Novel Separation Strategies in Industrial And Environmental Analysis

By

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Doctor of Philosophy

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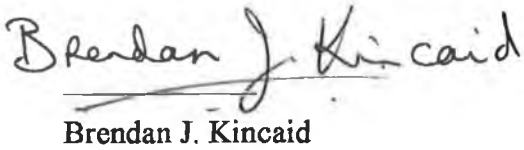
DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy (PhD) is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed *July 1999*



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Discovery consists of seeing what everybody has seen
and thinking what nobody has thought.

~ A. Szent-Gyorgi von Nagyrapolt.

For my Family

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ABBREVIATIONS

AES	atomic emmission spectrometry
APH	1-acetyl-2-phenylhydrazine
BF_3	boron trifluoride
$C_6H_5NHNH_2$	1-acetyl-2-phenylhydrazine
CE	capillary electrophoresis
CH_3CN	acetonitrile
$CHCl_3$	chloroform
CHP	cumene hydroperoxide
CTAB	cetyltrimethylammonium bromide
CV	cyclic voltammetry
DAD	diode array detection
DCM	dichloromethane
DHP	dihydropyridine
DME	dropping mercury electrode
DMF	dimethylformamide
DMNO	N,N-dimethyl-para-toluidine-N-oxide
DMOT	N,N-dimethyl ortho toluidine
DMPT	N,N-dimethyl-para-toluidine
DPP	differential pulse polarography
DSCL	differential scanning calorimetry
ECD	electrochemical detector
ELISA	enzyme-linked immunosorbent assay
EOF	electro-osmotic flow
FID	flame ionisation detector
FTIR	fourier transform infrared spectroscopy

GC	gas chromatography
GFF	glycine-l-phenylalanine-L-phenylalanine
H_3PO_4	phosphoric acid
HPLC	high performance liquid chromatography
HPSA	hydroxy propane sulphonic acid
IC	ion chromatography
ICP	inductively coupled plasma
ISE	ion selective electrode
ISRP	internal surface reversed-phase
KIO_3	potassium iodate
LIF	laser influenced fluorescence
LOD	limit of detection
MS	mass spectrometry
MSA	methyl sulphonic acid
PBDMA	poly(butadiene-maleic acid)
PFPA	penta-fluoro-propionic acid
PW	polymerisations warme (polymerisation exotherm)
RSD	relative standard deviation
SDS	sodium dodecyl sulphate
SO_2	sulphur dioxide
SPE	solid phase extraction
TAD	total acid determination (test)
TEA	triethyl amine

TFA	trifluoroacetic acid
THF	tetrahydrofuran
THQ	1,2,3,4-tetrahydroquinoline
TLC	thin layer chromatography
p-TSH	para-toluene sulphonyl hydrazine
TTAB	tetradecyl-trimethlammomium bromide

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ABSTRACT

This thesis is a study into the application of separation techniques to the analysis of both industrial and environmental sample matrices. Chapter 1 is a literature survey into the background of adhesives (both anaerobic and cyanoacrylic adhesives). The literature survey also details the composition of adhesives and their analysis.

Chapter 2 details an analytical study into the decomposition of the key reducing agents found in anaerobic adhesives, including acetyl phenylhydrazine, tetrahydroquinoline, dimethyl-*p*-toluidine and (*p*-toluene suphonyl) hydrazine. A suitable HPLC separation system was developed, which was then used to monitor any decomposition of the reducing agents. Investigation of the above was carried out in the presence of saccharin, maleic acid and cumene hydroperoxide (both in the presence and absence of the transition metals, copper and iron). Comparison of the rates of decomposition of the reducing agents revealed some information into the role of the acids and peroxide in the overall reaction mechanism. The products produced as a result of these reactions were also identified using preparative TLC and techniques such as IR, NMR and mass spectrometry.

Chapter 3 details the development of a suitable method of anion separation using capillary electrophoresis. A suitable extraction technique was also developed which could extract the anions from the adhesive mixture (both crude and distilled preparations), into an aqueous medium. The method developed was amenable to the analysis of the full range of cyanoacrylate adhesive mixtures (methyl-, ethyl-, butyl- and methoxy ethyl-cyanoacrylates).

Chapter 4 involved the development of an electrochemical system that was capable of detecting microcystins. The work also involved HPLC mobile phase optimisation, microcystin purification and microcystin extraction (both from 'pure bacterial cultures' and field samples). An electrochemical detector was developed which was capable of detecting microcystins, it was also capable of detecting other polypeptides (found in partially purified microcystins) which were not detected using UV detection. The main problem encountered was the detection of the microcystins in field samples as other proteins found in field samples were also electrochemically active.

Conclusions drawn from the work carried out in the thesis, and suggestions for future research are outlined in Chapter 5.

Chapter 1

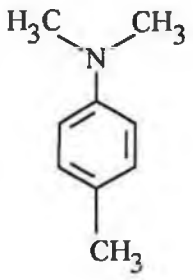
Introduction
to the Chemistry and Analysis
Adhesives

1.1 INTRODUCTION TO ANAEROBIC ADHESIVES

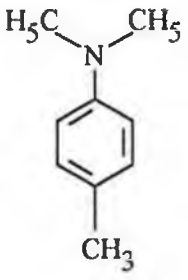
Adhesives, also known as glues or pastes, consist of a broad range of different types - each with its own particular application. Adhesives can be found for use in all areas from printed circuit boards to vehicle manufacture and general home use. One group of adhesives is the anaerobic adhesives.

Anaerobic adhesives are single component acrylic adhesives which cure rapidly at or below room temperature. Anaerobic adhesives are used primarily in industrial applications where they are used for locking and sealing threaded joints, pipe fittings and other rigid metal structures, such as metal flanges. Anaerobic adhesives are so named due to the characteristic need of an anaerobic environment for the initiation of the curing process. It is this requirement for an absence of oxygen that makes this group of adhesives so suitable for use in the sealing of tight fitting metal joints.

Anaerobics, broadly speaking, contain the following components : monomers, initiators, catalysts (accelerators), stabilisers/inhibitors and modifiers. Monomers are typically low molecular mass compounds which are involved in the polymerisation process. Initiators and accelerators are as their names suggests. There is a complex relationship between them. Initiators tend to be free radical in nature and hydroperoxides are one of the most important groups of initiators. Accelerators include compounds such as peroxides and oxides. Oxygen serves as an inhibitor of polymerisation in anaerobic adhesive mixtures; this alone, however, is not sufficient to provide all the required stabilisation. Other stabilisers/inhibitors used include chelating agents and nitrosamines. Modifiers are added to an adhesive to alter characteristics such as temperature performance - without altering the curing mechanism of the adhesive. Structures of typical anaerobic adhesive components are presented below in Figure 1.1.



N,N-dimethyl-*p*-toluidine



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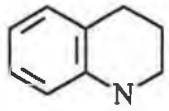
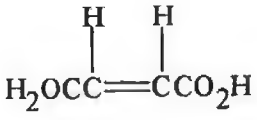
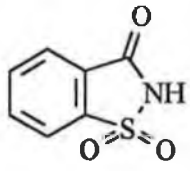


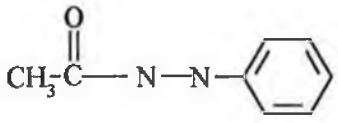
1,2,3,4-Tetrahydroquinoline



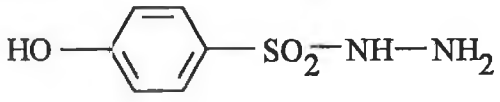
Maleic Acid



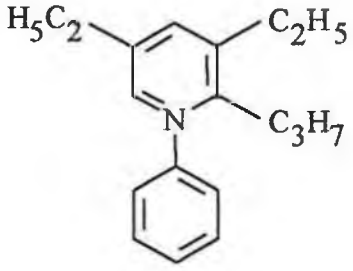
Saccharin



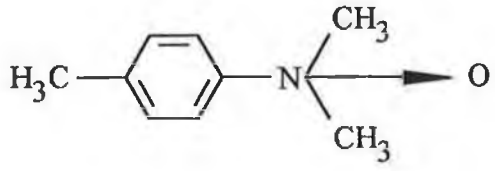
1-Acetyl-2-phenylhydrazine



p-Toluenesulphonylhydrazine



Dihydropyridine (DHP)



N,N-Dimethyl-*p*-toluidine-N-oxide

Figure 1.1. Structures of typical anaerobic adhesive components.

1.2. CHEMISTRY OF ANAEROBIC ADHESIVES

The fundamental step in the curing process of anaerobic adhesives is the generation of a free radical, which then adds to a monomer thus initiating free radical polymerisation. (Note that oxygen can suppress this propagation step). Polymerisation is then achieved via this propagation process. When the adhesive is confined between two tightly fitted metal surfaces, the resulting effect is twofold :

i) the exclusion of oxygen prevents the inhibition of the propagation step,

ii) the presence of the metal substrate promotes the decomposition of the initiator (via redox reaction), thus encouraging free radical propagation.

The metal substrate must contain an active metal surface, e.g. Fe^{3+} or Cu^{2+}. In contrast, however, metals such as zinc are relatively inactive. The oxidation state of the metal is also important as the hydroperoxide reacts faster with the lower oxidation state metals, e.g.

$$ROOH + Fe^{2+} \xrightarrow{\text{fast}} RO^{\cdot} + OH^{-} + Fe^{3+}$$

$$ROOH + Fe^{3+} \xrightarrow{\text{slow}} ROO^{\cdot} + H^{+} + Fe^{2+}$$

It has been found that tri-alkylamines could accelerate the polymerisation reaction, while having no detrimental effect on the storability of the adhesive. Further research has also identified that a co-accelerator combination of saccharin (*o*-benzoic sulphimide) and a dialkylarylamine, e.g. APH, can promote the cure speed on all surfaces.

It is presumed [1] that the amine serves to polarise the peroxide, thus assisting free radical generation. It does, however, appear that the dialkylarylamines undergo gradual auto-oxidation after long term storage thus resulting in a decreased cure speed. This also illustrates the important role of the amine in the cure process [2]. The

reactivity of the adhesives can be improved by the use of hydrazide accelerators [3], however, this in turn requires the use of stabilisers and inhibitors to prevent curing before use. It has been found that using larger quantities of the inhibitor resulted in reduced adhesive performance. Enhanced stability was achieved by the removal of trace amounts of metal ions to a level of 100 ppb or less. This removal was performed by using insoluble chelating agents. The resulting stability of the adhesive serves to illustrate the active role of the metal substrate in the curing process of anaerobic adhesives.

1.3. ANALYSIS OF ANAEROBIC ADHESIVES

There are primarily two methods by which anaerobic adhesives can be analysed, namely gas chromatography (GC) and high performance liquid chromatography (HPLC). In using these two techniques there are a number of detection modes ; these include GC-MS, GC-FTIR, GC-FID and HPLC-MS, -conductivity, UV - diode array detection (DAD). Studies have been performed using GC-MS / GC-FTIR [4] / GC-FID[5] and HPLC-UV[6].

1.3.1. GC-MS / GC- FTIR / GC-FID

Heatley [4] of Loctite Irl. developed a method using GC-MS-FTIR, which was suitable for the identification and quantification of cumene hydroperoxide (CHP), NN-dimethyl-para-toluidine (DMPT), NN-dimethyl-ortho-toluidine (DMOT), NN-diethyl-para-toluidine (DEPT) and tetrahydroquinilone (THQ). Sample preparation was performed by dissolving 1 g of sample in 4 g of dichloromethane (DCM). The GC conditions used were ;

Column : 25 m OV-17 fused silica.
Carrier : Helium at 12.8 psi, 3.0 ml min^{-1}.
Oven Temp : 80°C-200°C 0/15/0 and 200°C-310°C 0/35/7.7
Inj/Det temp : 300°C

Work previously carried out on GC at Loctite used a similar sample preparation although the GC operating conditions were slightly different [5]. The detector used was a FID system. This method was suitable for DEPT, DMOT, maleic acid, CHP, acetylphenylhydrazine (APH), Pegma, phosphate, glycol, phthalate and adhesive product samples.

Studies [6] have also been carried out using GC-MS to identify the reaction routes of APH in the presence of weak acids, CHP and transition metals. The solutions were prepared as 1% solutions in methanol and injected on to the column.

1.3.2. HPLC-UV

In a study [6] carried out by Loctite (Irl.), a HPLC method was developed for the analysis of APH, saccharin, maleic acid and CHP using a mobile phase of 40% tetrahydrofuran (THF) and 60% H_2O. The column used in this experiment was a reversed phase C_{18} column (Merck Lichrosphere, 5 μm). Detection mode used was UV with a wavelength of 254 nm.

A later study carried out by MacManus [7] used two mobile phases - one for the mixed "amine" cure system (this included saccharin, CHP, DMOT and DMPT) and one for the mixed "acid" cure system (this included saccharin, maleic acid, CHP and APH). The mixed amine system utilised an acetonitrile-water mix. A gradient elution of 40:60 (v/v) CH_3CN : H_2O to a final composition of 100% CH_3CN was used. The flow rate was 1.0 ml min^{-1}. The mixed acid system used a CH_3CN and buffer mobile phase. The buffer was 3% triethylamine in H_2O adjusted to pH 3.0 with *o*-phosphoric acid. A ratio of 45:55 (v/v) CH_3CN : H_2O was employed. The flow rate was 0.9 ml min^{-1}. Both systems were operated using a C_{18} μBondapak column. For the mixed amine analysis a 150 mm x 4.6 mm column was used and for the mixed acid analysis a 300 mm x 3.9 mm column. Samples were prepared in the respective mobile phases (approximately 0.2 g in 10 ml of solvent).

1.4. GENERAL ANALYSIS OF ADHESIVE COMPONENTS

There are a wide range of analytical techniques that may be applied to the analysis of anaerobic adhesive components. These techniques include HPLC, GC, infrared (IR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, MS and polarography. HPLC and GC-MS, -IR have been used by several groups [4,5,8,9] to identify, quantify and characterise the various components. The wide range of different detectors enhances the applicability and scope of the different techniques. The two techniques, HPLC and GC, provide the opportunity to analyse both non-volatile and volatile components of the adhesives. In cases of thermal decomposition of components, GC is unsuitable, and HPLC may be the preferred technique. A range of different mobile phases and chemically modified stationary phases also increases the capacity of HLPC, e.g. THF is suitable for dissolving adhesive compositions, thereby allowing the analysis of the same, whereas a methanol/H_2O mix or CH_3CN/H_2O/buffer is probably more suitable to the analysis of 'pure' amines and weak acids.

Voltammetry/polarography is ideally suited to analysis of trace metals and of oxidation products of the amines and hydroperoxides [10]. Other techniques such as thin layer chromatography (TLC), potentiometry and GPC have also been used successfully to characterise and quantify peroxides, amines and weak acids.

1.4. 1. Analysis of Amines

TLC has the advantage of rapid analysis times, and the use of sprays can distinguish between members of the same chemical group, e.g. amines. TLC has been applied to the analysis of amines, found in biological samples [11]. The amines were extracted into 20 mM HCl and then extracted in diethyl ether. The extract was eventually reconstituted in acetonitrile and analysed by TLC. The mobile phase that gave optimum separation was a mixture of benzene, triethylamine and acetone (10 : 2 : 1). Detection was by spectrofluorometry.

HPLC has also been applied to the analysis of amines, using UV as a means of detection[12]. A mixture of amines was analysed using a C_{18} column with a mobile phase consisting of ammonium acetate buffer and acetonitrile. The amines analysed in this particular method are not actually found in adhesive preparations; however, it is possible that the method may be amenable to the analysis of amines found in the adhesives. This is similar to the method developed by MacManus [7], which also uses an acetonitrile/ aqueous-based mobile phase.

1.4.2. Analysis of Peroxides

Analysis of peroxides have been carried out by numerous groups using differential pulse polarography (DPP), HPLC, GC, and indeed even a biosensor has been used [13]. Salvato et al. [10] carried out analyses of organic peroxides using DPP. The peroxides were dissolved in an organic medium such as ethanol, and a potential sweep of 2 or 4 mV/s was used.

A potentiometric biosensor was developed by Wollenberger et al.[13] to detect and analyse peroxides and hydroperoxides. An ion selective electrode (ISE) utilising an immobiliesd enzyme, namely horse-radish-peroxide (HRP), was developed. Although HRP is a rather unspecific enzyme, it can be used to detect different groups of organic hydroperoxides. The basic configuration of the ISE comprised of the enzyme in direct contact with the carbon electrode material, without any mediator. The response time of the ISE was 10 s, with an overall total measuring time of 30 s. The electrode surface was ~ 7 mm^2 , providing a relatively limited measurement reproducibilty of 9 %.

HPLC appears to be one of the more popular methods for the analysis of peroxides and hydroperoxides. Since HPLC can be carried out at room temperature it means that the samples do not have to be volatile. This makes it an ideal technique for peroxide analysis as they do, in fact, decompose at elevated temperatures. Baj and Dawid [8] utilised reversed-phase HPLC, to carry out a study on the correlation between the chemical structures of peroxides (dialkylperoxides) and their retention

times. In this study a C_{18} column was used with a mobile phase consisting of MeOH-H_2O mixtures with a flow rate 1.0 cm^3 min^{-1}. The detection system utilised was a UV lamp at a wavelength of 257 nm. The ratios of MeOH to H_2O ranged from 95:5 to 70:30 (v/v). This study determined the relevant factors for retention to be a result of non-specific, dispersive bulkiness-dependent and electrostatic interactions, involving solute molecules and molecules of both the stationary and mobile phase. It was concluded that the correlation obtained between the structural parameters and the retention indices for a variety of peroxides was very good and could be used to predict the retention properties of the investigated system of molecules.

Another study carried out by Patel and Lilly [9] used a mobile phase consisting of an acetonitrile and ammonium phosphate buffer mix, at a pH of 6.0. A colour developer (a combination of *o*-dianisidine and peroxidase) was also incorporated in the chromatographic system as a post-column mobile phase and the detector was set to a wavelength of 436 nm. A 25 cm x 4.6 mm Nucleosil Spherisorb CN column was used with a flow rate of 1.0 cm^3 min^{-1}. Samples were dissolved in MeOH. The method developed was designed to be suitable for hydroperoxide detection, but it can be used for peroxide detection e.g. for benzoyl peroxide.

Electrochemical detection can be used in conjunction with HPLC analysis of peroxides. An example of such a method was carried out by Funk and Baker [14] using a gold disc as a working electrode and a glassy carbon auxiliary electrode with a Ag/AgCl reference electrode. Due to problems such as poor reproducibility, however, investigations into the use of a dropping mercury electrode (DME) were carried out, with more success. The DME produces a new working electrode at regular intervals, has better reproducibly and it also avoids any carry over of sample, thus avoiding any contamination. An excellent LOD of one nanogram was obtained for benzoyl peroxide and cumene hydroperoxide, under optimum conditions. In this investigation it was found that the use of a buffered mobile phase was critical to the detection of peroxides. Without the buffered system, large negative peaks were observed. These negative peaks were discovered to be associated with the presence

of active silanol groups remaining on the surface of the chromatographic column packing.

Other chromatographic methods carried out for the analysis of hydroperoxides include GC and TLC. GC is less suited to hydroperoxide analysis due to the thermal decomposition that hydroperoxides can undergo upon injection on a GC system. However, should the hydroperoxides first be derivatised to produce a thermally stable compound they can then be analysed by GC.

TLC has also been used successfully for the analysis of hydroperoxides [15]. A number of hydroperoxides found in aerosols have been analysed using supercritical fluid extraction coupled to TLC. Following extraction with supercritical CO_2 and application of the hydrazines to the TLC plate, the plate was then developed in and dipped in a solution containing NN-dimethyl-p-phenylenediamine dihydrochloride. Detection was carried out spectrophotometrically at 554 nm.

Titrimetry can also be used in the quantitative measuring of peroxides. The method is based upon a back titration in which the peroxide is reacted with iodide ions (using potassium iodide or sodium iodide) under acidic conditions producing free iodine. The iodine can then be titrated with sodium thiosulphate. In order for this form of titrimetry to be used, it is essential that :

i) a suitable solvent mixture is selected which will solubilise both the peroxide and the iodide salt;
ii) oxygen is excluded from the reaction as the free radicals generated can react with oxygen to produce another peroxide. This exclusion can be achieved by the use of nitrogen or carbon dioxide gas;
iii) the reaction can be speeded up, as some reactions are slow at room temperature, this can be achieved by using higher temperatures or a catalyst.

A source of error, however, is the use of visual endpoints and this can lead to poor reproducibility. To avoid this, titrimetry can be performed using potentiometric or coulometric detection.

1.4.3. Analysis of Hydrazines

The analysis of hydrazines is quite important due to their widespread use in industry. The main method of hydrazine analysis depends on the redox reaction of the hydrazines as they have considerable reducing power. Malone [15] used potassium iodide, potassium permanganate, potassium bromide, mercuric chloride, potassium iodate and iodine as oxidants, e.g.

$$3C_6H_5NHNH_2 + 2KIO_3 + 3HCl \longrightarrow 3C_6H_5N_2Cl + 2KI + 6H_2O$$

APH is one of the primary hydrazines that is used in adhesive compositions. The adhesive can be solubilised in ethanol and the free hydrazine detected using differential pulse polarography (DPP).

Electrodes and ion selective electrodes (ISE's) have been used successfully to determine hydrazines in a variety of sample matrices. Wang and Pamidi [16] developed a screen-printed electrode (disposable type) which was based on the oxidation of the hydrazine. The hydrazines were quantified using chronoamperometry with a stepping potential from 0.1 to 1.0 V.

Spectrophotometry has also been used for the determination of hydrazines and phenylhydrazines by Yatsimirsky et al. [17] This method uses micellar catalysis (using sodium dodecyl sulphate, SDS) as it was found that the presence of micelles greatly increased the rate and the yield of the product formed. The method was applied to the determination of the product produced by the reaction between hydrazines and p-dimethylaminobenzaldehyde (DAB). The condensation product of the hydrazine and DAB, an azine, can then be determined spectrophotometrically.

Chromatography has also been utilised for the determination of hydrazines. Both TLC and HPLC have been used, although HPLC is the more common technique of the two. As for peroxides, TLC plates are used for hydrazine detection in conjunction with a dye or spray. Holtzclaw et al. [18] detected the hydrazines spectrophometrically after spraying with 4-chloro-5,7-dinitrobenzofurazan. The absorbance maximum was found in the wavelength range of 630-640 nm. In contrast to this, organic hydrazines absorbed between 500-510 nm once sprayed with 4-chloro-5,7-dinitrobenzofurazan. This reaction could even be used as a basis for the development of an optical sensor for the detection of hydrazines.

HPLC has been used successfully by Ravichandran and Baldwin [19], in the separation of hydrazines, using an electrochemical detection system. UV detection would be another viable detection system, the only requirement being the presence of a chromophore. Some hydrazines lacking chromophores, e.g. alkyl hydrazines, may require derivatisation, prior to UV detection. The hydrazine of interest in anaerobic adhesives is primarily APH, and this of course possesses the aromatic ring to act as a chromophore, which is a strong absorber of UV light.

Ravichandran and Baldwin [19] separated a number of hydrazines (methylhydrazine, 1,2-dimethylhydrazine and 1,1,-dimethylhydrazine), using a C_{18} reverse phase column with a mobile phase of 0.10 M potassium nitrate and 0.01 M di-sodium hydrogen phosphate, adjusted to a pH of 7.0. Electrochemical detection was performed using an electrochemically pretreated glassy carbon electrode. The purpose of pretreating the glassy carbon electrode is to enhance the analytical capacity of the actual electrode for hydrazine detection. Pretreated electrodes can detect hydrazines down to a range of 2 pmol to 50 pmol. This level of detection would be sensitive enough to detect the presence of free hydrazines in an adhesive sample. This was 10 to 800 times more sensitive than the limit of detection (LOD) of an untreated electrode. The reproducibility of this method was also quite acceptable (5%). In spite of the good detection limits of this method it is not applicable to adhesive analysis as the mobile

phase used (KNO_3 / $NaHPO_4$, pH 10.0) cannot dissolve the adhesive sample. In comparison to HPLC-UV, the HPLC-EC method is about 100 times more sensitive.

One other chromatographic means of hydrazine detection is GC [20]. GC has been successful in the application to hydrazine analysis, although it was necessary to derivatise the hydrazine in order to produce a more stable compound. The mode of detection used was a nitrogen specific detector (a thermionic detector in the nitrogen-phosphorous mode). The method was reported to have an overall precision better than 5 % R.S.D. for 90 ppb hydrazine, with a minimum detectable concentration of 4 ppb. The method was applied to the analysis of hydrazine, methylhydrazine and 1,1-dimethylhydrazine.

Titrimetry has also been used for the analysis of hydrazines using bromocreosol green as an indicator [21]. The sample is dissolved in methanol and then titrated against 0.1 M hydrochloric acid. It was reported that the results obtained using the titrimetric method agreed with the results obtained using GC.

1.4.4. Analysis of Toluidines

Toluidines are a group of weak bases, due to the presence of the N atom on the phenyl ring. Toluidines are suited to analysis by several chromatographic techniques, namely HPLC, GC and cation exchange chromatography. Due to their basic nature, toluidines can also be determined using titration techniques e.g. via iodination [22].

The toluidines that are utilised in the adhesive industry include DMPT and DEPT, the ortho- and meta- isomers are also sometimes used. Chang and Huang [23] carried out investigations into the separation of toluidines (and anilines) on a number of stationary phases including silica, amino and diamine bonded phases. Mobile phases consisted of varying mixtures of propan-2-ol and heptane. Investigations into the elution order of the compounds were carried out in an effort to elucidate the mechanisms that determine the retention mechanism. The elution order, using the silica column was ortho-isomers followed by meta-isomers and finally by para-isomers. It was proposed

that the toluidines interact with silica by means of hydrogen bonding, direct hydrogen bonding with the hydrogen of the silanol group or interactions with the alcohol hydrogen should 2-propanol be present. In the case of amino and diamine bonded phases, the retention of the toluidines in this case was greatly affected by the polar modifier (2-propanol). At low concentrations of modifier the retention was determined by the amine protons, whereas at higher alcohol concentrations the lone pair of electrons of the amino group had a more marked effect. Thus by careful control of the mobile phase composition and stationary phase it is possible to alter the selectivity of the method, with regard to the separation of toluidines.

McCrossen and Simpson [24] discovered that the addition of ion-pairing reagents and cyclodextrins to the mobile phase vastly improved the separation of ionic species. One such reagent added to the mobile phase of MeOH / H_2O mixture was valenonitrile (an aliphatic copper compound). A C_8 reversed phase column was used in the experiment and it was discovered that a small amount of valenonitrile added to the mobile phase could significantly improve the retention and chromatography of basic compounds. As mentioned previously, toluidines are basic in nature and thus are amenable to the above technique. It was found that addition of ~10 % (v/v) valenonitrile decreased the retention of *p*-toluidines, and also eliminated the peak tailing for toluidines, while simultaneously significantly improving the selectivity and resolution of the peaks.

Toluidines have been analysed by GC [25] after a derivatisation reaction was carried out, under acidic conditions. The toluidines were converted to N-permethyl derivatives by treatment with formaldehyde and sodium borohydride. The derivatisation process produced very good yields of derivatised products, typically resulting in a 96 - 100 % yield. By comparison it was found that these methyl derivatives displayed less tailing and gave an enhanced FID response and were more stable. Additionally, the separation of underivatised toluidine isomers is usually a difficult process ; however, separation of the derivatised compounds is relatively easy.

CE was used to separate toluidines (phenols and benzenethiols) using calixarenes [26]. A calixarene is essentially a molecule containing a number of aryl groups that forms a cavity into which other ions or molecules can fit. The deciding factor that determines which molecule will enter the cavity is size, the molecule or ion of the best fit, i.e. first to fill the cavity is the molecule of the most appropriate size. Selection of best cavity fit is not solely based on size but also orientation; thus *o*-, *m*- and *p*- isomers can differ in orientation and consequently each species will suffer a different level of steric stress and so the selectivity of the calixarene is determined. The calixarene used in this case was 4 mM *p*-sulfonate calix-{6}-arene, a macrocyclic oligomer. The analysis was performed on a capillary 80 cm x 50 μm i.d., and the detection wavelength was 220 nm. The electrolyte used was a phosphate buffer pH 7.0 although this was further modified by the addition of 0.01 M triethylamine (TEA) which enhanced the separation of the toluidines.

Toluidine separation has also been performed using a cation exchange column [27] using a Nucleosil 5-100 (5 μm pore size, 10 mm) with silica covered with a coating of poly(butadiene-maleic acid), PBDMA. The mobile phase used was methanol-H_2O mixtures, with a UV detector at 254 nm. By changing the amount of PBDMA and by careful control of the pH of the mobile phase it was possible to alter the selectivity of the system for the different isomers (meta-, ortho- and para-).

Toluidines can be determined quantitatively using titrimetry. Titrimetry can be carried out using a back titration with iodine and thiosulphate or by using potentiometry. Using iodination, the toluidines are iodinated in alkaline solutions (0.5 M sodium acetate) with iodine in chloroform and then the solution treated with bromine to liberate the iodine, which is then titrated with thiosulphate, the end-point detected visually. This method is, however, unable to distinguish between *o*-, *m*- and *p*-toluidines. The titration can also be monitored using potentiometry. In the case of toluidines, a strong acid is used e.g. perchloric acid, detection is carried out, using a glass reference electrode combination. Again, similar to the iodination method, the total quantity of toluidines is determined without distinguishing between *o*-, *m*-, *p*-isomers.

1.4.5. Analysis of Saccharin

The analysis of saccharin is of great importance in the food and drink industry where much of attention has been devoted to the development of various analytical techniques ranging from HPLC, TLC, to spectrophotometry. Most modes of analysis begin by dissolving the sample into a solution or extracting the sample into a suitable solvent, which can then be applied to the analytical system.

TLC has been used in conjunction with spectrophotometry [28] for saccharin analysis. The saccharin was extracted in $CHCl_3$, and after evaporation of the solvent, the residue was dissolved in ethyl acetate. This solution was applied to the TLC plate and the plate was then viewed under UV light at 254 nm. Quantitative information can also be obtained by extracting the saccharin from the silica plate and determining the amount of saccharin by spectrophotometry. Other modes of analysis have also been investigated including :

i) polarography (DPP),
ii) ion-pair HPLC [29],
iii) potentiometry [30] and ion selective electrodes [31].

The ion selective electrode was prepared using an ion exchanger coated on an Ag/AgCl electrode. The ion exchanger was prepared using picric acid, crystal violet solution and nitrobenzene. This exchanger was then coated onto a Ag/AgCl electrode. The sample (~0.4 g) is dissolved in 10 ml nitrobenzene and titrated against 5 mM crystal violet solution. The content of saccharin can then be calculated using a given formula. The method has been applied successfully to the analysis of saccharin in artificial mixtures and in an anaerobic acrylic adhesive formulation.

One titrimetric method which has been used successfully for saccharin analysis involves the use of mercurous nitrate [27]. The mercurous nitrate and saccharin react (producing mercurous saccharinate) and the remaining mercury, after the end point is

reached, is determined potentiometrically using a silver wire electrode. The method was reported to be both precise and inexpensive, with a detection limit of 0.5 mg/l and an acceptable coefficient of variation of 1.2 %.

The main concern with regard to the use of the above techniques for the analysis of saccharin in an anaerobic adhesive samples is that of solubility. All components of interest must be soluble before analysis can be performed. From the reports published, the two techniques that seem to show potential in the analysis of saccharin in (anaerobic) adhesive compositions are the TLC method [28] and the ISE method [31]. As mentioned above the ISE method has been applied to adhesive analysis, while in the TLC method the sample is dissolved in chloroform, a solvent which is amenable to the solubilisation of adhesive preparations.

1.4.6. Analysis of Maleic Acid

Maleic acid has been analysed by HPLC [32] using a Zorbax column C_8. The mobile phase used involved a gradient elution from 100 % 0.04 M H_3PO_4 to 25 % acetonitrile using a flow of 0.8 cm^3 min^{-1}. The wavelengths used for detection ranging from 235 to 294 nm. Peak shapes for maleic acid were well resolved and sharp.

1.5. INTRODUCTION TO CYANOACRYLATE ADHESIVES

Cyanoacrylate adhesives, commonly known as superglues, belong to a large group of adhesives which are single component in nature and which cure at room temperature. The manufacture of cyanoacrylate adhesives has progressed significantly since the first commercial cyanoacrylate adhesive was produced in 1958 by Eastman. Initially, the cyanoacrylate adhesives were subject to instability problems, both in the manufacturing process and in storage. Since then, these problems have been overcome and the manufacturing process has progressed to the point where cyanoacrylate adhesives have high tensile strength, high peel strength, high shear strength, and good chemical and heat resistance. Cyanoacrylate adhesives are also tolerant to surface contaminants such as oil [33]. New additives now allow curing of the adhesive on porous materials such as paper.

Although cyanoacrylates are typically expensive, they usually only require minute amounts of glue ('single drop' application) to form a bond. Additionally, they can cure without heating or exposure to radiation, which eliminates extra costs. Cyanoacrylate adhesives are now used in areas such as electronics (component bonding, circuit boards), the motor industry, medical device manufacture, plastics industry, jewellery bonding and general home use. One of the latest novel uses of cyanoacrylate adhesives is in medical applications. The purpose of these 'medical' adhesives is to replace sutures after surgery, whereby the skin can be glued together until the wound heals. 'Superglues' have now advanced to the point that their use is now so widespread that they account for 90% of the commercial volume of adhesives used in industry .

1.6. CHEMISTRY OF CYANOACRYLATE ADHESIVES

1.6.1. Cyanoacrylate Components and their Chemistry

Cyanoacrylate adhesives share some similarities with anaerobic adhesives, these include common components (monomers, initiators, catalysts, modifiers and stabilisers), free radical generation in the curing process and the inhibition of curing by oxygen. Oxygen is not used solely however, to inhibit the polymerisation process during storage rather an acid is used to stabilise the adhesive during storage.

Monomers in cyanoacrylate adhesives can be classified by their odour and two classes exist - high odour or low odour. High odour classification monomers are the more volatile of the two classes and these include methyl- or shorter alkyl chain methacyanoacrylates. Low odour monomers include methacrylates such as hydroxyethyl methacrylate, hydroxypropyl methacrylate and tetrahydrofurfuryl methacrylate. Due to overall cost considerations, availability and performance, high odour methacrylates are the most widely used, even though low odour methacrylates are cheaper. However, high odour methacrylates are of superior cure consistency, temperature resistance and plastic adhesion properties.

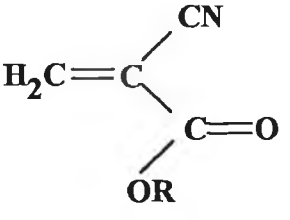
The catalytic system in cyanoacrylate adhesives comprises of the base component and the curative component of the formulation, forming a two part redox system. As with anaerobic adhesives a very common initiator is a hydroperoxide. The curative used in these formulations is a reductant produced by reaction of aniline and butyraldehyde [1]. Alternatively an initiator such as N,N-dimethylaniline can be used with a curative (an oxidant) such as benzoyl peroxide.

Modifiers include toughners which enhance the impact resistance and the peel strength of the adhesives. A rubbery compound such as chlorosulphonated polyethylene is sometimes added to toughen the adhesive. Plasticisers are added to improve the heat resistance of adhesives.

There are two types of cyanoacrylate adhesive stabilisers : anionic inhibitors and free radical inhibitors. The anionic cure process is initiated by anions and therefore acids are a suitable stabiliser. Acids used in preparations include SO_2, SO_3, BF_3 and HF. Other stronger acids, such as aromatic sulphonic acids, are used in lower concentrations as stabilisers. Too high a concentration of these will result in a very poor curing performance. Free radical stabilisers are not as effective as anion inhibitors, as polymerisation depends more on anionic mechanism than free radical generation. As a result, the level of free radical stabilisers required is relatively high. The high concentration of these stabilisers does not, however, have a deleterious effect on the cyanoacrylate adhesive properties. One of the most common inhibitors used in cyanoacrylate adhesive formulations is hydroquinone, although phenols are also sometimes used.

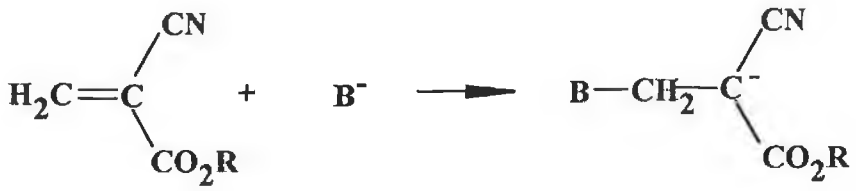
1.6.2. General Background Chemistry

The reactivity of cyanoacrylate adhesives is directly related to the basic chemical structure which consists of two strong electron withdrawing groups, CN and CO.

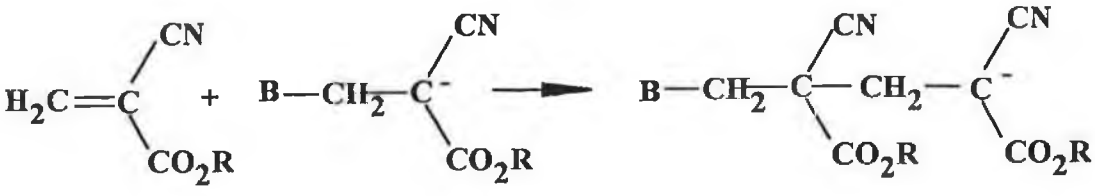


where R is an alkyl chain.

The presence of the two electron withdrawing groups means that the double bond is highly susceptible to attack by weak bases. Polymerisation of cyanoacrylate adhesives can occur in the presence of a weak base such as H_2O or an alcohol (via an 'anionic mechanism') ; hence initiation of the curing process can be started by anions found on the surface of the materials being bonded together. The anionic mechanism for polymerisation occurs as depicted below (Figure 1.2).

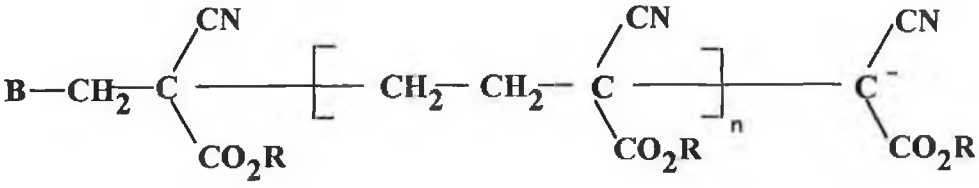


Initiation step



Propagation step

Figure 1.2 : The initiation and propagation step in the cyanoacrylate polymerisation process.



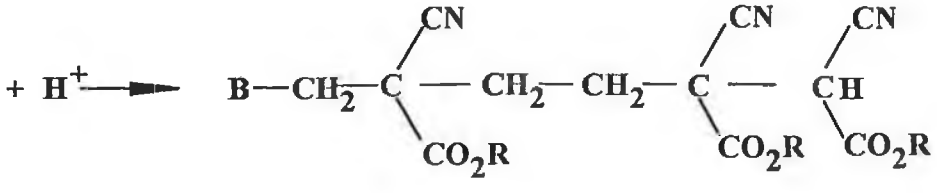


Figure 1.3 : The termination step of the cyanoacrylate polymerisation process.

As shown above (Figure 1.3.), the terminal step involves an acidic species. As acids will not initiate polymerisation, they are used as stabilisers in cyanoacrylate adhesives. The acids used include SO_2, BF_3 and sulphonic acids. The speed at which polymerisation can occur is due to the thin film of cyanoacrylate used and to the fact that the reaction involving the water is exothermic. The source of the water molecules is the thin layer of moisture adsorbed on the substrate surface.

1.7. ANALYSIS OF ANIONS IN CYANOACRYLATE ADHESIVES

Two separation techniques exist that are ideally suited to the analysis and separation of anions, namely ion exchange chromatography and capillary electrophoresis. Preliminary investigations were carried out by Loctite Irl.[34] into the use of ion-pair chromatography and its application to cyanoacrylic adhesive analysis. This preliminary study led to the later work using ion exchange chromatography. Capillary electrophoresis has not yet been applied to the analysis of cyanoacrylic adhesives. Raftery [35] developed a method to separate a number of anions in a typical cyanoacrylic adhesive, using ion exchange chromatography. The anions separated included the following ions; chloride, nitrate, sulphate, maleate, malonate, phosphate, formate, succinate, hydroxypropane sulphonic acid (HPSA), methane sulphonic acid (MSA) and cyanoacetate. The analysis was carried out on a Dionex Ion Pac AS-11 (4 mm x 250 mm) column, using a suppressed conductivity detection with 25 mM H_2SO_4 as reagent. The flow rate was 2 cm^3 min^{-1}. A gradient elution was used in the separation, as shown in Table 1, with an overall runtime of twenty minutes.

Time (min)	%E1	%E2	%E3	%E4
0	80	5	0	15
4	75	10	0	15
12	0	70	15	15
16	0	65	20	15
22	0	5	80	15

Eluent 1 :Deionised Water Eluent 3 : 50 mM NaOH
Eluent 2 : 5 mM NaOH Eluent 4 : Methanol

Table 1 : The mobile phase composition for the ion exchange system.

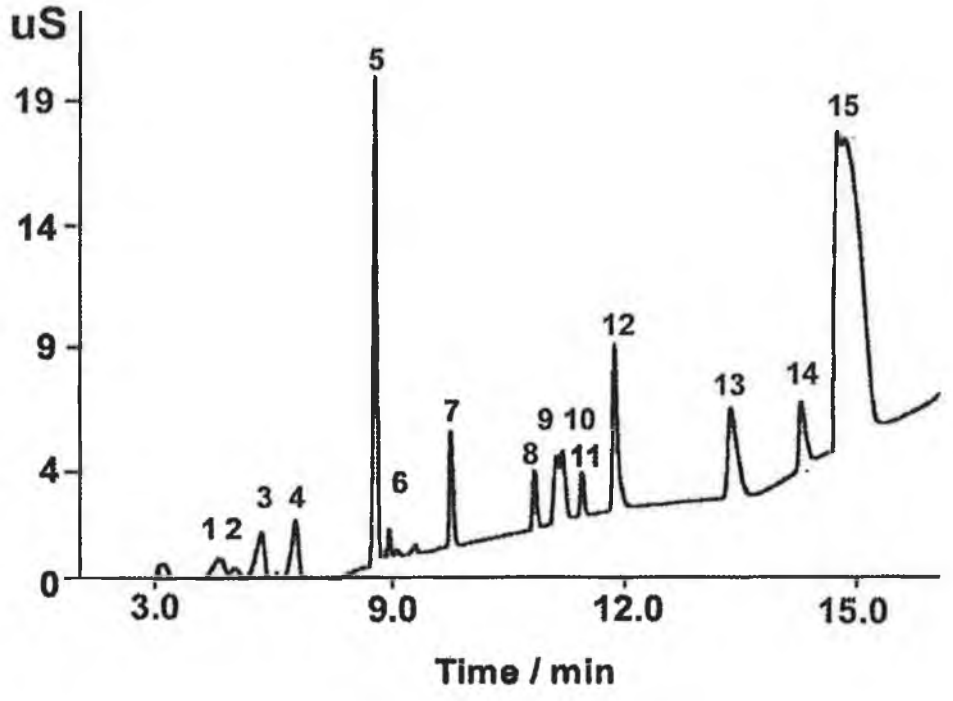


Figure 1.4. : Chromatogram of the standard anion run obtained using IC. Mobile phase is shown in Table 1. Flow rate of 2 cm^{-1}. Suppressed conductivity detection. Reproduced from ref. 35.

Although many papers have been published on the area of separation of anions using CE, there are no papers published based specifically on the analysis of anions found in cyanoacrylate adhesives. One of the most common buffer electrolytes that is used for anion separation in CE, is a chromate-based electrolyte. Several different concentrations of chromate electrolyte have been used, including 4 mM, 5 mM, 7 mM and 10 mM chromate. In addition to this a variety of EOF modifiers have been used in varying concentrations. Chromate is used due to its high absorbance at 254 nm (making it ideal for indirect UV detection) and its ionic mobility matches that of the majority of anions separated on CE [36, 37, 38].

In carrying out the analysis of anions, UV detection is usually employed, however due to the lack of chromophores in the majority of anions, it must be used in the indirect detection mode. Indirect detection requires the used of a buffer electrolyte with a high absorbance - when an anion of no or low absorbance passes the detector window the absorbance drops and thus produces a negative peak. The absorbance graph can then be inverted to display the typical graph with 'positive' absorbance peaks.

Jandik and Jones [39, 40] have produced a number of reports on the separation of anions. In all cases, a chromate buffer electrolyte was used in conjunction with an EOF modifier, in varying ratios. The modifier in this case was a long alkyl chain quaternary ammonium salt solution (named NICE-Pak OFM Anion-BT EOF modifier, Waters Corporation). In most of their studies a 75 μm i.d x 60 cm capillary was utilised (52 cm to the detector window) with a detection wavelength of 254 nm. They obtained good separation of a number of anions in a single run.

Other groups also used a chromate buffer electrolyte with an EOF modifier (OFM-BT) at working concentrations of 7 mM/0.7 mM (chromate/EOF modifier) [34], 5 mM/0.5 mM [41] and 4 mM/0.3 mM [42]. The capillary used was a 75 μm x 60 cm untreated fused silica capillary. Working voltages ranged from -15 kV to -30 kV. Wildman et al [43] in their research used a 50 μm x 60 cm capillary. There is little difference between the use of 50 μm or 75 μm capillary - the 50 μm capillary results in a smaller volume of sample being injected and with a small detector pathlength a

smaller signal is obtained. However, there is no benefit to be gained by using a much larger capillary, e.g. 100 μm capillary, as a loss of resolution occurs.

Another EOF modifier suitable for use in anion analysis is cetyltrimethylammonium bromide (CTAB). Jimider et al. [44] investigated the use of CTAB as an additive to the sodium chromate electrolyte for the separation of nitrates and nitrites in vegetables. CTAB is a surfactant, and so in order to enhance its solubility, 5% v/v of acetonitrile was added to the CTAB solution. The final buffer electrolyte was 10 mM chromate and 4.3 mM CTAB, pH 11.50. Voltage runs were performed at -15 kV and -20 kV.

Yet another surfactant that can be used as an EOF modifier is tetradecyltrimethylammonium bromide (TTAB). Stahl [45] used 5 mM chromate, 0.2 mM TTAB at pH 8.2 to analyse anions from soil samples. A longer capillary was used (75 μm x 120 cm) with a run voltage of -30 kV. The wavelength of the detector was 275 nm. Due to the long column length the higher voltage was required to reduce the end run time, yielding an overall end run time of ~10 minutes. This end run time does depend on the anions being analysed, but nevertheless the principle remains the same.

Another study was conducted using sodium chromate and TTAB as the buffer electrolyte with a capillary of 50 μm (and 75 μm) x 60 cm [46]; this time, however, a more complex buffer electrolyte was developed. It consisted of 5.0 mM boric acid solution (no volume given), adjusted to pH 8.0 with 0.2 M NaOH. To this solution 0.5 ml 0.1 M TTAB, 5.0 ml 0.1 M sodium chromate, was added. The electropherogram of the standard run of anions, obtained with this buffer electrolyte is shown in Figure 1.5.

This system was compared to a buffer electrolyte solution bought from Fluka and Dionex, consisting of 2.25 mM pyromellitic acid, 6.5 mM NaOH, 0.75 mM hexamethonium hydroxide and 1.0 mM triethanolamine, at pH 7.7 +/- 0.2. The former, 'homemade' buffer electrolyte developed did not, however, produce the same level of repeatability and reproducibility as the electrolyte solution from Fluka.

(Although the Fluka electrolyte had a noisier baseline compared to the 'homemade' electrolyte). The electropherogram of the standard run of anions, obtained with the commercial electrolyte is shown in Figure 1.6.

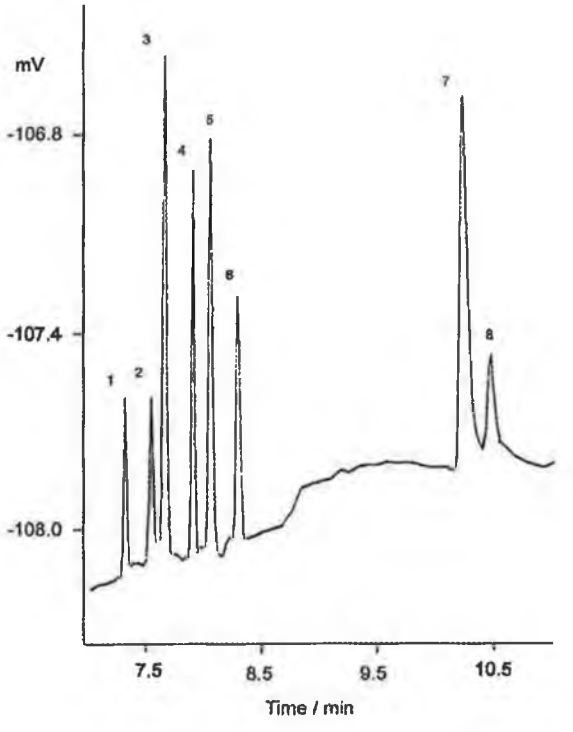


Figure 1.5. : Electropherogram of standard anions. Capillary 73 cm x 75 μm I.D. fused silica. Electrolyte : 5 mM sodium chromate, 0.5 mM TTAB, 5 mM boric acid, pH 8.0. Hydrodynamic injection, 6 s. Potential 20 kV. 1 = $S_2O_3^{2-}$ (4 ppm); 2 = Br^- (6 ppm); 3 = Cl^- (8 ppm); SO_4^{2-} (6 ppm); 5 = NO_2^- (7 ppm); 6 = NO_3^- (7 ppm); 7 = F^- (5 ppm); 8 = HPO_4^{2-} (2 ppm). Reproduced from ref 46.

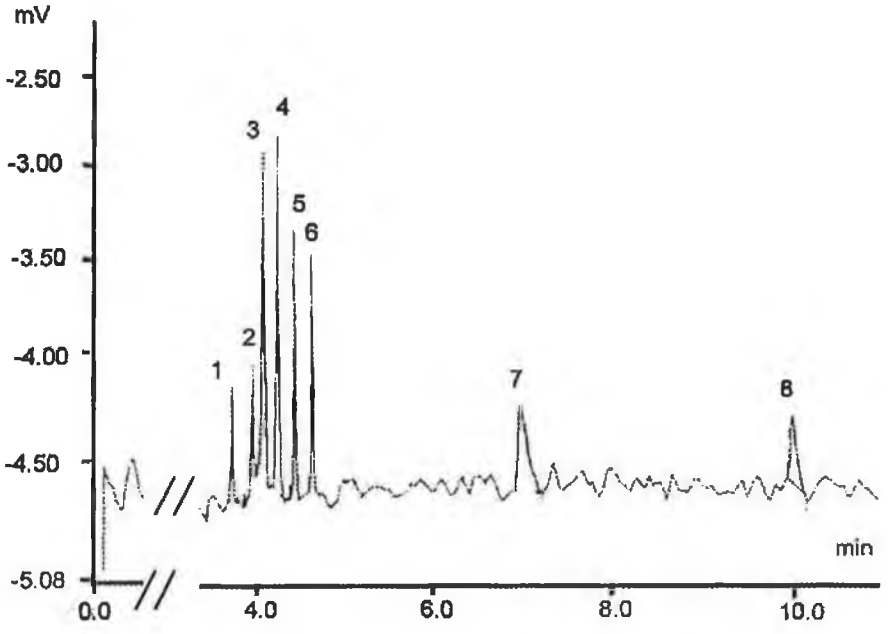


Figure 1.6. : Electropherogram of standard anions. Capillary 60 cm x 50 μm I.D. fused silica. Electrolyte : Trace anion buffer (Fluka). Injection : hydrodynamic, 6 s. 1 = $S_2O_3^{2-}$ (5 ppm); 2 = Br^- (5 ppm); 3 = Cl^- (6 ppm); SO_4^{2-} (7 ppm); 5 = NO_2^- (6 ppm); 6 = NO_3^- (6 ppm); 7 = F^- (2 ppm); 8 = HPO_4^{2-} (1 ppm). Reproduced from ref 46.

A paper was published by Stathakis and Cassidy [47], which dealt with the use of ionic polymers in the buffer electrolyte, these additives served as EOF modifiers. A number of cationic polyelectrolytes were used, namely; -poly(1,1-dimethyl-3,5-dimethylenepiperidine) chromate (PDDPiCr), -poly(1,1-dimethyl-3,5-dimethylenepyrrolidinium) chromate (PDDPyCr), -hexadimethrin chromate (HDMCr) -((diethylamino)ethyl) dextran chromate (DEAEDCr).

The above were added in concentration range of 0.004-0.6 % (w/v) to the electrolyte. All the polyelectrolytes were chromate salts, which suited the sodium chromate electrolyte. As mentioned previously, the chromate solution is ideal for the analysis of anions. The addition of the polyelectrolytes was found to change the analyte electrophoretic mobility, thereby altering the migration time of the anions. Depending on the additive used, the resolution between the fluoride ion and the phosphate was increased by as much as 15 %. The reproducibility of this method was found to quite high - in the range of 0.2-8.0 % RSD.

Benz and Fritz [48] compared the use of an ammonium salt and the use of 1-butanol as the EOF modifier. The modifier was added to a solution of 5 mM sodium chromate. It was found that the most efficient EOF modifier was a combination of both the ammonium salt and the organic solvent. A suggested mechanism for this observation is one in which both modifiers adsorb to the capillary wall in a dynamic equilibrium. The adsorbed butanol then causes a shift in the equilibrium of the salt, resulting in a net positive surface charge at a lower concentration of the salt. As can be seen from Figure 1.7.-1.8. the overall runtime for the separation of the anion mix can be reduced considerably by the introduction of some butanol, into the electrolyte buffer. Without the butanol the runtime required to separate out some of the anions was >60 min., however, once the butanol was added to the buffer electrolyte, the runtime was reduced to less than 5 min. This decrease represents a huge saving in time, however peaks 1-5 are not baseline resolved.

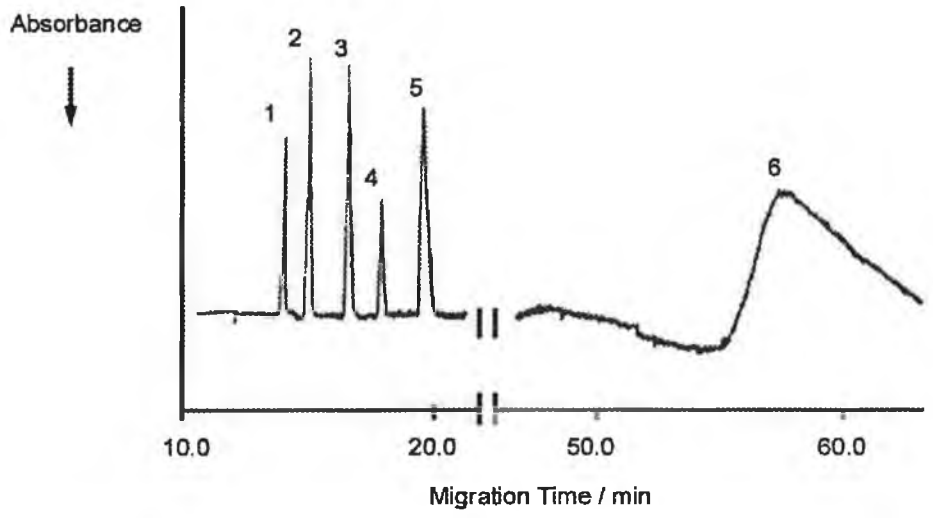


Figure 1.7. : Electropherogram of the separation of inorganic and organic acids. Voltage : -30 kV. Buffer : 5 mM chromate, 0.075 mM OFM-BT EOF modifier, pH 8.0. 1 = Br^- (5 ppm), 2 = Cl^- (5 ppm), 3 = SO_4^{2-} (6 ppm), 4 = NO_2^- (7 ppm), 5 = NO_3^- (8 ppm), 6 = F^- (8 ppm). Reproduced from ref 48.

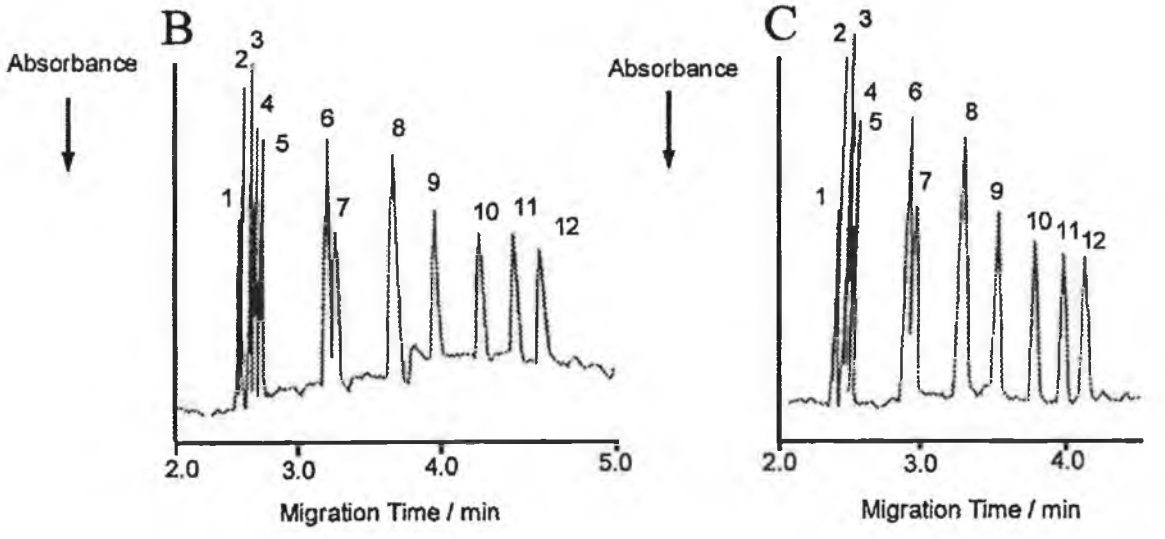
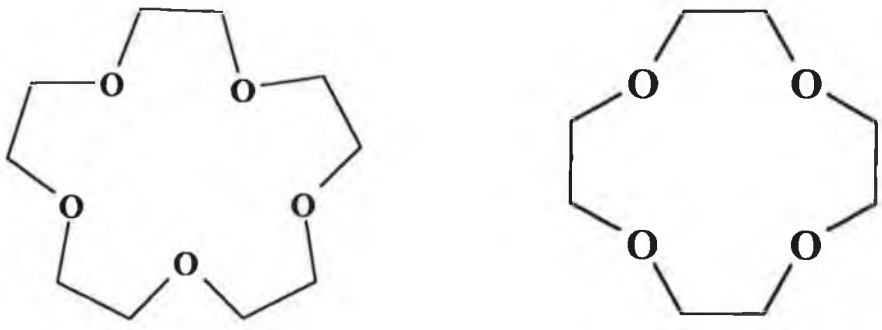


Figure 1.8. : Electropherograms showing the separation of several inorganic and organic acids. Voltage : -30 kV. (B) Buffer : 5 mM chromate, 0.075 mM OFM-BT EOF modifier, 3 % 1-butanol, pH 8.0. (C) Buffer : 5 mM chromate, 0.075 mM OFM-BT EOF modifier, 5 % 1-butanol, pH 8.0. 1 = Br^- (5 ppm), 2 = Cl^- (5 ppm), 3 = SO_4^{2-} (6 ppm), 4 = NO_2^- (7 ppm), 5 = NO_3^- (8 ppm), 6 = F^- (8 ppm), 7 = $HCOO^-$ (10 ppm), 8 = CO_3^{2-} (7 ppm), 9 = acetate (10 ppm), 10 = propionate (10 ppm), 11 = butyrate (10 ppm), 12 = valerate (10 ppm). Reproduced from ref 48.

A buffer electrolyte that does not contain chromate was used by Holderbeke et al. [49] for the analysis of anions in water. This buffer electrolyte consisted of trimesic acid with the addition of a polyamine. The trimesic acid is comparable to the chromate, while the polyamine acts as an EOF modifier. In this study, carried out on a 50 μm x 60 cm capillary, detection was at a wavelength of 230 nm. Good resolution was obtained between the ten anions separated.

The use of macrocycles as EOF modifiers was reported by Lamb et al. [50] This novel use of macrocycles compared the use of several different macrocyclic ligands in the separation of anions, namely 18-crown-6, 12-crown-5, 12-crown-4 and cryptand 2,2,2. Two of these modifiers are shown in Figure 1.9.



15-crown-5 12-crown-4

Figure 1.9. : The structures of the two macrocyclic ligands used as EOF modifiers in CE.

The EOF modifiers were used in conjunction with an electrolyte of 2.0 mM sodium borate, 1.0 mM sodium dichromate, 4.3 boric acid, pH 7.2. Electropherograms obtained using 15-crown-5 and 12-crown-4 as EOF modifiers are show in Figure 1.10. Careful selection of the ligand is required in order to maximise the resolution obtained between the different anions in the sample run. From the Figure 1.10. it can be seen that the nitrate and oxalate peaks can be partially resolved by using a different ligand.

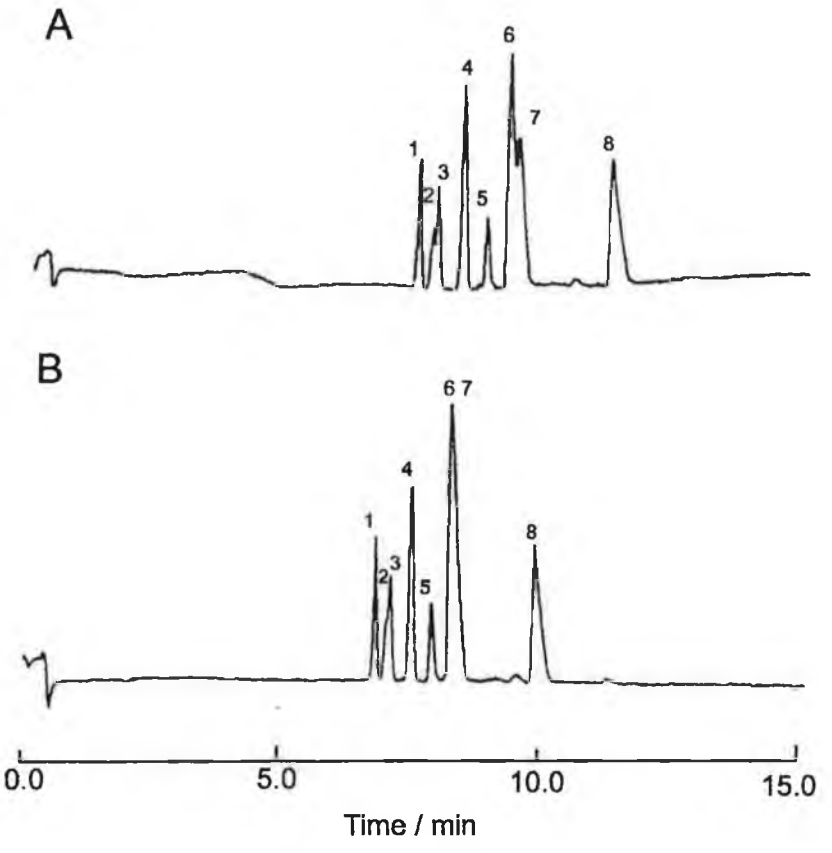


Figure 1.10. : Separation of 8 anion standards with the adddition of (A) 12-crown-4 and (B) 15-crown-5 as the electroosmotic flow modifiers. Buffer : 2.0 mM sodium tetraborate, 1.8 mM sodium chromate, 43.0 mM boric acid and 2.0 18-crown-6 at pH 7.2. Capillary : 75 μm i.d. x 60 cm. Indirect detection at 254 nm. 1) bromide, 2) chloride, 3) iodide, 4) sulphate, 5) nitrite, 6,7) nitrate and oxalate, 8) thiocyanate. Anion conc. Are 10 ppm except chloride which is 3 ppm.

The use of non-aqueous electrolytes was investigated by Salin-Moosavi and Cassidy [51]. In this study MeOH and dimethylformamide (DMF) were added to an aqueous electrolyte and their effectiveness for the separation of inorganic anions was assessed. The solvent DMF was chosen on account of its high dielectric constant, high boiling point and its widespread use in electrochemical studies. MeOH was chosen as it is a very common solvent in laboratories. Several aqueous electrolytes (with MeOH) were compared, namely chromate, benzoate and phthalate. Two detection modes were compared and contrasted, namely UV detection and electrochemical detection. Background electrolytes for electrochemical detection were tetrabutylammonium hexefluorphosphate (TBAHP) and tetraethylammonium perchlorate (TEAP). The organic solvents (MeOH/DMF) were added in concentrations of 0-100 % (v/v).

Results show that the mobility of the EOF decreased as the organic solvent concentration increased up to 75% v/v . Beyond this concentration, the mobility increased once again. This trend was seen for both solvents. Although the mobility did increase, it did not exceed the mobility for the electrolyte with 0 % organic solvent added. Another effect of the addition of organic solvents was a reversed separation order in contrast to the order in aqueous electrolytes. It was observed that the UV detection system gave linear calibration curves over a larger range when compared to the electrochemical detection. The detection limits were, however, much lower for the electrochemical detection system (1×10^{-9} to 6×10^{-8} mol/l) for some anions while the UV detection system produced limits of 2.0×10^{-5} to 3.4×10^{-5} mol/l.

Harakuwe and Haddad [52] illustrated the importance of pH control in CE, in the separation of fluoride and phosphate anions. The buffer electrolyte was chromate-TTAB based, with changes in the pH and/or the addition of small amounts of 1-butanol. It was shown that by careful control of the buffer electrolyte pH, separation of the two anions could be achieved.

1.8. CAPILLARY ELECTROPHORESIS

Capillary electrophoresis was the chosen method for the analysis of the acidic anions found in cyanoacrylic adhesive preparations. This technique is an ideal choice when it comes to the analysis of charged species, as discussed in the above sections.

1.8.1 Detection Methods

There are two types of detection systems which are employed in capillary electrophoresis, namely, optical and electrochemical. These two systems can be further subdivided. The optical systems include UV (both direct and indirect modes) and fluorescence. The electrochemical systems include conductimetric (both suppressed and non-suppressed modes), amperometric and potentiometric detection.

1.8.2. Optical based Detectors

Spectrophotometric detection is one of the most versatile and widely used forms of detection. Its applicability is found in all areas of biology, chemistry and biochemistry. Spectrophotometric detection is based upon the absorbance of light (visible or UV) by the analytes or the eluents. In the case of direct (UV) detection, it is the analytes which absorb the light, whereas in the case of indirect detection it is the background carrier or eluent which absorbs. Due to the limitations of the solvent cut-off, the analyte must absorb at wavelength above ~200 nm. Below this the solvent (or background carrier) will absorb the light. The extinction coefficient of many anions, both organic and inorganic anions is relatively low (less than 1000 mol^{-1} dm^3 cm^{-1}). The low extinction coefficient is indicative of a relatively low absorbance of UV light which makes ideally suited to indirect UV detection. Anions that fall into this category include ions such as chloride, nitrate and sulphate. The more strongly UV absorbing anions tend to be aromatic, heterocyclic or conjugated in nature.

Many groups have used indirect UV detection as a means of anion detection. All methods employed have used a high UV absorbing background buffer electrolyte system, typically but not always chromate based [30, 31, 36-38]. The most common wavelength used is 254 nm [30,31,38], although some methods use other wavelengths depending on the specific application in question. Holderbeke et al. [49] used a wavelength of 230 nm, but they also used a background chromophore of trimesic acid with a polyamine (unspecified). When detecting several halides, formate and carbonate, Stahl [45] used a wavelength of 275 nm.

Another means of detection at the disposal of the analyst is fluorescence. While this maybe a very useful technique in some areas, it is not of great value in the detection of anions, as there are few anions to be found that fluoresce. Fluorescence detection has been used successfully by Bazzanella et al.[53] to separate a number of inorganic anions, including chloride, nitrate, oxalate, malonate, fumarate, formate, succinate, tartrate, maleate, phosphate, glutarate, carbonate pyruvate, lactate, citrate and ascorbate. Indirect fluorescence was also used to detect anions such as chloride, sulphate, oxalate and fluoride [54]. To the capillary electrophoresis buffer electrolyte some fluorescein was added, which fluoresced, while the actual anions of interest did not. Laser induced fluorescence (LIF) has also been used successfully by Desbene et al.[55] as a means for indirect detection, again using fluorescein as a buffer additive.

One other final optical detection system is refractive index detection. This system is rarely used in capillary electrophoresis for anion analysis, due to problems with baseline noise and a poor level of sensitivity.

1.8.3. Electrochemical Detection

1.8.3.1. Potentiometric Detection

Potentiometric detection is based on the measurement of the ion in question using ion selective electrode (ISE). The potential measured by this electrode varies with the concentration of the ion under study. Potentiometric detection has been used to

detect anions such as halides, nitrate and nitrite, following capillary electrophoretic separation [56]. The ISE consisted of a Pt wire coated in a PVC membrane and depending on what type of ion was to be detected, other compounds were also incorporated. This method was successfully applied to both cation detection and anion detection. The potential measured by this electrode is measured with respect to that of a reference electrode, which is held at a constant potential. However, in comparison to conductimetric detection, potentiometric detection lacks sensitivity, and also has a slower response time.

1.8.3.2. Conductimetric detection

Conductimetric detection is based on the application of a potential to the solution which results in the production of a current; the current produced can then be related to the conductance of the solution between the two electrodes. There are two forms of conductimetric detection that are used to detection anions, namely non-suppressed and suppressed detection. In non-suppressed mode, the sensitivity is related to the difference between the equivalent conductivity of the eluent ions and the analyte ions. If the eluent consists of high conductivity ions, then analyte ions of lower conductivity will produce a decrease in the signal, thus producing a 'negative' peak. Conversely, if the eluent consists of low conductivity ions, then the sensitivity of the signal will increase as analyte ions of high conductivity are detected. Non-suppressed conductimetric detection suffers from the high background signal that can be produced by the buffer system. Therefore a means of reducing the conductivity of the eluent was developed which led to the development of suppressed conductimetric detection. By using a combination of resins, the ions of the eluent can be either neutralised or totally removed, thereby greatly reducing the actual conductivity of the eluent to a low value. The analyte ions then have greater conductivities than that of the eluent, and thus the signal produced is proportional to the conductivity of the analyte ions. Both forms of conductimetric detection have been applied to the detection of anions following capillary electrophoretic separation.

1.8.3.3. Amperometric Detection

Yet another means of electrochemical detection of anions, is amperometric detection. The basis of this technique is the oxidation or reduction of the analyte species. A fixed potential is applied to the flowcell and thus generates a current. The current generated is directly proportional to the concentration of analyte present. The main drawback with the application of this technique to the analysis of anions is that many anions, especially the inorganic anions, are difficult to oxidise or reduce. It is however, possible to detect these species after derivatisation. Salimi-Moosavi et al. [51] have applied amperometric detection to detect anions such as thiocyanate, azide and iodide, using a Pt ultramicroelectrode at a potential of 1700 mV (Vs SCE). The amperometric detector was not, however, used to detect several anions, including nitrate, chloride, fluoride and oxalate - some of the anions of interest in this project. These anions were detected using indirect UV detection. The sulphate anion has been detected amperometrically, using a potential of 900 mV and gold electrodes, by Tenberken et al. [57]

1.8.4. Enhancing Sensitivity

In spite of having several different detection modes available, efforts are constantly being made to improve CE sensitivity. One of the most popular methods is by cell geometry modification, e.g. bending of the capillary into the 'Z-cell' form. This form of cell geometry has been reported [58] to give a tenfold (or greater) increase in sensitivity. Other flattened types of capillary geometries are also available. In addition to capillary geometry modifications, it is also possible to use a method known as 'stacking', a type of on-line concentration technique [59, 60]. It has been reported [61] that stacking injection can result in a 10 to 20 fold sensitivity enhancement for dilute samples. It is possible to completely fill the capillary by using sufficiently long injection times, before pumping out the sample solvent. Burgi [60] reported a 100 fold improvement of detection of the fast moving ions (such as chloride, sulphate, nitrate and oxalate), by filling the whole capillary upon injection.

In order to perform large volume stacking, the electrophoretic mobility of the ions of interest must be negative or opposite with respect to the electroosmotic flow. In polarity switching large volume stacking, the configuration of the electrodes is switched after the sample is loaded into the column. Once the sample buffer plug is removed, the electrodes are switched back to their original configuration and the separation of ions occurs. In large volume stacking with an electroosmotic flow modifier, the electrode configuration is not changed after sample loading. The negative species move toward the ground electrode when the voltage, e.g. -20 kV, is placed on the injection end of the column. In large volume stacking, once the sample is introduced into the column and a voltage is applied, the local electroosmotic flow generated by the sample region will move the bulk solution toward the anode of the system. At the same time, the negative ions in the sample region will stack themselves up against the boundary between the sample region and the run buffer region. The ions will stay at the boundary until the water plug is pumped out of the column because almost all of the applied electric field is dropped across the run buffer region (which is now the whole column), the ions then begin to separate again. This process is shown schematically in Figure 1.11.

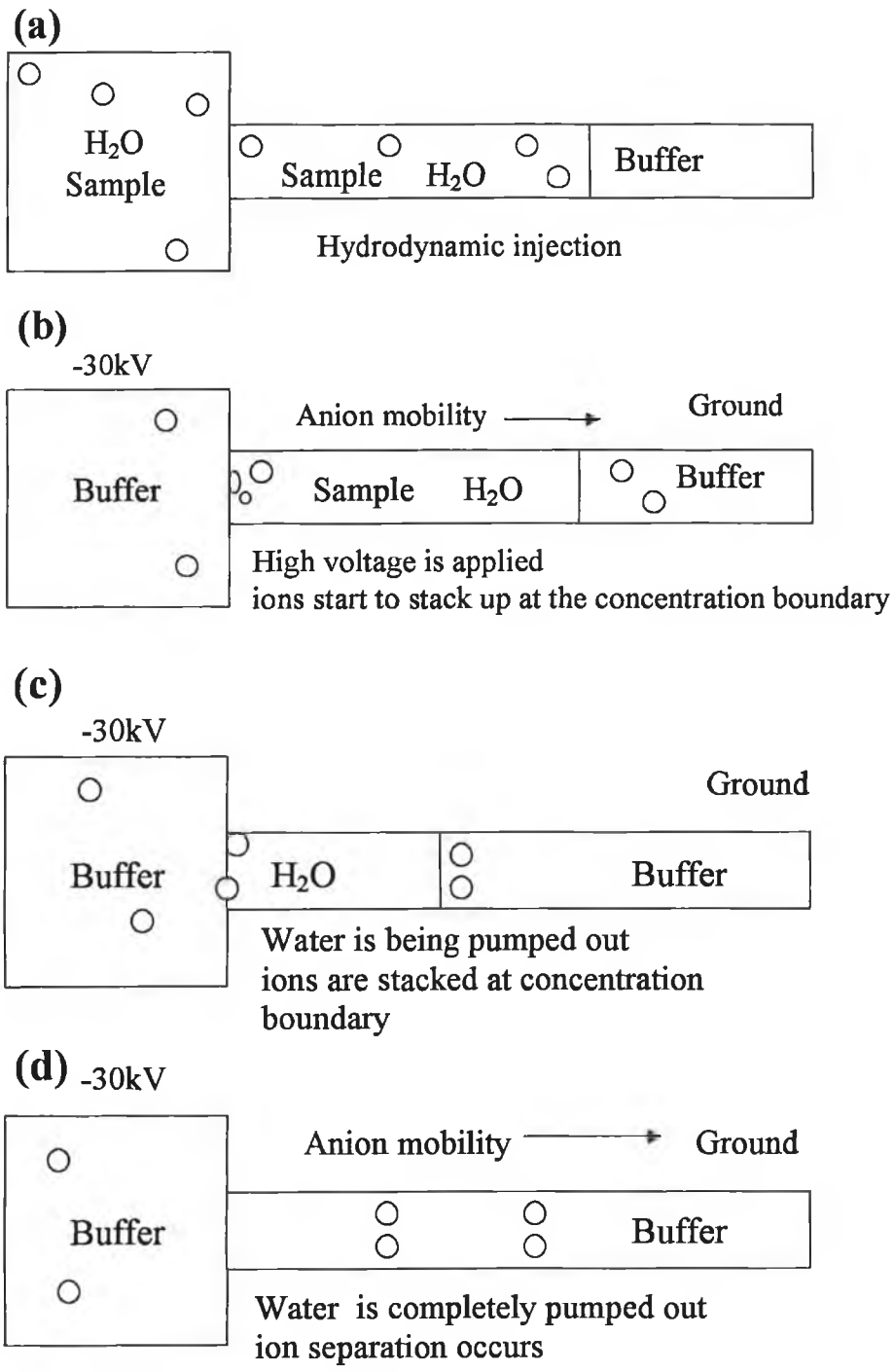


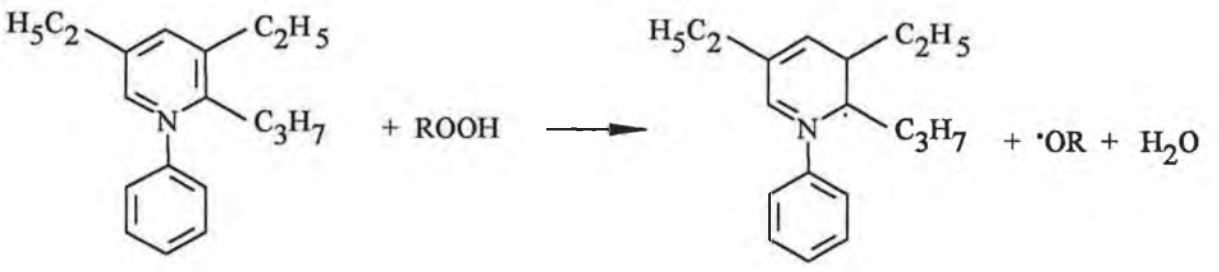
Figure 1.11. : The process of stacking : Once the sample is injected into the capillary, the buffer dissolves into the water of the sample (a) and after the voltage is applied the ions stay at the boundary until the water plug is pumped out of the column (b and c). Once the water is completely pumped out of the capillary the anions separate out under normal CE conditions (d).

1.9. ACRYLIC ADHESIVES

Another class of adhesives that exist in addition to anaerobic and cyanoacrylate adhesives are the acrylics. Acrylics are composed of catalysts, initiators and monomers. The most common application of acrylics is in the bonding of plastics and glass, where both flexibility and bond strength are required. The distinguishing feature of acrylics is the requirement of a primer component; that is they are a two-part adhesive mix. The monomers used in the the acrylic adhesive compositions are typically mono-functional methacrylates, which produce a lower crosslink density polymer, yet it is still a tough polymer. Methyl methacrylate and methacrylic acid are two examples of monomers used in the acrylic formulations. Curing of acrylic adhesives is initiated by a two-part redox system; one part is found in the base component of the adhesive and the second part found in the accelerator component. As in anaerobics, cumene hydroperoxide can be found in acrylics, in the oxidant part of the redox couple. The main additive to acrylics are toughners. The toughners are essential to prevent the formation of a brittle cured matrix. Typically a rubbery type component is introduced into the composition, e.g. a chlorosulphonated polyethylene.

1.9.1 Chemistry of Acrylic Adhesivs

The chemistry of acrylics share some similarities with that of the anaerobics; however the intiation of polymerisation involves some different reactions. There are two parts required in the curing system of acrylics, the base component and the curative (or accelerator) component. The most commonly used curative is a condensation product, N-phenyl-2-propyl-3, 5-diethyl-1, 2-dihydropyridine (DHP). This is one of the products produced by the condensation of aniline with n-butyraldehyde. The DHP functions in reducing the hydroperoxide to produce alkoxy free radicals, which are then involved in the polymerisation process. Transition metals, in the form of salts, are also employed in many low odour acrylic systems as initiators. As with the anaerobics, the cure of acrylics is inhibited by the presence of oxygen. Oxygen sensitivity is attenuated in acrylic adhesives due to the higher catalyst concentrations, which results in a greater rate of radical production (compared to anaerobic systems).



Dihydropyridine (DHP)

Figure 1.12 : The reaction that is believed to occur between DHP and the peroxide to produce alkoxy radicals.

Although the exact reaction that occurs is not known, it is believed that the first step involves hydrogen abstraction [62], as shown above in Figure 1.12. The other main cataytical system is based on the benzoyl peroxide and an aromatic redox couple, has been well studied and the formation of the benzoyl radicals has been well established.

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Chapter Two

Investigations into the Decomposition of Key Reducing Agents found in Anaerobic Adhesive Mixtures

2.1 INTRODUCTION

Anaerobic adhesives are single component systems, which can be stored in liquid form for long periods of time in the presence of oxygen, but harden rapidly in the absence of oxygen. The adhesive is therefore stored in polyethylene containers that allow small amounts of oxygen to permeate through to the adhesive. Anaerobic adhesives find applications in many areas of the engineering industry where they are used in sealing, threadlocking and retaining applications. The preparations generally consist of a monomer, initiator, accelerator and stabiliser. Other modifiers can be added that can affect performance, such as fillers and thickeners.

Although there is some knowledge as to the fundamental reactions that occur in the curing of the anaerobic adhesives, the complete chemistry is far from being fully understood. The complex relationship between the different components of the adhesive preparations only serves to further complicate the elucidation of the curing chemistry. In addition to this, there are a number of different accelerators and initiators that can be used in the formulations, all of which have different reactivities. The surface to which the adhesive is applied also has an effect on the speed of curing, as different transition metals have different effects on the curing of the adhesive, copper (II) being one of the most efficient of the metals in the curing process. The metals react with the hydroperoxide to generate free radicals, which go on to initiate the polymerisation process. As a result of the effect that some metals have on the adhesive, great care is taken to remove or at least chelate these metals in the formulations, thereby greatly increasing the storage stability of the adhesive.

2.2. PURPOSE AND AIMS OF RESEARCH

The focus of this research was to elucidate if the key reducing agents 1-acetyl-2—diphenylhydrazine (APH), N,N-dimethyl-p-toluidine (DMPT) and 1,2,3,4-tetrahydroquinoline (THQ) could possibly be consumed through reaction with the organic peroxide namely cumene hydroperoxide (CHP). It was also decided to investigate if this reaction (if any) could be catalysed by a 'co-accelerator' used in the adhesive preparations, such as an organic acid. These reactions were carried out at room temperature. This study was carried out in the presence of the acids typically used in anaerobic sealants, namely saccharin and maleic acid. The reactions were carried out in different ratios between CHP, a weak acid and the reducing agent. In order to establish if the time based reaction pathways differed in nature, to those which could be initiated by active metals, the reactions were repeated in the presence of metal salts of both copper (II) and iron (III). The copper salts used were copper (II) acetate and copper (II) sulphate. The iron salt used was iron (III) nitrate.

Preliminary investigations were carried out into the decomposition of other key reducing agents, namely (*p*-toluenesulphonyl) hydrazine (*p*-TSH), N,N-dimethyl-*p*-toluidine-N-oxide (DMNO) and N-phenyl-2-propyl-3, 5-diethyl-1, 2-dihydropyridine (DHP). These preliminary investigations involved reacting the reducing agents with CHP, both in the presence and absence of saccharin.

As these reactions were to be monitored using HPLC, a suitable method had to be developed, by which the decomposition product(s) of the reducing agents could be separated, detected and quantified.

The objective of the research programme was to elucidate as to whether, over the lifetime of the adhesive, any reactions were taking place between the various cure components. Identifying and understanding these reactions could assist in understanding the loss in performance with time, which is exhibited by certain adhesive formulations. This in turn could significantly impact on efforts to extend the effective lifetime of these formulations by countering or minimising these reactions.

As the curing systems embodied in these formulations typically incorporate an oxidant (i.e. an organic peroxide), a reducing agent (usually an amine or hydrazine) and an organic acid, it would be anticipated that time based reactions between these components is very likely. To date it would appear that no systematic studies on the nature and extent of these reactions have been carried out. This study aims to redress this deficiency and moreover to provide the basis for extending the active lifetime of these formulations.

2.3. EXPERIMENTAL DETAILS

2.3.1 Reagents and Apparatus

All reagents used were of the Analar grade. All adhesive components including APH, DMPT, THQ, CHP, (*p*-toluenesulphonyl) hydrazine, N,N-dimethyl-*p*-toluidine-N-oxide, dihydropyridine and saccharin were supplied by Loctite (Irl.) Ltd. The maleic acid was supplied by Aldrich and the copper and iron salts by Merck. The phosphate buffer was prepared using potassium dihydrogen phosphate (KH_2PO_4) and di-sodium hydrogen phosphate (Na_2HPO_4) both obtained from BDH.

HPLC studies were carried out using a Beckman system, including a Model 118 solvent delivery module and a Model 168 detector module equipped with a diode array detector. This was connected to a 486, 66-DX2 Elonex PC under the control of System Gold. A C_{18} RP column, Waters μBondapak, 10 μm, 300 x 3.9 mm was used in the separation. The flow rate was 1.2 cm^3 min^{-1}. The detection was in the UV range at 254 nm. The injection volume was 20 μl. Mobile phase was prepared from HPLC grade acetonitrile (from Labscan) and phosphate buffer using 18 ΩM deionised water (obtained from a Millipore, Milli-Q-system). Preparative TLC plates, 20 x 20 cm, 1000 μm thickness, silica gel on glass containing a fluorescent indicator, were supplied by Aldrich.

2.3.2. Methods

Before any analysis could be carried out using HPLC, a suitable analytical method had to be found whereby all components of interest could be separated. HPLC has been used previously in the analysis of some anaerobic adhesive components (HPLC separation followed by UV detection) [1]. In order to select the optimum wavelength at which to detect the adhesive components, solutions were prepared in MeOH of each of the components of interest, and their UV spectrum obtained. CHP had a maximum wavelength of 254 nm, and as it had relatively low absorbance, 254 nm was chosen as the wavelength at which to set the detector (to maximise the detection of CHP). The UV absorbance spectrum of each of the compounds showed that many of the compounds absorbed strongly in the lower UV range. This, however, is very close to the cut-off wavelength for many of the different solvents used in HPLC mobile phases and so a lower wavelength was not utilised.

Initially, an ODS Hypersil C_{18} RP column, 200 x 4.6 mm, 5 μm was used, in the method developed. The mobile phase 40 % THF / 60 % H_2O used by Heatley [2] was tried on the system but it proved ineffective in the separation of the acid components. The mobile phase developed by MacManus [1] was also investigated, but there was not complete resolution between the maleic acid and saccharin. This may possibly be due to the length of the column used. In this system, a 20 cm column was used while MacManus used a 30 cm column. The longer column would have allowed for a longer separation time thus increasing resolution between the two peaks.

As previously developed mobile phases could not be applied successfully to the separation of all the adhesive components, it was decided to develop a more suitable mobile phase. The development of the mobile phase first started with a non-buffered system of MeOH / H_2O followed by the use of acetonitrile instead of methanol. It was found that the two acids, saccharin and maleic acid, eluted as one peak immediately under the solvent peak, while the APH produced a broad peak. In order to separate the two acid peaks, and to sharpen the APH peak, it was necessary to use a buffered mobile phase, which also contained an ion-pairing agent, namely tri-ethyl

amine (TEA). The ion-pairing agent pairs with the ions (in this case saccharin and maleic acid) thus creating sufficient difference between the two, enabling them to be separated on the HPLC system. The optimum mobile phase was determined to be 35 % acetonitrile : 65 % buffer. The buffer was composed of 50 mM phosphate which contained 5 % TEA, adjusted to a pH of 4.50 with *o*-phosphoric acid. The phosphate buffer was prepared using 25 mM of potassium di-hydrogen phosphate and 25 mM di-sodium hydrogen phosphate, giving a 50 mM phosphate buffer solution. The flow rate was 1.00 ml min^{-1}.

Although this mobile phase was developed to separate saccharin, maleic acid, CHP and APH, it also proved suitable for the separation of other adhesive components, including DMPT, THQ, (*p*-toluenesulphonyl) hydrazine, NN-dimethyl-*p*-toluidine-N-oxide and dihydropyridine. The products formed by some of these compounds after reacting with saccharin and CHP were also separated from the reactant peaks.

Later in the study of the anaerobic adhesive components the column was changed to a Lichrosorb μBondapak, 10 μm, 300 x 3.9 mm column. The mobile phase composition was altered slightly to reduce the retention time without any loss in resolution. The flow rate was also increased to 1.20 ml min^{-1}, again without any loss in resolution. The final mobile phase composition was 40 % acetonitrile : 60 % buffer (composition as before), with a flow rate of 1. 20 ml min^{-1}.

With the exception of the copper (II) and iron (III) salts, all solutions were prepared as a stock solution in acetonitrile and then dissolved in a CH_3CN : H_2O (40 : 60) to give a final molar ratio (of reducing agent to component) of 1 : 1 or 4 : 1. All metal salt solutions were prepared in 18 MΩ de-ionised water. The stock solutions of all the components were prepared in concentrations of 250 mM. A number of such solutions were prepared in the ratios given in Table 2.1. The final concentration of reducing agent in each of the solutions was 0.1 mM – 0.75 mM depending on the absorbance of the reducing agent. All dilutions were prepared in a solvent mix of 40 % acetonitrile and 60 % water.

Reducing Agent	CHP	Saccharin	Maleic Acid	Metal Salt
1	1	0	0	0
1	1	1	0	0
1	1	0	1	0
1	1	1	1	0
1	1	1	1	1
4	4	4	4	1

Table 2.1. : Table showing the molar ratios of reducing agent to each of the other components in the reaction solutions.

2.3.6.1. Identification of Products

The products formed by the decomposition of the reducing agents could clearly be seen, and in some cases, such as for DMPT, two products were observed. In order to identify these products, it was first necessary to isolate them. By using the photodiode array HPLC detector, some information could be attained; however, more information was required in order to identify the structure of the product formed. The system developed could be directly applied to a preparative HPLC system, but as the mobile phase contained a phosphate buffer, the collected fractions could not be subjected to identification tests such as mass spectrometry. In order to obtain mass spectrometry or NMR data the products would have to be extracted from this mobile phase, into a more suitable solvent, such as an organic solvent. As the products were unknown, the development of an efficient extraction process would have been difficult; therefore, it was decided to turn to preparative TLC (thin layer chromatography). As the TLC plates were normal phase (i.e. silica coated - with no alkyl chains bound to the silica) non-aqueous solvents were employed. The solvents used include ethyl acetate, ethanol, hexane and petroleum ether. The latter two

solvents are the more non-polar, while ethanol is the most polar. Ethyl acetate falls midway in the polarity range. By using ethyl acetate and ethanol in varying ratios, it was possible to create a mobile phase that ranged in polarity from very polar to relatively non-polar. Conversely, by varying the amount of hexane or petroleum ether added to ethyl acetate it was possible to obtain a mobile phase that ranged in polarity from relatively non-polar to very non-polar.

The conditions developed for the isolation of the decomposition products for APH, DMPT and *p*-TSH were as follows :

APH :

Mobile phase consisted of 10 % ethyl acetate and 90 % hexane. One main product was isolated. Confirmation of the product's identity was performed by checking the retention time and UV spectrum of the isolated product on the HPLC system.

DMPT :

Mobile phase consisted of 5 % ethyl acetate and 95 % hexane. Although there were two products seen on the HPLC trace, only one major product was isolated. It was not possible to isolate the minor product in sufficient quantity to do any identification tests. Once again, confirmation of the product's identity was performed, by checking the retention time and UV spectrum of the isolated product on the HPLC system.

THQ :

Due to the multiple products formed by the decomposition of the THQ, it was not possible to isolate any product in sufficient quantity to obtain any acceptable identification test result.

***p*-TSH :**

The *p*-TSH and peroxide readily reacted in acetonitrile to produce a solid, which could be easily isolated by filtration. The sample product was formed when *p*-TSH was exposed to CHP only, and when it exposed to both CHP and saccharin. Due to the nature of the product formed by the *p*-TSH reaction product formed, no TLC

method was required for its separation. The product was only sparingly soluble in acetonitrile, but the starting compounds were readily soluble in acetonitrile, therefore the unreacted reactants could be easily removed, to produce a very pure product.

2.4. RESULTS

2.4.1. HPLC

The HPLC system was optimised for the separation and analysis of the APH containing solutions, although it was then applied to the solutions containing other reducing agents. A sample chromatogram shown in Figure 2.1., obtained from the APH solution, shows the two acids, the hydrazine, peroxide and product formed, (using a Lichrosorb C_{18} column).

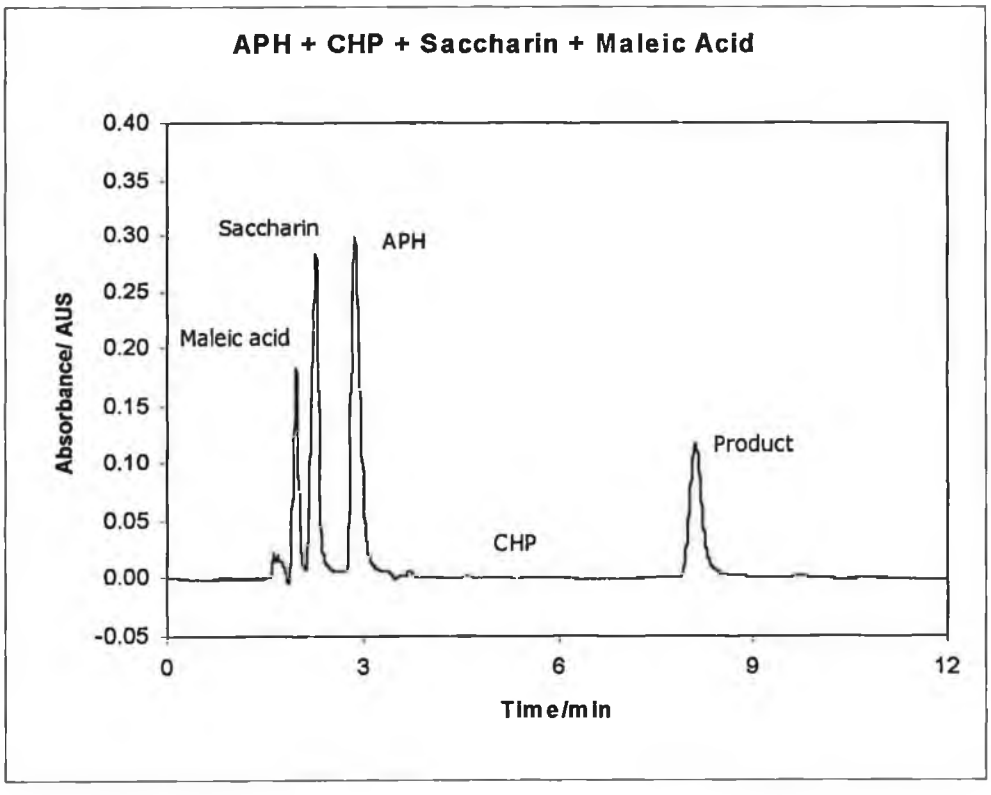

Figure 2.1 : A HPLC trace showing the separation obtained for a mixture containing the two acids, APH and CHP. Mobile phase consists of acetonitrile (40 %) and phosphate buffer (60 %). Detection at 254 nm.

It was discovered that CHP produced two peaks when analysed on the system. In fact, the CHP separates into two compounds, namely cumyl alcohol and acetophenone. This was confirmed using diode array detection. This separation or decomposition was not due to the solvent in which the CHP was dissolved. This was

confirmed by dissolving the CHP in a range of solvents (MeOH, MeOH/H_2O, CH_3CN, CH_3CN/H_2O, CH_3CN/buffer) and then monitoring the solutions over a period of two weeks to determine how stable the CHP was in the solution. It was discovered that the CHP decomposed in CH_3CN/buffer solution, probably due to the acidic environment (the buffer was at pH 4.50).

In order to determine the percentage decomposition of the reducing agent in each of the solutions, the following procedure was used.
A solution containing the reducing agent only was prepared at a given concentration. The area of the peak in the chromatogram was determined. The other components were added to the solution and then the area of the reducing agent peak was determined again. The difference between the initial peak area of the reducing agent peak and each successive reading was calculated, and this was taken to be the relative reduction in the concentration of the reducing agent in question. The relative decrease could then be expressed as percentage decomposition of the reducing agent. The percentage decomposition was then plotted against time, time being the age of the solution. Typically the solutions were kept for a period of 10-12 days. In order to determine if each of the individual components was stable in solution over time, an injection was run of each component over the two weeks. When the metal salts were added to the solutions, 'controls' were also prepared, consisting of the same solutions without the metal salts. These solutions were prepared and run simultaneously in order to make comparisons between the two.

As mentioned above the optimisation procedure was carried out using APH. Initially the metal salts were added the solutions in a ratio of 1:1 to APH. However, studies showed that the decomposition of APH occurred so rapidly that it was impossible to clearly distinguish between the different rates of decomposition in the presence or absence of the acid components. The ratio of copper (II) salts to APH was then changed to 1:4 and this decreased the rate of decomposition of APH sufficiently to allow the effect of the acids to be seen clearly. The graphical results of the reactions between APH, saccharin / maleic acid, CHP and the copper (II) salts in 1:1 ratio did not allow comparison between the effects of different acid components. The effect

could be seen more clearly in the graphs of the solutions containing the copper (II) salts in a 1:4 ratio to APH. It was therefore decided to carry out all the reactions involving the reducing agents and metals salts in this ratio. Similar solutions were prepared with iron (III) nitrate in the mixture instead of the copper (II) salts.

2.4.2. APH Chemistry

Preliminary investigations into the effects of differing concentrations of CHP or an acid on the decomposition of APH were performed. They revealed that in the absence of any CHP, the APH was stable in solution (even in the presence of saccharin or maleic acid). Adding increasing amounts of CHP resulted in an increase in the decomposition of the APH. Addition of low amounts of acid resulted in a decrease in the decomposition of the APH. In contrast adding higher concentrations of acid actually increased the decomposition of the APH. These observations are discussed in more detail later. While this preliminary investigations illustrated the effect of the peroxide and acid components, further investigations were performed using the above mentioned ratios.

The results obtained for the decomposition of APH in the presence of CHP and/or saccharin and/or maleic acid and in the presence or absence of a metal salt are all presented graphically in the Figures 2.2.-2.6. The graphs are intended to display the different trends seen, rather than being indicative of a direct relationship between the rate of decomposition and time.

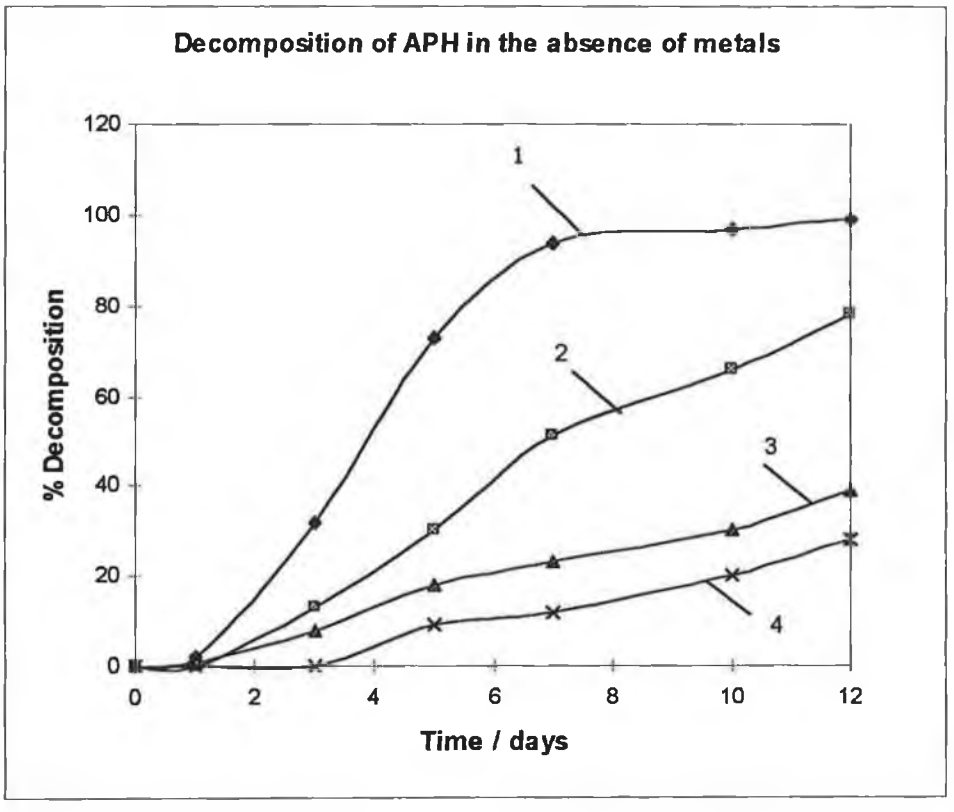

Figure 2.2 : Graph showing the rate of decomposition of APH in the presence of CHP and/or acids. Data was obtained from HPLC analysis, mobile phase of 40 % acetonitrile and 60 % phosphate buffer, detection at 254 nm. Flow rate = 1.2 ml min^{-1}. 1=APH + CHP, 2 = APH + CHP + maleic acid, 3 = APH + CHP + saccharin, 4 = APH + CHP + maleic acid + saccharin. All components are in a 1:1 ratio.

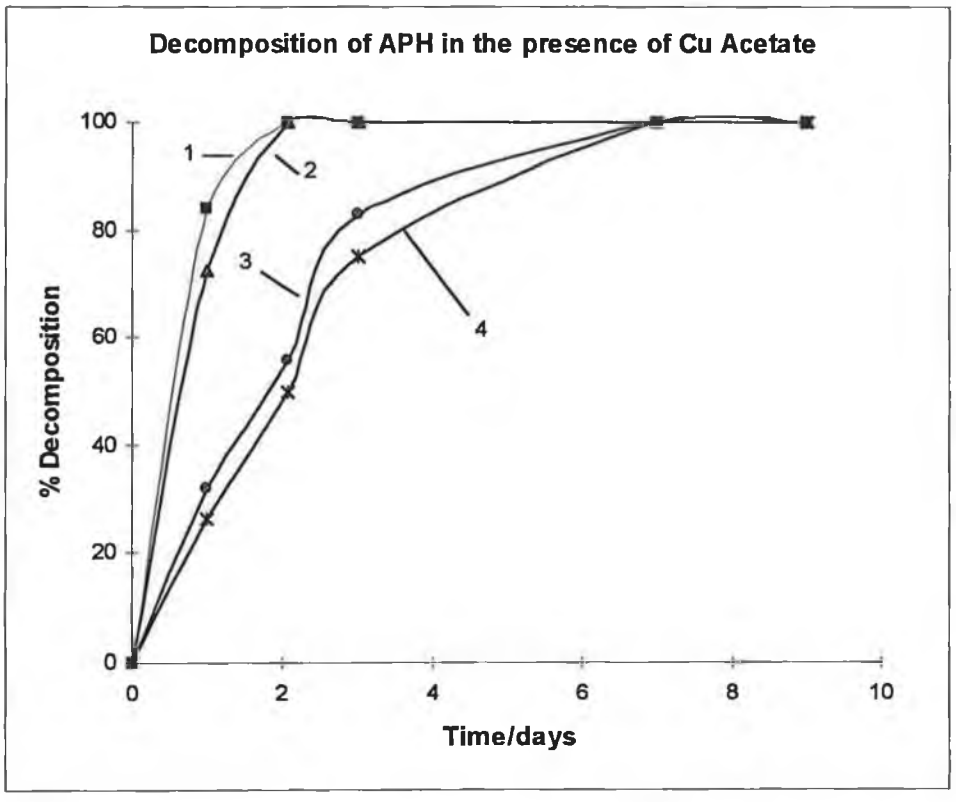

Figure 2.3 : Graph showing the rate of decomposition of APH in the presence of copper (II) acetate, CHP and acid components. Data obtained from HPLC runs, mobile phase of 40 % acetonitrile and 60 % phosphate buffer, detection at 254 nm. Flow rate = 1.2 ml min^{-1}. In addition to APH and copper (II) acetate, the solutions contain : 1 = CHP, 2 = CHP + maleic acid, 3 = CHP + saccharin, 4 = CHP + maleic acid + saccharin. The ratio of components : metal = 4:1.

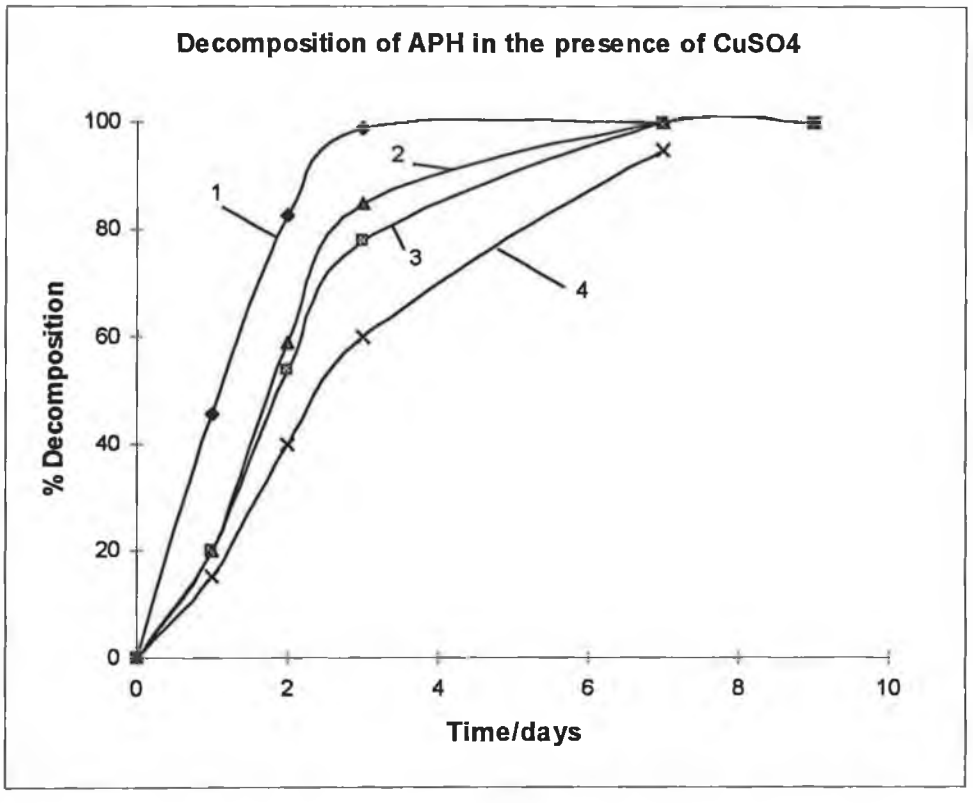

Figure 2.4 : Graph showing the rate of decomposition of APH in the presence of copper (II) sulphate, CHP and/or acid components. Data obtained from HPLC runs, mobile phase of 40 % acetonitrile and 60 % phosphate buffer, detection at 254 nm. Flow rate = 1.2 ml min^{-1}. In addition to APH and copper (II) sulphate, the solutions contain : 1 = CHP, 2 =CHP + maleic acid, 3 = CHP + saccharin, 4 = CHP + maleic acid + saccharin. The ratio of components : metal = 4:1.

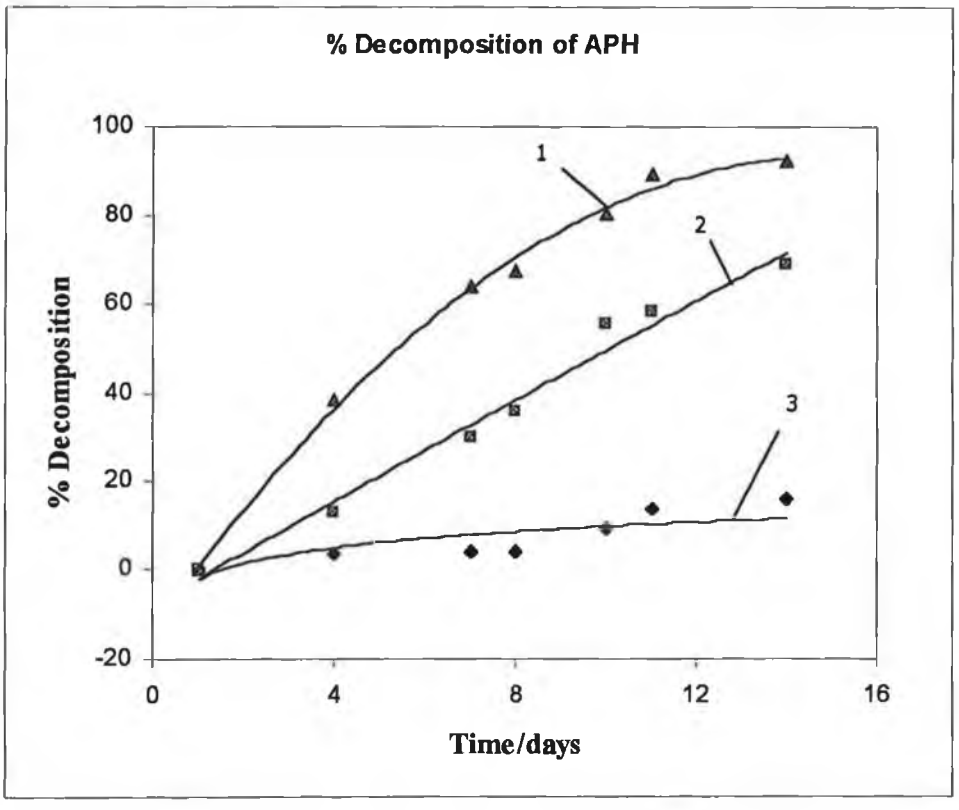

Figure 2.5 : Graph showing the rate of decomposition of APH in the presence of CHP and acid components. Data obtained from HPLC runs, mobile phase of 40 % acetonitrile and 60 % phosphate buffer, detection at 254 nm. Flow rate = 1.2 ml min^{-1}. 1 = APH + CHP + saccharin (1:3:3), 2 = APH + CHP + saccharin (1:1:3), 3 = APH + CHP (1:3).

When the solutions containing the APH, CHP and acid in 1:3 ratios were compared to those containing the compounds in a 1:1 ratio it was seen that the rates of decomposition differed between the two sets of solutions. From the Figures 2.2.-2.4., a comparison can be made between the rate of decomposition of APH in the presence and absence of the acid components. The fastest rate of decomposition occurs in solutions containing only APH and CHP in contrast to the solutions containing APH, CHP and an acid. However, as Figure 2.5. shows, the fastest rate of decomposition occurred when APH, CHP and saccharin (1:3:3 ratio) were present together in solution.

It is possible to explain these observations if one considers the actual reaction that is taking place. The protonation of the APH diminishes its reductivity potential, i.e. it is less effective in the protonated form at promoting homolysis of the peroxide. However in the presence of much higher amounts of acid, for example APH : CHP : saccharin (1:3:3), after any protonation of the APH, it is likely that the peroxide would become protonated. It has been reported that the protonated peroxide is much more reactive than the unprotonated one [3]. Hence in the presence of higher concentrations of acid, the protonated peroxide exerts a much greater influence on the overall reaction than any protonation of the APH. The difference observed between the acids used, i.e. maleic acid and saccharin, is difficult to explain. Although the exact chemistry involved has not yet been fully elucidated, it has been reported that the saccharin has a unique role in the overall chemistry of anaerobic adhesives [4].

It is proposed that at higher levels of CHP there is an increased tendency to form the hydroxyamine compound outlined in Figure 2.40. which would serve to suppress these homolytic reactions.

Similar trends to those displayed in Figure 2.2. are also seen in both Figures 2.3 and 2.4., obtained from the solutions containing both copper (II) sulphate and copper (II) acetate. When the solution containing APH, CHP and maleic acid is compared to that containing APH, CHP, saccharin and copper (II) acetate, a slightly faster rate of decomposition of the APH occurs in the presence of saccharin. Again this trend is to be seen throughout the solutions containing copper (II) sulphate. The HPLC retention time and the UV spectrum obtained for the APH decomposition product, both in the presence and absence of any copper (II) salts, is the same.

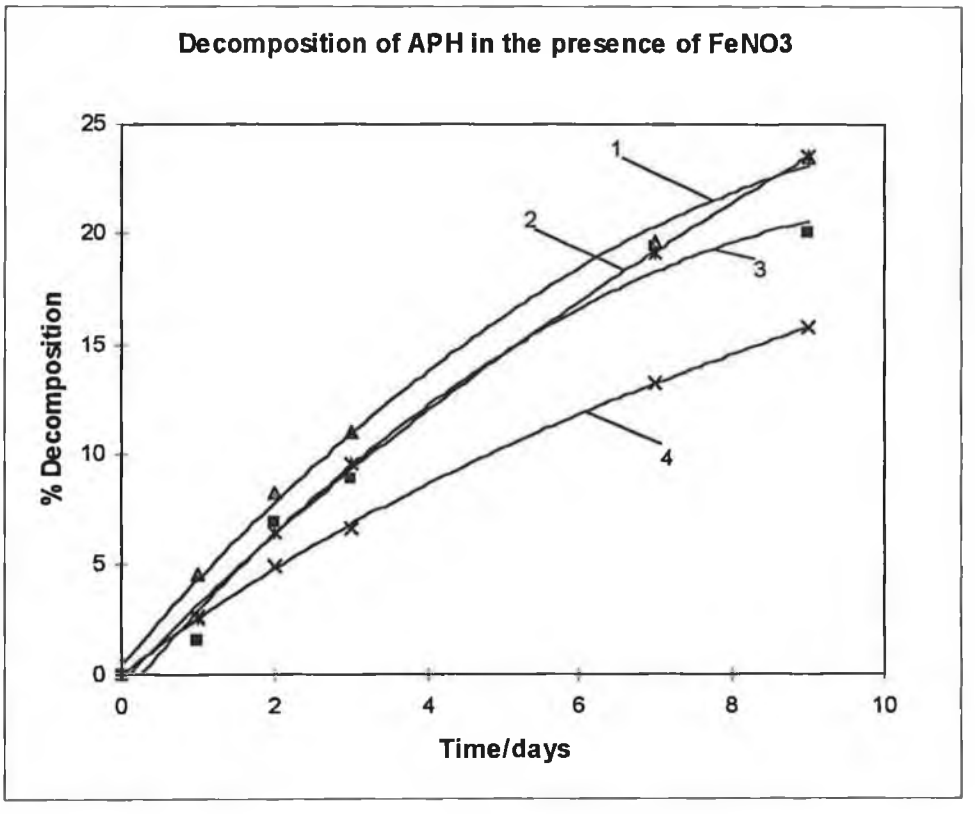

Figure 2.6. : Graph showing the rate of decomposition of APH in the presence of Iron (III) nitrate, CHP and acid components. Data obtained from HPLC runs, mobile phase of 40 % acetonitrile and 60 % phosphate buffer, detection at 254 nm. Flow rate = 1.2 ml min^{-1}. All solutions contain APH and iron (III) nitrate, in addition to : 1 = CHP + maleic acid, 2 = CHP + maleic acid + saccharin, 3 = CHP, 4 = CHP + saccharin.

Figure 2.6. shows the different rates of decomposition of APH in the presence both acids, CHP and iron (III). The reactions were carried out in the same ratio as that of the copper reactions, i.e. APH : metal, 4 : 1. However, in contrast to the 'copper' reactions the rates of decomposition in the 'iron' reactions are much slower. Even after the 10 days the percentage decomposition was 20-25 %. In the copper (II) solutions the APH was almost fully decomposed after 8 days. In addition to this there is a difference in the effect of the different acid components on the decomposition of the APH. In the presence of iron, the slowest reaction occurred with the saccharin and CHP. This observation is due to the unique role that saccharin plays in the overall chemistry of the reducing agents. This unique behaviour has been observed

previously [5] in the reaction involving DMPT, iron (III) and saccharin. In this reaction the iron (III) was reduced by 60 % to iron (II). However when the same reaction was carried out using other acids, including benzoic, acetic and maleic acid, no reduction of iron (III) was observed. It was concluded that the influence of saccharin on the reaction was not due to its acidic character, as other acids of similar pK_a did not exert the same effect on the reaction. It was not possible to further explain the unique reactivity of saccharin. Evidence to the unique role of saccharin and its effect on the Cu(II) reduction potential has been reported previously by Raftery [5]. Further differences between saccharin and maleic acid were found by Moane et al. [6], in a study concerning the decomposition of peroxides by transition metals, in the presence of acids and various accelerators. Again it was not possible to make any general conclusions with regard to the observed differences between the two different acids.

A possible reaction mechanism has been proposed (see ref. 2). In this study it a mechanism was proposed for the reaction between APH, CHP, saccharin and copper (II). This proposed mechanism yielded a product of 1-acetyl-2, 2-diphenylhydrazine, is shown in Figure 2.7. The structural data obtained for the major product isolated as a result of the APH reaction supports the proposed mechanism. A NMR spectrum, GC trace, mass spectrum and a FTIR spectrum were obtained for the isolated product, and are presented in Figures 2.8.–2.11. Additionally an NMR spectrum for the starting product (shown in Figure 2.8.) was obtained as a comparison to that of the final product. Each peak on the NMR spectrum can be identified with a part of the proposed product structure. The GC trace clearly shows a single peak, with no detectable impurities, as shown Figure 2.10. The mass spectrum gives a molecular mass, which matches that of the proposed product together with the appropriate fragmentation peaks. Peaks of interest are also marked on the FTIR spectrum. The peaks of interest are primarily the strong peaks produced by the aromatic rings (3270 cm^{-1}), the carbonyl group (1670 cm^{-1}), and those indicating monosubstitution of an aromatic ring (746, 694 cm^{-1}).

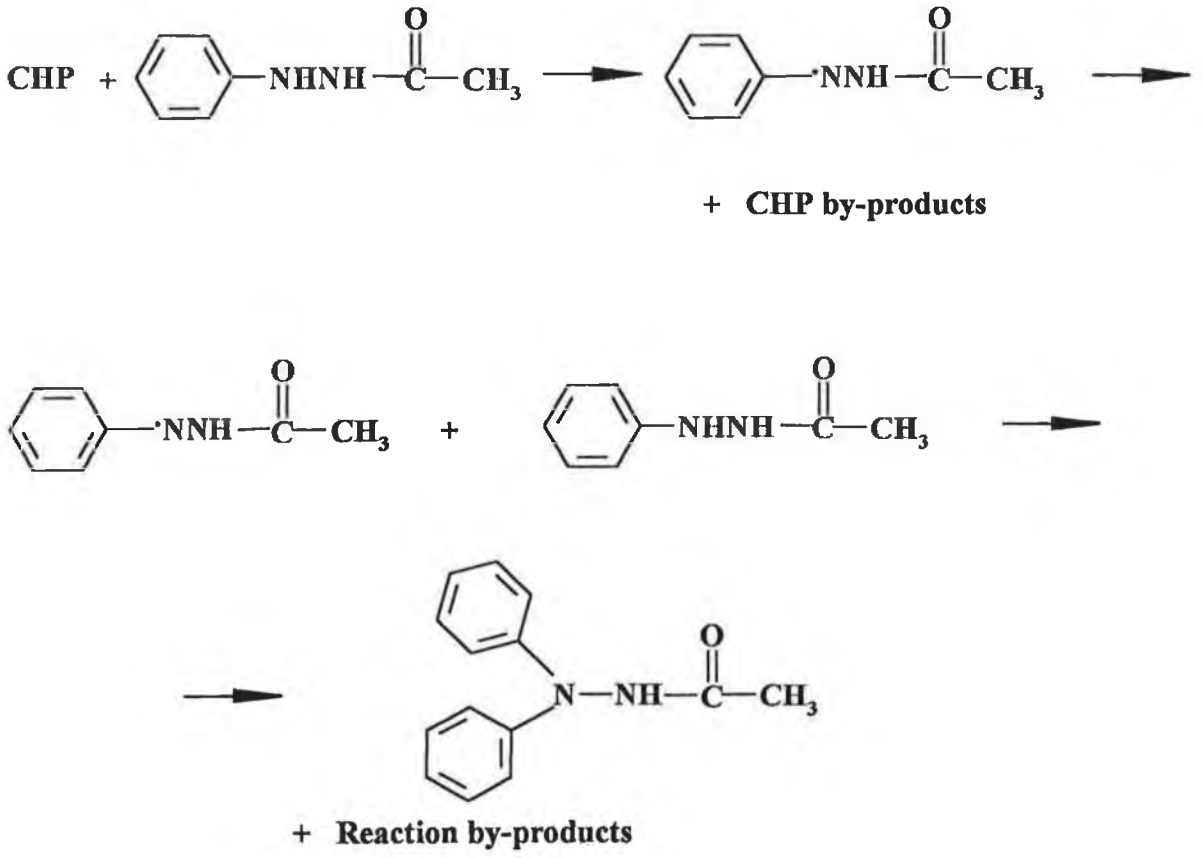

Figure 2.7. : Proposed reaction of APH with CHP. The major product is 1-acetyl-2,2-diphenyl hydrazine.

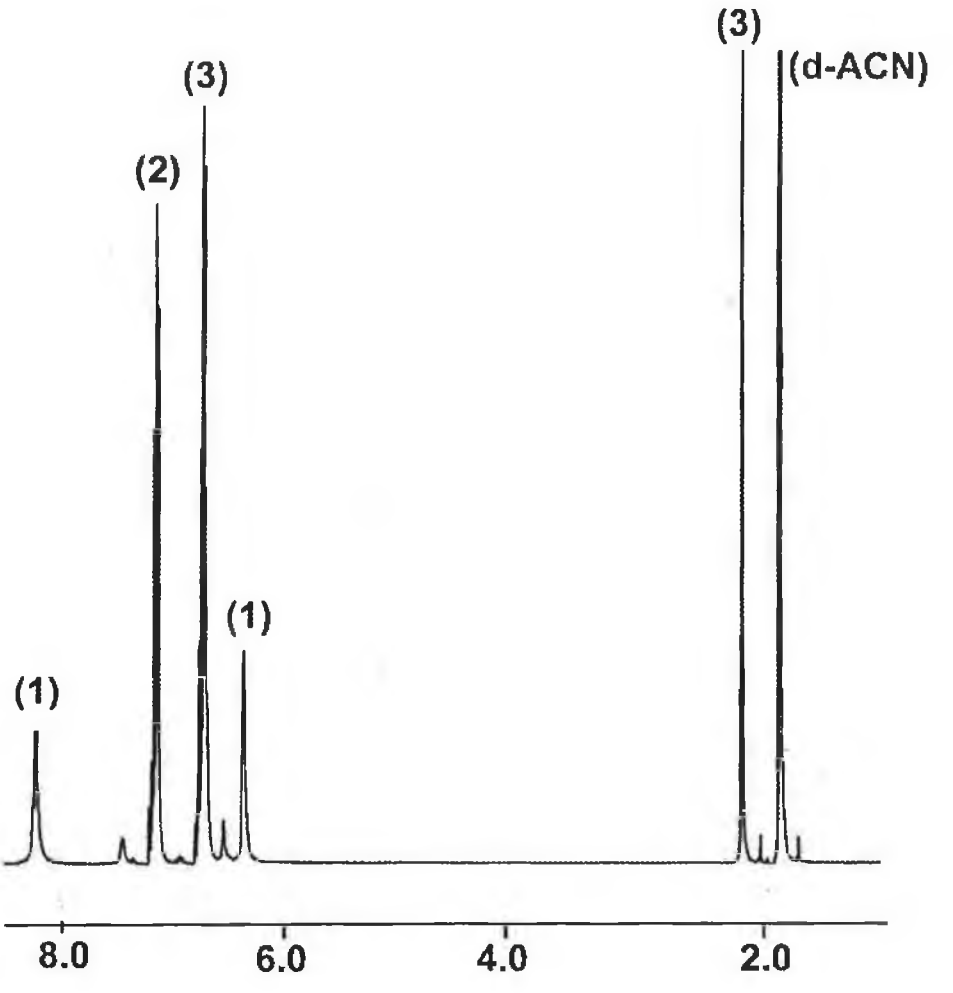

Figure 2.8. : NMR spectrum of the starting compound, APH. Indicated in parentheses is the number of protons that each peak represents. Going from left to right, the signal is produced by : amine proton, phenyl protons, phenyl protons, amine proton, and finally the methyl protons (of the acetate group).

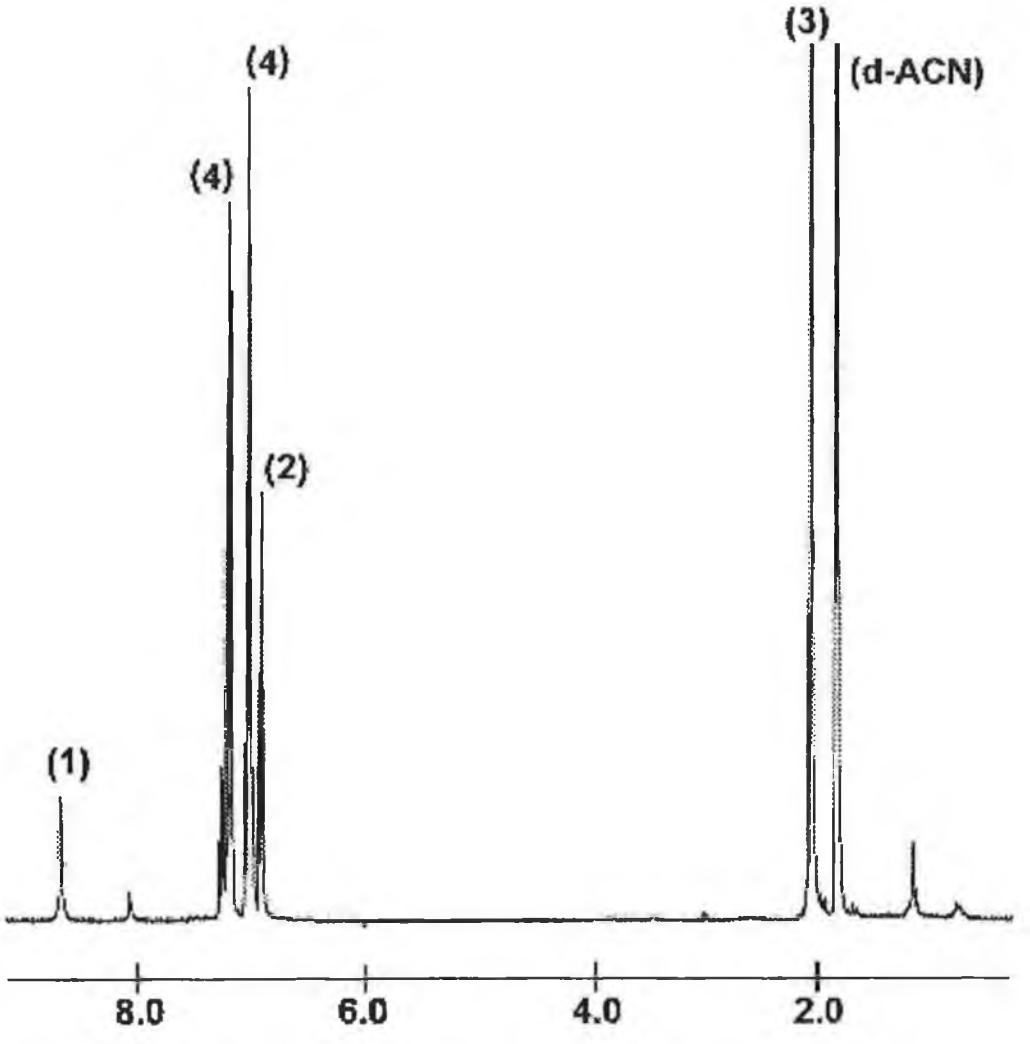

Figure 2.9. : NMR of the isolated product, 1-acetyl-2,2-diphenylhydrazine. Indicated, in parentheses, is the number of protons that each peak represents. Going from left to right, the signal is produced by : amine proton, phenyl protons, phenyl protons, phenyl protons and finally the methyl protons (of the acetate group).

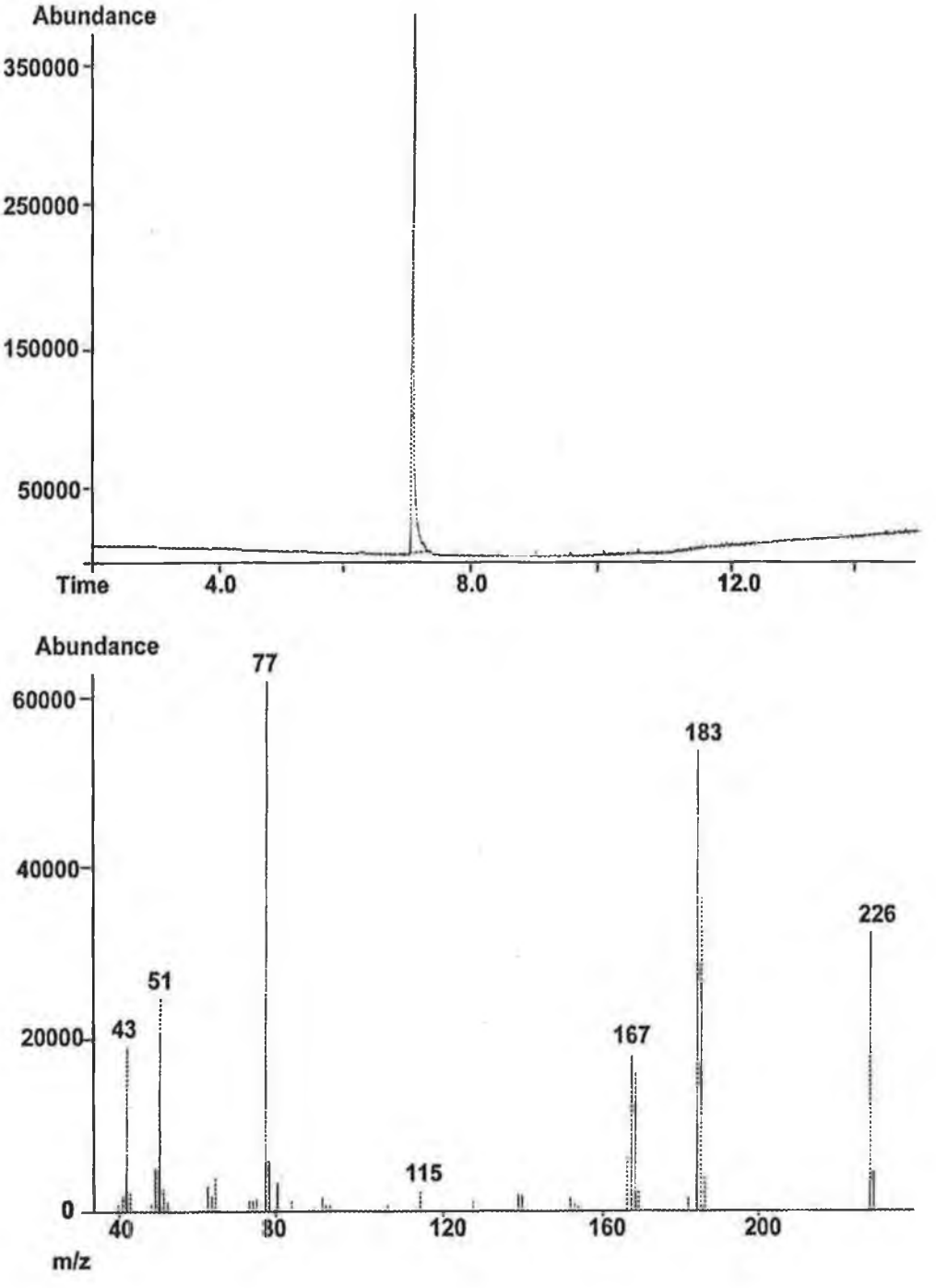

Figure 2.10. : The GC-MS results for the major APH decomposition product. The GC trace show a single peak produced by the isolated product. The mass spectrum shows the fragmentation product obtained for the product.

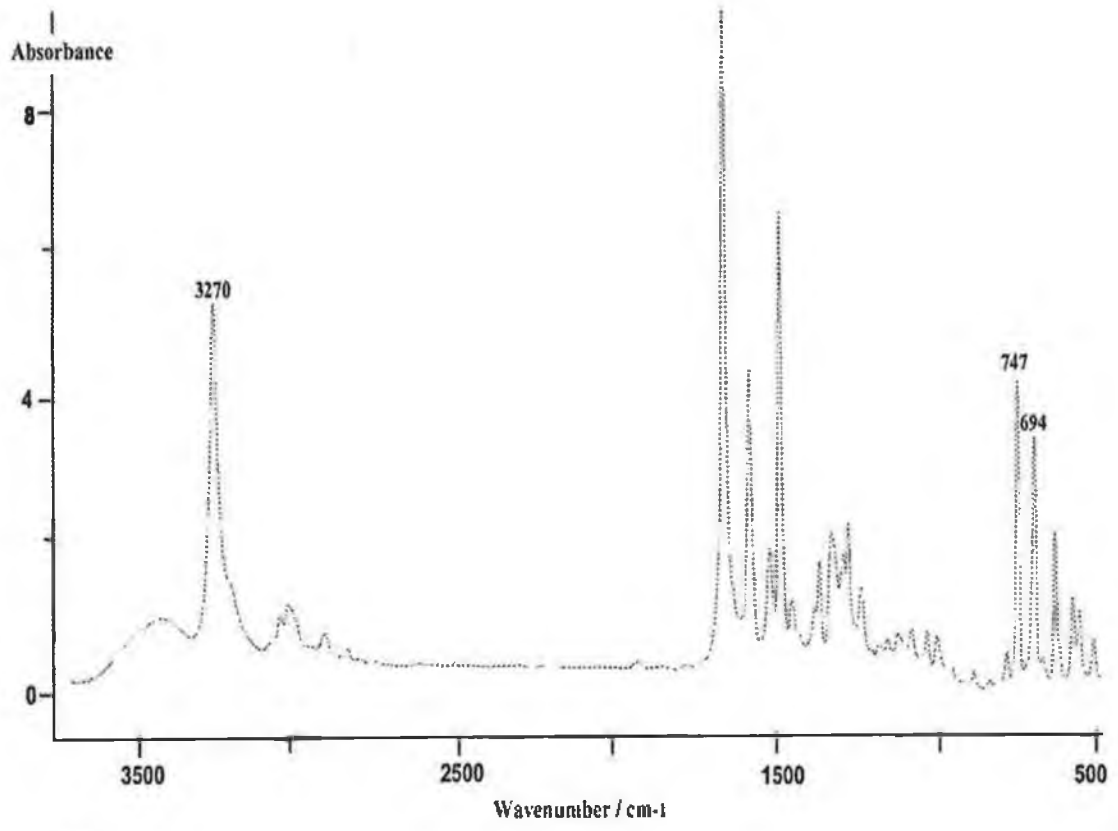

Figure 2.11. : The FTIR spectrum of the APH decomposition product.

2.4.3. DMPT Chemistry

Solutions containing various ratios of DMPT, maleic acid, saccharin and CHP were run under the conditions, as outlined in the Experimental section (2.3.)

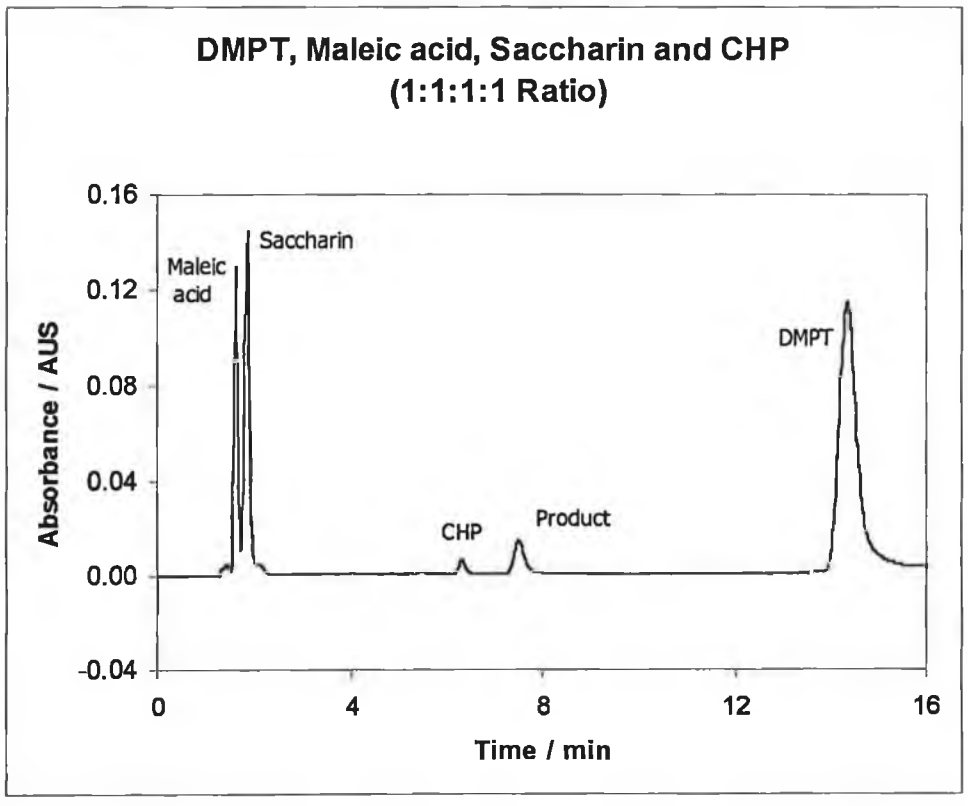

Figure 2.12.: Chromatogram of the reaction mixture of DMPT, saccharin, maleic acid and CHP. Also indicated on the trace is the major product formed as a result of the decomposition of the DMPT. Data obtained from HPLC runs, mobile phase of 40 % acetonitrile and 60 % phosphate buffer, detection at 254 nm. Flow rate 1.20 ml min^{-1}.

Solutions containing DMPT and either saccharin and/or maleic acid, showed no decomposition of the toluidine. However, decomposition of DMPT occurred once CHP was added to the solution. Although the rate of DMPT decomposition was quite low, even in the presence of CHP, (~ 10 % after 13 days) there was a noticeable difference between the solution containing DMPT and CHP, compared to the solution containing DMPT, CHP and both acid components. The percentage decomposition of the DMPT is shown graphically in Figure 2.13. The difference between the

decomposition of DMPT in the presence of saccharin compared to that in the presence of maleic acid was not as marked when compared to that seen in the APH chemistry. Similar reactions were carried out with copper (II) acetate and copper (II) sulphate added to the solutions. A similar trend was also seen whereby the fastest rate of decomposition of DMPT occurred where no acid components were present in solution.

In the reactions carried out in the absence of copper (II), there was a slight difference between the solutions containing an acid and those without any acids. The DMPT, maleic acid and CHP solution showed a slightly greater rate of decomposition of DMPT when compared to the DMPT, saccharin, CHP solution. Although the difference is small, it is discernible. When the reactions were carried out in the presence of copper (II), the same trend was seen, in the both copper (II) sulphate and copper (II) acetate solutions, Figures 2.14.-2.15. However, in both cases the difference between the saccharin and maleic acid solutions was more noticeable, again the rate of decomposition of the DMPT being greater in the solutions containing the maleic acid.

Similar type of solutions were prepared with DMPT in the presence of saccharin and/or CHP, (without any copper), except this time the solutions were prepared using higher ratios of the acid to that of the DMPT (3 : 1 ratio of acid to toluidine). Practically no decomposition of the toluidine was observed.

Different results were obtained when iron (III) nitrate was added to the DMPT solutions, again in the presence of acid components and CHP. In these solutions, the greatest rate of decomposition occurred when the DMPT was present in solution with both acids and CHP (~ 35 % decomposition of the toluidine occurred after 10 days). This is in contrast to the solutions with copper (II) where the greatest decomposition was seen to occur between the DMPT and CHP only (~80 % decomposition after 10 days). The least decomposition of the DMPT took place when the DMPT, iron (III), CHP and saccharin were present together in the solution, (< 5 % decomposition after 10 days). This once again highlights the unique role of saccharin in the chemistry of

anaerobic adhesive preparations. These results are presented graphically in Figure 2.16.

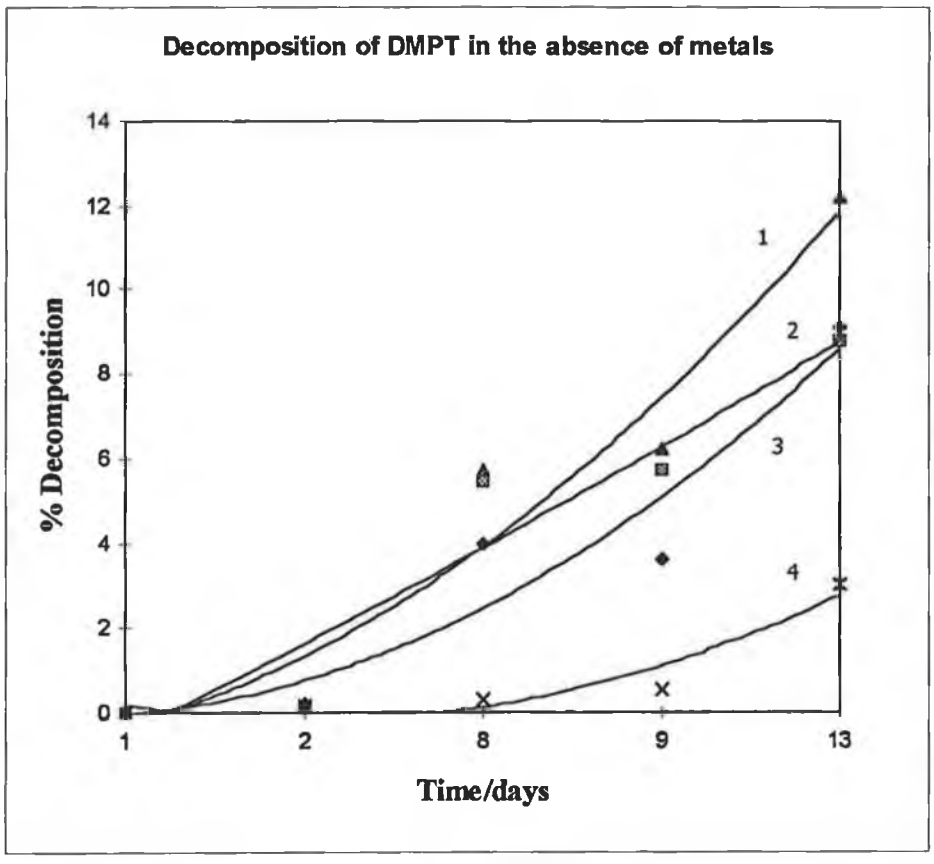

Figure 2.13. : Graph showing the rate of decomposition of DMPT in the presence of CHP and/or acid components. Data obtained from HPLC runs, mobile phase of 40 % acetonitrile and 60 % phosphate buffer, detection at 254 nm. Flow rate =1.20 ml min^{-1}. All solutions contained DMPT in addition to : 1= CHP, 2 = CHP + saccharin, 3 = CHP + maleic acid, 4 = CHP + maleic acid + saccharin.

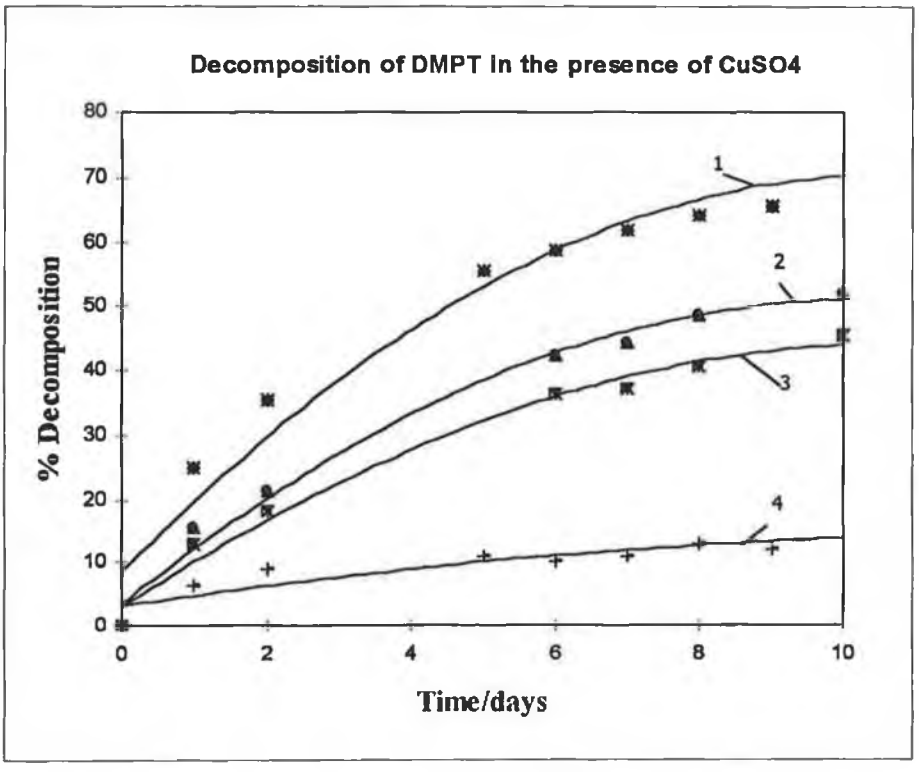

Figure 2.14. : Graph showing the rate of decomposition of DMPT in the presence of copper (II) sulphate, CHP and acid components. Data obtained from HPLC runs, mobile phase of 40 % acetonitrile and 60 % phosphate buffer, detection at 254 nm. Flow rate =1.20 ml min^{-1}. All solutions contained DMPT and copper (II) sulphate in addition to : 1= CHP, 2 = CHP + saccharin, 3 = CHP + maleic acid, 4 = CHP + maleic acid + saccharin.

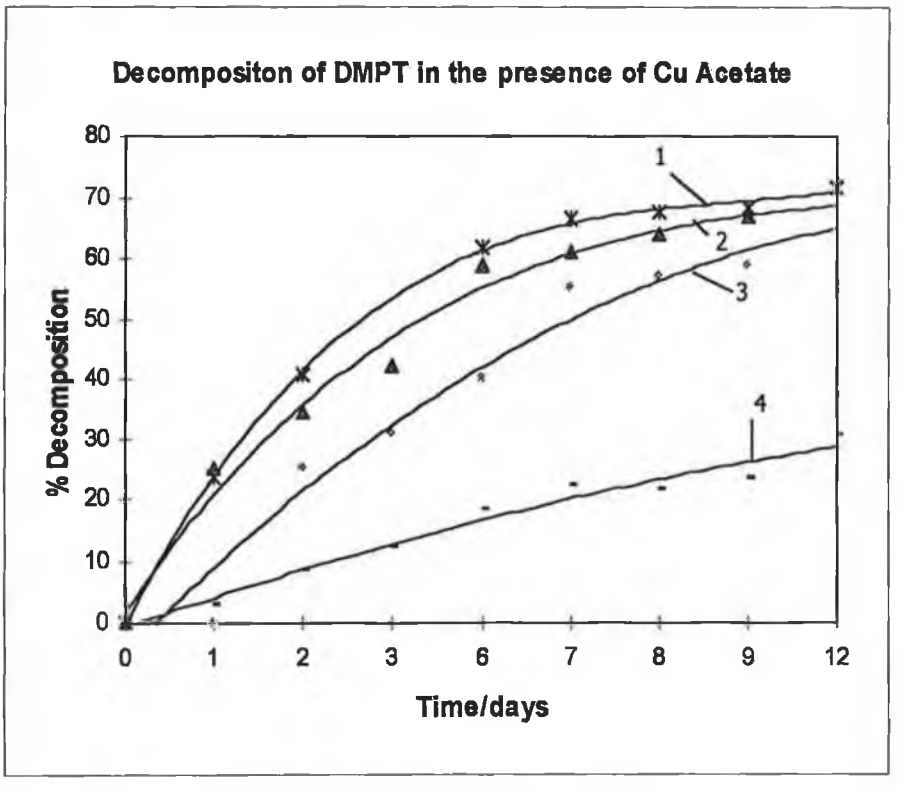

Figure 2.15. : Graph showing the rate of decomposition of DMPT in the presence of copper (II) acetate, CHP and acid components. Data obtained from HPLC runs, mobile phase of 40 % acetonitrile and 60 % phosphate buffer, detection at 254 nm. Flow rate =1.20 ml min^{-1}. All solutions contained DMPT and copper (II) acetate in addition to : 1= CHP, 2 = CHP + saccharin, 3 = CHP + maleic acid, 4 = CHP + maleic acid + saccharin.

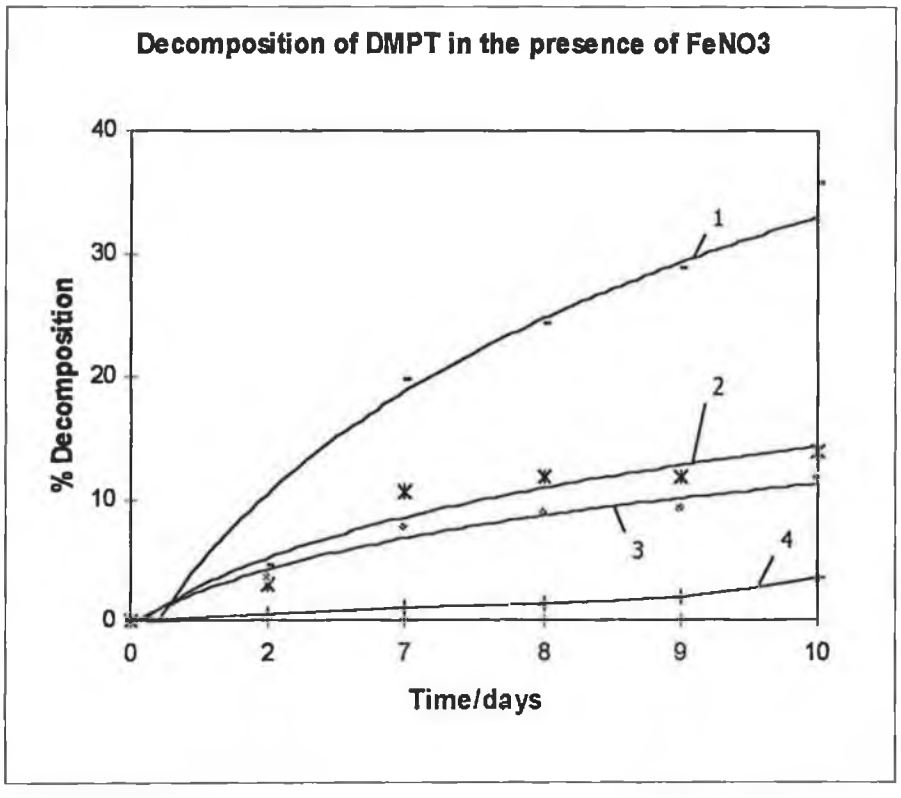

Figure 2.16. : Graph showing the rate of decomposition of DMPT in the presence of iron (III) nitrate, CHP and acid components. Data obtained from HPLC runs, mobile phase of 40 % acetonitrile and 60 % phosphate buffer, detection at 254 nm. Flow rate =1.20 ml min^{-1}. All solutions contained DMPT and iron (III) nitrate in addition to : 1 = CHP + saccharin, 2= CHP 3 = CHP + maleic acid, 4 = CHP + maleic acid + saccharin.

2.4.3.1. Identification of the DMPT reaction product

The reactions that the DMPT molecule can undergo have been proposed by Humpreys [7]. Three different reactions have been proposed, all of which involve oxidation of the amine : oxidative demethylation, formamide formation and oxazolidine formation. These reactions steps are shown in Figures 2.17.-2.19. The major reaction product that was isolated from the preparative TLC plates was subjected to ^{1}H NMR analysis, GC-MS and FTIR.

The proton NMR, (Figure 2.20.) produced peaks with chemical shifts that were assigned to a para-substituted aromatic ring, ~7.2 ppm, an aldehyde proton, ~8 ppm, and finally the peaks at ~3.6 and ~2.6 ppm were produced by the methyl groups. The integration of the peaks also supported the above assigned proton groups. Further evidence of the presence of the carbonyl group is the characteristic peak, found in the FTIR spectrum at 1680 cm^{-1}, (Figure 2.21). Evidence of the para-substitution is shown by the peak at 820 cm^{-1}. From the NMR data, a structure was assigned to the reaction product. The structure assigned is consistent with N-methyl, N-phenyl formamide, the product of the formamide formation reaction, Figure 2.19.

Other supporting evidence can be found in the GC-MS results and the UV spectrum for the compound. The mass spectrum, (Figure 2.22.), gives a molecular mass of 149 g, and this again supports the proposed structure of the formamide reaction product. In the GC chromatogram, (Figure 2.22.), two smaller peaks are seen to elute before the main peak. These peaks are assigned to trace amounts of mono-methyl-*p*-toluidine and di-methyl-*p*-toluidine, on the basis of the mass spectrometry results.

The photo diode array spectrum, (Figure 2.23.), shows maximum peak absorbances at ~214 nm and ~251 nm. Both wavelengths are consistent with the aldehyde group and the aromatic group, respectively.

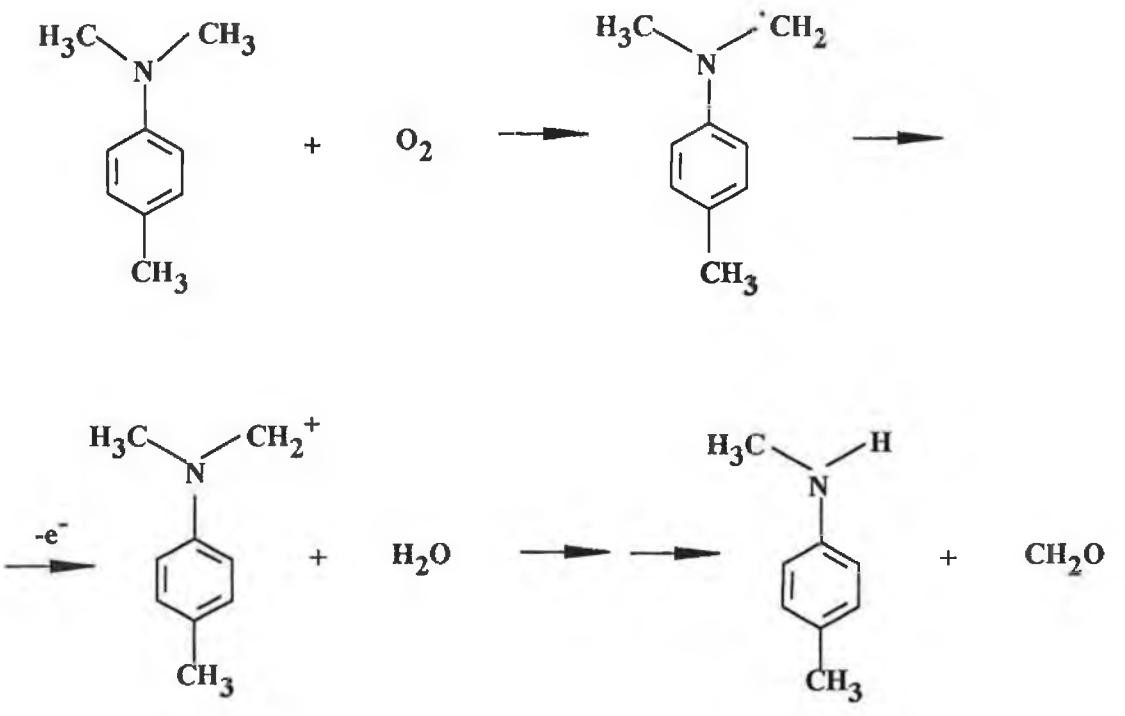

Figure 2.17. : One of the possible reaction pathways that a substituted aromatic amine may undergo. This reaction is an oxidative demethylation.

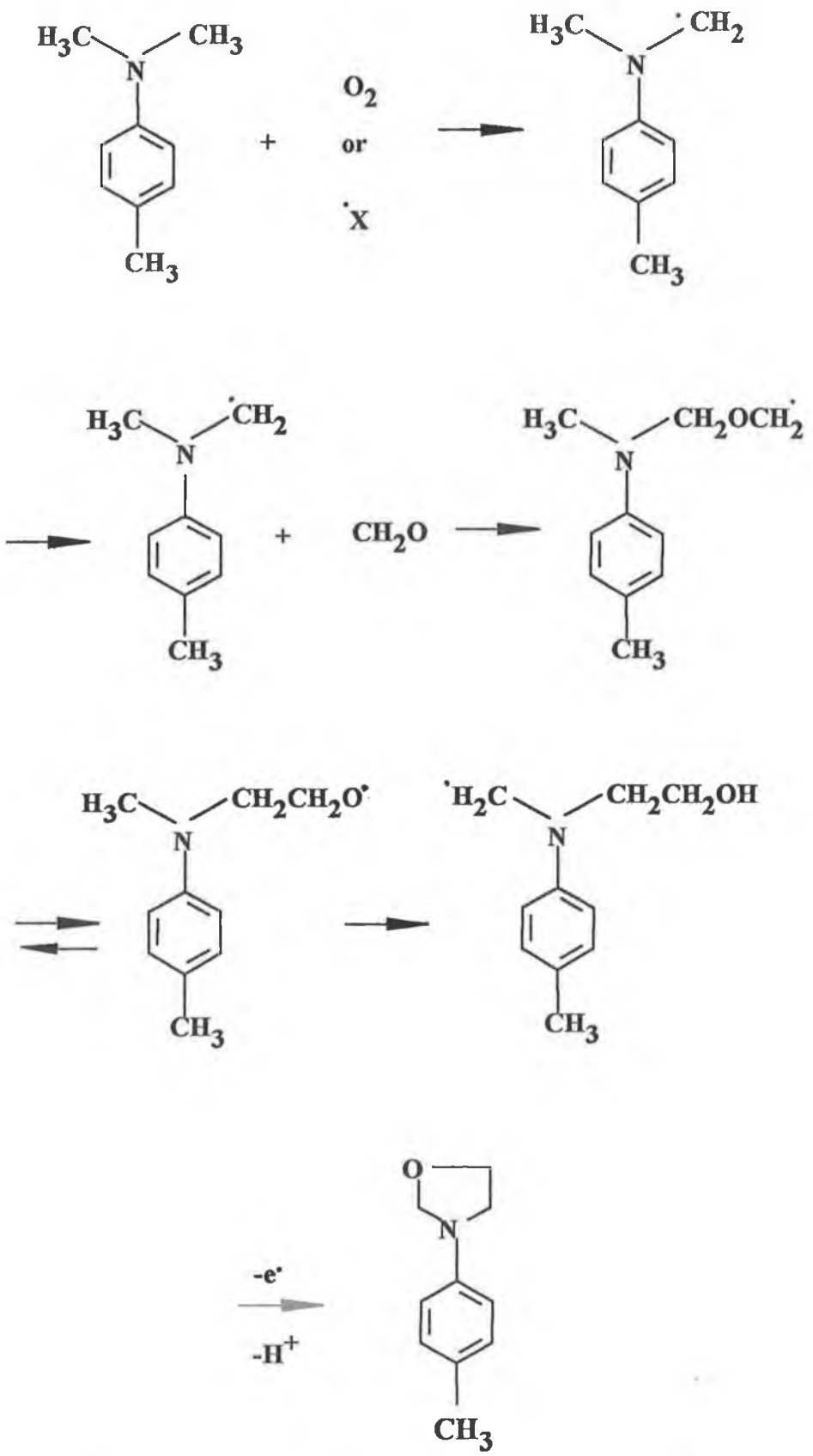

Figure 2.18. : Another of the possible reaction pathways that a substituted aromatic amine may undergo. This is an oxazolidine formation reaction.

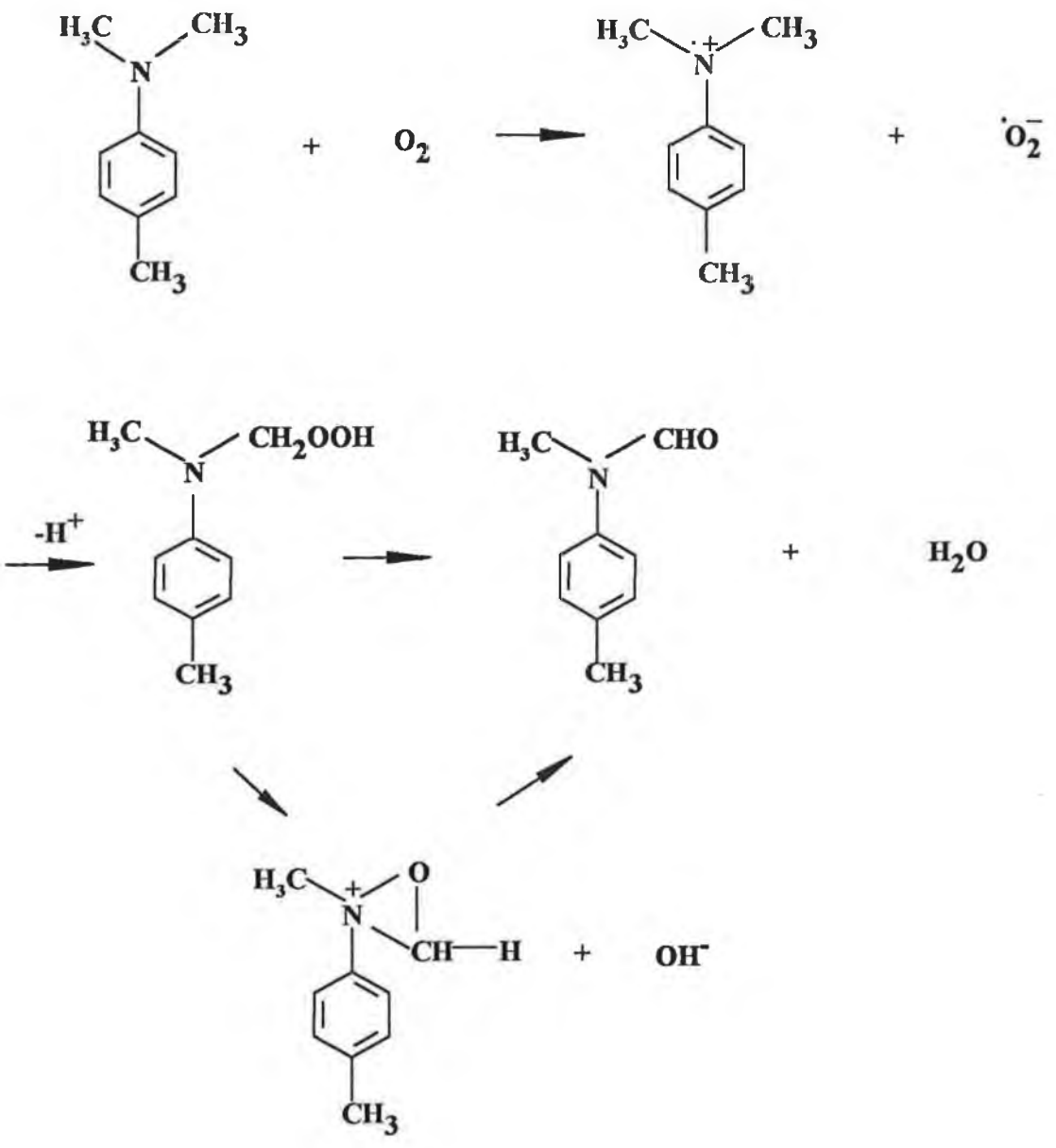

Figure 2.19. : Proposed reaction pathway that the substituted aromatic amine would have taken. This is known as a formamide formation reaction.

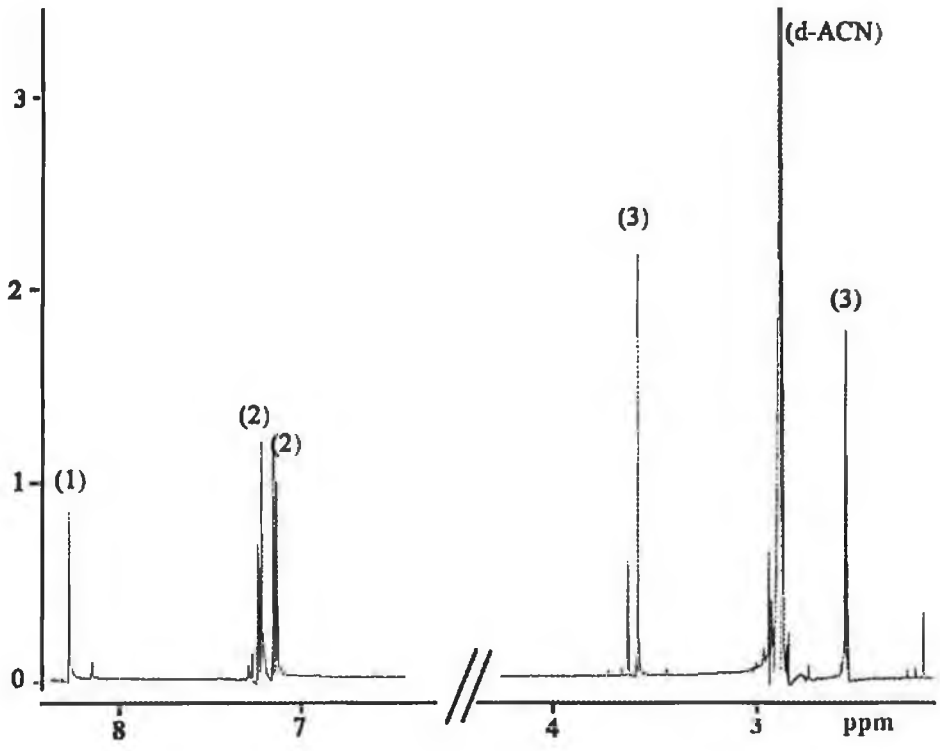

Figure 2.20. : The nmr spectrum obtained for the DMPT reaction product, showing peaks at chemical shifts consistent with the various substituents of the proposed reaction product. The integration values are given in the parentheses.

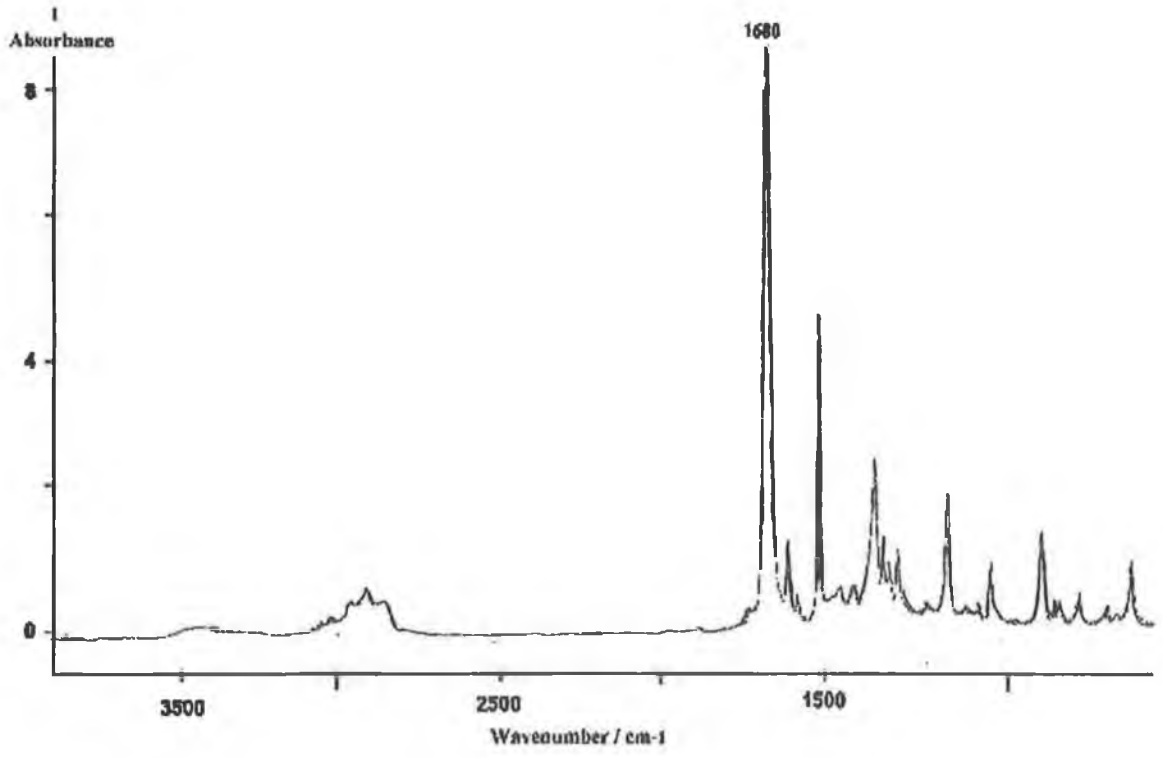

Figure 2.21. : FTIR spectrum for the DMPT reaction product. The peak at 1680 cm^{-1} is characteristic for the C=O bond.

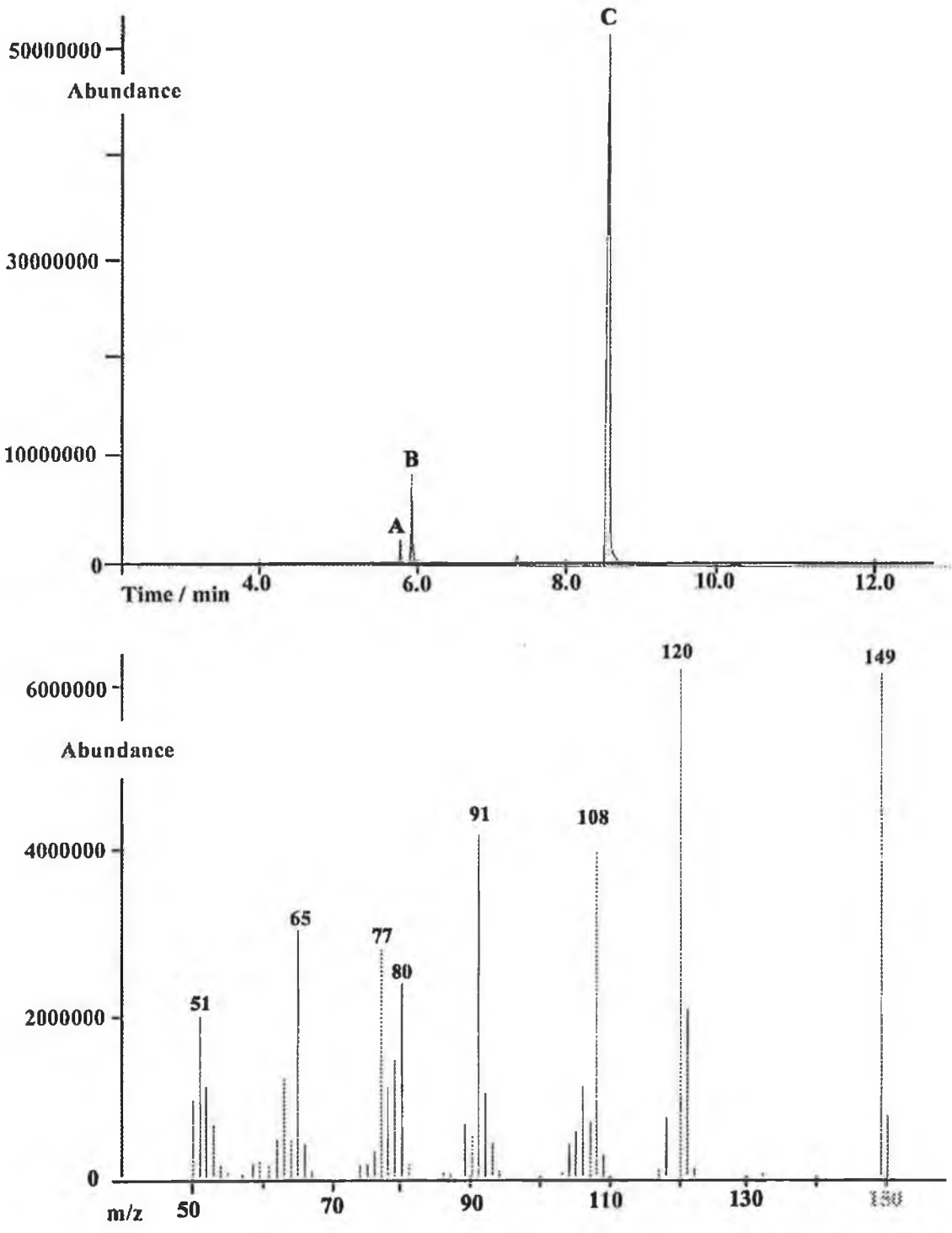

Figure 2.22. : The GC-MS traces of the isolated DMPT reaction product. The upper trace is the GC result obtained, showing the main product C and two other by products(A, B). A= mono-methyl-*p*-toluidine, B= di-methyl-*p*-toluidine and C= N-methyl, N-phenyl formamide

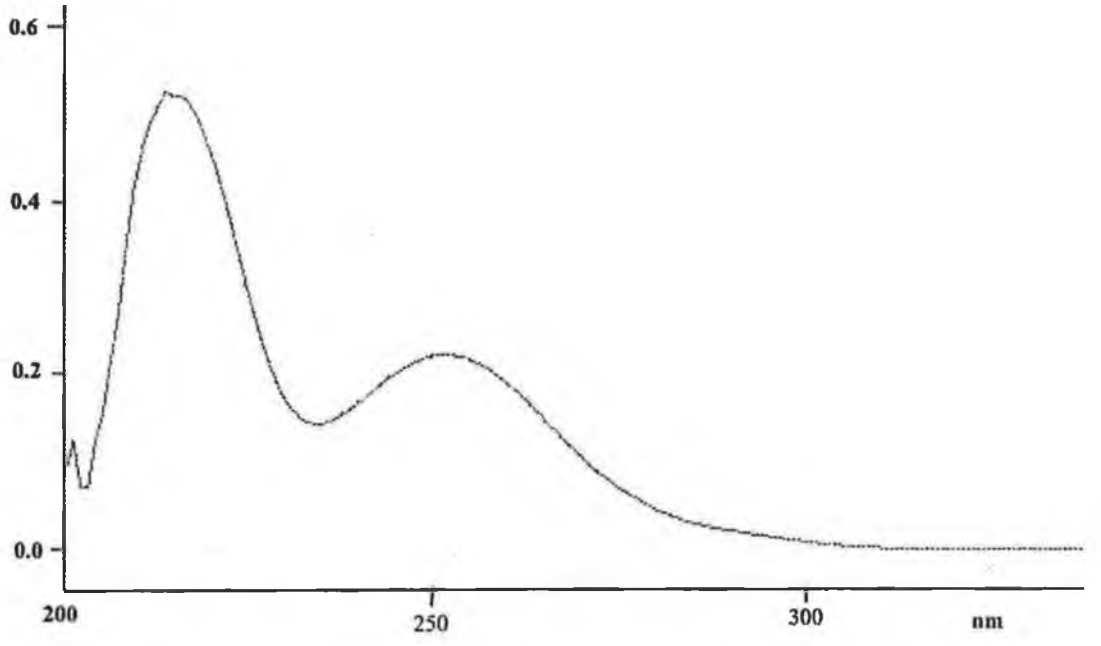

Figure 2.23. : The UV spectrum of the DMPT product shows two peak maxima, at ~214 nm and 251 nm, which are associated with the aldehyde and aromatic groups, respectively. The UV scan was obtained using the UV photodiode array HPLC detector.

2.4.4. THQ Chemistry

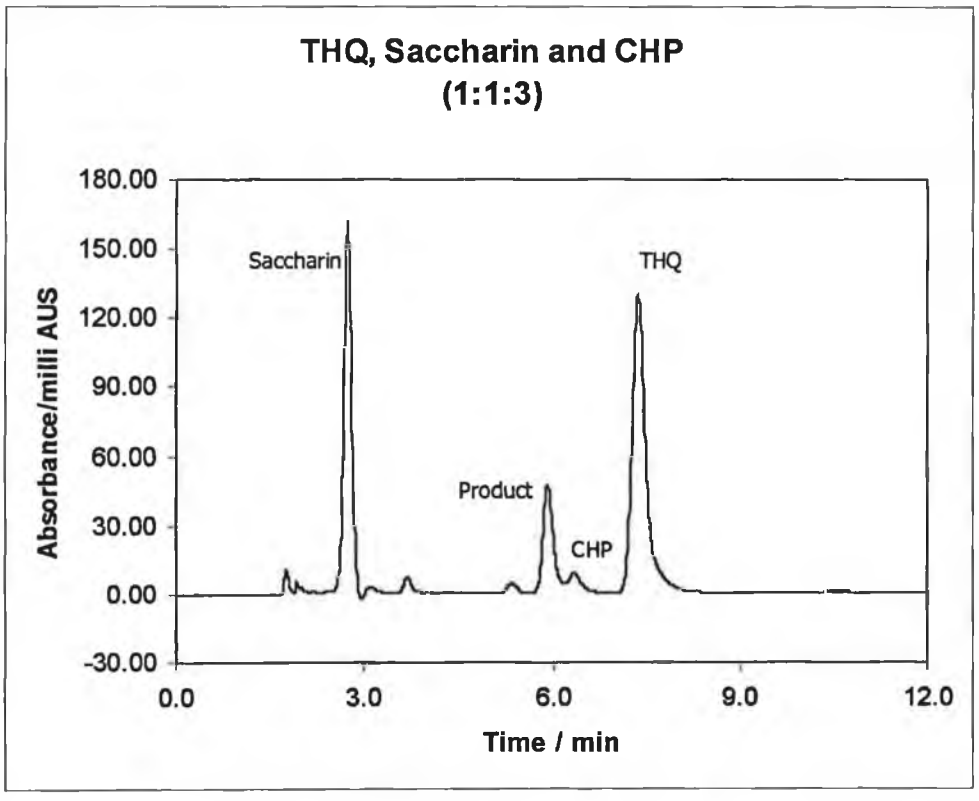

Figure 2.24. : Chromatogram of the separation obtained for the THQ mixture.

Figure 2.24. shows the typical trace obtained for the THQ mixture, using the same mobile phase developed for the separation of the APH-containing solutions. As with the above reducing agents, THQ was exposed to CHP and the acids dissolved in acetonitrile. After twelve days the THQ was seen to decompose slightly (by less than 15 %). There was very little difference in the decomposition rates between the different solutions, containing CHP only and CHP with the acids (without any metals present), as shown in Figure 2.25. In the absence of any CHP the THQ was stable. Once copper (II) salts were added to the solutions, there was a dramatic increase in the rate of THQ decomposition - see Figures 2.26.-2.27. However, once again there was very little difference in the rates of decomposition of the THQ in the various solutions.

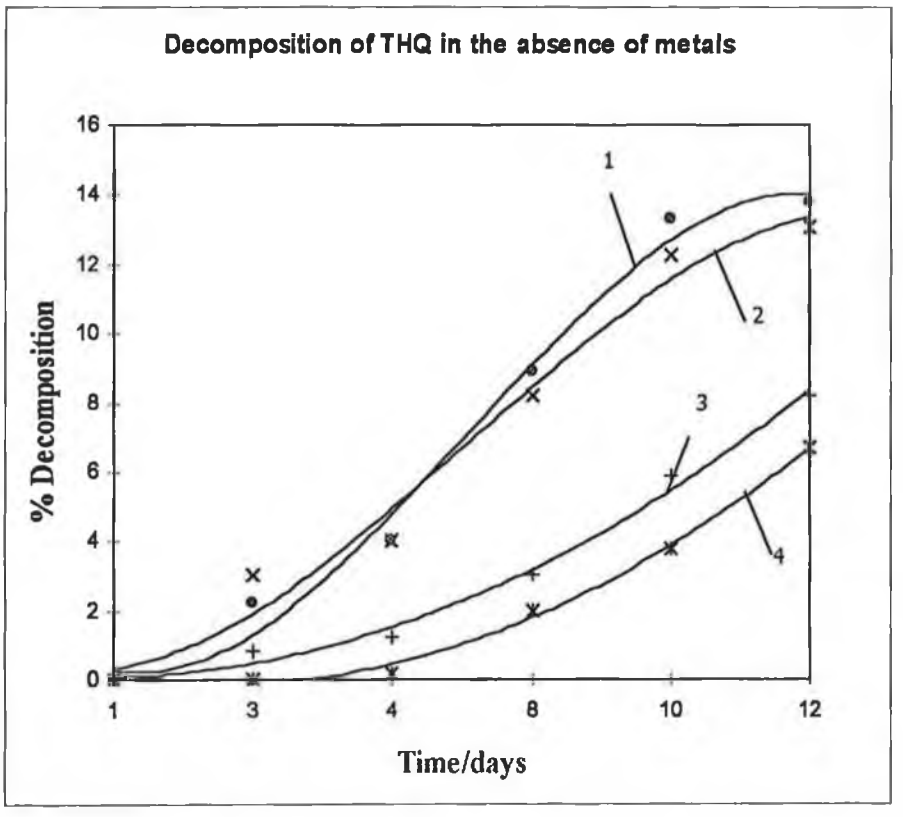

Figure 2.25. : The different rates of decomposition of THQ in the presence of CHP, saccharin and maleic acid but without any metals presence. Data obtained from HPLC runs, mobile phase of 40 % acetonitrile and 60 % phosphate buffer, detection at 254 nm. Flow rate = 1.2 ml min^{-1}. All solutions contained THQ in addition to : 1 = CHP + saccharin, 2 = CHP, 3 = CHP + maleic acid + saccharin, 4 = CHP + maleic acid.

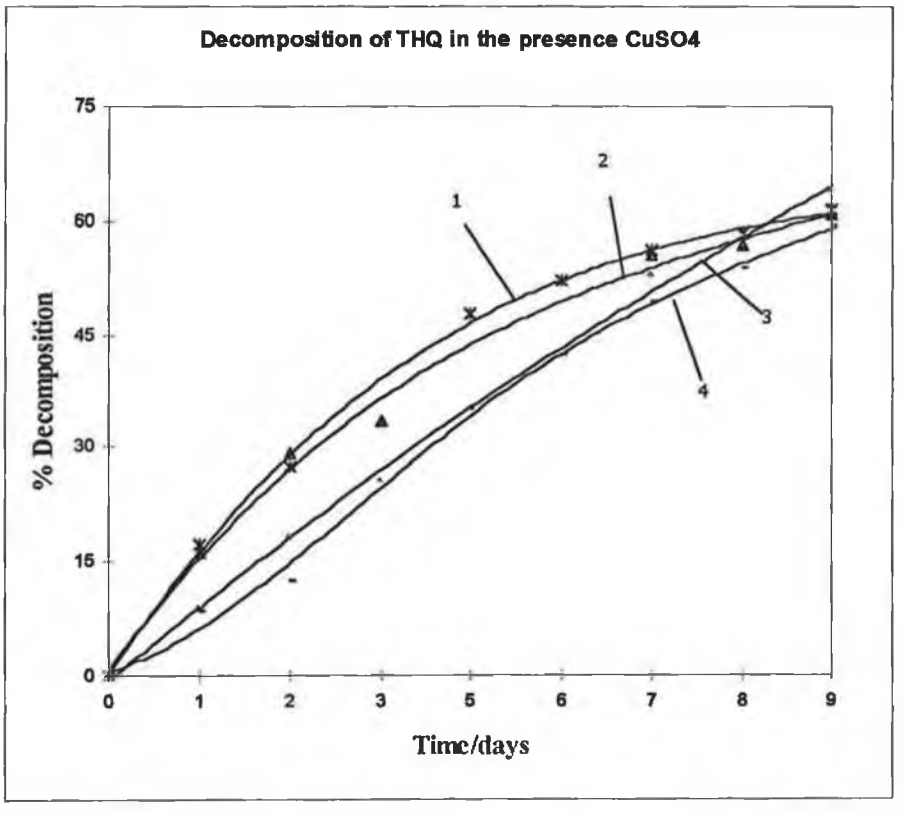

Figure 2.26. : The different rates of decomposition of THQ in the presence of CHP, saccharin and maleic acid in the presence of copper (II) sulphate. Data obtained from HPLC runs, mobile phase of 40 % acetonitrile and 60 % phosphate buffer, detection at 254 nm. Flow rate = 1.2 ml min^{-1}. All solutions contained THQ and copper (II) sulphate in addition to : 1 = CHP, 2 = CHP + saccharin, 3 = CHP + maleic acid, 4 = CHP + maleic acid + saccharin.

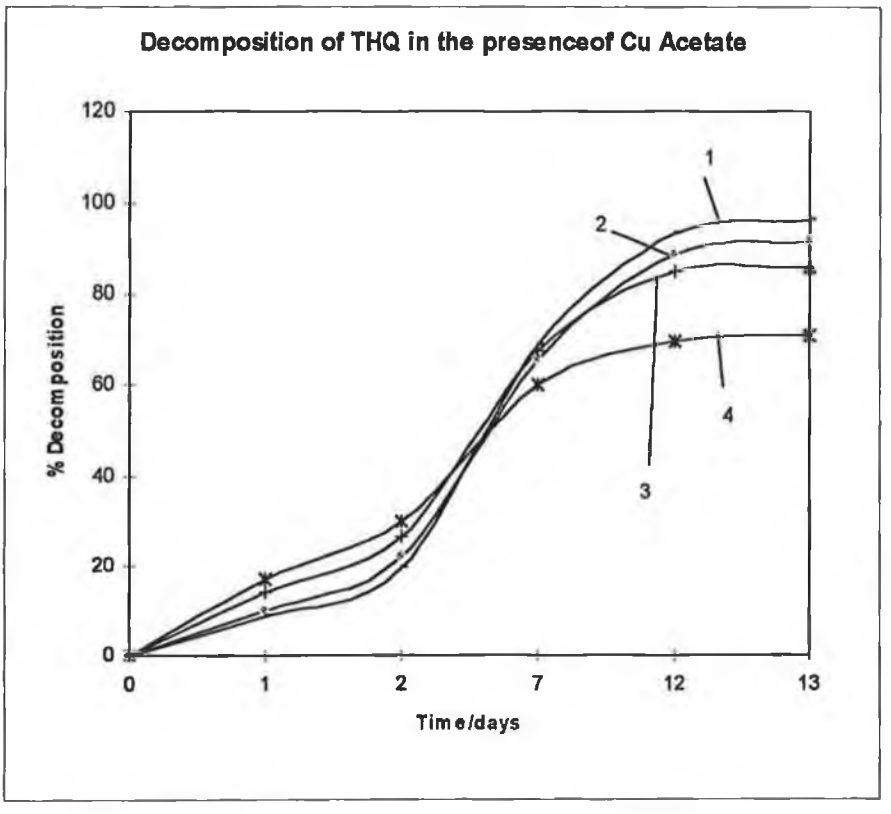

Figure 2.27. : The different rates of decomposition of THQ in the presence of CHP, saccharin and maleic acid presence of copper (II) acetate. Data obtained from HPLC runs, mobile phase of 40 % acetonitrile and 60 % phosphate buffer, detection at 254 nm. Flow rate = 1.2 ml min^{-1}. All solutions contained THQ in addition to : 1 = CHP + maleic acid + saccharin, 2 = CHP + maleic acid, 3 = CHP + saccharin, 4 = CHP.

Just as with the decomposition of APH, the effect of iron (III) on the decomposition rates is markedly different to those of copper. The THQ decomposed most rapidly in the presence of CHP, iron (III), saccharin and maleic acid, and the slowest in the presence of CHP and iron (III). There is a noticeable difference in the effect of saccharin on the decomposition of THQ and in the effect of maleic acid. Relative to the chemistry of the APH, the iron (III) has a more pronounced effect on the THQ, with up to an 80 percent reduction in the THQ concentration (compared to that of APH 20 – 25 % reduction). These results are shown graphically in Figure 2.28.

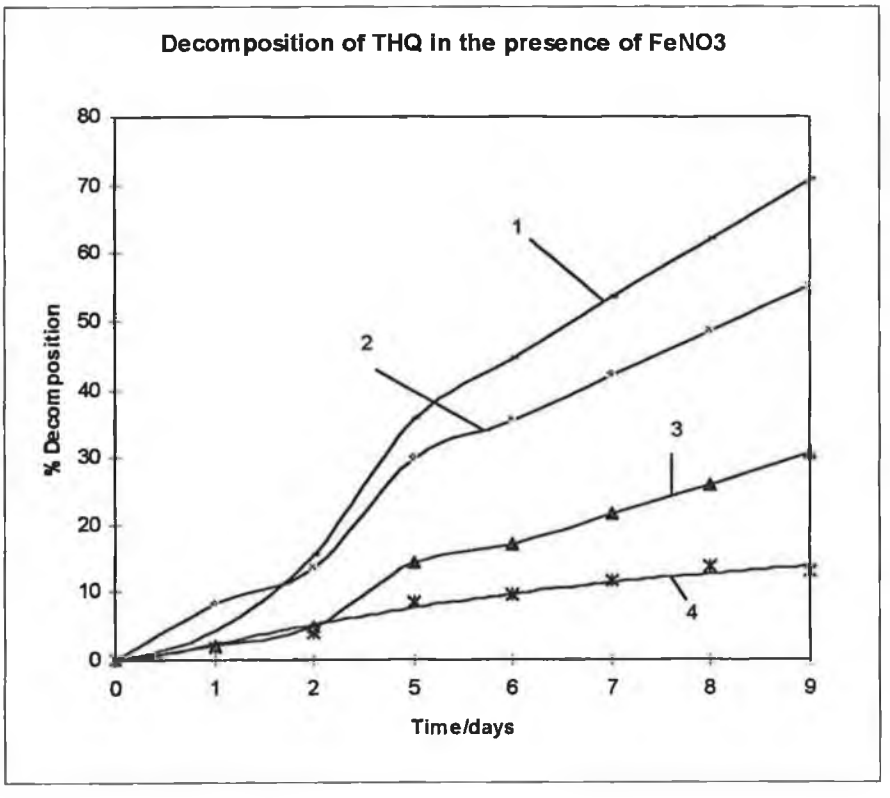

Figure 2.28. : The different rates of decomposition of THQ in the presence of CHP, saccharin and maleic acid in the presence of iron (III) nitrate. Data obtained from HPLC runs, mobile phase of 40 % acetonitrile and 60 % phosphate buffer, detection at 254 nm. Flow rate = 1.2 ml min^{-1}. All solutions contained THQ and iron (III) nitrate in addition to : 1 = CHP + maleic acid + saccharin, 2 = CHP + maleic acid, 3 = CHP + saccharin, 4 = CHP.

2.4.5. *p*-TSH Chemistry

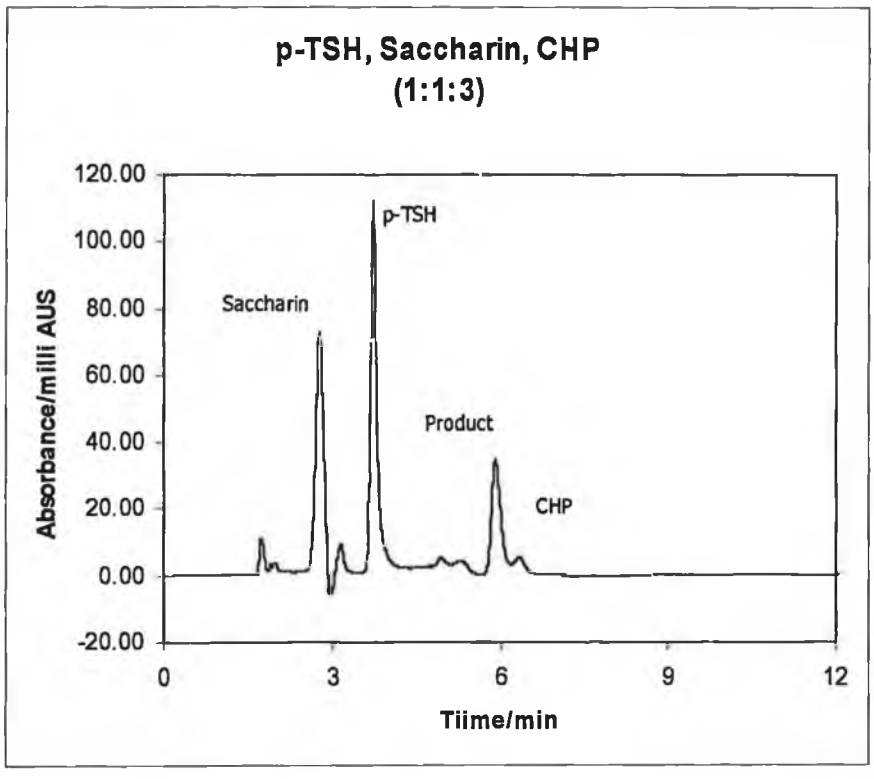

Figure 2.29. : Chromatogram showing the separation of *p*-TSH, its decomposition product, saccharin and CHP. Mobile phase composition : acetonitrile 40 %, phosphate buffer 60 %. UV detection at 254 nm. Flow rate 1.2 ml min^{-1}.

Preliminary investigations into *p*-TSH chemistry were carried out by reacting *p*-TSH with saccharin and/or CHP. Three different types of solutions were prepared, 0.2 mM *p*-TSH with CHP, (1:1), *p*-TSH, saccharin and CHP, (1:1:3) and *p*-TSH, saccharin and CHP, (1:3:3). As Figure 2.29. shows, the fastest rate of decomposition occurs in the presence of higher amounts of both saccharin and CHP. The decomposition product of *p*-TSH produced a clear solid which could be isolated and identified. The product was sparingly soluble in acetonitrile. The product formed in each of the solutions (in the presence and absence of any metal salts) had the same retention time and the same UV spectrum, when run on the HPLC. The same results were also obtained for the products when subjected to the structural elucidating tests.

A range of different analytical tests were performed on the product formed including, elemental analysis, mass spectrometry, NMR, thermal studies and IR spectroscopy. The results of the elemental tests are shown below, 2.30.

Elemental analysis results are as below :

Element	Reaction Product	p-TSH	Theoretical
	% found		%
Carbon	47.07	45.03	47.46
Hydrogen	5.02	5.35	5.08
Nitrogen	7.74	14.92	7.91
Sulphur	17.34	16.78	18.07

Figure 2.30. : Elemental analysis results for both the *p*-TSH and the related decomposition product.

The mass spectrometry fragmentation pattern is shown in Figure 2.31. Although the mass spectrum, obtained for the whole reaction product does not yield a molecular mass (it is split into two ions), the fragmentation pattern fits the proposed structure. The full range of NMR techniques were also performed on the *p*-TSH reaction product including ; proton NMR, carbon 13 , C-C correlation and H-H correlation.

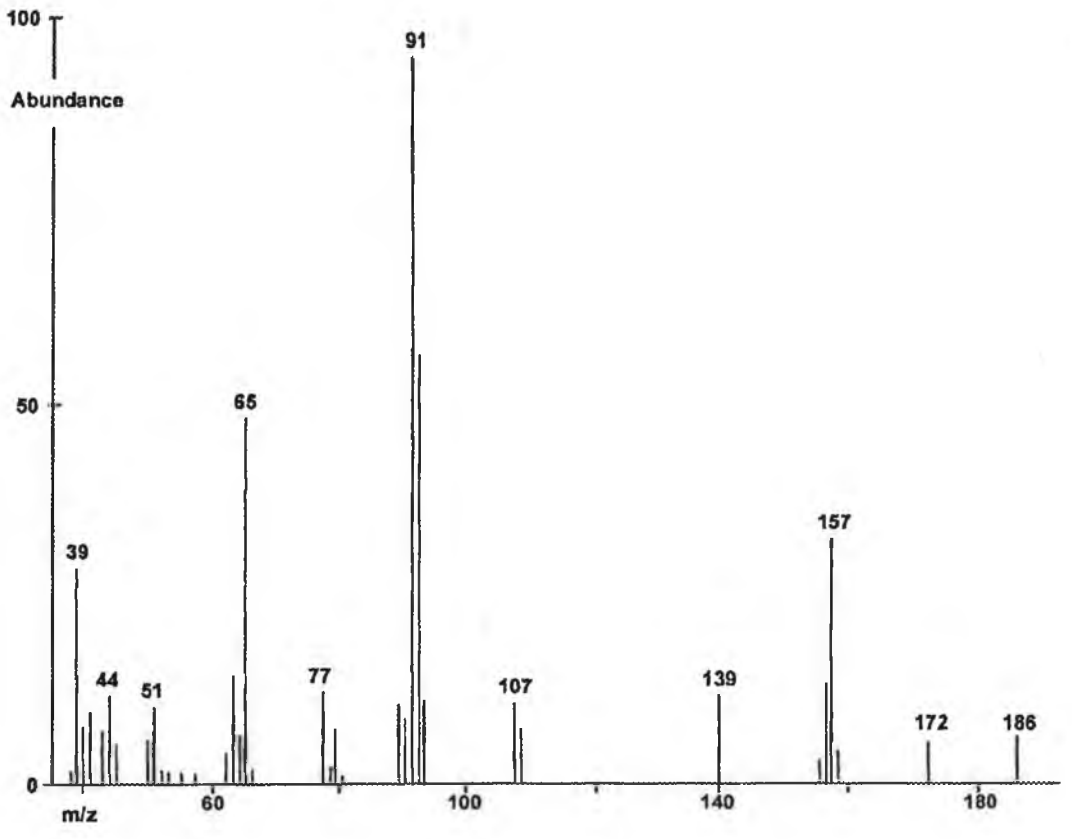

Figure 2.31. :The mass spectrum obtained for the *p*-TSH reaction product. The spectrum was produced using electrospray ionisation.

The Differential Scanning Calorimetry (DSC) studies, shown in Figures 2.32– 2.33., showed that the *p*-TSH melts at a temperature of ~190 °C . However the product (a salt) decomposes at a temperature of 178 °C. The trace obtained for the reaction product is typical for salts, which tend to decompose upon heating.

From the FTIR spectra in Figure 2.34.-2.35., several distinct peaks can be seen. The peak at ~1650 cm^{-1} in both spectra are indicative of an aromatic group, para-di-substituted. Amide 'bending' bands are found at ~1650 cm^{-1}, although any amide stretching bands are masked by the –OH band at 3500 cm^{-1}. The large OH band is due to the presence of water in the sample. In the spectrum of *p*-TSH there is a peak to be seen at 3500 cm^{-1} which is due to the NH stretching band.

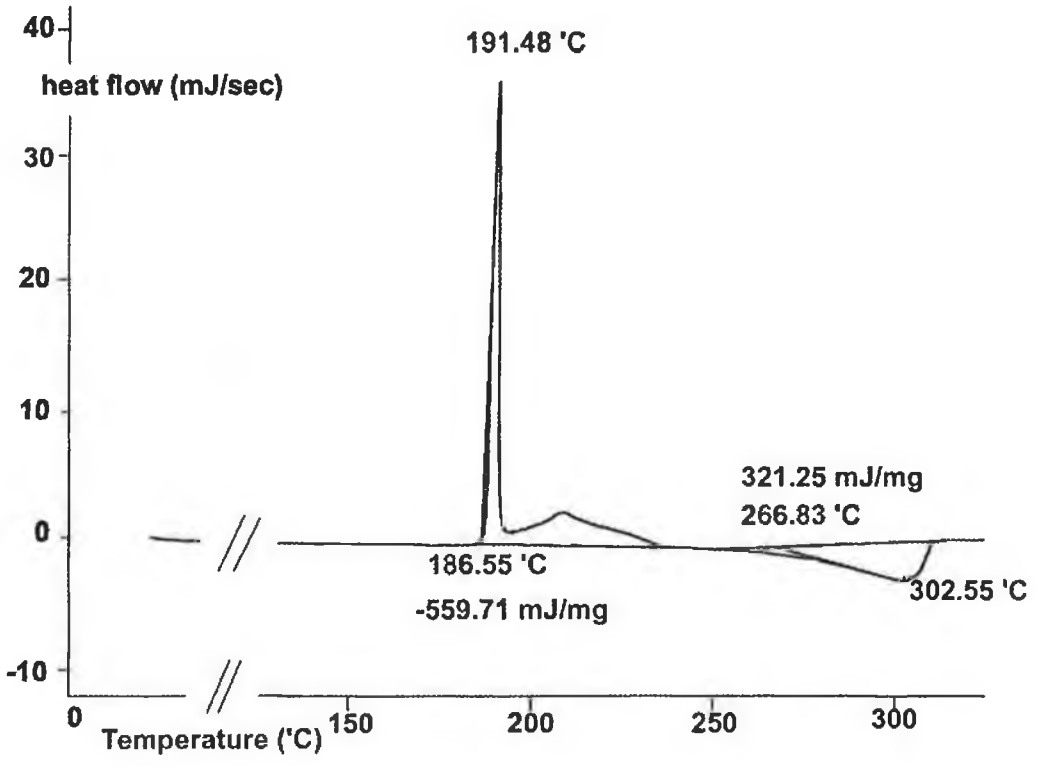

Figure 2.32. : DCS trace produced for the *p*-TSH reaction product, showing the sharp decomposition peak at 191.5 °C.

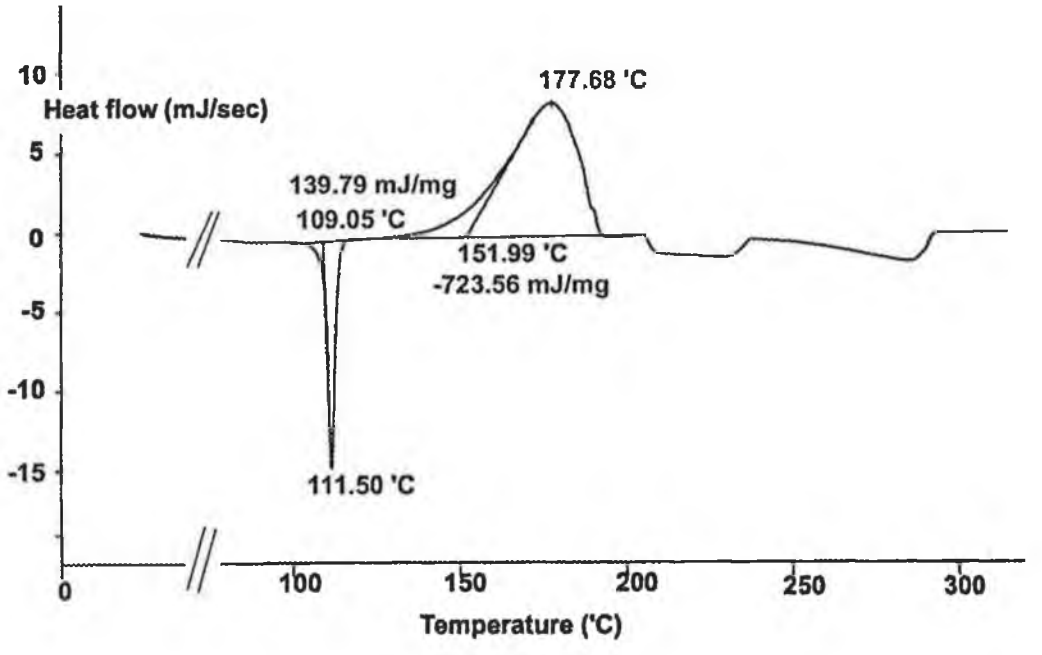

Figure 2.33. : The DSC trace obtained for TSH, showing a sharp melting point, at 111.5 °C followed by a decomposition peak at 178 °C.

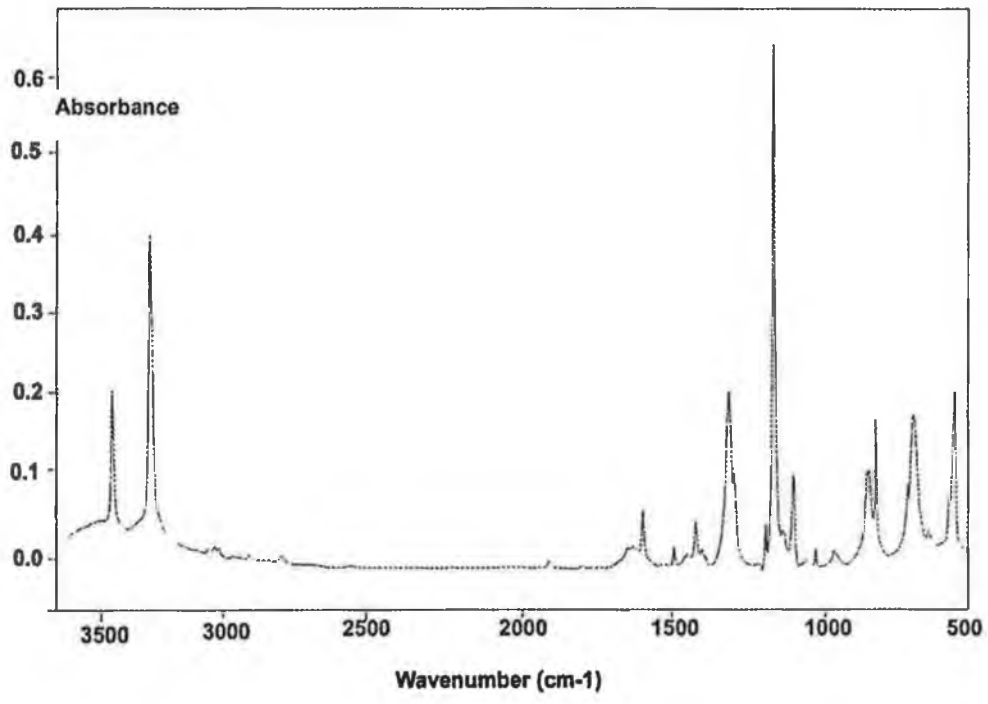

Figure 2.34 : FTIR spectrum of p-TSH.

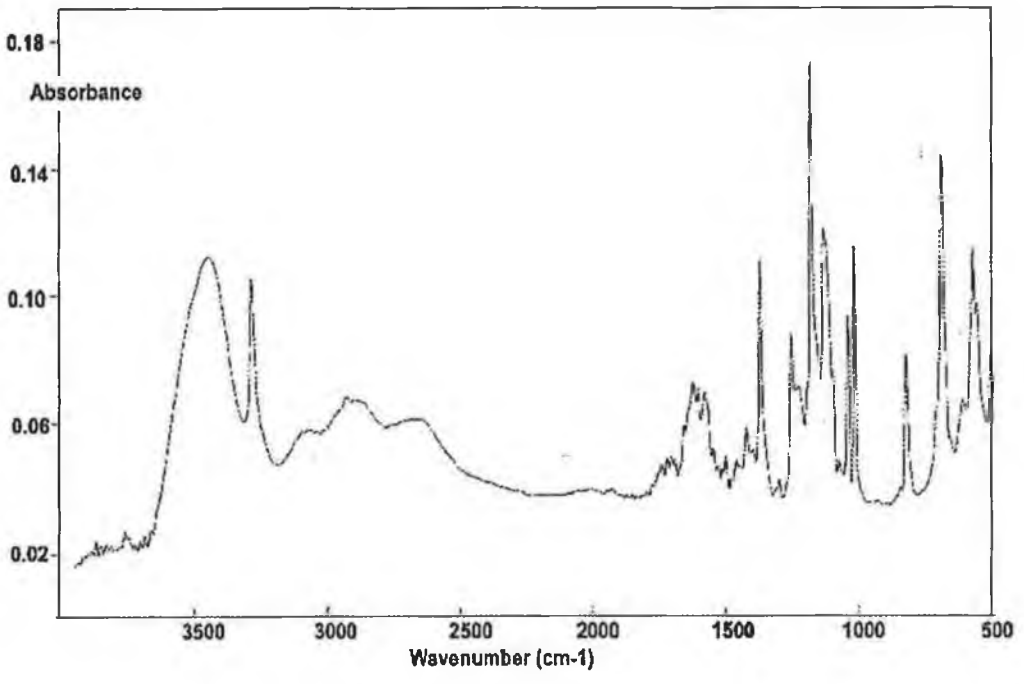

Figure 2.35 : FTIR spectrum of the *p*-TSH reaction product.

The proposed reaction mechanism is described schematically in Figure 2.36. The *p*-TSH reacts with the CHP in solution, yielding a diazene. Diazenes are typically unstable and this diazene would most likely decompose to produce toluene sulphinic acid, and simultaneously release nitrogen gas. The toluene sulphinic acid then goes on to react further with CHP, to yield toluene sulphonic acid. The toluene sulphonic acid is likely to protonate the *p*-TSH that still remains in solution, thus producing the final product, a toluene sulphonic salt. All the analytical tests performed on the product support the proposed structure.

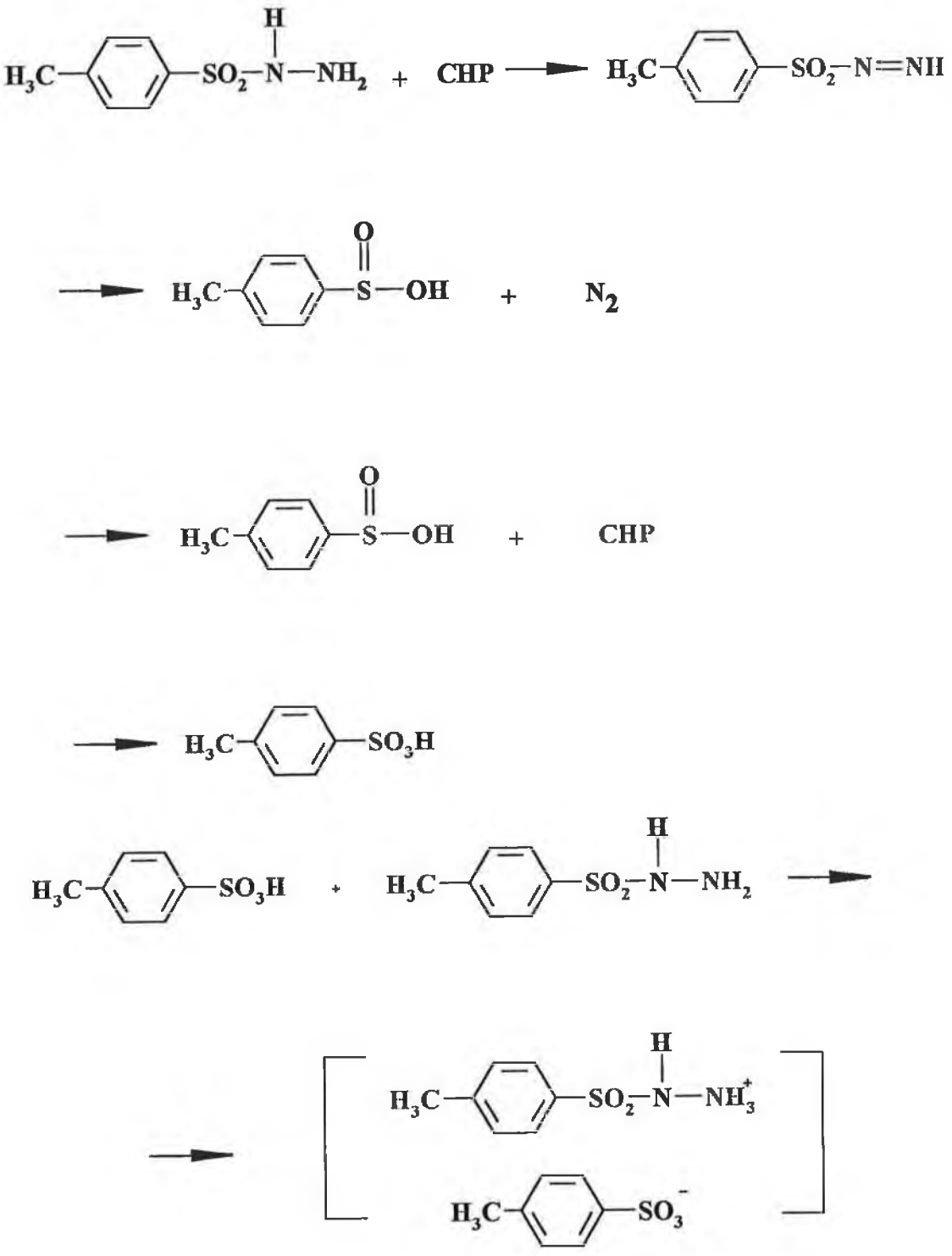

Figure 2.36. : The final product formed by the decomposition of *p*-TSH in the presence of CHP. The reaction was carried out in acetonitrile.

Further evidence, as to the production of the above compound, was obtained by reacting *p*-toluene sulphinic acid and *p*-TSH together. Each of the two salts were dissolved separately in acetonitrile and then added together. When both solutions were added together, a white product was formed which was isolated. An FTIR was obtained for the product and the spectrum produced was identical that of the *p*-TSH decomposition product.

2.4.5. DMNO Chemistry

Preliminary investigations into the decomposition of DMNO were carried out by preparing solutions of 0.75 mM DMNO, saccharin and CHP. (DMNO and CHP 1:1, DMNO, saccharin and CHP 1:1:3, DMNO, saccharin and CHP 1:3:3). Although the UV absorbance of the DMNO is relatively low, it was possible to detect the DMNO peak, as the chromatogram in Figure 2.37. shows. Higher concentrations of DMNO were required due to the lower UV absorbance of the compound. After 14 days there was no decomposition of the DMNO to be seen.

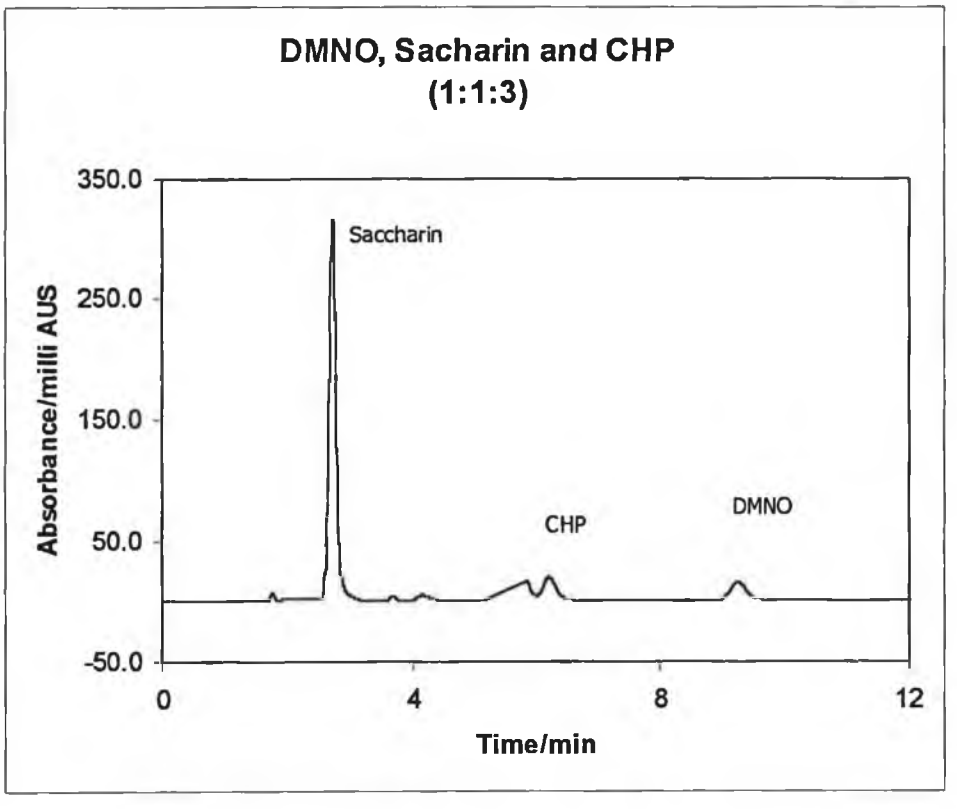

Figure 2.37. : Chromatogram of a mixture of DMNO (0.75 mM), saccharin and CHP in a 1:1:3 ratio. Separation conditions 40 % acetonitrile and 60 % phosphate buffer, pH 4.50. Detection at 254 nm. C18 reversed phase column. Flow rate : 1.20 ml min^{-1}.

2.4.6. DHP Chemistry

Preliminary investigations into the decomposition of DHP were carried out by preparing solutions of 0.75 mM DHP, saccharin and CHP. (DHP and CHP 1:1, DHP, saccharin and CHP 1:1:3, DHP, saccharin and CHP 1:3:3). Just as in the case of the DMNO, the UV absorbance of the DHP is relatively low. Again it was possible to detect the DHP, although the DHP had to be prepared in higher concentrations than in the case of APH or THQ. Figure 2.38. shows a sample chromatogram of the separation obtained for the DHP mixture. After 14 days there was no detectable decomposition of the DHP.

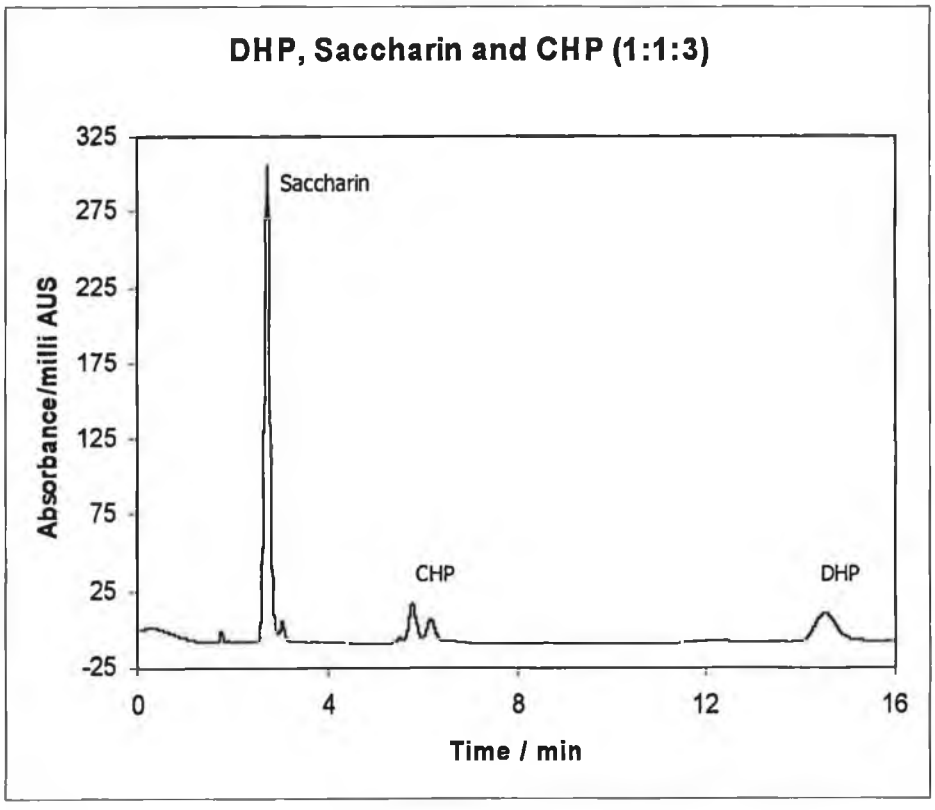

Figure 2.38. : Chromatogram of DHP (0.75 mM), saccharin and CHP (1:1:3 ratio) mixture. Separation conditions 40 % acetonitrile and 60 % phosphate buffer, pH 4.50. Detection at 254 nm. C18 reversed phase column. Flow rate : 1.20 ml min^{-1}.

2.5. DISCUSSION

In the study of the decomposition of the different reducing agents found in anaerobic adhesives, many different reactions has been found to occur. One of the observations that was made in this study was that of the apparent instability of the CHP in acidic conditions. A report by Okamoto [8] suggests that the acid may attack the oxygen of the CHP molecule, thus forming an intermediate complex which may then go on to form a cumyl cation and hydrogen peroxide, as shown in Figure 2.39.

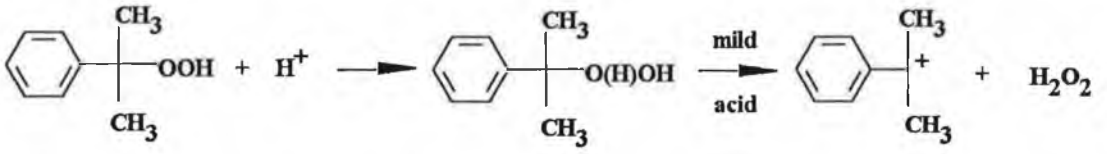

Figure 2.39. : The proposed reaction pathway of CHP in acidic conditions

The above reaction is reversible and the equilibrium can be forced towards the peroxide production under acidic conditions. This hypothesis may also support the observation that CHP decomposed in the acidic mobile phase solution when the sample solution was prepared, in contrast to a CH_3CN : H_2O sample solution.

Okamoto also studied the curing system of methyl methacrylate in the presence of APH, CHP and saccharin. It was observed that this system showed a long induction period in comparison to the same curing system using DMPT instead of APH. It was proposed that in the presence of APH another complex is formed, shown in Figure 2.40.

Figure 2.40. : Proposed reaction between APH and CHP in the presence of an acid.

The product formed is a hydroxyamine compound, which can act as a free radical inhibitor, which would explain the observed longer induction period, in this system. It is likely that the above hydroxyamine, is formed via an intermediate step, involving the formation of a APH free radical. As shown in Figure 2.7. one of the major products formed by the APH, CHP and saccharin system is 1-acetyl-2, 2-diphenylhydrazine. It is quite possible that under different reaction conditions that a different product is formed, or the hydroxyamine could be formed through a side reaction.

There is much less literature available on the chemistry of the other reducing agents studied. It is therefore hard to find any other supporting or contrasting evidence to the observations made as a result of the studies carried out on the reducing agents. Beaunez et al. [3] has done some research into the chemistry of DMPT, primarily kinetic studies, in the presence of CHP, saccharin and methyl methacrylate. Due to the difference in the reaction mixtures, it is difficult to make comparisons between their study and that carried out in this study. However, some support for the observations made with regard to the rates of decomposition can be found, and these are discussed in Section 2.4.2.

The reaction products formed in the presence and absence of any metal salts, produced the same UV spectra and had the same retention time. This was observed for each of the various components.

2.6. CONCLUSION

The development of an efficient mobile phase has allowed for the investigation into the decomposition of various reducing agents found in anaerobic adhesive formulations. The components separated include ; APH, *p*-TSH, DMPT, THQ, CHP, saccharin and maleic acid. In the case of three of the reducing agents, the main reaction product was isolated, successfully and consequently identified. Some supporting evidence as to the identity of the compounds could be found in the literature, as discussed previously. Although some reaction mechanisms have been proposed for the reactions carried out, the overall cure chemistry is undoubtedly more complex. The various observations that have been made throughout the course of this study only serve to highlight the complexity of the chemistry involved. Consequently, it is difficult to make any generalisations when dealing with the chemistry of anaerobic adhesives.

The importance of the reducing agent in the overall cure chemistry has been investigated previously by Raftery [5]. It is clear that any lowering of the concentration of the reducing agent in the adhesive formulation will have a detrimental effect on its performance. Hence in order to maintain the optimum performance of the adhesive upon storage, controlling the reactions of the reducing agents with the other cure components in the absence of the active metals is the key factor. The 'curing' activity or reducing ability of the reaction products formed from these reducing agents should also be evaluated. Furthermore changes in actual adhesive formulations based on these cure chemistries should be monitored by HPLC to ascertain as to whether the reactions identified in these studies are taking place in the anaerobic sealants.

APH :

In the case of APH when CHP is present in excess, the most usual situation prevailing in adhesive compositions, then the least decomposition occurs in the absence of the acid components, see Figure 2.5. Hence minimising the concentrations of the acids in such formulations should serve to retard the rate of decomposition.

DMPT :

In contrast to the above situation the least decomposition appears to arise in the most acidic formulations, see Figure 2.13. Hence relatively high levels of acid, particularly a combination of maleic acid and saccharin should serve to reduce the degree of decomposition of the reductant.

THQ :

In parallel to the situation with DMPT, the least decomposition of THQ occurs in the more acidic environment, in particular maleic acid only, or maleic acid and saccharin. Therefore in the presence of relatively high concentrations of acid the decomposition of the reductant should be minimised.

p-TSH :

Due to its chemical structure it would be excepted that its reactivity would be analogous to that of APH. Thus it would be expected that by minimising the level of acid components would minimise the decomposition of the *p*-TSH.

2.7. BIBLIOGRAPHY

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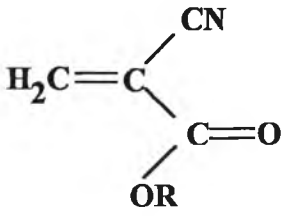
Chapter Three

Development and Application of a Capillary Electrophoretic Method for the Determination of Inorganic and Organic Anions found in Cyanoacrylate Adhesives

3.1. INTRODUCTION TO CYANOACRYLATE ADHESIVES

Alkyl cyanoacrylate adhesives are unique among the many classes of adhesives, in that they are the only single component, instant bonding adhesives that cure at room temperature without requiring an external energy source. It is this characteristic in addition to their ability to bond a very diverse range of materials that makes them an ideal adhesive for a number of bonding applications. Although these adhesives are relatively high in cost, they generally only require a single drop for most applications, thus making them very economical to use. Additionally there is no requirement for any heating or radiation sources in order to initiate the curing process.



(I)

The general structure for the cyanoacrylate monomer, I is shown above. Increasing the akyl chain length will result in a decrease in the cure speed and the strength of the adhesive. In recent years the volume of ethyl monomer produced has increased steadily relative to methyl monomer. Alkoxyl-alkyl esters are also finding application as low odour, low bloom adhesives.

The cyanoacrylate adhesives can be essentially made using one of two pathways. The first route involves the pyrolysis of an alkyl-3-acyloxy-2-cyanoproprionate to yield an alkyl-2-cyanoacrylate plus a carboxylic acid. The more popular route, nowadays, is the Knoevenagel pathway. This route involves the condensation of the alkyl cyanoacetate with formaldehyde to produce a poly(alkyl-2-cyanoacrylate), as shown schematically in Figure 3.1. The reaction is typically carried out in a non-aqueous organic solvent to aid in the removal of the water from the system and also to dissipate the heat evolved during the reaction exotherm. The polymerised cyanoacrylate is then heated to a temperature in the range of 140-260 °C. This

heating of the polymer forces the polycyanoacrylate back to a cyanoacrylate ester. Typically a yield in excess of 80 % can be achieved. For the reaction to occur smoothly and without repolymerisation occurring, the base catalyst must be removed. This is normally done by the addition of a small amount of acid, e.g. phosphoric acid or its anhydride. Additionally, a free radical inhibitor, such as hydroquinone, is added to prevent the repolymerisation, that could be initiated by the formation of free radicals at elevated temperatures.

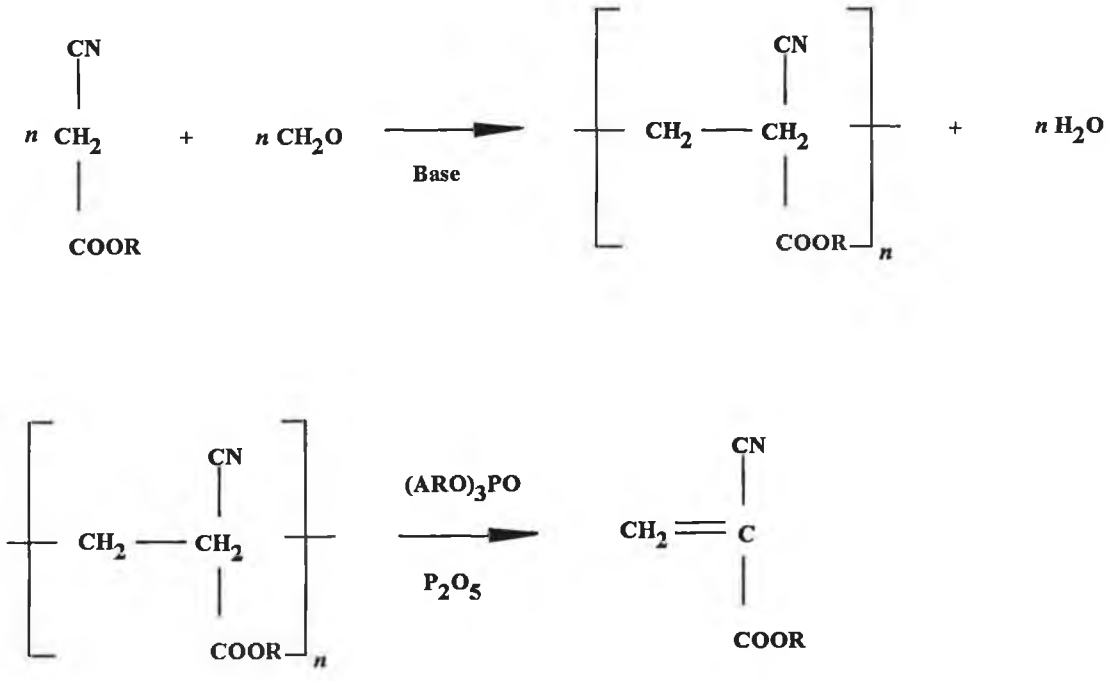


Figure 3.1 : The Knoevenagel cyanoacrylate synthetic route starts with a condensation reaction between an alkyl cyanoacetate molecule and formaldehyde molecule followed by a polymerisation step to yield a cyanoacrylate ester.

3.2. ANALYSIS OF CYANOACRYLATE ADHESIVES

There are several techniques that are used to specifically analyse for the presence of anions in cyanoacrylate adhesives. These techniques are used by Loctite in the analysis of their cyanoacrylate adhesive preparations. These approaches include plasma emission, both inductively-coupled (ICP) and direct current (DCP), non-aqueous titrimetry and 'total acid determination' tests, based on measurement of polymerisation exotherms or viscosity changes, and finally ion chromatography (IC). Titrations are used to determine the presence and concentration of stronger acids, such as sulphonic acids (e.g. MSA). Titrimetric analysis can also be used to analysis for the presence of MSA, HPSA, H_2SO_4 and SO_2.

3.2.1. ICP-AES

There are three main anionic stabilisers that are currently used in cyanoacrylate adhesives, namely MSA / SO_2, HPSA / SO_2 and more recently BF_3. The advantage of BF_3 is that it is very effective at minimising 'head space' polymerisation in the 'packed down' forms. Depending upon the application of the adhesive, different levels of stabilisers are needed in the preparation in order to ensure good product performance and good shelf life. As discussed later, IC fails to detect the fluoride/borofluoride anion, however, inductively-coupled plasma-atomic emission spectroscopy (ICP-AES) has been applied successfully to the detection of BF_3. This method involves an internal standard technique, where the level of boron is measured against the internal standard, gold. The internal standard method takes into account the associated variable parameters such as viscosity, plasma, flow rate and nebulisation efficiencies. By accounting for the above mentioned variable parameters, the precision of ICP-AES can be greatly improved.

3.2.2. Total Acid Determination (TAD) Tests

As the rate of polymerisation is greatly affected by the trace quantities of acid (anions) present, it provides a very sensitive method for acid determination. There are a

number of techniques that can be used to determine the amount of acid present, including viscosity and temperature related studies. The TAD test yields information regarding the total amount of acid content in the adhesive sample. The method is based upon the length of time taken for the sample to reach a certain viscosity, once an initiator is added to the solution. An associated test is the TAD polymerisation exotherm test, which is used to obtain TAD related data. The TAD measurements are carried out using a test tube containing a solution of N-oxydiethylene-benzothiazole-2-sulphenamide, di-n-butyl phthalate and the monomer or adhesive mixture. The test tube is placed in a water bath at room temperature and an applicator stick placed into the tube. After mixing the solution, the polymerisation process is initiated and the viscosity of the mixture gradually increases, until the tube eventually moves upwards when the applicator stick is drawn upwards. The viscosity TAD value is defined as the time taken for the TAD mixture to increase in viscosity, to the point when the tube is lifted upwards, by the movement of the applicator stick. The time starts from the moment the solution is mixed together. When a sample has a very rapid polymerisation process the endpoint is much more clearly defined and so the TAD value is quite accurate; however when the viscosity increases very gradually the endpoint is much more difficult to determine, thus leading to inaccuracy in the TAD value.

A similar method of analysis that is used for acid measurement is the thermal TAD technique. In this case the applicator stick is replaced by a thermocouple, (see Figure 3.2.). Once the solution is mixed thoroughly, no further mixing takes place. The temperature is measured, the values plotted and the point of inflexion is taken as the endpoint (see Figure 3.3).

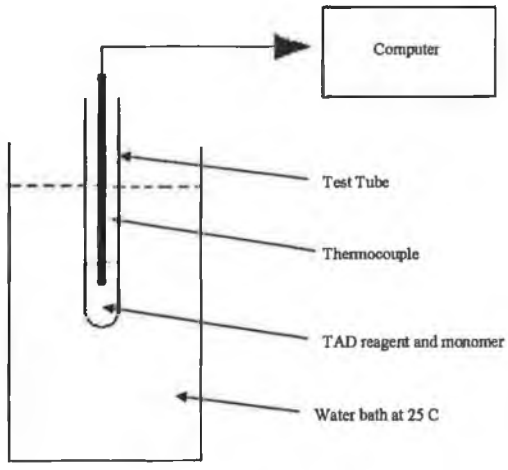


Figure 3.2 : The apparatus used in the thermal TAD test. In the viscosity TAD test the thermocouple is replaced by an applicator stick.

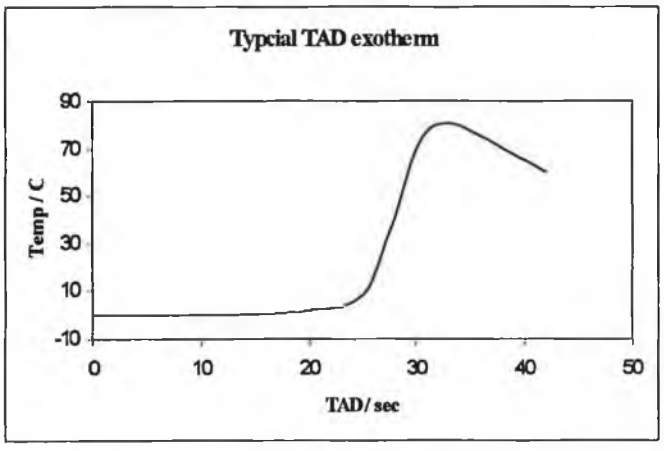


Figure 3.3. : A typical thermal TAD trace, where the point of inflexion is taken as the endpoint.

Comparisons between the TAD viscosity tests and the thermal tests have revealed differences in the acid concentrations. It has been found that the thermal TAD values are, typically ~ 10 % higher than the corresponding viscosity TAD values. The explanation for this observation lies in the differences in the shape of the TAD

exotherms, where the point of inflexion is taken as the endpoint. When dealing with low monomer conversions, ~5 % conversion is sufficient to increase the viscosity of the mixture to the point where the tube is lifted upwards by the applicator stick. However, in the case of the thermal TAD test, the point of inflexion usually occurs at the point where ~50 % of the monomer is converted. The differences between the two TAD tests is really only noticed when the polymerisation rates are rather slow or gradual. When the polymerisation rate is rapid the onset of the exotherm is also relatively rapid, thereby greatly reducing the differences between the viscosity and thermal TAD results. Instead of measuring the endpoint in the thermal TAD test at the point of inflexion, a point at say 5 or 10 % conversion, could be taken. This methodology could, however, lead to more reproducibility problems, as the thermocouple is not necessarily placed in the very centre of the test tube. If the thermocouple is placed near the side of the test tube, a smaller exotherm is recorded, thus leading to the reproducibility problems when taking endpoint readings at ~ 5 % conversion values.

3.2.3. Polymerisations Warme (PW) Methods

The PW method or 'Polymerisation Exotherm' method, developed by Henkel/ Sichel, is based upon the measurement of the time taken for a solution of cyanoacrylate sample to increase in temperature for 30 °C to 40 °C. The methodology of the PW method is as follows : 4 g of cyanoacrylate monomer or adhesive is dissolved in 4 g of ethyl cyanoacetate solution, and this is then placed in an insulated 10 ml polyethylene container. After the addition of 0.2 % N-ethyl-N-phenylethanolamine, the solution is mixed thoroughly and the temperature starts to increase. This increase in temperature is measured using a thermocouple and then the exotherm produced is measured. The time interval between 30 °C to 40 °C is measured using a computer. The starting temperature of the solution should be at an ambient temperature of ~20 °C. The experimental setup is very similar to that of the thermal TAD test (Figure 3.2.). The PW test shows high sensitivity to the stronger acids (e.g. sulphuric acid) found in cyanoacrylate preparations, but it does show rather low sensitivity to the weaker acids, such as cyanoacetic acid.

3.2.4. DSCL

Another thermal type of test that is used in acid determination is calorimetry. The test is the adiabatic dilute solution calorimeter (DSCL) test. The basic apparatus of the DSCL technique consists of a doubled walled glass reaction vessel, into which the sample is placed, as shown schematically in Figure 3.4. The sample is dissolved in THF and mixed thoroughly before the addition of the amine. The addition of the amine triggers the time switch (when the syringe plunger is fully depressed). The starting temperature should be approximately 20 °C, and the resulting increase in the temperature is measured by the thermistor probe. Different exotherm shapes are produced by different acids in the sample. Strong acids show an inhibition stage in the exotherms, while the weak or intermediate acids display a retardation period. Thus the DSCL exotherms can be used to determine the different types of acids present in the sample. This is in contrast to the TAD method where both the strong and weak acids produce inhibition periods. The sensitivities of the DSCL method to weak and strong acids are largely reversed when compared to the TAD method. The DSCL shows good sensitivity to weak acids, allowing changes of ~ 5 ppm to be detected, although the response decreases rapidly at higher levels of weak acids. This results in a widening of the concentration range but also in a loss of accuracy in the acid measurement at higher levels. In contrast, the DSCL inhibition periods for strong acids are approximately 11 times less sensitive, when compared to TAD inhibition curves.

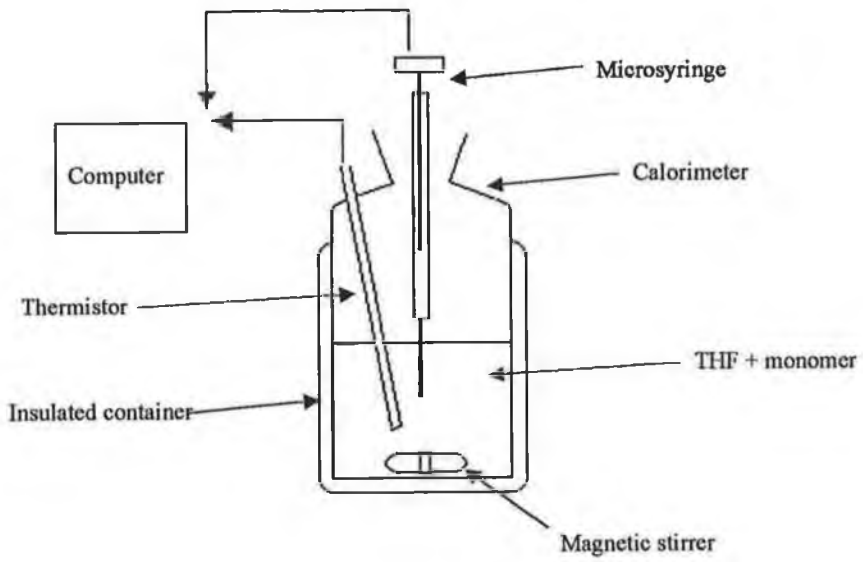
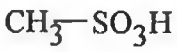


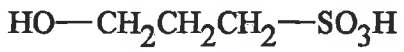
Figure 3.4. : The adiabatic dilute solution calorimeter (DSCL) and the associated data acquisition system.

3.3. PURPOSE AND AIMS OF RESEARCH

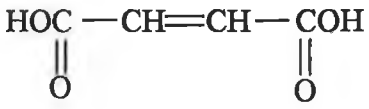
The aim of this research project was to develop a method by which anions found in a range of cyanoacrylate adhesives could be separated using CE. The anions to be separated included; chloride (Cl^-), nitrate (NO_3^-), sulphate (SO_4^{2-}), malonate ($^-O_2CCH_2CO_2^-$), formate (HCO_2^-), succinate ($^-O_2CCH_2CH_2CO_2^-$), phosphate (PO_4^{3-}), MSA or methyl sulphonate ($CH_3SO_3^-$), HPSA or hydropropane-sulphonate ($HOCH_2CH_2CH_2SO_3^-$) and cyanoacetate ($NCCH_2CO_2^-$). The chloride, nitrate, sulphate and formate anions were obtained from their corresponding sodium salt forms. The method developed had to be applicable to the analysis of ethyl cyanoacrylate adhesive samples. The structures of the main anions are illustrated in Figure 3.5. Additionally, anions of a lesser importance were run under the optimum conditions, such as monoethyl phosphate.



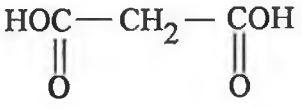
Methylsulphonic acid (MSA)



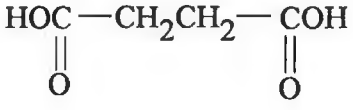
Hydroxypropanesulphonic acid (HPSA)



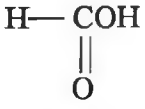
Maleic acid



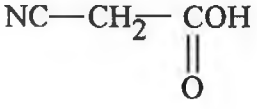
Malonic acid



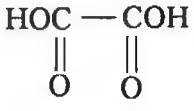
Succinic acid



Formic acid



Cyanoacetic acid



Oxalic acid

Figure 3.5. : Schematic diagrams of many of the anions found in cyanoacrylic adhesives. The anions are shown in their protonated form, i.e. as acids.

3.4. EXPERIMENTAL DETAILS

3.4.1. Reagents and Apparatus

The source of chemicals was as follows ;

chloride, nitrate, sulphate, sulphite, formate - Aldrich
malonate, phosphate - Sigma
succinate, CTAB - BDH
HPSA, MSA, cyanoacetate, cyanoacrylate, mono/di-ethyl phosphate, mono (methoxy ethyl) phosphate, tri (methoxy ethyl) phosphate - Loctite (Irl.)
acetonitrile - Labscan (HPLC grade).
NICE Pak OFM-BT Flow modifier - Waters Corp.
TTAB - Riedel-de Haen

The anions were obtained from the following compounds ;
chloride, nitrate, sulphate, formate - sodium salts.
malonic, succinate, HPSA, MSA, cyanoacetate, cyanoacrylate- corresponding acids.

Most of the anion solutions were made up as 500 ppm solutions using 18 MΩ deionised water (obtained from a Milli-pore Waters System). The solutions were prepared freshly every two weeks. The buffer electrolyte was, however, prepared fresh daily. The electrolyte consisted of 10 mM sodium chromate and 0.25 mM OFM-BT flow modifier, adjusted to pH 8.00 with 1 mM H_2SO_4. Some anions, due to their limited availability were just run as spikes and no calibration curves were prepared.

The CE instrument employed was a Beckman PACE 5000 CE system equipped with a high voltage power supply. Polyimide-coated, fused silica capillaries, 50 μm (i.d.) x 57 cm in length, were used with a length of 52 cm from the injector end to the detector window. Indirect UV detection was employed using a Hg lamp. The detector window aperature was 100 μm x 800 μm. Sample introduction was

performed using hydrodynamic (pressure) injection for 5 seconds. Sample vials were standard glass vials and were loaded in an autosampler carousel. All electropherograms were recorded and analysed using System Gold, Pace software using an Elonex 466-DX PC.

3.4.2. Method

Sample solutions were prepared using 18 MΩ deionised water. The buffer electrolyte consisted of sodium chromate buffer and EOF modifier. A stock solution of 50 mM chromate was prepared using sodium chromate (Na_2CrO_4), from which a 10 mM sodium chromate solution was prepared that was also 0.25 mM in EOF modifier concentration. The modifier used (which is currently under patent) is a 20 mM quaternary ammonium salt solution. The solution was adjusted to pH 8.0 using 1 mM H_2SO_4. The buffer electrolyte was filtered through a 0.45 μm swinny filter before use. The sample run was carried out at a voltage of -15 kV with an end run time of 8 minutes. This voltage was ramped up over 12 seconds and kept constant throughout the run. Previous to the above mentioned run conditions, other buffer electrolyte systems and voltages ramps were investigated. The effects and results of a number of other conditions will be discussed later.

3.4.2.1. Capillary Preparation

CE capillaries are quartz capillaries, coated in a polyimide coating to provide strength to the capillary. This coating is opaque and thus must be removed in order to create a detector window. The removal of the coating was achieved by using hot concentrated sulphuric acid, followed by rinsing with water. Also, before use, the capillary must be conditioned. The capillary was first flushed with 1 M HCl for 5 min, followed by H_2O for 2 min, 0.1 M NaOH for 20 min and H_2O for 5 min After conditioning, the capillary was equilibrated with the buffer electrolyte for 25 min Two 'blank' solutions were also injected to check on the baseline and to ensure complete equilibration of the capillary surface. Between each sample run the capillary was rinsed first with 0.1 M NaOH for 2 min followed by the buffer electrolyte, again for 2 min, in order to remove any residue of the sample from the capillary (including the water peak which eluted at ~15 min). As CE involves the migration of anions through the capillary, there is a constant movement of ions from the vial at the injection side to the vial at the detector end, thus leading to a build-up of 'extra' ions in the vial at the detector side compared to that at the injector side. After the

injection of many samples, this build-up of ions can lead to a difference in the composition of the two vial solutions, thus leading to variations between each sample run. Therefore the buffer electrolyte was replaced after every eight sample runs - before the build-up of ions became too great. The experimental conditions will determine the number of injections that can be made before the buffer needs to be replaced, e.g. in a publication by Stahl [1] it is recommended that the buffer electrolyte be replaced after no more than 15 injections.

3.4.2.2. Preparation of the Adhesive Sample

1.50 g of the cyanoacrylate monomer was dissolved in 20 ml chloroform (organic phase), and to this 5 ml of oxalic acid (aqueous phase) was added. The oxalic acid used was a 100 ppm solution at a pH of 3.5. This solution was then mixed thoroughly and then left to stand until complete separation of both the organic and the aqueous layers occurred. The (upper) aqueous layer, which contained the anions of interest, was then removed and filtered through a 0.45 μm swinny filter prior to analysis on the CE. In the case of crude monomer preparations, 0.75 g of sample was dissolved in 20 ml chloroform and extracted as described above.

3.4.2.3. Quantification of Anions in the Sample

In order to determine the quantity of anions present in the sample it was first necessary to construct a calibration curve for each anion. A range of anion concentrations were prepared by dissolving the anions in deionised water and then injecting on to the CE. Three replicate injections were performed. The concentration of the anions in the calibration curve was directly related to the peak area. Consequently the average peak area was used in the plotting of the calibration curves. The peak areas were integrated using the System Gold software. The equation for the calibration curve of each anion was then used to calculate its concentration from the peak areas in the actual cyanoacrylate samples. Oxalic acid (15 ppm) was used as an internal standard; however, phthalic acid would also serve as a suitable internal standard.

3.5. RESULTS

3.5.1. Sample Preparation

Adhesives in general are difficult to analyse, as the solubility of the adhesive is extremely limited. The analysis of anions found in the adhesive sample can only be analysed on a CE system if they are present in an aqueous solution. This fact in itself poses a problem as the adhesive sample cannot be simply dissolved in water, it must first be dissolved in an organic solution and then extracted into an aqueous environment. Due to the anionic mechanism by which cyanoacrylate adhesives polymerise, the aqueous media used in the extraction process, cannot be basic in nature. The extraction must therefore, be carried out using an acidic environment. Even in mildly alkaline conditions the polymer chains can form.

There are essentially only two organic solvents in which the cyanoacrylate adhesive can be dissolved : tetrahydrofuran (THF) and chloroform. The use of both solvents was investigated. Initially, the adhesive sample was dissolved in either 20 ml of THF or chloroform. Extraction was then performed using water, oxalic acid or boric acid. Although the sample is known to polymerise in water, the water-containing systems were investigated as a comparison to the acidic aqueous extraction systems. All of the THF solvent systems (THF and H_2O/oxalic acid/boric acid) proved very inefficient in anion extraction. Although the adhesive sample dissolved very readily in the THF, upon addition of the aqueous phase a thick polymer was formed, which proved impossible to filter. A polymer was also formed when using the chloroform/water solvent system. The boric acid extractions were performed using 200 ppm boric acid solutions, pH 3.0, although no insoluble polymer was formed, the borate solution proved ineffective in anion extraction. This was evident from the absence of any anion peaks in the electropherogram. The electropherogram baseline obtained from the borate extracted sample was also rather noisy, especially in comparison to the oxalic acid extracted sample. Oxalic acid is a suitable medium for the extraction process, as it is not present in the adhesive samples and its migration time does not interfere with any anions of interest. Citric acid had been used previously [2] in anion

extraction, but under these conditions the citrate masked other anion peaks of interest, included formate, phosphate and succinate.

A final approach in anion extraction was investigated, this involved adding ~5 g of cyanoacrylate sample slowly dropwise to 10 ml of buffer electrolyte. This procedure has previously be carried out on a chromatography system at Loctite (Irl.) [3]. Although the sample did form a polymer, the aim was to grind up the polymer in order to fully extract the anions, however due to the nature of the polymer formed, attempts at grinding it proved futile. The differences in buffer electrolyte used and the mobile phase used (tetrabutylammonium hydroxide/potassium hydrogenphthalate) undoubtedly has a marked effect on the type of polymer formed.

3.5.2. Separation of the Anions

Although many papers have been published on the separation and analysis of anions using CE, there are few, if any, to be found specifically on the separation of anions found in adhesives. In the separation of anions there are essentially three EOF modifiers used, TTAB, CTAB and OFM-BT modifier. Most of the anions could be separated on a wide variety of buffer electrolytes, however, the phosphate, formate and succinate anions were extremely difficult to separate. A number of electrolytes were investigated using all three EOF modifiers in varying ratios to the chromate buffer. Of all the flow modifiers used, TTAB proved to be the least efficient additive. Jimidar et al. have successfully used TTAB for the separation of nitrates and nitrites, but they did not investigate its use in the separation of several anions simultaneously. Figure 3.6. shows the trace obtained for the separation of a number of anions under the optimum conditions using TTAB (2 mM TTAB and 10 mM chromate buffer).

The CTAB solutions were prepared from a stock solution of 20 mM CTAB, prepared using 10 % ACN : 90 % H_2O. The use of the organic phase served to increase the solubility of the surfactant. CTAB did prove relatively successful in the simultaneous separation of the anions. However, even though a number of buffer electrolytes were investigated (including varying ratios of the CTAB EOF modifier to chromate, such as

10.0 mM/1.0 mM, 10.0 mM/2.0 mM, 5.0 mM/1.0 mM), it was not possible to fully resolve the phosphate and succinate anions. A trace showing the optimum separation of anions obtained, using 1 mM CTAB and 10 mM chromate buffer, is shown in Figure 3.7.

Another change in the buffer composition was that of pH. The chromate solution was adjusted to pH 8.0, 10.0 and 12.0, before making up the final buffer electrolyte. Although the pH 12.0 buffer solution did produce a slight separation between the two anion peaks (phosphate and succinate), it was not possible to obtain baseline resolution - even using voltage ramps. It was found that as the pH of the buffer electrolyte was increased the separation of the anions also increased, but there was no noticeable difference in the migration times.

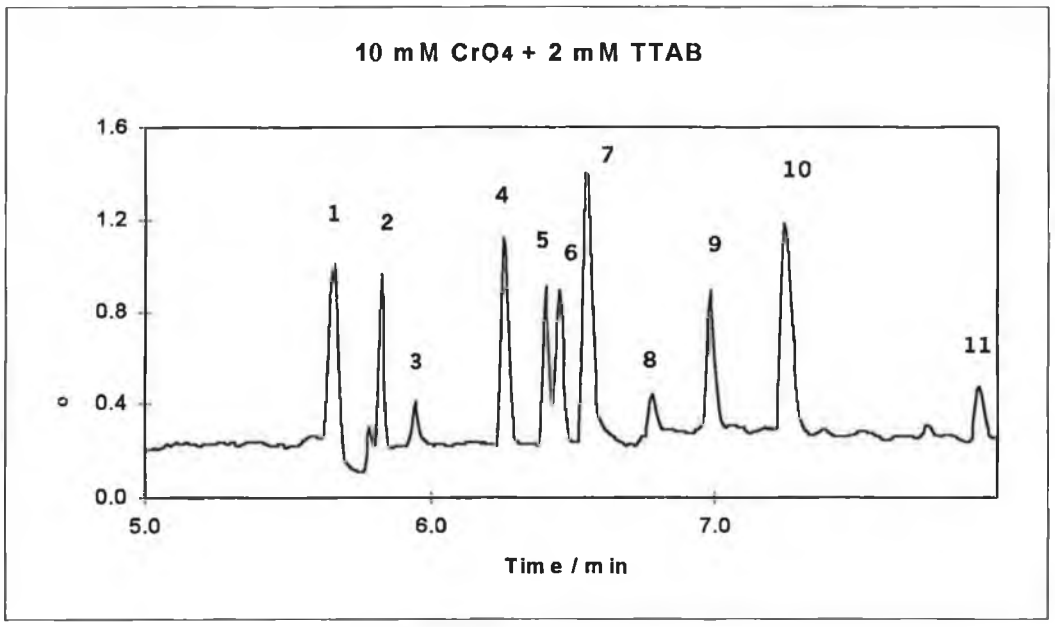


Figure 3.6. : Electropherogram of the separation of a mixture of anions. The buffer electrolyte is 2 mM TTAB and 10 mM chromate, indirect detection @ 254 nm, voltage 0-15 kV over 0.2 min. [1] chloride (7 ppm), [2] sulphate (9 ppm), [3] unidentified peak, [4] malonate (10 ppm), [5] maleate (10 ppm), [6] formate (6 ppm), [7] phosphate (25 ppm), [8] unidentified peak, [9] MSA (10 ppm), [10] cyanoacetate (10 ppm), [11] HPSA (10 ppm).

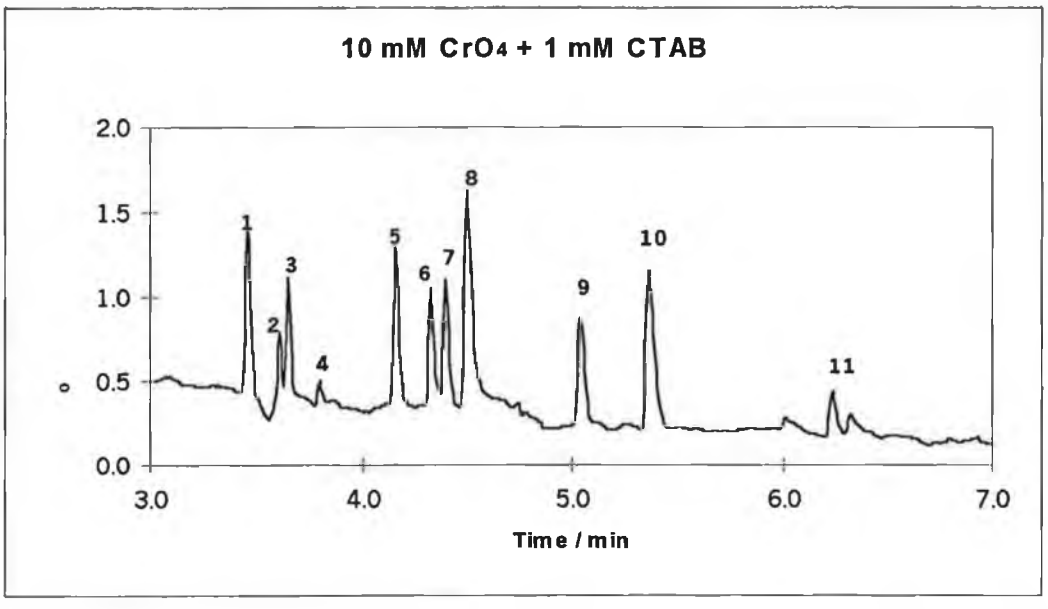


Figure 3.7. : Electropherogram of the separation of a mixture of anions. The buffer electrolyte is 1 mM CTAB and 10 mM chromate, indirect detection @ 254 nm, voltage 0-15 kV over 0.2 min. [1] chloride (7 ppm), [2] nitrate (7 ppm), [3] sulphate (9 ppm), [4] unidentified peak, [5] malonate (10 ppm), [5] maleate (10 ppm), [7] formate (6 ppm), [8] phosphate (25 ppm), [9] MSA (10 ppm), [10] cyanoacetate (10 ppm), [11] HPSA (10 ppm).

The OFM-BT modifier was added in similar concentrations to the CTAB and the solutions adjusted to the pH values as listed above. Better separation was obtained using the OFM-BT modifier when compared to the CTAB or TTAB. In contrast to the results obtained for the CTAB modified buffer, the buffer of lower pH produced better separation between the phosphate and succinate peaks. The lower pH buffer electrolyte also produced shorter migration times. Further investigations into the use of this modifier were carried out and it was found that the buffer electrolyte that gave the best overall separation, was 10 mM chromate and 0.25 mM EOF modifier, adjusted to a final pH of 8.0. Adjustments to pH values lower than 8.0 caused a precipitate to form. A similar observation was made by Harawuke and Haddad [4]. Better resolution between phosphate and succinate anions was attained by using a detector rise time of 0.1 sec., instead of 1.0 sec. The detector rise time is a measure of the rate of data collection, and although by using a faster detector rise time there was a slight increase in the baseline noise, the increase in resolution between the peaks was much improved.

The electropherogram shown below (Figure 3.8.) was obtained for the standard solution of the anions. The concentrations (ppm in w/v) of the individual anions were as follows

1) chloride (7 ppm),
2) sulphate (9 ppm),
3) nitrate (7 ppm),
4) oxalate (internal standard),
5) sulphite (10 ppm),
6) malonate (10 ppm),
7) maleate (10 ppm),
8) formate (6 ppm),
9) succinate (10 ppm),
10) phosphate (25 ppm),
11) phthalate (30 ppm),

12) MSA (15 ppm),

13) cyanoacetate (15 ppm),

14) HPSA (15 ppm).

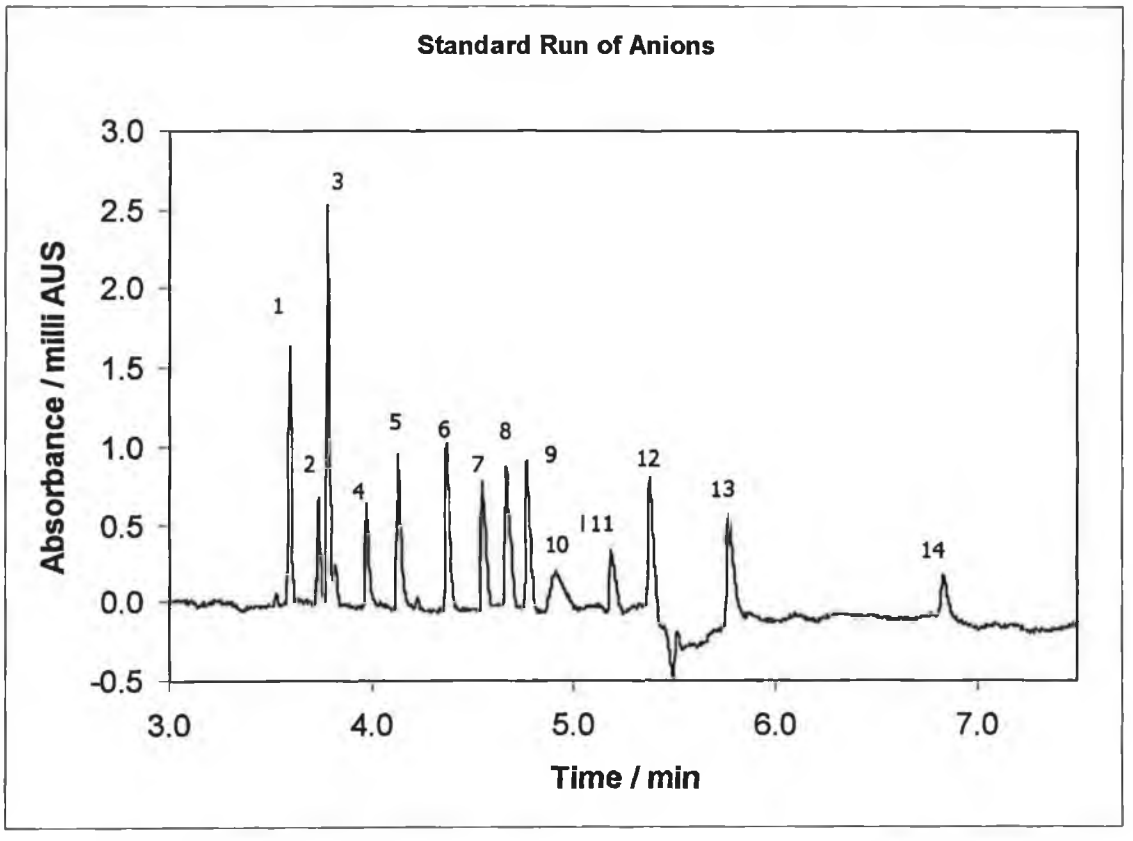


Figure 3.8. : Electropherogram showing the separation for a standard mixture of inorganic and organic acids. Buffer electrolyte : 10 mM chromate, 0.25 mM EOF OFM-BT modifier, adjusted to pH 8.0 with 1 mM H_2SO_4. Indirect detection @ 254 nm. Voltage 0-15 kV over 0.2 min, pressure injection 5 sec. The concentrations of the anions are : [1] chloride (7 ppm), [2] nitrate (7 ppm), [3] sulphate (9 ppm), [4] oxalate (int.std.), [5] sulphite (20 ppm), [6] malonate (10 ppm), [7] maleate (10 ppm), [8] formate (6 ppm), [9] succinate (10 ppm), [10] phosphate (25 ppm), [11] phthalate (30 ppm), [12] MSA (10 ppm), [13] cyanoacetate (10 ppm), [14] HPSA (10 ppm).

The linear working ranges of the anions are presented in Table 3.1., all have linear regression values greater than 0.998. The phosphate range is narrower than many of the other anions, due to the splitting of the phosphate peak at higher concentrations. The working range is, however, sufficient for most typical adhesive samples.

Anion	Working Range /ppm	L.O.D. * /ppm
Chloride	1 - 150	0.3
Nitrate	3 - 145	0.4
Sulphate	1 - 160	0.9
Oxalate	int. std.	-
Malonate	1 - 200	0.7
Maleate	1 - 200	0.3
Formate	1 - 200	0.9
Succinate	1 - 150	0.5
Phosphate	1 - 100	1.0
Phthalate	5 - 100	0.2
MSA	1 - 200	0.3
Cyanoacetate	1 - 100	0.8
HPSA	5 - 200	1.2
		* Based on a signal to noise ratio of 3

Table 3.1. : Table showing the working concentration ranges and limit of detection (L.O.D.) of the named anions.

The typical requirements for an effective internal standard were fulfilled by oxalic acid. The oxalic still served as an internal standard, even though it was used in the extraction process of cyanoacrylate adhesives. However, phthalic acid is also another suitable internal standard (it is not found in cyanoacrylate preparations), with a slightly later migration time. Many other weak acids were investigated as potential internal standards, such as citrate, boric acid and carbonate and acetate buffers. However, due either to poor peak shape or unsuitable migration times, none of the above proved suitable for use as an internal standard.

The use of UV (indirect) detection proved successful in the analysis of the inorganic and organic anions. Due to the lack of chromophores the anions were easily detected,

yet other compounds found in some adhesives were not detectable, thereby avoiding some interfering peaks. Some such compounds that occur in various cyanoacrylate adhesive samples are : MMBP (2,2-methylene-bis (4-methyl-6-tert butyl) phenol), a phenolic stabiliser and hydroquinone, neither which appear on the electropherogram. Spiking was used to test for the detection of these compounds.

Figure 3.9. shows an electropherogram of monoethyl/diethyl phosphate cyanoacrylate. Due to the ease with which the ethyl phosphates can be hydrolysed they had to be prepared immediately prior to analysis. They could not be incorporated into the overall standard mix as the weak acids such as maleic acid would result in the hydrolysis of the ethyl phosphates. The cyanoacrylate sample appeared to undergo some reaction when added to the standard mixture, and it was therefore run separately as a spike in water. The ethyl phosphates were prepared as a 0.01 % solution, again in water. The two traces are shown superimposed, as an indication of their migration times. Due to the limited amount of the samples, no calibration curves were prepared for these anions.

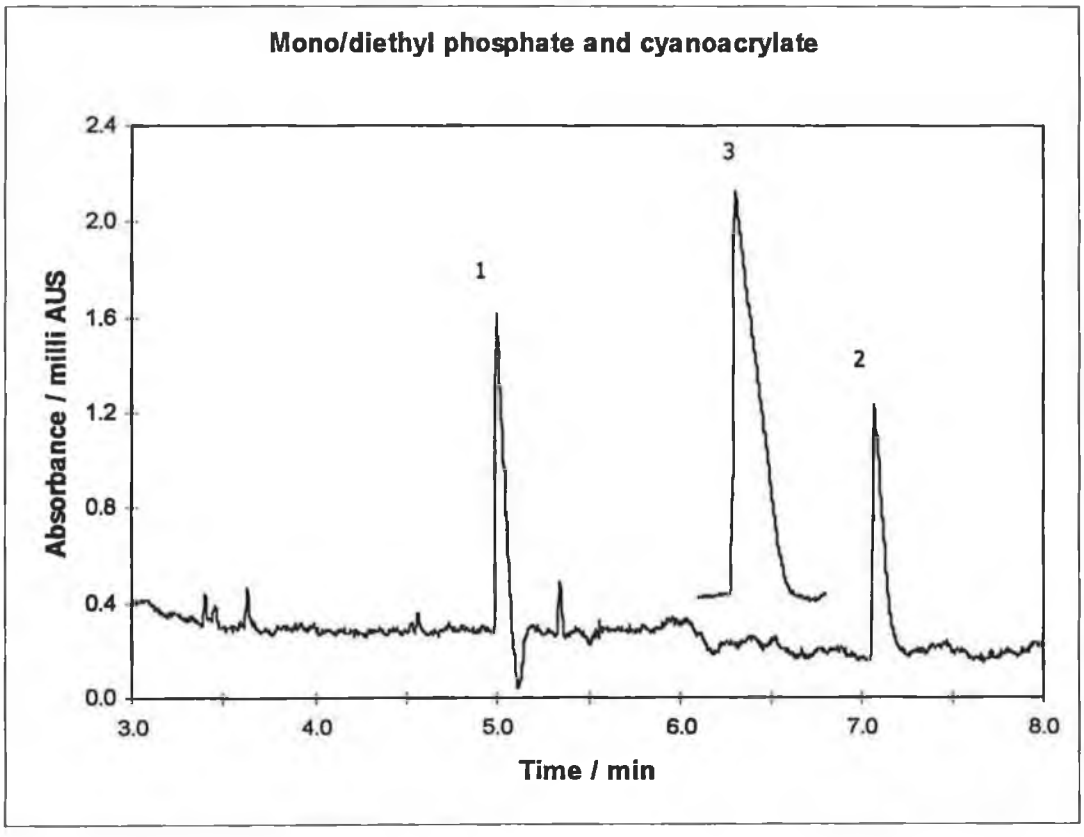


Figure 3.9. : Electropherogram obtained for a cyanoacrylate monomer adhesive sample. Buffer electrolyte : 10 mM chromate, 0.25 EOF OFM-BT modifier, adjusted to pH 8.0 with 1 mM H_2SO_4. Voltage 0-15 kV over 0.2 min, pressure injection 5 sec. 1) monoethyl phosphate 0.01 %, 2) diethyl phosphate 0.01 %, 3) cyanoacrylate spike.

Anion	A	B	C	BF_3	MSA	HPSA
	/ppm	/ppm	/ppm	/ppm	/ppm	/ppm
Chloride	148	159.8	154.1	75.5	62.7	63
Sulphate	131.2	121.6	125.1	12.5	12.8	15.7
Nitrate	n/d	< 1	n/d	n/d	< 1	< 1
Oxalate	i/s	i/s	i/s	i/s	i/s	i/s
Malonate	n/d	14.3	n/d	n/d	n/d	n/d
Maleate	n/d	n/d	n/d	n/d	n/d	n/d
Formate	15.2	16.9	24.4	14.5	16.4	19.9
Succinate	n/d	n/d	n/d	n/d	n/d	n/d
Phosphate	24	n/d	n/d	n/d	6.7	n/d
Phthalate	n/d	n/d	n/d	n/d	n/d	n/d
MSA	108.5	175.9	169.7	13.2	22.8	11.1
HPSA	624	391.6	443	9.9	n/d	16.1
Cyanoacetate	70.9	23.1	21.7	19.7	n/d	n/d

Table 3.2. : Table giving the concentrations of some anions found in cyanoacrylate adhesives (the traces of which are shown in Figures 3.7.- 3.10.). The samples A, B, C are crude monomer preparations. BF_3, MSA and HPSA are distilled preparations stabilised with that acid species. n/d = non-detectable, i/s = internal standard

Figures 3.10. shows an electropherogram obtained for a crude ethyl cyanoacrylate adhesive sample also known as crude monomer preparations. These crude samples undergo further purification through a distillation process, which results in the removal of many of the 'impurity' anions. Electropherograms obtained for several distilled fractions can be seen in Figures 3.11.-3.13. These pure monomer samples contain only trace levels of chloride, sulphate and formate in contrast to the crude samples in which 9-10 anions can be found in much higher concentrations, Table 3.2. Figure 3.11 shows a distilled monomer sample, BF_3 stabilised, which was spiked with potassium fluoride (KF), (source of fluoride), and with cyanoacrylic acid. Parallel samples of the BF_3 stabilised monomer were taken and then spiked with the named anions. The pure monomer preparations essentially differ only in the acid stabiliser used in the preparation (e.g. HPSA, MSA or BF_3). There is a noticeably higher concentration of chloride to be found in the distilled samples compared to that of the other anions found in the samples. This higher concentration is possibly due to the

fact that the cyanoacetate raw material may have been prepared from ethyl chloroacetate.

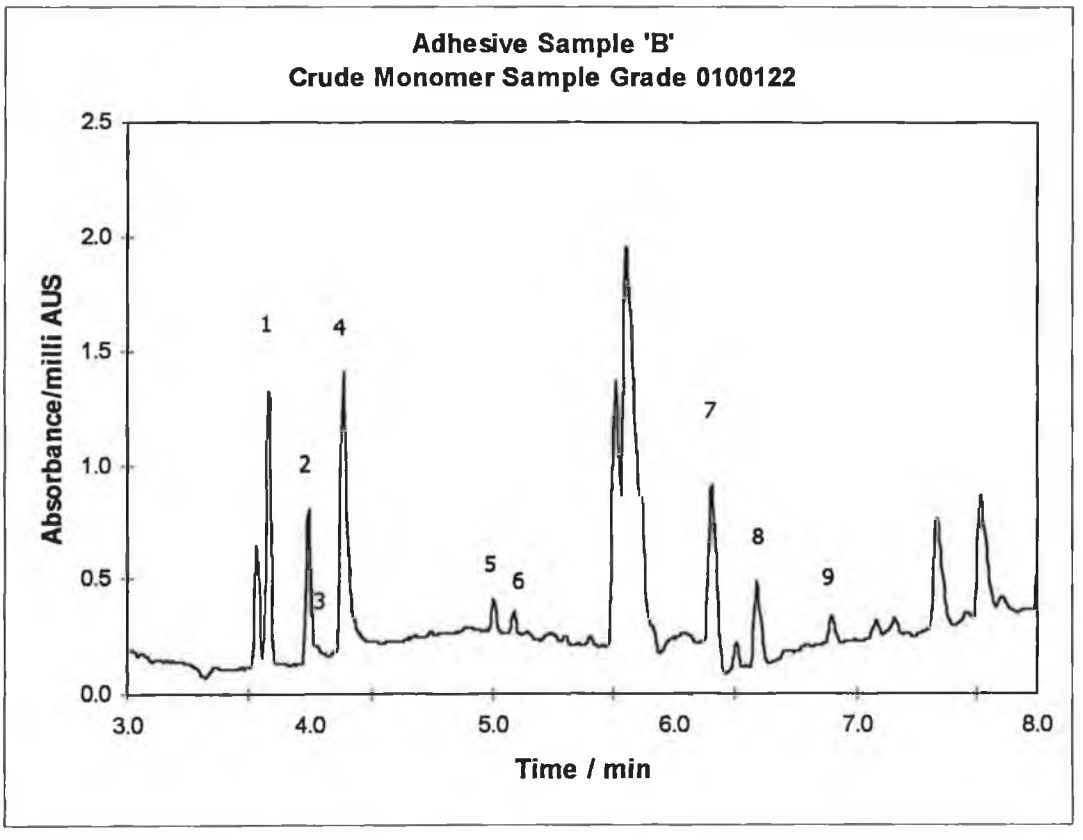


Figure 3.10. : Electropherogram obtained for a crude monomer adhesive sample. Buffer electrolyte : 10 mM chromate, 0.25 mM EOF OFM-BT modifier, adjusted to pH 8.0 with 1 mM H_2SO_4, indirect detection @ 254 nm. Voltage 0-15 kV over 0.2 min, pressure injection 5 sec. Anion concentrations are : [1] chloride (24 ppm), [2] sulphate (18 ppm), [3] nitrate (< 1.0 ppm), [4] oxalate (int.std.), [5] malonate (2.2), 6) formate (2.6 ppm), [7] MSA (26 ppm), [8] HPSA (59 ppm), [9] cyanoacetate (8 ppm). Non-labelled peaks are unidentified

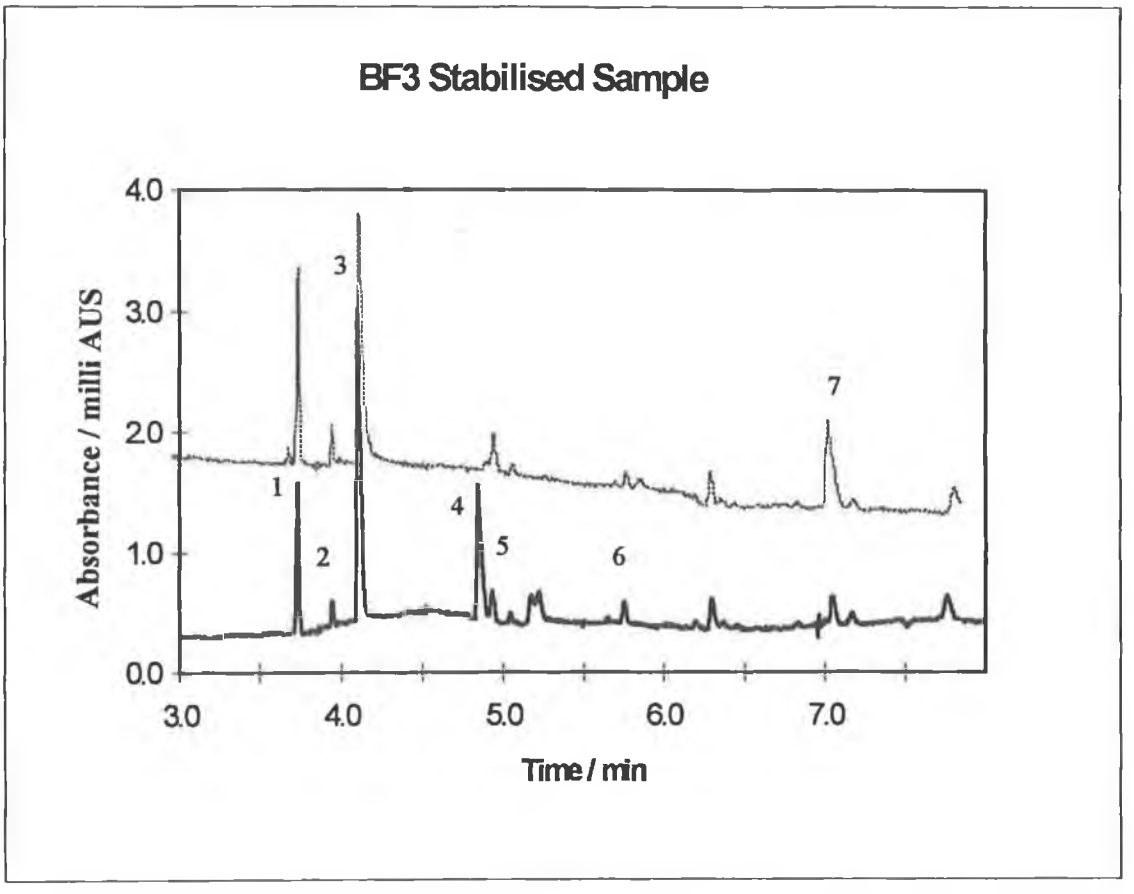


Figure 3.11. : Electropherogram of a 'pure' distilled monomer adhesive sample. Buffer electrolyte : 10 mM chromate, 0.25 mM EOF OFM-BT modifier, adjusted to pH 8.0 with 1 mM H_2SO_4, indirect detection @ 254 nm.. Voltage 0-15 kV over 0.2 min, pressure injection 5 sec. The upper trace shows a BF_3 stabilised sample spiked with cyanoacrylic acid. The lower trace show a similar sample spiked with fluoride (50 ppm). Anion concentrations are : [1] chloride (23 ppm), [2] sulphate (4 ppm), [3] oxalate (int.std.), [4] fluoride (spike), [5] formate (4 ppm), [6] MSA (4 ppm), [7] cyanoacrylate (spike)

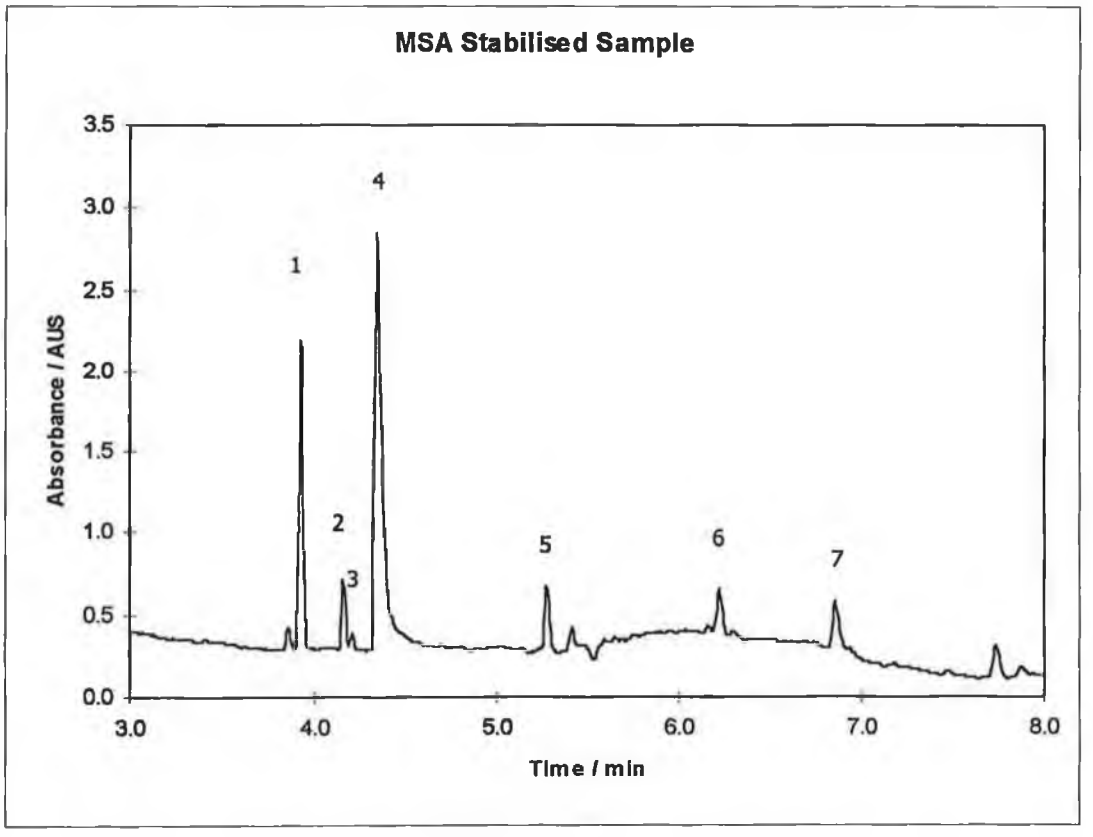


Figure 3.12. : Electropherogram of distilled monomer adhesive sample, MSA stabilised. Buffer electrolyte : 10 mM chromate, 0.25 mM EOF OFM-BT modifier, adjusted to pH 8.0 with 1 mM H_2SO_4, indirect detection @ 254 nm.. Voltage 0-15 kV over 0.2 min, pressure injection 5 sec. Anion concentrations are : [1] chloride (19 ppm), [2] sulphate (4 ppm), [3] nitrate (<1.0 ppm), [4] oxalate (int.std.), [5] formate (5 ppm), [6] MSA (7 ppm), [7] cyanoacetate (6 ppm)

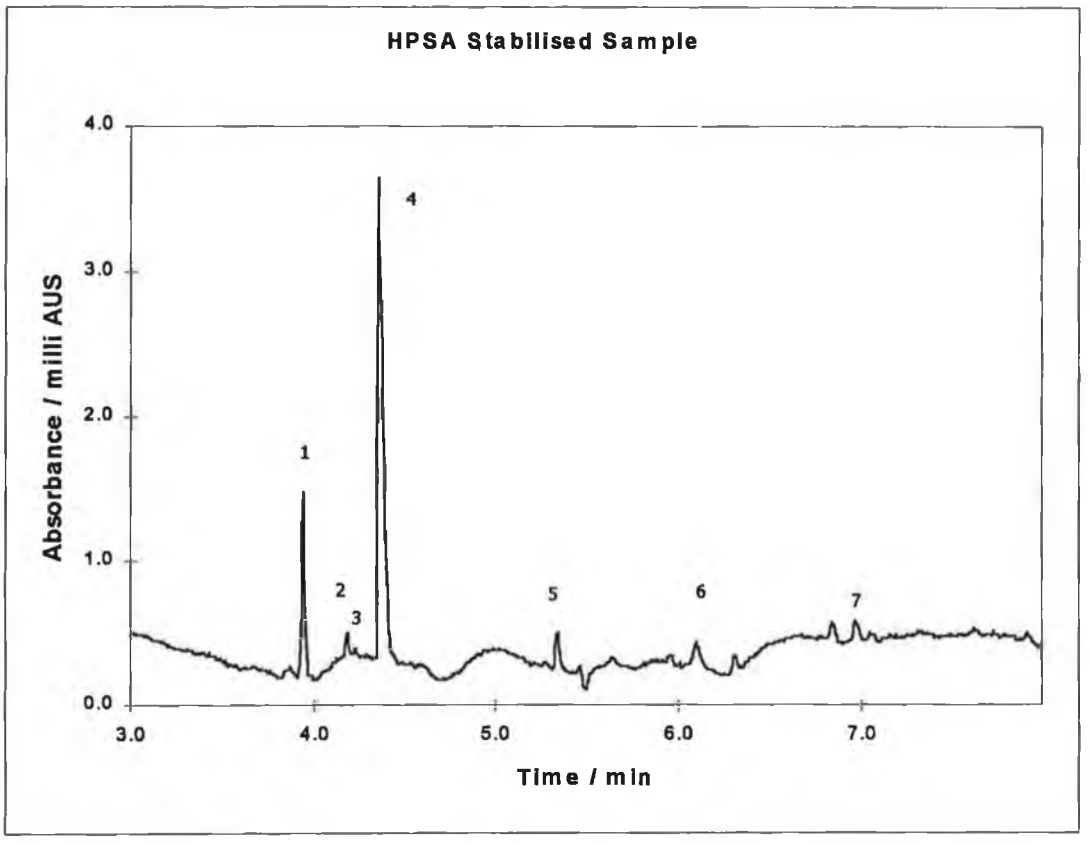


Figure 3.13. : Electropherogram of distilled monomer adhesive sample, HPSA stabilised. Buffer electrolyte : 10 mM chromate, 0.25 mM EOF OFM-BT modifier, adjusted to pH 8.0 with 1 mM H_2SO_4, indirect detection @ 254 nm. Voltage 0-15 kV over 0.2 min, pressure injection 5 sec. Anion concentrations are : [1] chloride (19 ppm), [2] sulphate (5 ppm), [3] nitrate (<1.0 ppm), [4] oxalate (int.std.), [5] formate (6 ppm), [6] MSA (3 ppm), [7] HPSA (8 ppm).

A study into the reproducibility of the adhesive extraction method was carried out, based on the figures obtained for the extraction of a crude monomer sample. The extraction procedure was carried out three times using sample 'B'. The main source of variation was in the measurement of the sample volume. As the sample matrix was extremely viscous, it was essential to thoroughly rinse the pipette used to measure out the volume of sample. The extraction procedure for the three samples was carried out simultaneously, to ensure there were no differences due to the length of time that the extraction process was carried out.

Anion	Mean Concentration(ppm)	R.S.D.
Chloride	148.0	2.5
Sulphate	131.2	1.9
Nitrate	n/d	n/d
Oxalate	i/s	i/s
Malonate	n/d	n/d
Maleate	n/d	n/d
Formate	15.2	1.0
Succinate	n/d	n/d
Phosphate	24.0	n/d
Phthalate	n/d	n/d
MSA	108.5	2.0
HPSA	624.0	3.0
Cyanoacetate	70.9	1.0

Table 3.3: Table giving the RSD values of some of the anions found in the crude monomer preparation 'sample A'.

The developed method was also applied to the analysis of other cyanoacrylate samples, including the analysis of methyl, butyl and methoxy ethyl cyanoacrylate samples. These in addition to the already analysed ethyl cyanoacrylate samples means that all available types of cyanoacrylate adhesives can be analysed by CE. Samples of methyl, butyl and methoxyethyl cyanoacrylates are shown in Figures 3.14-3.17.

Butyl cyanoacrylate samples were also analysed in a similar fashion and the results for samples from 1998, 1996 and 1995 are presented in Table 3.4. Sample electropherograms of the years 1995 and 1998 are shown in Figures 3.14-3.15. The later migrating peaks at 5.0 - 6.0 min do not appear in the electropherograms of the ethyl cyanoacrylate samples. These peaks were identified using mono/diethyl phosphate spikes, but due lack of mono/diethyl phosphate, they were not quantified.

Sample Year	Chloride	Sulphate	Formate	Phosphate	Cyanoacetate	MSA
	/ppm	/ppm	/ppm	/ppm	/ppm	/ppm
1998	50.7	18.6	3.3	0	3.4	10.6
1996	50.4	20.8	4.5	Trace	27.4	86.0
1995	49.3	21.4	6.1	Trace	68.0	88.7

Table 3.4. : Table of the concentrations of the anions identified in some butyl cyanoacrylate samples.

DSCL and titrations revealed that there was increasing amounts of strong acids, such as sulphonic acids, to be found in the older samples of butyl cyanoacrylate adhesives relative to fresher samples. These acids caused a decrease in the adhesive performance and so their identity was of importance. The titratable acid was thought to be a sulphonic acid and as the capillary electrophoresis results show, the content of sulphonic acid, MSA, in the older samples of adhesive is significantly higher. This result emphasises the strength of CE technique in the identification and quantification of acidic anions.

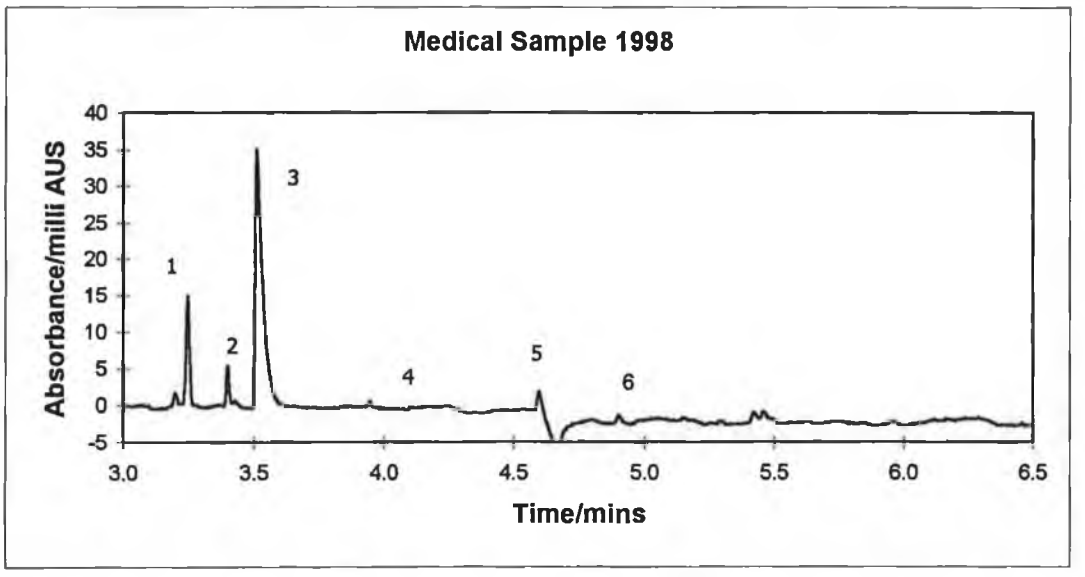


Figure 3.14. : An electropherogram of a 1998 butyl cyanoacrylate adhesive. Buffer electrolyte : 10 mM chromate, 0.25 mM EOF OFM-BT modifier, adjusted to pH 8.0 with 1 mM H_2SO_4, indirect detection @ 254 nm. Voltage 0-15 kV over 0.2 min, pressure injection 5 sec. Anion concentrations are : [1] chloride (15 ppm), [2] sulphate (6 ppm), [3] oxalate (int.std.), [4] formate (1 ppm), [5] MSA (3 ppm) , [6] cyanoacetate (1 ppm).

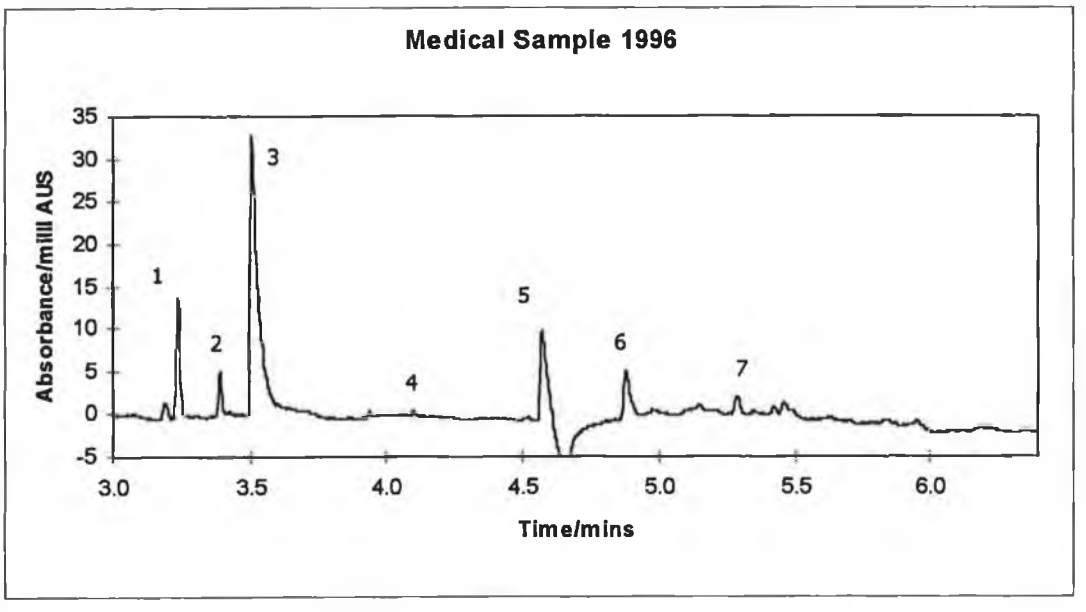


Figure 3.15. : An electropherogram of a 1995 butyl cyanoacrylate adhesive. Buffer electrolyte : 10 mM chromate, 0.25 mM EOF OFM-BT modifier, adjusted to pH 8.0 with 1 mM H_2SO_4, indirect detection @ 254 nm. Voltage 0-15 kV over 0.2 min, pressure injection 5 sec. Anion concentrations are : [1] chloride (15 ppm), [2] sulphate (6 ppm), [3] oxalate (int.std.), [4] formate (2 ppm), [5] MSA (8 ppm), [6], cyanoacetate (20 ppm), [7] unidentified peaks.

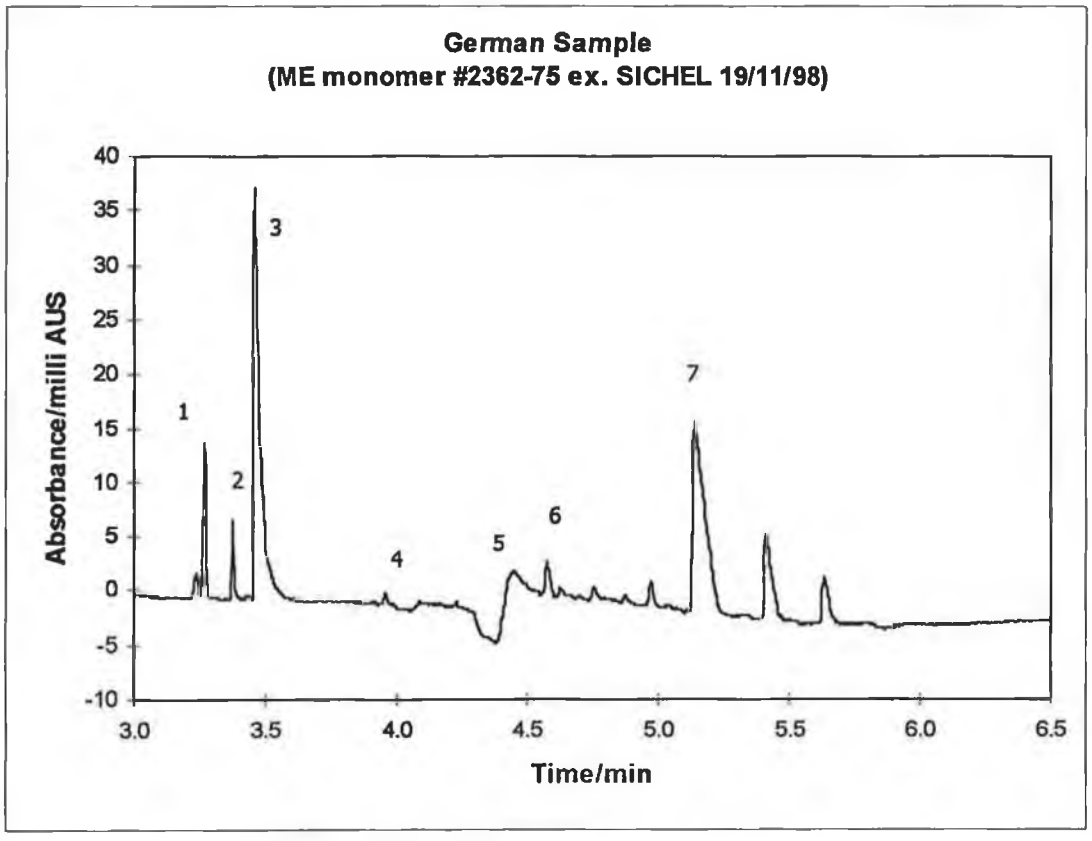


Figure 3.16. : The electropherogram shows the trace obtained for a methyl cyanoacrylate. Buffer electrolyte : 10 mM chromate, 0.25 mM EOF OFM-BT modifier, adjusted to pH 8.0 with 1 mM H_2SO_4, indirect detection @ 254 nm. Voltage 0-15 kV over 0.2 min, pressure injection 5 sec. The peaks are identified as : [1] chloride, [2] sulphate, [3] oxalate, [4] formate, [5] MSA, [6] cyanoacetate, [7] unidentified peak

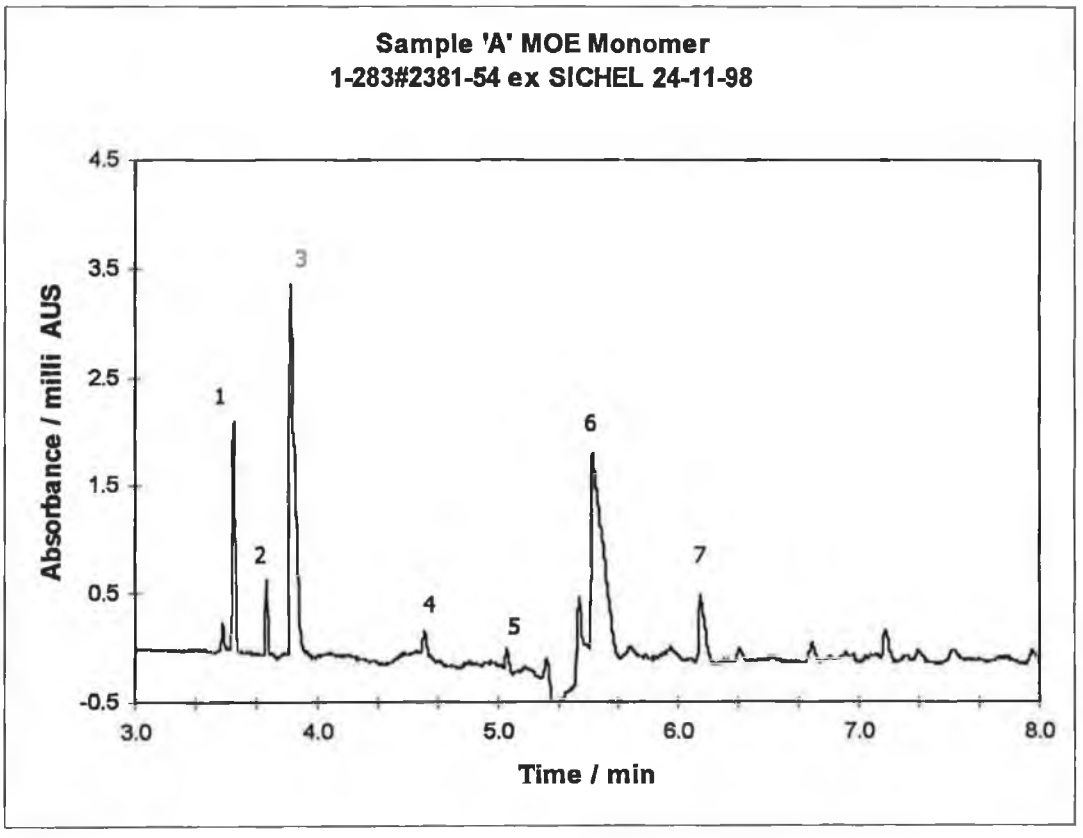


Figure 3.17. : An electropherogram of a German methoxy ethyl preparation. . Buffer electrolyte : 10 mM chromate, 0.25 mM EOF OFM-BT modifier, adjusted to pH 8.0 with 1 mM H_2SO_4, indirect detection @ 254 nm. Voltage 0-15 kV over 0.2 min, pressure injection 5 sec. The peaks are identified as : [1] chloride, [2] sulphate, [3] oxalate, [4] formate, [5] MSA, [6] cyanoacetate, [7] unidentified peak

Other lesser common components that are found in some cyanoacrylate adhesives include mono(methoxyethyl)phosphate and di(methoxyethyl)phosphate (Figure 3.18) and tri(methoxyethyl)phosphate (Figure 3.19).

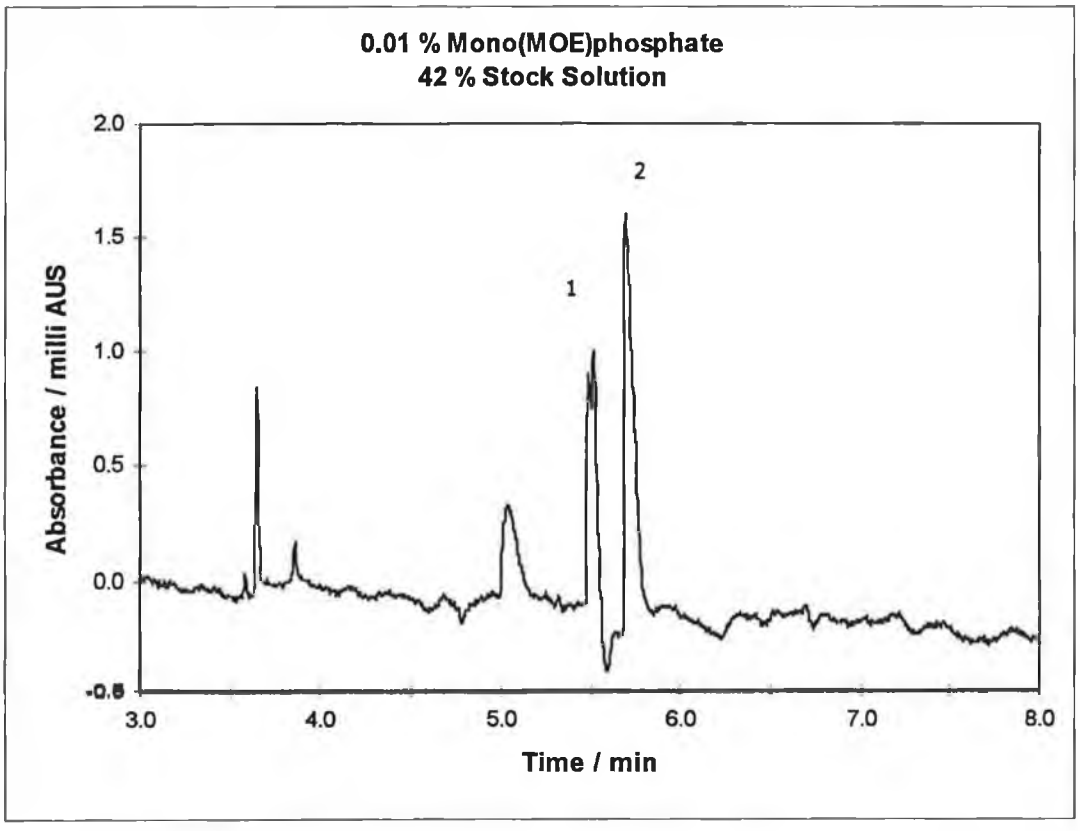


Figure 3.18 : The electropherogram shows a 0.01 % solution of a 42 % stock solution of Mono (methoxy ethyl) phosphate. The stock solution is composed of [1] mono (methoxy ethyl) phosphate and [2] di (methoxy ethyl) phosphate.

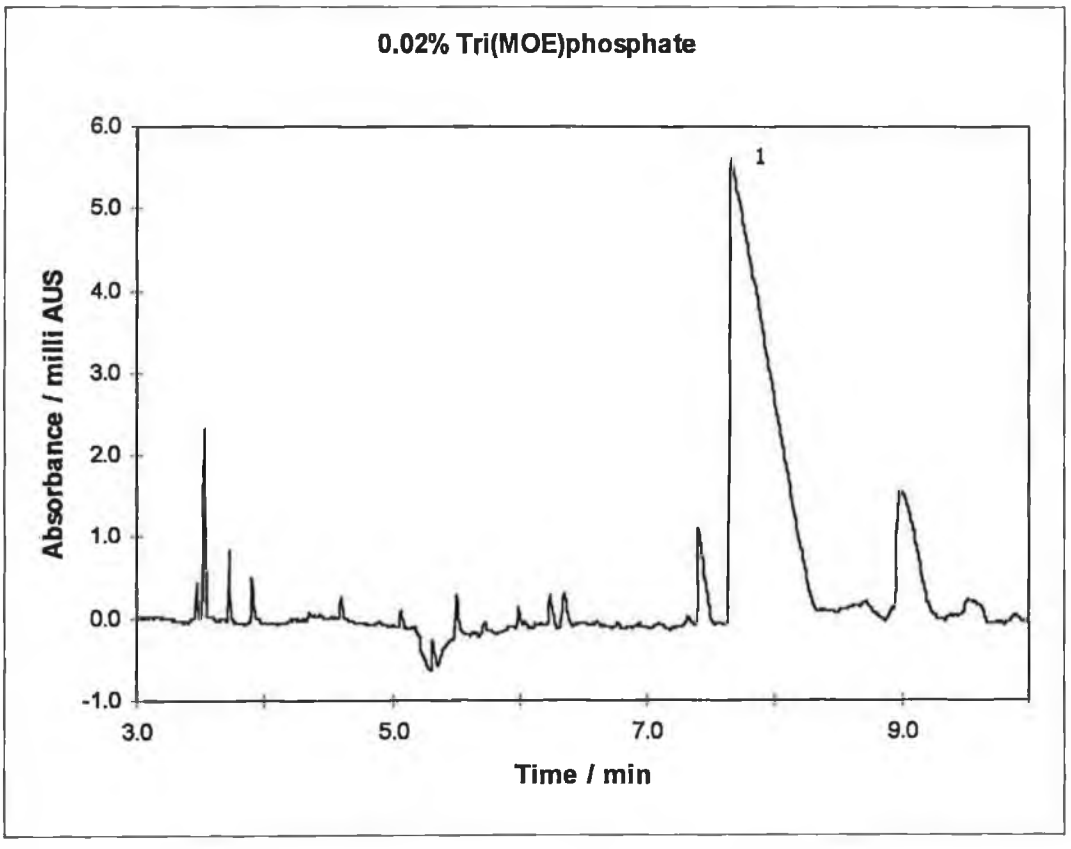


Figure 3.19 : The electropherogram shows a 0.02 % solution of a 90 % stock solution of Tri (methoxy ethyl) phosphate. The peak labelled [1] above is produced by the Tri (methoxy ethyl) phosphate.

3.5.3. Detection of Acidic Stabiliser Carryover

The production of cyanoacrylates consists of a number of different stages. The first stage, the Knoevenegel condensation, is depicted in Figure 3.1. This base-catalysed condensation reaction, between an alkyl cyanoacetate and formaldehyde, produces a cyanoacrylate oligomer, also known as a 'prepolymer'. This prepolymer has an average chain length of 12-16 units, with a molecular weight of approximately 1000 daltons. The second stage involves the thermal depolymerisation, also known as '*cracking*', of the oligomer under vacuum to produce the cyanoacrylate ester. In order to stabilise the highly reactive cyanoacrylate monomer, large amounts of acidic stabilisers are added to the vessel containing the oligomer prior to the depolymerisation process. The primary acidic species added is phosphoric acid (or in some cases phosphorous pentoxide).

The monomer that is produced as a result of this depolymerisation process is referred to as crude monomer. The next phase in the process involves the purification of the crude monomer. This is carried out via distillation or fractionation, under reduced pressure. The basic apparatus is depicted in Figure 3.20. The appropriate level of the final anionic stabiliser is also added at this point in the process.

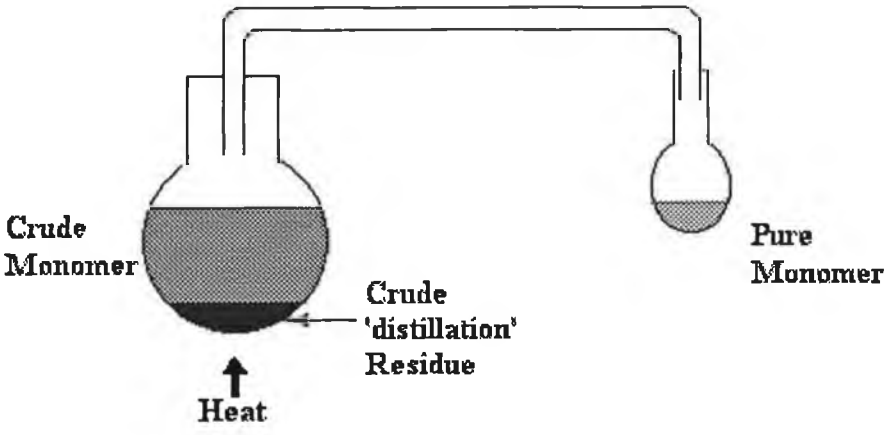


Figure 3.20. : Diagrammatic scheme of the distillation apparatus, also indicated are the different fractions.

The depolymerisation process, typically requires very high levels of phosphorous acid stabiliser, and hence there is concern over the possible carry over of the same, into subsequent stages. Should any phosphoric acid be carried over, it would also be of interest to determine in what form it exists. The possibility of phosphorous acid carry over is indicated by the presence of low levels of tri-ethyl phosphate in a purified monomer, when analysed by GC.

While tri-ethyl phosphate can be detected and analysed using GC, other more active forms, such as mono/di-alkyl esters of phosphorous acid, cannot be analysed by GC. These other forms are relatively involatile and are thus non-amenable to GC. HPLC-UV is ineffective in the detection of these compounds due to the lack of a chromophore. The lack of chromophores does, however, mean that indirect detection could be used to detect for the presence of these compounds, hence providing a possible application of the developed CE method in the detection of any acidic stabiliser carry over. IC equipped with a conductivity detector is another possible means of detection of the phosphate esters. Initial investigations revealed that the mono/di-ethyl phosphate gave discrete responses in the standard run of anions, completely resolved from all other peaks.

Due to the potential of the CE method, four Loctite world-wide samples were obtained and subjected to CE analysis. The four samples differed slightly from each other in terms of process chemistry and plant design, and also in terms of the phosphorous acid used. For example phosphorous pentoxide (P_2O_5) is used in one location, while the other three locations utilise phosphoric acid. Another difference between the samples is in the level of stabilisers used, Table 3.5. In each sample (from all locations) the distillation residue was recycled back into the initial condensation phase. The samples were analysed using the previously defined CE conditions. Samples analysed include 'crude' monomer fractions and distillation residues, as shown in Figure 3.21. –3.27. The anion concentrations were determined using the relevant calibration curve, with the exception of the diethyl phosphate. Due to limited sample availability, no calibration curve was prepared ; rather quantification was performed by comparison to a reference sample of diethyl phosphate.

Stage	IRL	PR	BR
Raw materials / kg			
Ethyl cyanoacetate	1.00	1.00	1.00
Para-formaldhyde	0.272	0.271	0.268
Distillation Residue	0.546	0.460	0.321
Pot/Crack / kg			
H3PO4	0.031	0.0	0.024
P2O5	0.0	0.036	0.0
PTSA	820 ppm	0.0	0.0
MSA	580 ppm	0.0	607 ppm
H2SO4	0.0	81 ppm	107 ppm
HQ	0.0017	0.0031	0.0013
Receiver/Crack / kg			
H2SO4	100 ppm	81 ppm	71 ppm
HQ	0.0008	0.0009	0.0012

IRL = Ireland, PR = Puerto Rico, BR = Brazil, HQ = Hydroquinone.

Table 3.5. : The different amounts of raw materials and stabilisers.

As only the diethyl phosphate concentrations were of interest, no concentrations for the other anions found in the samples are given. However, the anions are numbered in the electropherograms and identified in Table 3.6. The concentrations of the diethyl phosphate peaks are also given in Table 3.6.

Sample	IRL	IRL	BR	BR	PR	PR	PR
	Crude	Distillation	Crude	Distillation	Crude	Distillation	Reboiler
	Ireland	Ireland	Brazil	Brazil	P. Rico	P. Rico	P. Rico
Chloride	1	1	1	1	1	1	1
Sulphate	2	2	2	2	2	2	2
Oxalate	3	3	3	3	3	3	3
Formate	n/d	n/d	n/d	4	n/d	n/d	n/d
Succinate	4	4	4	5	n/d	n/d	n/d
Phosphate	5	5	5	6	n/d	n/d	n/d
MSA	6	6	6	7	n/d	n/d	n/d
Cyanoacetate	7	7	7	8	4	4	4
Diethyl	8	8	8	9	5	5	5
phosphate	2845	7382	1635	10111	115	1390	151
/ppm							

Table 3.6. : Peak identities and diethyl phosphate concentrations.

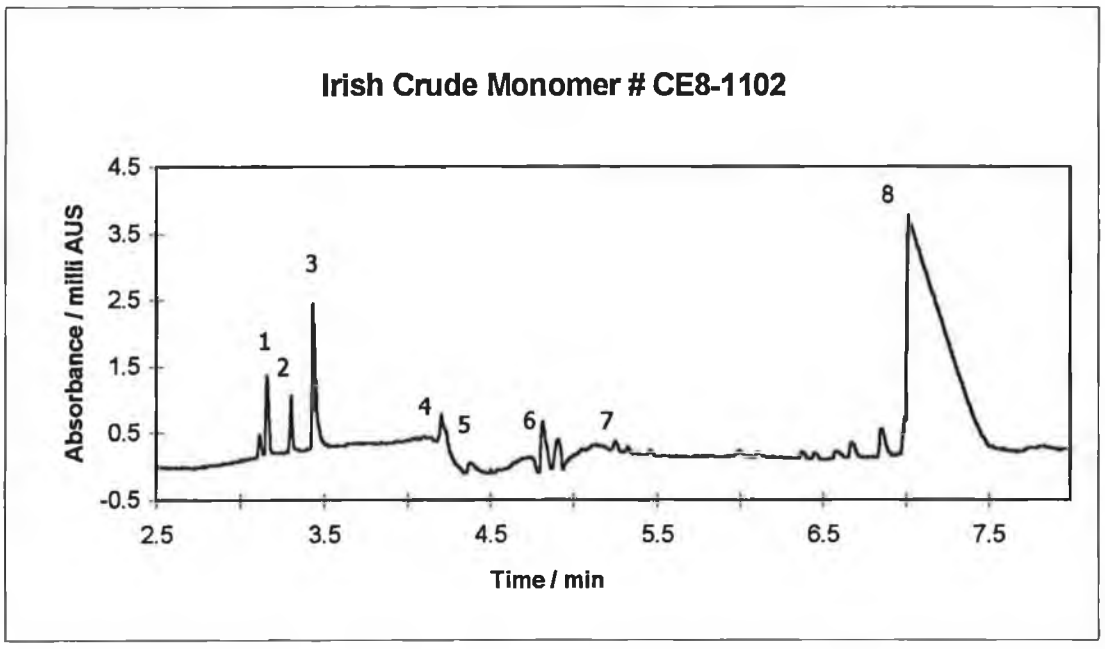


Figure 3.21. : CE analysis of an Irish Crude Monomer. Buffer electrolyte : 10 mM chromate, 0.25 mM EOF OFM-BT modifier, adjusted to pH 8.0 with 1 mM H_2SO_4, indirect detection @ 254 nm. Voltage 0-15 kV over 0.2 min, pressure injection 5 sec. The peaks are identified as : [1] chloride, [2] sulphate, [3] oxalate, [4] succinate, [5] phosphate, [6] MSA, [7] cyanoacetate, [8] diethyl phosphate.

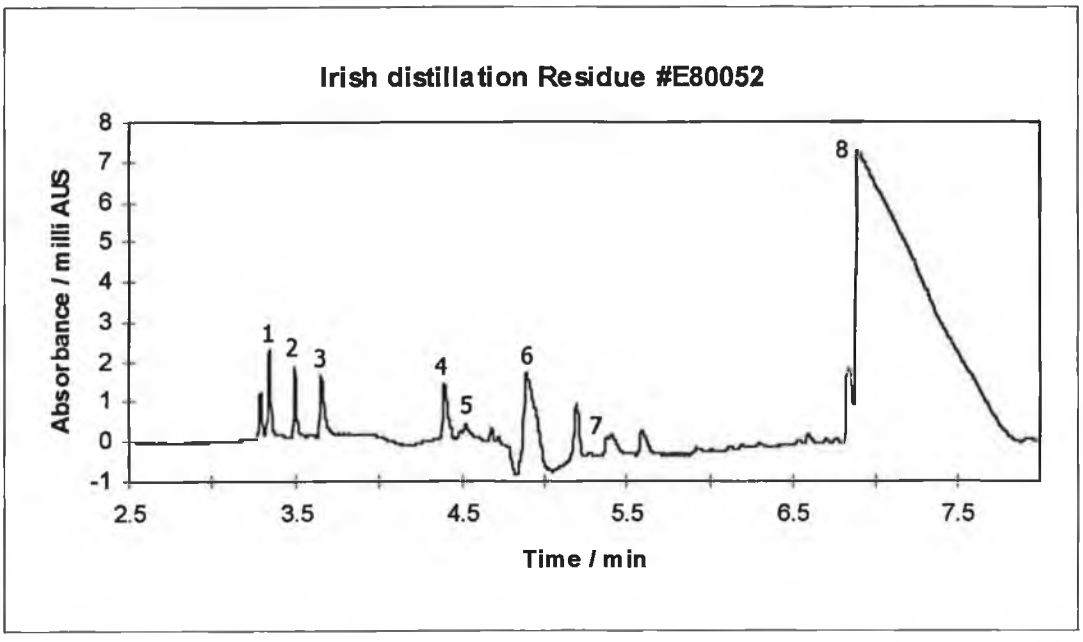


Figure 3.22. : CE analysis of an Irish Distillation Residue. Buffer electrolyte : 10 mM chromate, 0.25 mM EOF OFM-BT modifier, adjusted to pH 8.0 with 1 mM H_2SO_4, indirect detection @ 254 nm. Voltage 0-15 kV over 0.2 min, pressure injection 5 sec. The peaks are identified as : 1] Chloride, [2] sulphate, [3] oxalate, [4] succinate, [5] phosphate, [6] MSA, [7] cyanoacetate, [8] diethyl phosphate.

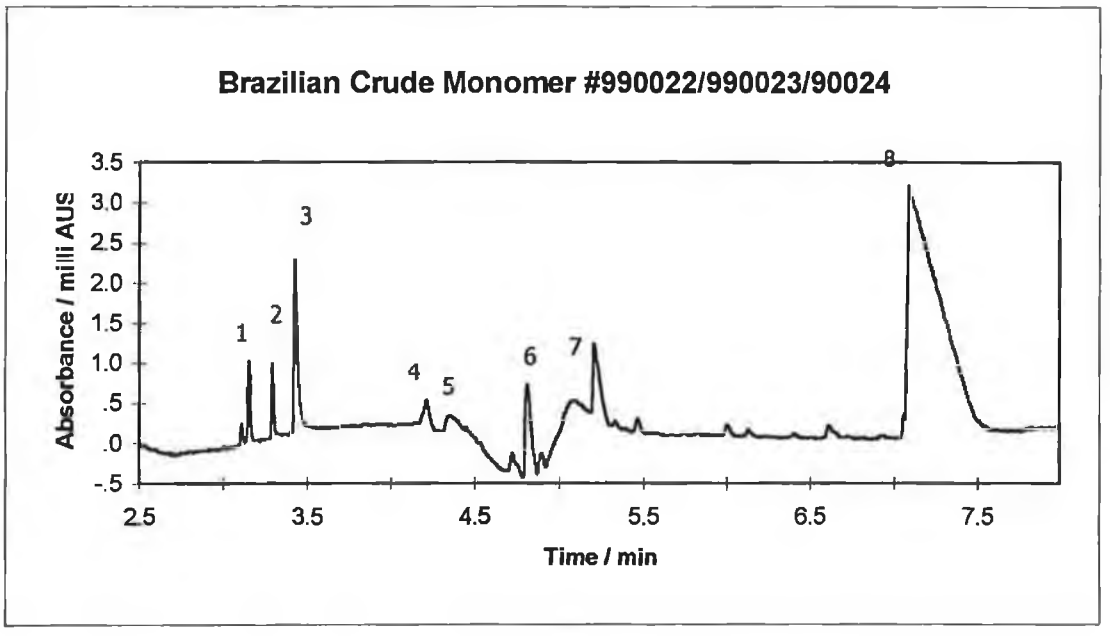


Figure 3.23. : CE analysis of a Brazilian Crude Monomer. Buffer electrolyte : 10 mM chromate, 0.25 mM EOF OFM-BT modifier, adjusted to pH 8.0 with 1 mM H_2SO_4, indirect detection @ 254 nm. Voltage 0-15 kV over 0.2 min, pressure injection 5 sec. The peaks are identified as : [1] chloride, [2] sulphate, [3] oxalate, [4] succinate, [5] phosphate, [6] MSA, [7] cyanoacetate, [8] diethyl phosphate.

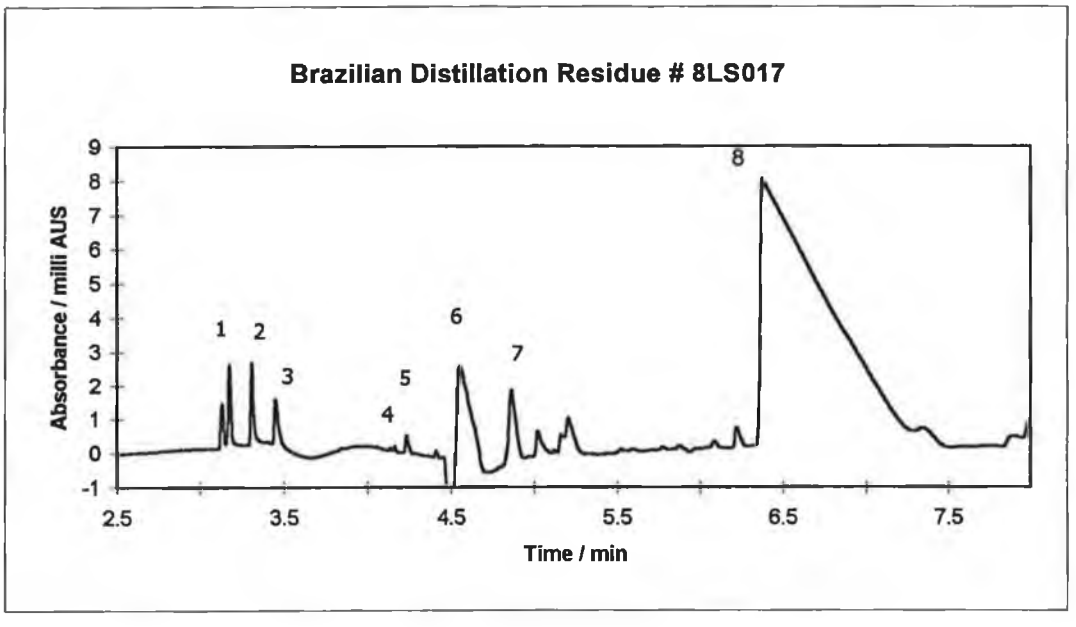


Figure 3.24. : CE analysis of a Brazilian Distillation Residue. Buffer electrolyte : 10 mM chromate, 0.25 mM EOF OFM-BT modifier, adjusted to pH 8.0 with 1 mM H_2SO_4, indirect detection @ 254 nm. Voltage 0-15 kV over 0.2 min, pressure injection 5 sec. The peaks are identified as : [1] chloride, [2] sulphate, [3] oxalate, [4] succinate, [5] phosphate, [6] MSA, [7] cyanoacetate, [8] diethyl phosphate.

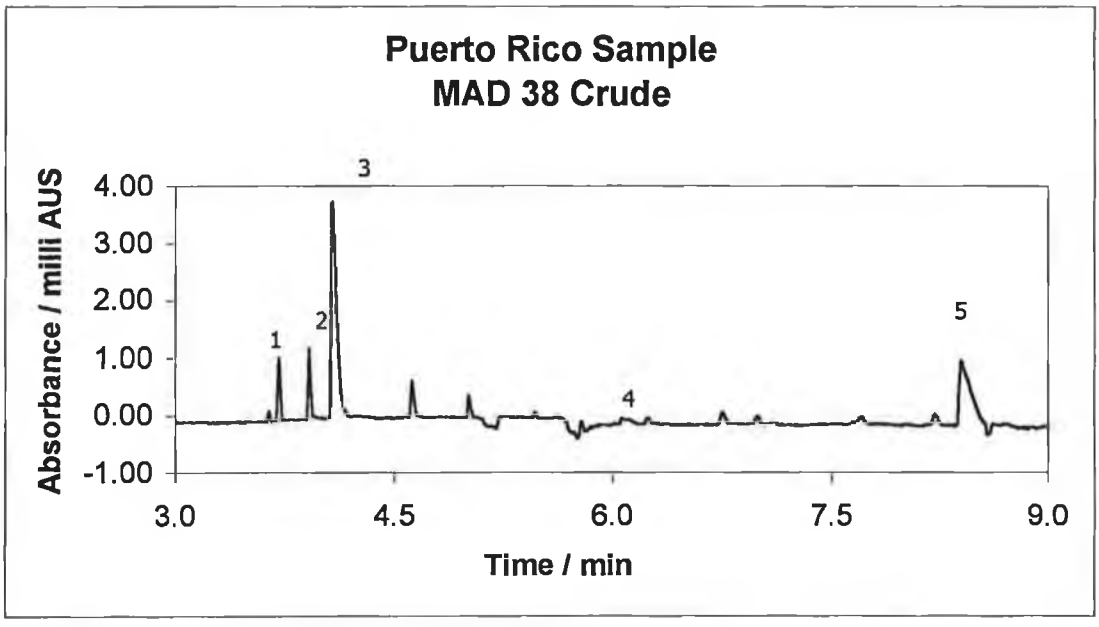


Figure 3.25. : CE analysis of a Puerto Rico Crude Monomer. Buffer electrolyte : 10 mM chromate, 0.25 mM EOF OFM-BT modifier, adjusted to pH 8.0 with 1 mM H_2SO_4, indirect detection @ 254 nm. Voltage 0-15 kV over 0.2 min, pressure injection 5 sec. The peaks are identified as : [1] chloride, [2] sulphate, [3] oxalate, [4] cyanoacetate, [5] diethyl phosphate.

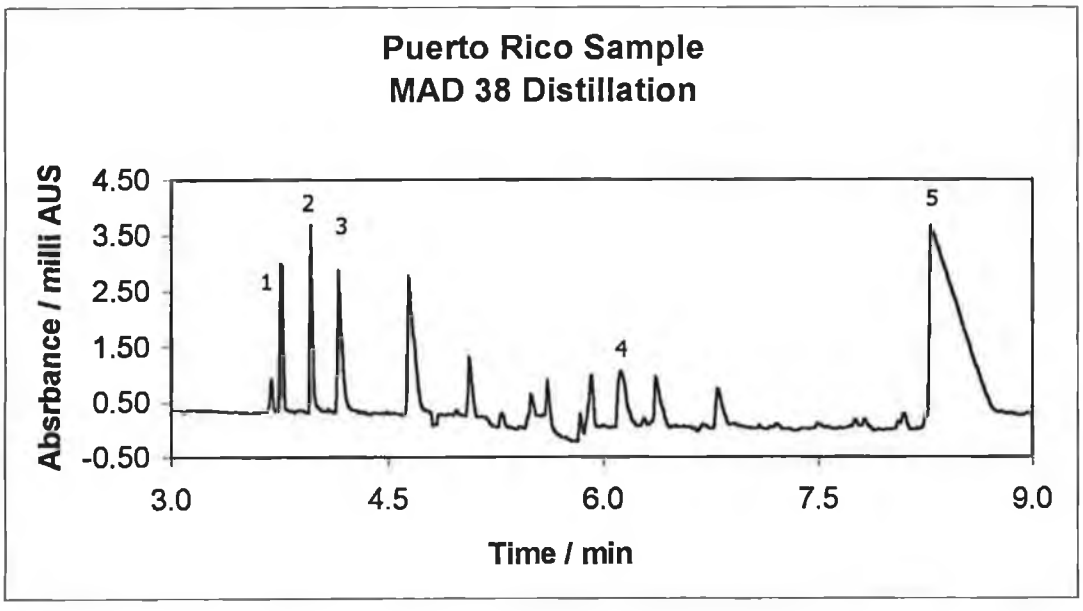


Figure 3.26. : CE analysis of a Puerto Rico MAD 38 Distillation Residue. Buffer electrolyte : 10 mM chromate, 0.25 mM EOF OFM-BT modifier, adjusted to pH 8.0 with 1 mM H_2SO_4, indirect detection @ 254 nm. Voltage 0-15 kV over 0.2 min, pressure injection 5 sec. The peaks are identified as : [1] chloride, [2] sulphate, [3] oxalate, [4] cyanoacetate, [5] diethyl phosphate

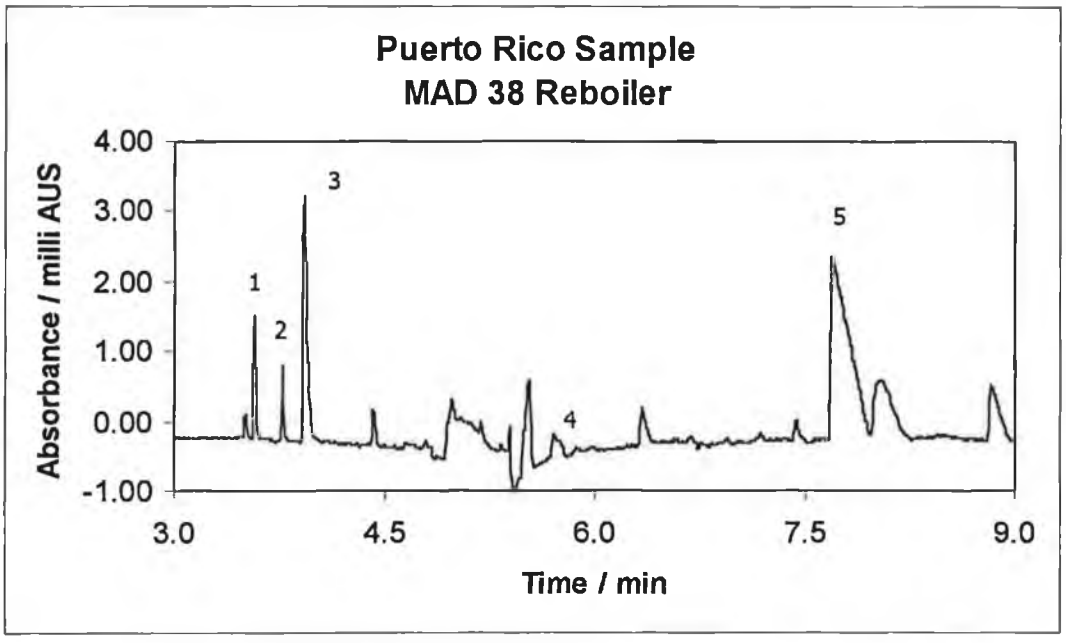


Figure 3.27. : CE analysis of a Puerto Rico MAD 38 Reboiler Residue. Buffer electrolyte : 10 mM chromate, 0.25 mM EOF OFM-BT modifier, adjusted to pH 8.0 with 1 mM H_2SO_4, indirect detection @ 254 nm. Voltage 0-15 kV over 0.2 min, pressure injection 5 sec. The peaks are identified as : [1] chloride, [2] sulphate, [3] oxalate, [4] cyanoacetate, [5] diethyl phosphate.

As can be seen from the concentration of diethyl phosphate in the Irish, Brazilian and Puerto Rican samples, there is a much higher concentration to be found in the Irish samples and in the Brazilian samples. There are a number of possible causes for the difference between the two sets of samples :

1) the Puerto Rico process uses phosphorous pentoxide instead of *o*-phosphoric acid which is used in both the Irish and Brazilian processes ;
2) a lesser amount of the distillation residue is recycled back into the Puerto Rico process ;
3) the depolymerisation process is terminated at a lower temperature in the Puerto Rico process, 185 °C compared to 190 °C in the Irish process ;
4) the Puerto Rico process uses significantly higher levels of hydroquinone at the depolymerisation phase. It has previously been established that the *o*-phosphoric acid can esterify the hydroquinone at the high temperatures used during the depolymerisation process.

3.6. DISCUSSION

The number of analytical techniques that are being investigated and used by Loctite in the analysis of anions found in cyanoacrylate adhesives, bears testimony to the importance of anion concentrations in the adhesive mixtures. The methods employed by Loctite (Irl.) in the analysis of anions include ICP-AES, polymerisation-related techniques, ion-chromatography and thermal studies.

The developed capillary electrophoretic method developed in this work compares well with the previously developed ion-exchange chromatography method [3]. In fact the CE method has a number of advantages over the IC method. For instance the overall run time in CE is 8 min, whereas in IC the run time is 22 min. In industrial applications, this saving of analysis time would be a considerable advantage. The CE method has proved to be capable of separating and detecting a greater number of anions than the IC method. Anions not resolved or detected by the IC method include fluoride, mono ethyl phosphate, diethyl phosphate and mono (methoxy ethyl) phosphate. The overall separation efficiency of the CE method is also superior to that of that of the IC method. All the anions analysed in the standard run (chloride, nitrate, sulphate, sulphite, oxalate, formate, phosphate, succinate, maleate, malonate, HPSA, MSA, cyanoacetate and phthalate) are baseline resolved; however, in the IC method a number of anion peaks were poorly resolved e.g. formate and HPSA peaks. Another consideration is that of cost; the quantity of solvents/chemicals used in CE is much less than in other forms of chromatography, such as IC, thus resulting in a considerable saving in cost. It has been found that the application of IC in the analysis of adhesives is rather costly, as the number of analyses that can be carried out using the one column before it must be replaced, is severely limited. Due to the high cost of these columns, this is a severe disadvantage. In contrast, the cost of the CE capillaries is relatively low. In terms of working ranges and limits of detection both techniques are quite comparable, with no major differences existing between the two techniques.

The applicability of CE to the analysis and quantification of the diethyl phosphate in the adhesive formulations is a major strength of the technique. IC has been found to be ineffective in the analysis of diethyl phosphate.

When compared to the other analytical techniques, such as the TAD test, DSCL and PW tests, the CE method has a number of advantages. The CE technique has the capacity to separate and detect, simultaneously, both strong and weak acids over a wide working range, with sufficient sensitivity for most acid concentrations found in cyanoacrylate samples. The sensitivity and concentration range for both types of acids are similar to each other. Furthermore the CE technique is a specific approach allowing both the identification and quantification of a considerable range of anions.

3.7. CONCLUSION

The method developed was found to be successful for the separation of a number of acids and a number of inorganic anions found in a typical cyanoacrylate adhesive. The developed method has been found to compare most favourably with the existing analytical techniques used in cyanoacrylate adhesive analysis. The advantages of the CE method include speed of analysis, lower cost and the ability to separate and quantify a wide range of organic and inorganic anions in a single run. Other methods, e.g. the TAD test, can only be used in the determination of the total amount of acid present, without distinguishing between the different types of acids.

The ability of the CE technique to identify and quantify the amount of diethyl phosphate in the formulation at different stages of the adhesive production process, as discussed previously, is potentially both useful and important. It presents the possibility to adjust the process conditions, such as reflux rates and pot temperature, to produce monomer with a significantly reduced level of process contaminant. Removal or reduction of these contaminants could results in a better quality of adhesive, in terms of stability and performance.

3.8. BIBLIOGRAPHY

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Chapter Four

Electrochemical Detection of Microcystins, Cyanobacterial Peptide Hepatotoxins, following High-Performance Liquid Chromatography

4.1. INTRODUCTION TO MICROCYSTINS

4.1.1. Background

Microcystins are a class of cyclic heptapeptides produced by some species of cyanobacteria (also known as blue-green algae), such as *Microcystis*, *Oscillatoria*, *Anabaena* and *Nostoc*. These cyclic heptapeptides are known to be potent hepatotoxins [1]. Related toxins are produced by the cyanobacterium species *Nodularia spumigena*, which is found in brackish water and by the sponge *Theonella swinhoei*, found in marine waters. Both of these species produce cyclic pentapeptides, which are also known hepatotoxins [2]. Both classes of hepatotoxins have a LD_{50} of 50-500 $\mu g\ kg^{-1}$, using the mouse i.p. [3, 4, 5] technique, in which the mice are injected intra-perinatally, i.e. injection into the lung area.

There is much concern over the presence of these microcystins in drinking water, both for human and animal consumption. Over the past few years there has been increasing evidence that these microcystins act as tumour promoters; their toxic effect being attributed to the inhibition of protein phosphatases, 1 and 2A [6, 7]. Lambert et al. [8] have carried out a comprehensive review into their effects on health. The inhibition of these phosphatases results in the collapse of the cell cytoskeleton and also interferes with the signal transduction mechanism in the cells (reversible phosphorylation) [9]. Reverse phosphorylation is responsible for controlling many biological processes, e.g. muscle contraction, metabolism and cell division. The pentapeptides produced by *Nodularia* are not only tumour promoters but are also liver carcinogens [2]. The occurrence of microcystins is world-wide, and there have been many reports of both human and animal illness as a result of the consumption of microcystin-containing waters. Consequently, it is important to remove microcystins from drinking water. There are, however, difficulties in the removal of microcystins from drinking water supplies, as conventional water treatment procedures (coaggulation, sedimentation and chlorination) have proven to be ineffective. Other means of microcystin removal are being investigated, and these methods include the use of activated carbon, ozone, UV light and free chlorine (for a review see reference [10]). Part of the difficulty in the

removal of microcystins is that they are extremely stable, e.g. microcystin-LR has been found to have a half life ($t_{1/2}$), at 40°C, of 3 to 10 weeks, in aqueous solutions of pH 1 to 9, respectively [11]. Watanabe et al. [12] investigated the stability of microcystins upon decomposition of the algae cells. The algae cells were grown in two types of media, distilled water and standard culture medium and then placed in the dark (at 24°C). Decomposition of the cells then began and the microcystins were released. It was found that the microcystins remained stable in the water for up to 42 days, after the decomposition of the cells. This decomposition occurred in the absence of any other bacteria. It is not known what effect, if any, they would have on the microcystin decomposition.

2.1.2. Structure

The general structure of microcystins is cyclo(-D-Ala-**X**-D-*erythro*-β-methylisoAsp-L-**Z**-Adda-D-iso-Glu-N-methyldehydroAla). The **X** and **Z** represent two variable amino acid residues found in these positions. The Adda moiety is an unusual C_{20} amino acid, 3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 6-dienoic acid [2,13]. Although, there are about 50 known analogues of microcystins, they all share the same cyclic backbone structure. The main structural changes, in the microcystins is to be found in the 2- and 4-amino acid residues. The established nomenclature of the microcystins refers to these variable amino acid residues by a two-letter suffix. For example microcystins containing the amino acid leucine is represented by -L, arginine by -R and tyrosine by -Y. Some microcystins occur with desmethylated residues at position 3 and 7, and these are referred to as their corresponding desmethylated forms e.g. 7-desmethyl microcystin-LR (Figure 4.1). Other variations that occur include variations in the Adda moiety both in terms of its stereochemistry and desmethylated forms. Serine containing moieties have been found at position 7, whereas alanine (-A) and methionine (-M) have been found in position 4. Alanine and methionine can be found in combination with the leucine and/or arginine and/or tyrosine residues e.g. microcystin-LA, microcystin-YA, microcystin-YM and microcystin-YR. The amino acid structures can be seen in Figure 4.2.

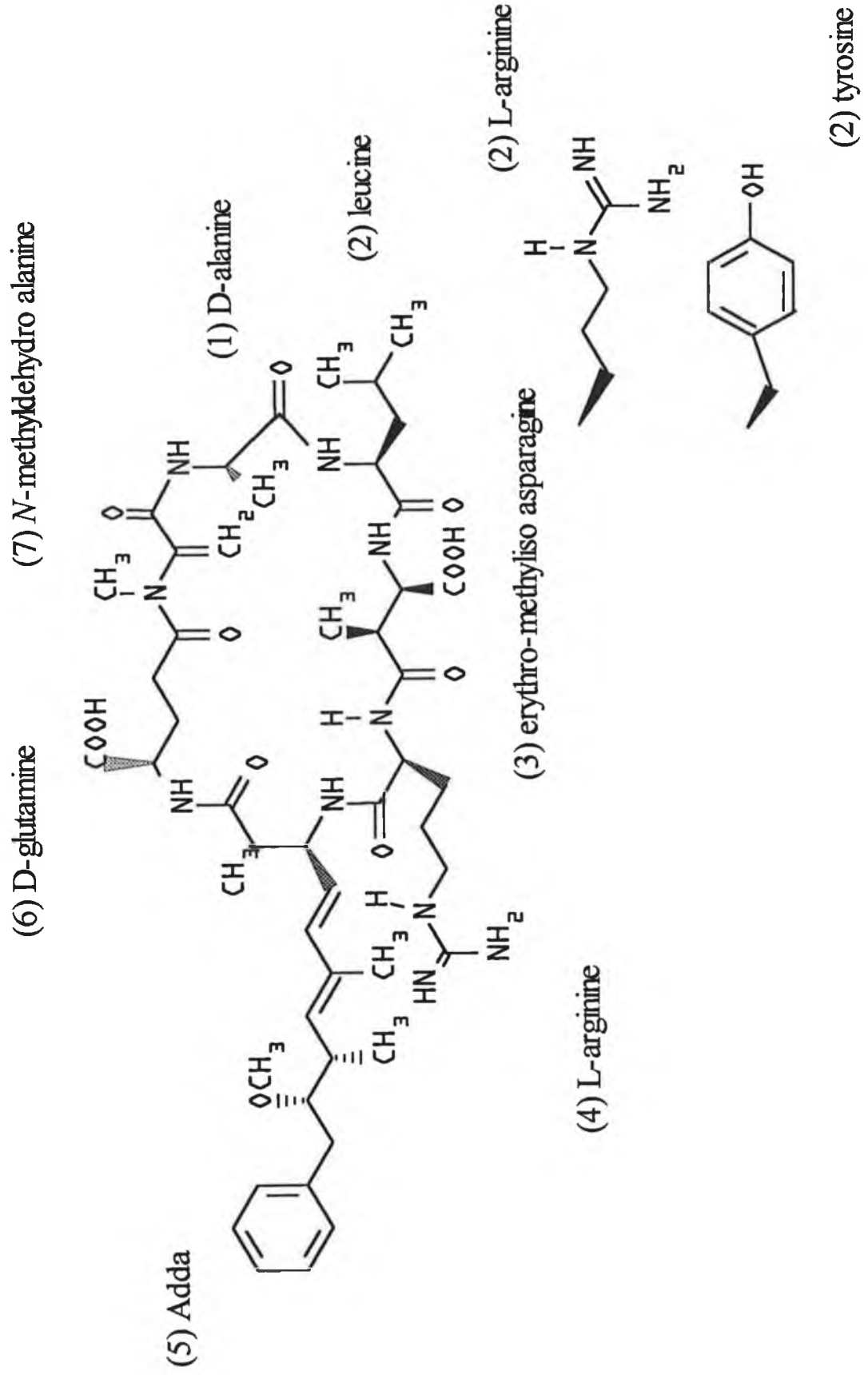

Figure 4.1 : Structure of microcystin-LR.

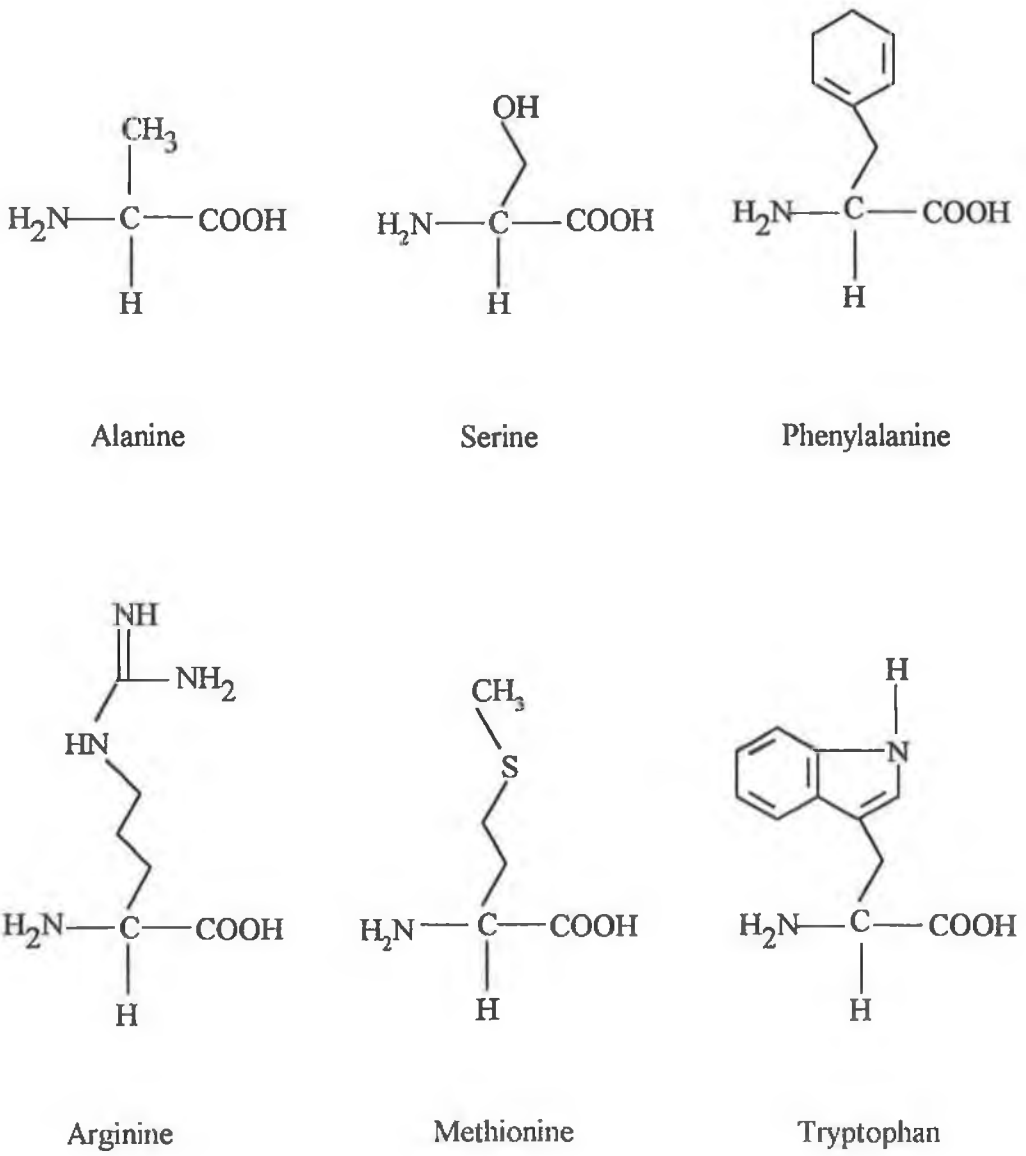

Figure 4.2 : Structures of the various amino acid residues found in the analogues of microcystin LR.

4.2. ANALYSIS OF MICROCYSTINS

4.2.1. Methods

There are numerous separation methods for the analysis of microcystins, including high performance liquid chromatography (HPLC) [14, 15] and capillary electrophoresis (CE) [16, 17], both equipped with a range of spectrometric detectors (e.g. ultra-violet (UV), mass spectrometric (MS) and fluorescence), thin layer chromatography (TLC) [5,18], enzyme-linked immunosorbent assay (ELISA) [19, 20, 21], mouse bioassay [22] and water bioassay tests [23]. Recently there has been some interest in the use of plants as a means of microcystin isolation and assay [24]. Prior to analysis of the microcystins from cyanobacteria samples it is often necessary to utilise some form of an extraction and clean-up procedure. Commonly used clean-up techniques include solid-phase extraction (SPE), preparative HPLC and flash chromatography.

4.2.1.1. Extraction/Clean-up Procedures

The first step in the clean-up procedure is the extraction of the actual microcystins from the bacteria cells. The bacteria cells are surrounded by a thick cell wall ; therefore the cells must be lysed in order to release the microcystins into solution. Commonly used lysing procedures include freeze-drying, freeze-thawing, stirring and sonication. Cyanobacteria samples are by definition protein rich and as high protein content samples are hard to deal with (chromatographically speaking), and their removal is an important aspect of the purification procedure. The aim of the lysing procedure is not only to extract the microcystins, but it should also minimise the amount of extraneous pigments and protein material extracted.

Harada et al.[25] used an extraction medium of 5 % (v/v) acetic acid in water, which is quite efficient in protein elimination (approximately three times less protein is extracted when compared to extraction with water [26]). Methanol extraction and *n*-butanol-methanol-water extraction has been used by various groups [27, 28]. Lawton et al.[28] compared the extraction performance of the above solvents and found that

methanol was the most effective extraction solvent. They advocated the use of methanol over *n*-butanol-methanol-water, not only due to the slightly higher extraction efficiency of microcystins, but also due to the ease of evaporation of methanol when concentrating the extract. 5 % (v/v) acetic acid was also found to be poor in extracting the hydrophobic microcystin variants (microcystin-LY, microcystin-LW, microcystin-LF). Although the published method involved repeated extractions (three extractions), freeze-thawed material was used without any sonication or stirring during the extraction procedure. Meriluoto [26] has found that the extraction of microcystins was more consistent using freeze-dried material with sonication. The absence of sonication in Lawton's method may also explain why the extraction efficiency of microcystins was observed to be biomass independent, as the extraction process was likely to be a cell leaching process (the filters containing the alga cells were simply soaked in the extracting solvent for one hour without any stirring or agitation). Another solvent system used in extraction of microcystins is distilled water. This was used by Jones et al.[29] when analysing dried algae bloom crusts.

Although the Lawton [28] paper reported that 100% methanol was the best extracting solvent, there are a number of other papers to be found that advocate the use of other solvent systems. As mentioned above, Harada's preferred extraction system was 5% (v/v) acetic acid in water. Distilled water was compared to *n*-butanol-methanol-water mix by Gathercole and Thiel [30], who found that the *n*-butanol-methanol-water mix was the better extraction medium. Interestingly, a similar comparison was made by Wicks and Thiel [38], and in contrast they preferred the water extraction.

Moolan et al.[31] performed a comparable study to that of Lawton et al.[28]; however they questioned the reliabilty of the method used by these workers. Points raised by Moolan et al. included, i) the possible appearance of additional peaks in the HPLC chromatogram as a result of the leaching of material from the trifunctional C_{18} SPE cartridge, and ii) the possibilty of coeluting organic compounds, which would make identification of microcystins difficult when using the UV spectra. In the analysis of microcystin-containing waters, chlorine must be removed, as the presence of chlorine can result in low microcystin recovery. Moolan et al. found that the use of sodium

sulphite and trifluoroacetic acid (TFA) to remove chlorine, as used by Lawton, resulted in inconsistent microcystin recoveries. The highest recovery percentage was obtained using sodium sulphite without any TFA. A slightly lower recovery (83%) was obtained when no soduim sulphite or TFA was used. It was not possible, however, to attribute the higher recovery to the omission of TFA, as microcystins are in fact stable in TFA solutions.

The second step in the clean-up procedure is usually based on solid-phase extraction (SPE). Although there are many different types of SPE cartridges available, including C_{18} ODS, cyano, trimethylamino, cyanopropyl, aminopropyl, diol, silica gel and carboxymethyl cartridges, the most commonly used are the C_{18} ODS cartridges. Tsuji et al.[32] investigated the use of the above cartridges (except C_{18} ODS) in microcystin purification, and it was found that with the exception of the diol and silica gel cartridges, the microcystins were not adsorbed onto the cartridge packing, and even then the microcystins absorbed only weakly onto the diol cartridges. Differences also exist between the different C_{18} ODS cartridges. For example Harada et al.[14] compared three different commercially available cartridges, namely Baker 10 C_{18} (J. T. Baker, USA), Bond Elut C_{18} (Analytichem International, USA) and Sep-Pak C_{18} (Waters Assoc., USA). It was reported that the Baker cartridges were superior in their microcystin adsorptive power, followed closely by Bond Elut cartridges and finally the Sep-Pak cartridges.

SPE cartridges consist of fine porous silica particles which allow particles less than a certain size to enter the pores; any particles greater than this size are excluded and pass through the cartridge more rapidly. The smaller particles enter the particles and so elute more slowly. It is thus possible to use SPE to remove the larger proteins from the microcystins in the sample. This makes SPE an ideal first step in the purification of microcystins, as there is a naturally high protein content present in samples due to the fragmentation of the algae cell wall. The main advantages of SPE is twofold: i) it serves to purify the microcystin samples, and ii) the microcystin-containing fractions can be collected and concentrated. Following SPE, the collected

fractions containing the microcystins are concentrated (using rotary evaporation) and the resulting solution then further purified using preparative HPLC.

4.2.2. Flash Chromatography

Edwards et al. [33] have developed a purification method involving the use of flash chromatography as an alternative for SPE. The starting material was 7 L of cyanobacterial scum which equated to approximately 300 g dry weight cyanobacterial cells. This sample was extracted in methanol, centrifuged and the pellet formed re-extracted (repeated twice). The extracts were then combined and rotary-evaporated. After dilution with methanol and water, the extract was filtered through glass fibre filters in preparation for flash chromatography loading. Two different stationary phases were investigated, namely spherical Hyperprep C_{18} (30 μm particle size, 120 Å) and irregular Bondapak C_{18} (37-55 μm particle size, 125 Å), both in 9 x 7.5 cm (i.d.) cartridges. The extract was then applied to the pre-conditioned column at a flow rate of 100 ml min^{-1} at a pressure of 80 psi. A step gradient eluent, from 0 to 100 % methanol, was utilised. The fractions were collected in 200 ml fractions - those containing microcystins were determined by absorbance at 238 nm (microcystins have a peak absorbance at 238 nm). The eluted fractions were pooled together into three fractions in accordance to their polarity. The fractions were then applied to a preparative HPLC C_{18} column (15 x 0.46 cm, 12 μm) with an isocratic mobile phase of ammonium acetate and trifluoroacetic acid, in order to further purify the microcystins. In this investigation, the Hyperprep stationary phase proved to be the best for the separation of the microcystins.

The performance of flash chromatography compares well to SPE ; in fact it would probably surpass it in terms of speed and ease of use, although the initial cost of the apparatus is relatively high (relative to SPE). This flash chromatographic method can also separate the microcystins into three broad groups based upon their polarities, a feature not available when using SPE. However, the main advantage of this technique is the speed and ease with which large samples can be dealt.

4.2.3. Preparative HPLC

Preparative HPLC is a popular technique for the final step in the purification of microcystins, as very pure microcystin samples can be obtained (to the order of 95% purity). Two of the most popular mobile phases in preparative HPLC are trifluoroacetic acid with methanol [11] and ammonium acetate with acetonitrile [34, 35, 36]. Harada et al. [11, 25] have advocated the use of SPE using 5% acetic acid for the extraction procedure, followed by preparative HPLC using a mobile phase of trifluoacetic acid and methanol.

Another liquid chromatography technique used in the purification of microcystins is fast protein liquid chromatography (FPLC). This is a medium pressure technique similar in concept to HPLC. Cremer and Henning [37] developed a purification method involving aqueous extraction, C_{18} SPE, quaternary methylamine anion exchange chromatography followed by FPLC. Their method was successfully applied to the purification and separation of microcystin-LR and 3-desmethyl-microcystin-LR. A novel mobile phase, in terms of microcystin purification, was used. It consisted of acetonitrile with water and pentafluoropropionic acid (PFPA) as an ion-pairing agent. This mobile phase was compared to a similar mobile phase containing TFA instead of PFPA. They found PFPA to be superior to TFA in the resolution of the above two microcystins (no chromatograms were shown).

4.3. ANALYTICAL CHROMATOGRAPHIC TECHNIQUES

Perhaps the most commonly used analytical technique for the analysis of microcystins is HPLC, due to the wide selection of operating conditions available, including different columns and mobile phases. Other analytical techniques available for microcystin analysis include capillary electrophoresis, gas chromatography and thin layer chromatography .

4.3.1. HPLC

HPLC has long been used in microcystin analysis, even as early as the 1970's. In the 1980's Guo et al.[38] developed a set of coefficients which could be used in the prediction of the retention times of peptides (based on the contribution of the different amino acids found in the proteins). This method, although successful in the prediction of retention times of short linear peptides, could not be extrapolated to microcystins due to their cyclic structure, which influences their retention times. The coefficients can be used, however, to give an indication to the retention order for the actual microcystins. Guo also compared the separation of microcystins using different mobile phase modifiers, e.g. iso-propanol (IPA), acetonitrile (ACN) and methanol (MeOH). It was found that an acetonitrile-based mobile phase was the best overall mobile phase to use. Consequently, the mobile phase used in their work consisted of water-acetonitrile-0.1% TFA, with gradient elution, carried out on a number of different reversed phase (RP) columns.

Another early study carried out by Siegelman et al.[5] used a HPLC method with a mobile phase of acetonitrile, 0.5 mM ammonium acetate (26:74 v/v) and a detection wavelength of 238 nm. The column used was a Hyersil column, (50 mm x 4.6 mm, 3 μm). The resultant chromatogram showed sharp well resolved peaks, although only microcystin-LR was analysed.

Harada et al.[14] performed work into the use of acidic mobile phases and their effect on the separation of microcystins. Three different mobile phases were tested namely, methanol : 0.05 % TFA (6 : 4), methanol : 0.05 M phosphate buffer (pH 3) (6 : 4), and methanol : 0.05 M sodium sulphate (1 : 1). They used two different columns, namely Nucleosil $3C_{18}$ (75 mm x 4.6 mm, 3 μm) and $5C_{18}$ (150 mm x 4.6 mm, 5 μm), with the usual detection wavelength of 238 nm. They found that the use of acidic mobile phase conditions influenced the retention of microcystins - the more acidic the mobile phase the longer the retention of the microcystins. In fact the capacity factors for both microcystins -LR and -RR at pH 2 were approximately 4 times as long as that at pH 6.

Work done with acetonitrile and TFA mobile phases has been carried out by many groups [20, 28, 39]. Lawton et al.[28] separated nine microcystins and one nodularin using a gradient mobile phase of water-0.05% v/v TFA to acetonitrile-0.05% TFA on a μBondapak C_{18} column (300 mm x 3.9 mm). The resolution between the peaks was good and the overall peak shapes were sharp, even though, the actual run time was a little on the long side (~40 min), but, the chromatographic was excellent (see Figure 4.3). The microcystins separated includes [D-Asp^3] microcystin-RR, microcystin-RR, -YR, -LR, -FR, -LA, -LY, -LW and finally microcystin-LF, (L, R = as before, Y = tyrosine, A = alanine, F = phenylalanine, W = tryptophan). It would be very interesting to see where the desmethylated microcystins would elute on their system, as there is certainly room for more peaks to elute and still allow for good separation between the peaks.

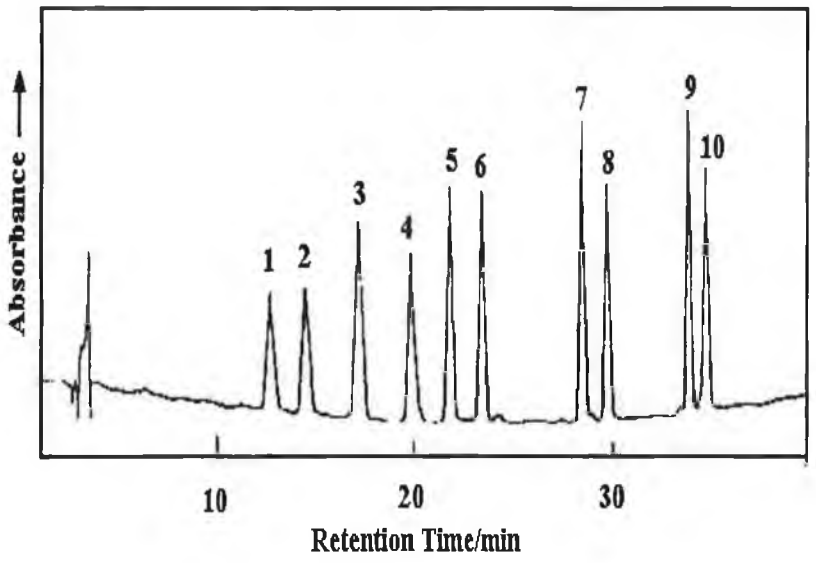

Figure 4.3 : Chromatogram of the separation of a mixture of microcystins and a nodularin. (Reproduced from ref. 5). 1=[D-Asp^3]microcystin-RR, 2=microcystin-RR, 3=nodularin, 4=microcystin-YR, 5=microcystin-LR, 6=microcystin-FR, 7=microcystin-LA, 8=microcystin-LY, 9=microcystin=LW, 10=microcystin-LF

Jones et al.[29] successfully separated a number of putative microcystins using a linear gradient mobile phase of acetonitrile : 0.008 M ammonium acetate, although good separation was obtained, the microcystin masses did not correspond to any previously identified microcystins.

4.3.1.1. Internal Surface Reversed-phase (ISRP) HPLC

The Internal Surface Reversed-phase (IRSP) column was originally developed by Pinkerton and Hagestam with a view to analyse drugs in plasma by direct injection onto the column. The packing material consists of narrow pore silica particles, internally coated with a glycine-L-phenylalanine-L-phenylalanine (GFF) partitioning phase, which is bound to the silica particles via glycerylpropyl moieties. The two phenylalanine residues have been removed from the external surfaces leaving just the hydrophilic glycine residue on the external surface (see Figure 4.4). The retention mechanism of the column is quite interesting as it is essentially multifunctional. Firstly, the narrow pores of the silica particles prevent the entrance of molecules of a

molecular mass greater than ~5000 Da (depending on their structure and shape); thus these large molecules will elute most rapidly. Secondly, the GFF phase resembles a reversed-phase surface and is selective for aromatic containing compounds. Finally, the free carboxylic group of the terminal phenylalanine group serves to act as a weak cation-exchanger within certain mobile phase environments. The typical mobile phases used with the column are neutral or slightly acidic in nature and usually contain 20 % or less organic modifiers. Phosphate buffer is typically used with a molarity of around 0.1 M [40].

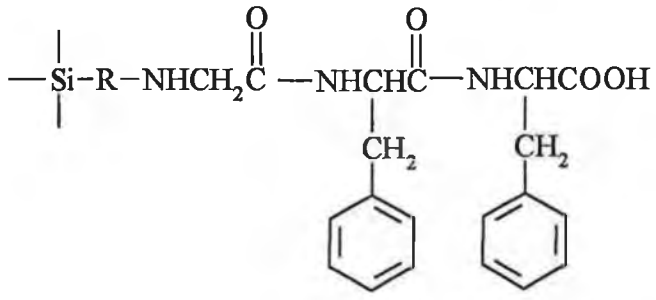

Figure 4.4 : The ISRP partitioning phase. The silane bound residue, on the left, is bound to the glycine moiety, which is in turn bound to the two phenylalanine residues.

Meriluoto et al.[40] have investigated the use of ISRP chromatography in analysing microcystins. The retention and selectivity of microcystins was studied in 0.1 M phosphate buffer with 10 %-15 % organic modifier (acetonitrile, tetrahydrofuran (THF) and isopropanol). This study also compared the retention of microcystins at neutral pH (6-8) and at acidic pH (2-4). At neutral pH good separation was achieved for microcystin-LA, -LR, -YR and -RR in that elution order. This shows that the more basic microcystins (those containing the arginine moiety) are retained longer on the column. Although the ISRP column is known to be more selective for phenyl groups, the microcystin-YR (contains two phenyl groups, one in the Adda chain and the other in the tyrosine,-Y, residue) elutes before microcystin-RR. This is due to the effect of the cation-exchange effect of the ISRP material at neutral pH values. In an acidic environment the free carboxylic group of the terminal phenylanaline is protonated and so the cation exchange effect is essentially suppressed and so the reversed-phase nature of the ISRP material is predominant. The elution order of the

microcystins in this case is microcystin-RR, -LR, -YR, -LA. However, when this result was compared to a similar experiment using a reversed-phase (RP) column, Nucleosil C_{18}, at pH 3, methanol : 0.1 M phosphate buffer, pH 3.0 (58 : 42), it was found that the relative elution order was different. On the RP system the microcystin-LR and -YR eluted in reverse order to that on the ISRP system. This indicates the selectivity of the ISRP for aromatics even in its 'reversed-phase mode'. A comparison between acetonitrile, THF and isopropanol found that the acetonitrile gave the highest number of theoretical plates for the column. This means that the use of acetonitrile in the mobile phase instead of THF or isopropanol will produce the sharpest peaks and the best resolution. This agrees with previous findings by Guo et al. [38] , using a C_{18} column. However, the best selectivity was achieved with THF at pH 2.0, with the separation of the three analogues : 7-desmethylated microcystin-RR, 3-desmethylated microcystin-RR and microcystin-RR.

4.3.2. Modes of Detection

There are several modes of spectroscopic detection that can be used in conjunction with HPLC and CE in order to detect microcystins, including UV, MS and fluorescence.

4.3.2.1. Ultraviolet Detection

The main chromophore of the microcystins has been determined to be the conjugated Adda residue [5]. The typical wavelength used in the detection of microcystins is 238 nm (wavelength of maximum absorbance); however, the microcystin-WR, containing tryptophan has a second absorption peak at 220 nm (Figure 4.5). There also exist isomers of microcystins known as 6(Z)-Adda isomers; these isomers of microcystin-LR and -RR have an absorption maximum at 242 nm instead of 238 nm. This difference is a very useful means of determining if any of these isomers are present in a sample (this is easily done using a photo diode array (PDA) detector, or

alternatively by using a fixed wavelength detector and running the sample at 242 nm and at 238 nm and comparing the absorbances).

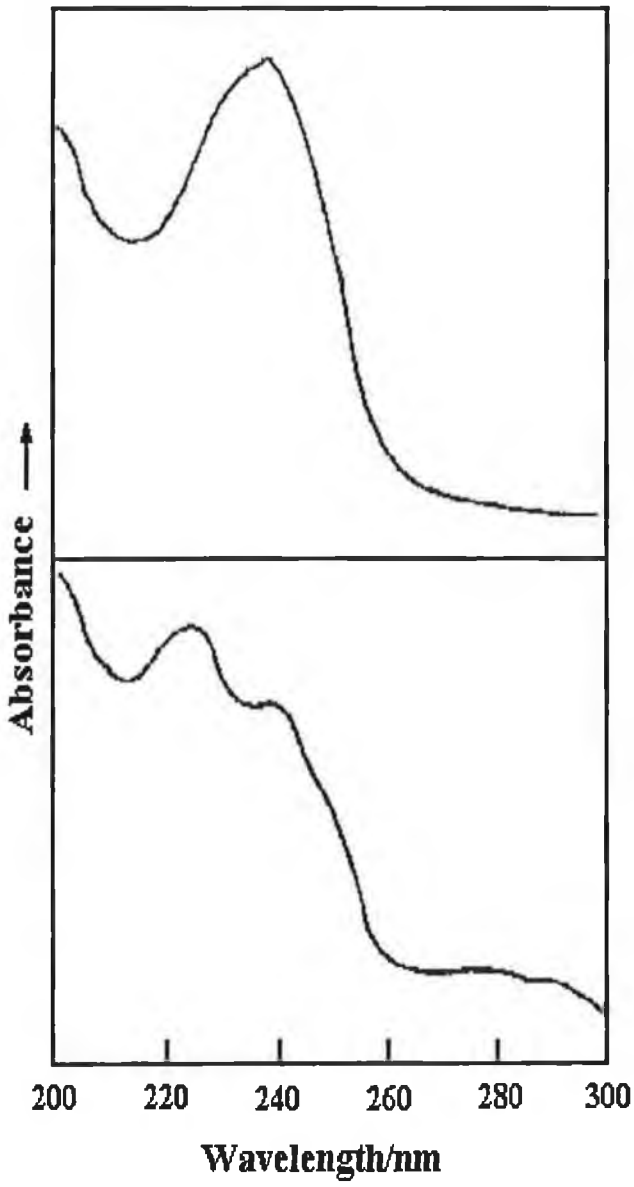

Figure 4.5 : UV spectra of some microcystins. The upper trace is typical for most microcystins, the lower trace is typical for microcystin variants containing tryptophan.

Harada et al.[41, 42] have done much work in the determination of molar extinction coefficients. The molar absorptivities of microcystin-LR, -YR and -RR is 39800 mol

l^{-1} cm at λ=238 nm. 3-desmethyl microcystin -LR has a molar extinction coefficient of 31600 mol l^{-1} cm at λ=238 nm while 7-desmethyl microcystin -LR has a coefficient of 46800 mol l^{-1} cm, again at λ=238 nm. All extinction coefficients are reported for microcystins in methanol.

UV absorbance is an extremely common means of detection for microcystins. Fixed wavelength detectors in HPLC systems can typically detect microcystin concentrations of a few nanograms, down to 0.50 μg ml^{-1}. In the case of field samples this may not be sensitive enough, and so some preconcentration may be required; however, if the sample is passed through a SPE cartridge, the eluent (methanol) can be evaporated off and the microcystin residue redissolved to give a more concentrated sample. This step is encorporated in the purification method involving SPE. In routine analysis of field samples it would be desirable to have as simple as possible a procedure while still having low a detection limit. When analysing for microcystins, care must be taken when using fixed wavelength detectors, as it is possible to have other coeluting compounds that absorb at the same wavelength, thus giving a higher signal. The use of a PDA detector provides a means to overcome this problem. Harada et al.[10] proposed the use of TLC with iodine detection in addition to UV detection in order to monitor for the presence of impurities. Lawton et al.[28] carried out some work using a PDA detector which is a good example of the applicability of the technique. However, Moolan et al.[31] in a critical review of this paper has warned against the use of fixed wavelength detection for critical microcystin analysis, and even when using a PDA the results should be examined carefully. Ideally a second detection method should also be used in critical microcystin analysis in order to check for the presence of non-UV absorbing impurities.

4.3.2.2. Mass Spectrometry

Mass spectrometry (MS) has been used extensively in the structural elucidation of microcystins (as well as in many structural analyses). MS detection can now be used in tandem with HPLC [43] and even in tandem with CE [16], in the analysis of microcystin

samples and standards. Kondo et al.[43] developed a LC-MS method to separate and identify microcystin-LR, -YR, -RR. In this method they utilised an isocratic mobile phase of methanol : 0.01 % TFA (61 : 39), (both volatile solvents containing 0.08 % glycerol) with a Chromatorex ODS column (250 mm x 4.0 mm, 5 μm). The actual MS technique used was fast atom bombardment MS (FAB-MS). The FAB technique produces both molecular ion species and characteristic fragment ions. Using this information, this method successfully allows for the identification of unknown microcystins without having standard samples. In addition to this, some impurity compounds were found, which were not detected by UV detection at 238 nm. In another publication, Kondo and Harada [44] carried out a number of MS techniques on microcystins, including liquid secondary ion MS (LSI-MS) and tandem FAB-MS. A review of other mass spectrometric analysis methods for other microcystins was also included.

Bateman et al.[16] compared both CE and RP-HPLC coupled to electrospray MS (ES-MS) in the analysis of microcystins. The detection limit for microcystin-LR using LC-ESMS was to the order of 20 μg l^{-1} while that using CE-ESMS was to the order of 200 μg l^{-1}. However, the main advantage that CE-ESMS has over the corresponding LC technique is its ability to separate the desmethyl forms of the microcystin-LR.

4.3.2.3. Other Detection Systems

Microcystins are not inherently fluorescent, so in order to detect microcystins fluorometrically some derivatisation procedure must be employed. Derivatisation of microcystins is, however, not easy to perform as microcystins lack reactive groups. This topic is covered later (see section 4.4.).

Although microcystins can be detected by other means, to date there has been no investigation into to the use of electrochemistry as a means to detect microcystins. The aim of this work was, therefore, to develop an electrochemical detector that can be added (in series) to an established HPLC system. The established HPLC

system is based on UV detection, a non-destructive detection method, and therefore the electrochemical cell can be added to the system in series following the UV detector. Two types of electrochemical detectors exist, namely amperometric and coulometric detectors. Amperometric detection typically involves the electrochemical conversion of a very low percentage of the sample, typically to the order of 1 %, while coulometric detection method converts practically 100 % of the sample. It was therefore envisaged to use an amperometric based detector as a detection method for microcystins -LR, -YR and -RR.

4.3.3. Thin-Layer Chromatography

TLC is a relatively simple technique to use and is not very time consuming. TLC also offers the added advantage of 2-dimensional analysis, which can separate co-eluting compounds. This is achieved by using a second mobile phase on the same TLC plate, the plate being run in a direction 90° to the direction used in the first run. There is also the option of normal and reversed-phase TLC systems, just as in HPLC. Poon et al.[18] and Jamel Al-Layl et al. [45] carried out TLC on microcystins using chloroform-methanol mobile phases. The chromatograms obtained with such mobile phases show rather broad peaks. Harada and co-workers have also carried out work on TLC of microcystins. They recommended the use of water-containing mobile phases for normal phase TLC as this has the effect of decreasing the activity of the silanol groups [10]. The absence of water may well have been the cause of the broad peaks obtained by the groups mentioned above. Harada et al. [25] used two solvent systems chloroform-methanol-water and ethyl acetate-isopropanol-water on normal phase TLC plates. They could use this mobile phase to successfully separate microcystin-RR, -YR, -LR, -LA (in that order with the first mobile phase), with the latter mobile phase the microcystin-YR and -LR were in the reverse order. For reverse phase TLC analysis the same mobile phase used for RP-HPLC can be used, e.g. the mobile phase used by Harada et al.[10] of methanol-0.1% TFA.

4.3.4. Capillary Electrophoresis

CE is one of the latest separation techniques to be applied to the analysis of microcystins. Bateman et al. [16] has applied CE to the analysis of microcystin-LR, using fused-silica capillaries coated internally with 5% (w/v) hexadimethrine bromide and 2% (w/v) ethylene glycol. The actual separation was carried out using 1 M formic acid buffer at -30 kV. Detection was by UV spectrometric and electrospray mass spectrometric (ESMS) detection. Detection limits using LC/ESMS was found to be 50 $\mu g\ l^{-1}$ while that using CE was 200 $\mu g\ l^{-1}$. The CE technique, however, had the advantage over LC as it was possible to separate microcystins differing only in a methyl group, e.g. 3-desmethyl microcystin variants differing in the Adda, Masp or Mdha residue.

Onyewuenyi and Hawkins [17] investigated the effects of buffer ionic strength, organic modifier solvents and pH in the capillary electrophoretic separation of microcystins. They used a CE method known as micellar electrokinetic capillary chromatography (MECC). This form of CE employs a surfactant in the buffer electrolyte. The surfactant must be present in a concentration greater than its critical micelle concentration, thus forming micelles in solution. MECC is typically used for the separation of neutral and uncharged molecules, however, this group found that MECC could be successfully applied to the separation of three microcystins (-LR, -RR, -YR) and nodularin (as an internal standard). With an arbitrary starting buffer electrolyte of 0.4 M boric acid, 0.1 M sodium tetraborate and 30 mM sodium dodecyl sulphate (SDS), and adjusted the pH with boric acid. pH values between 8 and 9 were tested and the optimum pH was found to be >8.8. The pH changes did not affect the elution order of the microcystins but an increase in pH did reduce their migration times. The buffer ionic strength was increased gradually from 0 to 25 mM borate. As the ionic strength increased, so too did the peak resolution. Increased additions of SDS affected migration times but the elution order and selectivity remained the same. Investigations into the use of organic modifiers revealed that the additions of methanol and acetonitrile changed the migration times, but again the elution order of the microcystins remained the same. Interestingly, increased

additions of isopropanol did change the elution order. When using methanol or acetonitrile, the elution order was microcystin-LR, -YR, -RR, but on addition of ~10% v/v isopropanol, the elution changed to microcystin-RR followed by the co-elution of microcystin-LR, -YR. The optimum conditions were determined to be 5% acetonitrile, 25 mM borate, 60 mM SDS and pH 8.85 using a 50 μm (i.d.) x 50 cm fused-silica capillary with a UV detector set at 230 nm. Alternatively, instead of acetonitrile, methanol could be used (10% v/v) with the SDS system. However, this buffer system increased the overall run time from 6 min to 8 min. Both systems gave rise to good peak shape and good resolution. Under optimum conditions this CE system had a linear calibration curve over the range of 66 mg l^{-1} to 300 μg l^{-1} for microcystin-LR, although the quoted detection limit was 0.12 μg l^{-1}. The lower end of this calibration curve corresponds to a typical calibration curve on a HPLC range. In practice, however, the working range on the HPLC is suitable for the analysis of microcystins from field samples. If necessary, the samples can be diluted in order for them to fall within the calibration curve. When all aspects are considered, the CE method compares extremely well with HPLC in terms of sensitivity, selectivity and in terms of the amount of solvents required. However, more work is required to investigate the separation of other microcystins on this CE system, especially of the more common microcystins such as the desmethylated microcystins.

4.3.5. Gas Chromatography of Microcystins

Sano et al. [46] published a paper on the determination of the total amount of microcystin in field samples of cyanobacteria. In their method they determined the amount of the oxidative product, 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB). This product was produced by the oxidative cleavage of the Adda moiety of the microcystin. As the Adda moiety is common to all microcystins this GC method is only suitable for the determination of the total microcystin content. The MMPB was also labelled with a fluorescent marker and this was analysed using HPLC, again for the total microcystin content.

4.4. DERIVATISATION OF MICROCYSTINS

Although microcystins can be detected easily by 'chemical' means, such as HPLC equipped with a UV detector, it is not quite as sensitive as some of the biological methods. Biological methods include techniques such as enzyme-linked immunosorbent assay (ELISA) which are discussed in the next section. It would therefore be desirable to derive microcystins in order to increase the sensitivity and selectivity of chemical methods. There is, however, some difficulty in chemical derivatisation as there is no obvious site available for derivatisation. Derivatisation of the arginine group is not ideal as the residue is not present on all microcystins. The carboxyl groups are another possibility. Meriluoto et al. [47] reacted microcystin-LR with 4-bromomethyl-7-acetoxycoumarin in accordance to a previously described method but failed to get a reaction. Reaction with 4-bromomethyl-7-methoxycoumarin also failed to produce a product.

The C=C double bond of the *N*-methyldehydroalanine residue has been utilised in the preparation of dansyl-cysteine adducts by Murata et al. [48]. Although the derivatised product has been reported to be detectable using HPLC, in concentrations as low as 0.12 μg l^{-1}, the working calibration curve has a lower limit of only 300 μg l^{-1}. Reduction of the double bond has been carried out by Meriluoto et al. [49] using sodium borohydride. This reaction was carried out in order to label the microcystins with tritium labels. This had been previously used by Botes et al. [50] in microcystin structure elucidation. As mentioned above, Sano et al. [46] did add a fluorescent label to the MMPB oxidative product of the microcystin, but this method lacks the selectivity required for many analyses.

4.5. BIOLOGICAL AND BIOCHEMICAL METHODS OF ANALYSIS

Perhaps the oldest and most traditional of biolological methods is the testing on animals. In the case of microcystins, mice are used. The mice are injected intraperitoneally with the microcystin extract and if enough toxic microcystin is present the mice die, typically within 1- 3 hours [3-5]. The mice die in a state of hypovolemic shock due to intraheptic bleeding [51]. When the mice are examined, the main finding is a blood engorged liver. In fact, in dead mice the liver is seen to have doubled its size. The mouse bioassay does, however, have a number of drawbacks including a high detection (e.g. 1 mg l^{-1} for microcystin-LR), a lack of specificity, and the ethical question of the killing of laboratory animals. On a cellular level, the hepatocyte membrane forms blebbs. This blebbing process can be used as a test for microcystins, whereby freshly isolated hepatocytes are incubated with microcystin extract and monitored for the formation of blebbs. The detection limit of this test is the same as that of the mouse bioassay.

Other biological tests include mosquito larvae, adult mosquitoes, brine shrimp larvae and plant tests [52, 53, 54, 24]. Once again there is a problem of lack of specificity with many of these biological tests as the invertebrates can react to other compounds and not just to microcystins. Kos et al. [24] used the mustard plant (*Sinapis Alba*) for their plant tests. They reported a 50 % inhibition of growth at 3 mg l^{-1} . One major drawback with the test is the overall length of time taken to obtain a result, approximately 5-8 days after exposure to the microcystin extract.

Biochemical tests are also available for the testing of microcystins, ELISA and protein phosphatase inhibition. ELISA has been performed by Chu et al. [20] using both polyclonal and monoclonal antibodies giving detection limits as low as 0.20 ng ml^{-1}. The exact epitope of the antibodies is not clear, but it appears that the Adda is required for activity. The antibodies showed a decrease in reactivity to the non-toxic microcystin-LR that contains the 6(Z)-Adda. One drawback is that the antibodies produced a positive result for the non-toxic microcystin-LR methyl ester.

Protein phosphatase inhibition has been applied as a means for the detection of microcystins, by An and Carmichael [55]. The test uses the recombinant protein phosphatase 1 (PP1) obtained from *E. Coli.* The developed test has a linear working range of 0.05 nM and 1 nM, which equates to approximately to 0.05 ng l^{-1} to 1 ng l^{-1} (or 1-25 ng ml^{-1} in the tested solution). The enzyme activity was monitored by colour production at 405 nm. The PP1 test was compared to an ELISA test and it was found that both tests compared well in terms of linear working ranges and detection limits.

4.6. PURPOSE AND AIMS OF RESEACH

The ultimate aim of the research project was to investigate the potential and possibility of developing an electrochemical detector that could be used in tandem with a HPLC system, following a UV detector. Other aims of the project were the optimisation of the microcystin extraction process, the microcystin purification process, and investigations into different mobile phases that could be used in the analytical HPLC method.

4.7. EXPERIMENTAL

4.7.1. Reagents and Toxins

Water was purified to 18 MΩ cm^{-1} on a Milli-Q plus PF apparatus (Millipore, Molsheim, France). HPLC grade acetonitrile was purchased from Rathburn (Walkerburn, Scotland). Analytical grade ammonium acetate, potassium dihydrogen phosphate and potassium hydroxide were purchased from Baker (Deventer, Holland). L-arginine (> 98 % purity) and glycine (> 99 %) were purchased from Sigma (St. Louis, MO, U.S.A.). Purum grade pentafluoropropionic acid (≥ 97 %) was obtained from Fluka. Peptide toxins, microcystin-LR and -RR and their desmethylated analogues (Figure 4.1) were purified from the cyanobacterium *Anabaena* sp. strain 90 grown in the laboratory, (see section 4.6.2.). Microcystin-YR was purchased from Calbiochem (La Jolla, CA, U.S.A.). Other equipment and supplies include a Branson B2210 (Danbury, CT, U.S.A.) ultrasonic bath Sorvall MC 12V, centrifuge (Du Pont, Newtown, CT, U.S.A.) and Whatman (Maidstone, England) GF/C filters.

4.7.2. Growing and Purification of Microcystins

The cyanobacteria species *Anabaena 90* was grown in the laboratory in Z8 standard growth medium, under fluorescent lighting. The culture was harvested

after approximately three weeks, by which time the culture had reached their stationary growth phase. Harvesting involved the filtering of the culture through Whatman (Maidstone, England) GF/C filters, which were then freeze dried. The extraction procedure used was based upon the method, as used by Harada [25], of extraction using 5 % acetic acid. The first step in the solid phase extraction (SPE) process, was to 'activate' the column packing, using a 10 % MeOH/90 % water mix, airdry, followed by a flush of acetic acid. (Failure to flush with the acetic acid would result in the non-binding of the microcystins to the column). The supernatant was then applied and the column flushed with a 10 % MeOH/90 % water mix. Different procedures were then applied in the removal of the microcystins form the column, with differing results. The three processes are shown in Table 4.1.

Procedure 1	Procedure 2	Procedure 3
Supernatant	Supernatant	Supernatant
10 % MeOH	10 % MeOH	10 % MeOH
Airdry	Airdry	-
100 % MeOH	10 % MeOH	100 % MeOH
Airdry	Airdry	-
100 % MeOH	10 % MeOH	100 % MeOH
Airdry	Airdry	-
100 % MeOH	10 % MeOH	100 % MeOH

Table 4.1. : The different processes used in the removal of the microcystins from the SPE column. The final three eluted fractions were pooled together and concentrated.

The toxins were further purified by C_{18} reversed-phase HPLC, firstly using penta-fluoropropionic acid (PFPA), mobile phase acetonitrile : 0.3 % PFPA in water (6 : 4) and secondly with ammonium acetate, according to a previously published protocol of acetonitrile : 0.0135 M ammonium acetate (27 :73) [35]. Increasing the percentage of PFPA in the mobile phase resulted in a reduction in the retention time of the microcystins. Microcystins were identified by their HPLC retention times, UV spectra and electrospray-MS masses. Four microscystins were isolated, namely microcystin-LR and microcystin-RR and their corresponding desmethylated analogues. The desmethylated toxins were likely to be 3-

desmethyl-microcystins rather than 7-desmethylated-microcystins as those have been previously isolated from the original culture of this strain [56].

4.7.3. HPLC Equipment and Mobile Phases

The HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-7A pump, Rheodyne (Cotati, CA, U.S.A.) 7125 injector equipped with a 20 µl sample loop, Shimadzu SPD-6A UV detector set at 238 nm, a Bioanalytical Systems (West Lafayette, IN, U.S.A.) LC-4B amperometric detector and a Shimadzu CR-5A integrator. The EC detector was equipped with a thin-layer flow cell (gasket thickness 0.05 mm) and operated at a potential of +1.20 V. The working electrode was glassy carbon, counter electrode stainless steel and reference electrode Ag/AgCl (3 M KCl). The column used in the microcystin purification work was a 250 mm x 10 mm i.d. Nucleosil-7 C_{18} column. The column for the analytical work was a 250 mm X 4.6 mm i.d. GFF-S5-80 internal surface reversed-phase column (Regis Chemical, Morton Grove, IL, U.S.A.) protected by a Rheodyne 7335 (0.5 µm) filter and a GFF precolumn. Two mobile phases were tested. Mobile phase A consisted of acetonitrile : 0.1 M ammonium acetate (15 : 85). Mobile phase B consisted of acetonitrile : 0.1 M potassium dihydrogen phosphate (15 : 85) adjusted to pH 6.5 with KOH. The flow rate was 1 ml min^{-1}.

4.7.4. Cyclic Voltammetry

A cyclic voltammetric investigation of the electrochemical behaviour of microcystin-LR on a glassy carbon electrode was made in a miniature laboratory-built electrochemical cell. A 0.1 mM solution of microcystin-LR in 1.0 M sulphuric acid solution was prepared and voltammograms obtained, using a BAS 100W electrochemical system. The cyclic scans were run between -0.40 V and +1.00 V (vs. Ag/AgCl, 3 M KCl). Figure 4.6 shows the cyclic voltammogram obtained.

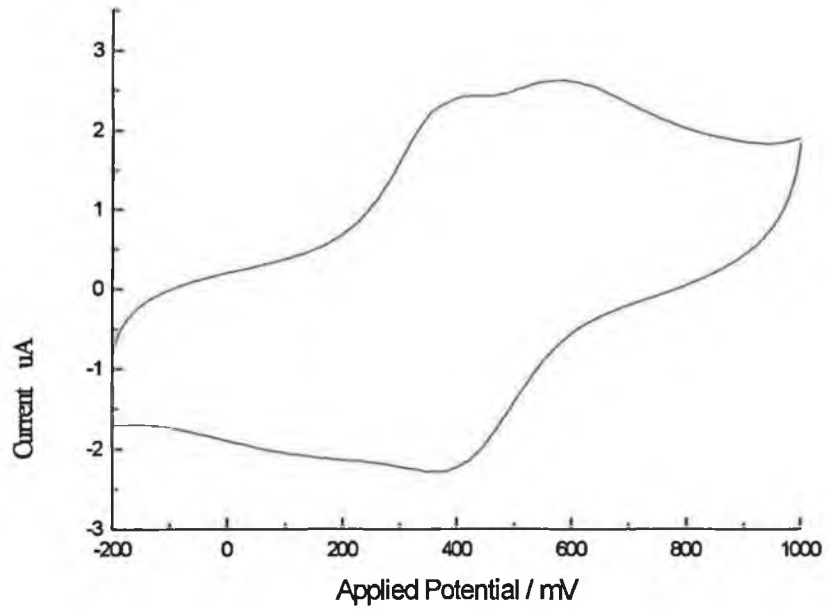

Figure 4.6 : The cyclic voltammogram obtained for 0.1 mM solution of microcystin-LR in 1.0 M sulphuric acid using a BAS 100W electrochemical system. The cyclic scans were run between -0.20 V and +1.00 V, using a glassy carbon working electrode (vs. Ag/AgCl, 3 M KCl).

4.7.5. Calibration curves

Microcystin-LR and -RR were dissolved in water and then diluted in methanol (1:5). As the toxins were both produced and purified within the laboratory, it was first necessary to determine their actual concentration. The molar extinction coefficients reported, previously by Harada and co-workers [41], as 39,800 for both microcystin-LR and -RR were used in the calculation of the microcystin concentrations. The absorbance of the methanolic solutions of microcystins was determined spectrophotometrically at 238 nm. Using the extinction coefficients, the concentrations of the stock solution of microcystins were determined and used to prepare the calibration curve solutions. Triplicate injections of 20 µl of the stock solutions diluted in acetonitrile : 0.1 M ammonium acetate (15 : 85) were then analysed in both mobile phases using both UV and EC detection. All microcystin solutions were kept in glass vials as some toxin loss was observed when pure toxin solutions were kept in polypropylene tubes, possibly due to

adhesion of the microcystin to the tube walls. The concentrations of 3-desmethyl-microcystin-LR and -RR were calculated according to the calibration curve for microcystin-LR and -RR, corrected with the molar absorptivity difference reported for a corresponding pair of toxins (microcystin-LR and the 3-desmethyl analogue) 39,800 vs. 31,600 [41].

Microcystin-YR was quantified using the microcystin-LR UV calibration curve corrected with the molecular weight difference (both have the same extinction coefficient). No microcystin-YR was produced by the alga culture and hence none was purified.

4.7.6. Extraction Efficiencies of Solvents

Investigations into the extraction efficiency of different mobile phases were carried out, to determine if any differences existed between the different solvents and their ability to extract the microcystins from the *Anabaena 90 cells*. Investigations were also carried out in order to determine the number of extractions required to extract at least 90 % of the microcystins. The samples were prepared in triplicate and extracted with the each of the solvents three times, using 100 µl per mg (dry weight) of algae cells. The amount of microcystin extracted was then quantified on the analytical HPLC system.

4.7.7. Preparation of Cyanobacterial Field Samples

Samples of the cyanobacterium *Oscillatoria agardhii* taken from a 5 m depth in Lake Vargsundet on Åland Islands (SW Finland) were prepared in order to assess the potential of the electrochemical detector in the detection of microcystins in natural samples. A 500 ml sample of lake water was filtered on a GF/C filter (diameter 47 mm) and freeze-dried. The freeze-dried filter was extracted twice in a polypropylene Eppendorf tube using 5 % acetic acid, 1 ml at a time with 5 min sonication in an ultrasonic waterbath. The collected extracts were pooled and centrifuged at 10,000 rpm for 10 min. The extracts were concentrated fivefold

using a 500 mg Varian (Harbor City, CA, U.S.A.) Bond-Elut C_{18} solid-phase extraction cartridge (protocol as described above) and redissolved in mobile phase A. The extracts were found to have trace amounts of microcystin-LR and 3-desmethyl-microcystin-RR. The extracts were then spiked with microcystin-LR and 3-desmethyl-microcystin-RR giving concentrations equivalent to 16.4 $\mu g\ l^{-1}$ and 26.0 $\mu g\ l^{-1}$, respectively (see Figure 4.7.). The spiked concentrations were reported, previously, to be typical for toxic *Oscillatoria agardhii* blooms found in lake water [57].

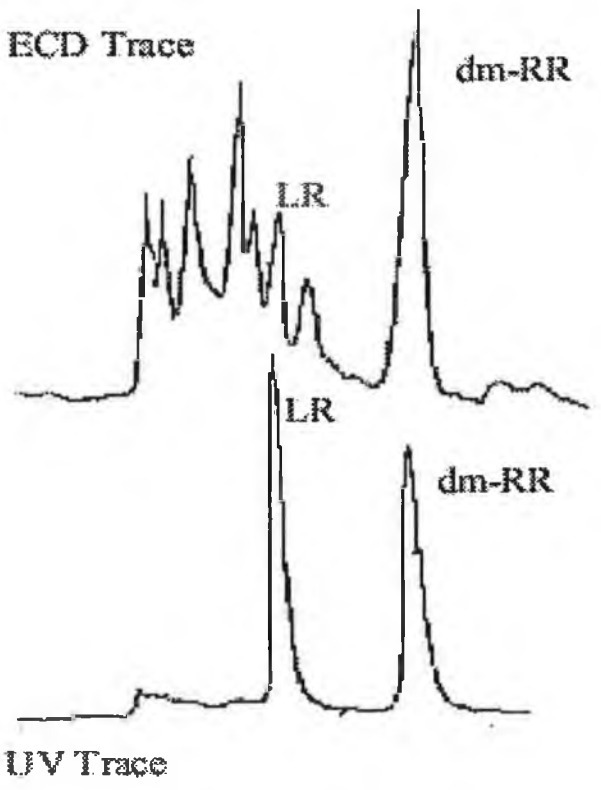

Figure 4.7 : The responses obtained for the spiked field samples, using both UV and ECD.

4.7.8. Electrochemical detection of Arginine and Glycine

As participation of the arginine residue in the electrochemical reaction was suspected, an experiment was set up in order to establish if arginine was

electrochemically active. Free amino acids arginine and glycine were dissolved in water in separate vials (10 µg per 20 µl and 4.3 µg per 20 µl, respectively), giving final concentrations of 57 nmoles of each amino acid per 20 µl of solution. The solutions then were injected onto the HPLC system (1 ml min^{-1} of mobile phase A as a carrier, column replaced by capillary tubing) and both detector responses recorded.

4.8. RESULTS

4.8.1. Purification of Microcystins

The SPE extraction process is outlined in section 4.2.1.1. It was found three extractions were required that in order to fully extract the microcystins from the filter paper. The efficiency of the removal of the microcystins, from the column, was monitored using the analytical HPLC-UV system. It was found that the most efficient SPE process involved airdrying the column between each MeOH flush. Process two proved to be the best in the microcystin removal, but only marginally better than process one, hence due to the ease of evaporation, (when concentrating the collected fractions), the 100 % MeOH, rather than the aqueous MeOH mix, process one was chosen as the best method.

The microcystins eluted in different order, depending on which mobile phase was used in the preparative HPLC purification process. With the PFPA mobile phase the elution order was, dm-RR, RR, dm-LR and finally LR. This was in contrast to that of the ammonium acetate based mobile phase in which the LR variants eluted first : LR, dm-LR, RR and finally dm-RR. The use of both mobile phases enabled very pure microcystin samples to be collected, to the order of >95 % purity, as determined by UV detection following HPLC separation. However, resolution between the microcystin peaks was comparable in both mobile phase systems, as were the overall run times in each mobile phase.

Figure 4.8. shows a trace obtained for the PFPA-based system and Figure 4.9. shows the trace obtained with the ammonium acetate based mobile phase (The figures show the separation obtained for an extracted mixture of microcystins). The fractions from the PFPA system were collected individually, the two desmethylated variants pooled, and separated using the second mobile phase system. As the elution order differed between the two systems, the resolution between the peaks was thus maximised in the second system, hence the high purity of the collected fractions.

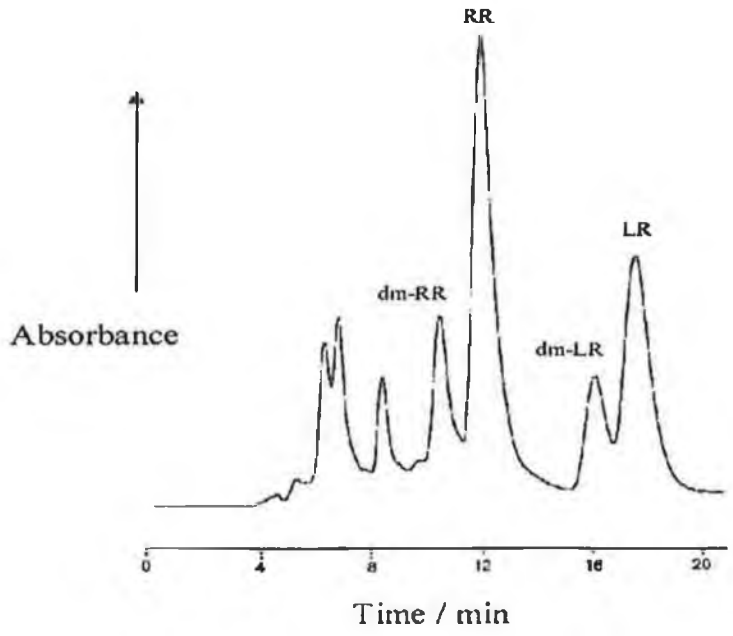

Figure 4.8 : The trace obtained from the PFPA based mobile phase system in the purification of the microcystins after SPE. The trace is produced by a mix of all four microcystins.

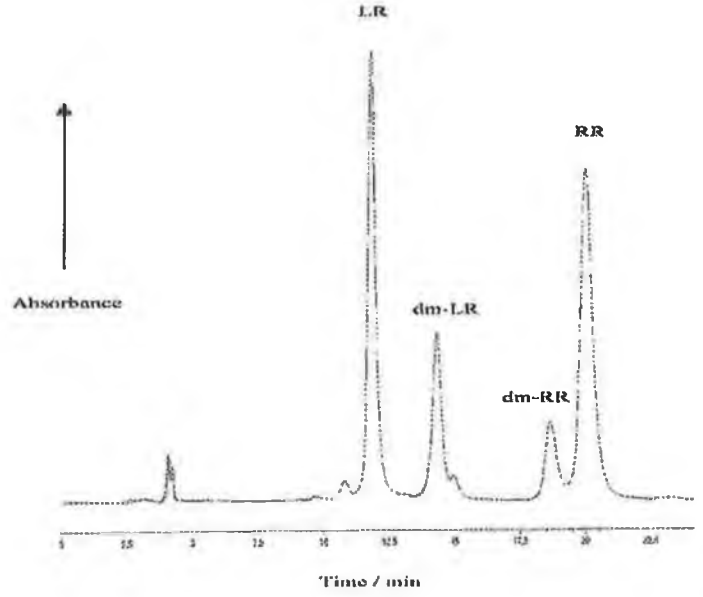

Figure 4.9.: The trace obtained using the ammonium acetate based mobile phase system in the purification of the microcystins after SPE. The trace is produced by a partially purified mixture.

4.8.2. Extraction Efficiencies

The extraction efficiencies are tabulated below in Table 4.2. As can be seen, two extractions were sufficient to extract >90 % of the microcystins from the cells. The calculations were based on the assumption that three extractions were sufficient to extract 100 % of the microcystins from the cells.

Extraction	Ammonium Acetate		KH_2PO_4	
	% Extracted	% Extracted	% Extracted	% Extracted
	LR	RR	LR	RR
First	81.1	85	81.5	91.6
Second	92.5	94.3	99.6	100
Third	100	100	100	100

Table 4.2. : Shows the percentage of microcystins extracted from the algae cells, using HPLC mobile phase solution.

When carrying out the analysis of field samples two extractions were carried out as speed of analysis was deemed to be of greater importance than carrying out the third extraction.

4.8.3. Cyclic Voltammetry

Cyclic voltammetric (CV) scans were run between -0.20 V and +1.00 V (vs. Ag/AgCl, 3 M KCl), scan rate of 50 mV / min. As can be see from Figure 4.6, the cyclic voltammogram obtained shows two oxidation peaks. When a faster scan rate was employed (as would be the case in an electrochemical detector in a HPLC system), only one oxidation peak was detected; this is indicative of a slow electron transfer rate. The reduction peak seen in the CV is also indicative of a reversible reaction. This reversibility is very important in an electrochemical detector as it means any oxidised species should essentially be reduced thereby preventing any build-up of any oxidised species on the working electrode surface.

4.8.4. Chromatography of toxins

The phosphate-based mobile phase B gave better separation of microcystin-LR, -YR and -RR than the acetate-based mobile phase A (Figures 4.10. & 4.11.). The desmethylated toxins eluted only a few seconds before the corresponding non-desmethylated ones and thus were not resolved. This was common to both mobile phases used. Generally speaking, the baseline in EC detection was more stable with mobile phase A than in mobile phase B. However, the electrochemical signals produced by the toxins in mobile phase B were consistently higher than those produced by the same sample in mobile phase A.

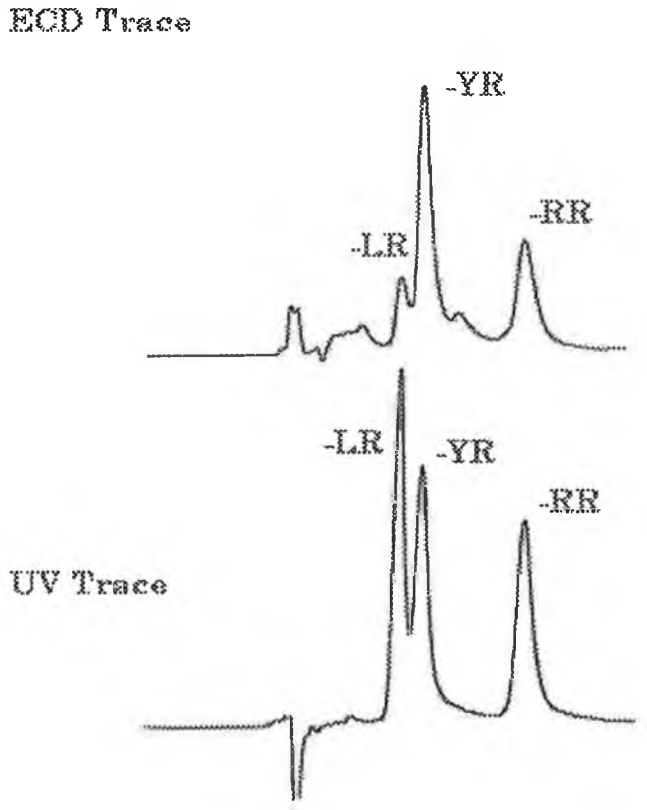

Figure 4.10. : UV and EC Detection of microcystin-LR, -YR and -RR following HPLC separation using the ammonium-acetate based mobile phase.

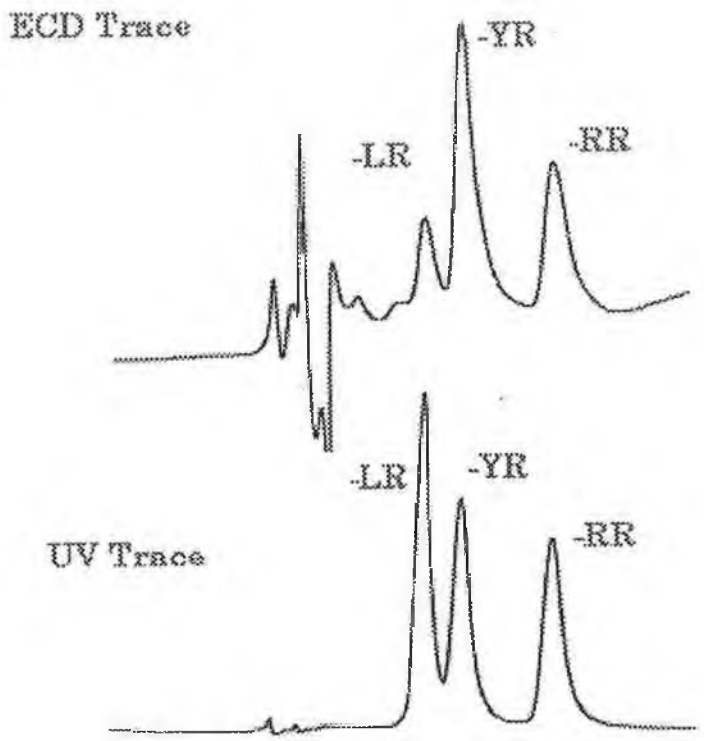

Figure 4.11. : UV and EC Detection of microcystin-LR, -YR and -RR following HPLC separation using the potassium phosphate-based mobile phase.

4.8.4.1. Calibration curves

Peaks on the UV chromatograms were quantified by peak area whereas noise and baseline disturbances in the EC detector trace necessitated manual peak height measurement. Both UV and EC calibration curves for microcystin-LR and -RR were linear in the range 13-250 ng (Figures 4.12.-4.13.). The regression coefficients for the linear regression lines were > 0.998. Although the UV detector calibration curve was linear at higher concentrations (up 500 ng inj^{-1}, the corresponding EC detector calibration curve was lowered by about 10 % at the higher concentrations (400-500 ng inj^{-1})). The UV detector responses were practically identical in both mobile phases (Figures 4.12.).

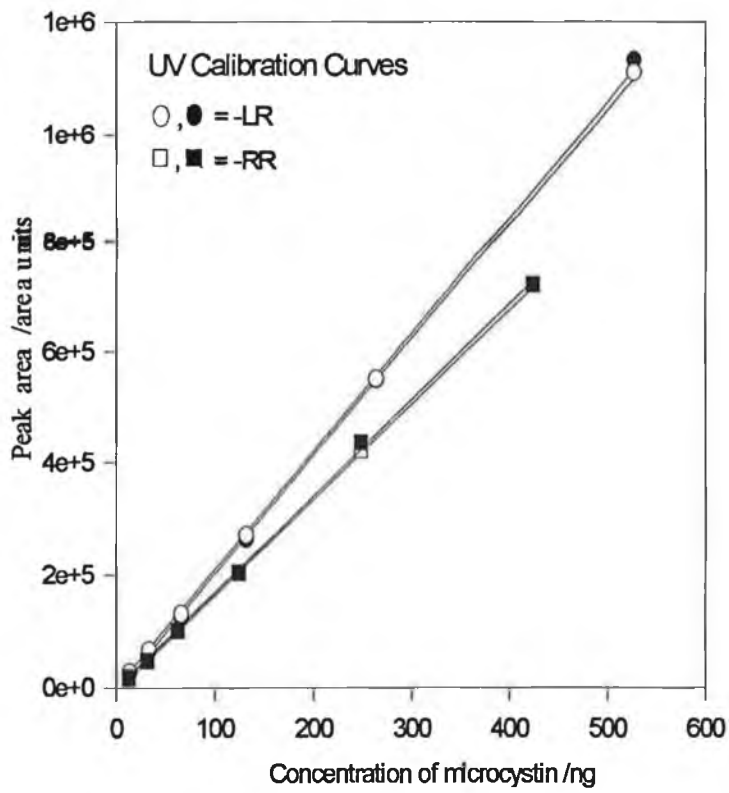

Figure 4.12 : The calibration curves obtained using the UV detector. Clear symbols are for ammonium acetate mobile phase, whereas black symbols are used for the phosphate mobile phase.

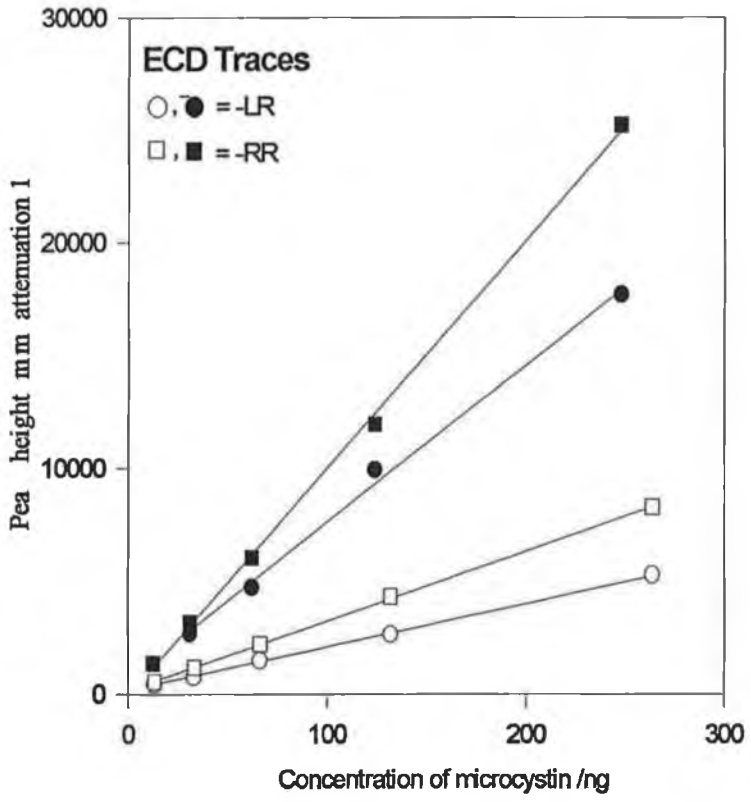

Figure 4.13. : The calibration curves obtained using the ECD detector Clear symbols are for ammonium acetate mobile phase, whereas black symbols are used for the phosphate mobile phase.

4.8.4.2. Electrochemically Active Residues in Microcystins

The EC detector responses were found to vary according to the working electrode condition and it was necessary to calibrate the EC detector against the UV detector on a daily basis. This was achieved by the injection of standard samples. The variation in EC detector response was due to the fouling of the working electrode. This was primarily caused by the injection of natural samples, which contained high concentrations of proteins and other particulate matter (in spite of the centrifugation). The EC calibration curves in Figure 4.13. show a comparison between the responses obtained for microcystin-LR and -RR. From this figure it can be seen that the molar EC response of microcystin-RR, relative to that of microcystin-LR, was about 1.8 (height-based calculation, mobile phase A) and 1.5 (height-based calculation, mobile phase B). This leads to the assumption that the electrochemical reaction occurs in the arginine residue (one arginine in microcystin-LR, two in -RR). The microcystin-RR peaks under the used HPLC conditions are the latter eluting peaks and consequently are broader in shape; in fact they are more than 30 % broader at half peak height than those of -LR. When this 30 % difference is taken into consideration and applied to the peak heights, both ratios become higher than two. This finding indicates that the Arg residue at position 2 (Figure 4.1.) is more easily oxidised than the Arg residue at position 4. One possible explanation for the difference in reactivity is steric hindrance from the Adda residue (position 5). Conformational studies have found that the Adda residue is quite flexible is solution and is also a relatively long residue [58].

Further evidence for the participation of the arginine residue in the electrochemical reaction was obtained by the comparison of the EC signals of Gly and Arg. Glycine is the simplest of all amino acids, possessing an electrochemically inactive hydrogen atom instead of a side chain. Equimolar injections of Gly and Arg (57 nmoles per 20 µl) showed that the area of the arginine-derived signal was 43 times larger than that of glycine. This finding

clearly shows that the guanidino chain of the arginine amino acid is oxidisable by electrochemical means.

The tyrosine-containing microcystin-YR gave the highest EC signal in comparison with the UV signal (Figures 4.12.-4.13.). This is likely due to a strong oxidation signal of the tyrosine residue. The height-based EC signal ratio between equimolar amounts of microcystin-YR and -LR was about 2.8 in mobile phase A and 2.3 in mobile phase B.

4.8.4.3. Natural Sample

The cyanobacterial sample from Lake Vargsundet contained several electrochemically active compounds (Figure 4.7.) which eluted mainly before microcystin-LR and 3-desmethyl-microcystin-RR. These electrochemically active compounds are not microcystins as they do not absorb at a wavelength of 238 nm, the λ_{max} for microcystins but they could be large or very hydrophilic peptides containing electrochemically active residues. The height-based EC quantification of microcystin-LR and 3-desmethyl-microcystin-RR was not affected by other electrochemically active substances. Although the quantification of microcystin-LR maybe difficult at lower concentrations due to the presence of these other electrochemically active residues, their detection may prove advantageous in certain circumstances.

4.9. DISCUSSION

The development of an electrochemical detector is an important addition to the available detection techniques as the family of microcystins is a difficult analytical challenge for chromatographers. Reliable identification of microcystins calls for a variety of techniques.

Electrochemical oxidation of the aromatic amino acids tyrosine, tryptophan and related bioactive compounds is well documented [59, 60, 61, 62, 63, 64] but much less has been reported on the electrochemical reaction of arginine. Arginine and other guanidino compounds have been determined by anion-exchange chromatography and electrochemical detection at 0.45 V vs. Ag/AgCl using a basic aqueous eluent and a nickel(III)oxide working electrode [65]. The authors speculated that other electrode types could improve the electrochemical selectivity for guanidines although this was not tested. The main pieces of our own evidence for the involvement of arginine in the electrochemical reaction are the relative ratios of EC/UV signals of microcystin-LR (one arginine residue) and -RR (two arginine residues) and the EC comparison of arginine and glycine.

It was also attempted to block the arginine residues with the guanidino-specific reagent benzoin, which has been reported to react with arginine residues in an alkaline medium. Microcystin-LR and -RR were derivatized according to a previously published method [66] with the exception that benzoin was dissolved in acetone instead of methylcellosolve. The desired benzoin-arginine reaction product could not be identified and/or isolated. It is assumed that the microcystins underwent some form of decomposition under the reaction conditions used (reaction conditions involved boiling and strong alkaline conditions).

The present electrochemical detection system can be used for example for verification of peak identities, especially in the context of a single-wavelength UV detector. So far the only real alternative detection technique for microcystins has

been mass spectrometry, a technique still too expensive for smaller laboratories. EC detection should also prove useful in the detection of other microcystins that contain arginine or tyrosine (e.g. microcystin-FR, -YM, -YA, -LY). Furthermore, the reported oxidation conditions for tryptophan (1.0 V vs. Ag/AgCl, carbon paste or glassy carbon electrode [60, 62]) indicate that microcystins with a tryptophan residue such as microcystin-WR could be detected electrochemically on the developed system. Due to lack of suitable microcystins this could not be tested. If the electrochemical detection system detects all microcystins containing arginine, tyrosine and tryptophan, then the only major non-detectable microcystin is microcystin-LA. Nodularins, microcystin-related pentapeptides from brackish and marine sources, have the general structure cyclo(-D-*erythro*-b-methylisoAsp-L-**Z**-Adda-D-isoGlu-*N*-methyldehydrobutyrine)[2, 13, 67, 68]. Some nodularins with desmethylations and biologically inactive Adda modifications have also been characterized[69]. The majority of the known nodularins have L-Arg as the variable amino acid (**Z**) and should thus be detectable electrochemically.

EC detection was also feasible in detecting some electrochemically active impurities (possibly of peptide nature) in purified microcystins. While short wavelength UV, MS or light-scattering detection might prove useful in the detection of impurities, the low UV transmission or involatile nature of some solvents and buffers preclude these possibilities. EC detection combined with the unique selectivity of the GFF phase revealed the presence of some impurities in 3-desmethyl-microcystin-LR purified on an ordinary C_{18} reversed phase column (Figure 4.14.). One further application for the electrochemical detection method is the individual identification of 3-desmethyl-microcystins and 7-desmethyl-microcystins which coelute in ISRP separation at neutral pH (they separate at pH 2 [40]). The molar absorptivity coefficient of 3-desmethyl-microcystin-LR has been reported to be 31,600 mol l^{-1} cm while the coefficient of 7-desmethyl-microcystin-LR has been determined to be 46,800 mol l^{-1} cm (39,800 mol l^{-1} cm for microcystin-LR). Preliminary results with the 3-desmethyl toxins show that while there was a difference in the UV response compared to the non-desmethylated toxins the electrochemical response remained the same. Therefore

it could be possible to use the ratio between the UV and EC responses to identify the site of desmethylation. This is a useful application of the EC detector as the only other means for the identification of the site of desmethylation is by MS fragmentation patterns or NMR spectra.

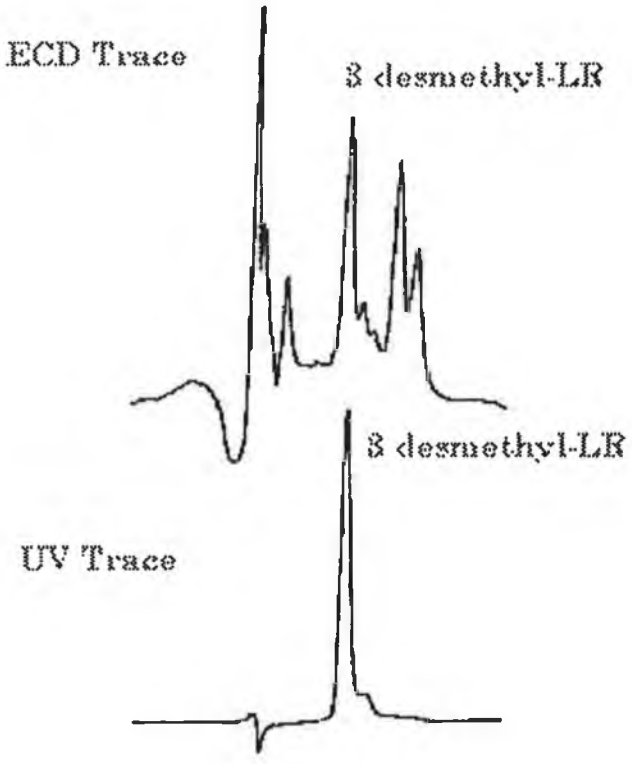

Figure 4.14. : UV and ECD traces obtained for a partially purified 3-desmethyl-LR, showing the presence of non-UV absorbing species.

Finally, some comments on the use of the electrochemical detector. It was found that passivation of the system was sometimes required. This was required in order to remove the high iron content present in the system, as it was oxidised thus causing many spurious peaks to appear in the electrochemical detector trace. Purification involved flushing the system with 5 M nitric acid, followed by water and finally by mobile phase. After the passivation process the ECD baseline was more stable. When running samples it was necessary to allow the ECD to equilibrate for 2-3 hours before use in order to reduce the amount of drift in the baseline. Polishing of the electrode was performed at an interval of 2-3 weeks,

depending on the use of the detector. Comparison of the ECD signal with the UV signal (obtained from a standard sample) indicated when the electrode required polishing.

The described HPLC detection system is essentially the first step in the electrochemical detection of microcystins. However, due to the relatively high background activity the applicability of this method is limited when analysing crude cyanobacterial extracts. It is necessary to investigate more effective ways of sample pretreatment to lower the amounts of impurities. A second objective is to derivatise microcystins with electrochemically active reagents that can be oxidised at a lower potential. Ideally these reagents should react with all known microcystins. At least three moieties will be subjected for derivatization trials: the α,β-unsaturated carbonyl group in the *N*-methyldehydroalanine residue, the carboxyl groups (primarily the in the Glu residue) and the conjugated diene system in the Adda residue. It would also be desirable to find alternative mobile phases and electrode materials giving improved sensitivity and electrochemical selectivity. The cell configuration used throughout the experiment was the thin layer cell. This could be improved upon by the use of the more efficient wall jet configuration. In the wall jet cell the eluting HPLC mobile phase impinges perpendicularly on the working electrode, resulting in better flow hydrodynamics, and increased sensitivity.

4.10. CONCLUSION

The developed EC detection system has proved to be a useful means of microcystin detection. It has good applicability to partially purified microcystins, especially in the detection of electroactive impurities. However, there are some limitations in the use of the EC detector when analysing field samples due to the presence of other electroactive species found in the field samples. The intention, however, was not to replace the UV detector, but rather to develop a complementary detector, to give more information than would otherwise be obtained using a single detector system.

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Chapter 5

Overall Conclusions

And

Future Work

5.1. GENERAL CONCLUSION

This thesis comprises of a number of studies concerned with separation strategies. The first two experimental chapters are designed to gain a further insight into the chemistry behind anaerobic adhesives and cyanoacrylate adhesives. While the third experimental chapter concentrates on the development of a novel detection system for the detection of microcystins.

5.1.1 Anaerobic Adhesive Study

The first anaerobic adhesives were developed in the 1950's, and the research at that time was focused on producing an adhesive that would be commercially successful. As a result there was less focus on the actual comprehension of the chemistry of the curing mechanism. Consequently, nowadays there is considerable research being conducted in an effort to elucidate the curing mechanism that occurs with anaerobics.

One of the main sources of the complexity of the cure chemistry is the different components of an adhesive (initiators, accelerators, stabilisers), and their different roles and functions in the curing mechanism. The reducing agents studied, function to reduce the transition metals from their higher oxidation state to their lower valency. Additionally they can act a complexing agents to metals in the decomposition of cumene hydroperoxide. The chemistry of the adhesives is further complicated by the difference in the effects of similar acids. Saccharin and maleic acid are both acids of similar strength, yet they show different effects in the curing mechnism. This is observed for each of the different reducing agents studied. This yet again stresses the complexity of the adhesive chemistry and emphasises the careful management of component concentrations that is required.

The reducing agents are shown to react in solution when present with cumene hydroperoxide (and acids). Of the four reducing agents studied, APH, DMPT and *p*-TSH reacted to produce one major product which was isolated and identified. THQ, however, produced several products which could not be separated. These results not only help in the elucdiation of the reaction pathways, but they are also important with

regard to the stability of adhesive compositions. As poor curing performance of the adhesive can occur should the reducing agents decompose over a period of time, it is essential that the adhesive preparation be such that the reducing agents be stable for a long period of time.

5.1.2. Cyanoacrylate Adhesive

The process of cyanoacrylate adhesive preparation involves the use of acid stabilisers. These stabilisers are required to neutralise the base catalyst that is used in the initial steps of the cyanoacrylate adhesive synthetic cycle. Any acids must be removed once the base catalyst has been neutratlised as they can cause hydrolysis of the cyanoacrylate monomer. Although there are methods that can be used to remove these acids, an analytical method is required that can accurately detect and quantify any residual acid species.

The methods which have been applied to the acidic anion analysis include the Total Acid Determination test, ion chromatography and potentiometric titration. Each of these tests suffers from some drawbacks, for example, the potentiometric titration cannot detect the weaker acidic species. Hence investigations were carried out into the suitablity of capillary electrophoresis in the analysis of cyanoacrylate adhesives. The capillary electrophoretic method developed for the analysis of the cyanoacrylate adhesives has proved to be reproducible and accurate in the detection and quantification of the anions found in cyanoacrylate adhesives. This method has proved capable of detecting all anions found in the adhesives, although some of the anions do have the same migration time. The anions that share the same migration times are however, rarely found together in the same cyanoacrylate preparation.

It is possible that the method developed could be applied as a routine analytical method in the future as a sensitive and reliable method in the determination of the acidic anion contaminants.

5.1.3. Detection of microcystins

There is increasing concern over the presence of microcystins found in rivers and lakes, due to the potential health hazard that they pose. Although a number of detection systems exist that can be used in their detection, it is always advantageous to have an alternative means of detection. The most common means of detection is that of UV, and it is a relatively sensitive means of microcystin detection. One disadvantage of this method is the interference that can occur due to co-eluting peaks, that have similar absorbance maxima. The use of a photodiode array detector can help overcome this problem. The electrochemical detector that has been developed has proved successful in the detection of non-UV active 'impurity' proteins in the sample matrix, and in a practically purified microcystin fraction. These results alone, highlight the potential of the electrochemical detector. The detection system can be used to detect all of the commonly found microcystins and most of the less common ones.

Improvements in the detector system can be carried out, in order improve the LOD and also to reduce the background interference from other electro-active proteins. This can be done using different electrode materials and more efficient extraction/purification procedures.

Appendix A : PUBLICATIONS

1. Electrochemical detection of microcystins, cyanobacterial peptide hepatotoxins, following separation by HPLC. J. Meriluoto, B. Kincaid, M. R. Smyth, M. Wasberg, *J. Chrom.*, 810, **1998**, 226.

2. Toxic algae and fish mortality in a brackish-water lake in Åland, SW Finland. T. Lindholm, P. Öhman, K. Kurki-Helasmo, B. Kincaid. & J. Meriluoto. *Hydrobiologia*, in press.