THE FRACTIONATION AND CHARACTERISATION OF LIPID FOULANTS ADSORBED ONTO ULTRAFILTRATION MEMBRANES DURING CONTACT WITH ABATTOIR EFFLUENT

by

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DECLARATION

I, the under signed, hereby declare that the work contained in this thesis is my own original work and has not been previously in its entire or in part been submitted at any university for a degree.

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SUMMARY

IN THIS STUDY:

1. Lipids present in abattoir effluent, as well as lipids that adsorbed onto UF membranes during contact with abattoir effluent, were separated chromatographically. The four major lipid classes were identified and quantified. The lipid classes present were, triglycerides, free and total cholesterol and phospholipids.

2. Using results obtained from the characterisation studies, various lipases and *phospholipase* A_2, were incorporated in existing membrane cleaning regimes. The cleaning efficiency of each enzyme was determined by the amount of lipids removed from fouled PSM. All three lipases used were effective in removing adsorbed lipids, although their efficiency in removing specific lipid types varied.

3. The effect of membrane pre-treatment with detergents such as Triton® X-100, Tween® 20 and Pluronics® F108, on the adsorption of lipid foulants onto UF membranes fouled in abattoir effluent, was investigated. It was found that membrane pre-treatment with each detergent had a different effect on lipid adsorption. Pre-coating with Triton® X-100 was the most effective method to reduce the adsorption of all the lipid types investigated. A spectrophotometric method was also developed to determine the amount of Pluronic® F108 in a solution. This could prove to be very useful in future studies.

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OPSOMMING

IN HIERDIE STUDIE IS:

1. Lipiede wat voorkom in abattoir afvalwater, sowel as lipiede wat adsorbeer aan ultrafiltrasie membrane tydens kontak met abattoir afvalwater, is chromatografies gefraksioneer. Vier lipied klasse is geidentifiseer en gekwantifiseer. Die lipiede teenwoordig was, triglisieriede, cholesterol esters en vry cholesterol, en fosfolipiede.

2. Met resultate verkry vanaf kwantifiseringstudies, is verskeie lipases asook *fosfolipase* A_2 in bestaande membraan skoonmaak prosedures inkorporeer. Die skoonmaak vermoë van elke ensiem is bepaal deur die hoeveelheid lipiede wat vanaf die polisulfoon membraan verwyder kan word. Alhoewel al drie ensieme wat getoets was, suksesvol was vir lipiedverwydering vanaf die membraanoppervlak, het hulle verskil ten opsigte van hulle aktiwiteit met die verskillende lipiede bespreek.

3. Die effek wat voorafbehandeling van die membrane met detergente soos Triton® X-100, Tween® 20 en Pluronics® F108 op die adsorpsie van lipiede aan membrane bevuil in abattoir afvalwater het, is ook ondersoek. Dit was bevind dat elke detergent aanleiding gee tot 'n verskillende effek op die adsorpsie van die verskillende lipiede. Voorafbehandeling van ultrafiltrasie membrane met Triton® X-100 was die mees effektiewe metode om die adsorpsie van alle lipiede aan die membraanoppervlak te verminder. 'n Spektrofotometriese metode was ook ontwikkel om Pluronic® F108 in 'n oplossing te meet. Dit kan van groot hulp wees in toekomstige studies.

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To My Grandmother

For everything you meant to me,

and for all the woman you were.

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ABBREVIATIONS:

PSM(s)	Polysulphone membrane(s)
UF	Ultrafiltration
DEAE	Diethylaminoethyl
TLC	Thin-layer chromatography
ATP	adenosine triphosphate
G-1-P	Glycerol-1-phosphate
ADP	Adenosine diphosphate
DAP	Dihidroxyacetone phosphate
AAP	Aminoantipyrine
ESPA	Sodium N-ethyl-N-(3-sulfopropyl)m-anisidine
rpm	Revolutions per minute
PLs	Phospholipids
GK	Glycerol kinase
GPO	Glycerol phosphate oxidase
PDO	Peroxidase

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CHAPTER 1

INTRODUCTION

All over the world industries like abattoirs, woolscouring processes and dairies produce excessive amounts of wastewater. Ultrafiltration has proven to be an effective filtration process towards the purification and recycling of biological effluents of such origin [1]. The content of these biological and industrial effluent streams, however, include animal fat, lanolin, protein, sand and clay which cause irreversible fouling of ultrafiltration membranes.

Ultrafiltration is a membrane process that retains macromolecules above a certain size from a solution [1]. Ultrafiltration is a very versatile and widely employed pressure-driven membrane operation [2]. This pressure-driven technique is widely utilized for fractionation, purification, separation and concentration of water-soluble solutes or water dispersible materials [3]. Applications for the technology can be found in the chemical industry, paper industry, pharmaceutical industry as well as food and dairy industries. It has been established that some of the macromolecules targeted for separation by ultrafiltration membranes, tend to adsorb onto the membrane surface and into the pores of the membrane material [3]. The extent of adsorption depends on the different types of macromolecule-membrane interactions such as hydrogen bonding, hydrophobic/hydrophilic interactions, Van der Waals interactions and electrostatic effects. Moreover, due to the retention of the macromolecules by the membrane, a layer of high concentration exists near the surface of the membrane during filtration. This phenomenon is known as concentration polarization. Concentration polarization contributes to the formation of a gel layer on the membrane surface. This layer acts as a resistance to transport and hinders product flow through the membrane [3].

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The most serious limitation of ultrafiltration membranes, especially those made from hydrophobic polymers, is the continuous decline of product flux through the membrane brought about by fouling. Permeation rates of solutions containing macromolecular species are much lower than pure-water permeation rates, and decrease rapidly as the filtration operation continues. Flux decline can have a significant negative impact on the feasibility and economics of the ultrafiltration process and for this reason measures must be taken to prevent or minimize its occurrence [3].

Various means are employed to minimize fouling. These are either (i) operational procedures, applied during the ultrafiltration process, such as:

- adjustment of operating conditions such as pH, temperature, operating pressure, water recovery etc.;
- employing appropriate pre-treatment strategies combined with proper selection of membrane modules;
- chemical cleaning of the membrane;

or (ii) fundamental modifications targeting the membrane itself, such as:

- membrane modifications [3].

Most commercial ultrafiltration (UF) membranes are manufactured from hydrophobic polymer materials as these materials are chemically, physically and mechanically more robust than their hydrophilic counterparts. Although the chemical resistance and mechanical properties of these membranes allow them to be used under sometimes harsh and hostile conditions, their hydrophobic properties can often be the cause of severe loss of flux due to fouling [4].

Effective chemical cleaning is the only way to remove adsorbed foulants to restore membrane performance. In some instances mechanical and thermal means were used to restore membrane performance loss associated with fouling. Chemical cleaning agents such as alkalis, detergents, sequestrants and

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acids were also employed in membrane cleaning techniques [5]. Although these methods proved to be successful in removing most of the foulants, the membrane surface was severely damaged by the harsh chemical cleaning conditions [5].

A need therefore exists to find effective and "membrane-friendly" cleaning agents for the removal of foulants. The use of enzymes to clean ultrafiltration membranes fouled in industrial effluents of biological origin, are potentially advantageous, because they are commercially available in large quantities and are very specific towards their substrates [6]. Substrate specificity has its advantages as well as disadvantages. Previous cleaning studies conducted on membranes fouled in abattoir effluent, employed random lipases to remove adsorbed lipids [7]. This was because the exact composition of the adsorbed lipids had not yet been identified. However, once the lipid classes that adsorbed onto the membranes are identified, more substrate specific enzymes, that may prove to be more effective in lipid removal may be chosen.

Previous studies by Maartens [7] on abattoir effluent, showed that lipids and proteins are the major contributors to membrane fouling. Membrane fouling studies performed with these effluents also indicated that multilayer adsorption occurred. The surface of polysulphone membranes (PSM) adsorbed lipids much more readily than proteins due to the hydrophobic nature of the membrane surface [7]. The adsorbed lipids provided the ideal surface for proteins, with both hydrophilic and hydrophobic groups, to adhere to the membrane surface causing irreversible membrane fouling.

The above information prompted a study in which the use of substrate specific enzymes, specifically lipases and proteases, were investigated in cleaning regimes[1]. A number of commercially available lipases and proteases were evaluated in cleaning regimes for PSMs statically fouled in abattoir effluent.

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[1] Z. Allie, Dept. Biochemistry, University of Stellenbosch, MSc. thesis submitted.

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Although the enzymes evaluated proved effective in obtaining a high lipid removal percentage, not all adsorbed lipids could be removed. Greater success was achieved with the removal of proteins from the membranes. Table 1 shows the specific activity of various lipases and proteases used in this study during cleaning trials performed on membranes fouled in abattoir effluent.

Table 1. Efficiency of lipase and protease enzymes.

Enzyme source	[Enzyme] (mg/ml)	Lipase activity *(U/ml)	Protease activity (U/ml)	[Enzyme] (g/600ml)
Candida lipase	1.00	**0.78 (± 0.28)**	***	0.39
Pseudomonas lipase	10.00	**0.59 (± 0.02)**	***	5.05
Aspergillus lipase	10.00	**0.43 (± 0.04)**	***	6.91
****Bacillus*** protease	0.0005	***	**2.46 (0.09)**	0.31
Protease A	0.005	***	**0.28 (0.02)**	0.11
Aspergillus protease	0.005	***	**0.22 (0.01)**	0.13

*One unit (U) of lipase activity is defined as the amount of enzyme that releases one μmole of fatty acid per minute.
Protease activity is defined as the amount of casein (mg) hydrolysed per one mg of protease under the specified assay conditions (0.5 U were used for all lipases and 10.0 U for all proteases except for the ***Bacillus* protease where 2500 U was used)
***These enzyme activities are insignificantly low

It was found that nearly 95% of the adsorbed protein material could be removed by these enzymes, while only 65% total lipid removal was achieved. Lipid adsorbance – and subsequent membrane fouling – thus still remained a problem. However, before the search for an enzyme with greater specificity towards lipids could begin, it was essential to first fractionate and characterise the adsorbed lipids in order to identify exactly which of the lipid classes were most responsible for fouling.

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Accordingly the intention of this study was to:

1. fractionate and characterise the lipids found in abattoir effluent;
2. identify those lipids that adsorb onto PSM during static adsorption tests, and identify those that remain on the membrane after enzymatic cleaning;
3. pretreat the membranes with non-ionic surfactants and identify those lipids that adsorb onto PSM during static adsorption tests;
4. after identifying the lipid class most responsible for fouling, establish whether a more substrate-specific enzyme would increase lipid removal.

In Chapter 2 of this thesis, the chemical and physical properties of lipids, as well as the various techniques of lipidology, are discussed. Chapter 3 describes the extraction and fractionation of lipids present in abattoir effluent and those found on fouled membranes. In Chapter 4 the use of lipases to clean membranes statically fouled in abattoir effluent is discussed. The cleaning potential of *Phospholipase* A_2 is discussed in Chapter 5. Chapter 6 deals with the pre-treatment of PSM and how this treatment affects subsequent fouling by the different lipid classes. In Chapter 7 the conclusions reached in this study is given.

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CHAPTER 2

LIPIDS

2.1 INTRODUCTION

Lipids are defined as substances which are: (a) insoluble in water, (b) soluble in non-polar organic solvents such as chloroform, ether or benzene, (c) contain long-chain hydrocarbon groups in their structure, and (d) are present in or derived from living organisms [8]. Lipids differ strikingly from the other two major classes of tissue components, the proteins and carbohydrates, especially with respect to their solubility characteristics.

Lipids can be classed into two major groups, neutral lipids and polar lipids. Polar lipids are those that contain a polar group. Examples of these polar groups are: (i) the phosphate group and "base" of the phospholipids, (ii) the sulphate group of the sulpholipids or (iii) the sugar moiety of the glycolipids [9]. Neutral lipids contain no such areas of polarity and they include free fatty acids, simple sterols, sterol esters and glycerides. The most readily observable differences between the two classes are in their solubility and chromatographic properties. Neutral lipids are much more readily soluble in completely apolar solvents such as hydrocarbons and during chromatography are much more easily eluted by these solvents than polar lipids [9]. In this study it was found that the three major lipid classes present in abattoir effluent were glycerides, cholesterol esters and phospholipids.

Glycerides are esters of the trihydric alcohol, glycerol, and fatty acids. In triglycerides all three glycerol hydroxy groups are esterified [Fig 2.1]. Glycerides

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are the chief constituents of natural fats (solids) and oils (liquids), although it is important to remember that natural fats and oils also contain minor proportions of other lipids [9].

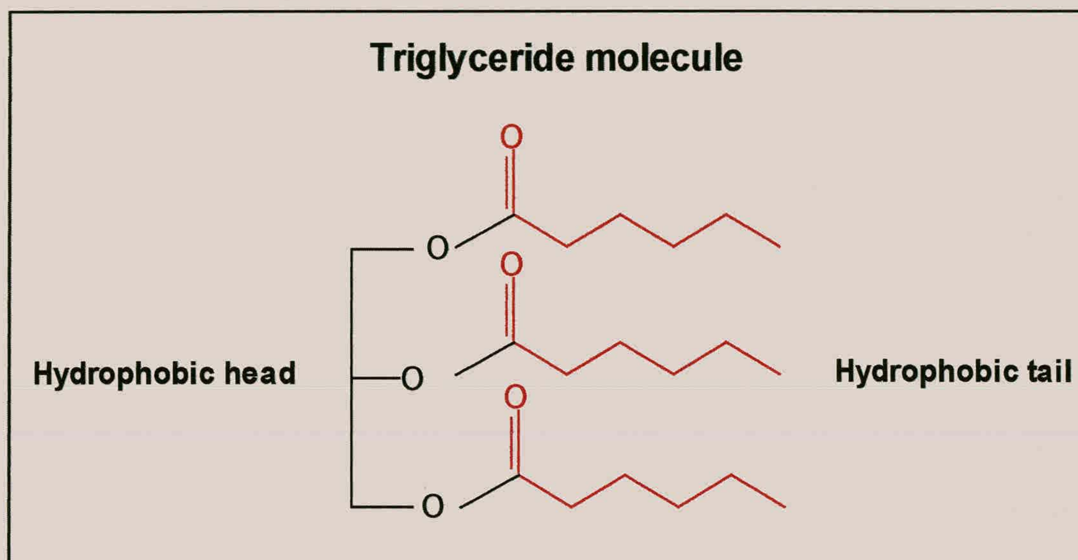

Fig 2.1. Structure of a triglyceride molecule with its non-polar/hydrophobic head and tail.

In many organisms and tissues, cholesterol exists as a mixture of the free alcohols and their long-chain fatty esters. Although cholesterol is present in most mammalian body tissues, the proportion of cholesterol ester to free cholesterol varies markedly. For example, blood plasma, especially that of man, is rich in cholesterol and like most plasma lipids are almost entirely found as components of the lipoproteins. About 60-80% of this cholesterol is esterified. In the adrenals too, where cholesterol is an important precursor of the steroid hormones, over 80% of the sterol is esterified. Only in the brain and other nervous tissue, where cholesterol is a major component of myelin, virtually no cholesterol esters are present [9].

Phospholipids are mixed esters of fatty acids and phosphoric acid with the alcohols glycerol or sphingosine. They derive their lipid properties from the long-

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chain fatty acid moieties, but also have a considerable polar character donated by the ionization of the phosphate and base groups. In physical properties they "bridge the gap" between the completely water insoluble neutral lipids and molecules which form true aqueous solutions [9]. The chemical structures of a phospholipid and cholesterol ester molecule can be seen in Fig 2.2.

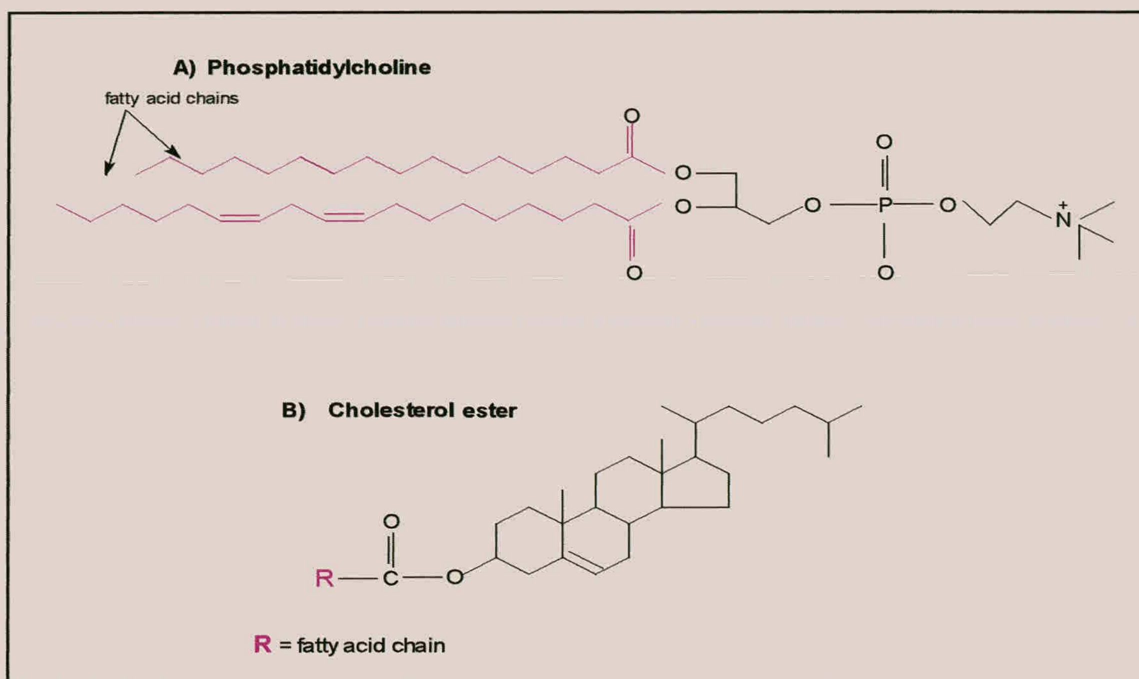

Fig 2.2 Chemical structures of A) phosphatidylcholine and B) cholesterol ester.

The low solubility and hydrophobic nature of lipids give rise to particular problems during the treatment of abattoir effluent with ultrafiltration and microfiltration membranes [6]. Abattoir effluent consists primarily of blood and tissue from slaughtered animals and it contains large amounts of proteins as well as fat. It has been shown in earlier studies that the forces which bind individual lipid molecules together in aggregates, and those stabilising large protein molecules and maintaining the secondary and tertiary structure, are of a similar kind. It is also known that discrete chemical complexes containing lipids and proteins, which we call lipoproteins, exist in cells. There are thus clearly certain forces

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present in cells that bind lipids to proteins. The main forces in play between lipids and proteins are hydrophobic forces and hydrogen bonds or electrostatic forces.

As discussed earlier, the hydrophobic surface of ultrafiltration PSM provides the ideal surface for lipid adsorption. Previous studies have shown that these adsorbed lipids cause irreversible fouling and concomitant flux decline [6]. The lipid classes found in blood are long-chain hydrocarbons, fatty acids, glycerides, phospholipids and cholesterol. The main question that arises now is which of these classes are most responsible for the fouling. Additionally, is there a notable difference in the extent at which these lipids are removed during enzymatic cleaning of the fouled membranes?

2.2 ENZYMATIC HYDROLYSIS OF LIPIDS

Lipases are lipolytic enzymes which are found in many microorganisms, plants and higher animals. They exist in solid as well as liquid form and the most unusual feature of all lipases is that, although they are water-soluble, they catalyze heterogeneous ester hydrolysis processes at the lipid-water interface. The reactions therefore occur exclusively at the interface generated by the hydrophobic lipid in a hydrophilic aqueous medium. The rate of lipolysis is directly determined by the concentration of the substrate molecules at this interface [10-13].

The initial step in lipase hydrolysis is the splitting of the fatty acids esterified to the primary hydroxyls of glycerol. This reaction is not stereospecific and the fatty acids in the 1- and 3-positions are initially removed at equal rates. Once one fatty acid has been removed, however, the resulting diglycerides and subsequently the monoglycerides are more slowly hydrolysed than the original triglycerides. The preference to remove the 1 and 3 fatty acids, together with the diminished rate of hydrolysis of partial glycerides, results in an accumulation of

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monoglycerides as the primary products of lipase hydrolysis. Lipase not only hydrolyses fatty acids on the outer position of glycerides, but will also liberate the esterified fatty acid in the 1-position of phosphoglycerides [9].

There is a dramatic increase in lipase activity when the substrate is presented in an insoluble phase; this is referred to as interfacial activation. The interfacial activation phenomenon is one of the most characteristic properties of lipase-catalyzed reactions. Interfacial lipase-catalyzed reactions involve binding of the enzyme to the emulsified substrate followed by catalysis at the interface. This fact is of particular importance when these enzymes are applied to remove lipids adsorbed onto ultrafiltration PSMs [7].

Lipase activity can be increased by the addition of Triton X-100. The detergent creates an oil-water interface which greatly enhances the activity of the enzyme. In addition, Triton® X-100 interacts with foulants causing a less dense foulant layer that is more accessible to the enzymatic reactants. Maartens *et al* [14] demonstrated that the addition of 0.1 % Triton® X-100 improved the effectiveness of lipases to remove lipids from fouled PSM.

2.2.1 Triglyceride specific enzymes

In another study (Z. Allie, MSc in preparation)[1], the use of three random lipases from *Candida cylindraceae, Pseudomonas mendocina* and *Aspergillus oryzea*, were investigated in cleaning regimes for ultrafiltration membranes statically fouled in abattoir effluent. The lipase *from Candida* is a random lipase which hydrolyzes the triglyceride molecule at all three ester bonds.

The hydrolysis reactions are rapid and complete. The lipases from *Pseudomonas* and *Aspergillus*, however, are 1,3-specific lipases which hydrolyze the triglyceride molecule primarily at the C1 and C3 atoms [15-16]. The reaction mechanism of the three lipases are depicted in Fig 2.3.

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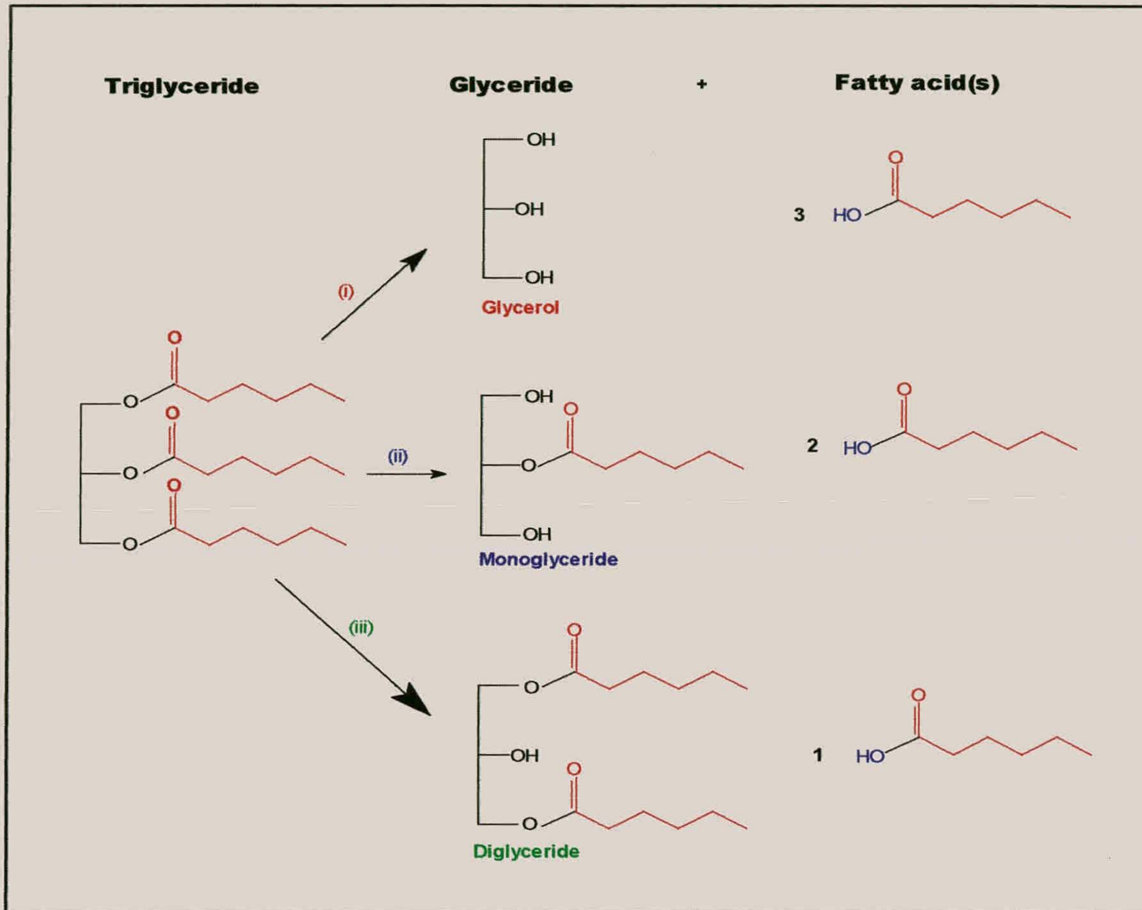

Fig 2.3. *Schematic presentation indicating the regioselectivity of lipases catalyzing the hydrolysis of a triglyceride (TAG) molecule with **(i)** a random lipase, **(ii)** a sn-1,3 specific lipase and **(iii)** a sn-2 specific lipase.*

These enzymes proved to be effective in removing adsorbed lipids from membranes fouled in abattoir effluent [14]. They are, however, specific for triglyceride hydrolysis, and because the type of lipids adsorbed onto PSM during contact with abattoir effluent were unknown, it could not be assumed that all adsorbed lipid classes were removed effectively by this enzyme.

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2.2.2 Phospholipase A

Phospholipase A is an enzyme that catalyses the hydrolysis of one fatty acid residue from lecithin and similar substrates. Early chemical analyses of the products of *phospholipase A* hydrolysis seemed to indicate that the enzyme released the fatty acid from the 1-position. When it was discovered that the enzyme also liberates fatty acid from plasmalogen (where it is known quite definitely to be located at the 2-position), the hydrolysis of lecithin was reinvestigated. It was determined that the enzyme required the presence of only one fatty ester linkage adjacent to the alcohol-phosphate bond and that the carbon atom to which this fatty acid is attached, must have a precise stereochemical configuration [9].

These enzymes are widely distributed and occur in the venoms of snakes, bees, scorpions and in several mammalian tissues. The *phospholipase A* from the venom of the common European honey bee (*Apis mellifica*), has been completely purified and was used in this study. Amino acid analysis of this enzyme shows the presence of large amounts of aspartic acid, threonine, glycine and serine. The stability of the enzyme can be explained by the six disulphide bridges present in its structure. In water, at pH 8.0, the enzyme is highly active towards the substrate and this activity is increased by the addition of calcium ions which is presumed to be the enzyme's natural activator. It is assumed that the calcium ions are involved in the binding of the substrate and enzyme. Use of the synthetic substrate, 1-oleoyl, 2-isolauroyl phosphatidyl ethanolamine, shows that the enzyme hydrolyses the ester linkage at the 2-position of the glycerol skeleton (Fig 2.4). The enzyme from bee venom is thus a phospholipase of the A_2 type [17].

Phospholipase A_2, like the microbial lipases mentioned earlier, also act on substrates that are part of macromolecular aggregates. *In vitro* kinetic studies have suggested that the lipid-water interface of these aggregates plays an

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important role in the enzymatic activity of *phospholipase* A_2 [17]. For this reason *phospholipase* A_2 from bee venom was employed in enzymatic cleaning trials to investigate its efficiency to remove lipids adsorbed onto ultrafiltration membranes.

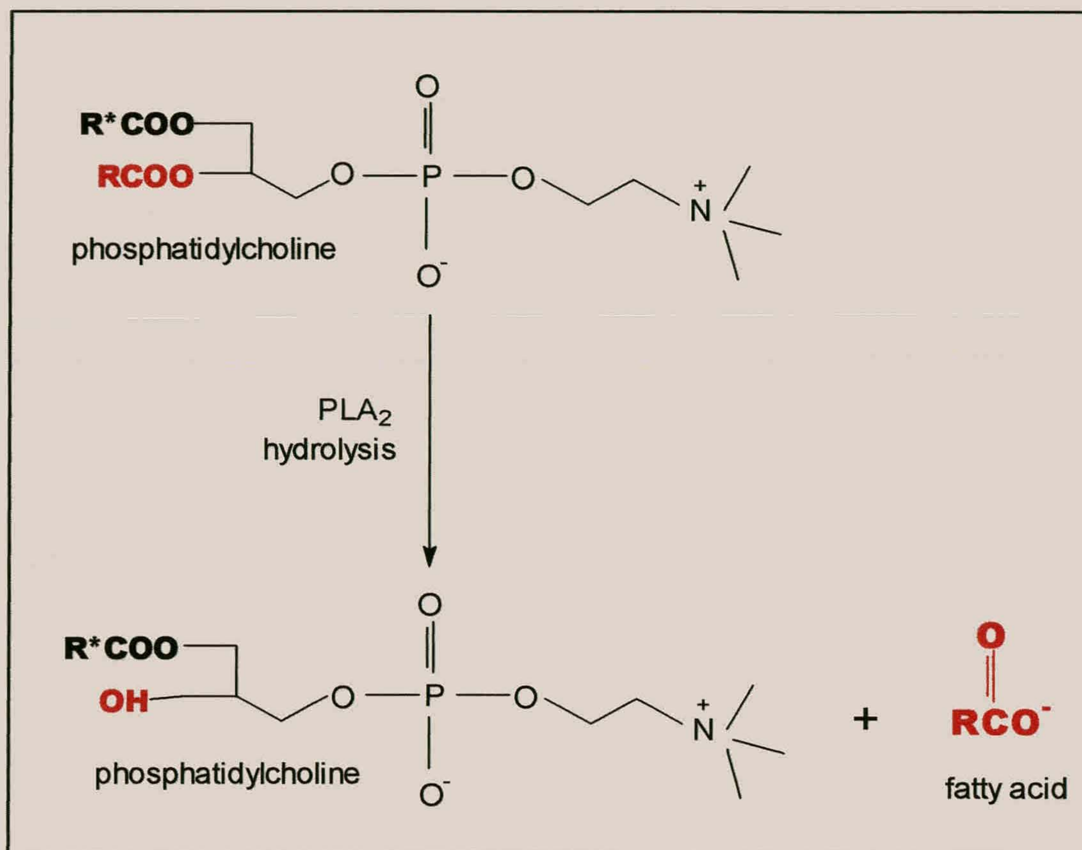

Fig 2.4. Schematic presentation of phosphatidylcholine hydrolysis by *phospholipase* A_2 *(PLA_2).*

The acyl chain composition of phospholipids depends on their origin, they are therefore non-homogenous materials with mainly saturated acyl chains at the sn1-position and unsaturated acyl chains at the sn2-position (Fig. 2.5) [18].

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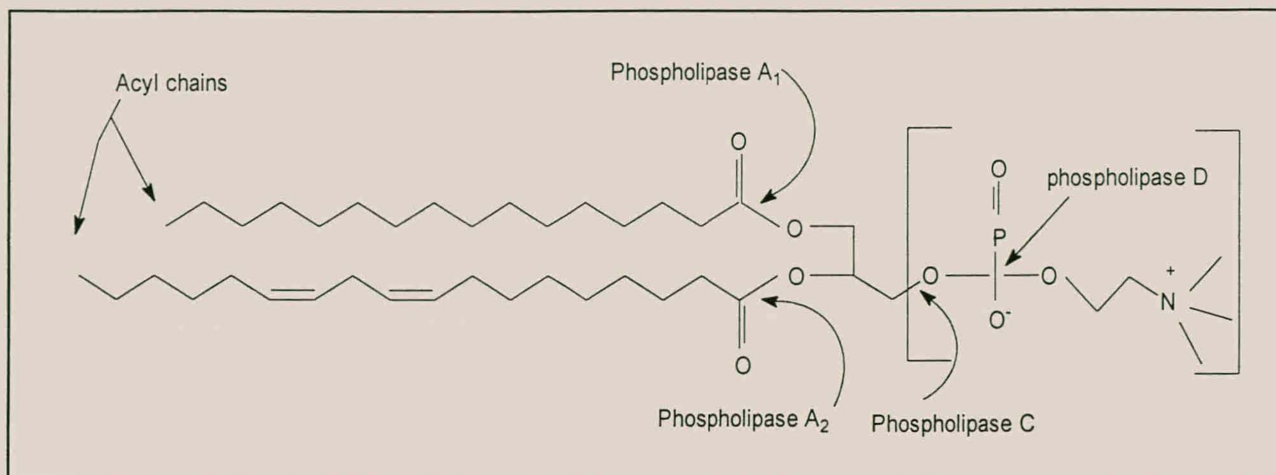

Fig 2.5. Phospholipases acting on phosphatidylcholine and their site of action. There is a specific enzyme catalysing the selective hydrolysis of each functionality [27].

Phospholipids (PLs) have two carboxylic ester bonds and two phosphate ester bonds. Each of the four major phospholipases selectively recognise each of the four ester bonds (Fig 2.5). Other enzymes can recognise the ester bonds in partially hydrolysed PLs, but these enzymes are less common and are not useful for biocatalytic applications [18,19]. All the phospholipases with potential applications are hydrolytic enzymes that are often secreted outside the cell. They can catalyse both the hydrolysis and the reverse reaction, i.e. the ester bond formation in the presence of a suitable acceptor or donor. The enzyme used in this study to hydrolyse the phospholipids adsorbed onto PSM will be described in Chapter 5.

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CHAPTER 3

SEPARATION AND CHARACTERISATION OF LIPIDS PRESENT IN ABATTOIR EFFLUENT AND ON FOULED MEMBRANES

3.1 INTRODUCTION

Lipases and proteases investigated in a previous study, proved to be successful in removing a fraction of adsorbed lipids[1]. However, these enzymes could not remove all the adsorbed lipids from the membrane surface. Before the search for a more effective enzyme could be carried out, the lipid classes present in abattoir effluent had to be identified and characterised. This entailed the extraction of lipids from abattoir effluent as well as from fouled membranes, and the subsequent separation of these lipids by silica gel chromatography. The extraction and separation procedures for lipids present in the effluent and on the membranes are discussed in detail in this chapter.

An ideal lipid extraction procedure should quantitatively extract lipids in an undegraded state, free of contamination with non-lipid components such as free sugars and proteins [8]. The effectiveness of the procedure will, to a large extent, depend on the chemical nature of the lipid components and the kind of complex or association in which they occur in the animal cell. There are three main types of associations in which lipids participate: (a) Van der Waals or hydrophobic association, (b) hydrogen bonding, electrostatic and hydrophobic association and

[1] Z. Allie, Dept. Biochemistry, University of Stellenbosch, M.Sc. Thesis submitted.

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(c) covalent bonding [8]. Complete extraction of lipids from any biological sample must take into account the fact that tissue structure may present formidable barriers to solvent access. It is therefore customary to first disrupt the native structure of the material mechanically, and then to employ a mixture of solvents, one of high and one of low polarity, in order to favour ready penetration of persistent biomembrane-enclosed domains. It has also been shown that a mixture of hexane and isopropyl alcohol is most effective for this purpose, and has the advantage of being less toxic than previously used diethyl ether-ethanol and chloroform-methanol mixtures [8].

After extraction of the tissue or cellular lipids, the next stage in the investigation of lipid composition involves the fractionation of the mixture into the various lipid classes and then into individual components [21]. The exact fractionation procedure to be used will depend largely on the particular classes of lipids present. Most animal lipids consist of about 60-85% phosphatides and glycolipids, the remainder being neutral or non-polar lipids such as glycerides, sterols, hydrocarbons and pigments [22].

A variety of techniques has been developed for the separation of lipids. These techniques include: thin-layer chromatography (TLC), gas-liquid chromatography (GLC) and high-pressure liquid chromatography (HPLC) [8]. The technique most applicable for this study, was one that could separate lipids into its major lipid classes, not giving a complete separation of polar and non-polar lipids. Silica gel chromatography was therefore used.

The development of chromatographic methods has made fractionation procedures in lipid chemistry and biochemistry more manageable [21,22]. The problem of resolving lipid extracts from natural sources into separate molecular species on a preparative scale, has been investigated most successfully by column chromatography in silicic acid. Whether separation would be achieved most successfully by using silicic acid or any other adsorbent is a matter for

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speculation, or rather, for future investigation. The work of Vance [23] established that a silicic acid column can separate a natural lipid mixture into two classes, "neutral lipids" and phospholipids, and can also resolve these into other classes like triglycerides and sterols. This ability to differentiate between lipid classes accounts chiefly for the present day popularity of silicic acid as an adsorbent. Moreover, since silicic acid adsorbents are unreactive at room temperature, 100% of the lipids can usually be recovered after chromatography [22].

In column chromatography studies a solid phase such as silica gel, DEAE cellulose or carboxymethyl cellulose is used. Depending upon the lipids being separated, solvent mixtures with different polarities such as ether and methanol are used. In silica gel chromatography, lipid compounds interact with the solid adsorbent by polar and ionic forces and to a smaller extent by non-polar forces. Separation of lipid mixtures thus takes place according to the relative polarities and charge of the individual lipid species. The lipid mixture loaded onto the column is eluted from the column with petroleum ether and diethyl ether mixtures in the following order of increasing polarity: stearyl esters, triglycerides, free fatty acids, sterols, diglycerides, monoglycerides, phospholipids [8].

Because of overlapping polarities, complete separation of the polar phospholipid classes is not normally achieved by a single column chromatography step [22]. Further fractionation, using preparative thin-layer chromatography (TLC) or another type of column chromatography, is usually necessary to isolate pure individual lipid classes.

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3.2 EXPERIMENTAL

Materials

The organic solvents used in this study, petroleum ether, diethyl ether and methanol, were of analytical grade and were obtained from NT Laboratory Supplies, Excom, Johannesburg, South Africa. All membranes used were flat sheet PSMs prepared from Udel P 3500 polysulphone cast from N-methyl 2-pyrrolidone solutions, obtained from the Institute of Polymer Science, University of Stellenbosch, South Africa. Membranes were stored in distilled water at 4°C with sodium azide (5mg/l) to prevent bacterial growth.

Silica gel (100-200 mesh) was obtained from Sigma Chemical Co., St Louis, USA. Lipid samples loaded for analyses were extracted from abattoir effluent and from PSMs fouled in abattoir effluent with a mixture of hexane:isopropanol (3:2 v/v).

Sigma Diagnostics Triglyceride Kit [GPO-Trinder], [Procedure No.337] was used for the measurement of true triglycerides. The kit contains a Triglyceride [GPO-Trinder] Reagent A and a Triglyceride [GPO-Trinder] Reagent B, as well as two glycerol standards of 250 mg/dL and 500 mg/dL [24].

Ammonium thiocyanate, ferric chloride and chloroform used in the phospholipid assay were all of analytical grade and were obtained from NT Laboratory supplies, Excom, Johannesburg, South Africa. L-α-phosphatidylcholine used in the same assay as a phosholipid standard, was obtained from Sigma Chemical Company, St. Louis, USA. Throughout the assay a standard solution of ammonium ferrothiocyanate, prepared by dissolving 27.03 g ferric chloride hexahydrate ($FeCl_36H_2O$) and 30.4 g ammonium thiocyanate (NH_4SCN) in deionized water (1L), was used. This solution was stable at room temperature

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for months [25]. A standard solution of 10 mg phosphatidylcholine in 100 ml chloroform was made in order to set up a calibration graph.

For the determination of the free cholesterol and cholesterol ester concentrations, a ferric chloride stock solution was made as follows; 2.5 g $FeCl_3 \cdot 6H_2O$ was dissolved in concentrated (85%) orthophosphoric acid. The resultant mixture was stored in a brown glass-stoppered bottle at room temperature and discarded when a precipitation occurred. The colour reagent was prepared by carefully diluting 4 ml of stock ferric chloride solution to 50 ml with concentrated sulfuric acid, cooling it down and storing it at room temperature. The colour reagent was discarded when the mixture became cloudy. The digitonin solution (1%) was prepared by dissolving 1 g of digitonin in 50 ml of 95% ethanol and diluting it to 100 ml with water. The solution was stored in a dark bottle and stored in a dark place. The standard cholesterol solution (0. 1%) was prepared by dissolving 100 mg of pure dry cholesterol in 100 ml glacial acetic acid [26].

3.2.1 Extraction of lipids from abattoir effluent

The effluent used for analyses was derived from the abattoir process effluent stream arriving from the slaughter process areas. Effluent was collected for a period of four hours from the Maitland abattoir in Cape Town and preserved at 4°C with sodium azide (1mg/200 ml) until analysis. Effluent samples were collected in 1L pyrex glass bottles [7].

Effluent samples (50 ml) were lyophilized and the total lipids extracted as follows: 50 ml hexane:isopropanol (3:2 v/v) was shaken up with the lyophilized residue and the resultant mixture boiled mildly, at about 70°C, for 15 minutes. The hot solution was filtered through a Whatman no 40 filter paper and the filtrate collected. The flask that contained the lyophilized residue was rinsed with

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another 30 ml of heated solvent mixture, filtered and recombined with the first extract. The amount of lipid present was determined gravimetrically after the volatile phase of the solution was removed under nitrogen at 40°C [7]. The lipid sample thus obtained was separated chromatographically and also used in further analyses.

3.2.2 Extraction of lipids from fouled membranes

Flat sheet PSMs were statically fouled in abattoir effluent for a period of 4 h. Rectangular membrane sections (10 x 32 cm) were affixed to a Perspex frame containing two 120 mm posts equipped with lead weights to keep the frame submerged. The PSM strips were clamped horizontally to the 120 mm posts and immersed into the abattoir effluent. After fouling, the membranes were washed thoroughly with tap water, placed in distilled water and preserved with sodium azide (5mg/l) at 4°C until analyses were done [7].

Fouled membranes were cut into 25 cm^2 squares and dried at room temperature. The dried membrane squares were shaken vigorously with 50 ml hexane:isopropanol (3:2 v/v) for 5 min and left at room temperature for 12 h. The extraction solution was subsequently boiled mildly at 70°C for 15 min and filtered through a Whatman No. 40 filter paper. The membrane squares, filter paper and flask were rinsed with an additional 20 ml preheated hexane:isopropanol (3:2 v/v), and the filtrate recombined with the first filtrate. The amount of the lipid residue was determined gravimetrically after the volatile phase of the solution had been removed under nitrogen at 40°C [7]. The lipid sample obtained was separated chromatographically and also used for further analyses.

3.2.3 Lipid separation with column chromatography

The plasma lipid classes were separated by the use of silica gel chromatography. In order to remove extremely small particles the silica was suspended in 250 ml

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methanol [27,28]. After 5 minutes of settling, the methanol was decanted. This procedure was repeated once with methanol and twice with diethyl ether. The remaining silica was dried in air and finally activated at 120°C for 24 hours.
Sixty g of the activated silica gel was suspended in ether and rapidly transferred to a glass column. The silica column, which was approximately 18 cm in height, was washed with the following dehydrated solvents: 100 ml of acetone-ethyl ether (1:1, vol/vol), followed by 100 ml of ether. Complete removal of the dehydrating solvents was then achieved by washing the column with 1 L of light petroleum (bp 60 to 80°C) for about 12 hours. A 60 g column was charged with approximately 1 g of lipid [27].

The 1g of lipid to be separated was dissolved in a small volume of light petroleum ether and pipetted on the column. Elution was carried out with solvent mixtures of increasing polarity. The following solvents were used: A) 792 ml light petroleum ether, bp 60 to 80°C with 8ml diethyl ether; B) 644 ml light petroleum with 56 ml diethyl ether; C) 375 ml light petroleum with 125 ml diethyl ether;
D) 250 ml diethyl ether and E) 1.25 L methanol. The flow rate of the solvents was regulated to 3 ml per minute with the stopcock at the lower end of the column. Fractions of the eluate, 25 ml each, were collected and used for gravimetric determination and colorimetric analysis [27].

The three solvents: petroleum ether, diethyl ether and methanol, span a range of solvent polarity and in different combinations provide any desired degree of eluting power for lipids of biological origin [28]. Although some other solvents of the same polarity might replace diethyl ether, Schroeder [28] found that petroleum ether and diethyl ether mixtures provide more complete separations than a number of other solvent pairs. The necessary changes of solvent polarity can be brought about by gradient elution or stepwise changes. Stepwise changing of eluant composition was adopted in this study. Hanahan *et al* [29] found that the use of a gradient did not improve lipid separations studied and in the case of phospholipids, the establishment of a gradient of methanol

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concentration in diethyl ether, actually resulted in a less sharp separation of the components than did an abrupt change.

Each 25 ml fraction was evaporated to dryness and weighed to the nearest milligram. Fraction weights were plotted against fraction number and fractions representing distinct lipid components were pooled in 10 ml petroleum ether for subsequent analysis.

CHARACTERISATION OF LIPID FRACTIONS

3.2.4 TRIGLYCERIDE ASSAY

Methods for triglyceride determination generally involve enzymatic or alkaline hydrolysis of triglycerides to glycerol and free fatty acids followed by either chemical or enzymatic measurement of the glycerol released. The procedure described below is a modification of the method of McGowan, *et al* [30]. McGowan's method does not differentiate between endogenous glycerol and glycerol derived by hydrolytic action of lipase on glycerides.

In the method described here, triglycerides are hydrolyzed by lipase to glycerol and free fatty acids. The glycerol produced is then measured by coupled enzyme reactions catalysed by glycerol kinase, glycerol, phosphate oxidase and peroxidase. The method of Sigma Diagnostics Triglyceride [GPO-Trinder] reagent (Procedure No. 337) can be used for the measurement of glycerol, true triglycerides, or total triglycerides in serum or plasma. The enzymatic reactions involved in the assay procedure are as follows:

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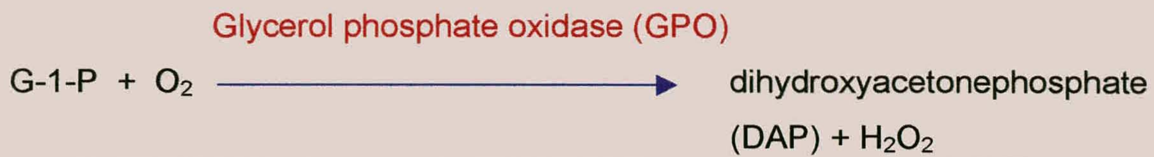
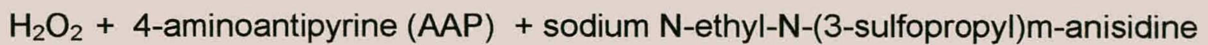
Triglyceride Assay





Peroxidase (POD)

The quinoneimine dye has an absorbance maximum at 540 nm [24]. The increase in absorbance at 540 nm is directly proportional to the triglyceride concentration in the sample.

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3.2.5 PHOSPHOLIPID ASSAY

Phospholipids were measured colorimetrically as L-α-phosphatidylcholine by forming a complex with ammonium ferrothiocyanate [25]. A calibration graph was obtained by adding different volumes from a standard phosphatidylcholine solution (between 0.1 ml and 1.0 ml), to 2 ml ammonium ferrothiocyanate solution in a test tube. Chloroform was added to a final volume of 4 ml. The biphasic system was mixed on a Vortex mixer for 1 min and the lower chloroform phase was removed with a Pasteur pipet. The optical density of the chloroform phase was determined at 488 nm and the average optical density plotted to obtain the standard curve, which was used in subsequent lipid concentration determinations (Fig 3.1). The linear relationship between mg/ml phospholipids and absorbance at 488nm with curve fit (r^2) = 0.9905 was:

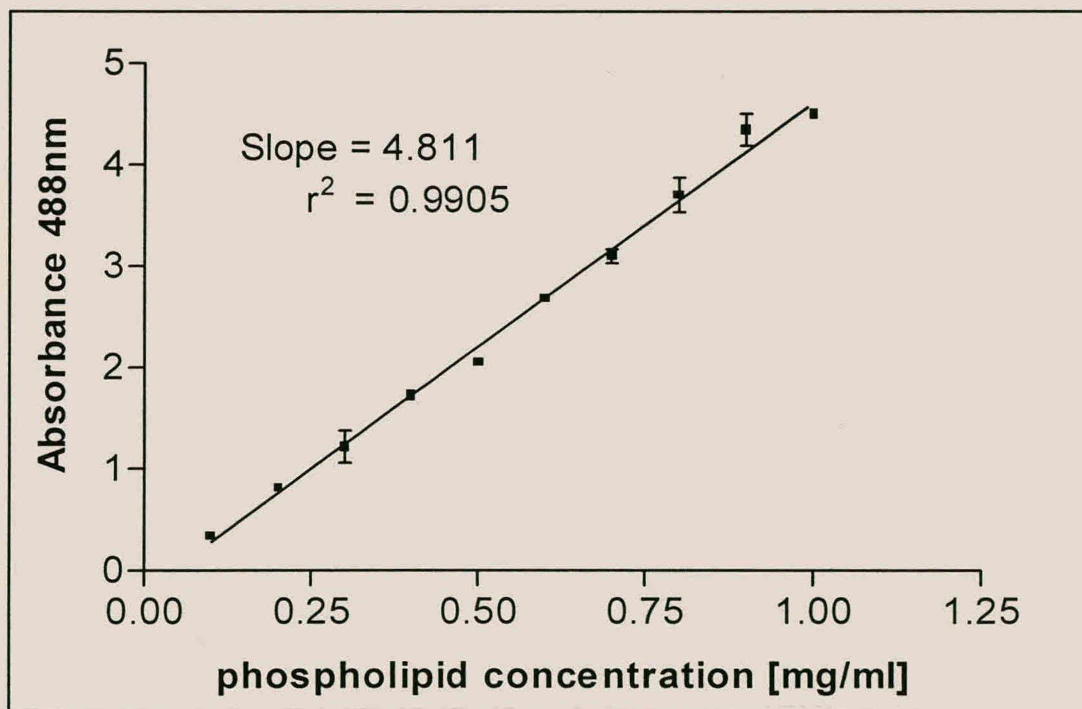

Fig 3.1. Standard curve of phospholipid assay. L-α- phosphatidylcholine concentration vs. absorbance at 488 nm. Each point is the mean of two determinations ± SE.

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The lipid sample to be analysed was dried under a stream of nitrogen and then dissolved in 2 ml chloroform. Two ml ammonium ferrothiocyanate was added to the lipid solution and the solution was thoroughly mixed for 1 minute. After phase separation, the lower chloroform phase was removed with a Pasteur pipette and the OD measured at a wavelength of 488nm [25].

3.2.6 FREE AND TOTAL CHOLESTEROL ASSAY

Free and total cholesterol may be performed on total lipid extracts as well as on purified components; the sum of free cholesterol and cholesterol esters is determined in the total cholesterol determination, and the free unesterified cholesterol is determined by the digitonin procedure [26].

Total cholesterol determination

The lipid sample (0.1g) was dissolved in chloroform and an aliquot of the chloroform solution placed in a test tube. The lipid solution was evaporated to dryness under a stream of nitrogen. Glacial acetic acid (3 ml) was added to the dried sample and the mixture was thoroughly stirred. Two ml of dilute ferric chloride colour reagent was also added to the solution and the resulting solution mixed by inversion or on a Vortex mixer. The assay solution was allowed to cool and after 10 min the absorbance of the purple-coloured solution, was determined at 560 nm against a reagent blank . The assay was calibrated with 0.1 mg, 0.2 mg and 0.3 mg cholesterol standards (Fig 3.2) [26].
The linear relationship between mg/ml cholesterol and absorbance at 560nm with curve fit (r^2) = 0.9744 was:

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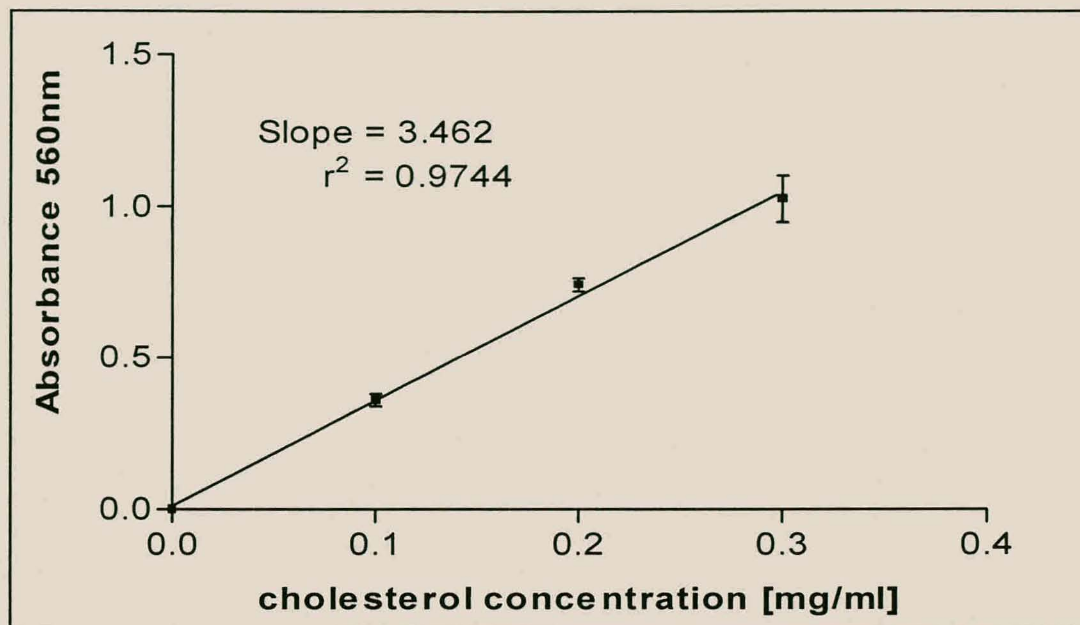

Fig 3.2. Standard curve of cholesterol assay. Cholesterol concentration vs. absorbance at 560 nm. Each point is the mean of three determinations ± SE.

An aliquot of a chloroform lipid solution was placed in a 15 ml conical centrifuge tube and evaporated to dryness under a stream of nitrogen. One ml of acetone-95% ethanol (1:1, v/v) and 1 ml of 1% digitonin solution (m/v) was added to the dried lipid sample whereafter the solution was mixed and left at room temperature for 10 min. The mixture was centrifuged at 1760g for 5 min, the supernatant discarded and the precipitate allowed to drain for 5 min. The residue was suspended in acetone (4 ml), mixed on a Vortex mixer and centrifuged as above. The supernatant was discarded, the precipitate drained for 5 min and finally the last traces of solvent was removed with a gentle stream of nitrogen. The digitonide precipitate was dissolved in glacial acetic acid (3 ml) and the same assay procedure was subsequently followed as for total cholesterol [26].

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3.3 RESULTS AND DISCUSSION

3.3.1 Chromatographic separation of lipids

The separation of lipids found in abattoir effluent and lipids adsorbed onto ultrafiltration PSMs during contact with abattoir effluent, resulted in a typical elution curve as shown in Fig 3.3.

Four major classes of lipids were identified during the separation of lipids found in abattoir effluent (Fig 3.3). This agrees with results of Hallgren, Stenhagen *et al* (1960) [27] and Hirsch and Ahrens, (1958) [31]. The same classes were found to be present in the adsorbed foulant layer of ultrafiltration membranes (Fig 3.4).

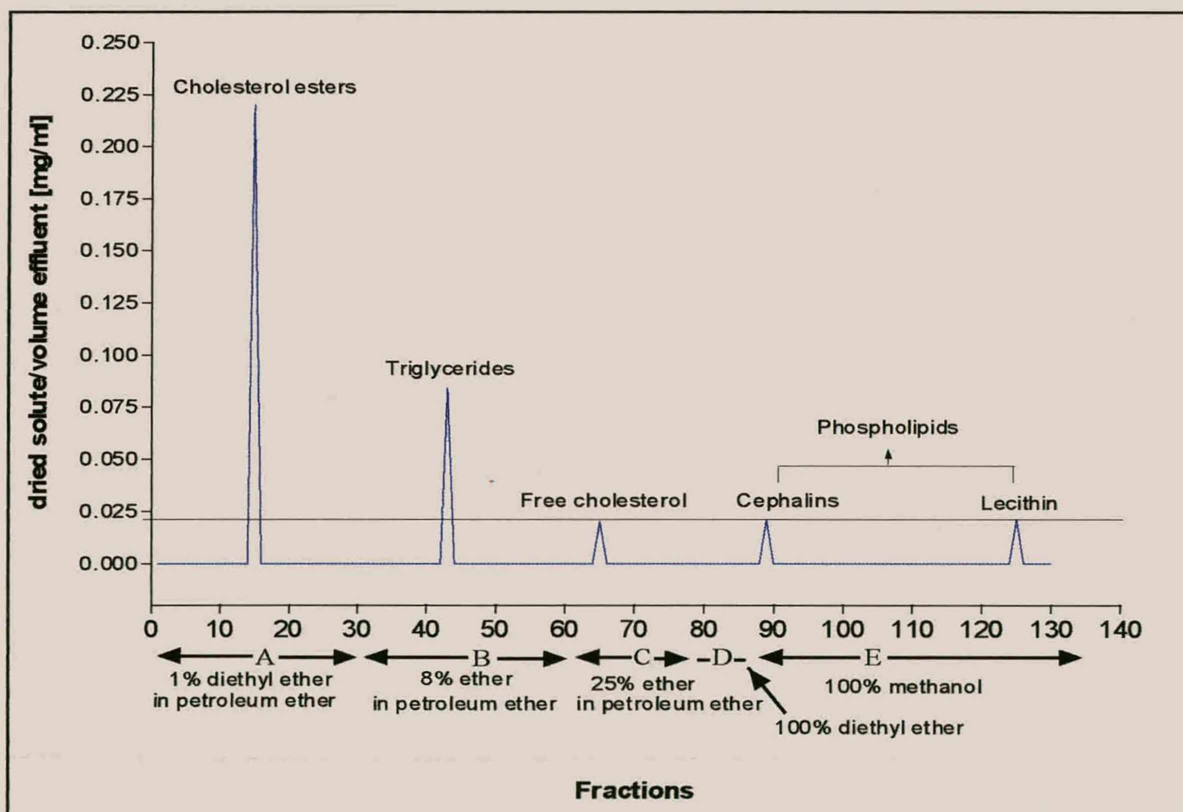

Fig. 3.3 Elution curve of lipids found in abattoir effluent.

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Each lipid class was eluted from the column by a different solvent, the solvents being used in order of increasing polarity. Cholesterol esters, the lipid class with the lowest polarity of the lipids present, eluted from the column first with solvent A. Solvent A contained petroleum ether with 1% (v/v) diethyl ether. The saturated long-chain triglycerides eluted second with solvent B, petroleum ether with 8% (v/v) diethyl ether. The next peak, eluted by 25% of diethyl ether in petroleum ether, contained free cholesterol. Solvent D, 100% diethyl ether, is added to the column to wash off any excess materials. Upon addition of solvent E, 100% methanol, the phospholipids were eluted from the column. The first peak represents the cephalins, as well as phosphorous free lipids. Lecithin leaves the column in the second peak. According to Hirsch and Ahrens [31], fatty acids elute from the column just before the free cholesterol with solvent C, but in this case no significant peaks were seen other than the free cholesterol peak, as is illustrated in Fig 3.3. No further fatty acid analysis was thus performed.

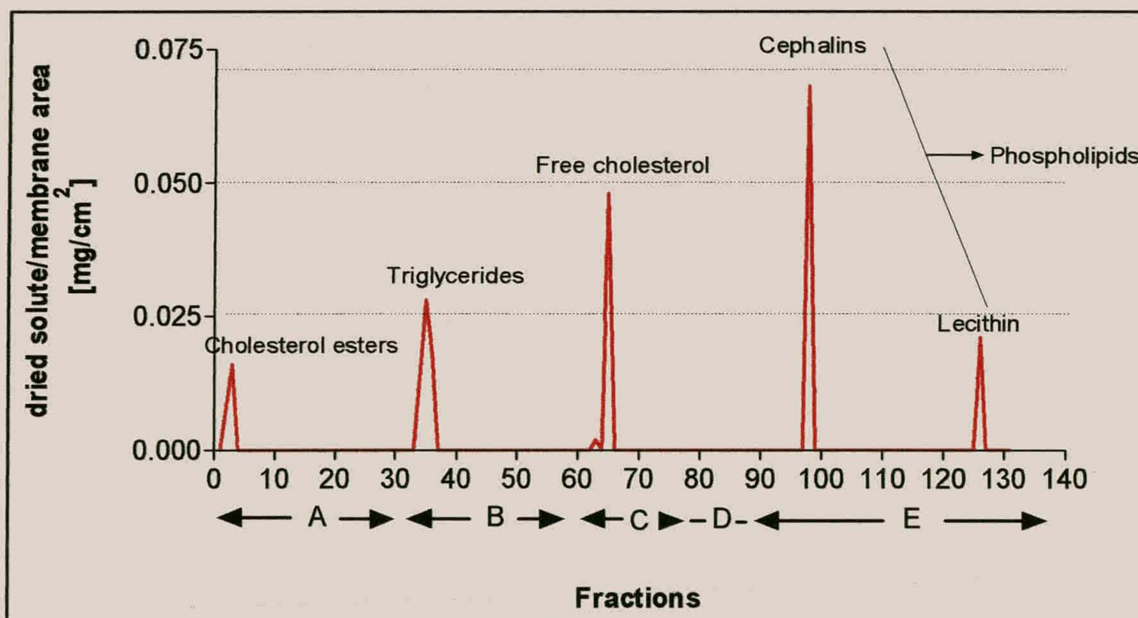

Fig 3.4. Elution curve of lipids adsorbed onto PSM during contact with abattoir effluent. The solvents used were A: 1% diethyl ether in petroleum ether , B: 8% diethyl ether in petroleum ether, C: 25% diethyl ether in petroleum ether, D: 100% diethyl ether and E: 100% methanol.

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It is clear that the lipids found in abattoir effluent and the lipids adsorbed onto PSM have the same composition. However, it is their concentrations that differ and might shed some light on the question of which lipid class adsorbs most onto the membranes. From the elution curve in Fig 3.4 it seems as if phospholipids is the most adsorptive lipid class. In abattoir effluent (Fig 3.3) two small peaks (D) representing phospholipids were obtained after lipid separation. When comparing these two peaks with the other lipids found in abattoir effluent, it seemed as if phospholipids made up only a small fraction of total lipids. In contrast, separation of adsorbed lipids resulted in 3 peaks upon methanol addition. These three peaks, when compared to the other lipids found in the adsorbed layer of PSM, indicates that it is indeed the phospholipids that are adsorbed onto PSM in greater quantities than the other lipid classes.

3.3.2 Phospholipid assay

The red inorganic compound, ammonium ferrothiocyanate, is insoluble in chloroform but forms a complex with L-α-phosphatidylcholine which is freely soluble in chloroform. When a solution of chloroform containing L-α-phosphatidylcholine is mixed thoroughly with ammonium ferrothiocyanate, a coloured complex is formed which partitions in the chloroform phase. The standard curve obtained for concentrations of L-α-phosphatidylcholine up to 0.7 mg in 2 ml of chloroform is shown in Fig 3.1. The standard curve was used to determine the phospholipid content (as L-α-phosphatidylcholine) of each sample.

3.3.3 Cholesterol assay (Free and Total)

The cholesterol assay is based on the same principle as the phospholipid assay, in the sense that here the colour reagent also forms a complex with the substrate which in turn can be monitored at a specific wavelength. Digitonin was added to the sample to determine the free cholesterol concentration. Digitonin forms an

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equimolecular insoluble complex with free cholesterol, making it easier to detect in the assay. Figure 3.2 represents the standard curve of the total, as well as free cholesterol, determinations. This curve was used to determine the total and free cholesterol concentrations of each sample.

3.3.4 Triglyceride assay

The true triglyceride concentration is calculated with the following formula:

$$\frac{FA_{sample} - (IA_{sample} \times F)}{FA_{standard} - (IA_{blank} \times F)} \times \text{concentration of standard}$$

FA = final absorbance

IA = initial absorbance

F = 0.81ml / 1.01ml = 0.80 ;

Where 0.81 = total volume of initial absorbance sample and

1.01 = total volume of final absorbance sample.

F = dilution factor.

3.3.5 Concentrations of lipid classes adsorbed onto PSM

After determining the concentration of each specific lipid class with the above assays, the various concentrations were compared. The lipids found present in abattoir effluent were first compared to the lipids found on fouled membranes. In this way one could determine exactly which and to what extent lipid classes adsorbed onto the PSM and contributed most to fouling.

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In another study[1], it was found that the highest percentage of lipid removal from fouled membranes was obtained with the three random lipases from *Candida cylindracea, Pseudomonas mendocina* and *Aspergillus oryzea.* These enzymes are all specific for triglyceride hydrolysis, but are random lipases in the sense that they can hydrolyse the triglyceride molecule at any of the three ester bonds present in the triglyceride structure (Fig 2.3). It was therefore expected that the triglycerides could be present in a high concentration on the fouled membranes, which was indeed what was found (Fig 3.5).

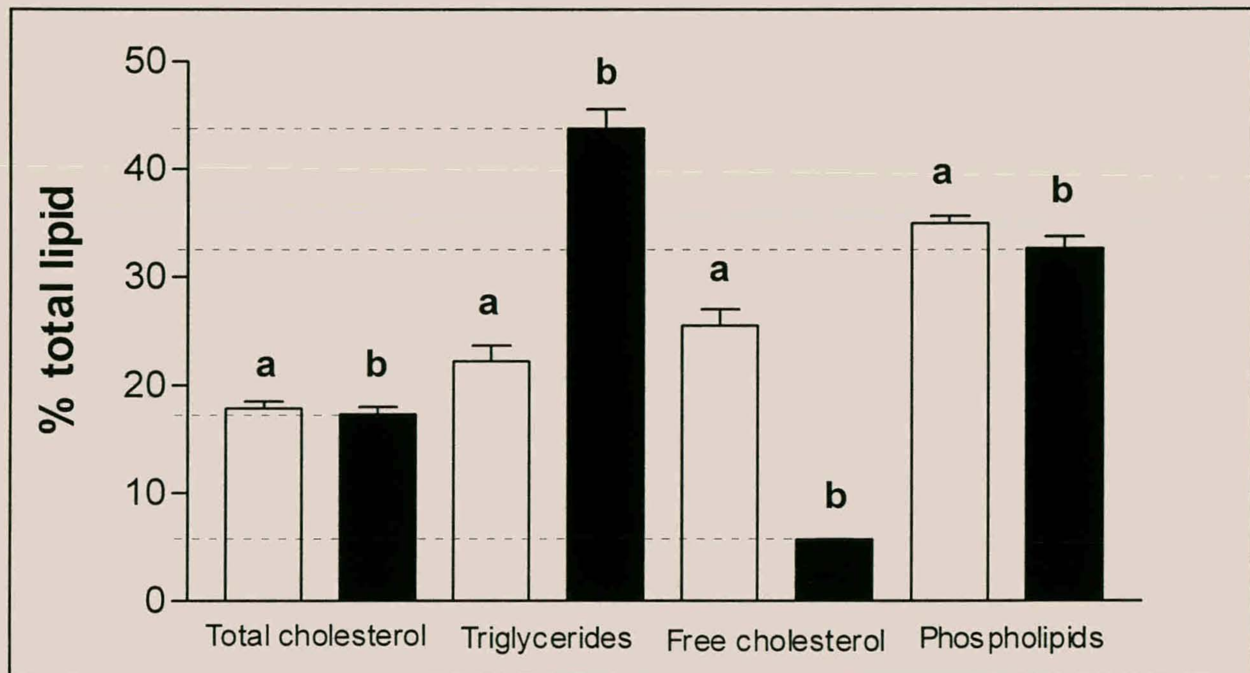

Fig 3.5. Comparison of the percentage of total lipid classes found in (a) abattoir effluent and (b) adsorbed onto UF membranes. Each bar represents the mean of 3 determinations ±SE.

Because lipids found in effluent is measured in mg/ml, compared to lipids found on PSM which is mg/cm^2, the lipid quantities are compared as percentage total lipid. The percentage of triglycerides that adsorbed onto PSM during static fouling, was almost double what was found in abattoir effluent. It was evident that phospholipids and cholesterol esters also played a significant role in UF membrane fouling, and although their relative concentrations were not as high as

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the triglycerides, they were also present in the adsorbed layer at high concentrations. Free cholesterol adsorption was minimal and would thus not be expected to be a major foulant in abattoir effluent.

When studying the chemical structure of a triglyceride molecule, the adsorption thereof onto PSM is quite comprehensible. A triglyceride molecule is non-polar or hydrophobic. The surface of the PSM is also hydrophobic and thus presents the ideal surface for triglyceride adsorption (Fig 2. 1). The cholesterol ester and phospholipid concentrations that also proved to be relatively high on the PSM, can be attributed to the fatty acids that are present in their structures (Fig 2. 2).

In the work discussed in this chapter, the following objectives were reached:

- A successful separation of lipids present in abattoir effluent, as well as lipids adsorbed onto PSM, was achieved with silica gel chromatography;
- Four major lipid classes were identified in the process effluent and in the adsorbed layer after separation: triglycerides, cholesterol esters, free cholesterol and phospholipids;
- The concentration of each lipid class was determined with specific assays and
- With these results a comparison of the percentage of each lipid class present in abattoir effluent and on the membrane could be made.

Having identified the four major lipid classes, an effective cleaning regime could now be developed to remove these foulant species from the membrane surface. In Chapter 4 the existing enzymatic cleaning regimes will be discussed, as well as its effectiveness in removing the four identified lipid classes from the membrane.

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CHAPTER 4

CHARACTERISATION OF LIPIDS FOUND ON ENZYMATICALLY CLEANED PSM

4.1 INTRODUCTION

Effluent streams from abattoirs contain large amounts of lipids and proteins that adsorb onto hydrophobic ultrafiltration membranes used for purification and recycling of waste water. Cleaning experiments conducted by Jacobs *et al* [32] and Maartens *et al* [6] have proved enzymes to be effective cleaning agents for the removal of adsorbed protein and lipid material from UF membranes.

Enzymes are ideal cleaning agents to remove foulants from ultrafiltration membranes as they are not only environmentally friendly, but they are also very economical. They also display a high specificity for the substrates that they bind and the reactions that they catalyse [32]. The use of enzymes as cleaning agents poses no threat of membrane damage, as enzymes act under very mild conditions of pH, temperature and ionic strength. Previous cleaning experiments proved that the enzymes indeed were very effective in removing 65% of the adsorbed lipids and more that 95% of adsorbed proteins [6].

These results prompted a further investigation into the use of proteases and lipases to find more effective enzymes or enzyme/detergent mixtures to remove all adsorbed lipid material. In another study, in which the cleaning efficiency of lipases from *Candida cylindracea*, *Pseudomonas fluorescens* and *Aspergillus,* was investigated, it was found that the random lipases were the most effective in reducing lipids adsorbed onto membranes [6]. Random lipases are lipases that are able to catalyse hydrolysis at all three positions of the triglyceride molecule to

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produce free fatty acids [20]. The adsorbed lipid material includes a number of different lipid classes, therefore it was not surprising that a random lipase, that has a broader substrate specificity, would be more effective than a more specific enzyme.

In the previous chapter the identification of the major lipid classes responsible for the fouling of PSM during contact with abattoir effluent, was described. In this chapter the effectiveness of the existing cleaning regimes for lipid removal will be discussed. The concentrations of the various lipid classes present on ultrafiltration membranes after enzymatic cleaning, could then be compared to the concentration of lipids present on the fouled membranes prior to enzymatic treatment.

4.2 EXPERIMENTAL

Materials

Lipases used in cleaning experiments are given in Table 2.

Table 2. Specific activity of each enzyme used in cleaning trials.

Enzyme source	Specific activity *(U/ml)	Optimum pH	Optimum temperature	Source
Aspergillus oryzea	0.4344	10.8	37°C	Enzymes SA, South Africa
Pseudomonas mendocina	0.5940	8.0	37°C	Genencor International
Candida cylindraceae	0.7800	8.0	37°C	Sigma Chemical Co., USA

* One unit of lipase activity (U), is defined as the amount of enzyme that releases one μmole of fatty acid per minute.

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Reagents and membranes were used as previously described in Section 3.2.1.

4.2.1 ENZYMATIC MEMBRANE CLEANING

Membranes fouled in abattoir effluent were washed in Clark and Lubs Buffer (pH 7.5). The membranes were incubated for an hour in a 600 ml solution of the lipase and the appropriate buffer at optimum pH and temperature for each enzyme to ensure maximum efficiency (Table 2). 0.5 units of each lipase was used in the cleaning experiments conducted. The non-ionic detergent, Triton X-100 (0.1% v/v), was also included in the enzymatic cleaning solutions. Following enzymatic cleaning, the membranes were washed thoroughly with distilled water to remove excess material, and analyzed for lipid content [6].

4.2.2 LIPID EXTRACTION

Enzymatically cleaned membranes were cut into 25 cm^2 squares and dried at room temperature. The dried membrane squares were shaken vigorously with 50 ml hexane:isopropanol (3:2 v/v) for 5 min and left at room temperature for 12 h. The extraction solution was subsequently boiled mildly for 15 min and filtered through a Whatman No. 40 filter paper. The membrane squares, filter paper and flask were rinsed with an additional 20 ml of preheated hexane:isopropanol (3:2 v/v) and the filtrate recombined with the first filtrate. The amount of the lipid residue was determined gravimetrically after the volatile phase of the solution had been removed under nitrogen at 40°C [7].

4.2.3 LIPID CHARACTERISATION

The triglyceride, phospholipid, and free and esterified cholesterol content of lipids that adsorbed onto PSM, were determined with the assays described in Section

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3.2.4 to 3.2.6. The same assays were performed after enzymatic cleaning of the fouled membranes.

4.3 RESULTS & DISCUSSION

4.3.1 Comparison of lipid concentrations found on fouled and enzymatically cleaned ultrafiltration membranes

In Figure 4.1 the concentrations of triglycerides adsorbed onto PSM before and after enzymatic cleaning, are compared. As mentioned earlier the triglycerides adsorbed onto the ultrafiltration membranes to a greater extent than the other three lipid classes. Although enzymatic PSM cleaning with all three random lipases proved successful, *Aspergillus oryzea* and *Pseudomonas mendocina*, bar B and D respectively, reduced the triglyceride concentration on the membranes most effectively.

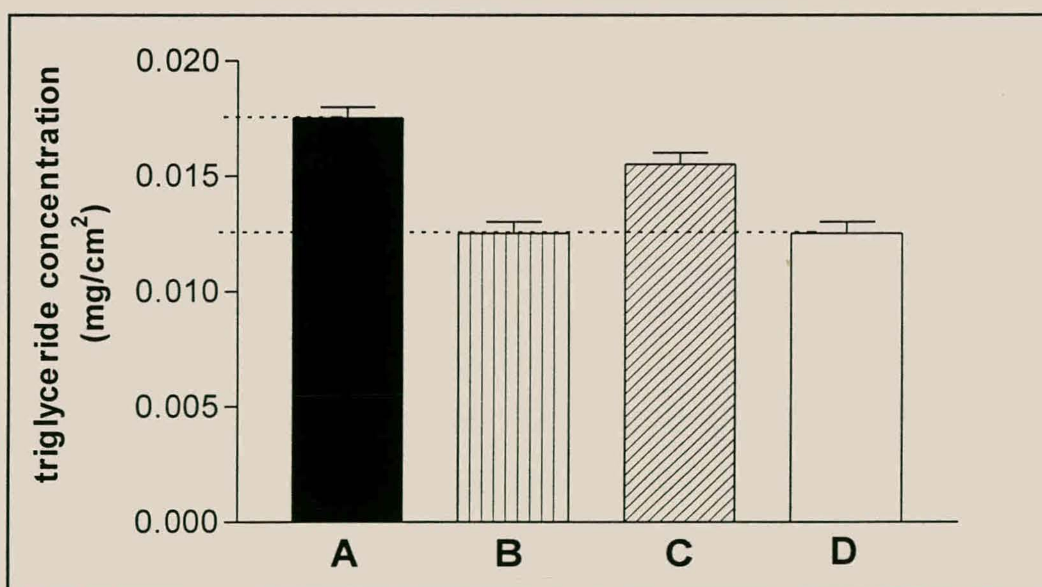

Fig 4.1. Triglyceride concentration on **A:** fouled PSM, **B:** fouled and cleaned with *Aspergillus* lipase, **C:** fouled and cleaned with *Candida* lipase and **D:** fouled and cleaned with *Pseudomonas* lipase. Each bar represents the mean of at least 3 determinations (± SE).

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The efficiency of these enzymes in triglyceride removal can be explained by the fact that all three lipases used in cleaning regimes are lipases that are specific for triglyceride hydrolysis. *Aspergillus* lipase is a sn-1,3 specific lipase, meaning that it will hydrolyse only primary ester bonds (C1 and C3 atoms of the triglyceride molecule). *Candida* and *Pseudomonas* are random lipases which will equally hydrolyse ester bonds at all three positions on the glycerol molecule and shows no positional specificity with respect to the chemical structure of the fatty acid. The lipids in abattoir effluent are a heterogeneous population of molecules and a somewhat less specific enzyme, *Pseudomonas*, would therefore be able to hydrolyse a wider range of esters than a more substrate specific enzyme. The results obtained in the triglyceride cleaning trials are in accordance with results from enzymatic cleaning regimes previously conducted[1].

Free cholesterol adsorbed onto the hydrophobic PSM to a lesser extent than the other lipid classes. The small percentage of free cholesterol that caused fouling though, was removed by the lipases used in the cleaning regimes and the cleaning efficiency was the same as that of triglyceride experiments (Fig 4.2). The *Candida* lipase was more efficient in free cholesterol removal than the other enzymes evaluated.

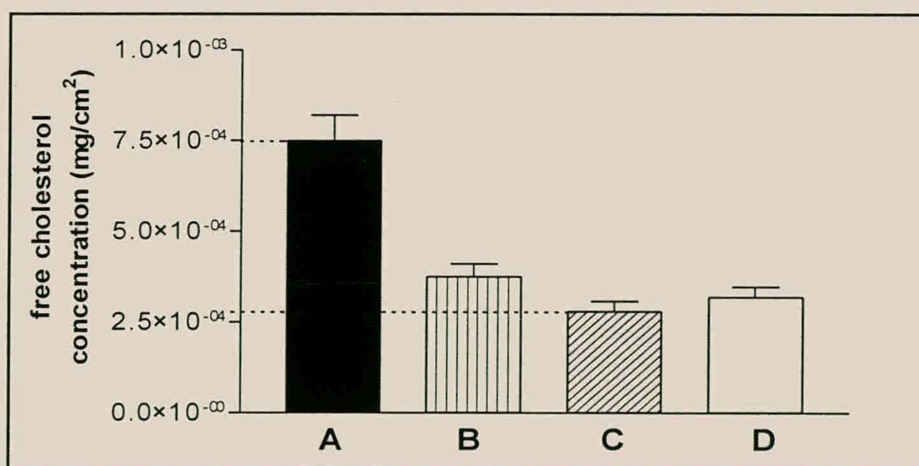

Fig 4.2. Free cholesterol concentration on **A:** fouled PSM, **B:** fouled and cleaned with *Aspergillus* lipase, **C:** fouled and cleaned with *Candida* lipase and **D:** fouled and cleaned with *Pseudomonas* lipase. Each bar represents the mean of at least 3 determinations (± SE).

[1] Z.Allie, Dept. Biochemistry, University of Stellenbosch, M.Sc Thesis submitted

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The *Candida* lipase also proved to be the more successful lipase with respect to total cholesterol removal. As seen with triglycerides and free cholesterol, success was achieved with all three random lipases applied to the fouled membranes. It is interesting to note though that *Candida* was the more effective lipase for removal of both cholesterol classes.

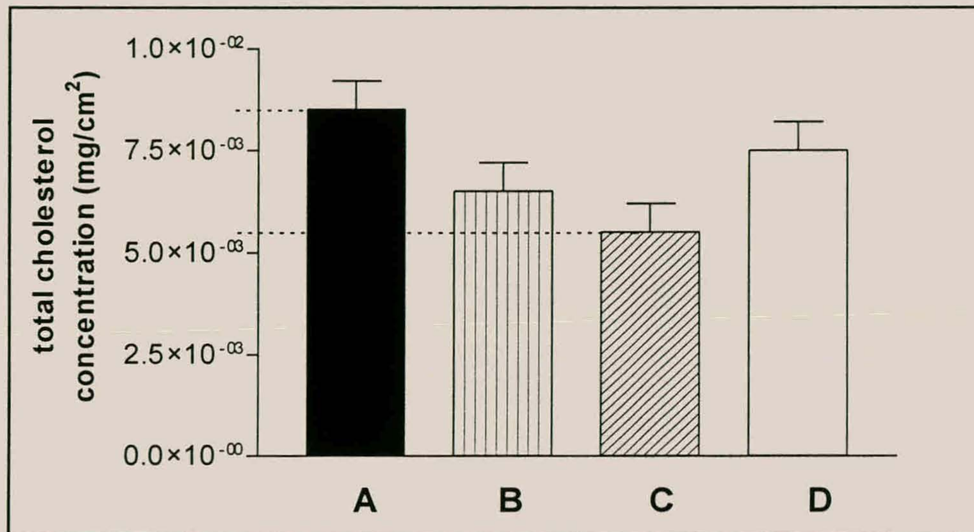

Fig 4.3. Total cholesterol concentration on **A:** fouled PSM, **B:** fouled and cleaned with *Aspergillus* lipase, **C:** fouled and cleaned with *Candida* lipase and **D:** fouled and cleaned with *Pseudomonas* lipase. Error bars represent the standard deviation of at least 3 determinations.

The three specific assays used for triglyceride, cholesterol and phospholipid determination, was also performed on unfouled enzymatically treated membranes to determine whether these enzymes might in any way have interfered with the assay mixture. It was then found that each of these enzymes also caused positive colour reactions when added to the specific assay mixtures. These positive colour reactions were used as background values and subtracted from the fouled enzymatically cleaned membranes values. It is these results that are represented in the figures discussed above.

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The three random lipases were successful in removing triglycerides and both total and free cholesterol from the membranes, but the phospholipids still remained a problem. It was then decided to employ an enzyme in the cleaning trials, that would hydrolyze phospholipids specifically. In the next chapter the use of *phospholipases*, specifically *phospholipase* A_2, will be discussed.

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CHAPTER 5

THE EFFECT OF PHOSPHOLIPASE A_2 ON MEMBRANE CLEANING

5.1 INTRODUCTION

In Chapter 4 it was shown that random lipases were successful in the removal of triglycerides and free and total cholesterol from ultrafiltration PSM. Phospholipids, however, remained a problem. The incorporation of *phospholipase A*, an enzyme specific for phospholipid hydrolysis, was subsequently investigated in cleaning trials and its effectiveness in removing phospholipids from PSMs was determined. This work will be described in this chapter.

Phospholipids are amphiphilic molecules that are ubiquitous in nature [33]. They are the basic components of natural membranes and cell walls, where they play a structural role, and function as cofactors and activators of several membrane-associated enzymes. Phospholipases are enzymes that regulate the hydrolysis of these compounds *in vivo* with the concomitant release of biologically active molecules. Phospholipids, substrates or starting materials for the phospholipases, can be obtained as mixtures of products with different polar heads and acyl chains from soy beans, many vegetable oils, egg yolk, biomass and other sources [33]. Phospholipids find applications as pharmaceuticals, food additives and in cosmetic formulations [34].

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EXPERIMENTAL

5.2.1 Phospholipase A_2 assay

Materials

L-α-phosphatidylcholine, and *Phospholipase A_2* from bee venom was obtained from Sigma Chemical Co., St. Louis, USA. Phenol red was used as pH indicator. Sodium chloride, calcium chloride, Triton X-100 and Tris-HCl was obtained from Merck Chemical Co.

Methods

Determination of the *Phospholipase A_2* activity by a spectrophotometric assay was performed in a total reaction volume of 1ml [35]. The reaction medium contained 100mM NaCl, 10 mM $CaCl_2$, 3.5mM phosphatidylcholine (substrate) dissolved in 7 mM Triton X-100, and 0.055 mM phenol red. For maximum activity the assay was initiated at the pK of phenol red, pH 7.6. Occasionally it was necessary to re-adjust the pH of the dye solution, because the solution had deviated from its original pH as a result of the buffering capacity of the assay sample.

The enzyme solution, in a volume less than 20 μl, was added to the sample cuvette containing 1 ml of reaction medium and the same volume of the solution without the enzyme was added to the reference cuvette [35]. The difference in absorbance between the reference and the sample cuvettes was monitored continuously. Enzymatic activity is expressed as a decrease in absorbance per min and per μg phospholipase. This activity can be converted to μmoles of fatty acid released per min and per mg of phospholipase by measuring the decrease in absorbance produced by known amounts of acid. A decrease in absorbance of 0.1 was obtained with 0.01 μmole of hydrochloric acid [35].

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5.2.2 Membrane cleaning

The membrane cleaning procedure was performed as described in Section 4.2.1.

The enzyme solution contained the appropriate concentrations of buffer and co-factors as described in the *phospholipase* A_2 assay, substituting the phenol red with 0.1M Tris-HCl, pH 8.0. In the first set of cleaning experiments fouled membrane squares of 25 cm^2 were incubated in the *phospholipase* A_2 reaction mixture for 1 hr at 34°C and then after rinsing, the lipids that remained after cleaning were characterised.

Secondly, the cleaning efficiency of *phospholipase* A_2, following cleaning with the *Pseudomonas* enzyme, was investigated. Fouled membranes were first cleaned with *Pseudomonas* lipase according to the procedure as described in Section 4.2.1. The same membranes were subsequently incubated with *phospholipase* A_2 and the remaining adsorbed lipids were characterised. The activity of the enzyme on lipid samples was also investigated.

In the third set of *phospholipase* A_2 activity determinations, the lipid sample extracted from membranes fouled in abattoir effluent, was emulsified with the reaction solution and incubated with the enzyme under the same conditions as the fouled membranes.

5.2.3 Lipid characterisation

After membrane cleaning with *phospholipase* A_2, the concentrations of triglycerides, cholesterol and phospholipids were determined with the same assays, as described in Sections 4.2.3, 4.2.4 and 4.2.5 of this thesis.

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5.3 RESULTS AND DISCUSSION

5.3.1 Phospholipase activity determination

Fig 5.1 shows the increase in absorbance with an increase in *phospholipase* A_2 concentration to a point of saturation. This result correlates well with the result obtained by Shipolini *et al* [35]. It must be noted that *phospholipase* A_2 activity was so high that with the addition of 0.1 μl of enzyme solution, maximum absorbance was measured.

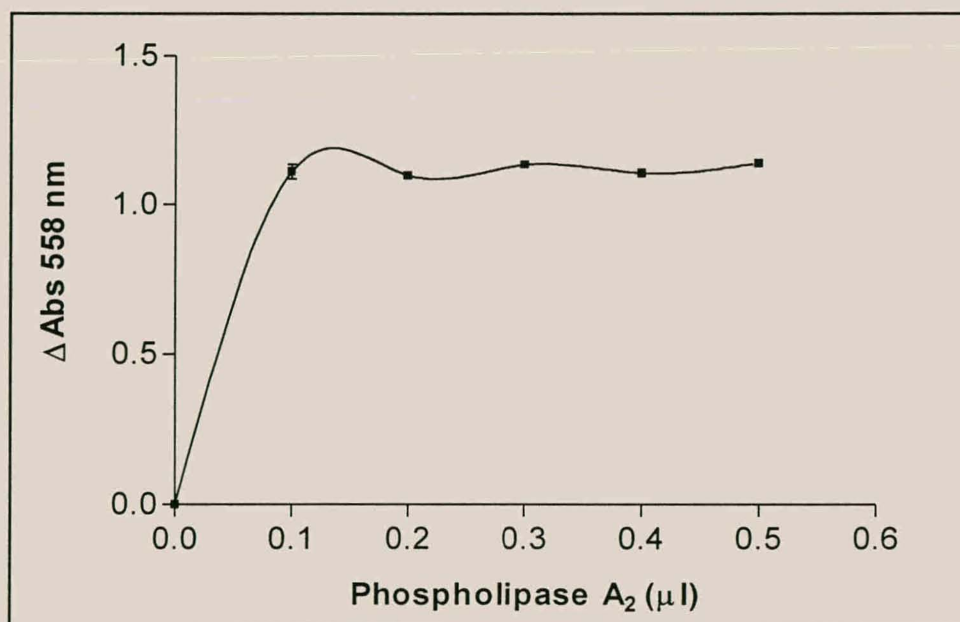

Fig 5.1. Determination of *phospholipase* A_2 activity by measuring the change in absorbance of the reaction solution with increasing *phospholipase* A_2 concentrations.

5.3.2 Concentrations of lipids on ultrafiltration membranes after enzymatic treatment

In Fig. 5.2 the changes in the triglyceride concentrations before and after cleaning trials are compared. It has been said in Chapter 3, that triglycerides

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adsorbed onto PSM to a greater extent than any of the other three lipid classes identified. Triglycerides is however, also the lipid class that could until now, be most successfully removed from fouled ultrafiltration. All three random lipases investigated in Chapter 4 proved to be effective in triglyceride removal. From Fig. 5.2 it is clear that triglycerides can be removed from fouled ultrafiltration membranes, with Triton X-100, *phospholipase* A_2 alone and *phospholipase* A_2 after *Pseudomonas,* with the same efficiency. There was no significant difference seen in the triglyceride concentration after the three different membrane cleaning steps.

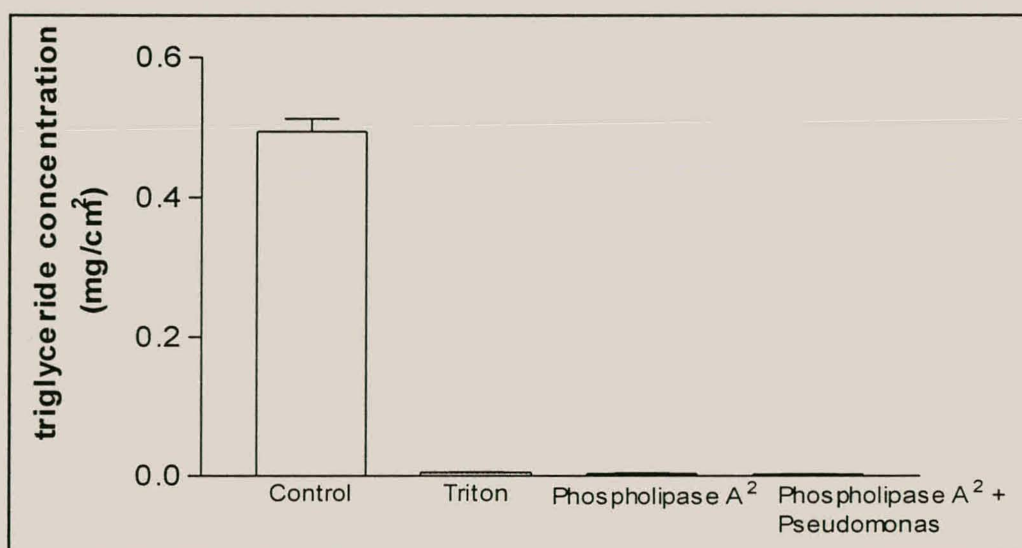

Fig 5.2. Triglyceride concentration on fouled membranes (control), and membranes cleaned with (a) Triton X-100-buffer solution, (b) *phospholipase* A_2-buffer solution and (c) *Pseudomonas* lipase prior to *phospholipase* A_2. Each bar represents the mean of at least three determinations (± SE)

Membrane cleaning with *phospholipase* A_2 did not have any effect on the total cholesterol and phospholipid concentrations. *Phospholipase* A_2, although highly specific, was thus not the most suitable enzyme to remove all adsorbed lipid classes from statically fouled PSM.

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5.3.3 Concentration of lipids found in lipid extracts from membranes fouled in abattoir effluent – before and after enzymatic treatment

Phospholipase A_2 was included in cleaning trials because of its specificity for phospholipid hydrolysis (Section 5.1). The enzyme did not prove, however, to be as effective in phospholipid removal from membranes fouled in abattoir effluent, as expected (Section 5.3.2). This prompted an investigation into the activity of this enzyme on extracted lipid samples. The following experiments were done to determine whether it was the membrane surface causing the inefficiency of *phospholipase* A_2 in phospholipid removal. Therefore, the enzyme was applied to lipid extracts directly, to investigate its effectiveness in the absence of the membrane surface.

Similarly to membrane cleaning with the random lipases discussed in Chapter 4 and the *phospholipase* A_2 studies done on fouled membranes, the triglycerides were nearly totally removed from the lipid extract. No significant difference was noted in the cleaning ability of Triton® X-100 and *phospholipase* A_2 for triglyceride removal [Fig 5.3] from solution. The same quantity of triglycerides could be removed from solution, as from the fouled membranes.

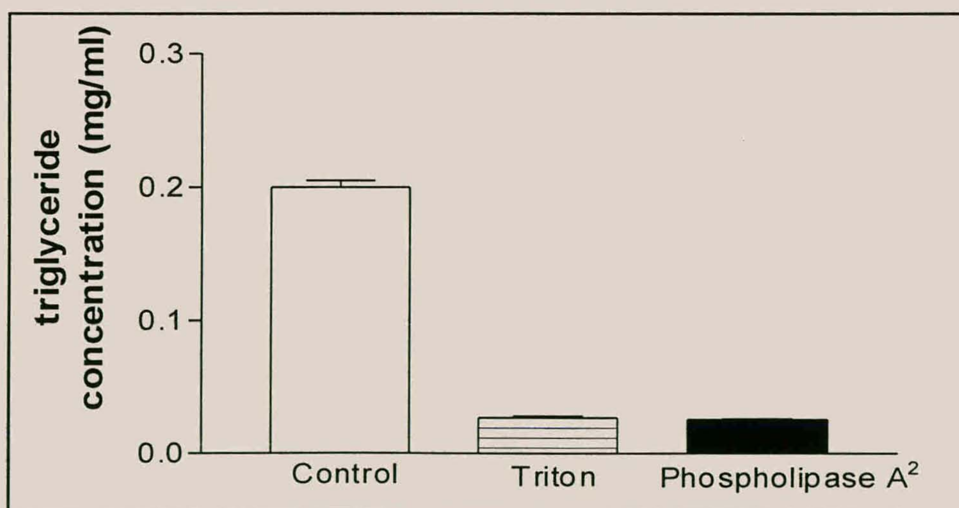

Fig 5.3. Triglyceride concentration in lipid samples extracted from abattoir effluent. The extracted lipids were emulsified with (a)Triton X-100 solution and (b)*phospholipase* A_2-buffer solution. Each bar represents the mean of at least three determinations (± SE).

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The triglycerides was the only lipid class that could successfully be removed with the random lipases, as well as with the more specific *phospholipase* A_2. Total cholesterol and phospholipid concentrations could not be reduced with these enzymes, indicating that some other method of foulant reduction might need to be investigated.

Recently Mustranta *et al* [36], conducted a study to determine the efficiencies of fungal lipases, which included an *Aspergillus* specie, and fungal phospholipases. They found that lipase preparations were more efficient than phospholipase preparations in the hydrolysis of the phospholipid substrate. The productivity of free fatty acids based on the amount of enzyme was also much higher with lipases than with phospholipases.

From these results it thus seems as though it's not the the phospholipases that needs further investigating as an effective phospholipid cleaning agent, but rather a different lipase. It is interesting to note though that the *phospholipase* A_1 from *Aspergillus niger*, exhibited similar activity to that of the lipase preparations in the hydrolyisis of phospholipids, but with a slower reaction rate [36]. *Phospholipase* A_1 could release fatty acids from both positions of the phospholipid molecule, compared to *phospholipase* A_2 where only partial selective hydrolysis of the 2-position fatty acids of phospholipids could be effected. Further cleaning studies with *phospholipase* A_1 could thus lead to more effective removal of phospholipids from fouled membranes.

The use of various enzymes as cleaning agents for membranes fouled in abattoir effluent was discussed in Chapter 3 and Chapter 4. Another method of foulant reduction is the pre-treatment of ultrafiltration membranes with surfactants or detergents. This possibility will be discussed in detail in the following chapter.

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CHAPTER 6

Characterisation of lipids adsorbed onto pre-treated ultrafiltration PSMs

6.1 INTRODUCTION

Numerous attempts have been undertaken to modify membrane materials in an atempt to produce membranes less susceptible to fouling. In previous studies adsorbed hydrophilic polymers were assessed as a possible means of reducing protein fouling in microfiltration and ultrafiltration membranes [37-43]. Polysulphone ultrafiltration and microfiltration membranes were modified by pre-adsorption of water-soluble polymers [43]. Pre-treatment of membranes with solutions containing hydrophilic surfactants, was found to hydrophilize the membrane surface and make it amenable for treating hydrophobic macromolecular solutions, though only temporarily [44]. Fouling has been widely believed to be reduced by incorporating hydrophilic sites on the membrane surface as the hydrophobic character of polysulphone is widely believed to correlate with protein fouling [45].

The ideal membrane for treating aqueous feed solutions, containing natural organic matter, would be homogeneously permeable and hydrophilic [46]. Most hydrophilic membranes are, however, not thermally stable and are susceptible to chemical degradation. The membranes used for most UF processes are therefore hydrophobic and mechanically robust and thus, as mentioned earlier, more susceptible to fouling [47]. Fane and Fell [39] showed that a reduction in

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foulant adsorption can be achieved by forming a hydrophilic monolayer on the membrane surface, leaving fewer hydrophobic sites for foulant adhesion [48].

Attempts have been made to investigate the effect of increasing membrane hydrophobicity on membrane performance in UF applications such as those involving oily/fatty water streams. It is not known how a more hydrophobic membrane surface will perform in treating aqueous solutions such as lipid/water emulsions that tend to acts as foulants. Maartens [46], showed that the pre-coating of ultrafiltration membranes with Triton® X-100 and Pluronic® F108, influenced foulant adsorption, colour retention and flux of water through a PSM [46]. The effect of pre-coating on lipid fouling, however, have up to now not been investigated. The experiments described in this chapter were therefore carried out to determine if membrane pre treatment could reduce lipid fouling of PSM.

As shown earlier, the use of enzymes in cleaning regimes proved to be quite successful in removing adsorbed lipids from ultrafiltration membranes. There are, however, still quite a few stumbling blocks to overcome and an enzyme with the ability to remove all adsorbed lipid species from ultrafiltration membranes, is still to be found.

Membranes were pre-coated with hydrophilic surfactants prior to fouling. The lipids that adsorbed onto the pre-treated UF membranes during contact with abattoir effluent, were then characterized and compared to lipids adsorbed onto untreated UF membranes.

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6.2 EXPERIMENTAL

Materials

Detergents used were Triton® X-100, Pluronic® F108 and Tween® 20. Membrane squares (25 cm^2) and cleaning reagents were used as described in Section 3.2.1.

6.2.1 Pretreatment of PSM

Unfouled PSMs were incubated for an hour in a 600ml solution of 0.1% detergent (m/v) and distilled water at 37°C. After pre-treatment, the membranes were washed thoroughly with distilled water and then subjected to fouling by abattoir effluent as described in Section 3.2.

6.2.2 Lipid extraction

The fouled UF membranes were cut into squares, after which a total lipid extraction was done as described in Section 4.2.2. The lipid sample obtained was used for lipid characterisation studies.

6.2.3 Lipid characterisation

The triglyceride concentration of lipids that adsorbed onto PSM after membrane pre-treatment, was determined as described in Section 4.2.3. The method for phospholipid determination is found in Section 4.2.4, and the cholesterol assay is explained in Section 4.2.5.

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6.3 RESULTS & DISCUSSION

Pre-treatment of membranes with Triton® X-100, Pluronics® F108 and Tween® 20, induced different effects in the adsorptive properties of the membrane. In Figure 6.1 the effect of membrane pre-treatment on the triglyceride concentration is shown. Triton® X-100 could reduce the adsorption of triglycerides most effectively, while Tween® 20 and Pluronics® F108 showed little or no effect.

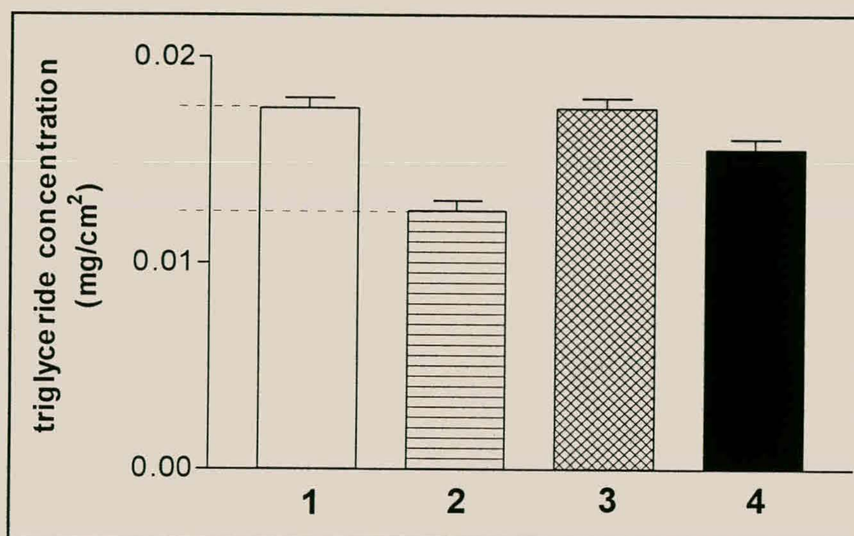

Fig 6.1. Triglyceride concentration on **1**: fouled membranes and membranes pre-treated with **2**: Triton® X-100, **3:** Tween® 20 and **4**: Pluronics® F108. Each bar represents the mean of at least three determinations (± SE).

When the phospholipid concentrations on fouled membranes were compared to that found on pre-treated membranes (Fig 6.2), it was found that membrane pre-treatment with all three surfactants used, could not significantly reduce phospholipid adsorption onto PSM.

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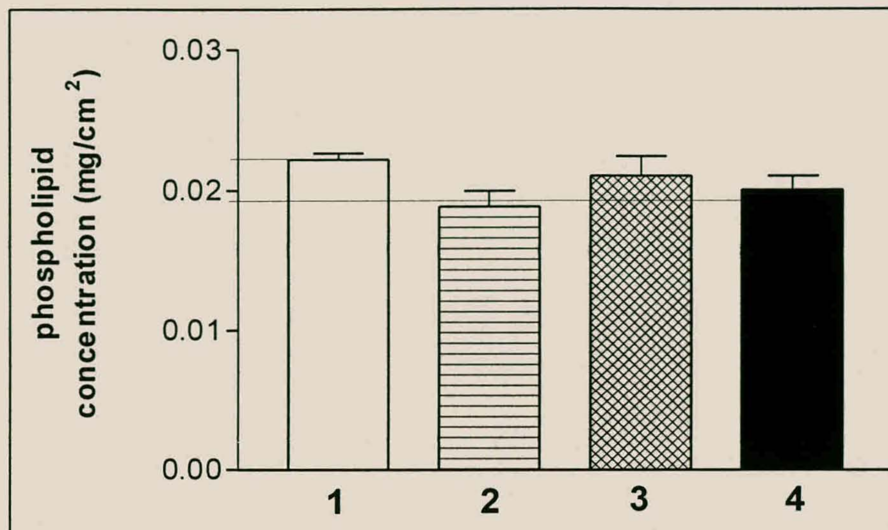

Fig 6.2. Phospholipid concentration on **1**: fouled membranes and membranes pre-treated with : Triton® X-100, **3**: Tween® 20 and **4**: Pluronics® F108. Each bar represents the mean of at least three determinations (± SE).

In Fig 6.3 the total cholesterol concentration before and after membrane pre-treatment is compared. Membrane pre-coating with Triton® X-100 resulted in a more than two-fold decrease in total cholesterol adsorption. In contrast, Tween® 20 did not cause any significant decrease in the cholesterol concentration and Pluronics® F108 showed only a slight decrease.

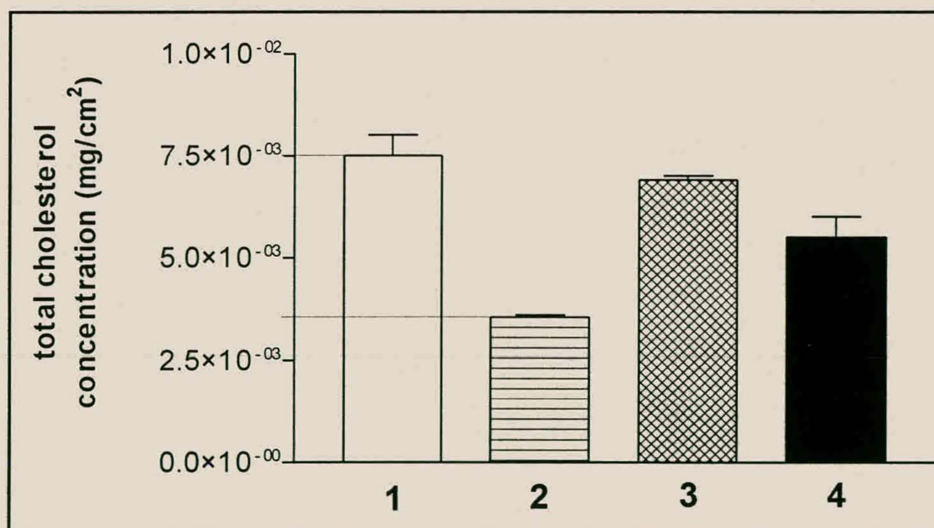

Fig 6.3. Total cholesterol concentration on **1**: fouled membranes and membranes pre-treated with **2**: Triton® X-100, **3**: Tween® and **4**: Pluronics® F108. Each bar represents the mean of at least three determinations (± SE).

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In Fig 6.4 the effect of membrane pre-treatment on free cholesterol adsorption can be seen. Again Triton® X-100 was found to be the most effective of the three surfactants used, substantially reducing the amount of adsorbed free cholesterol after treatment. Tween® 20 and Pluronics® F108 showed no significant effect on free cholesterol adsorption.

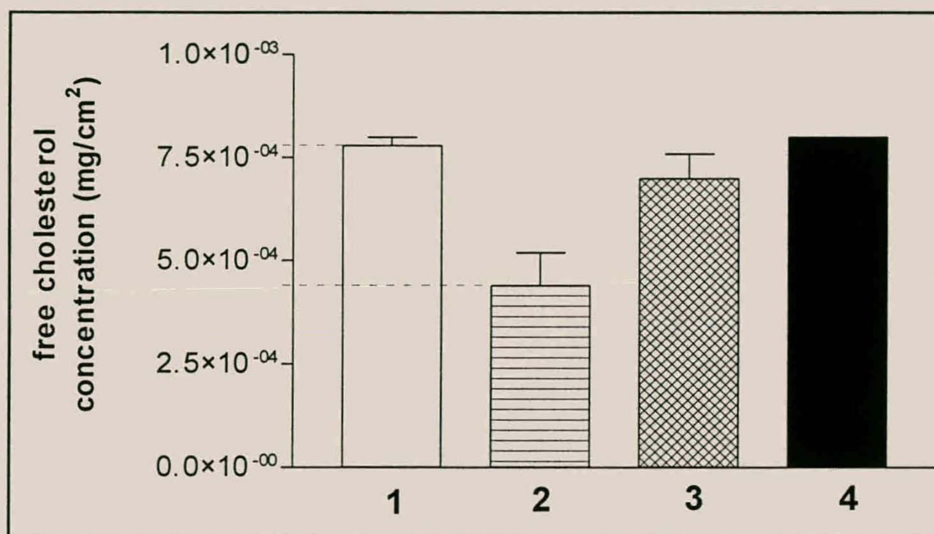

Fig 6.4. Free cholesterol concentration on **1**: fouled membranes and membranes pre-treated with **2**: Triton® X-100, **3**: Tween® and **4**: Pluronics® F108. Each bar represents the mean of at least three determinations (± SE).

It seems clear from the results obtained in this study, that Triton® X-100 was the more effective surfactant for membrane pre-treatment. Triton® X-100 could reduce the adsorption of all the lipid classes investigated substantially, with the exception of the phospholipids where less success was achieved. In Table 3 the percentage lipid reduction of the three surfactants used are compared.

The total lipid content on the fouled untreated PSM was firstly determined. The lipid reduction brought about by each surfactant used, was expressed as a fraction of that total lipid content with a value of 100%.

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Table 3. Percentage total lipid adsorption reduction obtained with surfactants.

SURFACTANT USED	% TOTAL LIPID REDUCTION
Triton® X-100	-26.918 (± 0.01)
Tween® 20	-5.309 (± 0.01)
Pluronic® F108	-13.374 (± 0.01)

In Table 4 the effect of membrane pre-treatment on the adsorption of each individual lipid class can be seen. It is evident from these results that Triton® X-100 was the most effective to reduce adsorption rates of each individual lipid class, while Tween® 20 and Pluronic® F108 had varied effects on adsorption.

Table 4. The percentage lipid reduction obtained for each individual lipid class.

% REDUCTION	Triton® X-100	Tween® 20	Pluronic® F108
Triglycerides	-29.091	------	-9.9091
Free cholesterol	-46.000	-32.800	------
Phospholipids	-16.617	-5.689	-13.174
Total cholesterol	-49.565	-13.913	-26.087

---- Percentage lipid reduction was insignificantly low.

When the concentrations of lipids adsorbed onto PSM after pre-treatment with surfactants were determined, it was found that in some cases increased lipid concentrations were observed on pre-treated PSMs compared to untreated PSMs. This result was very puzzling and it was then thought that there was some agent in the reaction mixtures of the assays performed, that resulted in a false positive result. Hexane-isopropanol extractions were subsequently performed on unfouled UF membranes, that had been pre-coated with Triton® X-100, Tween® 20 and Pluronic ® F108.

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It was found that these surfactants did indeed give positive reactions in the assays used, particularly in the phospholipid assay, thereby leading to an erroneously higher recorded lipid concentration. The values obtained have been subtracted as background from the results obtained above. Pluronic ® F108 was found to be particularly sensitive to the phospholipid assay. As a result the phospholipid assay was modified to serve as an assay for Pluronic® F108, seeing that there is not a recorded method up to date for Pluronic® F108 determination. The same possiblity was explored for Triton® X-100 and Tween® 20, but these two surfactants were not soluble in chloroform which is the solvent required for the assay.

6.3.1 Pluronic ® F 108 determination

Materials and Methods

The same reagents as described in the phospholipid assay were used (Section 3.2.5.). Phospholipid standards were substituted with Pluronic® F108. A standard curve was achieved as follows: A range of standards containing Pluronic® F108 dissolved in chloroform, of the following concentrations, 0.04 μg/ml, 0.02 μg/ml, 0.01 μg/ml, 0.005 μg/ml. 2ml of each standard was added to 2 ml of ammonium ferrothiocyanate reagent and the same method was then followed as for the phospholipid assay. Absorbance was also read at 488 nm.

Results

Fig 6.5 shows the standard curve obtained when the absorbance of various concentrations of Pluronic® F108 was measured at 488 nm. A linear increase in absorbance was observed with an increase in Pluronic® F108 concentration. To date there has not been a documented method for the quantitative determination of Pluronic® F108 in a solution. The method developed here proved to be very sensitive and was able to detect Pluronic® F108 at very low concentrations. This

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could prove to be a very useful tool in future and will be applied in further pilot studies to be conducted. The linear relationship between μg/ml Pluronic® F108 and absorbance at 488nm with curve fit (r^2) = 0.9930 was:

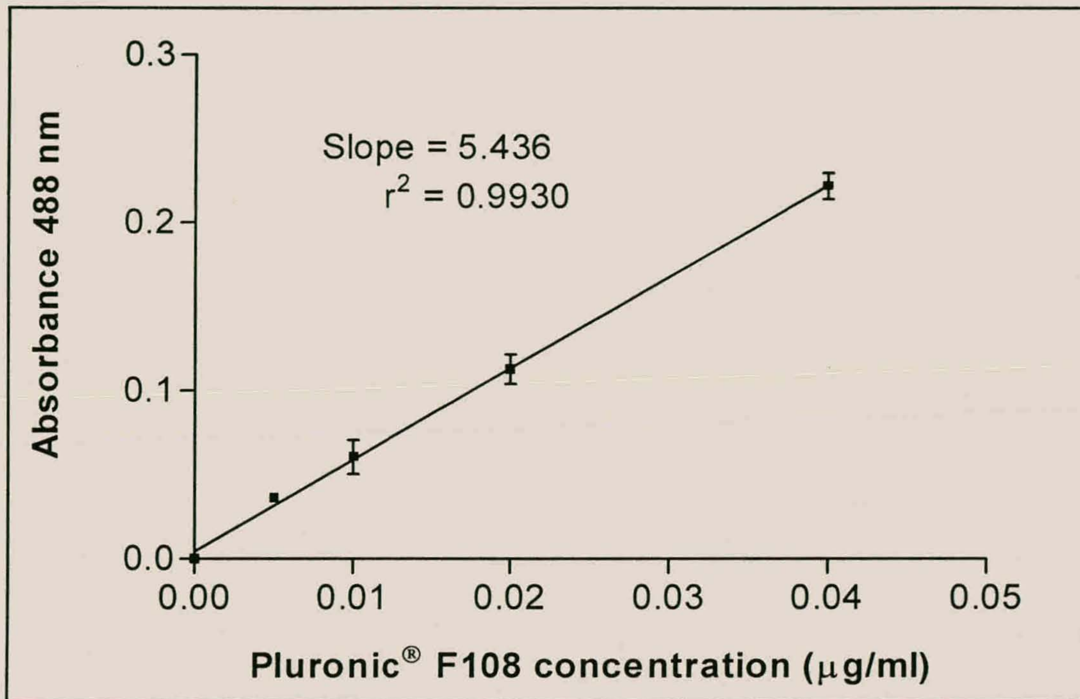

Fig 6.5 Standard curve of Pluronic® F108. Pluronic® F108 concentration vs. absorbance at 488nm. Each point is the mean of three determinations ± SE.

The results discussed in this chapter, indicate that pre-coating of the polysulphone membrane surface, does indeed have an effect on the membrane's adsorptive properties. However, this change in adsorptive properties is not the same for every lipid class identified in abattoir effluent. Triton® X-100 has clearly been identified as the most effective non-ionic surfactant to substantially reduce the adsorption of lipids onto PSM after membrane pre-treatment. Pre-coating alone is however, not the most efficient method of foulant reduction for ultrafiltration membranes fouled during contact with abattoir effluent.

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CHAPTER 7

CONCLUSIONS

7.1 INTRODUCTION

Cheryan, a renowned authority on membrane fouling, stated in 1986 that: "...fouling and inefficient cleaning are the main reasons why UF has not yet fulfilled its promise as an economical method for wastewater treatment...". Since this statement in 1986, tangible progress has been made to treat or minimise fouling. This progress has, however, proven to be insufficient as this statement is still valid in the application of ultrafiltration processes to purify biological effluents.

In recent years the emphasis of research in the field of membrane science has been on membrane and process development, and laboratory studies of fouling with model compounds. Very liitle work has been conducted on fouling studies with real effluent. Jacobs *et al* [4] and Maartens *et al* [7] set out to do fouling studies in a static system with abattoir effluent, identifying the major membrane foulant categories in that particular effluent as being proteins and lipids. Proteins and lipids adsorbed onto the hydrophobic membrane surface, causing flux decline and eventually a shorter membrane-life. Instead of trying to modify the membrane structure as such, attempts were made to treat the fouled membranes with cleaning solutions that would be more environmentally friendly and would not cause the membrane surface any damage.

Enzymes specific for protein and lipid degradation were employed in cleaning trials with great success. Proteins were removed from membranes fouled in abattoir effluent the most effectively, up to 95 %. Lipids, however, remained a

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problem with only about 65 % of the total lipid adsorbed onto the membrane being removed by enzymatic cleaning regimes. This study was continued with an investigation into a number of commercially available lipolytic enzymes. These lipolytic enzymes were included in cleaning trials and their efficiency determined. Three random lipases, *Aspergillus oryzea*, *Candida cylindracea* and *Pseudomonas mendocina* were identified as the most effective enzymes for lipid removal from fouled membranes. These enzymes were, however, not effective enough to remove the total lipid content from fouled membranes. Accordingly, this study was proposed to characterise lipids that adsorbed onto UF membranes during contact with abattoir effluent. These results would help to identify an enzyme most suitable for lipid removal and resolve the problem of membranes fouled in abattoir effluent.

7.2 CONCLUSIONS OF THIS STUDY

Fractionation of lipids found in abattoir effluent and lipids adsorbed onto UF membranes during contact with abattoir effluent, resulted in similar elution curves. The elution curve of lipids present in abattoir effluent, showed that the four major lipid classes were: triglycerides, free cholesterol and total cholesterol, and phospholipids. The elution curve of membrane adsorbed lipids indicated that the same lipid classes were present, though there relative concentrations differed.

The three assays used for the characterisation of lipids were very specific and each different assay measured only one specific lipid type. Results obtained from the characterisation studies indicated that the triglycerides and total cholesterol adsorbed onto ultrafiltration membranes to a greater extent than any of the other lipid classes present, and thus was responsible for the most of the fouling. The other two lipid classes identified in abattoir effluent that adsorbed onto UF membranes, free cholesterol and phospholipids, did not adsorb onto UF membranes as strongly as the triglycerides and total cholesterol did. It thus

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seemed as if these two lipid classes did not play a major role in UF membrane fouling.

After identifying the lipids that adsorbed onto UF membranes during contact with abattoir effluent, fouled membranes were cleaned with enzyme-detergent mixtures to investigate the enzymes' effectiveness. Three random lipases were identified as being effective in removing nearly 65 % of lipds adsorbed onto PSMs. These enzymes were lipases from the *Pseudomonas mendocina, Aspergillus oryzea* and *Candida cylindracea.* The enzymes were incorporated in cleaning regimes and used to remove adsorbed foulants from the membrane surface. After enzymatic cleaning, further characterisation studies were done to compare the lipid composition on the membrane surface before and after enzymatic treatment.

The lipases that were investigated, proved successful in cleaning regimes conducted in this study, by reducing the concentration of triglycerides and free and total cholesterol on the membrane surface. All three lipases used, differed in their cleaning efficiency of the various lipid types from the membrane surface. The lipases *from Aspergillus* and *Pseudomonas* reduced the triglyceride concentration on the membrane surface to the same extent. *Candida* lipase proved to be more effective in free and total cholesterol removal. The concentration of phospholipids, however, could not be reduced after enzymatic cleaning by any of the following three lipases used: *Aspergillus* lipase, *Candida* lipase and *Pseudomonas* lipase.

It was decided to incorporate an enzyme specific for phospholipid hydrolysis in existing membrane cleaning regimes. The choice of enzyme fell on *phospholipase* A_2 from bee venom, as it is the most economical phospholipase commercially available and it is easy to obtain. *Phospholipase* A_2 catalyses the hydrolysis of the ester linkage at the 2-position of the glycerol skeleton of the phospholipid molecule. This enzyme was incorporated in existing cleaning

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regimes and its efficiency for lipid reduction was investigated. Characterisation studies showed that *phospholipase* A_2 was also unsuccessful to reduce the phospholipid and total cholesterol concentration on the membrane. However, the triglycerides on the fouled membrane was nearly completely removed.

The inadequacy of the more specific *phospholipase* A_2 to remove phospholipids and total cholesterol from PSM, was unexpected and its weak effect during cleaning trials had to be explained. It was assumed that *phospholipase* A_2 was not able to catalyse phospholipid hydrolysis at the membrane interface and therefore *phospholipase* A_2 was applied to lipid samples extracted from fouled membranes. Lipid characterisation studies performed on the lipid samples after enzymatic treatment, indicated that *phospholipase* A_2had the same effect on lipid samples as on the membrane surface. No reduction in phospholipid and total cholesterol concentrations was observed, while the triglyceride concentration was reduced very effectively. The problem was thus not with the interfacial activation, but with the choice of *phospholipase* used. In future studies it would be interesting to include *phospholipase* A_1 in cleaning trials, as this enzyme has the ability to release fatty acids from both positions on the phospholipid molecule.

An alternative to enzymatic membrane cleaning as a method of fouling reduction, membrane pre-treatment, was also investigated in this study. Before contact with abattoir effluent, UF membranes were treated with one of the three non-ionic detergents, Triton® X-100, Tween® 20 and Pluronic® F108. After fouling of the pre-treated membranes, lipid characterisation studies were performed on the lipids that adsorbed onto UF membranes during contact with abattoir effluent. Each of the three detergents induced different effects in the adsorptive properties of the membrane. Only membrane pre-treatment with Triton® X-100 caused a significant reduction in triglyceride, free and total cholesterol, as well as phospholipid adsorption onto the membrane. Pre-coating with Tween® 20 and Pluronic® F108 showed only a slight decrease in the adsorption of the different

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lipid classes. Membrane pre-treatment is thus not a very effective method for the reduction of lipid adsorption onto UF membranes.

7.3 FUTURE RESEARCH

This study has laid the foundation for further investigation into improved methods for lipid removal from UF membranes fouled in abattoir effluent. It has been established in this study that enzymatic cleaning is the most effective method to date, for lipid removal. Further studies, however, need to be conducted to identify the most effective combination of random and specific enzymes to ensure maximum lipid removal from fouled UF membranes.

In addition, kinetic studies have to be performed on the activation of *phospholipase* A_2. *Phospholipase* A_2 is a highly reactive enzyme and could prove to be of great value in enzymatic cleaning regimes if its mechanism of action is firstly understood. *Phospholipase* A_1 could prove to be even more useful as a lipid removing agent and that possiblity would definitely be worth exploring.

The method developed in this study to measure Pluronic® F108, could be a breakthrough in the field of membrane science and could prove to be of great importance in future studies once fully developed.

Overall, results of this study contribute towards a better understanding of UF membrane fouling by lipids. Information contained in this document will also aid the further development of membrane cleaning techniques, membrane pre-treatment and ultrafiltration in general.

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