NEW LIQUID CHROMATOGRAPHIC METHODS FOR THE ANALYSIS OF CATIONIC TENSIDES

by

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Submitted in accordance with the requirements for the degree of Doctor of Philosophy

The University of Leeds

School of Chemistry

September 2001

The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others

ABSTRACT

Cationic tensides are important oleochemicals that are used to prevent adulteration of pharmaceutical preparations and personal care products, as well as being the conditioning agents in domestic fabric softeners. The widespread use of these materials requires quantitative methods for characterising them in raw materials and fully formulated products, whilst *"down-the-drain"* release necessitates similar methods for trace environmental analysis. Unfortunately, current methods used to quantify cationic tensides are generally non-specific, problematic, or are ill-suited to environmental analysis. There is currently no generic method that can be applied to the quantitation of these materials in all necessary matrices.

The aim of this work was to develop new liquid chromatographic (LC) methods for the analysis of the cationic preservative and fabric conditioner actives, before attempting to build the foundations of generic cationic tenside analysis. The development of a new normal phase LC method is reported for the quantitation of the cationic actives present in domestic fabric conditioners. The method yielded high resolution and repeatability, and allowed the quantitation of the homologues endemic in commercial samples. Subsequent hyphenation with mass spectrometry demonstrated the potential for the quantitation of these materials in environmental matrices.

The optimisation and validation of a reverse phase LC method for the analysis of cationic tenside preservatives is reported. Excellent repeatability and resolution were again attained, whilst the new method was also found to demonstrate the inherent sensitivity required for trace environmental analysis. Subsequent hyphenation unfortunately showed that method sensitivity was compromised by ion-suppression, highlighting the need for compromise in the development of LC/MS methods.

For both methods, stationary and mobile phase parameters were varied to assess the influence on analyte resolution, and also to gauge the potential for developing a generic liquid chromatographic method for the analysis of these materials. It was observed that many of the commonly held beliefs on the analysis of cationic tensides by reverse phase LC were misconceived. As a result, new insights were made into cationic tenside analysis, which should facilitate the development of a generic LC method applicable to the quantitation of cationic tensides and their hydrophilic biodegradation products in the future.

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LIST OF ABBREVIATIONS

ACN	***Acetonitrile***
APCI	***Atmospheric pressure chemical ionisation***
API	***Atmospheric pressure ionisation***
$C_{18:1}$	***Monounsaturated C_{18} fatty acid***
CELISA	***Competitive enzyme-linked immunosorbant assay***
CZE	***Capillary zone electrophoresis***
DSBAS	***Disulphine blue active substance***
ELSD	***Evaporative light scattering detector***
ELS	***Evaporative light scatterer***
ESI	***Electrospray ionisation***
GC	***Gas chromatography***
HPLC	***High performance liquid chromatography***
i.d.	***Internal diameter***
LC/MS	***Liquid chromatography / Mass spectrometry***
LOD	***Limit of detection***
LOQ	***Limit of quantitation***
MeOH	***Methanol***
MS	***Mass Spectrometry***
NP/LC	***Normal phase liquid chromatography***
Quats	***Quaternary ammonium surface active agent***
RIC	***Reconstructed ion chromatograms***
RP/LC	***Reverse phase liquid chromatography***
SIM	***Single ion monitoring***
TFA	***Trifluoroacetic acid***
TIC	***Total ion chromatogram***
TLC	***Thin layer chromatography***
UV/vis	***Ultra-violet / visible***

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank my supervisor Prof. Keith Bartle for giving me the chance to undertake this Ph. D. at Leeds, and for all his help, encouragement, and confidence throughout my studies. Thanks also to Dr's Graham Lawrence and Mark Burford from Unilever Research who were involved with the project from the start and who offered unlimited help and advice throughout. I would also like to acknowledge the RSC Analytical Division and Unilever for the provision of the joint SAC studentship, which funded this work.

I am indebted to the support staff at Leeds, who helped me throughout my four years. Thanks to Bernard, Mike, Francis, Peter and all the workshop crew, not forgetting the two Dave's, fellow followers of *"the greatest game on earth"*, the friendly banter was always appreciated, not least as Saints were beating all-comers!

My time at Leeds would have been much duller and less productive if not for the *"characters"* around me: Thanks to Rich and Richy for takin' Robbo's flack and for looking worse than I did after a heavy night! To Katherine, the only other blood-vessel burster I know, Sue (who scarred me mentally), Kate P, aka the boss who always put me in my place, Krys, Niki, Nee and Sarah, and not forgetting my personal secretary Stuart to whom I owe my first three months salaries for all the favours during writing up. To the French people who never did get the hangover cures! Dr Mark Robson and Prof. Peter Myers are thanked for their pearls of pure chromatographic wisdom, and the ritual humiliation, that must of cured me as it didn't quite kill me! Indeed to all my friends and colleagues at Leeds thanks for everything, 'cept the Barbie thing, that went a little too far!

Thanks to the members of Dragonfly who welcomed me as a student and then again when I came back to haunt them! In this motley crew special thanks goes to the Analytical team for all their efforts and help, to Mark, Dan and Paul for showing confidence in me (Dan I'm still waiting for the commission on your job in the States!), to James Moore for office and Bansai banter (and sports commentary), and thanks again to Dr Paul Beers for providing realism, laughs and a boot up the arse when it was needed...bloody biologists!

To the Gents from back home, Chris, Sid and Waz, who kept my feet on the ground when I was already knee deep in the mire. Three things: nope I still haven't finished, I have got a real job and Sid I never did find the secret to the third type of softness, I'll keep looking!

Most of all I would like to thank three people in particular: my long-suffering girlfriend, **KATE**, (and her family) who has been there throughout the good and bad times (and bore the brunt of the latter), thanks hon. I love you loads! and to **MY PARENTS** who, as always, have been there for me throughout, bailed me out again and again and ensured that I always knew there were people looking out for me. To the three of you: **THANK YOU VERY MUCH THIS IS AS MUCH YOURS AS IT IS MINE!**

To my grandparents,

Though my time with you was short, I am forever grateful. I hope someday you'll see this.

and

To the people who lost their lives on the 11th September 2001,

.....it puts everything into perspective.

CHAPTER ONE

General Introduction

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General Introduction

1.1 AMPHIPHILES, SURFACTANTS AND TENSIDES

I planted you like a choice vine from the very best seed. But look what you have become! You are like a rotten, worthless vine. Even if you washed with the strongest soap, I would still see the stain of your guilt.

Jeremiah 2. 21-22.

"Amphiphilic molecules" or *amphiphiles* is the generic term applied to organic compounds that contain at least two structural units, which demonstrate starkly contrasting physico-chemical properties, notably solubility. At least one region of the amphiphile will be seen to be *solvophilic*, or *"solvent-loving"*, and will thus dissolve readily, whilst at least one other will be *solvophobic*, or *"solvent hating"* (Garret, 1972; Hamley, 2000) **(Figure 1.1)**. Such ambivalence has led to them being referred to as *amphipathic*, as they simultaneously show sympathy and antipathy to the solvent (Tanford, 1980). When placed into solution such ambivalence leads to preferential accumulation at interfaces, which results in them having important advantageous properties that have been exploited by man for eons (Garret, 1972).

As amphiphiles are predominantely applied into aqueous systems the solubility of the solvaphilic and solvaphobic sub-units are related to water, and hence one region is seen to be *hydrophilic*, whilst at least one other is *hydrophobic*. Hydrophobic groups are most often based on a long hydrocarbon chain and are thus commonly referred to as "*tails*", whilst the hydrophilic group, or "*head*", can be charged or uncharged (**Figures 1.1** and **1.2**) (Garret, 1972; Tanford, 1980; Hamley, 2000).

The tendency of amphiphiles to accumulate at surfaces has led to them being referred to as *"surface active agents"* or *"surfactants"*. However, in the early 1960's a joint committee on nomenclature agreed on the adoption of the term *"tenside"* as the generic title for all amphiphilic molecules (Garret, 1972). Thus, tenside will be used

throughout this work to refer to compounds, which in many other texts are referred to as surfactants.

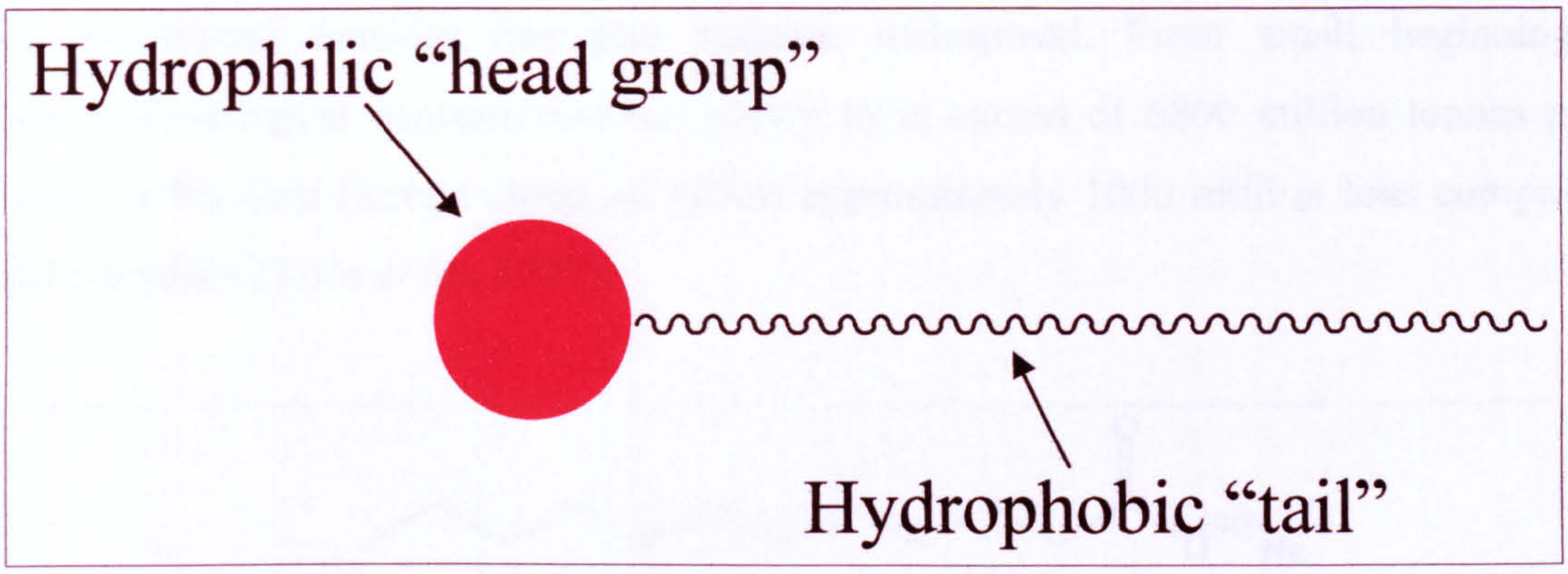

Figure 1.1: Representative structure of a generalised tenside showing the two distinct sub-units, which demonstrate contrasting solubility.

1.1.1 The historical development of the detergents industry

Whilst tensides have, and still do find widespread application as foaming, wetting, and dispersing agents (Garret, 1972; Hamley, 2000), history shows that they have been used primarily as *detergents*, for several millennia. Indeed, tracing the origins of the use of tenside detergents is almost impossible. Historians believe that the Babylonians may have been using *soap* (**Figure 1.2**), the earliest recognised tenside, to clean their garments as early as 2600 BC. There is clear evidence that the Phoenicians were using the product by 600 BC and the Celts had introduced the technique to the Romans some five hundred years on, but only later did the Romans utilise soap to clean themselves. However, whilst *saponification*, the change occurring in the formation of soap, derives from the Roman myth of it being discovered emanating from the sacrificial alters on Mount Sapo above the Tiber, it is much more likely that Pre-Historic Man was the first to recognise the detergent potential of tensides.

With the passing of four millennia, since the end of Babylonian civilisation, soap has retained its position as one of the principle products of the detergent industry. Soap bars and later, soap flakes formed the cornerstone of the domestic laundry market that developed in the late nineteenth century after mass production was pioneered by the Lever Brothers (Lever Brothers, 2000). Soap was eventually vanquished from its position as the principle laundry detergent in the second half of the twentieth century (Lever Brothers, 2000). At this point in time, alternative synthetic anionic tensides were

commercialised (Garret, 1972), and later, a number of other anionic tensides were developed for the use in domestic detergents. The use of non-ionic tensides like alcohol ethoxylates (AE's) (**Figure 1.2**) has shown dramatic growth, whilst the use of cationic and *amphoteric* tensides has also become widespread. From small beginnings, household detergent consumption has grown to in excess of 6800 million tonnes per annum in Western Europe alone, of which approximately 1000 million tons comprise active tensides (Trius *et al.*, 2000).

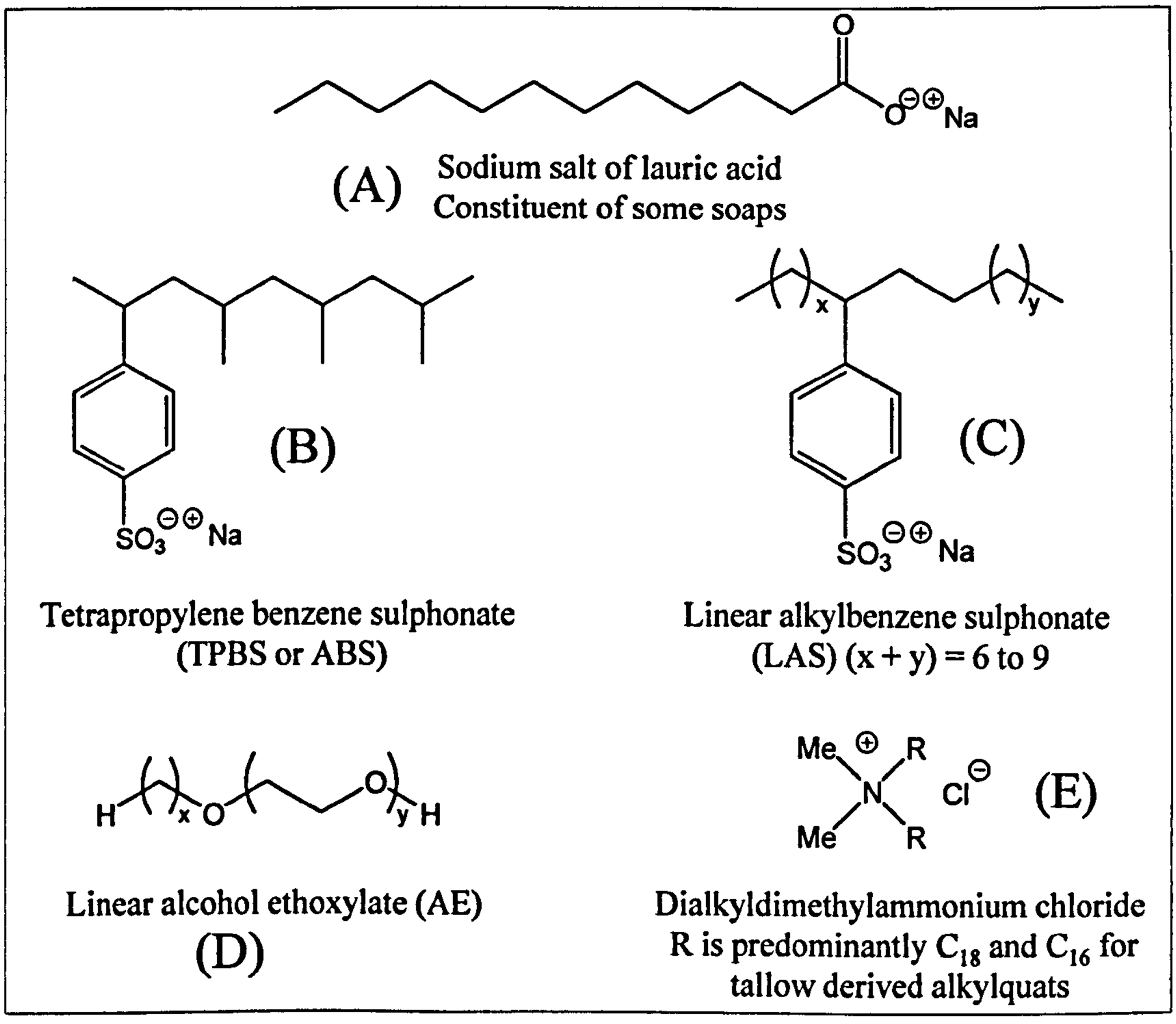

Figure 1.2: Representative structures of three typical anionic (A, B and C) tensides, one non-ionic (D) tenside and one cationic (E) tenside.

1.1.2 "Oleochemicals"

With the dramatic growth in the use of detergents, and tensides in general, increased demand has been placed on sourcing adequate supplies of the raw materials used in their production. As the hydrophobic unit of a tenside is normally based on a long hydrocarbon chain, tensides have traditionally been manufactured from natural or synthetic fats and oils (Dobson, 1996).

During the middle part of the twentieth century, the scale of the petroleum industry, particularly in North America led to many of the anionic and non-ionic tensides developed at this time, being manufactured from petroleum by-products (Garrett, 1972). However, historically, the majority of tensides have been manufactured from naturally occurring edible and inedible fats and oils of animal or plant origin (**Figure 1.3**). Indeed, in recent years there has been a notable decline in the use of petroleum-derived materials and an increase in the usage of renewable sources of raw materials, especially palm kernel oil (Dobson, 1996) (**Figure 1.3**). Currently, approximately 90% of all fat and oil raw materials used in tenside manufacture, are found above ground (Dobson, 1996; Trius *et al.*, 2000). As a result of their natural oil and fat origin, tensides are often referred to as *oleochemicals* (Dobson, 1996).

At this juncture it should be noted that the term fat or *fatty* is normally used to refer to alkyl chains with greater than or equal to twelve carbons within their structures (Garrett, 1972; Hamley, 2000). However, on occasions the term fatty is applied to alkyl chain lengths down to C_8.

Figure 1.3 shows the fatty acid distribution of the oils and fats commonly used in the manufacture of tensides. It is evident that there is a distribution of chain lengths present in the raw materials. As fractionation or purification of the oil or fat is not generally performed prior to manufacture, commercial tenside samples contain a distribution of alkyl chain homologues. As a result, a *"pure"* tenside sample will normally contain a number of active species, which contain hydrophobic groups of varying length (Lawrence, 1997). This can lead to some confusion as the detergents industry often class a pure tenside sample as *"that which is produced by the manufacturer"*, which does not necessitate that it contains only one active component.

In addition to natural fats and oils containing a distribution of alkyl chain lengths, it is evident from **Figure 1.3** that they also contain both saturated and mono- and poly-unsaturated fatty acids. As a result, commercial soap samples include a distribution of saturated and unsaturated fatty chains, which along with the chain length distribution has a definite effect on the structural properties and solubility of the final products (Garrett, 1972). The high percentage of palmitic, stearic and oleic acids present in tallow fat leads to the formation of hard, insoluble soaps, whilst the *Marseilles soap*,

which was renowned in the nineteenth century for being "pleasant on the skin", is soft, due to its manufacture from olive oil (Garret, 1972). As a result, blends of fats and oils are now utilised to impart the desired structural properties to modern soaps (Garrett, 1972). Whilst many other tensides are still manufactured from a single fat or oil (Garrett, 1972), hydrogenation of the raw material is commonly used to manipulate the properties of the final product (Lawrence, 1997). As a consequence, the saturated to unsaturated ratio of many tensides is very different to that of the fat or oil used during manufacture (Lawrence, 1997).

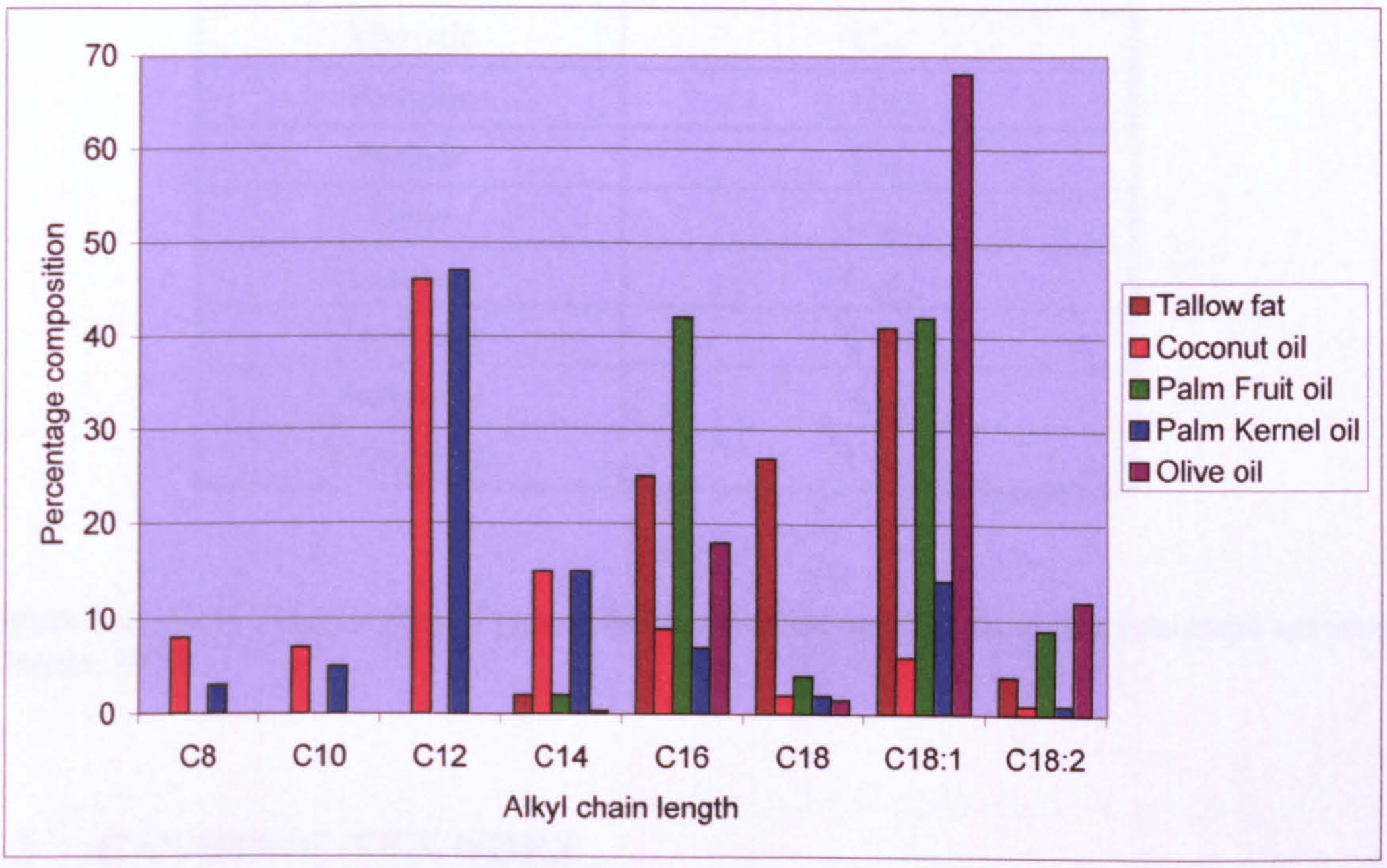

Figure 1.3: Fatty acid distributions of the natural fats and oils normally used in the production of tensides (Garrett, 1972).

Figure 1.3 also highlights an additional foible of the tenside industry: nomenclature. The IUPAC nomenclature used to name organic compounds is often circumvented by the industry in favour of trivial nomenclature relating to the corresponding fatty acid (**Figure 1.4**). As a result, lauryl and stearyl are commonly used to refer to saturated C_{12} and C_{18} hydrocarbon chains, rather than the IUPAC defined terms of dodecyl and octadecyl. However, further confusion arises from the continued use of other traditional trivial names, e.g. cetyl, which is still used in place of hexadecyl and palmityl. This problem is clearly evident in **Appendix One,** where a list of the tenside samples used during this study is presented. The names of the individual samples are given as seen on the characterisation data supplied with the sample, i.e.

benzyldimethylstearylammonium chloride dihydrate may also be referred to as benzyldimethyloctadecylammonium chloride dihydrate. For simplicity, the IUPAC method has been used to describe alkyl chain length during this work, whenever possible.

FATTY ACID	ALKYL CHAIN LENGTH
Caprylic	C_8
Capric	C_{10}
Lauric	C_{12}
Myristic	C_{14}
Palmitic	C_{16}
Stearic	C_{18}
Oleic	$C_{18:1}$
Linoleic	$C_{18:2}$
Linolenic	$C_{18:3}$
Arachidic	C_{20}
Eicosenoic	$C_{20:1}$

Figure 1.4: Alkyl chain lengths of typical fatty acid constituents present in commercial tensides (Garrett, 1972).

1.2 CATIONIC TENSIDES

Cationic tensides are positively charged amphiphiles that are predominantly based on a quaternised nitrogen atom. **Figure 1.5** depicts structural representations of some of the most important cationic tensides, along with a more generalised representation of a cationic tenside showing four alkyl and / or alkyl-aryl groups surrounding a central quaternised nitrogen atom that has an associated anionic counter ion. The association of the counter ion leads to the formation of a quaternary ammonium salt, which is seen to be surface active, due to it containing at least one fatty tail unit. As a result, the colloquialism *"quat"* is commonly applied to cationic tensides, which demonstrates this generalised form (Lawrence, 1997). It is important to note that whilst many industrially important cationic tensides have four distinct groups attached to the central nitrogen atom, others like the alkylpyridinium salts (**Figure 1.5**) **(Appendix One)** encapsulate the cationic nitrogen atom within a heterocyclic ring.

Nevertheless, these materials are still referred to as quats. For simplicity quat will be used throughout this work to refer to a fatty quaternary ammonium salt.

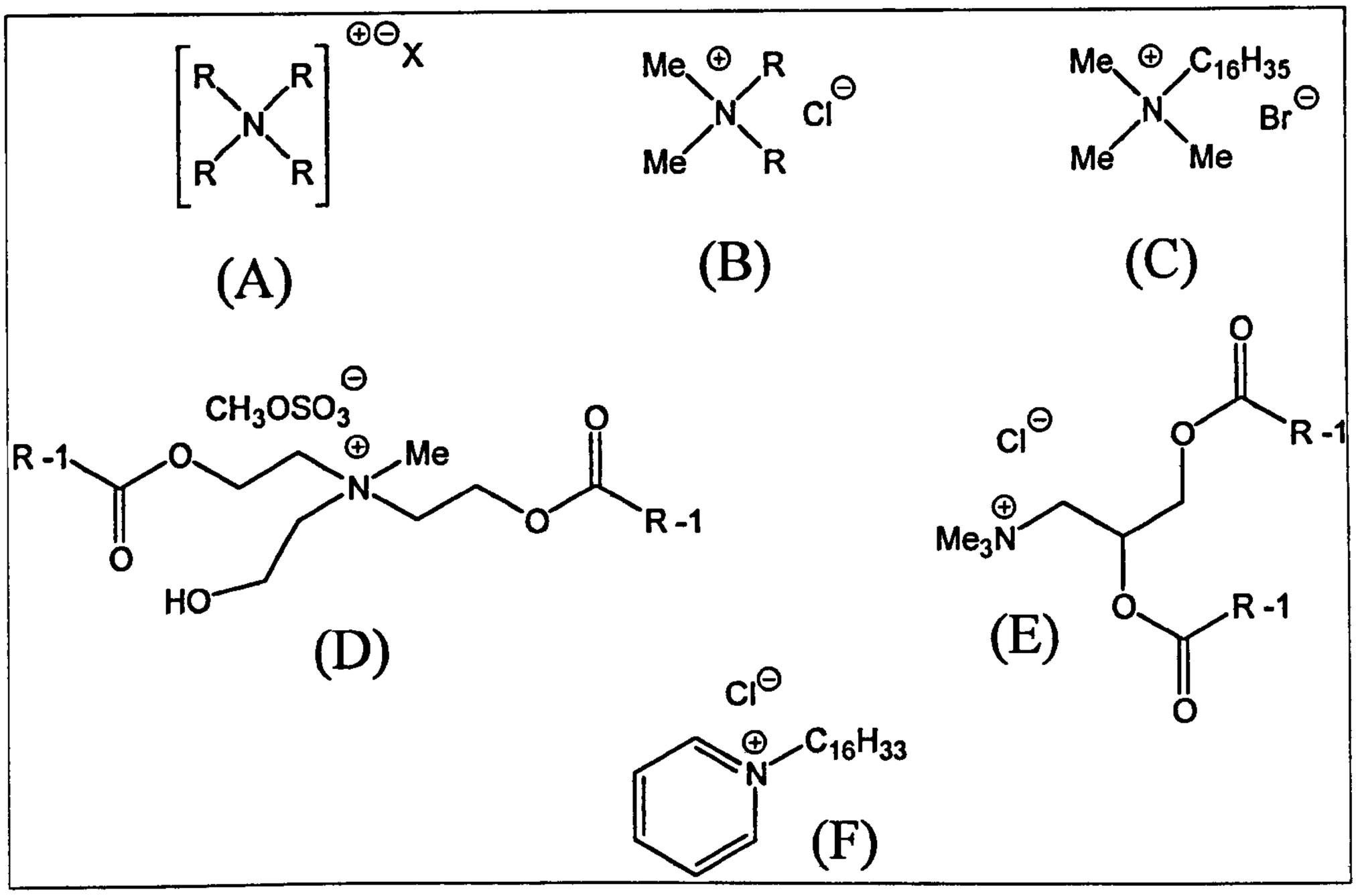

Figure 1.5: Representative structures of a generic *quat* tenside (A) and some industrially important cationic tensides (B – F).
For species B, D and E, R is normally C_{18} and C_{16} as they tend to be manufactured from tallow fat.

The rise to prominence of the cationic tensides came about in the early 1930's when Domagk recognised the commercial value of the bactericidal properties of these materials some thirty years after they were first reported (Lawrence, 1970). Since this time, the use of cationic tensides has become more widespread, with the materials now finding use in a number of different applications. Cationic tensides are applied to varying degrees as dye fixers (Domagk, 1987), corrosion inhibitors (Gough *et al.*, 1999; Branzoi *et al.*, 2000), oilfield extraction processes (Omar *et al.*, 1997) and in miceller and vesicular delivery systems for therapeutic drugs (Vievsky, 1997; Esposito *et al.*, 1999). However, whilst usage is growing in many of these areas, two traditional applications still dominate the cationic tensides market: softening agents in domestic fabric conditioners and antibacterial agents / preservatives in personal care products, pharmaceuticals and *cosmaceuticals* (Jungermann, 1970; Puchta, 1984; Boethling *et al.*, 1992).

1.2.1 Cationic fabric conditioner actives

The ability of cationic tensides to *condition* garments was initially discovered by accident. When clothing manufacturers applied solutions of these materials as dye-fixing agents, they were seen to impart softness, often referred to as *"hand"*, to the garments (Levinson, 1999). This observation was made in the mid-1950's, which proved timely as the increased use of mechanical washing machines, and the replacement of soap flakes with synthetic detergent powders, had led to improved stain removal and reduced re-deposition (Levinson, 1999). Whilst improved stain removal led to cleaner clothes, the lack of re-deposition led to greater removal of clay and oil residues that had built-up on the fibres over time. Unfortunately, the presence of these residues was found to improve the feel of the garment when in contact with the skin (Levinson, 1999) and, as a result, the new detergents where perceived to leave garments feeling coarse and uncomfortable making them less appealing to the consumers (Levinson, 1999). The subsequent utilisation of rinse-added cationic tensides was found to improve the feel of garments, even when washed with soap flake, and were thus acclaimed by consumers (Levinson, 1999).

Today cationic tensides have found widespread use as fabric *"softening"* agents in post-wash products that are perceived to impart softness and fragrance. However, to label these formulations as *softeners* does them a disservice. Research has shown that the use of cationic tensides improves the softness and bulk of the laundry, extends fabric lifetime, reduces electrostatic build-up, improves water and stain resistance, reduces the burden of post-wash mechanical processes (ironing and drying), and imparts that all-important *"freshness"* (Puchta *et al.*, 1993; Levinson, 1999).

Fabric conditioning products can be split into two main types, the liquid rinse-added formulations that are employed globally, and the sheet-based conditioners that are used in tumble dryers predominantly in North America (Levinson, 1999). Whilst the two groups of products have very different modes of application, the mode of action and structure of the active agents are identical (Levinson, 1999). Considering liquid rinse-added conditioners, cationic tensides account for less than 15% of the formulation in standard products, whilst the concentration can be up to 25% in concentrates (Levinson, 1999). The product is added to the laundry after removal of the anionic detergent to ensure that the cationic tensides can effectively adsorb onto the fibres of the garments

without being removed from the system through complex formation (Crutzen, 1995; Levinson, 1999). Whilst much of the cationic tenside is removed from the garments during final rinsing, a discrete amount of the material is left on the garments, imparting some water and stain resistance (Burford, 1997).

The primary compounds used in fabric conditioners can be divided into two main classes: the traditional, or 1st generation alkyl quats, like dihydrogenatedtallowdimethylammonium chloride, and the 2nd generation esterquats, such as the hydrogenated derivative of diethylesterdimethylammonium chloride (**Figure 1.5**) (**Appendix One**). There is a great deal of commonality in the two groups of tensides, not least in the fact that both groups tend to be manufactured from tallow fat, whether it fully, partially or non-hydrogenated (Waters *et al.*, 1991; Puchta *et al.*, 1993; Lawrence, 1997; Friedli *et al.*, 2000). In addition, the primary active agents present in fabric conditioner formulations are the di-chained species, the dialkyl and diester quats respectively (Waters *et al.*, 1991; Lawrence, 1997). However, due to the method of manufacture of both classes of cationic tensides, commercial samples are also seen to contain significant concentrations of the mono-chained species (Waters *et al.*, 1991; Lawrence, 1997) (**Figure 1.5**) (**Appendix One**). Indeed, in the case of the 1st generation alkylquats, and some esterquats, considerable concentrations of the tri-chained species are also present (Lawrence, 1997; Friedli *et al.*, 2000) (**Appendix One**). Both the mono- and tri-chained species are active impurities, in that they also add to the performance of a commercial formulation. However, their specific activities are different to that of the di-chained species.

The presence of tri-chained and / or mono-chained cationic tensides in commercial samples adds further complication to a so-called "*pure*" sample (**Section 1.1.2**). Therefore, some "*pure*" samples contain a distribution of di-chained actives corresponding to the fatty acids in tallow fat (**Figure 1.3**), which may or may not show unsaturation, a factor dependant on the processing of the tallow (**Section 1.1.2**), and two other classes of active quats, prior to use (Lawrence, 1999). To make matters worse, the mono- and tri-chained species show a distribution of alkyl chain lengths, and other impurities such as fatty acids and protonated fatty amines, which are also known to be present (Lawrence, 1997). If one considers that the concentration of active agents, and active and non-active impurities is carefully controlled to ensure the efficacy of the

commercial formulation, it is apparent that accurate characterisation data on the raw materials, and effective quality-control protocols for fully formulated products, are of paramount importance.

1.2.2 Cationic tenside preservatives

The second major area of application for cationic tensides are as anti-bacterial / fungicidal agents (Lawrence, 1970). Reference has already been made to the fact that the first reports of the beneficial properties of cationic tensides, and the first commercial application of these materials were as anti-bacterial agents (**Section 1.2**). In the sixty five years that have lapsed since the first commercialisation of these materials by Domagk, mono-chained quats have found widespread usage as antimicrobials, bacteriostatics and fungicidal agents (Lawrence, 1970; Boethling *et al.*, 1992; Lin *et al.*, 1991). Their potency and efficacy in these areas has led to their usage as preservatives in decongestive nasal sprays and most ophthalmic solutions (Santoni *et al.*, 1994; Price *et al.*, 1999), as well as other pharmaceutical and cosmetic products (Lin *et al.*, 1991; Kawakami *et al.*, 1998). Cationic tensides have also been applied as the active agents in throat sweets and lozenges (Taylor *et al.*, 1998), and have found widespread use as general anti-bacterial agents and sanitisers in the healthcare industry (Lawrence, 1970; Kümmerer *et al.*, 1997). In addition, their use as sanitisers within the food and drink industry, to prevent bacterial and microbial adulteration and fungal growth, is now becoming more widespread (Suortti *et al.*, 1990; Valladao *et al.*, 1994; Zhou *et al.*, 1999). Finally, in recent years the application of cationic tensides to prevent fungal growth and sapstain in the lumber industry, particularly in North America, has gained pace (Suortti *et al.*, 1990; Bull *et al.*, 1998b; Chen *et al.*, 1995).

Figure 1.6 shows representative structures of the three major classes of cationic tensides utilised as preservatives / sanitisers in the various applications listed above. All three classes contain only a single fatty alkyl chain, and commercial formulations are strictly controlled to ensure the removal of di-chained species. As a result, cationic preservative samples are much less complex than the corresponding fabric conditioner samples (Lawrence, 1970). An additional variation between the cationic preservatives and the cationic fabric conditioner actives is to be found in the choice of raw materials. Whilst the conditioner actives are generally only based on tallow fat, the fatty raw material used in the manufacture of the preservative actives is dependant on the

application. For example, the alkylbenzyl quats utilised as preservatives in healthcare and cosmetic products (**Figure 1.6**) (**Appendix One**) are primarily manufactured from coconut oil and thus, usually demonstrate an alkyl chain distribution that centres on C_{12} and C_{14} (Garrett, 1972) (**Figure 1.3**). However, the alkylpyridinium quats tend to be applied as single homologues with only the C_{12} and C_{16} species being of industrial relevance (Martindale, 1993; Zhou *et al.*, 1999). In contrast, the monoalkyltrimethyl quats can range in chain length from C_8 to C_{18}, dependant on the application (Lawrence, 1970). As a result, it is difficult to generalise the raw materials used in the manufacture of the cationic preservative actives.

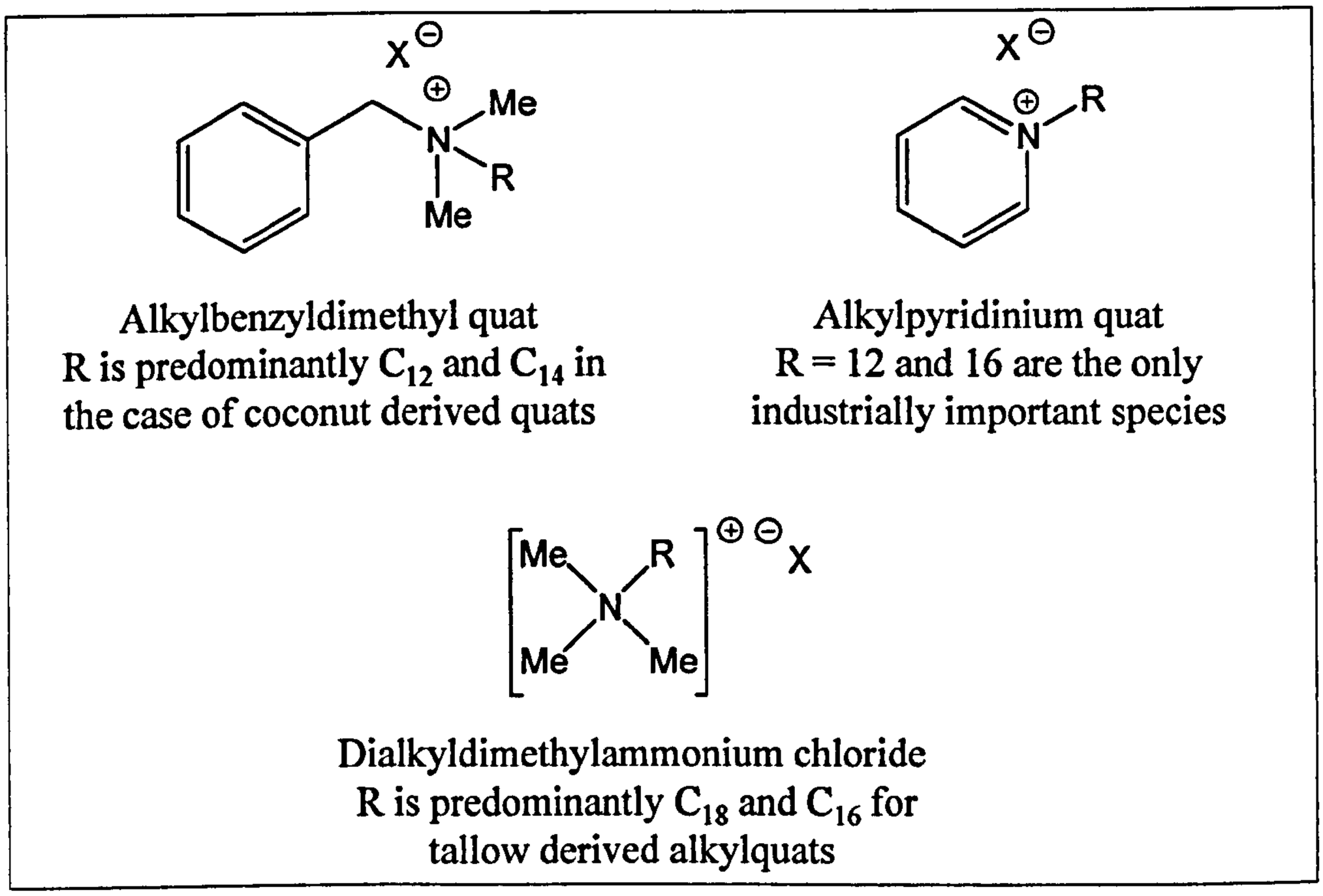

Figure 1.6: Representative structures of the three major classes of cationic preservatives.

It was mentioned above that different cationic tensides are utilised in different applications. Whilst the original choice of active was based on available materials, the choice is now dependant on the individual scenario in which the ingredient is to be applied, as different classes of tenside, and the individual homologues of each, demonstrate varying efficacy (Lawrence, 1970; Prince *et al.*, 1999). Such observed variation in the potency of individual homologues has led to strictly defined limits and controls being placed on the homologue distributions present in commercial samples. The United States Pharmacopoeia (USP), along with the British Pharmacopoeia (BP) and European Pharmacopoeia (Ph. Eur.), and other legislators, include clearly defined

boundaries on the nature and concentration of individual homologues in various products (Martindale, 1993; Prince *et al.*, 1999). The maximal and minimal concentration of active agent present in a commercial sample is stipulated, in addition to regulations on chain length ratio of the active materials (Martindale, 1993; Prince *et al.*, 1999). For example, the USP national formulary (USP/NF) guidelines stipulate that a commercial sample of *Benzalkonium Chloride* (**Appendix One**) must contain not less than 95% active C_8 to C_{18} alkylbenzyl quats. Of this, not less than 40% should be the C_{12} species, and not less than 20% should be C_{14} homologue. Additionally, the total C_{12} and C_{14} concentration should be greater than or equal to 70%. Any material not meeting these criteria will be blocked from application under the pseudonym Benzalkonium Chloride in pharmaceutical and healthcare products (Martindale, 1993). Similar restrictions are also enforced for the alkylpyridinium quats and the monoalkyltrimethyl quats (Martindale, 1993).

Whilst commercial cationic tenside preservative samples contain fewer active agents than are present in conditioner samples, strict legislation on composition and activity, forces formulators and raw materials suppliers to implement strict quality-control protocols. At the same time, the regulators must be able to test adherence to their legislation, which also requires the use of accurate analytical methods to quantify these materials as raw materials and in fully formulated products.

1.2.3 Environmental aspects of cationic tensides

In recent years there has been a marked increase in the environmental awareness of the world's population. In the developed world especially, there is widespread understanding of the potential effects that industrialisation and commercialisation have on the delicate balance of the World's Ecosystem. Whilst major environmental catastrophes like the Exxon Valdez oil spill in 1989, have made, and always will make headline news in the scientific and non-scientific press, this greater environmental awareness has led to greater emphasis being placed on *"environmental friendliness"* in all areas of life. As a result, strict environmental legislation now controls all of the activities of Man, which are perceived to influence the world around us.

1.2.3.1 Introduction to chemical risk assessment

The notion of environmental legislation is far from new in the control of chemical usage. The inception of laws regarding the safeguarding of potable water from sources of contamination can be found in the legislature of the Roman Empire, whilst others are to be seen in the laws of European countries during the middle ages (Ahmad *et al.*, 2001). Indeed, much of the basis of current environmental legislation grew out of human health legislation developed in the late nineteenth century (Ahmad, 2001). However, as the awareness of governments, and other authorised agencies, has developed, with regard to the potential effects of chemical usage on the environment, modern environmental legislature now runs in parallel to laws governing human health.

Today, the manufacture and usage of chemicals is strictly controlled by guidelines on both perceived and documented toxicological and environmental impact (Solbe, 2000). During the formulation of these guidelines two concepts are taken into account, *hazard assessment* and *risk assessment* (Solbe, 2000). A *hazard* is an inherent property of an action or compound, whilst *risk* is the likelihood of that effect / property being manifest. Death by drowning is a *hazard* of bathing, whilst electrocution is a hazard of using electricity, the *risk* associated with both is lessened if the two are not combined! From a chemicals perspective, a hazard of nitrogen gas is that it is an asphyxiant; the risk of asphyxiation being increased in confined areas.

Primarily, new legislation concerning chemical usage is based on risk assessment, as it is quantifiable, takes into account the likelihood of a detrimental effect occurring, and can also be applied to new compounds as it can be used to assess perceived as well as documented problems. As a result, approval of the use of all new chemicals, in addition to the use of known species for a role in which they were not originally specified, is controlled by a formal risk assessment procedure.

Whilst a risk-based assessment of chemical safety is welcomed by most, many environmental pressure groups believe that a hazard-based system should be adopted. Claims are often made that legislation is too lenient, as chemical manufacturers and formulators are thought to hold too much influence over the decisions of the lawmakers. Indeed, such groups often maintain that all unnaturally occurring chemicals are harmful and hazardous to the environment and / or human health, and, as a result, their usage

should be banned. However, adoption of a hazard-based system is often over simplified, as it cannot be used to assess indirect problems associated with some chemical species. For instance, an excess of phosphate in some confined lakes and rivers can lead to *eutrophication* and the ultimate death of the body of water (Moore *et al.*, 1976). Whilst the use of phosphate-based detergent formulations is claimed to have greatly increased the rapidity of eutrophication due to the occurrence of algal blooms, the process is naturally occurring, and is not a hazard, as such of phosphate. Only risk-based assessments can efficiently account for such secondary effects.

For environmental impact assessment, the formal risk assessment is based on a comparison of the lowest concentration at which a chemical will be seen to cause no harm to the environment, the so called *"predicted no-effect concentration" (PNEC)*, with the *"predicted environmental concentration" (PEC)*, the level expected in the different environmental compartments (**Figure 1.7**). The PNEC is calculated by assessing the effect of the compound on a base set of sensitive organisms present in each compartment (**Figure 1.8**). The conservative safety factor is then applied to ensure that the resulting concentration will have no discernible effect on the organisms present. Determination of the PEC is achieved by combining usage, removal, and environmental / geological data to determine the maximal concentration that the compound is likely to reach with prolonged use. It is apparent therefore that accurate risk assessments can only be based on accurate data, which details how much compound enters the environment, from where, and where it permeates to ultimately.

1.2.3.2 "Down the drain" and "rinse-off" chemicals

To have an effect on a specific environmental compartment, a chemical must have the ability to reach that compartment in the first instance. There are two primary modes by which a chemical can enter the environment, *use* and *misuse*. Of these, misuse is probably the most recognised for causing an environmental impact, as it is characteristic of the one-off pollution event that led to the Exxon Valdez disaster (Solbe, 2000). However, correct use of a chemical can also lead to its entry into the environment.

Cationic tensides, indeed many tensides in general, are examples of compounds that have the potential to be introduced into the environment through proper usage. If

one considers the two most important industrial applications of cationic tensides, fabric conditioners and preservatives (**Section 1.2**), their modes of action dictate that cationic tensides will have the ability to be transported to the environment. Fabric conditioners, and domestic and industrial surface cleaners are classed as *"down-the-drain"* chemicals (Burford, 1997). In the case of the developed world, the mode of action of a conditioner dictates that it will be expelled from the washing machine into the domestic sewage treatment system, (Versteeg, 1992). Surface cleaners are disposed of in a similar manner, as they are normally rinsed into the drainage system after application. If the local sewage treatment system is unable to completely remove the tensides, then they will be released into the aquatic environment through wastewater or into the terrestrial environment as sludge (Di Corcia, 1998). Cationic tenside preservatives also have a similar fate. Their usage to prevent adulteration of personal care, cosmetic and pharmaceutical *"rinse-off"* preparations, creams, pomades and ointments, also results in them being washed into the sewage treatment system (Berger, 1997).

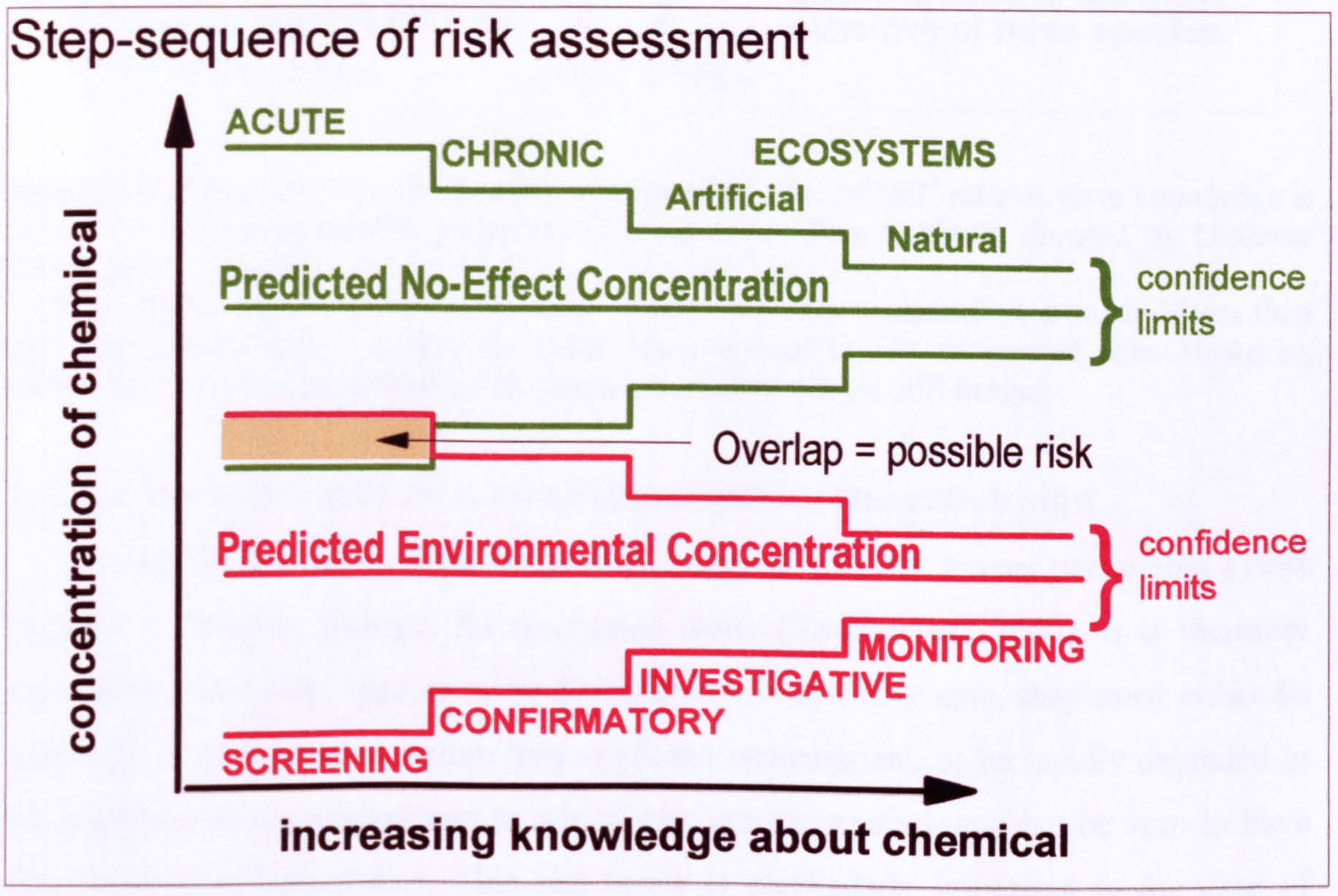

Figure 1.7: Figure showing the various stages of a conventional risk assessment process, demonstrating how the relationship between the PNEC and PEC affects the nature of the safety testing required (Picture kindly donated by Unilever Research).
As one moves towards the right hand side of the diagram, testing becomes more involved as the test becomes increasingly realistic of environmental conditions.

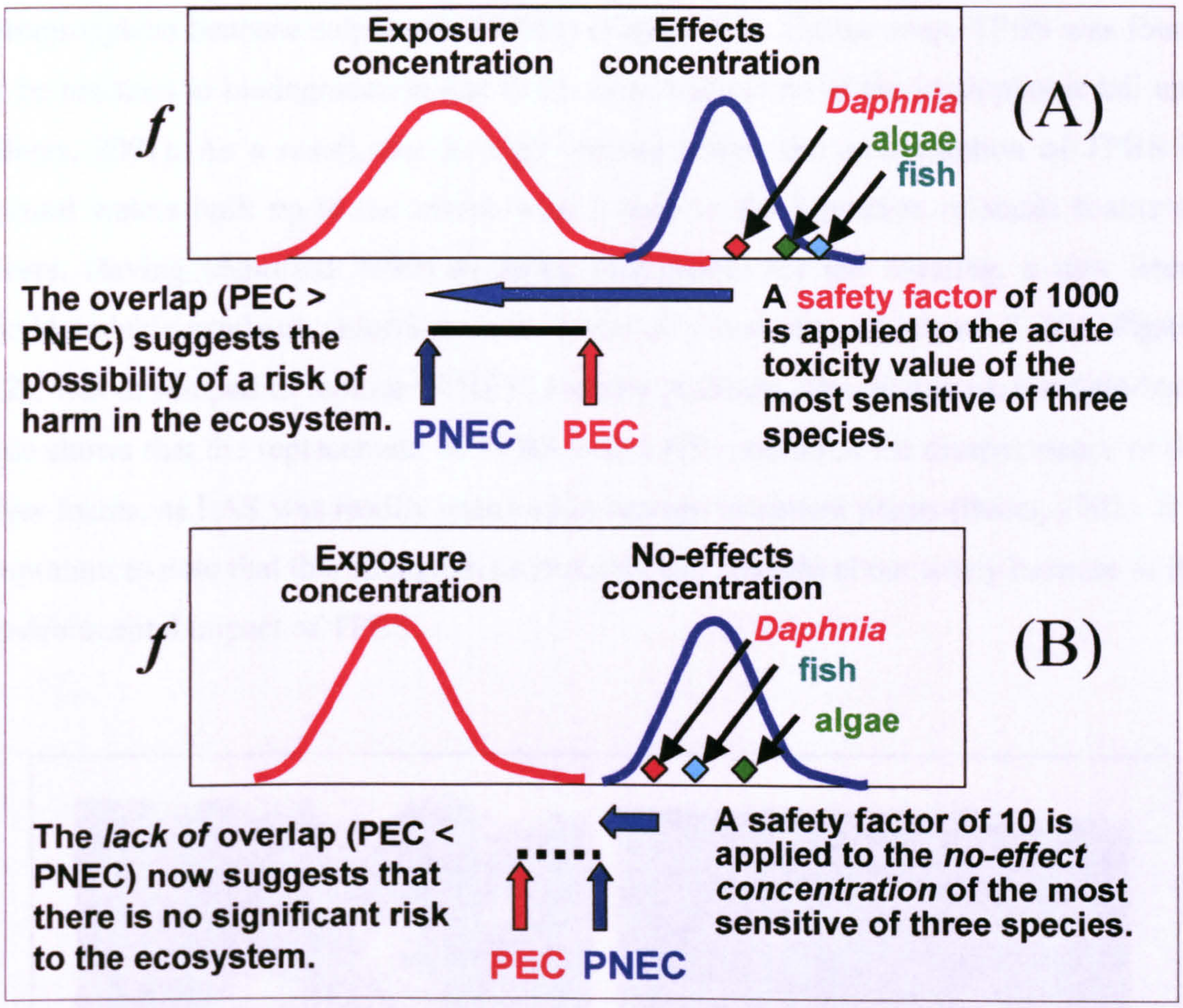

Figure 1.8: Figure showing the idealistic change in the PEC / PNEC ratio as more knowledge is gained on the environmental properties of a chemical (Picture kindly donated by Unilever Research).
As the methods used to calculate the PEC / PNEC ratio are conservative, a value of less than one represents a safety margin for using the chemical in it's designated role. However, additional work is often carried out to increase the safety margin still further.

1.2.3.3 Environmental fate, recalcitrant species and eco-design

The market for cationic tensides amounts to 135,000 tonnes per annum (1998 figures) in Western Europe, for detergents alone (Trius *et al.*, 2000). It is therefore evident that for these species to be deemed environmentally safe, they must either be removed to a great extent before they reach the environment, or be rapidly degraded in the environmental compartment to which they are transported, and not be seen to have the potential to accumulate. This last factor is particularly important in the case of relatively high production volume chemicals like the cationic tensides, and has been a historical problem with certain species. **Figure 1.9** shows two photographs taken at the outflow of a sewage treatment plant into the River Lea in Lemsford, in the United Kingdom in 1960 and 1964. The earlier picture (left hand side) coincided with the

replacement of soap flake in domestic laundry products for the synthetic tenside: tetrapropylene benzene sulphonate (TPBS) (**Figure 1.2**). Unlike soap, TPBS was found to be resistant to biodegradation due to the branched nature of the hydrophobic tail unit (Beers, 2001). As a result, due to high volume usage, the concentration of TPBS in natural waters built up to the extent were it lead to the formation of stable foams on rivers. Having identified TPBS as being responsible for the foaming, a new linear biodegradable synthetic anionic tenside, linear alkylbenzene sulphonate (LAS) (**Figure 1.2**), was developed to replace TPBS in laundry products. The picture on the right-hand side shows that the replacement of TPBS with LAS resulted in the disappearance of the river foams, as LAS was readily removed in sewage treatment plants (Beers, 2001). It is important to note that this change in ingredients was brought about solely because of the environmental impact of TPBS.

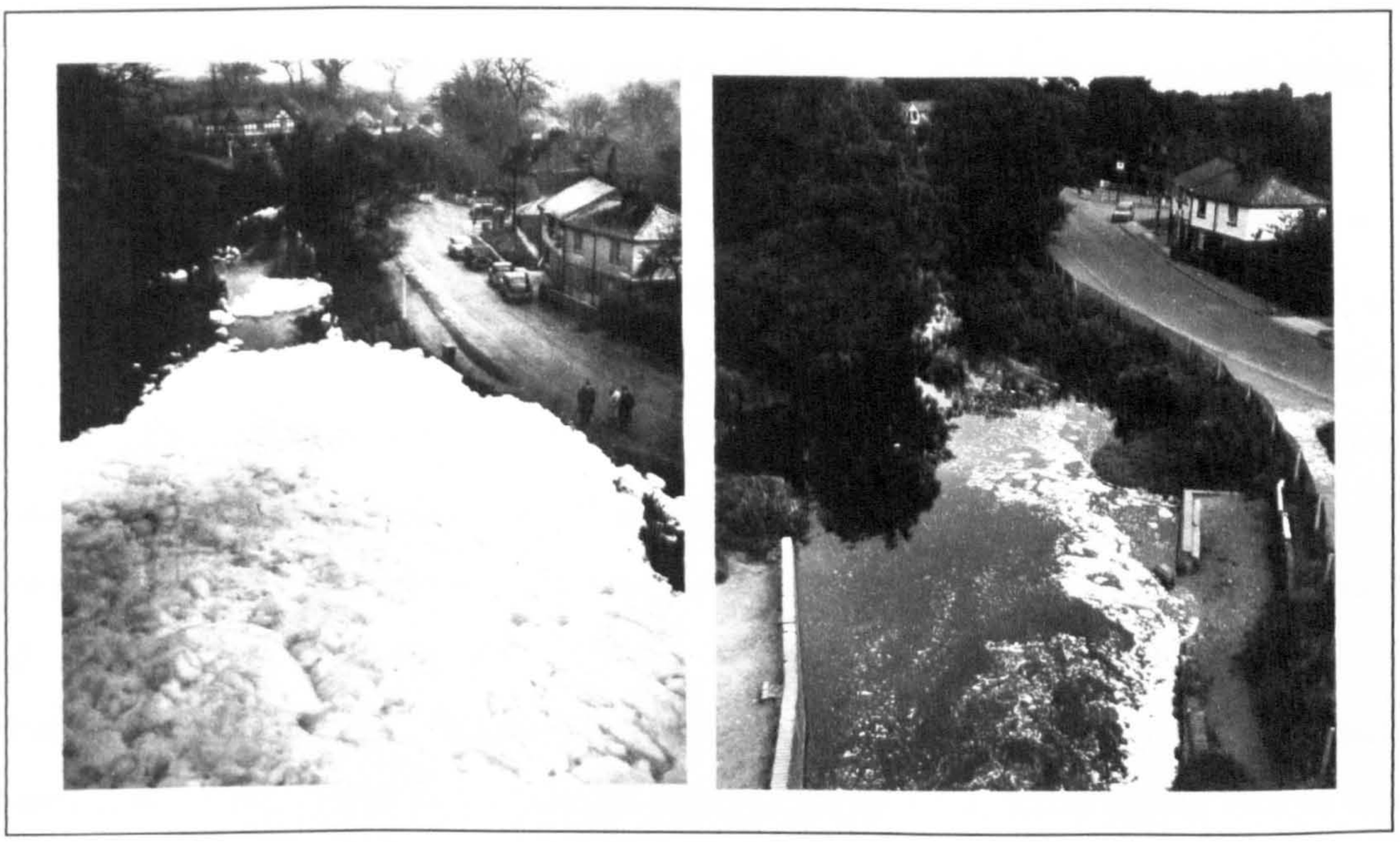

Figure 1.9: Photographs of the River Lea in Lemsford, UK, taken in (A) 1960 and (B) 1964. The figure shows the environmental impact caused by the replacement of soap with tetrapropylene sulphonate (TPBS) in domestic laundry products, and the subsequent adoption of linear alkylbenzene sulphonates in detergent formulations (LAS) (Pictures kindly donated by Unilever Research).

In addition to the problems caused by *persistent* parent tensides, another important consideration in the assessment of an environmental risk, posed by any chemical, is its propensity to form *recalcitrant metabolites*. Whilst, the parent tenside initially undergoes biodegradation, the process is halted at an inappropriate juncture, due to the tendency of the metabolite to resist further breakdown (Burford, 1997). This

process is characteristic of the nonylphenol ethoxylate tensides, which give rise to nonylphenol metabolites that are recalcitrant. These metabolites show higher aquatic toxicity compared to the parent species, and have been linked to inflicting endocrine disturbances (Beers, 2001). Factoring these risks into the formal assessment for this species has led to its voluntary replacement by the more readily biodegradable alcohol ethoxylates (**Figure 1.2**) (Beers,2001).

The propensity of many cationic tensides to be anti-bacterial and algaecidal, and to preferentially sorb onto negatively charged species has led to historical concerns over the widespread use of these species in "*down-the-drain*" and "*rinse-off*" products. The observation that the first generation fabric conditioner actives (**Section 1.2.1**) facilitated observable effects on sensitive species at low concentrations (Waters *et al.*, 1991) did not help to alleviate any of these fears. However, there was strong evidence that the alkyl quat tensides showed high removal within sewage treatment plants, and thus only low concentrations were observed in receiving waters, even before dilution occurred (Waters *et al.*, 1991). Nevertheless, in light of the concerns of legislative bodies over the environmental profile of these materials when used in fabric conditioners, the early 1990's saw formulators voluntarily replace the 1st generation alkylquats with new esterquat actives (**Figure 1.5**) (Waters *et al.*, 1991). These materials demonstrated much improved environmental profiles, owing to the presence of ester linkages between the cationic nitrogen atom and the fatty alkyl chain (Puchta *et al.*, 1993). These ester groups represented points of weakness in the structures of the new actives, which facilitated the rapid cleavage of the fatty acid groups from the parent tensides (**Figure 1.10**) (Lawrence, 1997), greatly increasing the observed rate of primary biodegradation (Waters *et al.*, 1991). As with the change from TPBS to LAS, some twenty five to thirty years earlier, the switch from the use of alkyl quats to esterquats was driven by environmental concerns (Burford, 1997). In the case of the cationic tensides, these concerns were primarily perceived rather than documented (Waters *et al.*, 1991).

As risk assessment of chemical usage must be performed separately for each environmental compartment into which the compound can ultimately pass, it is important to be able to quantify the path of a species through waste treatment and during its progress through the environment. Ultimately, the transport and movement of any chemical will be based on its characteristic physico-chemical properties. In the case

of cationic fabric conditioner actives, the limited water solubility and high octanol / water partition coefficient (K_{ow}), a measure of the propensity of a compound to reside in an aqueous or non-aqueous environment, which is often quoted as the logarithmic value (Log P) for convenience (Hansch *et al.*, 1979), indicates that the dialkyl quats preferentially partition out of the aqueous phase onto solids (Waters *et al.*, 1991). As a result, much of the removal of these species during sewage treatment is as a result of adsorption onto sludge solids (Beers, 2001). The process of sludge amendment onto agricultural land thus yields the introduction of these species into the terrestrial environment. Similarly, the residual di-chained quats, which pass into receiving waters, are seen to preferentially partition onto suspended and dissolved solids, which drop to the riverbed to form sediments (Scott, 2000) (**Figure 1.11**). As a result, the di-chained quats are introduced to the sediment compartment.

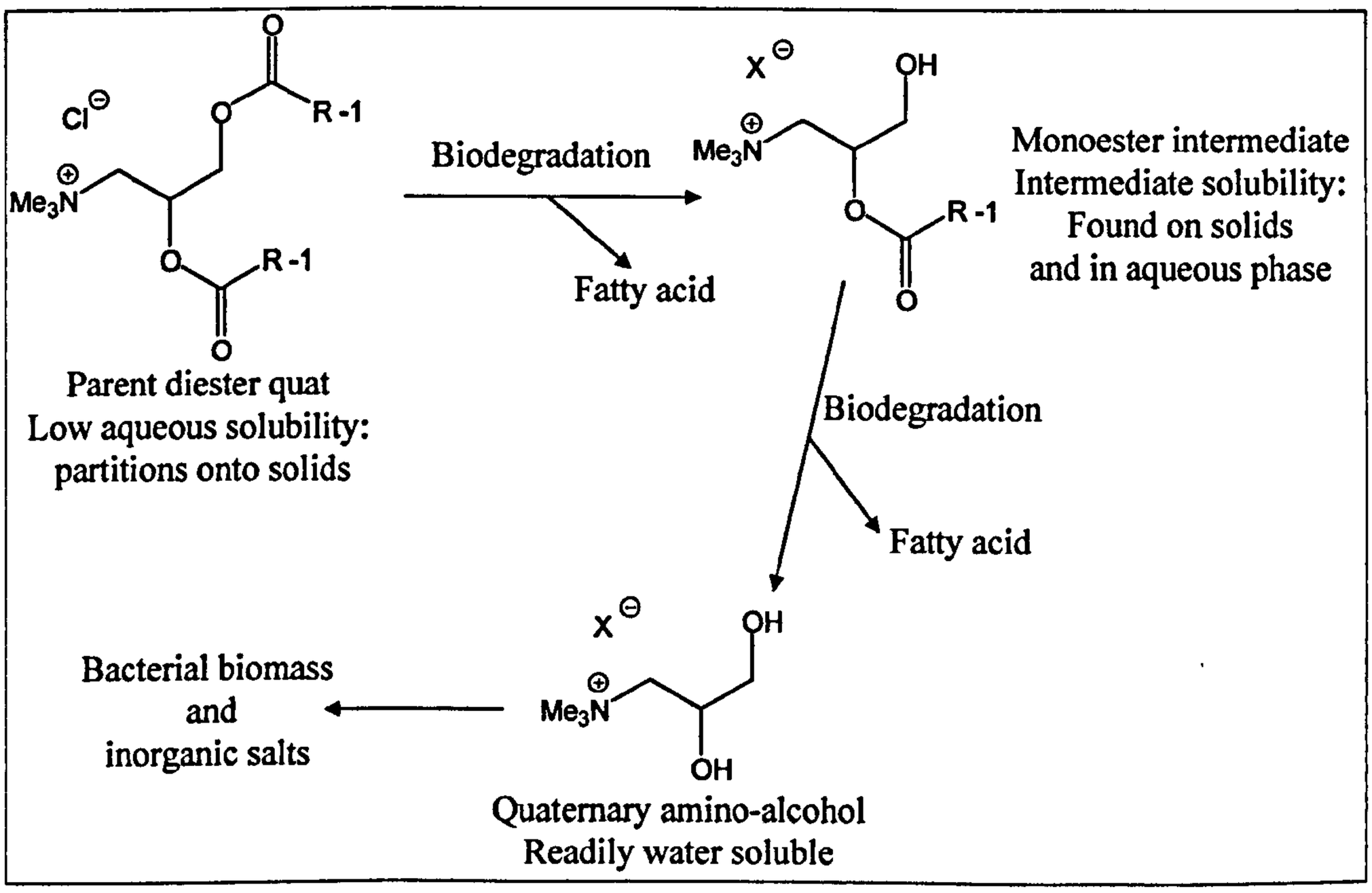

Figure 1.10: Representation of the biodegradation pathway of Hamburg diester quat (HEQ). The parent di-chained quat is very hydrophobic and readily partitions onto solids. Removal of the fatty acid groups increases aqueous solubility. As a result the quaternary amino-alcohol is readily water-soluble and is thus more *bioavailable*.

The partitioning behaviour of the mono-chain quats (**Figures 1.6** and **1.10**) is not as extensive as that of the di-chained quats, due to their increased aqueous solubility (Burford, 1997). As a result, there is increased likelihood of finding these species in their native forms in wastewater effluents i.e. solubilised rather than associated with solids (Burford, 1997). This is also true for the quaternary amino-alcohol metabolites of

the esterquats (**Figure 1.10**) (**Appendix One**), which are the most readily water soluble of the three groups of quats owing to the removal of the second hydrophobic group from the monoester quats (Burford, 1997). As a result of their high water solubility these species are unlikely to be found associated to solids or in sediments (Burford, 1997). Unfortunately, higher aqueous solubility leads to the species being more *bioavailable*, which in turn increases the potential for these species to *bioaccumulate* in the tissues of native organisms via absorption and / or ingestion (Beers, 2001). Extensive bioaccumulation could then lead to the concentration of the compound within the tissues of the organism approaching those at which a distinguishable effect is first witnessed (van Leeuwen, 1995). In view of recent concerns regarding metabolite formation during biodegradation, there has recently been an upsurge in regulatory interest in defining the future role that metabolite formation, transport, and accumulation should play in environmental risk assessment (Beers, 2001)

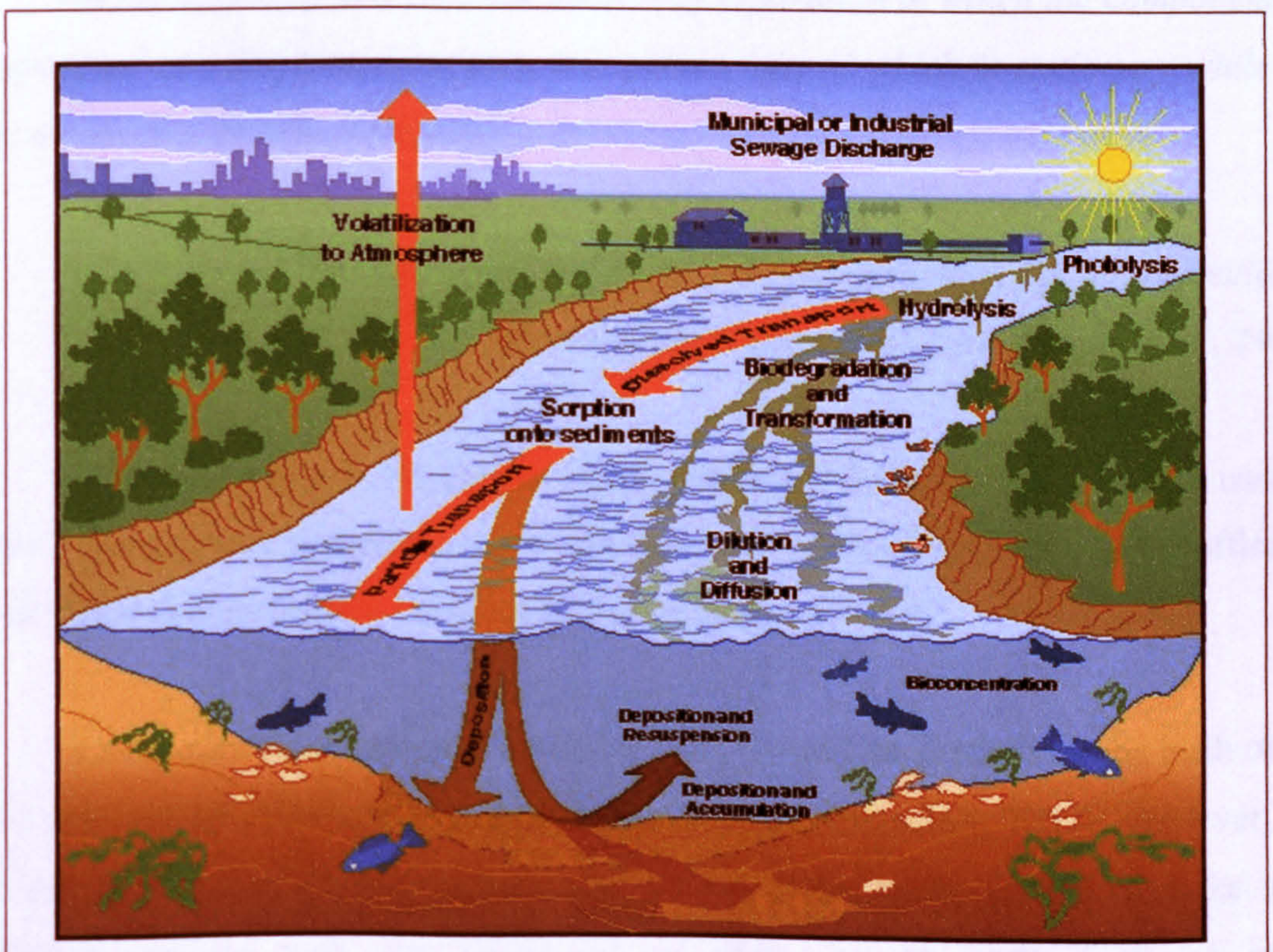

Figure 1.11: Figure showing some of the possible removal and transportation pathways that can be exhibited by a chemical after its release into a riverine environment (Source of picture United States Geological Survey website).

It is apparent that in order to carry out a formal risk assessment on the potential environmental impact of cationic tensides, accurate determination and quantitation is required in a number of different environmental matrices. In order that accurate measurement is attained, methods of analysis must be selective to minimise the interference caused by endogenous components of complex sample matrices. Not only does environmental assurance demand accurate quantitation of the parent cationic tensides, but there is increasing need to be able to quantify the hydrophilic metabolites resulting from primary biodegradation. In addition, the ability to monitor the transport and transformation of cationic tensides through the environment is of paramount importance if the environmental safety margins are to be maximised.

1.3 INTRODUCTION TO CHROMATOGRAPHY AND SEPARATION SCIENCE

Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary while the other moves in a definite direction.

"Unified Nomenclature on Chromatography", IUPAC definition of chromatography ca. 1993 (Braithwaite et al., 1996).

Chromatography is the generic name applied to techniques that can be used to separate two or more components of a mixture as a result of variations in the affinities of individual solutes for two immiscible phases (Ruthven, 1997).

Natural chromatographic phenomena have existed on Earth since the birth of the planet millions of years ago (Braithwaite *et al.*, 1996; Berezkin, 1997). However, the practical relevance of chromatographic techniques was not recognised until the mid-nineteenth century with the separation of dyes, by an early form of paper chromatography (Braithwaite *et al.*, 1996). Some ten years later Groppelsroeder reported the *"capillary analysis"* of pigments via separation on paper dipped into solvent (Braithwaite *et al.*, 1996). Groppelsroeder was aware that capillary action was bringing about the migration of the various pigments. However, he was unable to justify

the reasons behind the discrete coloured bands that he witnessed. Whilst these early *"Separation Scientists"* had reported observations that were fundamental to modern chromatographic separation, the birth and discovery of chromatography is normally attributed to the work of the Russian Botanist Tswett, at the start of the Twentieth century (Braithwaite *et al.*, 1996; Berezkin, 1997; Ruthven, 1997). Tswett demonstrated how it was possible to separate and isolate green and yellow plant pigments, by passing extracted plant material through a glass column packed with a fine powder (sucrose or calcium carbonate). After initially adding the plant material onto the *head* of the column in a fine band, Tswett added additional solvent and pressure, to move the pigments through the column (Braithwaite *et al.*, 1996; Start GC website). The addition of the solutes in a small focused band, and the subsequent addition of solvent, facilitated superior component *resolution,* compared to that achieved with the crude paper fractionation of dyes and pigments (Braithwaite *et al.*, 1996). In 1906, Tswett, during the reporting of his findings, first coined the phrase *chromatography* from the Greek words for colour, *"chroma"* and the verb "to write" or *graphein*, to describe how the new technique separated the plant material into discrete coloured bands on the column.

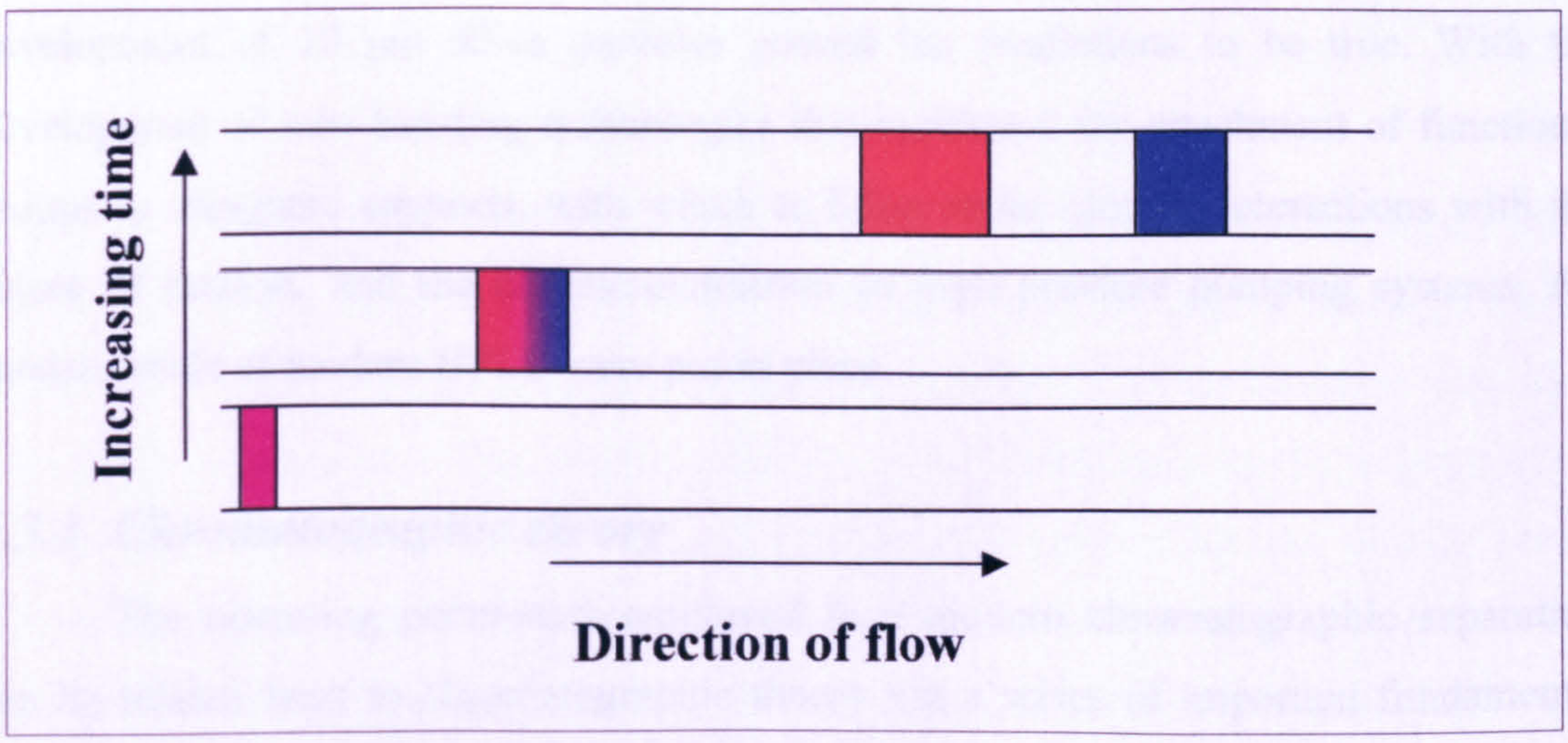

Figure 1.12: Stylised representation of a chromatographic separation.
The two components are separated over time due to their different affinities for the two, immiscible phases.

Some years later, the potential implications of Tswett's observations were recognised by chemists and led to the beginnings of chromatographic research. In 1941, Martin and Synge used chromatography to separate amino acids from wool. This evolved the technique of partition chromatography (Braithwaite *et al.*, 1996).

The commercial potential of *gas chromatography* (GC), particularly in the analysis of the complex samples characteristic of the petroleum industry, was realised during the pioneering work of Martin and James. These researchers were able to separate mixtures of short chain carboxylic acids on a support material, coated with a liquid film. By forcing the samples through a glass tube with the aid of compressed gas, it proved possible to isolate individual analyte bands. Some five years later, Golay published a theoretical treatise on optimising column efficiency and peak capacity in GC. His theories on the use of long, narrow columns, coated with a thin liquid film have subsequently given rise to the high resolving power, characteristic of modern GC analysis.

The development of modern day "*high performance liquid chromatography*" (HPLC), came about as a result of the predictions made by Giddings in 1963. Giddings suggested that the resolving power achieved with GC would only be forthcoming for LC separations, if very small diameter particles were packed into narrow columns. At the time, fabrication technologies could not provide the particles necessary to interrogate Giddings' theories, and some six years passed before the successful development of 10 μm silica particles proved his predictions to be true. With the development of new bonding technologies that facilitated the attachment of functional groups to inorganic supports, with which to bring about specific interactions with the solute of interest, and the commercialisation of high pressure pumping systems, the fundamentals of modern HPLC were put in place.

1.3.1 Chromatographic theory

The operating parameters employed in a modern chromatographic separation can be related back to chromatographic theory via a series of important fundamental parameters: *retention*, *efficiency*, *resolution* and *"solute band broadening"*. In the following section, these parameters will be considered with respect to their effect on experimentally derived separations. The information contained in this section has been obtained, to varying extents, from a small number of the many reference texts and information sources on the subject of chromatographic theory. References utilised at this time include Snyder *et al.* (1979), Braithwaite *et al.* (1996), Meyer (1993), Katz *et al.* (1998) and the Start GC website.

As mentioned previously, chromatographic separation is based on the variation in the affinity of solutes for two immiscible phases. The rate at which an *analyte* migrates through the system is therefore dependent on the length of time that the analyte spends in contact with the two distinct phases. Thus, in liquid chromatography (LC), the rate of migration is critically dependent on the intermolecular forces acting between the analyte and the stationary phase, and the analyte and the liquid mobile phase. For analytes that interact strongly, and thus have a high affinity for the stationary phase, their progress through the system is slow and retarded, in comparison to analytes that are only weakly attracted to the stationary phase. Chromatographic separation is therefore dependent on a variation in the extent of which analytes distribute, or *partition* between the two phases. For an analyte (solute) *i*, this behaviour can be described by the distribution or partition ratio **(Equation 1.1)**. The *"partition coefficient"*, *K*, shown in **Equation 1.2** is the equilibrium constant relating to this dynamic process,

$$i_{mobile} \leftrightarrow i_{stationary} \qquad \textbf{(Equation 1.1)}$$

$$K_i = \frac{C_{i,s}}{C_{i,m}} \qquad \textbf{(Equation 1.2)}$$

where $C_{i,s}$ and $C_{i,m}$ relate to the molar concentration of the solute in the stationary phase and mobile phase respectively. Therefore, the chromatogram that is achieved during analysis provides a pictorial representation of the history of the analytes migration through the column over time. Analytes that are seen to elute early in the chromatogram can be deduced as having spent the majority of their time within the mobile phase, whilst those that elute later in the trace, must have shown stronger affinity for the stationary phase. **Figure 1.13** shows a chromatogram depicting the separation of a two-component mixture. The baseline disturbance at the start of the trace is representative of the emergence of the solvent front from the column, and all unretained analytes. When an analyte is unretained it shows no affinity for the stationary phase and thus passes through the column unchecked. The time taken between the introduction of the mixture into the system, and the elution of an unretained component is known as the *"dead time"* or *"void time"* of the system, t_0. This parameter can then be used to quantify the residence time of the other components in the stationary phase. The values t_{R1} and t_{R2}

are the retention times of the two retained components. These are characteristic of the individual analyte, as they are derived from the fundamental interactions between the analyte and the stationary and mobile phases. Use of retention time data and knowledge of column length, L, can subsequently be utilised to determine the average linear velocity during the separation, $\bar{u}$ **(Equation 1.3)** and the average rate at which the solute migrated through the column, $\bar{v}$ **(Equation 1.4)**.

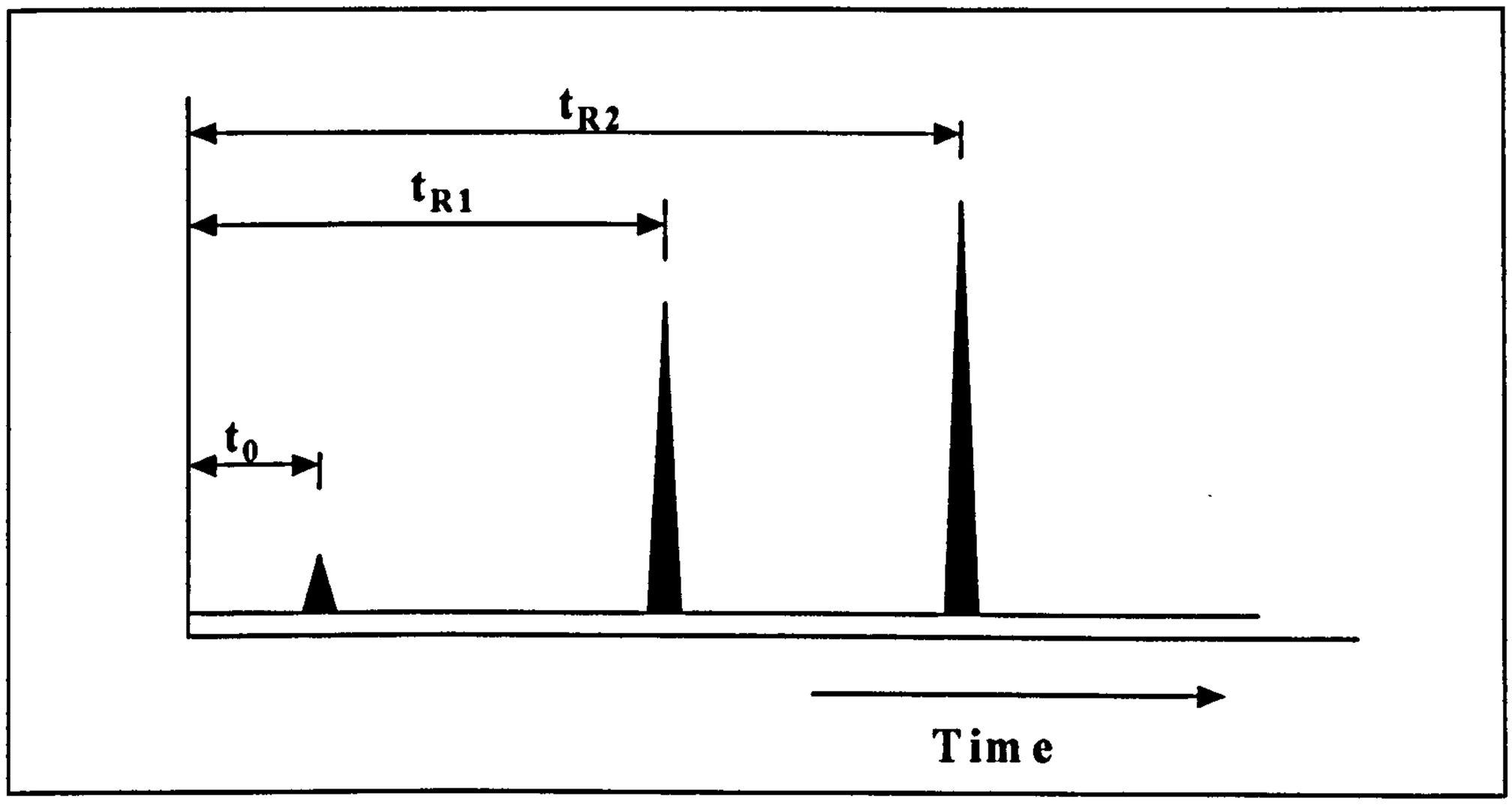

Figure 1.13: Typical chromatogram of a non-retained species and two retained components.

$$\bar{u} = \frac{L}{t_0} \qquad \textbf{(Equation 1.3)}$$

$$\bar{v} = \frac{L}{t_R} \qquad \textbf{(Equation 1.4)}$$

As $\bar{v}$ is related to residence time in the mobile and stationary phases, the parameter can also be calculated from comparing the number of moles of analyte in the stationary phase with the total number of moles in the system. As the number of moles of analyte present in the system is related to the molar concentration of the analyte in the stationary and mobile phases, $C_{l,s}$ and $C_{l,m}$ respectively, and the volume of the mobile and stationary phases, V_m and V_s, substitution and simplification results in $\bar{v}$ being described by **Equation 1.5**:

$$\bar{v} = u \cdot \frac{1}{1 + C_{i,s}V_s / C_{i,m}V_m} \qquad \textbf{(Equation 1.5)}$$

Substitution of the partition coefficient **(Equation 1.2)** into **Equation 1.5** leads to **Equation 1.6:**

$$\bar{v} = u \cdot \frac{1}{1 + K_i V_s / V_m} \qquad \textbf{(Equation 1.6)}$$

Simplification of **Equation 1.6** is facilitated by utilising the equation for the *"retention factor"*, *k*, **(Equation 1.7)**, which yields **Equation 1.8.** Replacement of the terms relating to ū and $\bar{v}$ **(Equations 1.3** and **1.4)** results in an equation for which, all components are determinable from an experimentally derived chromatogram **(Equation 1.9).**

$$k_i = \frac{K_i V_s}{V_m} \qquad \textbf{(Equation 1.7)}$$

$$\bar{v} = u \cdot \frac{1}{1 + k_i} \qquad \textbf{(Equation 1.8)}$$

$$k_i = \frac{t_R - t_0}{t_0} \qquad \textbf{(Equation 1.9)}$$

As *k* is proportional to T_R, a large increase in *k* is characteristic of a strongly retained analyte, and thus for convenience it is preferable to ensure that all *k* values are small to prevent excessive analysis time. However, if *k* is too small the analyte may be seen to elute close to the dead time. Control over the retention factor can be achieved by the variation of analytical parameters. In LC separations modification of mobile phase parameters can normally lead to the effective control of the retention factor.

During the analysis of a multi-component sample it is often convenient to utilise the retention factors of each of the analytes to quantify the *"selectivity"* of the method for two adjacent analyte peaks. The *"separation factor"*, α **(Equation 1.10)**, is a

descriptor which relates the relative retention of two components, *A* and *B* on a given stationary phase, under specified conditions.

$$\alpha = \frac{K_B}{K_A} \qquad \textbf{(Equation 1.10)}$$

where K_A and K_B are the distribution coefficients of solutes *A* and *B*. Substituting **Equation 1.7** into **Equation 1.10** highlights the relationship between selectivity and *k* **(Equation 1.11)**. It is evident that during the analysis of a multi-component mixture, if all *k* values are seen to be within a narrow range, selectivity may be compromised.

$$\alpha = \frac{k_B}{k_A} \qquad \textbf{(Equation 1.11)}$$

The ability of a column to produce narrow analytes bands is critical to high quality separations. Column *efficiency*, in liquid chromatography especially, is often quoted in terms of *"theoretical plates"*, a term whose basis lies in the fractionation towers of the petroleum industry. The higher the number of theoretical plates, the more efficient the separation is witnessed to be. This effect is apparent from observing the two forms of **Equation 1.12**. As the analyte bandwidth, *w*, decreases, efficiency is seen to increase; the column is better able to resolve individual components of a sample into discrete narrow bands.

$$N = 16 \cdot \left(\frac{t_R}{w}\right)^2 = 5.54 \cdot \left(\frac{t_R}{w_{1/2h}}\right)^2 \qquad \textbf{(Equation 1.12)}$$

It is apparent from **Equation 1.12** that *N* increases as the analytes residence time within the stationary phase increases, hence *N* is also seen be affected by column length, *L*. It is evident that as column length increases column efficiency also increases. **Equation 1.13** demonstrates the relationship between *N* and column length. The *H* term is a descriptor relating to the efficiency of the column per unit length. It is defined as *"the height equivalent to a theoretical plate"*, HETP, with rearrangement of **Equation 1.13** revealing that *H* decreases as *N* increases **(Equation 1.14)**, the distance between the theoretical plates decreases, which when related back to petroleum distillation

results in the isolation of a narrower boiling range of components. In terms of chromatography, the lower the plate height the narrower the resulting analyte band, which results in higher efficiency and thus better separation.

$$N = \frac{L}{H} \qquad \textbf{(Equation 1.13)}$$

$$H = \frac{L}{N} \qquad \textbf{(Equation 1.14)}$$

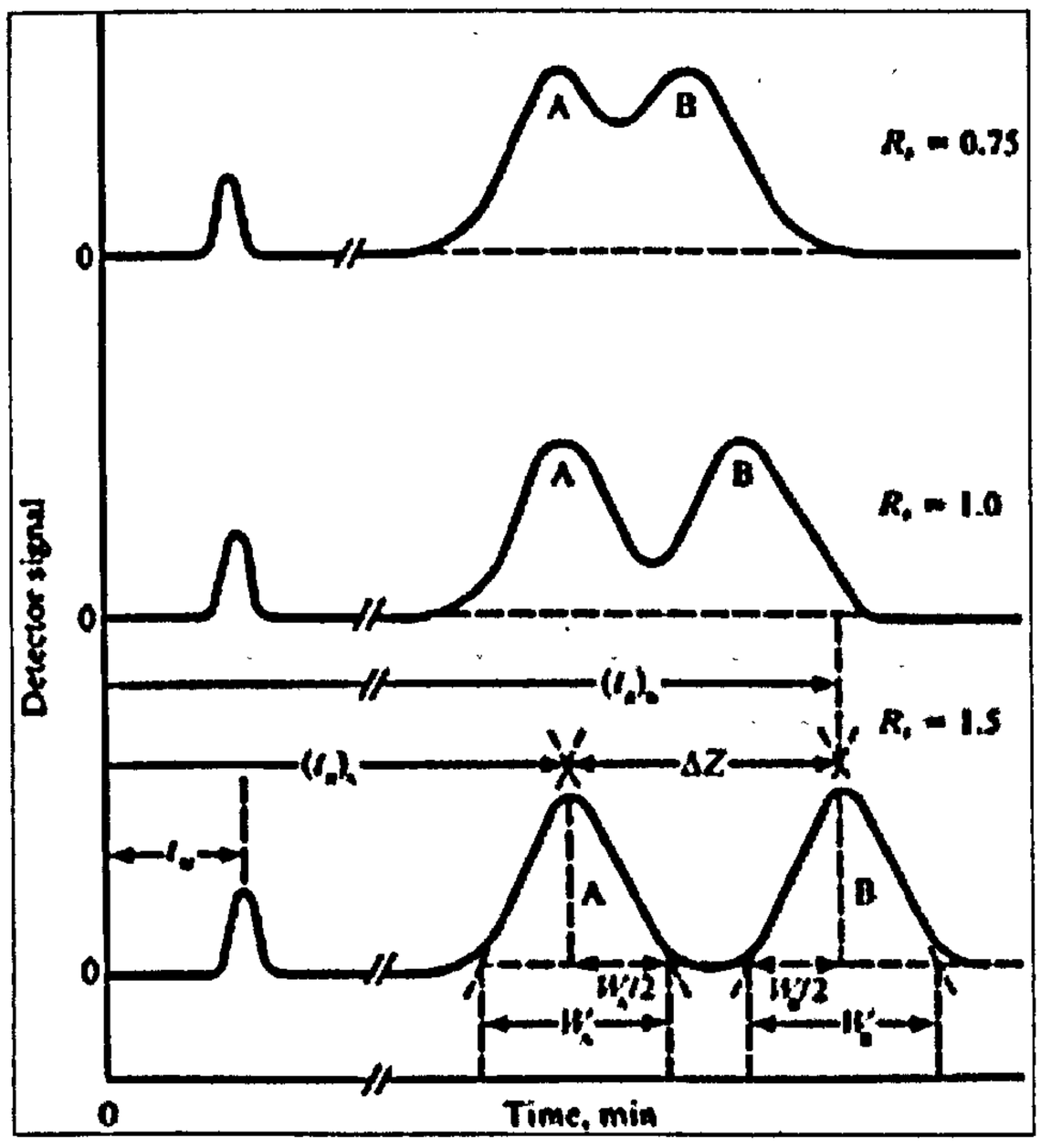

Figure 1.14: Figure demonstrating how the resolution of a two-component system can be manipulated in chromatography (Picture reproduced from Start GC website).
The terms shown are described in **Equations 1.10, 1.12, 1.21** and **1.22**. However, in the above example α is replaced by ΔZ, and components 1 and 2 in **Equations 1.21** are replaced by components A and B.

The processes by which multiple analytes are separated into discrete bands, and analyte bands broaden with time, witnessed by an increase in *w* as dwell time within the column increases, do not derive from the same origin. Instead the broadening of an analyte band on its journey through a column has been found to be a result of the physical effects represented pictorially in **Figure 1.15.**

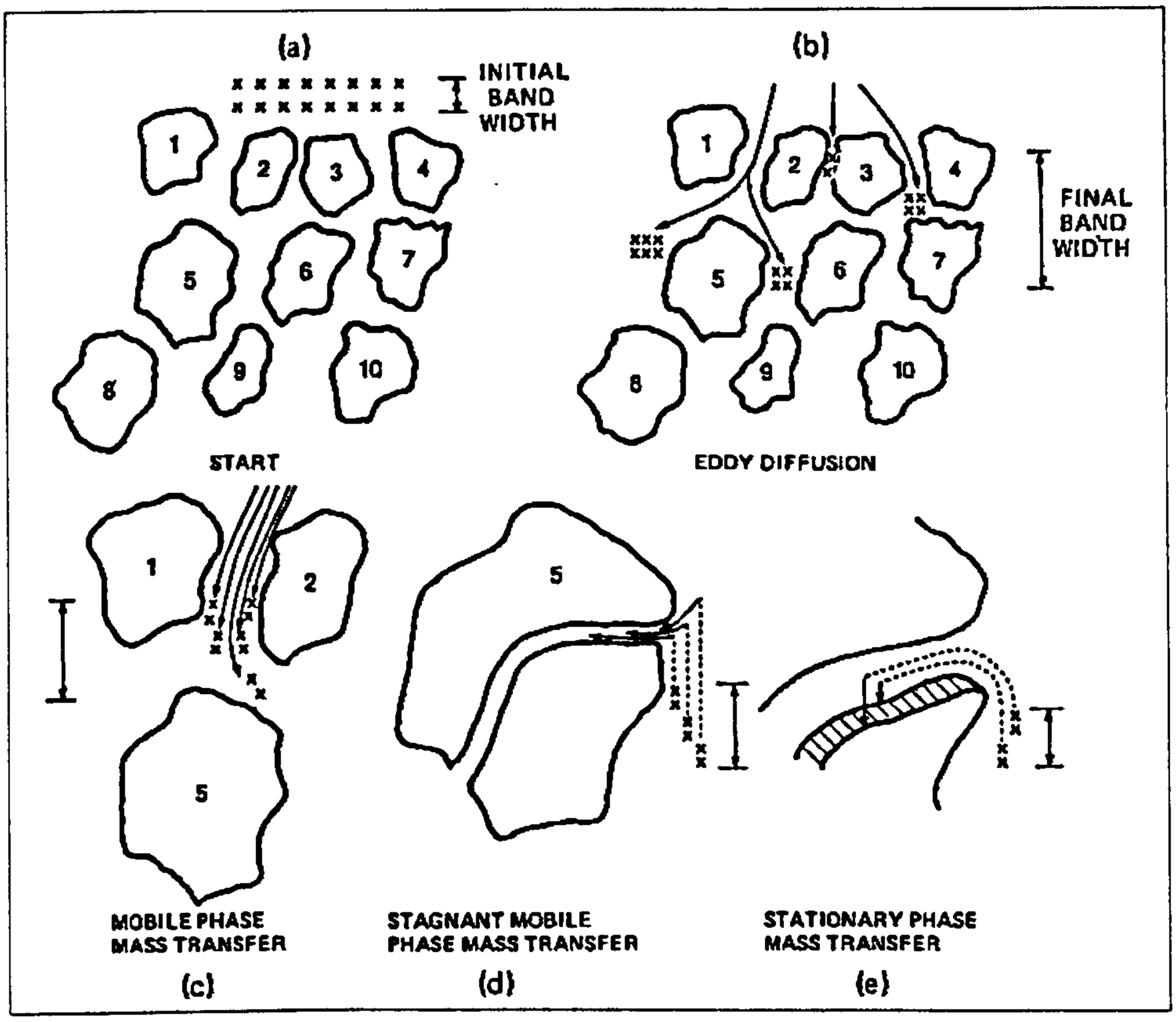

Figure 1.15: Individual contributions to the analyte band spreading witnessed in conventional high performance liquid chromatography (Reproduced from Snyder *et al.*, 1979).

Figure 1.15 (a) shows a cross-sectional view through the column as the analytes are introduced onto the head in a thin band. In **Figure 1.15 (b)**, the analyte molecules undergo the process of *"eddy diffusion"*, whereby the analyte band grows as a result of the analyte molecules taking distinctly different paths through the support. Those flowing through a wide channel will be seen to move faster than those moving through a narrow channel. The degree of spreading is seen to grow with time spent in the column. **Figure 1.15 (c)** highlights band spreading by *"mobile-phase mass transfer"* whereby the analytes in the middle of the band move much faster than those close to the particles due to the viscous drag being exerted band on the liquid at this pint. **Figure 1.15 (d)** provides a pictorial representation of the contribution of *"stagnant mobile phase mass transfer"* to band spreading. Differences in the rates to which analyte molecules partition in and out of the stagnant solvent present in the stationary phase pores also affect the overall size of the band. **Figure 1.15 (e)** shows the result of the final contribution *"stationary-phase mass transfer"*, whereby the analyte band is spread by some analytes permeating further into the stationary phase than others, which leads to

them showing additional retardation as they attempt to partition back into the bulk solvent.

The extent of which the processes seen in **Figure 1.15** affect band broadening, is dependant on the length of time that the analytes remain in the column. As a result, it can be seen that band broadening is inherently linked to the linear velocity at which the separation is performed. As a result, numerous studies have been performed to evaluate the effect of mobile phase velocity on separation efficiency. **Figure 1.16** shows the typical form of the graph obtained from studies characterising the change in *H* as a function of linear velocity. The data shown in **Figure 1.16** is characteristic of that obtained during GC analysis, and can be related to **Equation 1.15**, which was first reported by van Deemter.

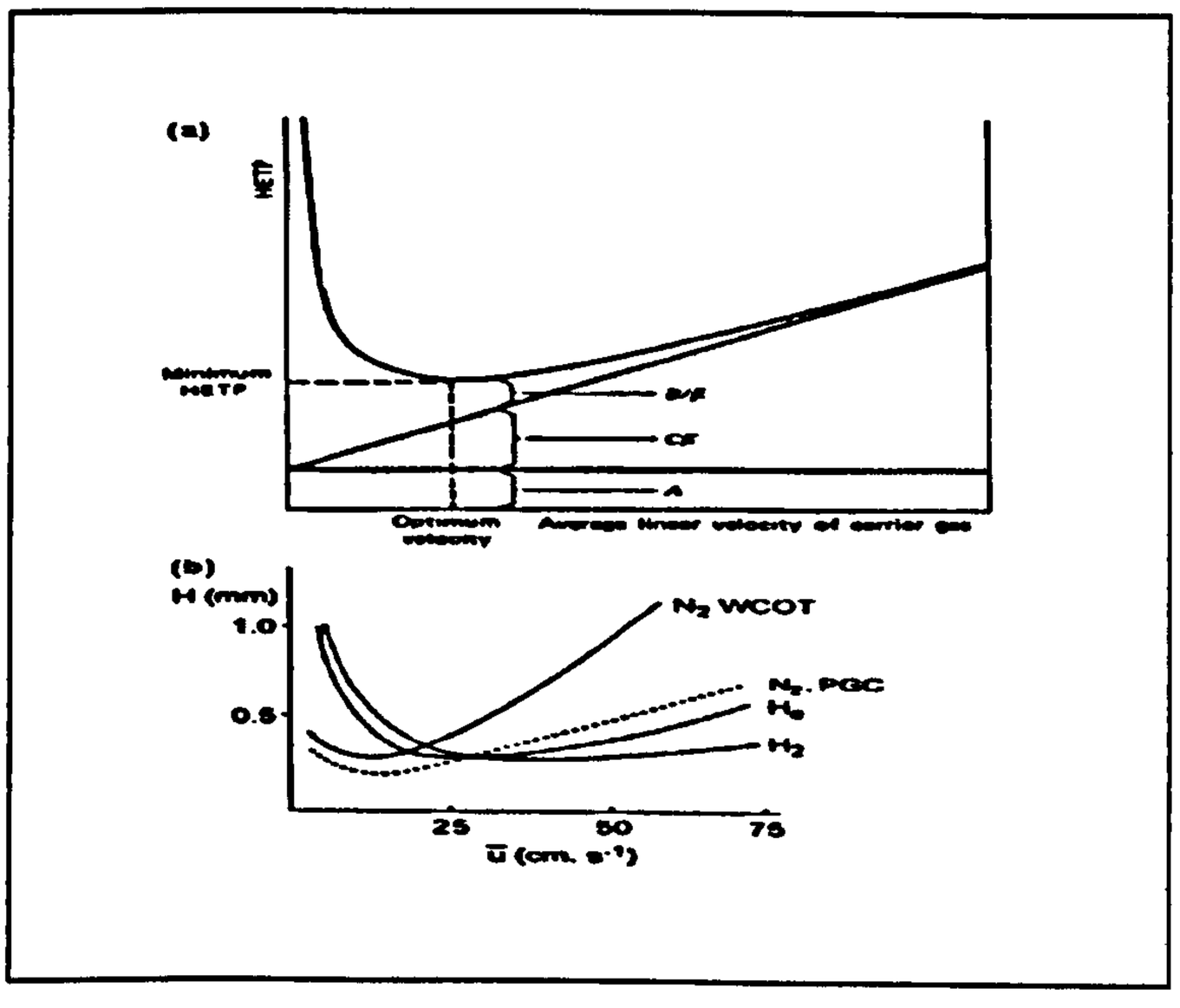

Figure 1.16: Figure showing how the linear velocity of a separation affects the individual contributions to band broadening, and how these changes subsequently affect the plate height *H*. The bottom diagram shows typical van Deemter plots obtained in gas chromatography.

$$H = A + \frac{B}{u} + Cu \qquad \textbf{(Equation 1.15)}$$

where *A*, *B* and *C* are the contributions to analyte band broadening characteristic of eddy diffusion, longitudinal diffusion and mass transfer processes, respectively.

Kennedy *et al.* (1972) later proposed a modified version of the van Deemter equation (**Equation 1.16**), which overcame the effects of particle size, column length and solute diffusion on separation efficiency, allowing the comparison of different columns being utilised under disparate experimental conditions. h is referred to as the reduced plate height, being obtained from the use of **Equation 1.19**, where d_p is the particle diameter. The reduced linear velocity is obtained from the relationship between u, d_p and D_m, the solute diffusion coefficient via **Equation 1.20**.

$$h = Av^{1/3} + \frac{B}{v} + Cv \qquad \textbf{(Equation 1.18)}$$

$$h = \frac{H}{d_p} \qquad \textbf{(Equation 1.19)}$$

$$h = \frac{H}{d_c} \qquad \textbf{(Equation 1.20)}$$

The final chromatographic parameter, which will be considered at this time, is the resolution R_s, which is used to quantify the degree of separation of adjacent analyte bans. Resolution can be defined equally well by either **Equation 1.21** or **1.22**. **Equation 1.22** is particularly noteworthy as it shows how modification of other chromatographic parameters affects the degree of resolution.

$$R_s = \frac{t_{R2} - t_{R1}}{\frac{1}{2}(w_2 + w_1)} \qquad \textbf{(Equation 1.21)}$$

$$R_s = \frac{N^{1/2}}{4} \times \frac{(\alpha - 1)}{\alpha} \times \left(\frac{k}{k+1}\right) \qquad \textbf{(Equation 1.22)}$$

1.3.2 Introduction to High Performance Liquid Chromatography

As with the general theory of chromatography, there are many excellent reference texts available on all facets of modern liquid chromatographic theory and practice. Amongst those used during the preparation of the following section are: Snyder *et al.*, 1979; Meyer, 1993; Braithwaite *et al.*, 1996 and Katz *et al.*, 1998.

In liquid chromatographic analysis, the resolution of a mixture of analytes is achieved as a result of their different affinities for a stationary phase, which is predominantly a solid surface, and a liquid mobile phase (Meyer, 1993). This so-called *liquid-solid chromatography* has in recent years made an analogous technique, *liquid-liquid chromatography*, a technique whereby the stationary phase is also a liquid, the two liquids being immiscible in order to create two discrete phases, almost obsolete. The adoption of the phrase *"High performance liquid chromatography" (HPLC)* comes from the fact that the stationary phase is made up of small particles, which give rise to efficient separations, a fact that was first predicted in the early 1940's by Martin and Synge (Meyer, 1993).

Over the last thirty years there has been a surge in the interest and application of HPLC to the separation and isolation of a host of organic and inorganic compounds. Liquid chromatography has a distinct advantage over gas chromatography (GC) as it is not limited in its application. Gas Chromatography can only be applied successfully to volatile analytes, or analytes that can be easily adapted through reaction to enhance their volatility, and as a result is only applicable to approximately 20% of the organic chemicals that have to date been characterised. In contrast liquid chromatography can be applied to many more compounds, as it requires only that the molecule is soluble in the chosen mobile phase (Meyer, 1993; Katz *et al.*, 1998). As the mobile phase parameters can be easily modified and adapted, they can be tailored to suit the needs of an individual analyte or series of analytes, enhancing solubility and making them fit for analysis by HPLC (Meyer, 1993; Katz *et al.*, 1998).

Modern liquid chromatographic can be performed in a number of different modes of operation, ranging from *"Size Exclusion Chromatography" (SEC)*, to the biologically relevant practice of *"Affinity Chromatography"* (Katz *et al.*, 1998). However only two modes of liquid chromatography will be considered at this time: *"normal phase liquid chromatography"* and *"reversed phase liquid chromatography"*.

In normal phase liquid chromatography separation is performed with the aid of a relatively non-polar mobile phase and a polar stationary phase, which is commonly bare silica (Katz *et al.*, 1998; Meyer, 1993). In this mode of operation polar analytes are

more strongly retained by the stationary phase than are non-polar analytes, which pass through the column relatively unchecked. As a result, analytes are seen to emerge from the column in order of increasing polarity. Retention in normal phase - liquid chromatography (NP-LC), is normally attributed to competitive adsorption of the analytes and solvent molecules onto the active sites on the stationary phase support (Katz *et al.*, 1998; Meyer, 1993; Dorsey *et al.*, 1994). In the case of silica, the most commonly used normal phase support material there are a number of different active sites on present on the silica substrate (**Figure 1.17**), with which the solute and solvent molecules can interact. It is generally assumed that solvent molecules form a mono-layer on the surface of the packing material (silica), and thus the solute molecules must displace one or more solvent molecules, dependant on the molecular size of the species, before they are able to interact with polar groups on stationary phase. Strong support for this model comes from the observation of a well-defined *eluotropic* series of normal phase solvents (Katz *et al.*, 1998; Meyer, 1993), whereby non-polar solvents like hexane and and carbon tetrachloride are found at the bottom of the series, and polar solvents like acetonitrile, isopropanol and methanol are found at the top of the series (Katz *et al.*, 1998). As the concentration of polar solvents within the mobile phase increases the competitive adsorption equibrium is driven towards the analyte remaining in the mobile phase as they are no longer able to dislodge sorbed solvent molecules.

In reversed phase liquid chromatography (RP-LC), analytes are eluted in the *"reverse"* order than would be expected under normal phase conditions, i.e. the most polar analytes elute first, whilst non-polar analytes are strongly retained. This reversal in elution order is due to the employment of a non-polar stationary phase, which is typically formed from bonding long hydrocarbon chains onto a silica support i.e. octadecylsilane and octylsilane (**Appendix One**), and a polar mobile phase, commonly consisting of water and a polar organic solvent. As a result, polar analytes tend to remain in the mobile phase, whilst non-polar analytes favour residence in the stationary phase (Katz *et al.*, 1998). Two principal models have been proposed to account for retention in reversed phase LC: *"the adsorption or solvophobic model"* and *"the partition model"*. Addressing and defining the mode of retention in RP-LC is one of the most contentious issues in modern chromatography. Whilst the partition model of analyte retention, whereby the solutes are fully encapsulated into the chains of the

bonded phase, is generally favoured, the solvophobic model still has strong support in some areas, most noticeably from Horvath.

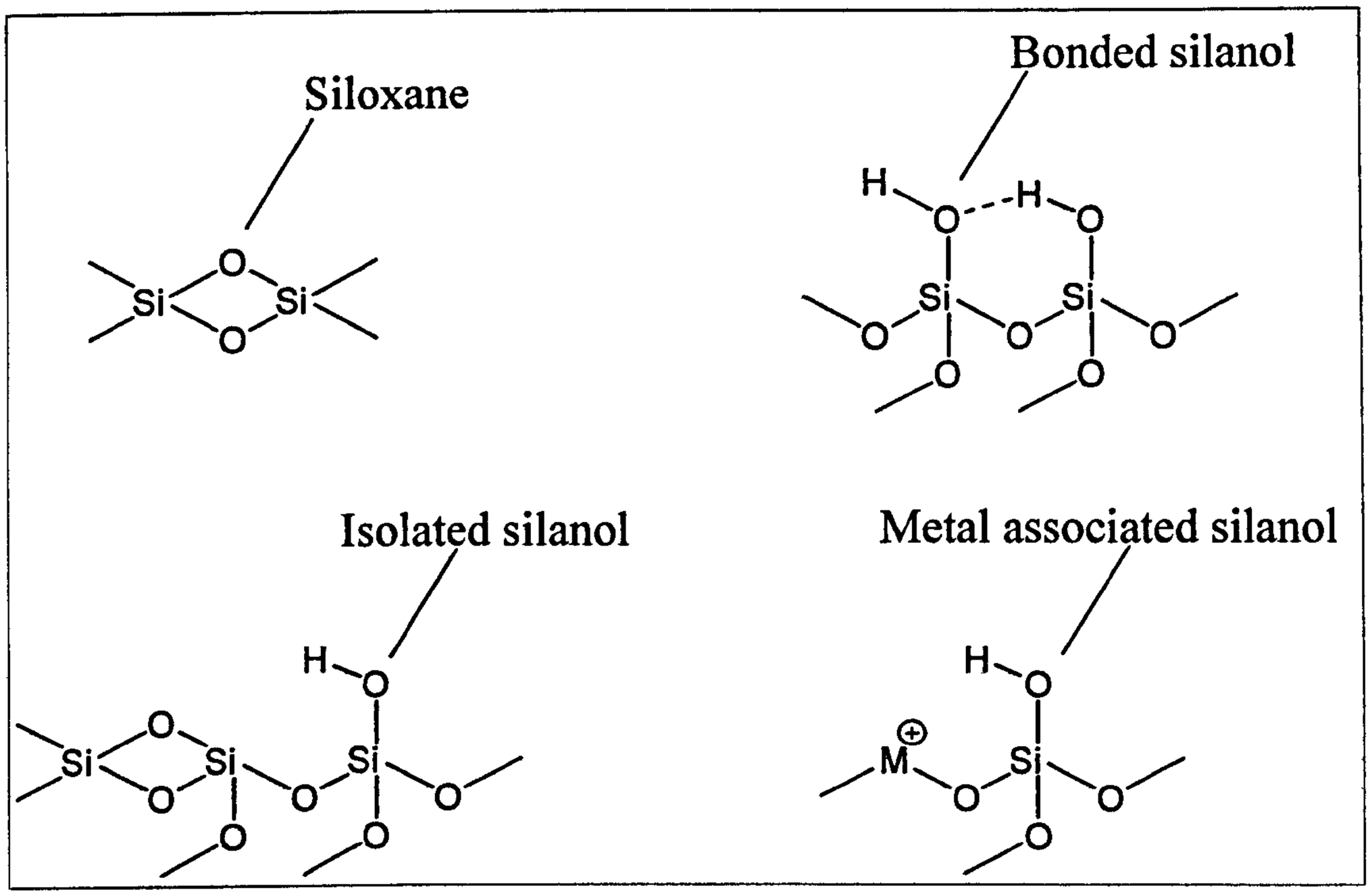

Figure 1.17: Representative structures of the *active* groups present on the surface of silica particles used as stationary phase supports in HPLC.
The acidity of the groups increases in moving from top left to bottom right. In normal phase LC, the acidic groups act as competitive adsorption targets for solutes and solvents, whilst in RP-LC interaction with isolated and metal-associated silanol groups is believed to lead to the tailing of basic analytes.

Reverse phase chromatography performed on a long alkyl-bonded stationary phase support is currently the most widely practised mode of liquid chromatographic analysis (Braithwaite *et al.*, 1996). Its suitability for the analysis of small polar organic analytes makes it readily applicable to the separation of therapeutic drug targets utilised by the pharmaceutical industry. Not surprisingly this sector of industry is currently the biggest user of liquid chromatographic products.

1.4 CRITICAL REVIEW OF CURRENT LITERATURE METHODS FOR THE ANALYSIS OF CATIONIC TENSIDES

Since the discovery at the turn of the century, of the beneficial properties of cationic tensides and their subsequent widespread exploitation (**Section 1.2**), a

significant amount of work has been invested into optimising methods for quantifying these materials in a range of matrices. As this work has encapsulated a number of decades, the types of methods used are numerous, and indeed, many have been superseded by more efficient modern instrumental techniques. However, a number of the older methods still remain, finding niches due to their ease of use and / or the limitations of modern techniques.

The following sections provide a detailed and critical review of the analytical techniques that are commonly applied to the determination of cationic tensides in a range of different matrices. In general, the historical perspective will be limited and attention will instead focus on current methods of analysis. Two recent reviews have included in-depth coverage of the historical methods used to determine cationic tensides (Boethling *et al.*, 1992; Schmitt, 2001), and both of these were utilised as source material during this review.

1.4.1 Non-specific methods

Many of the earliest methods for quantifying cationic tensides were based on non-specific measurement, typically involving the titration of the analytes after fractionation to determine the total cationic tenside active concentration (Schmitt, 2001). These methodologies often only provided crude estimates of the actual cationic tenside concentration due to cross reactivity with fatty amines and other matrix constituents. Of the earliest non-specific methods described for the analysis of cationic tensides, only the colorimetric methods remain of interest today. Of particular note is the so called *"disulphine blue active substances"* method that was optimised by Osburn in 1982, after originally being reported by Waters *et al.* in 1976. The methodology is based on the extraction into chloroform of the cationic tensides via complexation with an anionic dye-stuff. Association of the cationic tenside analyte to the anionic dye results in a change in the conjugation system, which allows the quantitative determination of the tensides by performing spectrometric analysis at a characteristic wavelength (Burford, 2000).

The disulphine blue active substances method is applicable to all cationic tensides, as it requires only the presence of one fatty alkyl group and a cationic head group, in order to form a strong ionic adduct with the . However, method selectivity and

sensitivity are dependant on the nature of the cationic tenside under investigation (Burford, 2000). Over years of use the method has been adapted on a number of occasions to improve sample through put and improve analyte specificity. Indeed recently, Unilever Research have managed to miniaturise the method described by Osburn (1982), allowing sample throughput to be greatly increased, and solvent waste to be minimised (Weston, 2000). Unfortunately due to the nature of the method, it is not possible to obtain structural information on the nature of the tensides that are present. In addition, the method also suffers from problems with cross-reactivity occurring in the presence of fatty amines and amphoteric tensides, and it cannot be applied to the analysis of cationic metabolites due to problems with back extracting into chloroform.

1.4.2 Thin layer chromatography (TLC)

Thin layer chromatography is commonly utilised in industry to determine cationic tenside levels in raw material supplies and fully formulated products (Lawrence, 1997). The methodology is applicable to the determination of cationic fabric conditioner actives and preservative actives (Paesen *et al.*, 1994; Lawrence, 1997). However, the developing conditions are very different. Whilst the separation of the preservative actives is performed with the aid of a very polar solvent system, with the hydrophilic quats migrating to the greater extent, separation of the cationic fabric conditioner actives is achieved with a non-polar elution solvent. In this scenario the hydrophobic quat species migrate furthest down the plate (Lawrence, 2000).

Whilst both methods have been used to quantify cationic tensides in industrial matrices TLC is known to be unsuitable for the analysis of environmental matrices due to the fouling of the separation plates by analyte interferences (Lawrence, 1997).

1.4.3 Gas Chromatography (GC)

Cationic tensides are non-volatile species, and thus are not directly amenable to GC analysis. As a result, there are few reports on the direct analysis of these compounds by GC. Instead, the cationic tensides are converted to their corresponding tertiary amines to improve their volatility. This process is usually achieved by the decomposition of the quaternary ammonium hydroxide species via Hoffmann Elimination. The quat sample is reacted with an excess of alkali to form the corresponding hydroxide species, and only once the reaction is completed, are the

tertiary amines extracted from the reaction mixture with a suitable organic solvent, and analysed. Takano *et al.* (1977a) first reported that alkylbenzyl quats could be analysed in this manner when they combined a quat sample with sodium methoxide and *N, N*-dimethylformamide. After refluxing for one hour at 180°C, the tertiary amines were extracted and then analysed by packed-column GC (Takano *et al.*, 1977a). In the same year, this research group performed Hoffmann degradation in a GC injector port, by combining the quat sample with methanolic potassium hydroxide, and injecting the reaction mixture into a GC injector port, maintained at 290°C (Takano *et al.*, 1977b). Another alkali that has been used in a Hoffmann elimination reaction is potassium *tert*-butoxide, to analyse the nature and concentration of benzalkonium chlorides in domestic sanitary wipes (Suzuki *et al.*, 1989). More recently, Hoffmann degradation with potassium *tert*-butoxide has been used in association with GC/MS to identify alkylbenzyl quat residues in Taiwanese sewage effluents and river water (Ding *et al.*, 2001). These researchers implemented a combination of solid-phase extraction and Hoffmann elimination methodologies to quantify individual alkylbenzyl quat homologues at 0.4 µg/l levels in natural river-water. Comparison of the method with a recently published HPLC-UV method (Prince *et al.*, 1999) showed that the GC/MS method offered a ten-fold increase in sensitivity and equivalent reproducibility. However, analysis time increased whilst sample preparation time doubled as a result of the Hoffmann procedure.

Although Hoffmann Elimination is the principal method for increasing the volatility of the cationic tensides, an early method described by Warrington, utilised hydrogenation to form the tertiary amines (Warrington, 1961). Abidi (1980) also used this method, but then went on to derivatise the tertiary amines to the corresponding cyanamide or trichloroethyl carbamate moieties in order to facilitate analysis at low column temperatures (Abidi, 1980).

The need for derivatisation of long chain quaternary amines, can by circumvented by the use of injector port pyrolysis GC and GC/MS (Ng *et al.*, 1986). Ng *et al.* (1986) employed this method to analyse cationic tensides samples by direct injection into a hot injector port (>250°C), with the separation of the resulting alkyldimethyl amines, alkylbenzylmethyl amines, and benzyl chloride impurities being performed on a DB-5 capillary column. The response of the alkyldimethyl amines was

then used to quantify the distribution and concentration of the alkylbenzyl quats in the original samples.

The main drawback with the application of GC analysis to the quantitation of cationic tensides is the difficulty experienced in quantifying the di- and tri-chained quat species (Lawrence, 1997). Few reports are available on the analysis of cationic tensides with more than one alkyl chain unit, and many of those that have, have shown that quantifying the origins of the amines formed after degradation is far from routine (Metcalfe, 1984). Indeed, quantitation of the mono-chained quats is also hampered in the presence of other cationic tenside analytes and / or fatty amine species, which can result in spiked recoveries in excess of 100 % (Metcalfe, 1984).

1.4.4 High Performance Liquid Chromatography (HPLC)

Today, HPLC is the preferred methodology for the analysis of quats, with separations being reported as early as 1974 (Nakae *et al.*, 1974), for the analysis of alkylpyridinium halides (Nakae *et al.*, 1974, 1977). In 1980, Meyer adapted this methodology by using a 300 x 4 mm i.d. μ-Bondapak CN column with a 60 : 40 acetonitrile : 0.1 M sodium acetate mobile phase, adjusted to pH 5.0 with acetic acid, to analyse benzalkonium chlorides (Meyer, 1980).

Nakamura *et al.* (1981) published the first of a series of papers on the separation of alkylbenzyldimethylammonium chlorides using a 250 x 4 mm i.d. column packed with 5μm TSK gel / ODS silica. ODS packed columns have subsequently been utilised by other research groups to enable the separation of quaternary ammonium species. However, they were found to be ill suited to the analysis of the cationic tensides, an observation that was attributed to the strong electrostatic interactions between the silica surface and the analytes of interest (Santoni *et al.*, 1993).

In 1988 de Schutter *et al.* used a novel mobile phase with two modifiers to separate a series of quaternary ammonium drugs. The addition of an amine and a sulphonate to an aqueous mobile phase system resulted in improved separation over that obtained with only the amine present. However, peak tailing was dramatically reduced, when only the sulphonate was present.

The use of indirect UV absorbance was reported by Helboe (1983) who evaluated the resolution offered by a series of Nucleosil column, including silica 5, C_{18}, C_8, and CN, and Nucleosil 7 phenyl. The CN column was reported to offer the most efficient separation. In the same year, Larson *et al.* (1983) used a Partisil SCX column to separate a group of alkyltrimethyl quats. Huang used a Zorbax C_8 column with a methanol : water mobile phase containing 5 mM *p*-toluenesulphonic acid and indirect UV detection to identify dihydrogenatedtallowdimethylammonium chloride (DHTDMAC). However, problems of co-eluting impurities were noted. Metcalfe reported routine analysis of the DHTDMAC compound Arquad HT on a μ-Bondapak C_{18} column with a 95 : 5 : 0.3 methanol : water : acetic acid mobile phase although no evidence of the attained separation was forthcoming.

Simon *et al.* (1987) identified the aliphatic and polyamine intermediates of quat manufacture using Nucleosil CN and C_{18} columns with an acetonitrile : water gradient programme, whilst Dowle *et al.* (1989) utilised a column packed with a similar polystyrene-divinylbenzene material to that used by Nakae *et al.* to separate amines and quaternary ammonium species. A 60 : 38 : 2 acetonitrile : water : acetic acid mobile phase being used for all separations.

In 1987 de Ruiter *et al.* published the first paper on the use of post-column ion-pair extraction in the detection of quat species. After separation of the mono-, di- and trihydrogenatedtallowmethylammonium chlorides utilising a mixed mode cyano-amino Partisil PAC 10 μm column, (a gradient chloroform : methanol : acetonitrile mobile phase system being employed) the analytes were mixed with methyl orange or 9, 10 dimethoxyanthracene-2-sulphonate, extracted into chloroform and passed through a sandwich phase separator into the detection cell of a UV or fluorescence detector. Detection limits of 10 ng/l for fluorescence detection were quoted. Subsequent papers have reported improvements in the efficiency of the system by the utilisation of a Partisil PAC 5 column with a chloroform : methanol : acetic acid mobile phase employed under gradient conditions (Gort *et al.*, 1993; Fernandez *et al.*, 1996). Alterations and improvements to the extraction system have also been reported.

Engelhardt *et al.* (1995) published a derivatisation method suitable for the analysis of quaternary aminoalcohols, the degradation products of the esterquats. The

addition of 9-fluoroenylmethylformamate resulted in the formation of a series of complexes, which were identified by fluorescence. Separations were performed on a Nucleosil SA 5 column with a acetonitrile : water : 0.1 M sodium acetate gradient system. The derivatisation step required 30 minutes to reach equilibrium, and in the analysis of unknown samples, a number of reactions needed to be performed, in order to obtain a constant ratio of complex formation.

Conductivity detection was first reported by Wee *et al.* (1982) who described the separation of four quat species with a Partisil PAC 10 column and a 92 : 8 chloroform : methanol mobile phase. Subsequent work by Gerike *et al.* (1994) and Breen *et al.* (1996) have improved peak resolution by using a PAC 5 column and altering the mobile phase to a 89 : 10 : 1 chloroform : methanol : acetic acid system. It is interesting to note that although the mono-, di- and tri- homlogous series were eluted as single peaks, the separation of the three classes is not as efficient as that obtained by de Ruiter *et al.* (1987).

Nitschke *et al.* (1992) used a similar system to identify a range of cationic surfactants including esterquats. Detection limits were reported to be in the low µg / l range.

Alternative detection-systems have been used previously by Spagnolo *et al.* (1987). These researchers employed a refractive index detector and a SCX column to separate a mix of aromatic and nonaromatic quats. Although separations in a homologous series are reported, they were only possible with the trimethylquats, as the other series were eluted as single peaks. Caeser *et al.* (1989) utilised a similar detection system to determine dialkyl quats in commercial personal care formulations. Two µ-Bondapak CN columns connected in series and a 55 : 45 : 0.1 water : acetonitrile : trifluoroacetic acid mobile phase were employed.

A series of papers published by Wilkes *et al.* (1992, 1994, 1996) reported the use of an evaporative light scattering (ELS) detector to analyse groups the mono-, di- and tristearyl- ammonium bromides separated on a 250 x 4.6 mm i.d RSil polyol column. Wilkes *et al.* highlighted that the retention times obtained with the system used by Gerike *et al.* and Breen *et al.* were very much dependent on the methanol

composition of the mobile phase and the analyte concentration. Using a gradient elution programme based on 5 mM trifluoroacetic acid in hexane and 5 mM trifluoroacetic acid in THF : methanol 3 : 1, Wilkes *et al.* were able to obtain stable retention times over a number of injections at various concentrations. The polyphenol column showed good separation of the mono-, di- and tri- constituents as well as being able to partially separate constituents of a homologous series.

Mass spectrometry (MS) is well suited to the identification of minor components present within a complex matrix. Cotter *et al.* (1982) published one of the first papers on the use of mass spectrometry in the analysis of cationic surfactants. Using direct-exposure chemical ionization MS they were able to identify the presence of a series of tenside molecules in a commercial sample. Lyon *et al.* (1984) extended this work with the analysis of a commercial dihydrogenated-dimethylammonium chloride. Fast atom bombardment (FAB) mass spectrometry gave rise to abundant ions at m/z 550, 522, 494, 466 and 438. Collision-activated desorption spectra revealed these ions to correspond to molecules with alkyl chain lengths ranging from C_{14} to C_{18}.

In 1988 Simms *et al.* (1988) published one of the first papers on the use of mass spectrometry for the quantitation of cationic surfactants in environmental matrices. Using isotopically labelled internal and external standards, FAB-MS and a signal averaging technique, the group were able to analyse cationic surfactants at concentrations approaching 100 ng/l. A subsequent paper by Simms *et al.* (1992) combined the use of FAB-MS with liquid scintillation counting, in order to characterise the biodegradation pathway of a newly synthesised ester quat. Quantitation of the ester quat in a sludge matrix and the rate of formation of a biodegradation intermediate were reported. Furthermore, in 1992, Lawrence (1992) used a continuous-flow fast atom bombardment interface to introduce analytes, which had previously been separated on standard 4.6 mm i.d. columns. A non-aqueous mobile phase system was utilised to quantify the concentration of ditallow-dimethylammonium chloride present in a commercial fabric conditioner formulation.

The use of ion-spray ionisation has also been reported in the analysis of cationic surfactants. In 1996 Ogura *et al.* (1996) identified the presence of a protonated dialkyl tertiary amine in a commercial fabric conditioner, using flow injection analysis ion-

spray MS. Kawakami *et al.* (1998) utilised a similar system for the trace analysis of benzalkonium chlorides on skin. A detection limit of 1.2 mg/l was reported. More recently, Di Corcia (1998) published a review on the use of ion-spray and electrospray MS in the analysis of surfactants. Although the paper is primarily concerned with the analysis of non-ionic surfactants, it highlights the suitability of these techniques in trace analysis studies.

Presently, Unilever Research, as with most major formulators of commercial products, which utilise alkylbenzyl quats, have a standard method for the determination of these species in their purified forms and also in fully formulated products. The separation is based on a reverse phase LC separation performed on a long cyanopropyl bonded stationary phase held at 35°C, from which the analytes are eluted, with a mobile phase containing a 0.2 mol/l sodium perchlorate. Unfortunately, this combination of column length, and high sodium perchlorate concentration appears to lead to long analysis times, and, as a result, the mobile phase is pumped at a high linear velocity, preventing the column from achieving optimum performance.

Whilst it is unclear as to the exact influence of sodium perchlorate on the behaviour of the analytes (this effect will be addressed in **Chapter 5**), what is clear, is that such a high concentration of a non-volatile salt, hinders or even prevents the hyphenation of the method with electrospray mass spectrometry. This presents a major problem for formulators when trying to draw up a risk assessment for the application of alkylbenzyl quats in a new market, as their detection in complex matrices is compounded by the fact that these species contain no fluorophore and show a maximum absorbance at 190-215 nm in the UV / vis range, the same range as many naturally and man-made organics ubiquitous in the environment. Therefore, in order to quantify these species in complex environmental matrices extensive sample preparation routines are required.

1.4.5 Capillary Electrophoresis (CE)

Capillary electrophoresis (CE) would appear well suited to the analysis of cationic tensides, as the separation is dependant on the variation in the behaviour of ions under the influence of an electric field (Issaq, 2000). As the analytes possess a permanent positive charge the variation in the charge to size ratios of the individual

homologues should allow for efficient resolution of the major species. The alkylbenzylquats are especially suited to analysis by capillary zone electrophoresis as they posses an exploitable chromophore, which facilitates detection of these analytes by UV analysis. As a result of this inherent chromophore there have been a number of reports appearing on the analysis of this particular class of cationic tenside. Two recent reviews (Vogt *et al.*, 1998; Heinig *et al.*, 1999) have demonstrated that since CE was first evaluated for the analysis of alkylbenzyl quats some nine years ago (Weiss *et al.*, 1992), the number of publications in this field has grown steadily to the present date.

Early methods for the analysis of alkylbenzyl quats utilised aqueous buffers and low organic solvent concentrations (Vogt *et al.*, 1998). However, these methods were soon replaced after the cationic surfactants were observed to favour formation of micelles and / or aggregates at the walls of the fused silica capillaries (Vogt *et al.*, 1998). Today, most CE methods used to analyse alkylbenzyl quats rely on the presence of high concentrations of organic solvent in the running buffer, or else the separation is performed in a completely non-aqueous environment (Heinig *et al.*, 1999). Both of these methods of analysis have been successfully applied to the analysis of alkylbenzyl quats in commercial products (Heinig *et al.*, 1999). In a recent report, the presence of α- or β-cyclodextrins, and a high concentration of organic solvent in a phosphate buffer system has been shown to afford excellent resolution of alkylbenzyl quat homologues (So *et al.*, 1999).

Unfortunately, the widespread applicability of CE to the analysis of cationic tensides appears limited at this moment in time for a number of reasons. Firstly there are limited detection systems available for use with commercial CE equipment. Whilst UV/ vis and fluorescence techniques are commonly applied to the analysis of other compounds (Issaq, 2000), these methods are either completely unsuitable or can only be applied to a small number of tenside classes. Whilst Heinig *et al.* (1997) have reported the determination of four monoalkyltrimethyl quats by indirect UV / vis analysis, the repeatability of the method was very low in comparison with a conventional ion chromatography method. Similar observations have also been made during the analysis

1.4.6 Indirect competitive enzyme-linked immunosorbant assays

Mention has already been made to the fact that the quantitation of cationic tensides in complex matrices is far from routine (**Section 1.3.2.1**). Labour intensive extraction and clean-up protocols, and / or derivatisation procedures are required in order to pre-concentrate and fractionate the samples in order to facilitate accurate determination of the analytes. Whilst such clean-up procedures are critical to attaining valid data, they also result in a significant increase in analysis time, reducing sample throughput. Another major limitation of all previously described methods is that they cannot be readily performed in the field, thus requiring that all samples be shipped back to the laboratory for analysis. For an on-going monitoring study, the necessity to ship samples back to the lab, and the difficulty in coping with large numbers of samples for a prolonged period, can lead to problems in obtaining a representative sample set. In addition, issues that could have been remedied in the field, had results been to hand, could be compounded by the delay in receiving study data.

The limitations described above are characteristic of those experienced by the lumber trade, where freshly felled timber is treated with fungicidal preparations containing cationic tensides, to prevent the growth of mould, and limit sapstain (Chen, 1995). Due to concerns over worker health and environmental impact, and to ensure optimal fungicidal treatment, rapid and quantitative methods of analysis are required for the determination of cationic tensides on lumber (Bull, 1998b). The scale of this task is put into perspective when one considers that in 1995, cationic tensides were employed in 95% of all sapstain products utilised by the massive Canadian lumber industry. In this situation where the sample set equates to hundreds per day, and where instantaneous results are required to ensure the integrity of the product, traditional methods of cationic analysis are found wanting. As a result, indirect competitive enzyme linked immunosorbant assays (CELISA's) have recently been developed for the determination of didecyldimethylammonium chloride (DDAC) (Chen *et al.*, 1995; Bull *et al.*, 1998b; Chen *et al.*, 1998) and benzyldimethyldodecylammonium chloride (Bull *et al.*, 1998a) residues. Such assays have been developed into field-portable kits for on-site analysis, and have satisfied the aforementioned criteria of high sample throughput rates, cost effectiveness, sensitivity, repeatability and ease of use (van Emon *et al.*, 1992).

Each of the assays that have so far been reported, involve the development of polyclonal antibodies, which is a multi-stage process. The analyte of interest is transformed into a *hapten* in order to produce highly selective and specific antibodies (Chen *et al.,* 1995). The structure of the hapten should retain the conformation of the analyte, especially those groups which impart the desired anti-fungal activity. In the case of DDAC, the two, fatty chains and the quaternised nitrogen cation are required (Chen *et al.,* 1995), whilst the use of a *"linker arm"* maximises antibody production and increases assay sensitivity (Chen *et al.,* 1995, Bull *et al.,* 1998a, 1998b). In order for the hapten to be recognised as an immunogen within a mammalian system, and induce an antibody response, it must be linked to a large immunogenic carrier protein. As the nature of quaternary ammonium salts prevents direct conjugation, a carboxylic acid group is often introduced onto the linker arm (Chen *et al.,* 1995, Bull *et al.,* 1998a, 1998b). Once a suitable immunogen has been developed, it is introduced into a mammalian species i.e. a rabbit (Chen *et al.,* 1995, Bull *et al.,* 1998a, 1998b), which then raises antibodies against the immunogen. By utilising the ELISA protocol, it is possible to gauge the titre of antibody in the rabbit's blood, and when sufficient levels are present, the antibodies are extracted and stored.

The sensitivity, specificity, and cross-reactivity of the antibodies produced by the rabbit are then characterised by performing an indirect competitive ELISA (CELISA) (Chen *et al.,* 1995, Bull *et al.,* 1998), in the same way as is used during analyte determination. The hapten-protein conjugate (antigen) is coated onto microtitration plates to form the sorbent base material. A mixture of antiserum and a known concentration of analyte is then added to each plate, and the competitive adsorption of the analyte and the antigen allowed to reach equilibrium. After washing the plate, a conjugated secondary antibody is added, which selectively binds to the hapten-antibody that is attached to the antigen, sorbed onto the plate. Addition of an enzymatic initiator causes rearrangement of the secondary antibody to facilitate the formation of a chromophoric group that can be observed at 490 nm. Utilising a range of analyte concentrations an inhibition curve can be set up, whereby increasing analyte concentration leads to decreased absorption by the secondary antibody. The resulting profile can then be used to quantify an unknown amount of analyte in a sample of interest. In terms of method sensitivity, the most selective hapten reported by Bull *et al.*

(1998b) was seen to be capable of quantifying DDAC at concentrations down to 25 parts per billion (ppb) in spiked samples.

Unfortunately, the CELISA methods do have limitations. A major disadvantage is that the organic solvent employed during extraction has been shown to affect the immunoreactivity of antibodies in immunoassays, with Bull *et al.* (1998a) reporting that antibodies raised against benzyldimethyldodecylammonium chloride lost > 10% reactivity in the presence of a number of organic solvents. Although not ideal, incorporation of the solvent into the samples used to generate the standard curve, can alleviate this problem (Kramer *et al.*, 1994).

Another downfall of the method is that the antibodies produced for a specific immunogen can recognise several epitopes of the antigen, with varying affinities. Thus, *"cross reactivity"* can occur whereby the polyclonal antibodies also recognise structurally similar compounds to the analytes of interest. In the case of cationic tensides, fatty amines and other quat homologues can result in cross reactivity. As mentioned previously, the sensitivity and specificity of an antibody is very much dependent on the structure of the hapten. Bull *et al.* (1998b) developed three haptens for DDAC with differing structures. It was observed that the hapten, which most resembled the structure of DDCA, showed a 600-fold increase in sensitivity and selectivity compared to that developed by Chen *et al.* (1995).

A final concern in the use of CELISA's for the analysis of cationic tensides comes from the observation that the analytes can interact non-specifically with proteins, and thus, may actually denature the antibody. However, Bull *et al.* (1998b) showed that the presence of three other industrially important tensides had no noticeable effect on the sensitivity of the method, and in addition, none of the three tensides were seen to cause significant cross-reactivity.

Though the use of CELISAs for the analysis of cationic tensides is still in its infancy, the technique may well prove to be a rapid and effective screening tool for the quantitation of cationic tensides in environmental matrices in the future. Certainly, the sensitivity witnessed by Bull *et al.* (1998b) suggests that the method has promise. However, ultimately, the rate at which ELISA's are incorporated into the suite of

analytical methods used to quantify cationic tensides will be dependant on reducing cross reactivity and limiting the sensitivity of commercial kits to matrix interferences.

1.5 CURRENT FAILINGS AND FUTURE NEEDS IN THE ANALYSIS OF CATIONIC TENSIDES

It was evident at the outset of this work that the suite of methods used to quantify cationic tensides in industrial and environmental samples were not sufficient to facilitate quantative and selective determination of all tenside components, of all tenside samples, in all matrices. Thin layer chromatographic methods were seen to be well suited to the analysis of cationic tensides in raw materials and formulated industrial products, yet the method could not be efficiently applied to environmental analysis due to plate fouling by matrix constituents. The application of gas chromatographic methods has been successfully applied to the environmental analysis of mono-chained quats. However, derivatisation or degradation was first required, and the problems experienced during the quantitation of the di- and tri-chained quats limited the widespread applicability of the method.

The non-selective disulphine blue active substances method and other colourimetric methods were seen to provide data on the total concentration of active substances in a sample. As structural information on the nature of the compounds present is unobtainable with the method, it cannot be used for studying the fate of cationic tensides in the environment. In addition, the cross reactivity of the method with fatty protonated amines and amphoteric tensides results in the calculated concentration of active substances being above that which is actually present. Similarly, development and application of competitive enzyme linked immunosorbant assays (CELISA's) to the quantitation of cationic tensides is still in its infancy due to problems with cross-reactivity and solvent effects.

Of the methods currently used to quantify cationic tensides high performance liquid chromatography is both the most widespread and the most applicable. Yet the range of methods that are available tend to be designed for specific classes of tenside, and even in light of this many still suffer from inherent problems. The reverse phase liquid chromatographic (RP-LC) methods that are generally applied to the analysis of

the alkylbenzyl and alkylpyridinium quats (Schmitt, 2001) are unsuitable for hyphenation with mass spectrometry, which limits the availability of structural information on these analytes. At the same time, the methods have been found to be unsuitable for the quantitation of the fabric conditioner actives, which instead rely on the use of normal phase LC methods for their quantitation, especially in environmental matrices (Schmitt, 2001). Yet these methods demonstrate poor repeatability, are unable to resolve the homologous series in each tenside class and are difficult to apply to the determination of the hydrophilic biodegradation products of the parent quats **(Figure 1.10)**.

The aim of this work was to develop new liquid chromatographic methods suitable for the determination and quantitation of cationic tensides in environmental matrices and industrial products. The initial aims at the commencement of the work were to optimise the resolution of the fabric conditioner actives and develop LC methodology capable of quantifying the parent analytes and their biodegradation products simultaneously. At the same time, hyphenation with electrospray ionisation mass spectrometry was also envisaged, in order that a method for studying the fate of the parent cationic fabric conditioner actives in environmental matrices could also be developed. The second aim of the work was to optimise the RP-LC methods of analysis used to quantify the alkylbenzyl quat preservative actives, and hyphenate the methodology with electrospray mass spectrometry in order to study the transport of these analytes through, and their removal from, the environment. Application of the suitability of the methodology to the other cationic preservative active would be performed subsequent to method validation.

It was predicted that the development and subsequent optimisation of liquid chromatographic methods for the quantitation of the two main types of cationic tenside would greatly enhance the suite of analytical methods available for these components. However, it was felt that the long-term benefits of two new methods for the specific determination of discrete analytes would be limited. Chromatographers would still be left with the question *"Which method do I use for the determination of a sample in matrix Y?"*

The plight of traditional analytical departments within the applied chemicals industry is becoming increasingly bleak. Analytical facilities of any kind are seen in many circles as an expensive luxury that can be circumvented by buying-in expertise, which is leading to a decrease in the funding available for many of these facilities. At the same time, management is becoming increasingly wooed by the promises and representations of contract research organisations (CRO's), who are able to use their inherent *"economies of scale"* to match or even undercut the costs associated with in-house support. In a buyers market, external competitors draw work from a broader client base, and can therefore afford the extra investment required for new automated equipment and laboratory space, which are required to meet customer demand. As a result internal facilities have been squeezed by both internal and external pressures, and therefore must find and provide, the *"added-value"* that only long-term experience of a raw material and / or fully formulated product can bring. However, expertise on the nature of the material, and experience in dealing with the matrix or matrices in which they are to be found is insufficient to guarantee survival, as a potential customer can achieve this with bought-in expertise. An internal facility must be able to deliver valid data in a shorter time frame than can be achieved by external competitors. From a chromatography perspective, this requires the development and implementation of generic methods of extraction and detection for similar compounds in order to speed method development, which is one of the bottlenecks in the data generation process. Whilst a generic methodology may not provide the *perfect* separation for all intended compounds, it will generate valid quantitative data without the need for method development. As a result, the development of two new LC methods for the analysis of specific classes of cationic tenside was deemed to represent an unsuitable conclusion to the work, in view of the current climate in industrial analytical facilities. Instead, a principal aim of the work was to further knowledge on the chromatographic behaviour of cationic tensides in general, in order that the road to a generic liquid chromatography method for the analysis of these materials may become visible on the horizon.

Generic analysis is not a new concept in chromatography. In GC analysis it is reported that approximately 80% of all separations can be performed with the aid of a 25m column of 0.25mm internal diameter. A stationary phase of 95% dimethyl polysiloxane and 5% diphenyl polysiloxane, together with a film thickness of 0.25 μm remain constant (M^{c}Nair, 2001), whereas oven parameters may be varied and tuned

according to the analyte of interest. In HPLC, truly generic methods of analysis are more difficult to come by. Whilst the use of an octylsilane or an octadecylsilane bonded stationary phase, operated under reverse phase conditions, accounts for the majority of separations currently performed, the disparate nature of stationary phase supports, and the variation in mobile phase parameters, makes the practice common but by no means generic. For a truly generic LC method, only the temperature at which the separation is performed and /or the solvent composition should be changed. Addition of modifiers and / or replacement of solvents are characteristics of a different method. Recently, reports have appeared which have begun to address the requirements for the development of generic methods of analysis in LC (Needham *et al.,* 1999 and 2000).

The two reports by Needham *et al.* (1999 and 2000) were based on the use of a generic set of mobile and stationary phase parameters for the analysis of basic drugs. Whilst it was observed that greater efficiency was possible on cyanopropyl, phenyl-based, and pentafluorophenyl-based stationary phases, compared to that achieved on traditional octadecylsilane bonded supports, the widespread applicability of the methodology was most impressive. By carefully selecting mobile phase parameters, the RP-LC methodology was seen to be applicable to a range of common basic drugs (Needham, 2000), offering equivalent efficiency in all cases. It was subsequently observed that the resolution and efficiency of the drug analytes was maintained during hyphenated LC/ESI-MS, due to the use of high organic solvent concentrations. As a result, it was predicted that the new methodology should allow the rapid separation and structural determination of novel basic drugs.

It was envisaged that by adopting many of the principles used by Needham *et al.* (2000), it could be possible to begin to develop generic methods of analysis for cationic tensides. The ultimate aim of the development would be to begin to progress down the path towards a single generic LC method for the quantitation of all cationic tenside components in all matrices. By utilising electrospray mass spectrometry, the method was envisaged to offer significant benefits over conventional LC methods for quantifying cationic tensides in environmental matrices. At the same, utilisation of gradient elution profiles were believed to offer promise for the determination of the hydrophilic cationic metabolites formed as a result of biodegradation. It was predicted that such a powerful method would also be applicable to the structural elucidation of

unknown metabolites and as yet unsynthesised parent cationic tensides. Initial steps on the road to such a method, the removal of the dilemma over which analytical methodology to choose, and an easing of the burden of cationic tenside method development would be the ultimate goals of this work.

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CHAPTER TWO

Experimental

CHAPTER TWO

Experimental

2.1 GENERAL

This chapter details the reagents, samples, equipment and techniques relevant to the practical work described in subsequent results chapters. General descriptions of the main instrumental and resource parameters utilised at the University of Leeds and Unilever Research Port Sunlight are detailed. Minor changes made during method optimisation are omitted at this time. For a more informative account regarding the optimisation of individual parameters, the reader is referred to the appropriate text and figure legends in the relevant chapters.

2.2 CHEMICALS, SOLVENTS AND GASES

2.2.1 University of Leeds

Glacial acetic acid (AcH) (AnalaR grade) and dimethyl sulphoxide (DMSO) (AnalaR grade) were purchased from BDH (Lutterworth, UK). Hydrochloric acid and ammonia solution (Specified reagents) were purchased from Fisher Scientific (Loughborough, UK). Spectrophotometric grade trifluoroacetic acid (TFA) (purity 99+%), formic acid (purity 88%, American Chemical Society (ACS) reagent), triethylamine (TEA) (purity 99% purity), ammonium hydroxide (NH_4OH) (24% ammonia in water), ammonium acetate (NH_4Ac) (purity > 98%), ammonium chloride (NH_4Cl) (ACS reagent), tetramethylammonium hydroxide (TMAOH) (25 % w/w solution in methanol), tetraethylammonium hydroxide (TEAOH) (35 % w/w in water) and tetrabutylammonium hydroxide (TBAOH) (40 weight % solution in water) were all purchased from Sigma-Aldrich Chemical Co. Ltd. (Dorset, UK). N-alkyl-N-benzyl-N,N-dimethylammonium chloride (purity > 90%) was purchased from Aldrich Chemical Co. Ltd. (Dorset, UK).

All solvents were of HPLC grade or higher. Chloroform ($CHCl_3$), methanol (MeOH), tetrahydrofuran (THF), propan-2-ol (IPA), dichloromethane (DCM), ethyl acetate, diethyl ether and acetonitrile (ACN) were purchased from Riedel-de-Haën

(Seelze, Germany). Riedel-de-Haën was also one of the suppliers of n-hexane, the others being Fisher Scientific Ltd. (Loughborough, UK), and Philip Harris Scientific (Lichfield, UK). All water used during the course of the reverse phase liquid chromatography work was double distilled.

Oxygen-free-nitrogen (OFN) (purity >90%) was purchased from BOC gases (Manchester, UK) and helium (purity >99%) was obtained from either BOC gases or Air Products Ltd. (Manchester, UK).

2.2.2 Unilever Research Port Sunlight

High purity reagent grade TFA and AcH, Fisher Specified reagent grade NH_4Ac and analytical reagent grade propionic acid were purchased from Fisher Scientific. HPLC reagent grade NH_4Ac was sourced from JT Baker (Deventer, Netherlands).

All solvents were of HPLC grade or higher. MeOH, hexane (hexane fraction from petroleum), THF, IPA, $CHCl_3$ and far UV grade ACN were purchased from Fisher Scientific. All water was obtained from a Milli Q plus system (Millipore Ltd.; Massachusetts, USA).

The nitrogen gas used for nebulisation within the Sedex 55 evaporative light scattering detector (Sedere; Alfortville, France) (See **Section 2.4.1.2**) was of >99% purity and was generated by a Domnick Hunter NG35 nitrogen generator (Domnick Hunter; Gateshead, UK). The nitrogen used as the sheath and auxiliary gas in the Finnigan MAT LCQ benchtop ion-trap mass spectrometer (Finnigan MAT; Hemel Hempstead, UK) (See **Section 2.4.2**) was provided by a Domnick Hunter Nitrox LCMSUHPN1101 nitrogen generator (Purity > 99.5%). Helium gas (purity > 99.995%) was obtained from Air Products.

2.3 SAMPLES

It was highlighted in **Chapter One** that many tensides are referred to as *"oleochemicals"* as they are downstream derivatives of natural fats and oils. It was noted that this origin results in commercial samples containing a number of active components, the number present being dependant on the distribution and range of fatty

acids present in the raw material. All of the samples studied during the course of this work were oleochemicals, and thus some of the *"pure"* samples contained in excess of ten active components. Characterisation data on chain length distribution, nature of the fat / oil used during manufacture and the main industrial / research use of each of the samples is provided in **Appendix One**.

2.4 INSTRUMENTAL PARAMETERS

It was observed early on in the experimental work that it would be inefficient to utilise a single instrumental set-up, or a generic set of instrumental parameters throughout the course of the study. As a result, the following sections describe the general resource parameters applicable to each different section of work.

2.4.1 Normal phase liquid chromatography

2.4.1.1 University of Leeds

The instrumental set-up employed for normal phase HPLC work at the University of Leeds comprised a Perkin Elmer (PE) Series Four quaternary HPLC pumping system (ex-Unilever Research), and a Rheodyne Model 7125 stainless steel injection valve (Phase Separations Ltd.; Clywd, UK) fitted with a 20 µl external injection loop unless otherwise stated. Two different detectors were utilised during this work: A Perkin Elmer LC-25 detector (no suppressor column) was used for conductivity experiments, and a Varex Mk II evaporative light scattering detector (Alltech Associates; Carnforth, UK) was used for light scattering analysis. Initially, helium (purity >99%) was chosen as the nebulising gas. However, this was replaced by OFN due to the cost implications of running the system for long periods of time. The nebulisation pressure was regulated at 55 pounds per square inch (psi.) (379.5 kPa), and the evaporation temperature at 95°C. The output from the detectors was linked to a PC running ProGC data capture software (ThermoUnicam; Hemel Hempstead, UK) for analysis and reprocessing.

Although a small number of separations were performed on a Partisil PAC column (Phase Separations; Clywd, UK), and a Bio-Sil polyol column (Bio-Rad; Hemel Hempstead, UK), much of the work in this section was carried out on a series of novel and commercially available Spherisorb based columns (see **Appendix Two** for column

details). A series of six novel supercritically bonded mixed-mode alkylamino / cyanopropyl bonded silica based columns were manufactured at the University of Leeds by Mark Robson and packed externally at Phase Separations (Carney, 1999; Dmoch, 1999; Robson, 1998). In addition to a number of commercial Spherisorb aminopropyl columns, the novel phases formed the basis of much of the work in this section. However, commercial Spherisorb cyanopropyl, mixed mode octadecyl silane (ODS) / aminopropyl, strong cation exchange (SCX) and bare silica columns were also evaluated. A guard column was not employed at any time.

All separations were performed at ambient laboratory temperature with a mobile phase flow rate of 1 ml/min. Initial mobile phase composition was identical to that currently used by Unilever Research, namely 90:10 $CHCl_3$: MeOH with 10 mmol/l AcH (Lawrence, 1997; Wee *et al.*, 1982). However, this mobile phase was quickly superseded by a ternary system of hexane, MeOH and THF modified with TFA.

The ratio of solvents and solvent mixing was controlled via the quaternary pump. Two solvent reservoirs were employed, one containing either chloroform modified with AcH or hexane modified with TFA, the other containing either MeOH modified with AcH or the polar constituents of hexane based solvent systems (normally THF, MeOH modified with TFA). The desired solvent ratio was drawn from the reservoirs, combined in the pumps' dynamic mixing chamber and then delivered to the rest of the analytical system. In this way any variation between the specified mobile phase composition and that in the analytical system was consistent. All solvents were degassed prior to the commencement of practical work by sparging with helium for thirty minutes.

2.4.1.2 Unilever Research Port Sunlight

Separations were driven by a PE Series 410 quaternary pump system (Perkin Elmer; Beaconsfield, UK). Injection was performed by using either a Rheodyne Model 7125 stainless steel injection valve (Fisher Scientific) that was fitted with a Phase Sep Event Marker (Phase Separations; Clywd, UK) to facilitate a synchronised start of the data capture software, or a Kontron 360 autosampler (Bio-Tek Kontron Instruments; Watford, UK). A Sedex 55 evaporative light scattering detector (Sedere; Alfortville, France) was used for all separations. Nitrogen was used as the nebulisation gas

throughout. The output from the Sedex detector was relayed via a PE 900 Series link box to a server running Perkin Elmer Turbochrom Client / Server software version 6.1.1. (Perkin Elmer, 1998). All method and sequence creation, along with integration, reanalysis and system suitability tests were performed with the aid of the Turbochrom software.

Normal phase LC separations were performed at ambient laboratory temperature on either a 150 × 4.6 mm i.d. 3 μm Spherisorb amino column (ex-University of Leeds) or a 150 mm Sphereclone amino column packed with 3 μm bonded silica (Phenomenex; Macclesfield, UK). Three different internal diameters of Sphereclone column were evaluated, namely 4.6 mm, 2.0 mm and 1.0 mm i.d., and all separations were performed with an equivalent linear velocity. For 4.6 mm columns a flow rate of 1 ml/min was used, for the 2.0 mm i.d. column the flow rate was reduced to 190 μl/min and for the 1.0 mm i.d. column the flow rate was reduced still further to 50 μl/min. For the validation exercise a SecurityGuard cartridge fitted with two 4 × 2.0 mm i.d. Sphereclone amino filters (all Phenomenex; Macclesfield, UK) was introduced into the inlet end of the column. The ternary hexane, MeOH and THF solvent system developed at the University of Leeds was employed for all separations. The polar and non-polar solvents were again held in separate reservoirs until needed, with the quaternary pump being used to control the solvent ratio.

Placement of a Jour X-Act 4 channel membrane degasser (Jour Research; Onsala, Sweden) between the solvent reservoirs and the pump inlet prevented dissolved gases from entering the analytical system.

2.4.2 Normal phase liquid chromatography / mass spectrometry

Normal phase liquid chromatography / mass spectrometry (LC/MS) experiments were performed with the aid of a HP1100 integrated LC system (Hewlett Packard; Waldbronn, Germany) consisting of a membrane degasser, a binary pump and a standard auto-sampler, and a Finnigan MAT LCQ benchtop ion-trap mass spectrometer fitted with an electrospray ionisation (ESI) interface (Finnigan MAT; Hemel Hempstead, UK) operating in the positive ionisation mode. Full scan spectra were obtained on the LCQ instrument across the range *m/z* 150 to 2000, with an ion time of 200 ms. The detector response was initially optimised with the aid of the automatic tune

function within the instrument software. However, additional optimisation of the Tube Lens Offset voltage, the capillary temperature and the sheath and auxiliary gas flows were performed manually.

During direct infusion studies and instrument tuning the integral syringe pump was used to ensure uniform metered delivery into the instrument. For LC/MS studies, front-end separations were performed on a 150 × 2.0 mm i.d. 3 μm Sphereclone amino column fitted with a SecurityGuard system at the column inlet (all Phenomenex; Macclesfield, UK). A one-piece column-coupler (Anachem, Luton, UK) was used to link the outlet of the column with the inlet of the ESI interface. The mobile phase again constituted hexane, MeOH and THF modified with TFA, and was delivered at a flow rate of 190 μl/min.

2.4.3 Reverse phase liquid chromatography

Reverse phase HPLC analysis was performed using a Waters Alliance HPLC system (Waters; Milford, USA), which comprised a model 616 binary pump controlled by a model 600s pump control module, and a model 486 tuneable wavelength detector. Injection was performed manually with a Rheodyne model 7125 stainless steel injection valve (Phase Separations Ltd.) fitted with a 10 or 5 μl external injection loop. The output from the 486 detector was connected via a Dionex advanced computer interface to a PC running AI-450 chromatography automation software (Dionex Corporation; Leeds, UK) for data capture, integration and reanalysis.

A number of silica based LC stationary phases were evaluated during the course of this practical segment. Separations were primarily performed on either a 150 × 4.6 mm i.d. 3 μm Spherisorb cyanopropyl column (Phase Separations; Clywd, UK) or a 150 × 4.6 or 2.0 mm i.d 3 μm Luna cyanopropyl column (Phenomenex; Macclesfield, UK)). However, Spherisorb silica, methyl, hexyl, octyl, ODS2, mixed mode ODS/cyanopropyl and SCX columns were also evaluated, along with a Zorbax stable bond cyanopropyl column (all Phase Separations; Clywd, UK)) (see **Appendix Two**).

Separations were performed with a binary mobile phase system comprising either ACN or MeOH as the strong solvent and either double distilled water or an aqueous solution of a strong base, both of which were modified to the required pH with

TFA, as the weak solvent. It is worthy of note at this time that in the case of all reverse phase LC and LC/MS work, modifiers were only added to the aqueous proportion of the mobile phase, and thus pH adjustment was only ever performed on that reservoir. As a result, the mobile phase passing through the analytical system would have demonstrated a higher *"apparent pH"* than that specified in the text.

Separations performed on 4.6 mm i.d. columns were achieved with a mobile phase flow rate of 1 ml/min. Flow rates were adjusted accordingly for columns with different internal diameters in order to maintain a constant linear velocity. Solvents were degassed throughout the course of the practical work by continuously sparging with helium gas.

2.4.4 Reverse phase liquid chromatography / mass spectrometry

An initial evaluation of reverse phase LC was performed at Unilever with a Perkin Elmer Integral 4000 HPLC system, fitted with an integral autosampler and diode array detector (DAD). The output of the DAD was linked via a PE 900 link box to a server running Turbochrom Client / Server software version 6.1.1. The Turbochrom software automated data capture, analysis and reanalysis. Chromatographic separations were performed on either a 150 × 4.6 mm i.d. 3 µm Spherisorb cyanopropyl column (ex-University of Leeds), or a 150 × 4.6 mm i.d. or a 150 × 2.0 mm i.d. 3 µm Luna cyano column (Phenomenex; Macclesfield, UK). Separations were performed with a binary mobile phase of ACN and Milli-Q water modified with a suitable base and adjusted to pH 2.0 with TFA. The flow rate was adjusted according to the internal diameter of the column in order to ensure separations were performed at a uniform linear velocity.

Reverse phase LC/MS analysis was performed with the aid of a HP1100 integrated LC system and a Finnigan MAT LCQ benchtop ion-trap instrument. The HP1100 consisted of four modules, a membrane degas unit, a binary pump, a peltier cooled autosampler and a diode array detector fitted with a standard flow cell. Full control of the instrument was achieved via the Chemstation software (Hewlett Packard; Waldbronn, Germany), the software was also used to control the output from the DAD, and for reanalysis. The LCQ instrument was fitted with an ESI interface and was operated in the positive ionisation mode. Both full scan and selected ion monitoring

(SIM) acquisition was used. In full scan mode a mass range of *m/z* 200-500 was acquired, whilst in SIM mode only *m/z* 303.5-304.5 was identified. In both modes of operation the maximum ion time was set to 200 ms. The detector response was initially optimised with the aid of the manual tune function within the instrument software. However, additional optimisation of the Tube Lens Offset voltage, the capillary temperature and the sheath and auxiliary gas flows were performed manually.

During direct infusion studies, and instrument tuning, analyte flow was delivered via the integral syringe pump on the LCQ instrument.

Front end LC separation was achieved on a 150 × 2.0 mm i.d. 3 μm Luna cyanopropyl column. The mobile phase composition was 50:50 ACN : 5 mmol/l ammonium acetate buffer adjusted to pH 2.0 with TFA. The flow rate of the mobile phase was set at 190 μl/min. Post-column addition of modifiers was achieved with the aid of a poly-ether-ether ketone (PEEK) T-piece (Fisher Scientific) positioned between the column outlet and the inlet of the ESI interface. This design allowed the constant infusion of solvent and/or acidic modifiers into the flowing effluent stream, the rate of delivery of the modifier again being controlled by the syringe pump on the LCQ.

2.5 REFERENCES

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CHAPTER THREE

Development of a new normal phase LC method for the analysis of cationic fabric conditioner actives

CHAPTER THREE

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3.1 INTRODUCTION

With the widespread use of cationic tensides as active agents in down-the-drain fabric conditioners there is a pressing need for methods of analysis capable of accurately quantifying the alkyl-chain distributions of the major and minor active agents in commercial formulations. The current methods used to quantify cationic tensides are non-specific, or they demonstrate other inherent problems that limit the application of the method to industrial or environmental analysis. To date there is no method available in the literature that is capable of quantifying all the major cationic tenside actives present in domestic fabric conditioners. Therefore, the aim of this section of work was to develop a new LC method capable of quantifying cationic fabric conditioner actives in bulk industrial formulations and environmental matrices.

3.2 LIMITATIONS OF STANDARD METHODOLOGY

Section 1.4 described the methods of analysis that are commonly used to determine cationic tensides in a range of different matrices. Though numerous methods are applicable to the quantitation of fabric conditioner actives, i.e. thin layer chromatography (TLC) and the disulphine blue active substances (DSBAS) method **(Section 1.4.1)**, normal phase liquid chromatography (NP-LC) is seen to be the most commonly utilised method in the literature (Schmitt, 2001). The method is preferable due to its suitability for use in quality control protocols, being as it is able to distinguish between different groups of tensides (Wee *et al.*, 1982). The ease of hyphenating the methodology with mass spectrometry has led to the method providing unequivocal structural information on the concentration of cationic tenside residues in complex environmental matrices (Fernandez *et al.*, 1996; Radke *et al.*, 1999).

The current standard operating procedure (SOP) used by Unilever Research for the analysis of the cationic fabric conditioner actives in environmental matrices, is characteristic of methods described in the literature (Wee *et al.*, 1982; Nitschke *et al.*, 1992; Norberg *et al.*, 2000). Separation is performed on a mixed-mode Partisil PAC phase (**Appendix Two**), with an isocratic mobile phase consisting of chloroform and methanol. Acetic acid is also included in the mobile phase to improve analyte peak shapes. Detection is brought about with the aid of a conductivity detector, which is commonly used without the aid of a suppresser (Cooper, 2000). Under these conditions, the major active agents, the di-chained quats, are eluted first as a single peak, followed by the mono-chained quats, which also elute as a single peak (**Section 1.4.4**).

Whilst this method is capable of resolving different groups of tensides the methodology is incapable of resolving the homologue distributions present in each of these groups (**Section 1.4.4**). As the alkyl chain homologues show different environmental profiles (**Section 1.2.3.3**), the ability to quantify the individual components is a principal requirement for the effective environmental profiling of these compounds. Aside from an inability to resolve cationic tenside homologues, the current Unilever SOP also suffers from poor reproducibility and the need for laborious conditioning of the column to the cationic analytes at the commencement of a study (Burford, 1997).

Before attempting to develop a new method of analysis for cationic fabric conditioner actives, the current Unilever SOP was utilised to assess the severity of the limitations described above. **Figure 3.1** shows a typical chromatogram obtained from the analysis of the HEQ sample (**Appendix One**) with the current Unilever method. The first peak eluting after ca. 8.2 minutes corresponded to the major active agents, the diester quats (**Section 1.2.1**), and the peak eluting after approximately thirteen minutes corresponded to the monoester quats (**Appendix One**). The two peaks were seen to be poorly efficient, and whilst much of the poor chromatography stemmed from the age and design of the conductivity detector employed during this work (**Section 2.4.1.1**), poor peak efficiency is endemic of the methodology (Lawrence, 1997).

It should be noted at this time that the chromatogram shown in **Figure 3.1** was obtained after first injecting a large *"slug"* of analyte onto the column to block the

active sites on the silica substrate. This approach was found to be necessary before commencing a study in order to avoid irreversible binding of the analytes to the stationary phase during use (Burford, 1997). This problem was witnessed in the form of excessive analyte retention and low peak area response (Burford, 1997). Over time, the active sites on the Partisil support were blocked giving rise to shortened retention and increased peak areas. Unfortunately, such dynamic modification was witnessed throughout the evaluation of the Unilever SOP, and thus retention time stability was poor, with the peak maximum being seen to change by up to ninety seconds over the course of five sequential injections. Instability of this kind makes it difficult to accurately define retention boundaries for the two groups of tensides in an automated reprocessing method, which leads to additional analyst time being required for reanalysis.

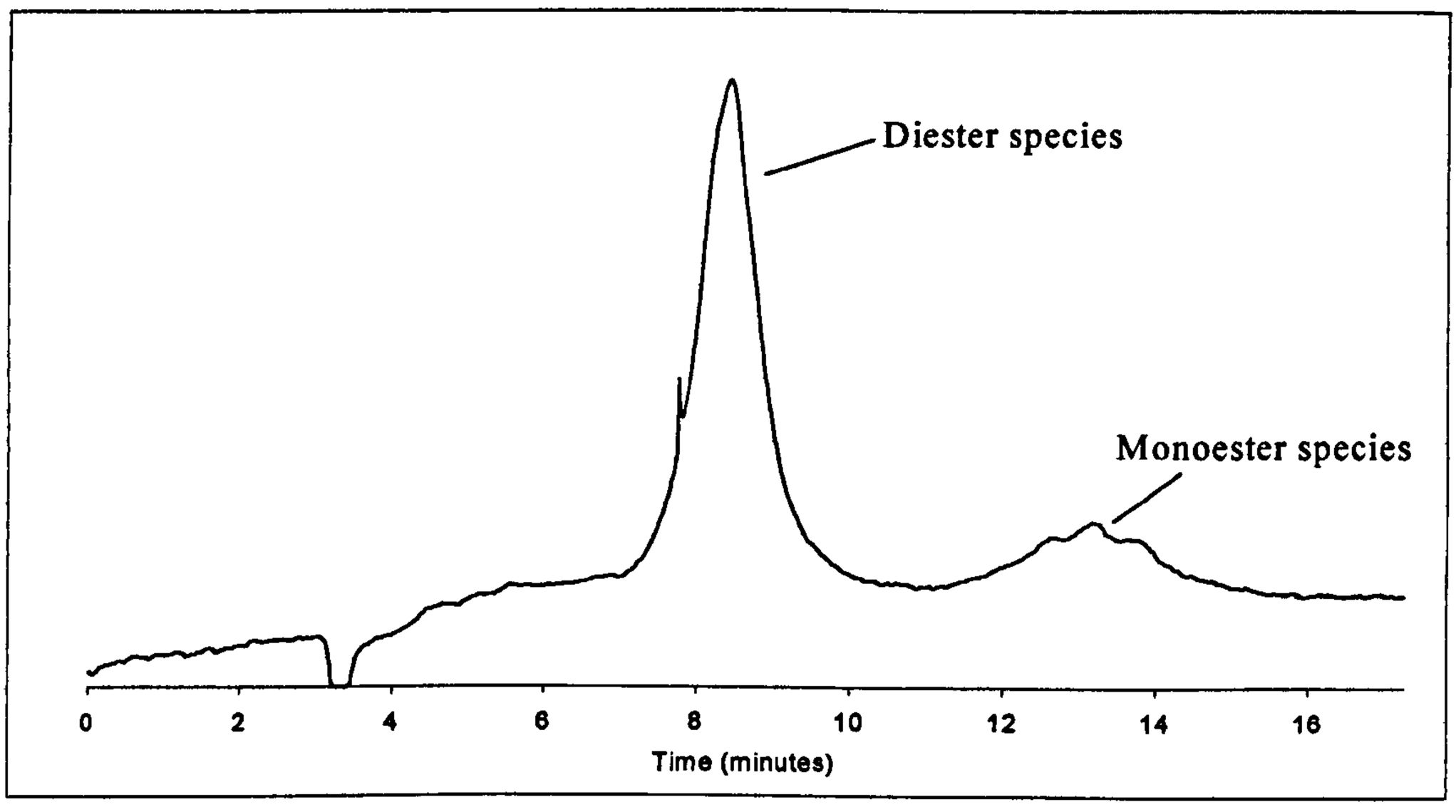

Figure 3.1: Chromatogram showing the analysis of the HEQ sample with the current Unilever method.
Conditions – Column: 250 × 4.6 mm i.d. 5 μm Partisil PAC; Mobile phase: 85:15 chloroform : methanol modified with 1% acetic acid; Flow rate: 1 ml/min; Detection method: Conductivity.

The problematic nature of the Unilever SOP highlighted the need for the development of alternative methodology that would afford improvements in analyte resolution and retention time stability.

3.3 EVALUATION OF AN ALTERNATIVE METHODOLOGY

During **Section 1.4.4** reference was paid to a series of papers that had reported the analysis of cationic and amphoteric tensides with hexane-based mobile phase systems (Wilkes *et al.*, 1992, 1994 and 1996). The three reports indicated that the use of hexane-based mobile phases could give rise to high efficiency separations of cationic tensides (Wilkes *et al.*, 1992). Significantly, Wilkes *et al.* (1992) observed that the peak corresponding to the monoalkyl quats was split, suggesting resolution of the homologous series (Wilkes *et al.*, 1992). Although this group did not assess the retention time stability of the method (Wilkes *et al.*, 1992, 1994 and 1996), previous reports have suggested superior reproducibility may be achieved with hexane-based systems, rather than with traditional chloroform-based systems (van Damme *et al*, 1986; Verzele *et al.*, 1986). In light of these observations the decision was taken to evaluate a hexane-based NP-LC method and compare its performance to that of the Unilever SOP.

3.3.1 Choice of the initial parameters

Wilkes *et al.* (1992) had utilised two polar solvents in the mobile phase system, tetrahydrofuran (THF) and methanol (MeOH), being used in a 3:1 ratio. As with the method of Wee *et al.* (1982), acid was added to the mobile phase to improve analyte peak shapes. However, Wilkes *et al.* (1992) used trifluoroacetic acid (TFA) rather than acetic acid at a concentration of 5 mmol/l, which improved mobile phase volatility making the method more compatible with evaporative light scattering detection **(Section 1.3.2.1)**. Separation was performed on a Bio-Sil polyol stationary phase **(Appendix Two)** with the aid of a gradient elution profile (Wilkes *et al.*, 1992).

The polyol phase was retained during the initial evaluation of the hexane-based method. However, variation was apparent in the choice of mobile phase parameters. Wilkes *et al.* (1992) employed a gradient elution profile of up to ninety percent polar solvent, and utilised copious amounts of THF. Having encountered instrumental problems with the literature solvent system, in the form of damaged pump seals, the ratio of the two polar solvents was changed. Methanol was utilised as the primary polar modifier, with the final ratio of MeOH : THF being 1.5:1. Whilst it was recognised that this solvent system could have led to incompatibility problems due to the limited miscibility of MeOH and hexane (Phenomenex, 2001), fears over instrument downtime and maintenance costs ultimately brought about the change. However, contingency

plans were put in place whereby the solvent system would have reverted back to the literature method if problems had been encountered during use.

TFA was maintained as the acid modifier in the mobile phase system. However, in another deviation from the previously reported literature method, the concentration of the acid was raised to 25 mmol/l. This change was not brought about consciously, but was rather the result of an error during the initial preparation of the mobile phase that was compounded over time.

In the first instance, isocratic elution of the cationic tensides was evaluated, as it had been recognised that Wilkes *et al.* (1992) may have limited resolution by utilising a rapid gradient elution profile. As a fundamental aim of the new methodology was to resolve the homologous series of tensides found in commercial samples (**Section 1.1.2**), it was envisaged that a reduction in the mobile phase strength might have facilitated increased resolution. An evaporative light scattering detector was used in the method after previously encountered problems with the use of the conductivity detector, (**Section 3.2**) and in the knowledge of its compatibility with gradient elution analysis (Wilkes *et al.*, 1992).

Figure 3.2 shows the chromatogram achieved from the analysis of the HEQ sample on the polyol bonded phase with an isocratic ternary solvent system based on hexane. Two groups of peaks were apparent eluting at eight and nineteen minutes. Whilst it was impossible to be certain of the identities of the peaks at this stage, the characterisation data available for the HEQ sample (**Appendix One**) indicated that the early eluting peaks corresponded to the diester quats, whilst the late eluting peaks corresponded to the monoester quats (**Section 1.4.4**). It was clear that both groups of peaks were being split, and it was tentatively hypothesised that resolution of the homologous series was therefore being witnessed.

Though a formal assessment of the reproducibility of the methodology was not performed at this time, the performance of a series of replicate injections showed that the retention time stability was much higher than that witnessed during the evaluation of the Unilever method. Additionally, it was observed that the conditioning of the new column was a much less involved process, compared to that with the Partisil PAC

phase. Although retention time drift was still observed at the commencement of a study, less time elapsed before reproducible retention times were obtained.

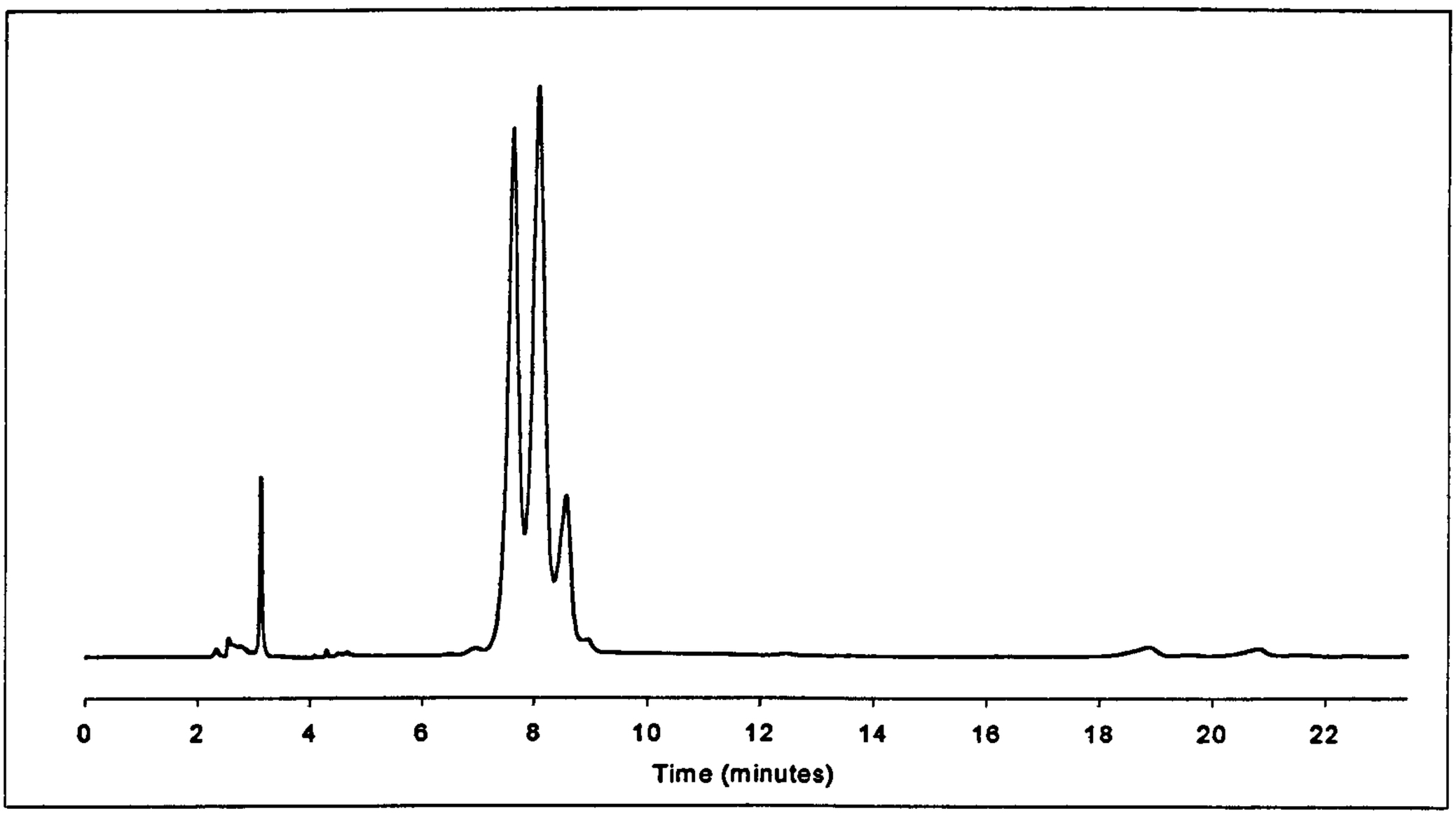

Figure 3.2: Chromatogram showing the separation of the HEQ sample that was achieved on the Bio-Sil polyol column with a ternary mobile phase system based on hexane.
Conditions – Column: 250 × 4.6 mm i.d. 5 µm Bio-sil poly-ol; Mobile phase: 80:12:8 hexane : MeOH : THF modified with 25 mmol/l TFA; Flow rate: 1 ml/min; Detection method: Evaporative light scattering.

3.3.2 Evaluation of alternative stationary phases

Whilst the new hexane based NP-LC methodology yielded significant improvements in peak efficiency and analyte retention time stability over the conventional Unilever SOP method, analysis time was seen to increase by over 50%. For a method that was ultimately aimed at routine usage, increased analysis time would have resulted in increased associated costs. A method was therefore sought, in which faster elution could be achieved.

Initial attempts to reduce the analysis time by increasing the eluting strength of the mobile phase were unsuccessful. Reducing the run-time to less than eighteen minutes resulted in co-elution of the two groups of peaks into two discrete peaks. Whilst increasing the mobile phase flow rate could have yielded improvements in run time, the LC methodology was being developed for hyphenation with electrospray ionisation mass spectrometry (ESI-MS). Therefore, an increase in mobile phase flow rate would

have adversely affected interface source dynamics and would have resulted in the sensitivity of the hyphenated methodology being compromised (**Section 1.3.2.1**).

An additional problem with the methodology was that the polyol column could only be sourced from one manufacturer and would soon be obsolete due to insufficient demand (Cooper, 1998). Whilst this initially caused inconvenience with delivery times, it was realised that a new SOP could not be based on an obsolete phase. In light of these problems, and the increased analysis time, alternative stationary phases were sought that would yield equivalent resolution and retention time stability in a shorter time period.

3.3.2.1 Evaluation of the Partisil PAC phase

With the original Unilever SOP yielding short analysis times, the Partisil PAC phase was assessed in association with the hexane-based mobile phase system. **Figure 3.3** shows the chromatogram that was attained from the analysis of the HEQ sample on the Partisil PAC phase with a mobile phase containing hexane, MeOH and THF modified with 25 mmol/l TFA. It was apparent that the PAC phase afforded less resolution than the polyol phase. However, the phase did appear to offer some benefits, as there was evidence of four major peaks and a shoulder in the first group of peaks, rather than the three major and two minor components seen in **Figure 3.2.** Having assumed that the peaks corresponded to the diester components, the larger number of peaks was more representative of what would be expected from the HEQ sample **(Appendix One).**

3.3.2.2 Assessment of a series of mixed-mode aminopropyl / cyanopropyl bonded phases

Whilst it was apparent that the commercial Partisil PAC phase yielded reduced resolution in comparison to the polyol phase, the increased number of partially resolved components led to a desire to investigate the resolution that could be achieved with similar phases. Whilst the exact nature of the PAC phase was unknown, literature reports suggested that aminopropyl and cyanopropyl groups were bound to the silica substrate, along with additional secondary amino groups **(Appendix Two).** In an attempt to optimise the resolution of the cationic tensides, a series of mixed-mode alkyl-amino / cyanopropyl phases were developed **(Section 2.4.1.1).** It was envisaged that by varying the nature of the bonded units on the silica substrate, it would be possible to

assess how resolution on the PAC phase came about, and ultimately maximise resolution. As the new phases were based on a spherical silica substrate, more uniform release of the analytes from the silica substrate was envisaged, leading to higher efficiency and increased resolution.

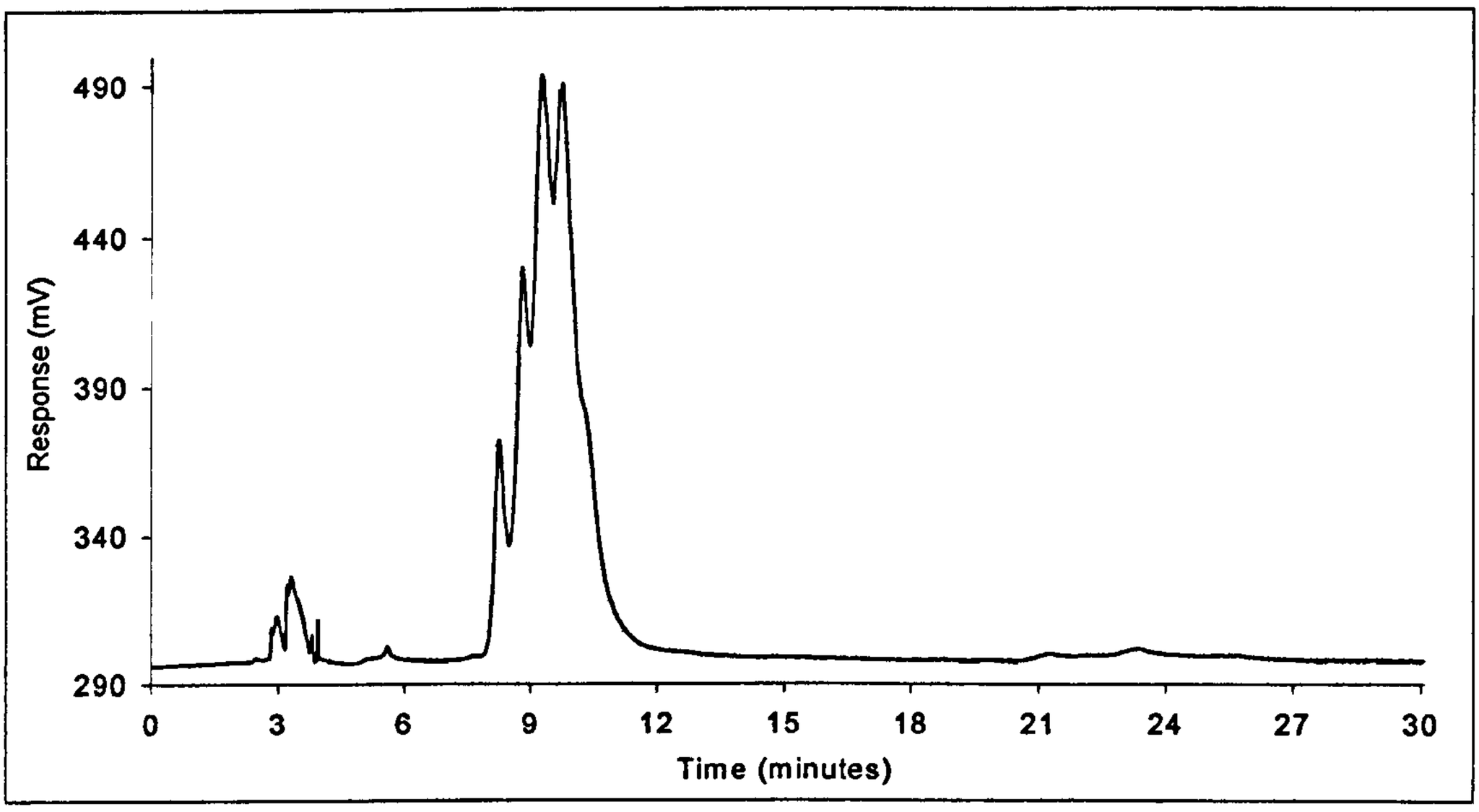

Figure 3.3: Chromatogram showing the separation of the HEQ sample on the Partisil PAC column.
Conditions – Column: 250 × 4.6 mm i.d. 5 µm Partisil PAC; Mobile phase: 80:12:8 hexane : MeOH : THF modified with 25 mmol/l TFA; Flow rate: 1 ml/min; Detection method: Evaporative light scattering.

Initially three phases were manufactured by a supercritical bonding method that had been previously shown to result in a high ligand density (Dmoch, 1999; Robson, 1998). The three columns, referred to collectively as *"Type A supercritically bonded mixed-mode columns"* (**Appendix Two**), contained varying ratios of aminopropyl and cyanopropyl units. Column M1 showed the highest percentage of cyanopropyl units, whereas M3 showed the lowest (**Appendix Two**).

Figure 3.4 shows the chromatogram obtained from the analysis of the HEQ sample on column M3. It was apparent that the separation was analogous to that seen on the Bio-Sil polyol phase (**Figure 3.2**) with an initial series of three major peaks, and two small additional peaks eluting as a group, prior to two late eluting peaks. Comparison of **Figures 3.2** and **3.4**, revealed that the resolution obtained on the new mixed-mode phase was higher than that achieved previously, and in addition, analysis time was seen

to haven fallen by approximately 33%, even though the polarity of the mobile phase had been significantly reduced.

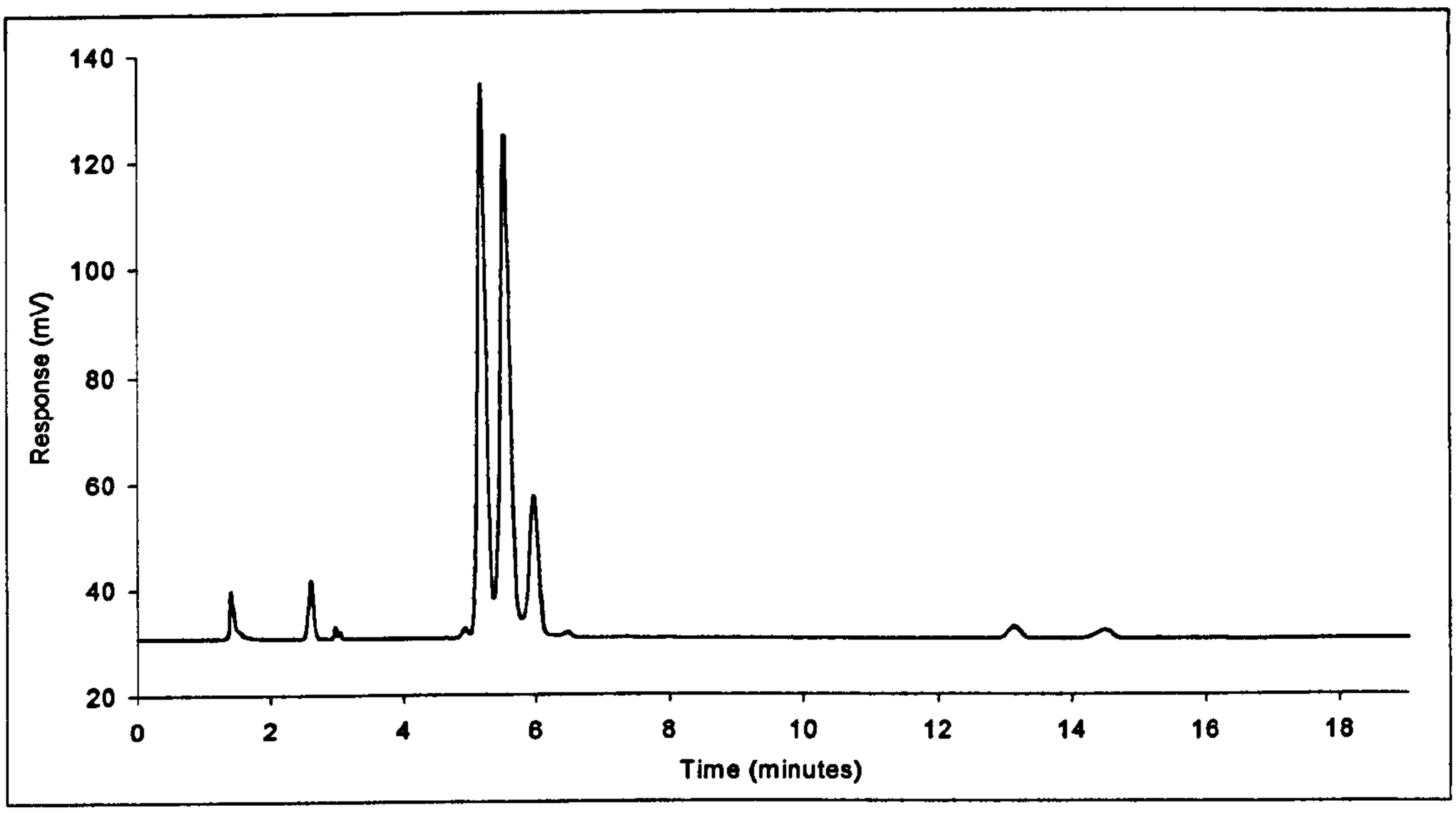

Figure 3.4: Chromatogram showing the separation of the HEQ sample on the M3 column. Conditions – Column: 150 × 4.6 mm i.d. 5 µm M3; Mobile phase: 85:9:6 hexane : MeOH : THF modified with 25 mmol/l TFA; Flow rate: 1 ml/min.

Whilst the resolution achieved with column M3 was superior to that observed on the poly-ol phase, the separations witnessed on columns M1 and M2 were very different. Both columns demonstrated the same retention characteristics as column M3, i.e. short analysis times with low polarity mobile phases. However, on both phases the resolution was lower than that achieved on column M3. **Figure 3.5** shows the variation in the resolution of the first group of peaks, thought to be the diesters, on columns M1 and M3. It was apparent that as the number of aminopropyl groups on the silica substrate increased, an improvement in the resolution of the three major components was witnessed. This trend was repeated on column M2, where the resolution was found to be intermediate of M1 and M3.

3.3.2.3 Assessment of a series of mixed-mode alkyl-amino / cyanopropyl bonded phases

Having witnessed a distinct effect upon changing the ratio of the aminopropyl and cyanopropyl units on the silica surface, a second series of columns were developed to test the influence of secondary amine groups on the resolution of the cationic

tensides. The three columns in this group, referred to collectively as *"Type B supercritically bonded mixed-mode columns"* (**Appendix Two**), contained varying ratios of alkyl-amino (**Appendix Two**) and cyanopropyl units bonded to the silica substrate. Column M4 showed the lowest percentage of alkyl-amino groups, and column M6 showed the highest (**Appendix Two**).

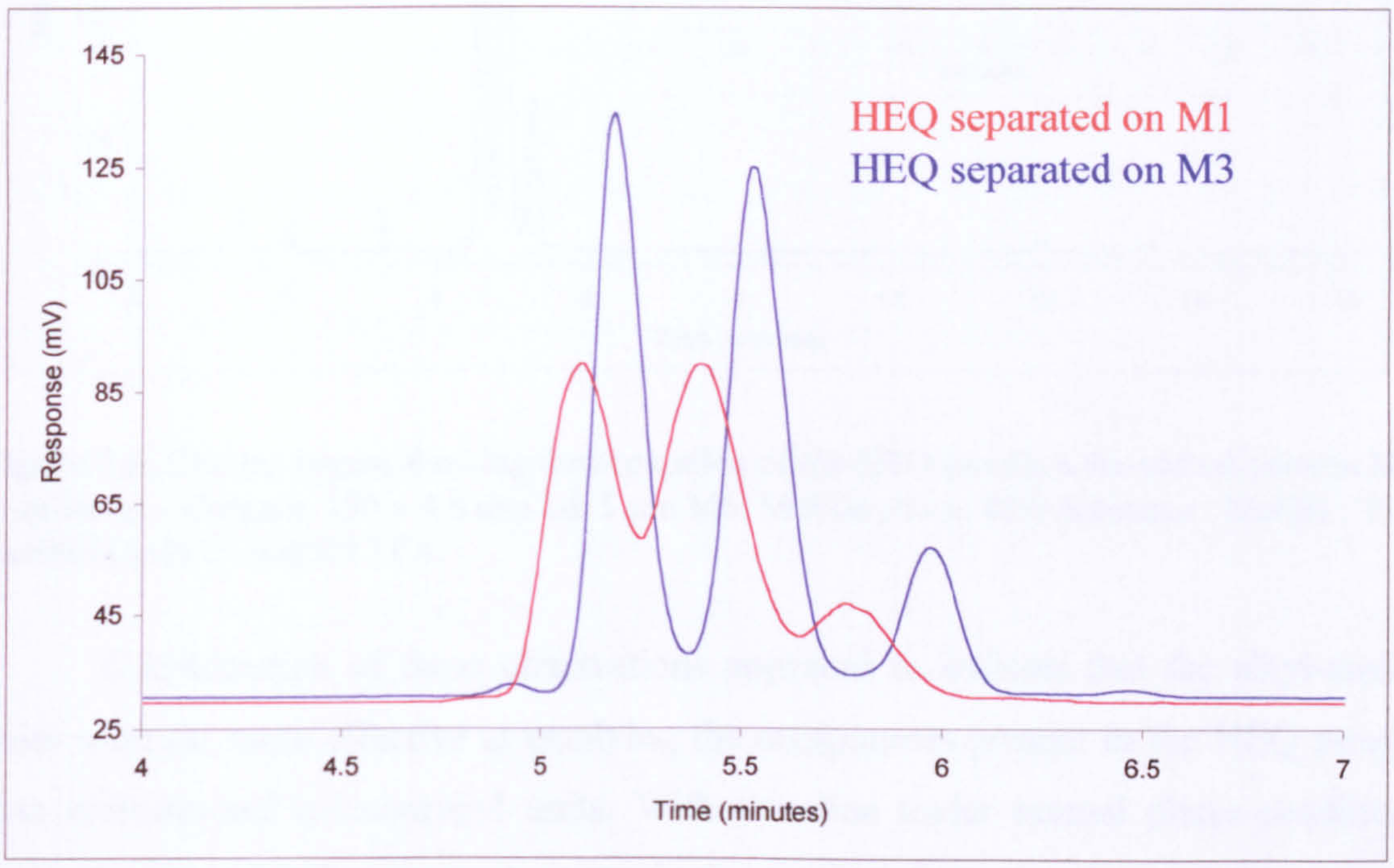

Figure 3.5: Chromatogram showing the variation in resolution of species present in the HEQ sample on columns M1 and M3.
Conditions – Column dimensions: 150 × 4.6 mm i.d. 5 µm packed with Spherisorb bonded silica; Column: Red trace – M1, blue trace M3; Mobile phase: 85:9:6 hexane : MeOH : THF modified with 25 mmol/l TFA.

Figure 3.6 shows a typical chromatogram obtained from the analysis of the HEQ sample on column M6. Resolution was superior to that achieved with column M3, and analysis time was reduced.

When the chromatograms achieved on the three Type B columns were compared, it was evident that the trend of increased resolution with increased concentration of aminopropyl units witnessed with the Type A columns, was not repeated to the same extent with the alkyl-amino phases. Whilst column M4 yielded the lowest resolution, the separation was still superior to that depicted in **Figure 3.4**. In addition, there was little variation apparent between the resolution achieved on column M5 and column M6.

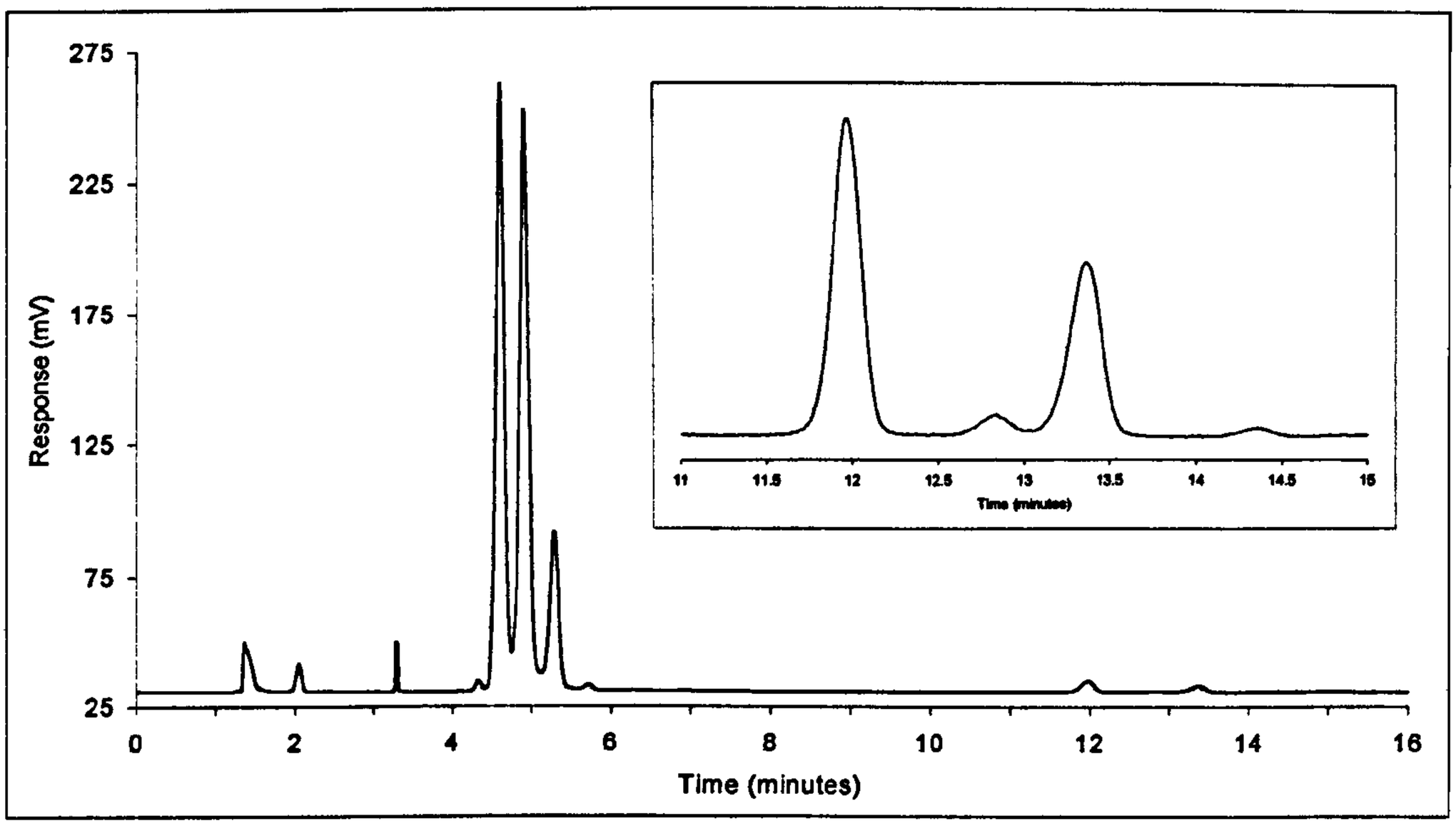

Figure 3.6: Chromatogram showing the separation of the HEQ sample achieved on column M6. Conditions – Column: 150 × 4.6 mm i.d. 5 µm M6; Mobile phase: 85:9:6 hexane : MeOH : THF modified with 25 mmol/l TFA.

Consideration of these observations appeared to indicate that the alkyl-amino units were far more effective at resolving the components present in the HEQ sample than conventional aminopropyl units. With retention under normal phase conditions normally being attributed to electrostatic interactions between the analytes and the stationary phase (Dorsey *et al.*, 1994), it was hypothesised that each alkyl-amino unit could interact with the analytes via the three amine groups. This would have amplified any variation in the electrostatic behaviour of the components of the HEQ sample, in comparison to the aminopropyl phases, and would account for the increased resolution witnessed on the Type B phases. These results led to the belief that the increased resolution witnessed with column M3, in comparison to M1, was due to the increased number of amino functionalities and not to the reduction in the number of cyanopropyl groups.

3.3.2.4 Attaining improved column performance

An observation that proved difficult to justify was the lack of a discernible trend in the resolution of the three Type B phases. After examining a number of hypotheses the most viable explanation appeared to be the complexity of the HEQ sample. It was predicted that if the first group of peaks corresponded to the diester quats, then at least twelve different components were eluted in a 120 second window. With the variation in

the electrostatic behaviour of the homologues expected to be small, especially in the case of the C_{20} / C_{16} and C_{18} / C_{18} components, it was envisaged that many of the components would co-elute. As a result, the resolution was tentatively hypothesised to plateau in the presence of an excess of amine groups bonded to the silica substrate.

Having observed that resolution generally improved with increasing numbers of amino groups, and that the alkyl-amino phases yielded higher resolution than the equivalent aminopropyl phases, a pure alkyl-amino phase appeared to provide the most effective method by which to test this hypothesis. Unfortunately, packing problems resulted in the new column being effectively useless. It was predicted, without strong evidence that limited improvement in resolution would have been witnessed from a full alkyl-amino bonded phase, compared to that witnessed on column M6.

One other additional stationary phase was evaluated at this time, a supercritically bonded gallic acid phase (**Appendix Two**). The development of this phase came about as a result of claims that poly-phenol phases could yield higher resolution than conventional silica, diol, aminopropyl, cyanopropyl and poly-ol phases (van Damme *et al.*, 1986; Verzele *et al.*, 1986 and 1987). In addition, the poly-ol phase had also afforded increased resolution over the PAC phase (**Section 3.3.2.1**). Though the chromatogram relating to the analysis of HEQ on the gallic acid phase is not shown, retention and resolution were equivalent to that witnessed on column M4, which was unexpected after the reports by van Damme *et al.* (1996) and Verzele *et al.*, (1996). Due to a lack of information on the nature of the phases used in literature reports, it was impossible to determine whether the variation in retention was due to variations in the bonded phase units that were employed. However, the increased resolution afforded by the alkyl-amino phases resulted in interest in the poly-phenol phases being dropped.

3.3.3 Evaluation of alternative mobile phases

Having attempted to optimise the stationary phase on which the separation of cationic fabric conditioner actives would be based, attention was turned to the mobile phase. In order to further optimise the methodology, the ratio of MeOH to THF was varied and alternative polar solvents assessed for their impact on resolution. It was evident that slight changes in the MeOH:THF ratio had a significant effect on the separation. When the ratio was increased, resolution of the first group of peaks suffered,

whilst increasing the THF content yielded long analysis times. In the same way, the replacement of THF with ethyl acetate, dichloromethane, and isopropanol resulted in a loss of resolution. It was evident that for isocratic elution purposes the initial starting parameters afforded the optimum separation.

In addition to evaluating alternative solvent systems, the choice of acid modifier was also reassessed in light of the well-documented problems of TFA-derived ion-suppression in hyphenated LC/ESI-MS methodologies (Kuhlmann *et al.*, 1995). Unfortunately, direct replacement of TFA with either formic acid or acetic acid led to a loss of resolution. As a result, it was concluded that TFA had to be maintained in the methodology.

3.4 PEAK ELUCIDATION BY CO-INJECTION ANALYSIS

One of the major limitations experienced when assessing the merits of the different stationary phases and solvent systems was the lack of information on the peak identities. Whilst it was tentatively hypothesised that the first group of peaks corresponded to the diester quats and the two late eluting peaks (**Figure 3.6**) were attributable to the monoesters (**Section 3.3.1**), confirmation of the peak identities was urgently required. Having not yet reached the stage where a hyphenated LC/MS methodology could be used for structural elucidation, the commercial tenside sample was instead enriched with individual components to evaluate the effect on peak areas.

Having been unable to obtain any of the single components present in the HEQ sample, a second commercial esterquat sample, diethylesterdimethylammonium chloride (DEEDMAC) (**Appendix One**) was analysed on the M6 phase (**Figure 3.7**). Like HEQ, DEEDMAC is employed as an active agent in some modern fabric conditioner formulations (**Section 1.2.1**). Having obtained samples corresponding to the major diester components, and the two principle monoester components present in the commercial sample, co-injection analysis was subsequently used to speciate the major peaks.

Figure 3.8 shows an overlay of the chromatograms produced when the commercial DEEDMAC sample was enriched with the C_{18} / C_{16} diester quat sample

(red trace) and the C_{16} / C_{16} diester quat sample (blue trace) (**Appendix One**). For clarity only the first group of peaks is shown, as no other change was apparent in the chromatogram. The peak area response of the second peak in the group showed a large increase after enrichment with the C_{18} / C_{16} diester quat sample, whilst enrichment with the C_{16} / C_{16} diester resulted in a similar increase in the third peak. When the sample was subsequently enriched with the C_{18} / C_{18} diester, an increase was witnessed in the area of the first peak of the group. Support had therefore been forthcoming in that the first set of peaks corresponded to the diester quats. More significantly, the methodology had been seen to partially resolve the homologous series present in the commercial sample.

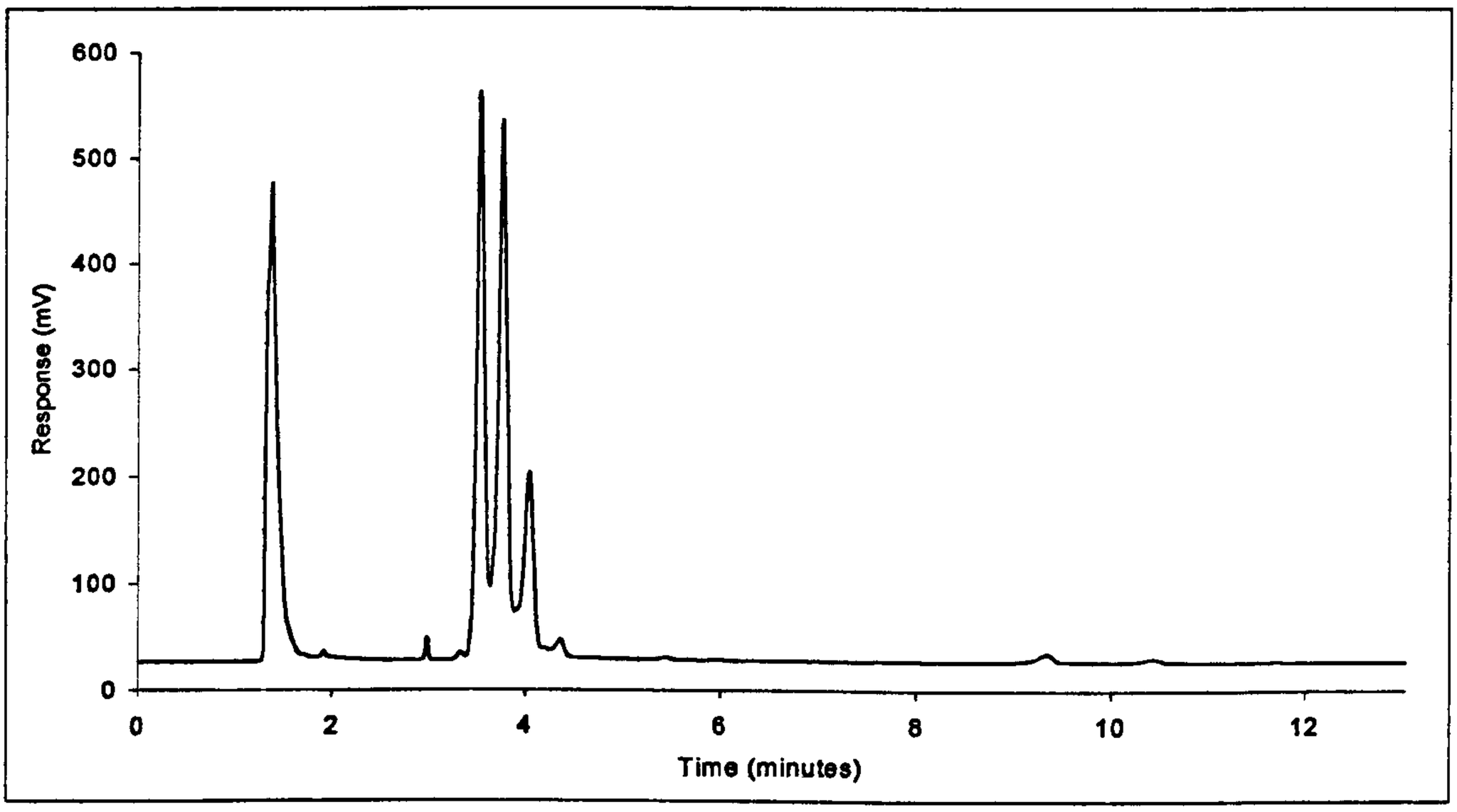

Figure 3.7: Chromatogram showing the analysis of the commercial DEEDMAC sample on the M6.
Conditions – Column: 150 × 4.6 mm i.d. 5 µm M6; Mobile phase: 85:9:6 hexane : MeOH : THF modified with 25 mmol/l TFA.

Figure 3.9 shows a comparison of the chromatograms achieved when the commercial DEEDMAC sample was enriched with the C_{18} monoester quat (red trace) and the C_{16} monoester quat (blue trace). The figure focuses on the two late eluting peaks as no further change occurred in the chromatogram. It was apparent that enrichment with the C_{18} and C_{16} monoesters led to an increase in the peak area of the first and second peaks respectively. Evidently the two late eluting peaks corresponded to the two major monoester components present in the DEEDMAC sample (**Appendix One**).

Sample enrichment provided strong evidence of the nature of the major peaks witnessed during the analysis of the commercial DEEDMAC sample, and indeed

additional evidence was forthcoming from the characterisation data provided with the sample (**Appendix One**). Previous analytical investigation had revealed that the diester quats were the major components of the sample, with the monoester components also having a significant presence. Assessment of the fatty acid composition showed that approximately 60% of the *"fatty"* chains were unsaturated octadecyl chains, with another 30% being unsaturated hexadecyl chains (**Appendix One**). It was therefore expected that the three major components of the DEEDMAC sample were the C_{18} / C_{18}, C_{18} / C_{16}, and C_{16} / C_{16} diester components. With the approximate ratio of C_{18} to C_{16} being 2:1, it was expected that the peak area of the C_{18} / C_{18} and C_{18} / C_{16} would be equivalent and approximately twice that of the C_{16} / C_{16} diester component, assuming no steric effects were experienced during manufacture. Subsequent evaluation of the peak areas of the three major peaks mirrored these predictions.

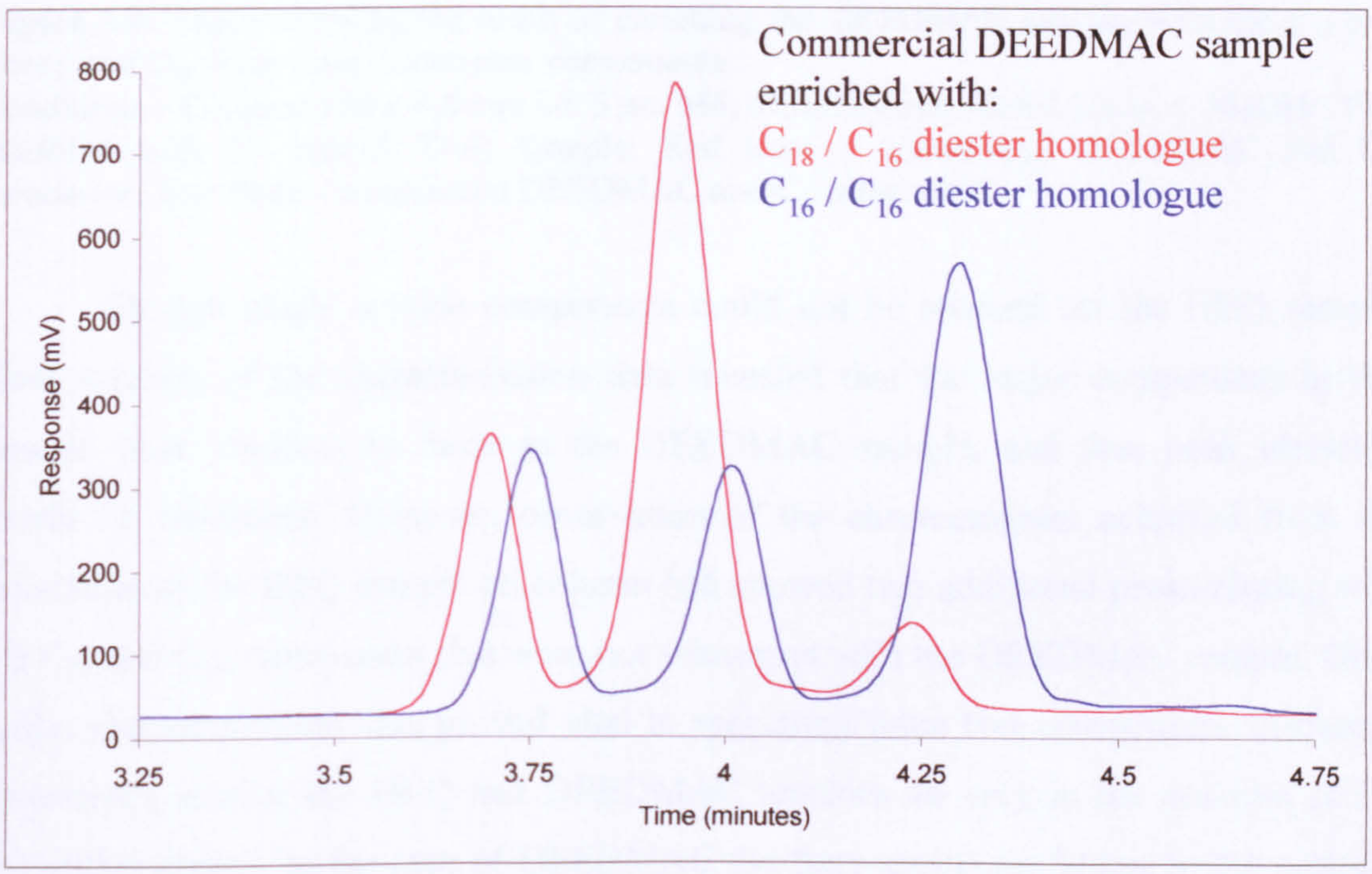

Figure 3.8: Figure showing the result of enriching the commercial DEEDMAC sample with the C_{18} / C_{16} (red trace), and the di C_{16} (blue trace) DEEDMAC diester samples.
Conditions – Column: 150 × 4.6 mm i.d. 5 µm M5; Mobile phase: 85:9:6 hexane : MeOH : THF modified with 25 mmol/l TFA; Sample: Red trace – commercial DEEDMAC and C_{18} / C_{16} DEEDMAC diester, blue trace – commercial DEEDMAC and C_{16} / C_{16} DEEDMAC diester.

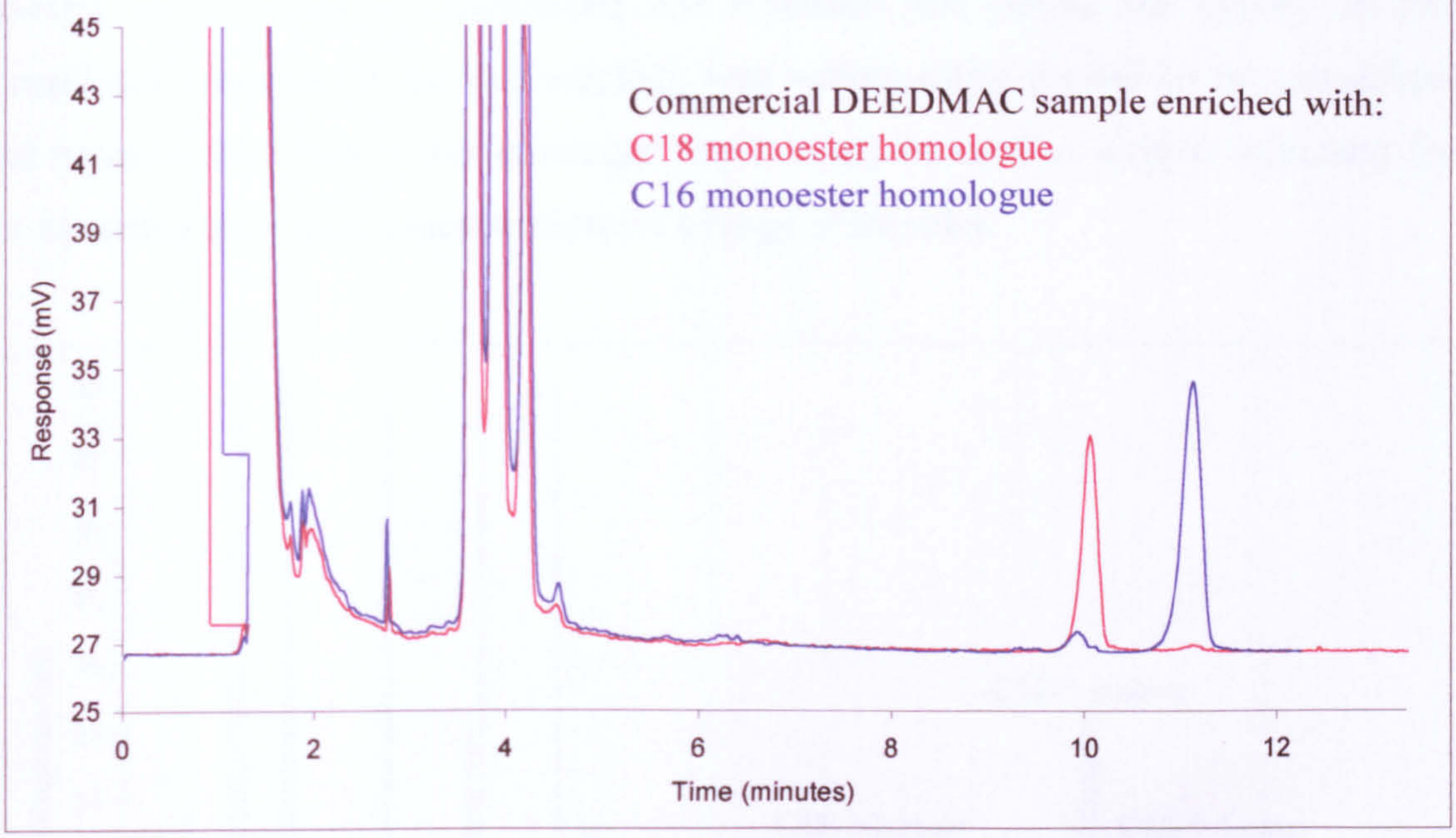

Figure 3.9: Figure showing the result of enriching the DEEDMAC sample, with the C_{18} (red trace), and C_{16} (blue trace) monoester components.
Conditions – Column: 150 × 4.6 mm i.d. 5 µm M6; Mobile phase: 85:9:6 hexane : MeOH : THF modified with 25 mmol/l TFA; Sample: Red trace – commercial DEEDMAC and C_{18} monoester, blue trace – commercial DEEDMAC and C_{16} monoester.

Though single tenside components could not be sourced for the HEQ sample, close scrutiny of the characterisation data revealed that the major components in this sample were identical to those in the DEEDMAC sample, and thus peak identities would be consistent. However, observation of the chromatogram achieved from the separation of the HEQ sample on column M6 showed two additional peaks eluting with the C_{18} and C_{16} monoesters that were not witnessed with the DEEDMAC sample. Once again, characterisation data proved vital in speciating these two components. Although structurally similar the HEQ and DEEDMAC tensides do vary in the position of the fatty-alkyl chains. In the case of DEEDMAC the fatty chains are linked to the nitrogen atom via an ethyl-ester group (**Appendix One**), and thus cleavage of the fatty-esters, yields an ethanolic moiety. In the case of HEQ the two fatty esters are contained on the same ligand. Removal of one of the fatty-ester groups would therefore yield either the 2- or the 3-isomer. Characterisation data suggests that the 3-isomer should be formed on most occasions (**Figure 3.10**).

The four discrete monoester peaks observed in **Figure 3.10** revealed that the new NP-LC method was also capable of resolving both positional isomers, as well as

members of a homologous series. It was therefore concluded that the new method showed the potential to characterise raw materials and ensure the quality of fully formulated products. If the methodology was subsequently proved to be quantifiable and reproducible, then it was envisaged that it could be used as a rapid screening tool for assessing ester quat concentrations in a range of samples.

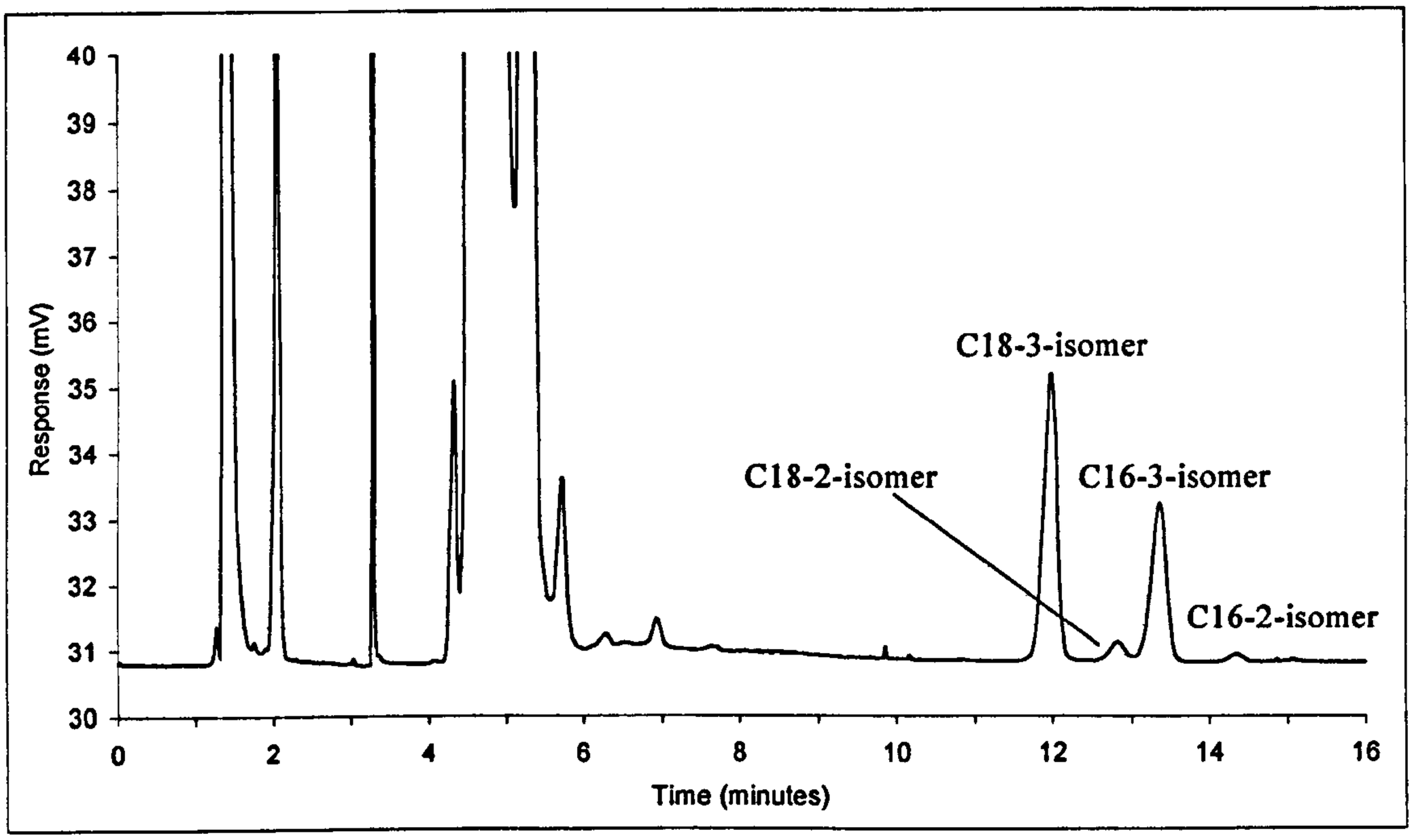

Figure 3.10: Figure showing the resolution of the monoester positional isomers present in the HEQ sample on column M5.
Conditions – Column: 150 × 4.6 mm i.d. 5 µm M6; Mobile phase: 85:9:6 hexane : MeOH : THF modified with 25 mmol/l TFA.

Until this point the effectiveness of the hexane-based NP-LC method had only been assessed for the analysis of the second-generation esterquats (**Section 1.2.1**). For the methodology to be proved effective for the analysis of all cationic fabric conditioner actives, assessment of the resolution of the first generation dialkyl quats (**Section 1.2.1**) was also required. **Figure 3.11** shows the chromatogram achieved from the analysis of the Arquad HT sample (**Appendix One**) on column M6. As was observed with the esterquats, a group of three principle peaks corresponding to the C_{18} / C_{18}, C_{18}/ C_{16}, and C_{16} /C_{16} dialkylquats dominated the trace (**Figure 3.12**). Two late eluting peaks were again evident, which were later confirmed as the C_{18} and C_{16} monoalkyl quats respectively.

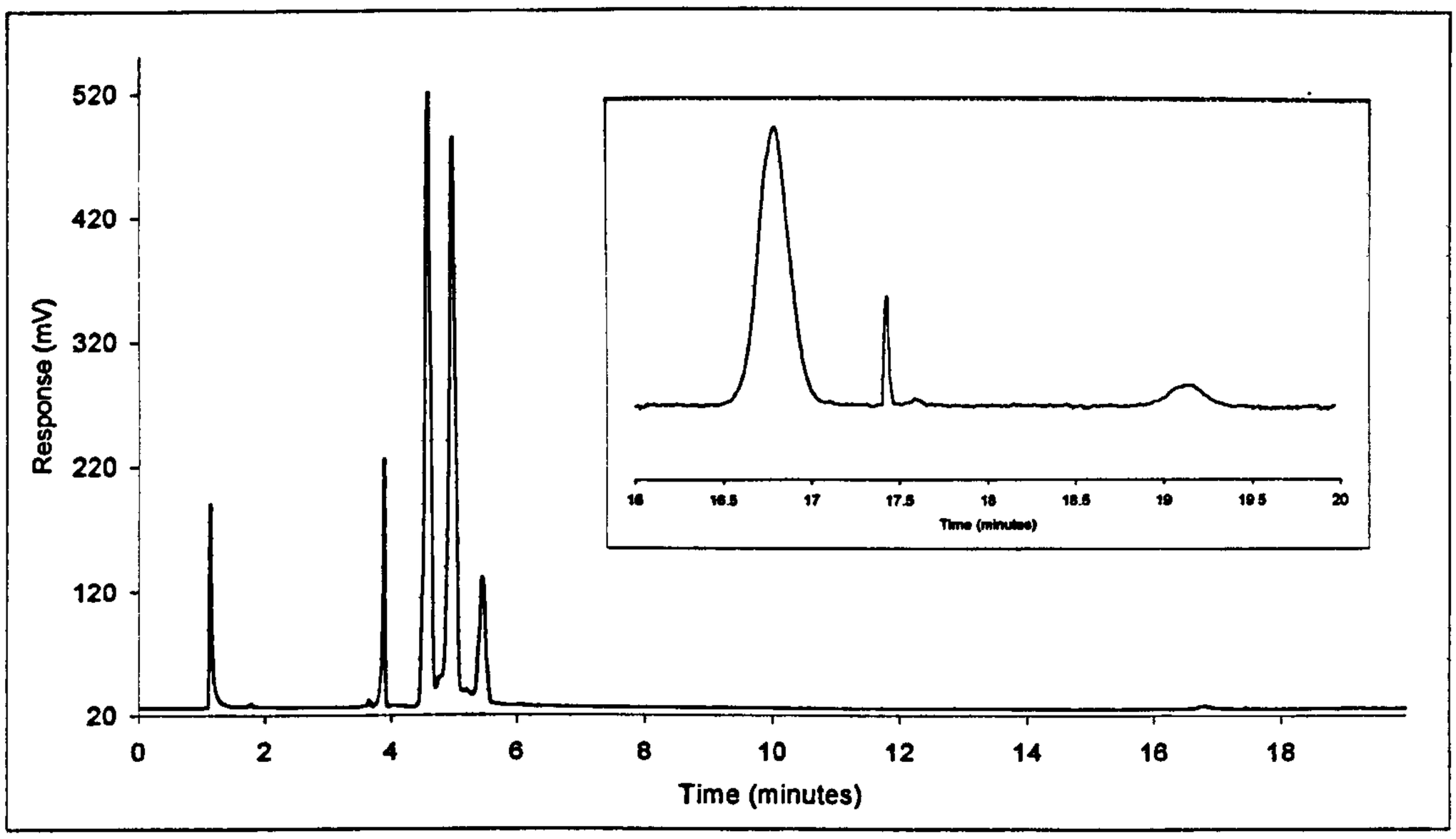

Figure 3.11: Chromatogram showing the analysis of the Arquad HT sample on the M6 column. Conditions – Column: 150 × 4.6 mm i.d. 5 µm M6; Mobile phase: 90:6:4 hexane : MeOH : THF modified with 25 mmol/l TFA.

Whilst the chromatogram obtained from the analysis of the Arquad HT sample was analogous to those obtained during the analysis of the HEQ and DEEDMAC, the major peak seen eluting after 3.9 minutes (**Figure 3.11**) had not been witnessed prior to this point. With the peak eluting earlier than the dialkyl quats, it was envisaged that the component or components showed inflated hydrophobicity compared to the dialkyl quats. Characterisation data indicated the sample comprised approximately 15% trialkyl quat (**Appendix One**), which was consistent with the observed peak area and the early elution time. However, only one peak was observed and the calculated number of trialkyl components was in excess of thirty. This apparent discrepancy was subsequently clarified when a reduction in the eluting strength of the mobile phase led to the single peak being split into a series of co-eluting peaks and shoulders. Ultimately, it proved impossible to accurately quantify any of the individual trialkyl quats present in the Arquad HT sample. However, with the trialkyl quats being *"active"* impurities it was sufficient to quantify the total trialkyl quat concentration. Similar problems were experienced during the analysis of Arquad T (**Appendix One**), a sample derived from non-hydrogenated tallow. The sample was known to contain a distribution of saturated and mono and poly-unsaturated fatty acids ranging in alkyl-chain length from C_{14} to C_{20}. As a result, the chromatogram was seen to consist of three peaks corresponding to the trialkyl, the dialkyl, and monoalkyl components. It was concluded that for cationic

tenside samples derived from non-hydrogenated fats and oils, only the total trialkyl, dialkyl and monoalkyl concentrations could be obtained with the new methodology.

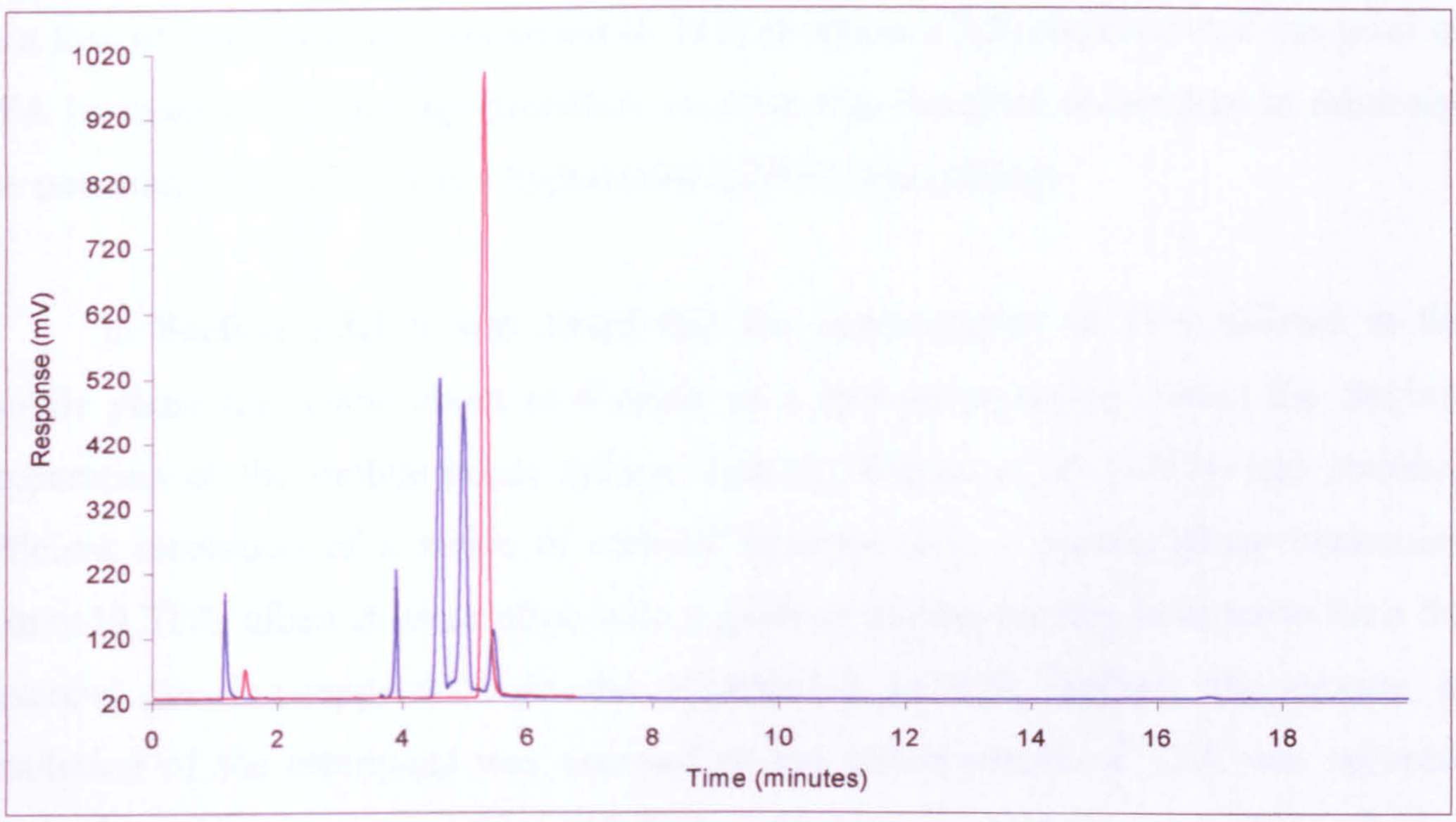

Figure 3.12: Chromatograms obtained from the analysis of the Arquad HT and dihexadecyldimethylammonium bromide samples on column M6. The two traces are overlaid to assist peak elucidation.
Conditions – Column: 150 × 4.6 mm i.d. 5 µm M6; Mobile phase: 90:6:4 hexane : MeOH : THF modified with 25 mmol/l TFA; Sample: Blue trace – Arquad HT, red trace - dihexadecyldimethylammonium bromide.

3.5 OPTIMISING THE METHODOLOGY FOR INDUSTRIAL AND ENVIRONMENTAL ANALYSIS

It was apparent that the new hexane-based NP-LC method offered significant benefits over the conventional Unilever SOP. However, a number of limitations were identified that were envisaged to limit the applicability of the method unless further optimisation was performed. The first of these limitations was the presence of TFA in the mobile phase. TFA is commonly used in the LC analysis of organic amines, as the acid associates with the analytes, forming stable pseudo-neutral adducts, which limit silanol-analyte interaction (**Section 1.3.2.1**). As a result, secondary retention is limited, facilitating increased resolution and reduced peak tailing. Unfortunately, whilst the use of TFA is beneficial in HPLC methods, it can lead to severe problems during LC/MS analysis (Kuhlmann *et al.*, 1995; Weston, 1999) (**Section 1.3.2.1**). It was envisaged that as the new LC methodology would ultimately be hyphenated with ESI-MS, in order to

quantify trace levels of cationic fabric conditioner actives in environmental matrices, it was necessary to limit future problems associated with the use of TFA in hyphenated LC/MS methodologies. The observation that the use of acetic and formic acid resulted in a loss of resolution in comparison to TFA (**Section 3.3.3**) required that the level of TFA be modified. A damage-limitation exercise was therefore undertaken to minimise the potential effect of TFA in a hyphenated LC/MS methodology.

In **Section 3.3.1** it was stated that the concentration of TFA utilised in the mobile phase had come about as a result of a mix-up occurring during the original preparation of the mobile phase system. Indeed, Wilkes *et al.* (1992), had obtained efficient resolution of a series of cationic tensides with a mobile phase containing 5 mmol/l TFA, albeit in association with a gradient elution profile. In order to limit the potential for ion-suppression in the hyphenated LC/MS method, the change in resolution of the esterquats was assessed as the concentration of TFA was reduced. Utilisation of 10 mmol/l and 5 mmol/l TFA yielded no apparent change in the resolution of the cationic analytes. However, analysis time was seen to increase by approximately 45% in the case of a mobile phase containing 5 mmol/l TFA. Further reduction in the acid concentration led to resolution being compromised and excessive retention of the monoesters. It was therefore concluded that in spite of the increased analysis time witnessed with 5 mmol/l TFA, the perceived reduction in ion-suppression, resulted in the lower acid concentration being maintained from here on in.

The second major limitation of the new methodology was the choice of stationary phase. The supercritically bonded phases had been developed at the University of Leeds to improve the resolution of the esterquats in comparison to two commercially available columns (**Section 3.2.2**). Whilst the novel alkyl-amino / cyanopropyl phases were suitable for peak elucidation studies, it was impractical to assume that they could be utilised in a method that was being groomed to become the industry standard. One of the main reservations with the use of the Bio-Sil poly-ol phase was that it could only be sourced from one manufacturer and would soon be obsolete (Cooper, 1998). This was far from ideal, as the method would need to be redeveloped when the phase became redundant. In the case of the novel supercritically bonded phases, the situation was far worse. Having been manufactured in-house at the University of Leeds, the columns had not undergone any quality control protocols to

ensure satisfactory performance and reproducibility. As the methodology would likely be employed in a Good Laboratory Practice and / or Good Manufacturing Practice (GLP and GMP) accredited environment, lack of documentation verifying the fitness for purpose of a new column would prohibit the use of the methodology. As a result, work was undertaken to identify a commercially available stationary phase that offered equivalent resolution to the novel mixed-mode phases.

Resolution of the cationic tensides had been maximised on mixed mode alkyl-amino / cyanopropyl bonded phases. In addition, during the evaluation of the three aminopropyl / cyanopropyl bonded stationary phases, resolution was seen to increase with an increase in the concentration of aminopropyl groups present on the silica substrate (**Section 3.3.2.2**). In light of these observations, the performance of a commercial Spherisorb aminopropyl phase was assessed in the new methodology. **Figure 3.13** shows a typical chromatogram obtained from the analysis of the HEQ sample on this phase. The resolution of the diester and monoester components was equivalent to that observed on column M5, with the monoester components still being resolved into the 2- and 3-isomers (**Section 3.4**). In light of the guaranteed performance and reproducibility of commercial stationary phases, and the ability to source a replacement column from a number of different suppliers, the Spherisorb aminopropyl phase was adopted as the column of choice for the new NP-LC methodology.

3.5.1 Analysis of Stepantex

The new normal phase LC methodology had been found to be suitable for the analysis of the HEQ and DEEDMAC esterquats. In order to test the applicability of the methodology to other commercial fabric conditioner actives, a commercial Stepantex sample (**Appendix One**) was analysed with the optimised methodology. The structure of Stepantex is closely related to that of DEEDMAC, but with one of the methyl groups attached to the nitrogen atom, being replaced by an ethanolic group (**Appendix One**). As a result, Stepantex components are more hydrophilic than corresponding components found in either HEQ or DEEDMAC. In addition, the ethanolic group is known to undergo esterification, which results in the commercial sample containing a high percentage of triester quats (Lawrence, 1999). With the commercial Stepantex material being manufactured from partially hydrogenated tallow, the commercial

Stepantex sample was also known to have a more complex fatty acid distribution than either HEQ or DEEDMAC **(Appendix One).**

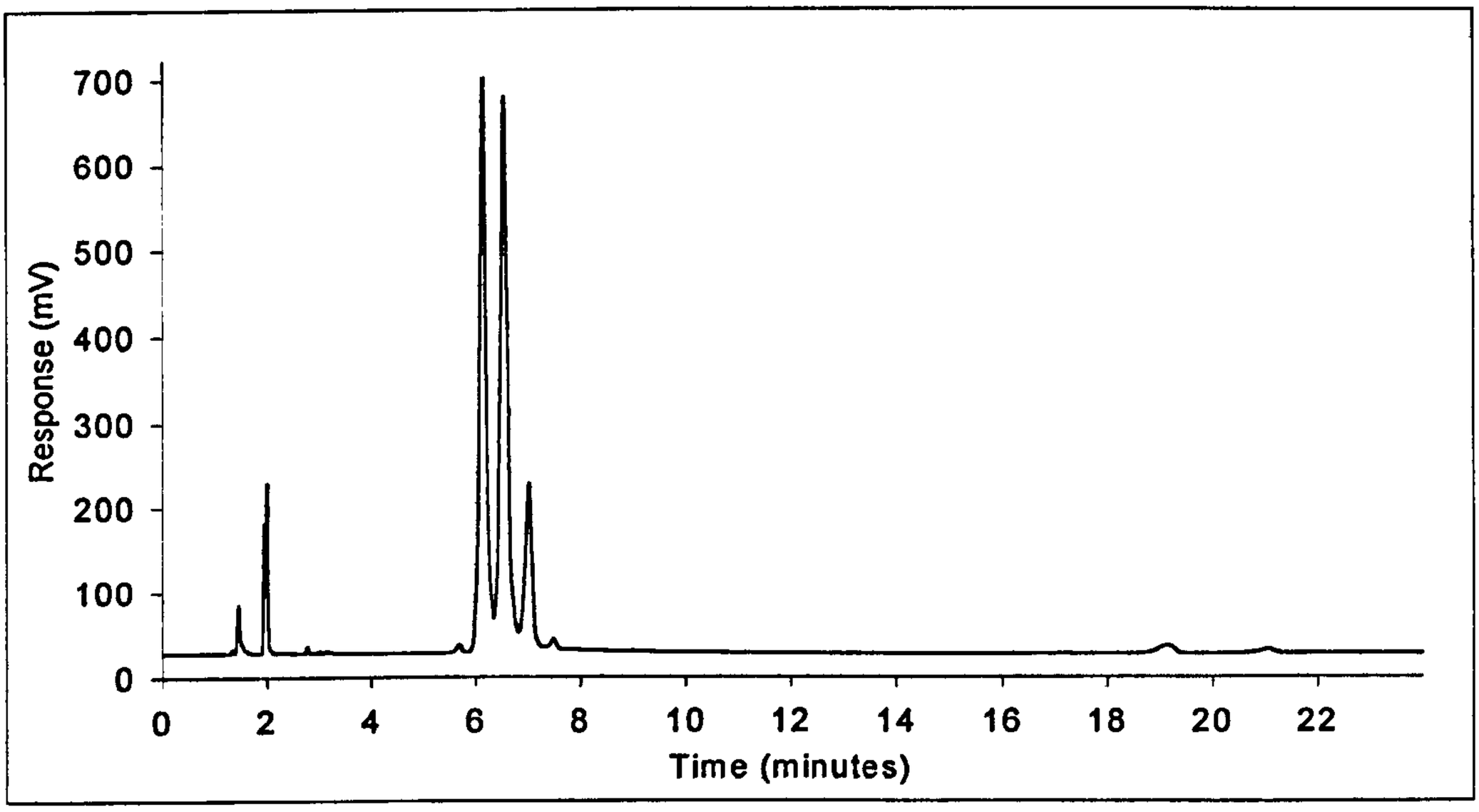

Figure 3.13: Chromatogram showing the analysis of the HEQ sample on a commercial Spherisorb aminopropyl column.
Conditions – Column: 150 × 4.6 mm i.d. 3 μm Spherisorb aminopropyl column; Mobile phase: 85:9:6 hexane : MeOH : THF modified with 5 mmol/l TFA.

Figure 3.14 shows the chromatogram that was achieved from the analysis of the commercial Stepantex sample on a Spherisorb aminopropyl stationary phase. The large peak eluting after five minutes corresponded to the total triester quat species, the large number of components present preventing resolution. The group of peaks eluting between eight and ten minutes were identified as the diester components. Again, due to the sample being only partially hydrogenated, it proved impossible to quantify individual components of this series, even after reducing the strength of the mobile phase. The method could only be utilised to calculate the total diester quat concentration.

During the initial analysis of the Stepantex sample, no evidence of the monoesters was apparent after twenty minutes from the point of injection. However, during subsequent analyses **(Figure 3.14)**, a broad peak was seen to elute prior to the solvent front, which had a similar peak area response to that expected for the C_{16} monoester components. It was hypothesised that due to the presence of the ethanolic group, the monoesters were eluting outside the limits set for the analysis, and thus were being carried-over to the start of the next injection. Further support for this theory was

apparent at the end of trace, where the C_{18} monoester components were seen to be eluting. Confirmation of the late elution of these components was subsequently forthcoming when the analysis time was increased to thirty minutes. Two broad peaks were witnessed eluting at ca. 25.5 minutes and 27.5 minutes, which corresponded to the three C_{18} monoester components, $C_{18:0}$, $C_{18:1}$, and $C_{18:2}$, and the two C_{16} monoester components, $C_{16:0}$ and $C_{16:1}$, respectively.

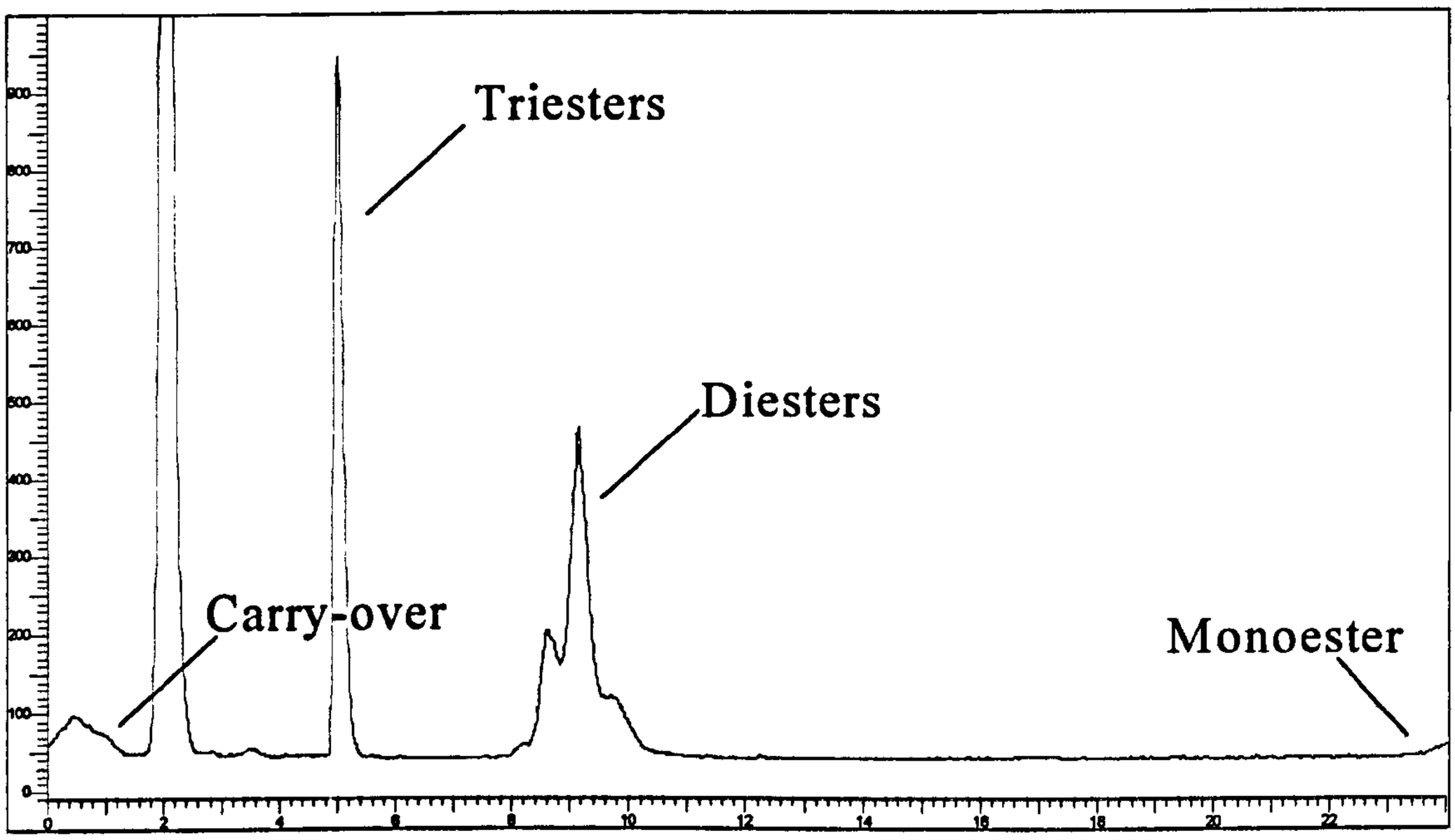

Figure 3.14: Chromatogram obtained from the analysis of the Stepantex sample on a Spherisorb aminopropyl phase.
Conditions – Column: 150 × 4.6 mm i.d. 3 µm Spherisorb aminopropyl column; Mobile phase: 85:9:6 hexane : MeOH : THF modified with 5 mmol/l TFA.

3.5.2 Resolution of the components of mixed esterquat samples

The late elution of the diester and monoester components of the Stepantex samples was known to facilitate the resolution of these components from those of either HEQ or DEEDMAC under the Unilever SOP method (Lawrence, 1999). Five peaks were witnessed corresponding to the Stepantex triesters, the HEQ or DEEDMAC diesters, the Stepantex diesters, the HEQ or DEEDMAC monoesters, and finally the strongest retained peak, the Stepantex monoesters (Lawrence, 1999; Cooper 2000). However, utilisation of the SOP method could not facilitate the resolution of the components of the HEQ and DEEDMAC samples. Instead, a single diester peak and a single monoester peak are witnessed (Cooper, 2000). A modified version of the NP-LC method published by Wee *et al.* (1982), has recently been developed, which results in

partial resolution of the total diester and monoester components of the two samples (Radke *et al.*, 1999), but with no resolution of the four separate homologous series **(Figure 3.15)**.

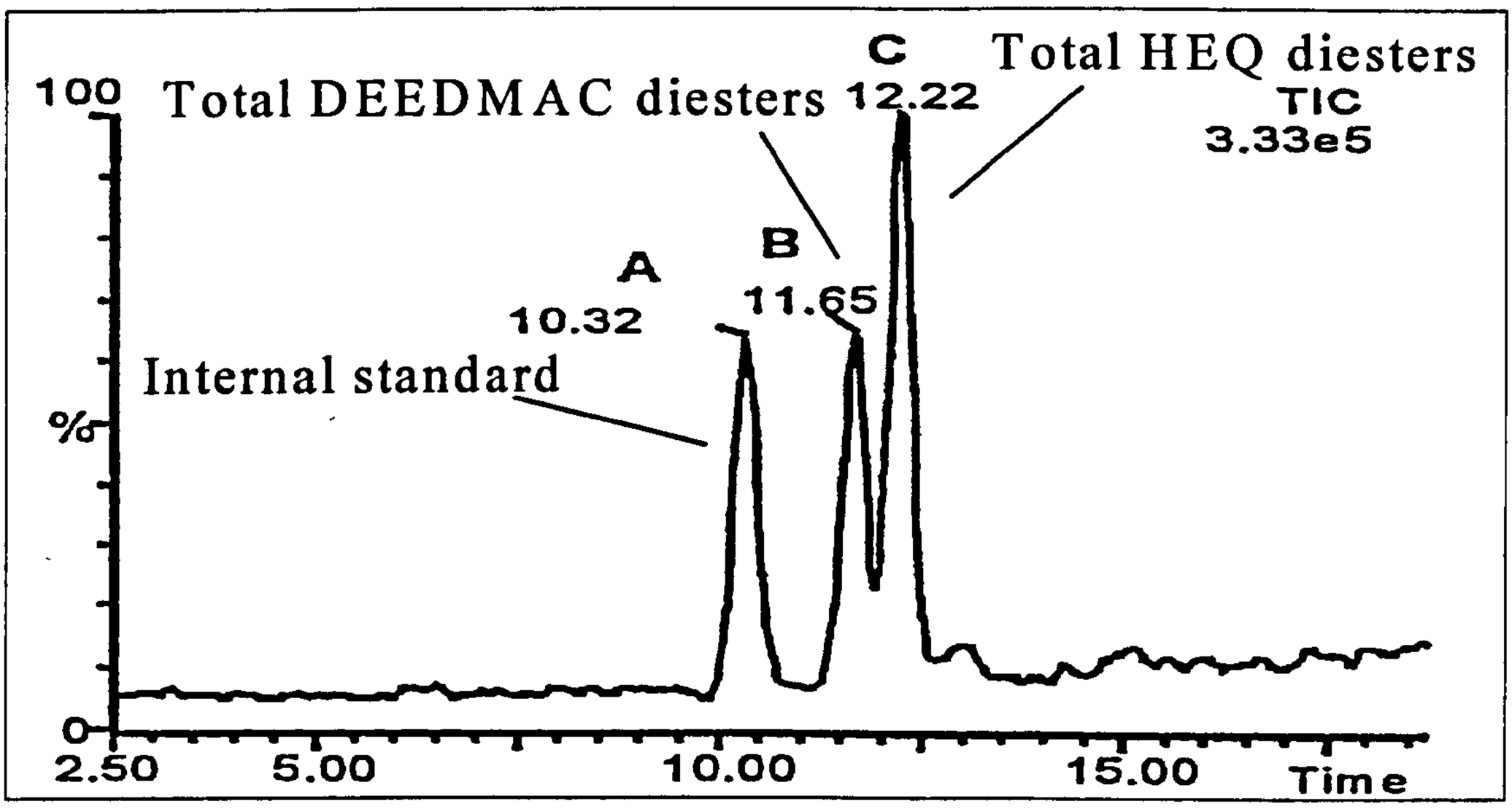

Figure 3.15: Figure showing the total ion chromatogram obtained from LC/ESI-MS analysis of a mixed sample containing HEQ and DEEDMAC diesters during a recent literature report (Radke *et al.*, 1999).
Conditions – Column: 150 × 1.0 mm i.d. 5 μm Partisil PAC column; Mobile phase: Gradient elution with chloroform, acetonitrile and methanol modified with 0.1% glacial acetic acid.

Comparison of the chromatograms achieved from the analysis of the commercial HEQ and DEEDMAC samples (**Figure 3.6** and **3.7**) have shown that the retention times of the major diester and monoester components of the two samples vary. It was therefore envisaged that it should be possible, during the analysis of a mixed sample, to resolve components from the different samples and partially resolve the homologous series of each group of cationic tensides. **Figure 3.16** shows the chromatogram that was achieved from the analysis of a mixed sample containing both the commercial DEEDMAC and HEQ samples. The resolution was found to be much higher than that reported by Radke *et al.* (1999), as the DEEDMAC and HEQ components were not only resolved from each other, but partial resolution of the homologous series was also witnessed. Subsequent addition of the commercial Stepantex sample to the mixed HEQ / DEEDMAC sample, studied in **Figure 3.16,** revealed that the new NP-LC methodology could be used to quantify components of the three esterquat samples in a single analysis. The Stepantex triesters eluted prior to the DEEDMAC diesters, which again partially co-eluted with the HEQ diesters. The Stepantex diesters were found to be

well resolved from all other components of the sample, and though the DEEDMAC and HEQ monoesters demonstrated partial co-elution, the Stepantex monoester eluted as two distinct peaks, separate from all others in the trace.

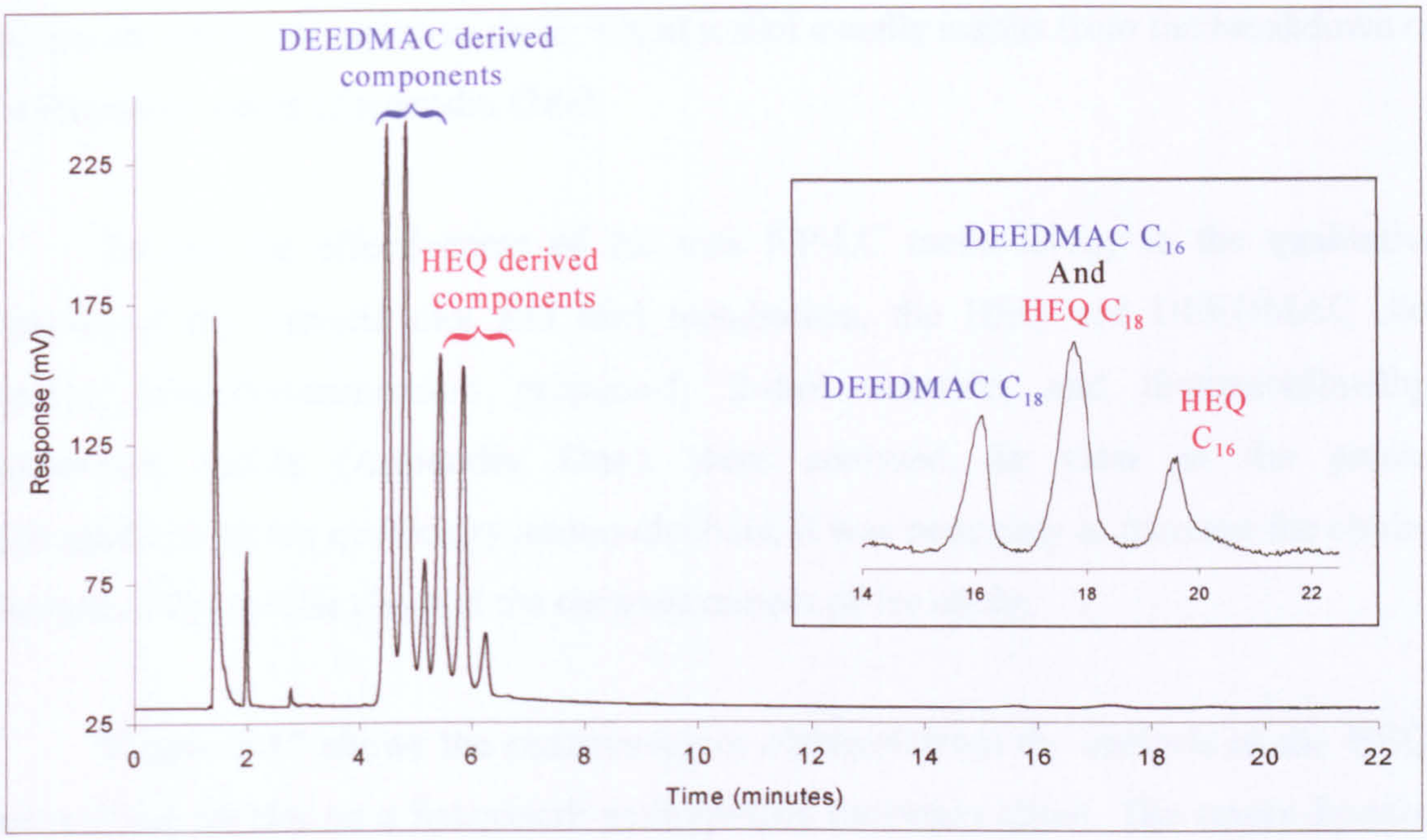

Figure 3.16: Chromatogram showing the separation of a mixed sample containing the commercial HEQ and DEEDMAC diester quat samples achieved on a Spherisorb aminopropyl phase.
Conditions – Column: 150 × 4.6 mm i.d. 3 μm Spherisorb aminopropyl column; Mobile phase: 85:9:6 hexane : MeOH : THF modified with 5 mmol/l TFA.

The observations made during the analysis of the mixed diester quat samples led to the realisation that the new methodology may have had the potential to determine individual cationic tenside residues in rivers, wastewater, and sediments. It was predicted that if the resolution witnessed in **Figure 3.16,** was maintained during the study of environmental matrices, it would be possible to quantify residual concentrations of individual components, derived from a known sample origin. In addition, the method could be used to assist in studies evaluating the removal rates of the different diester quats, in order to build a more accurate environmental profile of these analytes.

3.5.3 Analysis of the cationic metabolites

Studying the fate and biotransformation of the esterquats after their release into the environment requires the ability to quantify the major biodegradation products that are formed during the breakdown of the parent species. In **Section 1.2.3.3** an outline

was presented of the breakdown of the parent diester quats in the environment. Removal of one fatty acid group yields the monoester species, whilst subsequent removal of the second fatty acid results in the formation of the corresponding quaternary amino-alcohol (**Section 1.2.3.3** and **Appendix One**). In the case of HEQ and DEEDMAC the resulting species are often referred to as diols, whilst a triol usually results from the breakdown of the Stepantex esters (**Appendix One**).

To test the effectiveness of the new NP-LC methodology in the qualitative analysis of the cationic diol and triol metabolites, the HEQ and DEEDMAC diol species, trimethylammonium propane-1, 2-diol chloride, and diethanoldimethyl ammonium iodide (**Appendix One**), were analysed. In view of the greater hydrophilicity of the quaternary amino-alcohols, it was necessary to increase the eluting strength of the mobile phase at the commencement of the study.

Figure 3.17 shows the chromatogram obtained from the analysis of the HEQ-derived diol species on a Spherisorb aminopropyl stationary phase. The severe fronting demonstrated by this component was very unusual and was initially believed to be representative of sample overload, and / or sample-solvent incompatibility. Subsequent investigation revealed that sample concentration had little effect on peak shape, and thus the poor chromatography was attributed to an incompatibility between the analyte and the eluting solvent. As a consequence of the poor peak shape, the observed efficiency and peak height response were low, which made it difficult to assess the point at which the HEQ diol started to elute. Whilst this was a minor irritation with high analyte concentration (**Figure 3.17**), the low peak height response led to the belief that differentiating between the analyte and the baseline noise would compromise sensitivity during trace analysis.

Subsequent analysis of the DEEDMAC-derived diol species gave rise to an analogous peak to that witnessed during the analysis of the HEQ-diol (**Figure 3.18**). The component again demonstrated significant fronting, which could not be attributed to sample overload. In addition, a small secondary peak was also apparent eluting at the tail of the first peak. Unfortunately the origin of this peak could not be determined.

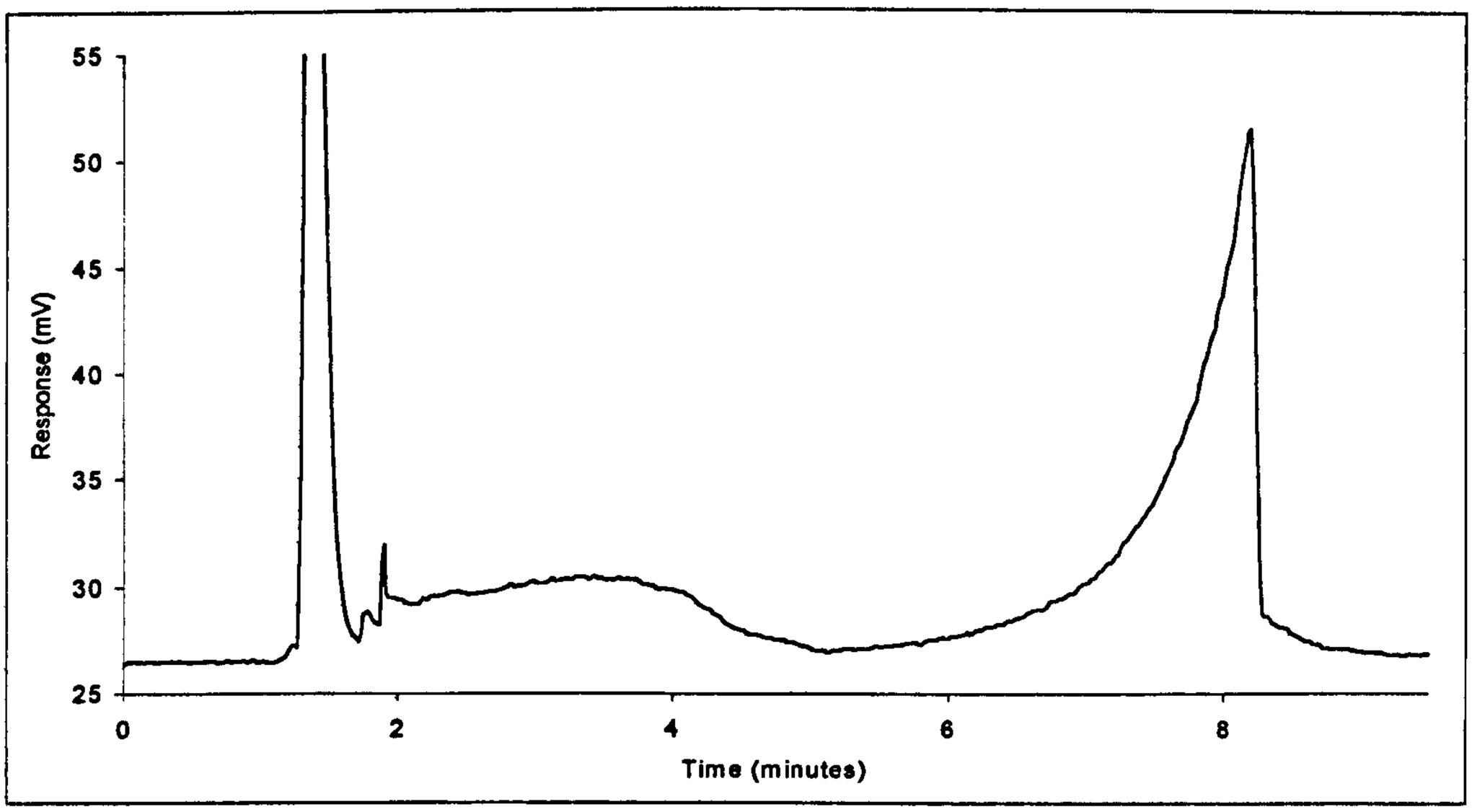

Figure 3.17: Chromatogram achieved from the analysis of the trimethylammonium propane-1, 2-diol chloride sample (HEQ diol) on a Spherisorb aminopropyl phase.
Conditions – Column: 150 × 4.6 mm i.d. 3 μm Spherisorb aminopropyl column; Mobile phase: 70:18:12 hexane : MeOH : THF modified with 5 mmol/l TFA.

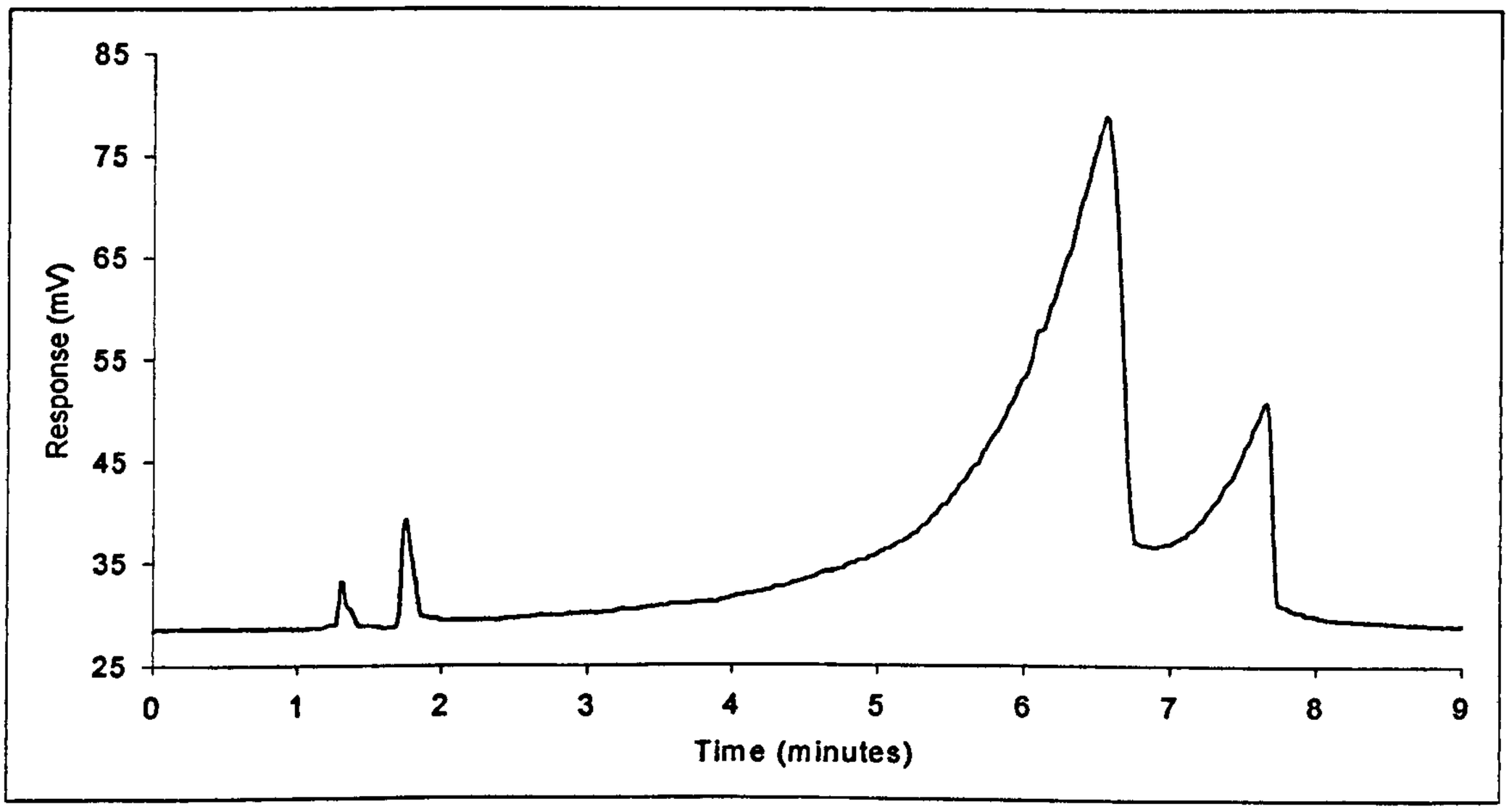

Figure 3.18: Chromatogram obtained from the analysis of the DEEDMAC derived diol sample on a Spherisorb aminopropyl phase.
Conditions – Column: 150 × 4.6 mm i.d. 3 μm Spherisorb aminopropyl column; Mobile phase: 70:18:12 hexane : MeOH : THF modified with 5 mmol/l TFA.

The strange peaks associated with the analysis of the two quaternary amino-alcohols were initially believed to provide experimental proof of the limited solubility

of the hydrophilic analytes in a hexane-based mobile phase system. However, additional observations made at other points during the development of the NP-LC methodology, suggested that the origin might have been more complex. Indeed, possible explanations to account for these, and other observations will be presented later in the chapter. Nonetheless, it was apparent that a methodology that had been found to offer excellent benefits over conventional methods for the analysis of the parent cationic fabric conditioner actives, had been found wanting during the analysis of their hydrophilic metabolites.

3.5.3.1 Gradient elution analysis for the simultaneous determination of parent diester quats and their biodegradation products

Utilising the Unilever SOP method for the quantitation of the esterquats and their cationic metabolites is known to be labour-intensive, as each sample has to be analysed under two different solvent systems in order to quantify the parent species and subsequently the diol or triol metabolites (Burford, 1997). At the outset of this work, one of the primary aims in developing new methodology was that it should be capable of quantifying parent analytes and metabolites simultaneously, as it was envisaged that this capability would greatly assist biotransformation and bioaccumulation studies performed on these species (**Section 1.2.3.3**). At the same time, it was predicted that a method capable of simultaneously quantifying parent analytes and their metabolites may ultimately lead to a generic method of cationic tenside analysis (**Section 1.5**).

The poor chromatography witnessed during the analysis of the quaternary amino-alcohols gave rise to scepticism that a single methodology capable of quantifying the esterquats and quaternary amino-alcohols could be developed from the new NP-LC method. However, it was recognised that the need to increase the eluting strength of the mobile phase during the analysis of the quaternary amino-alcohols (**Section 3.5.2**) would necessitate the use of a gradient elution profile for the simultaneous analysis of the cationic analytes. As gradient elution profiles are known to increase peak efficiency and method sensitivity (**Section 1.3.2.1**), it was hoped that the chromatography of the quaternary amino-alcohols could be improved. Gradient elution was thus evaluated for the simultaneous analysis of the esterquats and their corresponding quaternary amino-alcohol metabolites.

Figure 3.19 shows a chromatogram achieved from the gradient elution analysis of the commercial HEQ sample and its diol metabolite. The chromatogram shows the separation performed on the M6 column with the mobile phase being modified with 25 mmol/l TFA. Due to the nature of the gradient elution profile that was applied during the course of the analysis, the diester and monoester components were eluted under the isocratic conditions reported in **Section 3.3.1.** Initiating the onset of the gradient prior to the elution of the monoesters resulted in co-elution of the two principle components. Whilst it is clear from **Figure 3.19** that the gradient profile was not optimised for the elution of the diol component, some improvement was witnessed in peak shape and peak height response of the diol analyte. Further evaluation revealed that the analysis time could be reduced to twenty three minutes without observing a significant increase in baseline noise as a result of incomplete evaporation of the polar solvent.

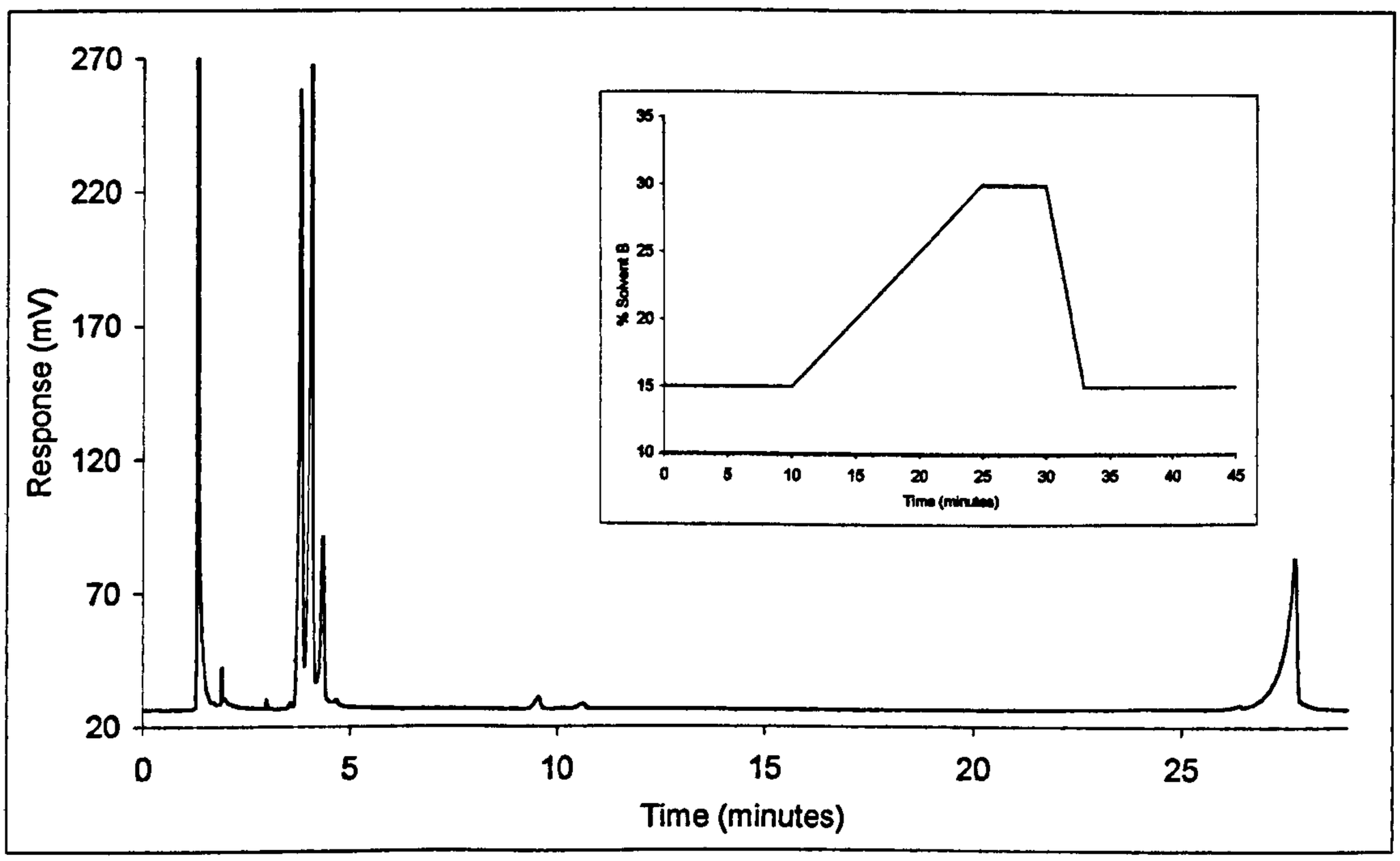

Figure 3.19: Chromatogram obtained from the gradient elution analysis of a standard containing the commercial HEQ sample and its diol intermediate.
Conditions – Column: 150 × 4.6 mm i.d. 5 µm M6; Solvents: A – hexane modified with 25 mmol/l TFA, B – 1.5:1: MeOH : THF modified with 25 mmol/l TFA.

Consideration of the above results showed that the methodology had potential to be adapted to quantify parent diesters and their biointermediates in a single analysis. However, it was clear that accurate quantitation of the diol species would be difficult

during trace environmental analysis due to the severe fronting that was still evident for the analyte.

3.5.4 Improving method sensitivity and compatibility with mass spectrometry

It was mentioned in **Section 1.3.2.1** that detection limits are improved in liquid chromatography by reducing the internal diameter (i.d.) of the column on which the separation takes place. The analyte bands elute in reduced peak volumes, giving rise to more concentrated bands and increased peak height responses (Hewlett Packard, 1997), and as the linear velocity is consistent, peak area response is also increased (Hewlett Packard, 1997). An additional benefit of performing separations on narrow-bore columns is that the reduced mobile phase flow rate improves the compatibility of the LC method with electrospray mass spectrometry (ESI-MS). The response of the detector is increased and interface dynamics are improved (Abian *et al.*, 1999) **(Section 1.3.2.1)**.

In order to improve the limit of detection (LOD) of the new NP-LC methodology and facilitate its hyphenation with ESI-MS, a study was undertaken to assess the effect of reducing column i.d. on the peak parameters of the diester and monoester quats. Three aminopropyl bonded stationary phases were evaluated with internal diameters of 4.6, 2.0 and 1.0 mm. Due to the cost implications of purchasing three Spherisorb columns an alternative stationary phase was sought that could be obtained at reduced expense. A review of commercial literature revealed that the Sphereclone material was marketed by Phenomenex (Macclesfield, UK) as a suitable replacement column for methods developed and validated on Spherisorb. Indeed the manufacturers went as far as to guarantee equivalent performance from this material, under identical conditions (Phenomenex, 2001). As the Sphereclone columns were found to retail for less than equivalent Spherisorb phases, three Sphereclone columns were used for the assessment of the effect of column i.d.

Whilst manufacturers' information may have shown that the Spherisorb and Sphereclone materials yielded identical chromatography, it was soon apparent that experimental observations did not support these claims in the case of the new NP-LC methodology. It was noted that a longer equilibration period was required for the

Sphereclone columns in comparison to the Spherisorb columns. In addition, the replacement of the Spherisorb material with the Sphereclone material led to a reduction in resolution and retention. As a result, the eluting strength of the mobile phase was reduced to bring the resolution and elution times of the tenside components in line with those witnessed with the Spherisorb material. Nevertheless, analogous separations of the three parent diester quat samples, and the two quaternary amino-alcohol samples **(Appendix One)** were ultimately achieved on the new Sphereclone aminopropyl phases.

Figure 3.20 shows a comparison of the chromatograms obtained on the 4.6 mm i.d. and 2.0 mm i.d. Sphereclone aminopropyl phases. It was evident that the peak areas of the three main diester components were significantly increased on the narrow bore column. Subsequent comparison of the peak area responses revealed that the use of the 2.0 mm i.d. column gave rise to an approximate five-fold increase in sensitivity with little associated loss in resolution.

The successful evaluation of the 2.0 mm i.d. column led to the hope that the use of the 1.0 mm i.d. would yield further improvements in both sensitivity and ESI-MS compatibility. Unfortunately, subsequent analysis of the commercial HEQ sample on this phase revealed that the diester components eluted as a broad inefficient peak, whilst the monoesters were barely visible above the baseline. It was subsequently discovered that the problems experienced with the 1.0 mm i.d. column could be traced to the use of instrumentation designed for industry-standard 4.6 mm i.d. columns. Whilst the quaternary HPLC pump **(Section 2.4.1.2)** was capable of delivering a reproducible flow of 50 µl/min, problems were experienced with the performance of the commercial autosampler and the evaporative light scattering detector.

The problems experienced with the autosampler came from an observed inability of the instrument to accurately meter uptake and delivery of a 1 µl injection plug. The utilisation of a 5 µl injection volume led to volume overload occurring on the column (Meyer, 1993), and excessive broadening of the analyte band within the injection port. However, the most serious problems experienced during the evaluation of the 1.0 mm i.d. column were traced to the evaporative light scattering detector. Although promotional literature suggested that the instrument could be utilised with mobile flow

rates of less than or equal to 100 µl/min, an inconsistent sputtering aerosol was witnessed emerging from the nebuliser. Advice obtained from the instrument vendor suggested that the response of the detector was known to fall-off when used in collaboration with a flow of below 150 µl/min (Anachem, 2000).

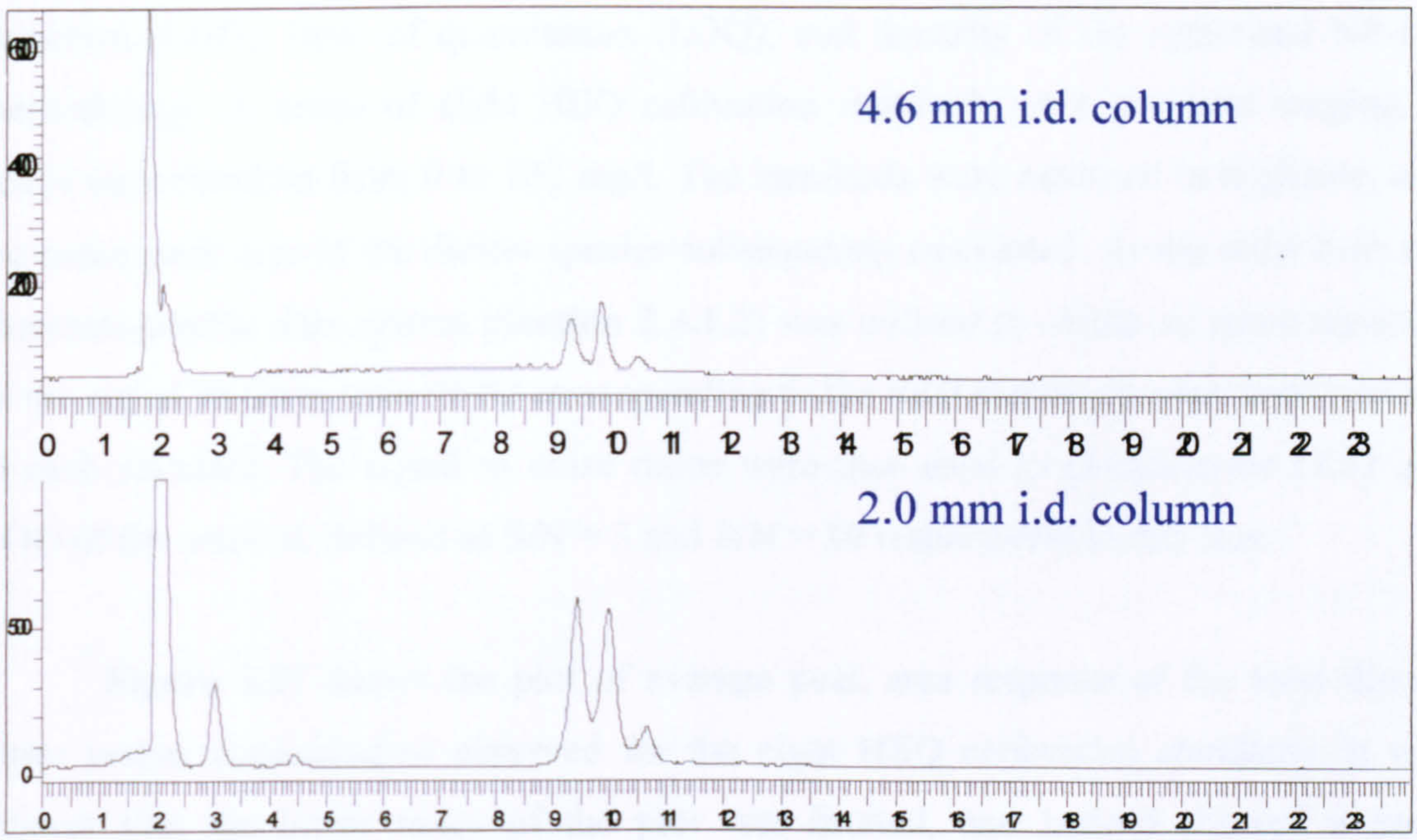

Figure 3.20: Figure showing the improvement in peak area response that was afforded by reducing the internal diameter of the aminopropyl column from 4.6 mm to 2.0 mm.
Conditions – Column: Top - 150 × 4.6 mm i.d. 3 µm Sphereclone aminopropyl, bottom - 150 × 2.0 mm i.d. 3 µm Sphereclone aminopropyl; Mobile phase: 90:6:4 hexane : MeOH : THF modified with 5 mmol/l TFA; Flow rate: 4.6 mm i.d. column – 1000 µl/min, 2.0 mm i.d. column – 190 µl/min.

In view of the problems experienced during the evaluation of the 1.0 mm i.d. Sphereclone aminopropyl column, and the increased sensitivity obtained in comparison with conventional 4.6 mm i.d. columns, 2.0 mm i.d. was adopted as the column dimension in the optimised method.

3.5.5 Evaluation of the sensitivity and robustness of the optimised liquid chromatographic methodology

The new normal phase LC methodology had been seen to offer significant benefits over both the Unilever SOP methodology and the updated methodology described by Radke *et al.* (1999). In order to test the validity of the methodology for environmental analysis and quality control protocols, the robustness, reproducibility, and sensitivity of the method were subsequently evaluated.

3.5.5.1 Determining the limit of detection and method linearity

Evaporative light scattering detectors are known to suffer from an inherent lack of sensitivity and linearity in comparison with more conventional UV / vis and fluorescent detectors. As a result, a study was undertaken to evaluate the limit of detection (LOD), limit of quantitation (LOQ), and linearity of the optimised NP-LC methodology. A series of eight HEQ calibration standards were prepared ranging in active concentration from 0 to 100 mg/l. The standards were analysed in triplicate, and the mean peak area of the diester species subsequently calculated. At the same time the chromatographic data system (**Section 2.4.1.2**) was utilised to obtain an approximation of the signal to noise ratio (S/N) corresponding to the total diester concentration present in each standard. The signal to noise ratios were then used to calculate the LOD and LOQ of the method, defined as S/N = 3 and S/N = 10 respectively in this case.

Figure 3.21 shows the plot of average peak area response of the total diester quats versus concentration observed for the eight HEQ calibration standards. It was evident that the linear range of the plot was limited, and instead showed a good approximation to a quadratic. Whilst the non-linear nature of the plot was not ideal, it was expected in light of previous work performed on the detector (Burford, 2000).

Observation of the signal to noise ratios of the eight calibration standards revealed that both the LOD and LOQ lay between 5 and 10 mg/l, further experimentation revealing the LOD to be 6 mg/l and the LOQ to be 9 mg/l. It must be noted that these values can only be considered as estimates, as the splitting of the diesters into three component peaks (**Section 3.4**) made accurate determination of the signal to noise ratio difficult. Nonetheless, it was apparent that in its current form, the new method lacked the inherent sensitivity necessary to make it useful for environmental analysis. A LOD of 1 mg/l is usually deemed necessary for an environmental methodology (Burford, 2000), as this facilitates a LOD in the low parts per billion range when used in combination with a suitable extraction and pre-concentration method. This level of sensitivity also accounts for the expected loss in method sensitivity witnessed during the analysis of complex environmental matrices (Burford, 2000).

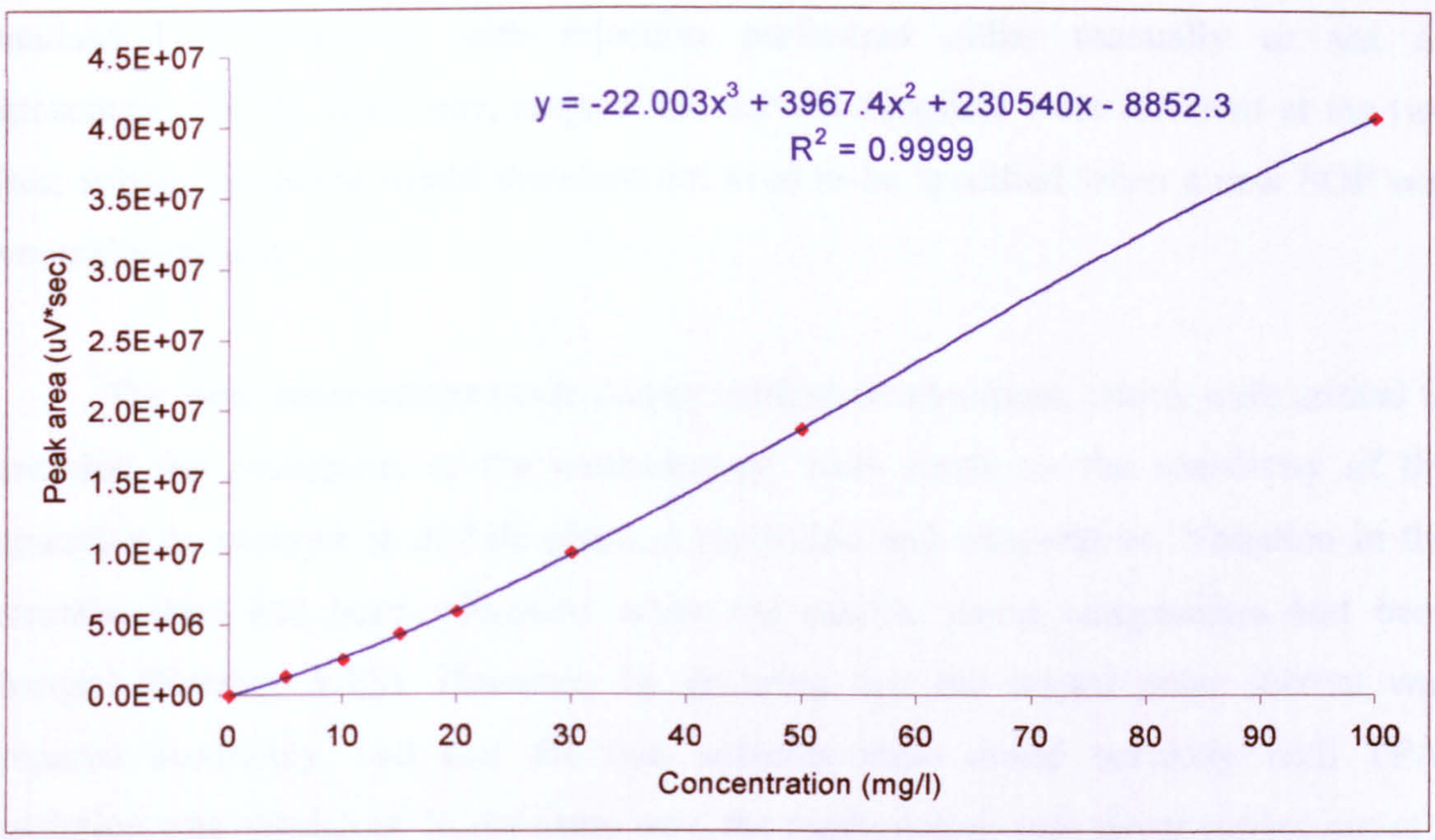

Figure 3.21: Graph showing the non-linearity of the Sedex 55 evaporative light scattering detector to increasing HEQ concentration.

3.5.5.2 Method validation

Assessment of the robustness and reproducibility of the new NP-LC methodology was made by means of a formal validation protocol. However, prior to its commencement it was predicted that the separation was reproducible and the method robust in view of the *"informal"* validation, which had been on going throughout the development of the method. In **Section 3.3.1** it was mentioned that during the original evaluation of the poly-ol stationary phase, retention time stability had been seen to be excellent over a number of weeks. Similar reproducibility was subsequently witnessed with the mixed-mode phases (**Section 3.3.2.2**) and with the commercial aminopropyl phase. Indeed the retention time stability of the aminopropyl phase was seen to be excellent throughout the six-month period that was dedicated to method optimisation. During this time a number of Spherisorb aminopropyl columns were utilised, all of which gave rise to equivalent separations, thus showing that the methodology was not based solely on the merits of a single *"deactivated"* column.

The second observation made during the informal validation process was that the separation achieved at the University of Leeds was recreated at Unilever Research without changes being made to the methodology. The methodology had therefore been seen to pass an inter-laboratory transfer test. At the same time, the instrumentation used

at Leeds and Unilever was very different, the method could apparently be performed on standard LC equipment, with injection performed either manually or via an autosampler. In the same way, reagent and solvent suppliers were different at the two sites; solvent suppliers would therefore not need to be specified when a new SOP was eventually created.

The final observations made during method development, which were critical to assessing the robustness of the methodology, were made on the sensitivity of the separation to changes in mobile phase composition and temperature. Variation in the retention time had been witnessed when the mobile phase composition had been changed (**Section 3.3.3**). However, by ensuring that the mixed polar solvent was prepared accurately, and that the two solvents were dosed correctly with TFA, resolution was consistent. In the same way, the methodology was never carried out at a fixed column temperature, and was instead performed in ambient laboratory conditions. As a change in retention time was only witnessed once during method development, and then only under extreme conditions in which laboratory temperature had fallen to less than 10°C, it was concluded that the methodology was insensitive to small changes in temperature.

Having observed excellent reproducibility and robustness during method development, the formal validation study aimed to mathematically quantify the degree of variation witnessed in the retention times and peak area responses of the major components. A series of six HEQ calibration standards were prepared ranging in concentration from 10 to 100 mg/l. Over three consecutive days a total of fifty two analyses of the various HEQ standards were performed. The chromatographic data corresponding to each analysis was then transferred into the System Suitability programme on the Turbochrom chromatographic data system (**Section 2.4.1.2**) to assess the variation in the retention times and peak area responses of each component.

The percentage relative standard deviation (%RSD) of the retention times of the three principle diester and two monoester components during the fifty two analyses was seen to be less than 1.10. This value highlighted the excellent reproducibility of the methodology. Retention was seen to be consistent over time and showed no variation with analyte concentration, a fact that was in stark contrast to the changing retention

times witnessed with varying analyte concentration in the Unilever SOP method (Lawrence, 1999). When the reproducibility of the peak area response was subsequently evaluated, it was found that the %RSD for the total diester concentration was less than 1.49, whilst the %RSD's for the three individual components were less than 2.40. It was apparent that the new methodology demonstrated reproducible peak area responses.

Though the new methodology facilitated high retention time stability and high resolution, the inherent lack of sensitivity prohibited the use of the methodology for environmental analysis in its current form. However, no such problems were envisaged during the evaluation of raw materials and the analysis of industrial samples. In both of these scenarios analyte concentration would be above trace levels and thus combination of the NP-LC methodology and a suitable extraction procedure was predicted to yield a method suitable for use in quality control protocols, assuming calibration was carefully controlled in light of the observed non-linearity.

3.5.6 Development of the hyphenated LC/MS methodology

The lack of sensitivity witnessed during the evaluation of the NP-LC-ELSD methodology limited its use in the quantitation of cationic fabric conditioner actives in environmental matrices. As a result, an alternative detection method was sourced that could offer greater sensitivity. In **Section 1.4.4** it was described how a number of groups had improved the sensitivity and selectivity of NP-LC methods for the determination of cationic fabric conditioner actives by hyphenating the methods with electrospray ionisation mass spectrometry (ESI-MS) (Fernandez *et al.*, 1996; Radke *et al.*, 1999). Utilisation of the hyphenated methodology facilitated structural confirmation of the components present in each of the samples studied, and also yielded quantitation of the tenside analytes at sub parts per billion concentrations (Radke *et al.*, 1999). Therefore, in an attempt to improve the selectivity, specificity and sensitivity of the methodology, particularly for environmental analysis, attempts were made to hyphenate the new LC methodology with electrospray mass spectrometry.

3.5.6.1 Direct infusion analysis

The initial mass spectral assessment of the cationic tenside samples was performed with the aid of direct infusion analysis (**Section 2.4.2**). **Figure 3.22** shows the mass spectrum obtained from the direct infusion of the commercial HEQ sample into the ESI interface at a flow rate of 3 µl/min. The mass spectral parameters used during the acquisition were obtained with the automatic tune facility in the LCQ instrument control software (**Section 2.4.2**).

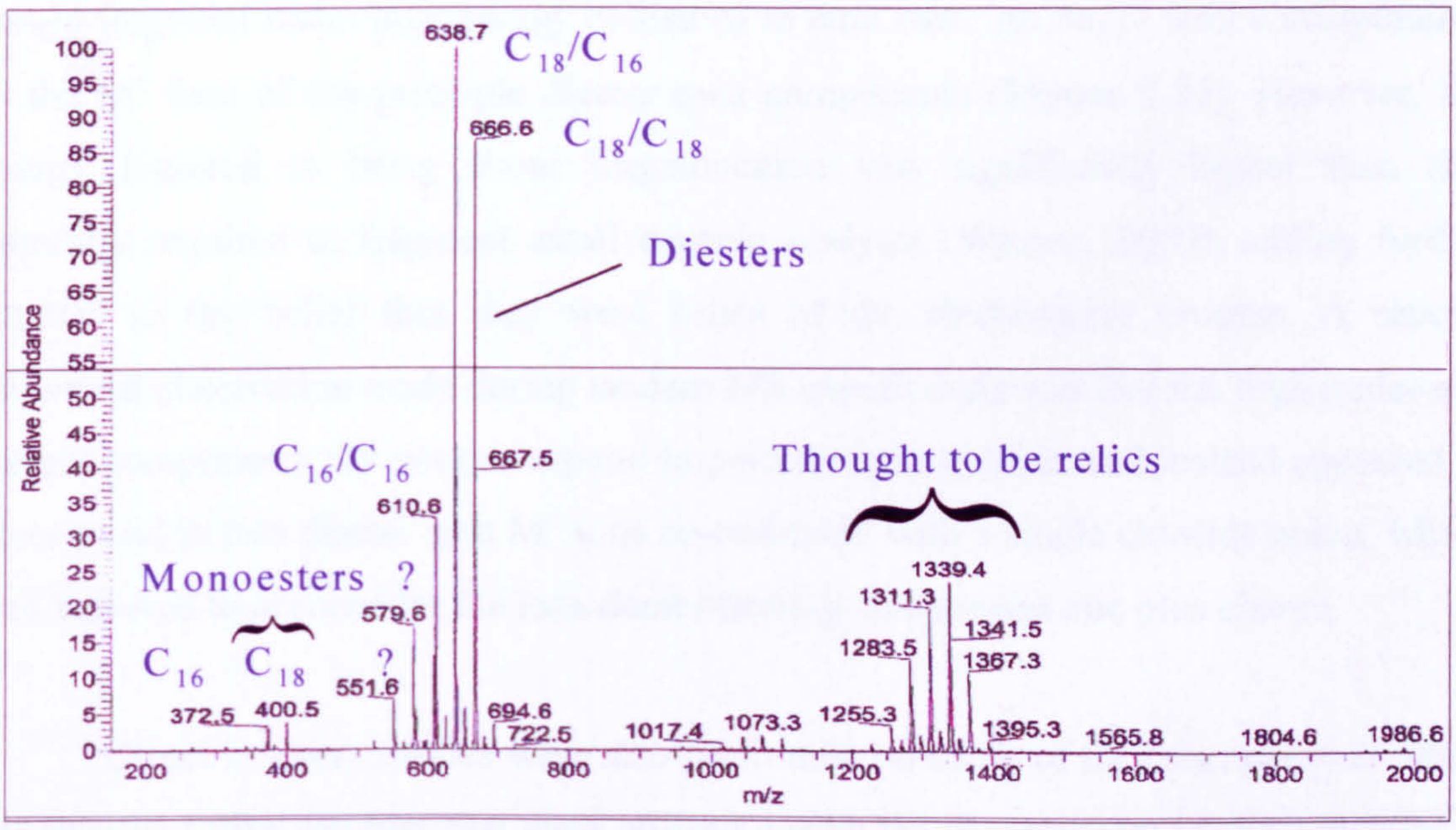

Figure 3.22: Mass spectrum obtained from the direct infusion of the HEQ sample into the Finnigan MAT LCQ instrument after MS optimisation.
Infusion rate: 3 µl/min.

The two principle ions witnessed in the mass spectra, the base peak at $m/z = 638.7$ and a ninety percent abundant ion at $m/z = 666.8$ were seen to correspond to the molecular ions (M^+) of the C_{18} / C_{16} and C_{18} / C_{18} diester components respectively, which had been expected from previous work carried out on the mass spectral analysis of HEQ (Burford, 1999; Radke *et al.*, 1999; Waters *et al.*, 2000; Weston, 2000). Similarly, the ions witnessed at m/z = 610.6, 400.5, and 372.5 were known to correspond to the C_{16} / C_{16} diester, the C_{18} monoester, and the C_{16} monoester respectively (Radke *et al.*, 1999). However, what was unexpected was the group of peaks witnessed between m/z = 1255 and 1395. These high molecular weight ions had not been observed previously during the analysis of HEQ by mass spectrometry (Weston, 2000), and indeed, characterisation data supplied by Unilever Research

suggested that components corresponding to this molecular weight range did not exist in the commercial sample (**Appendix One**). It was therefore hypothesised that these high molecular weight ions were relics of the electrospray process, being generated *in-situ*, and therefore were not native constituents of the sample (Weston, 2000). Further scrutiny of the distribution and mass to charge ratios of the ions revealed that they may have been dimeric adducts of the diester quats. However, the variation in the mass to charge ratios between adjacent ions showed that they were not doubly charged species. Tandem MS studies performed on the high molecular weight ions revealed that they would fragment under high energy collisions to emit two *"product"* ions corresponding to the M^+ ions of the principle diester quat components (**Figure 3.23**). However, the energy required to bring about fragmentation was significantly higher than that normally required to fragment small organic analytes (Weston, 2000), adding further support to the belief that they were relics of the electrospray process. A second important observation made during tandem MS experiments was that the high molecular weight components did not correspond to pure dimeric species, and instead appeared to correspond to two diester quat M^+ ions co-ordinated with a single chloride anion, which was believed to account for the ions demonstrating an apparent one plus charge.

Direct infusion studies were also performed on many of the other cationic fabric conditioner active samples that were utilised during the development of the new NP-LC methodology. Peak identities assigned during **Section 3.4** were subsequently confirmed by mass spectral identification.

3.5.6.2 Loop injection analysis

Prior to the assessment of the fully hyphenated LC/MS system, loop injection analysis of a number of cationic tenside samples was performed to assess the effect of the LC mobile phase system on the ionisation efficiency of the analytes and the electrospray interface.

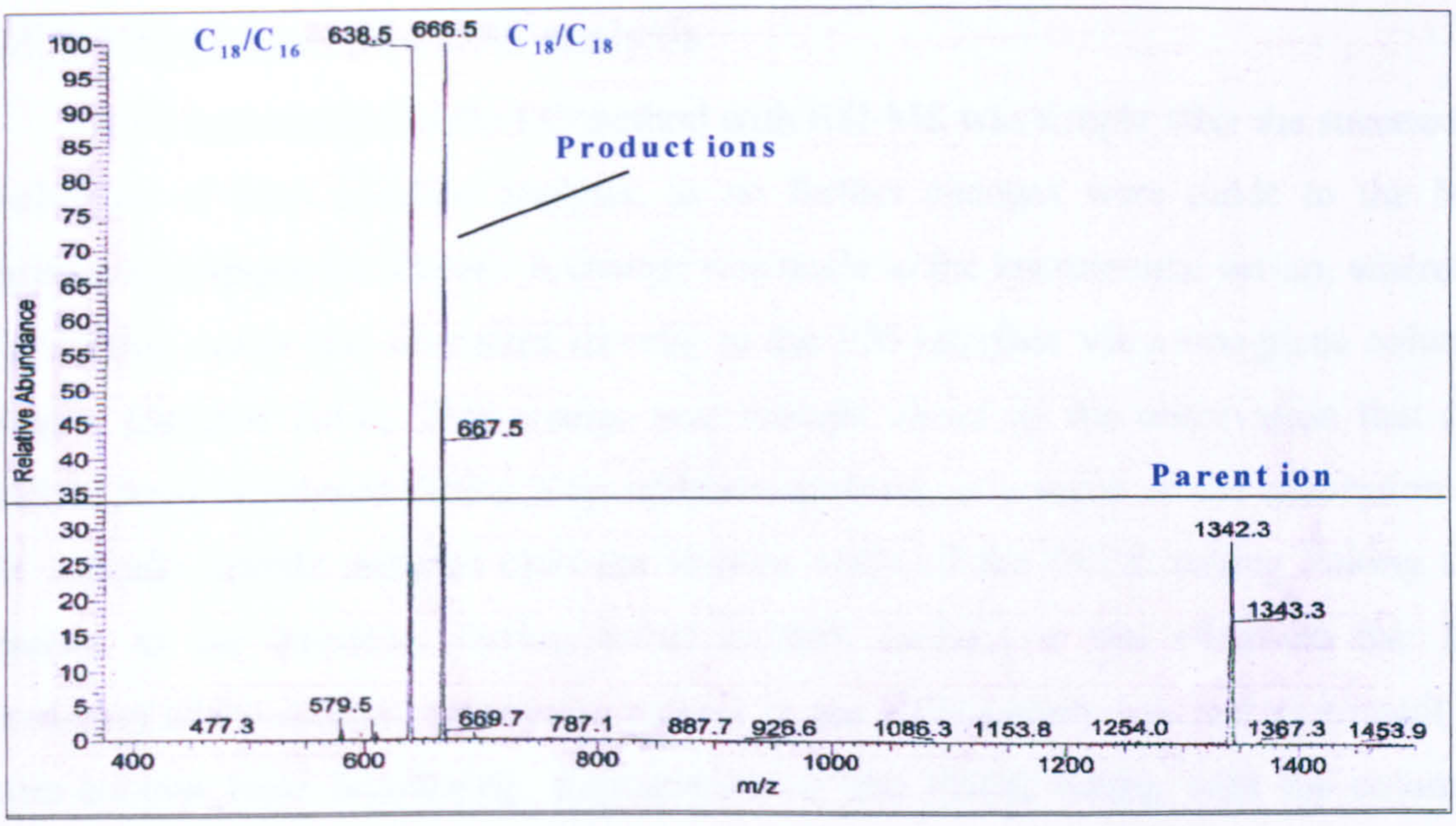

Figure 3.23: Figure showing the dimeric nature of the high molecular weight ions observed during the direct infusion analysis of the commercial HEQ sample.
Infusion rate: 3 µl/min; Trapped ions: m/z = 1340 – 1343; Relative collision energy: 100%.

For tuning purposes, a poly (ether (ether) ketone) (PEEK) T-piece was inserted into the instrumental set-up between the injector on the LC instrument and the inlet to the ESI interface. In this way, the integral syringe pump on the LCQ instrument (**Section 2.4.2**) could be used to constantly infuse the cationic tenside sample into the flowing LC mobile phase to efficiently optimise the MS response. The optimised MS parameters utilised throughout the loop injection studies are shown in **Appendix Three**.

Loop injection analysis was performed with the same instrumental set-up that would subsequently be used for the evaluation of the hyphenated LC/MS system (**Section 2.4.2**). However, the HPLC column was omitted from the set-up and thus the outlet of the injector port was connected directly to the inlet of electrospray interface via a length of PEEK tubing. Observation of the results obtained during loop injection analysis revealed analogous mass spectra were produced to those witnessed during direct infusion studies. However, it was evident that there was no longer any evidence of the high molecular weight ions detailed above, further support for the theory that these ions had been relics of the electrospray process. It was therefore concluded that the LC mobile phase was leading to subtle changes in the electrospray process. However, these changes did not appear to adversely affect the ionisation efficiency of the analytes.

3.5.6.3 Hyphenated LC/MS analysis

Full hyphenation of the LC method with ESI-MS was simple after the successful evaluation of loop injection analysis, as no further changes were made to the MS parameters (**Appendix Three**). A change was made to the instrumental set-up, whereby the column outlet was connected directly to the ESI interface via a one-piece column coupler (**Section 2.4.2**). This change was brought about by the observation that the analyte band broadened during loop injection analysis, as a result of the adsorption of the cationic tenside analytes onto the interior walls of the PEEK tubing linking the injector to the interface. During initial LC/MS analysis it was observed that the resolution of the diester components present in the HEQ sample was lost as a result of extra-column band broadening. Replacement of the PEEK tubing with the column-coupler was seen to resolve this problem.

Figure 3.24 shows the reconstructed ion chromatograms (RIC's) of the M^+ ions of the C_{18} / C_{18}, C_{18} / C_{16}, and C_{16} / C_{16} diester components (*m/z* = 666.5, 638.5 and 610.5) obtained from the LC/MS analysis of the HEQ sample. When the RIC's were compared with the total ion chromatogram (TIC) obtained from the same analysis (**Figure 3.25** shows the TIC from a later analysis when retention times had stabilised), it was apparent that the three main diester peaks separated by the front-end LC system, had been correctly identified in **Section 3.4**. In addition, the RIC's showed that the front-end LC separation was efficiently resolving the individual tenside components into discrete peaks, i.e. analyte carry-over and late elution were minimised. It was therefore concluded that the new NP-LC method represented an efficient means of resolving the individual diester and monoester components of the HEQ sample into discrete peaks. At the same time, it was envisaged that the use of the RIC's would provide an effective means of quantifying the individual cationic tenside components present in a commercial sample, if and when the sensitivity provided by ELS detection was found wanting.

Comparison of the RIC's of *m/z* = 694 and 582 (not shown), the M^+ ions of the C_{20} / C_{18} and C_{16} / C_{14} diester quats with the TIC, revealed that these two species corresponded to the two small peaks eluting prior to, and subsequent to the three principle diester components (**Figure 3.25**). Mass spectral evaluation of the two late

eluting peaks separated by the NP-LC method also confirmed that these were the C_{18} and C_{16} monoester quat components (m/z = 400.5 and 372.5) respectively.

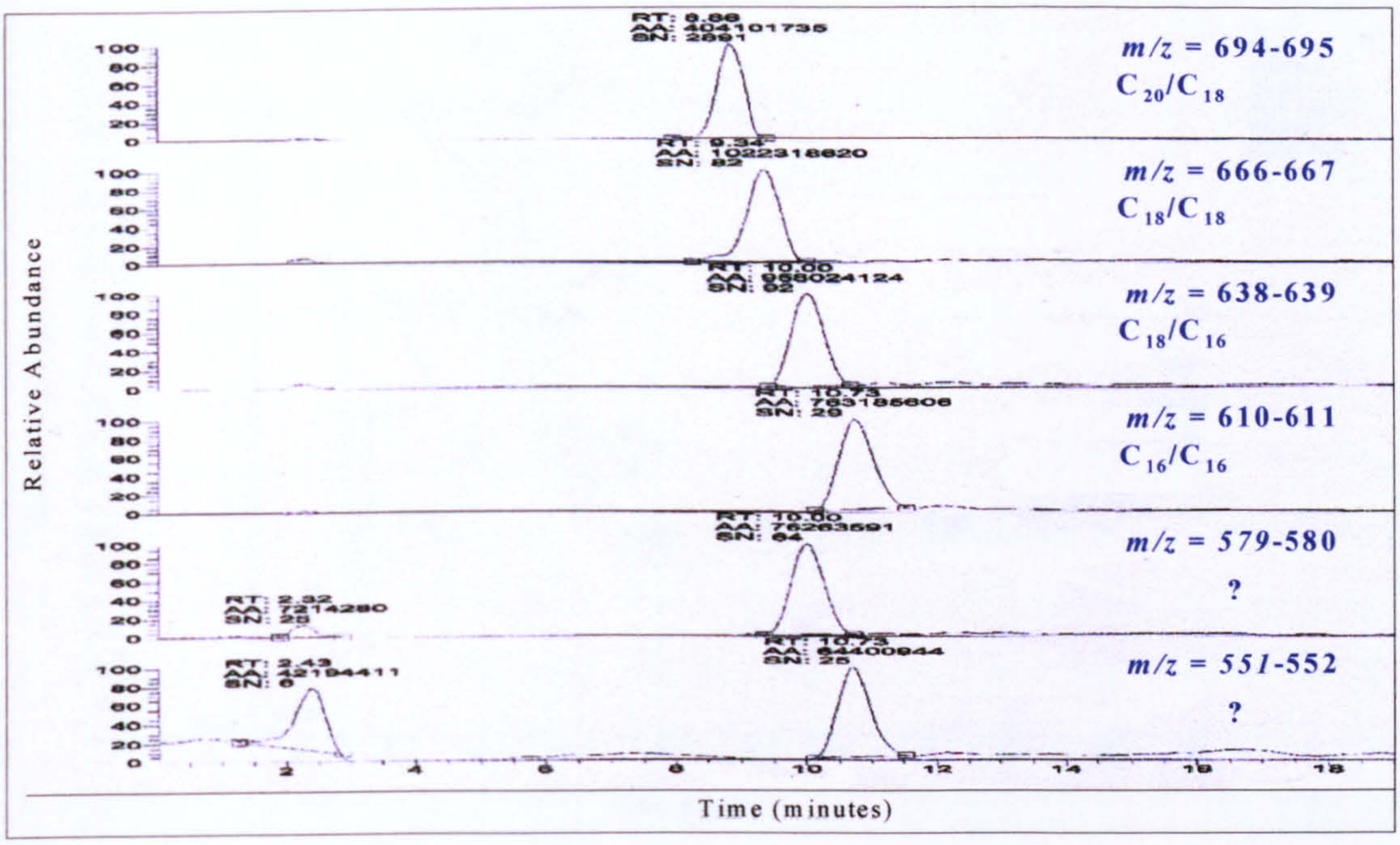

Figure 3.24: Reconstructed ion chromatograms of the major diester species and two unknown resulting from the LC/MS analysis of the commercial HEQ sample.
Conditions – Column: 150 × 2.0 mm i.d. 3μm Sphereclone aminopropyl; Mobile phase: 90:6:4 hexane : MeOH : THF modified with 5 mmol/l TFA; Flow rate: 190 μl/min.

The hyphenated LC/MS methodology was subsequently utilised to study many of the other cationic tenside samples listed in **Appendix One**. In each case, LC/MS analysis confirmed the validity of the pre-assigned peak identities (**Section 3.4** and **3.5.1**). It was observed that the hyphenated method was also well suited to the analysis of the DEEDMAC, Arquad and Stepantex samples, as well as the individual ester and alkyl quats (**Appendix One**). The quaternary amino-alcohols were not studied with the method.

The observations made during the evaluation of the new hyphenated NP-LC/ESI-MS methodology indicated that it might be well suited to the analysis of cationic fabric conditioner actives. Unfortunately due to time constraints it was not possible to evaluate the sensitivity and linearity of the methodology, or indeed to perform a formal validation. It was envisaged that a formal validation of the methodology, and assessment of the suitability of the methodology to the analysis of the

quaternary amino-alcohols, would be required in the near future to more fully assess its applicability to environmental analysis.

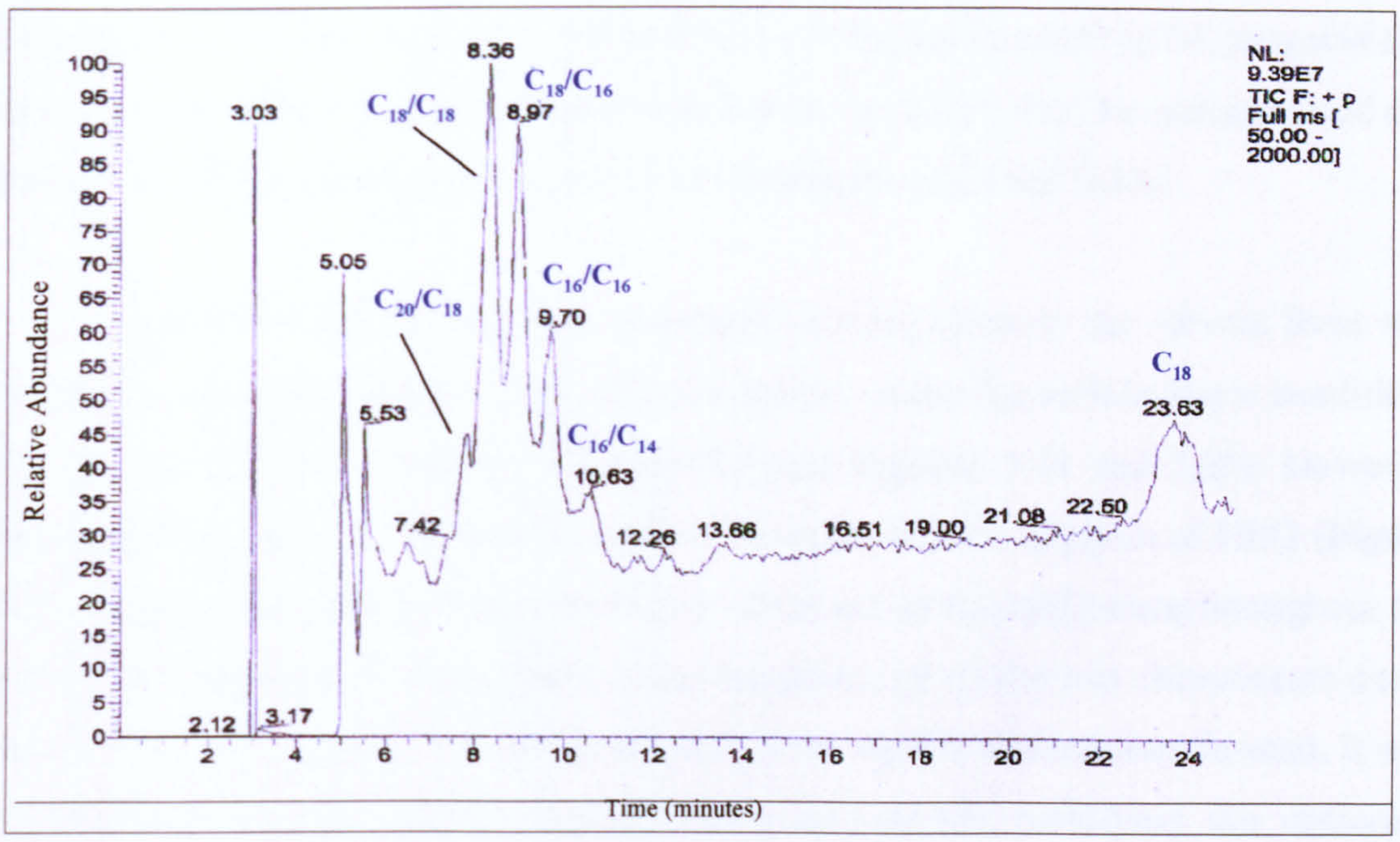

Figure 3.25: Total ion chromatogram resulting from the LC/MS analysis of the HEQ sample. Conditions – Column: 150 × 2.0 mm i.d. 3µm Sphereclone aminopropyl; Mobile phase: 90:6:4 hexane : MeOH : THF modified with 5 mmol/l TFA; Flow rate: 190 µl/min.

3.5.6.4 Dynamic modification of the stationary phase and analyte bleed

The two additional RIC's shown in **Figure 3.24** corresponded to two unknown ions witnessed during direct infusion studies (**Figure 3.22**), *m/z* = 579 and 551. As literature reports had not previously noted the existence of these two species in the commercial HEQ sample (Radke *et al.*, 1999; Waters *et al.*, 2000), it was originally assumed that the ions were additional relics of the electrospray process (**Section 3.5.6.1**). However, observation of these ions during loop injection and LC/MS analysis, where the high molecular weight ions (**Section 3.5.6.2**) were no longer evident, brought this hypothesis into doubt.

Having been unsuccessful in attributing the two ions with known impurities of the HEQ sample, attention turned to the origin of the ions being the presence of other cationic tenside analytes in the sample. It was soon realised that *m/z* = 579 and 551 corresponded to the M^+ ions of the C_{20} / C_{18} and C_{18} / C_{18} dialkyl quats (**Section 3.4**). Scrutiny of the characterisation data supplied with the HEQ sample suggested there was little evidence of the existence of these tensides in the HEQ sample (**Appendix One**),

and thus it was assumed that the sample of HEQ had become adulterated and / or the adsorption of the cationic tensides onto the interior of the ESI interface was leading to fouling and subsequent analyte carry-over (Cooper, 2000). However, subsequent comparison of the RIC's of *m/z* = 579 and 551 with the corresponding TIC revealed that the two components were eluting at two points in the trace; after the solvent front and between the three principle diester components (**Figures 3.22** and **3.24**).

The observation of the two components eluting close to the solvent front was attributed to the short elution times of these species under the mobile phase conditions utilised during LC/MS analysis (**Section 3.4** and **Figures 3.11** and **3.25**). However, examination of the chromatograms obtained from the NP-LC analysis of HEQ (**Figure 3.13**) revealed the same impurity had been witnessed in the HEQ trace throughout the method development. It was apparent that the existence of the two components could not be attributed to either the MS instrument or to sample adulteration. Instead, it was hypothesised that the cationic tensides were dynamically modifying the stationary phase, and in addition, a number of the analytes were liberating other bound analytes via an exchange process.

Whilst it proved impossible to ascertain whether this hypothesis was correct, it was believed that support for the theory was forthcoming from two areas. Firstly, it was observed that at the commencement of a study, a number of replicate injections were required before consistent retention was attained (**Section 3.5.4**). In the case of the Spherisorb material, it was observed that this process was only required during the use of a new column, whilst the Sphereclone material required this procedure to be performed whenever the system was halted. It was predicted that the shortening of retention times came about as a result of the dynamic modification of the active sites on the stationary phase (Nawrocki, 1997), stable retention being achieved when the most acidic sites were blocked. The difference in the equilibration time of the Sphereclone and Spherisorb materials was believed to provide evidence that the two silica supports behaved differently (**Section 3.5.4**).

Support for the dynamic exchange of the cationic tensides on the silica support and the continuous *"bleed"* of previously bound analytes was provided by the LC/MS work. The observation of the dialkyl quats in a trace corresponding to a commercial

esterquat sample could only have come about by the removal of a number of the dialkyl quats bound to the silica substrate during a previous analysis, having been confident that the sample had not been adulterated. More significantly the dialkyl quats were also seen eluting in the middle of the diester quats. The greater hydrophobicity of the dialkyl quats should have resulted in them demonstrating a much shorter retention time if they had been present in the analyte band at the point of injection. Their elution with the diester quats could therefore only be justified by the fact that the dialkyl quats were being desorbed from the silica substrate *in-situ*.

Validation of the hypotheses on dynamic modification of the stationary phase, and subsequent analyte bleed, was not forthcoming before work on the new NP-LC methodology ceased. However, in view of the fact that analyte bleed was probably occurring continuously, caution must be observed if and when the methodology is used for quantitation purposes. It is important that the extent to which the stationary phase is modified *in-situ* be fully assessed in the future.

3.6 PROBING THE RETENTION MECHANISM

The new NP-LC methodology developed for the analysis of cationic fabric conditioner actives had been shown to offer superior resolution (**Section 3.4**), reproducibility, and robustness (**Sections 3.5.5.1** and **3.5.5.2**) to that achieved with the Unilever SOP method (**Section 3.2**), the commonly used methodology of Wee *et al.* (1982), and a recent method developed by Radke *et al.* (1999). However, problems were experienced when the new methodology was applied to the analysis of the quaternary amino-alcohols (**Section 3.5.3**). Co-injection analysis and subsequent LC/MS analysis revealed that the new methodology could resolve the different groups of cationic tenside in a commercial sample (**Figure 3.14**), partially resolve the homologous series present in each group (**Figure 3.8**), resolve components from different tenside samples (**Figure 3.16**), and separate isomeric species (**Figure 3.10**). However, in spite of the efficient resolution of the cationic tensides questions still remained: *"How and why was the resolution occurring?" "Could the resolution be improved still further?"* and finally *"Could the methodology be optimised to yield efficient analysis of the quaternary amino-alcohols?"* It was envisaged that answers to the above questions would be

forthcoming from an understanding of the mechanism by which the cationic tenside analytes were being retained on column.

3.6.1 Assessing the influence of the stationary phase

During the development of the hexane-based NP-LC methodology a series of different stationary phases were evaluated. Whilst many of them were seen to give rise to analogous separations, it was realised that the subtle differences in resolution could be used to shed light on the retention mechanism. For example, during the evaluation of the supercritically bonded mixed-mode phases, it became apparent that superior resolution was achieved on the aminopropyl phases as the percentage of amino groups increased (**Section 3.3.2.2**). At the same time, resolution on the alkyl-amino phases (**Appendix Two**) was higher than that achieved on the corresponding aminopropyl phases (**Section 3.3.2.3**). It appeared that as the number of amine groups attached to the silica surface increased, there was a general increase in the resolution of the mono and di-chained cationic tensides. This apparent trend was supported by the observation that efficient resolution of the cationic tensides could be achieved on a commercial aminopropyl bonded phase (**Figure 3.13**), yet no evidence of the cationic tensides could be seen when the same sample was subsequently analysed on a commercial cyanopropyl phase. A reduction in resolution was subsequently observed when the HEQ sample was analysed on a mixed-mode aminopropyl / octadecylsilane (ODS) bonded phase (**Figure 3.26**). The diester quats were evidently eluting as a single peak.

Comparison of the observations made on all of the stationary phases revealed that the presence of hydroxyl groups or amine groups bonded to the silica substrate, facilitated the resolution of the homologous series present in each group of cationic tensides. As the number of amino and hydroxyl groups increased, a general increase was witnessed in resolution, yet consistent retention times were witnessed on most of the different phases. A change in the nature of the stationary phase led to negligible effect on analyte retention times but a large effect on resolution. Indeed, increased retention was only witnessed on the Bio-Sil poly-ol and Partisil PAC phases. Both of these phases were based on irregular silica supports (**Appendix Two**) and thus it was feasible that the increased retention came about as a result of the nature of the support material. It was hypothesised that the nature of the bonded phase was only affecting resolution of the analytes, and thus the mode of retention was equivalent on each.

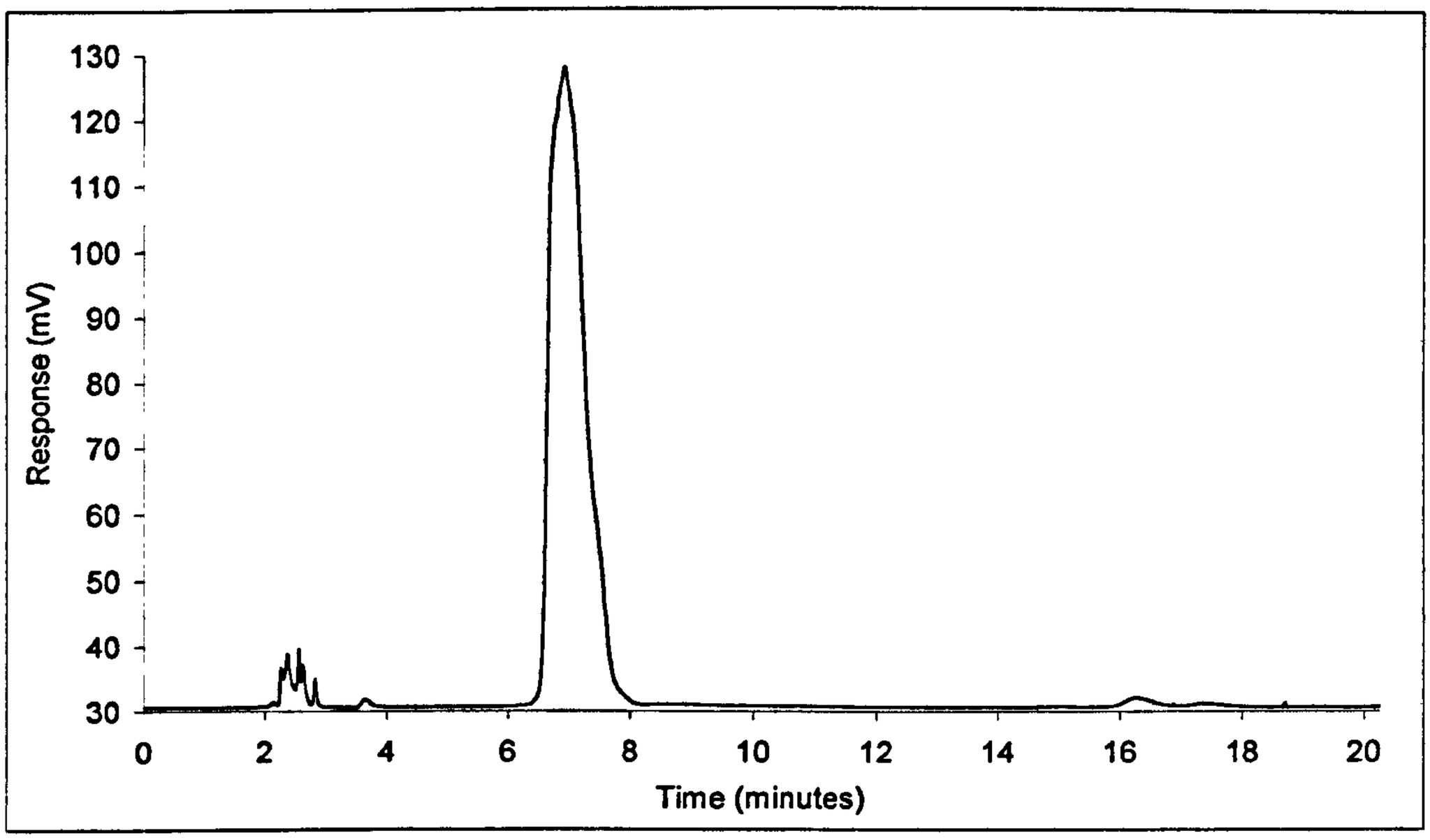

Figure 3.26: Chromatogram obtained from the analysis of the HEQ sample on the Spherisorb mixed mode ODS / NH2 phase.
Conditions – Column: 250 × 4.6 mm i.d. 5 µm Spherisorb mixed-mode aminopropyl / octadecylsilane; Mobile phase: 85:9:6 hexane : MeOH : THF modified with 25 mmol/l TFA; Flow rate: 1 ml/min; Detection method: Evaporative light scattering.

3.6.2 Assessing the influence of the mobile phase

It was stated in **Section 3.3.3** that an evaluation into the effect of varying the methanol : THF ratio and the direct replacement of THF with other organic solvents had led to changes in the resolution of the analytes and / or changes in analyte retention time. It was apparent that the replacement of THF with other organic solvents higher in the eluotropic series of normal phase solvents (Meyer, 1993) (**Section 1.3.2**) had a negligible effect on the retention time, and instead led to a reduction in the resolution of the analytes. This observation appeared to provide strong evidence that the mode of retention was different to the adsorption method usually attributed to normal phase LC (**Section 1.3.2**). In contrast, variation in the methanol : THF ratio led to very significant changes in both analyte retention and resolution. Increasing the proportion of methanol in the mixed polar solvent led to a reduction in analysis time and resolution, as was expected from the utilisation of a stronger solvent. However, when the proportion of THF was increased, a disproportionate increase in retention and resolution was witnessed. When combined, these observations appeared to indicate the need for both methanol and THF in the mobile phase to facilitate efficient resolution of the cationic tensides. The presence of methanol appeared to prevent excessive analysis time, whilst

the THF appeared to facilitate resolution of the tenside homologues. In order to test this hypothesis the HEQ and HEQ diol samples were analysed with a mobile phase system consisting of only hexane and methanol.

Figure 3.27 shows the chromatogram obtained from the analysis of the HEQ sample with a hexane and methanol binary solvent system. The diester quats were seen to elute as a single peak with an unusual band profile similar to those witnessed during the analysis of lycopene by NP-LC on a Partisil PAC phase (Piretti *et al.*, 1996). However, the monoester components were actually seen to show increased resolution in comparison to that witnessed with the ternary mobile phase system (**Figure 3.10**). Subsequent analysis of the commercial DEEDMAC sample resulted in an analogous separation.

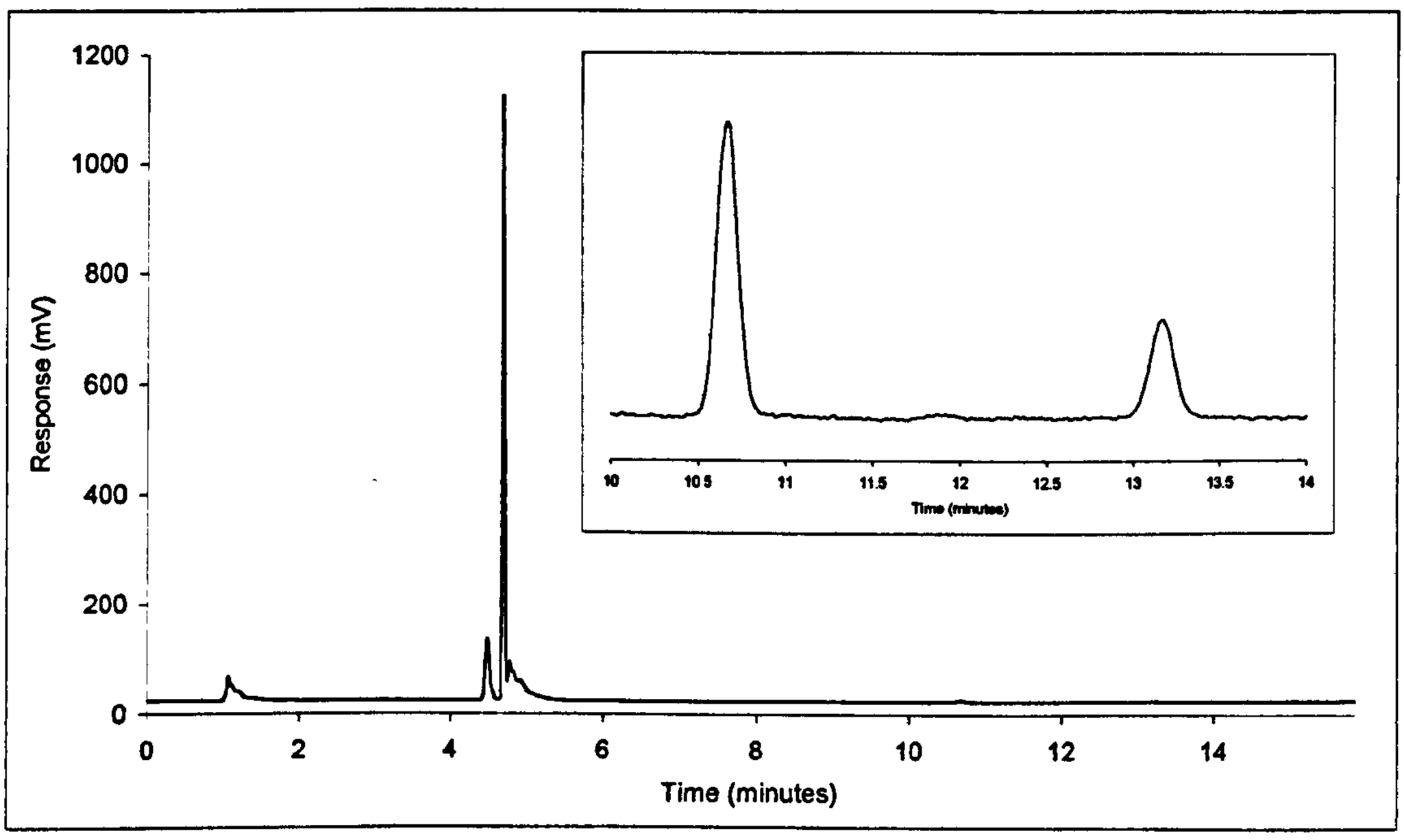

Figure 3.27: Chromatogram obtained from the analysis of the commercial HEQ sample on column M6 with a binary mobile phase system of hexane and methanol.
Conditions – Column: 150 × 4.6 mm i.d. 5 µm M6; Mobile phase: 90:10 hexane : MeOH modified with 25 mmol/l TFA; Flow rate: 1 ml/min; Detection method: Evaporative light scattering.

Figure 3.28 shows the chromatogram obtained from the analysis of the HEQ-derived quaternary amino-alcohol component (**Appendix One**) on the M6 column with a binary solvent system of hexane and methanol. The chromatography of the analyte was again seen to be very different to that witnessed with the ternary solvent system.

From showing severe fronting under the ternary mobile phase system, the analyte was seen to give rise to a tailing peak in the presence of the binary solvent system.

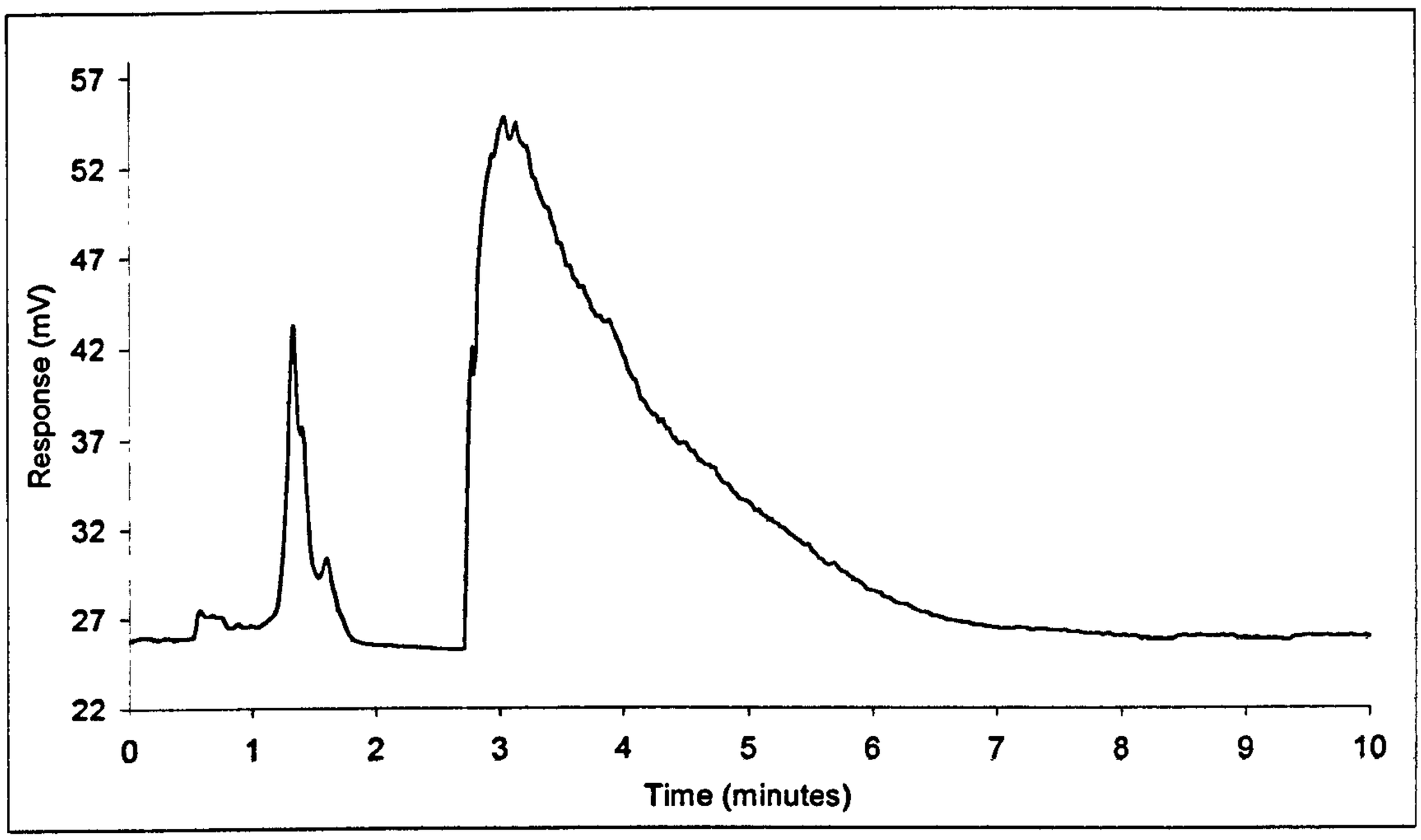

Figure 3.28: Chromatogram showing the analysis of the HEQ-derived diol species on column M6 with a binary mobile phase system of hexane and methanol.
Conditions – Column: 150 × 4.6 mm i.d. 5 µm M6; Mobile phase: 75:25 hexane : MeOH modified with 5 mmol/l TFA; Flow rate: 1 ml/min; Detection method: Evaporative light scattering.

It was evident from the results obtained with the binary solvent system that the diesters, monoesters, and diol components were affected differently by the change in the mobile phase system.

3.6.3 Mechanistic explanation of the experimental observations

Many observations were made during the development and optimisation of the normal phase LC methodology, especially regarding the effect that the nature of the stationary and mobile phases had on the chromatographic profiles of the cationic tenside analytes. Initially, it appeared that many of the observations were contradictory and it would thus prove impossible to develop a justifiable hypothesis to account for all of them. However, the results obtained during the evaluation of the hexane and methanol mobile phase system, implicated a retention mechanism previously described by Kažoka and co-workers (Kažoka *et al.*, 1992 and 1996; Kažoka, 2000).

The above authors studied the effect that changes to the mobile system had on the resolution of a series of purine and pyrimidine analytes separated on a bare silica phase under normal phase conditions (Kažoka *et al.*, 1992 and 1996). The group observed that improved separation was achieved when mutually immiscible solvent systems were employed, attributing these observations to the formation of a stationary solvent in the pores of the silica particles. Retention was thus believed to occur via adsorption onto the surface silanol groups of the silica and partitioning into and out of the stationary liquid (Kažoka, 2000). Importantly, as the ratio of the solvents varied, such that the system was pushed beyond its solubility limit, the extent of partitioning increased, facilitating resolution of the analytes of interest.

In view of the immiscibility of methanol and hexane at the levels used in the new NP-LC methodology (Meyer, 1993) it was hypothesised that a stationary liquid of THF and MeOH had accumulated in the pores of the silica during column equilibration. Whilst this proposal initially appeared to be a little too convenient, it was soon realised that many of the observations made during the development of the methodology supported this hypothesis.

Assuming the presence of stationary-liquid phase, the analytes would have first had to partition into this phase before interacting with the stationary phase. This prediction is evident from the fact that the majority of the bonded phase resides within the pores of the silica (Nawrocki, 1997). As a result, the rate at which the analytes partitioned into and out of the stationary liquid, and the degree to which they interacted with the stationary phase would have governed retention.

Having predicted that retention of the analytes relied on both adsorption and partitioning, it became apparent why a change in stationary phase was seen to have a major effect on the resolution, but minimal effect on the retention of the di-chained quats. The accumulation of polar solvent in the pores of the silica would have led to the solvent within the pores being more polar than that in the bulk, and thus a concentration gradient would have been set-up within the column With the analytes partitioning into and out of the stationary-liquid phase, hydrophobicity would have been seen to have a significant influence on the residence times of the analytes within this phase, which in turn would affect contact with the bonded phase. Under stationary-liquid conditions,

hydrophobic analytes such as the di-chained quats would spend less time in the pores of the silica than more hydrophilic species. Having minimal residence time in the pores, the di-chained quats would also have little potential to interact with the bonded phase, and thus changes in the nature of the bonded phase would have had little effect on the retention times of these analytes, which was evident throughout method development **(Figure 3.5)**.

In light of the mixed adsorption – partition model, the increased resolution of the di-chained analytes witnessed in the presence of increasing numbers of amino groups **(Section 3.3.2.2)**, was believed to derive from adsorption onto the bonded phase. Having shown that resolution of the ester and alkyl quats was maximised in the presence of large numbers of amine or hydroxyl groups **(Section 3.6.1)**, it was predicted that the quaternised nitrogen group would undergo electrostatic interaction with these respective groups. Thus, it appeared that variation in electrostatic interaction was giving rise to homologue resolution, whilst partitioning was bringing about class separation. This hypothesis was in direct contrast to normal theories on liquid chromatography (Dorsey, 1994), which assume that partitioning behaviour generally leads to homologue resolution under reverse phase conditions (Dorsey, 1994). Nevertheless, it was believed that the lack of resolution witnessed during the analysis of the diester quats, with the hexane - methanol solvent system **(Section 3.6.2)** provided support for this theory.

It was hypothesised that in the presence of the binary solvent system a stationary-liquid phase would still exist within the pores of the solvent. However, as methanol was the only solvent now present in the pores, residence times of the di-chained quats were expected to decrease. In addition, the bulk mobile phase was expected to demonstrate increased eluotropic strength due to the higher methanol concentration. These two predictions would then account for the shorter analyte retention times, whilst the reduced residence times of the di-chained quats in the stationary-liquid would have limited analyte-bonded phase interaction resulting in the observed loss of resolution **(Figure 3.27)**.

Additional support for the homologue resolution being derived from electrostatic interactions with the bonded phase, rather than partitioning, was believed to be forthcoming from the observed behaviour of the monoester quats in the two solvent

systems. The greater hydrophilicity of these analytes would have resulted in increased residence in the stationary-liquid phase, in comparison to the di-chained quats, in both solvent systems, accentuating the variation in the electrostatic behaviour of these components, resulting in greater resolution in comparison with the diesters (**Figure 3.10**). In addition, contrary to what was predicted for the diesters, it was envisaged that residence times of the monoester components would not have been affected when the mobile phase system was switched to hexane-methanol due to the observed solubility of these analytes in methanol. As a result, resolution would have been maintained, as was witnessed by experimentation (**Figure 3.27**).

Whilst it was not possible to conclusively justify how the resolution of the monoesters increased upon removal of THF, it was proposed that additional stationary phase interactions and / or solvent-induced retention might have been responsible. It was predicted that the monoesters would under-go hydrogen bonding with the stationary phase and the methanol in the stationary-liquid phase. With the methanol content of the stationary-liquid phase being far higher in the binary solvent system, hydrogen bonding would have increased, further accentuating any variations in the behaviour of the analytes. It was therefore hypothesised that increased hydrogen bonding facilitated increased resolution in the binary solvent system.

The mixed retention model and the observations made with the binary solvent system (**Figure 3.28**) could also be used to explain the severe peak fronting demonstrated by the quaternary amino-alcohols in the ternary mobile phase system. It was originally assumed that the fronting demonstrated by the diols derived from incompatibility of the analyte with the mobile phase. However, in light of the stationary-liquid hypothesis it was instead proposed that the diol analytes partitioned into the stationary liquid and resisted partitioning back out. It was envisaged that hydrogen bonding between the analytes, and between the analytes and the stationary phase and the methanol solvent, would stabilise their long-term residence in the stationary liquid. Having limited solubility in the bulk hexane solvent, partitioning of a diol analyte out the pores of the silica would only come about as a result of hydrogen bonding with a methanol molecule in the bulk liquid phase. As the analytes were predicted to interact with each other, it was hypothesised that the loss of one analyte into the bulk would promote the loss of others i.e. partitioning back into the bulk mobile

phase was co-operative. Assuming that transfer back into the bulk was a co-operative process, most analyte molecules would leave the stationary-liquid phase simultaneously. Though transfer would be slow at first, the rate would increase with time until all of the analyte molecules had been liberated to the bulk. This release pattern would be characterised by an analyte band that increased exponentially before falling back to the baseline as all of the molecules in the pores were exhausted. Such a band profile was analogous to the fronting peak witnessed during the analysis of the diol with the ternary solvent system.

Utilisation of the binary solvent system would have resulted in a significant increase in the concentration of methanol in the bulk liquid phase, which would have greatly increased the solubility of the diol component. As a result, a conventional band profile would be expected, where peak tailing would be likely to be severe as a result of uneven release of the analytes from the stationary phase and the stationary-liquid phase. Experimental observations for the diol species under the two solvent systems could therefore be justified.

Whilst experimental evidence is available to support the theory of a mixed adsorption - partition model of cationic tenside retention, a number of potentially important experiments need to be carried out in the future to resolve a number of unanswered questions. Firstly, a bare silica phase needs to be evaluated with both solvent systems to determine whether any resolution of the tenside homologues is forthcoming from the silanol groups on the surface of the support. Similarly, a strong anion exchange (SAX) column should also be evaluated, as this phase contains a permanently charged quaternary ammonium group, which could be used to determine whether charged groups on the bonded phase actually facilitate homologue resolution in the new NP-LC methodology. Finally, having predicted that the peak shape of the diol component was dependant on the concentration of methanol in the solvent system, gradual replacement of THF should allow one to study the decrease in fronting, and subsequent increase in peak tailing.

Until such a time occurs when these experiments are undertaken, it will be difficult to justify with any certainty the actual mode of retention in the new methodology. Nonetheless, it is apparent that whatever the retention mechanism, it

facilitates efficient reproducible resolution of first and second-generation cationic fabric conditioner actives.

3.7 CONCLUSIONS

This chapter has described the development of a new isocratic normal phase liquid chromatographic method for the analysis of cationic fabric conditioner actives, the method being developed from a previous literature report after problems were witnessed with the use of the current Unilever standard operating procedure. Evaluation of a range of stationary and mobile phase systems revealed that optimum resolution of the tensides was achieved with a ternary mobile phase system on an amino-bonded stationary phase.

Utilisation of co-injection analysis showed that the method was capable of separating the major classes of cationic tensides present in commercial samples, as well as partially resolving the homologous series present in each class. Subsequent analysis of mixed surfactant samples revealed that the new methodology could also resolve individual components from a specified sample origin, a feat that had not previously been possible with literature methods.

The capability of the new method to partially resolve the homologous series present in commercial samples was unusual for a normal phase LC method. As a result, extensive experimental and literal investigation led to the proposal of a mixed adsorption – partition model being responsible for the high resolving power of the new method. The resolution was therefore critically dependant on the formation of a stationary liquid in the pores of the silica substrate, yet all of the observed separations demonstrated excellent long-term reproducibility, and the method itself was found to be inherently robust.

Whilst the method was found to be well suited to the analysis of the parent cationic tensides, it was ill suited to the analysis of the quaternary amino-alcohol degradation products. Some improvement was gained in the chromatography of the diol species from utilisation of a gradient elution profile. However, it was ultimately

concluded that the poor chromatography resulting from the analysis of these analytes with the new methodology, would prohibit their efficient quantitation at trace levels.

Unfortunately, the new method also suffered from a number of other inherent limitations. Method sensitivity was low, even after inclusion of a a narrow-bore column, and the response of the evaporative light scattering detector was seen to be non-linear. It was envisaged that the lack of sensitivity in particular would limit the applicability of the methodology to environmental analysis. However, the potential to increase method sensitivity and selectivity with mass spectrometric detection was evident after the successful evaluation of a hyphenated methodology.

In its current form the new optimised NP-LC methodology appears to offer potential for routine use in the quality control assessment of the conditioner-active raw materials used in domestic fabric conditioners. However, further optimisation will be required before the methodology can be routinely applied to the analysis of industrial formulations and environmental matrices.

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CHAPTER FOUR

Development of a reverse phase LC/MS method for the routine analysis of alkylbenzyl quat preservatives

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4.1 INTRODUCTION

With the widespread application of alkylbenzyl quats as bactericides and preservatives in household and personal care products there is a requirement for methods of analysis to quantify these compounds in complex industrial and environmental matrices. Currently, reverse phase (RP) liquid chromatography (LC) separations performed on long cyanopropyl (CN) bonded columns are the primary methods used in industry. Separations utilise mobile phases containing high concentrations of non-volatile ion-pairing reagents and high linear velocities to facilitate short analysis times. While the homologue distributions present in commercial samples may be resolved by such methods, the mobile phase conditions prevent hyphenation with mass spectrometry making quantitation in environmental matrices troublesome. The aim of this section of work was to develop a hyphenated LC/MS method capable of analysing alkylbenzyl quats in fully formulated commercial products and environmental wastewaters, rivers and sediments.

4.2 DEVELOPMENT OF THE LC METHODOLOGY

4.2.1 Choice of initial parameters

For convenience, the method described in the current Unilever Research standard operating procedure (SOP) (**Section 1.4.4**) was chosen as an appropriate base from which to develop the new methodology. With the use of a CN column being characteristic of literature methods, and reported problems in the use of octadecylsilane (ODS) bonded stationary phases for the analysis of quaternary ammonium preservatives (Valladao *et al.*, 1994; Zhou *et al.*, 1999) (see also **Section 1.4.4**), a CN phase was maintained as the basis of the new method. However, column length was reduced from 250 mm to 150 mm to promote rapid analysis at reduced mobile phase velocities. (See **Section 1.3.1**).

ACN was retained as the strong solvent constituent of the mobile phase after the use of MeOH was found to result in lower efficiency, reduced resolution and longer analysis time. To improve compatibility of the LC methodology with electrospray mass spectrometry (ESI-MS) the non-volatile sodium perchlorate ($NaClO_4$) used in the Unilever SOP was replaced with volatile alternatives that would allow for efficient resolution and short analysis times of the quat species (Vervoort *et al.*, 1999; de Schutter *et al.*, 1988). In **Section 1.3.2.1** the advantages and disadvantages of using various organic modifiers were discussed in relation to the analysis of organic bases and quaternary ammonium species. It was noted how efficient resolution and short analysis times could be achieved when both an organic acid and base were present in the mobile phase. In light of such observations, two modifiers were incorporated into the new methodology, a competitor quaternary ammonium salt and a strong organic acid. Ammonium acetate (NH_4Ac) was chosen as the competitor species, due to its suitability for ESI-MS and its efficacy in the analysis of a number of other quaternary ammonium species (Barceló *et al*, 1991; Moyano *et al.*, 1996; van der Hoeven *et al.*, 1996; Evans *et al.*, 2000). Trifluoroacetic acid (TFA) was chosen as the organic acid due to the higher resolution that it had afforded in the NP-LC method in comparison with acetic acid and formic acid (**Section 3.3.3**), and its higher volatility and hence greater ESI-MS compatibility compared to octanesulphonic acid (de Schutter *et al.*, 1988). The choice of mobile phase pH was dictated by the durability of the Spherisorb CN stationary phase that was initially used in the methodology. Manufacturers' guidelines recommended the column be limited to a working range of pH 3-7 (Waters, 1999). However, the mobile phase was operated at pH 2.0 as previous work had shown only a small reduction in column lifetime at this pH (Myers, 2000), which was compensated by a reduction in secondary retention effects on surface silanol groups (**Section 1.3.2.1**). The strong acid properties of TFA also allowed pH adjustment to be achieved with lower volumes of reagent, reducing the likelihood of interface fouling and blockage (Lagerwerf *et al.*, 2000).

Of the instrumental parameters used in the Unilever SOP, only two were altered significantly during the initial development of the new methodology. The linear velocity was reduced by a third, the mobile phase flow rate was reduced from 1.5 ml/min to 1.0 ml/min, whilst the UV / vis detection wavelength was modified from 254 nm to 214

nm, following the observation that a ten-fold increase in sensitivity would be afforded at this new wavelength.

A chromatogram obtained from the analysis of a mixed alkylbenzyl quat sample with the modified methodology is shown in **Figure 4.1**. The sample comprised 250 mg/l C_{12}, C_{14}, C_{16} and C_{18} alkylbenzyl quat (**Appendix One**), and from reverse phase theory it was predicted that the first abundant peak (ca. 11.8 minutes) corresponded to the C_{12} homologue, whilst with the last corresponded to the C_{18} component. Evidence that the peak identities had been correctly assigned came *via* sequential enrichment of the sample with the individual homologues. The figure also shows that the new methodology afforded efficient resolution of the four quats, and that the homologous series actually appeared to commence at C_8 rather than C_{12}, indicating one or more of the individual quat samples contained short-chain impurities.

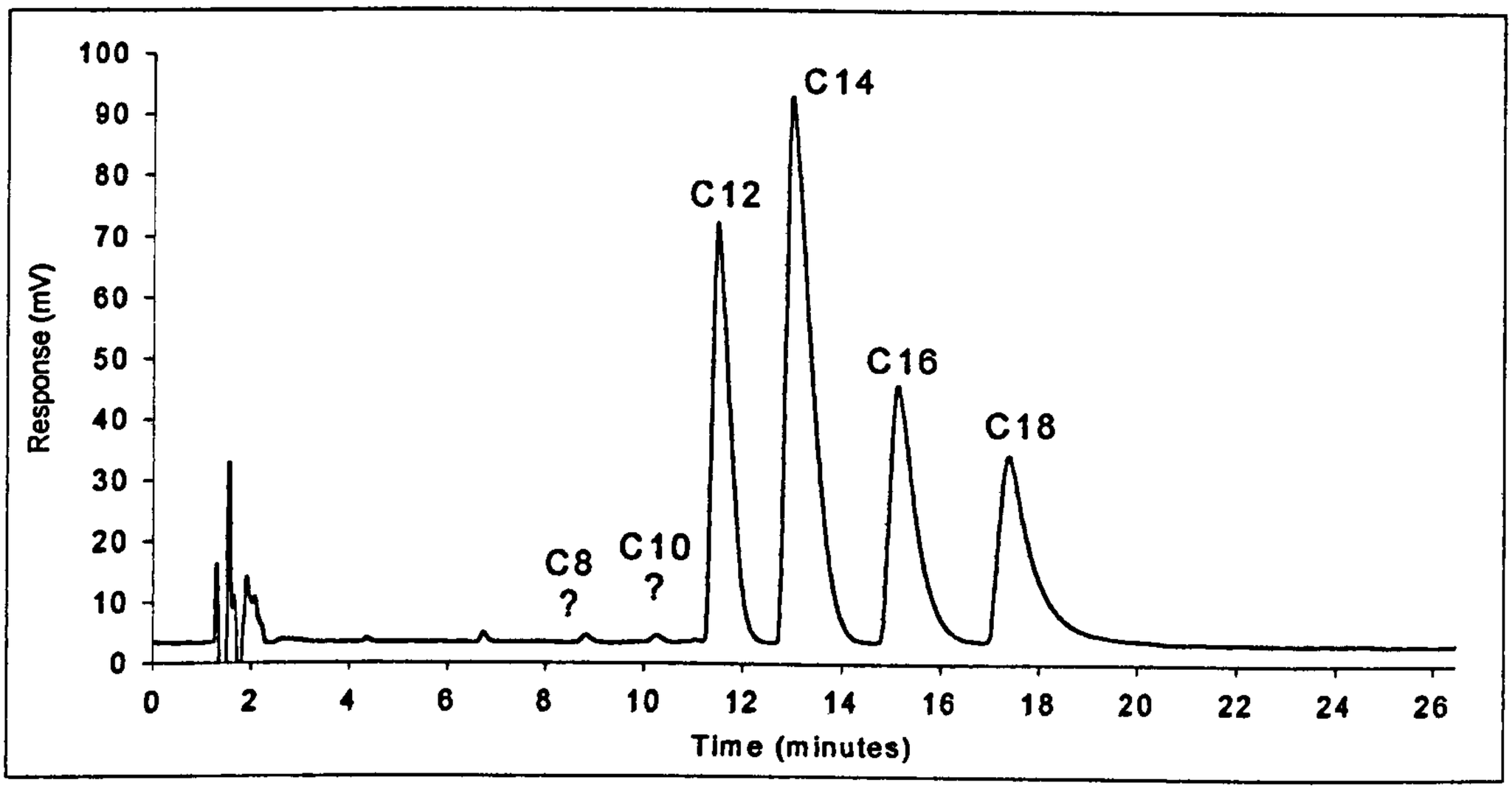

Figure 4.1: Chromatogram showing the separation of four alkylbenzyl quats achieved with the new RP-LC method parameters.
Conditions - Column: 150 × 4.6 mm i.d. Spherisorb 5 μm CN; Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac (pH 2.0); Flow rate: 1 ml/min; Detection wavelength: 214 nm.

4.2.2 Use of new silica technology

Whilst the new methodology facilitated quantitation of the individual alkyl benzyl quats, a number of limitations were evident. Firstly, analysis time had stretched to 21 minutes, an increase of over 260% on the Unilever method, and subsequent attempts to reduce the analysis time by increasing the eluting strength of the mobile

phase were unsuccessful as resolution was lost. Secondly, the method resulted in the quats demonstrating severe peak tailing as a result of uneven release from the silica surface brought about by additional interactions with surface silanol groups and / or retardation of the analytes within cusps in the Spherisorb material (Myers, 1999). Whilst peak tailing is an aesthetic problem in all LC separations, in trace analysis it leads to difficulties in differentiating between background noise and the tail of the analyte peak, which results in method sensitivity being compromised. During the analysis of complex matrices this problem is compounded by co-eluting interferences. The broader the peak the greater the number of possible interferences, and the greater the likelihood of further analyst and instrument time being taken up by reanalysis.

To overcome the problems of peak tailing witnessed in the analysis of basic analytes modern silica phases are based on high purity supports that are smoother, more spherical and have a more even distribution of surface silanol groups (**Section 1.3.2.1**). Such improvements give rise to even release of basic and / or cationic analytes from their surfaces (Myers, 1999; Phenomenex, 2000), and, whilst they do not eliminate the potential for secondary interactions they often improve efficiency and peak symmetry.

The separation of the mixed alkylbenzyl quat was thus repeated on a second CN material that was based on a modern high purity silica support (Luna from Phenomenex). **Figure 4.2** shows the variation in separation that was achieved on the two phases. The blue trace corresponding to the Luna phase clearly demonstrates that this material provided a reduction in peak tailing, analysis time and analyte peak volumes.

Manufacturers' guarantees on working pH range (1.5 to 7.0), column lifetime (guaranteed for > 1000 hours continuous running at pH 1.5) and the provision of a full certificate of analysis (Phenomenex, 2000), in addition to independent observations on the batch-to-batch reproducibility and robustness of the silica support (Kele *et al.*, 2000), as well as the observations detailed above, led to the Luna CN phase being adopted as the column of choice in the new methodology.

4.2.3 Improving sensitivity and MS compatibility

During the optimisation of the NP-LC method (**Chapter Three**) the use of a 2.0 mm i.d. column had provided a five-fold increase in method sensitivity, compared to an analogous 4.6 mm i.d. column. To improve the sensitivity of the RP-LC method, and ultimately improve MS response and interface dynamics (Abian *et al.*, 1999), the performance of a 2.0 mm i.d. Luna CN column was evaluated. **Figure 4.3** shows an overlay of the chromatograms resulting from the analysis of a 50 mg/l benzalkonium chloride standard on a 4.6 mm and 2.0 mm i.d. Luna 3 µm CN column. The two peaks corresponded to the C_{12} and C_{14} alkylbenzyl quats, with the ratio of the two species being characteristic of a sample derived from coconut oil. It is important to note that the linear velocity was constant in both analyses, and thus the observed variation in retention time was as a result of the influence of instrument and extra-column void volumes on narrow bore separations (Wehr, 2000; Hewlett Packard, 1997).

The chromatogram obtained on the 2.0 mm i.d. showed significant improvements in analyte peak area response compared to the 4.6 mm i.d. alternative, and thus the narrow-bore column was adopted for all further work.

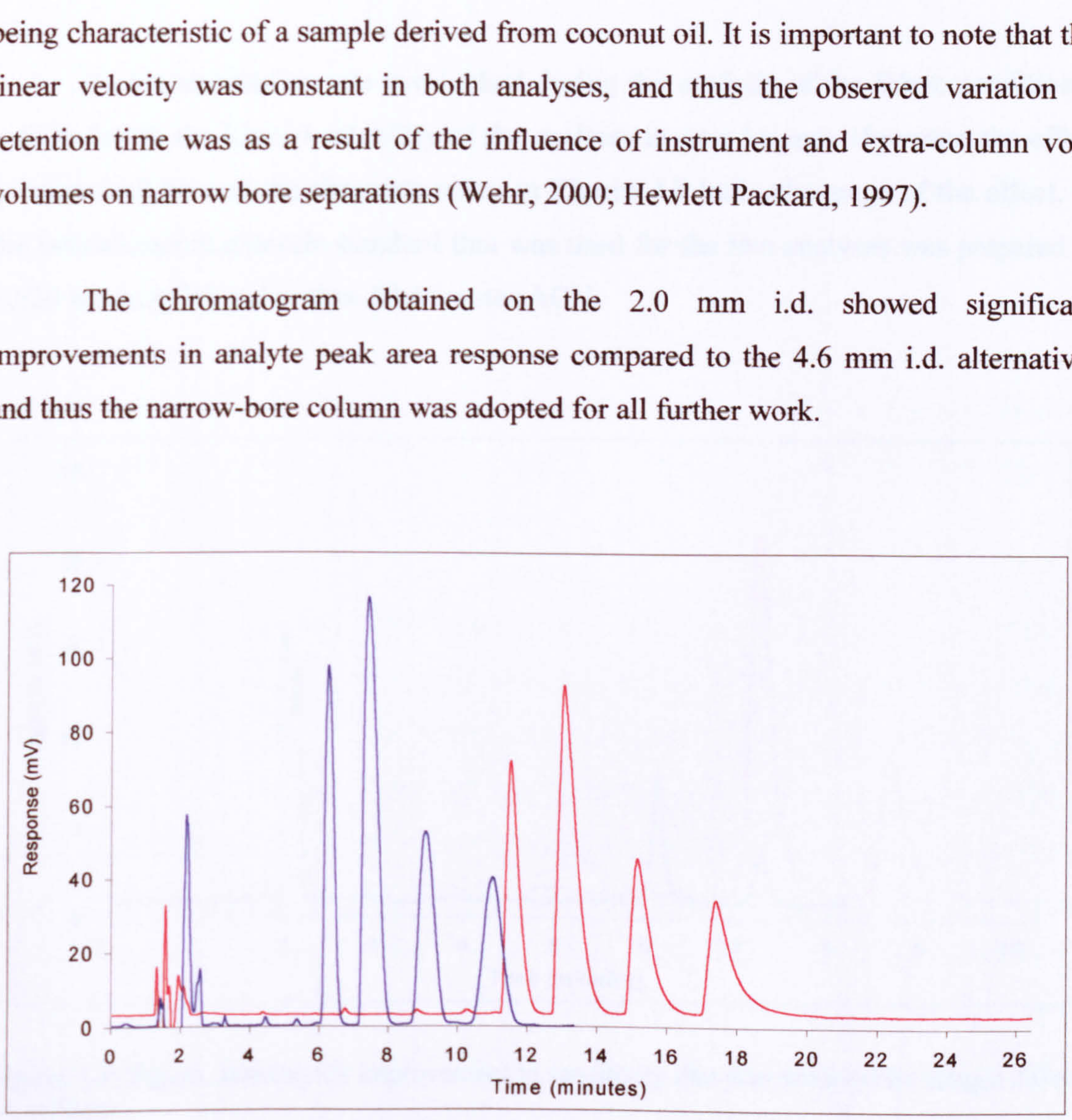

Figure 4.2: Figure showing the variation in separation efficiency afforded by the Luna CN (blue trace) and Spherisorb CN (red trace) materials.
Conditions - Column dimensions: 150 × 4.6 mm i.d. packed with 3 µm silica particles; Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac (pH 2.0); Flow rate: 1 ml/min.

4.2.3.1 The peak compression effect

"Analyte peak compression" is a recognised LC phenomenon in which the sample of interest is dissolved in a solvent of lower eluting strength than the LC mobile phase. When the sample is injected into the eluant stream the analyte plug is compacted, as the eluting strength at the rear of the band is stronger than that in the interior. Although the analytes undergo the same degree of band broadening during their passage through the column, having derived from a narrower band, peak volumes are reduced leading to an increase in efficiency and method sensitivity.

Peak compression was overlooked during the analysis of the fabric conditioner actives due to the limited solubility of the analytes in pure hexane. However, the effect is referenced here as the chromatograms in **Figure 4.3** took advantage of the effect, as the benzalkonium chloride standard that was used for the two analyses was prepared in 80:20 water:ACN, rather than 50:50 water:ACN.

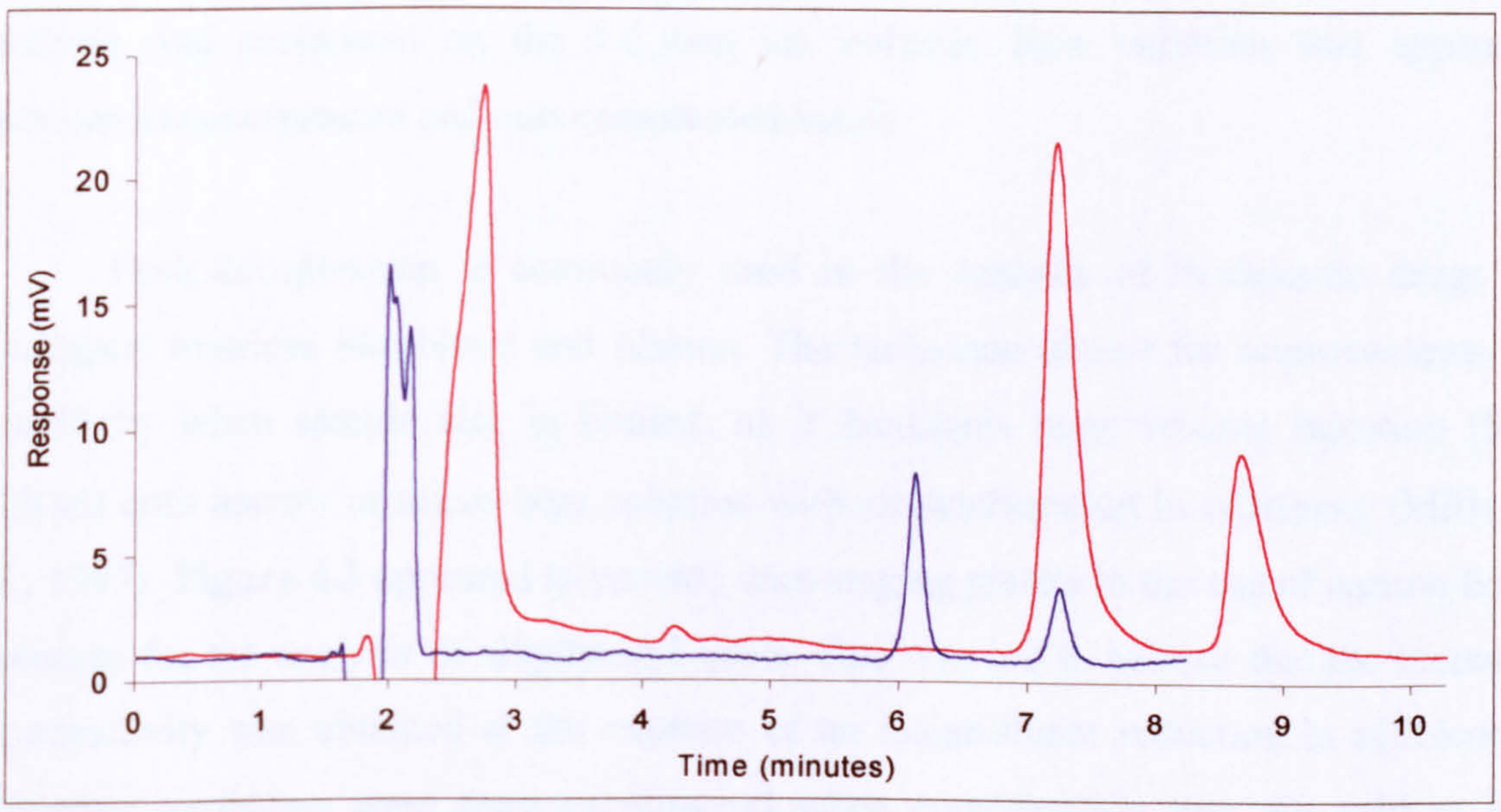

Figure 4.3: Figure showing the improvement in sensitivity that was achieved by using a 2.0 mm i.d. column.
Conditions – Column: 150 × 4.6 mm i.d. Luna 3 µm CN column (blue trace), 150 × 2.0 mm i.d. Luna 3 µm CN column (red trace); Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac (pH 2.0); Sample: 50 mg/l benzalkonium chloride in 80:20 water:ACN; Flow rate: 1000 µl/min (blue trace), 190 µl/min (red trace).

Peak compression was observed in both of the separations shown in **Figure 4.3.** However, the effect was more pronounced on the narrow-bore column due to the reduced mobile phase flow rate. In an HPLC injector, whether it be a manually actuated valve such as a Rheodyne model 7125 or a mechanically actuated valve like those found in commercial autosamplers, solute injection follows the switching of the valve from the load to the inject position. Eluant flow is diverted through the injection loop washing the solute band onto the analytical system. When a filled 10 µl loop is flushed with mobile phase at 1000 µl/min, the loop is emptied in 600 ms. However, when the flow rate is reduced to 190 µl/min the loop remains partially filled with sample for over 3000 ms. A broader analyte plug is formed, which results in larger peak volumes and ultimately lower efficiency. The severity of the band broadening occurring in the narrow-bore column is compounded when the sample diluent is of a higher eluting strength than the mobile phase eluant. Here, the front of band moves more rapidly than the tail leading to further expansion of the plug. This effect was witnessed with the optimised RP-LC methodology when ACN was used as the sample diluent. Instead of two discrete peaks, a single poorly efficient peak was observed on the 2.0 mm i.d. column, which demonstrated a baseline width in excess of four minutes. When the analysis was performed on the 4.6 mm i.d. column, little variation was apparent between the compressed and non-compressed bands.

Peak compression is commonly used in the analysis of therapeutic drugs in biological matrices like blood and plasma. The technique allows for improvements in sensitivity when sample size is limited, as it facilitates large volume injection (50-100 µl) onto narrow or micro-bore columns without deterioration in efficiency (Mills *et al.*, 1997). **Figure 4.3** appeared to provide encouraging results in the use of narrow bore columns for the analysis of alkylbenzyl quats. One was led to believe that the increase in sensitivity was obtained at the expense of an insignificant reduction in efficiency. However, problems were soon encountered when consideration was not paid to the manner in which a quat sample was prepared. Major problems were also envisaged in the use of the technique for the environmental analysis after discussions with Unilever Research (Sparham, 2000). Sample preparation protocols used to extract cationic tensides from environmental matrices include a 100 to a 1000 fold pre-concentration of the analytes, with the final step in the process being the resolvation of the analytes in the minimum volume of solvent. Whilst straightforward for most analytes, the propensity of

cationic tensides to sorb to the walls of a vessel has been seen to lead to inefficient recovery if resolvation is not carried out with extreme care.

Work performed at Unilever Research has shown that 80:20 water:ACN is an inefficient resolvation system for most cationic tensides (Sparham, 2000), and that pure ACN is required to guarantee efficient recoveries of the alkylbenzyl quats. When used in conjunction with the new methodology, efficient recoveries would be achieved at the expense of poor chromatography. As a result, although the 2.0 mm i.d. column and the use of a weak sample diluent were used for the remainder of the RP-LC work, observations made at Unilever Research suggest that it may be more effective to use a 4.6 mm i.d. column for environmental analysis.

4.2.4 Determination of the sensitivity, linearity and robustness of the new method

4.2.4.1 Method validation

A formal validation of the RP-LC-UV method was never performed due to time restrictions experienced during this segment of work. Instead, experience gained during the validation of the NP-LC method was applied to this RP-LC work:

During NP-LC method development an aged column could be replaced with a new column with no loss in performance. A similar observation was made during the RP-LC work with aged CN columns.

Transfer of the NP-LC method to Unilever Research yielded equivalent separation efficiency to that witnessed at the University of Leeds. Variations in instrumentation and reagent supplier apparently had no effect on the separation efficiency. These results were echoed when the RP-LC methodology was later transferred (**Figure 4.4**).

The above parameters form the basis of an inter-lab validation study, and are thus critical in determining the inherent robustness of new chromatographic methodology. Although the observations did not represent a rigorous validation of the method, it was predicted that the new methodology was sufficiently robust to allow it to be transferred between laboratories, and utilised by adequately qualified personnel, without resolution being compromised.

4.2.4.2 Method sensitivity and linearity

The sensitivity of an analytical method is ultimately dependant on the performance of the detector that is used. It is impossible to define an exact limit of detection for a new methodology without specifying that the same model of detector be used for all further work. Even then, equivalent performance will only be achieved if both detectors have been maintained to the same degree. For linearity, the same is also true.

To determine the linearity of the optimised RP-LC-UV methodology, a series of ten Querton KKBCL calibration standards were prepared, ranging in active concentration from 0 to 100 mg/l. The standards were analysed in triplicate, and the average peak areas of the two principle components, the C_{12} and C_{14} homologues, determined (for an indication of the separation see **Figure 4.5**). Calibration curves for the two components were created, and a first order polynomial regression fit applied to each data set. **Figures 4.6** and **4.7** show that both curves demonstrated excellent linearity, with the R^2 values being in excess of 0.995, and approaching 1.0 in the case of the C_{12} component.

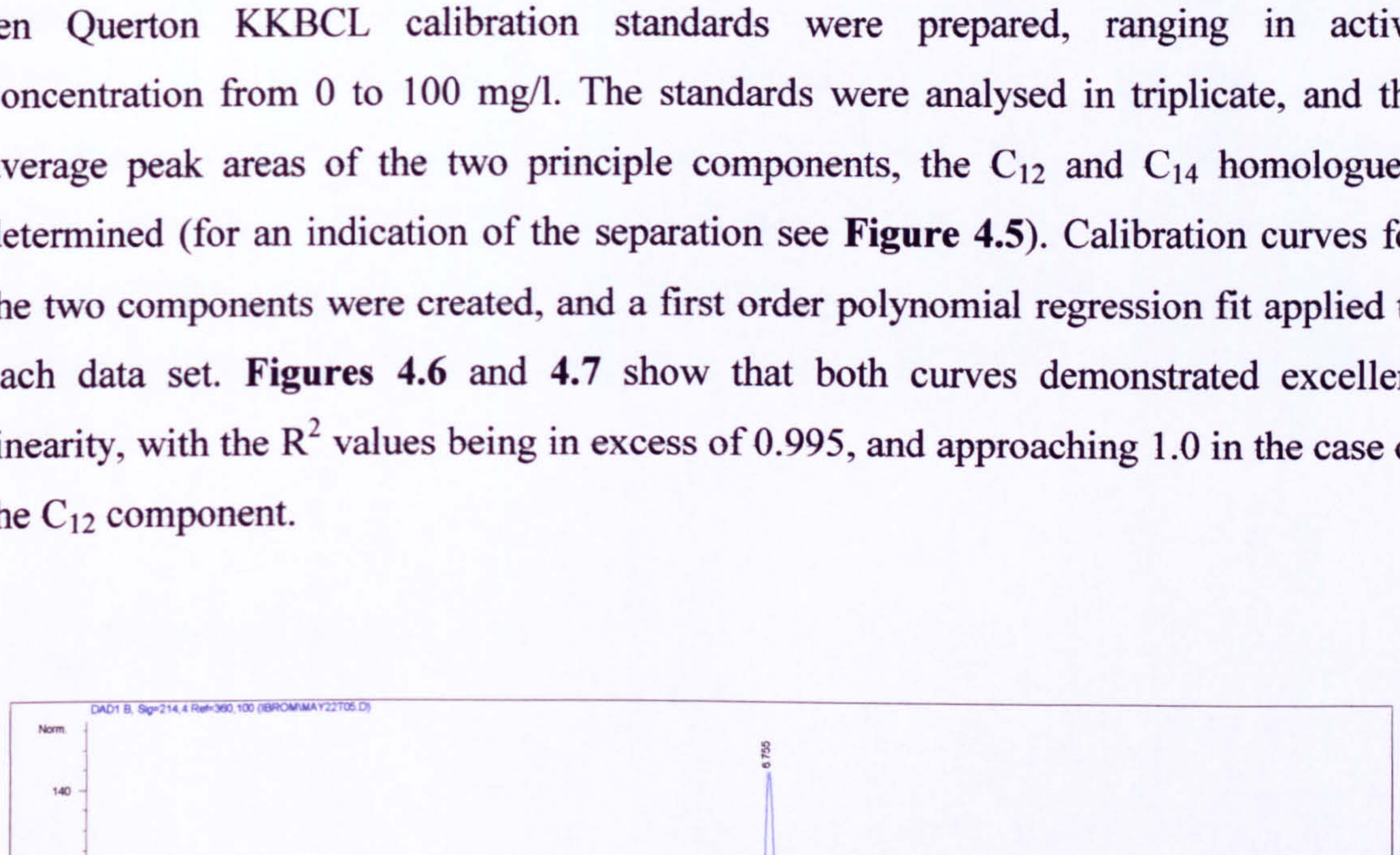

Figure 4.4: Chromatogram showing the analysis of a 100 mg/l benzalkonium chloride standard performed at the Port Sunlight laboratory.
Conditions – Column: 150 × 2.0 mm i.d. 3 µm Luna CN; Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac (pH 2.0); Sample diluent: 80:20 water:ACN; Flow rate: 190µl/min.

It was evident after the initial transfer of the RP-LC-UV method to Unilever Research that the diode array detector (DAD) present in the HP1100 system, would provide a lower limit of detection (LOD) than the detector used at Leeds. However, during the linearity study it was apparent that a number of standards were close to, or beyond both the limit of quantitation (LOQ), defined here as a signal to noise ratio (S/N) ratio of 10, and the LOD, defined as a S/N ratio of 3. When the 1 mg/l Querton KKBCL standard was analysed the peak area responses of the C_{12} and C_{14} components were seen to be above the LOQ (**Figure 4.8**). However, when the 500 µg/l standard was subsequently analysed the C_{14} component was no longer visible, and though the peak relating to the C_{12} component was present, the peak area response was below the LOD. Further work revealed that the actual limit of detection of the C_{12} component was 850 µg/l Querton (≈ 650 parts per billion (ppb) C_{12}), for a 10 µl injection. In **Section 3.5.5.1** it was stated that a limit of detection of 1 mg/l is required for an LC method to be acceptable for environmental analysis. It was therefore evident that the new LC-DAD methodology was suitable for the quantitation of alkylbenzyl quats in environmental matrices.

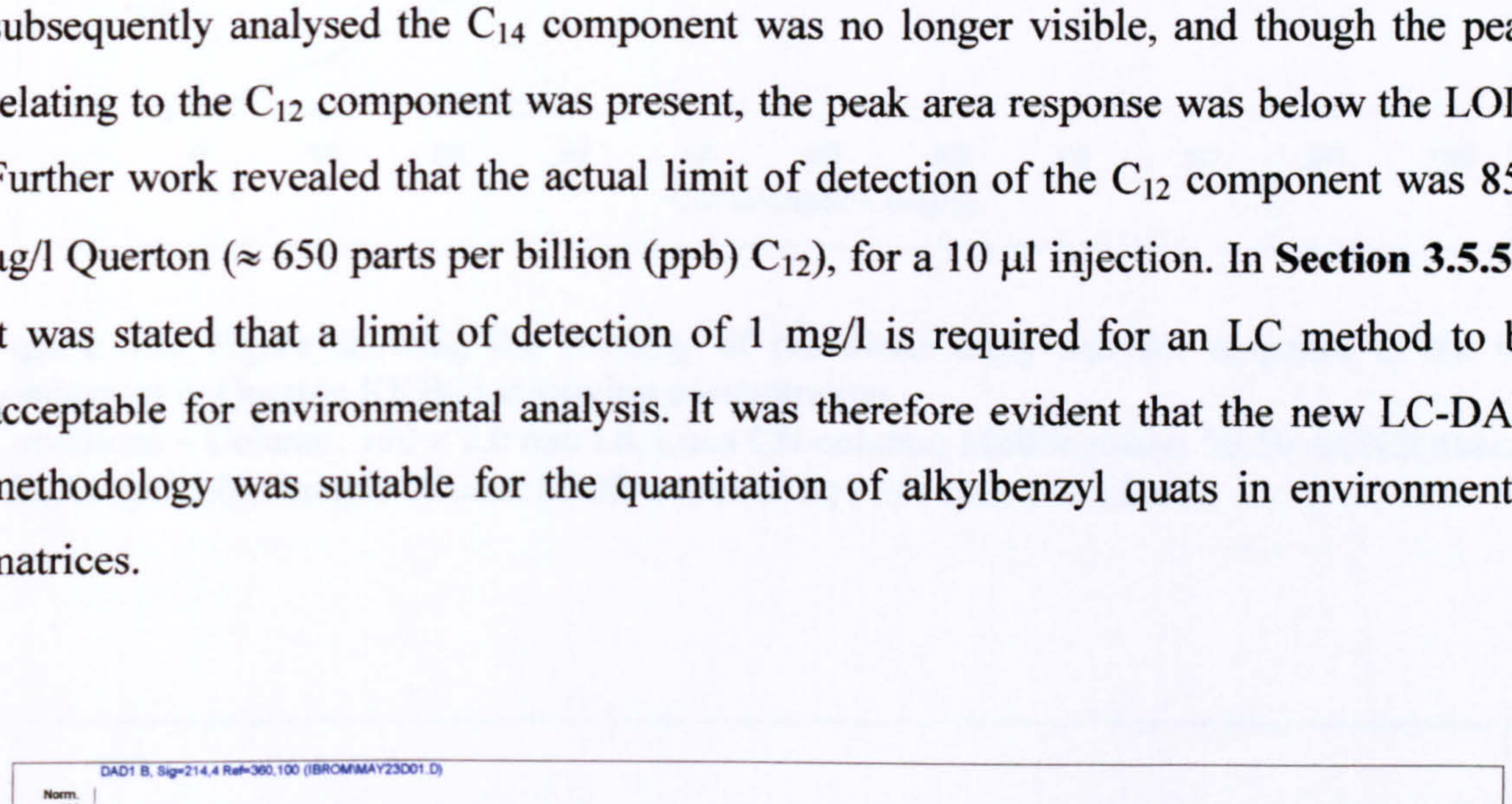

Figure 4.5: Chromatogram showing the analysis of the 50 mg/l Querton KKBCL standard. Conditions – Column: 150 × 2.0 mm i.d. 3 µm Luna CN; Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac (pH 2.0); Sample diluent: 80:20 water:ACN; Flow rate: 190µl/min.

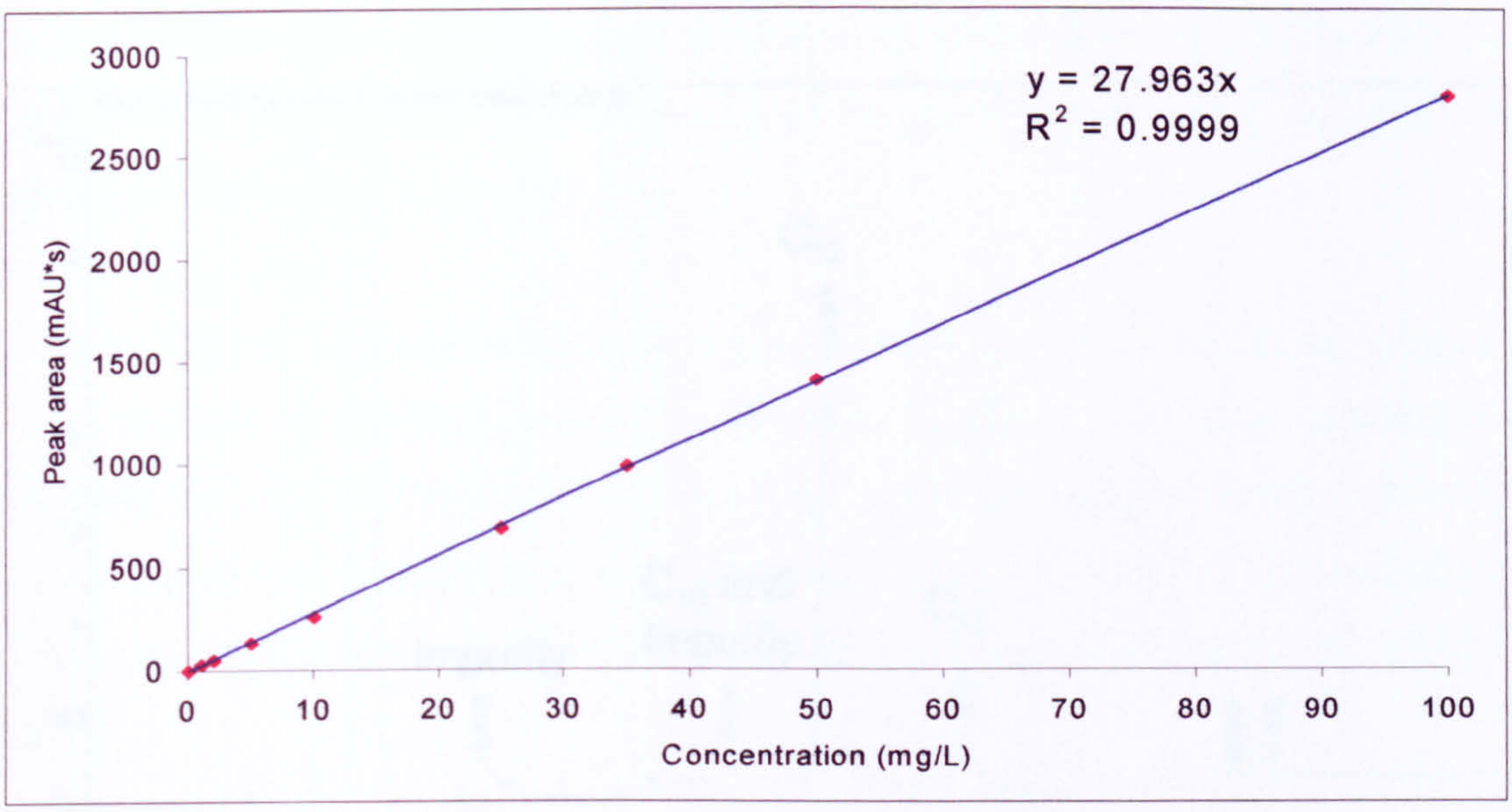

Figure 4.6: Figure showing the linearity of the diode array detector response to the C_{12} component in Querton KKBCl at varying concentration.
Conditions – Column: 150 × 2.0 mm i.d. Luna CN column; Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac (pH 2.0); Sample diluent: 80:20 water:ACN; Flow rate: 190 µl/min.

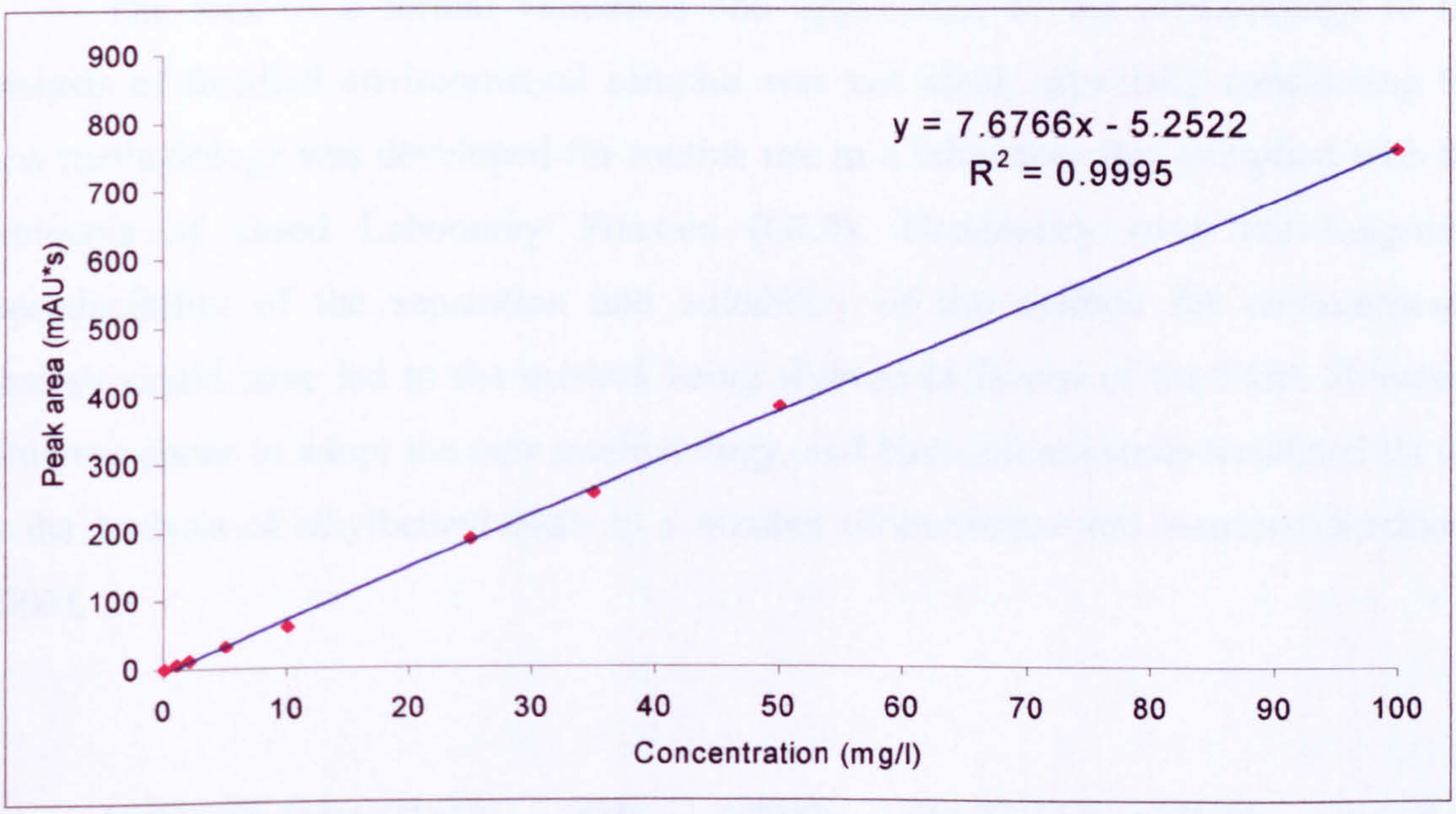

Figure 4.7: Figure showing the linearity of the diode array detector response to the C_{14} component in Querton KKBCl at varying concentration.
Conditions – Column: 150 × 2.0 mm i.d. Luna CN column; Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac (pH 2.0); Sample diluent: 80:20 water:ACN; Flow rate: 190 µl/min.

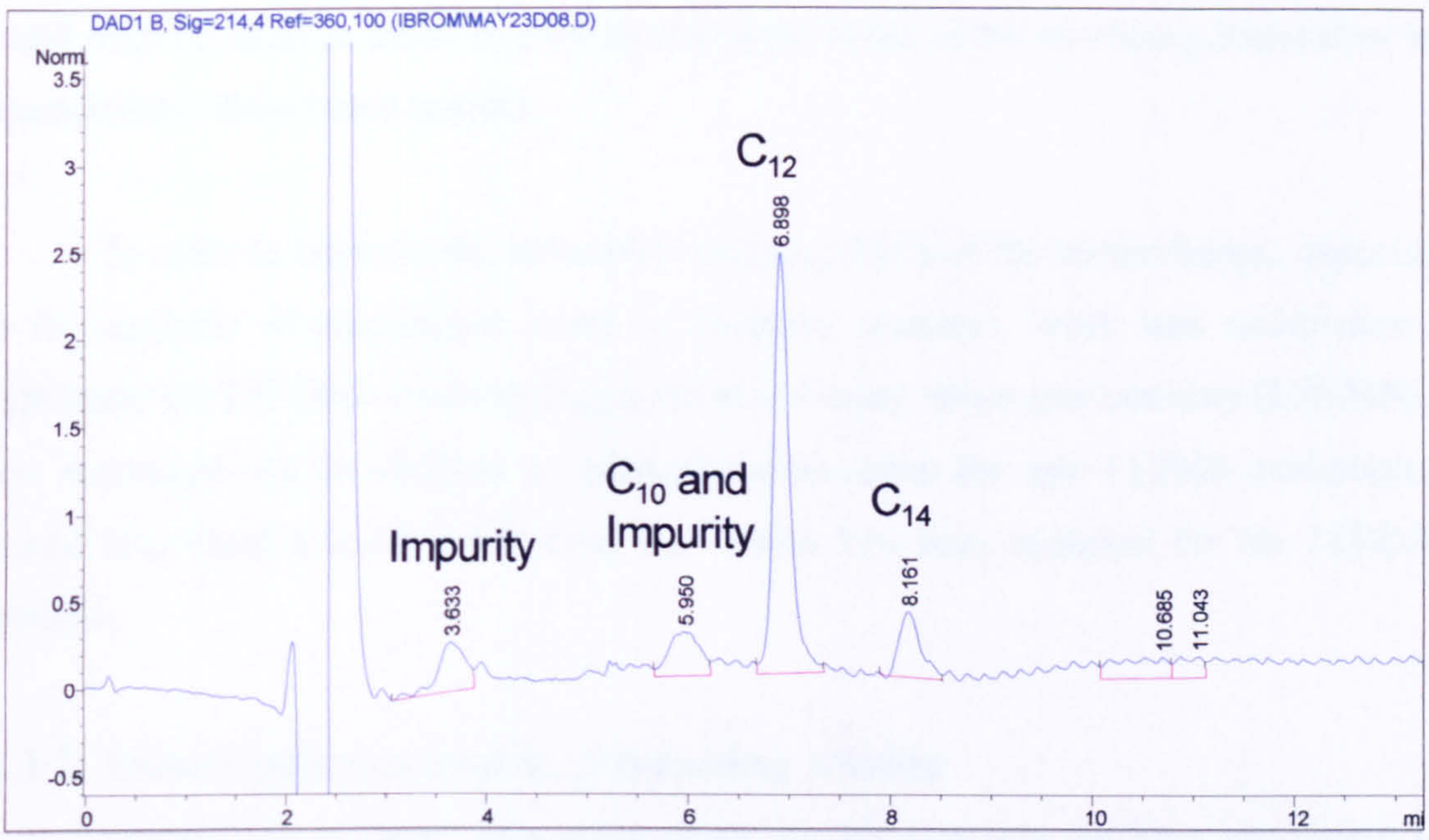

Figure 4.8: Chromatogram showing the analysis of the 1 mg/l Querton KKBCL standard. Conditions – Column: 150 × 2.0 mm i.d. Luna CN column; Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac (pH 2.0).

The lack of a formal validation and application of the methodology to the analysis of fortified environmental samples was not ideal, especially considering the new methodology was developed for routine use in a laboratory that complied with the protocols of Good Laboratory Practice (GLP). Uncertainty over the long-term reproducibility of the separation and suitability of the method for environmental analysis could have led to the method being shelved in favour of the SOP. However, Unilever chose to adopt the new methodology, and have subsequently validated its use in the analysis of alkylbenzyl quats in a number of environmental matrices (Sparham, 2000).

4.3 DEVELOPMENT OF THE HYPHENATED LC/MS METHODOLOGY

The unknown impurity that was seen to co-elute with the C_{10} component (**Figure 4.8**), and thus interfere with its quantitation, provides an example of the main limitation of the LC-DAD methodology; i.e. an absence of structural specificity to unambiguously confirm peaks identities. The UV spectra of the alkylbenzyl quats are

unremarkable at low wavelengths (190-260 nm), and have few if any characteristic bands at higher wavelengths. As a result, the spectral information provided by the DAD could only be used to assist in peak purity assessment when co-eluting impurities had characteristic absorbance spectra.

In order to improve the selectivity and specificity of the methodology, especially in the analysis of alkylbenzyl quats in complex matrices, work was undertaken to hyphenate the LC-DAD methodology with electrospray mass spectrometry (ESI-MS). It was envisaged that in addition to structural information the new LC/MS methodology would also yield a lower LOD than that which had been reported for the LC-DAD method.

4.3.1 Direct infusion and loop injection studies

Initial mass spectral assessment of the alkylbenzyl quat samples was performed with the aid of direct infusion and loop injection analysis (**Sections 3.5.6.1** and **3.5.6.2**).

In direct infusion mode a 100 mg/l benzalkonium chloride standard, prepared in 80:20 water:ACN, was used to tune the MS instrument (**Appendix Three**). Observation of the resulting spectrum (**Figure 4.9**), showed two principle ions, a base peak at $m/z = 304.3$ and a 50% abundant ion at $m/z = 332.2$, corresponding to the molecular ions (M^+), of the C_{12} and C_{14} alkylbenzyl quats. These ions had been expected after results observed with the fabric conditioner actives and previous RP-LC/MS analysis of quaternary ammonium species (Hau *et al.*, 2000; Evans *et al.*, 2000). Importantly, the ratio of the two ions correlated with the peak areas witnessed during the LC-UV work and with the manufacturers data (See **Appendix One**). In a repetition of observations made during **Section 3.5.6.1**, a series of high molecular weight ions were again apparent, fragmentation data revealing them to be derived from the presence of dimeric adducts of C_{12} and / or C_{14} quats. Whilst it was impossible to be certain of the origin of these ions, it was again hypothesised that they derived from the electrospray process **(Section 3.5.6.1)**.

During loop injection analysis tuning of the MS instrument was performed with the aid of a 100 mg/l Querton KKBCL standard. A poly (ether (ether) ketone) (PEEK) T-piece was inserted into the instrumental set-up (see **Section 3.5.6.2**) to facilitate

continuous infusion of the analyte into the eluant stream (infusion rate = 3 µl/min). Initial MS parameters were based on a tune file obtained previously (**Appendix Three**). The capillary temperature was increased from 220°C to 250°C, and the sheath gas being regulated to 35 arbitrary units in order to compensate for the higher volume of eluant entering the source.

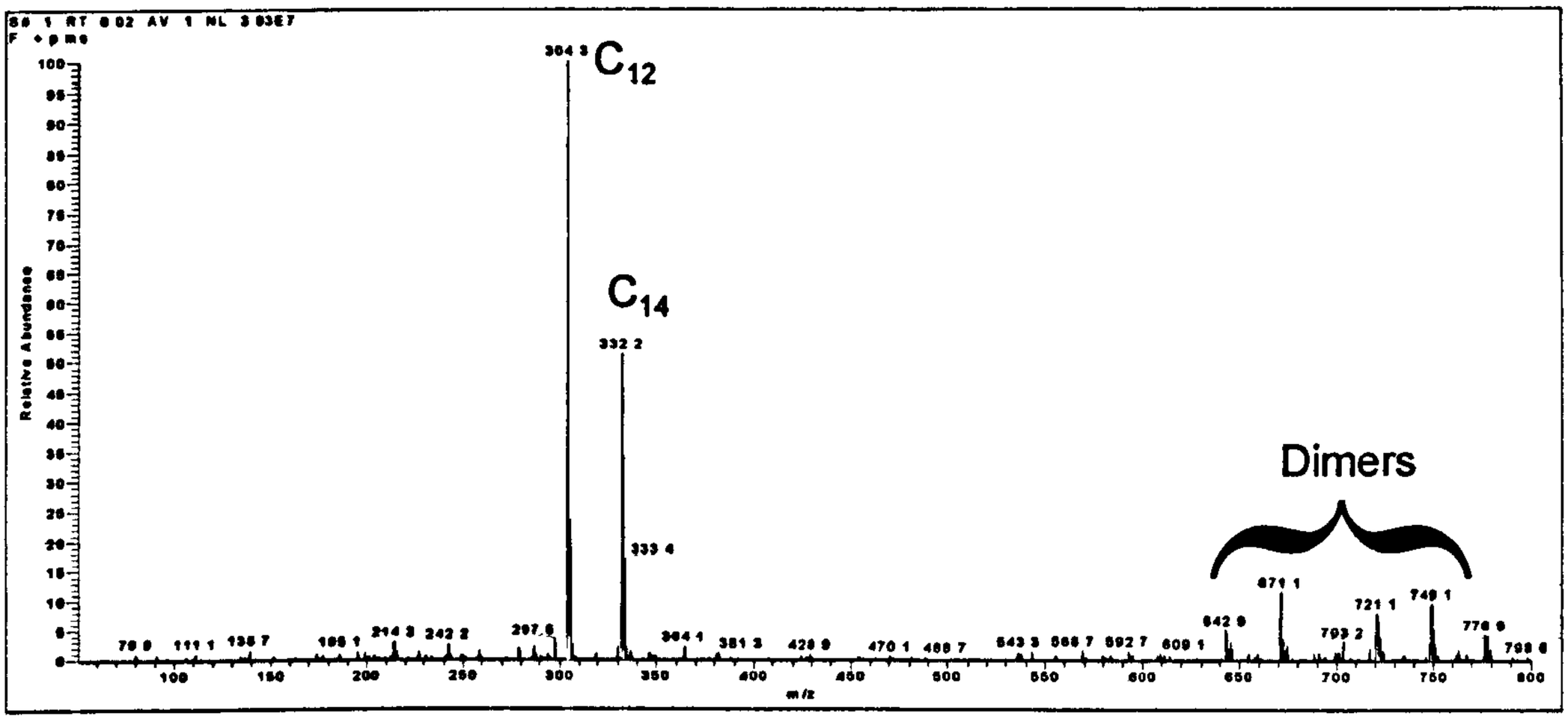

Figure 4.9: Mass spectrum obtained from the direct infusion of a 100 mg/l standard of benzalkonium chloride into the Finnigan MAT LCQ instrument after MS optimisation.
Infusion rate: 3µl/min.

When the analytes were initially infused into the flowing eluant, the molecular ion of the C_{12} component (*m/z* = 304.3) was dwarfed by an unknown ion at *m/z* = 242.3. Variation of the Tube Lens Offset voltage led to a variation in the abundance of the C_{12} molecular ion as more / less fragmentation occurred, but negligible effect was witnessed on the unknown ion. Previous direct infusion MS studies had shown low abundance of *m/z* = 242.3, and nuclear magnetic resonance (NMR) data provided by Unilever Research, indicated no major impurities were present in the sample (**Appendix One**). It was therefore hypothesised that the origin of the ion was the LC mobile phase, the hypothesis being subsequently confirmed when mobile phase was pumped directly into the ESI interface in the absence of any quat samples (**Figure 4.10**).

Having identified the origin of the ion, attempts were made to reduce its abundance. The capillary temperature and sheath gas flow rate were increased and auxiliary gas was added to facilitate droplet evaporation. Unfortunately, the

modifications had little effect, as too did the use of new batches of solvent and base and the replacement of TFA with AcH.

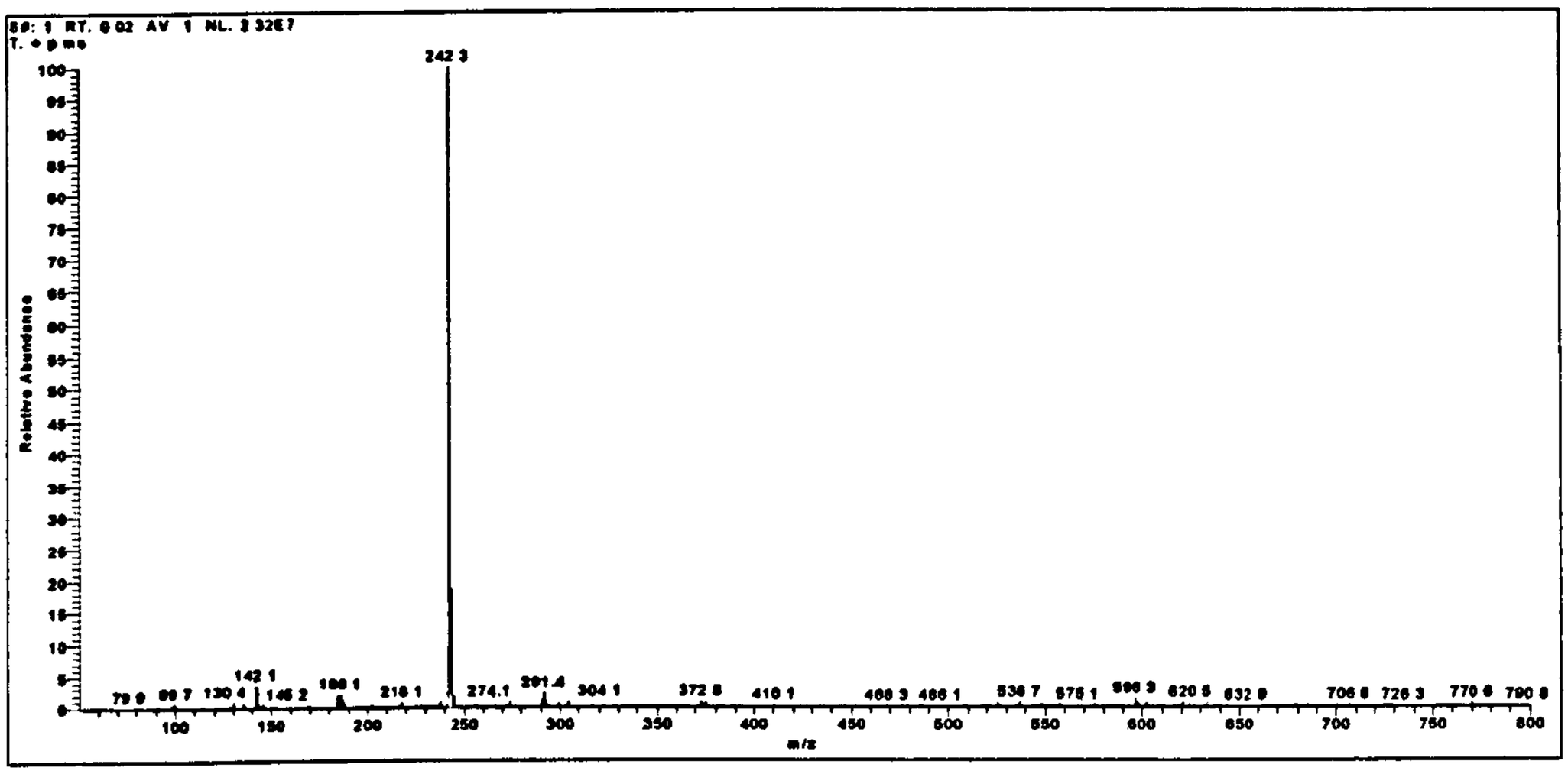

Figure 4.10: Mass spectrum of the RP-LC mobile phase system showing the unknown ion at m/z = 242.3.
Conditions – Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac (pH 2.0); Flow rate: 190 µl/min.

High abundancy impurities had been witnessed in a number of earlier LC/MS studies, in which ACN formed the basis of the mobile phase (Weston, 2000). In these studies the unknown ions were attributed to salt clusters formed from the co-ordination of ACN with Na^+ ions present in the HPLC solvent. In an attempt to confirm the origin and elucidate the structural composition of the ion, tandem MS experiments were performed. Unfortunately, the work was wholly unsuccessful, as fragmentation of the ion resulted in it being obliterated, liberating a host of product ions that were useless for elucidation purposes. Without structural information, it was impossible to categorise the unknown ion. However, a salt cluster origin remained the most viable hypothesis.

One method was identified to prevent the impurity ion from swamping the total ion chromatograms (TIC's) of the respective quat samples. Prior to each set of analyses a solvent spectrum was stored, which was subsequently subtracted from each of the analyte TIC's, removing the presence of the *m/z* = 242.3 ion, and yielding clean analyte spectra. **Figure 4.11** shows the mass spectrum that was obtained from the loop injection analysis of a 100 mg/l standard of Querton KKBCl, after the solvent spectrum had been subtracted from the trace. The abundances of the C_{10}, C_{12} and C_{14} components were

again in agreement with the characterisation data available for this sample, indicating a lack of MS detector bias. This problem had been witnessed at Unilever Research during the analysis of non-ionic alcohol ethoxylate surfactants (Weston, 2000), where qualitative and quantative data was found to be flawed as the detector struggled to identify the low molecular weight ethoxymers.

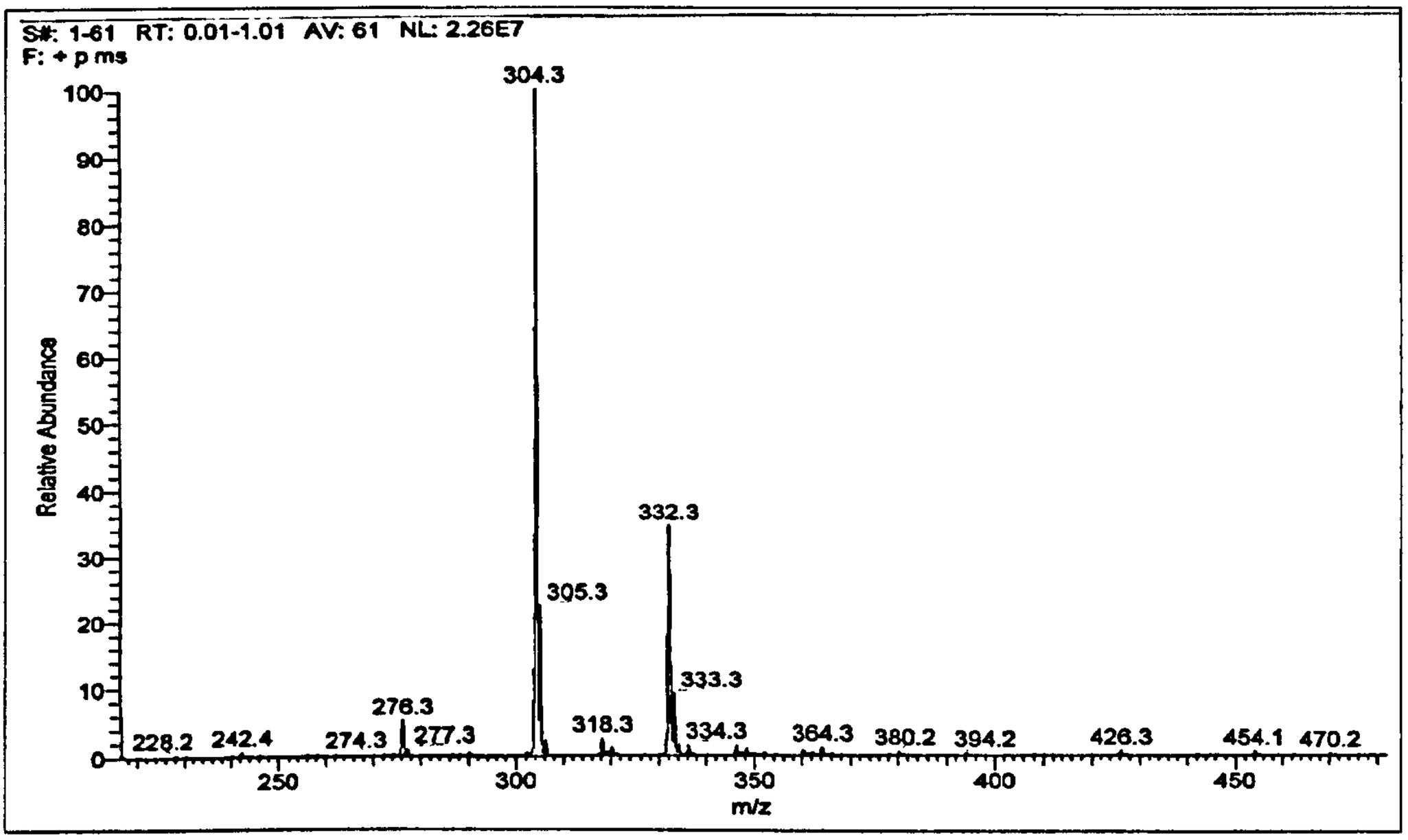

Figure 4.11: Mass spectrum of a 100 mg/l standard of Querton KKBCL analysed by loop injection analysis.
Conditions – Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac (pH 2.0); Flow rate: 190 µl/min; Acquisition time: 2 minutes.

4.3.2 Preliminary results of LC/MS analysis

After overcoming a number of problems during loop injection analysis, the transfer to a fully hyphenated LC/MS methodology was relatively simple. Preliminary work showed that the optimised tune parameters obtained from loop injection could be adapted for use in the hyphenated system with minimal modification. **Figure 4.12** shows the total ion chromatogram (TIC) obtained from the LC/MS analysis of a 100 mg/l Querton KKBCL standard. The reconstructed ion chromatograms (RIC's), corresponding to the M^+ ions of the major quat species are also shown, providing quantative data on each of the quat species, without external interferences. Information provided by the RIC's and the TIC, confirmed that the three principle peaks in the LC trace corresponded to the C_{12}, C_{14} and C_{10} alkylbenzyl quats respectively. At the same

time, confirmation of a C_{10} to C_{18} was also made. **Figure 4.12** shows that the RIC's corresponding to each of the alkylbenzyl quats only demonstrated ion accumulation at single discrete points within the trace, i.e. *m/z* = 276.3 (M^+ ion of the C_{10} component) was only observed between 6 and 6.5 minutes. The LC methodology was seen to be resolving the alkylbenzyl quats on the basis of alkyl chain length, with little sign of carry over of short chain species into the latter part of the trace, or early eluting long chain species.

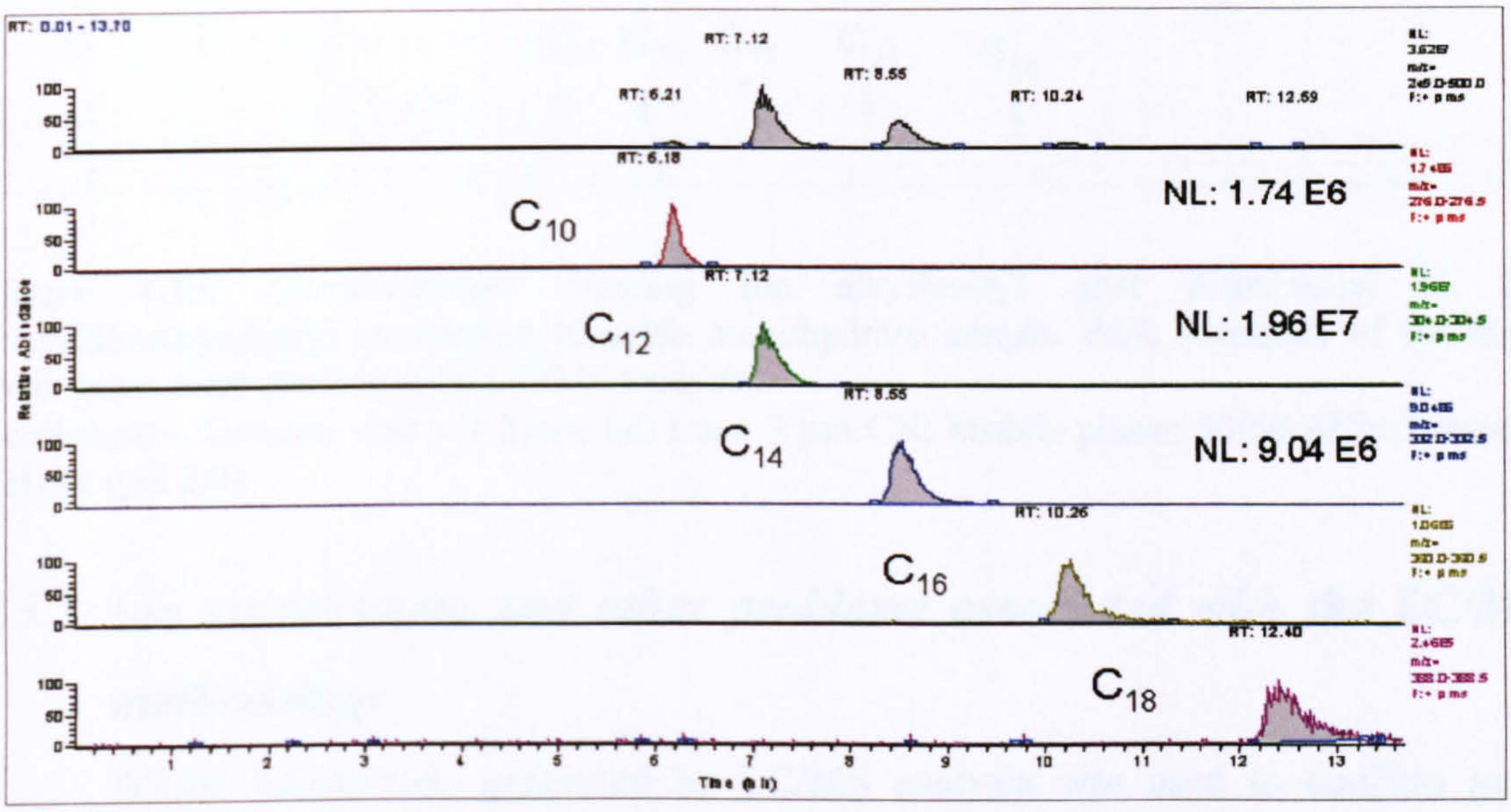

Figure 4.12: Total ion chromatogram (TIC) (top) and reconstructed ion chromatograms (RIC's) of the major alkylbenzyl homologues present in the Querton KKBCL sample.
Conditions – Column: 150 × 2.0 mm i.d. Luna 3 µm CN; Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac (pH 2.0).

Analysis of the remaining quat samples by LC/MS allowed confirmation of the alkyl chain distributions present in elsewhere. **Figure 4.13** shows that the analysis of a 100 mg/l benzyldimethylstearyl ammonium chloride monohydrate standard (**Appendix One**) revealed the presence of a quat distribution ranging from C_8 to C_{18}. It was therefore predicted that the short chain components witnessed in **Figure 4.1** were derived from the presence of this sample within the mixture.

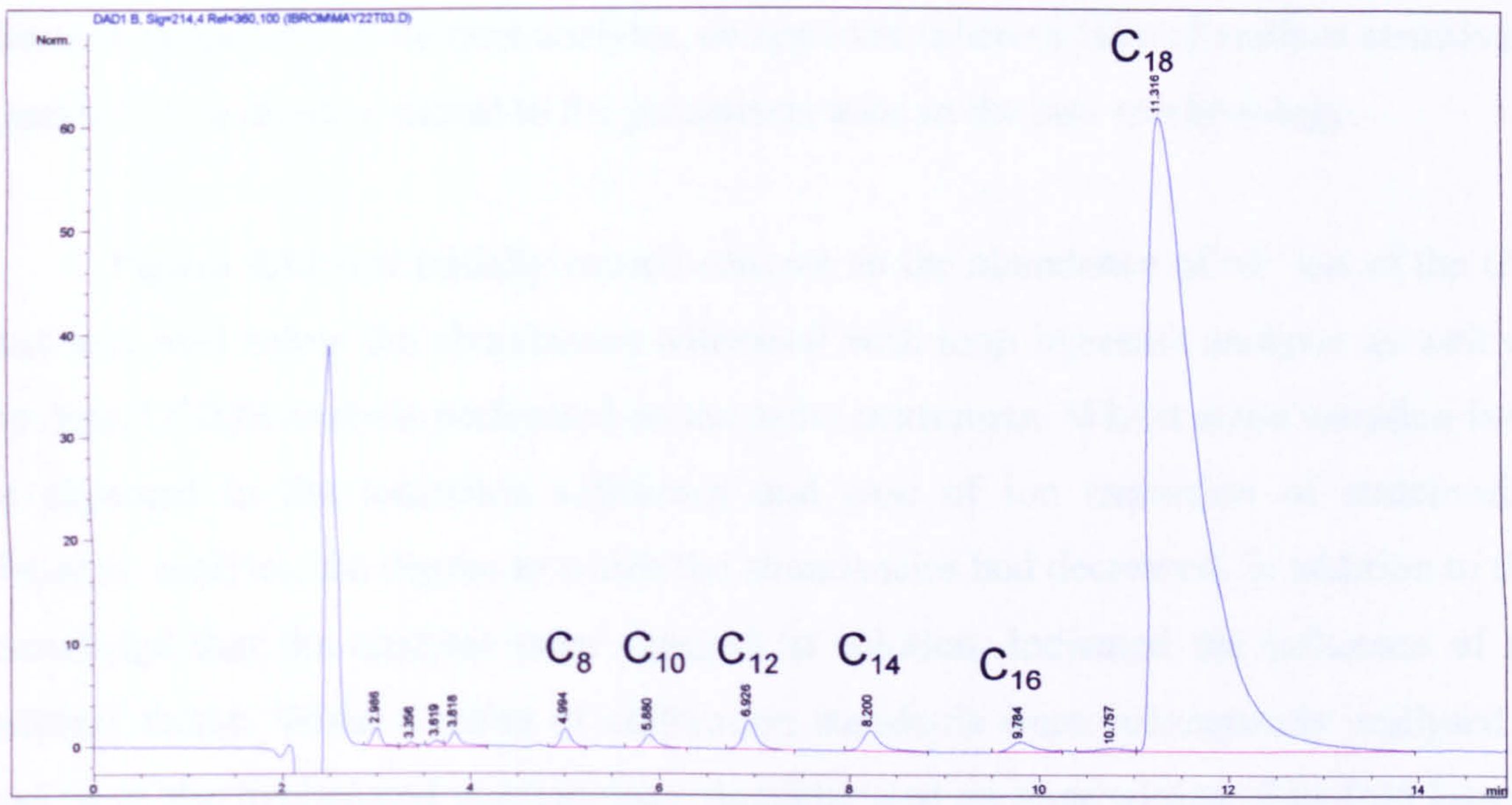

Figure 4.13: Chromatogram showing the alkylbenzyl quat distribution in the benzyldimethylstearyl ammonium chloride monohydrate sample. Peak identities of the short chain quats were confirmed by LC/MS analysis.
Conditions – Column: 150 × 2.0 mm i.d. Luna 3 µm CN; Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac (pH 2.0).

4.3.3 Ion suppression, and other problems associated with the LC/MS methodology

Whilst information generated by LC/MS analysis was used to confirm peak identities and characterise the minor quat impurities present in some samples, a number of inherent flaws were observed in the methodology. The propensity of quats to sorb to surfaces led to problems when the analytes were transferred from the outlet of the DAD to the inlet of the ESI interface. During previous LC/MS work (**Section 3.5.6.3**) column eluant had flowed into the ESI interface via a one piece column-coupler. Unfortunately, this design was not viable during the RP-LC/MS work, and as a result, a long length (≈ 50 cm) of 0.12 mm i.d. PEEK tubing was used as a transfer line between the DAD and the ESI inlet capillary. When the TIC's and RIC's were observed it was apparent that they showed severe band broadening, peak tailing and asymmetry (**Figure 4.12**) when compared to the LC-UV peak profiles seen earlier (**Figure 4.5**).

Problems were also witnessed with cross contamination; analyte carry-over being due to the fouling of the interface by sorbed quat species.

While the aforementioned problems were derived from the inherent physico-chemical properties of the quat analytes, an apparent inherent lack of method sensitivity appeared to be directly related to the parameters used in the new methodology.

Figure 4.12 had initially caused concern as the abundance of M^+ ion of the C_{12} quat was well below the abundances witnessed with loop injection analysis as well as previous LC/MS analysis performed on the same instrument. Whilst some variation is to be expected in the ionisation efficiency and ease of ion expulsion of structurally disparate analytes, the degree to which the abundancies had decreased, in addition to the knowledge that the analytes were charged in solution, indicated the influence of an external factor. When a series of calibration standards were subsequently analysed it was clear the hyphenated methodology demonstrated an approximate four-fold loss in sensitivity compared to the LC-DAD method, the LOD of the C_{12} component having risen to approximately 3 mg/l Querton.

4.4 OPTIMISATION OF THE HYPHENATED LC/MS METHODOLOGY

The limitations of using TFA as an ion-pair reagent in the LC/MS analysis of basic analytes were described in **Section 1.3.2.1**. A number of articles are referenced in which the presence of TFA led to significant ion-suppression of the organic bases, the effect being attributed to inefficient desolvation and ion expulsion occurring, as a result of the recalcitrant nature of TFA-base adducts (Apffel *et al.*, 1995). Severe ion-suppression has been reported to occur in negative ESI-MS mode in the presence of 100 mmol/l acetate ions, whilst the presence of TFA led to the effect being witnessed at much lower concentrations (Yamaguchi *et al.*, 1999; Kuhlmann *et al.* 1995). Closer inspection of the LC mobile phase revealed that approximately 0.5% TFA was present in the new methodology.

4.4.1 Reduction of TFA induced ion-suppression with post-column modifiers

Having previously observed that the use of AcH and formic acid resulted in reduced efficiency and increased peak tailing in the LC system in comparison to TFA, it was apparent that redevelopment of the methodology would be laborious and time

consuming. Having already assessed the sensitivity and linearity of the LC-UV method, in addition to building a profile of robustness and reproducibility, it was inconceivable to re-design the LC/MS methodology from scratch. Instead, a technique was sought that would allow the TFA problem to be *"fixed"* as an interim measure. A literature search revealed that the use of an organic and / or acidic sheath of liquid, infused into the eluant stream after the column outlet could lead to significant reductions in ion suppression (Kuhlmann *et al.*, 1995; see also **Section 1.3.2.1**). As a result, this method was subsequently evaluated in the LC/MS work.

Sheath liquid was infused into the column effluent via a PEEK T-piece in the same manner by which analyte was infused into the eluant stream during the tuning of the MS detector in loop injection mode. Initial attempts to reduce ion-suppression utilised a 100 µl/min sheath of HPLC grade propan-2-ol (IPA), and whilst most MS parameters were kept constant, the ESI capillary temperature was increased to 285°C, and the sheath and auxiliary gas flows were regulated to 45 and 10 arbitrary units respectively to assist solvent removal.

Figure 4.14 shows the chromatogram that resulted from the analysis of a 100 mg/l Querton standard in the presence of a sheath of IPA *"fixing solution"*. The abundancies of the major quat species were significantly higher than those witnessed above, with the calculated peak areas increasing three-fold on average. These improvements were higher than those reported previously in the literature (Apffel *et al.*, 1995).

It was predicted that the combined action of two factors was leading to the observed improvements in sensitivity. Increases in conductivity and surface tension of solvent systems containing TFA had been thought to be the root cause of a number of problems experienced during the ESI-MS of solvent systems containing this reagent. High droplet conductivity was thought to lead to interface shorting and an intermittent spray current. At the same time, high droplet surface tension was thought to limit analyte ion expulsion (Eshraghi *et al.*, 1993). Literature reports have therefore attributed improvements in analyte ion abundancy in the presence of IPA, to a reduction in the surface tension of the liquid droplets formed in the ESI interface. A significant reduction in droplet surface tension would greatly assist analyte ion expulsion and

would also yield greater volatilisation of TFA, which would to allow the formation of a stable spray current, reducing the likelihood of shorting, and also reduce the amount of reagent available in the interface to mop-up the generated charge.

It was predicted that the larger than expected improvements in analyte ion abundancy witnessed during this work, represented a more significant reduction in droplet surface tension as a result of excessive use of TFA in the new methodology, compared to previous literature reports.

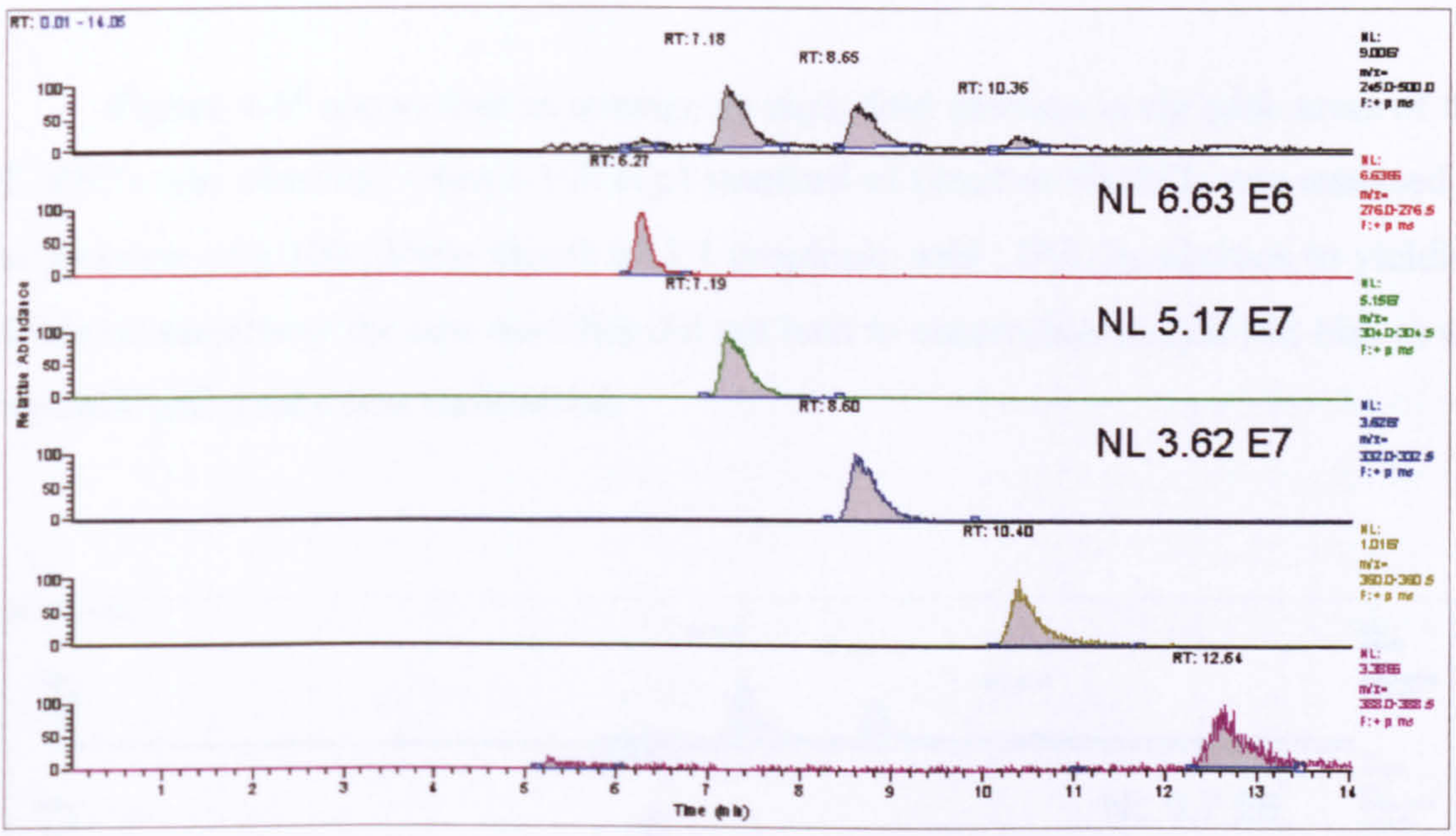

Figure 4.14: Total ion chromatogram (TIC) (top) and reconstructed ion chromatograms (RIC's) of the quat species present in the Querton sample, obtained after the addition of a fixing solution containing IPA.
Conditions – Column: 150 × 2.0 mm i.d. Luna 3 μm CN; Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac (pH 2.0); Fixing solution: IPA; Fixing solution flow rate: 100 μl/min.

One major drawback with the use of the sheath of IPA was that a change was witnessed in the ratio of the C_{12} and C_{14} components. It appeared that the M^+ ion corresponding to the C_{14} quat was being favoured as the C_{12}:C_{14} ratio fell from 3:1 to 2.2:1. It was hypothesised that the change in the C_{12}:C_{14} ratio reflected a variation in the adduct stability of the different analytes, as previous reports had demonstrated that the formation of TFA-base adducts was governed by the mutual accessibility of the ions, and the non-columbic interactions between them (Cai *et al.*, 1999).

To overcome the discrepancies witnessed with the C_{12}:C_{14} ratio, the composition of the fixing solution was adjusted to 3:1 propionic acid : IPA. This solvent system had been reported to yield a 30 fold increase in sensitivity in the abundances of small peptides in the presence of TFA compared to a system in which no fixing solution was used (Kulmann *et al.*, 1995). The presence of an excess of propionic acid was thought to split the TFA-base adducts, leading to the volatilisation of TFA and the formation of weak propionate-base adducts, which demonstrated a reversible equilibrium allowing them to be broken down in the ESI interface yielding higher abundancies of analyte ions within the detector and thus accounting for the observed increase in sensitivity (**Section 1.3.2.1**).

Figure 4.15 shows that on average an eight-fold increase in the peak areas of the M^+ RIC's was observed when a 100 mg/l standard of Querton KKBCL was analysed in the presence of a 100 µl/min sheath of 3:1 propionic acid : IPA. In addition to yielding additional sensitivity the new modifier did not lead to observable analyte MS bias as the original C_{12}:C_{14} ratio was maintained.

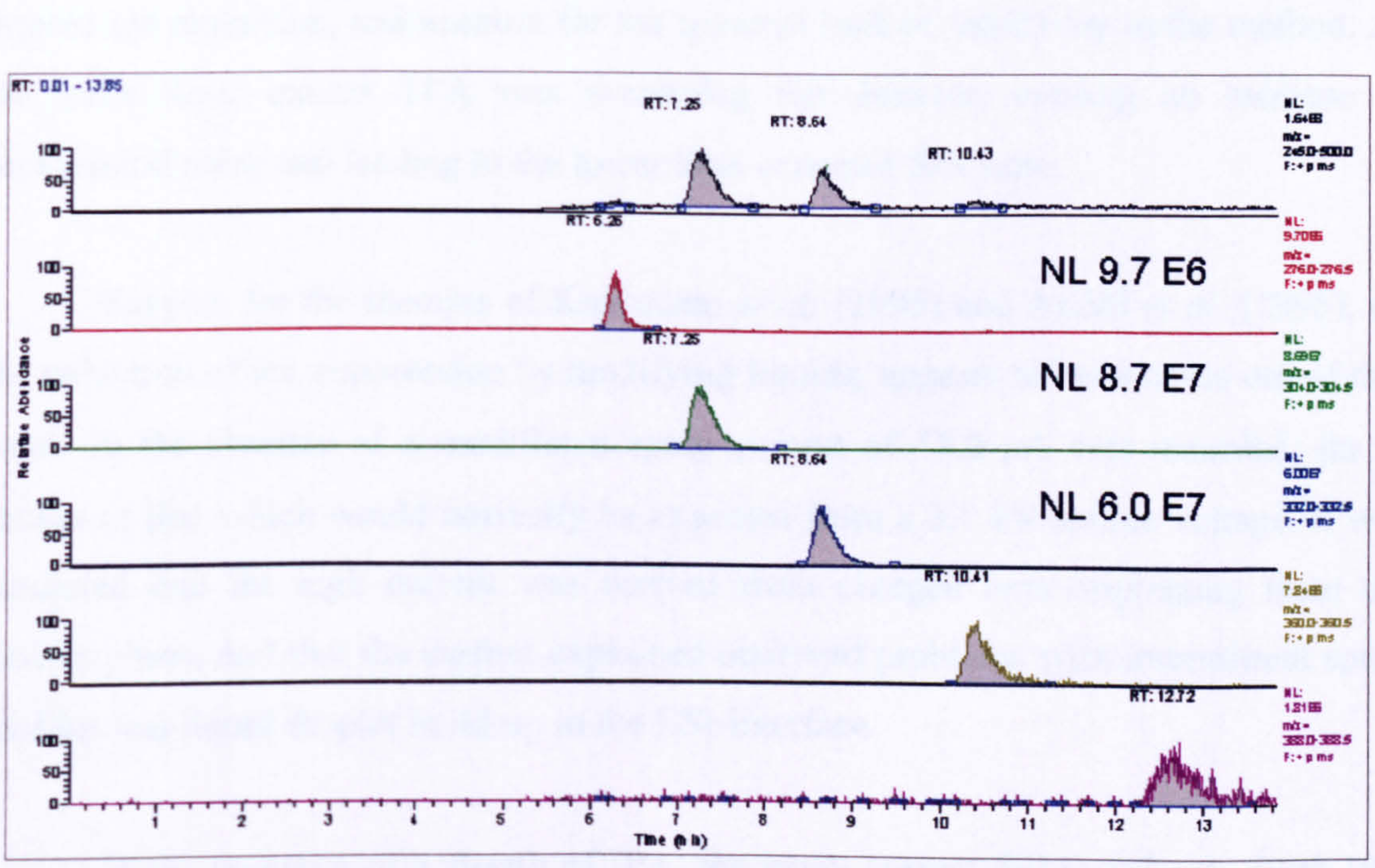

Figure 4.15: Total ion chromatogram (TIC) (top) and reconstructed ion chromatograms (RIC's) of the quat species present in the Querton sample, obtained after the addition of a fixing solution containing 3:1 propionic acid : IPA.
Conditions – Column: 150 × 2.0 mm i.d. Luna 3 µm CN; Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac (pH 2.0); Fixing solution: 3:1 propionic acid : IPA; Fixing solution flow rate: 100 µl/min.

Agreement has yet to be reached among mass spectrometrists as to the reasons why the presence of an excess of propionic acid / IPA leads to a reduction in the amount of ion-suppression, exhibited by basic and / or positively charged analytes, in the presence of TFA. The technique is in conflict with normal recommendations on the development of LC/ESI-MS methodologies, in that the concentration of modifying reagents entering the ESI interface is increased rather than reduced (Lagerwerf *et al.*, 2000). There is normally a general fear that the excessive use of reagents will increase the likelihood of interface fouling and / or that the excess reagent will mop-up the charge in the interface, leading to high background noise, and reduced analyte ionisation efficiency. With ionisation efficiency expected to decrease as background noise increases, a low S/N ratio is normally predicted.

Such a sequence of events was thought to have accounted for the low analyte ion abundances witnessed when the alkylbenzyl quats were analysed by LC/MS in the absence of a fixing solution. Although analyte ionisation efficiency was predicted to have had little influence on the system, the predicted increase in surface tension and the presence of stable TFA-analyte adducts (Kulmann *et al.*, 1995) was thought to lead to limited ion expulsion, and account for the inherent lack of sensitivity in the method. At the same time, excess TFA was swamping the detector, causing an increase in background noise and leading to the lower than expected S/N ratio.

Support for the theories of Kuhlmann *et al.* (1995) and Apffel *et al.* (1995), on the reduction of ion-suppression by modifying liquids, appears to have come out of this work. In the absence of a modifier a spray current of 13.2 μA was recorded, far in excess of that which would normally be expected from a 3.7 kV source voltage. It was predicted that the high current was derived from charged ions originating from the mobile phase, and that the current explained observed problems with intermittent spray profiles and liquid droplet build-up in the ESI-interface.

In the presence of a sheath of IPA, the spray current fell to 9.2 μA. With IPA predicted to only affect the surface tension of the spray droplets, the fall in spray current appeared to support the theory that as surface tension decreased, TFA was driven out of the droplets and volatilised into the gas phase. As a result, fewer TFA and TFA-induced

species were present in the interface to generate excess charge leading to the observed decrease in current.

When a modifying liquid of propionic acid - IPA (3:1 v/v) was used in the LC/MS methodology the spray current was seen to fall still further to 6.8 μA. Due to the respective strong and weak acid nature of TFA and propionic acid, the combination of the two reagents in an aqueous system would normally yield dissociated TFA and protonated propionic acid. However, Apffel *et al.* (1995) predicted the reverse to be true within the ESI interface. As acid dissociation was occurring at the same time as droplet size was decreasing, it was predicted that the higher volatility of TFA would lead to the equilibria being driven towards dissociation of the propionic acid, as the protonated TFA evaporated out of the droplet into the gas phase. As droplet evaporation was being facilitated by surface tension reducing IPA, it was thought that the effect would occur rapidly, leading to the removal of much of the TFA from the liquid droplets, and cleavage of many of the strong TFA-positively charged analyte adducts. Observation of an eight-fold increase in ion abundancy and a two-fold decrease in spray current in this work appears to suggest that the predictions regarding the acid equilibria were correct. TFA was being driven out of the source, leaving behind fewer charged species and fewer strong TFA-analyte adducts.

Whilst ion abundancy was seen to increase in the presence of a modifier containing propionic acid / IPA, the improvements witnessed during the course of this work did not approach those reported in the literature (Kuhlmann *et al.*, 1995). However, it was thought that due to the excessive use of TFA in the LC methodology, far more TFA remained in the ESI interface than in previous work. Although the spray current fell to 6.8 μA, this was still well above the levels witnessed during previous work on the instrument. It was hypothesised that an excess of charged species were still present in the interface.

4.4.2 Evaluation of the sensitivity and linearity of the optimised LC/MS methodology

Having observed significant improvements in analyte ion abundancy during LC/MS analyses performed in the presence of a 3:1 propionic acid : IPA fixing solution,

this modifier was incorporated into the optimised LC/MS methodology and was thus used in all further RP-LC/MS work.

To determine the sensitivity and linearity of the optimised methodology a series of Querton calibration standards was prepared ranging in concentration from 0 to 50 mg/l. Having previously observed that no adduct ions were formed between the alkylbenzyl quats and other species present in the solvent system, only the M^+ ions needed to be accounted for during quantative work. **Figure 4.16** shows a plot of the RIC peak area response of the LCQ instrument to the M^+ ion of the C_{12} alkylbenzyl quat component (*m/z* = 304.0 to 304.5), versus concentration of Querton. The detector showed good linearity over the specified calibration range, which was not fully expected in light of previous quantitation problems witnessed with ion trap instruments (Weston, 2000).

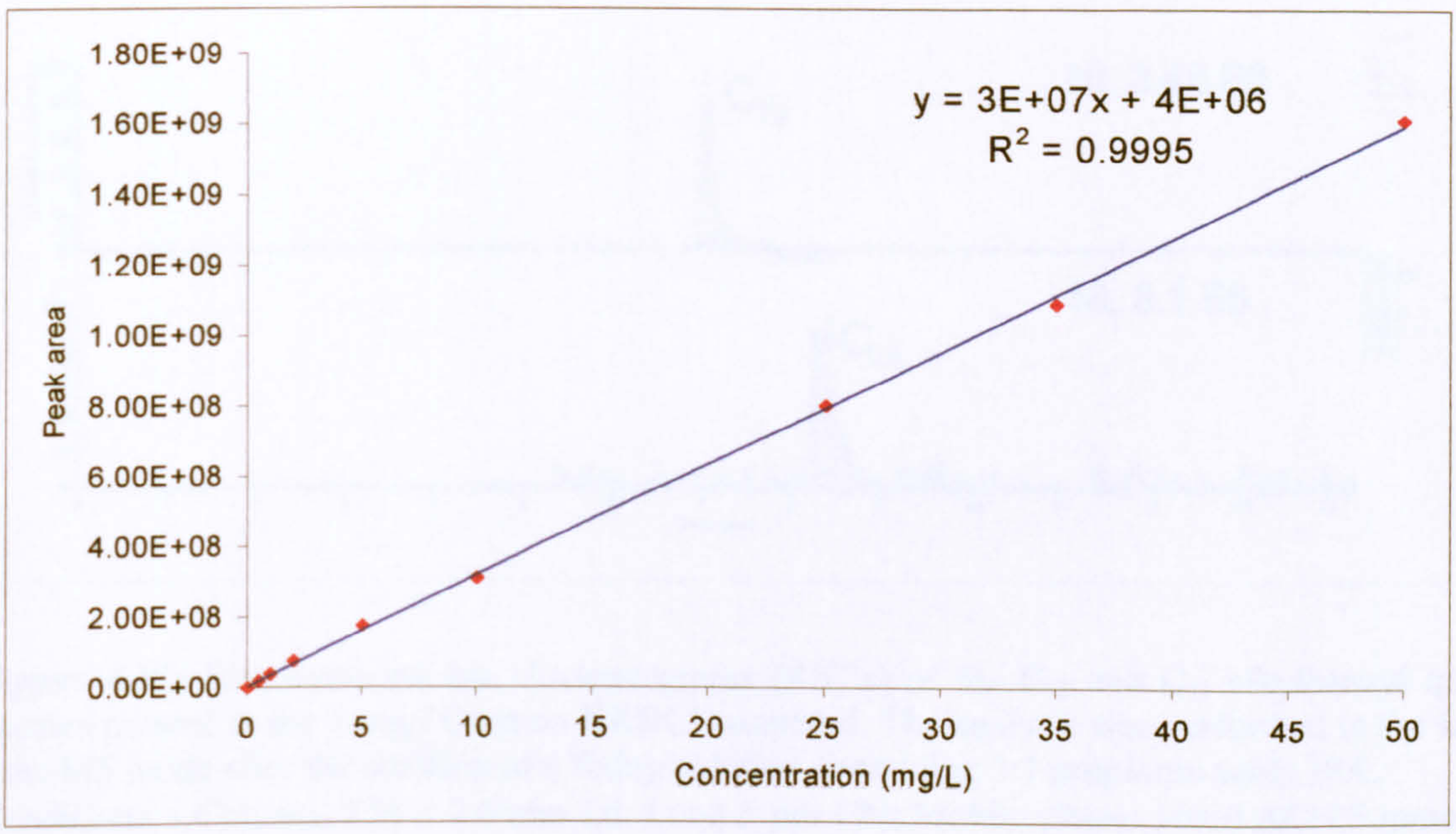

Figure 4.16: Plot of peak area response of the C_{12} quat species versus concentration of the Querton KKBCl sample performed on the LCQ instrument.
Conditions – Column: 150 × 2.0 mm i.d. Luna 3 µm CN; Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac (pH 2.0); Fixing solution: 3:1 propionic acid : IPA; Fixing solution flow rate: 100 µl/min.

In addition to good linearity, the optimised LC/MS methodology also demonstrated improved sensitivity compared to the LC-DAD method reported above. The LOD of the C_{12} component fell to 650 µg/l Querton, approximately 450 ppb of the

C_{12} component (see **Figure 4.17** for the RIC's of the C_{12} and C_{14} components obtained from the analysis of a 1 mg/l Querton standard).

4.4.3 Increasing the sensitivity of the LC/MS methodology – "Full scan" analysis versus "Single Ion Monitoring" (SIM)

The optimised LC/MS methodology had afforded an improvement in sensitivity over the previously reported LC-DAD method. However, application of the methodology to the study of the biodegradation of quats in environmental compartments could not be achieved without further improvements in sensitivity. Prior to this time all MS analyses had been performed in the *"full-scan MS mode"* (**Section 1.3.2.1**). However, having observed that the M^+ ions were the only abundant species present, a preliminary evaluation of *"single ion monitoring MS mode"* (SIM-MS) was performed.

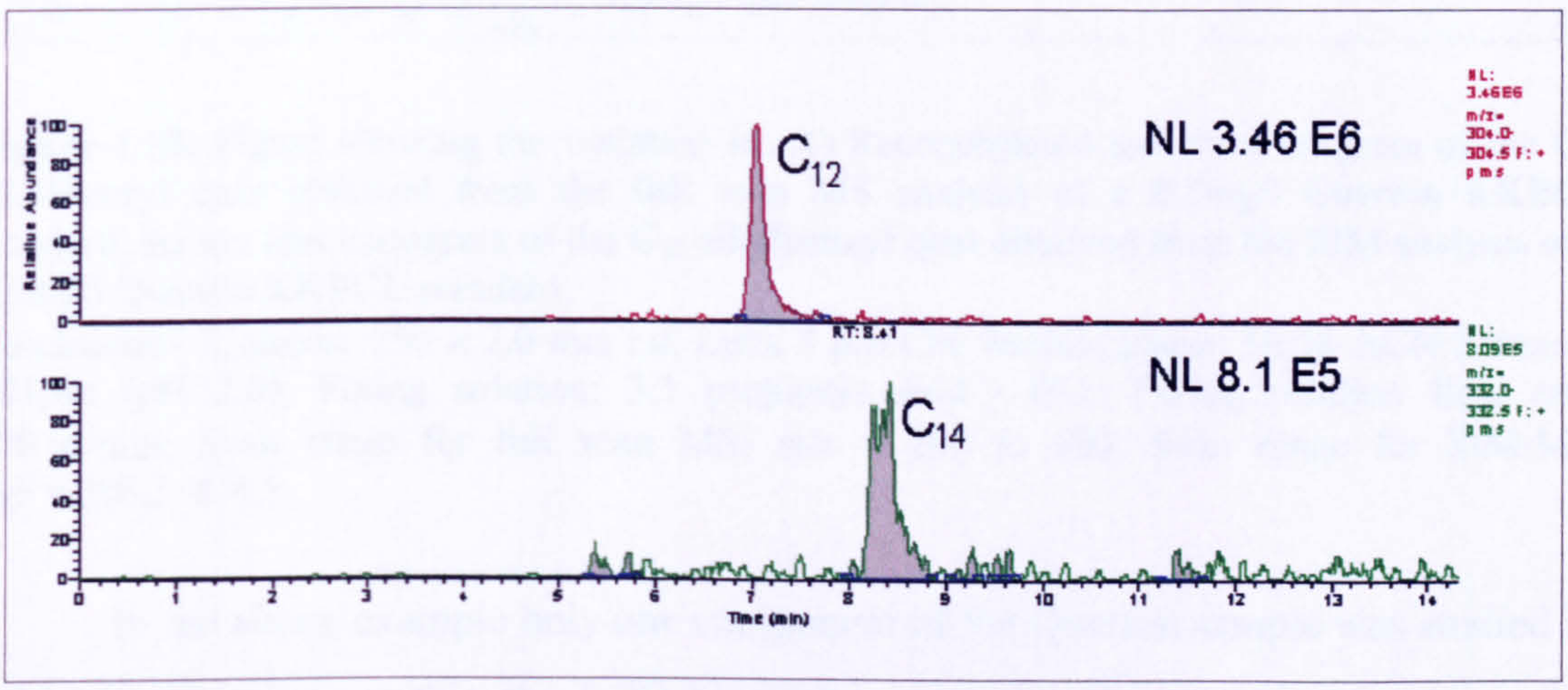

Figure 4.17: Reconstructed ion chromatograms (RIC's) of the C_{12} and C_{14} alkylbenzyl quat species present in the 1 mg/l Querton KKBCL standard. The analysis was performed in the full scan-MS mode after the addition of a fixing solution containing 3:1 propionic acid : IPA.
Conditions – Column: 150 × 2.0 mm i.d. Luna 3 µm CN; Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac (pH 2.0); Fixing solution: 3:1 propionic acid : IPA; Fixing solution flow rate: 100 µl/min.

Figure 4.18 shows the response of the MS instrument to the C_{12} alkylbenzyl quat present in a 500 µg/l Querton standard in A) full scan MS mode and B) SIM-MS mode. The ion chromatograms obtained from SIM-MS showed significantly higher ion abundancy compared to the corresponding RIC obtained during full scan MS. More detailed evaluation of SIM-MS revealed that the LOD for the C_{12} component was below 300 µg/l, and thus it was predicted that a methodology utilising solid phase extraction

(SPE) and LC/SIM-MS should allow quantitation of alkylbenzyl quats at ng/l levels (parts per trillion).

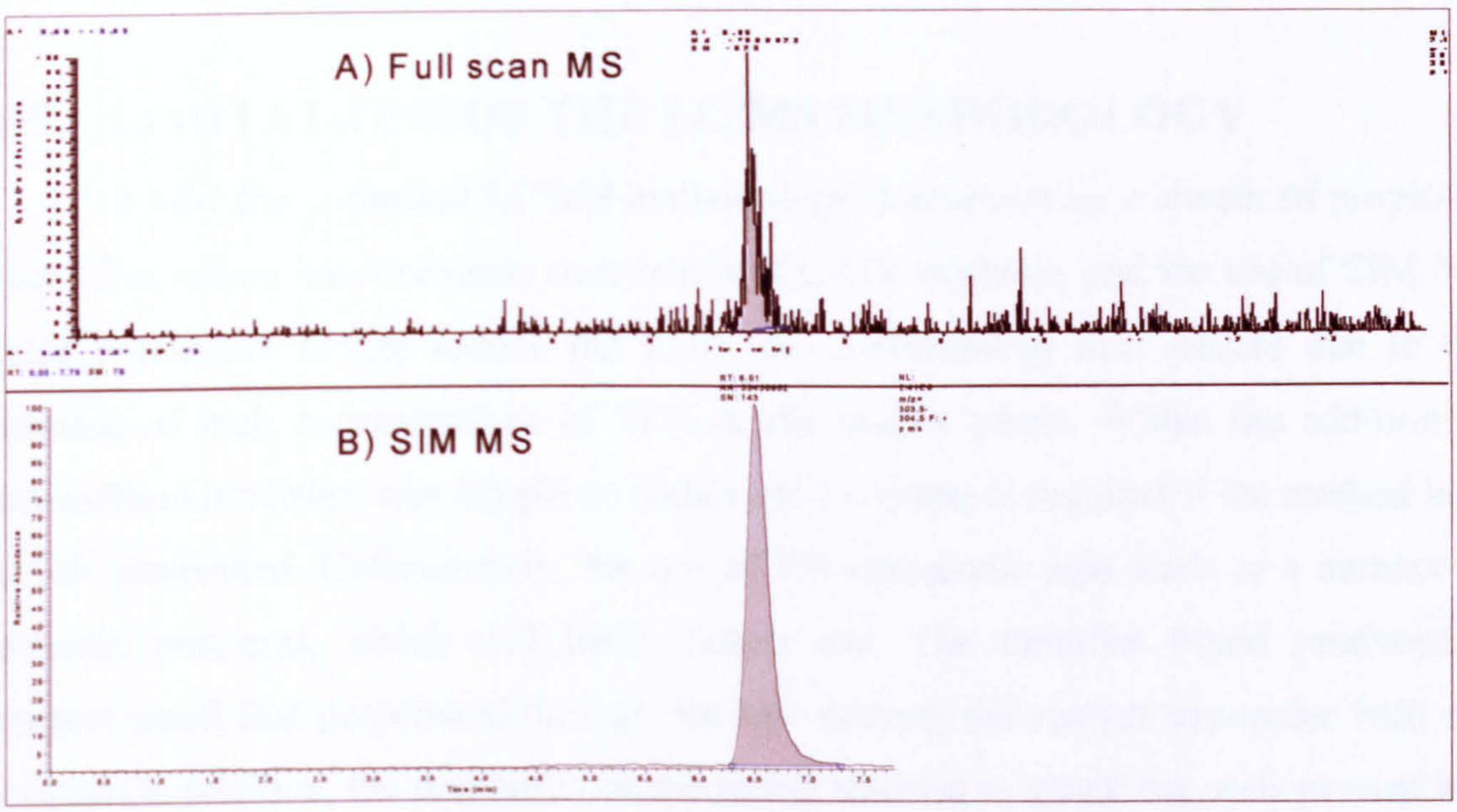

Figure 4.18: Figure showing the variation in: A) Reconstructed ion chromatogram of the C_{12} alkylbenzyl quat obtained from the full scan MS analysis of a 0.5mg/l Querton KKBCL standard; B) Ion chromatogram of the C_{12} alkylbenzyl quat obtained from the SIM analysis of a 0.5mg/l Querton KKBCL standard.
Conditions – Column: 150 × 2.0 mm i.d. Luna 3 µm CN; Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac (pH 2.0); Fixing solution: 3:1 propionic acid : IPA; Fixing solution flow rate: 100 µl/min; Scan range for full scan MS: *m/z* = 200 to 500; Scan range for SIM-MS: *m/z* = 303.5-304.5

In the above example only one component of the Querton sample was studied by SIM-MS. However, within the LCQ Navigator control software, as with many other modern benchtop instruments, the option was available to create multiple-segment MS acquisition methods, whereby SIM-MS at two or more mass ranges and / or SIM-MS and full scan MS could be performed at discrete points within an LC/MS run. Although not utilised during the course of this work, the use of multiple-segment MS analysis provides the capability to detect, by SIM-MS, a number of active components and /or metabolites, making it well suited to the analysis of quat samples. Having previously observed the excellent retention time stability of the LC methodology, SIM-MS segments could have been created for each of the individual components, improving the LOD for each active species present in the sample. Unfortunately, due to time restraints and a major instrumental breakdown, the application of multiple-segment MS methods and the subsequent integration of SIM-MS into the hyphenated methodology were not

evaluated during the course of this work. However, before SIM-MS can be adopted for routine use evaluation of the reproducibility, sensitivity, linearity and most importantly robustness must be performed.

4.5 LIMITATIONS OF THE LC/MS METHODOLOGY

Whilst the optimised LC/MS methodology, incorporating a sheath of propionic acid : IPA, offers improvements over current LC-UV methods, and the use of SIM-MS could potentially further reduce the LOD, the methodology still suffers due to the presence of high concentrations of TFA in the mobile phase. Whilst the addition of post-column modifiers was simple an additional LC pump is required if the method is to be left unattended. Unfortunately, the use of 75% propionic acid leads to a number of aesthetic problems, which will limit routine use. The modifier liquid produced a pungent smell that perpetrated through the lab, making the analyst unpopular with co-workers. In addition, the propensity of the fixing solution to attack the seals present in a syringe or a HPLC pump led to the belief that the method (and analyst) would quickly become unpopular with management, due to the prohibitive costs of purchasing new parts, and the increased frequency of instrument down-time associated with repairs and replacements.

Ultimately, the presence of high concentrations of TFA in the LC mobile phase will limit the applicability of the reported LC/MS methodology, and thus redevelopment of the front-end LC separation is paramount. An important question that needs to be addressed is *"Does the mobile phase need to be adjusted to pH 2.0?"* If a higher pH can be utilised without compromising the resolution of the alkylbenzyl quats, less acid will be needed in the system. As a result, the extent of ion suppression is expected to be reduced. This is one of a number of questions that will be considered in **Chapter Five.**

Since the completion of this work a report has appeared on the use of SPE-LC/MS and SPE-LC/MS/MS for the analysis of alkylbenzyl quats in wastewaters and rivers at sub µg/l levels (Ferrer *et al.*, 2001). The literature method has a number of inherent benefits over the method described above, as it does not require the use of a post-column fixing solution to obtain a high abundancy of analyte ions within the ion

trap. However, the literature method relies on the use of a gradient elution profile, as an ODS material was chosen as the stationary phase, yet the C_{16} alkylbenzyl quat still requires twenty five minutes to elute. If one also considers that a post time of ten minutes would also be required to re-equilibrate the column between successive analyses (potentially longer depending on the measures adopted to avoid void volumes in the HPLC system), then the literature method shows an approximate 3.5 fold increase in analysis time compared to the method reported above. The advantages and disadvantages of the individual parameters used in the two methodologies will be considered in **Chapter Five.**

4.6 CONCLUSIONS

This chapter has described the development of a new hyphenated RP-LC/MS method for the routine analysis of alkylbenzyl quats. The LC methodology was based on a Unilever Research standard operating procedure that is representative of methods available in the literature. Improvements in the LC separation were achieved by utilising a short cyanopropyl phase based on a high purity silica support. In comparison with traditional sol-gel silica-based stationary phases the high purity Luna phase yielded more symmetrical, faster eluting analyte peaks that showed reduced peak volumes.

Utilisation of a narrow-bore (2.0 mm i.d.) column improved analyte peak areas by a factor of five, and in addition to the replacement of non-volatile sodium perchlorate improved the compatibility of the method with electrospray mass spectrometry. However, problems were experienced with maintaining the resolution of the alkylbenzyl quat species on the narrower column, forcing the use of analyte peak compression. Recent problems at Unilever Research in the extraction of cationic tensides from environmental matrices suggest that the use of a narrow bore column in association with the peak compression effect will not be suitable for the application to environmental analysis, and thus a recommendation was made to revert to the use of a 4.6 mm i.d. column when problems are experienced.

The optimised LC-DAD methodology showed excellent linearity and demonstrated the necessary sensitivity to make it applicable to environmental analysis. In addition, whilst the method was not formally validated, long-term observations on

the reproducibility of the separation, its ease of transfer to an alternative laboratory, and subsequent observations made by Unilever Research, suggest the method is inherently robust.

Hyphenation of the LC methodology with mass spectrometry was more difficult. Problems were experienced with unknown ions swamping the detector, whilst hyphenation of the LC method with ESI-MS revealed a lack of method sensitivity, due to trifluoroacetic acid (TFA) induced ion suppression. The subsequent use of an acidic and / or organic fixing solution reduced the influence of TFA leading to increased ion abundancy and sensitivity. Observations made during this section of work also appeared to support previous theories on the improvements in signal to noise ratio that can be achieved with the use of post-column modifiers.

Evaluation of the optimised LC/MS methodology revealed that good linearity was shown in full scan mode, and that an approximate 25% reduction in the limit of detection could be achieved by using LC/MS rather than LC-UV. In addition, a preliminary investigation of the use of single ion monitoring MS revealed that it should be possible to develop a hyphenated LC/SIM-MS method that could be used in association with suitable selective extraction and pre-concentration techniques to study the fate of alkylbenzyl quats in wastewaters and rivers.

Whilst the LC-UV method reported here does provide benefits over other methods currently used by industry, the hyphenated LC/MS methodology ultimately suffers from the presence of an excess of TFA within the system. As a result, use and applicability of the new methodology will be limited. Of greater use are the observations made regarding the difficulties that can be experienced in the development of LC/MS methods for basic and / or cationic analytes.

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CHAPTER FIVE

The development of a generic chromatographic method for cationic tenside analysis

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5.1 INTRODUCTION

With the need to deliver analytical solutions on increasing numbers of parent cationic tensides and their specific metabolites, there is an urgent need to reduce the time taken for analytical method development. Such improvements in speed can be achieved by developing generic methods of analysis for structurally analogous compounds. However, for generic analysis to be successfully applied to environmental matrices, and / or the identification of metabolites, unambiguous structural data is required, which can only be provided by hyphenated chromatographic / mass spectrometric methods. The aim of this segment of work was to address the question *"Can generic methods of analysis be developed for the determination of cationic tensides in industrial and environmental matrices?"* This is achieved by building on the findings of previous chapters, with the experimental work setting out to provide a preliminary understanding of some of the problems that will be encountered on the path to a single generic method of analysis for cationic tensides.

5.2 DETERMINING A SUITABLE STARTING POINT FOR GENERIC ANALYSIS

Literature currently available on both separation science in general and the analysis of cationic tensides in particular, indicates that there are four prime starting points from which to develop a generic method for the analysis of cationic tensides:

1.) The technique of capillary zone electrophoresis (CZE).
2.) The disulphine blue active substances methodology (DBAS).
3.) Normal phase liquid chromatography / mass spectrometry (NP-LC/MS)
4.) Reverse phase liquid chromatography / mass spectrometry (RP-LC/MS)

In **Section 1.4** a critical review was presented of the common methods used to analyse cationic tensides, which included details of the inherent advantages and disadvantages of each of the methods. The review illustrated the major limitations that

the CZE and the DSBAS methods harbour, which prohibit their application to generic analysis (**Sections 1.4.5** and **1.4.1**). As a result, it was clear that a liquid chromatographic method represented the most appropriate starting point from which to develop a generic method of analysis for cationic tensides.

In **Chapters Three** and **Four** the development of two new hyphenated LC/MS methods are described for the analysis of specific cationic tensides. Both of these methods offered a number of inherent advantages over current literature methods, and thus it was concluded that the new methods should be evaluated for widespread applicability to cationic tensides.

5.3 EVALUATION OF THE NEW NP-LC/MS METHODOLOGY FOR GENERIC CATIONIC TENSIDE ANALYSIS

The new NP-LC/MS methodology described in **Chapter Three** was developed to improve the resolution of the cationic actives utilised in fabric conditioner formulations (**Section 1.2.1**). The LC methodology was shown to be suitable for the analysis of a range of diester, dialkyl, monoester, and monoalkyl quats, that are, or have been the primary active agents present in domestic fabric softeners (**Section 1.2.1**). The method was also successfully applied to the identification of the triester and trialkyl quat impurities present in a number of the samples (**Appendix One**). However, limited success was witnessed in the quantitation of the quaternary amino-alcohols, the degradation products of the esterquat tensides (**Section 1.2.3.3**). For the new NP-LC/MS methodology to be adopted for the generic analysis of cationic tensides, its applicability to other cationic tensides needed to be assessed, and the peak parameters of the quaternary amino-alcohols improved.

5.3.1 Application of the new method to the analysis of the alkylbenzyl quaternary ammonium tensides

Figure 5.1 shows the chromatogram obtained from the analysis of a mixed alkylbenzyl quat sample on a Spherisorb amino column with the ternary mobile phase system described in **Section 3.5.** Baseline resolution of the four homologues was achieved in thirty minutes, with the C_{18} alkylbenzyl quat eluting first and the C_{12} component eluting last **(Section 3.4)**.

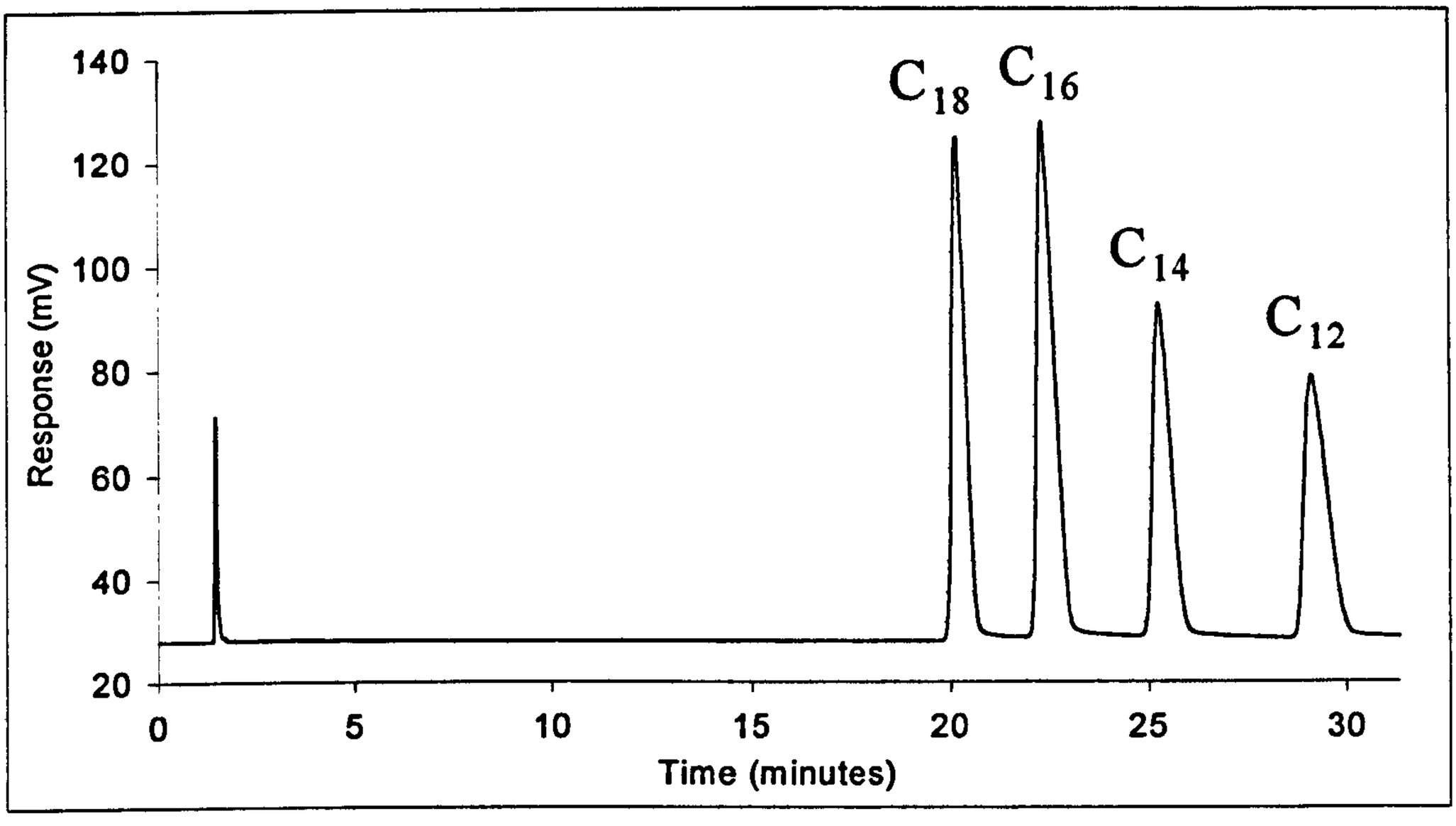

Figure 5.1: Chromatogram showing the resolution of a mixed alkylbenzyl quat sample under normal phase conditions.
Conditions – Column: 150 × 4.6 mm i.d. Spherisorb 3 µm amino; Mobile phase: 88:7.2:4.8 Hexane : MeOH : THF modified with 5 mmol/l TFA; Detection: Evaporative light scattering.

It was apparent after comparing the chromatograms obtained from the analysis of the octadecyltrimethylammonium bromide sample and the benzyldimethylstearylammonium chloride dihydrate sample under normal phase conditions (**Figure 5.2**) that the retention times of the alkylbenzyl quats were much greater than the corresponding alkyltrimethyl quats. Indeed, the increase in retention time was such that the calculated selectivity (see **Section 1.3.1** and **Equation 1.11**) of the two respective C_{18} species was seen to be approximately 1.6. This result was somewhat unexpected as the physico-chemical data available for the two species, in particular the predicted Log octanol / water partition coefficients (log K_{ow} or log P) (**Section 1.2.3.3**), suggested that the alkylbenzyl quat should have been more hydrophobic than the corresponding alkyltrimethyl quat (Hansch *et al.*, 1979; Syracuse Research Corporation).

It is usually accepted that analyte retention is brought about by electrostatic interactions between the analyte and the stationary phase under normal phase LC conditions (**Section 1.3.1**). After consideration of the mutual incompatibility of the ternary solvent system, and the chromatographic data collected during the analysis of

the fabric conditioner actives (**Chapter Three**), a hypothesis was proposed whereby the resolution of the cationic tensides was derived from the formation of a band of stationary polar solvent within the pores of the silica (**Section 3.6.3**). Under these conditions partitioning into the stationary liquid is thought to be the principal driver of analyte retention (Kažoka, 2000), rather than conventional electrostatic interactions. It was therefore envisaged that the hydrophobicity of the analytes should have influenced their retention characteristics; as hydrophobicity increased, retention was predicted to decrease due to preferential partitioning into the bulk mobile phase. Consideration of the two chromatograms shown in **Figure 5.2** revealed that experimentally derived data did not agree with these theoretical predictions.

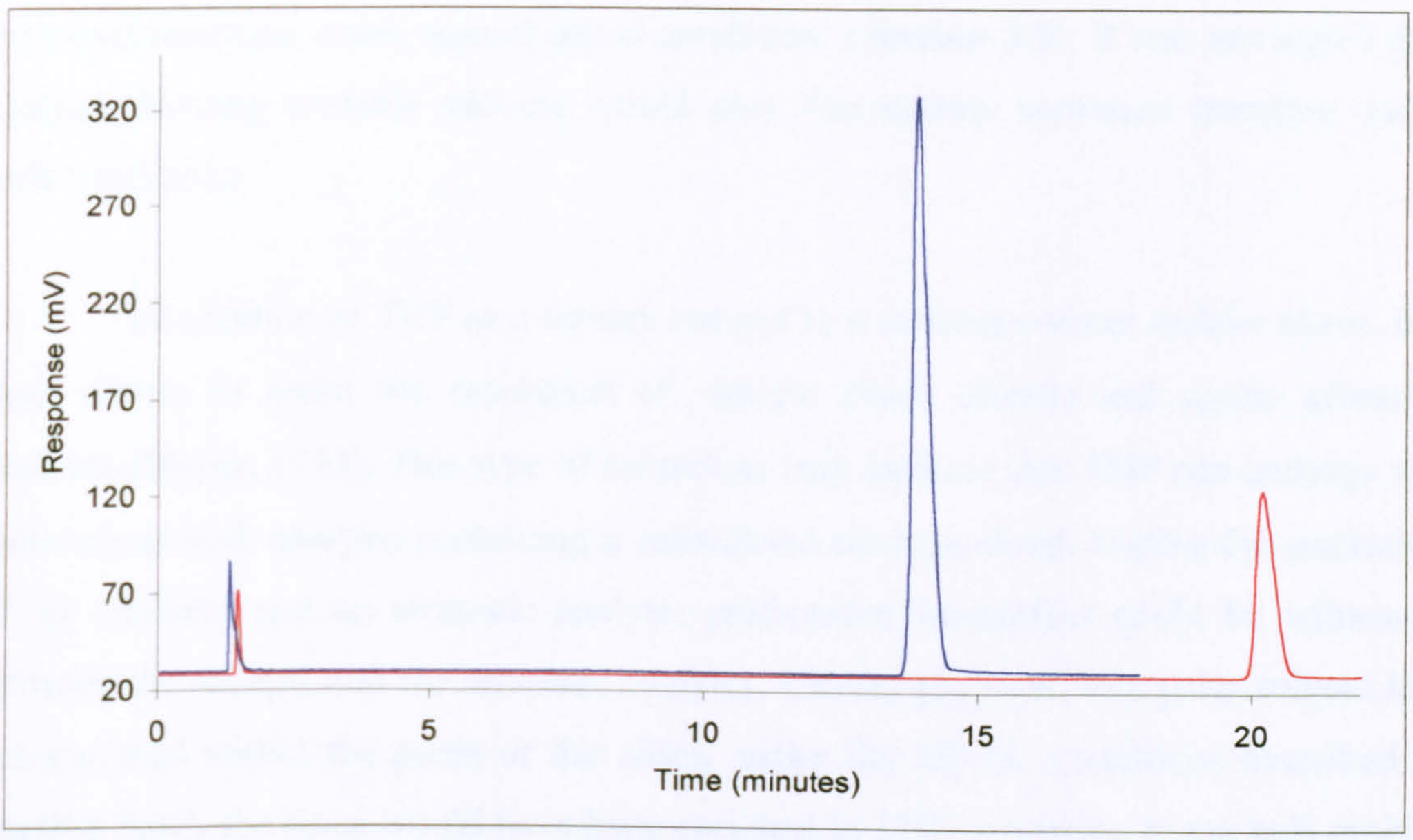

Figure 5.2: Figure showing the variation in the retention time between the octadecyltrimethylammonium bromide and benzyldimethylstearylammonium chloride dihydrate samples under normal phase conditions.
Conditions – Column: 150 × 4.6 mm i.d. Spherisorb 3 µm amino; Mobile phase: 88:7.2:4.8 Hexane : MeOH : THF modified with 5 mmol/l TFA; Detection: Evaporative light scattering.

Although it proved impossible to develop a definitive theory for the long retention times of the alkylbenzyl quats, it was predicted that one or both of the following was partly or wholly responsible for the long retention times:

1.) Stability of the TFA-analyte adducts

2.) Interactions between the stationary liquid and the phenyl unit present in the alkylbenzyl quats

1.) Reference has already been made to the fact that the association of TFA with basic and / or cationic analytes leads to the formation of pseudo-neutral adducts, which demonstrate greater hydrophobicity than the free analytes (**Section 1.3.2.1**). The formation of such adducts is governed by the mutual accessibility of the two ions (Cai *et al.*, 1999), and thus any steric bulk in close proximity to the cationic charge will hinder the formation and overall stability of the adducts. It was hypothesised that the increased retention of the alkylbenzyl quats could have indicated greater instability of the adducts formed by these quats compared to those of the corresponding monoalkyltrimethyl quats. Having already observed that a decrease in TFA concentration resulted in increased retention under normal phase conditions (**Section 3.5**), it was envisaged that analytes forming unstable adducts would also demonstrate increased retention under such conditions.

2.) The addition of THF as a ternary solvent to a methanol-water mobile phase, has been shown to assist the resolution of straight chain alkenes and cyclic aromatic analytes (Meyer, 1993). This type of behaviour may indicate that THF can undergo π-π interactions with analytes containing a delocalised electron cloud. During the resolution of an aliphatic and an aromatic analyte, preferential interaction could be witnessed between the solvent and the aromatic analytes. Having proposed that polar solvent had accumulated within the pores of the silica, under the NP-LC conditions described in **Section 3.6.3**, the silica would have been enriched in THF in relation to the bulk mobile phase. It is feasible that the THF in the stationary liquid would interact with the phenyl groups of the alkylbenzyl quats leading to an additional solvent-induced retention mode, which would not have been evident for the alkyltrimethyl quats. Whilst this theory appeared tenuous and perhaps contrived, support could be found in the original work of Wilkes *et al.* (1992), on the analysis of cationic tensides with hexane-based mobile phase systems. Contrary to the observations reported above, Wilkes *et al.* witnessed the C_{16} alkylbenzyl quat eluting prior to the C_{18} and C_{16} alkyltrimethyl quats, which was more in agreement with the estimates of log K_{ow} for these tensides (Hansch *et al.*, 1979). However, in the literature report, THF and MeOH had been employed in a 3:1 ratio, rather than the 1:1.5 ratio that was used during this work. Under these conditions,

the THF concentration in the bulk mobile phase and the stationary liquid would have been equal. As a result, the alkylbenzyl quats would not have undergone additional retention in the stationary liquid, and may even have been sped through the column due to the excess THF in the mobile phase, accounting for the earlier elution and the switch in elution order.

5.3.2 Analysis of the alkylpyridinium tensides

When a 500 mg/l standard of cetylpyridinium chloride monohydrate (**Appendix One**) was analysed under normal phase conditions a broad, poorly efficient peak was seen to elute after ca. 31 minutes. The peak showed severe tailing, which resulted in the baseline width being in excess of seven minutes. Whilst increasing the eluting strength of the mobile phase led to a reduction in the retention time of the analyte, the comparative change in peak symmetry was far less. When the 1-dodecylpyridinium chloride hydrate sample was subsequently analysed, peak symmetry and efficiency were seen to be even worse than that witnessed for the C_{16} homologue, showing that the normal phase methodology was not well suited to the analysis of these species.

Reliable physico-chemical data on the pyridinium quats was sparse. However, a predicted log K_{ow} value for the C_{16} homologue (Hansch *et al.*, 1979) suggested that it had similar hydrophilicity to the C_{16} alkyltrimethyl quat, with the variation being insufficient to account for the increase in the retention time of the alkylpyridinium quat. In view of the analytes partitioning into a stationary liquid, it was envisaged that hydrophobicity / hydrophilicity should have played some part in the retention mechanism, i.e. the alkylpyridinium quats would have been expected to have eluted much earlier than what was observed throughout the experiment. Indeed, it was predicted that if hydrophobicity were the only factor causing retention then the alkylpyridinium quats would have eluted close to the alkyltrimethyl quats.

In light of the predicted log K_{ow} values (Hansch *et al.*, 1979), the excessive retention and significant peak tailing observed during the analysis of the alkylpyridinium quats, provided further support for the theories regarding stability of the TFA-analyte adducts and the strong interaction between THF and quats containing aromatic functionalities (**Section 5.3.1.1**). With the quaternized nitrogen being constrained in a six-membered heterocyclic ring it was plausible that the approach of a

counter-ion would be severely limited, thus hindering adduct formation. A reduction in the rate of formation and / or the stability of the adducts was predicted to lead to lower apparent hydrophobicity and yield an increase in retention (**Section 3.5**). At the same time, free analytes would have more opportunity to interact with the silanol and bonded phase groups on the silica surface resulting in an increase in electrostatic interaction. The analytes therefore undergo partitioning and electrostatic interaction with the silica support, which is analogous to the mechanism ascribed to peak tailing under reverse phase conditions (**Section 1.3.2.1**).

The low peak efficiency, and problematic quantitation witnessed during the analysis of the alkylpyridinium quats and the quaternary amino-alcohols (**Section 3.5.3**) prohibited the application of the NP-LC methodology to these tensides. It appeared that as analyte hydrophilicity and / or instability of the TFA-analyte adducts increased, the applicability of the NP-LC method decreased. Unfortunately, the focus of environmental analysis has shifted in recent years to the study of hydrophilic analytes as their aqueous solubility increases the likelihood of them being found unassociated in rivers and environmental wastewaters, leading to high bioavailability (**Section 1.2.3.3**). An inability to deal effectively with these important analytes resulted in the methodology being abandoned at this point.

5.4 EVALUATION OF THE NEW RP-LC/MS METHODOLOGY FOR GENERIC CATIONIC TENSIDE ANALYSIS

In **Section 3.6.3** it was predicted that the unusual peak shapes witnessed during the analysis of the quaternary amino-alcohols indicated a non-linear adsorption / desorption isotherm (**Section 1.3.2.1**) for these analytes. A retention mechanism was predicted whereby the desorption of one analyte molecule from the surface of the silica promoted desorption of further molecules from the silica i.e. desorption appeared to be co-operative. The driving force for this unusual retention mechanism was predicted to be the low solubility of the diol and triol species in the bulk hexane-based mobile phase, as the analytes appeared to favour residence in the stationary liquid that was envisaged within the pores of the silica (**Section 3.6.3**). In order to obtain efficient peak shapes and short retention times for these analytes, a water and / or polar organic solvent-based mobile phase system was required. It was predicted that reverse phase LC (RP-LC)

presented a more suitable environment in which to obtain efficient resolution of the quaternary amino-alcohols.

5.4.1 Applicability of the new RP-LC/MS methodology to the analysis of alkylpyridinium quat preservatives

Whilst the optimised RP-LC methodology described in **Section 4.2.3** had been well suited to the rapid and efficient analysis of the alkylbenzyl quats, its wider applicability to other cationic tensides had yet to be evaluated. The second major group of cationic tenside preservatives, the alkylpyridinium quats (**Appendix One**) appeared to represent a suitable starting point from which to test the generic nature of the method, as these analytes had previously been analysed under similar RP-LC conditions to those described in **Section 4.2.1** (Taylor *et al.*, 1997; Zhou *et al.*, 1999).

Figure 5.3 shows a comparison of the chromatograms achieved during the analysis of 150 mg/l standards of 1-dodecylpyridinium chloride hydrate and benzyldimethyldodecylammonium bromide on the Luna CN phase (**Appendix Two**). The peak corresponding to the C_{12} pyridinium quat was seen to elute earlier than the equivalent alkylbenzyl quat species, which supported previous theories on the instability of the TFA-alkylpyridinium quat adducts (**Section 5.3.1.1**).

Aside from a shorter retention time, the C_{12} alkylpyridinium quat also showed a 290% increase in peak area response over that of the corresponding C_{12} alkylbenzyl quat, which was attributed to a variation in the extinction coefficients of the two species at 214 nm. It was envisaged that the increased response would facilitate a lower limit of detection for the alkylpyridinium quats than that reported for the alkylbenzyl quats in **Section 4.2.4.2.**

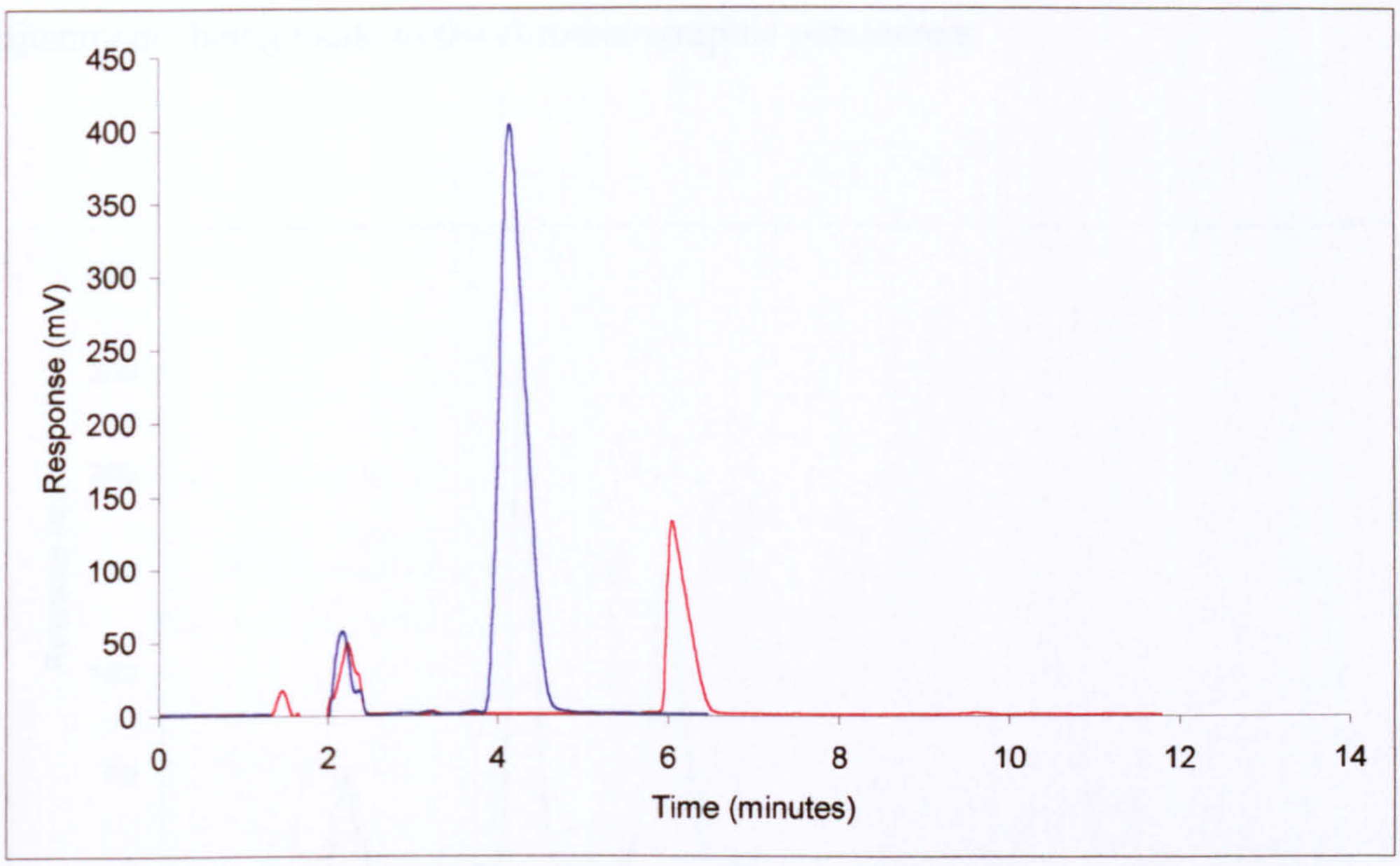

Figure 5.3: Figure showing the variation in retention of the 1-dodecylpyridinium chloride hydrate (blue trace) and benzyldimethyldodecyl ammonium bromide (red trace) samples on the Luna CN material.
Conditions - Column: 150 × 4.6 mm i.d. 3 µm Luna CN; Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac (pH 2.0).

5.4.1.1 Variation in separation efficiency on different cyanopropyl phases

It was previously reported that the retention times of the alkylbenzyl quats were different on the Spherisorb CN and Luna CN phases (See **Section 4.2.2** and **Figure 4.2**). Similar observations were also made during the analysis of the alkylpyridinium quats (**Figure 5.4**). The Luna CN material again afforded greater peak symmetry, reduced peak tailing and resulted in shorter analysis times compared to the Spherisorb material. However, when the retention times of the two groups of cationic tensides were compared, it became apparent that the C_{16} pyridinium quat eluted at a similar retention time to the C_{12} alkylbenzyl quat on the Luna material. **Figure 5.5** shows an overlay of the chromatograms resulting from the analyses of a mixed alkylpyridinium quat sample (blue trace) and a mixed alkylbenzyl quat sample (red trace) on A) the Spherisorb CN phase and B) the Luna CN phase. For the first time the Spherisorb material offered benefits over the high purity Luna material, as there was less overlap between the retention times of the C_{16} alkylpyridinium and C_{12} alkylbenzyl quats on this phase. It was predicted that the degree of overlap of the two species would increase during the analysis of environmental or industrial samples, due to the matrix effects (**Section**

1.3.2.1). As a result, the quantitation of the two species would be problematic without adjustments being made to the chromatographic parameters.

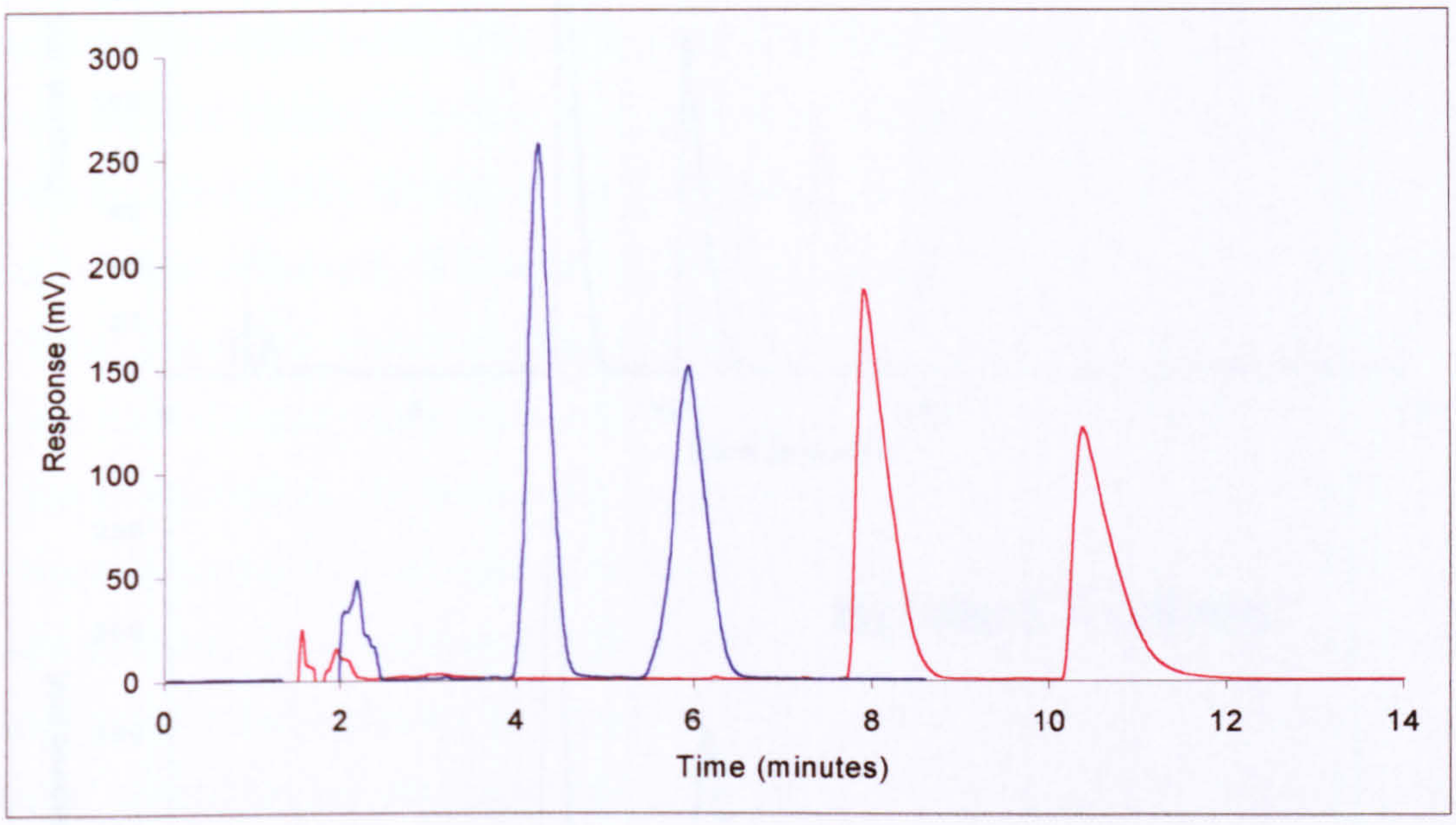

Figure 5.4: Figure showing the variation in retention times of a mixed pyridinium quat sample on the Luna (blue trace) and Spherisorb materials (red trace).
Conditions – Column dimensions : 150 × 4.6 mm i.d. packed with 3 μm CN bonded silica particles; Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac (pH 2.0).

5.4.2 Implications of alkylpyridinium quat analysis for the development of a generic methodology

The overlap of the quat species witnessed in **Figure 5.5** caused concerns over the analysis of the quaternary amino alcohols under RP-LC conditions. With their greater hydrophilicity and increased retention under NP-LC conditions, it was predicted that these analytes would show little retention under RP-LC conditions. With the C_{12} alkylpyridinium quat having demonstrated a retention factor (k) of approximately 1.0 on the Luna CN phase, it was envisaged that the diol and triol species (**Appendix One**) would be unretained on the Luna CN column, unless a reduction was made to the eluting strength of the mobile phase and / or the separation was performed on a different stationary phase.

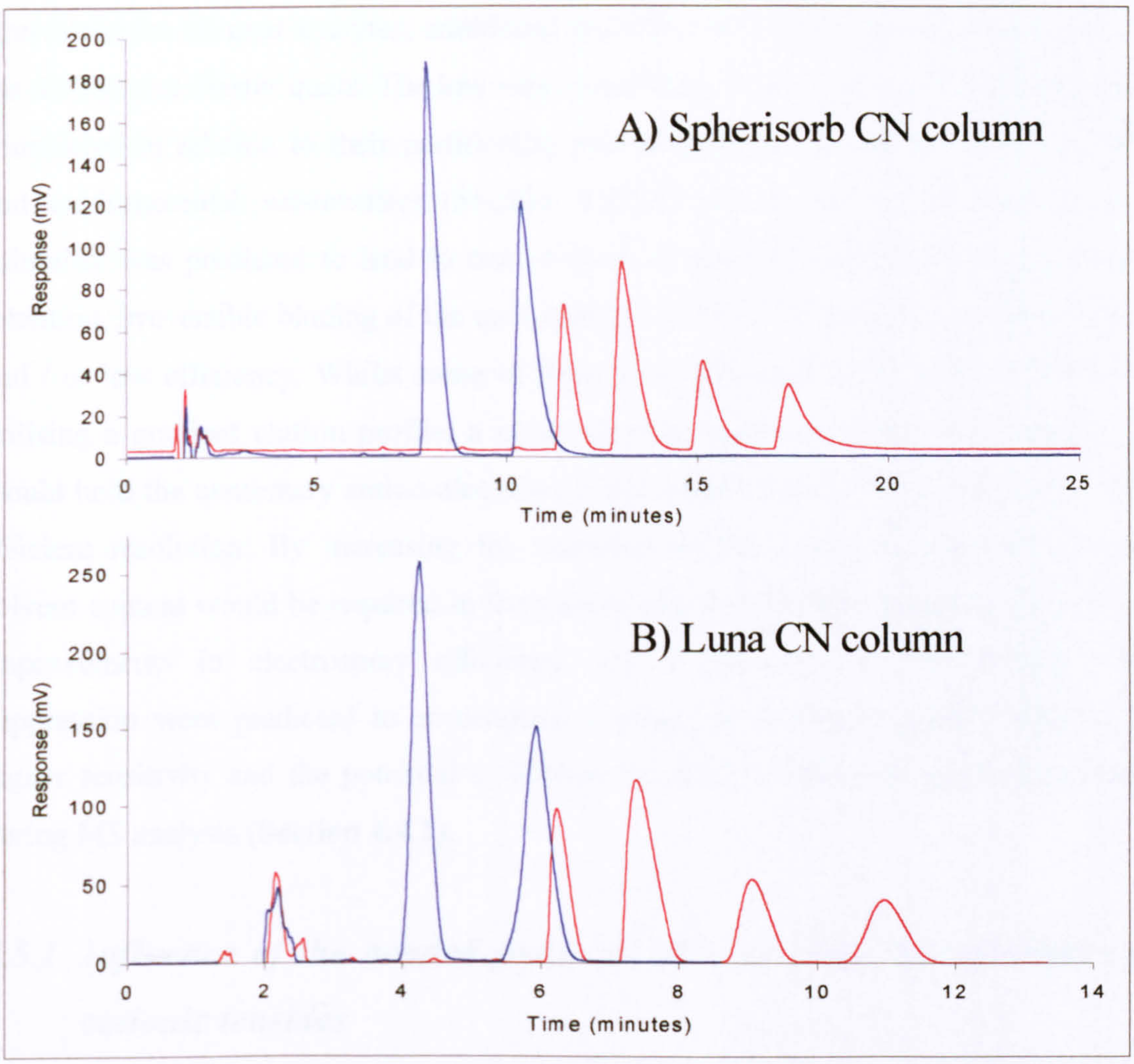

Figure 5.5: Figure showing the likelihood of co-elution of the C_{16} alkylpyridinium quat and the C_{12} alkylbenzyl quat species, on A) the Spherisorb CN phase and B) the Luna CN phase. Conditions – Column dimensions: 150 × 4.6 mm i.d. packed with 3 µm CN bonded silica particles; Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac (pH 2.0); Sample: Blue traces - mixed alkylpyridinium quat sample, red traces – mixed alkylbenzyl quat sample.

5.5 OPTIMISING THE RP-LC CONDITIONS FOR GENERIC ANALYSIS

Previous observations regarding the effect of the eluting strength of the mobile phase on the resolution of the alkylbenzyl quats have revealed that decreased elution strength resulted in increased peak tailing and lower efficiency. In addition, literature reports indicated that increasing the aqueous content of the LC mobile phase, resulted in reduced electrospray ionisation efficiency (Kostiainen *et al.*, 1996), due to the increased surface tension and lower volatility of water, in comparison to common reverse phase organic solvents (Eshraghi *et al.*, 1993) (**Section 1.3.2.1**).

Whilst the problems associated with high water content mobile phases were equivalent for all quat analytes, additional problems were identified in the analysis of the dialkyl and diester quats. The low water solubility of these species has already been considered in relation to their partitioning onto suspended / dissolved solids in rivers and environmental wastewaters (**Section 1.2.3.3**). As a result, their low aqueous solubility was predicted to lead to one or more of the following problems: excessive retention, irreversible binding of the quat analytes to the silica support, poor peak shape, and / or low efficiency. Whilst some of these problems could have been overcome by utilising a gradient elution profile, a more retentive stationary phase was sought, that would hold the quaternary amino-alcohols on the column for sufficient time as to allow efficient resolution. By increasing the retention of the quats, an increased organic solvent content would be required in the mobile phase to facilitate short analysis times. Improvements in electrospray efficiency and a reduction in TFA-induced ion-suppression were predicted to accompany a reduction in water content, resulting in higher sensitivity and the potential to obviate the need for the post-column modifiers during MS analysis (**Section 4.4.1**).

5.5.1 Influence of the bonded phase on the retention characteristics of cationic tensides

In order to maximise the retention of the quaternary amino-alcohols on a silica based stationary phase support, an octadecylsilane (ODS) (C_{18}) bonded phase was evaluated. However, when a 500 mg/l standard of benzyldimethyldodecylammonium bromide was analysed on a 250 × 4.6 mm i.d. 5 µm Spherisorb ODS2 column (**Appendix Two**), no peak was observed within one hundred and fifty minutes from the point of injection. This observation was in line with previous studies on the analysis of alkylbenzyl quats on long alkyl-bonded stationary phases, in which it was reported that such columns were unsuitable for this challenging analysis (van Liderkerke *et al.*, 1989; Santoni *et al.*, 1993; Zhou *et al.*, 1999). Most authors predicted that the elution problems were the result of secondary electrostatic interactions between the cationic charge and deprotonated silanol groups on the silica surface (Dowle *et al.*, 1989). Indeed, some groups went further, suggesting that the adoption of the cyano phase as the column of choice for the analysis of the alkylbenzyl quats was due to the cyano functionality interfering with silanol-analyte interactions, resulting in shorter elution times (Santoni *et al.*, 1993).

If electrostatic interactions were solely responsible for the excessive retention of the alkylbenzyl quats, then it should have proved impossible to elute other quaternary ammonium species from ODS bonded phases. Having seen literature reports that have shown that small biogenic quaternary ammonium compounds like choline, acetylcholine and carnitine (**Figure 5.6**) could be eluted from an ODS column in under five minutes (Liberato *et al.*, 1986; Tsai, 2000; Zhu *et al.* 2000), this was evidently not the case.

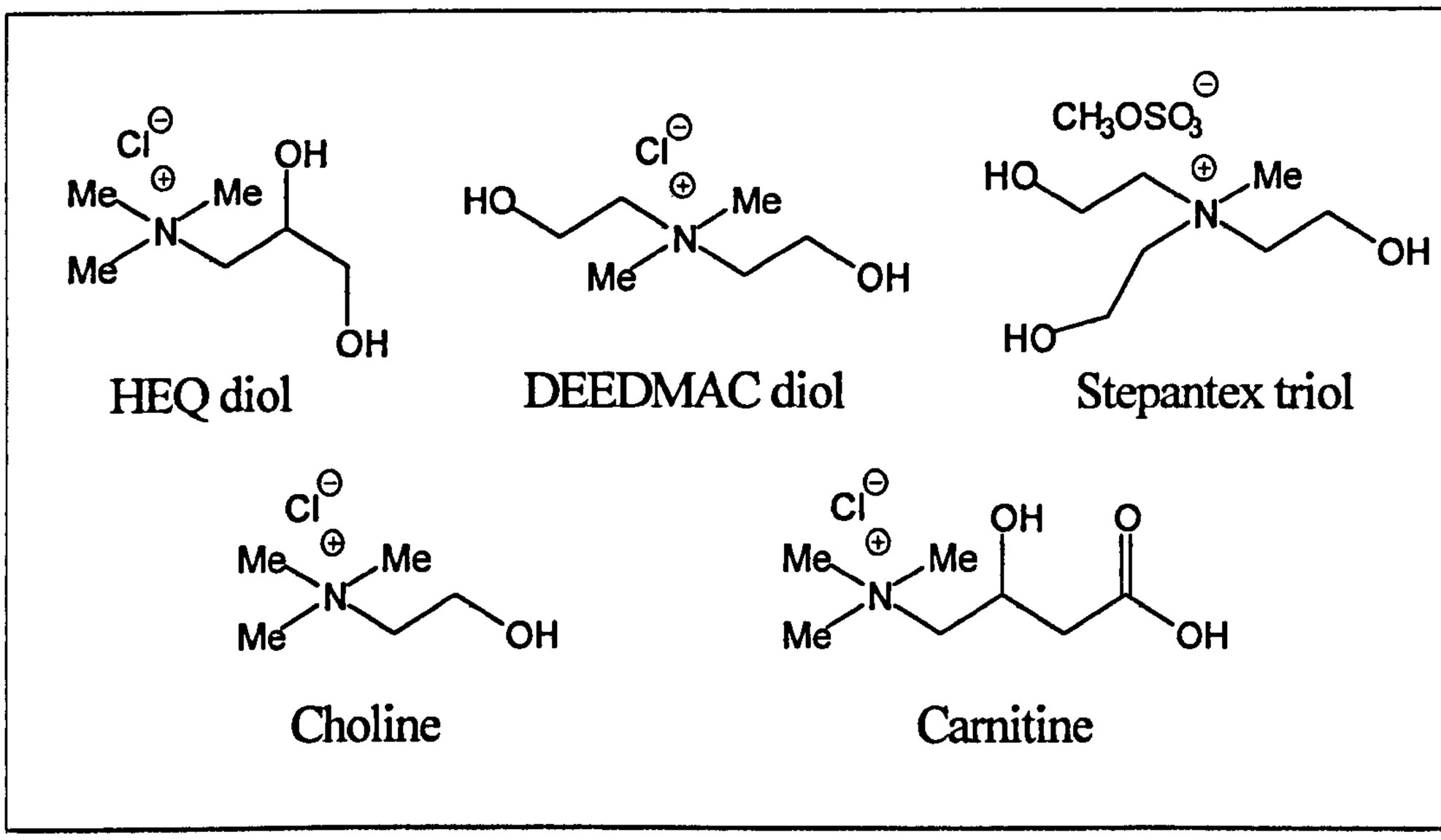

Figure 5.6: Figure showing a comparison of the structures of the quaternary amino-alcohols derived from three parent esterquat tensides and the biogenic amines choline and carnitine.

It was soon realised that the chromatographic parameters being utilised in the RP-LC methodology were limiting the potential for silanol-analyte interactions. Adjustment of the mobile phase pH to 2.0 was done deliberately to limit the numbers of charged silanol groups on the silica surface, as characterisation of the silica supports utilised in HPLC, had revealed that at pH 2.0, all but the most acidic silanol groups are protonated (Hill, 1990; or see Nawrocki, 1997 for a review) (**Section 1.3.2.1**). At the same time, the ammonium acetate modifier was believed to further limit the potential for secondary electrostatic interactions (de Schutter *et al.*, 1988; Nawrocki, 1997), by competing with the analytes for active sites on the silica substrate, and / or forming a mono-layer of cationic charge close to the silica surface, which repelled the approach of the analytes (de Schutter *et al.*, 1988; Vervoort *et al.*, 1992; Nawrocki, 1997). In

addition, the ion-suppression witnessed during the RP-LC/MS analysis of the alkylbenzyl quats (**Section 4.3.3**) confirmed the existence of stable TFA-analyte adducts within the analytical system. Under reverse phase conditions, association of cationic analytes with TFA leads to reduced peak tailing and increased retention, as the TFA effectively shields the cationic charge and results in the formation of pseudo-neutral adducts (Cai *et al.*, 1999).

Consideration of the above information indicated that the likelihood of silanol-tenside interactions was low. It was hypothesised that silanol-analyte interaction played a minor role in the retention of the alkylbenzyl quats on reverse phase supports at pH 2.0. Evidently an unidentified retention mechanism was responsible for excessive retention of the cationic preservatives on ODS bonded phases.

5.5.1.1 Retention of the alkylbenzyl quats on a standard ODS bonded stationary phase and a mixed-mode ODS / CN bonded phase

Having seen that elution of the C_{12} benzyl quat could not be achieved from an ODS bonded phase, yet it was achieved on a cyanopropyl bonded phase, a mixed-mode phase containing both ODS and cyanopropyl moieties was assessed to evaluate what effect, if any, the presence of both functional groups would have on the resolution of the quat species. **Figure 5.7** shows that the retention time of the 1-dodecylpyridinium quat increased by approximately 150% on the mixed-mode phase in comparison to the conventional Spherisorb CN phase, whilst the peak width increased by in excess of 400%. It was apparent that the presence of the cyano groups was reducing the retention of the alkylpyridinium quat in comparison to the standard ODS bonded phase. However, it was far from clear as to how or why the presence of the cyanopropyl groups was having such a significant effect on retention.

The work carried out on the ODS, cyano and mixed-mode ODS/CN phases had shown that the presence of two bonded units had a significant effect on the retention characteristics of the cationic tenside preservatives. However, due to the variation in the nature of the ligands and the bonding density (**Appendix Two**), it was unclear as to whether the cyanopropyl group was interfering with silanol-analyte interactions, or whether the variation in bonding density, alkyl chain length, or some other factor was responsible for the shorter elution time.

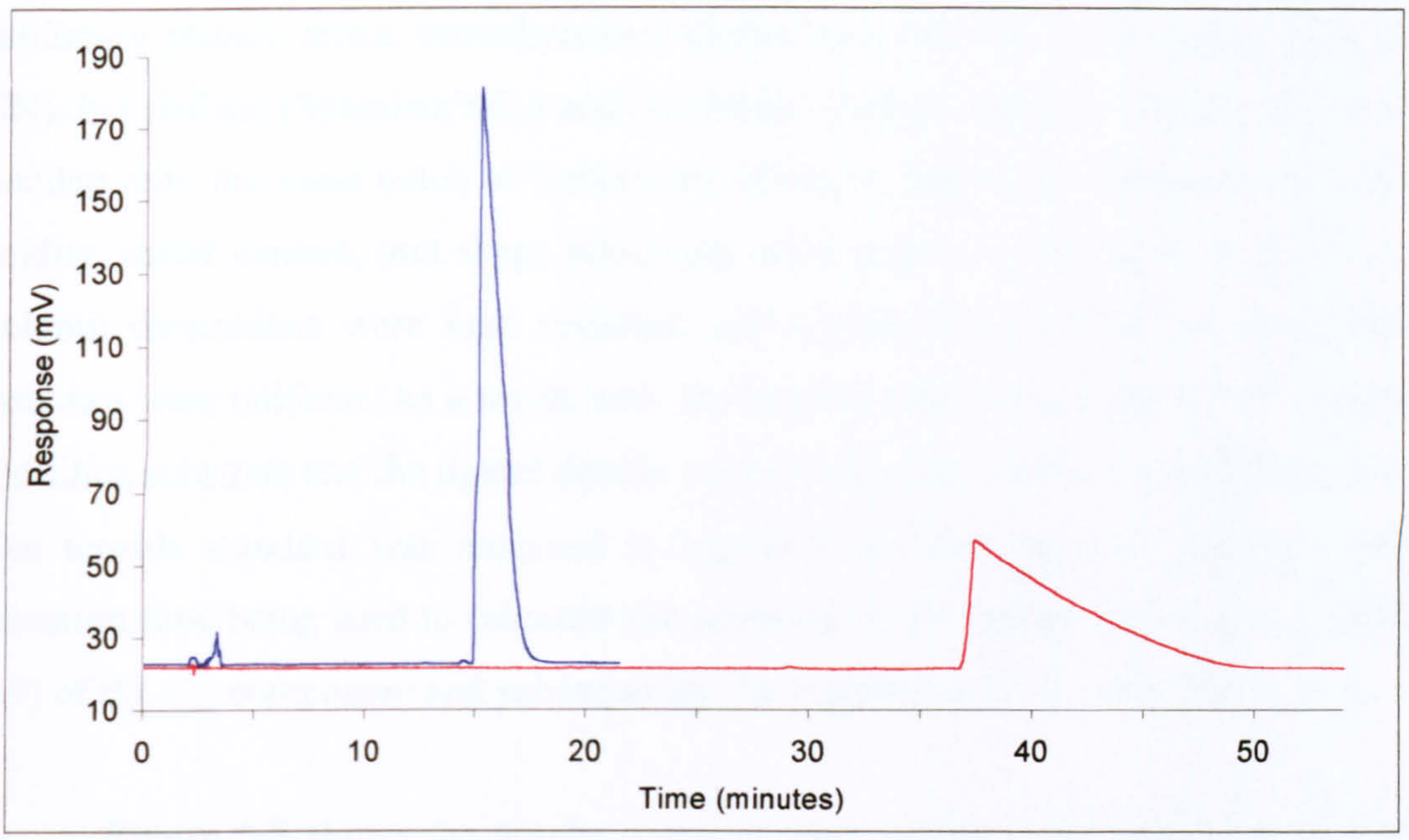

Figure 5.7: Figure showing the retention of the 1-dodecylpyridinium chloride hydrate analyte on a Spherisorb CN and mixed mode ODS / CN column.
Conditions – Column: Blue trace - 250 × 4.6 mm i.d. 5 µm Spherisorb CN, red trace - 250 × 4.6 mm i.d. 5 µm Spherisorb ODS/CN; Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac (pH 2.5).

5.5.1.2 Retention of benzyldimethyldodecylammonium bromide on a series of Spherisorb columns

There is still a significant controversy surrounding the actual method of retention in reverse phase LC (**Section 1.3.2**). However, it is now generally accepted that retention in RP-LC is brought about by the partitioning of analytes into the alkyl chains of the bonded phase (Dorsey *et al.*, 1994). A number of literature reports have studied the retention characteristics of alkyl-bonded phases, which varied in carbon chain length and / or ligand density, through the analysis of homologous series of analytes (Sander *et al.*, 1987; Lork *et al.*, 1988; Martin *et al.*, 1988). In general, as alkyl chain length and / or ligand density increased, a general increase in retention was witnessed (Lork *et al.*, 1988; Dorsey *et al.*, 1994).

If partitioning was the major retention mechanism affecting the cationic tensides on alkyl-bonded reverse phase supports, a general increase in retention should have been witnessed in moving from a trimethylsilane (C_1) bonded phase to an octylsilane (C_8) bonded phase. In order to determine the influence if any, of the nature of the

bonded phase on the retention of the alkylbenzyl quats, a 100 mg/l standard of benzyldimethyldodecylammonium bromide was analysed on five Spherisorb based stationary phases: silica, trimethylsilane (Spherisorb methyl), cyanopropyl (Spherisorb CN), hexylsilane (Spherisorb C_6) and octylsilane (Spherisorb C_8). The five phases were bonded onto the same batch of Spherisorb substrate, and thus variations in retentivity, acidity, metal content, and shape selectivity were kept to a minimum. In addition, the column dimensions were kept constant, and mobile phase conditions and injection volumes were uniform. As a result, only the length of the bonded phase unit attached to the silica substrate and the ligand density varied during the evaluation (**Appendix Two**). The tenside standard was analysed in triplicate on each column, with the average retention time being used to calculate the retention factor (k) (**Section 1.3.1**) (**Equation 1.9**) of the C_{12} component and subsequently the logarithm of the retention factor (log k).

Figure 5.8 shows the graphs obtained when A) the retention factor (k) and B) log k of the C_{12} alkylbenzyl quat were plotted against the number of carbons in the bonded unit on the silica support. For both graphs the three data points corresponding to the C_1, C_6 and C_8 phases were plotted first, and a suitable regression fit applied to the series. The data points corresponding to the silica and cyanopropyl phases were added later. It was immediately apparent that the plot of k versus carbon number was non-linear and instead showed good approximation to an exponential, whilst the plot of log k showed excellent linearity, with a correlation factor approaching 1.0.

The exponential increase in k and the linear increase of log k with increasing bonded unit length on the silica support indicated that under reverse phase conditions the main retention mechanism for the cationic tensides was partitioning. When the best-fit line of log k plot was subsequently extrapolated, it was found that the predicted retention factor of the C_{12} alkylbenzyl quat on a 250 mm ODS bonded column was 824.5, corresponding to a retention time of approximately 28 hours. This revelation made it very apparent why the analyte had not been seen to elute in **Section 5.5.1.1.**

A linear correlation between log k and bonded phase chain length had been witnessed during the analysis of groups of homologues on different alkyl-bonded phases (Tchapla *et al.*, 1994). Amongst the earliest of these reports was the work performed by Henion *et al.* (1978) who observed a linear increase in log k versus length of the bonded

phase for a series of poly-cyclic aromatic hydrocarbons. Having maintained a consistent ligand density on all of the columns, the authors interpreted these results as confirmation that the whole of the bonded phase interacted with the solute. It was hypothesised that the analytes were intercalating with the alkyl chains of the bonded phase, a process that could only be accounted for by a partitioning model of solute retention (**Section 1.3.2**).

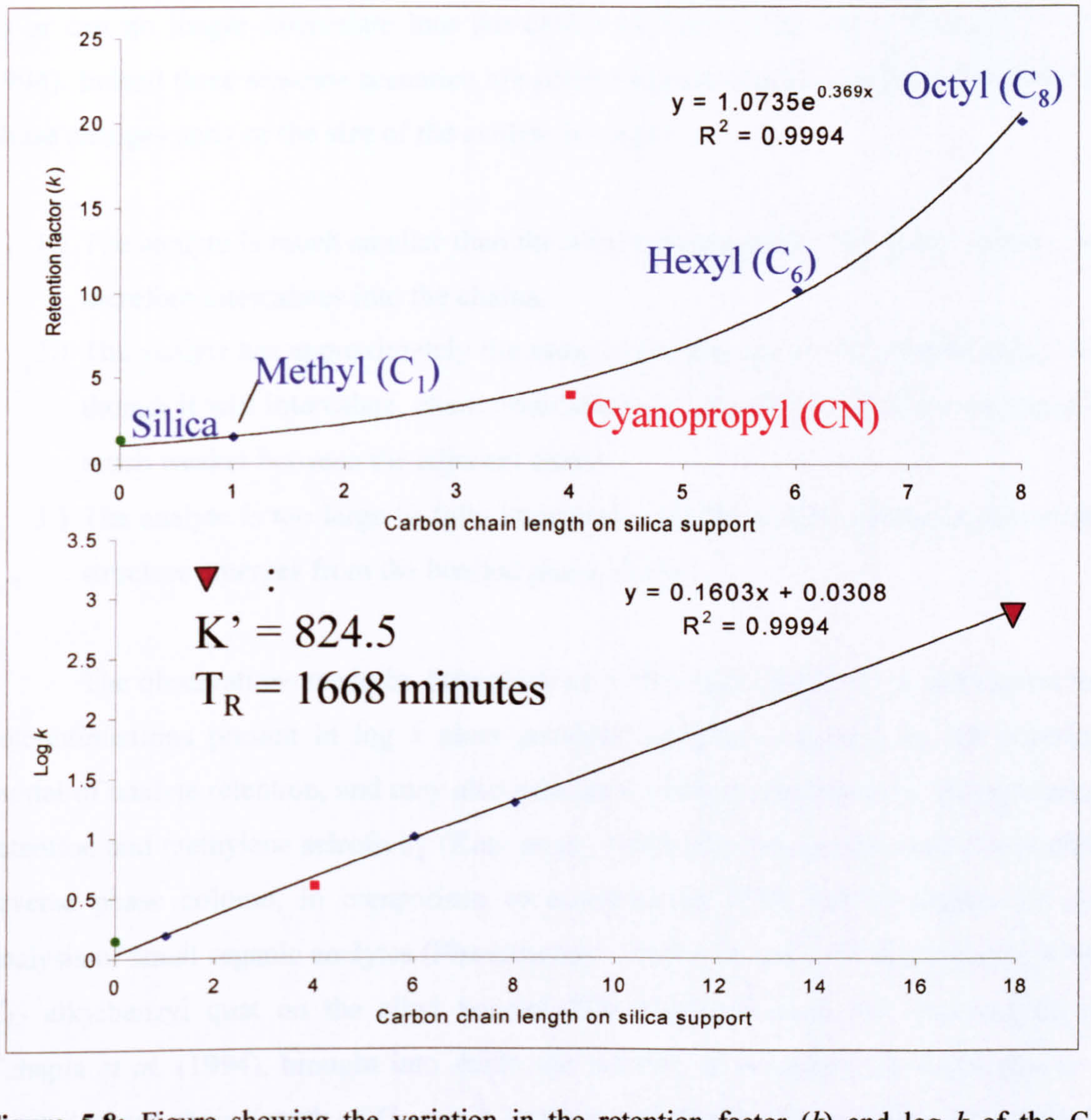

Figure 5.8: Figure showing the variation in the retention factor (k) and log k of the C_{12} alkylbenzyl quat versus the carbon chain length of the bonded phase unit on a series of Spherisorb based phases at pH 2.0. The best-fit curve of log k versus carbon number was extrapolated to C_{18} to demonstrate the predicted retention time of the C_{12} component on an ODS bonded phase.
Conditions – Column dimensions: 250 × 4.6 mm i.d. packed with 5 μm Spherisorb silica particles; Mobile phase: 70:30 ACN:5 mmol/l NH_4Ac (pH 2.0).

Though Henion *et al.* (1978) interpreted the linear correlation between log *k* and chain length of the bonded phase as confirmation of a partitioning mechanism, most subsequent authors have refuted these claims, suggesting the trend actually confirms the solvophobic theory of retention (**Section 1.3.2**) (Tchapla *et al.*, 1994; Barrett *et al.*, 1996a). However, supporters of the partitioning model of retention have shown that whilst a linear correlation of *k* is more likely to satisfy the partition theory (Barrett *et al.*, 1996a), so-called linear plots of log *k* versus bonded phase chain length actually show characteristic discontinuations at points in the graph where the analyte can begin to or can no longer intercalate into the chains of the bonded phase (Tchapla *et al.*, 1994). Indeed three separate scenarios are often recognised as the length of the bonded phase changes and / or the size of the analyte is varied:

1.) The analyte is much smaller than the alkyl unit bonded to the silica support and therefore intercalates into the chains.
2.) The analyte has approximately the same molecular size as the bonded phase and though it will intercalate, chain orientations are changed such that interaction is much weaker between the adjacent chains.
3.) The analyte is too large to fully intercalate into the bonded phase as part of the structure emerges from the bonded phase chains.

The observations made by Tchapla *et al.* (1994) and Barrett *et al.* (1996a) on the discontinuations present in log *k* plots provided additional support for the partition model of analyte retention, and may also provide a viable explanation for the equivalent retention and methylene selectivity (Katz *et al.*, 1998) of a new commercial C_{12} bonded reverse phase column, in comparison to conventional ODS bonded phases for the analysis of small organic analytes (Phenomenex, 2001a). In terms of the retention of the C_{12} alkylbenzyl quat on the alkyl bonded Spherisorb columns, the observations of Tchapla *et al.* (1994), brought into doubt the validity of extending the log *k* plot to a bonded phase chain length of C_{18}, as it became apparent that the gradient of the graph would change as the bonded phase length reached C_{12}. At C_{18} it was envisaged that the analyte may be able to fully intercalate into the bonded phase and thus the predicted retention time may have actually represented an under estimation of the actual elution time. This realisation provided additional justification for believing that an ODS bonded phase represents a poor stationary phase for the analysis of alkylbenzyl quat analytes.

With the fabric conditioner actives being significantly more hydrophobic than the monoalkyl quats, it was predicted that elution of these analytes from an ODS phase would not be achieved without utilising non-aqueous reverse-phase conditions, with copious amounts of chloroform, dichloromethane, or a similar elution strength solvent.

5.5.1.3 Retention of benzyldimethyldodecylammonium bromide on a bare silica column

Mention has been made to the fact that many authors who have witnessed the unsuitability of ODS bonded phases for the analysis of cationic tenside preservatives have hypothesised that problems result from electrostatic interactions between the analyte and surface silanol groups on the silica support (Santoni *et al.*, 1993). These theories had already been brought into doubt earlier in this chapter when the degree of silanol ionisation was predicted to be low at pH 2.0 (**Section 5.5.1.1**). The additional observation that the retention factor of the C_{12} alkylbenzyl quat increased exponentially with increasing alkyl chain length on the silica support, also implicated partitioning as the major retention mechanism of the quats, which added further weight to the theory that electrostatic interactions played only a minor role in the retention of cationic tensides. However, to better understand the degree to which electrostatic interactions at pH 2.0 influenced the retention of cationic tensides, elution of the C_{12} alkylbenzyl quat from the silica phase was probed.

With the degree of silanol-analyte interaction being directly related to the proportion of deprotonated silanol groups present on the silica, it should follow that maximum electrostatic interaction is witnessed when all of the original silanol groups are intact i.e. on bare silica. If electrostatic interactions had played a major role in the retention of the cationic tensides, then the silica column should have shown increased retention over that predicted for a C_0 column by extrapolation of the log k versus bonded chain length plot generated in **Figure 5.8**. It was apparent after comparing the predicted value for a C_0 column to the experimentally derived value for the silica phase that increased retention was occurring. However, whilst the silica column demonstrated a 16% increase in retention time over what was predicted, it was envisaged that much of the additional retention was due to interactions between the analytes and the bulk siloxane surface (Nawrocki, 1997) a factor that has been well documented in literature on a number of occasions (Bij *et al.*, 1981; Cox *et al.*, 1987; Cox, 1993). In the presence

of an alkyl bonded phase the analytes are thought to partition into the chains, limiting the influence of siloxane-analyte interaction. As a result, the extrapolated value for the C_0 column would not account for this mode of retention and would be lower than the actual experimental value.

Whilst this hypothesis may seem a little contrived, and could be viewed as an attempt to obviate the existence of electrostatic interactions between the analytes and deprotonated silanol groups, strong support for this theory can be witnessed from the equation of the best-fit regression plot shown in **Figure 5.8**. Whilst the log *k* versus carbon chain length plot shows excellent linearity, and a steep positive gradient, which shows that *k* and hence retention time increases with each methylene unit that is added to the bonded phase, the intercept value of the line is almost negligible. As the intercept provides an account of the retention that is not derived from partitioning, it can be seen that electrostatic interactions play a small part in the retention of the cationic tensides at pH 2.0. Strong dispersive interactions between the bonded phase and the cationic analytes (Carr *et al.*, 1993) **(Section 1.3.2)** appear to drive retention under reverse phase conditions at low pH. Electrostatic interactions between the analytes and deprotonated silanol groups on the silica support seem to have little influence under such mobile phase conditions.

5.5.2 Influence of mobile phase pH on the retention characteristics of cationic tensides

One of the main limitations of the RP-LC/MS methodology described in **Chapter Four** was the severe ion-suppression and hence lack of sensitivity observed with MS detection. Whilst addition of post-column modifiers was seen to improve ionisation efficiency and thus reduce the limit of detection **(Section 4.4.2)**, it would have been preferable if its use could have been avoided. With the ion-suppression believed to derive from the presence of TFA (Kuhlmann *et al.*, 1995), a reduction in the acid concentration should have been accompanied by reduced ion-suppression and thus increased sensitivity in LC/MS.

Figure 5.8 had provided a strong indication that retention of the C_{12} alkylbenzyl quat species under reverse phase conditions was due to the partitioning of the analyte into the bonded phase, with electrostatic interaction appearing to play a very minor role.

However, the proportion of deprotonated silanols at pH 2.0 is known to be low, with only the most acidic groups, potentially those associated with metal impurities (**Section 1.3.2.1**) remaining uncharged (Nawrocki, 1997). As a result, electrostatic interactions between the analytes and the silica support were likely to have been prohibited by the mobile phase pH.

Being acidic in nature (**Section 1.3.2.1**), the deprotonation of silanol groups is promoted at increased mobile phase pH (Nawrocki, 1997), which can result in an increased electrostatic interaction between the analytes and the silica support. Even so, having observed that this type of retention had little effect at pH 2.0, the possibility of reducing the TFA concentration without dramatically increasing analyte retention time was envisaged. The effect of increased mobile phase pH on the peak parameters of the cationic tenside preservatives was subsequently assessed. Whilst the average pK_a of the silanol groups on a silica substrate is predicted to be approximately 7.1 (Nawrocki, 1997), a cautious approach was adopted during this exercise, and thus peak parameters were assessed at pH 3.0 in the first instance.

5.5.2.1 Variation of the retention factor and the logarithm of the retention factor of benzyldimethyldodecylammonium bromide on a series of Spherisorb columns at pH 3.0

Figure 5.9 shows the graphs of retention factor (k) and log k versus bonded phase carbon chain length for the five Spherisorb columns utilised in **Section 5.5.1.2** at pH 3.0. The regression curves were again generated from the averaged retention time data of the C_1, C_6 and C_8 bonded phases, with the silica and cyanopropyl data points being added later. It was apparent that although the mobile phase pH had increased, the general forms of the graphs were identical to those observed at pH 2.0; the plot of k versus carbon number showed a good approximation to an exponential, whilst the log k plot again demonstrated good linearity.

With k still reflecting an exponential increase with increasing carbon number in the bonded phase, it initially appeared that partitioning was still the only major retention mechanism for the cationic tensides at pH 3.0. However, comparison of the equations of the best-fit curves for the two log k plots (**Figures 5.8** and **5.9**) showed that significant changes had accompanied the increase in pH.

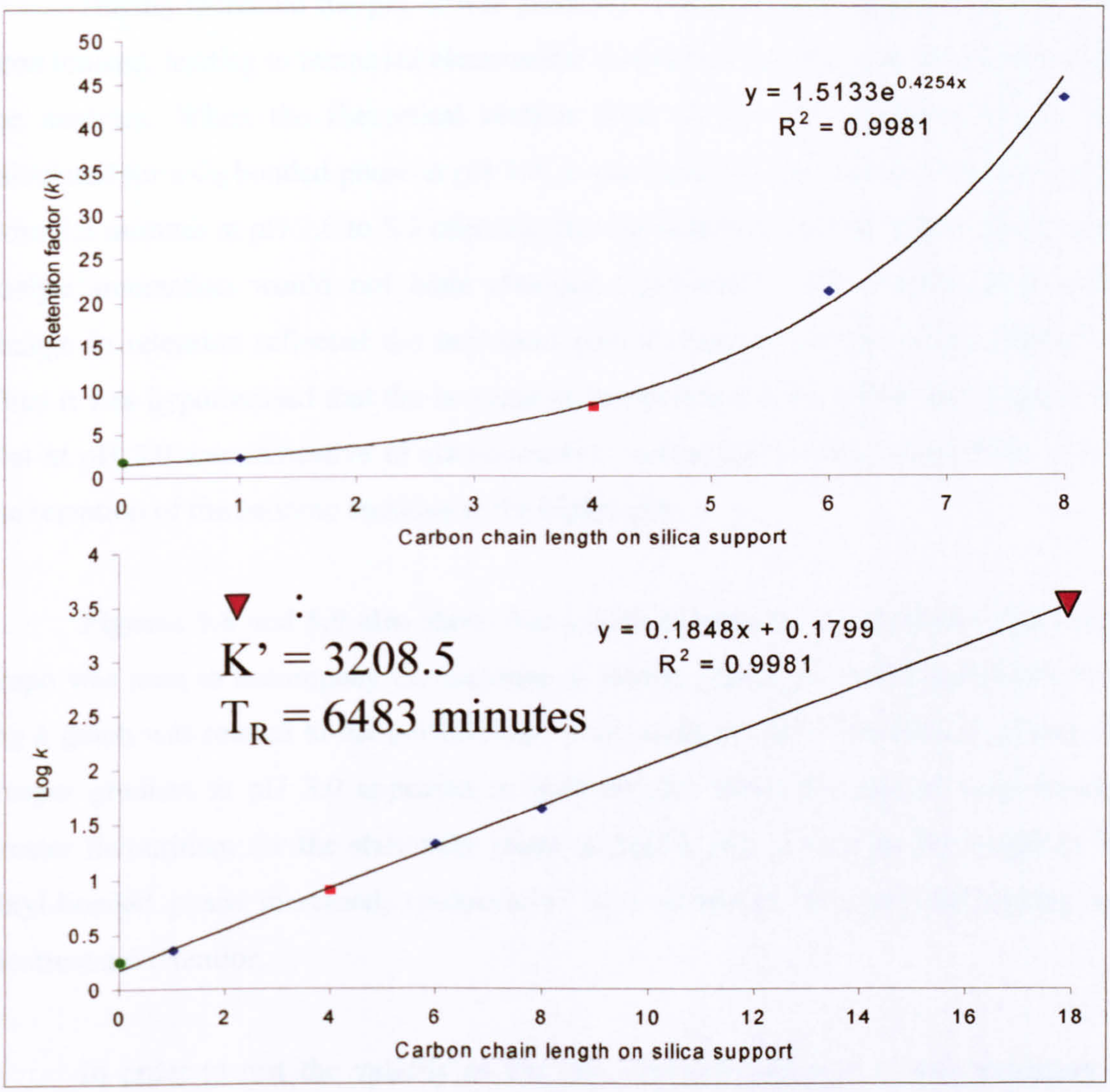

Figure 5.9: Figure showing the variation in the retention factor (k) and log k of the C_{12} alkylbenzyl quat versus the carbon chain length of the bonded phase unit on a series of Spherisorb based phases at pH 3.0. The best-fit curve of log k versus carbon number was extrapolated to C_{18} to demonstrate the predicted retention time of the C_{12} component on an ODS bonded phase.
Conditions – Column dimensions: 250 × 4.6 mm i.d. packed with 5 μm Spherisorb silica particles; Mobile phase: 70:30 ACN:5 mmol/l NH_4Ac (pH 3.0).

An increase in the intercept value of the regression plot was immediately apparent at pH 3.0. With the intercept having been recognised as providing an estimate of the degree of non-partition induced retention (**Section 5.5.1.3**), the increased intercept reflected an increased contribution from a second retention mechanism. At pH 2.0 the intercept value of log k was seen to be negligible, reflecting the minor contribution of secondary retention at this pH. However, at pH 3.0 the intercept had increased by approximately 480%, indicating that an additional retention mechanism was having a significant influence on the cationic analyte at this higher pH.

Having increased the pH, it was predicted that more silanol groups would have been ionised, leading to increased electrostatic interaction between the silica surface and the analytes. When the theoretical elution time of the C_{12} alkylbenzyl quat was calculated for a C_0 bonded phase at pH 3.0, it was observed that retention had increased from 4.2 minutes at pH 2.0 to 5.5 minutes. Having assumed that the degree of siloxane-analyte interaction would not have changed significantly with increasing pH, any change in retention reflected the increased contribution of silanol-analyte interaction. Thus it was hypothesised that the increase in the intercept value of the log *k* regression plot at pH 3.0 was indicative of silanol-analyte interaction having a significant role in the retention of the cationic tensides at the higher pH.

Figures 5.8 and **5.9** also show that a 15% increase in the gradient of the log *k* graph was seen to accompany the increase in mobile phase pH. As the gradient of the log *k* graph was related to the partitioning of the analytes into the stationary phase, the steeper gradient at pH 3.0 appeared to indicate that either the analyte was showing greater favouritism for the stationary phase at higher pH, or that as the length of the alkyl-bonded phase increased, co-operation was witnessed between partitioning and electrostatic retention.

In order to test the validity of the two above hypotheses, it was necessary to examine the peak parameters obtained for the C_{12} alkylbenzyl quat on each of the bonded phases, as it was recognised that for the increased residence theory to have been proved correct only the retention time of the analyte should have been seen to vary between the different phases at the two pH values. When the peak parameters were compared it was found that in addition to a change in retention time, peak tailing generally increased at pH 3.0, whilst a more significant increase in peak tailing was witnessed in going from the C_1 to the C_8 bonded phase. These observations seemed to put paid to the increased residence theory, and instead lent support to the co-operative retention theory.

In **Section 5.5.1.2** it was mentioned that a number of literature reports have appeared in the last four years, regarding the variation in the peak parameters of organic amines witnessed on a range of reverse phase materials (Barrett *et al.*, 1996a and b;

M^{c}Calley, 1999). In each of these reports it was observed that as the length of the bonded phase increased, there was also a general increase in the retention time of the analytes. At the same time, all the basic analytes studied, showed strong secondary retention, in the form of analyte peak tailing on the bonded phases (Barrett *et al.*, 1996a and b). Of more significance to the current discussion was the observation that the peak tailing was much more pronounced on the longer alkyl bonded phases i.e. C_8 and C_{18}, in comparison to the short alkyl phases i.e. C_1 and C_4 (Barrett *et al.*, 1996a). Barrett *et al.* (1996) concluded that a C_8 bonded phase provided superior performance than the conventional C_{18} bonded phases for the selected analytes, due to reduced peak tailing and increased peak efficiency.

Whilst the above reports demonstrated that increased peak tailing had been witnessed with increased bonded phase length during the analysis of organic amines, none of the reports could provide a suitable explanation to account for the experimental observations. Instead a sound hypothesis was found in a much earlier report on the analysis of a group of neutral, acidic, and basic organic analytes on two C_{18} bonded phases and a silica phase of the same support material (Bidlingmeyer *et al.*, 1982). The first important experimental observation reported in this paper was that benzocaine, which has a pK_a of 2.8, showed consistent retention across the pH range 3.0 to 9.0. This result provided the final proof that the increased residence theory was not responsible for the change in the gradient of the log *k* plot witnessed during this work. Bidlingmeyer *et al.* (1982) also showed that when four anaesthetics were analysed on a C_{18} bonded phase, a C_{18} bonded phase with half the ligand density of the conventional phase, and a bare silica column, the peak tailing of the four basic analytes was seen to decrease in the order C_{18} > 50% C_{18} > silica, i.e. maximum peak tailing was seen on the conventional C_{18} phase and maximum peak symmetry was witnessed on the bare silica phase. By utilising the same silica support for all three columns, variation in retentivity, acidity, and the number and distribution of silanol groups was kept to a minimum. Thus, the observed variation in peak tailing reflected a variation in the retention mechanism on the three columns. These observations led Bidlingmeyer *et al.* to conclude that the commonly held view that peak tailing is solely due to the interaction of the analytes with surface silanol groups (Daldrup *et al.*, 1984), and hence the notion of silanol groups as being *"bad"*, was misconceived. The authors instead proposed a mechanism whereby peak tailing is brought about by the inaccessibility of surface silanol groups,

and in particular the difficulties encountered by analytes as their attempts to move away from the surface are hindered by the bonded phase. It was envisaged that the reduced peak tailing demonstrated by the C_{18} phase with 50% coverage showed the analytes were less likely to come into contact with a bonded phase unit on their journey back into the bulk from the silica surface. Peak symmetry was therefore maximised on the silica phase, due to the lack of hindrance from bonded phase units as the analytes passed back into the bulk mobile phase. As a result, non-uniform peak shapes on the silica phase were probably representative of the retardation of the analytes in the pores of the silica **(Section 1.3.1)** and / or variation in the acidity of the silanol groups themselves **(Section 1.3.2.1).**

Support for the predictions and observations made by Bidlingmeyer *et al.* (1982) came five years later by Cox *et al.* (1987), who characterised the retention on silica and conventional reverse phase supports under pseudo-reverse phase conditions. Once again peak symmetry and efficiency was observed to be higher on silica than on the alkyl bonded phases.

It appeared that a suitable hypothesis had been found that could account for all of the observations made during the evaluation of the Spherisorb based columns under reverse phase conditions during this work. As the bonded phase length increased, peak tailing also increased as a result of retardation of the tenside analytes by the chains of the bonded phase. This observation could not simply have resulted from the variation in the ligand densities of the C_1, C_6 and C_8 phases, as the peak tailing observed on the C_8 phase was much more severe than that witnessed on the C_6 phase, yet the C_8 phase was reported to have a lower ligand density; 3.12 $\mu mol/m^2$ versus 3.36 $\mu mol/m^2$ **(Appendix Two)** (Phenomenex, 2001b).

Whilst peak tailing was in general, more severe at pH 3.0, it was also witnessed at pH 2.0, especially on the longer bonded phases. In addition to further substantiating the predictions made by Bidlingmeyer *et al.* (1982), these results showed that though few surface silanol groups were believed to be deprotonated at pH 2.0, their existence could be revealed by the analysis of cationic tensides.

5.5.2.2 Influence of a cyanopropyl bonded unit on the retention of benzyldimethyldodecylammonium bromide

Having observed that at low pH, partitioning of the analytes is primarily responsible for excessive retention on long bonded phases, and that secondary retention or peak tailing is also dependant on the length of the bonded phase unit, it was concluded that the hypothesis regarding the action of cyano-propyl head groups should be re-evaluated (**Section 5.5.1**). If the cyano groups were affecting retention to any significant degree, then the log *k* values for this column should have been significantly lower than those predicted for a C_4 phase, especially at pH 3.0, where this type of interaction was more prevalent. Inspection of **Figure 5.9** showed that the data point corresponding to the cyanopropyl column lay very close to the best-fit line obtained from the alkyl bonded phase data. When the predicted retention time for the C_4 column was compared to the experimental value for the CN phase it was found that there was less than 3% variation in the two results. Indeed, when the log *k* versus carbon chain plot was repeated to include the CN data the correlation factor of the regression fit was unchanged.

It appeared that under the reverse phase conditions developed in **Chapter Four** the cyanopropyl phase was actually behaving as a C_4 alkyl bonded phase, with the CN head group acting as a conventional terminal methyl group. It was apparent that the widespread adoption of the cyanopropyl phase had come about due to false assumptions and fears of the unknown, which had no strong foundation in experimental observation or chromatographic theory. For generic cationic tenside analysis the choice of stationary phase should no longer be restricted to one phase.

5.5.2.3 Revisiting the change in peak parameters of benzyldimethyldodecylammonium bromide witnessed on Spherisorb CN and ODS / CN phases

In addition to helping to explain the variation in the gradients of the log *k* plots at the differing pH values, the observations and subsequent hypotheses of Bidlingmeyer *et al.* (1982) (**Section 5.5.2.1**), could also be used in association with the results presented above to explain the variation in the peak parameters of the C_{12} alkylbenzyl quat witnessed on the Spherisorb CN and mixed-mode ODS / CN phases (**Figure 5.7** and **Section 5.5.1.1**).

The alkylbenzyl quat analyte demonstrated a significant increase in both retention time and peak tailing on the mixed-mode phase in comparison with the conventional cyanopropyl phase. The increase in retention time can be explained in terms of increased residence of the analyte in the stationary phase in the presence of the ODS bonded units, in the same way that increased retention was witnessed on the C_8 phase in comparison with the C_1 bonded phase. The explanation of the increased peak tailing had been much harder to justify. However, having recognised that the cyano group did not interfere with silanol-analyte interactions, neither did it interact preferentially with the cationic analytes, as had been witnessed previously in the analysis of organic amines (Barrett *et al.*, 1996a; M^{c}Calley, 1999; Needham *et al.*, 2000), the increase in peak tailing was attributed to the increased retardation of the analyte leaving the silica surface in the presence of ODS bonded units. Of greater significance than the actual peak tailing was the degree to which peak tailing had increased on the mixed-mode phase. The 400% increase had come about in spite of the ligand density of the column being half that of a conventional cyanopropyl phase **(Appendix Two)**. Although retardation by the ODS bonded moieties would have increased peak tailing **(Section 5.5.2.1)**, the poor surface coverage of the ligands appeared to accentuate the variation in the rates of release of the analytes from the surface, greatly enhancing peak tailing. The conclusion was reached that a mixed-mode phase of this nature should not be chosen as the stationary phase for the analysis of cationic tensides.

5.5.2.4 Retention of benzyldimethyldodecylammonium bromide on a Zorbax Stable Bond cyanopropyl column

Having witnessed that the logarithm of the retention factor of the C_{12} alkylbenzyl quat increased linearly on short alkyl bonded stationary phases at low pH **(Figures 5.8** and **5.9)**, it was predicted that a stationary phase which had two or more short alkyl units ($< C_4$) bonded to the silica support may demonstrate a series of discrete peaks for a single cationic tenside component. In the Zorbax Stable Bond CN phase **(Appendix Two)**, a suitable column was found to evaluate this theory, as the column has iso-butyl and iso-propyl groups at the base of the silica, which are designed to protect the surface from acid hydrolysis and thus extend column lifetime, in addition to the standard cyanopropyl unit (Phenomenex, 2001b). With a Spherisorb CN phase

having been seen to behave like a C_4 phase, it was envisaged that the Zorbax SB CN phase would act as a mixed-mode phase under the conditions described above.

Figure 5.10 shows the chromatogram obtained from the analysis of a 100 mg/l standard of benzyldimethyldodecylammonium bromide on a 150 × 4.6 mm i.d. 5 µm Zorbax SB CN column. The two peaks seen eluting at ca. 4.8 minutes and 6.4 minutes were both seen to correspond to the C_{12} species, as peak area response varied according to the concentration of the standard and the injection volume. It was proposed that the early eluting peak probably corresponded to the partitioning of the analyte into the cyanopropyl chains, whilst the later peak was probably brought about by additional retention on the Stable Bond ligands as the analytes attempted to dissociate from the silica substrate.

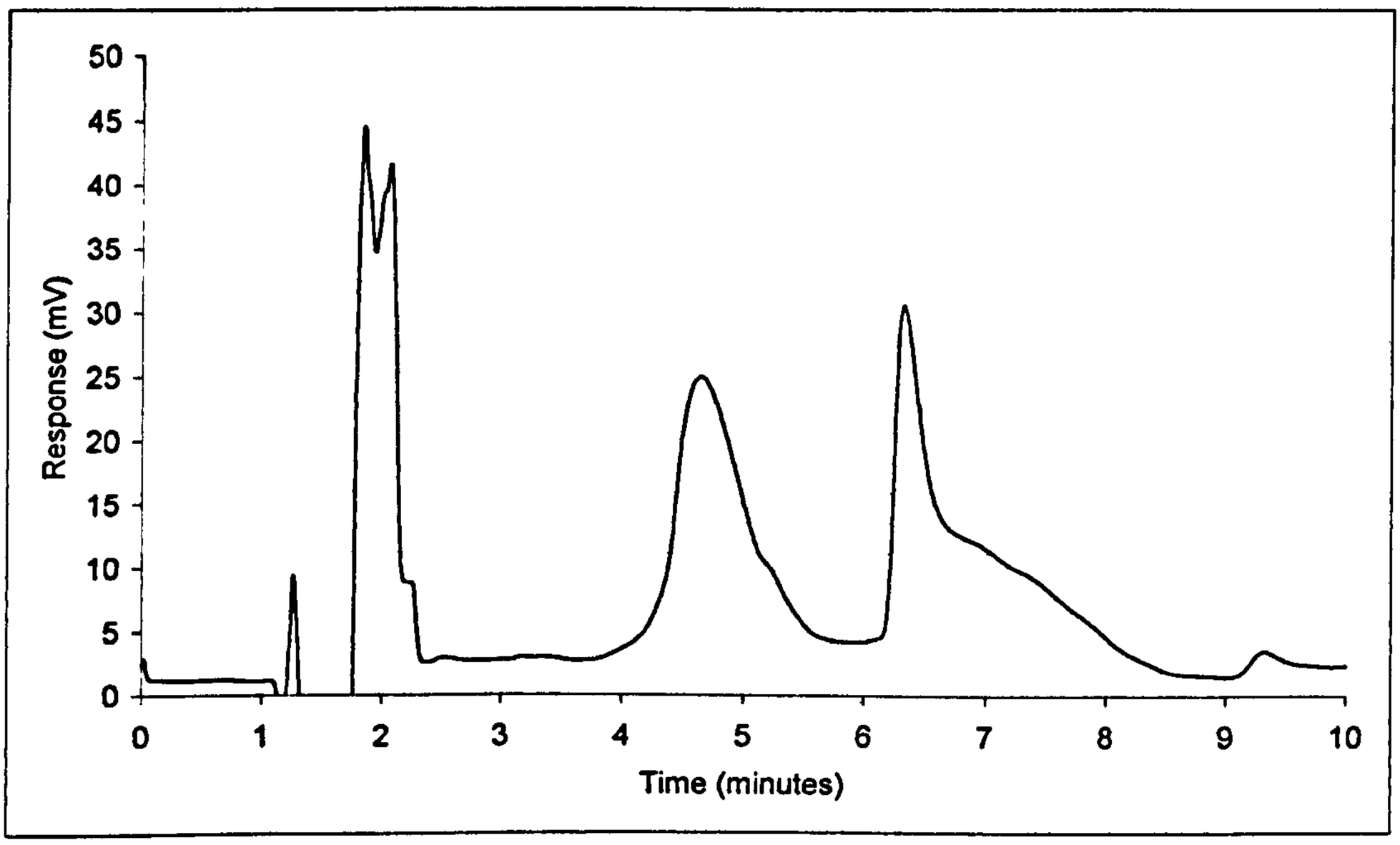

Figure 5.10: Chromatogram resulting from the analysis of a benzyldimethyldodecylammonium bromide standard on a Zorbax Stable Bond cyanopropyl column.
Conditions – Column: 150 × 4.6 mm i.d. 5 µm Zorbax SB CN; Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac (pH 2.0).

Recently, the use of a 50 × 4.6 mm 5 µm Zorbax SB ODS phase was also seen to give rise to multiple peaks during the analysis of single non-ionic tensides at Unilever Research (Cooper, 2000). At the same time, long chain tensides were observed to elute at two disparate points in the chromatogram. Whilst these results demonstrated that

Figure 5.10 was not an isolated event, it was still unclear as to why the two retention mechanisms had not been witnessed more often in light of the methyl groups being present on other reverse phase supports. On the Zorbax SB CN column at least, there was little difference in alkyl chain length between the main bonded unit and the steric groups at the silica support. In the case of the ODS bonded phase, it was thought that the high flow rate (2.0 ml/min) and short column length had combined to split the analyte band. Nonetheless it is clear that considerable work is required to evaluate whether these two observations were freak occurrences, or whether the Zorbax SB CN phase in particular may be able to shed important information on the fundamental method of retention under reverse phase conditions.

5.5.2.5 Influence of the silica substrate and mobile phase pH on the peak parameters of benzyldimethyldodecylammonium bromide

The RP-LC/MS methodology described in **Chapter Four** suffered from an inherent lack of sensitivity resulting from the ion-suppression witnessed in the presence of TFA. Therefore an increase in mobile phase pH would reduce the concentration of TFA and hence begin to limit ion-suppression. A brief evaluation of the effect of increased pH had already been performed on a series of Spherisorb columns (**Section 5.5.2.1**). However, to gain a greater understanding of the influence of pH on the peak parameters of the cationic tensides, a 100 mg/l standard of benzyldimethyldodecylammonium bromide was analysed in triplicate on the 150 × 4.6 mm i.d. Spherisorb and Luna 3 μm CN columns at varying pH.

Figure 5.11 shows the variation in the retention factor (k) of the C_{12} alkylbenzyl quat with changing pH on the Spherisorb CN phase. The retention factor was seen to increase markedly over the pH range 2.0 to 3.75, with a slower rate of growth between pH 3.75 and 5.0. Having maintained the ratio of acetonitrile to water at 1:1 throughout the duration of the experiment, changes in analyte retention must have been derived from increased electrostatic interaction between the analytes and the silica support, reflecting an increase in the number of deprotonated silanol groups. As a result, it became apparent that the Spherisorb material contained numerous silanol groups with low pK_a's and hence high acidity (Nawrocki, 1997). These observations went against previous findings made by Vervoort *et al.* (1992), who reported that peak asymmetry remained fairly constant between pH 3 and 6 on a μ-Bondapak column, and that no

variation was apparent between pH 2.5 and 3.5. The authors of this report hypothesised that these observations showed that silanol groups deprotonated at pH 3.5 would remain deprotonated at lower pH. A major limitation of the work performed by Vervoort *et al.* (1992) was that there was a lack of consistency in the mobile phase conditions employed at the different pH's, and thus the lack of variation in peak asymmetry, and the predictions regarding the nature of the silanols were probably attributable to mobile phase conditions. Indeed numerous reports are available in the literature, which demonstrate that silanols have been identified with pK_a values ranging from 1.0 to 10.0 (Nawrocki, 1997).

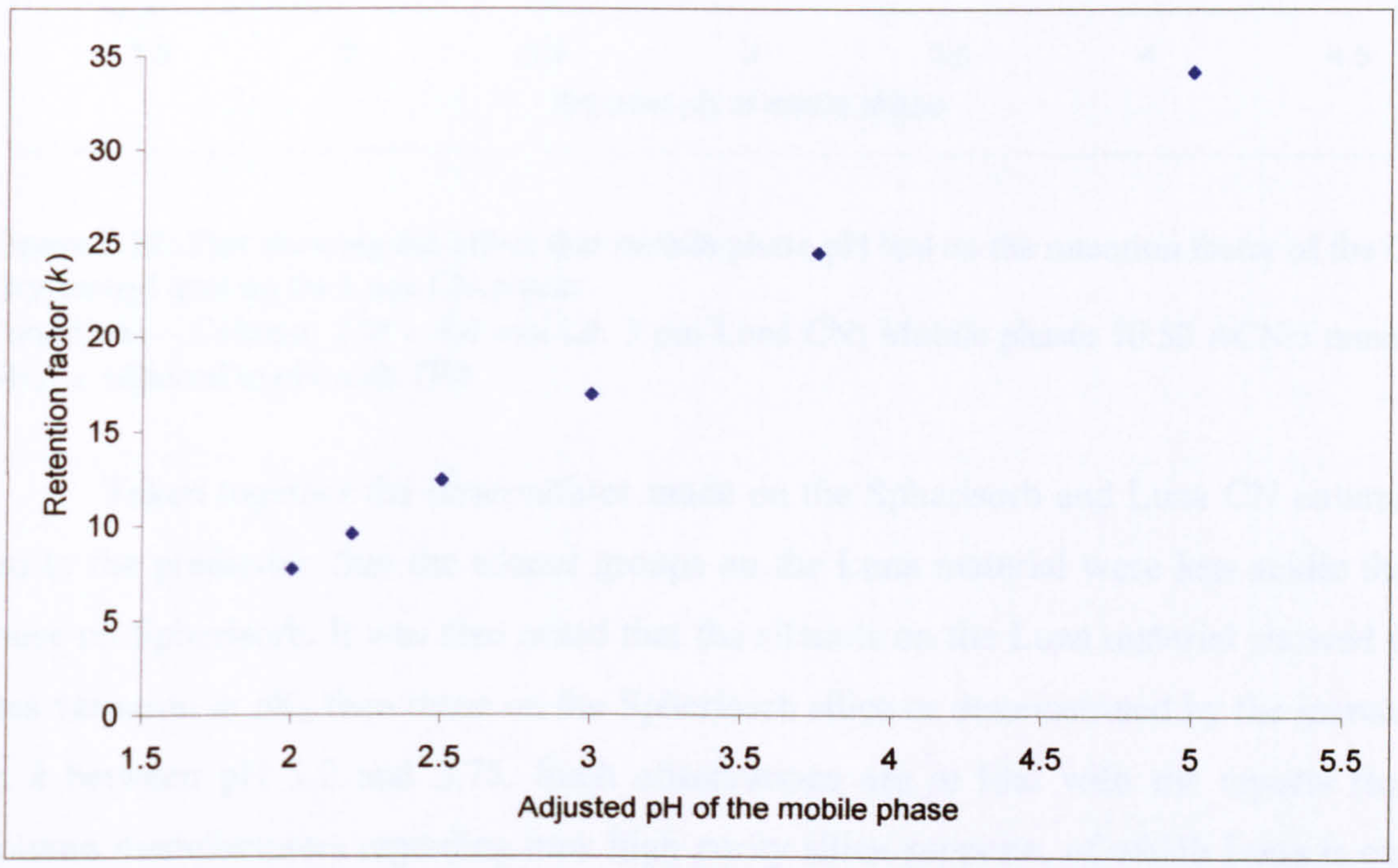

Figure 5.11: Plot showing the effect that mobile phase pH had on the retention factor of the C_{12} alkylbenzyl quat on the Spherisorb CN phase.
Conditions – Column: 150 × 4.6 mm i.d. 3 µm Spherisorb CN; Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac adjusted to pH with TFA.

When the variation in the retention factor was subsequently plotted against mobile phase pH for the Luna CN phase (**Figure 5.12**), it was observed that the silanol groups present on this material were very different to those on the Spherisorb material. Little increase in *k* was witnessed in going from pH 2.0 to 3.0 compared to what had been witnessed in **Figure 5.11**. It was therefore predicted that the Luna phase contained fewer silanol groups with pK_a values in the range 2.0 to 3.0 than its Spherisorb counterpart. However, the Luna phase did show a big increase in *k* in going from pH 3.0 to 3.75, which had not been observed with the Spherisorb material.

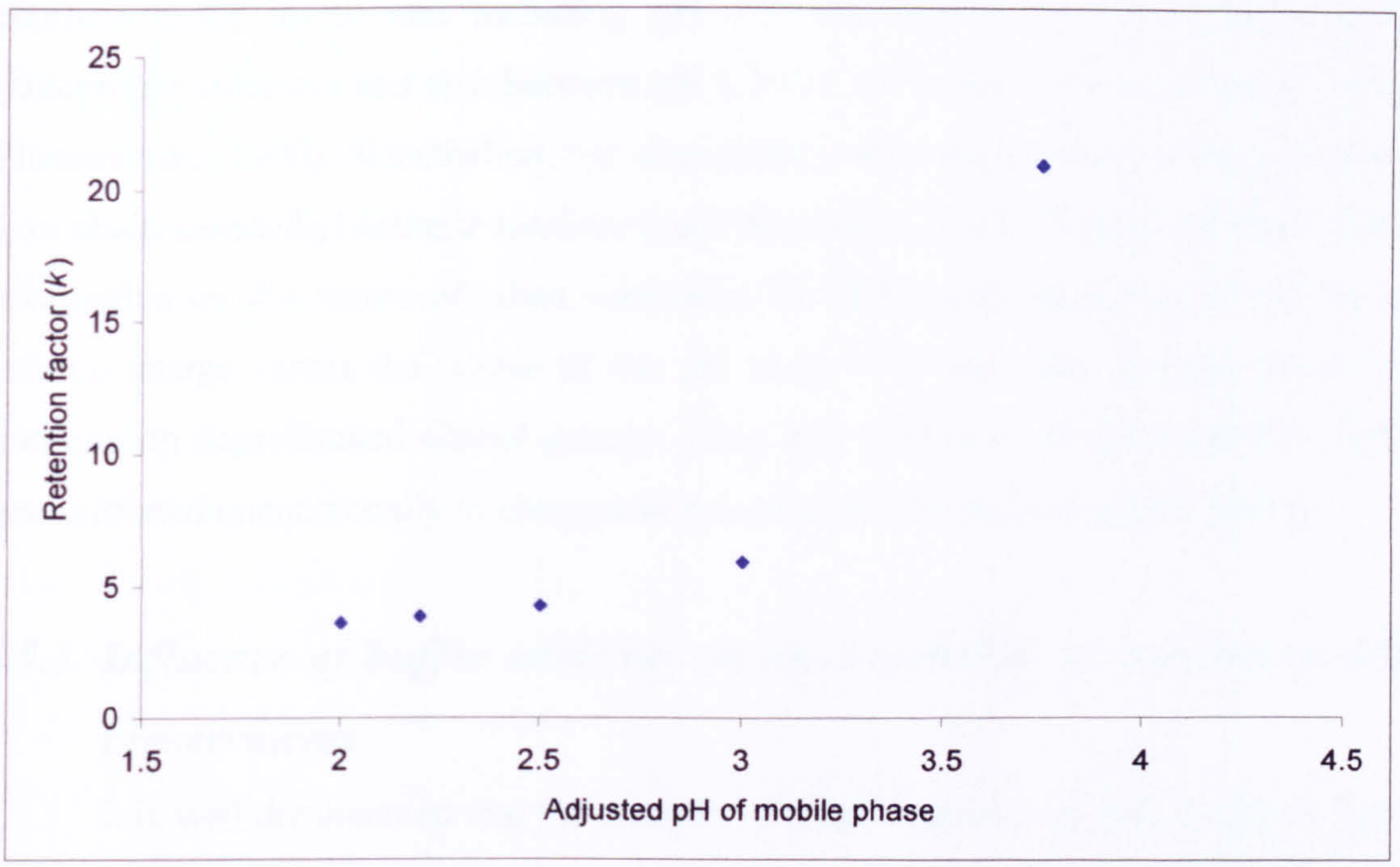

Figure 5.12: Plot showing the effect that mobile phase pH had on the retention factor of the C_{12} alkylbenzyl quat on the Luna CN phase.
Conditions – Column: 150 × 4.6 mm i.d. 3 µm Luna CN; Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac adjusted to pH with TFA.

Taken together the observations made on the Spherisorb and Luna CN columns led to the prediction that the silanol groups on the Luna material were less acidic than those on Spherisorb. It was also noted that the silanols on the Luna material showed far less variation in pK_a than those on the Spherisorb silica as demonstrated by the increase in k between pH 3.0 and 3.75. Such observations are in line with the reports from column manufacturers regarding new high purity silica supports, of which Luna is one, which claim that these materials have an even distribution of silanol groups that show less variation in their acidity (Phenomenex, 2000). The observation that the silanol groups on the Spherisorb material were more acidic than those on the high purity Luna silica also provided further support to the theory that silanol groups associated with metallic impurities demonstrate high acidity (**Section 1.3.2.1**), as the Luna material is reported to contain a lower concentration of residual metals than its Spherisorb counterpart (Phenomenex, 2001b).

A major limitation of the study on the change in retention factor with mobile phase pH was the limited range over which the work was performed. Useful information regarding the numbers, nature, and distribution of the silanol groups on the surface of the two supports could have been gained from evaluating the change in

retention factor up to and including pH 7.5, the limit of the working range of cyanopropyl columns, and also between pH 1.5 and 2.0 in the case of the Luna column (Phenomenex, 2000). Nonetheless, the observations described in this section show that short chain monoalkyl cationic tensides could be used to provide important fundamental information on the nature of silica substrates. As these analytes possess a permanent cationic charge across the whole of the pH range they will only undergo secondary retention on deprotonated silanol groups. Thus, any change in analyte peak parameters can be related unequivocally to changes in the nature of the surface silanol groups.

5.5.3 Influence of buffer additives on the resolution of cationic tenside preservatives

It is well documented that the analysis of organic amines is problematic in liquid chromatography due to secondary interactions between the analytes and surface silanol groups (Barrett *et al.*, 1996a). Indeed, the basis for the work described in **Sections 5.5.2.1** and **5.5.2.5** was to understand how the peak parameters of the cationic tensides change with chromatographic conditions, in order to minimise analysis time and maximise efficiency and sensitivity. One method of limiting secondary interactions between organic amines and the silica support, which had not been fully evaluated up to this point, was the addition of a basic and / or cationic modifier to the mobile phase (de Schutter *et al.*, 1988).

5.5.3.1 The effect of different mobile phase modifiers on the chromatographic parameters of cationic tenside preservatives

Whilst ammonium acetate had been used throughout the course of the reverse phase LC work to limit secondary retention, usage had been based on previous literature reports and ESI-MS compatibility (**Section 4.2.1**), rather than experimental observations. As a result, a brief evaluation of the suitability of a selected group of cationic modifiers was undertaken to assess their influence on the peak parameters of the alkylbenzyl quat tensides, and hence their ability to limit silanol-analyte interaction.

Four cationic modifiers were evaluated during the test, ammonium hydroxide (NH_4OH), ammonium acetate (NH_4Ac), tetramethylammonium hydroxide (TMAOH), and tetrabutylammonium hydroxide (TBAOH). Each modifier was added to the aqueous proportion of the mobile phase to a concentration of 5 mmol/l, before the pH was

regulated to 2.0 with TFA. In addition to the cationic modifiers, an assessment was made on the effect of excluding a modifier by regulating the pH of double-distilled water (**Section 2.2.1**) to 2.0 with TFA. Each of the mobile phases were then utilised for the analysis of a mixed alkylbenzyl quat sample on the Spherisorb CN phase (**Section 4.2.1**), to evaluate the resulting changes in peak shape and resolution.

Figure 5.13 shows an overlay of the chromatograms that were achieved with each of the five different mobile phases. It was apparent that as the strength and size of the quaternary ammonium cation increased, there was an associated decrease in analyte retention time and peak tailing. It was envisaged that these results indicated that the larger and more basic the cation, the more effective it was at blocking and / or competing with the analyte for the surface silanol groups i.e. the apparent trend was that $NH_3^+ < N(Me)_4^+ < N(Bu)_4^+$ at interfering with silanol-analyte interactions (Nawrocki, 1997).

In regard to which of the bases is best suited to the analysis of cationic tensides, TBAOH was seen to give rise to the shortest analysis time and the largest peak height response (**Figure 5.13**). However, when the analysis of the mixed alkylbenzyl quat sample was repeated with the Luna CN column, the use of TBAOH caused the C_8 alkylbenzyl quat to be eluted close to the solvent front (**Figure 5.14**). Whilst it was envisaged that increased retention time could probably be attained through the use of a reduced modifier concentration, the lower volatility of TBAOH in comparison to TMAOH and NH_4Ac led to the TBAOH being declared unsuitable for use in the generic analysis of cationic tensides.

Of the other modifiers that were evaluated TMAOH and NH_4Ac appeared to offer the optimum benefits for the analysis of cationic tensides, whilst being sufficiently volatile to be compatible with ESI-MS. In light of its widespread use in ESI-MS (**Section 4.2.1**), NH_4Ac was deemed to be the most suitable modifier of the four evaluated, for use in a generic methodology.

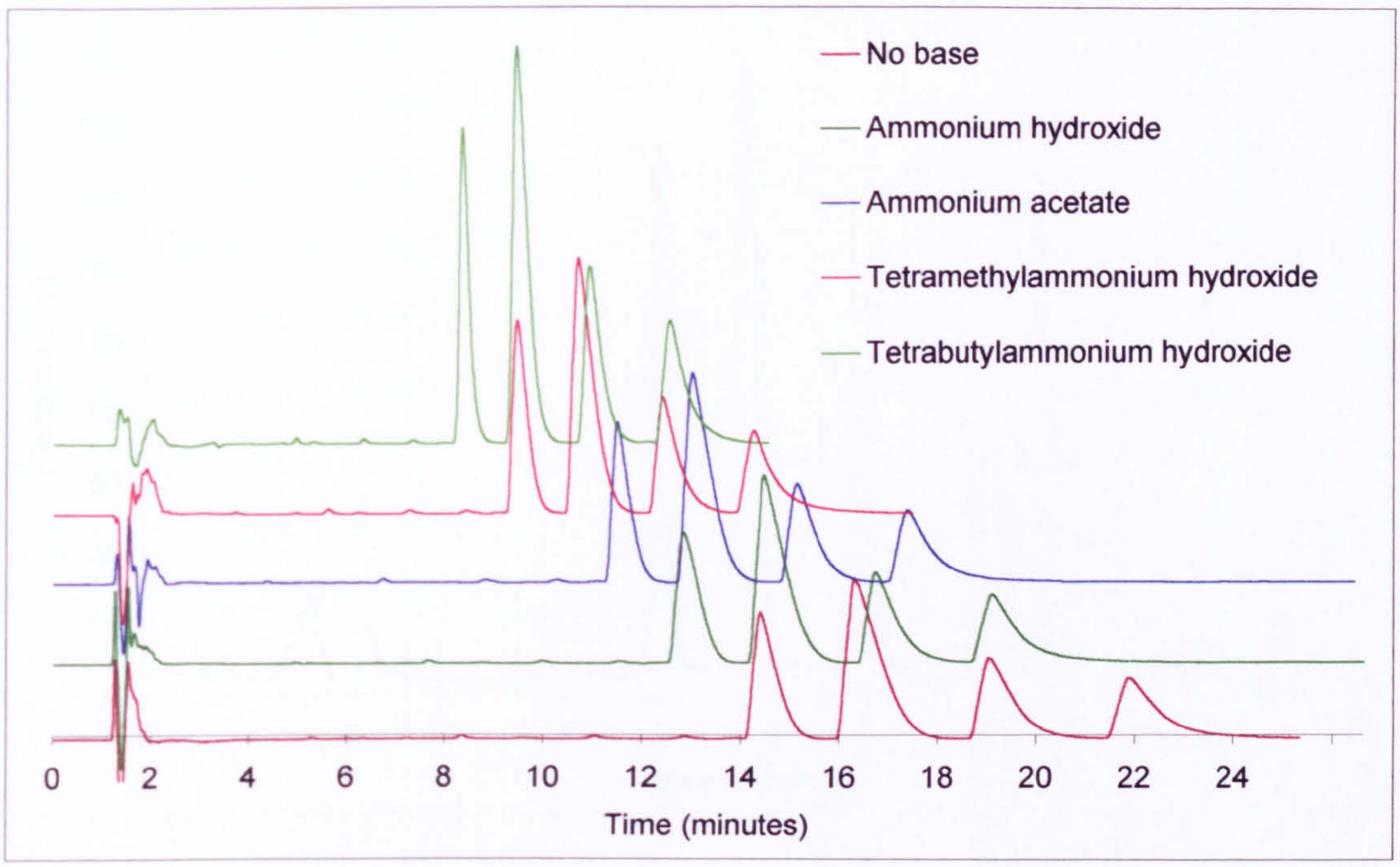

Figure 5.13: Figure showing how the nature of the basic and / or cationic modifier present in the mobile phase affects the retention times of a series of alkylbenzyl quats.
Conditions – Column: 150 × 4.6 mm i.d. 3 µm Spherisorb CN; Mobile phase: 50:50 ACN:5 mmol/l modifier (pH 2.0).

Whilst the size and basicity of the cations could explain some of the observations shown in **Figure 5.13**, the variation in the chromatograms achieved in the presence of NH_4OH and NH_4Ac could not be explained from the nature of the cation alone. These results implied that the nature of the anion also influenced the retention of the analytes, the presence of the much larger organic acetate anion leading to a reduction in the retention time in comparison with the smaller hydroxide anion. Whilst evidence had been obtained to support the theory that many of the analyte molecules were associated with TFA in pseudo-neutral adducts (**Section 4.4**), it was observed that silanol-analyte interactions were still evident at pH 2.0 (**Section 5.5.1.3**). It was hypothesised that the counter-ion may have associated with the free analytes in the system.

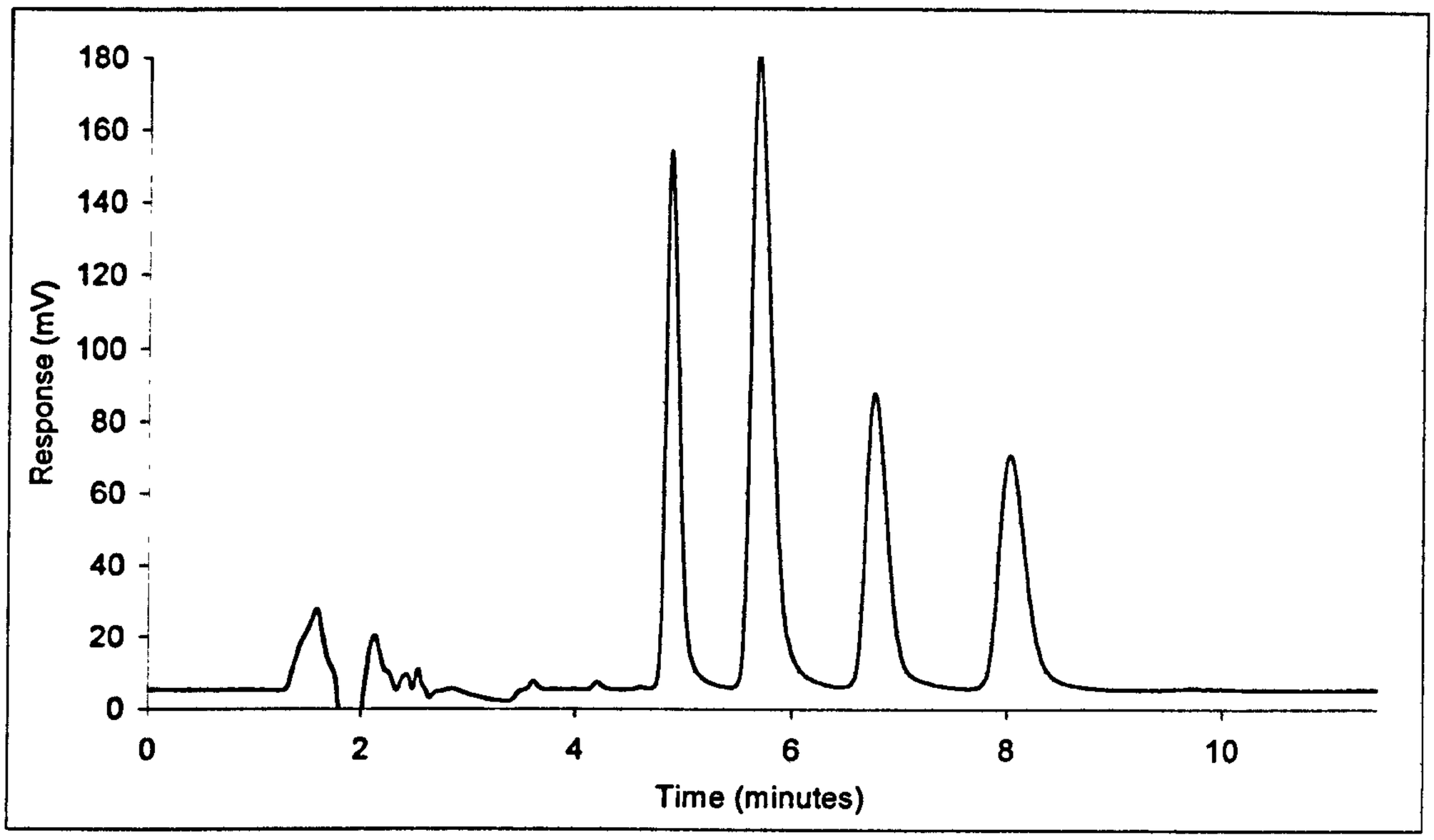

Figure 5.14: Figure showing the analysis of a mixed alkylbenzyl quat sample on the Luna CN column in the presence of TBAOH.
Conditions – Column: 150 × 4.6 mm i.d. Luna 3 µm CN; Mobile phase: 50:50 ACN:5 mmol/l TBAOH (pH 2.0); Flow rate: 1 ml/min.

Although it proved impossible to determine the influence of the anion on the peak parameters of the cationic tensides, a subsequent search through the available literature revealed that this phenomenon had been witnessed before during the analysis of quaternary ammonium analytes (Bluhm *et al.*, 1999). Two quaternary ammonium analytes, benzyltrimethylammonium bromide and a tri-quaternary drug gallamine triethiodide, had been analysed on a silica column with a series of mobile phases containing different competitor reagents that showed variation in the nature of the cation and the nature of the anion. It was reported that a change in the nature of the cation had a significant effect on the retention time and peak shapes of the analytes. However, it was also observed that when tetramethylammonium chloride and tetramethylammonium bromide were evaluated as mobile phase modifiers, the peak parameters of the two analytes showed a distinguishable variation. Bluhm *et al.* (1999) presented no explanations to account for their observations. However, when taken together with the results observed during this work, two possible theories would appear to account for the observations made during the two studies. Either a reduction in the molecular size of the anion increases the likelihood of it being able to interact with free analytes, due to reduced steric inhibition, leading to the formation of pseudo-neutral analytes, which

therefore show less interaction with the silica substrate, or it is the variation in Log P of the adducts, which leads to a change in the degree of partitioning of the analytes (Hansch *et al.*, 1979).

The above observations made it very apparent that the nature of the mobile phase additives has a significant influence on the peak parameters of the cationic tensides. As a result, work is needed to examine the influence of high concentrations of other cations and anions on the resolution of the tenside analytes. This work could yield important information on optimising mobile phase parameters, and at the same time begin to predict what changes are likely to be experienced when industrial, pharmacological and / or environmental samples are analysed with this methodology.

5.5.3.2 Irreversible binding of cationic tensides in the absence of mobile phase modifiers

In view of previous theories regarding the analysis of cationic tensides (Santoni *et al.*, 1993), the observation that good resolution of four alkylbenzyl quats could be achieved in the absence of a basic and /or cationic modifier (**Figure 5.13**) should have been a startling revelation. However, having observed that silanol-analyte interaction played only a minor role in the retention of cationic tensides at pH 2.0 (**Section 5.5.1.3**), this result was far less unexpected. Nevertheless, it was recognised that if efficient resolution of the tensides could be achieved in the absence of a modifier, LC/MS compatibility could be improved significantly.

The effectiveness of a mobile phase utilising no modifying reagents was therefore evaluated during the analysis of the alkylbenzyl quat preservatives on each of the bonded phase columns tested in **Section 5.5.1.2**. It was observed that although the alkylbenzyl quats did elute from each of the phases, with a high elution strength mobile phase, analysis time was excessive, peak shapes were poor, and co-elution of the homologues was often witnessed. However, of greater significance was the low retention time stability that was witnessed on each of the columns.

Numerous reports are available in the literature on the employment of cationic tensides to dynamically modify silica supports, in order to provide novel chromatography that cannot always be achieved with commercially available phases

(Hansen *et al.*, 1987; Cañas-Montalvo *et al.*, 1994; Lavine *et al.*, 1997). Each of the reports relies, to some extent, on the binding of the cationic tensides to the silica-support via strong electrostatic-interactions with surface silanol groups (Hansen *et al.*, 1987). Having read a number of reports on the dynamic modification of silica by cationic tensides, and having witnessed problems with the fabric conditioner actives during the normal phase work (**Section 3.5.6.4**), it was hypothesised that the inconsistent retention times witnessed in the absence of a modifier were derived from the irreversible binding of the analytes to the substrate. In order to test this hypothesis, a series of alkylbenzyl quats were analysed in succession on a Spherisorb silica column in the absence of mobile phase modifiers. The pH of the mobile phase was regulated to 3.0 with TFA to promote deprotonation of the surface silanol groups, prior to the mobile phase being flushed through the silica column for three hours to ensure complete removal of residual ammonium acetate.

Figure 5.15 shows the chromatogram achieved from the first analysis performed on the silica phase in the absence of a modifier. The peak area of the C_{12} alkylbenzyl quat was approximately 25% of that witnessed in the presence of NH_4Ac, and though retention had increased by over 450% (**Figure 5.9**), efficiency had fallen in excess of 25%. It was apparent from the retention time shift that interactions between the analytes and the silica substrate were much stronger in the absence of ammonium acetate.

When a Benzalkonium Chloride (**Appendix One**) standard was subsequently analysed in triplicate on the same phase, a decrease in the retention time of the C_{12} species was observed from approximately 33 minutes during the first analysis to 17 minutes during the fourth analysis (**Figure 5.16**). At the same time, an increase in the peak area response of the two cationic tensides was also observed between sequential injections. It was predicted that the observations made on shifting retention time and increasing peak area indicated that some of the analyte molecules were irreversibly binding to the silica support during each analysis, and hence blocking active sites on the support. As a result, analytes introduced to the system during subsequent injections were eluted faster due to the reduced interaction with the substrate. At the same time, fewer analytes were irreversibly binding to the surface during later injections due to the reduced numbers of strongly acidic sites.

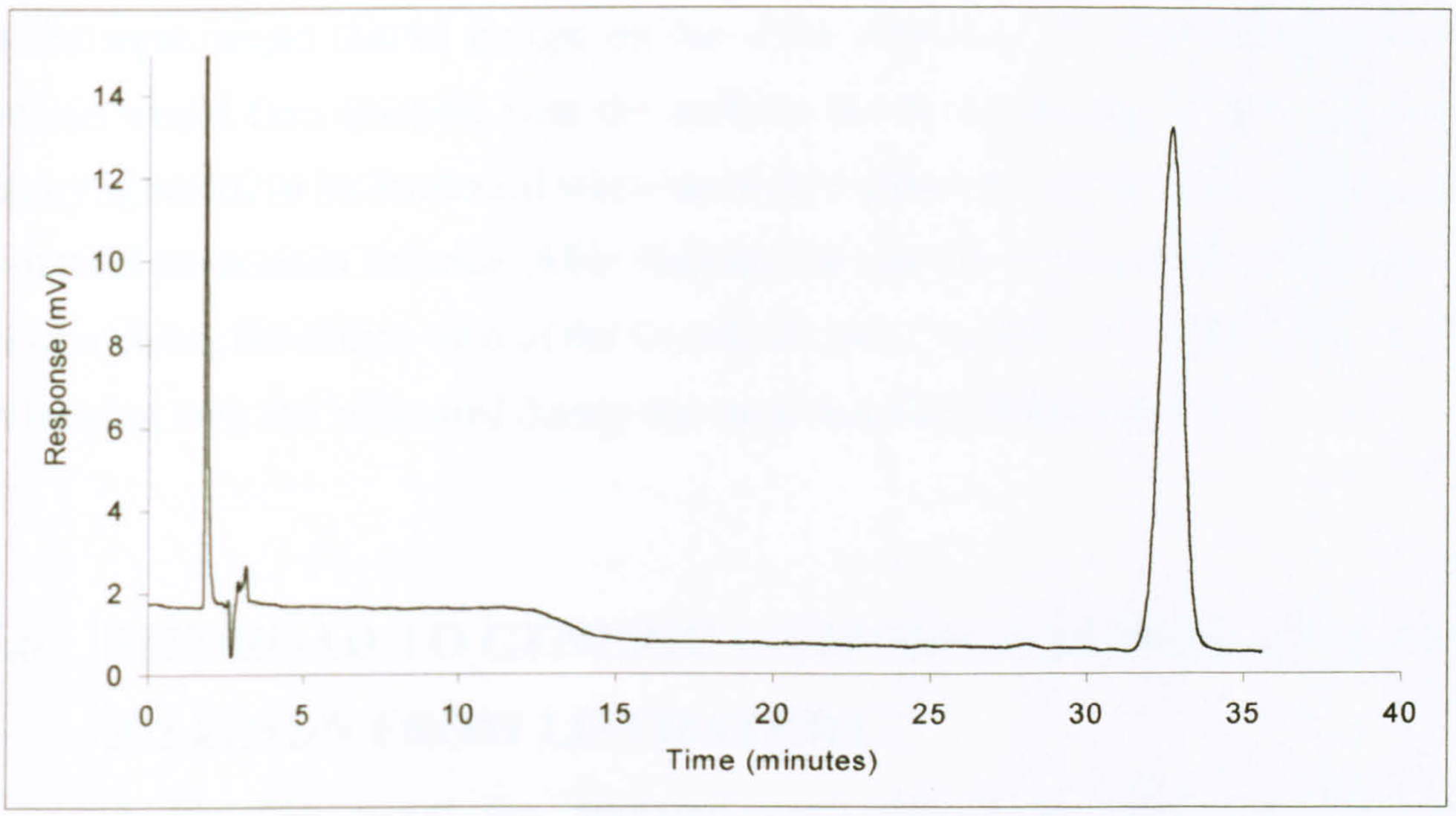

Figure 5.15: Chromatogram obtained from the initial analysis of a C_{12} alkylbenzyl quat standard on a Spherisorb silica column with a mobile phase containing no basic and / or cationic modifiers.
Conditions – Column: 250 × 4.6 mm i.d. 5 µm Spherisorb silica; Mobile phase: 70:30 ACN:H_2O (pH 3.0).

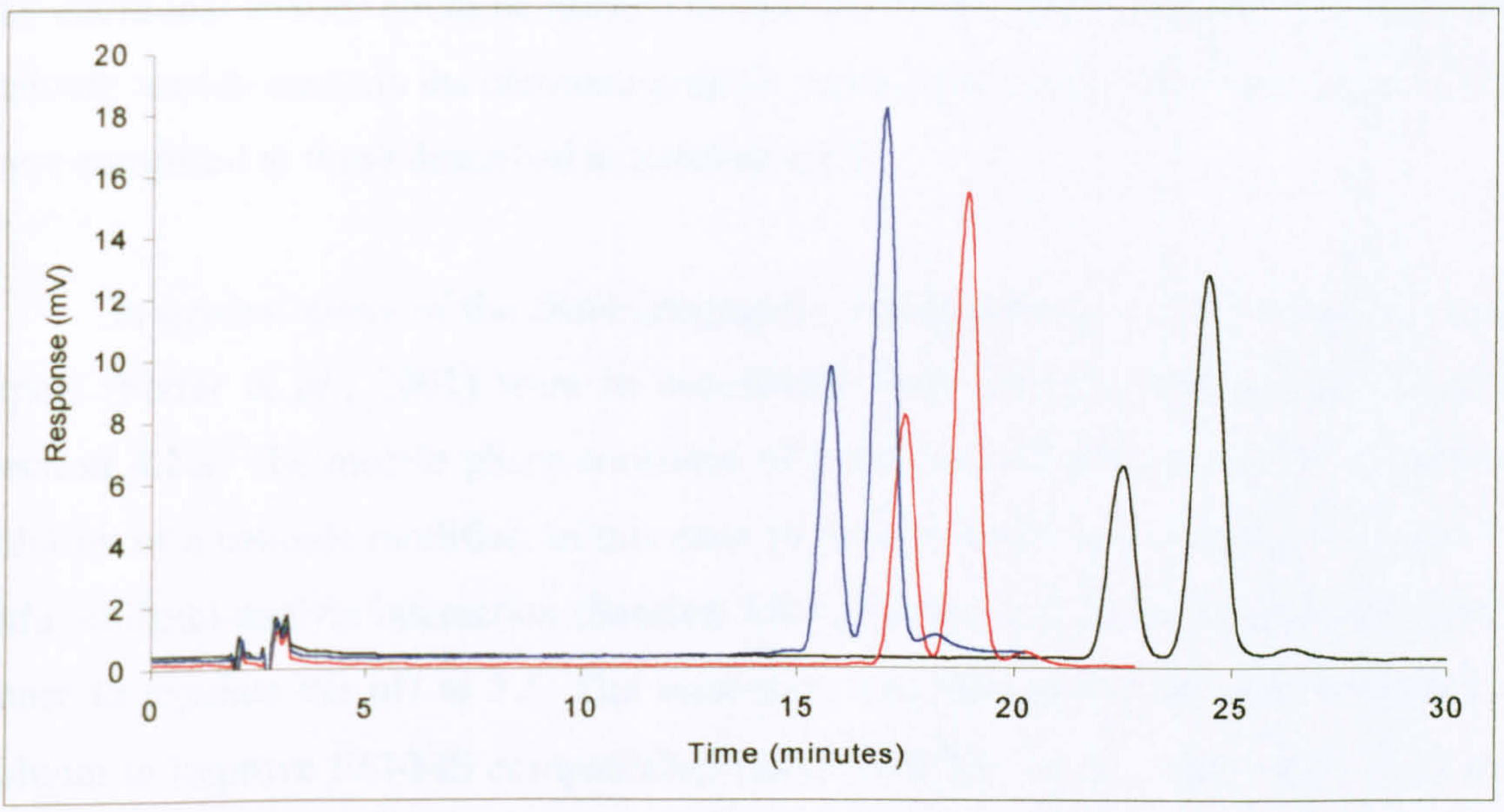

Figure 5.16: Sequential analysis of a 50 mg/l Benzalkonium chloride standard on a Spherisorb silica column with a mobile phase containing no basic and / or cationic modifiers, following the initial analysis of the C_{12} alkylbenzyl quat.
Conditions – Column: 250 × 4.6 mm i.d. 5 µm Spherisorb silica; Mobile phase: 70:30 ACN:H_2O (pH 3.0); Traces: Black - run one, Red – run two, Blue – run three.

It was hypothesised that the short retention time witnessed in the presence of ammonium acetate (**Section 5.5.2.1**) was brought about by ammonium cations binding to the most acidic silanol groups on the silica substrate. The remaining ammonium cations would then compete with the analytes for the remaining silanol groups. This theory appeared to be confirmed when the mobile phase was switched to one containing 5 mmol/l ammonium chloride. After flushing the column for five minutes with the new mobile phase, the elution time of the C_{12} alkylbenzyl quat fell to just over three minutes, in keeping with that witnessed during the work described **Section 5.5.2.1**.

5.6 THE ROAD TO GENERIC CATIONIC TENSIDE ANALYSIS: A LESSON FROM LITERATURE

A literature report has appeared very recently in which the analysis of alkylbenzyl quats was described in environmental wastewaters and rivers (Ferrer *et al.*, 2001). By utilising solid phase extraction followed by reverse phase LC/MS the authors were able to determine individual alkylbenzyl quat homologues at low part per billion levels in complex environmental matrices. To evaluate whether this report could provide any additional support to theories developed in this chapter, or indeed, whether any additional lessons could be learned to ease the development of a generic method for cationic tenside analysis the chromatographic parameters utilised by Ferrer *et al.* (2001), were compared to those described in **Section 4.2.3**.

In general many of the chromatographic parameters used in the recent literature report (Ferrer *et al.*, 2001) were in accordance with the methodology developed in **Section 4.2.3**. The mobile phase consisted of a mixture of acetonitrile and an aqueous solution of a cationic modifier; in this case 10 mmol/l ammonium formate was used to reduce silanol-analyte interaction (**Section 5.5.3**), formic acid being added to the mobile phase to regulate the pH to 3.5. The separation was also performed on a narrow-bore column to improve ESI-MS compatibility (**Section 4.2.3**), in this case a 250 × 3.0 mm i.d. mm long ODS bonded phase was chosen for the separation. At this point the similarities between the literature method and that reported in **Section 4.2.3** cease. By utilising formic acid instead of TFA, Ferrer *et al.* avoided the problems associated with ion-suppression in the electrospray interface, and thus obtained high MS sensitivity without the need for post-column modifiers (**Section 4.4.1**). This represented a distinct

advantage over the optimised LC/MS method reported in **Section 4.4.2,** as the method was simpler and avoided the problems described in **Section 4.5** that were observed during the use of post-column modifiers.

One of the main drawbacks of the literature methodology (Ferrer *et al.*, 2001) was the choice of the long ODS stationary phase. A gradient elution profile was required in which the acetonitrile concentration increased from 50% to 100% over fifteen minutes, with the system being maintained at this composition for a further ten minutes. In addition, a mobile phase flow rate of 600 µl/min was applied, equivalent to a flow rate of 1.4 ml/min on a conventional 4.6 mm i.d. column. In spite of these modifications, the C_{16} alkylbenzyl quat was still seen to demonstrate a 150% increase in retention time in comparison to that seen in **Section 4.5**. It was apparent that the method suffered from excessive retention, with the authors being unable to elute the C_{18} alkylbenzyl quat in the specified time frame. Having witnessed the excessive retention of the C_{12} alkylbenzyl quat on the Spherisorb C_8 at pH 3.0 (**Figure 5.8**), the long retention of the alkylbenzyl quats on the ODS bonded phase was not unexpected. However, examination of the retention time of the C_{12} component in the literature showed that there was a significant variation in the retentivity of the silica supports utilised in the two reports.

Having witnessed that the retention time of the C_{12} alkylbenzyl quat was in excess of eighty five minutes on the Spherisorb C_8 phase, and having been unable to elute this component from a Spherisorb ODS phase (**Section 5.5.1**), elution of the C_{12} component in under eleven minutes, as reported by Ferrer *et al.* (2001) was surprising. Even after accounting for the application of a gradient elution profile, and the higher flow rate used in the literature report, it was concluded that the Phenomenex column employed by Ferrer *et al.* showed less electrostatic retention than did the Spherisorb material, and / or the increase in the modifier concentration to 10 mmol/l must have led to a major reduction in silanol-analyte interaction, and hence the retention factor of the C_{12} alkylbenzyl quat.

It has been reported how the high purity Luna phase was seen to give rise to shorter retention times at pH 3.0 than did the Spherisorb material (**Figures 5.11, 5.12** and **5.17**). Although the nature of the silica used by Ferrer *et al.* was not specified

within the report, it was predicted that the stationary phase was based on a high purity silica support, which contained less acidic silanol groups than the Spherisorb material, and thus reduced analyte retention (**Section 5.5.2.5**). However, it was predicted that the changes made to the mobile phase reagents had a much more significant influence on the shift in analyte retention.

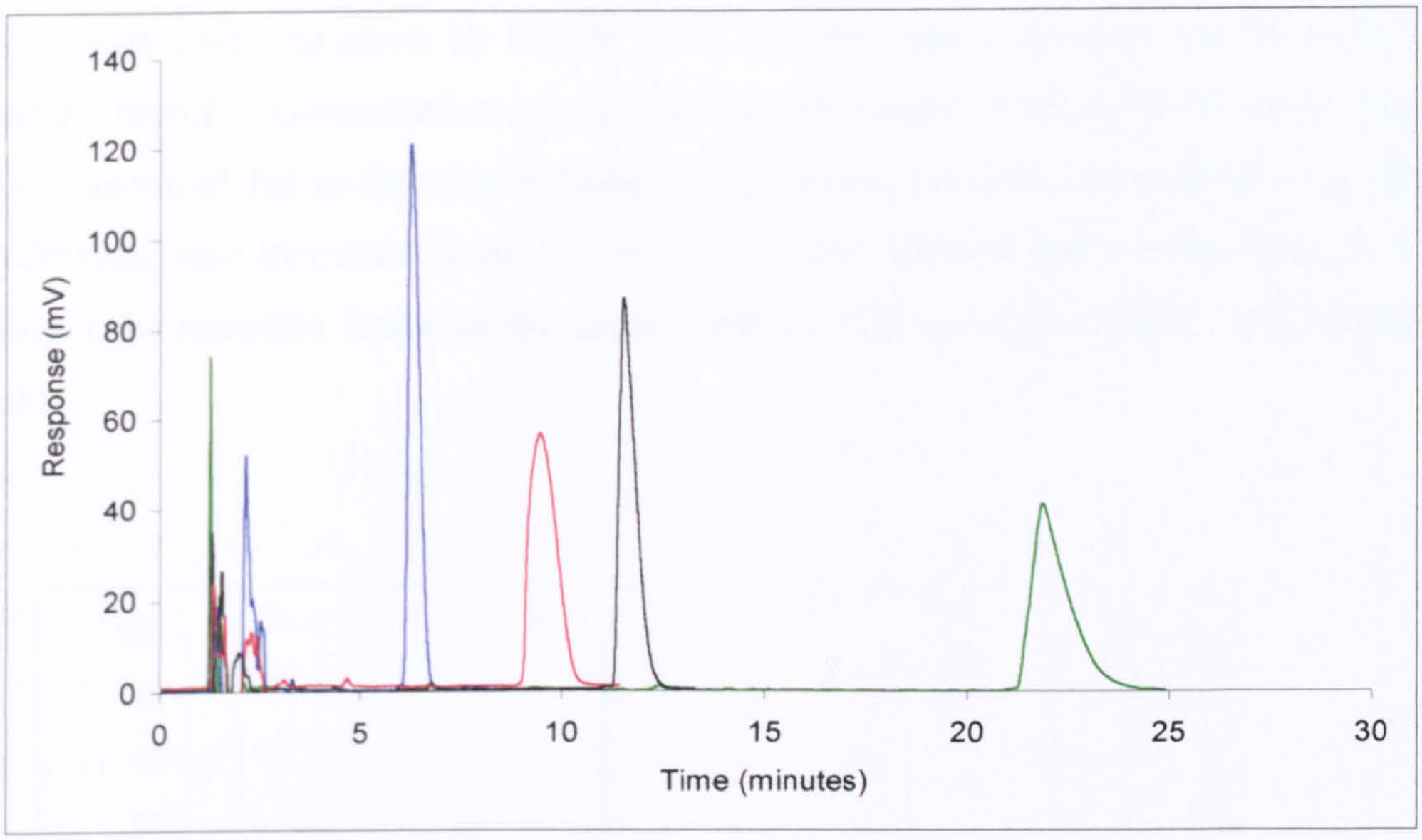

Figure 5.17: Figure showing the variation in the retention time of the C_{12} alkylbenzyl quat on the Luna CN and Spherisorb CN phases at pH 2.0 and 3.0.
Conditions - Column dimensions: 150 × 4.6 mm i.d. packed with 3 µm silica particles; Column: Red trace and blue trace – Luna CN, black trace and green trace – Spherisorb CN; Mobile phase: 50:50 ACN:5 mmol/l NH_4Ac adjusted to pH with TFA; pH of mobile phase: Blue trace and black trace – 2.0, red trace and green trace – 3.0; Flow rate: 1 ml/min.

Having observed that the use of ammonium acetate and ammonium hydroxide gave rise to some variation in the resolution of four alkylbenzyl quats (**Figure 5.13**), it was postulated that the use of ammonium formate (Ferrer *et al.*, 2001) would have given rise to a slight change in analyte peak parameters. However, as sound justification for a variation in the behaviour of the formate and acetate anions was not forthcoming, it was concluded that the increase in modifier concentration was responsible for the shift in analyte retention time. Whilst a full assessment of the variation in the peak parameters of the alkylbenzyl quats with changing modifier concentration, was overlooked during this work, comparison of the chromatograms achieved in the presence and absence of 5 mmol/l ammonium acetate (**Figure 5.18**) can begin to

provide some indication of the likely result of increasing the modifier concentration. **Figure 5.18** shows that a significant reduction in the retention of the C_{12} alkylbenzyl quat accompanied the introduction of the cationic modifier to the system. Whilst an increase in concentration from 5 to 10 mmol/l may not have yielded the same shift in retention as that witnessed in **Figure 5.18**, due to the fewer numbers of silanol groups left unassociated on the silica surface, it was predicted that a significant reduction in the analyte retention times would still be witnessed. Support for this hypothesis was found in a short communication by Bluhm *et al.* (1999), which assessed the influence of varying modifier concentration on the retention of benzyltrimethylammonium bromide. It was reported that as the concentration of the cationic modifier, tetramethylammonium hydroxide, was increased from 125 mmol/l to 250 mmol/l and subsequently to 500 mmol/l the retention factor of the analyte fell to 75% and then to 60% of its original value.

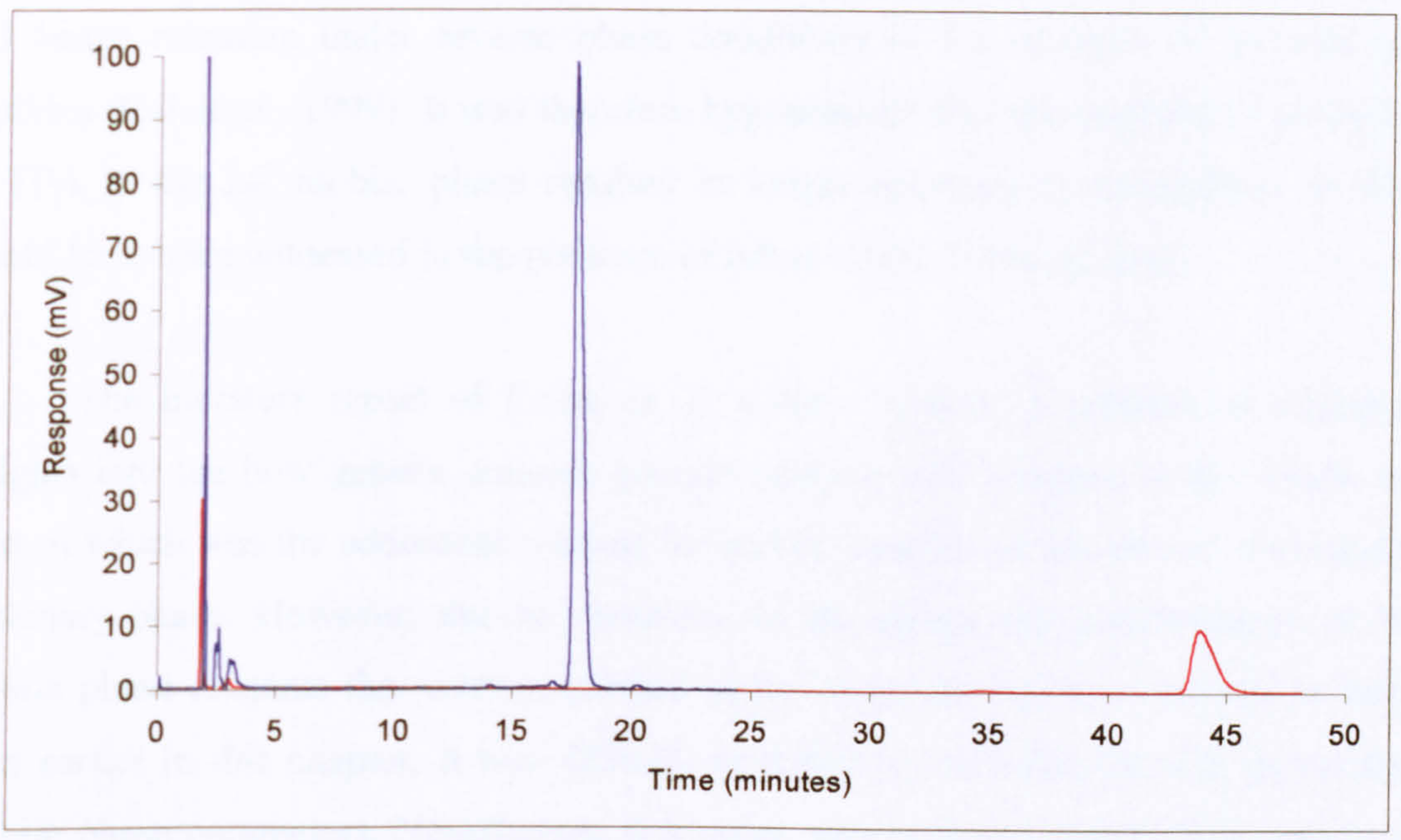

Figure 5.18: Figure showing the effect of 5 mmol/l ammonium acetate on the retention time of the C_{12} alkylbenzyl quat.
Conditions – Column: 250 × 4.6 mm i.d. Spherisorb 5 µm CN; Mobile phase: Blue trace - 70:30 ACN:5 mmol/l NH_4Ac (pH 3.0), Red trace - 70:30 ACN:H_2O (pH 3.0).

A second critical inference that could be deduced from the successful utilisation of 10 mmol/l ammonium formate by Ferrer *et al.* (2001), was that selected mobile phase modifiers could be utilised at concentrations in excess of 5 mmol/l in a hyphenated LC/MS methodology without affecting ESI-MS source dynamics and sensitivity. The

significance of this result was increased by the fact that the high mobile phase flow rate would have given rise to sub-maximal detector response and would have led to problems with source dynamics in comparison to the flow rate used in this work **(Section 1.3.2.1)**.

Whilst a change to the stationary phase and an increase in modifier concentration could together account for the shift in retention time between the work of Ferrer *et al.* (2001), and that presented in **Section 4.2.3**, the variation in the choice of acid used to regulate pH was also predicted to have some influence on analyte retention. The use of TFA in the methodology reported in this work was found to result in less peak tailing in comparison to acetic and formic acid (**Section 4.4.1**). However the effectiveness of TFA in reducing peak tailing may have also inadvertently led to an increase in analyte retention time, as the pseudo-neutral adducts formed from the association of TFA with cationic tensides, were believed to be more hydrophobic than the free analytes. This effect has previously been seen to lead to increased partitioning, and hence retention under reverse phase conditions in the analysis of proteins and peptides (Cai *et al.*, 1999). It was therefore hypothesised that the addition of an excess of TFA to the LC mobile phase resulted in longer retention in comparison to what would have been witnessed in the presence of either acetic or formic acid.

The literature report of Ferrer *et al.* (2001) revealed a number of important insights into the how generic cationic tenside analysis will progress in the future, not least of which was the additional support for earlier theories on the choice of a suitable stationary phase. However, due to variations in the nature and concentration of the mobile phase reagents that were employed in the literature report, compared to those used earlier in this chapter, it was difficult to develop justifiable theories on optimal mobile phase parameters. Nonetheless, it became very apparent that further studies on the choice and concentration of mobile phase modifiers would be needed in order to minimise silanol-analyte interaction and hence maximise peak efficiency. The variation in chromatographic peak parameters and MS response should be assessed as the concentration of ammonium acetate and ammonium formate is varied. At the same time, an analogous study should be performed with triethylamine as literature reports have recently revealed the greater efficacy of tertiary amines at blocking silanol-analyte

interaction than quaternary ammonium salts (Hill, 1990; Nawrocki, 1997; M^{c}Calley, 1999; Reta *et al.*, 1999).

5.7 CONCLUSIONS

Although this chapter may appear to consist of the discussion of a series of unconnected experiments, the theme and main challenge of all the work was *"the quest to develop a generic method of analysis for cationic tensides"*. Having recognised that liquid chromatographic methods offered the greatest potential for realising generic cationic tenside analysis, the broader applicability of the two new LC methods reported in **Chapters Three** and **Four** were assessed. The observation of poor chromatography during the analysis of the alkylpyridinium quats, in addition to previous problems with the analysis of the quaternary amino-alcohol metabolites showed that the inherent limitations of the new normal phase methodology would prohibit its use as a generic methodology.

Application of the new reverse phase LC method to the alkylpyridinium quats facilitated efficient resolution of two homologues of this quat series. However, comparison of the retention times of the alkylbenzyl quats and the alkylpyridinium quats revealed that co-elution was a problem on both of the cyanopropyl phases that were evaluated, which was interpreted as an indication that the quaternary amino-alcohols would be unretained on these types of support.

The search for a stationary phase that would provide stronger retention of the hydrophilic cationic tensides led to the evaluation of a series of commercially available reverse phase columns based on the Spherisorb silica. Elution of a short-chain alkylbenzyl quat could not be achieved on a conventional octadecylsilane bonded silica, yet the corresponding alkylpyridinium quat was seen to elute from a mixed-mode ODS / cyanopropyl bonded phase, albeit retention time and peak tailing were considerably increased in comparison to a conventional cyanopropyl bonded phase.

Further investigation into the retention of the cationic tensides on reverse phase supports revealed that at low pH, contrary to popular belief, silanol-analyte interactions had little effect on retention, and instead conventional reverse phase partitioning was

responsible for retaining the analytes on column. At higher pH, electrostatic interactions were seen to be much more prevalent in the retention mechanism, giving rise to long elution times and peak tailing. Interestingly, peak tailing was seen to be much more severe on the long-alkyl bonded supports which are predominantly chosen for the basis of reverse phase separations. This was thought to be as a result of the retardation of the analytes in the bonded phase as they desorbed from deprotonated silanol groups on the silica surface. Such observations revealed that the increased peak tailing witnessed on the mixed-mode ODS / CN column was due to variations in the rates of analyte transfer between the silica surface and the bulk mobile phase due to the presence of cyanopropyl and octadecyl silane bonded units on the silica surface.

Having assessed the influence of a series of quaternary ammonium salts on the resolution of four cationic tenside preservatives, it was apparent that silanol-analyte interaction fell as the basicity of the cation increased. An effect on the analyte peak parameters was also witnessed when the nature of the anion in the quaternary ammonium salt was varied. The change however, was much less pronounced than that observed with alternative cations. An interesting observation of the modifier work was the recognition that efficient resolution of the analytes occurred at low pH in the absence of a basic and / or cationic modifier. Unfortunately, dynamic modification of a silica column soon showed why the analysis of cationic tensides should be avoided under reverse phase conditions if a suitable basic modifier is unavailable.

Comparison of the results produced in this chapter with those documented in a recent literature report, has shown that high purity silica supports can yield significant reductions in analyte retention time and peak tailing, in comparison with older silica materials. The observation that the high purity Luna support appeared to have far fewer highly acidic groups on its surface than the corresponding Spherisorb material may go a long way to explaining these observations. Having witnessed a large increase in retention time on the Luna material over a short pH range, rather than an even increase in retention over a wider pH range, as was the case with the Spherisorb material, it was predicted that the silanol groups on the high purity phase demonstrated much more uniform pK_a's than those on the Spherisorb material.

Ultimately the results presented in this chapter show that more efficient analysis of monoalkyl cationic tensides can be achieved on short alkyl bonded or bare silica columns, operated under reverse or pseudo-reverse phase conditions, than can be achieved with normal phase LC and reverse phase LC, performed on conventional octadecylsilane-bonded stationary phases.

The most important realisation of this chapter is that many of the preconceived theories on the analysis of cationic tensides under reverse phase conditions are not based on either sound chromatographic theory or experimental fact. The path to generic cationic tenside analysis will be much simpler having removed many of the mist clouds surrounding these analytes.

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CHAPTER SIX

Conclusions and future work

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The widespread application of cationic tensides in pharmaceutical preparations, and home and personal care products, requires the capability to accurately quantify these analytes in a number of different classes of sample. As the nature of the matrix can range from *"pure"* raw material or formulated product, to natural water, sediment or soil, methods of analysis must be adaptable, unaffected by matrix interferences, and selectively speciate the analytes of interest at trace levels.

To date, most methods used to quantify cationic tensides demonstrate insufficient reliability, sensitivity, and / or selectivity for widespread use. In addition, they can often only be effectively applied to distinct matrices; no method is capable of speciating all of the cationic actives present in domestic fabric conditioners and pharmaceutical preparations, prior to use, and after *"down-the-drain release"*. As a result, the aim of this work was to fill some of the voids apparent in the current suite of methods used to quantify these important oleochemicals. Furthermore, it was envisaged that the foundations for generic cationic tenside analysis could also be built.

In **Chapter Three** a new normal phase liquid chromatographic method was developed to quantify the cationic actives present in domestic fabric softeners. The new method was seen to offer superior resolution and retention time stability compared to current methods used to quantify first and second generation fabric conditioner actives. It was observed that the new method could resolve different classes of cationic tenside, whilst unexpectedly for a normal phase LC method, partial resolution of the endemic homologues was also attained. In addition, the subsequent analysis of a tenside sample fabricated from a number of commercial samples, revealed that the new method also allowed the quantitation of an individual tenside homologue from a specified sample origin, without the need for mass spectrometry. This represented the first time such resolution had been achievable with a liquid chromatographic methodology.

Although the new method was thought to be suitable for the quality assurance of raw materials and formulated industrial products, an observed lack of sensitivity limited its applicability to the environmental analysis of cationic tensides. Furthermore, observation that the method was ill suited to the quantitation of hydrophilic cationic tensides (**Chapter Five**) and cationic biodegradation products (**Chapter Three**), led to the conclusion that the method could not be used for generic analysis.

In **Chapter Four,** experimentation centred on the development of a new analytical method capable of quantifying trace levels of cationic tenside preservatives in environmental matrices. Building on literature reports and current Unilever methodology, a reverse phase liquid chromatographic method was optimised for use with electrospray mass spectrometry. Utilisation of narrow-bore columns was seen to improve method sensitivity, whilst the employment of volatile organic modifiers improved the compatibility of the method with mass spectrometry. Although the new method was subsequently validated and found to demonstrate excellent linearity and reproducibility, the necessity to utilise peak compression was thought likely to lead to resolvation problems during environmental analysis.

Hyphenation of the LC method with mass spectrometry was found to be inefficient as sensitivity was compromised by ion-suppression, resulting from the presence of a high concentration of trifluoroacetic acid in the LC effluent. Whilst the use of a post-column modifier reduced ion-suppression, the optimised methodology proved unwieldy, contrived, and would result in increased instrument downtime. Experience highlighted the need for careful consideration of liquid chromatographic parameters prior to hyphenation with mass spectrometry. Compromise is evidently the key to the development of an efficient LC/MS methodology, and whilst bench-top MS instruments may have introduced LC/MS into many new laboratories, new lessons need to be learned before the technique is applied effectively and intuitively.

Following the observation that the new normal phase liquid chromatographic methodology showed little potential for the generic analysis of cationic tensides, efforts focused on optimising the reverse phase LC/MS method for its application to other groups of cationic tensides. Whilst success was forthcoming in the analysis of the alkylpyridinium preservatives, problems were anticipated for the determination of fabric

conditioner actives, especially in light of the well-documented problems of silanol-cationic tenside interaction. Preliminary results showed that the commonly used reverse phase supports, octadecylsilane and octylsilane, provided inefficient resolution of the cationic tenside preservatives, due to excessive retention and peak tailing. These results appeared to support previous theories on reverse phase LC analysis of cationic tensides, whereby retention was brought about by electroststic interactions between the analytes and surface silanol groups of the silica support. However, the observation that peak tailing was minimised on a bare silica phase, where such interactions should have been maximised, led to the hypothesis that strong partitioning and not electrostatic interaction was the fundamental reason for excessive analyte retention i.e. original theories were misconceived. It was later found that the severity of peak tailing was highest on long alkyl-bonded stationary phases as the analytes were strongly retarded on their path from the silica surface to the bulk mobile phase.

The use of competitive modifiers was found to be important in the analysis of cationic tensides, particularly on bare silica, where their presence prevented dynamic modification of the surface by the tenside analytes. Similarly, regulation of mobile phase pH was also found to be important in achieving efficient chromatographic separations. Regulation of the mobile phase to low pH conditions was found to limit the influence of silanol-analyte interaction on analyte retention. However, it was observed that under conditions normally perceived to protonate all the silanol groups on the silica substrate, electrostatic interactions were still evident between the analytes and the surface. The degree to which this interaction occurred was subsequently found to be reduced on modern high purity silica supports, which justified claims that these materials contain less highly acidic adsorption sites, and demonstrate greater uniformity in the nature of the silanol groups.

The work described herein was focussed on the development of new methodology for the analysis of cationic tensides. However, many of the lessons learned during **Chapters Four** and **Five** regarding peak tailing and LC/MS analysis will also be applicable to the analysis of small proteins and peptides, and to the amphoteric tensides, which also contain a quaternised nitrogen group within their structure.

Looking ahead, evaluation of the suitability of the new normal phase LC/MS and reverse phase LC/MS methods for the analysis of *"real-world"* samples, will be critical in determining the future potential of each of these methods. The normal phase methodology seems well suited to industrial applications due to the superior resolution that is offered in comparison to literature methods. Additional work is required on understanding the retention mechanism and the influences that adduct stability have on analyte retention times, as the findings should allow resolution to be maximised and the method to be made more applicable to the analysis of hydrophilic tensides. Whilst the sensitivity achieved with the evaporative light scattering detector was low, improvement could well be forthcoming from the use of conductivity detection, particularly when used with automated suppresser systems. However, the effect of gradient elution should be thoroughly assessed to ensure that baseline drift is not a major problem in the future.

The normal phase LC/MS method appears to demonstrate potential for the analysis of the parent ester and alkyl quats in environmental matrices. A formal evaluation of the effect that matrix constituents have on the resolution of the individual tenside homologues is required, and method sensitivity and linearity should subsequently be evaluated. The use of flow splitting, prior to the electrospray interface and single ion monitoring should also yield improvements in detector response and method sensitivity, which potentially could lead to the application of the methodology to environmental fate studies. Utilisation of MS instruments with an orthogonal interface design will be beneficial during the analysis of *"dirty"* samples that would be encountered at this point.

For the new reverse phase LC/MS method, the future would appear bleak. It is evident that the front-end LC separation needs to be redeveloped to improve its compatibility with electrospray mass spectrometry. It was apparent that the use of trifluoroacetic acid should be avoided or severely limited in the future to ensure the sensitive quantitation of cationic tensides by LC/MS. Nonetheless, the new reverse phase LC-DAD method has already shown worth in the routine analysis of cationic tenside preservatives in environmental matrices at Unilever Research.

From the perspective of the generic analysis of cationic tensides, the work reported herein is insufficient for a generic method to be immediately evident. However,

it is clear that for rapid and efficient quantitation of these analytes, chromatographers should focus on developing methods that utilise short (C_4 to C_8) alkyl bonded stationary phases that are based on high purity silica supports. The use of low concentrations of organic amines such as triethylamine and ammonium acetate will also facilitate short analysis times and speed sample throughput, whilst their inherent volatility will aid hyphenation with mass spectrometry.

The very different physico-chemical properties of the cationic metabolites and the dialkyl parent quats will require generic cationic tenside analysis to be performed with the aid of a gradient elution method. It is envisaged that a suitable mobile phase system would start from an aqueous-organic mixture similar to that used in **Chapters Four** and **Five,** and change to a fully non-aqueous system, based on a strong solvent such as dichloromethane over time. This system should facilitate elution of parent quats and cationic metabolites in a time frame suitable for routine analysis.

Utilisation of the new monolithic stationary phase technology should also be evaluated at the earliest convenience in the development of a generic methodology. The novel pore structure of this support material allows the utilisation of high linear velocities without the normal problems of high backpressure. The ability to vary separation velocity represents a powerful tool that could be used in conjunction with fast gradient elution analysis and elevated temperature, to speed analytical throughput and lift some of the pressure on entrenched analytical facilities.

Although generic cationic tenside analysis may still be some way off, many of the previous misconceptions that have led to irrational fears of irreversible binding and severe peak tailing have now been dispelled, and the road ahead is based on the foundation of chromatographic theory rather than preconceived ideas.

APPENDICES

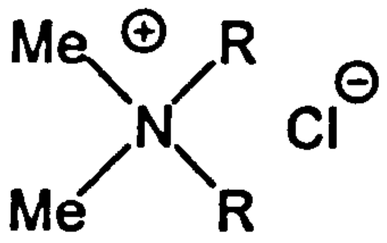
APPENDIX ONE

Sample characteristics

Arquad HT

General description: Hydrogenated tallow derived dialkyl quat. Used as the principal active agent in a number of fabric conditioners formulations prior to the mid 1990's, when it was replaced in Europe by the esterquat surfactants. Arquad HT and analogous products are still utilised in some fabric conditioners being sold outside of Europe.

Principal active: Dialkyl dimethyl ammonium chloride



Chain length distribution (%): R = $C_{20} \leq 2$, C_{18} = 60-80, C_{16} = 25-35, C_{14} = 1-5

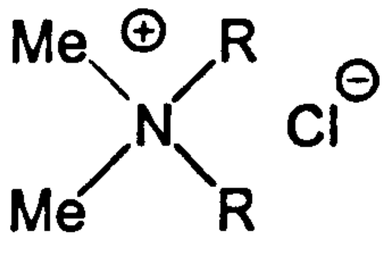
Impurities present: ≈ 15% monoalkyl quat; ≈ 10% trialkyl quat

Source: Unilever Research

Arquad T

General description: Tallow derived dialkyl quat. Has been used as an active agent in some fabric conditioner formulations.

Principal active: Dialkyl dimethyl ammonium chloride



Chain length distribution (%): Unknown but principally C_{16} and C_{18} saturated and unsaturated

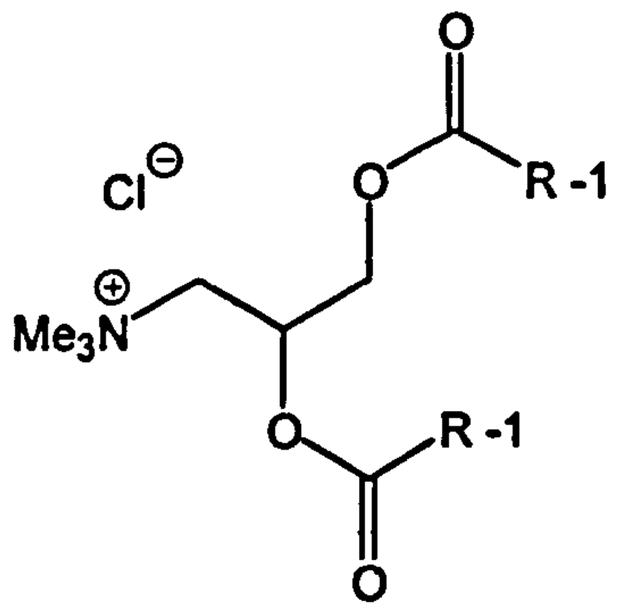
Impurities present: ≈ 17.5% monoalkyl quat; ≈ 15% trialkyl quat

Source: Unilever Research

Hamburg Ester Quat (HEQ)

General description: Hydrogenated tallow derived diester quat. Used as the principal active agent in some modern fabric conditioners formulations.

Principal active: Diester quat



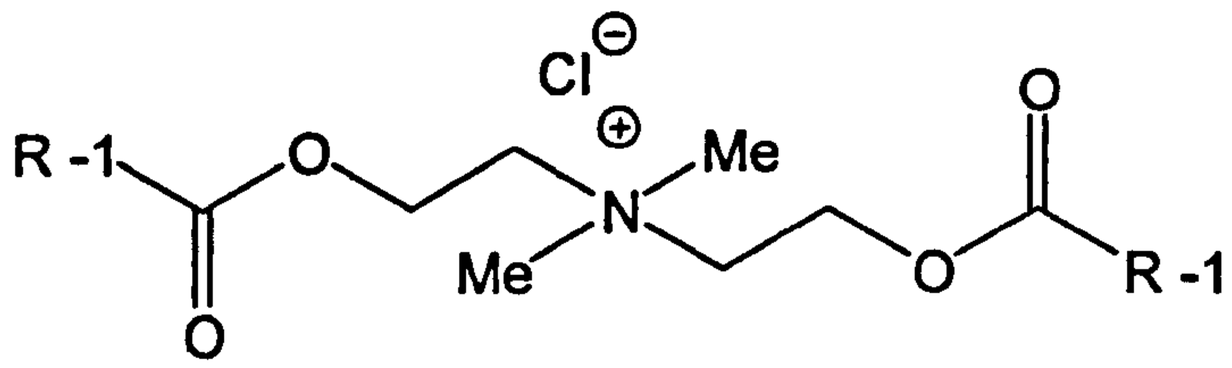
Chain length distribution (%): R = C_{20} = 1.4, C_{18} = 63.2, C_{17} ≈1, C_{16} = 31.1, C_{14} = 2
Impurities present: ≈ 5% monoester quat; ≈ 3% fatty amine; ≈ 10% free fatty acid

Source: Unilever Research

Diethylesterdimethylammonium chloride (DEEDMAC)

General description: Hydrogenated tallow derived diester quat. Used as the principal active agent in some modern fabric conditioners formulations.

Principal active: Diester quat



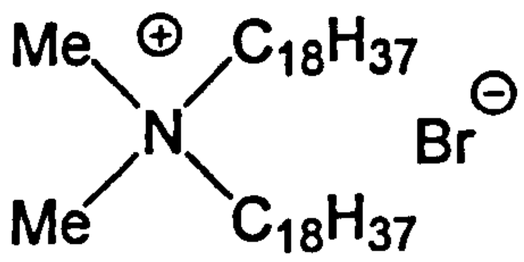
Chain length distribution (%): R = C_{18} = 64.2, C_{17} = 2.1, C_{16} = 28.8, C_{14} = 3.26
Impurities present: ≈ 3% monoester quat; ≈ 1.6% free fatty acid

Source: Unilever Research

Dimethyldioctadecylammonium bromide

General description: Hydrogenated dialkyl quat with two $C_{18}H_{37}$ alkyl chains. Representative of one of the main actives present in Arquad 2ht-75.

Principal active:



Chain length distribution (%): C_{18} / C_{18} ≈ 97

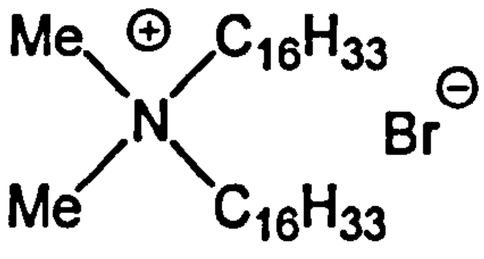
Impurities present: C_{18} / C_{16} ≈ 3%

Source: Sigma-Aldrich

Dihexadecyldimethylammonium bromide

General description: Hydrogenated dialkyl quat with two $C_{16}H_{35}$ alkyl chains. Representative of a major active component present in Arquad HT.

Principal active:



Chain length distribution (%): $C_{16} / C_{16} > 98$

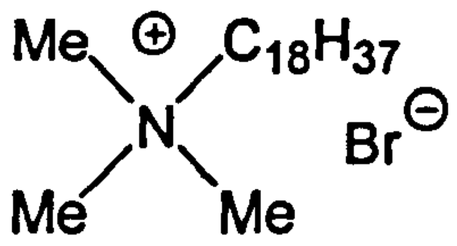
Impurities present: $C_{18} / C_{16} < 2\%$

Source: Sigma-Aldrich

Octadecyltrimethylammonium bromide

General description: Hydrogenated monoalkyl quat with a $C_{18}H_{37}$ alkyl chain. Representative of a minor impurity present in Arquad HT.

Principal active:



Chain length distribution (%): $C_{18} > 90$

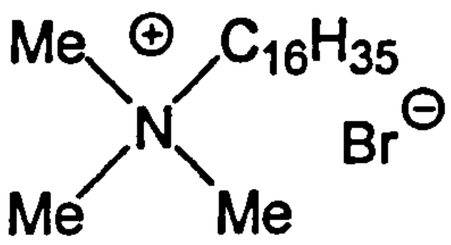
Impurities present: Mainly C_{16} but C_{14} and C_{12} are also present

Source: Sigma-Aldrich

Cetyltrimethylammonium bromide (Cetrimonium bromide or CTAB)

General description: Hydrogenated monoalkyl quat with a $C_{16}H_{37}$ alkyl chain.

Principal active:



Chain length distribution (%): $C_{16} > 95$

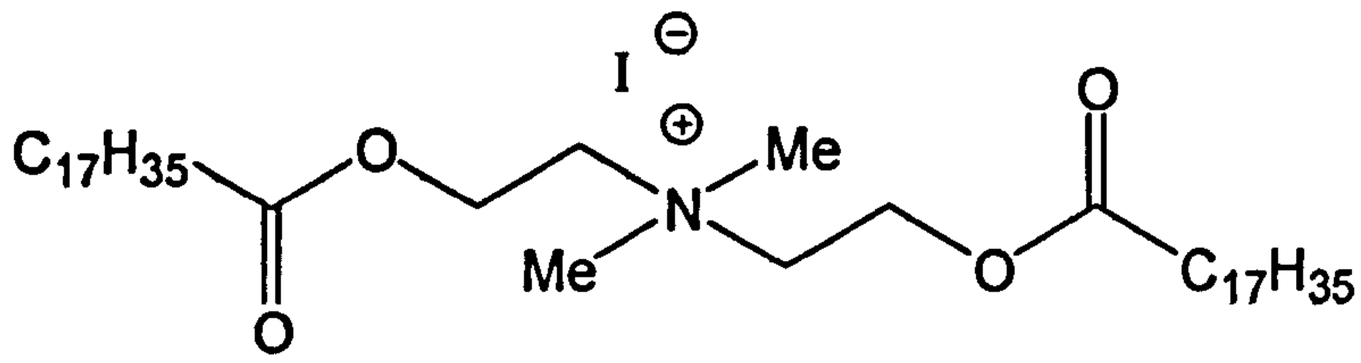
Impurities present: Mainly C_{18}

Source: Sigma-Aldrich

DEEDMAC C_{18} / C_{18} diester quat

General description: Hydrogenated tallow diester quat with two $C_{18}H_{35}O$ tail units. A principal active component of the commercial DEEDMAC sample.

Principal active:



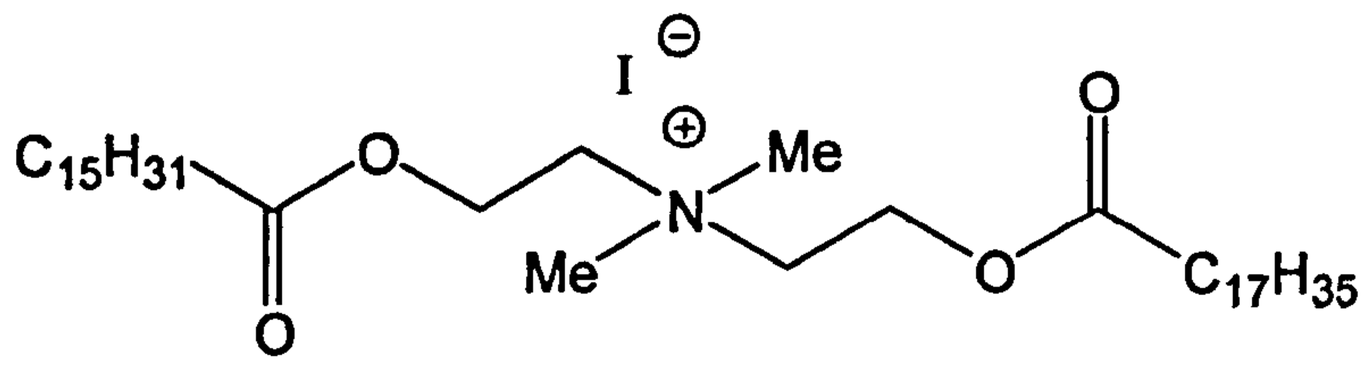
Chain length distribution (%): C_{18} / C_{18} > 95
Impurities present: Remainder is C_{18} / C_{16}

Source: Unilever Research

DEEDMAC C_{18} / C_{16} diester quat

General description: Hydrogenated tallow derived diester quat with one $C_{18}H_{35}O$ tail unit and one $C_{16}H_{31}O$ unit. A principal component of the commercial DEEDMAC sample.

Principal active:



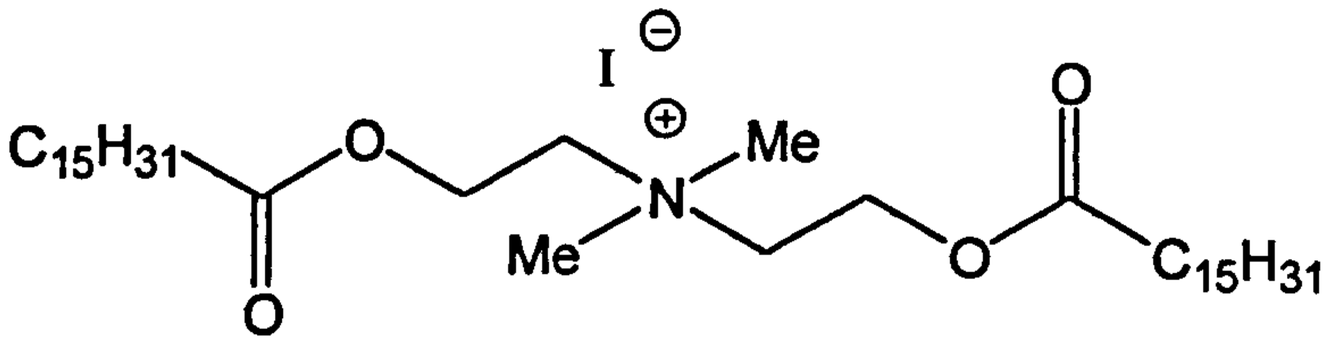
Chain length distribution (%): C_{18} / C_{16} > 95%
Impurities present: Remainder is C_{18} / C_{18} or C_{16} / C_{16}

Source: Unilever Research

DEEDMAC C_{16} / C_{16} diester quat

General description: Hydrogenated tallow derived diester quat with two $C_{16}H_{31}O$ units. A principal active component of the commercial DEEDMAC sample.

Principal active:



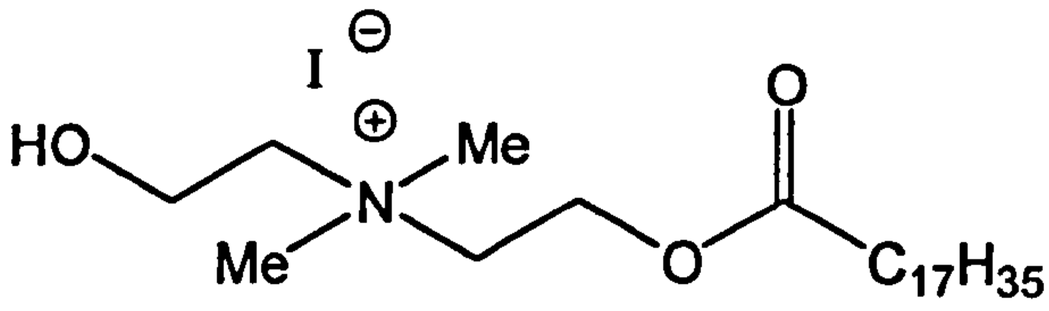
Chain length distribution (%): C_{16} / C_{16} > 95%
Impurities present: Remainder is C_{18} / C_{16}

Source: Unilever Research

DEEDMAC C_{18} monoester quat

General description: Hydrogenated tallow derived monoester quat with a $C_{18}H_{35}O$ tail. Active impurity present in the commercial DEEDMAC sample, and a biodegradation product of the parent diester species.

Principal active:



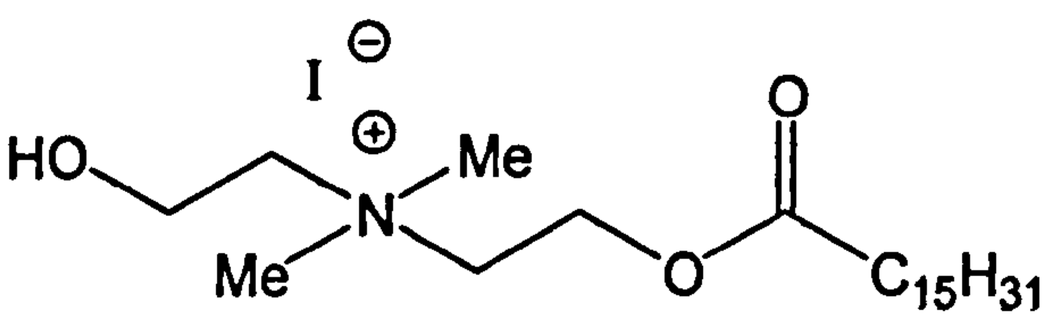
Chain length distribution (%): C_{18} > 98%
Impurities present: Remainder is C_{16}

Source: Unilever Research

DEEDMAC C_{16} monoester quat

General description: Hydrogenated tallow derived monoester quat with a $C_{16}H_{31}O$ tail unit. Active impurity present in the commercial DEEDMAC sample.

Principal active:



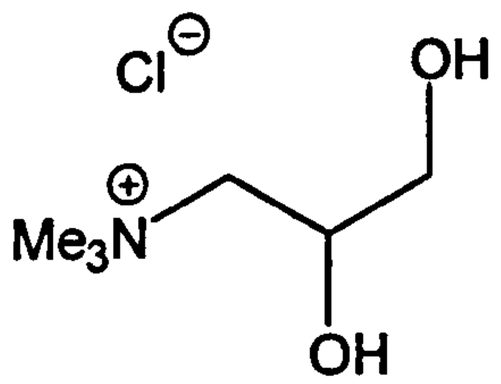
Chain length distribution (%): C_{16} > 98%
Impurities present: Remainder is C_{18}

Source: Unilever Research

Trimethylammonium propane-1,2-diol chloride (HEQ diol)

General description: Quaternary amino alcohol formed during the biodegradation of the HEQ derived monoester quat.

Principal active:



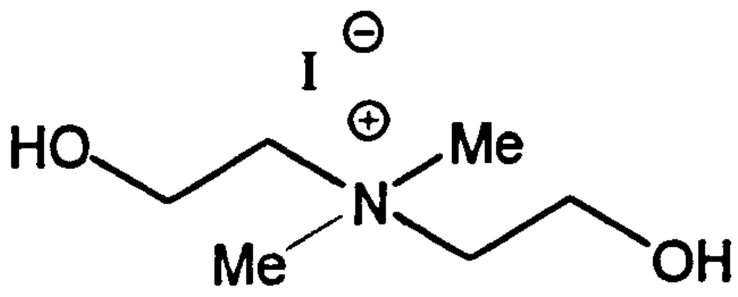
Impurities present: Unknown

Source: Unilever Research

Diethanoldimethylammonium iodide (DEEDMAC diol)

General description: Quaternary amino alcohol formed from the breakdown of the DEEDMAC derived monoester quat.

Principal active:



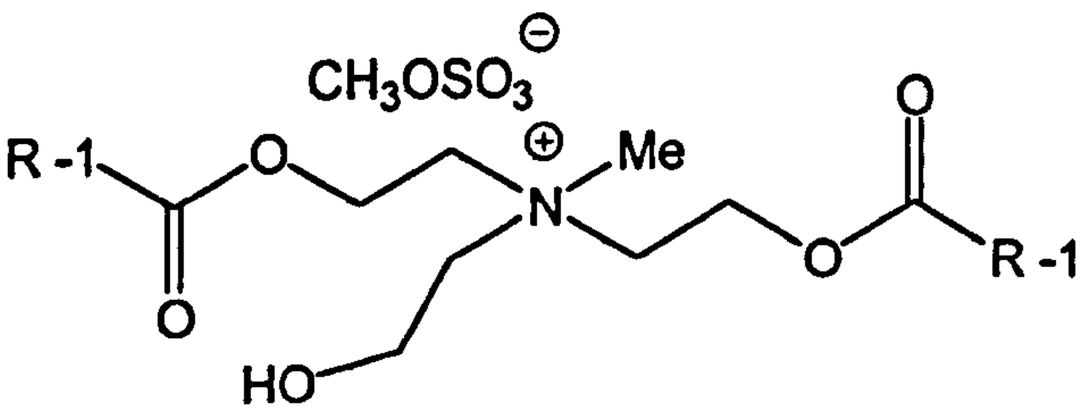
Impurities present: No information

Source: Unilever Research

Stepantex

General description: Tallow derived diester quat. Used as the principal active agent in some modern fabric conditioners formulations.

Principal active:



Chain length distribution (%): $C_{18} \approx 19$, $C_{18:1} \approx 39$, $C_{18:2} \approx 5$, $C_{16} \approx 26$, $C_{16:1} \approx 4$, $C_{14} \approx 2$

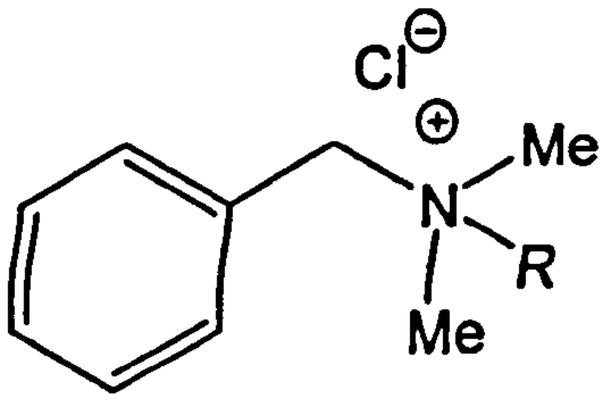
Impurities present: Triester, monoester, triol and free fatty acids are known to be present but the levels are unknown

Source: Unilever Research

Benzalkonium chloride

General description: Commercial alkyl benzyl quat derived from coconut oil. Samples of this kind are used as preservatives in personal care products and pharmaceutical preparations.

Principal active:



Chain length distribution (%): $C_{12} \approx 67$, $C_{14} \approx 32$

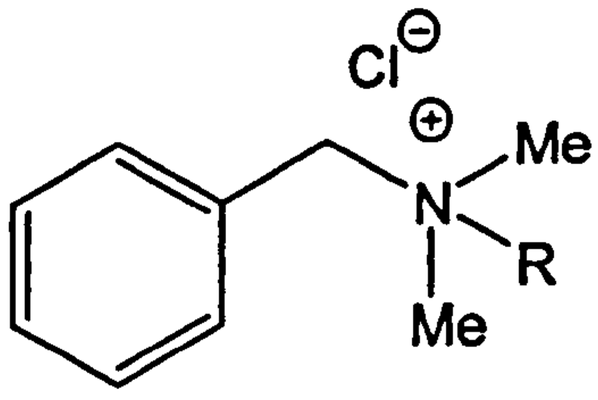
Impurities present: Traces of C_{16} and C_{10} are also present.

Source: Sigma-Aldrich

Querton KKBCL

General description: Commercial alkyl benzyl quat derived from coconut oil. Often used as a preservative in personal care products and pharmaceutical preparations.

Principal active:



Chain length distribution (%): $C_{16} \approx 1$, $C_{14} \approx 24$, $C_{12} \approx 71$, $C_{10} \approx 3$

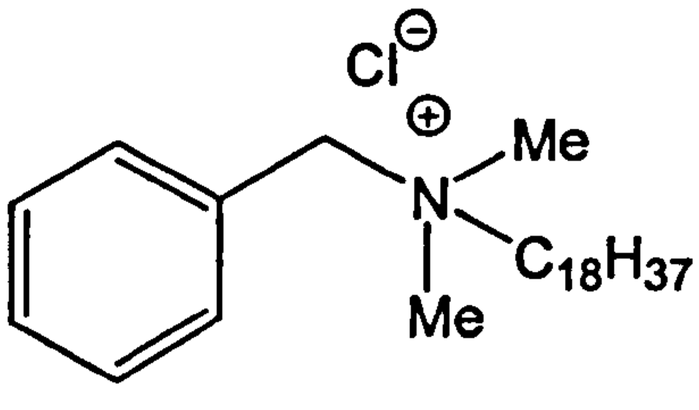
Impurities present: Traces of C_{18} are also present.

Source: Unilever Research

Benzyldimethylstearylammonium chloride dihydrate

General description: Alkyl benzyl quat with a $C_{18}H_{37}$ alkyl chain.

Principal active:



Chain length distribution (%): $C_{18} \approx 90$

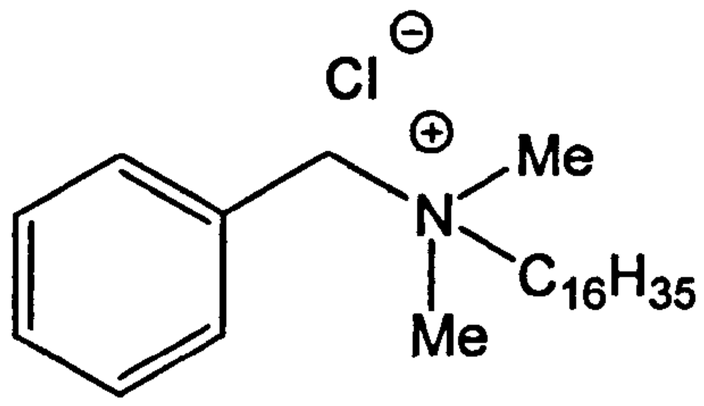
Impurities present: Remainder is C_{16}, C_{14} and C_{12}

Source: Sigma-Aldrich

Benzylcetyldimethylammonium chloride monohydrate

General description: Alkyl benzyl quat with a $C_{16}H_{33}$ alkyl chain.

Principal active:



Chain length distribution (%): $C_{16} > 95$

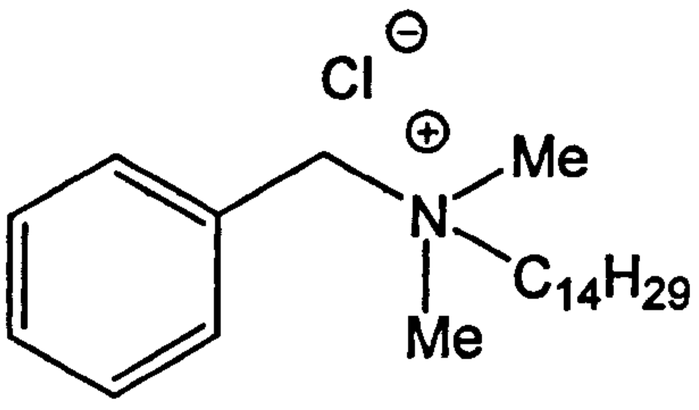
Impurities present: Remainder is C_{18}

Source: Sigma-Aldrich

Benzyldimethyltetradecylammonium chloride dihydrate

General description: Alkyl benzyl quat with a $C_{14}H_{29}$ alkyl chain.

Principal active:



Chain length distribution (%): $C_{14} > 99$

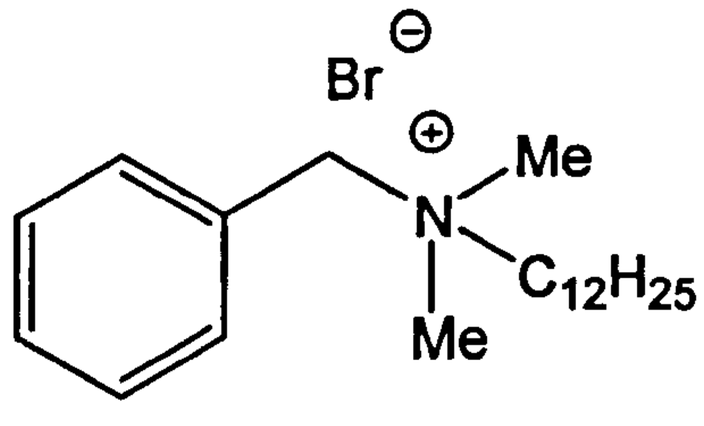
Impurities present: Remainder is C_{12}

Source: Sigma-Aldrich

Benzyldimethyldodecylammonium bromide

General description: Alkyl benzyl quat with a $C_{12}H_{25}$ alkyl chain. Analogous to the principal species in the commercial benzalkonium chloride sample

Principal active:



Chain length distribution (%): $C_{12} \approx 97$

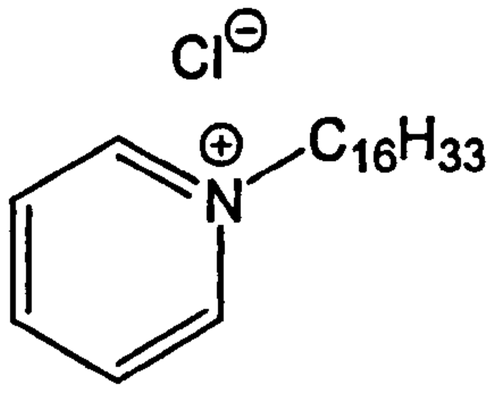
Impurities present: Remainder is C_{14}

Source: Sigma-Aldrich

Cetylpyridinium chloride monohydrate

General description: Alkyl pyridinium quat with a $C_{16}H_{29}$ alkyl chain. Pyridinium quats are utilised as preservatives and antibacterial agents in some personal care products.

Principal active:



Chain length distribution (%): $C_{16} = 98$

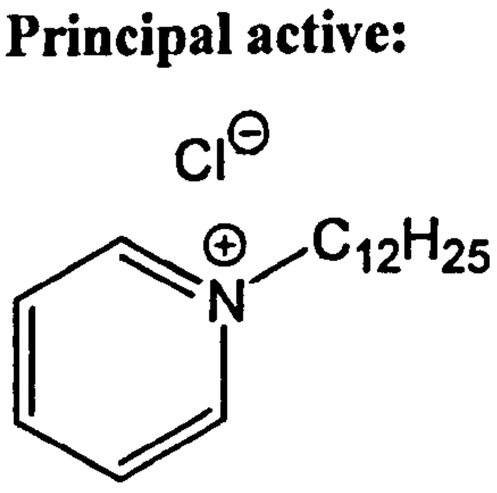
Impurities present: Unknown

Source: Sigma-Aldrich

1-Dodecylpyridinium chloride hydrate

General description: Alkyl pyridinium quat with a $C_{12}H_{29}$ alkyl chain.

Principal active:



Chain length distribution (%): $C_{16} = 98\%$

Impurities present: Unknown

Source: Sigma-Aldrich

REFERENCES

EcoLIMS, *SEAC Environment Ecotoxicology LIMS*, **1998,** Unilever Research Port Sunlight.

Lawrence J.G., *Personal communication*, **1999,** University of Leeds.

Sigma-Aldrich, *Online Product Reference Catalogue and MSDS Database*, <http://www.sigma-aldrich.com>.

Appendices

APPENDIX TWO

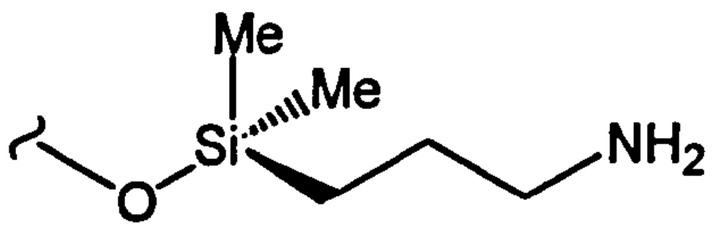
Column characteristics

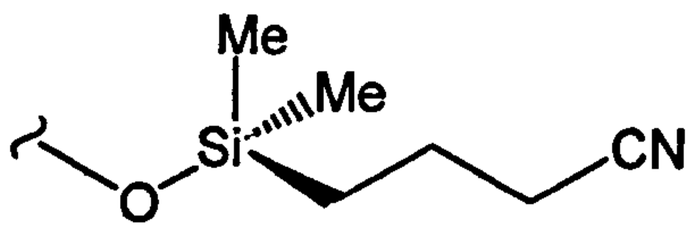
Partisil PAC

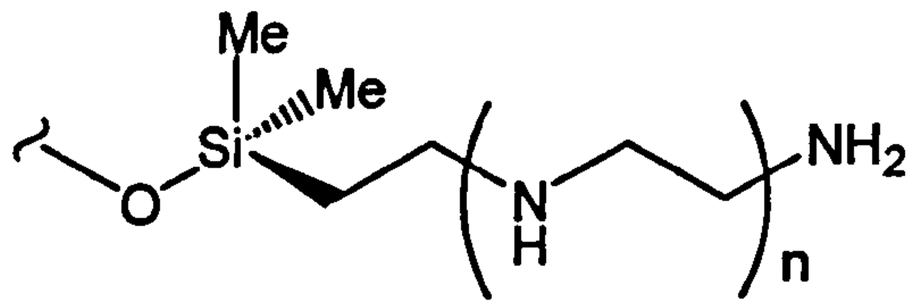
General: Mixed-mode alkylamino/cyanopropyl bonded irregular shaped silica produced by Whatman (Maidstone, UK).

Designed as a normal phase material, and acceptable for use as a L18 column following USP designation. Most current methods for analysing cationic fabric conditioner actives use this stationary phase (Wee *et al.*, 1982).

Bonded phase units:







Column dimensions: 250 × 4.6 mm i.d.

Particle size (μm) / pore size (Å) / surface area (m^2/g): 5 / 85 / 350

Carbon loading (%) / ligand coverage (μmol/m^2): 4.0 / 2.04

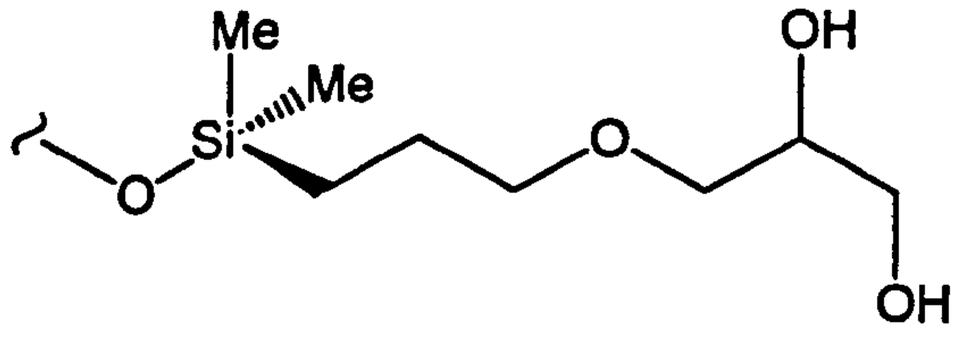
Source: Phase Separations

Bio-sil polyol

General: Poly-hydoxyl normal phase material that is claimed to offer higher selectivity for lipids than other NP materials.

This stationary phase has been used previously in the analysis of cationic fabric conditioner actives (Wilkes *et al.*, 1992).

Bonded phase unit: Unknown but thought to resemble a standard diol phase i.e.



Column dimensions: 250 × 4.6 mm i.d.

Particle size (μm) / pore size (Å) / surface area (m^2/g): 5 / 90 / unknown

Carbon loading (%): unknown

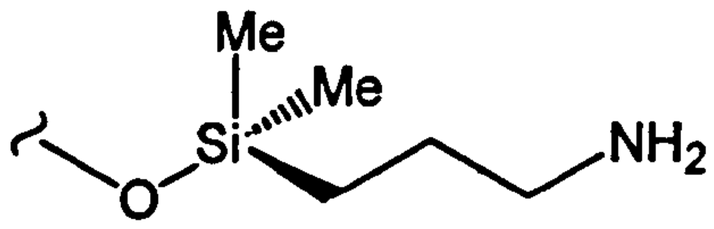
Source: Bio-Rad

Supercritically bonded mixed mode columns – Type A

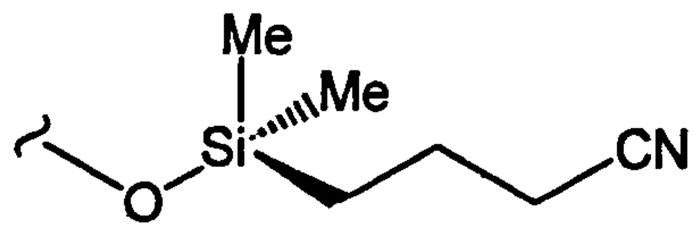
General: Mixed-mode aminopropyl/cyanopropyl bonded Spherisorb silica.

Manufactured at the University of Leeds by M.M. Robson to study the influence of the bonded phase units present on the Partisil PAC material on the resolution of the cationic fabric conditioner actives (Robson, 1998). The ratio of the amino/cyano functionalities was varied during the manufacturing process giving rise to columns M1, M2 and M3.

Bonded phase units:



A



B

	A	B
M1:	1	1.5
M2:	1	1
M3:	1.5	1

Column dimensions: 150 × 4.6 mm i.d.

Particle size (μm) / pore size (Å) / surface area (m^2/g): 5 / 80 / 200

Carbon loading (%): M1 = 2.8; M2 = 2.5; M3 = 2.1

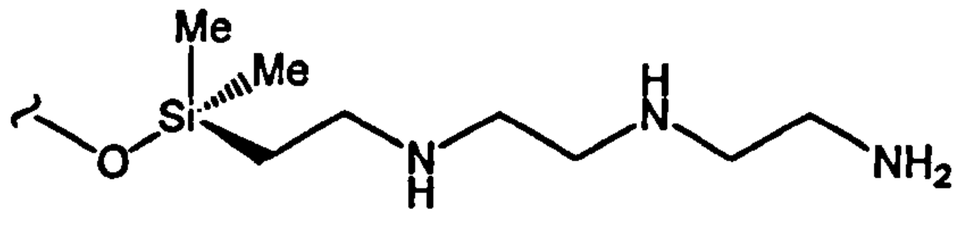
Source: University of Leeds

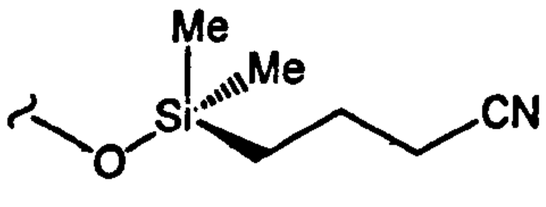
Supercritically bonded mixed mode columns – Type B

General: Mixed-mode aminopropyl / cyanopropyl bonded Spherisorb silica.

The ratio of the amino/cyano functionalities was varied during the manufacturing process giving rise to columns M4, M5 and M6.

Bonded phase units:





	Top: A	Bottom: B
M4:	1	1.5
M5:	1	1
M6:	1.5	1

Column dimensions: 150 × 4.6 mm i.d.

Particle size (μm) / pore size (Å) / surface area (m^2/g): 5 / 80 / 200

Carbon loading (%): M4 – 5.9

M5 – 5.7

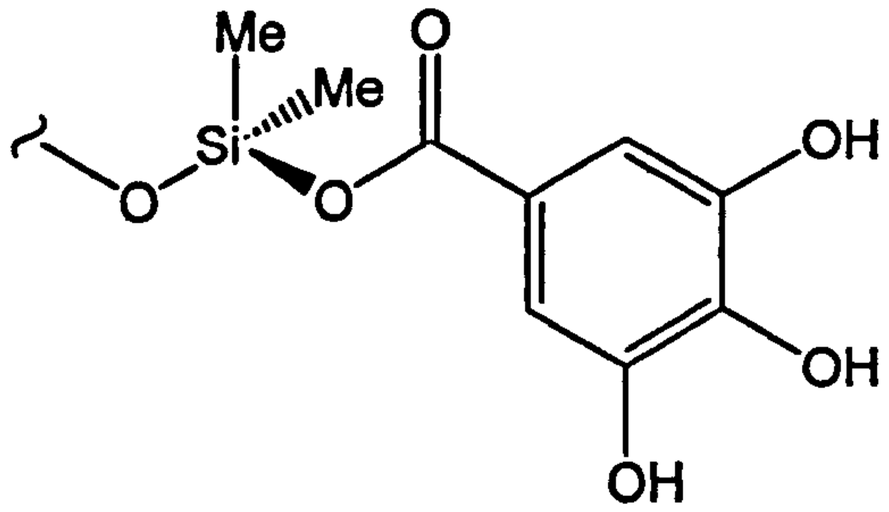
M6 – 5.3

Source: University of Leeds

Supercritically bonded gallic acid phase

General: Gallic acid-derived phase developed to test the resolution offered by a poly-hydroxyl / poly-phenol phase

Bonded phase units:



Column dimensions: 150 × 4.6 mm i.d.

Particle size (μm) / pore size (Å) / surface area (m^2/g): 5 / 80 / 200

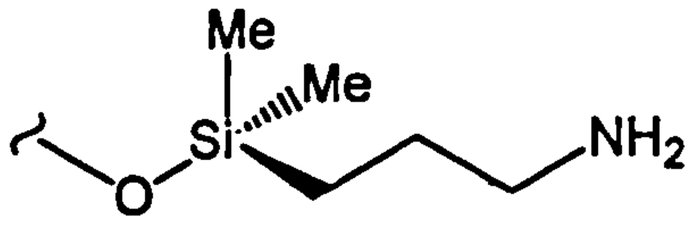
Carbon loading (%): 4.85

Source: University of Leeds

Spherisorb aminopropyl

General: Monofunctional aminopropyl bonded Spherisorb silica that meets the criteria for a USP L8 column. This column was employed as a normal phase support during this work.

Bonded phase unit:



Column dimensions: 250 × 4.6 mm i.d. and 150 × 4.6 mm i.d.

Particle size (μm) / pore size (Å) / surface area (m^2/g): 5,3 / 80 / 200

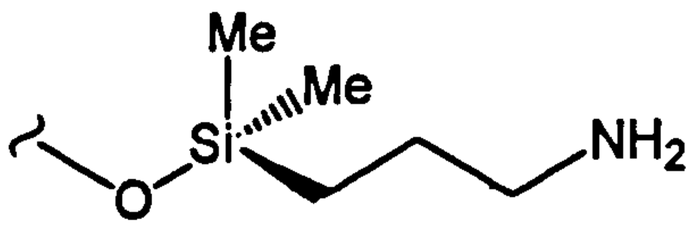
Carbon loading (%) / ligand coverage (μmol/m^2): 1.9 / 2.64

Source: Phase Separations

Sphereclone aminopropyl

General: Direct alternative to Spherisorb aminopropyl material. Manufacturer claims the phase offers equivalent resolution to the Spherisorb material.

Bonded phase unit:



Column dimensions: 150 × 4.6 mm i.d., 150 × 2.0 mm i.d. and 150 × 1.0 mm i.d.

Particle size (μm) / pore size (Å) / surface area (m^2/g): 3 / 80 / 200

Carbon loading (%): 2

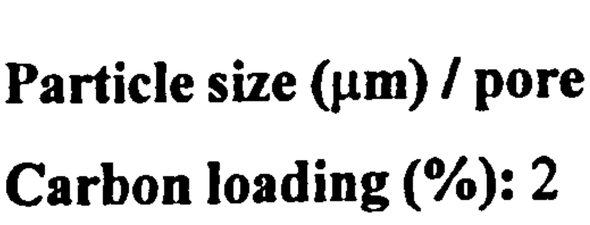
Source: Phenomenex

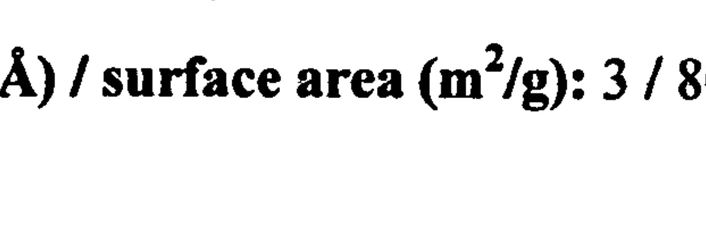
Spherisorb mixed mode octadecyl silane (ODS) / aminopropyl

General: Mixed-mode ODS/aminopropyl bonded Spherisorb silica.

Designed primarily as a reverse phase material the material was actually evaluated as a normal phase support during this work.

Bonded phase unit:





Column dimensions: 250 × 4.6 mm i.d.

Particle size (μm) / pore size (Å) / surface area (m^2/g): 5 / 80 / 200

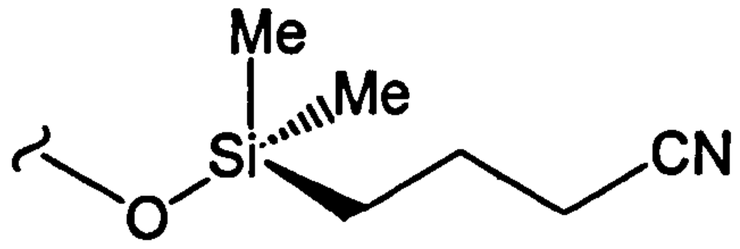
Carbon loading (%): unknown

Source: Phase Separations

Spherisorb cyanopropyl

General: Monofunctional cyanopropyl bonded Spherisorb silica that meets the criteria for a USP L10 column. This type of column was employed in both normal and reverse phase modes during the course of this work.

Bonded phase unit:



Column dimensions: 250 × 4.6 mm i.d. and 150 × 4.6 mm i.d.

Particle size (µm) / pore size (Å) / surface area (m^2/g): 5,3 / 80 / 200

Carbon loading (%) / ligand coverage (µmol/m^2): 3.1 / 3.29

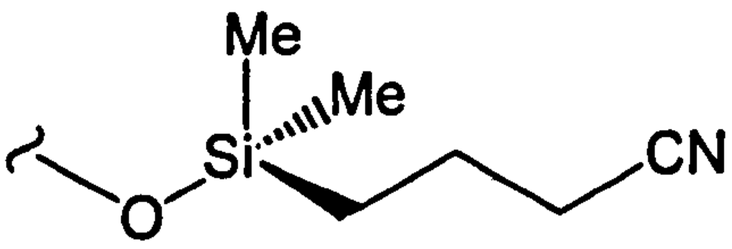
Source: Phase Separations

Luna cyanopropyl

General description: Cyanopropyl chemically bonded onto high purity Luna silica. Column meets the criteria for a USP L10 column.

This type of column was employed in the reverse phase mode during this work.

Bonded phase unit:



Column dimensions used: 150 × 4.6 mm i.d. and 150 × 2.0 mm i.d.

Particle size (µm) / pore size (Å) / surface area (m^2/g): 3 / 100 / 400

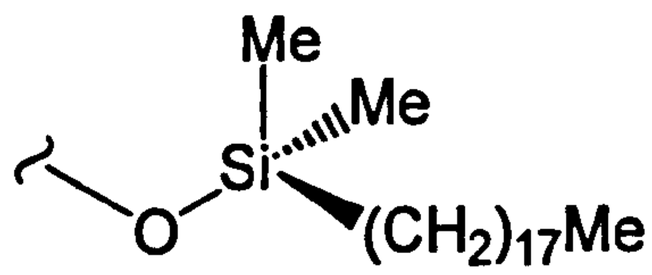
Carbon loading (%) / ligand coverage (µmol/m^2): 7 / 3.8

Source: Phenomenex

Spherisorb ODS 2

General description: Octadecylsilane (ODS) chemically bonded to porous Spherisorb silica. An end-capping group is present to reduce the number of free surface silanol groups on the silica support. This type of reverse phase material adheres to the USP criteria for a L1 stationary phase, and is the most common LC stationary phase currently in use.

Bonded phase unit:



Column dimensions used: 250 × 4.6 mm i.d.

Particle size (μm) / pore size (Å) / surface area (m^2/g): 5 / 80 / 200

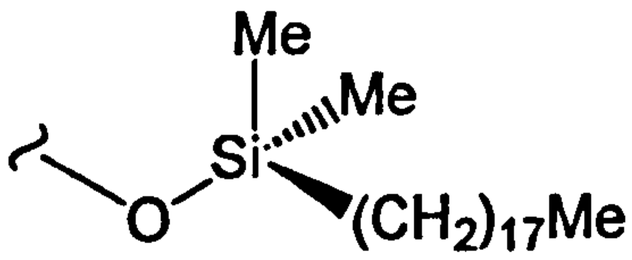
Carbon loading (%) / ligand coverage (μmol/m^2): 11.5 / 2.98

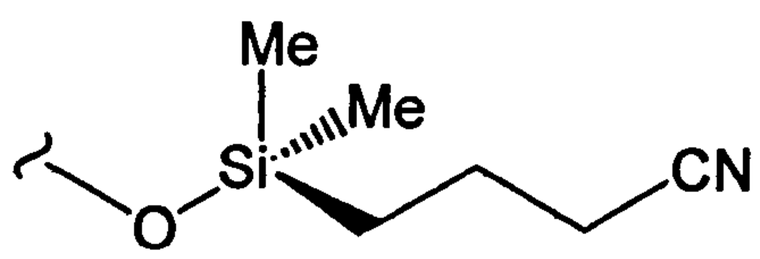
Source: Phase Separations

Spherisorb mixed mode ODS / cyanopropyl

General: Mixed-mode ODS/cyanopropyl bonded Spherisorb silica with an end-capping group that reduces the number of free surface silanol groups on the silica support.
Reverse phase material that offers increased polarity compared to a standard ODS bonded phase.

Bonded phase unit:





Column dimensions used: 250 × 4.6 mm i.d.

Particle size (μm) / pore size (Å) / surface area (m^2/g): 5 / 80 / 200

Carbon loading (%) / ligand coverage (μmol/m^2): 5 / 1.15

Source: Phase Separations

Spherisorb silica

General: Porous Spherisorb silica. This material meets the requirements of the USP L3 stationary phase notation.
Silica is primarily used as a normal phase material. However, it was evaluated as a reverse phase substrate during this work.

Bonded phase unit: None

Column dimensions: 250 × 4.6 mm i.d. and 150 × 4.6 mm i.d.

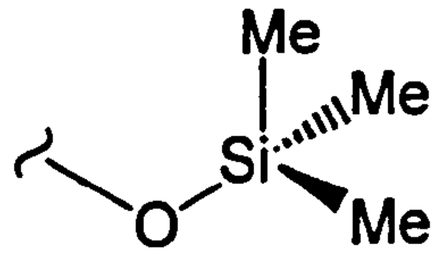
Particle size (μm) / pore size (Å) / surface area (m^2/g): 5 / 80 / 200

Source: Phase Separations

Spherisorb methyl

General description: Trimethylsilane chemically bonded to porous Spherisorb silica. This reverse phase material adheres to the USP criteria for a L13 stationary phase.

Bonded phase unit:



Column dimensions used: 250 × 4.6 mm i.d.

Particle size (μm) / pore size (Å) / surface area (m^2/g): 5 / 80 / 200

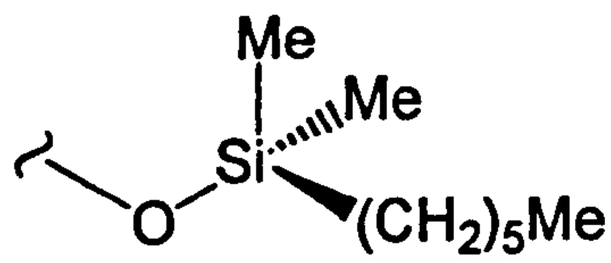
Carbon loading (%) / ligand coverage (μmol/m^2): 2.15 / 2.97

Source: Phase Separations

Spherisorb hexyl

General description: Hexylsilane chemically bonded to porous Spherisorb silica. An end-capping group is also bonded onto the silica support to reduce the number of free surface silanol groups. This reverse phase material adheres to the USP criteria for a L15 stationary phase.

Bonded phase unit:



Column dimensions used: 250 × 4.6 mm i.d.

Particle size (μm) / pore size (Å) / surface area (m^2/g): 5 / 80 / 200

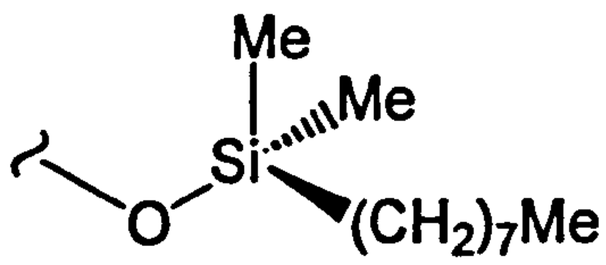
Carbon loading (%) / ligand coverage (μmol/m^2): 4.7 / 3.36

Source: Phase Separations

Spherisorb octyl

General description: Octylsilane chemically bonded to porous Spherisorb silica. An additional end-capping group is also present on the support to reduce the number of free surface silanol groups. This reverse phase material adheres to the USP criteria for a L7 stationary phase.

Bonded phase unit:



Column dimensions used: 250 × 4.6 mm i.d.

Particle size (μm) / pore size (Å) / surface area (m^2/g): 5 / 80 / 200

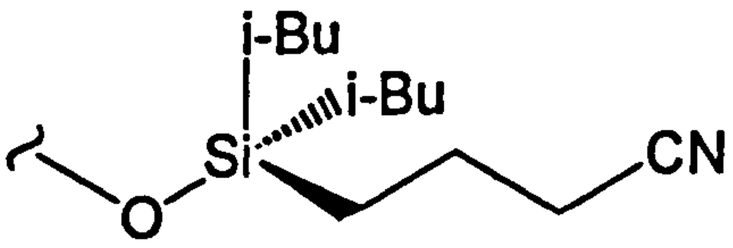
Carbon loading (%) / ligand coverage (μmol/m^2): 5.75 / 3.12

Source: Phase Separations

Zorbax stable bond cyanopropyl

General description: Monofunctional cyanopropyl bonded Zorbax silica manufactured by Agilent Technologies. This column was used in the reverse phase mode.

Bonded phase unit:



Column dimensions used: 150 × 4.6 mm i.d.

Particle size (μm) / pore size (Å) / surface area (m^2/g): 5 / 80 / 180

Carbon loading (%) / ligand coverage (μmol/m^2): 4 / 3.04

Source: Phase Separations

REFERENCES

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Wilkes A.J., Walraven G. and Talbot J.M., *J. Am. Oil Chem. Soc.*, **1992**, 69, 609-613.

Wee V.T. and Kennedy J.M., *Anal. Chem.*, **1982**, 54, 1631-1633.

APPENDIX THREE

Initial mass spectrometer parameters

Capillary Temperature (°C)	220.00
Source Voltage (kV)	3.7
Sheath Gas Flow	25.00
Auxiliary Gas Flow	0.00
Capillary Voltage (V)	3.00
Tube Lens Offset (V)	7.5
Octapole RF Amplifier (Vp-p)	400.00
Octapole 1 Offset (V)	- 4.00
Octapole 2 Offset (V)	- 8.00
Interoctapole Lens Voltage (V)	- 16.00
Trap DC Offset Voltage (V)	- 10.00
Maximum Ion Time (ms)	200.00
Ion Time (ms)	5.00
Source Type	Electrospray
Polarity	Positive
Zoom Micro Scans	10
Full Micro Scans	3
SIM Micro Scans	3