography	LF from whey	(Al-Mashikhi and Nakai 1987)
Extraction using sulphate compound	LF from defatted milk	(Kawakami *et al.* 1988)
Chelating chromatography	Immunoglobulins, LF from whey	(Al-Mashikhi *et al.* 1988)
IEX chromatography	LF	(Okonogi *et al.* 1988)
IEX chromatography	LP, globulin	(Yoshida 1988)
IEX chromatography	LP, LF from whey	(Burling 1989)
Hydrophobic interaction chromatography	LF	(Yoshida 1989)
Affinity chromatography (insolubilized bovine β-Lg)	Human LF	(Ena *et al.* 1990)
Microfiltration and affinity chromatography	LF, IgG from cheese whey	(Chen and Wang 1991)
Batch ion exchange (80% LP and 90% LP yields -60 min adsorptions)	LP, LF from whey	(Dionysius *et al.* 1991)
IEX chromatography (carboxymethyl cation)	LF from whey LP	(Yoshida and Xiuyun 1991a)
IEX chromatography (sulphopropyl cation)	LP, LF from whey	(Yoshida and Xiuyun 1991b)
Process (patent)	LF, LP from milk and milk products	(Kussendrager *et al.* 1994)
IEX chromatography (cation exchange resin)	LF, LP, secretory component	(Uchida *et al.* 1996)
IEX chromatography (cation exchange membrane)	LF, LP from whey	(Chiu and Etzel 1997)
IEX- column and expanded bed chromatography	LF from skim milk	(Etzel *et al.* 1998)
IEX chromatography (cation) evaluation of resins	LF, LP, IgG, BSA	(Hahn *et al.* 1998)
LF isolation from milk	LF from milk	(Nuyens and Van Veen 1999)

Table 2 continued

IEX chromatography (DEAE and sulphopropyl)	LP, LF, α-La, β-Lg A and B from whey	(Ye *et al.* 2000b)
IEX chromatography	LF A and B from colostrum	(Yoshida *et al.* 2000)
Foam fractionation (semi-batch foaming process)	BSA, α-La and LF	(Saleh and Hossain 2001)
Expanded-bed column chromatography (Streamline SP as adsorbent, 90% yield)	LF from skim milk	(Shiozawa *et al.* 2001)
CFF, cation exchange membranes (IEX)	LF from whey	(Ulber *et al.* 2001)
Transferrins, semi-large scale with HIC	LF from skim milk	(Tomita *et al.* 2002)
IEX chromatography (S Sepharose), and Marco Prep	Recombinant human LF in skim milk (transgenic cows)	(van Berkel *et al.* 2002 (van Berkel *et al.* 2002)
IEX chromatography (SP-Sepharose cation)	LF from colostrum	(Zhang *et al.* 2002)
IEX chromatography (sulphate-linked IEX)	LF, LP, secretory component	(Uchida *et al.* 2003)
IEX chromatography	LF, LP from raw whole milk	(Chand and Fee 2004)
IEX chromatography (SP BB)	Whey proteins: WPI, α-lac, β-Lg, IgG GMP, LF and LP	(Doultani *et al.* 2004)
Colloidal gas aphrons (CGAs) generated from anionic surfactant, AOT	LF, LP from whey	(Fuda *et al.* 2004)
Superparamagnetic anion- and cation exchangers (high-gradient magnetic fishing)	LF, LP, immuno-globulins from whey	(Heebøll-Nielsen *et al.* 2004)
IEX - Batch chromatography (cation-exchange phosphocellulose)	LP from acid whey	(Samsam and Naieri 2004)
IEX - chromatography (cation)	LF, LP from raw whole milk	(Fee and Chand 2005)
Reverse micelles formed by cationic surfactants	LF	(Noh *et al.* 2005)
Affinity chromatography Immobilised peptide as affinity ligands	LF from skim milk	(Noppe *et al.* 2006)

IEX – ion exchange chromatography; HIC – hydrophobic interaction chromatography, GMP glycomacropeptide

2.11.1 Chelation chromatography

Chelate-interaction-chromatography has been used to isolate immunoglobulins, LF and LP on non-sterilised skim milk (Vervaeck *et al.* 1992; Nikolov and Woodard 2004). Al-Mashiki and Li-Chan (1988) used a similar chelating technique using transition metals to isolate immunoglobulins and LF. Lonnerdal and Carlsson (1977) used metal chelate affinity chromatography to extract LF from human whey.

2.11.2 Affinity chromatography

Affinity chromatography has been described (Blackberg and Hernell 1979; Kawakami *et al.* 1988; Rejman *et al.* 1989; Chen and Wang 1991; Blomkalns and Gomez 1997). Some researchers used heparin immobilized on agarose and Sepharose to purify LF and immunoglobulins from whey. The heparin interaction is believed to

be by ion exchange of sulphate groups present in the glucosaminoglycan. Kawakami *et al.* (1988) used monoclonal LF antibodies to extract LF from human and bovine milks.

Single step affinity chromatography with heparin Sepharose was used to extract LF from cultured yeast (Paramasivam *et al.* 2002). Affinity chromatography combined with large scale ion-exchange (heparin chitosan bed, Sulphonated Chitopearl 3L) chromatography was used to isolate LF, LP and secretory compound from whey (Uchida *et al.* 2003). The LF and LP were eluted using citric buffer (pH 4.0) with 0.5 M NaCl, tightly bound-LF was eluted with 10 mM hydrogen carbonate (pH 7.6) with 0.7-1.0 M NaCl, and secretory compound was eluted at pH 6.0.

Grasselli and Cascone (1996) investigated the effects of pH (5, 7 and 9) and eight triazinic dyes using affinity chromatography to purify LF from rennet whey. Optimum conditions using Red HE-3B at pH 7.0 in a batch extraction gave a yield of 82% and a purity of 98%. Noppe *et al.* (2006) used selected phage clones expressing a peptide with high binding affinity coupled to macroporous poly(dimethylacrylamide) monolithic column to extract LF from skim milk. Bound LF of >95% purity was eluted using 1 M NaCl.

2.11.3 Hydrophobic interaction chromatography

Yoshida (1988) isolated globulin and LP from neutralized bovine milk acid whey using UF and hydrophobic interaction chromatography with Toyopearl 650M to extract LF. The LF was eluted with 0.25 M acetic acid. Tomita *et al.* (2002) used semi large-scale hydrophobic interaction chromatography to extract LF from cheese whey or skim milk.

2.11.4 Ion exchange chromatography

Extracting LF and LP using carboxymethyl ion exchange chromatography, as described by Johanson (1960), albeit other media, is still one of the most effective methods. Okonogoi *et al.* (1988) used a cation exchanger with carboxymethyl groups, which had with haemoglobin adsorption properties to extract LF from skim milk or whey. After desorption using salts, yields were >80%.

Ulber *et al.* (2001) used continuous crossflow filtration steps to remove particulate matter from whey before using an ion exchange membrane adsorber with a dynamic binding capacity of 0.2 mg/cm^2 to extract the LF. The process extracted 8 g of LF (feed 0.1 g/L) from two cycles in an hour (2 L/min), with 66% yields.

Dionysius *et al.* (1991) used CM Sephadex in a stirred tank to investigate the binding characteristics of LF and LP from cheese, acid and rennet whey. The LF recovery was not affected by whey type. The maximum binding capacity occurred with cheese whey at pH 7.0. More than 90% of the original amount of LF in the whey was recovered for a 60-minute contact time.

Heebøll-Nielson *et al.* (2004) used superparamagnetic anion- and cation-exchangers to fractionate bovine whey proteins as a model study for high-gradient magnetic fishing. The immunoglobulins were separated from other proteins by desorption with low concentration NaCl (≤0.4 M). The LF and LP were co-eluted to give 28-fold purification of LP over the starting material.

Chiu and Etzel (1997) used 3-5 μm cross-linked regenerated cellulose membranes with immobilized sulfonic moieties to extract LF and LP from whey. The reactor had 790 cm^2 of membrane in a 16-mL bed volume. After 12 cycles, the LF and LP were eluted with 0.9 and 0.3 M NaCl to give 50 and 72% recovery respectively.

Hahn *et al.* (1998) reported that SP FF, S-Hyper D-F and Fractogel EMD SO 650 (S) resins were suitable for large-scale purification of bovine whey proteins. The lower binding capacities (ca. 3.2-3.3 mg IgG per mL of gel) of the resins used were thought to be due to the low pH of the whey.

Gerberding and Byers (1998) describe a preparative ion exchange process for separating and recovering α-La, β-Lg, BSA, IgG and lactose from whey feed of pH between 5.5 and 5.8, using Q- and SP BB resins. The α-La and BSA were extracted with both anion and cation exchange resins and β-Lg with the anion exchange resin. The BSA, IgG and lactose were extracted mainly with cation exchange resin with varying degrees of recoveries (Table 2-6).

Table 2-6: Compositions of serum streams using anion and cation exchange columns for one CV (Gerberding and Byers, 1994).

	α-La	β-Lg	BSA	IgG	Lactose
Anion exchange					
Feed (% ^{w}/w)	1.68	7.31	0.40	0.82	9.72
Breakthrough (%)	63.6	6.1	78.8	94.2	100
Peak 1 fraction (%)	36.43	0.49	2.20	5.80	0
Peak 2 fraction (%)	-	93.4	19.0	-	-
Cation exchange					
Feed (%^{w}/w)	1.12	0.46	0.42	0.82	57.99
Breakthrough (%)	61.8	97.3	42.3	83.1	14.3
Peak 1 fraction (%)	38.2	2.7	57.7	16.9	85.7

Foley and Bates (1987) extracted LF from human whey using cellulose phosphate in a batch stirred tank system with a rapid, two-step procedure and obtained average yields of 80% and 96% purity.

Etzel (2004) used a prototype column chromatography to fractionate acid whey that had first been microfiltered (1.4 μm). The reported flow rates were low, giving long loading times (Burling 1989; Chiu and Etzel 1997; Etzel *et al.* 1998; Etzel 2004).

2.11.5 Gel filtration chromatography

Gel filtration has not been extensively used to separate LF and LP from milk and whey although Al-Mashiki and Nakai, (1987) used this method to separate LF and immunoglobulins from whey.

2.11.6 Other non chromatographic methods

Saleh and Hossain (2001) describe a semi-batch foaming process using a 530-mm long glass column fitted with a stainless steel sparger to generate bubbles. A yield of 87% was obtained from a mix of BSA, α-Lg and LF. Noh *et al.* (2005) selectively extracted LF from other whey proteins using reverse micelles formed by cationic surfactant, cetyldimethyl ammonium bromide in 50 mM berate buffer (pH 9).

Bargeman *et al.* (2002) isolated bioactive peptides from a αs_2-casein hydrolysate using electro-membrane filtration (EMF). They believed EMF to be more selective than membrane filtration, less costly than chromatography and an alternative for isolating bioactive proteins and peptides, especially those that carry strong charged components.

2.12 Other milk protein purifications

Milk proteins, other than LF and LP that could be of interest and possible targets for on-farm extractions exist. Although most of the literature discusses purification from skim milk and whey, direct purification from raw whole milk was demonstrated during this study (Chand and Fee 2004; Fee and Chand 2005).

There is interest in industrial-scale fractionation of casein for special applications. For example, β-casein has very high surface activity and could be used as a high-quality emulsifier or foaming agent. Human milk contains β- and κ-caseins but no α_s-caseins, so β-casein from bovine milk could be an attractive ingredient for bovine milk-based infant formulae. Ion exchange resins are effective for preparing high-quality casein and whey protein products with high purities (Mulvihill 1992).

Igarashi (1995) used differences in solubilities to fractionate milk proteins. Skim milk was first treated with 50% (^{v}/v) ethanol containing 0.4 M NaSCN and 0.15 M $CaCl_2$. Both the precipitate and the supernatant containing the γ-caseins and α-La were further fractionated using an urea-Ca phosphate system to yield κ-casein and β-Lg and an insoluble fraction of calcium-sensitive proteins (αs_1-casein, αs_2-casein and β-caseins). Tomasula *et al.* (1997) used high pressure CO_2 to isolate good quality casein from milk in two different reactors: a spray reactor where milk was sprayed into CO_2 and a tubular reactor to feed CO_2 into the milk.

Neystani *et al.* (2003) isolated α-La, β-Lg, and BSA from bovine whey by firstly removing casein from defatted milk using HCl. The globulins were then precipitated from the whey by half-saturated ammonium sulphate and β-Lg was purified further using Sephadex G-50 gel filtration. Dubois (1990) used UF with 40-kDa cut off membranes to produce retentates enriched in whey proteins.

Ye and Ng, (2000) isolated glycolactin from bovine acid whey, which had been obtained by precipitating out the casein (1.8 M $(NH_4)_2SO_4$), followed by centrifugation (10,000xg for 20 minutes at 4^{o}C). After dialysis, the whey was loaded onto CM-Sepharose cation exchange column at pH 7.7. A linear gradient elution (0-0.5 M NaCl) in phosphate buffer was used. Fractions were collected, lyophilized and

reconstituted in 50 mM phosphate buffer and then applied to Mono S HR 5/5 column and eluted under a linear gradient (0-0.5 M NaCl, 20 minute at 1 mL/min).

Caseins are usually isolated by isoelectric precipitation using lactic acid-producing bacteria or acidifying to pH 4.6 using mineral acids such as HCl or H_2SO_4. For example, Pearce (1999) adjusted whey to pH 4.2 and then heated it to denature, aggregate and precipitate α-La. Bounous (1994) produced un-denatured whey protein concentrate with at least 10% serum albumin content using MF to remove bacteria and UF to separate the casein proteins. Cheang and Zydney (2004) separated α-La and β-Lg from whey protein isolate using a two-stage membrane system (30 and 100 kDa) with recoveries of 70-95% for different setups. Vivekanand *et al.* (2004) showed that casein and whey proteins can be fractionated from skim milk using 0.1-μm MF membranes. This provided a non-thermal processing alternative to the more traditional coagulation operations for casein separation.

Pedersen *et al.* (2003) used whey proteins as a model system for chromatographic separations of proteins. They evaluated the anion exchangers Q-Sepharose XL, Source 30Q, Ceramic Q Hyper-D F, and Fractogel EMD 650 (S). After adsorption onto the anion exchangers, α-La, β-Lgs A and B, and BSA were eluted at pH 6 to 9 with various NaCl concentrations.

Avramescu *et al.* (2003) used different types of Lewatit ion exchange resins incorporated into a random copolymer of ethylene and vinyl alcohol as porous, mixed-matrix membranes to separate BSA and bovine haemoglobulin. Affinity cross-flow filtration, a technique that combines affinity adsorption and MF was used to isolate BSA from a mixture of IgG and serum albumin using Cibracron Blue with sodium octanoate as a desorber (Borneman *et al.* 2002). Gambero *et al.* (1997) used affinity chromatography in a packed column silica modified with β-diketoamine groups chelated with copper ions to separate α-La from whey. The single-step purification gave 66% α-La. Punidadas and Rizvi (1998) separated partially defatted skim milk into casein and whey-rich fractions using 0.05-μm ceramic membranes that retained the caseins and allowed α-La and β-Lg to pass through. Dimmer and Nussbaumer (1999) demonstrated that membranes could be added in parallel to increase flux and scaled the 21-m^2 pilot plant to 100-m^2 membrane surface for

separating haemoglobulin. Affinity-based MF membranes have been used to extract BSA (Avramesu *et al.* 2003) and have potential for other protein extractions by modifying the affinity ligands but the major limiting factor in most stages of milk fractionation is fouling (Brans *et al.* 2004).

Other components present in milk and colostrum such as such as hormones, growth factors (prolactin PRL, IGF-1), prosaposin (Craddock 2001), glycomacropeptide (Xu *et al.* 2000), casein glycomacropeptide (Nakano *et al.* 2002) and analogs such as glycolactin (Ye and Ng 2000), CD36 surface membrane protein (Wilcox *et al.* 2002), lysozyme (Owen and Chase 1997), milk-clotting enzyme (Iannucci *et al.* 2003), and whey growth factor extract (Colby *et al.* 1996) have also been extracted successfully in laboratory experiments (Pakkamen and Aalto 1997).

2.13 Large scale applications

Many reviews discuss processing technologies useful for the dairy and biopharmaceutical industry such as membrane adsorbers (Rosenberg 1995; Meindersma and Kuczynski 1996; Splitt *et al.* 1996; Zydney 1998; Demmer and Nussbaumer 1999; Kawai *et al.* 2003; Brans *et al.* 2004), and analytical and preparative chromatographic, and electrophoretic methods (Chisti and Moo-Young 1990; Wheelwright 1991; Strange *et al.* 1992; Levison 2003).

Treloar *et al.* (2000) used a whey feed stream and an already-published laboratory method to scale-up production of FBP (folate binding proteins). They report that although the laboratory-scale procedure was well documented, they had to optimize the process to scale to pilot plant operation. Liquid whey had various particulates that sometimes caused column blockages. They extracted 0.7 g of FBP per batch with an average yield of 40% and >90% purity.

Durham *et al.* (2003; 2004) patented a fractionation process based on ion exchange and NF to concentrate whey and recover mono ions which were then used to regenerate the ion exchange column. They reported 90% yield of high purity lactose.

Konecny *et al.* (1994) purified IgG from whey using thiophilic T-gel chromatography and found the process suitable for large scale isolation of IgG.

Colby *et al.* (1996) used LF and LP as model proteins to investigate the effects of compression on scaling SP BB resin in a commercially packed column and reported that compression was not an important factor in scale-up.

Cheng and Zydney (2003) reported on several methods that have been studied for whey protein fractionation including ion exchange chromatography (Uchida *et al.* 1996; Hahn *et al.* 1998) affinity binding (Bezwooda and Mansoor 1986; Kawakami *et al.* 1987; Chen and Wang 1991), and selective precipitation using salts, pH, and/or heating (Pearce 1983; Gesan-Guiziou *et al.* 1999). None of these processes or techniques has been effectively implemented on a large commercial scale due to inadequate yield/purification and/or poor overall economics.

Anon. (2003) reports of a prototype whey refinery process at Wisconsin University that uses milk as a starting material and uses MF to retain and concentrate the casein fraction. Membranes were used to separate α-La and ion exchange chromatography to separate α-, β- and κ-caseins.

Ion exchange chromatography has been developed and used successfully to separate whey proteins on a commercial scale. In a study of commercial extraction of LF from (defatted) milk, Buchanan (1994) evaluated several cation exchangers (not identified) and SP FF resin using column chromatography. Capacities ranged from 1.6 to 9.3 mg LF per mL of resin at pH 6.7 compared with 33 mg LF per mL of SP Sepharose FF resin. The high capacity cellulose-based resins were unsuitable for industrial applications due to their high compressibility. As a result of Buchannan's study, the industrial scale ion exchanger was replaced with one of the resins studied.

The Vistec process used by Davisco Co. (LeSuer, MN, U.S.A.) uses a cellulose-based exchanger in a stirred tank reactor. Whey pH is adjusted, lactose and the proteins are eluted. UF, evaporation and spray drying are used to give a whey protein concentrate with 95% ^{w}/w protein content. Tatua Dairy Co-operative (Tatanui, Morrinsville, New Zealand) uses cation exchanger column chromatography to extract LF, LP and other basic proteins from skim milk. Further ion exchange purifications give high purity LF, LP and other protein fractions. Details on the exchanger resin and the process are unavailable because the process is proprietary.

2.14 Effect of pre-processing on yield and activity of proteins

Proteins should be separated from a source material as fast and in as few steps as possible to minimise loss of activity and yield (Ladisch 2001; Harrison *et al.* 2003). Losses in individual steps and complex separation sequences can be large. For example, Nuyens and Van Veen (1999) report that acid precipitation of casein resulted in 4-8 times more LF entrapped in the casein pellet than in the whey fraction. Denman *et al.* (1991) lost 50% of the human tissue-type activator protein produced in transgenic goats milk in the acid precipitation step, and obtained an overall yield of only 25%.

Fast processing reduces proteolytic degradation of proteins (Jungbauer *et al.* 1988; Korhonen *et al.* 1998). Proteolysis increases heterogeneity of all species because milk contains a broad spectrum of proteins (Orthner *et al.* 1989).

High-temperature processing disrupts non-covalent bonds in macromolecules such as proteins and polysaccharides causing denaturation and gelling of milk components (Datta and Deeth 1999). Ultra heat temperature (UHT) pasteurization of skim milk causes interaction between casein and whey proteins, reduces solubility of casein at neutral pH or below, decreases chemically-available lysine in whey protein and causes a 56% denaturation of whey protein in the skim milk but no denaturation in the raw milk (Douglas *et al.* 1981).

2.15 Options for on-farm processing of milk proteins from raw whole milk

Chromatographic fractionation of proteins from milk at a commercial scale is quite different from analytical scale chromatography. For instance:

- all buffers must be food grade and inexpensive
- relative flow rates in column volumes must be 10-20 times greater than laboratory scale for economic viability
- columns must be 20,000 to 30,000 times larger in volume
- recovery must be >90%
- the column must operate for many cycles without loss in capacity before it needs to be cleaned.

The high solids content and fat globules with diameters up to 10 μm, and caseins present as a colloidal suspension of particles with diameters up to 0.1 μm (Bylund and TetraPak 2003) make raw whole milk a challenging matrix to process.

2.15.1 Membrane filtrations and adsorptive membranes

Large scale membrane processes have been successfully used in the dairy industry to remove bacteria and to control the fat and lactose content of various products (Zydney 1998). UF is the most widely-used commercial method to concentrate whey. However, it has the disadvantages of high capital and operating costs, membrane fouling, and low-molecular-mass solutes not being completely removed. In-place cleaning and sanitation are required to minimize microbial contamination. Also, large volumes of low protein content permeates are produced (Geberding and Byers 1998). The LF (80 kDa) and LP (77.8 kDa) have very similar molecular weights (which infers similar molecular size in MF), so membrane separation based on size is not possible.

Some researchers have used ion exchange membrane to selectively capture LF (Chen and Wang 1991; Chiu and Etzel 1997; Ulber *et al.* 2001). Whey was first clarified using MF before membrane adsorption. Chiu and Etzel (1997) recovered 72 and 50% of the LP and LF respectively, after repeated cycles using ion exchange membrane. Burling (1989) reported that the industrial processing of LF from whey in packed bed chromatography was only possible after MF (1.4 μm) and the flow rates were low (1.25 bed volumes/min) resulting in long loading time of 16.7 hours.

Etzel (2004) evaluated large-scale commercial whey refining and noted membrane filtration is not ideal for capturing individual whey fractions, especially LF, because membrane filtration capacity is mainly dictated by the volume of whey processed, whereas capacity of process chromatography is set mainly by the mass of LF recovered, making membrane processing (a volume dependant separation process) not economic. Mass transfer limitations using ion exchange membranes have also been reported (Sarfert and Etzel 1997).

There are many studies on membrane fouling and effective cleaning mechanisms (Brink *et al.* 1993; Chilukuri *et al.* 2001; James *et al.* 2003; Arguello *et al.* 2005). Chilukuri *et al.* (2001) used sodium dodecyl sulphate to reduce fouling during cross

flow-filtration of LF solutions and report that the protein aggregates play an important role in fouling. Significant fouling of the membranes with 0.2% (^{w}/v) LF occurred at high flux (200 L/m^2 hr).

Ion exchange membranes are a promising new technology that could overcome some of the difficulties encountered during ion exchange chromatography in packed beds and stirred tank systems. The interest in adsorptive membranes and their effectiveness for extracting LF and LP from whey, skim milk or reconstituted solutions shows that this emerging technology has potential and may be suitable for on-farm applications. The current prices of the adsorptive membranes make the process expensive, but this could change as more units are sold and the costs of disposable technology decreases.

2.15.2 Expanded bed adsorption chromatography

EBA chromatography has been demonstrated for biomolecules but current process times are too long. For example, two or more days can be required to process 250 L of mammalian cell culture broth (Wright *et al.* 1999). Process streams frequently encountered in biological applications of fluidized bed adsorptions often have viscosities of 1-2 cp (mammalian cell culture broths) and 10 cp or higher (*Escherichia coli* and *Pischia pastoris* broths). Most chromatographic resins suitable for product adsorption have low densities and unacceptably low settling velocities in this viscosity range. The viscosity of milk, which is temperature dependant, is usually 3 cp (25^{o}C), but the fat content in raw whole milk may cause fouling in EBA chromatography.

Etzel *et al.* (1998) used EBA to capture proteins from skim milk and demonstrated that crude feedstock can be processed in a fluidised bed. However, there were mass transfer limitations, which increased processing time. This would make EBA chromatography unsuitable for milk processing. Also, the long processing time may compromise milk quality by allowing microbial growth and/or protein degradation.

Houwing *et al.* (2003) found that mass-transfer effects also limited the performance of the simulated moving bed (SMB) chromatography during experimental fractionation of BSA and myoglobin on SP BB resin. The resin particle size selected for SMB was a compromise between productivity and mass transfer.

Noppe *et al.* (1999) recovered α-La from skim milk using hydrophobic Streamline Phenyl gel in EBA chromatography to avoid column clogging. They used defatted milk so the expanded bed would not be saturated with fat globules. Owen and Chase (1997) used continuous counter-current EBA chromatography to extract lysozyme from lysozyme-enriched fat-free milk and obtained 66% yields. The adsorbent was perfluorocarbon matrix coated with poly(vinyl alcohol) with triazine dye Procion Red HE-7B.

2.15.3 Ion exchange using column or batch stirred tank

Ion exchange has been used most widely used in the laboratory and industrially to extract LF and LP from skim milk and whey. It would be the preferred method for extracting LF and LP from raw whole milk. Raw whole milk contains large suspended particles that make it more difficult to process in column chromatography than whole (full-fat) processed milk, which has been homogenised in the factory to produce a uniform consistency. Raw or whole milk normally will not be expected to pass through a chromatography column because of its low processing temperatures. Milk is normally stored at 4-10°C to minimise bacterial growth during long holding periods and chromatographic extractions to fractionate milk proteins are also usually performed at these temperatures.

Limitations of batch stirred tank extractions include:

- low recoveries because of equilibrium considerations
- slow throughput because of long process times
- large equipment as the tanks must hold all the feed and the resin, and
- fractionation may be difficult because changing buffers usually requires emptying the tank

The major drawback of column chromatography is fouling, especially for milk, which has a high solids and particulate content. The low viscosity and presence of soluble aggregates in milk further complicates column process. These factors may generate high back-pressures in a column procedure. Batch procedures are more desirable for the process being investigated as many problems associated with packed bed chromatography, such as consolidation of packing material, formation of channels, and column blocking by particles in the feed solution are reduced.

Designing cost-effective separation processes requires knowledge and understanding of the equilibrium and kinetics of the adsorption process. Langmiur isotherms, based on single component adsorption, were used to determine maximum adsorption capacities of the resin (Chapter 3). Dynamic capacity would be a more useful characteristic to indicate capacity during ion exchange of raw whole milk and will be determined from breakthrough curves. The model of Rowe *et al.* (1999) is used to identify the parameters on the preparative chromatography and used for scale up (Chapter 5).

2.16 Summary

In this Chapter, minor components, LF and LP in milk that have potentially high value are discussed. Other components that were not discussed in detail but could be potential targets for on-farm extraction include components such as conjugated linoleic acid (anticarcinogenic fatty acid in milk fat), phospholipids, IgG, IgA, IgM, prolactin etc. LF and LP were widely extracted on a preparative scale as well as on industrial scale from skim milk and whey. Published purification technologies for their extraction were reviewed as well as a discussion on other techniques used in biopharmaceutical and dairy industry that may be suitable.

Product recoveries are not generally reported and there were no published data on industrial processes due to commercial sensitivity. Laboratory methods give variable yields and researchers highlight the importance of process optimization when considering large-scale processing (Treloar *et al.* 2000; Bensch *et al.* 2005) and that small-scale, established laboratory methods may not necessarily lead to similar performance at larger scale.

Even though raw whole milk was not universally used for fractionation, methods used to fractionation milk proteins and extract therapeutics from fermentation broths may be applicable to raw milk and are worthy of investigation. Column chromatography, after making allowance for fat content, and ion exchange membrane filtrations were methods worth investigating. The suitability of other techniques such as batch stirred tank and EBA for processing raw milk could also be investigated.

3 Laboratory Fractionation Materials and Methods

3.1 Introduction

An important pre-requisite when setting up a purification scheme is having an adequate assay procedure to follow changes in biological activity and protein content. Protein content can be measured by the Bradford method (Bradford, 1976), the Biuret method (Camara *et al.* 1991) with bincinchoninic acid (Smith *et al*. 1985) or by UV absorbance at 280 nm. Specific methods are used to quantify individual proteins and their activities. This chapter describes the materials and methods used to determine proteins and their activities. Techniques for protein purification, measuring resin performance (capacity, regeneration capability, capture efficiencies) and obtaining process parameters in laboratory experiments are also summarised.

3.2 Equipment

The major instruments/equipment used in laboratory experiments include:

- AKTA Explorer10™ with Autosampler 900 and Unicorn 4.0 control software - liquid chromatography system (GE Healthcare Technologies, Uppsala, Sweden).
- AKTA fplc ™ - fast protein liquid chromatography with Unicorn 4.0 control software (GE Healthcare Technologies)
- UltraSpec® UV-Vis Spectrometry (GE Healthcare Technologies)
- Scanning Electron Microscope (Hitachi S-4000 SEM, Hitachi Corporation, Japan)
- Surface plasmon resonance (Biacore® SPR 3000, Biacore International AB, Uppsala, Sweden)
- Masteriser S (Malvern Instruments Ltd., Worcestershire, United Kingdom), a dynamic light scattering (DLS) instrument
- Optical microscope (Olympus BH2-UMA, Olympus Corporation, Tokyo, Japan)
- Freeze dryer, Freeze Zone 2.5 (Labconco Corporation, Kansas, MO, U.S.A.)

- pH and conductivity meters (Cyberscan 100, Alphatech Systems, Auckland, New Zealand)
- Portable (digital) temperature probe (-50 to 150^{o}C) (Biolab Scientific, Albany, Auckland, New Zealand)
- ELX800 Universal Microplate Reader (Bio-Tek Instruments Inc., Winooski, VT, U.S.A.)
- Mini-centrifuge (Mini-Spin, Eppendorf, Hamburg, Germany)
- Sigma Laboratory centrifuge (Osterode am Harz, Germany)
- BioRad electrophoresis unit (BioRad Laboratories, Hercules, CA., U.S.A.)
- Phastgel system (GE Healthcare Technologies)
- Overhead stirrer (RW 20 DZMn, Labortechnik, Staufen, Germany)
- Multi-head stirrer system (Boltac Industries, Hamilton, New Zealand)

3.2.1 Standard proteins and buffers

Protein standards of >99% purity were used. LF standards were obtained from Sigma (Sigma-Aldrich, St Louis, Montana, U.S.A.), Bethyl (Bethyl Laboratories) and Fonterra (Fonterra Cooperative, Palmerston North, New Zealand). LP, lysozyme, α-La, β-Lg, BSA, IgG, α- and β-caseins were obtained from Sigma. The LF and LP of known purity and activity respectively were gifted by Tatua Dairy Cooperative (Morrinsville, New Zealand) and Fonterra Cooperative (Hautapu, New Zealand).

Prepared HBS-EP buffer [10 mM HEPES, 0.15 M NaCl at pH7.4 with 3 mM ethylene diamine tetra acetic acid (EDTA) and 0.005% (^{v}/v) P20 surfactant] was purchased from Biacore. The HBS-N used for HBS-EP consists of N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid); 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES).

Affinity purified goat polyclonal anti-bovine LF antibody (1 mg/mL) from Bethyl Laboratories (A10-126A) was used for ELISA and SPR analyses.

3.2.2 Resins (or media) for chromatographic adsorptions

SP Sepharose Big Beads™ (SP BB) and SP Sepharose Fast Flow™ (SP FF) were gifted by GE Healthcare Technologies (Uppsala, Sweden). Bio Rex 70 (BR 70) resin was obtained from BioRad (BioRad Laboratories, Hercules, CA., U.S.A.).

3.3 Chromatography columns

The following chromatography columns were used during laboratory studies:

- Superdex 75, a pre-packed size exclusion column (GE Healthcare Technologies)
- XK 16 columns (GE Healthcare Technologies) (1.6 cm id packed to required height)
- Resource S (a pre-packed 1-mL cation exchange column) (GE Healthcare Technologies)
- H5/5 column (0.5 mm id packed to required height)

3.4 Analytical chromatography

3.4.1 Size exclusion chromatography

The AKTA Explorer10 was used to analyze skim milk and standard proteins. A calibration curve for the Superdex 75 column was constructed using a gel filtration low molecular weight (LMW) standard kit (GE Healthcare Technologies). Aliquots (40 μL) of Sigma standards (1 mg to 2 mg/mL) were injected onto the Superdex column using the autosampler. Skim milk was diluted 1:40 and filtered with a 0.45-μm filter to remove particulate matter before analyses. Phosphate buffered saline (PBS, 10 mM, pH7.4 Sigma-Aldrich) was used as running buffer. Isocratic runs were done at 0.5 mL/min.

3.4.2 Cation exchange chromatography

A 1-mL Resource S (cation exchange) column was evaluated for quantifying LF, LP and lysozyme (LZ) using fplc. LF, LP and LZ standards of >99% purity (Sigma-Aldrich) were made to 0.05, 0.10, 0.20, 0.50 and 1.00 mg/mL for low level calibration and 1.0, 2.0, 5.0 10.0 and 20.0 mg/mL for higher level calibration. Calibration curves were constructed for LF and LP only; elution of LZ was to evaluate LZ's elution relative to LF and LP.

Raw whole milk was centrifuged at 4800x*g*, pre-filtered with a 0.45-μm filter to minimize column blockages, diluted 5 to 10 times (10 mM phosphate buffer) and then applied to the column. A flowrate of 4 mL/min was used. The column was equilibrated with 4 column volumes (CVs) of 10 mM phosphate buffer

(Na_2HPO_4 /NaH_2PO_4, pH7.4). The 0.5-mL samples were injected using a fixed loop (1 mL) and a Superloop was used for repeated injections. The column was washed with 2 CVs of phosphate buffer, followed by linear gradient elution using 0-100% of 1.0 M NaCl phosphate buffer over 20 CVs. Any residual protein was eluted using a further five CVs of 1.0 M NaCl.

3.5 LF assays

3.5.1 ELISA method

A bovine ELISA quantitation kit (Bethyl Laboratories Inc., Montogomery, TX, USA) was used to measure LF concentrations with the following modifications (Turner 2003 *et al.* 2003b). After incubation with goat anti-bovine LF antibody, plates were washed with Tris-buffered saline (TBS; 50 mM Tris, 0.14 M NaCl, pH 8.0) containing 2% ^{w}/v BSA. Goat anti-bovine LF horseradish peroxidase conjugate was diluted 1/100,000 in TBS containing 1% ^{w}/v BSA and 0.05% ^{v}/v Tween 20. The plates were developed by adding 100 µL 0.2 mg/mL 3,3',5,5'-tetramethyl-benzidine (Sigma-Aldrich) and 1 mg/mL urea hydrogen peroxidase (Sigma-Aldrich) and 11% ^{v}/v dimethyl-formamide (BDH Chemicals) in 0.1 M citrate buffer to each well. After 10-minute incubation at room temperature, the reaction was stopped by adding 100 µL/well of 1 M H_2SO_4. Absorbance at 450 nm was read in an ELX800 Universal Microplate Reader.

Calibration standards from the Bethyl ELISA kit were used for the standard curve (Figure 3-1). A 4-fit curve was used to calculate the unknown concentrations. This method is widely accepted for LF analysis (Martin *et al.* 2004, Takeuchi *et al*. 2004) and was used in the first half of the project before implementing the SPR assay.

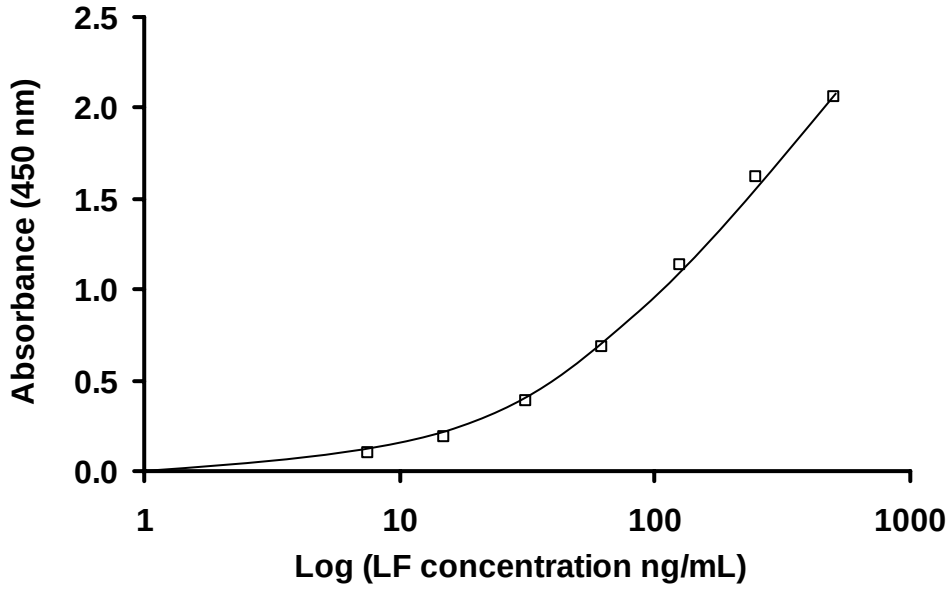


Figure 3-1: A calibration curve of LF using ELISA method and Bethyl standard.

3.5.2 LF concentrations using the surface plasmon resonance method

The surface plasmon resonance (SPR) method (Indyk and Filzoni 2005) was used to determine LF concentrations. The only significant modification was in sample preparation: raw whole milk samples were centrifuged at 4800xg for 2 minutes to remove fat and filtered through a 5-μm filter (Sartorious AG, Goettingen, Germany) before making serial dilutions (to 2000x) in 500 mM HBS-EP buffer. The 150-mM HBS running buffer was obtained from Biacore and NaCl concentrations were increased to 500 mM for sample and standard preparations to reduce non-specific interactions.

Goat anti-bovine LF (affinity purified) antibody was immobilised (Figure 3-2) on the CM5 sensorchip surface with N-hydroxy succinimide (NHS) and ethylene diamine (EDA) coupling reagents. The goat antibody was diluted to 50 μg/mL in 10 mM sodium acetate (pH 5.0). The antibody forms strong covalent bonds, which allow repeatable, quantitative measurements of LF in milk, eluates and processed samples. Ethanolamine-HCl (10 mM, pH 8.0) was used to flush the activated surface to remove loosely bound material and to block any unbound sites. This, together with the enhanced salt concentration of HBS-EP buffer (0.5 M NaCl), minimizes non-specific binding.

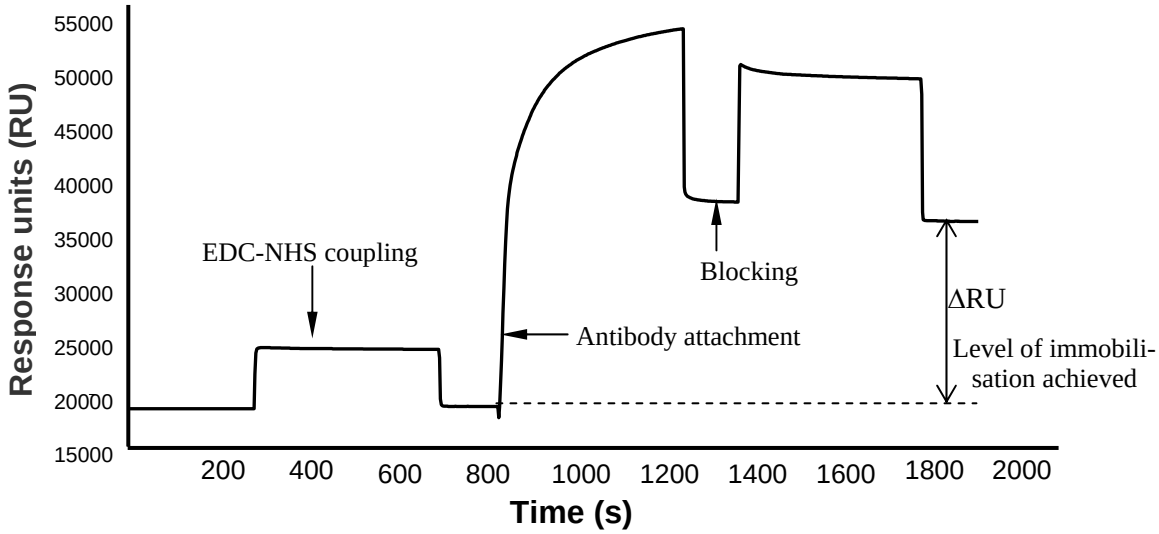


Figure 3-2: Typical immobilisation of goat anti-bovine LF antibody on a CM5 chip.

After a fresh CM5 chip was docked and primed, the immobilisation method was run at 50 μL/min for 7 minutes. Bethyl calibrator standards (1 mg/mL) were serially diluted to 0-1000 ng/mL and run on the SPR along with samples. A 4-fit calibration curve was generated and the unknown concentrations determined by matching their responses against the calibration curve (Figure 3-3).

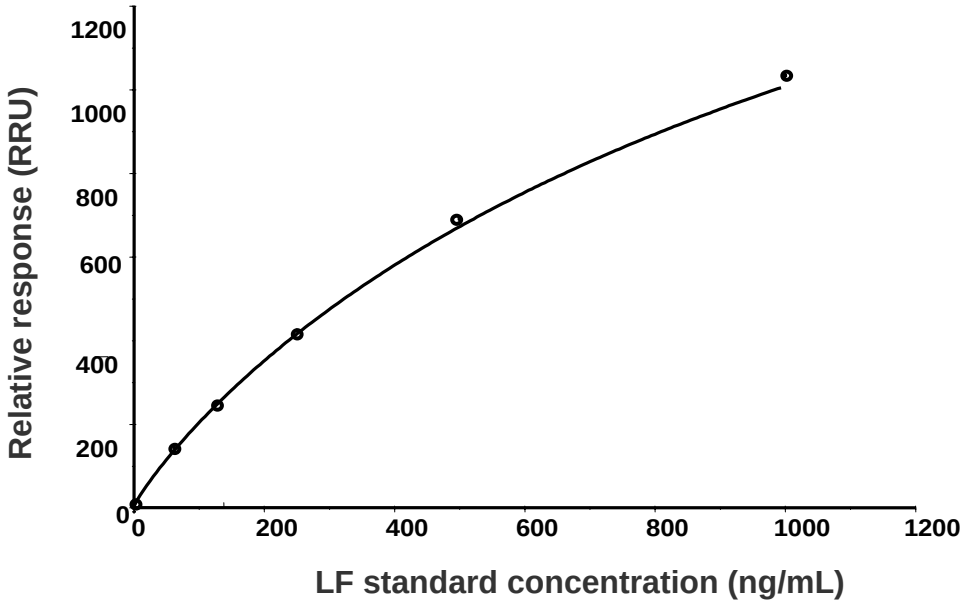


Figure 3-3: 4-fit calibration curve fit for LF standards analysed using SPR.

Up to 800 individual analyses were possible on one immobilized flow cell of a CM5 chip without a decrease in performance. A quality assurance sample (500 ng/mL LF) from a known LF source was routinely included in the analytical runs to monitor

performance of the method and the instrument. Relative responses of 1200 RU (response units) were obtained for the 1000 ng/mL standard.

3.5.3 Comparison of ELISA and SPR methods

The ELISA method is widely used for determining LF and was used during the first half of this project. When the SPR method became available, methods were compared before the SPR method was adopted for LF analyses. A set of 10 LF samples were analysed by both methods. The same Bethyl standard was used to construct the calibration curves for both analyses. Differences were within experimental error (Figure 3-4). Both methods agreed within 8% for all samples.

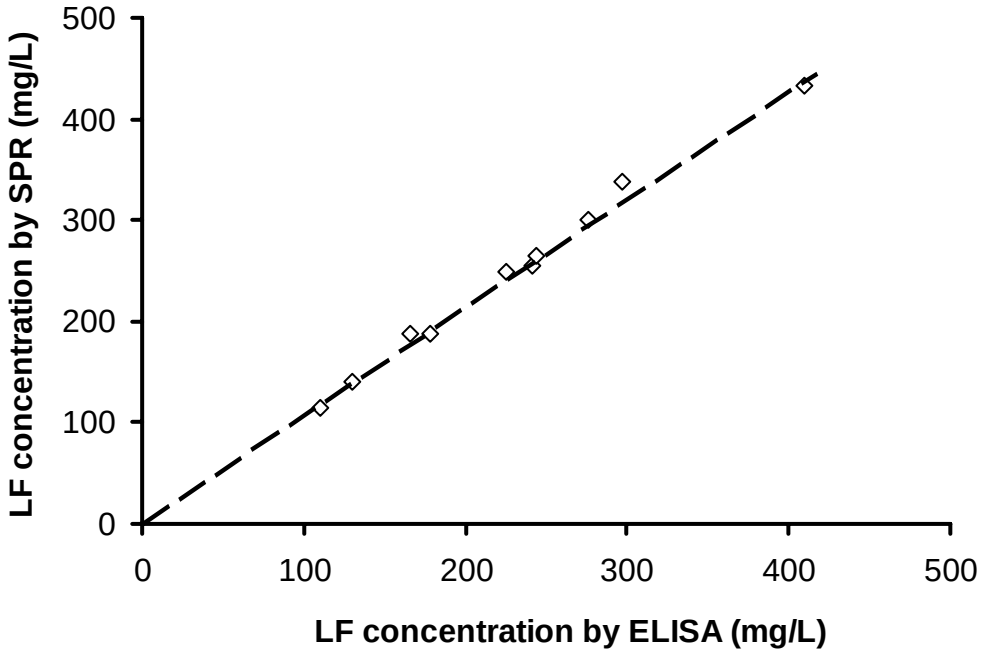


Figure 3-4: Comparison of LF concentrations obtained from SPR and ELISA methods.

3.5.4 Inter-laboratory comparison of LF determinations

To evaluate assay variability, an inter-laboratory comparison of LF in raw whole milk from 10 individual cows using the same batch of LF standards was undertaken. Raw whole milk samples were defatted by centrifugation before being sub-sampled and dispatched to participating laboratories. Samples and standards were supplied at the same time and run in one consecutive run by all laboratories. The methods used

were SPR (2 laboratories), Bethyl ELISA (1 laboratory) and HPLC (2 laboratories, with 1 running ELISA alongside HPLC), giving six data sets from five laboratories.

Data were within 10%, irrespective of the laboratory and method used (Figure 3-5). It is very important to obtain inter-laboratory data because the LF product has many claimed benefits and has significant commercial value. A validated analytical method assists in obtaining accurate values for yield and purity of the extracted protein.

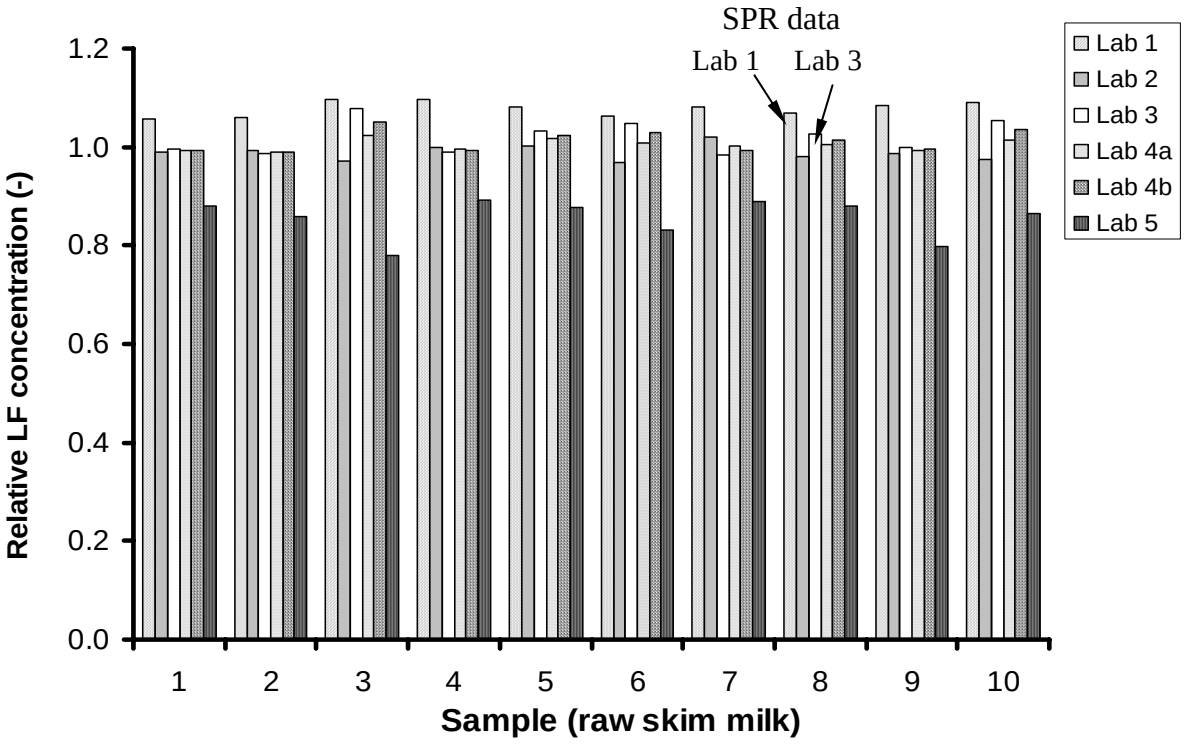


Figure 3-5: Inter-laboratory comparison of LF content in milk of 10 cows.

Data from the SPR at lab 1 were consistently higher than other methods. However, SPR data from lab 1 and lab 3 were within 5%. Although the trend is unrelated a factor can be applied to reflect true results since the trend is consistent.

3.5.5 Filtration for analytical cation exchange and SPR analyses

Initial SPR data for LF in raw whole milk samples varied widely. The sensor chip technology is very sensitive and any particles in the sample can cause variations and damage the chip surface. Particles in raw whole milk were measured using laser light scattering to determine the filtration required for the SPR analysis.

The mean diameter of particulates in the skim milk was 0.2± 0.1 µm, which corresponds to the mean diameter of casein micelles (0.03-0.3 µm). The fat globules in the raw whole milk were 1-12 µm in diameter (mean of 3 µm). Centrifugation removed most of the fat. Coarse filtration (about 5 µm) removed large particles and was used for samples for SPR analyses, or to remove resin from resin/milk slurries. Soluble LF and LP would not be affected by the filtration. Large variations in LF data for raw whole milk samples were eliminated by centrifuging (4000 rpm for 2 minute in a Mini-Spin centrifuge) to defat the milk and filtering.

3.6 LP Analysis

The 2,2'-azinobis[3-ethyl-benzothiazoline-6-sulphonic] diammonium salt (ABTS) enzyme was used to determine LP activity and concentration. The assay involved oxidizing the synthetic substrate, ABTS and comparison with a Sigma standard. The method was adapted from a published method (Chiu *et al*. 1997) and only measures active LP. It is a recognized method for testing level of preservation in pasteurized milk (EC 1.11.1.7, Keesey 1987).

After diluting the milk with 10 mM phosphate buffer, the conversion of ABTS per unit time was determined by photometric analysis. The following reaction occurs:

$$2\,ABTS + H_2O_2 + 2H^+ \longrightarrow 2ABTS^{\cdot +} + 2H_2O \qquad (3.1)$$

The amount of ABTS radical cations ($ABTS^+$) liberated per unit time is proportional to LP activity and is determined photometrically from the green colour at 420-436 nm. The following calculations were used to obtain the LP activity and concentration.

$$Units/mg\ \ solids = \frac{units/mL\ \ enzyme}{mg\ solid/mL\ \ enzyme} \qquad (3.2)$$

$$Units/mg\ \ protein = \frac{units/mL\ \ enzyme}{mg\ \ protein/mg\ \ enzyme} \qquad (3.3)$$

One unit of LP oxidizes 1.0 µmole of ABTS per minute at pH 5.5 at 25°C.

Sigma standard was used to monitor LP activity during analysis of samples. Typically 1:150 dilutions were applied to a stock Sigma standard of 1 mg/mL with an activity of 127 IU/mL (140 IU/mL Lowry). Results were usually between 120 to 140 IU/mL of standard. Data from the Sigma standard were used to calculate the protein content of the samples based on the activity obtained (i.e. sample activity was converted to mg LP).

3.7 Milk assays

3.7.1 SDS-PAGE

Sodium dodecyl sulphate - poly acrylamide gel electrophoresis (SDS-PAGE) analysis using Phastgels and 20 x 16 cm gels were done for purity analyses on proteins in the initial fractionation experiments. Phastgel SDS-PAGE is a rapid measurement to indicate protein in the purified and unpurified fractionation samples and was used according to Separation Technique File No.111 (Amersham Pharmacia Biotech AB, now GE Healthcare Technologies, 1998). This was a useful technique for rapid qualitative checks on purity of standards and of the purified proteins but was not quantitative.

The sandwiched gels were cast in the laboratory using a BioRad kit. 30% acrylamide bis and SDS were from BioRad Laboratories. A running gel was poured with a softer 25-mm stacking gel on top using the Laemmli method (1970). The 8-μL samples and standards were applied to the gels. Gel electrophoresis was run in a Hoefer ® SE 600 (GE Healthcare Technologies) electrophoresis unit. Buffer temperature was maintained between 8-10^{o}C using recirclating chilled bath. Gels were stained using Phastgel blue R stain overnight and destained overnight using 20% acetic acid/isopropanol mixture. The destain solution was changed three times until most of the background was removed.

The gels were visualized using an ultraviolet trans-illuminator (TFX 35M Gibco-BRL UV Transilluminator) and photographed using a UV gel camera. Images of the gels were scanned using Scion Imaging Software (Frederick, MD, USA). A typical SDS-PAGE gel (Figure 3-6) shows purity of LF and LP proteins along with milk proteins.

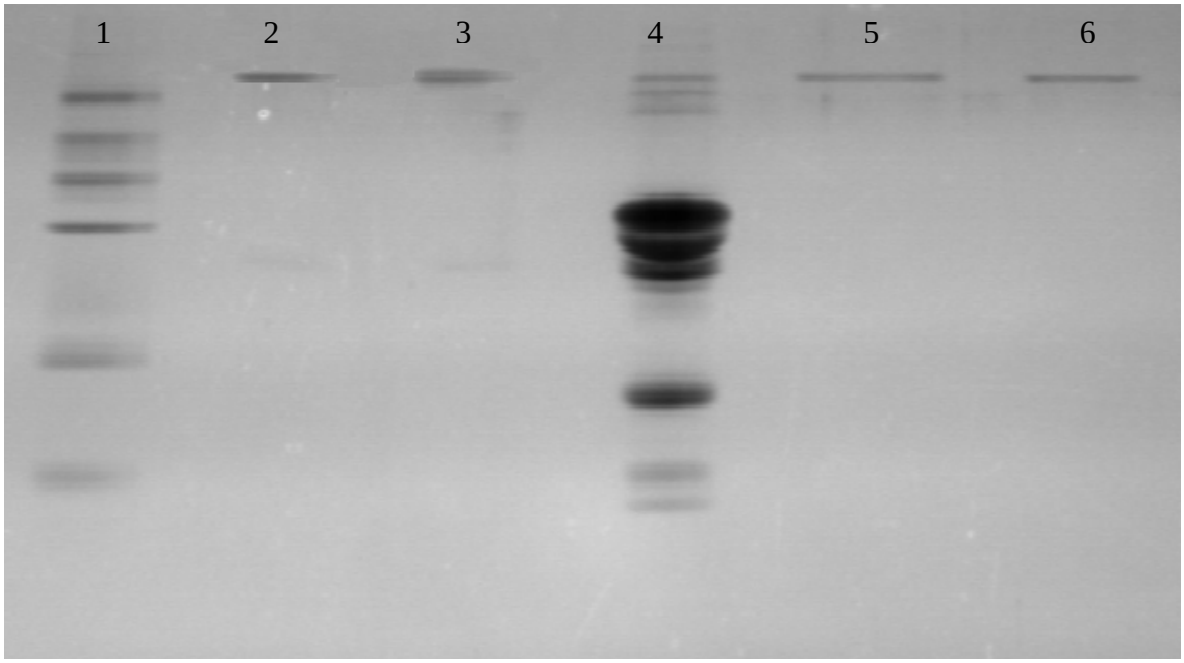


Figure 3-6: SDS-PAGE (20 x 16) gel electrophoresis with low molecular weight protein standard (GE Healthcare Technologies) (lane 1), LP fraction (lane 2), LF fraction (lane 3), skim milk (lane 4), LP standard (lane 5) and LF standard (lane 6).

3.7.2 Bincinchoninic acid (BCA) protein assay

The bincinchoninic acid (BCA) protein assay (Pierce, Rockfield, IL, U.S.A), sensitive between 20 and 1200 μg/mL, was used to measure LP and LF concentrations for equilibrium capacity and protein purity of initial and final products in fractionation experiments. The BCA reagent was purchased as a kit (Pierce). Working reagent was prepared by mixing 50 parts reagent A with 1 part reagent B. The standard curve was produced using 0, 100, 250, 500, 750, 1000 μg/mL BSA fraction V, >98% purity (Sigma-Aldrich) from a 1 mg/mL BSA stock solution. Protein samples or standards were prepared in 10 mM phosphate buffer in 1.5-mL microfuge tubes. One mL BCA working solution was added to 50-μL of sample or standard and vortexed. This was incubated at 60^{o}C for 15 minutes, then cooled to room temperature and immediately read at 562 nm. A zero correction was applied to the readings and the values for samples calculated after constructing the calibration curve with BSA standards. A typical calibration curve using BSA (Figure 3-7) was linear over the range used.

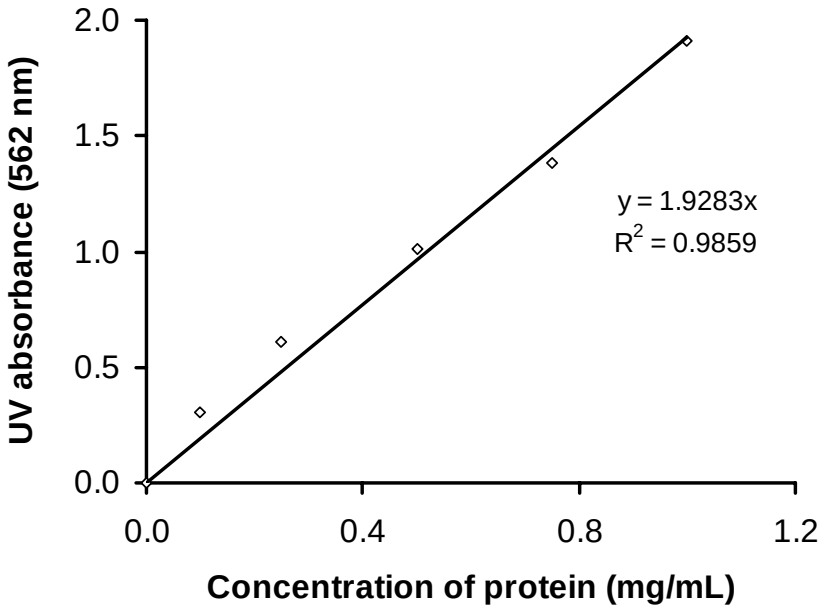


Figure 3-7: Calibration curve for BSA standard protein using the BCA assay.

3.7.3 FT-IR Spectroscopy (FT120 milk composition analyses)

The MilkoScan™ FT120 (Foss, Denmark) uses a Fourier-Transform Infrared Spectroscopy measuring principle and complies with IDF (International Dairy Federation) and AOAC standards for analysing fat, protein, lactose, crude protein, casein protein and total solids content of milk. Samples were analysed externally (Dexcel Limited, Hamilton, New Zealand). The FT120 was used to monitor bulk milk composition before and after fractionation by analyzing feed and outflow milk samples.

3.8 Laboratory-scale fractionation

3.8.1 Packed bed chromatography

Raw whole milk was obtained from the Greenfield's research farm (Dexcel Limited). SP BB and BR-70 ion exchange resins were packed into water-jacketed XK 16 columns to a bed height of 5.0 (following the manufacturer's instructions). The feed was maintained at 37^{o}C and a recirculating water bath was used to maintain column temperature of 37^{o}C. Fractionation was carried out using the AKTA fplc (Figure 3-8) with flowrates of 10 and 15 mL/min (linear velocities of 300 and 450 cm/hr respectively). The resin was equilibrated with 5 CVs of 10 mM phosphate buffer

(pH6.7). Several experiments were undertaken with varying amounts of raw whole milk (100-1000 mL). The captured LF and LP were washed using 2.0 CVs of phosphate buffer and 2.0 CVs of 0.1 M NaCl in phosphate buffer and then eluted using a 10-100% gradient of 1.0 M NaCl over 20 CVs. The elution stream was monitored at 280 nm and 5.0-mL fractions were collected. The fractions were analysed for LP activity using the ABTS assay. Both LF and LP content were measured by (Resource S) cation exchange chromatography. A process schematic for extracting LF and LP using packed bed chromatography is shown in Figure 3-9.

Figure 3-8: The AKTA fplc system for packed bed chromatography of raw whole milk at 37^{o}C.

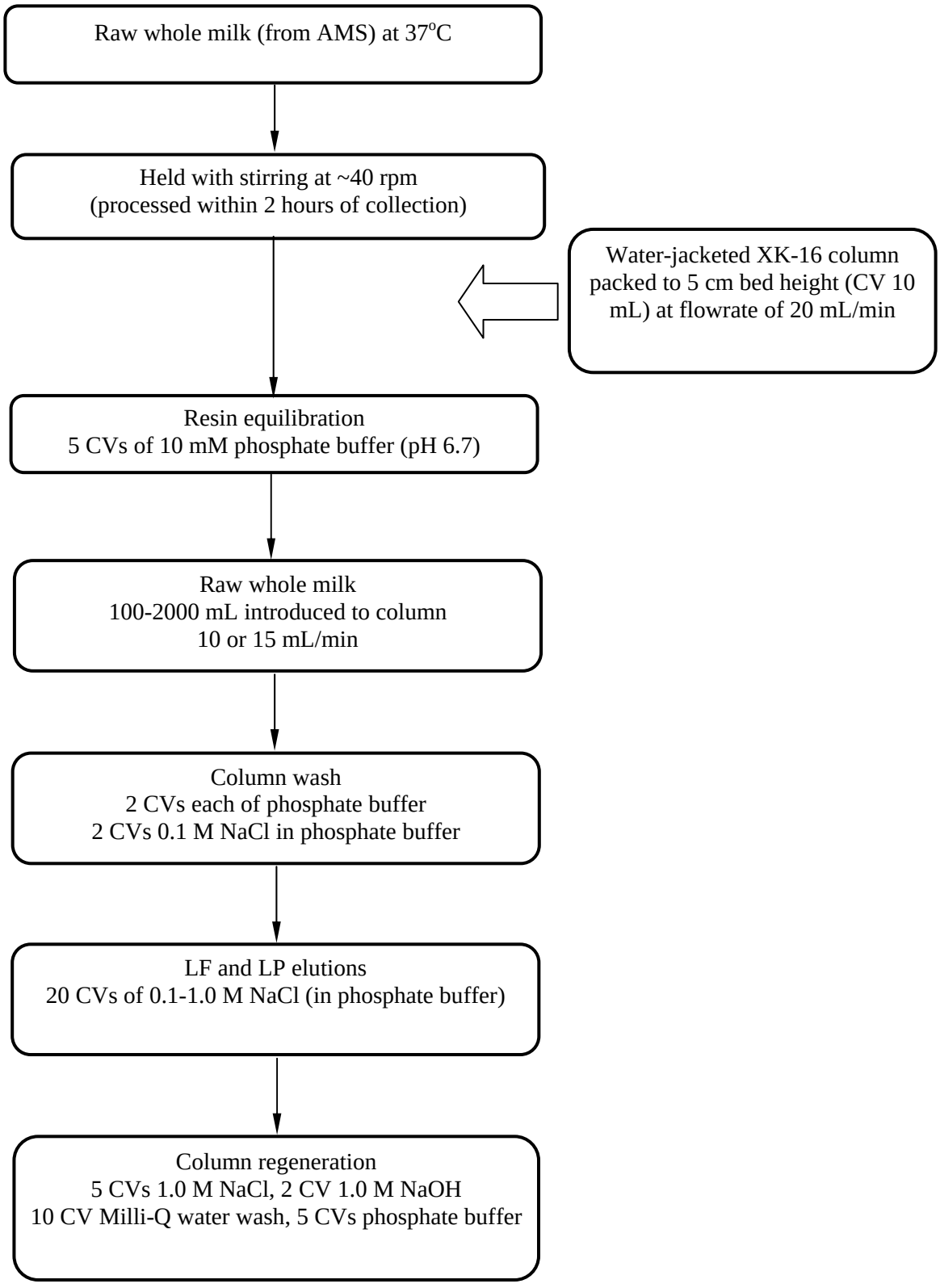


Figure 3-9: Procedure for extracting LF and LP from raw, whole milk.

3.9 Resin characteristics

3.9.1 Particle size distribution

Particle size distribution of chromatography media, raw whole milk and fat globules were determined by dynamic light scattering (DLS). The $d_v0.9$ and $d_v0.5$ (diameter below which 90 and 50% respectively of the volume of particles) and $d_v(m)$ (diameter of spheres of equivalent volume to measured particles) were determined.

SP FF was screened using a 42-µm sieve. Particle size distribution of the oversize and undersize fractions were determined.

3.9.2 Scanning electron microscopy (SEM)

Resin samples were freeze dried, placed on aluminum mounting disks, and platinum-coated before microscopy. Upon scanning, images were taken at resolutions of 750, 200 and 100 µm.

3.9.3 Optical microscopy

Resin particles were routinely observed by optical microscope to monitor the effects of processing and handling. This allowed resin appearance to be quickly evaluated before proceeding to the more rigorous experiments.

3.9.4 Effect of pumping on SP BB

Centrifugal pumps are normally used to pump milk in the milking parlours. Pumping may irreversibly damage the milk and may also damage the resin and reduce its practical lifetime. The effect of pumping SP BB was investigated by continuously recirculating 200 mL of SP BB suspended in 100 L of water through a centrifugal pump for 10 minutes at 20% power, a further 10 minutes at 50% power, and a further 10 minutes at 120% power representing volumetric flow rates of 0.6, 1.55 and 2.95 L/s respectively. A sample was taken at each pumping rate and drained through filter paper (Whatman No.41) to recover the resin. The resin was mounted on slides and observed under the optical microscope and the SEM.

3.9.5 Effects of high linear velocity on SP BB

SP BB resin was packed in a 1-mL HR 5/5 column (GE Healthcare Technologies) with a 42-μm end filter mesh. The column was rated at 1.5 MPa and resin at 0.5 MPa. Skim milk and ethanol were passed through the column at 5, 10, 15 and 20 mL/min. Subsamples of the resin were mounted and viewed under the SEM.

3.10 Extracting LF and LP from raw whole, skim milk and whey

The LF and LP were extracted from raw whole milk, skim milk and whey to mimic the effects of supply chain and dairy factory processing operations on LF and LP yields and activity. Processes applied to the skim milk and whey samples in the laboratory were: cooling, mixing, transportation, pasteurisation, defatting, acid coagulation (removal of casein); filtration (to produce whey); and ion exchange chromatography to capture LF and LP. Processing steps usually undertaken in the factory but not investigated in the laboratory were: homogenisation (to produce uniform fat and particles in milk); and ultrafiltration (clarifying the whey and removing lactose) before chromatographic capture (ion exchange step). A second chromatographic process used commercially to produce higher purity LF and LP was also not investigated in the laboratory. The shear forces applied in these trials were less extensive than in factory operations. The LF and LP were extracted using single-stage, stirred tank batch chromatographic capture with SP BB (pre-equilibrated with 10 mM phosphate buffer, pH 6.7). Factory capture of LF and LP is generally done using packed bed chromatography.

3.10.1 Milk treatment

Milk from cow 9201 was obtained from the AMS milking unit and transported to the laboratory. The LF and LP were then extracted from raw whole milk, skim milk and whey (Figure 3-10).

For each feed (raw whole milk, skim and whey), 20 mL of swelled, drained resin was added to 1.0 L of feed in a beaker in 37^{o}C (water bath). The milk/resin slurry was stirred at 150 rpm with an overhead stirrer. After 5 minutes, stirring was stopped and the slurry passed through Whatman No.41 (25 μm) filter to recover the resin. The

resin was washed with 500 mL of Milli-Q water at 37°C. Elutions were done with 100 mL of 0.4 M NaCl, and two 100 mL aliquots of 1.0 M NaCl. At each elution step the salt and resin slurry was stirred for 5 minutes before the resin was recovered by filtration. Subsamples from feed and eluates were analysed for LF and LP content.

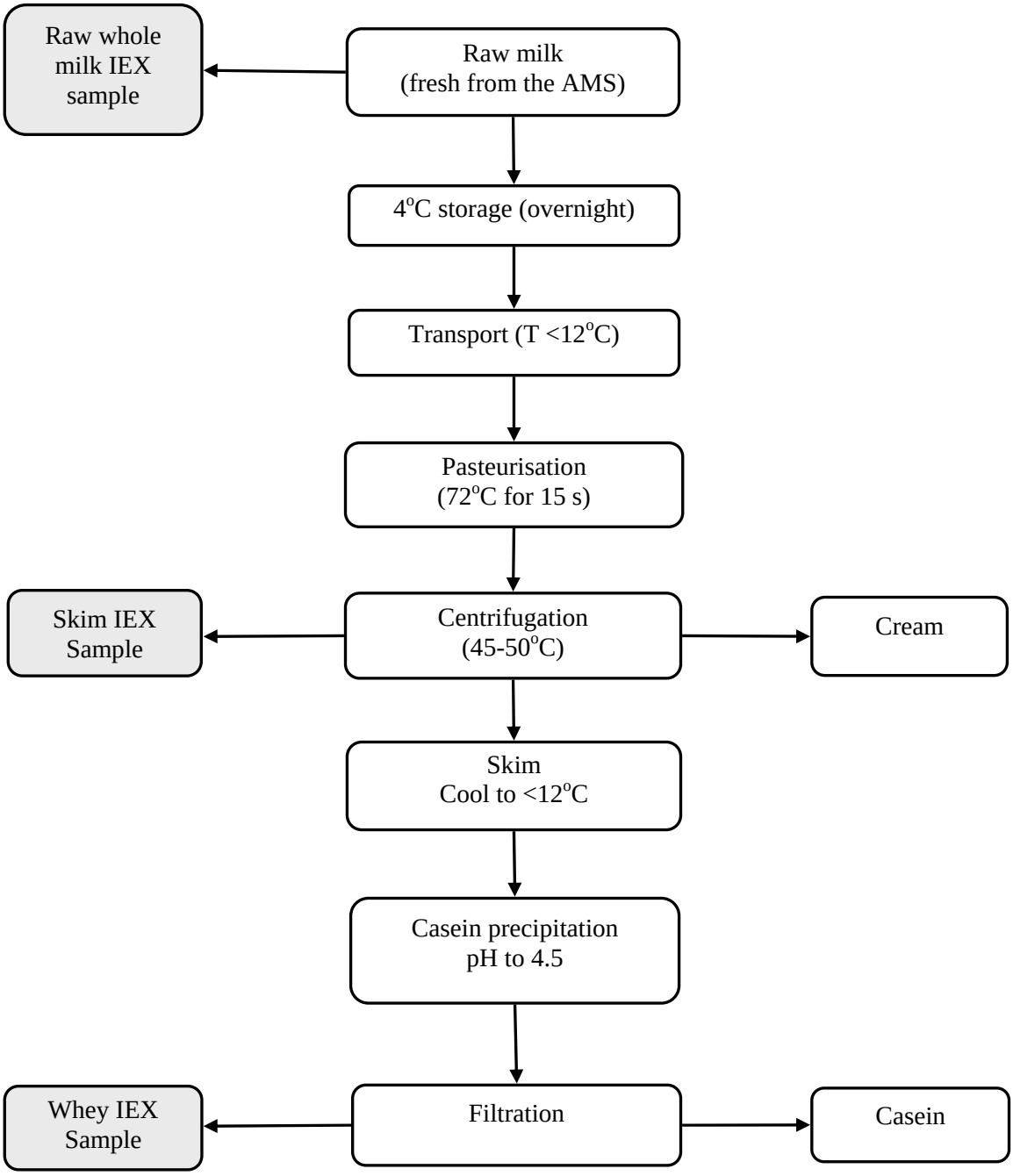


Figure 3-10: Process to produce samples for cation exchange of LF and LP from raw whole milk, skim milk and whey.

3.10.2 LF and LP extraction

- *Raw whole milk*

20 mL of equilibrated SP BB were added to 1.0 L of raw whole milk.

- *Pasteurized, skim milk*

Raw whole milk was centrifuged at 4000 rpm (Sigma Laboratory centrifuge) for 15 minutes in 250-mL centrifuge flasks. The centrifuged milk was left at 4^{o}C overnight to allow the cream to harden. It was then decanted to obtain skim milk.

A 4.0-L sample of skim milk was pasteurised at 72^{o}C with a 15-second holding time in a lab-made spiral core (coil) heat exchanger. The milk, initially at 18.8^{o}C, was heated to 72^{o}C by pumping at 0.25 L/min through a 2.4-m long, 5-mm i.d. coiled copper tube immersed in a 76^{o}C water bath. This flowrate gave a 15-second holding time at 72^{o}C. The LF and LP were extracted from 1 L of the pasteurised, skim milk.

- *LF and LP from whey*

A 2.0-L sample of pasteurised skim milk in a beaker was placed in a 40^{o}C water bath (40^{o}C helps minimise formation of fines, which make removal of coagulated casein by centrifugation or filtration difficult). The milk was gently stirred with an overhead stirrer while slowly adding 0.1 M HCl until skim milk was pH 4.6. The coagulated whey was then filtered through a Whatman No.1 filter to remove casein. The LP and LF were extracted from 1.0 L of filtered whey.

3.11 Adsorption studies

3.11.1 Equilibrium (static) capacity

To model preparative chromatography, the adsorption isotherm is required. The reversible adsorption in ion exchange chromatography is often described by the Langmuir isotherm (Equation 3.4). Values for the components in the equation can be calculated from batch adsorption to obtain equilibrium (static) capacity.

$$Q = Q^* \frac{K[A]}{1 + K[A]} \tag{3.4}$$

where Q is the desorption capacity in equilibrium with solute at concentration [A], Q* is the maximum adsorption capacity, and K is a rate constant. Solute concentration is expressed as mg/mL; adsorption capacities are expressed as mg protein per ml of resin.

Finite bath experiments were done to measure equilibrium adsorption capacities of BR 70, SP BB and SP FF resins. Aliquots (0.2 g) of equilibrated, swelled, drained resin were quantitatively weighed into 10-mL centrifuge tubes. The LF and LP standards from samples of known purity (Tatua Dairy Cooperative Limited) were re-constituted to 0.05 to 20.0 mg/mL. A 5-mL aliquot of each standard solution was added to the resin and left for 24 hours on a rotating plate in 37 ± 0.2^{o}C incubator. Tubes were then centrifuged to remove the resin and the supernatant was filtered in a 5-µm filter. Equilibrium LF (C_{LF}*) and LP (C_{LP}*) concentrations of the solutions were determined using the BCA protein assay. If required, samples were diluted to be within BSA calibration range. Equilibrium protein content in the resin was calculated as the difference between initial and final liquid-phase concentrations. A known weight of resin was settled in a measuring cylinder to obtain the equivalent volume for mass-volume conversion calculations.

The amount of protein bound to the resin was calculated from differences between initial and final solution protein concentrations. The equilibrium binding capacities for LF and LP (Q_{LF}* and Q_{LP}* respectively) were presented as the amount of protein bound per unit volume of resin (mg protein per mL resin). Langmuir isotherms were fitted to the experimental data.

3.11.2 Dynamic capacity

The effects of temperature on LF and LP extractions and breakthrough curves in packed bed chromatography were investigated. Dynamic capacities (breakthrough curves) for SP BB and BR 70 were determined using XK 16 columns packed to give a 10-mL CV. Raw whole milk (100 to 2000 mL) was processed (section 3.8.1 and Figure 3-9) at given flow rates and linear velocities (Table 3-1). Samples of the outflow were taken at 50-mL and then 100-mL intervals and analysed for LF and LP

content. The LF and LP in the feed were also determined. Breakthrough curves were generated. Yield and purity from the fractions collected after elutions were determined from selected experiments.

Table 3-1: Column dimensions, flowrate and linear velocities for breakthrough experiments.

Column			Flowrate	Linear velocity
diameter (cm)	height (cm)	volume (mL)	(mL/min)	(cm/hr)
1.6	5.0	10.0	10	298.4
1.6	5.0	10.0	15	447.6

The effect of temperature in packed bed 10 mL/min with SP BB were determined at 20, 25, 30, 35, 40 and 45^{o}C by maintaining feed and the column temperatures. 1000 mL feeds were used and samples taken at 100-mL intervals and analysed for LP activity and content and used to generate breakthrough curves. Pressure data was obtained from the Unicorn software and plotted for each of the processing temperatures.

3.12 LF and LP adsorption rates in a single-stage stirred tank

Dynamic capacities of SP BB and SP FF were determined using known concentrations of LF and LP in batch trials before undertaking more detailed kinetic adsorption experiments. Separate samples with 2.0 mg/mL LF or LP (Tatua Dairy Co-operative) at 36 ± 1^{o}C were continuously stirred at 150 rpm with an overhead stirrer (RW 20 DZ Mn). Samples were taken at regular intervals, filtered and analysed for protein content. Different resin:milk (^{v}/v) ratios (0.10 - 0.055) were used, and two separate raw whole milk samples were also used.

Detailed data for extracting LF from raw milk with SP BB and SP FF were determined using 2.0, 2.5, 3.3 and 5.0-mL aliquots of swelled, drained resin in 200 mL of raw whole milk in beakers in a water bath at 36 ± 1^{o}C. The resin to milk ratio (Φ) represented 250 mL media in 25, 20, 15 and 10.4 L of milk. Gentle, uniform stirring (150 rpm) was achieved using a multi-head stirrer system (Boltac, Figure 3-11). Approximately 1-mL aliquots were withdrawn from the milk/media slurry with a syringe at known times, passed through a 5-µm filter (Sartorious AG, Goettingen,

Germany) and analysed for LF content (SPR method). Data were used for kinetic modelling (Rowe *et al*. 1999).

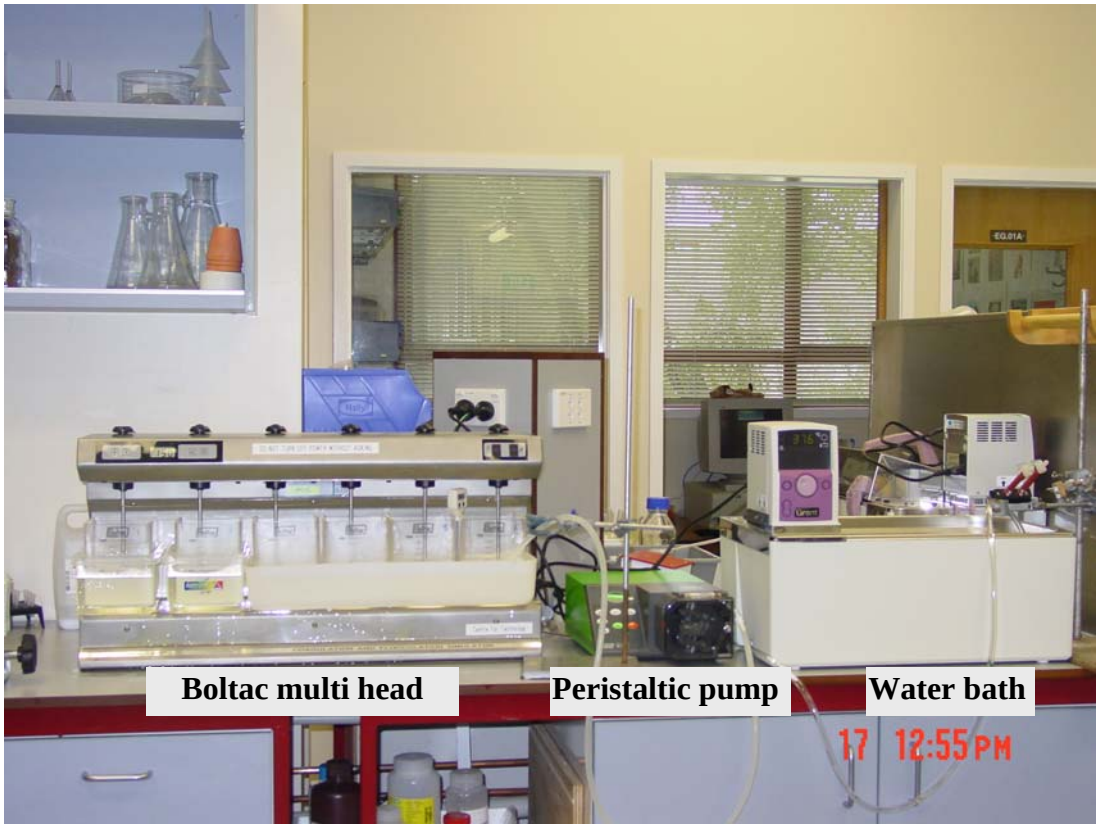


Figure 3-11: Single-stage stirred tank adsorption experiments for kinetic studies using a Boltac multi-head stirrer and a peristaltic pump to recirculate water for temperature control.

3.13 Lifecycle studies

Experiments were done to determine performance of SP FF resin after repetitive processing of raw whole milk and cleaning-in-place (CIP)/regeneration. Because resin is one of the major raw material costs in chromatographic separation, its lifetime (i.e. the number of times it can be used) is critical to this project's economic viability.

The experiments were designed to:

- evaluate LF yields over *n* cycles (50 cycles were chosen as a target)
- assess physical damage to resin by stirring (shear)
- obtain data for kinetic modelling

3.13.1 Controlled monitoring of performance using LF

Because raw whole milk must be processed immediately after collection to reduce degradation and LF concentrations vary between cows, reconstituted LF of known purity (Tatua Cooperative Limited) was used to measure resin performance after 1, 5, 10, 20, 30, 40 and 50 cycles. Using reconstituted LF ensured consistent feed. Fresh, raw whole milk was used for all other process cycles. Other components in milk such as fat, other proteins and lactose may also affect resin performance. Concentrations of these components also vary in individual cow's milk and it would have been unlikely that fresh milk samples would have consistent composition, especially using the voluntary milking system (AMS).

Samples (1 L) of fresh raw whole milk (from individual cows) was processed at ~37^{o}C in a single-stage stirred tank with an overhead stirrer (RW 20 DZMn) using a resin to milk ratio of 0.017. The resin milk slurry was stirred at 200 rpm. After processing, the resin was weighed, a SEM sample taken and fresh resin added to make up for any losses during processing.

The following processing sequence was used:

- Capture LF from feed (raw whole milk or reconstituted LF) on resin at 37^{o}C for 10 minutes. During this time take 1-mL samples at 1-minute intervals, pass though 5-µm filter and store at 4^{o}C for further analyses
- Drain resin on 25-µm paper filter (Whatman No.41) using suction to assist the draining process
- Wash with 3-5 CVs of distilled water or buffer
- Elute protein in two steps:

  4-5 CVs of 0.4 M NaCl

  4-5 CVs of 1.0 M NaCl
- Clean resin:

  Wash with 100 mL of 1.0 M NaOH

  Rinse with 200 mL of distilled water

  Wash with 100 mL of 1.0 M acetic acid

  Rinse with 200 mL of distilled water

- Regenerate resin

  Wash with 100 mL of 2.0 M NaCl (5.0 CVs)

  Rinse with 200 mL of distilled water

- Equilibrate resin

  Rinse with 10 mM phosphate buffer

  Check pH and adjust to to pH6.7-6.8 by repeated rinses with 10 mM phosphate buffer until desired pH is reached

Resin samples from the 1^{st}, 5^{th}, 10^{th}, 20^{th}, 30^{th}, 40^{th} and 50^{th} cycles were examined by optical microscopy and SEM. The LF concentrations in feed, and eluates were determined using the SPR method.

3.13.2 Solvent evaluation – physical effects of fat uptake

Resin used for raw whole milk extractions gradually accumulated fat and required a more rigorous cleaning protocol with a solvent that solubilised and removed the fat. The effectiveness of food grade solvents (ethanol, isopropanol and ethyl acetate widely used in the food industry and recommended by the resin manufacturer) was evaluated.

Fresh whole milk was centrifuged and kept at 4^{o}C for an hour to allow the cream to harden. The cream was then decanted off and rinsed with water. Samples (1.0 and 5.0 g) of cream were weighed into separating funnels and then 100 mL aliquots of 30%, 40% isopropanol or 70% ethanol was added to the separating funnel. The mixture was gently shaken to promote solvation. The amount of fat taken into the organic phase was inconclusive. Further analyses need to be done to determine the amount of fat dissolved in the organic phase. Gravimetric analyses were not possible because the cream and fat-containing organic phase could not be effectively separated. Ethyl acetate (approved for food usage) was also used to clean a small sample of resin.

Ethanol which has the highest polarity [(ethanol (C_2H_5OH) > isopropanol ($C(CH_3)_3OH$) > ethyl acetate ($C_2H_5COOC_2H_5$)] would be most effective in removing fat. A solvent cleaning step (200 mL of 40% isopropanol at ambient temperature, due to it's lower volatility relative to ethanol) was added to resin cleaning procedure.

3.13.3 Dairy cleaning solution

Optimum or Horolith Farm (Ecolab Limited, Hamilton, New Zealand) is a non-quaternary ammonium compound (QAC) acid detergent-sanitiser used in the food processing and dairy industries. New Zealand Food Safety Authority has approved its use for food contact surfaces. Optimum contains 0.1 M sulphuric acid and 0.1 M orthophosphoric acid. A 1:1000 dilution is used for cleaning milking equipment, followed by water rinses. This procedure can be an alternative to 1.0 M acetic acid.

3.13.4 Simplified cleaning protocol

The LF standards used to monitor resin performance were initially not fully recovered. The cleaning procedure was modified. Initially, each cleaning step had a two-minute desorption period. This was increased to at least ten minutes to allow reversible reactions to go to completion (e.g. recovering proteins using NaCl). The cleaning cycle, especially using NaCl and NaOH were undertaken at 37^{o}C. The modified procedure included rinsing with water at 37^{o}C for fat malleability.

In on-farm trials, protein was eluted from the resin, which was then pooled for cleaning and regeneration.

The following modified protocol as used:

Cleaning

- Wash to remove lipids (40% isopropanol, 37^{o}C for 10 min)
- Wash with 5 CV of 1.0 M NaOH (15 min in bath)
- Rinse with 10 CV of distilled water (water at 37^{o}C)
- Wash with 5 CV of 0.1% Tween in 1.0 M Acetic acid (15 min in water bath)
- Rinse with 10 CV L of distilled water (37^{o}C)

Regeneration

- 5 CV of 2.0 M NaCl (5.0 CV)
- Rinse with 10 CV of distilled water

Equilibration

- Equilibration with 10 mM phosphate buffer
- pH checked to be within 6.7-6.8 (else repeat rinses with 10-20 mM phosphate buffer)

4 Laboratory Fractionation Results and Discussion

4.1 Analytical chromatography

4.1.1 Size exclusion chromatography

Good separation for most proteins typically present in milk such as α-casein, β-casein, α-La, β-Lg, BSA, IgG and LP could be obtained with the Superdex 75 column (Figure 4-1).

The peak for the LF standard was diffused and the detection level was low (1 mg/mL). Sensitivities of 0.05 to 0.50 mg/mL are desired because these are the usual LF levels in milk. Using higher concentrations (up to 4 mg/mL) of LF standard did not improve the response. The LF may have been interacting with the size exclusion media because it was eluted during the 1.0 M NaOH wash cycles. Repeat injections of LF standards after rigorous cleaning did not improve the results.

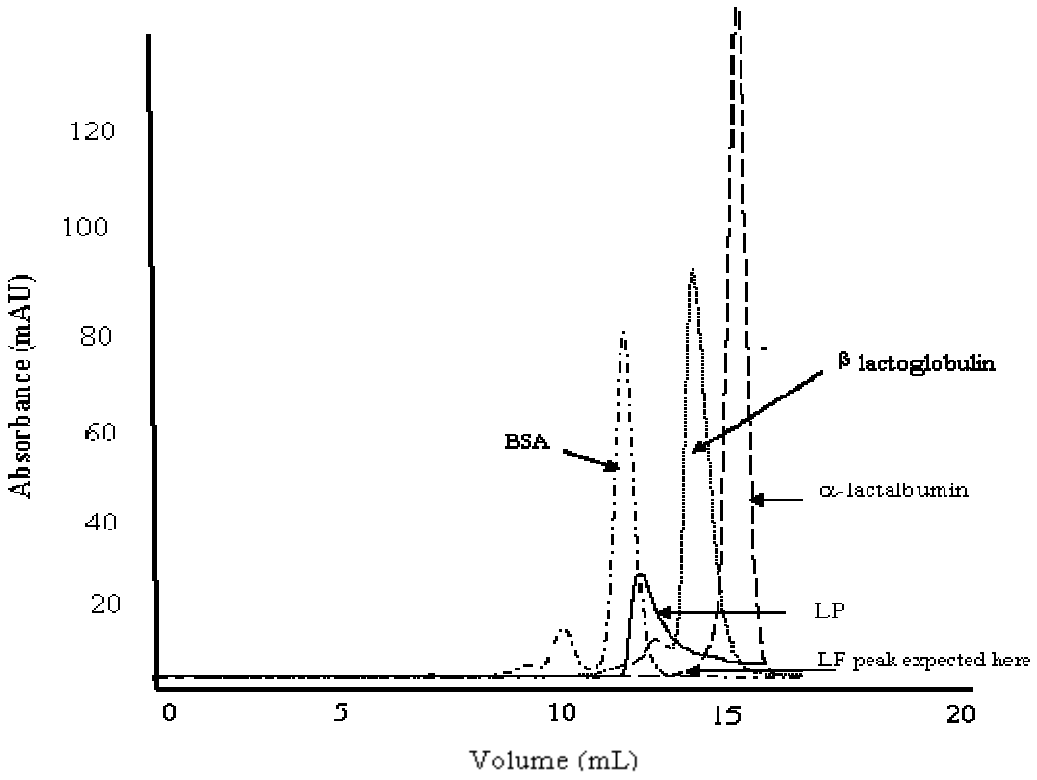


Figure 4-1: Size exclusion chromatogram of milk proteins using the Superdex 75 column.

4.1.2 Cation exchange chromatography

Detection level for LF and LP on cation exchange chromatography was 0.025 mg/mL. The area of the peaks in the UV traces were integrated and used to indicate the total mass, and hence concentration, in the samples. Replicate analyses (n=5) were used to construct the 0-20 mg/mL calibration curves, which had a linear regression of >0.999 (Figure 4-2).

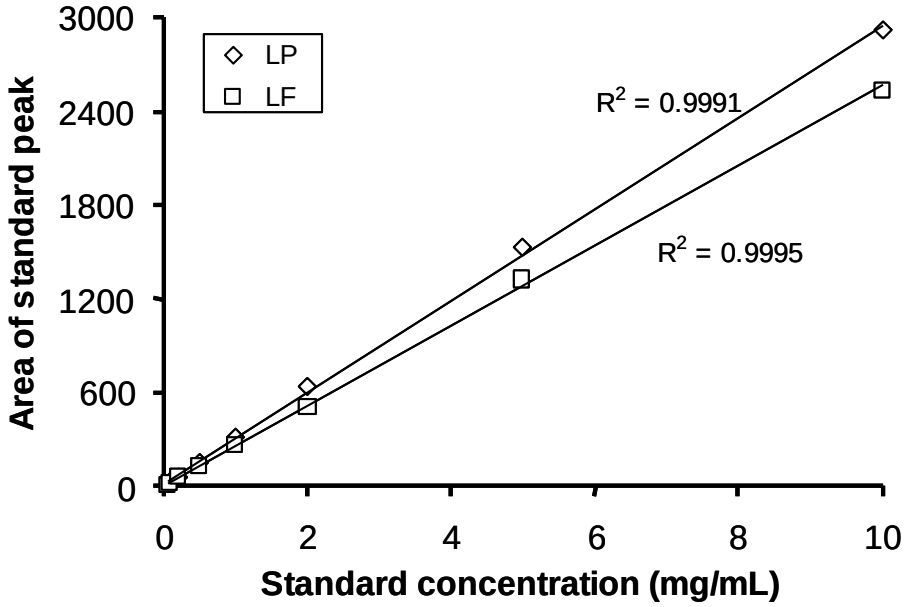


Figure 4-2: Standard calibration curves for LF and LP using a 1-mL Resource S column.

A typical chromatogram showed good resolution of LF and LP peaks but the LZ and LP peaks overlapped (Figure 4-3) and could not be separated even when the gradient was changed. Because the LZ content in milk is low, it will not be routinely detected. Capture effects of competing proteins such as LZ when fractionating LF and LP from raw whole milk was not evaluated but is expected to be insignificant at the normal target LF and LP purities of around 90%.

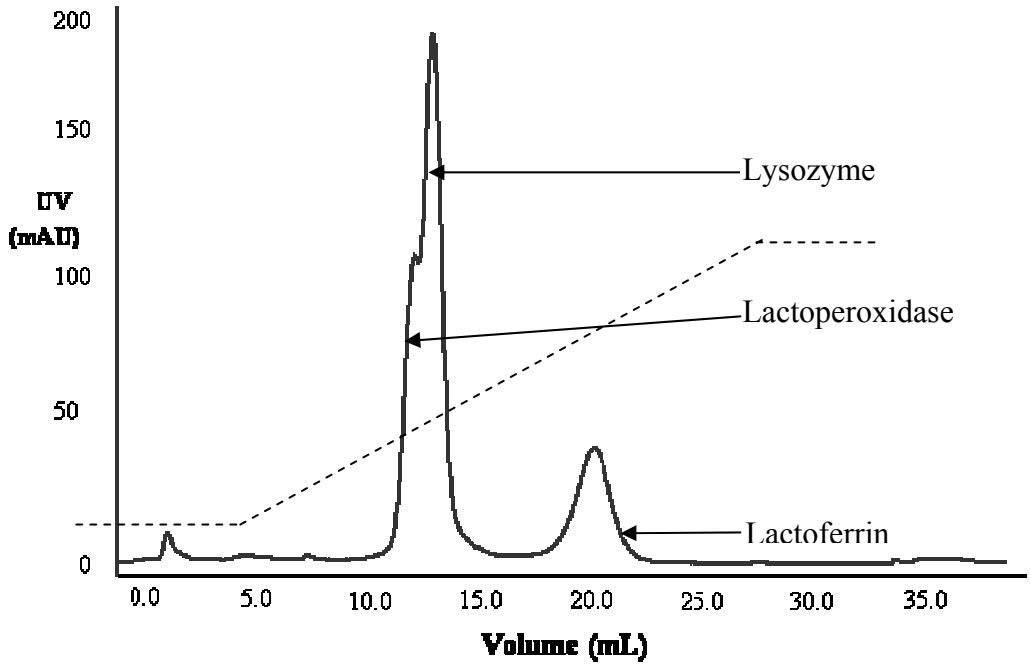


Figure 4-3: Typical resolution of LF, LP and LZ using a Resource S column on fplc with UV monitoring at 280 nm.

4.2 pH and conductivity

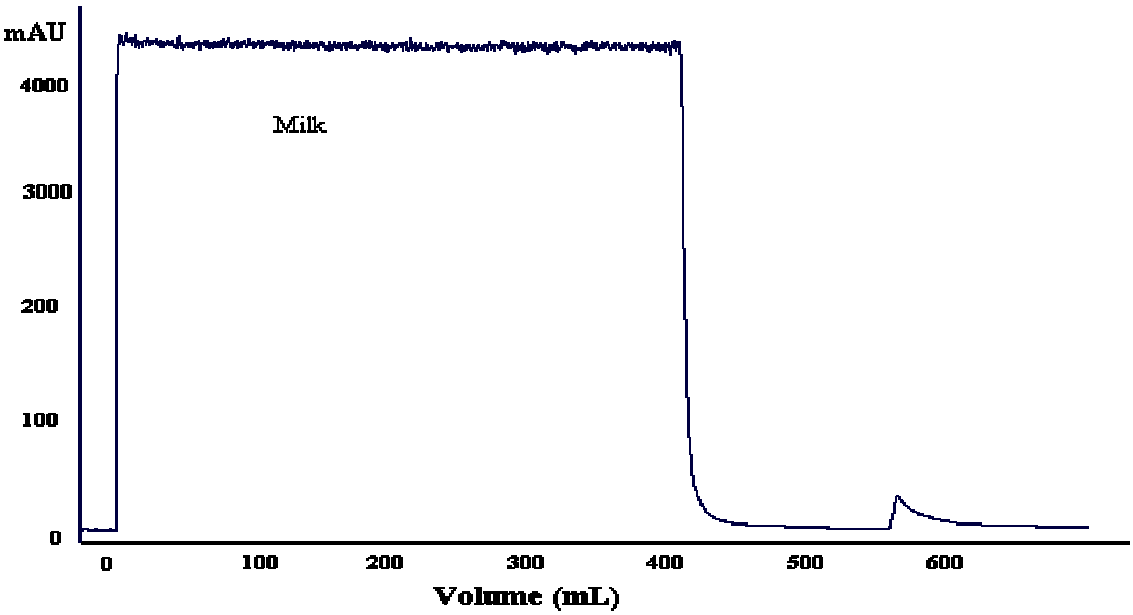
Raw whole milk used in the study normally had a pH between 6.5 and 6.8 and a conductivity between 4.8 and 6.2 mS/cm. These values are similar to those reported for bovine milk (Coulon *et al.* 1998; Mabrook and Petty 2002; Norberg 2005; Huppertz and Fox, 2006).

4.3 Laboratory-scale fractionation

4.3.1 Packed bed chromatography

A typical UV trace of cation exchange of raw whole milk has two distinct peaks (Figure 4-4 a and b). The first peak was confirmed as LP using the ABTS activity assay and the second peak was identified as LF after analysis on the Resource S column. This elution order agrees with published studies (Yoshida, 1991; Etzel *et al.* 1998). The LP activity in the collected fractions correlated well with the UV peak profile. Fractions 12-20 had the maximum LP activity (Figure 4-4b). Capture and elution profiles for BR 70 and SP BB resins were similar.

a. Adsorption



b. Elution

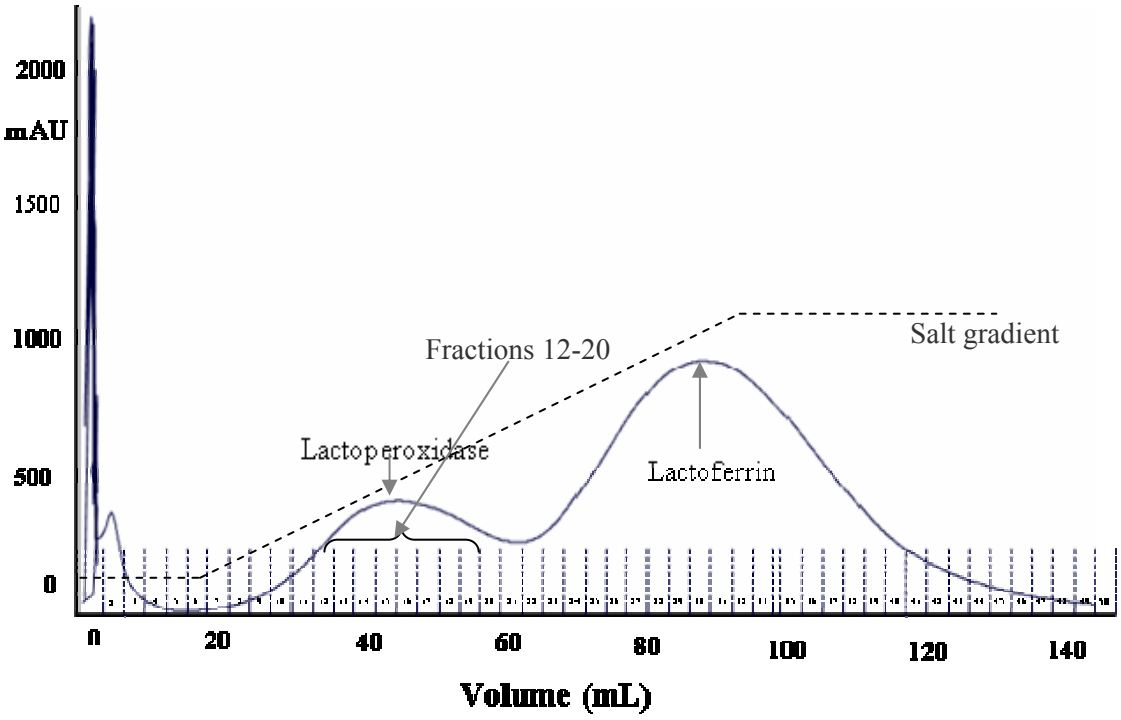


Figure 4-4: A typical fractionation UV trace of raw whole milk using FPLC.

When fractionating raw whole milk, no LP activity was detected in the outflow, indicating that most of the LP was captured by the resin in the 10-mL columns for feeds up to 1000 mL. Typical LP and LF content in bulk milk were calculated to be

10-40 mg/L and 120-300 mg/L respectively. These values compare well to reported LP and LF values (Kawakami *et al*. 1990; Elgar *et al*. 2000; Indyk and Filonzi 2005).

Early work in the study (Chand and Fee 2004) demonstrated that fat globules or casein micelles did not need to be removed before extracting LF and LP from raw whole milk using SP BB in a packed bed provided processing temperature was sufficiently high (section 4.6.2). More than 100 column volumes of raw whole milk at 35^{o}C could be passed through a 5-cm packed bed at 300 cm/hr (10 mL/min) without exceeding the maximum backpressure of the media (0.5 MPa) and column (Figure 4-5). The dynamic media capacity for LF was approximately 48.6 mg/mL. Flow rates of 10 mL/min or 15 mL/min (linear velocities of 300 or 450 cm/hr) were achieved within the recommended pressure maximum of 0.5 MPa for the XK 16 column (Figure 4-5).

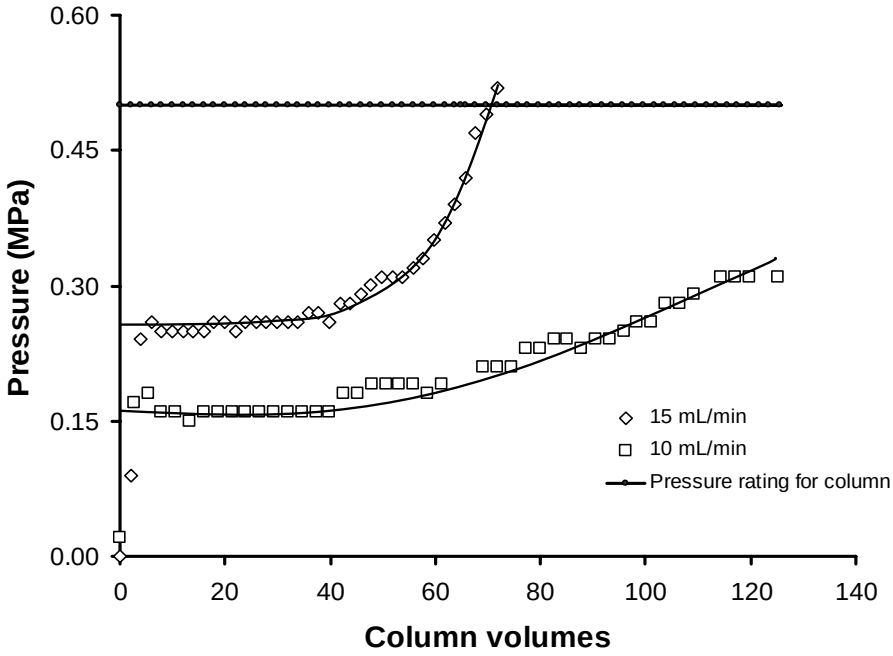


Figure 4-5: Pressure profiles when fractionating LF and LP from raw whole milk (37^{o}C) in a 10-mL XK 16 column packed with SP BB resin.

The capacities obtained for packed bed chromatography were similar to the capacity of 34 g LF per L skim milk for the SP BB resin reported by Etzel *et al*. (1998). Etzel *et al*. recovered 80% of the feed LF and suggested that other components may compete with LF for binding sites on the resin, reducing the net recovery. Trials showed that LF and LP could be successfully extracted from fresh whole milk by ion

exchange chromatography without affecting overall milk composition and the resultant milk could still be used in the traditional processes (Table 4-1).

Table 4-1: Composition (%) of bulk raw whole milk before (feed) and after (outflow) LF and LP extraction.

	Fat (% ^{w}/v)	Protein (% ^{w}/v)	Casein (% ^{w}/v)	Lactose (% ^{w}/v)	Total solids (% ^{w}/v)
Sample 1					
Feed	4.11	3.27	2.61	4.84	13.2
Outflow	4.04	3.29	2.63	4.84	13.1
Sample 2					
Feed	3.64	3.15	2.44	4.21	11.8
Outflow	3.55	3.07	2.35	4.35	11.6

4.4 Resin characteristics

4.4.1 Particle size characterization

Suspended particles in 10 individual raw milk samples had a $d_v(m)$ of 2.91±0.9 μm, a $d_v(0.9)$ of 5.52 μm and a $d_v(0.5)$ of 2.91 μm. These values compare well with published values for milk fat globules (Etzel 1998; Michalski *et al*. 2005). The size of casein micelles was not measured but published values report that casein micelles in raw milk have a diameter of 0.15 μm. The SP BB resin had a $d_v(m)$ of 154±67 μm, a $d_v(0.9)$ of 219 μm and a $d_v(0.5)$ of 155 μm (Figure 4-6).

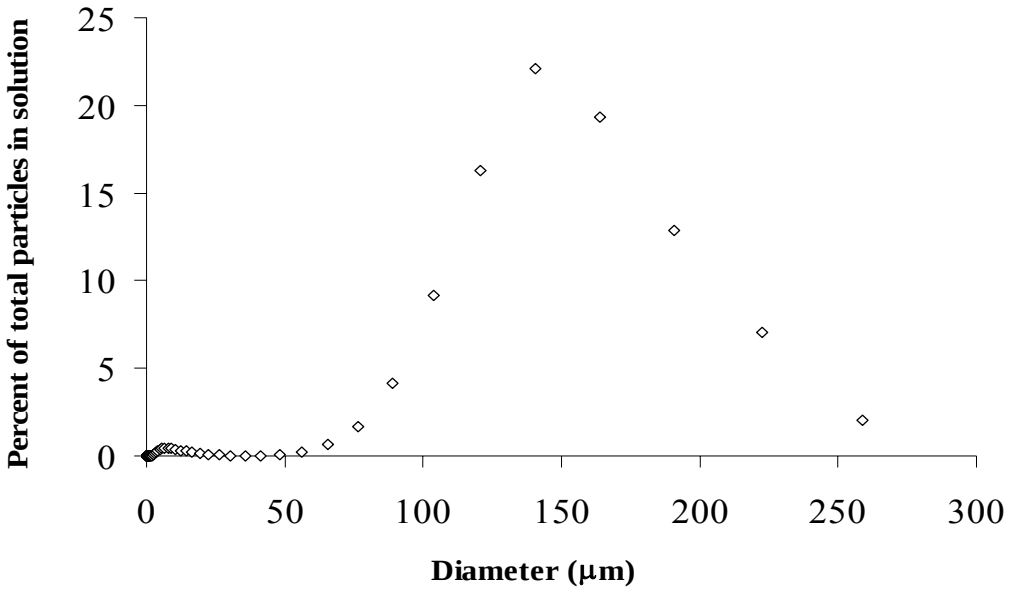


Figure 4-6: Laser size distribution of SP BB particles.

4.4.2 Screening to remove fines from SP FF

Smaller resin particles (fines) could be effectively removed by screening the SP FF resin with a 42 μm sieve (Table 4-2). Fines will increase the potential to clog the column and increase draining time when recovering resin in a stirred-tank system. Removing fines could make on-farm extractions with SP FF feasible.

Table 4-2: Effect of sieving SP FF with a 42-μm filter on particle size distribution (μm).

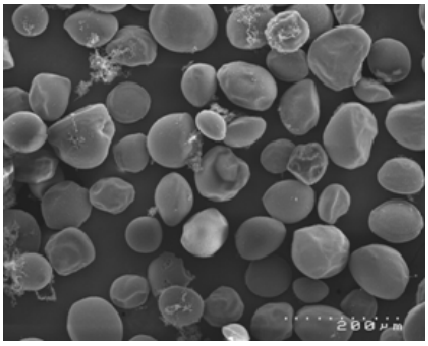
Source of sample	d_v (0.1)	d_v(0.5)	d_v(0.9)	d_v(m)
SP FF (fresh)	36.57	79.22	129.73	80.89
Oversize	46.49	89.19	136.30	89.14
Undersize (fines)	17.49	43.86	63.17	42.44

4.4.3 Effect of pumping on SP BB

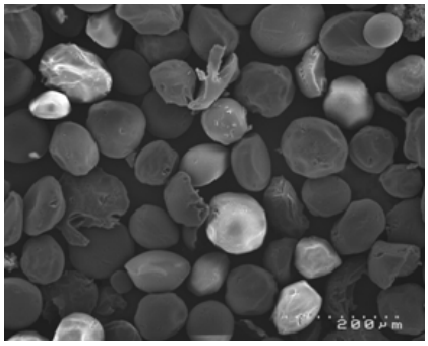
SEM images showed that resin subjected to centrifugal pumping at 1.55 or 2.95 L/s contained broken and cracked particles (**Error! Reference source not found.**ii-iii). Resin pumped at 0.60 L/s showed little or no sign of damage. Thus, the fastest rate that an individual volume of milk should be processed without undue resin damage is 0.6 L/s.

4.4.4 Effect of high linear velocity on SP BB

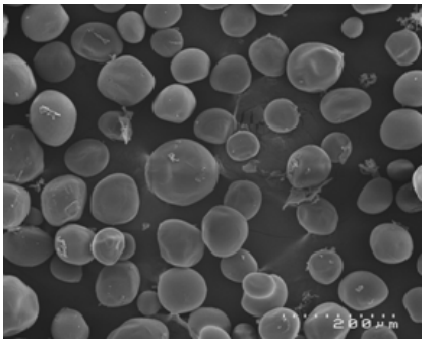
The maximum back pressure on a 1-mL HR5/5 column for fplc when using feeds of skim or ethanol at flow rates between 5and 20 mL/min was 0.31 MPa (column rated at 1.5 MPa). The SEM images of the SP BB resin (**Error! Reference source not found.**iv and v) showed no evidence of any damage at linear velocities of up to 6000 cm/hr.



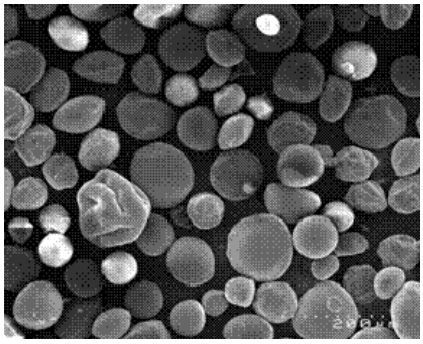
i. Fresh unused resin



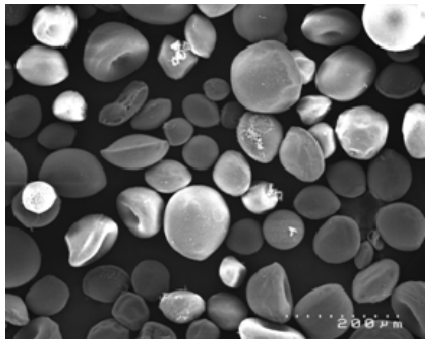
iii. After being pumped (2.65 L/s).



ii. Exposed to 1.5 MPa in a packed column (HR 5/5)



iv. After 6000 cm/hr with standard filter (CV 0.55 mL; HR 5/5)



iii) After 6000 cm/hr with 200 μm filter (CV 0.3 mL; HR 5/5)

Figure 4-7 (i-v): SEM images of SP BB resin subjected to various physical stresses.

Data from this series of experiments indicated that it would be best if the resin was not pumped as most damage was sustained through centrifugal force. The resin was not very susceptible to high linear velocities in packed columns and earlier work using packed bed chromatography showed no evidence of the SP BB resin compacting. However, at these high flow rates, damage may occur at the filter interface even though samples taken from the bulk part of bed were not damaged. Ion exchange column chromatography is normally operated at 300-600 cm/hr, and SP BB can be operated to at least 1000 cm/hr provided adsorption rates are suitable.

4.5 Equilibrium (static) capacity

Langmuir isotherms fitted to experimental data (Figure 4-8) showed the that maximum equilibrium capacities for swelled resin after 24 h adsorption were 200 mg LF and 225 mg LP per g of SP BB resin and 100 mg LF and 125 mg LP per g of BR 70 resin.

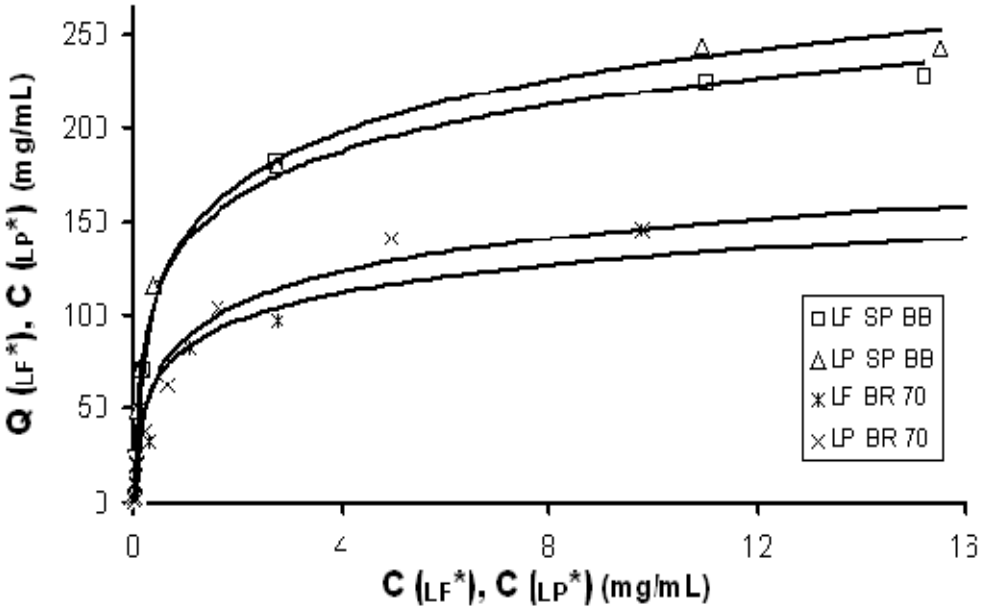


Figure 4-8: Langmuir isotherm fit for the experimental equilibrium adsorption data of SP BB and BR 70 resins for LF and LP after 24 h.

The SP FF resin, which has a similar average particle size to BR 70 resin (90 μm) also has similar maximum equilibrium capacity of 100 mg LF (Figure 4-9) or LP per g of SP FF resin.

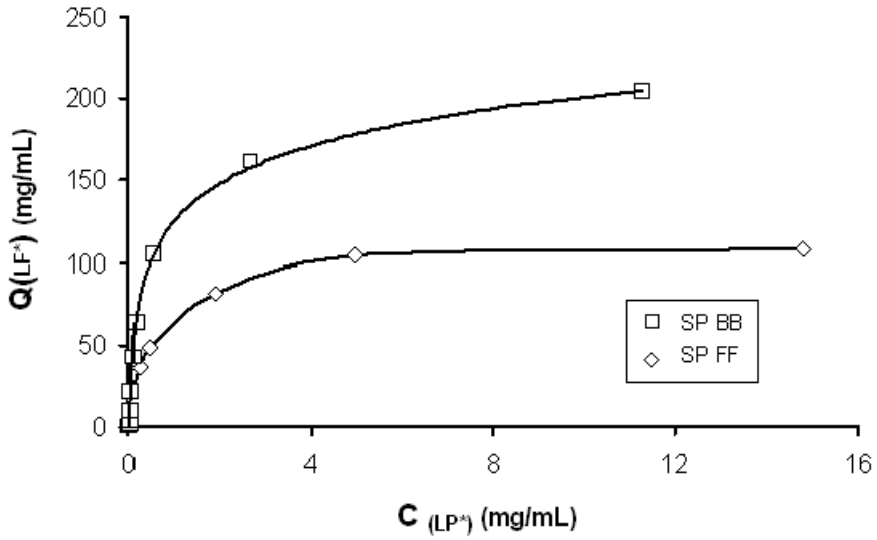


Figure 4-9: Langmuir isotherm fit for the experimental equilibrium adsorption data of SP BB and SP FF resins for LF after 24 h.

The measured maximum static binding equilibrium capacity that SP BB resin had for LF (Mr 77 kDa) is very similar to the manufacturer's claims for its dynamic capacity of approximately 200 mg BSA (Mr 67 kDa) per mL resin at 12 cm/h rate (GE Healthcare Technologies, 2005). Similarly, the maximum capacity measured for SP FF resin is comparable to the manufacturer's claim for human serum albumin (Mr 67 kDa) of 110 mg/mL.

The maximum capacity of media is very high for standard solutions but Q_{LF}* is strongly dependant on C_{LF}* below 1 mg/mL. Although the maximum equilibrium capacity of the resin is 100 to 200 mg/L for high solution concentrations (>5 mg/mL) of LF and LP, the equilibrium resin capacity at the normal concentrations of these proteins in milk will be much lower than the maxima. Also, during processing, the maximum capacity of the resin cannot be achieved as the high contact times required would compromise milk quality by allowing microbial growth. Processing ideally should be completed in the average time for milking (a few minutes). Other milk components may adversely affect the LF binding but this was not investigated.

The experimental data showed that the cation exchangers investigated (BR 70, SP BB or SP FF) could capture LF and LP from raw whole milk. If the adsorption process is rate dependant, the smaller bead size of BR 70 and SP FF is advantageous because there is more surface area per unit volume of resin. However the higher

static adsorption capacity of SP BB resin, together the lower back-pressure due to its larger size, make it more suitable for milk processing.

4.6 Dynamic capacity of resins

4.6.1 Breakthrough curves

Trials showed that for LP and LF feed concentrations of 12.5 and 207 mg/L, 100 CVs of raw milk could be processed at a linear velocity of 300 cm/h (10 mL/min) in 10-mL XK 16 columns before LP and LF breakthrough occurred (Figure 4-10 and 4-11 respectively). Column pressures remained below 0.5 MPa during processing. Protein adsorption by the resin depended on linear velocity and protein concentration in the feed. Virtually all the LP and up to 95 % of LF could be extracted using packed bed chromatography of raw whole milk at 37^{o}C before breakthrough occurred.

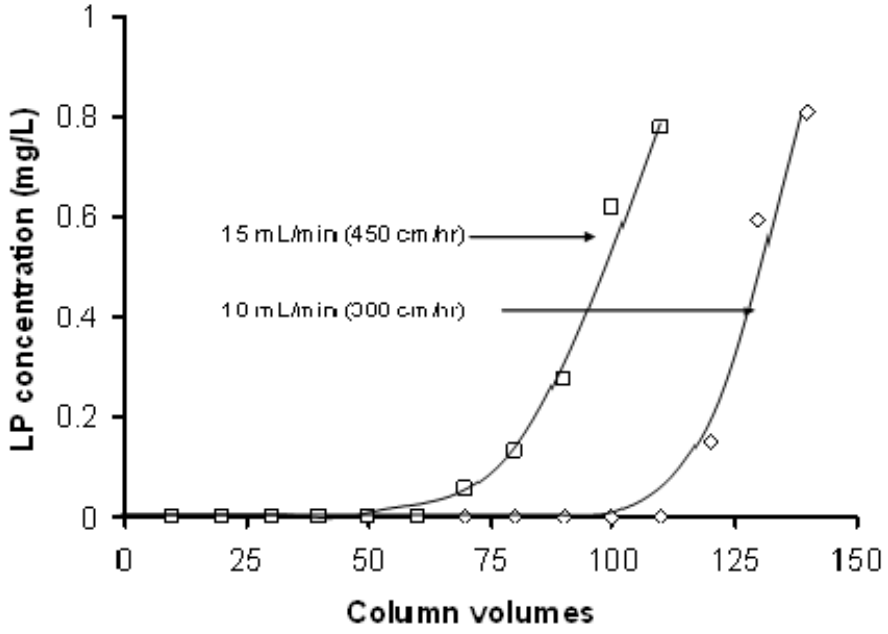


Figure 4-10: Effect of linear velocity on LP breakthrough when processing raw whole milk in a 10-mL XK 16 column packed with SP BB resin.

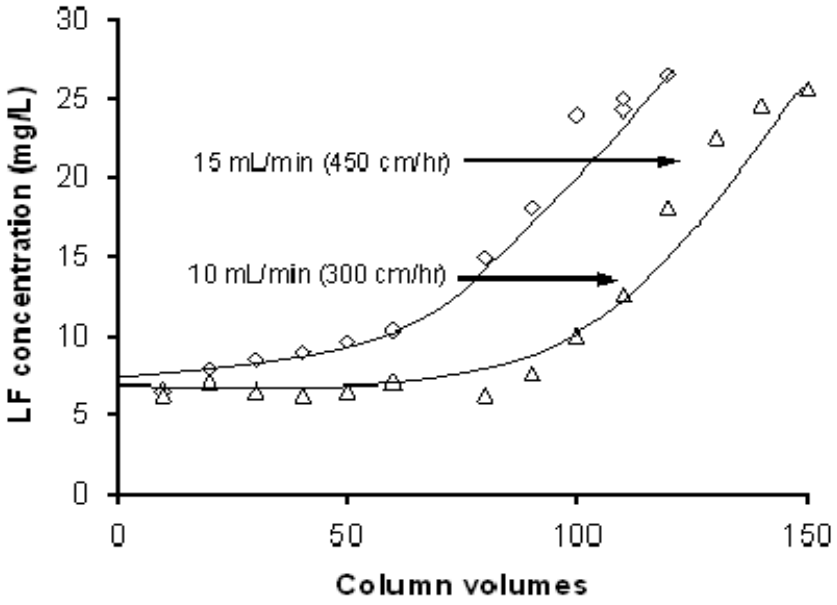


Figure 4-11: Effect of linear velocity on LF breakthrough when processing raw whole milk in a 10-mL XK 16 column packed with SP BB resin.

Etzel *et al*. (1990) investigated the effects of processing parameters in packed bed chromatography with SP BB and EBA with Streamline™ SP (density 1.2 g/L) using skim milk with enhanced levels of LF (0.5 g/L) to reflect levels present in transgenic milk and reported that SP BB had a dynamic capacity of 34 mg LF per mL resin, which compares well with the dynamic capacities (35 and 48.8 mg LF per mL SP BB) observed during this study using raw whole milk.

4.6.2 Effects of temperature on breakthrough

Increasing the temperature improved processing and adsorption kinetics. More milk passed through the column before allowable pressure limits were reached (Figure 4-12) and more protein was adsorbed onto the resin before breakthrough occurred (Figure 4-13). Also, while freshly obtained milk could be processed at 20^{o}C, the raw whole milk that had been refrigerated or left standing for a few hours could not, mainly because some of the fat had separated out.

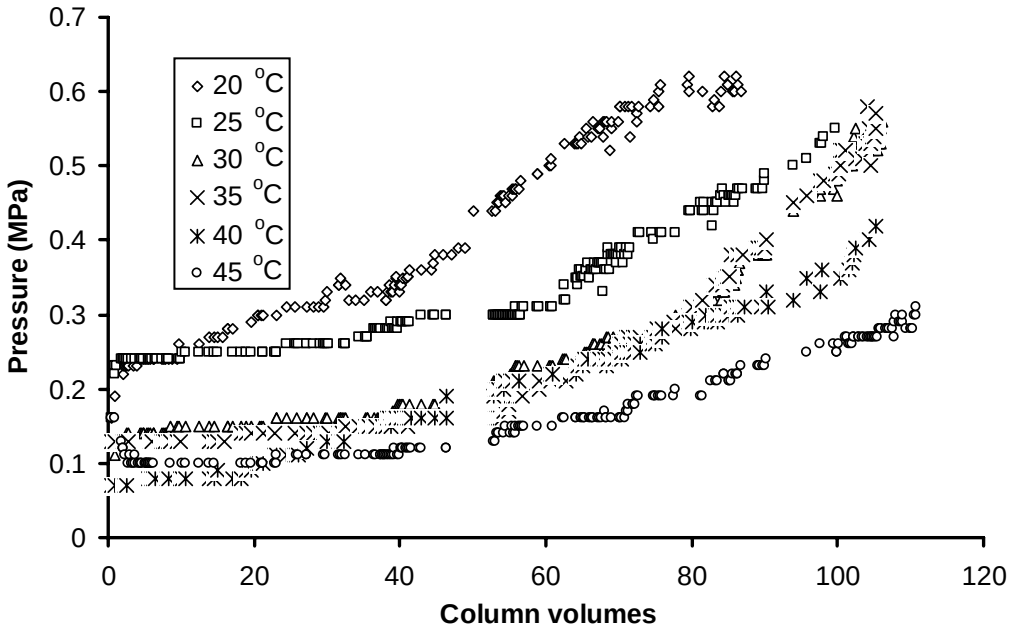


Figure 4-12: Effect of temperature of feed (raw whole milk) on the backpressure on column during processing using packed bed chromatography with SP BB at 20, 25, 30, 35, 40 and 45°C.

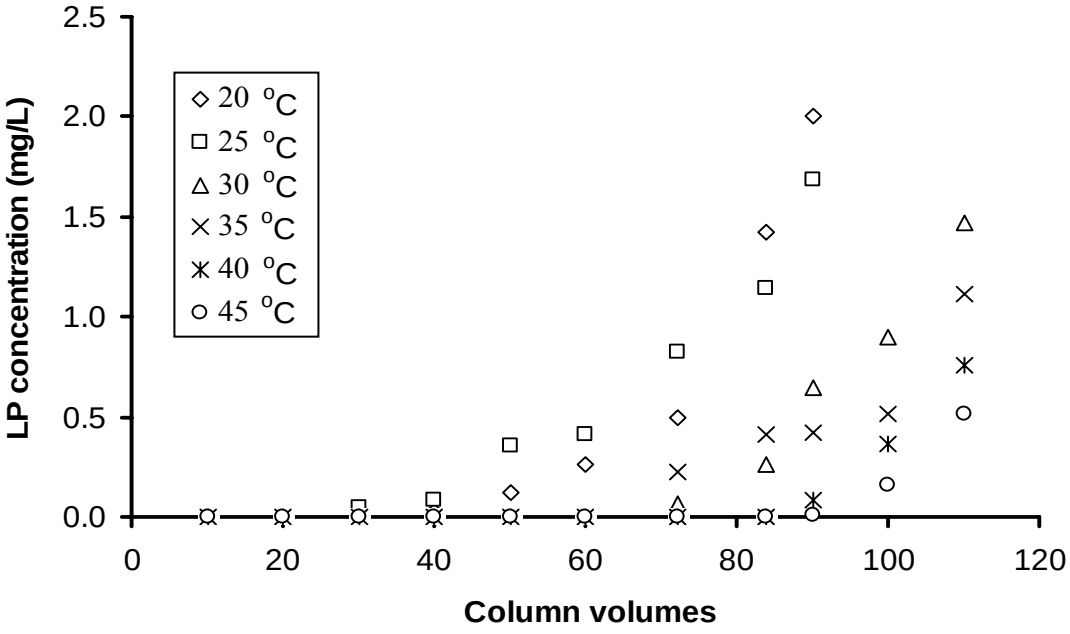


Figure 4-13: The effects of temperature on breakthrough of LP at 20, 25 30, 35, 40 and 45°C using SP BB resin.

The size of the milk fat globules (up to 10 μm), effect of temperature on fat viscosity, and the strong influence of processing temperature on column backpressure may make milk fat globules become more malleable as temperature increases, allowing

them to pass through the bed. At lower temperatures, the fat hardens or solidifies, preventing passage. The larger size of SP BB resin compared to FF and BR 70 resins assists in processing raw whole milk by allowing larger particles to pass through before the resin becomes clogged. Etzel *et al*. (1998), while processing skim milk at a miniature whey refinery, reported that robustness improved slightly by increasing temperature (which decreased viscosity) from 10°C to 20°C but felt that the risk to process hygiene possibly outweighed the improvement achieved.

4.6.3 Effects of temperature, milk and fat retention on column processing

An analysis of data (Figure 4-12) shows that the number of CVs that could be processed before pressure exceeds on arbitrary value of 0.3 MPa increases with temperature (

Figure **4-14**).

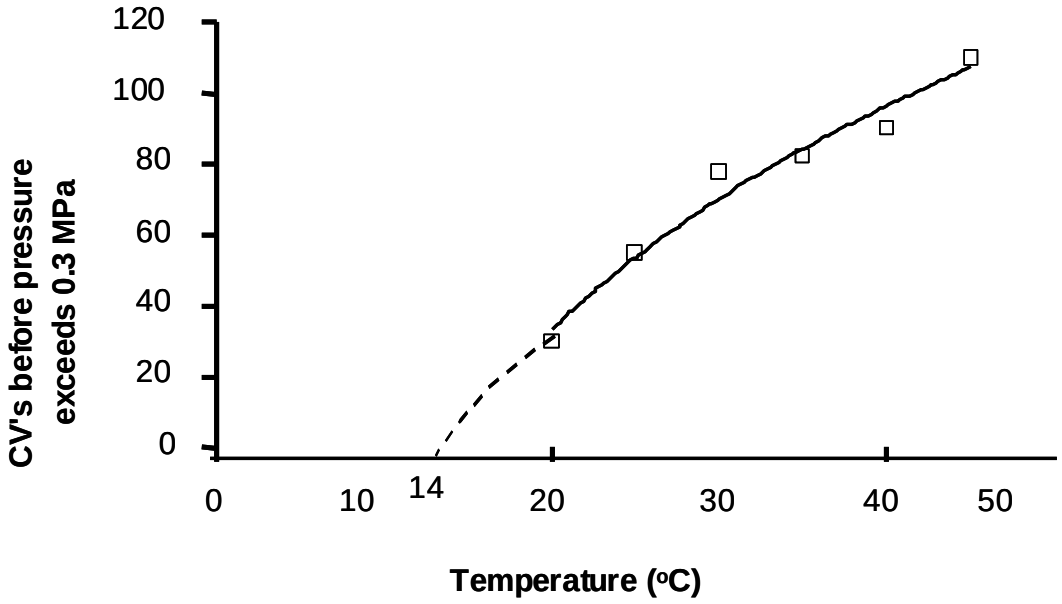


Figure 4-14: Effect of temperature on CVs that can be processed in a packed bed of SP BB resin before pressure exceeds 0.3 MPa.

Particles in milk typically have diameters from 0.1 μm (caseins) to 10 μm (fat globules) (Bylund and TetraPak 2003). Fat globules usually cause problems in chromatographic separations because they block packed columns as soon as the feed is introduced. Therefore, fat is normally removed before cation exchange capture of

LF and LP from milk. Raw whole milk contains larger suspended particles than whole (full fat) processed milk, which has been homogenized in the factory to a uniform consistency. Thus, raw whole milk might be more difficult to process using column chromatography. The generally accepted view that raw whole milk can not pass through a packed bed chromatography column is likely caused by low processing temperatures.

Milk is processed nominally at 4^{o}C to minimise microbial growth. At approximately 14^{o}C, very little milk can pass through the packed bed column. An investigation of the composition of the fatty acids in milk fat triglycerides, along with their individual melting points (Table 4-3) shows that oleic acid (C18:1), which is up to 40% of the fatty acid in milk fat, and has a melting point of 14^{o}C. This temperature corresponds well with the temperature at which processing through packed bed becomes impossible.

Table 4-3: Composition and melting points of the major fatty acids in milk fat (Bylund and TetraPak 2003).

Fatty acid	% of total fatty acid content	Melting point (◦C)
Butyric	3.0–4.5	−7.9
Caproic	1.3–2.2	−1.5
Caprylic	0.8–2.5	16.5
Capric	1.8–3.8	31.4
Lauric	2.0–5.0	43.6
Myristic	7.0–11.0	53.8
Palmitic	25.0–29.0	62.6
Stearic	3.0–7.0	69.3
Oleic	30.0–40.0	14.0
Linoleic	2.0–3.0	−5.0
Linolenic	<1.0	−5.0
Arachidonic	<1.0	−49.5

Fresh raw milk, immediately after milking is about 35-37^{o}C. Although this temperature is below the melting points of several fatty acids, notably myristic and palmitic acids, it is above the melting point of oleic acid. Fat globules hardness will be affected by the relative fatty acid compositions of the milk fat triglycerides. At 35-37^{o}C, milk fat globules may be soft enough to easily deform and pass through a packed bed if the chromatography resin has a sufficiently large particle diameter. Also, milk viscosity decreases with increasing temperature so column backpressure for low fat milk decreases at higher temperatures. The lower viscosity, combined

with higher protein diffusivities at higher temperatures can enhance chromatographic separations.

4.7 LF and LP extractions from raw whole milk, skim milk and whey feeds

Milk (12.8 L) from cow 9201 was used in trials to investigate extraction of LP from raw whole milk, skim milk or whey. The highest LP recovery in the eluates (72%) was from skim milk (Table 4-4). This recovery, after accounting for LP concentration due to cream removal, represented 64% of the original LP in the whole milk compared with 59% yield from raw whole milk. The lower yield may indicate that fat in raw whole milk affects LP recovery. The lower capture of LP present in the whey (26.8%) indicates that extensive processing may adversely affect the extraction process; only 22% of LP present in whole milk was recovered from the whey fraction. Another reason could be that the protein (LP) is longer present in the form to be captured under the processing and elution conditions used in the column (where almost all LP was captured).

Table 4-4: Activity, recovery and yields of LP extracted from 1 L of raw whole milk or the equivalent skim milk and whey with SP BB after 10-minute adsorption in single-stage stirred tank.

Stream and process	Volume (mL)	LP (IU/mL)	Total LP (mg)	LP mass recovery (%)	LP yield %	Yield in eluates (%)	LP relative to original (%)
Fresh whole milk	1000	2.2	15.7		100.0		
Eluate 1	100	9.24	6.6		42.0		
Eluate 2	100	3.66	2.6		16.6		
Outflow	1000	0.9	6.4		40.9		
				99.5		58.6	58.6
Skim milk	889	2.89	16.3		100.0		
Eluate 1	100	18.17	10.3		62.9		
Eluate 2	100	2.64	1.5		9.1		
Outflow	889	0.73	4.1		25.3		
				97.3		72.0	64.0
Whey	738	0.98	3.8		100.0		
Eluate 1	100	0.91	0.4		9.3		
Eluate 2	100	1.72	0.7		17.6		
Outflow	738	0.7	2.7		71.4		
				98.3		26.8	22.3

The trend for the amount of LF extracted from raw whole milk, skim milk and whey was similar to that observed for LP, (Table 4-5) but less was extracted (25.3, 32.4

and 19.9% respectively). The higher capture of LF from skim milk than from raw whole milk could be due to fat content impacting on adsorption. Removing the cream increases concentrations of milk components in the skim. For example, the LF concentration increased from 1590 mg/mL in raw whole milk to 1716 mg/mL in the skim milk. Having higher feed concentrations will increase the adsorption kinetics (i.e. rate) which increases binding rate and hence the yield obtained in a given time. The low pH of whey (4.6 compared with 6.8 of skim and whole milk) can also affect LF yields. Hahn *et al*. (1998) attributed acidity of the (mineral) whey to the low LF yields they obtained.

Table 4-5: Recovery and yields of LF extracted from raw whole milk, skim milk and whey with SP BB after 10-minute adsorption in single-stage stirred tank.

Stream and process	Volume (mL)	Total LF (mg)	LF mass recovery (%)	LF yield (%)	Yield in eluate (%)	LF relative to original
Fresh whole milk	1000	1590		100.0		
Mill-Q wash	500	21		1.3		
Eluate 1	100	13		0.8		
Eluate 2	100	369		23.2		
Outflow	1000	1241		78.1		
			103.4		25.3	25.3
Skim milk	889	1526		100.0		
Milli-Q wash	500	11		0.7		
Eluate 1	100	7		0.5		
Eluate 2	100	477		31.3		
Outflow	889	999		65.5		
			98.0		32.5	28.9
Whey	738	889		100.0		
Milli-Q wash	500	29		3.3		
Eluate 1	100	13		1.5		
Eluate 2	100	134		15.1		
Outflow	738	669		75.3		
			95.2		19.9	14.7

Further investigations are needed to optimize adsorption of both LF and LP in a single-stage stirred tank system before the process can be adopted for on-farm capture. The SP FF resin which has smaller particles (average 90 μm) may give better adsorption and yields.

Extraction time and temperature effects were also investigated and mathematical modelling assistance used to enhance understanding the kinetics of adsorption (section 4.9 and Chapter 5).

4.8 Lifecycle studies

Because resins are very expensive, economic performance of chromatographic separations are very dependent on resin stability over repeated extractions. Ideally, SP Sepharose resin may be recycled for at least 500 cycles provided adequate cleaning and regeneration protocols are used (GE Healthcare, 2004). The lifecycle studies carried out showed that SP FF could be used for at least 50 cycles without any decrease in performance (Figure 4-15). Further work is required to confirm the maximum lifetime of the resin.

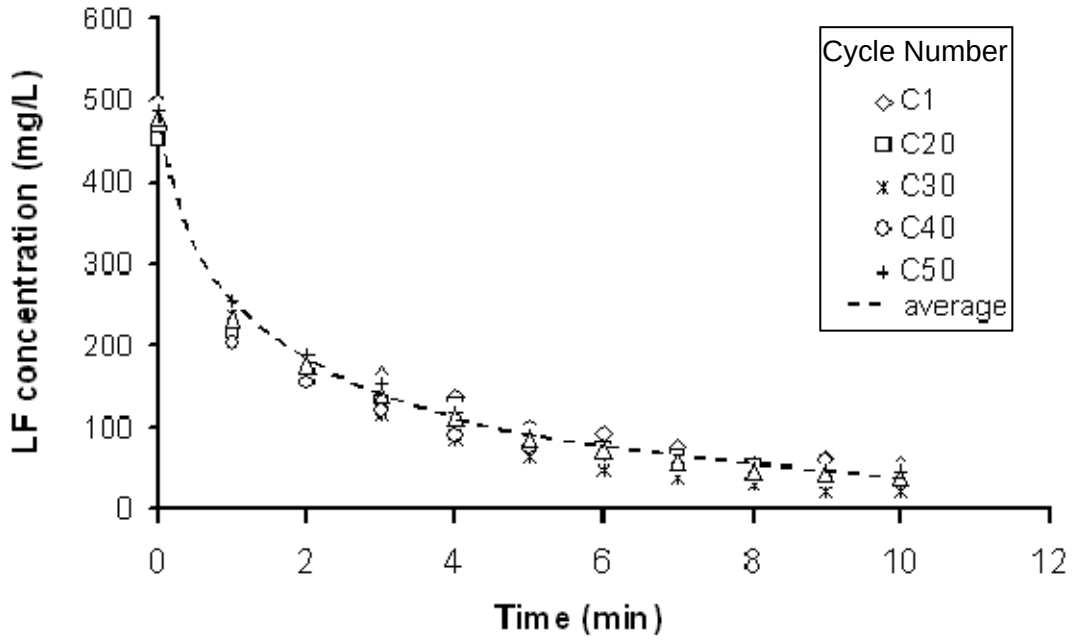
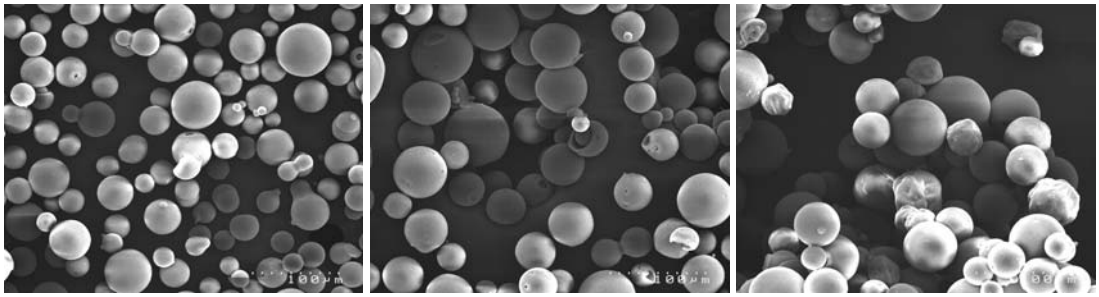
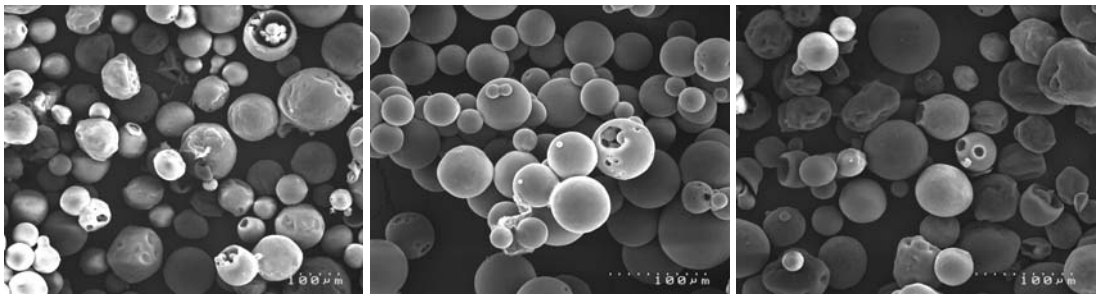


Figure 4-15: Effect of resin reuse (up to 50 cycles) on LF adsorption from a LF standard solution (0.5 mg/mL).

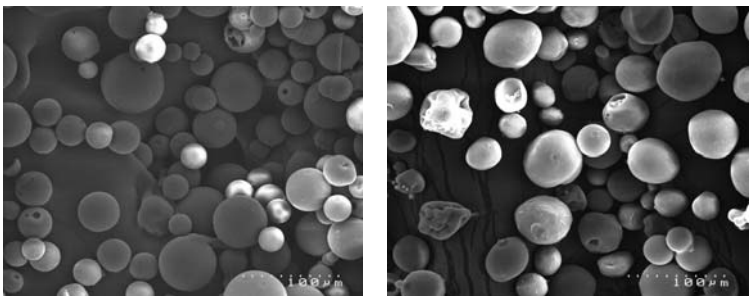
Data from cycles 5 and 10 have not been included in Figure 4-15. The feed/resin slurry during these runs was in a Perspex container in a water bath (37^{o}C). However, due to the slow heat transfer, the temperature of the slurry was only at 24^{o}C after 10 minute. Thus, the lower performance for cycles 5 and 10 was attributed to procedural error rather than lower resin performance. For the remaining trials, a glass container was used and the milk and reconstituted LF were pre-warmed to 37^{o}C before being placed in the bath. The SEM images (Figure 4-16 a-h) of fresh resin and resin after various absorption cycles show no evidence of damage or physical distortion of resin particles.



a. Fresh resin b. After cycle 1 c. After cycle 5



d. After cycle 10 e. After cycle 20 f. After cycle 30



g. After cycle 40 h. After cycle 50

Figure 4-16: SEM images of SP FF resin before (a) and after cyclic processing (b-h).

4.9 Kinetics of adsorption

4.9.1 Batch extraction of LF in a single-stage stirred tank

Data on the amount of LF extracted from standard solutions showed that SP FF resin has faster rate uptake than SP BB resin (Figure 4-17). The uptake rate increased with increased resin:milk ratios.

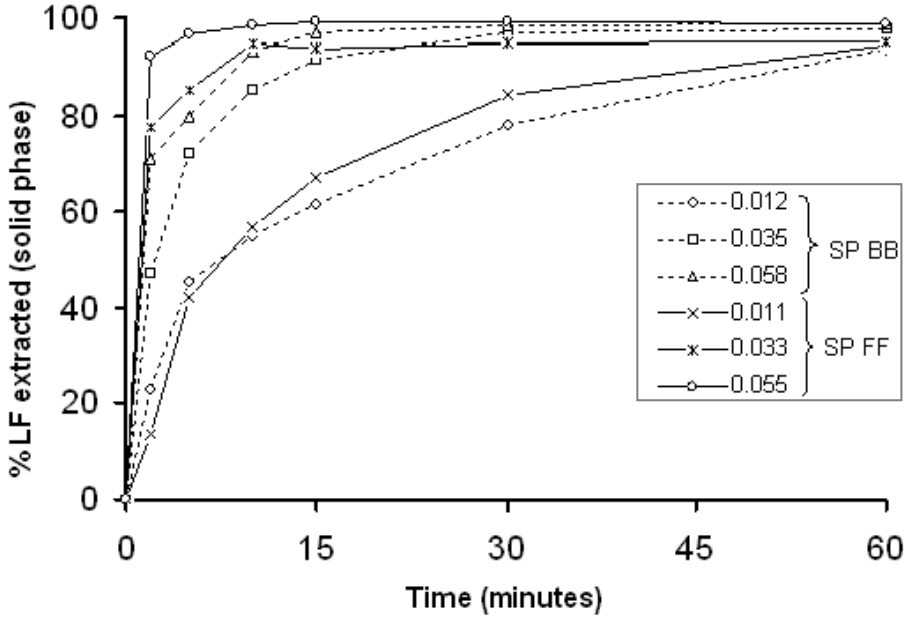


Figure 4-17: Effects of time and resin amount on LF adsorption with SP BB and SP FF resins with standard LF (0.5 mg/mL) in a single-stage stirred tank at 36^{o}C.

The approach to equilibrium is fast enough to absorb significant amounts of LF within 10 minutes. After 45 minutes adsorption, uptake appears to be at or near equilibrium. Laboratory experiments were done at a nominal temperature of 36 ±1^{o}C and the effect of temperature on kinetics of adsorption during batch extraction was not studied because on-farm extractions were expected to be at nominal milk secretion temperatures (approximately 37^{o}C). However, temperature had a favourable effect on adsorption in packed bed chromatography (section 4.6.2).

Further detailed investigations of the effects of initial LF concentrations and resin:milk ratio values that would be encountered in the proposed on-farm system were done using milk from individual cows (Chapter 5). The LF adsorption was influenced by resin:milk ratio (Φ), time and initial concentration (Figure 4-18).

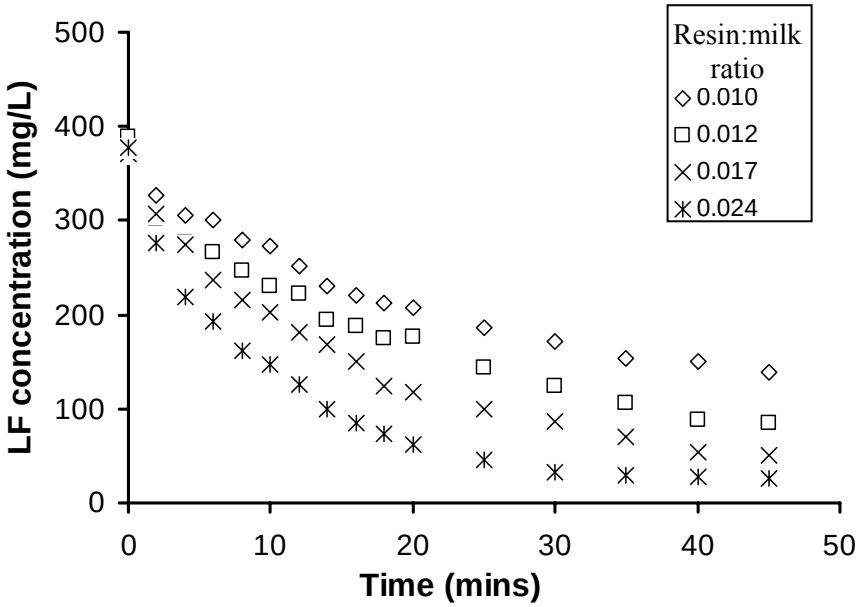


Figure 4-18: Effect of time and resin:milk ratio on LF adsorptions using SP BB (milk sample from cow 3423).

4.10 Summary

This chapter investigated the most practical and easily scale-up method to extract LF and LP from raw whole milk. Conventional column chromatography with SP BB and BR 70 were used for processing raw whole milk at 35-37^{o}C. Equilibrium and dynamic capacities for the resins indicated that both resins could be used to effectively capture LF and LP. It was found that ideally the processing temperature at close to milk secretion temperature was most suitable for the adsorption process. Results from single-stage stirred tank system showed that LF and LP could be captured in stirred tank but optimisations were necessary.

The resin lifetime studies showed that SP Sepharose could be effectively reused for 50 cycles. This infers that it could be possible to use the resin for much longer than 50 cycles without compromising performance.

5 Protein Adsorption Kinetic Modelling and Analysis

5.1 Introduction

Modelling simulates a process for prototyping and is useful if scale-up data are not available. Once a model for a protein purification process is experimentally verified, it can be used to predict performance and yields. An objective in this study was to model adsorption data so protein purification could be understood, and to use the model to predict process performance if feed concentration; amount of resin, and adsorption time required for processing was known.

Rowe *et al*. (1999) developed a novel composite non-linear (CNL) kinetic model with four independent parameters to describe batch adsorption that could simulate protein concentration-time profiles over a wide range of initial (feed) protein concentrations and phase ratios (resin:feed volume ratio, Φ). This approach, unlike Langmuir and solid film linear kinetic models, does not require an adsorption isotherm (Skidmore *et al*. 1990). The advantage of the CNL model is that kinetic capacity (q_k) is linearly related to the physically measurable quantity of feed concentration (initial and changes in feed concentration over time). After a certain finite time, the relationship ln(1-q/q_k)ln(C/Co) for protein adsorption on suspended anion exchange resin particles is linear with time i.e.

$$\ln\left[1-\frac{q}{q_k}\right]\ \ln\left[\frac{C}{C_0}\right] = \quad kt + y(0) \tag{5. 1}$$

where q is protein absorbed on the solid phase (mg/mL resin); q_k is a kinetic parameter; C is protein concentration in solution (mg/mL); Co is initial concentration (mg/mL); k is a rate constant (min^{-1}), and $y(0)$ is the zero-time intercept.

More generally:

$$\ln\left[1-\frac{q}{q_k}\right]\ \ln\left[\frac{C}{C_0}\right] = \quad kt + y(0)\left[1-e^{-at}\right] \tag{5.2}$$

where *a* is a parameter for deviation from the straight line at times less than 5 minutes (Figure 5-1).

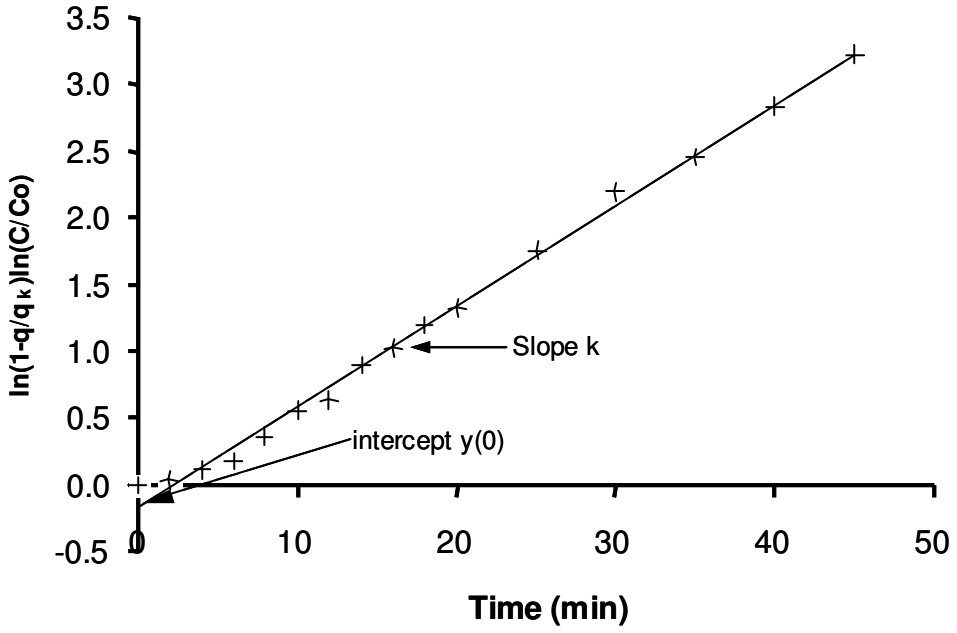


Figure 5-1: The CNL model for batch ion exchange adsorption of proteins. Data points deviate from a straight line below 5 minutes.

Adsorption data for SP BB and SP FF resins were obtained in laboratory extractions. However, because on-farm trials were only done with SP BB, only these data were used to obtain values for q_k, *k, a* and *y(0)* in the CNL model. The model would allow adsorption uptake to be predicted from feed and operating conditions, ideally *Co* and Φ. Data obtained for extracting LF from raw whole milk over 45 minutes was transformed using steps in section 5.2. The limiting factors of the parameters (parameterisation) and their application are discussed.

5.2 Methodology for determining CNL model parameters

5.2.1 Determining q_k, *k* and *y(0)*

Feed concentrations were converted to mg/mL and *q* was calculated as:

$$q = \frac{(Co * \ Feed \ volume)\left(1 - \frac{C}{Co}\right)}{Resin \ volume} \qquad (5.3)$$

An initial value was given to q_k (a useful value is one slightly higher than the experimentally observed last, or final, value of q). Then the left hand side of Equation 5.1 was plotted with time and the linear portion of the plot used to determine k (gradient) and $y(0)$ using Excel Solver. The initial guess for q_k was then refined to maximize the regression coefficient for the fit to Equation 5.1.

5.2.2 Determining a

For lower resin:feed ratios ($0.05<\Phi<0.15$), a was assigned an initial value between 0.25-0.5. Higher values were assigned for higher Φ values. Once q_k was determined, the estimate of a was adjusted to minimize the sum of difference of squares of errors between a complete set of adsorption data and results using Equation 5.2.

5.2.3 Fitting the CNL model and experimental data

The original methodology of Rowe *et al*. (1999) included differentiating Equation 5.2 with time to give:

$$\frac{dC}{dt} = -\Phi \frac{dq}{dt} = \frac{\left[k + a \cdot y(0) \cdot e^{-a \cdot t}\right] \cdot C \cdot (q_k - q)}{(q_k - q) \cdot \ln(1 - q/q_k) + (1/\Phi) \cdot C \cdot \ln(C/Co)} \qquad (5.4)$$

At t=0, q=0 (no absorption), so Equation 5.4 is undefined. The initial adsorption rate was therefore determined by twice differentiating Equation 5.2 and assuming that adsorption begins after immediately initial contact, taken as 0.01 min.

$$\frac{dC}{dt} = -\Phi \frac{dq}{dt} = -a\sqrt{\frac{-\Phi\ q_k\ y(0) Co}{2}} \qquad (5.5)$$

Equations 5.4 and 5.5 were solved simultaneously using the Ordinary Differential Equation solver (ODE) function ("rkadapt") in MathCad™ (Mathsoft, Cambridge, MA, USA). The values for a and k, obtained using Excel Solver, were adjusted slightly (within 10%) to obtain the best fit in MathCad. Values for parameters $y(0)$, a and k were optimised by trial and error so the model fitted the experimental data.

5.3 Assessing parameters of the CNL model

5.3.1 Kinetic capacity, q_k

For each initial feed concentration used, q_k was asymptotically related to Φ (Figure 5-2). When Φ was >0.017, q_k approached a constant value for each initial protein concentration. Although the actual meaning of q_k in the Rowe *et al*. (1990) model is unclear, it may represent the approach to equilibrium adsorption capacity, which is demonstrated by the data obtained.

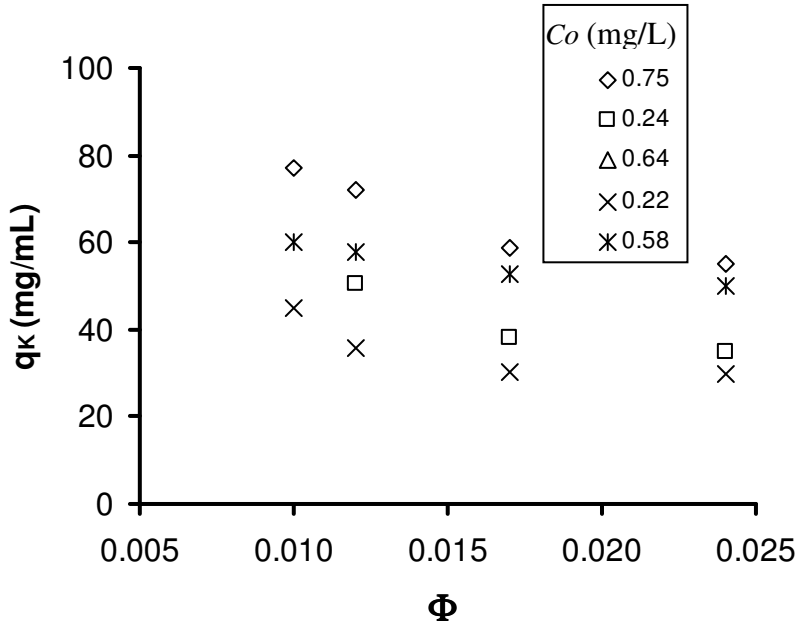


Figure 5-2: q_k vs Φ for milk from individual cows.

The data can also be presented as the effect of initial protein concentration (*Co*) on q_k for various resin:milk ratios, (Φ).

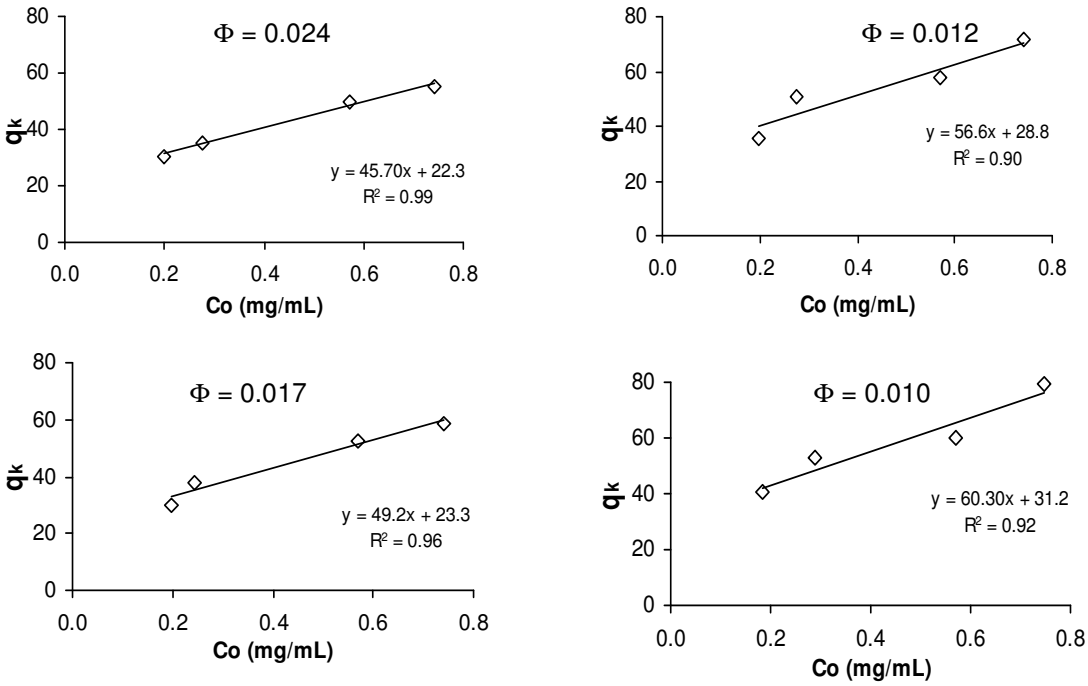


Figure 5-3 Effect of Φ and Co on q_k value.

A sensitivity analysis showed that q_k values significantly affected the best fit for the CNL model. Once values of *qk* had been determined, changing *a* and *k* had relatively little effect on the fit of the model.

After the model has been obtained, information on milk volume (measured as a cow is milked), and its LF content (from information on the particular cow), together with the amount of resin being used to capture the protein, means that q_k can be predicted and then the expected yield can be predicted. This information could be used in a dynamic on-farm scenario to selectively accept or reject protein capture from specific milk based on predicted yield.

Values for the intercept and gradient of the relationship between q_k and *Co* for different Φ values (0.010 - 0.024) show that q_k can be related with *Co* by:

$$q_k = xCo + c; \tag{5.6}$$

where $x = 66.1 - 882\Phi$ and $c = 33.9 - 516\Phi$

or $$q_k = (66.1 - 882\Phi)\ Co + (33.9 - 516\Phi) \tag{5.7}$$

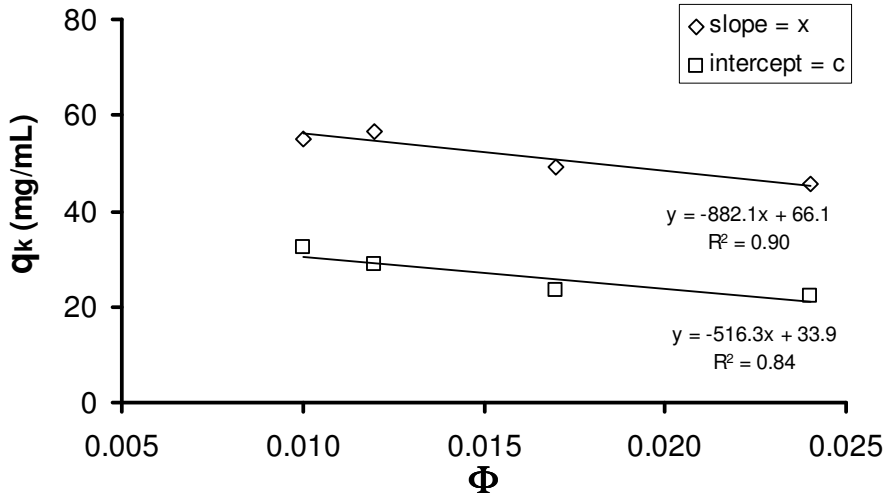


Figure 5-4: Effect of Φ on the intercept and gradient for the linear relationship between q_k vs *Co* (slope and intercepts obtained from Figure 5.3).

5.3.2 Rate constant, *k*

For all Φ values investigated there was a positive linear relationship between the rate constant, *k*, and *Co* (Figure 5-5).

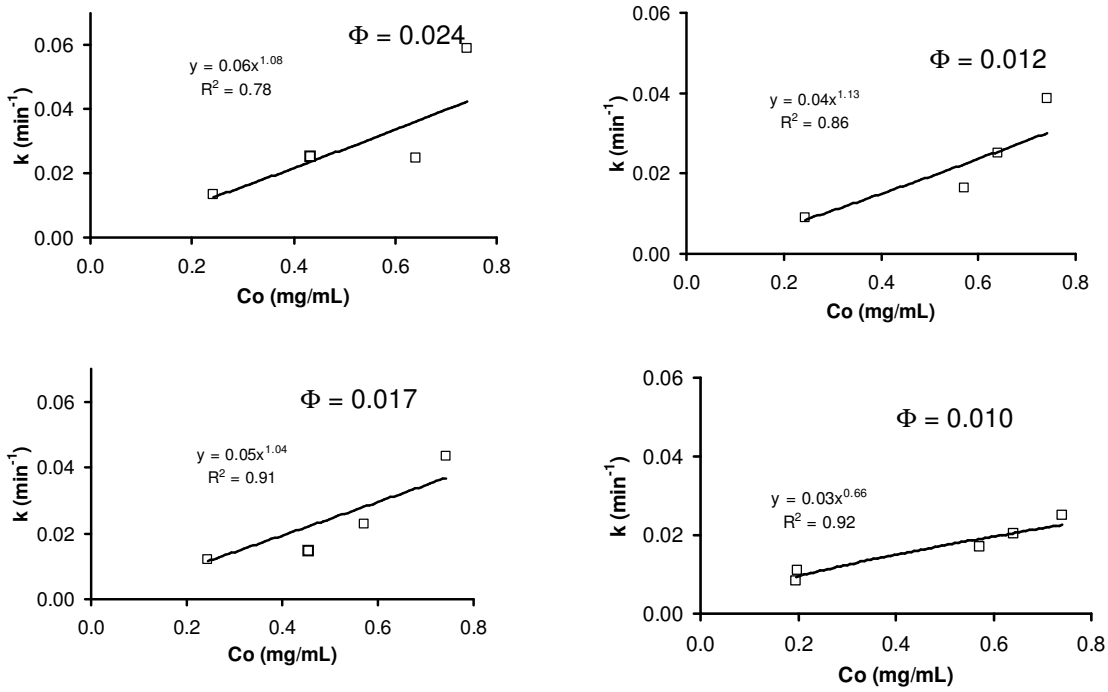


Figure 5-5: Effect of Φ and *Co* on *k* value.

A summary of the gradient for relationship between k and Co at various Φ values (Table 5-1) show:

$$k = 0.05\ Co^{1.05}$$

If $Co^{1.05}$ can be approximated to Co, then:

$$k = 0.05\ Co \qquad (5.6)$$

Table 5-1 Power log fit of k and Co for given resin:milk ratios (Φ).

Φ	Gradient (k vs Co)
0.010	0.03
0.012	0.04
0.017	0.05
0.024	0.06

A sensitivity analysis showed that $0.03<k<0.06$ did not significantly affect the CNL model. A plot of k vs Φ (Figure 5-6) shows that k is constant for low Co and then increases with Φ for higher Co values.

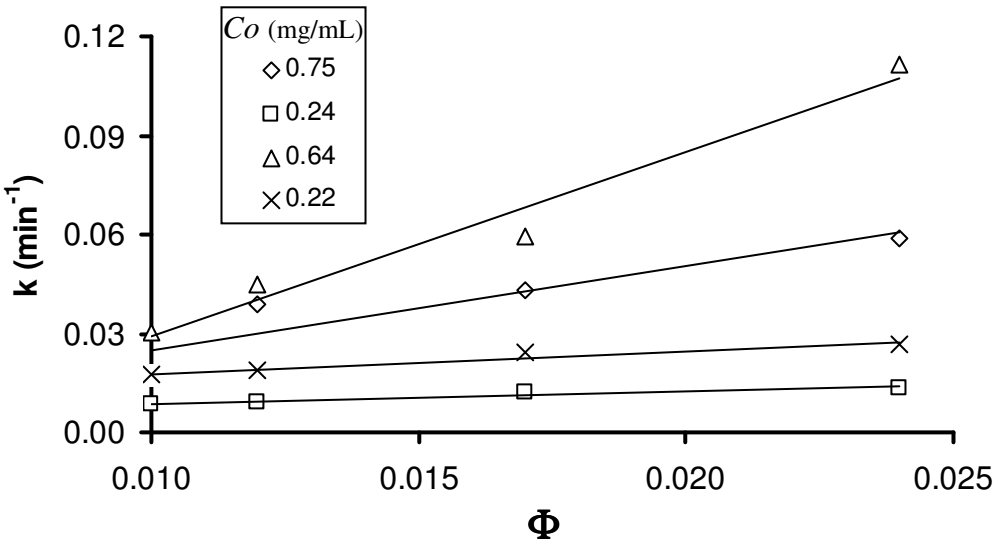


Figure 5-6: Effect of Co on the relationship between Φ and k.

The characteristics for the curves are summarised in Table 5-2.

Table 5-2: Summary of relationships between k and Φ for given Co values.

Co	Relationship to k	Regression (R^2)
0.220	0.6871Φ + 0.011	0.9514
0.240	0.3629Φ + 0.005	0.9213
0.640	5.5837Φ - 0.027	0.9679
0.750	2.5248Φ - 0.0001	0.8465

There is a linear relationship between Φ and the gradient of the relationship between k and Φ (at given Co) (R^2=0.95), which can be expressed as:

$$k = (2.01\,\Phi + 0.013)\,Co \qquad (5.7)$$

5.3.3 *a* for adsorption

Values for the time constant, *a,* were generally small (0.12 to 0.30; average of 0.20) at the Φ values used. For given Φ, *Co* did not affect *a* (Figure 5-7), therefore Co could be expressed in terms of Φ. There was no significant relationship between *a* and Φ (Figure 5-8) and most of the variation is likely to be due to experimental (measurement) error (or random scatter).

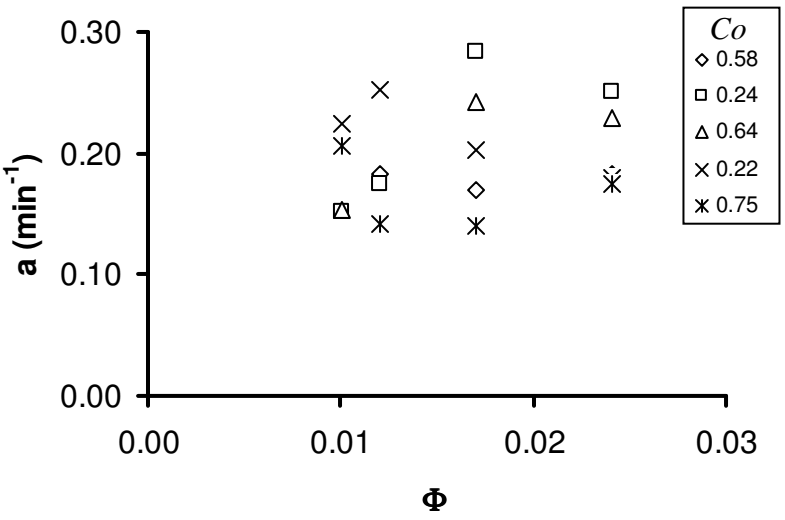


Figure 5-7: Effect of Φ on *a.*

The sensitivity analysis showed that the CNL model is not affected if 0.18<a<0.22 so *a* could be approximated as 0.2. This was lower than the 0.3 - 0.8 obtained by Rowe *et al*. (1999) for anion exchange of BSA at concentrations up to 3 mg/mL. They used

a second order equation to relate the effect of Φ on *a* whereas data obtained in the current experiments on cation exchange of LF can be approximated as a single value.

Raw whole milk is a natural product with a LF concentration between 0.07 and 1.0 mg/mL. Therefore it was not possible to investigate LF concentrations above 1.0 mg/mL because 1.0 mg/mL is the maximum concentration expected in milk from individual cows during majority of the lactating period.

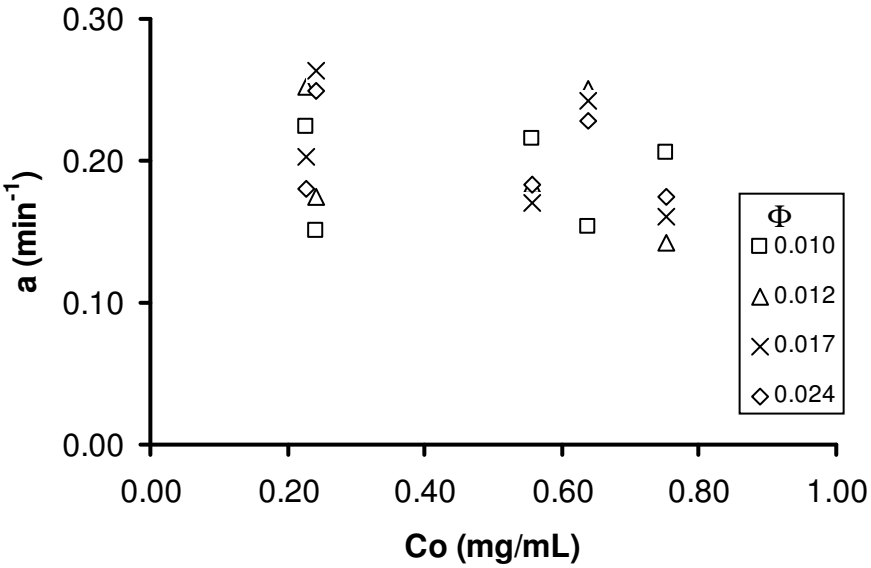


Figure 5-8: Effect of *Co* and Φ on *a*.

5.4 Effect of *a*, *Co* and Φ on *y(0)*

Values for *y(0)*, obtained by extrapolation of plots from left side of Equation 5.2 were then used to generate a CNL model fit experimental data using MathCad. The parameter *y(0)* is an artefact due to deviation from straight line adsorption and could be related to *a* and *k*. The *y(0)* values were generally small (-0.07≤y(0)≤-0.01). If *y(0)* was set at smaller negative or positive values, a solution could not be obtained using the ODE (MathCad).

As *a* is relatively constant (~0.2) and *k* depends on *Co* and Φ, *y(0)* may also be affected by values of *Co* and Φ. However, no obvious relationships between *y(0)* and Φ or *Co* (Figure 5-9) were observed, probably because *y(0)* is very small compared with *a* and *k*.

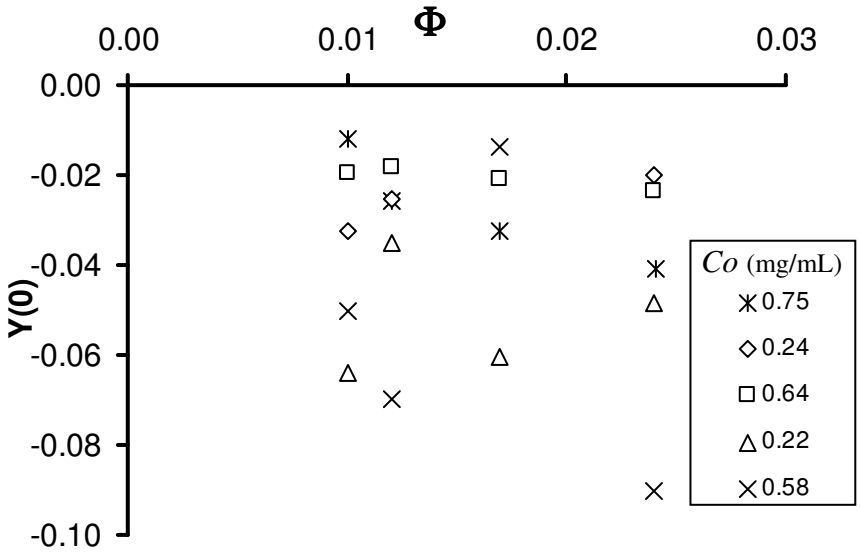


Figure 5-9: Investigation on whether Φ affects *y(0)* for LF adsorption from individual cow's milk.

The linear relationships between *y(0)* and *a* for milk from individual cows over the range of *Co* tested (Figure 5-10) can be expressed as regression equations (Table 5-3). If the value a = 0.2 is substituted into the overall composite regression equation (Equation 5.9), then *y(0)* = -0.023.

Table 5-3: *y(0)* vs *a* relationship for milk from individual cows with regression data.

Cow Id	*y(0)* vs *a* relationship	R^2
3423	0.2333*a* - 0.077	0.9708
1607	0.085*a* - 0.0405	0.7581
324	0.2421*a* - 0.0642	0.8975

$$y(0) = 0.1868a - 0.0606 \qquad (5.8)$$

Sensitivity analysis showed that as for the constant *a*, value of *y(0)* did not affect the CNL fit significantly.

Rowe *et al.* (1999) found that there was a relationship between *y(0)* and *k/a.* However, the scatter plots showed no obvious relationship between *y(0)* and *a* (Figure 5-10) or *y(0)* and *k/a* (Figure 5-11). This may be because a much smaller range (0.1 to 1.0 mg/mL) of *Co* values were used in the LF adsorption studies compared with 0.0 to 3.0 mg/mL BSA used by Rowe and co-workers.

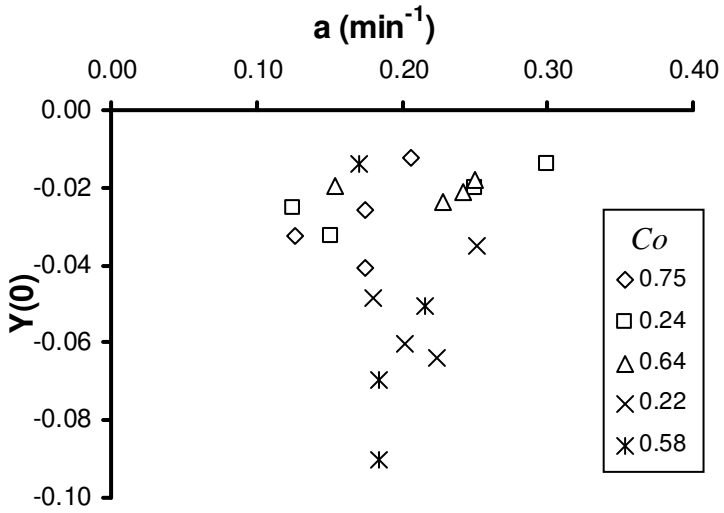


Figure 5-10: Effect of *Co* and *a* values on *y(0)*.

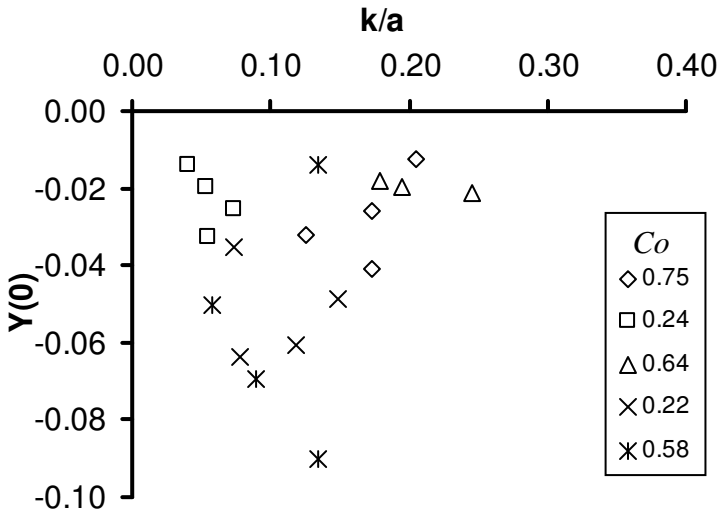


Figure 5-11: Effect of *Co* and *k/a* values on *y(0)*.

5.5 Practical use of the CNL model

The CNL model could be fitted to batch adsorption of LF from raw whole milk. The amount of LF extracted, over the range of Φ values tested for extraction times of 30 minutes or longer, was almost independent of Φ. The data indicate that overall adsorption rates were more dependant on q_k than the rate constants k and a. The value of q_k was affected by *Co* but this effect decreased as *Co* increased.

Correlations obtained in sections 5.2-5.4 show that the CNL model for cation exchange adsorption of LF from whole milk can be represented by:

$$\ln\left[1-\frac{q}{q_k}\right]\ \ln\left[\frac{C}{C_0}\right] = \quad kt\ -0.0234\left[1-e^{-0.2t}\right] \tag{5.9}$$

where $q_k = (66.1-882\Phi)\ Co\ +\ (33.9-516\Phi)$

and $k\ =\ (2.01\ \Phi\ +\ 0.013)\ Co$ for $0.18 \le a \le 0.22$ and $y(0) = -0.023$

This CNL model can be used to predict the amount of LF that would be extracted for a known volume of resin and volume of milk with known initial LF concentration. Milk from six individual cows was used for laboratory-scale LF adsorptions in a single-stage stirred tank over 45 minutes for different Φ. The *Co* and Φ values were used to obtain the CNL model parameters.

There was good agreement between predicted values for LF adsorption with time for Φ=0.017 (being Φ for average volume of milk produced by an individual cow and 250 mL of resin) by the CNL model (Equation 5.10) and experimental data (Figure 5-12).

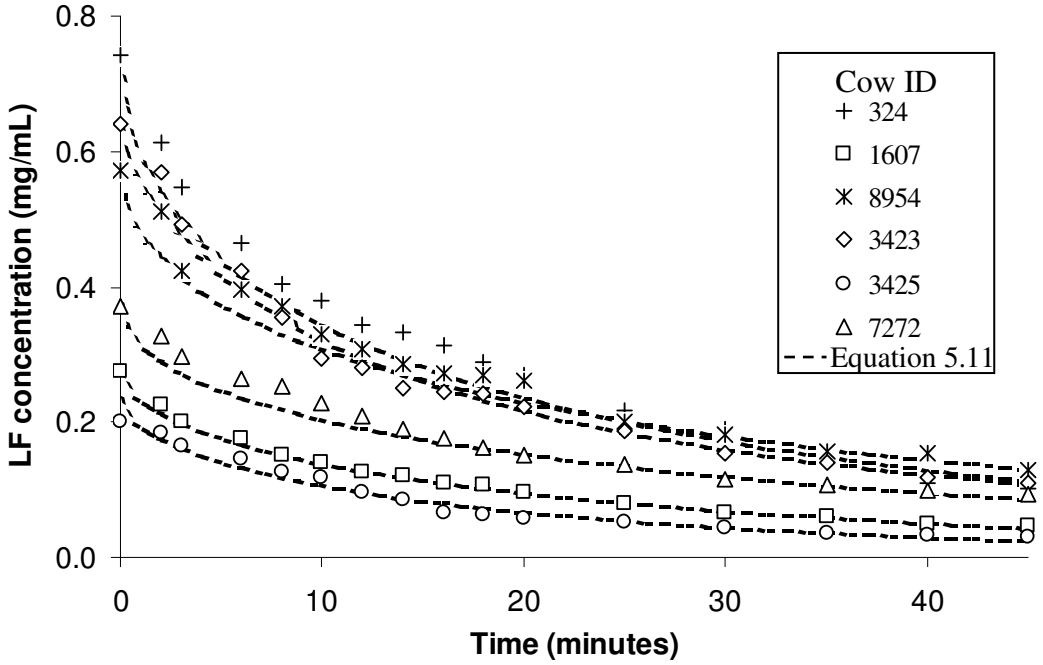


Figure 5-12: Comparison of experimental and simulated data (Equation 5.10) for Φ=0.017.

5.6 Data from on-farm experiments

The *Co* values in milk from individual cows used in on-farm fractionations were between 0.08 and 0.48 mg/mL and the Φ values were between 0.010 and 0.030 (section 7.3.3). These *Co* and Φ values were used to predict LF extraction with time using Equation 5.10 (Figure 5-13). Some trends observed in the on-farm studies were noted on the simulated graphs. For example, higher Φ values (e.g. when Cow 3532, 3022 and 9570 produced less milk, resulting in Φ = 0.030, 0.028 and 0.024 respectively) gave faster absorption rates and therefore higher yields for 10-minute extraction (Figure 5-14). Because of the limited range (0.08 to 0.048 mg/mL) of initial LF in the milk used, effects of concentration were not observed.

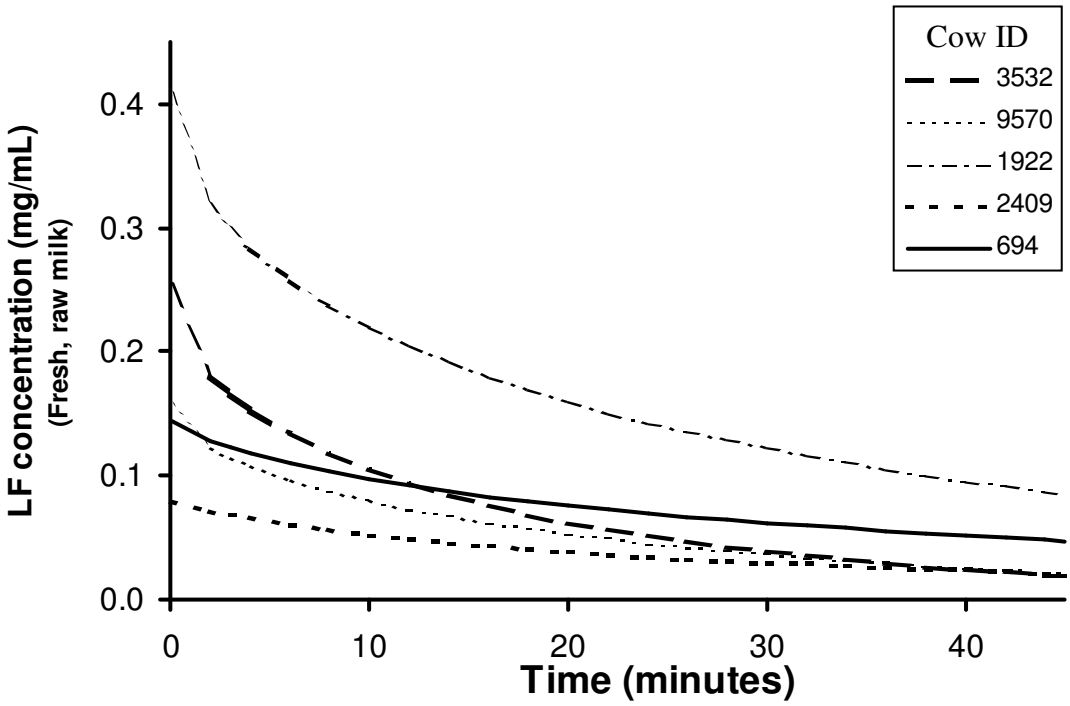


Figure 5-13: LF adsorption simulations using *Co* and Φ from on-farm experiments and Equation 5.10.

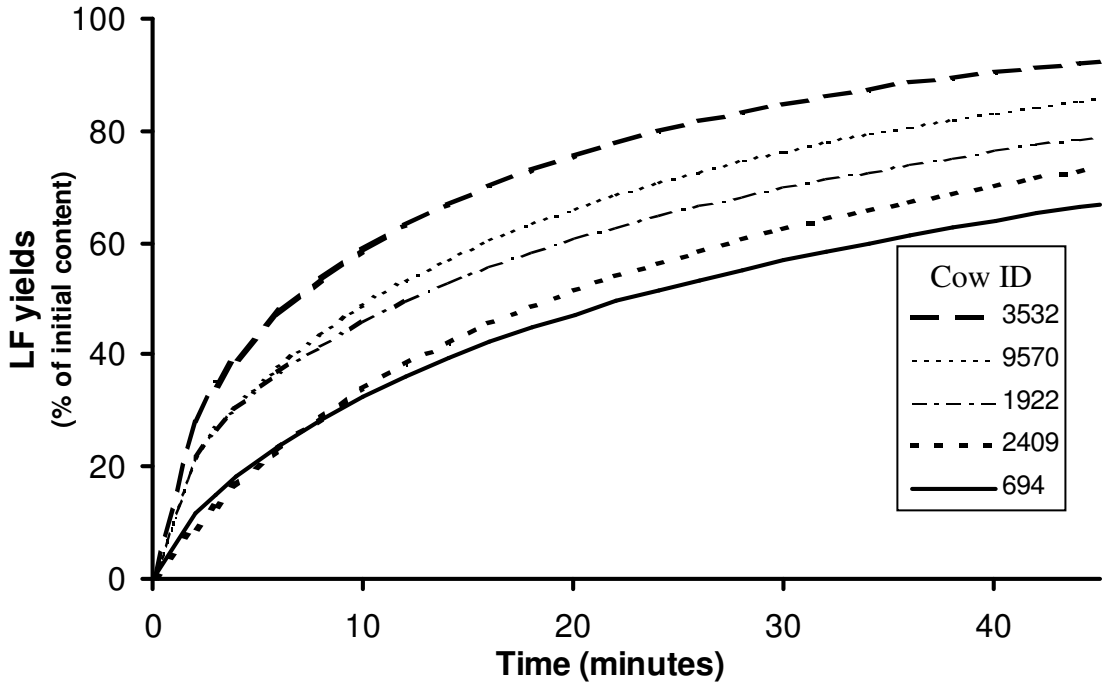


Figure 5-14: Predicted LF yields using Co and Φ from on-farm experiments and Equation 5.10.

The CNL model does not include elution efficiency (i.e. recovering protein from the resin). Simulations for on-farm extraction represent complete recovery of protein that has been absorbed onto the resin concentrated into small volumes.

Data from on-farm experiments differed from the simulations by an average of 14% (Table 5-4), indicating that the model developed can be used to predict protein yields at a specific time in the process. Experimental yields are full recovery of protein but simulated yields are based on adsorption only. Therefore, the latter overestimates yield, which can be treated as the losses that occur during elution. This infers that the fit between experimental and predicted yields is better than 14%. A factor that may affect values predicted by the model that was not studied in the current trials is the effect of fat on protein absorption.

Table 5-4: Experimental (on-farm) and predicted (using Equation 5.10) yields of LF after 10-minute adsorption.

Cow Identification	LF (mg/mL)	% Yield		Variation†
		Predicted	Experimental	
3532	0.254	58.7	55.2	0.94
3022	0.373	60.1	46.0	0.77
9570	0.157	48.9	48.9	1.00
3109	0.374	46.8	25.6	0.55*
1922	0.239	41.2	27.6	0.67
1922	0.408	46.1	47.8	1.04
3402	0.392	44.6	23.4	0.52*
1607	0.330	40.6	36.1	0.89
2409	0.078	33.9	36.7	1.08
9564	0.240	36.8	43.5	1.18
694	0.145	32.5	35.3	1.09
5710	0.333	39.6	29.5	0.74
480	0.478	40.4	34.9	0.86
1401	0.260	34.0	28.5	0.84
7656	0.101	26.7	19.7	0.74
Overall difference*				0.91±0.16

† Expressed as experimental over predicted. * Excluding Cow 3109 and 3402 data

5.7 Summary

The most valuable aspect of modelling is being able to predict the amount or yield of target protein (in this case LF) that can be extracted from a given a volume of milk, with a known initial feed concentration using a known amount of resin and extraction time. The kinetic model described by Rowe *et al*. (1999) assisted in understanding the effect of contact time between feed and resin when developing relationships between feed concentrations, amount of resin, contact time and expected yields. The model can be used to show typical extractions profiles for initial feed LF concentrations of 0.1-1.0 mg/mL in the average milk volume (15 L/cow), with a resin: milk ratio of 0.017 over 45 minute (Figure 5-15).

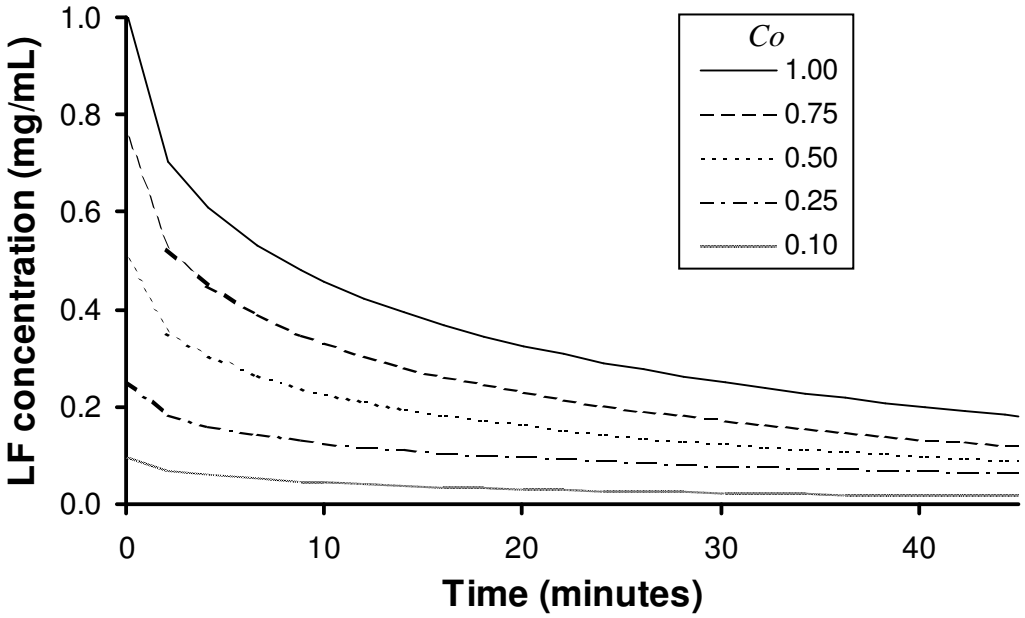


Figure 5-15: Simulated LF extraction for *Co* of 0.10 to 1.00 mg/mL over 45 minutes with $\Phi = 0.017$.

An investigation of the effects of initial feed concentration and volume (10-20 L) on LF yield using 250 mL of SP BB resin showed that yield increases as initial LF concentration increases and that the highest yield was obtained when processing 10 L because this was at the highest Φ (Figure 5-16). Therefore, in an optimised system, higher process yields (and hence productivity) will be obtained when processing milk with a higher content of the target protein (high *Co*). A targeted system for LF would be ideal when milk has highest LF levels just after calving (i.e. in the colostrum) and total milk volume is low. This also occurs at the end of the season, where milk volume per cow is decreasing and LF concentration is increasing.

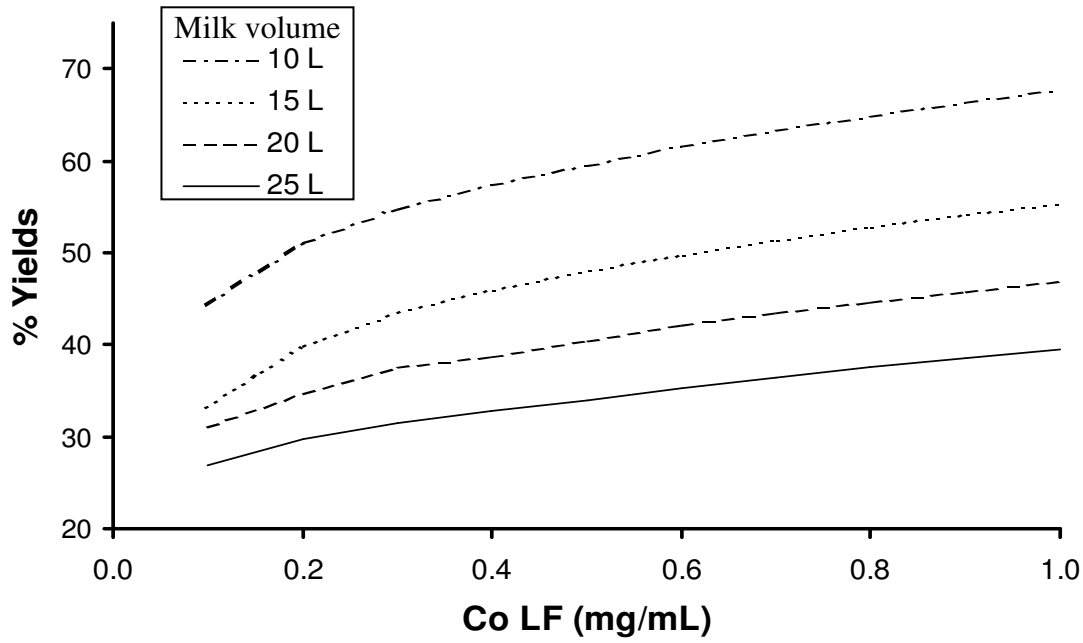


Figure 5-16: Effect of Co (initial LF content) and milk volume on yields when using 250 mL of SP BB resin and 10-minute contact time in a single-stage stirred tank.

6 On-Farm Fractionation Materials, Methods and Prototype Design

6.1 Introduction

Two on-farm trials for capturing target milk proteins were done at the Greenfield dairy unit (Dexcel Limited). The first experiment (section 6.2), done during the early stages of the project, evaluated the concept of on-farm fractionation. The data showed that manual on-farm fractionation was possible and a patent was subsequently granted (Claycomb, 2004). The second trial (section 6-13) used the fully automated prototype Protein Fractionation Robot (PFR), which was connected to the AMS. The AMS has full automated control, a data management system, and traffic control (Fusion Electronics, Diksmuide B-8600, Belgium). Design considerations and developing the PFR are described in sections 6.4-6.10.

6.2 Proof of concept for on-farm fractionation

The aims of this experiment were to:

- demonstrate that proteins could be captured on-farm using freshly harvested raw whole milk
- test feasibility of capture using pleated filter cartridges filled with resin and
- obtain preliminary data for intellectual property protection

6.2.1 Pleated cartridge filter capture

Two Aqua-Pure^{TM} (AP11T, CUNO, Blacktown, N.S.W., Australia) water filter housings with AP1020 20-μm polyester pleated cartridges (Figure 6-1) were filled separately with approximately 600 mL of swelled cation exchanger resin (SP BB or BR 70). A peristaltic pump (Watson-Marlow Bredel Inc, Wilmington, MA, U.S.A.) was used to pack the slurries into the cartridges. Phosphate buffer flow rates of up to 3 L/min through the packed cartridges were achieved with the peristaltic pump without significant back pressure.

Figure 6-1: AP11T Aqua-Pure™ water filter housing with AP1020-20 µm polyester pleated cartridge.

Milk from an individual cow was diverted from the AMS using a centrifugal pump at 2 L/s into a secondary receiving can (Figure 6-2). The milk was then pumped at 1 L/min (peristaltic pump) through the cartridge filter containing BR 70 resin.

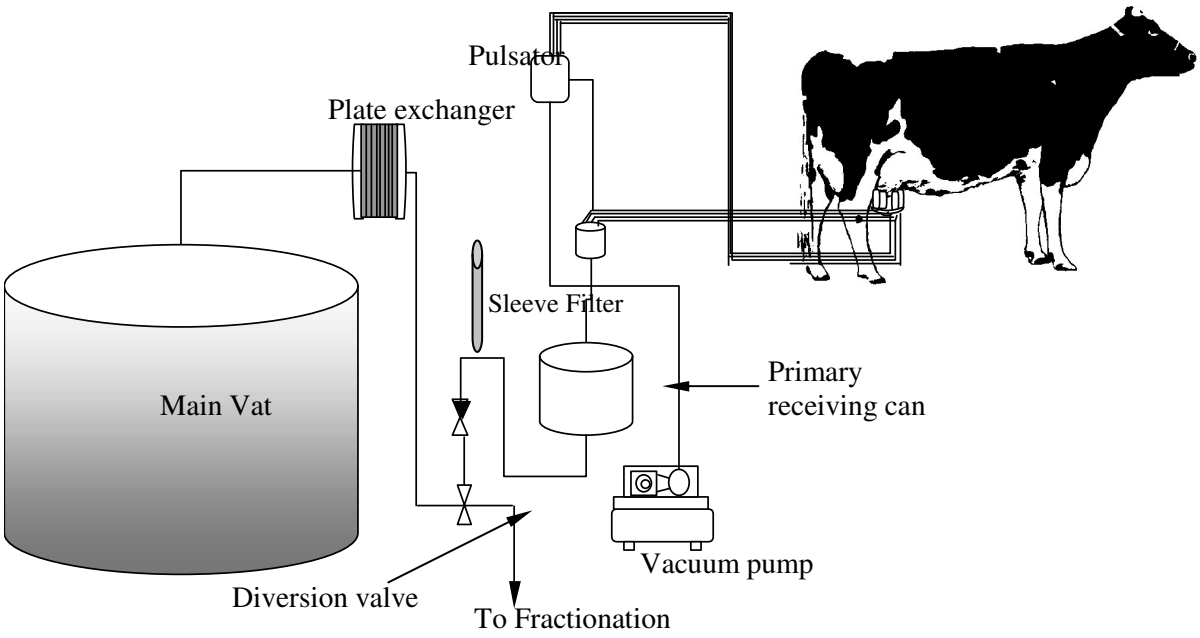


Figure 6-2: Process flow sheet for milking using the AMS showing the standard milk route and diversion for on-farm fractionations.

6.2.2 Batch stirred-tank capture

The LF and LP in fresh raw whole milk were captured with SP BB resin immediately after milking (harvesting). The resin (600 mL of SP BB, pre-equilibrated to pH 6.7 with 10 mM phosphate buffer) was placed in the secondary processing can.

Milk (8.1 L, pH 6.88) from an individual cow was diverted from the AMS into the can and the milk/resin slurry was manually stirred for 10 minutes. The milk/resin slurry was then passed at 1 L/min through a fresh cartridge filter using a peristaltic pump. The filter cartridge was then rinsed with approximately 3 L of phosphate buffer and transferred to the laboratory to elute the proteins.

Protein was eluted by batch washes. The resin was first washed with 2 L of 10 mM phosphate buffer before eluting the LF and LP in two steps. The LP was the eluted with 1.0 L of 0.25 M NaCl in phosphate buffer and then the LF was eluted with 1 L of 0.8 M NaCl in phosphate buffer. Resin was then cleaned by consecutive washes of 0.5 L of 1.0 M NaCl, 0.5 L of 1.0 M NaOH and 2 x 1-L washes of Milli-Q water. Cleaned resin was stored in 20% ethanol.

Total protein in feed and eluates were determined by the BCA protein assay. The pH, fat, protein, lactose, total solids, LP activity, and LF and LP content of samples from raw whole milk (feed) and processed milk (outflow) were determined. Five-mL samples of eluates were desalted on a 5-mL HiTrap desalting column on the AKTA fplc and then the LF and LP content were analysed using a Resource S column.

6.3 Contactor design considerations

The experiments (section 6.2) for processing volumes of milk expected in a robotic milking system, indicated that it was possible to process fresh, raw whole on the farm and that an engineering solution was needed for the efficient capture of LF and LP.

Observations from on-farm fractionation (section 6.2) and other laboratory experiments (Chapter 4) indicated that:

- the concept of using a cartridge filter, pre-coated with resin, is not viable. Non-uniform coating and redistribution of resin during milk loading resulted in milk by-passing the resin almost entirely

- the size of fat globules in the raw whole milk, combined with the milk's viscosity, contributed to resin fouling and decreased processing efficiency
- raw whole milk at 37^{o}C could pass through a packed bed containing SP BB or BR 70 resin
- SP BB resin had better flow characteristics, less back pressure, less bed compaction and better absorption capacity (perhaps due to the larger particle size) than BR 70 resin
- SP BB resin has the added advantage that it is approved as Generally Regarded As Safe (GRAS) by the FDA (GE Healthcare Technologies 2004, 2005).

6.4 PFR design considerations for a prototype

6.4.1 Prototype specifications

A design brief was created and a team assembled to design, fabricate and assemble a PFR. The overall approach was to design a system that could transfer a laboratory-based fractionation operation to an on-site processing, that is to couple a chromatographic extraction unit onto an existing AMS so that proteins could be extracted, directly from raw, untreated milk, immediately after each milking. The prototype had to be able to process milk from individual cows as they were being milked, without disrupting the normal milking process. A protein purity target was not set for the prototype. However, yields greater than 80% were desirable. The prototype had to be robust enough to withstand the dairy parlour environment, be reliable and work without needing a technician on-site. In other words, the principle of minimal human intervention, as for the AMS, was to be maintained and applied to this system.

6.4.2 Design constraints

The PFR system ideally, had to be able to consecutively process from each cow that came to be milked by the AMS. The AMS takes an average of 8 minutes (range of a few to 20 minutes) to milk one cow. This time is similar to the reported average milking time (6-7 minutes) for a cow producing 20 L of milk (Mein 1998). This meant the prototype had to be able to complete one process cycle within 6-7 minutes,

including preparing the system to accept milk from the next cow. Thus, the time for actual adsorption had to be no more than 5 minutes.

All materials, buffers, chemicals, fittings, etc had to be approved for dairy or food usage. Losses in milk volume processed due to void volumes and transfers had to be minimal. Temperature affects adsorption rate and pressure drop through the resin during recovery when straining through a fine mesh, so milk temperature had to be maintained as close to the secretion temperature as possible. The PFR has to have a facility for CIP and sanitisation so it could be cleaned and hygiene maintained.

Fresh, raw whole milk harvested using the AMS had to meet the following specifications:

- its quality had to be maintained throughout the process. For example, increases in somatic cell counts or spoilage of milk should not occur during processing
- able to be further processed (i.e. transported to a factory)
- not kept at ambient temperature for prolonged periods (e.g. > 30 minutes)
- contain no residues (i.e. no foreign matter could be introduced during processing).

The PFR was to be:

- manufactured from food-compliant dairy-approved materials and components
- simple yet versatile so variations in processing time, volumes, etc could be accommodated
- automated with minimal operator assistance
- user-friendly, with an interface for the farmer or operator

Because the AMS is operating in a commercial dairy unit, coupling the PFR to the AMS had to be done without disrupting daily milking operations or causing down-time.

6.4.3 Feed stream and temperature effects

Milk secretion temperature is nominally 37^{o}C. However, the final temperature of batches of harvested milk depends on ambient temperature, the time it takes to milk

(some cows milk faster than others) and the volume of milk produced. Small volumes of milk cool quickly because the surface area to milk volume is higher.

Milk is a regulated food material in New Zealand. General manufacturing practice means that milk is usually cooled as quickly as possible to minimize microbial and chemical deterioration. Milk must be pasteurized before any further processing is done. However, milk is legally defined as such only after it enters the factory; current pasteurisation regulations do not apply on the farm.

Earlier studies indicated (section 4.6.2-4.6.3) that protein uptake by the resin increases with temperature. However, cation-exchanger containing the captured LF and LP must be stored at low temperature (e.g $4^{o}C$), to minimize microbial growth and to maintain product bioactivity. It would also be desirable to store fresh resin at $4^{o}C$ until processing.

6.4.4 Selecting the chromatographic media

In food processing, chromatography resin (media) is generally treated as a raw material. Resins used in the food processing industry must be approved by the appropriate regulatory authority such as FDA (for food being exported to U.S.A.) and/or Food Standards Australia and New Zealand (FSANZ) for product being used in New Zealand and Australia. Two resins (BR 70 and SP BB) were used in the initial packed bed trials in the laboratory. The BR 70 resin had been used in previous experiments and was included for comparison. SP BB and SP FF resins have GRAS status. This dextran-based matrix is already used in the New Zealand dairy industry for similar applications such as processing LF and LP from skim milk and whey.

Fractionating LF and LP from raw whole milk is not considered downstream processing. They are only minor components in milk and it is envisaged that the milk would essentially be unchanged and suitable for normal consumption or further processing after the extractions had been done. Thus, any extraction process will leave the milk as near to normal state as possible. Hence, only food grade approved resin can be used.

Physical stability of SP BB

Trials showed that high centrifugal forces and contact with fast moving impellers damaged the resin. Peristaltic pumps are gentler but may not provide uniform flow rates due to tube fatigue. High linear velocities (up to 6000 cm/hr) in a packed column did not damage the resin (refer to section 4.4.3).

6.4.5 Traceability

The AMS has Crystal management software and can identify each animal from radio frequency identification tags, control milking events, collect milking data for each animal, interface with online sensors, and control ancillary equipment. If the adsorption device was linked to the AMS, it would be possible to control the process and optimise extraction from each animal. Data on product yield and activity for individual animals and information from the AMS would allow the farmer to identify high-producing animals and to relate various farm management practices (milking frequency and time, feeding regimes, etc) to yield of specific proteins.

The food value chain may also benefit from advanced, rapid product traceability. For example, if there was a disease alert (such as *Bovine Spongiform Encepthalphy*), this traceability would allow affected batches to be easily and quickly isolated. Also, the regulatory process for producing recombinant proteins for human therapeutic use from milk of transgenic animals may be more readily satisfied if the protein could be traced back to individual animals. If an animal develops ill-health or infection, product from that animal could be traced and withdrawn from further processing or disposed of. Likewise, any problems with individual product identified during quality checks could be tracked to the individual animal rather than the whole herd.

6.5 Assessing the available adsorption processes

The design brief included fast, effective and rapid protein captures. The contact mode for on-line fractionation is one of the critical steps. After evaluating the resin and the delivery mode, it was necessary to scale-up, modify, and incorporate the system on the AMS. The amount of resin required was based on laboratory experiments with 1-L samples of milk. Because resin is one of the major raw material costs in this process, it is important that the adsorption process is optimised and the minimum amount of resin used.

6.5.1 Packed bed chromatography

Column chromatography was first evaluated, as it is the most widely-used technique for protein extraction. Cation-exchange resins are currently used for commercial production of LF and LP from whey and skim milk. Laboratory experiments demonstrated that proteins could be separated chromatographically, directly from untreated raw, whole milk. The SP BB resin was selected because it has a relatively large particle diameter, which gave lower column back pressures than smaller diameter resin. No attempt was made in the current study to compare the performance of SP BB with resins other than BR 70.

Approximate dimensions of a scaled-up, packed bed chromatographic extraction unit were investigated. Two options for packed bed chromatography were considered:

- One or two large columns to process a defined number of cows or the milk collected over a long period (e.g. 24 hr)

If the linear velocity between laboratory and farm was maintained, a 5-cm high, 36-cm diameter column was needed to extract almost all of the LP and 95% of LF from the average milk volume (15 L) of 33 cows. However, the milking process is voluntary in the AMS; and cows arrive throughout the 24-hr day and sometimes there are periods when there is no milking. Microbial growth becomes an issue if a column is repeatedly loaded with raw milk and maintained at 35^{o}C for extended times. To manage contamination, the column would need sanitation between milkings. The adsorbed protein will probably deteriorate with prolonged storage at 35^{o}C and/or repeated cleaning cycles. These aspects could be dealt with by eluting the protein between milkings but would require a column sized for single milking and equipment to elute, store and retrieve the captured protein.

- Single, packed bed columns to process milk from individual cows

A 15-L batch of milk contains approximately 8 g of LF and LP. About 150 mL of resin with a dynamic capacity of 50 mg/mL would be required per milking. If total processing time is arbitrarily set to 3 minutes per milking to minimise the time milk is at 35^{o}C, a 36-cm diameter fixed bed is required to maintain the linear velocity at 300 cm/hr. The bed height for 150 mL of resin is only 1.5 mm, which is not practical. Reducing column diameter to obtain a practical bed height will increase flow rates

(which increases back pressures, reduces dynamic capacity and blocks column faster) or increase processing time (which increases microbial growth and means longer times than the time to milk an individual cow).

Column diameter affects three design parameters: bed height, time required to process at a linear velocity of 300 cm/hr and the flow rate required to process a given volume e.g. 15 L in three minutes (Figure 6-3). Bed height only reaches a practical (though still very small) value (2 cm) if column diameter is reduced to 10 cm but it will take 40 minutes to process 15 L of milk at 300 cm/hr. To complete processing in three minutes with this column diameter requires a flow rate of 5 L/min. Also, insufficient adsorption will occur at the high flow rates.

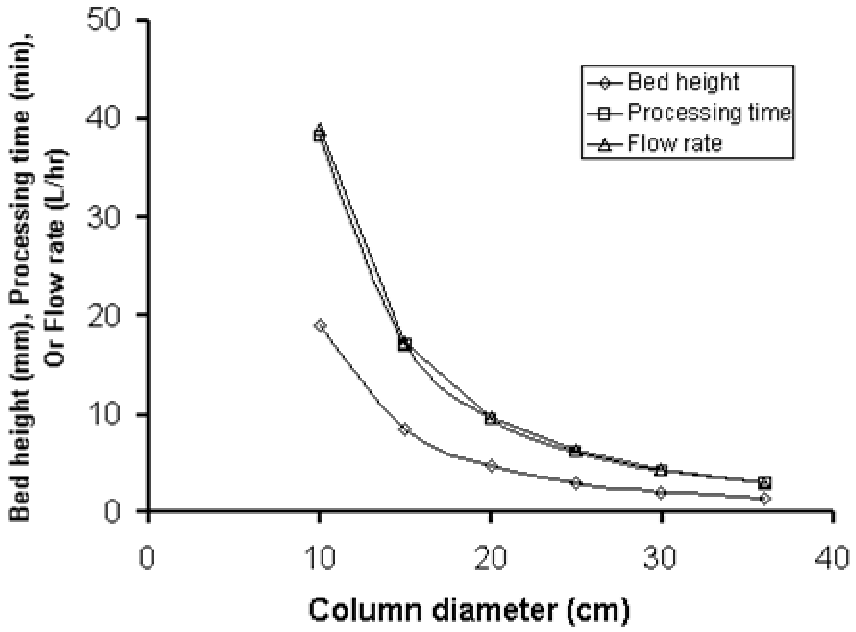


Figure 6-3: Effect of column diameter on design parameters while maintaining the linear velocity of 300 cm/hr.

A packed bed column was not a suitable process to meet the design brief for capturing proteins from milk of individual cows. A second column chromatography option is a multi-column system such as a simulated moving bed on a carousel, with a column for each cow. Ion exchange systems generally operate at 300-600 cm/hr to obtain appreciable protein adsorption. To process milk within the average milking time would require linear velocities of 2000-4000 cm/hr, making column design impractical. Also, most preparative-scale protein chromatographic resins cannot

withstand the back pressure generated if linear velocities are above 1000 cm/hr. (Prolonged exposure to high linear velocities were not tested for SP BB resin).

6.5.2 Ion exchange membranes

Because column-based chromatography had many disadvantages, other processes were investigated. The membrane ion exchange systems currently available have small pores (0.2-0.5 μm) and no prototype membranes with bigger pore sizes were available for trial. The manufacturers' dairy specialists advised that raw whole milk could not be processed with these membranes because the fat globules and other suspended matter would cause excessive fouling. In hindsight, the membranes available probably could process raw milk at 35-37^{o}C but the membrane suppliers have not considered this operating temperature when advising on process feasibility.

The cost for 10-mL and 100-mL ion exchange membranes was NZ$980 and $4300 respectively (2005 prices, Sartorius Sartobind). Some ion exchange membranes are available as disposable cartridges for pharmaceutical use where validation, hygiene and contamination are important process considerations. If the 100-mL membrane could be reused for 1000 cycles, the membrane cost would reduce to $4 per cycle, but this is considered too expensive for on-farm processing. The effect of membrane pore size and the cost for processing milk from individual cows needs to be investigated.

6.5.3 Batch chromatography in a single-stage stirred tank

Batch processes are less efficient than column processes but have advantages if feed viscosity is high and particles in the feed make column chromatography unsuitable. A batch process has many advantages in this study, especially because of the high fat content in the feed.

These considerations led into investigating protein adsorption from individual animals using a single-stage stirred tank. Laboratory trials on the effect of LF feed content on protein capture using SP BB resin in a batch process showed that adequate LF and LP yields could be obtained provided the resin to milk ratio was appropriate and sufficient time was allowed for adsorption.

Most of LP and up to 95% of LF was captured at acceptable linear velocities in column chromatography but similar levels of capture were only possible in a batch process if a 30-minute adsorption time was used. An alternative resin, SP FF (average bead size of 90 μm) gave better yields (section 4.9.1).

The simplest method to recover ion exchange resin in a compact volume after adsorption is by straining (sieving). Milk should be at or near the temperature it comes from the cow to maintain flow through the resin being collected on the strainer. Adsorption uptake, which should ideally be fast enough to be done within the time to milk each animal, is also higher at the secretion temperature. The efficiency of straining was explored (section 6.9).

6.6 Protein uptake rate

Detailed adsorption studies showed that the rate of protein uptake and final product yields depended on initial feed LF concentrations, adsorption temperature, type of resin (e.g. 200 μm vs 90 μm diameter), the resin:milk ratios (Φ), and adsorption time. The effects of other milk components were not evaluated. Yields could be predicted from the CNL model (section 5.5 – 5.7). Results from modelling showed that milk volume had little effect on adsorption above a Φ of 0.02. Resin is the major raw material cost (Chapter 8) so it is important to operate at the minimum Φ.

Laboratory trials showed that SP FF resin, with smaller beads and higher dynamic capacity, gave higher protein yields at 10 minutes than an equivalent volume SP BB resin, which has a higher equilibrium capacity (Chapter 4.5). Because yield within 10 minutes processing was is the most important process factor, the dynamic capacity rather than equilibrium capacity was the most relevant resin characteristic.

6.7 Cleaning, sanitisation and sterilisation procedures

Equipment and resin must be sanitised between adsorption cycles to reduce cross contamination. Any fat on the resin will reduce adsorption. Dairy equipment must be sterilised after cleaning to destroy micro-organisms in the system. Caustic soda (NaOH), a very efficient sanitiser and cleaner that solubilises precipitated proteins and lipids, can be effectively combined with solvent or detergent-based cleaning

methods. Dairy equipment is usually sterilised with steam after NaOH sanitisation. Resin manufacturers supply recommended cleaning procedures (Table 6-1).

Table 6-1: Suggested cleaning protocol for SP Sepharose resin.

Procedure	Reagent amount and concentrations
Removing precipitated proteins	1.0 M NaOH (2-3 bed volumes)
Removing lipids, hydrophobic proteins, lipoproteins	70% ethanol or 30% isopropanol (3 to 4 bed volumes) 0.5% non ionic detergent in 1.0 M acetic acid (3 to 4 bed volumes)
Sanitisation 1.0 M NaOH	
Sterilsation Autoclave media at 121^{o}C for 15 min.	

Cleaning-in-place (CIP) allows equipment to be sanitized without disassembling the equipment. The AMS CIP procedure includes an acid wash using Optima™ (Ecolab Limited, Hamilton, New Zealand) and NaOH hot rinses followed by water rinses, which are standard washing protocols used in the dairy industry. Protocols for cleaning and regeneration of resin were evaluated in section 3.13.3.

6.8 Capacity of the PFR

One cow at the AMS dairy unit had produced 37 L of milk, which was unusually high compared with the average of 15 L. To allow for the maximum likely milk production, a 37-L processing can was selected for processing. Volume of milk produced by a cow at each milking in the AMS dairy unit can be altered by changing the milking frequency in a 24-hour period.

Simultaneous processing of several cows using multiple receiver cans in the extraction unit would increase flexibility. However, this requires a more sophisticated system and will increase costs. The system had to be designed, constructed and evaluated within one year; incorporating the possibility of multiple processing would have taken too long to implement.

6.9 Mixing and resin recovery (straining)

Mixing suspends resin in the raw whole milk. Fines are generated if the resin is stirred too vigorously, which can increase filtration time, so upper stirring speeds were limited. Speeds of up to 200 rpm have been used during adsorption without

damaging the resin (Huppertz *et al*. 2003), although this will depend on the impeller blade and tank geometry. Having an adjustable stirrer would be useful for controlling stirring rates.

After the adsorption cycle, the resin must be recovered. This can be done by straining the resin on a polymeric membrane or a stainless mesh. Stainless steel mesh is safe, robust, chemically inert, corrosion resistant, and comes in wide range of sizes. Stainless steel mesh such as T316 is designed for use in the food industry.

The SP BB and SP FF resin had to be sieved. While being drained by gravity through a mesh in a dead-end filtration mode, a cake of retained resin quickly builds up. Flow resistance increases and draining rate drops rapidly. The decrease in drainage rate is very high if the milk/resin slurry drains too quickly initially (e.g. by suddenly opening a valve), presumably because resin particles are driven into, and then plug, the mesh. However, if drainage rate is controlled and the resin remains suspended during draining by stirring near the mesh surface, mesh blockage is minimised, and drainage rates can be maintained.

Slurry draining experiments were done in a 0.5-m, 70-mm i.d. Perspex tube with a 44-μm stainless steel filter (mesh 325, Mounts Wire Industries, Auckland, New Zealand) across the bottom. The tube was inserted into a tightly-fitting funnel and sealed with silicon sealant. A 12-mm PVC ball valve was attached to outlet of the funnel and a three-blade turbine impeller was placed 5 cm above the mesh (Figure 6-4). Raw milk at 35^{o}C was poured to a height of 35 cm and then 48.5 mL of swelled, drained resin was added (Φ = 0.016). After 30 seconds mixing, the outlet valve was opened carefully and the height of the milk/resin slurry recorded with time.

Figure 6-4: Modified Perspex-tube used to study draining characteristics of resin/milk slurry.

Stirrer speed affected slurry draining time (Figure 6-5) but the slurry drained quickly enough at the higher stirring rates for the planned process conditions. A stirrer with two impellers was best. The upper impeller was placed to give gentle stirring at 150 rpm during adsorption and the lower impeller assisted both suspension during adsorption and minimised resin build-up on the mesh during draining. The variable speed motor allowed stirrer speed to be increased during the draining process.

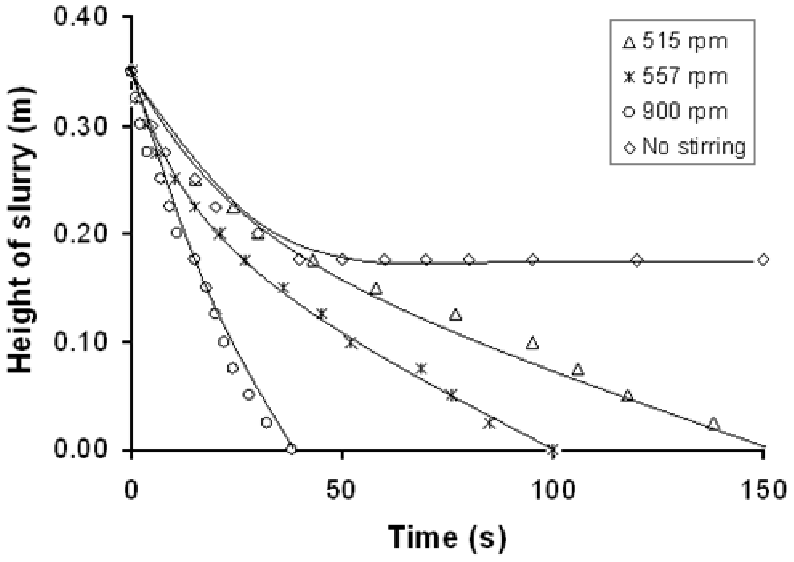


Figure 6-5: Effect of stirring speed on draining of a SP FF/milk slurry.

Liquid level sensors (Honeywell Control Systems Limited, Scotland, U.K.) were placed in the process vessel to sense milk level during processing and draining so stirrer speed could be controlled and the rinse step started.

6.10 The PFR mechanical design and assembly team

Over the summer period (2004/2005), the mechanical design of the PFR was undertaken by a group of students from The University of Waikato Florian Kern (a Mechanical Engineering PhD student and overall project team leader), with Andrew Hall (a 4th year BE student), and Nathan Scott (a recent BE graduate). The team was given various concepts to evaluate in terms of costs and time to manufacture a single-stage stirred tank reactor operation. Once design drawings were completed, parts were contracted for manufacture and components were ordered. The PFR was assembled, semi-automated, tested and transferred to the Greenfield's site (Figure 6-6). A process schematic for the PFR is given in Figure 6-7. Detailed design drawings are given in Appendix A.

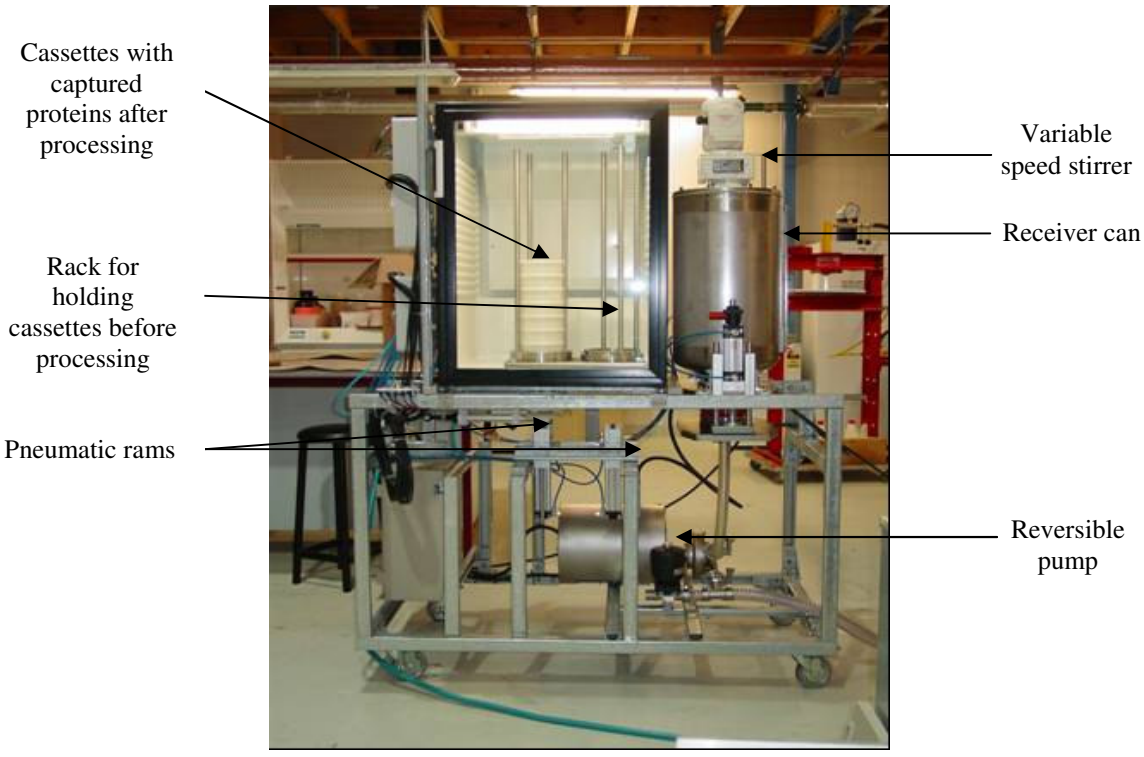


Figure 6-6: The Protein Fractionation Robot prototype for on-farm capture of LF and LP.

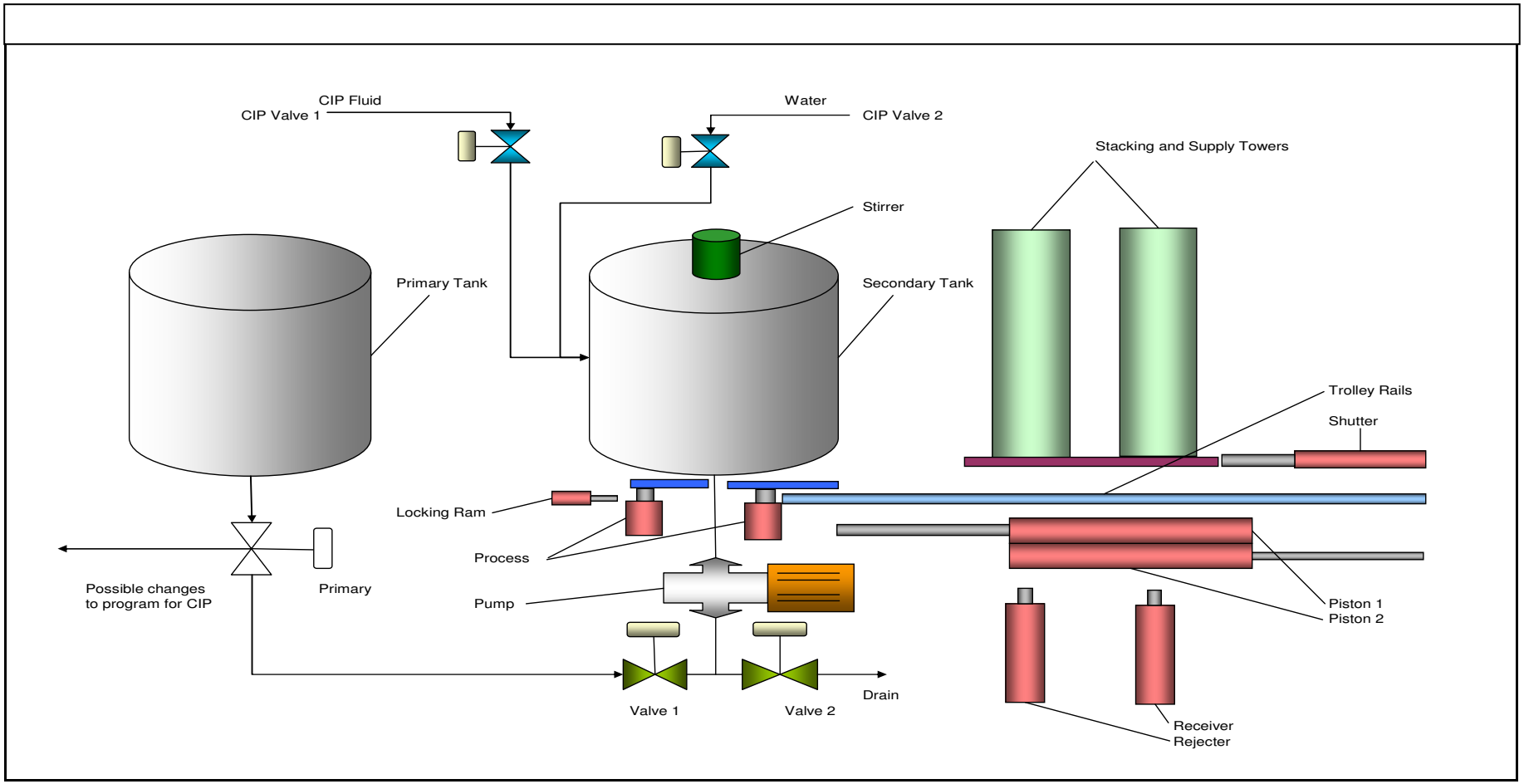


Figure 6-7: Process schematics for the Protein Fractionation Robot prototype.

6.11 On-farm fractionation using the PFR

Samples of milk, taken via the in-line autosampler on the AMS, were used to determine LF in milk from cows milked over a 24-hr period.

Not all aspects of the extraction purification process could be accomplished on-farm (Figure 6-8). A complete fractionation system should ideally include all aspects of protein purification, with most of the processing done on-farm. The PFR was designed to extract and store the proteins at 4°C before further processing was done.

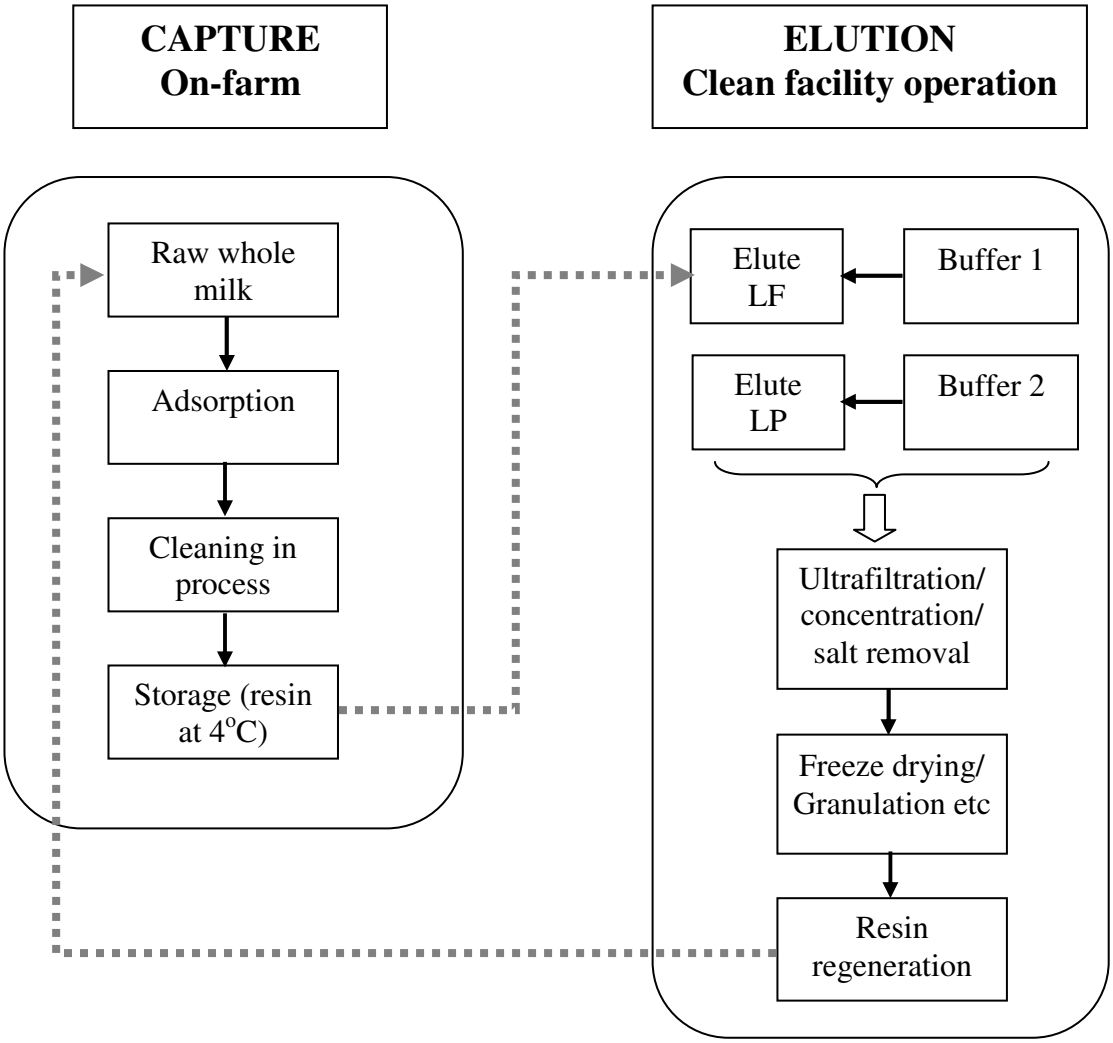


Figure 6-8: Protein fractionation process to capture and recover high-value proteins.

Individual cows usually produce similar milk volumes from day to day. As cows go through the drying-off period, milk volumes gradually decrease to between 2 and 5 L. As the lactation season progresses, milking frequency (number of visits to the

milking parlour) can be reduced or increased based on milk volumes. The Greenfield No 4 dairy operates on a 4-season calving basis. At any time there are cows at early, mid and late stages of lactation.

Extra Programmable Logic Controllers (PLCs) were put on the AMS to allow the PFR to be coupled in. Milk was pumped from the AMS receiver can into the prototype using a reversible, liquid ring pump (Fristam Pumps Inc. Middleton, Wisconsin, U.S.A.) on the prototype. The system was set so that once the "selected cow" (specific cow selected based on unique identification), "correct cow" (cow selected on milk volume or other variable such as colostrum), or "any cow" (any cow reporting for milking) conditions were met the harvested milk would be immediately diverted to the PFR for processing. The system was able to process (a) any cow consecutively or (b) any selected cows provided the PFR was ready to accept milk (i.e. not in a process or cleaning cycle).

6.11.1 Preliminary runs

The control algorithm is summarized in Figure 6-9. The first runs were done with empty cassettes. The processing (adsorption period) for single-stage batch adsorption was set to five minutes. Milk from the AMS was set for acceptance by the PFR, regardless of volume of milk produced. Once a cow reported for milking and the AMS successfully attached all teat-cups, it sent a signal to the PFR requesting the PFR to load an empty cassette and send a ready signal back to the AMS. The PFR picked up a cassette and loaded it into place, then sent a signal to the AMS for milk acceptance. A 30 second interval was provided for receipt of the ready signal before milk diversion was abandoned. Once the PFR was ready and the AMS was notified, the AMS would notify the PFR when that particular milking was complete. This allowed the PFR to divert the milk for processing using the reversible pump. The stirrer was set at 150 rpm. After the adsorption period was completed, the processed milk was pumped from the tank using the same pump, with the cassette still in place (load position). The tank was rinsed twice with water (~ 40^{o}C). The cassette was then stacked in the second rack (processed cassettes).

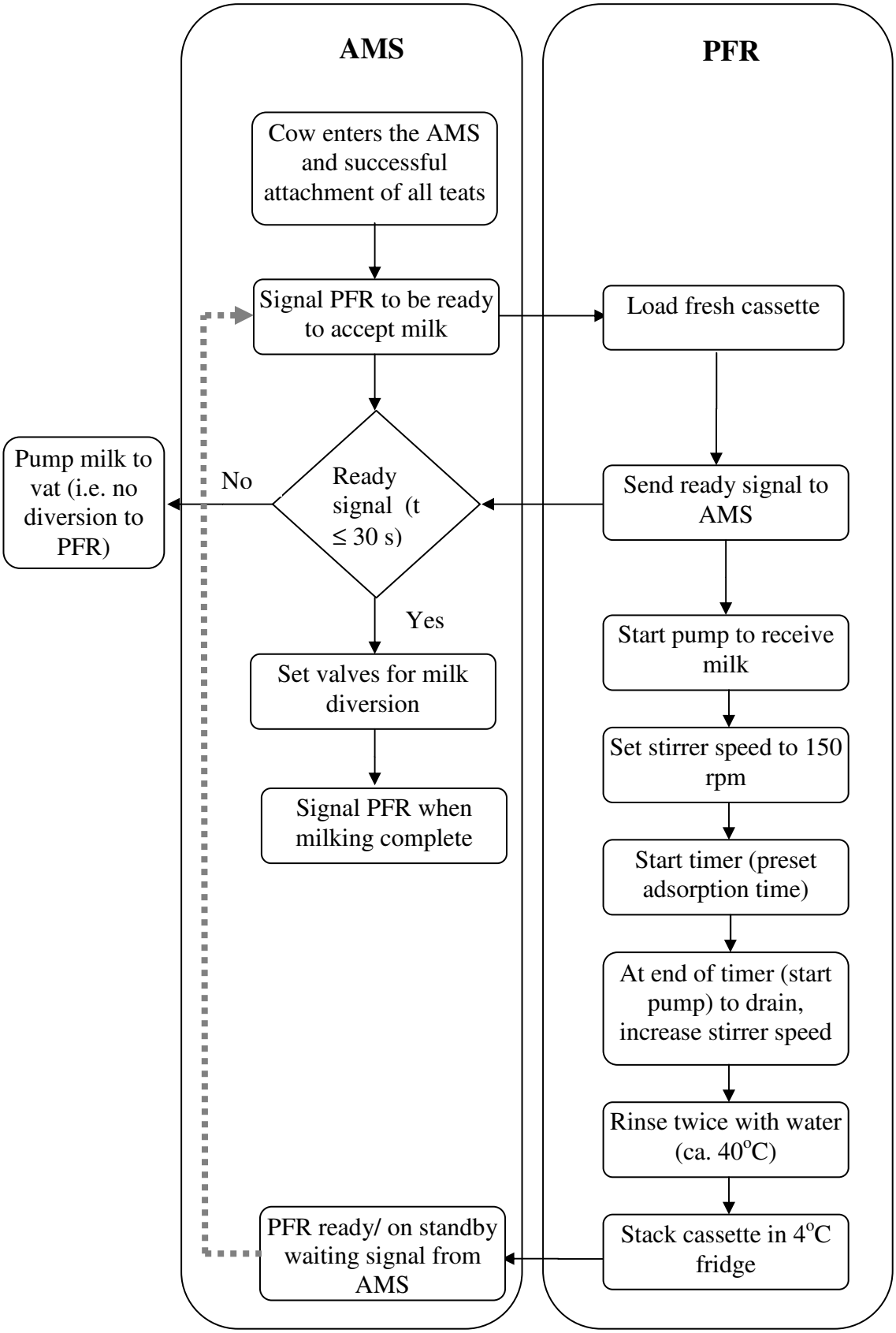


Figure 6-9: Procedure for on-farm capture using the PFR and the AMS.

6.11.2 Comparing SP BB and SP FF resins

One of the design challenges for on-farm extraction was the decrease in drainage rate as resin cake formed on the sieve. The drainage characteristics of SP BB and SP FF resins were compared by diverting milk from individual cows (cow 9705, 18.1 L for SP BB; cow 8730, 18.6 L for SP FF) to the PFR and adding 200 mL of resin. After 5 minute adsorption, the resin/milk slurry was drained to recover the resin. The SP BB particles were recovered from the resin/milk slurry in 45 seconds. Most of the milk drained from the SP FF/milk slurry in 5 minutes but it took a further 6 minutes to drain the next 1 L of milk, and some milk was still present when draining was stopped. The outflow temperature during the draining was 32.7^{o}C for SP BB resin and 27^{o}C for SP FF resin.

Bead size was an important factor on draining times. Prolonged stirring and exposure to the elements (such as ambient temperature) makes processing inefficient and can cause irreversible damage to the resin. It could also affect milk quality. The temperature observed during SP FF trials (27^{o}C) may have adversely affected results. However, time constraints meant the effect of temperature was not investigated.

The milk cools further if drainage times are extended. Fat solidifies and milk viscosity increases. The stirrer had a maximum speed of 450 rpm. Trials had shown that it took at least 100 seconds to drain 1 L of SP FF/milk slurry at 515 rpm and at 37^{o}C (Figure 6-5). High stirrer speeds were needed to drain SP FF quickly and it was not practical to achieve complete milk recovery within a minute. Although milk is secreted at 37^{o}C, it cooled to 25-32^{o}C (depending on ambient temperature and milk volume) during milking and storage in the un-insulated primary receiving can.

6.11.3 On-farm adsorption using the PFR

The SP BB resin was equilibrated in 10 mM phosphate buffer and 250 mL of swelled drained resin (also weighed) was packed into cassettes and loaded into the PFR (which was 4^{o}C). A contact time of 10 minutes was allowed for adsorption, followed by up to 1 minute draining to recover resin. The resin was rinsed twice with water at approximately 40^{o}C. Cassettes with the captured proteins were stored at 4^{o}C for 0.5 to 4 hours before being transferred to the laboratory for further processing.

The acceptance criteria for processing was set on the AMS as a minimum volume of 8.0 L of milk, partly because the AMS could not automatically sub-sample volumes less then 5.0 L. Cow identifications were noted and samples (milk feed and outflow) were analysed for bulk milk composition, total protein, and LF and LP content. Milk feed sample (i.e. before processing) was obtained from the automatic sampler on the AMS (except when the autosampler was not working, so manual sampling were taken). Samples of outflow milk (i.e. after processing) were obtained by inserting a 5-mm i.d. sampling tube into the outflow milk-line and taking continuous sub-samples during the draining process. Sampling variations may have occurred because sometimes sampling was not stopped during rinse cycles or before all the milk had drained.

The LF and LP were recovered using two 10-minute step elutions at 37^{o}C (water bath). The LP was eluted with 750 mL of 0.4 M NaCl, with a further 250 mL used for rinsing (total eluted volume 1 L). The LF was eluted with 2 x 750 mL of 1.0 M NaCl. The resin was cleaned, regenerated and stored in 20% ethanol.

7 On-farm Fractionation Results and Discussion

The results from the on-farm experiments with manual capture and trials using the PFR prototype are presented and discussed.

7.1 Pleated filter cartridge capture

When milk was passed through a cartridge filter pre-loaded with resin, milk filled the region with resin, flowed into the central region and then through the exit chamber. After the initial flow regime was established, the bed compacted and the rest of the milk bypassed the resin entirely. This meant no adsorption occurred because milk was not contacting the resin so this method was discarded.

7.2 Batch stirred-tank capture

Milk temperature before and after the 10-minute processing was 36.7 and 32°C respectively. Milk composition (% ^{w}/v) before (feed) and after (outflow) processing was similar (Table 7-1).

Table 7-1: Gross milk composition (% ^{w}/v) before and after batch adsorption.

Milk	Fat	Protein	Casein	Lactose	Total solids
Feed	4.53	3.67	2.73	4.45	13.5
Outflow	4.43	3.68	2.77	4.35	13.4

Of the original LP in the feed, 13.8 and 61.5% were in eluates 1 and 2 respectively (Table 7-2).

Table 7-2: LP concentration in feed and eluates.

Sample	Volume (L)	LP (mg/mL)	Total LP (mg)	LP yield (%)
Milk (feed)	8.1	0.010	77.5	--
Eluate 1	0.97	0.011	10.7	13.8
Eluate 2	0.98	0.048	47.7	61.5

Purity (Equation 7.1) and purification factor (Equation 7.2) indicate effectiveness of the capture and separation. The greater the purification factor, the more effective the operation (a value of 1 indicates no change in purity).

$$Purity = \frac{mg\ of\ target\ protein\ (eg\ LP)}{mg\ of\ total\ protein} \quad (7.1)$$

$$Purification\ factor = \frac{purity\ in\ eluate}{purity\ in\ feed} \quad (7.2)$$

The high purification factors (Table 7-3) show that LP and LF had been successfully captured from raw whole milk and that protein in the eluates was of high purity.

Table 7-3: LF and LP purity and total yields in feed and eluates.

	LF & LP (mg/mL)	Total LF & LP (mg)	Total protein (mg/mL)	Purity (%)	Purification factor
Milk (feed)	0.08	1119.4	36.4	0.2	--
Eluate 1	0.34	338.1	0.343	99.1	451
Eluate 2	0.38	380.0	0.424	89.5	407

7.3 On-farm fractionation with the PFR

7.3.1 LF concentrations in raw whole milk

Average milk production from 48 cows, milked by the AMS over a 24-hr period, was 14.7±5.7 L, with 38 cows producing >10 L. The LF concentrations ranged from 115 to 1317 mg/L. High concentrations of LF (e.g. 1317 mg/L) could be due to milk being from cows in early stages of lactation. The LF concentration varies during lactation (Turner 2002; Turner *et al.* 2003); it is highest in colostrums (milk immediately postpartum), is low during normal lactation, and increases in the drying-off (late lactation) period when milk production is low (<5 L /day) (Figure 7-1).

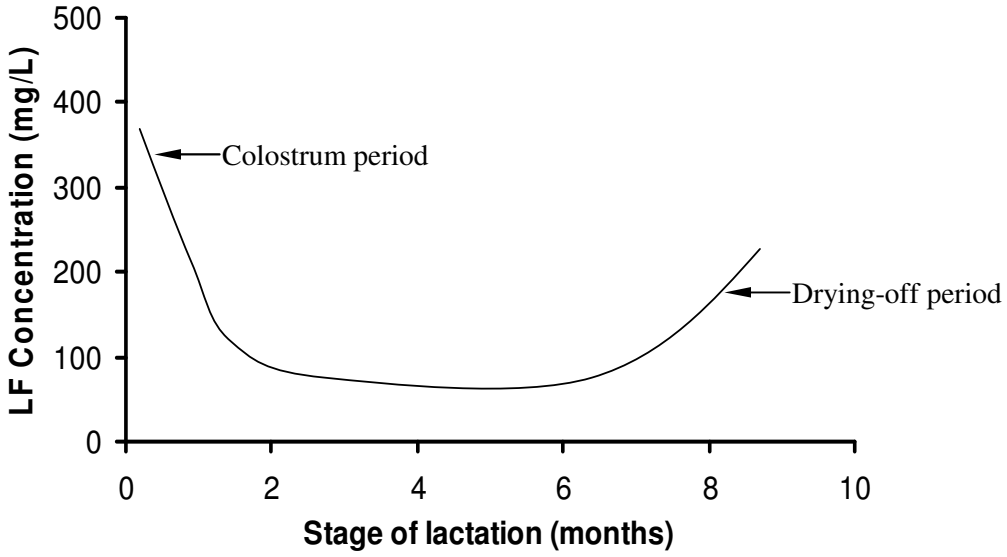


Figure 7-1: The LF concentration in cow's milk over a lactation period.

7.3.2 Processing milk from consecutive individual cows

In dummy runs with an adsorption cycle time of 5 minutes, it was possible to process all milkings from the AMS. If a resin with fast protein uptake were available, then contact time for adsorption will not be the limiting factor when processing and the PFR would be able to process milk from all cows, regardless of milk volume produced or time taken to milk an individual cow.

7.3.3 On-farm capture of LF and LP

The average milk volume from individual cows in the on-farm trials was 16.0 ± 4.6 L (Table 7-4). The milk feed contained 7-18 mg/L and 78-480 mg/L of LP and LF respectively.

The average volume of fresh, clean resin before processing and after regeneration was 250.2 ± 1.8 and 248.2 ± 13.6 mL respectively, indicating minimal loss of resin.

Milk from a Jersey cow (cow 480) had a high fat content (8.09%), which may have adversely affected LP recovery (only 51% compared with average of 87%), although LP yields generally were not particularly low (Table 7-4). Therefore, this data was excluded from statistical analyses.

Table 7-4: Milk characteristics and LF and LP recoveries (yields) from individual cows using on-farm capture with the PFR.

Cow ID	pH	Conductivity	Temperature (°C)		Milk	Resin:milk	Protein recovery	
		(mS/cm)	Feed	Outflow	(L)	ratio (v/v)	(% LF)	(% LP)
480	7.08	5.69	29.9	30.0	19.2	0.013	34.9	51.2
694	6.82	4.09	33.6	31.6	17.8	0.014	35.3	92.4
1401	6.74	4.65	31.5	30.8	21.1	0.012	28.5	81.6
1607	6.88	4.77	31.9	31.6	16.6	0.015	36.1	94.6
1922	6.70	5.45	32.0	30.2	14.4	0.017	27.6	77.1
1922	6.72	5.51	31.8	30.8	14.8	0.017	47.8	92.0
2409	6.74	4.07	31.7	30.2	16.8	0.015	36.7	90.4
3022	6.99	4.42	28.3	25.8	9.0	0.028	46.0	91.0
3109	6.81	5.06	32.0	30.0	13.6	0.018	25.6	84.7
3402	6.66	4.71	30.9	30.9	15.6	0.016	23.4	92.9
3532	6.70	4.68	30.1	29.3	8.2	0.030	55.2	99.5
5710	6.70	5.01	31.7	31.5	18.4	0.014	29.5	98.8
7656	6.70	5.25	32.0	31.8	26.0	0.010	19.7	81.9
9564	6.67	4.61	31.0	30.7	17.4	0.014	43.5	95.9
9570	6.79	4.50	32.0	30.2	10.6	0.024	48.9	82.9
Mean	6.78	4.83	31.4	30.4	16.0	0.017	35.6	87.1
Std Dev*	0.12	0.49	1.2	1.4	4.6	0.006	10.2	12.0

*standard deviation

Although there were insufficient data (n=14) to obtain conclusions, trends were noticed that are further discussed. The variability of individual milk components between individual cows meant the effects of individual feed characteristic or processing conditions on the amount of LF and LP extracted were not conclusive but indicated aspects that could be examined in a controlled environment (e.g. laboratory experiments). Preliminary correlation coefficients were obtained (Table 7-5).

Table 7-5: Correlation coefficients for feed characteristics and LF and LP yields.

Factor	% LF extracted	% LP extracted
Milk volume (L)	-0.619*	-0.048
LF feed (mg/L)	-0.168	0.186
LP feed (mg/L)	0.063	-0.544
Fat (% w/v)	0.619*	0.517
Protein (% w/v)	0.465	0.439
Casein (% w/v)	0.423	0.447
Lactose (% w/v)	-0.104	-0.050
Total Solids (% w/v)	0.610*	0.528
Resin:milk ratio (v/v)	0.677*	0.115
pH	0.181	-0.082
Conductivity (mS/cm)	-0.171	-0.219
Temperature feed (°C)	-0.370	-0.240

* $p = 0.05$, $r \geq 0.553$

Usually at least 80% of the LP (mean of 87.1%) was extracted but LF yields were much lower (35.2±10.2% of input LF) and very variable (Table 7-4). The higher LP

extraction may be because (a) the original LP concentration in the milk is lower and (b) preferentially binding of LF to the resin. In laboratory packed-bed chromatography experiments, almost all the LP but only 95% LF was extracted (sections 4.3.1 and 4.7.1). Etzel (1998) also reported incomplete LF extraction from skim milk during packed bed chromatography with SP BB resin.

There is a high correlation (p=0.05) between LF extracted and milk volume, resin:milk ratio (Φ), fat, and total solids. Because a fixed amount (250 mL) of resin was used in each extraction cycle in the PFR, Φ is influenced by the volume of milk produced per cow. Higher LF yields were achieved at higher Φ values (Figure 7-2), but Φ did not affect LP yields. Again this could be due to the lower LP concentrations and a different interaction between LP and the cation (SO_3^-) on the resin. The LP elutes at lower NaCl concentration, but LF binds more strongly and requires up to 1.0 M NaCl for desorption.

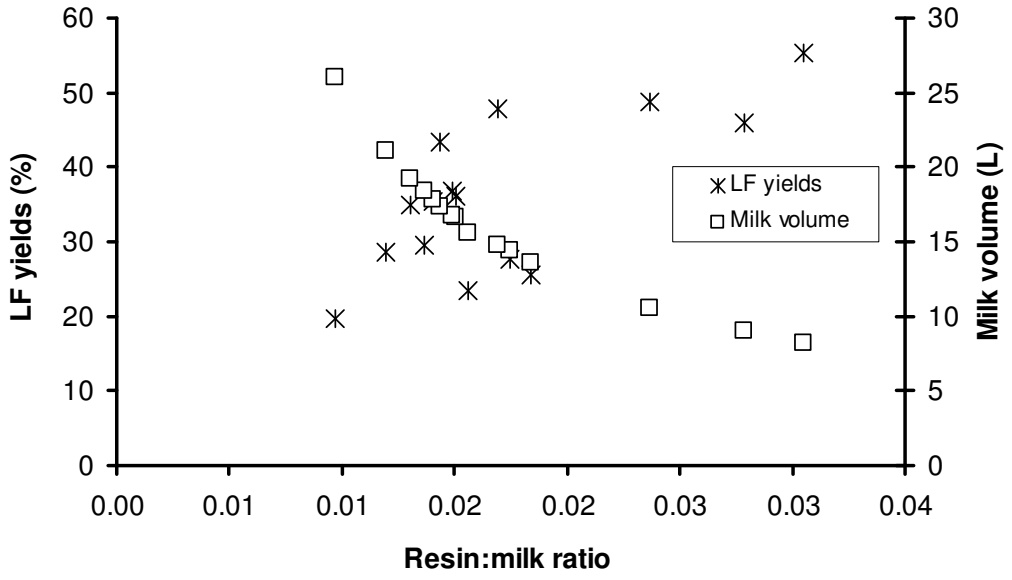


Figure 7-2: Effect of resin:milk ratio on LF extraction using SP BB resin (250 mL), in the PFR for milk volumes of 8.2 - 26.0 L.

Milk volume and LF yield were negatively correlated (-0.619) but there was no relationship (r= -0.048) between milk volume and LP yield (Figure 7-3). A fixed resin volume was used for on-farm experiments. Therefore, processing an increased milk volume from an individual cow will reduce the Φ value.

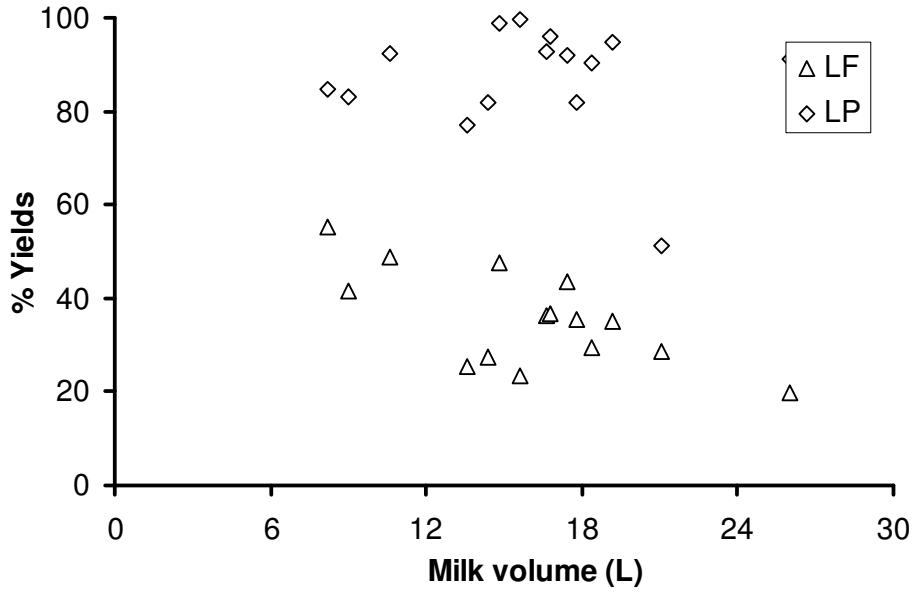


Figure 7-3: Effect of milk volume on LF and LP yields.

The LF and LP yields were not influenced by initial LF and LP content or milk volume (Figure 7-4 and 7-5) and LP yields were high irrespective of initial concentration (Figure 7-5). The Rowe *et al*. (1999) extracted BSA (initial concentrations of 0.1 to 3.0 mg/mL) with anion exchange resin and reported that initial protein concentration influences yield. However, milk in the on-farm studies contained only 0.07 to 0.48 mg LF/mL. The laboratory studies for kinetic modelling used a limited range (up to 1.0 mg/mL) of initial LF concentrations (section 5.5), which covered the levels normally found in cow milk. Perhaps the initial feed LF concentration range studied was not wide enough to see the trends observed by Rowe *et al*.

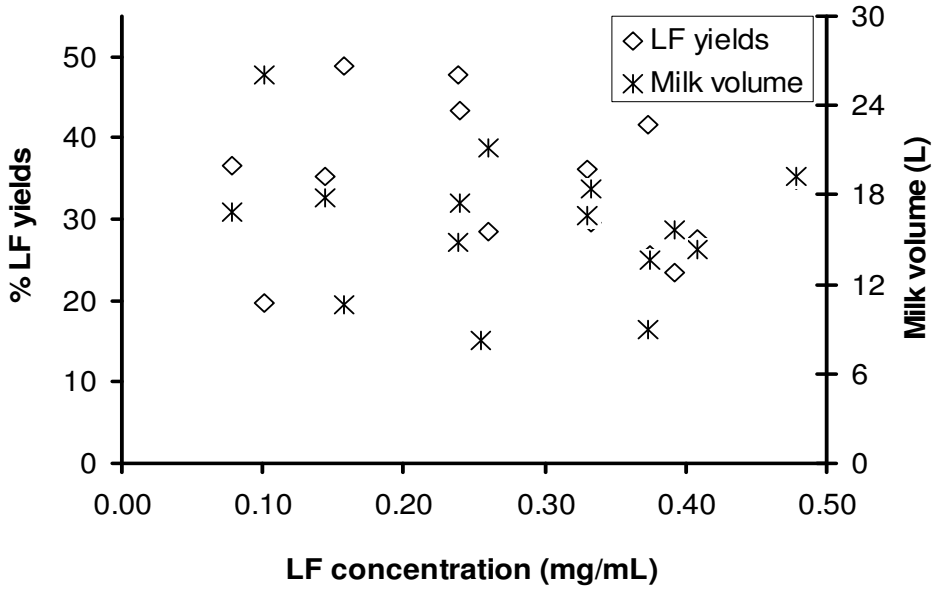


Figure 7-4: Effect milk volume and milk LF content on LF yields.

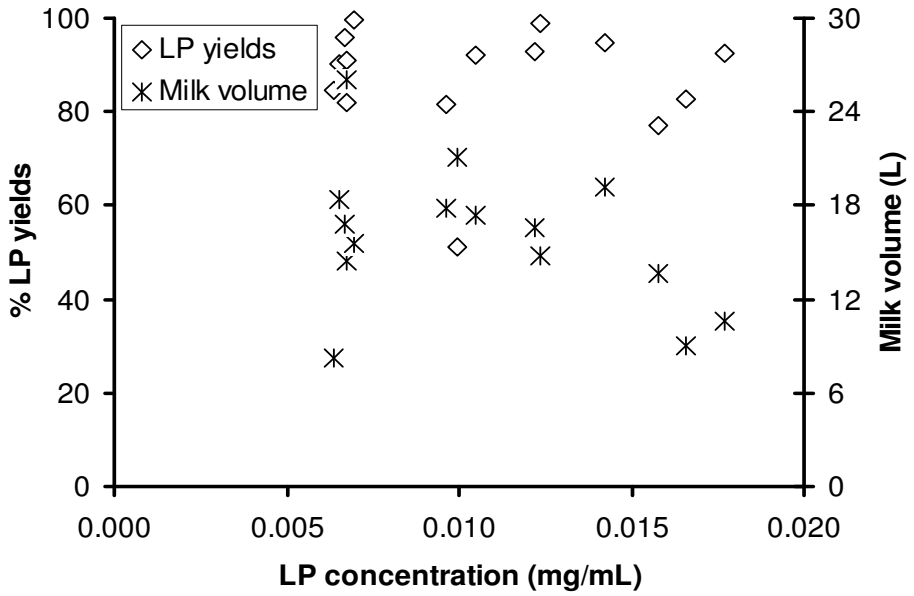


Figure 7-5: Effect of milk volume and milk LP content on LP yields.

Extraction with resin significantly ($p<0.05$) affected composition of the milk (Table 7-6) (Student's t-test, Excel 2003, Microsoft Corporation, U.S.A.).

Table 7-6: Effect of on-farm LF and LP extraction on milk composition (% ^{w}/v).

	Feed					Outflow				
Cow Id	Fat	Protein	Casein	Lactose	Total solids	Fat	Protein	Casein	Lactose	Total solids
480	7.83	3.82	3.01	3.74	15.9	8.09	3.74	2.95	3.71	16.1
694	4.59	3.75	2.93	4.90	13.8	4.67	3.68	2.85	4.74	13.8
1401	5.13	3.09	2.45	4.91	13.7	4.94	3.08	2.39	4.79	13.4
1607	3.91	3.40	2.68	4.72	12.7	3.78	3.27	2.50	4.51	12.2
1922	2.94	2.87	2.22	4.47	11.0	2.97	2.84	2.15	4.32	10.9
1922	5.51	2.92	2.31	4.37	13.3	5.17	2.91	2.21	4.37	13.0
2409	4.07	3.40	2.74	5.18	13.2	4.13	3.34	2.62	5.17	13.1
3022	4.09	3.46	2.59	4.74	13.0	4.35	3.34	2.42	4.48	12.8
3109	1.91	2.78	2.09	4.46	10.0	1.85	2.64	1.88	4.15	9.5
3402	4.34	3.34	2.62	4.94	13.2	4.12	3.25	2.46	4.71	12.7
3532	6.38	3.48	2.68	4.50	15.0	6.08	3.37	2.51	4.35	14.4
5710	5.00	3.41	2.67	4.59	13.7	4.77	3.27	2.50	4.41	13.1
9564	4.63	3.84	3.03	4.79	13.9	4.98	3.74	2.92	4.72	14.0
9570	4.90	3.87	3.00	4.96	14.4	3.93	3.20	2.36	4.31	12.3
Mean	4.66	3.39	2.64	4.66	13.3	4.56	3.26	2.48	4.48	13.0
Std dev*	1.36	0.35	0.29	0.34	1.42	1.38	0.31	0.29	0.33	1.49

* standard deviation

The effects of milk pH and conductivity on adsorption were not investigated. Milk pH ranged from 6.66 to 6.99 (average pH 6.78, Table 7-4) and conductivity from 4.09 to 5.69 mS/cm (average 4.83 mS/cm, Table 7-4). Milk processing temperature was expected to be near 37^{o}C. However, significant cooling occurred in the receiver can and milk temperature after the milking was completed was 31.4 (±1.2^{o}C) (Table 7-4). The trials were done in winter and ambient temperature could also affect milk temperature at the end of milking. For example, milk temperature was 36.7^{o}C immediately after milking in earlier experiment during the summer (section 7.2).

7.3.4 Overall LF yield

During the test period, 239 L of milk was processed in the PFR and 44.2% of LF in the raw milk was captured (11.6 g/100 L raw whole milk). After elution from the resin, 21.6 g of LF (or 35.6% of LF the raw milk) was obtained.

Industrial LF extractions report yields of 10 g/100 L of skim milk (Nimmo-Bell, 2003). Without any optimisation, yields with the PFR were 15% higher than those from centralised, commercial extraction of feed such as skim or whey.

The PFR extraction can be optimised to give better yields. For example, SP FF resin (section 4.9.1) has better protein uptake and can absorb 65-75% of LF after 10 minute contact. This represents 42-48 g of LF from 239 L of raw whole milk (or 17.6-20.1 g LF per 100 L of raw milk), or twice that reported for an optimised commercial process. Because laboratory performance of SP BB resin was transferred to the on-farm situation, it is expected that SP FF resin performance in the laboratory can also reliably be extrapolated to the on-farm situation.

7.4 Summary and future prospects for the PFR

The PFR met most of the design objectives and performed well against project objectives. Several aspects could be improved to enhance PFR's functionality.

Currently, the CIP on the PFR is operated semi-automatically and independent of the AMS. This could be automated for ease of operation. The CIP program could be controlled by using the AMS software to CIP the PFR and AMS at the same time using same cleaning solutions. However, because the CIP on the AMS is a scheduled operation, its access is difficult, which limits flexibility and availability. An independent, automated CIP facility is the preferred option.

There was unnecessary dead volumes in pipes (0.5 to 0.6 L) connecting the PFR to the AMS. Positioning the PFR closer to the AMS would reduce pipe length and hence volumes needed to transfer milk.

The reversible pump was self-priming. However, it did not prime sufficiently at the start of the run. Rinsing the system with hot water would pre-clean and assist priming. It would also warm the processing can and thus help maintain processing temperature of the first lot of milk received by the can.

The stainless steel processing can could be lagged or have a heating jacket or heating tape to maintain milk at the secretion temperature (35-37^{o}C) for a short time (10-15 minutes depending on the process time). A higher temperature assists adsorption and may increase overall yields.

The effects of temperature (section 4.6.2) and adsorption time (section 4.9) were studied in the laboratory. On-farm trials indicated that milk components (e.g. fat, other proteins, etc) may also affect yields. These factors could be considered when selecting cows for processing or for yield calculations.

Aeration during milking and/or pumping results in frothy milk, which increases draining times and could decreases process efficiency. This was not a problem for most milkings but could be an issue if it occurred frequently. The reasons for frothing could be introduction of air into milk-line during milking process and/or milk components (the causes were not investigated). The control system can identify cows individually; any cow that produces milk that is difficult to process could be excluded.

The PFR prototype is mounted on a skid for portability. The cassette LOAD position under the processing can must be aligned to obtain a good seal. Any leakages could result in some resin loss.

The smaller diameter resin (SP FF, 90 μm) had long draining times. However, it captures more LF and LP than the larger SP BB (200 μm) in a given process time, resulting in better yields. Aspects that would make extractions with SP FF possible include: maintaining milk and processing can temperature at 35^{o}C; increasing stirrer speed (without damaging the resin) during draining; redesigning the impeller blade and adjusting its position in the processing can; and removing the finer resin beads (<40 μm) before using the resin.

The following are some aspects worthy of investigation:

- whether processing from selected cows milk with enhanced LF and LP (or other target concentrations) would enhance process economics
- optimising impeller design and flow through the cartridge holding the resin so smaller and more efficient beads (resin) can be used
- identify a better resin with higher binding capacity to achieve better yields
- replacing expensive pharmaceutical-grade resins with cost-effective capture media (beads, resin or other means), which can be used for food processing
- other forms of capture on-farm that may enhance yield and purity (such as membranes, activated filter system)

- exploring whether the process can be used to capture and purify transgenic proteins
- investigating whether other valuable proteins such as immunoglobulins, prolactin, CD14, etc can be processed. For example, Protein A media could be used to recover immunoglobulins directly from standard or hyperimmune milk or from colostrum
- determining quality attributes of LF and LP and other proteins extracted from raw whole milk

8 On-farm Fractionation Economic Analysis

8.1 Introduction

Data from the on-farm trial using the PFR were used to calculate profitability of on-farm capture. Costs of raw materials, capital for the PFR, and labour for elutions, together with LF and LP yields and market prices were used for the economic feasibility study. Process simulations and economic analysis was done using SuperPro Designer® (version 5.1, Intelligen Inc., MIT, U.S.A.). An example of the inputs and outputs from SuperPro Designer (case 19) is included in Appendix C. Sensitivity analyses were done on key variables such as raw material costs (especially milk), resin binding capacities and yields, and product price to assess robustness of the economic analysis on profitability, net present value (NPV) and payback period.

The effect of the following factors on profitability and rate of return were investigated:

Scenario 1: Variable LF and LP prices reflecting market and end-user prices where:

- on-farm fractionation does not markedly affect milk properties and milk retains its value to the farmer (cases 1-6)
- milk is purchased at cost and is discarded after LF and LP extraction because it has no value (cases 7-10)
- milk retains 80% of its value and can be further processed (cases 11-15)

Scenario 2: Average LF and LP yields achieved on-farm using the PFR (section 7.3) and realistic and conservative LF and LP prices where:

- on-farm fractionation does not markedly affect milk properties and milk retains its value to the farmer (cases 16)
- milk is purchased at cost and is discarded after LF and LP extraction because it has no value (case 17)
- milk retains its value and a better resin (for example SP FF) is used for the extraction, (case 18)

Scenario 3: Milk retains its value and on-farm LF and LP extraction is optimized by using high (1000 mg/L) LF milk and resin with fast adsorption, for example, SP FF (case 19)

Scenario 4: Extracting proteins worth $5000 to $20000 per kilogram from transgenic milk, where price of raw whole milk is higher and includes a disposal cost (cases 20-23)

The costs, parameters and assumptions of each case are described and sensitivity analyses based on different scenarios are tabulated.

8.2 Lactoferrin market and prices

The current world market for LF is around 100 tonnes per year. Japan is both the largest producer and consumer. Increased demand could easily be met by processing more whey and it is feasible to produce 1000 to 2000 tonnes worldwide (Anon 2005; Thiersch 2004). However, the elasticity of LF price is unknown. Producing such high quantities could decrease the prices from the usual US$500/kg. The growth of the market is slower than the increase in production. It is relatively easy to produce LF with greater than 95% purity with reported wholesale prices of US$300/kg (Fonterra, NBR 2004), US$350-400/kg (Tatua Nutaceuticals, Nimmo-Bell 2003) or US$350-500/kg (Gloy 2004). Retail and end-user prices average US$1500/kg (range US$1.13- $3.50 per gram). Gloy (2004) reported that the proprietary nature of new products makes it challenging to identify market prices on most milk protein fractions.

Most milk in New Zealand is processed for export. New Zealand dairy farmers receive NZ$3.50 and $5.00 per kilogram milk solids (MAF, 2005). At conservative selling prices (US$300/kg), the LF in milk (1000 mg/L) represents the value farmers currently get for their raw milk (NZ$0.24 to $0.40/L).

8.3 Costs and variables used in economic analyses

All prices are in New Zealand dollars, unless otherwise specified. Processing costs include raw material, buffer and chemicals, costs for eluting captured proteins into concentrated solutions, and resin regeneration. Processing costs do not include diafiltration and freeze drying to obtain protein powder.

8.3.1 Raw material costs

The costs of materials such as buffers, mineral acids, base, and salt were obtained from Harrison *et al.* (2003). Current resin costs (from New Zealand agents for litre amounts) were used because SP BB resin is contract manufactured and not readily available.

Phosphate buffer (10 mM)	$0.0784 /L
Sodium hydroxide (1 M)	$0.0560 /L
Hydrochloric acid (1 M)	$0.0650 /L
Isopropanol (40%)	$0.8680 /L
Sodium chloride (1.0 M)	$0.0420 /L
Resin	$1.00 /mL
Milk solids (2005/06 season)	$4.00 /kg milk solids
Raw whole milk (L)	$0.24-0.40 /L

8.3.2 Production capacity

The PFR operating capacity was estimated from actual PFR operation. Between 11 am to 5.00 pm, milk from 16 individual animals was successfully diverted to the PFR for LF and LP capture. At approximately 1 pm, a scheduled 35-minute CIP for the AMS occurred. The PFR operation was uninterrupted and there was no malfunction or downtime during processing. Limitations to the number of units processed through the PFR, if any, were due to asynchronisation of the AMS milking and PFR cycles, because the 10-minute time for processing (adsorption) made the PFR cycle longer than an average milking time.

Processing vessel volume	37.22 L
Dead volume (pipes)	0.65 L
Production capacity of prototype	40 units or individual cows per 24 hr

8.3.3 Costs of major capital items

Costs for major capital items, based on actual cost of components and materials purchased, and assembling the PFR are summarized in Table 8-1. Manufacturing cost should decrease if multiple units were produced.

Table 8-1: Cost of major capital items and assembling the PFR.

Item	Amount ($)	
Tank assembly	12 000	
PLC	2 000	
Pump (lobe)	5 500	
Motor	500	
Variable speed drive	1 000	
Trolley	1 000	
Total equipment purchase cost (PC)		22 000
Assembly, wiring, fabrication, polishing, valves, dummy cassette		26 000
Programming, auxillary etc (not included in above) (DC)		$58 000
Total direct fixed capital cost (DFC)		$119 250 (incl GST)

PC - Equipment purchase cost, DC - Direct cost in equipment costs, DFC – direct fixed capital costs (consistent with terms used in SuperPro Designer)

Factors (between 10-30% of PC) were added to the equipment cost for piping, plumbing, electrical components, etc, installation (Lang, 1948), and the initial cost of chromatography resin to obtain total investment. Working capital in the economic analysis covers the cost for 30 days of labour, raw materials, utilities and waste treatment and depends on the assumed cost of milk.

8.3.4 Process and economics consideration

Project time	20 years
Inflation	2%
Net present value interest	7%
Income tax	33.0%
Days in operation	250 days per annum (or milking season)
Cycles per annum	10000 cycles (250 days @ 40 cassettes per day i.e. milk from 40 cows)
Processing (adsorption) time	10 minutes
Resin per cycle	250 mL
Resin retention/cycle	99.5 %

8.3.5 Market prices

LF (wholesale price)	$450-500/kg
LP (wholesale price)	$300-350/kg
Retail price (end-user products)	$2000-3000/kg (Appendix B)

The LF market price used in the economic analyses reflects current prices ($NZ450-500/kg), a deflated price (NZ$300/kg), end-user based prices (NZ$1000-3000/kg) and a projected price ($NZ600/kg) with a premium for LF extracted on-farm from milk that has not been pre-treated.

8.3.6 Resin characteristics

Resin capacity (static capacity)	30 mg protein/mL resin
Binding	80% LF, 80% LP
Yield (relative to initial feed)	95% LF, 95% LP
Resin life	500 cycles

8.3.7 Feed

Volume of raw whole milk	16.0 L/cycle
LP content in raw milk	30 mg/L
LF content in raw milk	500 mg/L
Transgenic protein content	500 mg/L

In scenarios 1-4 evaluations were based on resin dynamic capacity (30 mg/mL) and 80% binding. These characteristics may be expected for SP FF resin. Even though transgenic protein content is expected to be much higher than 500 mg/L, similar concentrations were maintained so the same production capacity is used throughout the analysis to give comparative values.

8.4 Profitability and rate of return for on-farm processing

Parameters used to calculate the effect of LF prices on profitability are the same (section 8.3) in all analyses unless otherwise specified.

8.4.1 Effect of product price on profitability and rate of return

Economic analyses show that if the processed milk can be sold at its normal price, the breakeven point for on-farm extration occurs when LF can be sold for

approximately about $850/kg. At high LF selling price (e.g. $2000/kg) a very short payback time (1.3 years) is achieved for the for the investment (Table 8-2)

Table 8-2: Effect of product price on profitability if milk was bought and sold for $0.33/L; total investment = $125 280.

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
LF price ($/kg)	3000	2000	1000	875	500	300
LP price ($/kg)	1000	700	350	305	150	100
Revenue ($/yr)	174 420	116 394	58 197	50 992	29 013	17 442
Operating costs ($/yr)	36 135	36 135		36 135	36 135	36 135
Production (kg/yr)	57	57	57	57	57	57
Production cost ($/kg)	633	633	633	633	633	633
Gross margin (%)	79.3	69.5	38.0	29.0	-24.3	-107.2
ROI (%)	78.7	47.48	16.4	12.5	1.1	-10.3
Payback time (years)	1.27	2.11	6.12	8.02	--	--
IRR (%) (after tax)	141.9	62.7	12.4	7.1	--	--
NPV (7%) ($)	862 978	456 321	48 466	270	-199 810	-320 367

8.4.2 Effect of product price on profitability if milk has no resale value

If the milk has no residual value, the breakeven price occurs if LF can be sold for $1760/kg (Table 8-3), which is near the current retail price of end-user products ($2000-3000/kg). Therefore, the processed milk must have a value to ensure the process is profitable.

Table 8-3: Effect of product price on profitability if milk purchased at $0.33/L with no resale value; total investment = $143 585.

	Case 7	Case 8	Case 9	Case 10
LF price ($/kg)	3000	2000	1760	1000
LP price ($/kg)	1000	700	600	350
Revenue ($/yr)	174 420	116 394	102 389	58 197
Operating costs ($/yr)	85 233	85 233	85 233	85 233
Production (kg/yr)	57	57	57	57
Unit production cost ($/kg)	1495	1495	1495	1495
Gross margin (%)	51.2	26.7	16.7	-46.2
ROI (%)	45.8	18.5	12.0	-14.8
Payback time (years)	2.19	5.40	8.4	--
IRR (%) (after tax)	57.11	15.86	7.11	--
NPV (7%) ($)	505 654	98 998	840	-420 528

8.4.3 Effect of product price on profitability if milk retains 80% of its value

If the processed milk retains 80% of its original value (an arbitrary value), the breakeven selling price occurs if LF can be sold for about $1100/kg (which is above the current wholesale price) (Table 8-4).

Table 8-4: Effect of product price on profitability if milk retains 80% of its value (milk purchased at $0.33 and sold at $0.25 L; total investment = $143 575)

	Case 11	Case 12	Case 13	Case 14	Case 15
LF price ($/kg)	3000	2000	1070	1000	500
LP price ($/kg)	1000	700	380	350	150
Revenue ($/yr)	213 699	155 673	102 138	97 476	68 292
Operating costs ($/yr)	85 233	85 233	85 233	85 233	85 233
Production (kg/yr)	57	57	57	57	57
Unit production cost ($/kg)	1495	1495	1495	1495	1495
Gross margin (%)	60.0	45.5	16.6	12.6	-24.8
ROI (%)	64.2	37.0	11.8	10.3	7.8
Payback time (years)	1.56	2.71	8.4	10.3	--
IRR (%) (after tax)	93.1	42.2	6.6	4.3	--
NPV (7%) ($)	780 927	374 270	-600	-31 568	-315 351

8.4.4 Profitability if PFR operation is optimised

Several factors can increase economic viability of the PFR operation. For example, if a better resin with high capacity (80%) (and ideally with a high adsorption rate is used) the production capacity is increased 3 times (case 18, Table 8-5). The production capacity increases 10 times (109 kg compared to 10.5, for example, if binding capacity is increased from 50% to 80% in addition to processing from selected cows milk that has LF concentration of 1000 mg/L. In this case the payback time for investing into a PFR system decreases to 2.6 years (i.e. 1000 mg/L compared with the average of 278 mg/L is processed). This can be achieved by identifying higher producing cows and/or cows in the correct stage of lactation cycle (case 19, Table 8-5). One of the disadvantages of this system include not having enough milk to process because only a fraction of the normal herd produces milk with the required LF content (e.g. only 3 of the 48 cows tested, produced LF over 1000 mg/L, section 7.3.1). Therefore, a specialised herd will be needed. However, this introduces the possibility of breeding or selecting a herd that expresses high levels of a target component in their milk.

Table 8-5: Effect of resin binding capacity and concentration of LF and LP in milk on process profitability (Investment = $125 281).

	Case 16	Case 17	Case 18	Case 19
LF price ($/kg))	400	400	400	400
LP price ($/kg)	150	150	150	150
Resin capacity (mg protein/mL resin)	50	50	80	80
Binding (%)	50	50	95	95
Yield (%)	80	80	95	95
LF feed (mg/L)	278	278	278	1000
LP feed (mg/L)	10.5	10.5	10.5	10.5
Milk volume procesed (L)	16	16	16	16
Milk purchase price ($/L)	0	33.0	0	0
Milk sell price ($/L)	33	0	0.33	0.33
Revenue ($/yr)	53 400	4290	61 368	92 868
Operating costs ($/yr)	28 501	77 611	28 501	28 501
Production (kg/yr)	10.5	10.5	30.2	109.2
Unit production cost ($/kg)	2714	7391	942	261
Gross margin (%)	46.6	-1709	53.6	69.3
ROI (%)	18.0	-47.1	22.1	39.0
Payback time (years)	5.6	--	4.5	2.6
IRR (%) (after tax)	14.5	--	20.0	46.6
NPV (7%) ($)	68 350	-902 774	124 191	344 947

8.4.5 Profitability of processing transgenic protein

Recombinant human proteins can be expressed in the milk of transgenic cows (van Berkel *et al*. 2002) in g to kg quantities. Although it is difficult to predict the price of transgenic proteins, some therapeutics are worth billions of dollars (van Berkel *et al.* 2002). Conservative prices of $5 000 to $20 000 per kg were used in the analyses. The raw milk would cost much more than normal milk because strict quality assurance regulations will apply. The economic analysis assumes that the raw milk will cost three times that of normal milk and an extra cost of $NZ0.25/L has been included for milk disposal because milk from transgenic animal will not be accepted in the food chain nor can it be dumped because of environmental problems. The economic analysis shows that the PFR operation is economically viable, even if the transgenic protein is only worth $5000/kg (case 23, Table 8-6). If the protein can be sold for $10,000/kg, the payback time is less than 1 year (case 22, Table 8-6).

Table 8-6: Effect of transgenic protein price on profitability with milk price ($0.98/L) and disposal cost (-0.25/L) and investment = $198 158.

	Case 20	Case 21	Case 22	Case 23
Transgenic protein price ($/kg)	20 000	15 000	10 000	5 000
Milk purchase price ($/L)	0.98	0.98	0.98	0.98
Milk sell price ($/L)	0	0	0	0
Disposal costs ($/L)	-0.25	-0.25	-0.25	-0.25
Investment ($)	198 158	198 158	198 158	198 158
Revenue ($/yr)	1 140 000	855 000	570 000	285 000
Operating costs ($/yr)	232 103	232 103	232 103	232 103
Production (kg/yr)	57	57	57	57
Unit production cost ($/kg)	4071	4071	4071	4071
Gross margin (%)	80.8	74.1	60.8	21.8
ROI (%)	320.4	222.3	122.6	25.0
Payback time (years)	0.31	0.45	0.81	4.00
IRR (%) (after tax)	818.36	441.64	187.42	19.77
NPV (7%) ($)	6 264 933	4 275 438	2 285 944	292 658

8.5 Summary

Economic analyses show that the PFR process for extracting LF and LP from milk can be viable but depends on the farmer receiving a premium for a specialty protein such as LF as well as being paid for total milk solids. This is based on the assumption that a farmer invests in an optimised PFR system. At current wholesale LF and LP prices, the system will have a payback of approximately five years. Targeting high-producing cows (1000 mg/L LF) and using a resin that can capture 80% of the LF in the milk, increases profitability substantially. If LF and LP obtained immediately after milking has better quality (in terms of functionality) than similar product obtained from bulk processing, a premium may be possible.

The profitability analysis can be incorporated into a business plan, which could encompass the likely commercial and supply chain model for establishing on-farm fractionation. The trade-off between manufacturing cost, target extraction percentage, processing time, media volume per batch, final activity and yield of target protein(s) vary widely, depending on objectives of each extraction. For instance, on-farm extraction of LF and LP from milk that can be further processed into other products is different from extracting a recombinant protein from transgenic milk that cannot be used in the normal food chain.

9 Conclusions and Recommendations for Future Work

9.1 Laboratory studies

This study demonstrated that significant quantities of raw whole milk can pass through a shallow chromatography column packed with commercially available resin provided the processing temperature is kept at or near the temperature of freshly collected milk (35-37^{o}C). Adsorption rate at these temperatures were higher than those at normal milk chromatography temperatures (4-8^{o}C).

Design considerations led to using a single-stage stirred tank for extracting the model proteins, LF and LP, in an on-farm environment. The cation exchange resins SP FF and SP BB were chosen as candidates for on-farm capture because they are approved for food use by the FDA. Equilibrium capacities were 200 and 110 mg LF per mL of resin for SP BB and SP FF respectively. For designing the system, resin dynamic capacity was more relevant than its equilibrium capacity because faster protein uptake rates were most important in achieving high extraction yields due to the limited time available for adsorption between milking individual cows.

9.2 Kinetic modelling and analysis

Data from batch adsorption experiments with raw whole milk showed that the new composite non-linear (CNL) model of Rowe *et al*. (1999) could be used to predict extraction yields as a function of time, feed concentration and milk volume for a fixed volume of resin. The four independent parameters q_k, k, a and $y(0)$ in the CNL model to describe batch adsorption dynamics, could be obtained from Co and Φ (resin:milk volume) and related to LF extracted with time or for a pre-determined adsorption period.

An investigation of the modeling showed that kinetic capacity parameter, q_k was the most important factor in adsorption kinetics. Higher feed LF and higher Φ ratios gave greater yields for a given amount of resin. The LF obtained from on-farm

extraction using SP BB resin agreed to within 14% of the values predicted from the CNL model. The CNL model can be used to evaluate performance of other resins.

9.3 Protein Fractionation Robot prototype

Design data obtained from laboratory trials together with a design brief was successfully used to produce a PFR unit. The unit mounted on a skid for transport and consisted of:

- a single-stage 37-L stirred tank system which had an overhead stirrer with two impellers to maintain resin suspension and to aid draining when recovering resin after an adsorption cycle,
- a 4^{o}C chiller to store cassettes with fresh resin and resin with captured protein
- cassettes with a 44-μm sieve base to hold resin, allow resin suspension with positive milk flow, and to capture suspended resin after processing
- a reversible pump to transfer milk from the AMS to the PFR and to remove milk after the adsorption process
- variable speed drives to control stirring speeds and pumping rate
- a timer to control the adsorption time, PLCs for automation
- pneumatic rams for loading and unloading of cassettes
- an automated warm water (40^{o}C) rinse cycle
- a semi-automated CIP cycle

9.4 On-farm fractionation studies

Valuable milk components were successfully extracted in the PFR, on-farm, from fresh, raw whole milk collected immediately after milking. The LF and LP could be extracted using SP BB cation exchanger without disrupting the milk harvesting process. Milk yield and composition were slightly lower after fractionation but the processed milk would be acceptable for consumption or further processing.

Extractions cycles could be achieved within the time to milk an individual cow. Milk required no pre-treatment and its temperature, as obtained from the cow, was very suitable for the extraction process.

Average LF and LP yields from 16 individual cows were 35.6 and 87.1% feed LF and LP respectively. Although the experiment did not selectively target high-LF milk, the LF yield of 9.1 g/100 L is 15% higher than current practices used in centralised processing from skim milk. Selective milk processing and further optimisation will enhance LF yields.

9.5 Benefits from this research

Processing raw whole milk is not limited to ion exchange; other chromatographic techniques such as affinity or reverse-phase chromatography could be used, which would allow extraction of other minor milk components such as immunoglobulins, and lysozyme.

A significant aspect of on-farm processing is that bioactive proteins in their native state and at their maximum activity can be obtained from fresh, raw whole milk. The repeated cycles of heating, cooling and shear that occur during transportation and bulk processing are bypassed. On-farm processing provides an opportunity to extract minor but high-value proteins before their value is lost by combining milk in big silos and bulk processing. The value of using farm management practices that produce milk with elevated levels of specific components can be maximised by on-farm processing.

Economic studies showed that the stand-alone process is profitable provided the unit operations are optimized. Profitability is markedly improved if milk from selected animals was processed for target protein(s) and the farmer is to be paid for high-value protein as well as the bulk milk.

On-farm extraction was successful for producing minor, high-value proteins from conventional milk. The system would be very suitable for processing recombinant proteins from milk of transgenic animals. Coupling the AMS and the PFR allows milk from individual cows to be identified, provides explicit traceability, and contains all raw materials and products. An extra benefit is that immediate processing maximises yields and retains bioactivity. This approach allows a new business model in dairy processing, where the farmer can produce crude, high-value protein fractions as well as a liquid (or milk solids) for commodity dairy manufacturers.

9.6 Recommendations for future work

Although many separation processes are technically feasible, extraction efficiency is a key determinant of commercial success. It is recommended that closely monitored extractions and processing are done using the PFR to give more data for assessments. This was not possible because of constraints on available testing time during this project. The effects of bulk milk composition (protein, lactose, total solids and especially fat) on extractions also need to be further studied as well as whether on-farm processing affects bulk milk composition. Data from laboratory experiments indicated that extractions did not affect bulk milk composition but data from on-farm experiments were inconclusive.

The following work, which would assist in developing and validating the concept and identifying technology associated with on-farm processing, is highly recommended:

- examining whether bioactivity is retained during processing and distribution. *In vitro* trials could be routinely used to measure bioactivity. *In vivo* trials may then be needed to satisfy authorities that claimed effects are real
- assessing whether heat-sensitive bioactive proteins can be produced economically
- identifying other minor, high-value milk proteins or components that could be extracted on-farm (i.e. possible commercial targets) and their extraction characteristics
- optimising PFR mechanical operations. For example, adding insulation to maintain the processing can at 37^{o}C (and hence increase adsorption), designing and optimizing the impeller blade to improve resin mixing and suspension during draining, developing a method to recover smaller resins (such as 90-µm SP FF) and other rigid resins (e.g. Capto™) with high adsorption characteristics
- optimise the adsorption process and overall yields by using resins that bind protein more quickly. This could be combined with processing milk only from individual cows that have high initial target protein concentration
- modelling the effects of other milk components (fat, protein, lactose) on the extraction process. These may be more pronounced when processing milk

from individual cows. Understanding and quantifying these effects will help optimise the adsorption process and hence increase overall yield

- evaluating other resins for on-farm protein extraction of from raw milk (e.g. Protein A media, special affinity media for IgA, IgM etc) and identifying cheaper resins. Resin cost used in the economic analyses was based on the expensive (~$1/mL), commercially-available resins manufactured for pharmaceutical use. Opportunity exists for developing robust, cheap, food safe resins, and activated ceramic membranes for specialist food applications
- investigating other suitable process options such as ion exchange membranes, activated filters, etc. and testing them on-farm. For example, the effect of membrane pore size and the cost for processing milk from individual cows needs to be investigated
- assessing the impact of this on-farm technology on transportation costs, which are high for dairy industry
- developing a value-chain and a business model for implementing on-farm processing
- extending this technology to situations such as recovering pharmaceuticals from fermentation broths

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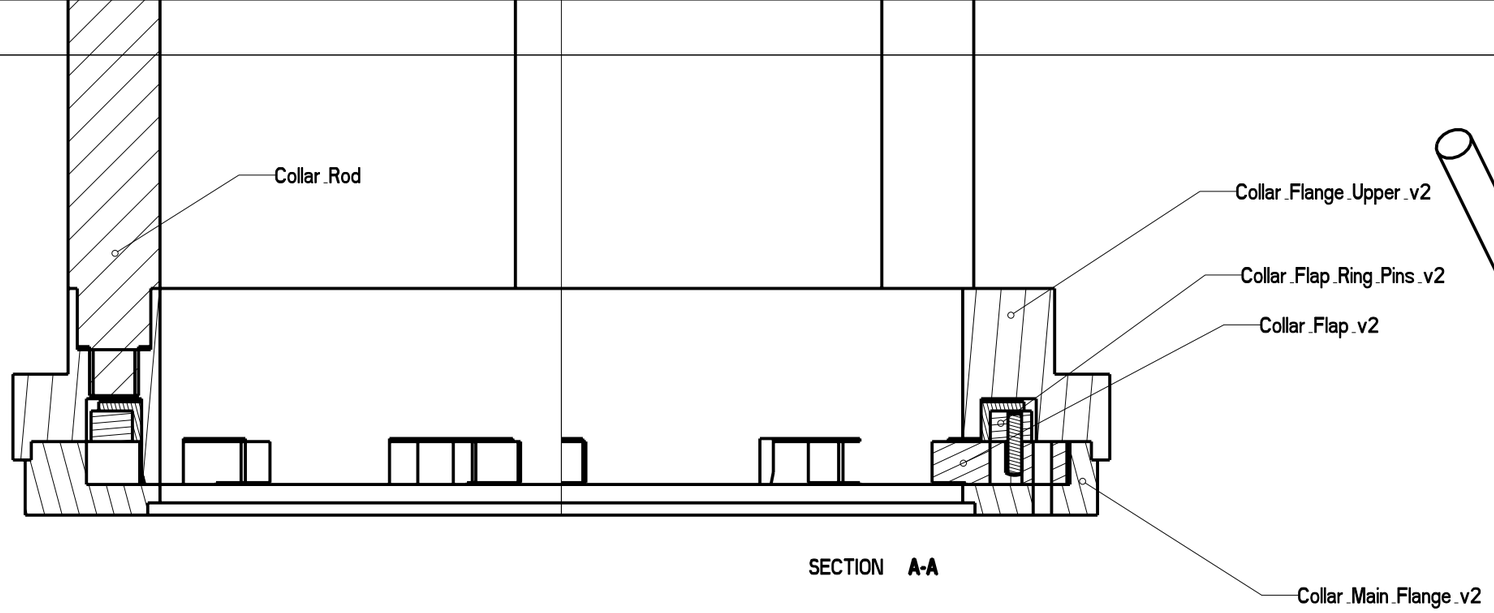
Appendix A

Detailed Engineering Drawings

By Florian Kern

The University of Waikato

2005



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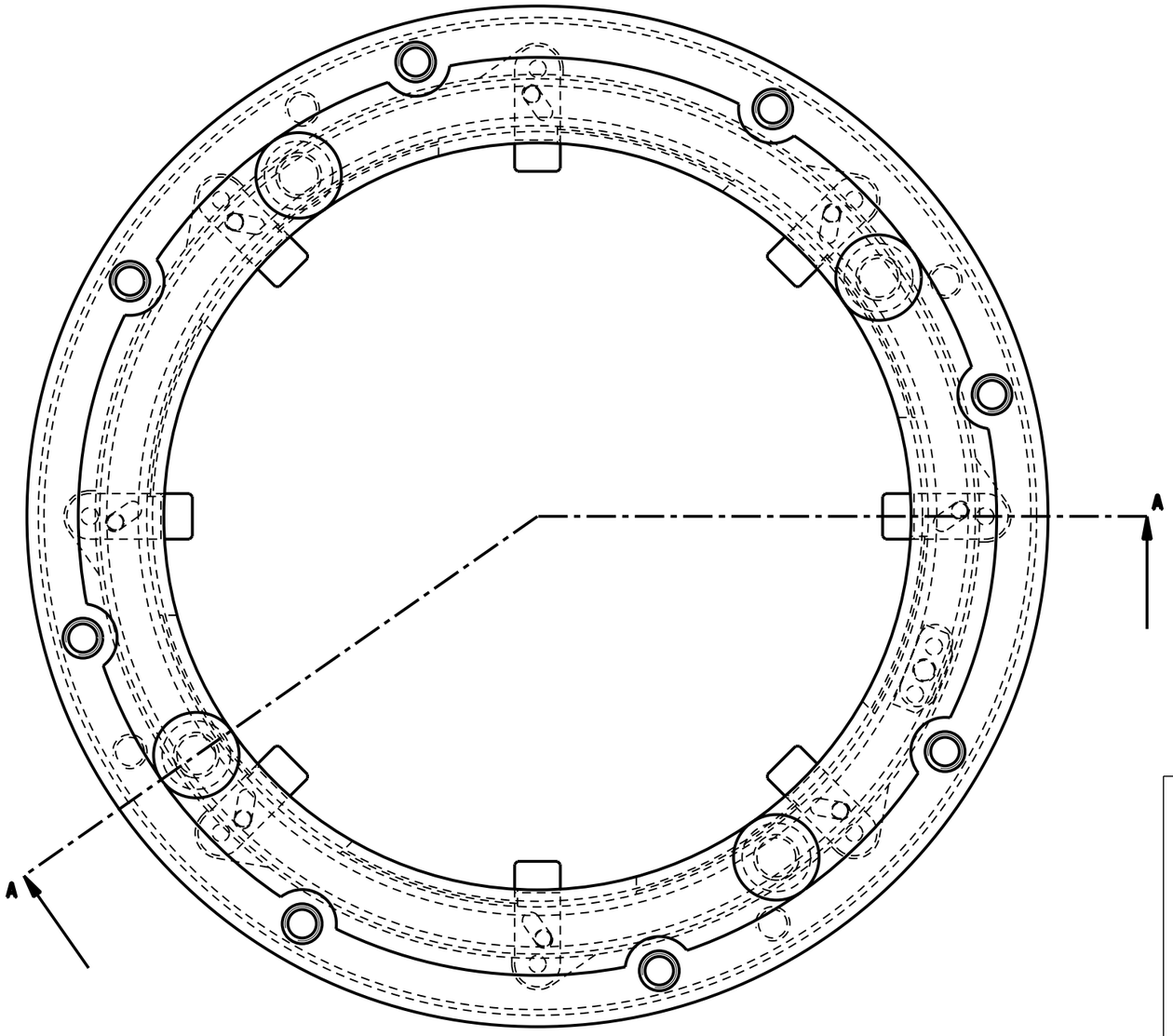

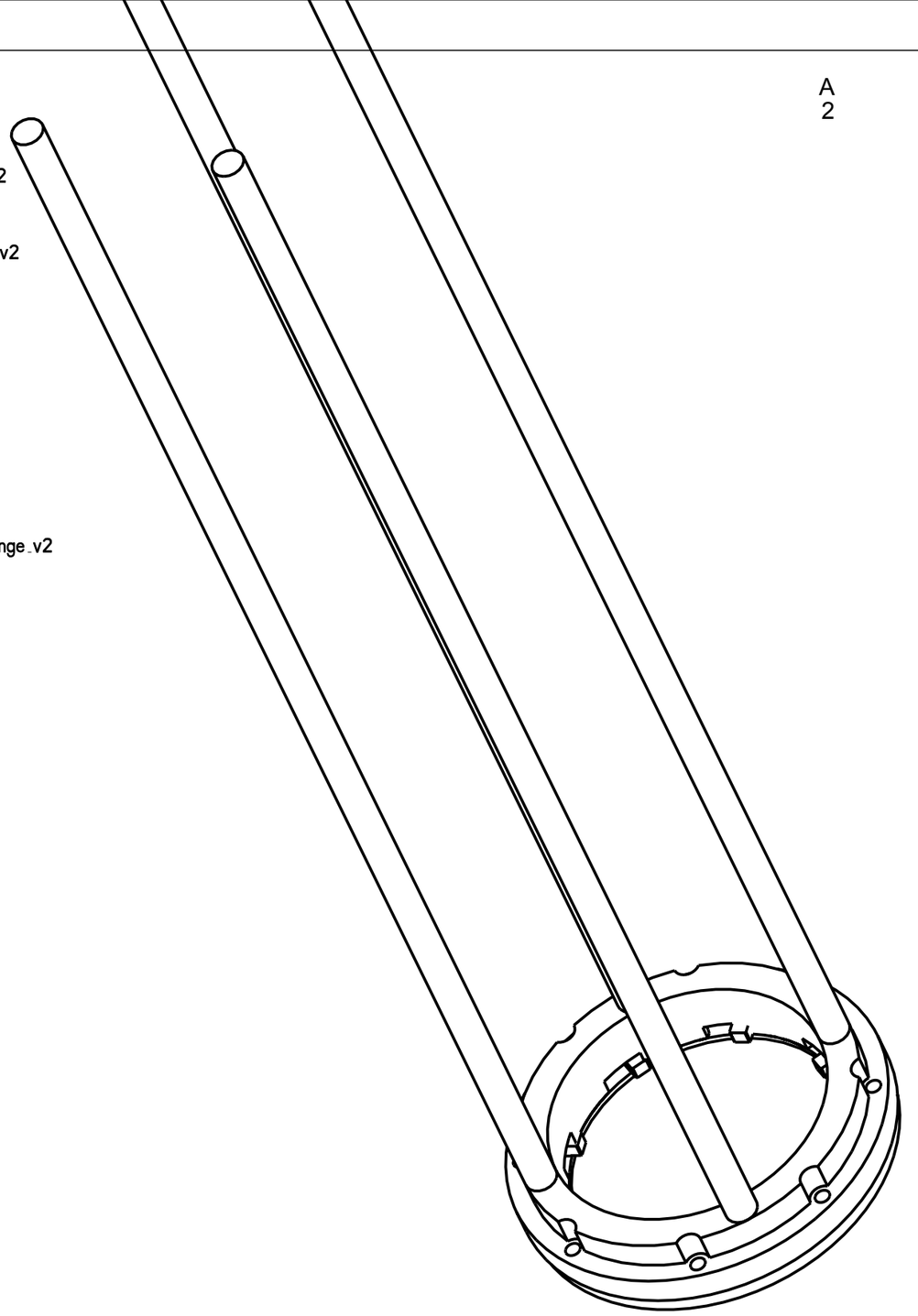

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REMOVE SHARP EDGES

UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

SURFACE ROUGHNESS ✓

ALL DIAMETERS TO BE UNLESS OTHERWISE STATED ⌀

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Model

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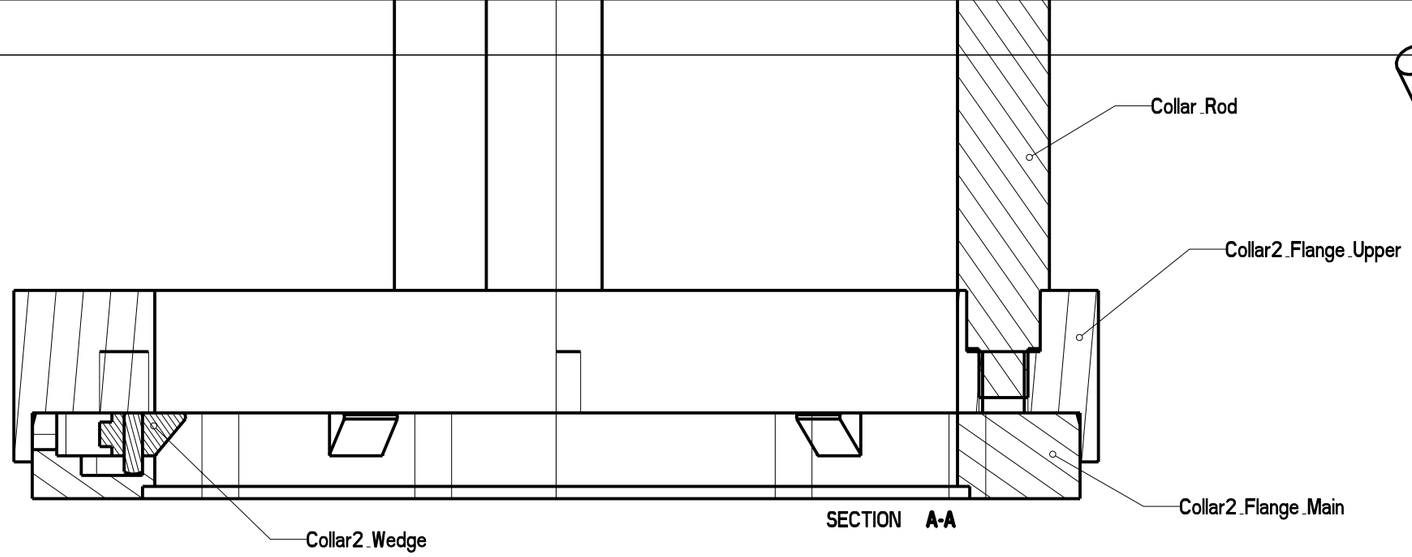

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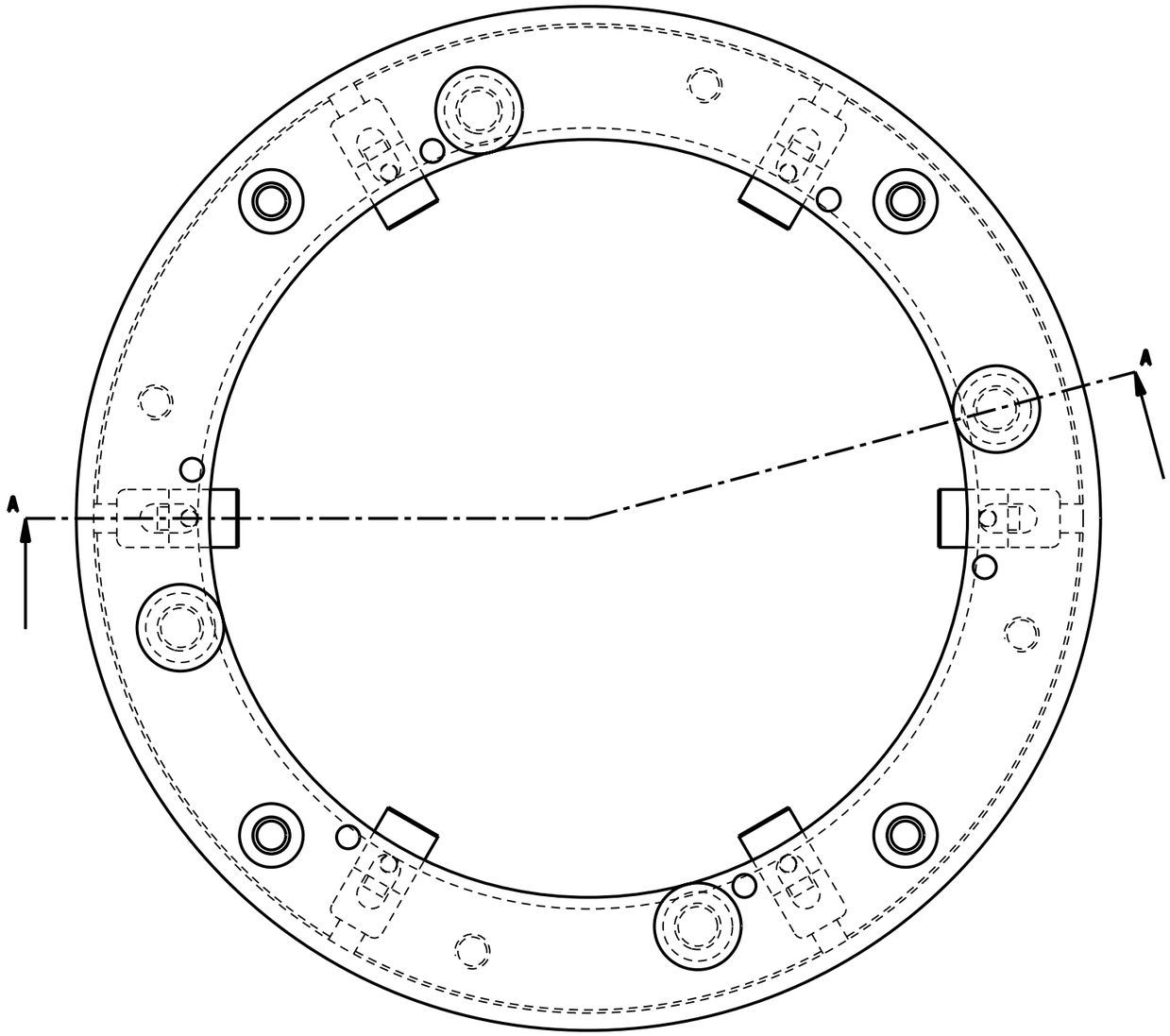

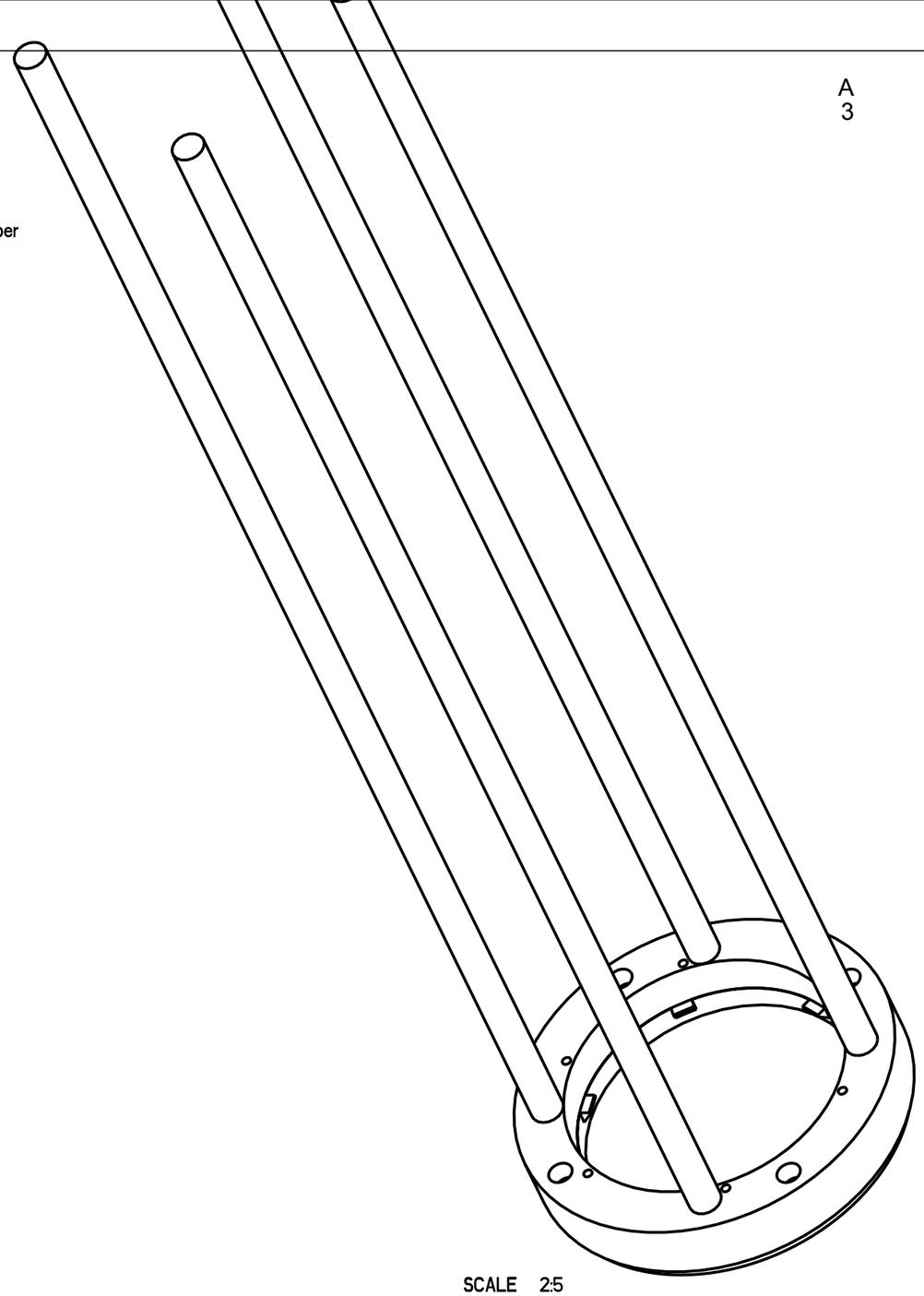

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DIMENSIONS IN MILLIMETRES (mm)
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UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

SURFACE ROUGHNESS ✓

ALL DIAMETERS TO BE UNLESS OTHERWISE STATED ⌀

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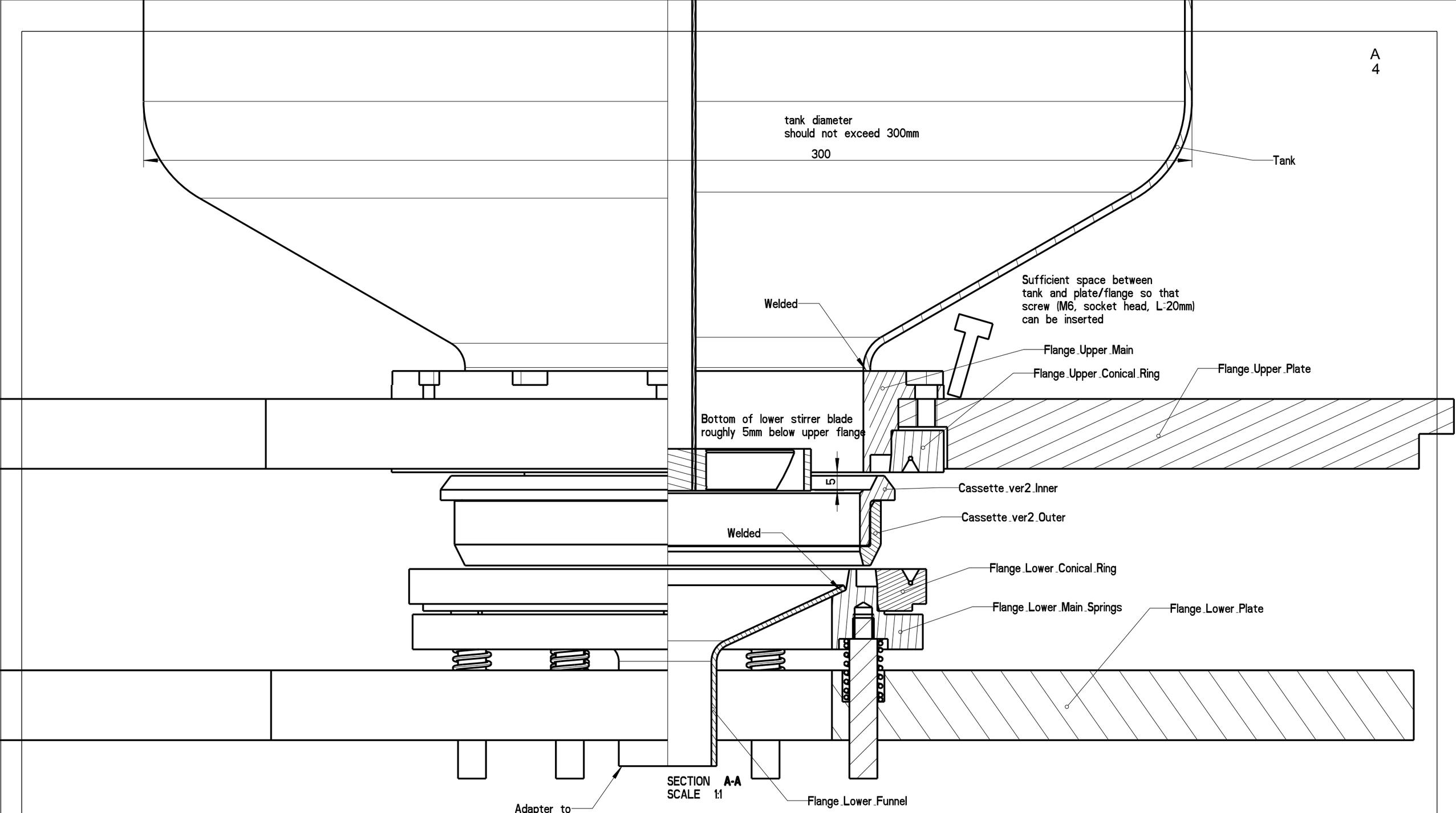

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UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

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ALL DIAMETERS TO BE
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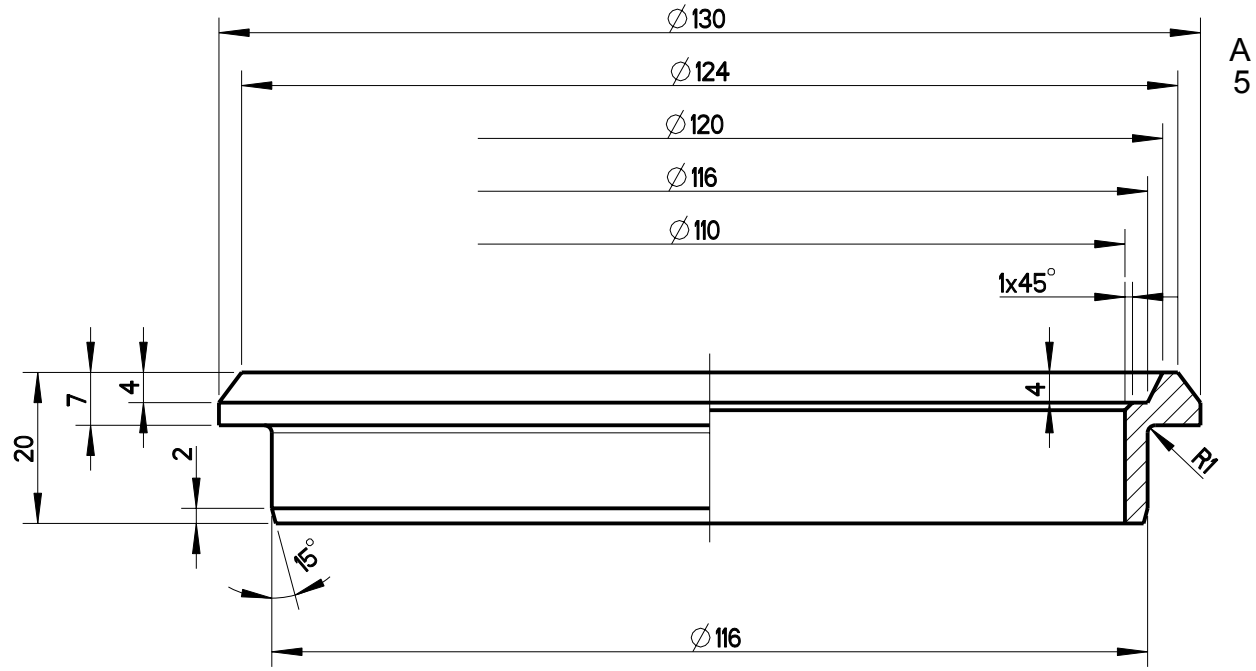

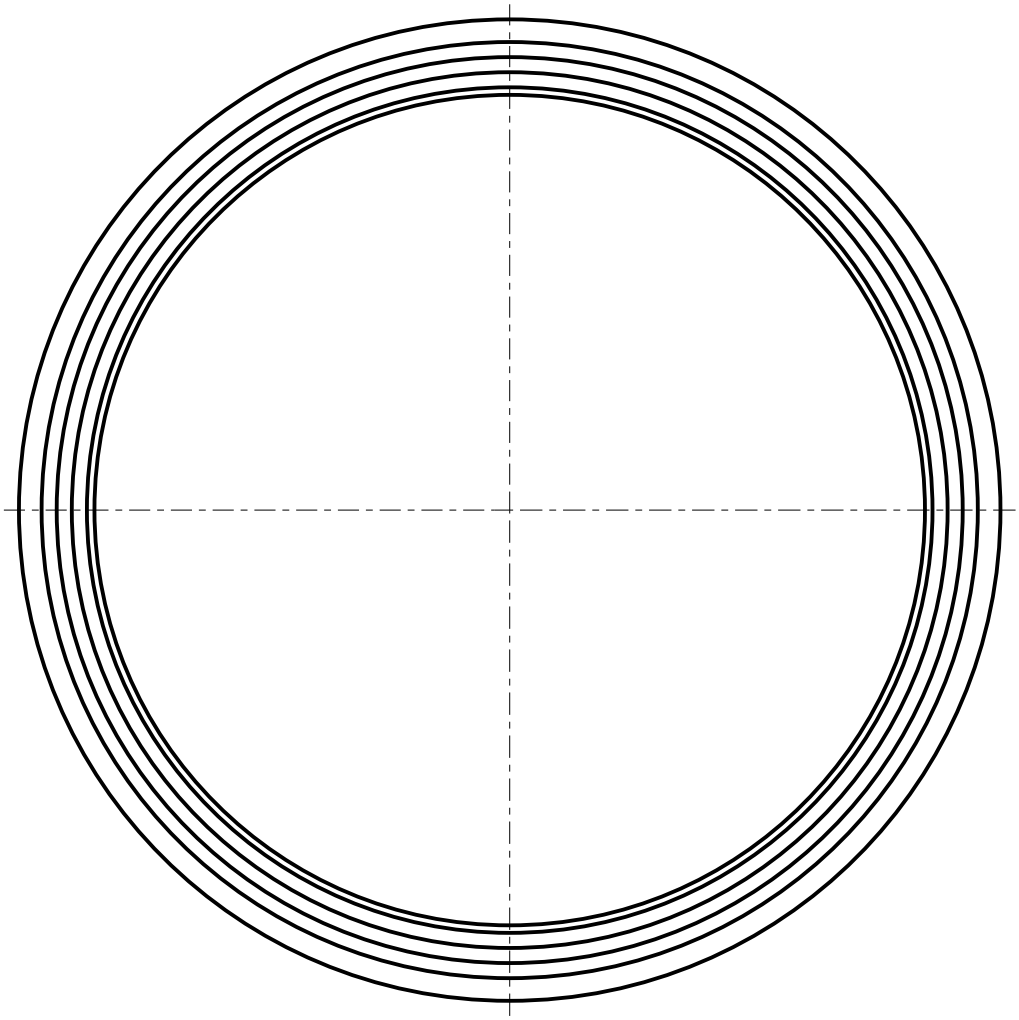

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UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

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ALL DIAMETERS TO BE UNLESS OTHERWISE STATED

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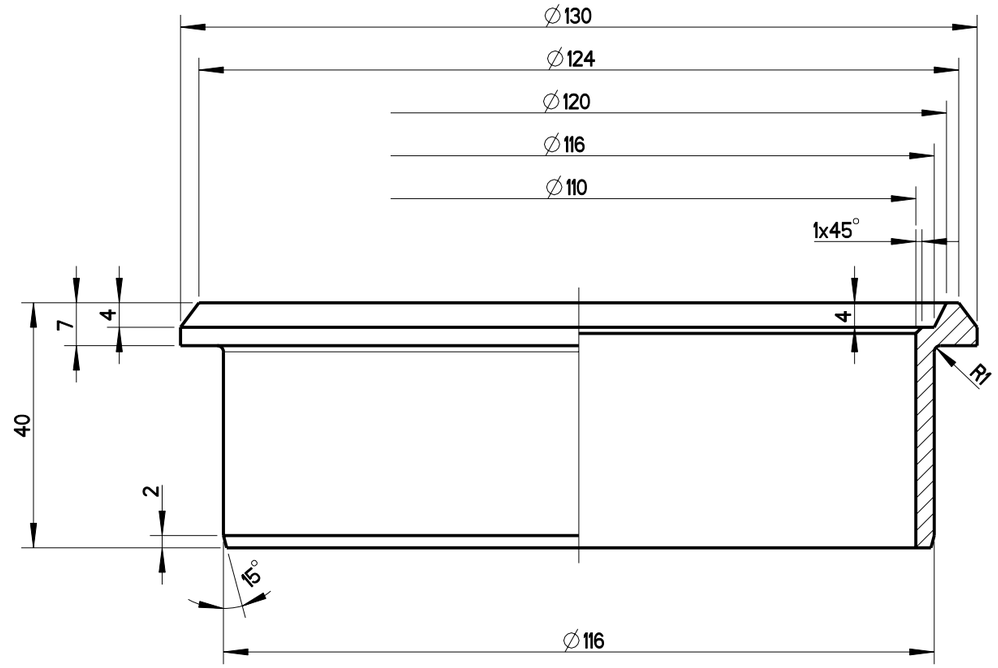

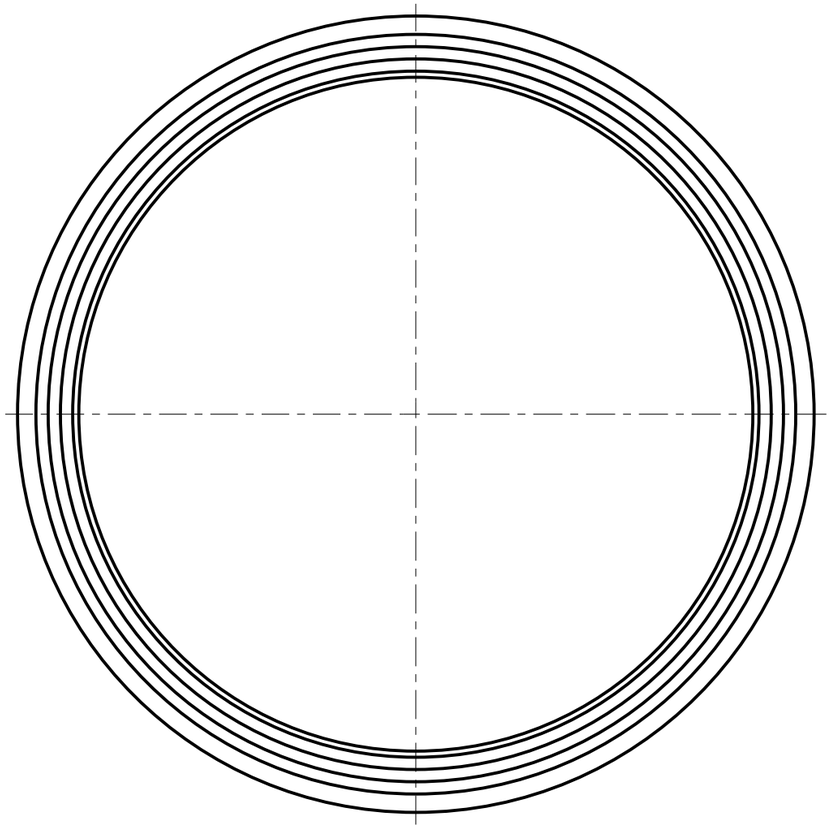

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UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

SURFACE ROUGHNESS

ALL DIAMETERS TO BE
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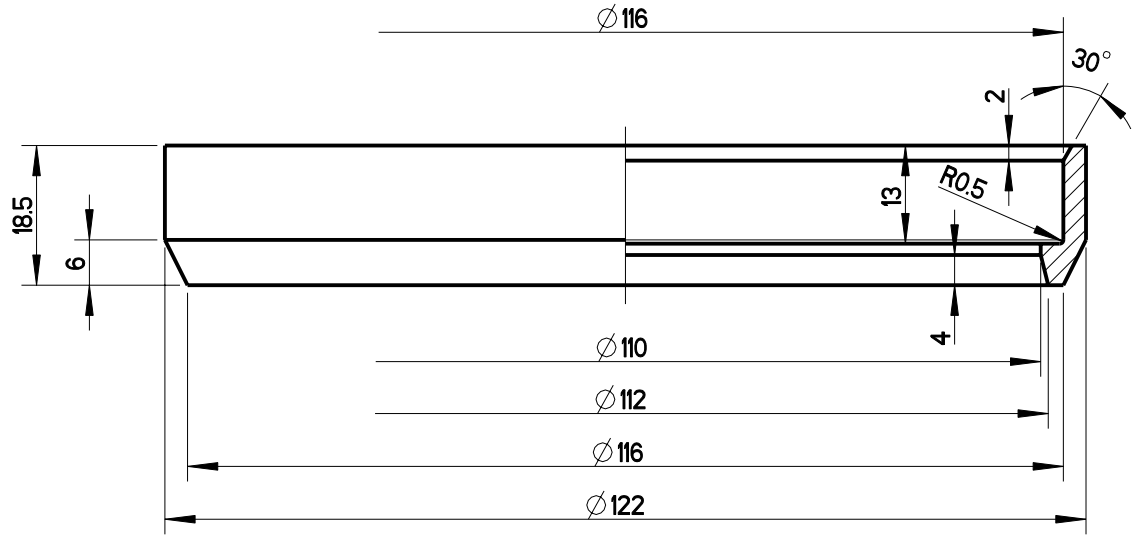

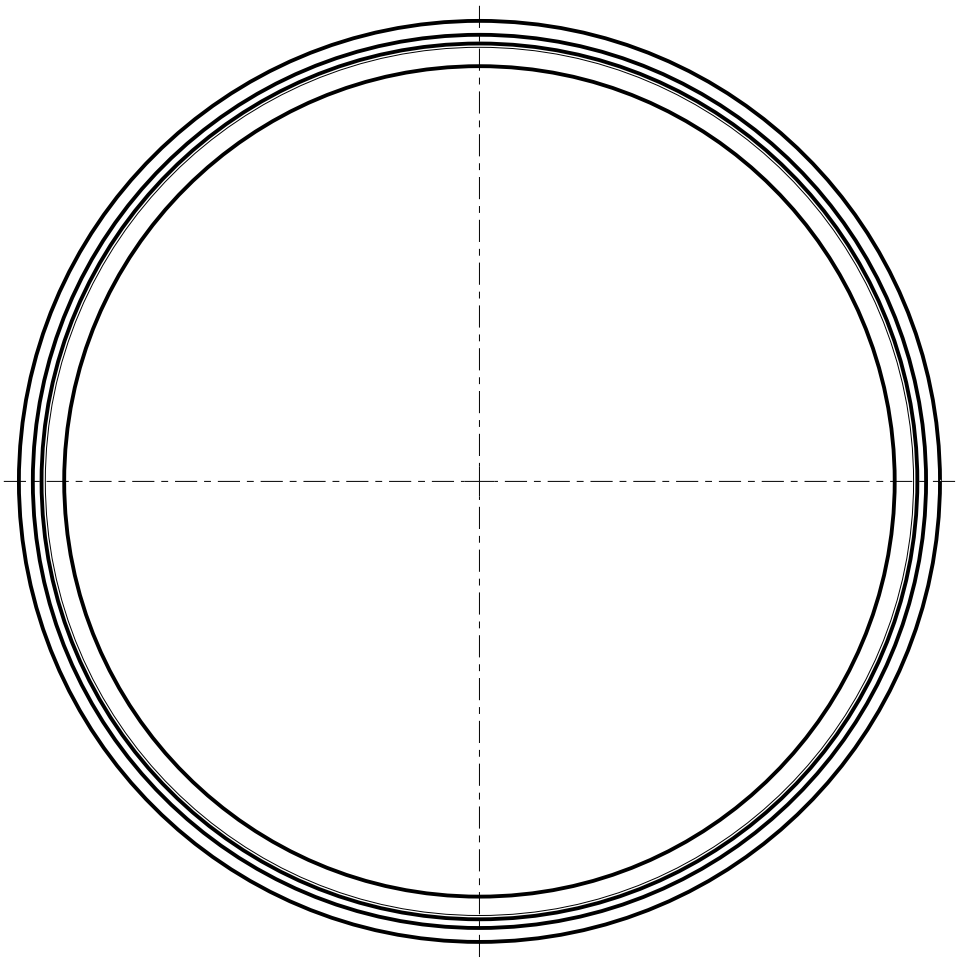

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UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

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ALL DIAMETERS TO BE
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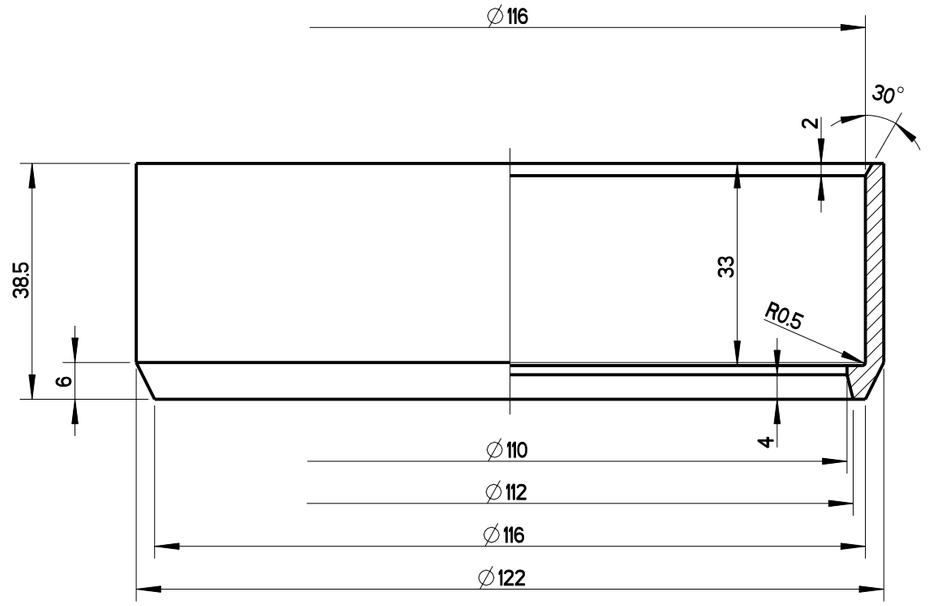

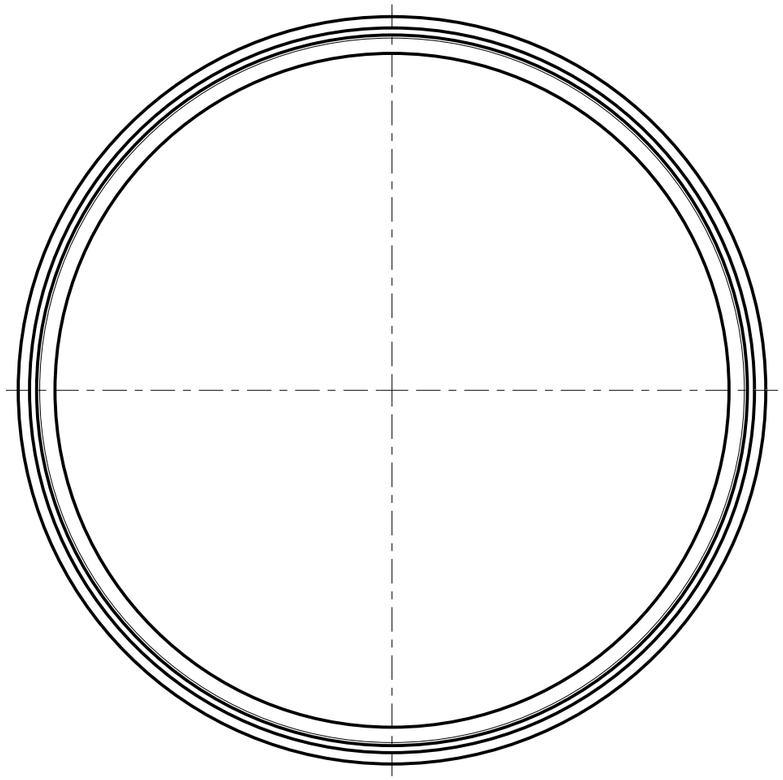

DIMENSIONS IN MILLIMETRES (mm)
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ALL DIAMETERS TO BE
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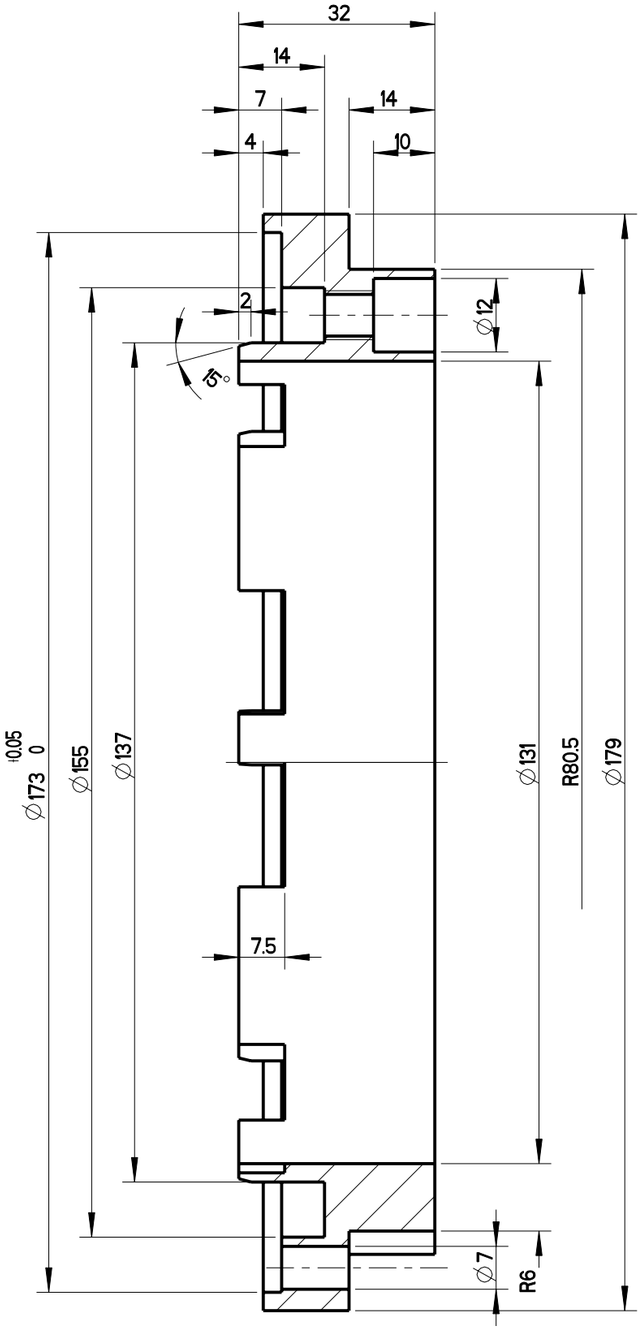

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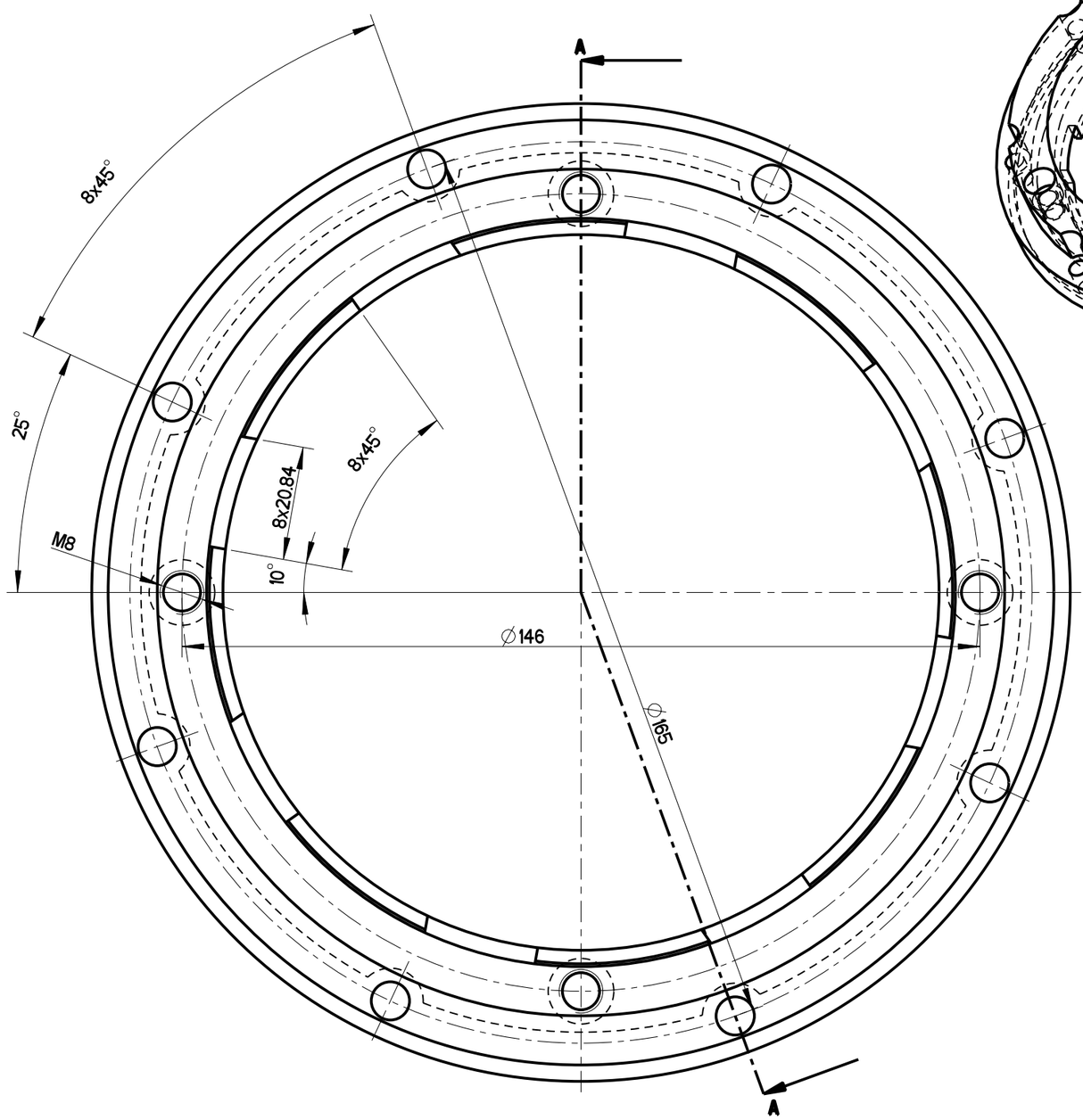

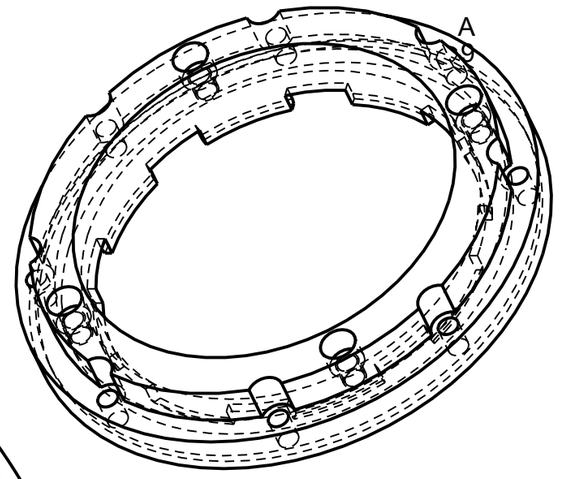

DIMENSIONS IN MILLIMETRES (mm)
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UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

SURFACE ROUGHNESS ✓

ALL DIAMETERS TO BE UNLESS OTHERWISE STATED Ø

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Scale 1:1 | Weight

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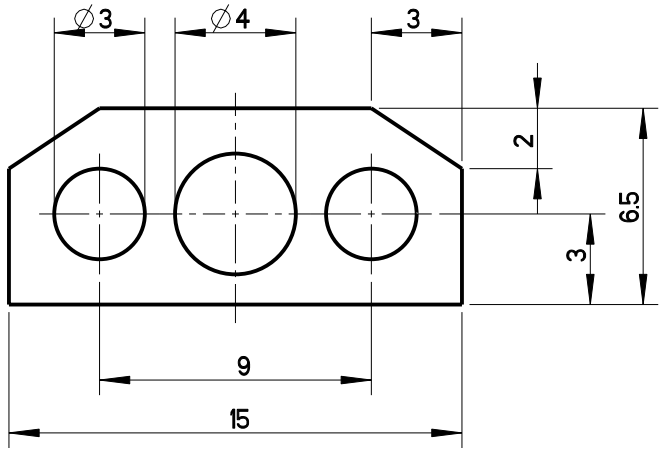

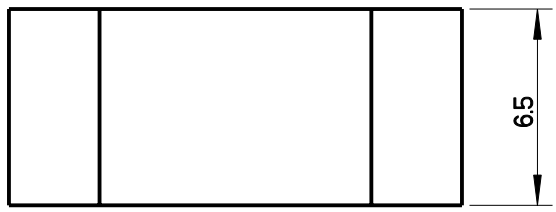

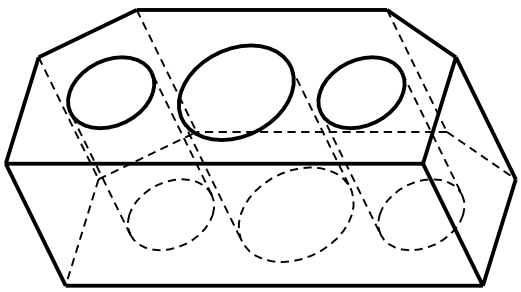

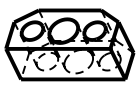

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Drawn	14-Feb-05	

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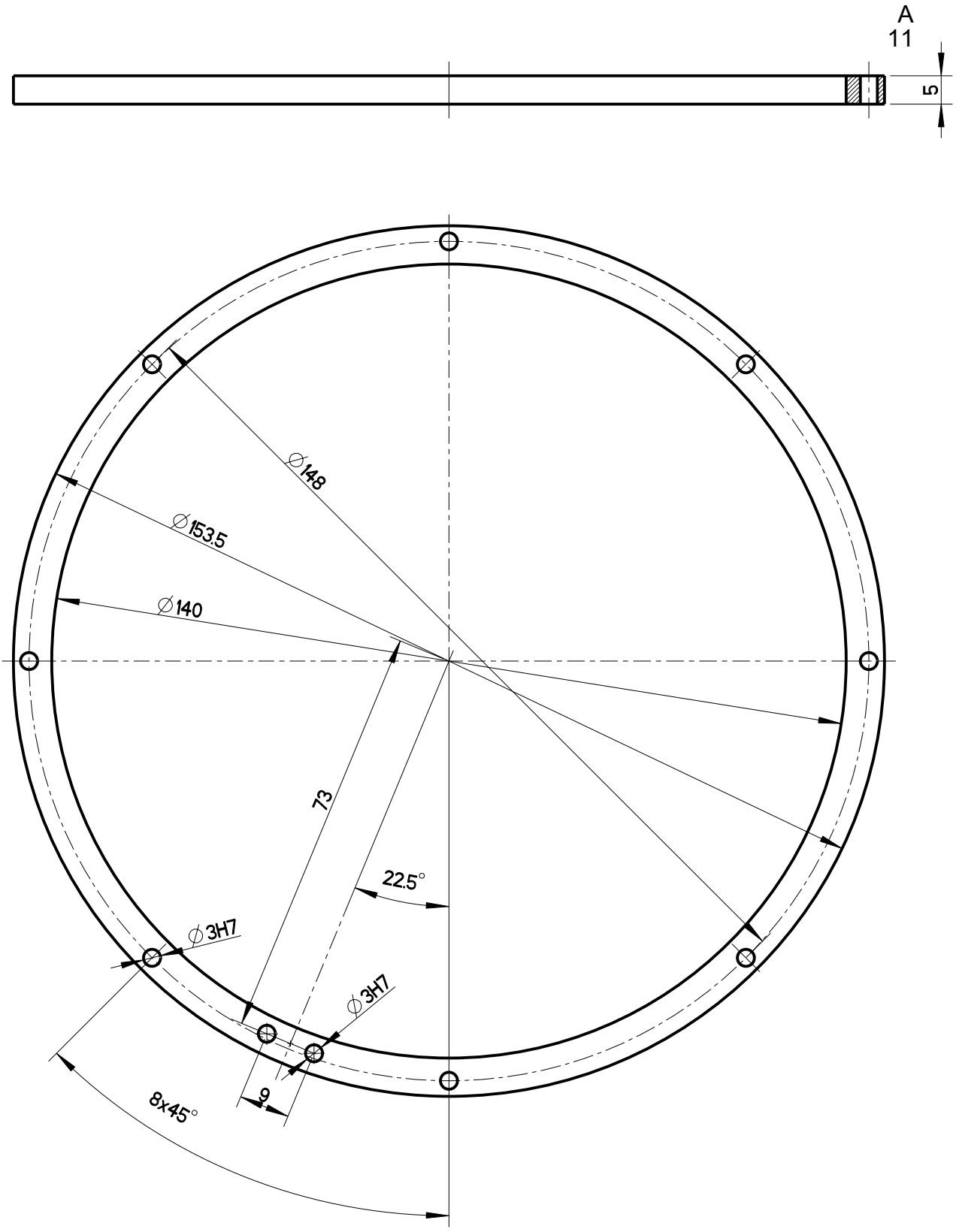

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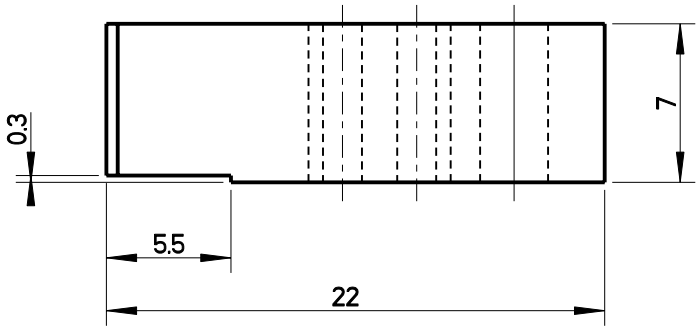

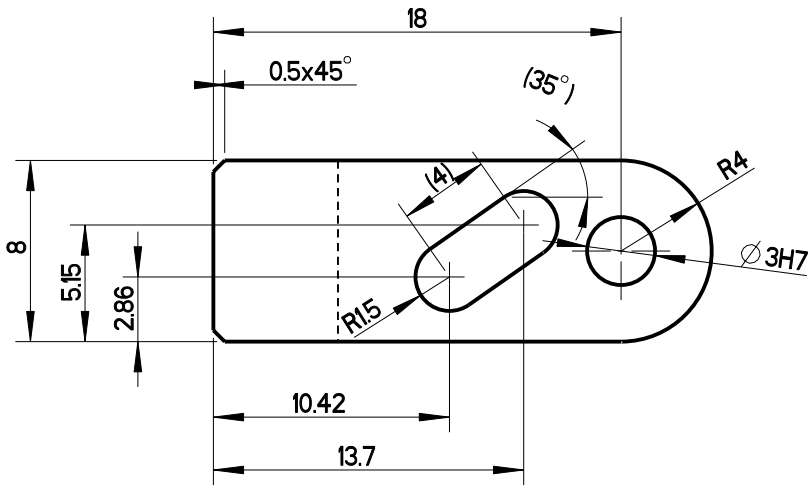

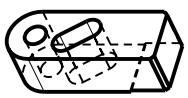

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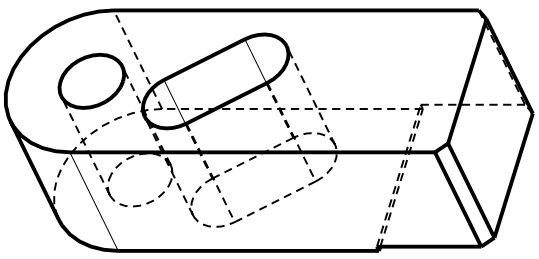

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UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

SURFACE ROUGHNESS ✓

ALL DIAMETERS TO BE UNLESS OTHERWISE STATED ⌀

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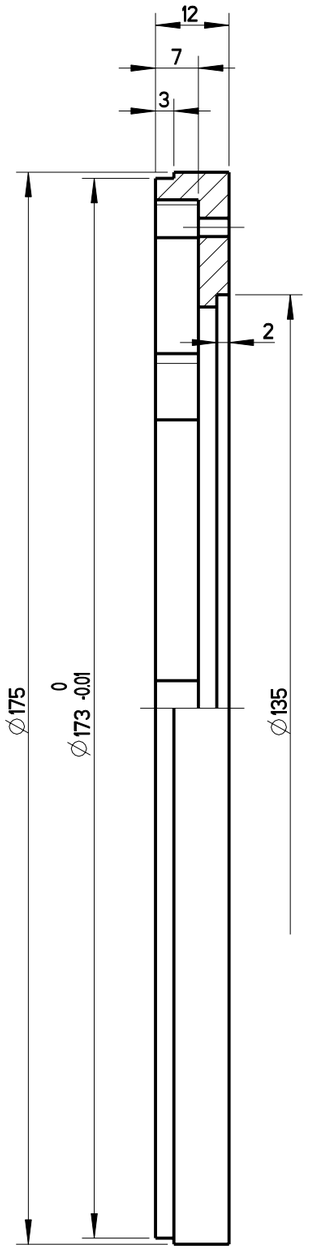

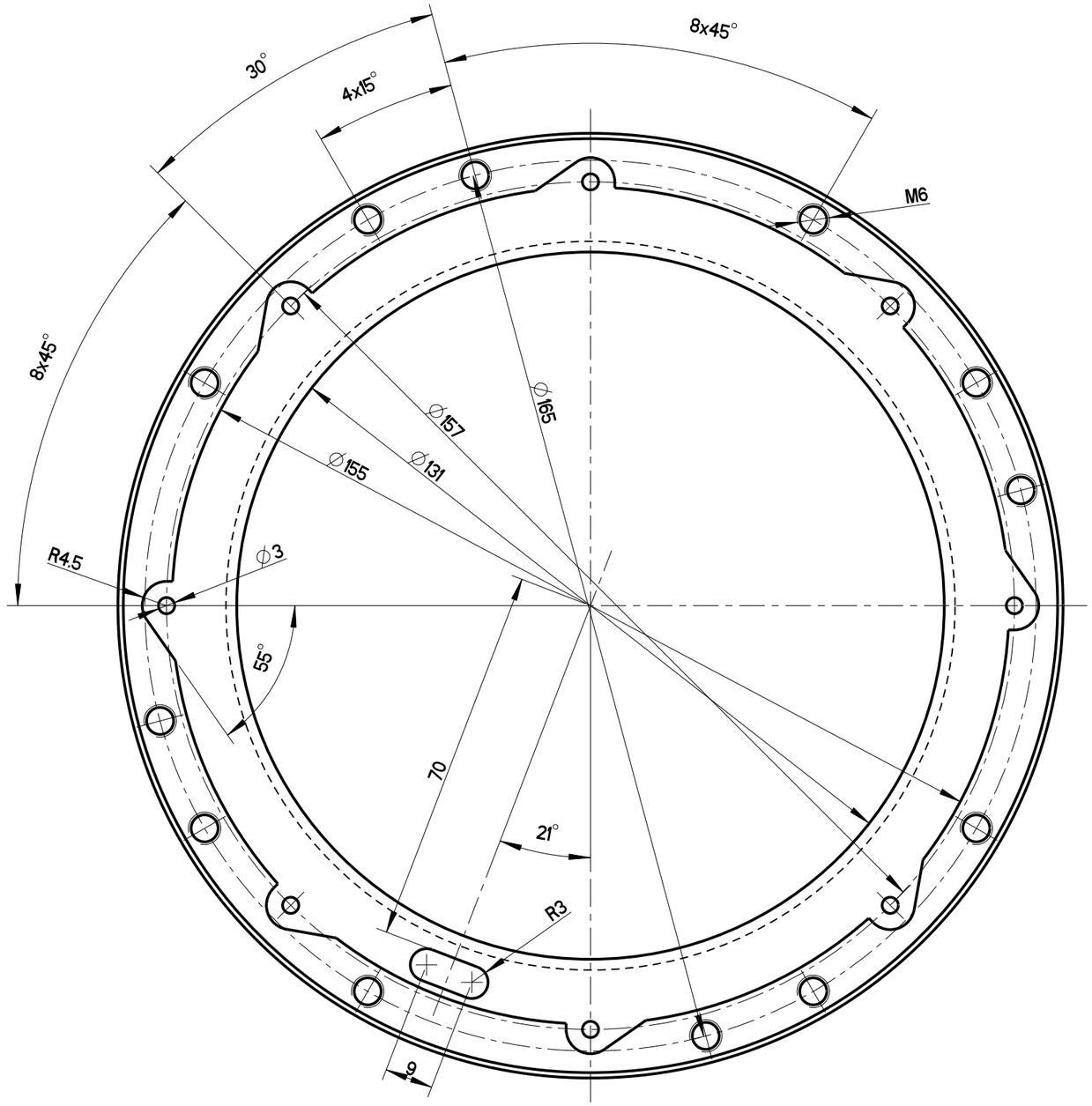

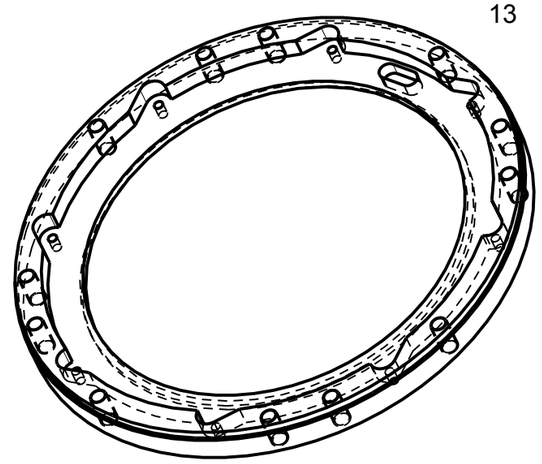

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SURFACE ROUGHNESS

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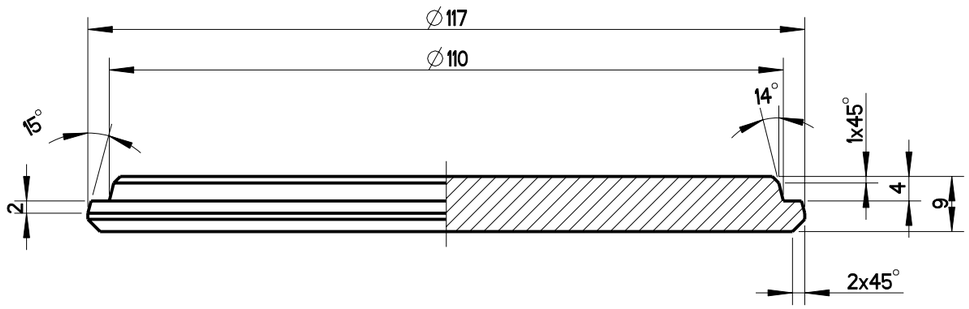

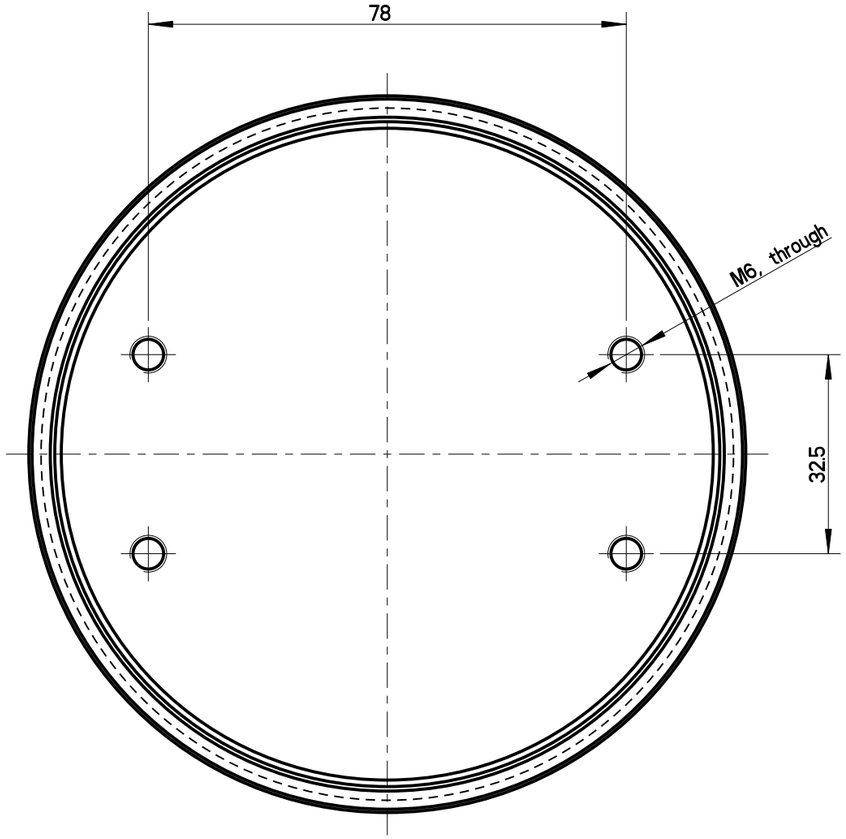

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ALL DIAMETERS TO BE
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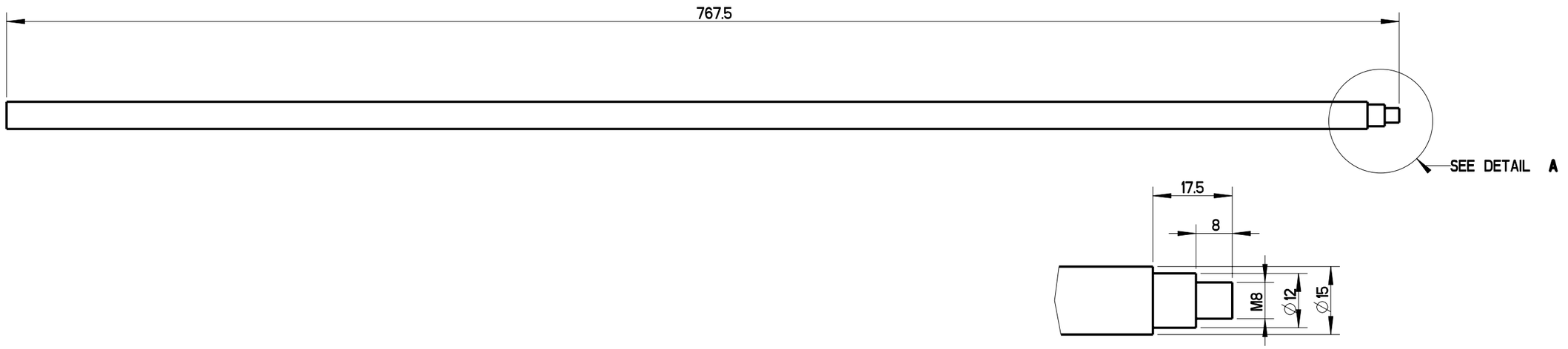

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SURFACE ROUGHNESS

ALL DIAMETERS TO BE UNLESS OTHERWISE STATED

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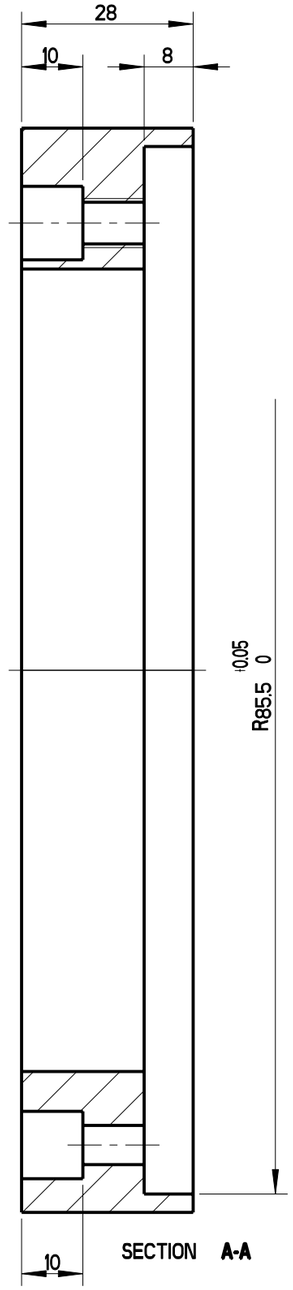

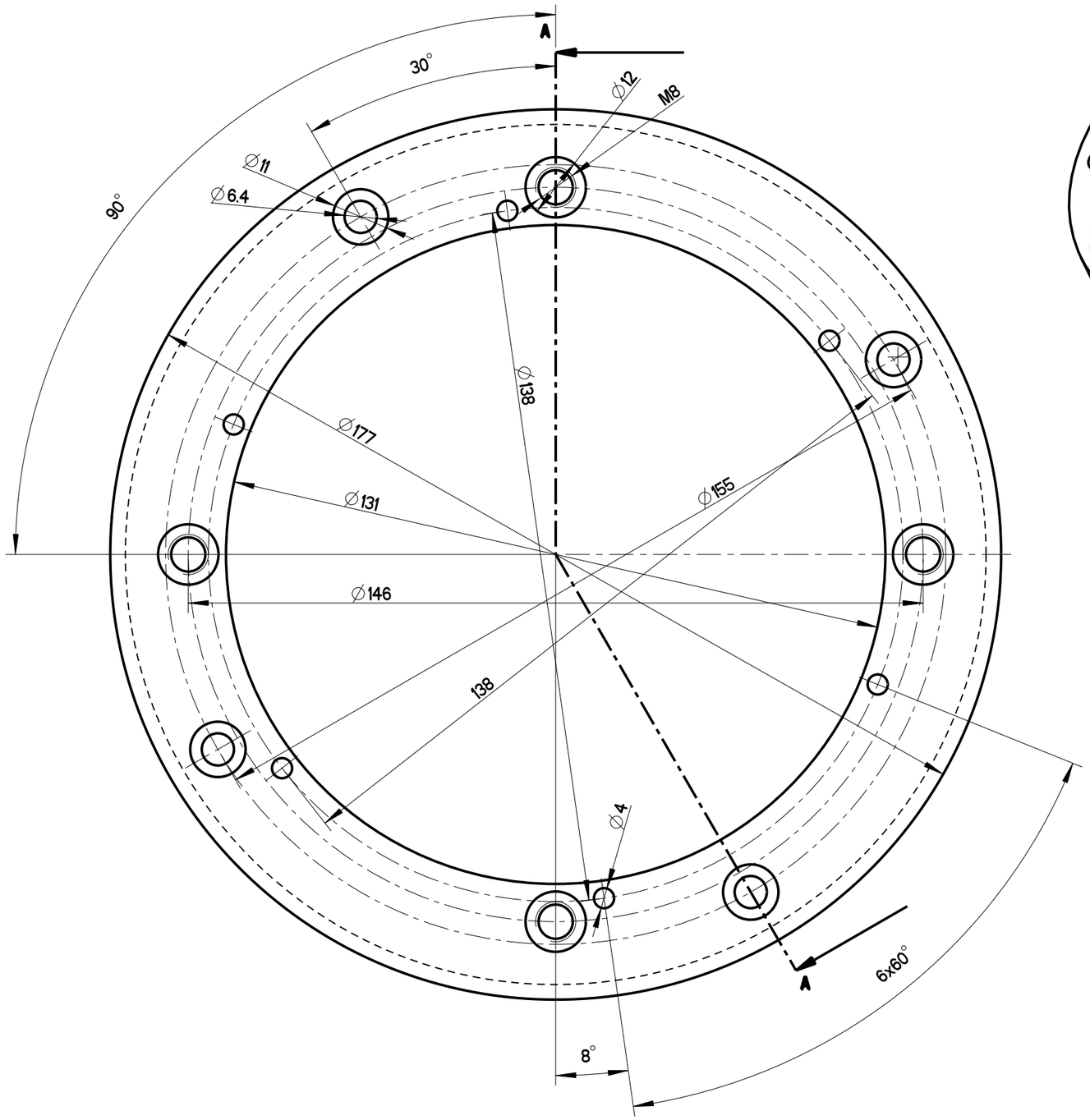

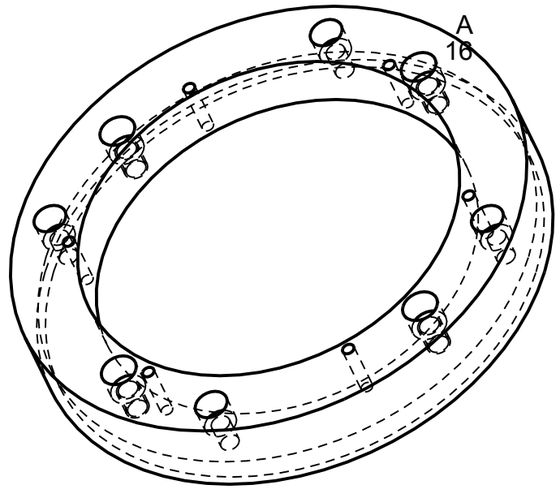

DIMENSIONS IN MILLIMETRES (mm)
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SURFACE ROUGHNESS

ALL DIAMETERS TO BE UNLESS OTHERWISE STATED

Drawn	Date	Name
	14-Feb-05	

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Model

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File: COLLAR2_FLANGE_UPPER

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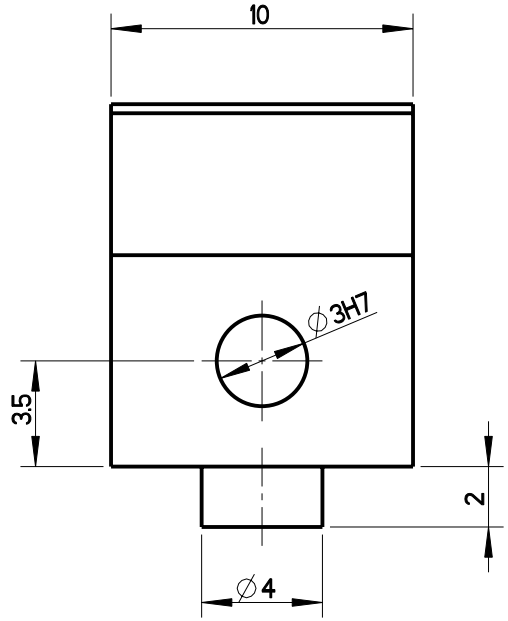

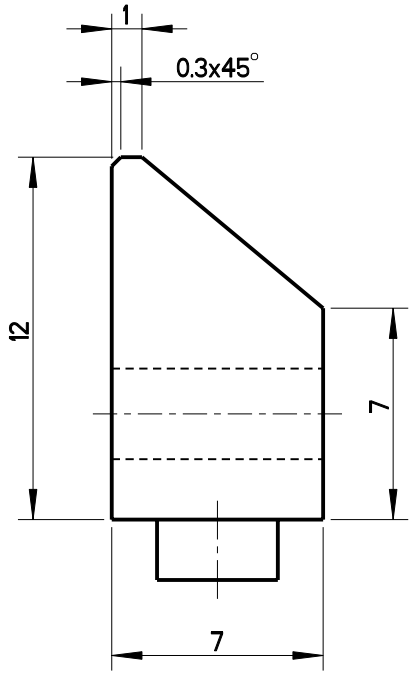

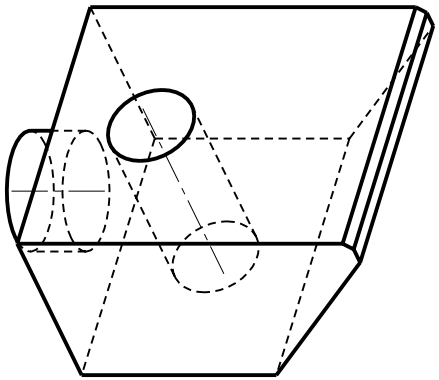

TRUE SCALE 1:1

DIMENSIONS IN MILLIMETRES (mm)
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UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

SURFACE ROUGHNESS ✓

ALL DIAMETERS TO BE
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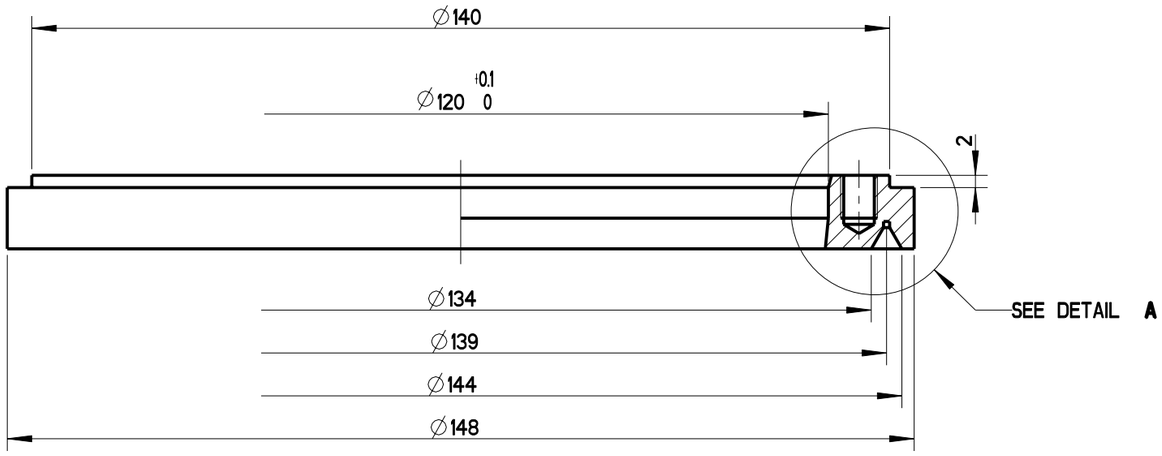
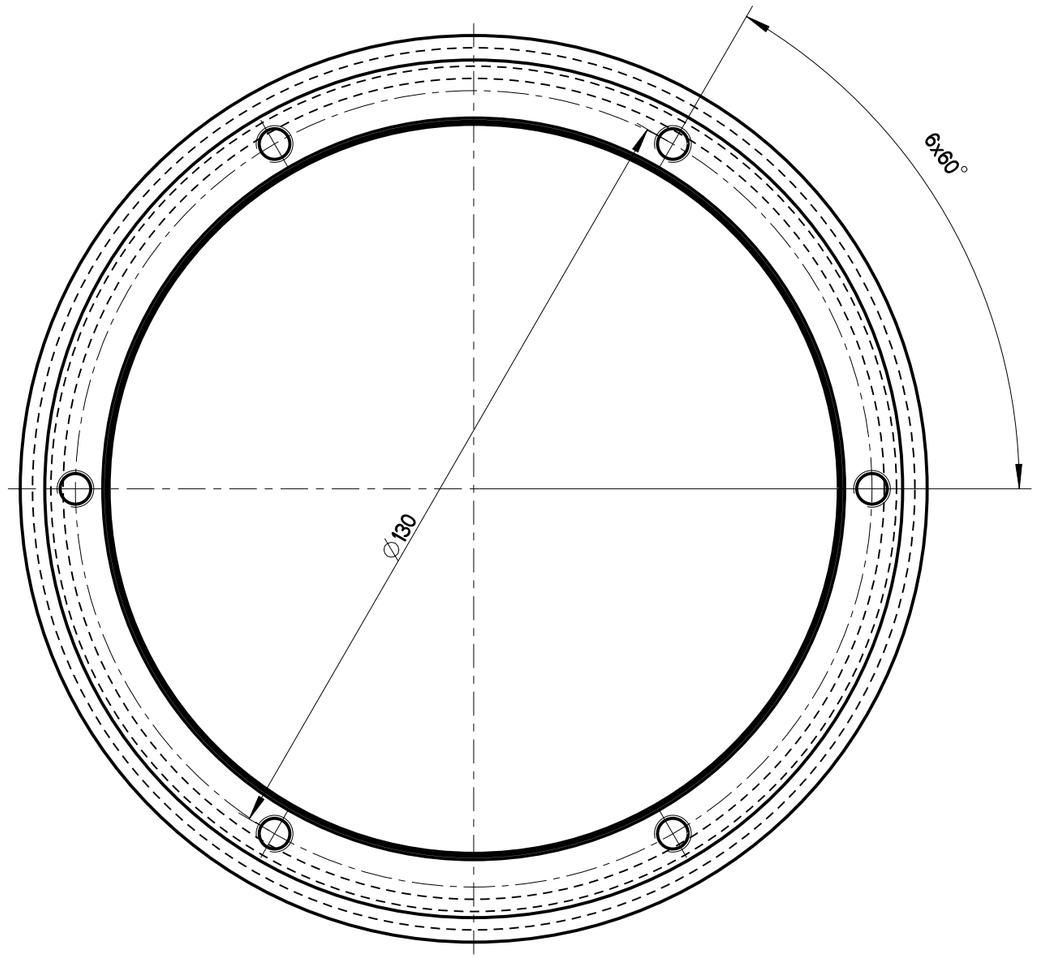
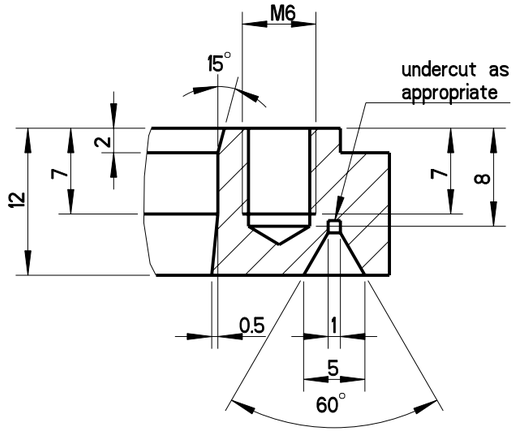
Sheets 1





DETAIL A
SCALE 2:1



DIMENSIONS IN MILLIMETRES (mm)
REMOVE SHARP EDGES

UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

SURFACE ROUGHNESS ✓

ALL DIAMETERS TO BE UNLESS OTHERWISE STATED ⌀

Drawn	Date	Name
	14-Feb-05	

Scale 1:1 | Weight

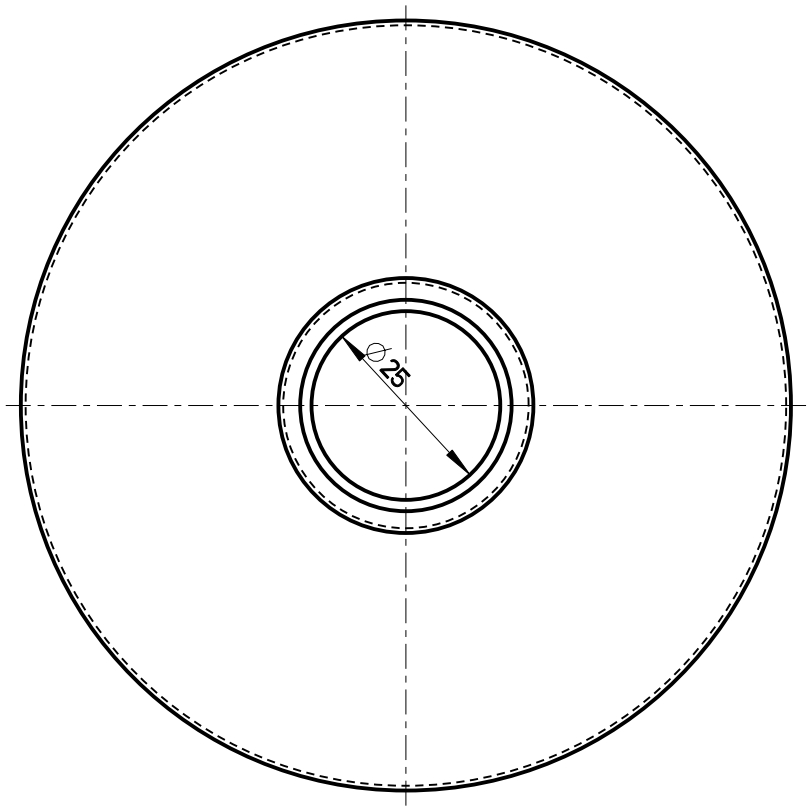
Material: Stainless 304

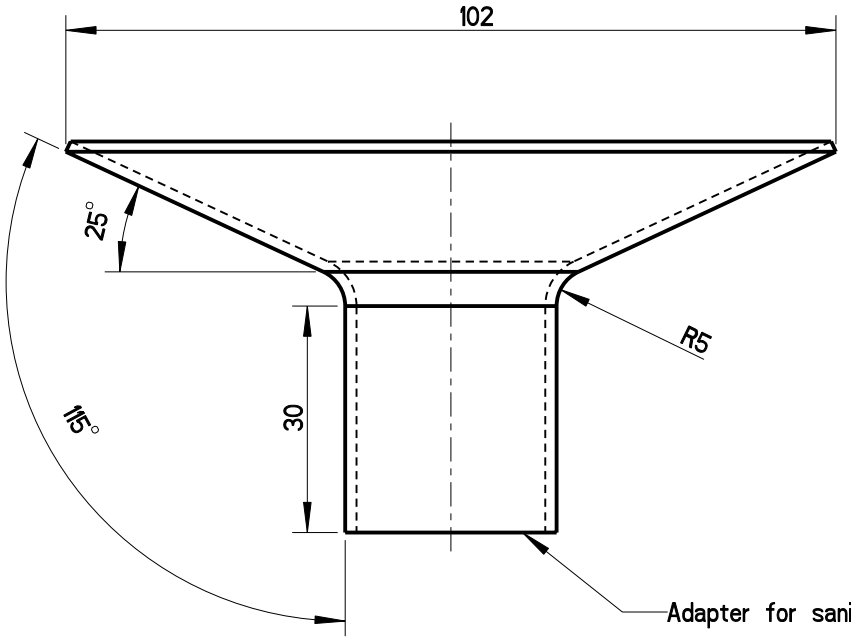
Model: FLANGE_LOWER_CONICAL_RING_V5

File: FLANGE_LOWER_CONICAL_RING_V5

Sheet 1
Sheets 1

A
19





Sheet thickness
circa 1.5mm

Adapter for sanitary clamp welded on

DIMENSIONS IN MILLIMETRES (mm)
REMOVE SHARP EDGES

UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

SURFACE ROUGHNESS

ALL DIAMETERS TO BE
UNLESS OTHERWISE STATED

	Date	Name
Drawn	14-Feb-05	

Scale 1:1 | Weight

Material: Stainless 304

Model

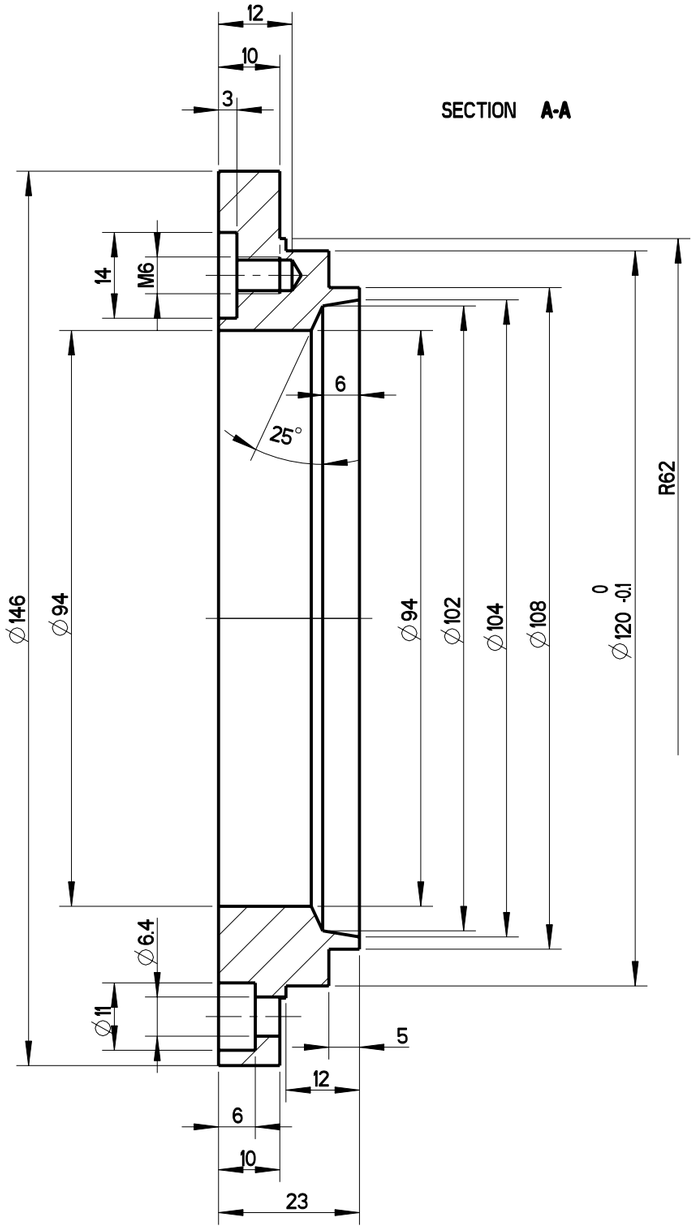
FLANGE_LOWER_FUNNEL

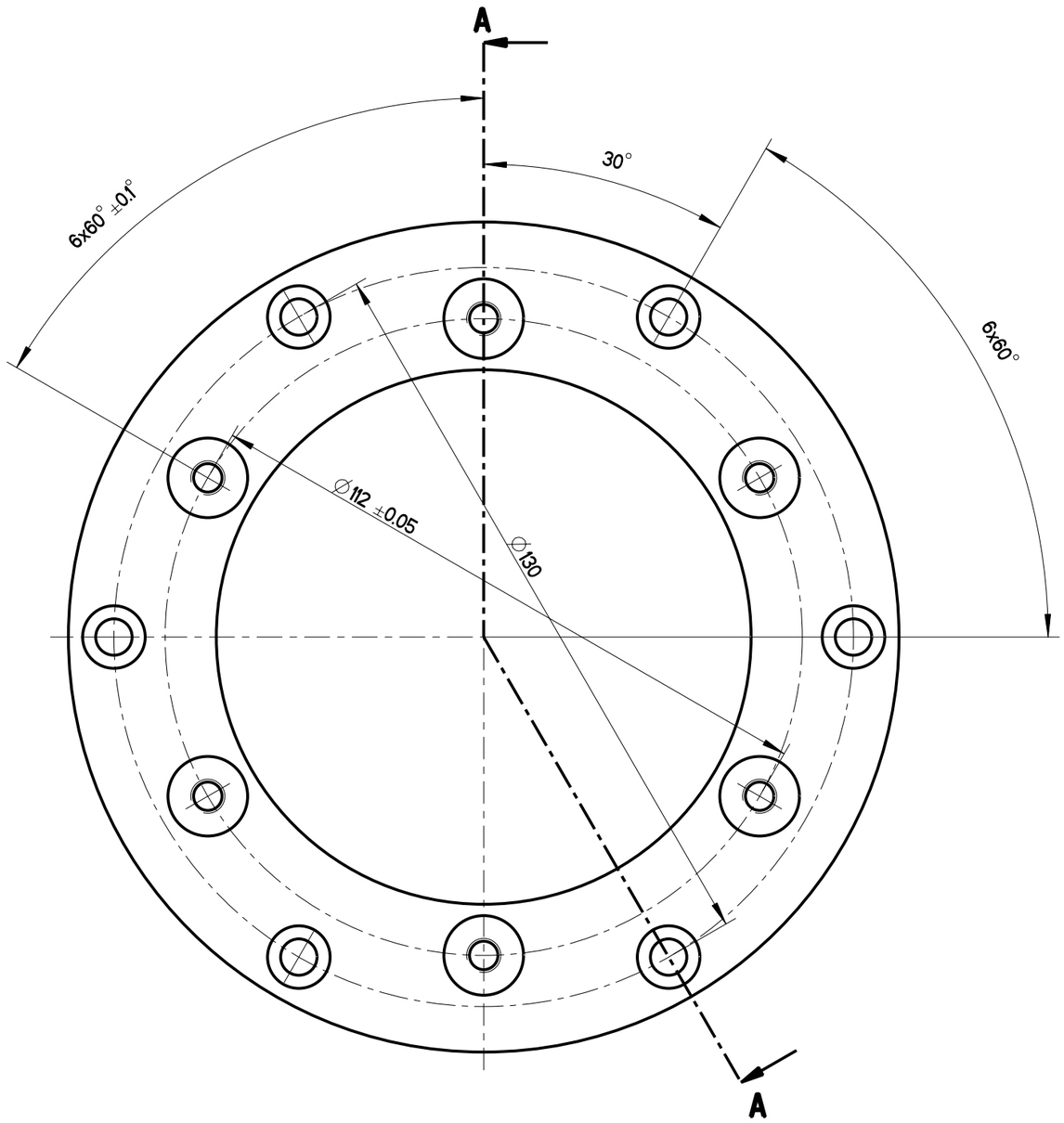
File: FLANGE_LOWER_FUNNEL

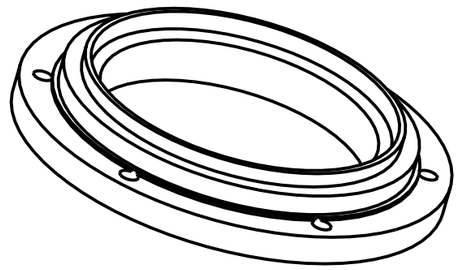
Sheet 1
Sheets 1

A
20

SECTION A-A







SCALE 1:2

DIMENSIONS IN MILLIMETRES (mm)
REMOVE SHARP EDGES

UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

SURFACE ROUGHNESS ✓

ALL DIAMETERS TO BE UNLESS OTHERWISE STATED ∅

Drawn	Date	Name
	14-Feb-05	

Scale 1:1 | Weight

Material: Stainless 304

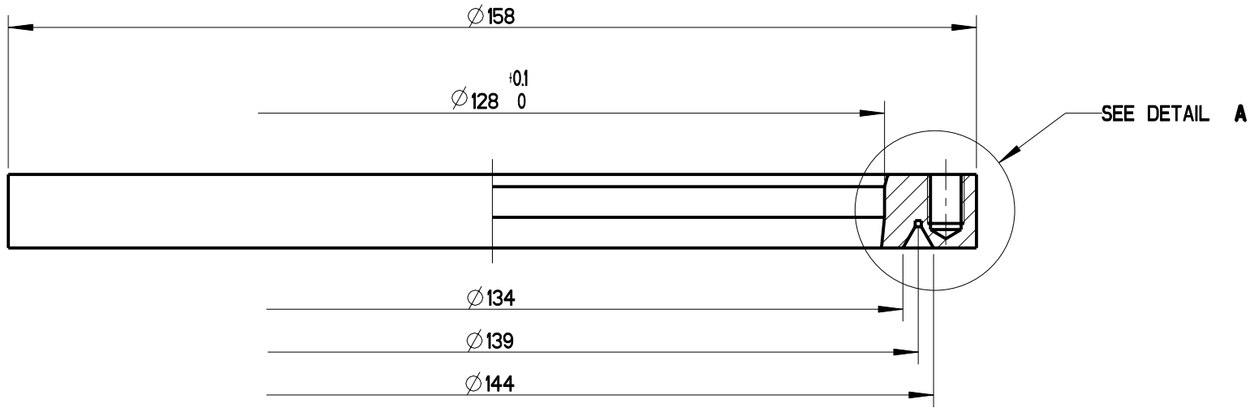
Model: FLANGE_LOWER_MAIN_SPRING_V5

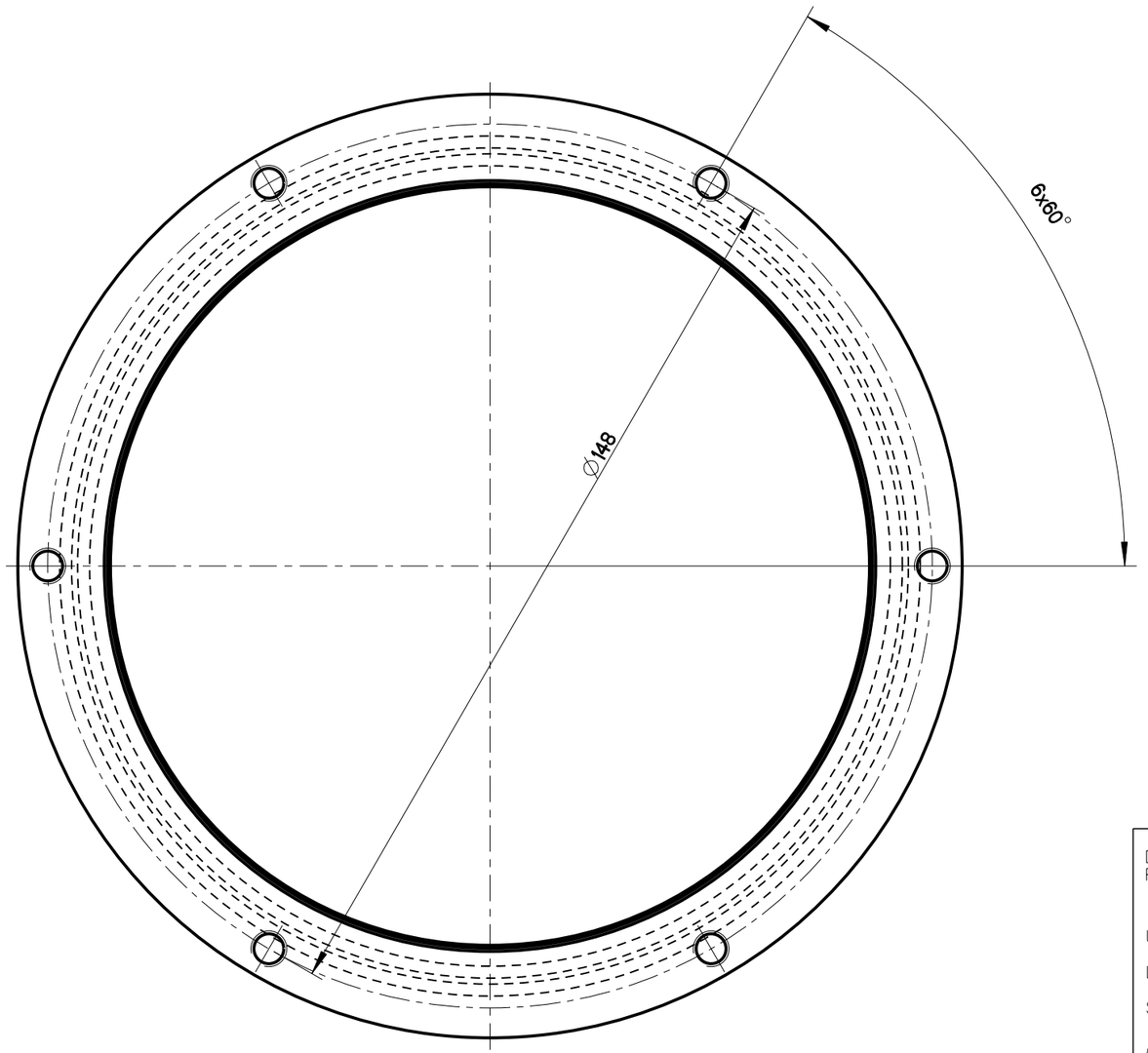
File: FLANGE_LOWER_MAIN_SPRING_V5

Sheet 1

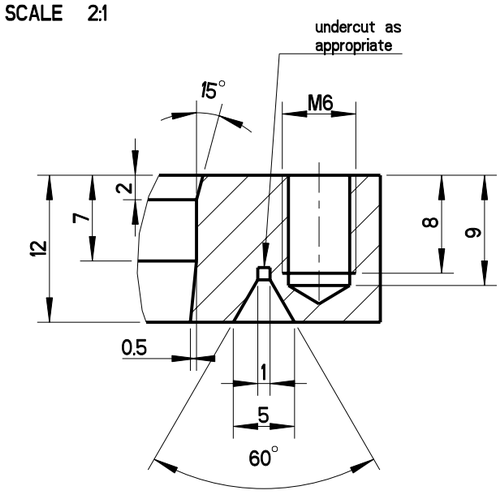
Sheets 1

A
21





DETAIL A
SCALE 2:1



DIMENSIONS IN MILLIMETRES (mm)
REMOVE SHARP EDGES

UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

SURFACE ROUGHNESS

ALL DIAMETERS TO BE UNLESS OTHERWISE STATED

Drawn	Date	Name
	14-Feb-05	

Scale 1:1 | Weight

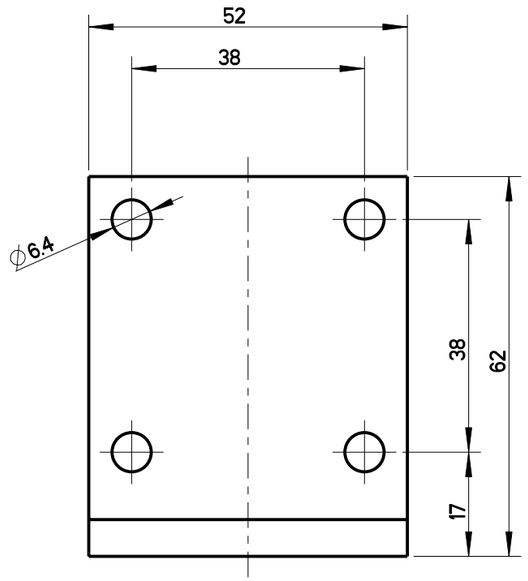
Material: Stainless 304

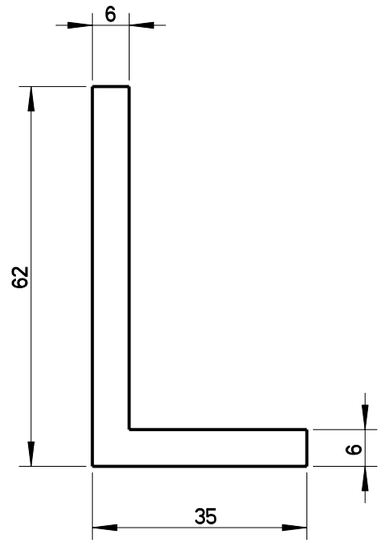
Model: FLANGE_UPPER_CONICAL_RING_V5

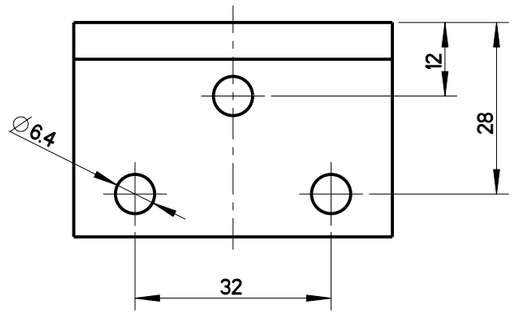
File: FLANGE_UPPER_CONICAL_RING

Sheet 1
Sheets 1

A
22







DIMENSIONS IN MILLIMETRES (mm)
REMOVE SHARP EDGES

UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

SURFACE ROUGHNESS

ALL DIAMETERS TO BE UNLESS OTHERWISE STATED

Drawn	Date	Name
	14-Feb-05	

Scale 1:1 | Weight

Material: Stainless 304

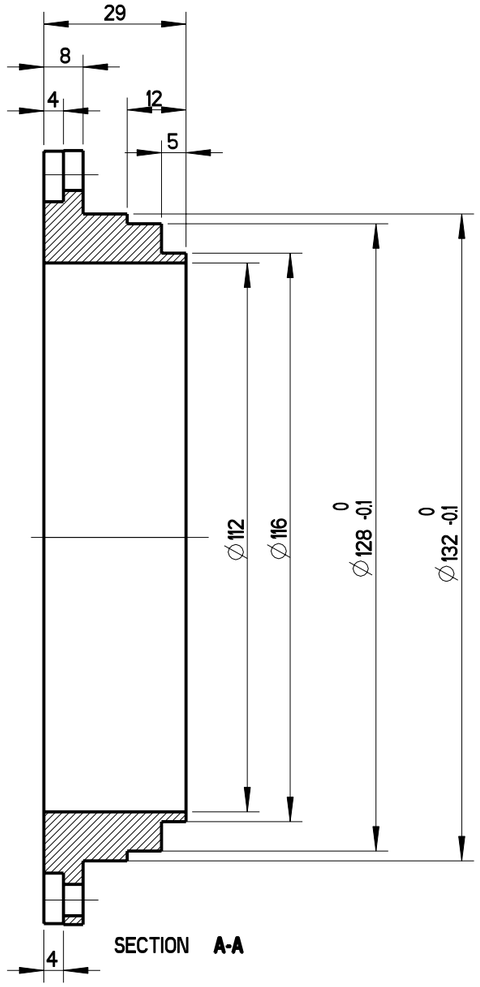
Model: FLANGE_UPPER_CYL_MOUNT

File: FLANGE_UPPER_CYL_MOUNT

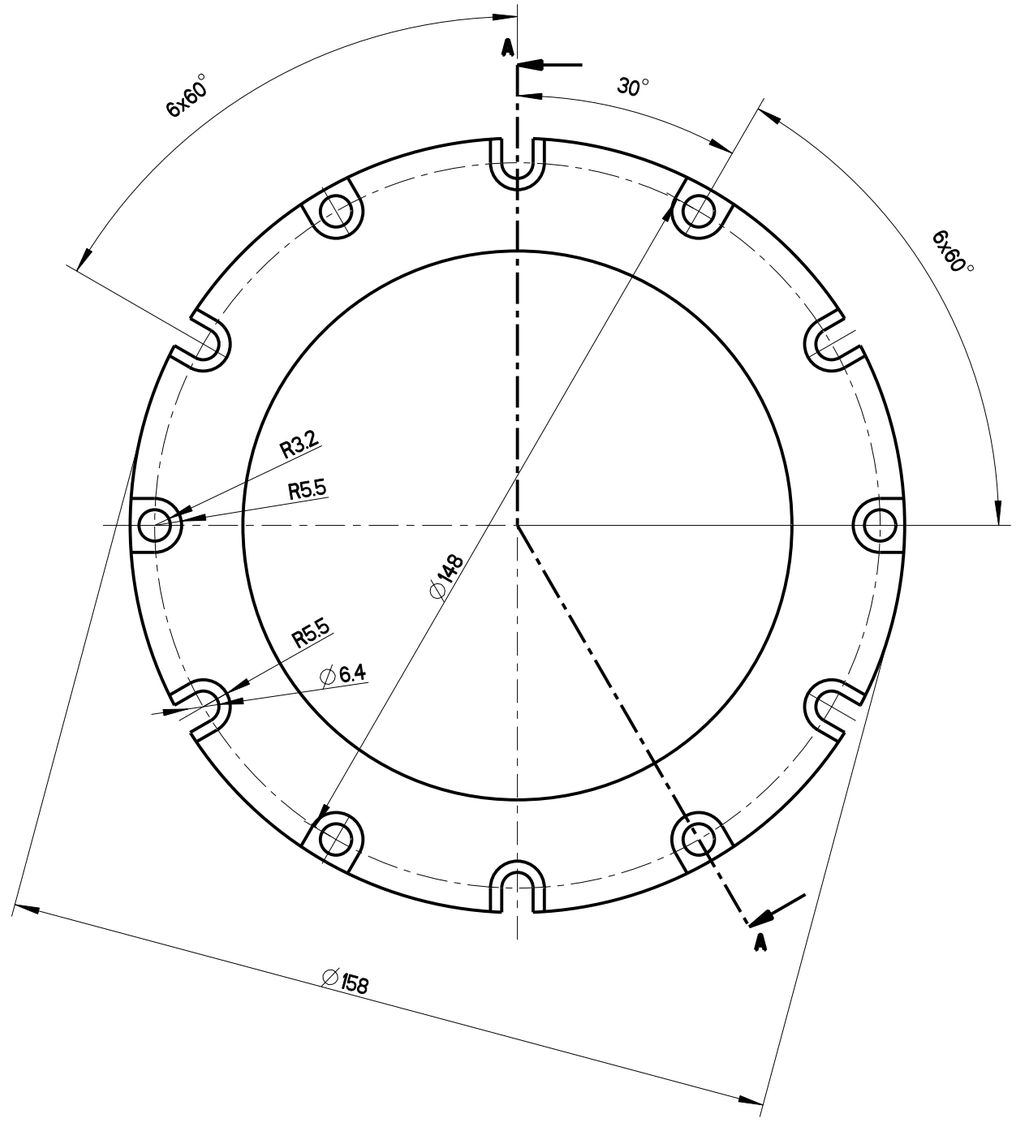
Sheet 1

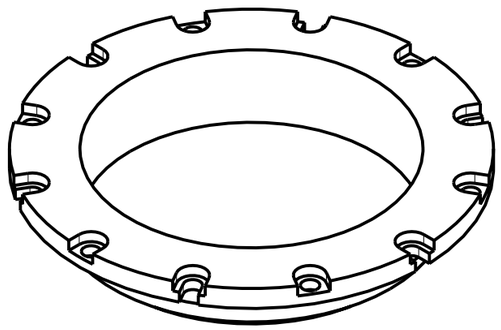
Sheets 1

A
23



SECTION **A-A**





SCALE 1:2

DIMENSIONS IN MILLIMETRES (mm)
REMOVE SHARP EDGES

UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

SURFACE ROUGHNESS ✓

ALL DIAMETERS TO BE UNLESS OTHERWISE STATED ⌀

Drawn	Date	Name
	14-Feb-05	

Scale 1:1 | Weight

Material: Stainless 304

Model: FLANGE_UPPER_MAIN_V5

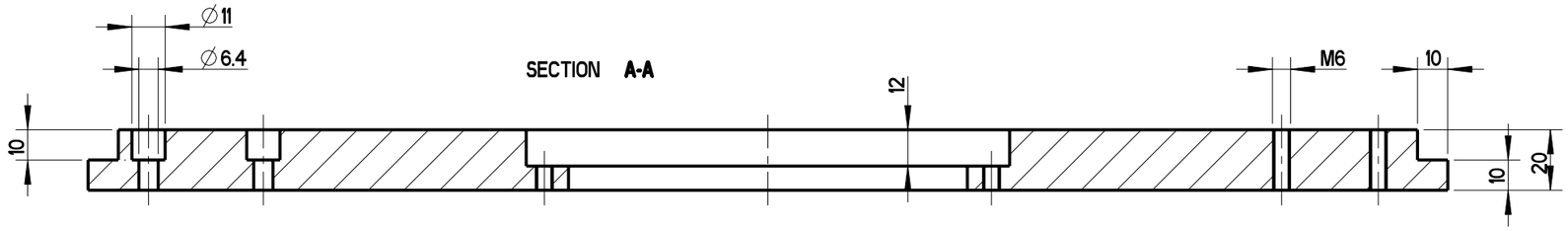
File: FLANGE_UPPER_MAIN_V5

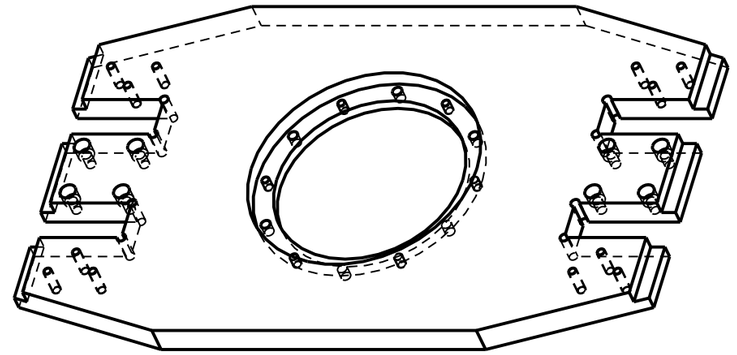
Sheet 1

Sheets 1

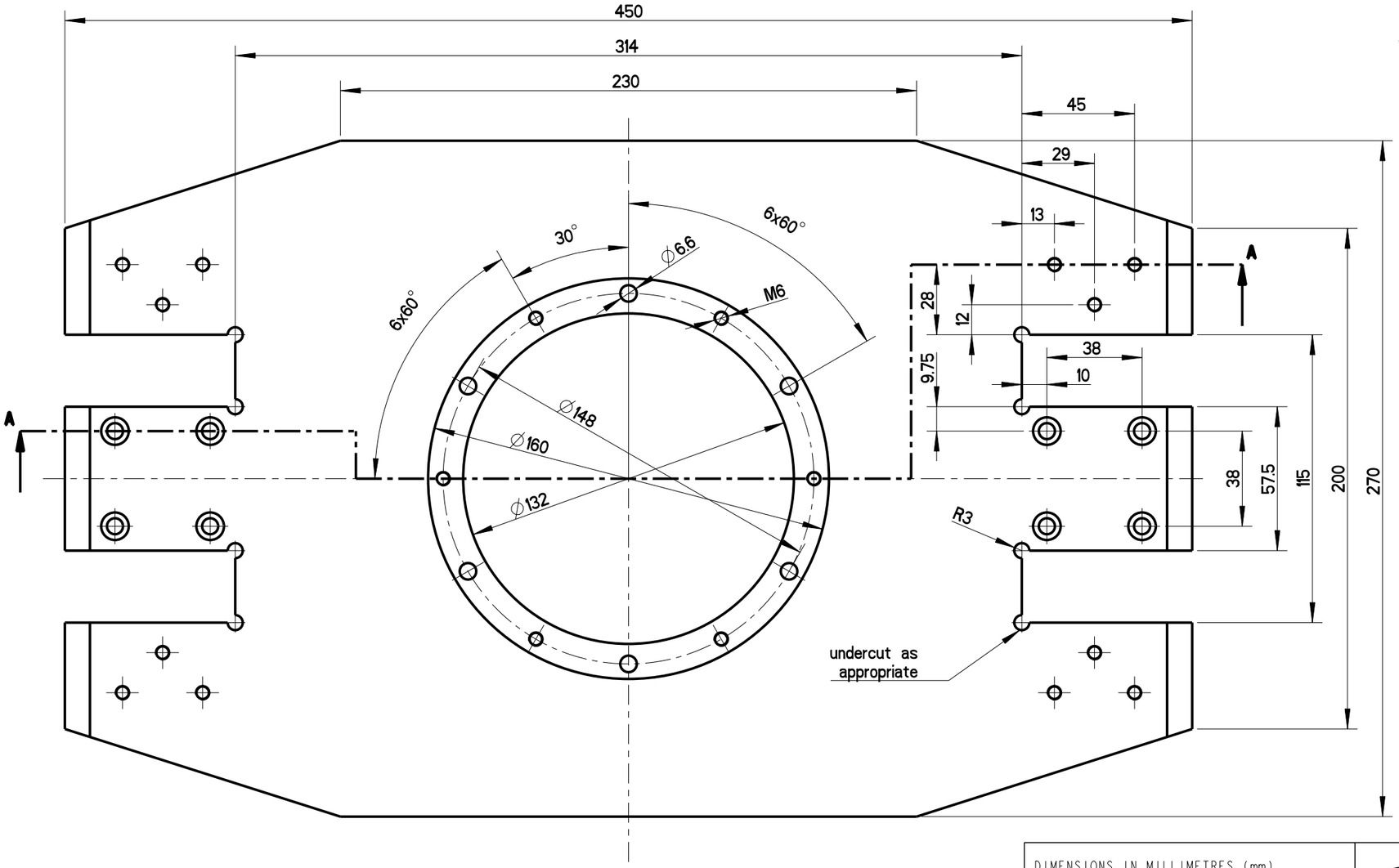
A
24

SECTION **A-A**





SCALE 3:10



DIMENSIONS IN MILLIMETRES (mm)
REMOVE SHARP EDGES

UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

SURFACE ROUGHNESS ✓

ALL DIAMETERS TO BE
UNLESS OTHERWISE STATED ⌀

Drawn	Date	Name
	14-Feb-05	

Scale 1:2 | Weight

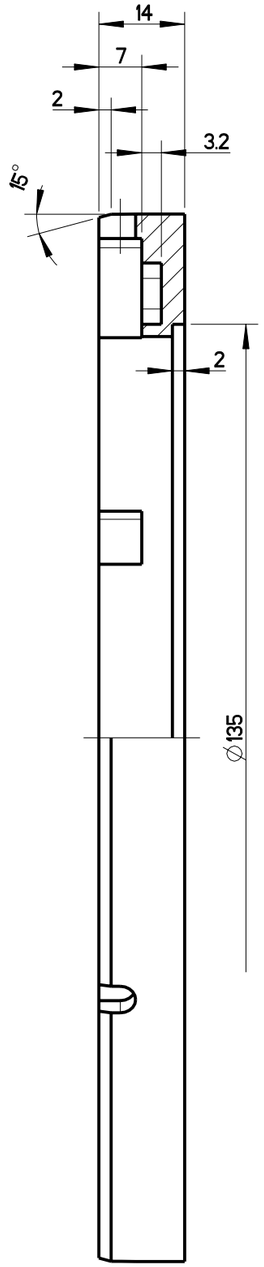
Material: Stainless 304

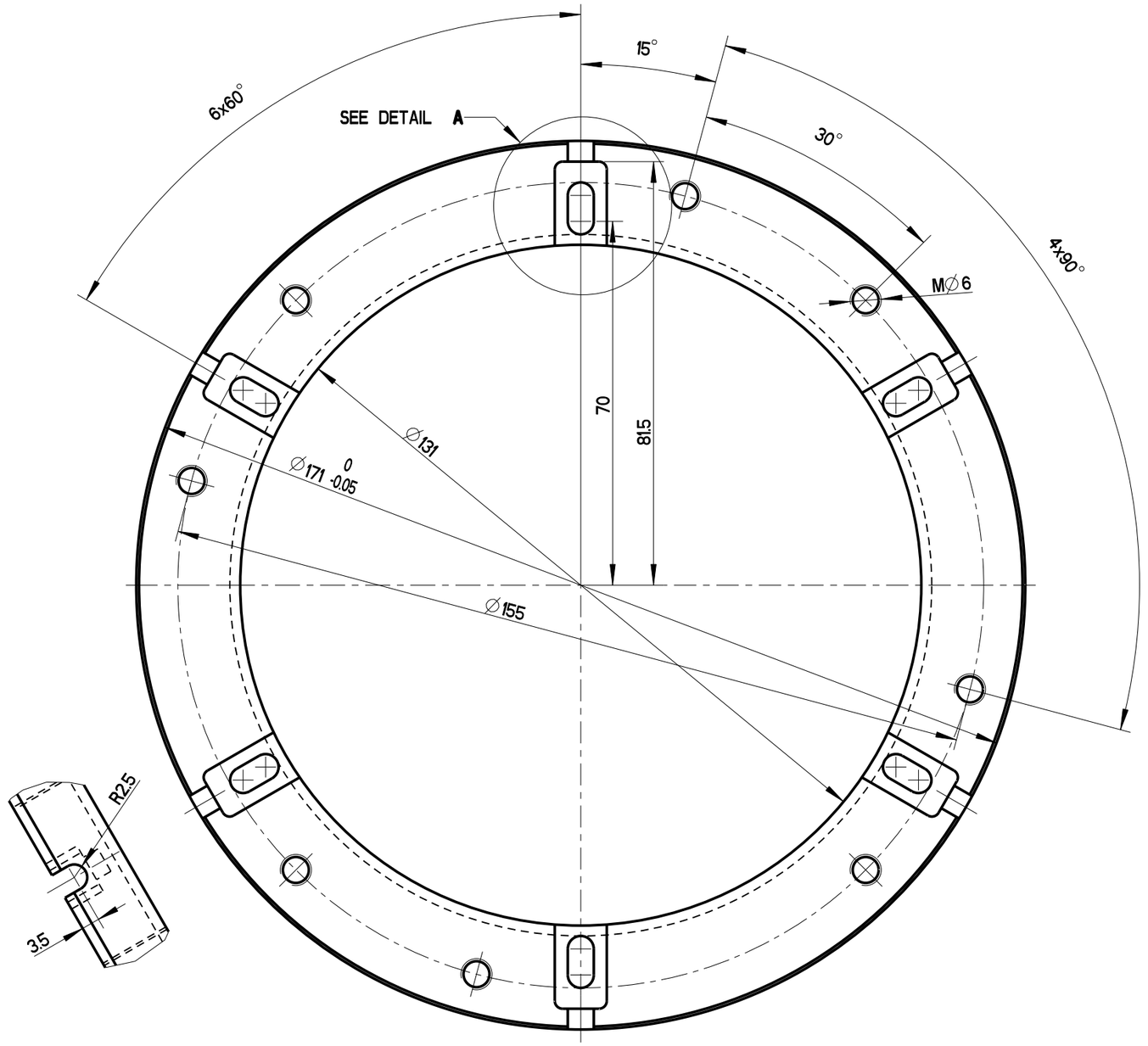
Model: FLANGE_UPPER_PLATE_GUIDE

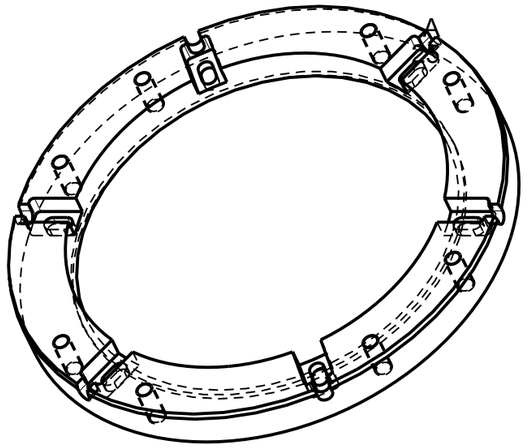
File: FLANGE_UPPER_PLATE_GUIDE

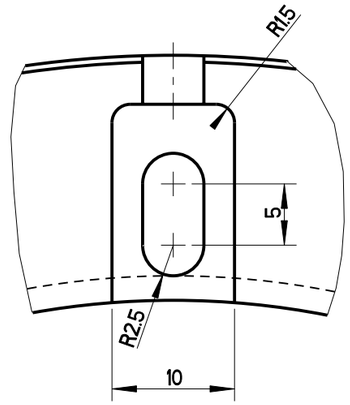
Sheet 1

Sheets 1









DETAIL A
SCALE 2:1

DIMENSIONS IN MILLIMETRES (mm)
REMOVE SHARP EDGES

UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

SURFACE ROUGHNESS ✓

ALL DIAMETERS TO BE UNLESS OTHERWISE STATED ⌀

Drawn	Date	Name
	14-Feb-05	

Scale 1:1 | Weight

Material: Stainless 304

Model: COLLAR2_FLANGE_MAIN

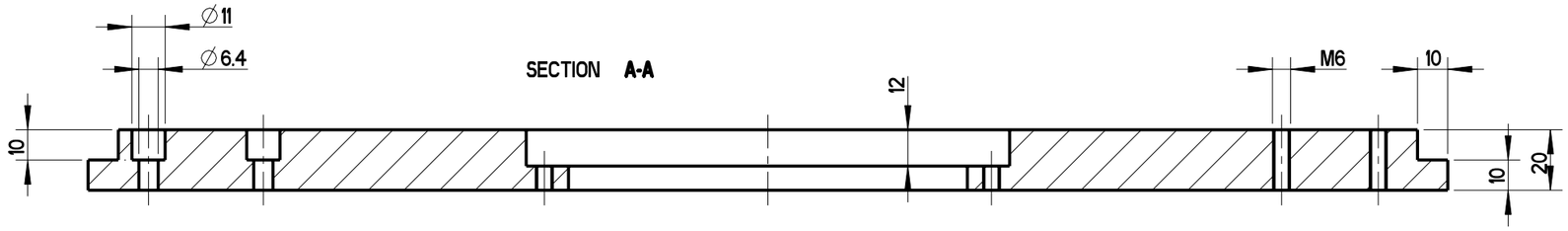
File: COLLAR2_FLANGE_MAIN

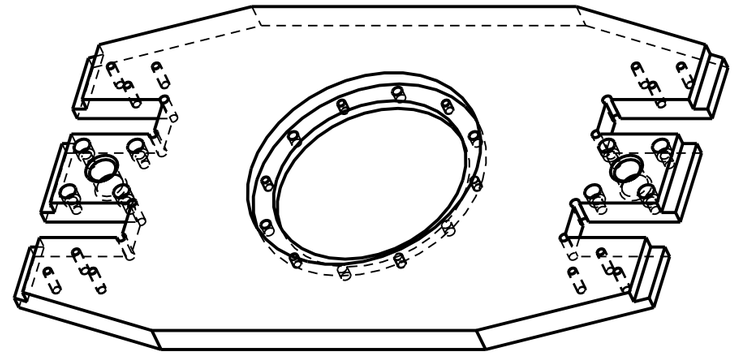
Sheet 1

Sheets 1

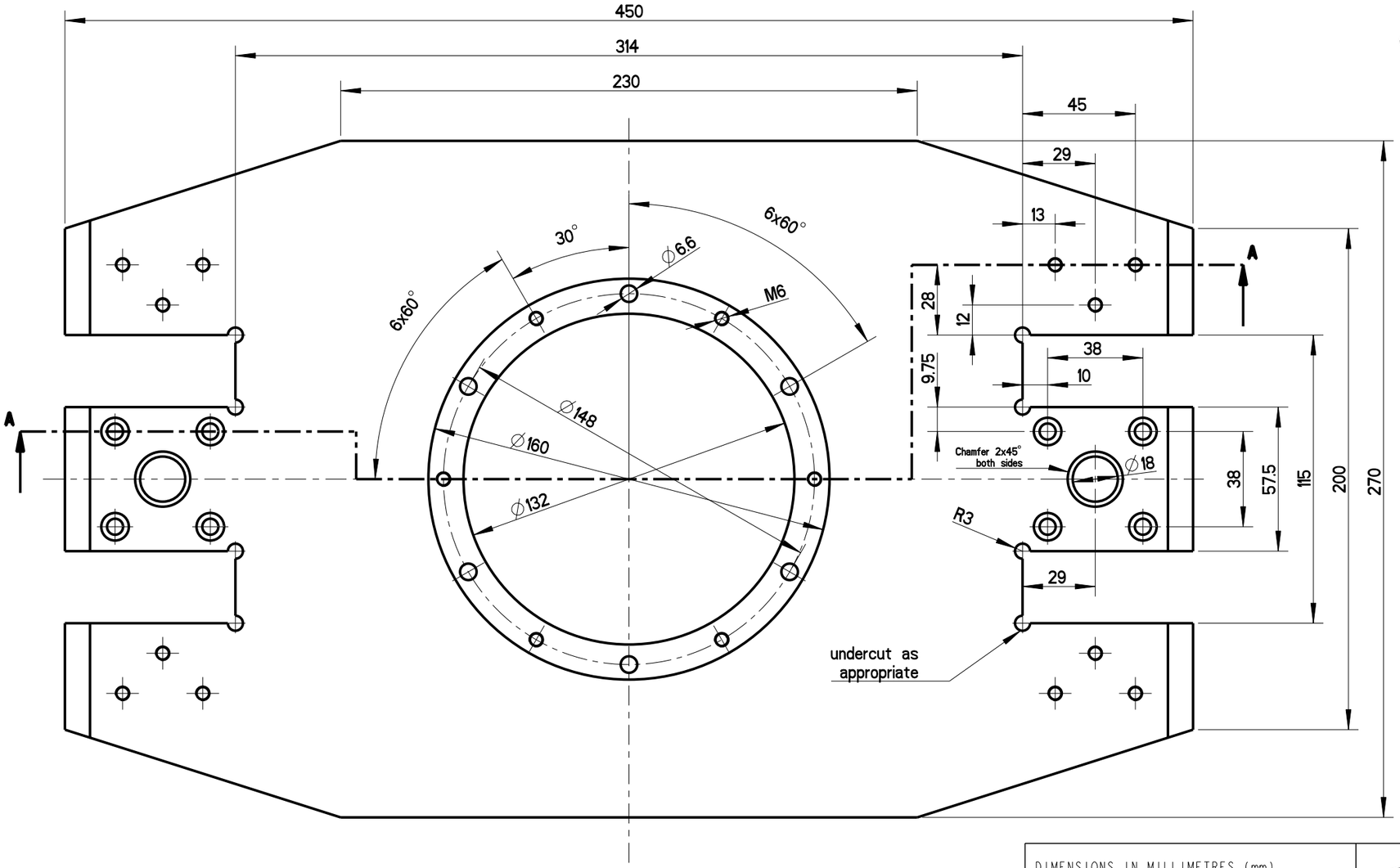
A
26

SECTION **A-A**





SCALE 3:10



DIMENSIONS IN MILLIMETRES (mm)
REMOVE SHARP EDGES

UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

SURFACE ROUGHNESS ✓

ALL DIAMETERS TO BE UNLESS OTHERWISE STATED ⌀

Drawn	Date	Name
	08-Mar-05	

Scale 1:2 | Weight

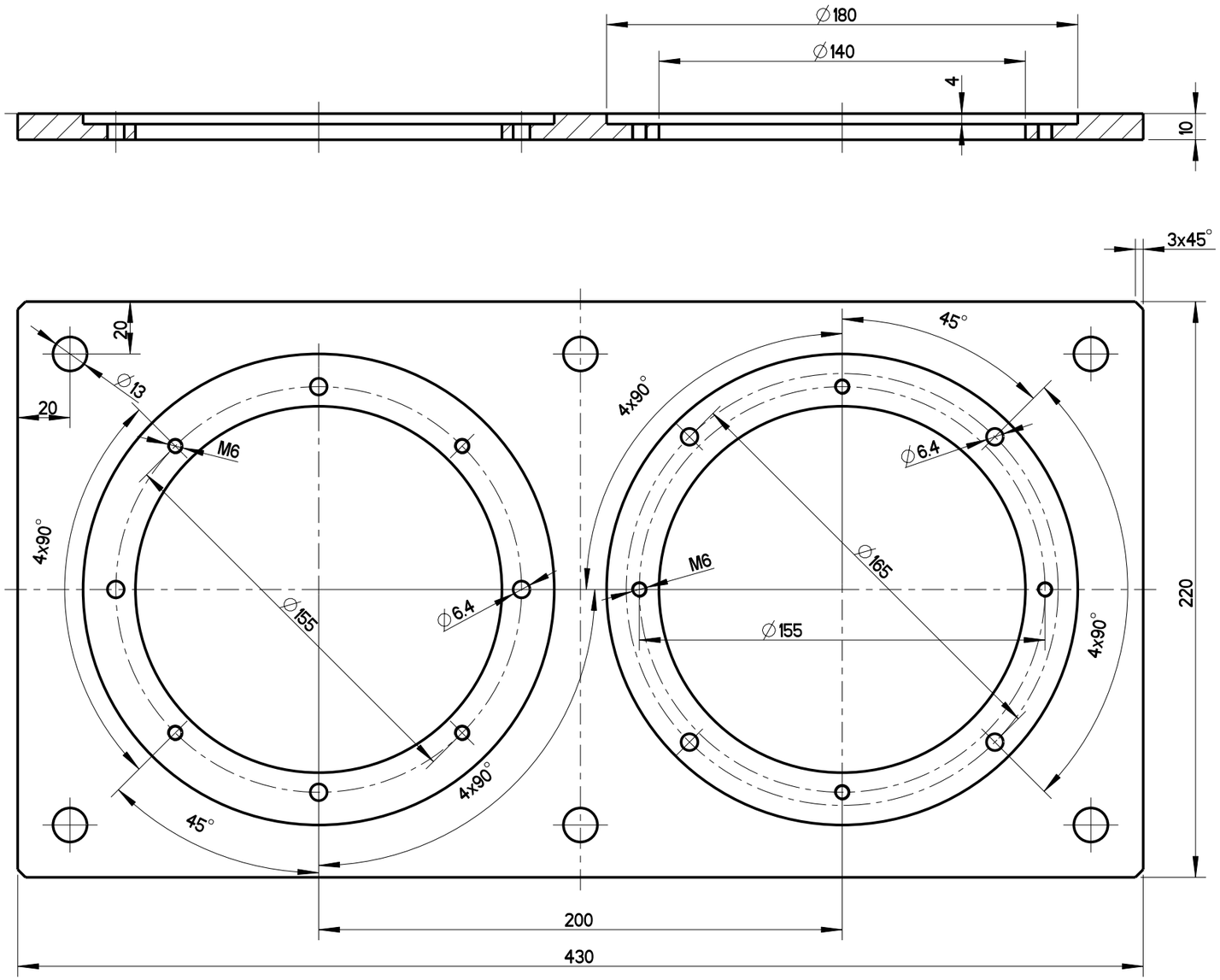
Material: Stainless 304

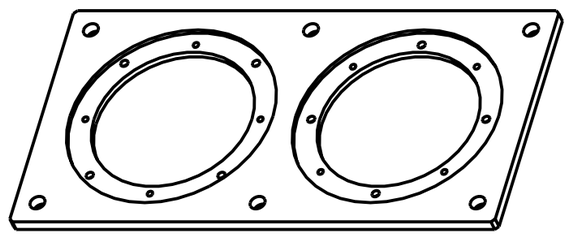
Model: FLANGE_UPPER_PLATE_GUIDE

File: FLANGE_UPPER_PLATE_GUIDE

Sheet 1

Sheets 1





DIMENSIONS IN MILLIMETRES (mm)
REMOVE SHARP EDGES

UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

SURFACE ROUGHNESS ✓

ALL DIAMETERS TO BE UNLESS OTHERWISE STATED ⌀

Drawn	Date	Name
	11-Mar-05	

Scale 1:5 | Weight

Material: Stainless 304

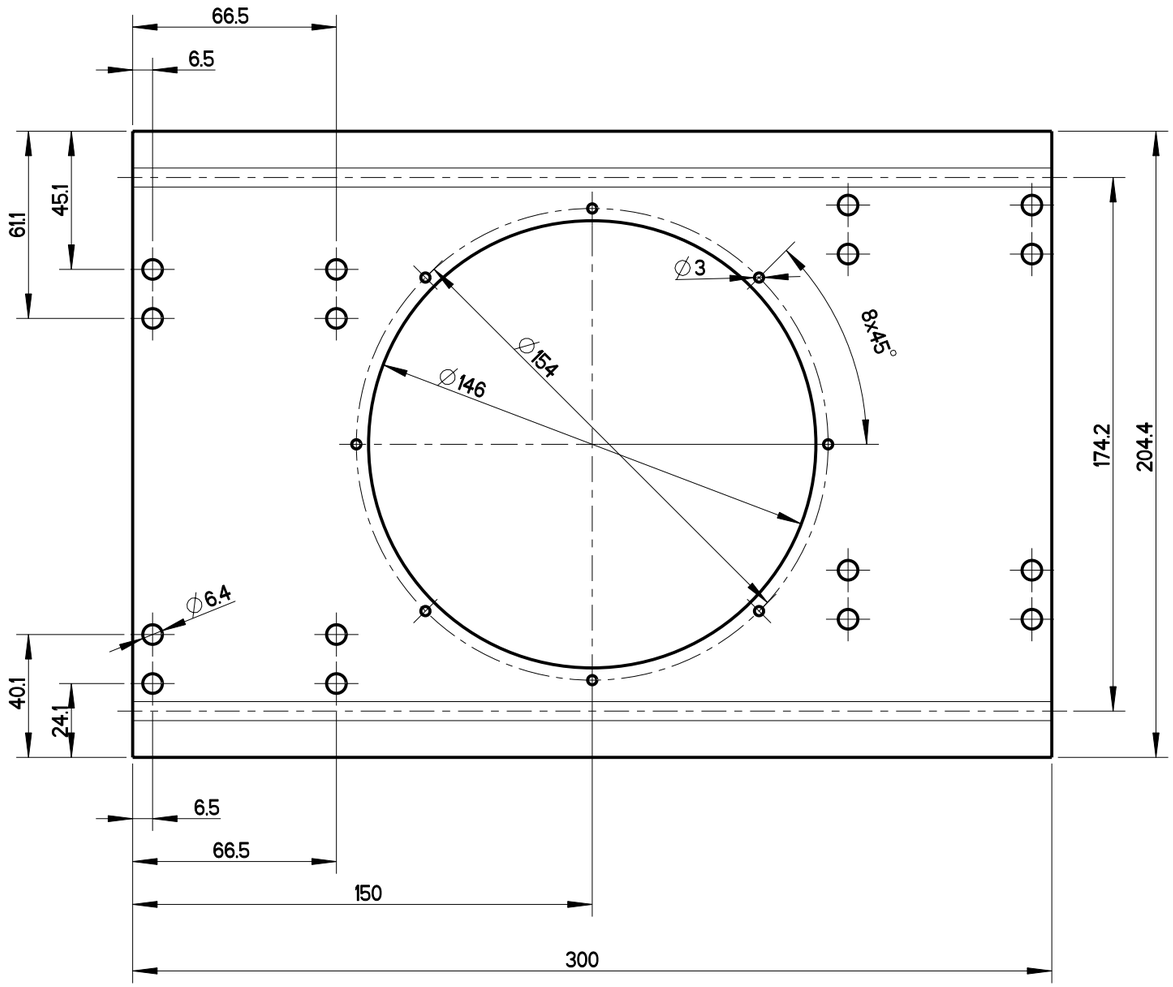
Model: COLLAR_PLATE

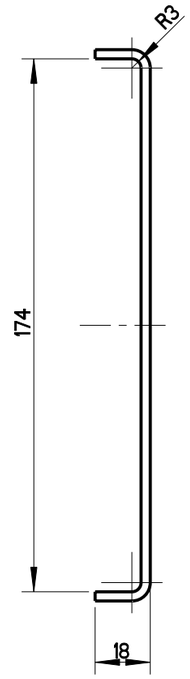
File: COLLAR_PLATE

Sheet 1

Sheets 1

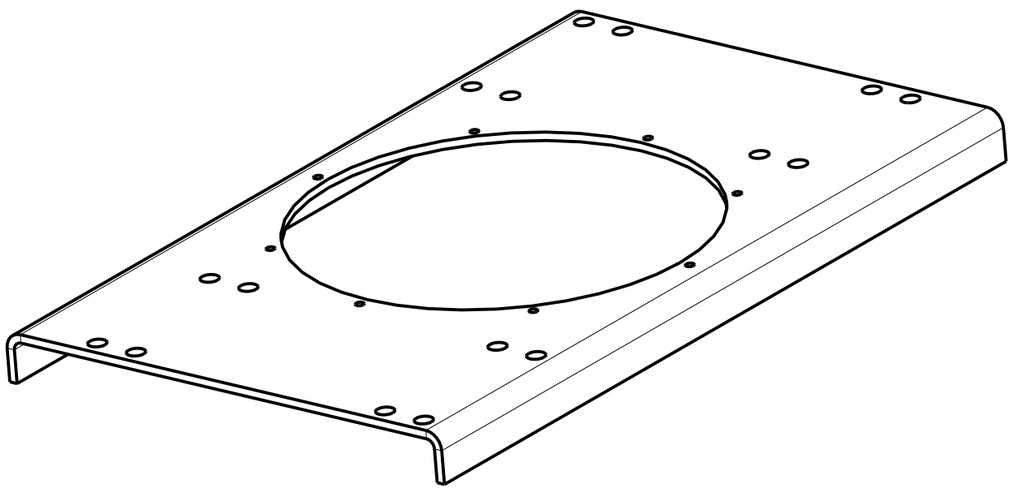
A
28





Bending radius as small as possible
Drawing assumes inside radius of 3mm

Laser cut surfaces are sufficient



DIMENSIONS IN MILLIMETRES (mm)
REMOVE SHARP EDGES

UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

SURFACE ROUGHNESS

ALL DIAMETERS TO BE UNLESS OTHERWISE STATED ⌀

Drawn	Date	Name
	10-Mar-05	

Scale 1:2 | Weight

Material: Stainless 304, 3mm

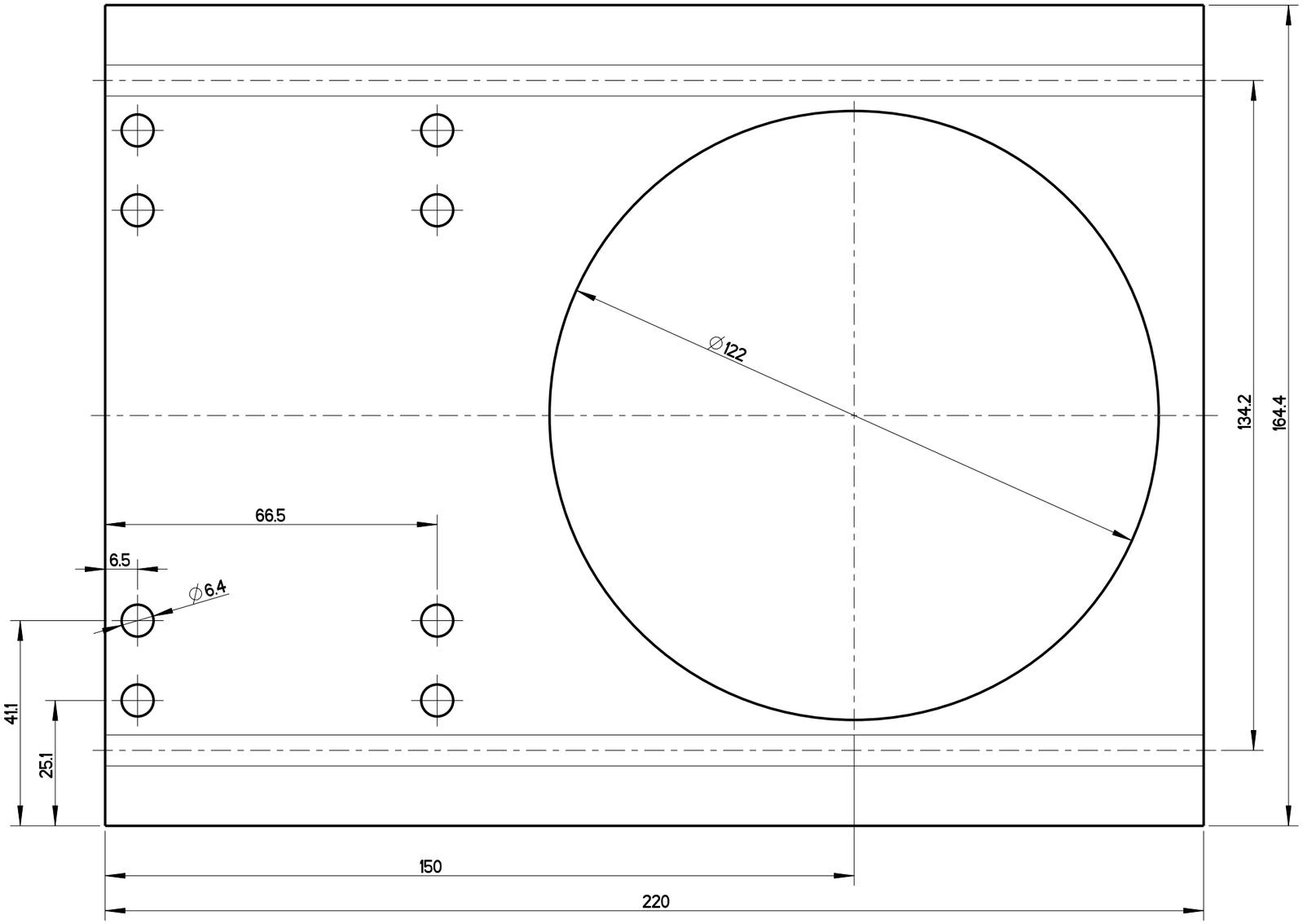
Model: DUMMY_SHEET_V2

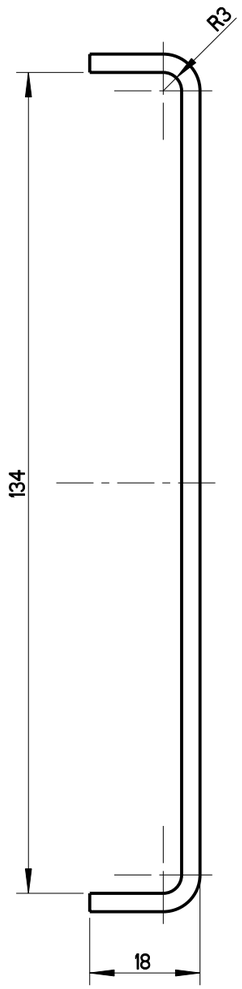
File: DUMMY_SHEET_V2

Sheet 1

Sheets 1

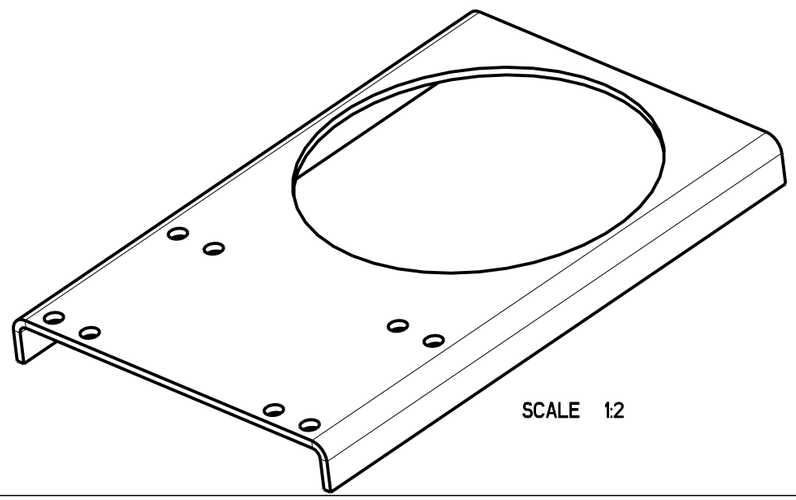
A
29





Bending radius as small as possible
Drawing assumes inside radius of 3mm

Laser cut surfaces are sufficient



SCALE 1:2

DIMENSIONS IN MILLIMETRES (mm)
REMOVE SHARP EDGES

UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

SURFACE ROUGHNESS

ALL DIAMETERS TO BE
UNLESS OTHERWISE STATED

Drawn	Date	Name
	10-Mar-05	

Scale 1:1 | Weight

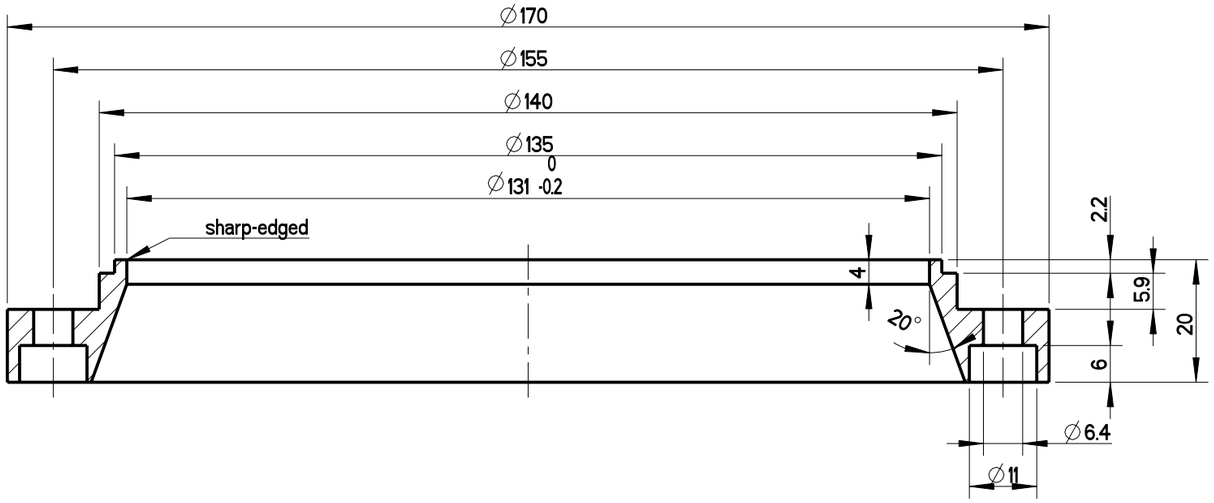
Material: Stainless 304, 3mm

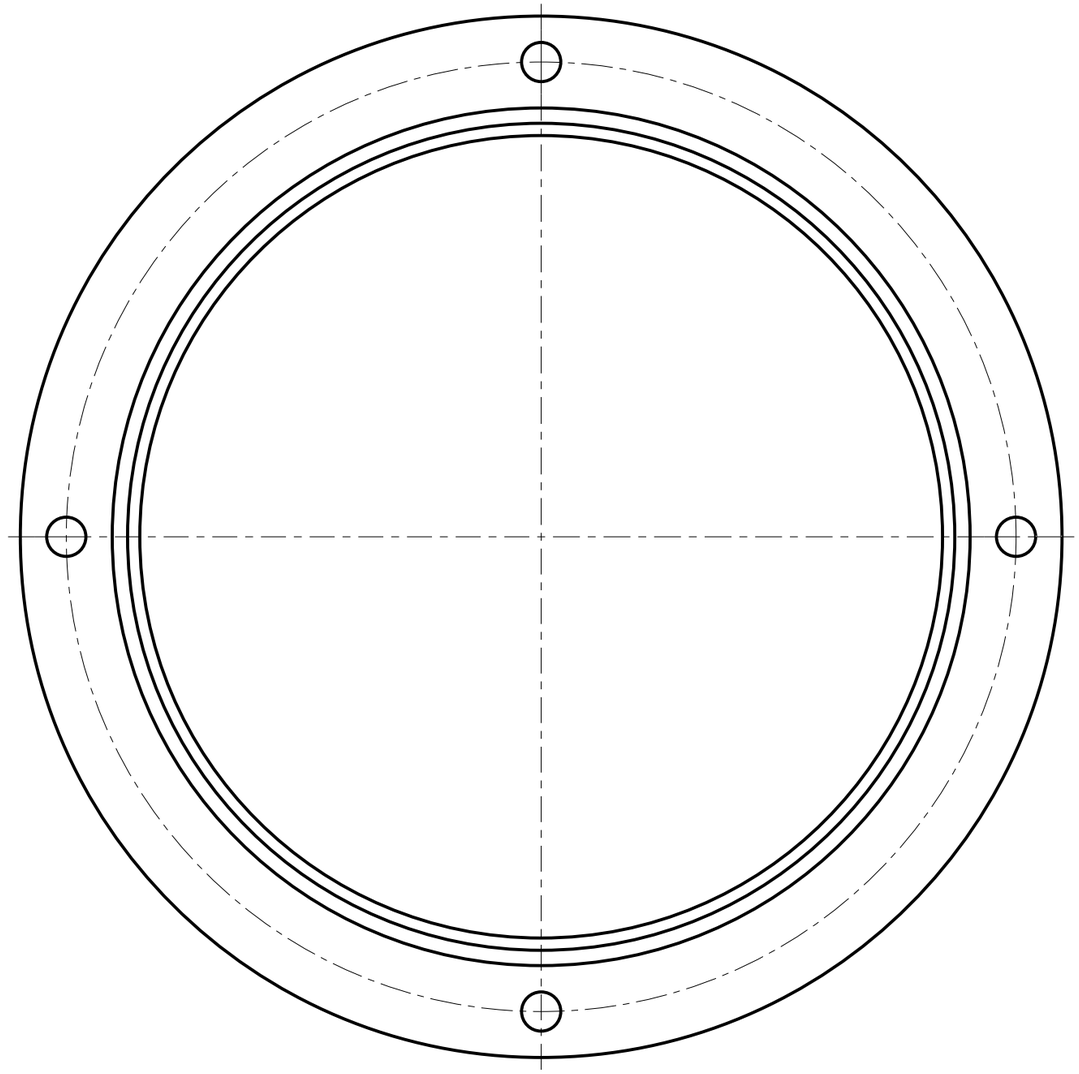
Model: TROLLEY_SHEET_V2

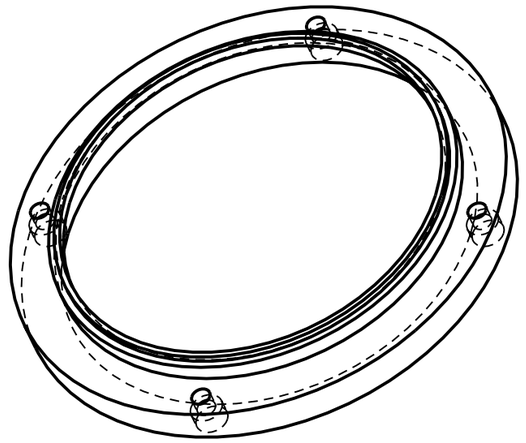
File: TROLLEY_SHEET_V2

Sheet 1
Sheets 1

A
30







SCALE 1:2

DIMENSIONS IN MILLIMETRES (mm)
REMOVE SHARP EDGES

UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

SURFACE ROUGHNESS ✓

ALL DIAMETERS TO BE
UNLESS OTHERWISE STATED ⌀

Drawn	Date	Name
	14-Mar-05	

Scale 1:1 | Weight

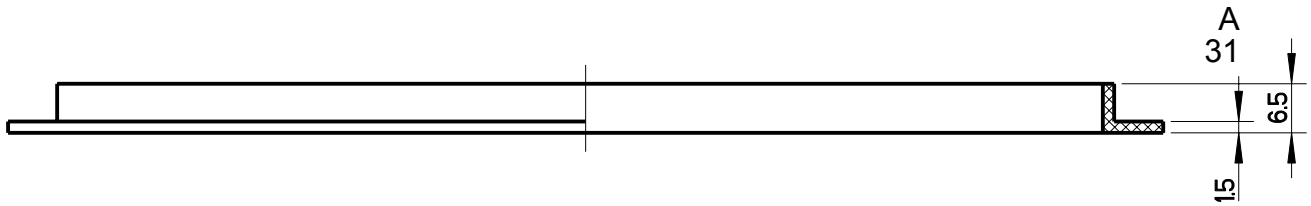
Material: UHMWPE

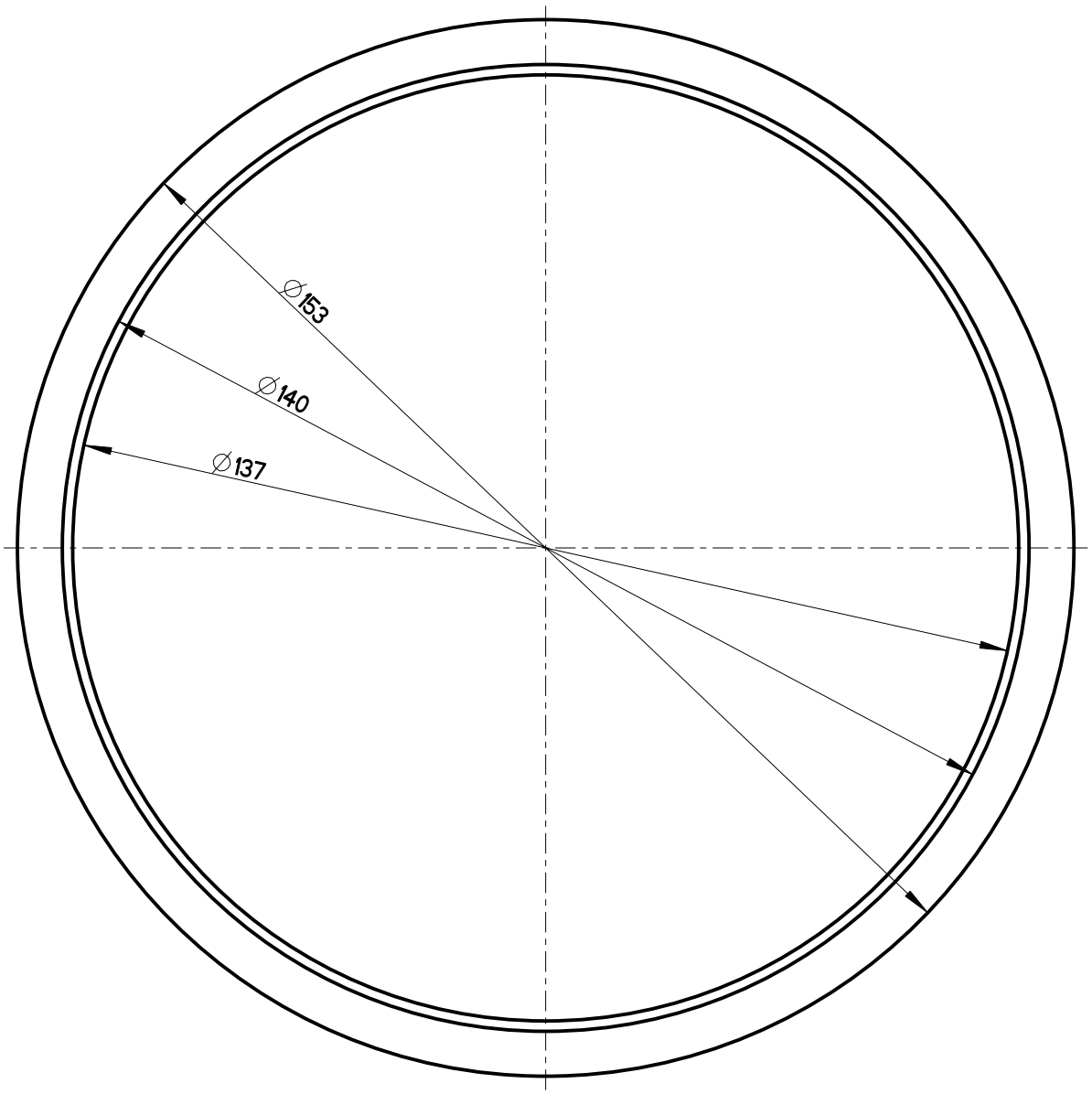
Model: COLLAR_CENTRE_RING

File: COLLAR_CENTRE_RING

Sheet 1

Sheets 1





DIMENSIONS IN MILLIMETRES (mm)
REMOVE SHARP EDGES

UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

SURFACE ROUGHNESS

ALL DIAMETERS TO BE
UNLESS OTHERWISE STATED

	Date	Name
Drawn	11-Mar-05	

Scale 1:1 | Weight

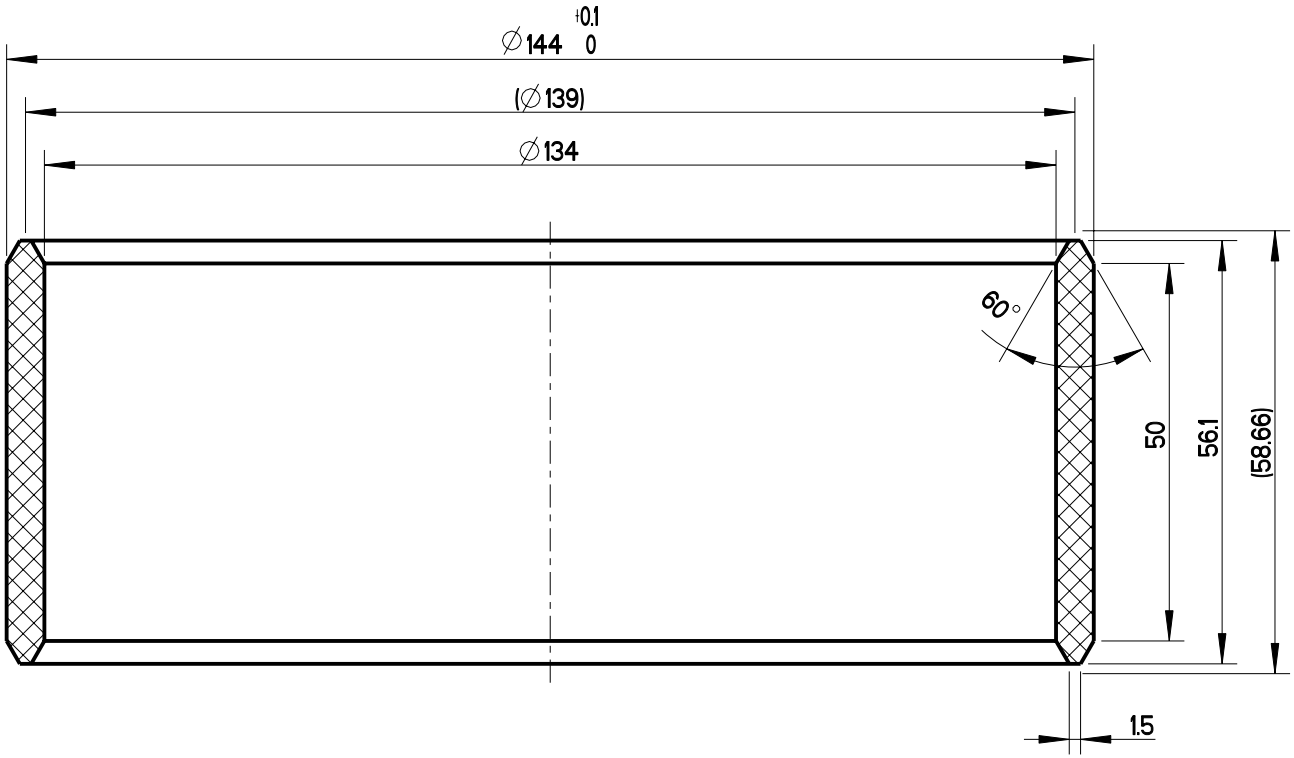
Material: PTFE

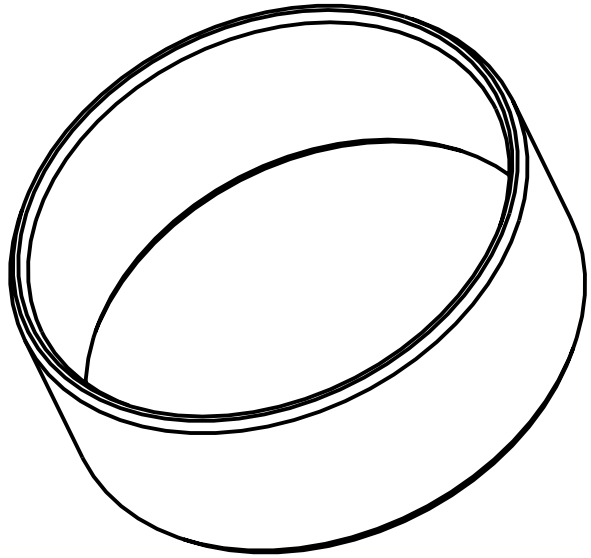
Model: COLLAR_GLIDE_RING_V2

File: COLLAR_GLIDE_RING_PTFE_V2

Sheet 1
Sheets 1

A
32





SCALE 1:2

DIMENSIONS IN MILLIMETRES (mm)
REMOVE SHARP EDGES

UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

SURFACE ROUGHNESS ✓

ALL DIAMETERS TO BE
UNLESS OTHERWISE STATED ⌖ ∅

	Date	Name
Drawn	12-Mar-05	

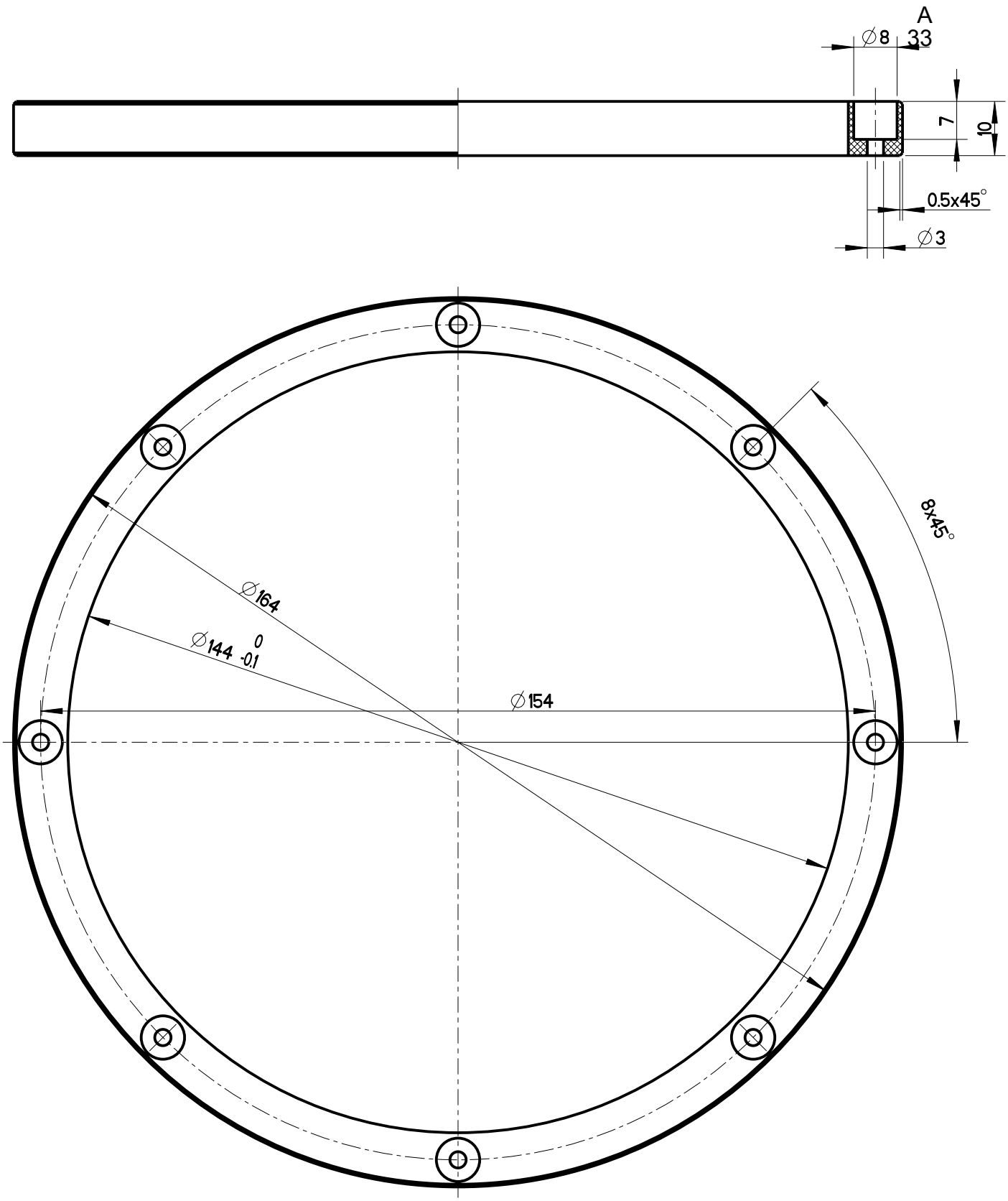
Scale 1:1 | Weight

Material: PU, Hardness to be specified

Model: DUMMY_DUMMYDISC_INNER

File: DUMMY_DUMMYDISC_INNER

Sheet 1
Sheets 1



DIMENSIONS IN MILLIMETRES (mm)
REMOVE SHARP EDGES

UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

SURFACE ROUGHNESS ✓

ALL DIAMETERS TO BE UNLESS OTHERWISE STATED ⌀

	Date	Name
Drawn	12-Mar-05	

Scale 1:1 | Weight

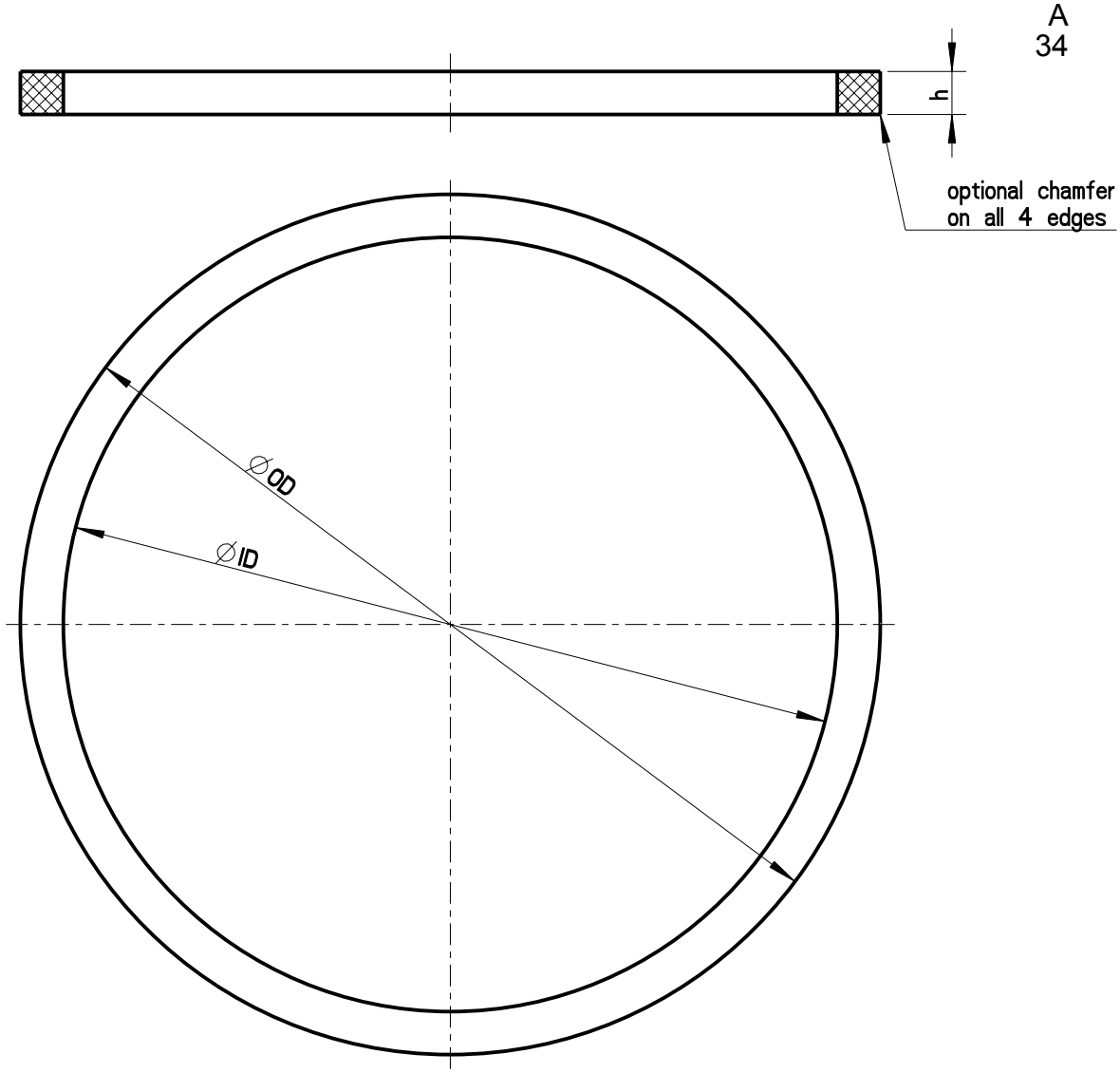
Material: UHMWPE

Model: DUMMY_DUMMYDISC_OUTER

File: DUMMY_DUMMYDISC_OUTER

Sheet 1
Sheets 1

A
34



Seal No	Qty	ID	OD	h	Chamfer
1	1	116.0	128.0	6.0	0.5x45°
2	1	115.5	128.0	5.0	none
3	1	115.5	128.0	6.0	0.5x45°
4	1	107.5	120.0	5.0	none
5	1	107.5	120.0	6	0.5x45°

DIMENSIONS IN MILLIMETRES (mm)
REMOVE SHARP EDGES

UNLESS OTHERWISE STATED TOLERANCES

LINEAR ± ANGULAR ±

SURFACE ROUGHNESS √

ALL DIAMETERS TO BE UNLESS OTHERWISE STATED

	Date	Name
Drawn	11-Mar-05	

Scale 1:1 | Weight

Material: to be specified

Model

FLANGE_LOWER_SEAL

File: FLANGE_LOWER_SEAL

Sheet 1
Sheets 1

Appendix B

Retail (end-user) prices for LF

2005

Table B1: Some of the lactoferrin products that are available through internet with respective prices.

Product name	Company	price			Retail prices
				NZ$/g	NZ$/kg
60 caps, 250 mg Immunecare	ImmuneCare UK	75.59	NZD	5.04	5039.33
60 Capsules 250 mg each	EINC.inc USA	16.99	USD	1.62	1619.71
60 Capsules 300 mg each	Health-n-Energy	24.95	USD	1.98	1982.14
300 mg 60 capsules, Code 513	Healthmarketplace.com	39.95	USD	3.17	3173.81
Lactoferrin 60 Caps (250 mg)	Herbal Nutrition	19.50	USD	1.86	1859.00
Denta Shield 60 Chewable Tabs	Herbal Nutrition	20.12	USD	1.92	1918.11
Laktoferrin, 120 Caps (350 mg)	Herbal Nutrition	50.00	USD	1.70	1702.38
Laktoferrin, 90 Caps (350 mg)	Herbal Nutrition	39.00	USD	1.77	1770.48
Lactoferrin 250mg - 60 Caps	Worldwidehealthcenter.net	31.95	USD	3.05	3045.90
Symbiotics New Life					
100% Lactoferrin 60 Capsules	Rite Care Pharmacy	21.88	USD	2.09	2085.89
Lactoferrin 60 capsules, 250 mg	Jarrow Formulae	19.29	USD	1.84	1838.98
Lactoferrin 250 mg, 60 capsules	Vitamin Research Products	29.95	USD	2.86	2855.23
Average retail price (excluding ImmuneCare)				2.17	2168.33

Source: internet (prices valid as at December, 2005).

Retail prices ranged from $1.62 - $5.04 per g for capsule form of lactoferrin. These are generally marketed as nutraceuticals and/or functional food.

Appendix C

SuperPro Designer®
Process layout and input, output summary of economic analysis from Case 19
(section 8.4.3)

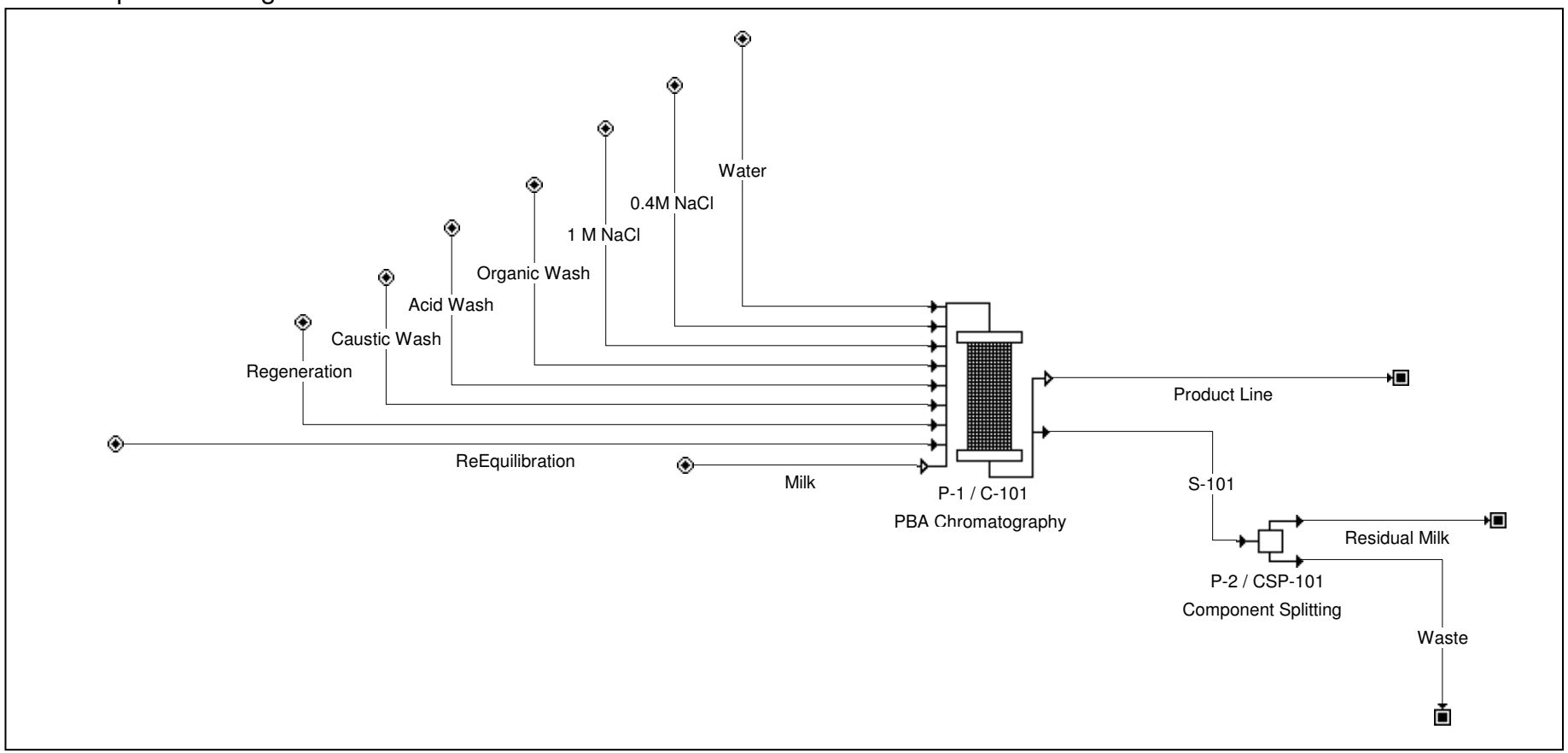


Figure C1: A simple process layout from SuperPro Designer, showing the capture and elution process of on-farm fractionation that was used in feasibility studies.

Example of economic feasibility report based on Case 19.

EXECUTIVE SUMMARY (2005 prices)

TOTAL CAPITAL INVESTMENT	125000	$
CAPITAL INV. CHARGED TO THIS PROJECT	125000	$
OPERATING COST	29000	$/year
PRODUCTION RATE	109.1	kg/year of LF (in Product Line)
UNIT PRODUCTION COST	261.3	$/kg of LF (in Product Line)
TOTAL REVENUES	93000	$/year
GROSS MARGIN	69.31	%
RETURN ON INVESTMENT	38.98	%
PAYBACK TIME	2.57	years
IRR AFTER TAXES	46.64%	
NPV (at 7.0 % interest)	345000	$

MAJOR EQUIPMENT SPECIFICATION AND FOB COST (2005 prices)

Quantity/ Stand-by		Description	Unit Cost ($)	Cost ($)
3/0	C-101	Chromatography Column Column Diameter = 0.04 m Column Height = 0.25 m	6000	18000
1/0	CSP-101	Component Splitter Throughput = 2881.63 kg/h	0	0
		Cost of Unlisted Equipment		4000
TOTAL EQUIPMENT PURCHASE COST				22000

OVERALL PROCESS DATA

Annual Operating Time	1932.3	h
Annual Throughput	109.08	kg MP
Batch Throughput	0.01	kg MP
Recipe Batch Time	0.19	h
Recipe Cycle Time	0.19	h
Number of Batches Per Year	10000	

MP = Main Product Flow of LF (in Product Line)

FIXED CAPITAL ESTIMATE SUMMARY (2005 prices in $)

A. TOTAL PLANT DIRECT COST (TPDC) (physical cost)			
1. Equipment Purchase Cost		22000	
2. Installation		3000	
3. Process Piping		8000	
4. Instrumentation		9000	
5. Insulation		1000	
6. Electricals		2000	
7. Buildings		10000	
8. Yard Improvement		3000	
9. Auxiliary Facilities		9000	
	TPDC =	65000	
B. TOTAL PLANT INDIRECT COST (TPIC)			
10. Engineering		16000	
11. Construction		23000	
	TPIC =	39000	
C. TOTAL PLANT COST (TPDC+TPIC)	TPC =		104000
12. Contractor's fee		5000	
13. Contingency		10000	
	(12+13) =	16000	
D. DIRECT FIXED CAPITAL (DFC) TPC+12+13			120000

LABOR REQUIREMENT AND COST SUMMARY

Section Name	Labor Hours Per Year	Labor Cost $/year	%
Main Section	183	5000	100
TOTAL	183	5000	100

RAW MATERIALS COST SUMMARY

Raw Material	Unit Cost ($/kg)	Annual Amount (kg)	Cost ($/yr)	%
LP	0	1.5	0	0
Milk	0	149845.5	0	0
LF	0	151.5	0	0
Water	0	97480.3	0	0
Sodium Chloride	0.042	3623.79	152	1.75
Isopropanol	0.868	5479.58	4756	54.77
HCl (1 M)	0.065	15420.82	1004	11.56
NaOH (1 M)	0.056	15924.67	892	10.27
NaCl (2 M)	0.042	16413.25	689	7.94
Phosphate Buffer	0.078	15187.28	1191	13.71
TOTAL		319528.19	9000	100

VARIOUS CONSUMABLES (2005 prices)

CHROMATOGRAPHY RESINS

Procedure Name	Equipment Name	Unit Cost ($/L)	Annual Amount (L)	Cost ($/yr)
P-1	C-101	1000	7.63	8000
TOTAL				8000

WASTE TREATMENT / DISPOSAL (2005 prices)

Stream Name	Unit Cost ($/kg)	Annual Amount (kg)	Cost ($/yr)
a. SOLID WASTE			
a. Subtotal (Solid Waste)			0
b. LIQUID WASTE			
Waste	0.00E+00	138317.46	0
b. Subtotal (Liquid Waste)			0
c. EMISSIONS			
c. Subtotal (Emissions)			0
WASTE TREATMENT/DISPOSAL TOTAL COST (a+b+c)			0

UTILITY REQUIREMENTS (2005 prices)

TOTAL	0

ANNUAL OPERATING COST - SUMMARY (2005 prices)

Cost Item	$/Year	%
Raw Materials	9000	30.47
Labor-Dependent	5000	18.33
Facility-Dependent	7000	24.23
Laboratory/QC/QA	0	0.18
Consumables	8000	26.79
Waste Treatment/Disposal	0	0
Utilities	0	0
Transportation	0	0
Miscellaneous	0	0
Advertising and Selling	0	0
Running Royalties	0	0
Failed Product Disposal	0	0
TOTAL	29000	100

PROFITABILITY ANALYSIS (2005 prices) $

A. DIRECT FIXED CAPITAL	120000	
B. WORKING CAPITAL	5000	
C. STARTUP COST	0	
D. UP-FRONT R&D	0	
E. UP-FRONT ROYALTIES	0	
F. TOTAL INVESTMENT (A+B+C+D+E)	125000	
G. INVESTMENT CHARGED TO THIS PROJECT	125000	
H. REVENUE STREAM FLOWRATES		
kg/year of LF (in Product Line)	109.079	
kg/year of total flow (in Residual Milk)	149846	
I. PRODUCTION (UNIT) COST		
$/kg of LF (in Product Line)	261.289	
J. SELLING/PROCESSING PRICE		
$/kg of LF (in Product Line)	401.485	
$/kg of total flow (in Residual Milk)	0.328	
K. REVENUES ($/year)		
Product Line	44000	
Residual Milk	49000	
Total Revenues	93000	
L. ANNUAL OPERATING COST	29000	
M. GROSS PROFIT (K-L)	64000	
N. TAXES (33 %)	21000	
O. NET PROFIT (M-N + Depreciation)	49000	
GROSS MARGIN	69.31	%
RETURN ON INVESTMENT	38.98	%
PAYBACK TIME (years)	2.57	

LOAN INFORMATION ($'000)

	Direct Fixed Capital	Working Capital	Up Front R&D	Up Front Royalties
Amount	120	5	0	0
Equity (%)	100	100	100	100
Debt (%)	0	0	0	0
Interest (%)	9	12	12	12
Loan Time (yrs)	10	6	6	6

BREAKDOWN OF CAPITAL OUTLAY ($)

YEAR	DIRECT FIXED CAPITAL	WORKING CAPITAL	START-UP COST	UP FRONT R&D	UP FRONT ROYALTIES	TOTAL
1	-36029	-5183	0	0	0	-41212
2	-48039	0	0	0	0	-48039
3	-36029	0	0	0	0	-36029
4-19	0	0	0	0	0	0
20	6005	5183	0	0	0	11188

BREAKDOWN OF LOAN PAYMENT ($)

YEAR	DIRECT FIXED CAPITAL	WORKING CAPITAL	UP FRONT R&D	UP FRONT ROYALTIES	TOTAL
1-20	0	0	0	0	0

CASH FLOW ANALYSIS ($'000)

YR	CAPITAL INVESTM	DEBT FINANCE	SALES	OPERAT COST	GROSS PROFIT	LOAN PAYMENT	DEPREC.	TAXABLE INCOME	TAXES	NET PROFIT	NET CASH FLOW
1	-41	0	8	13	-6	0	6	0	0	-6	-47
2	-48	0	93	29	64	0	6	59	19	45	-3
3	-36	0	93	29	64	0	6	59	19	45	9
4-19	0	0	93	29	64	0	6	59	19	45	45
20	11	0	93	29	64	0	6	59	19	45	56

IRR BEFORE TAXES	74.609	%	INTEREST	7.00%	9.00%	11.00%
IRR AFTER TAXES	46.641	%	NPV	345	284	235

Depreciation Method:	Straight-Line
DFC Salvage Factor	0.05