DEVELOPMENT OF RESIN-BOUND REAGENTS FOR ANALYSIS OF CARBOXYLIC ACIDS AND AMINES

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CERTIFICATION

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Finally to God that gives me the guidance to know when to hold on and when to let go and grace to make the right decision with dignity.

DEDICATION

To the memory of

My Mother,
Mrs. V.A. Adewuyi

and

My Grandmother,
Mama Rachel Adeoye.

EPIGRAM

Tough times do not last, but

Tough people do.

ABSTRACT

Resin-bound-reagents made up of ionically-bound 2-naphthalene-methanol and covalently bound and sodium-benzoxazole-2-sulfonate have been synthesized and utilized as analytical reagents for the pre-chromatographic derivatization of fatty acids (i.e. acetic, lauric, capric, hexanoic, octanoic, nonanoic, palmitic, docosanoic linoleic), 1° and 2° aliphatic amines and amino acids respectively.

The reagents have been designed to contain fluorescent moieties attached to the anionic and cationic resin backbones through sulfonated ester linkages. These moieties imparted UV and FL detector properties to the final derivatives.

The derivatization reactions were performed before the thin-layer chromatographic separations. Standards were prepared and were used in monitoring the extent of reactions on the resin support.

The derivatives were chromatographed and fluorescent spots were observed under UV light.

These solid phase derivatizations have led to preliminary investigation of these two functionalities before embarking on instrumentation analysis such as high performance liquid chromatography with UV or fluorescence detection and HPLC-MS identification.

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CHAPTER ONE

1. INTRODUCTION

Fatty acids and amines are of immense importance. Some fatty acids are known precursors of the biologically important prostagladins.[1] Some other aliphatic amines and polyamines which are odorous substances are known precursors of the carcinogenic and toxic N-nitrosamines.[2]

The ability to monitor these two groups of compounds is very important and several techniques to detect or confirm their presence are needed. A number of analytical methods have been used for their analysis ranging from classical to instrumental methods.[3] However, each of these methods has its own limitations and disadvantages.

Classical methods of analysis have the disadvantages of low sensitivity of detection, non-selectivity and non-specificity. The limitations of the classical approach have been overcome by modern chromatographic techniques, in particular gas liquid chromatography and high performance liquid chromatography.

Problems that are encountered in chromatographic analysis of these two functionalities can, for example, be illustrated with the determination of aliphatic amines. Gas chromatographic determination of these amines at low concentration is hampered by adsorption and decomposition, column ghosting phenomena, tailed elution

peaks and low detector sensitivity.[4] To different degrees, one or more of these problems are encountered in the analysis of carboxylic acids using any of the available chromatographic techniques.

A general approach to these problems has been the preparation of suitable derivatives of the analytes, because derivatization can be used to improve the chromatographic behaviour as well as the sensitivity and specificity of detection.

1.1 Derivatization in Homogeneous Media

The importance of chemical derivatization techniques in chromatographic analysis cannot be exaggerated. However, most of the derivatizations performed have involved the use of homogeneous reactions in which the reagent is present in solution and is mixed with a solution of substrate of interest.[4] Many books have been published on homogeneous derivatization reaction that can be performed prior to chromatographic analysis of trace organic compounds. When an analyte is derivatized in an homogeneous medium, a large excess of derivatizing reagent would be present in reaction solution. The earliest approach to the problem of removal of excess reagents during pre-chromatographic derivatization was the development of volatile reagents which could be easily evaporated after the derivatization process.

Use of such reagents seems to have been limited to gas chromatographic applications. There may be many stages involved in such a reaction including purification and recovery of desired derivatized product. A major drawback of homogeneous derivatization techniques is associated with the need to remove excess of reagent because the reagent often has similar behaviour to the derivative in terms of its response to the chromatographic detection. In the presence of the excess of reagent, therefore, the response of the system to their derivative may be swamped by that of the excess reagent.

This problem of excess reagent is one that could be conveniently overcome if derivatization was done in a heterogeneous medium.

1.2 Derivatization in Heterogeneous Media with Resin Bound Reagent

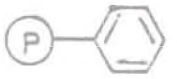
To avert problems present in homogeneous derivatization, heterogeneous derivatization have been employed. In heterogeneous derivatization, the reagent is insoluble in the reaction medium. This is possible because it is attached to a preformed polymer of high molecular mass. Such attachments may be at remote points along a polymer chain or anchored in close proximity. There are a number of significant advantages in using heterogeneous

reactions to derivatise; for instance, it allows excess of reagent and substrates to be separated from the reaction product by simple filtration thereby avoiding complex chromatographic procedure,[5] which are tedious and time-consuming. In addition, reactions that are not possible in solution because of the low solubility of one or more of the reagents may be carried out in high effective reagent concentration on a polymeric support. Also, heterogeneous reactions often are more selective and give fewer side products. In some instances, the by-products can remain attached to an insoluble polymer, these can then be reconverted into the original valuable reagents that can later be reused.

As a result of all the possible advantages enumerated above, there is therefore a need to develop more readily accessible reagents for heterogeneous derivatizations of carboxylic acids, amines and other commonly encountered functionalities.

1.3 Resin-Bound Reagents

Resin-bound reagents are formed when low molecular weight reagents are attached to preformed resin. The bound reagents will possess a combination of the physical properties of a high polymer and chemical properties of the attached reagent.[6]



= polymer or resin

r = low molecular weight reagent.

Fig. 1.0: Resin-bound reagent

1.3.1 Polymers

These are large molecules consisting of repeating small units called mers, the small units are covalently bonded together to form the large molecules. The simple molecule from which a polymer is made is called a monomer. For example, styrene, a monomer, can repeatedly react with itself and be transformed into polystyrene, a large polymeric chain[7] as shown in Figure 1.1 below.

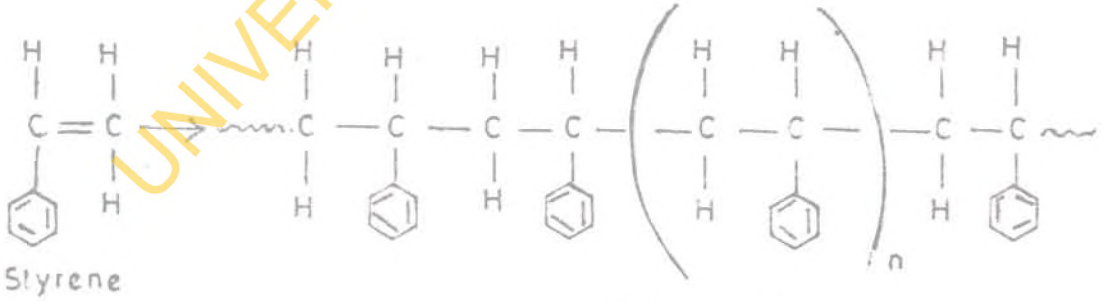


Fig. 1.1: Transformation of styrene to polystyrene.

Chemically, there are fundamentally two modes of polymerizations, namely addition and condensation polymerizations. In addition to polymerization, the product is theoretically an integral multiple of the monomeric molecule, e.g. polythene and polystyrene. Condensation process always result in the formation of copolymer such as dimers, trimers and tetramers, examples of such copolymer are organosilicon polymers (silicones); phenol-formaldehyde resin, Nylon 66. The initial part of condensation polymerization, reaction, involve the conversion of all the monomers to low molecular weight polymers, and no high molecular weight polymers are present. Thereafter, the lower molecular weight species condense in a stepwise manner forming polymer of large degree of polymerization.[7] This process can be further explained in the Figure 1.2 below.

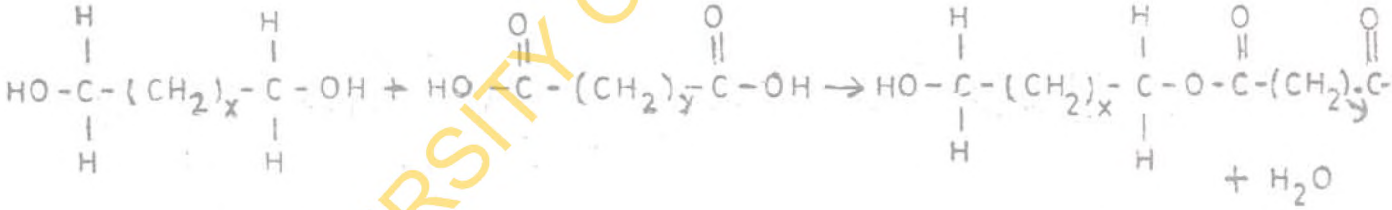


Fig. 1.2: Condensation reaction between a dialcohol and an organic diacid

A massively cross-linked structure is also formed if one of the reactants is more than bifunctional, in this form, polymerization occurs in the three dimensions.

In polymers, monomeric units can be joined together in different forms. In linear polymers, monomeric units are joined together in a straight open-chain fashion as seen in the figure below:

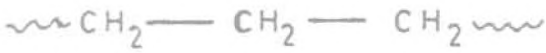


Fig. 1.3: Linear polymer e.g. polyethene.

In linear alternating copolymer, two different types of monomers are arranged in a straight open-chain alternating fashion as in the figure to follow:

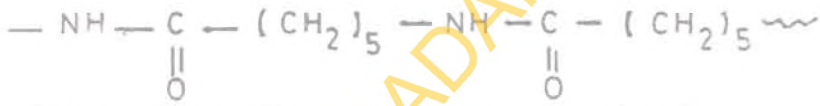


Fig. 1.4: Linear alternating copolymer e.g. nylon 6.

In minor cross-linked polymer, there exists a limited number of cross-linkages between each polymeric chain as seen in the figure below:

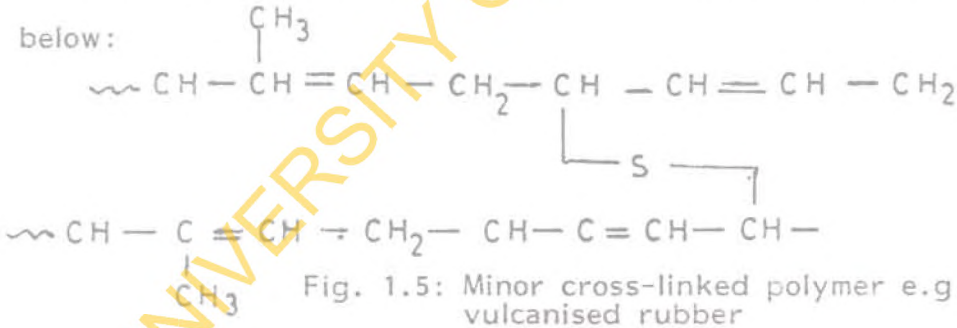


Fig. 1.5: Minor cross-linked polymer e.g. vulcanised rubber

There is also the massively cross-linked polymer as observed in urea-formaldehyde resin in the figure below:

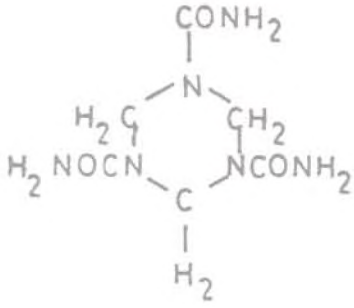


Fig. 1.6: Massively cross-linked polymer e.g. urea formaldehyde.

Finally, there is the block polymers made up of monomers in block of individual monomers.

1.3.2 Properties of Polymers

Polymeric substances whether they are of natural origin such as cotton, wool, natural rubber, proteins, polysaccharides, nucleic acids or produced synthetically in the laboratory e.g. polyethylene, polymethylmethacrylate are easily recognised by their physical appearance and some specific properties;[9] these properties include low or negligible solubilities in common solvents, mechanical strength, elasticity, fibre-forming properties and dimensional stability; however, they may still be different when their physical properties are considered.

Polymers may be of various forms ranging from readily soluble liquids, or low-melting, waxy, or very hard and brittle solids.

The anomalous properties of polymers in comparison to the low-molecular weight compound can be explained in terms of molecular size and stabilizing forces.

There are two major bonding forces in polymers, they are the primary chemical bonds which occur along polymer chains and secondary bond forces such as vander waals, dipole interactions, and hydrogen bonding. Unlike the small molecules, the secondary bond forces play an extremely important role in polymers, this is because the high molecular weight of the polymer permits these forces to build up sufficient strength to impart mechanical strength and rigidity in the polymers. These intermolecular forces also influence other properties of polymers such as swelling, gelation, miscibility and solubility in certain solvents.

Crystalline regions are formed by polymer molecules possessing symmetry. Though polymers may be amorphous, yet have regions of crystallinity.

The crystalline and amorphous regions of polymer are considerably different in properties, for instance the former has increased mechanical strength.

Crystallinity and other physical properties of a polymer are dependent upon the substituent's configuration. Considering the mode of polymerization, polymers can be either isotactic in which

substituents around the polymer backbone are in an ordered configuration, or atactic, that is when substituents are fashioned in a random manner.

Syndiotactic polymers have their substituent groups like alternately above and below the plane. The isotactic, syndiotactic and atactic configurations of a polymer chain are shown in the figure below:

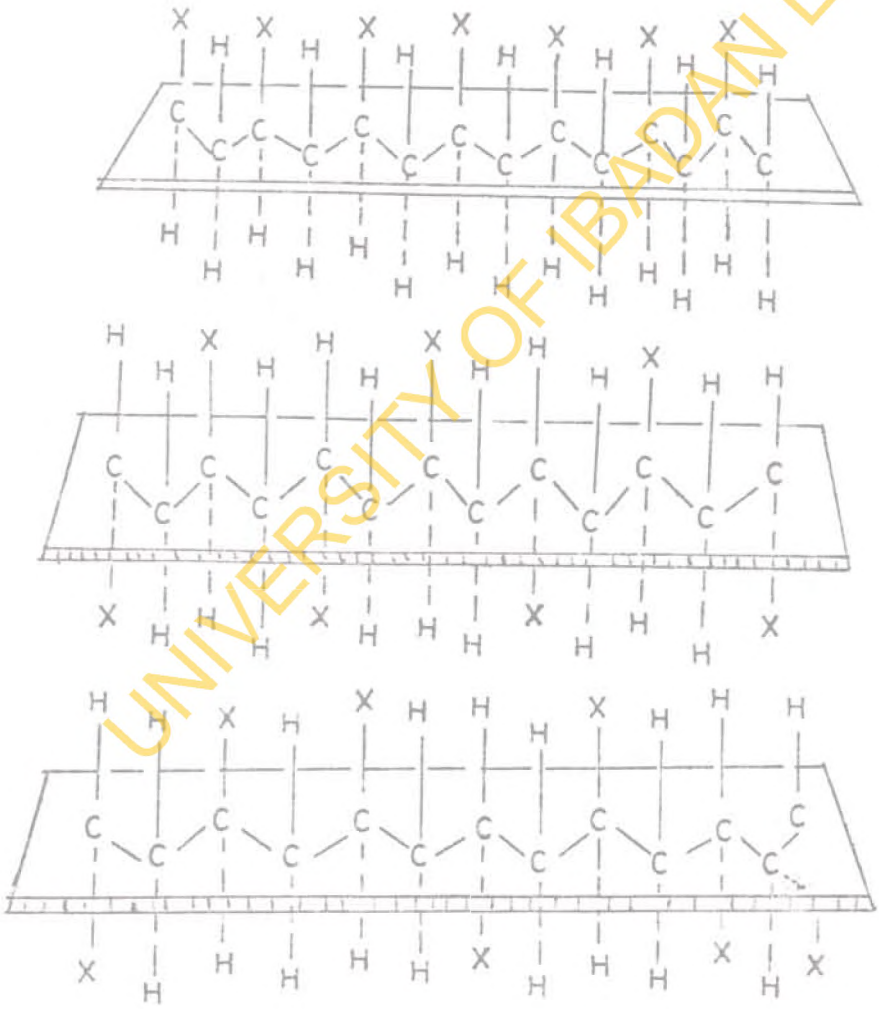


Fig. 1.7: Isotactic, syndiotactic, atactic configurations of a polymer chain.

Of equal importance are the first- and second-order transition temperatures. These temperatures are not as "sharp" as those of low-molecular-weight solids. In crystalline polymers, these temperatures refer to the melting temperatures of crystal regions, and to the softening temperature in amorphous regions. Polymers are softening when there is increase kinetic energy of the molecules as it becomes large enough to overcome secondary bond forces.

Other properties that are determined by intermolecular forces are miscibility and solubility respectively. Polymers dissolution unlike the low-molecular weight substances is a slow process occurring in two stages. In the first stage, the solvent molecules slowly diffuse into the polymer leading to swelling and gelation. This may be the only stage if strong polymer-polymer intermolecular forces are present. This may be due to cross-linking, crystallinity, and strong hydrogen bonding. However, in linear polymer, the first stage is accompanied by a second stage in which a truly homogeneous solution results from diffusion of solvated polymer molecules into the solvent.

Polymers that are used as insoluble reagents possess the required property of swelling rather than solubility.

The usual colligative properties of solutions are exhibited by dilute solutions of completely soluble polymers. The properties have been employed in the determination of polymer molecular weights.

The chemical reactions of polymers can be classified as follows: The first class are those affecting the degree of polymerization (D.P) which include further polymerization of already formed polymers and the synthesis of a graft or a block co-polymer and degradation reaction classified as macromolecule. The second class are those not affecting the degree of polymerization (DP), but involve the reaction of functional group already contained in the polymer-mediated organic synthesis.

1.3.3 Polymer-Supported Reagents

Polystyrene has been the most widely used polymer for various reactions. Polystyrene can be functionalized by either converting the polymer directly into the desired reagent or it serves as a handle to which a low-molecular weight reagent is then attached.[9]

Reagent attachment to the polymer backbone can be by ionic bond referred to as ionically bound reagent, while those that are attached by covalent bonding are known as covalent bound reagents respectively.

1.3.3.1 Ionically bound reagents

These are based on ion-exchange resins and are easy to prepare. In ion-exchange ions of like charges are exchanged between solution and an insoluble solid in contact with the solution. For exchange to occur, the ion-exchanger (solid) must carry its own ion and must be structured to facilitate movement of the ions. In addition, no appreciable physical change must occur in the materials during exchange process. The ion-exchanger is complex and polymeric and carries an electric charge that is exactly neutralized by charges on the counter ions. Some exchangers are used as solution in a solvent immiscible with water; these are liquid ion-exchangers.

In cation exchangers, the active or counter ions are cations and the resins or polymers are anionic while in anion exchangers, the counter or active ions are anions while the resins or polymers are cationic.[10]

1.3.3.2 Cation-exchange resins

These are high molecular-weight cross-linked polymers containing sulphonic, carboxylic, phenolic groups as an integral part of the resin and equivalent amount of cations like hydrogen or sodium. There are strongly acidic cation exchangers and weakly acidic exchangers.

Strongly acidic cation-exchangers are usually supplied in the hydrogen or sodium forms as in polystyrene sulphonic acid resin written as $(Res\ SO_3^-)H^+$ for the hydrogen form and as $(Res\ SO_3^-)Na^+$ for the sodium form.

Weakly acidic cation-exchangers such as polymethylacrylic acid resin also come in hydrogen form. In addition, some weak acid cation exchangers contain the carboxylate group, i.e. $(Res\ COO^-)H^+$. The exchange capacity for strongly acidic cation resins is pH independent.[10] While for weak acid cation exchangers, exchange only occurs in alkaline solution.

The equation for the exchange capacity are shown in Figure 1.8 below.

ph independent

$$(Res\ SO_3^-)H^+ + Na^+Cl^-_{SOln} \rightleftharpoons (Res\ SO_3^-)Na^+ + H^+Cl^-_{SOln}$$

$$(Res\ COO^-)H^+ + Na^+OH^-_{SOln} \underset{\text{medium}}{\overset{\text{alkaline}}{\rightleftharpoons}} (Res\ COO^-)Na^+ + H_2O$$

Fig. 1.8: Equations for exchange capacity.

A widely used cation-exchange resin is prepared by co-polymerization styrene and a small proportion of divinylbenzene as shown in Fig.1.9 below.

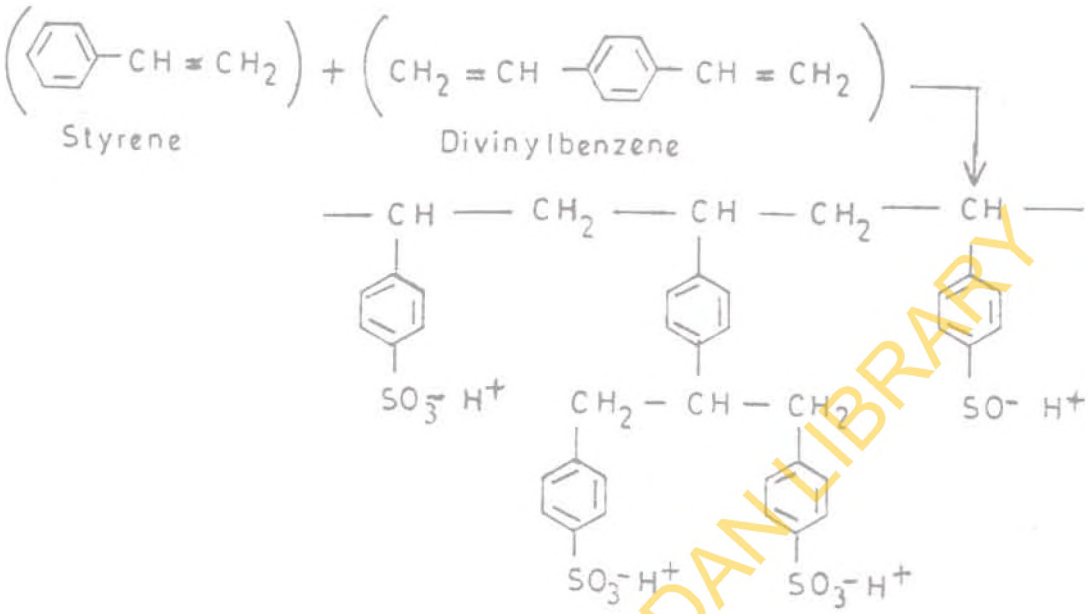


Fig. 1.9: Preparation of cation exchange resin.

The polymeric skeleton in the above structure is held together by linkings crossing from one polymer chain to the next; the ion exchange groups are carried on this skeleton. The degree of linking determines the physical properties. Thus, highly cross-linked resins are harder, more brittle, and impervious than lightly cross-linked counterpart.

Swelling and preference of resin for one ion over another are influenced by degree of cross-linkings. In the above figure, divinylbenzene units weld the polystyrene chain together, this prevents it from swelling indefinitely. The dispersing and

sponge-like network arrangement provides a framework in which the negatively charged sulphonate ions are attached. The fixed negative charges are balanced by an equivalent number of cations such as hydrogen ions or sodium ions respectively. These ions are mobile and move freely within the water filled pores. The ions can exchange with other ions, as seen in a situation where a cation exchanger containing mobile ions X^+ is brought into close contact with a solution containing cations Y^+ which diffuse into the resin structure and cations X^+ diffuse out until equilibrium is attained. At the end of exchange process, the solid and the solution contain both cations X^+ and Y^+ in numbers depending upon the position of equilibrium. The same process holds for exchange of anions in an anion exchanger. The exchange process is explained in Figures 1.10a and 1.10b below.

$$(Res.A^-)X^+ + Y^+(Soln) \rightleftharpoons (Res.A^-)Y^+ + X^+(Soln)$$

Fig. 1.10a: Exchange process of ion exchange resin

a typical example is the displacement of potassium ions in a sulphonate resin by magnesium ions

$$2(Res.SO_3^-)K^+ + Mg^{2+}(Soln) \qquad (Res.SO_3)_2^- Mg^{2+} + 2K^+ Soln$$

Fig. 1.10b: Displacement of potassium ions by magnesium ion

1.3.3.3 Anion exchange resins

This is prepared by co-polymerizing styrene and a little divinylbenzene followed by chloromethylation (i.e. introducing $-CH_2Cl$ group) in the free para position and interaction with a base such as triethylamine in Figure 1.11 below.

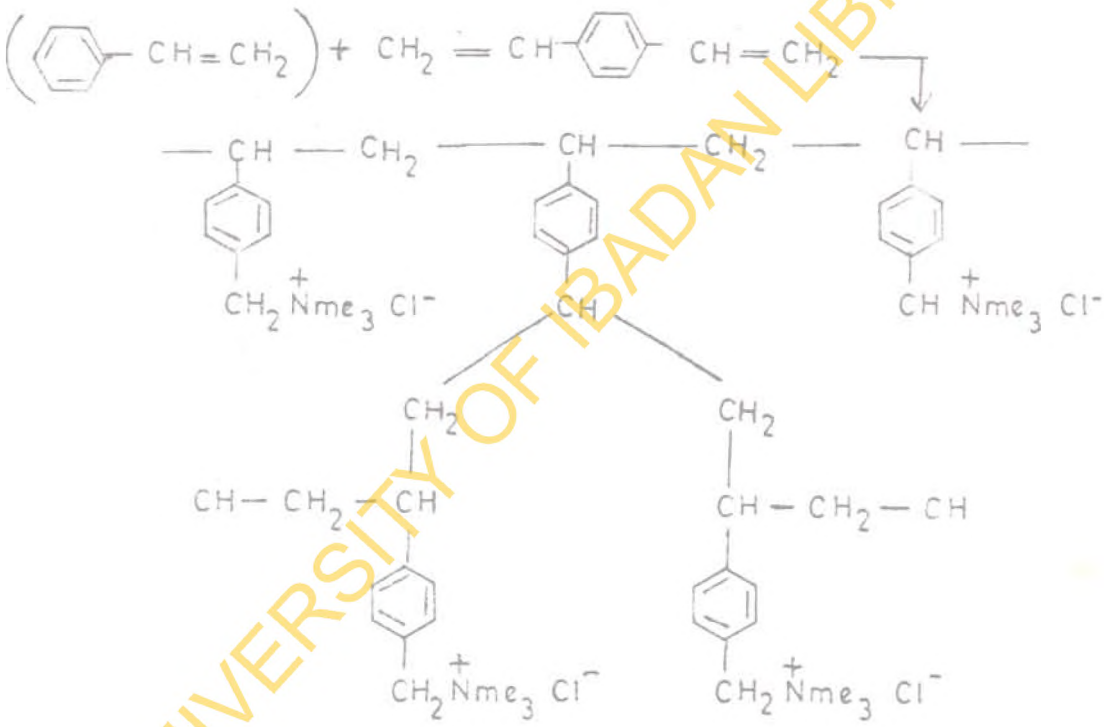


Fig. 1.11: Structure of an anion-exchange resin.

1.3.3.4 Covalent-bound-reagents

These are reagents attached to the polymeric support by simple covalent bonding. They are classified according to types and functions of reagents that are attached to the polymer support. Table 1 below gives an explanation on their classification.

1.3.3.5 Functionalization of polystyrene

Apart from attaching reagents to resins, resins can directly be converted to the desired reagent; such a conversion involves primary transformation of polystyrene through Friedel Crafts reaction[11] with chloromethylation being the starting step. Chloromethylation is a process whereby a hydrogen atom of an aromatic compound is replaced by a chloromethyl group, CH_2Cl. The introduction of a chloromethyl group is very useful since this group is readily converted into other groups such as aldehydes alcohol, ethers and amines.

Table 1: Functions and Classification of Covalent-Bound Reagents

Function	Reagents	Application
Oxidizing reagents	Peracids, chromium containing polymer supported chromic acid chemisorb chromylchloride (CrO_2Cl_2) on silica alumina) silver carbonate-celite. Polymeric thio-anisolyl resin.	In epoxidation of alkene oxidation of primary and secondary carbonyl compound. Preparation of aldehyde and ketone from allylic and benzylic halides oxidation of alcohol and lactones. Selective oxidation of alcohol.
Oxidation-reduction reagents	Polymeric quinones.	Dehydrogenation and oxidation of organic compounds.
Polymeric-reducing reagents	Alumina-supported material.	Reduction of disulphides of thiols. Reduction of carbonyl compounds.
Polymeric-reducing reagents	Polymeric tin hydride.	Selective reduction of halides in the presence of other functional groups e.g. bromoaceto phenone to acetophenone.
	Silica-gel absorbing tri-butyltinhydride.	Selective oxidation of aldehyde.
Polymeric group transfer reagents	Halogenating agents.	Introduction of halogen into molecule either by nucleophilic displacement or electrophilic addition.
Polymeric group transfer agents	Anion-exchange resin carrying fluorine, chlorine bromine iodine anions.	Use in exchange of one halide from another in alkalhalide.
Acylating agents	Polymers incorporating mixed anhydride of carboxylic and benzoic acids.	Used in peptide synthesis.

Table 1 (Contd.)

Function	Reagents	Application
Alkylating agents	N-alkyl-naryltriazine supported on polymer material.	Alkylation of carboxylic acid.
Polymer supported nucleophiles	CN^- etc supported on anion-exchanger.	Nucleophilic substitution as in preparation of benzylcyanide by the reaction of benzyl-bromide with an anion exchange resin. Separation of main product from by product is easily achieved.
	Witting reagents and other ylids polymeric witting reagents polymer incorporating a triphenyl phosphine group.	Conversion of low-molecular weight-carbonyl group to alkene.
	Polymeric sulphur ylid reagents containing dimethyl sulfonium methylide synthesized from a co-polymer of vinylmethyl thioether, styrene and DVB (Divinylbenzene).	Synthesis of epoxides from carbonyl compound product were easily isolated.
Polymeric coupling agents	Carbodimides polymeric carbodimides	Use in peptides synthesis and in the preparation of carboxylic anhydrides used in oxidation of alcohol to aldehydes, starting reagents can be regenerated through less active than the original reagents.
Polymeric coupling agents	Sulfonyl chlorides polymeric-benzene sulfonyl chloride.	Peptide synthesis via the polymeric mixed carboxylic sulfonyl anhydrides.
	Polymeric aryl sulfonyl chlorides	Synthesis of oligonucleotides.

Such conversions can be carried out in organic solvents most often swelling solvents and the reaction can also proceed under phase transfer conditions.

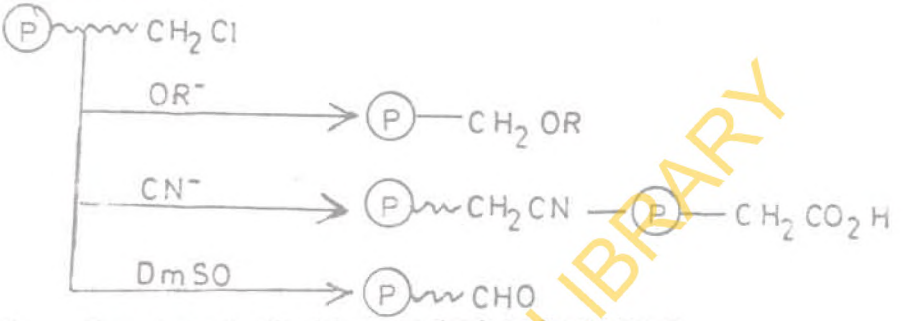


Fig. 1.12: Treatment of chloromethyl polystyrene with nucleophiles

The phase transfer process is illustrated in Fig. 1.13 below.

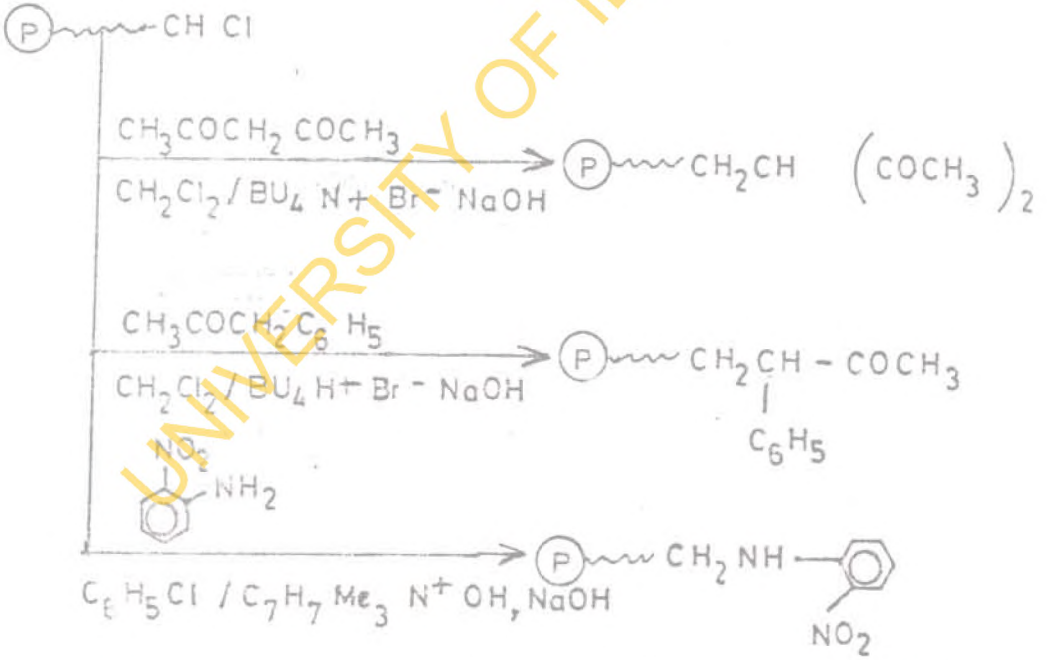


Fig. 1.13: Phase transfer process involving chloromethyl polystyrene.

Polystyrene can also be alkylated under Friedel-Crafts conditions The reaction is versatile in converting polystyrene to various reagents. This route is coupled to the polymer through a stable C-C bond as shown in Figure 1.14.

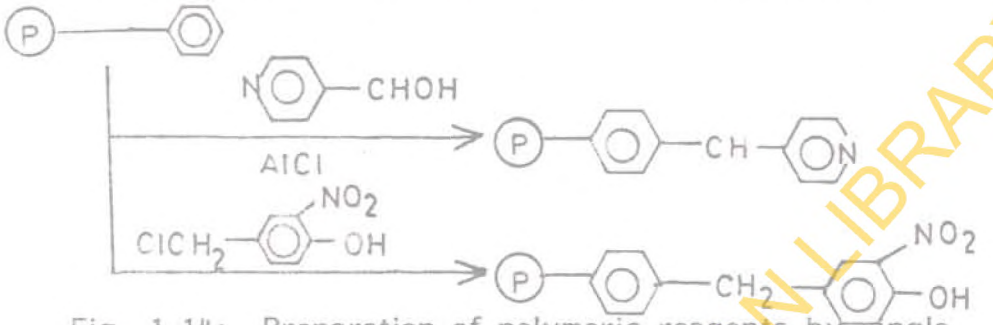


Fig. 1.14: Preparation of polymeric reagents by single step Friedel-Crafts alkylation of polystyrene

Polymeric sulfonate esters and tosylazide were prepared from chlorosulfonated polystyrene as shown in Figure 1.15.

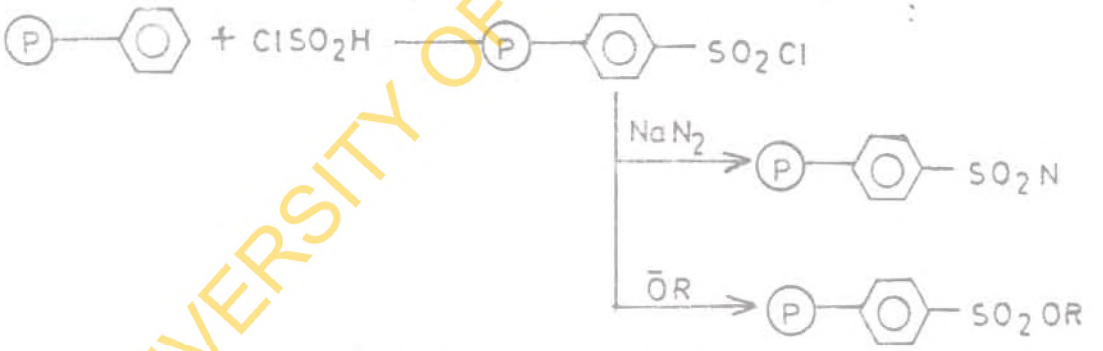


Fig. 1.15: Preparation of polymeric sulfonate esters and toxylazide from chlorosulfonated polystyrene.

In forming the lithiated polymer, polystyrene is halogenated to obtain an intermediate that produces the lithiated polymer. Butylithium N,N,N,^{1}N,^{1}-tetramethylene diamine is a good metalating agent for cross-linked polystyrene. The metalated

polystyrene intermediate is a good route in the preparation of polymeric reagents. This is shown in Figure 1.16.

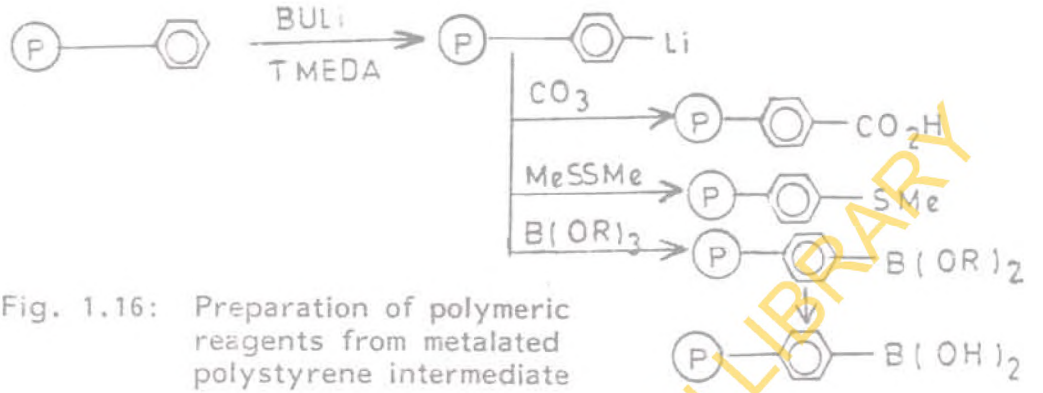


Fig. 1.16: Preparation of polymeric reagents from metalated polystyrene intermediate

Various research workers have endeavoured to use some of the above mentioned reagents for their works while some have modified the reagents to suit their purposes. The application of these reagents in heterogeneous derivatization has been exploited by Krull and co-workers.[4] In one of their works they used polymer-bound anhydrides as acylating agents for prechromatographic derivatization of amines. An interesting aspects of their work is that they prepared polymer-bound reagents which could be used for either off-line or on-line derivatization for high performance liquid chromatography. This has prompted us to look into the possibility of developing readily available reagents not only for amines but for other commonly encountered functionalities such as alcohols, amino acids and carboxylic acids.

Moreover, literature survey revealed that in the analysis of carboxylic acid such heterogeneous derivatization techniques have not been fully exploited. Most workers tend to carry out their derivatization through homogeneous processes.

1.4 Analysis of Carboxylic Acids

A number of liquid chromatographic methods have been employed for the determination of fatty acids. For instance, Pei et al[12] used methylester derivatives for high performance liquid chromatogrphic analysis of carboxylic acids. In addition, methylester derivatives of 11- and 12-hydroxylauric acid produced by microsomal metabolism of sodium laurate have been analysed by reverse phase high performance liquid chromatography.

Another worker[13], also investigated the separation of fatty acid methylesters using corail 11 with a stationary phase of silver-nitrate ethylene glycol.

In preparing methyl ester derivatives of the acids, carboxylic acids are esterified in methanol with hydrogen chloride or boron trichloride as the catalyst. In all cases, detection levels were poor although such derivatives are certainly useful for preparative work. Hence in an attempt to produce suitable derivatives for high performance liquid chromatographic analysis, acids were

esterified with aromatic derivatizing agents, this produced derivatives with improved chromatographic properties and increase sensitivity of detection. Some workers[14] were able to prepare the phenacyl ester derivatives by reacting the acid with 2 bromoacetophenone using triethylamine as base. Borch in his work reported the separation of long chain fatty acids as phenacyl esters by HPLC. Cooper and Ander[1] used 2-naphthacyl-bromide to derivatize carboxylic acids to obtain their 2-naphthacyl ester that was analysed by high performance liquid chromatography and mass spectrometry. In his derivatization scheme, mixtures of the acid (10 μmoles), 2-naphthacyl bromide (20 μmoles) and N,N-diisopropylethylamine (40 μmoles) all in 1ml dimethyl-formamide were heated at 60°C for 10 minutes. The aliquot was then analysed by liquid chromatography. He however observed good chromatographic properties of the naphthacyl esters using a reversed phase column but α- and γ-linolenic acids could not be resolved.

Other workers,[15] reported the determination of carboxylic acids by liquid chromatography after phase-transfer catalysed fluorogenic labelling. In their work, tetrabutylammonium mediated transfer of analyte as an anion into ethylene dichloride (organic phase) containing the fluorogenic reagent. Even though

derivatization proved successful, there was the need to eliminate excess of derivatization reagent which might interfere with the determination.

Fatty acids have also been separated by adsorption and partition methods.[16] While the chromatographic separation by adsorption proved difficult, that of partition yielded excellent results.

Though each worker tended to modify and improve on the work of the previous worker, no attempt had been made to obtain derivatives for fatty acid determination through heterogeneous techniques, so also, some of the reagents employed for homogeneous derivatization by some workers could not be easily obtained. Thus there is the need to develop reagents which are accessible and can be used to prepare fluorescent derivatives through heterogeneous techniques for the analysis of carboxylic acid.

1.5 Analysis of Amines

Amines are ammonia derivatives in which one or more hydrogen atoms have been replaced by alkyl and or aryl groups attached to the nitrogen atom,[8] Aliphatic amines can be present or can be produced through natural decomposition processes in a variety of systems such as in many daily foods.[17]

Aliphatic amines can by themselves be toxic or become toxic via chemical reactions as in reaction with nitrites, forming nitrosamines. Some nitrosamines are highly toxic carcinogenic compounds. Some microorganisms produce nitrosamines 'in vivo' during infection.[18] In the field of medicine, and the industry, the study of aliphatic polyfunctional amines are of great importance, for instance, the presence of spermine, spermidine, the putrescine detected at trace levels in urine allowed cancer diagnosis at an unusually early stage.

1.5.1 Methods of Analysis

Many analytical methods have been employed for the analysis of amines by many workers. However, most of the methods have been chemical derivatization techniques in which the amine substrate of interest is reacted with reagents to form derivatives that can be monitored by spectrophotometry fluorimetry or colorimetry.

Some workers[17] used dansylchloride as the derivatising agent and detection was by fluorimetry. Such a reaction provides an opportunity for identification and separation of aliphatic monamines, diamines and polyamines with fluorescence detectors.

1.5.2 Dansylation Reaction

Dansylchloride which is 5-dimethylamino-naphthalene-1-sulfochloride has been used to form amides that are characterized by high absorbivity as well as fluorescence emission.

In the reaction, mixture of 5.0ml of 0.25M sodium hydrogen carbonate solution, 1.0ml of dansylchloride 0.02m and 0.5ml of each amine solution of desired concentration were reacted in about 10.5ml acetone. The reaction was carried out in a sealed reaction vessel at 60°C for 20 minutes after which the derivatives were obtained and analysed.

The major limitation of this method is the need to avoid the use of excess dansylchloride which can fluorescence as the derivatives thus interferring with the analysis by swamping the response of the system to the derivatives to be determined.

In addition, Schwedt and Bossemas[19] converted dopamine and noradernaline to fluorescent derivatives by the reaction with dansylchloride also, nitrogeneous compounds related to amines such as carbamates have been examined as the dansyl derivatives of N-methylcarbamates and monitored by high performance liquid chromatography (HPLC).[20] Furthermore, barbiturates have been derivatized by dansylchlorides and detected by fluorescence.

1.5.3 Other Reagents

Other useful reagents that have been used in converting amines and related compounds into fluorescent derivatives include fluorescamine and O-phthaldehyde (OPA).[21]

Fluorescamine which was substituted for ninhydrin has been used for both post and precolumn derivatization of catecholamines, dopamines and noradrenaline.[22,23]

Also of equal importance is O-phthaldehyde and a unique aspect of this reagent is that itself and the product have entirely different electronic spectra, thus separation of fluorescent products from equally fluorescent excess reagent is avoided in the reaction. OPA undergoes condensation reaction with the amine substrate in the presence of a strong nucleophile (i.e. athiol) to form an isoindole that fluorescences intensely.[21]

However, the limitation of this reaction is that the product i.e. isoindole, is highly unstable with respect to light, acid attack or air oxidation.

P-Nitrobenzoyl chloride, chloroacetaldehyde have been employed as derivatizing agents[24,25] for amines and they formed derivatives that were easily detected by fluorescence or spectrophotometer.

Diastereoisomeric derivatives were produced by reacting optically active amines with (s)-o-methyl mandeloyi chloride. The resolution of the R- and S-methyl benzylamine derivatives revealed the optical purity of the amines.[26]

Amines have also been identified by spectrophotometric method. In this aspect, amines and related compounds were reacted with reagents to produce coloured species that could be determined spectrophotometrically.

Anthony Benson and William Spillance[27] determined amines and sulfamates (i.e. artificial sweetner) with 1,4-benzoquinone using spectrophotometry. In their reaction, the amine substrate in chloroform was reacted with 1% ethanolic, 1,4-benzoquinone at 60°C for different reaction times to obtain reaction products that were determined spectrophotometrically by measurement of absorbance at λ max (wavelength of absorption) 478-510nm.

Most of the reported works have been carried out through homogeneous derivatization techniques, however, the application of heterogeneous derivatization in the analysis of amines has been limited to the reported work of Krull and co-workers.[4] The workers prepared different bound anhydrides. The polymeric anhydride contains 0-acetyl salicyl as the labelled moiety to derivatize secondary and primary amines. The structure of the

group together with its reaction with an amine compound is presented in Figure 1.17.

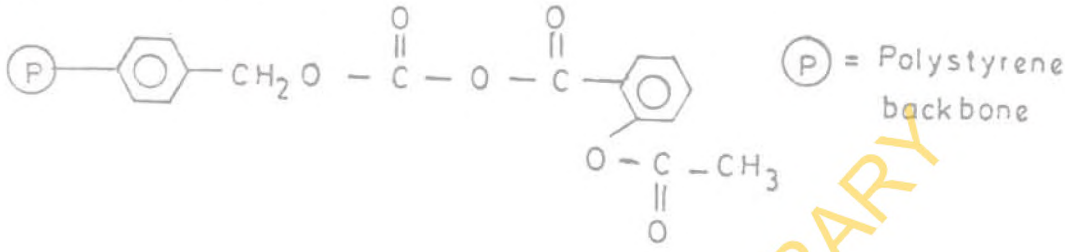


Fig. 1.17: Structure of polymeric anhydridge containing O-acetyl as the labelled moiety.

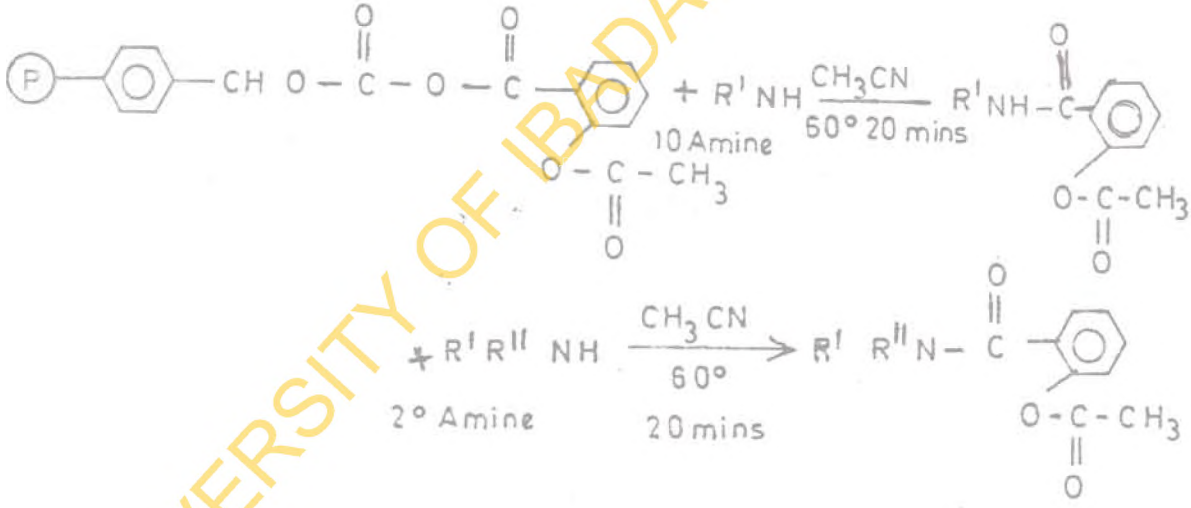


Fig. 1.18: Derivatization reaction of primary and secondary amines with a polymer-bound anhydride reagent.

The same group of workers[28] also investigated the use of (a) a polymeric benzotriazole activated ester reagent, and (b) a polymer fluorenylester activated by 0-nitro-benzophenone for the derivatization of amines for HPLC analysis with UV/FL detection. The limitations of the polymeric benzotriazole reagents are its sensitivity to moisture and elevated temperature due to the unstable triazole ring. For these reasons it is not easily used in on-line derivatization, though it can be used successfully for the off-line derivatization.

The continual use of these reagents in a precolumn on-line manner leads to gradual degradation of the materials and loss of all tagging properties because of the instability of the reagents. On the other hand, the 0-nitrobenzophenone activated ester reagent is more stable to moisture at both room and elevated temperature. It is highly reactive towards nucleophilic attack and can therefore be used for on-line derivatization in HPLC.

The structure of the fluorenyl attached polymeric o-nitrobenzophenone reagents in Fig. 1.19, together with its reaction with an amino compound is represented in Figure 1.19 and Fig. 1.20.

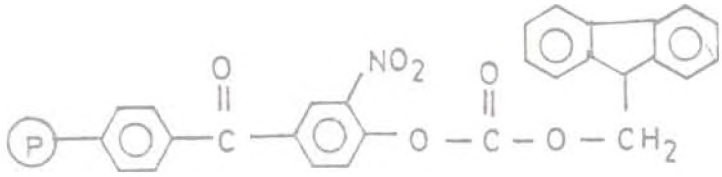


Fig. 1.19: Polymer-3-nitro-4 [(9-fluorenylmethoxy)-carboxyl]-oxy benzophenone.

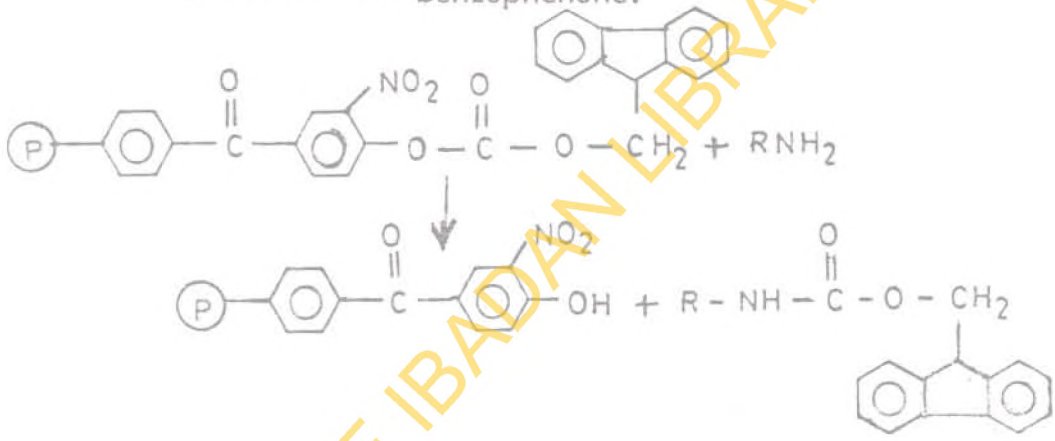


Fig. 1.20: Derivatization of typical amines with the polymer bound nitrogenzophenone activated ester

Also Chun, Gao, Grinberg and Kruil[29] have utilized a polymer which is covalently attached to a Chiral molecule such as amino acid in resolving and quantitating biologically active enantiomers. In their approach, 9-fluorenylmethyl moiety (FMOG), a detector senstivie molecule, was covalently bonded to an amino acid (a chiral molecule) which has already been covalently attached

to a polymer. This approach had been mainly applicable in the determination of enantiomeric purity and composition as well as chemical purity of virtually all strong nucleophiles such as primary and secondary optically active amines and amino like compounds. But the limitations of the approach is that the particular reagents employed have not been successful for soft nucleophiles such as alcohols, thiols, carboxylate anions.

The structure of polymer bound 4-hydroxyl-3-nitrobenzophenone containing FMOC L-proline is shown in Figure 1.21 below.

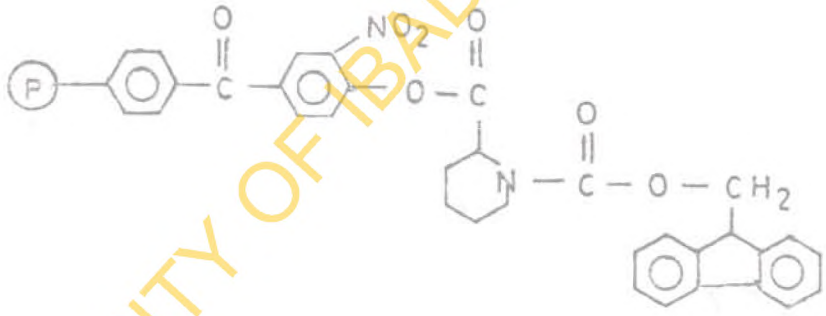


Fig. 1.21: Structure of polymer bound 4-hydroxyl-3-nitrobenzophenone containing FMOC-L-proline.

Though a lot of synthetic work has been carried out by various workers with this approach, the reagents developed by these workers cannot be easily prepared in a routine laboratory. There is therefore a need to develop more readily accessible

reagents, not only for amines, but also for other commonly encountered functionalities such as carboxylic acids, alcohols, carbonyl compounds and so on. Such reagents could be based on a variety of other ideas apart from the activated anhydride approach repeatedly adopted by Krull and coworkers.[2,4,28,29] Also, because of the ready availability of ion-exchange resins, bound reagents based on these resins as support would be expected to be more readily accessible than the previously reported reagents based on the use of specially prepared polymers as support.

1.6 Aim and Objective

The aim of the present work is therefore the development of novel ionically or covalently bound reagents for the pre-chromatographic derivatization of carboxylic acids and amines. These reagents will be based mostly on the use of readily available ion-exchange resins as supports.

CHAPTER TWO

EXPERIMENTAL

2.1 Preparation of Sodium-benzoxazole-2-sulphonate[30]

2.1.1 Preparation of 2-mercaptobenzoxazole

O-aminophenol (15.0g) was mixed with 75ml of methanol, 10ml of potassium hydroxide solution prepared by dissolving 10g of the pellets in 10ml of distilled water, was added. 25ml of carbon disulphide was carefully added to the mixture and refluxed for two and half hours, after which a further 10ml of carbon disulphide was carefully added and refluxed for further 30 min.

After 3 hrs, the mixture was allowed to cool for 5 minutes, decolorizing charcoal was added and further refluxed for 10 mins before filtering hot through fluted filter paper. The filtrate was evaporated to dryness in boiling water bath. The residue was redisolved in 75-100ml of distilled water and the solution treated carefully, with vigorous shaking, with 30ml of glacial acetic acid mixed with an equal volume of water. 2-mercaptobenzoxazole was immediately precipitated as a whitish solid. This was filtered by suction and dried in air. The melting point was determined.

2.1.2 Preparation of 2-chlorobenzoxazole and sodium-benzoxazole-2-sulphonate

2-mercaptobenzoxazole (5.0g) was mixed with about 15g of phosphorus pentachloride. Mixture was heated on a water bath for 1 hour and then on a hot plate for 1-1½ hours, to reflux. The mixture was allowed to cool to room temperature and treated with 50ml of 25% sodium sulphite solution.

After effervescence, the mixture was treated with a further 50ml of saturated sodium sulphite solution. It was then refluxed for about 2 hours on hot plate, after which it was filtered hot through fluted filter paper. Crystals appeared as white needles. This was filtered by suction and dried in air. Thionylchloride may be used in place of phosphorus pentachloride. When 2-mercaptobenzoxazole (4.5g) was treated with 20ml of thionyl chloride ($SOCl_2$), a vigorous reaction with evolution of gas and a dark green solution was obtained. The solution was refluxed in water bath for 2 hours during which three 10ml portion of thionylchloride were added at intervals. The excess thionyl chloride was distilled off and a brown oil was left. The brown oil was poured carefully into 50ml of 25% W/V sodium sulphide solution. This was then refluxed for 2-2.5 hours. At the end of reflux, solution was filtered hot and white crystals precipitated. The brown solid in the round botton flask was

repeatedly digested 4 times with 20-30ml of distilled water by heating over a hot plate for 15-20 minutes on each occasion, the hot solution was filtered into the original liquid, more of the whilte product precipitated. This was then filtered by suction to obtain white solid.

2.2 Preparation of Resin-Bound-Benzoxazole-2-Sulfonate

2.2.1 Determination of exchange capacity of an ion-exchange resin (anion exchange resin((Cl) form

1.0g of air dried anion exchange resin (chloride form) was weighted into a watch glass, and this was transferred carefully through a dry clean funnel into a 50ml clean burrette. Sufficiently distilled water was added to cover the resin and all traces of air bubbles that stick to the resin and the burette were dislodged.

0.24M of sodium nitrate ($NaNO_3$) solution was prepared and transferred into a 250cm^3 separating funnel which was mounted on the top of the burette such that the tip of the funnel made close contact with the opening of the burette as the solution dropped into the column at the rate of about 2cm^3 per minute, the effluent was collected in a 500cm^3 conical flask and titrated with standard 0.1M silver nitrate solution ($AgNO_3$) using potassium chromate as indicator.

The following reaction occurred as explained in Fig. 1.22 below.

$$R^+Cl^- + NO_3^- \qquad R + NO_3^- + Cl^-$$

R^+ = resin

Cl^- = counter ion

NO_3 = exchanging ion

Fig. 1.22: Equation showing the exchange of chloride ion by nitrate ion in an anion-exchange resin

2.2.2 Titration of effluent (NaCl) against standard 0.1M silver nitrate

Titration: Effluent (NaCl) vs 0.1M $AgNO_3$

Pipette ($25cm^3$) contains 0.25M NaCl

Burrette ($50cm^3$) contains 0.1M $AgNO_3$

Indicator $0.5cm^3$ for each $25cm^3$ of the final volume of the test solution

Average results of three different titrations _ 29.70 cm^3

Indicator blank = 1st $1.65cm^3$, 2nd $1.25cm^3$.

Exchange capacity of resin = Milli-equivalent per gram = bV/W where VCM^3 of $BMAgNO_3$ are required by Wg of the resin. The calculation is shown in Fig. 1.23 below.

$$\therefore \quad bV/M = \frac{0.1 \times 29.70}{1} = 2.970 \text{ Meq/g}$$

$\therefore$ 2.97×10^{-3} Moles $\equiv$ 1g

2.97 Moles = 100g

0.00297 = 1g

$\therefore$ Meq/g of anion exchange resin (chloride form)

= 0.00297

Fig. 1.23: Equation expressing the determination of exchange capacity

2.2.3 Coupling sodium-benzoxazole-2-sulfonate with resin (chloride form)[30]

Resin (2.97meq/g) used for preparation of resin bound agent as follows.

The equivalent amount of sodium-benzoxazole-2-sulphonate that will couple with 1g of resin (chloride form) is obtained as follows.

0.00297 x 221g (molar mass sodium benzoxazole-2-sulphate = 0.69615g.

0.5g of resin was stirred with sodium-benzoxazole-2-sulfonate (0.35gms) in solution of warm distilled water. To establish the optimum time for exchange, mixture was stirred for 3 hours, solution from mixture was collected at time intervals of 15 minutes, 30 mins; 1 hour; 1 hour 30 mins; 2 hours; 2 hours 30 mins; and 3 hours, and titrated against a standard solution of silver nitrate

Table 2: Determination of optimum time of exchange between chloride ion and benzoxazole moiety in anion exchange resin (chloride form)

Time Interval	15 mins	30 mins	1 hr	1hr 30m	2 hr	2hr 30m	2hr 45m	3.0 hr
Final readings cm^3	11.20cm^3	11.60cm^3	12.60	22.65	11.60	25.00cm^3	36.60	12.70cm^3
Initial reading cm^3	0.0	0.0	1.00	11.40	0.00	13.35	25.00	1.50
Volume of $AgNO_3$ used in cm^3	11.20	11.60	11.60	11.25	11.60	11.65	11.60	11.20

using potassium dichromate/chromate mixture as indicator. Same procedure was carried out for blank. The procedure used in determining optimum time is summarized in Table 2 below.

Correction was also done for blank, i.e. distilled water. This is shown in Fig. 1.2 below.

Blank distilled water

Final readings cm^3	13.55
Initial readings cm^3	12.00
Volume of $AgNO_3$ used cm^3	0.55

Fig. 1.24: Blank correction.

The optimum time for exchange was 30 minutes. Equation for coupling is shown in Fig. 1.25 below.

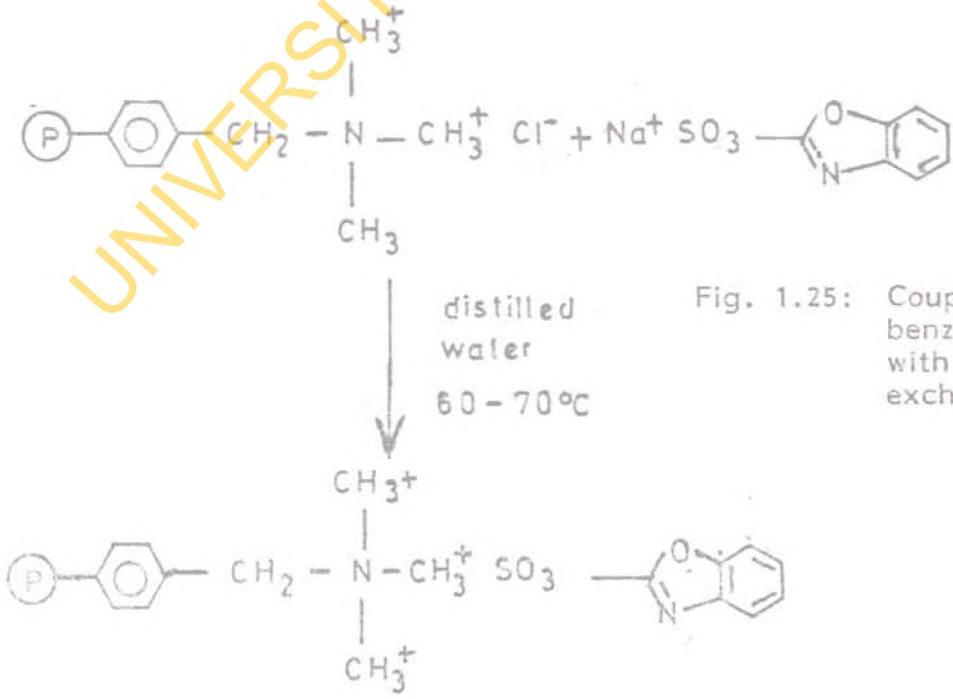


Fig. 1.25: Coupling of benzoxazole with anion exchange resin

2.3 Reaction of Amines with Sodium-Benzoxazole-2-Sulfonate

10µl of the following amines, Di-n-butylamine, diethylamine, Di-n-propylamine, 4 nitroaniline and blank were added to five different test tubes. Sodium-benzoxazole-2-sulfonate (0.1g) was added to each of the test tubes. The mouth of the test tubes were sealed with masking tape, and mixtures were warmed at 60°C for 5 minutes in a water bath. At the end of 30 minutes, the reaction product were each observed under ultra-violent light (254nm and 360nm). After cooling, the mixture was extracted with 5ml of chloroform by shaking on a vortex mixer for 1-2min. The aqueous layer was removed with a pasteur pipet and the chloroform dried with anhydrous sodium sulfate. The chloroform extract was then transferred to another test tubes and the chloroform removed under a stream of nitrogen. Some of the residue obtained was redissolved in methanol for TLC; HPLC and mass spectrometric analysis. The methanol solution was also examined under UV light. The same procedure was also carried out using acetonitrile as medium for reaction instead of distilled water.

2.4 Optimization of Solvent, Temperature and Time

Among the reaction performed was to investigate which other solvent apart from water could be used as a good medium for derivatization reactions of the analyte. In this regard optimal reaction conditions for the formation of the derivatives were determined by comparing the fluorescence of the products under UV light as a function of solvent. All other reactions conditions were held constant; solvents tested included methanol, ethylacetate, hexane and acetonitrile.

Temperature was optimized by varying from 30°C (laboratory temp.) to 100°C holding time constant at 30 minutes.

Next to be optimized was time in the process, the optimum temperature at 60°C was held constant and time varied from 0 minutes to 1 hour.

2.5 Derivatization of Amines with Resin-Bound Benzoxazole-2-Sulfonate

To a solution of 100µl of diethyl- or di-n-propyl or di-n-butylamine or 4-nitroaniline in 1.0ml of methanol was added 1.0g of resin tagged with benzoxazole-2-sulfonate. After warming in a water bath (60°C) for 5 min the solution was examined under UV light and analysed by TLC, HPLC and HPLC-MS. The procedure

was repeated using acetonitrile in place of methanol. The procedure was repeated, with the mixture being kept at room temperature before chromatographic analysis.

The same procedure was done for blank.

2.6 Reaction of Amino-Acids with Benzoxazole-2-Sulfonate

To 100mg of glycine (or l-lysine or l-cysteine) dissolved in 1.0ml of water was added a solution of 100mg of sodium benzoxazole-2-sulfonate in 2.0ml of water. The mixture was examined under UV light and then warmed in a water bath (60°C) for 5 mins before being re-examined under UV light. A portion of the reaction mixture was basified by dissolving about 200mg of sodium bicarbonate in it and solution re-examined under UV light.

The same procedure was done for blank.

2.7 Derivatization of Amino-Acids Using Resin-Bound Benzoxazole-2- Sulfonate

To 100mg of glycine (or 1 lysine or 1 cysteine) dissolved in 1.0ml of methanol was added a 1.0g of resin-tagged with benzoxazole-2-sulfonate. After warming in a water bath (60°C) for 5 min the solution was examined under UV light. An aliquot

of the reaction mixture was treated with 200mg of sodium carbonate and observed under UV light and analysed by TLC.

The same procedure was done for blank.

2.8 Derivatization Using Resin, Sodium Benzoxazole-2-Sulfonate, Amine Substrates

To 100µl of diethyl or di-n-propyl or di-n-butyl or 4-nitroaniline in 1.0ml of methanol was added solution of 0.1g sodium benzoxazole in methanol and 0.1g of anion exchange resin chloride form. The mixture was warmed in a water bath at 60°C for 5 minutes before being observed under UV light. The same procedure was carried out for blank.

2.9 Derivatization Using Resin, Sodium Benzoxazole-2-Sulfonate, Amino Acids Substrate

To 100mg of glysine or 1 lysine or 1 cysteine in methanol was added solution of 0.1g sodium benzoxazole in methanol and 0.1g of anion exchange resin chloride form. The mixture was warmed in a water bath at 60°C for 5 minutes before being observed under UV light. An aliquot of reaction mixture was treated with 200mg of sodium carbonate and examined under UV light. The same procedure was performed for blank.

2.10 Thin-Layer Chromatographic Analysis of Amine Derivatives Obtained Through Homogeneous Reaction

Working Procedure

1-2µl of diethylamine, di-n-butylamine, di-n-propylamine, 4 nitroaniline derivatized in aqueous medium (standard amine derivatives) were spotted along with blank on a thin layer plate coated with silica gel. Spots were allowed to dry in an oven after which the plate was developed in a tank containing acetonitrile:water (85:15).

At the end of development the plate was removed from the bank and allowed to dry in air; after which the spots were observed under a UV light. The same procedure was also carried out for standard amine derivatives prepared using acetonitrile as the medium for reaction.

2.11 Thin Layer Chromatography of Amino Acid Derivatives Obtained From Homogeneous Reaction

Working Procedure

Aliquots of L-lysine, cystine, glycine derivatized in aqueous medium were spotted along with the appropriate blank. The procedure above as described for amines was followed.

2.12 Thin-Layer Chromatography of Amine Derivatives Obtained Through Heterogeneous Reaction

Aliquot of the derivatized products (diethylamine, di-n-butylamine, di-n-propylamine, 4 nitroaniline) and blank were spotted on a high layer coated with silica. Spots were allowed to dry after which plate was developed in a tank containing acetonitrole:water (85:15). At the end of development the plate was removed and solvent was allowed to dry after which it was observed under a UV light.

2.13 Thin Layer Chromatography of Derivatives from Resin (Chlorideform), Sodium Benzoxazole-2-Sulfonate and Amine Substrates i.e. Diethylamine, Di-n-butylamine, Di-n-propylamine, 4 nitroaniline and Blank

The procedure described above was followed. Plate was also observed under a UV light.

2.14 Thin Layer Chromatography of Resin Bound Derivatives of Lysine, Glycine, Cystein and Blank

Aliquot of the derivatized products (lysine, glycine, cystein) and blank were spotted on a thin layer coated with silica. Spots were allowed to dry after which plate was developed in a tank containing acetonitrile:water (85:15). At the end of

development, the plate was removed and solvent was allowed to dry after which spots were observed under ultra-violet light.

2.15 HPLC-UV-FL Analysis

HPLC was performed on a Waters liquid chromatography system consisting of a water model 510 solvent delivery unit, a injector and a Waters model 440UV detector set at 254nm. A Waters Ubondapak C_{18} column (4.6mm x 30cm) was used altogether with a mobile phase of acetonitrile:water (65:35v/v) at a flow rate of 2.0ml/min. Data acquisition and chromatography control was done using a Waters 860 data station.

2.16 Mass Spectrometric Identification

Mass spectrometric identification of the derivatives was performed using a HPLC-MS system consisting of a Hewlett Packard 1090 Liquid Chromatography system linked to a Hewlette Packard HP5989A mass spectrometer through a HP 59980B particle beam interface. The HPLC mobile phase was a 50:50 (v/v) mixture of acetonitrite and 0.1M ammonium acetate (adjusted to pH 4.5) at a flow rate of 0.5 ml/min. There was no column on-line. The conditions of the mass spectrometer was set as follows for positive EI mode:desolvation temperature, 65°C

source temperature, 150°C; electron energy 230eV; helium pressure, 60psi; acceleration potential, 7KV computerised background substration was carried out.

2.17 Preparation of 2-Chloromethyl Naphthalene

Naphthalene (30g), paraformaldehyde (9g), glacial acetic acid (90ml), phosphoric acid (30ml) and conc. hydrochloric acid (30ml) were mixed. The mixture was heated by immensing in a water bath at 90°C - 100°C for 3 hours; with shaking at intervals.

A yellowish oil was formed on the top of the mixture after heating for 2½ hours, the oil layer was carefully decanted and the yellowish mixture heated for further 30 minutes, more oily layer emerged and was carefully decanted. The oil removed soon solidified and this was digested with acetone by heating on a water bath to give a pale yellowish solid. Mixture of the pale yellow solid separates as the main bulk of the reaction mixtures cooled. The melting point was determined and yield calculated.

2.18 Preparation of 2-Naphthalene Methanol

2-Chloromethyl naphthalene (2g) obtained as described above was dissolved in acetone, and solution of silver nitrate (1.8g) dissolved in 40ml of acetone containing 10ml of water (i.e. 30ml

acetone _ 10ml distilled water) was prepared. Both solutions were mixed and there was an immediate reaction and silver chloride being precipitated. The solution was filtered to remove the silver chloride residue after which the filterate was treated with distilled water to precipitate 2-naphthalene methanol.

The melting point was determined and yield calculated.

2.19 Preparation of Resin-Supported Sulphonylchloride

The sulfonated ion-exchange resin (sodium form) was dried in an oven at 135°C for 3 hours. The dried resin (85g) and powdered phosphorus pentachloride (45g) were mixed in a 500ml round bottomed flask quick fit with a reflux condenser. The mixture was heated in an oil bath at 175°C for 13 hours, with shaking at interval of 3 hours. At the end of reflux, product was allowed to cool to room temperature, after which it was washed with cold water, followed immediately by acetone. The product was allowed to dry in air.

2.20 Preparation of Resin Bound Naphthalene Methanol

2-Naphthalene methanol (1g) was dissolved in an inert solvent (acetone). The solution was added to 4g of resin-supported sulfonyl chloride in a 250ml round bottomed flask quick

fitted with a reflux condenser, 5ml of trimethylamine was added to the mixture which was then refluxed for 3 hours. At the end of reflux, the mixture was allowed to cool to room temperature and filtered by suction. The resin-sulfonate ester was washed many times with acetone to remove traces of unreacted 2-naphthalene methanol. The washing continued until the filterate after observation under ultra-violet light did not fluorescence.

The resin was then allowed to dry in air.

2.21 Reaction of Carboxylic Acids with 2-Chloromethyl Naphthalene

Stoichometric amount of mixtures of fatty acids, 2-chloromethyl naphthalene and triethylamine were refluxed in an oil bath at different temperatures and different reaction times. The Table 3 shows the stoichometric quantities for various fatty acids and 2-chloromethyl naphthalene in triethylamine. The various temperature and reaction time were also indicated.

Table 3: Stoichometric Preparation of Ester Derivatives of Fatty Acids

2-Chloromethyl Naphthalene $C_{11}H_9$ F.W(176.5g) 1g of $C_{11}H_9C_1$ (0.00566 moles)

	Fatt Acids	Structural Formula	Molecular Formula	Formula Weight	Amount to react	Temp range (°C)	Time range (hours)
1.	Lauric	$CH_3(CH_2)_{10}COOH$	$C_{12}H_{24}O_2$	188.0	1.064g	140°	3-4
2.	Palmitic	$CH_3(CH_2)_{14}COOH$	$C_{16}H_{32}O_2$	256.0	1.448g	165°	
3.	n-Docosanoic	$CH_3(CH_2)_{20}COOH$	$C_{22}H_{44}O_2$	340.0	1.924g		
4.	n-Valeric	$CH_3(CH_2)_3(COOH)$	$C_5H_{10}O_2$	102.0	0.577g		
5.	n-Nonanoic	$CH_3(CH_2)_7COOH$	$C_9H_{18}O_2$	158.0	0.894g		
6.	n-Hexanoic	$CH_3(CH_3)_4COOH$	$C_6H_{12}O_2$	116.0	0.656g		
7.	Capric	$CH_3(CH_2)_8COOH$	$C_{10}H_{20}O_2$	172.0	0.973g		
8.	Octanoic	$CH_3(CH_2)_6COOH$	$C_8H_{16}O_2$	144.0	0.815g		
9.	Linoleic	$CH_3(CH_2CH=CH)_3$	$(CH_2)_7COOH$	278.44	1.575g		
10.	Acetic	CH_3COOH	$C_2H_4O_2$	60.0	0.3396g		

Triethylamine $(C_2H_5)_3N$ = 101.19 0.592g.

Equation for reaction is shown in Figs. 1.26 and 1.27.

$$R^1COOH + R^{11}CH_2 \times \xrightarrow[3-4 \text{ hours}]{140 - 165^\circ C} R^{11}CH_2O\ C\text{-}R^1 + HX$$

$$(C_2H_5)_3N$$

Fig. 1.26: Equation of reaction between carboxylic acids and chloromethyl naphthalene

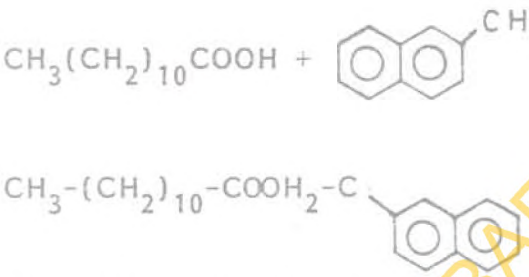


Fig.1.27: Equation to show the reaction of 2-chloromethyl naphthalene with lauric acid

At the end of reflux, the oily products obtained were each dissolved in hexane and filtered into sodium sulphate crystals. The filterate was then removed and solvent evaporated off on a water bath. The oily product crystallized to a solid mass as product cooled. The product was stored in a refrigerator.

2.22 Determination of Carboxylic Acids with Resin-Bound Reagents

As an application, the resin-bound reagent was used to derivatize the acids as follows. In a 25ml quick fit round bottom flask. 0.25g of a carboxylic acid was added to .5g of the resin-bound reagent. 5ml of acetone was added to the mixture followed by 2ml of triethylamine. The mixture was refluxed at 78-80°C for 1 hour 30 minutes after which the mixture was allowed to cool to room temperature. The supernatant was decanted off into specimen bottle and acetone evaporated off. Distilled water 0.5ml was added to the product followed by 2ml of chloroform or methylene chloride. The mixture was shaken thoroughly and the aqueous layer removed with a siphoning pipette. Few quantities of sodium-sulphate was added to the organic phase and the filterate was removed after some minutes (3-5). The chloroform was evaporated off to obtain the oily product which later solidified. 0.5g of the resin-bound reagent and 5ml of acetone followed by 2ml of triethylamine were refluxed for 1 hour 30 minutes at 78-80°C. At the end of reflux the mixture was allowed to cool and the supernatant decanted off into a specimen bottle.

The blank was also cleaned up as mentioned above and observed under ultra violet light.

2.23 Thin-Layer Chromatographic Analysis of Esters

(i) Preparation of sample

Few quantities of stand ester derivatives of lauric, capric, hexanoic, octanoic nonanoic, palmitic lindeic, desanoic, valeric were each dissolved with 0.05ml of chloroform. Aliquots of the derivatives were then spotted along with blank on a coated plate. The spot was allowed to dry in air after which the plate was developed in a 63% alcohol prepared by adding 1 volume of distilled water to 2 volume of 95% ethanol. At the end of development the plate was allowed to dry in air, after which it was observed under ultra violet light.

(ii) To validate the result further

Thin layer chromatography was carried out in another solvent medium i.e. 1ml ammonia solution and 100ml of 95% ethanol. The plate was also observed under UV light.

The chromatographic procedure was performed 3 times and each reaction mixture was spotted in triplicate for a total (b=9).

HPLC quantitation to be carried out in an outside laboratory.

2.24 Melting Point Determination of Standard Ester Derivatives

The melting points of the various ester derivatives were determined by two methods. The first involved the use of paraffin and capillary tubes attached to a celsuis thermometer (100°C). The second procedure involved the use of an instrument (electrothermal). In both cases with limits of experimental error the result obtained were reproducible when compared.

The various melting points are listed under results and discussions section.

2.25 Thin-Layer Chromatographic Analysis of Derivatives Prepared Using the Resin Bound Reagent

Following the same procedure applied for chromatographic analysis of standard ester derivatives. The thin layer chromatographic analysis of resin-bound derivatives and the appropriate blank were carried out. The results obtained were compared with those obtained for standard ester derivatives. This is discussed under the section for results and discussion.

2.26 Thin-Layer Chromatographic Analysis of Standard Ester Derivatives and Each Corresponding Derivatives Obtained from Resin-Bound Reaction

On same plate each standard ester derivatives was spotted alongside its corresponding resin-bound derivatives. This was also done for the blank. Plates were developed as explained previously and observed under UV light. The results are discussed under the section for result and discussion.

CHAPTER THREE

RESULT AND DISCUSSION

3.1 Synthesis of Sodium Benzoxazole-2-Sulfonate

For derivatization reagents to be ideal for HPLC on TLC with UV or FL detection it should possess UV or FL characteristics that are completely different from that of the derivatives, this will take care of excess reagent that may interfere with the detection of the derivative.

However, if reagent and the derivative have similar spectra characteristics, their chromatographic behaviour should be widely different; this will allow easy separation of excess reagent from the derivative if this cannot be easily achieved by simple solvent extraction. The reagent should react readily with the analyte without any complicating side reactions.

In addition derivatives obtained from reaction should be stable, and derivatization reaction should be possible in a variety of solvents and solvent combinations that are likely to be encountered during the intended chromatographic applications. These are important requirements if the reagent is to be applicable to on-line pre- or post-column derivatization in a possible automation of the analytical method. The reagent should be cheap.

Most analytical reagents previously used by various workers either have close similarity in their spectra or fluorescent characteristics when compared to the derivatives they formed, with amine, amino acids and carboxylic acid e.g. Dansylchloride (5-dimethylamino-naphthalene-1-sulfochloride).

As a result, the inevitable presence of excess reagent in the reaction mixture, therefore, causes high background response and hence an inadequate detection limit for the analyte. Introduction of steps to remove excess reagent often makes the method more tedious and more prone to error.

Some reagents such as fluorescamine, NBD chloride (7,chloro-4-nitrobenzo-2-oxa-1, 3-diazole), OPA (o-phthalaldehyde are non-fluorescent or poorly fluorescent; though they react with amines to give fluorescent derivatives, are in this respect better than a fluorescent reagent such as dansyl chloride. These reagents, however, share the disadvantage that they can only be used in homogeneous media, moreover, they sometimes undergo undesirable hydrolytic decomposition during derivatization reactions with amine; besides, their fluorescent derivatives are highly unstable to light, temperature and moisture.

Amines are known as fairly strong nucelophiles, chromogenic derivatives of amines have been prepared. Such preparation was based on their reaction with reagents bearing sulfonate groups activated towards nucleophilic substitution. The reagent that were used for such preparation included sodium 2,4-dinitrobenzene sulfonate[35-38] and sodium 2,6-dinitro-4-trifluoromethylbenzene sulfonate.[39]

These reagents have the desirable property of being usable in aqueous media. The reagents, however, give non-fluorescent derivatives since the electron-withdrawing nitro groups required for the activation of the sulfonate moiety towards nucelophilic substitution are also strong inhibitors of fluorescence.

For the preparation of fluorescent derivatives of amines, it was thought that a liabile sulfonate group attached to a fluorescent aromatic nucleus would give a water-soluble reagent possessing the desirable characteristic outlined.

Azoles are monocylic aromatic compound with more than one heteroatoms. They are 5 membered rings with 2-heteroatoms with nitrogen as one of the heteroatoms.

In oxazole, the 3rd carbon atom of a furan is replaced by nitrogen and when this oxazole is joined to a benzene ring a benzoxazole results as shown in the Figure 1.28 below.

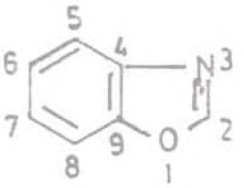


Fig. 1.28: Structure of Benzoxazole.

The nitrogen atom is highly unsaturated, and as Longuet and co-workers[31] have shown, the 2 or α-position is relatively low in electro density. Thus the reactivity of a methylene group in this position is similar to that of activated methylene group of 2,4,6,trinitrotoluene or ethyl acetoacetate.

Thus 2-methyl benzoxazole will participate in knoevenagel reactions[32] i.e. a general method of preparing α, β-unsaturated acids as shown in Figure 1.29 below.

$$RCHO + CH_2(CO_2C_2H_5)_2 \xrightarrow{\text{base}} RCH{=}C(CO_2C_2H_5)_2 + H_2O$$

$$RCH = C(CO_2C_2H_5)_2 \xrightarrow[\text{(ii) HCl}]{\text{(i) KOH}} RCH = C(CO_2H)_2 \xrightarrow{150\text{-}200^\circ C}$$

$$RCH = CHCO_2H + CO_2$$

Fig. 1.29: Reaction between aldehydes and compounds with active methylene groups in the presence of an organic base.

In addition, 2 methylbenzoxazole will take part, in Ortoleva King reaction[33] just like methyl or methylene groups in ketones as shown in the example below.

Fig. 1.30: Reaction of methyl groups in ketones with pyridine

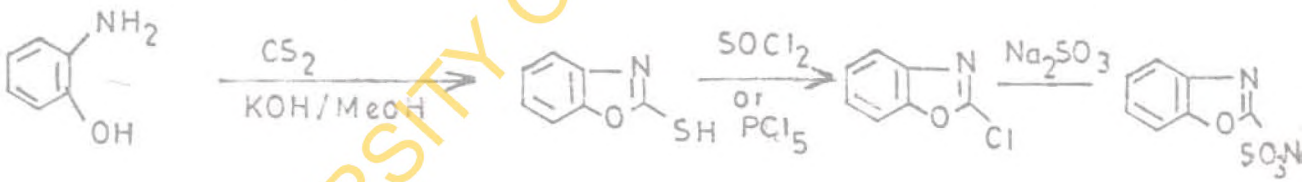
Also it has been shown that the activated sulfonic group in 2,4,6,trinitrobenzene sulphonic acid can be subjected to nucleophilic substitution. Besides, the reagent was used to determine amino compounds spectrophotometrically[34]. Such a reagent cannot however be used for analysis of amines by spectrofluorimetry due to the presence of nitro groups that is fluorescence inhibiting.

Thus from all the enumerated analogies, it was imagined that a sulphonic acid group in the activated 2-position of benzoxazole would be liable to nucleophilic substitution. Besides, benzoxazole is potentially a fluorescent moiety which will be non-fluorescent or weakly fluorescent when a sulfonate group is attached due to electron-withdrawing/fluorescent inhibiting effect of this group, however, substitution by an amine will give a fluorescent product.[30]

In conclusion, due to anticipated high reactivity of the activated sulfonic group towards amines and the potential fluorescence of the benzoxazole moiety, sodium benzoxazole-2-sulfonate was prepared and investigated as a possible water soluble reagent for fluorimetric analysis of amines and amino acids.

Sodium benzoxazole-2-sulfonate has only been mentioned in an old patent literature.[40] It is readily obtained pure from 2-chloro benzoxazole as described in Figure 1.31 below. Although we had to prepare 2-chlorobenzoxazole, this compound is apparently available commercially.

The reagent was prepared as seen in the equation[30] as shown in Figure 1.31 below.



Each stage of the reaction was monitored by thin-layer chromatography. When thin layer chromatography was carried out in chloroform. Single but different spots were obtained for 2 mercaptobenzoxazole, 2 chlorobenzoxazole and sodium benzoxazole 2-sulfonate.

3.1.1 Observation of sodium-benzoxazole solution under UV

100mg of the sodium-benzoxazole-2-sulfonate was dissolved by warming in distilled water. This solution was observed under UV light there was no fluorescence. Then about 100µl of N-butylamine was added to the solution and observed under UV light. The solution gave an intense blue fluorescence. This indicated that the amine will react very fast with benzoxazole solution to give a flurescent product. The equation for such reaction is shown in Figure 1.32 below.

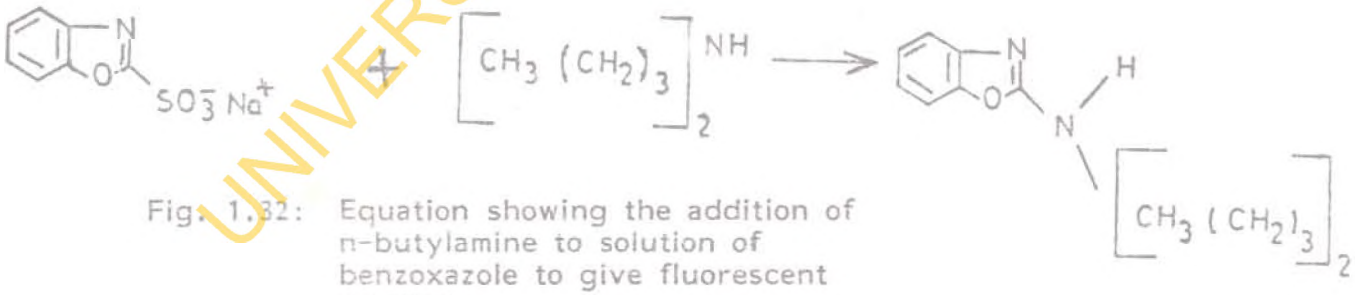


Fig. 1.32: Equation showing the addition of n-butylamine to solution of benzoxazole to give fluorescent product

One of the initial experiment was to determine the exchange capacity of the anion exchange resins. Based on the determination, it was established that 0.69615g of benzoxazole reagent will exchange for chloride ions in 1g of anionic resins. Moreover, comparison of titre values showed that the amount of sodium chloride released into solution as a result of the displacement of chloride ions by the benzoxazole-2-sulfonate ion remained constant after 30 min of stirring the resin with sodium benzoxazole-2-sulfonate. This was taken to mean that the exchange was complete within 30 min, and subsequent preparations of the resin-bound benzoxazole-2-sulfonate simply involved stirring a warm solution of the reagent with the anion-exchange resin for 30-45min. The equation for coupling is explained in Figure 1.33 below.

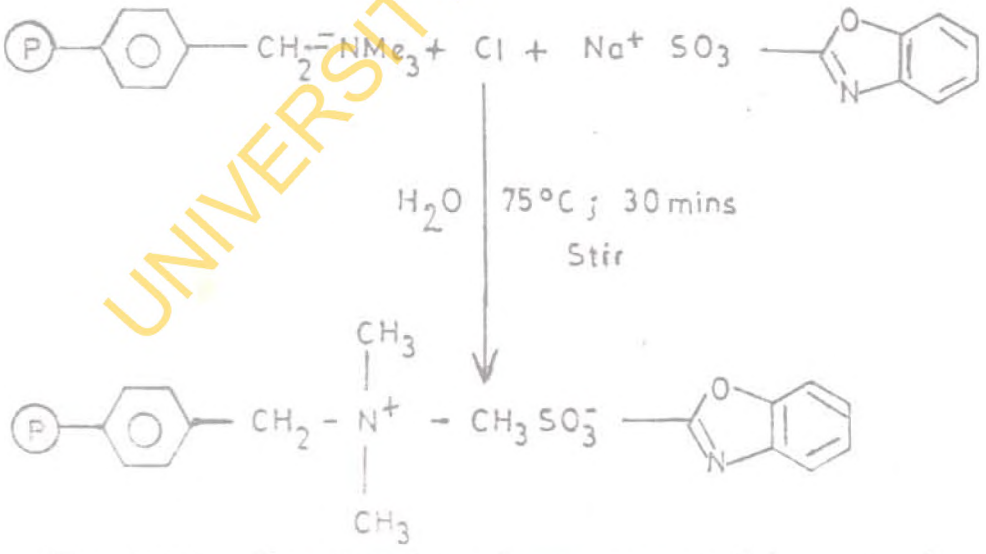


Fig. 1.33: Equation for polymer supported benzoxazole moiety (analytical reagent)

The optimization of solvent, temperature and time experiments showed that methanol and acetonitrile solvents gave the best fluorescent product. Also the derivatives obtained at 60°C and above fluoresced intensely with no significant difference in the fluorescence at higher temperature.

The time indicated that the fluorescent derivatives have been formed in 5 minutes of reacting the benzoxazole with the substrate.

As a result of the above observations, derivatization reaction can be performed in the following media:

(a) distilled water

(b) methanol

(c) acetonitrile

at temperature between 60°-65° holding time constant at 5 minutes or 10 minutes.

3.1.2 Effects of reaction of amines with the resin-bound benzoxazole-2-sulfonate

All further reactions of the amines were performed under optimized time, temperature and solvent as determined. The volume of sample derivatized was 100µl as reported in the experimental section. The following amine were derivatized:

1. Diethylamine
2. Di-n-butylamine
3. Di-n-propylamine
4. 4 nitroaniline.

The equation for derivatization on the polymeric support is shown in Figure 1.34 below.

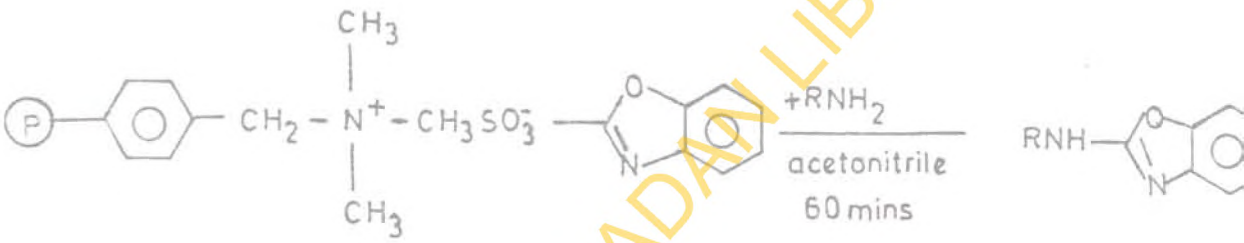


Fig. 1.34: Equation for derivatization of amine on resin support

The same reaction procedure was carried out for the blank and all the supernatant were collected each in different test-tubes and observed under the UV. The result obtained are presented in Table 4.1 below.

Table 4.1 Observation of fluorescent derivatives of amines under UV light

Analytical Reagent	Amine substrate	Supernatant Observation under UV
Resin-bound benzoxazole	Diethylamine	Blue fluorescence weak
	Di-n-butylamine	Intense blue fluorescence strong
	Di-n-propylamine	Blue fluoresence
	4 nitro-aniline	Dark red fluorescence
	Blank	No fluorescence

The result obtained are presented in Table 5 below.

Table 5: Thin-layer chromatographic result of amine derivatives obtained by heterogeneous reaction

Derivatives of	Colour of spot under UV	Spot centre RF value=Solvent front
2-(N,N-diethylamino) benzoxazole	Blue FL weak	4.3/7.2 = 0.597cm
2-(N,N-di-n-butylamino) benzoxazole	Intense blue FL strong	3.7/7.2 = 0.514cm
2-(N,N-di-n-propylamino) benzoxazole	Blue	3.9/7.2 = 0.542cm
2-(N,N-4 nitroanilino) benzoxazole	Dark red	5.7/7.2 = 0.792cm
Blank (sodium benzoxazole 2-sulfonate)	No fluorescence	No RF value

The reaction of amines with sodium benzoxazole-2-sulfonate is illustrated in Figure 1.35 below. Formation of the derivatives was confirmed from their mass spectra. The mass spectra of the derivatives of diethyl-di-n-propyl and di-n-butylamine are shown in Figures 1.35a, 1.35b and 1.35c respectively.

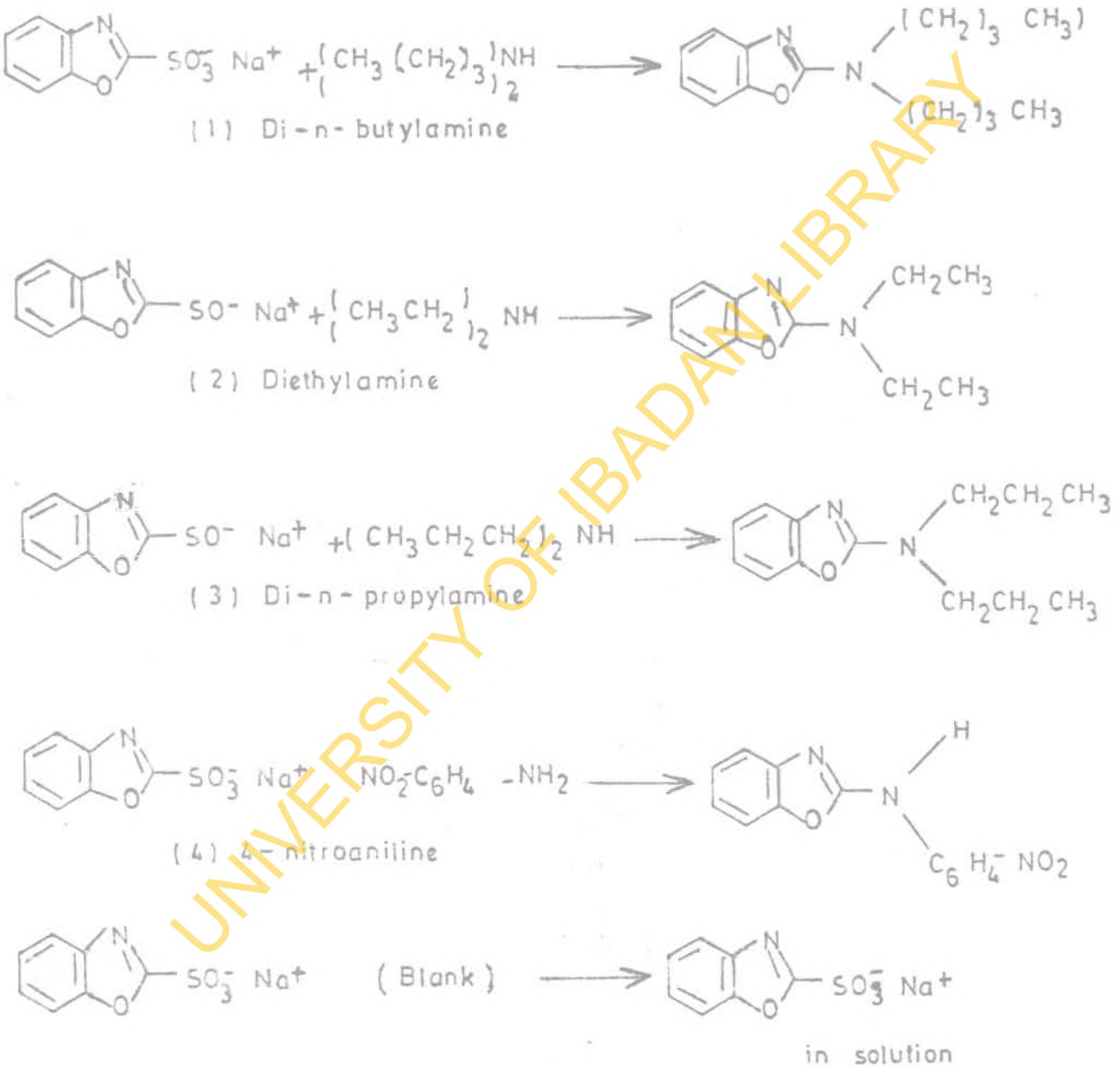


Fig. 1.35: Illustration of reaction of sodium benzoxazole-2-sulfonate with amines (homogeneous approach)

All the 2-(N,N-dialkylamino) benzoxazole, gave spectra which exhibit intense $[M+NH_4]^+$ ions due to the presence of acetate in the mobile phase used for the HPLC-MS system. The other peaks in the mass spectra of these compounds may be readily rationalised as shown in Figure 1.35d. The ion with m/z 135 is common to the mass spectra of 2-(N,N-dialkylamino) benzoxazoles and apparently results from loss of the two alkyl groups from the $[M+H]^+$ ion as the corresponding olefines. That is, loss of the alkyl groups accompanied by H-rearrangement. Fission of the bond between the O-atom and the 2-carbon atom is known to be the primary fragmentation step in the mass spectra of oxazoles.[41]

This seems to be true for these 2-dialkylamino benzoxazoles too as shown in Figure 1.35d. The apparent specificity of the fragmentation modes leading to the ions bearing the alkyl groups that the benzoxazole derivatives may also be useful in the qualitative identification of unknown amines. The mass spectrum of the reagent is shown in Figure 1.35d. Apparently the base peak ion (m/z 136) results from elimination of SO_2 from the sulfonic acid.

Sodium benzoxazole-2-sulfonate was found to be non-fluorescent, probably because of the fluorescence inhibiting effect

of the sulfonate group. The 2-(N,N-dialkylamino) benzoxazole however, exhibits an intense blue fluorescence when their solution were observed under UV light. Reaction of the amines with sodium benzoxazole-2-sulfonate occurs rapidly, with the blue fluorescence of the 2-(N,N-dialkylamino) benzoxazoles appearing within a minute or two of adding an aqueous or methanol solution of the amine to an aqueous or methanol solution of the reagent. The derivatives were detectable at low microgram levels by TLC followed by examination of the plate under UV light. The rf of the derivatives of diethyl-, di-n-propyl- and di-n-butylamine were 0.60, 0.54, 0.51 respectively. Direct HPLC analysis of the reaction mixture was found possible without interference from excess reagent which, being a salt, is poorly retained on the reversed phase column. A chromatogram of a reaction mixture of amines with sodium benzoxazole-2-sulfonate is shown in Figure 1.35e. If need be, the presence of excess reagent in the solution to be chromatographed may be avoided altogether by adding a little of a strong anion-exchange resin in the chloride form to the reaction mixture. Any excess reagent is sequestered or "mopped up" by the resin. The derivatives may also be extracted with chloroform without interference from the reagent.

To examine the possibility of using the reagent for on-line pre- or post-column derivatisation of amines, the resin-bound form of the reagent was prepared as described. In this form the reagent still reacted as readily with amines as when the reaction was carried out in a homogeneous medium. The reaction of amines with resin-bound benzoxazole-2-sulfonate has been illustrated in Figure 1.34 above.

When the tagged resin was packed in a pasteur pippet and a solution of the amines passed through the column of the resin, the eluate exhibited the blue fluorescence of the 2-(N,N-dialkylamino)benzoxazoles. A chromatogram of an eluate containing a mixture of the derivatives is identical as shown in Figure 1.35e, except that there is no peak due to excess reagent. The resin-bound benzoxazole-2-sulfonate would therefore be useful for on-line HPLC derivatization of amines.

The inavailability of water-soluble reagents is a problem in the derivatization of amino acids for HPLC-fluorescence analysis. Sodium benzoxazole-2-sulfonate is water soluble, and was found to react with amino acids to give products which exhibit a blue fluorescence under UV light. It was observed that the fluorescence of the reaction mixtures of the amino acids (except lysine) were not as intense as those of the aliphatic amines.

This was thought to be due to the possibility that under the neutral reaction conditions some of the amino acid would exist as the Zwitterion which would not react with benzoxazole-2-sulfonate thereby lowering the yield of the fluorescent derivative. Furthermore, a proportion of the amino acid derivative itself would exist as the Zwitterion as illustrated in Figure 1.35f. The electron-withdrawing effect of the quarternary ammonium group would cause a lowering of the fluorescence of the derivative. These suggestions would explain why lysine, which was a second primary amine group, gave a reaction mixture which had a fluorescence intensity comparable to those of the reaction mixture of the dialkylamine. As expected, therefore, the fluorescence of the reaction mixture of the amino acids immediately intensified on dissolving some sodium bicarbonate in the mixture.

The equations for the homogeneous reaction of amine and benzoxazole are presented in Figure 1.35 already shown.

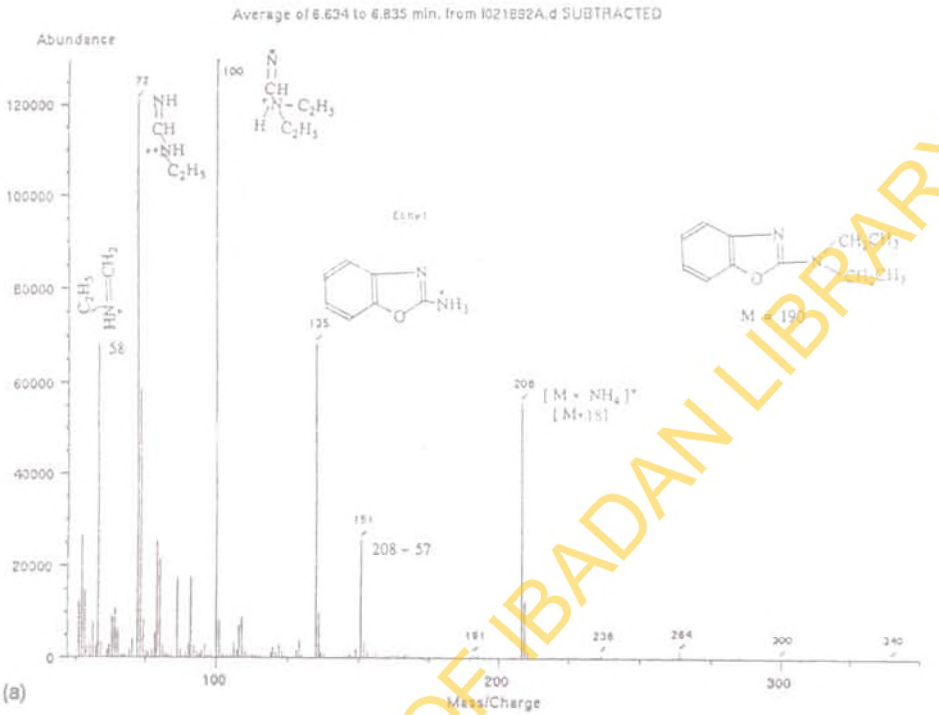


Fig. 1.35a-c: Mass spectra of 2-(N,N-dialkylamino) benzoxazole formed from the reaction of dialkylamines with benzoxazole-2-sulfonate

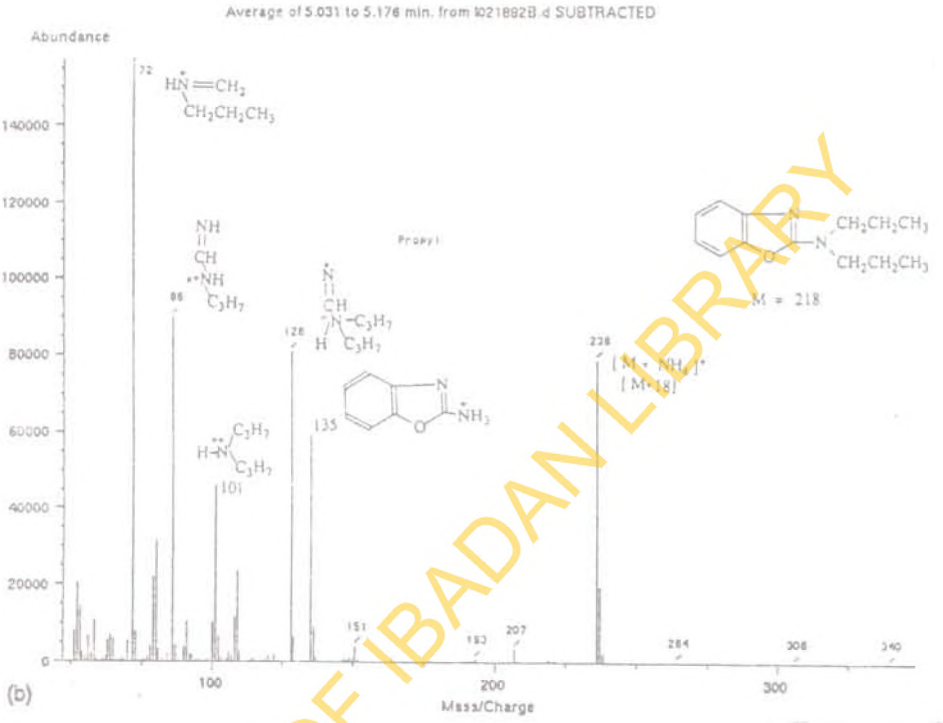


Fig. 1.35b

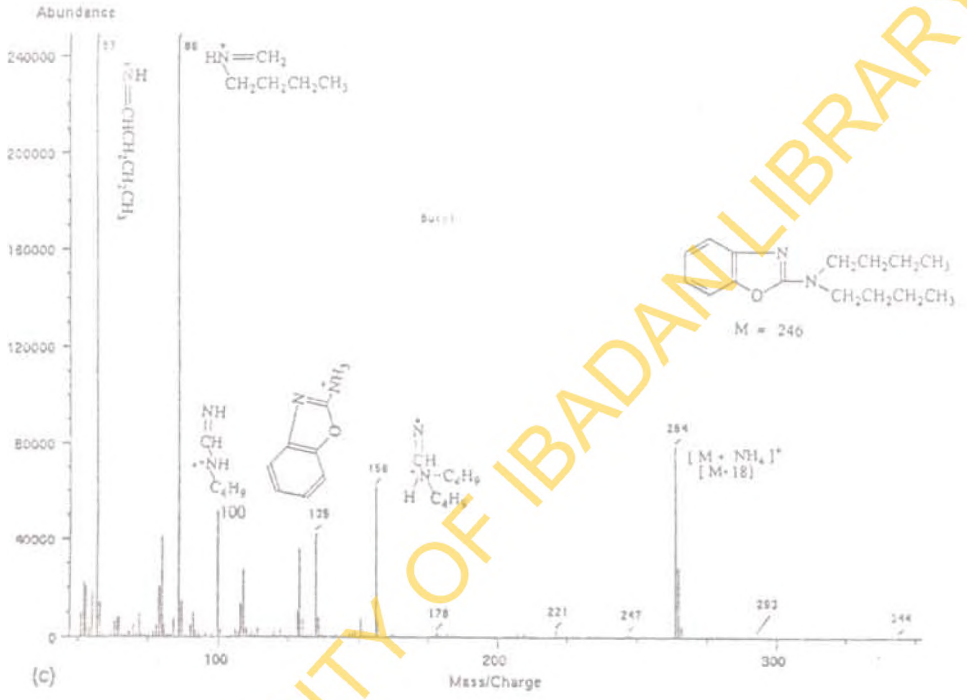


Fig. 1.35c

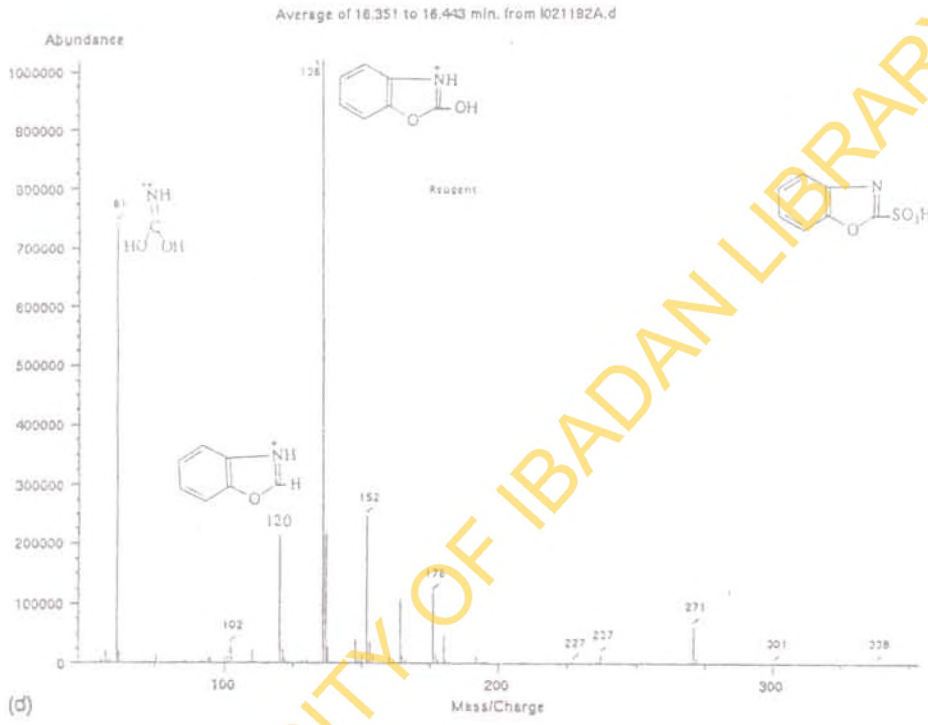


Fig. 1.35d: Mass spectrum of benzoxazole-2-sulfonate

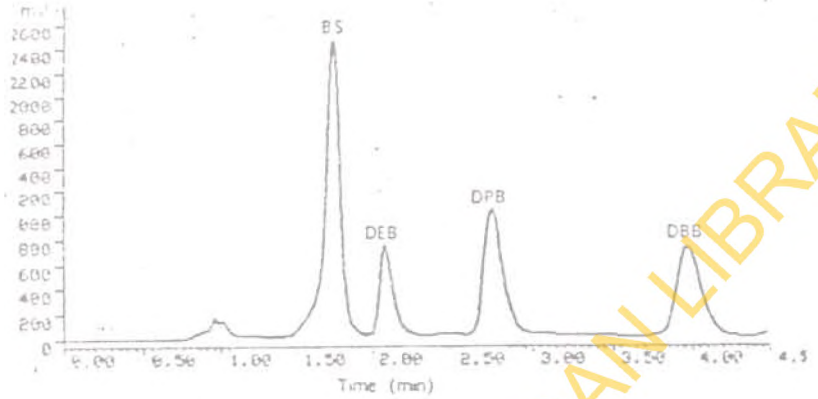


Fig. 1.35e: A chromatograph of a reaction in mixture of amines with sodium benzoxazole-2-sulfonate.

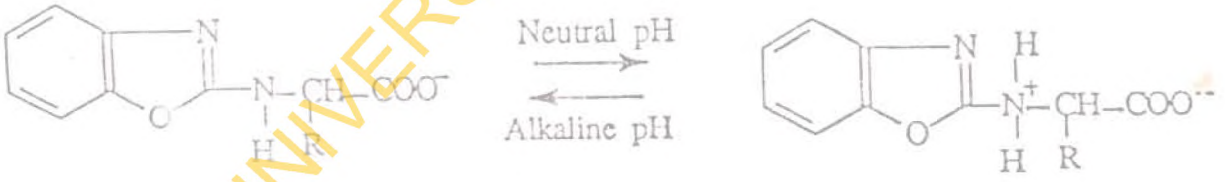


Fig. 1.35f: Zwitterion illustration in amino acid derivatives.

Observing the product under UV light we have the following in the Table 6 below:

Table 6: UV observation of the (a) amine derivatives obtained by homogeneous reaction of amines with sodium benzoxazole-2-sulfonate, and (b) underivatized amine

Derivatives	Observation under UV	Solution in water	Observation
2-(N,N-di-n-butylamino) benzoxazole	Intense blue fluorescence very strong	Di-n-butylamine	No Blue FL
2-(N,N-diethylamino) benzoxazole	Very weak blue fluorescence	Diethylamine	No Blue FL
2-(N,N-di-n-propylamino) benzoxazole	Blue fluorescence	Di-n-propylamine	No Blue FL
2-(N,N-4 nitroanilino) benzoxazole	Almost non-fluorescent	4 nitro-aniline	No FL
Blank Sodium benzoxazole-2-sulfonate	No fluorescence	Blank	No FL

The above results thus suggest that the reaction of the amines (1° and 2°) with benzoxazole 2-sulphonate in solution resulted in a fluorescent product. The intensity of fluorescence increases with increase carbon chain length as was noticed with di-n-butylamine which gave a very intense blue fluorescence.

Moreover, the reagent has the advantage of reacting rapidly with the amines (1° and 2°) hence it can be used to trap volatile amines. We can therefore measure the fluorescence of the reaction product directly without isolating the product or removing the excess reagents.

3.1.3 Thin-layer chromatography of homogeneous reaction product and comparing with derivatives from resin-bound reagent

Thin-layer chromatography was carried out as explained under experimental section. It was found that each of the spots fluoresced under UV light and they had different RF values. To ascertain that the reaction took place, the derivatizing reagents, which was sodium benzoxazole, was spotted on same plate. The reagent did not give a fluorescent spot under UV. When the RF values for each of the derivatized amines obtained through homogeneous method were compared with those that were obtained using resin-bound reagents, the RF values were almost identical, with limits of experimental error, it showed that the reaction on the resin-support occurred. This is shown in the Table 7 below.

Table 7: Comparison of thin-layer chromatographic analysis of derivatives obtained by homogeneous approach and those obtained by heterogeneous approach

Derivatives from resin-bound A	Derivatives from benzo-xazole B	Spots A under UV	Spots B under UV	RF Value A	RF Value B
Diethylamine	Diethylamine	Blue FL(W)	Blue FL(W)	0.590cm	0.597cm
Di-n-butylamine	Di-n-butylamine	Intense Blue (S)	Intense Blue (S)	0.510cm	0.514cm
Di-n-propyl-amine	Di-ni-propyl-amine	Blue FL	Blue FL	0.539cm	0.542cm
4-nitroaniline	4-nitroaniline	Almost non-FL	Almost non-FL	0.685cm	0.792cm
Blank	Blank	No FL	No FL	No FL	No FL

W = Weak S = Strong FL = Fluorescence

3.1.4 Conducting reactions through one-way process

The resin (Cl form), sodium benzoxazole 2-sulphonate and amine solution were all mixed together in a test-tube and reaction was carried out as explained under the experimental section. The supernatant from each test-tubes including the blank were collected and observed under the UV light. The reaction products, with the exception of the blank, fluoresced under UV lights. When compared with the result obtained from derivatives of the resin-bound reagents they were similar.

The results are compared in Table 8 below.

3.1.5 Effect of reaction of amino acids with sodium benzoxazole-2-sulfonate

The amino acids were cystein, lysine and glycine. The benzoxazole derivatives of the amino acids gave very weak blue fluorescence, however when the solution was made basic with sodium carbonate blue fluorescence became intensified. Basified solution of the sulfonate (blank) did not show fluorescence under UV light. No fluorescence was also observed for each solution of the amino acids.

The result is presented in Table 9 below.

Table 8: Comparison of ultraviolet light observation of resin-bound benzoxazole derivatives

Resin + Benzoxazole + Amine Derivatives	Observation Under UV Light	Resin-bound Derivatives	Observation under UV
Diethylamine	Blue FL weak	Diethylamine	Blue FL weak
Di-n-butylamine	Intense blue FL	Di-n-butylamine	Intense blue
Di-n-propylamine	Blue FL	Di-n-propylamine	Blue FL
4-nitroaniline	Almost non-FL	4-nitroaniline	Almost non-FL
Blank	No FL	Blank	No FL

Table 9: Comparison of UV light observation of amino acids and amino acids benzoxazole derivatives

	Observation under UV	Solution of sulfonate + amino acids addition of Na_2CO_3	Observation under UV
Solution of sulfonate and amino acids			
Cystein	Weak Blue FL	Cystein	Blue
Lysine	Weak Blue FL	Lysine	Blue
Glycine	Weak Blue FL	Glycine	Blue
Blank	No FL	Blank	No FL
Solution of amino acid			
Cystein	No FL	Cystein	No FL
Lysine	No FL	Lysine	No FL
Glycine	No FL	Glycine	No FL
Distilled water	No FL	Distilled water	No FL

3.2 Preparation of 2-Chloromethylnaphthalene

The method for preparation are discussed in the experimental section. The yield obtained for reaction product was 26.50g and a melting point of 56-57°C. The 2-chloromethylnaphthalene was used for the preparation of ester derivatives of lauric acids, palmitic acids, n-dosanoic acid, n-valeric acid, n-nonanoic acid, n-hexanoic acid, capric acid, octanoic acid, linoleic acid and acetic acid respectively. The equation for the preparation discussed in the experimental section is as follows in Figures 1.36 and 1.37 below.

$$R^1COOH + R^{11}CH_2\ x \xrightarrow[\text{3-4 hours}]{140°C\ -\ 165°C} R^{11}CH_2O\text{-}\underset{O}{C}\text{-}R^1 + HX$$
$$(C_2H_5)_3N$$

Figure 1.36: Equation for the preparation of ester derivative of carboxylic acid

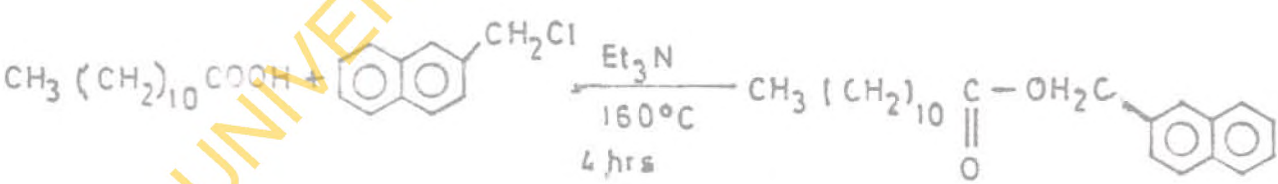


Figure 1.37: Equation illustrating the reaction between lauric acid and 2-chloromethylnaphthalene

3.2.1 Thin-layer chromatographic analysis (TLC) of ester derivatives

The developing solvent was 63% alcohol[16] as prepared under the experimental section. The chromatograms were monitored under the UV light and qualitative separation of the ester derivatives were obtained. The result from the determined RF values allowed the identification of the acids. The position of spots were not always constant for three analyses (n=3). This was due to the amount of samples spotted. The ester derivatives separated on the basis of the increasing chain-length which indicated increase in molecular mass. In order to ascertain that reaction took place 2-chloromethyl naphthalene (derivatizing reagent) was spotted alongside the ester derivatives. This reagent gave an entirely different RF values which did not correspond to the RF values of any of the ester derivatives.

To validate the method further, developing solvent was changed to 1ml ammonia solution and 100ml 95% ethanol.[16] The same result was obtained. This indicated that the method employed for their determination was reproducible.

Table 10: Thin layer chromatographic analysis of ester derivatives of carboxylic acids and 2-chloromethyl naphthalene showing retentive factors values

Standard Ester Derivatives	Observation under UV light	RF Values = $\frac{\text{Spot centre}}{\text{Solvent front}}$
1. Acetic $C_2H_4O_2$	Blue	0.815cm
2. Valeric $C_5H_{10}O_2$	Blue	0.775cm
3. Hexanoic $C_6H_{12}O_2$	Blue	0.705cm
4. Octanoic = $C_8H_{16}O_2$	Blue	0.735cm
5. Nonanoic = $C_9H_{18}O_2$	Blue	0.700cm
6. Capric = $C_{10}H_{20}O_2$	Blue	0.632cm
7. Lauric = $C_{12}H_{24}O_2$	Blue	0.542cm
8. Palmitic = $C_{16}H_{32}O_2$	Blue	0.432cm
9. Donosanoic = $C_{22}H_{44}O_2$	Blue	0.315cm
10. Linoleic = $CH_3(CH_2)_4CH=CHCH_2=CH(CH_2)_2CO_2H$	Intense yellow	0.571cm
11. 2-Chloromethyl naphtalene	Intense blue	0.900cm

3.2.2 Preparation of 2-naphthalene methanol

2-chloromethyl naphthalene was hydrolysed to the alcohol with a view of coupling to a cationic resin support. Equation for the hydrolysis as carried out in the experimental section is shown in Figure 1.38 below.

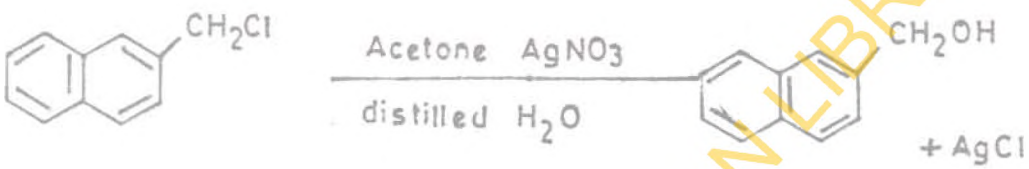


Fig. 1.38: Equation illustrating conversion of 2-chloromethylnaphthalene to 2-naphthalene methanol

Melting point determined to be 79°C - 80°C.

Literature melting point is 79 - 81°C.

3.2.3 Preparation of resin-supported sulponyl chloride and coupling with 2-naphthalene methanol

Sulphonated ion exchange resin (sodium form) was converted to the sulphonyl chloride (experimental section).

Equation for reaction is shown in Figure 1.39 below.

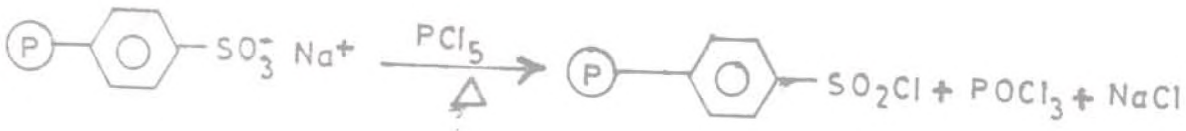


Fig. 1.39: Equation illustrating conversion of sulphonated ion exchange resin (sodium form) to the sulphonyl chloride form

The ion exchange resin, sulphonyl chloride form was condensed with 2-naphthalene methanol (method explained under the experimental section). The equation for the conversion is shown in Figure 1.40 below.

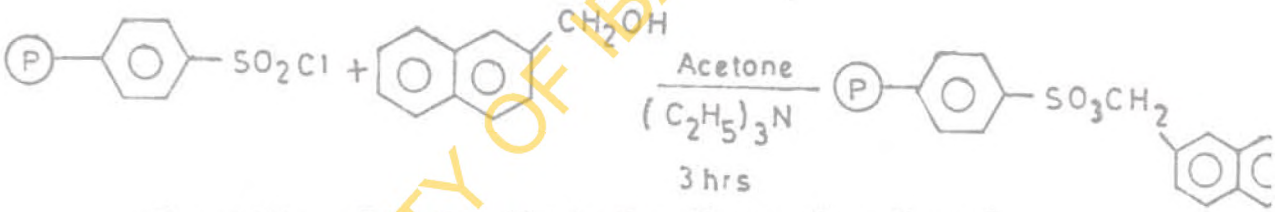


Fig. 1.40: Equation illustrating the condensation of ion exchange resin in sulfonyl chloride form with 2-naphthalene methanol

The resin-bound alcohol (analytical reagent) was used to derivatize the fatty acids, i.e. Acetic, Valeric, Hexanoic, Octanoic, Nonaxoic, Capric, Lauric, Palmitic, Dosanoic, Linoleic. The equation for the derivatization procedure under experimental section is shown below in Figure 1.41 below.

as an example -

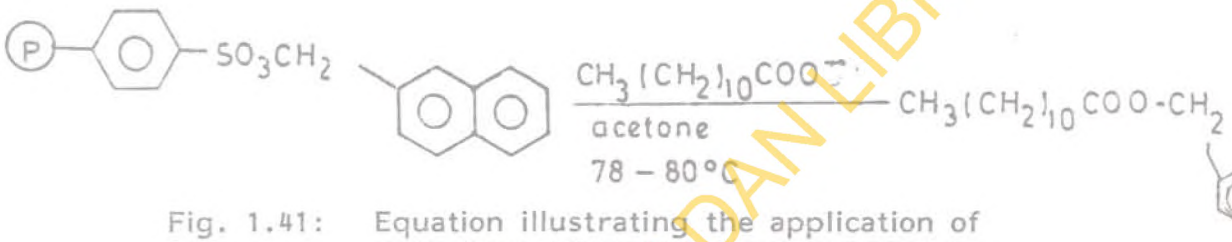


Fig. 1.41: Equation illustrating the application of resin-bound 2-naphthalene methanol in derivatizing carboxylic acid e.g. lauric acid

The reactions were carried out for all the fatty acids and the blank, reaction products were observed under UV light and they all flurescend. The blank did not fluorescence.

3.2.4 Thin-layer chromatography of resin-bound derivatives

The resin-bound derivatives, blank and 2 naphthalene methanol were spotted on same plate. The chromatographs were observed under UV light. The spots corresponding to each of the derivatives gave blue fluorescence; there was no fluorescence

for the blank. 2-naphthalene methanol gave blue fluorescence spot. From the RF values it was found that the derivatives prepared with resin-bound reagent had different RF values because they exhibited different mobilities depending on the carbon chain length. In addition 2-naphthalene methanol also gave a different RF value not corresponding to any of the derivatives. The RF values are recorded in Table 11.

Comparing the RF values of the resin-bound derivatives within the standard ester derivatives that were prepared in solution, similar results were obtained. This is an indication that derivatization reactions carried out on resin supports have been successful.

3.2.5 RF values of standard ester derivatives and resin-bound derivatives

The RF values of the standard ester derivatives and the resin-bound derivatives for each of the acids were found to be the same. From the results on Table 11, it was shown that reactions on the resin support were successful and could be employed for post column derivatization of the acids.

The product from solution phase reactions (i.e. standard ester derivatives of lauric, capric, valeric, nonanoic, octanoic, palmitic, acetic dosanoic, linoleic hexanoic acids) have been shown

Table 11: Thin-layer chromatographic analysis of deivatives of carboxylic acids obtained through homogeneous and heterogeneous approaches

Developing Solvent	Characterisation (MP, TLC, UV/FL)	Melting Point (°C)	Observation under UV light	RF Values = Spot centre / Solvent front
A. 63% alcohol i.e. 1 volume of water to 2 volume of 95% ethanol	a STD ester derivative of acetic	72°C (determined)	Blue FL	0.814cm
	b Resin-bound derivative of acetic	70°C (determined)	Blue FL	0.800cm
	c Parent fatty acid valeric	(Literature)	No FL	No RF
	a STD ester derivative of valeric	68°C (determined)	Blue FL	0.775cm
	b Resin bound derivative of valeric	67°C (determined)	Blue FL	0.782cm
	c Parent fatty acid valeric		No FL	No Rf
B. 1ml of ammonia solution and 100ml of 95% ethanol	a STD ester derivative of hexanoic	78.5°C (determined)	Blue FL	0.705cm
	b Resin bound derivative of hexanoic	78°C (determined)	Blue FL	0.709cm
	c Parent fatty acid hexanoic	3°C (literature)	No FL	No Rf
	a STD ester derivative of octanoic	82°C (determined)	Blue FL	0.735cm
	b Resin-bound derivative of octanoic	81°C (determined)	Blue FL	0.738cm
	c Parent fatty acid	32°C (literature)	No FL	No Rf
	a STD ester derivative of nonanoic	79.5°C (determined)	Blue FL	0.700cm
	b Resin bound derivative of nonanoic	79.0°C (determined)	Blue FL	0.742cm
	c Parent fatty acid nonanoic	32°C (literature)	No FL	No Rf
	a STD ester derivative of capric	83.5°C (determined)	Blue FL	0.632cm
	b Resin bound derivative of capric	83°C (determined)	Blue FL	0.550cm
	c Parent fatty acid capric	31°C (literature)	No FL	No FL
	a STD ester derivative of lauric	81.5°C (determined)	Blue FL	0.542cm
	b Resin bound derivative of lauric	81°C (determined)	Blue FL	0.530cm
	c Parent fatty acid lauric	46°C (literature)	No FL	No Rf
	a STD ester derivative of palmitic	74.5°C (determined)	Blue FL	0.432cm
	b Resin-bound derivative of palmitic	75°C (determined)	Blue FL	0.350cm
	c Parent fatty acid palmitic	64°C (literature)	No FL	No Rf
	a STD ester derivative of docosanoic	63.5°C (determined)	Blue FL	0.315cm
	b Resin bound derivative of docosanoic	63°C (determined)	Blue FL	0.300cm
	c Parent fatty acid docosanoic	82°C (literature)	No FL	No Rf
	a STD ester derivative of linoleic	84.5°C (determined)	Intense yellow FL	0.571cm
	b Resin-bound derivative of linoleic	83°C (determined)	Intense yellow FL	0.570cm
	c Parent fatty acid linoleic	5°C (literature)	No FL	No FL
	Blanks	No Melting Point	No Fluoresence	
	2- Chloromethylnaphtalene	56°C (determined)	Blue FL	0.900cm
	2- Naphthalene methanol	80°C (determined)	Blue FL	
		79°C - 81°C (literature)		

to be different from their corresponding fatty acids by melting point determination and observing chromatograms which spots fluorescence significantly under ultraviolet light used. The above results are presented in Table 11.

CHAPTER FOUR

CONCLUSION

The analytical reagents that were developed i.e. Benzoxazole-2-sulfonate and 2-naphthalene methanol may be considered an excellent reagents for homogeneous on heterogeneous derivatisation of amino compounds and carboxylic acids for HPLC-fluorescence (or UV) analysis. Though there is the need to further concentrate on the reagent for carboxylic acid which had not produced an excellent derivative, the reagent for the amino compounds is excellent. The principle described for the synthesis and derivatisation of benzoxazole seems quite versatile and may be extended to the use of other N-heteroaromatics-2-sulfonates. For instance, sodium naphthoxazole 2-sulfonates was found to react with amines too, though the compound is not as attractive as sodium benzoxazole-2-sulfonate because it is itself fluorescent and it is poorly soluble in water.

Finally, the principle have opened up room for future research on development of bound reagents for functionalities such as carboxylic acids, carbonyls and thiols.

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