The Nature and Fate of Ocular Lipids in Contact Lens Wear

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Doctor of Philosophy

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THESIS SUMMARY

The main objectives of this project were to adapt existing analytical techniques, for the examination of lipids extracted from subject-worn contact lenses and tear samples. A great amount of research has been done in the area of meibomian gland lipid analysis; however, there has been little research investigating *ex vivo* lens deposits and more importantly the effect of lens wear on the lipid layer. Since the development of silicone hydrogel contact lenses, lipid deposition and problems that relate to lipid deposition have increased. Discontinuation of lens wear is often related to discomfort, particularly symptoms of dryness. This research was therefore based on the analysis of changes in lipid structure as a result of contact lens wear. The development of an array of techniques enabled different aspects of lipid structure to be examined. Chromatographic techniques such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas chromatography (GC) were utilised to separate individual lipid species and provide structural information. GC analysis of *ex vivo* lens extracts showed significant differences in lipid profiles between daily wear and continuous wear lens extracts. As well as assessment of lipid conformational changes, the presence of lipid oxidative products in the ocular environment were investigated. Malondialdehyde (MDA), a secondary lipid oxidative end product has previously been linked with contact lens intolerance and discomfort. This research showed that oxidative end products were building-up within the contact lens matrix.

To my family

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Publications

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List of abbreviations

Lipids – PLs = phospholipids (PE = phosphatidyl ethanolamine, PC = phosphatidyl choline, PI = phosphatidyl inositol, PS = phosphatidyl serine), DMPC = dimyristoyl phosphatidylcholine, MGs = monoglycerides, DGs = diglycerides, TGs = triglycerides, CE = cholesteryl esters (CM = cholesteryl myristate, CP = cholesteryl palmitate, CO = cholesteryl oleate, CS = cholesteryl stearate), CH = cholesterol, FFA = free fatty acid, SL = sphingolipids, FAMEs = fatty acid methyl esters, PUFAs = polyunsaturated fatty acids

Analytical techniques and assays – TLC = thin layer chromatography, HPLC = high performance liquid chromatography, UV = ultraviolet, FS = fluorescence spectrophotometry, GC = gas chromatography, MS = mass spectrometry, GCMS = gas chromatography mass spectrometry, IR = Infrared, NMR = nuclear magnetic resonance, TBARS = thiobarbituric acid reactive substances, NMPI = N-methyl-2-phenylindole, ELISA = enzyme linked immunosorbant assay, TBA = thiobarbituric acid, TEP = 1,1,3,3 – tetraethoxypropane, TMOP = tetramethoxypropane

General – CW = continuous wear, DW = daily wear, SiHy = silicone hydrogel, CoHy = conventional hydrogel, MDA = malondialdehyde, MeOH = Methanol, $CHCl_3$ = chloroform, MGD = meibomian gland dysfunction, mins = minutes, ml = millilitres, nm = nanometres, PBS = phosphate buffered saline, HEMA = hydroxyethyl methacrylate, MMA = methyl methacylate

Lenses – PV = PureVision, F.N&D = Focus Night & Day, AO = air optix, AOA = air optix aqua, Acu = Acuvue, AcuOas = Acuvue Oasys, S66 = Soflens 66

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Chapter 1 – Introduction

1.1 Introduction

Contact lenses are widely used throughout the world as visual aids, either therapeutically or cosmetically. A large proportion of contact lens wearers experience discomfort (particularly at the end of the day) and in particular symptoms of dryness, which can sometimes lead to discontinuation of wear (1). The main aim of this research was to better understand certain aspects of contact lens intolerance and discomfort. The interaction between the lens and the ocular environment is important to understand; as the lens is approximately ten times thicker than the tear film it can cause instability and irregular function. One of the main objectives for biomaterials scientists is to try to understand this complex interaction between a biomaterial and the host tissue.

This research was based on the development of analytical techniques to enable the study of ocular lipids in contact lens wear. Knowledge of the range of lipid structures involved is therefore important in order that the function and fate of lipids in contact lens wear can be understood.

1.2 Lipid nomenclature

The term lipid is given to molecules which are located in cells and tissues that are soluble in organic solvents but not in water (2). Although there is no widely accepted definition for lipids, Christie's description *"fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds"* is useful in the context of this research (3). It encompasses a whole array of compounds which vary in chemical composition, structure, polarity and function. Lipids can be hydrophobic or amphipathic (i.e. can possess aspects of both hydrophilic and hydrophobic behaviour) and have many biological functions. Lipids are conventionally separated into specific classes and each class consists of many individual lipids.

1.2.1 Hydrocarbons

Hydrocarbons can vary in chain length and unsaturation, as seen in Figure 1.1 to Figure 1.4. They are non-polar lipids, which mean they are largely insoluble in polar solvents.

Figure 1.1 – Structure of decane

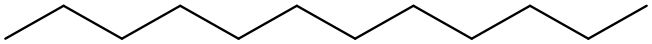


Figure 1.2 – Structure of dodecane

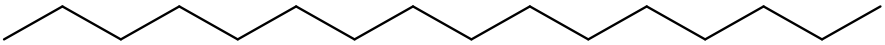


Figure 1.3 – Structure of hexadecane

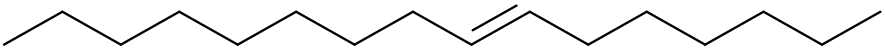


Figure 1.4 – Structure of hexadecene

1.2.2 Fatty acids

Perhaps the most important lipid building blocks are fatty acids which are hydrocarbon chains of varying length and unsaturation with carboxylic functional groups. The individual characteristics of the fatty acids are determined by the chain length and degree of unsaturation. The longer the carbon chain of the fatty acid the higher the melting point and the more hydrophobic the fatty acid. The more double bonds the lower the melting point and the more fluidic the fatty acid (4). The conventional naming of fatty acids is based on the number of carbons atoms in the chain and the number of double bonds. The position of the double bond can also be used in the naming of fatty acids. There are two ways to identify the position of the double bond – one way is to count from the carboxylic functional group end and this is the α numbering system and the other way

is to count from the methyl group end and this is the ω numbering system. There are several ways in which the same fatty acid can be named; for example, 18:1 can also be named octadecenoic acid or oleic acid, 16:0 can be named hexadecanoic acid or palmitic acid.

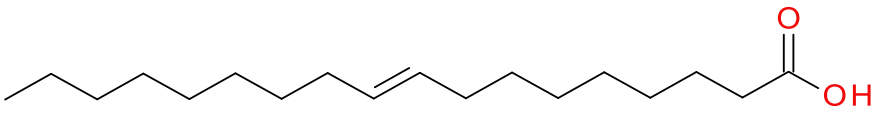


Figure 1.5 – Structure of oleic acid (18:1)

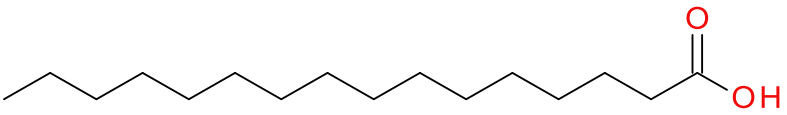


Figure 1.6 – Structure of palmitic acid (16:0)

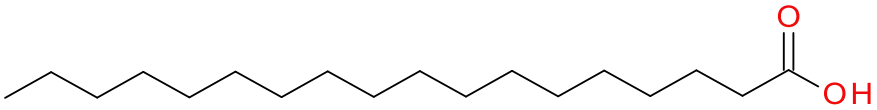


Figure 1.7 – Structure of stearic acid (18:0)

1.2.3 Sterols

The second important building block is cholesterol, which is a relatively non-polar lipid as its structure demonstrates (Figure 1.8). It contains a small polar hydroxyl group attached to a large non-polar substrate. Cholesterol is the major sterol found in meibomian gland secretions.

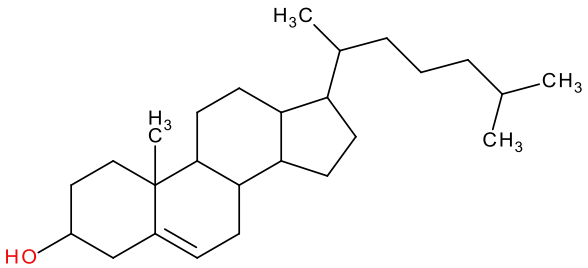


Figure 1.8 – Structure of cholesterol

1.2.4 Cholesteryl esters

Cholesteryl esters are simply cholesterol molecules which have been esterified with fatty acids of varying chain length and unsaturation. The general structure of a cholesteryl ester is shown in Figure 1.9.

Where R1 is a fatty acid of varying chain length and unsaturation

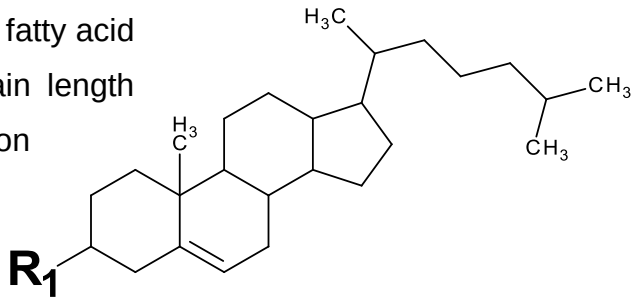


Figure 1.9 – Structure of a cholesteryl ester

1.2.5 Acyl glycerides

Triglycerides (TGs) are glycerol molecules esterified with three fatty acids of varying chain length and unsaturation. They are classed as neutral lipids as the polar groups are sterically hindered by the non-polar hydrocarbons chains.

Monoglycerides (MGs) and diglycerides (DGs) also have glycerol backbones and are esterified with one and two fatty acids respectively and the fatty acids vary in saturation and chain length. MGs and DGs are slightly more hydrophilic than TGs respectively.

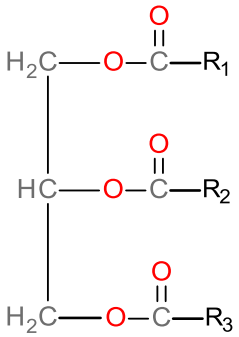


Figure 1.10 – General structure of a triglyceride; where R1, R2 and R3 are fatty acids of different chain lengths and unsaturation)

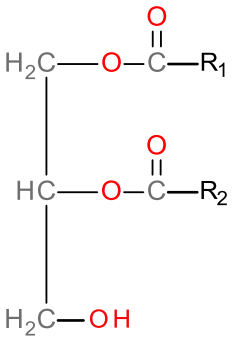


Figure 1.11 – General structure of a diglyceride; where R1 and R2 are fatty acids of different chain length and unsaturation

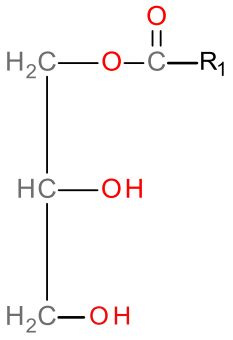
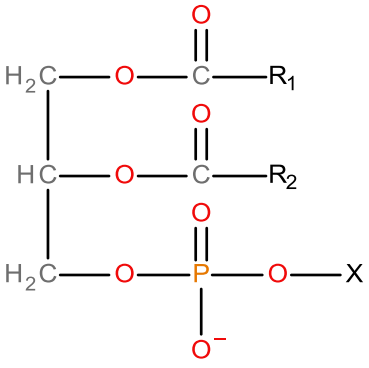


Figure 1.12 – General structure of a monoglyceride; where R1 is a fatty acid of different chain length and unsaturation

1.2.6 Phospholipids

Phospholipids have similar structures to TGs but they have a phosphate group attached to an alcohol head-group making them polar lipids.



Where: R1 and R2 are fatty acids of varying chain length and unsaturation and X is an alcohol head group either ethanolamine, choline, serine or inositol

Figure 1.13 – General structure of a phospholipid

The polar group at the X position is usually an alcohol head group. There are several alcohol head groups which can be at the X position.

Figure 1.14 to Figure 1.17 show the structures of phospholipids with different alcohol head groups that are present in interfacial fluids in the body.

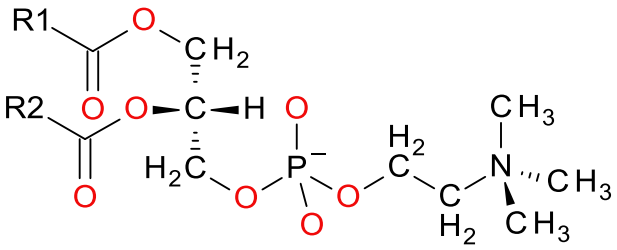


Figure 1.14 – General structure of phosphatidyl choline (PC)

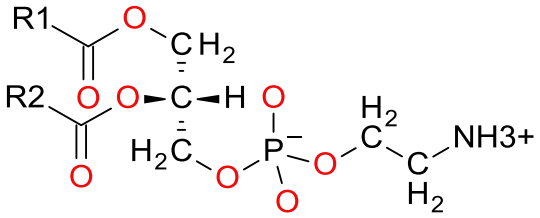


Figure 1.15 – General structure of phosphatidyl ethanol amine (PE)

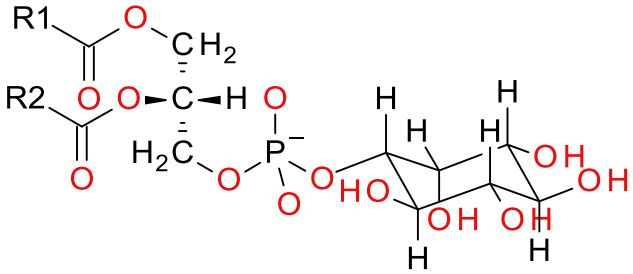


Figure 1.16 – General structure of phosphatidyl inositol (PI)

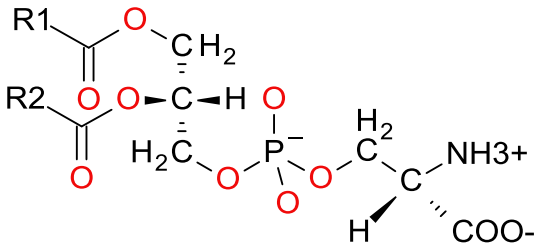


Figure 1.17 – General structure of phosphatidyl serine (PS)

1.2.7 Sphingolipids

Sphingolipid is the general name given to a group of lipids that are based on the sphingosine structure (structure shown in Figure 1.18) and are polar molecules.

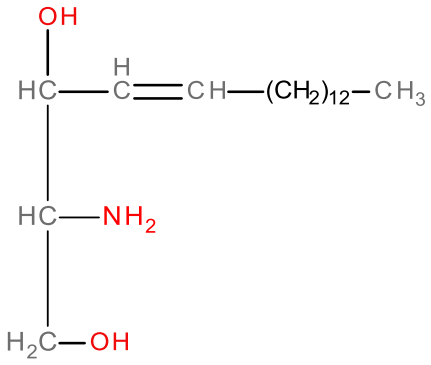
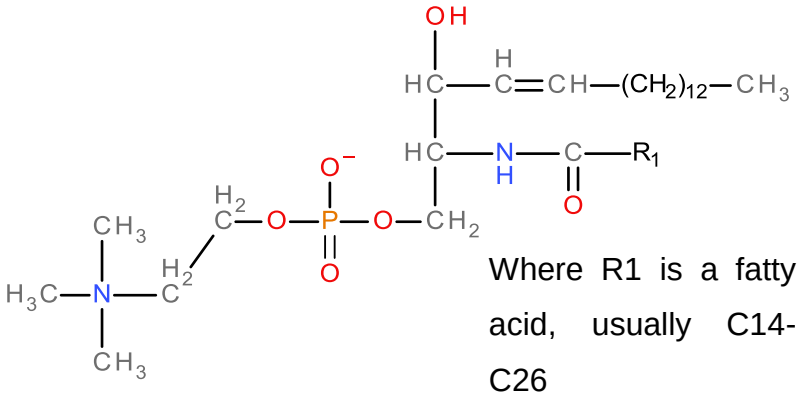


Figure 1.18 – Sphingosine structure



Where R1 is a fatty acid, usually C14-C26

Figure 1.19 – General structure of a sphingomyelin (SM)

1.2.8 Wax esters

Wax esters are long chain fatty acids that have been esterified to very long chain fatty alcohols and are non-polar as the general structure demonstrates (Figure 1.20)

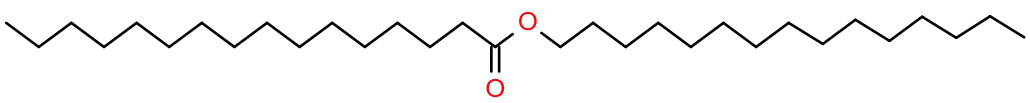


Figure 1.20 – General structure of a wax ester

1.3 The tear film

The tear film is the thin watery layer that covers the anterior ocular surface. The general and accepted model for the tear film is that it is made up of three layers – the inner mucous layer, middle aqueous layer and the outer lipid layer (5). The three layer structure is still believed to be correct, however the nature of the interfaces are still uncertain (6). The layers and components of each layer in the tear film help ensure the regular function of the ocular environment. Each of the tear film layers will be discussed in detail. Each section will describe their function and composition going into further in-depth description of the tear film lipid layer.

Figure 1.21 – Cross section of the tear film (the old concept based on the (7, 8) model and the new concept based on (9)).

1.3.1 The Aqueous layer

The aqueous layer is reported to be ~6.5-7.5µm in thickness. It makes up approximately 98% of the overall tear film and it contains a wide range of proteins, electrolytes and neutral metabolites (5). Many tear proteins are locally produced and are mainly secreted by the lacrimal glands and to a lesser extent the accessory glands of Krause and Wolfring. The number of identified proteins has increased following recent research. Gachon *et al.* (10) suggested there were approximately 60 identified proteins.

However, more recently, De Souza *et al.* (11) identified 491 proteins in the tear film. Over the years there have been several more proteins identified with the aid of newer and more sensitive analytical techniques. Table 1.1 shows some of the major tear proteins found in the tear film and their average concentrations from literature.

Table 1.1 – An average of protein concentrations in literature (12, 13)

Protein component	*Average concentration (mg/ml)*
Total protein	7.51
Lysozyme	2.36
Albumin	1.30
Lipocalin	1.23
Lactoferrin	1.84
IgA	0.30
IgG	0.126
IgM	0.00086
IgE	0.0001

The general role of tear proteins is to act as a defence mechanism for the ocular environment. For example, lysozyme which is one of the most abundant tear proteins, acts to destroy cell membranes of bacteria. Each individual protein acts in different ways to protect the ocular environment against infection.

1.3.2 The mucous layer

The main function of the mucous layer is to provide a lubricious surface for the ocular environment. The mucous layer coats any foreign particles that may have entered the eye with mucin, which ultimately protects the cornea and conjunctiva from damage (14). The mucous layer composition is only recently starting to be understood in any detail. It is mainly composed of glycoprotiens, which are proteins with a high ratio of carbohydrate to protein. More and more mucins are being characterised such as MUC5AC and MUC5b (5). Mucins are secreted mainly by goblet cells of the cornea and conjunctiva and can be soluble or insoluble (15).

1.3.3 Tear lipid layer

As previously stated, the lipid layer is the outer most layer of the tear film. It is also the thinnest layer, with an average thickness of ~100nm thickness, however this is known to vary from subject to subject (16). This project is mainly based on the tear lipid layer; although it is important to understand how this layer interacts with the other tear film layers.

1.3.3.1 Lipid layer composition and function

The main functions of the tear lipid layer are to prevent the evaporation of the underlying aqueous layer and provide a smooth surface for the eyelid to slide over (6, 17). It is the unique composition of the lipid layer which makes its functions possible. The lipid layer is mainly composed of lipids secreted by the meibomian glands. However, it is still uncertain as to whether the tear lipid layer is composed solely of meibomian gland lipids or whether other sources contribute to the overall composition of the tear lipid layer (explained in further detail on Page 33). According to Nagyova *et al.* (18) tear lipid composition is very different to meibomian gland lipids, however the composition of tear lipids is still under considerable debate. Certain publications state that the tear film lipid layer is actually two layers, an outer hydrophobic layer and an inner hydrophilic layer composed of mainly phospholipids. Phospholipids, due to their amphipathic nature act as an interface between the hydrophobic lipids and the hydrophilic aqueous layer (19).

1.3.3.2 Composition of meibomian gland secretions

The majority of tear lipids are secreted by meibomian glands which are located in the upper and lower eyelids. They secrete lipid and the action of blinking spreads the lipid across the surface of the cornea.

The lipid composition of meibomian gland secretions has been well reviewed in literature and the general lipid classes are mainly agreed upon. However, the percentage composition of each lipid class varies from researcher to researcher perhaps as a result of many advances in analytical methods and/or the tear sampling technique used. The percentage compositions of meibomian gland lipids has been summarised

in the review by Wojtowicz *et al.* (20). The major lipids secreted by meibomian glands are predominately non-polar lipids such as wax esters and sterol esters such as cholesteryl ester. Other lipids are secreted as minor compounds such as free fatty acids, fatty alcohols, triglycerides and polar lipids such as phospholipids. Figure 1.22 illustrates the mix of lipids secreted by the meibomian glands as an average from various authors.

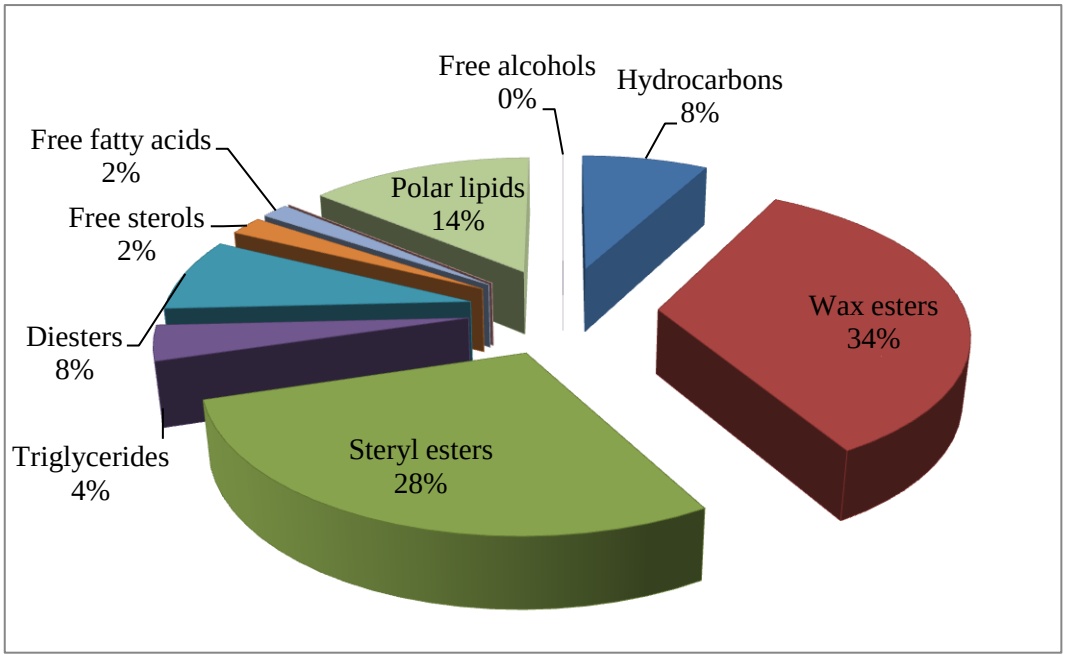


Figure 1.22 – Average percentage composition for meibomian gland secretions (from (21-24)).

1.4 Lipid compositional differences between meibomian gland secretions and the tear lipid layer

The tear lipid layer is made up of predominately lipids secreted by the meibomian glands, however tear lipids are believed to be more complex than meibomian gland secretions (25). Other possible sources of lipid which contribute to the tear lipid layer are the conjunctiva, cellular debris, lacrimal glands and the lid margins (26). There have been significant levels of research in the area of lipid composition of tears and meibomian glands. There is some general agreement in literature with regards to meibomian gland secretion lipid composition; however the major debate is regarding the levels of polar lipids. The level of phospholipids secreted by meibomian glands has been disputed by several authors. Many authors

have reported the presence of several phospholipids from meibomian gland secretions, some as minor compounds and others as major compounds (16, 19, 23, 27-29). However, there have been several recent publications which report little to undetectable levels of phospholipids secreted by the meibomian glands (30, 31). Butovich (31) and Borchman *et al*. (30) have stated that the meibomian glands do not secrete detectable levels of phospholipids using high performance liquid chromatography (HPLC) and infrared (IR) respectively. Interestingly, Butovich has stated that there are phospholipids in tears, but not secreted by the meibomian glands which suggests there are other sources of lipid that contribute to the tear lipid layer. Cellular debris, conjunctiva and lacrimal gland secretions are all possible sources of lipids which contribute to the tear lipid layer (26).

Campbell *et al.* (32) have shown that phospholipids are not detected in tears because they are enzymatically degraded by certain phospholipases. They reported the main enzyme; phospholipase C was responsible for the degradation of phospholipids in to by-products such as DGs and lysophospholipids.

Recent publications by Saville *et al.* (25, 33) report the presence of several phospholipids in the tear film, these include varying chain length PCs and SMs. Saville *et al.* (33) report that the PCs detected in meibomian glands were also detected in tears which suggested that tear lipids originate from meibomian gland secretions.

As analytical techniques have improved, a wider range of lipoidal species have been more accurately identified in meibomian secretions. Butovich *et al*. (34-36) have shown that very long chain fatty acids are present within the family of cholesteryl esters. Although the presence of long chain fatty acids (C16 to C26) had previously been reported (37, 38), the lipid class with which they are combined had not been clearly determined because of the limitations of the analytical technique used.

1.5 Function and composition of lipids at biological interfaces

To help better understand the function of tear film lipids, the composition and role of lipids at other body interfaces is important to review.

Pulmonary surfactant and synovial fluid have similar functions to the tear film, they help reduce surface tension and do so under a load. However, the composition of pulmonary surfactant and synovial fluid differ significantly from the composition of the tear film.

1.5.1 Pulmonary surfactant: lipid composition and function

Pulmonary surfactant is the fluid lining the lungs. It is a mixture of proteins and lipids which coats the lungs and reduces surface tension at the air/water interface (39). It was discovered that it was the role of the lipids which reduced the surface tension and allowed the lung to expand and contract without the alveolar collapsing. Phospholipids have been recognised for some years as the major component of pulmonary surfactant together with the fact that a phosphatidyl choline (PC) – dipalmityl phosphatidyl choline (DPPC) plays a major role in the function of this complex fluid (40-42). The total lipid composition of pulmonary surfactant is shown in Figure 1.23. Phosphosphatidyl cholines (PCs) represent approximately 80% of the total and DPPC accounts for approximately half of this total. The other 40% is made up of PCs with fatty acids of varying chain length and unsaturation at Sn1 and Sn2 positions on the glycerol backbone. The fatty acids at the Sn1 and Sn2 positions of the glycerol backbone are usually mono or di-unsaturated but rarely polyunsaturated. It is interesting that lung surfactant fatty acids resemble those found in meibomian gland lipids in that both very small and polyunsaturated fatty acids are virtually absent. Cholesterol, on the other hand which is believed to aid in the spreading of the phospholipids and increase the fluidity of lung surfactant is also present in meibomian gland secretions (43, 44). Other non-polar lipids such as monoacylglycerols, diacylglycerols and triglycerides are also present in pulmonary surfactant at low levels (45, 46).

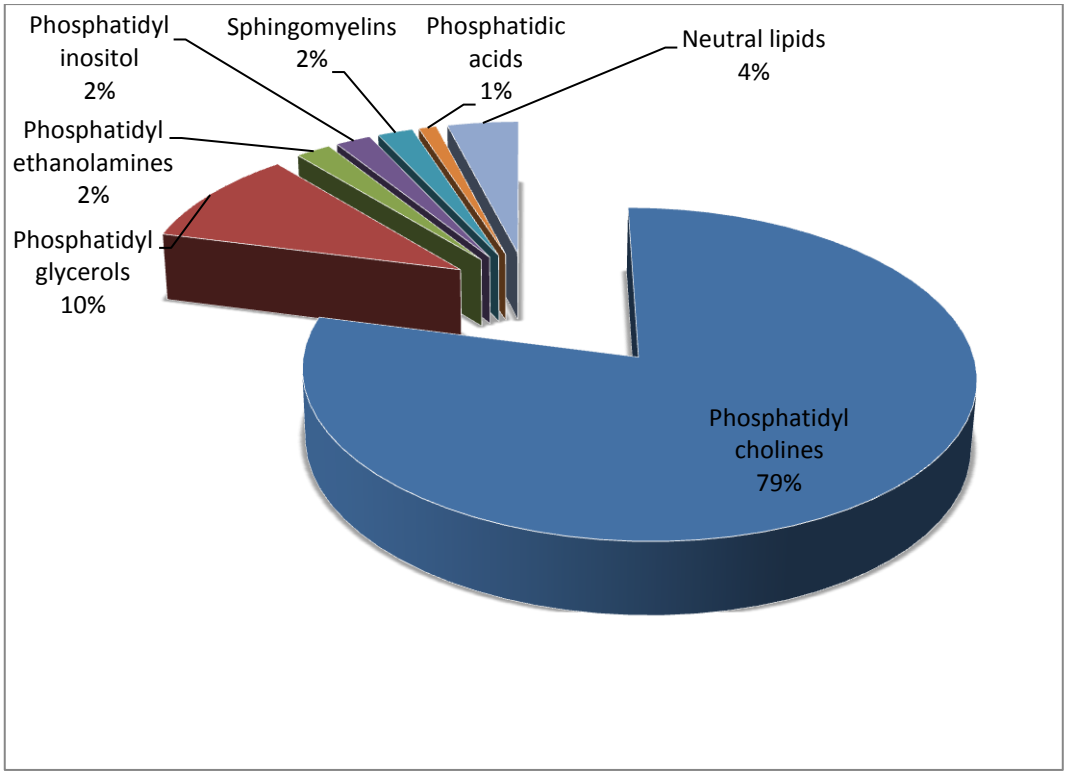


Figure 1.23 – Pulmonary surfactant phospholipid composition

1.5.2 Synovial fluid: lipid composition and function

The synovial fluid (SF) of joints functions as a biological lubricant, providing low-friction and low-wear properties to articulating cartilage surfaces through the combined contribution of hyaluronic acid (HA), lubricin and surface active phospholipids (SAPL). These species are secreted by chondrocytes in articular cartilage and synoviocytes in synovium, and concentrated in the synovial space by the semi-permeable synovial lining. The predominant contributor to fluid film lubrication is HA whereas SAPL play a major role in boundary layer lubrication.

The two major functions of synovial fluid are firstly to create a smooth surface for the articulating joints and the second is being able to do so under considerable loads over a substantial time period. HA was the first component found to be present in significant amounts in synovial fluid and was believed to be the component most responsible for lubricity in joints. It was later discovered that HA failed under any load and was therefore not the component responsible for the lubricity. Earlier studies failed to

recognise the lubricating role of lipids but subsequent work established their key role as boundary lubricants under load (47-50).

The important physicochemical point is that HA has no significant surface activity and will therefore not function effectively at interfaces. Lubricin and especially phospholipid (hence SAPL) are surface active and provide the surface and boundary layer functionality needed to provide the complete lubrication capability that is essential to the functionality of synovial fluid. This interfacial activity of phospholipids is even more evident in their function in the lung (51).

1.5.3 Comparison of lipid composition and function at biological interfaces

The overall lipid compositions of synovial fluid, pulmonary surfactant and the tear film are different to each other. The tear film is made up of predominately non-polar lipids such as cholesteryl esters and wax esters whereas pulmonary surfactant and synovial fluid are made up of mostly phospholipids. It is clear that the tear film lipid layer is unique in its composition. Although the functions of each biological fluid may be similar, their lipid compositions are not. The tear film lipid layer, not only has to aid in the ocular surface lubrication, it has to prevent evaporation of the aqueous. It is the unique mix of non-polar lipids which helps this function be fulfilled.

1.5.3.1 Surfactant proteins at biological interfaces

It is the amphipathic nature of phospholipids which promotes their ability to function at a range of interfaces. Although phospholipids can function at interfaces, there are certain proteins present in pulmonary surfactant and the tear film which also act as surfactants. Certain proteins have been found in pulmonary surfactant and are their presence is being researched in the tear film. These surfactant proteins include surfactant protein A (SP-A), surfactant protein B (SP-B), surfactant protein C (SP-C) and surfactant protein D (SP-D). All four of these proteins have recently been identified in the ocular environment, more specifically in the lacrimal glands (52, 53).

SP-B and SP-C are hydrophobic proteins and their main functions in pulmonary surfactant are adsorbing phospholipids to an air/liquid interface and the formation of surface active monolayers. Their role in the ocular environment is still unknown, but is thought to be similar to that of pulmonary surfactant (54).

The commonality of spreading function and boundary layer lubrication associated with the combination of phospholipids and the surfactant proteins; surfactant protein B (SP-B) and surfactant protein C (SP-C) in lung and articular joints is well established. Both SP-B and SP-C have been identified in the ocular environment and particularly in the lacrimal glands (52). The expansion and compression of the lipid layer during the blinking process has apparent similarities to the function of lung surfactant (55) and it is logical to expect that the interfacial combination of phospholipids and surfactant proteins plays a part in this process.

SP-C is a very small protein approximately 4-6kDa. The role of SP-C in pulmonary surfactant is to reduce surface tension between the phospholipid and the soluble interface (56). The role of SP-C in the tear film is believed to be the same, acting as a surfactant between the phospholipids and the aqueous layer. SP-B has a similar role of reducing the surface tension of lipids.

Brauer *et al*. (53) had already reported the presence of SP-A and SP-D in human tear fluid and other parts of the ocular environment and later reported the presence of SP-B and SP-C in human tear fluid (57). SP-A and SP-D are both hydrophilic and their major roles in pulmonary surfactant are improving the host defence and the reuse of surfactant (54).

There is still considerable contradiction as to whether the SP-A or SP-D or both are present in the ocular environment. Certain publications have identified SP-A and SP-D in the ocular environment (57), however others have only reported the presence of SP-D but not SP-A (58, 59). Both of these proteins are present in lung surfactant and they have very important roles in aiding surface activity.

SP-A is 28-36kDa and its function is to aid the function of SP-B in reducing surface tension at an air/liquid interface (60-62) SP-D is a 43kDa protein and its function in pulmonary surfactant is the interaction with bacteria and viruses (63). Less is known about the function of these surfactant proteins in ocular environment, but they are believed to function in a similar manor to pulmonary surfactant.

The tear film lipid layer is composed of mainly non-polar lipids such as cholesteryl and wax esters. The lipid compositions of synovial fluid and pulmonary surfactant differ significantly from the tear film lipid layer as they are composed mainly of polar lipids such as phospholipids. Although their functions are similar, their lipid compositions are very different. The main focus of the research in this project was the tear film lipid layer but more importantly, the effects of contact lens wear on the composition of lipids. Certain ocular disorders can also be a cause of or cause tear lipid compositional changes and these are discussed in detail in section 1.5.

1.6 Dry eye – compositional changes to meibomian gland lipids

The definition for dry eye given by the definition and classification subcommittee of the international dry eye workshop (DEWS) : '*dry eye is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface'* (64-73).

Dry eye has been separated into two main classes in literature – deficiency of aqueous production and increased rate of evaporation. One of the major causes of increased rate of evaporation is meibomian gland dysfunction (MGD), of which there are many types. The causes of MGD include obstructive MGD, which is a physical blockage of the glands or non-obstructive MGD. There many types of MGD such obstruction of the glands, over-secretion of meibum, under-secretion of meibum. Obstruction of the glands can cause low levels of meibum to be secreted

by the glands. However, low levels of meibum secretion may not be caused by obstruction.

MGD can lead to changes in the tear film dynamics, irritation of the eye and disease of the ocular surface (74). Other ocular disorders, which can be caused by MGD, have been previously confused with MGD and these include blepharitis. Blepharitis is generally described as inflammation of the entire eyelid. Because MGD can be the cause of many ocular symptoms such as inflammation, other disorders have been confused with it; however MGD can be the cause of many other ocular disorders such as blepharitis.

MGD often causes changes to the tear film such as increased evaporation of the aqueous as a result of decreased meibum production. Obstruction of the glands is the main type of MGD which often leads to decreased secretion of meibum. There are many causes of obstructive MGD which include changes in secretion composition or an obstruction of the duct of the gland. C18:1 (oleic acid) has been found to activate epidermal keratinocytes which could lead to blockage of the meibomian glands (75).

Changes in lipid composition can often cause MGD. There are reported differences in lipid composition between normal subjects compared to subjects with MGD, in particular changes in the fatty acid structure. A reduction of unsaturated lipids results in a thicker secretion. Saturated lipids can pack together which produces a more viscous secretion. Therefore, a reduction in the levels of unsaturated lipids could result in blockage of the meibomian glands (38).

A recent study analysed the differences in fatty acid composition between subjects with MGD and aqueous deficient dry eye compared to normal subjects and found differences in the fatty acid composition between normal subjects and subjects with MGD (38). There was however no change in fatty acid composition between normal subjects compared to aqueous deficient dry eye subjects (38). Significantly lower levels of C16:0 and C18:0 were observed for subjects suffering with MGD

compared to normal subjects. Therefore, the ratio of saturated to unsaturated fatty acids is important for regular function of the tear film.

There are reports that levels of phospholipase A2, an enzyme that degrades phospholipids (described in detail in section 1.11.2) are higher in dry eye patients (76). Phospholipids in the tear lipid layer are thought to act as an interface between the aqueous and the non-polar lipids, increasing tear film stability (19). Therefore, an increase in the levels of phospholipases that degrade phospholipids could lead to decreased tear film stability and dry eye.

1.6.1 Contact lens wear, MGD and dry eye

Many of the disorders discussed above can prevent the wear of contact lenses by certain individuals. Certain ocular disorders can be caused by contact lens wear, known as contact lens induced dry eye. There have been some reports that link contact lens wear to an increased risk of MGD. However, the International workshop on meibomian gland dysfunction: report of subcommittee on epidemiology of, and associated risk factors for MGD have reviewed past literature and reported there was no significant correlation between contact lens wear and MGD (77-80), even though earlier studies had reported a significant correlation between contact lens wear and MGD (81, 82). Arita *et al*. have investigated the morphological changes to meibomian glands in contact lens wearers vs. non-contact lens wearers and found significant changes to the structure of the glands in contact lens wearers (83). This led to a decrease in the number of functioning glands which resulted in decreased meibum production which ultimately caused dry eye.

1.6.1.1 MGD and dry eye therapies

Current MGD therapies include warm compresses of the eyelids, the use of ocular lubricants, topical antibiotics and tetracycline derivative treatment (84).

There are many therapies for contact lens induced dry eye and these include the use of lubricating eye drops. Other treatments include the use

of contact lenses which release drugs to the eye over an extended period of time (85).

Recent studies have investigated dietary essential fatty acid supplements to decrease the symptoms of dry eye and improve the tear lipid layer composition. A review by Rosenberg *et al*. (86) stated that some previous studies using various essential fatty acids for dry eye treatment reported improvements in dry eye symptoms (87-89) whilst others did not show any significant improvements in symptoms (90, 91). However, this was not a significant number of studies to conclusively state that increasing dietary intake of essential fatty acids decreases symptoms of dry eye disease. Further work by Wojtowicz *et al.* (92) revealed that there was no change in meibum lipid composition after a dietary intake of an essential omega-3 fatty acid. The main treatment for contact lens and non-contact lens induced dry eye is mainly the use of lubricating eye drops.

1.6.1.2 Dry eye and contact lens wear strategies

Dry eye as a result of contact lens wear is a common complaint and one of the most prevalent causes of discontinuation of wear (93). There are many contact lens strategies for dry eye and there has been a review on this topic by Sindt *et al*. (94). Some of these contact lens features which are designed to improve dryness symptoms are discussed in section 1.7.1.

A new daily disposable silicone hydrogel lens (Dailies Total 1 – delafilcon A), loaded with a phospholipid – DMPC (dimyristoyl phosphatidylcholine) was developed to try to improve symptoms of dryness. The principle of the lens was to release the DMPC into the tear film throughout the wear time, increase tear film stability and therefore reduce the symptoms of dryness (95). Chapter 6 investigates both *in vitro* and *ex vivo* studies of DMPC-containing lenses.

1.7 Contact lenses materials

A brief introduction to the history of contact lens materials is important to discuss as they will have an effect on tear film dynamics. The interaction

between the lens and tear film will be effected by a change in contact lens properties such as the lens material, surface properties and water content and each of these properties are discussed below.

1.7.1 Types of contact lenses

To the date of writing this thesis, there had been five generations of contact lenses. The first generation of contact lenses were polymethyl methacrylate (PMMA) based lenses. These were very glassy in nature and did not allow any oxygen to pass to the cornea.

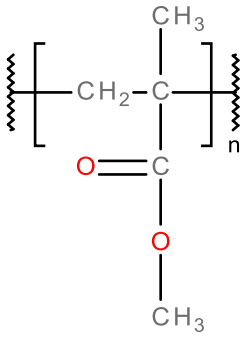


Figure 1.24 – Structure of PMMA

The second generation of contact lenses were the conventional hydrogels. They were based on the hydrophilic polymer, poly (hydroxyethyl methacrylate) (polyHEMA). Other conventional hydrogel materials were developed based on copolymers of polyHEMA with other polymers. The ionicity and the water content of conventional hydrogels varied according to the nature of the polymers. There are four main groups of conventional hydrogel.

Group I lenses = Low water content, non-ionic

Group II lenses = High water content, non-ionic

Group III lenses = Low water content, ionic

Group IV lenses = High water content, ionic

Division between low and high water content is set at 50%. An ionic material is defined as one that contains more than 0.2% ionic material (96, 97).

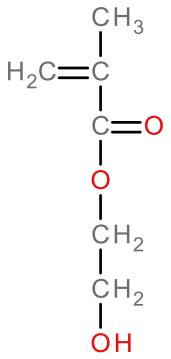


Figure 1.25 – Structure of HEMA (hydroxyethyl methacrylate)

Conventional hydrogels have limited oxygen permeability and are therefore used predominantly as daily disposable lenses.

The third generation of lenses were therefore developed to improve oxygen permeability to the cornea by the introduction of silicone to the lens. Silicone has a very high Dk (oxygen permeability) value, which indicates high oxygen permeability. Third generation lenses were made predominantly from silicone rubber which was bonded to oxygen (siloxane). The lenses allowed high levels of oxygen to reach the cornea but they were very hydrophobic in nature which meant they adhered to the cornea and increased lipid deposition was a problem.

The fourth generation of contact lenses were rigid gas permeable lenses that were based on copolymers of a monomer referred to as TRIS (tris [trimethylsiloxy] – methacryloxy – propylsilane).

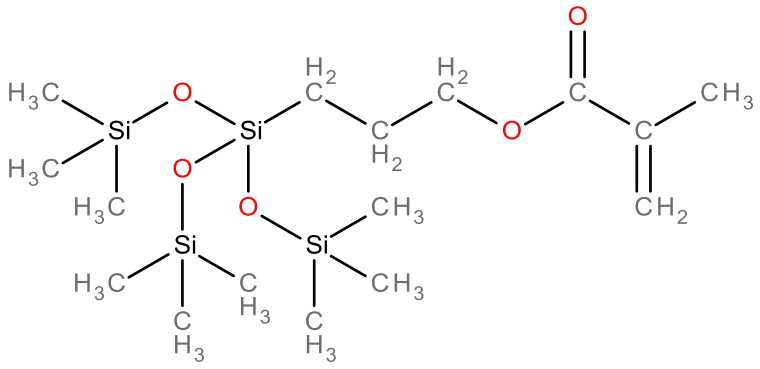


Figure 1.26 – Structure of tris (trimethylsiloxy)-methacryloxy-propylsilane (TRIS monomer)

The fifth and current generation of contact lenses were a hybrid of conventional hydrogels and silicone lenses. They incorporated monomers such as hydroxyethyl methacrylate (HEMA) and methyl methacrylate (MMA) with silicone-containing monomers which produced silicone hydrogels. Silicone hydrogels could be worn on an extended wear basis as they had greater oxygen permeability.

There have been many developments in the area of silicone hydrogel materials. One of the areas of development has been the surface modification of silicone hydrogel materials. This is because the surface of the lens should be hydrophilic so the aqueous tear film can easily spread over the lens and to reduce the level of lipid deposition.

1.7.2 Surface modification of contact lenses

The first generation of silicone hydrogel materials such as Focus Night & Day (lotrafilcon A, CIBA Vision) and PureVision (balafilcon A, Bausch and Lomb) are surface treated in two different ways.

Focus Night & Day lenses are coated by using plasma polymerisation whereas PureVision lenses are plasma oxidised. Both of these methods are used to make the contact lens surface more hydrophilic to prevent increased attraction of lipids. Plasma oxidation and plasma polymerisation have different effects on the surface of these lenses. PureVision is surface treated using plasma oxidation however; this surface treatment does not create a uniform coating which leads to hydrophobic patches on

the surface of the lens. As the lens is exposed to air (hydrophobic), the polymer chains rearrange themselves towards a hydrophilic nature within the matrix of the lens which results in hydrophobic areas being created on the surface of the lens. Focus Night & Day lenses on the other hand have relatively uniform hydrophilic surfaces and subsequently have lower levels of gross lipid deposition (98).

The next generation of silicone hydrogels such as Acuvue Advance (galyfilcon A, Vistakon) and Acuvue Oasys (senofilcon A, Vistakon) lenses are not surface modified. They contain a molecule called polyvinyl pyrollidone (PVP). PVP is a long chain hydrophobic molecule which acts as a wetting agent for the lens.

Newer lens materials have been developed which are not surface modified or use wetting agents. Instead they use certain polymers which are wettable, materials include Biofinity (comfilcon A, CooperVision) (94).

The surface modification of the lenses effect the way in which tear film components interact with the lens, in particular the interaction of lipids with the lens. This will affect the wettability of the lens surface.

1.8 Factors which influence lipid deposition on contact lenses

There are many factors which may influence the rate of lipid deposition on contact lens materials. These include: the lens material, the surface characteristics of the lens, the length of wear, the wear schedule, the cleaning regime but ultimately the subject wearing the lens. In many cases the individual is the greatest variable as lipid profiles vary from subject-to-subject (16), which could be as a result of individual diet and climate. Variables such as age and health can significantly affect lipid composition (16, 99).

1.8.1 Contact lens material and lipid deposition

Tear film components will readily deposit on contact lenses. The interaction of lipids with contact lenses is affected by several factors that are discussed below.

The lens material characteristics change the way lipids and other tear film components are attracted to the surface of the lens (100). Lipid does not only adsorb on the surface of contact lenses but can also penetrate within the lens matrix, particularly with hydrogels which are semi-permeable (100). Lipid adsorption has been shown to be greater on contact lenses that contain the monomer N-vinylpyrrolidone (NVP) (101). The addition of NVP to lenses was to try and increase lens water content. However, due to the hydrophobic backbone of NVP it led to the increased lipid deposition.

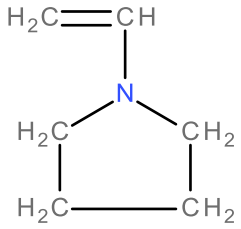


Figure 1.27 – Structure of N-vinyl pyrrolidone (NVP)

The ionicity of the lens does not greatly affect lipid deposition but has been shown to have a greater effect on protein deposition (102).

Experiments performed by Bontempo *et al*. (103) showed that 'group IV lenses (high water content, ionic matrix) are more likely to be prone to higher levels of protein deposition than lipid deposition. They also showed that lipid deposition was more likely to occur on group II lenses (high water content, non-ionic matrix). This was the case when unworn contact lenses had been spoiled in either a protein solution or lipid solution. However, Bontempo *et al.* (103) demonstrate that in a lipid and protein combination

solution, this is not the case. When group II lenses were spoiled in a lipid-protein mix, they now attracted more protein than lipid. This is because certain charged proteins in the solution compete with the polar lipids and therefore bind to the hydrophilic sites on the contact lens.

For group I, III and IV lenses, the presence of proteins in the artificial tear solution caused an increase in lipid adsorption (normally non-polar lipids). Rigid gas permeable (RGP) contact lenses were more likely to have higher levels of lipid deposition than protein deposition due to their hydrophobic nature. But when both protein and lipid were present in the artificial tear solution, the lipid made the contact lens surface more hydrophilic. This is because the hydrophobic sites of the contact lens attracted the lipid and the hydrophilic sites repelled lipids, this therefore created a hydrophilic surface for the proteins bind. Lipid deposition on RGP contact lenses was shown to be material dependant, whereas protein deposition was shown to be minimal (104).

Levels of lipid deposition are known to be greater than protein deposition on many silicone hydrogel materials because of the hydrophobic nature of tear lipids (105).

The nature of the monomers used to make contact lenses can determine which individual lipids are deposited on the lens (97). Carney *et al.* (97) have demonstrated that different lipid classes will have a greater affinity for different contact lens materials. This was determined from the incubation of different lens materials in a non-polar lipid solution (cholesterol) and a polar lipid solution (PE – a phospholipid). The lens materials chosen were a mix of conventional and silicone hydrogel materials. Their results showed that after the 20 days incubation period, PureVision (balafilcon A), Acuvue Oasys (senofilcon A) had greater levels of cholesterol deposition. This was most likely due to the surface treatments of these lenses. Acuvue Advance (galyfilcon A) had higher levels of PE adsorption over the 20 day period compared with Acuvue Oasys and PureVision. However, the concentration of polar lipid adsorption was lower for all lenses compared with non-polar lipid

adsorption. The monomers used to make the lens and the water content of the lens were determining factors for the levels of lipid deposition as well as the surface modification of the lens. This study investigated lipid adsorption using an *in vitro* spoliation model but this does not mimic *in vivo* conditions. Although *in vitro* lipid spoliation models are useful, they do not mimic *in vivo* conditions. There are a limited amount of studies which investigate the nature of lipid deposition from *ex vivo* lenses. However, there have been recent studies which investigate the nature of lipid composition from *ex vivo* contact lenses that have been cleaned with various lens solutions and these publications are reviewed in section 1.7.2.

1.8.2 Contact lens solutions and lipid deposition

A recent study investigated and quantified the levels of different non-polar lipid species deposited on *ex vivo* Acuvue Oasys (senofilcon A) lenses by using HPLC for the separation of the lens extracts (106). Heynen *et al*. (106) found that different cleaning solutions and regimes performed differently, and removed varying amounts of lipid from the lens. Cholesteryl esters were the predominant lipid class deposited on the Acuvue Oasys lenses as determined by HPLC separation, with a lesser amount of cholesterol and free fatty acids. The cholesterol ester used was cholesteryl oleate and the free fatty acid used was oleic acid. Heynen *et al.* (106) reported that solutions that contained particular preservatives such as Polyquad (Polyquarterniums) and Aldox were more effective for the removal of cholesteryl esters (the major lipid class deposited on the lens) than solutions that contained hydrogen peroxide. This study focused on the cleaning efficacy of solutions for the removal of non-polar lipids from lenses, whereas a study by Saville *et al.* (25) investigated the propensity of various phospholipids (polar lipids) for *ex vivo* contact lenses. The lens materials chosen were PureVision (balafilcon A) and Acuvue Oasys (senofilcon A). Groups of subjects were asked to wear the lenses for a thirty day period and clean the lenses every night with their allocated solution (one of the three solutions tested). The *ex vivo* contact lenses were spiked with a phospholipid standards, extracted and the

extracts were analysed by mass spectrometry. The results showed that there were high levels of phospholipids and cholesterol present on Acuvue Oasys lenses. PureVision lens extract analysis revealed that the levels of lipid deposition were not affected by the cleaning regime used which indicated that the cleaning solutions were not effective at the removal of lipids deposited on the lens throughout the wear schedule. This accounts for the build-up of lipids on lenses over the wear schedule of the lens.

1.9 Tear lipid and meibomian gland secretion collection techniques

As it is still unclear whether the meibomian glands are the sole contributor to the tear lipid layer (18), it becomes difficult to find a suitable tear lipid sampling technique. There are many techniques used for the collection of lipids from the ocular environment, but each technique is suited to the collection of lipids from a certain area. For example, hard expression of meibomian glands can provide a pure meibomian gland secretion sample and is not contaminated by tear lipids. Hard expression is often used to sample meibomian gland lipids as a large amount of lipid can be collected the use of this method (23). The meibomian glands are squeezed and the meibum secreted is collected using platinum spatula. This method of collection is used solely for meibomian gland lipids. This method of collection has the advantages of being able to obtain a pure meibum sample and can be done on cadaver eyes as well as live patients. The main disadvantage of this method is that it can be quite painful. However, trying to obtain a pure tear lipid sample is much more difficult. The many techniques used in literature for meibomian gland lipid collection have been microcapillary tube (107), Schirmer strips (26, 30) and hard expression of the glands (23) and each technique has its advantages and disadvantages.

Microcapillary tube method is often used to collect a tear sample. The microcapillary is placed in the meniscus of the eye and tears are drawn up the tube by capillary action. This method has the advantages of being a very rapid collection method and being pain free. However, the

disadvantages of this method are that there is only a small sample volume and that there is a great risk of contamination from tear lipids and cellular lipids (107).

Schirmer Strips have also been used for tear lipid collection by Borchman *et al*. (26, 30). The strip is placed in the lower eyelid and the sample is collected. This method is a suitable method for tear lipid collection, but not very good for meibomian gland secretion collection. This method is also more invasive compared with the microcapillary collection method.

Ophthalmic sponges are another method used to collect tear lipids. The sponge is made of cellulose and is placed in the meniscus of the eye to collect tear lipid sample. The advantages of using this method for collecting tear lipid samples is the large sample volume obtained and it is a pain free method for the collection of tear lipids. However, the disadvantages are the risk of contamination from cellular debris and skin lipids. There are no reports in literature about the use of ophthalmic sponges to collect tear lipid samples. However, this technique has been used previously in this research group for the collection of tear lipids and was therefore used for the research in this thesis (108).

The contact lens is also a method for tear lipid collection. The analysis of deposits on worn contact lenses is an advantageous method for the collection of tear lipid samples as it can provide a 'snapshot' of what is occurring at a particular point in time. The disadvantages of this method are that certain contact lenses do not adsorb that much sample volume and the contact lens can induce changes to the lipid composition (to be discussed in further detail in later chapters).

In summary, the most advantageous methods for tear lipid sampling, according to literature are the Schirmer strips and the ophthalmic sponges. The best method for collecting a pure meibomian gland secretion sample is hard expression of the meibomian glands.

1.10 Analytical techniques for meibomian gland, tear lipids and lipid deposits on contact lenses analysis

Many analytical techniques have been used by several researchers. They have enabled the characterisation of lipids from meibomian gland secretions, tear lipid samples and contact lens extracts.

1.10.1 Thin layer chromatography (TLC)

TLC has been used many by many researchers for the separation meibomian gland secretions into their relative lipid class. TLC separates the lipid classes according to their relative polarity. TLC is often used in conjunction with gas chromatography (GC) as it was by Nicholaides *et al*. (37) and Harvey *et al*. (109). Lipid classes were separated by TLC then analysed in further detail by methods such as GC (which separates individual fatty acids). TLC was also used by Zhao *et al.* (110) for the analysis of *ex vivo* contact lens extracts and the cholesterol band was analysed by mass spectrometry.

1.10.2 Gas chromatography (GC)/gas chromatography mass spectrometry (GCMS)

GC and GCMS can be used after TLC separation of lipid classes or independently. GC, GCMS and TLC with GC and GCMS have been used by for fatty acid separation by several researchers (23, 38, 111-113).

Joffre *et al*. (38) used GC with flame ionisation detection (FID) and GCMS to separate and identify the fatty acid moieties of lipids from meibomian gland samples. They investigated the differences in the fatty acid composition of meibomian gland dysfunction patients and aqueous deficient patients compared to normal patients.

Nicolaides *et al*. (23) used GCMS for fatty acid separation after TLC separation of lipid classes from the meibomian glands from cadavers.

Shine *et al*. (111, 112) also used GCMS after lipid class separation by TLC to investigate the changes in fatty acids of normal patients compared to those with certain cases of chronic blepharitis.

1.10.3 High performance liquid chromatography (HPLC)

HPLC has been used for tear lipid and meibomian lipid separation by various researchers. HPLC is used for the separation of lipids into either lipid class or individual lipids and is used in conjunction with various detectors such as UV, fluorescence and different types of MS. HPLC with UV detection was used by Shine *et al*. (19, 27) for the separation of polar lipids from meibomian gland secretions. Both of the studies separated lipids from meibomian gland secretions by TLC and then used HPLC with UV detection to separate the individual lipids. The individual lipids were identified by the comparison of their retention time with standards (19, 27).

Butovich *et al.* (29, 31, 34, 35, 114) also used HPLC for the separation of meibomian gland lipids and a MS detector was used. There are various ionisation methods in MS such as electron ionisation (EI), chemical ionisation (CI), atmospheric pressure chemical ionisation (APCI), and electrospray ionisation (ESI) (discussed in detail in section 10.3.1). Butovich *et al*. (29, 114) used different ionisation methods as they were each suited to the identification of different lipids.

Jones *et al*. (115) and Haynen *et al*. (106) have also used HPLC but in conjunction with UV detection for the separation of *ex vivo* contact lens deposits.

1.10.4 Types of mass spectrometry (MS)

There are many types of ionisation which provide mass to charge data for unknown compounds but they each fragment the sample differently. The methods described below are examples of the ionisation methods most suited for use in conjunction with HPLC.

Electron ionisation (EI) applies a positively charged ion to the separated sample which can provide information such as the molecular weight of the analyte. Chemical ionisation (CI) is similar to EI; however CI uses gases to produce ions of the analytes. The main advantage of CI over EI is that CI uses less energy than EI and therefore CI is a much softer ionisation

method. Atmospheric pressure chemical ionisation (APCI) is a soft ionisation method and is useful for the analysis of non-polar lipids. It is most often used in tandem with HPLC as it can tolerate high solvent flow rates. APCI is similar to CI but the ionisation occurs at atmospheric pressure. Electrospray ionisation (ESI) is particularly useful for the analysis of polar analytes. ESI provides molecular weight information as the mass spectrum provides the positive ion of the analyte.

1.10.5 Mass spectrometry (MS)

Saville *et al*. (25, 33) used MS for the detection and quantification of phospholipids and cholesterol from contact lens extracts and from tear samples.

1.10.6 Infrared (IR)

Borchman *et al.* (26, 30, 99, 116) used fourier transform IR spectroscopy to investigate the conformational changes in the hydrocarbon chains of meibomian gland lipids which may have occurred with age, disease or temperature. By using IR they were able to identify conformational changes in the hydrocarbon chain of meibomian gland secretions comparing normal subjects to subjects with MGD or older subjects.

1.10.7 Cholesteryl esterase enzyme reaction

This method, in simplistic terms works by the conversion of cholesteryl esters to cholesterol by hydrolysis and the oxidation of free cholesterol to choles-4-en-3-one and this binds it to an aminopyrine, which forms a coloured complex (117). This method was used by Pucker *et al.* (118) to determine the concentration of cholesterol and cholesteryl esters on *ex vivo* and *in vitro* spoiled silicone hydrogel contact lenses. Lenses were extracted and hydrolysed, oxidised and reacted with a reaction buffer and the absorbance was read at 500nm.

1.11 Lipid oxidation and degradation

A major part of this project involved the development of existing lipid analytical techniques, but also the measurement of lipid oxidation products by the adaptation and use of commercial assays. Lipid oxidation can be

an enzymatic or non-enzymatic process. Non-enzymatic lipid oxidation is caused by initiators such as heat, light, metals, increased levels of oxygen and is referred to as autoxidation. Enzymatic oxidation is caused by particular enzymes that cause degradation of the lipid species. Therefore, there are two major pathways of oxidation; autoxidative and enzymatic.

1.11.1 Autoxidation

Autoxidation is a spontaneous reaction whereby lipids react with oxygen present in the atmosphere and form free radicals. There are usually three stages involved: initiation, propagation and termination steps. The initiation step involves the abstraction of a hydrogen atom from the carbon atom next to the double bond (known as the allylic hydrogen) and this process is promoted by one or several of the initiators mentioned above. This creates a radical species that can react with oxygen. This can then self-propagate and can repeat several times until termination. Termination is achieved when two radical species react together to terminate the process.

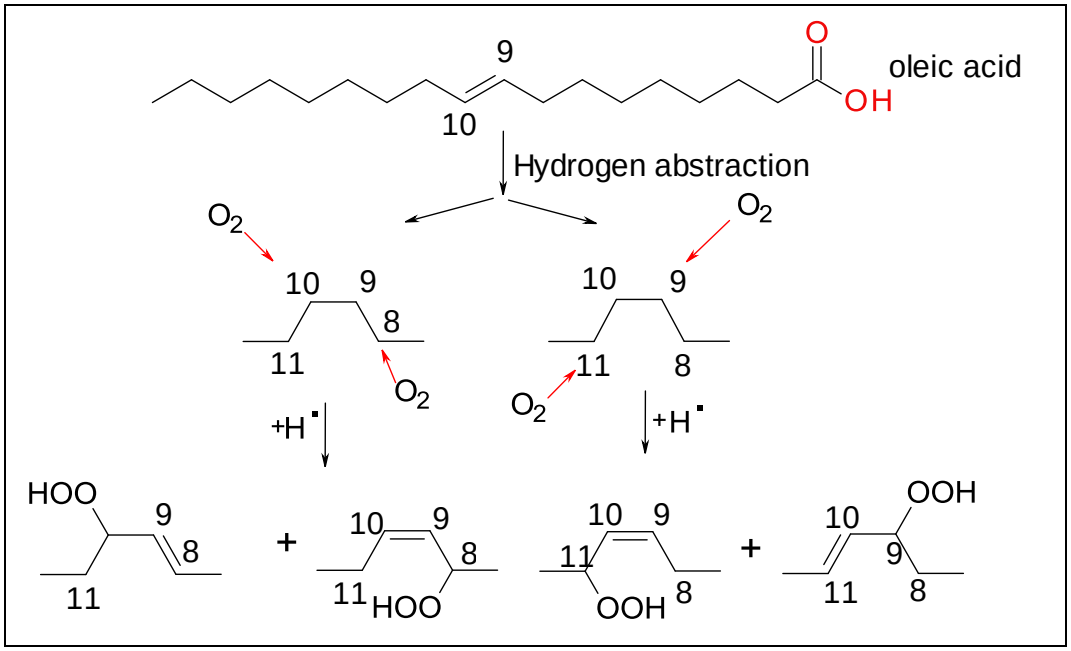


Figure 1.28 – Autoxidation of oleic acid (119)

Figure 1.28 demonstrates how oleic acid (C18:1) can undergo autoxidation which leads to the production of several hydroperoxy species.

Lipids with more than one double bond undergo oxidation more rapidly. Polyunsaturated fatty acids therefore undergo autoxidation more rapidly than monounsaturated fatty acid because there are more allylic hydrogens susceptible to abstraction (shown in Table 1.2).

Table 1.2 – Rate of oxidation of various fatty acids (120)

Fatty acid	*Number of double bonds*	*Rate of oxidation*
Stearic acid (18:0)	0	1
Oleic acid (18:1)	1	40
Linoleic acid (18:2)	2	1200
Linolenic acid (18:3)	3	2500

Polyunsaturated fatty acid oxidation yields many primary and secondary products. The primary products of oxidation include hydroperoxides which are not stable, which makes it very difficult to monitor their presence. However, secondary products of PUFA oxidation such as particular aldehydes and ketones are commonly measured as a biomarker of lipid oxidation. These include aldehydes such as malondialdehyde (MDA), which is one of the most commonly studied lipid oxidation products.

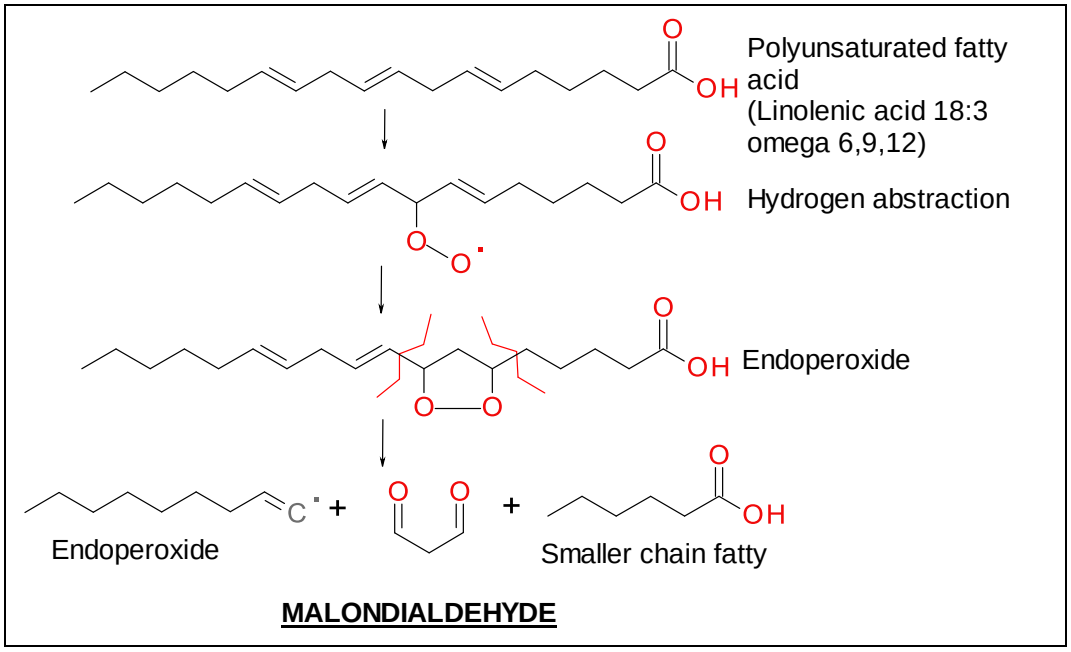


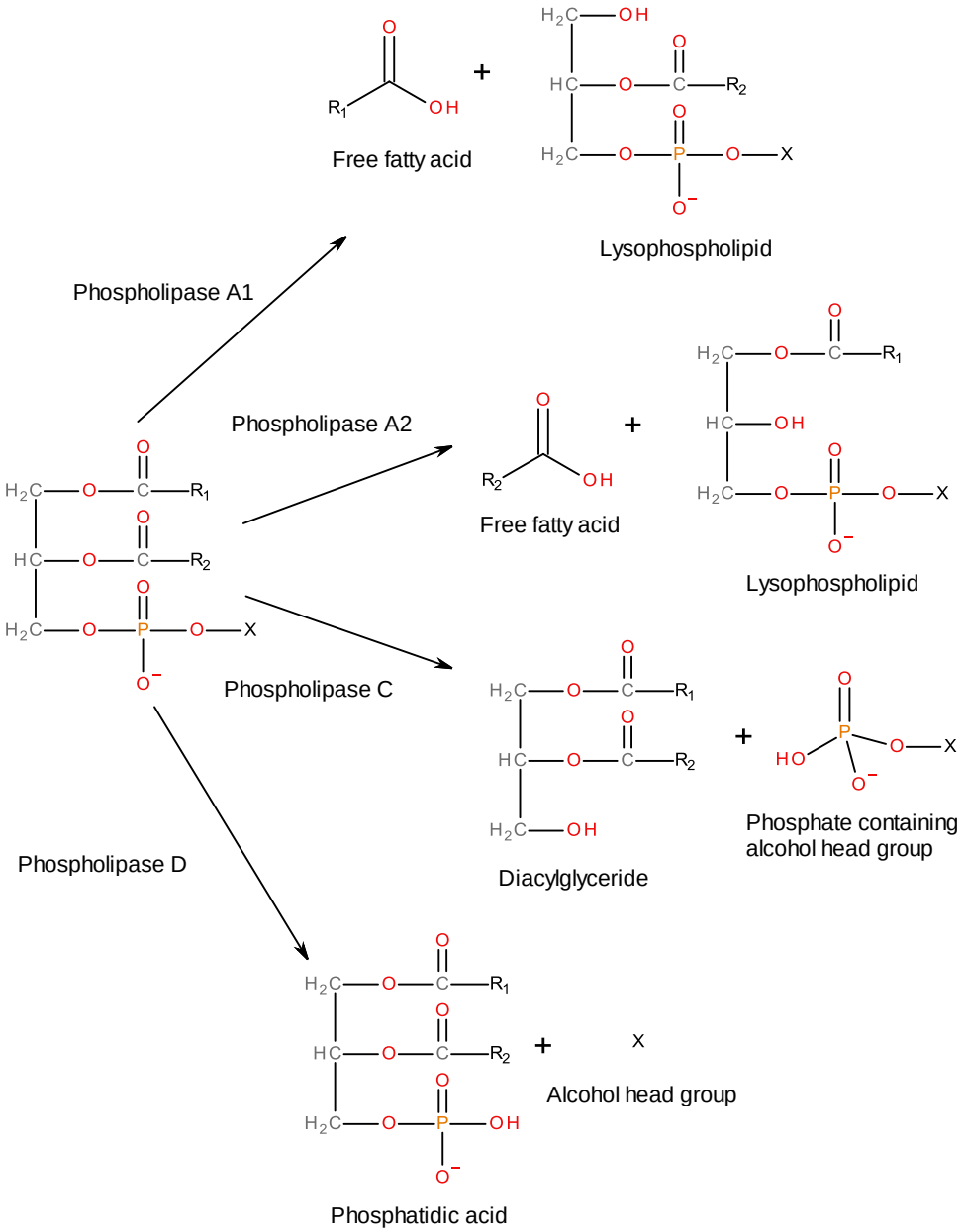
Figure 1.29 – PUFA oxidation resulting in the production of MDA

Figure 1.29 demonstrates how a PUFA such as linolenic acid (C18:3) can oxidise and produce MDA, as well as other shorter chain fatty acids. The presence of MDA been researched in literature and has been linked with contact lens intolerance (121). MDA could therefore be a potential biomarker for contact lens intolerance or discomfort. Therefore, the occurrence of lipid oxidation in the ocular environment is important to monitor.

The assessment of lipid peroxidation products can be difficult especially the analysis of these products in tears. The various methods used to detect secondary products of lipid oxidation in tears in this project will be discussed in detail in Chapter 5.

1.11.2 Enzymatic hydrolysis

Enzymatic hydrolysis is a pathway of lipid degradation whereby lipids are targeted by particular enzymes which break the lipid into by-products. Examples include the enzymatic degradation of phospholipids by different phospholipases.



Where: R1 and R2 are fatty acids of varying chain length and unsaturation
and X is an alcohol head group either: choline, serine, ethanolamine or inositol

Figure 1.30 – Enzymatic hydrolytic pathways of a phospholipid with different phospholipases

The presence and role of phospholipids in the tear film has been investigated by several researchers (21, 122-125). As mentioned in the section 1.3, the presence of phospholipids in the tear film has been debated by several authors. For those authors that report their presence, McCulley *et al*. (21) report that phospholipids act as the interface between the non-polar lipid layer and the aqueous layer of the tear film.

The polar layer acts a surfactant between the non-polar lipids and the aqueous layer. This suggests that phospholipids are therefore vital for the stability of the tear film. A lack of phospholipids has been linked to tear film instability and increased tear break-up (126, 127). Phospholipids are susceptible to enzymatic degradation by various enzymes called phospholipases at various positions of the phospholipid (shown in Figure 1.30). There have been reports of increased levels of certain phospholipases that enzymatically degrade phospholipids as a result of contact lens wear. The presence of phospholipids in tears is still under debate as certain authors suggest they are secreted by meibomian glands and are present in tears (21). Others suggest they are secreted by the meibomian glands and are degraded by enzymes present in tears (32, 126). Butovich *et al*. report there are no phospholipids secreted by meibomian glands, but are detected in tear samples (29, 31). As meibomian glands are holocrine glands, the entire cell is secreted and cell membranes are made up of phospholipids suggesting the meibomian glands may actually secrete phospholipids. In the normal eye these phospholipids act as a surfactant, however in contact lens wear, increases in phospholipases can lead to phospholipid degradation. This degradation in phospholipids could be the cause of an increase in dryness related symptoms.

1.11.3 Cholesterol oxidation

Cholesterol is also susceptible to oxidation due to the ease of hydrogen abstraction on the allylic hydrogen. The pathway of cholesterol oxidation is far less complicated than fatty acid oxidation and it yields far fewer by-products compared with fatty acid oxidation (128). Cholesterol oxidation has not been fully investigated in this project but as cholesteryl esters are a major component of the tear film, it is a future direction of this project.

There are three pathways of cholesterol oxidation – autoxidation, photoxidation and enzymatic oxidation and each pathway yield various forms of oxysterol.

1.11.3.1 Autoxidation of cholesterol

Autoxidation of cholesterol produces various forms of oxysterol based around the 7- position on the cholesterol molecule and the predominant products are shown in Figure 1.31 and Figure 1.32. The reaction of cholesterol with free radicals will most likely produce the products shown in Figure 1.31 and Figure 1.32 (129).

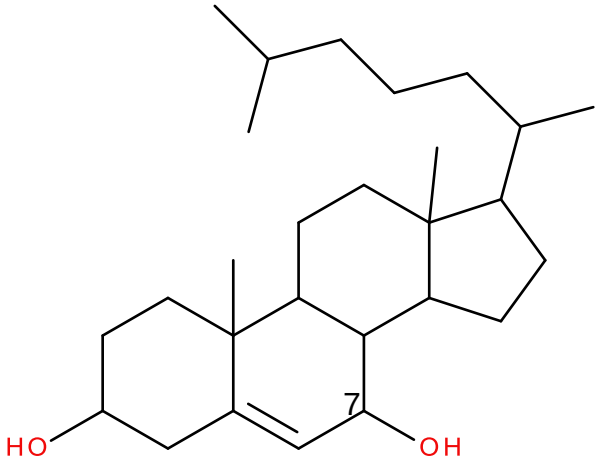


Figure 1.31 – Structure of a 7α-OH or 7β-OH cholesterol

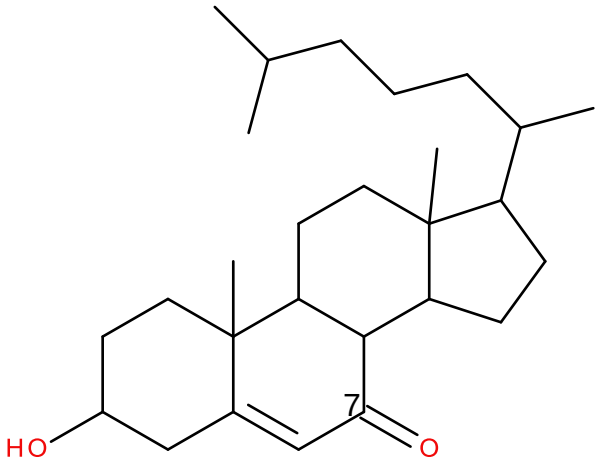


Figure 1.32 – 7-keto cholesterol

1.11.3.2 Photoxidation of cholesterol

Photoxidation of cholesterol produces hydroperoxy-cholesterol molecules based around the 5-position on the cholesterol molecule. The major product of cholesterol photoxidation is shown in Figure 1.33.

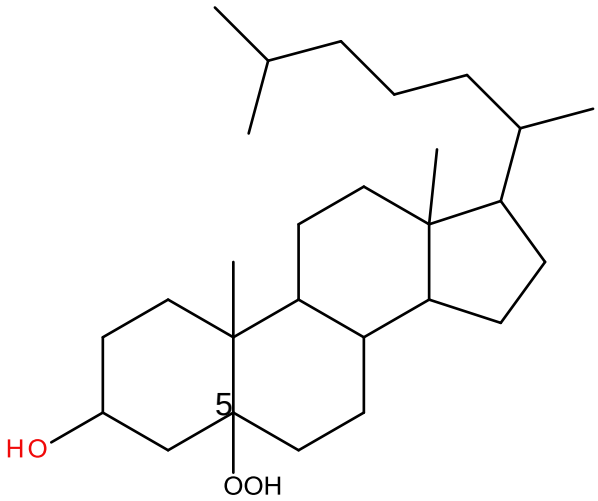


Figure 1.33 – 5α hydroperoxy-cholesterol

1.11.3.3 Enzymatic Cholesterol Oxidation

Enzymatic oxidation is more complicated than autoxidative and photoxidation of cholesterol. Enzymatic oxidation can lead to the production of many products such as epoxycholesterols at various carbons on the cholesterol molecule. Enzymatic cholesterol oxidation occurs in the brain as this is where certain enzymes are produced (130).

1.11.4 Factors influencing lipid oxidation

There are many other factors, other than the level of fatty acid unsaturation which may affect the rate of oxidation. The position of the fatty acid can affect the rate of oxidation. For example, if the same fatty acid was attached to the SN1 and SN2 position of a glycerol backbone, (either a TAG or phospholipid) the fatty acid at position SN1 would have a higher rate of oxidation. This is most likely because the fatty acid chain at the SN2 position is sterically hindered (131).

The lipid class can also affect the rate of oxidation. Free fatty acids have the fastest rate of oxidation compared with other lipid classes. However, phospholipids also have a fast rate of oxidation and this may be due to the level of unsaturation of fatty acids attached to phospholipids. Phospholipids make up cell membranes and will have polyunsaturated fatty acids attached to keep the cell membrane fluidic. The level of unsaturation ultimately affects the rate of oxidation.

1.12 The methods used for the measurement of lipid oxidation

There are several methods used in literature to measure either the extent of lipid oxidation or the production of lipid oxidation products. Many of the currently available assays were adapted and used in this research to measure the presence of lipid oxidation products in tears and on contact lenses. The most commonly used method to detect MDA (secondary lipid oxidation product) is the thiobarbituric acid reactive substances (TBARS) assay. TBA binds to MDA in the sample or standard under acidic conditions to produce a colour complex. This coloured complex can then be measured by UV or fluorescence absorbance and the concentration of MDA in the sample can be determined. The TBA method was developed in 1957 to determine the presence of lipid oxidation in food (132). This method is consistently used to determine the presence of MDA in various samples such as blood, urine, plasma and food products (133). The TBARS assay has its limitations as TBA can bind to many other substances such as aldehydes. The TBARS assay can therefore exaggerate the levels of MDA. HPLC methods have therefore been developed as TBA – MDA complex has a characteristic retention time and is therefore a more accurate method for the determination of MDA concentration in the sample (134, 135).

Other methods, for the measuring the concentration of MDA or other aldehyde in various unknown samples have been developed. A method similar to the TBARS assay, works by a hydrazine complex binding to

MDA forming a colour complex. The TBARS and other colorimetric assays were used in the work discussed in Chapter 5.

As well as MDA analysis, there are several other methods for the measurement of lipid oxidative end products which include using GC or HPLC to assess the levels of conjugated dienes, which are primary products of lipid oxidation. However, these methods were not used in this research because additional hardware for analytical techniques was required.

1.13 Ocular antioxidants

It is important to consider the presence and levels of antioxidants when lipid oxidation is discussed. The eye is exposed to UV light, oxygen and environmental factors which may cause oxidation of proteins and/or lipids of the tear film. The tear film is the first line of defence for the ocular environment. There have been several studies which have analysed the levels of antioxidants in tears and have found that predominant antioxidants in tears are ascorbic acid and uric acid (136, 137). Ascorbic acid (Vitamin C) is a dietary antioxidant and uric acid is not. The origin of these antioxidants was thought to be the cornea, however, Choy *et al.* (138) state that these antioxidants are present in lacrimal gland secretions. Gogia *et al*. (139) detected several other antioxidants in human tears such as cysteine, glutathione and tyrosine, however the presence of these antioxidants has not been investigated by others.

1.14 Consequences of lipid oxidation for contact lens wear

1.14.1 Lipid degradation and discrete deposits on contact lenses

The first point to make is that the autoxidation of the unsaturated fatty acid moieties, whether present as free acids or esters can lead to oligomerisation, cross-linking and consequent immobilisation. In this state the lipid is not extractable without destroying the lens matrix in which it is

immobilised. This leads to two clinically undesirable problems. The first is the formation of hydrophobic domains driven, for example, by the oligomerisation of unsaturated cholesteryl esters. These domains attract the more hydrophobic components which lead to regions of severe dewetting which are anchored into the lens matrix. The second problem arises from autoxidation and immobilisation of lipids in the formation of discrete elevated deposits which are white in colour because of sequestered calcium. Several authors have previously identified the presence of 'white spots' or 'jelly bumps' on hydrogel contact lenses (140, 141). Bowers *et al.* (141) described their formation as a combination of the oligomerisation of the unsaturated fatty acids which then complex with tear – borne calcium ions resulting in the formation of insoluble deposits that are both on and in the lens matrix. These deposits are readily seen with the naked eye but can be more effectively examined using microscopy. Confocol microscopy is especially useful in that it enables the visualisation of the deposit structure not only at the surface but also within the lens matrix. The technique has the ability to scan across sections of the lens allowing the images to be stacked providing a three – dimensional image. Figure 1.34 shows 'slices' taken through a white spot deposit (142-144). The images show sections through deposits and illustrate that the structure continues to grow through the matrix. It is important to recognise that there would be no white spot deposit formation without lipid degradation (autoxidation) and that, additionally the level of calcium ions in the tear of individual subjects plays an important part in driving the rate of deposit formation.

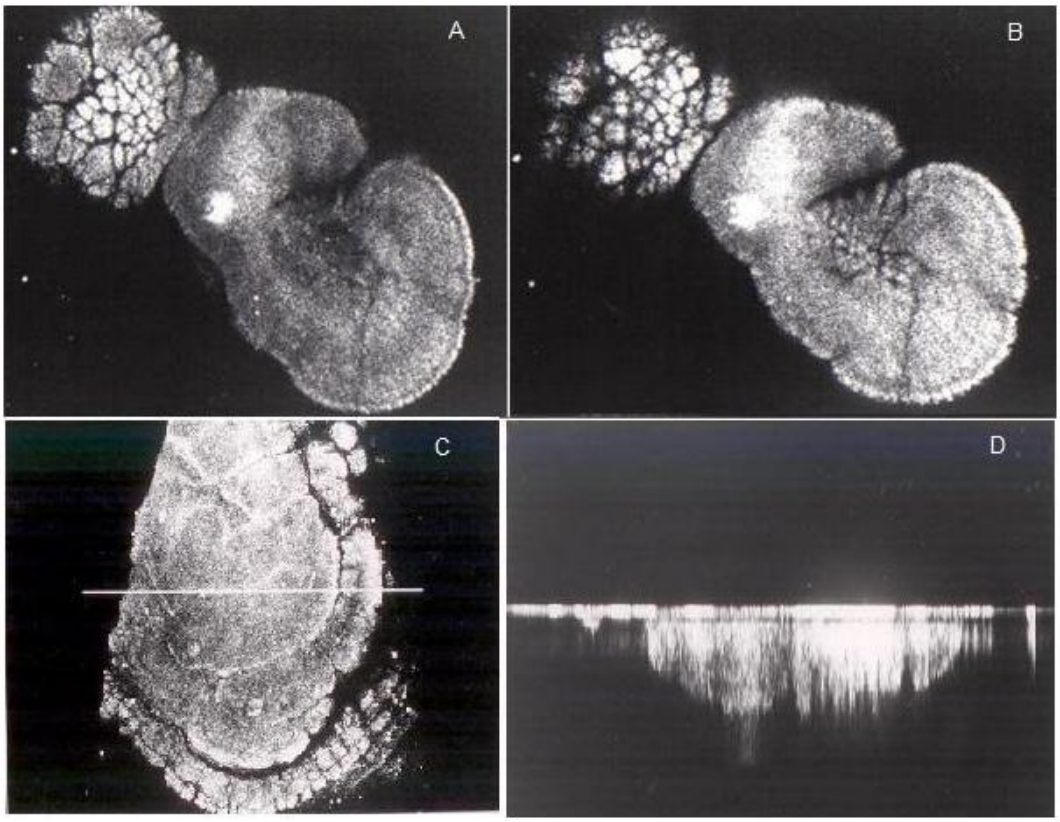


Figure 1.34 – Scanning confocol microscopy images of white spots on a conventional hydrogel, showing:
Optical "slices" though a white spot in the X-Y (horizontal) plane (A and B); an X-Y white spot image (C) indicating (transverse white line) the section reconstructed in the Z direction (D) by stacking optical images at that point. The horizontal line in D results from the fact that the lens was placed anterior surface down on a microscope slide to collect the images.

1.14.2 Lipid oxidation products and ocular discomfort

One possible contribution to the 'end of day' discomfort phenomenon may be found in the lipid oxidation and degradation processes that arise due to exposure of immobilised lipids on the anterior surface of the lens for long periods. Lipid turnover in general is much slower than aqueous tear flow and turnover in contact lens wear (145). The lipid profile of individual subjects and ocular dynamics affecting tear break-up and inter-blink period will ultimately determine the rate of oxidation. For example, if the subject has an unusual fatty acid profile containing more unsaturated fatty acids, or a significant level of unsaturated fatty acids from plasma leakage, these

will increase the level of oxidative degradation. Similarly, longer inter-blink periods and greater exposure of the lipid layer to light and atmospheric oxygen will accelerate oxidative processes. It is important therefore to assess the potential contribution of these processes to environmental changes brought about by contact lens wear.

Build-up of polyunsaturated fatty acids leads to oxidation which can ultimately result in the production of oxidative end products such as MDA (malondialdehyde, Figure 1.29). Although there are few studies of this molecule in tears (assays are not straight forward), MDA has been detected in the tears of intolerant contact lens wearers (121). In this study, Glasson *et al*. (121) analysed tears for MDA of two separate groups of subjects known to be either contact lens intolerant or contact lens tolerant. They observed a statistically significant difference in MDA levels for the two subject groups and identified the intolerant subjects as having higher levels of MDA in their tears. One important feature of MDA is the ability to react with proteins or DNA disrupting their regular function. Thus MDA (and other aldehydes) can be toxic in tissues because of reactivity with thiol and amino groups of proteins which can therefore cause tissue damage (133).

In addition to increased levels of oxidised lipid products in contact lens intolerant subjects, there have been several reports of increased levels of enzymes which specifically degrade phospholipids. Glasson *et al.* for example reported increased levels of phospholipase A2 (PLA2) in the tears of contact lens intolerant patients (121). Phospholipids are believed to be responsible for the stability of the tear film and increased levels of enzymes which degrade phospholipids may decrease tear film stability. Additionally, there have been several reports of PLA2 in tears of contact lens wearers and in the tears of subjects with ocular disorders (76, 126, 146-148). Campbell *et al*. also reported the presence of phospholipase C and identified DGs as the end product of the cleavage reaction. This is potentially important because the degradation of phospholipids by different phospholipases yields different end products. All of which have significant polarity, which suggests that phospholipases may have a

significant role in modulating aspects of lipid composition that influence spreading and tear film integrity (32).

These various factors, taken together, indicate considerable scope for the range of subject specificity encountered in reports and studies of contact lens induced discomfort. It is clear that the lipid layer, although regarded as relatively inert for many years, is now seen as a potentially important and reactive family of compounds in the search for improvements in contact lens comfort.

This literature survey has helped identify what is already known about ocular lipids and what areas still require a significant amount of research. There have been several publications which have investigated meibomian gland secretions and differences between meibomian gland secretions compared to tear lipids. There have also been a significant number of publications which have investigated *in vitro* lipid spoliation models on contact lenses and methods for analysing the extracts. There have been far fewer publications which investigate the nature of lipid deposition on *ex vivo* contact lenses.

It is apparent from the literature review that there has not been much research investigating the fate of lipids on lenses and in particular what effect lens material, wear schedule and subject-to-subject variability have on the nature and fate of lipids.

The work in this project was therefore based on firstly the development of an array of analytical techniques and a process of *ex vivo* lens analysis and secondly using the results generated to better understand lipids on contact lenses.

Chapter 2 – Materials and Methodology

2.1 Introduction

The work in this project involved the modification of existing analytical techniques to investigate lipid composition of lipids from *ex vivo* contact lenses and tear samples. These techniques were then used to investigate changes in lipid structure as a result of contact lens wear.

2.2 Contact lens materials used

Table 2.1 – General information about the contact lenses used in this project

Commercial name (abbreviation) /USAN name/manufacturer	*SiHy/CoHy*	*Principle components of the lens*
PureVision (PV)/balafilcon A/Bausch & Lomb	SiHy	N-vinyl pyrrolidine, tris-vinyl carbamate, N-carboxyvinyl ester
Focus Night & Day (F.N&D)/lotrafilcon A /CIBA vision	SiHy	Dimethyl acrylamide, TRIS, siloxane
Air Optix (AO)/lotrafilcon B/CIBA vision	SiHy	Dimethyl acrylamide, TRIS, siloxane
Air Optix Aqua (AOA)/lotrafilcon B/CIBA vision	SiHy	Dimethyl acrylamide, TRIS, siloxane
Acuvue/ etafilcon A/ Johnson & Jonhson	CoHy	Hydroxyethyl methacrylate, methacrylic acid
Acuvue Oasys (AcuOas) /senofilcon A/ Johnson & Johnson	SiHy	Polydimethylsiloxane, dimethyl acrylamide, hydroxyethyl methacrylate
Soflens 66 (S66)/ alphafilcon A/ Bausch & Lomb	CoHy	Hydroxyethyl methacrylate, vinyl pyrrolidone
Focus Dailies/ nelfilcon A/ CIBA vision	CoHy	Polyvinyl alcohol
Acuvue TruEye/ narafilcon A/B/ Johnson & Johnson	SiHy	Polydimethylsiloxane, dimethyl acrylamide,

		hydroxyethyl methyacrylate, polyvinyl pyrrolidone
Dailies Total 1/ delafilcon A/ CIBA vision	SiHy	Contains dimethyl phosphatidylcholine

The commercial names will be referred to throughout the thesis.

2.3 Contact lenses and tear samples

The *ex vivo* contact lenses used throughout this thesis were all harvested from clinically controlled trials. Individual lenses were extracted using the extraction protocols described in section 2.6 and the extracts were subjected to an array of analyses (discussed in Chapter 3).

The two methods used for tear collection throughout this thesis were both the microcapillary tube method and the Visispear ophthalmic sponge technique. The Visispear ophthalmic sponge tip is made from cellulose, which is an absorbent material. It was therefore suitable for the collection of lipids in tears. Tears were collected by placing the tip of the sponge in the lower meniscus of the eye, taking care to minimise contact with the conjunctiva. Tears were collected by the sponge; the tip of the sponge was then cut off and placed in a glass vial for extraction. The sponge was then extracted using the extraction protocol described in section 2.9. The volume of tears collected from individuals varied from subject-to-subject.

The microcapillary method is used for the collection aqueous tears. This method of tear collection was suitable for the work in Chapter 5 (investigating lipid peroxidative products in the ocular environment). The microcapillary tube is placed in the lower meniscus of the eye and tears are drawn into the tube by capillary action. Varying volumes of tears are collected from individuals depending on individual subjects tear flow. In certain cases, no tears were collected because of reduced to no tear flow, which was related to dry eye in certain individuals.

2.4 Tear envelope

A tear envelope is a tear film sample which has been in direct contact with the anterior and posterior surfaces of a contact lens. A tear envelope sample is collected by placing a freshly worn contact lens into a 0.7ml eppendorf that has a small hole at the base. Various volumes of water (or other solvents, the choice of which depended on how the sample was to be analysed) are added to either side of the lens. The smaller eppendorf, containing the lens, is then placed inside a larger eppendorf (2ml). The eppendorfs are centrifuged for 5 minutes at 3300rpm which draws the tear envelope sample into the large eppendorf. Tear envelope samples were analysed by various techniques (used mainly for the work discussed in Chapter 5).

2.5 Lipid analysis: Preparation of FAMEs

Samples must be volatile for GCMS analysis; therefore fatty acids (not volatile) are converted to fatty acid methyl esters (FAMEs) which are volatile. This reaction is known as a tranesterification or transmethylation. Lens extracts and tear samples were transmethylated for GCMS analysis. The transmethylation process is described below.

950µl methanol and 5µl concentrated sulphuric acid were added to amber vials that contained lens extracts or tear sample extracts. The vials were then placed in a heating block for 90mins at 70°C. The vials were then allowed to cool for 5mins. 400µl isohexane followed by 100µl water were added to the vials. The vials were shaken and the solutions were allowed to stand to phase separate. The fatty acid methyl esters (FAMEs) partition into the upper layer (isohexane layer). The upper layer was collected using a Pasteur pipette and transferred to 2ml amber vials. Another 400µl isohexane was added to the first vial and it was shaken. The upper layer, which contained the FAMEs was collected. The process was repeated another two times until the final volume of FAMEs collected was 1.6ml. The solvent containing the FAMEs was evaporated to dryness under nitrogen and resuspended in 100µl isohexane. The solution was transferred to a glass insert (100µl glass insert designed to sit inside a 2ml

amber vial), evaporated to dryness under nitrogen and resuspended in 25µl isohexane for GCMS analysis.

2.6 Protocols used for the extraction of lipids from subject-worn contact lenses

HPLC grade solvents were used for extraction and for mobile phase preparation.

There are several protocols used by different researchers in the literature for the extraction of lipids from lenses. They are mainly based on extraction using different ratios of chloroform: methanol for various amounts of time (25, 102, 110, 118, 126, 149, 150). Some of the protocols are used for the extraction of lipids from lenses which have been spoiled *in vitro* and some are used on *ex vivo* lenses. The extraction of lipids from lenses which have been spoiled *in vitro* would be very different to the extractability of lipid from subject-worn contact lenses. *In vitro* spoliation does not mimic the blink action of the eyelid over the contact lens. There are also other components in the tear film which have an effect on lipid deposition on lenses. Whilst the use of *in vitro* models provides useful information, they do not mimic the extractability of lipids from *ex vivo* lenses.

2.6.1 Extraction protocol 1:

Extraction protocol 1 was the main protocol used for lipid extraction from *ex vivo* lenses because it was an effective lipid extraction procedure. Single lenses were placed in glass vials that contained 1:1 chloroform: methanol (1.5ml). The glass vials were covered with aluminium foil and then parafilm and placed on a flat bed shaker for at least 30mins. The extract medium was transferred to an amber vial and evaporated to dryness. The extract was resuspended in a solvent, the selection of which depended on the technique to be used for analysis. Sample preparations for each technique are detailed under the appropriate headings (section 2.10).

2.6.2 Extraction protocol 2:

Single lenses were placed in 2ml amber vials. 1:1 chloroform: methanol (600µl) was added to the vials and they were then placed on a flat-bed shaker for 30mins. The vials were also vortexed for 30 seconds at 0mins, 15mins and 30mins time points in the extraction. The lens was removed and discarded and the solvent extract was evaporated to dryness under nitrogen. The extract was then resuspended in a solvent, the choice of which depended on the technique to be used for analysis.

2.6.3 Extraction protocol 3 (double extraction):

Lenses were extracted using extraction protocol 1. Fresh 1:1 chloroform: methanol (1.5ml) was added to the vials that contained the already extracted lens. The vials were placed on a flat-bed shaker for at least another 30mins. The extracts were either pooled or kept separate depending on the nature of investigation. The extract medium was evaporated to dryness and resuspended in a solvent, the choice of which depended on the technique to be used for analysis.

2.6.4 Extraction protocol 4 (double extraction):

Lenses were extracted using extraction protocol 2. Fresh 1:1 chloroform: methanol (600µl) was added to the vial containing the already extracted lens. The vial was placed on a flat-bed shaker for another 30mins. In addition, the vial and lens were vortexed for 30 seconds at 0mins, 15mins and 30mins time points in the extraction. The extracts were either pooled or kept separate depending on the nature of investigation. The solvent extract was evaporated to dryness under nitrogen and resuspended in a solvent, the choice of which depended on the technique to be used for analysis.

2.6.5 Extraction protocol 5:

A single lens was added to a 20ml stopperred measuring cylinder that contained 10ml methanol, 5ml chloroform and 3.75ml 0.15M acetic acid. The cylinder was shaken by hand for 5mins. 2.5ml chloroform and 2.5ml water were added to the cylinder. The solution was allowed to stand to

phase separate for 40mins and the lower organic phase was collected using a Pasteur pipette and transferred to 2ml amber vial. The solvent extract was evaporated to dryness under nitrogen whilst the extraction procedure was repeated another two times. The extracts were pooled together and the extract medium was evaporated to dryness. The extract was resuspended in a solvent, the choice of which depended on the technique to be used for analysis.

2.7 Extraction of dimyristoyl phosphatidylcholine (DMPC) from phospholipid-containing contact lenses

In order to try and mimic *in vivo* extraction of DMPC from phospholipid-containing lenses, several extraction procedures were tested. The results are shown in Chapter 6 and the extraction procedure details are described below.

2.7.1 100% methanol

A lens was taken straight from the blister pack and placed in a glass vial that contained 1.5ml methanol. The vial was covered with aluminium foil and then parafilm and then placed on a flat-bed shaker for 40mins. The solvent extract was transferred to an amber vial. The solvent was evaporated to dryness under a steady stream of nitrogen. The extract was then transmethylated (described in section 2.5) and analysed by GCMS.

2.7.2 Hexane: methanol extraction

A lens was taken straight from the blister pack and placed in a glass vial that contained 1.5ml 1:1 hexane: methanol (not miscible). The lens was extracted for 40mins. The solvent extract was evaporated to dryness under nitrogen and transmethylated as described in section 2.5.

2.7.3 Ethyl acetate extraction

A lens was removed from the blister pack and placed in a glass vial that contained 1.5ml ethyl acetate. The vial was placed on a flat-bed shaker for 40mins. The extract was transmethylated as described in section 2.5 of this chapter.

2.7.4 Butanol extraction

A lens was removed from the blister pack and transferred to an amber vial that contained 100µl butanol. The vial was placed on a flat-bed shaker for 1hr and vortexed for 30 seconds at 0mins, 30mins and 60mins time intervals. The extraction solvent was evaporated to dryness under nitrogen and transmethylated as described in section 2.5.

2.7.5 Octanol extraction

A lens was removed from the blister pack and transferred to a glass vial that contained 750µl octanol. The lens was extracted for 2 hours on a flat-bed shaker. 200µl of the extract medium was transferred to an amber vial and transmethylated as described in section 2.5 of this chapter. The FAMEs were resuspended in 100µl isohexane and analysed by GCMS.

2.7.6 Isopropyl palmitate (IPP) extraction

10µl IPP was transmethylated in order to validate the volume of IPP the lenses should be extracted in. The C16 peak (which was the main peak for IPP) was very strong; therefore no more than 10µl was transmethylated. A lens was removed from the blister pack and placed in a glass vial that contained 2ml IPP. The vial was placed on a flat-bed shaker and 10µl aliquots were taken at 30mins, 60mins, 4 days, 5 days and 6 days. The 10µl aliquots of the solvent extracts were transmethylated directly (without evaporation under nitrogen). The FAMEs were resuspended in 100µl isohexane. Samples were injected using a split injection (where only part of the sample is transferred to the column).

The second IPP extraction method used involved placing a lens in a 1.5ml eppendorf that contained 200µl IPP. The eppendorf was vortexed for 30 seconds and placed on a flat-bed shaker for 1 hour. After 1 hour on a flat-bed shaker, the eppendorf was vortexed for 30 seconds. 10µl IPP was transferred to an amber vial and transmethylated as previously described (section 2.5). The FAMEs were resuspended in 100µl isohexane. The samples were injected using a split injection.

2.7.7 IPP extraction of glass slides

This extraction method involved dipping a lens in IPP. This lens was then placed between two glass slides and the glass sides were rubbed at periodic intervals. The glass slides were then extracted in 1:1 chloroform: methanol (1.5ml) for 40mins, transmethylated and analysed by GCMS. The lenses were also kept and extracted in 1:1 chloroform: methanol (1.5ml) for 40mins, transmethylated and analysed by GCMS to evaluate how much DMPC remained in the lens.

2.7.8 Glycerol extraction

A lens was removed from the blister pack and placed in an amber vial that contained 100µl glycerol. The lens was extracted for 30mins on a flat-bed shaker and vortexed for 30 seconds at 0mins, 15mins and 30mins time intervals. The lens was removed and the extract was transmethylated as described in section 2.5.

2.8 MDA extraction from *ex vivo* lenses: extraction protocols (used in Chapter 5)

2.8.1 Extraction procedure 1

Acetonitrile: 0.2% trifluoroacetic acid (1ml) was added to single lenses (daily wear and continuous wear PV and F.N&D) in glass vials covered entirely with foil. The vials were kept in a dark cupboard overnight and the lens removed. 10µl of each extract was added to 1ml 0.2% TBA buffer (preparation of TBA buffer is described in section 2.11.5.1.1) in individual eppendorfs. The eppendorfs were placed in the oven for 1hr at 90°C. The solutions were cooled in a water bath for 5mins. 200µl of each solution was transferred to 96 well plate and the UV absorbance measured at 532nm.

2.8.2 Extraction procedure 2

1ml PBS was added to single lenses (daily wear and continuous wear PV and F.N&D lenses) and placed on a flat-bed shaker overnight. The lenses were removed and the extracts were prepared for analyses. 10µl of each

extract was added to 1ml 0.2% TBA buffer (preparation of TBA buffer described in section 2.11.5.1.1) in individual eppendorfs. The eppendorfs were placed in the oven for 1hr at 90°C. The solutions were cooled in a water bath for 5mins. 200µl of each solution was transferred to 96 well plate and the UV absorbance measured at 532nm.

2.8.3 Extraction procedure 3

1:1 chloroform: methanol (1ml) was added to individual lenses (daily wear and continuous wear PV and F.N&D lenses) in glass vials and the vials were placed on a flat-bed shaker for 2 hours. The lenses were removed and the solvent evaporated to dryness under a steady stream of nitrogen. The extracts were resuspended in 1ml PBS. 10µl of each extract was added to 1ml 0.2% TBA buffer (preparation of TBA buffer described in section 2.11.5.1.1) in individual eppendorfs. The eppendorfs were placed in the oven for 1hr at 90°C. The solutions were cooled in a water bath for 5mins. 200µl of each solution was transferred to a 96 well plate and the UV absorbance measured at 532nm.

2.9 Extraction of tear samples taken using Visispear ophthalmic sponges

As described in section 2.3, tears were collected using the Visispear sponge by another researcher. The tips of the sponges that had collected tears were cut and placed in glass vials that contained 1.5ml chloroform. The vials were covered with aluminium foil and then parafilm and placed on a flat-bed shaker for at least 2 hours. The extraction solvent was transferred to an amber vial and evaporated to dryness under nitrogen. The extract was then either transmethylated for GC analysis (as described in section 2.5 of this chapter) or resuspended in mobile phase for HPLC analysis (as described in section 2.10.4.1 of this chapter).

2.10 Analytical techniques methodology

The techniques used for lipid detection and analysis throughout this thesis were fluorescence spectrophotometry (FS), thin layer chromatography (TLC), high performance liquid chromatography (HPLC) with UV and

fluorescence detection and gas chromatography mass spectrometry (GCMS). FS was used for the measurement of the levels of gross lipid deposition on contact lenses. TLC and HPLC were useful for general lipid class separation and GCMS was used for fatty acid separation.

2.10.1 Fluorescence spectrophotometry (FS)

Certain molecules possess natural fluorescence. These molecules are usually aromatic or have conjugated double bonds. Lipids such as cholesteryl esters are fluorescent molecules. The fluorescence of lipids is visible when excited at 360nm and a peak is observed in the emission spectra at approximately 440nm.

For the work in this thesis, fluorescence spectrophotometry was used to provide a measure of the gross levels of lipid on the anterior and posterior surfaces of worn contact lenses. FS was an effective method to non-destructively measure the levels of gross lipid on lenses. The model used in this research project was a modified Hitachi F4500 spectrophotometer. The contact lens was placed in a cylindrical cuvette and the cuvette was filled with distilled water. The cuvette was placed in the holder with the convex side of the lens facing the beam of light. The wavelength was set at 360nm excitation and the emission spectra was analysed 440nm (representing the lipid peak). The intensity of the peak at 440nm provided a measure of the gross levels of lipid on the lens.

2.10.2 Thin layer chromatography (TLC)

The principle of this technique is to separate lipids according to their relative polarity. TLC was used to separate lipids extracted from lenses or tear samples.

The TLC plates used for this research were made of silica gel, they were 20cm x 20cm and the silica layer was 250μm in thickness.

Single lens extracts (protocol 1, section 2.6.1 was used for extraction) and tear samples extracts (extraction protocol described in section 2.9) were resuspended in 100μl chloroform. The samples were spotted on a line drawn 2cm from the bottom of the TLC plate. 3 x 10μl aliquots of each

sample were spotted on the plate. The plate was placed in a TLC tank that contained the mobile phase consisting of hexane: diethyl ether: acetic acid (70:30:1 v/v). These conditions allowed for general lipid class separation where non-polar lipids eluted to the top of the plate with the non-polar solvent and polar lipids eluted quickly, nearer the bottom of the plate. The plate was taken from the tank when the solvent line was approximately 2cm from the top of the plate.

The plate was allowed to dry for approximately one minute and it was placed in an iodine tank to stain the separated spots. The separated species were identified from the comparison of their retentions times compared to lipid standards.

2.10.3 High performance liquid chromatography (HPLC)

HPLC is used for the separation, identification and quantification of individual species in unknown samples. In this project, both reverse phase and normal phase HPLC columns were used for the separation of lipids collected from tears samples and contact lens extracts.

An Agilent 1100 HPLC system, connected to a diode array detector (DAD) and fluorescence detector was used in this project.

2.10.4 Normal phase chromatography

The column used for general lipid class separation was a LiChrospher 100 diol 250mm length x 4mm (inner diameter ID) with 5µm packing. The mobile phase consisted of hexane: propan-2-ol: acetic acid (1000: 5: 1 v/v). This combination of non-polar solvents and polar column allowed for the separation of lipids according to their relative polarities. Non-polar lipids would elute quicker than polar lipids because of their interaction with the polar column.

8µl of each sample was injected using the autosampler and the flow rate was set to 1ml/min. The wavelengths selected on the DAD were 205nm, 254nm, 280nm, 360nm. An excitation wavelength of 360nm and emission wavelength of 440nm were set on the fluorescence detector.

2.10.4.1 Sample preparation for normal phase HPLC

Both, tear samples and lens extracts were analysed by HPLC. Lenses were extracted using mainly extraction protocol 1 (1:1 chloroform: methanol as described in section 2.6.1) and tear samples were extracted using the protocol described in section 2.9. The extracts were evaporated to dryness under nitrogen and resuspended in 100µl mobile phase and transferred to a glass insert. The solution was evaporated to dryness under nitrogen and resuspended in 25µl mobile phase.

2.10.4.2 Normal phase HPLC calibration curve preparation

The column and mobile phase details are outlined in Chapter 2 section 2.10.4. Both UV and fluorescence detectors were used in conjunction with HPLC. In contrast to mass spectroscopy, UV and fluorescence detectors do not provide any structural information for separated species but are extremely useful for the detection of individual species. Unknown peaks were identified by the comparison of their retention times to lipid standards. Calibration curves were prepared with known concentrations of lipid standards to assist with quantification. Lipid classes known to be secreted by meibomian glands, including cholesteryl esters, cholesterol, free fatty acids and triglycerides were used as standards.

The calibration curve data and calibration curve for a cholesteryl ester (cholesteryl myristate) are shown in Table 2.2 and Figure 2.1 respectively. The calibration curves for other lipid standards (cholesterol, free fatty acid and TAG) are shown in Appendix 1.

Table 2.2 – Calibration curve data for cholesteryl myristate

Concentration of cholesteryl myristate ($moldm^{-3}$)	*Area under peak at approx 1.8mins*
0	0
0.00041875	291.78
0.0008375	577.05
0.001675	1032.31
0.00335	2145.12
0.0067	4003.48

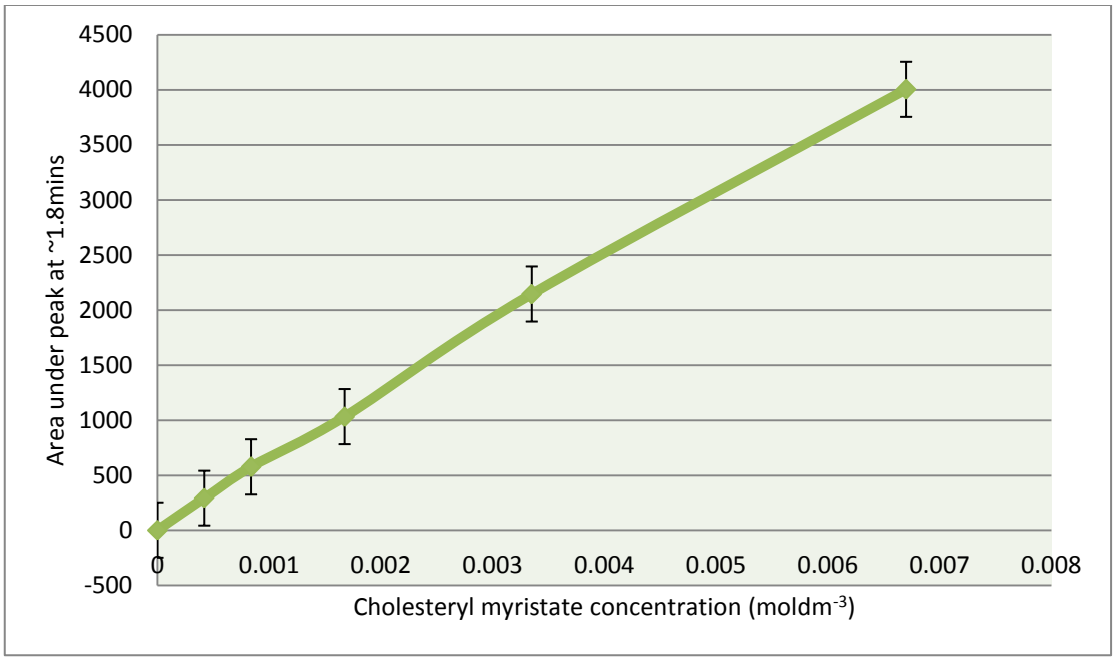


Figure 2.1 – Calibration curve of cholesterol myristate (cholesteryl ester)

2.10.4.3 Retention times of lipid standards measured by normal phase HPLC

Using the normal phase column and conditions described in section 2.10.4, the following retention times were observed for lipid standards (Table 2.3).

Table 2.3 – Relative retention time of lipid classes

Lipid class	*Retention time (minutes)*
Cholesteryl ester	1.5-1.8
Cholesterol	8.6-8.9
Fatty acids	6.0-6.3
Phospholipids	Too polar to detect in reasonable run time
Triglycerides	1.9-3.0
Diglycerides	3.4-3.8
Wax ester	1.5-1.7

It was difficult to distinguish between a wax ester and a cholesteryl ester using normal phase HPLC because they both had the same retention time. Cholesteryl esters and wax esters have similar polarity which made it difficult to separately identify them using a normal phase column. An equal mix of cholesteryl ester (cholesteryl myristate) and a wax ester (behenyl oleate) standard was analysed by HPLC and it produced a single peak that centred at 1.533mins.

Contact lens extracts and tear samples could now be analysed by HPLC. The concentration of lipid classes in unknown samples were determined from the relative calibration curves (Appendix 1).

2.10.5 Reverse phase chromatography

A reverse phase column was used for the separation of lipid species that had similar retention times when separated by a normal phase column (wax esters and cholesteryl esters). Normal phase columns are generally used for the separation of non-polar compounds because non-polar compounds will be soluble in non-polar solvents, which are compatible with the polar column. One particular problem encountered with normal phase separation was that the separated species all had similar retention times because they all had similar polarities. Therefore a reverse phase column was used to try and separate lipid species with similar polarities.

The reverse phase column used was a Zorbax Eclipse XDB C18 2.1(ID) x 150mm, 3.5μm particle size from Agilent. The mobile phase used for this work was 700: 300(v/v) acetonitrile: water. The flow rate was set at 300μl/min and sample injection volume set at 8μl. The same wavelengths that were used in normal phase separation were used in reverse phase separation (205nm, 254nm, 280nm and 360nm. Fluorescence detection wavelengths were - excitation at 360nm and emission at 440nm). The analysis time was initially set at 35mins (same as the normal phase analysis time), however the standards were non-polar and were expected to have a very long retention time, therefore and the analysis time was increased to 60mins.

2.10.5.1 Reverse phase chromatography sample and standard preparation

Lenses were extracted using mainly extraction protocol 1 (described in section 2.6.1) and extracts were resuspended in hexane because hexane does not have a UV absorbance at the wavelengths chosen for lipid analysis. For polar lipid analysis (fatty acids and phospholipids), lipids (standards and lens extracts) were resuspended in reverse phase mobile phase (ACN: H_2O). For non-polar lipid analysis, lipids (standards and lens extracts) were resuspended in hexane. However, after initial investigation, reverse phase C18 column was not used for non-polar lipid separation because standards did not elute after 60mins of analysis time. Reverse phase separation was therefore used for polar lipid class (fatty acids) separation. Shorter chain fatty acids eluted quicker as they were more hydrophilic than longer chain fatty acids.

Fatty acid standard separation by reverse phase HPLC column showed lipids eluted in the order of ascending molecular weight: C16:0, C16:1, C18:0, C18:1, C20:0 and C20:4. Although reverse phase chromatography achieved separation of fatty acids, lens extracts were not analysed. GCMS was the preferred technique for separation and identification of individual fatty acids from contact lens extracts.

2.10.6 Gas chromatography mass spectrometry (GCMS)

GCMS can both separate and identify individual species within an unknown sample. In this project, GCMS was used to separate and identify individual fatty acid species from worn lens extracts and tear samples. A Varian Saturn 2000 GCMS with an 8400 autosampler was used in this project for analysis.

Chromatography is the term for a range of techniques used for separation. Each chromatographic technique works by mobile phase passing the unknown sample through a stationary phase. The mobile phase in GC is a gas, which in this research was helium and the stationary phase was the column. A VF-5ms column with dimensions of 30m x 0.25mm (inner diameter ID) and DF of 0.25 was used for all GCMS analyses carried out throughout this thesis. The packing in the column consisted of 5% phenyl and 95% dimethyl polysiloxane. This was a slightly polar column which allowed the separation of fatty acid methyl esters. The injector temperature was set at 250°C. The GC column oven temperature sequence was 100°C hold for 2mins. Increased temperature to 180°C at 10°C/min, temperature was held for 5mins. The temperature was increased to 240°C at 5°C/min, with no temperature hold, which made a total analysis time of 35mins.

The mass spectrometer was set on electron ionisation (EI) mode. The helium flow rate was set at 1ml/min. The ionisation range was set at 40m/z to 650m/z.

2.10.6.1 GCMS calibration curve preparation and method development

The GCMS conditions chosen for this research are described in section 2.10.6. An isocratic temperature at the beginning of the run provided good peak shape and resolution. The temperature was raised to ensure separation of all species over a range of melting points. The column oven temperature was set as described in Chapter 2 section 2.10.6, which enabled efficient separation of peaks.

A calibration curve was constructed of known concentrations of heptadecanoic acid (C17:0) to assist with quantification.

Table 2.4, Figure 2.2 and Figure 2.3 show the data, calibration curve and gas chromatograms of heptadecanoic acid at different concentrations respectively.

Table 2.4 – Heptadecanoic acid (17:0) calibration curve data

Heptadecanoic acid concentration (mg/ml)	*Area under peak at 13.949*
0.00012	178760
0.0009765	432594
0.001953125	562284
0.00390625	14580000
0.0078125	21560000
0.015625	38970000
0.03125	62190000

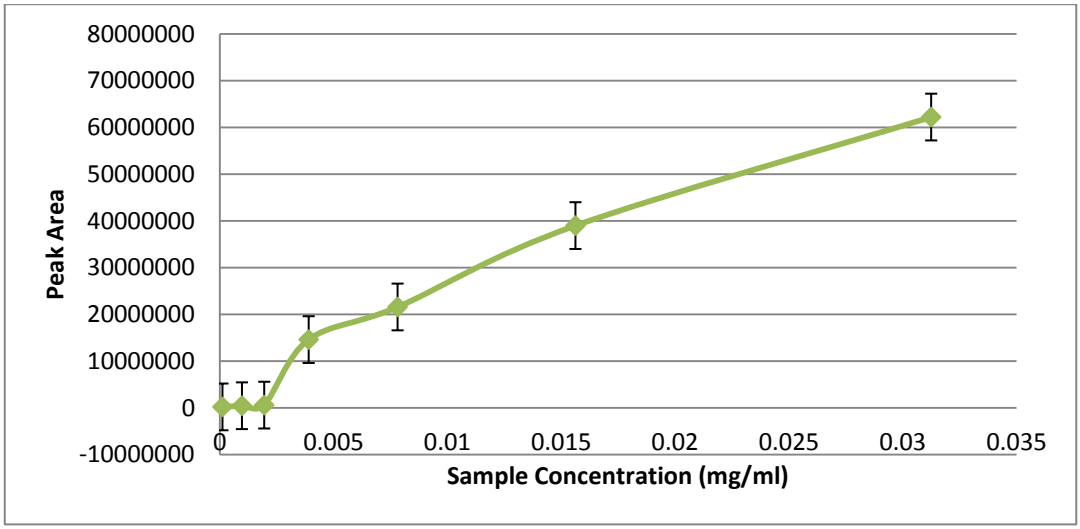


Figure 2.2 – Calibration curve for heptadecanoic acid (C17:0)

The calibration curve was not linear at very low concentrations of C17:0.

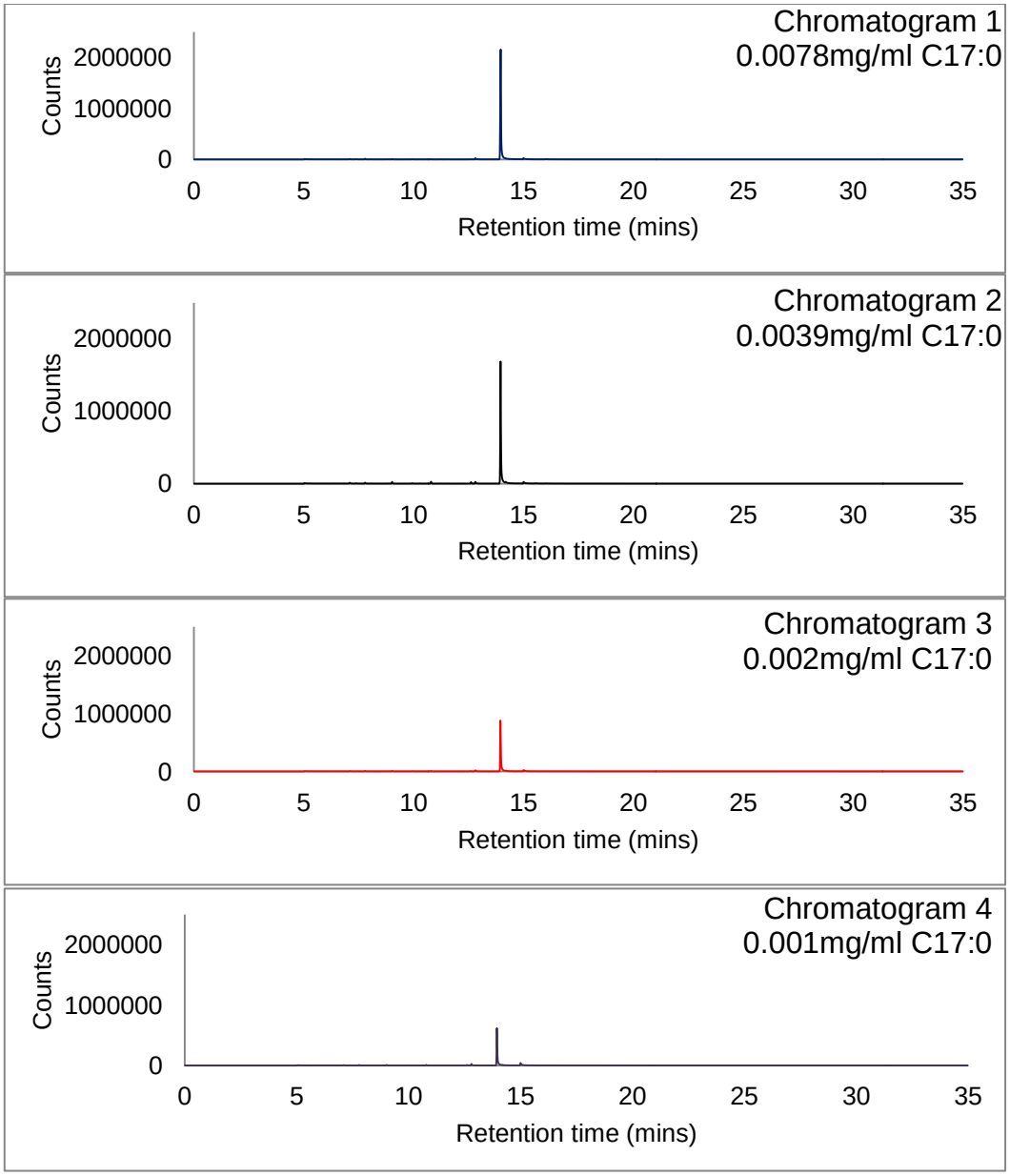


Figure 2.3 – GC traces of C17:0 calibration curve

Very long chain fatty acids (C20:0 and C20:1) could also be analysed by GCMS. C20:0 produced a peak which centred at 17.090mins and C20:1 produced a peak which centred at 17.11mins. The identity of individual lipid species was determined from the retention times of lipid standards. The identity of individual peaks was also determined by comparing the individual mass spectra against a library of standards.

2.10.7 Mass spectra for standard fatty acid methyl esters

Each peak was identified by its retention time on the gas chromatogram and their mass spectra. Mass spectra for representative unsaturated (C18:1) and saturated (C18:0) fatty acids are shown in Figure 2.4 and Figure 2.5 respectively. These fatty acids are predominant fatty acids known to be secreted by the meibomian glands (as well as C16:1 and C16:0). The mass spectra for other fatty acid standards are shown in Appendix 2.

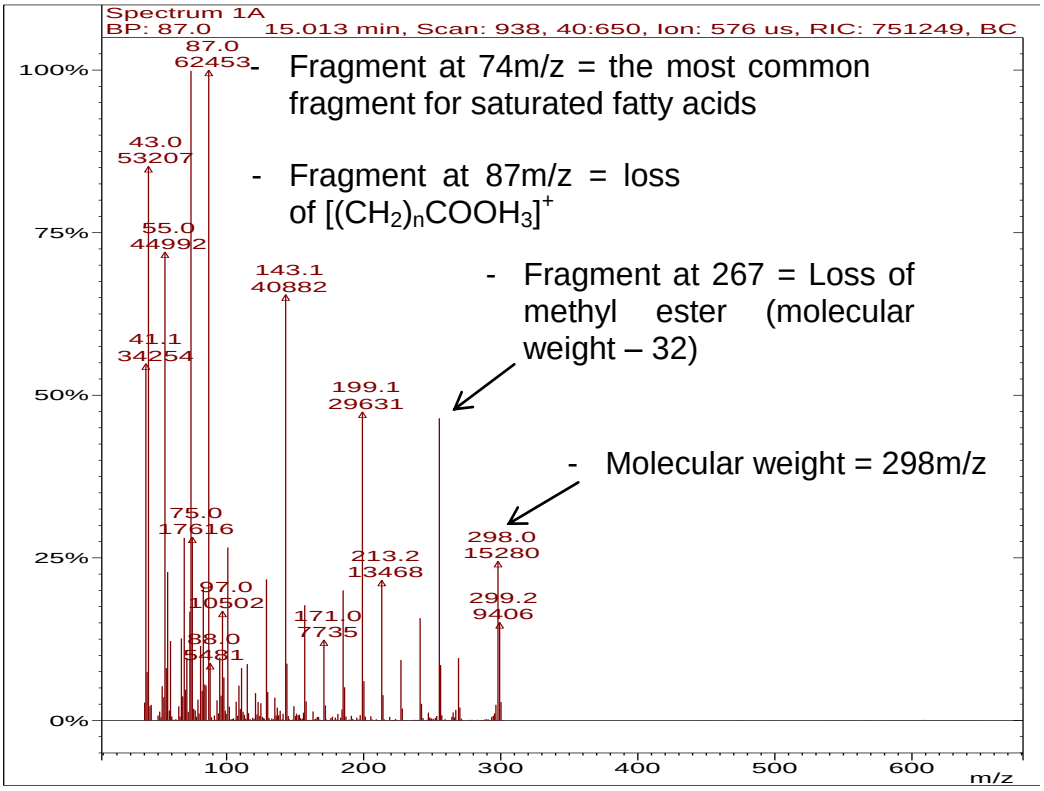


Figure 2.4 – MS of stearic acid (C18:0) methyl ester

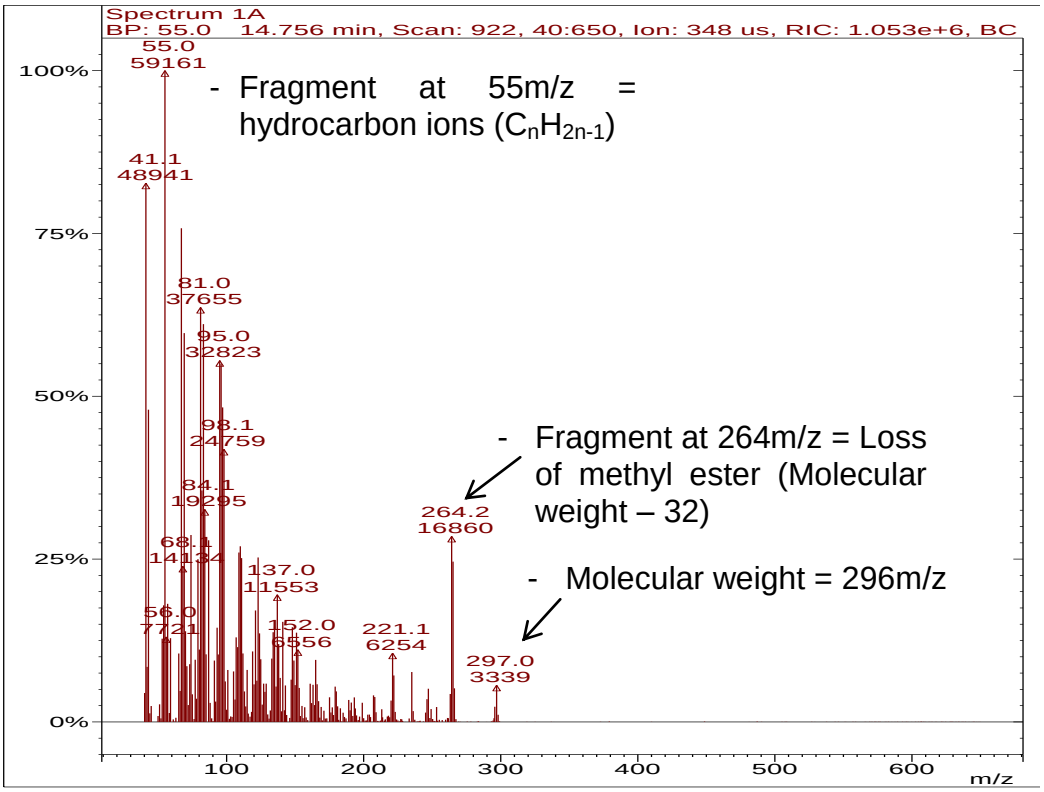


Figure 2.5 – MS of oleic acid (C18:1) methyl ester

The mass spectral fragmentation patterns of saturated fatty acids differ from those obtained from unsaturated fatty acids. Mass spectroscopy can also provide molecular weight information for each separated species. The combination of gas chromatography with mass spectroscopy makes the identification of separated species more accurate than GC alone.

The analysis of standards and the preparation of calibration curves enabled separated species from unknown samples to be identified and their concentrations determined. Both normal phase HPLC and GCMS are used for the analysis of lens extracts and tear samples, this is discussed in Chapter 3.

2.11 Oxidative assays methodology

Several oxidative assays kits were used in this research for the analysis of lipid peroxidative products in the ocular environment. Many of the assay kits available were not designed for the analysis of tears so were therefore adapted. For example, many of the assays required 100µl or more sample volume for analysis. This volume of tears could not be collected from

individuals in one go, therefore tears were either pooled or made-up to the required volume with water or PBS. The sample preparation details for each assay are discussed in Chapter 5.

The assays and analytical techniques used were the thiobarbituric acid reactive substances (TBARS) assay, N-methyl-2-phenylindole (NMPI) assay, capillary electrophoresis, high performance liquid chromatography (HPLC) with fluorescence detection and enzyme linked immunosorbant assay (ELISA). The TBARS, NMPI and ELISA assays were commercially available assays, currently used for malondialdehyde (MDA – an end product of lipid oxidation, discussed in Chapter 1, section 1.11) detection from blood, urine and plasma samples.

2.11.1 TBARS assay protocol (Oxi Select TBARS assay kit MDA quantitation from Cell Biolabs Inc.)

This is a commercially available assay kit and all reagents required for sample preparation are supplied in the kit. The general assay theory is that MDA, in unknown samples (tear samples, lens extracts and tear envelope samples) or standards binds to thiobarbituric acid (TBA) to form an MDA-TBA adduct which has UV absorbance at 523nm and fluorescence absorbance at 590nm emission wavelength when an excitation at 540nm is applied. The reaction scheme is shown in Figure 2.6. The sample volume required for this assay was 100µl.

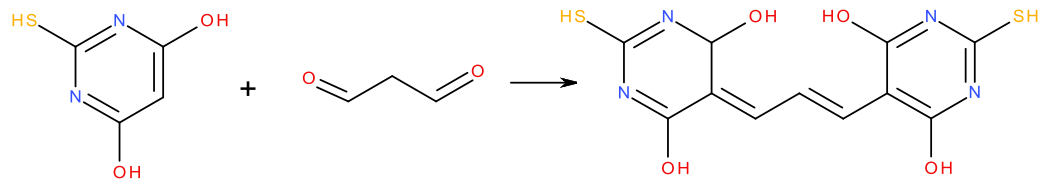


Figure 2.6 – TBA reaction with MDA forming an MDA - TBA adduct under acidic conditions

2.11.1.1 TBARS method:

The supplied assay protocol was followed. The reagents supplied with the assay kit were prepared as described below.

The 1xTBA acid diluent was prepared from the dilution of 2xTBA acid diluent (supplied) with distilled water. For 10 assays, 6ml 2xTBA acid diluent was diluted with 6ml distilled water. For more assays, more 1xTBA acid diluent was prepared.

SDS lysis (supplied) was prepared by placing the SDS lysis bottle in a warm water bath until the crystals had dissolved.

5.2mg/ml TBA reagent was prepared from the addition of the appropriate amount of TBA (appropriate weight for 10 samples was 13mg) to the 1xTBA acid diluent (2.5ml for 10 samples). The pH of the TBA reagent was adjusted to pH3.5 by the addition of sodium hydroxide to the solution and the use of the pH meter.

Once all of the above reagents were prepared, MDA standards over a range of concentrations (for the calibration curve) were prepared.

A 1mM MDA standard is supplied with the assay. From this standard, 125µM to 0.98µM MDA standards were prepared by double dilution in water. 100µl of each MDA standard was transferred to individual 2ml eppendorfs, 100µl SDS lysis (supplied) and 250µl TBA reagent (previously prepared, as described above) were added to each standard and unknown sample. The eppendorfs were placed in the oven at 95°C for 60mins. The eppendorfs were then placed in an ice bath for 5mins and then centrifuged for 15mins at 3300rpm to collect any unwanted debris at the bottom of the eppendorf. The upper 200µl of each standard or unknown sample was transferred to a 96 well plate for spectroscopic analysis at 532nm UV absorbance or fluorescence absorbance with an excitation wavelength of 540nm and emission wavelength of 590nm. The concentration of MDA in unknown samples was determined from the calibration curve of MDA standard. The calibration curves obtained by using this assay are shown in Chapter 5 (Figure 5.1).

2.11.2 MDA – Specific assay protocol (ALDetect Lipid Peroxidation assay kit from Enzo Life Sciences)

The principle of this assay is similar to the TBARS assay. MDA binds to N-methyl-2-phenylindole (NMPI) to produce a coloured complex that can be detected by UV absorption (Figure 2.7). The maximum absorbance for the adduct was found to be at 572nm, although the assay protocol suggested it was at 586nm. The sample volume required for this assay was 200µl. The advantage of this assay over the TBARS assay is that it gives a more accurate measure of MDA levels. TBA can bind to other aldehydes and can exaggerate the true level of MDA.

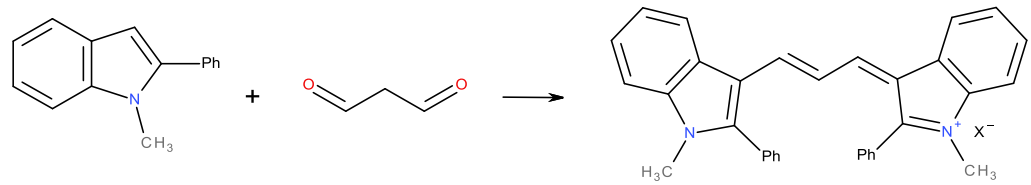


Figure 2.7 – MDA reaction with NMPI forming a MDA – NMPI adduct

2.11.2.1 NMPI method:

The protocol supplied with this assay was followed. Briefly, the supplied reagents were prepared in the following ways. R1 reagent (supplied) was prepared from the addition of 18ml R1 reagent to 6ml methanol. Tetramethoxypropane (TMOP) was used as an MDA substitute, TMOP was supplied as a 10mM stock solution. TMOP was diluted 1/500 with water to prepare a 20µM stock solution. The preparation of TMOP standards over a range of concentrations is shown in Table 2.5.

Once the TMOP standards (over a range of concentrations) were prepared, 200µl of each TMOP standard or test sample was transferred to a 15ml centrifuge tube. 10µl probucol (supplied) was added to the centrifuge tube. 640µl of the diluted R1 reagent (previously prepared) was then added. 150µl R2 reagent (supplied) was also added and the centrifuge tubes were vortexed to mix the solution. The centrifuge tubes were then incubated in the oven for 60mins at 45°C. They were then

centrifuged (1.1rpm for 10mins) until a clear solution was obtained. Each solution was added to a cuvette and UV wavelength scans were taken to determine the maximum absorbance. The maximum UV absorbance was found to be at 572nm. Therefore, all subsequent standards and unknowns were analysed at 572nm. The calibration curve is shown in Chapter 5.

Table 2.5 – Preparation of TMOP standards for MDA-NMPI calibration curve

Volume of 20µM stock (µl)	*Volume of water (µl)*	*Concentration of TMOP (µM)*
0	200	0
25	175	0.5
50	150	1.0
100	100	2.0
150	50	3.0
200	0	4.0

2.11.3 ELISA-MDA assay protocol (from Cell Biolabs Inc.)

MDA and 4-hydroxynonenal have the ability to bind to proteins and form stable MDA-protein adducts. An enzyme immunoassay was used to quantitatively detect this MDA-protein adduct. The sample volume required for this assay was 100µl.

2.11.3.1 Method:

MDA-BSA standards were prepared at the concentrations shown in Table 2.6. (The 1µg/ml standard is diluted by half to prepare a stock solution of 0.5µg/ml MDA-BSA standard for the calibration curve). 100µl standards or unknowns were added to the 96-well plate provided in the assay kit and incubated overnight at 4°C. The plate was then washed with a non-reacting blocking buffer (supplied) which prevented non-specific binding of primary antibody. The plate was washed with several buffers supplied in the assay kit and the plate was then patted dry on tissue paper. 100µl of the secondary horseradish peroxidise conjugated antibody (supplied) was

added the plate and it was incubated for 1hr at room temperature. The plate was washed with the wash buffer provided with the assay. 100µl substrate solution (supplied) was added to the wells of the plate and a colour change was observed (from clear to blue). The reaction was stopped by the addition of the stop solution (which was supplied) and a change of colour from blue to yellow was observed. The absorbencies of the samples and standards were analysed at 450nm and a calibration curve of standards was obtained. The concentration of MDA in unknown samples was determined from the calibration curve (shown in Chapter 5).

Table 2.6 – Preparation of standards for MDA-ELISA assay

Tube Number	*Volume of MDA-BSA (µl)*	*Volume of reduced BSA (µl)*	*MDA adduct concentration (pmol/mg)*
1	1000	0	120
2	500µl of no.1	500	60
3	500µl of no.2	500	30
4	500µl of no.3	500	15
5	500µl of no.4	500	7.5
6	500µl of no.5	500	3.75
7	500µl of no.6	500	1.875
8	0	500	0

2.11.4 Capillary Electrophoresis with UV detection

Capillary electrophoresis was used to detect MDA as it had previously been used for MDA detection in tears by Georgakopoulosa *et al.* (151). Capillary electrophoresis is a combination of electrophoresis and HPLC, it can therefore separate components based on polarity and charge.

The following conditions were used for the detection of MDA – Capillary: bared fused-silica capillary with 50µm ID, 56cm effective length and 65cm total length. Separation: 25mM borate buffer, pH10.0 which contained 100mM sodium dodecyl sulfate (SDS) at the temperature of 25°C and

voltage at 20kV. Detection: 266nm and 290nm wavelengths using a diode-array detector. Samples were pressure injected at 50mbars at the anode (normal polarity). The conditions used in this research were similar to those used in the literature.

To determine a lower limit of detection, MDA standards were analysed by capillary electrophoresis. A 2.5µM MDA solution was analysed and it produced a peak at approximately 20mins, however the peak had a very low intensity. Tear samples, tear envelope samples and lens extracts were subsequently analysed however, MDA was not detected in any sample.

2.11.5 High Performance Liquid Chromatography (HPLC) with fluorescence detection

HPLC with fluorescence detection had been used for MDA separation and identification in blood plasma samples by Fukunaga *et al*. (134). The methodology used by Fukunaga *et al.* was followed in this research. The method is based on an MDA-TBA reaction. The MDA-TBA adduct is then separated by HPLC with fluorescence detection. TBA can bind with other aldehydes; however only TBA bound to MDA will have a characteristic retention time. MDA-TBA adduct has a characteristic retention time which makes this method more accurate than the TBARS assay alone.

An MDA substitute was used because MDA is not stable and was therefore not commercially available. 1,1,3,3 tetraethoxypropane (TEP) was used as the substitute (Figure 2.8). The theory is that TEP changes to MDA in a hydrolysis reaction when the TBA buffer and TEP are heated.

A Hypersil ODS column with 5µm packing, 150mm x 4.6mm ID (Kinesis UK) was used for the separation of MDA-TBA. The column was also fitted with a guard column with 5µm packing and 10mmx4.6mm ID. The mobile phase was 700/300 (v/v) acetonitrile: water with a flow rate of 0.5ml/min. The MDA-TBA adduct had a fluorescence absorbance at the following wavelengths - excitation of 515nm and the emissions were analysed at

553nm. The sample volume required for this assay, as described in the literature was 5µl (134).

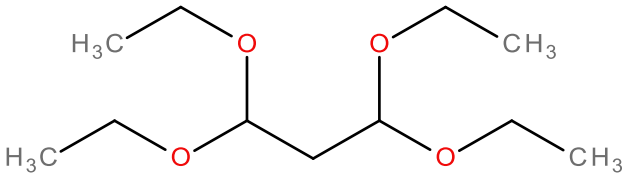


Figure 2.8 – Structure of 1,1,3,3 tetraethoxypropane (TEP)

2.11.5.1 MDA-TBA separation and detection by HPLC with fluorescence detection method:

2.11.5.1.1 Preparation of TBA buffer

0.1M sodium acetate buffer was prepared by firstly, dissolving 1.36g sodium acetate in 100ml HPLC grade water. Secondly, 8.5ml was transferred to a 100ml volumetric flask, 0.1M acetic acid was added until the final volume was 100ml. The pH of the sodium acetate buffer was adjusted to pH3.5 by adding sodium hydroxide.

0.2g TBA was then added to the 0.1M sodium acetate buffer solution, which made a 0.2% (w/v) TBA solution in 0.1M sodium acetate buffer.

2.11.5.1.2 Preparation of standards

10µmol/ml TEP stock solution was prepared by addition of 24µl TEP to 10ml HPLC grade ethanol. Other concentrations of TEP were prepared by serial dilution of the 10µmol/ml stock solution with dilution in HPLC grade water.

Table 2.7 – Preparation of TEP standards for HPLC separation

Standard tubes	*TEP standard*	*Amount of water*	*Concentration of TEP*
Stock	24µl	10ml	10µmol/ml
1	200µl of stock	9800µl	200nmol/ml
2	100µl of stock	9900µl	100nmol/ml
3	50µl of stock	9950µl	50nmol/ml
4	40µl of stock	9960µl	40nmol/ml
5	30µl of stock	9970µl	30nmol/ml
6	20µl of stock	9980µl	20nmol/ml
7	10µl of stock	9990µl	10nmol/ml
8	1ml of tube 7	9ml	1nmol/ml
9	1ml of tube 8	1ml	0.5nmol/ml
10	1ml of tube 8	9ml	0.1nmol/ml
11	1ml of tube 10	9ml	0.01nmol/ml

2.11.5.1.3 Preparation of samples for HPLC analysis

5µl TEP standard or 5µl unknown sample was added to 1ml of 0.2% (w/v) TBA buffer in 0.1M sodium acetate buffer in 2ml eppendorfs. These eppendorfs were then placed in an oven for 60 minutes at 90°C. The solutions in the eppendorfs were then cooled by placing them in a cold water bath for 5mins. The eppendorfs were centrifuged for 15mins at 6600rpm. The upper 750µl were transferred to amber vials for analysis by HPLC.

2.12 Method used for inducing lipid oxidation

100µl linolenic acid standard was transferred to a glass vial and left exposed to atmospheric oxygen on a shaker for 24hrs to promote oxidation.

5ml chloroform was added to the remaining unused linolenic acid standard and stored at -20°C.

10µl of each standard (the oxygen exposed linolenic acid and the fresh linolenic acid standard from the freezer) was added to 500µl TBA buffer and placed in an oven for 1hr at 90°C.

Chapter 3 – Technique development and analysis of lenses, lens extracts and tears

3.1 Introduction

After the insertion of a contact lens, tear film components begin to adsorb on to its surface and in some cases begin to absorb into the lens matrix. To monitor the progression of lipid build-up, a range of analytical techniques were used. A process of analysis (shown in Figure 3.1) was developed and followed in order to, firstly, measure gross lipid non-destructively and, secondly, analyse lipids by destructive techniques. Fluorescence spectrophotometry is a non-destructive technique and it was used to measure gross lipid on the both anterior and posterior surfaces of single worn lenses. To gain structural information about the lipids, lenses were extracted and analysed by an array of chromatographic techniques. The results generated by each technique provide information about different aspects of the lipid structure.

Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) were used to separate lipid classes in accordance to their relative polarities. Gas chromatography (GC) was used for the separation of individual fatty acids (in the form of their methyl esters) from lens extracts and it also provided information about the state of oxidation of each fatty acid. To determine the extent of oxidation, various assays were used to quantify the levels of oxidative end products in tears, or on contact lenses (details of each analytical technique are explained in detail in Chapter 2 and results are discussed in Chapter 5).

The work discussed in this chapter will show that a single lens extract can be analysed by various techniques, each technique providing information about lipid structure. Unworn lenses will be analysed by GCMS and HPLC to provide a baseline for *ex vivo* lens extracts. Tear samples, taken using Visispear ophthalmic sponges were also analysed by HPLC and GCMS.

3.2 Analytical technique development

Figure 3.1 – Analytical technique flow diagram

The process of analysis for a single subject-worn lens is shown in Figure 3.1. This process shows how a single lens extract, subjected to an array of techniques can provide a vast amount of information about lipid structure.

3.3 Non-destructive analysis: *ex vivo* lens analysis by fluorescence spectrophotometry

Fluorescence spectrophotometry (FS) was used to non-destructively analyse the levels of gross lipid deposition on lens surfaces. FS enables differences in lipid deposition between anterior and posterior surfaces of lenses to be analysed. It also provides data for lipid deposition on different lens materials. The details for this technique are explained in Chapter 2, section 2.10.1.

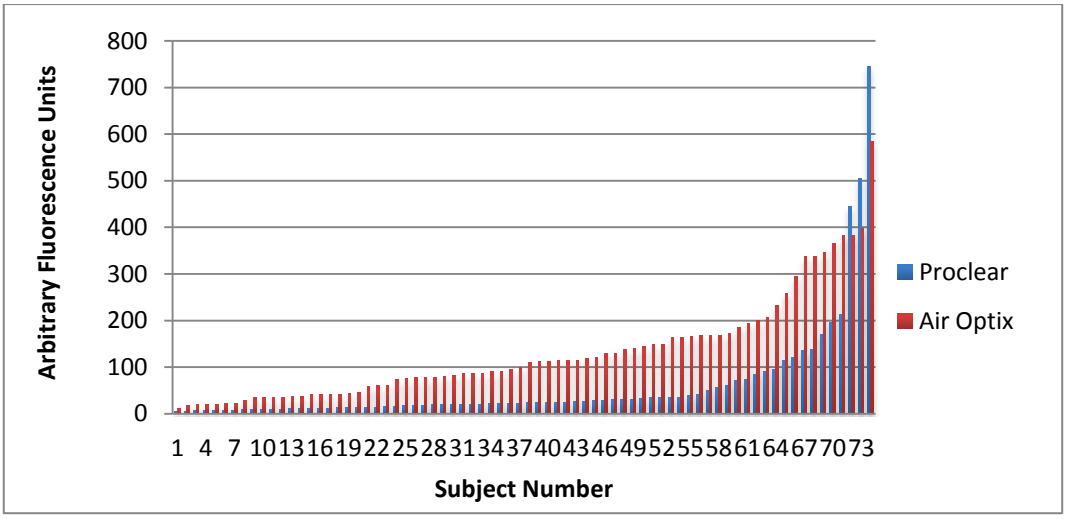


Figure 3.2 – Lipid subject-to-subject variability on two contact lens materials over a range of subjects measured by fluorescence spectrophotometry

Figure 3.2 compares lipid deposition across a range of subjects wearing either Proclear (a conventional hydrogel) or Air Optix lenses (a coated silicone hydrogel). Over the range of subjects, it is clear that the level of lipid deposition on Air Optix lenses is greater than on the Proclear lenses. Additionally, it can be seen that there is marked subject-to-subject variation in the level of deposited lipid. For a small subset of subjects, lipid deposition can be described as abnormally high. This technique shows that lipid deposition on lenses is to an extent material dependant, but it is ultimately subject dependant.

Once lenses had been analysed by fluorescence spectrophotometry for gross lipid levels, individual lenses were subjected to destructive analysis. Single lenses were extracted (using various extraction protocols described in section 2.6) and subjected to an array of techniques.

3.4 Destructive analysis: lens extraction and process of analysis

Although the fluorescence absorbance results for worn PureVision (PV) lenses are not shown, they usually have an absorbance above 500 fluorescence units, which is considerably higher than the Air Optix lenses analysed in Figure 3.2. This was the reason a PV lens extract was

separated by an array of techniques. In order to show that a single lens could be extracted and analysed by several techniques, a single PV lens was extracted using extraction protocol 1 (described in section 2.6.1) and was separated by TLC, HPLC and GCMS. The results generated by each technique showed different aspects of lipid structure. TLC separation provided a general lipid class separation based on polarity. HPLC also provided a general lipid class separation; the ratios of non-polar to polar lipids could also be determined by this method. GCMS enabled the separation and identification of individual fatty acids. Subjecting a lens extract to this process of analysis validated both the choice of extraction protocol and the technique conditions chosen.

3.4.1 TLC separation of an ex vivo PureVision lens extract

A single PureVision (PV) lens (known for high levels of lipid deposition) was extracted using extraction protocol 1 (described in section 2.6.1) and resuspended in chloroform for TLC analysis. TLC separation of this PV lens extract is shown in Figure 3.3. Using the conditions described in section 2.10.2, individual lipid classes, extracted from a single lens were separated. Polar lipids eluted quickly, nearer the bottom of the plate and non-polar lipids eluted more slowly, nearer the top of the plate. The separated bands were identified by the comparison of separated species against lipid standards. The spots on the line near the bottom of the plate were identified as phospholipids, however, analysis of worn lens extracts by HPLC showed no peaks for phospholipids. The non-polar lipids, such as the sterol esters and TGs ran to the top of the plate with the non-polar mobile phase.

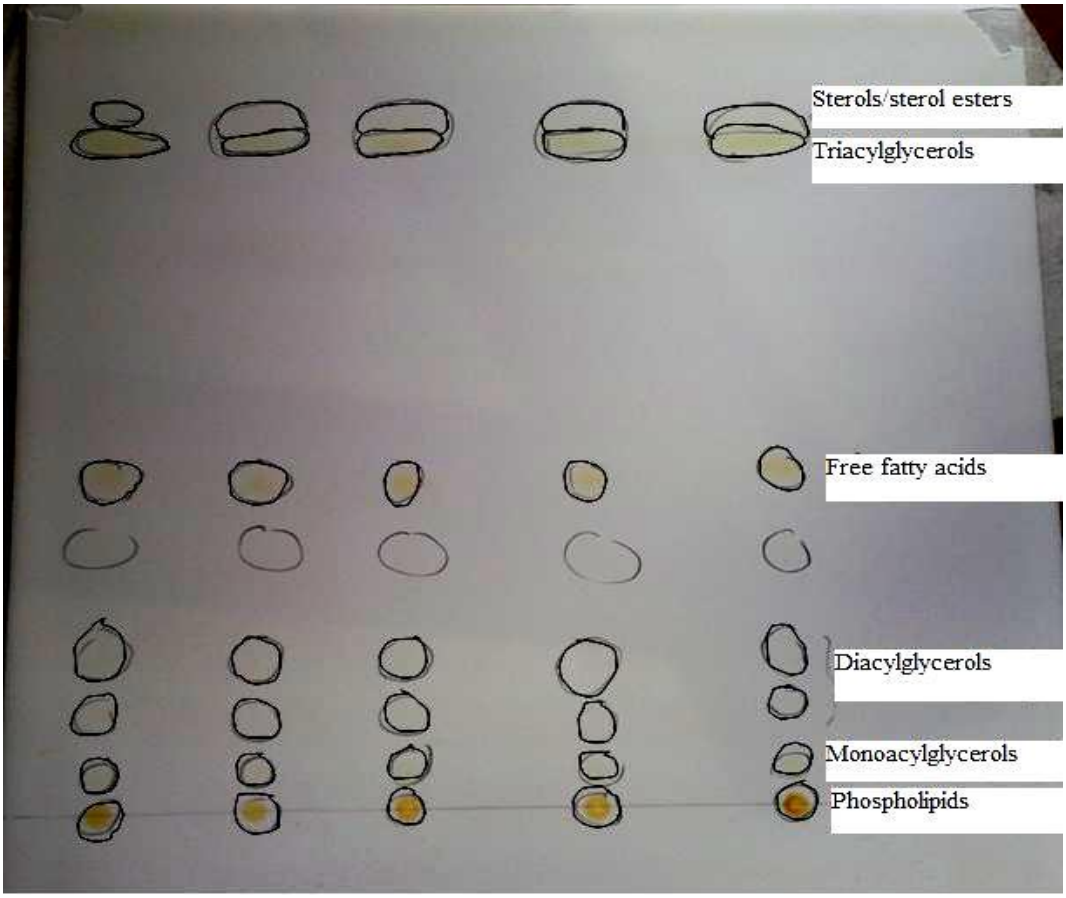


Figure 3.3 – TLC separation of a single worn PureVision lens extract

3.4.2 Normal phase separation of an ex vivo PureVision lens extract

The remainder of the extract medium (from TLC analysis) was evaporated to dryness under nitrogen and resuspended in mobile phase for normal phase HPLC analysis. An analysis time of 35mins was chosen to ensure that no other polar lipids eluted (Figure 3.4).

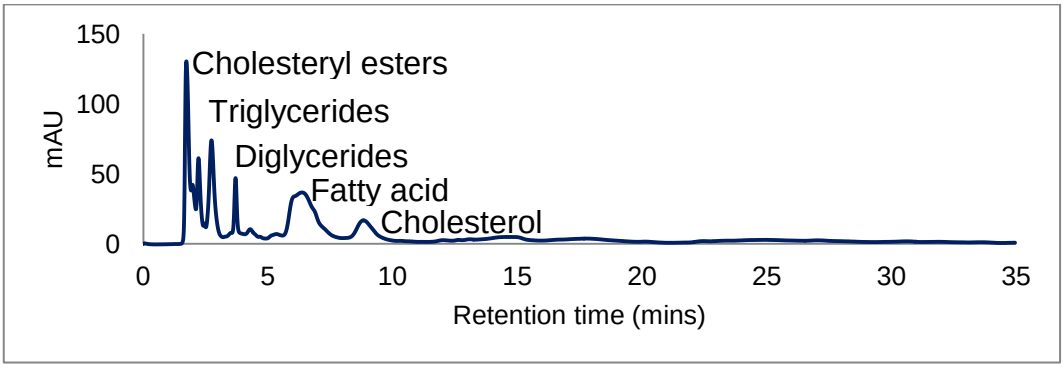


Figure 3.4 – HPLC trace of a single ex vivo PureVision lens extract (205nm)

The HPLC trace of a single *ex vivo* PV lens extract shown in Figure 3.4 demonstrates how powerful normal phase chromatography is for the separation of lipids. This HPLC trace shows the separation of the major lipid classes adsorbed on the contact lens. The trace for this particular lens extract showed peaks corresponding to CEs, TGs, DGs, FFA and cholesterol. The concentration of individual lipids was determined from the calibration curves shown in Appendix 1.

Table 3.1 – Analysis of the HPLC trace shown in Figure 3.4

Lipid class	*Concentration of lipid class*
Cholesteryl esters (CEs)	$1.67x10^{-3}moldm^{-3}$
Triglycerides (TGs)	$1.40x10^{-3}moldm^{-3}$
Diglycerides (DGs)	$0.8x10^{-3}moldm^{-3}$
Free fatty acids (FFAs)	$4.0x10^{-3}moldm^{-3}$
Cholesterol	$9.5x10^{-4}moldm^{-3}$

3.4.3 GCMS separation of an ex vivo PureVision lens extract

Although normal phase HPLC separation provided information about the general lipid classes deposited on and in the lens, GCMS was able to separate and identify the fatty acid groups from single lens extracts. The analysis of lens extracts by GCMS is important to investigate because the levels of unsaturated fatty acids and the ratio of unsaturated to saturated fatty acids can be determined. GCMS is used in Chapter 4 to investigate changes in individual fatty acid profiles between lenses worn on a daily wear schedule compared to a continuous wear schedule.

The remainder of the contact lens extract (from Figure 3.4) was evaporated to dryness under nitrogen and then transmethylated (as described in Chapter 2 section 2.5). The individual fatty acids were separated and the GC trace is shown in Figure 3.5.

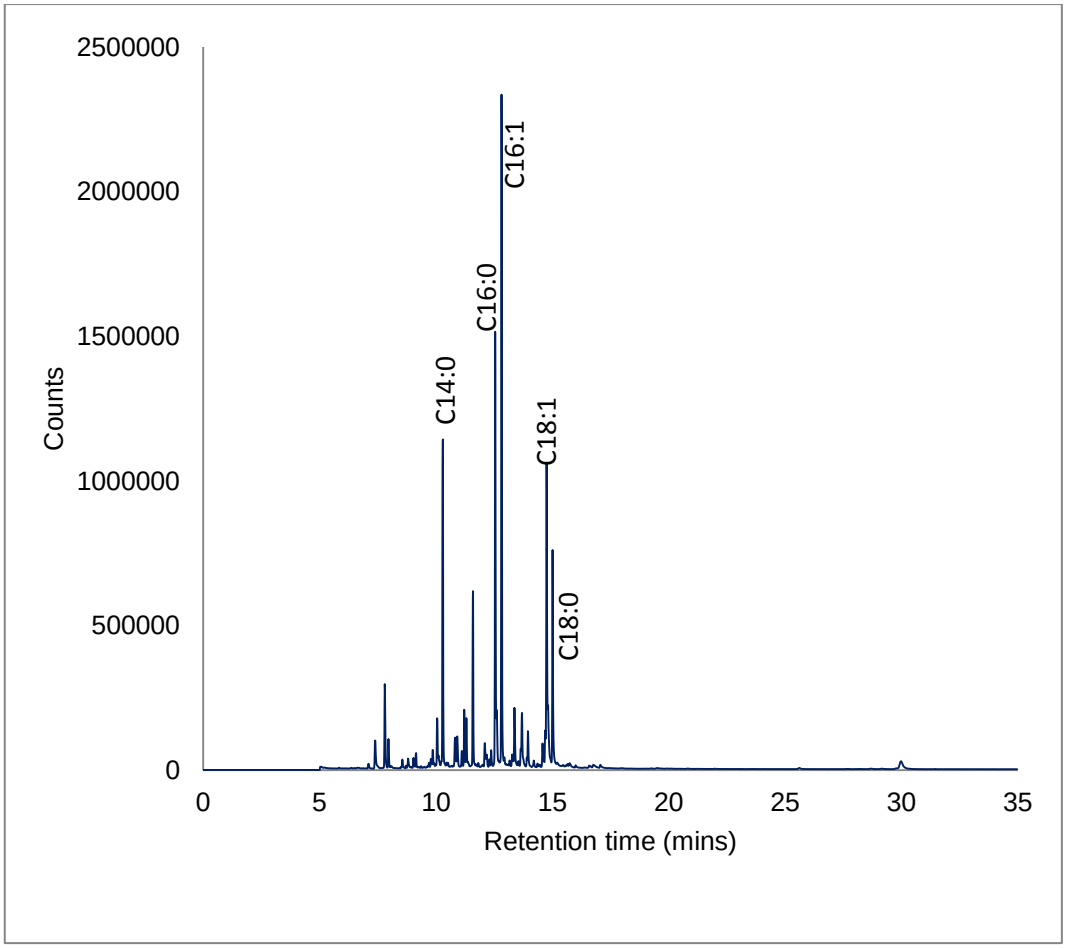


Figure 3.5 – Gas chromatogram of a single ex vivo PV lens extract

The GC trace shown in Figure 3.5 represents a worn PV lens extract. The major peaks observed correspond to the major tear film fatty acids as reported in literature (23). C14:0, C16:1, C16:0, C18:1 and C18:0 are observed for this PV lens extract (as shown in Table 3.2). GCMS has not previously been used for the analysis of fatty acids from contact lens extracts. These results show that single lenses can be extracted and analysed by GCMS. The concentrations of individual fatty acids could be determined from the calibration curve (shown in Figure 2.3).

Table 3.2 – Fatty acid retention times, separated by GCMS

Peak retention time (mins)	*Fatty acid*	*Saturated/unsaturated*
10.3	C14:0	saturated
12.5	C16:1	unsaturated (1 double bond)
12.8	C16:0	saturated
14.8	C18:1	unsaturated (1 double bond)
15.0	C18:0	saturated

Unworn PureVision and other unworn lens materials were also analysed by GCMS (shown in section 3.5.1). This allowed the differentiation between extractable material from lenses and tear lipids to be made. Although the peaks for unworn lenses were at the same retention times as certain fatty acids (mainly C16:0 and C18:0), the peaks were less intense for unworn lens materials compared to worn extracts. Tear samples were also analysed to show both C16:0 and C18:0 are common tear lipids and do deposit on lenses (shown in Figure 3.16).

3.5 Unworn lens extraction results

In addition to the extraction of worn lenses and the analysis of the deposits, unworn (fresh lenses) were extracted (using extraction protocol 1) and analysed by both GCMS and normal phase HPLC. Several *ex vivo* lens materials including PureVision and Focus Night & Day were extracted and the deposits analysed throughout this thesis. It was therefore necessary to extract and analyse unworn (blank) lenses to provide a baseline. Several unworn lens materials were extracted and analysed by both GCMS and HPLC (Figure 3.6 to Figure 3.11). Unworn lenses were extracted and treated in the same way *ex vivo* lenses were, to provide a direct comparison between worn and unworn lenses.

Prior to the extraction of unworn lenses, empty vials were extracted (with no lens) using extraction protocol 1 (section 2.6.1). The empty vial extracts were also analysed by GCMS to ensure the vials were clean. Unworn lenses were then extracted in these vials to ensure the vials were

not contaminated. The gas chromatograms for empty vial extracts are shown in Appendix 3.

3.5.1 Gas chromatograms of unworn lens extracts

The gas chromatograms in Figure 3.6 represent various unworn lens extracts. Unworn PureVision, Focus Night & Day, Air Optix, Air Optix Aqua and Soflens 66 lenses were analysed by GCMS. These particular lenses were chosen because they represented a range of both silicone and conventional hydrogels from the major contact lens manufacturers (Ciba Vision and Bausch & Lomb). Also, because *ex vivo* PureVision and worn Focus Night & Day lens extracts are analysed by GCMS and HPLC (in Chapter 4), baseline chromatograms were required.

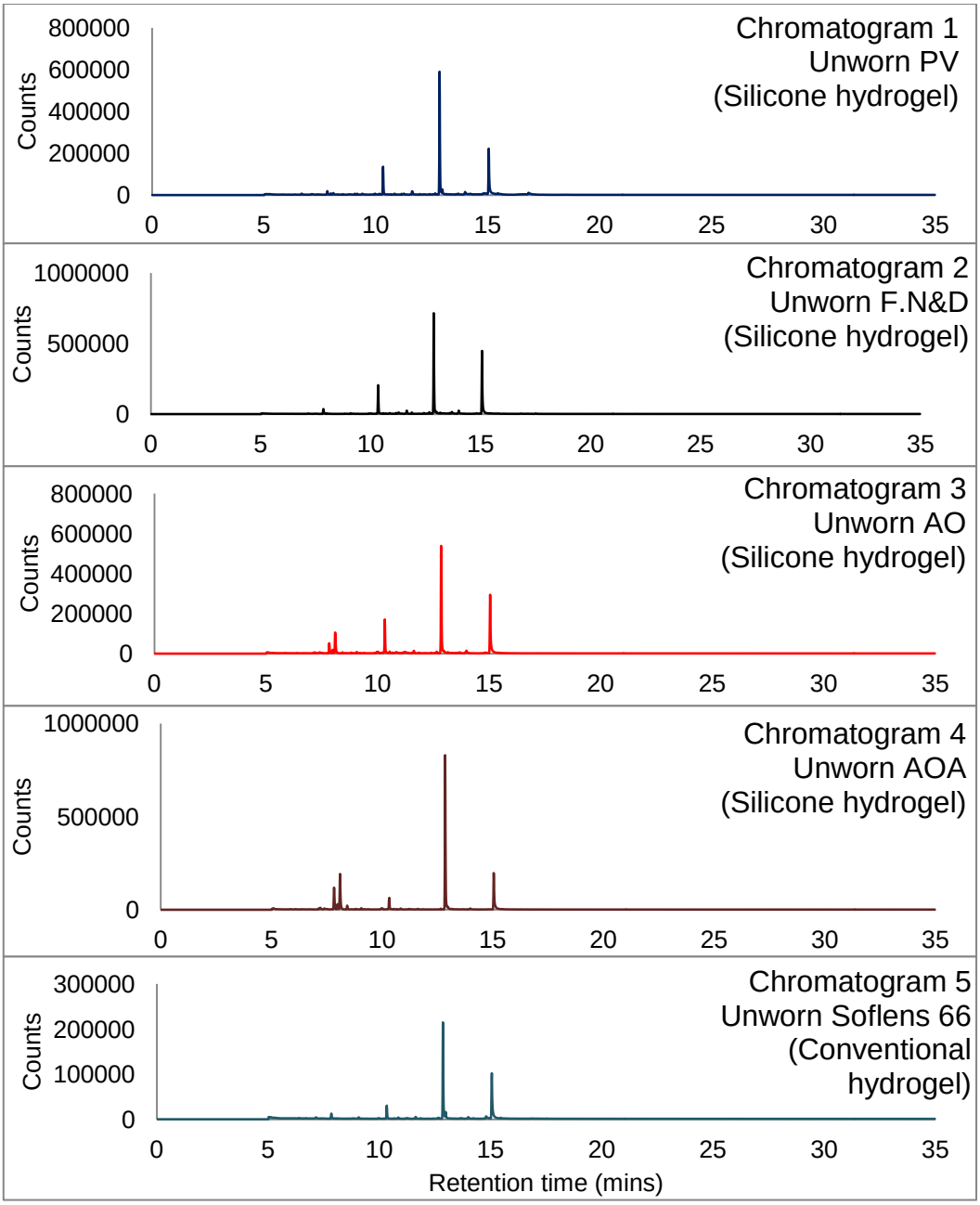


Figure 3.6 – Gas chromatograms representing unworn lens extracts, where: Chromatogram 1 – unworn PV extract, Chromatogram 2 – unworn F.N&D extract, Chromatogram 3 – unworn AO extract, Chromatogram 4 – unworn AOA extract and Chromatogram 5 – unworn Soflens 66 extract.

The peaks observed for all unworn contact lenses analysed corresponded to C16:0 and C18:0 (at 12.8 and 15.0mins respectively). These peaks were identified from their retention times and their mass spectra. The peak intensities for the silicone hydrogel materials did not differ significantly from one another. The C16:0 peak height for the silicone hydrogel materials was ~700kcounts (equivalent to approximately

3.5x10^{-4}mg/ml, determined from the calibration curve shown in Figure 2.2). The peak intensities for the unworn Soflens 66 lens extract (chromatogram 5) were very low in comparison to all other silicone hydrogel materials analysed. The C16:0 peak height was ~200kcounts (equivalent to approximately 1.1x10^{-4}mg/ml).

The C16:0 peak height for a worn PureVision lens extract (Figure 3.5) is approximately 2.5Mcounts (approximately 8.0x10^{-4}mg/ml), which is considerably higher compared to an unworn PureVision lens extract.

Chromatogram 1 and 2 represent unworn PV and F.N&D lens extracts respectively. Both of the chromatograms for each lens extracts look similar, there are peaks at 10.3mins, 12.8mins and 15.04mins. The C16:0 peak intensities are also similar (approximately 700kcounts for both lens materials). The C18:0 (15.04mins) peak intensities differed, 400kcounts (2.7x10^{-4}mg/ml) for the F.N&D lens extract and 200kcounts (1.4x10^{-4}mg/ml) for the PV lens extract.

Chromatograms 3 and 4 represent unworn Air Optix and Air Optix Aqua lens extracts. Both of the chromatograms showed peaks at 8.1mins, 10.3mins, 12.8mins and 15.04mins. The peak intensities differed between these two lenses. The peak intensities were higher for the Air Optix Aqua lens extract compared to the Air Optix extract.

Chromatogram 5 represents an unworn Soflens 66 lens extract. The chromatogram showed peaks at 12.8mins (peak height of ~200kcounts, equivalent to 1.1x10^{-4}mg/ml) and 15.04mins (peak height of ~100kcounts equivalent to 0.75x10^{-4}mg/ml).

Although the peak intensities differed significantly between the conventional and silicone hydrogel materials tested, there was no difference in the retention times of the extractable components.

The mass spectra for each of the peaks were analysed and compared to the NIST (National Institute of Standards and Technology) database. The peak at 12.8mins corresponded to C16:0, which was identified by its retention time and its mass spectrum. The peak at 15.04mins

corresponded to C18:0 by the retention time and the mass spectrum. The peaks at 8.1mins and 10.3mins were identified as benzoic acid, 4-ethoxyethyl ester and tridecanoic acid methyl ester respectively. These were identified by the use of the NIST database which gave a 97% match to the spectrum for the peak at 8.1mins and a 59% match to the peak at 10.3mins. Both C16:0 and C18:0 peaks are present on gas chromatograms of worn lens extracts and are common tear fatty acids. The analysis of *ex vivo* lens extracts by gas chromatography also shows peaks which corresponded to C16:0 and C18:0. These fatty acids are believed to be from tear lipids as they are present in tear sample analysis.

The peaks observed for unworn lens extracts were thought to be from un-reacted monomers used in the manufacturing process of lenses.

3.5.1.1 GCMS separation of unworn lenses: Summary

- GCMS analysis of unworn lenses showed that there was extractable material from the particular lenses analysed
- The peaks on GC traces for all unworn lenses tested corresponded to C16:0 and C18:0
- C16:0 and C18:0 peaks are observed for worn lens extracts but the peaks are more intense ($3.5x10^{-4}$mg/ml for unworn lenses and $1.0x10^{-3}$mg/ml for worn lenses)
- C16:0 and C18:0 were also observed for tear samples, therefore they correspond to fatty acids from tears not from the lens itself

3.5.2 Normal phase HPLC separation of unworn lens extracts

The same lens materials as those analysed by GCMS (Figure 3.6) were extracted and analysed by HPLC. Whilst GCMS analysis of unworn lenses showed there was extractable material from unworn lenses, the samples had to be transmethylated prior to analysis. Therefore, HPLC was used for unworn lens extract analysis because the extract could be analysed directly without any sample preparation. Also, only volatile species can be separated by GCMS, therefore, HPLC was used for analysis because the entire extract can be separated.

Figure 3.7 to Figure 3.11 represent chromatograms of unworn lens extracts. The same wavelengths used for worn lens analysis were chosen for unworn lens analysis.

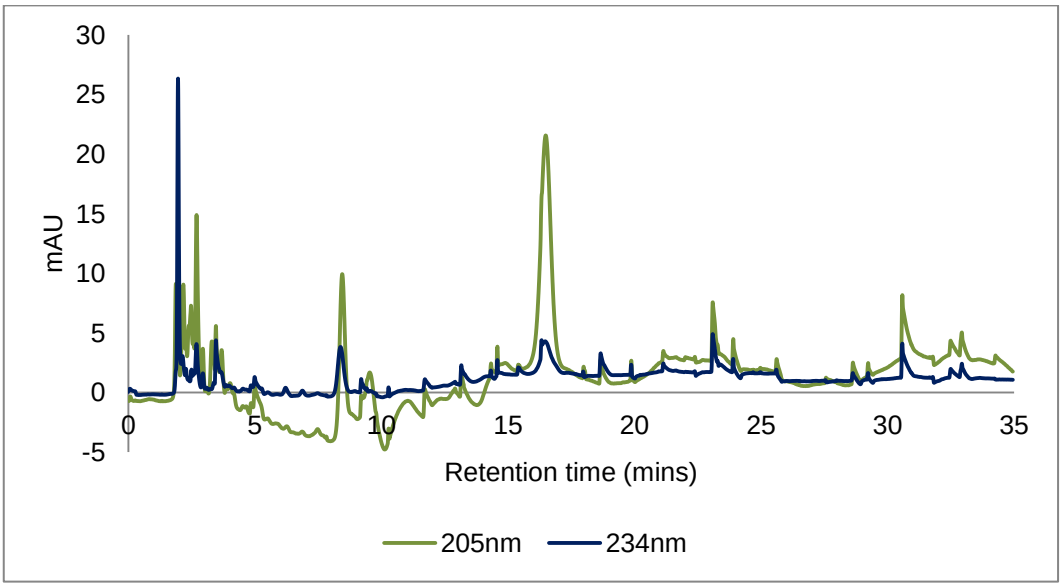


Figure 3.7 – HPLC traces at 205nm and 234nm of an unworn PV lens extract

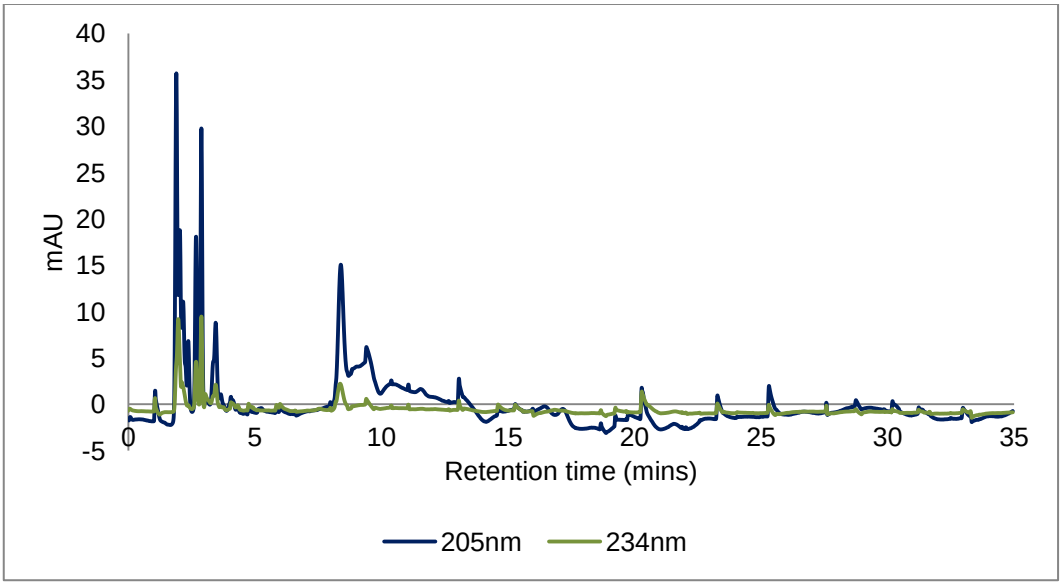


Figure 3.8 – HPLC traces at 205nm and 234nm of an unworn F.N&D lens extract

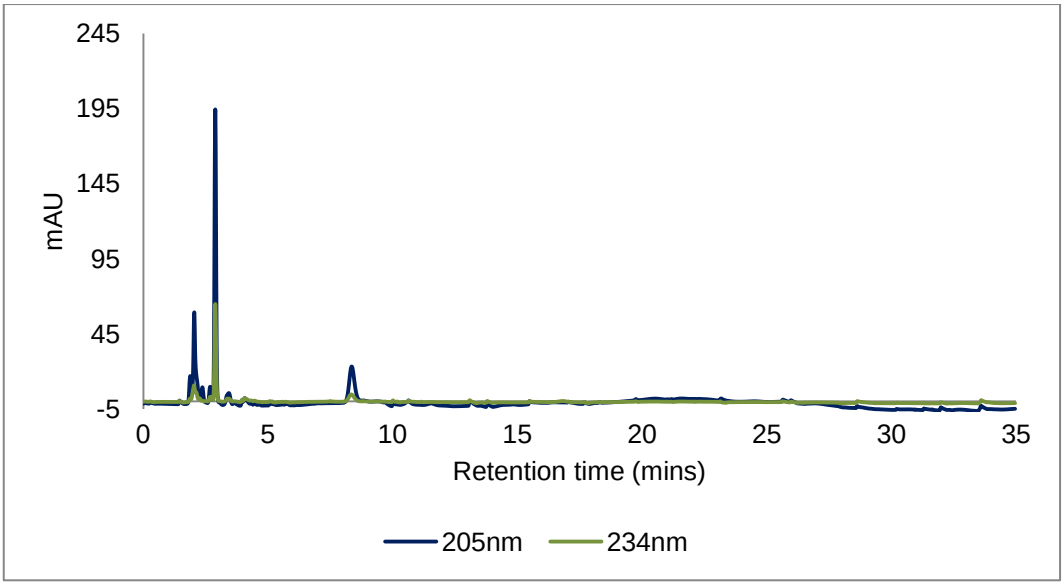


Figure 3.9 – HPLC traces at 205nm and 234nm of an unworn Air Optix lens extract

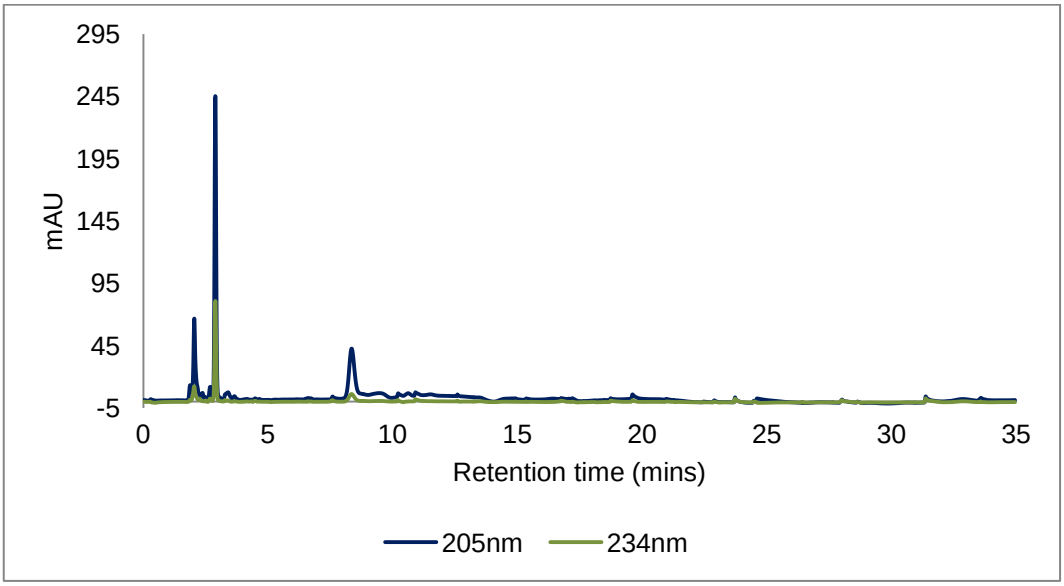


Figure 3.10 – HPLC traces at 205nm and 234nm of an unworn Air Optix Aqua lens extract

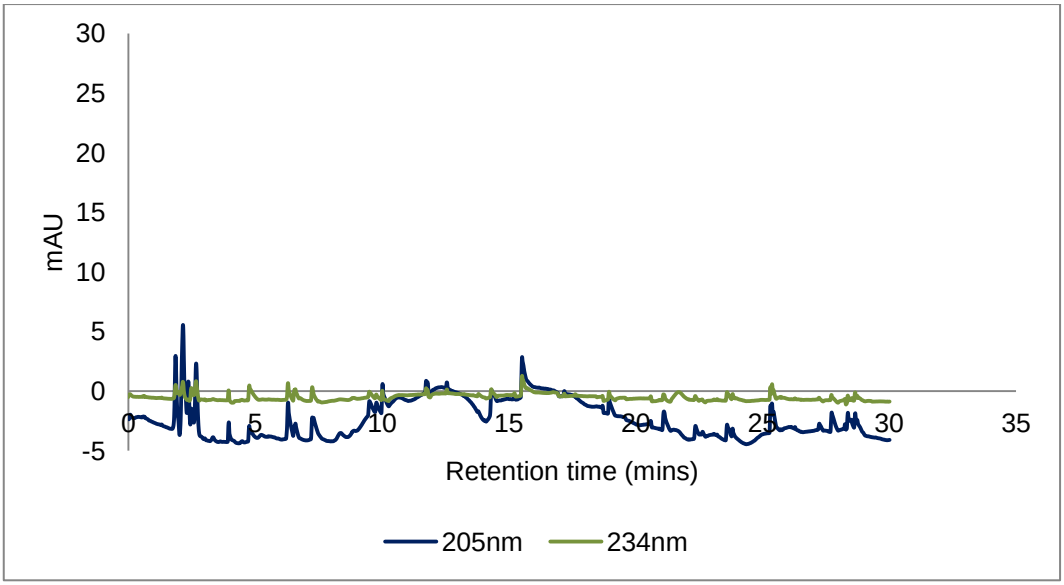


Figure 3.11 – HPLC traces at 205nm and 234nm of an unworn Soflens 66 lens extract

As well as GCMS analysis of unworn lenses, HPLC with UV and fluorescence detection were used to separate and detect the extractable material from unworn lenses. The results are shown in Figure 3.7 to Figure 3.11.

The HPLC traces in Figure 3.7 represent an unworn PV lens extract. A predominant peak at 16.5mins (205nm absorbance) was observed. This peak had an intensity of ~20mAU which is considerably lower than the intensity of the peak observed for *ex vivo* PureVision lens extracts (120mAU for CE peak, Figure 3.4). The peaks were not identified, but the peak at 1.9mins was very hydrophobic as it has a short retention time.

The HPLC trace for AO lens extract (Figure 3.9) at 205nm showed a strong peak at 2.881mins with a peak intensity of 180mAU. The HPLC trace for an AOA lens extract showed a similar trace to the AO extract. There was a strong absorption at 2.881mins of over 200mAU at 205nm (Figure 3.10).

The HPLC trace for an unworn S66 lens extract shown in Figure 3.11 showed very small peaks not more than 20mAU.

The separation of unworn contact lens extracts by HPLC produced many peaks. The retention times of the peaks on HPLC traces of unworn lens

extracts differed to worn lens extracts. The retention times of the peaks for unworn lens extracts did not correspond to lipids. This meant that peaks observed for worn lens extracts corresponded to lipids and not extractable components of the lens itself.

The GC and HPLC traces both showed the most extractable material was from AO and AOA lenses. Both of these lens extracts showed high absorbencies on both the GC and the HPLC traces. The peaks were not identified but they were very hydrophobic as they had a short retention time. When both AO and AOA lenses had been extracted and evaporated to dryness there was a white solid that coated the vial observed for both lens extracts.

Zhao *et al.* (110) used TLC to separate lipids extracted from lenses and also analysed unworn lens extracts. They found that there were separated species observed for unworn lens extracts. However, these bands did not correspond to any lipid standards and were therefore extractable components of the lens itself.

3.5.2.1 Unworn lens extraction: Summary

The HPLC results showed that there was extractable material from the unworn contact lenses that were tested. Tear samples and *ex vivo* contact lens extracts were analysed by HPLC to distinguish between tear lipids and extractable components of the lens.

- The HPLC traces for single unworn lens extracts showed that there are extractable components from unworn lenses
- The HPLC traces for the materials tested differed from one another
- Air Optix and Air Optix aqua lenses had similar traces as they are essentially the same lens material (Figure 3.9 and Figure 3.10)
- There was less extractable material from Soflens 66 lenses compared to all the silicone hydrogel materials analysed (Figure 3.11)
- Although the GC traces for the unworn lens materials analysed had similar profiles to one another (Figure 3.6), the HPLC traces for the same set of materials differed from one another

- GC separation only allowed volatile species to be separated, however HPLC allowed the separation of all extracted species
- The HPLC profile for unworn and worn lenses differed
    - Peaks were more intense for worn lens extracts compared with unworn extracts (for PV lenses, 25mAU for unworn lens extracts compared to 125mAU)
    - The retention times of peaks for unworn lenses were different to the retention times of lipids
- Components from unworn lenses are believed to be extracted by the tear film during wear, however the effect on the tear film is yet unknown

3.6 HPLC and GCMS analysis of tear samples

HPLC and GCMS were both used for lens extract analysis. However, these techniques were very useful for the analysis of tear samples. This would enable the differences in lipid profiles between tear samples and lens extracts to be analysed.

3.6.1 Normal phase separation of tear samples

HPLC was used for the separation of tear samples which were collected using the Visispear sponge method (as described in section 2.3). Tear samples were analysed to establish whether there were any differences in lipid profiles between tear samples and lens extracts.

The HPLC traces of tear samples are shown in Figure 3.12 and Figure 3.13.

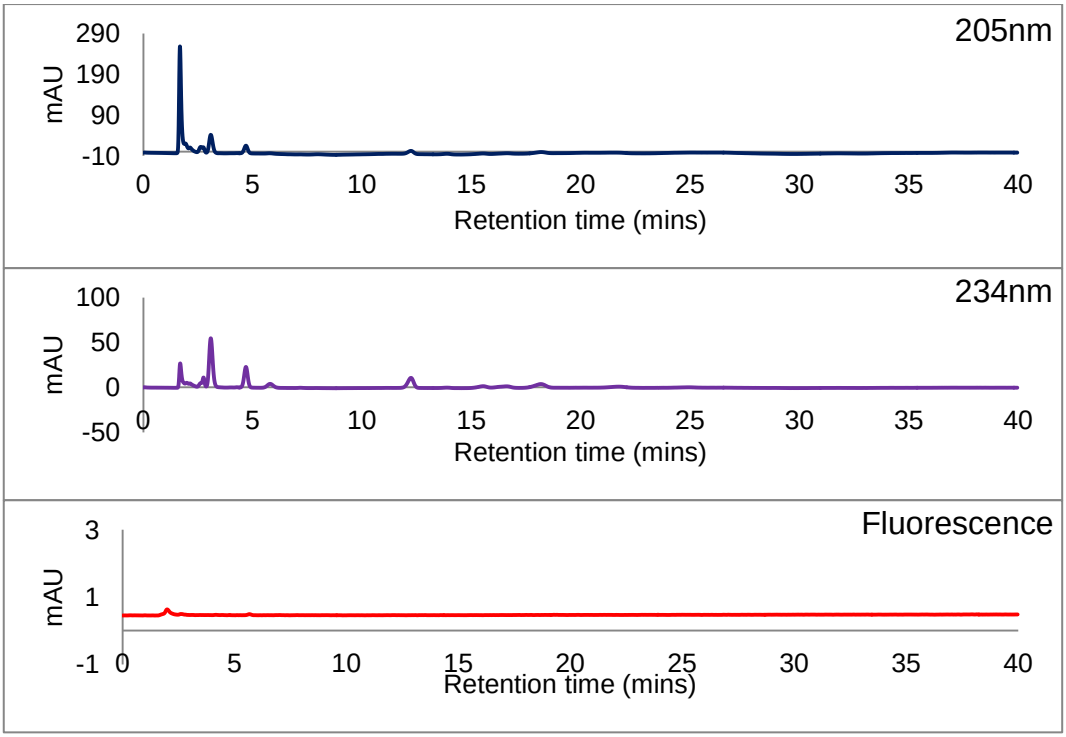


Figure 3.12 – HPLC trace of subject (AM) tear sample taken with a Visispear ophthalmic sponge

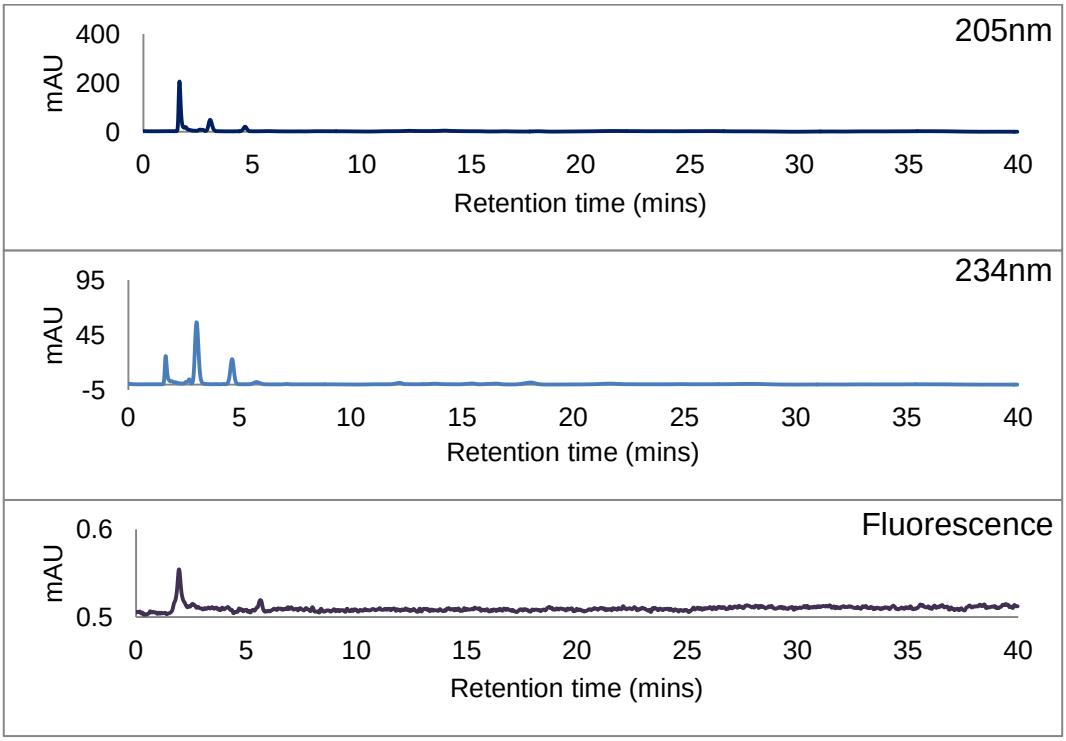


Figure 3.13 – HPLC trace of subject (AM2) tear sample taken with a Visispear ophthalmic sponge

Figure 3.12 and Figure 3.13 represent Visispear tear samples collected from two different subjects. The traces for both subjects tear samples are very similar as they both show predominant peaks at 1.6mins which correspond to CEs. However, the HPLC trace of the worn lens extract (Figure 3.4) differed significantly from the traces of tear sample extracts (Figure 3.12 and Figure 3.13).

The HPLC trace for the worn lens extract showed significantly higher levels of TGs, free fatty acids and cholesterol compared to both HPLC traces for tear samples (Figure 3.12 and Figure 3.13). The major lipid class extracted from tear samples was cholesteryl esters. This clearly demonstrates that lipids build up on the contact lens throughout the wear-time of the lens. The PV lens extract in Figure 3.3, Figure 3.4 and Figure 3.5 was worn on a daily wear basis for a period of 30 days. The lens was cleaned with contact lens solution every night and re-inserted every morning. The results also show that this particular contact lens solution is not effective at removing lipid deposits from the lens. Whilst it is known that lipids deposit on lenses, these HPLC traces clearly show the extent of lipid build-up. Lipids deposited on and in the contact lens are left immobilised within the lens matrix and are susceptible to oxidative attack. This forms the basis for our proposition that tear film lipids are susceptible to degradation and structural change as a result of contact lens wear.

3.6.1.1 Normal phase HPLC separation: Summary

Normal phase HPLC separation of contact lens extracts and tear samples showed:

- lipid class separation of tear samples and single lens extracts
- extent of lipid build-up on contact lenses (Figure 3.4)
- differences in lipid classes from lens extracts compared with tear samples (Figure 3.4 compared with Figure 3.12)
- the Visispear ophthalmic sponge is an effective tear lipid sampling tool
- ratios of non-polar to polar lipids can be determined

- lipids are immobilised within the lens and are susceptible to degradation

Although the HPLC traces for tear samples and lens extracts differed (shown in Figure 3.12 and Figure 3.5 respectively), the peaks observed for worn lens extracts corresponded to known tear lipids and not extractable components of the lens. Certain lipids such as cholesterol, TGs and free fatty acids are known tear lipids but they were not observed for certain subjects tear samples (Figure 3.12 and Figure 3.13), however they were observed for the contact lens extract shown in Figure 3.5. This is because particular lipids will build up on certain lenses. Therefore, lipids that are present in the tear film at low levels will build up on lenses and will be present at higher levels when analysed.

3.6.2 Normal phase HPLC analysis of lipid samples from the lens-wearing eye

A Visispear ophthalmic sponge was gently wiped over the anterior surface of an *in vivo* contact lens and care was taken not to move the lens. The tip of the sponge was cut off, placed in a vial and extracted as described in Chapter 2, section 2.9. The sponge lipid sample and the contact lenses were extracted and analysed by HPLC. This experiment was undertaken to determine whether the Visispear sponge would collect lipids from the anterior superficial lipid layer. By extracting the lens and the Visispear sponge, would indicate the level of lipid build-up on and in this lens.

The chromatograms representing the Visispear sponge sample and lens extracts are shown in Figure 3.14.

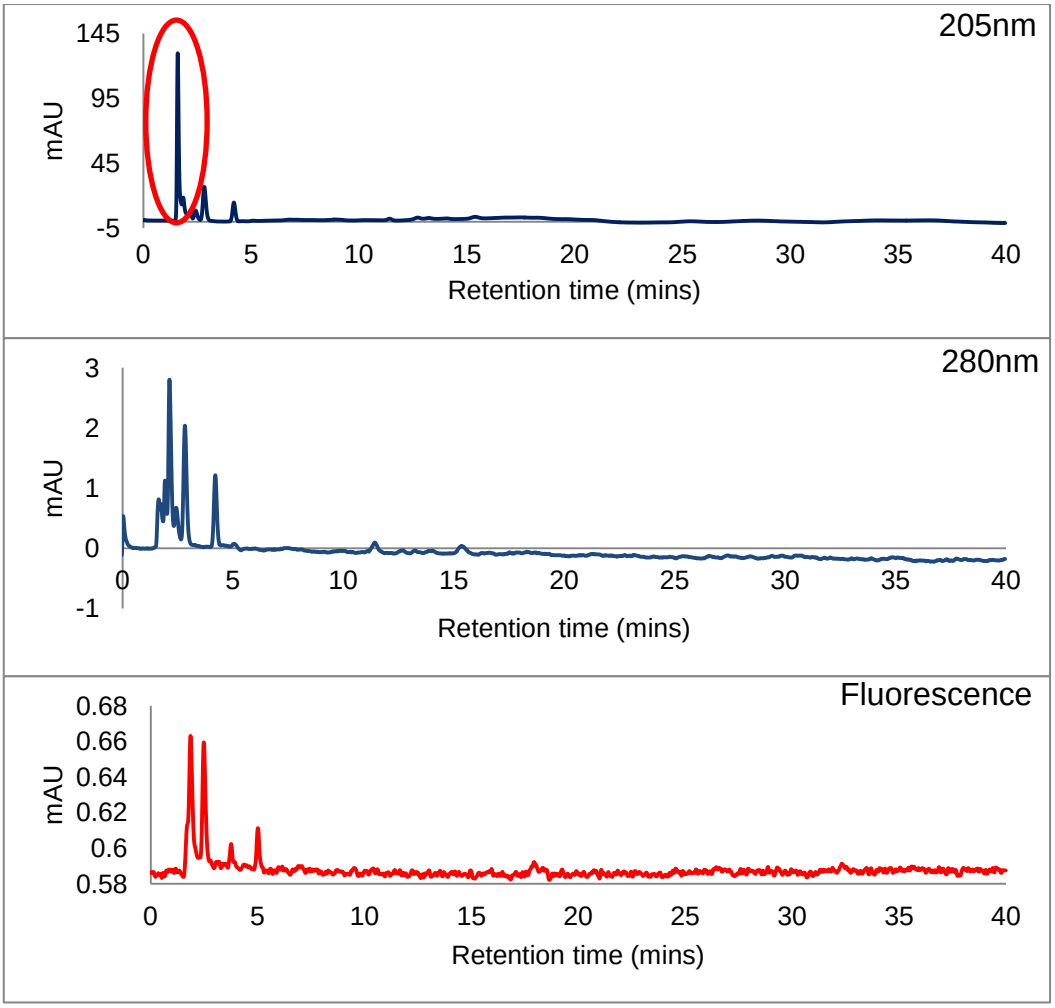


Figure 3.14 – HPLC traces of a lipid sample of the lens-wearing eye. A Visispear sponge was wiped over the anterior surface of two Acuvue Oasys lenses in vivo. Trace 1: UV absorbance at 205nm, Trace 2: UV absorbance at 280nm. Trace 3: Fluorescence absorbance, excitation 360nm and emission 440nm. Peak at 1.5mins = cholesteryl esters

Figure 3.14 shows that the sponge had adsorbed lipids, mainly CEs from the anterior superficial lipid layer. Peaks corresponding to CEs and TGs were observed. The circled peak on Figure 3.14 (trace 1: 205nm) corresponds to cholesteryl esters and its intensity was ~120mAU. This shows that significant levels of CEs were absorbed by the sponge from the superficial lipid layer.

The contact lenses that had been wiped by a Visispear sponge were also extracted and analysed by normal phase HPLC. The chromatograms are shown in Figure 3.15. To ensure the sponge did not have any extractable

components that would interfere, a blank sponge was extracted and analysed by HPLC and no peaks were observed.

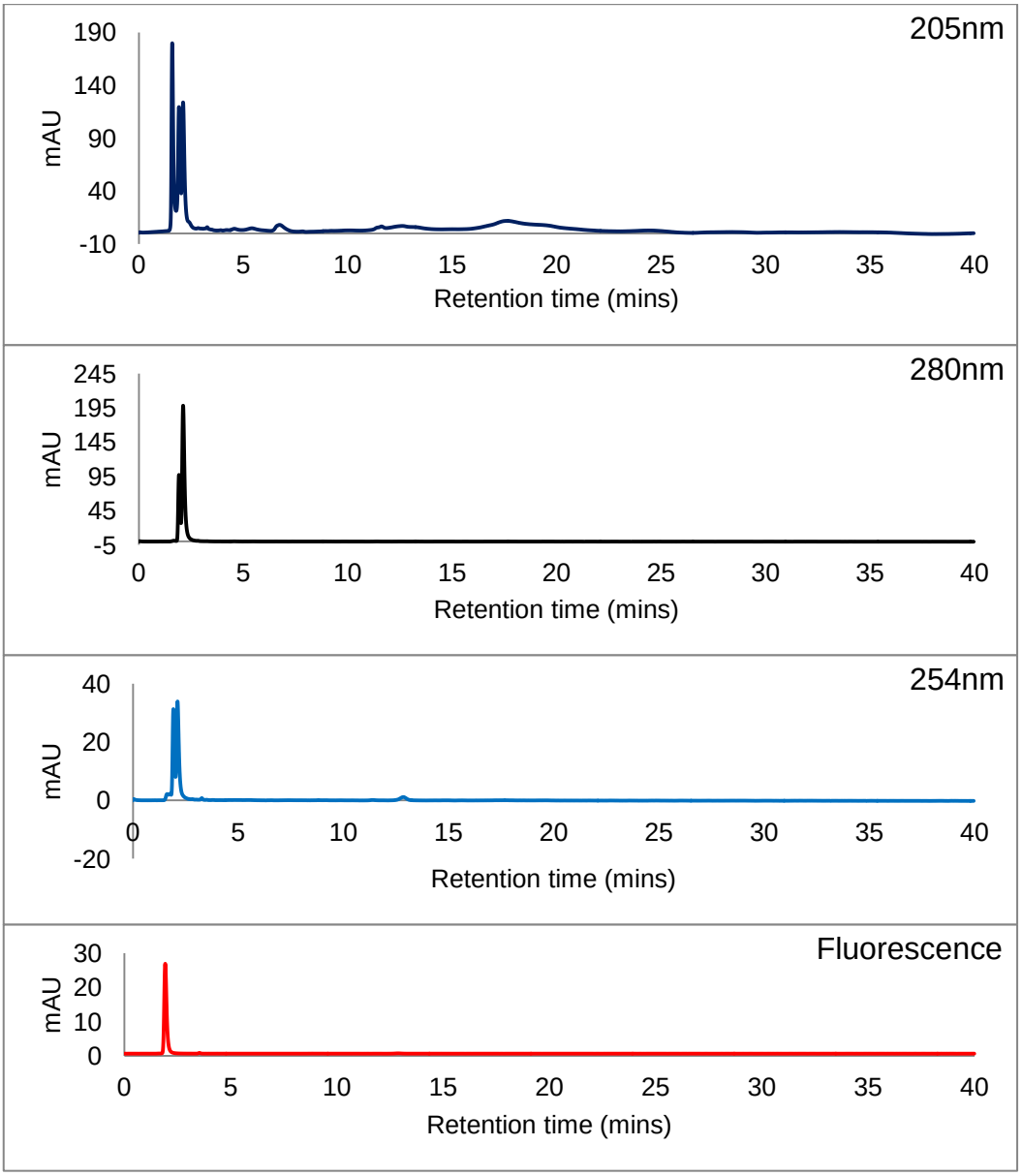


*Figure 3.15 – HPLC traces of ex vivo contact lens extract (Acuvue Oasys **left** lens) after the lens was wiped with ophthalmic sponge. Trace 1: UV absorbance at 205nm, Trace 2: UV absorbance at 280nm, Trace 3: UV absorbance at 254nm, Trace 4: Fluorescence absorbance, excitation 360nm, emission 440nm. Peaks at 1.5mins = cholesteryl esters, peaks at 2.1mins = triglycerides*

Although the sponge absorbed lipids (Figure 3.14), in particular CEs from the anterior superficial lipid layer, there were still significant levels of CEs extracted from the lens (Figure 3.15). This is shown in the chromatograms in Figure 3.15, which represent the lens extracts. The height of the peak that correspond to CEs (~1.6mins) was much more intense for the lens extracts compared to the tear sample.

This suggested that these lipids were not easily removed from the lens surface because they were absorbed into the lens matrix. Although wiping a sponge over the surface of the lens did remove some lipid, there was still a considerable amount still left on and in the lens. It is these non-polar lipids which penetrate into the lens matrix and are difficult to be removed by contact lens cleaning solutions (as discussed in the literature overview – section 1.8.2). Again, it is these immobilised lipids that are susceptible to oxidative attack and have been shown in literature to produce 'jelly bumps' or 'white spots' (140, 141). These highly non-polar lipids also make the surface of the contact lens less wettable, leading to contact lens discomfort.

3.6.2.1 Lipid samples from the lens-wearing eye: Summary

HPLC analysis of tear samples taken from the lens-wearing eye demonstrated that:

- lipids were absorbed by the sponge from the superficial lipid layer from the lens-wearing eye
- high levels of lipids were still extracted from lenses even though they had been wiped by an ophthalmic sponge
- lipids become immobilised in the lens and they are therefore not absorbed by the ophthalmic sponge
- immobilised lipids make the surface of the lens less wettable and are susceptible to oxidative attack, which has links to contact lens discomfort.

3.6.3 GCMS analysis of tear samples

GCMS was also used for the analysis of tear samples taken with Visispear ophthalmic sponges. The GC trace of a sponge tear sample extract is shown in Figure 3.16. A blank Visispear ophthalmic sponge was also extracted to ensure there was no contamination. No peaks were observed on the GC trace for a blank Visispear extract.

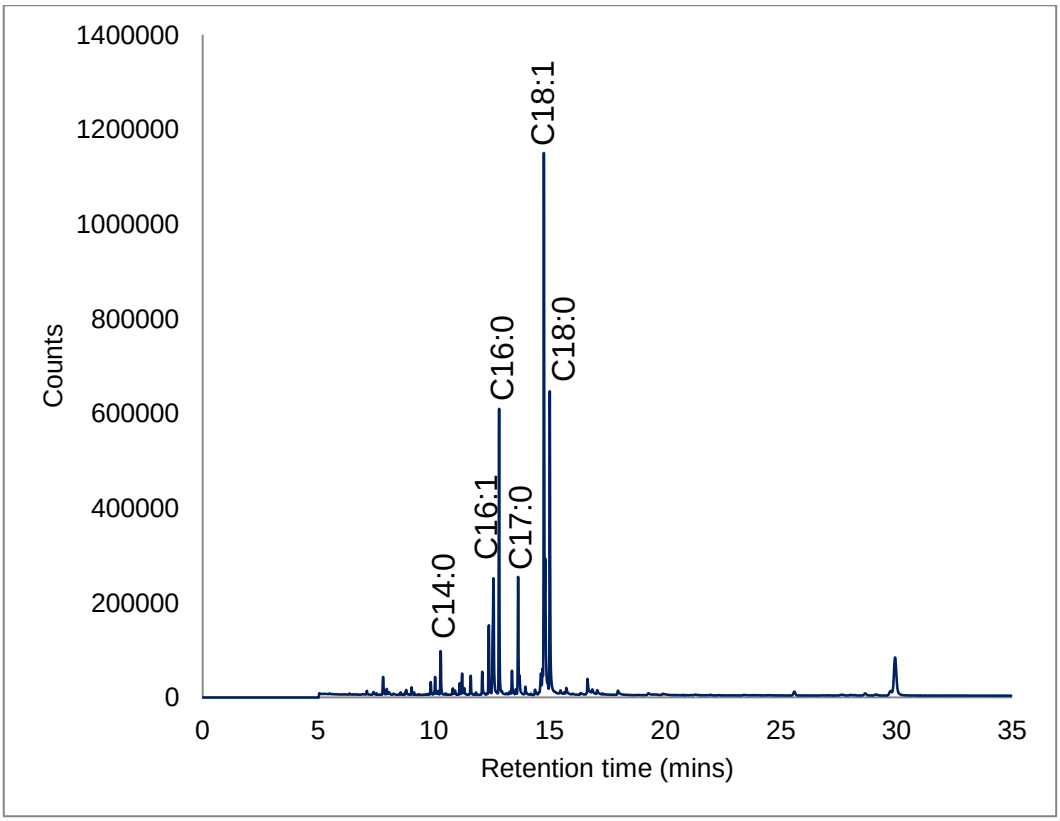


Figure 3.16 – Gas chromatogram of a Visispear ophthalmic sponge tear sample

The GC traces for worn lens extracts (Figure 3.5) and tear samples (Figure 3.16) showed a similar profile, but the intensity of the peaks differed significantly. The peak heights were more intense on the gas chromatogram representing the PureVision lens extract (Figure 3.5) compared to the tear sample trace (Figure 3.16). This again shows that lipids accumulate on, and in the contact lens. It is these immobilised lipids that are susceptible to oxidative attack. Therefore, certain lens extracts were analysed by various oxidative assays to investigate the presence of lipid oxidative products such as MDA (assay details explained in further detail in Chapter 2 and results discussed in Chapter 6).

3.6.3.1 GCMS separation of lens extracts and tear samples: Summary

GCMS analysis of worn lens extracts showed:

- single lenses extracts can be analysed by GCMS and compared to tear samples
- results in Figure 3.5 and Figure 3.16 show lipids build-up on and in the contact lens (peak intensity lower for tear samples compared with lens extracts)
- lipids become immobilised in the lens result in decreased wettability of the lens, which leads to decreased comfort

3.7 Conclusions

Overall, the work discussed in this chapter was important because it demonstrated that a single lens could be extracted and subjected to various chromatographic analyses. Each technique provided different information about lipid structure.

Normal phase HPLC was extremely useful for general lipid class separation and was able to separate individual lipid classes from a single lens extract. It was also useful for the separation of tear lipids. HPLC separation of lens extracts and tear lipids demonstrated that various lipid classes accumulate on and in the lens throughout the wear schedule.

GCMS was able to separate and identify individual fatty acid species from single lens extracts and from tear samples. GCMS has not been previously used for fatty acid analysis from lens extracts. The work in this chapter showed that single lens extracts could be analysed using this technique.

Both GCMS and HPLC have been shown to be very useful techniques for the analysis of tear lipid chemistry. Each of these techniques is used throughout this research to investigate various aspects of lipid structure.

Chapter 4 – Changes in lipid composition during overnight contact lens wear

4.1 Introduction

The aim of the work discussed in this chapter was to investigate the changes in lipid structure during overnight contact lens wear. GCMS was used for the separation of individual fatty acids and HPLC was used for individual lipid class separation.

PureVision (PV) and Focus Night & Day (F.N&D) lenses were harvested from a clinically controlled study. The clinical study involved 43 subjects wearing either PV or F.N&D lenses for 30 days on a daily wear or continuous wear schedule. Those subjects wearing lenses on a daily wear schedule wore the lenses throughout the day and used OptiFree Express for disinfection of the lens. The subjects wearing lenses on a continuous wear schedule wore the lenses for 30 days and nights. The study details are outlined in Appendix 4.

GCMS and HPLC were used to investigate the changes in the structure of lipids deposited on lenses as a function of wear schedule. GCMS was chosen for the analysis of lens extracts because of its ability to separate and identify individual fatty acid profiles for single lens extracts (shown in Chapter 3). The length and unsaturation of the fatty acid moieties for individual lipid classes can dictate the susceptibility to oxidation (Table 1.2). The analysis of single lens extracts was important because individual subject variability in lipid deposition could be monitored. It has been demonstrated in Chapter 3 that a single lens can be extracted, transmethylated and the individual fatty acids separated and identified by GCMS.

During initial method development stages, the analysis of certain lens extracts revealed differences in fatty acid profiles relating to wear schedule. The work presented in this chapter, therefore, elaborates on these initial findings and provides further evidence that there are changes in fatty acid profiles for lenses that are worn for different wear schedules.

HPLC separation was used to investigate whether there were any differences in individual lipid classes between lenses worn for different wear schedules, different lens materials and subject-subject variability.

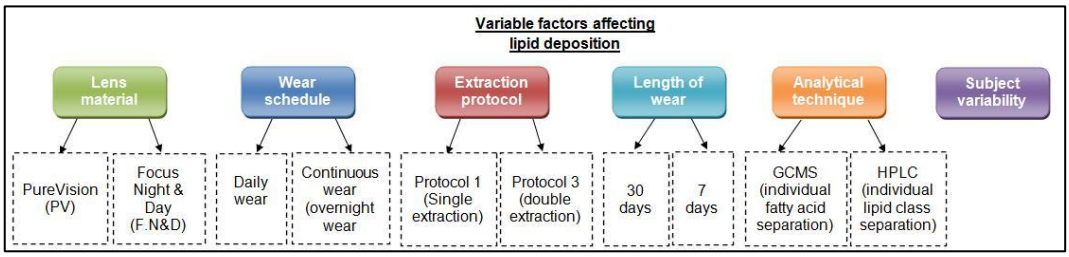


Figure 4.1 – The variable factors discussed throughout Chapter 4

Figure 4.1 shows the many variables that were investigated throughout this chapter such as wear schedule, lens material, extraction protocol and length of wear.

4.1.1 Lens material – PureVision and Focus Night and Day

The two lens materials harvested from a clinically controlled study were PureVision (PV) and Focus Night & Day (F.N&D). They are both approved by the FDA to be worn on a daily wear and continuous (overnight) wear schedule. The properties of both of these lenses are discussed in Chapter 1 (section 1.7.2). Briefly, they are both surface-treated silicone hydrogel materials. PV lenses are known for high levels of lipid deposition because not only are they silicone hydrogels but they also contain the monomer NVP.

4.1.2 Wear schedule – Daily wear or continuous wear

Lenses were worn by subjects on a continuous wear (overnight) or daily wear schedule. Those subjects wearing lenses on a daily wear schedule used OptiFree Express for disinfection of their lenses. The lenses were collected from individuals after wear and stored at 4°C until required for analysis in vials that contained saline.

4.1.3 Length of wear – 7 days or 30 days

The study involved subjects wearing lenses for a 30 days period. However, a smaller study involving two subjects wearing lenses for 7days

was carried out (section 4.2.6). Only two subjects were recruited for this part of the work because it was difficult finding subjects to wear lenses on a continuous wear schedule.

4.1.4 Extraction protocol – single extraction or double extraction

To investigate whether a change in extraction protocol affected the levels or composition of lipids extracted, lenses were extracted using either:

- extraction protocol 1: single extraction in 1.5ml 1:1 chloroform: methanol),
- extraction protocol 2: single extraction in 600µl 1:1 chloroform: methanol)
- extraction protocol 3: double extraction in 1.5ml 1:1 chloroform: methanol)
- extraction protocol 4: double extraction in 600µl 1:1 chloroform: methanol)

4.1.5 Subject-to-subject variability

To investigate subject-to-subject variation in lipid profiles several subjects lenses were analysed.

4.1.6 Analytical technique – GCMS and HPLC

Lens extracts were analysed by GCMS for fatty acid separation and HPLC for general lipid class separation.

4.2 Results and analysis

Representative gas chromatograms of both daily wear and continuous wear PV lens extracts are shown in Figure 4.2. Although many subjects lenses were extracted and analysed, the gas chromatograms were similar for all; however, ratios of saturated to unsaturated lipids differed from subject-to-subject. Therefore, representative gas chromatograms for both daily wear and continuous wear PV and F.N&D lenses are displayed in this chapter (Figure 4.2 and Figure 4.3 respectively) and all other additional gas chromatograms are shown in Appendix 5 and 6. The gas chromatograms shown in Appendix 5 represent additional traces for

continuous wear and daily wear PV lens extracts that had been worn by various subjects. The gas chromatograms shown in Appendix 6 represent additional traces for continuous and daily wear F.N&D lenses extract that had been worn by various subjects.

To ensure complete separation of individual fatty acid species by GC, 35mins elution time was chosen. However the peaks of interest were observed between the retention times of 10mins to 20mins. The retention times for the peaks of interest and corresponding fatty acids are shown in Table 3.2.

4.2.1 Gas chromatograms of daily wear and continuous wear PV lens extracts-30 days wear

Figure 4.2 shows gas chromatograms of continuous wear and daily wear PV lens extracts worn by two subjects. Gas chromatograms of daily wear lens extracts clearly show both saturated and unsaturated lipids whereas continuous wear lens extracts do not. These traces are representative of almost all of the PV lenses analysed, however ratios of unsaturated (for example C18:1) to saturated (for example C18:0) differed from subject-to- subject. Additional gas chromatograms for the all other PV lens extracts are shown in Appendix 5.

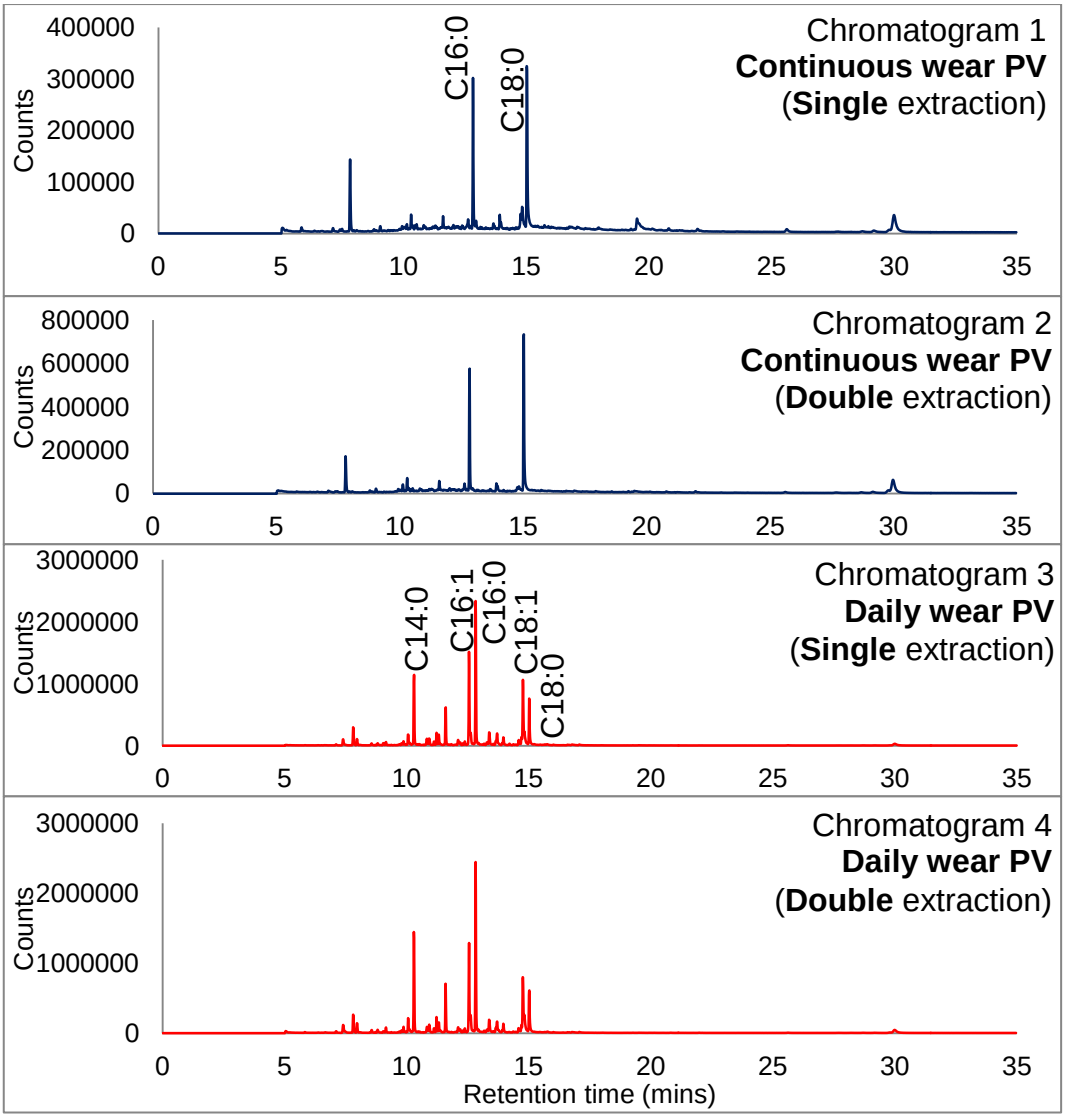


Figure 4.2 – Gas chromatograms of PV lens extracts:

Chromatogram 1 – Continuous wear PV lens extract (extraction protocol 1)
Chromatogram 2 – Continuous wear PV lens extract (extraction protocol 3)
Chromatogram 3 – Daily wear PV lens extract (extraction protocol 1)
Chromatogram 4 – Daily wear PV lens extract (extraction protocol 3)

The gas chromatograms in Figure 4.2 show:

- A single extraction was efficient for lipid extraction as the fatty acid profiles for single vs. double extraction were the same
- No unsaturated fatty acids were observed on GC traces representing continuous wear PV lens extracts (Chromatograms 1 and 2)

4.2.2 Gas chromatograms of daily wear and continuous wear F.N&D lens extracts-30days wear

Figure 4.3 shows gas chromatograms of continuous wear and daily wear F.N&D lens extracts for two subjects. These traces are representative of almost all of the F.N&D lenses analysed. The gas chromatograms for the other F.N&D lenses analysed are shown in Appendix 6.

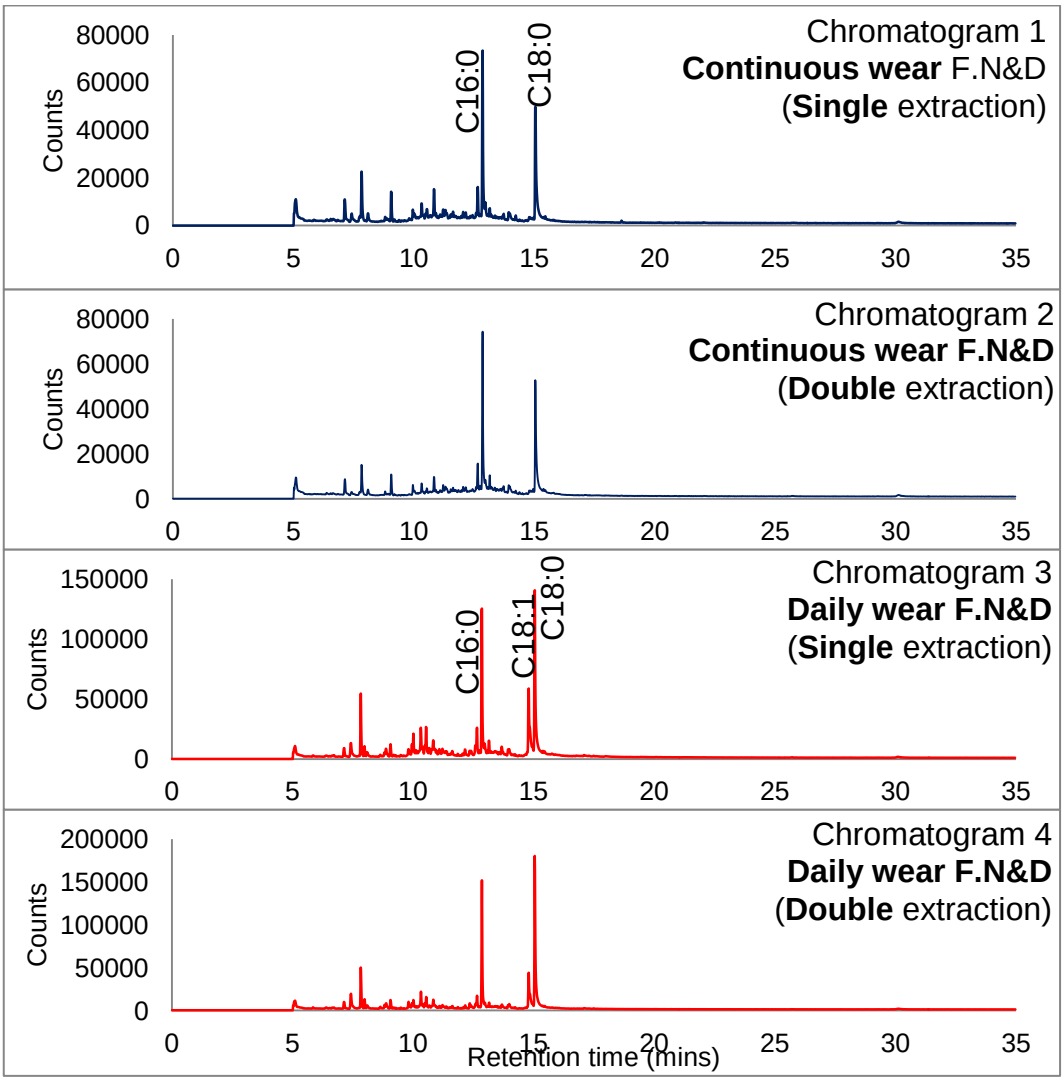


Figure 4.3 – Gas chromatograms of F.N&D lens extracts where:

Chromatogram 1 – Continuous wear F.N&D lens extract (extraction protocol 1)
Chromatogram 2 – Continuous wear F.N&D lens extract (extraction protocol 3)
Chromatogram 3 – Daily wear F.N&D lens extract (extraction protocol 1)
Chromatogram 4 – Daily wear F.N&D lens extract (extraction protocol 3)

The gas chromatograms in Figure 4.3 show:

- A single extraction was efficient for the extraction of lipids from F.N&D lenses as the fatty acid profiles for single and double extraction were the same
- There were no unsaturated lipids observed on the GC traces representing continuous wear lenses (Chromatograms 1 and 2)
- There was less gross lipid, therefore lower levels of C16:1 observed for F.N&D lens extracts compared with PV lens extracts

4.2.3 GC results analysis – lenses worn for 30 days (Figure 4.2 and Figure 4.3)

4.2.3.1 Differences in fatty acid profiles relating to wear schedule

One of the major variables in this chapter was the wear schedule of the lenses (Figure 4.1). As previously stated, lenses were either worn on a continuous wear basis or a daily wear basis. There were obvious differences in fatty acid profiles observed between lenses worn on a daily wear schedule compared to a continuous wear schedule.

The gas chromatograms in Figure 4.2 (PV lens extracts) and Figure 4.3 (F.N&D lens extracts) show clear differences in fatty acid profiles between lenses which were worn on daily wear schedule compared to those worn on a continuous wear schedule.

In general, the chromatograms representing lens extracts worn on a continuous wear schedule (Figure 4.2 chromatograms 1 and 2; Figure 4.3 chromatograms 1 and 2) showed no detectable levels of unsaturated fatty acids (no C16:1 and C18:1). However, those lenses worn on a daily wear schedule (Figure 4.2 chromatograms 3 and 4; Figure 4.3 chromatograms 3 and 4) showed both unsaturated and saturated fatty acids. The differences in fatty acid profiles relating to wear schedule were also observed for the majority of lenses analysed (additional chromatograms shown in Appendix 5 and Appendix 6).

4.2.3.2 Differences in fatty acid profiles related to contact lens material

The major difference observed between PV and F.N&D GC traces were the levels of lipid deposition. The intensities of the peaks for PV lens extracts were higher than F.N&D lens extracts.

Table 4.1 – Comparison in C16:0 concentration between ex vivo PV and F.N&D lenses

Lens material	*Peak identity*	*Peak intensity*	*Concentration of fatty acid*
PureVision (PV)	C16:0 (12.8mins)	2.5MCounts	$1.0x10^{-3}$mg/ml
Focus Night & Day (F.N&D)	C16:0 (12.8mins)	150kCounts	$0.9x10^{-4}$mg/ml

The peak height for the C16:0 (12.8mins) in Figure 4.2, chromatogram 3 (daily wear PV lens extract) is approximately 2.5MCounts, equivalent to $1.0x10^{-3}$mg/ml. However, the peak height for the C16:0 (12.8mins) in Figure 4.3, chromatogram 3 (daily wear F.N&D) is 150kCounts equivalent to $0.9x10^{-4}$mg/ml. Because of the difference in overall lipid deposition between PV and F.N&D lenses, the levels of individual lipids was also lower. The C16:1 peak (usually observed at 12.6mins) was not observed for daily wear F.N&D.

4.2.3.3 Extraction procedure variable

Figure 4.2, chromatograms 1 and 2 represent continuous wear PV lens extracts for one subject. One lens was extracted using extraction protocol 1 (single 1:1 chloroform: methanol extraction) and the other lens was extracted using extraction protocol 3 (double 1:1 chloroform: methanol extraction). To ensure the majority of lipids were extracted, a double extraction was used. Both chromatograms 1 and 2 showed two predominant peaks at 12.8mins and 15.0mins. They were identified as C16:0 and C18:0 respectively by their retention time and mass spectra.

Figure 4.2, chromatograms 3 and 4 represent daily wear PV lens extracts for one subject. Again, one lens was extracted using extraction protocol 1 (single extraction) and the other was extracted using extraction protocol 3

(double extraction). Both chromatograms looked identical, which again proved that a single extraction was effective for the removal of the majority of the lipid. Both chromatograms 3 and 4 showed peaks at 10mins, 11.5mins, 12.5, 12.8mins, 14.8mins and 15.0mins. These peaks were identified as C14:0 (at 10.33mins), C16:1 (at 12.5mins), C16:0 (at 12.8mins), C18:1 (at 14.8mins) and C18:0 (at 15.0mins) from their retention times and their mass spectra (shown in Chapter 3, section 2.10.7).

There was no difference in fatty acid profile relating to extraction protocol for the both the PV or the F.N&D lenses analysed (Figure 4.3 and Appendix 6).

4.2.4 Daily wear and continuous wear PV and F.N&D, 30days wear: Summary

The fatty acid profiles for continuous wear and daily wear lens extracts were very different from each other (as seen in Figure 4.2 and Figure 4.3). This was regardless of contact lens material or extraction protocol used. PV and F.N&D lenses have very different properties. The two lenses differ in equilibrium water content (EWC); PV lenses have an EWC of 35% and F.N&D of 24%. They are both silicone hydrogel lenses that have been surface treated in two different ways. Both lenses have different surface properties to one another because of the method of surface treatment. PV lenses are known for higher levels of lipid deposition compared with F.N&D lenses. Although PV and F.N&D lenses have different characteristics, the variations in fatty acid profile between continuous wear and daily wear lens extracts were still observed. There were no unsaturated lipids observed on the gas chromatograms representing lenses worn on a continuous wear schedule.

From the gas chromatogram shown in Figure 4.2 and Figure 4.3, it was concluded that the lenses worn on a continuous wear schedule had little to no unsaturated lipids on the lens compared to the lenses worn on a daily wear schedule. Further analysis of other subjects lens extracts was carried out in order to prove that the differences in fatty acid profiles

between daily wear and continuous wear lipid deposits were as result of wear schedule and not material or extraction procedure. Even with a double extraction, there were no detectable levels of unsaturated lipid observed.

The gas chromatograms representing PV and F.N&D lens extracts from various subjects are shown in Appendix 5 and Appendix 6. For the majority of lens extracts analysed, unsaturated lipids were not present on lenses that were worn on a continuous wear basis. The lenses worn on a continuous wear schedule had been worn overnight. The composition of tear components in the overnight tear film is very much different in the open eye. However, this has never previously been studied because it is difficult to obtain a 'true' overnight tear sample. The work discussed in this chapter used the contact lens as a tear sampling tool and it provided a snapshot of the overnight tear film.

4.2.4.1 PV and F.N&D continuous and daily wear results: Key observations

The main observations from GC analysis of 30 days continuous and daily wear PV and F.N&D lens extracts (Figure 4.2 and Figure 4.3) are listed below:

- The most significant finding was that there were no unsaturated lipids observed on the GC traces of continuous wear lens extracts
- the differences in fatty acid profile relating to wear schedule was seen for both lens materials even though they have such different properties
- the difference in fatty acid profile relating to wear schedule was observed even when the lens was extracted for a second time (i.e. using extraction protocol 3 – double extraction)
- there was less gross lipid extracted from F.N&D lenses which resulted in less C16:1 extracted from daily wear lenses
- a single extraction was efficient for the extraction of lipids from daily wear and continuous wear PV and F.N&D lenses

All of the evidence collected for lenses worn for 30 days shows that unsaturated lipids degrade during overnight wear of lenses. However, these lenses were worn for 30 days and nights. Would this degradation occur on lenses worn for a shorter time period? To try to answer this question, subject were recruited and they wore PV and F.N&D lenses for 7 days, again either on a daily wear schedule or a continuous wear schedule. The results are discussed in the next section.

4.2.5 Gas chromatograms of PV lens and F.N&D lens extracts worn for 7 days

To establish whether degradation of unsaturated lipids occurs after a shorter wear period than 30 days, subjects wore lenses for 7days. Again PV and F.N&D lenses were worn on both a daily and continuous wear schedule. However, only two subjects were recruited for this study and Subject 1 was only able to wear the PV lens material.

The gas chromatograms in Figure 4.4 to Figure 4.6 represent both PV and F.N&D lens extracts worn for 7 days on a continuous and daily wear basis. All of the lenses collected were extracted using extraction protocol 1 (single extraction) as previous work had established it was an effective extraction procedure. Due to the small number of subjects recruited to wear lenses for 7days, lenses were analysed by only GCMS and not HPLC.

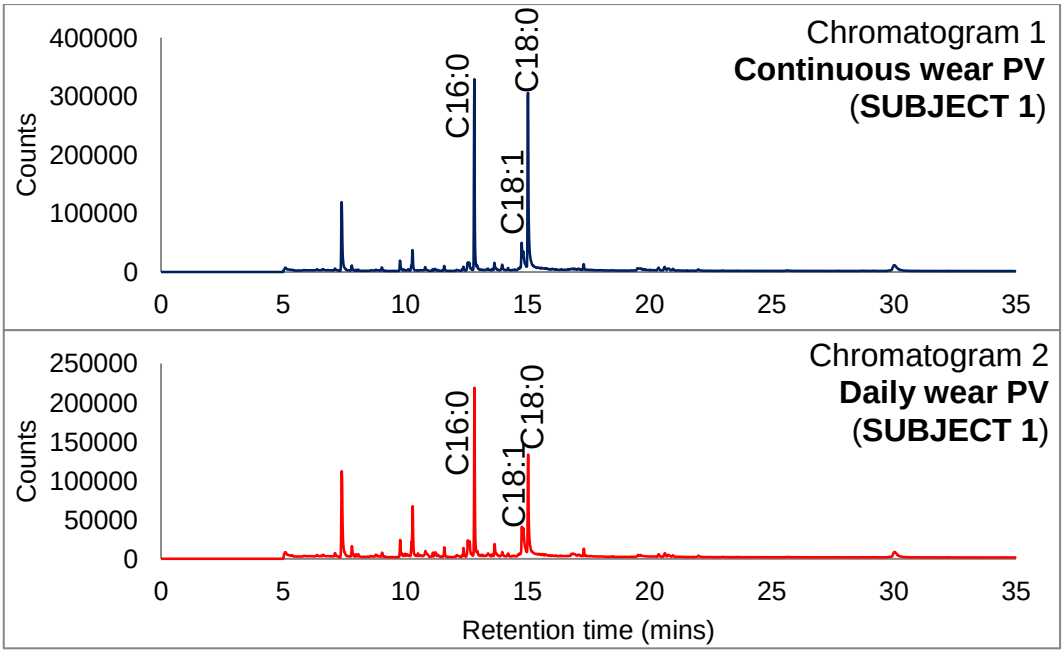


*Figure 4.4 – Gas chromatograms of 7 days wear **PV** lens extracts (**SUBJECT 1**) where: Chromatogram 1 – Continuous wear PV lens extract (extraction protocol 1) and Chromatogram 2 – Daily wear PV lens extract (extraction protocol 1)*

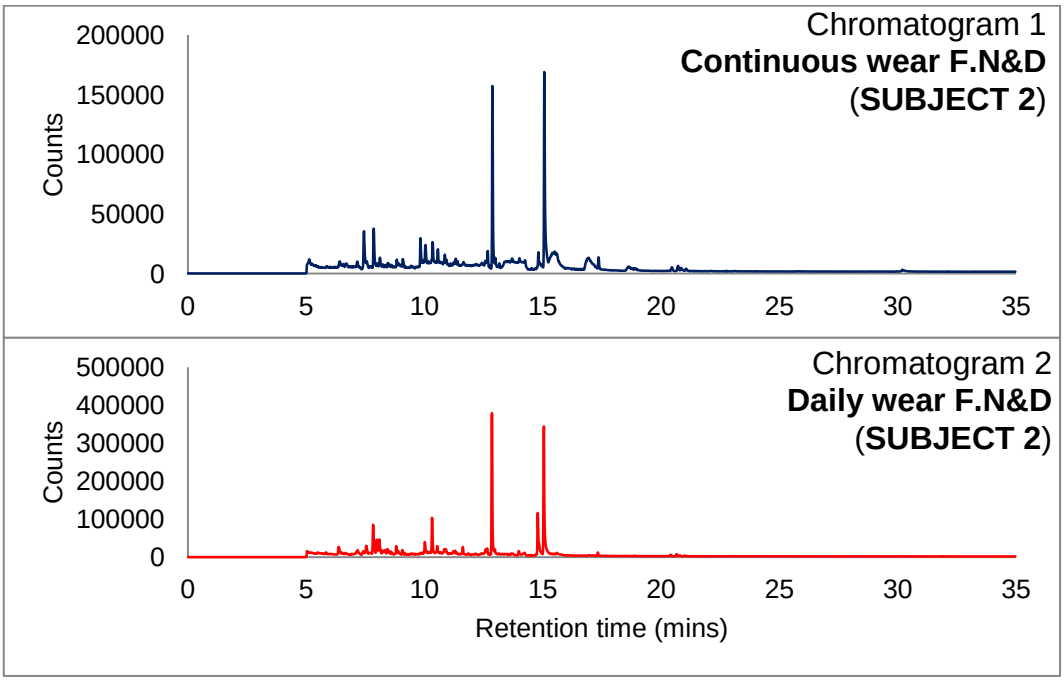


*Figure 4.5 – Gas chromatograms of 7 day wear **F.N&D** lens extracts (**SUBJECT 2**) where: Chromatogram 1 – Continuous wear F.N&D lens extract and Chromatogram 2 – Daily wear F.N&D lens extract*

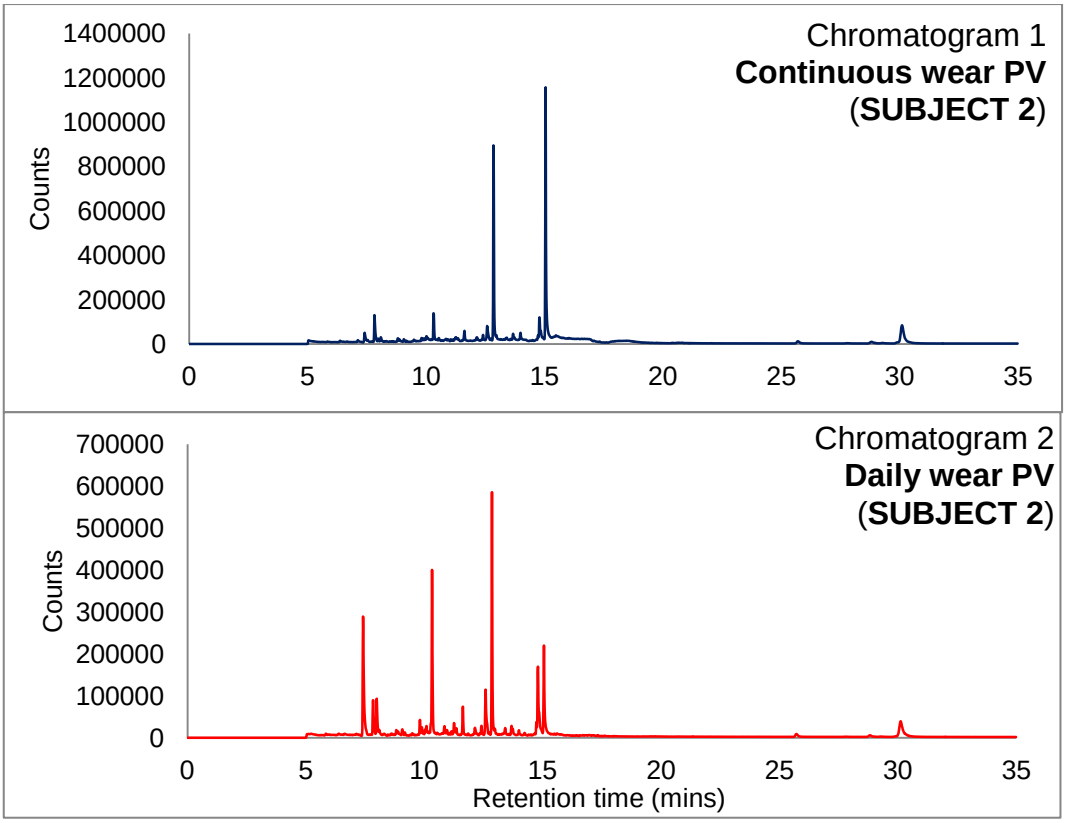


*Figure 4.6 – Gas chromatograms of 7 day wear **PV** lens extracts (**SUBJECT 2**) where: Chromatogram 1 – Continuous wear PV lens extract (extraction protocol 1) and Chromatogram 2 – Daily wear PV lens extract (extraction protocol 1)*

4.2.6 GC results analysis – lenses worn for 7 days

The gas chromatograms representing Subject 1, daily wear and continuous wear PV lens extracts are shown in Figure 4.4. There were low levels of unsaturated fatty acids (18:1 in particular) extracted from Subject 1 continuous wear PV lenses. The intensity of the C18:1 is 60kCounts which is equivalent to 5.71×10^{-5}mg/ml (for continuous wear PV extract). The concentration of the C18:1 peak for Subject 1, daily wear PV lens extract (Figure 4.4, chromatogram 2) was determined to be 5.75×10^{-5}mg/ml. Therefore, a similar concentration of C18:1 was extracted from both the continuous and daily wear lenses for Subject 1 (shown in Table 4.2).

*Table 4.2 – Analysis of **subject 1**, PV, daily wear vs. continuous wear GC traces (shown in Figure 4.4)*

Wear schedule	*Peak identity*	*Peak intensity*	*Concentration of fatty acid*
Continuous wear	C18:1 (15.0mins)	~60kCounts	5.71×10^{-5}mg/ml
Daily wear	C18:1 (15.0mins)	~60kCounts	5.75×10^{-5}mg/ml

However, the gas chromatograms for Subject 2 continuous wear PV lens extract showed no detectable levels of unsaturated lipid (Figure 4.6, chromatogram 1). However, the concentration of C18:1 extracted from Subject 2, daily wear PV lens extract (Figure 4.6, chromatogram 2) was approximately 1.89×10^{-4}mg/ml. The amount of C18:1 extracted from Subject 2 daily wear PV lens was higher than the amount extracted from Subject 1 daily wear PV lens. This showed subject-to-subject variability in the levels of lipid deposition.

The gas chromatograms for Subject 2 continuous wear and daily wear F.N&D lens extracts are shown in Figure 4.5. The gas chromatogram of Subject 2 continuous wear F.N&D lens extract did not show peaks corresponding to unsaturated fatty acids (i.e. no peaks corresponding to C18:1 and C16:1). However, the concentration of C18:1 extracted from the daily wear F.N&D lens (Figure 4.5, chromatogram 2) was determined to be 1.06×10^{-4}mg/ml (shown in Table 4.3).

*Table 4.3 – Analysis of **subject 2**, PV and F.N&D, daily wear vs. continuous wear GC traces (shown in Figure 4.5 and Figure 4.6)*

Wear schedule	*Lens material*	*Peak identity*	*Peak intensity*	*Concentration of fatty acid*
Continuous wear	PureVision (PV)	C18:1 (15.0mins)	Below lower limit of detection	Below lower limit of detection
Daily wear	PureVision (PV)	C18:1 (15.0mins)	~200kCounts	$1.89x10^{-4}$mg/ml
Continuous wear	Focus Night & Day (F.N&D)	C18:1 (15.0mins)	Below lower limit of detection	Below lower limit of detection
Daily wear	Focus Night & Day (F.N&D)	C18:1 (15.0mins)	~130kCounts	$1.06x10^{-4}$mg/ml

4.2.6.1 Summary

- the results in Figure 4.4 to Figure 4.6 suggested that degradation of unsaturated fatty acids was subject-dependent in this first week of wear
- the unsaturated fatty acids had fully degraded on one subjects continuous wear lens extracts (Figure 4.5), but not for the other subject
- degradation had occurred for the majority of 30 days wear lens extracts analysed (see Figure 4.2 and Figure 4.3). Degradation during the first week of wear seemed subject-dependent
- ratios of lipids classes differ from subject-to-subject; therefore rate of lipid deposition on lenses will also vary
- the extent of degradation after 7 days continuous wear will therefore depend on the rate of lipid deposition, this is ultimately subject dependant

Overall, GCMS proved to be a very useful technique for the analysis of changes in fatty acid profiles from contact lens extracts. It clearly showed changes in fatty acid profiles from those lenses worn on different wear schedules. GCMS was very useful as it provided information about the fatty acid moiety.

4.3 Normal phase HPLC results: daily and continuous wear PureVision and Focus Night and Day lens extracts

HPLC was used to provide information about the lipid classes. GCMS analysis of daily wear and continuous wear lens extracts had shown significant differences in fatty acid profiles relating to wear schedule. Therefore, normal phase separation was used to investigate whether there were any differences observed in lipid class profiles between daily wear and continuous wear lens extracts. To examine whether a second extraction would remove more lipid, extraction protocol 3 (double extraction in 1:1 chloroform: methanol 1.5ml) was used for certain lenses.

The HPLC column, mobile phase and flow rate details were described in section 2.10.4. In brief; a normal phase column was used, the mobile phase consisted of hexane: isopropanol: acetic acid (1000: 5: 1 v/v) and the flow rate was set at 1ml/min. The UV wavelengths selected were at 205nm, 254nm, 280nm and 360nm. An excitation wavelength of 360nm and emission wavelength of 440nm were set on the fluorescence detector.

4.3.1 Normal phase HPLC separation results: continuous wear and daily wear PureVision lens extracts (extraction protocol 1)

The HPLC traces, shown in Figure 4.7 and Figure 4.8 represent continuous wear and daily wear PV lens extracts respectively. These lenses were extracted using extraction protocol 1 (1.5ml 1:1 chloroform: methanol for at least 30mins).

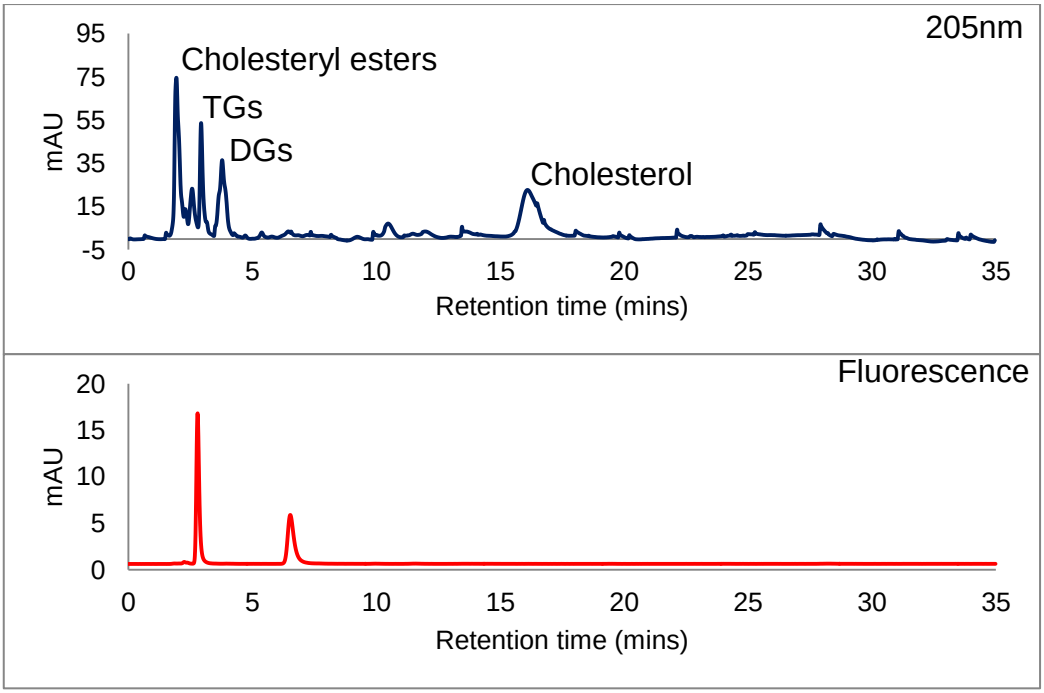


Figure 4.7 – HPLC trace of a daily wear PV lens extract (Subject 61)

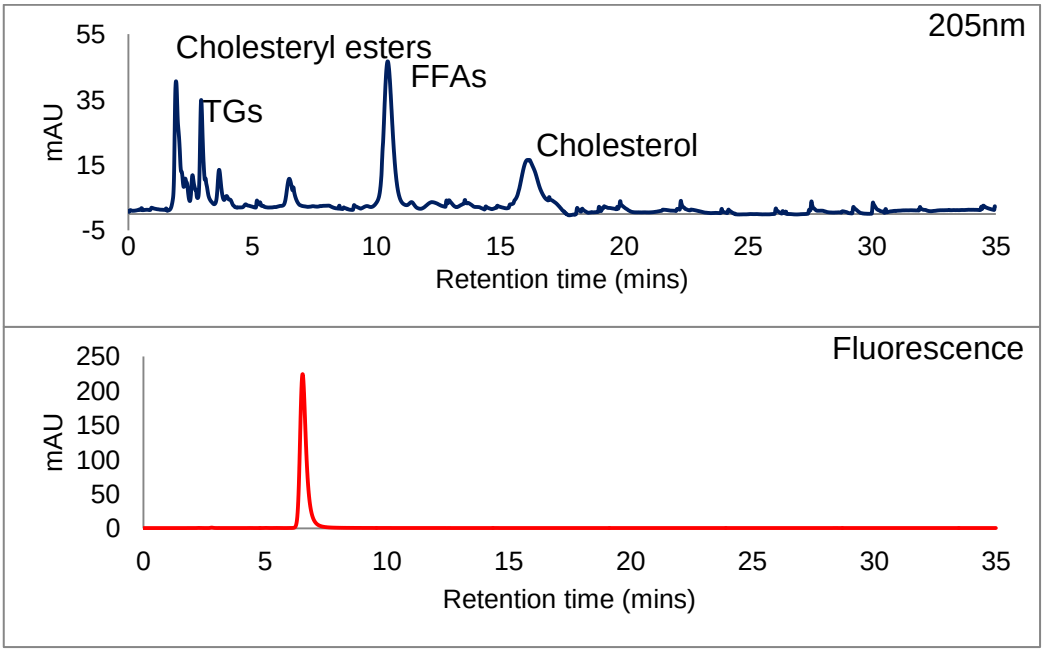


Figure 4.8 – HPLC trace of a continuous wear PV lens extract (Subject 56)

The HPLC trace in Figure 4.7 represents a daily wear PV lens extract. The peaks observed for this particular subject's daily wear lens extract correspond to CEs, TGs, DGs, FFAs and cholesterol.

The HPLC traces in and Figure 4.8 represent a continuous wear PV lens extract. The peaks observed for this particular subject's continuous wear PV lens extract correspond to CEs, TGs, DGs, FFAs and cholesterol. Particular lipids classes such as cholesterol and cholesteryl esters have natural fluorescence, therefore fluorescence spectra are shown. The fluorescence spectra for the continuous wear lens extract (Figure 4.8) shows strong peak at 6.5mins. The retention time of this peak did not correspond to retention time of cholesteryl esters or cholesterol. Also due to the intensity of the peak, it suppressed all other peaks. This peak was not observed for all lens extracts analysed by HPLC, but it was observed for certain subjects lens extracts. It was therefore concluded that this peak corresponded to an unknown extractable component from particular subject's lenses. This peak did not correspond to extractable material from the lens as unworn PV and F.N&D lens extracts did not produce a fluorescence signal.

There were clear observable differences in lipid profile between the two lens extracts analysed in Figure 4.7 and Figure 4.8. The differences did not correspond to differences in wear schedule. The peaks representing the major lipid classes (CE, TGs, DGs, FFAs, cholesterol) were all present, although the ratios of each differed which is due to subject-to-subject variability in lipid deposition.

HPLC was used for the analysis of various subjects PV lens extracts and the traces are shown in Appendix 7. It can be seen from the HPLC traces displayed in Appendix 7 that there are no clear differences in lipid class profiles for lens extracts relating to wear schedule (daily wear vs. continuous wear). The majority of the lenses analysed showed peaks that corresponded to the major tear lipid classes such as cholesteryl esters, TGs, FFAs and cholesterol. However, the ratios of lipid classes differed from subject-to-subject.

The HPLC traces shown in Appendix 7 were obtained using the same column and column conditions as the results shown in Figure 4.7 to Figure 4.12 . However, the column was removed and the same column replaced.

The HPLC traces in Appendix 7 were all obtained after the column had been replaced. Fresh lipid standards were used to re-calibrate the column to ensure all peaks were correctly identified. The peak that corresponds to cholesterol eluted at approximately 16-18mins in Figure 4.7 to Figure 4.12 (before the column change), but eluted at 7-9mins in the HPLC traces shown in Appendix 7 (after the column change). Again, there were no significant differences in lipid profiles for relating to a change in wear schedule, although there were subject-to-subject differences.

4.3.1.1 Figure 4.7 and Figure 4.8: Summary

- There were no observable differences in lipid profiles relating to wear schedule (daily wear vs. continuous wear) for the PV lens extracts
- However, the differences in the ratios of lipid profiles was subject-dependent
- The peak at 6.5mins (Figure 4.8, fluorescence signal) did not correspond to lipids or extractable material from the lens, it was subject-dependent.

4.3.2 Normal phase HPLC separation results: continuous wear and daily wear extracts (extraction protocol 4)

Extraction protocol 4, 1:1 chloroform: methanol (600µl) was used to investigate whether extracting in a smaller volume of solvent would improve the extractability of lipids from lenses. The vials were also vortexed to try and improve the extraction of lipids which were absorbed within the lens matrix. The HPLC traces shown in Figure 4.9 to Figure 4.12 represent PV lens extracts which were extracted using extraction protocol 4. Extraction protocol 4 was either a single or double extraction in 1:1 chloroform: methanol (600µl), as outlined in sections 2.6.2 and 2.6.3. The first and second extracts were analysed separately to investigate whether: a) lipids were extracted by a second extraction and b) whether there was a difference in lipid classes extracted by a first extraction compared to a second extraction.

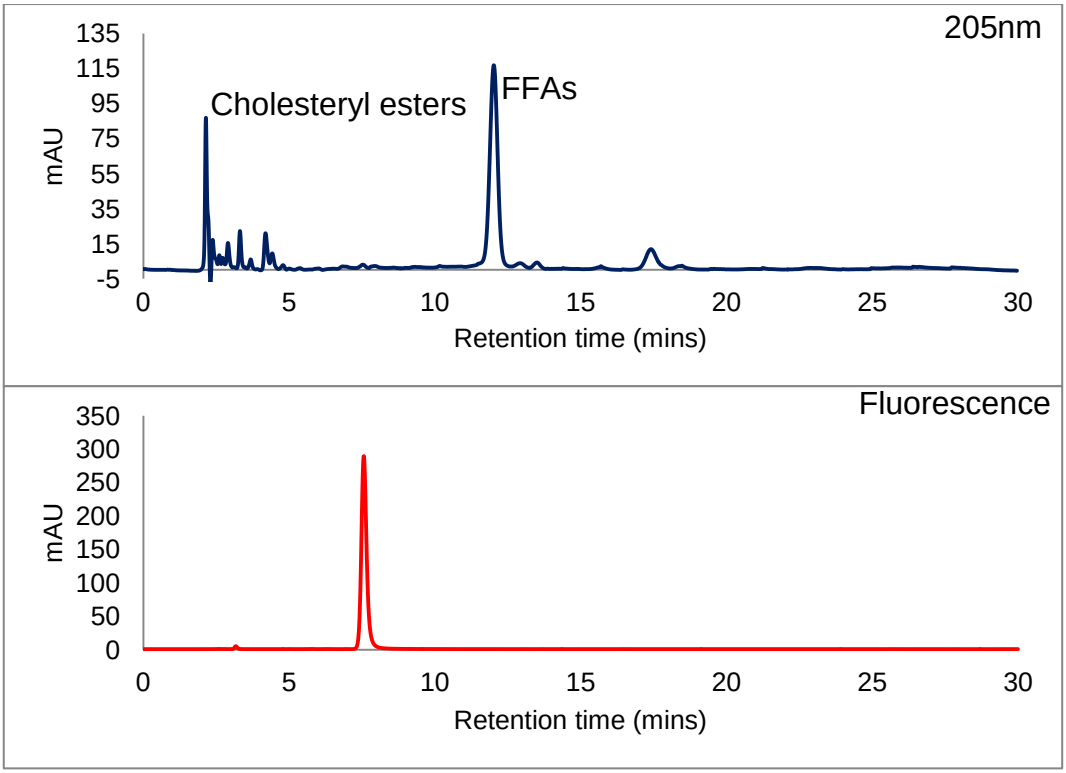


*Figure 4.9 – HPLC trace of a continuous wear PV lens extract (extraction protocol 4, **1st extract**) (subject 24)*

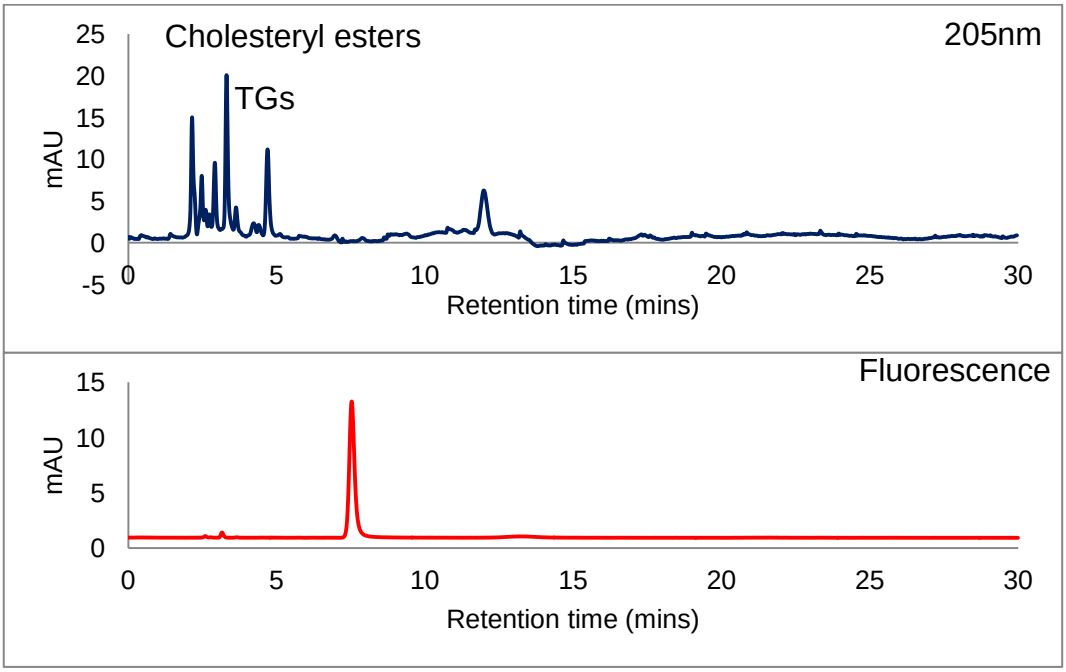


*Figure 4.10 – HPLC trace of a continuous wear PV lens extract (extraction protocol 4, **2nd extract**) (subject 24)*

4.3.2.1 Analysis of HPLC traces in Figure 4.9 and Figure 4.10 (continuous wear PV, single vs. double extraction)

The HPLC traces shown in Figure 4.9 represent the first extraction of a continuous wear PV lens. The peaks corresponded to CEs, FFAs and free cholesterol (at 2.1, 12.01 and 17.0mins respectively). The peak which corresponds to FFAs (at 12.01mins) is the predominant peak, with an intensity of ~100mAU which is equivalent to approximately $3.67x10^{-3}moldm^{-3}$. The predominant peak seen for most lens extracts corresponds to cholesteryl esters. However, for this particular subject, the peak corresponding to FFAs was the most predominant. This again demonstrates that the ratios of individual lipid classes vary from subject-to-subject.

The traces shown in Figure 4.10 represent the second extraction of the same continuous wear PV lens (as in Figure 4.9). The trace at 205nm shows peaks that correspond to CEs, TGs, DGs and FFAs (at 2.1, 3.5, 4.5 and 11.9mins respectively). However, all peaks had lower intensities than the peaks on the HPLC traces of the first extract (100mAU first extract compared to 17.5mAU from the second). This showed that the first extract was successful for the extraction of the majority of the lipid in this case. The concentration of cholesteryl esters extracted in the second extract was $1.32x10^{-4}moldm^{-3}$. This was significantly lower than in the first extract. For this particular lens, the first extract was effective for the removal of the majority of the lipid; however a second extract did still extract a lower amount of lipid.

Table 4.4 – Analysis of HPLC traces in Figure 4.9 and Figure 4.10 (continuous wear PV, single vs. double extraction)

Extract	*Peak retention time (mins)*	*Peak identity*	*Peak height*	*Concentration*
1st extract	12.0	FFAs	~100mAU	3.67×10^{-3} moldm^{-3}
2nd extract	2.1	CEs	~16mAU	1.32×10^{-4} moldm^{-3}

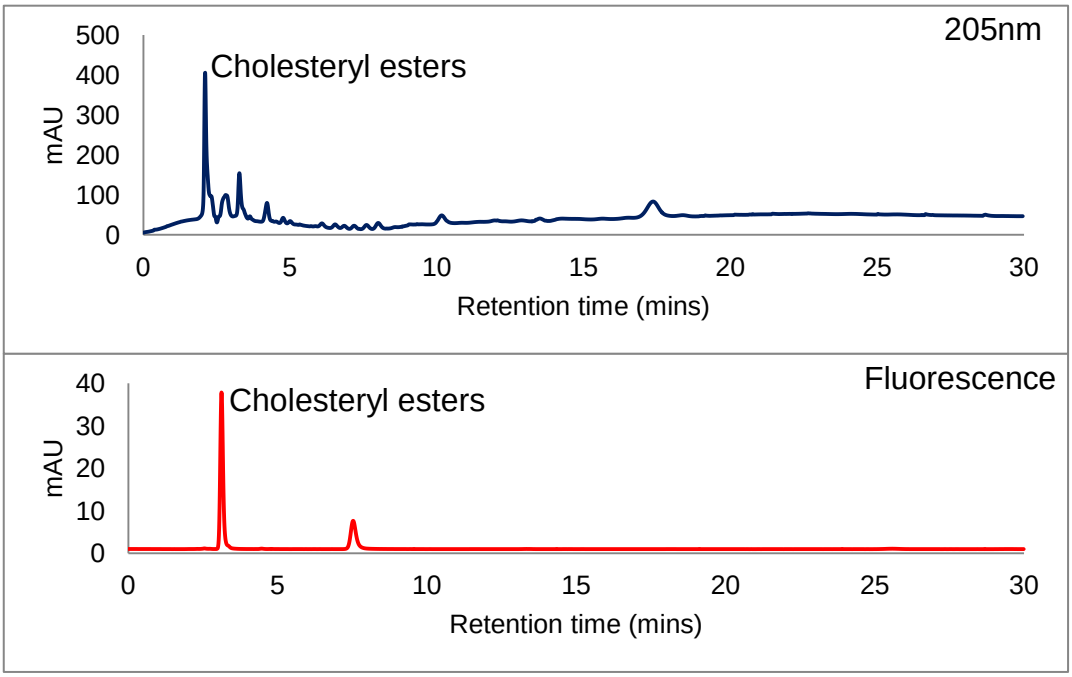


*Figure 4.11 – HPLC trace of a daily wear PV lens extract (extraction protocol 4, **1st extract**) (subject 51)*

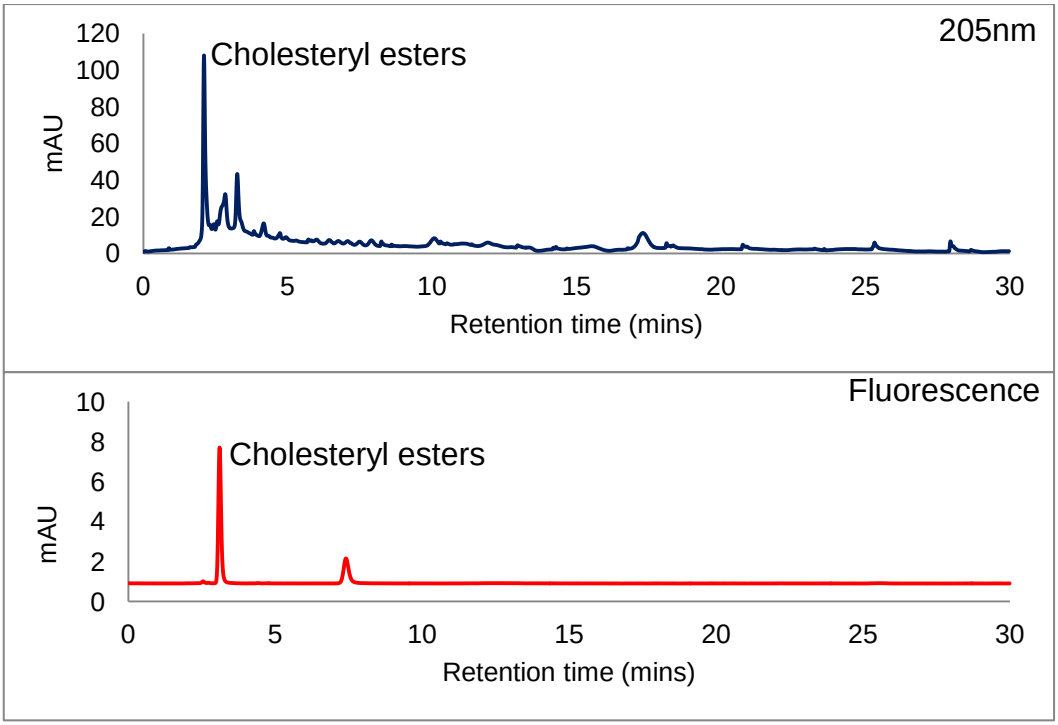


*Figure 4.12 – HPLC trace of a daily wear PV lens extract (extraction protocol 4, **2st extract**) (subject 51)*

4.3.2.2 Analysis of HPLC traces in Figure 4.11 and Figure 4.12 (daily wear PV, single vs. double extraction)

The HPLC traces shown in Figure 4.11 represent the first extraction of a daily wear PV lens. The peaks at 2.1, 3.7, 10.1, 17.3mins (at 205nm) correspond to CEs, TGs, FFAs and cholesterol respectively. The peak that corresponds to CEs had a very strong absorbance of ~400mAU, which is equivalent to 8.49×10^{-3}moldm^{-3}. CEs also had a fluorescence absorbance at 3.1mins.

The HPLC trace of the second extraction of the same lens showed peaks representing CEs, TGs and cholesterol (Figure 4.12). The peak that corresponds to CEs (at ~2.1mins) had a strong absorbance of ~100mAU, which is equivalent to 1.08×10^{-3}moldm^{-3}. This showed that a single extraction was not effective for the removal of the majority of the lipids in this case. This particular subject deposited high levels of non-polar lipids on the lens after 30 days daily wear.

Table 4.5 – Analysis of HPLC traces in Figure 4.11 and Figure 4.12 (daily wear PV, single vs. double extraction)

Extract	*Peak retention time (mins)*	*Peak identity*	*Peak height*	*Concentration*
1st extract	2.1	CEs	~400mAU	$8.49x10^{-3}moldm^{-3}$
2nd extract	2.1	CEs	~100mAU	$1.08x10^{-3}moldm^{-3}$

4.3.2.3 Figure 4.9 to Figure 4.12: Summary

- A single extraction using extraction protocol 4 was not efficient at removing most of the lipid in the first extract
- There was a considerable amount of CEs extracted in the second extract (shown in Figure 4.11 and Figure 4.12)
- There was still no observable difference in lipid profiles relating to wear schedule for PV lenses using different extraction protocols
- Extraction protocol 1 was used for all future extractions

4.3.3 HPLC results of daily wear and continuous wear F.N&D lens extracts for various subjects

Daily wear and continuous wear F.N&D lenses were analysed by GCMS, they were therefore also analysed by normal phase HPLC. Daily wear and continuous wear PV lenses were analysed by normal phase HPLC and no differences in lipid profiles relating to wear schedule were observed. Figure 4.13 to Figure 4.14 represent HPLC traces of both continuous and daily wear F.N&D lens extracts worn by two different subjects.

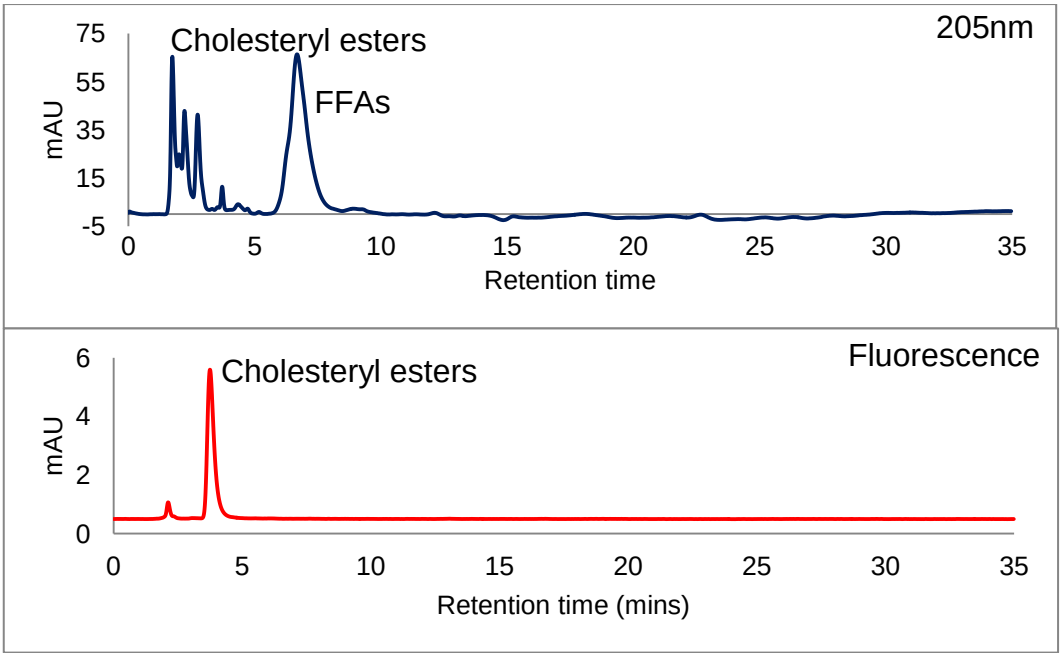


Figure 4.13 – HPLC traces of a daily wear F.N&D lens extract (extraction protocol 1) (subject 33)

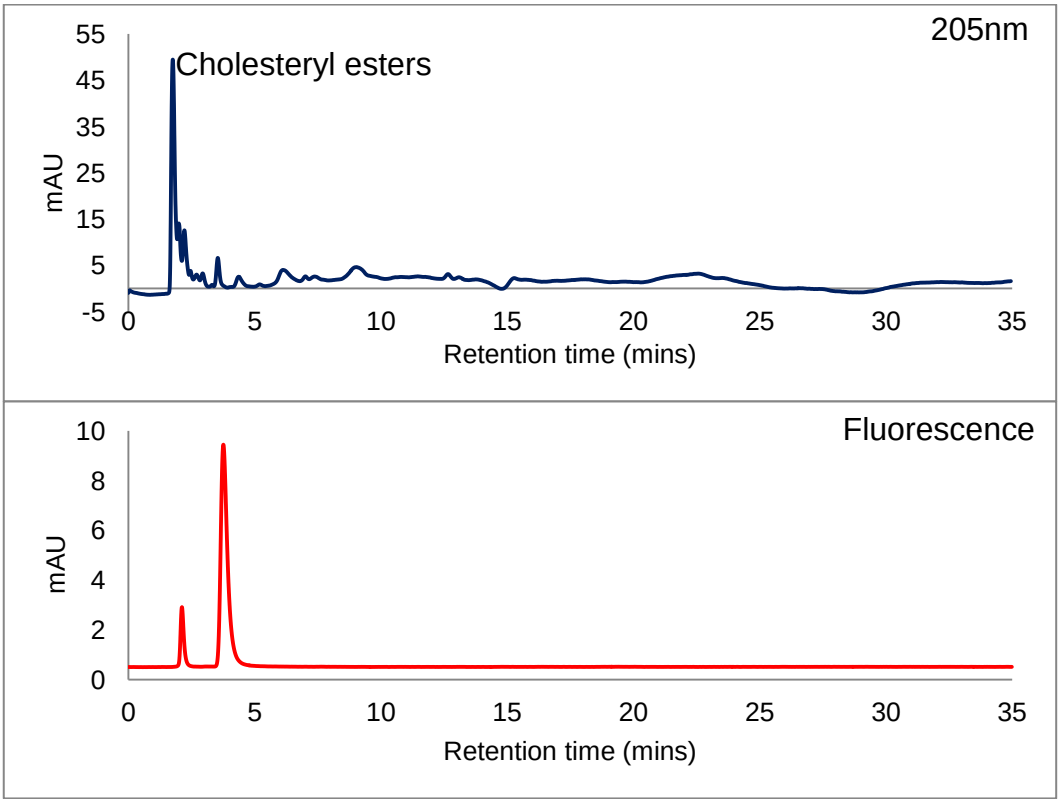


Figure 4.14 – HPLC traces of a continuous wear F.N&D lens extract (extraction protocol 1) (subject 38)

The HPLC separation of daily wear and continuous wear F.N&D lenses did not show any significant differences in lipid profile between relating to wear schedule. However, there was definite subject-to-subject variability observed.

The HPLC traces in Figure 4.13 represent a daily wear F.N&D lens extracts for worn by one particular subject. The predominant peaks observed on the chromatogram correspond to CEs and FFAs (at 1.7mins and 6.6mins). The concentration of CEs extracted from this particular daily wear F.N&D lens was $1.15x10^{-3}moldm^{-3}$. The concentration of FFAs extracted was $5.07x10^{-3}moldm^{-3}$. CEs were the most predominant lipid class extracted from the majority of lenses analysed. Many other F.N&D lens extracts that had been worn by various subjects were analysed by HPLC, the results are shown in Appendix 8.

The HPLC traces in Figure 4.14 represent a continuous wear F.N&D lens extract worn by one particular subject. The predominant peak at 1.7mins corresponds to CEs (equivalent to $9.64x10^{-4}moldm^{-3}$). Various other subjects continuous wear F.N&D lens extracts were analysed by HPLC and the traces are shown in Appendix 8. There were no differences in lipid class profiles between relating to wear schedule. The same general lipid classes were observed for all F.N&D lens extracts, such as CEs, TGs, FFAs and cholesterol. However, subject-to-subject variability in the ratios of individual lipid classes were observed.

Table 4.6 – Analysis of HPLC traces in Figure 4.12 and Figure 4.13

Wear schedule	*Peak retention time (mins)*	*Peak identity*	*Peak height*	*Concentration*
Daily wear	1.7	CEs	~70mAU	$1.15x10^{-3}moldm^{-3}$
Continuous wear	1.7	CEs	~50mAU	$9.64x10^{-4}moldm^{-3}$

4.3.3.1 Figure 4.13 and Figure 4.14: Summary

- There were no observable differences in lipid profile between daily wear and continuous wear F.N&D lens extracts relating to wear schedule
- Subject-to-subject variability in individual lipid profiles were observed
- For the majority of F.N&D lens extracts analysed, the predominant peak for both daily wear and continuous wear F.N&D lenses corresponded to CEs

4.4 Discussion and overall observations

The overall observations from the GCMS results of various lens extracts were:

- there were no unsaturated fatty acids in the extracts of the majority of continuous wear lens extracts, regardless of lens material, subject-to-subject variability in lipid deposition and extraction procedure
- ratios of unsaturated to saturated lipids did vary from subject-to-subject (for daily wear lens extracts only)
- a single extraction was effective for the removal of the majority of lipids deposited on and in the lens matrix (above 90%)
- initial results for lenses worn for a 7day period showed degradation of unsaturated lipids for one subject and not the other, suggesting degradation is dependent on the individual wearing the lens

The overall observations from HPLC results of various lens extracts showed:

- subject-to-subject variation in ratios of individual lipid classes, however, these changes did not have any correlation with wear schedule
- for certain lenses, a single extraction did not remove all lipid deposited on and within the lens matrix (Figure 4.10 and Figure 4.12) although the majority of GCMS traces suggested a single extraction was effective for lipid extraction

The GCMS results revealed that there were significant differences in fatty acid profile for lenses worn on a daily wear schedule compared to those worn on a continuous wear schedule for 30 days. This could be seen in Figure 4.2 and Figure 4.3 which represent PV and F.N&D lens extracts respectively. There were no peaks that corresponded to the major unsaturated tear fatty acids, C16:1 (at 12.5mins) and C18:1 (at 14.8mins) observed on gas chromatograms of continuous wear PV and F.N&D lens extracts (Figure 4.2, chromatograms 1 and 2; Figure 4.3, chromatograms 1 and 2). However, both unsaturated and saturated lipids were extracted from both lens materials that were worn on a daily wear schedule for 30 days (observed in Figure 4.2, chromatograms 3 and 4; Figure 4.3, chromatograms 3 and 4).

Additional gas chromatograms which represent daily wear and continuous wear PV lens extracts for various subjects are shown in Appendix 5. Additional gas chromatograms which represent daily wear and continuous wear F.N&D lens extracts for various subjects are shown in Appendix 6. For the majority of the lenses analysed, there were no unsaturated lipids on the lenses worn on a continuous wear schedule. This indicated that the unsaturated lipids had been degraded during overnight contact lens wear.

This was the most significant finding of the research discussed in this chapter. Changes in lipid structure during sleep have never been previously studied. This work was therefore novel in showing that there are significant changes occurring in the overnight tear film to lipids.

As mentioned in the introduction, the overnight tear film is stagnant which leads to increase in levels of certain tear components due to reduced tear flow. Certain enzymes are believed to be responsible for the degradation of unsaturated lipids during eye closure.

The preliminary results for the lenses worn for 7 days on either a daily wear schedule or a continuous wear schedule indicated that the degradation of unsaturated lipids on continuous wear lenses was subject-dependent. Certain subjects gas chromatograms showed no detectable levels of unsaturated lipids after 7 days continuous wear. However, other

subjects lens extracts showed there were still detectable levels of unsaturated lipid on the continuous wear lenses analysed (Figure 4.4 to Figure 4.6). This was believed to be as a result of subject-to-subject variation in lipid deposition. This work therefore clearly showed that unsaturated lipids degrade during overnight contact lens wear and that this degradation occurs at different rates.

As well as using GCMS to investigate any possible differences in fatty acid profile, HPLC was used for individual lipid class separation.

Many of the HPLC traces for the lens extracts analysed (daily wear and continuous wear) were similar to one another. The use of GC enabled the analysis of individual fatty acid composition and the results showed significant differences between lenses worn daily to those worn continuously. HPLC identified differences in ratio of different lipid classes, however, the differences observed had no correlation to wear schedule. The differences in lipid profiles observed varied from subject-to-subject.

This chapter showed that there are significant differences in lipid profiles from contact lens extracts worn on two different wear schedules. This chapter also showed that GCMS is a powerful technique which provided results that allowed these changes to be observed.

The work discussed in this chapter showed the degradation of unsaturated lipids during overnight wear of contact lenses.

The work discussed in Chapter 5 is therefore based on investigating the presence of lipid oxidative end products in tears and contact lens extracts.

Chapter 5 – Lipid oxidation and biomarkers of lipid oxidation

5.1 Introduction

The presence of MDA in tears has previously been linked with contact lens intolerance (121). The purpose of the work discussed in this chapter was to adapt existing methods, currently used for malondialdehyde (MDA) quantification in blood, urine and plasma samples to enable the measurement of MDA in the ocular environment. As discussed in Chapter 1 (section 1.11), MDA is a secondary product of lipid oxidation and is also a biomarker for lipid oxidation. MDA is only produced from the oxidation of polyunsaturated fatty acids (e.g. C18:3); the scheme is shown in Figure 1.29 (Chapter 1, section 1.11.1). Although the most predominant fatty acid found in tears is C18:1 and polyunsaturated fatty acids (such as C18:3) are found at very low levels, MDA was chosen for analysis because it provides a measure of oxidation. It is important to note that MDA can be produced from other sources such as carbohydrate oxidation.

5.2 Assays and techniques

Several commercially available assay test kits were adapted to enable the measurement of MDA in tear samples and contact lens extracts. The assays used for MDA measurement were the thiobarbituric acid reactive substances assay (TBARS), MDA-specific assay (using N-methyl-2-phenylindole) and ELISA-MDA assay (enzyme linked immunosorbant assay). All of the assay kits were used in accordance to the individually supplied protocols. Each of the assay protocols are described in Chapter 2.

Analytical techniques such as HPLC and capillary electrophoresis have been used for MDA analysis and have been discussed in the literature. Capillary electrophoresis has been used previously by Georgakopoulos *et al*.(151) for the measurement of MDA in tears, therefore a similar methodology was followed in this research.

High performance liquid chromatography (HPLC) with fluorescence detection was used by Fukunaga *et al*. (134) to separate and quantify

levels of MDA in plasma samples. The HPLC and capillary electrophoresis conditions are described in detail in Chapters 2.

5.3 Sample acquirement

Various samples were analysed by an array of assays and techniques in order to determine the location of MDA in the ocular environment. Tear samples were collected from individual subjects using the microcapillary tear collection method. Tear envelope samples (method described in section 2.4) were collected from stored lenses which were obtained from clinically controlled studies. Tear envelope samples were also collected from freshly worn contact lenses. Contact lens extracts that were extracted using various extraction protocols were also analysed.

5.4 TBARS: method development, sample preparation and results

5.4.1 Tear sample (TS) preparation

Microcapillaries were used for the collection of tear samples from individuals throughout a day. The tear samples were pooled together until the required volume of 100μl was obtained. Certain subjects tear samples were up to 100μl with PBS.

5.4.2 Tear envelope (TE) preparation

Tear envelope samples were taken by the addition of 100μl of either water or PBS to the lens, centrifuging and the collection of the tear envelope sample (as described in Chapter 2, section 2.4).

5.4.3 Contact lens preparation

Worn contact lenses were extracted (for 2hrs) by using the standard 1:1 chloroform: methanol protocol 1. The extracts were then resuspended in 200μl PBS for ready for analysis.

The method was developed by the preparation of a calibration curve as described in the assay protocol (Chapter 2, section 2.11.1.1) and all unknowns were compared to this calibration curve.

5.4.4 TBARS assay preparation

Briefly, MDA standards were prepared from the reaction of MDA (at different concentrations) with the TBA diluent at 95°C for 60 minutes. The absorbencies of these standards were read at 532nm and a calibration curve was prepared. All unknown samples were treated in the same way as MDA standard, and the absorbance values were compared to the standard curve.

The concentrations of the standards are shown in Table 5.1 and the calibration curve is shown in Figure 5.1.

5.4.5 TBARS calibration preparation data

The standards for the calibration curve were prepared as described in Chapter 2, section 2.11.1.1. The calibration curve data and standard curves are shown in Table 5.1, Figure 5.1 and Figure 5.2 respectively.

Table 5.1 – Calibration curve data for MDA TBARS assay

Concentration of MDA (µM)	*Absorbance of MDA at 532nm*
250	2.693
125	1.413
62.5	0.745
31.25	0.383
15.63	0.195
7.81	0.095
3.91	0.050
1.95	0.027
0.98	0.012

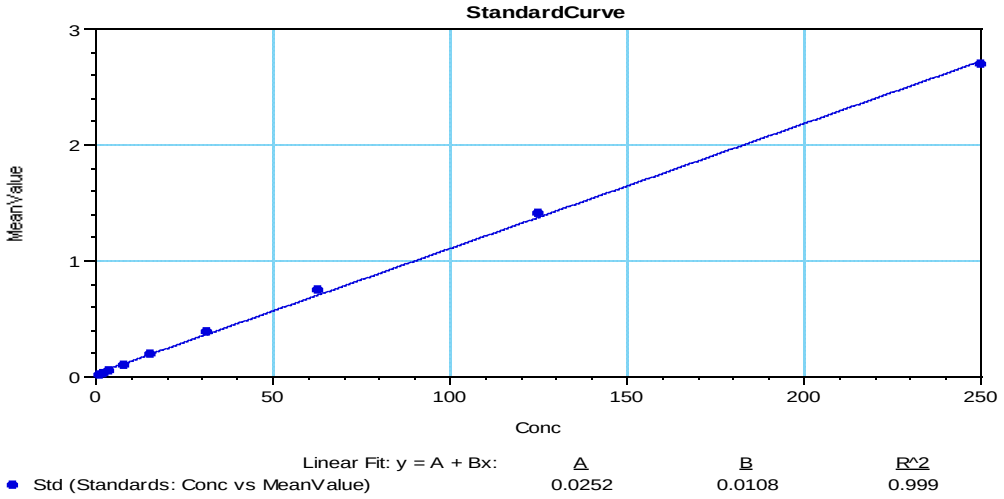


Figure 5.1 – Standard curve of MDA standard using the TBARS assay at 532nm UV absorbance

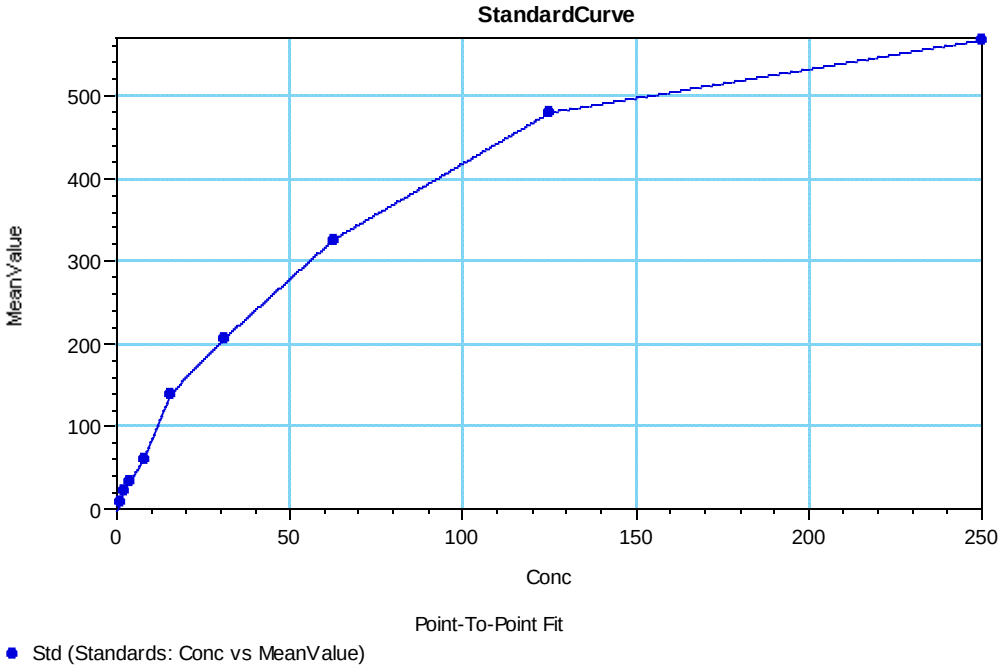


Figure 5.2 – Standard curve of MDA using TBARS measured by fluorescence. Excitation of 540nm and emission at 590nm

A linear standard curve of MDA standard measured at 532nm UV absorbance was achieved. Known concentrations of MDA standard were also measured by fluorescence absorbance. The calibration curve (Figure 5.2) was as shown in the assay protocol. All unknown samples were measured by UV absorbance at 532nm.

5.4.6 TBARS results

The values displayed in Table 5.2 are the preliminary results obtained by the TBARS assay. The concentration of MDA in unknown samples was determined by using the calibration curve shown in Figure 5.1. The samples analysed all showed low concentrations of MDA. Tear samples were collected from individual subjects throughout a day and pooled together to achieve the volume required for this particular assay (100µl). *Ex vivo* PureVision lenses were extracted using extraction protocol 1 (1:1 chloroform: methanol) and resuspended in 200µl PBS.

Table 5.2 – Preliminary TBARS results

Sample	*Values*	*Results (µM)*
Tear sample (TS) subject 1	0.019	1.05
Tear sample (TS) subject 2	0.029	1.91
Tear envelope (TE) subject 3	0.024	1.50
Tear sample (TS) subject 3	0.036	2.53
(10x) worn PV lens extracts 1	0.241	19.81
(10x) worn PV lens extracts 2	0.218	17.90

Where: 100µl TS were collected and then pooled, TE from 2x ex vivo Focus Dailies lenses, 10xPV lenses extracted in 1:1 chloroform: methanol and resuspended in 200µl PBS

The preliminary results obtained from the TBARS assay showed positive results for tear samples, TE samples and lens extracts. These results revealed that tear samples collected from certain subjects had higher levels of MDA than others. As only three subjects tears were analysed by the TBARS assay, further work was required to determine the validity of this assay. Therefore, more samples were collected including tear

samples, tear envelope samples and contact lens extracts. They were all analysed by the TBARS assay and the results are shown Table 5.3 and Table 5.4. The sample details are given below Table 5.3.

Table 5.3 – TBARS results measured by UV absorbance at 532nm

Sample	*Value*	*Result (µM)*
Subject 1 TS LE	-0.008	-1.21
Subject 1 TS RE	0.021	1.25
Subject 1 TS RE *(repeat)*	0.01	0.37
Subject 2 TS LE	0.032	2.19
Subject 2 TS RE	0.003	-0.28
Subject 2 TS RE *(repeat)*	0.009	0.26
Subject 3 TS LE	0.008	0.13
Subject 3 TS RE	0.002	-0.31
Subject 3 TS RE *(repeat)*	0.038	2.67
Unworn Focus Dailies soaked in MDA, extracted in PBS *(1)*	0.487	40.54
Unworn Focus Dailies lens soaked in MDA with addition of BHT, extracted in PBS *(1)*	0.485	40.37
Unworn Focus Dailies lens soaked in MDA, extracted in PBS *(2)*	0.564	46.99
Unworn Focus Dailies lens soaked in MDA with addition of BHT, extracted in PBS *(2)*	0.491	40.88
Unworn Focus Dailies lens soaked in MDA, extracted in Renu *(1)*	0.109	8.68
Unworn Focus Dailies lens soaked in MDA with the addition of BHT, extracted in Renu *(1)*	0.065	4.96
Unworn Focus Dailies lens soaked in MDA, extracted in Renu *(2)*	0.061	4.64
Unworn Focus Dailies lens soaked in MDA with the addition of BHT, extracted in Renu *(2)*	0.1	7.95
MDA standard saline *(1)*	0.505	42.07
MDA standard saline *(2)*	0.484	40.25

MDA standard saline BHT *(1)*	0.47	39.11
MDA standard saline BHT*(2)*	0.61	50.90

*Where: **TS LE/RE**= pooled tear samples from right/left eye of subjects 1,2 and 3. Unworn Focus Dailies lens soaked in MDA standard overnight, extracted in PBS for 2hrs (with and without the addition of antioxidant butylated hydroxytoluene - BHT) and assayed. **MDA standard saline**= 2mg/ml MDA in PBS with/without BHT. Unworn Focus Dailies lens soaked in MDA standard (2mg/ml) overnight, with or without BHT and extracted with multipurpose solution – Renu.*

The concentrations of the unknown samples in Table 5.3 were determined by using the calibration curve in Figure 5.1. The concentration of MDA in tear samples showed variable results. Some subjects showed positive values, however some subjects showed negative results.

Focus Dailies lenses were soaked in MDA standard overnight and extracted in either PBS or Renu (contact lens multipurpose solution). The extraction of MDA from contact lenses had not previously been studied in literature. This work was therefore based on trying to find an effective extraction procedure and to establish the reproducibility of this assay. The PBS and Renu extracts of lenses soaked in MDA standard showed positive results. This means that both PBS and Renu were efficient for the extraction of MDA from lenses. Renu was less efficient for the extraction of MDA from lenses because the concentration of MDA in Renu extracts was considerably lower than the PBS extracts.

The results shown in Table 5.4 are the same samples as those shown in Table 5.3. The fluorescence absorbance of the unknown samples was measured and the concentration of MDA determined from the calibration curve shown in Figure 5.2.

Table 5.4 – MDA TBARS results measured by fluorescence

Sample	*Value*	*Result (µM)*
Subject 1 TS LE	1.856	0.43
Subject 1 TS RE	1.133	0.37
Subject 1 TS RE *(repeat)*	0.95	0.36
Subject 2 TS LE	1.68	0.42
Subject 2 TS RE	1.855	0.43
Subject 2 TS RE *(repeat)*	1.205	0.38
Subject 3 TS LE	1.165	0.38
Subject 3 TS RE	1.075	0.37
Subject 3 TS RE *(repeat)*	1.418	0.40
Unworn Focus Dailies lens soaked in MDA, extracted in PBS *(1)*	128.60	14.48
Unworn Focus Dailies lens soaked in MDA with the addition of BHT, extracted in PBS *(1)*	127.00	14.32
Unworn Focus Dailies lens soaked in MDA, extracted in PBS *(2)*	150.56	18.04
Unworn Focus Dailies lens soaked in MDA with the addition of BHT, extracted in PBS *(2)*	128.40	14.46
Unworn Focus Dailies lens soaked in MDA, extracted in Renu *(1)*	29.12	3.05
Unworn Focus Dailies lens, soaked in MDA with the addition of BHT, extracted in Renu *(1)*	20.54	1.80
Unworn Focus Dailies lens soaked in MDA, extracted in Renu *(2)*	17.80	1.60
Unworn Focus Dailies lens soaked in MDA with the addition of BHT, extracted in Renu *(2)*	28.74	3.00
MDA standard saline *(1)*	126.60	14.28
MDA standard saline *(2)*	128.67	14.48
MDA standard saline BHT *(1)*	135.80	15.19
MDA standard saline BHT *(2)*	133.38	14.95

These samples were the same as those tested in Table 5.3

The tear samples all showed positive results for MDA as measured by fluorescence absorbance. However, the concentration of MDA in the tear samples measured by fluorescence absorbance was higher than the same tear samples measured by UV absorbance. The concentration of MDA from *in vitro* spoiled lenses was lower when measured by fluorescence absorbance compared with UV absorbance. For example, the unworn Focus Dailies lens soaked in MDA, extracted in Renu showed an absorbance of 29.12 which was equivalent to 3.047μM MDA when measured by fluorescence absorbance. However, the same sample measured by UV absorbance showed an absorbance of 0.109, equivalent to 8.675μM MDA. As the results varied depending on the calibration curve used, the TBARS assay was not very accurate for the measure of MDA in the ocular environment.

Also, according to the assay protocol a new calibration curve had to be prepared each time the assay was used. The absorbance values for known concentrations of MDA standard varied each time they were measured, therefore the calibration curve varied. This meant that the true concentration of MDA in unknown samples varied depending on the calibration curve used. All unknown samples were therefore compared to one calibration curve (shown in Figure 5.1). This assay was therefore not very accurate for the purposes of this research.

5.4.7 TBARS assay results: Summary

- the results obtained using the TBARS assay showed that MDA was present in some tear samples, however, this was not very accurate because the results depended on the calibration curve used
- the assay protocol recommended a new calibration curve be prepared each time the assay was used, which altered the results
- fluorescence absorbance of samples showed lower levels of MDA compared with UV absorbance
- PBS was efficient at extracting MDA from lenses that had been soaked in an MDA solution *in vitro*

5.5 MDA – specific N-methyl-2-phenylindole (NMPI) assay results

The assay methodology described in Chapter 2, section 2.11.2.1 was followed. Briefly, a dilution series of tetramethoxypropane (TMOP, a MDA substitute), in the concentration range 0-4μM was prepared from the dilution of TMOP in water. The concentration of MDA in unknown samples was determined from the calibration curve of TMOP standard. The calibration curve is shown in Figure 5.3.

5.5.1 Tear sample preparation

Tear samples were taken using microcapillaries (described in chapter 2 section 2.3) and either pooled together to obtain a final volume of 200μl required for the assay or made up to the final volume using PBS.

5.5.2 Tear envelope sample preparation

100μl PBS was placed on either side of the lens and the tear envelope collected. This was made up the final volume of 200μl for analysis.

5.5.3 Contact lens extracts preparation

Single lenses were extracted in 500μl PBS for a minimum of 2 hours on a slow shaker and the extract was used directly for analysis.

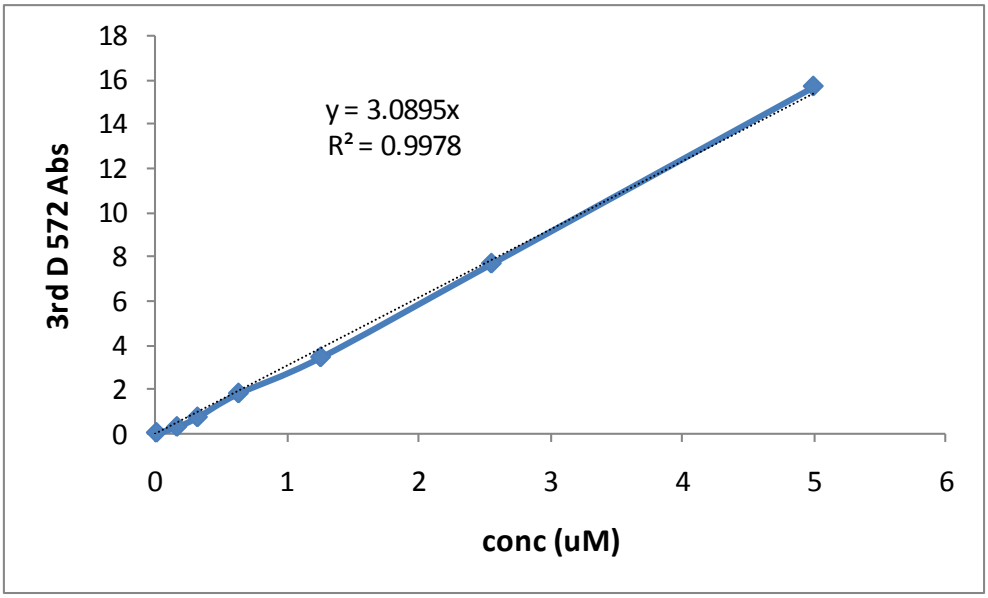


Figure 5.3 – Calibration curve of TMOP standard using the MDA – specific assay UV absorbance at 572nm

5.5.4 MDA-specific NMPI: Summary

The assay protocol recommended reading the samples at a wavelength of 586nm (maximum absorbance), however the calibration curve was not very linear. Therefore, the standards were analysed at 572nm as this produced a linear calibration curve. All of the samples were below the calibration range and this assay was not used for this research.

5.6 MDA – ELISA

This assay methodology was followed as described in Chapter 2, section 2.11.3. The sample volume required was 100µl. The concentration of MDA in unknown samples was compared to the standard calibration curve (full assay details are described in Chapter 2).

5.6.1 Tear sample preparation

Tears that were collected using microcapillaries from individuals were pooled and analysed by the assay.

5.6.2 Tear envelope sample preparation

100µl PBS was added to the lens and centrifuged to collect the volume required for the assay (as described in section 2.4).

5.6.3 Contact lens extracts preparation

Single contact lenses were extracted in 1:1 chloroform: methanol and resuspended in 100µl PBS for analysis by the assay.

The results for various contact lens extracts, TE samples and TS are shown in Table 5.5. The concentration of MDA in unknown samples was established from the calibration curve shown in Figure 5.4.

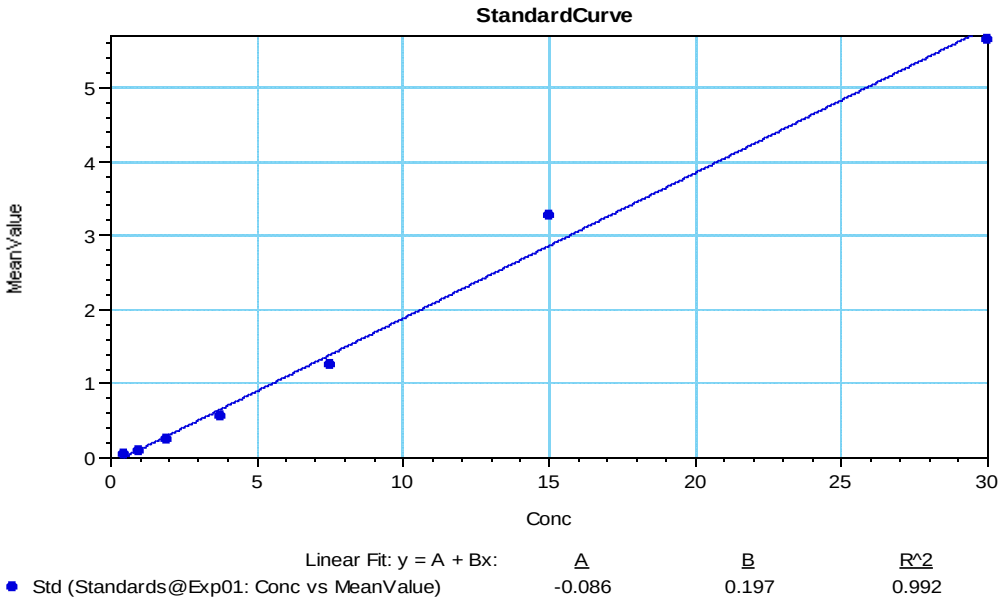


Figure 5.4 – MDA-BSA ELISA standard curve, absorbance read at 450nm

Table 5.5 – The concentration of MDA in various samples (lens extracts, TS and TE samples) measured by the MDA – ELISA assay

Sample	*Amount of MDA (pmol/ml)*
Stored Air Optix lens extract (using extraction protocol 1, resuspended in PBS)	21.0
Fresh Air Optix lens extract (using extraction protocol 1, resuspended in PBS)	4.0
Unworn Air Optix lens extract (using extraction protocol 1, resuspended in PBS)	0.5
Fresh Focus Dailies lens extract (using extraction protocol 1, resuspended in PBS)	3.0
Unworn Focus Dailies lens extract (using extraction protocol 1, resuspended in PBS)	0.5
Fresh PV lens extract (using extraction protocol 1, resuspended in PBS)	1.0
Unworn PV lens extract (using extraction protocol 1, resuspended in PBS)	0.5
Fresh Air Optix TE (pooled LE and RE using PBS)	1.0
Focus Dailies TE (pooled LE and RE using PBS)	2.5
PV TE (pooled LE and RE-using PBS)	0.7
Pooled TS (using capillary collection method)	0.5

The results shown in Table 5.5 represent various samples that were assayed using the MDA-ELISA technique. Various samples including lens extracts, tear samples and tear envelope samples were analysed.

The concentration of MDA in the TS analysed was very low (0.5ρmol/ml). MDA was extracted from worn lenses, with *ex vivo* stored Air Optix lens extracts showing higher levels of MDA than fresh lens extracts. Unworn lens extracts were also analysed and these did not show significant levels of MDA. Therefore, extractable material from the lens did not interfere

with the results. The lenses were extracted using 1:1 chloroform: methanol extraction and the extracts were resuspended in PBS.

MDA was also present in tear envelope samples. PBS was used to obtain tear envelope samples from worn Air Optix, Focus Dailies and PureVision lenses. The Focus Dailies TE sample showed the highest levels of MDA compared with the TEs of the other lenses.

5.6.4 MDA-ELISA results: Summary

- the highest levels of MDA were observed for stored Air Optix lens extracts
- fresh lens extracts also showed significant levels of MDA
- tear samples showed very low levels of MDA
- this assay was the most sensitive assay of those tested
- future work is required to validate this assay, however these initial findings were very interesting and unique

5.7 Capillary electrophoresis with UV detection

Capillary electrophoresis was used for MDA detection and the method conditions and parameters are described in Chapter 2, section 2.11.4. A calibration curve was not obtained using capillary electrophoresis because a high concentration of MDA standard was required to produce a peak with a low intensity. The capillary electrophoresis trace for MDA standard is shown in Figure 5.5. Samples were not analysed using this method because a high concentration of MDA was required to produce a peak with low intensity. Therefore this method was not sensitive enough for the purposes of this research.

Georgokopoulos *et al*. (151) used capillary electrophoresis to separate and identify MDA in tear samples. A very similar method followed in this research; however the same results were not achieved. Georgokopoulos *et al*. state their method has a lower limit of detection of 2.5μM MDA. In this research, a 250μM MDA standard was separated by capillary electrophoresis and this produced a peak with very low intensity, as seen in Figure 5.5. Georgokopoulos *et al*. were able to detect MDA in the tears

of 7 out of 11 healthy subjects; however the levels of MDA were below the lower limit of quantitation for all tear samples.

Tear samples, tear envelope samples and contact lens extracts were not analysed using this method because it was not considered to be sensitive enough.

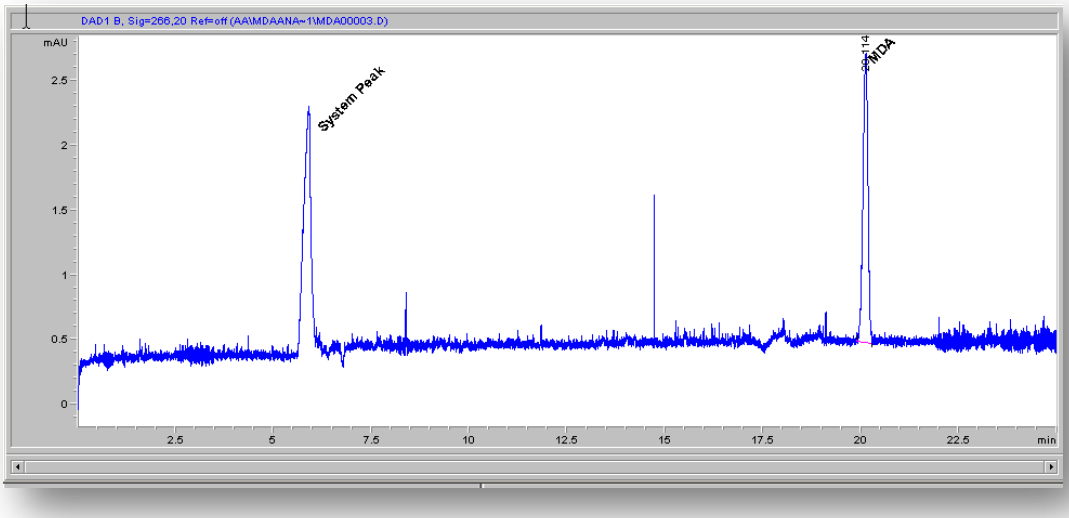


Figure 5.5 – Capillary electrophoresis trace of MDA standard (250µM)

5.7.1 Capillary electrophoresis results: Summary

- although this method had been used for the detection of MDA in tears in the literature, similar results were not achieved in this research
- MDA standards were separated by capillary electrophoresis but a high concentration of standard produced a peak of low intensity
- this method was therefore not used for the analysis of MDA in unknown samples

5.8 MDA-TBA HPLC with fluorescence detection results

For this assay, 5µl of standard MDA or unknown samples were reacted with 100µl TBA buffer for 60 minutes at 95°C in eppendorfs. These were then placed under cold water for 5 minutes and centrifuged for 15 minutes at 6600rpm. The upper 750µl were transferred to amber vials for analysis by HPLC. The column and mobile phase details were used as described

in Chapter 2, section 2.11.5. Briefly, TEP (tetraethoxypropane), a MDA substitute, was reacted with 0.2% TBA buffer (prepared as described in 2.11.5.1.1). Dilution series of TEP over a range of concentrations was prepared by serial dilution of TEP in water (concentration range shown in Table 2.7). All standards were separated by HPLC with fluorescence detection and the area under the peak that corresponded to TEP-TBA was measured (at approximately 2.4mins as shown in Figure 5.8 to Figure 5.10). Calibration curves of TEP (at various concentrations) were plotted (Table 5.6, Table 5.7, Figure 5.6 and Figure 5.7). The concentration of MDA in unknown samples was determined from the calibration curves. Throughout this section both TEP and MDA are interchangeable.

Table 5.6 – Calibration curve data for MDA measured by HPLC

Concentration of MDA (µmol/ml)	*Area under peak at ~2.4 minutes*
0.625	21.97
1.25	61.13
2.5	111.92
5.0	208.62
10.0	412.12

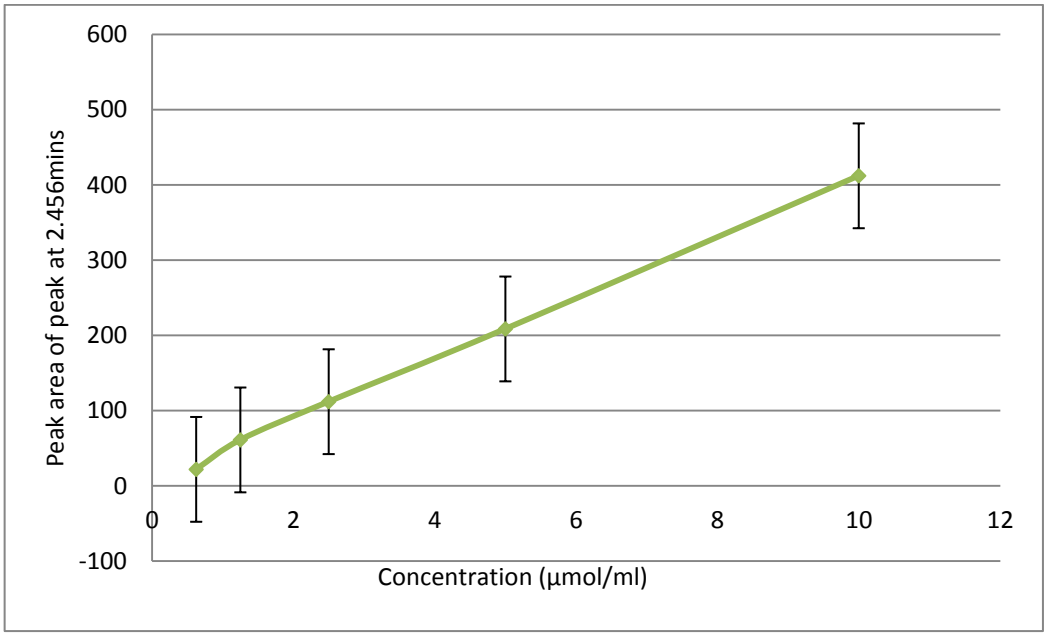


Figure 5.6 – Calibration curve for MDA measured by HPLC

Calibration curve of MDA at lower concentrations was also prepared and the curve is shown in Figure 5.7.

Table 5.7 – Calibration curve data for MDA measured by HPLC at lower concentrations

Concentration of MDA (nmol/ml)	*Area under peak at ~2.4mins*
0	0
20	2.16
30	2.18
40	2.55
50	2.98
100	5.89
200	10.09

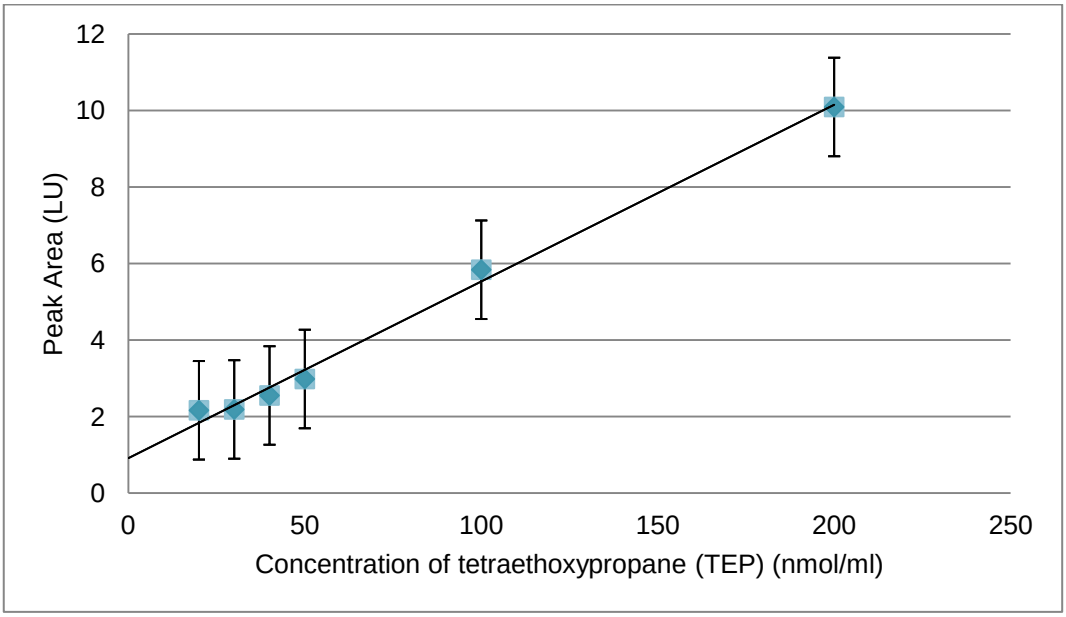


Figure 5.7 – Calibration curve for MDA measured by HPLC at lower concentrations

It can be seen that the calibration curves at higher and lower concentrations of MDA are both linear.

The HPLC traces for TEP standard at various concentrations are shown in Figure 5.8 to Figure 5.11. TBA buffer was also separated by HPLC with fluorescence detection to determine whether it would interfere with the

MDA-TBA peak (shown in Figure 5.11). Unknown samples were also separated by HPLC and the traces are shown in Figure 5.11.

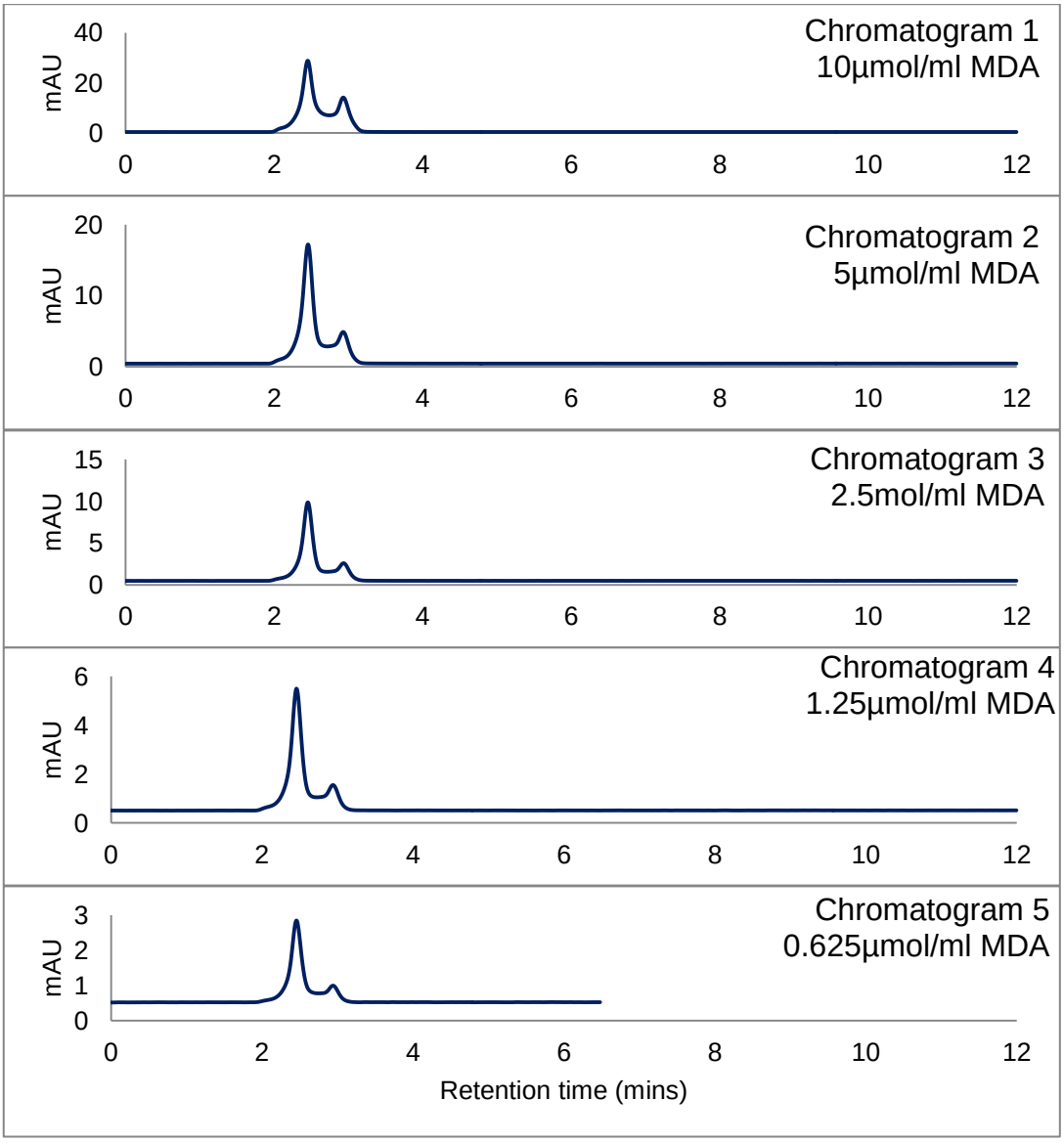


Figure 5.8 – HPLC fluorescence scans for MDA-TBA adduct for concentrations of 10µmol/ml – 0.635µmol/ml TEP standard

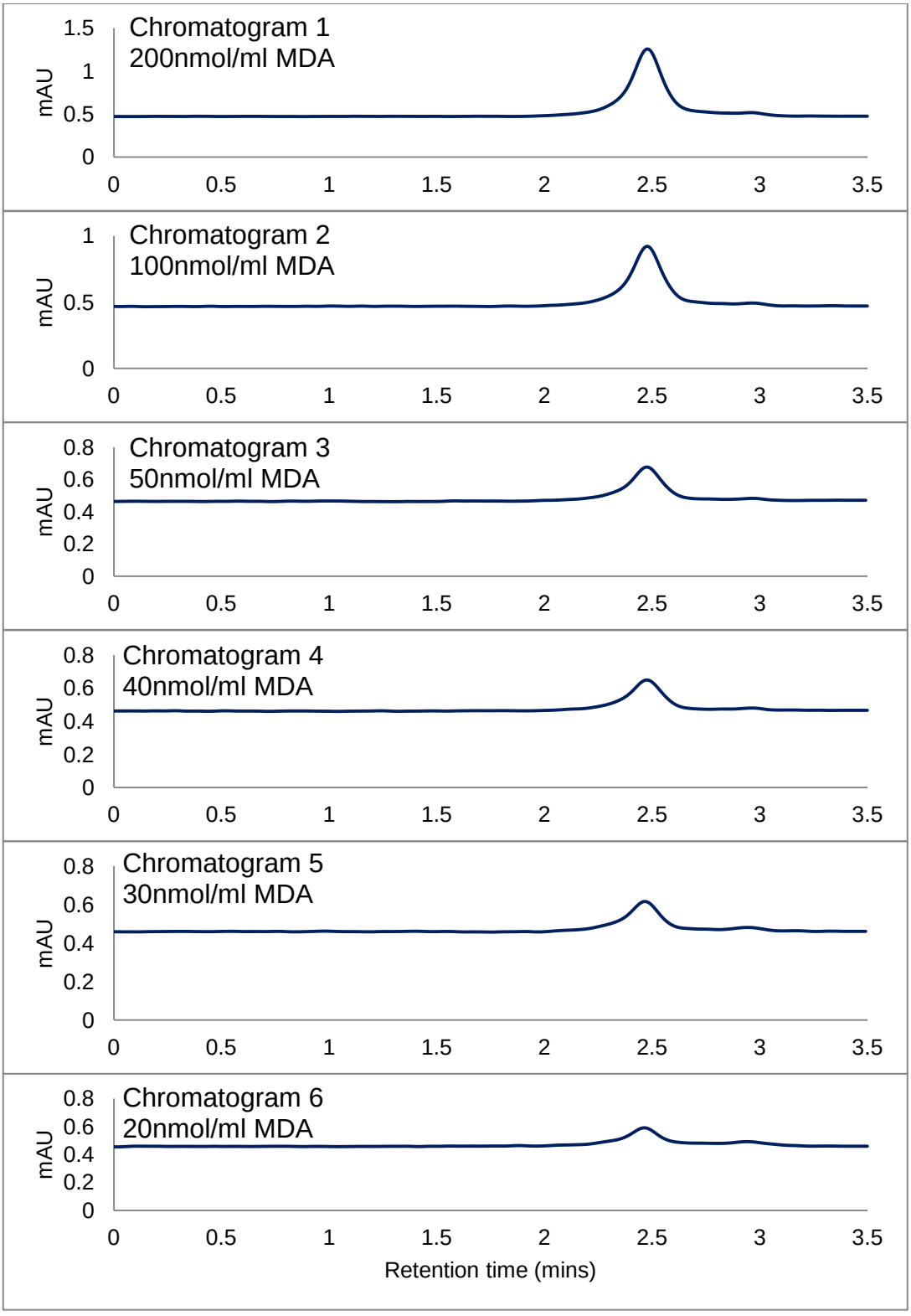


Figure 5.9 – HPLC fluorescence scans for MDA-TBA adduct for concentrations of 200nmol/ml – 20nmol/ml TEP standard

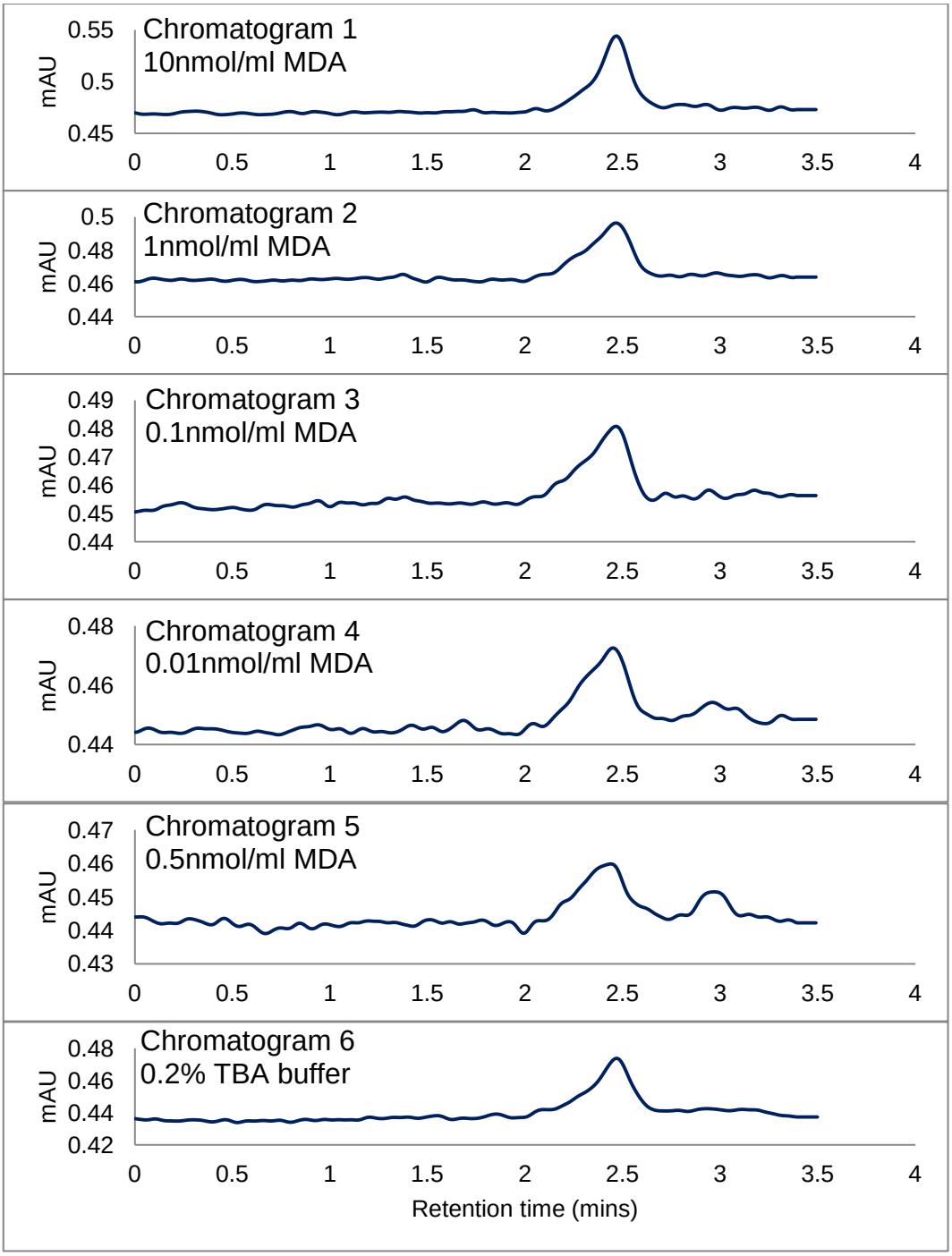


Figure 5.10 – HPLC fluorescence scans for MDA-TBA adduct for concentrations of 10nmol/ml – 0.5nmol/ml TEP standard

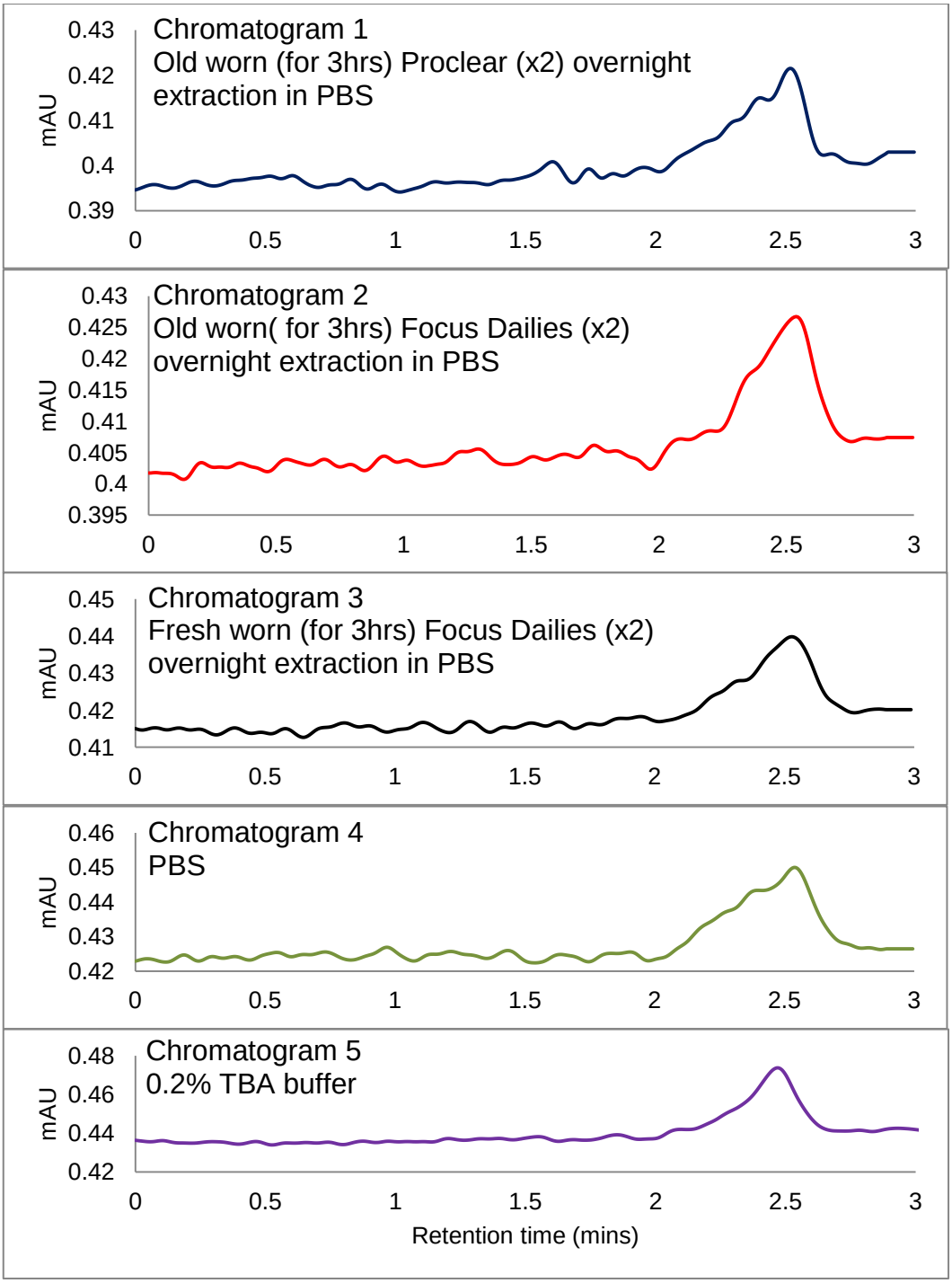


Figure 5.11 – HPLC fluorescence scans for MDA-TBA adduct for unknown samples

The HPLC method did not produce any positive results for the samples tested. The lower limit of detection was approximately 1nmol/ml standard. However, the TBA buffer also showed an absorbance at a very similar retention time as MDA-TBA. It was therefore difficult to distinguish between MDA and the TBA buffer at lower concentrations.

Although this method was not sensitive enough to detect MDA in the samples tested, it was used for the measurement of MDA uptake and release from lenses. This technique was chosen as it was a more cost effective method compared to the commercial assays and was a sensitive method and more accurate technique than the TBARS assay alone.

5.8.1 HPLC with fluorescence detection results: summary

- A linear calibration curve was prepared, with a lower limit of detection of 1nmol/ml
- TBA buffer had an absorbance at a similar retention time to the MDA-TBA buffer, it was therefore difficult to distinguish between the TBA buffer and MDA-TBA at low concentrations of MDA
- The samples analysed did show an absorbance at ~2.4mins (Figure 5.11), however the TBA buffer had an absorbance at the same retention time and a similar peak intensity
- This method therefore lacked the sensitivity required to analyse MDA in the ocular environment
- Unknown samples were therefore not analysed using HPLC, but HPLC was used to determine the levels of MDA uptake and release from various lenses because a linear calibration curve was established

5.8.2 The use of HPLC to determine the uptake and release of MDA from contact lenses

The principle of *in vitro* uptake and release of active species with particular contact lenses is well established. However, the behaviour of MDA with any lens material has not been studied.

MDA is a small molecule; it can therefore be absorbed into the lens matrix. The chemical structure of MDA suggests it is water soluble. The *in vitro* uptake and release of MDA from various lenses was therefore investigated.

The lenses chosen for investigation were PureVision (PV) and Acuvue Oasys (Oasys) as these lenses are known to adsorb high levels of lipid.

They are both silicone hydrogel materials but PureVision lenses have been surface treated whereas Acuvue Oasys lenses have not (as described in section 1.7.2).

5.8.2.1 Preparation of solutions and reagents for the measurement of uptake and release of 1,1,3,3- tetraethoxypropane (TEP) from lenses:
A stock solution of 10µmol/ml TEP (MDA substitute) solution was prepared by adding 24µl TEP to 10ml ethanol. From the stock solution, 5µmol/ml and 100nmol/ml standard solutions were prepared by serial dilution in water. Single lenses (either PV or Oasys) were soaked in 2ml of a 5µmol/ml or a 100nmol/ml TEP solution.

All lenses were blotted with filter paper to prevent packaging solution contamination prior to soaking in the TEP solutions.

The lenses were added to 2ml of the two concentrations of TEP (5µmol/ml or a 100nmol/ml) in centrifuge tubes. These were placed on a flat-bed shaker at 172rpm for 2hrs, 4hrs and 24hrs. After 2hrs, 4hrs and 24hrs; 5µl of the TEP solution was transferred to a 1.5ml eppendorf. 1ml 0.2% TBA buffer was added to the eppendorfs and they were incubated for 60mins at 95°C. After the incubation, the eppendorfs were cooled in a water bath for approximately 5mins, vortexed and centrifuged at 12.5rpm for 15 minutes. The upper 750µl was transferred to amber vials and analysed by HPLC with fluorescence detection.

The lenses that had been soaked in MDA solutions for different periods of time were transferred to new centrifuge tubes containing 1ml HPLC grade water to allow the release of MDA. The tubes were left on a flat-bed shaker for 24 hours to release any MDA in the lens. 5µl aliquots of the release solution was taken from each tube and transferred to 1.5ml eppendorfs. 1ml 0.2% TBA buffer was added and the solution was heated for 60mins at 95°C. The eppendorfs were cooled under cold water, vortexed and centrifuged at 12.5rpm for 15mins. The upper 750µl was transferred to amber vials for HPLC with fluorescence detection analysis.

The HPLC traces for standard MDA-TBA over a range of concentrations are shown in Figure 5.8, Figure 5.9 and Figure 5.10.

5.8.2.2 Results of the uptake and release of TEP from PureVision lenses measured by HPLC

The data in Table 5.8 shows values for the area under the peak at 2.4mins, corresponding to MDA-TBA complex. PureVision lenses were soaked in individual 5μmol/ml MDA solutions. The solutions were separated by HPLC with fluorescence detection after 0, 2, 4 and 24hours soaking and the area under the peak at 2.4mins (which represents MDA-TBA complex) was measured for each solution. The area under the peak for the release solutions were also measured.

Table 5.8 – Absorbance values for MDA uptake and release for PureVision lenses from a 2ml solution of 5μmol/ml MDA over time

Time (Hours)	*PureVision uptake from 5μmol/ml MDA solution (LU) (area under peak at 2.44mins)*	*Repeat of 'uptake' solutions*	*Release after 24 hours*
0	184.99	-	-
2	205.45	230.75	16.99
4	228.52	281.66	14.09
24	145.25	366.02	14.17

The results shown in Table 5.8 demonstrated many things. Firstly, the absorbance value for the uptake solution after 0hours soaking was higher than the absorbance value after 24hours soaking (Table 5.8). These results suggested MDA was not absorbed by the lenses, even after 24 hours of soaking in a 5μmol/ml MDA solution. The HPLC separation of the uptake solutions was repeated and these solutions produced significantly different results for the same samples. The absorbance values for the repeated uptake solutions were much higher than the values of the original solutions. For example, the absorbance for the 24hour uptake solution was measured and it was 145.25LU. However, when this same solution was re-analysed, the absorbance value was 366.02LU. This was more

than double the absorbance for the same solution. The uptake results varied considerably. The repeated absorbance values for the uptake solutions were all considerably higher than the original measurements. Therefore, the concentration of MDA up-taken by PV lenses could not be accurately calculated.

The absorbance values of the release solutions were also measured for PV lenses and they all showed positive results. The absorbance values for the release solution after 2hours soaking in MDA solutions was 16.99LU. This is the equivalent of approximately 295nmol/ml MDA.

Although the concentration of MDA absorbed by the lens could not be accurately determined, the release results proved that MDA was absorbed by PV lenses and subsequently released.

Both, the results obtained by HPLC separation and visual observation of the solutions after they were reacted with TBA showed that MDA was released from PV lenses after a 2hour soak. When the release solutions were reacted with the TBA buffer at 90°C for 60mins, all of the solutions were slightly pink in colour. The solution will only turn pink if MDA is present. Therefore, MDA was either absorbed into the lens matrix or adsorbed on the surface after 2hours soaking because there was definitely MDA in the release solutions.

As well as soaking the lenses in 5μmol/ml MDA solutions, fresh PV lenses were also soaked in 100nmol/ml solutions. This was to establish whether PV lenses still absorbed MDA even when soaked in lower concentrations of MDA. The solutions were separated by HPLC and the absorbance values were again obtained by measuring the area under the peak at 2.4mins and the absorbance values are shown in Table 5.9.

Table 5.9 – Absorbance values for MDA uptake and release for PureVision lenses from a 2ml solution of 100nmol/ml MDA over time

Time (Hours)	*PureVision uptake from 100nmol/ml MDA solution (LU) (area under peak at 2.44mins)*	*Repeat of 'uptake' solutions*	*Release after 24 hours*
0	4.34	-	-
2	4.40	5.10	1.42
4	4.87	6.50	1.44
24	12.4	6.12	1.34

Again, the absorbance values for the uptake solutions varied (shown in Table 5.9). The absorbance value for the 24hours uptake solution was significantly higher than the 0hour uptake solution (4.34 compared to 12.4). This suggested there was more MDA present in the solution after 24 hours than at 0hours, which is not possible. This showed that the results were not reproducible. Therefore, the concentration of MDA absorbed by PV lenses from a 100nmol/ml MDA solution could not be accurately calculated.

The absorbance values for the release solutions show MDA was released after a 2hour soak in a 100nmol/ml MDA solution (the absorbance value was 1.42LU, equivalent to 10nmol/ml MDA). All of the release solutions were pink once they had been reacted with TBA, which meant MDA was present. Therefore, MDA was absorbed by the lens even from a lower concentration solution.

5.8.2.3 PureVision uptake and release: Summary

- MDA was absorbed by PV lenses from both a 5μmol/ml MDA solution and a 100nmol/ml solution
- subsequently, MDA was released from PV lenses, although the uptake results did not show that MDA had been absorbed by PV lenses, the release values suggest that MDA was absorbed and then released

- This research is unique in showing that MDA can be absorbed by PV lenses and then released

5.8.2.4 Results for the uptake and release of TEP from Acuvue Oasys lenses measured by HPLC

The uptake and release of MDA from Acuvue Oasys lenses was investigated to determine whether a material change would affect the results.

The results shown in Table 5.10 and Table 5.11 represent the uptake and release absorbance values for Oasys lenses. The lenses were soaked in 5μmol/ml and 100nmol/ml MDA solutions for various amounts of time. After 0, 2, 4 and 24hours 5μl aliquots were taken, reacted with TBA and separated by HPLC. The area under the peak at 2.4mins was measured for each sample. The absorbance values for the uptake and release solutions are given in Table 5.10 and Table 5.11.

Table 5.10 – Acuvue Oasys uptake and release results in a 5μmol/ml solution

Time (Hours)	*Acuvue Oasys uptake from 5μmol/ml MDA solution (LU) (area under peak at 2.44mins)*	*Repeat of 'uptake' solutions*	*Release after 24 hours*
0	185.00	-	-
2	209.32	339.84	11.97
4	259.15	459.88	12.37
24	227.48	574.62	18.93

Table 5.11 – Acuvue Oasys uptake and release results in a 100nmol/ml solution

Time (Hours)	*Acuvue Oasys uptake from 100nmol/ml MDA solution (LU) (area under peak at 2.44mins)*	*Repeat of 'uptake' solutions*	*Release after 24 hours*
0	4.34	-	-
2	4.37	6.04	1.34
4	5.57	7.27	1.33
24	4.43	11.15	1.20

The results shown in Table 5.10 and Table 5.11 suggest that TEP is being released by Acuvue Oasys lenses but not up-taken by the lens.

TEP was released by the lens as the solution was slightly pink after the MDA-TBA reaction. The solution will turn pink only if MDA is present as it binds to TBA and forms a coloured complex. The results also show that TEP is released, but the amount up taken could not be accurately determined.

There were no observable differences between the two lens materials in their affinity for MDA. The absorbance values for all of the solutions tested were not very accurate.

The results in Table 5.8 to Table 5.11 indicated that this method of the measurement of MDA uptake and release from lenses was not very accurate. Therefore, repeat experiments were carried out on fresh solutions. The reproducibility was also checked without the added complication of having the contact lens in the solution.

5.8.2.5 Acuvue Oasys uptake and release: Summary

- The absorbance values suggest that MDA is released by Acuvue Oasys lenses but not up-taken
- MDA has to be absorbed by the lens to be released, therefore MDA is absorbed by Acuvue Oasys lenses, however the concentration up-taken cannot be accurately determined

- MDA is up-taken from both 5µmol/ml and 100nmol/ml MDA solutions
- There were no differences observed in the amount of uptake and release of MDA between PV and Oasys lenses

5.8.2.5.1 Reproducibility of the HPLC uptake and release technique

Further reproducibility tests were carried out to determine how useful the HPLC technique would be for the measurement of uptake and release of MDA from various lens materials.

Fresh 100nmol/ml standard was prepared by firstly preparing a 10µmol/ml tetraethoxypropane (TEP) in ethanol (24µl TEP in 10ml ethanol) in a 15ml centrifuge tube and diluting it with HPLC grade water. 6x100nmol/ml TEP solutions were prepared in 6 x 15ml centrifuge tubes. 5µl of each 100nmol/ml solution was transferred to 1.5ml eppendorfs containing 1ml TBA reagent. These were placed in an oven at 95°C for 1hr. They were then cooled for 5mins in cold water bath and then centrifuged. The upper 750µl from each sample was used for HPLC analysis. 5µl aliquots were taken from each 100nmol/ml standard and analysed at time intervals. The results are shown Table 5.12.

Table 5.12 – Reproducibility results of 100nmol/ml solutions

100nmol/ml Sample	*Area under 2.4 peak (LU) after:*			
	0hrs	***2hrs***	***4hrs***	***24hrs***
1	6.02	5.75	6.05	6.07
2	6.71	6.52	6.50	6.17
3	6.70	6.69	7.38	6.74
4	7.39	6.50	***9.01***	6.23
5	6.80	6.91	***9.08***	6.43
6	6.94	6.43	***9.36***	5.84

The absorbance values in the highlighted boxes represent samples that were not injected by the HPLC immediately after being centrifuged. They were analysed after 24hrs and this may have affected results, causing them to have a higher absorption value.

The average area under the peak for a 100nmol/ml solution was 6.51 LU (excluding shaded results) 6.84LU (including shaded results). The absorbance value for each sample was quite reproducible; therefore further uptake and release experiments were conducted. PureVision and Acuvue Oasys lenses were only soaked in 100nmol/ml TEP solutions and not 5μmol/ml solutions. The absorbance values for lower concentrations of TEP were more reproducible.

Uptake and release experiments were repeated to determine whether the results generated by HPLC separation were reproducible. A stock solution of 10μmol/ml (24μl TEP in 10ml ethanol) was prepared. 9900μl HPLC grade water was added to six 15ml centrifuge tubes. 100μl of the stock solution was added to each centrifuge tube making 6 x 100nmol/ml TEP solutions. 2ml of each solution was transferred to fresh centrifuge tubes ready for the lenses to be added.

The lenses used for these experiments were PV and Oasys because they are both known for high levels of lipid deposition. The lenses were removed from the blister packaging, blotted on filter paper and placed in HPLC grade water. The lenses were then removed from the water and blotted on filter paper. The lenses were then placed in the centrifuge tubes that contained 2ml 100nmol/ml TEP solutions. Individual PV lenses were added to 3 centrifuge tubes and Acuvue Oasys lenses were added to the other 3 centrifuge tubes.

5μl was taken from the tubes at 0hrs, 2hrs, 4hrs and 24hrs. This was reacted with TBA as described in Chapter 2, section 2.11.5.1.3.

The lenses from the 24hrs uptake solutions were removed and placed in separate centrifuge tubes each contained 1ml HPLC grade water for release.

Table 5.13 – PureVision uptake and release results in a 100nmol/ml solution

Time (Hours)	*PureVision uptake from 100nmol/ml MDA solution (LU) (area under peak at 2.44mins*	*Release after 24 hours*
0	5.38	-
2	5.31	-
4	4.87	-
24	5.23	1.58

Table 5.14 – Acuvue Oasys uptake and release results in a 100nmol/ml solution

Time (Hours)	*Acuvue Oasys uptake from 100nmol/ml MDA solution (LU) (area under peak at 2.44mins)*	*Release after 24 hours*
0	5.62	-
2	5.87	-
4	4.87	-
24	5.30	1.27

The absorbance values in Table 5.13 and Table 5.14 represent the uptake and release values for PV and Oasys lenses respectively. The absorbance values for both lenses (shown in Table 5.13 and Table 5.14) show that MDA is being absorbed by the lens and then released.

The release of MDA was only measured after 24hours uptake. The absorbance values suggest MDA was released after 24 hours uptake, for both lens materials. The absorbance values for uptake and release for both lens materials were similar. The results suggested that both lens materials (PV and Oasys) had a similar affinity for MDA.

Overall, the results obtained from HPLC separation were not very reproducible. In particular, the absorbance values corresponding to

uptake, for both lenses were not reproducible. Although the results were not very reproducible, it was clear that MDA was absorbed by the both lens materials and subsequently released.

As the results for the uptake and release of MDA obtained by HPLC separation were not very reproducible, this method was not used for further analysis. However, the results did reveal that MDA is absorbed by both lens materials and then released. Therefore, uptake and release of MDA was now measured directly by UV absorbance rather than HPLC separation and then fluorescence detection. The methodology and results are shown in section 5.9.

5.8.3 Uptake and release of MDA using HPLC: Summary

- The HPLC method for the measurement of MDA uptake and release was not very accurate
- However, it was still determined that MDA was up-taken and released by both lens materials
- MDA was absorbed by both lens materials from both a 5µmol/ml MDA solution and a 100nmol/ml MDA solution
- There were no significant differences in uptake and release results between PureVision and Acuvue Oasys lenses. Both lenses seem to have a similar affinity for MDA

5.9 UV and fluorescence absorbance of MDA

The HPLC methodology for the separation the MDA-TBA adduct was not a very reproducible method and was not sensitive enough to detect MDA in tear samples or contact lens extracts. Therefore, the same method was used but without the chromatographic separation (i.e. direct UV absorbance).

0.2% (w/v) TBA buffer in 0.1M sodium acetate buffer was prepared as described in Chapter 2 (section 2.11.5.1.1).

MDA standards were prepared at the same concentrations as shown in Chapter 2, Table 2.7. 10µl of each standard was added to 1ml TBA buffer and placed in an oven for 60mins at 90°C. The solutions were allowed to

cool in a water bath for 5mins. The UV absorbance of each standard was measured. As the maximum absorbance was not known, a full wavelength scan was taken. The maximum UV absorbance was found to be at between 530-535nm. A wavelength scan of the TBA buffer was taken to ensure its absorbance did not interfere with the MDA peak (shown in Figure 5.12).

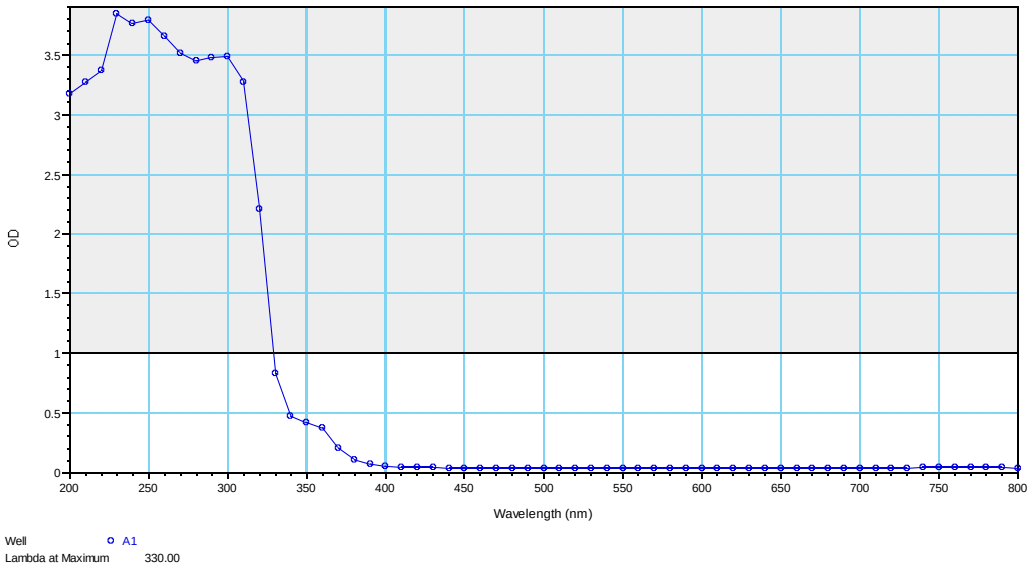


Figure 5.12 – Wavelength scan between 200nm and 800nm of 0.2% TBA (w/v) in 0.1M sodium acetate buffer

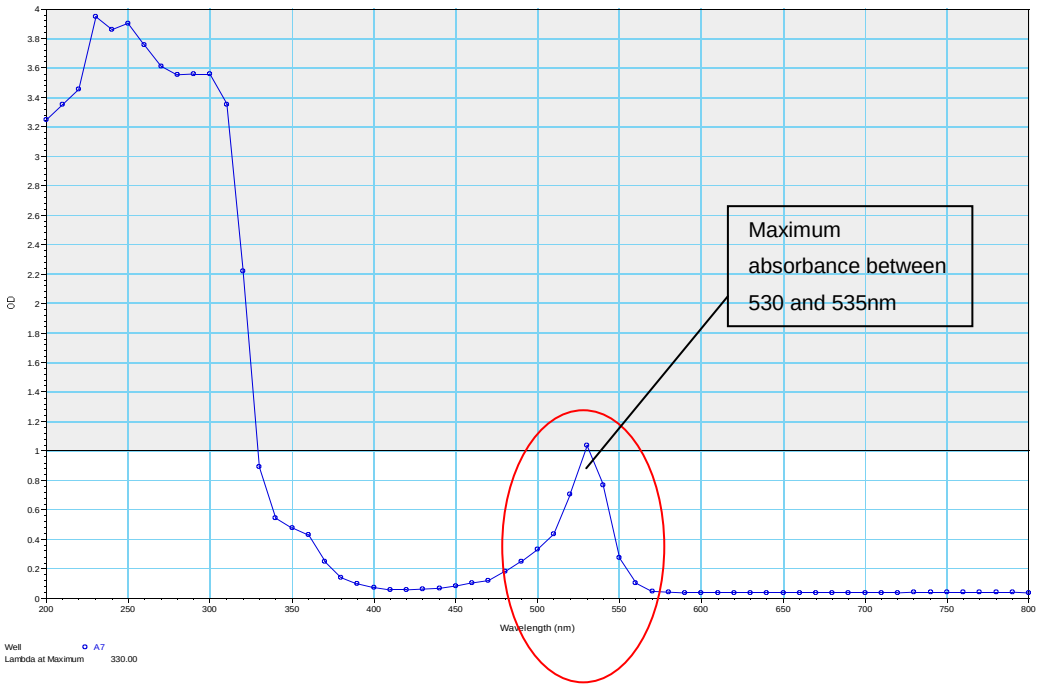


Figure 5.13 – Wavelength scan of 20nmol/ml TEP reacted with 0.2% TBA (w/v) in 0.1M sodium acetate buffer between 200nm and 800nm

The maximum absorbance for TEP-TBA adduct was found to be between 530 and 535nm. Therefore, the UV absorbance for all standards and unknowns were measured at this wavelength. The calibration curve shown in Figure 5.14 represents the UV absorbance for TEP-TBA over a range of concentrations.

Table 5.15 – 10µl TEP standards reacted with 1ml TBA buffer absorbance data at 532nm

TEP concentration (nmol/ml)	*Absorbance*
200	0.179
100	0.092
50	0.059
40	0.033
30	0.026
20	0.019
1	0.006
0.5	0
0.1	0
0.01	-0.001

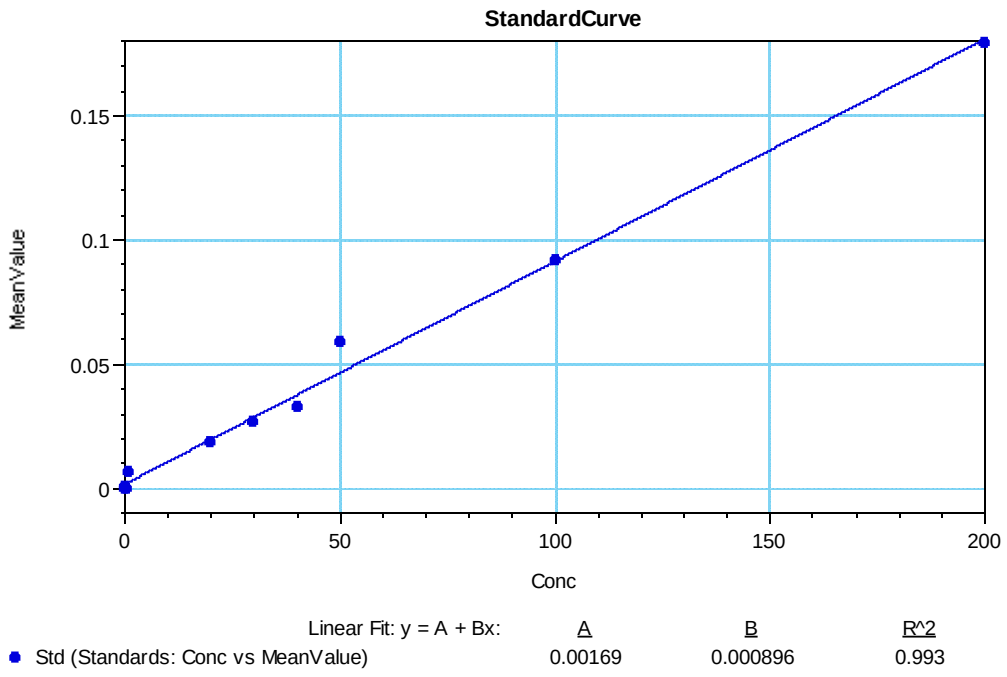


Figure 5.14 – 10µl TEP standard calibration curve measured at 532nm

The concentration of MDA in unknown samples was determined from the calibration curve shown in Figure 5.14. The samples tested were *ex vivo* daily wear and continuous wear PureVision (PV) and Focus Night and Day (F.N&D) lens extracts. These particular lens materials were used because the work discussed in Chapter 4 had shown that there were clear differences in fatty acid profile between wear schedules. GCMS results showed that unsaturated fatty acids had degraded on lenses that were worn on a continuous wear schedule. Therefore, it was important to investigate these lenses for the presence of MDA or lipid oxidative products.

5.9.1 UV absorbance technique for measurement of MDA

Daily wear and continuous wear PureVision and Focus Night & Day lenses were analysed for MDA using the UV absorbance. The three extraction protocols described in Chapter 2, sections 2.8.1 to section 2.8.3 were used to extract both daily wear and continuous wear PV and F.N&D lenses.

None of the *ex vivo* PV or F.N&D lens extracts analysed (daily wear or continuous wear) produced positive results for the presence of MDA. The UV absorbance method was not sensitive enough for the measurement of MDA in these particular extracts.

5.10 Induced lipid oxidation

To test the reproducibility of the UV absorbance technique, linolenic acid (18:3 ^{9,12,15}) standard was left exposed to oxygen. This induced the oxidation of linolenic acid which would produce MDA. This oxidised linolenic acid was analysed by the UV absorbance technique for the presence of MDA. The method used for inducing oxidation is described in Chapter 2, section 2.12.

The oxidised linolenic acid solution had turned a dark pink colour indicating MDA had been produced. The un-oxidised linolenic acid was a clear solution. 200µl of each sample was transferred to a 96 well plate and the absorbance read at 532nm. The un-oxidised lipid had an

absorbance value of 0.173 which corresponded to approximately 75nmol/ml MDA. The oxidised linolenic acid samples had an absorbance value of 2.981 which would correspond to more than 200nmol/ml MDA (the most concentrated standard analysed). The results showed that the so called 'un-oxidised' linolenic acid sample produced MDA, which meant it had also oxidised. But not to the extent of the linolenic sample which was left exposed to oxygen for 24 hours. Although attempts were made to try and prevent the oxidation of the linolenic acid sample, the slightest exposure to oxygen caused oxidation to occur.

This experiment proved that the concentration of MDA could be measured by the UV absorbance technique. This technique combined elements of the TBARS assay and the HPLC method. The UV absorbance method was therefore useful for measurement of MDA at higher concentrations. However, it was not sensitive enough to detect MDA in the samples analysed.

5.11 Discussion

The aims of the work discussed in this chapter were to find a single or multiple methods for the measurement of lipid peroxidation products in the ocular environment (either tears or from contact lens extracts).

Many commercial available assays that are used for the measurement of MDA in various blood, urine and plasma samples were used in this work to measure MDA in the ocular environment. These assay kits were not designed to measure lipid peroxidation products in tears. They included the TBARS, NMPI, ELISA assays kits.

Analytical techniques such as HPLC and capillary electrophoresis were also used to enable the measurement of MDA. Both, HPLC and capillary electrophoresis have been used by other researchers for the measurement of MDA (134, 151). Although HPLC was not used for MDA measurement in tears in the literature, the small sample volume requirement (5μl) made this method ideal for the analysis of tears.

Capillary electrophoresis was used for the separation of MDA from tear samples in the literature.

Many of the assays used required at least 100μl of sample. Therefore all of the samples collected; tear samples, tear envelope and lens extracts had to be either pooled or made up to the required volume with either water or PBS.

From the initial results, both the TBARS and ELISA assays provided positive results for certain samples (Table 5.3 and Table 5.5 respectively). The initial ELISA assay provided positive results for contact lens extracts which indicated that MDA was in the contact lens matrix. Further MDA-ELISA results showed that *ex vivo* stored lenses showed a higher concentration of MDA compared with freshly worn *ex vivo* contact lens extracts (21pmol/ml – stored Air Optix lens compared to 4pmol/ml freshly worn Air Optix lens extract). This was a significant finding as it showed that the concentration of MDA was lower in lenses which had just been worn and extracted immediately. This was compared to lenses which had been worn and then stored and these lenses showed a significantly higher concentration of MDA (21pmol/ml). This work was novel, as this assay had not previously been used to assess the levels of MDA in the ocular environment. It was also novel as the concentration of MDA had not been measured from contact lens extracts.

TBARS results in Table 5.2 and Table 5.3 show that there were detectable levels of MDA from particular subjects tear samples and tear envelope samples. The initial results (shown in Table 5.2) showed levels of MDA between 1.0-2.5μM for tear samples.

The follow-up results (shown in Table 5.3) showed positive and negative results for tear samples and tear envelope samples. The results for the tear sample collected from Subject 1 showed positive levels of MDA from one eye and negative from the other. Many of the tear samples analysed by TBARS showed negative results, however some showed positive results.

The TBARS assay recommended preparing a new calibration curve each time the assay was used. However, the absorbencies for known concentrations of MDA varied each time the calibration curve was prepared. Therefore, the absorbance results for unknown samples were compared to one calibration curve, shown in Figure 5.1. Therefore, the true concentration of MDA in unknown samples was not very reproducible and this assay was not very accurate. Although the TBARS assay was not very sensitive, it did provide a measure of general lipid oxidation, even though it may not have been specific for MDA levels.

The NMPI technique produced negative results for tear samples, tear envelope samples and contact lens extracts. This assay used third derivative spectroscopy which was to increase specificity and sensitivity of the data. As this assay provided negative results for all unknown samples, it was not used for any further experiments. However, a similar assay was used by Glasson *et al.* (121). They analysed the tears of contact lens tolerant and intolerant subjects using a similar assay to the one used in our research. Glasson *et al*. discovered that the levels of MDA were higher in the tears of contact lens intolerance subjects.

All of the tear samples analysed using the NMPI assay in this work did not produce any positive results.

Both of the analytical techniques; capillary electrophoresis and HPLC did not produce any positive results for test samples. Capillary electrophoresis was chosen for MDA analysis as had been previously used for the measurement of MDA and antioxidant levels in tears and therefore a similar protocol was followed (151). The MDA standard, from the TBARS assay, was used to establish a standard peak on the capillary electrophoresis. There was a small peak that corresponded to MDA at 20mins with an absorbance of ~2.5mAU. However, the concentration of MDA standard used was very high and it produced a peak with a small absorbance, therefore capillary electrophoresis was not considered a sensitive enough technique. Georgakopoulos *et al*. (151) used capillary electrophoresis for the separation and identification of MDA in tears. The

MDA peak was detected at ~22mins. MDA was detected in the tears of 7 out of 11 tear samples analysed, however the levels were below the lower limit of quantification for all subjects. Also, the intensity of the peak that corresponded to MDA was very low (approximately 1mAU).

The capillary electrophoresis method that was used in this research was similar to the method used by Georgakopoulos *et al*. (151). However this method did not prove to be very sensitive for the analysis of MDA.

The other analytical technique used for the separation and identification of MDA was HPLC with fluorescence detection. This method was used because a small sample volume of 5µl could be used for analysis. This method provides a more accurate measure of the levels of MDA in unknown samples than TBARS assay alone. The HPLC method is based on a reaction between MDA and TBA. TBA can bind to other molecules such as aldehydes. Therefore, the TBARS assay can over-estimate the levels of MDA. However, only MDA bound to TBA will produce a peak at a characteristic retention time by HPLC separation. Therefore, the HPLC method provides a more accurate measure for the actual concentration of MDA.

HPLC separation of MDA showed a lower limit of detection of approximately 10nmol/ml. However, the TBA buffer used in this method had an absorbance at approximately the same retention time as the MDA-TBA adduct. It was therefore difficult to establish the between the TBA buffer and MDA-TBA at low concentrations. Tear samples and contact lens extracts were analysed using the HPLC method, but they did not produce any positive results. This method was therefore not further used for the analysis of worn contact lens extracts.

HPLC was used to enable the measurement of the *in vitro* uptake and release of MDA of various lens materials. The area under the peak was used to determine the concentration of MDA up-taken and released by various lenses. The lenses chosen were PV and Oasys. The HPLC method was not very accurate and therefore a difference was not observed in the levels of uptake and release between the two lens

materials. There were low levels of MDA absorbed by both lens types as there was a low amount released. This was evident from the absorbance values of the release solutions shown in Table 5.13 and Table 5.14.

Visual observation of the solutions after they had been reacted with the TBA buffer showed that MDA was released by both lenses as the solutions had turned pink (indicating the presence of MDA).

However, this method was not sensitive enough to determine an accurate measure of the concentration absorbed by the lens and subsequently released by the lens. This was the case for both lens materials. The HPLC method was therefore not used for further work.

As the HPLC separation technique was not very accurate, a similar procedure was followed, but the samples were not separated by HPLC. Instead, the unknown samples and MDA standards were reacted with the TBA buffer and the UV absorbance measured directly. This method had a lower limit of detection of 1-10nmol/ml. Worn PV and F.N&D lens extracts that were either worn on a continuous wear or daily wear schedule did not produce any positive results by the UV absorbance technique. This meant that either there was no MDA present or that it was below the detection limit. These samples were kept at -20°C for analysis using the MDA-ELISA assay as it was proved to be the most sensitive. The same samples did not produce any positive results when analysed by the MDA-ELISA assay.

5.12 Conclusions

MDA is one of the most studied biomarkers of lipid oxidation. It is produced from the oxidation of polyunsaturated fatty acids (PUFAs) and the pathway of MDA production is shown in Figure 1.29. High levels of PUFAs have not been previously detected in meibomian gland secretions, however, as already shown, lipids build-up within the contact lens matrix and are exposed to oxidative attack.

The presence of MDA in tears has been previously linked to contact lens intolerance in the literature. Therefore, the aims of the work discussed in

this chapter were to find a single or multiple methods for the analysis of MDA in the ocular environment. The most significant findings from the work discussed in this chapter are listed below:

- several commercially available assays and analytical techniques were used for the analysis of MDA, overall, the MDA-ELISA technique was the most sensitive technique
- although capillary electrophoresis has been used to detect MDA in tears in the literature, this technique was not sensitive enough for the detection of MDA in any sample tested in this research
- the TBARS assay is widely used for MDA detection in various samples (blood, urine, plasma); however it was not as sensitive as the MDA-ELISA technique
- the NMPI method did not detect MDA in any sample tested in this research, although it had previously been used for MDA detection in tears by Glasson *et al*. (121)
- a major finding of the research discussed in this chapter was that MDA was found to build-up in the contact lens matrix. This had never previously been shown
- the most effective extraction protocol for MDA from lenses was extraction protocol 1 (1:1 chloroform: methanol extraction). The structure of MDA suggested it would be water soluble; however MDA was not extracted by water or saline. Because MDA was only extracted using solvents, this accounted for MDA build-up within the lens matrix

Further work is required to determine whether increased levels of MDA for particular subjects correlate with contact lens intolerance and/or contact lens comfort. However, the work discussed in this chapter has shown that MDA and can build-up in the lens matrix and this has not been previously studied.

Chapter 6 – Phospholipid-containing contact lenses: *in vitro* and *in vivo* studies

6.1 Introduction

A large proportion of contact lens wearers report symptoms of discomfort, which are similar to those symptoms experienced by dry eye patients. This type of dry eye is often called contact lens induced dry eye (1, 152). Dry eye can be a pre-existing condition or can be caused by the contact lens and this is discussed in detail in Chapter 1 section 1.6. Contact lens induced dry eye is often associated with the disruption of the tear film and in particular, the disruption of the tear lipid layer (127). Correlations have also been made between a lack of phospholipids and irregular function of the lipid layer, ultimately leading to signs of dryness (95, 121, 127, 153, 154). There are many therapies used for the treatment of dry eye such as dietary intake of omega-3 fatty acids (86), physical stimulation of the meibomian glands, drug therapy, topical treatments (84, 86) and the use of eye drops (discussed in detail in section 1.6),

A new type of contact lens has been developed for the treatment of dry eye. Dailies Total 1 (delafilcon A) lenses, produced by CIBA vision are a new daily disposable silicone hydrogel contact lens material. They contain a phospholipid, dimyristoyl phosphatidylcholine (DMPC) which is said to be released into the tear film lipid layer and enhance comfort throughout the day.

There has been a recent publication by Pitt *et al*. (95) which investigates the release of phospholipid from a phospholipid-containing contact lens into an artificial tear fluid. The work by Pitt *et al.* used radiolabelled DMPC to monitor the release of DMPC from the lenses into an artificial tear fluid. However, these *in vitro* conditions did not necessarily mimic *in vivo* conditions.

The work discussed in this chapter was therefore based on trying to establish whether DMPC would be released to the tear film during wear. In order to do this, the work involved trying to find a suitable extraction procedure that would mimic *in vivo* conditions.

GCMS was used for analysis of worn and unworn phospholipid containing lenses because it was able to separate the C14:0 (the fatty acid moiety) of the DMPC.

6.2 Batches of lenses

All three batches of lenses were supplied by the manufacturer.

Batch 1 lenses: Clinical lenses with and without DMPC.

Batch 2 lenses: Non-clinical lenses with and without DMPC. With the addition of two different additives: Processing aid 1 and 2 (IPC-3C and IPC-4A lenses).

Batch 3 lenses: Non-clinical lenses with and without DMPC and processing aid 3 (LPEG – a polyethylene glycol like molecule)

Dailies Total 1 clinical lenses: CIBA vision daily wear silicone hydrogel lens, Dailies Total 1 (delafilcon A) that contain DMPC

This work involved the development of an extraction procedure which would mimic the extraction of DMPC into the tear film. GCMS was used for the analysis of lens extracts as it could monitor the presence of the C14:0 peak at the retention time of approximately 10.3mins.

Several extraction protocols were tested in order to try to mimic the extraction of DMPC which may occur in the eye, however this proved very difficult to do for several reasons. If an artificial tear like fluid was used for the extraction of the lens, the entire extract solution would have to be transmethylated and analysed by the GC. As the GC is a very sensitive technique, this had the possibility of over-loading the column. This was the case for many solvents or chemicals used for the extraction of DMPC from the lens. Therefore, the solvents chosen for extraction had to be volatile so they could be evaporated to dryness and the extract could be transmethylated, rather than the transmethylation of the entire solution. However, these solvents are considered to be harsh and they do not mimic the *in vivo* extraction conditions of DMPC.

The main analytical technique used for this work was GCMS. GCMS enabled the separation and identification of the peak that corresponded to C14:0 (from DMPC) from lens extracts. The area under the C14:0 peak was quantified to determine the concentration of C14:0 from unworn and worn lens extracts. A calibration curve of DMPC standard at different concentrations against C14:0 peak area was prepared which allowed the concentration of DMPC extracted from the lenses to be calculated. After this, non-clinical lenses were extracted by various extraction protocols and they were analysed by GCMS. *Ex vivo* clinical lenses were extracted and analysed as well as tear samples taken after the wear of DMPC-containing lenses to establish whether DMPC had been released into the tear film.

6.3 Results

6.3.1 Calibration curve of DMPC separated by GCMS

The gas chromatograms shown in Figure 6.1 represent different concentrations of standard DMPC. The predominant peak at approximately 10.3mins corresponds to C14:0. When DMPC is transmethylated, the C14:0 fatty acid is converted to a C14:0 fatty acid methyl ester and this is separated by GCMS. The area under each peak was measured and a calibration curve was prepared (shown in Figure 6.2).

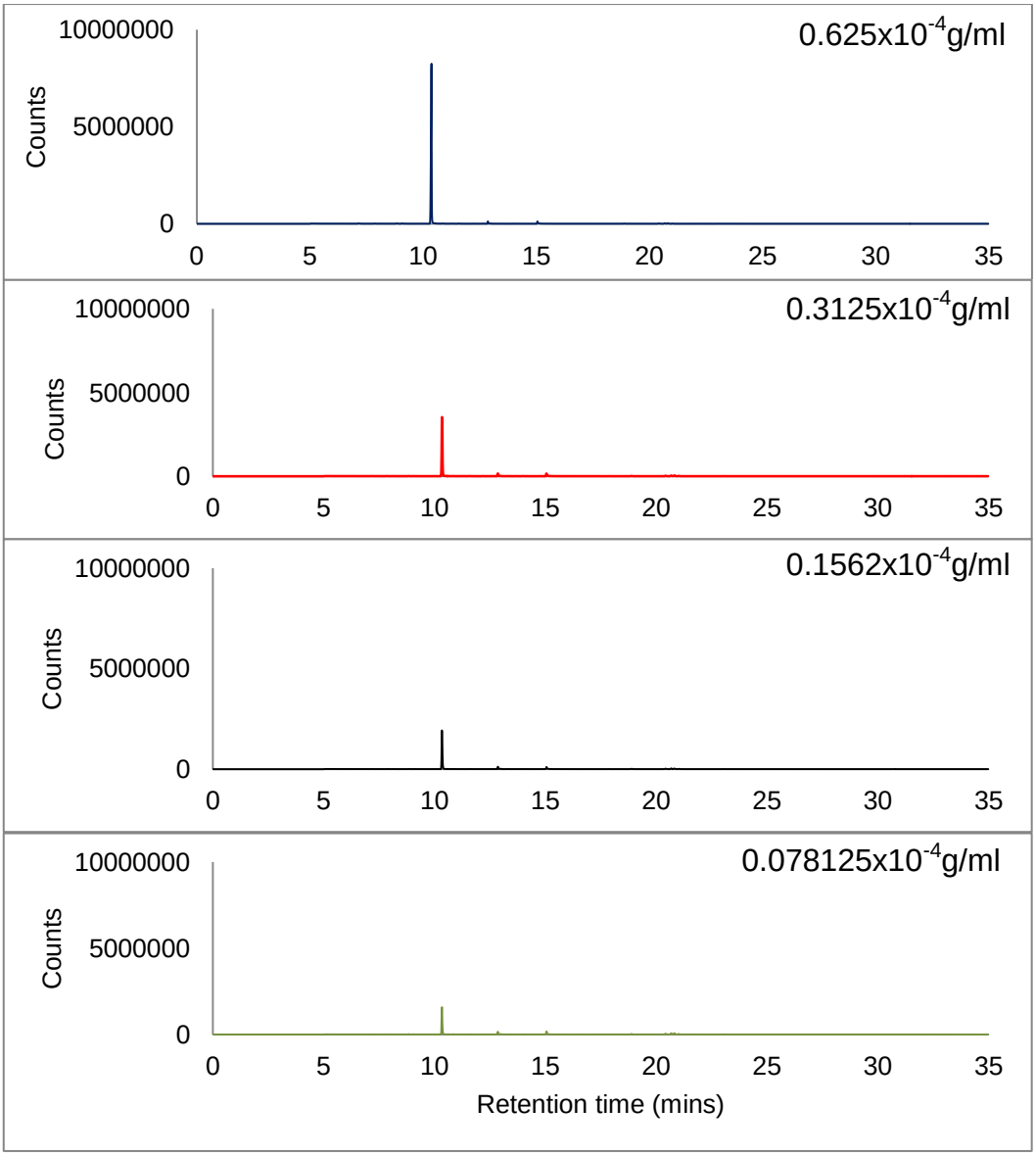


Figure 6.1 – Gas chromatograms of standard DMPC at various concentrations

Table 6.1 – Calibration curve data for standard DMPC measured by area under peak at 10.3mins measured by GC

DMPC concentration (g/ml) ($x10^{-4}$)	*Area under peak at 10.3mins*
0.625	18600000
0.3125	9250000
0.1562	5217000
0.078125	3921000

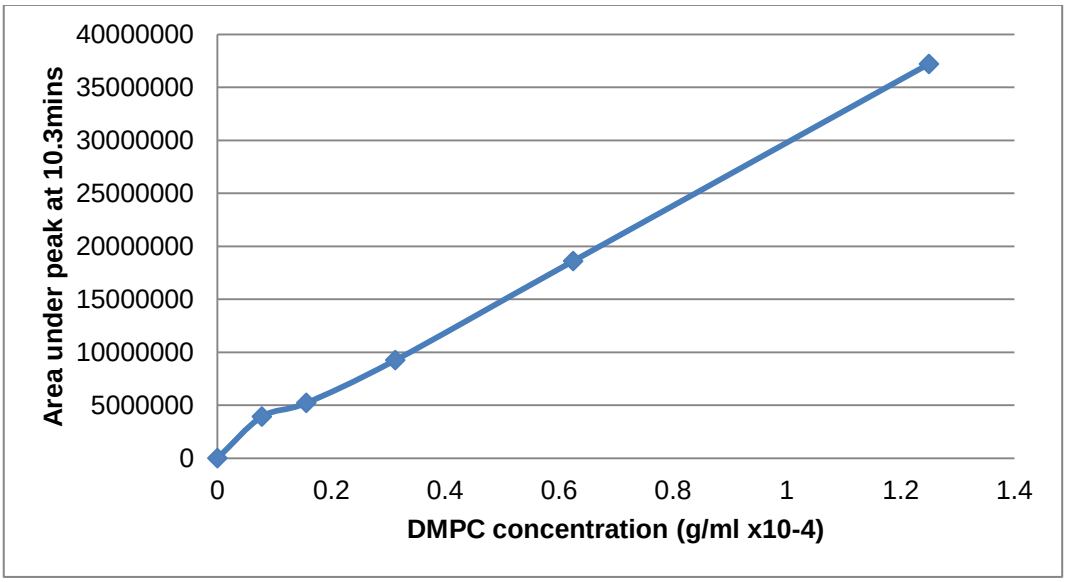


Figure 6.2 – Calibration curve for DMPC measured by area under peak at 10.3mins on gas chromatogram

6.3.2 Batch 1 results:

The chromatograms shown in Figure 6.3 represent batch 1 DMPC-containing lens extracts (unworn and worn for 2hrs). The circled peaks correspond to C14:0.

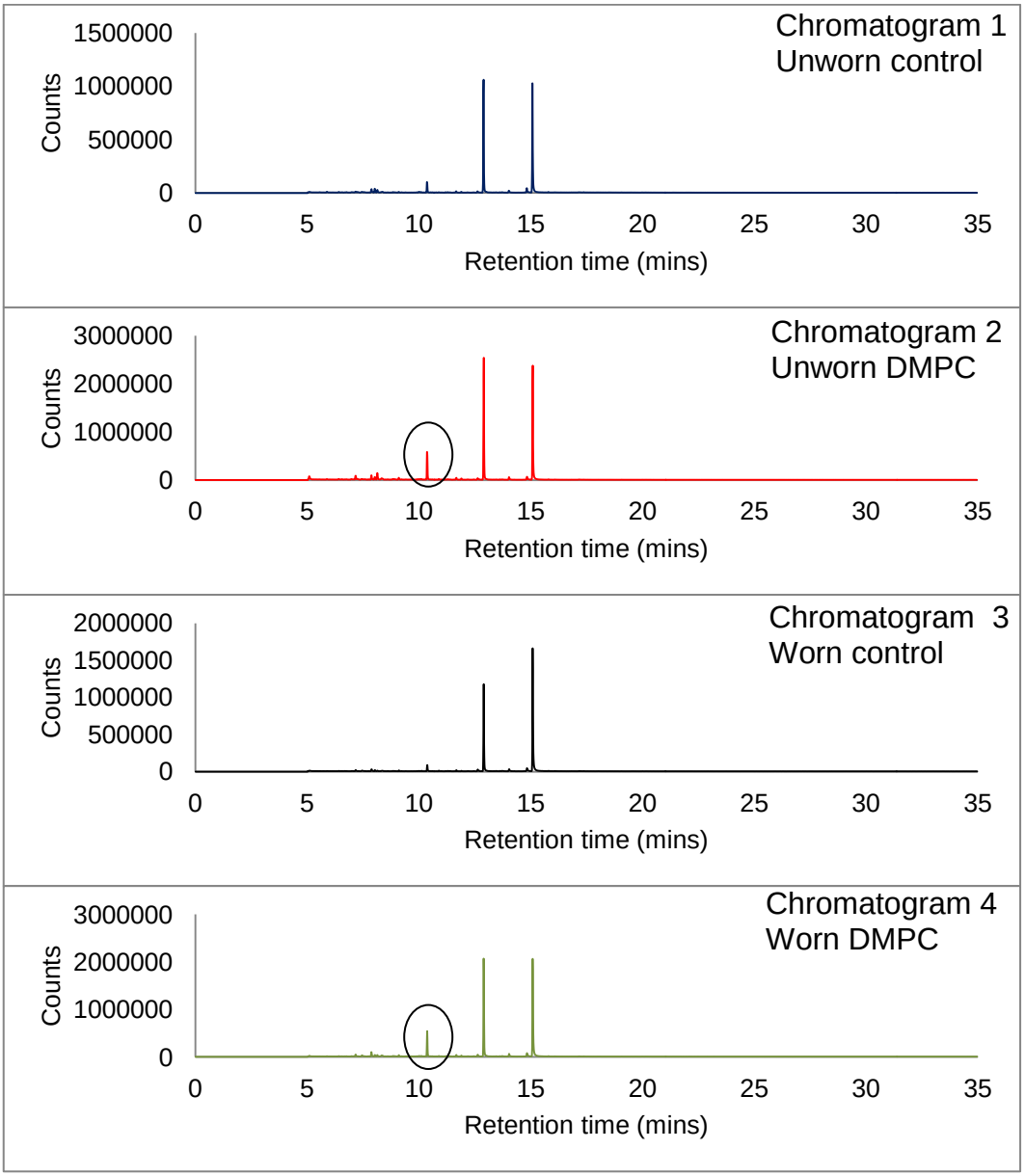


Figure 6.3 – Gas chromatograms of batch 1 lens extracts extracted in 1:1 chloroform: methanol where: chromatograms 1 and 2 are unworn control lenses without and with DMPC respectively and chromatograms 3 and 4 are worn control lenses without and with DMPC respectively

The circled peak on chromatograms 2 and 4 corresponds to C14:0 from the DMPC. There was a slight difference in the area under the peak between worn and unworn lens extracts. The concentration of DMPC in

unworn lenses was $0.025x10^{-4}$g/ml. The concentration of DMPC remaining in an *ex vivo* lens was $0.020x10^{-4}$g/ml. This suggested that a small amount of DMPC was delivered to the tear film; however the majority of DMPC remained in the lens after wear.

The other two peaks observed on the chromatograms in Figure 6.3 correspond to C16:0 and C18:0. These are common peaks observed for unworn silicone hydrogel contact lens extracts (see Figure 3.6).

Table 6.2 – Analysis of the GC traces shown in Figure 6.3

Chromatogram	*Concentration of C14:0*
Unworn lens extract (chromatogram 2)	$0.025x10^{-4}$g/ml
Worn lens extract (chromatogram 4)	$0.020x10^{-4}$g/ml

6.3.3 Batch 2 (non-clinical) results:

Batch 2 lenses were non-clinical lenses (not approved to be worn). Batch 2 lenses were extracted using several extraction protocols to find a suitable procedure to mimic *in vivo* conditions.

The chromatograms in Figure 6.4 represent lens extracts of batch 2- with/without DMPC lenses. Extraction protocol 1 (1:1 chloroform: methanol) was used for the extraction. The circled peaks in chromatograms 2, 4 and 6 correspond to C14:0.

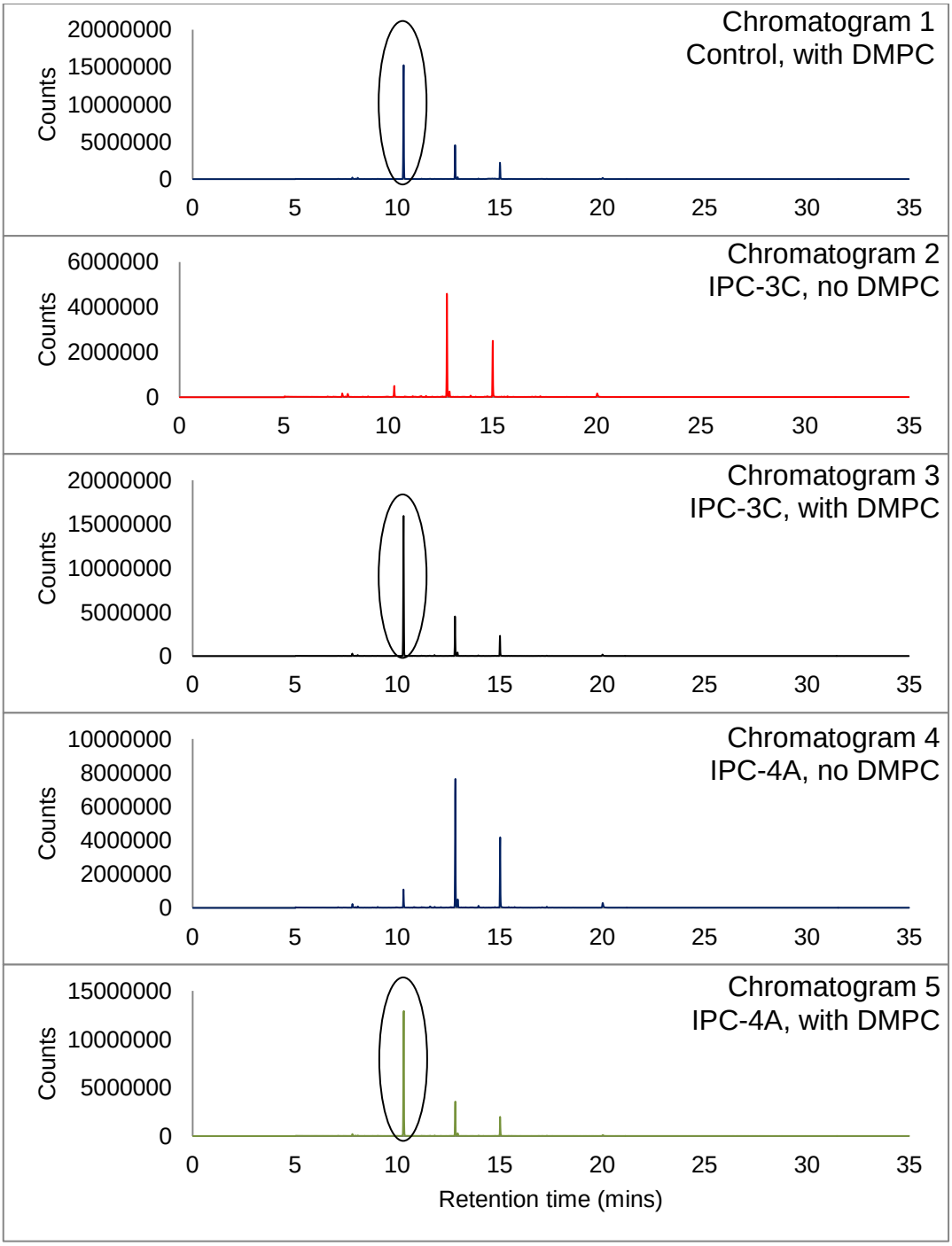


Figure 6.4 – Gas chromatograms of batch 2 lens extracts extracted in 1:1 chloroform: methanol. Where: chromatograms 1 and 2 are control lens extracts without and with DMPC respectively, chromatograms 3 and 4 are IPC-3C lens extracts without and with DMPC respectively and chromatograms 5 and 6 are IPC-3C lens extracts with and without DMPC respectively

The circled peaks in chromatograms 2, 4 and 6 represent C14:0. There is considerably more DMPC in these lenses compared with batch 1 lenses. This can be seen from the intensity of the C14:0 peak. The concentration

of DMPC in this particular control lens was approximately $0.90x10^{-4}$g/ml (chromatogram 2). The concentration of DMPC in the IPC-3C lens was determined to be $0.97x10^{-4}$g/ml (chromatogram 4). The concentration of DMPC in this IPC-4A lens was worked out to be $1.08x10^{-4}$g/ml (chromatogram 6). The concentration of DMPC in batch 2 lenses was considerably more than in batch 1 lenses ($0.025x10^{-4}$g/ml).

Batch 2 lenses were extracted using different extraction protocols. The IPC-3C lenses, with and without DMPC were used to investigate the efficacy of various extraction procedures because there were several of these lenses available for analysis.

Although the chromatograms for different extraction protocols are not shown, the peak areas representing the C14:0 peak were analysed and the results are shown in Table 6.3. Butanol was successful for the extraction of some of the DMPC, however the results show that further DMPC was extracted from the lenses that had already been extracted in butanol.

Octanol was used for extraction, however it is not volatile, therefore part of the extract was transmethylated and analysed by GCMS. The chromatograms did not show the peak corresponding to C14:0, therefore octanol was not effective for DMPC extraction. These lenses were then extracted in chloroform: methanol and the chromatograms showed that the majority of DMPC was extracted in this extraction and not in the octanol extraction ($1.77x10^{-4}$g/ml).

Table 6.3 – Analysis of batch 2 GC traces

Lens description	*Extraction solvent*	*Concentration of C14:0 extracted from batch 2 lenses*
Control	Chloroform: methanol	$0.90x10^{-4}$g/ml
IPC-3C	Chloroform: methanol	$0.97x10^{-4}$g/ml
IPC-4A	Chloroform: methanol	$1.08x10^{-4}$g/ml
IPC-3C	Butanol	$0.20x10^{-4}$g/ml
IPC-3C	glycerol	Below limit of detection
IPC-3C	methanol	$0.5x10^{-4}$g/ml
IPC-3C	hexane	Below limit of detection

6.3.3.1 Batch 2 results: Summary

The main aim of the work in section 6.3.3 involved trying to find a suitable extraction procedure to mimic *in vivo* conditions. This was difficult to achieve because the extraction solvent needed to be volatile so that it would evaporate under nitrogen in order for the extract to be transmethylated for GC analysis. However, if a tear-like fluid was used for extraction, the entire extract would have to be transmethylated for GC analysis as it would not be volatile. The fatty acids, in the tear-like fluid used for extraction would be converted to methyl esters and would be separated by GC. It would be difficult to detect C14:0 corresponding to the DMPC because the intensity of the peaks from the tear-like fluid would suppress any extracted material from the lens. This is why a tear-like fluid was not used because it had the potential to over-load the column.

- Butanol was used because it has a slightly longer hydrocarbon chain length than methanol but is still volatile. It was effective for the extraction of DMPC from DMPC-containing lenses; however it did not extract all of the DMPC from the lens.
- Octanol was used for the extraction of DMPC because it has a long hydrocarbon chain (trying to mimic *in vitro* extraction), but it was not volatile enough. The whole extraction was transmethylated and analysed, but octanol was not effective for DMPC extraction

- Ethyl acetate and glycerol were not effective for the extraction of DMPC
- The concentration of DMPC in batch 2 lenses was much higher than in batch 1 lenses (Figure 6.3).

6.3.4 Batch 3 results:

The next set of DMPC-containing lenses were extracted and analysed by GCMS. These lenses were not clinical lenses, and were not approved for wear.

The chromatograms in Figure 6.5 represent batch 3 with/without DMPC lens extracts with/without additive 3. The lenses were extracted in 1:1 chloroform: methanol (extraction protocol 1) or 100% methanol.

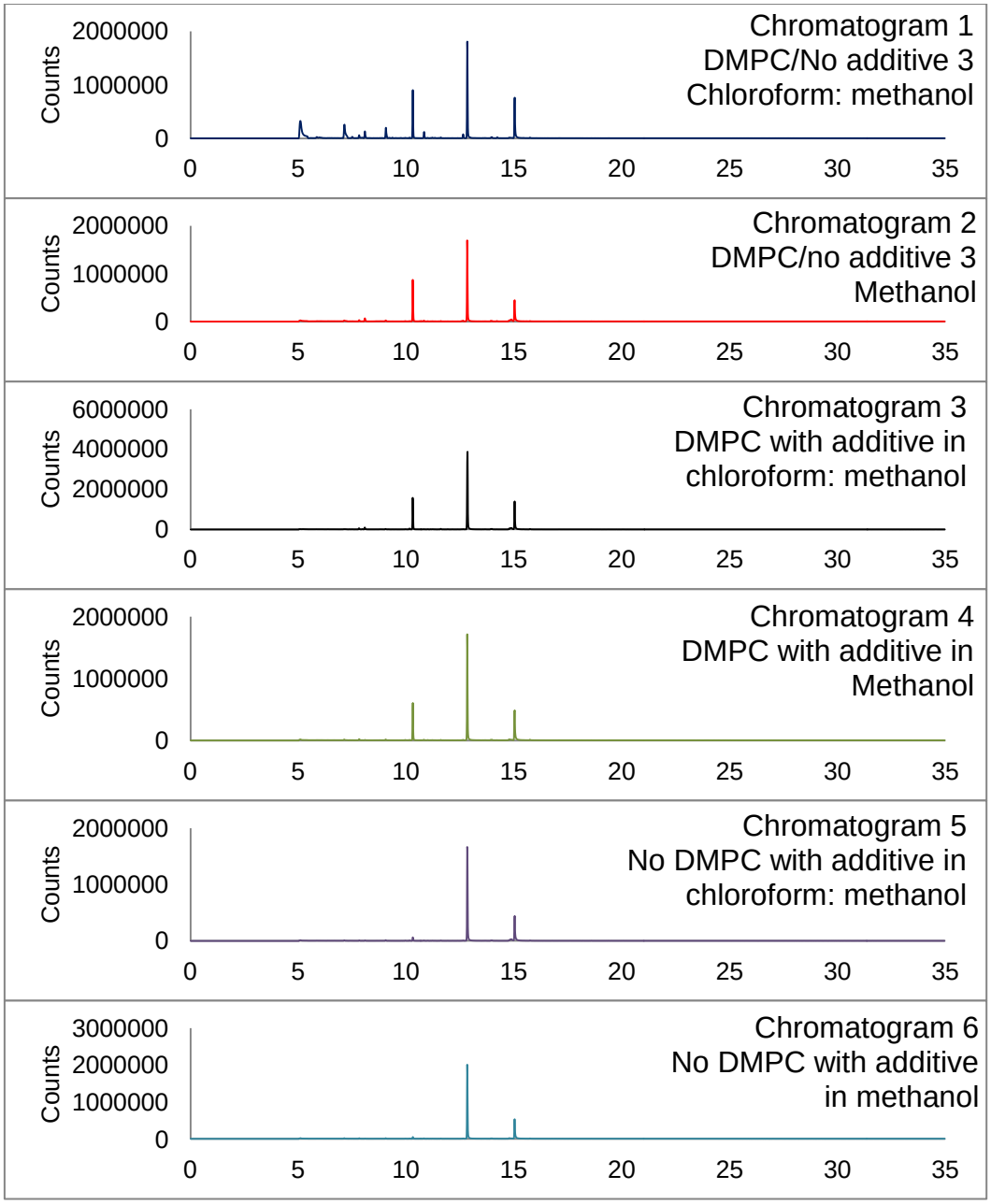


Figure 6.5 – Gas chromatograms of batch 3 lenses extracted in either 1:1 chloroform: methanol or 100% methanol

The chromatograms in Figure 6.5 show that 1:1 chloroform: methanol and 100% methanol extraction procedures were effective at extracting DMPC from batch 3 lenses. The C14:0 peak was present on all chromatograms representing DMPC-containing lens extracts. The concentration of DMPC in batch 3 lenses was between 0.038×10^{-4}g/ml and 0.10×10^{-4}g/ml. This was less than the batch 2 lenses. Peaks corresponding to C16:0 and

C18:0 (at 12.8 and 15.0mins) were present for all extracts. These peaks are common for unworn silicone hydrogel lenses (see Figure 3.6).

6.3.5 Dailies Total 1and Acuvue TruEye extraction results

The chromatograms in Figure 6.6 represent unworn Dailies Total 1 and Acuvue TruEye lens extracts. Acuvue TruEye (narafilcon A) lens extracts were analysed because they are also daily disposable silicone hydrogels lenses, however they do not contain DMPC.

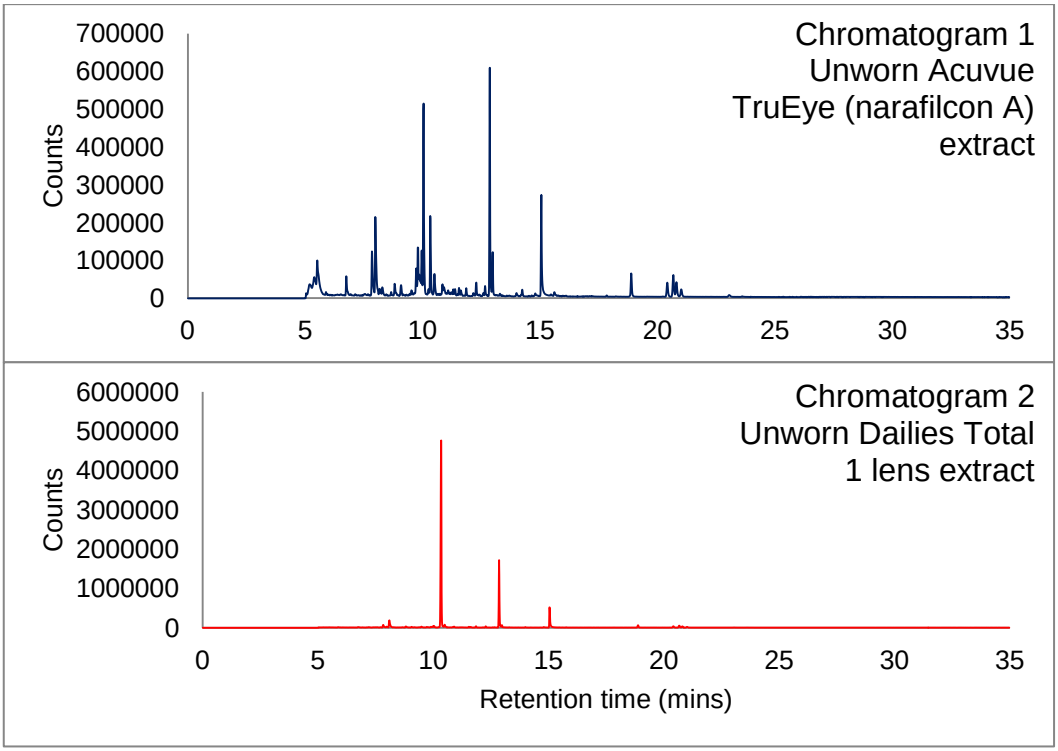


Figure 6.6 – Gas chromatograms of lenses extracted using protocol 1 where: Chromatogram 1 is an unworn Acuvue TruEye lens extract and Chromatogram 2 is an unworn Dailies Total 1 lens extract

The two chromatograms in Figure 6.6 represent an Acuvue TruEye lens extract and Dailies Total 1 lens extract. The two traces are very different from each other. There are many peaks on the gas chromatogram for an Acuvue TruEye lens extract (chromatogram 1). The peak at 7.9mins corresponded to C10:0 and it was identified by its retention time and mass spectra. This lens is known to contain low levels of decanoic acid (C10:0). The peaks at 10.0 and 10.3mins were not identified as their mass spectra did not correspond to known structures. The peaks at 12.8mins and 15.0mins correspond to C16:0 and C18:0 respectively. These are

common fatty acids seen observed on the chromatograms of many unworn contact lens extracts (see Figure 3.6 for gas chromatograms representing unworn contact lens extracts).

Figure 6.6, chromatogram 2 represents an unworn Dailies Total 1 lens extract. The predominant peak observed at approximately 10.3mins corresponds to C14:0, which is the fatty acid moiety of DMPC. There are also peaks at 12.8mins and 15.0mins which correspond to C16:0 and C18:0 respectively. These peaks were observed for many unworn lens extracts (see Figure 3.6 for gas chromatograms of unworn contact lenses). The concentration of DMPC extracted from this particular unworn Dailies Total 1 lens was $0.38x10^{-4}$g/ml.

6.3.5.1 Gas chromatograms of ex vivo Dailies Total 1 lens extracts and tear samples

Both and Dailies Total 1 and Acuvue TruEye lenses were worn by a group of subjects for 16hours. Their lenses were collected and *ex vivo* Dailies Total 1 lens extracts were analysed by GCMS to establish the levels of DMPC remaining in the lens. *Ex vivo* Acuvue TruEye lens extracts were analysed to determine differences in fatty acid profiles between worn and unworn extracts.

The chromatograms in Figure 6.7 represent *ex vivo* Dailies Total 1 lens extracts that have been worn by various subjects. *Ex vivo* lenses were extracted and analysed to determine whether DMPC had been released into the tear film.

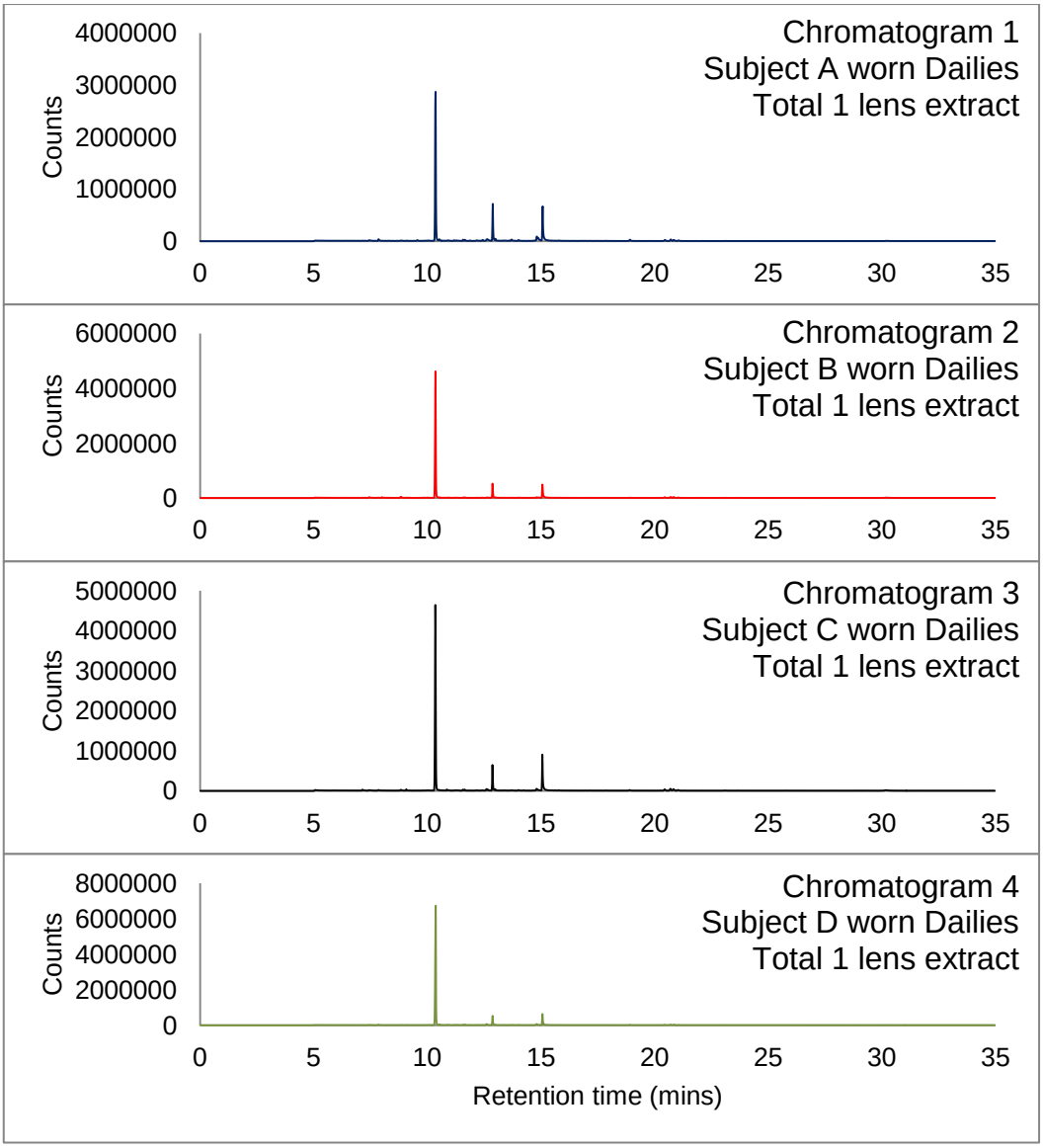


Figure 6.7 – Gas chromatograms of worn Dailies Total 1 lens extracts where: chromatograms 1-4 are subjects A-D respectively

The chromatograms in Figure 6.7 represent subjects A-D Dailies Total 1 lens extracts. The predominant peak, at approximately 10.3mins corresponds to C14:0. Chromatograms 1-4 look identical to the chromatogram of an unworn Dailies Total 1 lens extract (shown in Figure 6.6). These results suggested that little to no levels of DMPC were actually released into the tear film throughout wear. The intensities of the peaks corresponding to C14:0 were similar to the intensity of the C14:0 peak of an unworn Dailies Total 1 lens.

The concentration of DMPC extracted from *ex vivo* Dailies Total 1 lenses was measured to determine the level of DMPC extracted by the tear film

during wear. The concentration of DMPC extracted from subject A *ex vivo* lens was $0.231x10^{-4}$g/ml, from subject B was $0.33x10^{-4}$g/ml, subject C was $0.35x10^{-4}$g/ml and from subject D was $0.52x10^{-4}$g/ml. The concentration extracted from an unworn Dailies Total 1 lens was $0.38x10^{-4}$g/ml, however this value changed from lens to lens. Therefore, it was difficult to determine whether DMPC had been extracted by the tear film during wear. However, it was clear that the majority of the DMPC had not been released into the tear film and remained in the lens.

Table 6.4 – Analysis of GC traces shown in Figure 6.7

Subject	*Concentration of C14:0 extracted from subject-worn Dailies Total 1 lenses*
A	$0.23x10^{-4}$g/ml
B	$0.33x10^{-4}$g/ml
C	$0.35x10^{-4}$g/ml
D	$0.52x10^{-4}$g/ml
Unworn Dailies Total 1	$0.38x10^{-4}$g/ml

To determine whether DMPC was released into the tear film throughout wear, tear samples were taken from subjects immediately after they wore Dailies Total 1 lenses. This was to observe if there was an increase in the C14:0 peak. Tear samples were collected using the Visispear ophthalmic sponge collection method (as described in section 2.9). The gas chromatograms in Figure 6.8 represent tear samples taken from subjects A-D.

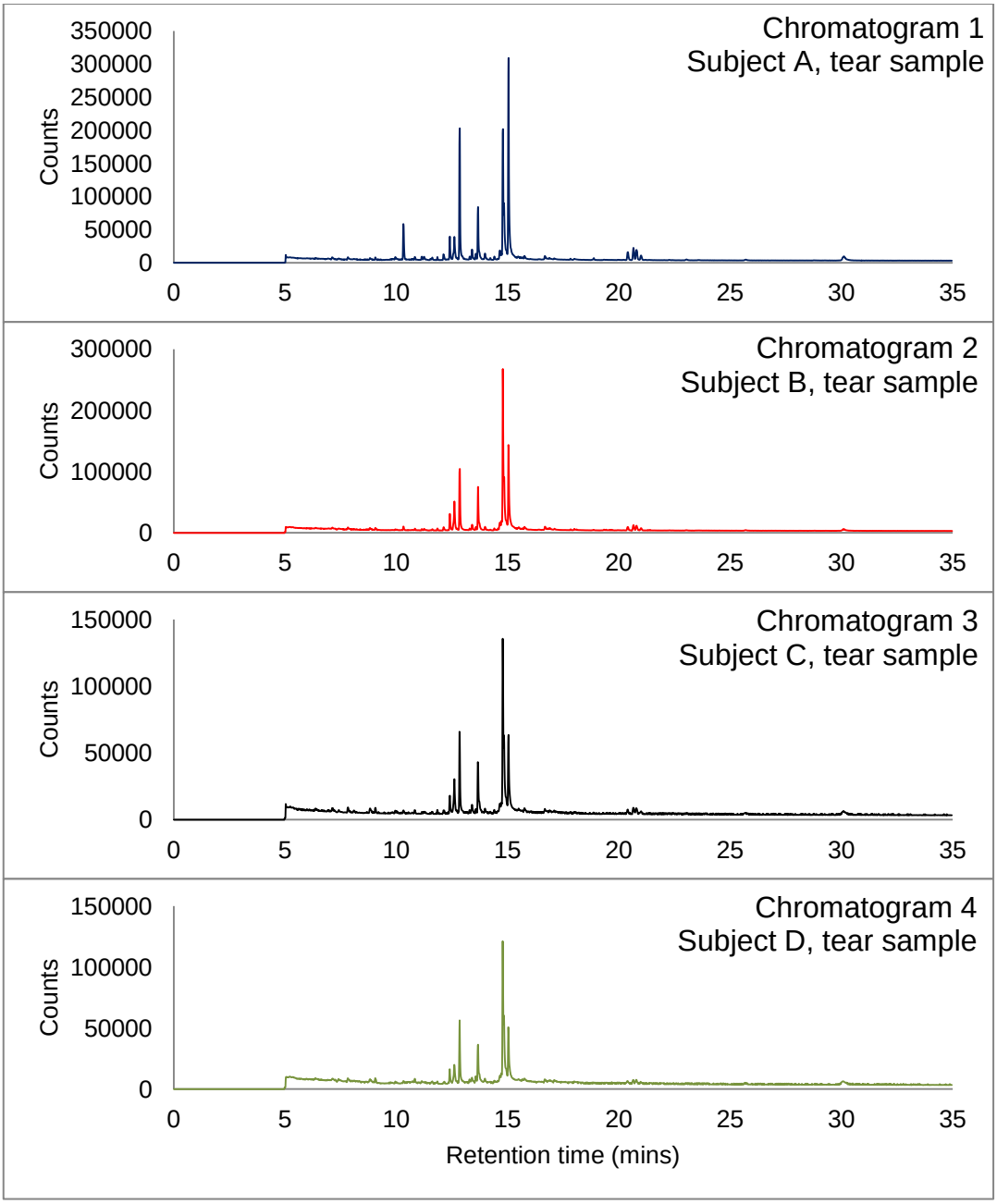


Figure 6.8 – Gas chromatograms of Visispear ophthalmic sponge extracts (after 12hours wear of Dailies Total 1 lenses) where: Chromatograms 1-4 are sponge extracts of subjects A-D respectively

The peaks observed for subjects A-D tear samples represent common tear fatty acids such as C14:0, C16:0, C18:1 and C18:0 (at 10.3, 12.9, 14.8 and 15mins respectively). There did not appear to be high levels of C14:0 observed on the gas chromatograms representing tear samples from each subject. This suggested that DMPC was not being released into the tear film from the contact lens.

The next set of subjects Dailies Total 1 lens extracts were extracted and analysed by GCMS. The gas chromatograms representing subject 4, 5, 12, 13 and 14 are shown in Figure 6.9.

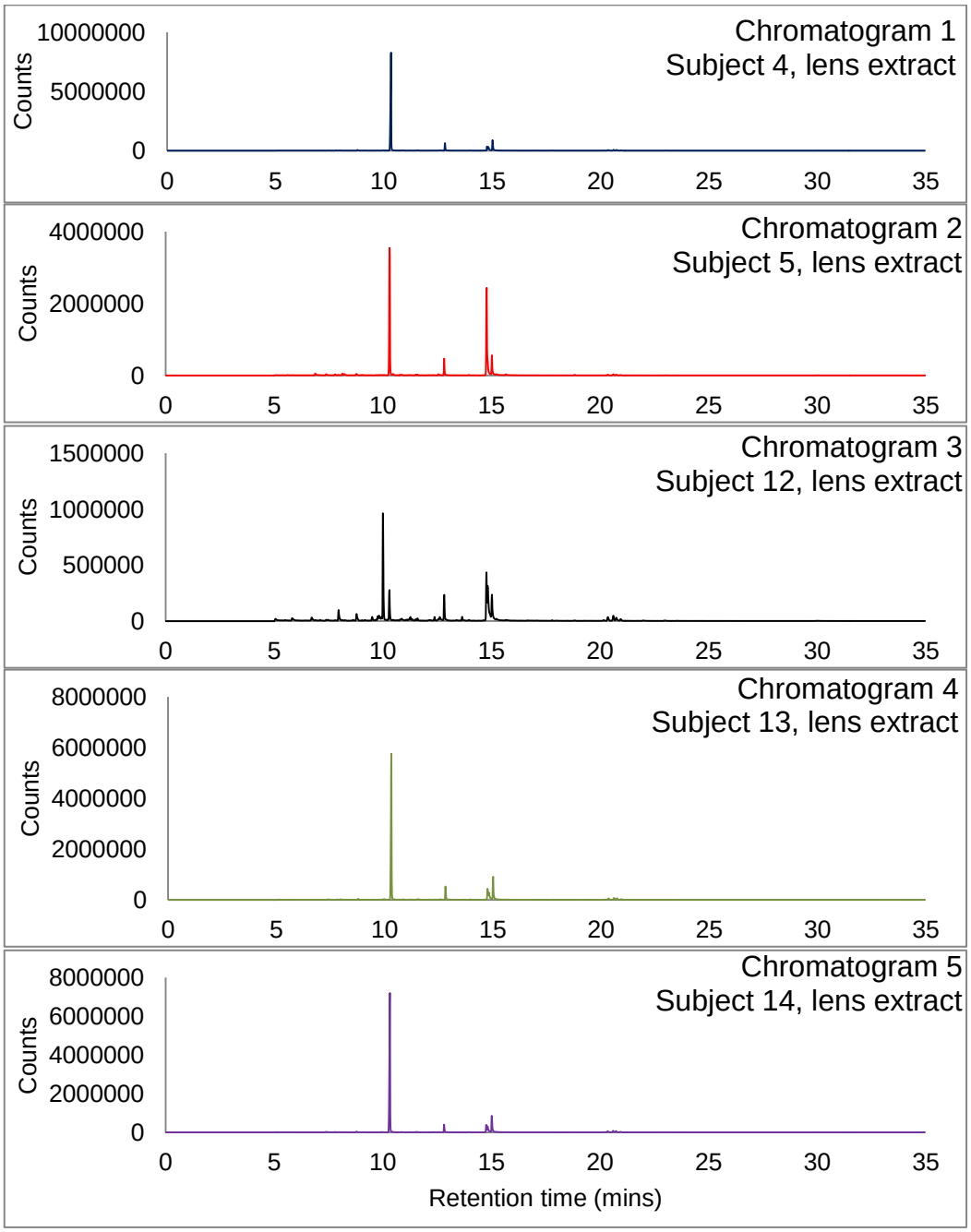


Figure 6.9 – Gas chromatograms of Worn Dailies total 1 lens extracts, where: Chromatogram 1=Px4, Chromatogram 2=Px5, Chromatogram 3=Px12, Chromatogram 4=Px13, Chromatogram 5=Px14

Each of the chromatograms in Figure 6.9 showed a predominant peak at 10.3mins which corresponds with C14:0. These chromatograms were similar to an unworn lens extract. However, the peaks corresponding to

C16:0 and C18:0 were less intense compared with the unworn lens extracts shown in Figure 6.6, chromatogram 2. This suggested that these components may have been extracted from the lens into the tear film. Chromatogram 3, subject 12 lens extract was incorrectly labelled and is an Acuvue TruEye lens extract. It is clearly different to the other chromatograms because the predominant C14:0 peak is not present and the peak at 10.3mins is characteristic of a Dailies Total 1 lens extract.

Subjects 4-5, 12-14 lenses were removed and a Visispear sponge was wiped over the anterior surface of the lens prior to extraction. The sponges were extracted and analysed by GCMS. The GC traces are shown in Figure 6.10.

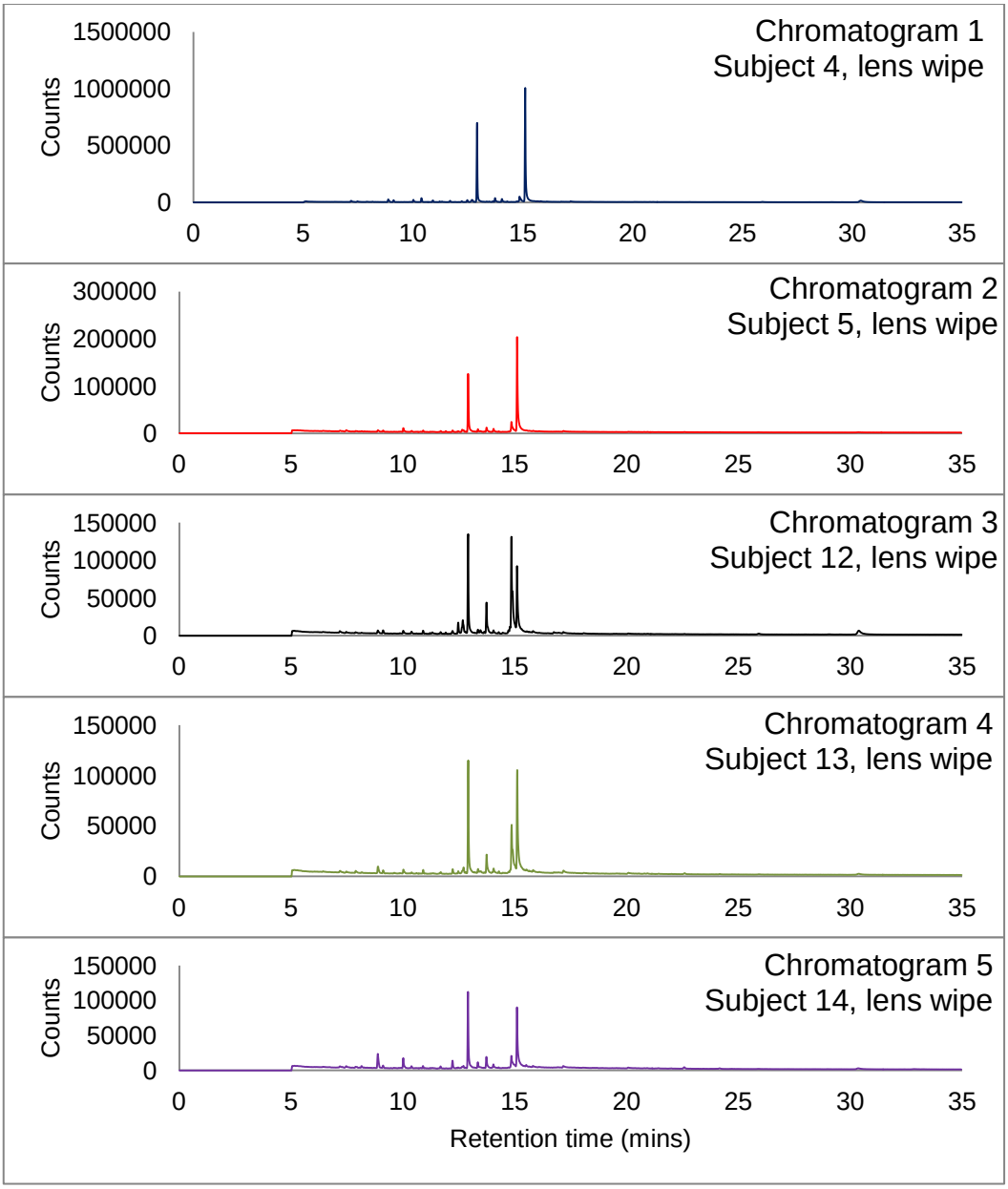


Figure 6.10 – Gas chromatograms representing Subjects 4-5, 12-14 Visispear Ophalmic sponge lens wipe samples. Lenses were removed and an ophthalmic sponge was wiped over the anterior surface of the lens.

Low levels of lipid were extracted from Visispear ophthalmic sponges that had been wiped over *ex vivo* Dailies Total 1 lenses (very low peak intensities were observed). The C14:0 peak was not observed on any chromatogram representing lens wipe samples. The predominant peaks observed at 12.8, 14.8 and 15.0mins correspond to C16:0, C18:1 and C18:0 respectively.

Tear samples, from the lens-wearing eye wear were also taken using Visispear ophthalmic sponges. The tear samples were extracted and analysed by GCMS. The gas chromatograms are shown in Figure 6.11.

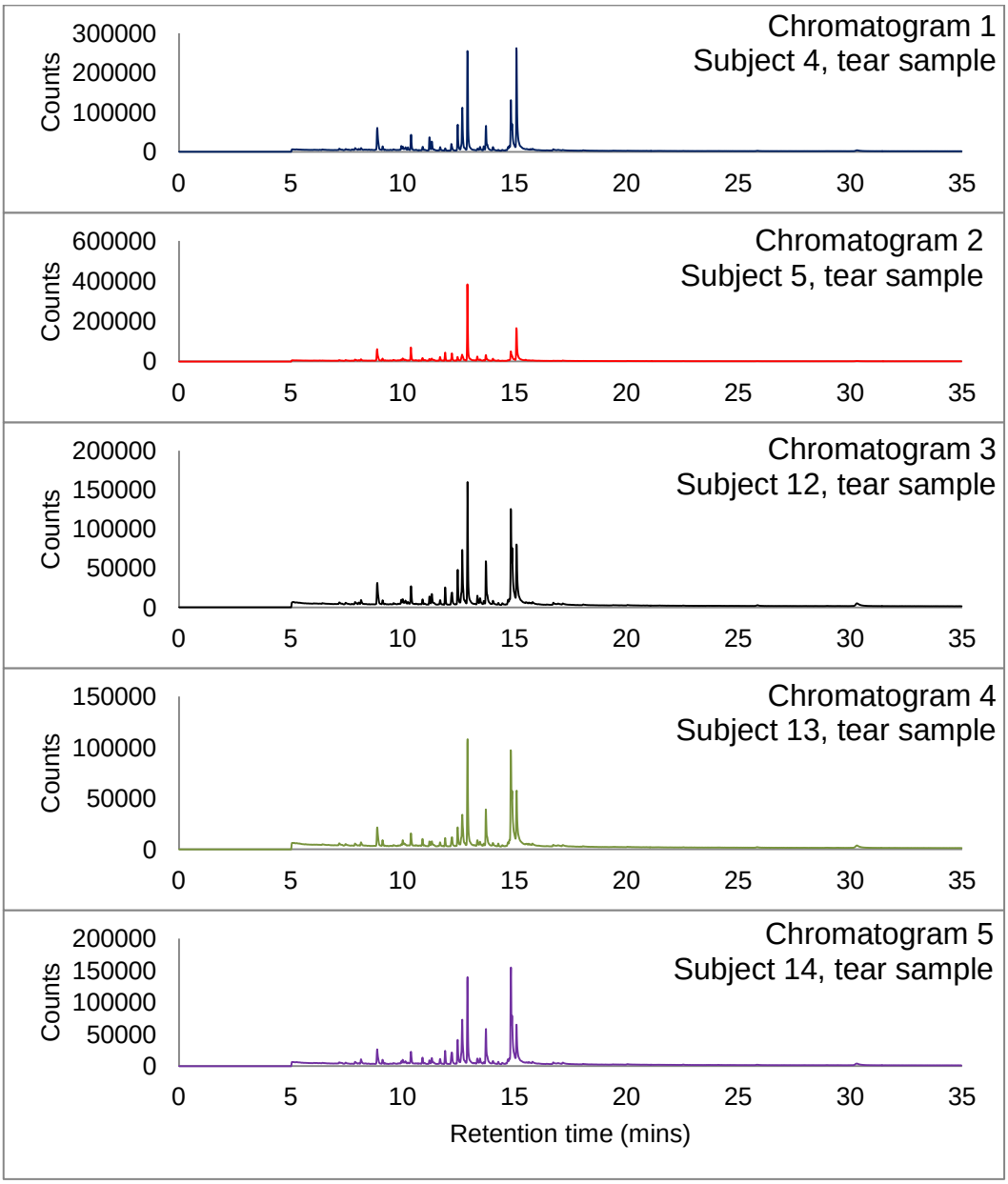


Figure 6.11 – Gas chromatograms representing Subjects 4-5, 12-14 tear samples taken using the Visispear ophthalmic sponges. Tear samples were collected whilst subjects wore Dailies Total 1 lenses and the sponge was gently wiped over the lens.

The chromatograms in Figure 6.11 represent tear samples that were taken whilst the subjects were wearing Dailies Total 1 lenses. The predominant peaks observed corresponded to C16:1 (12.6mins), C16:0 (12.8mins), C18:1 (14.8mins) and C18:0 (15.0mins). The sample volumes

collected were very small, therefore the peak intensities were low. The C14:0 peak was not present in all of the chromatograms in Figure 6.11.

More *ex vivo* Dailies Total 1 lenses were analysed by GCMS and the gas chromatograms are shown in Figure 6.12.

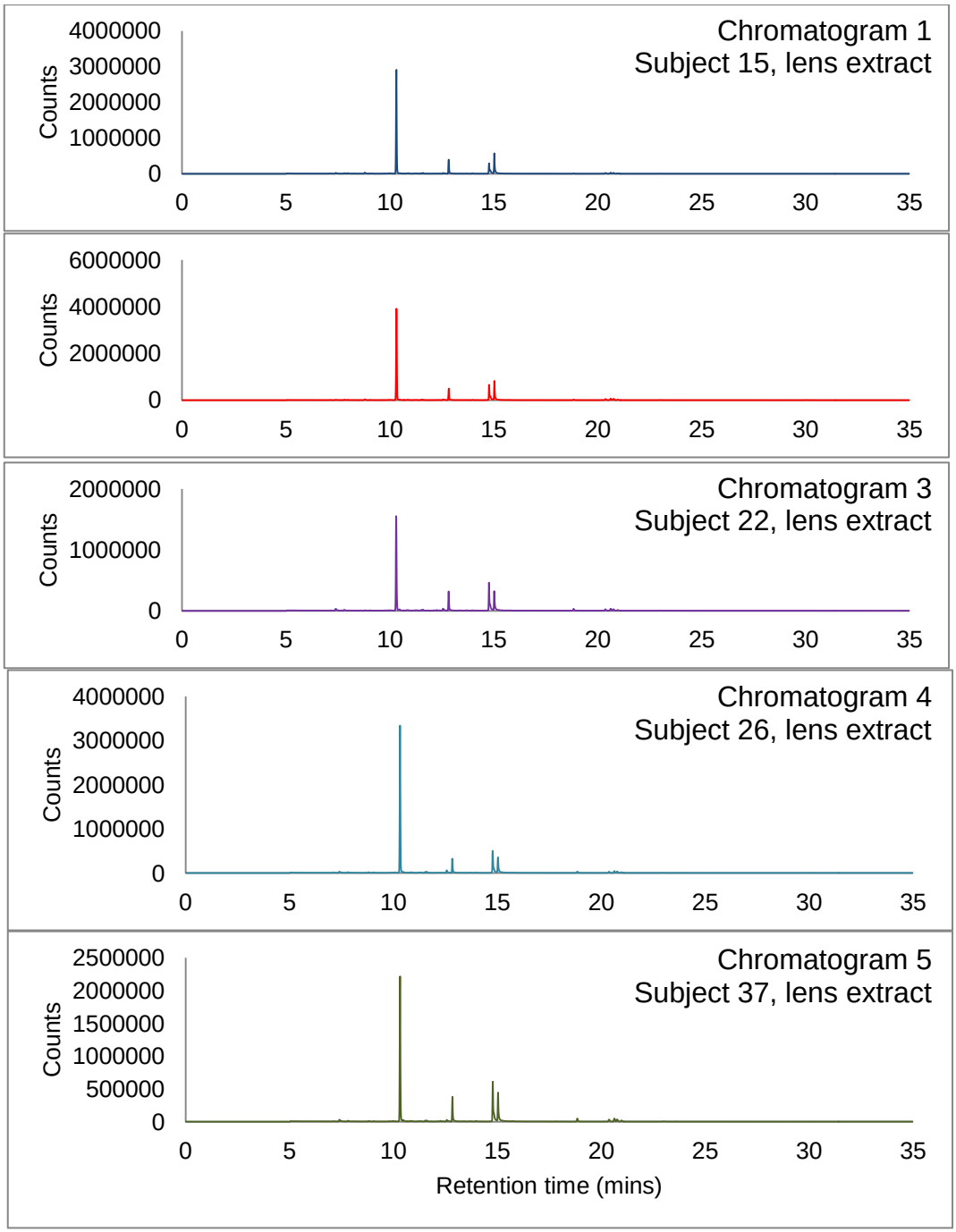


Figure 6.12 – Gas chromatograms subject worn Dailies Total 1 lens extracts. Where: Chromatogram 1=subject 15, Chromatogram 2=subject 20, chromatogram 3=subject 22, Chromatogram 4=subject 26, Chromatogram 5=subject 37

The chromatograms in Figure 6.12 represent Dailies Total 1 lens extracts for various subjects. The most predominant peak observed was at 10.3mins and corresponded to C14:0 from the lens. There were low levels of C18:1 and C18:0 at 14.8mins and 15.0mins respectively. Both C18:1 and C18:0 are common tear fatty acids.

The concentration of DMPC that was extracted from subject 15, 20, 22, 26 and 37 *ex vivo* lenses were: Subject 15 = $0.21x10^{-4}$g/ml, Subject 20 = $0.31x10^{-4}$g/ml, Subject 22 = $0.13x10^{-4}$g/ml, Subject 26 = $0.24x10^{-4}$g/ml and Subject 37 = $0.17x10^{-4}$g/ml. There were lower concentrations of DMPC remaining in these particular subjects lenses compared with an unworn Dailies Total 1 lens ($0.37x10^{-4}$g/ml). This would suggest that some DMPC was released into the tear film during wear. However, the concentration of DMPC in unworn Dailies Total 1 lenses varied from lens to lens. It was therefore difficult to conclusively state that DMPC was released into the tear film or not.

Table 6.5 – Analysis of the GC traces shown in Figure 6.12

Subject	*Concentration of C14:0 extracted from subject-worn Dailies Total 1 lenses*
15	$0.21x10^{-4}$g/ml
20	$0.31x10^{-4}$g/ml
22	$0.13x10^{-4}$g/ml
26	$0.24x10^{-4}$g/ml
37	$0.17x10^{-4}$g/ml
Unworn Dailies Total 1	$0.38x10^{-4}$g/ml

6.3.5.1.1 Dailies Total 1 GCMS results: Summary

It was difficult to mimic the way in which the tear film would extract DMPC from the lens using *in vitro* conditions.

- Many extraction solvents were used, however these were ‘harsh’ and did not mimic the way tears would extract DMPC from the lens

- If a tear-like fluid was used for extraction, the whole extract would have to be analysed by GCMS, this had the problem of over-loading the column
- It was therefore decided to analyse *ex vivo* lenses to determine whether there was any decrease in the levels of C14:0 compared with unworn lenses
- Tears were collected after wearing Dailies Total 1 lenses and analysed to investigate the presence of C14:0
- The analysis of *ex vivo* lenses showed similar levels of C14:0 as the unworn lens extracts, this suggested DMPC was not being released into the tear film. Also, tear samples did not show any increases in the levels of C14:0, suggesting that DMPC was not released into tears
- It was difficult to assess the exact concentration of DMPC released into the tear film by analysing *ex vivo* lenses because the concentration in unworn Dailies Total 1 lenses varied from lens to lens.

6.3.6 Ex vivo Acuvue TruEye (narafilcon A) lens extracts

Acuvue TruEye lenses were used as a comparator lens to Dailies Total 1 becasue they are both daily disposable silicone hydrogel lenses.

The gas chromatograms in Figure 6.13 represent subject worn Acuvue TruEye lens extracts. The gas chromatograms in Figure 6.13 represent subjects A-D *ex vivo* Acuvue TruEye lens extracts.

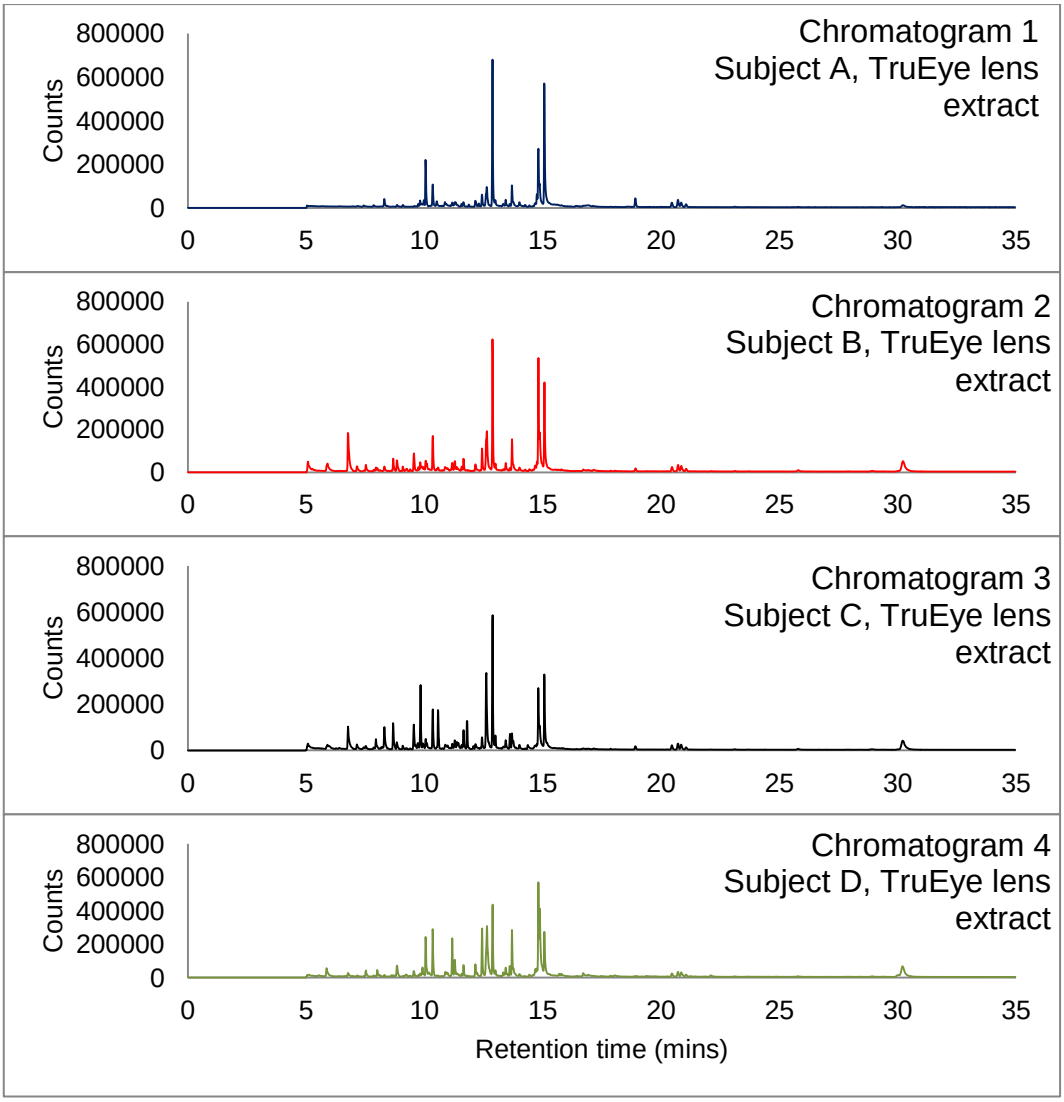


Figure 6.13 – Gas chromatograms of worn Acuvue TruEye lens extracts where: Chromatogram 1-4 are subjects A-D respectively

The chromatograms in Figure 6.13 represent subjects A-D Acuvue TruEye lens extracts. The predominant peaks observed for subjects A and B lens extracts (chromatograms 1 and 2) corresponded to C16:0, C18:1 and C18:0 (at 12.8mins, 14.8mins and 15.1mins respectively). The peak corresponding to C18:1 (at 14.8mins) was not observed for unworn Acuvue TruEye lens extracts, it therefore corresponds to lipids from the tear film. However, both C16:0 and C18:0 have been identified in meibomian gland secretions and are therefore considered to be from the tear film and not extractable components of the lens.

The most predominant peaks observed for subjects C and D lens extracts (chromatograms 3 and 4) correspond to C16:1, C16:0, C18:1, C18:1 (at

12.4mins, 12.8mins, 14.8mins and 15.1mins respectively). These are all common fatty acids known to be secreted by the meibomian glands.

The chromatograms representing subjects A-D Acuvue TruEye lens extracts were different to the chromatogram of an unworn Acuvue TruEye extract (Figure 6.6, Chromatogram A). For example, the peaks at 10.03mins and 7.9mins on the chromatogram representing an unworn Acuvue TruEye lens extract (Figure 6.6, Chromatogram A) are not present at all or at the same intensity on subject A-D Acuvue TruEye lens extracts. These two species were most likely extracted by the tear film during wear.

The gas chromatograms in Figure 6.14 represent unworn narafilcon A, narafilcon B and worn narafilcon B lens extracts. Narafilcon A is available to the European market and narafilcon B is only available from the USA market. Narafilcon B lenses differ from narafilcon A because they have a higher proportion of hydrogel to silicone.

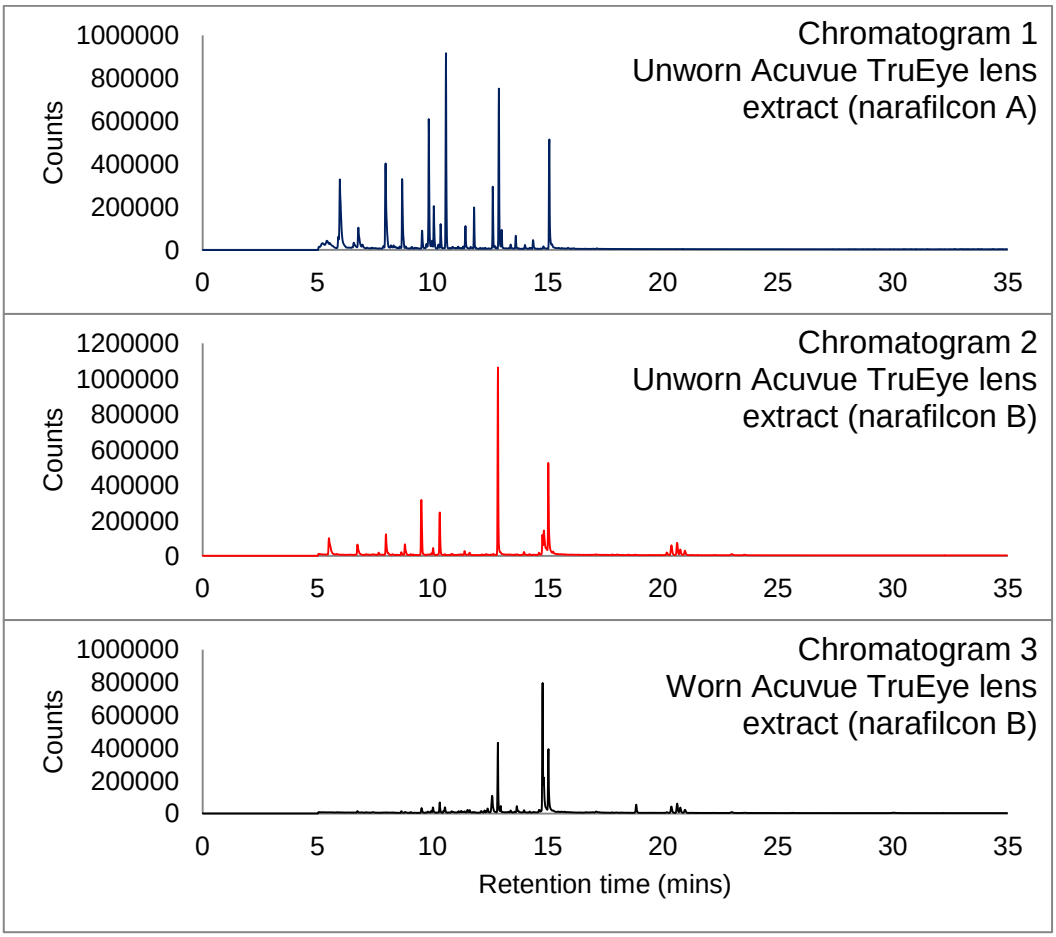


Figure 6.14 – Gas chromatograms of an unworn narafilcon A lens extract, unworn and worn narafilcon B lens extracts

The GC trace for an unworn narafilcon A lens extract was very different to an unworn narafilcon B lens extract. The C10:0 peak at 7mins was not present on the chromatogram representing the unworn narafilcon B extract (chromatogram 2) but was a characteristic peak on the narafilcon A extract chromatogram. The peak at ~10mins on chromatogram 1 (unworn narafilcon A extract) was not present on chromatogram 2 (unworn narafilcon B extract). Peaks representing C16:0 and C18:0 (at 12.8mins and 15.0mins) were present on chromatograms 1 and 2 (unworn narafilcon A and unworn narafilcon B respectively).

The GC trace for the worn narafilcon B lens extract (chromatogram 3) was not very different to the GC trace of an unworn extract. A major difference was the presence of the peak corresponding to C18:1 at 14.8mins on chromatogram 3 (worn narafilcon B extract). The GC trace for the unworn narafilcon B extract did not show this peak. C18:1 is a common tear fatty acid and is therefore not present on the trace of an unworn extract.

Also, the GC trace of the unworn narafilcon B lens extract (Figure 6.14, chromatogram 2) showed peaks at 9.5 and 10.3mins. These peaks corresponded to extractable species from the lens. These peaks were not observed on the gas chromatogram representing the worn narafilcon B lens extract (chromatogram 3). These species had most likely been extracted from the lens by the tear film.

6.3.6.1 Acuvue TruEye lens extraction: Summary

- There were clear differences in the GC traces between worn and unworn narafilcon A lens extract (Figure 6.6 chromatogram A and Figure 6.14)
- Certain peaks observed for unworn narafilcon A extracts were not observed for worn lens extracts, therefore the tear film had extracted them
- There were differences in GC traces between unworn narafilcon A and narafilcon B lens extracts
- There was no peak corresponding to C10:0 observed for unworn narafilcon B lens extracts, the peak at 10.5mins on GC traces representing narafilcon A lens extract (Figure 6.14, chromatogram 1) was not present for the narafilcon B extract (Figure 6.14, chromatogram 2)
- There were differences in GC traces between worn and unworn narafilcon B lens extracts
- Certain peaks present on the GC trace of an unworn narafilcon B extracts were not present on the GC trace of the worn extract, which suggested these components had been extracted by the tear film during wear

6.4 Conclusions

The aim of this work was to establish whether DMPC would be delivered to the tear film during wear. Initially, non-clinical contact lenses were extracted using various extraction protocols. The results showed DMPC was extractible from these lenses using *in vitro* conditions (Figure 6.3). However, many of the extraction protocols did not mimic *in vivo* conditions. Once clinical lenses were obtained, they were extracted (post wear) and analysed using GCMS. The concentration of DMPC that remained in *ex vivo* lenses in the lens was determined. In addition to the analysis of *ex vivo* lens extracts, tear samples were taken to establish whether DMPC had been released into the tear film. The results show that unworn and worn lenses had similar levels of DMPC after the lenses had been worn for one day, which indicated low levels or no DMPC had been extracted by the tear film. There was no C14:0 peak present on the gas chromatograms representing tear samples taken directly after wearing DMPC-containing lenses (see Figure 6.8, Figure 6.10 and Figure 6.11). It was difficult to ascertain the exact concentration of DMPC released into the tear film (if any) because the amount of DMPC in unworn lenses varied from lens-to-lens. However, the results suggested that if any DMPC was released, it was a very small amount.

- In summary, it is unclear how much DMPC is released into the tear film by using the techniques discussed in this chapter.
- The concentration of DMPC extracted from several unworn DMPC-containing lenses varied from lens to lens, which made it difficult to monitor whether DMPC was released into the tear film.
- It could not be conclusively stated whether DMPC was released into the tear film or not, however from the work conducted in this chapter, it was observed that the majority of the DMPC could still be extracted from *ex vivo* lenses.

Pitt *et al*. showed DMPC was being released after 20hrs using an *in vitro* model (95). They loaded lenses with radio-labelled DMPC and then measured its release, however the DMPC standards were prepared in n-

propanol and lenses were soaked in this solution and this may have swollen the lenses which allowed DMPC to be released.

The work undertaken in this chapter was to ultimately establish whether DMPC was released into the tear film from phospholipid-containing contact lenses. The aim was also to try and find a suitable *in vitro* extraction protocol which would mimic the tear film extraction of DMPC from the lens. The research discussed in this chapter successfully established that little to no DMPC was actually released into the tear film from the phospholipid-containing contact lenses. After additional research of Acuvue TruEye lens extracts, it was shown that components of this lens were extracted by the tear film.

The consequences on the tear film as a result of tear film lipid layer extraction of species from lenses are yet to be investigated. The research in this chapter shows that tear film is capable of extracting components from certain lens materials. However, it is unknown whether the extraction of components of the lens into the tear film will be beneficial or detrimental to tear film dynamics.

Chapter 7 – Summary, conclusions and future work

7.1 Concluding discussions

Contact lenses are worn by millions of individuals all over the world. Consequently, the contact lens industry is worth an estimated $6.1 billion dollars (155). The development of contact lens materials is on-going to ultimately produce more comfortable lens materials. Contact lens comfort is a very important aspect for wearers, clinicians and manufacturers. Many contact lens wearers suffer from a phenomenon known as 'end of day discomfort'. Trying to understand certain areas of lens comfort were therefore the major aim of this research.

The evolution of silicone hydrogel lenses has been discussed in section 1.7. Some of the major problems with silicone hydrogel lenses are related to high levels of lipid deposition. It is well established that lipids deposit on contact lenses throughout wear and this has been shown throughout this thesis, particularly in section 3.6.1.1, however the nature and fate of lipids on contact lenses has not previously been studied in great detail. The correlations between the tear film lipid layer and contact lens comfort are only now starting to be examined. The work in this thesis was based on investigating the changes in lipid structure as a consequence of contact lens wear.

7.1.1 Major developments achieved in this work

- Analytical techniques, extraction techniques and tear sampling techniques were developed for the examination of lipids.
- The Visispear ophthalmic sponge was used to successfully sample tear lipids (shown in Chapter 3).
- Development of an effective contact lens extraction protocol was achieved and single lens extracts were examined. Therefore, variations in lipid structure for individual subjects were investigated.
- The development of HPLC and GCMS to examine both lipid classes and fatty acid profiles from lens extracts and tear samples.

A major part of the work discussed in this thesis was the development of analytical techniques, HPLC and GCMS. HPLC had been used by some researchers for the analysis of tear lipids and lens extracts but it had never

been used in conjunction with GCMS. Both HPLC and GCMS proved to be powerful techniques which provided individual lipid class and fatty acid profiles from single lens extracts and tear samples. HPLC was useful for determining polar to non-polar ratios for individual subjects tears. The properties, including spreading and wetting of individual lipid classes vary and this is discussed in the next section.

7.1.1.1 Spreading and wetting of lipids on contact lenses

Combined aqueous and lipoidal lubrication is common to several biological interfaces (e.g. lung, articulating joint and anterior eye) and there are both commonalities and differences between them. It is clear, for example, that the lipid layer associated with the tear film is unique in its composition. Whereas the lipoidal content of pulmonary surfactant and synovial fluid are primarily phospholipids, the tear film lipid layer consists of mainly non-polar lipids such as cholesterol, cholesteryl esters and glyceryl esters.

Although the level of phospholipids is now generally agreed to be much lower in the tear film than was previously thought (31, 32) it remains likely that they have an active role at the aqueous-lipid interface. Saville *et al*. suggest the concentration of phosphatidyl cholines in meibum is 18±5ng/mg (33).

The properties of non-polar lipids are significantly different from those of polar lipids. Phospholipids tend to form bilayers and this is a particular feature of lung surfactant, whereas the tear film lipid layer is necessarily much thicker because of its function in reducing tear film evaporation. In order to fulfil this function it is essential that the lipid layer should maintain a so-called duplex film rather than forming lenses or a monolayer. Additionally, because of the rapidity and regularity of eyelid movement it is essential that the lipid film spreads rapidly over the tear aqueous.

The equilibrium stability of the lipid layer on tear aqueous can be estimated by calculation of the Harkins spreading coefficient. In order to calculate this it is necessary to know the surface tension parameters of the lipid components, the surface tension parameters of the tear aqueous and the interfacial tension between the two layers. The underlying

thermodynamic principle is that the lowest energy configuration (fully spread lipid film or separate droplets or lenses of lipid) will be favoured. The same principles apply to the spreading of tear aqueous on a lipid-coated lens and the same data set can be used to calculate the spreading coefficients for this configuration.

The phenomenon of surface tension arises from the excess molecular force between molecules at an air interface. The absence of similar molecules in the air phase means that bonding and interactive forces between molecules in the surface layer is enhanced. Where two immiscible phases meet, a similar concept can be applied. The sum of the surface tensions of the two individual phases contributes to an interfacial tension. The magnitude of this combined value is reduced by any molecular forces that can operate across the interface. A reasonable approximation of this interaction can be obtained by separating the total surface tension (γ_T) into polar (γ_p) and non-polar (commonly known as dispersive (γ_d)) components. The interfacial tension is calculated from these individual components by use of Equation (1).

$$\gamma_T = \gamma a + \gamma b - 2(\gamma a_p \gamma b_p)^{1/2} - 2(\gamma a_d \gamma b_d)^{1/2} \qquad \text{Equation (1)}$$

Information on the surface tension parameters of different lipid classes is not readily available. The effect of hydrocarbon chain length on the surface tension parameters of fatty acids, fatty alcohols and tracyliglycerides has been collected, as have available data on the surface energy parameters of cholesterol. Phospholipids, because of their greater polarity and asymmetry, the tendency to form bilayers is more difficult. Useful information on the surface free energy of DPPC multilayers on different substrates has been obtained, which emphasises the great tendency of this molecule to align its polar head group with polar interfaces. Additionally, useful direct measurements of the interfacial tension between tear aqueous and lipid-like components have been reported. Data from these various sources enable the necessary calculations to be made (13, 156-160).

Table 7.1 – Spreading coefficients for various lipid classes

Lipid class	Surface tension of tears (γ_{aq})		Surface tension of lipid classes (γ_L)	Interfacial tension at the lipid, tear interface (γ_{aqL})		Spreading Coefficient of lipid on tear $S=\gamma_{aq}-(\gamma_L + \gamma_{aqL})$		Spreading Coefficient of tears on lipid $S=\gamma_L-(\gamma_{aq} + \gamma_{aqL})$	
	Min	Max		Min	Max	Min	Max	Min	Max
Cholesterol*	43.6	46.0	36.0	13.4	16.6	-9.6	-3.4	-24.2	-22.4
TAG	43.6	46.0	31.0	10.6	13.2	-0.6	+4.0	-25.8	-23.6
PLs	43.6	46.0	32.0	9.0	11.0	+0.6	+5.0	-23.0	-22.6

*Cholesteryl esters give similar values (161)

Since a positive spreading coefficient denotes that the conditions are favourable for spreading, the following conclusions can be drawn:

- The most hydrophobic tear lipids will not form a stable spread layer on the tear aqueous
- As the lipid components become more polar there is a transition to favourable spreading conditions
- The calculations indicate that polar lipids are required at the aqueous interface in order to promote formation of a spread lipid layer over the tear aqueous
- Tear aqueous is unable to form a spread layer over a lipid-coated substrate (i.e. contact lens) that remains stable as the film thins and surface forces dominate regardless of the lipid composition.

The data also indicate why lipid spreading is not uniform and is subject-dependent. The tendency to form colour fringes, striations, meshwork

patterns and in some cases homogeneous and amorphous films are quite consistent with the variation in spreading coefficient of individual lipid components (162).

It is important to note that there are additional factors that affect lipid spreading behaviour that will not be dealt with in detail here. These include temperature-dependant phase transitions which form the basis of so-called liquid crystal behaviour. Since many of these occur around 30°C they exert a considerable influence on lipid viscosity and consequently flow patterns. A second important aspect of lipid layer formation is the rate at which the lipid layer spreads so far we have considered only the equilibrium condition which reflects the tendency, rather than the rate. The rate of lipid spreading is affected by a complex inter-relation of various factors including surface tension gradients and viscosity, but made more complex because spreading is an unstable and dynamic situation (163). In experimental studies the spreading coefficient has been found to be a useful predictor of relative rates of spreading (163).

The majority of tear lipids are made up of fatty acids such as cholesteryl esters, phospholipids, triglycerides, wax esters as well as free fatty acids themselves. The fatty acid chain length and level of unsaturation affects the structure and function of the lipid.

7.1.2 Use of GCMS to examine fatty acid profiles from single lens extracts

- GCMS analysis of lenses worn on a daily wear schedule compared with lenses worn on a continuous wear schedule revealed significant differences in fatty acid profiles related to wear schedule.
- Unsaturated lipids had degraded during overnight wear of contact lenses after a 30 day wear period.
- The degradation was independent of lens material and extraction protocol.
- GCMS had never previously been used to examine fatty acid profiles from lens extracts. The work discussed in Chapter 4 showed that

GCMS was an extremely powerful technique for the analysis of fatty acids from single lens extracts.

- The use of GCMS enabled the differences in fatty acid profiles between daily wear and continuous wear lens extracts to be examined. This was a significant finding of this work and not been previously investigated.

7.1.2.1 Changes to the tear film protein levels during eye closure

Publications by Sack *et al*. (164) revealed that levels of certain proteins increased when the tears were collected straight after sleep. Stapleton *et al*. (165) investigated the changes in tear protein composition during sleep in contact lenses. They showed that levels of certain proteins such as sIgA and complement proteins increased after overnight wear of contact lenses. However, they also stated that similar increases had previously been showed without contact lens wear. They also showed that certain proteins have an affinity for depositing on the contact lenses which could deplete tears of these proteins. Stapleton *et al*. (165) extracted and analysed the worn contact lenses as well as analysing tears prior to and post sleep in lenses to show that certain proteins deposit on the lens which depleted their concentrations in tears. There have been no studies up to this date and to our knowledge which examine changes in lipid composition post sleep. The work carried out in Chapter 4 provides evidence that there are significant changes to the structure of lipids during sleep.

The consequences of changes in tear lipid composition as a result of overnight contact lens wear are to be fully investigated; however degradation of lipids does produce oxidative products.

7.1.3 Production of lipid oxidative end products in the ocular environment

The unique composition of the tear film lipid layer is such that it is fairly oxidatively stable. There are only low levels of polyunsaturated fatty acids in the tear film because they are more prone to oxidation. However, the introduction of a contact lens to the eye, as already discussed causes a

huge disturbance to the ocular environment. Therefore, the stability of the tear film is compromised by the presence of a contact lens.

The work discussed in Chapter 5 was therefore based on investigating the presence of lipid oxidative end products in the ocular environment. MDA, a commonly studied biomarker of lipid oxidation was found to be present in the tears of contact lens intolerant patients by Glasson *et al*. (121). Other than this publication, there has been no other research investigating lipid oxidative end products in contact lens wearers.

- Therefore, the research in Chapter 5 was mainly based on establishing a sensitive and suitable assay for the measure of MDA in the ocular environment.
- The MDA-ELISA assay kit was found to be the most sensitive assay for the detection of MDA in the ocular environment.
- The results using this assay showed that MDA was building up within the lens matrix and this had never been shown before. This work demonstrates that lipid oxidation can produce MDA, which has been previously linked with contact lens intolerance.

There is another pathway of lipid degradation causing problems of decreased wetting and subsequent discomfort and this is the production of discrete deposits on contact lenses.

There have been previous reports of lipid degradation as a result of increased lipid deposition on lenses. Increased lipid deposition can lead to the oligomerisation of lipids (conformational change in structure) and can produce white spots. Several authors have previously identified the presence of ‘white spots’ or ‘jelly bumps’ on hydrogel contact lenses (140, 141). Both Hart and Bowers and Tighe (141) found the presence of calcium within these deposits, all be it at a very small concentration compared to the amount of lipid. Bowers and Tighe (141) found that not only did the lens material have an effect on deposition but also that subject variability affected the rate of deposition. The composition of these ‘white spots’ were identified as being predominantly non-polar cholesteryl esters with unsaturated fatty acids esterified to the cholesterol (140). These

unsaturated fatty acids had oligomerised and therefore produced ‘white spots’ or ‘jelly bumps’ on the lens. Due to the non-polar nature of these lipids, they become very difficult to remove using cleaning surfactants. The wettability of the lens surface also becomes very difficult because of the build-up predominantly non-polar lipids. The degradation of lipids can therefore have two effects: it can either lead to the production of lipid oxidation products such as MDA (as discussed in Chapter 5) which can bind to proteins and/or cause deposits on the lens which can lead to decreased visual acuity and decreased wetting of the lens. Both, the production of deposits on lenses and/or the production of lipid oxidative end products have a major effect on contact lens comfort. As already discussed, contact lens comfort is important for the contact lens industry.

The spreading and wetting of lipids is vital for the lubrication of the ocular surface over the contact lens. The role and function of the lipid layer is to aid lubrication of the eyelid over the ocular surface. Its function is even more important in contact lens wear as it has additional interfaces to cope with. The comparison of the tear film with other biological interfaces helps understand the function of the tear film. Chapter 1, section 1.5.3 is a review of literature regarding the comparison in lipid compositions of pulmonary surfactant and synovial fluid to the composition of the tear film. The key piece of information is that whilst the function at each interface is very much similar, the composition of the tear film is unique. The tear film lipid layer is composed of mainly non-polar lipids whereas both synovial fluid and pulmonary surfactant are made up of predominately polar phospholipids. Different lipid classes have different properties, not only in their interaction with other lipid species, but their spreading properties are also different.

7.2 Conclusions

The major developments of the work carried out in this thesis are discussed below.

- Single lenses were and analysed by various analytical techniques.
- Visispear ophthalmic sponges were successfully used to collect tear lipids from individual subjects.
- HPLC and GCMS were both used for lipid class and fatty acid profiles for individual lens extracts and tear samples, the build-up of lipids on contact lenses was observed (Chapter 3).
- A range of unworn lenses were extracted and analysed by HPLC and GCMS. Many extractable components were observed. These extractable components from unworn lenses can be extracted by the tear film; however their effect on the tear film is unknown.
- GCMS analysis of phospholipid-containing lenses (Dailies Total 1) showed little to no DMPC was extracted into the tear film
- GCMS showed that even monounsaturated unsaturated fatty acids degrade with overnight wear of contact lenses (Chapter 4).
- Although the tear film lipid layer is known to have low levels of polyunsaturated fatty acids (PUFAs), and MDA is only produced by PUFAs oxidation, contact lens wear changes tear film dynamics and lipids from other sources enter the tear film. Therefore, MDA can be produced in the ocular environment. MDA has been linked with contact lens intolerance in the literature.
- MDA was assayed by various commercial assays and MDA-ELISA was the most sensitive technique. For the first time, it was shown that MDA builds up within the contact lens. A chloroform: methanol extraction was the most effective method of MDA extraction.

The work carried out in this project was to try to understand and possibly monitor the effects on the lipid layer as a result of contact lens wear. Although there is still some future work to be carried out, the research undertaken provided evidence that lipid composition and structure is affected by lens wear and that these changes could be a cause of contact lens discomfort.

The possible implications of the research discussed in this thesis are listed:

- understanding individual subjects tear chemistry is important to help chose suitable lenses for those individuals
- the interaction and consequences of contact lens wear on the tear film are important to understand in order to for the development of newer, better contact lens materials.

7.3 Future directions

- The future directions of this work involve further investigations into the presence of lipid oxidation and MDA, in particular linked with length of wear, wear schedule and use of solutions.
- Investigating the levels of MDA in correlation with tear proteins derived from plasma leakage (such as tear albumin). Albumin can be found in tears, it passes into the tear film from plasma leakage. One of the functions of albumin in serum is the transport of free fatty acids (166). Therefore, increased levels of albumin could correlate to increased levels of MDA.
- Other future work would involve linking HPLC with other detectors such as mass spectroscopy to provide structural identification for separated species.
- The link between analytical and surface techniques (such as the measurement of coefficient of friction and Langmuir) is also another future aspect of the work discussed in this thesis.
- It was shown in Chapter 3 and Chapter 6 that non-polar solvents extract material from unworn silicone hydrogel materials. Investigating the effects that these extractable components have on the tear film lipid layer will be of interest in the future.
- The recruitment of subjects to wear lenses for 7days on a daily wear and continuous wear basis to elaborate on the initial findings in Chapter 4.
- As well as the analysis of lipid oxidative end products, the analysis of the levels of antioxidants in contact lens wear would be of interest in the future.

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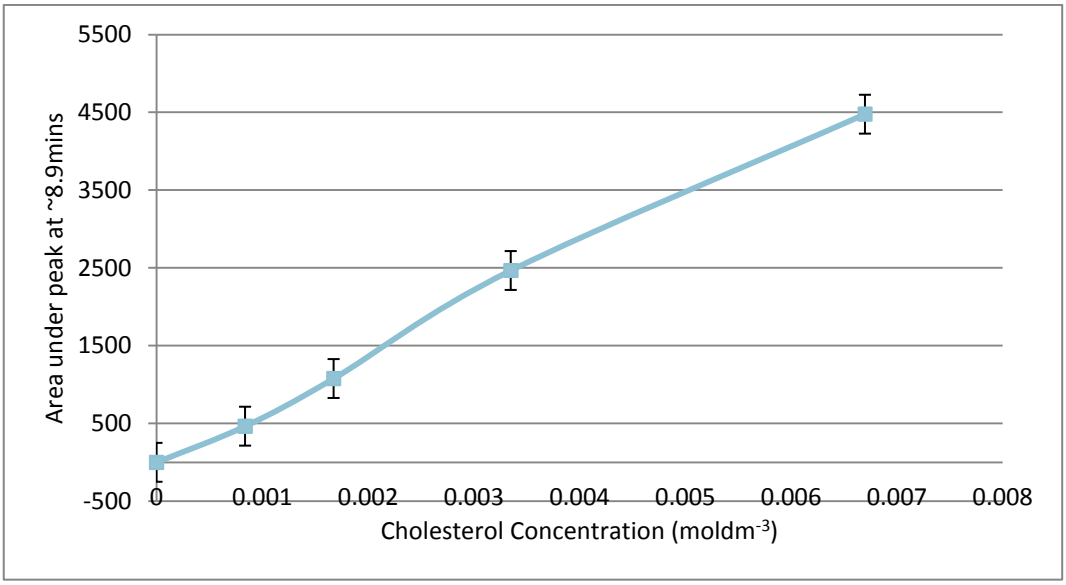
Appendices

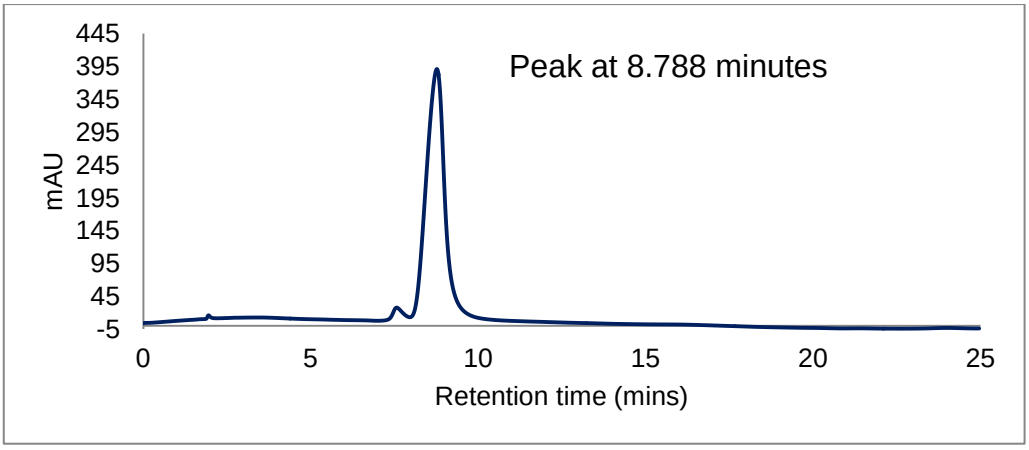
Appendix 1: Calibration curve data for lipid classes measured by normal phase HPLC

Calibration curve data for cholesterol standard

Concentration of cholesterol ($moldm^{-3}$)	*Area under peak at approx 8.9mins*
0	0
0.0008375	464.64053
0.001675	1077.4406
0.00335	2466.45459
0.0067	4476.15723



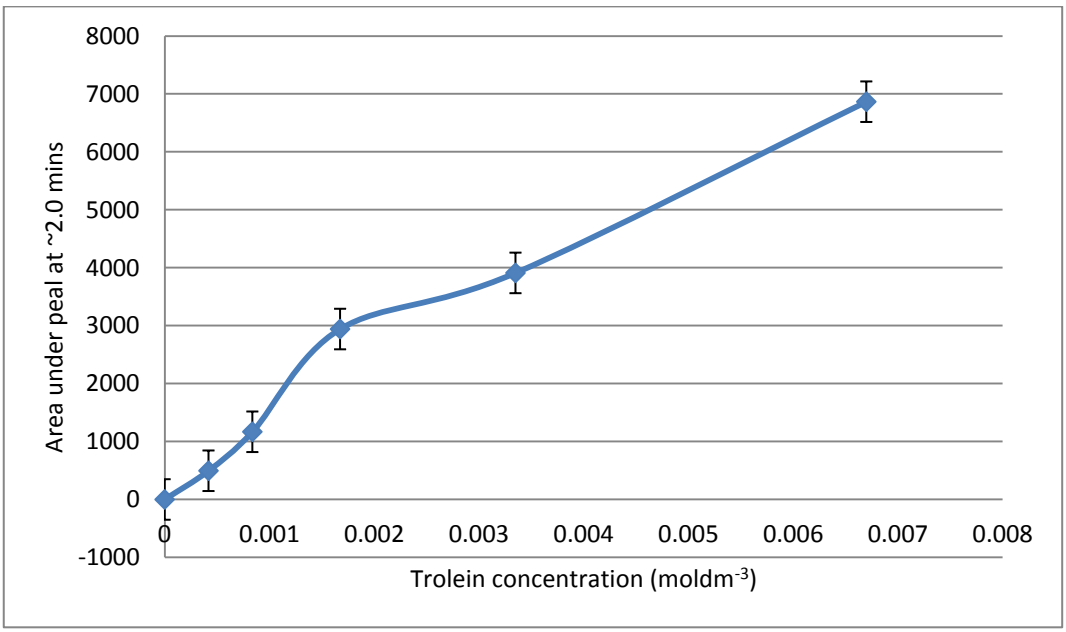
Calibration curve for cholesterol standard measured by HPLC



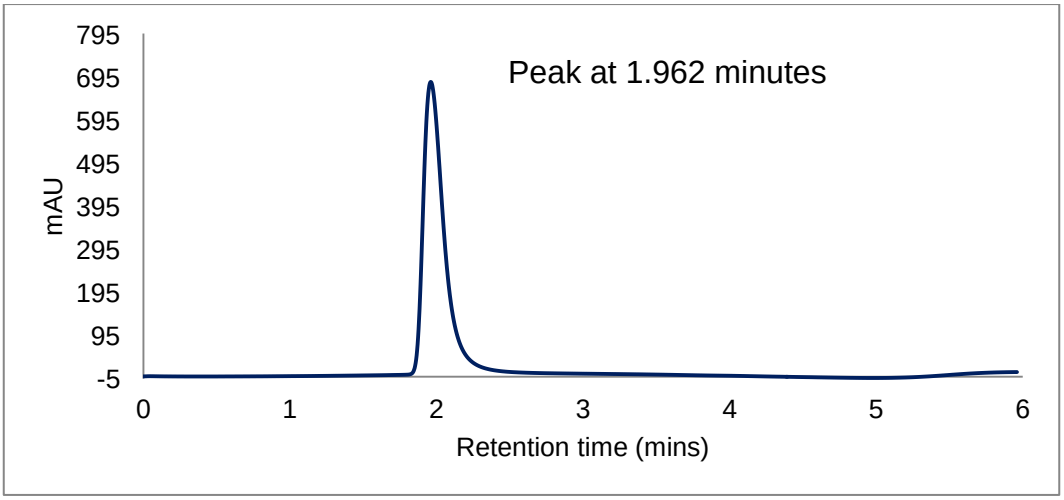
Primary peak for cholesterol standard measured by normal phase HPLC

Calibration curve data for triolein

Concentration of triolein (moldm^{-3})	*Area under peak at 1.962 minutes*
0	0
0.00041875	495.807
0.0008375	1168.66052
0.001675	2942.1212
0.00335	3910.76563
0.0067	6866.19727



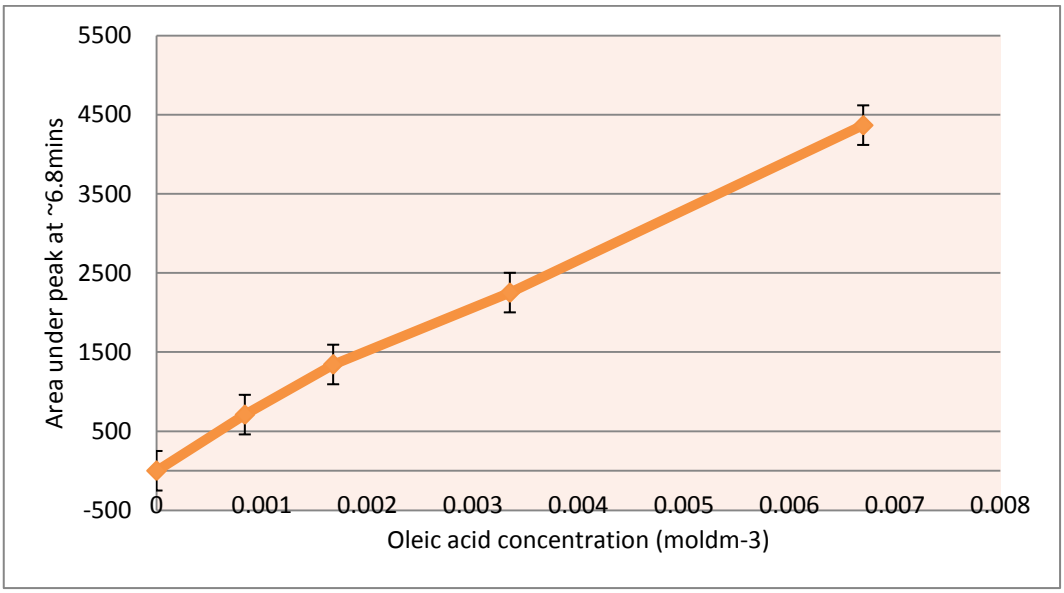
Calibration curve for triolein (TAG)



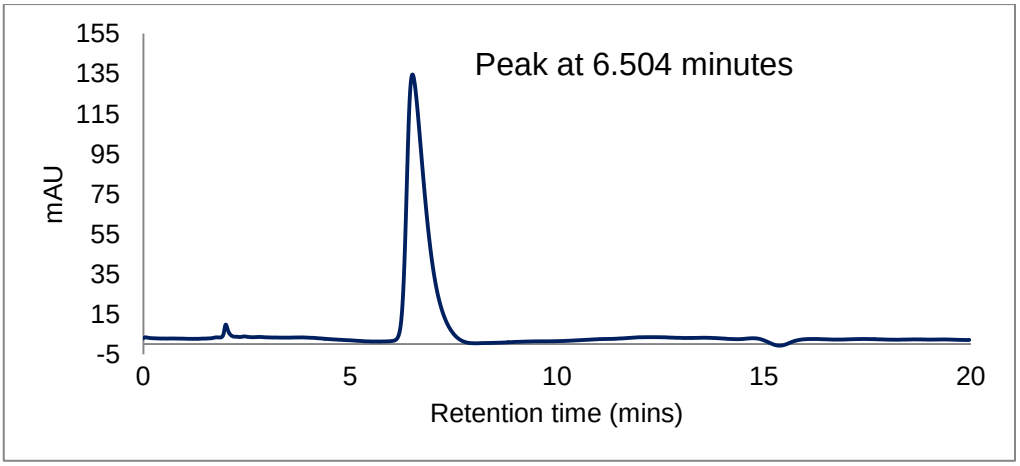
Primary peak for triolein (a TAG)

Calibration curve data for oleic acid

Concentration of oleic acid (moldm^{-3})	*Area under peak at 6.504 minutes*
0	0
0.0008375	709.35547
0.001675	1342.91541
0.00335	2251.34546
0.0067	4366.9842



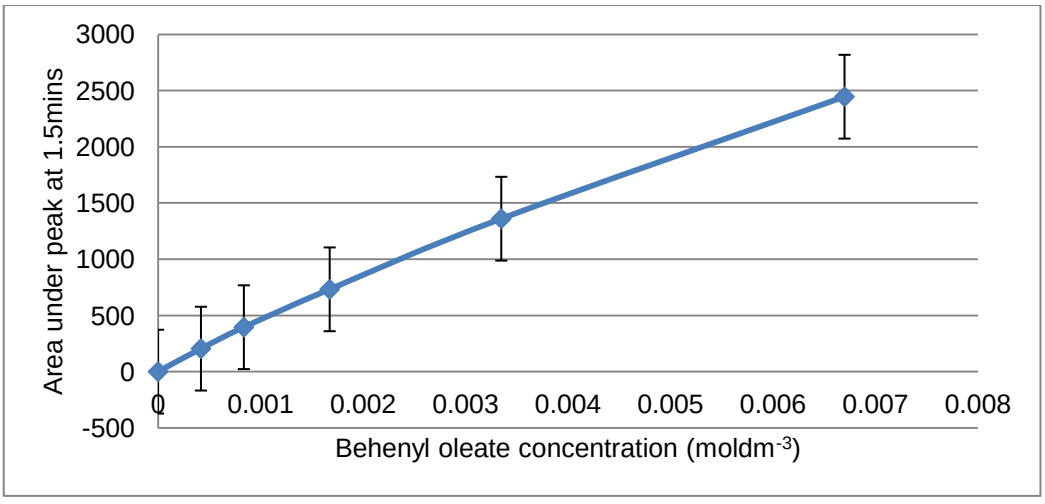
Calibration curve for oleic acid measured by normal phase HPLC separation



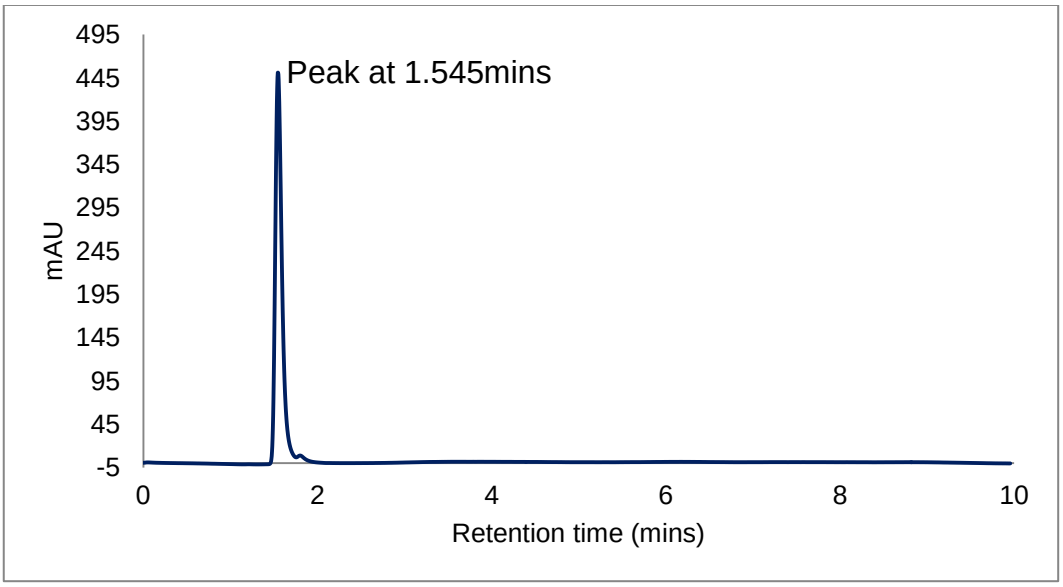
Primary peak for oleic acid standard separated by normal phase HPLC

Calibration curve data for behenyl oleate (a wax ester)

Concentration of behenyl oleate ($moldm^{-3}$)	*Area under peak at 1.545 minutes*
0	0
0.00041875	204.69272
0.0008375	395.40240
0.001675	732.32935
0.00335	1360.37585
0.0067	2445.56860

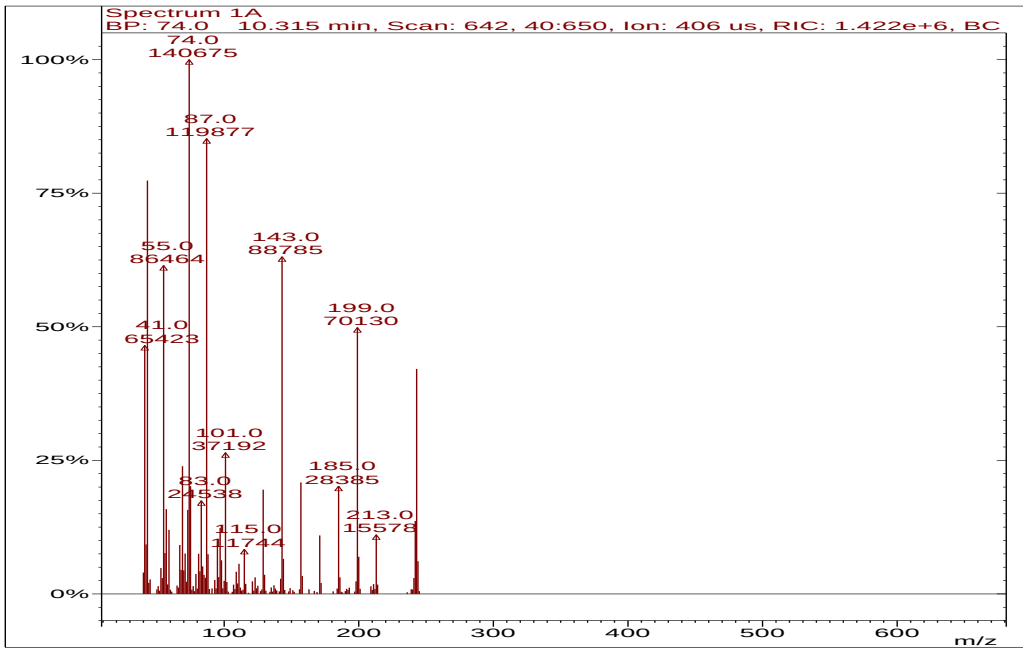


Calibration curve for behenyl oleate (a wax ester)

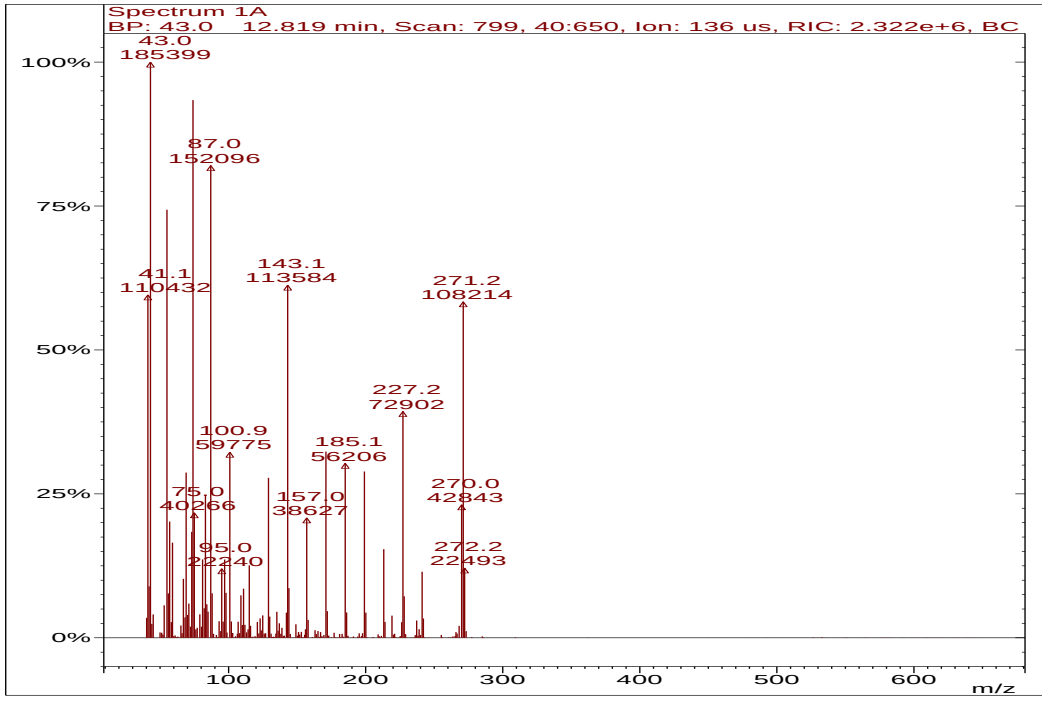


Primary peak for behenyl oleate standard separated by normal phase HPLC

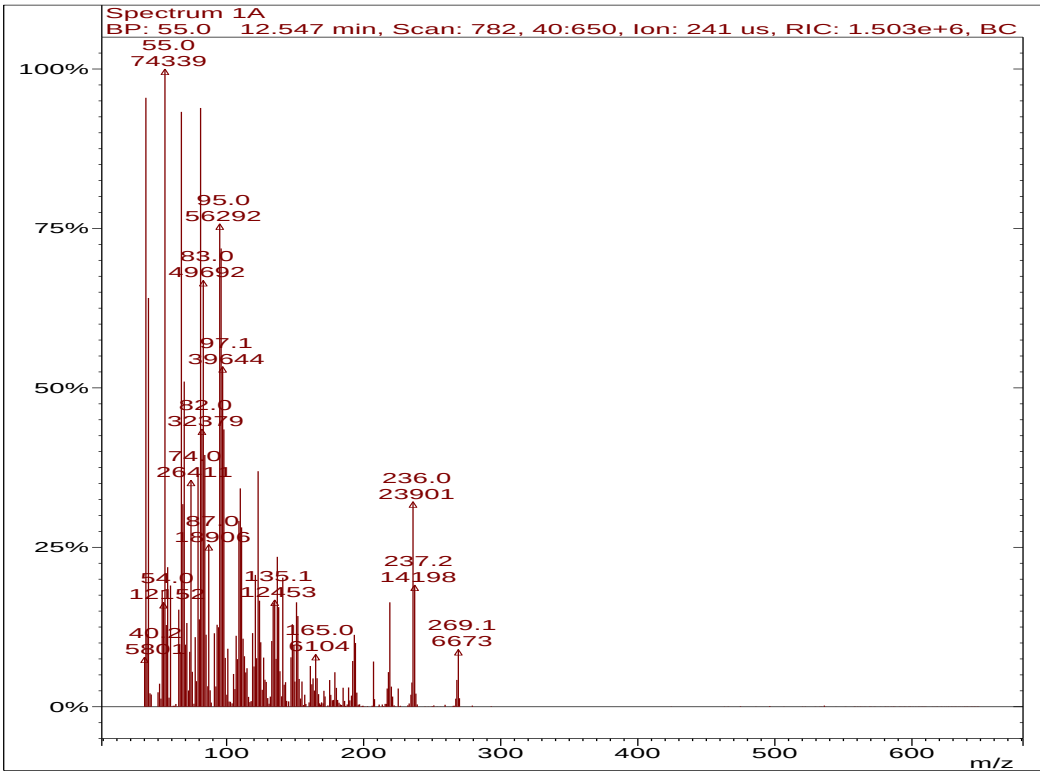
Appendix 2: Mass spectra of various fatty acid standards



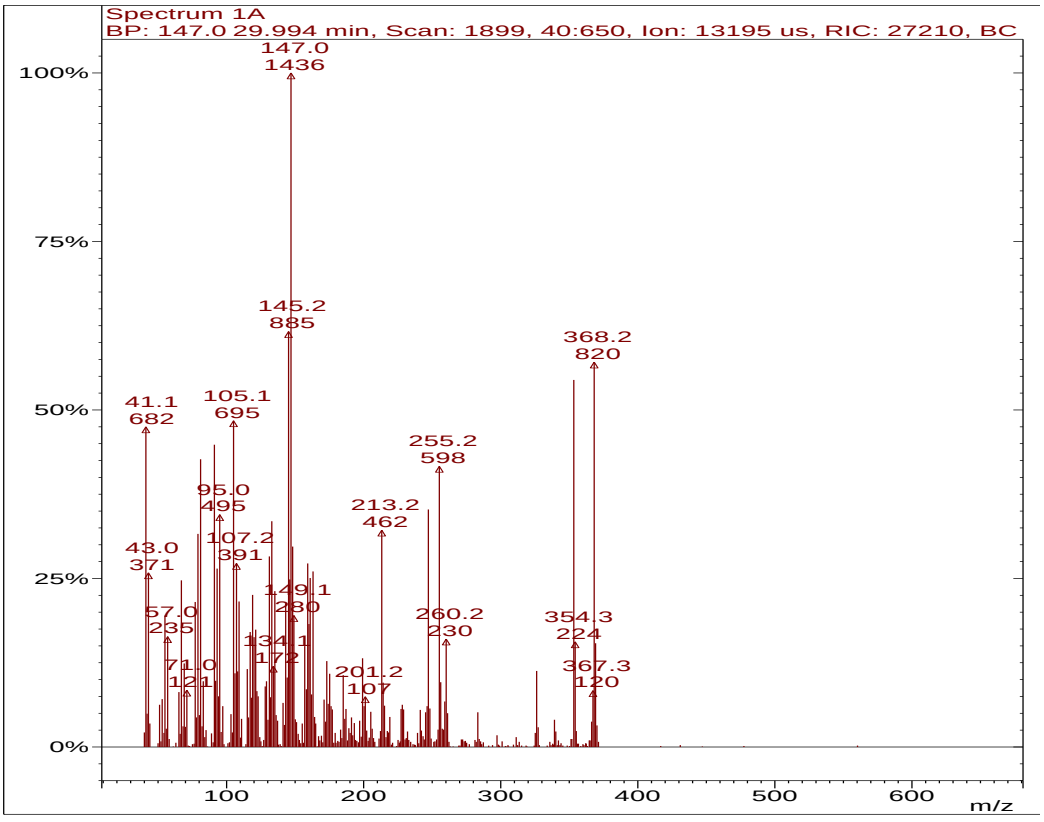
Mass spectra for C14:0 (myristic acid methyl ester)



Mass spectra for C16:0 (palmitic acid methyl ester)

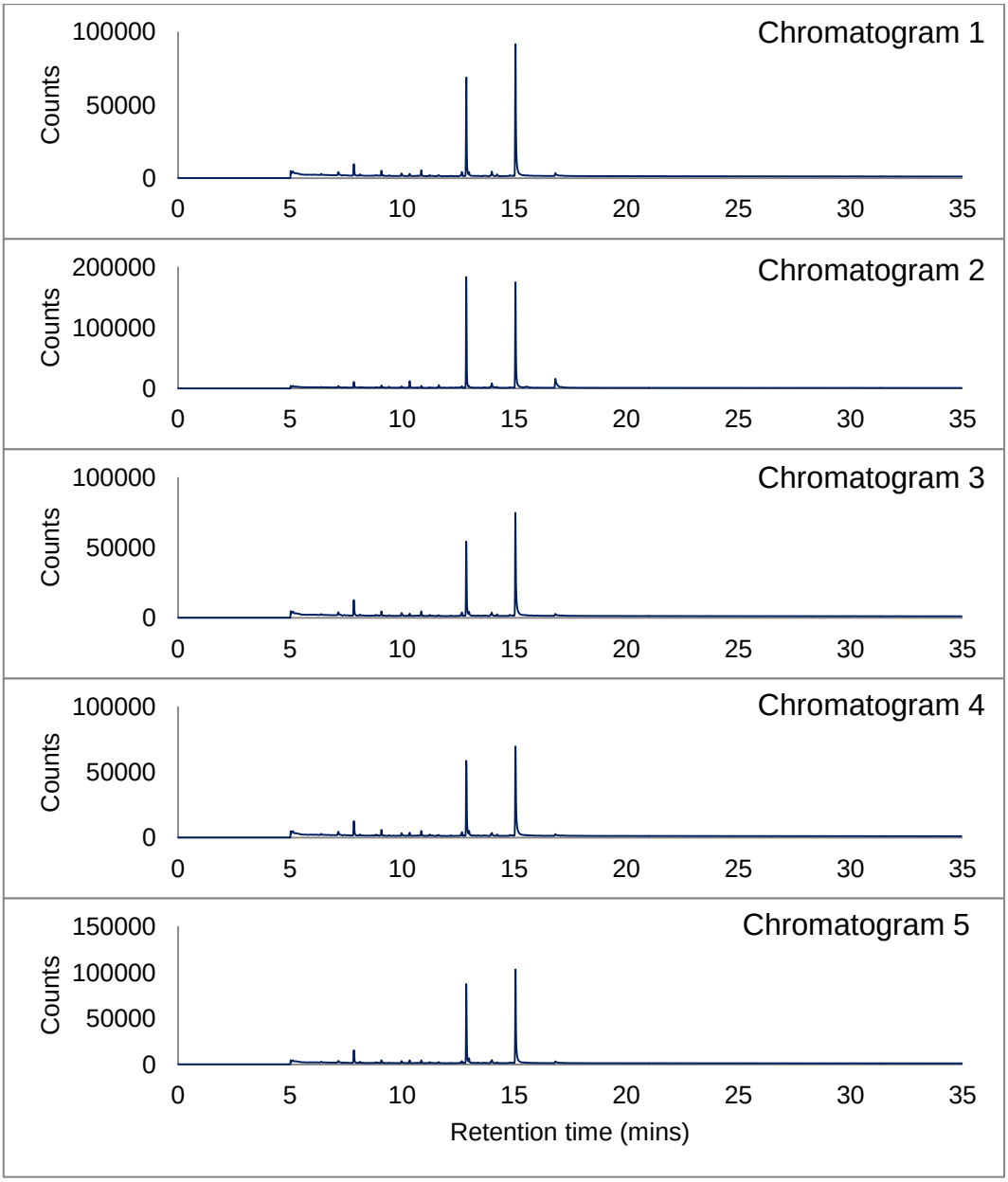


Mass spectra for C16:1 (palmitoleic acid methyl ester)



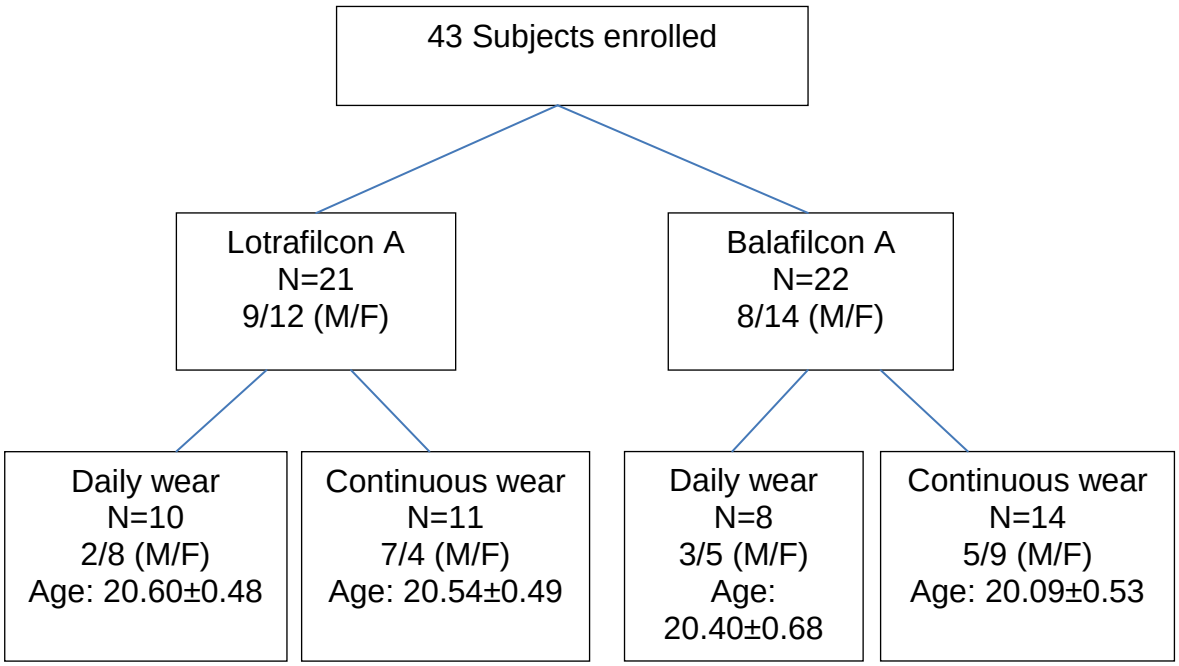
Mass spectra of cholesta-3,5-diene

Appendix 3: Gas chromatograms of empty vial extracts used for unworn lens extraction



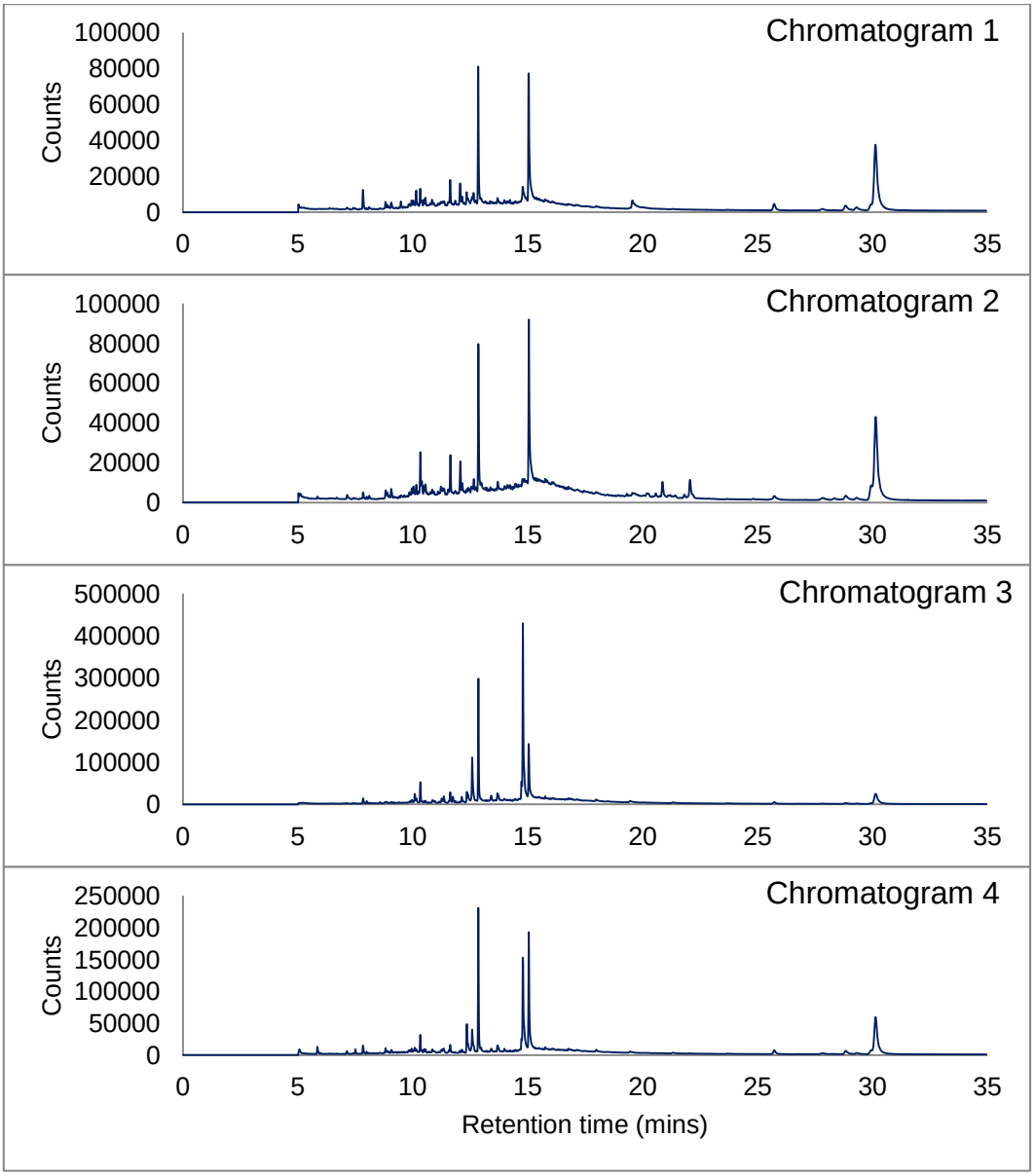
Gas chromatograms of empty vial extractions where: Chromatogram 1: empty vial extraction, vial used for unworn PV extraction, Chromatogram 2: empty vial extraction, vial used for unworn F.N&D extraction, Chromatogram 3: empty vial extraction, vial used for unworn AO extraction, Chromatogram 4: empty vial extraction used for AOA extraction, Chromatogram 5: empty vial extraction used for Soflens 66 extraction

Appendix 4: Study details for clinical trial (lenses used in Chapter 4)



M/F = male: female ratio

Appendix 5: Gas chromatograms of daily wear and continuous wear PureVision (PV) lens extracts for individual subjects

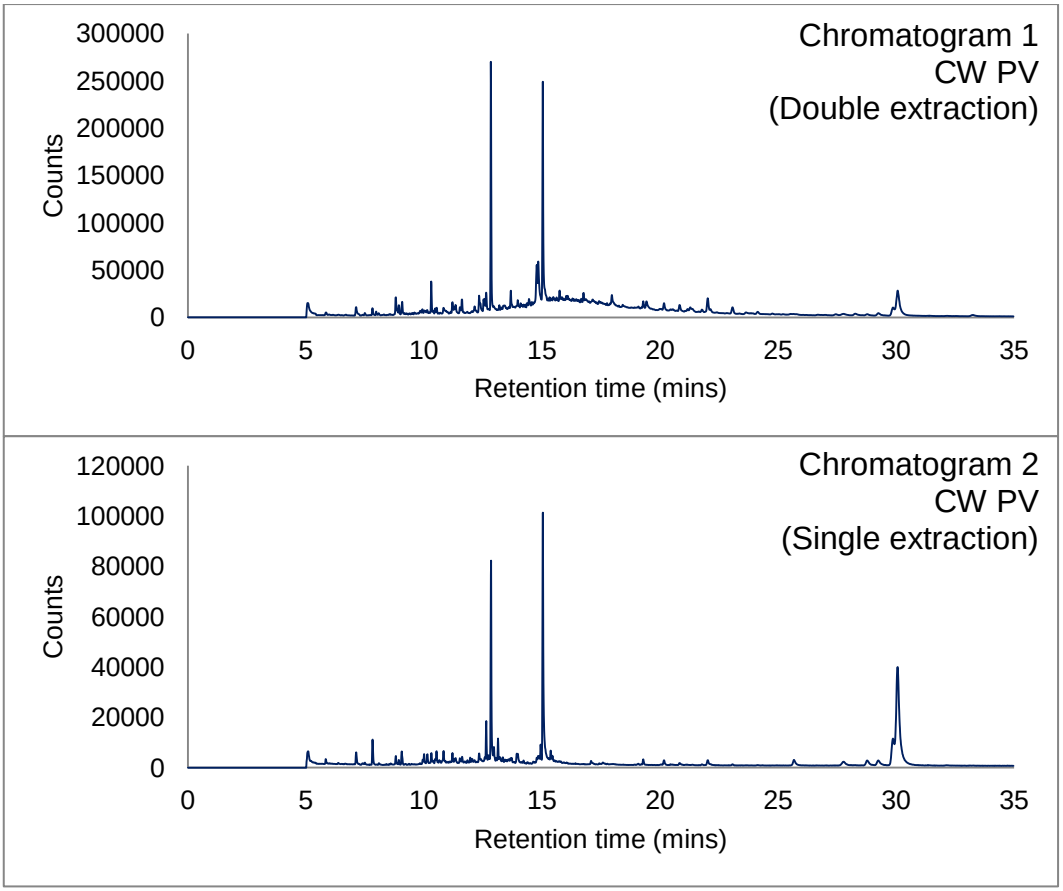


Chromatogram 1 – Continuous wear PV lens extract (extraction protocol 1)

Chromatogram 2 – Continuous wear PV lens extract (extraction protocol 3)

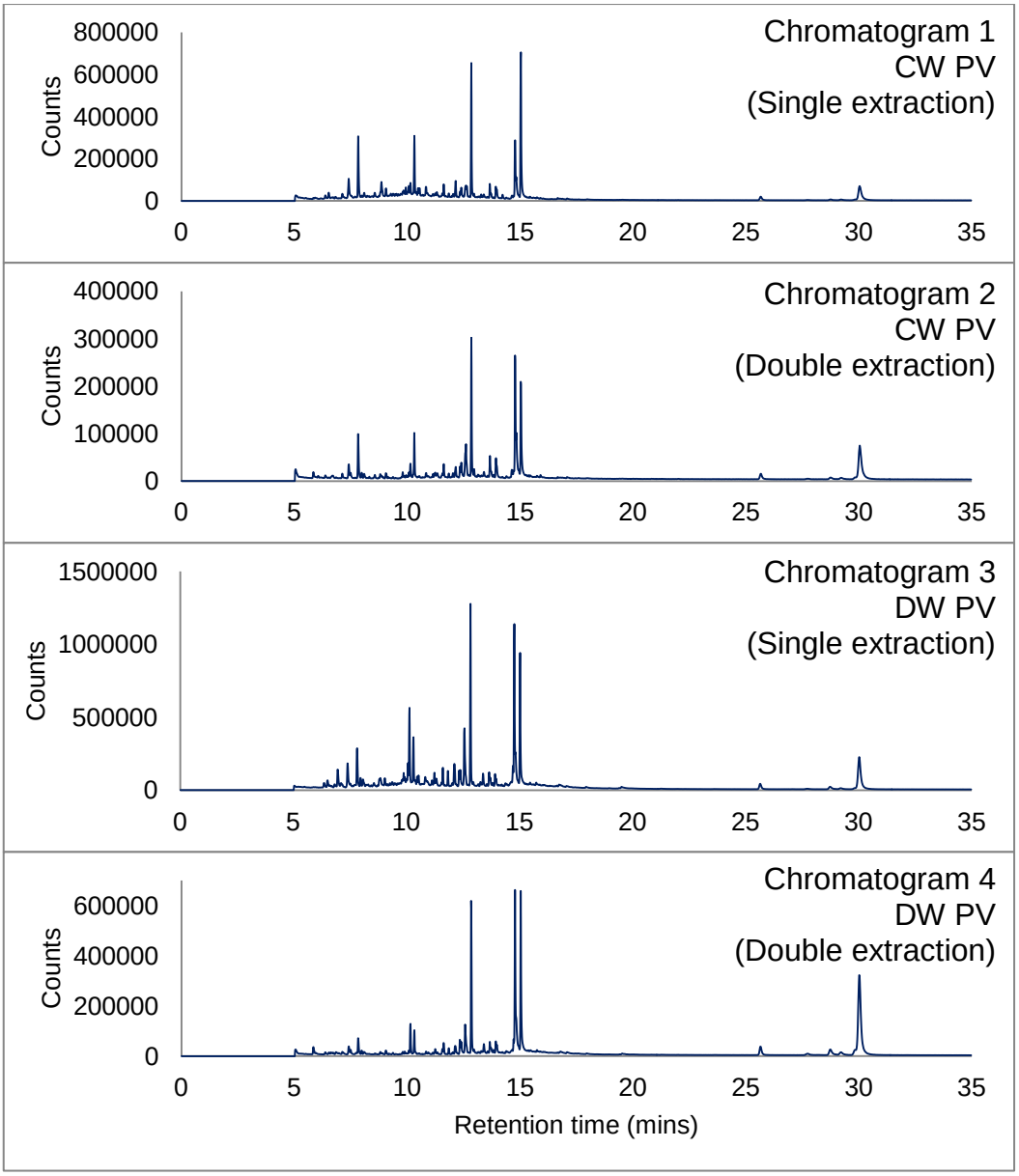
Chromatogram 3 – Daily wear PV lens extract (extraction protocol 1)

Chromatogram 4 – Daily wear PV lens extract (extraction protocol 3)



<u>Chromatogram 1</u> – Continuous wear PV lens extract (extraction protocol 3)

<u>Chromatogram 2</u> – Continuous wear PV lens extract (extraction protocol 1)

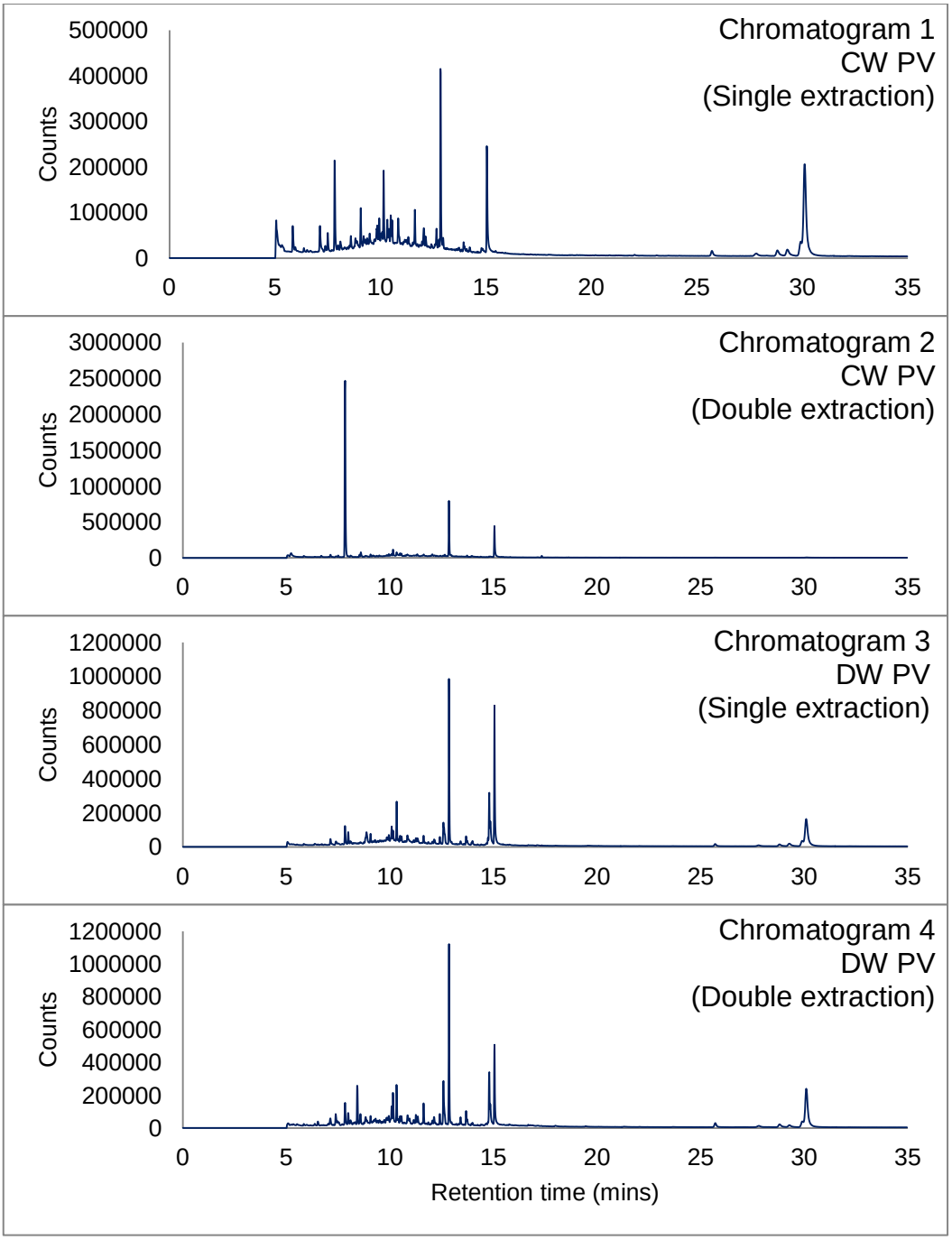


<u>Chromatogram 1</u> – Continuous wear PV lens extract (extraction protocol 1)

<u>Chromatogram 2</u> – Continuous wear PV lens extract (extraction protocol 3)

<u>Chromatogram 3</u> – Daily wear PV lens extract (extraction protocol 1)

<u>Chromatogram 4</u> – Daily wear PV lens extract (extraction protocol 3)

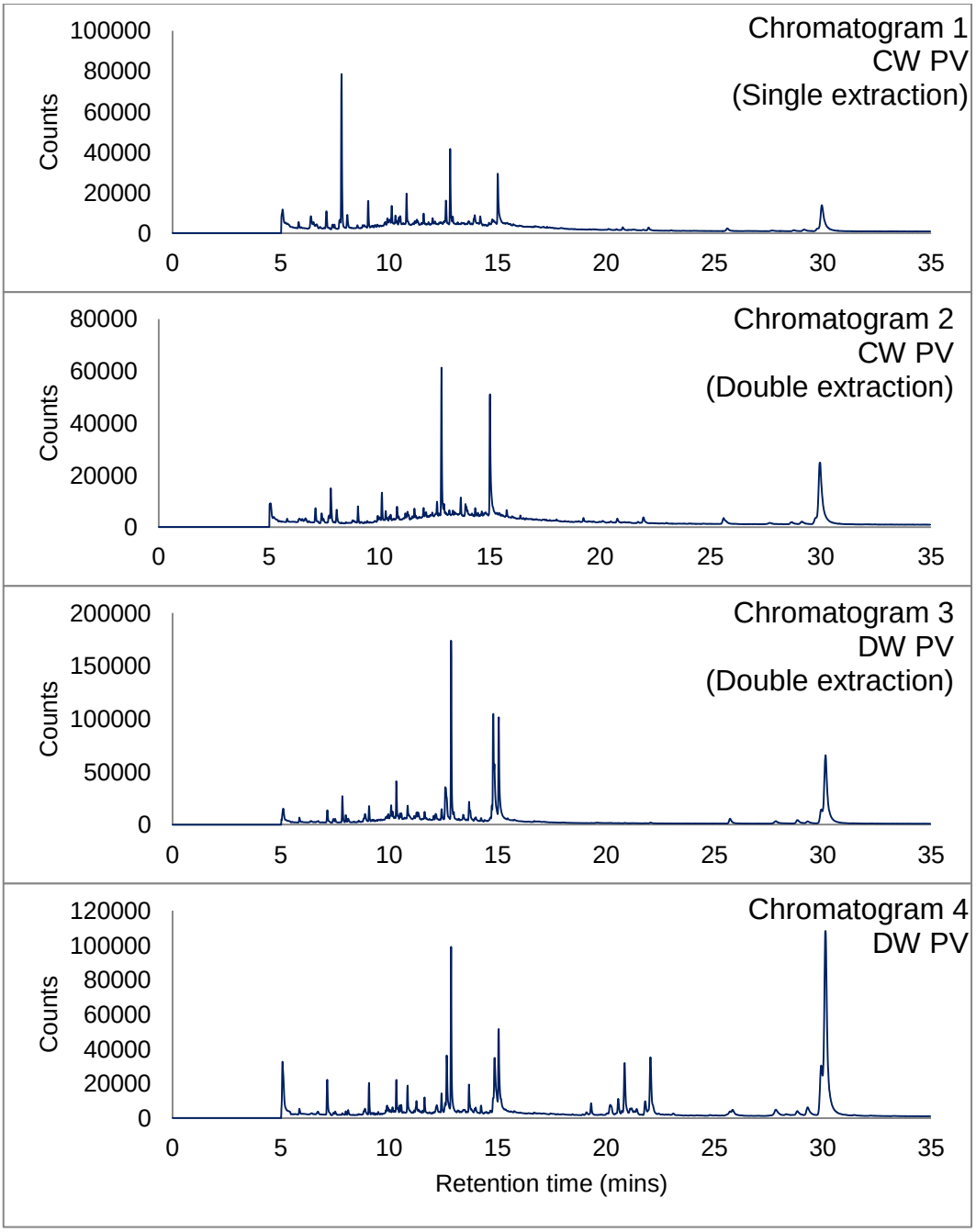


<u>Chromatogram 1</u> – Continuous wear PV lens extract (extraction protocol 1)

<u>Chromatogram 2</u> – Continuous wear PV lens extract (extraction protocol 3)

<u>Chromatogram 3</u> – Daily wear PV lens extract (extraction protocol 1)

<u>Chromatogram 4</u> – Daily wear PV lens extract (extraction protocol 3)

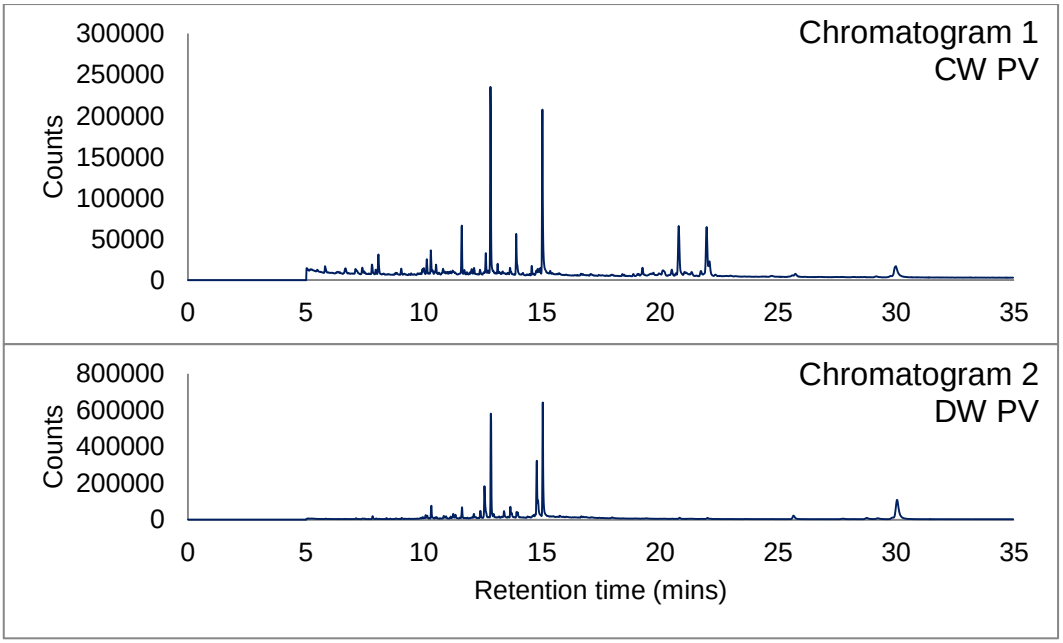


<u>Chromatogram 1</u> – Continuous wear PV lens extract (extraction protocol 1)

<u>Chromatogram 2</u> – Continuous wear PV lens extract (extraction protocol 3)

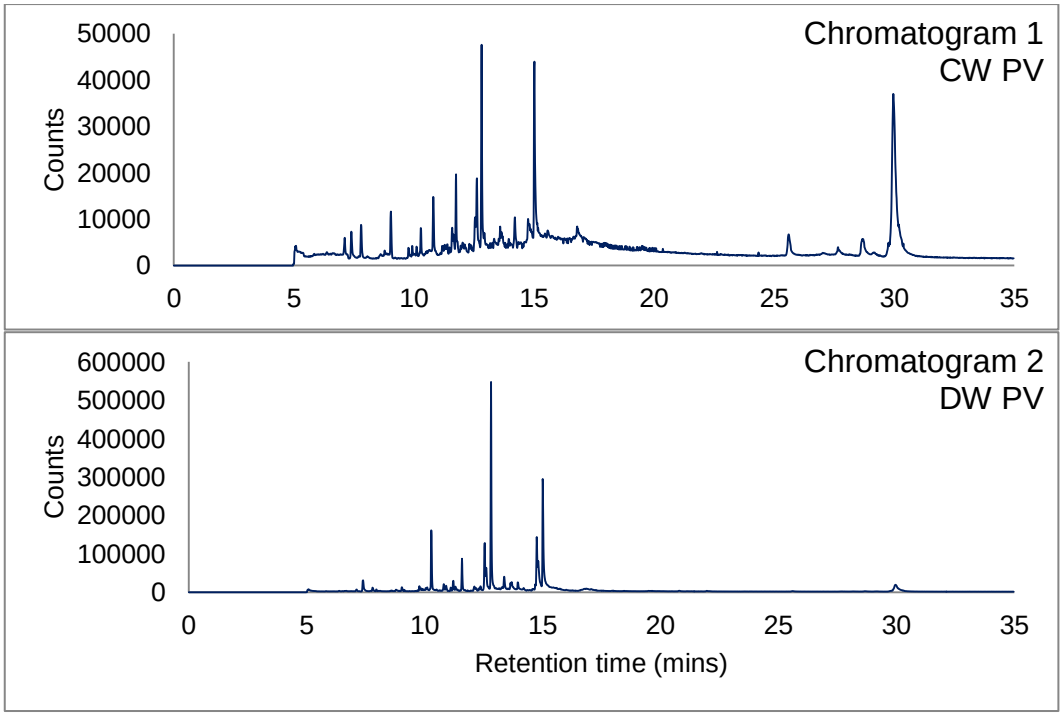
<u>Chromatogram 3</u> – Daily wear PV lens extract (extraction protocol 1)

<u>Chromatogram 4</u> – Daily wear PV lens extract (extraction protocol 3)



<u>Chromatogram 1</u> – Continuous wear PV lens extract (extraction protocol 1)

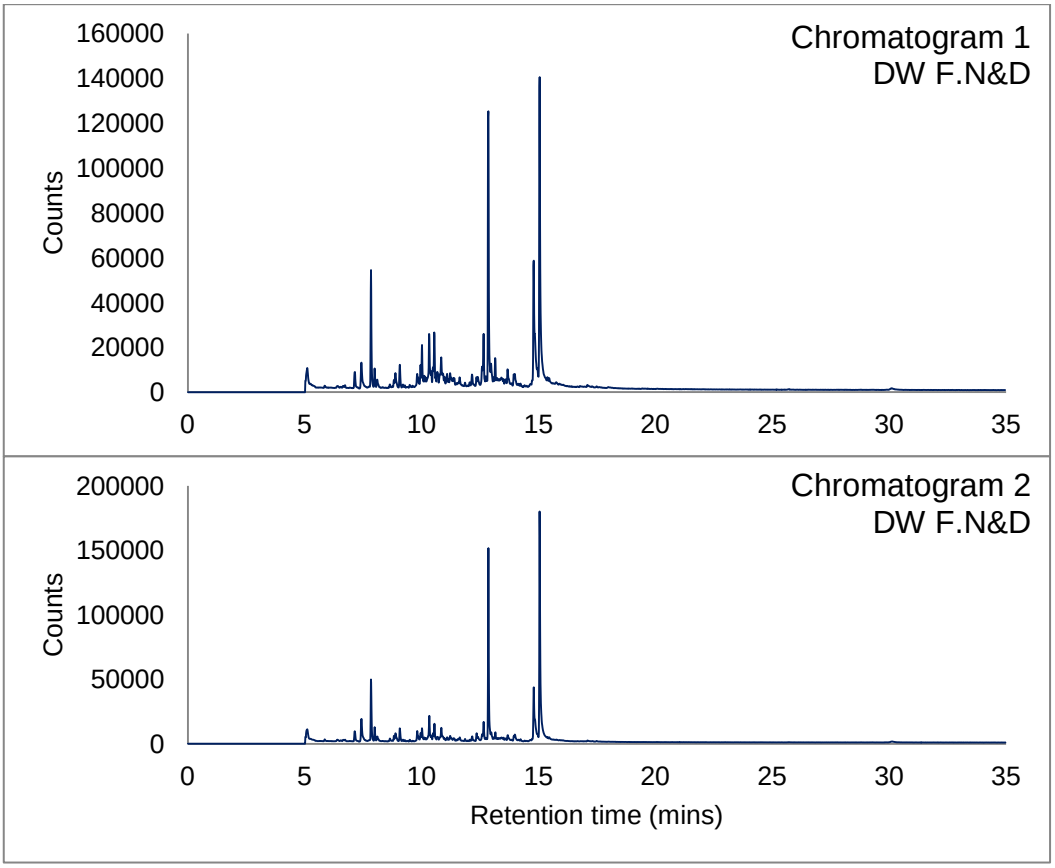
<u>Chromatogram 2</u> – Daily wear PV lens extract (extraction protocol 1)



<u>Chromatogram A</u> – Continuous wear PV lens extract (extraction protocol 1)

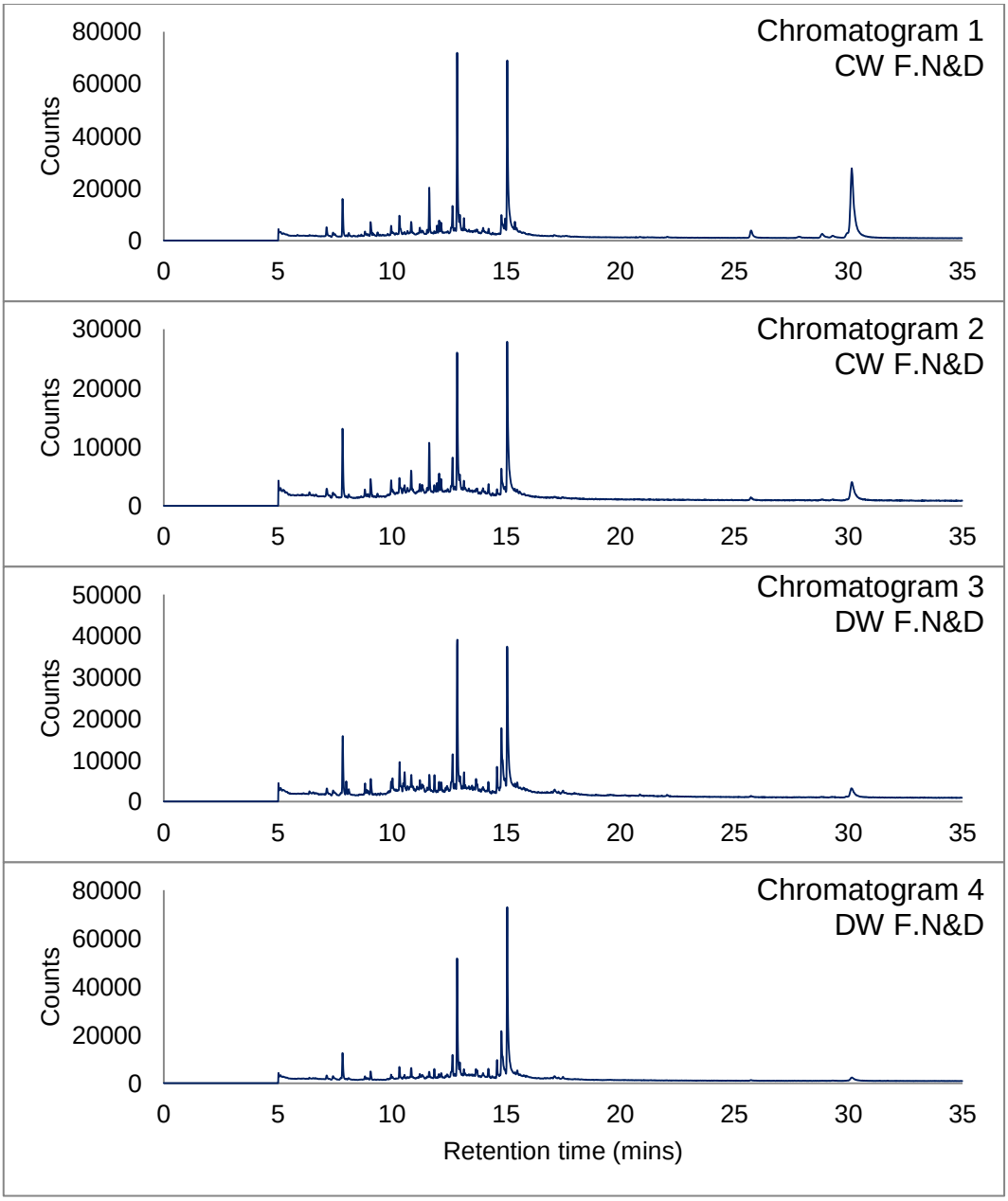
<u>Chromatogram B</u> – Daily Wear PV lens extract (extraction protocol 1)

Appendix 6: Gas chromatograms of daily wear and continuous wear Focus Night and Day lens (F.N&D) extracts



Chromatogram 1 – *Daily wear F.N&D lens extract*

Chromatogram 2 – *Daily wear F.N&D lens extract*

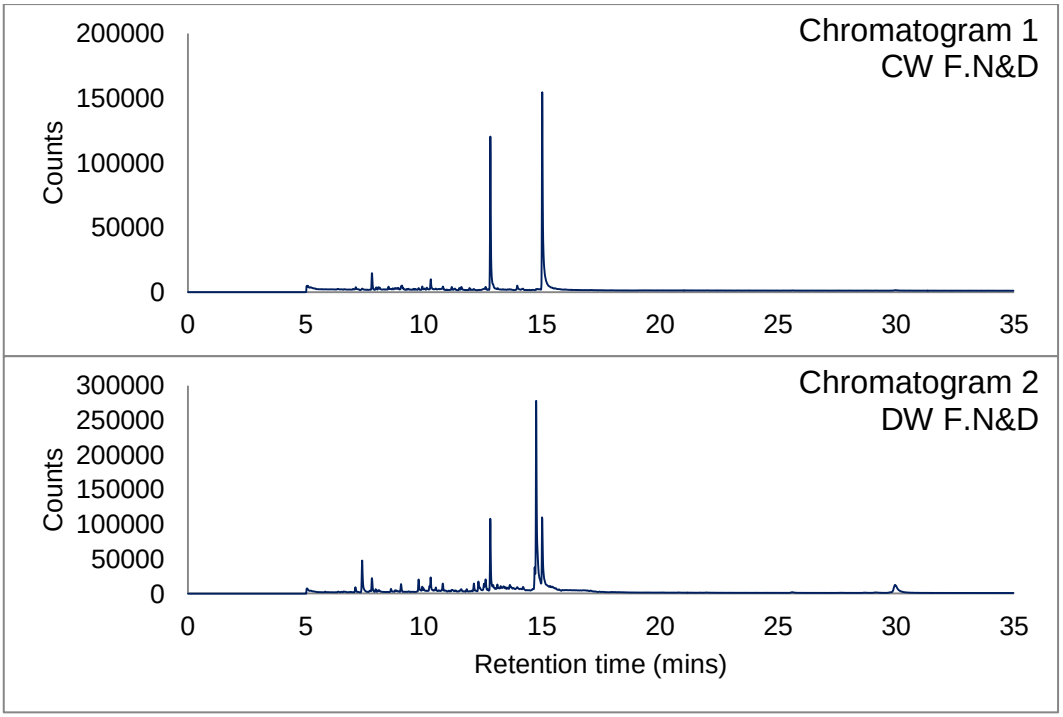


<u>Chromatogram 1</u> – Continuous wear F.N&D lens extraction (extraction protocol 3)

<u>Chromatogram 2</u> – Continuous wear F.N&D lens extraction (extraction protocol 1)

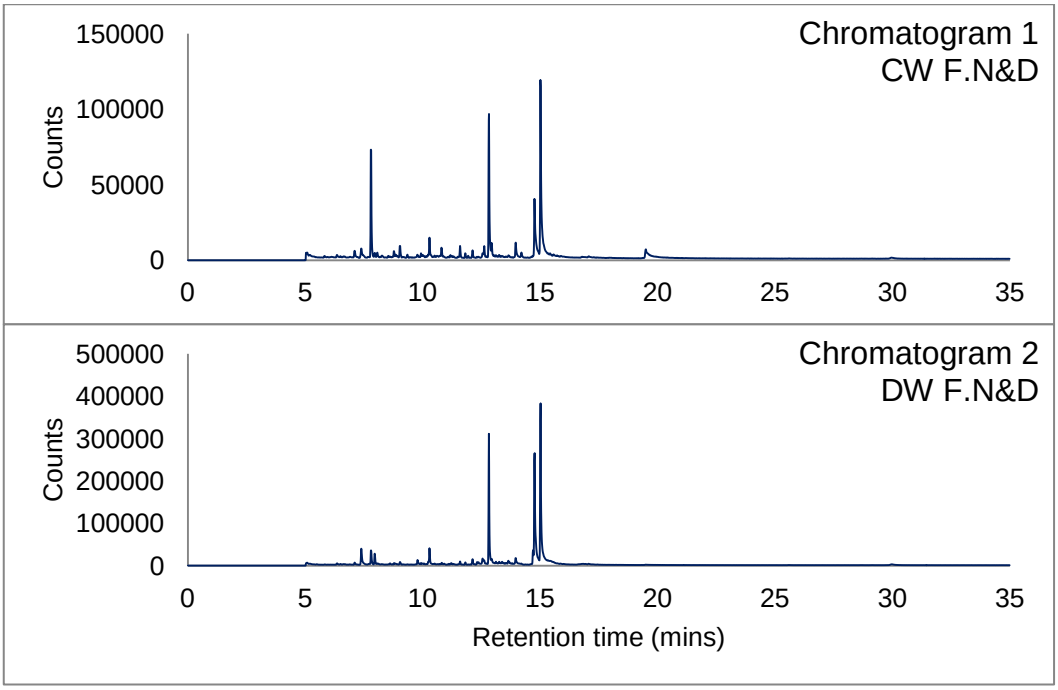
<u>Chromatogram 3</u> – Daily wear F.N&D lens extraction (extraction protocol 1)

<u>Chromatogram 4</u> – Daily wear F.N&D lens extraction (extraction protocol 3)



<u>Chromatogram 1</u> – Continuous wear F.N&D lens extract

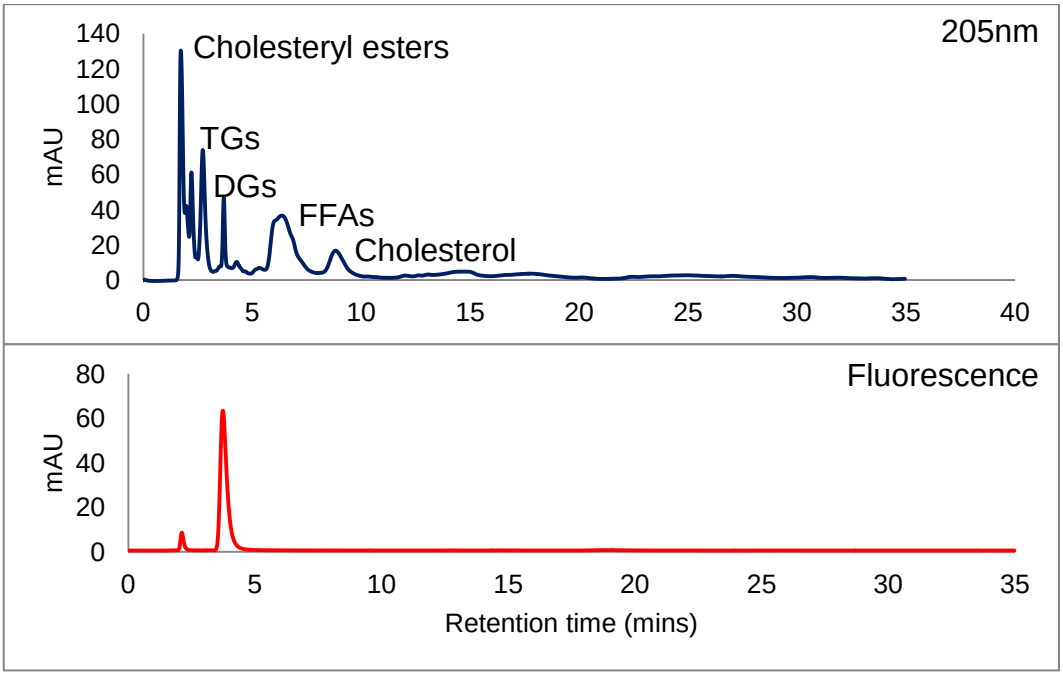
<u>Chromatogram 2</u> – Daily wear F.N&D lens extract



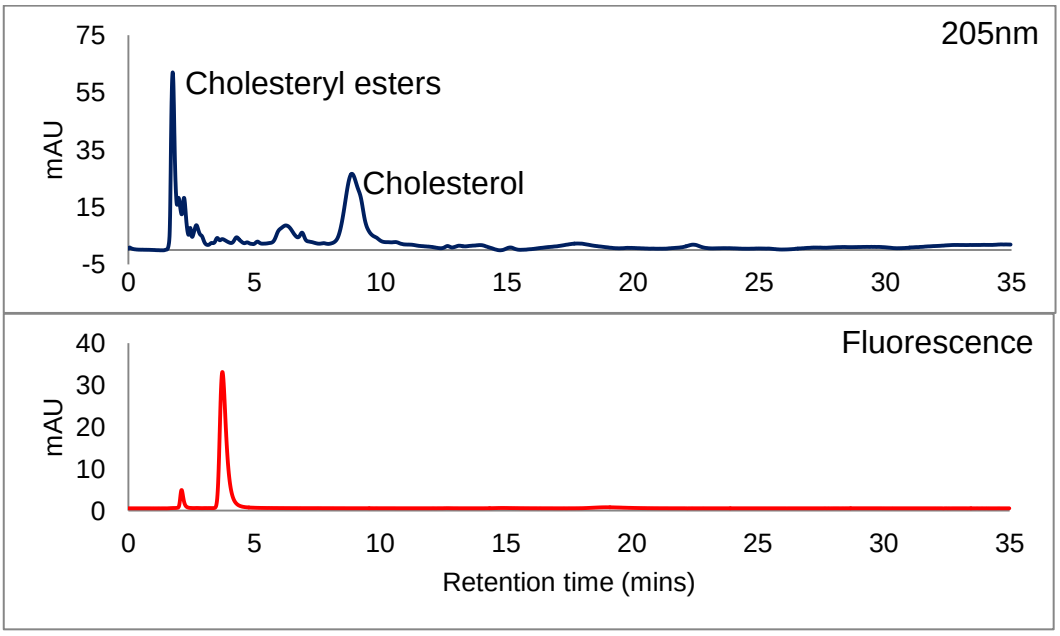
<u>Chromatogram 1</u> – Continuous wear F.N&D lens extract

<u>Chromatogram 2</u> – Daily wear F.N&D lens extract

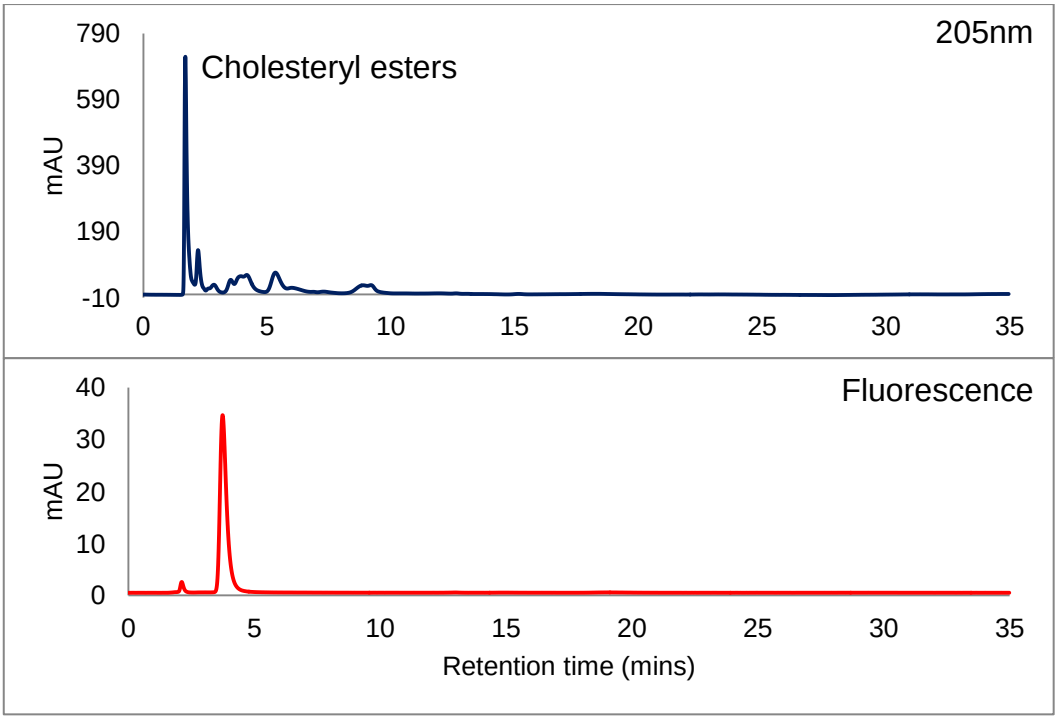
Appendix 7: HPLC traces of daily wear and continuous wear PV lens extracts worn by different subjects (post column change)



HPLC trace of a daily wear PV lens extract (extraction protocol 1) (subject 53)



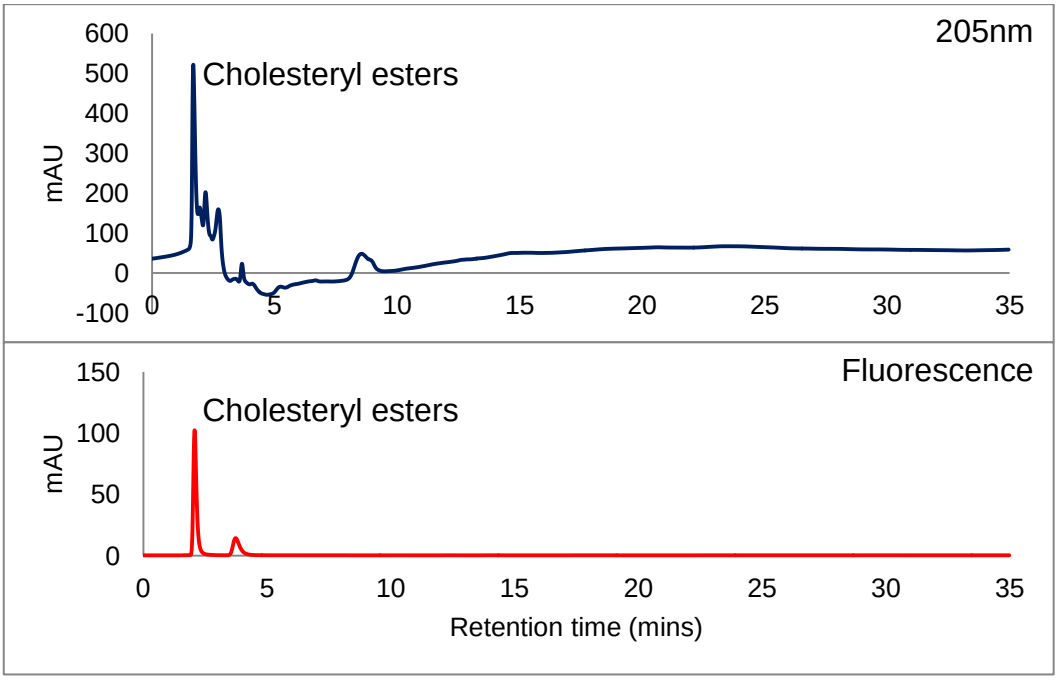
HPLC trace of a daily wear PV lens extract (extraction protocol 1) (subject 54)



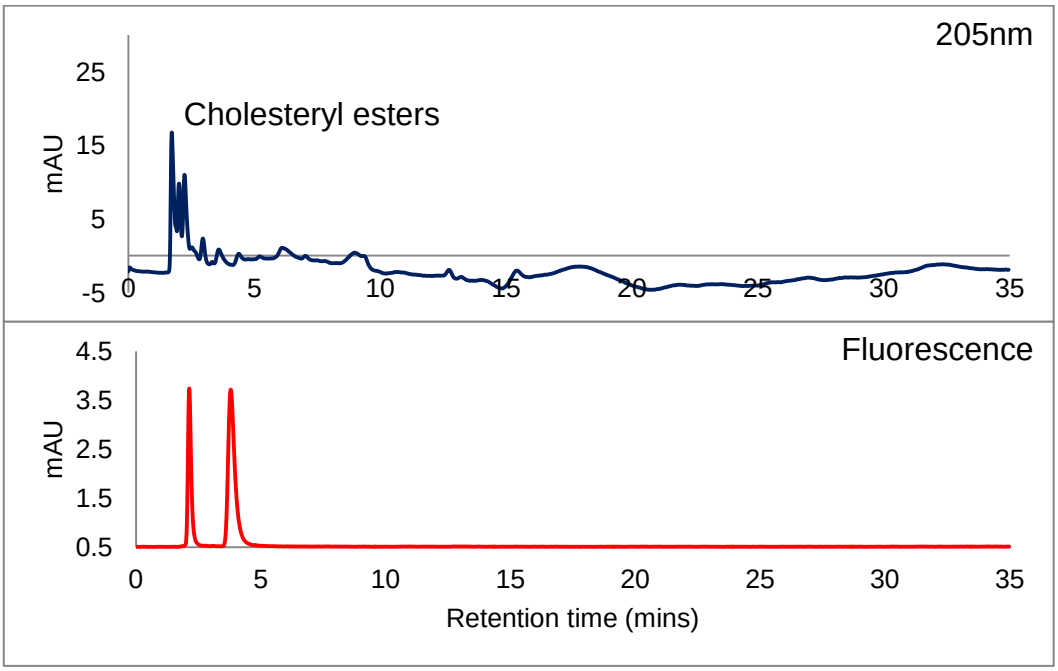
HPLC trace of a continuous wear PV lens extract (extraction protocol 1) (subject 29)

HPLC trace of a continuous wear PV lens extract (extraction protocol 1) (subject 59)

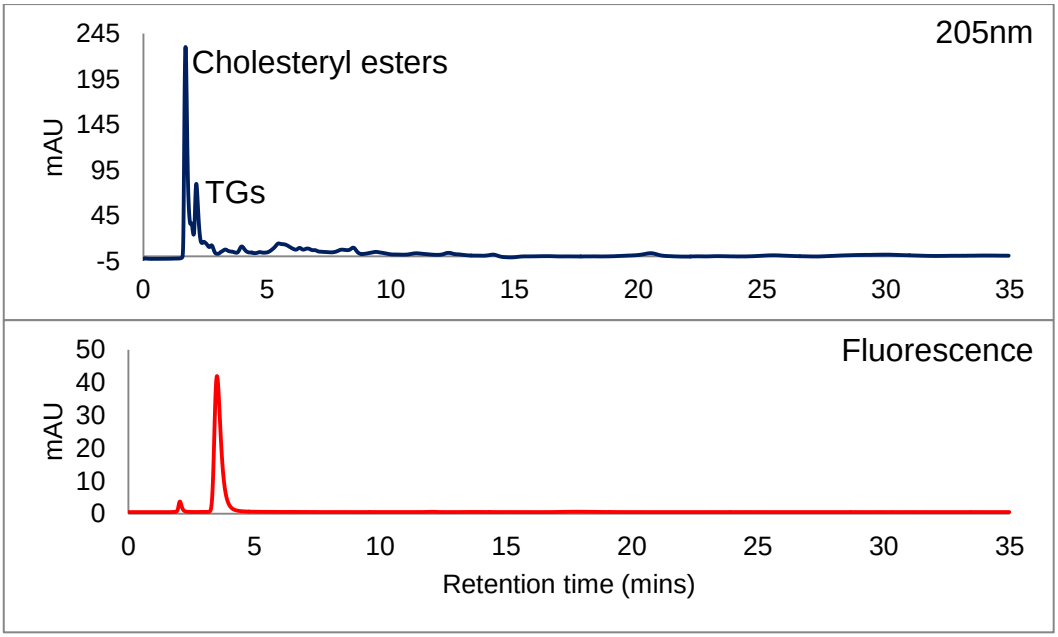
Appendix 8: HPLC traces of daily wear and continuous wear Focus Night and Day lens extracts worn by various subjects



HPLC traces of a daily wear F.N&D lens extract (extraction protocol 1) subject 27)

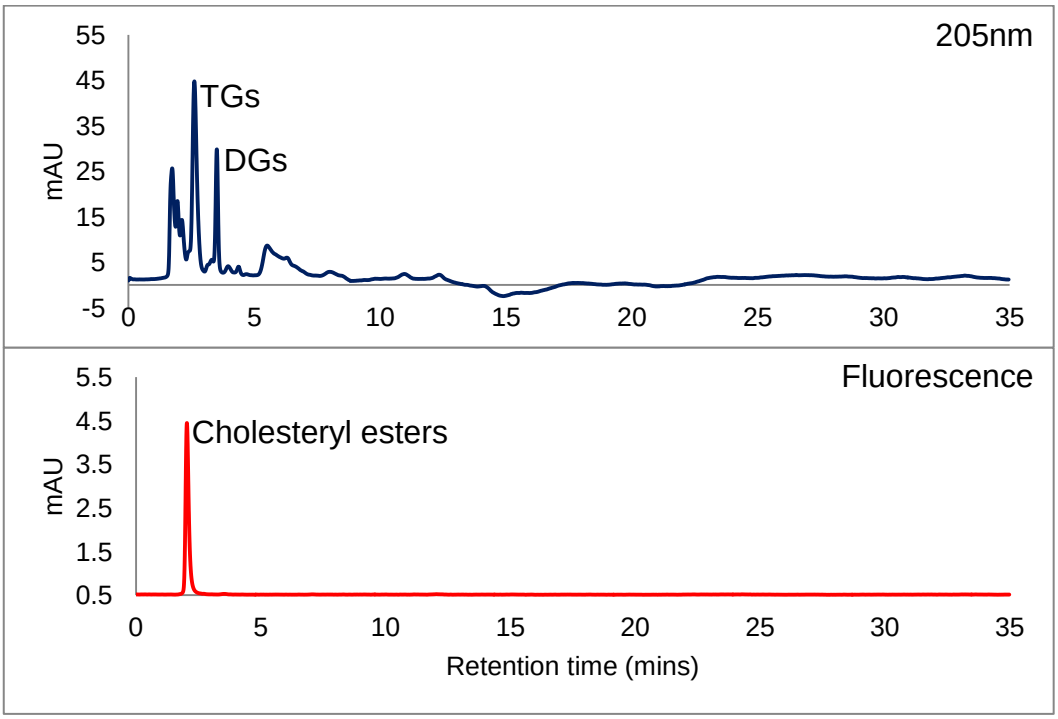


HPLC traces of a daily wear F.N&D lens extract (extraction protocol 1) (subject 11)



HPLC traces of a daily wear F.N&D lens extract (extraction protocol 1) (subject 61)

CEs



HPLC traces of a continuous wear F.N&D lens extract (extraction protocol 1) (subject 56)