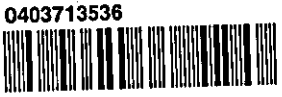

LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY ANALYSIS OF OLIGOLACTIC ACIDS

By Monica Dolci

A Doctoral Thesis submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy of Loughborough University

August 2008

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Dr. Maggi G. Tebrake, 3M Health Care Limited

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Abstract

The project has demonstrated the application of liquid chromatography coupled to mass spectrometry (LC-MS) for the characterisation of oligolactic acids (OLAs), employed as pharmaceutical excipients in metered dose inhalers.
OLAs proved to be difficult to characterise because of their complexity, which was ascribed to the presence of repeated structural monomeric units and a non-repeating moiety (the head group). Furthermore, during the course of method development the potential presence of degradation products and impurities had to be considered for quality control purposes.
Various LC-MS methods were developed to target both oligomeric distribution and head group functionalities of OLAs.

Liquid chromatography at critical conditions (LCCC), aimed at addressing the head group distribution of OLAs, led to the separation of the cyclic impurities from the parent linear molecules. However, to successfully achieve a complete characterisation of OLAs, a second separation targeting the oligomeric distribution was investigated.
Hydrophobic and polar interactions and possible solvation effects, which regulate RP-HPLC separation mechanisms, proved to be able to offer the selectivity necessary to resolve OLAs in terms of their size and their head groups, leading to the simultaneous separation between the linear molecules and their cyclic impurities and the determination of oligomeric distribution.

Acknowledgments

I would like to thank my supervisor Prof. Roger M. Smith for his guidance and invaluable support throughout the course of this project. His knowledge and experience have been inspirational and it has been an honour for me to be able to share ideas and time with him.

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This project would have been unsuccessful without the financial and technical support of my sponsor, 3M Health Care.

A very special thank you to Peter Irwin, for his continued support and friendship and for making me believe I could make it.

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Many thanks also to my friend and colleague Lee Derbyshire for his IT help during the last stretch of this incredible journey.

Last but not least, I wish to thank my parents for always directing me in the right way.

Finally I wish to dedicate this work to the memory of Paolo and my grandparents.

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1. Introduction

This research describes the characterisation of a group of synthetic oligomers employed as surfactants in pharmaceutical formulations used in metered dose inhalers (MDIs). The compounds investigated are a family of hydrofluoroalkane (HFA)-compatible excipients based on oligolactic acid (OLA, Figure 1.1).

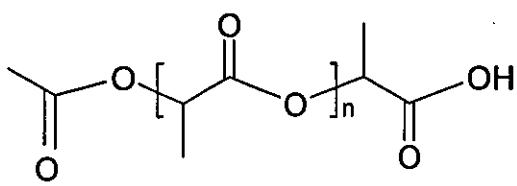


Figure 1.1: Structure of Oligolactic Acid

OLAs are biodegradable and biocompatible excipients, which interact with the drug to form drug/excipient complexes. These complexes offer advantages in either solution or suspensions in sustained release aerosol formulations. Their main advantage is a significant increase in the drug delivery efficiency, making MDIs an ideal device for the treatment of systemic diseases as well as a variety of diseases of the lung.

The regulatory guidances do not provide specific requirements for the chemical characterisation of excipients but it has been proposed that the analytical characterisation of OLAs should mimic those of active pharmaceutical ingredients (as stated in Q6A of ICH guidelines) [1]. However, oligomeric materials, such as OLAs, are difficult to characterise because they are not single chemical entities. They contain a number (n) of repeated structural groups (monomer units), representing regular molar mass differences, and a non-repeating moiety (the head group) and sometimes a tail group.

The presence of process impurities and related substances further complicates the characterisation of OLAs, (see Chapter 1.1 "Oligolactic acids and their importance in metered dose inhalers"). Because of the two types of distribution of characteristic structural units, OLAs present a property defined by Giddings as 'sample dimensionality' [2] (i.e. 'regular structural distribution of sample components'). In virtue of this definition OLAs are two-dimensional samples; this definition is of fundamental importance for targeting the analysis of the complexity of OLAs.

1.1. Oligolactic acids (OLAs) and their importance in metered dose inhalers (MDIs)

OLAs are a class of oligomers with a wide molecular weight range. They are derived from the oligomerisation of D,L-lactic acid:

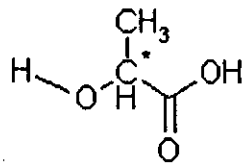


Figure 1.2: Structure of D,L-lactic Acid

Racemic mixtures of D- and L-lactic acids are used as the starting material to avoid crystallisation so that the final material is amorphous in order to improve its solubility.

1.1.1. Structural types

The simplest OLA structure is the free acid (Figure 1.3), which is oligomerised to give mixtures of products in the range of n= 2-100.

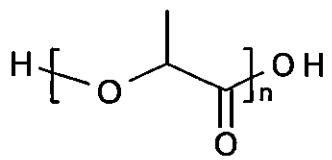


Figure 1.3: Structure of OLA free acid (n = 1)

During the lactic acid oligomerisation cyclic impurities (Figure 1.4) with different ring sizes can also be formed [3].

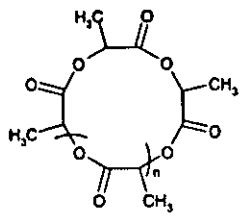


Figure 1.4: Structure of OLA cyclic impurities (typically n = 6-10)

The OLAs are then usually derivatised to provide excipients designed to be used as suspension aids or solubilising agents in formulations containing HFA propellants. Typically, within a suspension aid or a solubilising agent there are two distinctive portions.

A portion of the molecule (known as the 'head group'), which must interact with the drug but not with the HFA. In a typical surfactant, the head group is a hydrophilic moiety, e.g. PEG, carboxylate and sulfonate.

The other portion of the molecule (known as the 'tail') must interact with the HFA propellant. Typically in surfactants, the tail is a hydrophobic moiety, e.g. fatty or alkyl section. HFAs have high dielectric constant and dipole moment and proton donor capabilities. Molecules with electron donor capabilities will interact with the HFAs, such as ethers, carbonates, amides and esters. OLAs have a high density of esters on the backbone, ensuring multiple interactions with the propellant.

The effects of structural variations in a series of OLAs on their utility as suspension (and solubilising agents) in HFA-based MDI formulations were researched by Stefely *et al* [4]. They found that OLAs with an average of 5 to 15 repeat units functioned well as suspension aids, leading to improved suspension formulations.

They also investigated a series of substituents of the head/tail groups, in view of the fact that suspension characteristics can be improved by the presence of one or more

head groups and/or one or more tail groups. The simplest OLA structure (the free acid) with two head groups (i.e. OH and COOH) is a mediocre suspension agent but they demonstrated that acetylation of the hydroxy functionality resulted in a mono-functional suspension aid (acetyl OLA, or ac-OLA, Figure 1.5), with improved suspension qualities:

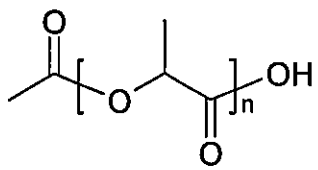


Figure 1.5: Structure of acetyl OLA

Similarly, a propionyl group CH_3CH_2CO- could be substituted on the hydrogen atom of the free hydroxyl to give propionyl-OLA (pr-OLA) (Figure 1.6) [3]:

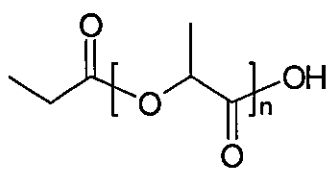


Figure 1.6: Structure of propionyl-OLA

A third substituent was the pyruvyl group, CH_3COCO-, which led to pyruvyl-OLA (pyr-OLA) (Figure 1.7) [3]:

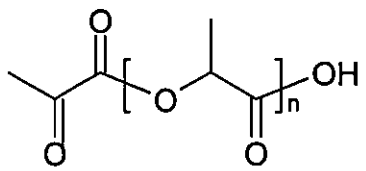


Figure 1.7: Structure of pyruviyl-OLA

Stefely *et al.* also showed that amidation of the carboxylic acid head group with glycine provided a further improvement in the formulation quality with several drugs [4].

When a diamine was reacted with a propionyl OLA, double amidation could take place, leading to the formation of diamide diester forms of OLA, (such as OLA-81) (Figure 1.8) [3]:

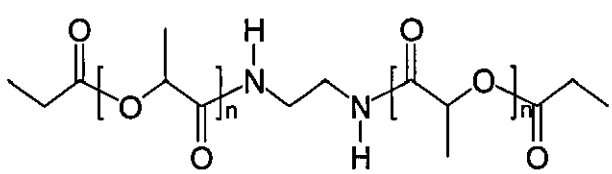
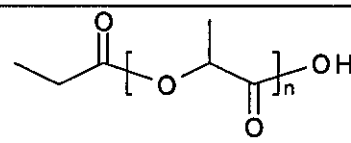
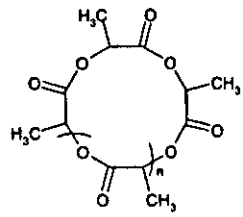
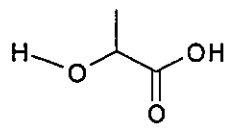
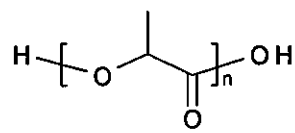
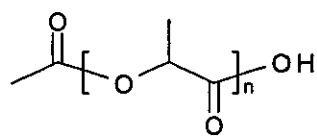
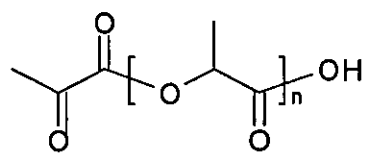
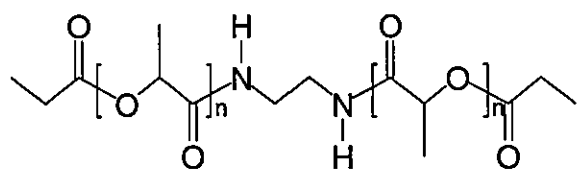
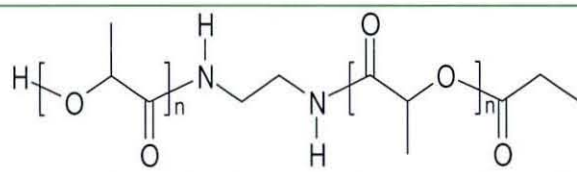


Figure 1.8: Structure of OLA-81, ethylene bis (propionyl oligolactyl amide) (n = 8-25).

Because there may be incomplete formation of these derivatives or hydrolysis of the esters or amide groups, other impurities may be present in materials being used for formulation including chains with terminal free alcohol and acids, such as: pr-OLAs, acyl free acid, acetyl free acid and free acid OLAs (Table 1.1).

Table 1.1: Some typical substituted OLAs and their impurities and degradation products

Name	Structure	OLAs Chemical entities
Pr-OLA		Degradation product
Cyclic		Process impurity
Free lactic acid		Degradation product
Free oligolactic acid		Degradation product
Ac-OLA		Degradation product
Pyr-OLA		Degradation product
Diamide diester		Main excipient

Diamide monoester		Degradation product

1.1.2. Role of OLAs in metered dose inhalers (MDIs)

MDIs are drug delivery systems that use gases or highly volatile liquid propellants to atomize a precisely metered volume of a medicinal formulation into particles/ droplets, which are small enough to penetrate deep into the patient's lungs. Figure 1.9 below shows a schematic of a typical MDI configuration.

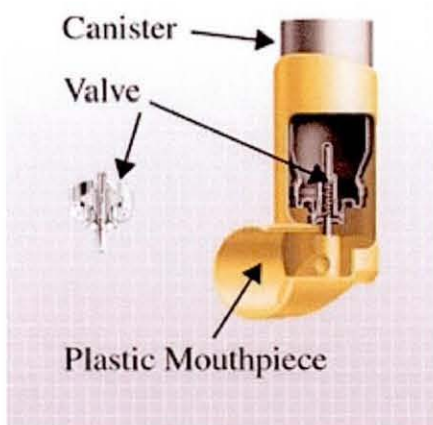


Figure 1.9: Metered dose inhaler configuration. Ref. [4].

When an MDI is actuated, the formulation is atomized into very small droplets, which rapidly decrease in size due to evaporation of the highly volatile formulation. For the drug to be delivered efficiently, the droplets must rapidly decrease in size to less than 5 microns, in order to penetrate through the oropharynx and into the lung [5]. The drug can either be in solution or as a suspension. Generally, MDIs

developed as a solution formulation utilise ethanol as a co-solvent to solubilise the drug. The advantage of solution MDIs is that the size distribution of the drug particles delivered to the patient is significantly smaller than the size of particles delivered from typical suspension MDIs [6-8]. Originally chlorofluorocarbons (CFCs) were widely used as propellants because of their inertness and low toxicity. However, since the introduction of the Montreal Protocol, which limited and then banned the use of CFCs even for medicinal purposes, alternative propellants have been investigated. The two main groups currently used are hydrocarbons, such as pentane, which tends to be used in domestic products such as hairspray and polishes, and hydrofluoroalkanes (HFAs), such as 1,1,1,2-tetrafluoroethane (fluorocarbon 134a) [9]. The introduction of HFAs represented a main challenge for the pharmaceutical industry, since many drugs are relatively polar and are only poorly soluble in HFAs; as a consequence, some drugs need a co-solvent in order to be solubilised.

However, not all drugs can be efficiently delivered using conventional co-solvent-based solution MDIs [5]. This is because the co-solvents, such as ethanol, decrease the volatility of the formulation, thus decreasing the efficiency of the drug delivery. Poorly soluble drugs, or drugs that require a large delivered dose, would require large amounts of co-solvent to solubilise the drug and can not be efficiently delivered using conventional HFA solution formulations.

HFA MDIs developed as a suspension formulation almost always need surfactants to obtain acceptable dosing reproducibility due to the rapid flocculation of most drugs in HFA propellants [5].

Traditional surfactants are not soluble in HFAs and therefore co-solvents are needed to solubilise the surfactants, thus leading to the issue of decreased drug delivery efficiency. One alternative approach has been to add solubilising agents such as

OLAs, which have been shown to provide stable suspension formulations, with little or no co-solvent, and can therefore optimize the drug delivery efficiency [4, 10, 11]. The role of OLAs is to interact with drug molecules to form drug/excipient complexes that are highly soluble in HFA propellants because of the electron density of the oligomeric ester groups.

As shown in Figure 1.10, when a suspension formulation containing OLAs is actuated, the propellant evaporates from the aerosol droplets, leaving a homogeneous microsphere composed of drug and OLA.

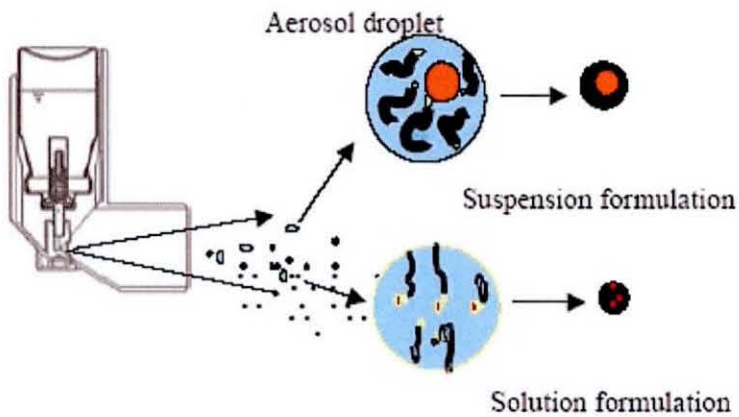


Figure 1.10: *In-situ* generated microspheres on actuation of MDI. Ref. [12].

1.1.3. Analysis of oligolactic acids and lactic acid

Because of the different chains lengths of the oligomers, and potential presence of degradation products and impurities, any of which could alter the physical properties of the formulation and hence the efficacy of the MDIs, it has been necessary to develop methods to determine not only the total quantity of the OLAs but also their composition for quality control purposes. A number of analytical techniques have been deployed, including GPC (gel permeation chromatography), HPLC, NMR

spectroscopy and MALDI-TOF [3]. However, relatively few of the methods have been published.

GPC, on a PLgel Mixed-E column with a THF mobile phase, allowed the calculation of the % weight of each oligomer [13]. A theoretical distribution (expected from a random condensation reaction of the same average degree of oligomerisation) was compared to the measured data (Figure 1.11). A deviation was observed at low molecular weights, but the two curves overlapped when n> 20.

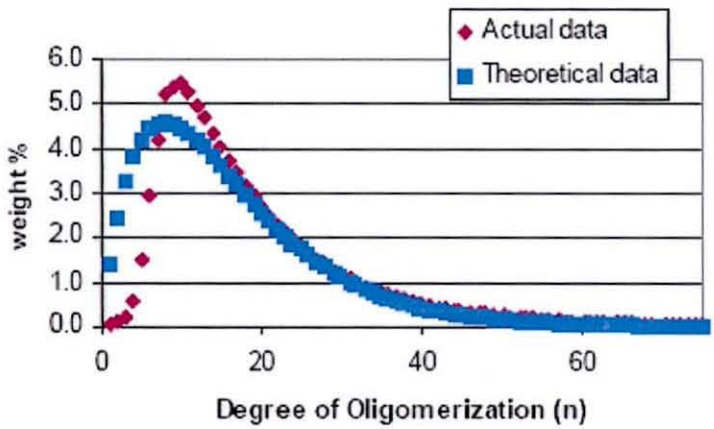


Figure 1.11: Theoretical weight % distribution of step-condensation oligomers vs. experimental data for Ac-OLA-OH, n= 7.8. Ref. [13].

The authors also examined whether GPC would be sensitive to low levels of degradation, if hydrolysis led to a cleavage of the chain,

In a RP-HPLC (reversed-phase HPLC) investigation, Capecchi *et al.* [13] employed a C_8 column with an aqueous acetonitrile mobile phase. They found that the method separated the low molecular weight oligomers, but the resolution was lost as the molecular weight increased. Furthermore, the separation was complicated when the polymerisation was carried out with increasing proportions of D-lactic acid as the individual oligomer peaks were each separated into stereoisomers (Figure 1.12). They concluded that although RP-HPLC allowed characterisation of Ac-OLA-OH, it

would not be suitable for impurity profiling, since the large number of oligomer peaks would reduce the ability to identify impurity peaks.

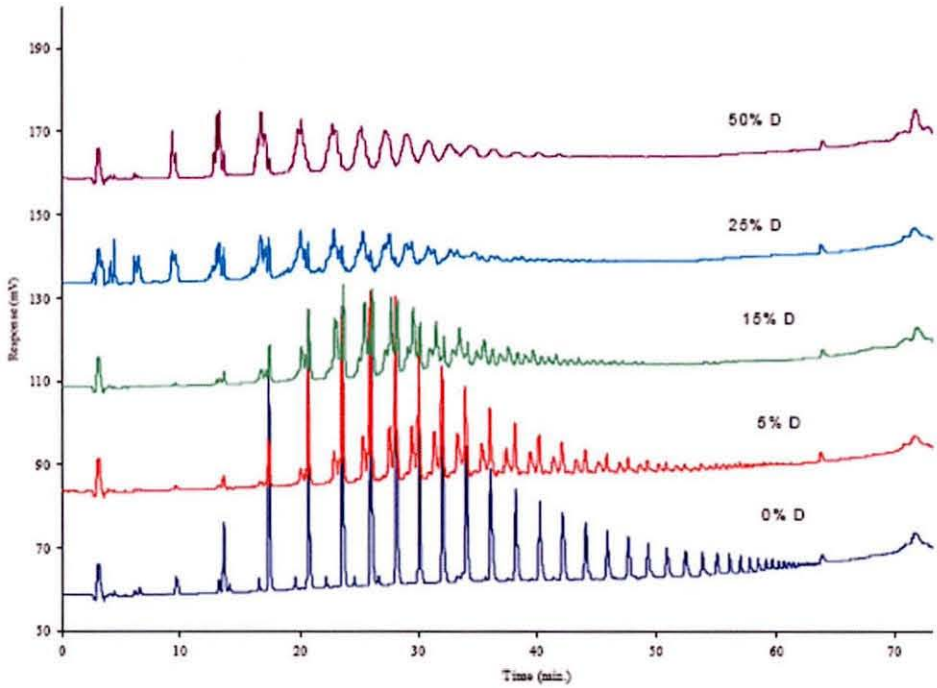


Figure 1.12: RP-HPLC chromatograms of acetyl oligo-D,L-lactic acid prepared with varying D/L ratios. Ref. [13]. Mobile phase A: 95% water, 5% MeCN, 0.1% phosphoric acid. Mobile phase B: 5% water, 95% MeCN, 0.1% phosphoric acid. Mobile phase delivered in a gradient: 25 to 60% B in 25 min, then taken to 75% in 25 min and 100% in 20 min. Flow rate: 1 ml/min; injection volume: 10 µl; UV: 210 nm. Column: Zorbax C_8 250 x 4.6 mm, 5µm.

More recently Capecchi and his group [14] developed a NP-HPLC (normal-phase HPLC) method coupled to MS that enabled the quantitation of OLAs impurities to 0.05% w/w. The chromatographic method was based on a silica column with hexane/ethyl acetate mobile phase and separated Ac-OLA-OH and some of its impurities. The compounds not separated chromatographically were separated and detected by MS, by virtue of their unique masses. However, the NP-HPLC method did not provide resolution between oligomers.

H^1-NMR of OLAs enabled Capecchi and co-workers [13] to measure the relative proportion of end-groups and repeat units and hence the number average molecular weight. The number average molecular weights obtained by NMR were compared to the values obtained by GPC. Although a good correlation was observed, the authors noted that the GPC values were higher than NMR values. NMR was also used to detect potential impurities, such as the pyruvate terminated OLA. However, the NMR methodology had low sensitivity and did not allow the quantification of impurities at less than about 1% w/w.

Mass spectroscopic analysis of Ac-OLA-OH showed the presence of series of peaks separated by 72 amu, the mass of the lactate repeat unit [13]. The series were assigned to Ac-OLA-OH, to their sodium and potassium adducts, and to the pyruvate-impurities. Although MS is an invaluable tool for characterisation work, the authors concluded that it could not be used to quantify the various species present because:

i. MS methods exaggerate the concentration of low molecular weight species (due to variability of ionisation efficiency and volatility of different chemical structures);

ii the high fragmentation energy can create new species or alter the concentration of existing species.

The OLAs could also be examined by the methods used for the shorter-chain members of the polylactides. Staniforth *et al.* [15] carried out a comparative study of three analytical techniques, which could be applied to lactic acid and polylactides, in consumer healthcare products. The methods investigated were: ion-exclusion HPLC, headspace GC-MS (gas chromatography-mass spectroscopy) and on-column injection GC-MS. The headspace GC method employed two coupled columns of a non-polar phase and a medium polar phase, to separate methylated lactic acid. The methylation conditions were 120 °C for 20 minutes, but under these conditions the polylactides present were converted back to lactic acid.

An effective HPLC analysis of lactic acid was described by Cheng and Gadde [16]; using two approaches, RP-HPLC with ion pairing and ion-exclusion HPLC. The ion-exclusion method comprised an amine column and acetonitrile/water mobile phase with 3.2 mM phosphate buffer. The method allowed separation of the lactic acid peak from the excipient peaks and quantitation of lactic acid.

Other methods include the use of ion-exclusion HPLC for lactic acid but the stationary phase used was polystyrene-divinylbenzene (PS-DVB) [17, 18].

GC analysis of the lactic acid monomer has required derivatisation (typically using t-butyldimethylsilyl) or oxidation to acetaldehyde [19-21]. To circumvent derivatisation, Heitfuss *et al.* [22] developed a method using static headspace GC, with methylation of lactic acid in the headspace vial; McCalley [23] employed an on-column GC method with water/oxalic acid as the sample solvent.

1.2. Theoretical aspects and considerations in synthetic oligomers analysis

In polymer chemistry oligomers are loosely defined as compounds consisting of a finite number of repeat units (monomers), generally between 10-100 units. As the individual molecules vary in the number of monomers, a molecular weight distribution (MWD) can be described. When functional groups are present on the oligomeric structure, either as end-groups or elsewhere along the backbone, a functionality-type distribution (FTD) will be present. Therefore, oligomers are not homogeneous chemical species; rather they feature simultaneous MWD and FTD [24]. Polymers also feature molar mass distribution (MMD) and copolymers have chemical-composition distribution (CCD). For the precise characterisation of synthetic polymers (and indeed oligomers), all the different distributions must be determined.

The traditional analytical techniques, such as IR (infrared absorption spectroscopy), UV (ultraviolet spectroscopy), NMR (nuclear-magnetic-resonance spectroscopy), light scattering and viscometry, can give important information on the average of certain polymeric distributions and on the functional groups [25]. However, they do not provide any knowledge on the widths and shapes of the distributions. Information on the distribution is necessary, since two polymers with the same average molecular weight but different distributions can have very different properties. Ideally, some form of separation is applied that is sensitive to one molecular characteristic only (the effect of other molecular characteristics can suppressed). Liquid chromatography, in its many different forms, has been a very successful technique for separating polymers and oligomers. For example, size exclusion chromatography (SEC) (Section 1.2.1) has been used for the separation of oligomers species (in terms of their size) from low molecular weight polymer fractions. Interactive chromatography (RP-HPLC and NP-HPLC) allows

oligomeric separations in terms of chemical and structural features. Other chromatographic techniques have been explored for oligomer applications. If the oligomers of interest have sufficient thermal stability and are sufficiently volatile, then gas chromatography (GC) can be used. Thin layer chromatography (TLC) has been evaluated for the separation of oligomers, but it must be said that it is not extensively used. A powerful separation method for substances that are not amenable to GC analysis is supercritical fluid chromatography (SFC); SFC has been employed for oligomer analysis [25]. SFC, TLC and GC will be discussed in section 1.2.3, 1.2.4 and 1.3 respectively.

Multidimensional chromatography, bringing together two of the separation methods is being increasingly used for the separation of complex mixtures and the analysis of polymers and oligomeric species (where different, mutually dependent distributions can be simultaneously addressed) (Section 1.5).

1.2.1. Size exclusion chromatography (SEC)

SEC is also referred to as gel-permeation chromatography (GPC), when applied to synthetic polymer analysis, or as gel-filtration chromatography (GFC) when applied to biopolymer analysis. SEC has been the most commonly applied technique for separating macromolecules on the basis of their molecular size, since its development in the late 1950s [26, 27]. According to the separation mechanism large molecules can be partially or completely excluded from the volume of the pores of the column packing materials [28], thus leading to larger molecules eluting earlier than smaller molecules. The entropic effect dominates the separation process in SEC.

SEC does not reflect molecular distributions other than indirectly through size; differences in chemical composition or functionality will be overlooked. However,

the molecular weight range for SEC is usually higher than would be applicable to the OLAs (of 1200 to 5000) and will not be considered further for this study.
For these reasons SEC was not chosen as a potential analytical technique to be employed in the course of this research, and no past literature was reviewed.

1.2.2. Interactive liquid chromatography (LC)

Interactive LC is based on molecular interactions (adsorption for NP-HPLC and partition for RP-HPLC) between the analyte and the mobile and stationary phases. For the analysis of oligomers, interactive LC provides better separation selectivity and peak capacity than SEC [29]. The retention of polymers (and oligomers) in interactive LC usually increases exponentially with the number of repeat units. Furthermore, the dependence of retention on the mobile phase composition implies that in most cases gradient-elution LC is necessary. In gradient elution [30] a very small change in the mobile phase composition has much stronger effect on the retention of large molecules than on the retention of small compounds. Consequently only a narrow composition range of the mobile phase will provide elution of large molecules. The gradient LC of polymers was first introduced in 1978 by Maeden *et al.* [31], and since then it has been widely applied to polymers and oligomers separations using both aqueous and non-aqueous mobile phases.

1.2.2.1. Interactive RP-HPLC and NP-HPLC of oligomers

The application of interactive LC to the characterisation of polymeric/oligomeric materials in both reversed-phase [32-39] and normal-phase [40-44] has been important during the 1980s and 1990s. Past publications [45-48] showed that in reversed-phase separation is mainly in terms of molar mass and in normal-phase is mainly in terms of functional groups [49].

Researchers started to realize that it could be possible to obtain simultaneous information on the average molecular structure as well as on the underlying distributions of polymers and oligomers if multidimensional separations were applied.

In 1990 Glöckner and Barth [50] applied interactive, gradient LC to SEC-fractionated samples, achieving characterisation of copolymers. They found that without pre-fractionation, the copolymers were not baseline resolved. Their work was prompted by Balke and Patel [51, 52], who carried out pre-fractionation by SEC, followed by a second SEC separation. These were early examples of multidimensional separations. Since its beginning, multidimensional LC has gained increasingly popularity and it has certainly become the selected methodology for polymer analysis (Section 1.5).

Jandera proposed the use of several equations to describe the simultaneous dependence of retention on both mobile phase composition and the degree of polymerisation [53-60]. From these equations, the contributions of the polymer chain size and end groups could be distinguished and related to physical properties [61].
Selected examples of gradient LC (in both RP and NP) from recent publications will be given in the next paragraphs. Mobile phase and temperature effects on selectivity will be examined and reviewed in more detail.

1.2.2.2. Examples of the Interactive RP-HPLC of oligomers

In the early 1990s Bodmeier and Paevatakul [62] applied RP-HPLC to identify plasticizers used with polymers present in pharmaceutical applications (polymer-coated sugar beads and polymeric films from simulated intestinal fluids). RP-

HPLC coupled to evaporative light-scattering detection was employed by Mengerink *et al.* [63] for analysing oligomeric surfactants.

In a paper by Rissler [64] an efficient separation of polyesters composed of a large number of oligomers was described. The chromatography was quite innovative at the time, because of the use of 1.5 μm particles (at the time, i. e. late 1990s, 1.5 μm particle columns were rarely used). The mobile phase (acetonitrile/tetrahydrofuran) was delivered in a gradient. Separation was achieved not only with respect to molecular weight but also to chemical composition and functionality type. Subsequently, Rissler [65] carried out the separation of polybutylene glycols (non-ionic surfactants) on monolithic, polymeric and silica-based materials, using ternary gradients of acetonitrile, tetrahydrofuran and water. The best separations were achieved on the monolithic and on the polymer-based columns.

Philipsen *et al.* [46] reported that the separation of end groups are affected by both stationary phase and mobile phase, and showed that the order of elution of oligomers could be reversed by selecting a different mobile phase.

Rogers *et al.* [66] showed the variation in selectivity towards the stereoisomers of polystyrene oligomers brought about by different stationary phases (various allyl, phenyl and fluorinated materials). The mobile phase was a mixture of water/acetonitrile, delivered in a gradient. All the allyl phases (from C_1 to C_{18}) provided a separation of the stereoisomers, whereas phenyl and fluorinated materials gave no isomer selectivity. More recently Sweeney *et al.* [67] separated the diastereoisomers from oligomers of low molecular weight polystyrene by using a carbon clad zirconia stationary phase and an acetonitrile mobile phase.

1.2.2.3. Examples of the Interactive NP-HPLC of oligomers

The adsorption chromatography of polystyrene oligomers was successfully used by Mourey [68], where a mixture was eluted with n-hexane/dichloromethane on a silica column. Mourey *et al.* [69] showed that a mobile phase containing tetrahydrofuran and ethyl acetate led to separations according to the number of oligomer units and a mobile phase containing dichloromethane separated the stereoisomers of individual oligomers.

Lai *et al.* [70] have employed a cyano stationary phase with polar solvents, such as dichloromethane, to separate styrene oligomers in PS600 and PS730 standards. Lai *et al.* [71] achieved partial stereoisomer resolution of the individual oligomers present in PS600, on a phenyl column with n-hexane as mobile phase.

Jandera and Rozkošná [72] compared the separations of styrene oligomers on silica material using 1,4-dioxane/n-heptane and tetrahydrofuran/n-heptane mobile phases (delivered in both isocratic and gradient modes). From this work the authors were able to derive an expression, which described the dependence of the oligomeric retention factors, k on the degree of polymerisation and on the mobile phase composition.

Marquez *et al.* [73] showed that on a silica column, isocratic elution allowed the separation of surfactant oligomers up to 10 repeat units; gradient elution led to the separation of 15 units. An amino column extended the limit to 25 units. Higher repeat units were resolved when the silica and amino columns were coupled.

1.2.2.4. Organic solvents and their effect on selectivity in interactive RP-HPLC and NP-HPLC of oligomers

Many researchers have investigated the retention changes of analytes in RP-HPLC, with binary aqueous mobile phases [74-82]; in the present review, attention will be given to polymer/oligomer applications in both RP-HPLC and NP-HPLC.

Lemr *et al.* [83] investigated the effect of mobile phase composition on the retention of linear alcohol polyethoxylates (LAE), non-ionic surfactants used in detergent formulations in RP-HPLC. An elution order inversion was observed for the ethoxy units, when the composition of aqueous acetonitrile (MeCN) mobile phase was changed. At low MeCN content (expressed as φ, the organic solvent fraction in the mobile phase, (v/v), where φ= 0.940), LAEs with a large number of ethoxy units eluted first. The elution order was inverted (i.e. small number of ethoxy units eluted first) at higher MeCN content (φ= 0.960). At intermediate MeCN content (φ= 0.948) they observed co-elution of LAEs, independent of ethoxy unit chain length.

The RP-HPLC inversion of elution order for polyethoxylic oligomers had first been reported by Melander *et al.* [84], followed by Okada [85]; the inversion of elution order was explained in terms of conformational changes of the ethoxymers.

The key role of organic modifiers on selectivity was also investigated by Cho *et al.* [86], for non ionic surfactants. They studied fatty alcohol ethoxylates (FAEs), a class of compounds which shows a distribution in alkyl and polyethyleneoxide (PEO) chain length. The PEO distribution could be analysed by NP-HPLC [87-92], and the alkyl chain distribution could be analysed by RP-HPLC [92-98].

Jandera *et al.* [92] reported on the peculiar retention behaviour of FAEs; they concluded that a combined effect of the mobile phase polarity and the solvation of

EO groups took place. The EO solvation, in turns, depended on the water content in the mobile phase. The authors also observed different solvation effects in MeOH-water and in MeCN-water mobile phases. EO groups were subject to proton-acceptor interactions with OH groups of water and methanol. These interactions were not possible with MeCN. Consequently, aqueous MeOH was a 'better' eluent than aqueous MeCN for the separation of ethoxylated alcohols into groups with different alkyl lengths.

Trathnigg and Gorbunov [97-99] worked on FAEs separations and concluded that the longer FAEs eluted first because of size-exclusion (SE) mechanisms. Lochmüller *et al.* [100] found an inverted elution order in RP-HPLC of PEO samples. The entropy-enthalpy compensation point they obtained was far from the total void volume of the column (which would indicate SE mode), and would explain the early elution of larger FAEs. The separation mechanism depended instead on temperature (see following paragraph).

1.2.2.5. Temperature and its effect on retention and selectivity in interactive LC

Temperature has been shown to have a key role in the separation and in the reversal of the elution order of some polymers. Cho *et al.* [86] found that at a fixed mobile phase composition the elution order of PEOs on NP-HPLC changed with temperature. At low temperature they obtained resolution, with individual peaks. As the temperature increased, the retentions decreased and the peaks of the oligomers merged into a single peak. At high temperatures the smaller PEOs eluted first. As the temperature was lowered, the authors observed a change in elution order, which passed through a co-elution point and lead to the larger PEOs eluting first at low temperatures. This peculiar behaviour was investigated by studying enthalpy (ΔH) and entropy (ΔS) changes. The two moieties of FAEs (PEOs and alkyl chains) appeared to contribute to the retention in a thermodynamically opposite manner.

The authors showed that PEOs retention in RP-HPLC was energetically unfavourable and entropy-driven ($\Delta H>0$ and $\Delta S>0$). The retention was based on hydrophobic interactions and increased with temperature increases. The entropy gain was brought about by the release of water molecules from the solvated PEO chain.

The sorption of the alkyl chains showed $\Delta H<0$ and $\Delta S<0$, so it was energetically favourable and entropically unfavourable. Their retention decreased with temperature increases. The inverted elution order was caused by the positive ΔH. The entropy increase associated with the hydrophobic interactions was larger than the entropy loss of the alkyl chains in the sorption process, so overall, the entropy gain had to be large enough to overcome the enthalpy penalty of the endothermic sorption process. PEO stayed retained on the stationary phase. At low temperature (15 °C) the resolution of EO units was better, while at higher temperature (40 °C) their resolution decreased and the alkyl chain retention increased.

Other researchers considered temperature as an active parameter in the LC of non ionic surfactants. Vanhoenacker and Sandra [79] observed significant selectivity changes in RP-HPLC when temperature was increased; the oligomers elution order was reversed by temperature increase, in systems with MeCN/water as the mobile phase and their retention decreased with increased temperature.
In RP-HPLC, with aqueous MeCN as mobile phase, a chain length distribution separation was obtained (Figure 1.13):

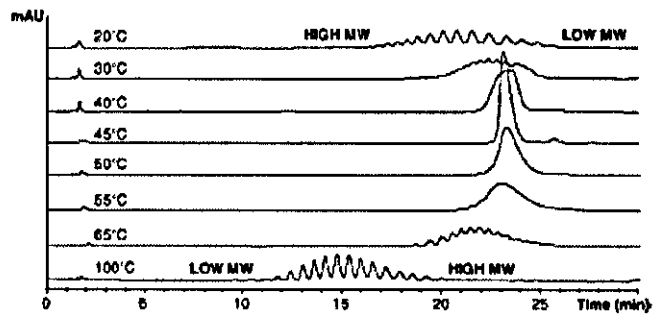


Figure 1.13: Temperature effect on elution of surfactants, on a Selerity Blaze C_8 column (150 x 4.6) mm, 3 µm particles, 50/50 water/MeCN mobile phase. Ref. [79].

At room temperature (about 20 °C) the separation was based on hydrophobic interactions: the larger oligomers, being less hydrophobic, were eluting in front of the smaller, more hydrophobic oligomers. At 50 °C, the separation according to chain length did not take place: there was no oligomeric separation ("critical" temperature). At higher temperature (90 °C), the chain length distribution separation re-appeared but, with inversed elution order: the smaller oligomers were at the front.
Their investigation of the thermodynamic parameters, ΔH and ΔS, at 20 and 50 °C, confirmed Cho *et al.* [86] results.

However, Vanhoenacker and Sandra went above 50 °C and up to 100 °C. In the 65-100 °C region they observed that sorption was enthalpy driven, resulting in a

reversal of elution order. From working at high temperature the authors could also gain:

i. reduced mobile phase viscosity and therefore decreased pressure drop over the column;

ii. higher flow rate and therefore a reduced analysis time (by a factor of 3);

iii. enhanced solute transfer between mobile phase and stationary phase and therefore an improved efficiency.

However, when MeCN was substituted by MeOH, a reversal of elution order was not achieved on increasing temperature. During the study, mobile phase and column temperatures were controlled, to eliminate thermal mismatch between mobile and stationary phase. The authors also used stationary phases with high temperature stability: graphitised carbon, zirconium oxide and polystyrene/divinyl benzene phases. When working in NP-HPLC, the reversal of elution order was not achieved; the only clear trend, the authors could observe, was a reduced retention of all oligomers when temperature was increased. The thermodynamic parameters obtained ($\Delta H<0$ and $\Delta S<0$) indicated that the separation process was enthalpy-driven, across the 15-150 °C temperature range.

Choosing a non-aqueous system, at high temperature, had the advantage over aqueous systems at high temperature of not causing hydrolysis of the stationary phase. The authors reported that they could have chosen reversed phase material or polar material for the stationary phase. In both materials high temperature is responsible for an increase in column efficiency and a decrease in analysis time. Reversed phase materials are often preferred because of the poor reproducibility offered by silica columns.

Other two liquid chromatography methodologies employed for oligomeric analysis and based on polar interactions are supercritical fluid chromatography and thin liquid chromatography; they will be discussed in the next two paragraphs.

1.2.3. Separations of oligomers using supercritical fluid chromatography (SFC)

Separation of oligomers showed that SFC chromatograms gave better separations compared to HPLC. SFC optimisation strategies were shown by Schmitz and co-workers [101], with respect to temperature and pressure programming and gradient elution. The retention behaviour of samples having different polarities was investigated by Hirata [102], who employed silica and C_{18} columns and n-hexane/ethanol mobile phase.

However, alkanes above their critical points are hazardous and after the early work most studies concentrated on carbon dioxide. Schmitz *et al.* [103] separated oligomers, over a wide molecular weight range, by gradient SFC. For oligomers that absorb at low wavelength they employed a CO_2/acetonitrile mobile phase, allowing UV detection to be performed at 200 nm.

SFC can be regarded as a specialised case of normal phase chromatography in which liquid carbon dioxide above its critical point is usually used as the mobile phase. Prior to entering the chromatographic column the mobile phase is pressurised and then heated above the critical temperature. Standard HPLC columns can be used, as well as capillary columns (for higher efficiency and sensitivity values). At the column outlet the mobile phase is decompressed and allowed to reach its gaseous state and can be re-cycled. The most common detectors employed with SFC are UV and flame ionisation detectors.

One of the earliest separations of oligomers by SFC was reported by Klesper and Hartmann [104] in 1978. However, it was only when gradient elution techniques became established that SFC started to be employed for low molecular weight polymers and oligomers analysis. Klesper and Schmitz [105] were able to reach good separations of oligomeric species on a silica column, with supercritical pentane/1,4-dioxane gradients. Schmitz *et al.* [106] investigated the effect of a number of mobile phase compositions on the separation of oligostyrenes by gradient SFC, with a combination of an alkane with dioxane giving the best resolutions. Just *et al.* [107] successfully applied SFC (coupled to MALDI-TOF, Matrix-assisted laser desorption ionisation time of flight mass spectrometry) for the analysis of oligomeric ethylene oxides and their adducts. Bartle *et al.* [108] also employed SFC for the analysis of lower oligomers from poly(ethylene terephthalate) films. They extracted the sample prior to chromatographic analysis by both on-line and off-line SFE (supercritical fluid extraction). In off-line SFE, the authors subjected a series of samples to a flow of fluid and then collected the eluting fluid. Off-line chromatographic analysis followed the extraction step. In on-line SFE-SFC the sample was extracted by a flowing stream of fluid and then the extract was directly deposited on the SFC column inlet. Both on-line and off-line SFE set ups proved to be successful, although the results derived from the on-line extraction were not suitable for quantitative analysis.

1.2.4. Oligomers separations by thin layer chromatography (TLC)

Although 'manual spotting' TLC is less accurate and reproducible than NP-HPLC, it offers good selectivity and the apparatus is simple and cheap. Hudgins *et al.* [109] described a number of TLC separations on silica gel for oligomers of PET (polyethylene terphthalate). The authors achieved separations between cyclic and linear oligomers of equal molar mass. Cserháti *et al.* [110] employed TLC for separating a number of ethylene oxide oligomers, according to their chain length.

Cserháti [111] investigated the effect of solvent strength on TLC selectivity, for ethylene oxide oligomers applications. Bui *et al.* [112] obtained TLC separations of low molecular weight polyisoprene, polystyrene and poly (ethylene oxide); they also compared the TLC separations with HPLC and SEC separations. They found that TLC had a higher resolving power than SEC, but a lower resolving power than HPLC.

1.2.5. Oligomers separations using hydrophilic interaction chromatography (HILIC)

In recent years a new approach to separate oligomeric analytes has been based on hydrophilic interaction chromatography (HILIC). This technique was first described by Alpert [113] to describe a chromatographic technique where the analytes interact with a hydrophilic, polar stationary phase and are eluted with a relatively hydrophobic binary eluent. It is a variant of normal-phase chromatography, although it uses water in an aqueous-organic solvent mixture as mobile phase. Water is the stronger eluting member of the binary mobile phase. This means that the retention order in HILIC is roughly the opposite of the retention order observed in RP-HPLC and polar compounds are more retained than non-polar compounds.

The distinction between HILIC and NP-HPLC is debated. There is an on-going discussion on the separation mechanisms involved in HILIC, which was summarised by Hemström and Irgum [114] in a comprehensive review. However, there seems to be an agreement that both adsorption (which mainly governs retention in NP-HPLC) and partitioning (retention mechanism suggested by

Alpert) could be involved. The partitioning is between the bulk mobile phase and a layer of mobile phase enriched with water and partially immobilized on the stationary phase (Figure 1.14).

Historically NP-HPLC has been employed for separation of polar molecules, which lack the hydrophobic character necessary for suitable retention on a C_{18} stationary phase [115]. However, its application has been limited because of problems associated with dissolving the hydrophilic materials in the non-aqueous eluents typically used in NP-HPLC [116]. Initially it was applied to carbohydrates and peptides analysis [113, 117-121]; it was also reported to provide a unique selectivity for separating impurities and degradation products [122].

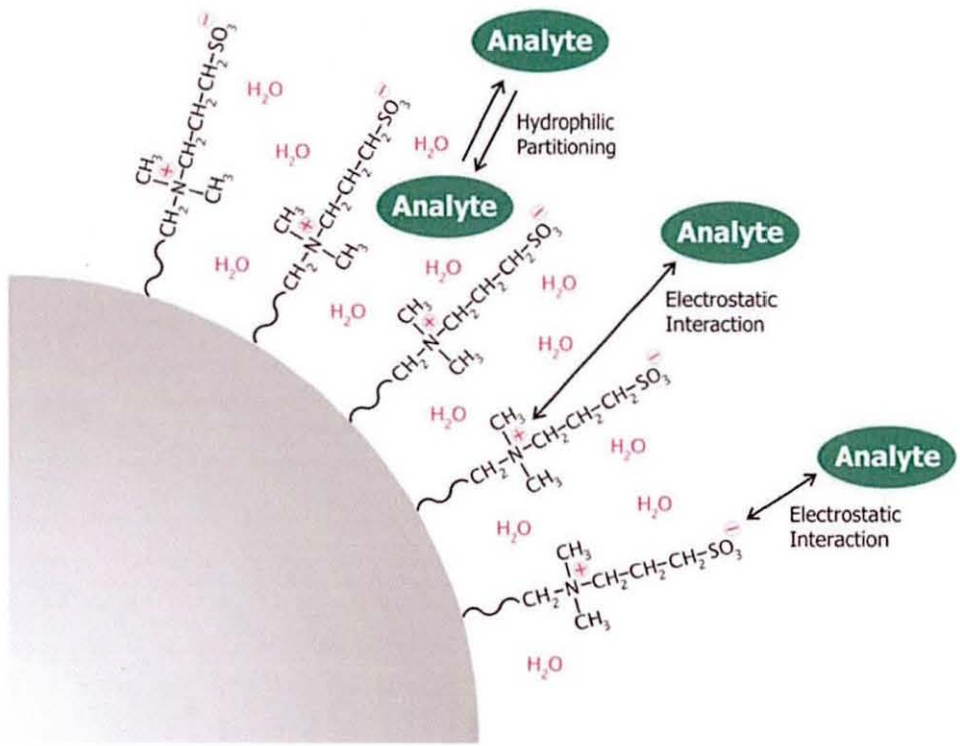


Figure 1.14: Illustration of the separation mechanism involved in HILIC. Ref. [123]

Stationary phases, which have been employed in HILIC, are silica, amino or cyano phases traditionally used in NP-HPLC [117, 119, 120, 122, 124-126]. Other phases used for small polar compounds are amide, aspartamide and diol bonded phases [113, 126-133]. Recently some column manufacturers have started to market stationary phases specifically designed for HILIC [114], such as Atlantis HILIC Silica, ZIC HILIC, Cosmosil HILIC and YMC HILIC columns.

As in RP-HPLC, HILIC employs water and acetonitrile as mobile phase but, with a high percentage of organic (generally higher than 60%) [113] and the percentage of organic solvent is probably the most important factor to affect retention. Guo and Gaiki [134] investigated the effects of various parameters on the retention in particular the effect of acetonitrile content, buffer pH, salt type and concentration in the mobile phase. They observed a decreasing retention as the water content was increased. They also studied the effect of column temperature (in the range 20-70 °C), and observed a decrease in retention as the column temperature increased and a linear relationship between ln k' and 1/T. Guo and Gaiki chose the mobile phase salts, ammonium acetate, formate and bicarbonate, because of their solubility in high percentage of acetonitrile and their compatibility with the MS detector. Increased concentrations of ammonium acetate caused increased retentions. Probably this was because the salt prefers to be in the water-rich liquid layer of the partitioning model for HILIC. Olsen [122] showed that HILIC has NP behaviour, since increases in the aqueous content of the mobile phase caused a decrease in retention of polar compounds. He also showed that substituting alcohols in place of water in the mobile phase gave longer retentions and found that n-propanol was a stronger eluting solvent than acetonitrile. Strege [120] evaluated several cyano and amino columns for polar analytes and tested mobile phases containing MeCN/water, with or without buffers and acceptable reproducibility was achieved when the ammonium acetate concentration was at least 6.5 mM.

Jandera *et al.* [135] successfully employed HILIC for the separation of ethylene oxide-propylene oxide (co)oligomers. Svec *et al.* [136] analysed poly(vinyl pyrrolidinone) on a polymeric diol phase with a gradient of 0-50% water in acetone. They also analysed dextran oligomers, which were baseline resolved by utilising a gradient of 10-40% water in acetone.

1.2.6. Liquid chromatography under critical conditions (LCCC)

As well as SEC and interactive chromatography there is a specific mode of isocratic LC in which retention is solely influenced by the chemical composition or functionality of the molecules. This approach, generally used for the analysis of complex polymeric mixture, is termed liquid chromatography under critical conditions (LCCC). Macko and Hunkeler [137] have presented a review in which all known LC systems under critical conditions reported since the early '80s were summarized.

Under certain conditions (which are critical with respect to temperature and mobile phase composition) both entropic exclusion and enthalpic adsorption compensate each other and a mixture of differently substituted polymer molecules can be separated by LCCC solely according to the number of functional groups (FTD), the chain length does not contribute to the retention. For this reason LCCC can be used to determine a particular oligomeric series with a specific functionality by causing all the oligomers to have the same retention factor [138]. For example LCCC has been used to successfully separate cyclic structures of polyamides from their parent linear matrixes [139, 140].

Critical chromatography has proved an invaluable tool to separate polymers based on their end-group functionality, for both low (up to 10000 [141-145]) and higher molecular mass oligomers (although problems have been encountered in obtaining

critical conditions [146].) Most of published critical separations dealt with the separation of polymer itself as the main component. In the majority of cases, LCCC is carried out in NP, since most functional groups are more polar than the polymer backbone. Examples have been described for polyethers [147-151], polyesters [152, 153] and polyamides [154].

However, as Mengerink *et al.* [138] demonstrated, LCCC can also be applied to determine small amounts of a particular polymer in a sample matrix. They compared the selectivity of two LCCC systems (RP-HPLC and NP-HPLC) for the analysis of polyols. They used a C_{18} column for the RP-HPLC set up and a silica column for the NP-HPLC experiment; the mobile phase constituents were water, formic acid, THF and 1-propanol. Linear and cyclic pTHF could be separated by RP LCCC, but only the cyclic pTHF was separated from the coating. In NP LCCC, cyclic and linear pTHF co-eluted but both were separated from the coating material.

However, most examples found in the literature rely on LCCC separation mechanisms only and mass spectrometric detection/identification. For example Mengerink *et al.* [140] separated the linear and cyclic structures of polyamide-6 by LCCC, independently of molar mass. Retention was based solely on end-group functionality, while retaining the critical conditions of the backbone units. Separation was optimized by using different parameters: percentage modifier, temperature and pressure. Quantification was carried out by evaporative light scattering detection (ELSD) and identification by MS.

The separation of functional poly(n-butyl acrylate) polymers, carried out by Jiang *et al.* [155] was also based on the number of carboxylic end-groups under LCCC. The authors used an unbonded silica column, and mixtures of acetonitrile, acetic (or formic) acid and dichloromethane of varying composition. ELSD calibration curves were obtained and were used for quantitative analysis of carboxyl groups, whereas MS data confirmed that the critical separation was based on the carboxyl

functionality. Similarly, Peters *et al.* [156] developed a method for an end-group based separation of low molecular mass poly(methyl methacrylate). Phillips *et al.* [157] applied LCCC to the separation of water-soluble poly (acrylic acid) and polystyrene sulfonate; the variations in buffer concentration and in the proportion of organic modifier in the mobile phase were used to achieve the critical conditions for the two polymers.

Pasch and his group [158] characterised a series of linear and star-shaped poly-L-lactide), by LCCC. A silica gel column and a mixture of 1,4-dioxane/n-hexane as mobile phase at 50 °C were employed.

Critical conditions are often found after optimising mobile phase constituents and column temperature [140]. Pressure also can influence the critical conditions [159], and so can the choice of stationary phase and mobile phase. Since so many different parameters must be taken in consideration, many experiments are required in order to achieve critical conditions.

1.2.6.1. Theoretical aspects of Liquid Chromatography under critical conditions

The first theory describing the chromatographic behaviour of macromolecules was given by Cassaca [160], which was then followed by Gorbunov and Skvortsov [161, 162]. Their basic idea was the concept of interaction energy, $-\varepsilon$, a measure of the affinity of a monomer unit towards the adsorbent; which is related to:

Chemical nature of polymer
Type of adsorbent

Type of solvent

Temperature

Below a certain value $-\varepsilon_{CT}$ (critical interaction energy) there is no interaction with the substrate and macromolecules will undergo endothermic entropic exclusion. Thermodynamically this can be expressed as (Equation 1.1):

$\Delta H = 0$ and $\Delta G = -T\Delta S > 0$ **Equation 1.1**

Where:

ΔH is the enthalpic energy of transfer of the analyte with the stationary phase;

ΔS is the entropic energy of transfer;

ΔG is the Gibbs free energy of transfer;

T is the absolute temperature.

The chromatographic system will be in SEC mode.

When $-\varepsilon$ increases (because of stronger solvent concentration) enthalpic interactions will start to show their effect on the macromolecules distribution; when $-\varepsilon_{CT}$ is exceeded the macromolecules will be dominated by exothermic enthalpic adsorption effects.

Thermodynamically this can be expressed as (Equation 1.2):

$\Delta S = 0$ and $\Delta G = \Delta H < 0$ **Equation 1.2**

When $-\varepsilon = -\varepsilon_{CT}$

$\Delta G = 0$

The enthalpic interaction effects are exactly compensated by the entropic exclusion effect. Critical conditions are reached and the distribution is independent of the chain length

$\Delta H = T\Delta S$

According to the Martin rule, the Gibbs free energy of a polymer is a summation of the Gibbs free energy of the end groups and the backbone unit (Equation 1.3):

$\Delta G_{polymer} = \Delta G_{endgroup\ 1} + \Delta G_{endgroup\ 2} + n\Delta G_{backbone\ unit}$ **Equation 1.3**

To achieve the critical conditions for a polymer with a specific substitution, the retention must be independent of the number of backbone units, so $n\Delta G_{backbone\ unit} = 0$. This can be accomplished by compensating the enthalpic interaction effects with the entropic exclusion effects at a certain temperature T (Equations 1.4, 1.5):

$\Delta G_{backbone\ unit} = \Delta H_{backbone\ unit} - T\Delta S_{backbone\ unit} = 0$ **Equation 1.4**

$\Delta H_{backbone\ unit} = T\Delta S_{backbone\ unit}$ **Equation 1.5**

Under these condition polymers with different substitutions (end groups) can then be separated if they have different enthalpies.
To achieve this, both normal phase and reversed phase can be used. When working in normal phase (and using silica columns), silica can interact with polar functional groups, for example, interactions were obtained for the carboxylic end-groups [163].

1.2.6.2. Strategy for obtaining critical conditions

Many experiments are often needed to obtain the critical conditions (CC) of a specific system. A method has been described by Gorshkov *et al.* [164-167]. Cools *et al.* [168] conceived an elegant method to reach CC. They obtained the retention times of a limited number of polymer standards with different chain lengths at different percentage of non-solvent (NS,) water, starting with 100% solvent (THF) at constant temperature (30° C). By increasing the NS percentage the retention time increased (they were using C_{18} columns; therefore they were operating in RPLC).

The polymers retention times were then plotted against the NS composition (Figure 1.15).

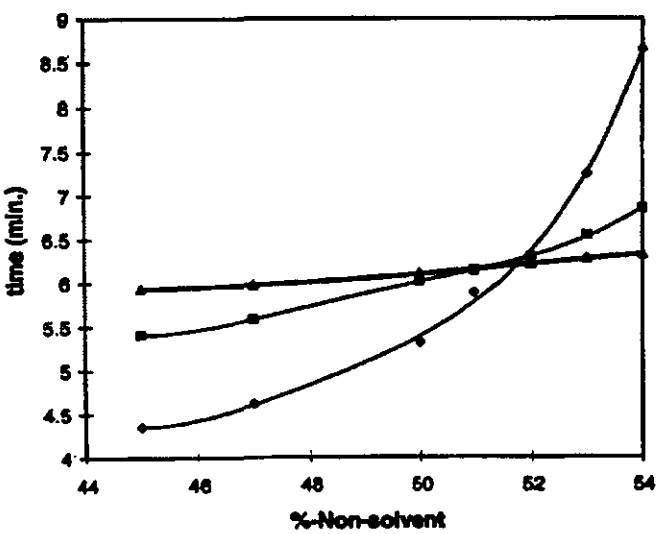


Figure 1.15: CSC plot. Ref. [146]. The three curves represent the retention behaviour of three standard polystyrenes (PS) of different molecular mass: ♦= PS 35000, ■= PS 8500, ▲= PS 1800.

The intersection point of the retention curves for the different chain lengths represented the NS composition at which the polymer standards with the same chemical structure but with different molar mass should be eluted simultaneously.

The accuracy of the method was about ± 1% NS. The authors observed that especially near the CC, small variations in the percentage of NS had a great influence on the retention behaviour. They also suggested that to obtain fine tuning (since solvent composition can not be established more accurately than 1%), temperature could be used. This method offered the advantage that only a few solvent-non-solvent compositions were required for identification of the CC.

A similar method was used by Philipsen *et al.* [146] who determined the retention times of three polystyrene samples (of different molar masses) at different elution composition, holding the temperature at 30 °C. The intersection point represented the critical solvent composition (CSC). They also tested the effect of column type, solvent and temperature. They found that for low molecular mass polymers, an increase in temperature led to a decrease in retention time (in reversed phase mode). This was not always the case for macromolecules. They also observed that changes in retention time as a function of temperature were more pronounced at higher molecular mass (in reversed phase mode). Temperature changes mainly affect the adsorption process, with limited effect on the exclusion process. Since retention of large molecules is more influenced by adsorption than that of small molecules, the effect of temperature changes is more pronounced in the former case. In normal phase mode, retention changed non-monotonously (not in a predictable manner) as a function of temperature, and this was explained in terms of adsorption itself varying non-monotonously with temperature [169].

Trathnigg *et al.* [170] employed LCCC for the analysis of functional polymers and block copolymers. They adjusted the CC by varying the temperature. The authors calculated ΔH and ΔS from the slope and the intercept of van't Hoff plots and used the following equation to calculate the 'compensation temperature', Tc (Equation 1.6):

$\Delta H = Tc\ \Delta S$ **Equation 1.6**

Many authors [171-178] used a plot of ΔH versus ΔS to investigate the separation mechanisms. From the slope in this plot they obtained the compensation temperature. However, ascribing the meaning of critical temperature (in the case of homopolymers) to the compensation temperature is meaningful only if ΔS and ΔH changes are measured at the critical conditions. If the conditions (mobile phase and temperature) are already close enough to the CC, the slope in the plot ΔH versus ΔS can be used for fine adjustments of the critical temperature.

Trathnigg and co-workers [170] found that in MeOH/water the temperature dependence in the 15-35 °C range was almost negligible, but it was more pronounced in acetone/water.

Generally temperature variations are preferred as a mechanism to fine-tune critical conditions over changing the eluent composition, because the accuracy of most HPLC pumps does not allow the needed changes in eluent composition (sometimes as small as 0.1%). However, it is debateable if temperature can be controlled accurately either.

Phillips and Olesik [179] used a different approach to reach critical conditions by employing enhanced-fluidity liquid mobile phases (of an organic solvent and supercritical carbon dioxide), whose higher compressibility provided manipulation of solvent strength by changing the temperature or the pressure of the solution.

1.3. Separation of oligomers by gas chromatography (GC)

Most oligomers are relatively involatile because of their size but GC can be applied to smaller non-polar oligomers although its application is limited and it will not be discussed in detail. For example, Gilbert *et al.* [180] applied GC-MS for the analysis of vinyl chloride oligomers, compounds that can migrate from PVC used in the food packaging industry and Abrantes [181] identified 20 polysterene oligomers (from polysterene plastic used for milk packaging) by headspace GC-MS. Garner *et al.* [182] demonstrated that capillary GC gave good separations of oligomer mixtures of low molecular weight tertiary butoxide and bornyl oxide, which were only partially resolved on packed columns.

1.4. Mass spectrometric characterisation of synthetic oligomers

Mass spectroscopy (MS) has become a very important tool in polymer and oligomer analysis [183-186], especially since the development of soft ionisation techniques, such as MALDI (Matrix Assisted Laser Desorption Ionisation) and ESI (Electrospray Ionisation). Both enabled the ionisation of large non-volatile compounds and gave little molecular fragmentation. Mass spectra complexity is therefore reduced, although multiple ionisation and adduct formation can contribute to a certain degree of complexity. By preserving the integrity of the molecules, these techniques allow the determination of molecular weight values. APCI (Atmospheric Pressure Chemical Ionisation) [79, 92, 187] has also found application for oligomer analysis. It tends to be used for compounds with medium to high polarity, and when coupled to NP-HPLC (where eluents have low dipole moments).

ESI-MS and APCI-MS are easy to couple on-line to an LC system, because the sample is introduced in solution at atmospheric pressure. Furthermore, LC-MS provides a continuous detection and therefore it can facilitate the understanding of LC separation mechanisms [188-190].

1.5. Multidimensional separations and oligomer analysis

Because of the complexity of oligomer sample one potentially valuable technique would be to use the enhanced capacity of multidimensional chromatography. The origin of multidimensional chromatography lay in planar chromatography; paper chromatography developed in parallel with liquid-liquid partition chromatography. Martin *et al.* [191] in 1944 evaluated the possibility of different eluents in different directions and later, in the early 50's Kirchner *et al.* [192] pioneered two-dimensional thin-layer chromatography but most modern methods used two linked column systems (Figure 1.16).

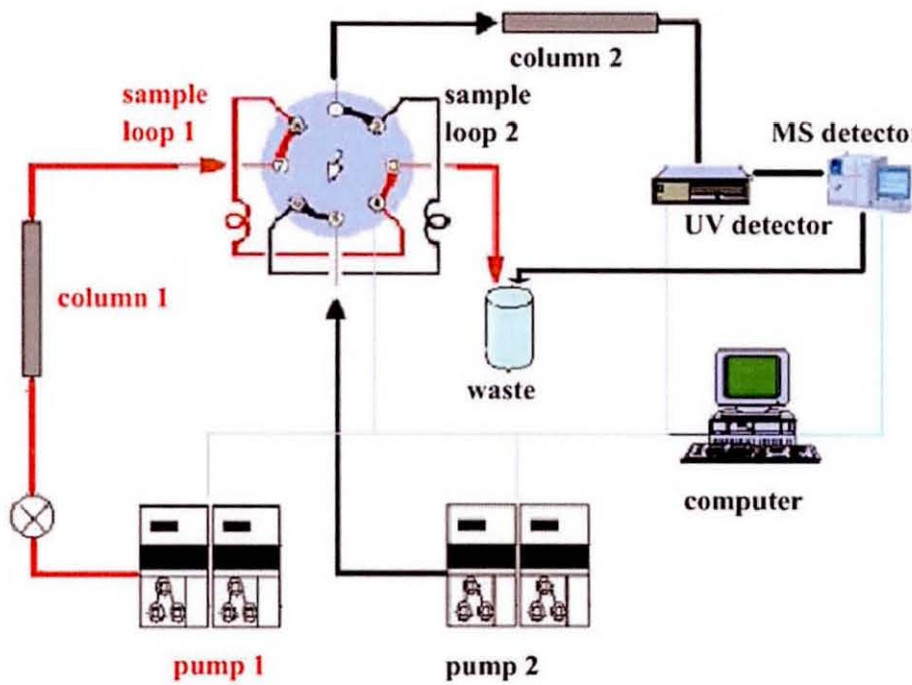


Figure 1.16: LC x LC layout. Ref. [193].

Such multidimensional analyses, which involves two or more separations in different dimensions linked together, are playing an increasingly important role in polymer and oligomer analysis. The interest has come because one dimensional chromatography of complex samples is limited in its resolving power. Giddings [2] predicted that approximately four million theoretical plates would be required to separate 82 of 100 components randomly distributed in one dimensional separation, whereas in multidimensional systems the resolving capabilities are considerably increased. When all the eluent from the first dimension is transferred in packages to a second dimensional separation the system is termed comprehensive chromatography (Blomberg *et al.* [194]), whereas transferring selected fractions results in heart cut or a partially comprehensive analysis

1.5.1. Peak resolution capacity

In order to describe the effectiveness of separation systems to resolve a multicomponent mixture Giddings introduced the concept of peak capacity [195]. His work was based on isocratic chromatography and he defined peak capacity as "the maximum number of peaks to be separated on a given column". This publication was then followed by a short note by Horváth and Lipsky [196], where the concept of peak capacity was applied to gradient chromatography. Since then peak capacity has been used as a measure of the performance of a gradient separation. For example, Stadalius *et al.* [197] and Gilar *et al.* [198] used it to assess peptide separation; similarly Stout *et al.* [199] used it for the separation of proteins. More recently Neue [200] has derived the equations that describe the peak capacity in gradient chromatography both for reversed phase and ion-exchange chromatography. He defines the peak capacity Pc as "the number of

peaks that can be separated within a retention window" (t_0-t_r), or in other words "the retention time measured in peak width units". The peak width is defined as 4σ (σ is the standard deviation of the retention times within a peak) of a peak, and if it changes with the retention time, t_r, Pc can be expressed as (Equation 1.7):

$$P_c = 1 + \int_{t_0}^{t_r} \frac{1}{4\sigma}\, dt \qquad \textbf{Equation 1.7}$$

t_0= retention time of an unretained peak.

σ describes the distribution of the molecules in a band as it elutes in the column. The overall broadness of the band is measured in terms of its base width. Classical chromatography theory considers that a separation takes place by a succession of equilibration steps. The more steps in a column, the more efficient the separation will be. The peak width is a function of the retention factor k_e at the point of elution t and the column plate count N. It is also a function of time, t; the longer the band takes to elute, the more time the molecules have to spread out. All this can be summarised in the following equation (Equation 1.8):

$$\sigma = \frac{t}{\sqrt{N}}(k_e+1) \qquad \textbf{Equation 1.8}$$

If we substitute equation 1.8 into equation 1.7 we obtain Equation 1.9:

$$P_c = 1 + \frac{\sqrt{N}}{4}\int_{t_0}^{t_r} \frac{1}{t}\frac{1}{k_e+1}\, dt \qquad \textbf{Equation 1.9}$$

Integration yields Equation 1.10:

$$P_c = 1 + \frac{\sqrt{N}}{4}\frac{1}{k_e + 1}\ln\frac{t_r}{t_0} \quad \textbf{Equation 1.10}$$

This equation implies that the plate count N is not a function of the gradient.

In gradient chromatography peak capacity can be calculated from the peak width ω (measured at 13.4% of peak height) by using Equation 1.11:

$$P = 1 + \frac{t_g}{\sum_1^n \omega\, {}^1\!/_n} \quad \textbf{Equation 1.11}$$

Where:

n= number of peaks

t_g= gradient run time

From Equation 1.11 it can be appreciated that peak capacity is simply the gradient run time divided by the average peak width. It follows that peak capacity is generally higher in gradient elution than in isocratic mode because of narrower peak widths ω in gradient elution [201]. Under both elution modes peak capacity increases proportionally to the square root of the column plate number, N (as shown in Equation 1.10). In turn, N is directly proportional to the column length L, therefore peak capacity increases in direct proportion to the square root of L. However, Gilar *et al.* [198] found that peak capacity reached a maximum for 150 mm column length, but decreased for longer columns.

1.5.2. Peak capacity of columns connected in series

If n columns are used in series (i.e. the sample components migrate the same distance), the total peak capacity P_n can be calculated from the Pi, peak capacity of the individual columns, as Equation 1.12:

$$P_n = \sqrt{\sum_{n=0}^{i} P_i^2} \qquad \textbf{Equation 1.12}$$

The fact that resolution and Pn increase in direct proportion to the square root of the column number can be illustrated by an example. Jandera *et al.* [201] carried out non-aqueous reversed phase gradient separation of triacylglycerols from a Dracocephalum moldavica oil, using Novapack C_{18} columns. The experimental increase in the resolution on three and two identical columns connected in series with respect to a single column was very close to the theoretical values, i.e. square root of three, 1.73 or square root of two, 1.41, respectively.

1.5.3. Peak capacity in multidimensional systems

If instead, n columns are used in parallel (i.e. the sample components migrate two different distances), the total theoretical peak capacity PnD is equal to the product of the peak capacities of n columns:

$$P_{nD} = P_1 \; P_2 \ldots \; .P_n \qquad \textbf{Equation 1.13}$$

This is what, is provided in theory by multi-dimensional chromatography: a significant increase in peak capacity. In real terms the peak capacity increase is lower than what Equation 1.13 predicts, because of:

i. the degree of similarity of the coupled columns (this concept will be expanded in the orthogonality section)
ii. the overlapped components, as demonstrated by Davis and Giddings [202].

When the increase in peak capacity and the number of components that can be separated is lower than predicted by Equation 1.13, peak capacity can be estimated as the weighted average of the two limiting cases:
i. completely dissimilar (orthogonal) two dimensional systems
ii. two fully identical systems, connected in series:

$$P_{2D=}P_1\ P_2\ (1\text{-}R) + (P_1^2 + P_2^2)^{1/2}\ R \quad \textbf{Equation 1.14}$$

Where: R is the weighting factor, or measure of correlation between the retention in the separation systems in the first and in second dimensions. If the two systems are not correlated, R = 0 and equation 1.14 becomes equal to equation 1.13, whereas if the two systems are completely correlated, R= 1 and equation 1.14 becomes identical to equation 1.12.

1.5.4. Peak overlap

Davis and Giddings [202] showed that the peak capacity is the maximum number of mixture constituents, which a chromatographic system may resolve. If for example, Pc= 100, this value means that 100 single component peaks can fit into

the allowed retention volume range, assuming that the peaks are evenly distributed, following one another at exactly the right distance to yield the minimum stated resolution. But components of a mixture are not always uniformly distributed and can appear randomly, overlapping each other. Peak capacity is therefore an "ideal" number, and expresses the maximum number of resolvable components; it exceeds the real number by some factor, determined by operational conditions (e. g. the allowable separation time) [202]. Davis and Giddings estimated that a mixture of 50 random components would appear as about 18 single peaks; many of which are actually composite peaks. As a result of this overlap there is a considerable loss of analytical information. The authors proposed a statistical method of overlap to show how the number of resolved single peaks, S, is related to the peak capacity, Pc and the number of components in the mixture, m (Equation 1.15):

$$S = m^{\frac{-2m}{P_c}} \qquad \textbf{Equation 1.15}$$

The fraction of the peaks resolved, also represents the probability, P that a component will be separated as single peak. So, if Equation 1.15 is re-arranged:

$$P = \exp\left(-\frac{2m}{P_c}\right) \qquad \textbf{Equation 1.16}$$

Where: m: 0, 1, 2,...

By applying a theoretical analysis to equation 1.16, Davis and Giddings could draw the following conclusions:

i. with random spacing one can't expect to see more than 37% of the peaks;

ii. the number of single component peaks cannot exceed 18% of the peak capacity.

1.5.5. Orthogonality of separations

Multidimensional analysis, which involves two or more different separation dimensions, offers significant advantages with respect to the enhanced differentiating power that can be achieved. Both dimensions must be compatible towards the analysis of the sample components and the coupling interface must consider the possible different nature of the fluids used in each dimension.

The multidimensional analytical method combines independent displacement processes with x and y axes oriented at right angles. Along each axis the peak capacity will respectively be Pc x and Pc y. The orthogonality criterion will be satisfied when the two separations are based on two independent and unrelated retention mechanisms; synentropy across dimensions (cross information) is minimised (Figure 1.17), resulting in multiplicative peak capacity.

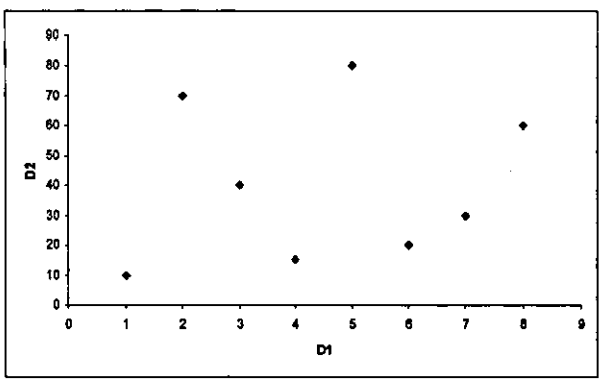


Figure 1.17: Retention space coverage in 2D separation of hypothetical samples showing low synentropy. The graph is a schematic illustration.

The importance of orthogonality in multidimensional separations is critical because it determines the magnitude of the separation space that is utilised [203]. Retention correlation across dimensions reduces the maximum peak capacity to some fraction of that which is theoretically available. A high degree of retention correlation can reduce a multidimensional separation to an essentially one-dimension separation, with peaks distributed along a diagonal [204] (Figure 1.18).

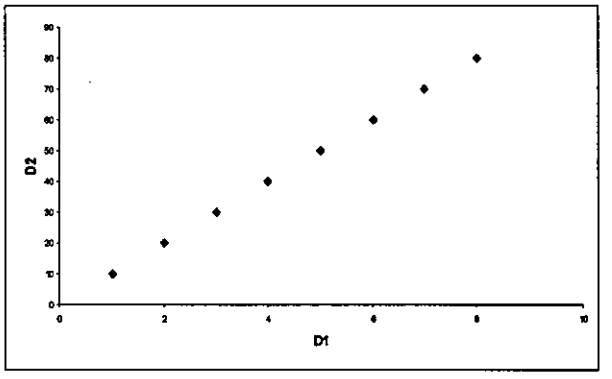


Figure 1.18: Retention space coverage in 2D separation of hypothetical samples showing a high retention correlation. The graph is a schematic illustration.

Orthogonality in coupled column chromatography can be realised either by coupling dissimilar techniques [205, 206] or by selectively tuning the operational parameters which can influence retention, like temperature [204] and mobile phase composition [207].

One very important observation must be made at this point. It is generally accepted that coupled methods in hyphenated instruments can be made independent of each other by combining methods that are as different as possible [205-208]. For example GC/MS appears to be a good hyphenated instrument because the two analytical methods are very different and they provide different types of information, and might be expected to be orthogonal. However, since both chromatographic retention and mass-to-charge ratio are correlated with molecule size, the two techniques are not orthogonal and the synentropy is bigger than zero. Furthermore, coupling dissimilar techniques (e.g. LC/CZE and SEC/CZE) could prove to be challenging and complicated, because the mode of operation varies with increase in dissimilarities between retention mechanisms. On the other hand, one might expect, for example, GC x GC to be far from orthogonal, because the two dimensions are closely related. But applying fine adjustments to the second

column temperature can eliminate any retention mechanism in common, making the entire retention space accessible.

In recent years GC x GC, had attracted considerable interest and has been shown to be a powerful technique which provides excellent results in terms of separation power, compounds classification, speed of analysis and compound identification. Its methods and applications [209-212], especially in the petrochemical field [213-215], have been widely reviewed but have seen little application for oligomer analysis because of the analytes limited volatility.

1.5.6. Orthogonality in LC x LC

Oligomer separations are more compatible with liquid phase methods and a number of different two-dimensional (2D) systems have been developed, including LC x LC, LC x capillary zone electrophoresis (CZE), size exclusion chromatography (SEC) x CZE, gel electrophoresis x LC and IE x LC [206, 216-220]. Although coupling orthogonal techniques minimizes synentropy, the challenges, such as sampling criteria and the mode of operation, are considerable. On the other hand, operating in reversed-phase LC in both dimensions and concentrating the orthogonal efforts on column selectivity only can provide minimal synentropy. This is because sample hydrophobicity is the key separation factor in both cases and analytes molecules retained on one stationary phase will still be the more retained analytes on a different stationary phase [207].

In the following section, examples on how orthogonality can be achieved in LC x LC separations are described with emphasis on oligomer analysis.

1.5.6.1. Normal phase HPLC (NP-HPLC) coupled to reversed phase HPLC (RP-HPLC)

Of all LC x LC approaches, NP-HPLC x RP-HPLC is most probably the most orthogonal [221] and therefore potentially able to provide high resolution. However, because of mobile phase incompatibility, interfacing the two dimensions is a fundamental limitation. Aqueous-organic mobile phases used in the first dimension (1D) RP-HPLC usually showed limited miscibility with purely organic solvents used in the second dimension (2D) NP-HPLC; furthermore, aqueous-organic mobile phases strongly de-activated the stationary phase employed in the 2D NP-HPLC. Even 1µl of an aqueous acetonitrile mobile phase fraction transferred from a RP column to a silica gel one, has completely destroyed the NP resolution [222].

An example of normal phase and reversed phase chromatography coupled in a comprehensive system was given by the analysis of alcohol ethoxylates [223], which are used as non ionic surfactants in many industrial products and processes. Murphy and his group [223] characterised the polyethylene oxide (constituents of alcohol ethoxylates) on a silica column with water-acetonitrile gradient. The alkyl distribution was achieved on a C_{18} column with isocratic methanol-water. The mobile phases were miscible, resulting in the stepwise injection of the entire first dimension eluent into the second dimension. Although the eluent used on the silica column was aqueous acetonitrile, the authors still referred to the techniques as NP, since retention was in order of increasing number of ethylene oxide units.

Jandera *et al.* [222] investigated the separation selectivity in aqueous and non-aqueous RP systems and NP-HPLC system, for the analysis of triacyl glycol (from plant oil samples) and ethylene glycol-propylene glycol oligomers (EO-PO, used

as surfactants). EO-PO polymers varying in polarity were expected to show different selectivity in RP and NP modes. Mono-, di- and triacyl glycerols, saturated and unsaturated fatty acids and their esters were contained in the natural oil samples. The first dimension (1D) separation was on a C_{18} column with acetonitrile/water or methanol/water. In the second dimension (2D) propanol/hexane was used as the eluent on an aminopropyl bonded silica column. Gradient elution starting at 50% MeCN was used for the full separation of EO-PO cooligomers. Fractions from the 1D were cut and sent to the 2D system. The resulting 2D RP-NP chromatograms enabled the calculation of the numbers of the EO and PO units in the individual fractions.

Another example of 2D RP-HPLC x NP-HPLC has been reported by Park *et al.* [224], who separated polystyrene (PS)-block-polyisoprene (PI) diblock copolymers using RP-HPLC, which fractionated the PI block, and NP-HPLC, which separated the PS block. In other studies, RP x NP systems have been applied to pharmaceutical analysis [221] and food analysis [225, 226].

1.5.6.2. RP-HPLC coupled to hydrophilic interaction chromatography (HILIC)

Jandera *et al.* [187] also analysed ethylene oxide (EO)-propylene oxide (PO) oligomers. RP-HPLC was used in the first dimension and HILIC in the second dimension. The two systems proved to be orthogonal with respect to the separation selectivity for EO and PO units. In 1D resolution of the PO oligomers was obtained on a C_{18} microbore column, using a gradient of acetonitrile and water. The HILIC NP used an ethanol-dichloromethane-water mobile phase on an aminopropyl silica column, which provided separation according to the EO distribution.

Wang *et al.* [227] developed an HILIC method orthogonal to a RP-HPLC method to separate a pharmaceutical compound and three of its related impurities, using a XTerra C_{18} column with 0.09% phosphoric acid/MeCN mobile phase in RP and a diol column and MeCN/salt solution (95:5, v/v), (10 mM ammonium chloride) mobile phase for the HILIC separation. The elution order of the four compounds was much different in HILIC from that in RP-HPLC (Figure 1.19).

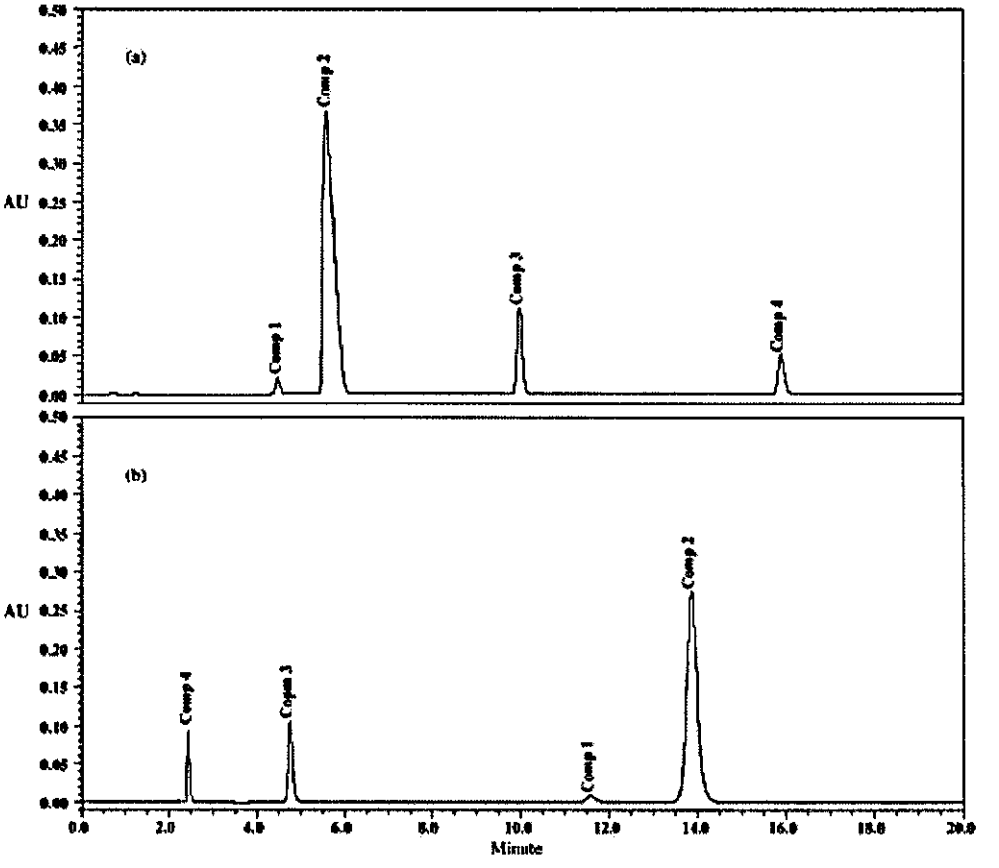


Figure 1.19: Chromatograms of the specificity solution on (a) RP-HPLC and (b) HILIC conditions. Ref. [227].

1.5.6.3. Liquid chromatography at critical conditions (LCCC) or SEC coupled to RP-HPLC

LCCC has been coupled to RP-HPLC [228-232] for the separation of polymers and has the advantage that in the first dimension the separation is solely based on functionality and in the second dimension is based on molecular mass, leading to complementary information that couldn't have been obtained using the techniques separately [233]. Recently Im *et al.* [234] used a different approach for the 2D-LC analysis of branched polystyrenes (PS) using a RP temperature gradient LC for the 1D, which allowed separation in terms of the molecular weight, and LCCC in the 2D which led to a separation in terms of the number of branches. Since the same eluent (chloroform/MeCN) was used in both dimensions, the resulting chromatograms were free from possible 'break through' and large system peaks.

SEC has often been a preferred choice for the 2D (to separate according to the molecular weight) and recent applications of SEC x NP and SEC x RP applied to synthetic polymers, oligomers and biopolymers have been reviewed [60].

1.5.6.4. RP-HPLC coupled to RP-HPLC

A procedure for the development of a RP-HPLC separation orthogonal to a pre-existing 1D RP-HPLC was proposed by Pellett *et al.* [235]. The procedure involved changes of some of the experimental conditions that affect RPLC selectivity (Table 1.2).

Table 1.2: Experimental conditions that affect RP-HPLC selectivity. Ref. [235].

Condition	Comment
Solvent strength (%B)	Usually a maximum change in %B≤10%
Gradient steepness, t_G	Usually a maximum change in t_G ≤10-fold
Temperature T	Maximun change in T is limited by a need to maintain 1≤k≤10 and avoid temperature degradation of the column
Solvent type	
• Acetonitrile	Preferred initial solvent
• Methanol	Acceptable alternative, unless detection at ≤210 nm is required
• Tetrahydrofuran	Less desirable alternative, avoided by many laboratories
Column type	Very wide range of columns available. Selectivity cannot be changed continuosly
Mobile phase pH	Potential large change in selectivity for ionisable compounds
Mobile phase buffer type concentration	Moderate change in selectivity for ionised bases
Mobile phase amine additives	Moderate change in selectivity for protonated bases
Ion-pair reagents	Potentially change in selectivity for ionised compounds

In particular, the authors proposed changing the mobile phase organic solvent, the column and the mobile phase pH. They improved the resolution and peak spacing by adjusting the column temperature and by using gradient instead of isocratic runs.

With this procedure they were able to develop methods for nine RPLC methods from six pharmaceutical laboratories.

RP-HPLC x RP-HPLC systems have also been used for polymer separations; the necessary selectivity differences were obtained with different columns and eluents. Trathnigg *et al.* [236, 237] analysed fatty acid methyl ester ethoxylates (FAMEEs), using a Zorbax 300 C_{18} column/85% aqueous methanol in the first dimension and a Prodigy ODS(3) column/acetone-water in the second dimension.

The separation of oligostyrenes was achieved by Gray *et al.* [238, 239], and by Sweeney *et al.* [67, 240] (coupling off-line a C_{18} column/methanol system to a carbon-clad zirconia column/acetonitrile system). The same approach was then adapted for comprehensive analysis of isomers in a mixture of oligostyrenes [241, 242]. The first dimension C_{18} column allowed separation of the oligomers according to their molecular weight, whereas the carbon clad zirconia (CCZ) stationary phase used in the second dimension allowed the expression of the isomeric sample dimensionality. Although it is arguable that the carbon clad column is responsible for RP separation mechanisms (a surface electronic interaction would be a more accurate description), these systems are still classed as RP-HPLC x RP-HPLC. Gray *et al.* [243] developed a similar separation system, using C_{18} stationary phase with methanol as mobile phase in the first dimension and CCZ stationary phase with acetonitrile as mobile phase in the second dimension. They analysed 32 oligostyrene structural isomers and stereoisomers; they theoretically assessed the parameters that could give an optimum performing system (i.e. percent synentropy, orthogonality, peak capacity, etc), using Information Theory and Factor Analysis.

RP-HPLC x RP-HPLC have also been used for other groups of analytes by Gilar *et al.* [244], Ikegami *et al.* [245], Venkatramani and Zelechonok [207], Opiteck *et al.* [220] and Holland and Jorgenson [219].

1.6. Present study

The current study sets out to characterise the OLA 81 (ethylene bis (propionyl oligo lactyl amide), (mean n = 10), and its process impurities by liquid chromatography and mass spectrometry. As it appeared that a two dimensional separation should be able to provide the required resolution of the oligomers and the different functional groups, this will be examined by two stages.

a. Firstly, reversed-phase liquid chromatography (RP-HPLC) will be examined for its ability to determine the oligomeric distribution (chain length).

b. A second separation method will be developed to achieve the head group distribution and to resolve the OLAs from other components of the sample such as cyclic impurities, using liquid chromatography at critical conditions (LCCC) in either normal-phase (NP-HPLC) or HILIC (hydrophilic interaction liquid chromatography).

These methods will be examined with the intention of combining them into a comprehensive two dimensional separation so that compatibility of the mobile phase will be an important criterion.

Because the OLAs have only a limited chromophore and hence poor detectability, mass spectroscopy will be examined as the main detector which will have the advantage of providing a further orthogonal assay.

2. Experimental work

2.1. Materials and reagents

2.1.1. General chemicals

Formic acid and trifluoroacetic acid (TFA) were purchased from Sigma, Poole, Dorset, UK. Acetonitrile (HPLC grade, Far UV), methanol (HPLC grade), THF (HPLC grade), n-hexane (analytical reagent grade) and ammonium formate were purchased from Fisher, Loughborough, UK. Acetone (HPLC grade) was from BDH, VWR International, Poole, UK. Water was de-ionized in-house, to $_{18}$.2 MΩ using Elga Pure Water system (Elga, Wycombe, UK).

2.1.2. Standard chemicals

Samples of ethylene bis (propionyl oligo lactyl amide, nominally n=10) and their cyclic and linear impurities were provided by 3M Healthcare (St Paul, MN USA). Samples of OLAs were prepared in acetonitrile or THF

2.1.3. Mobile phases

The mobile phases consisted of different ratios of the components listed in 'general chemicals'. All mobile phases contained 0.1% of either formic acid or trifluoroacetic acid.

2.1.4. Uracil solution (dead volume marker)

Uracil was from Sigma (Poole, Dorset, UK). A 64 μg/ml uracil solution was prepared in acetonitrile.

2.1.5. Chromatographic columns

Table 2.1: Summary of chromatographic columns used for LC studies (the polar materials are highlighted in green)

Column name	Phase type	Dimension (mm)	Particle size (μm)
Spherisorb NH2	Amino	250 x 4.6mm	5
Luna NH2	Amino	150 x 2.0mm	3
Zorbax CN	Cyano	250 x 4.6 mm	7
Spherisorb C_6	Hexyl	250 x 4.6 mm	10
Spherisorb S5W	Silica	250 x 4.6 mm	5
Symmetry C_{18}	Octadecyl	150 x 3.9 mm	5
Symmetry C_4	Butyl	150 x 4.6 mm	5
Eclipse XDB C_8	Octyl	150 x 4.6 mm	5
Prodigy ODS (3)	Octadecyl	150 x 4.6 mm	3

Spherisorb NH2 was purchased from Supelco Inc, Sigma-Aldrich Company Ltd, Dorset, UK.

Luna NH2 and Prodigy ODS (3) came from Phenomenex, Macclesfield, Cheshire, UK.

Symmetry C_{18} and Symmetry 300^{TM} C_4 columns were purchased from Waters, Manchester, UK.

Zorbax CN and Spherisorb S5W were purchased from Phase Separations Ltd, Deeside, Clwyd, UK; at the time of writing, Phase Separations Ltd have become part of Waters, Manchester, UK.

Spherisorb C_6 was from FSA Laboratory Supplies, Loughborough, UK; at the time of writing, FSA Laboratory Supplies have become part of Thermo Fisher Scientific, Loughborough, UK.

HP Zorbax Eclipse XDB-C_8 was purchased from Agilent, UK.

2.2. Instrumentation and instrumental settings

2.2.1. LC/UVD system

A LC HP1090 series II (Hewlett-Packard, Waldbronn, Germany) fitted with variable wavelength UV detector was used for some of the temperature related studies and some of the selectivity studies. When temperature studies were carried out the chromatographic columns were inserted in a Jasco 860 column oven (Great Dunmow, Cambridge, UK). All analysis were carried out in isocratic mode.
The software was ChemStation (Pascal series).

2.2.2. Fraction collector

The fraction collector employed was Frac-100, from Pharmacia Fine Chemicals.

2.2.3. LC/UVD/MSD system

The LC/UVD/MS Quadrupole used was a HP1100 (Agilent Technologies, Waldbronn, Germany), equipped with a UV detector. An atmospheric pressure chemical ionization source (APCI) (Agilent Technologies, Waldbronn, Germany) was employed. An electrospray ionisation source (ESI) (Agilent Technologies, Waldbronn, Germany) was also used.
Software employed was ChemStation rev. B. 01.03.

Quadrupole Mass spectrometer detector conditions were as follows:
Ionisation mode: APCI
Polarity: positive and negative;

Scan parameters: 100-2500;
LC-MS interface settings: drying gas temperature = 350 °C, nitrogen drying gas flow = 10.0 l/min;
Spray chamber: vaporizer temperature = 450 °C, nebuliser pressure = 45 psig, Capillary voltage (positive) = 4000 V, capillary voltage (negative) = 5500 V, Corona (positive) = 10 μA, Corona (negative) = 15 μA.

Ionisation mode: API-ESI
Polarity: positive
Scan Parameters: 50-2000 Mass range
LC-MS interface settings: drying gas temperature = 350 °C, nitrogen drying gas flow = 8.0 l/min;
Spray chamber: nebuliser pressure = 50 psig; Capillary voltage (positive) = 6000 V.

2.2.4. LC/MSD system

A MS Ion trap Finnigan LTQ was employed (Thermo Electron Corporation, Hemel, Hempstead, Hertfordshire, UK), with Electrospray ionisation source (Thermo Electron Corporation, Hemel, Hempstead, Hertfordshire, UK), equipped with a Surveyor LC pump/autosampler. Software used was Finnigan XCalibur, High Chem Mass Frontier (XCAL1-97113 rev. A).

Ion trap Mass spectrometer conditions were as follows:
Ionisation mode: ESI;
Polarity: positive;
Scan range: 50-2000;
Sheath gas: 46;
Auxiliary gas: 15;

Sweep gas: 5;
Spray voltage: 5.50 kV;
Capillary temperature: 180 °C;
Capillary voltage: 30 V;
Tube lens: 65 V.

2.2.5. LC/ELSD system

A LC HP1100 (Agilent Technologies, Waldbronn, Germany) equipped with a light scattering detector (PL-ELS 1000, Polymer Labs, Church Stretton, Shropshire, UK) was also used.
Software employed was Varian Galaxie rev. 1.9.3.2.
ELSD conditions were as follows:
Evaporator: 95;
Nebuliser: 70;
T. line: 30;
Gas flow rate: 1.7;
Autozero offset: 0;
Time const: 0.

2.3. Experimental work on RP-HPLC

2.3.1. Experimental procedure

2.3.1.1. Mobile phase preparation

0.1% formic acid in organic solvent or in water was prepared. Formic acid was chosen to favour ionisation during MS analysis.

2.4. Experimental work on LCCC

2.4.1. Experimental procedure

2.4.1.1. Mobile phase preparation

0.1% formic acid in organic or in water was prepared. Formic acid was chosen to favour ionisation during MS analysis. Ammonium formate buffer was used over the 5-50 mM range. 5, 10, 15, 20 and 50 mM ammonium formate solutions were prepared by dissolving respectively 0.32, 0.64, 0.96, 1.28 and 3.20 g of ammonium formate into a litre of deionised water, which contained 1 ml of concentrated formic acid. THF was mixed with MeCN over the 10-30% range.

2.5. Calculations

The chromatographic retention factor, k was evaluated by using the following relationship:

$$k = \frac{(t_r - t_0)}{t_0}$$ **Equation 2.1**

Where:

t_r: solute retention time (in minute);

t_0: dead time (in minute, $t_0 = V_0$ (dead volume), when eluent flow rate= 1 ml/min).

The chromatographic separations were evaluated by using the following resolution equation (half-width method):

$$Rs = 1.176\frac{Rt_2 - Rt_1}{w_{1h} + w_{2h}}$$ **Equation 2.2**

Where:

Rt_2: retention time of second component;

Rt_1: retention time of first component;

w_{1h}: width of first component at half peak height;

w_{2h}: width of second component at half peak height.

3. Results and Discussion: preliminary investigations

3.1. Mass spectral analysis

Mass spectroscopy was chosen as main form of detection, since OLA molecules do not have significant chromophores and UV detection in the low wavelength region would be impaired by mobile phase absorption. In addition, mass spectroscopy (MS) should provide high sensitivity, broad range, specificity and selectivity. Two forms of ionisation were employed: electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI).

3.1.1. Electrospray Ionisation, ESI MS data analysis

Mass spectral interpretation of OLAs and their degradation products and impurities was achieved with the aid of Table 3.1 (table of molecular masses of OLAs and their related substances).

The mass spectra obtained from ESI experiments are made up of:

- A protonated molecular ion $[M + H]^+$,
- a doubly charged ion $[M + 2H]^{2+}$,
- an ion $[M + 23]^+$ that can be attributed to sodium adduct formation $[M + Na]^+$,
- a doubly charged adduct ion $[M + 2Na]^{2+}$.

The detection of an $[M + H]^+$ ion in the positive ion mode is a result of the electrospray ionisation (ESI) process. Cationisation with alkali metal ions instead

of proton addition is also often observed when working in ESI, in the positive ion mode [246]. The sodium is derived from possible trace level presence in the water used as mobile phase or from glassware or column materials. Multiply charged molecular species ($[M + nH]^{n+}$) are also common in ESI MS, particularly with compounds that have molecular weights larger than 1200 [246]. In the present study multiply charged species up to pentacharged ions were observed.

Overall, the mass spectra present a series of pseudo molecular ions, with a regular molar mass increment of 72. This incremental value confirms the monomer repeating unit, which is typical of lactic oligomers (-$OCOCHCH_3$-). Each OLA diamide diester (dd) oligomer was identified on the basis of the mass of its $[M + Na]^+$ ion (Figure 3.1a), and for large oligomers from its $[M + Na]^+$ ion and $[M + 2Na]^{2+}$ion (Figure 3.1b), the latter being separated by 36 units.

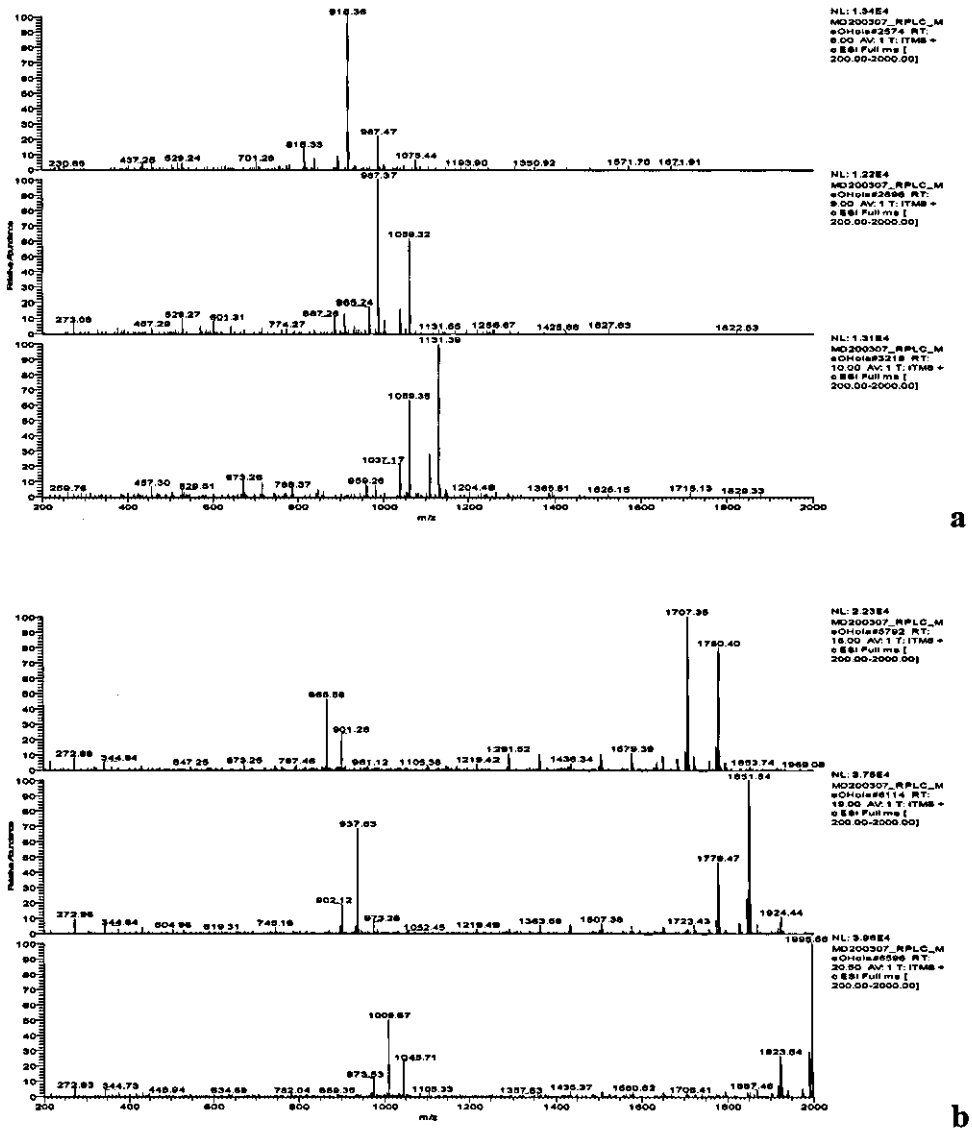


Figure 3.1: ESI mass spectra of OLAs, obtained on LC-MS: Thermo Finnigan LTQ. Injection volume: 10 µl; OLAs excipient solution concentration: 1082.0 µg/ml; chromatographic column: Symmetry C_4 (150 x 4.6) mm, 5 µm; mobile phase A: 0.1% formic acid in water, mobile phase B: 0.1% formic acid in MeCN; starting conditions: 60% mobile phase B, taken to 70% in 25 min and then ramped to 100% at 30 min; LC flow rate: 0.5 ml/min. a: mass spectra made up of ions $[M + Na]^+$; b: mass spectra made up of doubly charged ions $[M + 2Na]^{2+}$.

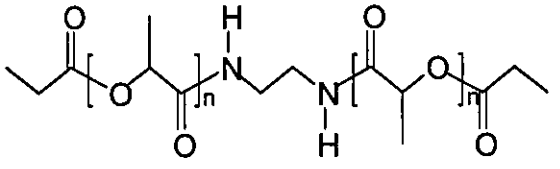
Mass Spectra in Figure 3.1A could be explained as coming from the oligomers as follows:

915 = $[dd + 23]^+$, corresponding to diamide diester n = 10;
987 = $[dd + 23]^+$, corresponding to diamide diester n = 11;
1059 = $[dd + 23]^+$, corresponding to diamide diester n = 12;
1131 = $[dd + 23]^+$, corresponding to diamide diester n = 13.

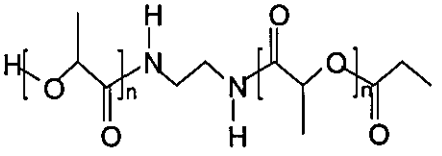
Mass Spectra in Figure 3.1B could be explained as coming from the single and doubly charged ions oligomers as follows:

1707, 1780 = $[dd + 23]^+$, corresponding to diamide diester adduct n = 21, 22;
865, 901 = $[dd + (2 \times 23)]^{2+}$, corresponding to doubly charged diamide diester adducts n = 22, 23;
1851, 1779 = $[dd + 23]^+$, corresponding to diamide diesters n = 23, 22;
937, 902 = $[dd + (2 \times 23)]^{2+}$, corresponding to diamide diesters n = 24, 22;
1923, 1995 = $[dd + 23]^+$, corresponding to diamide diesters n = 24, 25;
973, 1009, 1045 = $[dd + (2 \times 23)]^{2+}$, corresponding to diamide diesters n = 25, 26, 27.

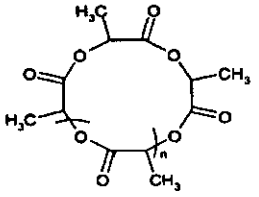
The following OLAs chemical structures refer to the OLAs whose oligomeric masses are reported in Table 3.1.



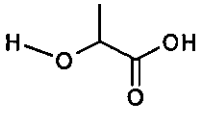
Diamide diester



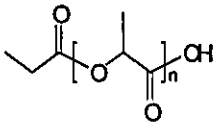
Diamide monoster



Cyclic



Free acid



Propionyl OLA

Table 3.1: Molecular masses of oligomers (of n repeat monomer units) of OLAs excipient (diamide diester) and their related substances.

n	diamide diester	diamide monoester	Cyclic	free acid	pr-OLA
1	244	188	72	90	146
2	316	260	144	162	218
3	388	332	216	234	290
4	460	404	288	306	362
5	532	476	360	378	434
6	604	548	432	450	506
7	676	620	504	522	578
8	748	692	576	594	650
9	820	764	648	666	722
10	892	836	720	738	794
11	964	908	792	810	866
12	1036	980	864	882	938
13	1108	1052	936	954	1010
14	1180	1124	1008	1026	1082
15	1252	1196	1080	1098	1154
16	1324	1268	1152	1170	1226
17	1396	1340	1224	1242	1298
18	1468	1412	1296	1314	1370
19	1540	1484	1368	1386	1442
20	1612	1556	1440	1458	1514
21	1684	1628	1512	1530	1586
22	1756	1700	1584	1602	1658
23	1828	1772	1656	1674	1730
24	1900	1844	1728	1746	1802
25	1972	1916	1800	1818	1874

As the mass range of the LC-MS systems in use was limited to 2000, for species whose singly charged adduct ion was above that size only the multiply charged ions could be detected. For each of the nominal molecular ions there were also isotopic species, as shown in figure below:

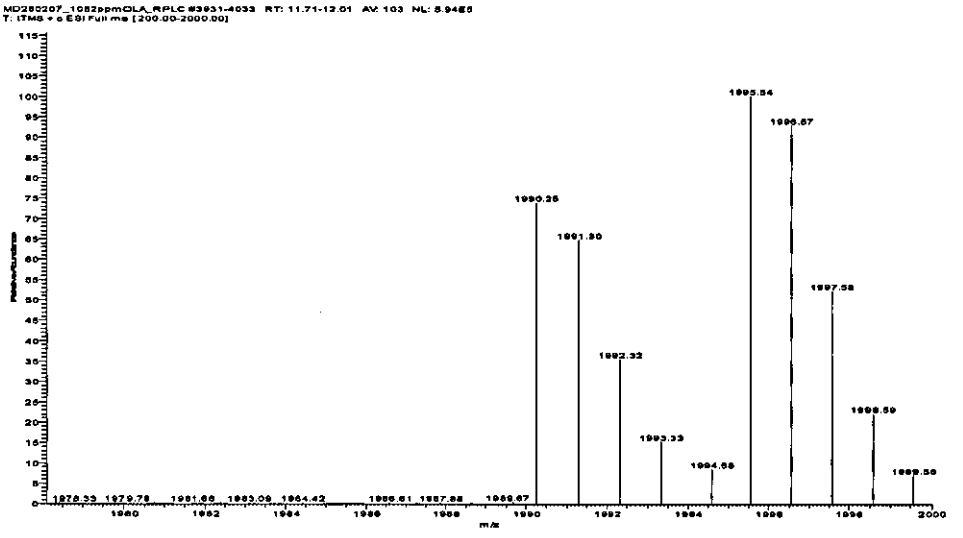


Figure 3.2: Mass spectrum (expanded) of OLAs (n = 25), obtained on LC-MS: Thermo Finnigan LTQ. Injection volume: 10 µl; OLAs excipient solution concentration: 1082.0 µg/ml; chromatographic conditions as for Figure 3.1.

The spectra reported in Figure 3.2 show other ions of higher intensity (e. g. ca. 25 % of the m/z 1995.54 ions at m/z 1994.58). These represent the molecules in which one ^{12}C atom has been replaced by a ^{13}C atom, because carbon has a naturally occurring isotope one atomic mass unit higher.

The intensities of these isotopic ions relates to the relative abundance of the naturally occurring isotope (1.1%) multiplied by the total number of carbon atoms in the molecule. The presence of an isotope signal one mass unit higher is an indication that the sample ions are singly charged (z = 1). If the sample ions had been doubly charged, then the m/z values would only differ by 0.5 mass unit.

3.1.2. Atmospheric Pressure Chemical Ionisation, APCI MS data analysis

In the APCI-MS spectra the detected ions could be either an ion $[M - H]^-$, or an ion $[M + H]^+$, according to whether negative or positive ionisation was chosen. Concurrent generation of positive and negative ion mass spectra enabled pr-OLAs and diamide diesters (dd) to be detected. This was possible because:

- the amide functionality carried by dd, which supports the positive charge responsible for the APCI signal obtained under these conditions;
- the carboxyl group in the pr-OLA, can readily lose a proton to give a negatively charged species.

In the positive ionisation mode spectra for the cyclic and free acid impurities were also detected.

Each OLA cyclic (cyc) oligomer and free acid oligomer was identified on the basis of the mass of its $[M + H]^+$ or $[M]^+$ ion (for example Figure 3.3), with the aid of Table 3.1.

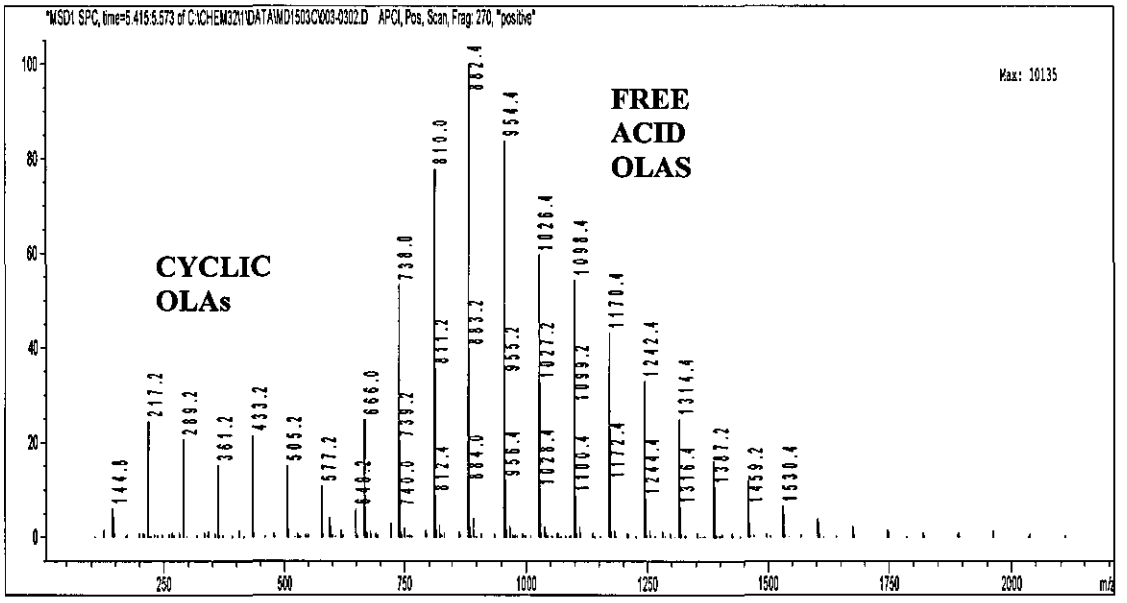


Figure 3.3: Mass spectrum (positive ionisation) obtained on: LC-MS: HP 1100, equipped with APCI source. Injection volume: 20 µl; OLA cyclic solution concentration: 3000.0 µg/ml; chromatographic column: Spherisorb NH2 column (250 x 4.6) mm, 5µm, at 100% 0.1% formic acid in acetonitrile, flow rate: 0.5 ml/min. MS of OLA cyclic series, with range of oligomer masses spanning from n = 1 (145) to n = 9 (649) and free acid series, with range of oligomer masses spanning from n = 10 (739) to n = 20 (1459).

Mass Spectra in Figure 3.3 could be interpreted as follows:

145 = $[cyc + H]^+$, corresponding to cyclic n = 2;

217 = $[cyc + H]^+$, corresponding to cyclic n = 3;

289 = $[cyc + H]^+$, corresponding to cyclic n = 4;

361 = $[cyc + H]^+$, corresponding to cyclic n = 5;

433 = $[cyc + H]^+$, corresponding to cyclic n = 6;

505 = $[cyc + H]^+$, corresponding to cyclic n = 7;

577 = $[cyc + H]^+$, corresponding to cyclic n = 8;

649 = $[cyc + H]^+$, corresponding to cyclic n = 9;

738 = $[free\ acid]^+$, corresponding to free acid n = 10;

810 = $[free\ acid]^+$, corresponding to free acid n = 11;

882 = $[free\ acid]^+$, corresponding to free acid n = 12;

954 = $[free\ acid]^+$, corresponding to free acid n = 13;

1026 = [free acid]$^+$, corresponding to free acid n = 14;

1098 = [free acid]$^+$, corresponding to free acid n = 15;

1386 = [free acid]$^+$, corresponding to free acid n = 19;

1458 = [free acid]$^+$, corresponding to free acid n = 20.

The 738, 810, 882 etc, peaks are possibly directly ionised species $[M]^+$ of the free acids. The 739, 811, 883 etc, peaks are probably $[M + H]^+$ ions of the free acids.

Each pr-OL oligomer was identified on the basis of the mass of its $[M - H]^-$ ion (Figure 3.4), with the aid of Table 3.1.

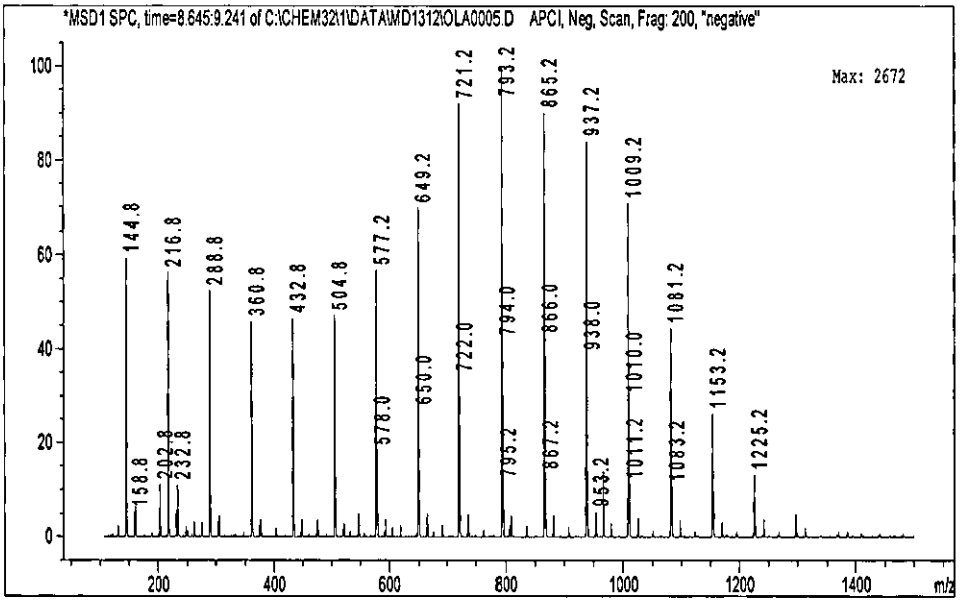


Figure 3.4: Mass spectrum (negative ionisation) obtained on: LC-MS: HP 1100, equipped with APCI source. Injection volume: 5 µl; OLA main excipient solution concentration: 520.0 µg/ml; chromatographic column: Symmetry C_{18} (150 x 3.9) mm, 5 µm; mobile phase: 60% 0.1% formic acid in THF/40% 0.1% formic acid in water (isocratic run); flow rate: 0.5 ml/min. MS of pr-OLA series, with range of oligomer masses spanning from n = 1 (145) to n = 16 (1225).

Mass Spectra in Figure 3.4 could be explained as follows:

145 = [pr-OLA - H]$^-$, corresponding to pr-OLA n = 1;
217 = [pr-OLA - H]$^-$, corresponding to pr-OLA n = 2;
289 = [pr-OLA - H]$^-$, corresponding to pr-OLA n = 3;
361 = [pr-OLA - H]$^-$, corresponding to pr-OLA n = 4;
433 = [pr-OLA - H]$^-$, corresponding to pr-OLA n = 5;
505 = [pr-OLA - H]$^-$, corresponding to pr-OLA n = 6;
577 = [pr-OLA - H]$^-$, corresponding to pr-OLA n = 7;
649 = [pr-OLA - H]$^-$, corresponding to pr-OLA n = 8;
721 = [pr-OLA - H]$^-$, corresponding to pr-OLA n = 9;
793 = [pr-OLA - H]$^-$, corresponding to pr-OLA n = 10;
865 = [pr-OLA - H]$^-$, corresponding to pr-OLA n = 11;
937 = [pr-OLA - H]$^-$, corresponding to pr-OLA n = 12;
1009 = [pr-OLA - H]$^-$, corresponding to pr-OLA n = 13;
1081 = [pr-OLA - H]$^-$, corresponding to pr-OLA n = 14;
1153 = [pr-OLA - H]$^-$, corresponding to pr-OLA n = 15;
1225 = [pr-OLA - H]$^-$, corresponding to pr-OLA n = 16.

3.2. The oligomeric distribution approach by RP-HPLC

In order to be able to characterise the composition oligomeric mixture of the OLAs and their impurities, it is necessary to examine:

- the oligomeric distribution (or chain length, number of repeat units) and
- the head group distribution.

The initial hope was to combine these two requirements into a comprehensive two dimensional assay, in which one dimension would determine the oligomeric profile, based on a reversed-phase liquid chromatography (RP-HPLC) and the second orthogonal dimension would separate the oligomers, which had different

substituents and the impurities, such as the cyclic compounds. This dimension would probably use normal phase or HILIC separation.

The investigation of the OLAs in terms of their chain length considered the following aspects which will be described in the following sections:

- column temperature;
- stationary phase;
- mobile phase.

The initial aim was to obtain the isolation of specific fragments of the OLAs main component. The separation process principally used an hexyl column with an aqueous acetonitrile mobile phase. The resulting fractions were subsequently subjected to a second analysis using RP-HPLC-MS to determine the purity and resolution of the oligomers. Some of these fractions were then available for the LCCC method development (refer to later paragraph). Temperature related studies were investigated in aqueous mobile phases, eluting on a hexyl stationary phase (30-75 °C temperature range used) and on a C_{18} column (over a 30-60 °C temperature range). Butyl, hexyl, octyl and octadecyl materials were compared in terms of their retention capabilities for OLAs. Separation selectivity for the three more widely used organic modifiers in RP-HPLC, acetonitrile, THF and methanol, was investigated.

3.3. Preliminary investigations into OLAs characterisation

3.3.1. Collection of OLAs fractions

Because it can be difficult to work with a mixture of oligomers an initial study was carried out to obtain samples of individual identifiable oligomers which could be used to monitor changes in their relative positions.

It must be also pointed out that since racemic mixtures of lactic acid were used as the starting materials for the oligomerisation, the final product is a racemic mixture of diastereoisomeric oligomers. Although aware of OLAs chirality, it was decided not to investigate this aspect. The original mixture of the OLAs ethylene bis (propionyl oligo lactyl amide) was reported to have a nominal mean value of n = 10 lactic acid units.

The RP-HPLC separation process used a C_6 column with an aqueous acetonitrile mobile phase, which was chosen because it represents a typical RP-HPLC eluent and because OLAs are soluble in acetonitrile, so that sample diluent and mobile phase would be as close as possible to each other in terms of composition.
A number of test runs was conducted in order to select a solvent gradient which would elute the more highly retained larger oligomers within reasonable time scale.

The selected optimum separation of OLAs into oligomers is shown in Figure 3.5:

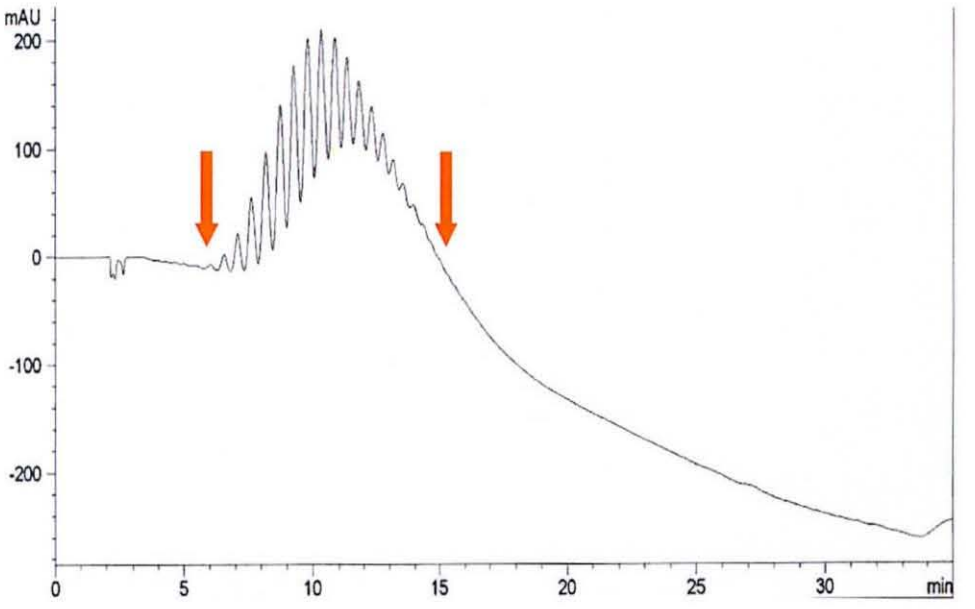


Figure 3.5: Chromatographic separation of 575.0 µg/ml OLAs solution, run on a Spherisorb C_6 column; Mobile phase A: 95% water, 5% Acetonitrile, 0.1% TFA; Mobile phase B: 95% Acetonitrile, 5% water, 0.1% TFA; Gradient starting from 40% mobile phase A and taken to 20% in 25 min and to 0% in 30 min; UV: 210 nm; flow rate: 1 ml/min; injection volume: 25 µl. LC: HP1100. The two arrows represent the starting and stopping points set to collect fractions, as explained in section below.

The base line drift observed in the chromatography was possibly due to a difference in absorption between mobile phase A and mobile phase B.

A fraction collector was employed, to collect fractions starting at 6 min and stopping collecting at 16 min (as indicated by arrows in figure above). The collecting interval was of 30 sec and gave a total of 22 fractions, which were re-examined by re-injection to confirm purity. Their chromatograms are reported in Appendix 1 and their retention values are compiled in Table 3.2.

The fractions were then examined by MS (Table 3.2), to confirm that the RP-HPLC separation had achieved an oligomeric separation (refer to Appendix 1 for mass spectra relevant to the 22 fractions). Most fractions contained only one or two oligomers, indicating a reasonably efficient resolution especially as the

fractionation had for simplicity been timed based rather than matching the collection to peaks intensities.

Table 3.2: MS interpretation summary of OLAs fractions, with specific diamide diester oligomers (of n repeat monomer units) assigned to nominal fractions, labelled 1-22. For mass spectra corresponding to individual fractions refer to Appendix 1. For oligomer masses refer to Table 3.1.

Fraction	Retention time (min)	Oligomer, n=	Oligomer, n=	Oligomer, n=
1	5.43			
2	5.40			
3	6.54	8	9	10
4	6.98	9	10	11
5	7.13	10	11	12
6	7.67	11	12	13
7	7.79	12	13	14
8	8.28	14	15	
9	9.39		16	17
10	10.29	15	16	17
11	10.81	17	18	
12	11.07	18	19	20
13	11.33	19		
14	11.87	20		
15	11.93	20		
16	12.53	21	22	
17	12.49	22		
18	13.04	22	23	
19	13.23	23		
20	13.52	24		
21	13.83	24	25	
22	13.92	25		

3.3.2. Conclusions on RP-HPLC 'fractionation' of OLAs

From the MS analysis of the OLAs fractions it was possible to conclude that the RP-HPLC separation, using a C_6 stationary phase and an aqueous acetonitrile mobile phase, was based on hydrophobic interactions, and had led to an oligomeric separation with elution in order of increasing size. The chain length distribution was therefore successfully targeted in RP-HPLC. These fractions were used in subsequent studies, when carrying out LCCC method development.

During these preliminary experiments the column temperature was not controlled. Subsequently it was decided to monitor whether temperature variations could have an impact on OLAs retention selectivity.

3.4. Further investigations into RP-HPLC: effects of column temperature on OLAs oligomeric distribution

This study investigated the effects of temperature changes on the retention and elution order of OLA main components in aqueous/organic systems, with non polar columns. The stationary phases were a hexyl bonded material (30-75 °C temperature range used), and an octadecyl material (over a 30-60 °C temperature range). This line of investigation followed a study carried out by Vanhoenacker and Sandra [79] who observed a reversal in elution order of a mixture of oligomers when the temperature was increased from 20 to 100 °C. At a certain temperature, critical conditions (CC) of elution had been achieved (i.e. all the oligomers elute together as a single peak).

In the present study, with water/acetonitrile (with added formic acid to aid MS ionisation) a chain length distribution separation (oligomeric separation), controlled by hydrophobic interactions was achieved on a C_6 column at 30 °C.
The chromatogram obtained (Figure 3.6) is comparable to the earlier separation at ambient temperature (Figure 3.5). Peaks 5, 7 and 10 contained respectively mainly n = 10, 11, 12, n = 12, 13, 14 and n = 15, 16, 17 oligomers and were used to monitor retention changes.

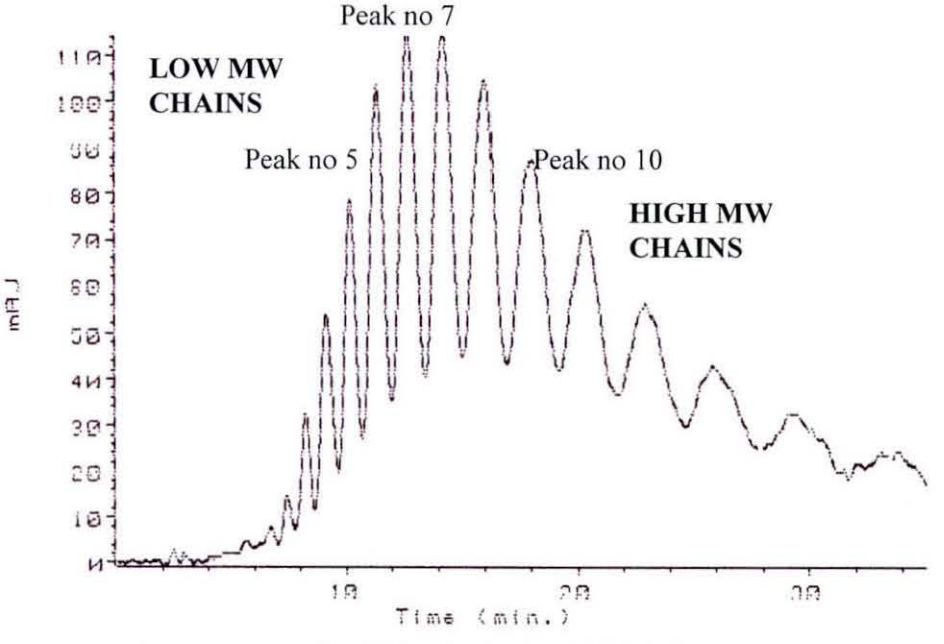


Figure 3.6: Chromatogram for OLAs obtained at 30 °C. LC: HP 1090. UV: 210 nm. Injection volume: 10 µl; main excipient solution concentration: 40.8 mg/ml; chromatographic column: Spherisorb C_6 (250 x 4.6) mm, 10 µm; mobile phase: 40% 0.1% formic acid in water/60% 0.1% formic acid in MeCN (isocratic run); flow rate: 1 ml/min.

On raising the temperature, the OLAs oligomer retentions decreased and the peaks shapes were sharper (up to 75 °C, Figure 3.7). The confirmation of the order of elution is shown by the mass spectral traces (Figure 3.8a at the low temperature and Figure 3.8b at the high temperature). This decrease could be explained if

partitioning mechanisms took place. Because partition is an exothermic process, an increase in temperature generally speaking will lead to a decrease in retention.

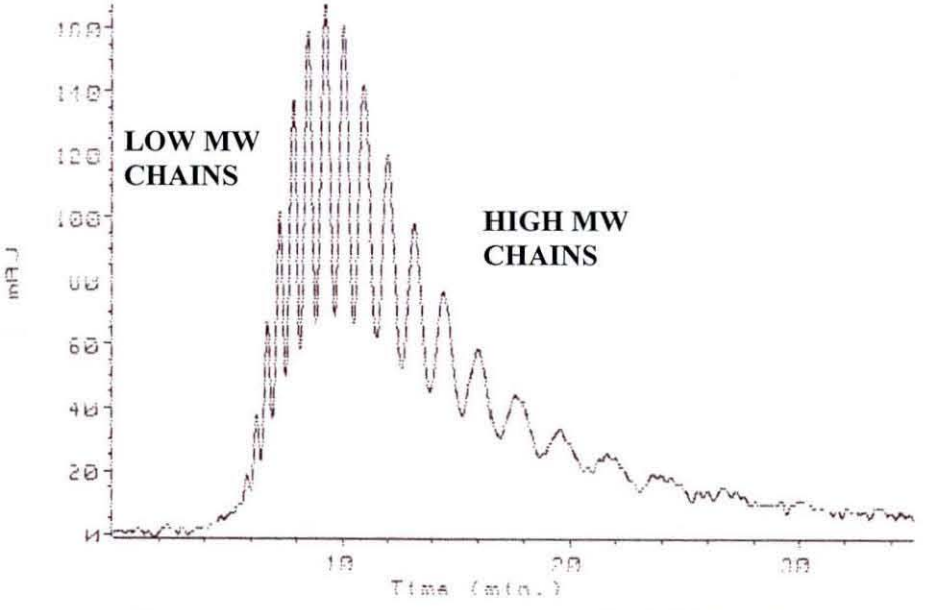
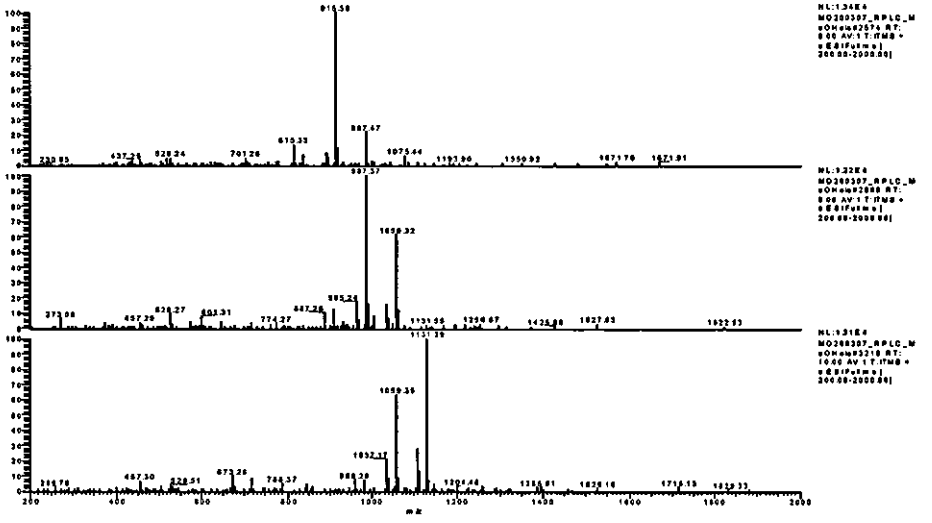


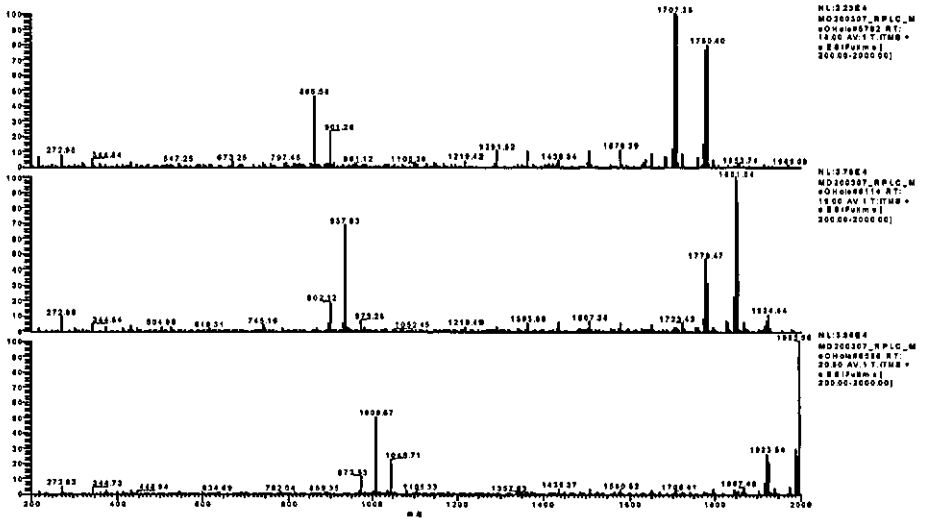
Figure 3.7: Chromatogram for OLAs obtained at 75 °C. LC: HP 1090. UV: 210 nm. Injection volume: 10 µl; main excipient solution concentration: 40.8 mg/ml; chromatographic column: Spherisorb C_6 (250 x 4.6) mm, 10 µm; mobile phase: 40% 0.1% formic acid in water/60% 0.1% formic acid in MeCN (isocratic run); flow rate: 1 ml/min.

The opposite behaviour had been observed by Cho *et al.* when analysing non-ionic surfactants. They reported an increase in retention of an alkyl chain and PEO copolymer when temperature was increased [86].

They suggested that sorption of the FAE to the stationary phase was energetically favourable for alkyl chains but unfavourable for PEO blocks, while it was entropically favourable for PEO blocks but unfavourable for alkyl chains. Increasing temperature led to an increase in retention of FAE with long PEO blocks.



a



b

Figure 3.8: ESI mass spectra of OLAs, obtained on LC-MS: Thermo Finnigan LTQ. Injection volume: 10 µl; OLAs excipient solution concentration: 1082.0 µg/ml; chromatographic column: Symmetry C_4 (150 x 4.6) mm, 5 µm; mobile phase A: 0.1% formic acid in water, mobile phase B: 0.1% formic acid in MeCN; starting conditions: 60% mobile phase B, taken to 70% in 25 min and then ramped to 100% at 30 min; LC flow rate: 0.5 ml/min. Temperature: 30 °C in a and 75 °C in b.

The thermodynamics of the chromatographic process were investigated over the 30-75 °C temperature range.

The retention factors, *k* (for peaks 5, 7 and 10, numbered as Figure 3.6) were plotted against the reciprocal absolute temperature (Figure 3.9).

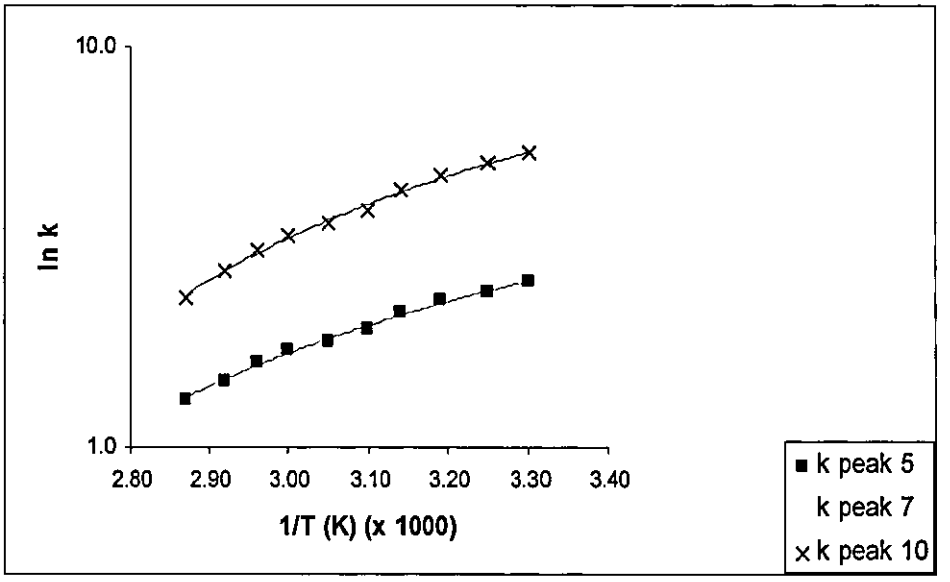


Figure 3.9: van't Hoff plots for peaks 5, 7 and 10, over the 30-75 °C range. LC: HP 1090. UV: 210 nm. Injection volume: 10 µl; OLAs main excipient solution concentration: 40.8 mg/ml; chromatographic column: Spherisorb C_6 (250 x 4.6) mm, 10 µm: mobile phase: 40% 0.1% formic acid in water/60% 0.1% formic acid in MeCN (isocratic run); flow rate: 1 ml/min. Peak 5 corresponds to dd oligomers 10, 11 and 12, peak 7 to dd oligomers 12, 13 and 14, and peak 10 to dd oligomers 15, 16 and 17, as reported in Table 3.2.

There was a systematic dependence between ln *k* and 1/T.

The three estimated straight lines (because of non-linear isothermic response) obtained from Figure 3.9 had the following equations:

y= 2.96x-7.17 (for peak 5) **Equation 3.1**

y= 4.27x-10.57 (for peak 7) **Equation 3.2**

y= 7.15x-18.11 (for peak 10) **Equation 3.3.**

The intercepts and the slopes of Equations 3.1, 3.2 and 3.3 were substituted in the van't Hoff relationship (Equation 1.6), to give the ΔS and ΔH values:

1. ΔH= -24.61 kJ/mol; ΔS=- 59.61 J/Kmol (for peak 5).

2. ΔH= -35.50 kJ/mol; ΔS=- 87.88 J/Kmol (for peak 7).

3. ΔH= -59.44 kJ/mol; ΔS=- 150.57 J/Kmol (for peak 10).

These are not accurate values, because of the curved relationship between ln *k* and 1/T.

Several authors [172-179] have used a plot of ΔH versus ΔS in investigations of separations mechanisms. From the slope in this plot they obtained the compensation temperature, i.e. the temperature at which ΔH = ΔS, which for a homopolymer is the same as the critical point (i.e. CC of elution).
However, this is meaningful only if ΔS and ΔH changes are measured very close to the critical conditions.

The mobile phase composition used in the present temperature-related experiments (40% 0.1% formic acid in water/60% 0.1% formic acid in MeCN) was too far from a composition that would allow oligomeric co-elution (i.e. CC), as shown by the oligomeric distribution in the above chromatograms.

However, a theoretical calculation was followed, according to Trathnigg *et al.* [170] line of work. By plotting ΔS against ΔH (according to equation 1.6), Tc, the predicted compensation temperature, could be computed from the slope of y = 0.38x-1.88 (graph shown in Figure 3.10), resulting Tc= 107 °C.

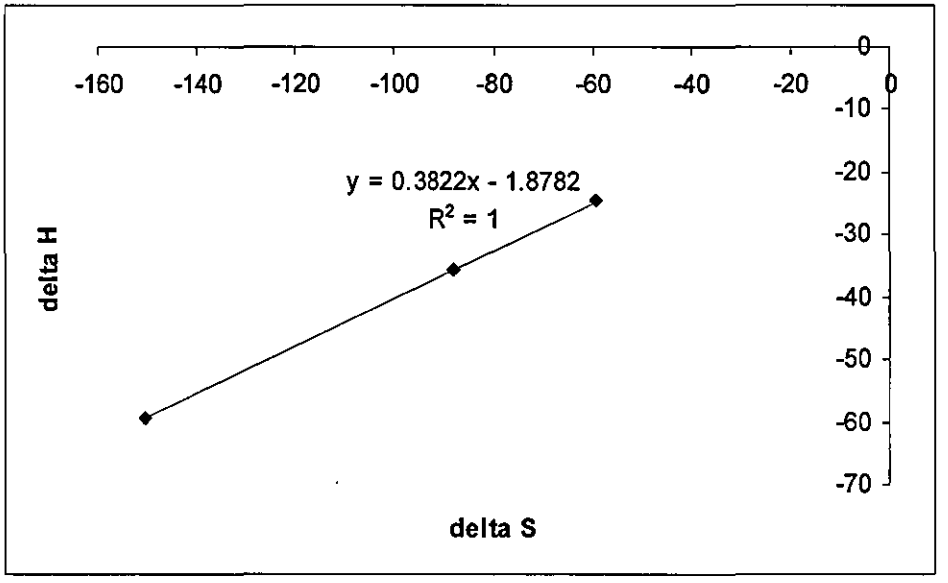


Figure 3.10: Plot of ΔS versus ΔH for peaks 5, 7 and 10, over the 30-75 °C range. LC: HP 1090. UV: 210 nm. Injection volume: 10 µl; OLA solution concentration: 40.8 mg/ml; chromatographic column: Spherisorb C_6 (250 x 4.6) mm, 10 µm; mobile phase: 40% 0.1% formic acid in water/60% 0.1% formic acid in MeCN (isocratic run); flow rate: 1 ml/min.

A compensation temperature of 107 °C, at the conditions stated in Figure 3.6 could lead to critical conditions of elution (i.e. all oligomers elute together as a single peak and the chain length selectivity disappears). However, it was decided not to increase the temperature up to 107 °C because of concern over the stability of the stationary phase.

Temperature-related investigations were also carried out on a second reversed-phase chromatographic system, employing an aqueous/organic eluent.

A Symmetry C_{18} column was used, with a mobile phase consisting of 40% 0.1% formic acid in water/60% 0.1% formic acid in THF. Over the temperature range 30-60 °C, the oligomers of different degrees of polymerisation merged into a single peak, as shown by the mass chromatograms (see examples in Figures 3.11 and 3.16). The mass chromatograms showed that at the front of the main peak a minor peak was present (labelled as 'front' peak in figures below).

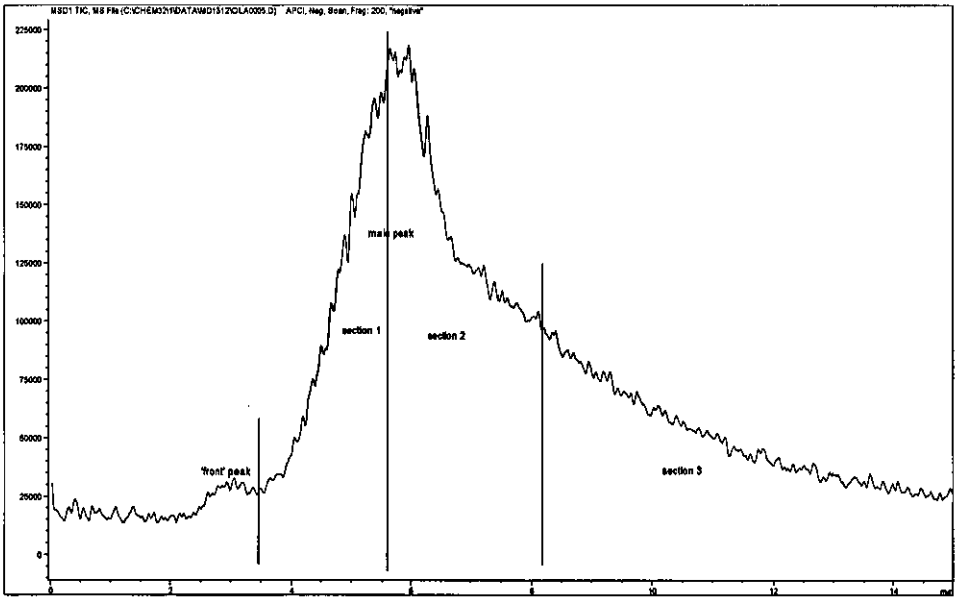


Figure 3.11: Mass chromatogram obtained at 30 °C. Chromatographic conditions: LC-MS: HP 1100, equipped with APCI source. Injection volume: 5 µl; OLA main excipient solution concentration: 520.0 µg/ml; chromatographic column: Symmetry C_{18} (150 x 3.9) mm, 5 µm; mobile phase: 60% 0.1% formic acid in THF/40% 0.1% formic acid in water (isocratic run); flow rate: 0.5 ml/min.

MS data (in negative ionisation mode) identified pr-OLA oligomers and free acid oligomers in the peak at the front of the main peak (see Figure 3.12 for mass spectrum of 'front' peak). Each pr-OLA oligomer and free acid oligomer was identified on the basis of the mass of its $[M - H]^-$ ion, with the aid of Table 3.1.

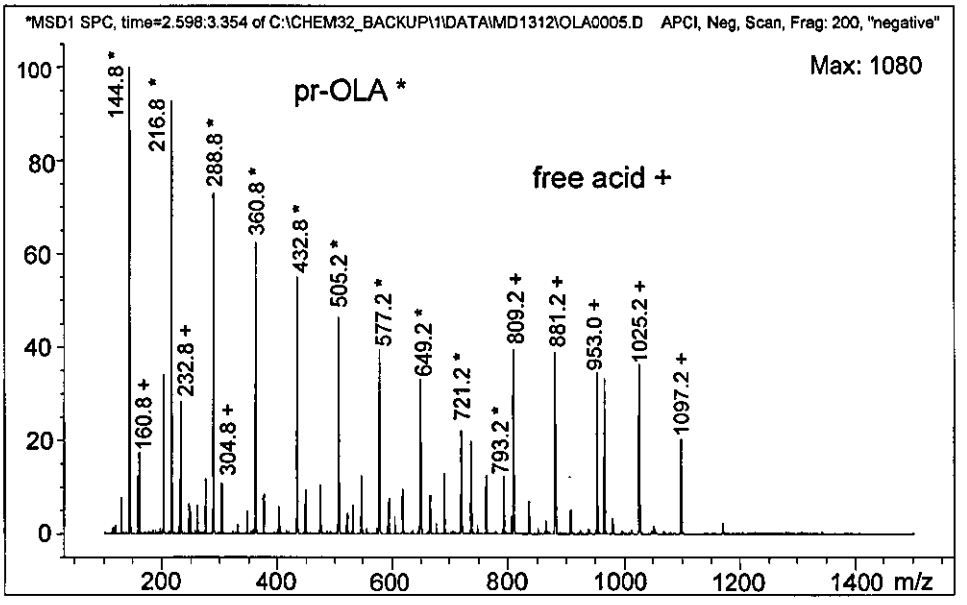


Figure 3.12: Mass spectrum (negative ionisation) obtained at 30 °C, relative to 'front' peak in Figure 3.11 (time: 2.79-3.13 min); OLA main excipient solution concentration: 520.0 µg/ml. Chromatographic conditions as for Figure 3.11.

MS data analysis identified pr-OLA oligomers in the main peak of chromatogram reported in Figure 3.11 (see Figure 3.13 for mass spectrum from the main peak):

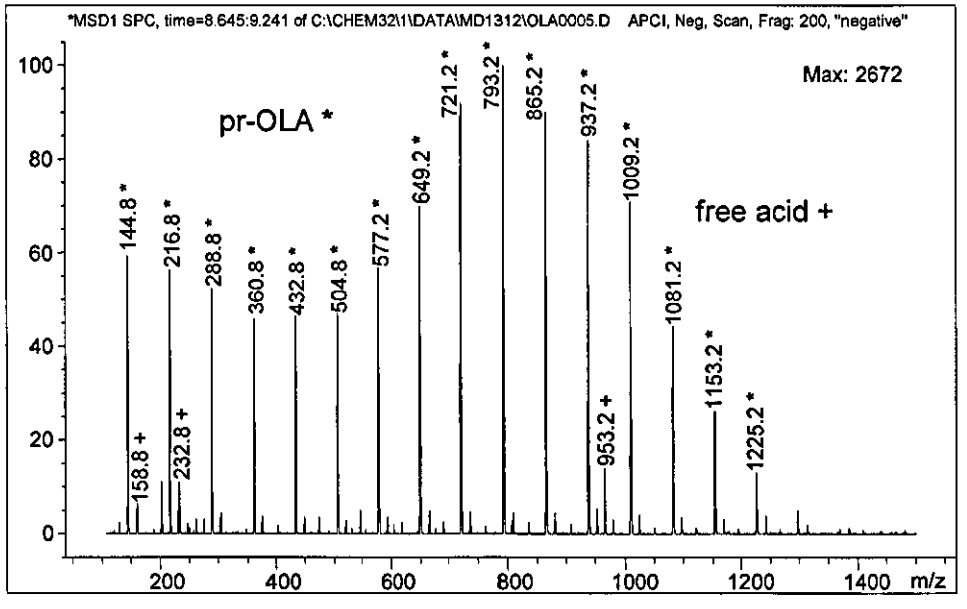


Figure 3.13: Mass spectrum (negative ionisation) obtained at 30 °C, of main peak in Figure 3.11 (time: 8.65-9.24 min); OLA main excipient solution concentration: 520.0 µg/ml. Chromatographic conditions as for Figure 3.11. MS of pr-OLA series, with range of oligomer masses spanning from n = 1 (145) to n = 16 (1225).

Furthermore, MS data (see Figure 3.14) showed the smaller pr-OLA oligomers eluting at the front of the larger pr-OLA oligomers:

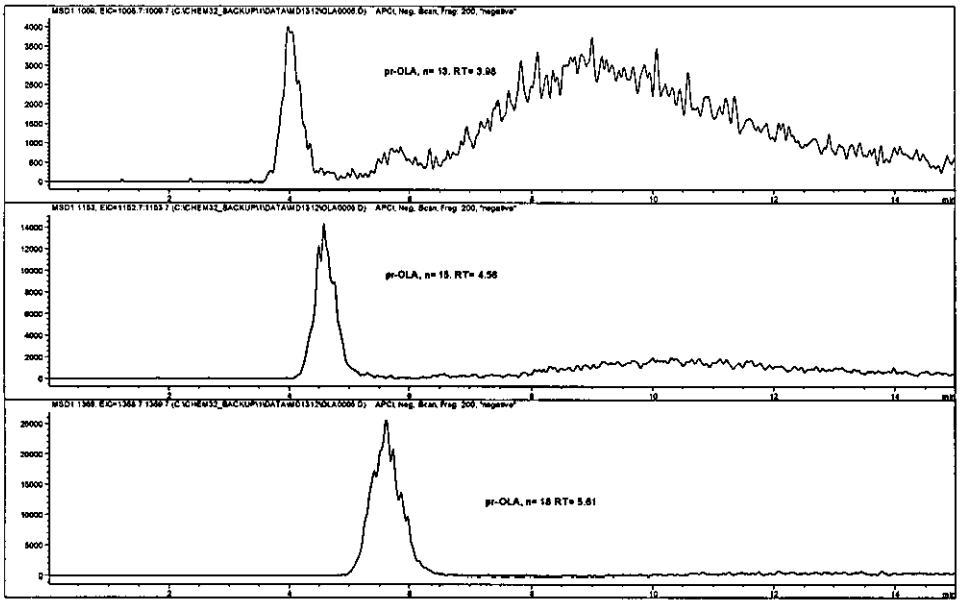


Figure 3.14: EICs corresponding to pr-OLA oligomers, obtained at 30 °C. Chromatographic conditions as for Figure 3.11. OLA solution concentration: 520.0 µg/ml.

Increasing temperature, up to 60 °C led to a sharper chromatography, as shown by Figure 3.15:

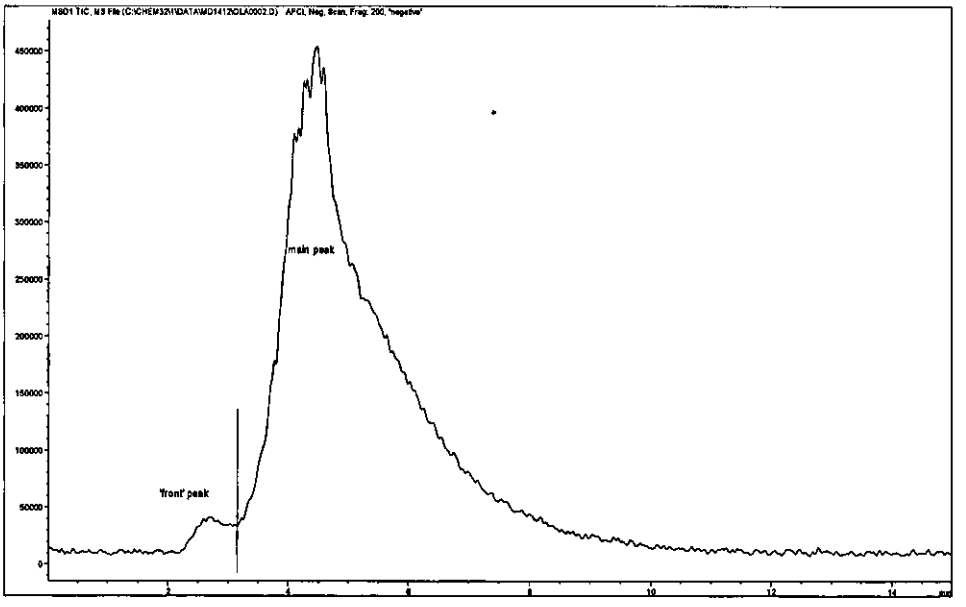


Figure 3.15: Mass chromatogram obtained at 60 °C. Chromatographic conditions as reported in Figure 3.11. OLA solution concentration: 520.0 µg/ml.

However, the oligomeric elution order of pr-OLA was not affected by temperature changes, as shown by EICs obtained at 60 °C, where the smaller oligomers are eluting in front of the larger oligomers:

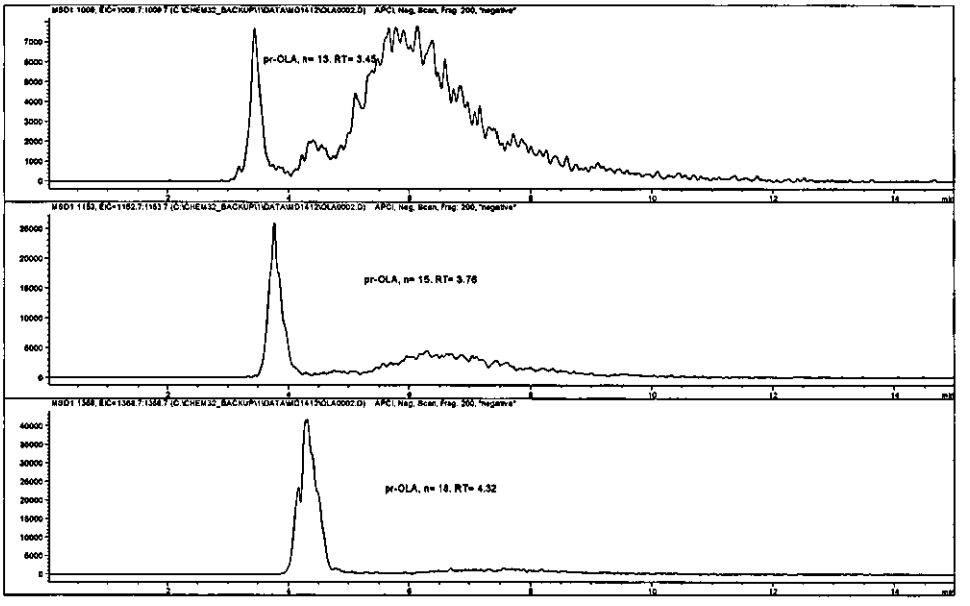


Figure 3.16: EICs relative to pr-OLA oligomers, obtained at 60 °C. Chromatographic conditions as for Figure 3.11. OLA solution concentration: 520.0 µg/ml.

Gradual temperature increases decreased the retention time of pr-OLAs, see Figure 3.17. This is expected reduction in retention with increased temperature.

Data for the C_{18} material were in disagreement with results obtained by Cho *et al.* [86] and Vanhoenacker and Sandra [79] who had suggested changes of elution order of oligomers on temperature variations.

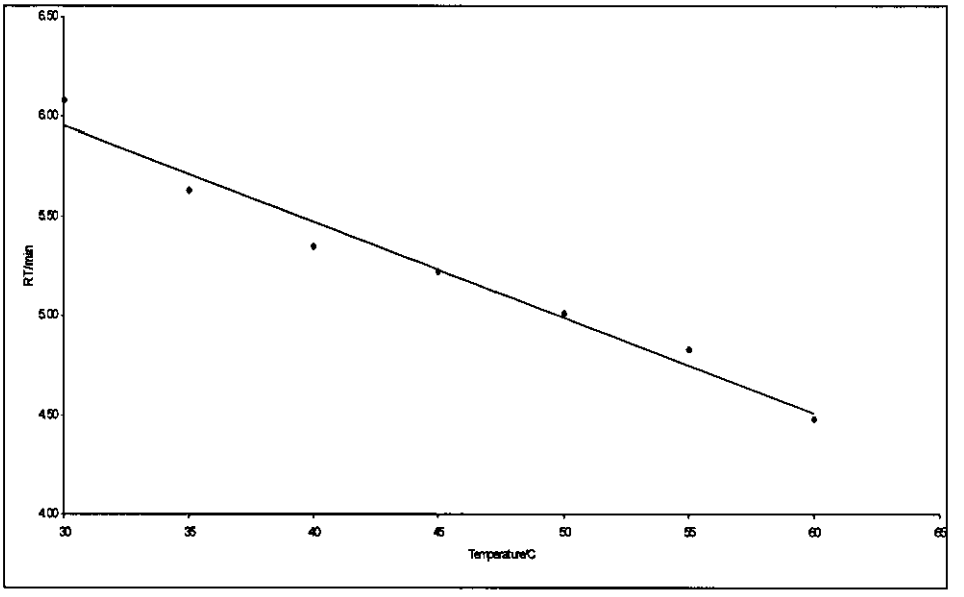


Figure 3.17: Temperature effect on retention of pr-OLAs, over the 30-60 °C range, for a C_{18} column with water/THF mobile phase. OLA main excipient solution concentration: 520.0 µg/ml. Chromatographic conditions as reported in Figure 3.11.

The results of the temperature studies were that although a temperature range up to 75 °C was examined and an attempt to predict whether CC of elution for OLAs could be achieved by means of temperature variations was made, the predicted value was outside the feasible range of operation for the columns that were being used.

3.5. Further investigations into RP-HPLC: effects of mobile phase composition on OLAs retention

Another important parameter that needed consideration was the choice of organic solvent in the mobile phase. Acetonitrile was the first organic solvent chosen. Subsequently it was decided to monitor whether THF/water and acetonitrile (MeCN)/water could have an impact on OLAs retention selectivity, using a Spherisorb C_6 column.

Aqueous THF and aqueous MeCN, on a Spherisorb C_6 column, provided similar retention mechanisms for OLAs dd: reduced retention values with increasing organic percentage (as summarised in Figure 3.18).

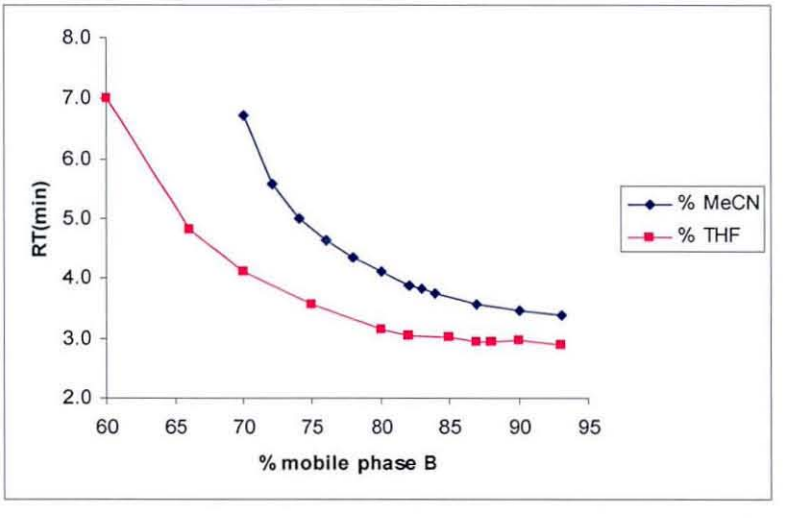


Figure 3.18: Elution of 40.8 mg/ml OLA dd solution; LC: HP 1090; UV: 210 nm; Mobile phase A: 0.1% formic acid in water; Mobile phase B: 0.1% formic acid in THF or in MeCN (delivered in isocratic mode). Injection volume: 25 µl; flow rate: 1 ml/min. Column: Spherisorb C_6 (250 x 4.6) mm, 10µm.

From the above chart it can be said that THF was a stronger eluent than MeCN for OLAs eluting on a C_6 material, as it would be expected from its higher strength in the eluotropic series.

During this experiment, OLA cyclic impurities could not be detected, possibly because these structures do not have chromophores, making UV detection problematic. For this reason ELSD was employed as an alternative form of detection to UV. ELSD is also typically used in polymer and oligomer analysis [98, 247-249]. In the course of this experimental work aqueous methanol was used as eluent.

The analysis of OLAs dd is shown in Figure 3.19:

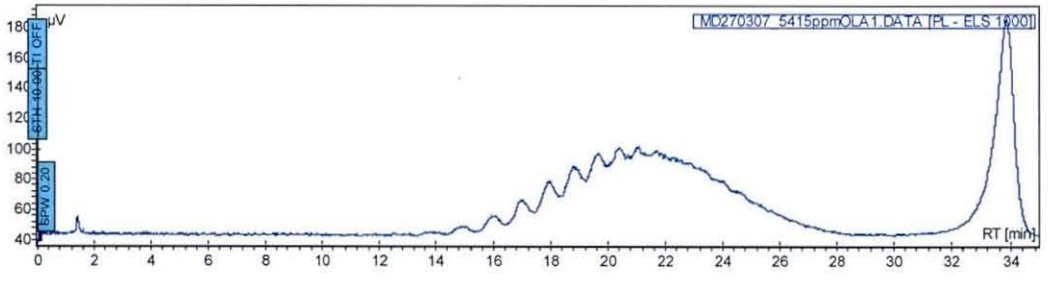


Figure 3.19: Elution of 5415 µg/ml OLA dd solution; LC/ELSD: HP 1100/PL-ELS 1000; Mobile phase A: water; Mobile phase B: MeOH; starting conditions: 60% mobile phase B, taken to 70% in 25 min and then ramped to 100% at 30 min. Injection volume: 10 µl; flow rate: 1 ml/min. Column: Spherisorb C_6 (250 x 4.6) mm, 10µm. ELSD conditions as stated in paragraph 2.2.5.

Although the ELSD conditions were optimised (i. e. flow rate of the carrier gas, temperature of evaporator and temperature of nebuliser), its sensitivity was poor, and a very highly concentrated solution of OLAs dd had to be injected (about 100 times more concentrated than when using UV and 5 times more concentrated than when using MS).

The ELSD response to the OLA cyclic impurities was non-existing, regardless of the efforts made towards the optimisation of the detector conditions.
A typical profile obtained upon injection of cyclic solution is presented in Figure 3.20:

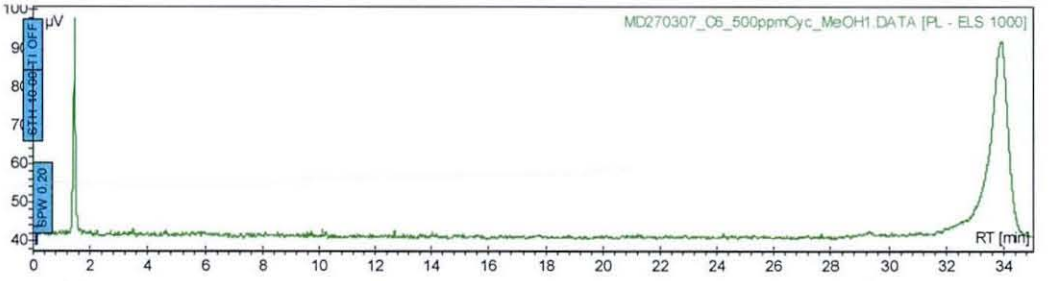


Figure 3.20: Elution of 300 µg/ml OLA cyclic solution; LC/ELSD: HP 1100/PL-ELS 1000; Mobile phase A: water; Mobile phase B: MeOH; starting conditions: 60% mobile phase B, taken to 70% in 25 min and then ramped to 100% at 30 min. Injection volume: 10 µl; flow rate: 1 ml/min. Column: Spherisorb C_6 (250 x 4.6) mm, 10µm. ELSD conditions as stated in paragraph 2.2.5.

In a study carried out by Trathnigg *et al.* [250], it was demonstrated that lower oligomers of fatty alcohol ethoxylates ($n < 4$) had very small response factors and lower fatty alcohols ($< C_{14}$) were not detected at all. This could have been due to the ELSD design, as more modern systems can handle fairly volatile analytes. Given the PL-ELS 1000 used in the current study and the vaporisation temperature (95 °C) needed to evaporate the eluent, it could have been possible that this system could not handle the volatile cyclic OLAs.

3.6. Conclusions on the oligomeric distribution approach of OLAs by RP-HPLC

The first dimension separation of OLAs on the basis of chain length was accomplished in RP-HPLC, using a C_6 stationary phase and an aqueous acetonitrile mobile phase. The oligomers elution was in terms of increasing size: smaller oligomers eluted before larger oligomers and the order was unaffected by the analytical temperature. As temperature increased, a decrease in retention of all OLAs oligomers was observed. Similar results were found on a C_{18} column.

THF and MeCN were compared, as organic components of mobile phases for eluting OLAs; this comparison resulted in no changes in order of elution. From this investigation it can be said that THF was a stronger eluent than MeCN for OLAs eluting on a C_6 material.

4. Results and Discussion: the head group distribution approached by LCCC

The next step towards a two-dimensional analysis of OLAs was the investigation of the head/tail group distribution by LCCC.

If the critical conditions (LCCC) (see section 1.2.6) for an oligomeric series can be achieved then the effect of differences in the oligomeric chain length will be negligible and all oligomers with the same functionality should elute together, so that compounds with different functionalities are separated.

4.1. LCCC method development

4.1.1. Experimental approach in LCCC

The following aspects were examined to achieve LCCC:

- Effects of column temperature;
- Effects of stationary phase;
- Effects of mobile phase;
- Effects of buffer salt concentration.

Following the approach of Cools *et al.* [168] the retention times of three OLAs fractions (from Section 3.3.1, containing predominately n = 8, 13, and 18), were measured on a Spherisorb C_6 column with different eluents starting from 60 % (0.1% TFA in MeCN) and adding an increasing percentage of 0.1% TFA in water

(namely the 'non-solvent'). After each change in percentage of non-solvent the system was equilibrated by eluting mobile phase at 1 ml/min for about 30 minutes.

By plotting the retention time versus percentage of non-solvent (water), for each fraction, the predicted critical point was calculated as the intersection point of the three curves (see figure below). This is the composition at which the three oligomers would be predicted to have the same elution time.

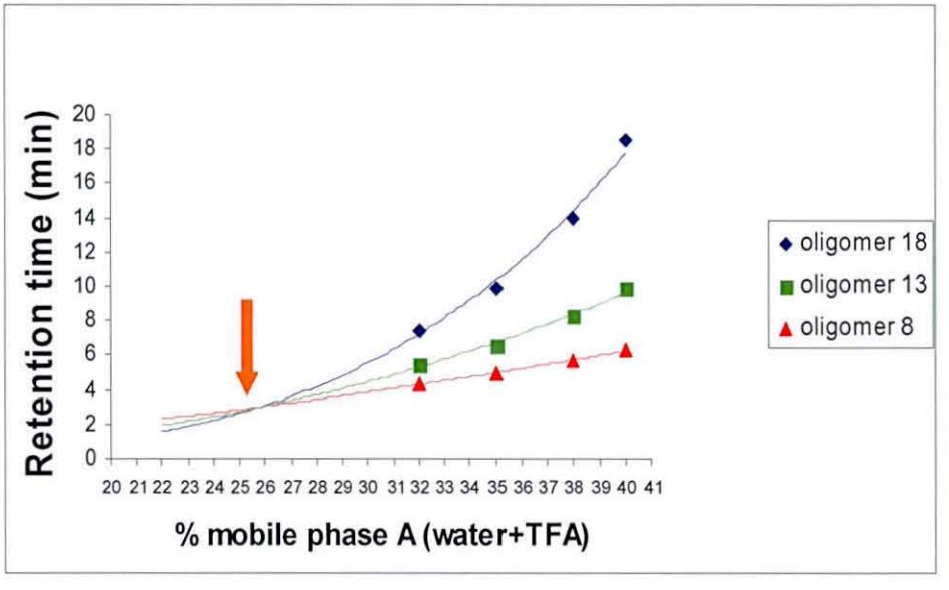


Figure 4.1: Elution of three OLA oligomers at different mobile phase composition; Spherisorb C_6 column (250 x 4.6) mm, 10 µm; water/MeCN mobile phase; Injection volume: 10 µl. UV: 210 nm; flow rate: 1 ml/min. LC-MS: HP1100, equipped with ESI source. The arrow indicates the composition at which the three oligomers are predicted to have the same elution time.

The intersection point corresponded to 26% mobile phase A, but the corresponding retention time was approximately 3 min, which was very close to that of the void volume of t_0= 3.16 min (retention time for a 10 µl injection volume of 64 µg/ml uracil solution). This means that under these conditions the three oligomers 8, 13 and 18 are not retained, indicating that this approach is probably not suitable for oligomers of relatively low molecular mass, such as OLAs; it was however

examined in a little more detail as it might have produced separation from the different head groups.

4.1.2. LCCC: from method development to preliminary investigations

In an attempt to separate OLAs from their cyclic impurities, some preliminary experiments were run with the dd (diamide diester) oligomeric mixture and the cyclic impurities mixture (injected in separate runs), at the critical conditions extrapolated from Figure 4.1. The chromatographic system represented by the C_6 column with 26% mobile phase A (0.1% TFA in water) and 74% mobile phase B (0.1% TFA in acetonitrile), caused oligomeric co-elution of the OLAs dd at 5.6 min, and oligomeric co-elution of their cyclic impurities at 5.5 min. Fig 4.2 represents single ion chromatograms, showing three representative oligomers of each material with equal retention values.

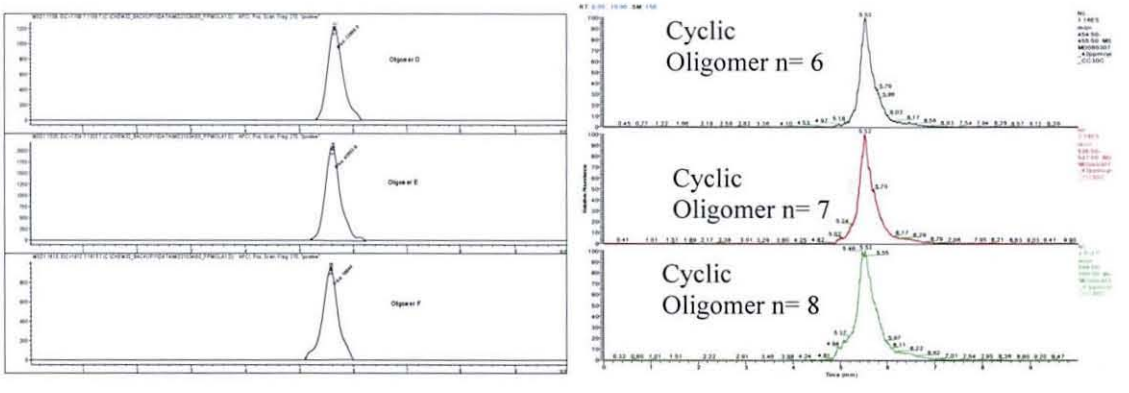


Figure 4.2: single ion chromatograms (SIC), obtained on LC-MS: HP1100, equipped with APCI source (on the left) and LC-MS: Thermo Finnigan LTQ, equipped with ESI source (on the right). Injection volume: 10 µl; OLAs dd excipient solution concentration: 1082.0 µg/ml, SIC on the left; OLA cyclic solution concentration: 42 µg/ml, SIC on the right; chromatographic column: Spherisorb C_6 (250 x 4.6) mm, 10 µm; 26% mobile phase A (0.1% TFA in water), 74% mobile phase B (0.1% TFA in MeCN); LC flow rate: 0.5 ml/min.

The three dd oligomers, represented on the left-hand side of Figure 4.2 were identified on the basis of Table 3.1 and the following:

1109 = $[dd + H]^+$, corresponded to diamide diester n = 13 (labelled oligomer D in Figure 4.2);
1325 = $[dd + H]^+$, corresponded to diamide diester n = 16 (labelled oligomer E in Figure 4.2);
1613 = $[dd + H]^+$, corresponded to diamide diester n = 20 (labelled oligomer F in Figure 4.2).

The three cyclic oligomers, represented on the right-hand side of Figure 4.2 were identified on the basis of Table 3.1 and the following:

455 = $[cyc + 23]^+$, corresponded to cyclic n = 6;
527 = $[cyc + 23]^+$, corresponded to cyclic n = 7;
599 = $[cyc + 23]^+$, corresponded to cyclic n = 8

At the conditions reported above, the dd excipients and their cyclic impurities could not be resolved. Other parameters that could affect selectivity and that could lead to OLAs separation in terms of their head groups were therefore investigated. In the next section, the effects of stationary phase and mobile phase on OLAs functional groups separation selectivity will be investigated.

4.2. Discussion of the effects of stationary phase, column temperature and mobile phase polarity on OLAs functional groups selectivity

Because there was little retention at the critical conditions on the hydrophobic C_6 material, alternative selectivities were examined on amino and cyano phases, materials traditionally used in NP-HPLC and hydrophilic interaction chromatography (HILIC). Since HILIC has been shown to be successful for the study of polar compounds [120] and since the aim of the current study was to target the polar functional groups of OLAs, it appeared that polar columns might lead to suitable retentions. HILIC and NP-HPLC mobile phases systems were therefore examined at different temperatures to see if critical conditions could be obtained.

4.2.1. Retention of OLAs on cyano and amino materials in NP-HPLC systems: a quest for LCCC

The first aspect was attempted to find the critical solvent composition in NP-HPLC systems, which could result in the separation of functional amides and their non-functional amide cyclic components. A range of organic solvents was considered, on the basis of their different influences on solute-solvent interactions; subsequently, the combined effect of the mobile phase polarity and the solvation of OLAs offered by aqueous/organic solvents was examined. Acetone, THF and n-hexane were considered because of their differences in dipole moment (see table below for polarity values) and therefore differences in selectivity.

Table 4.1: List of solvents and their dipole moment and dielectric constant values. Taken from http://www.usm.maine.edu

Name	Dipole moment	Dielectric constant
water	1.85	80
MeCN	3.92	36.6
Acetone	2.88	21
THF	1.63	7.52
hexane	...	2.02

Additionally, temperature can reduce the selectivity between oligomers with different chain lengths. At the 'critical' temperature no oligomeric separation takes place and functional entities can be differentiated. In an attempt to find 'critical' temperature, the column temperature was gradually increased and changes in the retention and elution order of OLA dd excipients were monitored. The stationary phases employed were a polar amino material (30-80 °C temperature range used) and a silica sorbent (30-55 °C temperature range used), typical NP-HPLC materials. Non-aqueous organic solvents were used as eluents.

4.2.1.1. Cyano bonded material

Separation between OLA diamide diester (dd) excipients and cyclics, was examined in terms of the number of head-groups (where the cyclics do not have head groups) on a cyano bonded stationary phase. The starting mobile phase was 100% acetonitrile. The mobile phase polarity was altered by gradually introducing (in isocratic runs) respectively 10, 20 and 30% of n-hexane, in an attempt to find the critical solvent composition and cyclic and diamide diester components were identified on the basis of their mass spectra. At 100% acetonitrile and 0.1% formic acid, cyclics and dd excipients co-eluted, at approximately 5.5 min, (Figure 4.3, representing the overlaid chromatograms, with cyclics labelled as 'cyc' and diamide diester labelled as 'dd'):

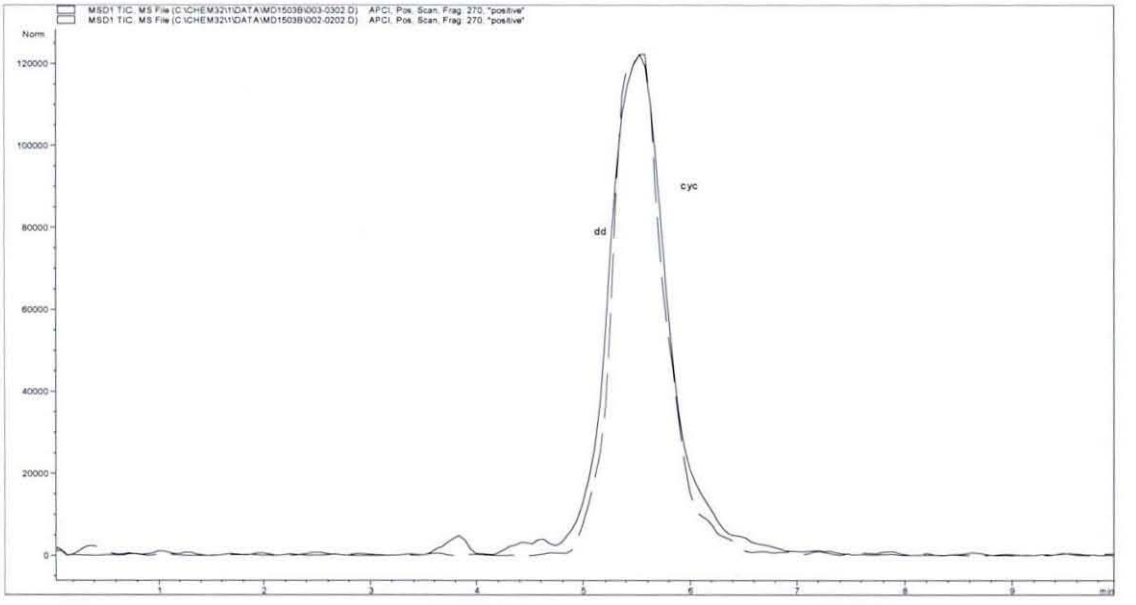


Figure 4.3: Overlaid mass chromatograms, obtained with Zorbax cyano column (250 x 4.6) mm, 7µm, at 100% 0.1% formic acid in MeCN (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 50 µg/ml. Retention time: 5.5 min. LC-MS: HP1100, equipped with APCI source.

At 90% acetonitrile/10% n-hexane, the two components were not completely overlapped, with cyclics eluting at 5.51 min and dd eluting at 5.80 min (Figure 4.4).

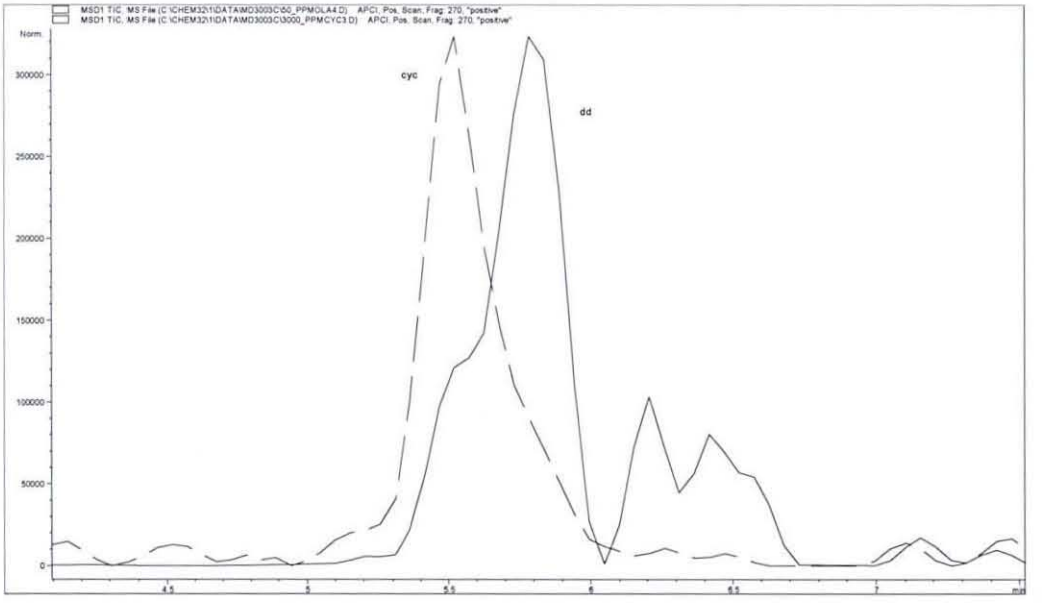


Figure 4.4: Overlaid mass chromatograms, obtained with Zorbax cyano column (250 x 4.6) mm, 7µm, at 90% 0.1% formic acid in MeCN/10% n-hexane (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 50 µg/ml. LC-MS: HP1100, equipped with APCI source.

At 80% acetonitrile/20% n-hexane, the resolution of cyclics and dd excipients decreased (Figure 4.5), with retention values of 5.82 min for dd and 5.02 min for cyclics. The peak due to the cyclic component showed pronounced tailing, and therefore having a detrimental effect on the resolution. At a later stage in the overall study it was discovered that the tailing was possibly due to the fact that the cyclic material consists of cyclics and long chain free acid OLAs [3]. This aspect will be discussed more in detail in chapter 6.

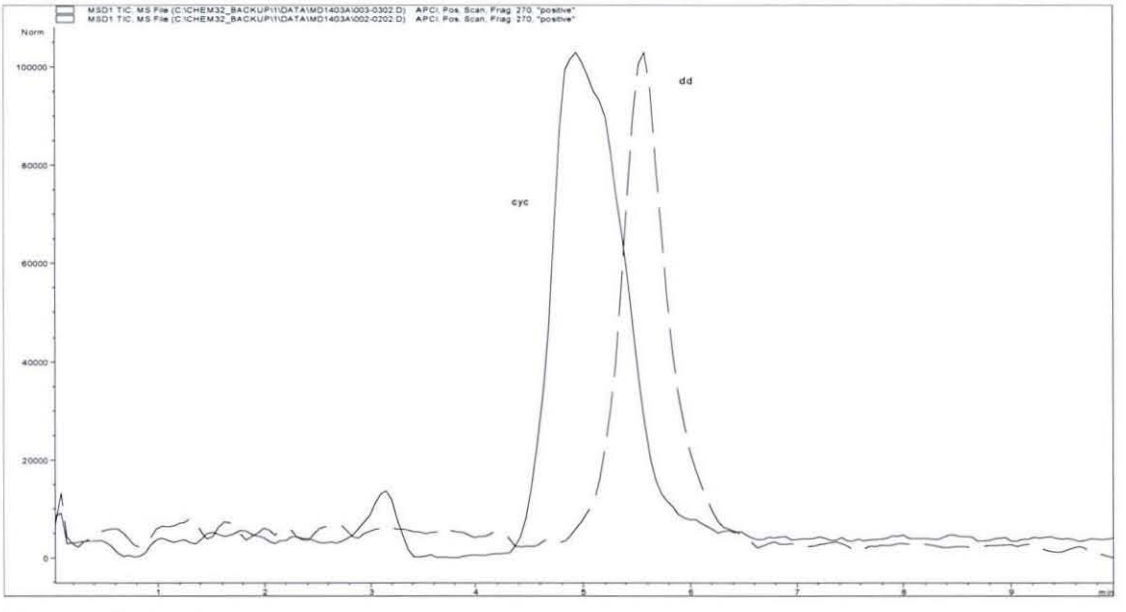


Figure 4.5: Overlaid mass chromatograms, obtained with Zorbax cyano column (250 x 4.6) mm, 7µm, 20% n-hexane, 80% 0.1% formic acid in MeCN (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 50 µg/ml. Dd retention time: 5.82 min, cyclic retention time: 5.02 min. LC-MS: HP1100, equipped with APCI source.

At 70% acetonitrile/30% n-hexane, a very poor chromatography was obtained, with very broad dd (RT= 6.24 min) and cyclics peak (RT= 5.35 min), as shown in Figure 4.6. The observed 'poor-quality' for the total ion chromatograms (TICs), could be due to a decrease in the ionisation efficiency with increased modifiers; a similar effect was reported by Montaudo and Lattimer for polymer analysis [183].

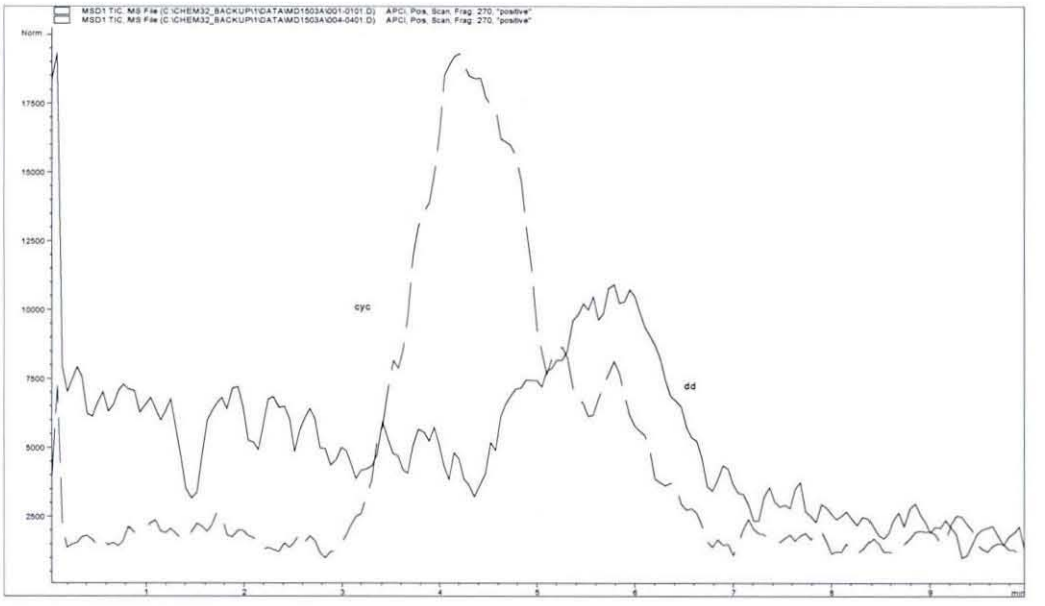


Figure 4.6: Overlaid mass chromatograms, obtained with Zorbax cyano column (250 x 4.6) mm, 7µm, 30% n-hexane, 70% 0.1% formic acid in MeCN (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 50 µg/ml. LC-MS: HP1100, equipped with APCI source.

These changes in mobile phase polarity did not lead to a 'systematic trend' in terms of retention selectivity for cyclics and diamide diester components. However, the cyclic impurities were consistently eluting in front of the dd. MS monitoring of elution profiles of individual OLA oligomers was used to investigate the effect of the chain lengths of the OLAs on the elution behaviour.

At 100% acetonitrile the longer chains were eluted in front of smaller chains; this elution order was unaffected by n-hexane addition. Figure 4.7 and Figure 4.8 show the elution order for dd and cyclic oligomers, respectively, at 80% acetonitrile 20% n-hexane.

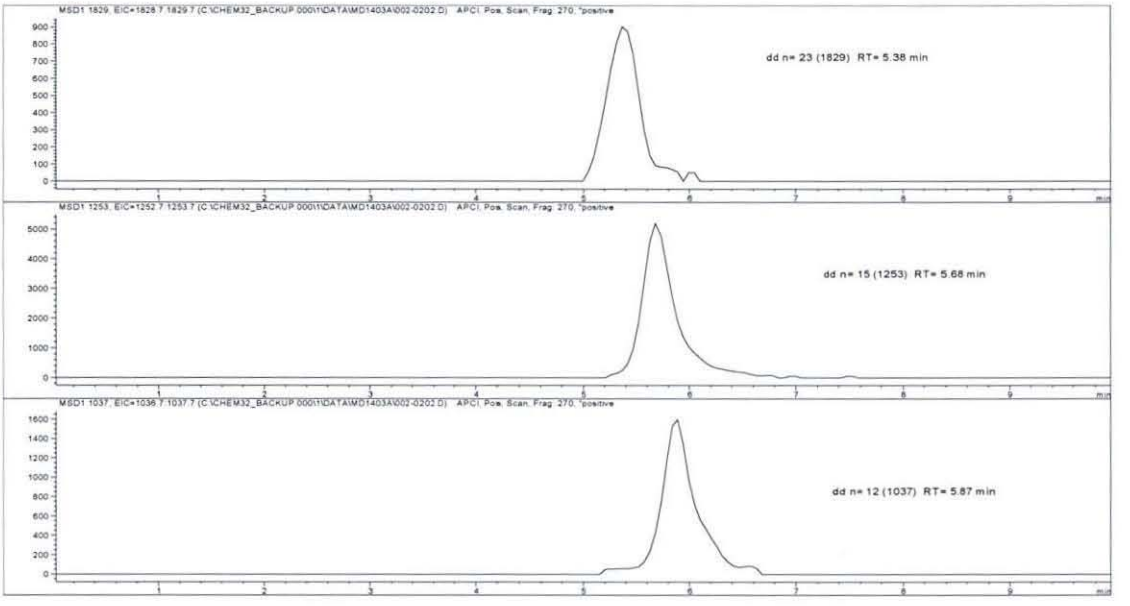


Figure 4.7: EIC (positive mode) for 50 mcg/ml OLA dd solution relative to Zorbax cyano column (250 x 4.6) mm, 7µm, 20% n-hexane/80% 0.1% formic acid in MeCN. LC-MS: HP1100, equipped with APCI source. Injection volume: 20 µl; flow rate: 0.5 ml/min. EICs corresponding to dd, n = 23, 15 and 12.

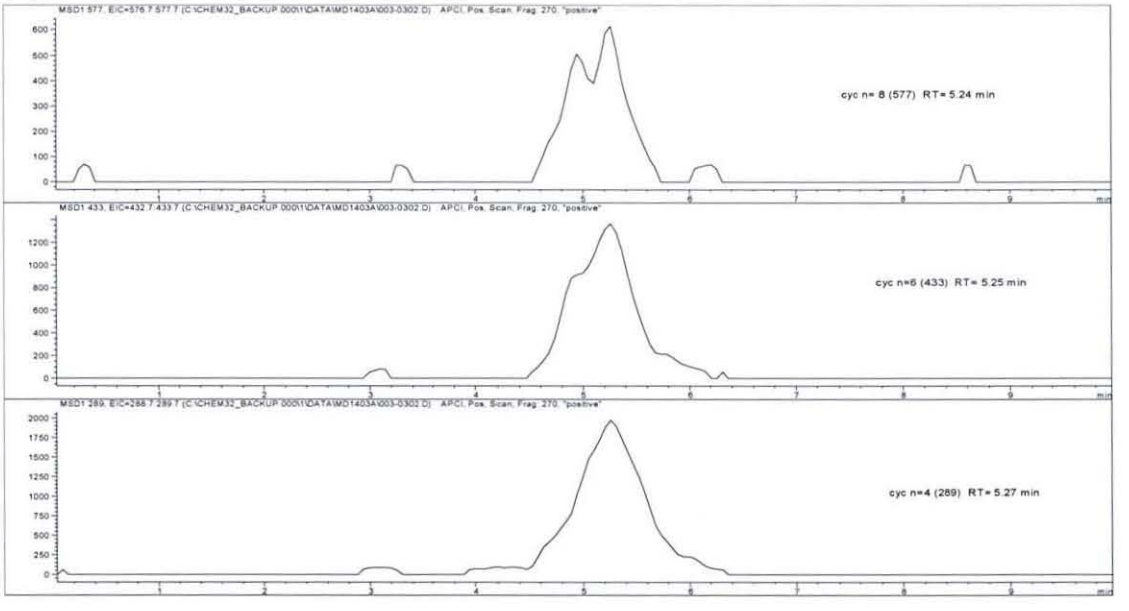


Figure 4.8: EIC (positive mode) for 3000 mcg/ml OLA cyclic solution relative to Zorbax cyano column (250 x 4.6) mm, 7µm, 20% n-hexane/80% 0.1% formic acid in MeCN. LC-MS: HP1100, equipped with APCI source. Injection volume: 20 µl; flow rate: 0.5 ml/min. EICs corresponding to cyc, n = 8, 6 and 4.

From the selected mass chromatograms reported above it was also observed that the various oligomers had similar, but not exactly the same, retention times. In order to further confirm the oligomeric suppression, selected mass chromatograms of 7 sequential dd oligomers were measured (Figure 4.9).

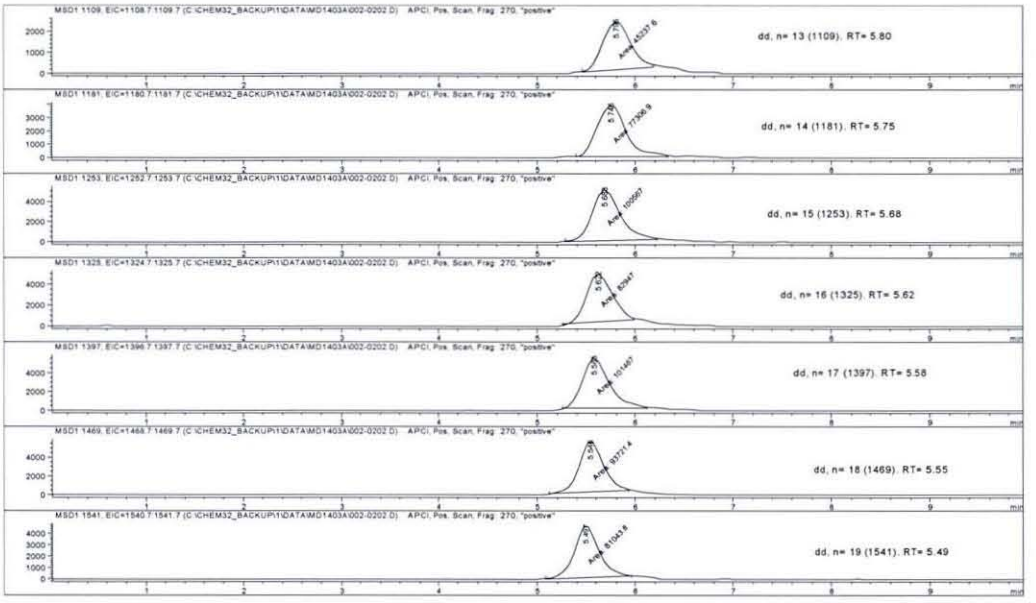


Figure 4.9: EIC (positive mode) for 50 mcg/ml OLA solution relative to Zorbax cyano column (250 x 4.6) mm, 7µm, 20% n-hexane/80% 0.1% formic acid in MeCN. LC-MS: HP1100, equipped with APCI source. Injection volume: 20 µl; flow rate: 0.5 ml/min. EICs corresponding to dd, n = 13, 14, 15, 16, 17, 18 and 19.

It can be seen that the retention time of the selected oligomers decreased slightly with increasing molecular weight (from 5.80 min to 5.50 min for diamide diester with n increasing from 13 to 19). This variation in retention values indicated a slight adsorption effect of OLA dd excipients, so that the conditions of elution were not perfectly critical. This was also true for the cyclic components (Figure 4.10). The retention times of the selected oligomers decreased slightly with increasing molecular weight (from 5.33 min to 5.06 min with n increasing from 2 to 7). A decrease in retention with increasing chain length was also observed by Jandera *et al.* [92] when analysing ethoxylated alcohols on silica and amino columns with 2-propanol in n-hexane as mobile phase

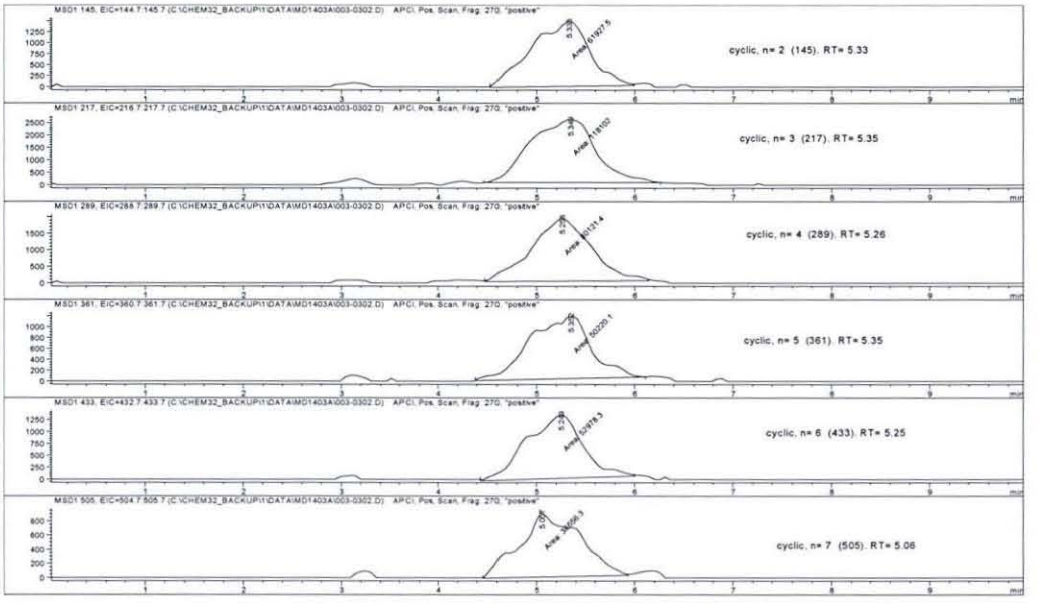


Figure 4.10: EIC (positive mode) for 3000 mcg/ml cyclic solution relative to Zorbax cyano column (250 x 4.6) mm, 7µm, 20% n-hexane/80% 0.1% formic acid in MeCN. LC-MS: HP1100, equipped with APCI source. Injection volume: 20 µl; flow rate: 0.5 ml/min. EICs corresponding to cyc, n = 2-7.

The conclusion from this approach on the cyano column was that although near to critical conditions were obtained, there was incomplete separation of the dd and cyc oligomers on the cyano column with acetonitrile/n-hexane; an amino column was then examined.

4.2.1.2. Amino bonded material

Separation between OLAs main oligomers and cyclics, in terms of their head-groups was investigated in a chromatographic system consisting of an amino stationary phase. The starting mobile phase was 100% acetonitrile. The mobile phase polarity was altered by gradually introducing (in isocratic runs) respectively 10, 20 and 30% of n-hexane. Standard solutions consisting of OLA dd oligomers (diamide diester) and their cyclic impurities were injected separately in individual runs

At 100% acetonitrile, the dd oligomers eluted after 5.87 min, and the cyclics retention time was 5.52 min, resulting in partial overlapping of the two peaks, as shown in Figure 4.11:

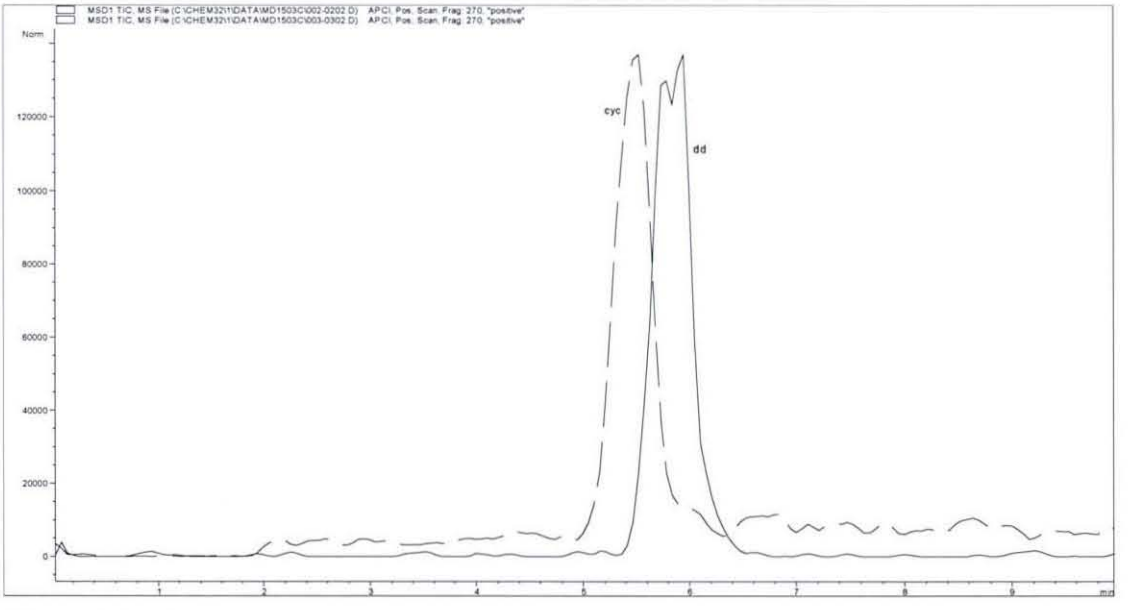


Figure 4.11: Overlaid mass chromatograms, with cyclics labelled 'cyc' and diamide diester labelled 'dd', obtained with the Spherisorb amino column (250 x 4.6) mm, 5 µm, at 100% 0.1% formic acid in MeCN (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 50 µg/ml. LC-MS: HP1100, equipped with APCI source.

At 90% acetonitrile/10% n-hexane, cyclics eluted at 5.51 min and dd oligomers eluted at 6.19 min (Figure 4.12), with resolution, Rs, of 0.8.

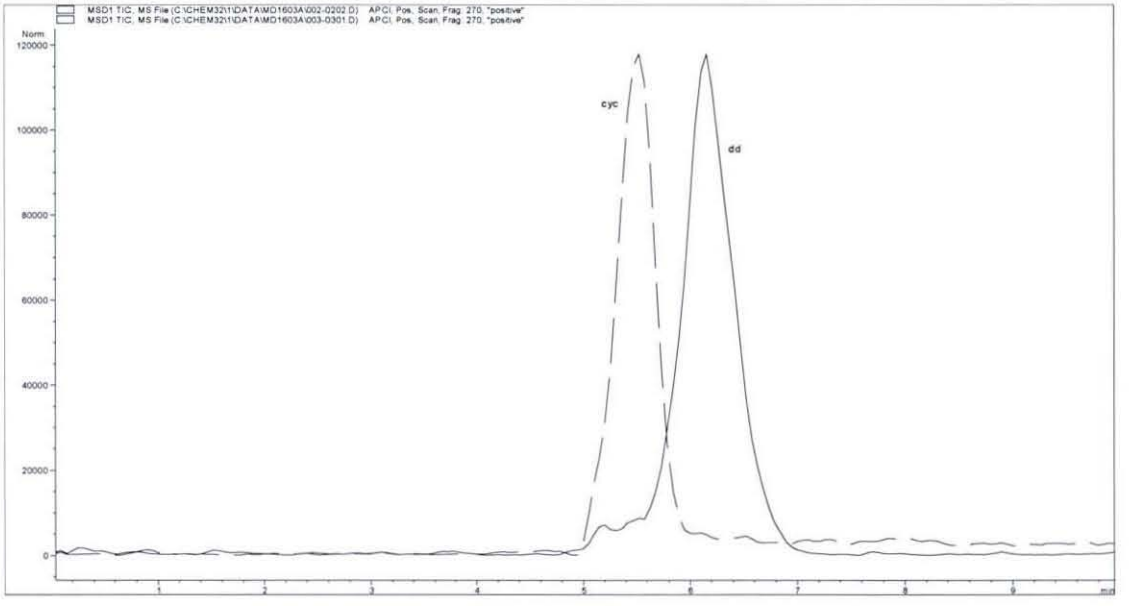


Figure 4.12: Overlaid mass chromatograms of dd and cyc oligomers, obtained with the Spherisorb amino column (250 x 4.6) mm, 5 µm, at 10% n-hexane/90% 0.1% formic acid in MeCN (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 50 µg/ml. LC-MS: HP1100, equipped with APCI source.

Further increase of n-hexane percentage in the mobile phase to 20% lead to a further decrease in retention times: the dd oligomers (5.82 min), and the cyclics (5.37 min), but no improvement in the resolution (see Figure 4.13).

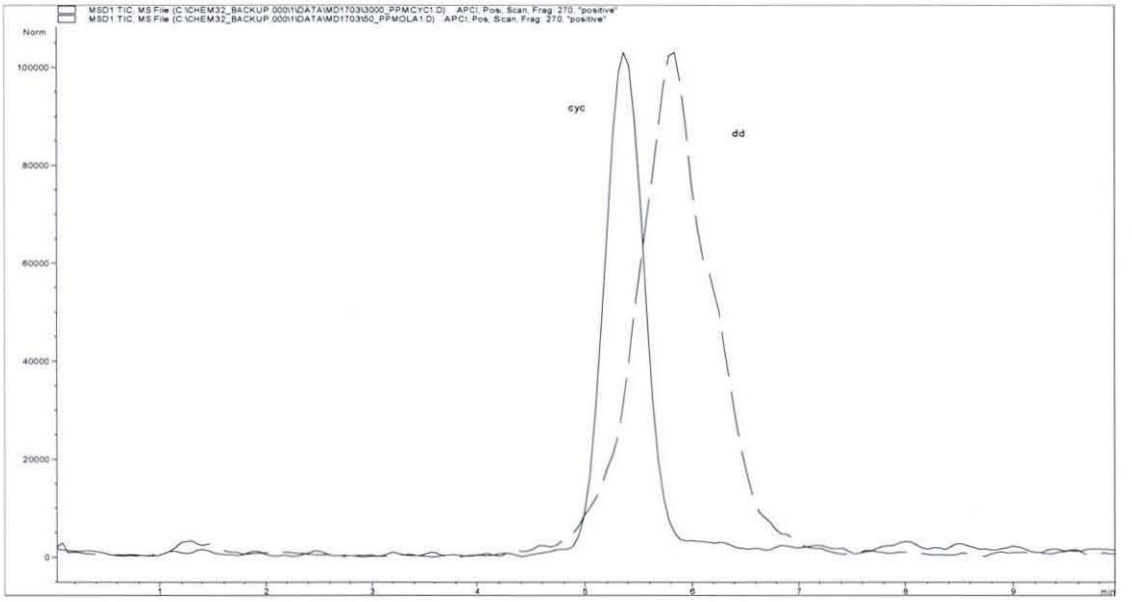


Figure 4.13: Overlaid mass chromatograms of dd and cyc oligomers, on a Spherisorb amino column (250 x 4.6) mm, 5 µm, at 80% 0.1% formic acid in MeCN/20% n-hexane (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 50 µg/ml. LC-MS: HP1100, equipped with APCI source.

The further increase to 30% n-hexane caused the two components to elute at 6.18 min (dd) and 5.42 min (cyc), but also to broaden and to reduce the resolution (Figure 4.14):

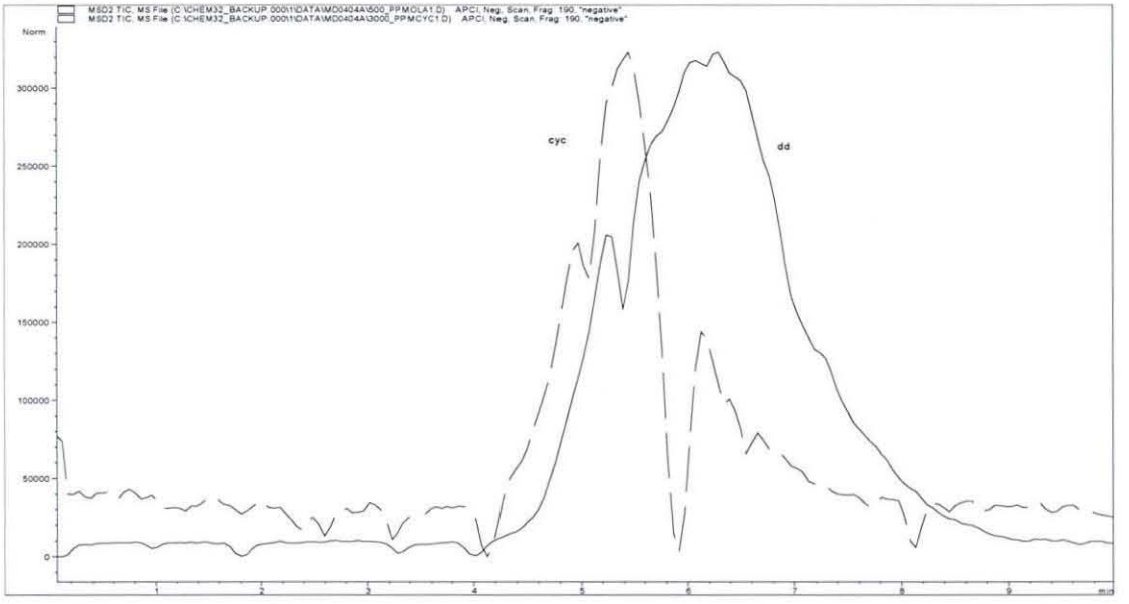


Figure 4.14: Overlaid mass chromatograms of dd and cyc oligomers, obtained with the Spherisorb amino column (250 x 4.6) mm, 5 µm, at 70% 0.1% formic acid in MeCN/30% n-hexane (negative ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 50 µg/ml. LC-MS: HP1100, equipped with APCI source.

The decrease of mobile phase polarity (brought about by n-hexane addition to acetonitrile) did not lead to a 'trend' in terms of retention selectivity for cyclics and diamide diester components. However, the cyclic impurities were consistently eluting in front of the dd, which led to the conclusion that the dd oligomers were more polar than the cyclic impurities.

MS analysis allowed the elution order of individual diamide diester and cyclic oligomers to be monitored. With the mobile phase of 100% acetonitrile, in both cases, longer chains were eluted before shorter chains (Figure 4.15 and Figure 4.16).

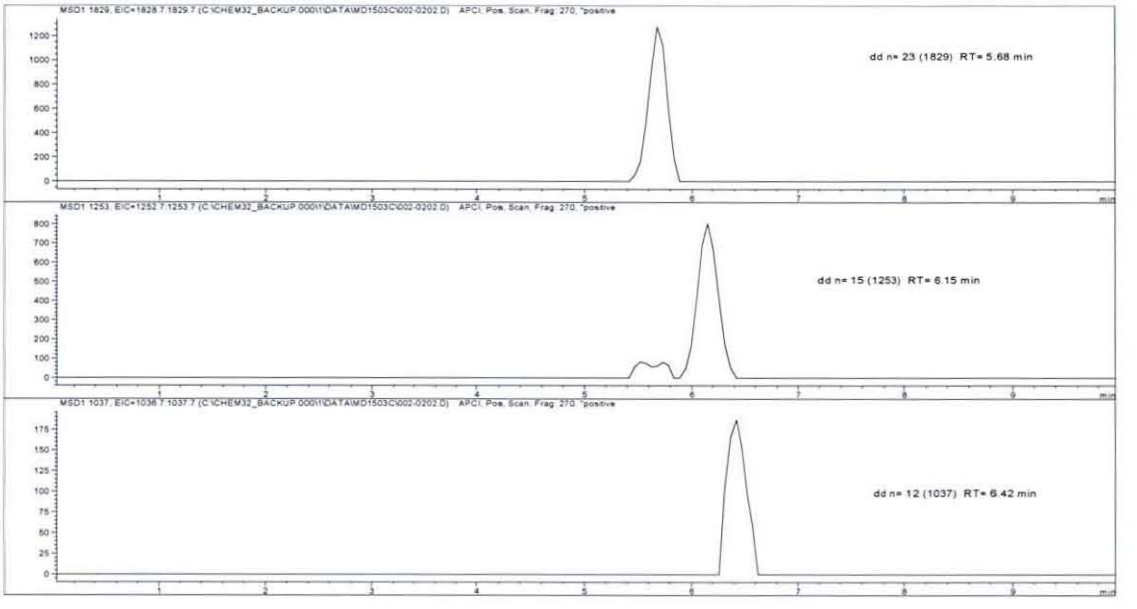


Figure 4.15: EICs (positive mode) for 50 mcg/ml OLA dd excipient solution on a Spherisorb amino column (250 x 4.6) mm, 5 µm, 100% 0.1% formic acid in MeCN, (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. LC-MS: HP1100, equipped with APCI source.

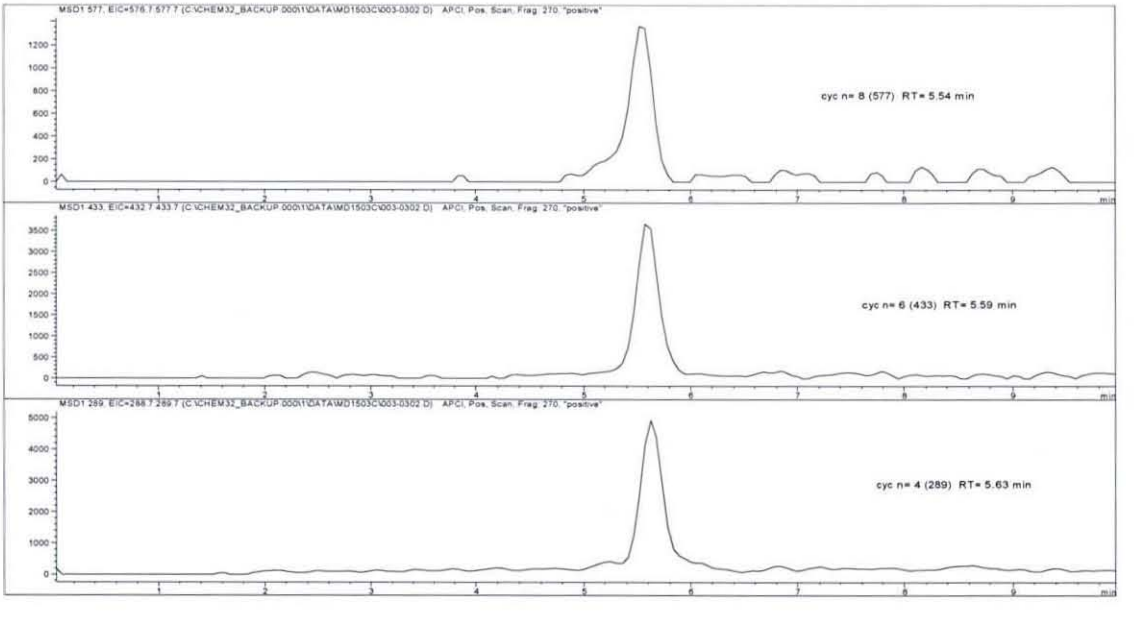


Figure 4.16: EICs (positive mode) for 50 mcg/ml OLA cyclic solution relative to Spherisorb amino column (250 x 4.6) mm, 5 µm, 100% 0.1% formic acid in MeCN, (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. LC-MS: HP1100, equipped with APCI source.

The same phenomenon was observed at 90% acetonitrile/10% n-hexane, with larger oligomers eluting before smaller oligomers. The mass dependent elution behaviour of OLAs was not affected by addition of n-hexane (up to 30%) to acetonitrile in the mobile phase. From the extracted ion chromatograms (EICs) relative to OLAs dd excipient (Figure 4.17), it can be seen that the retention time of the selected oligomers decreased slightly with increasing molecular weight (from 6.57 min to 6.09 min for diamide diester with n increasing from 13 to 19).

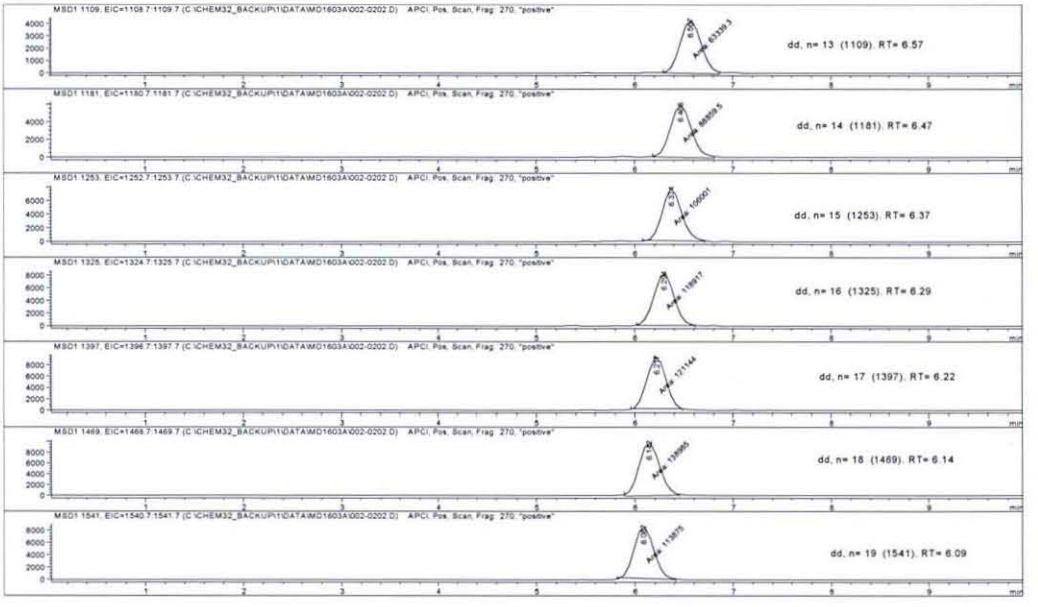


Figure 4.17: EIC (positive mode) for 50 mcg/ml OLA solution relative to Spherisorb amino column (250 x 4.6) mm, 5 µm. 10% n-hexane/90% 0.1% formic acid in MeCN Injection volume: 20 µl; flow rate: 0.5 ml/min. LC-MS: HP1100, equipped with APCI source.

This variation in retention values indicated a slight adsorption effect of OLA main excipients, so that the conditions of elution were not perfectly critical. This was also true for the cyclic components. From the extracted ion chromatograms (EICs) relative to OLAs cyclic impurities (Figure 4.18), it can be seen that the retention time of the selected oligomers decreased slightly with increasing molecular weight (from 5.70 min to 5.62 min with n increasing from 2 to 7).

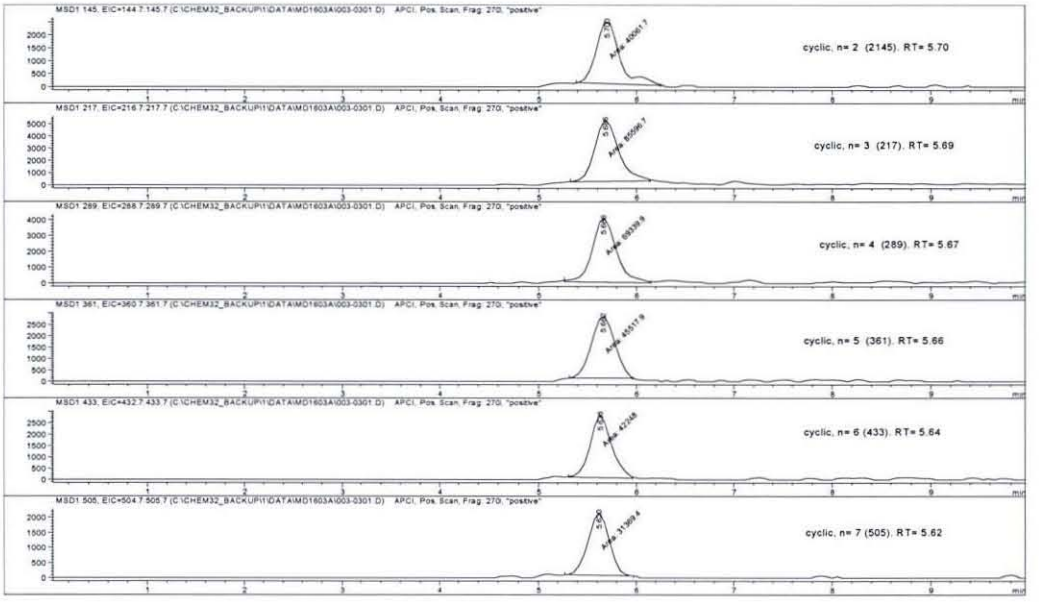


Figure 4.18: EIC (positive mode) for 3000 mcg/ml cyclic solution relative to Spherisorb amino column (250 x 4.6) mm, 5 µm. 10% n-hexane/90% 0.1% formic acid in MeCN Injection volume: 20 µl; flow rate: 0.5 ml/min. LC-MS: HP1100, equipped with APCI source.

In the light of the results obtained using the amino material and n-hexane/acetonitrile as mobile phase, it was concluded that this approach would not lead to a suitable system for OLAs characterisation. Although separation between main excipient and cyclics, in terms of their head-groups took place, the two peaks were insufficiently resolved and the LC conditions were not 'perfectly critical'.

4.2.1.2.1. The effect of coupling two amino columns on resolution of OLAs

In order to increase the resolution between the two groups, two amino bonded columns were coupled together in an attempt to maximize the resolution between the diamide diester and their cyclic oligomers. Standard solutions consisting of OLA oligomers (diamide diester) and their cyclic impurities were injected separately in individual runs.

At 100% acetonitrile, the cyclics eluted at 6.30 min and dd oligomers eluted at 6.80 min, resulting in partial overlapping of the two peaks (Figure 4.19).

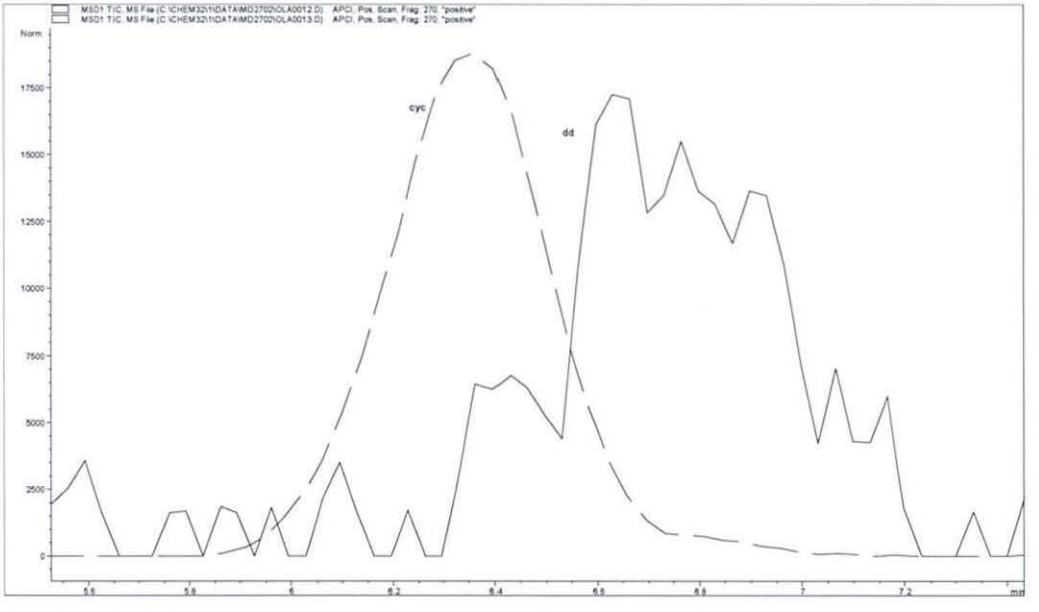


Figure 4.19: Overlaid mass chromatograms (expanded), (representing the overlaid chromatograms, with cyclics labelled as 'cyc' and diamide diester labelled as 'dd'), obtained with NH2-NH2 columns, Spherisorb (250 x 4.6) mm, 5µm-Luna (150 x 2) mm, 3 µm, at 100% 0.1% formic acid in MeCN (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 5 µg/ml. LC-MS: HP1100, equipped with APCI source.

The chromatography reported above is poor, especially for the diamide diester run and this affects the resolution.

At 90% acetonitrile/10% n-hexane, the cyclics eluting at 6.41 min and dd oligomers eluting at 6.85 min (standard solutions injected separately in individual runs) were partially separated (Figure 4.20).

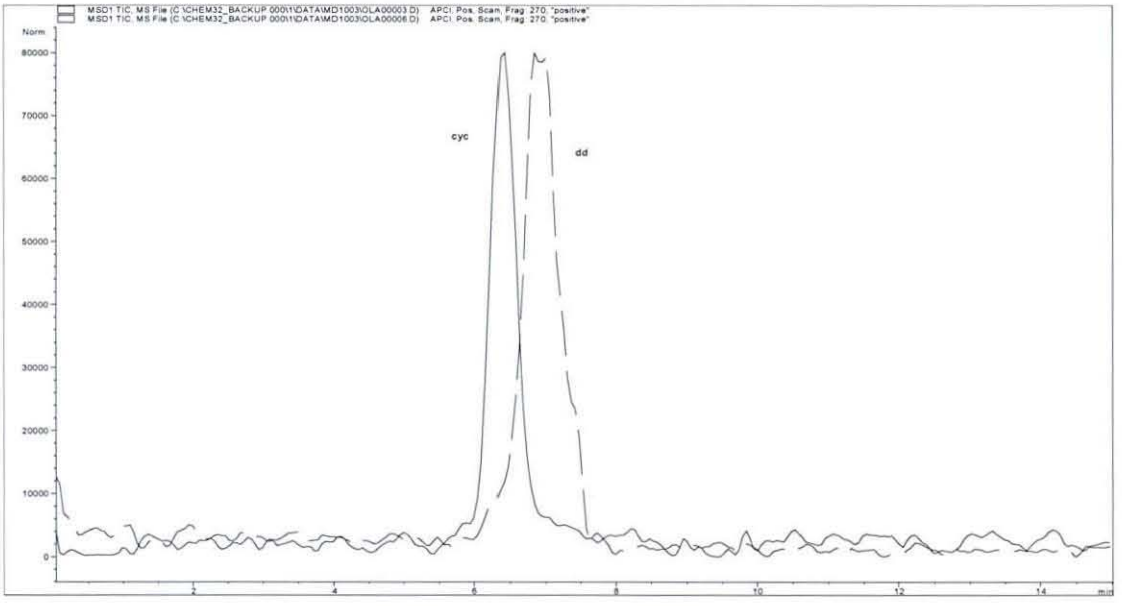


Figure 4.20: Overlaid mass chromatograms, obtained with NH2-NH2 columns, Spherisorb (250 x 4.6) mm, 5µm-Luna (150 x 2) mm, 3 µm, at 10% n-hexane/90% 0.1% formic acid in MeCN, (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 5 µg/ml. LC-MS: HP1100, equipped with APCI source.

Further increase of the percentage of n-hexane to 20% lead to a decrease in retentions: the cyclic retention time was 5.90 min and the dd oligomers retention time was 6.61 min, with resolution Rs of 1.1 (Figure 4.21).

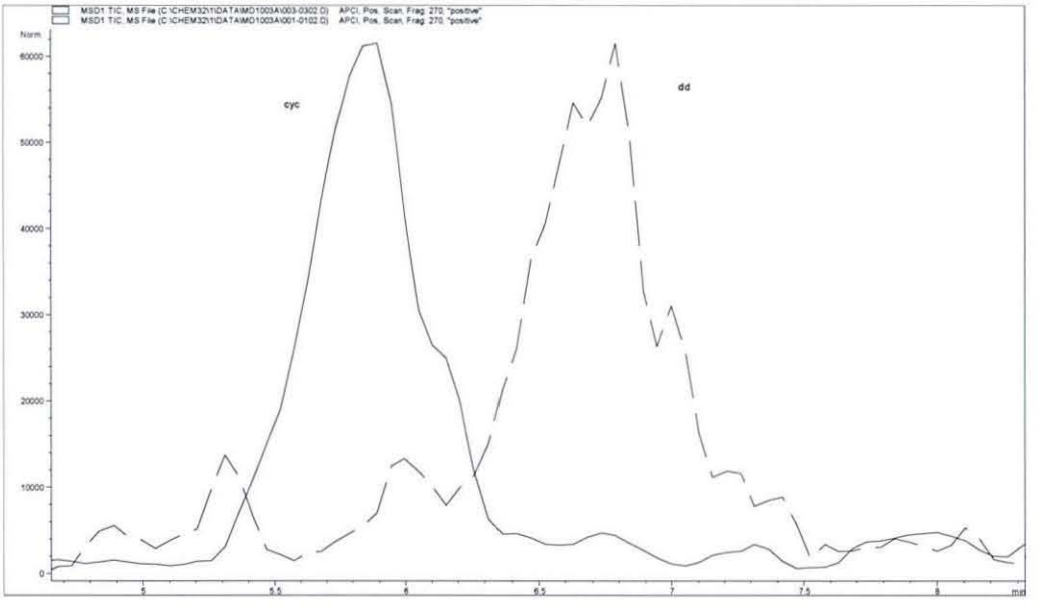


Figure 4.21: Overlaid mass chromatograms (expanded), obtained with NH2-NH2 columns, Spherisorb (250 x 4.6) mm, 5µm-Luna (150 x 2) mm, 3 µm, at 20% n-hexane/80% 0.1% formic acid in MeCN, (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 5 µg/ml. LC-MS: HP1100, equipped with APCI source.

The further increase to 30% n-hexane in the mobile phase caused the two components to elute at 6.49 min (cyc) and 7.04 min (dd), and they were therefore partially overlapped (Figure 4.22).

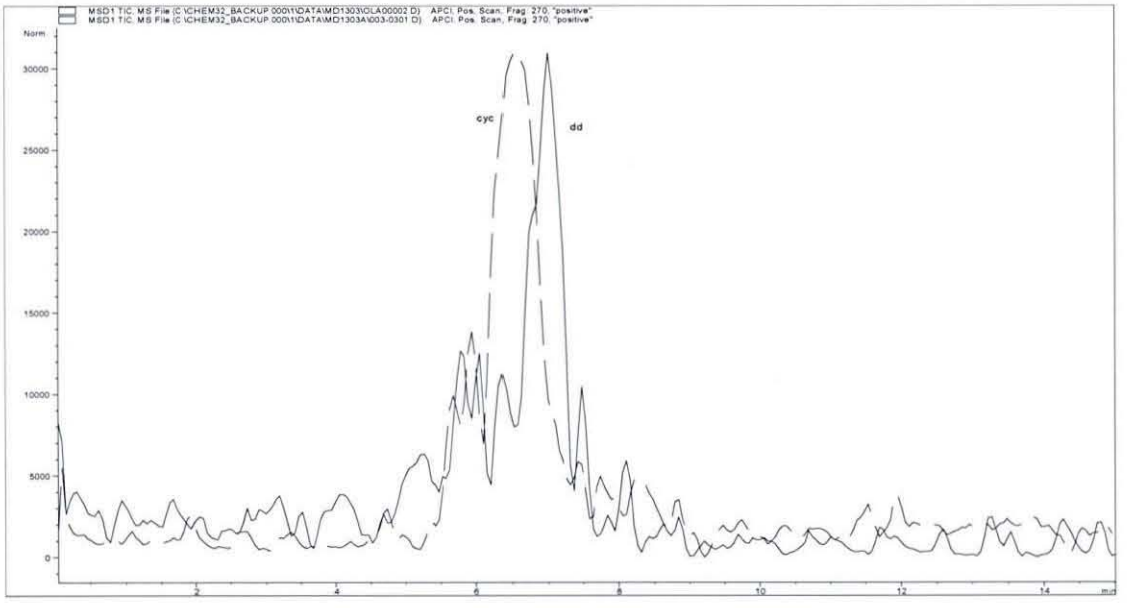


Figure 4.22: Overlaid mass chromatograms (expanded), obtained with NH2-NH2 columns, Spherisorb (250 x 4.6) mm, 5µm-Luna (150 x 2) mm, 3 µm, at 30% n-hexane/70% 0.1% formic acid in MeCN, (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 5 µg/ml. LC-MS: HP1100, equipped with APCI source.

The single ion traces showed an elution order of longer diamide diester oligomers first, followed by shorter oligomers (Figure 4.23).

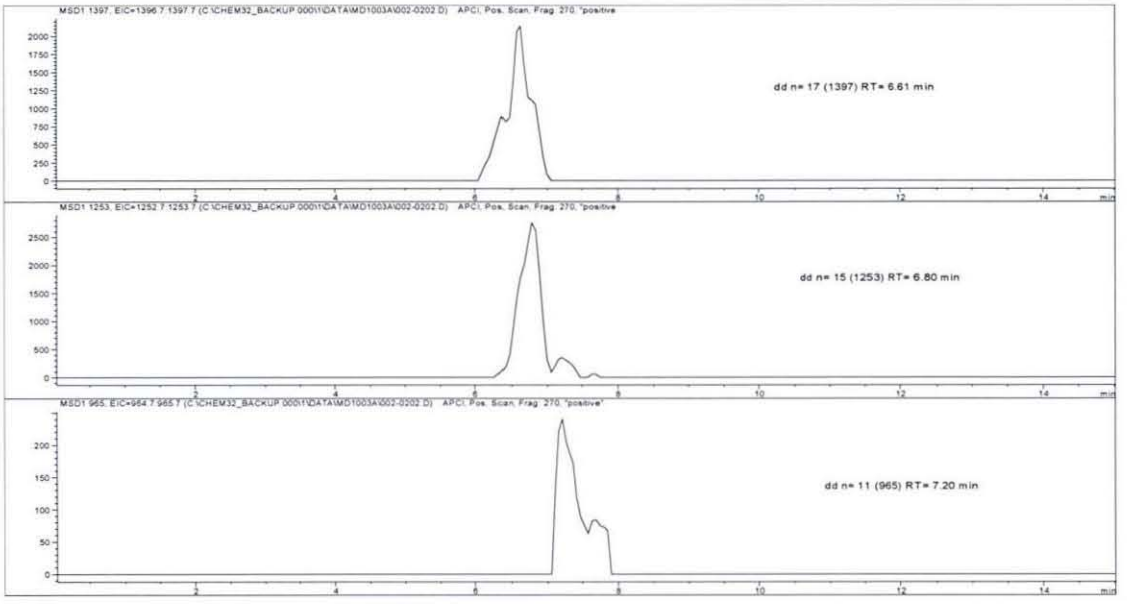


Figure 4.23: EIC (positive mode) for 50 mcg/ml OLA solution relative to NH2-NH2 columns, Spherisorb (250 x 4.6) mm, 5µm-Luna (150 x 2) mm, 3 µm, at 20% n-hexane/80% 0.1% formic acid in MeCN, (positive ionisation). Injection volume: 20 µl; flow rate: 0.5 ml/min. Diamide diester 5 µg/ml. LC-MS: HP1100, equipped with APCI source.

This variation in retention values indicated a slight adsorption effect of OLA main excipients, so that the conditions of elution are not perfectly critical. This was also true for the cyclic components. From the extracted ion chromatograms (EICs) relative to OLAs cyclic impurities (Figure 4.24), it can be seen that the retention time of the selected oligomers decreased slightly with increasing molecular weight (from 6.25 min to 6.16 min with n increasing from 2 to 7).

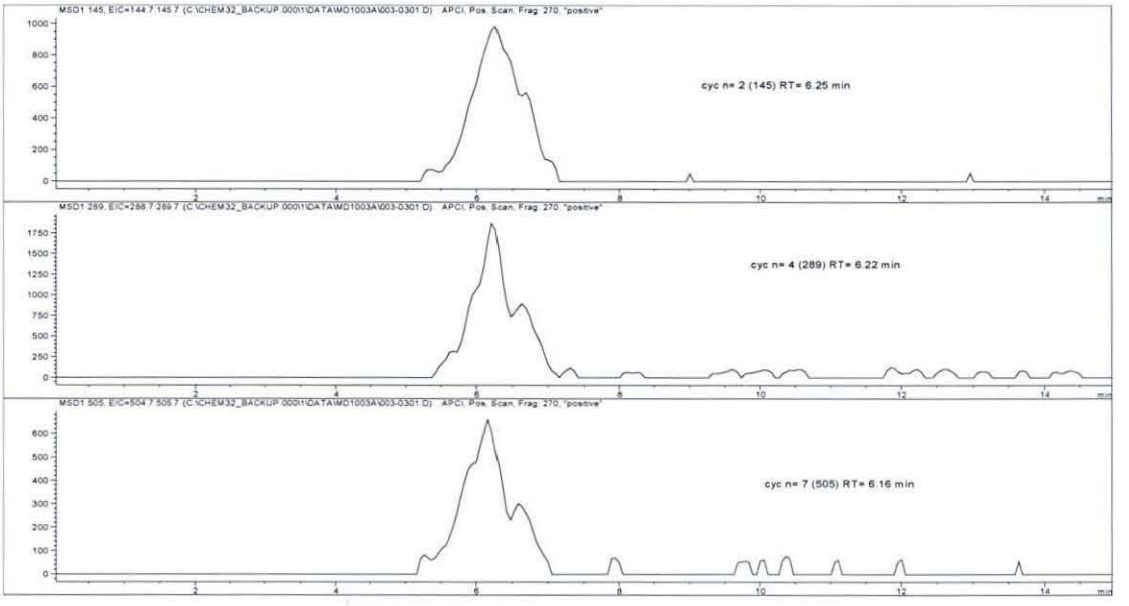


Figure 4.24: EIC (positive mode) for 50 mcg/ml OLA solution relative to NH2-NH2 columns, Spherisorb (250 x 4.6) mm, 5µm-Luna (150 x 2) mm, 3 µm, at 20% n-hexane/80% 0.1% formic acid in MeCN, (positive ionisation). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml. LC-MS: HP1100, equipped with APCI source.

4.2.2. Conclusions on the retention of OLAs on amino and cyano materials in NP-HPLC systems

Figure 4.25 represents a summary of OLAs retention changes with the proportion of n-hexane content in acetonitrile on the amino and cyano columns:

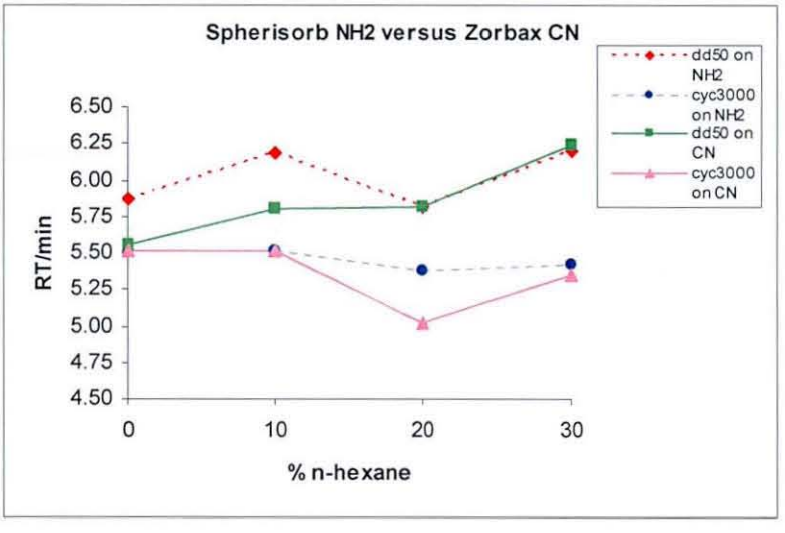


Figure 4.25: Elution of cyc3000 (3000 µg/ml cyclic solution) and dd50 (50 µg/ml OLA solution); Spherisorb amino column (250 x 4.6) mm, 5µm, and Zorbax cyano column (250 x 4.6) mm, 7µm, hexane/0.1% formic acid in MeCN. Injection volume: 20 µl; flow rate: 0.5 ml/min. LC-MS: HP1100, equipped with APCI source.

The proportion of n-hexane did not provide trends in the retention behaviour. On the amino stationary phase, 10% n-hexane caused an increase in retention for both cyclic and dd; 20% n-hexane decreased their RT and 30% gave an increase in retention. On the cyano column, 10% n-hexane didn't cause any variation in the cyclics retention time, whilst the dd increased their retention. 20% n-hexane gave a decrease in RT for the cyclics, whereas the dd did not vary. For 30% n-hexane both cyclics and dd increased retention.

Thus a mobile phase of n-hexane and acetonitrile, on either amino or cyano stationary phases did not provide critical conditions of elution for OLAs and was

therefore not a suitable eluent for OLAs characterisation. This was also the case for two amino columns coupled together.

Coupling two amino columns did add extra retention power to the system only when 20% n-hexane/80% acetonitrile was used as mobile phase (Figure 4.26). The addition of n-hexane to acetonitrile did not provide a trend in the retention behaviour of cyclics and dd oligomers.

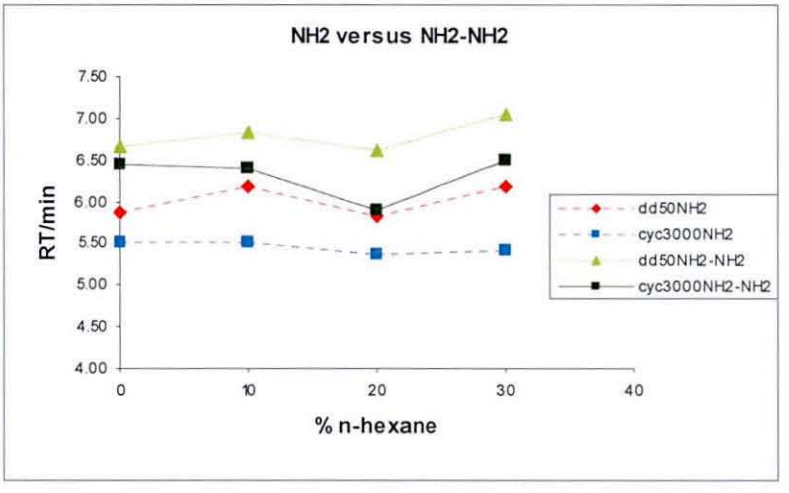


Figure 4.26: Elution of cyc3000 (3000 µg/ml cyclic solution) and dd50 (50 µg/ml OLA solution) on the Spherisorb amino column (250 x 4.6) mm, 5µm and on the Luna (150 x 2) mm, 3 µm, coupled to the Spherisorb column . Injection volume: 20 µl; flow rate: 0.5 ml/min. Retention time variations are due to n-hexane addition to 0.1% formic acid in acetonitrile.

4.2.3. Effects of column temperature on OLAs selectivity in NP-HPLC

Normal-phase separations of OLA main oligomers at different column temperature were then investigated, to check whether other temperature could reduce the oligomeric selectivity and hence leading to critical conditions of elution.

Previously, Cho *et al.* [86] and Vanhoenacker and Sandra [79] have demonstrated that at high temperature there was a decreased retention of oligomers, in NP-HPLC systems, which could lead to the suppression of oligomeric elution.

In the current study a polar amino material at 30-80 °C and a silica sorbent at 30-55 °C were used as the stationary phases. When the amino column was tested over the temperature range 30-80 °C, with acetonitrile as mobile phase, the oligomers with different degrees of polymerisation were unresolved (Figure 4.27 at 30 °C).

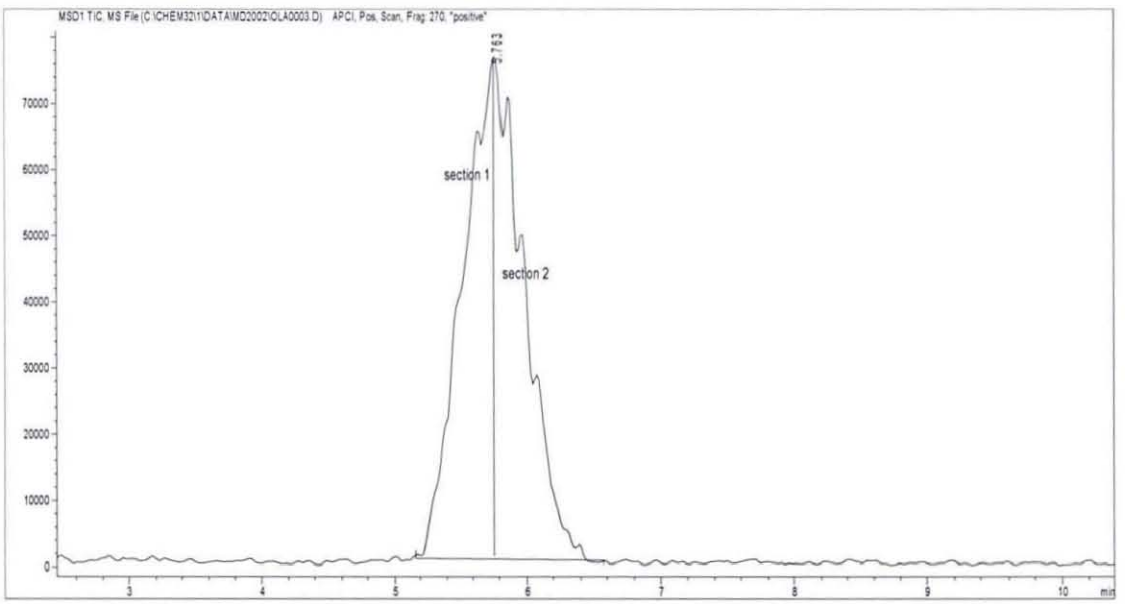


Figure 4.27: Mass chromatogram obtained at 30 °C. LC-MS: HP 1100, equipped with APCI source. Injection volume: 5 µl; OLAs dd excipient sample solution concentration: 52.0 µg/ml; chromatographic column: Spherisorb NH2 (250 x 4.6) mm, 5 µm; mobile phase: 0.1% formic acid in MeCN (isocratic run); flow rate: 0.5 ml/min; (positive ionisation). RT= 5.76 min.

EIC analysis (Figure 4.28) showed the OLAs oligomers n = 12-23 eluted with decreasing different retention times, between 6.22 and 5.48 min.

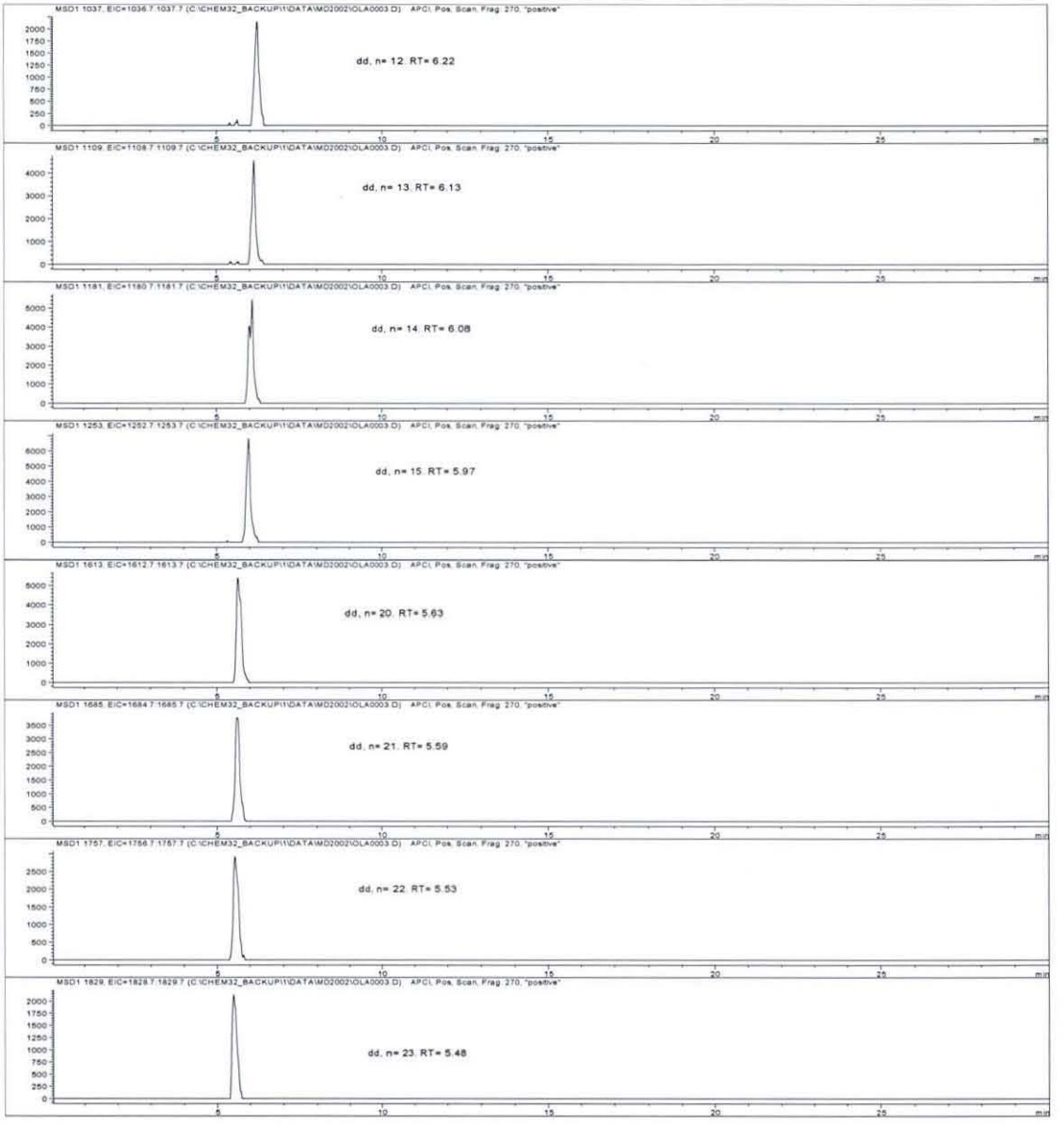


Figure 4.28: Extracted Ion Chromatograms (EICs) obtained at 30 °C. LC-MS: HP 1100, equipped with APCI source. Injection volume: 5 µl; OLAs dd excipient sample solution concentration: 52.0 µg/ml; chromatographic column: Spherisorb NH2 (250 x 4.6) mm, 5 µm; mobile phase: 0.1% formic acid in MeCN (isocratic run); flow rate: 0.5 ml/min; (positive ionisation).

This variation in retention values indicated a slight adsorption effect of OLAs, so the conditions of elution were not perfectly critical.

Throughout the temperature range (up to 80 °C, Figure 4.29), the oligomers maintained the same elution order, with larger oligomers eluting in front of the smaller oligomers.

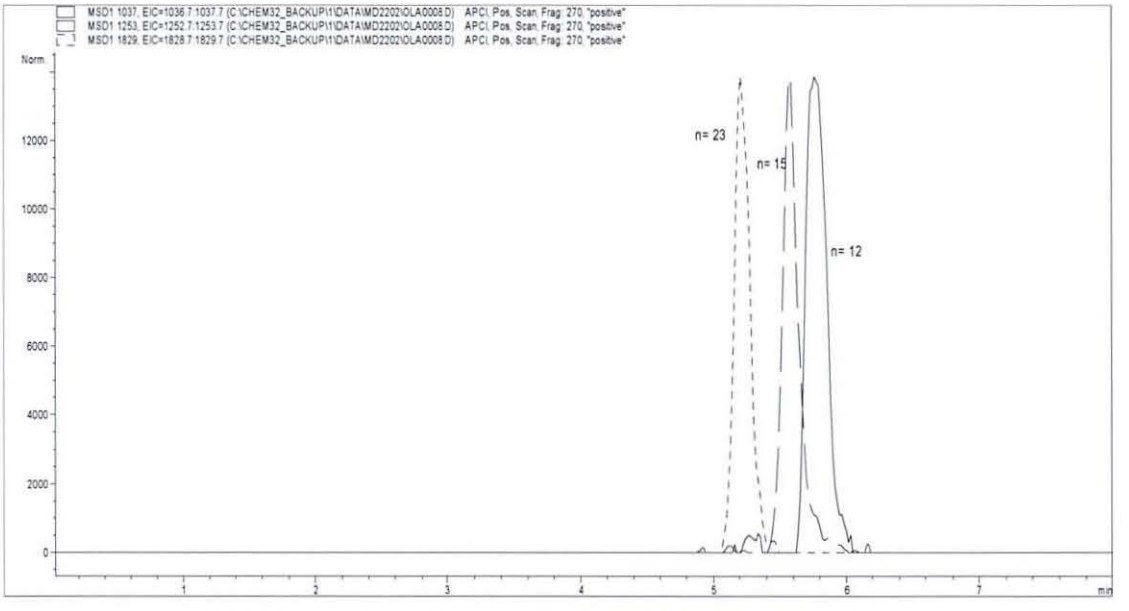


Figure 4.29: Overlaid Extracted Ion Chromatograms (EICs) obtained at 80 °C. LC-MS: HP 1100, equipped with APCI source. Injection volume: 5 µl; OLAs dd excipient sample solution concentration: 52.0 µg/ml; chromatographic column: Spherisorb NH2 (250 x 4.6) mm, 5 µm; mobile phase: 0.1% formic acid in MeCN (isocratic run); flow rate: 0.5 ml/min; (positive ionisation).

This elution order was not affected by the analytical temperature, and this would lead to the conclusion that the temperature increases on an amino column with acetonitrile as mobile phase, would not yield a significant selectivity change.

The only clear trend observed associated with the temperature increase was the reduced retention of all oligomers, (Figure 4.30).

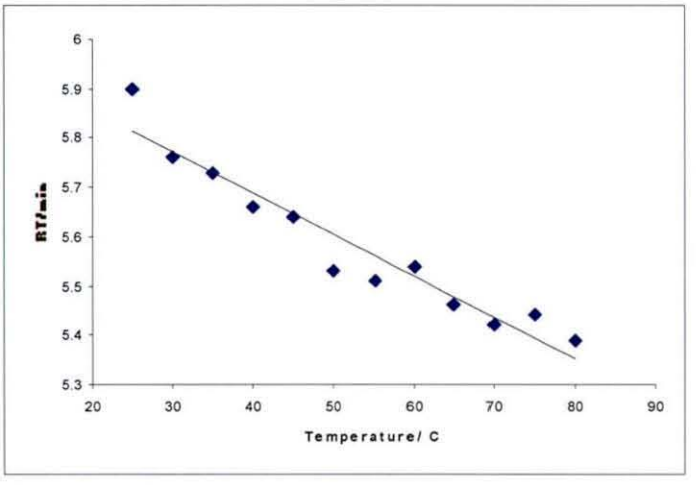


Figure 4.30: Temperature effect on retention of OLAs (dd), over the 30-80 °C range. LC-MS: HP 1100, equipped with APCI source. Injection volume: 5 µl; OLA sample solution concentration: 52.0 µg/ml; chromatographic column: Spherisorb NH2 (250 x 4.6) mm, 5 µm; mobile phase: 0.1% formic acid in MeCN (isocratic run); flow rate: 0.5 ml/min.

These changes were in agreement with the results obtained by Cho *et al.* [86] for poly (ethylene oxide) (PEO), in NP-HPLC. Vanhoenacker and Sandra [79] also found that temperature did not cause a change in selectivity, in NP-HPLC. In both cases the increase in temperature only reduced retention.

Similarly on the silica material co-elution of OLAs oligomers was observed, resulting in a single chromatographic peak, over the 30-55 °C range (Figure 4.31 for 50 °C). The chain length distribution caused by hydrophobic interactions did not take place on this column.

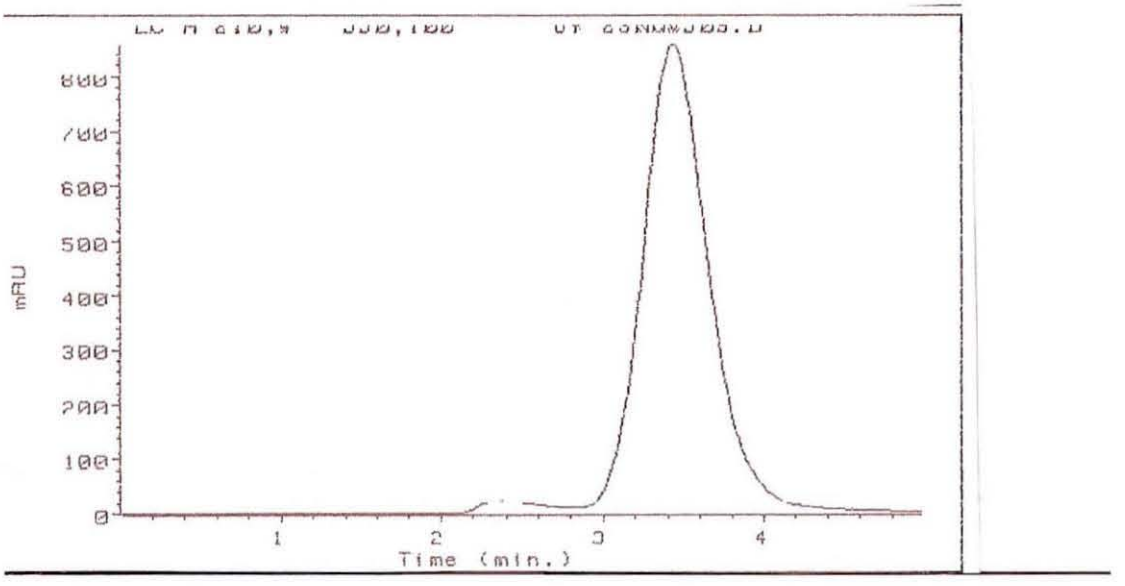


Figure 4.31: Chromatogram obtained at 50 °C. LC: HP 1090. Injection volume 10 µl; UV: 210 nm. Injection volume: 10 µl; OLAs main excipient solution concentration: 26.0 mg/ml; chromatographic column: Spherisorb S5W (250 x 4.6) mm, 5 µm; mobile phase: 0.1% formic acid in THF (isocratic run); flow rate: 1 ml/min.

The increase in temperature led to decreased retention time of OLAs oligomers (Figure 4.32).

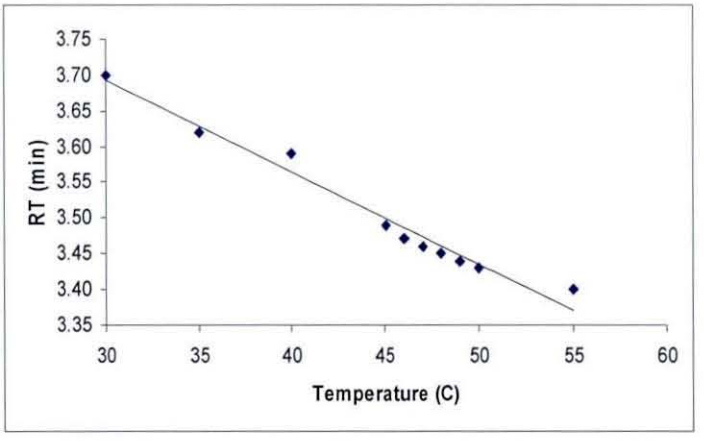


Figure 4.32: Temperature effect on retention of OLAs, over the 30-55 °C range. LC: HP 1090. UV: 210 nm. Injection volume: 10 µl; OLAs main excipient solution concentration: 26.0 mg/ml; chromatographic column: Spherisorb S5W (250 x 4.6) mm, 5 µm; mobile phase: 0.1% formic acid in THF (isocratic run); flow rate: 1 ml/min.

From the data obtained with the NH2 column and the silica column it can be said that both materials exhibited polar interactions with the OLAs and typical NP retention behaviour when temperature changes were applied (of decreasing retention with increasing temperature). Thus, temperature had a limited effect on the OLA retention selectivities offered by the two polar columns and on the oligomer elution order, in non aqueous mobile phases.

4.2.4. Conclusions on effects of column temperature on OLAs selectivity in NP-HPLC

The retention behaviour of OLAs in normal phase LC, on an amino column (with temperatures ranging from 30 to 80 °C) and on a silica column (with temperatures ranging from 30 to 55 °C), was only marginally affected: an increase in temperature caused a decrease in retention time of OLAs (on the amino column, retention varied from approx. 5.9 min, at 30 °C to 5.4 min at 80 °C, which corresponds to about 0.5 min for a 50 °C variation; on the silica column, retention varied from approx. 3.7 min, at 30 °C to 3.4 min at 55 °C, which corresponds to about 0.3 min for a 25 °C variation).

The MS identified the various oligomers of the diamide diester component. The larger oligomers eluted earlier than the smaller oligomers, indicating a NP retention mechanism. Increased temperature did not lead to an inversion of the oligomers elution order. Oligomeric separation was not suppressed by temperature and therefore critical conditions of elution could not be achieved.

4.2.5. Retention of OLAs on cyano and amino materials in HILIC: a quest for LCCC

Strege [120] has shown HILIC to be successful for the study of polar compounds and as the aim of the current study was to target the polar functional groups of OLAs, aqueous eluents on polar sorbents were therefore considered as alternative chromatographic systems, whose selectivity could lead to oligomeric co-elution of the OLAs.

4.2.5.1. Cyano bonded stationary phase

The separation between OLAs oligomers and cyclics, in terms of their head-groups was investigated on a cyano stationary phase using a relatively polar eluent to induce a hydrophobic interaction effect in contrast to a normal phase separation.

The starting mobile phase was 100% acetonitrile. The mobile phase polarity was altered by gradually introducing (in isocratic runs) successively 10, 20 and 30% of 50 mM ammonium formate. Standard solutions consisting of OLA (diamide diester) or their cyclic impurities were injected separately in individual runs

At 100% acetonitrile, cyclics and main oligomers eluted at approximately 5.5 min. At 90% acetonitrile/10% 50 mM ammonium formate, cyclics eluted at 5.47 min and diamide diester eluted at 5.19, showing a certain degree of separation (Figure 4.33):

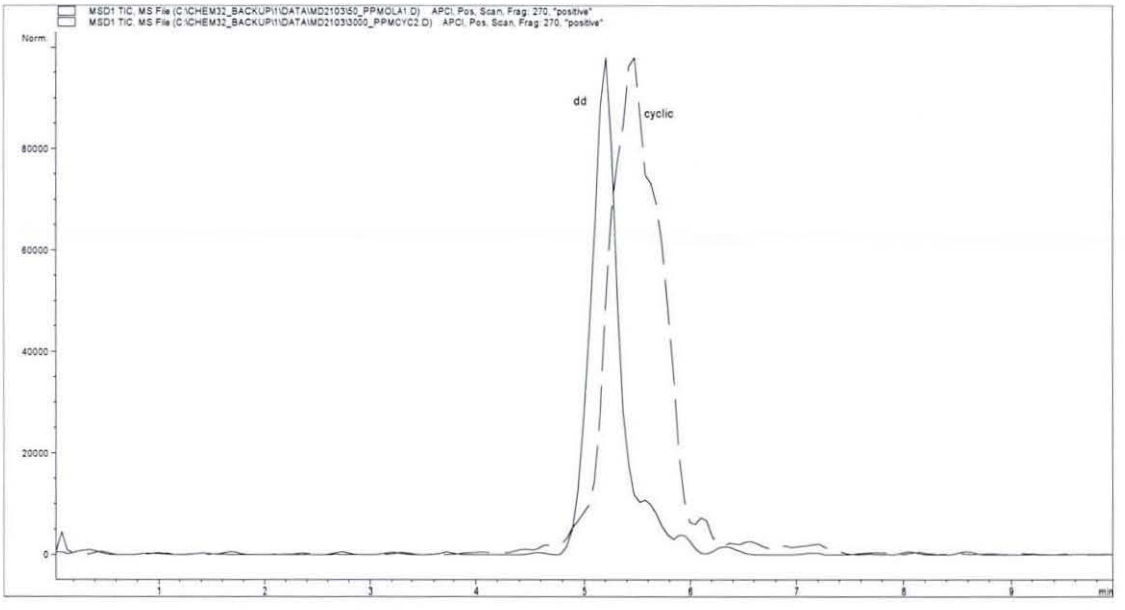


Figure 4.33: Overlaid mass chromatograms, obtained with Zorbax cyano column (250 x 4.6) mm, 7µm, 10% 50 mM ammonium formate, 90% 0.1% formic acid in MeCN (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 50 µg/ml. Dd retention time: 5.19 min, cyclic retention time: 5.47 min. LC-MS: HP1100, equipped with APCI source.

With 80% acetonitrile/20% 50 mM ammonium formate, the cyclics and dd were separated, but not base-line resolved, resolution Rs= 0.9 (Figure 4.34):

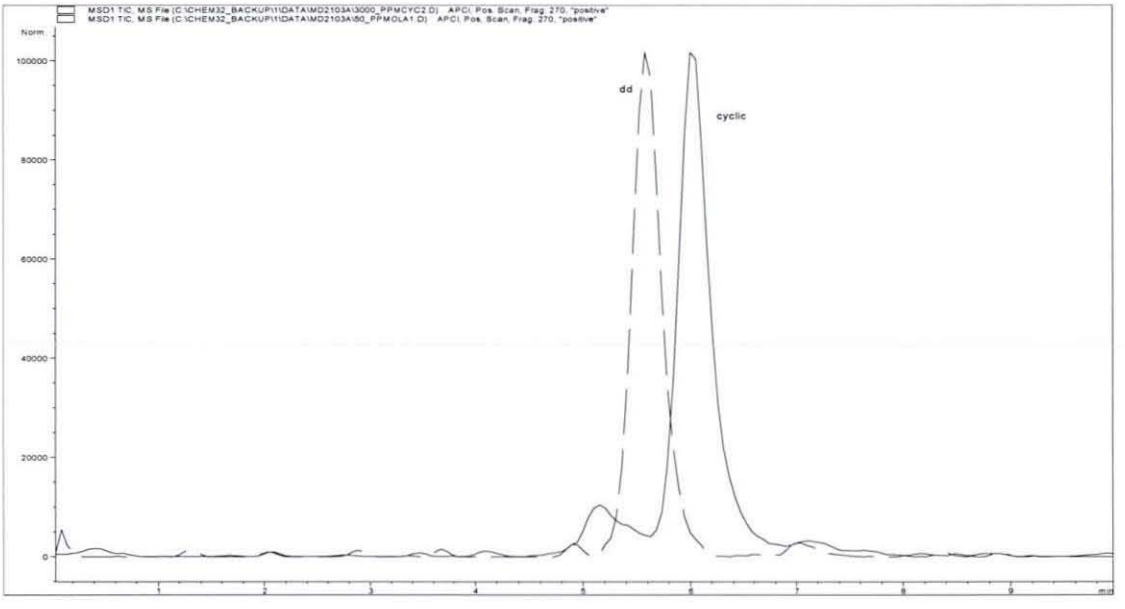


Figure 4.34: Overlaid mass chromatograms, obtained with Zorbax cyano column (250 x 4.6) mm, 7µm, 20% 50 mM Ammonium formate, 80% 0.1% formic acid in MeCN (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 50 µg/ml. Dd retention time: 5.58 min, cyclic retention time: 6.02 min. LC-MS: HP1100, equipped with APCI source.

A further increase of ammonium formate percentage -70% acetonitrile/30% 50 mM ammonium formate- did not improve the resolution between cyclics and dd excipients. Instead the two peaks co-eluted (dd: 7.2 min, cyclics: 7.4 min, Figure 4.35).

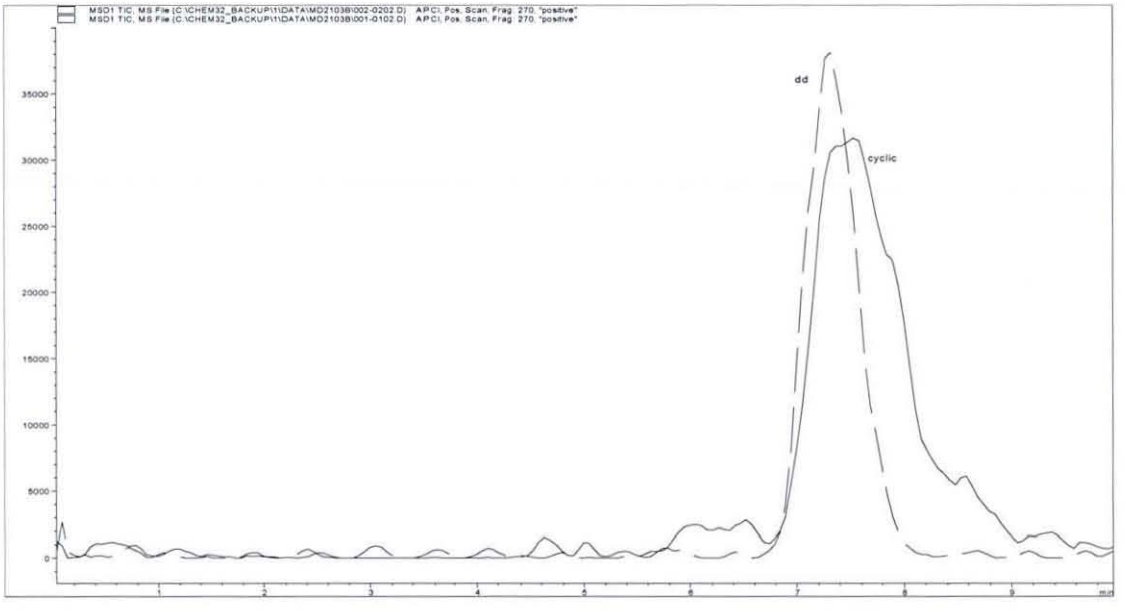


Figure 4.35: Overlaid mass chromatograms, obtained with Zorbax cyano column (250 x 4.6) mm, 7µm, 30% 50 mM Ammonium formate, 70% 0.1% formic acid in MeCN (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 50 µg/ml. Dd retention time: 5.58 min, cyclic retention time: 6.02 min. LC-MS: HP1100, equipped with APCI source.

The gradual increase in mobile phase polarity was responsible for the dd oligomers eluting in front of the cyclic impurities. From this it could be concluded that the dd oligomers were more polar than the cyclic impurities.

MS analysis monitor showed the elution order of diamide diester oligomeric longer chains before shorter chains (Figure 4.36).

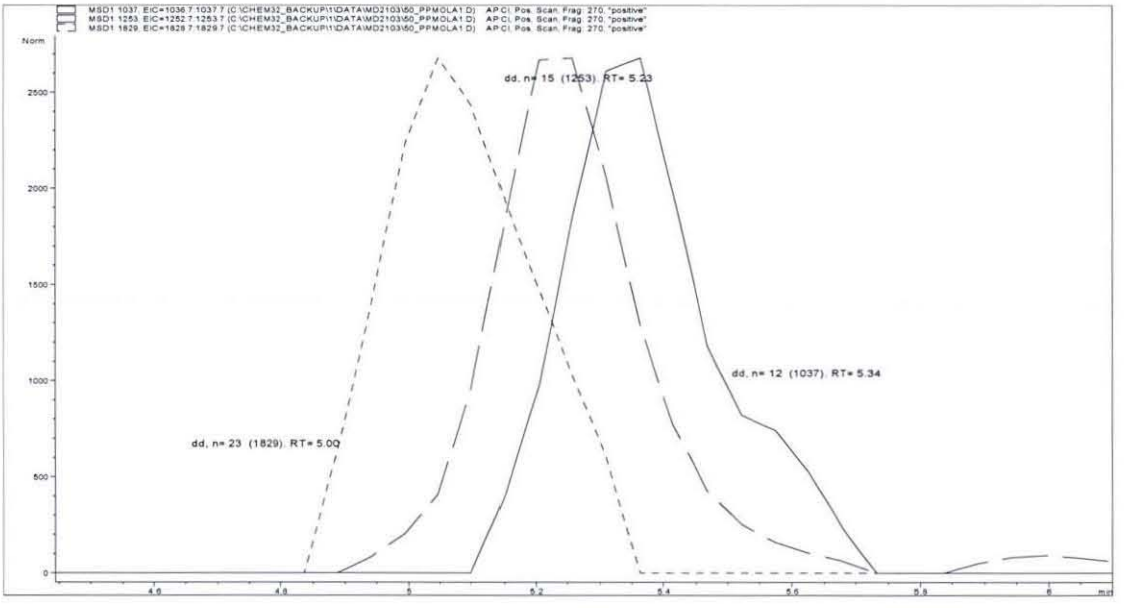


Figure 4.36.: EICs (positive mode) for 50 mcg/ml OLA dd excipient solution relative to Zorbax cyano column (250 x 4.6) mm, 7µm, 90% 0.1% formic acid in MeCN /10% 50 mM Ammonium formate, (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. LC-MS: HP1100, equipped with APCI source.

The phenomenon of larger chains eluting before shorter chains was observed by Sandra and Vanhoenacker [79] when analysing alkylphenol ethoxylates and by Jandera [92] when analysing ethoxylated alcohols. This phenomenon was also reported by Cho *et al.* [86] and Lemr *et al.* [83] when analysing FAEs. The authors advocated polar interactions to justify the observed retention mechanisms.

However, in the current study, when the percentage of ammonium formate was increased to 20%, all oligomers eluted with the same retention time (RT: 5.6 min, approximately, Figure 4.37).

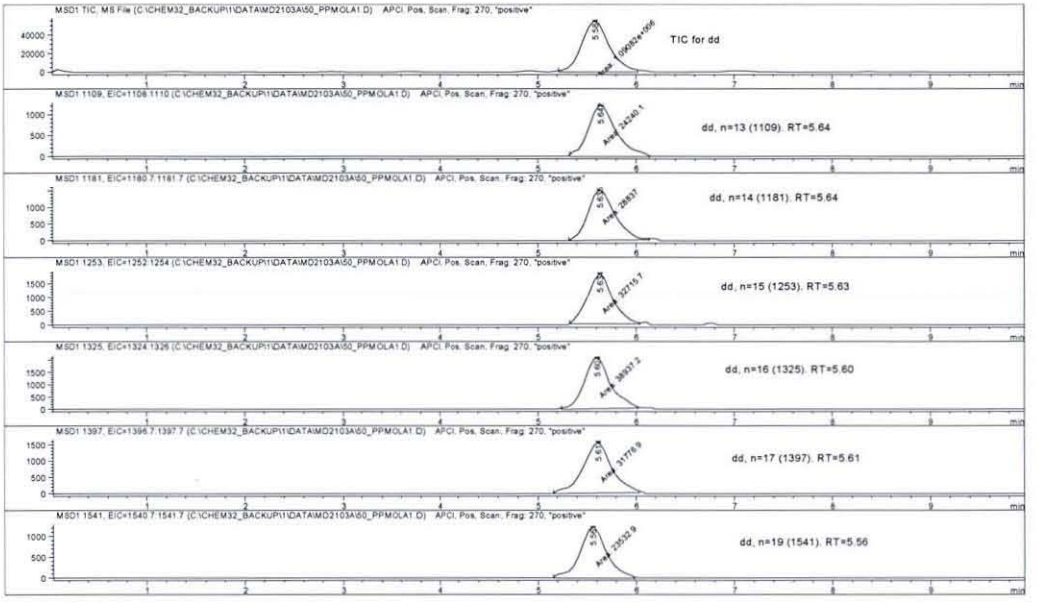


Figure 4.37: Total ion chromatogram (TIC) and EICs (positive mode) for 50 mcg/ml OLA dd excipient solution relative to Zorbax cyano column (250 x 4.6) mm, 7µm, 80% 0.1% formic acid in MeCN /20% 50 mM Ammonium formate, (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. LC-MS: HP1100, equipped with APCI source.

Since all the oligomers had the same retention, it was concluded that critical conditions of elution existed at 80% acetonitrile /20% ammonium formate. Because a specific functionality of an oligomeric series can be determined in liquid chromatography at critical conditions (LCCC), OLAs main excipient and their cyclic impurities were expected to be resolved at 80% acetonitrile/20% ammonium formate. Extracted ion chromatograms (EICs) of the cyclics (Figure 4.38) showed that this was the case, with dd eluting at approximately 5.6 min and the cyclics eluting at approximately 6.1 min.

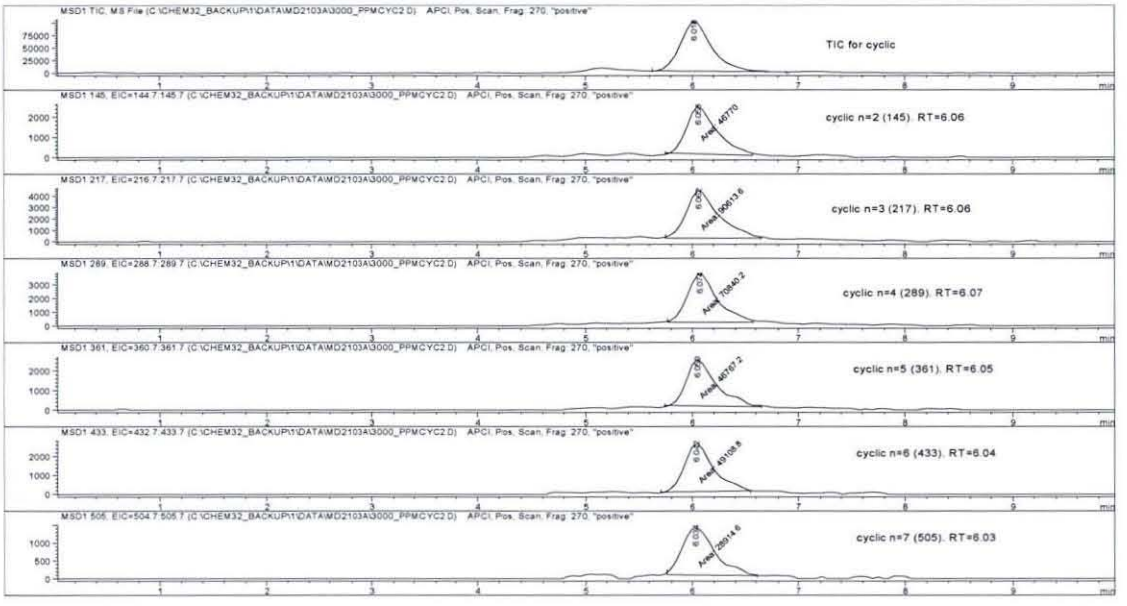


Figure 4.38: TIC and EICs (positive mode) for 3000 mcg/ml cyclic solution relative to Zorbax cyano column (250 x 4.6) mm, 7µm, 80% 0.1% formic acid in MeCN /20% 50 mM Ammonium formate, (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. LC-MS: HP1100, equipped with APCI source.

MS analysis of separation of dd oligomers obtained at 70% acetonitrile/30% ammonium formate showed a conventional reversed phase elution with smaller oligomers eluting in front of the larger oligomers (Figure 4.39):

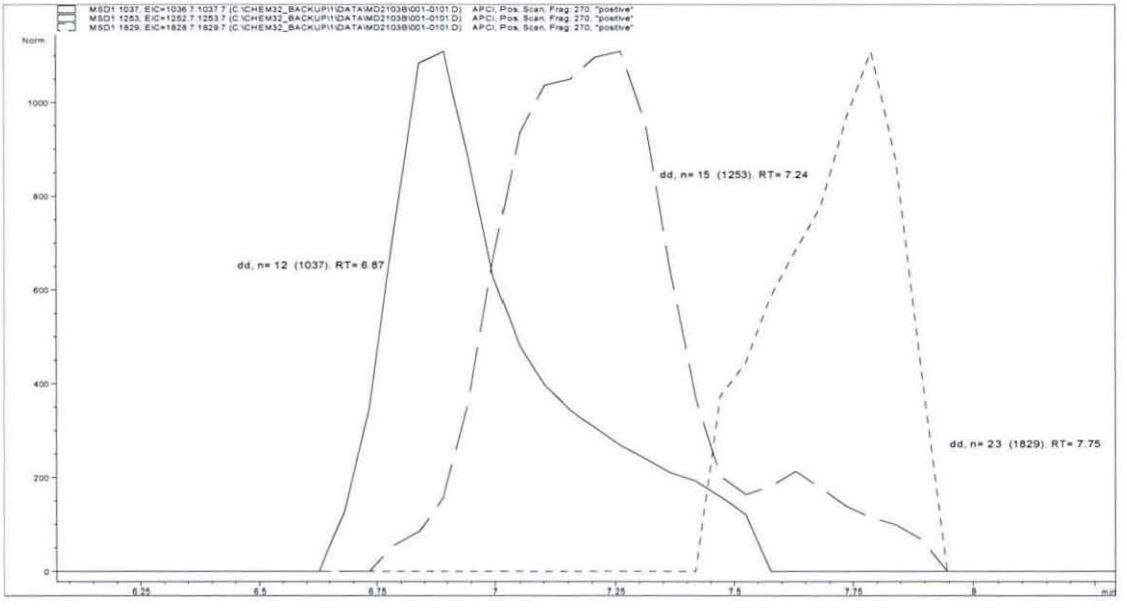


Figure 4.39: EICs (positive mode) for 50 mcg/ml OLA dd mixture solution relative to Zorbax cyano column (250 x 4.6) mm, 7µm, 70% 0.1% formic acid in MeCN /30% 50 mM Ammonium formate, (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. LC-MS: HP1100, equipped with APCI source. the dd, have the smaller oligomers eluting in front of the larger oligomers.

A similar inversion of the elution order was also observed by Lemr *et al.* [83], in the course of the investigation of the effect of mobile phase composition on the retention of FAEs in RP-HPLC. The authors noted that at low acetonitrile percentage, FAEs with a large number of ethyleneoxide (EO) units eluted first. The elution order was inverted (i.e. small number of EO units eluted first) at high acetonitrile percentage. At intermediate acetonitrile percentage they observed co-elution of FAEs, independent of EO unit chain length (indicating critical conditions of elution).

It therefore appeared that the cyano material, with ammonium formate/acetonitrile as mobile phase could be a suitable system for OLAs characterisation.
An HILIC environment was provided by the cyano column when up to 10% ammonium formate was present in the mobile phase, (retention values decreased with increasing mobile phase aqueous content and larger oligomers eluting first). The cyano column behaved as a RP-HPLC column when the mobile phase contained a percentage above 20% of ammonium formate (retention values increased with increasing mobile phase aqueous content, and smaller oligomers eluted first). At intermediate ammonium formate percentage (20%) critical conditions of elution took place and a separation was observed between dd oligomers and cyclics.

Although the 20% ammonium formate/80% MeCN eluent composition led to a resolution of 0.9 between dd and cyclic peaks, the corresponding total ion chromatograms (TICs) show that the critical pair was not baseline resolved.
It was therefore decided to substitute the aqueous component of the mobile phase with THF (aprotic solvent) and check whether reduced solvation effects on OLAs dd (due to proton-acceptor interactions with OH groups of water) would delay their elution and increase their spacing from the cyclic components.

Adding 10% THF to acetonitrile (10% THF/90% MeCN) did not make any difference to the resolution of cyclics and dd components (see Figure 4.40 and compare it with Figure 4.33).

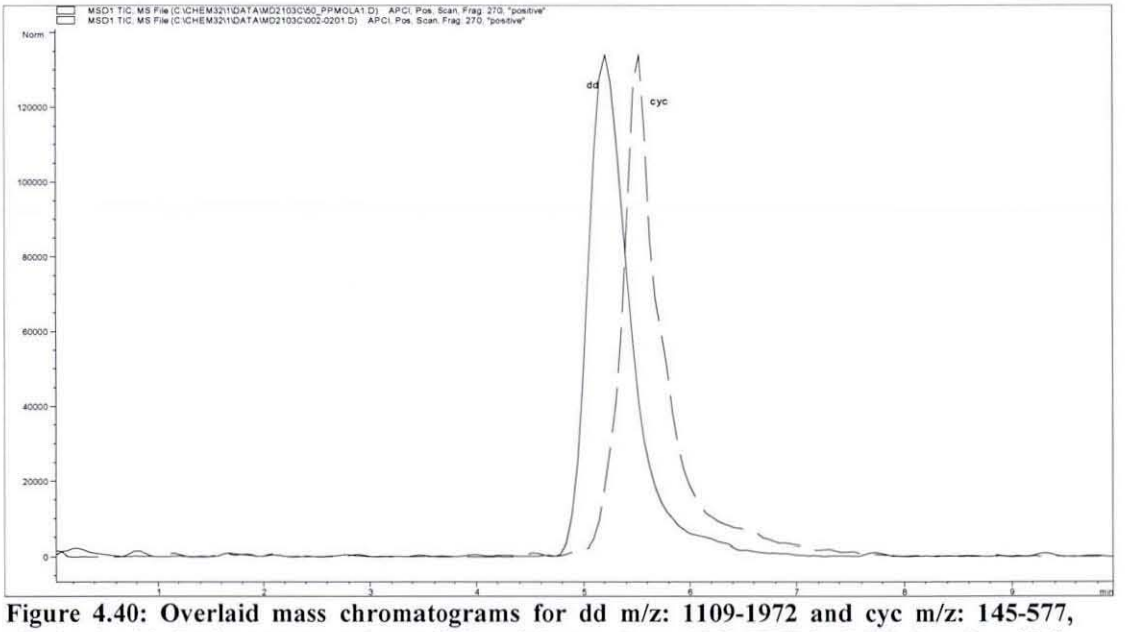


Figure 4.40: Overlaid mass chromatograms for dd m/z: 1109-1972 and cyc m/z: 145-577, obtained with Zorbax cyano column (250 x 4.6) mm, 7µm, 10% THF in 0.1% formic acid in MeCN, (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 50 µg/ml.

Increasing THF from 10 to 20% and 30%, in the THF/MeCN mobile phase mixture, did not cause an increase in resolution. As can be seen from the mass chromatograms below (Figure 4.41 and Figure 4.42), the dd and the cyclics are still partially overlapped.

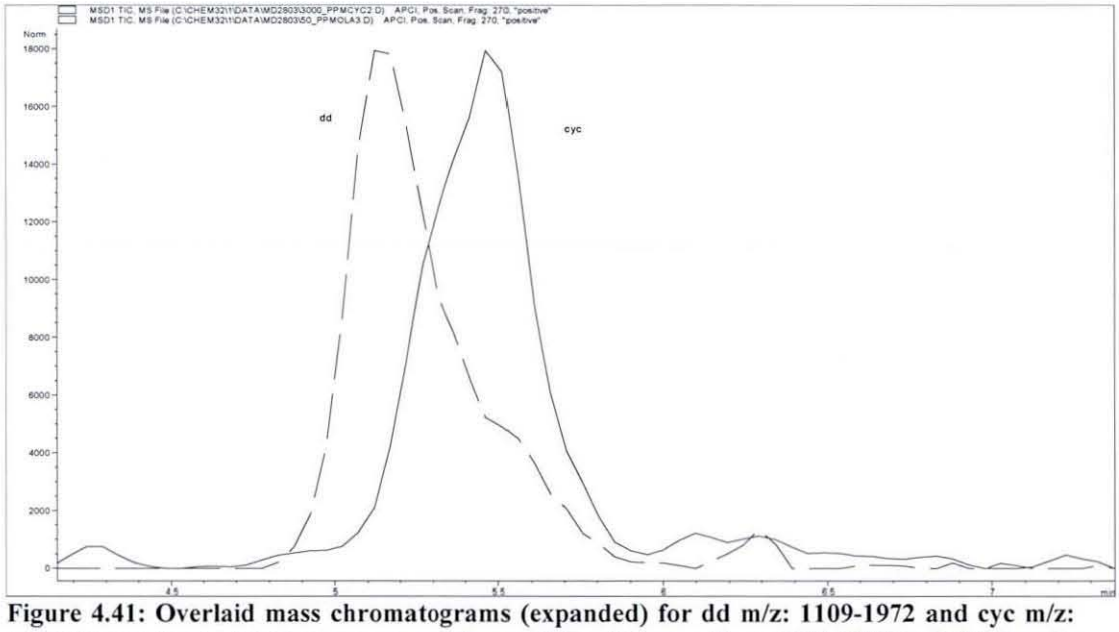


Figure 4.41: Overlaid mass chromatograms (expanded) for dd m/z: 1109-1972 and cyc m/z: 145-577, obtained with Zorbax cyano column (250 x 4.6) mm, 7µm, 20% THF in 0.1% formic acid in MeCN (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 50 µg/ml solution.

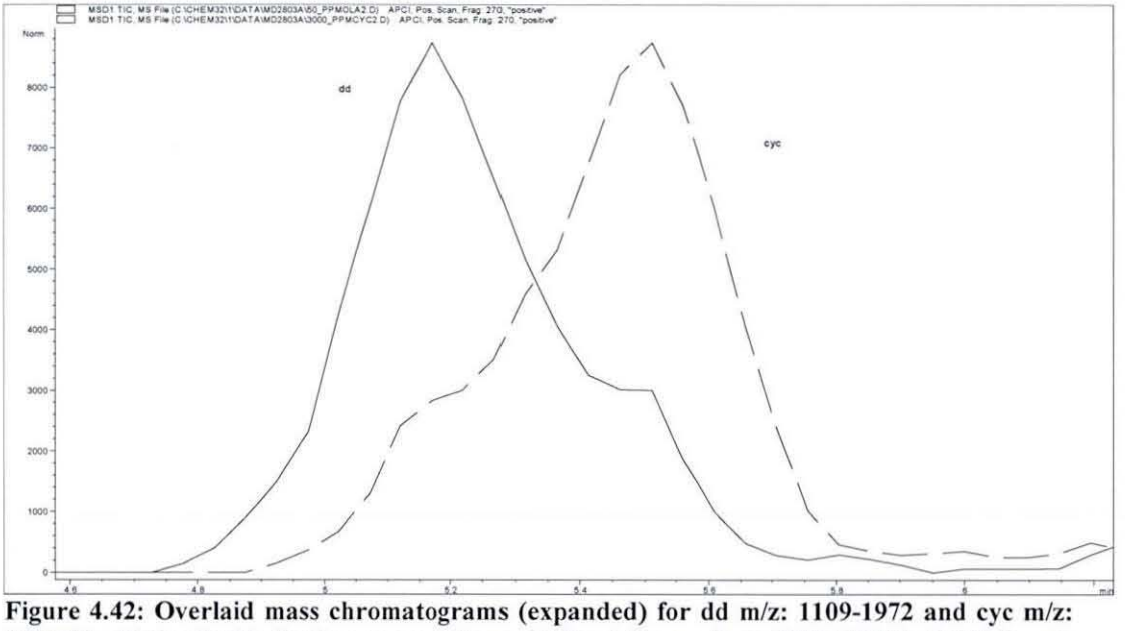


Figure 4.42: Overlaid mass chromatograms (expanded) for dd m/z: 1109-1972 and cyc m/z: 145-577, obtained with Zorbax cyano column (250 x 4.6) mm, 7µm, 30% THF in 0.1% formic acid in MeCN (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 50 µg/ml solution.

Since the substitution of the aqueous component of the mobile phase with THF did not increase resolution, it was accepted that a value of 0.9 was the maximum resolution achievable on a cyano bonded phase.

The methodology comprising cyano bonded material with a 20% ammonium formate/80% MeCN eluent could represent the second orthogonal dimension of the original comprehensive two dimensional assay for OLAs. However, before proceeding towards the coupling of the two separations, it was deemed that further investigation into HILIC systems employing amino bonded material would be interesting. In particular it was decided to monitor whether a variation in ammonium formate percentage in the mobile phase would cause a 'switch' from HILIC to RP-HPLC, with a LCCC cross over, as witnessed on the cyano material.

4.2.5.2. Amino bonded stationary phase

The separation between OLAs and cyclics, was investigated on an amino stationary phase with acetonitrile mobile phase. The mobile phase polarity was altered by gradually introducing (in isocratic runs), respectively, 10, 20 and 30% of 50 mM ammonium formate. Standard solutions consisting of OLA oligomers (diamide diester) and their cyclic impurities were injected separately in individual runs

At 100% acetonitrile, dd oligomers eluted after 5.87 min, and the cyclics after 5.52 min, resulting in partial overlapping of the two peaks (Figure 4.43).

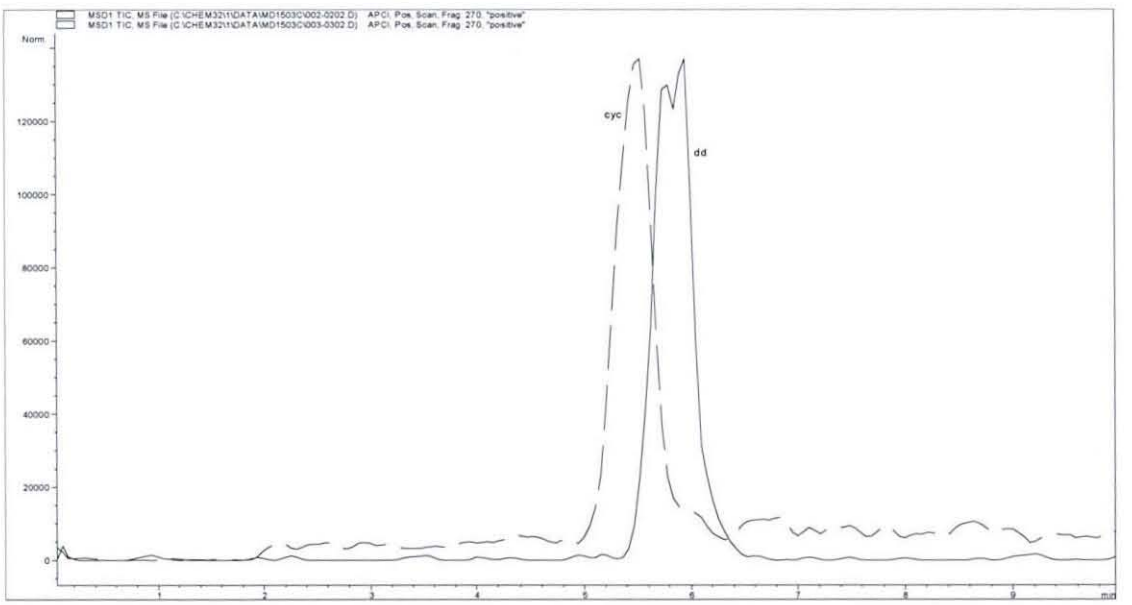


Figure 4.43: Overlaid mass chromatograms, obtained with the Spherisorb amino column (250 x 4.6) mm, 5 µm, at 100% 0.1% formic acid in MeCN (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 50 µg/ml. LC-MS: HP1100, equipped with APCI source.

With 90% acetonitrile/10% 50 mM ammonium formate, the separation of cyclics and diamide diester (standard solutions injected separately in individual runs) was reversed. The dd oligomers were eluted after 4.73 min and the cyclics after 4.91 min, still resulting in partial co-elution (Figure 4.44):

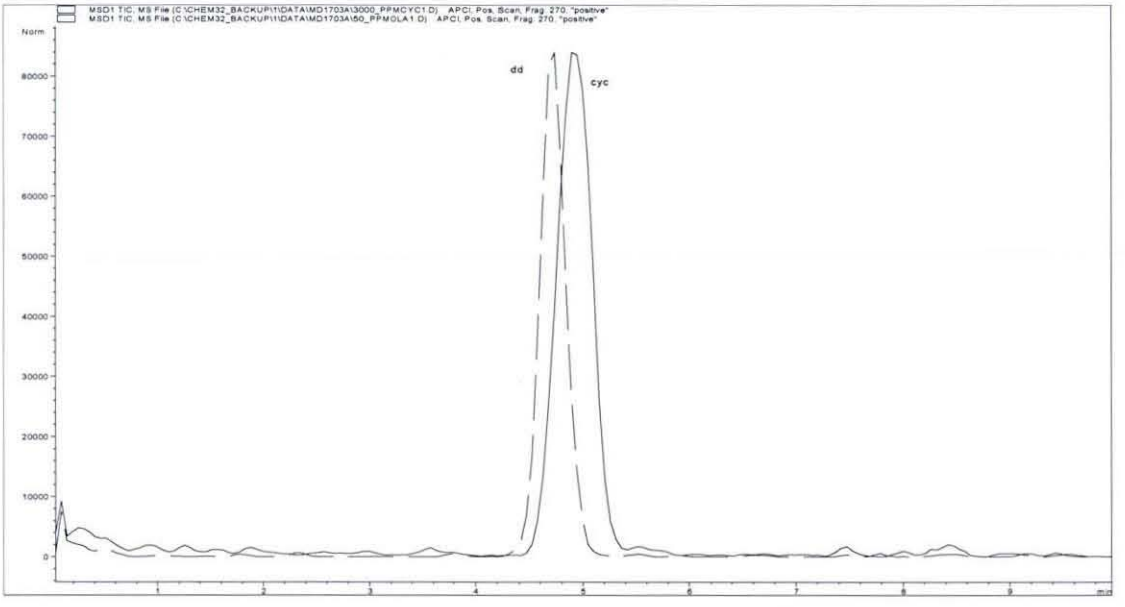


Figure 4.44: Overlaid mass chromatograms for dd m/z: 1109-1972 and cyc m/z: 145-577, obtained with the Spherisorb amino column (250 x 4.6) mm, 5 µm, at 90% 0.1% formic acid in MeCN/10% 50 mM ammonium formate (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 50 µg/ml. Dd retention time: LC-MS: HP1100, equipped with APCI source.

A further increase in ammonium formate to 20% lead to a further decrease in retentions: the dd oligomers retention time was 4.30 min, and the cyclic retention time was 4.50 min, with no improvement in terms of resolution (Figure 4.45).

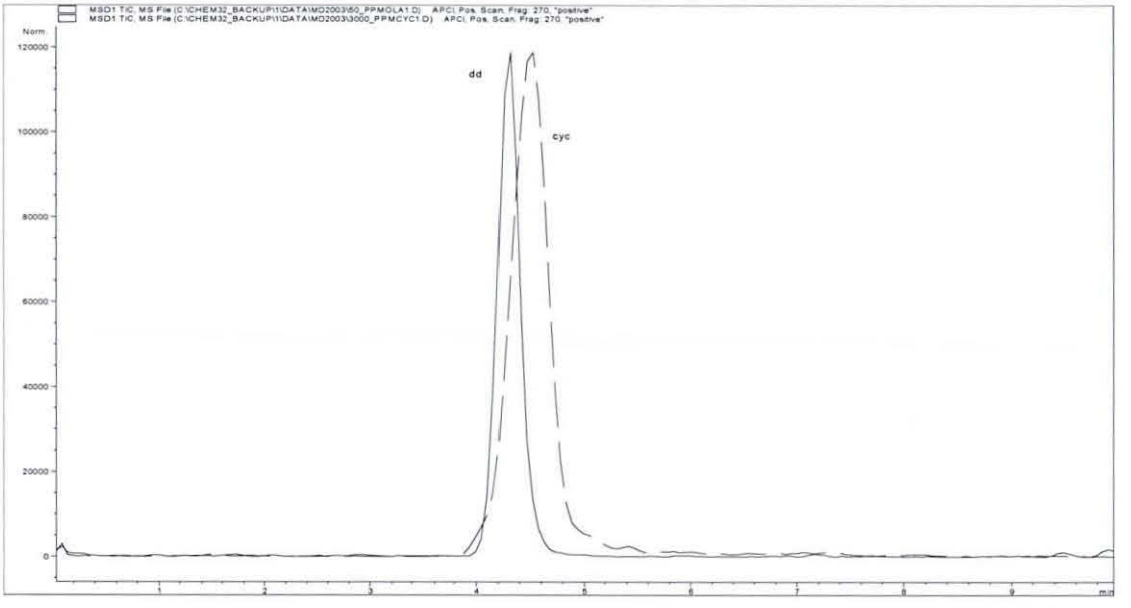


Figure 4.45: Overlaid mass chromatograms, obtained with the Spherisorb amino column (250 x 4.6) mm, 5 µm, at 80% 0.1% formic acid in MeCN/20% 50 mM ammonium formate (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 50 µg/ml. LC-MS: HP1100, equipped with APCI source.

The final increase to 30% ammonium formate caused the two components to elute at 4.0 min (dd) and 4.4 min (cyc), still partially overlapped (Figure 4.46).

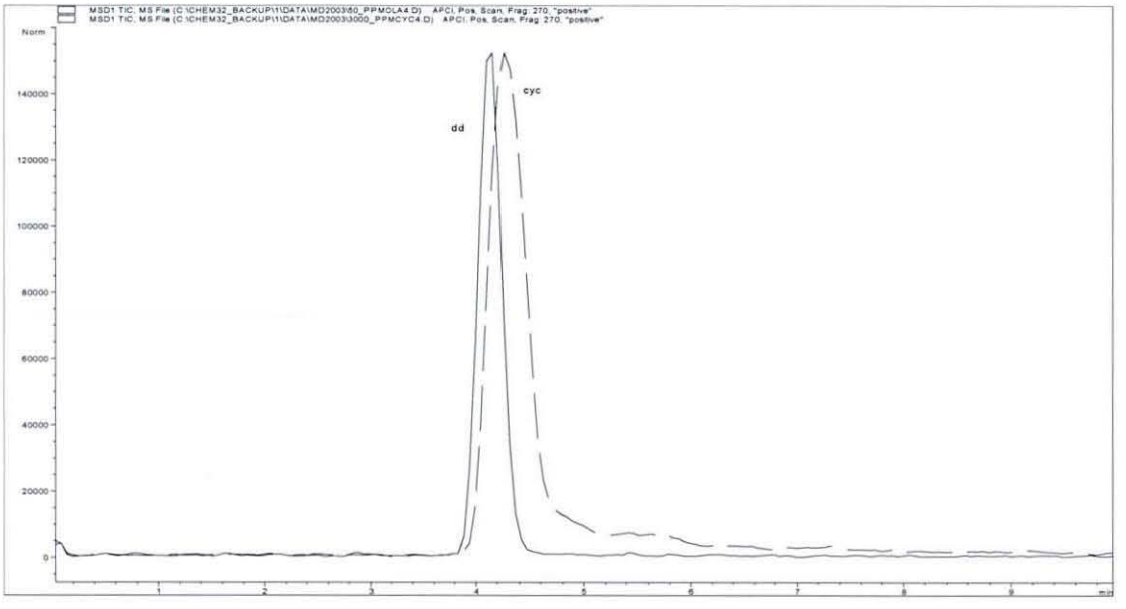


Figure 4.46: Overlaid mass chromatograms, obtained with the Spherisorb amino column (250 x 4.6) mm, 5 µm, at 80% 0.1% formic acid in MeCN/20% 50 mM ammonium formate (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 50 µg/ml. LC-MS: HP1100, equipped with APCI source.

The results obtained in this study were in agreement with the results obtained by Guo and Gaiki [134] and Olsen [122], who found that the retention of analytes decreased as the water content of mobile phase increased.

In the current study the MS analysis allowed the elution order of diamide diester and cyclic oligomers to be monitored. With 100% acetonitrile the longer chains were eluted before the shorter chains.

The same order was observed at 90% acetonitrile/10% ammonium formate (Figure 4.47 and Figure 4.48), with longer oligomers eluting before shorter oligomers, even though the order of elution of the dd and the cyc was reversed:

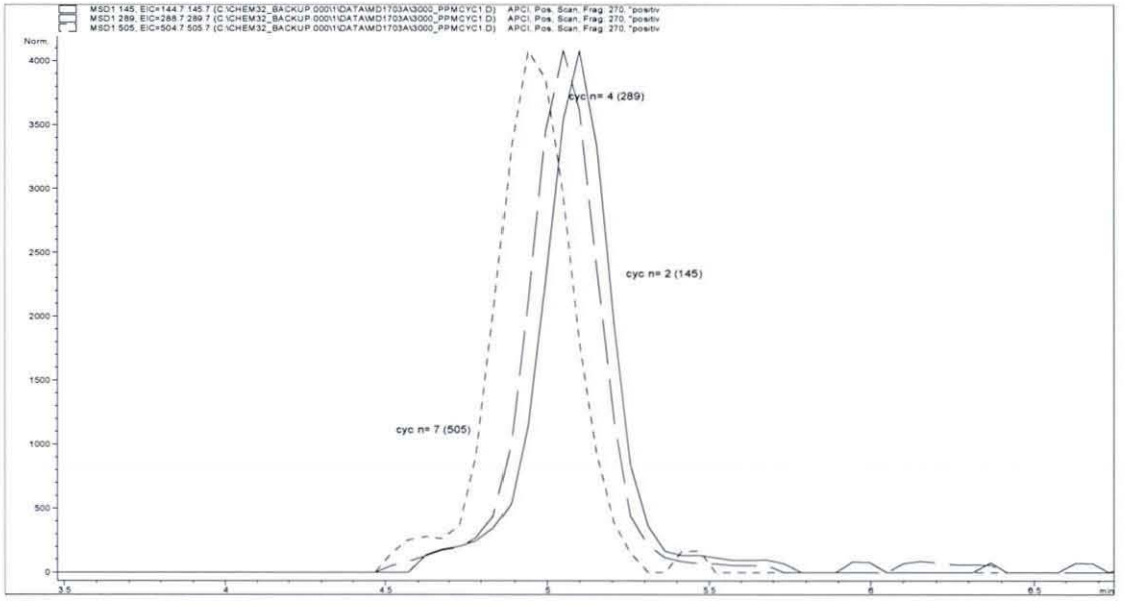


Figure 4.47: EICs (positive mode) for 300 mcg/ml OLA cyclic solution relative to Spherisorb amino column (250 x 4.6) mm, 5 µm, 90% 0.1% formic acid in MeCN /10% 50 mM Ammonium formate, (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. LC-MS: HP1100, equipped with APCI source.

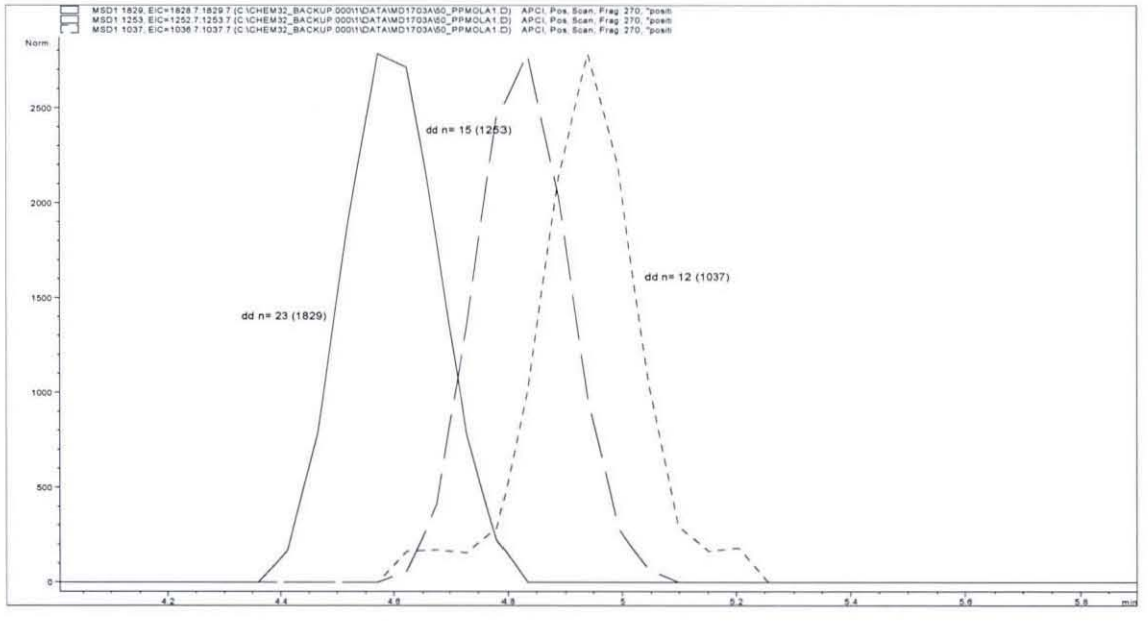


Figure 4.48: EICs (positive mode) for 50 mcg/ml OLA dd excipient solution relative to Spherisorb amino column (250 x 4.6) mm, 5 µm , 90% 0.1% formic acid in MeCN /10% 50 mM Ammonium formate, (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. LC-MS: HP1100, equipped with APCI source.

The oligomeric elution order was left unchanged by an increase in the percentage of ammonium formate to 20% or to 30% (Figure 4.49 and Figure 4.50).

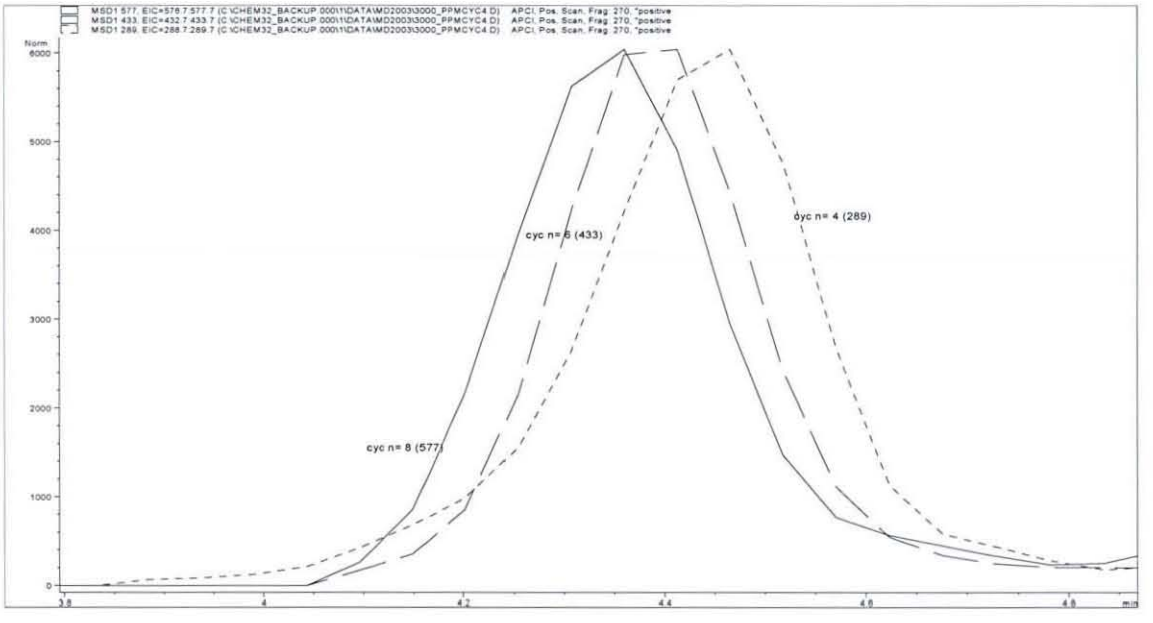


Figure 4.49: EICs (positive mode) for 300 mcg/ml OLA cyclic solution relative to Spherisorb amino column (250 x 4.6) mm, 70% 0.1% formic acid in MeCN /30% 50 mM Ammonium formate, (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. LC-MS: HP1100, equipped with APCI source.

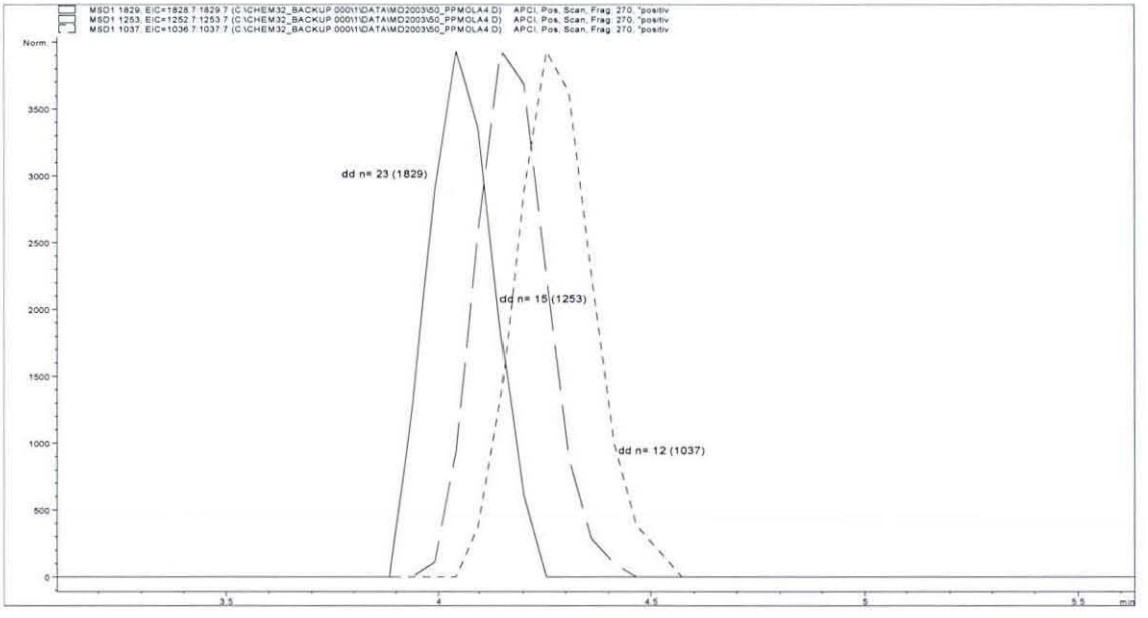


Figure 4.50: EICs (positive mode) for 50 mcg/ml OLA dd excipient solution relative to Spherisorb amino column (250 x 4.6) mm, 70% 0.1% formic acid in MeCN /30% 50 mM Ammonium formate, (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. LC-MS: HP1100, equipped with APCI source.

The observation of an elution order of longer OLAs in front of shorter OLAs was in agreement with the results obtained by Jandera *et al.* [92] with ethoxylated alcohol surfactants. The authors explained this phenomenon in terms of the different conformation of the longer and shorter oxyethylene (EO) chains in mobile phases containing small amount of water and on amino bonded materials.

Jandera and his group proposed that the oligomers would be adsorbed by the more polar oxyethylene parts of the molecules, with the chains oriented towards the mobile phase. The oligomers with lower number of EO units would be more retained because they would stick more closely to the adsorbent surface than the oligomers with longer chains.

From the MS data analysis of OLAs, it was concluded that the retention behaviour governing the NH2 stationary phase with ammonium formate/acetonitrile mobile phase was that of typical HILIC separations.

However, in light of the results obtained in the current study, using the amino material and ammonium formate/acetonitrile as mobile phase, it was concluded that this approach would not provide a suitable system for OLAs characterisation, due to the poor resolution between the dd and cyclics oligomers.

4.2.5.2.1. Acetone as organic solvent on the amino material

It was decided to replace acetonitrile with acetone (which, like acetonitrile is a polar aprotic solvent, although less polar than MeCN) and check whether the reduced polarity of the mobile phase would delay OLAs dd elution and increase their separation from the cyclic components.

However, no significant resolution between the cyclics and dd was observed (Figure 4.51).

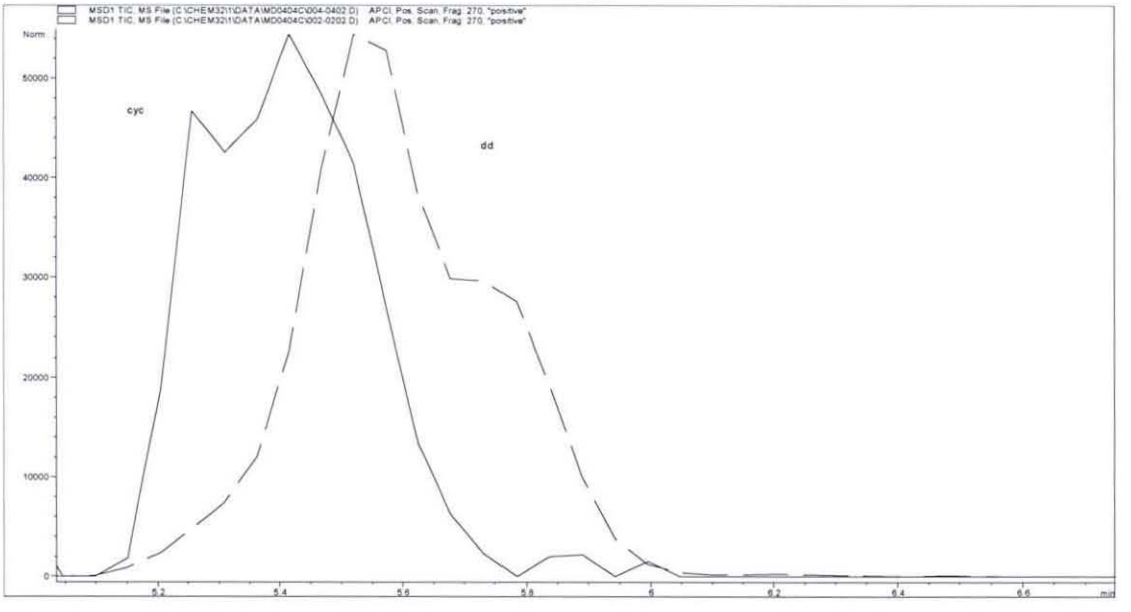


Figure 4.51: Overlaid mass chromatograms (expanded), obtained with Spherisorb NH2 column (250 x 4.6) mm, 5µm, at 100% 0.1% formic acid in acetone (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 510 µg/ml.

Various percentages of ammonium formate were added to the acetone, but no effect on the resolution between the two peaks was observed. Again, there was a reversal in elution order as more ammonium formate was added (Figure 4.52).

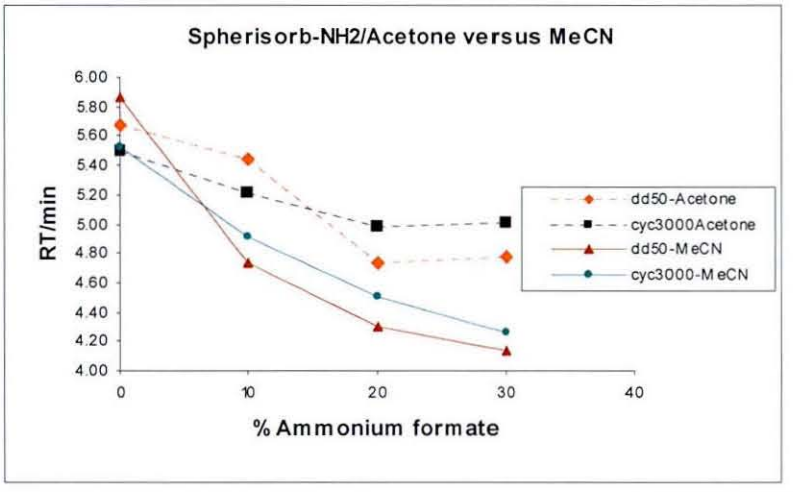


Figure 4.52: elution of cyc3000 (3000 µg/ml cyclic solution) and dd50 (50 µg/ml OLA solution) on the Spherisorb amino column (250 x 4.6) mm, 5µm. Injection volume: 20 µl; flow rate: 0.5 ml/min. Retention time variations are due to 50 mM ammonium formate solution addition to acetonitrile and acetone.

It was concluded that these mobile phases were not suitable for OLAs characterisation because dd excipient and cyclics peaks were insufficiently resolved.

4.2.5.2.2. The effect of coupling two amino columns on OLAs resolution

In order to increase the resolution two amino columns were connected in series. Using 100% acetonitrile, the cyclics (cyc) eluted before the diamide diester (dd) (Figure 4.53). A similar separation was achieved when coupling two amino columns in NP-HPLC, using 100% acetonitrile (figure 4.19).

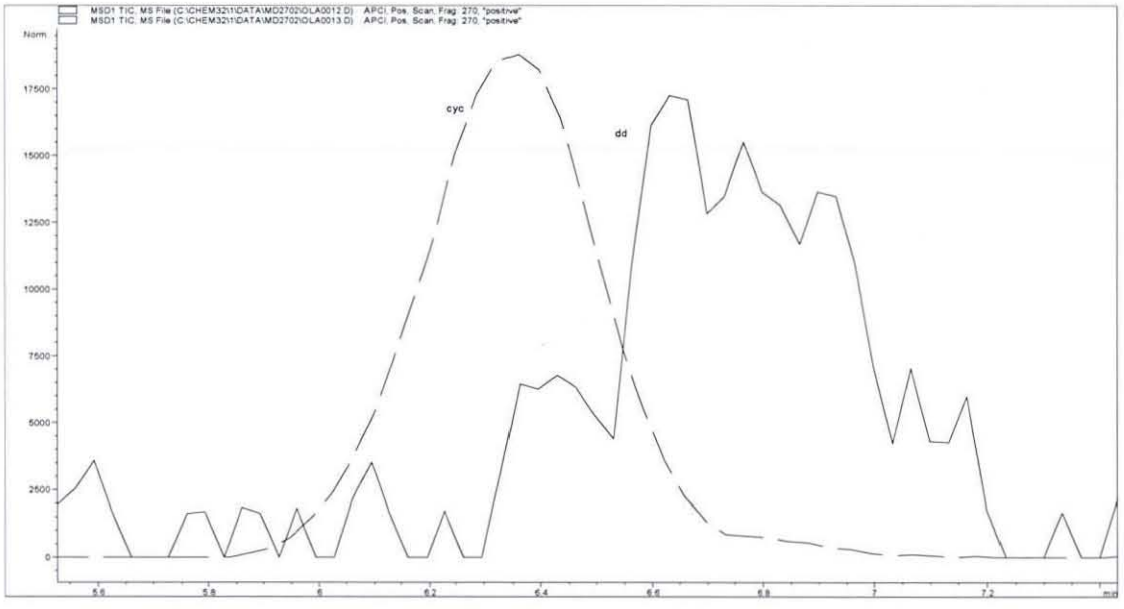


Figure 4.53: Overlaid mass chromatograms (expanded), obtained with NH2-NH2 columns, (250 x 4.6) mm, 5µm-(150 x 2) mm, 3 µm, at 100% 0.1% formic acid in MeCN (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 3000 µg/ml solution, diamide diester 5 µg/ml. LC-MS: HP1100, equipped with APCI source.

The polarity of acetonitrile was gradually increased, by adding 10, 20, 30 and 35% of 50 mM ammonium formate stock solution, and this reversed the elution order (Figure 4.54), with the cyclics eluting after the dd. Overall, increasing the aqueous proportion eluted the polar dd earlier, which was in agreement with HILIC retention behaviour.

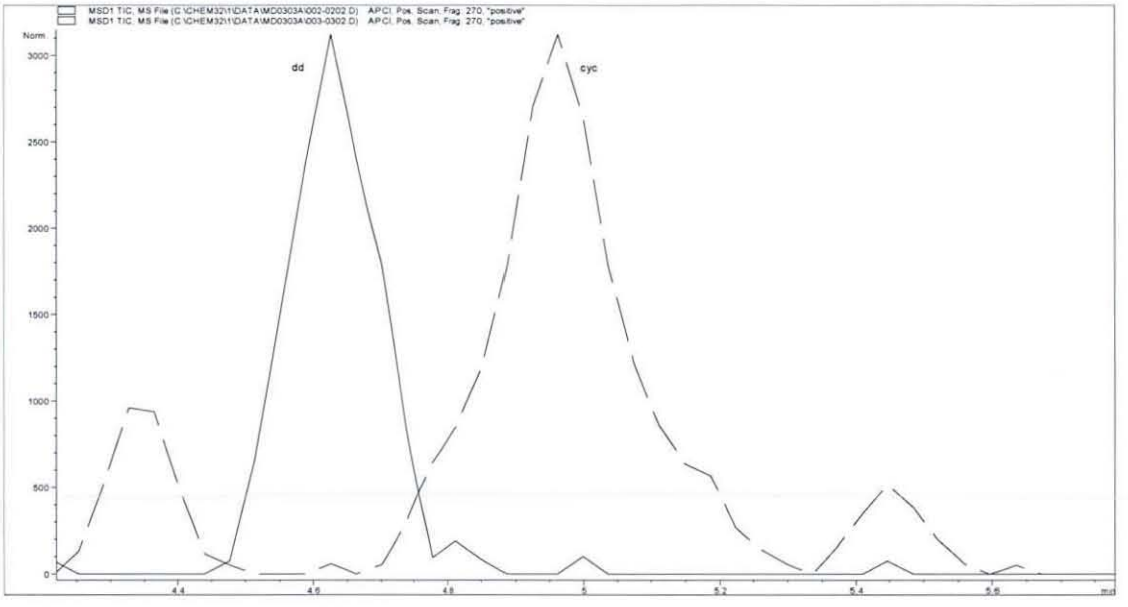


Figure 4.54: Overlaid mass chromatograms (expanded), obtained with NH2-NH2 columns, (250 x 4.6) mm, 5µm-(150 x 2) mm, 3 µm, at 25% 50 mM ammonium formate/75% 0.1% formic acid in MeCN, (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 300 µg/ml solution, diamide diester 5 µg/ml. LC-MS: HP1100, equipped with APCI source.

The MS data for the cyc and the dd oligomers indicated that the larger oligomers eluted earlier than the smaller oligomers (Figure 4.55 and Figure 4.56).

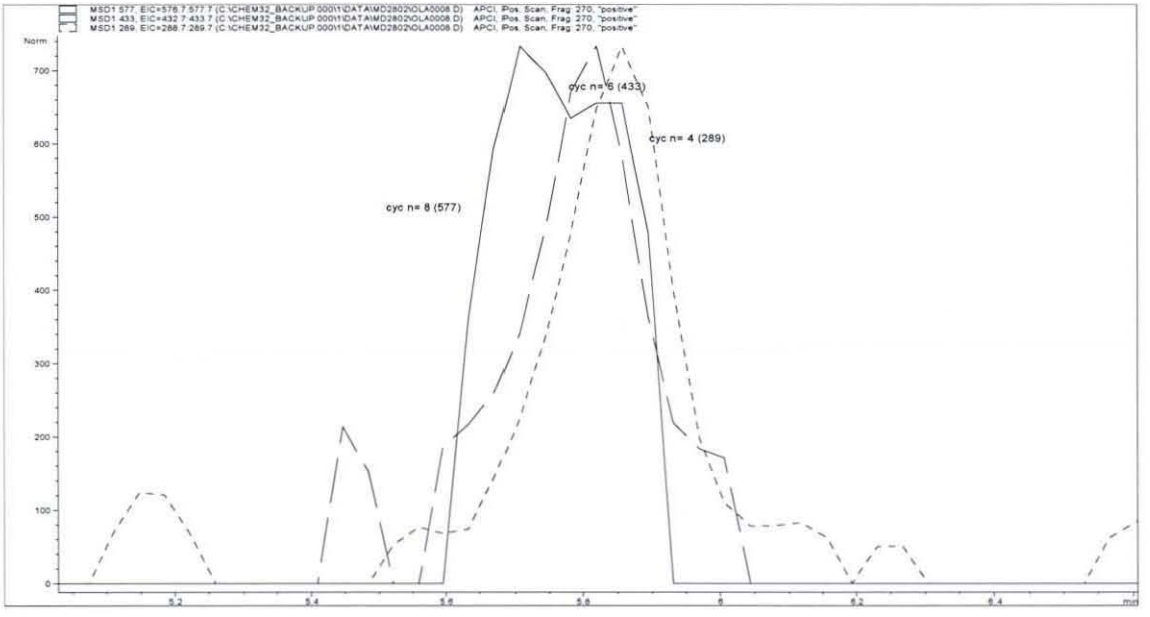


Figure 4.55: Extracted ion chromatograms (expanded), obtained with NH2-NH2 columns, (250 x 4.6) mm, 5µm-(150 x 2) mm, 3 µm, at 10% 50 mM ammonium formate/90% 0.1% formic acid in MeCN, (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. Cyclic 300 µg/ml solution.

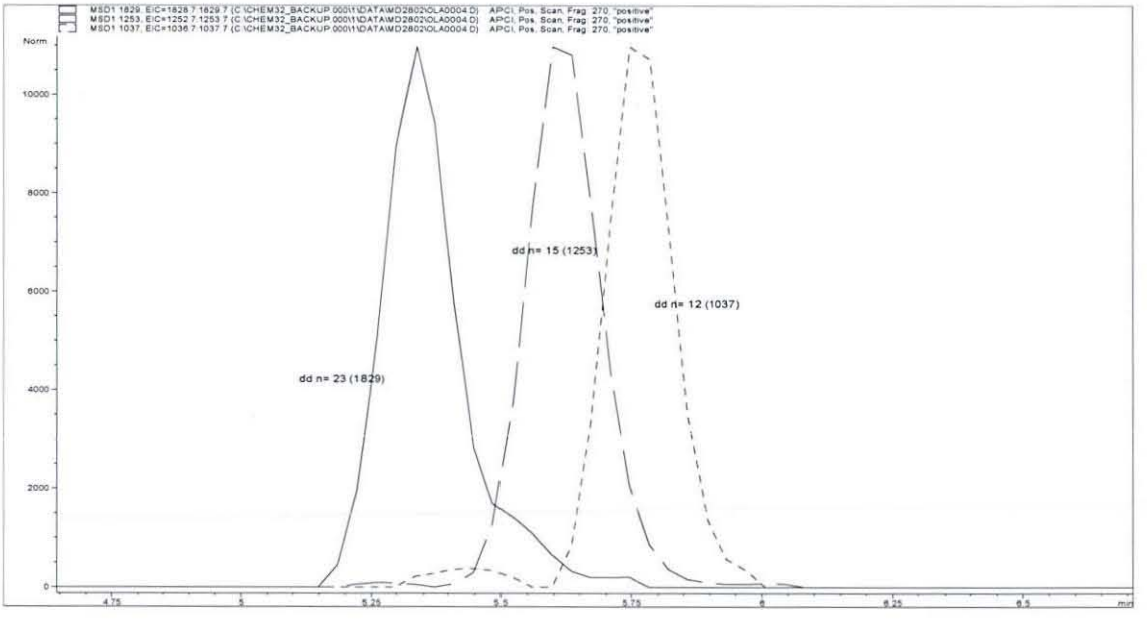


Figure 4.56: Extracted ion chromatograms (expanded), obtained with NH2-NH2 columns, (250 x 4.6) mm, 5µm-(150 x 2) mm, 3 µm, at 10% 50 mM ammonium formate/90% 0.1% formic acid in MeCN, (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. OLA dd 50 µg/ml solution.

4.2.6. Conclusions on retention of OLAs on amino and cyano materials in HILIC/ RP-HPLC

The retention changes of the OLAs with the changes in aqueous content on the amino and cyano columns are summarized in Figure 4.57.

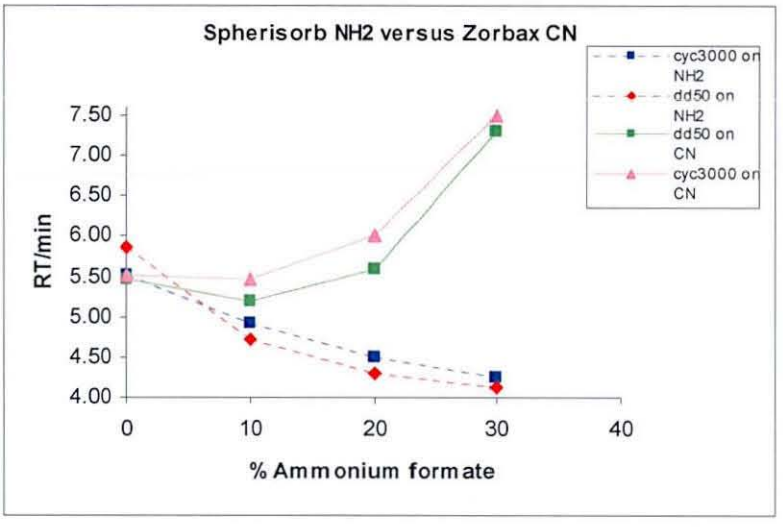


Figure 4.57: Change in retention times of cyc3000 (3000 µg/ml cyclic solution) and dd50 (50 µg/ml OLA excipient solution); Spherisorb amino column (250 x 4.6) mm, 5µm, and Zorbax cyano column (250 x 4.6) mm, 7µm, with proportion of 50 mM ammonium formate/0.1% formic acid in MeCN. LC-MS: HP1100, equipped with APCI source.

On both columns, the addition of 10% of the aqueous solution lead to a decrease in retention for OLAs and their impurities; a 20% aqueous/80% organic led to an increase in retention of both cyclics and dd on the cyano column and a decrease on the amino stationary phase (typical HILIC behaviour shown by the amino sorbent: decreasing retention with increasing aqueous content in mobile phase). Thus, the cyano column provided a HILIC environment when less than 10% ammonium formate was present in the mobile phase, (retention values decreased with increasing mobile phase aqueous content). The cyano column behaved as a RP-HPLC column when the mobile phase contained more than 10% ammonium formate (retention values increased with increasing mobile phase aqueous

content). MS data confirmed the cyano material 'dual' behaviour and at 10% ammonium formate (Figure 4.36) the longer OLAs diamide diester oligomers eluted in front of the shorter oligomers. MS data obtained at 30% ammonium formate (Figure 4.39), showed the longer oligomers eluting after the shorter oligomers. Increasing the aqueous content of mobile phase eluting on the amino stationary phase caused a decrease in OLAs retentions. These results were in agreement with the results obtained by Oyler *et al.* [124] and by Olsen [122].
There was also a reversal in the order of elution of cyclics and main excipient (dd) on addition of an aqueous component to the mobile phase.
The difference in retention between dd and cyc were slightly higher with the cyano than with the amino column, leading to a better separation selectivity.

Coupling two amino columns added extra retention power to the system (Figure 4.58), and as with one column, the addition of ammonium formate to acetonitrile caused a switch in the retention behaviour of cyclics and dd excipients.

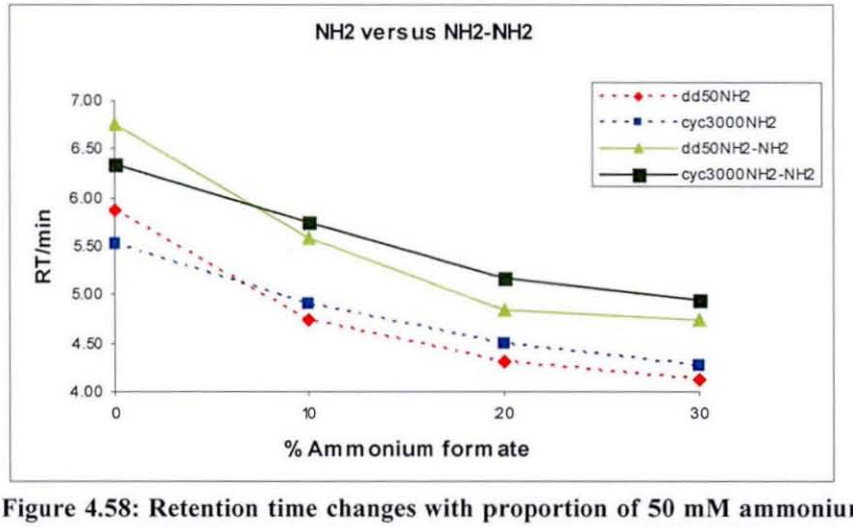


Figure 4.58: Retention time changes with proportion of 50 mM ammonium formate solution addition to 0.1% formic acid in acetonitrile for the cyc3000 (3000 µg/ml cyclic solution) and dd50 (50 µg/ml OLA solution) on the Spherisorb amino column (NH2) and on the Luna and Spherisorb columns (250 x 4.6) mm, 5µm-(150 x 2) mm, 3 µm (NH2-NH2). Injection volume: 20 µl; flow rate: 0.5 ml/min.

Generally the aqueous mobile phase on the cyano column favoured elution of the more hydrophilic dd, with the cyclics being more retained. Thus the cyano stationary phase with acetonitrile/ammonium formate as mobile phase proved capable of separating OLAs main oligomers and their cyclic impurities, in terms of their functional groups, providing critical conditions of elution.

4.3. Effects of buffer salt concentration in HILIC

In the light of the work of Guo and Gaiki [134], who showed that an increase in buffer concentration caused an increase in retention of polar analytes, it was considered important to determine whether OLAs selectivity would be affected by variations in buffer concentration and whether exchanging ammonium formate with water would cause variations on their retention behaviour.

Ammonium formate was of interest because it is compatible with MS detection and because of its high solubility at high organic levels. However, in order to check whether the level of organic affects the salt solubility, the MeCN content was varied from 100 to 65% by adding 0, 10, 15, 20, 25, 30 and 35% of the ammonium formate solutions. Both the water and ammonium formate (at different concentrations) showed similar effects on the retention of OLAs on the Zorbax cyano column (Figure 4.59).

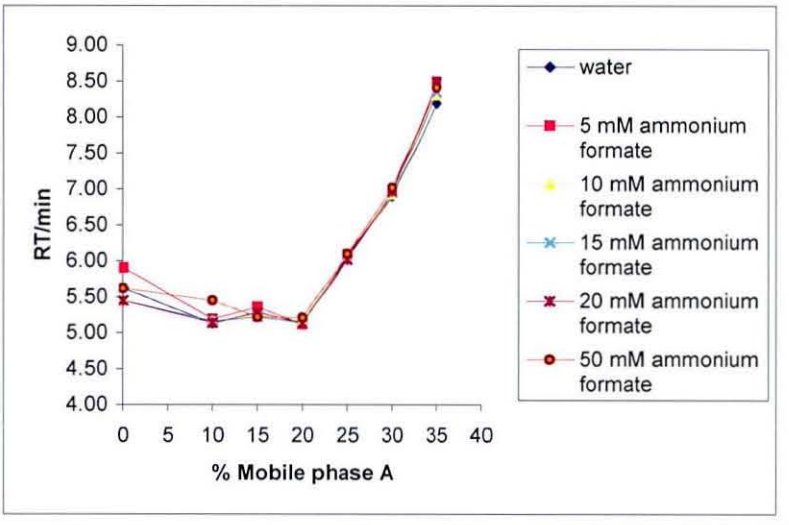


Figure 4.59: Retention times of OLAs with several ratios of mobile phase A/ 0.1% formic acid in MeCN. Column: Zorbax CN column (250 x 4.6) mm, 7µm, injecting 20 µl of 5 µg/ml OLAs main excipient solution; flow rate: 0.5 ml/min. Runs were isocratic. LC-MS: HP1100, equipped with APCI source.

An initial increase to 10% of either water or ammonium formate solution caused a decrease in retention. These results agreed with earlier work by Guo and Gaiki [134], who observed that polar analytes exhibited typical HILIC behaviour on decreasing retentions with increasing water content in the mobile phase and by Jandera *et al.* [135], who reported that (co)oligomers experienced an increase in retention when the aqueous content of the mobile phase was decreased. The MS data analysis of OLAs dd showed that longer dd chains eluted in front of shorter chains. The more polar shorter chains were more retained.

Aqueous solution or ammonium formate solutions up to 20% caused a generally small increase in retention, apart from 50 mM ammonium formate. MS data (20% water or ammonium formate) showed co-elution of oligomers, therefore indicating critical conditions of elution (Figure 4.60 and Figure 4.61).

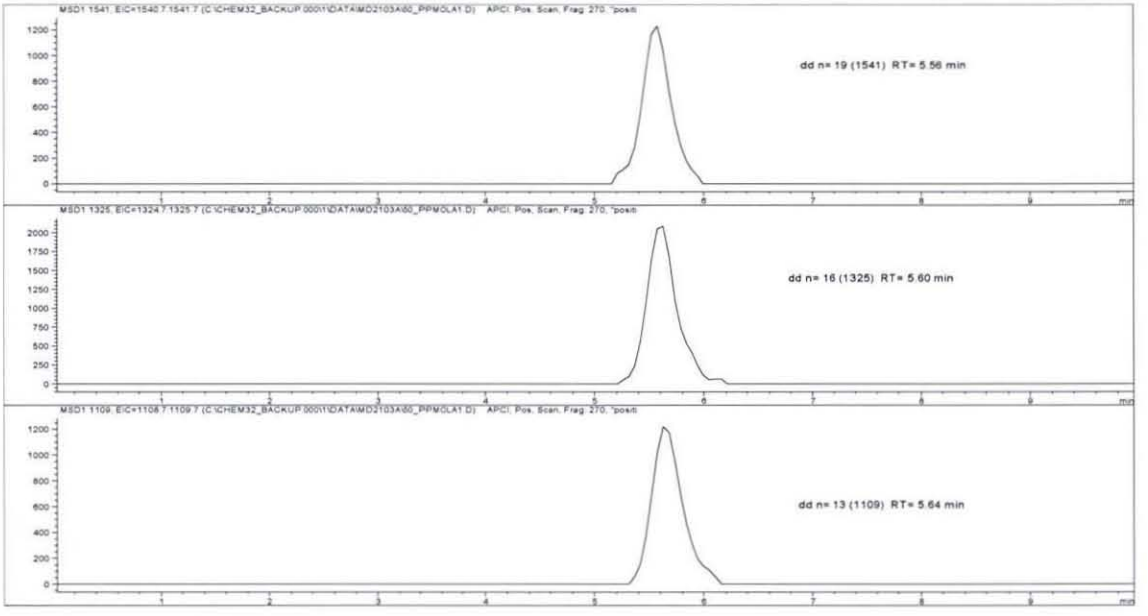


Figure 4.60: EICs (positive mode) for 50 mcg/ml OLA dd excipient solution relative to Zorbax cyano column (250 x 4.6) mm, 7µm, 80% 0.1% formic acid in MeCN /20% 50 mM Ammonium formate, (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. LC-MS: HP1100, equipped with APCI source.

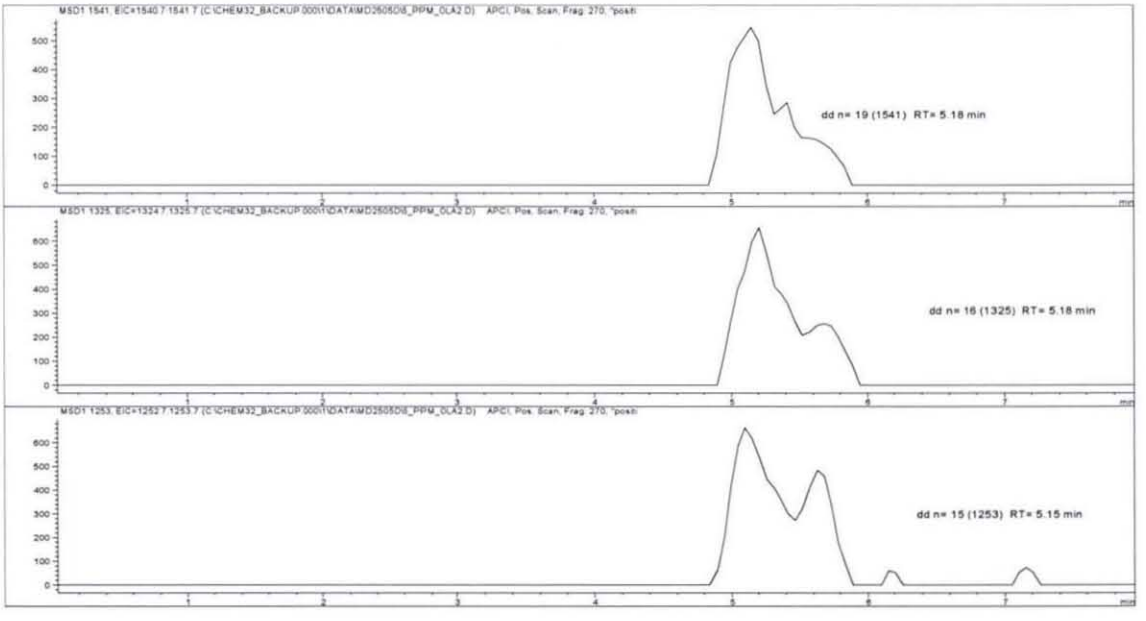


Figure 4.61: EICs (positive mode) for 50 mcg/ml OLA dd excipient solution relative to Zorbax cyano column (250 x 4.6) mm, 7µm, 80% 0.1% formic acid in MeCN /20% 0.1% formic acid in water, (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. LC-MS: HP1100, equipped with APCI source.

Above 20% aqueous or ammonium formate solutions the retention increased. This is typical RP-HPLC behaviour: increasing retention values with higher aqueous content; MS data (Figure 4.62 for dd eluting with 30% 50 mM ammonium formate and Figure 4.63 for dd eluting with 30% water) confirmed a RP mechanism, as shorter chains were eluting in front of longer chains, suggesting that a selectivity change had occurred. These results contrasted with Guo and Gaiki [134], Jandera *et al.* [135], Oyler *et al.* [124] and Olsen [122], who found decreasing retention values with higher aqueous content.

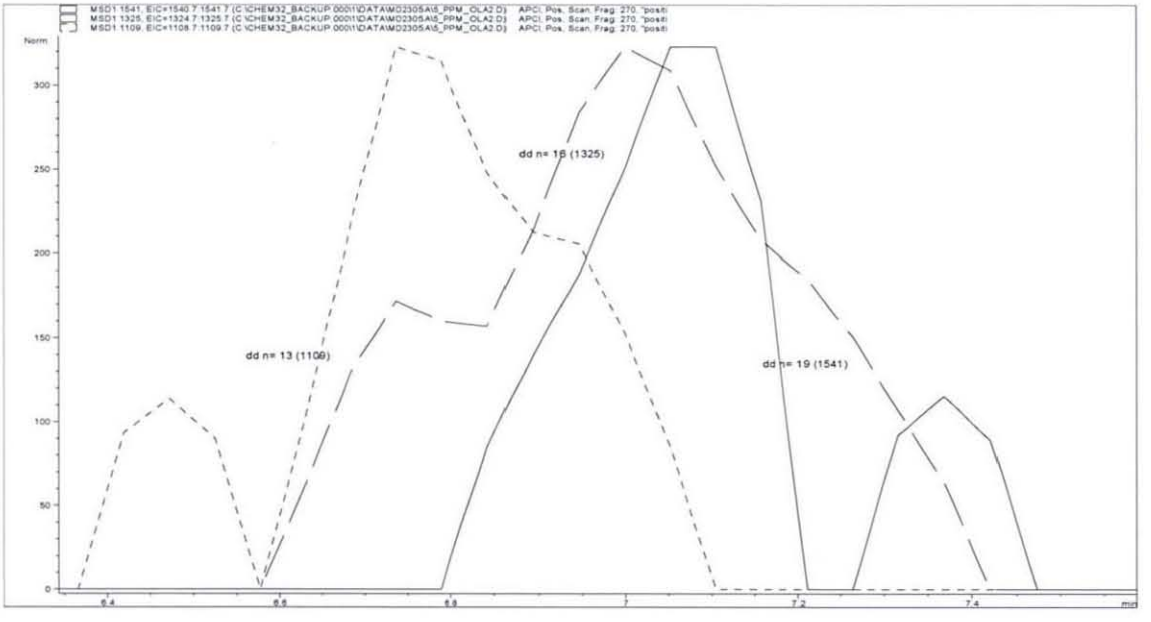


Figure 4.62: EICs (positive mode) for 50 mcg/ml OLA dd excipient oligomers on Zorbax cyano column (250 x 4.6) mm, 7µm, 70% 0.1% formic acid in MeCN /30% 50 mM Ammonium formate, (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. LC-MS: HP1100, equipped with APCI source.

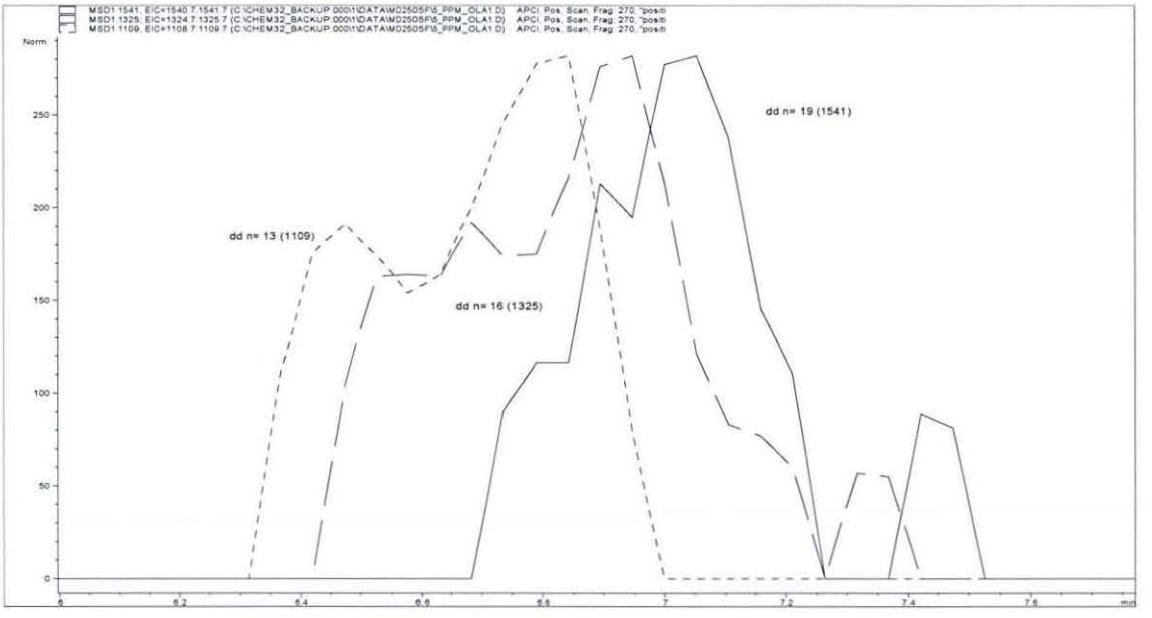


Figure 4.63: EICs (positive mode) for 50 mcg/ml OLA dd oligomers on Zorbax cyano column (250 x 4.6) mm, 7µm, 70% 0.1% formic acid in MeCN /30% 0.1% formic acid in water, (positive ionisation, each signal in full scale). Injection volume: 20 µl; flow rate: 0.5 ml/min. LC-MS: HP1100, equipped with APCI source.

The inversion in elution order of OLA oligomers (passing 'through' critical conditions of elution), with a change in mobile phase composition was in agreement with the results obtained by Lemr *et al.* [83] for the separation of FAEs in RP-HPLC. At low MeCN percentage, FAEs with a large number of ethyleneoxide (EO) units eluted first, but the elution order was inverted (i.e. small number of EO units eluted first) at high MeCN percentage. At intermediate MeCN percentage they observed co-elution of FAEs, independent of EO unit chain length. Jandera *et al.* [92] also reported that a combined effect of the mobile phase polarity and the solvation of EO groups took place.

Salt concentration did not have a major effect on the elution strength of MeCN, for the OLA dd oligomers on a Zorbax cyano material (Figure 4.64). This was in disagreement with Guo and Gaiki [134] who found an increase in retention (of small polar compounds) as the concentration of ammonium acetate in the mobile

phase was increased from 5 to 20 mM on TSK gel Amide-80, HILIC Silica and ZIC-HILIC. When the authors tested an YMC-Pack NH2, they observed a decrease in retention for salicylic acid and aspirin and an increase in retention for cytosine when the concentration of ammonium acetate was increased from 5 to 20 mM.

They concluded that, as the effect was seen on stationary phases with different functional groups, it could have been related to hydrophilic partitioning process instead of specific interactions between the stationary phase and analytes. This was based on the proposed hydrophilic partitioning model for HILIC [113], where it is assumed that high levels of organic content in the mobile phase make the salt prefer to be in the water-rich liquid layer and this would consequently result in an increase in volume of the liquid layer, leading to stronger retention of the analytes.

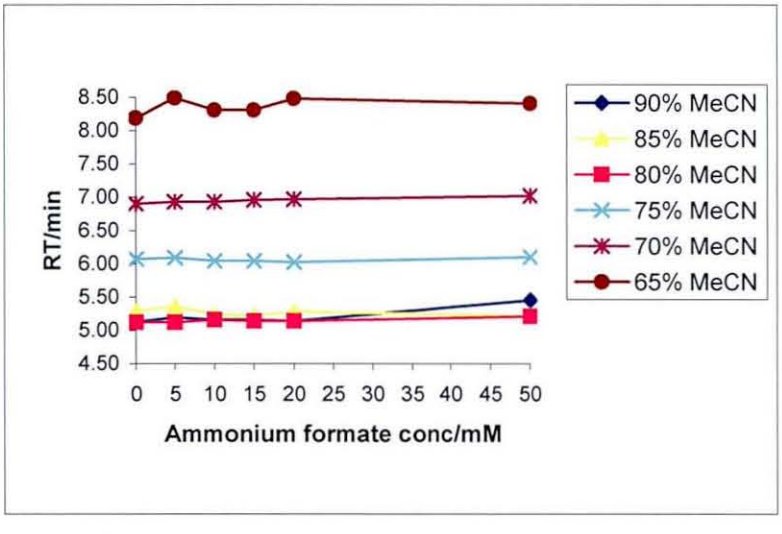


Figure 4.64: Comparison of eluting strength of ammonium formate/MeCN mixtures. Chromatography obtained on a Zorbax CN column (250 x 4.6) mm, 7µm, injecting 20 µl of 5 µg/ml OLAs main excipient solution; flow rate: 0.5 ml/min. LC-MS: HP1100, equipped with APCI source.

The current study also showed that on a Zorbax cyano column, HILIC retention behaviour (of decreasing OLAs retention with increasing aqueous content) changed to RP-HPLC retention behaviour (of increasing OLAs retention with increasing aqueous content), passing through critical conditions of elution (oligomeric co-elution of chain of same size).

4.4. Conclusions on the head group distribution approach by LCCC

The potential second dimension for a two-dimensional separation of OLAs was therefore possible by separating the main oligomers from their cyclic impurities according to their amide head groups, by operating at or near to critical conditions. Using a CN stationary phase and an aqueous acetonitrile mobile phase (20% aqueous/80% acetonitrile), at 30° C, led to a suppression of the oligomeric distribution.

Since the initial aim of this work was to apply a two-dimensional approach to the analysis of OLAs, the next step would be coupling the first dimension RP-HPLC based on chain length with the second dimension LCCC, so that each oligomer would be separated respectively in terms of its chain size and its head groups.

However, it was uncertain whether a two-dimensional analysis would considerably improve the separation of OLAs and cyclic oligomers, or whether the necessary selectivity changes could be brought about on one-dimension system. Since the study on the effects of buffer salt concentration in mobile phase had shown that the level of aqueous solution was the factor with highest influence on OLAs

selectivity, it was considered whether a change in the choice of solvent used as mobile phase would be more effective than adding a second dimension column.

5. Results and discussion: the effect of the mobile phase and temperature on the separation of diamide diester and cyclic oligomers

Rather than employing a two dimensional separation, which could be time consuming because of the complexity of the oligomeric mixture, it was decided to investigate whether the resolution of the two groups could be obtained by altering the mobile phase composition or temperature or the length of the side chain on the RP-stationary phase.

5.1. Effects of solvent type on OLAs selectivity

The separation selectivity for the two most widely used organic modifiers in LC, acetonitrile and methanol, were examined under gradient conditions, using a Symmetry C_4 column. Although earlier (Chapter 3) it had been found that a water and acetonitrile gradient separated the individual oligomers, this eluent did not resolve the cyclic impurity oligomers and main OLA oligomers (Figure 5.1). The EICs, reported in Figure 5.1, show three dd and cyclic oligomers eluting within the same retention window (7.2-8.6 min).

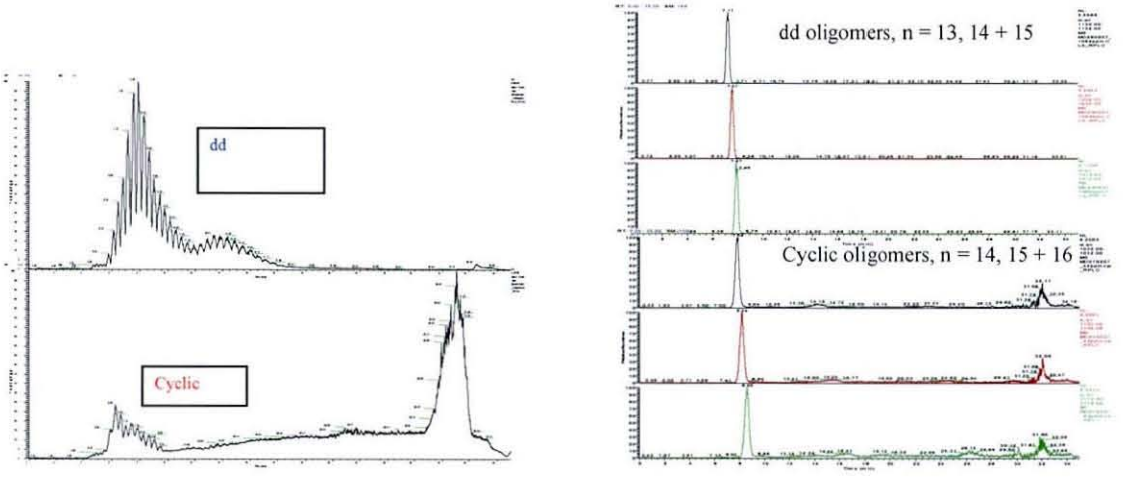


Figure 5.1: OLAs mass chromatograms and single ion chromatograms, obtained on LC-MS: Thermo Finnigan LTQ, equipped with ESI source. Injection volume: 10 µl; OLAs dd solution concentration: 1082.0 µg/ml; OLA cyclic solution concentration: 42 µg/ml; chromatographic column: Symmetry C_4 (150 x 4.6) mm, 5 µm; mobile phase A: 0.1% formic acid in water, mobile phase B: 0.1% formic acid in MeCN; starting conditions: 60% mobile phase B, taken to 70% in 25 min and then ramped to 100% at 30 min; LC flow rate: 0.5 ml/min.

However, if MeOH was used as the organic modifier instead of MeCN, the main oligomers started to elute later than in MeCN, at about 12 min, leading to separation between OLAs and their cyclic impurities (which were still eluting between 6-10 min) (Figure 5.2). An oligomeric separation was also obtained.

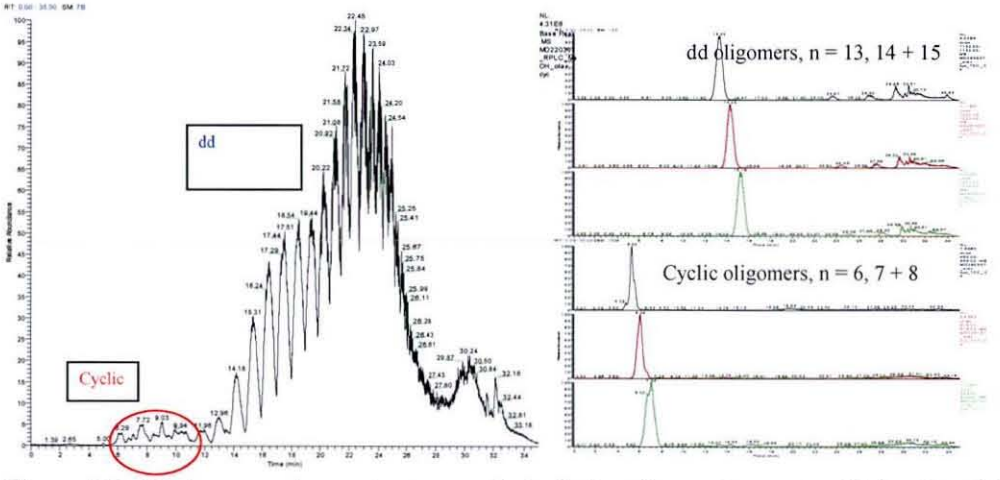


Figure 5.2: OLAs mass chromatogram and single ion chromatograms, obtained on LC-MS: Thermo Finnigan LTQ, equipped with ESI source. Injection volume: 10 µl; OLAs solution containing a 50:50 mixture of dd solution (1082.0 µg/ml) and cyclic solution (42 µg/ml); chromatographic column: Symmetry C_4 (150 x 4.6) mm, 5 µm; mobile phase A: 0.1% formic acid in water, mobile phase B: 0.1% formic acid in MeOH; starting conditions: 50% mobile phase B, taken to 70% in 25 min and then ramped to 100% at 30 min; LC flow rate: 0.5 ml/min.

A similar change was reported by Jandera [92], who ascribed the effect to different solvation effects in MeOH-water and in MeCN-water mobile phases, during the analysis of ethoxylated surfactants. The oxyethylene groups were thought to be subjected to proton-acceptor interactions with OH groups of water and methanol, methanol having good hydrogen bond-donating capability. These interactions were not possible with acetonitrile, having weak hydrogen bond-accepting ability. This difference between methanol and acetonitrile in RP-HPLC of ethoxylated surfactants was also described by Rissler *et al.* [80-82].

In Figure 5.2 it can be appreciated that the chromatography exhibits a decrease in band spacing for the later dd oligomers. This is possibly due to the steeper later

gradient. Reducing its steepness could lead to an increase in resolution for the later components. The resolution of oligomers could also be improved, possibly to baseline level, by coupling two C4 columns in series. This line of work should be investigated in future work, if quantitative analysis of OLAs is required.

It was also noticed that in the methanol-water separation (Figure 5.2), the cyclic sample was showing a late 'peak', between 28-33 min. A similar shoulder is also apparent in the acetonitrile-water separation (Figure 5.1) of the mixture of the two oligomers. MS analysis (Figures 5.3-5.5) identified these peaks as being due to multiply charged ions $[M + nNa]^{n+}$, of very long chain free acid OLAs (Figure 1.3) (Table 5.1). These long chain entities were being formed during the synthesis of the cyclic oligomers, in which OLA polymerisation was taken to a high level to enable a sufficiently high yield [3].

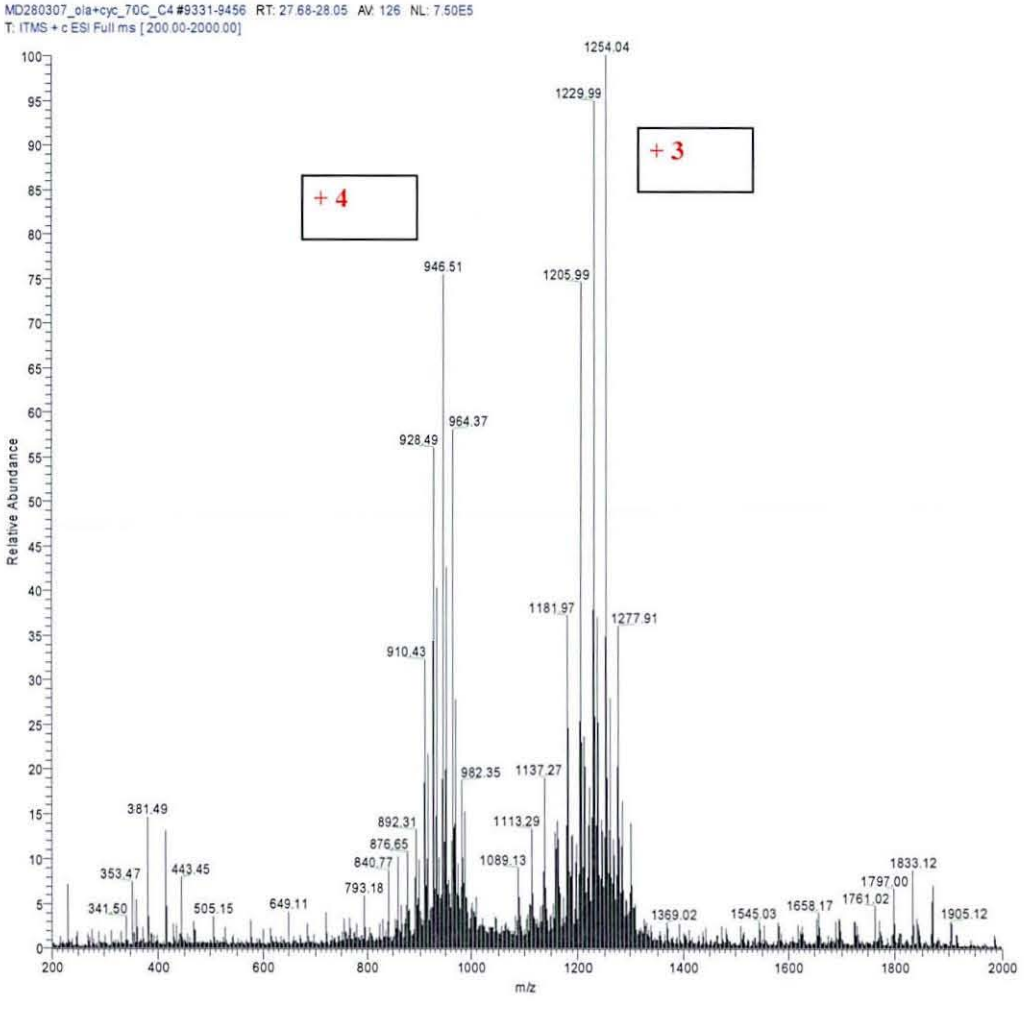


Figure 5.3: MS of late peaks in Figure 5.1, taken between 27.7-28.1 min. First series: 18 units difference = quadruply charged free chain ions OLAs (highlighted in orange in Table 5.1), corresponding to oligomers 49-52. Second series: 24 units difference = triply charged free chain ions OLAs (highlighted in green in Table 5.1), corresponding to oligomers 48-52.

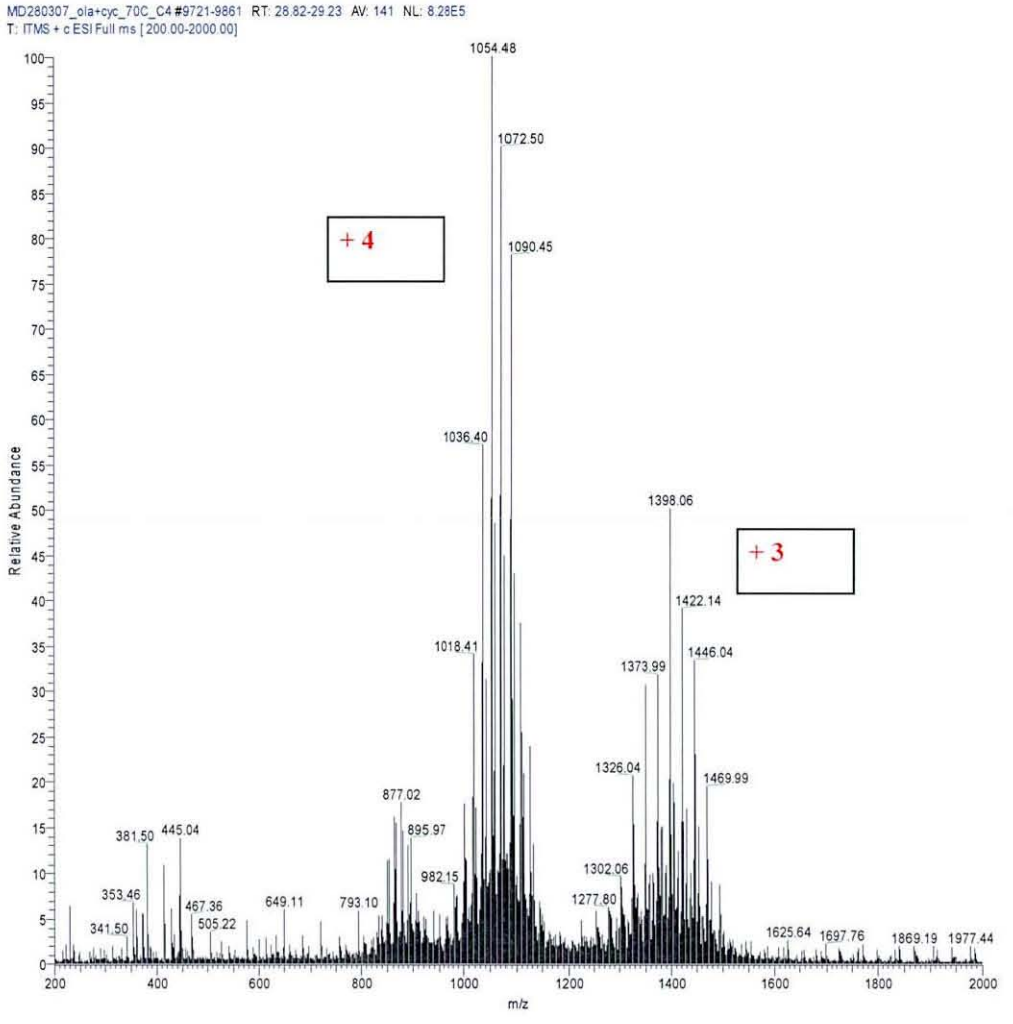


Figure 5.4: MS of late peaks in Figure 5.1, taken between 28.8-29.2 min. First series: 18 units difference = quadruply charged free chain ions OLAs (highlighted in red in Table 5.1), corresponding to oligomers 56-59. Second series: 24 units difference = triply charged free chain ions OLAs (highlighted in lime green in Table 5.1), corresponding to oligomers 54-60.

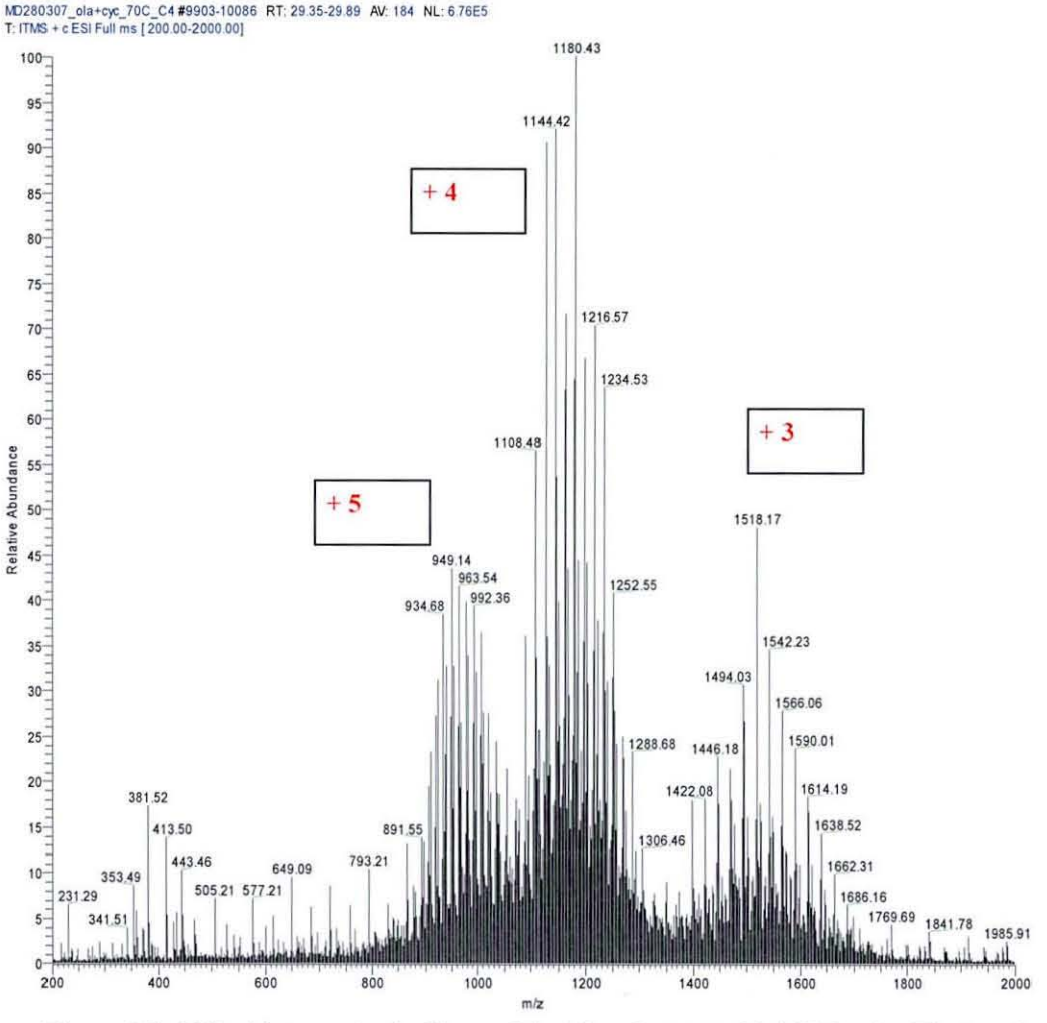


Figure 5.5: MS of late peaks in Figure 5.1, taken between 29.4-29.9 min. First series: 14.4 units difference = penta charged free chain ions OLAs (highlighted in pink in Table 5.1), corresponding to oligomers 60-67. Second series: 18 units difference = quadruply charged free chain ions OLAs (highlighted in mauve in Table 5.1), corresponding to oligomers 60-68. Third series: 24 units difference = triply charged free chain ions OLAs (highlighted in blue in Table 5.1), corresponding to oligomers 61-67.

Table 5.1: Masse to charge ratios (m/z) of multiply charged ions $[M + nNa]^{n+}$ relative to free acid OLAs. Masses highlighted in various colours refer to oligomer masses reported in mass spectra (Figures 5.3/5.5).

n	free acid	free acid +23	doubly charged+2 Na (72/2=36)	triply charged+3Na (72/3=24)	quadruply charged+4Na (72/4=18)	penta charged+5Na (72/5=14.4)
40	2898	2921	1472.0	989.0	747.5	602.6
41	2970	2993	1508.0	1013.0	765.5	617.0
42	3042	3065	1544.0	1037.0	783.5	631.4
43	3114	3137	1580.0	1061.0	801.5	645.8
44	3186	3209	1616.0	1085.0	819.5	660.2
45	3258	3281	1652.0	1109.0	837.5	674.6
46	3330	3353	1688.0	1133.0	855.5	689.0
47	3402	3425	1724.0	1157.0	873.5	703.4
48	3474	3497	1760.0	1181.0	891.5	717.8
49	3546	3569	1796.0	1205.0	909.5	732.2
50	3618	3641	1832.0	1229.0	927.5	746.6
51	3690	3713	1868.0	1253.0	945.5	761.0
52	3762	3785	1904.0	1277.0	963.5	775.4
53	3834	3857	1940.0	1301.0	981.5	789.8
54	3906	3929	1976.0	1325.0	999.5	804.2
55	3978	4001	2012.0	1349.0	1017.5	818.6
56	4050	4073	2048.0	1373.0	1035.5	833.0
57	4122	4145	2084.0	1397.0	1053.5	847.4
58	4194	4217	2120.0	1421.0	1071.5	861.8
59	4266	4289	2156.0	1445.0	1089.5	876.2
60	4338	4361	2192.0	1469.0	1107.5	890.6
61	4410	4433	2228.0	1493.0	1125.5	905.0
62	4482	4505	2264.0	1517.0	1143.5	919.4
63	4554	4577	2300.0	1541.0	1161.5	933.8
64	4626	4649	2336.0	1565.0	1179.5	948.2
65	4698	4721	2372.0	1589.0	1197.5	962.6
66	4770	4793	2408.0	1613.0	1215.5	977.0
67	4842	4865	2444.0	1637.0	1233.5	991.4
68	4914	4937	2480.0	1661.0	1251.5	1005.8
69	4986	5009	2516.0	1685.0	1269.5	1020.2
70	5058	5081	2552.0	1709.0	1287.5	1034.6
71	5130	5153	2588.0	1733.0	1305.5	1049.0
72	5202	5225	2624.0	1757.0	1323.5	1063.4
73	5274	5297	2660.0	1781.0	1341.5	1077.8
74	5346	5369	2696.0	1805.0	1359.5	1092.2

Because of these results, which highlighted the presence of long chain free acid molecules in the cyclic impurity standard, there was concern over whether the separation recorded using the total ion current between main oligomers and cyclics obtained on the CN column, at critical conditions of elution (20% aqueous/80% acetonitrile, 30 °C) would have shown a greater resolution if the cyclic solution had not contained the long chain free acid components.

5.1. Effects of stationary phase on OLAs selectivity

Having obtained a reasonable separation of cyclics and OLAs on a Symmetry C_4 column, it was decided to determine if a higher resolution could be obtained using a longer bonded alkyl chain. The separation was then repeated on an Eclipse C_8 column using a water and methanol gradient.

Again the cyclics were eluted before the OLAs, as shown in Figure 5.6:

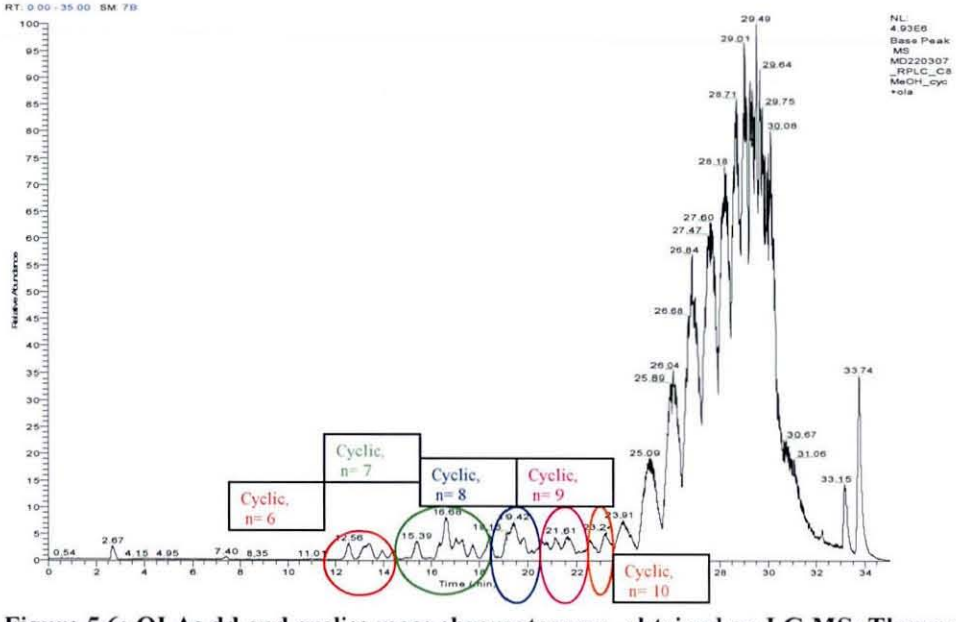


Figure 5.6: OLAs dd and cyclics mass chromatogram, obtained on LC-MS: Thermo Finnigan LTQ, equipped with ESI source. Injection volume: 10 µl; OLAs solution containing a 50:50 mixture of dd solution (1082.0 µg/ml) and cyclic solution (42 µg/ml); chromatographic column: Eclipse C_8 (150 x 4.6) mm, 5 µm; mobile phase A: 0.1% formic acid in water, mobile phase B: 0.1% formic acid in MeOH; starting conditions: 50% mobile phase B, taken to 70% in 25 min and then ramped to 100% at 30 min; LC flow rate: 0.5 ml/min. Cyclic oligomers circled in different colours, each circle represents an oligomer; red corresponds to n =6, green corresponds to n = 7, blue corresponds to n = 8, pink corresponds to n = 9 and orange corresponds to n = 10).

Overall the C_8 column was more retentive than the C_4 column. The cyclics eluted between 12-23 min (6-10 min on C_4 column); dd oligomers started to elute at about 24 min on the C_8 column and at about 12 min on the C_4 column.

However, the main dd oligomers lost their clear oligomeric resolution (Figure 5.6), possibly because they were being eluted with a higher concentration of methanol at a later stage of the gradient profile. MS analysis confirmed the presence of about 3 oligomers in each chromatographic peak (Figure 5.7). This suggested that flatter gradient would be preferable. In polymers/oligomers gradient elution is typically observed that small changes in mobile phase composition have a large effect on retention.

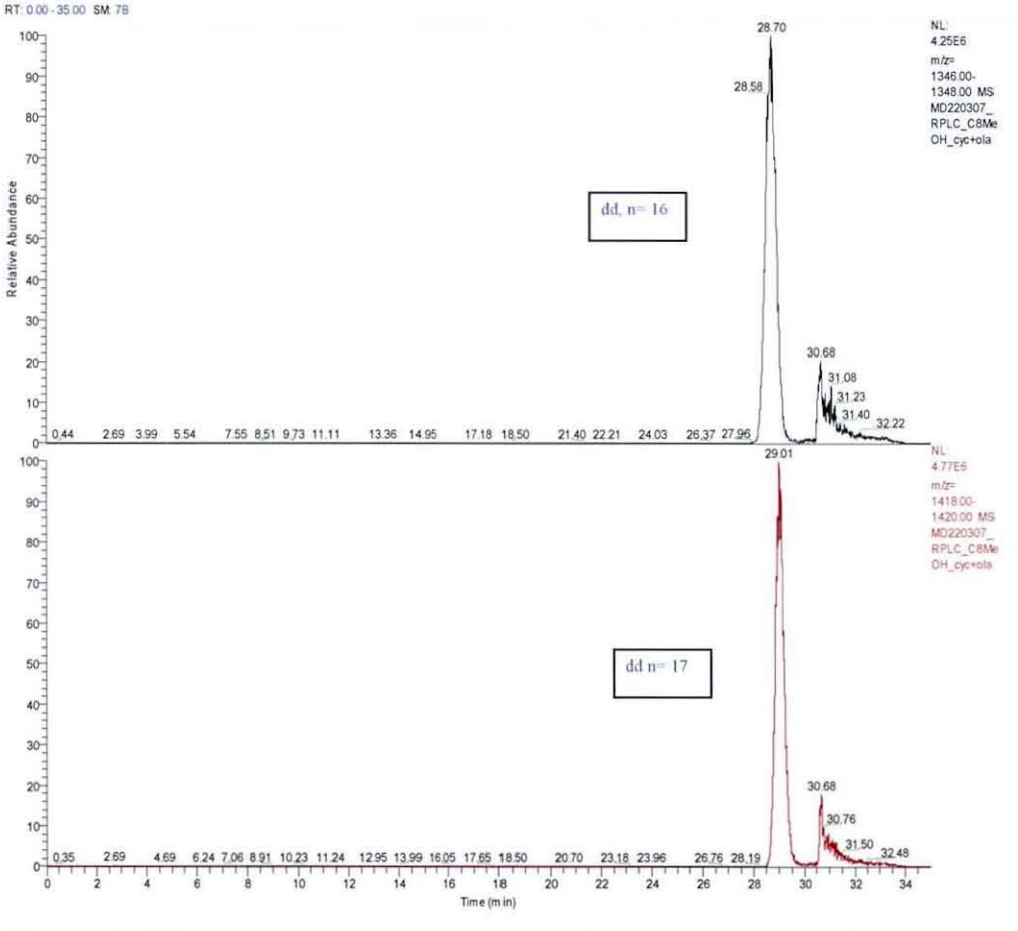


Figure 5.7: EICs of OLAs, obtained on LC-MS: Thermo Finnigan LTQ, equipped with ESI source. Injection volume: 10 µl; OLAs solution containing a 50:50 mixture of dd solution (1082.0 µg/ml) and cyclic solution (42 µg/ml); chromatographic column: Eclipse C_8 (150 x 4.6) mm, 5 µm; mobile phase A: 0.1% formic acid in water, mobile phase B: 0.1% formic acid in MeOH; starting conditions: 50% mobile phase B, taken to 70% in 25 min and then ramped to 100% at 30 min; LC flow rate: 0.5 ml/min. Dd oligomers n = 16 (1347) and n = 17 (1419) eluting at very close retention values.

The separation of the cyclic material, on the other hand, showed an increased number of peaks (Figure 5.6). MS analysis (Figure 5.8) showed that 3 or 4 chromatographic peaks had the same mass, which was interpreted as the result of separation of diastereoisomeric cyclic compounds with the same number of oligomeric units, formed from the racemic mixture of lactic acids used as starting material for the production of OLAs and cyclic oligomers.

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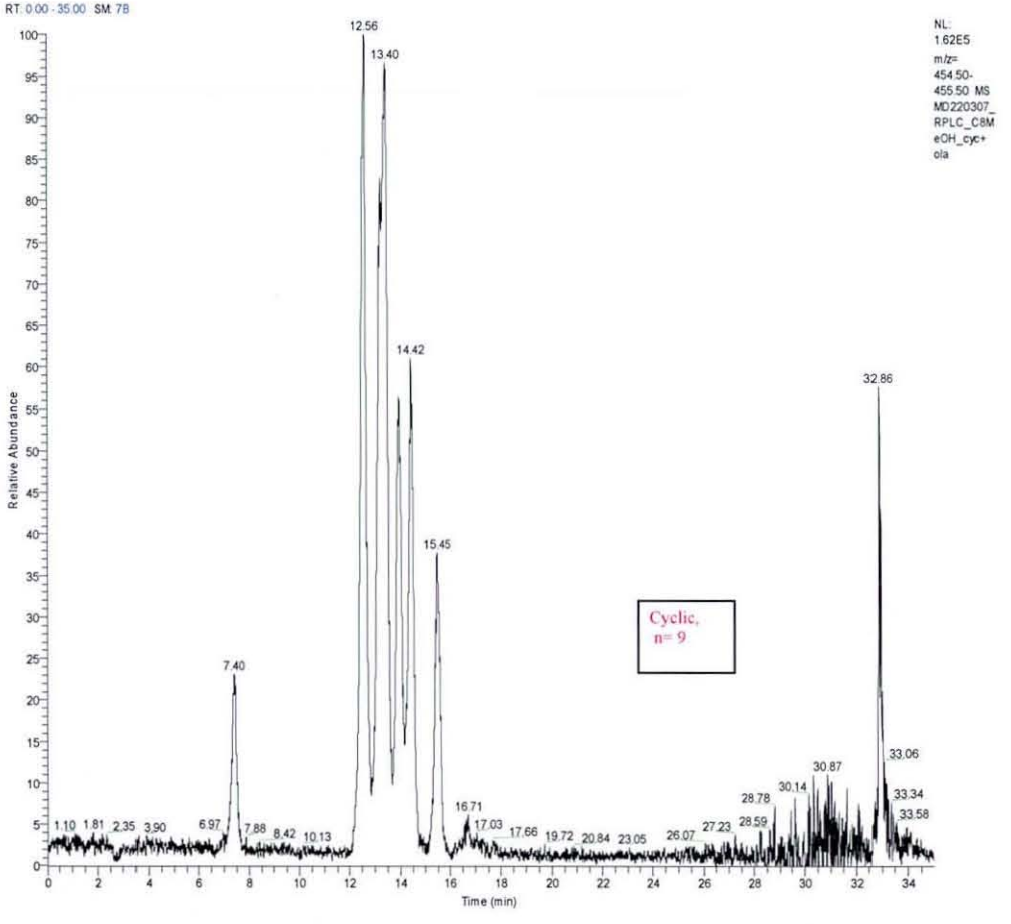

Figure 5.8: OLAs EIC, obtained on LC-MS: Thermo Finnigan LTQ, equipped with ESI source. Injection volume: 10 µl; OLAs solution containing a 50:50 mixture of dd solution (1082.0 µg/ml) and cyclic solution (42 µg/ml); chromatographic column: Eclipse C_8 (150 x 4.6) mm, 5 µm; mobile phase A: 0.1% formic acid in water, mobile phase B: 0.1% formic acid in MeOH; starting conditions: 50% mobile phase B, taken to 70% in 25 min and then ramped to 100% at 30 min; LC flow rate: 0.5 ml/min. Same cyclic oligomer (455, n = 9) with different retention values.

5.2. Effects of column temperature on OLAs selectivity

Because it was desirable to achieve a good resolution, the effect of temperature changes on the retention of OLAs in water/methanol mobile phase was examined using a Symmetry C_4 column. The temperature was gradually increased from 30 to 70 °C and OLAs diamide diester (dd) and cyclic solutions were injected as a 50:50 mixture.

The chromatogram for the separation at 40°C (Figure 5.9) showed cyclic oligomers eluting between 6-14 min and main oligomers eluting between 14-28 min. MS data analysis (Figure 5.10 and Figure 5.11) confirmed that cyclics eluted in front of main excipients.

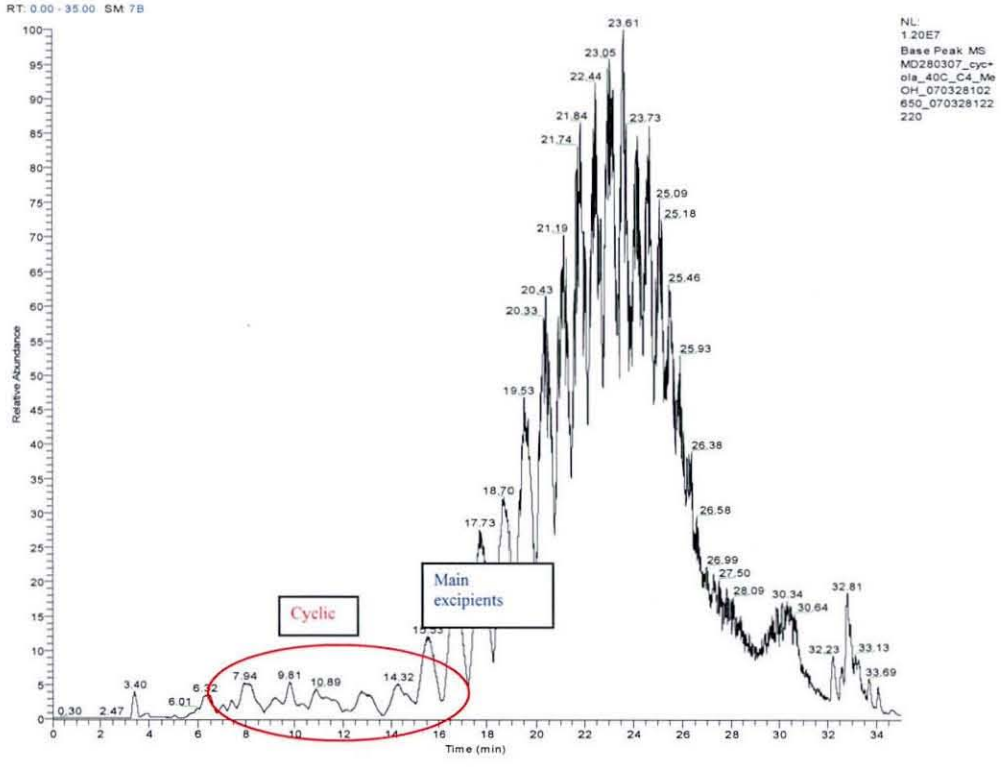

Figure 5.9: OLAs mass chromatogram, obtained on LC-MS: Thermo Finnigan LTQ, equipped with ESI source. Injection volume: 10 µl; OLAs solution containing a 50:50 mixture of dd solution (1082.0 µg/ml) and cyclic solution (42 µg/ml); chromatographic column: Symmetry C_4 (150 x 4.6) mm, 5 µm; mobile phase A: 0.1% formic acid in water, mobile phase B: 0.1% formic acid in MeOH; starting conditions: 50% mobile phase B, taken to 70% in 25 min and then ramped to 100% at 30 min; LC flow rate: 0.5 ml/min. column temperature: 40 °C.

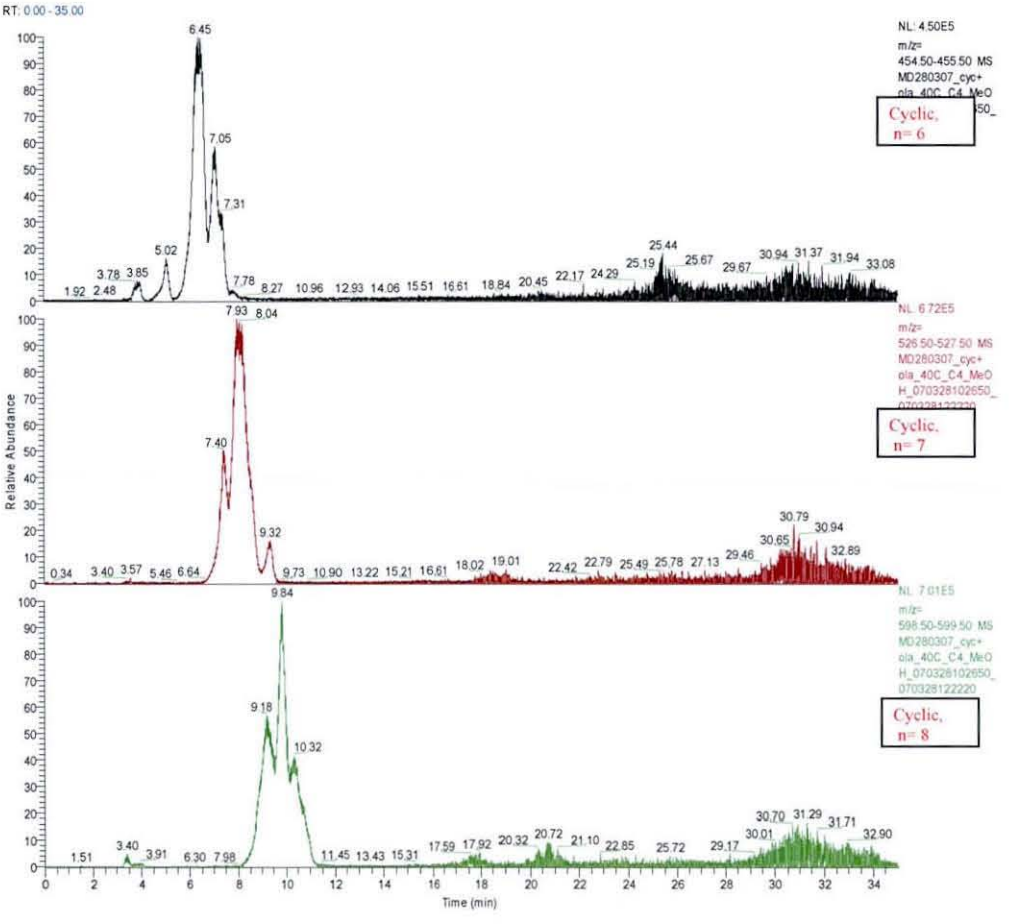

Figure 5.10: OLAs EICs, obtained on LC-MS: Thermo Finnigan LTQ, equipped with ESI source. Injection volume: 10 µl; OLAs solution containing a 50:50 mixture of dd solution (1082.0 µg/ml) and cyclic solution (42 µg/ml); chromatographic column: Symmetry C_4 (150 x 4.6) mm, 5 µm; mobile phase A: 0.1% formic acid in water, mobile phase B: 0.1% formic acid in MeOH; starting conditions: 50% mobile phase B, taken to 70% in 25 min and then ramped to 100% at 30 min; LC flow rate: 0.5 ml/min. column temperature: 40 °C. Cyclic oligomers 6, 7 and 8.

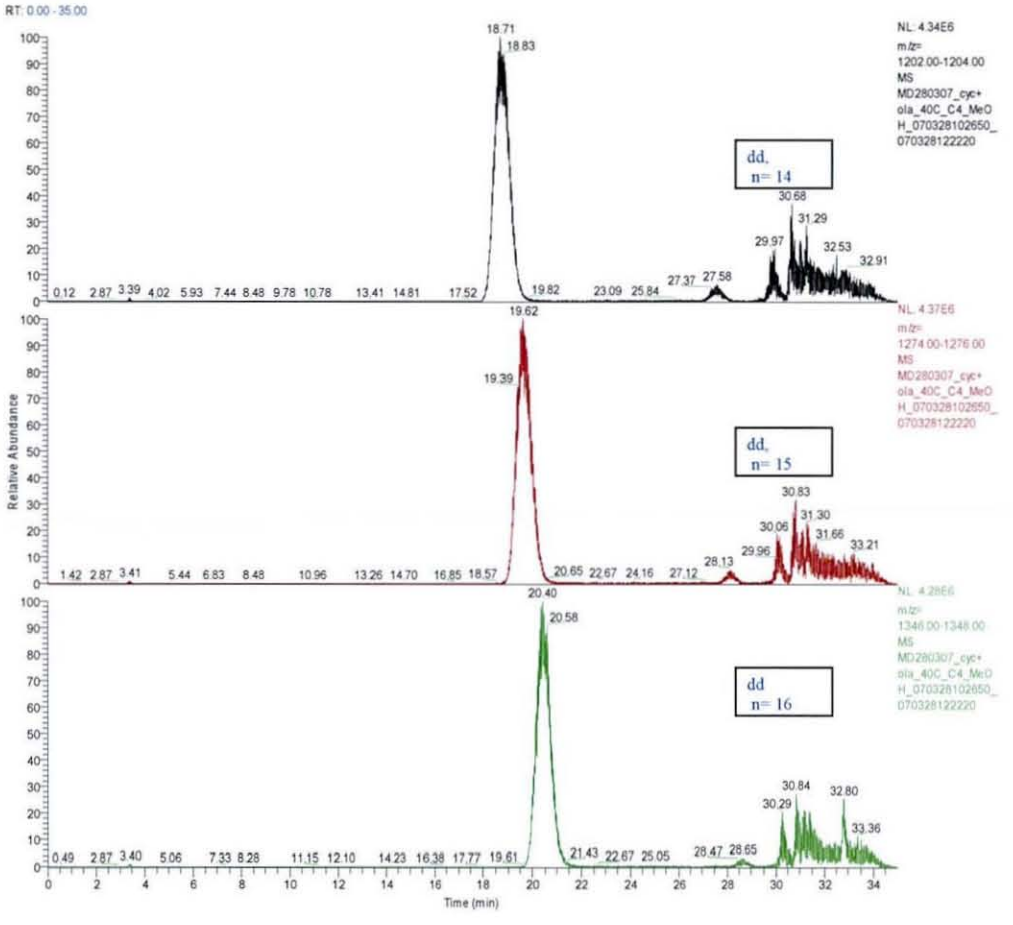

Figure 5.11: OLAs EICs, obtained on LC-MS: Thermo Finnigan LTQ, equipped with ESI source. Injection volume: 10 µl; OLAs solution containing a 50:50 mixture of dd solution (1082.0 µg/ml) and cyclic solution (42 µg/ml); chromatographic column: Symmetry C_4 (150 x 4.6) mm, 5 µm; mobile phase A: 0.1% formic acid in water, mobile phase B: 0.1% formic acid in MeOH; starting conditions: 50% mobile phase B, taken to 70% in 25 min and then ramped to 100% at 30 min; LC flow rate: 0.5 ml/min. column temperature: 40 °C. Dd oligomers 14, 15 and 16.

On increasing the temperature, the retention times decreased, for both cyclics and dd, (70°C, Figure 5.12), where cyclic oligomers eluted between 5-11 min and main oligomers eluted between 11- 24 min. The chromatography obtained at 70°C also had sharper peaks than the chromatography obtained at 40°C.

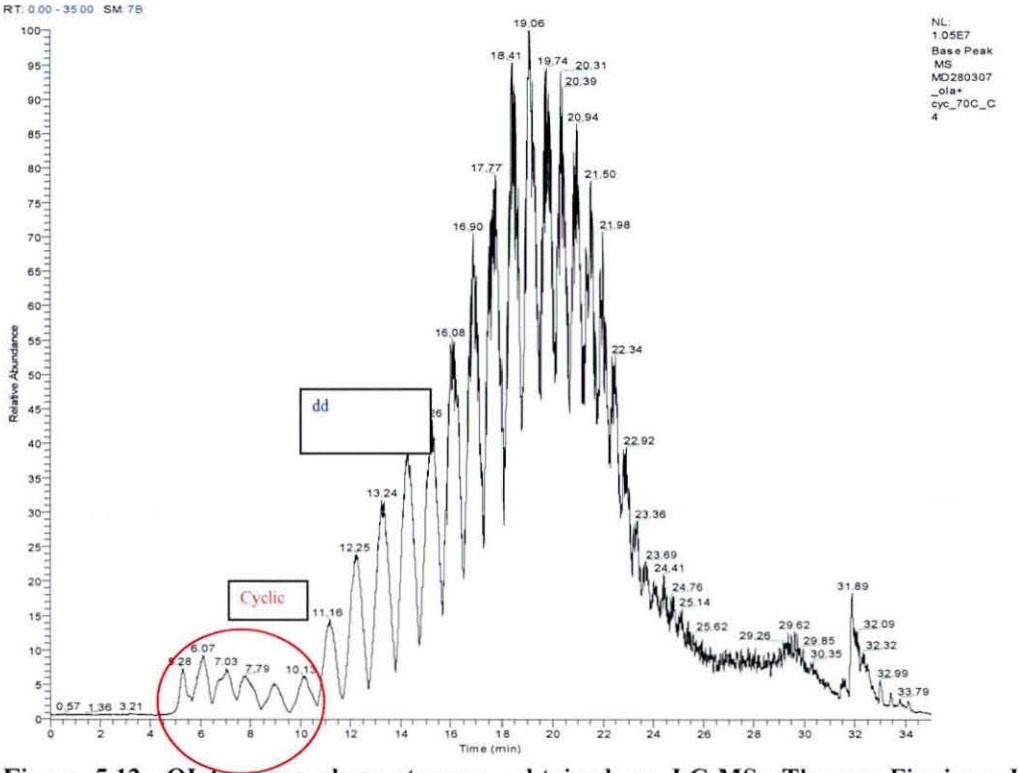

Figure 5.12: OLAs mass chromatogram, obtained on LC-MS: Thermo Finnigan LTQ, equipped with ESI source. Injection volume: 10 µl; OLAs solution containing a 50:50 mixture of dd solution (1082.0 µg/ml) and cyclic solution (42 µg/ml); chromatographic column: Symmetry C_4 (150 x 4.6) mm, 5 µm; mobile phase A: 0.1% formic acid in water, mobile phase B: 0.1% formic acid in MeOH; starting conditions: 50% mobile phase B, taken to 70% in 25 min and then ramped to 100% at 30 min; LC flow rate: 0.5 ml/min. Column temperature: 70 °C.

Overall, the temperature studies carried out (on butyl bonded column material with an aqueous/methanol eluent) did not lead to significant selectivity changes, but higher temperatures gave sharper peaks and decrease retention values.

5.3. Conclusions on the use of different organic modifiers

This study suggested that resolution of the head group distribution and the oligomeric distribution of OLAs could be approached in a single chromatographic dimension. The Symmetry C_4 column with an aqueous/methanol mobile phase achieved separation between OLA dd and their cyclic impurities and increasing the temperature from 30 to 70 °C provided a sharper chromatography.

An Eclipse C_8 column with an aqueous/methanol mobile phase enabled the diastereoisomeric separation of the cyclic impurities. However, a longer C_4 column (250 mm rather than a 150 mm) could possibly lead to a higher resolution of OLAs oligomers, making a C_4 material a better choice than a C_8 column.

Furthermore, it would be advisable to use a shallower eluent gradient, in order to improve the resolution of the later dd oligomers.

6. Conclusions and future work

This study has demonstrated for the first time the full characterisation of OLAs (ethylene bis (propionyl oligo lactyl amide), n= 10). RP-HPLC coupled to MS provided the selectivity required to simultaneously resolve OLAs and their cyclic process impurities, in terms of chain size and head groups.

The analysis of OLAs in reversed-phase LC, on a C_6 column, acetonitrile/water mobile phase, with temperature ranging from 30 to 75 °C, led to a separation based on chain length distribution. MS data showed earlier elution of low molecular weight chains, followed by elution of higher molecular weight chains. The increase in temperature caused a decrease in peak retention and a sharpening of peaks. The elution order was left unaltered by temperature increase. Thermodynamic data analysis led to a theoretical compensation temperature (i.e. temperature at which LCCC are reached), Tc = 107 °C.

The retention behaviour of OLAs in normal phase LC, on an amino column and acetonitrile mobile phase (with temperature ranging from 30 to 80 °C) and on a silica column and THF mobile phase (with temperature ranging from 30 to 55 °C), was very different from RP-HPLC. The separation of the oligomers according to chain length was suppressed, resulting in one single peak. Temperature variation marginally affected OLAs retention in NP-HPLC: an increase in temperature caused a decrease in retention time of OLAs (on the amino column, retention varied from approx. 5.9 min, at 30 °C to 5.4 min at 80 °C, which corresponds to about 0.5 min for a 50 °C variation; on the silica column, retention varied from approx. 3.7 min, at 30 °C to 3.4 min at 55 °C, which corresponds to about 0.3 min for a 25 °C variation). The MS data for the chromatographic system that employed the amino column identified the various oligomers of the diamide diester component. The longer oligomers eluted in front of the shorter oligomers,

indicating a NP retention mechanism. Temperature did not change the elution order of the oligomers.

Although the amino and cyano columns proved to be both selective, with respect to OLAs analysis, different retention behaviours were exhibited by the two adsorbents. The Spherisorb amino stationary phase exhibited typical HILIC behaviour: decreasing retentions were observed with increasing aqueous content in mobile phase. Additionally, the MS data showed an elution order of longer oligomers (less polar) first, followed by shorter oligomers (more polar). The retention mechanism was based on polarity, indicating NP environment.

The cyano column provided a HILIC environment when less than 10% ammonium formate was present in the mobile phase, (retention values decrease when increasing mobile phase aqueous content). The cyano column behaved as a RP-HPLC column when the mobile phase contained more than 10% ammonium formate (retention values increased on increasing mobile phase aqueous content).

Additionally, the MS data showed a change in elution order of the various oligomers. The typical elution, based on polarity, of longer oligomers first, observed at low aqueous content (and indicating NP environment) 'switched' to an elution of shorter oligomers first, observed at higher aqueous content (and indicating RP environment). The exact mobile phase composition (20% 50 mM ammonium formate/80% MeCN) that led to critical conditions of elution on the cyano column was also established.

It can also be concluded that resolution between main oligomeric components and cyclic impurity was higher on the cyano than on the amino column, when aqueous/MeCN was used as mobile phase.

A mobile phase of n-hexane and acetonitrile, on either amino or cyano stationary phases did not contribute to critical conditions of elution for OLAs. Variations in concentration of n-hexane in the mobile phase did not provide a trend in the OLAs retention behaviour, neither on the cyano, nor on the amino materials.

However, none of the chromatographic systems investigated (in HILIC or in NP-HPLC) provided a baseline resolution between the main oligomers and the cyclic peaks.

The results suggested that a 2D system could be feasible; employing a combination of two orthogonal and compatible separation systems (RP-HPLC mechanisms in the first dimension for oligomeric resolution and LCCC in the second dimension for resolution of the head group distribution) would lead to base-line resolution between cyclics and OLAs oligomers. Salt concentration did not have major effect on the eluting strength of MeCN, for OLAs on a Zorbax cyano material. Water and ammonium formate showed similar effects on the retention of OLAs on a Zorbax cyano column. This study confirmed the chromatographic conditions for which NP retention behaviour changed to RP, passing through CC.

However during a study to enhance the separation of the oligomeric mixture it was found that methanol and acetonitrile, in RP-HPLC were responsible for different selectivity separation of OLAs. Methanol/water led to separation between OLAs dd excipient and their cyclic impurity. Such separation was not observed in acetonitrile/water. An improved solvation of OLAs head-groups, due to proton-acceptor interactions between the head-groups and OH groups of methanol could be responsible for the observed selectivity change. The resolution of oligomers could be improved, possibly to baseline level, by coupling two C_4 columns in series. This line of work should be further investigated, if quantitative analysis of OLAs will have to be undertaken. A convex gradient might also be desirable in

which the rate of organic increase is reduced as the elution continues to improve the separation of the higher oligomers.

Such a single dimension separation would be preferable to the mechanical complexities of a 2D comprehensive separation and the added data handling difficulties. Throughout the study of the components of the 2D system there was always a complexity because of the incompatibility of the mobile phases used for the two dimensions. However, a single dimension analysis might still have difficulties resolving other components of the mixture, although with the increase in the power and reduction in costs of LC-MS using MS as the second dimension in the separation could lead to a full component analysis and it could for example readily resolve the free acids and amide by using negative and positive ionisation APCI modes.

Further analytical work on the determination of the OLAs should also include the cleaning up of the cyclic standard. Solid phase extraction could be employed to isolate the long chain free acid components present with the cyclic impurities, making the cyclic chromatography free of interfering peaks.

References

[1] ICH guidelines, Specifications section, Q6A, www.ich.org

[2] J.C. Giddings, J. Chromatogr. A 703 (1995) 3.

[3] J.T. Capecchi personal communication. J.T. Capecchi is a scientist at 3M.

[4] J.S. Stefely, D.C. Duan, P. B. Myrdal, D.L. Ross, D.W. Schultz, C.L. Leach, In: R.N. Dalby, P.R. Byron, S.J. Farr, J.A. Peart, Editors, Respiratory Drug Delivery VII Proceedings, Serentec Press, Inc., Raleigh (2000).

[5] J.S. Stefely, S.W. Stein, Drug Delivery Technology 3 Issue 1 (2003).

[6] C.L. Leach, P.J. Davidson, R.J. Boudreau, Euro Respir J. 12 Issue 6 (1998) 1346.

[7] C.L. Leach, Respir. Med. 92 (1998)3.

[8] T.S. Purewal, H. Dahya, B.J. Gabrio Metered Dose Inhaler Technology Improvements Introduced by a Non-CFC Beclomethasone Dipropionate Inhaler. Paper presented at World Asthma Meeting, Barcelona, Spain;1998.

[9] http://ptcl.chem.ox.ac.uk/MSDS/TE/1,1,1,2-tetrafluoroethane.html

[10] J.S. Stefely, B. Brown, D.M. Hammerbeck, S.W. Stein, In: R.N. Dalby, P.R. Byron, S.J. Farr, J.A. Peart, Editors, Respiratory Drug Delivery VIII. Raleigh, NC: Davis Horwood Int. Pub. Ltd (2002) 207.

[11] J.S. Stefely, Drug Delivery Technology 2 Issue 6 (2002) 62.

[12] 3M poster: 'Sustained release suspension aerosol formulations with oligolactic acid (OLA)' J.S. Stefely, Z. Lee (http://solutions.3m.com).

[13] 3M poster: 'Characterization of oligomeric materials developed for use as excipients in metered dose inhalers' J.T. Capecchi, J.S. Stefely, A. Reese, P.M. Seiler, K. Bechtold, J. Zurowski, N. Queque-Larson, J. L. Battiste, D. Weil, L.E. Schallinger, D.A. Hanggi (http://solutions.3m.com).

[14] 3M poster: 'LC/MS Characterization of oligomeric materials developed for use as excipients in metered dose inhalers' K. Hansen, J. Drake, J. Osterheim, J.T. Capecchi, D. Dohmeir (http://solutions.3m.com).

[15] M. Staniforth, M. O'Hanlon, T.M. Khong, J. Chromatogr. A 833 (1999) 195.

[16] H. Cheng, R.R. Gadde, J. Chromatogr. 355 (1986) 399.

[17] R. Andersson, B. Hedlund, Z. Lebensm, Unters. Forsch. 176 (1983) 440.

[18] S.H. Ashoor, J. Welty, J. Chromatogr. 287 (1984) 452.

[19] D.L. Schooley, F.M. Kubiak, J.V. Evans, J. Chromatogr. Sci. 23 (1985) 385.

[20] K.R. Kim, M.K. Hahn, A. Zlatkis, E.C. Horning, B.S. Middleditch, J. Chromatogr. 468 (1989) 289.

[21] P.G. Brotz, D.M. Schaefer, J. Microbiol. Methods 6 (1987) 139.

[22] S. Heitefuss, A. Heine, H.S.H. Seifert, J. Chromatogr. 532 (1990) 374.

[23] D.V. McCalley, J. High Resolut. Chromatogr. 12 (1989) 465.

[24] T. Chang, Adv. Polym. Sci. 163 (2003) 1.

[25] A.H. Celik Doctoral Thesis 'Characterisation of oligomers and additives from polymeric materials', Loughborough University, 1997.

[26] Handbook of size-exclusion chromatography, C. Wu (Ed), Chromatographic Science Series, Volume 69, Marcel-Dekker, New York (1995).

[27] H.G. Barth, in: Handbook of HPLC, E. Katz, R. Eksteen, P. Schoenmakers, N. Miller (Eds), Chromatographic Science Series, Volume 78, Marcel-Dekker, New York (1998).

[28] X. Jiang, A. van der Horst, V. Lima, P.J. Schoenmakers, J. Chromatogr. A 1076 (2005) 51.

[29] Y. Mengerink, Doctoral Thesis 'Non-exclusion separation techniques for polyamides', Eindhoven University of Technology, 2001.

[30] P. Jandera, in: P.R. Brown, E.Grushka, S. Lunte (Eds), Advances in Chromatography, Volume 43, Marcel-Dekker, New York (2005).

[31] F.P.B. van der Maeden, M.E.F. Biemond, P.C.G.M. Janssen, J. Chromatogr. 149 (1978) 539.

[32] L.R. Snyder, M.A. Stadalius, M.A. Quarry, Anal. Chem. 55(1983) 1413A.

[33] R.A. Shalliker, P.E. Kavanagh, I.M. Russell, D.G. Hawthorne, Chromatographia 33 (1992) 427.

[34] K. Rissler, H.P. Kunzi, H.J. Grether, J. Chromatogr. 635 (1993) 89.

[35] T.C. Schunk, J. Chromatogr. A 656 (1993) 591.

[36] G. Glöckner, D. Wolf, H. Engelhardt, Chromatographia 39 (1994) 557.

[37] S.P. Reid, E. Meehan, J.V. Dawkins, J. Liq. Chromatogr. 17 (1994) 3045.

[38] T. Kawai, M. Akashima, S. Teramachi, Polymer 36 (1995) 2851.

[39] S. Teramachi, Macromol. Symp. 110 (1996) 217.

[40] C. Kuo, H.T. Provder, R.M. Holsworth, A.F. Kah, in: Liquid Chromatography of Polymers and Related Materials, J. Cazes (Ed), Marcel-Dekker, New York (1981).

[41] L. Shihtse, J. Chromatogr. 321 (1985) 2.

[42] I. Zeman, J. Chromatogr. 363 (1986) 223.

[43] M. Augenstein, M. Stickler, Makromol. Chem. 191 (1990) 415.

[44] S. Mori, J. Chromatogr. 541 (1991) 375.

[45] H.J.A. Philipsen, B. Klumperman, A.L. German, J. Chromatogr. A 746 (1996) 211.

[46] H.J.A. Philipsen, M.R. de Cooker, H.A. Claessens, B. Klumperman, A.L. German, J. Chromatogr. A 761 (1997) 147.

[47] H.J.A. Philipsen, M. Oestreich, B. Klumperman, A.L. German, J. Chromatogr. A 775 (1997) 157.

[48] H.J.A. Philipsen, B. Klumperman, A.L. German, Proc. Int. GPC. Symp., San Diego, September1996, 368.

[49] H.J.A. Philipsen, H.A. Claessens, M. Bosman, B. Klumperman, A.L. German Chromatographia 48 (1998) 623.

[50] G. Glöckner, H.G. Barth, J. Chromatogr. 499 (1990) 645.

[51] S.T. Balke, R.D. Patel, Adv. Chem. Ser. 203 (1983) 281.

[52] S.T. Balke, Sep. Purif. Methods 11 (1982) 1.

[53] P. Jandera, Chromatographia 19 (1984) 101.

[54] P. Jandera, J. Chromatogr. 314 (1984) 13.

[55] P. Jandera, J. Chromatogr. 449 (1988) 361.

[56] P. Jandera, J. Urbanek, J. Chromatogr. 689 (1995) 225.

[57] P. Jandera, Chromatographia 26 (1988) 417.

[58] P. Jandera, J. Urbanek, B. Prokes, J. Churacek, J. Chromatogr. 504 (1990) 297.

[59] P. Jandera, J. Urbanek, B. Prokes, H. Blazkova-Brunova, J. Chromatogr. A 736 (1996) 131.

[60] P. Jandera, J. Sep. Sci. 29 (2006) 1763.

[61] H.J.A. Philipsen, H.A. Claessens, P. Jandera, M. Bosman, B. Klumperman, Chromatographia 52 (2000) 325.

[62] R. Bodmeier, O. Paevatakul, J. Liq. Chromatogr. 14 (1991) 365.

[63] Y. Mengerink, H.C.J. Deman, S.Vanderwal, J. Chromatogr. 552 (1991) 593.

[64] K. Rissler, J. Chromatogr. A 871 (2000) 243.

[65] K. Rissler, Chromatographia 59 (2004) 669.

[66] L.B. Rogers, J.J. Lewis, R.E. Pauls, J. Chromatogr. 299 (1984) 311.

[67] A.P. Sweeney, V. Wong, R.A. Shalliker, Chromatographia 54 (2001) 24.

[68] T.H. Mourey, Anal Chem. 56 (1984) 1777.

[69] T.H. Mourey, G.A. Smith, L.R. Snyder, Anal. Chem. 56 (1986) 1773.

[70] S.T. Lai, D.C. Locke, J. Chromatogr. 252 (1982) 325.

[71] S.T. Lai, L. Sangermano, D.C. Locke, J. Chromatogr. 312 (1984) 313.

[72] P. Jandera, J. Rozkošná, J. Chromatogr. 362 (1986) 325.

[73] N. Marquez, R.E. Anton, A. Usubillaga, J.L. Salager, J. Liq. Chromatogr. 17 (1994) 1147.

[74] T.H. Dzido, T.E. Kossowski, D. Matosiuk, J. Chromatogr. A 947 (2002) 167.

[75] E.H. Slaats, W. Markowski, J. Fekete, H. Poppe, J. Chromatogr. 207 (1981) 299.

[76] R.K. Gilpin, M. Jaroniec, S. Lin, Anal. Chem. 62 (1990) 2092.

[77] Y.V. Kazakevich, R. LoBrutto, F. Chan, T. Patel, J. Chromatogr. A 913 (2001) 75.

[78] T.H. Dzido, H. Engelhardt, Chromatographia 39 (1994) 51.

[79] G. Vanhoenacker, P. Sandra, J. Chromatogr. A 1082 (2005) 193.

[80] K. Rissler, U. Fuchslueger, H. Grether, J. Chromatogr. A 654 (1993) 309.

[81] K. Rissler, J. Chromatogr. A 667 (1994) 167.

[82] K. Rissler, U. Fuchslueger, J. Liq. Chromatogr. 17 (1994) 2791.

[83] K. Lemr, M. Zanette, A. Marcomini, J. Chromatogr. A 686 (1994) 219.

[84] W.R. Melander, A. Nahum, Cs. Horváth J. Chromatogr. 185 (1979) 129.

[85] T. Okada, Anal. Chim. Acta 281 (1993) 85.

[86] D. Cho, J. Hong, S. Park, T. Chang, J. Chromatogr. A 986 (2003) 199.

[87] G.R. Bear, J. Chromatogr. 459 (1988) 91.

[88] T.C.G. Kibbey, T.P. Yavaraski, K.F. Hayes, J. Chromatogr. A 752 (1996) 155.

[89] N.M.A. Ibrahim, B.B. Wheals, J. Chromatogr. A 731 (1996) 171.

[90] C. Sun, M. Baird, H.A. Anderson, D.L. Brydon, J. Chromatogr. A 731 (1996) 161.

[91] B. Trathnigg, B. Maier, A. Gorbunov, A. Skvortsov, J. Chromatogr. A 791 (1997) 21.

[92] P. Jandera, M. Holcapek, G. Theodoridis, J. Chromatogr. A 813 (1998) 299.

[93] M. Kudoh, S. Konami, S. Fudano, S. Yamaguchi, J. Chromatogr. A 234 (1982) 209.

[94] P.L. Desbene, F.I. Portet, G.J. Goussot, J. Chromatogr. A 730 (1996) 209.

[95] G. Cretier, C. Podevin, J.-L. Rocca, J. Chromatogr. A 874 (2000) 305.

[96] T. Kamiusuki, T. Monde, K. Omae, K. Morioka, T. Konakahara, Chromatographia 51 (2000) 390.

[97] B. Trathnigg, A. Gorbunov, J. Chromatogr. A 910 (2001) 207.

[98] B. Trathnigg, J. Chromatogr. A 915 (2001) 155.

[99] Gorbunov, B. Trathnigg, J. Chromatogr. A 955 (2002) 9.

[100] C.H. Lochmüller, M.A. Moebus, Q.C. Liu, C. Jiang, M. Elomaa, J. Chromatogr. Sci. 34 (1996) 69.

[101] F.P. Schmitz, D. Leyendecker, D. Leyendecker, B. Gemmel, J. Chromatogr. 395 (1987) 111.

[102] Y. Hirata, J.Chromatogr. 315 (1984) 39.

[103] F.P. Schmitz, B. Gemmel, Fresenius Z. Anal. Chem. 330 (1988) 216.

[104] E. Klesper, W. Hartmann, Eur. Polym. J. 14 (1978) 77.

[105] F.P. Schmitz, E. Klesper, Makromol. Chem., Rapid Commun. 2 (1981) 735.

[106] F.P. Schmitz, H. Hilgers, E. Klesper, J. Chromatogr. 267 (1983) 267.

[107] U. Just, H.R. Holzbauer, M. Resch, J. Chromatogr. A 667 (1994) 354.

[108] K.D. Bartle, T. Boddington, A.A. Clifford, N.J. Cotton, Anal. Chem. 63 (1991) 2371.

[109] W.R. Hugins, K. Theurer, T. Mariani, J. Appl. Polym. Sci. 34 (1978) 145.

[110] T. Cserháti, A. Somogyi, J. Chromatogr. 446 (1988) 17.

[111] T. Cserháti, J. Chrom. Sci. 31 (1993) 233.

[112] K.H. Bui, W.D. Armstrong, J. Liq. Chromatogr. 7 (1984) 29.

[113] A.J. Alpert, J. Chromatogr. 499 (1990) 177.

[114] P. Hemström, K. Irgum, J. Sep. Sci 29 (2006) 1784.

[115] M.A. Strege, Anal. Chem. 70 (1998) 2439.

[116] E.S. Grumbach, D.M. Diehl, D.R. McCabe, J.R. Mazzeo, U.D. Neue, LC GC N. Am. Suppl. (2003) 53.

[117] V.V. Tolstikov, O. Fiehn, Anal. Biochem. 301 (2002) 298.

[118] S.D. Garbis, A. Melse-Boonstra, C.E. West, R.B. van Breemen, Anal. Chem. 73 (2001) 5358.

[119] M.A. Strege, Am. Pharm. Rev. 2 (1999) 53.

[120] M.A. Strege, Anal. Chem. 70 (1998) 2439.

[121] B.Y. Zhu, C.T. Colin, R.S. Hodges, J. Chromatogr. 548 (1991) 13.

[122] B. Olsen, J. Chromatogr. A 913 (2001) 113.

[123] SeQuant ZIC® –HILIC product sheet 27xx-103-031120-TJN.

[124] R. Oyler, B.L. Armstrong, J.Y. Cha, M.X. Zhou, Q. Yang, R.I. Robinson, R. Dunphy, D.J. Burinsky, J. Chromatogr. A 724 (1996) 378.

[125] R. Li, J. Huang, J. Chromatogr. A 1041 (2004) 163.

[126] B.Y. Zhu, C.T. Mant, R.S. Hodges, J. Chromatogr. 594 (1992) 75.

[127] M. Lafosse, B. Herbreteau, M. Dreux, L. Morin Allory, J. Chromatogr. 472 (1989) 209.

[128] A.J. Alpert, M. Mukta, A.K. Shukla, L.R. Ziekske, S.W. Yuen, M.A.J. Ferguson, A. Mehlert, M. Pauly and R. Orlando, J. Chromatogr. A 676 (1994) 191.

[129] T. Yoshida, Anal. Chem. 69 (1997) 3038.

[130] T.J. Higley and T. Yoshida, LCGC N. Am. Suppl. (2003) 20.

[131] P. Dallet, L. Labat, E. Kummer and J.P. Dubost, J. Chromatogr. B 742 (2000) 447.

[132] H. Schlichtherle-Cerny, M. Affloter and C. Cerny, Anal. Chem. 75 (2003) 2349.

[133] Normal Phase Chromatography: TSKgel Amide-80, Tosoh BioScience 2003 Laboratory Products Catalogue, www.tosohbiosep.com.

[134] Y. Guo, S. Gaiki, J. Chromatogr. A 1074 (2005) 71.

[135] P. Jandera, J. Fischer, H. Lahovská, K. Novotná, P. Česla, L. Kolářová, J. Chromatogr. A 1119 (2006) 3.

[136] Svec, F., Petro, M., Fréchet, J. M. J., Coll. Czech. Chem. Commun. 66 (2001) 1047.

[137] T. Macko, D. Hunkeler, Adv. Polym. Sci. 163 (2003) 61.

[138] Y. Mengerink, R. Peters, Sj. Van der Wal, F.Derk, T. Ho, J. Julian, R. Domingo, Chromatographia 58 (2003) 523.

[139] Y. Mengerink, R. Peters, Sj. Van der Wal, H.A. Claessens, C.A. Cramers, J. Chromatogr. A. 949 (2002) 337.

[140] Y. Mengerink, R. Peters, C.G. deKoster, Sj. van der Wal, H.A. Claessens, C.A. Cramers, J. Chromatogr. A 914 (2001) 131.

[141] G. Schultz, H. Much, H. Kruger, C. Wehrstedt, J. Liq. Chromatogr. 13 (1990) 1745.

[142] A.V. Gorshkov, H. Much, H. Becker, H. Pasch, V.V. Evreinov, S.G. Entilis, J. Chromatogr. 523 (1990) 91.

[143] B. Trathnigg, M. Kollroser, Int. J. Pol. Anal. Char. 1 (1995) 301.

[144] H. Yun, S.V. Olesik, E.H. Marti, J. Microcol. Sep. 11 (1999) 53.

[145] R. Kruger, H. Much, G. Schultz, J. Liq. Chromatogr. 17 (1994) 3069.

[146] H.J.A. Philipsen, B. Klumperman, A.M. Herk, A.L. German, J. Chromatogr. A 727 (1996) 13.

[147] A.V. Gorshkov, H. Much, H. Becker, H. Pasch, V.V. Evreinov, S.G. Entilis, J. Chromatogr. 523 (1990) 91.

[148] B. Trathnigg, M. Kollroser, Int. J. Pol. Anal. Char. 1 (1995) 301.

[149] Günter Schulz; Helmut Much; Hartmut Kruger; Carla Wehrstedt J. Liq. Chromatogr. 13 (1990) 1745.

[150] B. Trathnigg; D. Thamer; X. Yan; S. Kinugasa, J. Liq. Chromatogr. 16 (1993) 2439.

[151] H. Pasch, I. Zammert, J. Liq. Chromatogr. 17 (1994) 3091.

[152] R.P. Krüger; H. Much; G. Schulz, J. Liq. Chromatogr. 17 (1994) 3069.

[153] T. B. Tennikova, I. V. Blagodatskikh, F. Svec, M. B. Tennikov, J. Chromatogr. 509 (1990) 233.

[154] Y. Mengerink, R. Peters, Sj. Van der Wal, H.A. Claessens, C.A. Cramers, J. Chromatogr. A. 949 (2002) 337.

[155] X. Jiang, P.J. Schoenmakers, X. Lou, V. Lima, J.L.J. van Dongen, J. Brokken-Zijp, J. Chromatogr. A. 1055 (2004) 123.

[156] R. Peters, Y. Mengerink, S. Langereis, M. Frederix, H. Linssen, J. van Hest, Sj. van der Wal, J. Chromatogr. A 949 (2002) 327.

[157] S.L. Phillips, L. Ding, M. Stegemiller, S.V. Olesik, Anal. Chem. 75 (2003) 5539.

[158] T. Biela, A. Duda, K. Rode, H. Pasch, Polymer 44 (2003) 1851.

[159] D. Berek, M. Chalanyova, T. Macko, J. Chromatogr. 286 (1984) 185.

[160] E.F. Cassaca, J. Polym. Sci. 773 (1967) 773.

[161] A.M. Skvortsov, A.A. Gorbunov, Polym. Sci. USSR 21 (1979) 371.

[162] A.A. Gorbunov, A.M. Skvortsov, Polym. Sci. USSR 30 (1988) 439.

[163] P.J.C.H. Cools, Thesis, University of Eindhoven, 1999 59.

[164] S.G. Entelis, V.V. Evreinov, A.V. Gorshkov, Adv. Polym. Sci. 76 (1985) 129.

[165] A.V. Gorshkov, T.N. Prudskova, V.V. Gur'yanova, V.V. Evreinov, Polym. Bulletin 15 (1986) 465.

[166] A.V. Gorshkov, V.V. Evreinov, B. Lausecker, H. Pasch, H. Becker, G. Wagner, Acta Polymerica 37 (1986) 740.

[167] A.V. Gorshkov, S.S. Verenich, V.V. Evreinov, S.G. Entelis, Chromatographia 26 (1988) 338.

[168] P.J.C.H. Cools, A.M. van Herk, A.L. German, W. Staal, J. Liq. Chromatography 17 (1994) 3133.

[169] G. Glöckner, Polymer Characterization by Liquid Chromatography in: Elsevier, Amsterdam (1987) 86.

[170] B. Trathnigg, C. Rappel, S. Fraydl, A. Gorbunov, J. Chromatogr. A 1085 (2005) 253.

[171] Y.C. Guillaume, C. Guinchard, Anal. Chem. 68 (1996) 2869.

[172] A. Vailaya, C. Horvath, J. Phys. Chem. 100 (1996) 2447.

[173] K. Miyabe, G. Guiochon, Anal. Chem. 72 (2000) 1475.

[174] K. Miyabe, S. Sotoura, G. Guiochon, J. Chromatogr. A 919 (2001) 231.

[175] K. Miyabe, G. Guiochon, Anal. Chem. 74 (2002) 5754.

[176] M. Balcan, D.F. Anghel, A. Voicu, D.C. Balcan, Colloids Surfaces A: Physicochem. Eng. Asp. 204 (2002) 141.

[177] M. Balcan, D.-F. Anghel, Colloids and Surfaces A: Physicochem. Eng. Asp. 221 (2003) 1.

[178] H.J.A. Philipsen, H.A. Claessens, H. Lind, B. Klumperman, A.L. German, J. Chromatogr. A 790 (1997) 101.

[179] S. Phillips, S.V. Olesik, Anal. Chem. 74 (2002) 799.

[180] J. Gilbert, M.J. Shepard, J.R. Startin, M.A. Wallwork, J. Chromatogr. 237 (1982) 249.

[181] S. Abrantes, HRC – J. High Res. Chromatogr. 16 (1993) 113.

[182] G.M. Garner, Y. Ma, J. Barks, Abstracts of the papers of the American Chemical Soc. 203 (1992) 19.

[183] Mass spectrometry of polymers, G. Montaudo, R.P. Lattimer (Eds), CRC press, Cleveland, OH, USA (2002).

[184] J.H. Scrivens, A.T. Jackson, Int. J. Mass Spectrom. 200 (2000) 261.

[185] S.D. Hanton, Chem. Rev. 101 (2001) 527.

[186] C.N. McEwen, P.M. Peacock, Anal. Chem. 74 (2002) 2743.

[187] P. Jandera, M. Holcapek, L. Kolářová, J. Chromatogr. A 869 (2000) 65.

[188] M.W.F. Nielen, F.A. Buijtenhuis, Anal. Chem. 71 (1999) 1809.

[189] R. Murgasova, D.H. Hercules, Anal. Bioanal. Chem. 373 (2002) 481.

[190] W.M.A. Niessen, Liquid Chromatography-Mass Spectrometry, Third Edition,Chromatographic Science Series Vol. 97.

[191] R. Consden, A.H. Gordon, A.J.P. Martin, Biochem. J. 38 (1944) 224.

[192] J.G. Kirchner, J.M. Miller, G.J. Keller, Anal. Chem. 23 (1951) 420.

[193] X. Chen, L. Kong, X. Su, H. Fu, J. Ni, R. Zhao, H. Z, J. Chromatogr. A 1040 (2004) 169.

[194] J. Blomberg, P.J. Schoenmakers, J. Beens, R. Tijssen. J. High Resolut. Chromatogr. 20 (1997) 539.

[195] J.C. Giddings, Anal. Chem. 39 (1967) 1027.

[196] C.G. Horváth, S.R. Lipsky, Anal. Chem. 39 (1967) 1893.

[197] M.A. Stadalius, M.A. Quarry, L.R. Snyder, J. Chromatogr. 327 (1985) 93.

[198] M.Gilar, A.E. Daly, M. Kele, U.D. Neue, J.C. Gebler, J. Chromatogr. A 1061 (2004) 183.

[199] R.W. Stout, S.I. Sivakoff, R.D. Ricker, L.R. Snyder, J. Chromatogr. 353 (1986) 439.

[200] U.D. Neue, J. Chromatogr. A 1079 (2005) 153.

[201] P. Jandera, K. Novotna', L. Kolarova', J. Fischer, Chromatographia 60 (2004) S27.

[202] J.M. Davis, J.C. Giddings, Anal. Chem. 55 (1983) 418.

[203] D. Ryan, P. Morrison, P. Marriott, J. Chromatogr. A 1071 (2005) 47.

[204] C.J. Venkatramani, J. Xu, J.B. Phillips, Anal. Chem. 68 (1996) 1486.

[205] M.M. Bushey, J.W. Jorgenson, Anal. Chem. 62 (1990) 161.

[206] M.M. Bushey, J.W. Jorgenson, Anal. Chem. 62 (1990) 978.

[207] C.J. Venkatramani, Y. Zelechonok, Anal. Chem. 75 (2003) 3484.

[208] F. Erni, R.W. Frei, J. Chromatogr. 149 (1978) 561.

[209] P.Q. Tranchida, P. Dugo, G. Dugo, L. Mondello, J. Chromatogr. A. 1054 (2004) 3.

[210] J. Xu, J.B. Phillips, J. Chromatogr. A 703 (1995) 327.

[211] M. Dunn, R. Shellie, P. Morrison, P. Marriott, J. Chromatogr. A 1056 (2004) 163.

[212] L. Mondello, A.C. Lewis, K.D. Bartle, Multidimensional chromatography, John Wiley & Sons, Ltd. Chichester, England (2002).

[213] C.J. Venkatramani, J.B. Phillips. J. Microcol. Sep. 5 (1993) 511.

[214] R.B. Gaines, G.S. Frysinger, M.S. Hendrick-Smith, J.D. Stuart. Environ. Sci. Technol. 33 (1999) 2106.

[215] P.J. Schoenmakers, J.L.M.M. Oomen, J. Blomberg, W. Genuit, G. van Velzen. J. Chromatogr. A 892 (2000) 29.

[216] M.M. Bushey, J.W. Jorgenson, J. Microcolumn. Sep. 2 (1990) 293.

[217] A.V. Lemmo, J.W. Jorgenson, J. Chromatogr. A 633 (1993) 213.

[218] D.J. Rose, G.J. Opiteck, Anal. Chem. 66 (1994) 2529.

[219] L.A. Holland, J.W. Jorgenson, Anal. Chem. 67 (1995) 3275.

[220] G.J. Opiteck, J.W. Jorgenson, R.J. Anderegg, Anal. Chem. 69 (1997) 2283.

[221] I. François, A. de Villiers, P. Sandra, J. Sep. Sci. 29 (2006) 492.

[222] P. Jandera, M. Halama, L. Kolářová, J. Fischer, K. Novotná, J. Chromatogr. A 1087 (2005) 112.

[223] R.E. Murphy, M.R. Schure, J.P. Foley, Anal. Chem. 70 (1998) 4353.

[224] S. Park, D. Cho, J. Ryu, K. Kwon, W. Lee, T. Chang, Macromolecules 35 (2002) 5974.

[225] P. Dugo, O. Favoino, R. Luppino, G. Dugo, L. Mondello, Anal. Chem. 76 (2004) 2525.

[226] P. Dugo, T. Kumm, B. Chiofalo, A. Cotroneo, L. Mondello, J. Sep. Sci. 29 (2006) 1146.

[227] X. Wang, W. Li, H.T. Rasmussen, J. Chromatogr. A 1083 (2005) 58.

[228] P. Kilz, R.P. Kruger, H. Much, G. Schulz, ACS Adv. Chem. 247 (1995) 223.

[229] A. van der Horst, P.J. Schoenmakers, J. Chromatogr. A. 1000 (2003) 693.

[230] S.M. Graef, A.J.P. van Zyl, R.D. Sanderson, B. Klumperman, H. Pasch, J. Appl. Polym. Sci. 88 (2003) 2530.

[231] J. Adrian, E. Esser, G. Hellman, H. Pasch, Polymer 41 (2000) 2439.

[232] A. Siewing, J. Schierholz, D. Braun, G. Hellmann, H. Pasch, Macromol. Chem. Phys. 202 (2001) 2890.

[233] L. Coulier, E.R. Kaal, T. Hankemeier, J. Chromatogr. A. 1070 (2005) 79.

[234] K. Im, Y. Kim, T. Chang, K. Lee, N. Choi, J. Chromatogr. A 1103 (2006) 235.

[235] J. Pellett, P. Lukulay, Y. Mao, W. Bowen, R. Reed, M. Ma, R.C. Munger, J.W. Dolan, L. Wrisley, K. Medwid, N.P. Toltl, C.C. Chan, M. Skibic, K.l Biswas, K. A. Wells, L.R. Snyder, J. Chromatogr. A 1101 (2006) 122.

[236] B. Trathnigg, M. Kollroser, C. Rappel, J. Chromatogr. A 922 (2001) 193.

[237] B. Trathnigg, C. Rappel, R. Raml, A. Gorbunov, J. Chromatogr. A 953 (2002) 89.

[238] M. J. Gray, G. R. Denis, P. J. Slonecker, R. A. Shalliker, J. Chromatogr. A 1015 (2003) 89.

[239] M. J. Gray, G. R. Denis, P. Wormell, R. A. Shalliker, P. J. Slonecker, J. Chromatogr. A 975 (2002) 285.

[240] A. P. Sweeney, S. G. Wyllie, R. A. Shalliker, J. Liquid Chromatogr. Rel. Technol. 24 (2001) 2559.

[241] M. J. Gray, A. P. Sweeney, G. R. Denis, P. J. Slonecker, R. A. Shalliker, Analyst 128 (2003) 598.

[242] E. P. Toups, M. J. Gray, G. R. Denis, N. Reddy, M.A. Wilson, R.A. Shalliker, J. Sep. Sci. 29 (2006) 491.

[243] M.J. Gray, G.R. Dennis, P.J. Slonecker, R.A. Shalliker, J. Chromatogr. A. 1041 (2004) 101.

[244] M. Gilar, P. Olivova, A.E. Daly, J.C. Gebler, J. Sep. Sci. 28 (2005) 1694.

[245] T. Ikegami, T. Hara, H. Kimura, H. Kobayashi, K. Hosoya, K. Cabrera, N. Tanaka, J. Chromatogr. A 1106 (2006) 112.

[246] K. A. Mortier, G.F. Zhang, C.H. Van Peteghem, W.E. Lambert, J. Am. Soc. Mass Spectrom. 15 (2004) 585.

[247] M. Dreux, M. Lafosse, Analysis 20 (1992) 587.

[248] M. Dreux, M. Lafosse, L. Morin-Allory, LC-GC Int. 9 (1996) 148.

[249] M. Lafosse, C. Elfakir, L. Morin-Allory, M. Dreux, J. High Resolut. Chromatogr. 15 (1992) 312.

[250] B. Trathnigg, M. Kollroser, D. Berek, S. Nguyen and D. Hunkeler, in: Chromatography of Polymers: Hyphenated and Multidimensional Techniques, T. Provder (Ed), American Chemical Society, Washington, DC (1999).

Poster/Oral Presentations

1. Monica Dolci, Roger M. Smith and Maggi G. Tebrake *Fundamental evaluation of separation orthogonality in off-line two-dimensional liquid chromatography.* Presented at the Analytical Research Forum 2005, organised by the Analytical Division of the Royal Society of Chemistry, University of Plymouth, 18-20 July 2005.

2. Monica Dolci, Roger M. Smith and Maggi G. Tebrake *Fundamental evaluation of separation orthogonality in off-line two-dimensional liquid chromatography.* Presented at the 2005 Younger European Chemists' Conference, Brno, 30 August-04 September 2005.

3. Monica Dolci, Roger M. Smith and Maggi G. Tebrake *Fundamental evaluation of separation orthogonality in off-line two-dimensional liquid chromatography.* Presented at PASG 2005, Marriott Forest of Arden Hotel, Solihull, 11 October 2005.

4. Monica Dolci, Roger M. Smith and Maggi G. Tebrake *Fundamental evaluation of separation orthogonality in off-line two-dimensional liquid chromatography.* Presented at HTC-9, York, 06-10 February 2006.

5. Monica Dolci *Multidimensional Liquid Chromatography Characterisation of Oligolactic acids* Presented at 3M HealthCare EP+T Comms Meeting, Loughborough, April 2007.

6. Monica Dolci *Multidimensional Liquid Chromatography Characterisation of Oligolactic acids* Presented at 3M HealthCare University Forum, Loughborough, May 2007.

7. Monica Dolci, Roger M. Smith and Maggi G. Tebrake *Screening of LC methods for the separation of surfactants employed as pharmaceutical excipients.* Presented at HPLC-07, Ghent, 17-21 June 2007.

8. Monica Dolci *LC method screenin for the separation of surfactants employed as pharmaceutical excipients.* Presented at PASG 2007, Whittlebury Hall Northamptonshire, 08-09 October 2007.

Postgraduate Research Student Skills Training Record

Training summary **Years** 2004/07

Name of Student	ID	FULL-TIME OR PART-TIME
Monica Dolci	A 47 40 76	Full time

Name(s) of Supervisor(s)	Department
Prof. R. Smith	Chemistry

Type of training	Number of days
Department-based training	8
Professional Development courses (data from PD database)	36
Other activities (validated by PD or research supervisor)	7
UK GRAD courses (Research Council funded students)	3
Overall training total in days	54

Department-based training **Year** **2004/05**

(including external training approved by the Department, but not UK GRAD courses, Professional Development courses or other activities validated by Professional Development or supervisor)

Name of Student	**ID**	**FULL-TIME OR PART-TIME**
Monica Dolci	A 47 40 76	Full time

Name(s) of Supervisor(s)	**Department**
Prof. R. Smith	Chemistry

Activity	Time claimed in days	Date completed
Safety induction	1/2	10/04
PD Induction day	1	08/10/04
RSC talk	1/2	14/10/04
Laboratory News Forum	1	18/10/04
9th Desty Memorial Meeting	1	20/10/04
Gas Phase Separation lectures	1	26/10/04
Liquid Phase Separation lectures	5	09/11-01/12/04
Plagiarism, Citation and RefWork	1/2	10/11/04
Lecture on Catalysis, Nottingham University	1/2	17/11/04
Teaching Skills A	1/2	18/11/04
Teaching Skills B	1/2	25/11/04
Presentation Skills A	1/2	19/11/04
Presentation Skills B	1/2	26/11/04
Office 2003, Power Point	1	23, 26/11/04
Managing your Project	1/2	09/12/04
Chairing Meetings	1/2	17/01/05
Assertiveness A	1/2	18/01/05
Assertiveness B	1/2	25/01/05
Conference Presentation Skills	1	19/11/04, 03/12/05
Essential Intr Skills for PhD	2	29,30/11/04
Exam invigilator training	1/2	25/01/05
Word: large documents	1/2	10/02/05
Pharmaceutical analysis	1/2	15/02/05
Teaching Skills C	1/2	19/11/04
Dealing with difficult people	2	17, 24/02/05
Keeping research up to date	1/2	17/02/05
Asbestos	1/2	28/02/05
Risk Assessment in research	1/3	07/03/05
Safety in research	1/3	07/03/05
Report writing	1/3	07/03/05
Statistic courses	2	03-04/05
Anova	2	6-13/04/05
Getting the most out of supervision	1/2	18/04/05
EYP (RSC)	2	13-15/05/05
Managing your Project	1/2	09/12/04

Department-based training **Year** **2005/06**

(including external training approved by the Department, but not UK GRAD courses, Professional Development courses or other activities validated by Professional Development or supervisor)

Activity	Time claimed in days	Date completed
Organic Synthesis Symposium (Dept)	1/2	05/10/05
PASG (External Conference)	1	11/10/05
RSC talk: Chemistry, friend or foe (Dept)	1/2	25/10/05
Managing personal stress (PD)	1	17/11/05
Writing your PhD (PD)	1/2	06/12/05
Report writing (PD)	1/2	14/12/05
CV and application form (PD)	1/2	23/01/06
Safe surfing (PD)	1 hr	25/01/06
Job interviews (PD)	1/2	30/01/06
LC x LC course (External course)	1	06/02/06
Keepin your research up to date (PD)	1/2	02/02/06
Data protection issues (PD)	1/2	08/03/06
Career managment	1/2	10/03/06
Taking minutes (PD)	1/2	16/03/06
Workshop on Method Validation (LGC course)	1	23/03/06
Viva, what happens? (PD)	1/2	27/03/06

Department-based training **Year** **2006/07**

(including external training approved by the Department, but not UK GRAD courses, Professional Development courses or other activities validated by Professional Development or supervisor)

Activity	Time claimed in days	Date completed
The emotionally intelligent manager	1/2	13/11/06
Recruitment and selection	1/2	14/11/06
Interviewing skills	1/2	23/11/06
Excel	1	30/11/06
Networking skills	1/2	07/12/06
Making an impact	1/2	11/12/06
MOSAIC	5	Jan-Mar/07
Career managment	1	31/01/07
Solution to stress	1/2	13/09/07

Appendix 1: LC-MS of OLAs fractions

Fraction 1

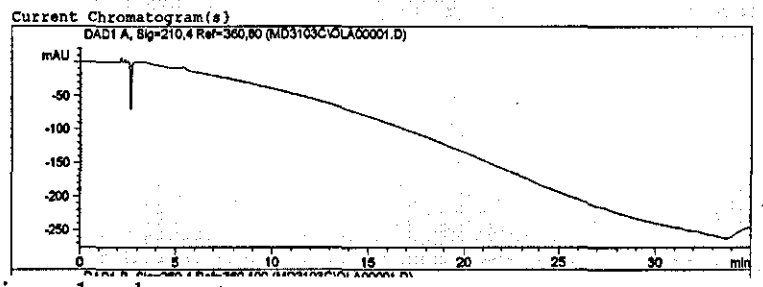

Figure 1a: chromatogram

of window 80: Apex Mass Spectrum of Peak 5.43 of OLA00001.D

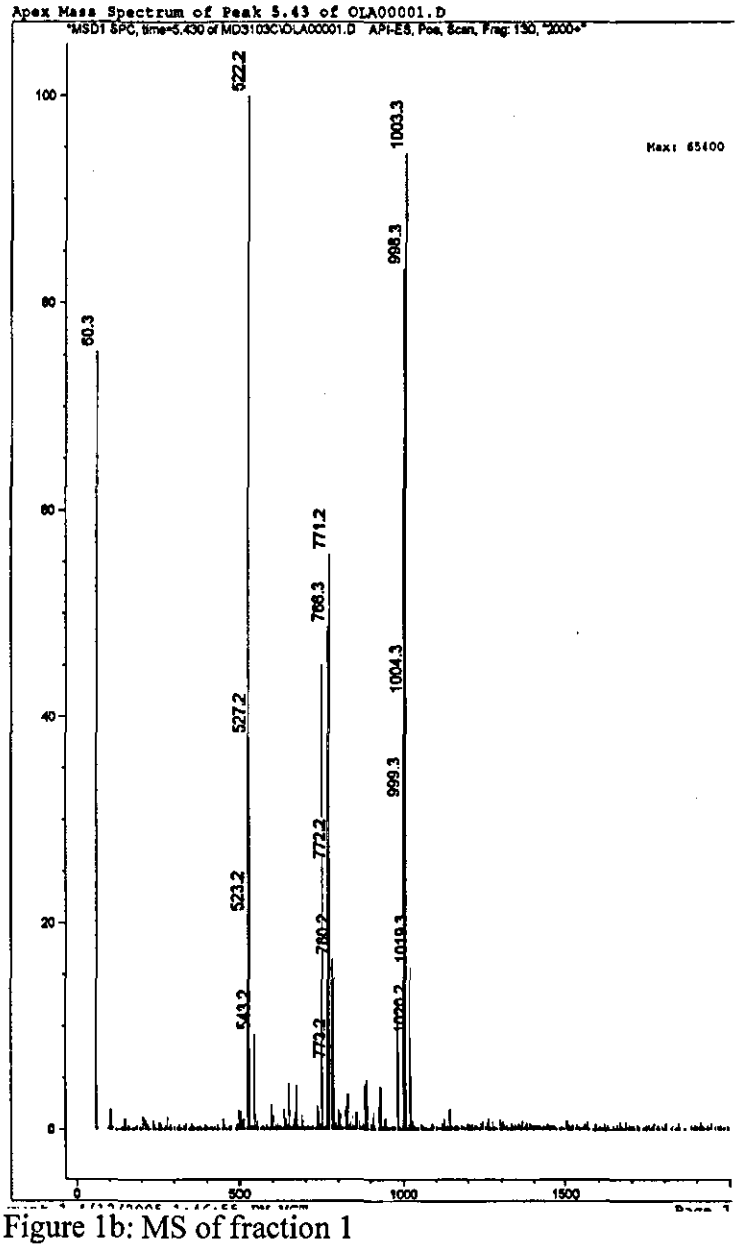

Figure 1b: MS of fraction 1

Possibly we see a cyclic with n= 13 (13 x 72= 936) which has lost a cleaved monomer (936+44= 980), which has lost a (H+OH) group, (980+18= **998**)

Fraction 2

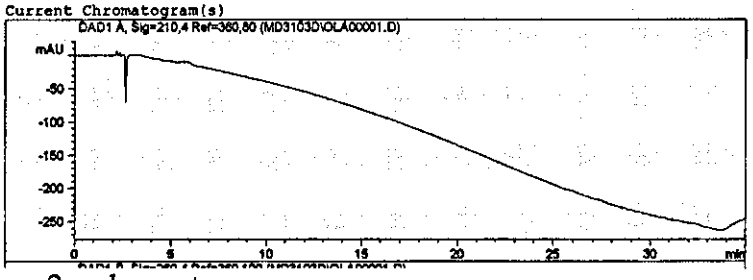

Figure 2a: chromatogram

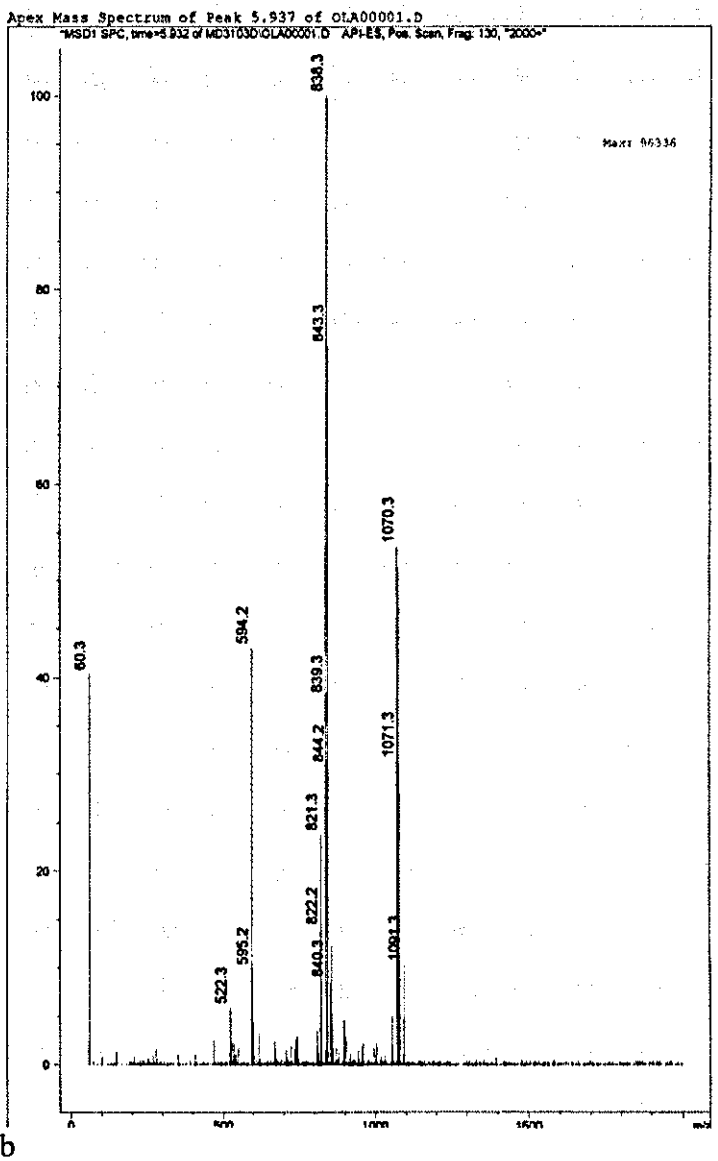

Figure 2b

This fraction could contain a cyclic with n= 14 (14 x 72= 1008), which has lost a cleaved monomer and a (H+OH) group, (1008+18+44= **1070**).

Also, it could contain a cyclic with n= 11 (11 x 72= 792) which has lost a cleaved monomer and 2 protons (792+44+2= **838**).

Fraction 3

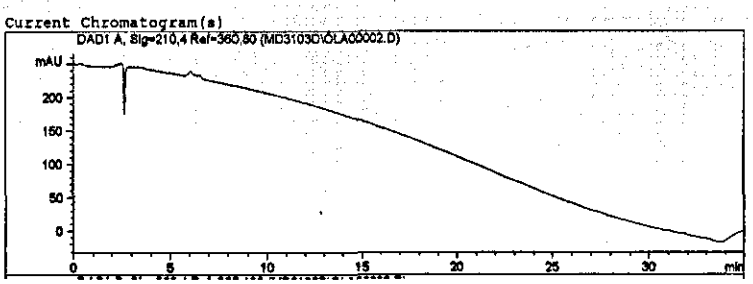

Figure 3a

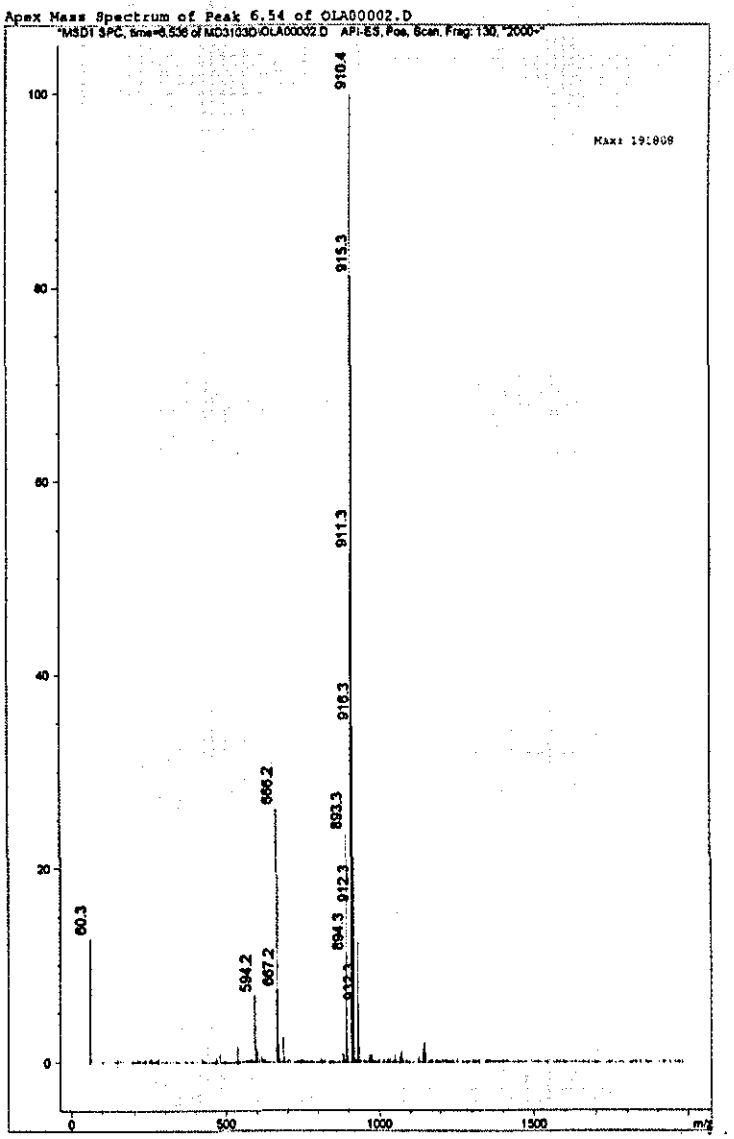

Figure 3b

In this fraction we have three different oligomers, with n= 10 (**910**-18-172= 720; 720:72= 10); n= 9 (**666**-18= 648; 648:72= 9) and n= 8 (**594**-18= 576; 576:72= 8).

Fraction 4

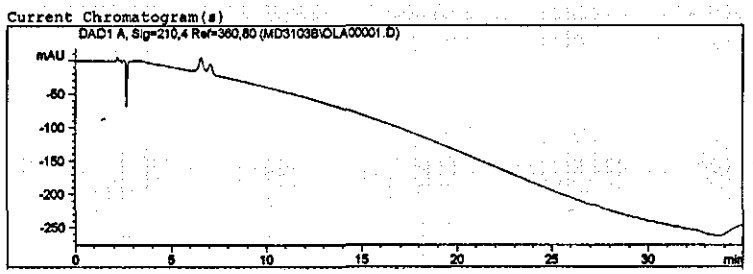

Figure 4a

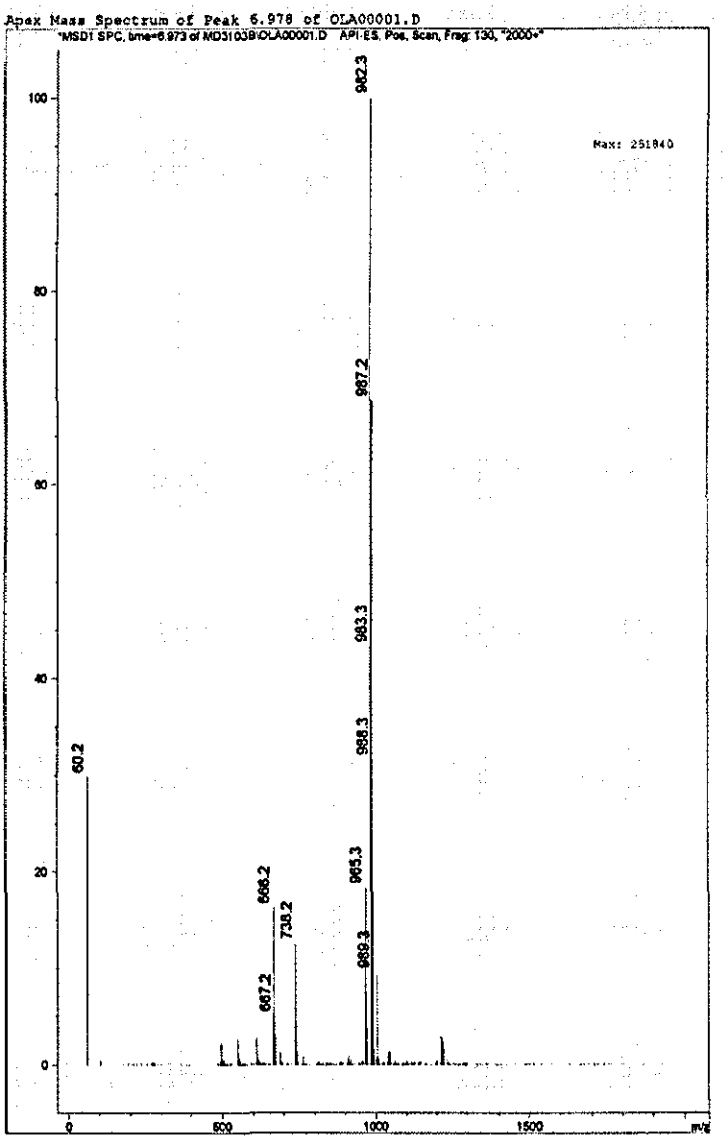

Figure 4b

In this fraction we have the oligomers n= 9 (**666**), n= 10 (**738**) and n=11 (**982-18-172= 792; 792:72= 11**).

Fraction 5

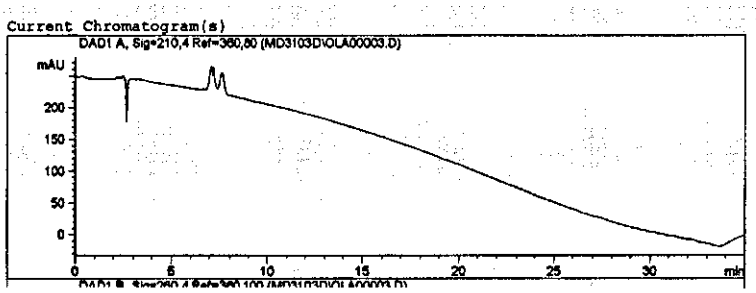

Figure 5a

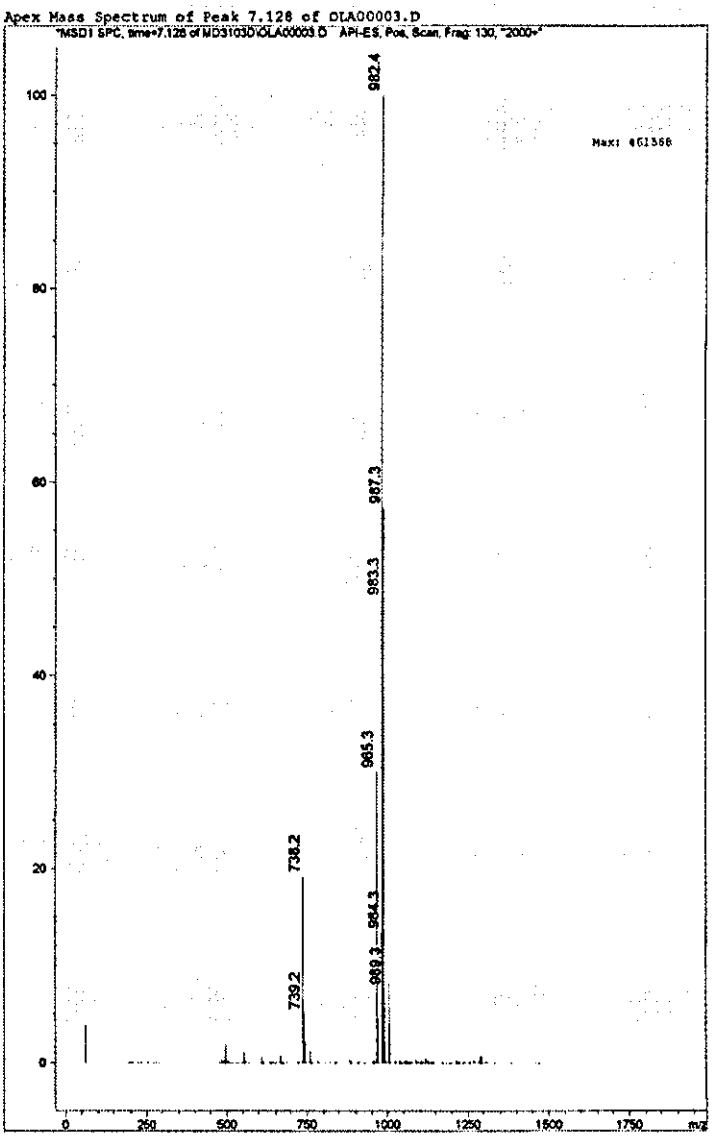

Figure 5b: MS of first peak shown in figure 5a

This part of fraction 5 still contains the units n= 10 (**738**) and n= 11 (**982**)

Fraction 5

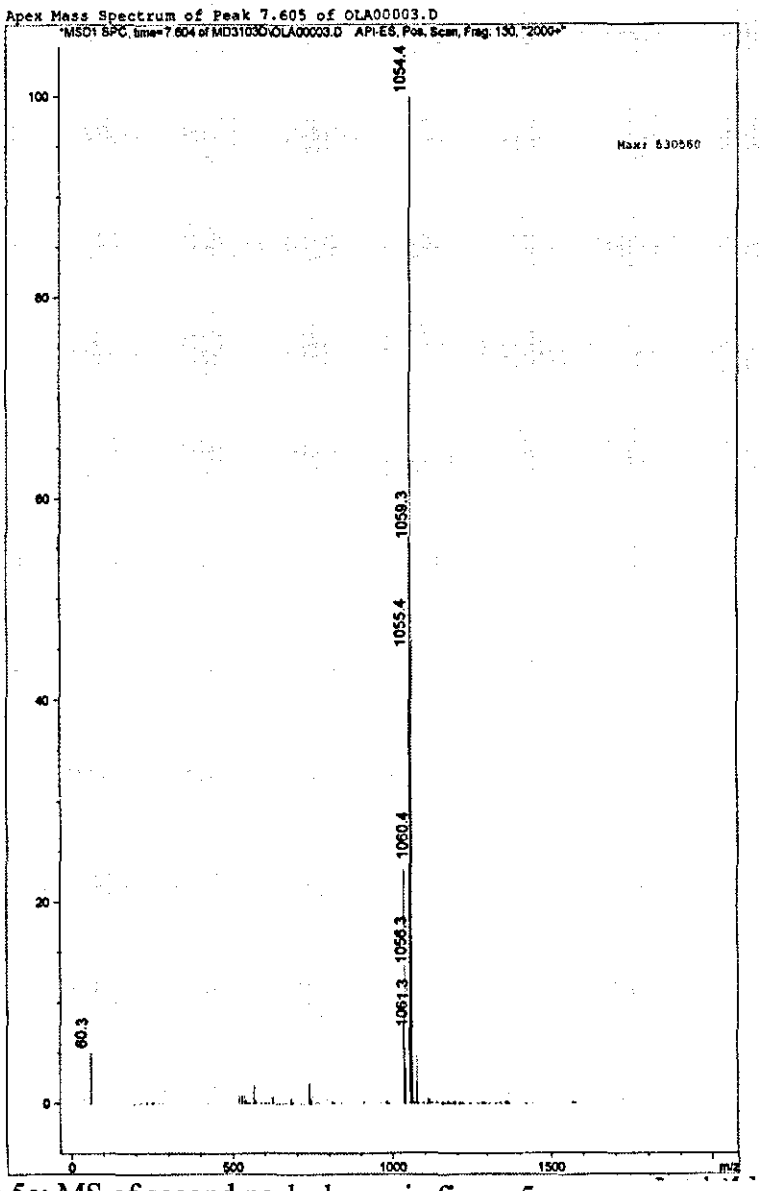

Figure 5c: MS of second peak shown in figure 5a

This part of fraction 5 has oligomer n= 12 (**1054**-18-172= 864; 864:72=12).

Fraction 6

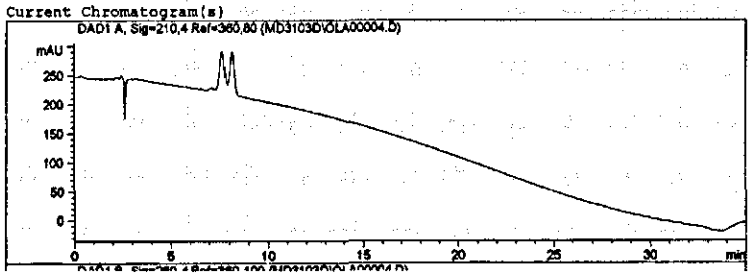

Figure 6a

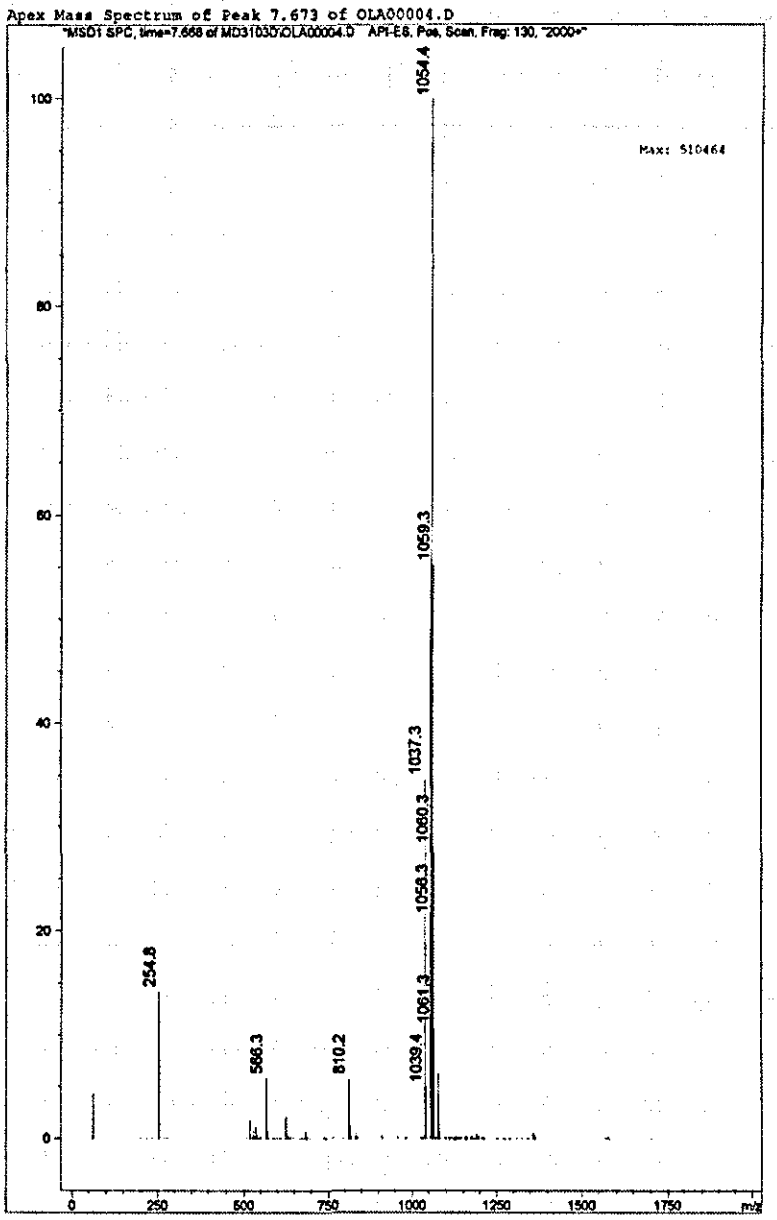

Figure 6b

We can still see monomer n=11 (**810**-18= 792; 792:72= 11) and n=12 (**1054**)

Fraction 6

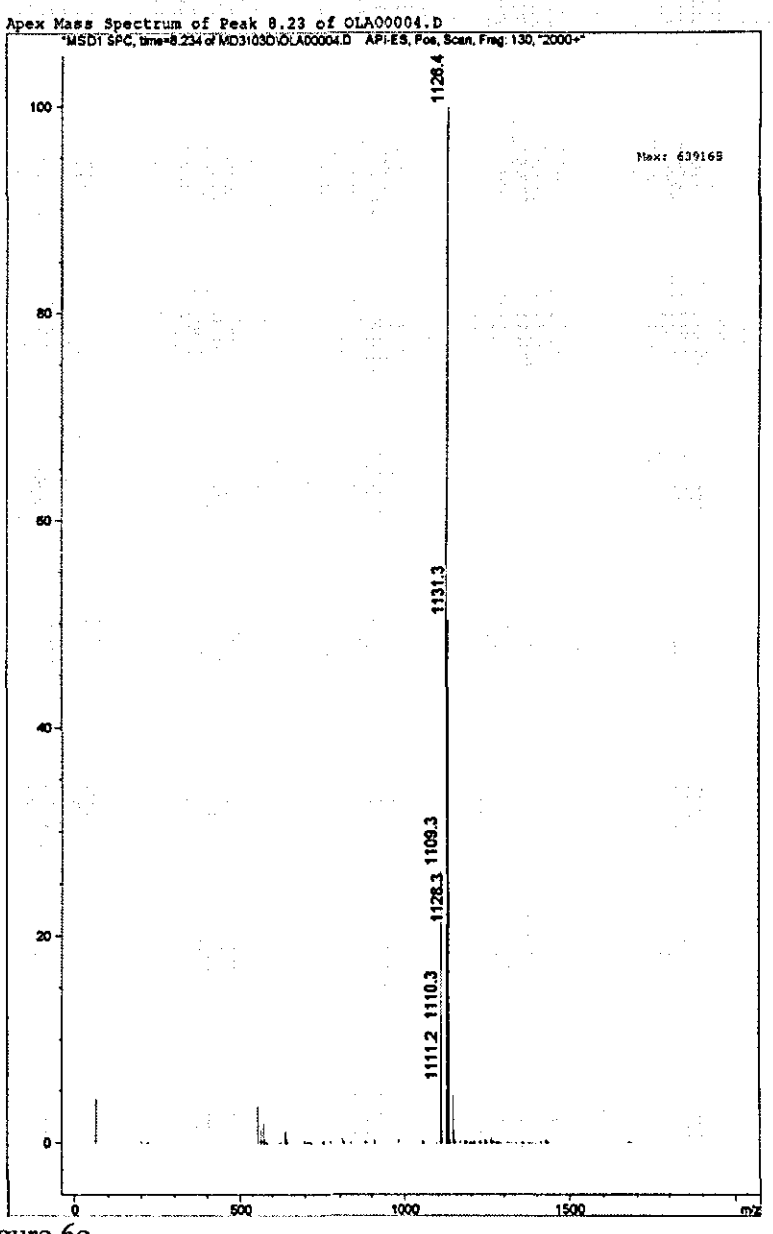

Figure 6c

We can see monomer n=13 (**1126**-18-172= 936; 936:72= 13).

Fraction 7

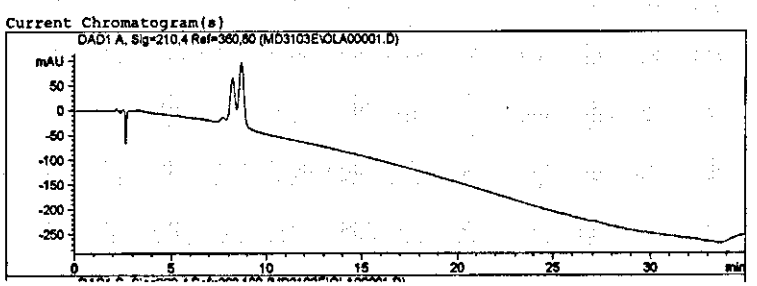

Figure 7a

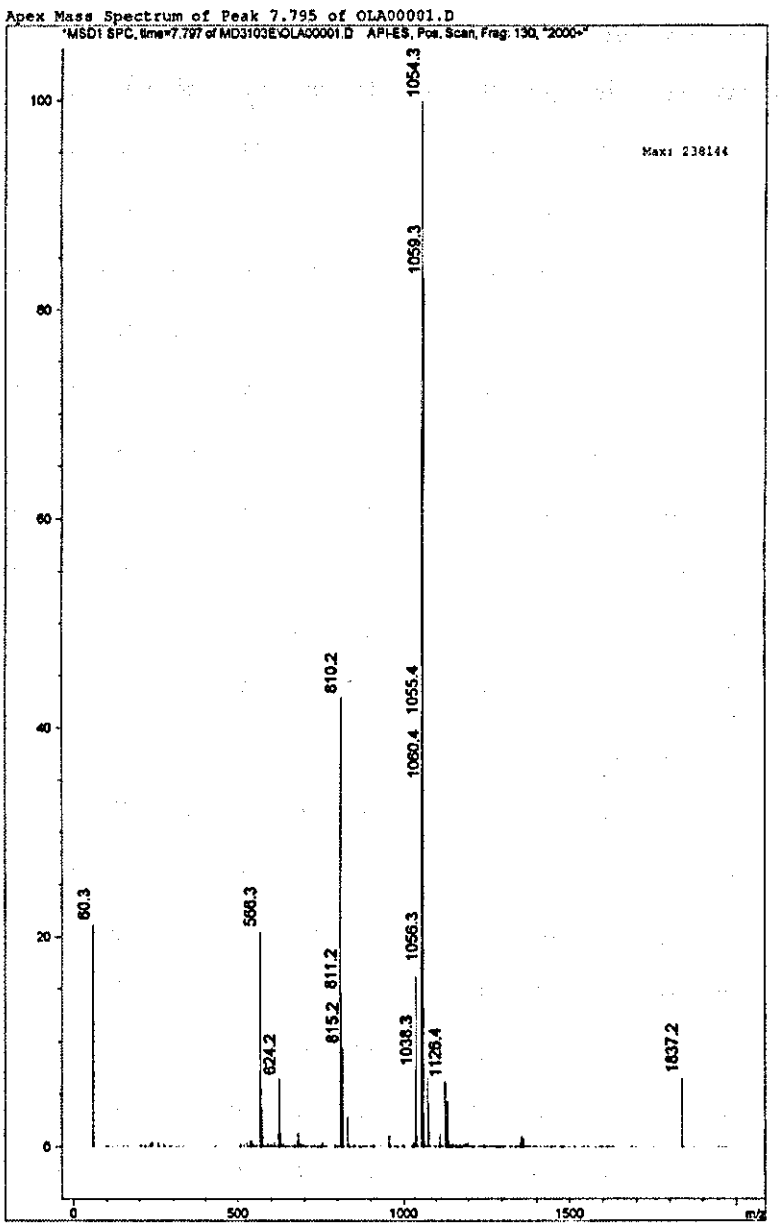

Figure 7b

We can see monomer n=12 (**1054**-18-172= 864; 864:72= 12). Doubly charged species 624 is also present (624 x 2= 1248; 1248-18-172= 1058, protonated **1059).**

Fraction 7

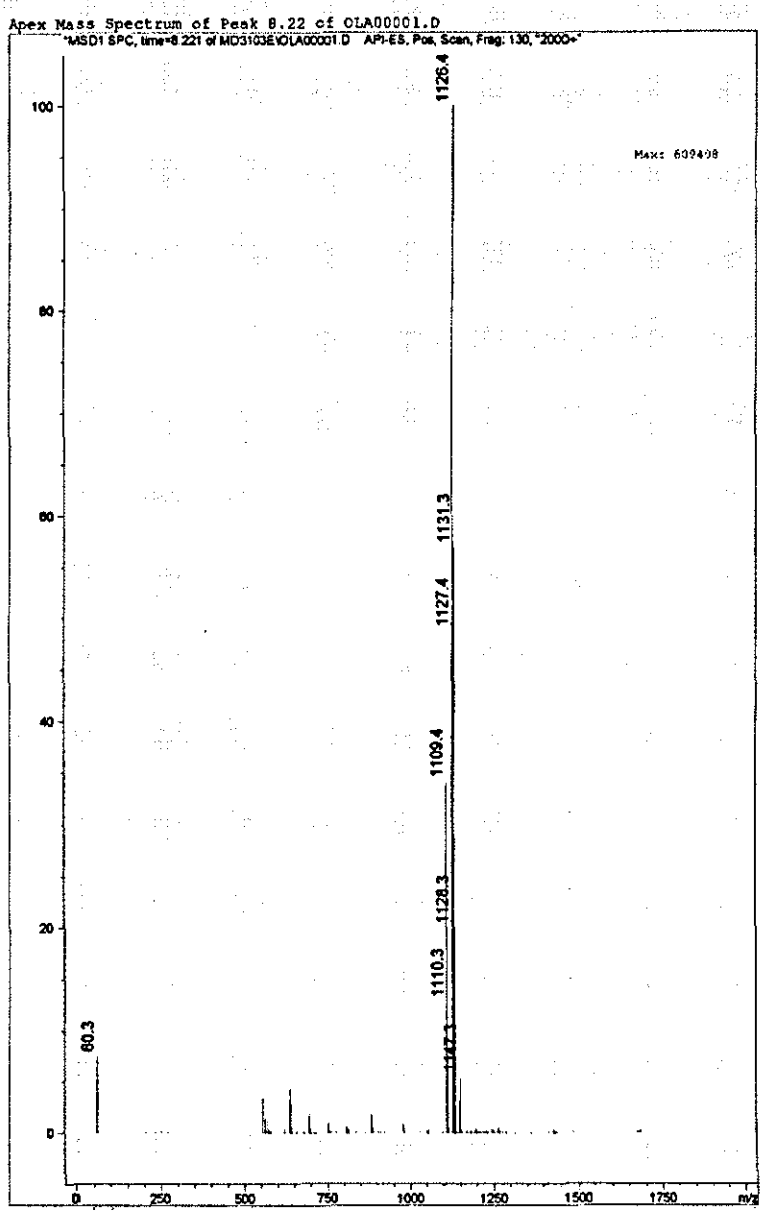

Figure 7c

We can see monomer n=13 (**1126**-18-172= 936; 936:72= 13).

Fraction 7

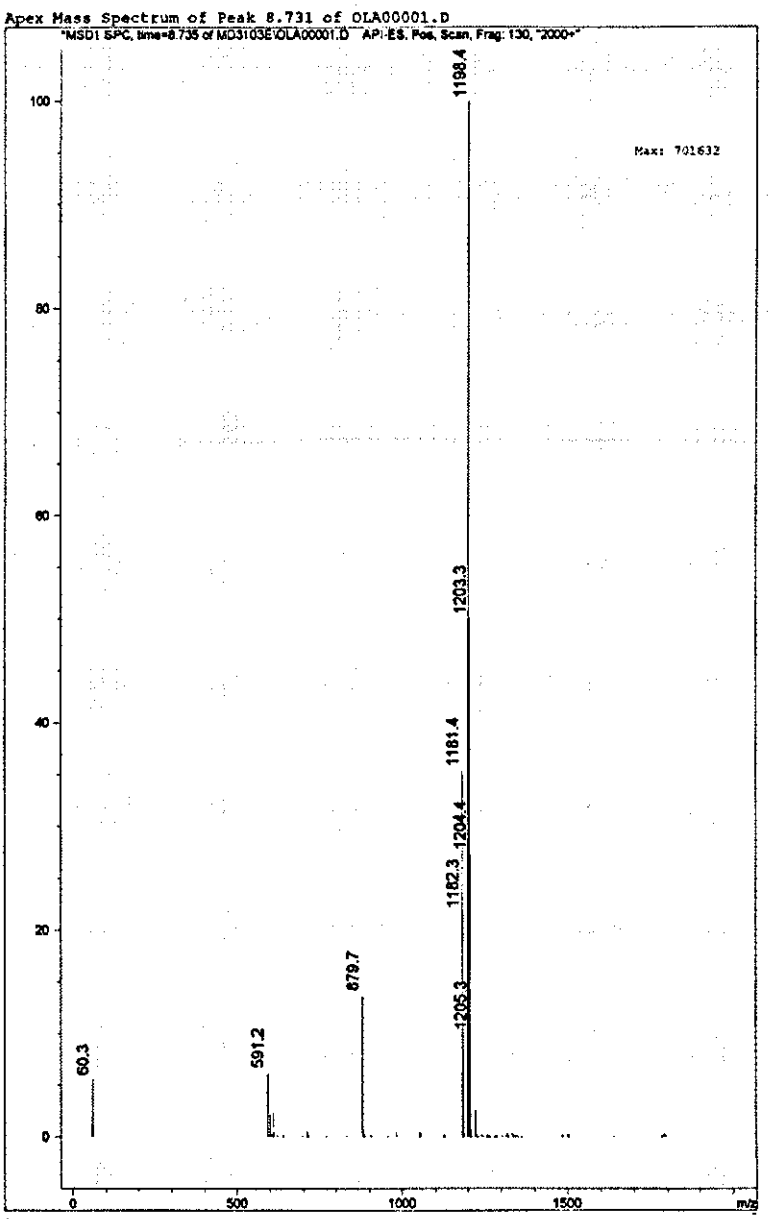

Figure 7d

We can see monomer n=14 (**1198**-18-172= 1008; 1008:72= 14). Doubly charged species 591 is also present (591 x 2= 1182).

Fraction 8

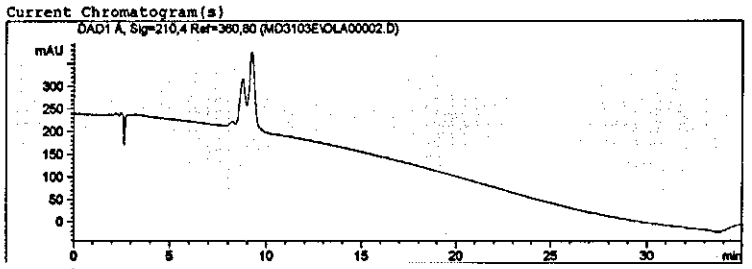

Figure 8a

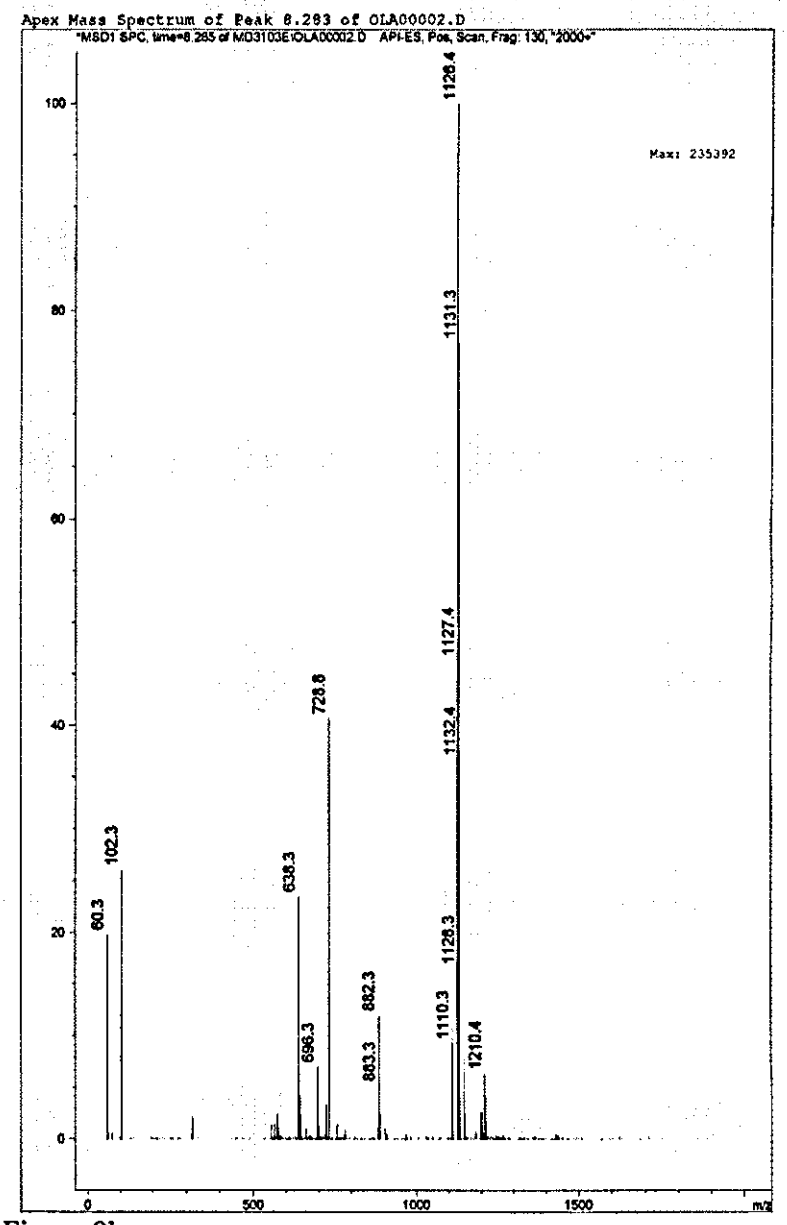

Figure 8b

We can see monomer n=13 (**1126**-18-172= 936; 936:72= 13). Doubly charged species 638 (638 x 2= 1276; 1276+57+57+18+18=1126).

Fraction 8

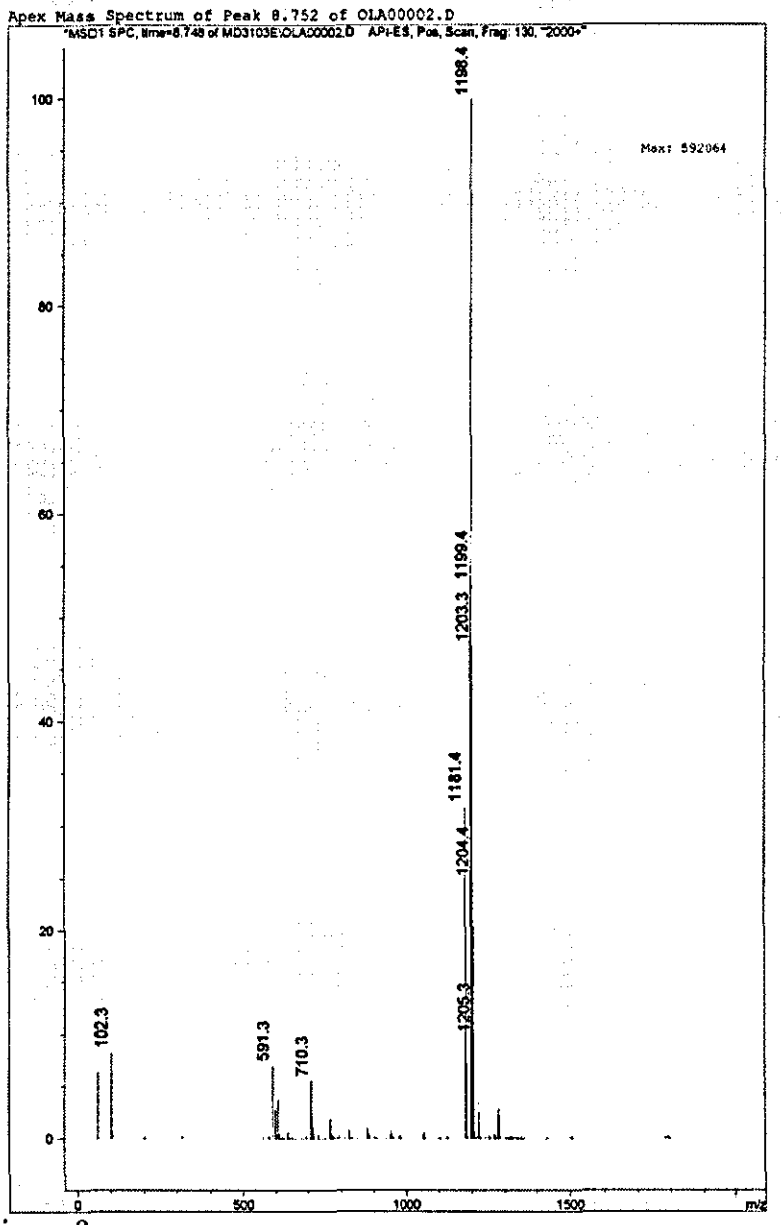

Figure 8c

We can see monomer n=14 (**1198**-18-172= 1008; 1008:72= 14) and doubly charged species 591.

Fraction 8

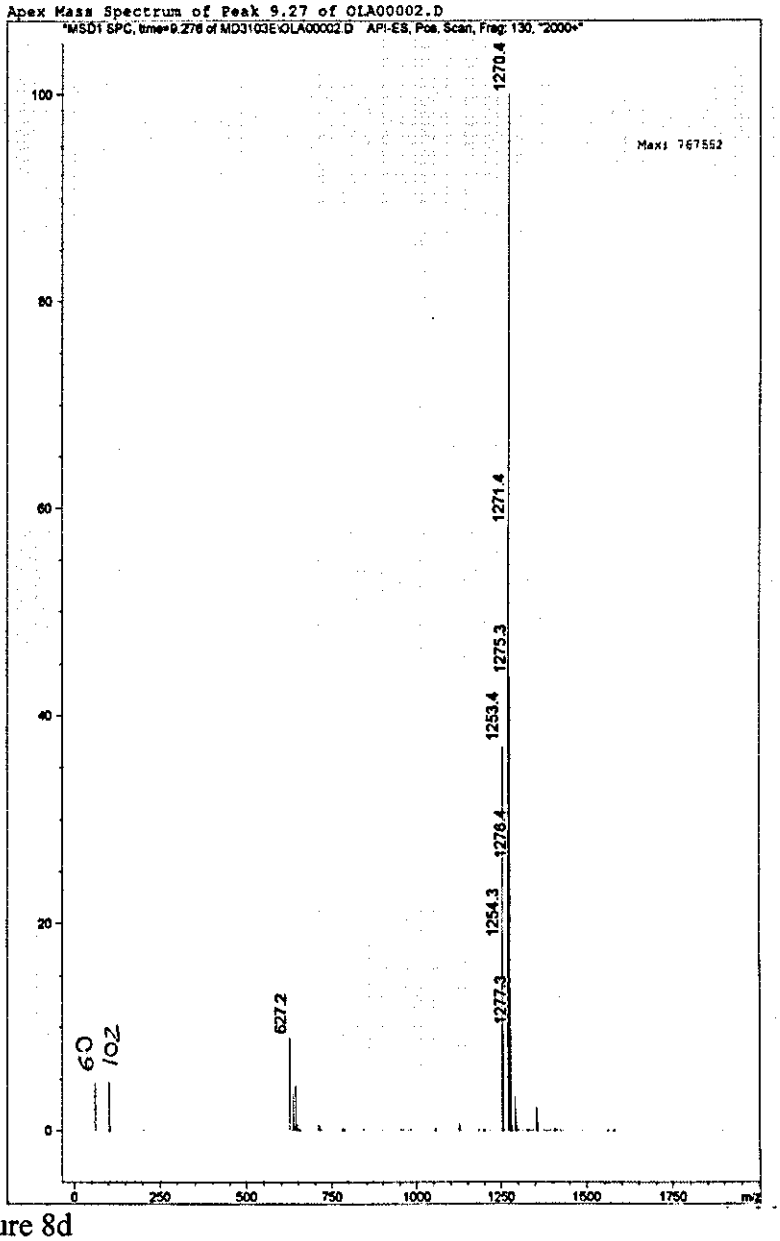

Figure 8d

We can see monomer n=15 (**1270**-18-172= 1080; 1080:72= 15) and doubly charged species 627 (627 x 2= 1254).

Fraction 9

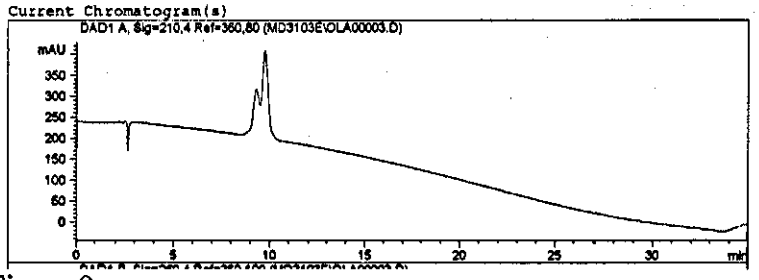

Figure 9a

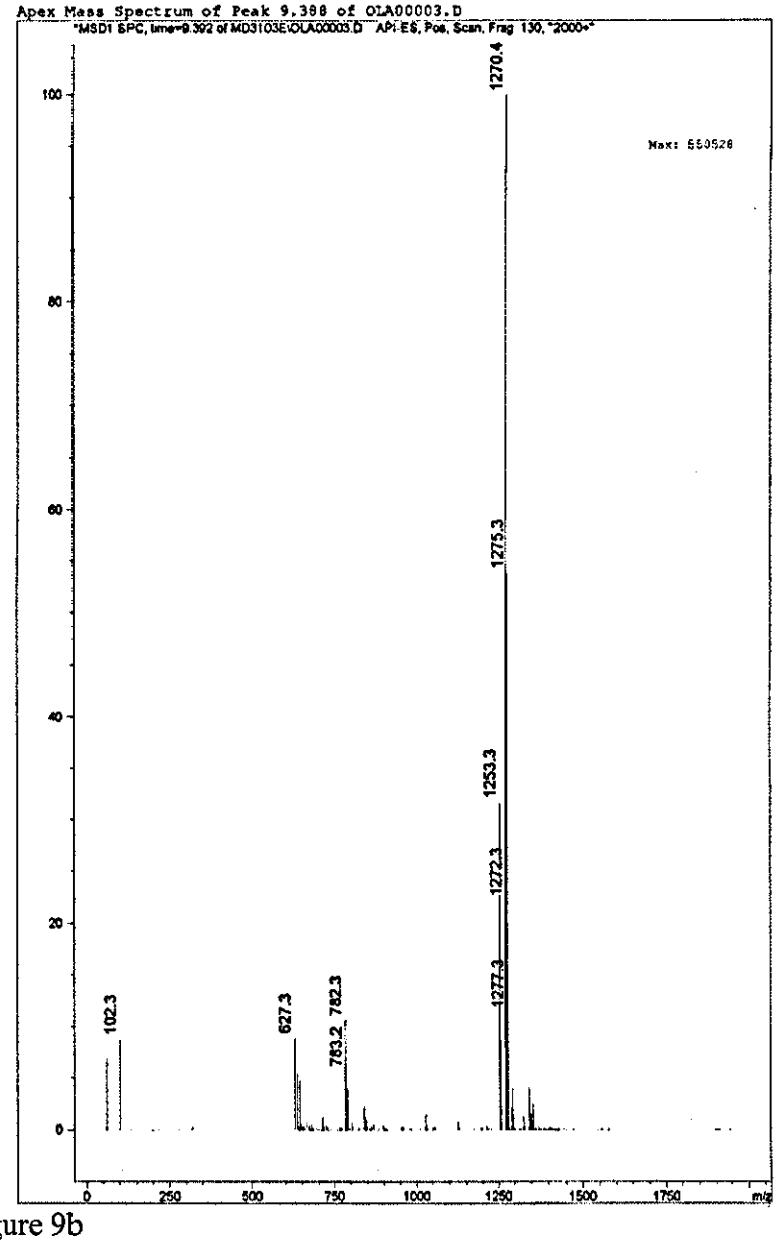

Figure 9b

We can see monomer n=15 (1270-18-172= 1080; 1080:72= 15) and doubly charged species 627.

Fraction 9

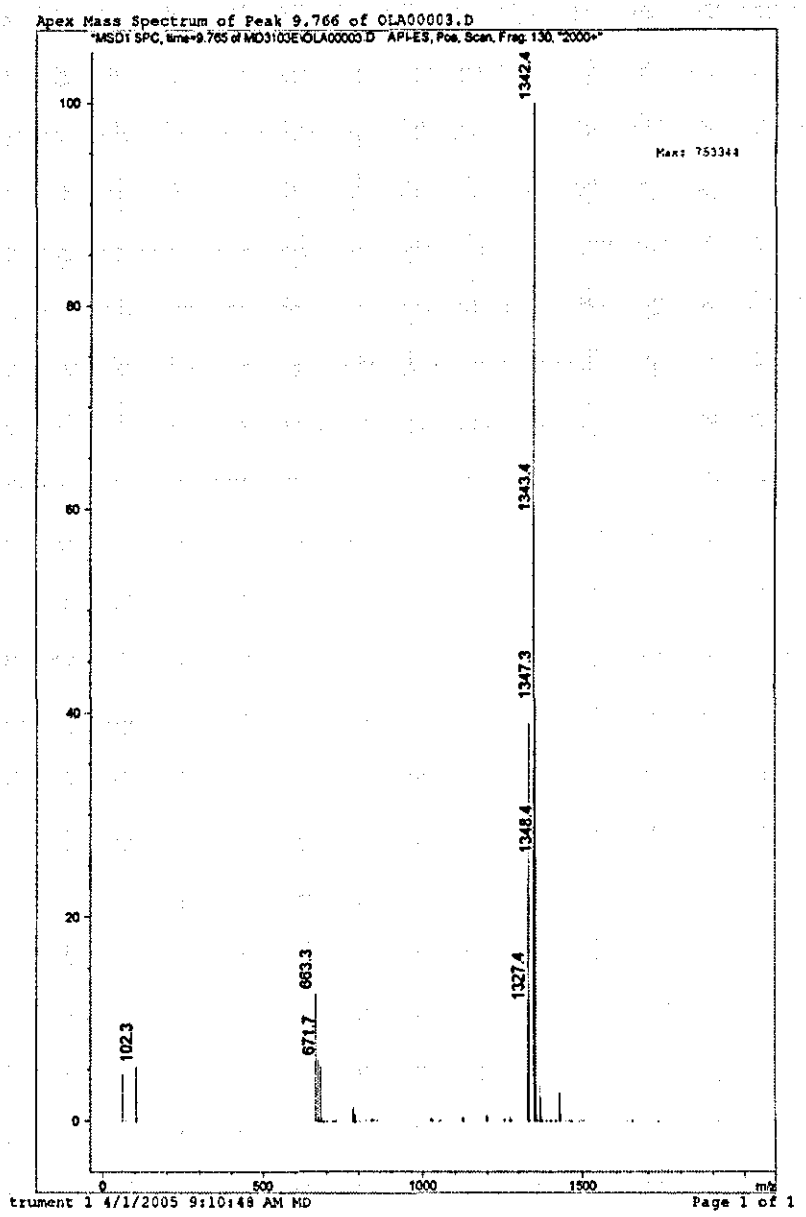

Figure 9c

We can see monomer n=16 (**1342**-18-172= 1152; 1152:72= 16). Two doubly charged species: 663 (663 x 2= 1326, protonated to give the fragment 1327) and 671 (671 x 2= 1342).

Fraction 10

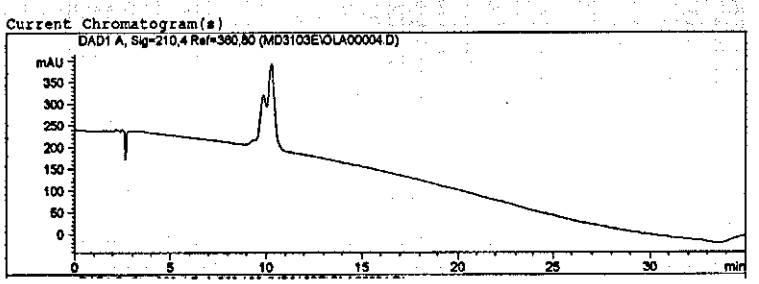

Figure 10a

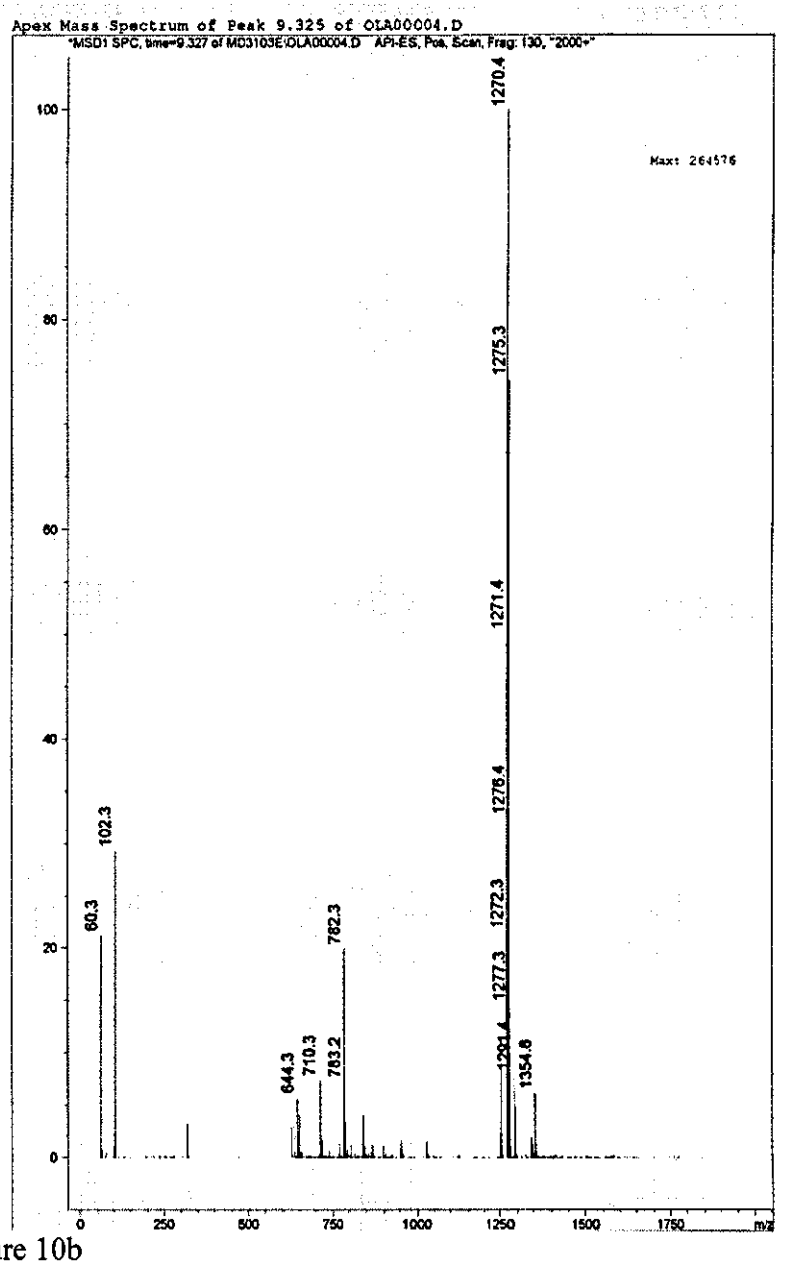

Figure 10b

We can still see monomer n=15 (**1270**-18-172= 1080; 1080:72= 15).

Fraction 10

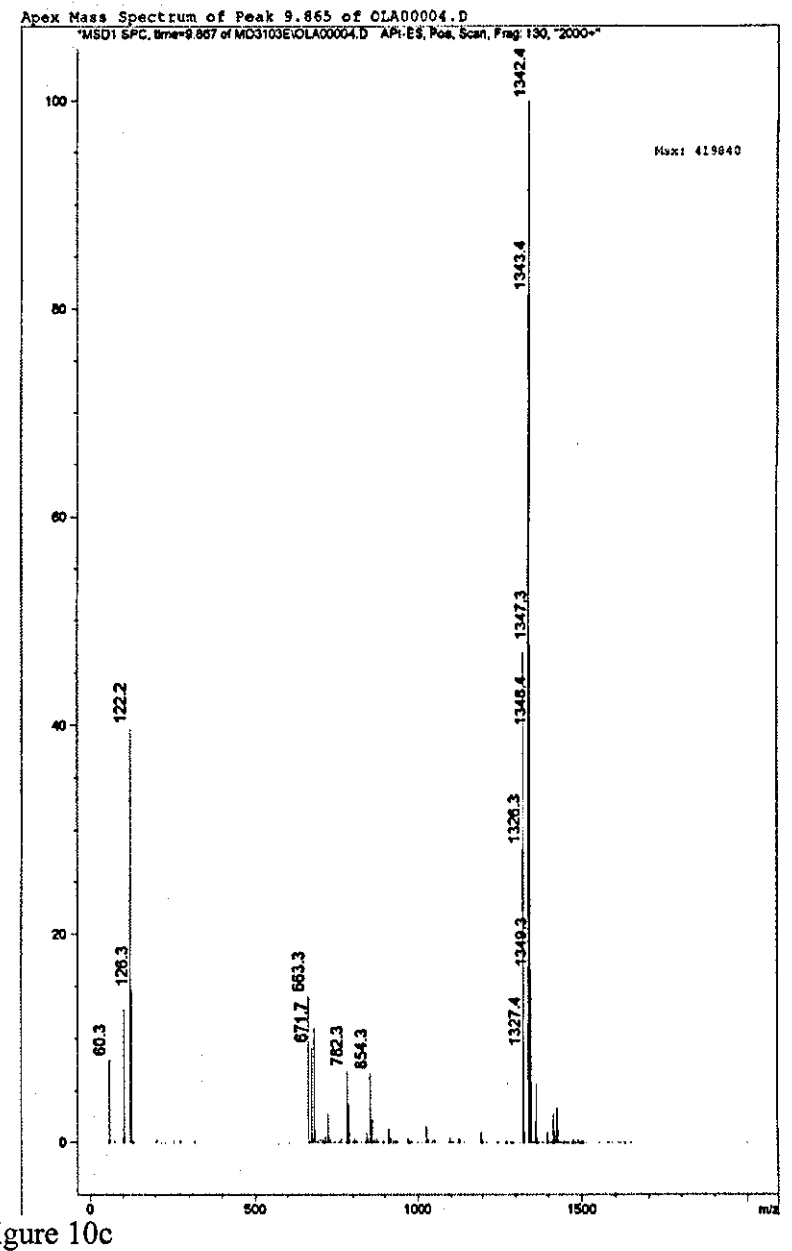

Figure 10c

We can see monomer n=16 (**1342**-18-172= 1152; 1152:72= 16) and doubly charged ions 663 (663 x 2= 1326) and 671 (671 x 2= 1342).

Fraction 10

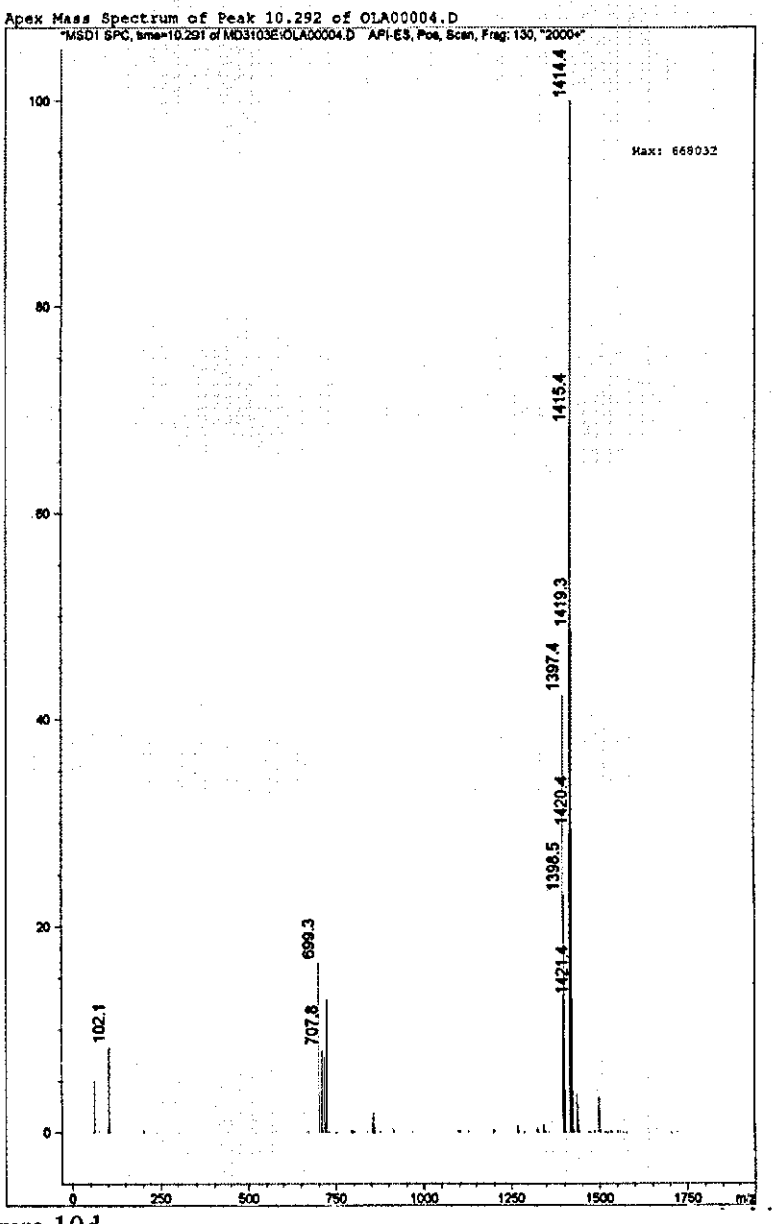

Figure 10d

We can see monomer n=17 (**1414**-18-172= 1224; 1224:72= 17) and doubly charged species 699 (699 x 2= 1398) and 707 (707 x 2= 1414).

Fraction 11

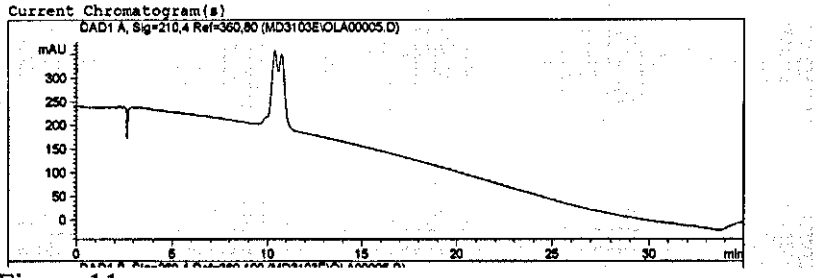

Figure 11a

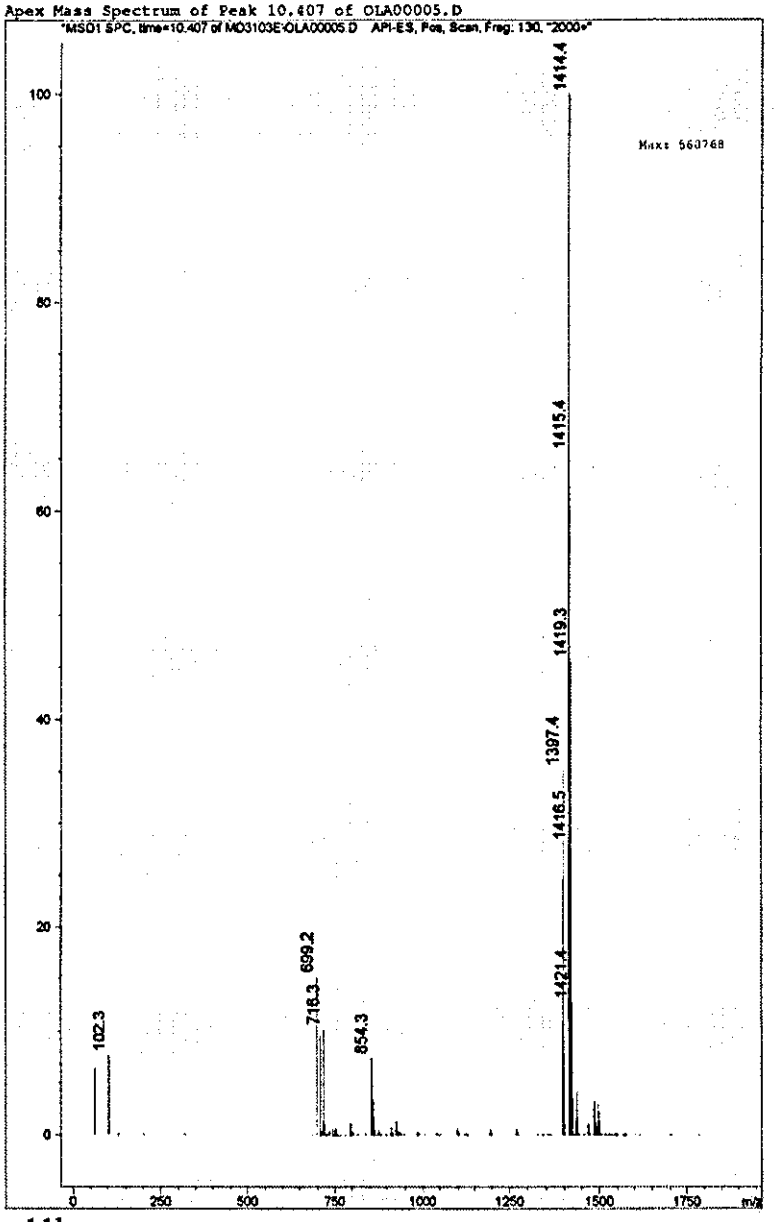

Figure 11b

We can see monomer n=17 (**1414**-18-172= 1224; 1224:72= 17) and doubly charged species 699 (699 x 2= 1398; 1398+18= 1416) and 716 (716 x 2= 1432; 1432-18= 1414).

Fraction 11

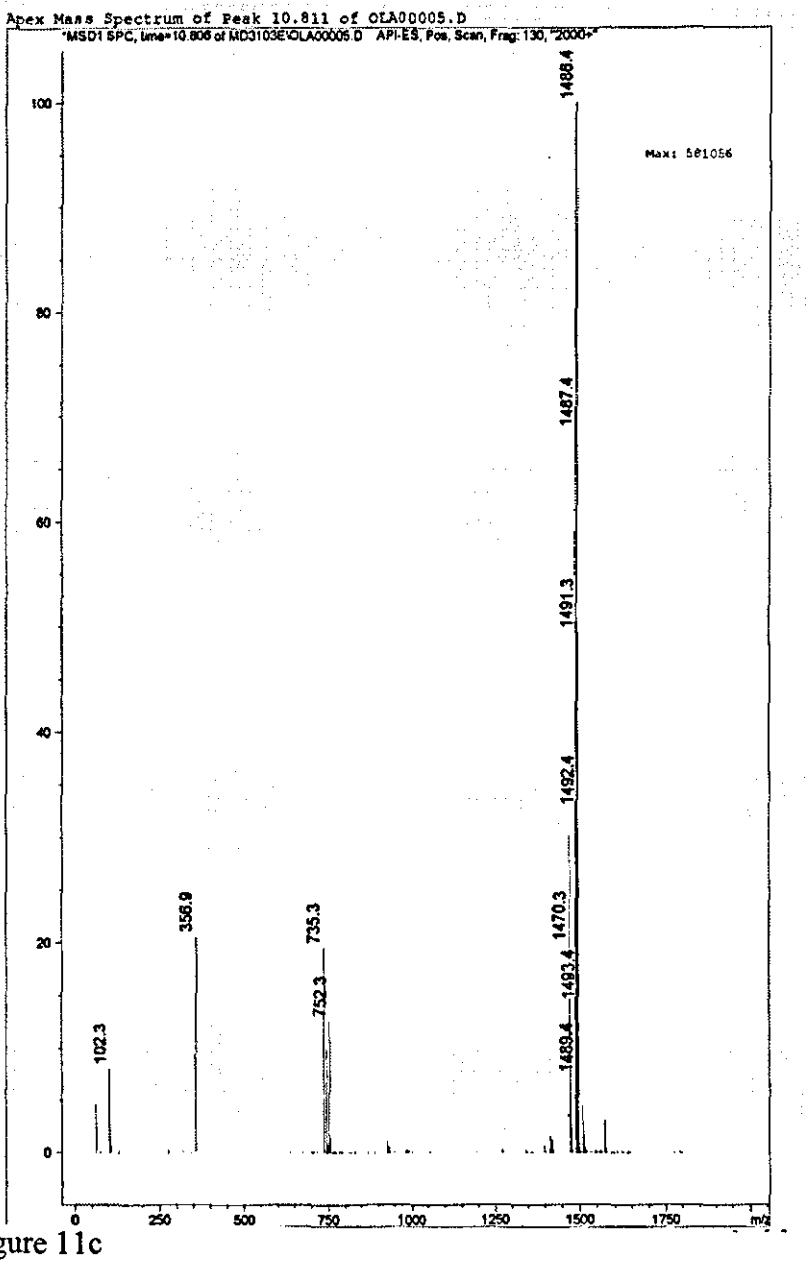

Figure 11c

We can see monomer n=18 (1486-18-172= 1296; 1296:72= 18) and doubly charged species 735 (735 x 2= 1470) and 752 (752 x 2= 1504; 1504-18= 1486).

Fraction 12

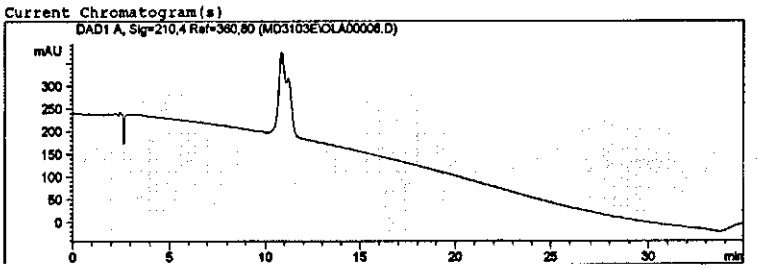

Figure 12a

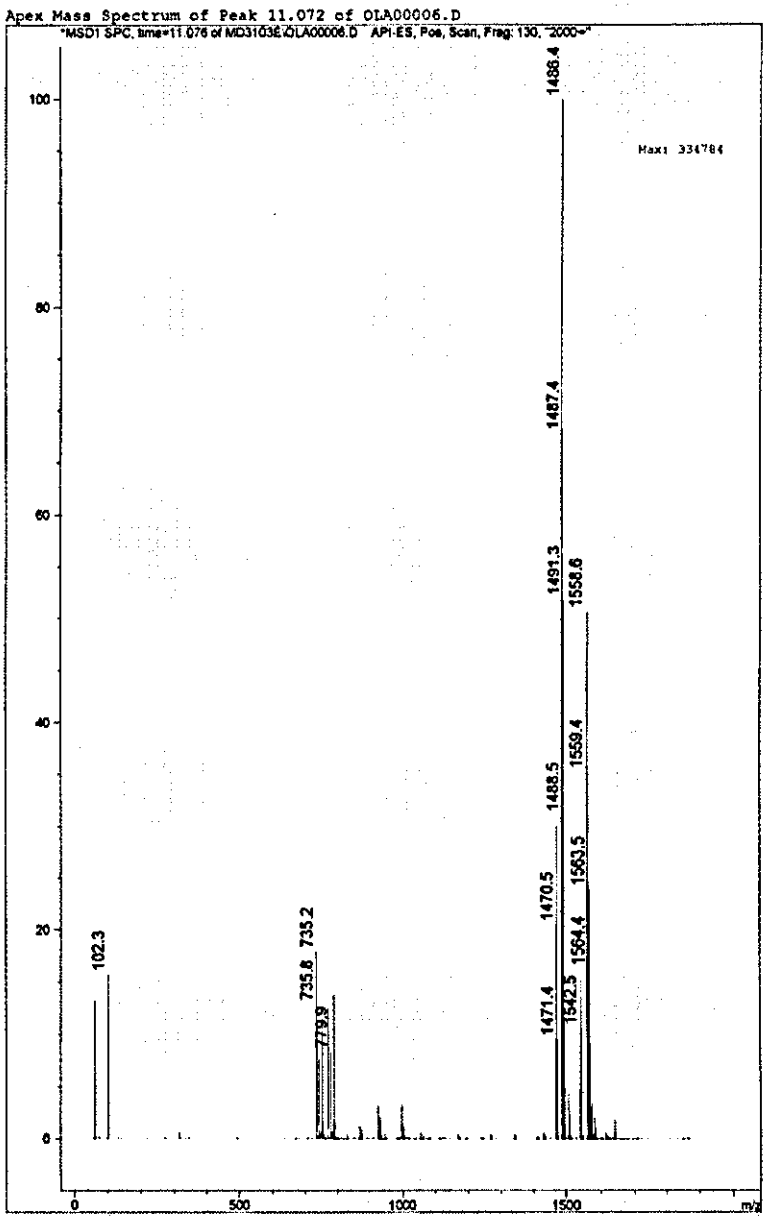

Figure 12b

We can still see monomer n=18 (**1486**-18-172= 1296; 1296:72= 18) and doubly charged species 735 (735 x 2= 1470), 771 (771 x 2= 1542) and 779 (779 x 2= 1558).

Fraction 13

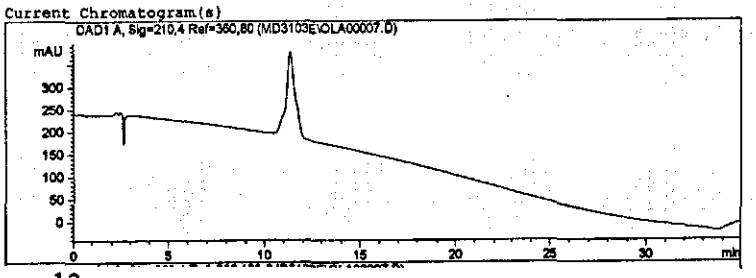

Figure 13a

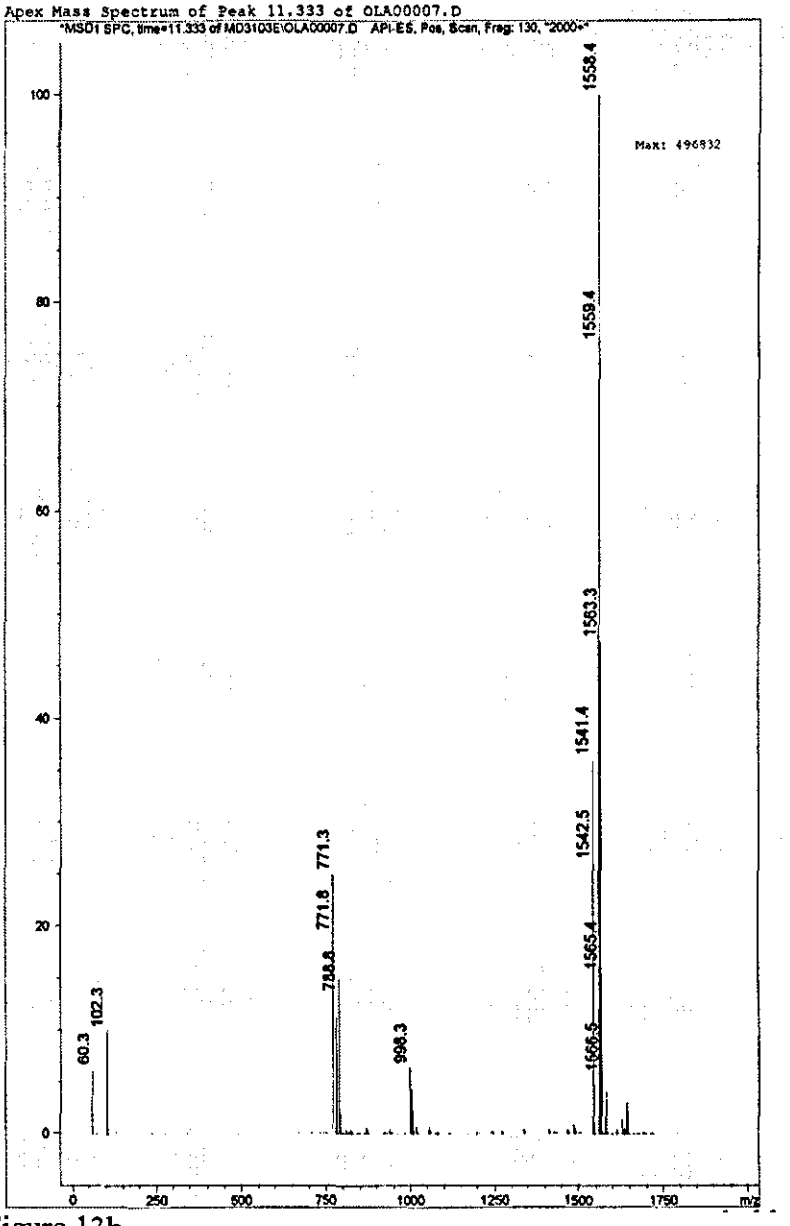

Figure 13b

We can see monomer n=19 (1558-18-172= 1368; 1368:72= 19) and doubly charged species 771 (771 x 2= 1542) and 788 (788 x 2= 1576; 1576-18= 1558).

Fraction 14

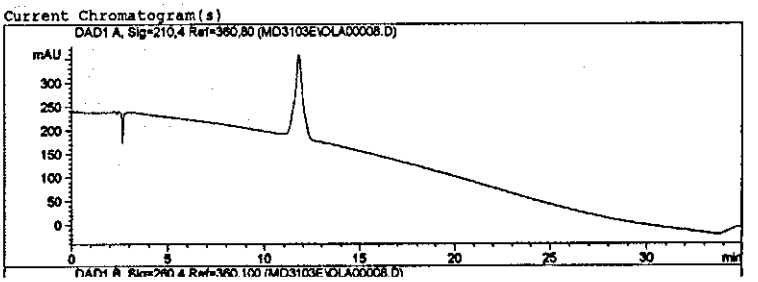

Figure 14a

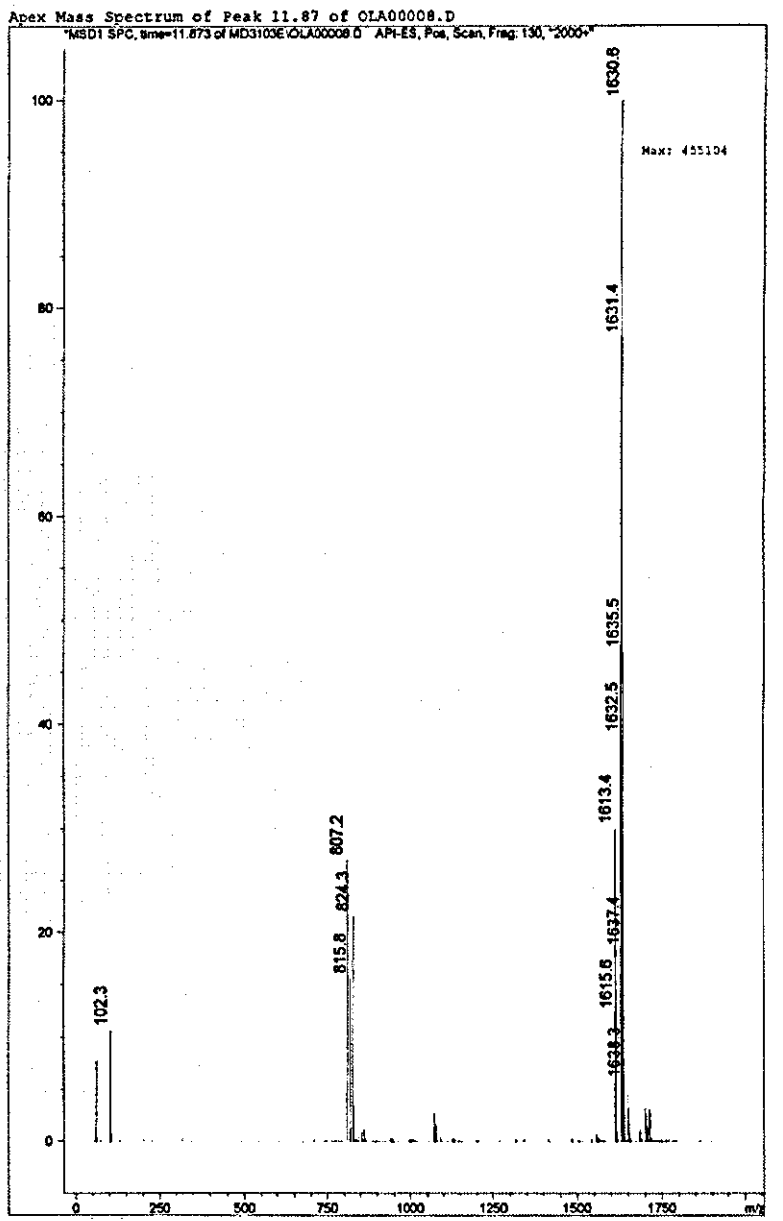

Figure 14b

We can see monomer n=20 (1630-18-172= 1440; 1440:72= 20) and doubly charged species 807 (807 x 2= 1614, protonated to 1615), 815 (815 x 2=1630) and 824 (824 x 2= 1648; 1648-18= 1630).

Fraction 15

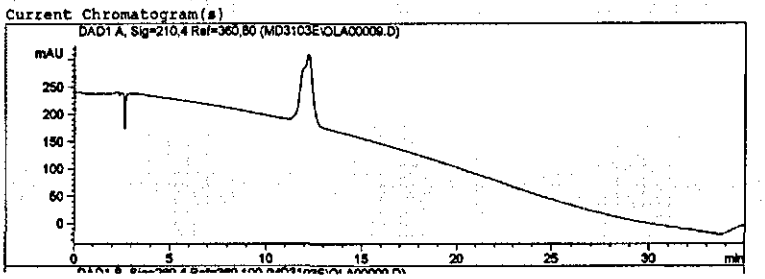

Figure 15a

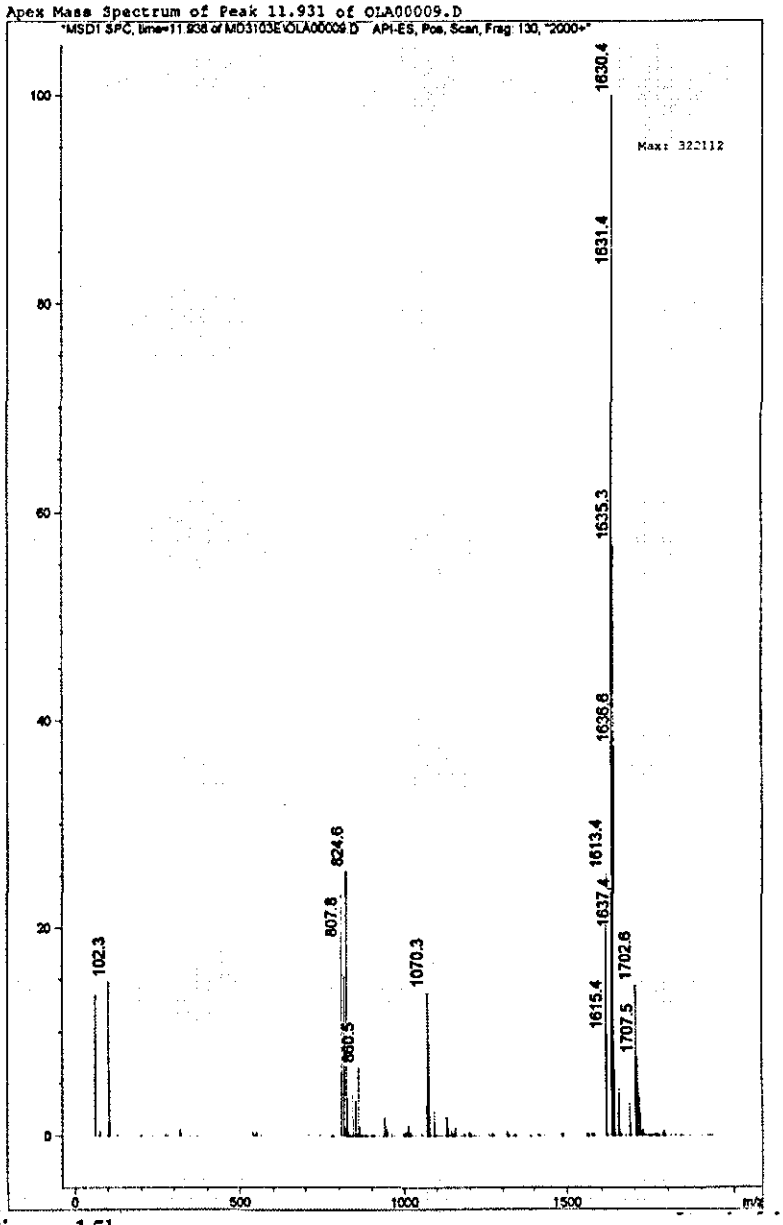

Figure 15b

We can still see monomer n=20 (1630-18-172= 1440; 1440:72= 20) and doubly charged species 815 (815 x 2=1630) and 824 (824 x 2= 1648; 1648-18= 1630).

Fraction 16

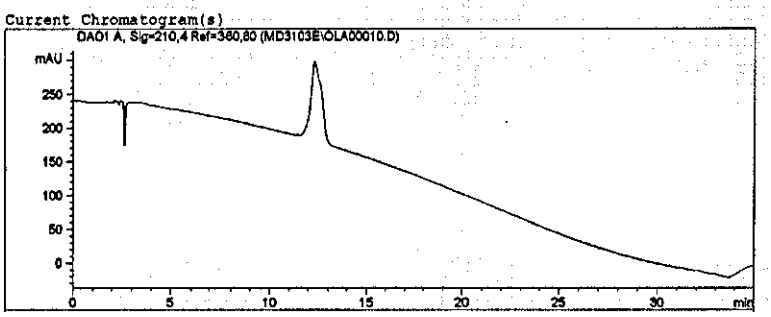

Figure 16a

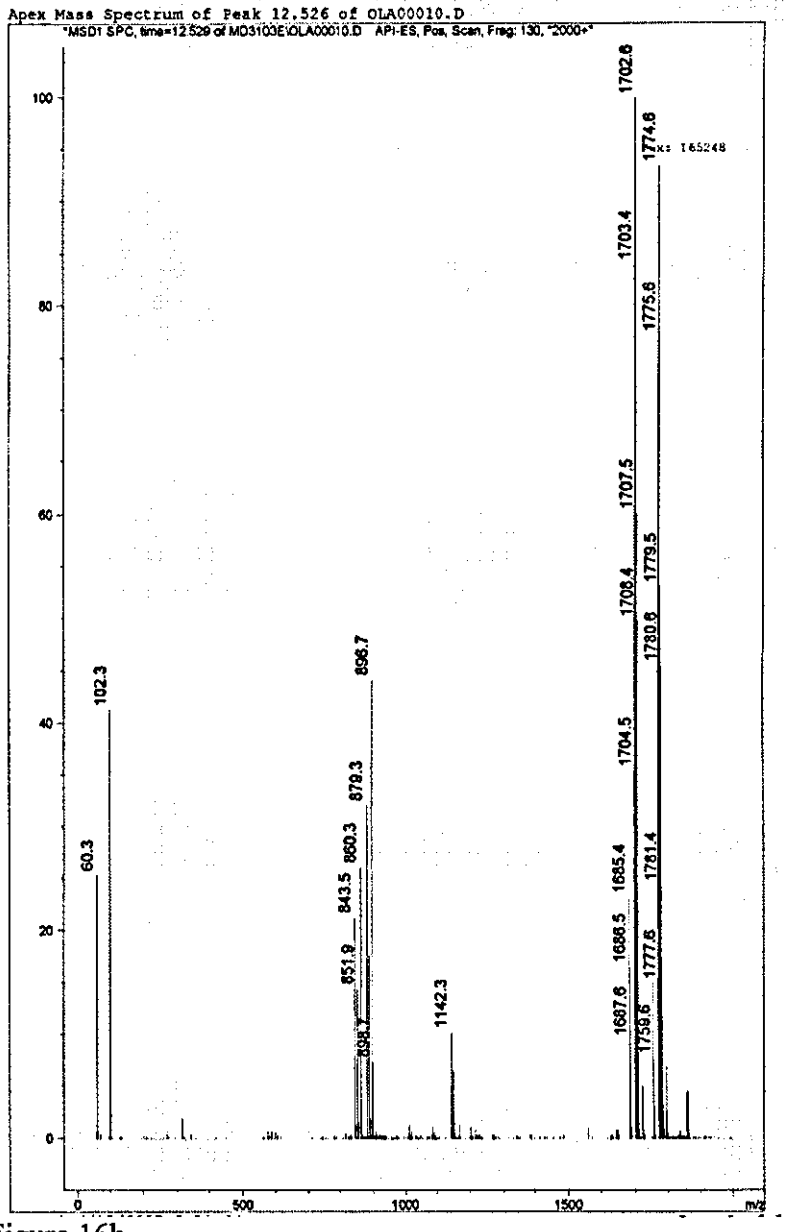

Figure 16b

We can see monomers n=21 (1702-18-172= 1512; 1512:72= 21) and n= 22 (1774-18-172=1756; 1756:72= 22). The following doubly charged species are observed: 851 (851 x 2= 1702) and 860 (860 x 2=1720; 1720- 18= 1702).

Fraction 17

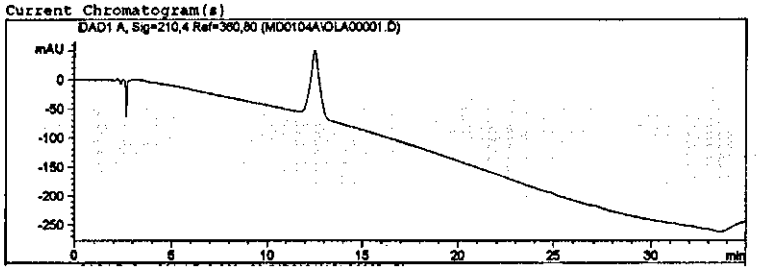

Figure 17a

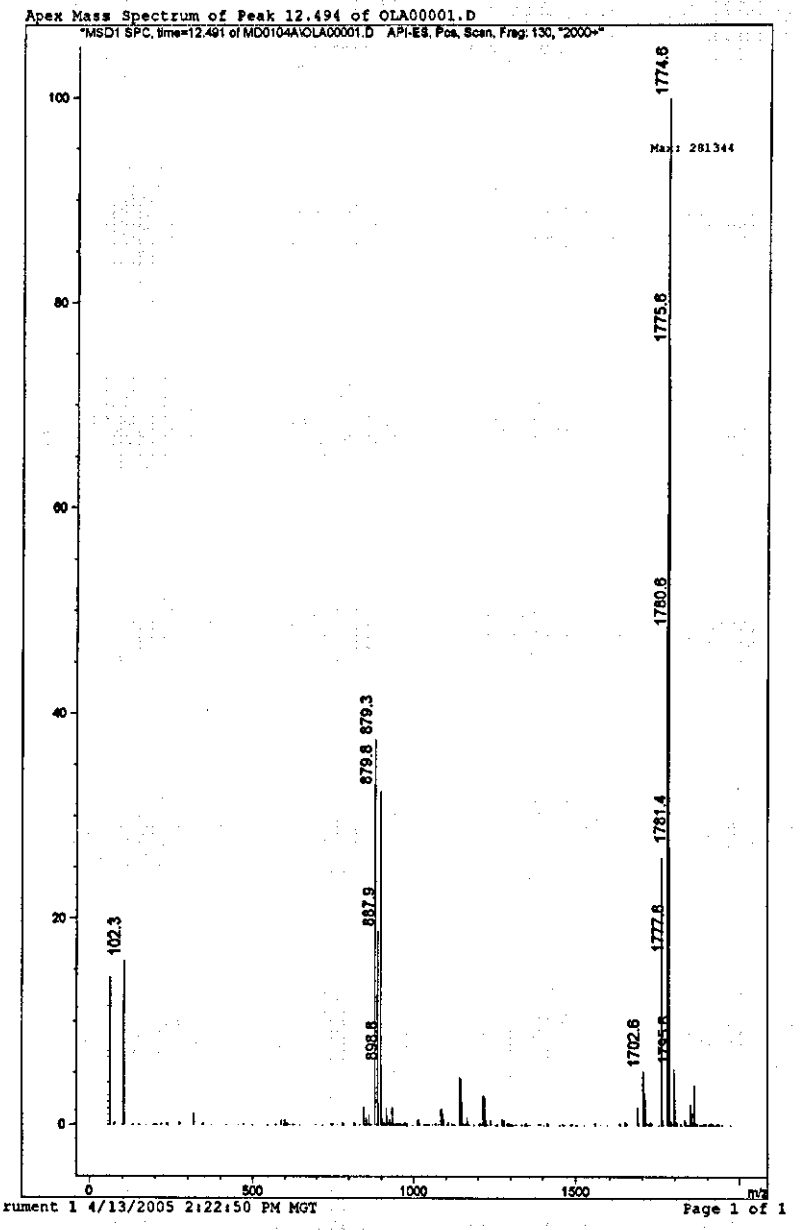

Figure 17b

Monomer n= 22 is still present (1774-18-172=1756; 1756:72= 22). The following doubly charged species are observed: 879 (879 x 2= 1758; 1758 + 18= 1776, protonated to 1777), 887 (887 x 2=1774) and 898 (898 x 2= 1796, deprotonated to give 1795).

Fraction 18

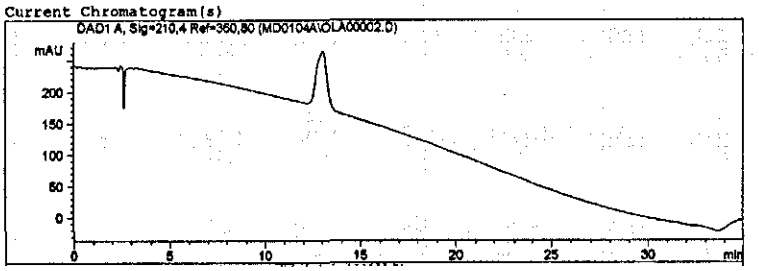

Figure 18a

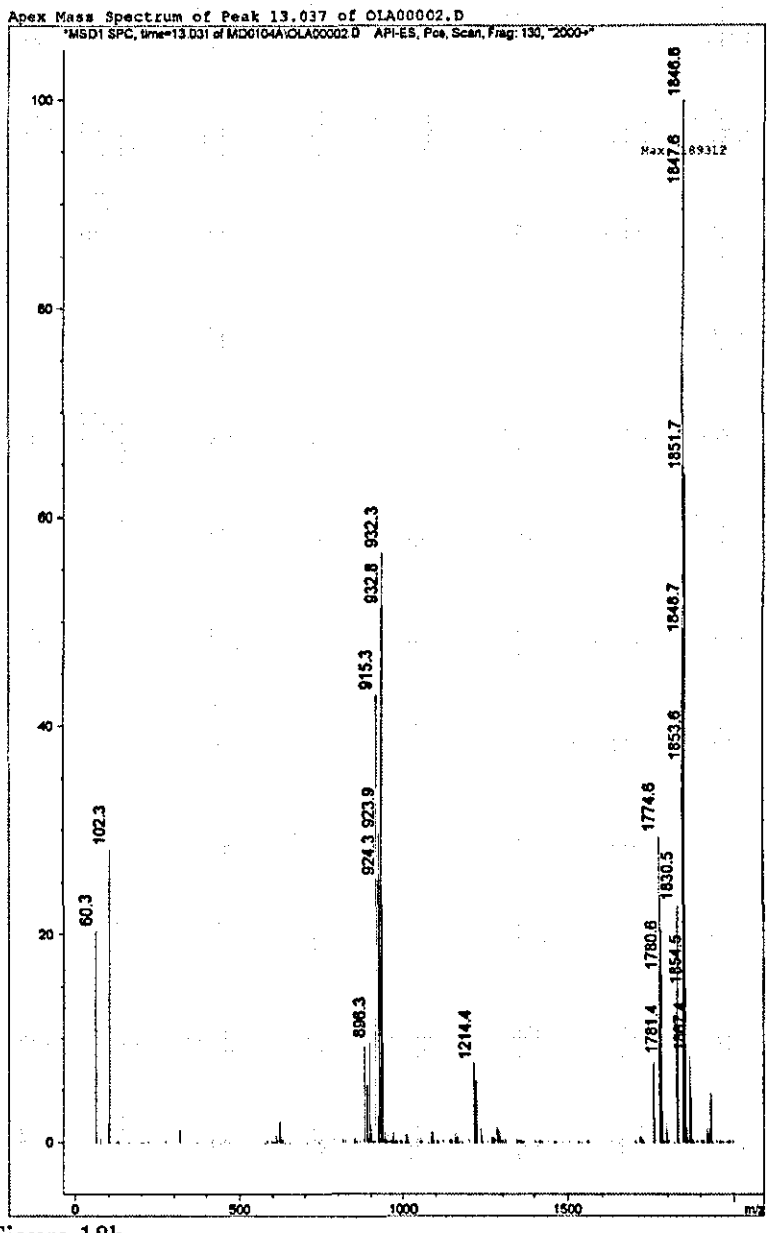

Figure 18b

Monomer n= 22 is still present (**1774**-18-172=1756; 1756:72= 22). Also, monomer n= 23 is present (**1846**-18-172=1656; 1656:72= 23).). The following doubly charged species are observed: 896 (896 x 2= 1792; 1792 - 18= 1774), 915 (915 x 2=1830), 923 (923 x 2= 1846) and 932 (932 x 2= 1864; 1864-18= 1846).

Fraction 19

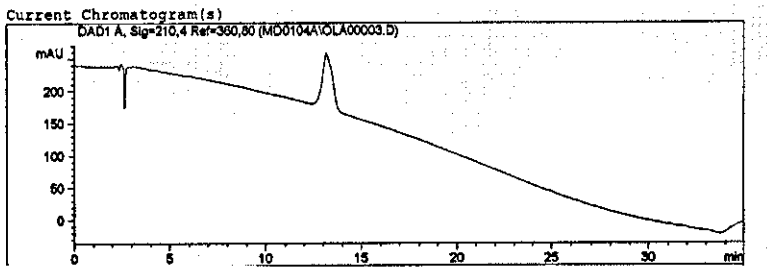

Figure 19a

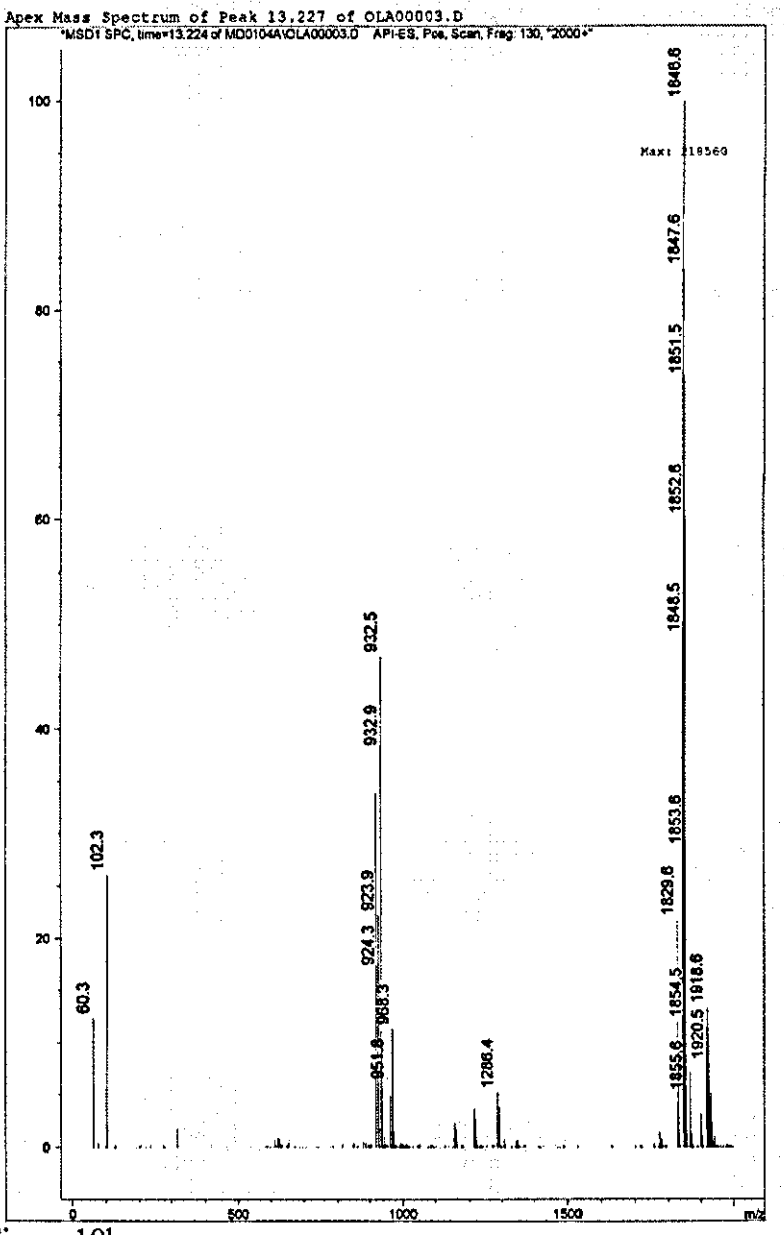

Figure 19b

Monomer n= 23 is shown (**1846**-18-172=1656; 1656:72= 23). Doubly charged species: 923 (923 x 2= 1846) and 932 (932 x 2= 1864; 1864-18= 1846).

Fraction 20

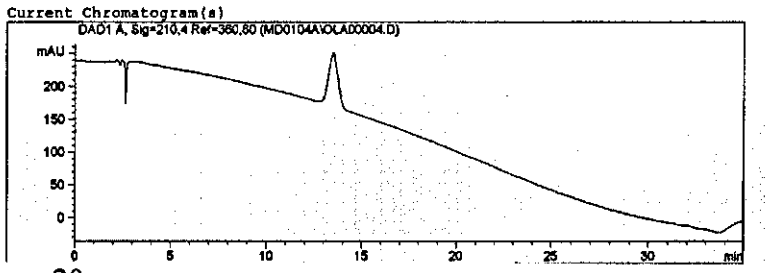

Figure 20a

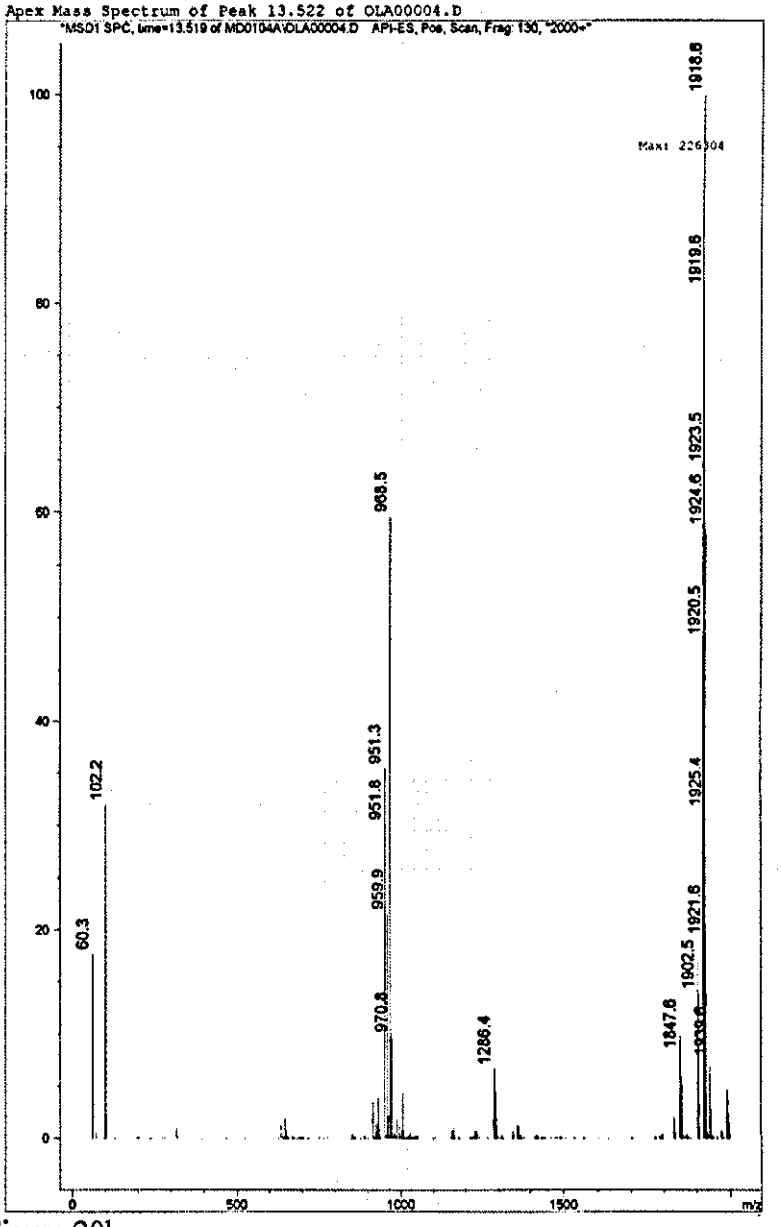

Figure 20b

Monomer n= 24 is shown (**1918**-18-172=1728; 1728:72= 24). Doubly charged species: 951(951 x 2= 1902, protonated to 1903), 959 (959 x 2=1918) and 968 (968 x 2=1936; 1936-18= 1918).

Fraction 21

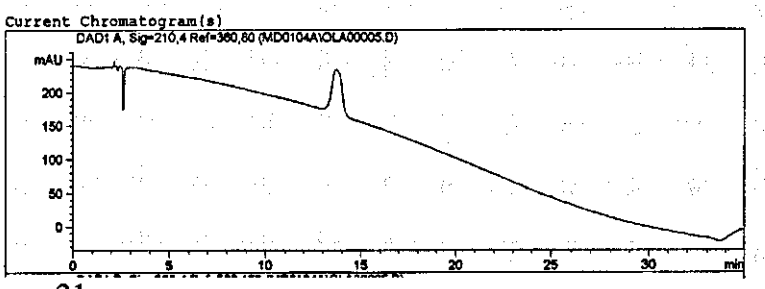

Figure 21a

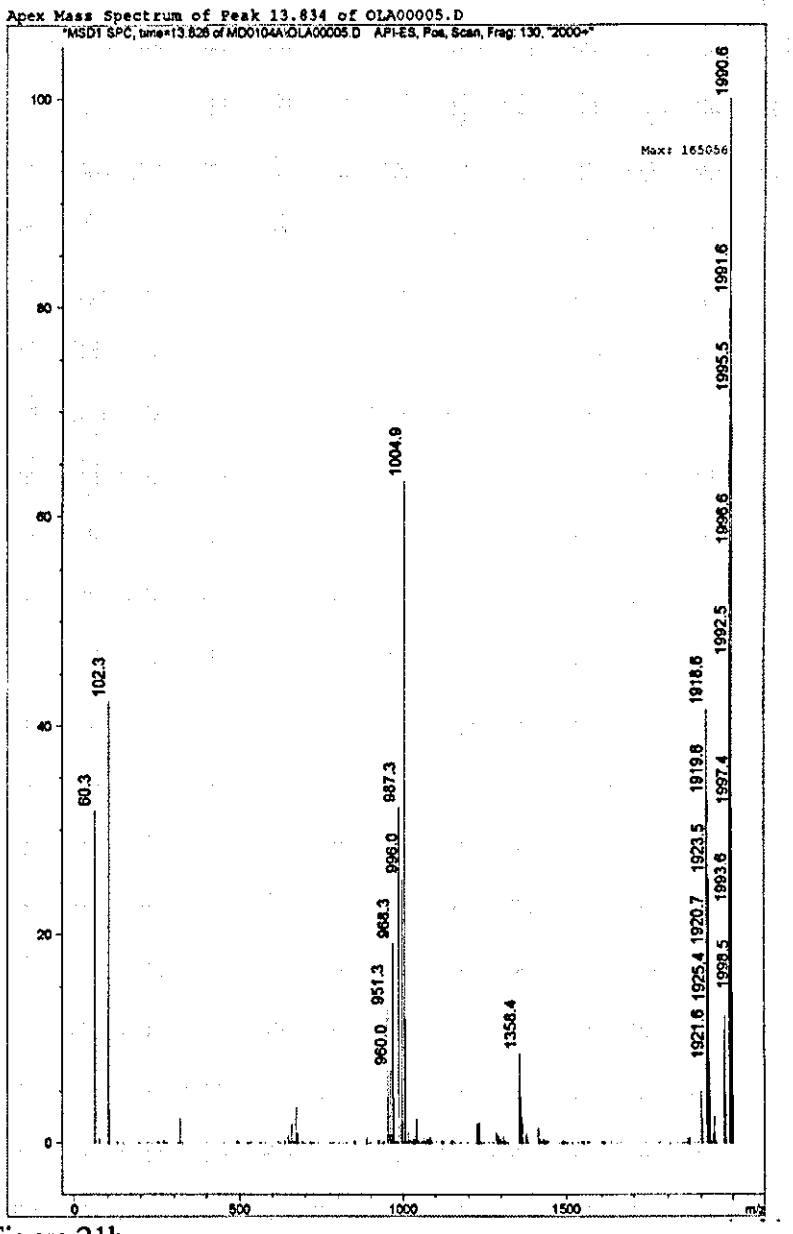

Figure 21b

Monomers n= 24 and n= 25 are shown (**1918**-18-172=1728; 1728:72= 24); (**1990**-18-172= 1800; 1800:72= 25). Doubly charged species: 951(951 x 2= 1902; 1902+18= 1920), 960 (960 x 2=1920) and 968 (968 x 2=1936; 1936-18= 1918). Also, 987 (987 x 2= 1974; 1974+18= 1992), 996 (996 x 2= 1992) and 1004 (1004 x 2= 2008; 2008-18= 1990).

Fraction 22

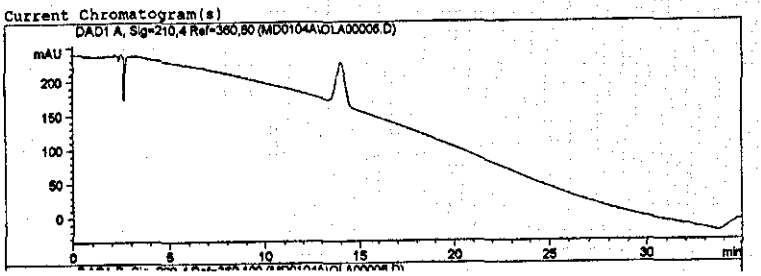

Figure 22a

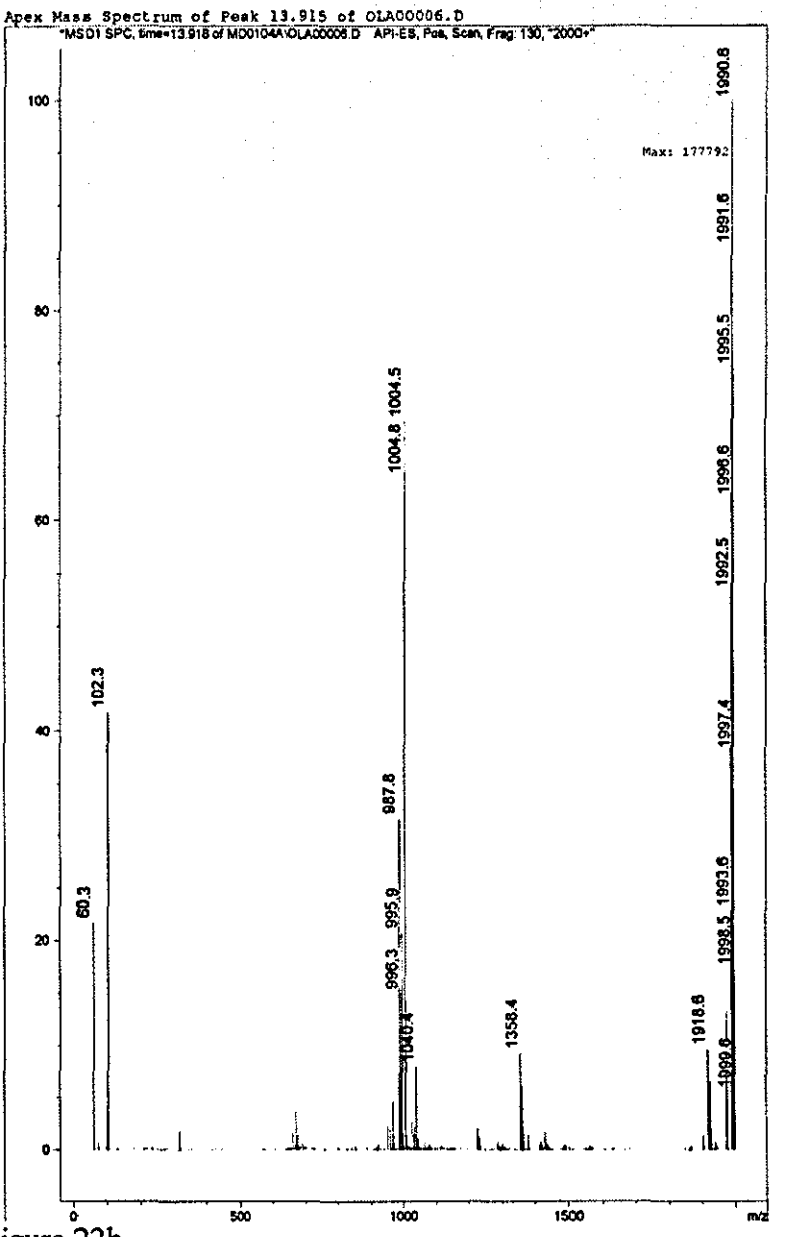

Figure 22b

Monomer n= 25 is still seen here (**1990**-18-172= 1800; 1800:72= 25).