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THE CHARACTERISATION OF ENGINE OILS BY LIQUID CHROMATOGRAPHY WITH LUMINESCENCE DETECTION

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THE CHARACTERISATION OF ENGINE OILS BY LIQUID CHROMATOGRAPHY WITH LUMINESCENCE DETECTION

by

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ABSTRACT

THE CHARACTERISATION OF ENGINE OILS BY LIQUID CHROMATOGRAPHY WITH LUMINESCENCE DETECTION

SIMON WYNDHAM LEWIS

This thesis describes the development of pre column derivatisation reactions for the selective determination of organic functional groups in oxidised oils by liquid chromatography (LC) with luminescence detection. The testing of oils for oxidative stability and the current methods of oil analysis are reviewed in chapter one. An overview of LC and luminescence spectrometry, with particular regard to chemiluminescence (CL) is also presented.

Chapter two is a flow injection investigation of the experimental variables of the peroxyoxalate CL reaction. Two aryl oxalates; bis(2,4-dinitrophenyl)oxalate and bis(2,4,6-trichlorophenyl)oxalate were compared for their suitability to LC detection. A charge coupled device (CCD) was used to obtain the CL spectra of fluorophores and to investigate the CL emission background.

Chapter three and four describes procedures for the determination of carboxylic acids in oils by pre column esterification with 9-anthracenemethanol, reversed phase LC and either peroxyoxalate CL or fluorescence detection. The oils were dialysed prior to derivatisation to remove high molecular weight species which were found to interfere with the reaction. Carboxylic acids were present in the oxidised oils and their concentrations reflected the degree of degradation of the oils.

The determination of aldehydes in oxidised oils is described in chapter five. The aldehydes in oil dialysates are labelled by reductive amination with 3-aminofluoranthene and the derivatives separated by isocratic reversed-phase LC with peroxyoxalate CL detection using TCPO-hydrogen peroxide. Aldehydes were also found to be produced by the oxidation of oils in car engines.

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For Alexander

AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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SAC' 92, Reading, UK, 1992

Luminescence Spectrometry V, Gent, Belgium, 1993

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Date.....11/2/94.....

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Chapter 1

Introduction

1.1 OIL OXIDATION AND OIL ANALYSIS

1.1.1 The oxidation of engine oils

Modern automotive oil formulations consist of a complex mixture of hydrocarbons with a package of additives to enhance the oils performance. The main functions of an engine oil are:

Lubrication; preventing metal-to-metal contacts that lead to wear of engine components.

Cooling; assisting in removal of excess heat from the engine mechanism.

Sealing; preventing the blow back of combustion gases past the cylinders, pistons and rings.

Cleaning; dispersion within the oil of contaminants that would otherwise form harmful deposits within the engine.

Protection; preventing chemical attack upon the metal surfaces of the engine.

An oil formulation must be capable of performing these tasks in the extreme environment of the modern internal combustion engine [1]. However, the solubility of oxygen in oil, the presence of metals and the high operating temperatures give conditions which are conducive to the oxidation of the lubricating oil [2, 3]. Oil oxidation leads to the formation of sludges which degrade the performance of the oil by blocking filters, oil lines and lubrication grooves. This reduces the efficiency of lubrication and leads to excessive engine wear. It is therefore desirable to have a mechanistic understanding of the oxidation pathways, which necessitates analytical procedures for monitoring chemical parameters in the oils.

The oxidation of hydrocarbon based engine oils occurs by a radical chain mechanism [4-6]. After an induction period, the length of which depends on the oil formulation, initiation occurs. The overall oxidation mechanism is well known but the mechanisms for the initiation reaction have not been fully established. There is evidence that hydroperoxides are formed [6].

Oxidation then proceeds with the following propagation and termination reactions [5, 6];

Propagation;

$$R^{\bullet} + O_2 \rightarrow RO_2^{\bullet}$$

$$RO_2^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$$

Termination;

$$R^{\bullet} + R^{\bullet} \rightarrow R\text{-}R$$

$$R^{\bullet} + RO_2^{\bullet} \rightarrow ROOR$$

$$RO_2^{\bullet} + RO_2^{\bullet} \rightarrow ROOR + O_2$$

The primary products of the oxidation are oil soluble aldehydes, ketones and carboxylic acids. Polymerisation and condensation reactions also occur, leading to the formation of oil-insoluble compounds which precipitate from the oil as gums and sludges [2, 3, 7, 8].

The sludges from the oil, along with other contaminants e.g. metal particles, soot and water, increase the viscosity of the oil, degrading the lubricating action of the engine oil. The performance of the oil is further degraded by the sludges blocking filters, oil lines and lubrication grooves leading to increased wear [2]. Lacquers form on the engine walls interfering with heat transfer and engine components are corroded by the acid oxidation products [3, 9, 10].

The hydrocarbon mineral oil which is the backbone of an engine oil is obtained from crude oil by fractional distillation under vacuum. The vacuum distillate cannot be used directly as an engine oil as its ability to carry out the functions listed above would degrade rapidly. This is due to the presence of a variety of undesirable components [11] including acids and sulphur compounds which are corrosive, and asphaltenes, resins and waxes which assist the formation of sludges.

To improve the oxidative stability of the oil, the distillate is further refined by treatment with sulphuric acid or oleum (acid refining), extraction with selective solvents e.g. phenol, furfural (solvent refining) and catalytic hydrogenation (hydrotreating) [11].

In addition to undesirable compounds however, oils contain compounds that are useful e.g. natural oxidation inhibitors like thiophenes, sulphur containing heterocycles. Weakly refined oils are more prone to forming sludges than acids since the natural oxidation inhibitors are still present, while over refined oils have an increased acid-forming tendency as the inhibitors have been removed (Fig 1.1)[2].

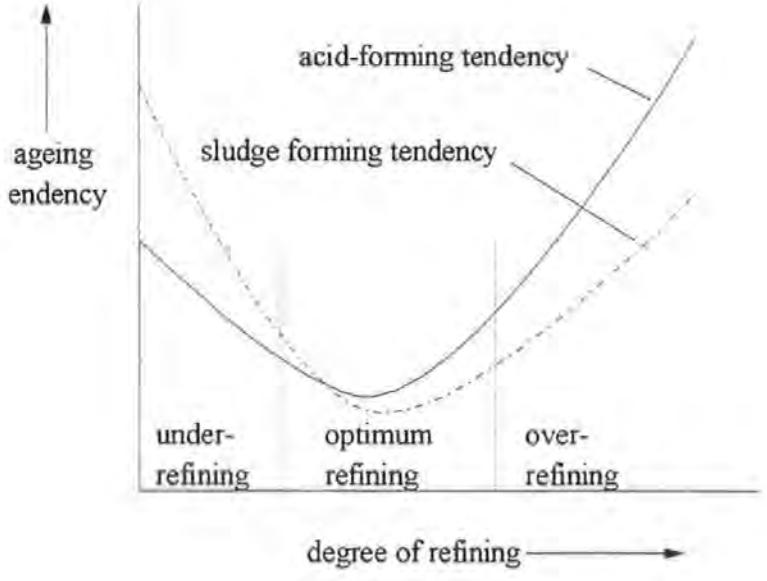


Fig 1.1 The effect of refining upon the oxidation of a hydrocarbon lubricant base oil

Several different base oils are blended together with an additive package to give an engine oil which carries out the functions listed above. Additives are generally classified by the property they are intended to treat, for example "anti-oxidant", "anti-foaming" (Table 1.1) [12]. It is important to note that many additives are multifunctional, e.g. a pour point depressant may also act as a viscosity index improver, and they can also have synergistic or antagonistic effects on each others properties.

Table 1.1 Lubricating oil additives		
Type	Reason for use	Typical compounds
Dispersants, detergents	Keep sludge and other deposit precursors suspended in oil	Neutral metallic sulphonates, polymeric detergents
Basic metal compounds	Neutralise acids, prevent corrosion from acid attack	Overbased metallic sulphonates and phenates
Oxidation inhibitors	Prevent or control oxidation of oil	Zinc dialkyldithiophosphates
Extreme pressure antiwear additives	Reduce wear by forming protective film on engine parts	Zinc dialkyldithiophosphates
Friction modifiers	Reduce or modify friction, improve fuel economy	Long chain polar compounds e.g. amides, phosphates,
Rust inhibitors	Prevent the formation of rust on metal surfaces by formation of surface film or neutralisation of acids	High base additives, sulphonates, phosphates, organic acids or esters, amines
Viscosity index improvers	Reduce the rate of viscosity change with temperature, allow easy cold starting	Polyisobutylene, methacrylate and acrylate polymers, olefin copolymers
Metal deactivators	Prevent metal surfaces catalysing oil oxidation	zinc diakyldithiophosphates
Pour point dispersants	Assure free flow at low temperatures	Low molecular weight methacrylate polymers
Antifoamants	Reduce foam in crankcase	Silicone polymers

1.1.2 Oil oxidation testing and analysis

Oil oxidation tests

The stability of an engine oil towards oxidation is an important parameter to determine for an engine oil, as other factors such as corrosion, wear and deposit formation depend on it. An engine oil which lacks oxidative stability will degrade rapidly and require frequent changes if damage to the engine is to be avoided.

The oxidation stability of a particular engine oil formulation is determined by engine testing [13-15]. As such tests are extremely expensive, laboratory based oxidation tests simulating engine conditions, are widely used to pre-screen oils [16-18]. Oils are oxidised and the degree of degradation measured by analysis of the oxidised residue of the oil sample.

Examples of such tests are the Institute of Petroleum (IP) Standards 280/89 [19], 307/93 [20], 335/93 [21] and the Sinclair Railroad Oil Oxidation Test [16]. These involve passing oxygen through the test oil, which may contain metal catalysts e.g. iron and copper, in an oxidation cell held at an elevated temperature. This treatment corresponds to the conditions that are found in the sump. Thin film tests, such as the Penn State Micro oxidation Test, have been developed to simulate the more extreme conditions found in "hot-spots" in the engines, such as piston rings and cylinder walls [7, 22]. These tests involve air oxidation of a thin film of oil , deposited on metal pellets or in a metal cup, at an elevated temperature [22].

Continuous monitoring of the oxidation stability of oils has been achieved using inverse phase gas chromatography (GC) [18]. Oxygen was passed over a GC support coated with the test oil, packed in a GC column and the oil condition was monitored by measurement of retention indices of a series of probe compounds. As oxidation of the oil proceeded it produced polar oxidation products which altered the retention characteristics of the oil coated support and increased the retention indices. Use of a GC allowed precise control over the temperature and the flow rate of oxygen.

Pressurised differential scanning calorimetry (PDSC) measures the net heat evolved as a result of exothermic and endothermic reactions in a sample as it is heated under controlled conditions. It has been applied to the simulation of deposit formation in engines. Oils were characterised and ranked according to their deposit forming tendencies [15].

Oil analysis

The degradation of an oil is measured by the acidity, viscosity, and carbon residue of the oxidised oil. These traditional approaches have been augmented by a variety of instrumental techniques which are summarised in. Table 1.2.

Carboxylic acids, aldehydes and ketones are among the primary products of oil oxidation in car engines. They are therefore important indicators of the degree of oxidation. Infra-red (IR) spectroscopy [23, 24] can give an indication of the presence of particular functional groups, gel permeation chromatography (GPC) [25, 26] can give an indication of the range of molecular weights of oxidation products and thin-layer chromatography with flame ionisation detection (TLC-FID) [27] the increase in polar oxidation products due to oxidation. None of these methods are selective for a particular group of products. More selective analysis of the polar primary products would provide useful information with which to elucidate the oxidation pathways, in relation to the operating characteristics of the oil. For routine application in a process environment, the procedures are required to be rapid with a minimum of sample pre-treatment.

Gas chromatography (GC) and liquid chromatography (LC) have been applied widely to the determination of organic species in non-aqueous media. GC has been used to determine carboxylic acids in crude oils edible oils [28, 29] and the determination of aldehydes in car exhausts [30]. However, many organic species are too thermally unstable or in-volatile to be analysed by GC without chemical modification (derivatisation) [31]. In addition, the analysis of used engine oils is aggravated by the presence of additives and the oil matrix, which would lead to poor resolution and complex chromatograms. LC has a number of advantages over GC for monitoring organic species in oils. In GC, separations are largely dependant on the type of stationary phase, while in LC they are also strongly effected by the wide choice of mobile phase. Analysis times are generally shorter and there are also a greater number of selective detectors available for LC, a separation therefore does not need to be complete if the detector is only monitoring the species of interest [31].

Table 1.2 Methods for the analysis of oxidised engine oils		
Method	Procedure	Application
Titrimetry	Sample dissolved in toluene-isopropyl alcohol-water and titrated with alcoholic potassium hydroxide. The endpoint is detected by a colour change of an indicator (*p*-naphtholbenzein which is orange in acid and green-brown in base)[32] or, as used oils are highly coloured, by potentiometry [33].	Results quoted as Total Acid Number (TAN), which is the quantity of potassium hydroxide (mg) required to neutralise all acidic constituents in 1 g of sample [34]. Standard method for determining acidity of an oil, e.g. in Penn State Micro-oxidation Tests [7, 22].
Thin-layer chromatography with flame ionisation detection (TLC-FID)	TLC carried out using a rod of silica stationary phase, rod is then passed through the flame of an FID [27].	A typical chromatogram showing increase in the polar fraction as a result of oxidation is shown in Fig. 1.2. When *n*-hexane is used as the mobile phase, the saturated hydrocarbon fraction elutes first, followed by the aromatic fraction, with the polar fraction remaining at the origin.
Infrared (IR) spectroscopy	Absorbance of IR by organic functional groups leads to characteristic spectra, difficult to interpret due to complexity of sample [23].	Has been used to monitor degradation of oils during oxidation by following additive depletion [23], also used to examine deposits on engine surfaces [24].

Table 1.2 (continued) Methods for the analysis of oxidised engine oils		
Method	Procedure	Application
Chemiluminescence (CL)	CL is generated during the oxidation of hydrocarbon oils from the recombination of peroxy radicals [6, 22]; $RO_2^{\bullet} + RO_2^{\bullet} \rightarrow RO^{*} + ROH + O_2$ The excited state ketone, RO*, relaxes to the ground state emitting light. The low quantum efficiency of the reaction (10^{-7}-10^{-8}), due to deactivation and matrix quenching, can be improved by addition of energy transfer agents e.g. dibenzoanthracene [13]. Instrument consists of heated reaction cell in a light-tight box with a light detector e.g. photomultiplier tube.	CL has been used to probe the oxidation mechanism, as the square of the concentration of the peroxy radicals is proportional to the intensity of CL [22]. It has also been shown that there is a correlation between the amount of sample oxidised and the maximum intensity of CL [13]. CL cannot be used to directly compare oxidation rates of different oils, as the reactions giving CL may be suppressed by additives present [5, 22].
Electrical conductivity	Electrical conductivity measured with a probe, direct current measured with an electrometer [35]. Designed for field testing.	Electrical conductivity has been shown to correlate with TAN and has been suggested as the basis for a real-time sensor for monitoring oil condition [35].
Gel permeation chromatography (GPC)	GPC separates mixtures according to molecular size.	GPC has been used to study the oil insoluble oxidation products in used engine oils [25, 26].

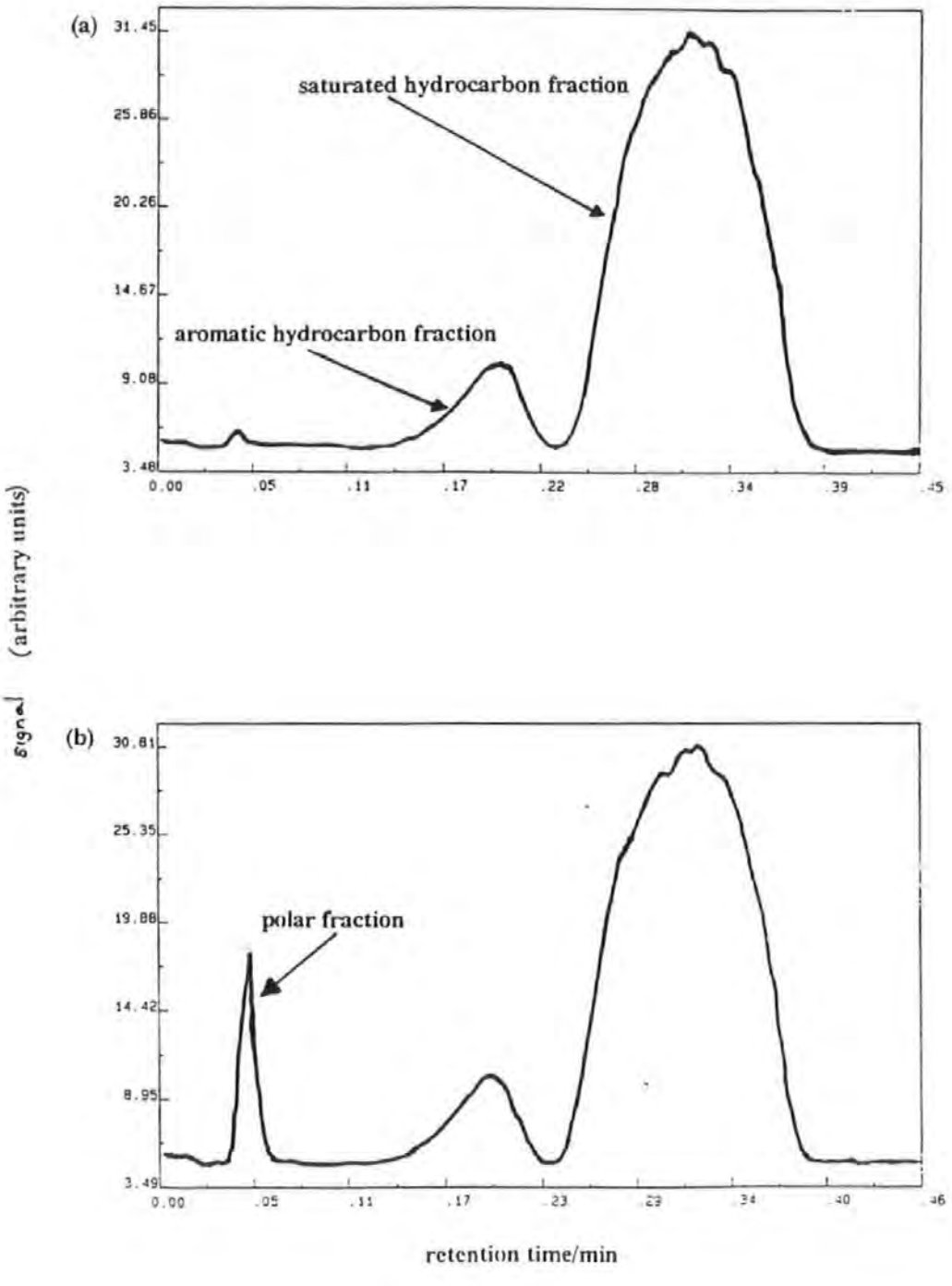


Fig 1.2 TLC-FID traces of an engine oil
(a) before oxidation (b) after oxidation
n-hexane mobile phase, silica stationary phase, the time axis denotes the time for the TLC rod to pass through the flame of the FID

1.2 LUMINESCENCE SPECTROMETRY

1.2.1 The scope of luminescence spectrometry

Luminescence spectrometry encompasses a range of techniques, all of which involve the emission of ultraviolet (UV), visible or near infrared (NIR) radiation from an electronically excited state. The means by which this excited state is reached defines the type of luminescence occurring, as indicated in Table 1.3. Luminescence measurements have found widespread application to the selective and sensitive determination of molecular species.

The major types of luminescence most widely used for analytical purposes are fluorescence, phosphorescence, chemiluminescence and bioluminescence. At present fluorescence methods far outnumber other luminescence procedures. The theory and applications of luminescence spectrometry have been reviewed in a number of publications [36-40]

Luminescence methods are analytically attractive due to their inherent sensitivity and wide dynamic ranges. Balanced against this is their low applicability compared with absorbance methods due to the relatively limited number of species that show luminescence. This means that the use of luminescence for detection in LC often requires the derivatisation of the analyte species.

1.2.2 Photoluminescence

The electronic transitions occurring in a molecule after absorption of UV or visible radiation can be represented by a molecular energy level diagram (Fig 1.3).

The occupied orbitals of most organic compounds in the ground state contain a pair of electrons. According to the Pauli Exclusion Principle each of the electrons has opposing spin, this condition is known as a singlet state. On absorption of UV or visible radiation the molecule is raised to an excited singlet state. This transition is fast enough (10^{-15} s) for the nuclei of the molecule to be considered stationary (Franck-Condon Principle).

Table 1.3 Nomenclature of luminescence	
Luminescence	The emission of UV, visible or NIR radiation from a molecule or an atom resulting from the transition of an electronically excited state to a lower energy state (usually the ground state).
Photoluminescence	Luminescence in which the mode of excitation is the absorption of UV, visible or NIR radiation.
Fluorescence	Short lived (10^{-9}-10^{-7} s) photoluminescence from a singlet electronically excited state.
Phosphorescence	Long lived photoluminescence (10^{-4}-10 s) from a triplet electronically excited state.
Chemiluminescence	Luminescence in which the electronically excited state is produced by a chemical reaction.
Bioluminescence	A type of chemiluminescence in which visible radiation is emitted from living organisms.
Electrochemiluminescence	A type of chemiluminescence, occurring in solution, in which the electronically excited state is produced by high energy electron transfer reactions. Also known as electrogenerated chemiluminescence.
Thermoluminescence	Luminescence from solids on mild heating.
Pyroluminescence	Luminescence from metal atoms in flames.
Candoluminescence	Luminescence from incandescent solids.
Cathodoluminescence	Luminescence arising from irradiation by β particles.
Anodoluminescence	Luminescence arising from irradiation by α particles.
Radioluminescence	Luminescence arising from irradiation by γ- or X-rays.
Electroluminescence	Luminescence from electrical discharges.
Sonoluminescence	Luminescence arising from exposure to ultrasonic sound waves in solution.
Triboluminescence	Luminescence arising from structural rearrangements in solids e.g. by crushing.

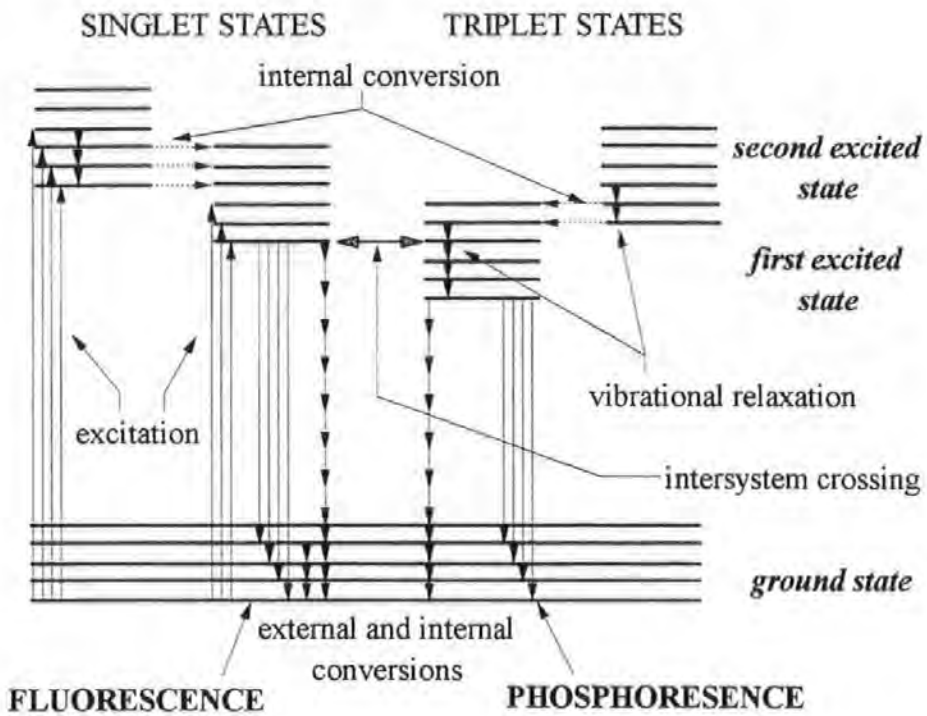


Fig 1.3 Partial energy level diagram for a photoluminescent molecule

Relaxation of an excited species can occur by several mechanistic pathways, both radiative and non-radiative. The most favoured route is that which keeps the lifetime of the excited state species to a minimum. For photoluminescence to be observed, deactivation by radiative pathways must be rapid with respect to the non-radiative pathways.

Fluorescence

For fluorescence the molecule first relaxes to the lowest vibrational energy level within the lowest singlet excited state (10^{-12} s) before relaxing to the ground state, emitting photons. As this process does not involve a change in spin it is termed an allowed transition and occurs rapidly, the lifetime of a singlet excited state is of the order of 10^{-9}-10^{-7} s.

In the simplest type of fluorescence, exhibited by dilute atomic vapours, the excited state relaxes to the lowest vibrational level in the ground state with the radiation being emitted without alteration of wavelength (resonance fluorescence). For

molecules in solution the emitted radiation for a particular transition is displaced to a higher wavelength. This displacement is known as the Stokes Shift and is due to the loss of vibrational and rotational energy by collision of the excited state molecules with solvent and solute molecules.

Non-radiative transitions that compete with fluorescence are internal conversion, external conversion and inter system crossing. Internal conversion is a very efficient deactivation pathway and occurs if two energy levels are close enough for vibrational energy levels to overlap, the exact processes involved are not clearly understood but probably involve vibrational relaxations. External conversion is caused by energy transfer between the excited state molecule, solvent molecules and other solute molecules.

Inter system crossing describes the non-radiative transfer from singlet excited state to a triplet excited state, which is where the spins of the electron pair are parallel. As it involves a change in spin, this process is spin-forbidden, however it can take place due to spin orbital coupling. This is enhanced by molecules containing heavy atoms e.g. bromine and iodine (the heavy atom effect) and paramagnetic species e.g. molecular oxygen.

Phosphorescence

Phosphorescence arises from relaxation to the ground state from a triplet excited state. As this is a forbidden transition it has a low probability of occurring. Because of this, the rate of relaxation for phosphorescence is slow with lifetimes in the range 10^{-4} to 10 s. Excitation to the triplet excited state from the ground state rarely happens, the singlet excited state passes to a lower triplet excited state by inter system crossing. As the lowest triplet excited state has a lower energy than the lowest singlet excited state, phosphorescence is observed at longer wavelengths than fluorescence.

The non-radiative deactivation processes are very fast in relation to the rate of deactivation through phosphorescence and unless they are slowed down phosphorescence is not usually observed. Analytically useful phosphorescence can

be achieved by cooling to very low temperatures (< 77 K)and by the use of viscous media, micellar media or adsorption onto solid surfaces to trap the excited state molecule [41, 42].

1.2.3 Variables effecting photoluminescence

The efficiency of a fluorescent process can be described by the term fluorescence quantum efficiency (quantum yield), Φ_F, the fraction of excited state molecules which produce a photon. In the absence of photochemical reactions;

$$\Phi_F + \Phi_P + \Phi_I = 1$$

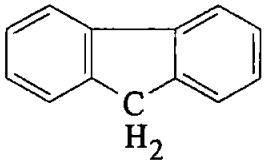
where Φ_P and Φ_I are the quantum efficiencies for phosphorescence and non-radiative processes respectively.

In most fluorescent compounds, emission arises from π bonding, π anti bonding (π-π^*) transitions, although emission is possible from n-π^* transitions. Transitions of the type π-π^* have greater quantum efficiencies than n-π^* transitions as they have shorter lifetimes and competing deactivation processes are less likely to occur. Hence compounds exhibiting the most intense fluorescence will contain low energy π-π^* systems, the largest group of these are aromatic compounds. A small number of aliphatic and alicyclic carbonyl structures and species with highly conjugated double bond structures are also fluorescent.

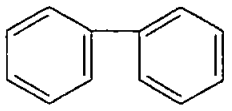
Most unsubstituted aromatic hydrocarbons are fluorescent in solution, the fluorescence quantum efficiency increasing with the number of rings and the degree of condensation. Substitution on the aromatic rings will cause shifts in emission wavelength and effect the intensity of fluorescence. Electron donating substituents, e.g. -OR and $-NR_2$, enhance fluorescence intensity, while electron-withdrawing substituents, e.g. $-NO_2$, -COOH and halogens decrease the intensity observed emission. A summary of the effects of substituents on luminescence is given by Hurtubise [36].

Structural rigidity has a strong effect on fluorescence, with emission from rigid molecules being favoured e.g. when the quantum efficiencies of fluorene and

biphenyl are measured under similar conditions they are found to be 1.0 and 0.2 respectively.



fluorene



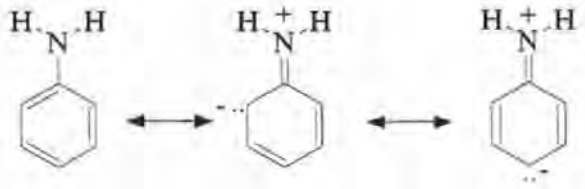
biphenyl

The increased rigidity of the fluorene is supplied by the bridging methylene group [43].

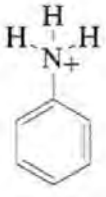
The environment of a fluorescent species has a great effect upon the intensity of emission observed. Increasing temperature and decreasing solvent viscosity increase the likelihood of deactivation by external conversion, due to the higher frequency of collisions between solvent molecules and the excited species. Halogenated solvents e.g. carbon tetrabromide and ethyl iodide cause an increase in inter system crossing to the triplet excited state due to the heavy atom effect, giving decreased fluorescence. Dissolved oxygen also assists non-radiative deactivation by inter system crossing, it may also quench fluorescence by photochemically induced oxidation of the fluorescent species.

The polarity of the solvent can also have an effect on luminescence, molecules having lowest excited singlet states of the n-π^* type e.g. quinoline and acridine, rarely fluoresce in non-polar solvents while in polar solvent e.g. ethanol, hydrogen bonding leads to the lowest excited state being of the π-π^* type and the molecules exhibit fluorescence [44]. Micellar media can enhance fluorescence by shielding the excited state species from deactivation processes [45].

The fluorescence of acidic or basic fluorescent compounds will depend on the pH, e.g. aniline and its ionised form, the anilinium ion.



resonance forms of aniline



anilinum ion

The resonance forms of aniline lead to a more stable excited state, due to increased conjugation and the species is fluorescent, the anilinum ion has only one structure, which is not highly conjugated and is not fluorescent [43].

1.2.4 Luminescence spectra

Conventional fluorescence and phosphoresence spectra are determined by measuring the intensity of luminescence relative to wavelength at a fixed excitation wavelength. Excitation spectra are similarly determined at a fixed emission wavelength (Fig 1.4). Molecular luminescence spectra are broad due to the large number of electronic transitions taking place. "Synchronous" scanning with a fixed wavelength difference between the excitation and emission monochromators is possible and has been shown to give spectra that are simpler, narrower and therefore more characteristic than conventional spectra [46].

1.2.5 Chemiluminescence

Chemiluminescence (CL) is luminescence in which the source of excitation is a chemical reaction. It can occur in the gas-, liquid- or solid-phase. In its simplest form a CL reaction can be represented by;

$$A + B \rightarrow P^{*} \rightarrow P + LIGHT$$

where P* is an excited state product (direct CL). In some cases the excited state product is either weakly or non-fluorescent and the emission is enhanced by addition of a sensitiser, which becomes the emitting species after energy transfer;

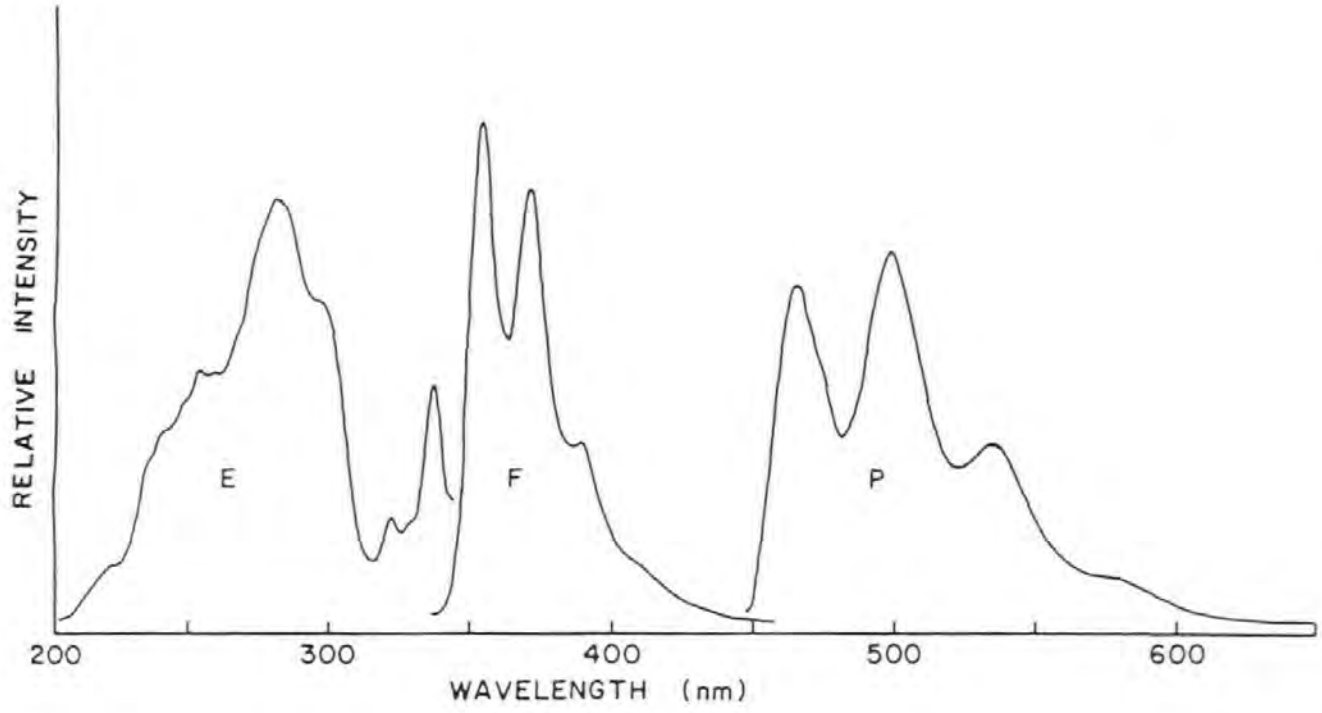
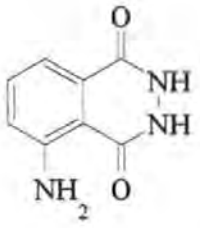


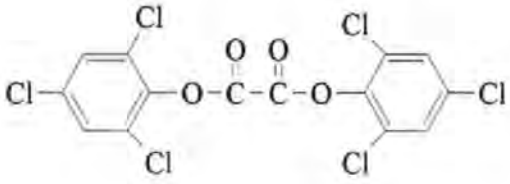
Fig 1.4 Examples of excitation and emission spectra for a fluorophore (benzo[*f*]quinoline in ethanol [36] E; excitation, F; fluorescence and P; phosphoresence

$$A + B \rightarrow P^* + F \rightarrow P + F^* \rightarrow P + F + LIGHT$$

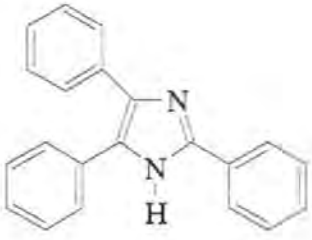
where F is the sensitiser. This indirect CL is also called energy transfer or sensitised CL [47]. If the CL reaction involves a living organism, the emission is referred to as bioluminescence (BL). CL can also be produced from electrochemical reactions, electrogenerated CL [48]. As yet this is of restricted analytical application and so is not considered here. The structures of a selection of chemiluminescent compounds is presented in Fig 1.5.



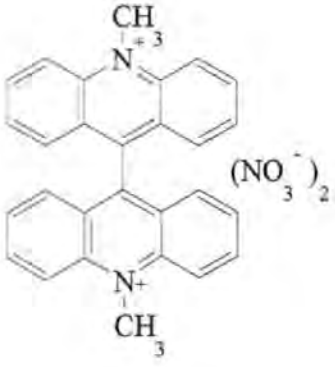
Luminol



Bis(2,4,6-trichlorophenyl)oxalate



Lophine (2,4,5-triphenylimidazole)



Lucigenin (bis-*N*-methylacridinium nitrate)

Fig 1.5 A selection of chemiluminescent compounds

For the generation of CL in the visible region of the spectrum the following conditions must be met;

1. There should be an energetically favourable reaction pathway for the production of the excited state species. Of the total number of molecules participating in the reaction a significant number should reach the excited state.

2. The reaction is required to be exothermic, with the free energy change being in the range 170-300 kJ mol^{-1}.

3. There should be a favourable deactivation pathway for CL emission. In this regard the competing processes are the same as those for molecular fluorescence.

The energy necessary to generate a photon of light of a particular wavelength may be calculated using the Planck-Einstein equation [49];

$$E = hc/\lambda$$

where E is the energy of the photon (J), h is Planck's constant (6.63 x 10^{-34} J s), c is the velocity of light (3 x 10^{-8} m s^{-1} in a vacuum) and λ is the wavelength of light (m). Therefore for green light (λ = 500 nm);

$$E = L \cdot hc/5\times10^{-7} = 240 \text{ kJmol}^{-1}$$

where L is Avagadro's constant (6.023 x 10^{23}).

For red light (650 nm) and blue light (425 nm) it can be shown that the energy required is 184 kJ mol^{-1} and 281 kJ mol^{-1} respectively. Therefore for a chemical reaction to produce luminescence in the visible region (400-700 nm) it must supply energy, i.e. be exothermic, with a free energy change in the range 170-300 kJ mol^{-1}.

The intensity of CL emission from a reaction is dependant upon the rate of reaction and the efficiency of the process generating excited state species. The latter can be described by the CL quantum efficiency, Φ_{CL}, which is defined as;

Φ_{CL} = (total number of photons emitted)/(number of molecules reacting)

It is the product of the efficiencies of the excitation Φ_{EX} and emission Φ_F;

$$\Phi_{CL} = \Phi_{EX} \cdot \Phi_F$$

CL quantum efficiencies vary widely from 10^{-15} to nearly 1, however most of the reactions used in analysis fall in the range 0.01-0.1 [47]. The use of very sensitive detectors and the almost complete absence of background emission has allowed the

monitoring of even inefficient CL reactions, e.g. ultra weak CL, that is systems with quantum efficiencies < 0.001 [50].

The quantum efficiency and colour of a CL emission are greatly affected by the environment in which the reaction takes place. For solution phase CL the factors that will effect the reaction are similar to those affecting normal fluorescence. For example the CL quantum efficiency and colour of emission for luminol in dimethyl sulphoxide and water are 0.05, blue-violet (425 nm) and 0.01, blue-green (480-502 nm) respectively [51]. Table 1.4 presents the properties of a number of CL reactions in the gas-, liquid- and solid-phase [47].

For analytical purposes it is the CL emission intensity (I_{CL}) that is measured, either as an integral over the lifetime of the emission or as a transient response. It is a function of both the efficiency and the rate of the reaction;

$$I_{CL} = \Phi_{CL}(dC/dt)$$

where dC/dt is the rate of reaction (molecules reacting s^{-1}). CL reactions can occur very rapidly (<1 s) or extremely slowly (> 1 day), according to the reaction and the conditions. CL reactions used analytically tend to be very fast.

1.2.6 The application of CL reactions to chemical analysis

CL and BL reactions have gained wider acceptance for analytical applications, as illustrated by recent reviews [51-55] and conference proceedings [56-58]. Until recently the lack of commercially available instrumentation and reagents has restricted the widespread application of CL.

In general the most successful analytical application area for liquid phase CL reactions to date has been the biomedical field, particularly as a means of detection in immunoassay and for the direct analysis of various species of clinical interest by BL reactions. Gas phase reactions have found application in the determination of sulphur and oxides of nitrogen by their reaction with ozone [59, 60] and as the basis for sulphur- and phosphorus-selective gas chromatography detectors [60]. (Gas phase CL reactions are not discussed here).

Table 1.4 Properties of chemiluminescent reactions		
Reaction	Colour (λ_{max})	Quantum yield
Solid		
Rubrene peroxide dissociation by heat	red	
Oxidation of siloxene	red	
Liquid		
Oxidation of *p*-chlorophenyl magnesium bromide (Grignard reagent)	475 nm	10^{-6}-10^{-8}
Oxidation of luminol in dimethyl sulphoxide	blue-violet (480-502 nm)	0.05
Oxidation of luminol in aqueous alkali	blue (425 nm)	
Oxidation of lucigenin in alkaline H_2O_2	blue-green (440 nm)	0.016
Oxidation of lophine in alcoholic NaOH	yellow (525 nm)	
Pyrogallol in alkaline H_2O_2	reddish pink	
Peroxyoxalate reaction	sensitiser dependant	0.05-0.5
ATP-dependant oxidation of D-luciferin with firefly luciferase:		
pH 8.6	yellow-green (560 nm)	0.88
pH 7.0	red (615 nm)	
Bacterial luciferase/oxidoreductase	blue, blue-green (480-490 nm)	0.1-0.2
Gas		
Nitric oxide + ozone	600 nm	
Reduced sulphur compounds + ozone	300-400 nm	

Instrumentation

The instrumentation required for monitoring CL reactions is relatively simple and inexpensive. The most important component of the instrument is the light detector which traditionally has been the photo-multiplier tube (PMT).

A PMT consists of a photo cathode and a series of dynodes at progressively higher positive potentials contained in a glass vacuum tube, exposure to light causes the emission of electrons from the photo cathode by the photoelectric effect which are amplified down the chain of dynodes to an anode. The resulting current is proportional to the light intensity impinging on the cathode surface. PMTs have fast response times (typically nanoseconds), good linearity and low noise (particularly if cooled) and are supplied with a variety of photo cathode materials sensitive to different sections of the visible spectrum. Major drawbacks in the use of PMTs are their fragility, high voltage supply requirements (>1.0 kV) and the length of time to return to maximum sensitivity after exposure to daylight [61].

Solid state devices for the detection of light, e.g. the photodiode and the charge coupled device (CCD) are more robust than PMTs. Photo diodes consist of a silicon pn junction which is reverse biased creating a depletion layer that reduces the conductance of the junction to near zero. When light falls on the junction, free electrons are formed which under the influence of an external potential produce a current proportional to the intensity of light. Photo diodes are inexpensive, small, rugged and have low power requirements (<15 V DC) [62]. Red and blue/UV sensitive photo diodes are available and with careful instrument design they can give sensitivity comparable to PMTs, a photo diode based detector has been applied to CL detection in FI and LC [63].

CCDs have found application for imaging, particularly in astronomy, and their use for spectroscopy is increasing [64, 65]. They consist of arrays of metal oxide semiconductor capacitors on a single silicon chip (Fig 1.6). Photo generated electrons are collected in detector elements (pixels) as charge packets which are transferred to an on-chip amplifier by passing them vertically in sequence from one

pixel to the next adjacent pixel into a serial register and then horizontally into the amplifier [64]. The transfer is controlled by electrodes, the potential of which provides barriers that separate the individual charge packets.

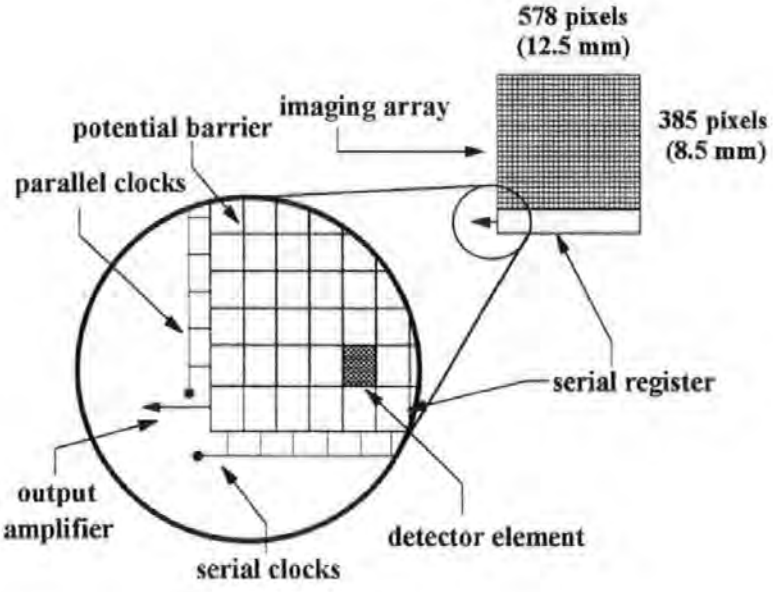


Fig 1.6 Schematic diagram of a CCD chip

CCDs exhibit good signal-to-noise ratios due to the on-chip amplifier and the multiplex advantage of an array detector [64]. The multiplex or Felgett's signal-to-noise advantage is that a array detector gives a signal-to-noise advantage of $\sqrt{N_{pixels}}$, where N_{pixels} is the number of pixels in the array [65]. The dark current of a CCD when cooled with liquid nitrogen (140 K) is as low as 1 e^-/pixel/h and dynamic ranges are wide ($1\text{-}10^5$).

It is possible to read the CCD by a process called binning where the charge contained in several vertical elements is summed before passing to the amplifier. The noise associated with this is of the level of reading a single pixel in contrast to summation in computer memory where the noise associated with reading each pixel separately contributes to the total noise [64]. CCDs show excellent sensitivity at the red/infrared end of the spectrum and their sensitivity at the blue/UV end may be improved by the use of UV sensitive dye coatings.

Spectral information for CL reactions can be obtained by placing a monochromator between the sample cell and the PMT, however instantaneous measurement of spectra are not possible due to the time taken to make a wavelength scan. As a CCD is an array detector it is possible to measure the entire spectrum simultaneously. CCDs therefore have the potential to be sensitive and selective detection systems for chemiluminescence.

Batch luminometers are widely available with various degrees of automation. In a typical instrument a fixed volume of reagent is injected into a tube containing the sample. This type of instrument is well suited to monitoring reactions that are selective and have a high quantum yield and/or a long lifetime e.g. BL reactions. The majority of CL reactions are, however, not highly selective and require chemical or physical separation prior to detection.

Flow injection (FI) has been applied to monitoring CL reactions. It has been defined as the injection of a liquid sample into a moving, non-segmented continuous carrier stream of a suitable liquid The injected sample forms a zone which is then transported to the detector [66]. The basic principles of FI, practical aspects of instrumentation and applications have been described in a number of specialised texts [66, 67]. It is now well established as a powerful sample handling technique for laboratory analysis that is compatible with a wide range of detection systems.

FI is ideally suited to monitoring CL reactions because of the rapid and reproducible mixing of sample and reagent that can be achieved in close proximity to the detector, which results in maximum sensitivity and reproducibility for weak and short-lived emissions. Basic single line and multi-channel FI-CL manifolds have been used to study a number of reactions but these have primarily been applied to synthetic standards only.

For applications involving real sample matrices the facility of FI to accommodate on-line chemical treatment, to remove the matrix or chemically modify the analyte, can be used to provide a certain degree of selectivity. The application of FI to CL and BL assays has been reviewed recently [68].

For most chemical applications, CL detection combined with liquid chromatography is potentially the best option. In practice the sensitivity of CL and the selectivity of LC have to be balanced against the incompatibility of the best mobile phase for separation and the best conditions for the CL reaction.

There are several advantages of CL detection for LC over other methods e.g. UV-visible absorption and fluorescence. CL reactions are inherently sensitive, this is due especially to the absence of a radiation source which reduces or eliminates Raman and Rayleigh scattering and source noise. The CL emission is therefore measured against a darker background than fluorescence, allowing very low limits of detection, e.g. limits of detection of 1 x 10^{-11} mol l^{-1} hydrogen peroxide [69] and 1 x 10^{-10} mol l^{-1} morphine [70]. In addition wide dynamic ranges are possible for many analytes, e.g. the log-log calibration graph for morphine is linear over the range 10^{-4}-10^{-10} mol l^{-1}[70].

1.2.7 Liquid-phase chemiluminescence

Of the CL reactions that occur in the liquid phase, the luminol and peroxyoxalate reactions have found the most widespread application. Other reactions that have been utilised analytically include the lucigenin, lophine and tris(2,2'-bipyridine)ruthenium (III) reactions. The reaction principle and a selection of applications for these reactions (excluding the peroxyoxalate reaction which is discussed in Chapter 2) are presented in Table 1.5.

There are several less well known CL reactions that have been used analytically, these tend to involve the oxidation of organic compounds. The compounds can be oxidised directly, e.g. the oxidation of morphine with permanganate in an acidic tetraphosphate solution [70], in the presence of a catalyst, e.g. the determination of Co (II) by its catalysis of the oxidation of gallic acid by alkaline hydrogen peroxide [71] or in the presence of a sensitiser e.g. the oxidation of triethylamine by sodium hypochlorite in the presence of Rhodamine B [72]. Other systems that have been used have been reviewed by Townshend [73].

Table 1.5 Analytical applications of solution phase chemiluminescent reactions

Reaction	Principle	Applications
Luminol (5-aminophthalhydrazide)	Oxidation of luminol under alkaline conditions in the presence of a catalyst (transition metal ions, horseradish peroxidase) to give excited state 3-aminophthalate anion.	1. Determination of oxidant e.g. hydrogen peroxide in rain water by flow injection [74]. 2. Determination of catalyst e.g. Cr (III) and Cr (IV) in fresh water by ion chromatography [75]. 3. Determination of species that suppress luminol CL e.g. alkanolamines by ion-interaction chromatography [76].
Lucigenin (bis-*N*-methylacridinium nitrate)	Reaction in alkaline solution, primary emitter is *N*-methylacridone. Net reaction is an oxidation, but involves oxidation and reduction steps. In the presence of dissolved oxygen CL can be produced by reaction with reductants.	1. Determination of ascorbic acid and dehydroascorbic acids by ion exchange chromatography [77]. 2. Determination of corticosteriods and *p*-nitrophenacyl esters of carboxylic acids after separation by reversed-phase LC [78].
Lophine (2,4,5-triphenylimidazole)	Oxidation of lophine under alkaline conditions.	Determination of metal ions; Co (II), Cr (III), Cu (II) [79, 80].
Tris(2,2'-bipyridine)ruthenium (III) ($Ru(bpy)_3$)	Reaction of $Ru(bpy)_3^{3+}$ with $Ru(bpy)_3^{+}$ to yield $Ru(bpy)_3^{2+}$. $Ru(bpy)_3^{+}$ and $Ru(bpy)_3^{3+}$ may be electrogenerated from $Ru(bpy)_3^{2+}$ (the stable species in solution). The opposite species, and CL emission, is produced by reaction with suitable oxidant or reductant.	1. Determination of aliphatic trialkylamines by LC [81]. 2. Determination of amino acids, peptides, proteins and isoniazid by flow injection [82]. 3. Determination of alicyclic tertiary amines by LC with electrochemical reactor for on-line generation of $Ru(bpy)_3^{3+}$ [83].

The CL reactions of 1,2-dioxetanes and 1,2-dioxetanones are of great interest due to their possible key role as intermediates in CL reactions. McCapra [84] postulated in the 1960's that many CL reactions, e.g. luminol, lucigenin, and lophine reactions, involved intermediates containing these four-membered peroxide rings (Fig 1.7). Ring strain due to the -O-O- would render these intermediates prone to spontaneous cleavage to yield excited state carbonyl products (Fig 1.7).

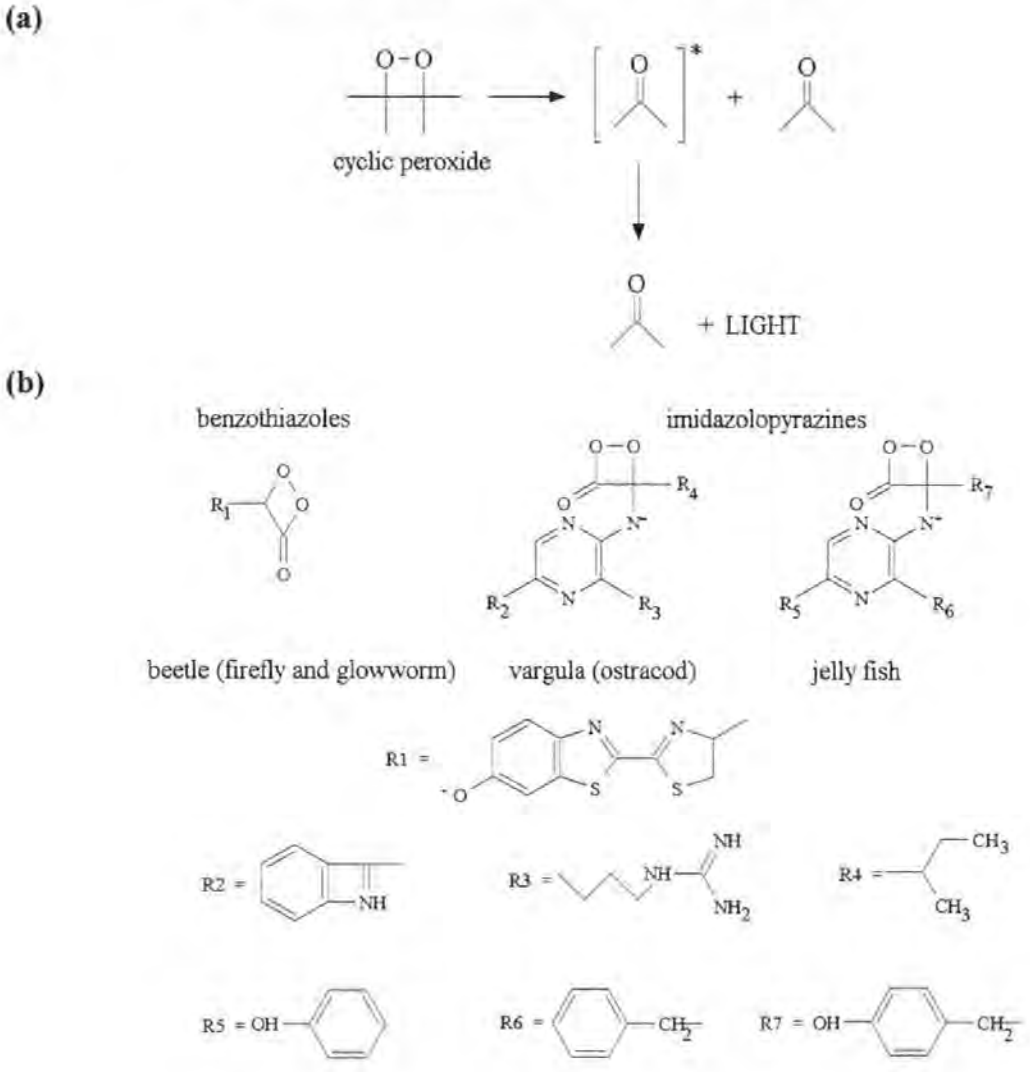


Fig 1.7 1,2-Dioxetane and 1,2-dioxetandione CL (a) emission of CL from decomposition of cyclic peroxides (b) Dioxetanone intermediates in BL reactions

In 1969 it was shown that stable compounds of this type could be synthesised [85], since then many have been synthesised, all of which are chemiluminescent, the CL being produced by gentle heating [86]. 1,2-Dioxetanes have been used in immunoassays [87] and a preliminary investigation into their use for LC has been reported [88].

1.3 LIQUID CHROMATOGRAPHY

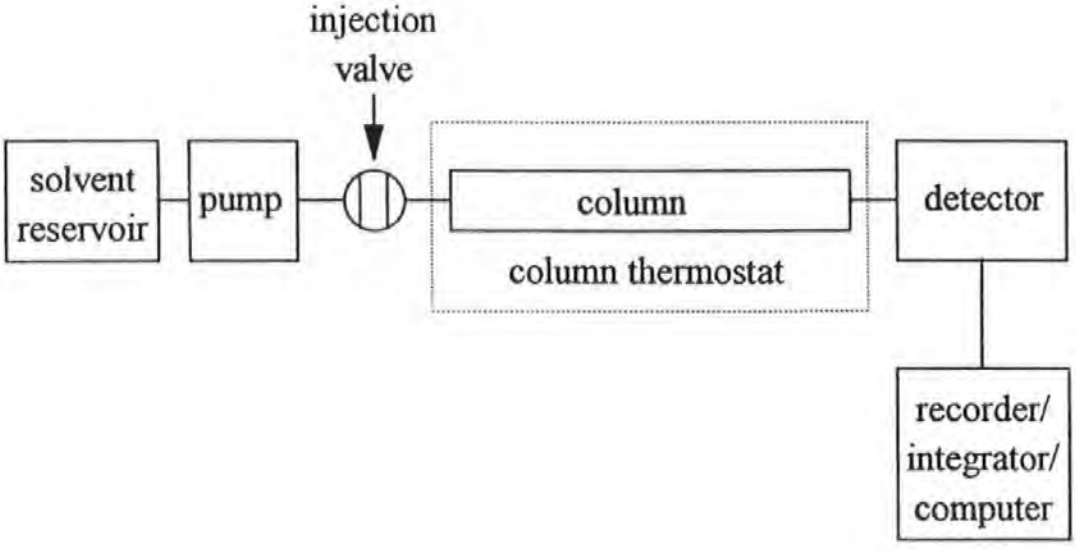
1.3.1 Introduction

Chromatography is a technique for the separation of components in a chemical mixture, and their subsequent identification and quantification, based on their chemical and physical properties. The principle was first described the Russian botanist Michael Tswett (1872-1919) in 1906, and the history of the evolution of liquid chromatography has been reviewed by Ettre [89]. Methods based on chromatographic techniques are very widely applied throughout contemporary chemical analysis.

Chromatographic separations are achieved by exploiting the differences between species of a variety of properties including e.g.; volatility, polarity, charge, size, and mass. These properties affect the rate of migration of the species in a mixture passing under the influence of a moving fluid (the mobile phase) through a bed of stationary material (the stationary phase).

In liquid chromatography (LC) the mobile phase is a liquid and is commonly referred to as the eluent. The stationary phase through which it passes is often, thought not exclusively, packed in stainless steel columns. Samples are injected into a flowing stream of eluent and transported to the column where the components of the mixture are separated by interaction with the stationary phase. As the components elute from the column, the change caused to a property of the eluent is registered at a detector and monitored by a chart recorder, or increasingly by a computer.

A plot of the detector response with time is called a chromatogram, Fig 1.8 shows a block diagram of the basic components for a liquid chromatograph and a typical chromatogram with the parameters which may be used to characterise it [90].



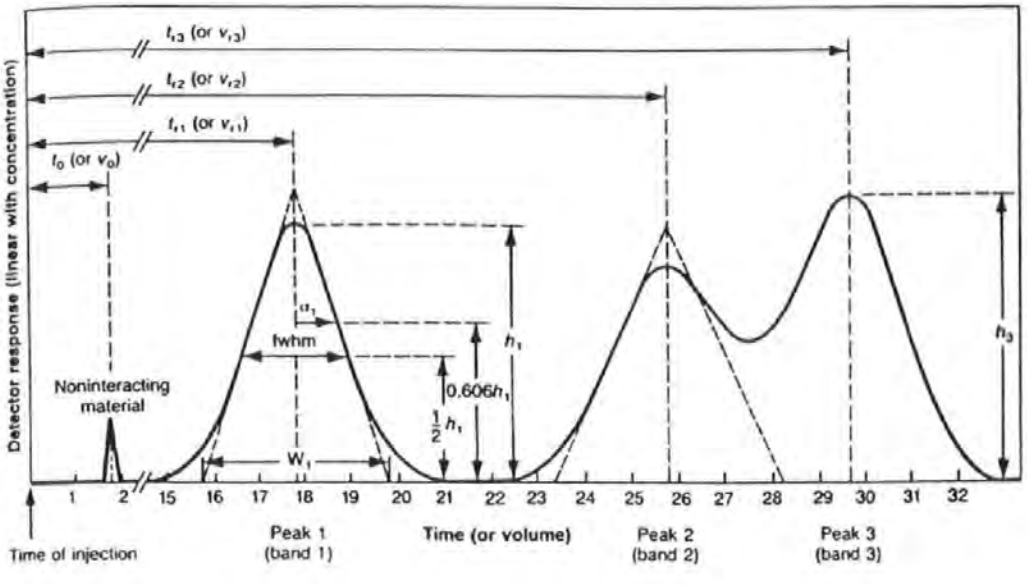


Fig 1.8 Block diagram of the components of a liquid chromatograph and a hypothetical chromatogram with derived parameters

The selectivity of a LC system towards a particular species can be split into two components;

1. Chromatographic; the ability of the column to separate components with small differences in structure or molecular weight.

2. Detector; the ability of the detector to respond specifically to the components of interest without interference from other compounds present.

1.3.2 Modes of Liquid Chromatography

Several different modes of chromatography are available, which give selectivity by the different interactions that are required for separation. Separations in LC are strongly dependant upon the nature of the mobile phase, unlike GC. A small change in mobile phase will have a large effect upon a separation [26]. If two bands overlap, the separation can be made larger by changing the chemical nature of the mobile phase. The exact characteristics of the mobile phase will depend upon the sample and the type of stationary phase used.

The classification of LC modes is summarised in Table 1.6. It is important to note that no LC separation occurs by the means of only one kind of interaction, as for example a separation carried out by liquid-liquid chromatography is likely to have a contribution from adsorption at least [91].

An important parameter of a solvent for use in a mobile phase is its polarity. These are derived from dipole moments and dielectric constants. Solvents can be placed in order of polarity in an eluotropic series, e.g. Table 1.7 [91].

Solvents are also be described as strong or weak, solvent strength can be defined as a solvent's ability to speed up a separation and depends upon the chemical interactions used between mobile phase and stationary phase to achieve a separation.

Table 1.6 Classification of modes of LC		
LC Mode	**Description**	**Separation**
Adsorption	Solid stationary phase, e.g. silica and alumina, non-polar organic mobile phase e.g. hexane.	Adsorption
Liquid-liquid and bonded phase	Liquid physically adsorbed to support or chemical species bonded to a solid support can either be *normal-phase* (polar stationary phase, non-polar mobile phase e.g. hexane) or *reversed-phase* (non-polar mobile phase, polar mobile phase e.g. methanol, acetonitrile, water).	Partition, adsorption
Ion-exchange	Ion-exchange resin stationary phase, mobile phase of buffer and water.	Ion-exchange
Size exclusion	Liquid in interstices of a polymeric solids.	Partition, sieving

Table 1.7 Solvent strength parameters, ε°, for LC solvents; the eluotropic series		
Solvent	ε°, alumina support	ε°, silica support
Hexane	0.01	-
Cyclohexane	0.04	0.03
Carbon tetrachloride	0.18	0.11
Toluene	0.29	0.23
Chloroform	0.40	0.26
Tetrahydrofuran	0.45	-
Acetonitrile	0.65	0.5
Methanol	0.95	0.73
Water	large	-

Adsorption chromatography

This was the original mode of chromatography as practised by Michael Tswett. Another term for it is liquid-solid chromatography. The stationary phase is a polar solid, silica and alumina are the most common. The mobile phase used is relatively non-polar, for example hexane.

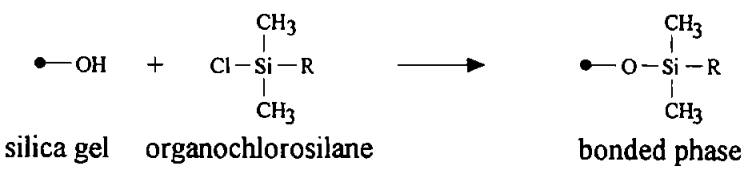
Separations are due in the most part to interactions between sample polar functional groups and adsorption sites on the solid stationary phase. For this mode a strong solvent is a more polar one, modifying the non-polar mobile phase of hexane with a more polar solvent e.g. methanol will shorten retention times.

The retention behaviour of individual sample solutes is governed by functional groups. The relative position of the functional groups interacts with the spatial arrangement of hydroxyl groups on the surface of the solid support. Because of this adsorption LC is good at resolving isomers [92].

Liquid-liquid and bonded phase chromatography

Through the application of bonded phases this has become the most widely used of LC modes. Components in a sample are distributed between the mobile phase and a stationary "liquid" phase immobilised upon a comparatively inert solid support, usually silica. The "liquid" can be a true liquid physically adsorbed to the support or a layer chemically bonded to the silica [93]. Liquid-liquid LC is limited by the choice of truly immiscible solvent pairs and has almost been entirely superceded by bonded-phase LC.

The usual solid support for bonded phases is silica, groups are bonded to its surface by silanisation with organochlorosilanes [94].



Residual hydroxyl groups on the silica are undesirable as they lead to mixed retention mechanisms, peak tailing with polar solutes (particularly basic molecules) and possible chemical reactions with the sample or the mobile phase. This problem can be combated by the further silanisation with chlorodimethylalkylsilane and chlorotrimethylsilane to "end-cap" and deactivate the residual hydroxyl groups [95].

Bonded phase LC can be split into two main categories, normal- and reversed-phase. For reversed-phase applications R is based on a hydrocarbon, e.g. octadecyl (C_{18}), octyl (C_8), phenyl, while for normal-phase, amino and diamino are examples of functional groups that have been used.

In normal-phase, the stationary phase is polar and the mobile phase is non-polar, the least polar components are eluted first and increasing the polarity of the mobile phase leads to a decrease in retention time. Mobile phases used are similar to those used for adsorption LC, the separations obtained are similar as well although there are selectivity differences. In general, bonded normal-phase packings are less retentive than adsorbents, but they do not suffer to the same extent as adsorbents from chemisorption, peak tailing and catalytic activity. In addition there is less chance of irreversible retention of a sample component and more rapid equilibration of the column when using solvent gradients [93].

In reversed-phase LC the stationary phase is non-polar and the mobile phase is relatively polar, the most polar components are eluted first and an increase in mobile phase polarity results in an increase in retention times. The basic mobile phase is water with water miscible organic solvents added to modify the elution characteristics of the samples e.g. methanol, acetonitrile and tetrahydrofuran. The range of sample applicability can be increased by using totally organic mobile phases (for low polarity solutes) or more aqueous mobile phases modified by the addition of neutral salts, buffers or surfactants, as in ion-pair LC (see below).

Reversed-phase LC is widely used and it is estimated that over 65 % of all LC separations are carried out in this mode and that it has been applied to the separation of virtually all molecules of relative molecular masses below 2000 daltons [96]. The

mechanism of reversed phase LC is not fully understood, with several phenomena effecting retention and selectivity. These include interactions between solute molecules and residual silica hydroxyls, interaction of the solutes with metal contaminants in the silica support, steric effects, specific interactions with additives in the mobile phase and solvation of the solutes and/or the stationary phase by the organic component of the mobile phase [97].

In general, the predominant factor for retention in reversed-phase LC is the non-specific hydrophobic (solvophobic) interactions of the solute molecules with the stationary phase. This explains the wide applicability of the mode, as practically all organic molecules have a region in their structure which is hydrophobic. Assuming other factors are constant, bonded reversed-phase packings with the longest hydrocarbon chain are more retentive for non-polar compounds [97].

Reversed phase packing materials can also be used for ion-pair chromatography using mobile phases consisting of aqueous buffers containing organic solvent, e.g. methanol, and a counter ion of opposite charge to the analyte capable of forming an ion pair with the analyte [98]. The ion pair is neutral and soluble in non-aqueous solvents. The counter-ion has a hydrophobic portion which allows it to partition into the stationary phase forming, in-situ, a dynamic ion-exchange medium. Analyte ions (R^+) are attracted to the counter ion and an equilibrium is set up;

$$R^+_{aq} + X^-_{org} \rightleftharpoons (RX)_{org}$$

Where X^-_{org} is the counter ion in the stationary phase.

Retention of analytes is increased by increasing the concentration of the counter ion. Ion-pair chromatography has begun to replace ion-exchange chromatography because of its advantages of speed, efficiency and reproducibility.

Ion-exchange Chromatography

This mode of LC is based upon separations achieved through ionic equilibria [99]. The stationary phase consists of high molecular weight solids, synthetic resins, cross linked for strength, with charged groups on their surface. Mobile phases are aqueous

solutions containing organic modifiers and buffers. Assuming no secondary factors, retention and selectivity are governed by pH, the concentration of the buffer and the presence of any counter-ion that may compete with active sites on the stationary phase. For a sulphonic acid anion exchange resin separating cations (M^{x+}), the ionic equilibria involved in an ideal case can be represented by;

$$\underset{\text{solid}}{x\,RSO_3^-H^+} + \underset{\text{solution}}{M^{x+}} \rightleftharpoons \underset{\text{solid}}{(RSO_3^-)_xM^{x+}} + \underset{\text{solution}}{xH^+}$$

In practice many separations cannot be fully explained by ionic equilibria, other factors also effect retention including adsorption, partition and hydrogen bonding.

Size exclusion chromatography

This is also called gel filtration (aqueous mobile phases, hydrophilic stationary phases) and gel permeation chromatography (non-polar mobile organic phases, hydrophobic stationary phases). It is applied to the separation of high molecular weight species according to molecular size [100].

The mechanism of separation depends upon the different rates of diffusion of molecules through a porous matrix. The packing material consists of silica or polymer particles with very precisely controlled pore cross sections; molecules become trapped in the pores and are removed from the mobile phase. The residence time in the pore is reliant upon the effective size of the molecule, larger molecules are eluted first as they are not retained in the pores. Separations are according to molecular size, not mass and in the case of polymers, molecular shape can influence retention.

Mobile phase selection is such that the solvent used is of a similar polarity to the stationary phase, so as to avoid interaction between sample components and the surface of the gel.

1.3.3 Chromatographic Parameters

A number of parameters can be derived from the chromatogram (Fig 1.8) to describe the component peaks (or bands) individually and relative to each other, these are summarised in Table 1.8.

Table 1.8 Chromatographic parameters

Measured parameters	Symbol
Column void volume (time)	v_0 (t_0)
Retention volume (time)	v_r (t_r)
Peak base width	W
Peak width at half the peak height	$W_{½}$
Peak height	h
Derived parameters	**Symbol and derivation**
Retention; capacity factor	$k' = \frac{v_r - v_0}{v_0} = \frac{t_r - t_0}{t_0}$
Selectivity; selectivity factor (for two peaks *a* and *b*)	$\alpha = \frac{k'_b}{k'_a} = \frac{(t_{rb} - t_0)}{(t_{ra} - t_0)}$
Column efficiency; number of theoretical plates (calculated from a peak *a*)	$N = 16(t_{ra}/W_a)^2$, $N = 5.52(t_{ra}/W_{½a})$
Height equivalent to a theoretical plate (for *a* column length *L*)	$H = L / N$
Resolution; resolution factor (for two peaks *a* and *b*)	$R = 2(t_{rb} - t_{ra})/(W_b - W_a)$

Retention

Retention is the term used to describe the interaction of sample components with the stationary phase. For a particular component the degree of retention is characteristic of the component, and is described by the retention volume, v_r. Components which do not interact with the stationary phase are eluted in the dead volume of the column, also called the column void volume, v_0. Non-interacting material appears as a small peak at the beginning of a chromatogram with a time of t_0, using this and the

flow rate, v_0 can be calculated. Occasionally the first peak of the chromatogram may not appear at the void volume if the detector is not sensitive to the non-interacting species or if all the components of the mixture interact with the stationary phase.

Retention volumes of components derived from a particular chromatographic system are absolute values, as such they will vary with flow rate. A relative term for retention which does not vary with flow rate is the capacity factor, k', which is calculated for each peak as described in Table 1.8. At constant flow rate retention times may be used instead of volumes. Useful separations typically involve capacity factors in the range 1-10, capacity factors greater than 10 can lead to excessive analysis times [101].

The capacity factor of a component can be related to its distribution coefficient K [102]. K is defined as;

$$K = \frac{C_S}{C_M}$$

where C_S and C_M are the molar concentrations of a solute in the stationary phase and mobile phase respectively. The capacity factor for the component can be defined as;

$$k' = \frac{M_S}{M_M} = \frac{C_S}{C_M} \cdot \frac{V_S}{V_M} = K \cdot \frac{V_S}{V_M}$$

where M_S and M_M are the masses of the component contained in the stationary phase and mobile phase, V_S and V_M are the total volumes of stationary phase and mobile phase respectively. The treatment above is for liquid-liquid chromatography, but it also applies to bonded phase LC and the other modes of LC, except that surface area (adsorption LC) ion-exchange capacity (ion-exchange LC) and total pore volume are used in place the volume of stationary phase [102].

Ideally K is constant over a wide range of solute concentrations, this situation can occur over limited concentrations, particularly low ones and the relationship between C_S and C_M can be assumed to be linear, most chromatography tends to be

carried out under these conditions [103]. Non-linear behaviour leads to variation in retention time with sample concentration [104].

Selectivity

In a mixture the various components will have different values of k'. The difference between the degrees of retention of the different species can be described by the selectivity factor, α, which is always calculated so that its value is greater than or equal to unity. If the value is unity, the components have the same interaction with the stationary phase and cannot be separated by that particular set of chromatographic conditions. The selectivity factor does not have to vary too much from unity for a separation to be achieved, typically it falls in the range 1.1-2.0 [102]. However a selectivity factor greater than unity does not mean that separation of two components is possible, as band broadening will cause the peaks to overlap.

Efficiency

Samples can be thought of as being injected on to a column as a band, which moves through the column under the influence of the mobile phase. Under ideal conditions the sample will be eluted as discrete bands whose total volume will be equal to the original sample volume and the peak profiles will be rectangular in profile [105]. In practice mixing and diffusion lead to dispersion of the sample bands giving peaks which approximate to gaussian distributions.

The width of the bands will greatly effect the separation of a mixture on a column. The mixing and diffusion phenomena that lead to band broadening must therefore be kept to a minimum, to enable good separations in short times. The ability of a column to minimise band broadening is termed the column efficiency and can be expressed as the number of theoretical plates, N of a column. Several methods have been used for the calculation of column efficiencies which vary with point where the peak width is measured. If a chromatographic peak is not symmetrical the base width is not a true reflection of the dispersion of the peak. The width at half the peak height (full width at half maximum, $W_{½}$) can be used to calculate column efficiency [90].

The number of theoretical plates contains no information about the dimensions of a column. Efficiency may also be determined as length of column per unit number of theoretical plates. The term used is height equivalent to a theoretical plate, *H*, the lower its value, the more efficient the column.

Band broadening in LC is considered as arising from four sources; eddy diffusion, longitudinal diffusion, mass transfer and extra-column diffusion [106]. Eddy diffusion arises from the different flow path lengths present in a column due to the lack of uniformity of the packing material, it is independent of mobile phase velocity. Longitudinal diffusion is caused by molecular diffusion from random molecular motion and is unimportant in LC except at very low mobile phase flow rates. Band broadening due to mass transfer arises from two sources; the speed of diffusion between the mobile phase and stationary phase, diffusion between mobile phase and mobile phase trapped in pores in the solid support particles. These sources of band broadening within the column can be related to *H* by the van Deemter equation [107];

$$H = A + \frac{B}{u} + Cu$$

Where *A*, *B* and *C* are constants determined by the physical properties of the stationary and mobile phases, *u* is the mobile phase velocity (cm s^{-1}). *A* characterises the eddy diffusion, *B/u* the longitudinal diffusion and *Cu*, mass transfer. As can be seen longitudinal diffusion can be reduced by increasing the velocity of the mobile phase. Eddy diffusion can be reduced by using smaller diameter particles in the packing material consistent with producing a uniform packing. Mass transfer effects are made worse at higher mobile phase velocities, they can be minimised by reducing the diameter of the support particles, eliminating long narrow pores that trap mobile phase and by using thin coatings of stationary phase upon the solid support.

Extra column band broadening is caused by the dead volume associated with the detector flow cell and the fittings connecting it to the column. To combat this, the

dead volume has to be kept to a minimum. This is major problem, particularly when post-column reactors are being used [108].

Resolution

With the width of the peak defined, a real measure of the resolution of two peaks is possible using the resolution factor R, when this equals is unity there is still overlap of the two peaks (2 %), which is reduced at R = 1.5 (0.03 %) [109]. In symmetrical peaks of similar size this is not a problem, however if one of the peaks is smaller than the other, quantification may be difficult, particularly at trace levels. This is exacerbated if the peaks are not symmetrical e.g. peak tailing.

General resolution equation

The expressions for resolution, column efficiency, selectivity and capacity factors can be combined in one equation to describe resolution. This is termed the general resolution equation [102, 110];

$$R = \sqrt{N}[(\alpha - 1)/\alpha\,][k'_a/(k'_b + 1)]/4$$

On inspection of the equation it can be seen that R is directly related to selectivity (nature of interactions)and capacity (the extent of phase interaction). Altering these by manipulation of the mobile and the stationary phases will therefore be the best way of improving resolution of two peaks, very large changes in efficiency are required to achieve the same result.

1.3.4 Detectors for liquid chromatography

The properties of an ideal detector for LC can be defined as; sensitivity, good stability and reproducibility, linear response to solutes over several orders of magnitude, short response times independent of flow rate, high reliability and ease of use, similarity of response to all solutes or a predictable response to one or several classes of solutes, minimal internal volume of sample cell to prevent band-broadening and non-destructive to the sample. No detection method can fulfil all of these requirements [111]. However the range of detection modes available means that most cases can be covered.

Detectors can be mass sensitive or concentration sensitive, the latter is the most common. The output of the detector can be linear, integral or differential. For a detector with linear output, the signal is directly proportional to solute concentration;

$$y = Ac$$

where y is the signal, c is the concentration of the solute and A is a constant. Detectors are designed to give linear outputs as the chromatograms from integral and differential output detectors are hard to interpret and quantification of solutes is difficult [112].

Two response ranges may be defined for LC detectors; dynamic and linear dynamic range. The dynamic range is the solute concentration range over which a detector will give a concentration dependant output. The linear dynamic range is the range of solute concentrations over which the response of the detector is linear relative to the solute concentration. The two ranges are not the same as response to a solute can vary at high and low concentrations of solute.

The dynamic range for a detector is limited at the lower end by detector noise. Detector noise is defined as any perturbation of the detector output not related to an eluted solute [112]. It is an important parameter as it ultimately determines the sensitivity of the detector and is measured by its amplitude (Fig 1.9). The different types of detector noise are illustrated in Fig 1.9.

Among these, long term noise is the most serious. It arises from the susceptibility of the detector to small changes in ambient conditions and from instabilities of detector components e.g. refractive index detectors are sensitive to temperature and pressure while chemical noise is a significant problem when using CL detection.

The detectors for LC can be split into three categories. General detectors which respond to modulation of a mobile phase bulk property by solutes, e.g. refractive index and conductivity, specific detectors responding to properties of solutes e.g. absorbance and fluorescence, detectors responding to solutes after removal of the mobile phase e.g. mass spectrometry. The most common methods of detection are

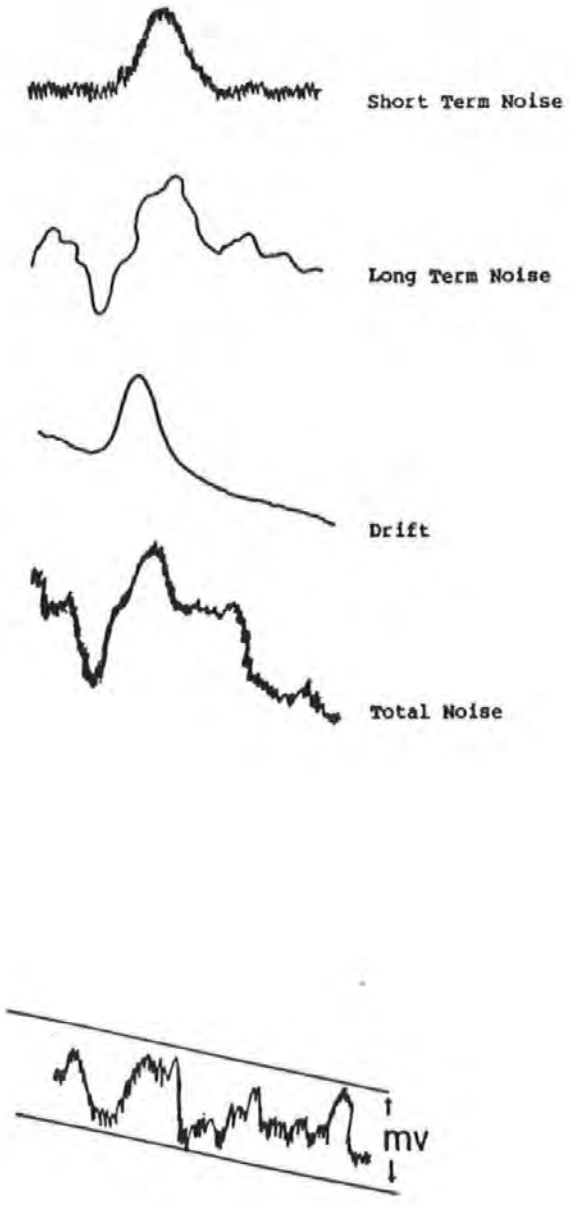


Fig 1.9 Illustration of the types and measurement of detector noise

summarised in Table 1.9. Other methods of detection have also been applied to LC, including flame ionisation detection, using a moving belt interface similar to that used for mass spectrometry, inductively coupled mass spectrometry (ICP-MS), atomic absorption and nuclear magnetic resonance (NMR) [113].

Each of the methods of detection described in the table has drawbacks, and none approach the applicability of the flame ionisation detector. Refractive index detectors have the advantage of responding to all solutes, although in complex mixtures components may cover a wide range of index values and some may be close to that of the mobile phase and not be detected. They are also limited by temperature dependence and cannot be used with solvent gradients. For electrochemical detection the response of the detector is dependant upon the mobile phase, gradient elution is therefore not normally possible. Mass spectrometric detectors for LC are near universal detectors but they are specialised and very expensive pieces of equipment. UV absorbance has found the widest application, however detectability and sensitivity can be greatly effected by the absorbance due to the sample matrix. Fewer species are luminescent, therefore luminescence detection for LC should lead to simpler chromatograms with less matrix effects.

Luminescence detectors

Fluorescence has the advantage of higher sensitivity and selectivity compared with absorbance measurements. The theory and application of fluorescence and other luminescence techniques has been discussed in an earlier section. As mentioned, the applicability of this mode of detection is restricted by the number of naturally fluorescent compounds. In addition, linear dynamic range is limited in fluorescence detection due to inner filter effects at high analyte concentrations. Mobile phase composition can also have a significant affect upon fluorescence, due to solvent effects.

Table 1.9 Detectors for LC	
Mode of detection	Description
Absorbance	Absorbance of ultraviolet (UV), visible and infra-red radiation, UV absorbance is the most common. Species with one or more double bonds, unshared lone bonding electrons absorb UV e.g. olefins, aromatics, compounds containing >C=O, >C=S, -N=O, -N=N-, therefore of wide applicability. Detector consists of a flow cell, a beam of UV light is focused through the cell on to a photodiode.
Refractive index	The first on-line detectors for LC, they are bulk property detectors. Pure mobile phase is passed through one half of the cell while column eluent passes through the other. The incident beam of a light source shining through the cell is bent by the difference in the refractive indices of the two solvent streams due to the presence of solutes in the mobile phase. Refractive index detectors have found application in the food industry for sugar analysis and in the polymer industry as a detector for size-exclusion chromatography.
Electrochemical	Conductivity, polarographic, amperometric and coulometric detectors have been used. Conductivity is applicable to ionic compounds, while the other electrochemical methods are applied to compounds which can be electrolytically oxidised or reduced e.g. phenols, mercaptans, aromatic nitro and halogen compounds, catecholamines, heterocylic compounds, aldehydes and ketones. Mobile phases are required to be electrically conducting.
Mass spectrometric	Major problem of marrying large solvent volumes of LC and the vacuum requirements of mass spectrometry has been overcome by the use of several interfaces e.g. the moving belt and thermospray. Direct liquid introduction is possible with microbore LC, which uses narrow bore columns with very low flow rates (10-50 $\mu l\ min^{-1}$).

Only a few applications of phosphorescence detection in LC exist, as phosphorescence requires frozen matrices to reduce non-radiative modes of deactivation. However room-temperature phosphorescence in the liquid phase is possible for a few species under special conditions, and it has been utilised in the detection of mixtures of polychloronaphthalenes and polychlorobiphenyls following separation by LC [114].

CL has also been applied to LC, particularly the peroxyoxalate system which is discussed in chapter 2.

1.3.5 Derivatisation in liquid chromatography

An analyte may not be detected with sufficient selectivity and/or sensitivity due to the nature of the species of interest or the complexity of the sample matrix. In a position such as this, a chemical manipulation (derivatisation), of the analyte is used to improve detectability. This approach is widely used and has been very fully reviewed in a number of publications [115-119]. As in GC, derivatisation may be used to alter the chromatographic properties of analytes in order to improve separation, although this is not such a factor in LC due to the wide variety of separation modes. The main use of derivatisation in LC is to improve detectability [120]. The principle exception to this is use of chiral derivatisation reagents in the resolution of racemic mixtures [121].

Derivatisation reactions can be classified into labelling and non-labelling reactions [122]. The former involves the formation of a bond between the analyte and a species which improves the detection characteristics of the analyte e.g. chromophores, fluorophores, radiophores, electrophores. All other reactions that are used are termed non-labelling as they do not involve the attachment of another molecule to the analyte and include redox, solvolytic, photochemical, ion-pair formation and micellar formation reactions [123]. Derivatisation reactions have been reviewed according to the functional group involved [124] and to the mode of detection used [119].

Derivatisations may be performed prior to separation (pre-column derivatisation) or after separation (post-column derivatisations), the advantages and disadvantages associated with these two approaches are discussed below.

Pre-column derivatisation

Pre-column derivatisation, particularly off-line, is the major technique for the conversion of species in to those with improved detectability. Labelling reactions pre-dominate, but others have been used. The advantages of off-line pre-column derivatisations are;

- the reaction kinetics of the chosen reaction are not important as long as the reaction goes to completion within a reasonable time. This allows optimisation of the reaction yield.
- the reaction conditions e.g. reaction solvent, are not required to be compatible with the conditions.
- complex multi-step reactions are possible.

The disadvantages may be summarised as follows;

- the possibility of multiple products from one analyte.
- the formation of side-products can produce interference especially at trace levels in complex matrices. This can be improved by the use of cleanup techniques such as solid phase extraction.
- multistep procedures can lead to contamination and loss of the sample leading to poor recoveries.
- the procedures are labour-intensive and time consuming, particularly if cleanup is required.

In an ideal situation the derivatisation reagents and the reaction solvent would not affect the separation and detection of the derivatives, this is not always the case and their removal is required prior to analysis. It may also be the case that the derivative is not stable under the conditions required for separation.

Post-column derivatisation

Post-column derivatisation is invariably carried out on-line in a reactor between the column and the detector. This has also has also been called chemical reaction detection (CRD) [125]. A comprehensive overview of theory and applications has been edited by Krull [118]. Advantages of its use include;

- interference from reaction artefacts is minimal.
- reduced sample pre treatment compared with pre-column derivatisations.
- the reaction is not required to go to completion, as long as it is reproducible.

Disadvantages are;

- an increase in band broadening. The dispersion that is the cause of this has three sources; reagent addition, mixing and the length of reaction. The design of the reactor is therefore critical, the effect of this and mixer geometry have been discussed by Frei [126] and more recently Lillig and Englehardt [125]. Although additional band-broadening is inevitable with on-line post-column derivatisation, it can be ignored if the selectivity of the reaction is high.
- the number of reactions that may be utilised is restricted, due to reagent in-compatibility and reaction kinetics. If reaction kinetics are slow, a prohibitively long residence time in the reactor is required leading to severe band broadening.
- the complexity of the instrument is increased due to the addition of at least one extra pump for the post-column reagents. The possibility of using "pumpless chemistries" has been investigated by several groups. Procedures that have been applied include solid state reagents [127], immobilised enzymes [128], and photolysis [129].

The first application of on-line post-column derivatisation was by Spackman et al. in 1958 [130]. A post column reaction with ninhydrin was used in the determination of amino acids . Since then the principle has been applied to many different analyses [127, 131], Table 1.10 summarises some recent applications of post-column derivatisation.

Table 1.10 Recent applications of post-column derivatisations for detection in liquid chromatography

Analyte	Separation	Post-column Reaction	Detection	Ref.
Transition metals in stainless steels	Ion chromatography	Complexation with 4-(2-pyridylazo)resorcinol (PAR)(for V, Zr, Cu, Ni, Mn, Co, Pb, Ti, Nb, Ta) or arsenazo III (for Zr, Cr, Cu, Al, Pb).	Absorbance	132
Oligosaccharides in beer	Ion-exchange chromatography	Co-immobilised enzyme reactor; amyloglucosidase, mutarotase and glucose dehydrogenase co-immobilised on control pore glass packed in low dead volume columns, action of enzymes produces NADH.	Absorbance	133
Carbohydrates in wine	Ion-exchange chromatography	Reaction with ethanolamine-boric acid after post-column hydrolysis with *p*-toluenesulphonic acid.	Fluorescence	134
N-methylcarbamate pesticides and metabolites in surface waters	Reversed-phase LC	Hydrolysis on a solid-phase (anion-exchange) catalyst, reaction of methylamine formed with *o*-phthaldehyde-2-mercaptoethanol.	Fluorescence	135
Glutathione in plasma	Ion-pair LC	Reaction with *o*-phthaldehyde.	Fluorescence	136
Sulbitiamine and disulphide derivatives in plasma	Reversed-phase LC	Reduction by cysteine to free thiamine derivatives followed by oxidation to corresponding thiochromes with ferricyanide.	Fluorescence	137

1.4 RESEARCH OBJECTIVES

The primary aim of this research was to investigate pre-column labelling reactions with luminescence detection, with a view to developing sensitive and selective assays for organic functional groups in automobile engine oils. Present procedures for the analysis of oils are unsuitable for the selective determination of polar oxidation products, pre-column derivatisation and liquid chromatography with luminescence detection can supply this information which would be used to monitor oil condition during the course of engine tests.

Specific aims of this research were;

1 The design and construction of a flow through CL detector and the FI investigation of peroxyoxalate CL for the determination of fluorescent species in non-aqueous media.

2. The development of LC procedures for the determination of carboxylic acids and aldehydes in oil matrices by selective pre-column derivatisation and luminescence detection.

3. The analysis of oxidised oil samples from engine tests for carboxylic acids and aldehydes to evaluate the oxidative stability of the oils .

Chapter 2

Peroxyoxalate Chemiluminescence

2.1 INTRODUCTION

Peroxyoxalate chemiluminescence (POCL) involves the hydrogen peroxide induced oxidation of aryl oxalate esters in the presence of a suitable fluorophore. It is an example of sensitised (energy transfer) CL. As the reaction takes place almost exclusively in non-aqueous media, it is compatible with reversed-phase LC and therefore has found greater application as a post-column reaction detection system than other CL reactions.

This chapter describes a FI investigation of the experimental conditions that affect the application of the POCL reaction to the analysis of oils by reversed-phase LC.

2.2 EXPERIMENTAL

2.2.1 Reagents

High quality de-ionised water from a Milli-Q system (Millipore) and analytical grade reagents were used unless otherwise stated. Acetone, acetonitrile (ACN) and tetrahydrofuran (THF) were of HPLC grade (Rathburn).

Stock imidazole solution (2.0 mol l^{-1}) was prepared by dissolving imidazole (Fluka) in water. Imidazole buffer was prepared by dilution of the stock with water and adjusting pH with nitric acid (1.0 mol l^{-1}). Citrate buffer (5.0×10^{-2} mol l^{-1})was prepared from citric acid (Merck) and the pH adjusted with sodium hydroxide (Merck, 0.1 mol l^{-1}).

Stock perylene (Fluka), 9-anthracenemethanol (Fluka) and 3-aminofluoranthene (Janssen) solutions (all 1.0×10^{-3} mol l^{-1}) were prepared in acetone. Working standards of perylene (2.5×10^{-7} mol l^{-1}), calibration standards of 9-anthracenemethanol (1.0×10^{-7}-1.0×10^{-5} mol l^{-1}) and 3-aminofluoranthene (1.0×10^{-9}-1.0×10^{-7} mol l^{-1}) were prepared by dilution of stock with ACN.

Solutions of bis(2,4-dinitrophenyl)oxalate (DNPO; Fluka) and bis(2,4,6-trichlorophenyl)oxalate (TCPO; Fluka) were prepared in ACN; sonication was required to assist solubilisation. Hydrogen peroxide solutions were prepared by

dilution of a 100 volume stock solution (aqueous; Fluka) with ACN. Mobile phase was prepared on a volume/volume basis and was degassed by sonication for 1 minute. The chemiluminogenic reagents and mobile phase were prepared daily.

Standard conditions

The following reaction conditions were used unless otherwise stated;

1. aryl oxalate solution, 1×10^{-3} mol l^{-1}
2. hydrogen peroxide solution, 0.1 mol l^{-1}
3. mobile phase, ACN-THF-water 80:10:10 v/v/v
4. analyte, 2.5×10^{-7} mol l^{-1} perylene

Emission intensity was measured as the signal-to-noise.

2.2.2 Instrumentation

Univariate and simplex optimisation of peroxyoxalate CL

A schematic diagram of the experimental configuration used is given in Fig. 2.1. The carrier, aryl oxalate and hydrogen peroxide streams were pumped at equal flow rates by a peristaltic pump (Gilson). Standards (20 µl) were injected (Rheodyne 5020 teflon rotary valve) into the carrier stream which merged at a teflon Y-piece (Omnifit) with the chemiluminogenic stream containing hydrogen peroxide and aryl oxalate solutions previously merged at a teflon Y-piece (Omnifit).

The merged streams passed into a lamina flow cell (volume 234 µl) [138]. This consisted of a PTFE spacer with an elliptical orifice sandwiched between a stainless steel block (with inlet and outlet ports) and a glass plate, all of which were held in place by a stainless steel pressure plate with an orifice of the same dimensions as the spacer.

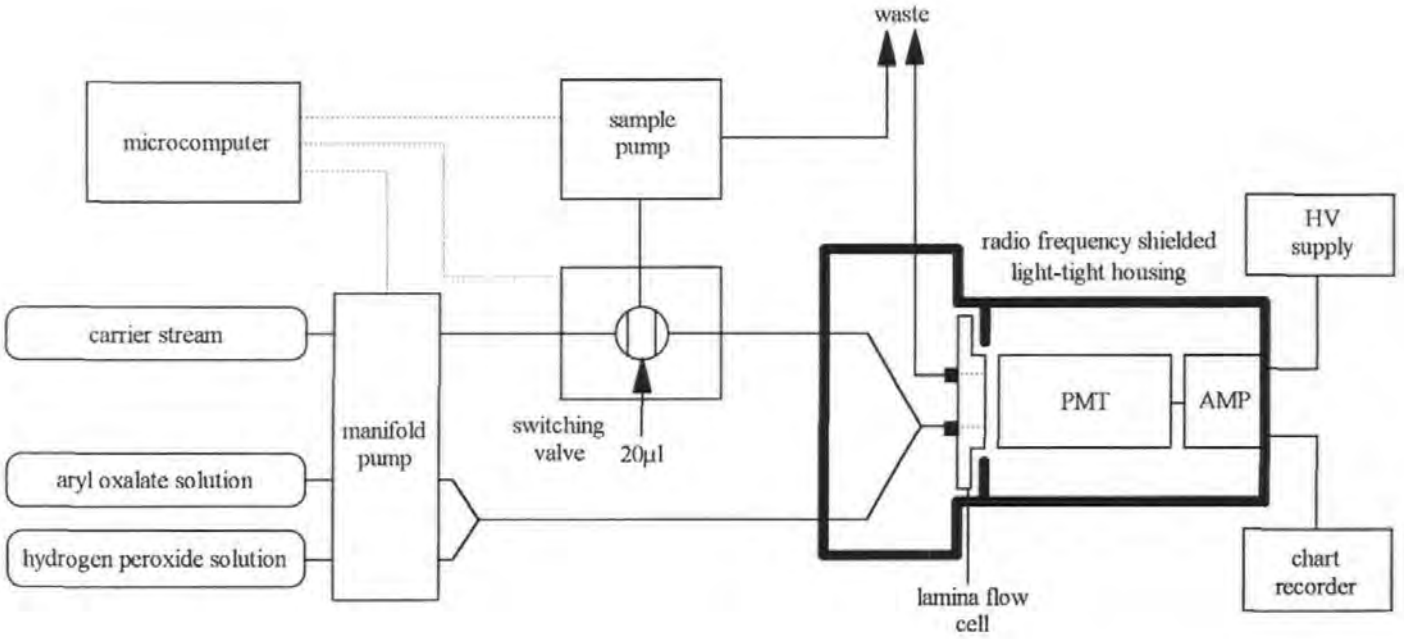


Fig 2.1 FI-CL manifold for the investigation of the POCL reaction.

The length of tubing between the second Y-piece and the flow cell was kept as short as possible (5.6 cm).

The sample loop was filled by a peristaltic pump (Watson-Marlow) and the rotary valve switched by a valve switching module (Anachem). The sample pump, switching valve and manifold pump were all controlled by a microcomputer (BBC B+) via the user port (set for output).

Detection was by an end window photomultiplier tube (PMT; Thorn EMI 9789 QA operated at 1.1 kV) with the window placed flush against the flow cell. The detector assembly, PMT and flow cell were encased in a radio frequency shielded, light-tight housing. Pump tubing was silicone rubber (Labsystems), all other tubing was PTFE (0.8 mm i.d.) (Anachem). All lines were sheathed in black silicone rubber to prevent light piping. The output from the PMT was amplified by an inverting amplifier (Thorn EMI C634) and recorded on a strip chart recorder (Chessell BD 4004).

Chemiluminescence spectra

The instrumental set-up for the measurement of CL spectra is outlined in Fig 2.2. Peristaltic pumps (Gilson Minipuls 2) were used to propel the carrier (0.4 ml min^{-1}) and CL reagent streams (0.9 ml min^{-1} each). CL detection was carried with the following reagent conditions;

TCPO;

TCPO = 2.0×10^{-3} mol l^{-1} hydrogen peroxide = 1.75×10^{-1} mol l^{-1}

carrier = ACN-THF-imidazole buffer (2.5×10^{-2} mol l^{-1}, pH 7.5) (80:10:10)

DNPO;

DNPO = 0.5×10^{-3} mol l^{-1} hydrogen peroxide = 1.00×10^{-3} mol l^{-1}

carrier = ACN-THF-citrate buffer (5.0×10^{-2} mol l^{-1}, pH 3.0) (80:10:10)

Standards (20 µl) were injected (Rheodyne 5020 teflon rotary valve) into the carrier stream which merged at a teflon Y-piece (Omnifit) with the chemiluminogenic stream containing hydrogen peroxide and aryl oxalate solutions previously merged at a teflon Y-piece (Omnifit).

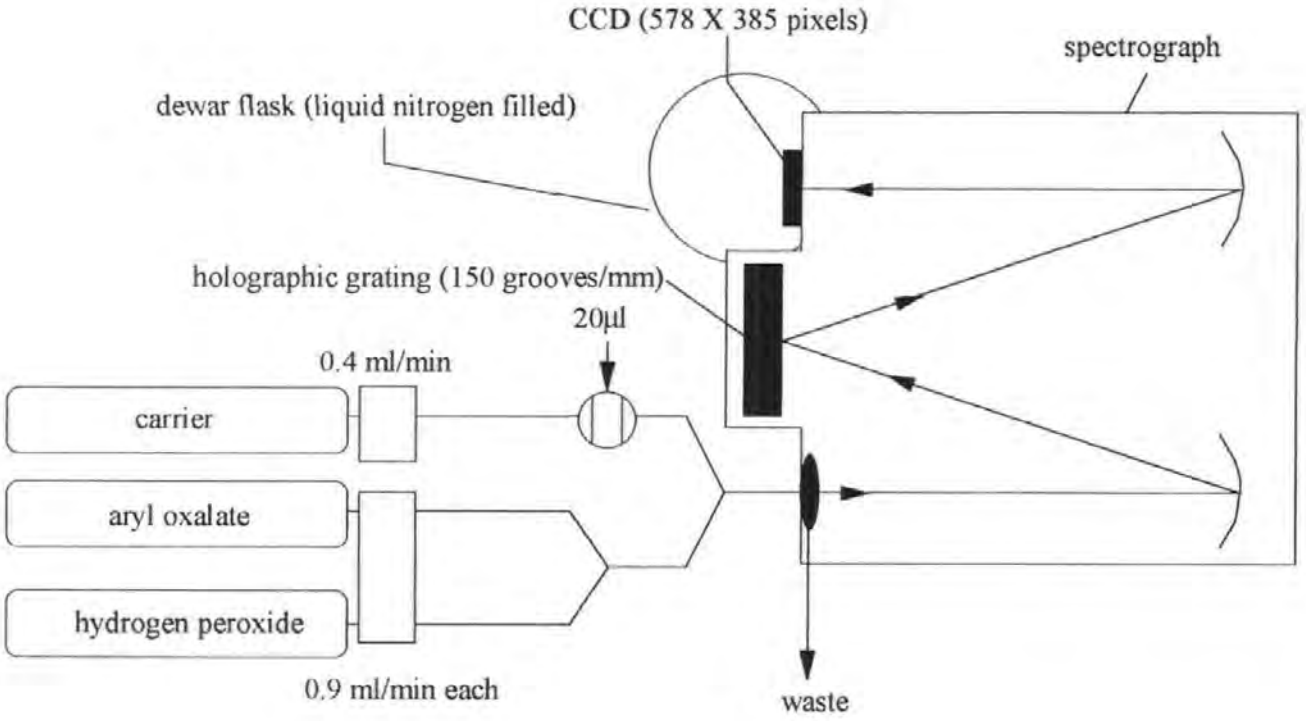


Fig 2.2 FI-CL manifold with spectrograph and CCD for the determination of CL spectra.

The merged stream passed into a glass coil flow cell (1.5 mm i.d., volume 240 µl) placed flush against the entrance slit of a spectrograph (270M imaging spectrograph, Spex Industries). Pump tubing was silicone rubber (Labsystems), all other tubing was PTFE (0.8 mm i.d.; Anachem). All lines were sheathed in black silicone rubber to prevent light piping.

In the spectrograph (focal length = 0.27 m, entrance aperture ratio f/4) incident radiation was dispersed with a blazed holographic grating (150 grooves/mm) and focused across a CCD chip (Spectrum One CCD, 578 x 385 pixels (12.5 x 8.5 mm), Spex Industries) placed at the focal plane in the exit port. The CCD was cooled to 140 K with liquid nitrogen.

DM 3000 spectroscopy software (Spex Industries) on a personal computer was used to control the spectrograph and acquire and manipulate the data generated.

2.3 RESULTS AND DISCUSSION

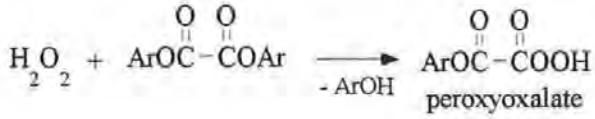
2.3.1 Peroxyoxalate chemiluminescence

CL from the reaction between oxalyl chloride and hydrogen peroxide in dioxane or benzene was first reported by Chandross in 1963 [139139]. Several fluorophores e.g. 1,10-diphenylanthracene (DPA) and *N*-methylacridone (NMA), were shown to sensitise the reaction. This lead Rauhut and co-workers to investigate the reactions of aryl oxalate esters and hydrogen peroxide, which resulted in the development of long-lived light sources for military and civilian use [140, 141140, 141].

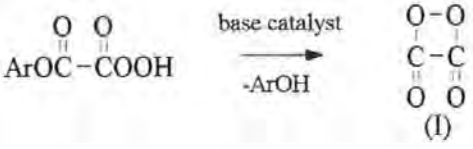
A wide range of fluorophores were found to act as sensitisers for the reaction and the quantum yields for the reaction were found to be higher than those for other synthetic CL reactions e.g. the reported quantum yield for the reaction of DNPO and hydrogen peroxide in dimethylphthalate as solvent and rubrene as a sensitiser was 0.23, compared to the quantum yields for the luminol and lucigenin reactions which are of the order of 0.01 [140, 142142]

Reaction mechanism

The mechanism for POCL is the subject of continuing debate [143]. A mechanism was originally proposed by Rauhut involving the formation of an excited state intermediate, 1,2-dioxetanedione, which excited the fluorophore by formation of a charge transfer complex [141]. This mechanism was modified by McCapra based upon chemically initiated electron exchange luminescence (CIEEL) [144-146].

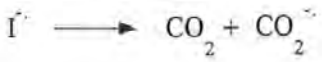


Step 1: oxidation of aryl oxalate to give peroxyoxalate

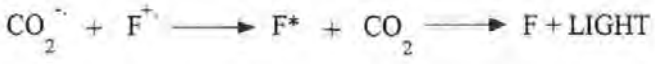


Step 2: formation of 1,2-dioxetanedione intermediate (I)

Step 3: formation of radical ion pair



Step 4: decomposition of 1,2-dioxetanedione to yield reduced carbon dioxide



Step 5: generation of excited state fluorophore

Fig 2.3 The CIEEL modified mechanism for POCL, where (I) is the 1,2-dioxetanedione and F is the fluorophore.

The CIEEL mechanism involves the formation of an ion pair by a bimolecular oxidation-reduction reaction (Fig 2.3). The ion pair consists of a 1,2-dioxetanedione radical anion and a fluorophore radical cation. The dioxetanedione decomposes to

yield a carbon dioxide radical anion which transfers its electron to the fluorophore radical cation generating the fluorophore in its singlet excited state.

This mechanism indicates that molecules with good fluorescence quantum yields and low oxidation potentials would give high CL efficiencies with the POCL reaction, this has been shown to be true with polycyclic aromatic hydrocarbons [147]. The Rauhut mechanism, with or without the McCapra modification has been used as the basic model for POCL by most authors. However the key 1,2-dioxetanedione intermediate has not been isolated, despite a number of attempts [148, 149]. Other intermediates and mechanisms have also been suggested [150-154].

Birks and co-workers have carried out extensive investigations on the kinetics of light production from the POCL reaction [155]. They found that in aqueous based solvents, the POCL reaction mechanism could be described in the simplified form;

$$A \xrightarrow{k_r} [B] \xrightarrow{k_f} C$$

where A and C represent "pools" of reactants and products respectively. [B] represents a "pool" of intermediates capable of exciting the fluorophore. In aqueous based solvents, the time-intensity profile is very closely described by a kinetic treatment which assumes that [B] is a single intermediate, k_r and k_f represent the rate constants for the rise and fall portions of the profile respectively. The kinetic expression for the time-dependant CL intensity, I_{CL} is given by the following equation;

$$I_{CL} = \frac{k_r M}{k_f - k_r}(e^{-k_r t} - e^{-k_f t}) \qquad \text{(equation A)}$$

where M represents the concentration of the intermediate(s). Expressions for the efficiency (Φ_{CL}), intensity at the maximum (J) and time required to reach maximum (τ_{max}) can be derived from equation A;

$$\Phi_{CL} = M/k_f$$

$$J = M(k_f/k_r)^{(k_f/[k_r - k_f])}$$

$$\tau_{max} = \{\ln(k_f/k_r)\}/(k_f - k_r)$$

This "pooled intermediate" kinetic approach was used to investigate the effect of five variables on the POCL reaction; (1) the nature and concentration of the aryl oxalate, (2) the concentration of the hydrogen peroxide, (3) the nature and concentration of the catalyst, (4) the pH and (5) aqueous-organic solvent mixtures. For each variable, values for k_r, k_f and M were obtained and those for J, τ_{max} and Φ_{CL} calculated [155].

From their results Birks and co-workers postulated a mechanism for POCL, shown in Fig 2.4 using the TCPO-hydrogen peroxide reaction with 9,10-diphenylanthracene (DPA) as the fluorophore [155]. They found that imidazole base catalyst played a key role in conversion of the aryl oxalate into the peroxyoxalate. Two routes to the peroxyoxalate intermediate are possible, competing with hydrolytic reactions of the oxalate. There are also a number of routes possible from the intermediate, either light-producing or non-light-producing.

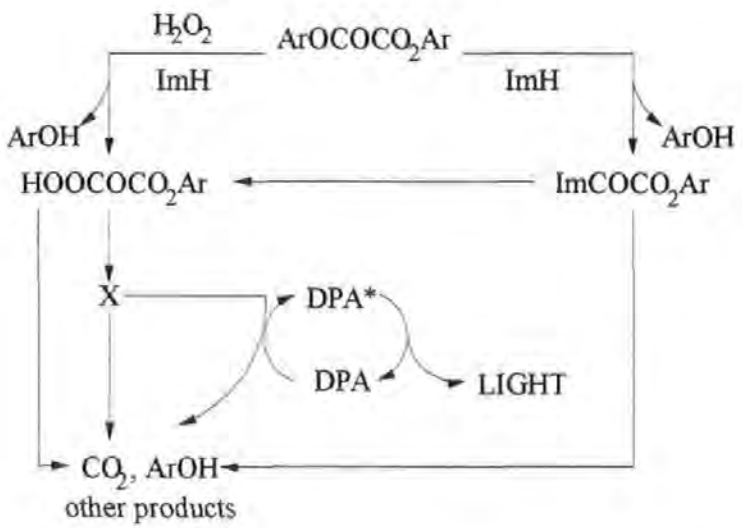


Fig 2.4 POCL mechanism as proposed by Givens *et al.* [155]. ImH = imidazole, Ar = 2,4,6-trichlorophenyl.

While this work has provided mechanistic understanding of the POCL reaction, further research is still required to determine the nature of the intermediate(s). New aryl oxalates could be synthesised on the basis of this information leading to the development of POCL systems with higher quantum yields and thus higher sensitivities.

Analytical applications

Hydrogen peroxide, fluorescent species and species which quench the emission may been determined with POCL. As the emission has a relatively long lifetime it is compatible with batch analysers. However it is more often used with flowing streams, i.e. FI and LC. Determinations using POCL are summarised in Table 2.1.

Direct application of POCL to the determination of species in real samples poses problems due to potential interferences from other sample components. Selectivity can be introduced by on-line treatments with FI, however POCL has found wider use as a post column reaction detection system for LC. POCL with LC can be used for the direct analysis of fluorescent species e.g. polycyclic aromatic hydrocarbons [156156] and polycyclic aromatic amines [147]. It is more commonly coupled with pre-column selective fluorescence derivatisation. POCL is most efficient for easily oxidised sensitisers like polycyclic aromatic amines, making it more selective than fluorescence and hence giving less complex chromatograms [147].

Reaction conditions

Optimisation of the POCL reaction for FI or LC requires the "fine-tuning" of reaction conditions to capture the maximum intensity of emission in the flow cell with minimum light loss before and after the cell. The following factors have been established as having the greatest effect upon emission intensity and the speed of the reaction [155], and therefore should be investigated in optimisation of the reaction for detection in flowing streams;

1. the nature and concentration of the aryl oxalate.
2. concentration of hydrogen peroxide.
3. the nature of mobile phase, mobile phase modifiers and CL reagent solvents.
4. the presence and concentration of base catalyst.
5. pH.

Table 2.1 Analytical applications of the POCL reaction

Analyte	Comments	Matrix	Limits of detection[a]	Ref.
Liquid chromatography				
Dansyl amino acids	TCPO, hydrogen peroxide	synthetic	10 fmol	157
Polycyclic aromatic hydrocarbons (PAH)	TCPO, hydrogen peroxide	coal tar	perylene 0.77 pg	156
Amino-PAH	TCPO, hydrogen peroxide	shale oil, coal oil, coal gasifier tar	1-aminoperylene 0.11 pg	147
Nitro-PAH	On-line zinc reductor column, reduces nitro-PAH to corresponding amino-PAH, TCPO, hydrogen peroxide	shale oil	> 3 pg	158
Aldehydes and ketones	Pre-column derivatisation with 3-aminofluoranthene, TCPO, hydrogen peroxide	synthetic	fmol	159
PAH	DNPO, hydrogen peroxide	biomass fuels	benzo[*a*]pyrene 10 ng ml^{-1}	160
Carboxylic acids	Pre-column derivatisation with 3-aminoperylene, TCPO, hydrogen peroxide	synthetic	1-10 fmol	161

[a]limits of detection as specified in original paper

Table 2.1 (continued) Analytical applications of the POCL reaction				
Analyte	Comments	Matrix	Limits of detection[a]	Ref.
Flow injection				
Hydrogen peroxide	TCPO solid state reactor, immobilised 3-aminofluoranthene	rain water	1.5×10^{-8} mol l^{-1}	162
Glucose	METQ, rhodamine B, hydrogen peroxide generated enzymatically from glucose	urine	7×10^{-8} mol l^{-1}	163
Histamine	TDPO, sulphorhodamine 101, hydrogen peroxide	fish	7×10^{-10} mol l^{-1}	164
NO^{2-}, SO^{2-}, organosulphur compounds, anilines	TCPO solid state reactor, immobilised 3-aminofluoranthene, hydrogen peroxide, detection by quenching of CL reaction	synthetic	2.8 ng NO^{2-} 1.1 ng thiourea 1.4 ng 3-ethylaniline	165
Dansylalanine	TCPO/DNPO, hydrogen peroxide	synthetic	5 fmol	166
Pyrimido(5,4-*d*)pyrimidines, e.g. dipyridamole	TCPO, hydrogen peroxide	synthetic	not given	167
Amino-PAH	TCPO, photo initiated	synthetic	9 pg 6-aminochrysene 6.6 pg 1-aminopyrene 8.5 pg 1-aminoanthracene	168

[a]limits of detection as specified in original paper

Table 2.1 (continued) Analytical applications of the POCL reaction				
Analyte	Comments	Matrix	Limits of detection[a]	Ref.
Miscellaneous				
Glucose	Stopped flow, TCPO, perylene, hydrogen peroxide generated enzymatically from glucose	urine	not given	169
NADH	Air segmented flow, TCPO, perylene, in presence of methylene blue NADH reduces oxygen to hydrogen peroxide	synthetic	2×10^{-7} mol l^{-1}	170
Dansyl-amino acids	TLC, plate sprayed with solutions of TCPO and hydrogen peroxide	synthetic	not given	171
Glucose	Batch analyser, TCPO, perylene, hydrogen peroxide generated enzymatically from glucose, CL enhanced by surfactant	synthetic	6×10^{-7} mol l^{-1}	172
Urinary oxalate and porphyrins	Batch analyser; 1. urinary oxalate-hydrogen peroxide-diphenylanthracene, 2. urinary porphyrins-DNPO-hydrogen peroxide	urine	1. oxalate 10 nmol l^{-1} 2. porphyrins 200 µg l^{-1}	173
Dansyl amino acids	Capillary electrophoresis, TCPO, hydrogen peroxide	synthetic	average 1.2 fmol	174
Urinary coproporphyrin	Batch analyser, TCPO, DNPO, hydrogen peroxide	urine	not given	175

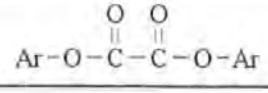
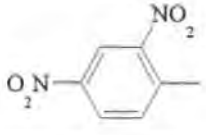
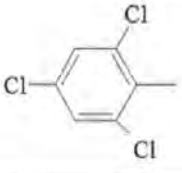
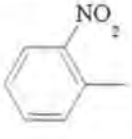
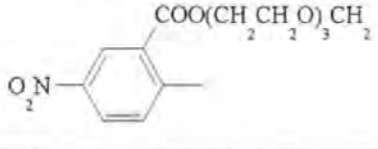
[a]limits of detection as specified in original paper

A number of studies of POCL reaction conditions have been reported using batch [157, 166, 176], stopped flow [177, 178] and FI [179-182] procedures. While batch and stopped flow approaches can give useful fundamental data, they do not simulate the conditions found in LC as well as FI.

Aryl oxalates are best used in non-aqueous media as they are relatively insoluble and unstable under aqueous conditions. This influences the choice of solvents for the mobile phase. Choice of solvent has also to be considered for the solubilisation of the derivatised oil samples. Oils are generally insoluble in the polar solvents commonly used for reversed-phase LC, e.g. ACN, they are however soluble in THF and isopropyl alcohol. Therefore addition of these solvents to a mobile phase based on ACN will improve the solubility of the oil sample in the mobile phase.

TCPO and DNPO are the most widely used aryl oxalates, although alternative aryl oxalates have been investigated [178, 183, 184]. The structures of aryl oxalates that have been used analytically are presented in Table 2.2.

Table 2.2 Structures of analytically useful aryl oxalates

aryl oxalate general structure	
aryl oxalate	Ar =
bis(2,4-dinitrophenyl)oxalate DNPO	
bis(2,4,6-trichlorophenyl)oxalate TCPO	
bis(2-nitrophenyl)oxalate 2-NPO	
bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl]oxalate TDPO	

The advantages and disadvantages of DNPO and TCPO are summarised below in Table 2.3. The faster reaction kinetics for DNPO are due to the nitro substituent groups, which are more electron withdrawing than the chlorine groups for TCPO [185], they lead to a "chemical band-narrowing effect" in flow cells [179].

Table 2.3 Comparison of DNPO and TCPO detection in flowing streams

DNPO	TCPO
Advantages	
fast reaction kinetics	stable in solution when mixed with hydrogen peroxide
intense emission	lower background CL emission compared with DNPO
soluble in solvents compatible with reversed-phase LC	
Disadvantages	
unstable in solution due to hydrolysis	less soluble than DNPO in reversed-phase LC solvents
expensive	

A variety of solvents have been used for the dissolution of post-column CL reagents including ACN, acetone, ethyl acetate and dioxane, of these ACN is the most compatible with reversed-phase LC mobile phases.

Weak bases, such as triethylamine, tris(hydroxymethyl)aminomethane and imidazole, have been used to catalyse the peroxyoxalate reaction. As discussed above, imidazole plays a key role in the reaction mechanism as a nucleophilic base catalyst for the hydrolysis of the aryl oxalate esters to peroxyoxalate intermediates [155]. CL emission using TCPO [177] and bis(4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl)oxalate (TDPO) [186] was shown to be greatly enhanced by imidazole.

2.3.2 Univariate optimisation of the POCL reaction

To reduce the number of variables, the following conditions were selected; aryl oxalates (TCPO and DNPO), CL reagent solvent (ACN), mobile phase (ACN-THF-water (aqueous buffer) mixtures) and base catalyst (imidazole). The mobile phase was selected to simulate a typical mobile phase for the analysis of oil samples [187]. Perylene was used as the fluorophore as it gives a strong emission with the POCL reaction. The ranges over which the parameters were investigated were established using previously published studies [157, 166, 176-182].

Effect of imidazole

To investigate the effect of imidazole on TCPO and DNPO, the water content of the mobile phase was replaced by imidazole solutions (0-150 x 10^{-3} mol l^{-1}). As can be seen from Fig 2.5, imidazole enhances the emission from the TCPO CL reaction, the enhancement is dependant upon the concentration of imidazole.

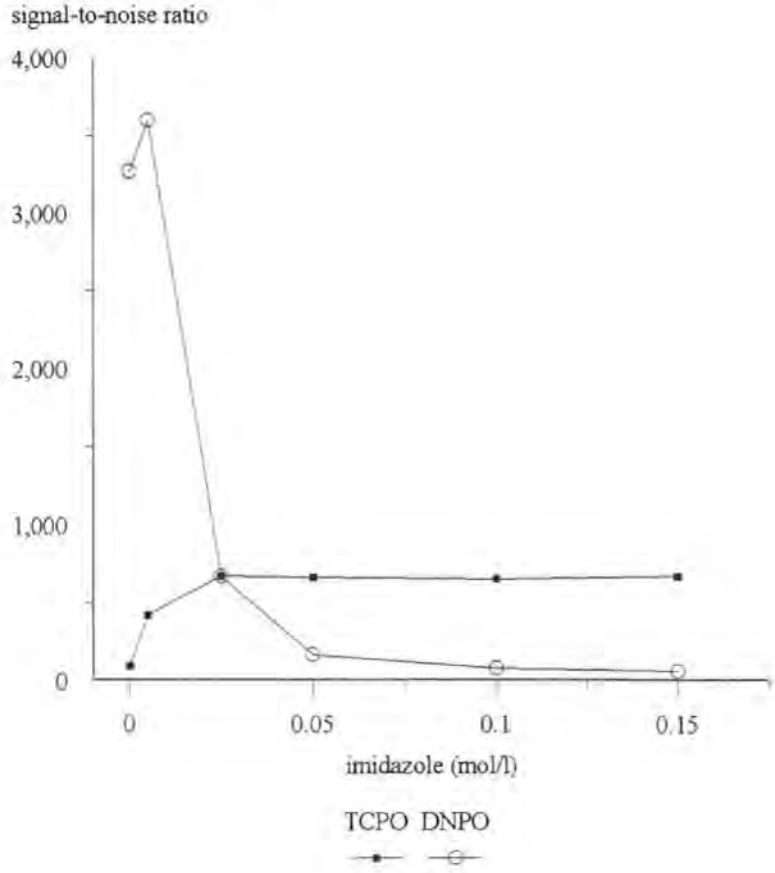


Fig 2.5 Effect of imidazole on the CL emission intensity for TCPO and DNPO

The optimum concentration of imidazole was 25 x 10^{-3} mol l^{-1}, this compares favourably with the reported results of a FI study, where the optimum imidazole concentration was determined to be 20 x 10^{-3} mol l^{-1}[177]. A concentration of 25 x 10^{-3} mol l^{-1} imidazole was used in subsequent experiments with TCPO.

No such enhancement was observed with DNPO, while higher concentrations of imidazole lead to a dramatic decrease in the signal-to-noise. This reduction of emission observed with DNPO may be a result of the maximum emission occurring before the flow cell, as imidazole has been reported to speed up the kinetics of POCL reactions [177].

pH

In addition to acting as a catalyst, imidazole acts as a buffer over the pH range 6.0-8.0 [177]. The effect of pH on TCPO was investigated over this range, the pH of the imidazole being adjusted with nitric acid as NO_3^- has been shown not to effect the TCPO reaction [166]. The optimum pH was found to be 7.5, which is in good agreement with previously reported studies [181]. DNPO has been reported as having a more acidic optimum pH of 3.5. Using citrate buffer (5.0 x 10^{-2} mol l^{-1}) the optimum pH for DNPO was found to be 3.0. These optimum pH of 7.5 for TCPO and 3.0 for DNPO were used in all subsequent experiments.

Concentration of CL reagents

The effect of aryl oxalate concentration on the CL emission intensity was investigated for TCPO and DNPO, the results are shown in Fig 2.6 (a), the narrower concentration range for TCPO was due to the insolubility of TCPO in ACN above 2.0 x 10^{-3} mol l^{-1}. As can be seen from Fig 2.6 (a), after reaching a maximum at 1.0 x 10^{-3} mol l^{-1} for both aryl oxalates, the signal-to-noise ratio levels off, and in the case of DNPO decreases. This is due to increased noise caused by higher concentrations of phenolic products of the reaction, trichlorophenol (TCP) and dinitrophenol (DNP). DNP has been reported to give more interference with POCL than TCP [188], which would explain the fall in signal-to-noise that has been observed.

Hydrogen peroxide concentrations over the range 1.0 x 10^{-2} - 0.15 mol l^{-1} were investigated, and the results are shown in Fig 2.6 (b). The decrease in response for DNPO at higher concentrations of hydrogen peroxide has also been observed by De Jong et al [179], who attributed it to the instability of DNPO after mixing with the hydrogen peroxide stream. However it has been shown that an increase in the aqueous fraction of the reaction mixture will speed up the reaction kinetics of POCL reactions [177], therefore it is more likely that the maximum emission intensity occurs before the flow cell due to the increase in the water content of the reaction mixture from the hydrogen peroxide solution.

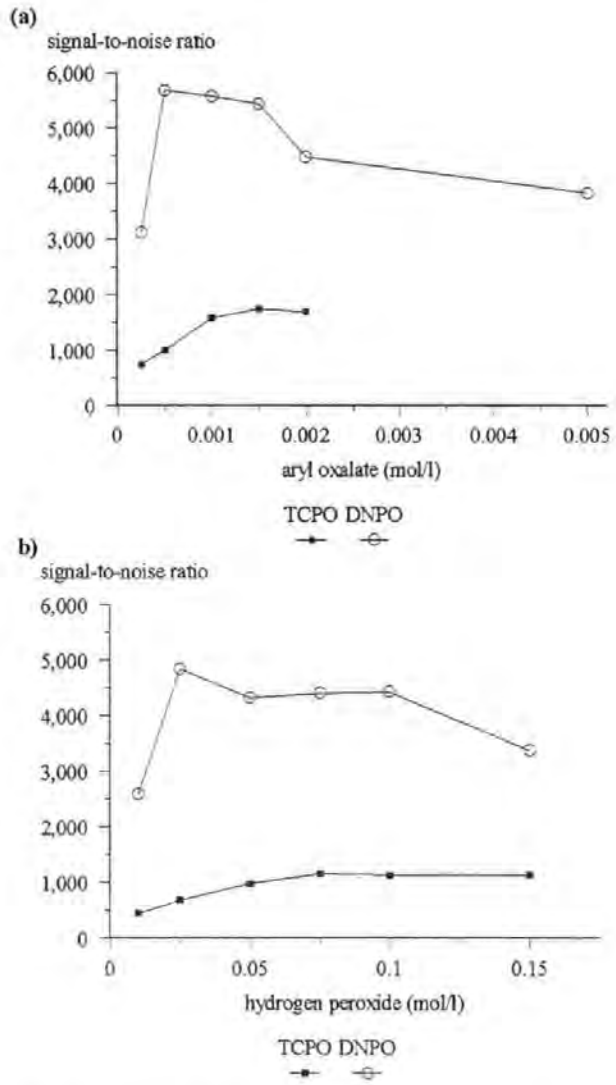


Fig 2.6 Effect of CL reagent concentration on POCL
(a) aryl oxalate (b) hydrogen peroxide (for experimental conditions see p 51).

Effect of mobile phase modifiers

Methanol and THF were both investigated for their effect on the POCL reaction. Methanol and THF were separately added to the mobile phase, replacing acetonitrile. It was found that both solvents reduce the emission intensity for TCPO and DNPO (Table 2.4). The amount of solvent is quoted in the final reaction solvent to allow comparison with other reports. Methanol is a nucleophilic solvent and is therefore able to consume the aryl oxalate in non-chemiluminescent side reactions. It has been reported by Hanoaka and co-workers to halve the CL emission intensity at concentrations as low as 1 % [177]. Their investigations were carried out under highly aqueous conditions (20 % v/v in final reaction mixture), but with the higher percentage of organic solvent used for this work, the quenching was not as severe.

Table 2.4 Effect of mobile phase modifiers on POCL emission intensity

Methanol		
Methanol in final reaction solvent (% v/v)	signal-to-noise TCPO	signal-to-noise DNPO
0.0	1231	3817
1.7	1207	3455
3.3	1048	3113
5.0	410	2277
THF		
THF in final reaction solvent (% v/v)	signal-to-noise TCPO	signal-to-noise DNPO[a]
3.3	1390	1424
5.0	1149	1342
6.7	796	1075
10.0	717	787

[a]the measurements for DNPO for the investigation of THF are low due to a temporary loss in sensitivity of the PMT

2.3.3 Simplex optimisation of the POCL reaction

The previous experiments are univariate optimisations, i.e. one parameter is investigated while the others are kept fixed. This method is unsatisfactory if interaction occurs between two or more of the parameters. An alternative multivariate approach is simplex optimisation [189, 190].

The relationship between response and the levels of parameters may be plotted to give a response surface. For two parameters this can be represented by a contour diagram (Fig 2.7). A simplex is a geometrical figure with N+1 vertices with respect to N parameters, i.e. for two parameters the simplex is a triangle. For two parameters the initial simplex is defined by three points which represent three sets of experimental conditions. The response is measured at each of these points. The point associated with the worst response is reflected through the centroid of the other points. The response is then measured for the new set of experimental conditions. This process is repeated and the simplex migrates towards the optimum (Fig 2.7).

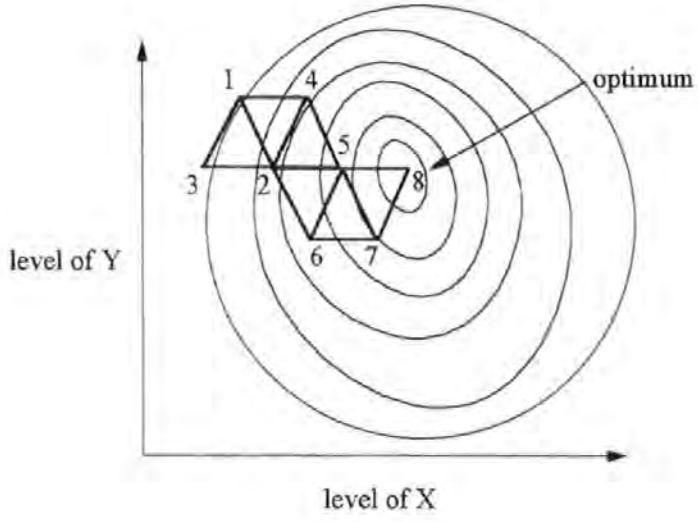


Fig 2.7 Simplex optimisation of two variables, X and Y

To improve the performance, the basic simplex method has been modified [191]. The modified procedure uses a simplex which expands or contracts depending on the comparison of the response of the new point with previous points. Simplex

optimisations have found wide application in many areas of analytical chemistry [189].

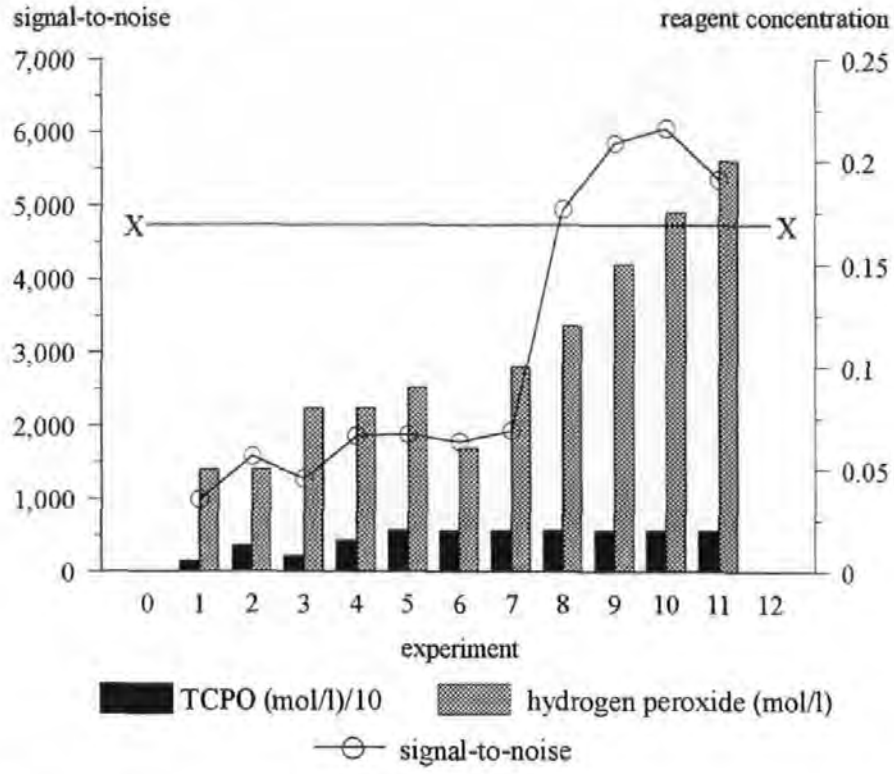
A modified simplex program [191] was used to optimise the TCPO and DNPO reaction conditions for the determination of perylene by FI. For simplicity the flow rates, pH and buffer concentration were kept constant, at the optimum values determined in the previous experiments, only the concentrations of the CL reagents were altered. The simplex variables used for TCPO and DNPO are detailed in Table 2.5.

Table 2.5 Simplex variables for the optimisation of POCL detection for FI

TCPO				
Variable	Precision	Maximum	Minimum	Step size
TCPO (mol l^{-1})	0.25×10^{-3}	2.00×10^{-3}	0.50×10^{-3}	0.75×10^{-3}
Peroxide (mol l^{-1})	10×10^{-3}	120×10^{-3}	50×10^{-3}	30×10^{-3}
Response = signal-to-noise			Precision of measurement = 0.25	
DNPO				
Variable	Precision	Maximum	Minimum	Step size
DNPO (mol l^{-1})	0.5×10^{-3}	5.00×10^{-3}	0.50×10^{-3}	1.5×10^{-3}
Peroxide (mol l^{-1})	20×10^{-3}	200×10^{-3}	20×10^{-3}	60×10^{-3}
Response = signal-to-noise			Precision of measurement = 0.25	

These parameters were chosen on the basis of the univariate experiments. The upper limit for the TCPO concentration was fixed due to the insolubility of the aryl oxalate in acetonitrile above this concentration. The simplex histories for TCPO and DNPO are presented in Fig 2.8 (a) and Fig 2.8 (b) respectively.

(a)



(b)

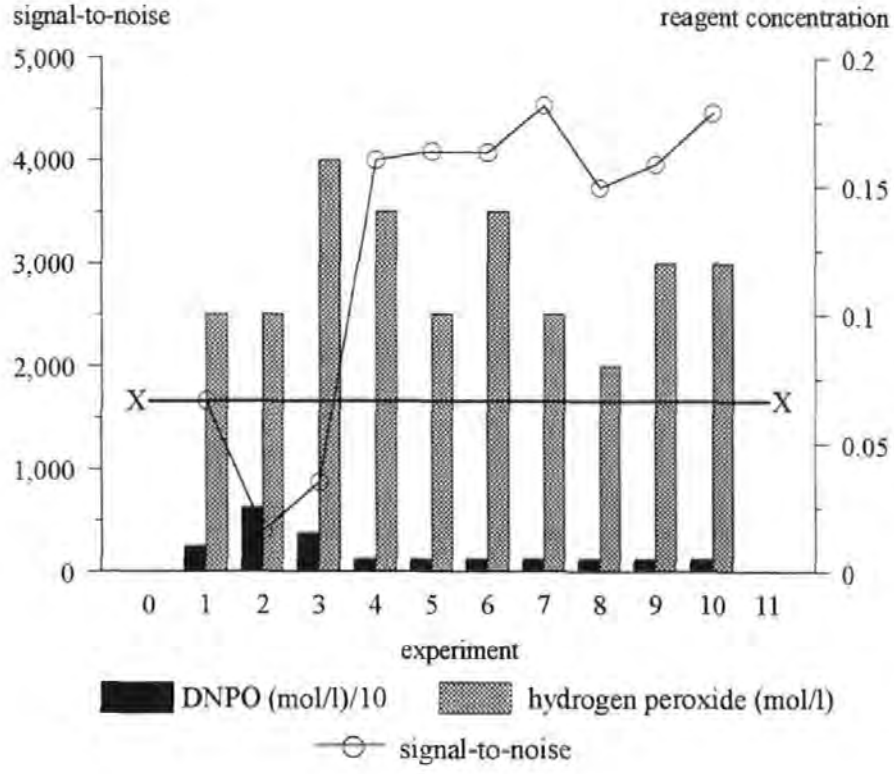


Fig 2.8 Simple histories (a) TCPO (b) DNPO, in both cases X-X is the signal-to-noise under the modified standard conditions (see text)

The optimum conditions for TCPO and DNPO are shown in Table 2.6. Hydrogen peroxide concentration has the greatest effect upon the size of the response.

Table 2.6 Optimised conditions for POCL detection for FI		
	TCPO	DNPO
Concentration of aryl oxalate (mol l-1)	2.0×10^{-3}	0.5×10^{-3}
Concentration of hydrogen peroxide (mol l-1)	1.75×10^{-1}	1.0×10^{-1}
Improvement in signal-to ratio (%)[a]	28	173

[a]the optimum conditions were compared with the signal-to-noise obtained under the following modified standard conditions; aryl oxalate 1×10^{-3} mol l^{-1}, hydrogen peroxide 1×10^{-1} mol l^{-1}, mobile phase for TCPO; ACN-THF-imidazole buffer (pH 7.5), mobile phase for DNPO; ACN-THF-citrate buffer (pH 3.0).

2.3.4 Calibration Data

The optimum conditions for TCPO and DNPO were used to carry out calibrations with the fluorophores 9-anthracenemethanol (1.0×10^{-7}-1.0×10^{-5} mol l^{-1}) and 3-aminofluoranthene (1.0×10^{-9}-1.0×10^{-7} mol l^{-1}). These fluorophores were chosen as they have been reported as CL labels for carboxylic acids and aldehydes respectively. Regression data is presented in Table 2.7 while calibration data is presented in Table 2.8. For both fluorophores DNPO was found to give the lower limits of detection, but the linear range for DNPO was restricted (Table 2.7)

Table 2.7 Regression data for FI determination of fluorophores with POCL detection				
Fluorophore	Linear fit, x = fluorophore, (mol l^{-1})	r^2	limit of detection (s/n=3, mol l^{-1})	linear range (mol l^{-1})
TCPO				
9-anthracenemethanol	$s/n=2.52 \times 10^{-7}x + 0.13$	0.9998	1.1×10^{-7}	$0\text{-}1.0 \times 10^{-5}$
3-aminofluoranthene	$s/n=6.47 \times 10^{-9}x + 0.88$	1.0000	6.4×10^{-10}[a]	$0\text{-}1.0 \times 10^{-7}$
DNPO				
9-anthracenemethanol	$s/n=6.64 \times 10^{-7}x + 0.30$	0.9982	4.1×10^{-8}	$0\text{-}5.0 \times 10^{-6}$
3-aminofluoranthene	$s/n=9.84 \times 10^{-9}x + 4.1$	0.9991	9.3×10^{-11}[a]	$0\text{-}2.0 \times 10^{-8}$

[a]s/n = 5

Table 2.8 Calibration data for FI determination of fluorophores with POCL detection				
Aryl oxalate	**TCPO**		**DNPO**	
Fluorophore concentration (mol l^{-1})	Signal-to-noise	Relative standard deviation (%, n=3)	Signal-to-noise	Relative standard deviation (%, n=3)
9-anthracenemethanol				
1.0×10^{-7}	3.1	3.1	10.5	2.7
2.0×10^{-7}	4.9	4.5	15.7	1.6
5.0×10^{-7}	12.2	2.4	-	-
1.0×10^{-6}	24.7	1.8	64.2	2.5
3.0×10^{-6}	75.1	2.2	190.4	1.2
5.0×10^{-6}	128.3	2.8	337.7	2.6
1.0×10^{-5}	250.7	0.5	1558.5	4.7
3-aminofluoranthene				
1.0×10^{-9}	5.4	8.9	14.3	6.0
4.0×10^{-9}	26.0	1.6	44.3	4.8
8.0×10^{-9}	53.5	4.9	79.3	1.4
1.0×10^{-8}	66.5	3.4	104.7	3.5
2.0×10^{-8}	131.4	3.3	200.9	3.7
1.0×10^{-7}	647.2	7.1	2834.9	0.3

2.3.5 Chemiluminescence spectra

CL background spectra

The main source of noise for POCL is the background emission which is always present when an aryl oxalate and hydrogen peroxide are mixed. The CL spectra obtained with the flow-system and CCD show broad emission bands centred on 600 nm for TCPO and 525 nm for DNPO, with DNPO giving a more intense signal than TCPO (Figs 2.9 and 2.10).

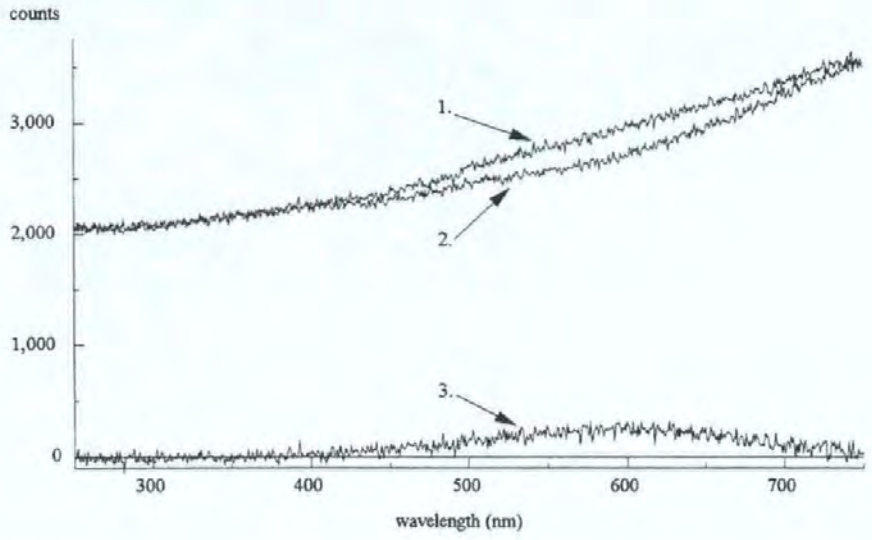
The spectra of the emission has been investigated by a number of workers using modified fluorimeters [156, 192]. It was established that the background was independent of the solvent and it was hypothesised that the emission was caused by intermediates in the reaction [156]. Grayeski and Mann investigated the background emission of four aryl oxalates including TCPO and DNPO; they found that all of the oxalates gave a maximum emission around 450 nm, with TCPO a second peak was observed at about 540 nm [192]. The spectra were measured by taking the average of ten scans of a static reaction solution, the work reported here gives a more accurate picture of the background emission that would be observed in a flow cell.

The analytical implication of these results is that sensitivity can be improved by use of a fluorophore emitting above 600 nm in conjunction with filters.[185]. An alternative to filters is to use the CCD detector with the monochromator set to monitor the CL wavelength of interest.

CL spectra of fluorophores

The spectrograph was used to measure the CL spectra of 9-anthracenemethanol (1 x 10^{-3} mol l^{-1}) and 3-aminofluoranthene (2 x 10^{-6} mol l^{-1}), which are presented in Fig 2.11. The spectra were acquired in 0.5 s as the CL peak reached a maximum in the flow cell.

(a)



(b)

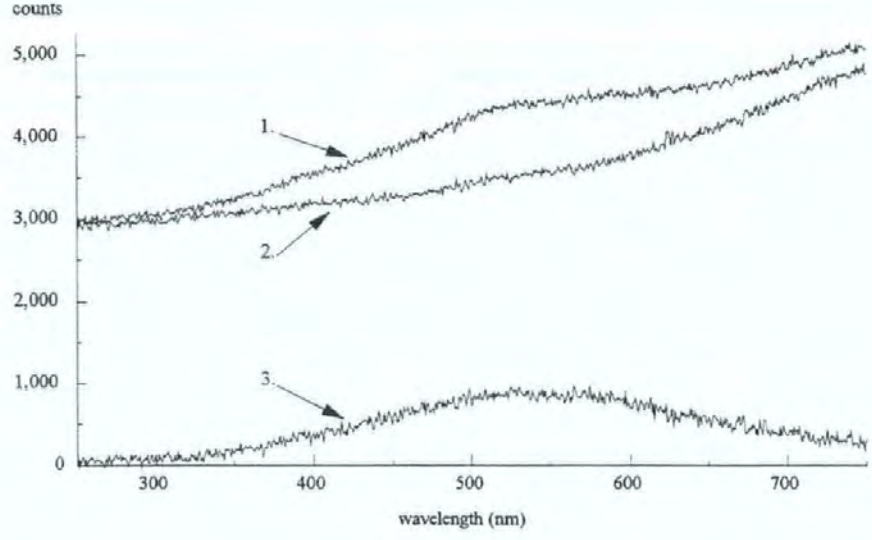


Fig 2.9 CL spectra of POCL background emission

(a) TCPO (2.0×10^{-3} mol l^{-1}) (b) DNPO (0.5×10^{-3} mol l^{-1}).
For both: 1. spectra of aryl oxalate + hydrogen peroxide + carrier stream, 2. spectra of hydrogen peroxide + carrier stream, 3. background subtracted CL spectra of aryl oxalate CL emission
(all spectra were acquired for 100 s)

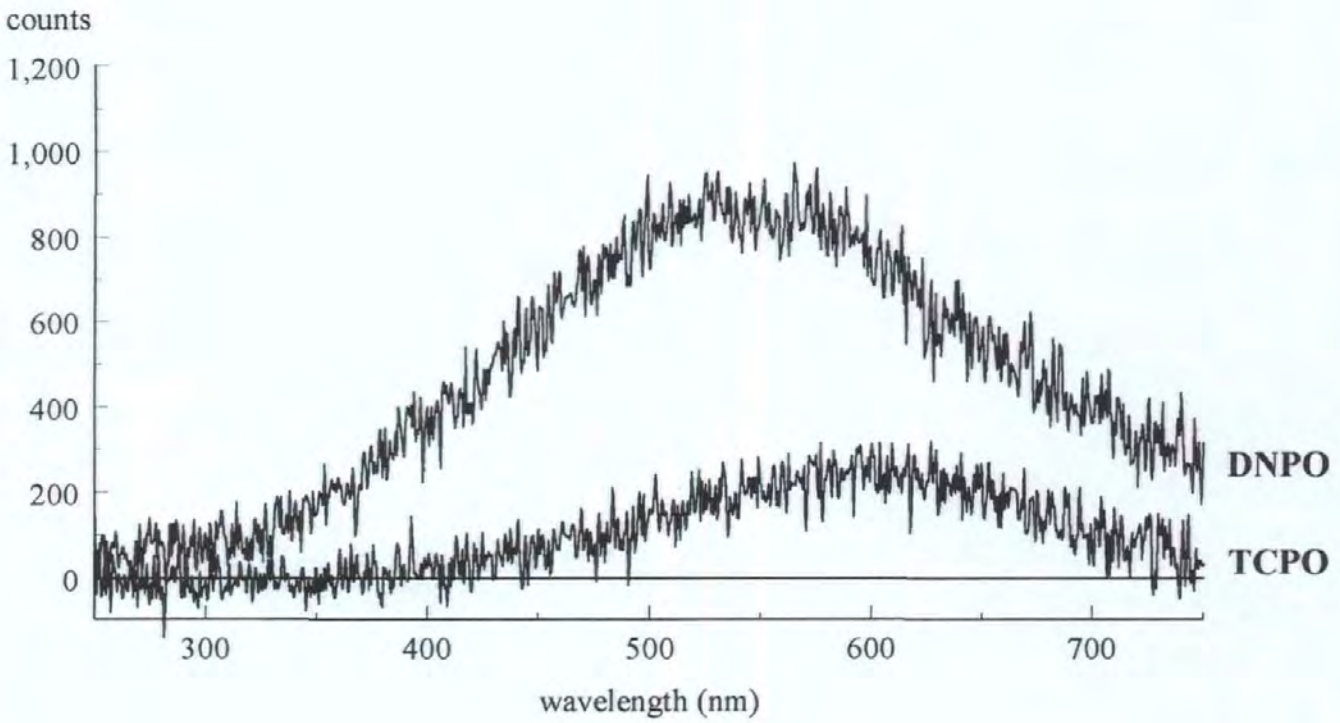
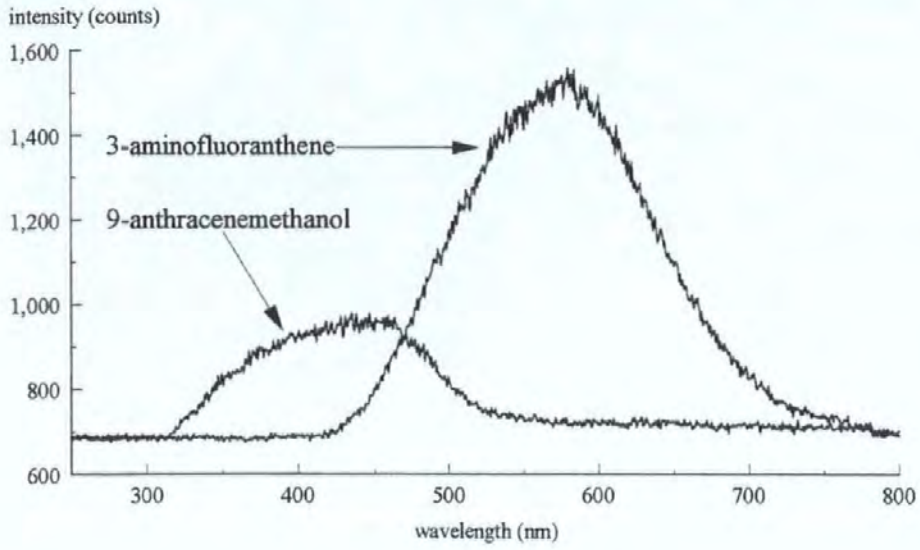


Fig 2.10 CL spectra of the background emission for TCPO and DNPO

(a)



(b)

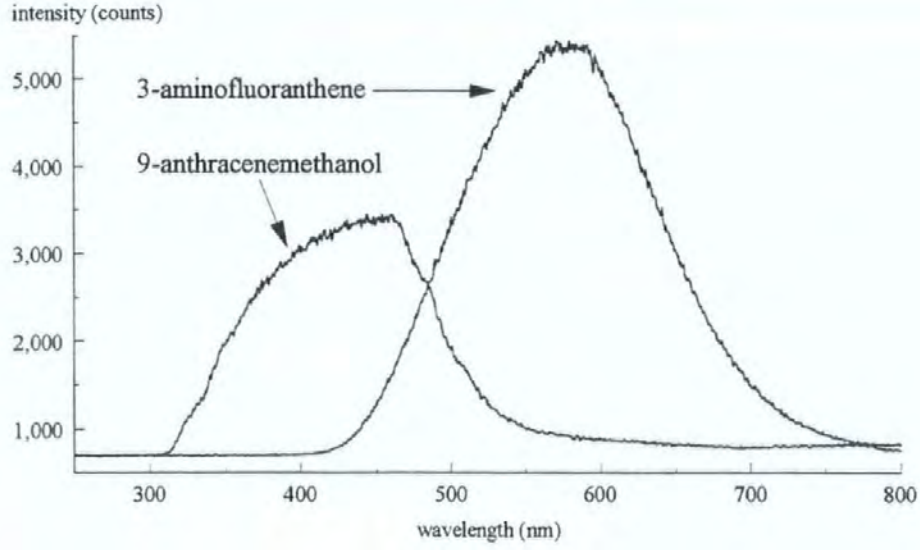


Fig 2.11 CL spectra of 3-aminofluoranthene and 9-anthracenemethanol (a) TCPO (b) DNPO

As can be seen the spectra obtained for the two fluorophores using DNPO and TCPO only differ in intensity, with the strongest emission being observed with DNPO. In addition, 3-aminofluoranthene gives a much more intense response at its maximum wavelength (about 600 nm) than the 9-anthracenemethanol. This is due to the more efficient excitation of amino PAH by POCL as discussed above. The wavelength of maximum emission for 3-aminofluoranthene is at the red end of the spectrum while interference from the matrix and native fluorescence is more significant at shorter wavelengths at the blue/UV end of the spectrum. This fluorophore therefore has potential as a label for the selective and sensitive determination of organic species by monitoring POCL emission with the spectrometer/CCD.

2.4 CONCLUSIONS

1. The presence of imidazole in the mobile phase greatly enhances the CL emission for TCPO.

2. The optimum pH for TCPO was 7.5, and for DNPO 3.0.

3. Methanol and THF were found to quench the emission for both aryl oxalates.

4. DNPO was more sensitive than TCPO for the determination of the fluorophores 9-anthracenemethanol and 3-aminofluoranthene, however TCPO gave wider linear ranges.

5. The use of a spectrometer with a CCD detector allows the rapid acquisition of CL spectra (0.5 s- 100 s).

Chapter 3

Determination of Carboxylic Acids in Used Engine Oils by Liquid Chromatography with Chemiluminescence Detection

3.1 INTRODUCTION

This chapter describes a procedure for the determination of carboxylic acids (benzoic and C_8-C_{16} straight chain aliphatic acids) in non-aqueous matrices. It is based on a simple and selective derivatisation of the acids with a fluorescent label followed by reversed phase liquid chromatography (LC) and post-column reaction detection with peroxyoxalate chemiluminescence (POCL).

The procedure was applied to the determination of carboxylic acids in oils before and after laboratory simulated engine oxidation. Carboxylic acids are thought to be major primary end products of oil oxidation in engines and, as such, are important indicators of the degree of degradation. A method to monitor individual carboxylic acids in the presence of other acidic species is therefore desirable. The information provided by such an analysis would be useful as an aid to the determination of the oxidation pathways taking place within the engine, as well as giving a more selective indicator of oil condition compared with traditional procedures.

3.2 EXPERIMENTAL

3.2.1 Reagents

High quality de ionised water from a Milli-Q system (Millipore) and analytical grade reagents were used unless otherwise stated. Acetonitrile (ACN), dichloromethane (DCM), *n*-heptane, and tetrahydrofuran (THF) were of HPLC grade (Rathburn).

Solutions of 9-anthracenemethanol (Fluka) and 4-pyrrolidinopyridine (Aldrich) were prepared daily in DCM. *N,N'*-Dicyclohexylcarbodiimide (DCC) was used as the solid. Solutions of octanoic (C_8), dodecanoic (C_{12}), hexadecanoic (C_{16}) acids (all Aldrich) and benzoic acid (BDH) were prepared in *n*-heptane. All carboxylic acid standards were of reagent grade.

Working solutions of bis(2,4-dinitrophenyl)oxalate (DNPO; Fluka) were prepared in ACN. Hydrogen peroxide solutions were prepared by dilution of a 100 volume aqueous stock solution (BDH) with ACN. Both chemiluminogenic reagents were

prepared daily. The LC mobile phase was prepared daily on a volume/volume basis and was degassed with helium immediately before use. Fresh and oxidised oil samples were supplied by Thornton Research Centre, Shell Research Limited, Chester, UK.

3.2.2 Instrumentation

A schematic diagram of the experimental configuration is given in Fig. 3.1. Samples (20 µl) were injected (Rheodyne 7010) into a mobile phase of ACN-THF-water (40:40:20 v/v/v) pumped at 0.7 ml min^{-1} (Altex 110A). Separation was achieved using a Spherisorb S5 ODS2-5 analytical column (5 µm, 250 x 4.6 mm i.d.) with a Spherisorb S5 ODS2-5 guard column (both PhaseSep).

The post-column reagents, DNPO (5.0×10^{-3} mol l^{-1}) and hydrogen peroxide (2.2×10^{-1} mol l^{-1}), were each pumped at 0.4 ml min^{-1} by a peristaltic pump (Gilson Minipuls 2) fitted with silicone pump tubing (Labsystems), through pulse dampers (2 m of PTFE tubing coiled around a steel rod) before being merged at a T-piece. The combined reagent stream was merged with the column eluate at a low dead volume stainless steel T-piece (Anachem) before passing into a lamina flow cell (volume 234 µl). Detection was by an end window PMT (Thorn EMI 9789QA operated at 1.1 kV) with the end window placed flush against the flow cell. The detector assembly, PMT and flow cell were encased in a radio frequency shielded, light-tight housing.

Stainless steel tubing was used for the connections between the LC pump and the columns; PTFE tubing was used for all other connections. The chemiluminogenic lines prior to the first T-piece were 0.8 mm i.d. and all other tubing was 0.15 mm i.d.. All PTFE lines were sheathed with black silicone rubber tubing to prevent light piping. The length of tubing between the second T-piece and the flow cell was kept as short as possible (6 cm). The output from the PMT was amplified by an inverting amplifier (Thorn EMI C634) and recorded on a strip chart recorder (Chessell BD 4004). Response was measured manually as peak heights, and noise measured as the amplitude of the baseline.

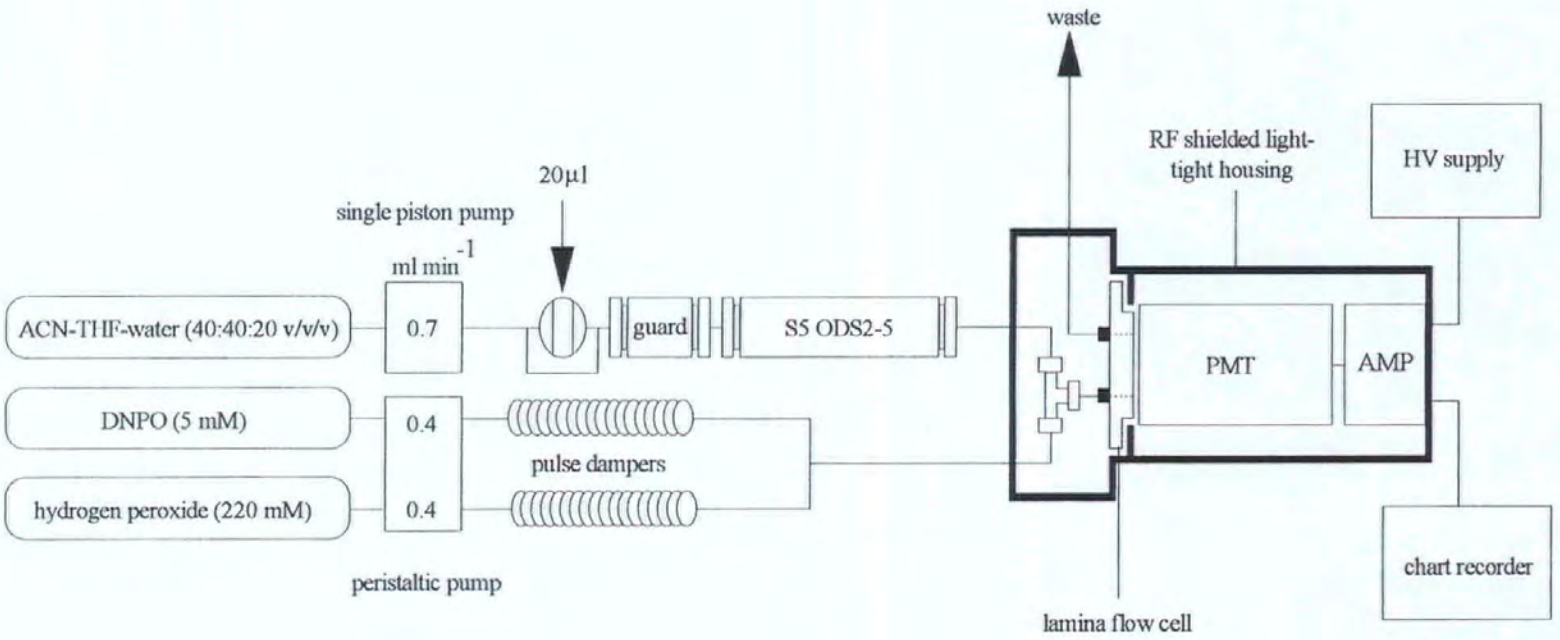


Fig 3.1 LC-CL manifold for the determination of carboxylic acids in non-aqueous media

3.2.3 Pre column derivatisation

The following reaction conditions were used unless otherwise stated. Acid standards in *n*-heptane, oil samples and oil samples spiked with acids (250 µl) were added to a mixture of DCC (0.25 g), 9-anthracenemethanol in DCM (2.1×10^{-2} mol l^{-1}, 1 ml), 4-pyrrolidinopyridine in DCM (1.0×10^{-2} mol l^{-1}, 1 ml) and *n*-heptane (15 ml) and heated under reflux for 15 min. The reaction mixture was allowed to cool for 5 min before the reaction solvent was removed by rotary evaporation. The residue was dissolved in ACN-THF (40:60 v/v, 25 ml).

3.3 RESULTS AND DISCUSSION

All concentrations relate to the original sample

3.3.1 Pre column derivatisation of carboxylic acids

As aliphatic carboxylic acids do not possess a chromophore or a fluorophore, derivatisation is needed to achieve the required selectivity and sensitivity for their determination. Derivatisation of carboxylic acids normally involves the formation of esters or amides with a derivatising reagent consisting of a chromophore or a fluorophore with a reactive end group, e.g. halogen, alcohol or amine. Tables 3.1 and 3.2 detail a selection of derivatising reagents for the determination of carboxylic acids by LC.

Esterification normally takes place by ionisation of the carboxylic acid followed by nucleophilic substitution. The carboxylate anion is a weak nucleophile and solvation of the anion in a polar protic solvent (water, methanol) would reduce its nucleophilicity. Esterifications are therefore generally carried out in polar aprotic solvents (acetone, ACN) [124]. The nucleophilic nature of the anion is often enhanced by neutralising the carboxylic acid with a base e.g. potassium carbonate and complexing the companion cation with a crown ether. This gives a "naked anion" which is extremely reactive (Fig 3.2) [124].

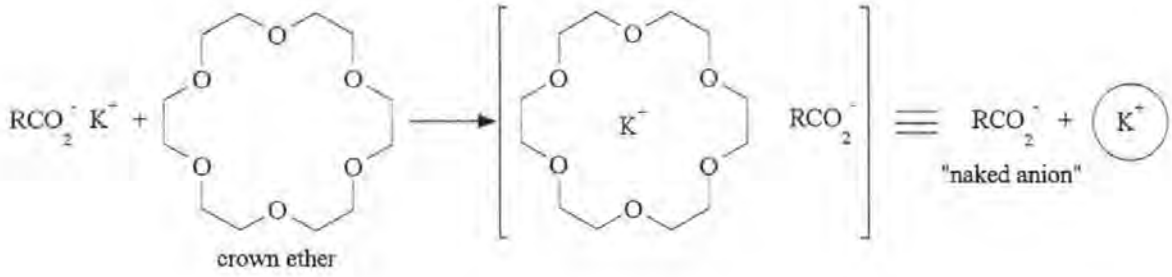


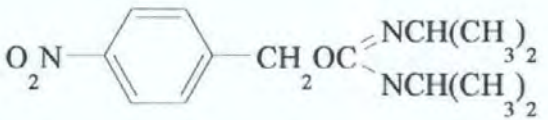
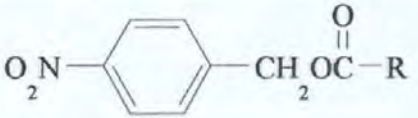
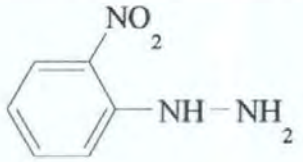
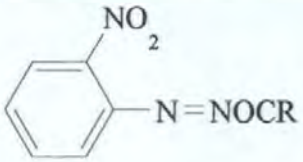
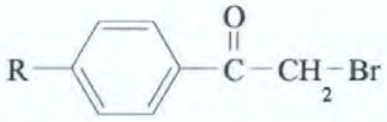
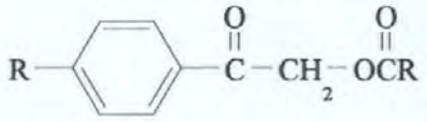
Fig 3.2 Catalysis of carboxylate anion alkylation by selective complexation of potassium cation with crown ether 18-crown-6

Phenacyl and naphthacyl halides have been widely employed for the esterification of carboxylic acids for ultraviolet absorbance detection. Commercial preparations containing label and catalyst are readily available [193].

Reagents with active halogens for carboxylic acid derivatisation based on 7-methoxycoumarin have been most widely used for fluorescence detection. 4-Bromomethyl-7-methoxycoumarin (Br-Mmc) [194] is the most common of the reagents of this type. Other coumarin derivatives have been developed to improve on the performance of Br-Mmc including 4-hydroxy-7-methoxycoumarin (Hy-Mmc) [195] and 4-bromomethyl-6,7-dimethoxycoumarin (Br-Mdmc) [196]. Post-column hydrolysis of 4-bromomethyl-7-acetoxycoumarin (Br-Mac) esters to yield 7-hydroxycoumarin has also been used [197]. Esterification of carboxylic acids has been carried out with reagents based on the fluorescent 6,7-dimethoxy-1-methyl-1(1H)-quinoxalinone nucleus [198].

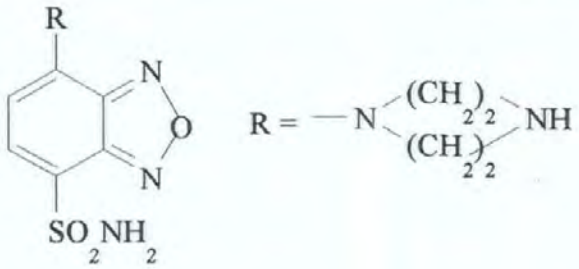
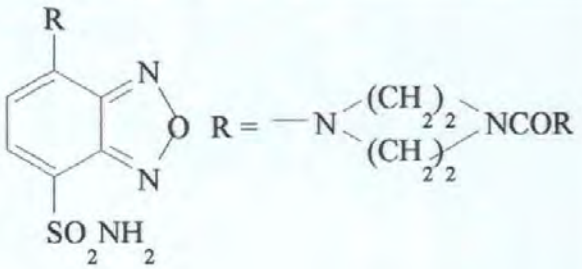
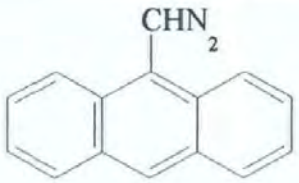
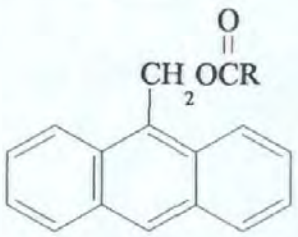
Reactive groups other than halogens have been used to esterify carboxylic acids. *O*-alkyl isoureas have been applied e.g. *o,p*-nitrobenzyl-*N,N'*-diisopropylisourea for UV detection [124, 199], *N,N'*-dicyclohexyl- and *N,N'*-diisopropyl-*O*-(7-methoxycoumarin-4-yl)-methylisourea [200] for fluorescence detection. Diazoalkane derivatives, e.g. 9-anthryldiazomethane, are highly reactive towards carboxylic acids in the absence of a catalyst [201].

Table 3.1 Derivatising reagents for the LC determination of carboxylic acids with UV absorbance and fluorescence detection

Label, mode of separation and detection, limits of detection[a]	Structure	Derivative (of carboxylic acid $R-\overset{O}{\overset{\|}{C}}-OH$)
UV absorbance detection		
o,p-nitrobenzyl-*N,N'*-diisopropylisourea [124, 199], heating in DCM for 2 h, normal-phase LC, UV detection (254 nm), detection limit 100 ng		
2-nitrophenylhydrazine [202], heating in aqueous/ethanolic solution with label (as hydrochloride) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride coupling reagent, reversed-phase LC, visible detection (400 nm) and UV detection (230 nm), 2.5-15 pmol per injection		
Phenacyl bromides, napthacyl bromides [203, 204] heating in ACN with label and crown ether catalyst for 15 min with stirring, normal or reversed-phase LC, UV detection (254 nm), nanogram level	 R = H, phenacylbromide R = Br, p-bromophenacylbromide	

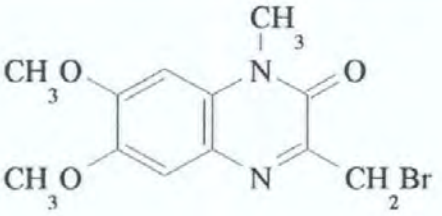
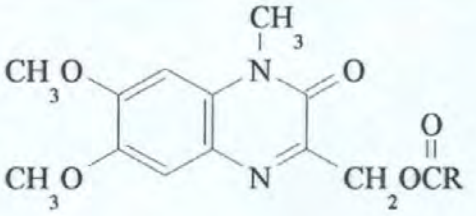
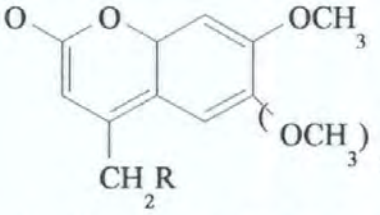
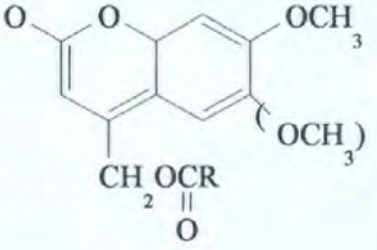
[a]limits of detection as quoted in original paper

Table 3.1 (continued) Derivatising reagents for the LC determination of carboxylic acids with UV absorbance and fluorescence detection

Label, mode of separation and detection, limits of detection[a]	Structure	Derivative (of carboxylic acid $R-\overset{O}{\overset{\|}{C}}-OH$)
Fluorescence		
4-(aminosulphonyl)-2,1,3-benzoxadiazole amine derivatives [205], dimethylformamide reaction solvent, diethyl phosphorocyanidate to activate carboxylic acid group, stand at room temperature for 6 h, reversed-phase LC, fluorescence detection (λ_{ex} = 440 nm, λ_{em} = 580 nm for arachidic acid derivative), 10-50 fmol.	 e.g. 4-(aminosulphonyl)-7-(1-piperizinyl)-2,1,3-benzoxadiazole (ABD-PZ)	
9-anthryldiazomethane [201], acid plus label in methanol, stand for 60 min, fluorescence detection (λ_{ex} = 365 nm, λ_{em} = 412 nm), pmol		

[a]limits of detection as quoted in original paper

Table 3.1 (continued) Derivatising reagents for the LC determination of carboxylic acids with UV absorbance and fluorescence detection

Label, mode of separation and detection, limits of detection[a]	Structure	Derivative (of carboxylic acid $R-\overset{O}{\overset{\|}{C}}-OH$)
3-bromomethyl-6,7-dimethoxy-1-methyl-1(1H)-quinoxalinone [198], heat in ACN with solid potassium carbonate and crown ether catalyst for 20 min in the dark, reversed-phase LC, fluorescence detection (λ_{ex} = 370 nm, λ_{em} = 450 nm), 0.3-1 fmol		
7-methoxycoumarin fluorophore with various reactive groups attached [194-196], heating in acetone with crown ether and potassium carbonate catalysts, reversed-phase LC, fluorescence detection (λ_{ex} = 365 nm, λ_{em} = 420 nm for Br-Mmc derivatives [124], fmol-low pmol.	 for Br Mmdc R = Br, Br-Mmc, Br-Mmdc, R = OH, Hy-Mmc	

[a]limits of detection as quoted in original paper

For reaction with amines, activation of the carboxylic acid is required by conversion to a compound with a better leaving group [44]. Amine derivatives of fluorescent benzofurazans (2,1,3-benzoxadiazoles) have been used to derivatise carboxylic acids using diethylphosphorocyanidate (DEPC) as an activator [205]. The DEPC converts the -COOH group to -COCN.

Chemiluminescence derivatisation

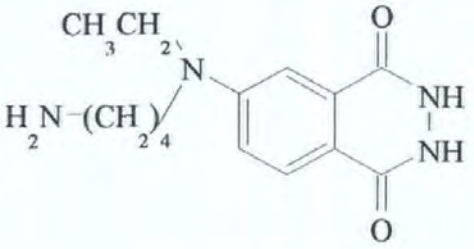
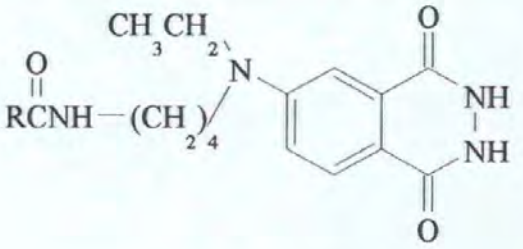
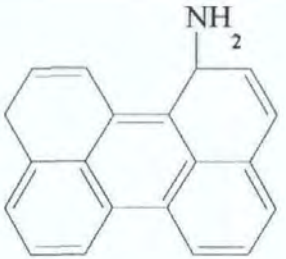
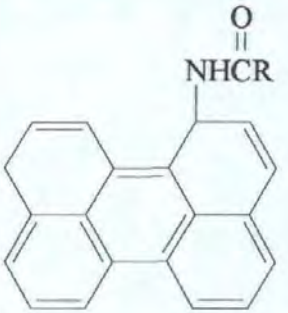
Compared with fluorescence and UV absorbance detection, CL has not been widely applied to the determination of carboxylic acids (Table 3.2). Most derivatisations for CL detection involve pre column labelling with a fluorescent tag that yields a CL response with the POCL reaction [161, 206-208]. An exception is the use of N-(4-aminobutyl)-n-ethylisoluminol (ABEI) [209].

ABEI is an derivative of luminol that reacts with hydrogen peroxide in the presence of metal catalyst in alkaline solution to produce CL. Carboxylic acids can be labelled with ABEI, using 2-chloro-1-methylpyridinium iodide (CMPI) to activate the carboxylic group. CL is produced post column by oxidation with hydrogen peroxide in the presence of potassium hexacyanoferrate (III) catalyst.

Carboxylic acids can be labelled with the amino substituted polycyclic aromatic hydrocarbon, 3-aminoperylene using DCC as a activation agent [161]. Amino-PAH, as discussed in section 2.3.1, give very intense emission with POCL. Dansyl derivatives are also very sensitively detected by POCL due to their low oxidation potentials and low singlet excitation energies [161]. *N*-(bromoacetyl)-*N'*-[5-(dimethylamino)naphthalene-1-sulphonyl]piperazine (Dansyl-BAP) has been used for the derivatisation of carboxylic acids with POCL detection [206]. Reagents based on coumarin and benzofurazan nuclei have also have been applied [207, 208].

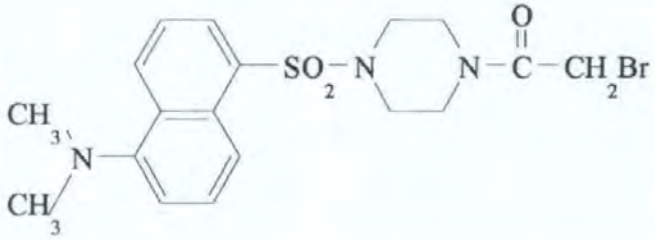
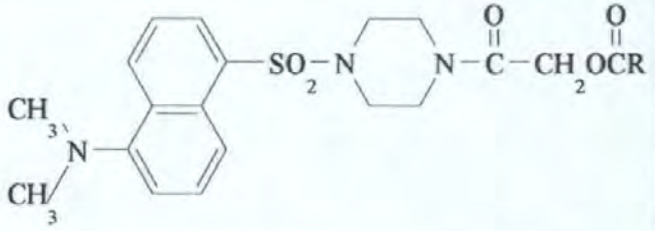
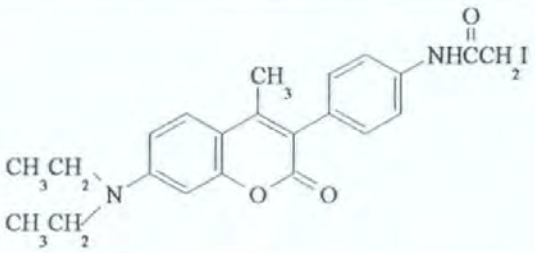
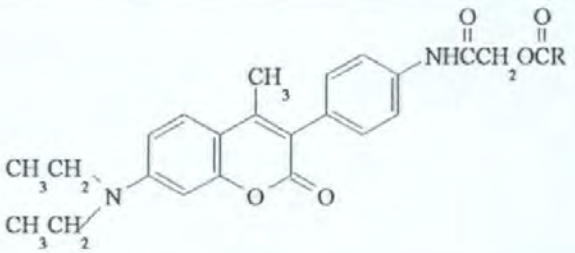
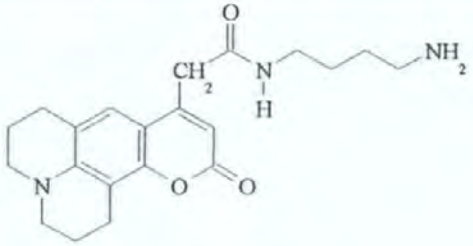
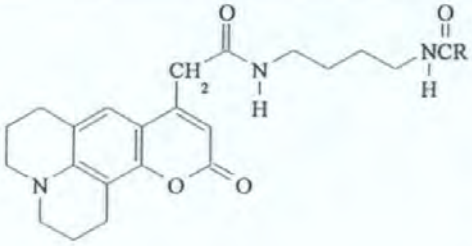
UV absorbance derivatisations are unsuitable for the LC analysis of oxidised oils due to the complexity of the oil matrix. As discussed in Chapter 2, POCL detection can give less complex chromatograms, e.g. it has been applied to the determination of fluorescent species in shale oil, coal oil and gas gasifier tar [147].

Table 3.2 Derivatising reagents for the LC determination of carboxylic acids with CL detection

Label, mode of separation and detection, limits of detection[a]	Structure	Derivative (of carboxylic acid $R-\overset{O}{\overset{\|}{C}}-OH$)
N-(4-aminobutyl)-n-ethylisoluminol (ABEI) [209], heating in ACN with 2-chloro-1-methylpyridinium iodide (to activate carboxylic acid) and base catalyst for 2 h, reversed-phase LC, CL detection by reaction with hydrogen peroxide-potassium hexacyanoferrate (III), cholic acid 20 fmol		
3-aminoperylene [161], heating in benzene with DCC and pyridine (base catalyst) for 2 h, reversed-phase LC, POCL detection (2-NPO-hydrogen peroxide), 0.1 fmol		

[a]limits of detection as quoted in original paper

Table 3.2 (continued) Derivatising reagents for the LC determination of carboxylic acids

Label, reaction, mode of separation and detection, limits of detection	Structure	Derivative (of carboxylic acid $R-\overset{O}{\overset{\|}{C}}-OH$)
N-(bromoacetyl)-*N'*-[5-(dimethylamino) naphthalene-1-sulphonyl]piperazine [206], potassium carbonate, crown ether catalyst, room temperature in ACN for 30 min, reversed-phase LC, POCL detection (2NPO-hydrogen peroxide), 25 fmol		
7-(diethylamino)-3-[4-((iodoacetyl)amino)phenyl]-4-methylcoumarin (DCIA) [207], heat in ACN with potassium carbonate and crown ether catalyst for 70 min, reversed phase LC, POCL detection (TCPO-hydrogen peroxide), low fmol detection limits		
Luminarin 4, quinolizinocoumarin fluorophore with amino reactive group [208], DCC, dimethylformamide solvent, 12 h at room temperature, reversed-phase LC, POCL detection (TCPO-hydrogen peroxide), detection limits 50 fmol		

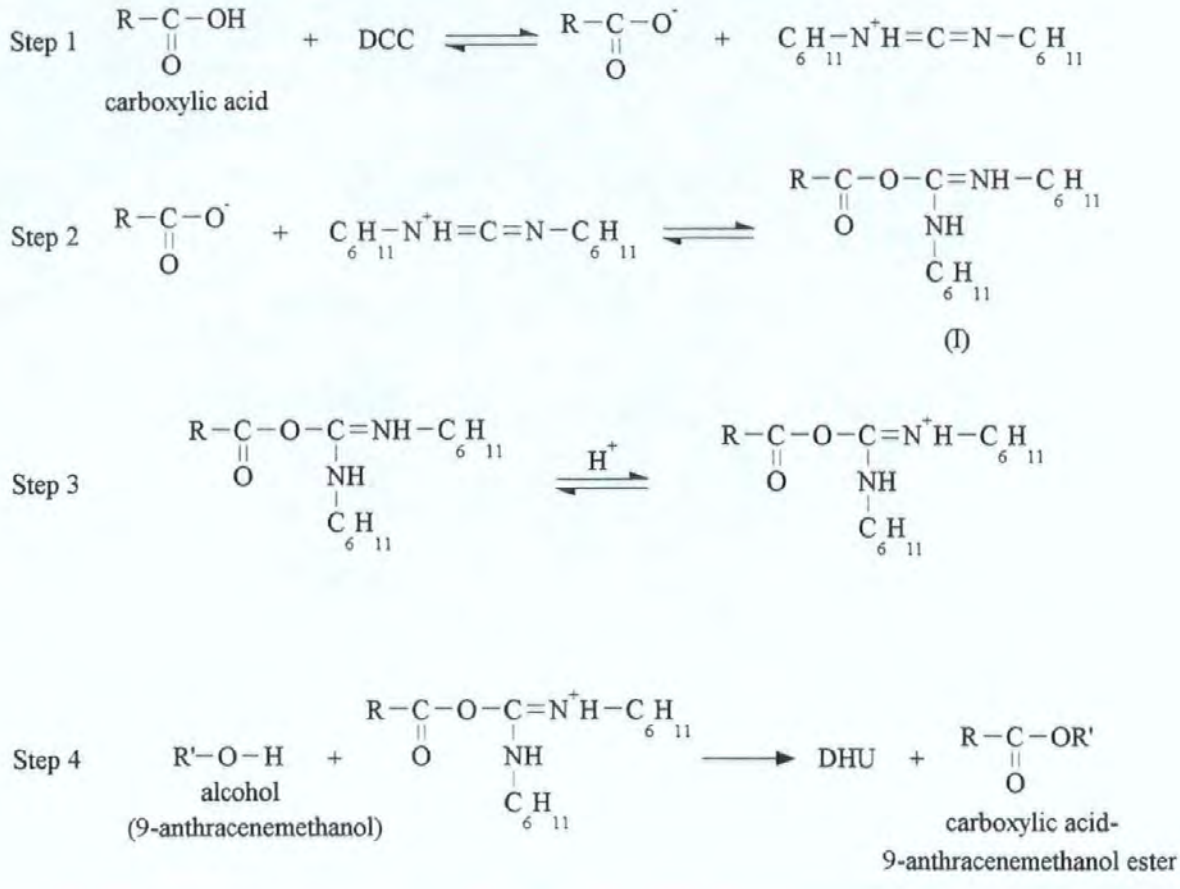
However the reactions detailed in Table 3.2 are unsuitable for the derivatisation of oil samples for a number of reasons. They either use toxic solvents (e.g. benzene) or polar solvents which are incompatible with oil samples. Most of the derivatisation procedures are lengthy and complex therefore rendering them unsuitable for regular use in a process environment.

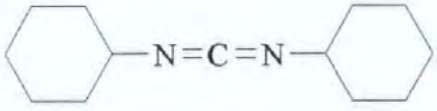
3.3.2 Esterification with 9-anthracenemethanol as a pre column derivatisation procedure

The fluorescent alcohol 9-anthracenemethanol can be coupled to carboxylic acids after activation of the carboxyl group and has been used for the fluorescence and laser-induced fluorescence detection of carboxylic acids [210, 211].

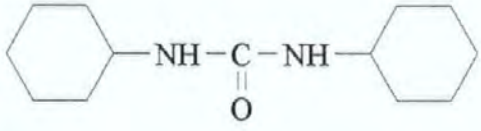
A procedure for the derivatisation of carboxylic acids in non-aqueous media with 9-anthracenemethanol and detection after separation by POCL has been described [212]. To attach the fluorophore to the acid, activation of the carboxyl function is achieved with DCC, which allows the esterification with primary and secondary alcohols under mild conditions [213]. Previously DCC has been used for peptide synthesis [214, 215] and for the isolation of fatty acids from oils and fats [216]. It has found along with other carbodiimides wide application in the determination of carboxylic acids e.g. labelling with 2-nitrophenyl hydrazine [217], fluorescent amines [202], 3-aminoperylene [161] and luminarin 4 (a quinolizinocoumarin derivative) [208].

The esterification reaction occurs according to a four step mechanism, which is outlined in Fig. 3.3 [218]. The carboxylic acid is converted to compound (I), an *o*-acylisourea. This has a better leaving group than the carboxyl acid, i.e. a substituted urea. Nucleophilic substitution of the 9-anthracenemethanol on (I) takes place eliminating dicyclohexylurea. The structure of the derivatives were confirmed by mass spectrometry to be the 9-anthracenemethyl esters (Fig. 3.3)[187].

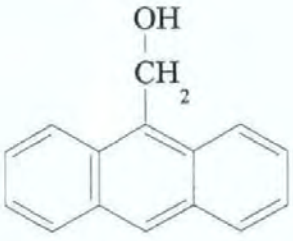




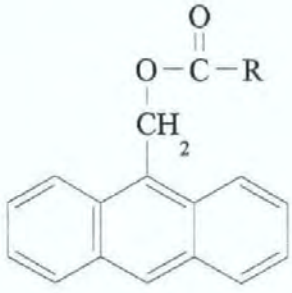
dicyclohexylcarbodiimide (DCC)



dicyclohexylurea (DHU)



9-anthracenemethanol



9-anthracenemethanol derivative of carboxylic acid, RCOOH

Fig 3.3 Esterification of carboxylic acids with activation by DCC. Step 1: protonation of N atom of DCC, Step 2: acyl O of carboxylic acid conjugate base of acid attacks DCC to form carboxylic acid-DCC derivative (I), Step 3: protonation of N atom of (I), Step 4: nucleophilic substitution of fluorescent alcohol on carbonyl C of (I) eliminating substituted urea DHU

3.3.3 Optimisation of pre column derivatisation

Sample solvent

An important requirement for oil analysis by LC is a suitable solvent for the solubilisation of the derivatised oil sample which does not adversely affect the separation. Oils are generally insoluble in the polar solvents commonly used as mobile phases for reversed-phase LC, e.g. ACN and methanol. Direct injection of an aliquot of the derivatised sample in the reaction solvent (*n*-heptane and DCM) was found to lead to peak splitting and tailing [187].

This problem was caused by solvation of the derivatives by the reaction solvent, and was overcome by removing the solvent, dissolving the residue in a minimum volume of DCM, then making up with ACN to a volume of 25 ml [187]. When applied to oil samples for this work the procedure resulted in the formation of emulsions unsuitable for injection onto the chromatographic column.

As oils are soluble in THF, which is compatible with reversed-phase LC mobile phases, mixtures of ACN with THF over the range 0-100 % THF were investigated. 100 % THF was not suitable as it resulted in poor peak shapes and resolution. Derivatised oils were found to be soluble in a mixture of ACN and THF (40:60, v/v), which was sufficiently similar to the optimum mobile phase (ACN-THF-water, 40:40:20, v/v/v) to give good peak shapes and resolution.

Reaction time

To investigate the effect of reaction time on derivatisation, mixtures of octanoic and dodecanoic acid were derivatised by heating under reflux for 15-90 min. Each sample was allowed to cool for 5 min before the reaction solvent was removed. The results showed that the reaction had gone to completion for both acids within 15 min (Table 3.3).

Table 3.3 Effect of reaction time on the derivatisation of carboxylic acids with 9-anthracenemethanol		
Reaction time (min)	Signal, octanoic acid derivative (mV)	Signal, dodecanoic acid derivative (mV)
15	2128.2	1037.5
30	2115.7	946.9
45	2068.8	931.3
60	2013.8	841.8
75	2112.5	1009.4
90	2018.8	940.7

Label concentration

The 9-anthracenemethanol label should be present in excess to ensure complete derivatisation but any unreacted label will degrade the resolution of early eluting peaks, particularly the benzoic acid derivative. A mixture of benzoic acid and octanoic acid was derivatised with varying concentrations of 9-anthracenemethanol to determine the optimum concentration of label to be used.

An equimolar mixture of label and total carboxylic acid concentration gave the best compromise between sensitivity and resolution (Table 3.4). Therefore, for oxidised oil samples some idea of total acid concentration is useful when choosing the label concentration. This could be obtained from TAN measurements and/or TLC-FID.

Table 3.4 Effect of 9-anthracenemethanol label concentration on the derivatisation of carboxylic acids		
Concentration (mol in reaction flask) octanoic acid = 1.0×10^{-5}, dodecanoic acid = 0.9×10^{-5}, total carboxylic acid concentration in flask (mol in reaction flask) = 1.9×10^{-5}		
Concentration label (mol in reaction flask)	Octanoic acid derivative signal (mV)	Dodecanoic acid derivative signal (mV)
4.6×10^{-6}	650	85.1
9.3×10^{-6}	1645	151.3
1.9×10^{-5}	1431.3	793.8
5.6×10^{-5}	1512.5	665.7
9.3×10^{-5}	1212.5	378.2

3.3.4 Liquid chromatography and chemiluminescence detection

Chemiluminescence detection

DNPO was the aryl oxalate used since its rapid reaction kinetics leads to a "chemical band narrowing" effect in flow cells [179]. DNPO also gives a more intense emission, and is more readily soluble in ACN than the other commonly used ester, bis(2,4,6-trichlorophenyl)oxalate, although it is more expensive and is less stable, due to an increased susceptibility to hydrolysis.

The optimum DNPO concentration, hydrogen peroxide and post-column reagent flow rate to giving the maximum response (peak height) for dodecanoic acid 9-anthracenemethanol ester (0.17 % m/v) were determined by a multivariate procedure, using the modified simplex optimisation program (section 2.3.3).

The simplex variables are presented in Table 3.5. The optimisation was terminated after seven experiments.

Table 3.5 Simplex variables for optimisation of CL reaction detection of carboxylic acid-9-anthracenemethanol esters				
Variable	Precision	Maximum	Minimum	Step size
DNPO (mol l^{-1})	2.0×10^{-3}	1.0×10^{-2}	2.0×10^{-3}	4×10^{-3}
H_2O_2 (mol l^{-1})	5.0×10^{-2}	3.5×10^{-1}	1.0×10^{-1}	1.0×10^{-1}
Flow rate	0.05	0.80	0.20	0.10
Response = signal (mV)			Precision of measurement = 0.25 mV	

The major factors affecting the response were DNPO concentration and the flow-rate of the reagent streams; both parameters were directly related to the CL emission intensity. Increasing the reagent flow rate above the flow rate of the column eluate lead to a decrease in resolution due to band broadening. The conditions selected for routine application were a DNPO concentration of 5.0×10^{-3} mol l^{-1} and a peroxide concentration of 2.2×10^{-1} mol l^{-1} pumped at a combined flow rate of 0.8 ml min^{-1}. These values were chosen to give the best sensitivity commensurate with reasonable reagent cost and minimal band broadening.

Liquid chromatography

As discussed in Chapter 2, the solvent requirement for maximum CL emission precludes the use of certain mobile phases e.g. methanol, water. An ACN-THF-water gradient for the separation of carboxylic acid-9-anthracenemethanol esters has been described [212], however an isocratic method would be preferable for a process environment.

Mobile phases with differing proportions of ACN-THF-water were investigated using a dodecanoic acid-9-anthracenemethanol derivative (0.17 % m/v) as the analyte (Table 3.6 and Fig. 3.4) to determine the optimum solvent blend for separation of the esters.

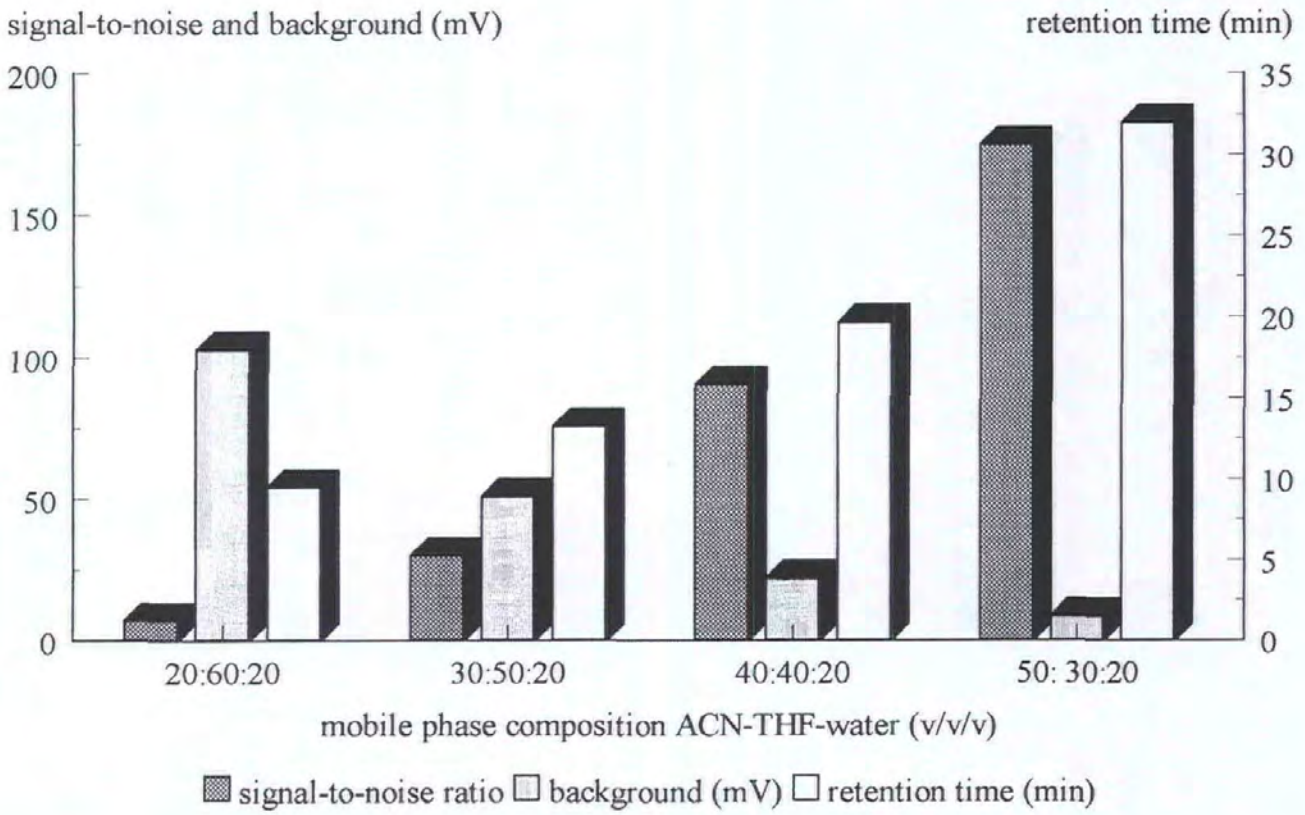


Fig 3.4 Effect of mobile phase composition on DNPO CL detection for a 0.17 % (m/v) dodecanoic acid ester

Table 3.6 Effect of variation of mobile phase on DNPO CL detection using the 9-anthracenemethanol ester of dodecanoic acid as a model analyte			
Mobile phase ACN-THF-water (v/v/v)	Background (mV)	Signal-to-noise ratio	Retention time (min)
20:60:20	103.0	7.2	9.5
30:50:20	51.1	30.1	13.2
40:40:20	22.1	90.7	19.7
50:30:20	8.5	175.0	32.0

THF is the least polar of the mobile phase solvents (Table 1.8) and increasing its relative concentration will therefore increase the eluting power of the mobile phase and lead to shorter retention times. Retention can be related to the concentration of a particular solvent in a mobile phase by plotting log (k') against log (solvent concentration) [219]. The plot for log (k') against log (% THF v/v) was linear (r^2 = 0.9976) with an equation of best fit of log (k') = -2.15 log (% THF v/v) + 4.18 (Fig. 3.5). The capacity factors, k', were calculated according to the equation described in Section 1.3.3.

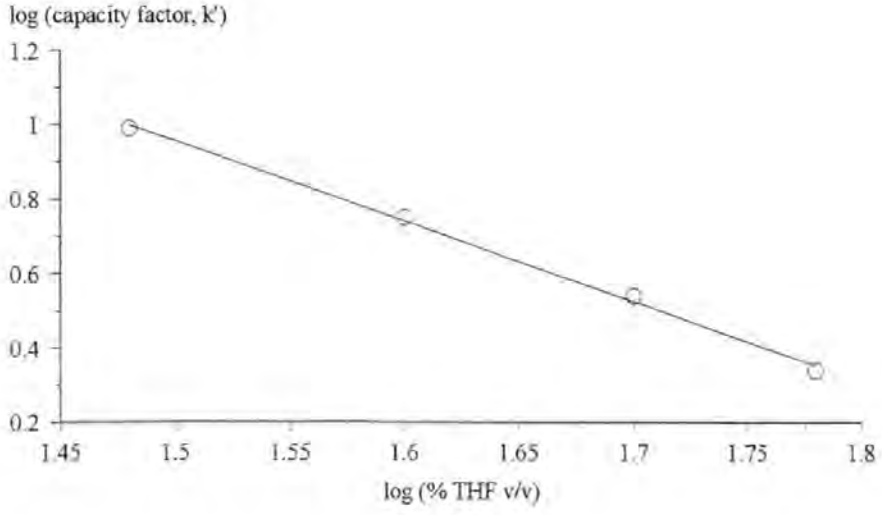


Fig 3.5 Log-log relationship between capacity factor of a dodecanoic acid-9-anthracenemethanol ester and the concentration of THF in the mobile phase

However THF also has a marked effect on the CL emission intensity, with higher concentrations reducing the signal-to-noise ratio and increasing the reagent background signal. Therefore on the basis of the data, a mobile phase of ACN-THF-water (40:40:20 v/v/v) was used.

3.3.5 Oil Analysis

9-Anthracenemethanol esters of benzoic, octanoic, dodecanoic and hexadecanoic acids were analysed individually to determine their capacity factors, which are presented in Table 3.7.

The derivatives were completely resolved within 45 min, except for the benzoic acid derivative. This derivative was not fully resolved from the excess label peak, which might create difficulties when analysing low levels of benzoic acid.

Table 3.7 Retention data for carboxylic acid-9-anthracenemethanol esters (p 80 for conditions)

Acid derivative	Capacity factor, k'^{a}
Benzoic acid	1.2
Octanoic acid (C_8)	2.4
Dodecanoic acid (C_{12})	5.4
Hexadecanoic acid (C_{16})	12.4

$^{a}t_0 = 3$ min

A plot of the log of the capacity factor of the straight chain aliphatic acid-9-anthracenemethanol derivatives against the carbon number of the respective acids was linear ($r^2 = 0.9998$) (Fig. 3.6). This is characteristic of a homologous series and can be used to predict the retention of any member of that series [220]. As can be seen from Fig. 3.7, log (k') of the benzoic acid derivative does not lie on the line of best fit.

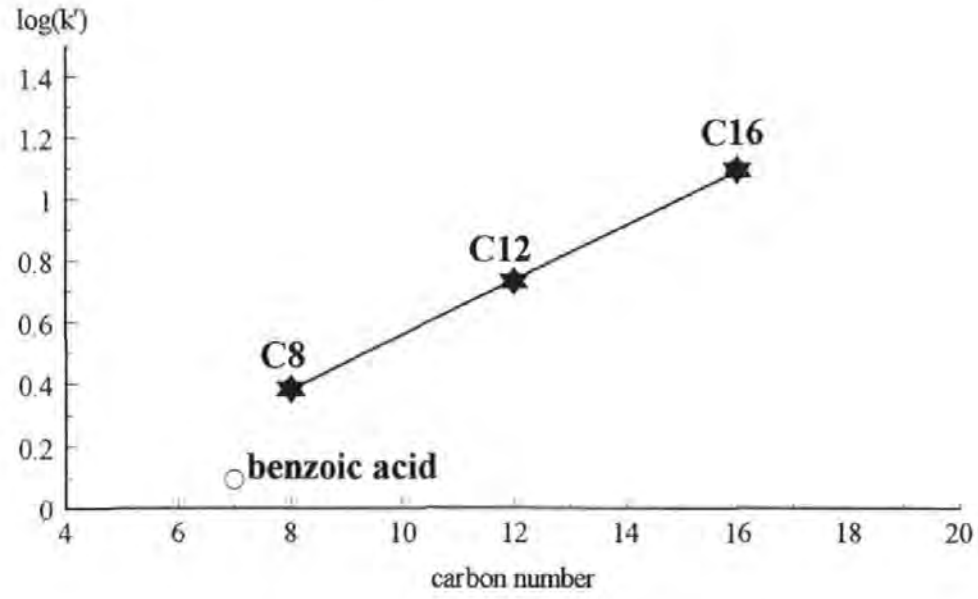


Fig 3.6 Log-linear relationship between capacity factor and structure for 9-anthracenemethanol esters of carboxylic acids

To investigate the effect of the sample matrix on the derivatisation reaction, a mixture of three acids, benzoic, dodecanoic and hexadecanoic at varying concentrations (0-0.4 % m/v), was used to spike pure heptane, a fresh oil and the same oil after laboratory simulated engine oxidation prior to derivatisation. The calibration graphs obtained were non-linear, with the oil matrix calibrations showing a marked decrease in response relative to the heptane matrix (Figs. 3.7-3.9). Calibration data are presented in Table 3.8.

Table 3.8 Calibration data for carboxylic acids in heptane and oil matrices

Calibration data for benzoic acid in heptane and oil matrices

Concentration of acid (% m/v)	Signal, heptane matrix (mV)	Signal, fresh oil matrix (mV)	Signal, oxidised oil matrix (mV)
0.0	0.0	0.0	20.0
0.1	47.5	15.5	33.8
0.2	212.5	62.5	69.0
0.3	512.5	108.8	128.4
0.4	723.8	245.7	177.3

Calibration data for dodecanoic acid in heptane and oil matrices

Concentration of acid (% m/v)	Signal, heptane matrix (mV)	Signal, fresh oil matrix (mV)	Signal, oxidised oil matrix (mV)
0.0	0.0	0.0	0.0
0.1	89.0	27.3	17.7
0.2	240.0	67.5	55.3
0.3	487.5	165.6	94.0
0.4	692.5	298.2	178.5

Calibration data for hexadecanoic acid in heptane and oil matrices

Concentration of acid (% m/v)	signal, heptane matrix (mV)	signal, fresh oil matrix (mV)	signal, oxidised oil matrix (mV)
0.0	0.0	0.0	0.0
0.1	31.3	15.7	9.8
0.2	100.0	38.2	24.5
0.3	188.8	83.8	46.5
0.4	276.8	118.2	75.7

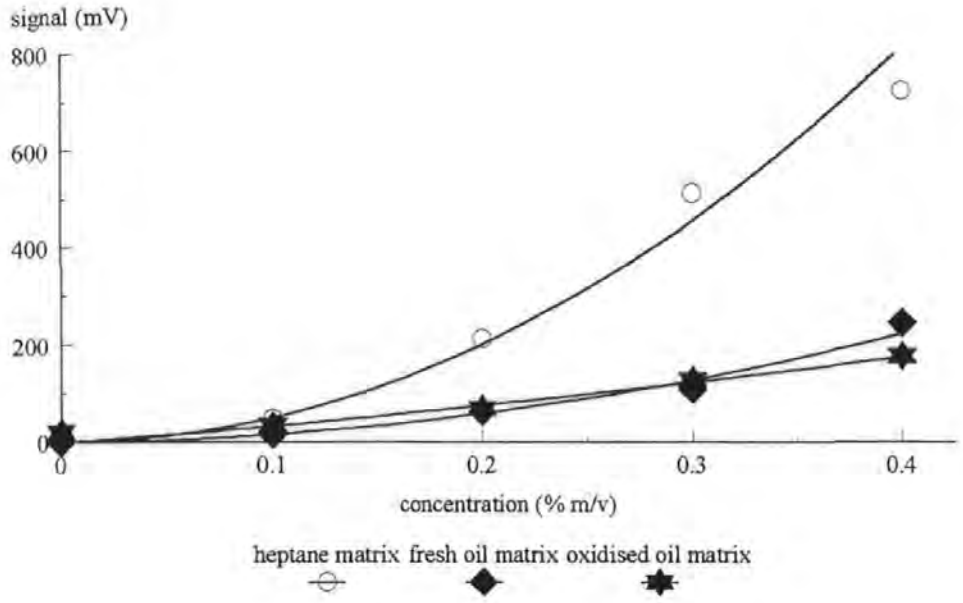


Fig 3.7 Effect of matrix on the determination of benzoic acid in n-heptane, an unoxidised oil and in the same oil after oxidation

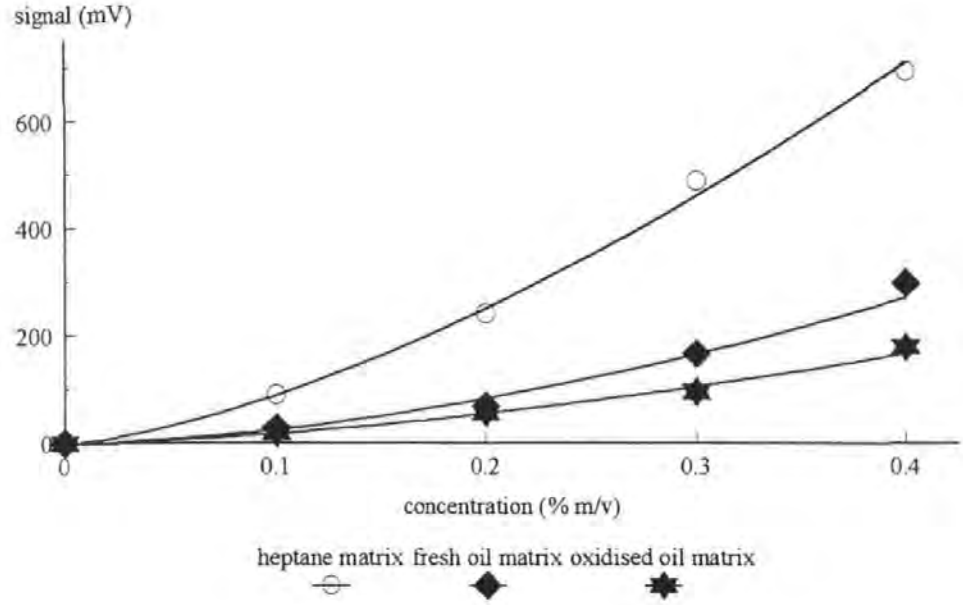


Fig 3.8 Effect of matrix on the determination of dodecanoic acid in n-heptane, an unoxidised oil and in the same oil after oxidation

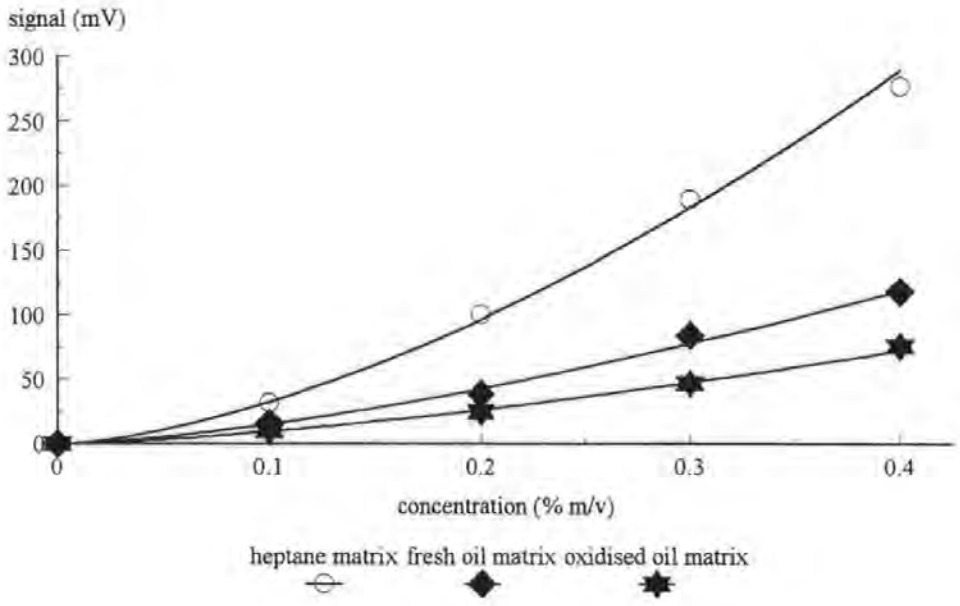


Fig 3.9 Effect of matrix on the determination of hexadecanoic acid in n-heptane, an unoxidised oil and in the same oil after oxidation

There is a less marked attenuation of the CL response with the oxidised oil matrix compared with the fresh oil matrix. The attenuated response and the non-linear nature of the calibrations are due to a combination of competing side reactions during derivatisation and probable quenching of the CL emission by the oil matrix. These calibrations were repeated and were found to be reproducible.

Side reactions have been reported with DCC, particularly the formation of *N*-acylureas [215, 218, 206]. Sulphonic acid and phenol, which may be present in oils, have been shown to interfere with derivatisation by reacting with the label or the acids [187]. Other components in the oils, such as high molecular weight polymeric additives and in the oxidised oils, organometallic species and solid debris, may also affect the derivatisation reaction. The linearity and magnitude of the response could be improved if these components were removed from the oil samples prior to derivatisation, possibly by dialysis.

No carboxylic acids were detected in the unoxidised oil but there was a component with the same retention time as the benzoic acid ester derivative in the oxidised oil. Chromatograms of the oxidised oil and the same oil spiked with a mixture of

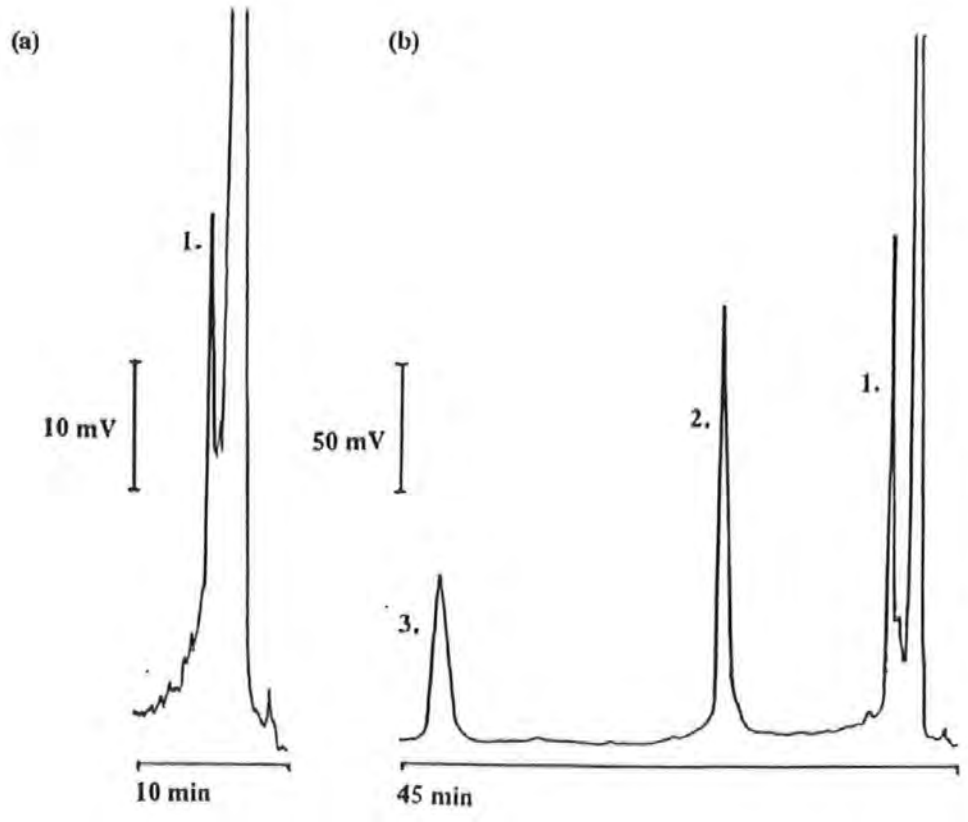


Fig 3.10 Chromatograms of an oxidised oil sample; (a) unspiked oxidised oil showing the presence of benzoic acid (1), (b) the same oil sample spiked at the 0.4 % m/v level with a mixture of ; (1) benzoic, (2) dodecanoic and (3) hexadecanoic acid

benzoic, dodecanoic and hexadecanoic acids (0.4 % (m/v)) are shown in Figs. 3.10 (a) and 3.10 (b) respectively. A standard additions procedure for benzoic acid indicated a concentration of 0.016 % (m/v) in the oil, although no reference method was available to corroborate this data (Fig. 3.11). Salicylic acid derivatives are used as oil additives, and the benzoic acid found in the oxidised oil is probably formed from these additives during the oxidation process.

A low-level calibration (0-0.1 % m/v) in the oxidised oil matrix for dodecanoic acid was linear (r^2 = 0.9968), data for which are presented in Table 3.9. The limit of detection, defined as twice the peak-to-peak noise, was calculated from the equation of the regression line to be 0.013 % (m/v) in the original solution, which corresponds to an on-column detection limit of 26 ng (130 pmol).

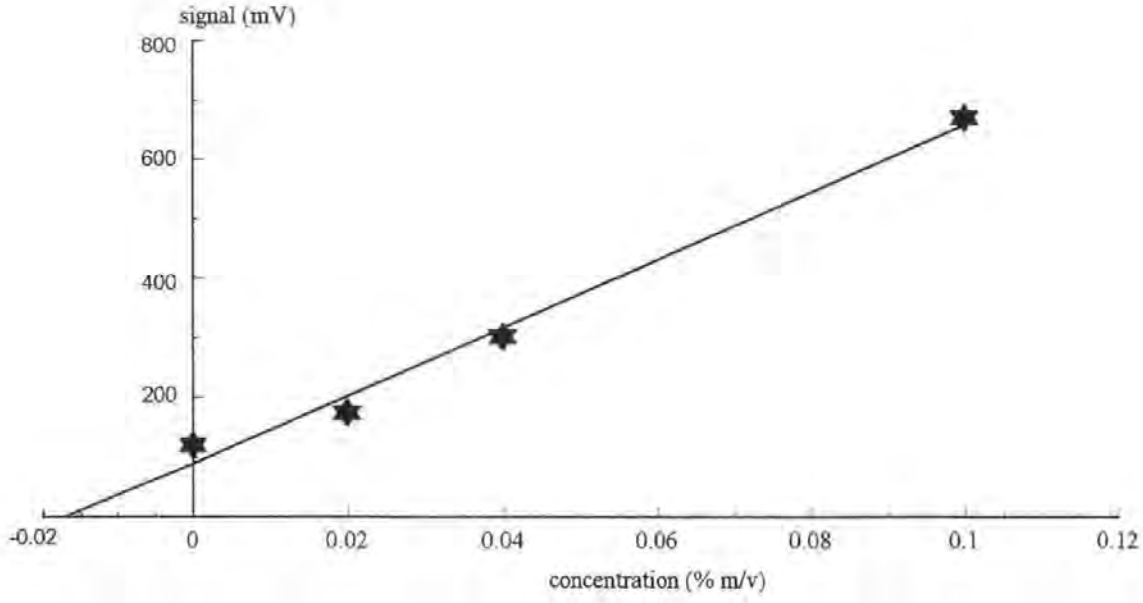


Fig 3.11 Standard additions calibration for benzoic acid in oxidised oil matrix (signal = 5696 (% (m/v)) + 89, r^2 = 0.9887)

Table 3.9 Low level calibration data for dodecanoic acid (n=5)	
Concentration of acid (% (m/v))	signal (mV)
0.00	0.0
0.02	8.8
0.04	15.9
0.06	26.9
0.10	38.1
signal (mV) = 387 (% (m/v)) + 0.9, r^2 = 0.9968, peak-to-peak noise = 0.3 mV limit of detection (signal equivalent to 2 x noise) = 0.013 % (m/v) in the original solution	

3.4 CONCLUSIONS

The results show that benzoic acid and straight chain aliphatic carboxylic acids (C_6-C_{18}) in non-aqueous media can be selectively derivatised with the fluorescent label 9-anthracenemethanol and separated by reversed phase LC. Detection is achieved by a post column CL reaction with DNPO-hydrogen peroxide.

The procedure was applied to the determination of carboxylic acids in oil before and after laboratory simulated engine oxidation. Fresh and oxidised oil matrices were found to reduce the sensitivity of the procedure. The probable causes of this reduction are competing side reactions during the derivatisation and quenching of CL emission by the oil matrix. Determination is possible over the range 0-0.4 % (m/v) for individual acids with a detection limit of 0.013 % (m/v) (26 ng, 130 pmol on-column with a 20 µl injection) for dodecanoic acid in an oxidised oil matrix. An oil oxidised in a laboratory simulated engine test was found to contain benzoic acid at a level of 0.015 % (m/v) in the oil.

Chapter 4

Monitoring Carboxylic Acid Formation in Used Engine Oils by Liquid Chromatography with Fluorescence Detection

4.1 INTRODUCTION

The previous chapter described a procedure for the selective derivatisation of carboxylic acids in non-aqueous media with reversed-phase separation and peroxyoxalate CL detection. The isocratic LC method developed however has a longer runtime (45 min) than would be desirable for routine analysis (30 min). The separation of the esters may be achieved in a shorter period of time by changing the stationary phase used for the separation and by utilising gradient elution rather than isocratic elution. However the peroxyoxalate CL detection used is sensitive to mobile phase composition and therefore unsuitable, but as 9-anthracenemethanol is highly fluorescent an alternative mode of luminescence detection, fluorescence, is possible.

This chapter describes a procedure for monitoring carboxylic acids in oxidised engine oils by LC with gradient elution and fluorescence detection. Polymeric additives, organometallic oxidation products and solid debris, that may interfere with the derivatisation reaction, were removed from samples prior to analysis by dialysis. The method was applied to the analysis of several series of oxidised oils sampled from an engine after different periods of operation.

4.2 EXPERIMENTAL

4.2.1 Reagents

High quality de ionised water from a Milli-Q system (Millipore) and analytical grade reagents were used unless otherwise stated. Acetonitrile (ACN), dichloromethane (DCM), *n*-heptane, hexane, petroleum spirit (60/80 °C) and tetrahydrofuran (THF) were of HPLC grade (Rathburn).

Solutions of 9-anthracenemethanol (Fluka) and 4-pyrrolidinopyridine (Aldrich) were prepared daily in DCM. Dicyclohexylcarbodiimide (DCC; Fluka) was used as the solid. Solutions of hexanoic (C_6), heptanoic (C_7), octanoic (C_8), nonanoic (C_9), decanoic (C_{10}), dodecanoic (C_{12}), hexadecanoic (C_{16}), octadecanoic (C_{18}),

eicosanoic (C_{22}) and docosanoic (C_{20}) acids (all Aldrich) were prepared in *n*-heptane. All carboxylic acids were of reagent grade.

4.2.2 Sample pre treatment

A scheme of analysis of the oils is shown in Fig. 4.1.

The oil samples were initially fractionated using a continuous dialysis system. as shown in Fig 4.2. Solutions of oil in petroleum spirit (60/80 °C) were contained within a semipermeable membrane around which warm petroleum spirit (60/80 °C) was continually circulated. The membrane was a dry, hypo-allergenic incontinence sheath rubber membrane (London Rubber Company; product number Q100-251, C10103900)

The dialysis proceeded for 24 hours to allow low molecular weight material to diffuse through the membrane (nominally the molecular weight cut-off is 1000). Solvent was removed from the dialysate by rotary evaporation.

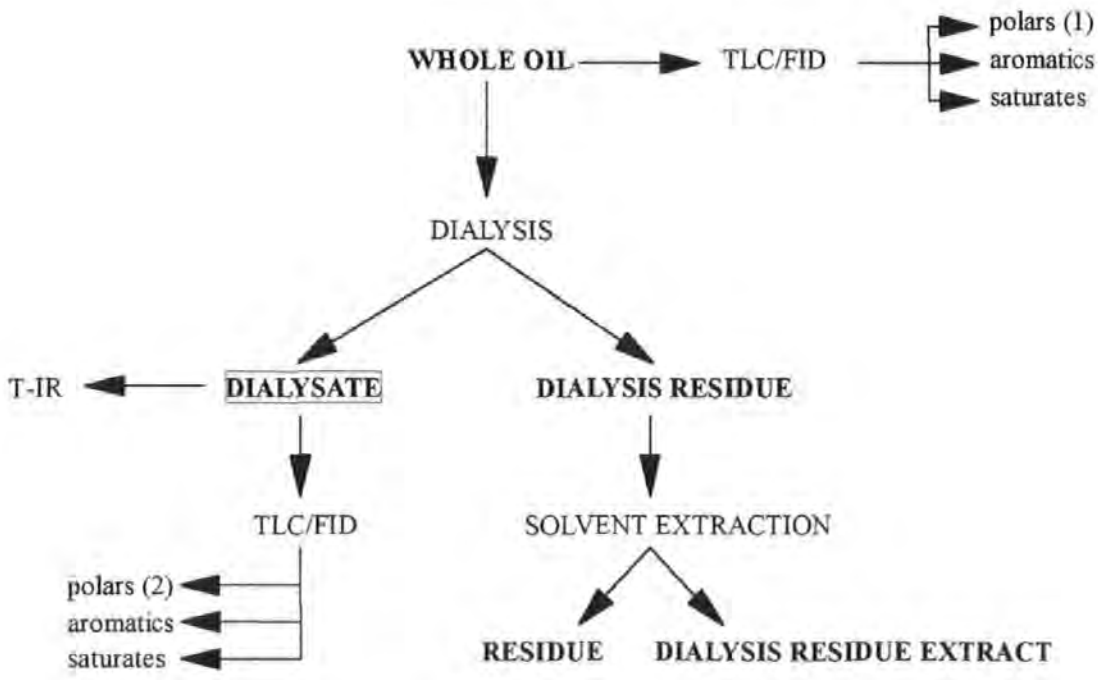


Fig 4.1 Scheme of analysis for engine oils.

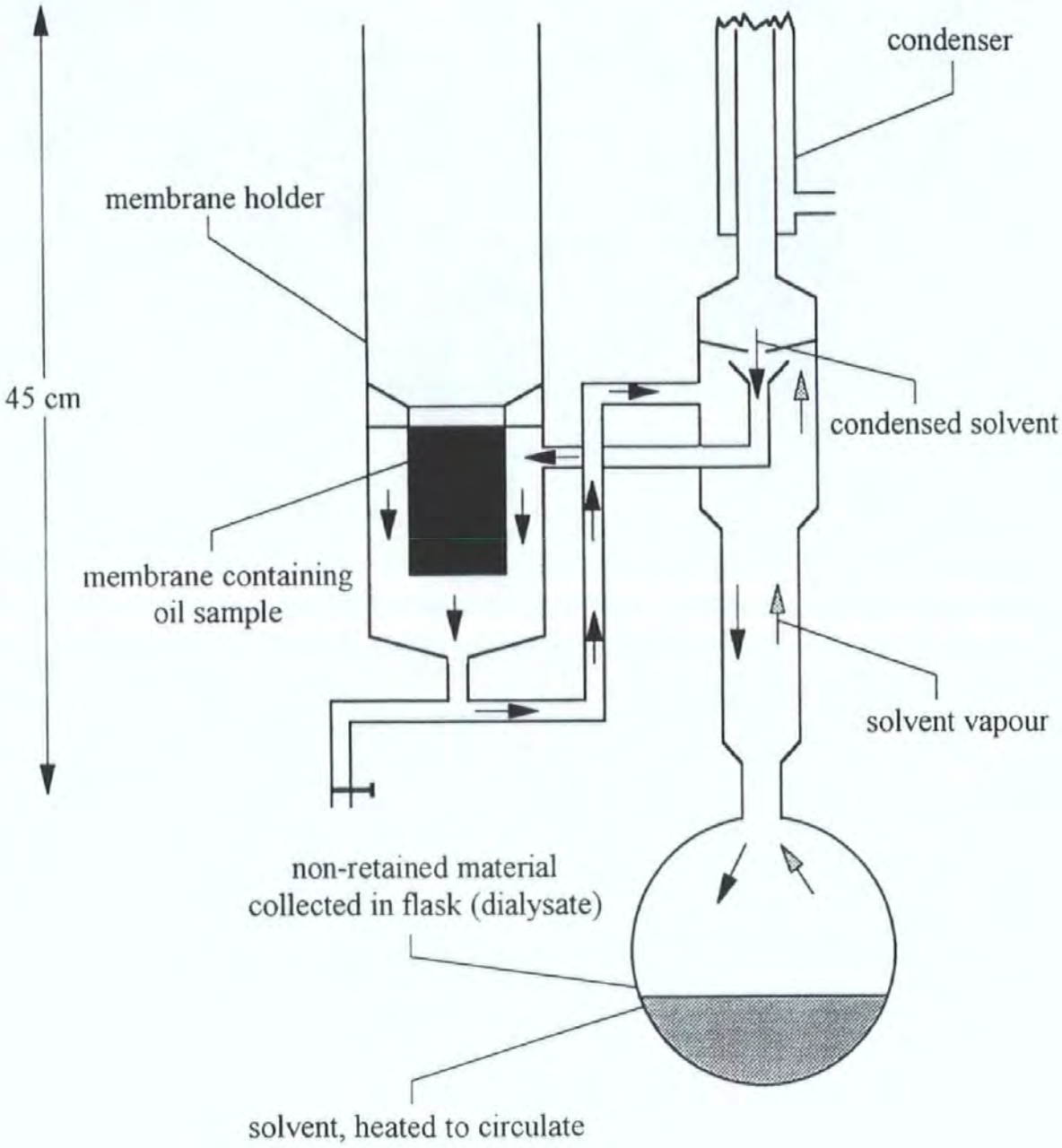


Fig 4.2 Schematic diagram of continuous dialysis system

4.2.3 Pre column derivatisation

Carboxylic acid standard solutions and oil dialysate samples, unspiked and spiked with mixtures of acids (250 µl), were added to a mixture of dicyclohexylcarbodiimide (0.25 g), 9-anthracenemethanol in DCM (2.1×10^{-2} mol l^{-1}, 1 ml), 4-pyrrolidinopyridine in DCM (1.0×10^{-2} mol l^{-1}, 1 ml) and n-heptane (15 ml) which was heated under reflux for 15 min. The reaction mixture was allowed to cool for 5 min before the solvent was removed by rotary evaporation. The residue was dissolved in ACN-THF (40:60, v/v) (25 ml).

4.2.4 Label removal by solid phase extraction

Silica Sep-Pak cartridges (Waters, part number 51900) were used to remove excess 9-anthracenemethanol label. The cartridge was precleaned with hexane (10 ml). The sample was derivatised as in the previous section, except that the residue was dissolved in hexane (5 ml). Derivatised sample in hexane (200 µl) was loaded on the silica cartridge and the carboxylic acid-9-anthracenemethanol derivatives were eluted with DCM-hexane (20:80 v/v; 20 ml). The solvent was removed by rotary evaporation and the residue dissolved in ACN-THF (60:40 v/v; 1 ml).

4.2.5 Liquid chromatography

Liquid chromatography was carried out using a Hewlett-Packard HP1090M liquid chromatograph with a Hewlett-Packard HP1046A dual grating fluorescence detector.

To measure the excitation and emission spectra of the label and the derivatives, peaks were isolated in the fluorescence detector flow cell by a manual valve (Rheodyne). The optimum wavelengths for excitation and emission were determined to be 251 nm and 412 nm respectively.

The instrument, containing an integral ternary solvent pump with helium sparging, an automated injector and a column oven (40 °C) was controlled by a Hewlett-Packard Chemstation. The Chemstation was also used to control the detector and acquire and manipulate the data generated.

Separation of the carboxylic acid esters was achieved with two separate columns (stainless steel, 150 x 4.6 mm i.d.; Hichrom) packed with; 1. Spherisorb S5 C8 (5 μ m) and 2. Hichrom RPB (5 μm), a reversed-phase base deactivated column (both supplied by Hichrom). Isocratic and gradient elution with ACN-THF-water mobile phases were investigated. The optimum gradient profile is described in Table 4.1.

Table 4.1 Solvent gradient for the separation of carboxylic acid-9-anthracenemethanol esters

Time	Mobile phase composition	
0.0 min	water/ACN/THF	40/50/10
5.0 min	water/ACN/THF	40/50/10
15.0 min	ACN/THF	60/40
20.0 min	ACN/THF	60/10
21.0 min	water/ACN/THF	40/50/10
25.0 min	water/ACN/THF	40/50/10

The injection volume was initially 20 μl for the isocratic elution experiments and then 5 μl for the gradient elution experiments. The flow rate of the mobile phase for both elution modes was 1.0 ml min^{-1}

4.3 RESULTS AND DISCUSSION

All concentrations quoted relate to the original sample

During the course of this work three oils, A, B and C were used. B and C are identical formulations except that C contains an anti-oxidant additive not present in B. All three oils were subjected to testing in a car engine and were sampled at intervals during the test (0, 16, 24 and 32 h for oil A, 0, 16, 40 and 64 h for oils B and C).

4.3.1 Dialysis

From the results of the work described in Chapter 3, it was suspected that polymeric additives, organometallic oxidation products and solid debris were interfering with the derivatisation reaction.

A lubricating oil comprises of a base oil plus additives and dialysis is used for the bulk fractionation of these components into a dialysis residue (high molecular weight species, usually > 1000 daltons) and a dialysate (base oil plus low molecular weight species).

Dialysis is commonly used as a de-oiling process, but in this case it was used to remove higher molecular weight compounds and debris which could interfere with the pre-column derivatisation of the carboxylic acid oxidation products.

4.3.2 Liquid chromatography

Liquid chromatography with isocratic elution

9-Anthracenemethanol esters have been separated by reversed-phase LC with isocratic elution using Spherisorb S5 ODS-2, a stationary phase with octadecyl (C_{18}) hydrocarbon chains bonded to silica particles (5 μm). Separation of the esters (C_6-C_{16}) was achieved within 45 min but for routine analysis separations achieved within 30 min are preferable.

In reversed-phase LC retention is increased proportionally with the increasing chain length of the bound hydrocarbon of the stationary phase [97]. Spherisorb S5 C8 has a bonded phase of octyl (C8) hydrocarbon chains and is less retentive than the S5 ODS2 packing material. In addition modern manufacturing techniques mean that separation of mixtures are possible on 150 mm length columns in shorter times with equivalent efficiency to the 250 mm length columns which have been traditionally used.

A mixture of octanoic (C_8), dodecanoic (C_{12}) and hexadecanoic (C_{16}) acids spiked with the dialysate of fresh oil A was derivatised and the esters separated using a

Spherisorb S5 C8 column (150 mm x 4.6 mm i.d, 5 μm). The optimum mobile phase composition was found to be ACN-THF-water (65:10:25), the chromatogram of the separation of the esters is shown in Fig. 4.3; the retention data for the esters is presented in Table 4.2.

Table 4.2 Retention data for carboxylic acid-9-anthracenemethanol esters, isocratic elution on S5 C8 column

Acid derivative	Capacity factor (k')[a]
Octanoic acid (C_8)	3.5
Dodecanoic acid (C_{12})	8.0
Hexadecanoic acid (C_{16})	18.8

[a] t_0=1.24 min

A plot of log (factor, k') against carboxylic acid carbon number was linear (r^2 = 0.9998) with the equation of best fit of;

$$\log (k') = 0.0907 \text{ carbon number} - 0.180 \quad \text{(equation 4.1)}.$$

The 32 h used oil dialysate of oil A was derivatised and analysed using isocratic elution with ACN-THF-water (65:10:25). The chromatogram obtained is shown in Fig. 4.4. The peaks in the chromatogram were not fully resolved and although the chromatogram shows peaks up to 30 min, there were still components eluting after this time.

Octanoic acid, dodecanoic acid and hexadecanoic acid were identified in the oil by the retention times of their esters. The peaks have the appearance of a series and predictions of carbon numbers calculated from the equation 4.1, of the components from the peak eluting at 4.8 min to the hexadecanoic ester at 24.7 min are presented in Table 4.3.

Oxidation of the oil has resulted in the formation of an homologous series of straight chain aliphatic acids, with hydrocarbon chain lengths up to and greater than hexadecanoic acid (C_{16}).

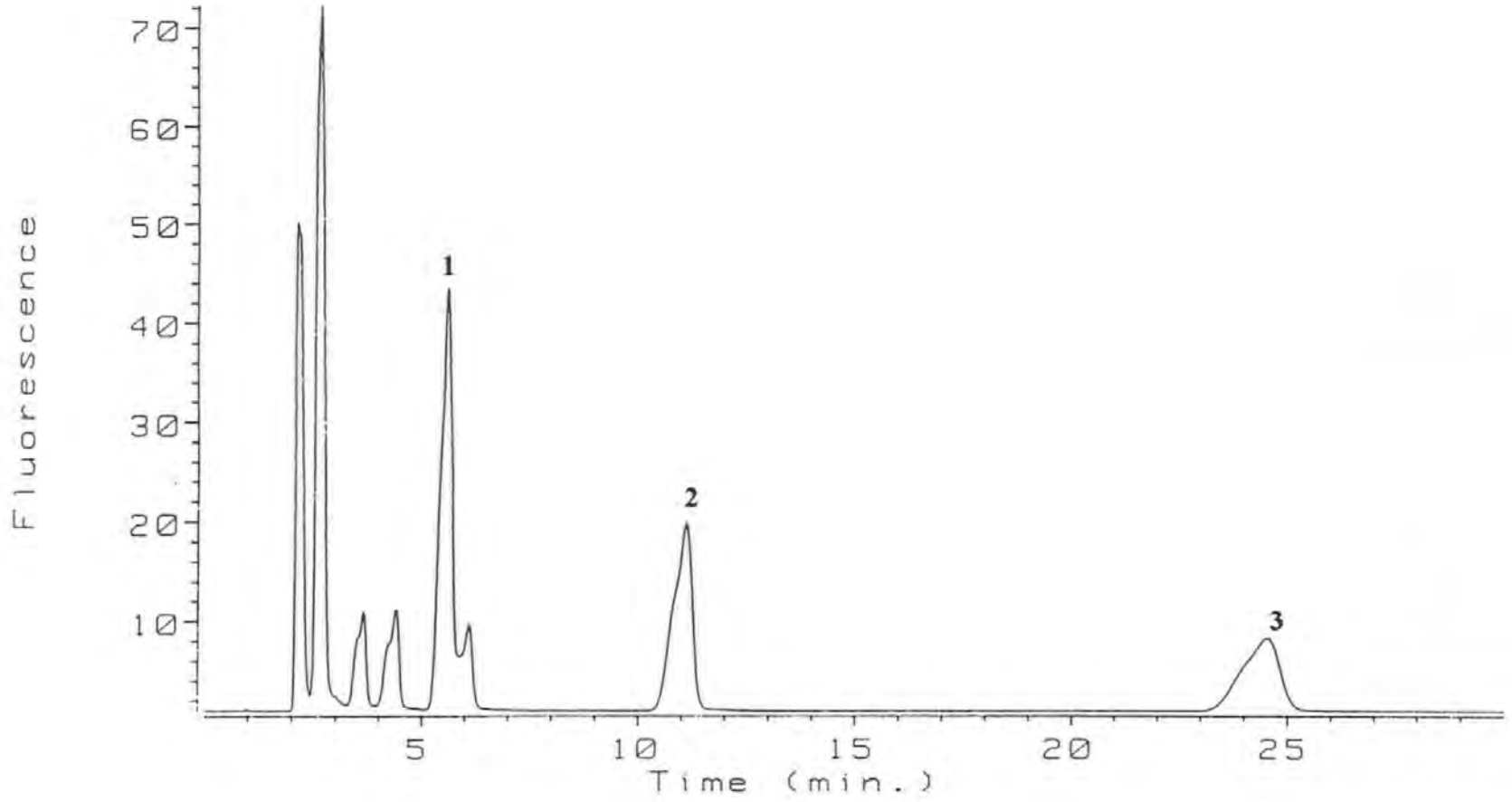


Fig 4.3 Chromatogram of carboxylic acid-9-anthracenemethanol esters, fresh oil A dialysate matrix, isocratic elution, S5 C8 column Peak assignment; 9-anthracenemethanol (1) octanoate (C_8),(2) dodecanoate (C_{12})and (3) hexadecanoate (C_{16})

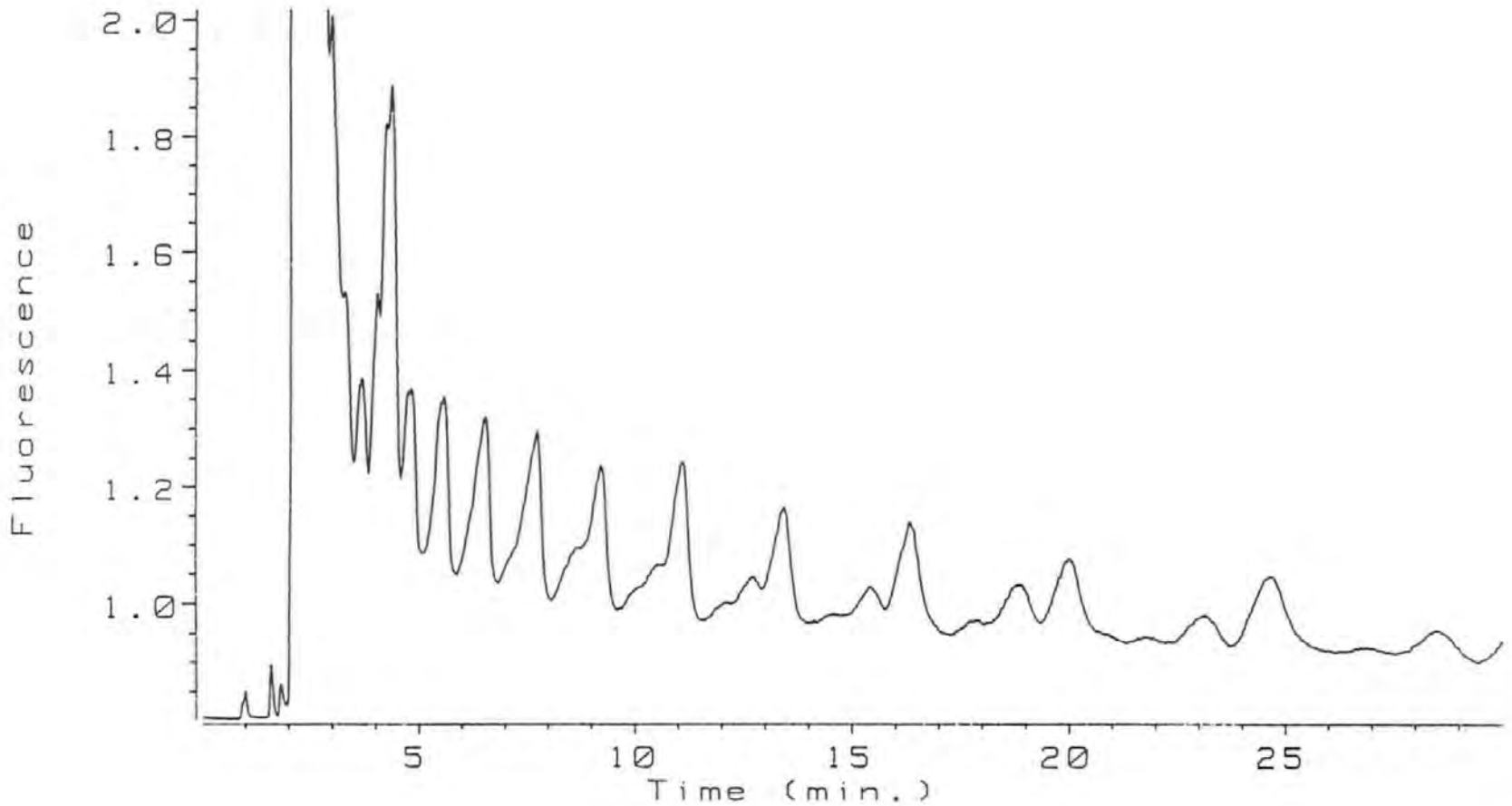


Fig 4.4 Chromatogram of derivatised 32 h used oil A dialysate, isocratic elution, S5 C8 column

Table 4.3 Identification of carboxylic acid-9-anthracenemethanol esters in 32 h used oil dialysate, oil A		
Peak	Capacity factor (k')[a]	Predicted carbon number
1	2.88	7.1
2	3.50	8.0
3	4.28	8.9
4	5.24	9.9
5	6.42	10.9
6	7.93	11.9
7	9.83	12.9
8	12.17	14.0
9	14.19	14.7
10	15.14	15
11	18.90	16.1

[a] t_0=1.24

Liquid chromatography with gradient elution

As isocratic elution had proved inadequate, gradient elution was investigated. Separation of 9-anthracenemethanol-carboxylic acid esters on an ODS (C18) column using a ACN-THF-water gradient has been reported [212]. The gradient profile separated acid derivatives over the range C_2-C_{20} in 45 min, however as with isocratic elution this is too long for routine use.

Separation of the carboxylic acid-9-anthracenemethanol derivatives on the S5 C8 column was investigated with several water-ACN-THF gradients, the optimum gradient is described above (section 4.2.5). The column was kept at 40 °C and decreasing the volume of sample injected from 20 µl to 5 µl enhanced the resolution of the derivatives due to the lower mass of material being separated (Fig 4.5)

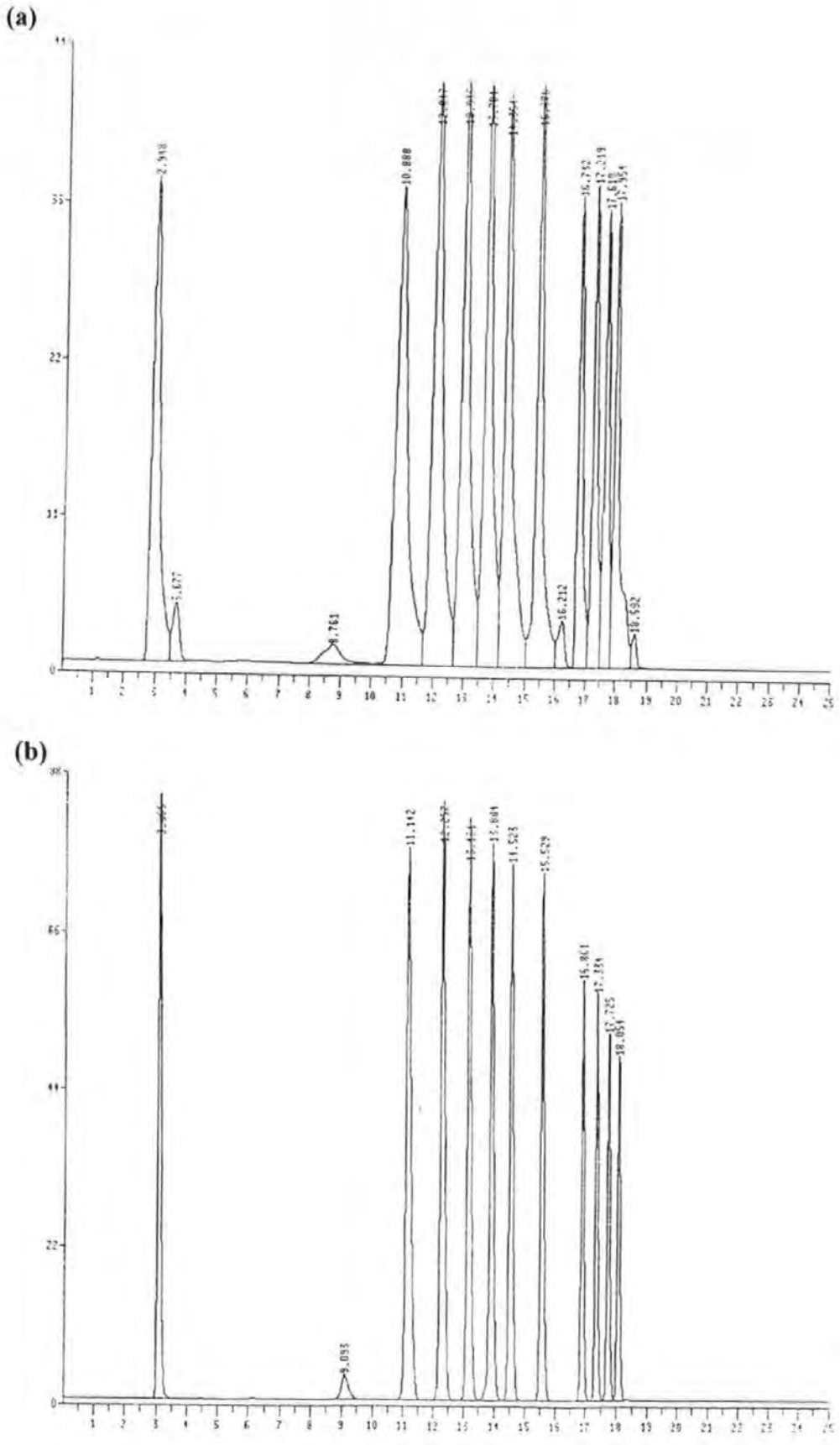


Fig 4.5 Chromatograms showing improvement in resolution on reducing injection volume, C_6-C_{22} carboxylic acid-9-anthracenemethanol esters (each 4 mg ml^{-1}) (a) 20 µl injection (b) 5 µl injection

4.3.3 Oil analysis

Two oil formulations, oil B and oil C, differing only in the presence of an anti-oxidant additive in oil C, were tested in a car engine. The oil in the sump of the engine was sampled after 0, 16, 40 and 64 hours continuous running of the engine. At 64 h, oil B had thickened and degraded to beyond the point of use while oil C still held its lubricating properties.

When analysed by TLC-FID, the concentration of polar oxidation products in the dialysates of both oils was shown to increase with time from a negligible amount at 24 h to a high proportion (30 % m/m) at 64 h. FT-IR spectra of the 64 h dialysates indicated the presence of acid carbonyl groups.

Calibration data

The fresh oil (0 h) dialysate of oil B was spiked at varying concentration levels (0-4 mg ml^{-1}) with a mixture of aliphatic straight chain carboxylic acids (C_6-C_{22}) in n-heptane prior to derivatisation. Separation of the esters was achieved on the Spherisorb S5 C8 column within 20 min, with negligible interference from the oil dialysate matrix (except on the hexanoic acid ester).

Three components with retention times between 10.5 and 12.0 min interfered with the hexanoic acid derivative. These peaks were not present in the reagent blank (derivatisation mixture without a spike of oil dialysate or acid standard) and so were probably due to acidic additives in the oil, e.g. salicylic acid based detergents. The reagent blank is compared with the fresh oil dialysates in Fig. 4.6. whilst the fresh oil B dialysate and the fresh oil B dialysate spiked with the mixture of acids at the 2 mg ml^{-1} level are compared in Fig. 4.7. Retention data for the acid esters in the fresh oil dialysate and in pure solvent are presented in Table 4.4. As can be seen by comparison of the capacity factors (k'), the oil dialysate matrix does not affect the retention times of the esters.

Calibration graphs for all of the acids over the range of interest (1-4 mg ml^{-1}) were linear (Table 4.5), with correlation coefficients (r^2) over the range 0.9987 - 1.0000.

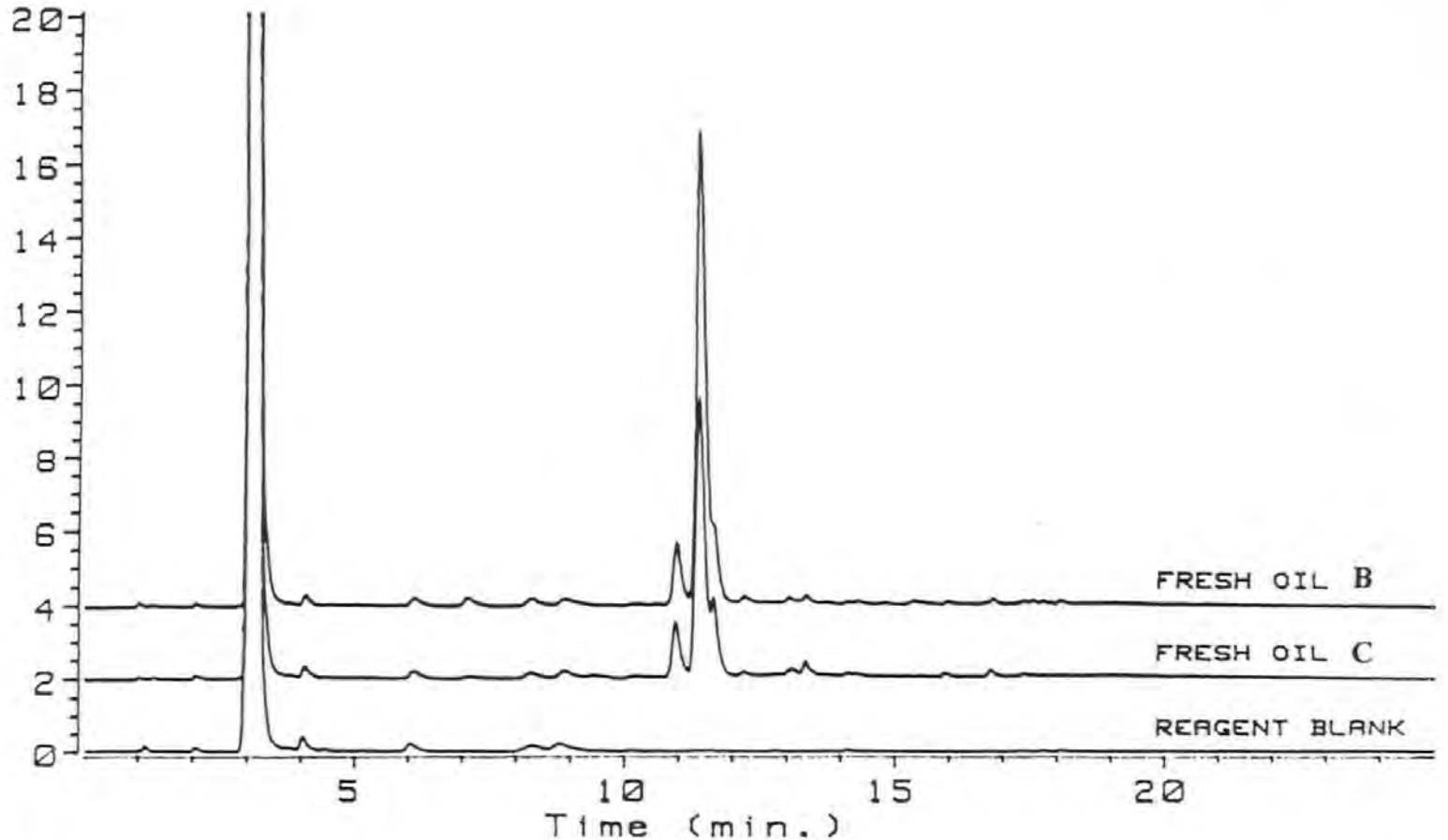


Fig 4.6 Chromatogram of a reagent blank (derivatisation mixture without spike of oil dialysate or acid standard) compared with chromatograms of derivatised fresh oil dialysate of oils B and C

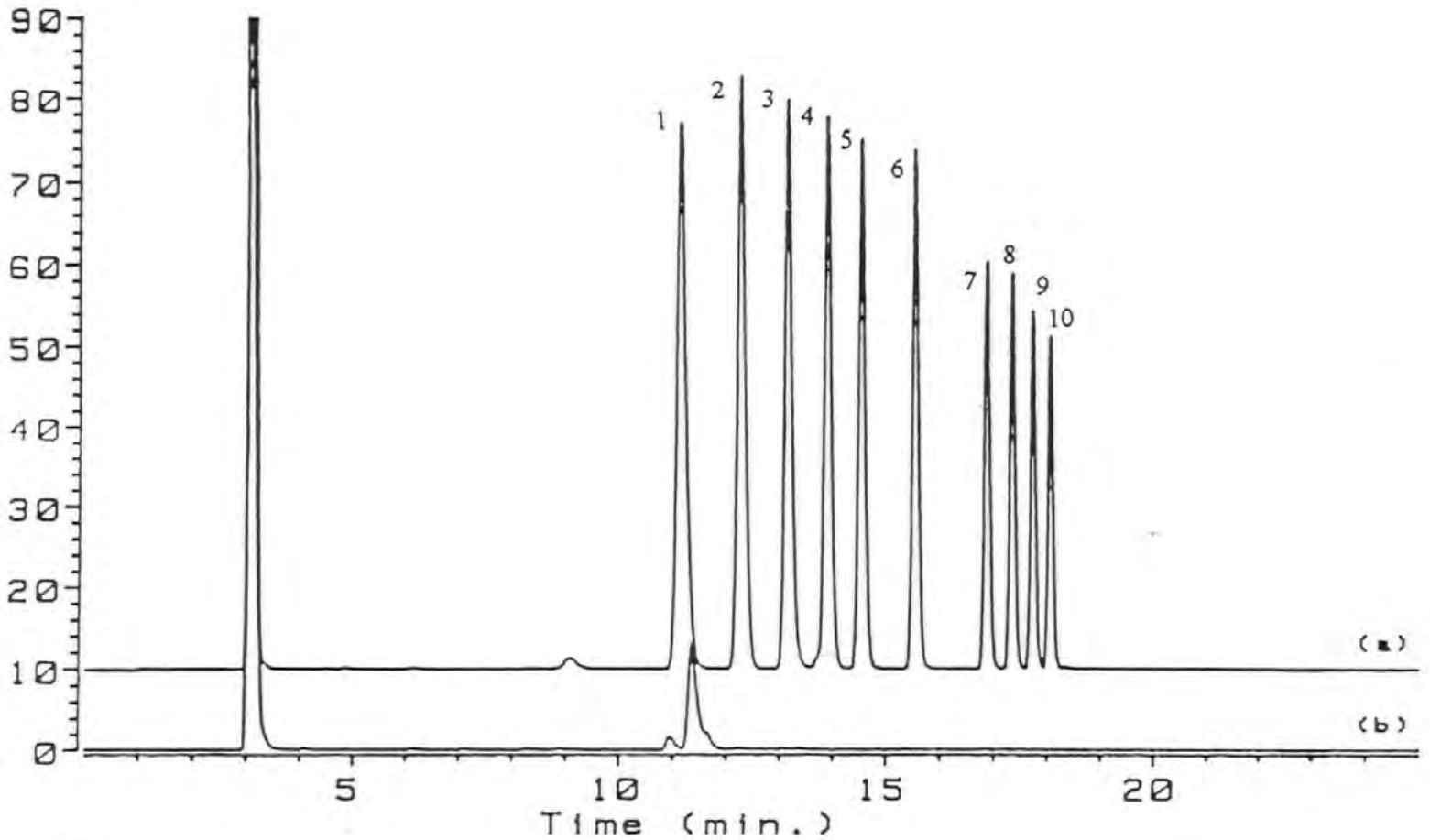


Fig 4.7 Comparison of chromatograms derivatised; (a) fresh oil B dialysate and (b) the same oil dialysate spiked with a mixture of carboxylic acids (2 mg ml^{-1} each). Peak assignment; (1) C_6, (2) C_7, (3) C_8, (4) C_9, (5) C_{10}, (6) C_{12}, (7) C_{16}, (8) C_{18}, (9) C_{20} and (10) C_{22}

Table 4.4 Retention data for carboxylic acid-9-anthracenemethanol esters in *n*-heptane and fresh oil B dialysate matrices, identification of carboxylic acids in 64 h used oil dialysates of oil B and oil C, gradient elution on S5 C8 column

Acid Derivative	Carbon Number	Capacity factor (k')[a] solvent matrix	Capacity factor (k')[a] fresh oil dialysate matrix	Capacity factor (k')[a] oil B, 64 h dialysate	Capacity factor (k')[a] oil C, 64 h dialysate
Hexanoic acid	6	8.0	8.0	not found	not found
Heptanoic acid	7	8.9	8.9	not found	not found
Octanoic acid	8	9.6	9.6	9.6	not found
Nonanoic acid	9	10.2	10.2	not found	not found
Decanoic acid	10	10.7	10.7	10.7	10.7
Dodecanoic acid	12	11.5	11.5	11.5	11.5
Hexadecanoic acid	16	12.6	12.6	12.6	12.6
Octadecanoic acid	18	13.0	13.0	13.0	13.0
Eicosanoic acid	20	13.3	13.3	13.3	13.3
Docosanoic acid	22	13.6	13.6	13.6	13.6

[a] t_0=1.24 min

Table 4.5 Linear fit and recovery data for carboxylic acids in fresh oil B dialysate matrix					
Acid	Slope (concentration (mg ml^{-1}))	Intercept	Concentration found fresh oil dialysate spiked with mixture of acids (mg ml-1)	Concentration of acid standard spike (mg ml-1)	Recovery (%)
Hexanoic acid	318.5	-185.5	3.0	2.9	103
Heptanoic acid	277.6	-168.5	3.2	3.2	100
Octanoic acid	257.6	-148.0	3.1	3.0	103
Nonanoic acid	232.9	-141.0	3.2	3.1	103
Decanoic acid	216.6	-125.7	3.0	3.0	100
Dodecanoic acid	185.2	-108.1	3.2	3.1	103
Hexadecanoic acid	143.2	-81.9	3.0	2.9	103
Octadecanoic acid	128.5	-76.2	3.1	3.1	100
Eicosanoic acid	116.6	-67.8	3.0	3.0	100
Docosanoic acid	105.7	-62.2	3.0	3.0	100

The detection limit (s/n = 3) for octadecanoic acid (determined using standards covering the range 0-0.15 mg ml^{-1}) was 85 μg ml^{-1} in *n*-heptane (4.3 ng on-column). Recoveries for the derivatisation reaction where in the range 100-103 % (Table 4.5) for the fresh oil dialysate (oil B) spiked with the mixture of aliphatic acids (C_6-C_{22}) at the 3 mg ml^{-1} level.

To determine whether the derivatives have a similar detector response, the calibration data were recalculated with the concentrations of the acids converted from mg ml^{-1} to mol l^{-1}. The slopes of the lines of best fit for 10 acids (C_6-C_{22}) are very similar (in the range 35495-37367, mean = 36640) with a relative standard deviation of 1.8 %. It can be inferred from this that the detector response of the 9-anthracenemethanol esters of straight chain aliphatic carboxylic acids is independent of chain length and the solvent gradient.

Comparison of engine test oils

The two series of dialysates for oil B and oil C over the time course of the engine test were derivatised and analysed and gave very similar results; the chromatograms for and the two 64 h samples (B and C) and the entire series for oil B are presented in Fig. 4.8 (a) and 4.8 (b) respectively. As expected the peaks tentatively identified as additives disappear from the oil dialysates between 0 h and 24 h of the test as they are used up in the course of carrying out their functions. No measurable carboxylic acids appear until 64 h, at which time an homologous series of acids starting at C_8 and going beyond C_{22} can be clearly seen superimposed on a "hump" of unresolved components. The carboxylic acids identified by capacity factors (and confirmed by spiking) in the two 64 h samples are shown in Table 4.4.

The difference between the two 64 h samples was calculated by summing the areas of the peaks with retention times between 15.07 and 18.31 min and dividing by the mass of oil dialysate taken. This time window was selected because it contained the bulk of the carboxylic acids and was free of interference from the label and oil additives. It gives an indication of total carboxylic acid content and allows direct comparison of different oils.

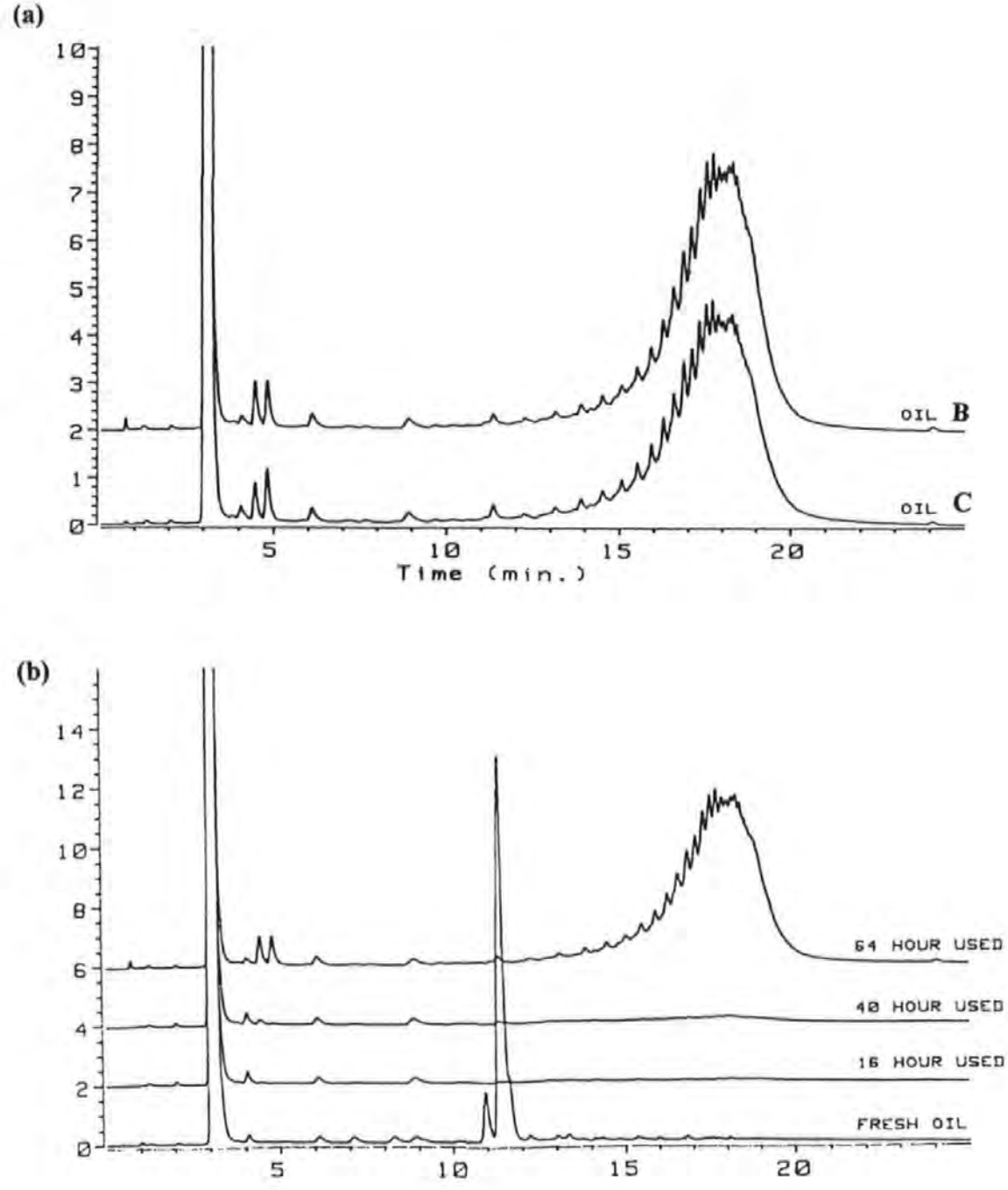


Fig 4.8 Chromatograms of derivatised engine oil dialysates; (a) 64 h used engine oils and (b) oil B engine test

The area/mg is greater in oil B (2.8 area units mg^{-1}) than oil C (2.3 area units mg^{-1}), indicating that the hydrocarbon fraction of oil B has been oxidised to a greater extent than that of oil C. This is in agreement with the physical characteristics of the two oils. The most important physical characteristic is thickening (change in viscosity) and the cut-off point is a 270 % increase in viscosity compared to that of the fresh oil at the start of the engine test. In the case of oil B there was a 950 % increase in viscosity after 64 h as compared with a 215 % increase for oil C.

4.3.4 Removal of excess label

Interference from excess label prevents sensitive determination of the lower chain length carboxylic acids, removal of the label is possible by silica column chromatography [187], but this is labour intensive and lengthy to perform for many samples. Solid phase extraction with disposable cartridges has been developed for rapid sample clean-up procedures and has found wide application to complex sample matrices e.g. separation of petroleum hydrocarbons from crude oil and product oil [221]. Removal of excess 9-anthracenemethanol label is possible by solid phase extraction with silica cartridges [63]. As 9-anthracenemethanol is more polar than the carboxylic acid esters, it is retained on a silica solid phase extraction cartridge while the esters are eluted. The recovery of carboxylic-acid-9-anthracenemethanol esters was shown to be 100 % [63].

A mixture of hexanoic (116 $\mu g\ ml^{-1}$), decanoic (172 $\mu g\ ml^{-1}$)and octadecanoic (284 $\mu g\ ml^{-1}$) acids was derivatised in *n*-heptane and the label removed using a silica solid phase extraction cartridge. The chromatogram obtained is compared with the chromatogram of the same mixture of acids without the label removed in Fig. 4.9. As can be seen, the chromatogram of the sample after label removal is less complex and identification of the carboxylic acid esters is made easier.

4.3.5 Method Modification

All of the above work was carried out with a Spherisorb S5 C8 column, to improve the separation a recently developed base deactivated reverse phase packing, Hichrom RPB, was investigated. Hichrom RPB, a mixed bonded phase packing of

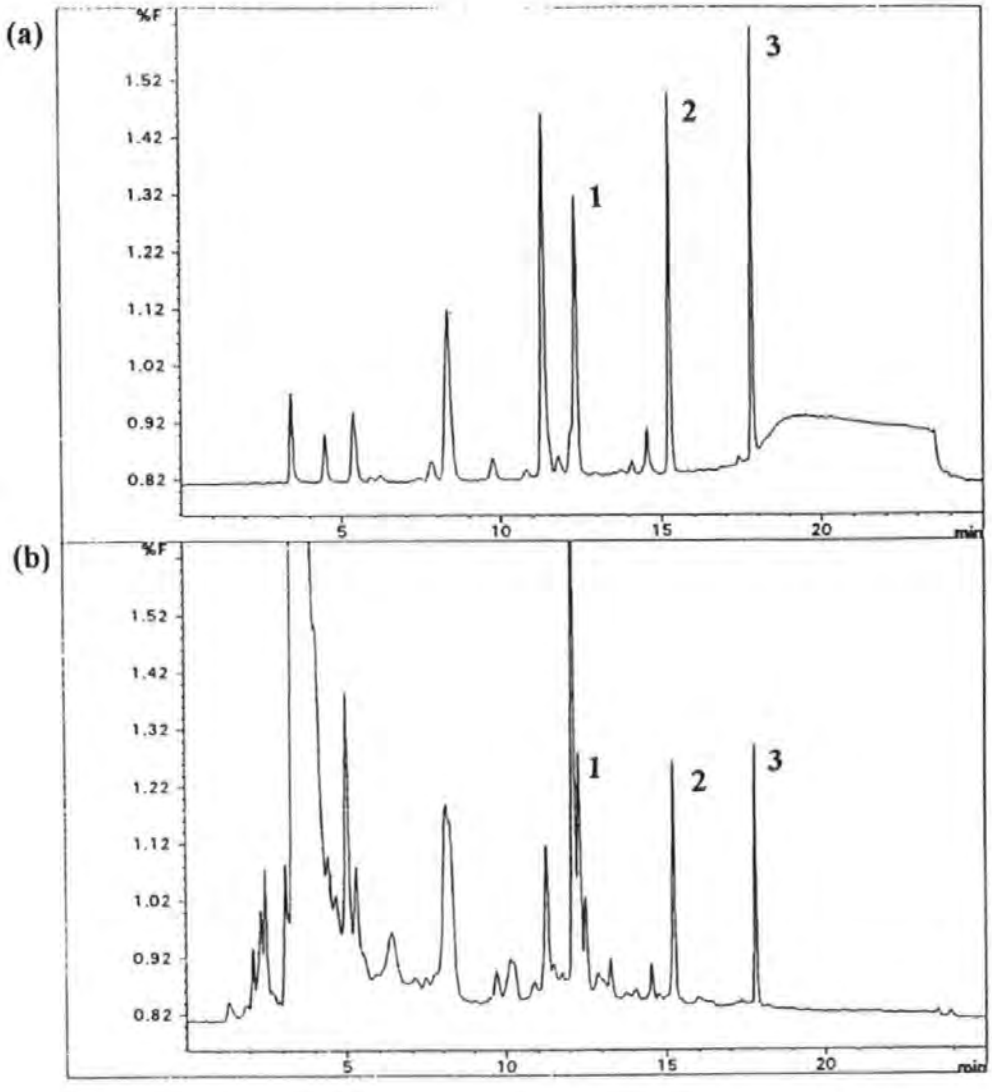


Fig 4.9 Separation of carboxylic acid-9-anthracenemethanol esters (solvent matrix); (a) without label removal and (b) with label removal
Peak assignment for both; (1) C_6, (2) C_{10} and (3) C_{18}

both octyl-(C_8) and octadecyl- (C_{18}) silanes based upon a high purity silica, was used with the same gradient profile to separate a derivatised mixture of acids (hexanoic (58 μg ml^{-1}), decanoic (86 μg ml^{-1}) and octadecanoic (142 μg ml^{-1})). The metal content of the silica and residual silanols adversely affect reversed-phase separations [97], use of a high purity silica will therefore give improved performance.

Preliminary results showed that the Hichrom RPB column separated the acid derivative peaks of interest from the worst of the interference without increasing the total analysis time (Fig 4.10). The retention data for the separation on the Hichrom RPB column is compared with the data obtained using the S5 C8 column in Table 4.6, peak shape was also improved as can be seen by comparison of the baseline peak widths (Table 4.6).

Table 4.6 Retention data for carboxylic acid-9-anthracenemethanol esters, gradient elution on Hichrom RPB column

Acid derivative	Capacity factor (k') Hichrom RPB[a]	Capacity factor (k') S5 C8[b]
Hexanoic acid	6.4	8.0
Decanoic acid	8.1	10.7
Octadecanoic acid	9.7	13.0
Acid derivative	Baseline peak width (min), Hichrom RPB	Baseline peak width (min), S5 C8
Decanoic acid	0.10	0.21
Octadecanoic acid	0.09	0.15

$^{a}t_0$ = 1.67 min, $^{b}t_0$ = 1.24 min

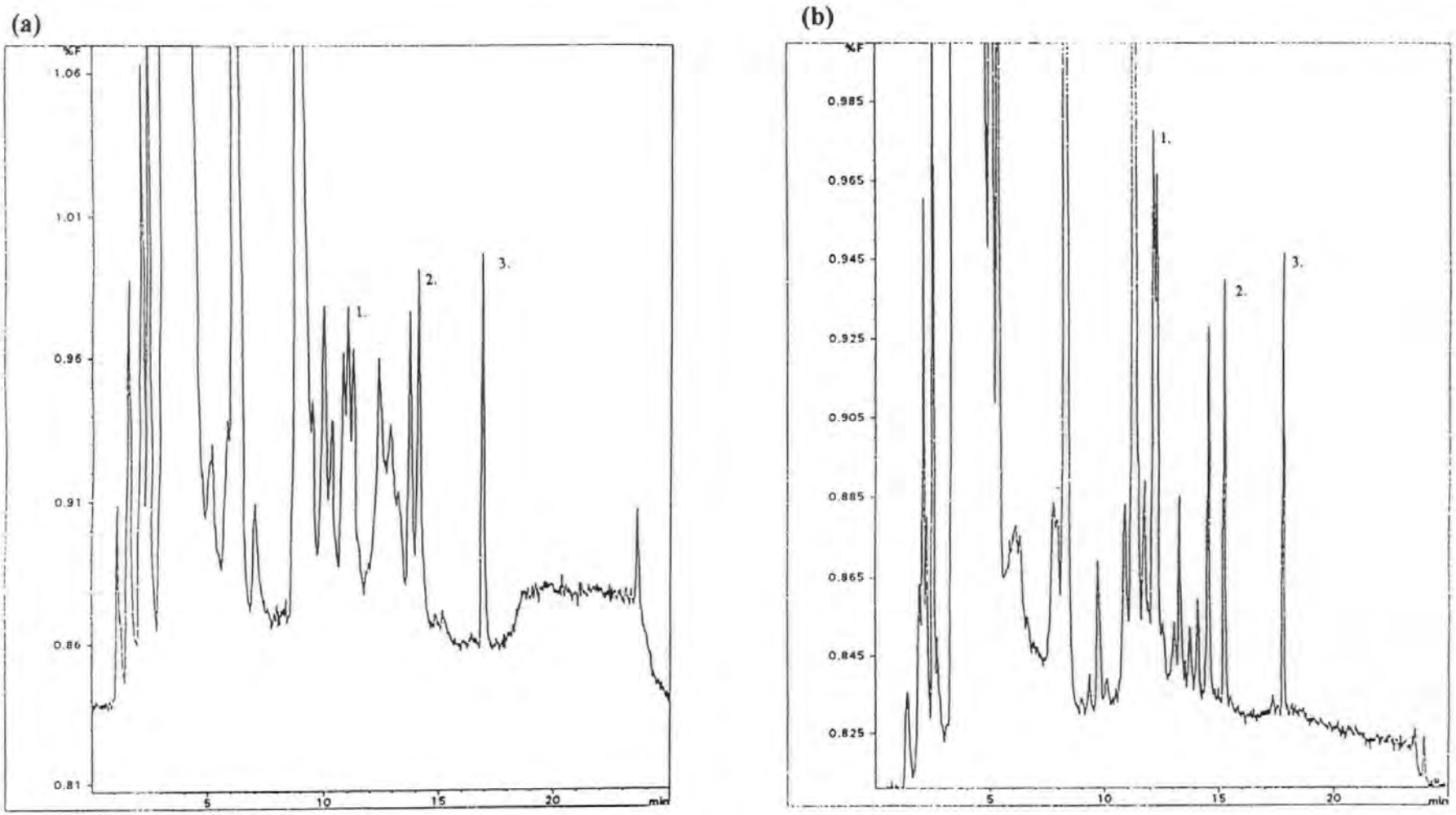


Fig 4.10 Separation of carboxylic acid-9-anthracenemethanol esters (solvent matrix) on; (a) Spherisorb S5 C8 and (b) Hichrom RPB Peak assignment for both; (1) C_6, (2) C_{10} and (3) C_{18}

4.4 CONCLUSIONS

This procedure allows for the rapid and simple determination of aliphatic carboxylic acids (C_6-C_{22}) in used oil matrices by selective derivatisation with 9-anthracenemethanol and gradient reversed-phase LC with fluorescence detection. Calibrations over the range 1-4 mg ml^{-1} were linear. The limit of detection (s/n = 3) for octadecanoic acid was 85 μg ml^{-1} in *n*-heptane (4.3 ng on-column). More sensitive determination of earlier eluting derivatives was achieved after removal of excess label by solid phase extraction. It was shown by analysis of two series of oils from engine tests, that carboxylic acids are formed during the oxidation of engine oils and that their concentrations in the oils reflect the degree of degradation.

Chapter 5

Determination of Aldehydes in Used Engine Oils by Liquid Chromatography with Chemiluminescence Detection

5.1 INTRODUCTION

The previous two chapters described procedures for the determination of carboxylic acids in engine oils. As discussed in Chapter 1, aldehydes are intermediates in the oxidation pathway, it would therefore be advantageous to be able to monitor them in conjunction with the carboxylic acids in order to obtain a greater understanding of the oxidation process.

This chapter describes a procedure for the determination of aliphatic straight chain aldehydes (C_6-C_{14}) in non-aqueous matrices. It is based on a simple and selective derivatisation of the aldehydes with a fluorescent label, 3-aminofluoranthene, followed by reversed-phase LC and post-column reaction detection with POCL. The method was applied to the analysis of a series of oxidised oils sampled from an engine after different periods of operation.

5.2 EXPERIMENTAL

5.2.1 Reagents

High quality de ionised water from a Milli-Q system (Millipore) and analytical grade reagents were used unless otherwise stated. Acetonitrile (ACN), tetrahydrofuran (THF) and toluene were of HPLC grade (Rathburn).

Solutions of 3-aminofluoranthene (Janssen) were prepared daily in acetic acid (glacial; BDH)-toluene (2:7, v/v). Borane-pyridine complex (BAP; Aldrich) was used as the liquid. Solutions of hexanal (C_6), octanal (C_8) (both Aldrich), decanal (C_{10}), dodecanal (C_{12}) and tetradecanal (C_{14}) (all Fluka) were prepared in toluene. All aldehydes were of reagent grade.

Configuration 1

Working solutions of bis(2,4,6-trichlorophenyl)oxalate (TCPO; Fluka) were prepared in ACN, sonication was required to ease solubilisation. Hydrogen peroxide solutions were prepared by dilution of a 100 volume stock solution (aqueous; BDH) with ACN.

Configuration 2

A mixed CL reagent of TCPO and hydrogen peroxide was prepared by dissolving TCPO in ACN. To this solution was added 100 volume stock hydrogen peroxide solution. The combined solution was well mixed and left to stand for one hour before use.

Optimum concentrations of CL reagents are detailed in section 5.2.2.

Imidazole buffer (3.0×10^{-2} mol l^{-1}, pH 7.5) was prepared by dissolving imidazole (Fluka) in water; the pH was adjusted with nitric acid (Merck; 0.1 mol l^{-1}). LC mobile phase was prepared on a volume/volume basis and was degassed with helium immediately before use. CL reagents and LC mobile phase were prepared daily. Fresh and oxidised oil dialysate samples were supplied by Thornton Research Centre, Shell Research Limited, Chester, U.K.

5.2.2 Instrumentation

Configuration 1

The instrumental set-up was as described in Section 3.2.2 and in Fig 3.1. The mobile phase of ACN-THF-imidazole buffer (75:15:10 v/v/v) was pumped at 1.2 ml min^{-1}. The post column reagents, TCPO (1.0×10^{-3} mol l^{-1}) and hydrogen peroxide (0.1 mol l^{-1}) were each pumped at 0.4 ml min^{-1}.

Configuration 2

A schematic diagram of the instrumental configuration is given in Fig 5.1. Samples (5 µl) were injected (Rheodyne 7010) into a mobile phase of ACN-THF-imidazole buffer (75:15:10 v/v/v) pumped at 0.6 ml min^{-1}. Separation was achieved using a stainless steel column (250 x 3.2 mm i.d) packed with Spherisorb S5 ODS2-5 (5µm) (Phenomenex).

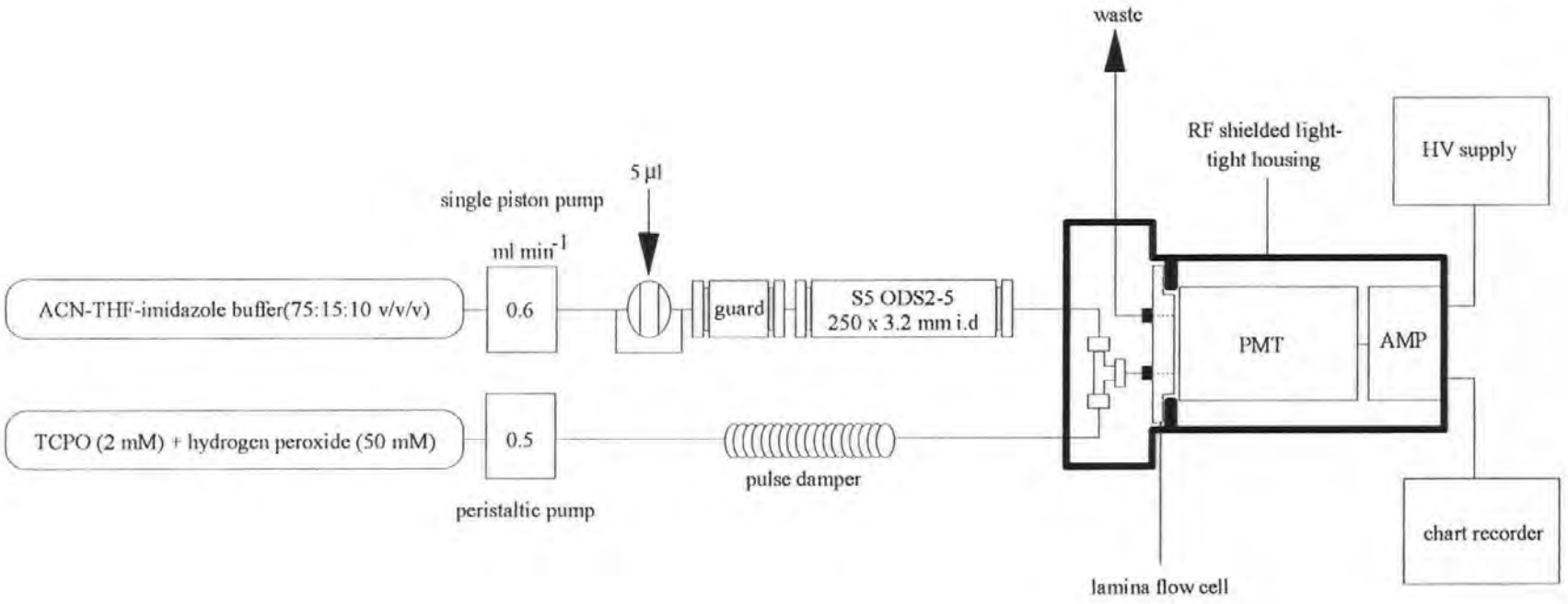


Fig 5.1 LC-CL manifold for the determination of aldehydes in non-aqueous media

A Spherisorb S5 ODS2-5 cartridge guard column (Hichrom) was used. A mixed CL reagent containing TCPO (2.0×10^{-3} mol l^{-1}) and hydrogen peroxide (5.0×10^{-2} mol l^{-1}), was pumped at 0.5 ml min^{-1}. All other experimental details were as described in section 3.2.2.

Response was determined as the signal-to-noise ratio. Signals were measured manually as peak heights and noise as the amplitude of the baseline.

5.2.3 Pre column derivatisation

Polymeric additives, organometallic oxidation products and solid debris which could interfere with the pre-column derivatisation were removed by dialysis at Thornton Research Centre as described in section 4.2.2. The following reaction conditions were used unless otherwise stated.

Aldehyde standards in toluene, oil dialysate samples and oil dialysates spiked with aldehyde standards (100 µl) were added to a mixture of 3-aminofluoranthene (1.0×10^{-3} mol l^{-1} in glacial acetic acid-toluene (2:7 v/v); 2 ml) and BAP (5 µl). Toluene was added to bring the final volume of the reaction mixture to 2.25 ml. The reaction mixture was shaken and an aliquot (250 µl) diluted with ACN-THF (20:5 v/v; 25 ml), the derivatised sample was then ready for immediate analysis.

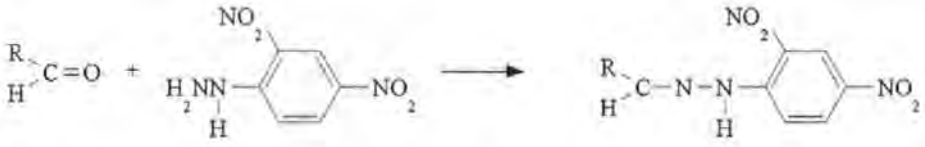
5.3 RESULTS AND DISCUSSION

All concentrations relate to the original sample.

5.3.1 Pre column derivatisation

Liquid chromatography has been applied to the determination of aldehydes, particularly in environmental samples. Aldehydes may be separated by ion chromatography and detected by pulsed amperometric detection [222], however derivatisation with a chromophore or a fluorophore prior to chromatographic analysis is more common. Nucleophilic addition with nitrogen containing nucleophiles, e.g. oximes, hydrazines and semicarbazides, has been the main reaction used [124].

The most common derivatising reagent for aldehydes is dinitrophenylhydrazine (DNPH) [124]. DNPH reacts with aldehydes to give hydrazones which are strongly UV absorbing.

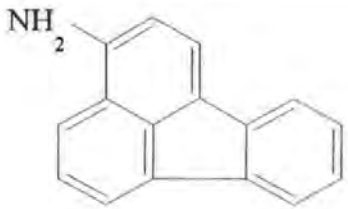
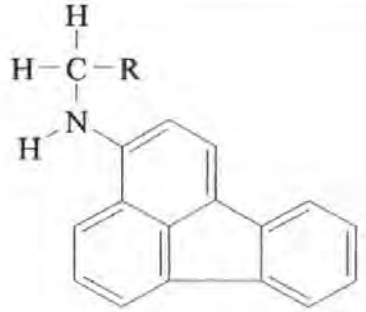
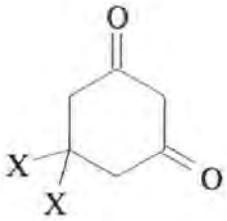
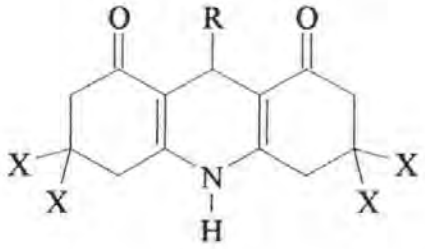


aldehyde DNPH hydrazone

The method has found wide application to the determination of aldehydes in environmental samples, e.g. rain and river water [223], automobile exhaust [224] and atmospheric air [225]. The derivatisation usually takes place under acidic conditions in a polar solvent e.g. methanol [124]. For gaseous samples, the aldehydes may be collected on filters [226] or silica solid phase extraction cartridges [225] coated with DNPH. The presence of nitrogen in the hydrazone derivatives allows determination by GC with thermionic specific detection (GC-TSD) [227], detection of the hydrazones is also possible by reduction at an electrode [228].

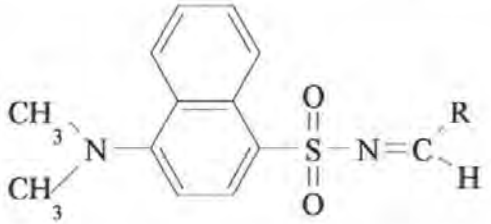
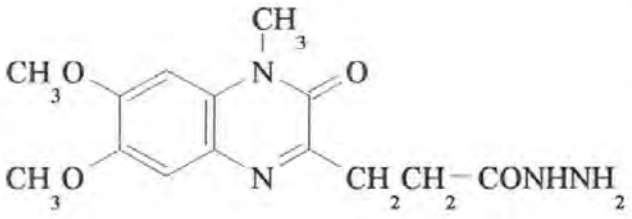
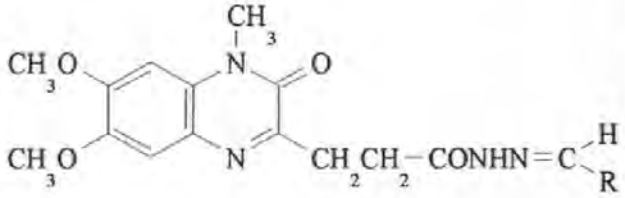
Aldehydes form azines with 3-methyl-2-benzothiazolone hydrazone (MBTH) which when oxidised give highly coloured cations. This has been used for colourimetric detection (640 nm) for the determination of aldehydes in cloud- and fog water [229].

Fluorimetric derivatising reagents that have been applied to the determination of aldehydes are presented in Table 5.1, several of them utilise fluorophores that have been applied to the determination of carboxylic acids e.g. benzofurazans [230], quinolizinocoumarins [231] and quinoxalinones [232]. Hydrazine derivatives of benzofurazans (2,1,3-benzoxadiazoles) have been applied to the determination of aldehydes in whisky. As the derivatives are not fluorescent in the aqueous reversed-phase solvents used for separation, a post-column on-line liquid-liquid extraction into an immiscible organic solvent e.g. chloroform in which the derivatives are fluorescent is carried out [230].

Table 5.1 Derivatisation reactions for the LC determination of aldehydes		
Label, mode of separation and detection, limits of detection[a]	Structure of label	Derivative
3-aminofluoranthene aldehydes and ketones in non-aqueous media, reversed-phase LC, fluorescence (λ_{ex} = 305 nm, λ_{em} = ≥389 nm), and peroxyoxalate CL (TCPO) detection, fmol limits of detection, CL 30 x improvement on fluorescence [159]		
1,3-cyclohexanedione (X = H) aldehydes in wine, automobile exhaust , reversed-phase LC, fluorescence detection (λ_{ex} = 305-395 nm, λ_{em} = 450 nm) [233] **5,5-dimethylcyclohexanedione (X = CH_3)** aldehydes in perfume and automobile exhaust , reversed-phase LC, fluorescence detection (λ_{ex} = 385 nm, λ_{em} = 460 nm), fmol limits of detection [234]		

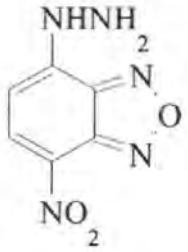
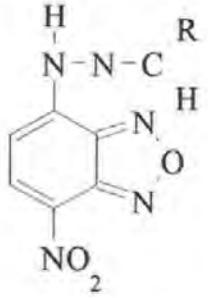
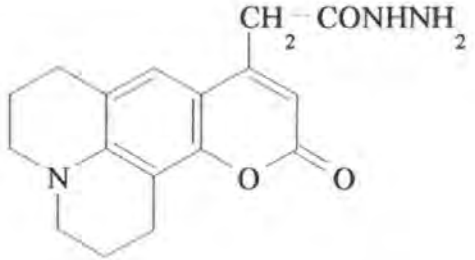
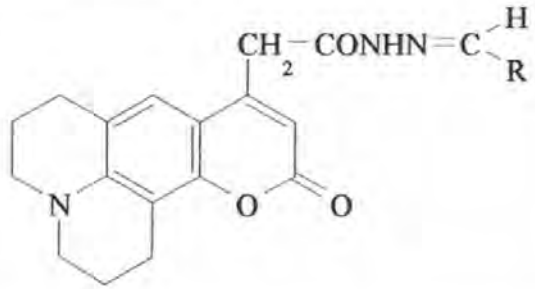
[a]limits of detection as quoted in original paper

Table 5.1 (continued) Derivatisation reactions for the LC determination of aldehydes

Label, mode of separation and detection, limits of detection[a]	Structure of label	Derivative
Dansylhydrazine aldehydes in air, reversed-phase LC, fluorescence (λ_{ex} = 240 nm, λ_{em} = >470 nm) and photo initiated peroxyoxalate CL (TCPO) detection[235], sub ng detection limits for both modes of detection.		
DMEQ-hydrazide pharmaceutical analysis, reversed-phase LC, fluorescence detection, (λ_{ex} = 362 nm, λ_{em} = 442 nm), fmol detection limits[232]		

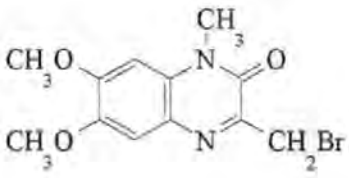
[a]limits of detection as quoted in original paper

Table 5.1 (continued) Derivatisation reactions for the LC determination of aldehydes

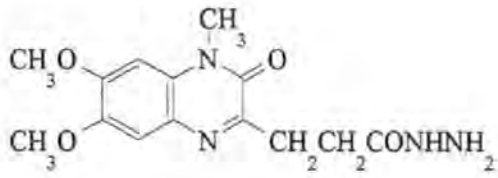
Label, mode of separation and detection, limits of detection[a]	Structure of label	Derivative
7-Hydrazino-4-nitrobenzo-2,1,3-oxadiazole food analysis, reversed-phase LC, post-column on-line liquid-liquid extraction into chloroform-carbon tetrachloride (50:50 v/v), fluorescence detection (λ_{ex} = 470 nm, λ_{em} = 530 nm) [230]		
Luminarin 3 biochemical analysis, quinolizinocoumarin fluorophore with carboxylic acid hydrazide reacting group, reversed-phase and normal phase LC, fluorescence detection, fmol detection limits [231]		

[a]limits of detection as quoted in original paper

Quinolizinocoumarin fluorophores have been modified for use with aldehydes by the addition of a carboxylic acid hydrazide reacting group [231] while the 3-bromomethyl-6,7-dimethoxy-1-methyl-1(1H)-quinoxalinone [232] has been modified by replacement of the halogen with a reactive group possessing a hydrazino functionality for application to the selective derivatisation of aldehydes;



3-Bromomethyl-6,7-dimethoxy-1-methyl-1(1H)-quinoxalinone



6,7-dimethoxy-1-methyl-2-oxo-1,2-dihydroquinoxalin-3-ylpropionohydrazide (DMEQ-hydrazide)

Photo initiated POCL has been used to detect dansylhydrazone derivatives of airborne aldehydes. UV irradiation in a photochemical reactor of the aryl oxalate solution in the presence of a hydrogen atom donor (e.g. 2-propanol) and dissolved oxygen forms high energy intermediate(s) that excite the fluorescent derivatives [235].

Reductive amination of aldehydes with 3-aminofluoranthene

Amino-substituted polycyclic aromatic hydrocarbons (amino-PAH) have been shown to be among the most efficient sensitisers for POCL [147]. A procedure for the labelling of aldehydes and ketones with the amino-PAH fluorophore 3-aminofluoranthene via reductive amination has been reported (Fig. 5.2) [159]. Reduction is possible in acid and neutral aqueous media with sodium cyanoborohydride, this is unsuitable for derivatisation of engine oils as the reaction conditions are incompatible with the oil matrix. In addition the reductant is toxic and generation of sodium cyanide and hydrogen cyanide may occur during the reaction [236].

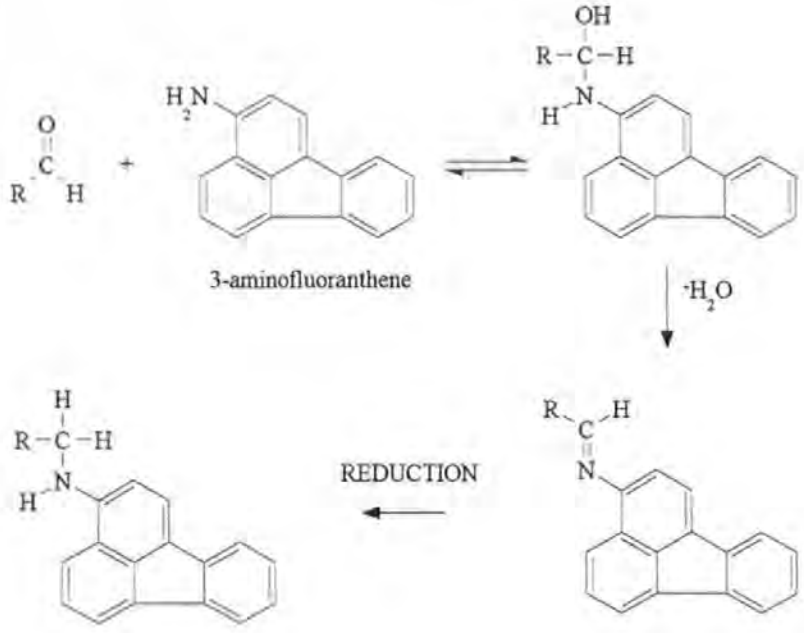


Fig 5.2 Reductive amination of aldehydes by 3-aminofluoranthene

The derivatisation of the aldehydes in the non-aqueous media which are compatible with the oil matrix may be carried out using acid catalysed reduction of the aldehydes with 3-aminofluoranthene in the presence of BAP.

BAP was suggested by Pelter *et al.* [236] as a cheap, less toxic and readily available alternative to sodium cyanoborohydride. For high yields to be obtained from the reductive amination of an aldehyde, at equilibrium there should be a sizeable concentration of the imine intermediate. Formation of the imine is assisted by acid catalysis [237, 238]. In addition as imines are much more basic than the starting aldehyde, reduction will be directed towards the imine [239]. A nucleophilic, acid stable, reductant is therefore required for this reaction and BAP fulfils these requirements.

Aldehydes react immediately with 3-aminofluoranthene in the presence of BAP, while ketones require heating for up to 14 hours. Therefore if no heating is applied this procedure is selective for aldehydes.

5.3.2 Liquid chromatography and chemiluminescence detection

Configuration 1

Grayeski and Mann separated the 3-aminofluoranthene derivatives on a C18 column using a mobile phase of ACN-aqueous tris(hydroxymethyl)aminomethane buffer (4.0×10^{-3} mol l^{-1}, pH 7.5), 85:15 v/v. In this work the derivatives were separated on an ODS2-5 column using a mobile phase of ACN-THF-imidazole buffer (3.0×10^{-2} mol l^{-1}). The inclusion of THF ensures that the mobile phase is compatible with the derivatised oil dialysate samples (Chapter 3). Imidazole not only buffers the TCPO CL reaction, but has also been shown to give a marked catalytic effect (Chapter 2 and references therein).

THF is relatively non-polar compared with the other mobile phase components, increasing the relative concentration will increase the eluting power of the mobile phase with the aldehyde-3-aminofluoranthene derivatives. As stated in section 3.3.4, the maximum concentration of THF is restricted as it quenches POCL emission. Using a mixture of hexanal- and octanal-3-aminofluoranthene derivatives, the ACN and THF concentrations in the mobile phase were varied whilst keeping the imidazole buffer concentration constant at 10 % (v/v). The effect of mobile phase composition on retention of the derivatives is presented in Table 5.2.

Table 5.2 Effect of mobile phase composition on aldehyde-3-aminofluoranthene derivative retention

Mobile phase composition	Hexanal (C_6)	Octanal (C_8)
ACN-THF-imidazole buffer (v/v/v)	Capacity factor (k')[a]	Capacity factor (k')[a]
80:10:10	4.0	6.6
77.5:12.5:10	3.7	5.9
75:15:10	3.3	5.1
72.5:17.5:10	2.8	4.6

[a] t_0 = 1.8 min

The mobile phase containing the highest proportion of THF, gave the lowest retention times, but there was increased interference from the peak associated with excess label. On the basis of this data, a mobile phase of ACN-THF-imidazole buffer (75:15:10 v/v/v) was selected. A mixture of straight chain aldehyde derivatives (hexanal, octanal, decanal, dodecanal and tetradecanal) was analysed using this mobile phase composition and separation of the derivatives was achieved in 40 min. Retention data is presented in Table 5.3.

Table 5.3 Retention data for aldehyde-3-aminofluoranthene derivatives separated on 4.6 mm i.d. column

Aldehyde derivative	Capacity factor (k')[a]
Hexanal (C_6)	3.1
Octanal (C_8)	4.9
Decanal (C_{10})	7.8
Dodecanal (C_{12})	12.9
Tetradecanal (C_{14})	21.5

[a] t_0 = 1.8 min

Configuration 2

Microbore LC involves columns with diameters of the order of 1-2 mm i.d. [240, 241] and has been reported with POCL detection [242, 243]. As the volume of solvent required to maintain a given linear velocity is less in micro-bore columns, lower flow rates are possible leading to reduced solvent consumption [244]. Greater sensitivity is possible as the peak volumes are lower, leading to less dilution of the injected sample by mobile phase [241, 242]. However microbore LC requires specialised equipment e.g. pumps capable of delivering very low flow rates accurately (10 - 800 µl) and low dead volume flow cells [241].

An alternative is mid bore 3.2 mm i.d. columns, this is the diameter necessary to reduce the flow rate of a 4.6 mm i.d. conventional column by half, and still retain an equivalent linear velocity [245]. No specialised pumps, fittings or flow cells are

required. In addition lower flow rates for the CL reagents will be possible, leading to reduced solvent and reagent consumption.

Aldehyde-3-aminofluoranthene derivatives were separated on a 3.2 mm i.d. column, (250 mm, packed with Spherisorb ODS2-5) using the same mobile phase, ACN-THF-imidazole buffer (75:15:10 v/v/v), at a flow rate of 0.6 ml min^{-1}. In order not to overload the column, the sample injection had to be reduced to 5 µl. In addition a mixed TCPO-hydrogen peroxide post-column reagent was used, allowing for simpler instrument geometry.

The use of a mixed aryl oxalate-hydrogen peroxide reagent to simplify instrumental set-ups has been shown with TCPO and TDPO [246]. DNPO cannot be used in this manner as it is unstable in the presence of hydrogen peroxide [179]. The stability of the mixed reagent was assessed by repeat injections of 3-aminofluoranthene (1×10^{-6} mol l^{-1}) over a period of 8 hours. The concentration of TCPO was 1.0×10^{-3} mol l^{-1} and that of hydrogen peroxide was 0.1 mol l^{-1}. The flow rate of the CL reagent was set at 0.4 ml min^{-1}. The signal-to-noise ratio was found not to vary significantly over this time period.

A mixture containing straight chain aldehyde derivatives was separated on the 3.2 mm i.d. column and detected with the mixed TCPO-hydrogen peroxide reagent. The derivatives were all separated within 30 minutes, an improvement over the conventional column of 10 minutes. Retention data are presented in Table 5.4. Capacity factors for the derivatives separated on the 4.6 mm i.d. column are included for comparison. A chromatogram of the separation is presented in Fig. 5.3.

The logarithms of the capacity factors plotted against the carbon number of the aldehydes for both columns gave straight lines, the linear fit and regression data are shown in Table 5.5.

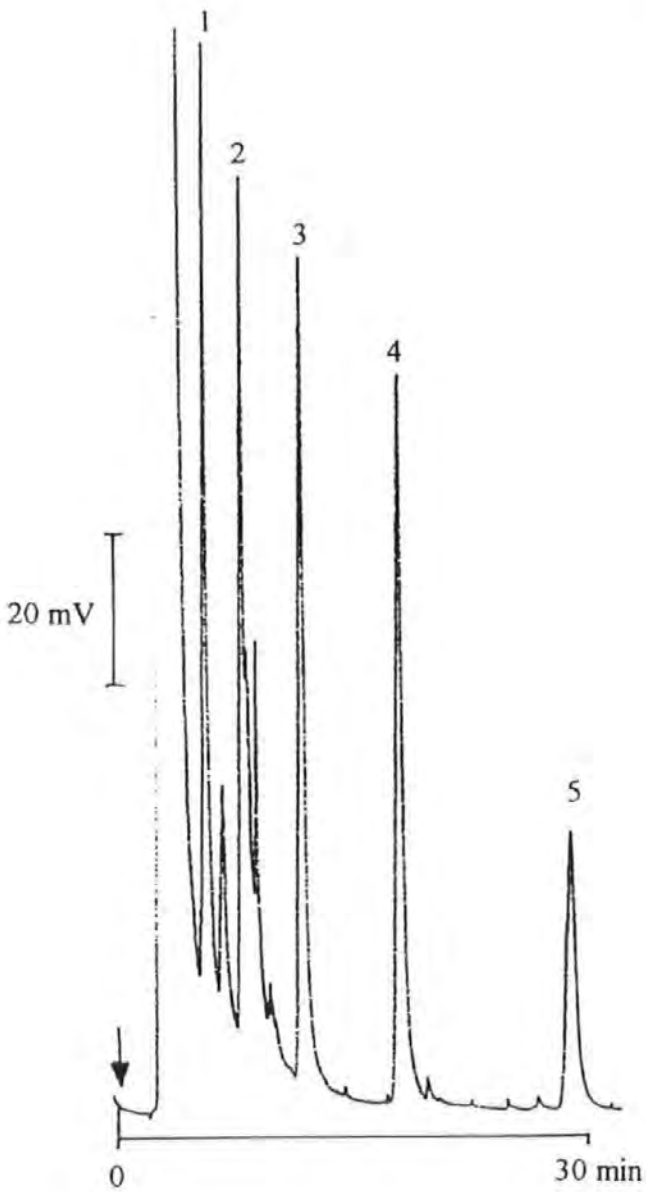


Fig 5.3 Separation of aliphatic straight chain aldehyde-3-aminofluoranthene derivatives using 3.2 mm i.d. S5 ODS2-5 column
Peak assignment; (1) C_6, (2) C_8, (3) C_{10}, (4) C_{12} and (5) C_{14}

Table 5.4 Retention data for aldehyde-3-aminofluoranthene derivatives separated on 3.2 mm i.d. column		
Aldehyde derivative	Capacity factor (k')[a] 3.2 mm i.d. column	Capacity factor (k')[b] 4.6 mm i.d. column
Hexanal (C_6)	1.9	3.1
Octanal (C_8)	3.0	4.9
Decanal (C_{10})	4.8	7.8
Dodecanal (C_{12})	8.0	12.9
Tetradecanal (C_{14})	13.3	21.5

[a] t_0 = 2.0 min, [b] t_0 = 1.8 min

Table 5.5 Linear fit data for log(capacity factor, k') vs. carbon number		
Column	Linear fit (C = carbon number)	r^2
4.6 mm i.d.	log(k') = 0.105 C-0.145	0.9994
3.2 mm i.d.	log(k') = 0.106 C -0.364	0.9991

Configuration 2 (3.2 mm i.d. column, mixed CL reagent) was used for all subsequent work.

5.3.3 Optimisation

Chemiluminescence detection

CL reagent flow rate and CL reagent concentration were investigated using a hexanal derivative (5×10^{-4} mol l^{-1}) as a representative compound.

The flow rate of CL reagent was varied over the range 0.35 - 0.60 ml min^{-1}. The CL reagents were present at the following concentrations; TCPO, 0.5×10^{-3} mol l^{-1} and hydrogen peroxide, 5.0×10^{-2} mol l^{-1}. The CL emission intensity was directly

related to flow rate up to an optimum of 0.5 ml min^{-1}. Flow rates greater than this lead to lower observed CL emission intensities due to the point of maximum intensity occurring after the analyte had passed through the flow cell.

The concentration of TCPO was investigated over the range 2.5 x 10^{-4} mol l^{-1}- 2.0 x 10^{-3} mol l^{-1}, hydrogen peroxide was kept at 5.0 x 10^{-2} mol l^{-1} and the CL reagent flow rate was 0.5 ml min^{-1}. Aryl oxalate concentrations above 2.0 x 10^{-3} mol l^{-1} were not investigated as the TCPO was not completely soluble at these levels. CL emission intensity was found to be directly related to TCPO concentration, as was found to be the case with the separate CL reagent flow injection experiments described in Chapter 2. The optimum concentration of TCPO was determined to be 2.0 x 10^{-3} mol l^{-1} (Fig 5.5 (a)).

The concentration of hydrogen peroxide was investigated over the range 1.0 x 10^{-2}- 7.5 x 10^{-2} mol l^{-1}, TCPO concentration was 2.0 x 10^{-3} mol l^{-1} and the CL reagent flow rate was 0.5 ml min^{-1}. Concentrations of hydrogen peroxide above 7.5 x 10^{-2} mol l^{-1} lead to the precipitation of aryl oxalate from the mixed reagent. This is due to the increase in the percentage of water (70 % w/v) associated with the hydrogen peroxide stock solution.

Over this range CL emission intensity is directly related to hydrogen peroxide concentration, with an optimum at a hydrogen peroxide concentration of 7.5 x 10^{-2} mol l^{-1} (Fig. 5.4 (b)). On close examination of the CL reagent reservoir however it was seen that TCPO began to come out of solution over a period of 30 min when hydrogen peroxide was present at this level.

The optimum conditions which were used in all subsequent experiments were; TCPO, 2.0 x 10^{-3} mol l^{-1}, hydrogen peroxide, 5.0 x 10^{-2} mol l^{-1}, CL reagent flow rate, 0.5 ml min^{-1}. With these conditions the mixed CL reagent was stable for a period of 24 hours, but were not fit for use after 48 hours.

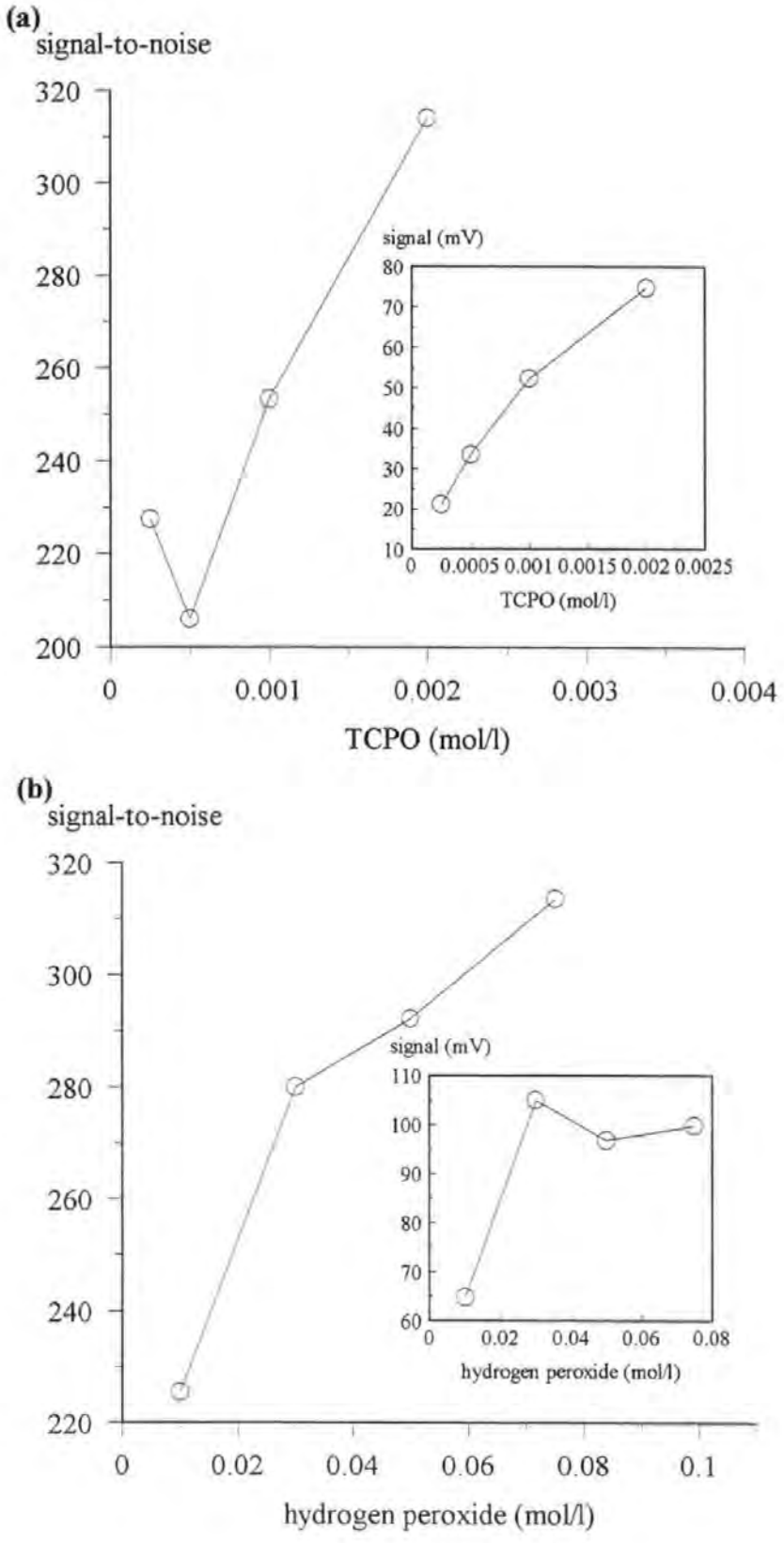


Fig 5.4 Optimisation of CL reagent concentrations (a) TCPO (b) hydrogen peroxide

Pre-column derivatisation

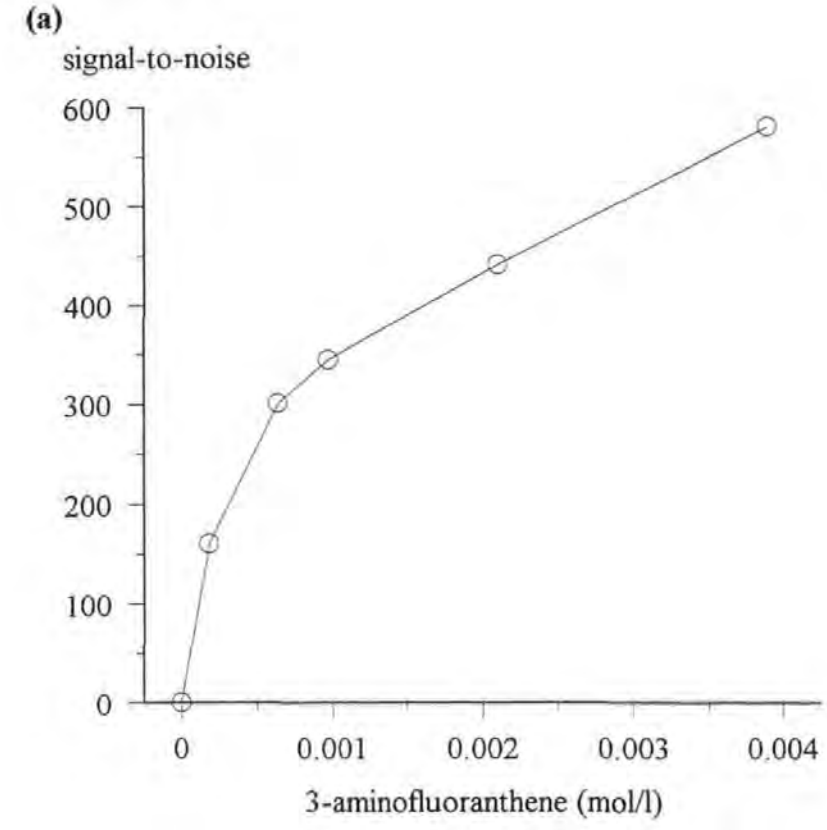
Hexanal (5.0×10^{-4} mol l^{-1}) was derivatised with varying concentrations of 3-aminofluoranthene over the range 0 - 4.0×10^{-3} mol l^{-1}. The yield of the reaction, measured by the detector response, was found to be directly related to the concentration of label (Fig 5.5 (a)). Excess label interfered with the early eluting peaks at higher concentrations of label, a concentration of 1.0×10^{-3} mol l^{-1} was therefore selected for all further work.

The volume of BAP added to the reaction mixture was varied over the range 0-100 µl, using hexanal (5.0×10^{-4} mol l^{-1}) as the model compound. When no catalyst was present, no detectable quantities of derivatised aldehyde were seen. The optimum level of BAP was found to be 5 µl; increasing the amount of BAP lead to a decrease in the yield of derivative (Fig 5.5 (b)). An increase in other peaks was not observed, therefore the drop in yield was due to the formation of non-fluorescent by-products.

5.3.4 Calibrations

Calibration data for hexanal, octanal, decanal, dodecanal and tetradecanal were obtained over the range 0 - 5.0×10^{-4} mol l^{-1} in toluene, and are presented in Table 5.6. The calibrations were all linear over the range investigated ($0.9980<r^2<0.9997$) and the linear fit data are presented in Table 5.7. Limits of detection (Table 5.8) fall in the range 0.7 - 75 fmol (on column, 5 µl injection) . These results are an order of magnitude lower than those reported by Mann and Grayeski [159], this is due to the increased sensitivity of the TCPO CL reaction when catalysed by imidazole.

Recovery data was obtained by derivatising a mixture of aldehydes (C_6-C_{14} at the 2.5×10^{-4} mol l^{-1} level), all the recoveries fall in the range 83-104 % except for octanal which had a recovery of 77 % (Table 5.7). This low recovery was due to an interfering peak from the derivatisation mixture. The precision of the CL detection was determined at the lowest concentration level used for the calibration by repeated injections of a derivatised mixture of decanal and dodecanal (2.5×10^{-5} mol l^{-1}) (Table 5.9).



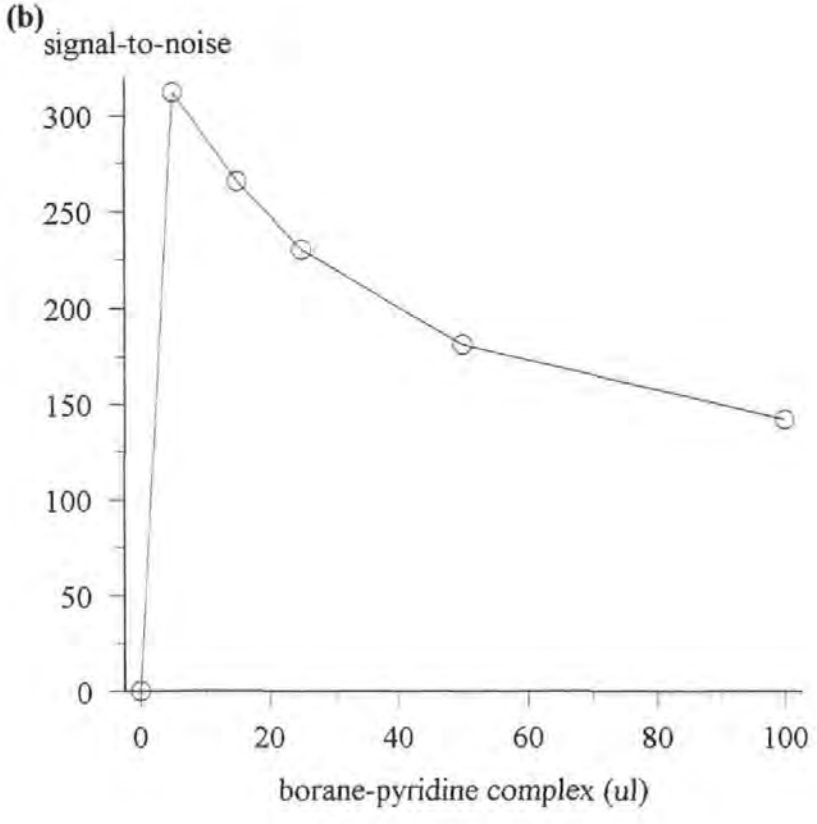


Fig 5.5 Optimisation of derivatisation reagent concentrations (a) 3-aminofluoranthene (b) borane-pyridine complex (BAP)

Table 5.6 Aldehyde calibration data

Hexanal		Octanal		Decanal		Dodecanal		Tetradecanal	
Conc. (mol l^{-1})	Response (s/n)	Conc. (mol l^{-1})	Response (s/n)	Conc. (mol l^{-1})	Response (s/n)	Conc. (mol l^{-1})	Response (s/n)	Conc. (mol l^{-1})	Response (s/n)
0.0	0.0	0.0	7.3	0.0	0.0	0.0	0.0	0.0	0.0
-	-	2.6×10^{-5}	11.1	3.0×10^{-5}	7.1	2.6×10^{-5}	5.9	2.5×10^{-5}	2.5
5.2×10^{-5}	21.5	5.2×10^{-5}	17.3	6.0×10^{-5}	13.8	5.1×10^{-5}	10.9	5.0×10^{-5}	4.3
1.0×10^{-4}	42.5	1.0×10^{-4}	27.6	1.3×10^{-4}	25.9	1.0×10^{-4}	21.1	1.0×10^{-4}	7.4
5.2×10^{-4}	191.3	5.2×10^{-4}	125.2	6.0×10^{-4}	158.8	5.1×10^{-4}	114.5	5.0×10^{-4}	31.5

Table 5.7 Linear fit and recovery data for aldehyde calibrations						
Aldehyde	Slope (x 10^5 mol l^{-1})	Intercept	r^2	Conc. standard (mol l^{-1})	Conc. found (mol l^{-1})	Recovery (%)
Hexanal	3.67 ± 0.18	2.2 ± 4.8	0.9995	2.6×10^{-4}	2.7×10^{-4}	104
Octanal	2.31 ± 0.11	5.5 ± 2.6	0.9993	2.6×10^{-4}	2.0×10^{-4}	77
Decanal	2.66 ± 0.22	-2.4 ± 6.0	0.9980	3.0×10^{-4}	2.5×10^{-4}	83
Dodecanal	2.23 ± 0.07	-0.5 ± 0.5	0.9997	2.6×10^{-4}	2.3×10^{-4}	88
Tetradecanal	0.62 ± 0.05	0.8 ± 1.0	0.9984	2.5×10^{-4}	2.6×10^{-4}	104

Table 5.8 Limits of detection (s/n = 3)			
Aldehyde	mol l^{-1}	mol on-column	g on-column
hexanal	3.0×10^{-7}	6.7×10^{-16}	6.7×10^{-14}
decanal	2.5×10^{-6}	5.6×10^{-13}	8.7×10^{-12}
dodecanal	1.8×10^{-6}	4.0×10^{-13}	7.4×10^{-12}
tetradecanal	3.4×10^{-6}	7.5×10^{-13}	1.6×10^{-11}

Table 5.9 Precision of CL detection assessed using lowest concentration aldehyde standard (2.5×10^{-5} mol l^{-1})		
Aldehyde derivative	Response (s/n)	Relative standard deviation (%), n= 6
Decanal	4.6	5.6
Dodecanal	3.7	7.1

5.3.5 Oil analysis

Oil A was sampled from the sump of a car engine after 0, 16, 24 and 32 hours continuous running of the engine. From analysis of the 32 h used oil dialysate, oxidation of the oil resulted in the formation of a homologous series of straight chain aliphatic acids, with hydrocarbon chain lengths from C_7 to above C_{16}. No carboxylic acids were detectable in the 0, 16 and 24 hour oil dialysates.

The series of oil dialysates for oil A over the time course of the engine test was derivatised and analysed. Dodecanal was identified by retention time in the 0 h fresh oil dialysate at a level of 1.5×10^{-5} mol l^{-1} (Table 5.10); no other straight chain aldehydes were detectable. Recoveries in the fresh oil dialysate obtained by spiking the fresh oil dialysate prior to derivatisation with a mixture of aldehydes (C_{10}, C_{12}, C_{14} at the 5×10^{-5} mol l^{-1} level) were in the range 104-112 % (Table 5.10).

Table 5.10 Analysis of 0 h fresh oil dialysate

Aldehyde	Conc.0 h (mol l^{-1})	Conc. 0 h ($\mu g\ g^{-1}$)	Conc. 0 h + spike (mol l^{-1})	Conc. found (mol l^{-1})	Recovery (%)
Decanal	not detected	not detected	6.0×10^{-5}	6.7×10^{-5}	112
Dodecanal	1.5×10^{-5}	3.2	5.1×10^{-5}	6.8×10^{-5}	104
Tetradecanal	not detected	not detected	5.0×10^{-5}	5.4×10^{-5}	108

As can be seen from the chromatograms (Fig 5.6), two distinct series of peaks appear in the 16 h used oil dialysate and the peak heights of these increase in magnitude with the time of test duration, these two series are labelled ♥ and ♣ on the chromatogram of the 32 h used oil dialysate (Fig 5.7). Several peaks of the series labelled ♥ for the 32 h used oil dialysate were identified as straight chain aldehydes by capacity factors (Table 5.11). This was confirmed by spiking the used oil dialysate with a mixture of the aldehydes prior to derivatisation. These aldehydes

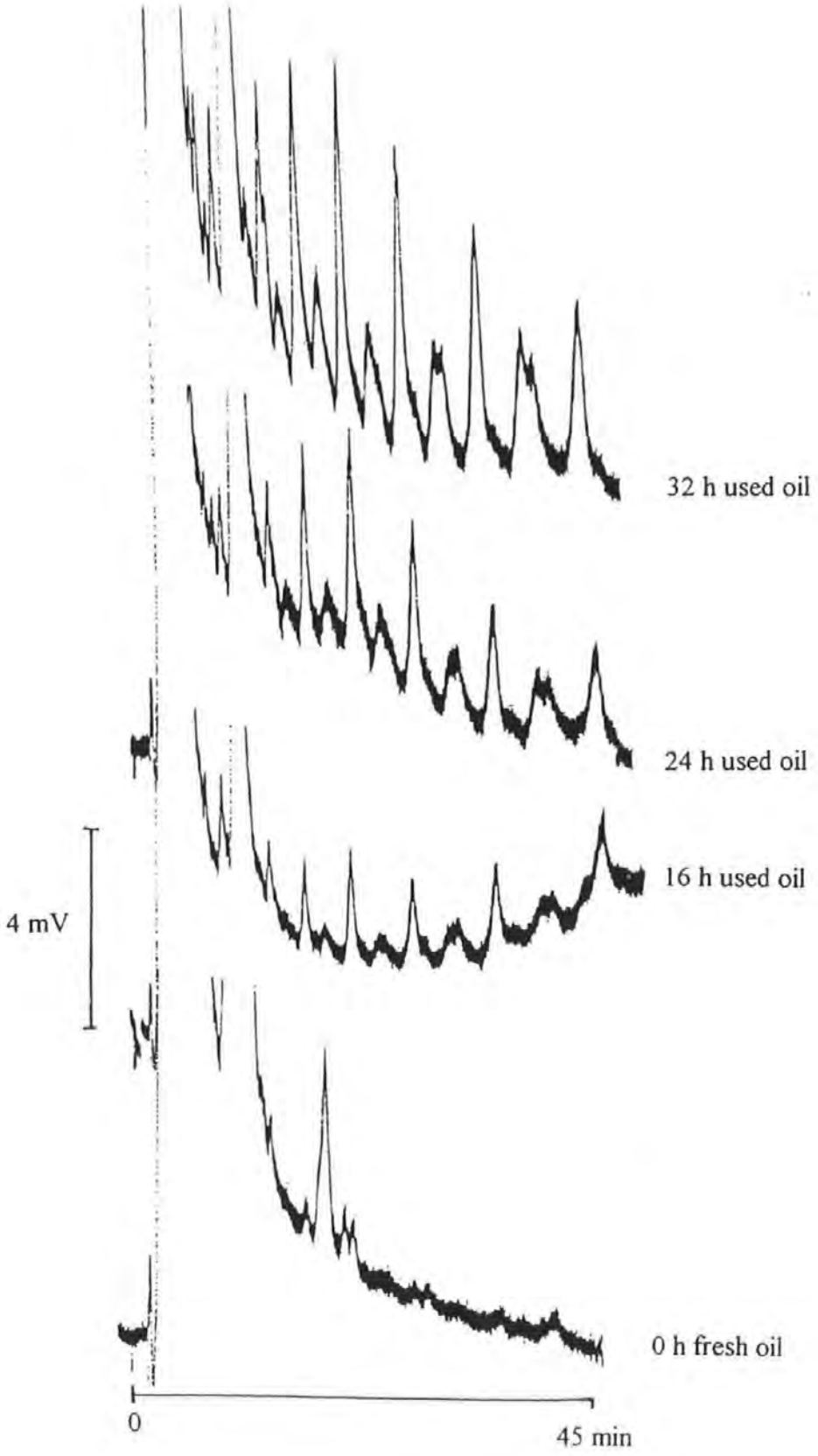


Fig 5.6 Chromatograms showing the formation of aldehydes in an engine oil over the time course of an engine test

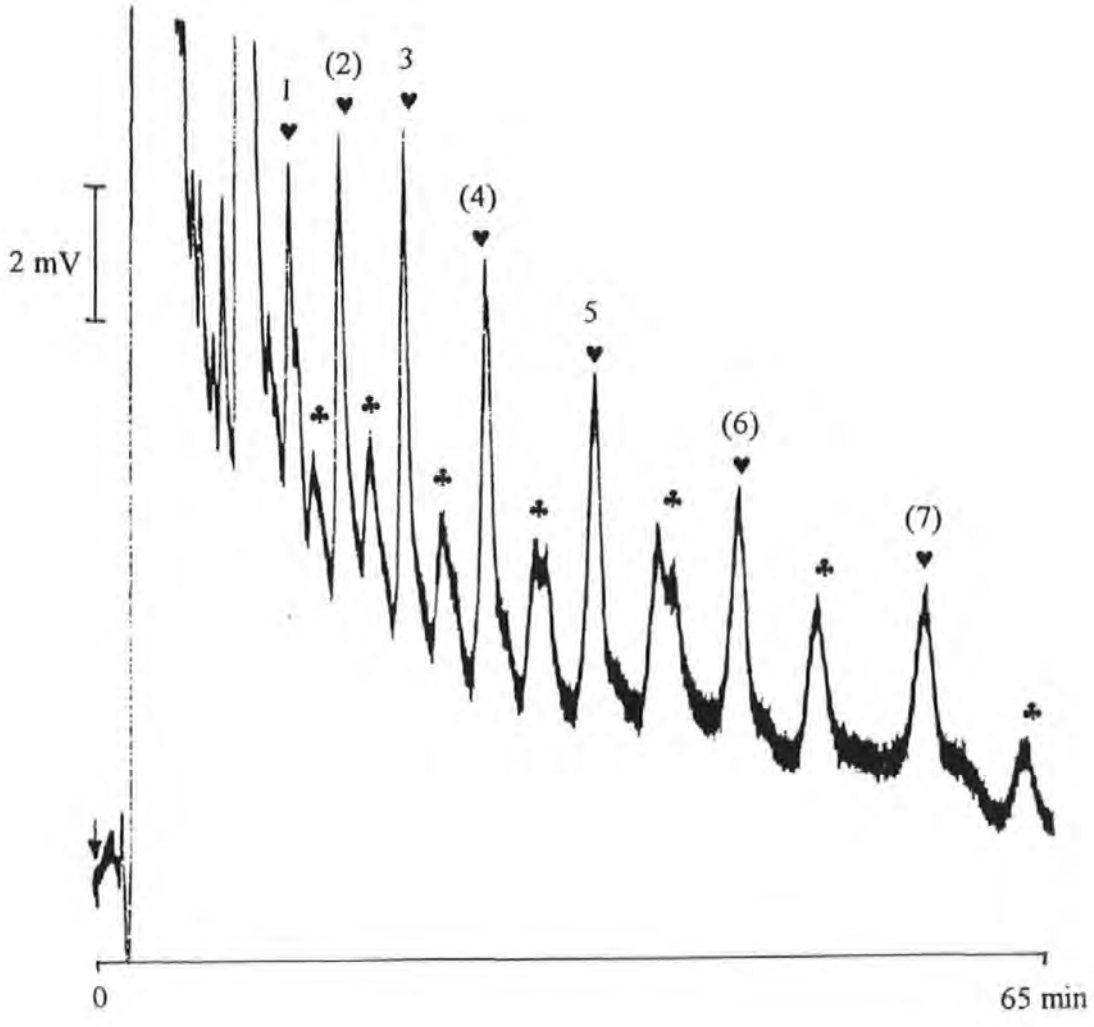


Fig 5.7 Chromatogram of 32 h used oil A dialysate
♥ = aliphatic straight chain aldehydes, ♣ = unidentified derivatives, possibly branched chain aldehydes
Peak assignment; (1) C_{10}, (2) C_{11}, (3) C_{12}, (4) C_{13}, (5) C_{14}, (6) C_{15} and (7) C_{16}

were present at concentrations in the range 1.1 x 10^{-5}-8.6 x 10^{-5} mol l^{-1} (Table 5.12).

Recoveries were carried out by spiking the 32 oil dialysate with a mixture of aldehydes (C_6-C_{14} at the 2.5 x 10^{-5} mol l^{-1} level) prior to derivatisation (Table 5.12). The lower recoveries obtained for hexanal and dodecanal were due to the interference in the unspiked oil dialysate, which in the case of hexanal was due to interference from excess label.

Table 5.11 Identification of aldehydes in 32 h used oil dialysate

Aldehyde derivative	Aldehyde standard (k')[a]	32 h used oil dialysate (k')[a]
Hexanal	1.9	1.8
Octanal	3.0	-
Decanal	4.8	4.9
Dodecanal	8.0	8.0
Tetradecanal	13.3	13.4

[a] $t_0 = 2.0$ min

Table 5.12 Analysis of 32 h used oil dialysate

Aldehyde	Conc. 32 h (mol l^{-1})	Conc. 32 h (μg g^{-1})	Conc. spike (mol l^{-1})	Conc. 32 h + spike (mol l^{-1})	Recovery (%)
Hexanal	1.1 x 10^{-5}	1.3	2.6 x 10^{-5}	3.3 x 10^{-5}	85
Decanal	2.6 x 10^{-5}	4.7	3.0 x 10^{-5}	5.6 x 10^{-5}	100
Dodecanal	4.0 x 10^{-5}	8.6	2.6 x 10^{-5}	5.8 x 10^{-5}	69
Tetradecanal	8.6 x 10^{-5}	21.3	2.5 x 10^{-5}	11.3 x 10^{-5}	108

The identity of the other peaks of the series as straight chain aldehydes was established by using the linear relationship between log (k') and carbon number determined from the analysis of the standard mixture of aldehydes (Table 5.13).

Table 5.13 Identification of straight chain aldehydes in 32 h used oil dialysate by calculation from equation log (k') = 0.106 carbon number - 0.364		
Capacity factor (k')[a]	Calculated carbon number	Aldehyde
6.3	11.0	undecanal (C_{11})
10.2	13.0	tridecanal (C_{13})
17.2	15.1	pentadecanal(C_{15})
22.0	16.2	hexadecanal (C_{16})

[a] t_0 = 2.0 min

The formation of the aldehydes in the engine oil is shown graphically in Fig 5.8. The aldehydes are detectable at an earlier stage of the engine test compared to carboxylic acids, which were not detected earlier than the 32 h sample.

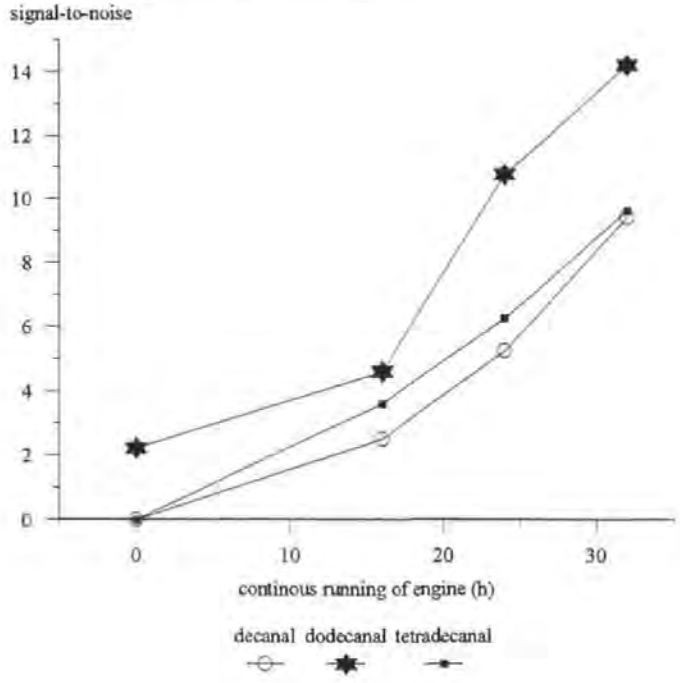


Fig 5.8 Formation of aldehydes in oil A over the time course of an engine test

Investigation of the aldehydes eluting after tetradecanal and identification of the derivatives labelled ♣ (possibly branched chain aldehydes) requires development of a gradient elution procedure in conjunction with removal of the label by solid phase extraction.

5.4 CONCLUSIONS

Aliphatic straight chain aldehydes (C_6-C_{14}) in non-aqueous media can be rapidly determined by selective derivatisation with 3-aminofluoranthene and reversed-phase LC with CL detection. The CL reaction manifold was simplified by the use of a mixed TCPO-hydrogen peroxide reagent. Calibrations over the range 0-5 x 10^{-4} mol l^{-1} were linear. The limits of detection (s/n = 3) were in the range 0.7-75 fmol on-column. It was shown by analysis of oil from an engine test that aldehydes are formed during the oxidation of engine oils. Aldehydes are detectable in the oil at an earlier stage of the engine test in comparison with carboxylic acids.

Chapter 6

General Conclusions and Suggestions for Future Work

6.1 GENERAL CONCLUSIONS

In addition to the specific conclusions at the end of each chapter, the following general conclusions can be drawn from the work described in this thesis;

1. Peroxyoxalate CL is suitable for the sensitive determination of fluorescent species in non-aqueous media. Flow injection studies showed that bis(2,4-dinitrophenyl)oxalate (DNPO) is more sensitive than bis(2,4,6-trichlorophenyl)oxalate (TCPO), however TCPO gives wider linear ranges and is more stable in solution than DNPO. The use of a spectrometer with a CCD detector allows the rapid acquisition of chemiluminescence spectra in flowing streams.

2. Carboxylic acids (C_6-C_{22}) can be determined in oils by derivatisation with 9-anthracenemethanol after dialysis has removed polymeric additives, organometallic oxidation products and solid debris which are suspected of interfering with the derivatisation reaction. Gradient elution with fluorescence detection is appropriate as the range of carboxylic acids present in oils is not separated by isocratic elution in a suitable time (< 30 min). The limit of detection (s/n = 3) for octadecanoic acid in *n*-heptane was 15 pmol (on column, 5 µl injection).

   Aldehydes (C_6-C_{14}) can be determined in oil dialysates by isocratic elution with peroxyoxalate CL detection, utilising a simplified post column reaction set-up with a mixed CL reagent of TCPO-hydrogen peroxide. The limits of detection (s/n = 3) were in the range 0.7-75 fmol (on column, 5 µl injection). This derivatisation is very rapid and sensitive and therefore could be used for monitoring oil condition during the course of engine tests.

3. The procedures described for carboxylic acids and aldehydes are suitable for monitoring the oxidative stability of engine oils. Homologous series of straight chain aliphatic carboxylic acids and aldehydes are formed during the oxidation of oils in a car engine

Aldehydes are formed at an early stage while the formation of carboxylic acids does not proceed until a later stage of the oxidation.The concentration of carboxylic acids in oxidised oils reflects the degree of degradation of the oil and is in good agreement with physical data (change in viscosity).

6.2 SUGGESTIONS FOR FUTURE WORK

Possible future work arising from this research can be divided into three separate areas;

Chemiluminescence

- The investigation of red and infrared fluorophores for peroxyoxalate chemiluminescence by flow injection with CCD detection. The background CL emission from the reactions of TCPO and DNPO is most significant at wavelengths < 600 nm, the use of red and infrared fluorophores with a spectrometer and the CCD detector would give improved sensitivity by allowing the isolation of individual bands of wavelengths.

- Application of spectrometer with CCD detection to liquid chromatography. This would allow wavelength discrimination so improving selectivity and sensitivity, particularly if red/infrared fluorophores are used.

Pre-column derivatisation

- Removal of excess label for the derivatisation of aldehydes with 3-aminofluoranthene by solid phase extraction would improve the sensitivity of this reaction towards lower chain length aldehydes that would otherwise be obscured by the label.

- As the 3-aminofluoranthene derivatisation reaction is very fast this allows the possibility of on-line derivatisation for liquid chromatography, thus improving analysis precision and reducing contact with the samples and derivatisation reagents, which are a potential health hazards .

Oil analysis

- There is evidence that there are aldehydes with chain lengths > C_{14} in the oxidised oil dialysates, a gradient elution procedure is required for examination of these aldehydes and the unidentified 3-aminofluoranthene derivatives.

- A figure for total aldehydes would be a useful parameter to monitor the oxidation of engine oils in tests. The derivatisation procedure is more rapid than carboxylic acid esterification with 9-anthracenemethanol and the aldehydes appear at an earlier stage of oxidation, so allowing observation of the onset of oil degradation. An approach to supplying this information in a rapid manner is flow injection with on-line derivatisation with 3-aminofluoranthene and on-line label removal.

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