FURTHER IMPROVEMENTS OF THE FLOW INJECTION GLUTAMATE OXIDASE-BASED ASSAY FOR β-ODAP IN GRASS PEA

A Thesis

Submitted to

The School of Graduate Studies

Addis Ababa University

In Partial Fulfilment of

The Requirement for the Degree of

Master of Science in Chemistry

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June, 1996

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ACKNOWLEDGMENTS

I wish to express my profound gratitude to my advisors Dr. Ghirma Moges and Prof. Theodros Solomon for their guidance and encouragement in the course of the project.

I would like to express my deepest gratitude to Ato Belete Tegbaru, (Junior Immunologist, EHNRI), Ato Oumer Yaynie (AAU, Mathematics Department), Ato Adane Bitew (Dean of Medical Laboratory Technology Training School, EHNRI), and Ato Bitew Fisseha (Head of National Referral AIDS Laboratory, EHNRI) for their material and moral support throughout my study.

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LIST OF ABBREVIATIONS

4-AP	4-Aminophenazone (4-aminoantipyrine)
BOAA	β-N-oxalylamino-L-alanine
CPG	Controlled Pore Glass
DAP	L-α,β-diaminopropionic acid
DCN	2,4-dichloronaphthol
DCNS	2,4-dichloronaphthol sulphonate
DCP	2,4-dichlorophenol
DCPS	2,4-dichlorophenol-6-sulphonate
FI	Flow Injection
FIA	Flow Injection Analysis
GlOD	Glutamate Oxidase
GOD	Glucose Oxidase
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidase
IMERs	Immobilized Enzyme Reactors
β-ODAP	β-N-oxalyl-L-α,β-diaminopropionic acid
OPT	o-phthaldehyde
PITC	phenylisothiocyanate
SPE	Solid Phase Extraction
TCA	trichloroacetic acid
UF	Ultrafiltration

ABSTRACT

The content of β-N-oxalyl-L-α,β-diaminopropionic acid (β-ODAP) in thirty grass pea seed samples were determined by a FI system incorporating a glutamate oxidase reactor. The assay of β-ODAP was made via the oxidation product, hydrogen peroxide, which was detected spectrophotometrically at 512 nm using Trinder chromogenic reagent. The same samples were assayed by the Rao's modified colorimetric procedure at 476 nm. The concentrations obtained by the enzymatic method was 10-50% higher than that obtained by the colorimetric method. The results of another variation of Rao's method (Campbell procedure) was 53-66% lower than that of the FI method.

Separations of proteins from β-ODAP in grass pea extracts were made by two precipitation methods with trichloroacetic acid (1%) and perchloric acid (0.46%) and by ultrafiltration. Sample injections of glucose spiked extracts after the three sample clean-up procedures enhanced stability of glucose oxidase reactors in a FI system. The response of a FI system with a small GOD reactor to glucose-spiked crude extract gradually decreased to zero after 36 injections.

Synthesis and characterization of sulphonated 2,4-dichloronaphthol, as substitute for 2,4-dichlorophenol-6-sulphonate in Trinder reagent, was studied. The molar absorptivities for hydrogen peroxide at 512 nm (λ_{max}) were 15,220 and 11,380 M^{-1} cm^{-1} at pH 4.2-5 and at pH 7 respectively. This reagent was used in the determination of hydrogen peroxide and glucose in both batch and flow injection systems.

1. INTRODUCTION

Lathyrism is a disease which causes an irreversible paralysis of the legs in humans due to the ingestion of the seeds of *grass pea, Lathyrus Sativus* [1]. It has been known since the days of Hipocrates though the term was coined by Cantani in 1873 [2].

The word "Lathyrism" is originated from the Greek word for vetchlings, "Lathyros", and was used to describe either of the two different pathogenic entities that result from the consumption of the peas of the *Lathyrus* species. Hans Selye in 1957 proposed the use of the term Neurolathyrism to describe the syndrome which affects the nervous system of man (Human Lathyrism) and Ostheolathyrism (otherwise known as odoratism) to designate the syndrome which predominantly affects connective tissues of certain animals other than man [3].

The *Lathyrus* species commonly identified as causing neurolathyrism is the peas of *Lathyrus Sativus,* although *Lathyrus Cicera* and *Lathyrus Clymenum* have also been implicated [4].

1.1 NEUROLATHYRISM

The grass pea also known as 'Guaya' in Ethiopia, is a legume widely cultivated in Ethiopia, India, Syria, Pakistan, Bangladesh, China and Nepal. It is a nutritious pulse with a protein content of 26-30% [1]. The plant is still widely cultivated and consumed in the mentioned regions particularly by the poor people, because it is a

hardy crop that requires very little agricultural attention and is resistant to drought and water-logging.

The symptoms of neurolathyrism are: muscular rigidity, weakness and paralysis of the leg muscles, convulsions, head retraction and stiffening of the neck. There may be a burning sensation and incontinence of urine in some of the severely affected cases [5]. Epidemic cases of the disease have been reported in Northwestern Ethiopia when famine caused by drought, prevailed in the region [6]. Consumption of a diet consisting of more than one-third of Lathyrus seed meal for over three months was considered to cause neurolathyrism [5,7]. Lathyrism affects the male, especially young males, much more frequently than it does the female, the child and the older man [2,5].

1.2 HISTORICAL BACKGROUND

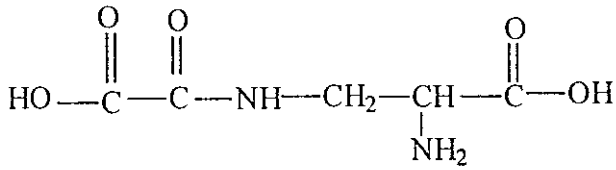
Stockman in 1917 stated that the active principle was an alkaloid but latter changed his opinion to an acid salt of phytic acid and described the poisoning mechanism as the inefficiency of the bowel to convert the organic phytates to harmless inorganic phosphates [8-10]. Horvath regarded the pyrimidine base divicine as the toxic principle and Palasi said that the poisoning was due to the deficiency of certain amino acids in the protein of *L.Sativus* [11,12].

Rudra and Bhatacharya suggested that the increase in serum alkaline phosphatase was indicative of lathyrism [13]. Some other authors tried to relate the disease with

metal poisoning such as selenium which was believed to interfere in methionine metabolism [14] and high levels of serum magnesium which was considered as marker of lathyrism [15].

In 1962, β-cyano-L-alanine and a water insoluble aliphatic amino acid glycoside with a nitrile group, which was latter proposed as N-α-L-arabinosyl-N-β-D-glucopyranosyl-α,β-diaminopropionitrile, were claimed to be the toxic factor [16-18].

Adiga and coworkers in the same year found that the toxic principle could be a ninhydrin positive amino alcohol [19] and they finally postulated and identified the neurotoxic non-essential amino acid β-N-oxalyl-L-α,β-diaminopropionic acid (β-ODAP) or its synonym β-N-oxalylamino-L-alanine (BOAA) [20,21] the structure of which is



Yields of 0.5-2.5% of β-ODAP (dry weight) have been reported from seeds of *L. Sativus* [22]. Naturally this non-essential amino acid exists in the plant as the two isomeric forms: α- and β-. The α- isomer is reported to occur to about 5% but isomerization of the latter takes place at neutral pH and elevated temperatures raising the amount to about 40% [23, 24].

1.3 METHODS OF DETERMINATION OF β-ODAP

Researchers in different fields of study have been trying to minimize or eliminate the content of β-ODAP in foods made from *L. Sativus* seeds. Methods to detoxify Lathyrus foods before ingestion and searches for non- or low-ODAP varieties of *L.Sativus* have been the main important approaches to eradicate Lathyrism [25-27]. This entailed the search for a fast and selective method of assaying the neurotoxin since large varieties of samples are brought from the field.

The first known method is the ninhydrin method. A few microliter of the extract of grass pea seed was spotted on a filter paper strip, electrophoresed at pH 3.6 or 1.9 for 2 hours at 400 V, sprayed with 0.5% ninhydrin in acetone and heated at 80°C for 15 min. The ninhydrin positive band was eluted with 75% ethanol containing copper sulphate and the absorbance read at 510 nm. The method however,was not sensitive [28, 29].

Rao proposed an indirect colorimetric assay method for the toxin which involves its alkaline hydrolysis to L-α,β-diaminopropionic acid (DAP) and oxalic acid [29]. DAP was determined colorimetrically at 420 nm after a reaction with o-phthaldehyde (OPT). Since its first report in 1978, there has been a number of modifications of the method including the period of extraction, medium of extraction, change of some of the constituents of the OPT reagent, change of the buffer system, etc. A number of modifications of the OPT method have so far been reported [30]. Due to these modifications used in various laboratories, widely varied contents in grass pea are

reported.

A recent report by Hussien and co-workers describes that six modifications practised in six different laboratories showed significant discrepancy in the assay of β-ODAP for the same sample [30]. According to this report the result of one modification is more than four times higher than the other. Moreover, repeated determinations of the sample over a long period of time resulted in a high fluctuation. Besides the Rao method lacks selectivity since the non-toxic isomer, α-ODAP, also hydrolyzes to DAP. It is not also rapid since, after extraction, assaying the toxin involves a one-hour hydrolysis and colour reaction. Despite these drawbacks the OPT method continues to be used for assaying β-ODAP in grass pea.

In the search for more selective and rapid assay, high performance liquid chromatographic (HPLC) methods for ODAP which involves an off-line precolumn derivatization with dansyl chloride and 9-fluorenyl methyl chloroformate were developed [31,32]. Again these methods are not selective [31,32]. Another HPLC separation of both isomers using phenylisothiocyanate (PITC) derivatization was reported. This method is experimentally inconvenient because of the difficulty to isolate the derivatization reagent [24]. Hence, the need for a fast, β-isomer selective method has been considered important.

This led to a further search for a more rapid and selective detection method of the neurotoxin, including possible application of enzymes. In a screening of seven

enzymes for catalytic activity towards ODAP, Moges et al. found that glutamate oxidase (GlOD) catalyzes the oxidation of β-ODAP [33]. Based on the common typical oxidation products of amino acids, they tentatively proposed that the oxidation product of β-ODAP (catalyzed by GlOD) is an α-keto acid, β-N-oxalyl-α-keto-β-aminopropionic acid, hydrogen peroxide and ammonia. This entails characterization of the proposed product by screening and testing enzymes that may act on α-keto acids.

This report was followed by another paper on the application of immobilized GlOD to a flow injection (FI) system for the determination of the neurotoxin in grass pea [34].This FI system contained a 20-μL GlOD-catalase reactor to destroy the major substrate, glutamate. Hydrogen peroxide, the oxidation product of ODAP, reacted with Trinder reagent in Horse Radish Peroxidase (HRP) reactor in the presence of 4-aminophenazone (4-AP) to form a red coloured quinoneimine dye, which was detected spectrophotometrically at 512 nm. The results of the new method are important since GlOD is specific to the β-isomer and doesn't act on the non-toxic α-isomer. As stated in the aforementioned report direct injection of the extracts of *L. Sativus* seeds gave erratic and unreliable results because of adsorption of proteins and other macromolecules in the reactors. To avoid this problem the work involved an ultrafiltration method for protein separation from the extract [34]. But the system for this separation technique cannot be afforded in laboratories of the countries severely affected by lathyrism (e.g., Ethiopia and Bangladesh). Therefore, examining relatively inexpensive and simpler methods such as solid phase extraction (SPE) and

precipitation is very much desired.

It has recently been shown that sulphonated 2,4-dichloronaphthol can replace one of the reagents in the Trinder reaction, 2,4-dichlorophenol sulphonate (DCPS) [35]. The objectives was to use possibly a more sensitive H_2O_2-detecting reagent system that could be used to monitor substrates via oxidase-catalyzed reactions which include that of glucose oxidase and glutamate oxidase. But characterization and further optimization of the synthesis of sulphonated 2,4-dichloronaphthol is required. The reagent could be further tested for alternative enzymatic colorimetric determination of the neurotoxin β-ODAP, both in batch and FI modes.

Thus, the objectives of this thesis research are:

1. Optimization of methods of protein separation from the extracts of *L.Sativus* seeds, prior to injection.

2. Determination of ODAP using FI methods in different *Lathyrus Sativus* seeds, food and biological samples using a GlOD reactor and HRP-based colorimetric reactions.

3. Characterization and optimization of the synthesis of sulphonated 2,4-dichloro-naphthol that can alternatively be used in Trinder's chromogenic reagent.

4. Enzymatic characterization of the oxidation product of β-ODAP.

In this thesis, separation of proteins from grass pea seed extracts via acid precipitants, determination of the ODAP contents of thirty grass pea samples and comparison with that of Rao method as well as optimization of the synthesis of 2,4-dichloronaphthol sulphonate and its characterization will be presented. The enzymatic characterization of the oxidation product of β-ODAP could not be investigated due to unavailability of suitable enzymes.

2. LITERATURE REVIEW

2.1 Enzymatic Analysis

Enzymatic analysis is a branch of analytical chemistry which started to exert its impact on analytical chemistry in the 1840's when hydrogen peroxide was detected with peroxidase [36]. However, scarcity, high cost, instability and general lack of familiarity of the behaviour of enzymes limited their use in analysis till the introduction of optical measuring instruments and highly purified enzymes in the mid 1960's [36,37].

Enzymes are complex, high molecular weight molecules that catalyze numerous specific biochemical reactions in a living cell. They are members of a class of compounds called proteins, but are distinguished from others of the class by the existence of an active site in the structures which facilitate catalysis. Enzymes accelerate the rate of chemical reactions by factors of 10^{12}-10^{20} over that of uncatalyzed reactions at body temperature. Industrial catalysts are not at all as effective as enzymes under comparable conditions. In addition to their catalytic power another important characteristic of enzymes is their specificity. Hence, enzymes are used as sensitive analytical reagents in medical, agricultural and food sciences [36].

There are more than 2000 enzymes that have been isolated and characterized. Previously it was the custom for the individual who isolated and characterized the

enzyme to also name it. However, in many instances the same enzyme was given different names or two different enzymes were given the same name. Consequently, the nomenclature for enzymes became so chaotic that The International Union of Biochemistry instituted a Commission on Nomenclature and Classification of Enzymes to prepare a system of nomenclature that has become standard [38].

According to this system of nomenclature an enzyme is coded with four numerals the first of which is the main category to which the enzyme belongs, namely: (1) oxidoreductases, (2) transferases, (3) hydrolases, (4) lyases, (5) isomerases, and (6) ligases; the second is the subclass that identifies the enzyme in more specific terms; the third precisely defines the types of enzymic activity; and the fourth numeral is the serial number of the enzyme in its sub-subclass [39].

Enzymes are often expensive and difficult to obtain. An example is glutamate oxidase (from Yamsa corporation, Japan). 15 mg (100 units) of the enzyme costs about $500. Hence, it is important that they are used efficiently as analytical reagents. Once used, free enzymes are disposed after reactions in solution, rendering them expensive. One way of minimizing such wastage is by immobilizing them [40].

2.2 Immobilized Enzymes

Immobilized enzymes are enzymes constrained one way or another within the limited confines of a solid support. The solid support can be polysaccarides (e. g. sephadex,

DEAE-cellulose), fibrous proteins (e.g. collagens and keratin), inorganic supports(such as porous glass, porous ceramics, alumina), synthetic polymers, hydrogels and hollow fibers [41]. Enzymes can be constrained or held to these supports by adsorption, crosslinking, entrapment, microencapsulation, covalent attachment or by various combination of the foregoing methods [41,42].

Immobilization by adsorption is the most economical and the simplest procedure of all though the reactions involved are complex. Here, an aqueous solution of the enzyme is placed in contact with an inert material for some time and the excess enzyme is washed off.

Intermolecular covalent linkages can be formed between macromolecules with the aid of bifunctional cross-linking agents (e.g. di-isothiocyanates, alkylating agents, dialdehydes such as glutaraldehyde). A major disadvantage is that some systems lose biological activity when three-dimensional structure of the enzyme is altered. Furthermore, one has to prevent intramolecular crosslinking by controlling the pH, ionic strength, temperature and reaction time [43].

Entrapment techniques involve creating a barrier through which substrate and product molecules will pass freely but which is impermeable to the enzyme. Some of the disadvantages are restriction to small size of substrate and products, leakage of the enzyme from the matrix and control of several experimental factors [41,44].

Microencapsulation is a process by which an enzyme is enveloped within various forms of semipermeable membranes. The enveloped enzymes cannot leak out, but external substrate can dialyze freely across the membrane to be acted by the enveloped enzymes.

Immobilization by covalent attachment is the most frequently used method. There are several methods of preparing covalent conjugates, but the most popular techniques involve the reaction of an aqueous solution of an enzyme with an activated, derivatized water insoluble support. Triethoxysilane derivatization and glutaraldehyde activation was used in this work.

The advantages of immobilized enzymes with respect to their free counterparts are: the ability to use the enzyme many times more than the soluble enzyme can be used, easy recovery of the reaction mixture from the enzyme, increased operational and thermal stability, and improved enzyme behaviour (e.g. pH range) [45]. The initial cost of immobilization of enzymes is high but this is usually more than compensated because of its use for several weeks (even months).

The system in which an immobilized enzyme is used is often important in determining the productivity of the system as the actual method of immobilization itself. It can be a reactor, or biosensor, i.e. immobilized enzyme in close proximity to a transducer. Enzyme reactors in flow systems are particularly more common because of their ability to be interfaced with almost any type of detector.

The main function of an enzyme reactor is to retain the immobilized complex and to ensure efficient and controlled contact between the enzyme in order to maximize product formation. In order to increase the conversion efficiency of immobilized enzyme reactors (IMER) one has to use high enzyme loading so that it cannot be affected by a slight change in pH, ionic strength, concentration of inhibitors and activators, and temperature [45]. The conversion efficiency, however, is dependent on the reactor configuration used. The possible configurations are: packed beds, single-bed-stirring, fluidized beds, open tubular and sheet reactors. The most commonly used device is packed bed reactor in which the particles are packed in a small column fitted with an inlet and outlet at opposite ends. Fluid flow may be downwards or upwards. The biocatalyst is packed at its maximum density within such a reactor and the degree of conversion of the substrate increases with the length of the column [46].

The kinetics of immobilized enzyme systems may be influenced by conformational and steric, microenvironmental, and diffusional (mass transfer) effects [47]. External and internal diffusional resistances has to be taken into account in order to apply the well known Michaelis-Menten equation for an immobilized enzyme on a porous support.

Based on the Michaelis-Menten equation for one substrate reaction, Johansson and coworkers have deduced the following first order relationship for a reaction of substrate catalyzed by an enzyme reactor [48].

$$\frac{-d[S]}{dt} = K_{ps}^{app}[S] \quad \ldots\ldots\ldots (1)$$

where K_{ps}^{app} is the pseudo-first order rate constant identical to V_{max}/ K_M^{app} and K_M^{app} is the Michaelis constant. K_M^{app} is greater than K_M because the effective concentration at the catalytic site is smaller than in the bulk due to the chemical reaction. Integration of equation (1) yields

$$-\ln(1-X) = K_{ps}^{app}\tau \quad \ldots\ldots\ldots (2)$$

where τ is the residence time and X the fraction of the substrate which reacted in the reactor.

Equation (2) can be used to predict the fractional conversion efficiency of the reactor at a given mean residence time. If the apparent rate constant is sufficiently high, the mean residence time can be kept low (high flow rate) to achieve complete substrate conversion. This equation can also be used to select a reasonable flow rate and reactor size.

Immobilized enzyme reactors have found numerous applications in analysis and biochemical synthesis. Especially, packed-bed reactors have found extensive applications in flow injection analysis. They can easily be used in a flow system in conjunction with almost any type of detector which can record the concentration of

a product, a cosubstrate, a coenzyme or the reacted substrate. Their utility can be increased by a combination of different enzyme reactors in series or by coimmobilization of two or more enzymes [49].

2.3 Flow Injection Analysis

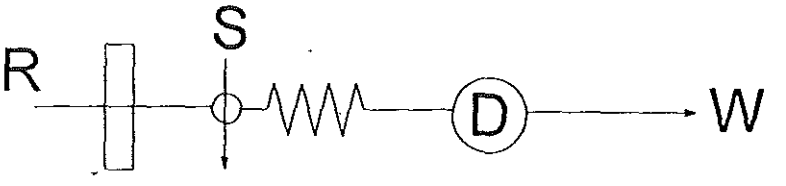
In the mid 1970's Ruzicka and Hansen in Copenhagen and Stewart and coworkers in Washington, D.C., independently abandoned Skeggs' idea of air segmented (SFA) flow analysis and injected the sample directly into the carrier stream without air segmentation. This kind of nonsegmented continuous flow analysis is called *Flow Injection Analysis*, FIA [49].

FIA is a method "based on the injection of a liquid sample into a moving, nonsegmented continuous carrier stream of a suitable liquid. The injected sample forms a zone, which is then transported towards a detector that continuously records the absorbance, electrode potential, or other physical parameter as it continuously changes due to the passage of the sample material through the flow cell" [50].

The simplest FIA analyzer (Fig.1a) consists of a propulsion unit (such as a peristaltic pump), injection port (S), by which a well-defined volume of sample solution is injected into a carrier stream, and a coil in which the sample zone disperses and reacts with the components of the carrier stream, forming a species (e.g. coloured) to be sensed by a flow-through detector (D). A typical recording has the form of a

sharp peak (Fig. 1b) the height of which is related to the concentration of the analyte [50-52].

a)



b)

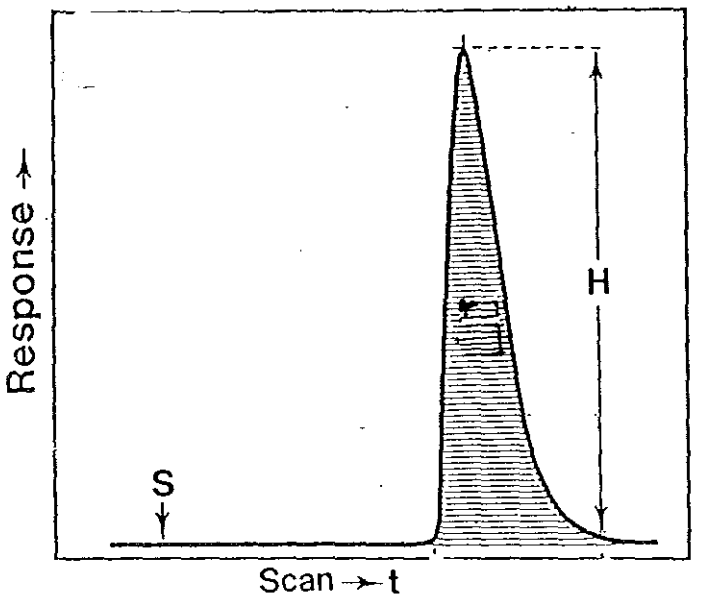


Figure 1 a)The components of simplest single-line FIA system.

b)Typical recording showing one sampling cycle, commencing at the point of injection (S) when valve has been turned and terminating when sample zone has passed into the waste (W).

The cornerstones or basic principles of FIA are injection of fixed volume of sample, controlled dispersion, and exact timing [53]. Since the chemical reactions are taking place while the sample material is dispersing within the reagent, dispersion, controlled within space and time, are the main factors that characterize the high reproducibility of FIA.

The quantitative description of dispersion simply relates the original concentration of the sample material, C°, to its concentration C in an element of fluid which has undergone dispersion: $D = C^{\circ}/C$. For $C=C^{max}$, $D=C^{\circ}/C^{max}$, where C^{max} is the concentration at the peak maximum. In this way the dispersion coefficient D describes the dilution ratio of sample material prior to injection to that measured by the detector [54].

Depending on the intended application, the flow injection systems can be designed to yield a limited (D=1-2), medium (D=2-10) or large (D>10) dispersion, by selecting the geometry and dimensions of the flow channel and volume of the injected sample [55].

Limited dispersion is preferred when measuring the property of the analyte directly in the absence of chemical reaction (e.g. measurement of pH, pCa, etc.). The residence time is so short that convection is prevailing over radial mixing. Thus, the flow rate must be high and large sample volume must be used in order to minimize dispersion.

Medium dispersion is employed when the analyte mixes and reacts with the reagent to form a detectable product. By using long tubes, low flow rates, and several channels with various mixing points, partial mixing between the injected sample zone and the reagent stream can be effected.

Large dispersion is used only when the sample is diluted to bring it into measurement range. In such systems where a very long reactor tube is used, the residence time is too long.

The simplest way of measuring the dispersion coefficient is to inject a well-defined volume of a dye solution into a colourless carrier stream and to monitor the absorbance of the dispersed dye zone continuously by a colorimeter. To obtain the D^{max} value, the height (i.e.absorbance) of the recorded peak is measured and compared with the distance between the baseline and the signal obtained when the cell has been filled with the undiluted dye. Provided that the Lambert-Beer law is obeyed, the ratio of respective absorbance yields a D^{max} value that describes the FIA manifold, detector, and method of detection [50]. The degree of dispersion, and therefore the recorded peak height, is determined by a number of factors including the injected sample volume, the flow rate as well as the channel length and geometry.

Changing the injected sample volume is a powerful way to change dispersion. An increase in peak height and in sensitivity of measurement is achieved by increasing

the volume of the injected sample solution. Conversely, dilution of overly concentrated sample material is best achieved by reducing the injected volumes.

The dispersion of the sample zone increases with the square root of the distance travelled through the tubular conduit and decreases with decreasing flow rate. Thus, if dispersion is to be reduced and the residence time to be increased, the tube dimensions should be minimized and the pumping rate should be decreased. The most effective way to increase the residence time and to avoid further dispersion is to inject the sample into a flowing stream and stop the stream's forward movement, then resuming pumping after a sufficient reaction time has elapsed [56].

There are a number of channel geometries used in FIA such as straight tube,coiled tube, mixing chamber, single-bead string reactor, 3-D or "knitted" reactor, and imprinted meander or combinations of these geometries. The function of these reactors is to increase the intensity of radial mixing, by which the parabolic velocity profile in the axial direction, formed when the sample zone is injected into a laminar flow of carrier stream, is reduced [50]. A coiled tube is the most frequently used reactor geometry since it can conveniently accommodate any length of tubing in an experimental set up and also because secondary flow within the coiled tubing promotes radial mixing [56].

In addition to simple assays, employing one or several reagents, FIA systems may be designed to dilute or to preconcentrate the analyte; to perform separations based

on solvent extraction, ion exchange, gas diffusion, or dialysis; to generate unstable reagents to the concentration suitable for a given assay [50-53]

2.4 Applications of IMERs in FIA

Enzyme reactors with FIA have been used in assaying species of biomedical, biochemical, food and agricultural interest. Their application in FIA can be categorized in three main parts.

2.4.1. Single analyte (substrate) determination

This is the most common and simplest application of an enzyme reactor. The analyte is allowed to react selectively with a coenzyme or coreactant to produce detectable species which then is carried into a flow cell to be measured. Coimmobilization or insertion of another reactor between the analytical reactor and detector is possible to indicate the reaction [37].

In such determinations the detection limit can be lowered even to femttomolar levels [57] by enzyme recycling reactions or amplification.The analyte is recycled by choosing the enzymes in such a way that the product of the first enzyme reaction is in turn the substrate for the second enzyme which will then convert it back to the original substrate [58,59]. This allows the analyte to be reconverted (recycled) several times and, at each cycle, a cosubstrate of one of the enzymes is transformed into a detectable product, resulting in an amplified response at the detector.

appropriate pre-reactor, to give non-detectable species. Most frequently encountered interferences and the pre-reactors used to avoid the interferants are listed elsewhere [37].

In cases where the reagent stream contains a substrate which is identical to the analyte, insertion of a reactor along the reagent channel may destroy the interference. Therefore, the sample is injected after a carrier passed through the reactor that destroys the undesired component and hence, the method is called an on-line elimination method.

If the relative activity of an enzyme with respect to two different substrates differs widely, selectivity for the substrate to which the enzyme is much less active can be achieved by using a small reactor to destroy the substrate to which the enzyme is very active [34].

2.5 Determination of Hydrogen Peroxide

Reactions catalyzed by a subclass of enzymes called oxidases produce hydrogen peroxide. For instance, the oxidation products of glucose catalyzed by glucose oxidase are gluconic acid and hydrogen peroxide. The hydrogen peroxide which is directly proportional to the concentration of the substrate, can be monitored by several techniques.

Techniques for the determination of peroxides find application in diverse analytical

situations. This is exemplified by the multitude of procedures found in the literature [61-63] ranging from those designed for industrial purposes to clinical assays employed in monitoring the composition of body fluids. However, few have been suitable or reliable when dealing with peroxides of low concentration. Among these few, peroxidase-coupled oxidation of donor substrate systems gained popularity due to its use in monitoring oxidase catalyzed reactions.

$$\text{Substrate} + O_2 \xrightarrow{\text{oxidase}} \text{product} + H_2O_2$$

$$H_2O_2 + \text{donor substrate} \xrightarrow{\text{peroxidase}} \text{coloured product}$$

Horse radish peroxidase (HRP E.C.1.11.1.7) coupled with chromogenic reagents have been used in determination of hydrogen peroxide and other substrates converted by oxidases [33-35, 64]. The ferriprotoporphyrin prosthetic group, present in all peroxidases, catalyzes the oxidations of electron donor substrates with hydrogen peroxide to produce coloured species [65].

Several chromogens, organic (o-tolidine, o-toluidine, o-dianisiidine, phenols, substituted aniline, etc.) and inorganic (titanium (IV) sulphate, iodide, hexacyanoferrate(II), etc.) have been used in the oxidative coupling reaction and the inorganics suffer from poor sensitivity and hence are less suited for the determination of biological samples [64-66].

Before the discovery of the potent mutagenic and carcinogenic nature of benzidine, o-toluidine and o-dianisidine, those compounds were widely used as chromogens for peroxidase-catalyzed reactions. Several less hazardous chromogenic systems have since been developed [64,65]. In 1969 Trinder applied the Emerson colour test for phenolic compounds to the enzymatic determination of glucose and glucose oxidase [64]. The Trinder-Emerson system has been used to measure a number of metabolites which can be oxidised in the presence of oxygen and an oxidase to yield an oxidised product and hydrogen peroxide. The latter can then be measured via a peroxidase-coupled reaction.

The Trinder-Emerson system which consists of a phenol reagent and 4-AP has, however, a few problems. The phenol reagent, is not stable, tends to polymerize due to air oxidation, and it is an enzyme-denaturating agent [67]. Improvements have been made by substituting a sulphonated 2,4-dichlorophenol for the phenol reagent [65]. The kinetics of peroxidase-catalyzed oxidation of halogen-substituted phenols, p-cresol and pyrocathechol with 4-AP have been thoroughly studied [68].

Replacement of the phenolic compound with dimethylaniline was not successful because the dimethylaniline is sparingly soluble, toxic, and leave the used glasswares with a lasting stench. The use of 5-aminosalicylic acid and o-phenylenediamine are also problematic because the product produced by the enzyme is light sensitive and partially insoluble, causing turbidity and it is mutagenic [67].

Shiga and co-workers have used five water soluble diphenyl methane derivatives, which gave coloured products by condensation with 3-methylbenzothiozlinon-hydrazone in the presence of hydrogen peroxide and peroxidase, from which Di[4-(3-sulphopropyl ethyl -amino)phenyl]methane, disodium salt, was the best of all reagents tested [69]. Eventhough the molar extinction coefficient is sufficiently high in the wavelength range 500-600 nm, it has difficulties to be used in FIA because of the incubation periods before and after the addition of hydrogen peroxide.

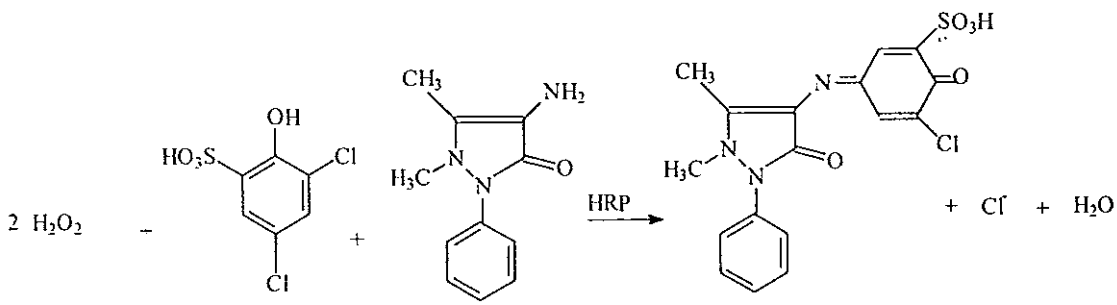
2,2-Azinobis (3-ethylbenzthiazoline-6-sulphonate) in the presence of HRP have been used to determine the hydrogen peroxide generated from cholesterol passing through cholesterol esterase-cholesterol oxidase reactor [70]. The drawback of using this chromogenic reagent is that it cannot be used to detect micromolar amounts.

Fernandez-Romero and Luque-de-Castro have used 4-AP and 2-(N-ethyl-m-tolylamino) ethanol solutions which mixes with the carrier before reaching the analytical reactor [71]. The main disadvantage is that the latter compound is an enzyme denaturating agent.

According to Ngo and co-workers, a good chromogenic system should have the following properties: (a) the reagents should be stable; (b) they should have negligible colour in the absence of peroxidase; (c) the components of the reagent should be water soluble and readily available from commercial sources; (d) the enzymatic oxidation product should be a single, stable, water soluble compound; (e)

the oxidation product should have a high extinction coefficient, preferably with a broad absorption maxima between 500 and 600 nm; (f) the colour formation should be instantaneous so that the change in absorbance can truly reflect the activity of the enzyme or the concentration of the substrate being assayed; (g) the reagents should not be carcinogenic, flammable, or volatile [67].

The most widely used reagent is that of Trinder which consists of 4-AP, 2,4-dichlorophenol sulphonate (DCPS) and HRP. The product, in the presence of hydrogen peroxide is a quinone imine dye which absorbs at 512 nm with molar absorptivity of 22000 M^{-1} cm^{-1}.



In a preliminary investigation, Ashagre has shown that 2,4-dichloronaphthol sulphonate (DCNS) could substitute DCPS in Trinder reagent [35]. In the present work, attempts have been made to optimize the synthesis and further characterize the product, DCNS. An account of sulphonation of aromatic compounds is described below. The experimental section and the results and discussion deal with sulphonation of 2,4-dichloronaphthol (pp 42-43 and 55-61).

2.6 Sulphonic Acids and Sulphonation

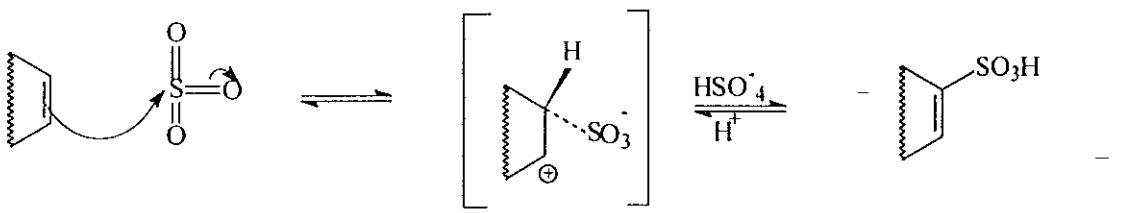
DCPS has been used in Trinder reagent to determine hydrogen peroxide colorimetrically. Before its synthesis was known, phenolic compounds were used but their insolubility, their low sensitivity as colorimetric reagents, and other factors discussed above (2.5) lead to their replacement by the water soluble DCPS. 2,4-dichloronaphthol is quite insoluble in water and to fulfil one of the requirements of a good chromogenic reagent (solubility in water) [67], sulphonation reaction is necessary.

Sulphonation reactions are usually effected by sulphur trioxide (concentrated H_2SO_4, oleum, SO_3 alone, SO_3 adducts with dioxane, thioxane and pyridine), sulphuryl chloride, sulphites and bisulphites, chlorosulphonic, fluorosulphonic, and aminosulphonic acids, acid sulphates and polysulphates. Since the electrophile involved is SO_3 or a sulphur trioxide complex, sulphonating agents act as sources of SO_3 and they vary in strength depending on the effective concentration of SO_3 that they provide. SO_3 itself is, as would be expected, an extremely active sulphonating agent [72].

Oleum and H_2SO_4 are by far the most generally used sulphonating agents. They are effective for the direct sulphonation of practically all classes of aromatic compounds. The ease with which aromatic hydrocarbons react with H_2SO_4 to form sulphonic acids is one of the characteristics that differentiate them from the paraffin hydrocarbons [73].

The sulphonation mechanism is as follows [72,74].

$$2H_2SO_4 \rightleftharpoons SO_3 + H_3O^+ + HSO_4^-$$



Sulphonic acids, because of the resonance stabilization possible for the anion resulting from the loss of the proton on the hydroxyl group, are strong acids comparable to H_2SO_4 with pK_a values of approximately -0.5 and are readily determined by titration [75,76]. But their strength persists if the base molecule is not too large and the group is not neutralized by e.g.an amino group inside the molecule [77].

The products resulting from sulphonation depends on a number of factors such as concentration of the sulphonating agent, temperature of the reaction, time of reaction, catalyst and solvents [74].

From a practical view point, benzene can be sulphonated with an acid stronger than 78.4% H_2SO_4, naphthalene with an acid stronger than 63.7% H_2SO_4, and anthracene is readily converted to the monosulphonic acid with 53% H_2SO_4 [73].

The characteristic IR absorption bands of sulphonic acids are at 1420-1333 cm^{-1}

and at 1200-1145 cm^{-1} [78].

Thus, the sulphonic acids of naphthalene, naphthol, and naphthylamine are numerous and important. In the course of years, these compounds have acquired a wide technical application and are among the most important intermediates in the preparation of azo dyes [73].

2.7 Methods of Separation of Proteins

The term protein, derived from the Greek word Proteois (first importance or primary) was coined by the Dutch chemist Gerardus Mulder [79]. Proteins are macromolecules with molecular weights ranging from approximately 5000 to many millions. They are the most abundant of cellular components constituting more than 50% of their dry weight [80].

Depending on their conformation, proteins can be placed in two major classes, fibrous and globular. The former are physically tough (hence play structural role) and are insoluble in water or dilute salt solutions. Examples are α-keratin of hair, nails, and feathers, and collagen of bone matrix and tendons. In contrast, the latter proteins are mostly soluble in aqueous systems and have functional role in the cell. Almost all known enzymes are globular proteins [80,81].

Normally protein containing samples cannot be directly injected into a flow injection (FI) system because accumulation of proteins on the surface of the immobilized

enzyme reactor results in a rapid increase in back-pressure and a deterioration of its performance [34,82]. Moreover, the sample clean-up helps to increase the accuracy, reproducibility, precision, and the selectivity of the measurement [83]. Thus, sample clean-up before injection is necessary.

In developing a suitable sample pretreatment procedure a number of features should be considered, such as the physicochemical properties of the analyte; the chemical composition of the sample; the stability of the analyte during sample pretreatment; the simplicity, reproducibility, accuracy and precision of the sample pretreatment procedure which should be as high as possible; the enrichment of the analyte in the final step; etc.

There are several methods of separation and purification of proteins based on their molecular size (dialysis, ultrafiltration, and gel-exclusion chromatography), solubility (isoelectric precipitation, salting in and salting out), electric charge (electrophoresis and ion-exchange chromatography), difference in adsorption characteristics, and biological affinity for other molecules (affinity chromatography) [83,84]. Among these precipitation of proteins using organic and inorganic precipitants, isolation of the analyte with a (disposable) solid phase cartridge (solid phase extraction, SPE) and ultrafiltration are the most widely employed sample pretreatment procedures.

Precipitation of proteins from biological samples can be performed with a great number of reagents, e.g. acetonitrile, acetone, ethanol, methanol, trichloroacetic acid

(TCA), perchloric acid, tungstic acid, ammonium sulphate, ammonium chloride, sulphosalicylic acid basic lead acetate or hydrochloric acid [82,85].

When inorganic salts, such as ammonium sulphate and ammonium chloride, are applied a reversible protein precipitation occurs i.e. the biological activity of the proteins persists after dilution of the sample. Thus, ammonium sulphate precipitation is found to be satisfactory to concentrate urine proteins [86]. However, by using acids or organic solvents for protein precipitation the biological activity of the proteins is destroyed irreversibly.

According to Blanchard 10% (w/v) TCA and 6% (w/v) perchloric acid are needed to remove more than 98% of the protein present in plasma and furthermore, TCA, perchloric, tungstic, and metaphosphoric acids are found to be the best precipitants for plasma proteins [82].

The major disadvantage of protein precipitation reactions is that in the majority of analyses a second sample pretreatment should be incorporated in the procedure to concentrate the sample or to increase the reactor's lifetime [83].

Ultrafiltration (UF) is based on the selective filtration or retainment of analytes, as the case may be, by convective solvent flow through a membrane. It proceeds by forcing the liquid in a protein solution through a membrane and retains the protein. Elevated pressure has been largely superceded by centrifugal force as the means

of forcing the liquid through the membrane [85]. If the membrane pores are such that smaller protein molecules can pass through with the ultrafiltrate, a separation is achieved. Larger molecules are retained and concentrated relative to the starting solution.

Nowadays, UF membranes are present with a cut-off values ranging from a molecular weight of 500 to 300,000. Since there is no absolute cut-off point, a proportion of molecules of sizes close to the stated cutoff size will pass through, the remainder staying behind. But if the protein of interest has a much smaller or larger than the quoted cutoff size, virtually all of it will appear in either the ultrafiltrate or the retentate [87]. For instance, suppose the protein of interest has a molecular weight of 300,000 mixed with other proteins which are distributed in size and amount, using a membrane with cutoff of 200,000 most of the proteins of size less than 150,000 passes through the membrane.

To achieve the desired UF, the equipment must be designed to obtain a high transport flow over the membrane and to diminish the effect of concentration polarization (increase of macromolecular concentration just above the membrane), which is the major problem in UF.

One of the major advantages of the UF technique is its speed. Moreover, protein removal by UF avoids the interference and low recoveries which are inherent to most of the chemical precipitation methods [82,88]. Furthermore, the combination of

several UF membranes allows the isolation of a group of analytes with a very narrow molecular weight range. This technique was employed by Moges and Johansson [34] to remove proteins and other macromolecules from the grass pea seed extracts. Since the UF cartridges are expensive and are not easily available in every laboratory the search for alternative cheaper protein separation techniques is sought for.

Solid phase extraction (SPE) is a means of isolation of an analyte or a group of solutes with in a narrow polarity range of a solvent, while all the other compounds are totally unretained or completely retained. The sequence of manipulation starts with the activation (wetting) and conditioning (equilibration) of the sorbent. The wetting, especially for the chemically bonded hydrophobic columns, is very important because it opens the hydrocarbon chains by solvation and increases the surface area of the sorbent [89]. Subsequently the sample is applied to the column and the interfering compounds are washed off the column (clean-up). Finally, the analyte is eluted from the column and, if necessary, the column is reconditioned. For the analysis of small amounts of the analytes it may be advantageous to use polypropylene or polyethylene columns to avoid irreversible adsorption of the analytes onto glass surfaces [83].

The polarity of the sorbent and the choice of eluting and washing solvents is very critical when SPE procedures are applied. A number of physicochemical and other parameters should therefore be taken into consideration: the nature of the analyte

(e.g. molecular weight, solubility), nature of functional group(s) of the analyte (e.g. polarity, acidity), nature of the sorbent (e.g. particle size), nature of the matrix, the forces of the different interactions, and the recoveries of the analyte from the aqueous phase as well as from the biological matrix.

The use of SPE for sample pretreatment has gained increased popularity because it is efficient, fast and easy to automate. Moreover, the procedures have high precision with a high degree of selectivity achieved by the wide variety of sorbents and cleaner samples are obtained because of the minimal introduction of impurities.

2.8 Protein Assay Methods

Once the sample pretreatment is applied in any of the procedures discussed above, the protein content of the eluent or the retentate has to be estimated. The most widely used protein quantitiation methods are: Biuret, Lowry, UV absorption at 280 nm and dye binding methods.

Absorption measurement at 280 nm is a rapid method of determining whether sample solutions contain protein. Proteins absorb at 280 nm solely because of tyrosine (Tyr), tryptophan (Trp) and phenylalanine (Phe) residues (unless they also contain UV-absorbing prosthetic groups) and hence it is not strictly quantitative. Different proteins may therefore have widely varying molar absorptivities; if a protein contains no Phe, Tyr, or Trp, it will be undetected [90]. The method needs correction due to nucleic acid and nucleotide interferences [91].

Biuret reaction involves a strongly alkaline copper reagent which produces a purple coloration with protein. The alkaline copper reagent reacts with the peptide chain of the protein and the absorbance read at 540 nm. The principal reason why it is not widely used is its low sensitivity; several milligrams of sample must be sacrificed for a reliable measurement [92].

The dye binding method, also known as Bradford assay, is a rapid and reliable dye-based assay for determining protein content in a solution. Coomassie Blue G-250 dissolved in acid at a pH below 1 turns a red-brown colour, but when it binds to protein the blue colour is restored, due to a shift in the pK_a of the bound Coomassie Blue. The procedure is simply to add a sample of protein to the reagent and measure the blue colour at 595 nm. Although there are relatively few interfering substances, the dye interacts more or less strongly with different purified proteins and thus is not strictly quantitative. Moreover, the dye adsorbs to glassware and to cuvettes (and to skin) [93-97].

Lowry assay is based on a combination of the copper reaction used in the Biuret method and the Folin-Ciocalteau phenol reagent, the active constituent of which is phosphomolybdic-tungstic mixed acid. Proteins effect reduction of the mixed acid by loss of 1, 2, or 3 oxygen atoms from tungstate and/or molybdate, thereby producing one or more of several possible reduced species which have a characteristic blue colour at 750 nm. Copper apparently chelates in the peptide structure and facilitates electron transfer to the mixed acid chromogen, particularly in the vicinity of amino

acid functional groups, thereby increasing the sensitivity to protein. The principal chromogenic amino acids to which the method is sensitive are tyrosine and tryptophan and, to a lesser extent, cystine, cysteine, and histidine. The peptide linkages are also chromogenic as reduction of the mixed acid occurs from dipeptides consisting of otherwise nonchromogenic amino acids [98]. It is a standard quantitative assay for determining protein content in a solution. Unfortunately there are a number of interfering substances reported and different modifications are required to overcome each problem. But since it is a sensitive method interfering compounds are often diluted out to levels where there effect is insignificant [99].

In this thesis, estimation of the protein content after precipitation of the protein in grass pea was made with Lowry method.

3. EXPERIMENTAL

3.1 Chemicals and Reagents

4-Aminoantipyrine, AP (BDH), 2,4-dichlorophenol, DCP (Riedel-DeHaën), and anhydrous 2,4-dichloronaphthol, D-(+)-glucose and monosodium salt of L-glutamic acid (Sigma) were used as received. DCPS was synthesized from DCP and concentrated sulphuric acid according to the method of Barham and Trinder [65]. Hydrogen peroxide was standardized by permanganate titration [100].

DAP.HCl and ODAP.HCl were synthesized according to the method of Rao [101]. o-Phthaldehyde (OPT) reagent was prepared as follows: 100 mg of OPT (BDH) in 95% ethanol and 0.2 mL of ethanethiol (Riedel-DeHaën) were added to 99 mL of 0.5 M potassium tetraborate buffer [102]. For the Campbell procedure the same preparation was made except the replacement of ethanethiol by mercaptoethanol (Sigma) [30].

The Lowry protein reagent consisted of reagent A: 0.5 g copper sulphate and 1 g sodium citrate dissolved in 100 mL of water; reagent B: 20 g sodium carbonate and 4 g sodium hydroxide dissolved in 1 L water; reagent C: 50 mL reagent B mixed with 1 mL reagent A; and reagent D: 1:1 diluted Folin-Cicalteau reagent (Sigma) [87].

The reagent for the FI system was prepared in 0.1 M phosphate buffer, pH 7, and consisted of 2.5 mM DCPS, 0.5 mM 4-AP, 0.5 mM EDTA and 0.01 mg/mL horse radish peroxidase (HRP, E.C. 1.11.1.7, 290 purpurogallin U/mg solid, Sigma)

unless otherwise specified. The carrier in the flow system was 0.1 M phosphate buffer (pH 7) with 500 µM EDTA.

3.2 Immobilization of Enzymes

Controlled pore glass (CPG-10 with 515Å pore size, Serva) was treated with concentrated nitric acid for 2 h in a boiling water bath and then thoroughly washed with doubly distilled water and dried at 190°C in an oven overnight. The CPG was then silanized by refluxing in a boiling water bath with 10% 3-aminopropyl-triethoxysilane (Sigma) in sodium-dried toluene for 40 min and thoroughly washed with toluene over a G-3 filter. The product was dried in an oven at 115°C overnight. The silanization was tested by adding 3 drops of 1.5% 2,4,6-trinitrobenzene sulphonate solution in ethanol into a test tube containing small amount of the derivatized CPG mixed with saturated sodium borate solution. A deep orange coloured surface appeared a confirming positive test [103]. 180 mg of silanized CPG was activated at reduced pressure and 4°C with 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7 for 30 min. The product was thoroughly washed with doubly distilled water over a G-3 filter. Before activation, the stock 25% glutaraldehyde solution (Sigma) was treated with activated charcoal to remove any polymeric products.

7 mg of glutamate oxidase (GlOD, Streptomyces species, E.C. 1.4.3.11, 6.8 units/mg; from Yamsa Corp., Japan) was dissolved in 3 mL of 0.1 M phosphate buffer at pH 7. The enzyme solution was added to 180 mg of the glutaraldehyde-activated CPG according to earlier procedure [104]. The immobilized enzyme-support was packed in 250 µL plexiglass tube (i.d. 2.0 mm) for flow injection

applications and, when not in use, it was stored at 4°C in 0.1 M phosphate buffer at pH 7.

1 mg Catalase (Cat, Bovine liver, E.C. 1.11.1.6, 19,900 units/mg solid, Sigma), dissolved in 3 mL 0.1 M phosphate buffer, was immobilized on 50 mg of CPG using the same method used for GlOD. 25 µL of each of immobilized GlOD and Cat were mixed and packed in a 50 µL reactor.

Glucose Oxidase (GOD, Penicillium notatum, E.C. 1.1.3.4, 767 mmol/s_protein, VEB LCA-Feinchmie Sebnitz) was also immobilized on CPG (10 mg/100 mg of glass in the first case and 1 mg/50 mg of glass in the other) by the same method.

3.3 Flow Injection Apparatus

The assembly of the FI system for β-ODAP assay is basically the same as that reported elsewhere [34, 105]. It consisted of a two channel propulsion unit, P (Gilson peristaltic pump, Model M312 France), an injection port, S (Rheodyne injection valve with a 20 µL-sample loop), a pre-reactor, PR, (GlOD/Cat reactor), analytical reactor (GlOD reactor), a mixing tee, M, a coiled tube for effective mixing, C, a flow through detector, D (LKB 2151 UV-Vis spectrophotometer) and a recorder, R (Pederson Strip Chart recorder Model 27MR, California). The carrier flow rate was 0.3 mL/min and that of the reagent was 0.12 mL/min.

The above reactors were replaced by 150 µL or 50 µL GOD reactor to study reactor stability after glucose-spiked grass pea extracts were injected. The flow

rate was double that of the GlOD-based FI system. The system with 50 µL GOD reactor was also used to study the reaction of sulphonated 2,4-dichloronaphthol as reagent in the FI system (replacing DCPS) for calibrating with glucose or hydrogen peroxide.

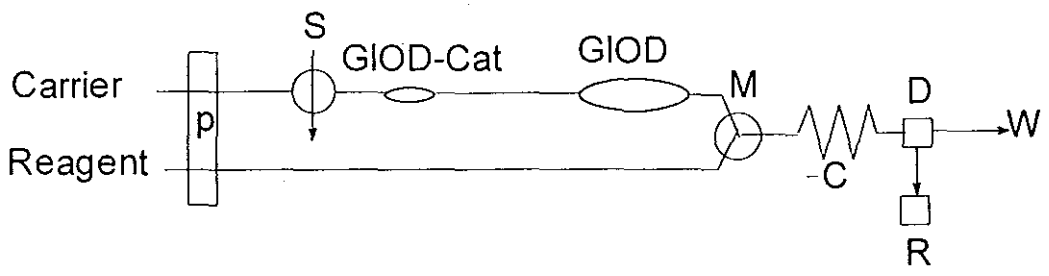


Figure 2. Schematic representation of the FI system used; For ODAP determination PR was 50 µL GlOD/Cat reactor and AR was 250 µL GlOD reactor; for glucose determinations, both reactors were removed and replaced by 150 or 50 µL GOD reactor.

3.4 Extraction of β-ODAP from Grass Pea

For FI determination β-ODAP was extracted from 40 mg of grass pea powder in 10 mL of 0.1 M phosphate buffer containing 500 µM EDTA, pH 7. The extraction was effected over an ice bath by agitation with a magnetic stirrer for 2 h. For the water extract the same procedure was followed except replacement of 10 mL of double distilled water by the 0.1 M phosphate buffer. Particulate matter were removed by filtration through a 0.45-µm membrane filter. The extraction using a sonicator was made from 80 mg of *LS* seed powder in 8 mL of double distilled water by agitation with a sonicator for an hour [102]. The extraction was made

over a water bath thermostated at 55°C. Whatman No 542 filter paper was used to remove particulate matter.

3.5 Isomerization of β-ODAP

500 µL of each of the ultrafiltrates of four buffer extracts were placed in five different Eppendorf tubes and heated in a water bath at 80°C for one hour. The samples were cooled to room temperature. The unisomerized β-ODAP was determined (in duplicates) by injecting the sample at room temperature into the flow system. As control, the same procedure was applied for 300 µM glutamate.

3.6 Protein Precipitation

To 4 mL of buffer extract of grass pea seed 1 mL of 5% TCA was added and kept for thirty minutes at 4 °C. The mixture was centrifuged and the supernatant solution was used for protein assay. Similarly, to 12 mL of crude extract 1 mL of 6% perchloric acid was added, kept at 4 °C, and centrifuged.

The protein in the supernatant was quantitiated by Lowry reaction [87]. To 0.5 mL sample, 2.5 mL reagent C (section 3.1) was added. After 10 min 0.25 mL reagent D was added and absorbance at 750 nm was read after 30 min, using the ultrafiltrate of the extract as a blank.

3.7 Colorimetric Assay forβ-ODAP

The colorimetric procedure used was that modified by T.Haimanot and co-workers [102]. To a 0.1 mL of filtrate of a sonicated extract, 0.2 mL of 3 mol/L KOH was

added and the mixture heated in a water bath at 84°C for 30 min. 0.2 mL of 3 mol/L HCl was added to the cooled mixture and the volume made to 1 mL. 2 mL of freshly prepared OPT reagent was added and the absorbance read at 476 nm against a reagent blank.

In the Campbell procedure 500 mg of sample and 5 mL of 60% ethanol were shaken for 30 min at room temperature and the mixture centrifuged for 30 min. The extraction was repeated with a further volume of 60% ethanol and the two extracts combined. An aliquot (2 mL) of the extract was mixed with 3 mol/L KOH (4 mL) and hydrolyzed in a test tube held in a boiling water bath for 30 min and then cooled. An aliquot (0.25 mL) of the hydrolyzed extract (or the unhydrolyzed extract as blank) was mixed with distilled water (0.75 mL) and 2 mL of OPT reagent. The absorbance of the solution was determined at 426 nm after 30 min [30].

3.8 Sulphonation of 2,4-Dichloronaphthol

To 6.6594 g (0.06 mole) of 2,4-dichloronaphthol 10 mL of concentrated H_2SO_4 (98%, BDH) was added and heated in a boiling water bath for 7 h. After cooling to room temperature 200 mL of doubly distilled water was added to it and then neutralized by 10 mol/L NaOH. The final volume was made to 250 mL and stored overnight in a refrigerator. The supernatant was decanted after centrifugation and the precipitate filtered, washed with 10 mL of 95% of ethanol, dried in the dark and stored in a brown bottle.

The ^1H NMR spectra were recorded using 90 MHz (Joel Fx 90Q) instrument, the

IR using Perkin-Elmer FTIR 1600 series and the UV using Beckman DU-65 spectrophotometer. The melting point was determined using Bock-Monosc op. melting point apparatus. The analytical TLC was silica gel (F_{254} Fluka) coated on aluminium sheet.

4. RESULTS AND DISCUSSION

4.1 Performance of the GIOD Reactor

Flow rate is inversely proportional to the residence time of the substrates and products within the enzyme reactor [106]. Thus, decreasing the flow rate will increase the conversion efficiency of the reactor. There is an optimum flow rate required for equilibrium to be reached, depending on the activity of the enzyme in a reactor. The optimum total flow rate in the two-channel FI system obtained by injecting 150 μM β-ODAP into a 20 μL sample loop in the presence of 250 μL GIOD reactor without the GIOD-Cat prereactor was 0.25 mL/min which is in close agreement with previous reports [34,105]. Due to the low sample throughput routine measurements were made at a total flow rate of 0.42 mL/min. The linear range of the FI system was 1-1000 μM for glutamate and hydrogen peroxide and 1-200 μM for β-ODAP. This range was narrower than that reported before (10-650 μM and 10-300 μM) for the same reactor size [34.105]. This might be due to the lower enzyme loading in this work. The immobilization yield was not, however, estimated in the present work. Moreover, there might be a difference in geometry and length of the two FI systems.

4.2 Comparison of Extraction Efficiency

Extraction of ODAP in the original Rao method was made in 60% ethanol [29]. Extraction of the neurotoxin in water is also reported [102]. Recently Moges and Johansson showed that the extraction of β-ODAP for the FI determination was efficient in 0.1 M phosphate buffer [34].

Three different extraction procedures were investigated to examine the efficiency of extraction of β-ODAP. Five grass pea seed samples were extracted in distilled water, in phosphate buffer and in water using a sonicator (at 55°C) and filtered (section 3.4). All filtrates were then ultrafiltered by membrane (Amicon, molecular weight cutoff 10,000) using a centrifuge at 4000 rpm. Prior to injection the ultrafiltrate from the sonicated extract was diluted in such a way that it contained the same amount of sample as in the other two cases and all ultrafiltrates were injected into the GlOD reactor. The peak height was recorded and is shown in Table 1.

Table 1. Comparison of the response of GlOD reactor towards β-ODAP extracted using three different extraction methods.

Sample GP GPE/ Adet G/94	P E A K H E I G H T (AU)		
	Buffer Extracts	Water Extracts	Extraction with sonication
Coll. No 71	0.062	0.060	0.056
72	0.058	0.061	0.056
74	0.055	0.057	0.055
75	0.047	0.046	0.050
76	0.048	0.052	0.050

As can be seen from the table the results are almost the same for the three

cases. Thus, the extraction in distilled water is as effective as the two other methods. The results in the last two columns show that the toxin in the seed powder can be quantitatively extracted into water which implies that effective removal of the toxin can be made under such conditions. The slightly lower results observed in the case of extraction with sonicator at 55°C is expected to be due to isomerization of β-ODAP.

In a thesis work by Belay, attempt was made to extract β-ODAP in a phosphate buffer containing 500 μM EDTA that gave a less turbid extract. As discussed in his work, the decrease in turbidity might be due to the complexing action of EDTA on the metal ions that would otherwise precipitate and possibly adsorb on the reactor and deactivate the enzyme [105]. However, the β-ODAP content of the two extracts were not compared. In the present study three grass pea seed samples were extracted in a buffer containing 500 μM EDTA and in a buffer only. The observed difference in turbidity was not apparent. Each extract was filtered (0.45-μm membrane), ultrafiltered and the ultrafiltrates injected into GIOD reactor. The percentage of β-ODAP in each case is presented in Table 2.

The results of this observation shows that the presence of EDTA does not have any advantage during the extraction. Therefore, it is not necessary to use a buffer containing EDTA for the extraction of β-ODAP in grass pea. The percentages of β-ODAP for these three samples were found to be the same as those obtained by Belay using the same FI system [105].

Table 2. Determination of β-ODAP extracted in a buffer with and without EDTA (n = 2).

Sample code (Acc/sei.)	% of β-ODAP found by the FI system	
	In buffer extract	In buffer + EDTA
385/504	0.696	0.694
392/505	0.672	0.696
462/527	0.684	0.681

4.3 Thermal Isomerization of β-ODAP in Grass Pea

As described in the experimental section (3.5) the ultrafiltrates of four samples were heated in a water bath at 80°C for one hour to effect isomerization. As a control 300 μM glutamate was also treated in the same way. Table 3 shows the peak height recorded and the percentage β-ODAP unisomerized.

These results show that the unisomerized concentration of the toxin after heating lies in the range 57.8-62.8%. Isomerization data at 80°C for β-ODAP standards using FI system showed 59-60% β-ODAP at equilibrium [34,105], which was in good agreement with the present data in Table 3.

The response for the control, 300 μM glutamate, was the same before and after heating confirming that no change occurred in the solution. If glutamate were present in the sample the results would have been higher than the values shown in Table 3. It also confirms that β-ODAP in the grass pea extracts was isomerized

during the heating.

Table 3. Peak height for β-ODAP in grass pea before and after isomerization and percent of β-ODAP unisomerized (n = 2).

Sample code GP GPE(Adet) G/94	Peak height before isomerization	Peak height after isomerization	% β-ODAP unisomerized
Coll. No 71	0.063	0.044	57.8
72	0.059	0.039	62.4
74	0.049	0.038	61.8
75	0.045	0.034	62.8

4.4 Determination of β-ODAP

Since the development of the FI enzymatic assay method for β-ODAP, no analysis has been made so far with the method for a relatively large number of grass pea samples.

Using the flow injection set-up described in Fig. 2 the β-ODAP content of thirty grass pea samples, obtained from the *Lathyrus* Research Unit (LRU), AAU, Department of Chemistry, were determined. The samples were extracted in a phosphate buffer containing 500 μM EDTA, membrane-filtered, ultrafiltrated and injected to the FI system (3.4). The reactor's response with and without the prereactor was the same indicating the absence or very low level of glutamate in

the samples and this has also been shown earlier [34,105] .

The ODAP content of these samples were also determined by the modified form of Rao method [102]. The results of the FI and the Rao method are tabulated (Table 4).

The results of the FI determination were found to be 10-50% higher than that obtained by the Rao method. For the same thirty samples using the Rao method by two independent workers, deviations between 2-30% was observed (present work and records of LRU, not shown).

Using another modified form of Rao method (Campbell procedure) four samples were analyzed and their results was 53-66% lower than that obtained by the FI-enzymatic procedure (Table 5).

Hussein and co-workers showed that the β-ODAP content for the same sample assayed by six modified forms of the Rao method practised in six different laboratories gave results that are significantly different [30]. In the report, the result of one variation of the method was up to 4.6 times as much as of the other. The conflicting results show that some unambiguous method is sought for future comparisons. But the isomerization result in Table 3 indicates that the FI response was due to β-ODAP only. Evidently, the high results due to the FI method in Table 5 cannot be due to the major substrate, glutamate. Previous reports also show that the GlOD-based assay is β-ODAP selective [34,105].

Table 4. The analysis results of thirty grass pea samples by FI system and Rao method.

Sample GP GPE/Adet G/94	% ODAP analyzed by FI system	% ODAP by Rao method (modified)
Coll. No. 71	0.739	0.522
72	0.996	0.576
74	0.939	0.586
75	0.797	0.468
76	0.541	0.622
77	0.797	0.571
78	0.638	0.471
79	0.744	0.658
80	0.744	0.524
81	0.797	0.643
82	0.797	0.591
83	0.611	0.414
84	0.664	0.452
85	0.799	0.393
86	0.849	0.668
87	0.854	0.602
88	0.854	0.542
89	0.739	0.499
90	0.683	0.519
91	0.654	0.519
92	0.768	0.617
93	0.882	0.638
94	0.739	0.491
95	0.711	0.427
96	0.768	0.560
97	0.655	0.486
98	0.427	0.560
99	0.683	0.509
100	0.739	0.512
101	0.683	0.539

The earlier investigations on the β-α equilibrium after isomerization further supports the β-selectivity of the method [34,107].

Table 5. Comparison of the results of FI, Campbell, and LRU-accepted procedures.

Sample GP GPE/Adet G/94	% ODAP by FI system	% ODAP by LRU-accepted procedure	% ODAP by Campbell procedure
Coll.No. 79	0.744	0.658	0.349
80	0.744	0.524	0.253
81	0.797	0.643	0.296
82	0.797	0.591	0.283

4.5 Separation of Proteins from Grass Pea Seed Extracts

As discussed before the major problem in the FI determination of β-ODAP is deterioration of the reactor's performance due to accumulation of proteins and other macromolecules on the surface of the reactor. Ultrafiltration was used to overcome this problem. The unavailability of the ultrafiltration system entailed a search for other common and simpler separation techniques. Thus, precipitation methods employing trichloroacetic acid (TCA) and perchloric acid were investigated to this effect.

Protein precipitants such as trichloroacetic, perchloric and metaphosphoric acids are believed to function by forming insoluble salts with the positively charged amino groups of the protein molecules at a pH below their isoelectric point, pI [82]. The pI of β-ODAP is 2.45 [21,108] and it seems reasonable to use these precipitants in such a way that the pH of the final solution is above the pI to safeguard the β-ODAP from its hydrolysis.

Based on this hypothesis, 5% TCA and 6% perchloric acid were used in different amounts (and hence different final concentration) to precipitate the proteins from grass pea seed extracts. The optimum final concentration (to keep the pH above the pI of β-ODAP) was found to be 1% with respect to TCA and 0.46% with respect to perchloric acid. After centrifugation the pH values of the supernatant solutions were 2.6 and 2.5 respectively.

Different methods were utilized to determine the protein content before and after the acid-treatment. Assaying the protein by measuring the absorbance at 280 nm was not selective since other absorbing species that would not be affected by the acids could possibly affect the measurement. Hence, the most widely accepted and sensitive method of Lowry [98,99] was used and the amount of protein removed was estimated to be 78% by TCA and 73% by perchloric acid as compared to the initial protein content of the crude extract.

To test the stability of a 150 µL GOD reactor (a) 400 µM aqueous solution of pure glucose (50 injections); (b) 500 µM glucose spiked in the ultrafiltrate of grass pea extract (40 injections); (c) 400 µM glucose spiked in TCA-treated sample (50

injections); (d) 300 µM glucose spiked in perchloric acid-treated extract (50 injections); and (e) 400 µM glucose spiked in a crude grass pea extract (50 injections) were sequentially injected to the FI system. The response was unaffected in each case after a total of 250 injections. Even the peak heights for the glucose spiked in the crude extract was practically constant for the fifty injections. This is because of the high enzyme loading in the GOD reactor and any decay in activity remained unnoticed. The fact that the response of the FI system with the 150 µL GOD reactor is unaffected by the 50 injections of the crude grass pea extracts containing 400 µM glucose shows that stability of a reactor can be elongated by high enzyme loading.

It was necessary, therefore, to employ a reactor with small GOD loading to observe the effect of the crude extract. Thus, the size of the GOD reactor was reduced to 50 µL and 46 units was applied for immobilization onto 50 mg CPG (which is about the same activity of glutamate oxidase used for immobilization onto CPG). The same FI runs were made for pure glucose (40 injections), glucose spiked extracts treated with the acid precipitants, TCA and $HClO_4$ (40 and 50 injections respectively), ultrafiltrate (40 injections) as well as with the crude extract using the small reactor.

All except that spiked in the crude extract gave practically constant peak height for a total of 170 injections (Fig. 3 and 4). The response of FI system to the glucose spiked crude extract decreased gradually and no response was recorded after thirty six injections. The plot in Fig. 4 reflects the decaying peak heights against number of injections which is because of the accumulation of proteins and

other macromolecules on the reactor's surface.

Initially, the contents of glucose in five different crude extracts of grass pea samples were assayed by the Trinder colorimetric method in a batch mode using glucose oxidase to oxidize any glucose in the sample. The same samples were injected into a two channel-FI system incorporating a 150 µL GOD reactor. The results in both the FI and batch modes show the absence of β-D-glucose in grass pea seeds.

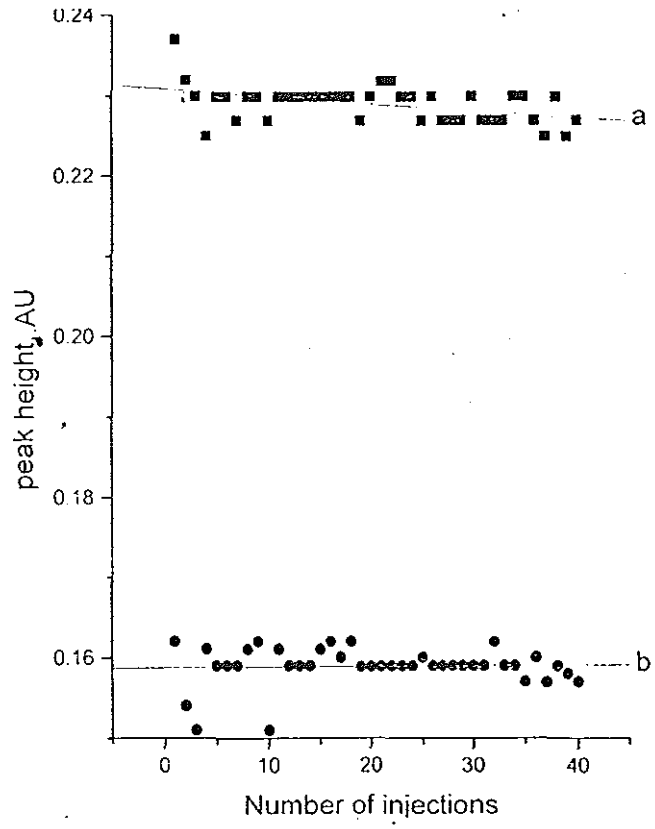


Figure 3. A plot of peak height against number of injections for a) pure glucose of concentration 400 µM; and b) TCA-treated sample spiked in 300 µM (final) glucose (reactor size 50 µl and flow rate 0.84 mL/min).

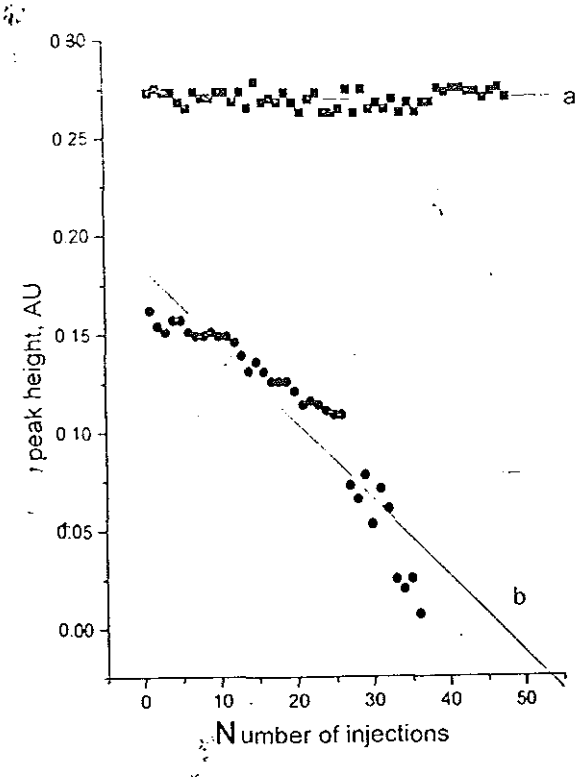


Figure 4. A plot of peak height versus number of injections for a) perchloric acid-treated sample spiked in 500 μM (final) glucose; and b) crude extract spiked with 300 μM (final) glucose.

4.6 Synthesis and Characterization of 2,4-Dichloronaphthol Sulphonate

The earliest chromogenic reagents used to monitor oxidase catalyzed reactions, namely: benzidine, o-tolidine, o-toluidine and o-dianisidine coupled with 4-aminoantipyrine were abandoned because of their carcinogenic effects [67]. Therefore, the less hazardous phenolic compound, DCPS, has been used for years. But the starting material in the synthesis of 2,4-dichlorophenol-6-

sulphonate, 2,4-dichlorophenol, is very toxic. If the cap of the container is opened for a minute its irritating and disturbing smell lasts for a long time in the lab and to the clothings and lab jacket of the worker. This has prompted the search for alternative and less hazardous chromogen.

The basis of this synthetic procedure is the work of Barham and Trinder [65]. The sulphonation was done using concentrated sulphuric acid. The temperature at which sulphonation of DCN was 80°C over water bath as investigated earlier [35]. Lower amounts of DCN are not recommended for the same amount of sulphuric acid because of the charring of the former and bumping during addition of water and/or neutralization by a base.

Attempts to recrystallize the product from toluene, 96% ethanol, acetone, methanol, chloroform and glacial acetic acid was not successful. To optimize the period of synthesis two different syntheses were made: one heated for 5 h and the other for 7 h. The yield of the solid precipitate in the latter was double of that of the former. The two products were, however, the same as confirmed by their TLC (equal R_f values). Moreover, the IR spectra of these two products were the same.

When water was added to the pasty material obtained after heating, a grey precipitate resulted which in turn appeared as a red-brown solution after neutralization by NaOH. The red-brown solution showed different behaviour at different pH. In very acidic medium a faint red-brown precipitate resulted which dissolved at pH greater than 7. At pH 11 the colour of the solution turned

greenish brown.

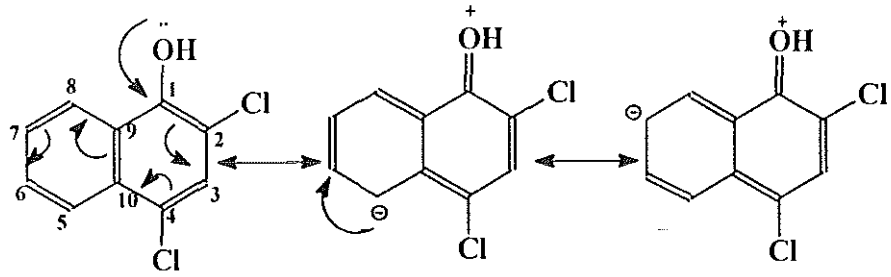
The neutralized solution was kept overnight in a refrigerator, centrifuged, and washed with a limited amount of water. The precipitate was air-dried and stored in a brown bottle. Some physical tests of this solid product and starting material, DCN, were made (Table 6).

Table 6. Some physical tests of the sulphonated product and comparison with the starting material, DCN

	SOLUBILITY IN			M.P. (°C)
	Water	Buffer	NaOH	
Product	soluble	soluble	soluble	above 350
DCN	insoluble	insoluble	oily black ppt.	105-106

The solubility of the sulphonated product was found to be 0.0105 g/mL at room temperature. To know whether the product was a mixture of sulphonated compounds a TLC was run using methanol:chloroform (1:1) solvent system and only one spot (R_f 0.658) that fluoresce at 366 nm was observed.

The resonance structure depicted below shows that position 7 and 5 are susceptible to electrophilic attack. But the 5th position is highly unlikely due to steric reasons.



The ^1H NMR spectrum (90 MHz) of the product was not so descriptive to determine the exact position of sulphonation due to the lower sensitivity of the instrument. However, compared to the spectrum of DCN it shows a disturbance in the splitting pattern of the protons of the ring on which sulphonation is highly probable. The IR spectrum shows absorption bands that are absent in that of DCN. The IR bands at 1336, 1332 and 1245 cm^{-1} are characteristics of sulphonic acids [78]. The UV-spectrum also shows increase in the λ_{max} and intensity of absorption compared to that of DCN due to introduction of other chromophoric group to the system.

2.5 mM of the sulphonated naphthol was used instead of DCPS in Trinder reagent, and its chromogenic property was studied in the presence of 50 µM H_2O_2. The maximum absorption wavelength (λ_{max}) of the resulting red-coloured dye was the same as that obtained using DCPS (512 nm) [65].

The possible chromogenic reaction is as follows:

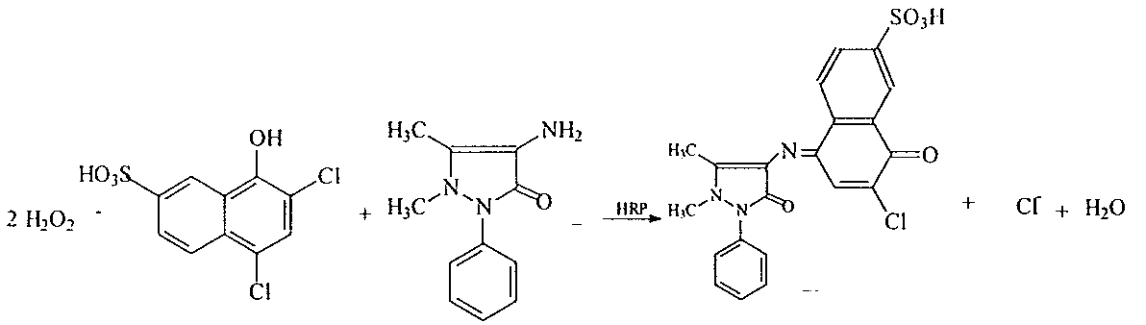


Figure 5 shows a plot of absorbance against pH using the chromogenic reagent in the presence of 50 µM H_2O_2. The maximum absorbance was recorded at pH 4.2 and the minimum at pH 3. The decrease at pH 3 should be due to the decline of activity of HRP since enzyme activity is pH dependent. The molar absorptivity was found to be 15,220 at pH 4.2-5 and 11,380 $M^{-1}cm^{-1}$ at pH 7. The molar absorptivity for the DCPS-4-AP based assay of hydrogen peroxide at pH 7 is 22,000 $M^{-1}cm^{-1}$. The red-coloured dye formed after the addition of H_2O_2 was stable and showed no change in absorbance for over 2 h. Only about 3% of the absorbance decreased after 24 h. The absorbance of the DCPS-based product from Trinder reaction decreases the colour intensity at a rate of 10% per hour [65]. This is another advantage of the sulphonated 2,4-dichloronaphthol over DCPS although the former is less sensitive.

This chromogenic reagent was used to determine H_2O_2 and glucose both in batch and FI modes. In the FI system the size of the GOD reactor was 50 µL. The calibration curve in each case and comparison to that obtained using DCPS is shown below (Figure 6 and 7).

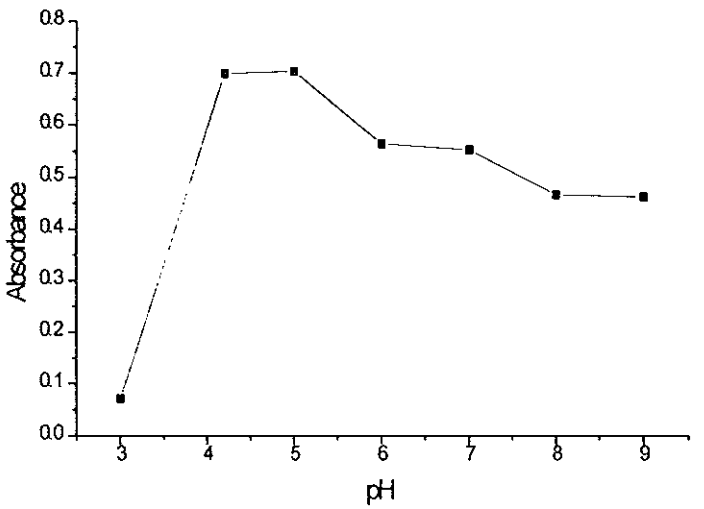


Figure 5. Plot of maximum absorbance versus pH for the new chromogenic reagent.

In both cases the slope of the curve using DCNS is fifty percent of that using DCPS. Despite this, sulphonated 2,4-dicloronaphthol fulfils the requirements for a good chromogenic reagent discussed in section 2.5.

Due to time constraints this reagent could not be used in this work for determination of β-ODAP. As shown for the determination of glucose and hydrogen peroxide it can evidently be used for the assay of β-ODAP, after its GlOD-catalyzed oxidation.

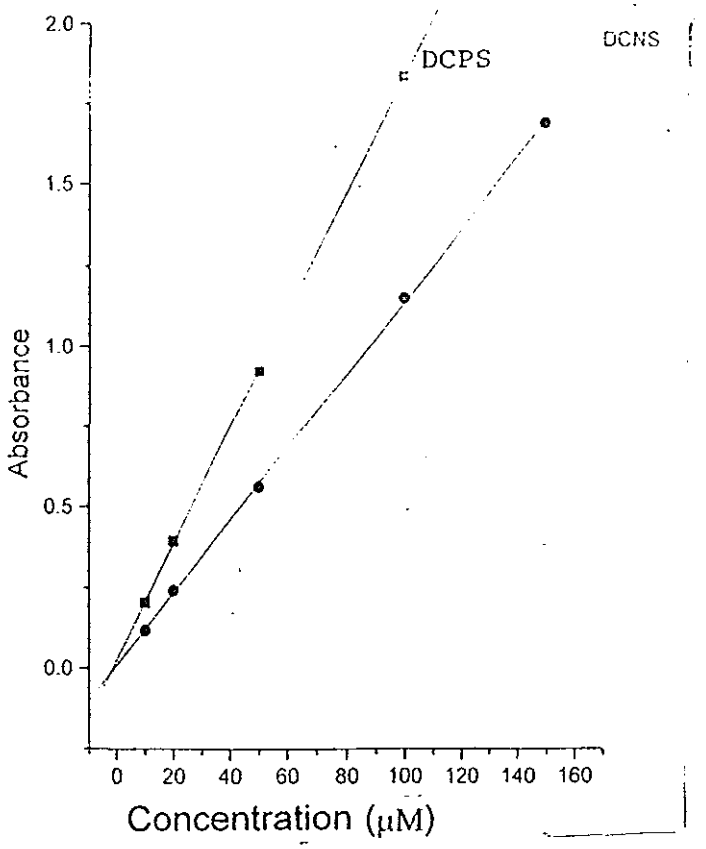


Figure 6. Plot of absorbance versus concentration for glucose determined by the batch mode at pH 7.

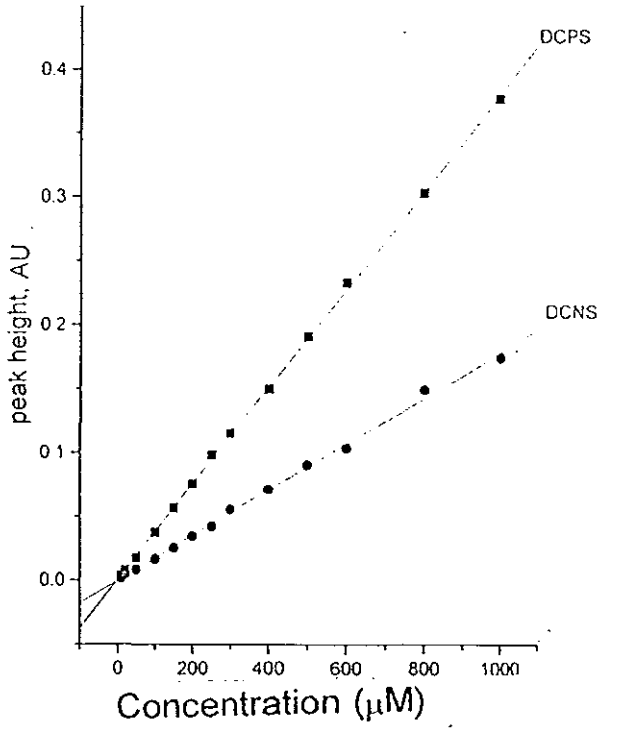
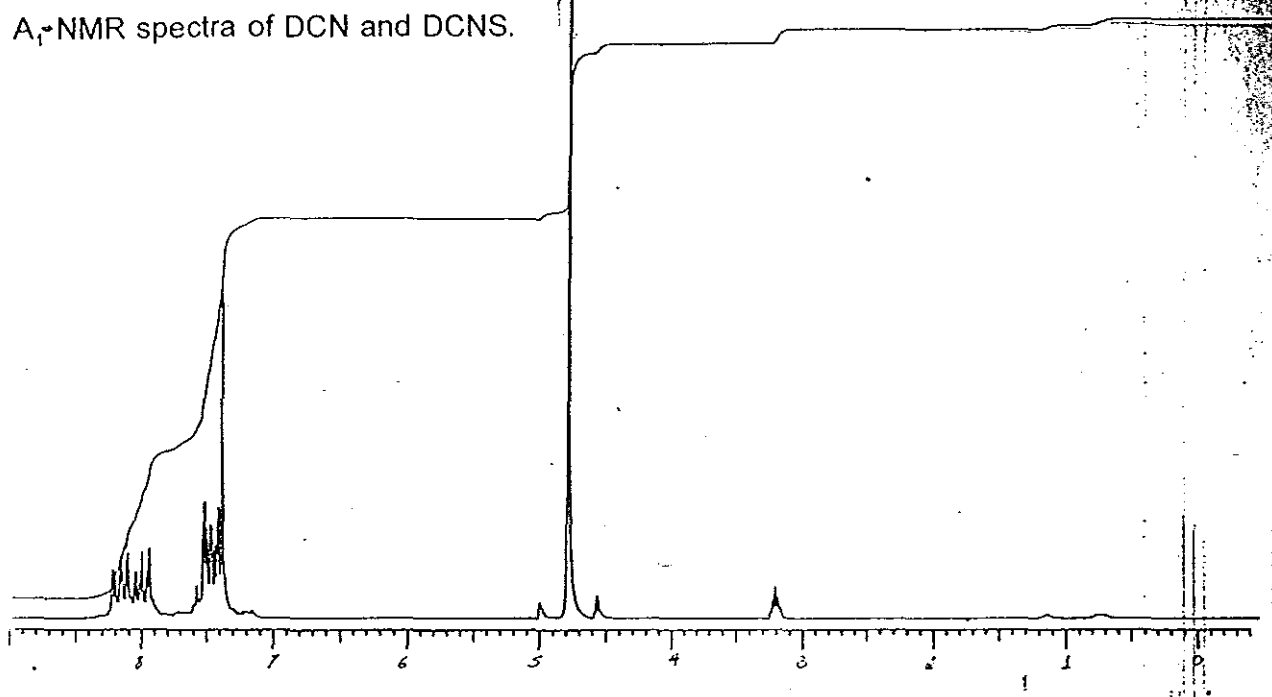


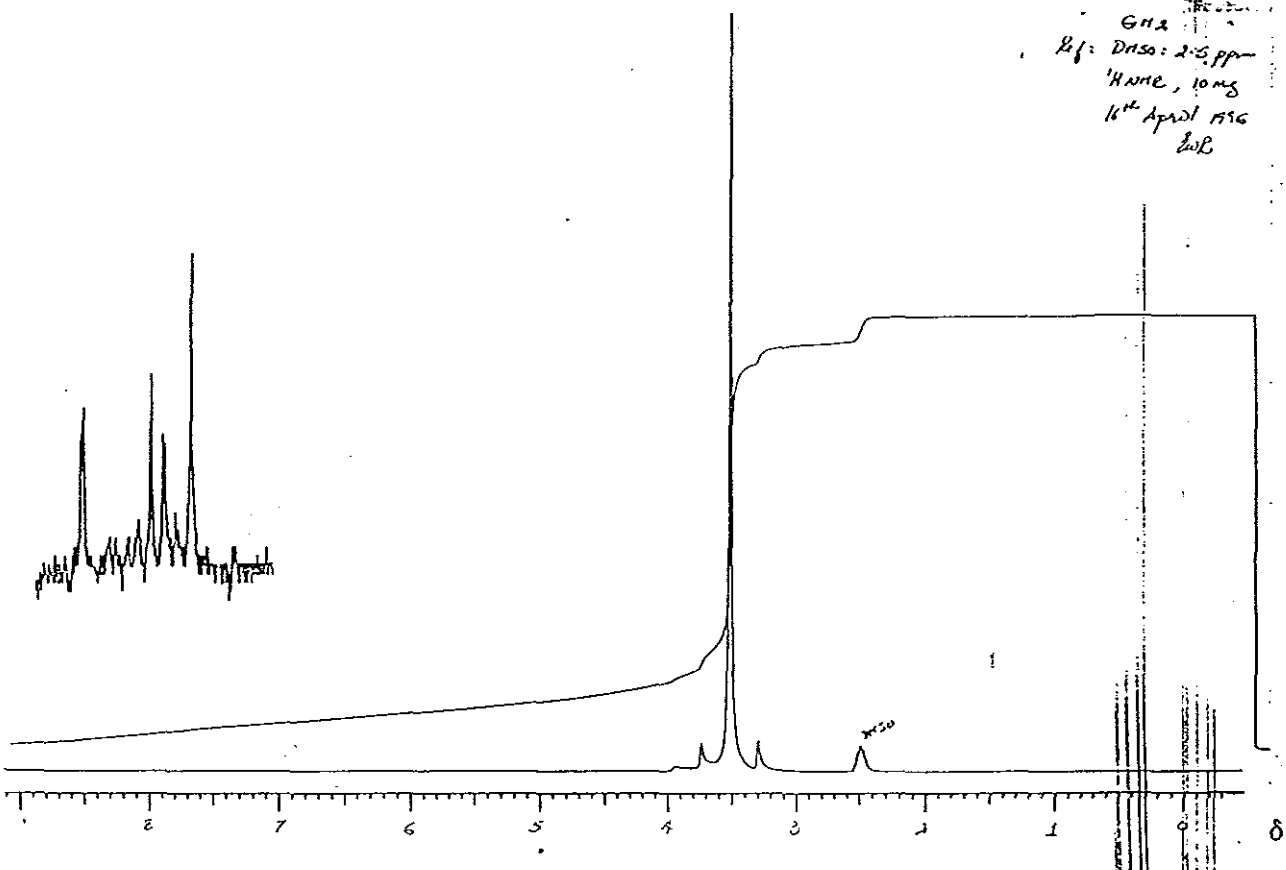
Figure 7. Plot of peak height versus concentration for glucose via the FI system at pH 7; 50 μL GOD reactor; flow rate 0.84 mL/min.

5. CONCLUSION

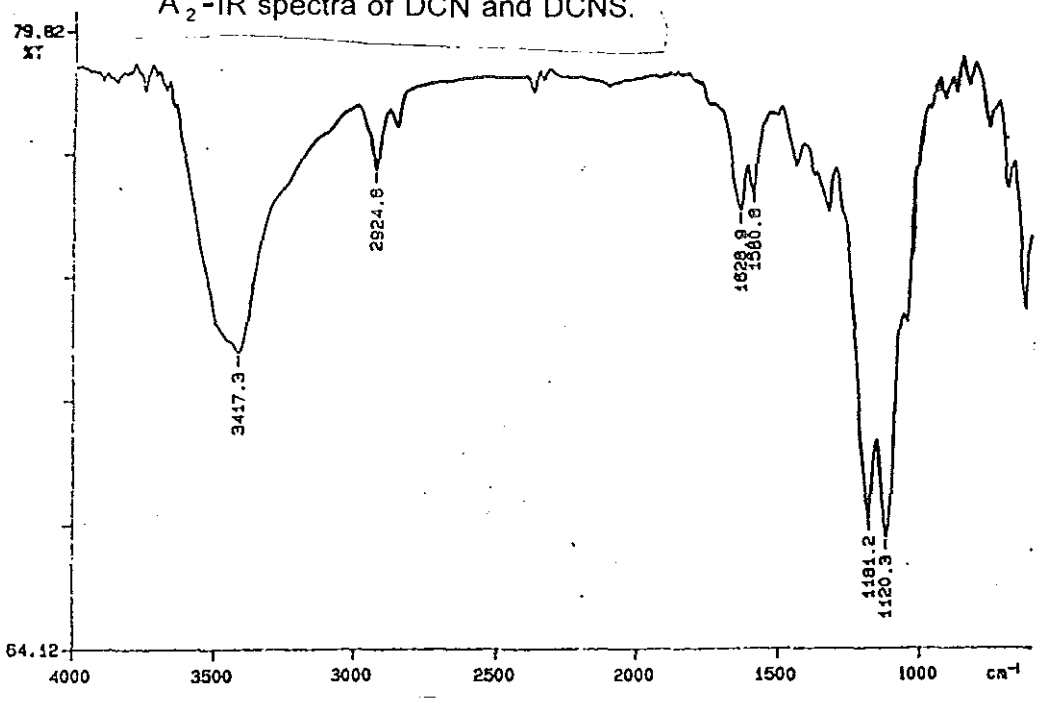
The difference in the assays of β-ODAP between the Rao and FI-glutamate oxidase methods suggests further comparisons should be made with other methods, e.g. HPLC. Despite this, the FI methods still confirms that β-ODAP is selectively detected without interference from the non-toxic amino acid, α-ODAP. TCA and perchloric acid can be used to remove proteins from *LS* seed extracts before injecting into an enzyme reactor. The sulphonated 2,4-dichloronaphthol can be employed for the determination of substrates catalyzed by oxidases, including β-ODAP via its glutamate oxidase catalyzed oxidation.

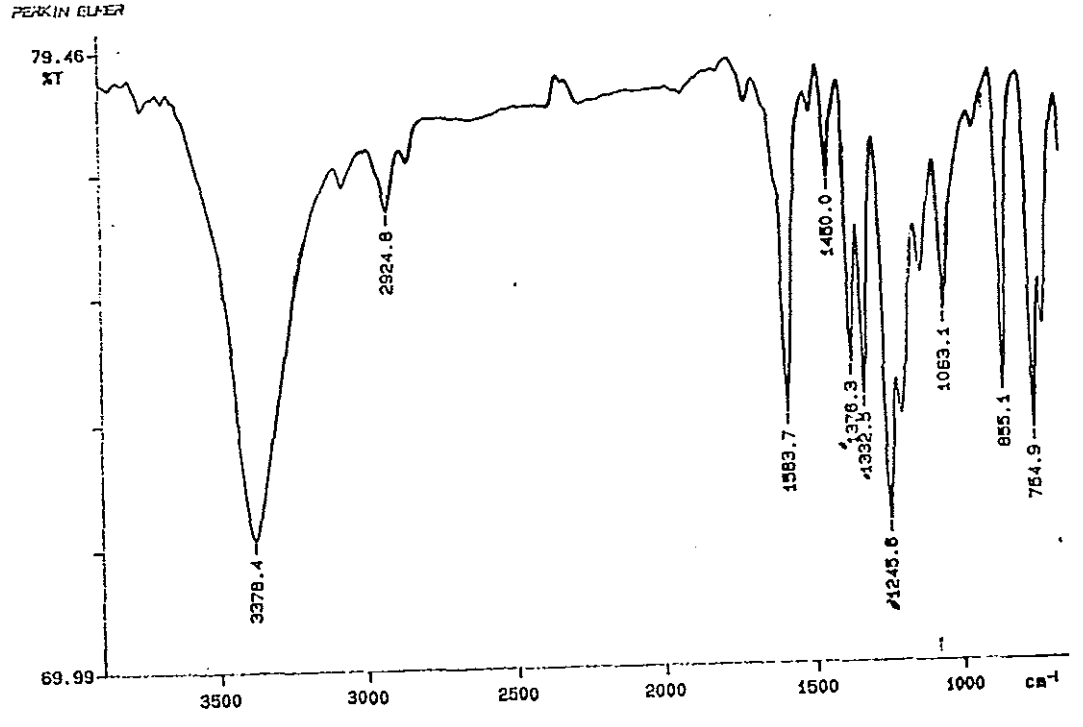
A_1-NMR spectra of DCN and DCNS.





A_2-IR spectra of DCN and DCNS.





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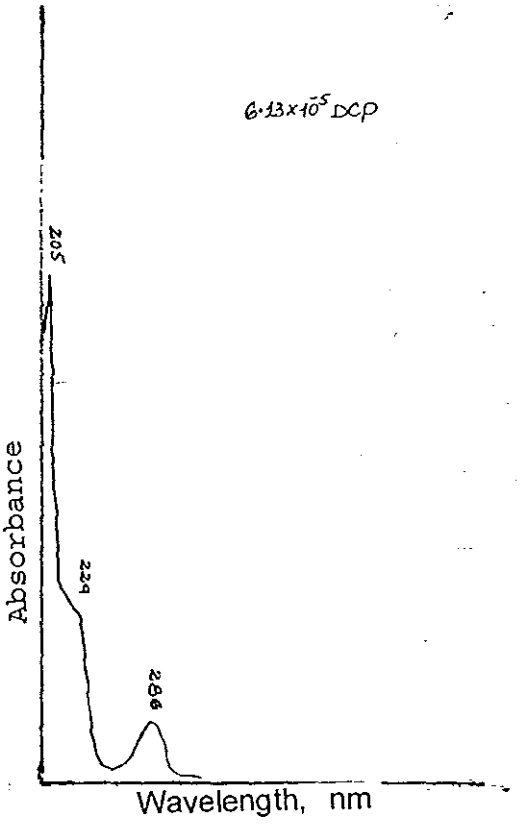
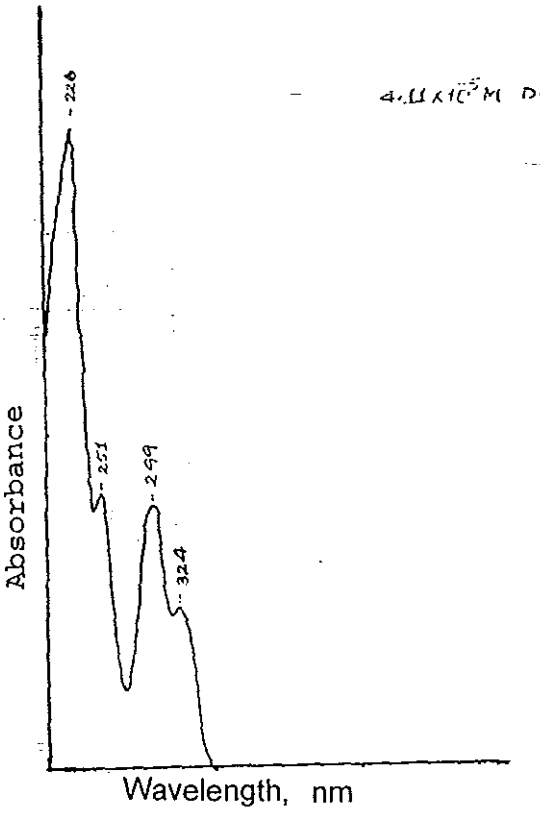
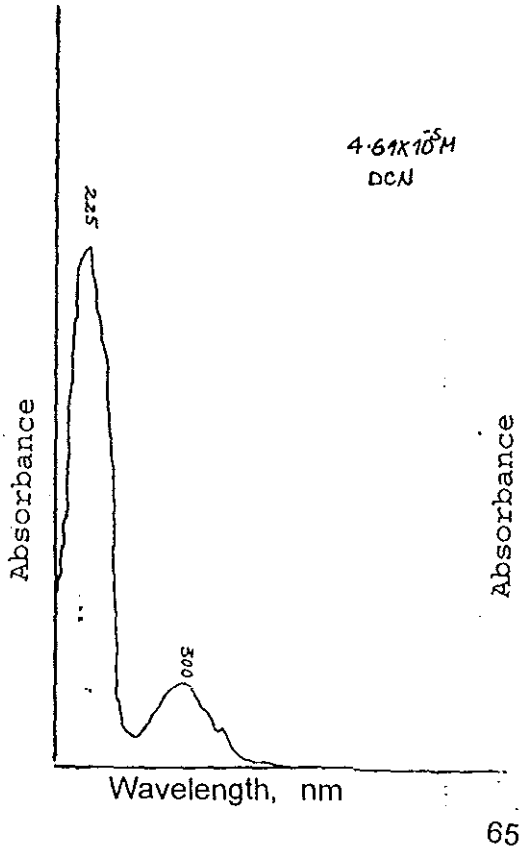
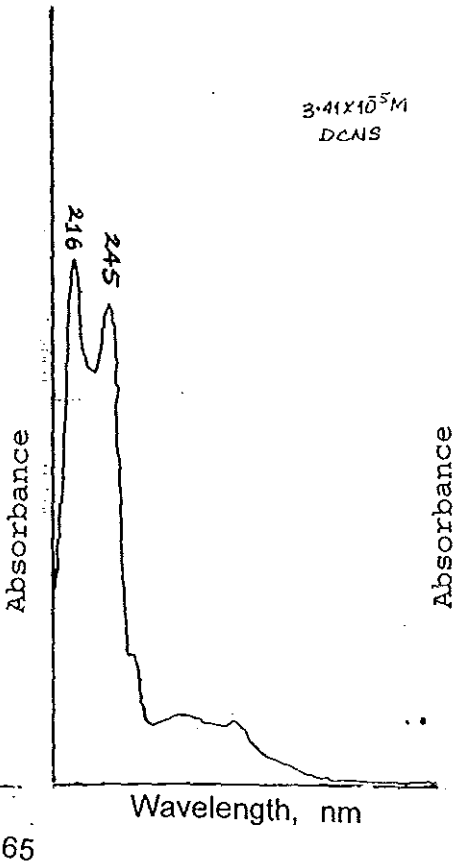
X: 4 scans, 4.0cm-1, diff, flat, smooth
ftir-65 GMOGES1 sulphonated diCl.naphtol, syn. KBr

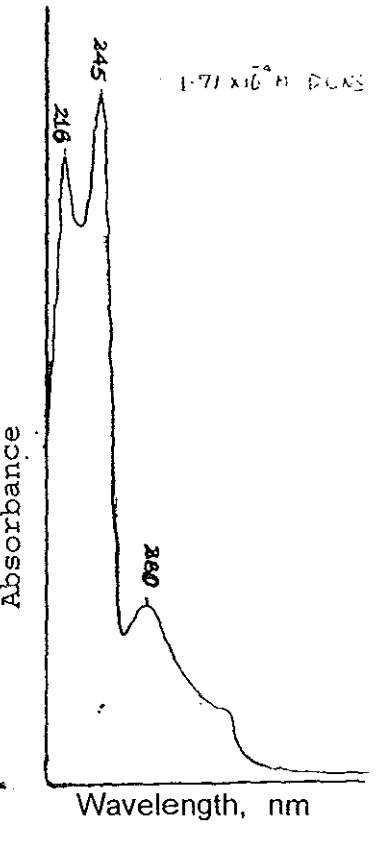
A 3 UV spectra of DCP, DCPS, DCN and DCNS at different concentrations.











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