FLUORESCENCE DETERMINATION OF MONOSACCHARIDES AND CATECHOLAMINES BY USING DANSYLAMINOPHENYL BORONIC ACID

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

Z. EBRU SEÇKİN

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
IN
CHEMISTRY

DECEMBER, 2004

Approval of the Graduate School of Natural and Applied Sciences.

_____
Prof. Dr. Canan ÖZGEN
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

_____
Prof. Dr. Hüseyin İŞÇİ
Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

_____
Prof. Dr. Mürvet VOLKAN
Supervisor

Examining Committee Members

Prof. Dr. O. Yavuz Ataman (Chairman) _____

Prof. Dr. Mürvet Volkan _____

Prof. Dr. E. Hale Göktürk _____

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ABSTRACT

FLUORESCENCE DETERMINATION OF MONOSACCHARIDES AND CATECHOLAMINES BY USING DANSYLAMINOPHENYL BORONIC ACID

SEÇKİN, Z. Ebru

MS, Department of Chemistry

Supervisor: Prof. Dr. Mürvet VOLKAN

December, 2004, 87 pages

In sugar industry, determination of invert sugars (fructose and glucose) provides information about the quality, process control and characteristics of the produced fructose and glucose syrups.

Determination of invert sugar is also important for wine industries. In wine process, fructose and glucose are converted to ethanol by fermentation and the type of wine is designated by the amount of invert sugar remained. Fast and reliable invert sugar detection techniques are required to check the quality of wine throughout the fermentation process.

Cathecholamines (eg. dopamine and epinephrine) are diol containing compounds which play important roles in higher animals' physicomotor activities, learning, sleeping, memory and immune system. They also affect brain functions. Inbalances in dopamine level in brain result in a number of psychiatric disorders, particularly schizophrenia and Parkinson disease.

Catecholamines are present in relatively high amounts in drugs. Many efforts have been made to develop analytical procedures for their rapid, simple and accurate determination. For these reasons, catecholamine quantification is important in the field of pharmacy and medicine.

Boronic acids interact with 1,2 or 1,3-diol containing compounds, such as fructose, glucose and dopamine, rapidly and reversibly. Hence, boronic acids are used as the recognition moeity in the construction of photoinduced electron transfer (PET) fluorophores specific for saccharides and catecholamines.

In this study, a flow injection analysis system has been developed for the rapid and selective recognition of fructose and glucose in wine and in commercial sugar syrups; dopamine and epinephrine in pharmaceutical injections by using dansylaminophenyl boronic acid (DAPB) which is a fluorescent PET molecule.

Keywords: Fructose, Glucose, Dopamin, Flow Injection Analysis, Reverse Photoinduced Electron Transfer, Fluorescence.

ÖZ

DANSİLAMİNOFENİL BORONİK ASİT KULLANARAK MONOSAKKARİTLERİN VE KATEKOLAMİNLERİN FLORESANS METODU İLE TAYİNİ

SEÇKİN, Z. Ebru

Yüksek Lisans, Kimya Bölümü

Tez Yöneticisi: Prof. Dr. Mürvet VOLKAN

Aralık, 2004, 87 sayfa

İnvert şekerlerin (fruktoz ve glikoz) tayini, şeker endüstrisinde üretilen fruktoz ve glikoz şuruplarının kalitesi, süreç kontrolü ve özelliği hakkında bilgi vermektedir.

İnvert şeker tayini şarap endüstrisinde de önemlidir. Şarap üretimi sürecinde, fruktoz ve glikoz fermentasyon yoluyla etil alkole dönüşür ve şarabın türü kalan invert şeker miktarına göre belirlenir. Fermentasyon sürecinde şarap kalitesini kontrol etmek için hızlı ve güvenilir invert şeker tayin tekniklerine ihtiyaç duyulmaktadır.

Katekolaminler (örneğin dopamin ve epinefrin), yüksek metabolizmalı canlıların fizikomotor aktiviteleri, öğrenme, uyuma, hafıza ve bağışıklık sisteminde önemli rol oynayan diol içerikli bileşiklerdir. Ayrıca beyin fonksiyonlarını da etkilerler. Beyindeki dopamin düzeyindeki dengesizlikler özellikle şizofreni ve Parkinson hastalığı gibi bazı psikiyatrik rahatsızlıklara neden olmaktadır.

Katekolaminler ilaçlarda oldukça yüksek miktarlarda bulunur. Hızlı, basit ve kesin tayin edilebilmeleri için pek çok analitik yöntem geliştirilmeye çalışılmıştır. Bu nedenlerden ötürü, katekolamin tayini eczacılık ve tıp alanlarında önem taşımaktadır.

Boronik asitler fruktoz, glukoz ve dopamin gibi 1,2 ya da 1,3 diol gruplarına sahip bileşiklerle hızlı ve tersinir reaksiyon verirler. Bundan dolayı, boronik asitler sakkarit ve katekolaminlere özgü fotoindüklenmiş elektron transfer (PET) floroforlarının oluşturulmasında kullanılırlar.

Bu çalışmada, şaraplarda ve ticari şeker şuruplarında fruktoz ve glikozun, ilaç enjeksiyonlarında dopamin ve epinefrinin seçici tayinleri için floresans bir PET molekül olan dansilaminofenil boronik asit kullanılarak bir akışa enjeksiyon sistemi geliştirilmiştir.

Anahtar Sözcükler: Fruktoz, Glikoz, Dopamin, Akışa Enjeksiyon Analizi, Ters Fotoindüklenmiş Elektron Transferi, Floresans.

TO MY FAMILY

ACKNOWLEDGEMENTS

I would like to express my gratitude thanks to Prof. Dr. Mürvet Volkan for her encouragement, understanding and care throughout this study but also throughout the years I spent in METU.

The supports of Jale Leblebici, M. Füsun Kavas, Gülin Sezer from Şeker Enstitüsüare gratefully acknowledged.

I am very grateful to Prof. Dr. Erdal Bayramlı for providing the sugar samples.

Very special thanks go to Serap Tekin for her sincere help at any time. Special thanks are extended to all analytical chemistry group members.

I would like to thank my examining commitee members for their advice, time and interest in this study.

Grateful thanks are due to my dearest friend Deniz İnam for everything we shared.

Finally, my sincere thanks go to my father, mother and brother for their patince and support throughout my life.

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INTRODUCTION

1.1 Carbohydrates

Carbohydrates occur in all plants and animals and are essential to life. They are nature's conveyors of energy and therefore essential for cell survival [1]. Through photosynthesis, plants convert atmospheric carbon dioxide to carbohydrates, mainly cellulose, starch and sugars. Carbohydrates are polyhydroxy aldehydes, or ketones, or substances that produce such compounds when hydrolyzed [2]. Carbohydrates constitute one of the three major chemical compound classes that are ingredients of nutrient products; they play a very important role in the life cycle and form the base of many foodstuffs. The quality and the caloric value of many foodstuffs and diet products are partly dependent on the quantity and type of their carbohydrate content. In contrast, the presence of excess of sugar in the blood gives evidence of serious malfunction of the human organism. Carbohydrates are constructed from the atoms of carbon, oxygen, and hydrogen, occurring in proportion that approximates that of a "hydrate of carbon". They exist in three major classes: the monosaccharides, the oligosaccharides, and the polysaccharides [2].

1.2 Monosaccharides

Monosaccharides are structurally the simplest form of carbohydrate, in that they cannot be reduced in size to smaller units by hydrolysis. For this reason, they are sometimes referred to as simple sugars [2]. Another characteristic of monosaccharides is that they can act as mild reducing agents. This is because the aldehyde group that is present can be oxidized to form a carboxylic acid group, or in the presence of a base, carboxylate ion group [3].

The most abundant monosaccharide in nature is the 6-carbon sugar glucose (6-membered cyclic sugar), is an essential component of blood. Common names include glucose, grape sugar, and blood sugar [4]. Another common monosaccharide is fructose (5-membered cyclic sugar), also known as fruit sugar. Fructose can also act as a reducing sugar, even though it has a ketone group instead of an aldehyde group. Under basic conditions, fructose molecules can, essentially, have the location of the carbonyl bond switched to convert them into a glucose molecule [3]. Fructose is %50 sweeter than sucrose (table sugar) and is the major sugar in honey. Galactose is another example for monosaccharide. It is found in naturally in milk and milk products. It is hydrolysis product of lactose (which is composed of galactose and glucose) [2].

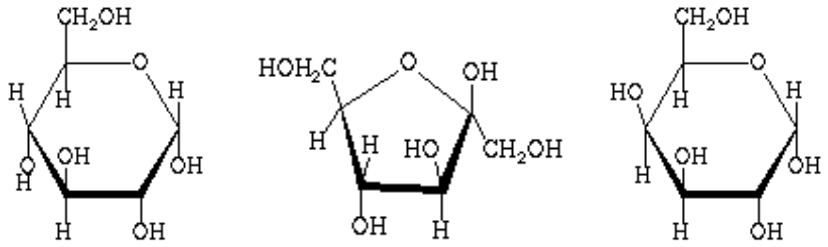

Figure 1.1 Chemical Structures of Glucose, Fructose and Galactose.

1.3 Oligosaccharides

Oligosaccharides consist of short chains of monosaccharide units joined by covalent bonds. The number of units is designated by the prefixes di-, tri-, tetra- , and so on . Among the oligosaccharides, the disaccharides, having just two monosaccharide units, are the most abundant. Within this group, sucrose, consisting of a glucose and a fructose residue, is nutritionally the most significant [2].

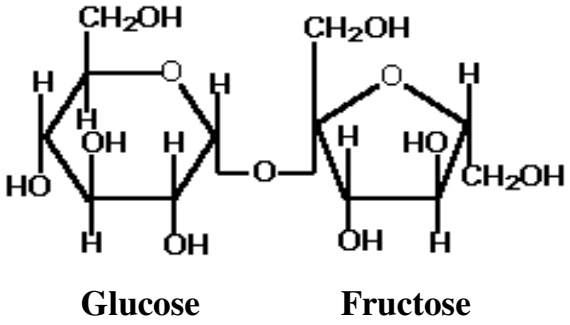

Figure 1.2 Chemical Stucture of Sucrose

1.4 Invert Sugar

Invert sugar is described as a mixture which have equal quantity of fructose and glucose [5]. Invert sugar is prepared by the hydrolysis of sucrose to glucose and fructose. This is achieved by subjecting a sucrose solution to acid and heat. Invert sugar syrups can be fully or partially inverted to leave part of the original sugar unchanged. These syrups are known as medium or partial invert sugar syrups.

The term “invert” originates from the effect on the polarimeter instrument traditionally used to analyse sucrose solutions. Compared to pure sucrose, a mixture of glucose and fructose “inverts” the plane of polarised light, and so this is known as invert sugar [7].

World-wide, high fructose syrups (HFS) are produced from a variety of starch sources. World-wide production of starch based sweeteners is over 15 million tons per year. These sweeteners are used in baking, candy making, beverages, fruit canning, etc. In producing fructose syrup products, starch, a polymer of glucose, is heated and processed with enzymes that hydrolyse starch into glucose. Glucose is further processed in the presence of an isomerase enzyme. This enzyme acts on the glucose and converts it to it’s isomer, fructose. The conversion is limited by equilibrium in the isomerisation process. Ideally, given a 100% glucose stream, a 50% represents the equilibrium point.

However, the realities of producing a commodity product and the limitations inherent in chemical processing originally reduces this to approximately 42-43%. This therefore became the fructose

concentration used for a commercial product, 42 HFS, in the early 1970s. The product as collected from the isomerisation process is not a saleable product. It is further refined to remove colours, odours, and ash introduced in the reaction processes, and to adjust the concentration to something suitable for shipping and storage. If it is desired to make a 55 HFS product, chromatographic separation is used to concentrate the fructose to 90; this 90 HFS is then blended with 42 HFS at the appropriate ratio to produce 55 HFS [8].

Glucose and fructose (invert sugars) are commonly present in fruits and fruit drinks. There is a demand for the determination of invert sugars for quality control, nutritional purposes and for monitoring the ripening of fresh fruits [6]. Precise information about the content of food stuff ingredients is currently required or demanded by external quality control bodies for checking [9]. The frequent determination of the content of invert sugars (glucose plus fructose concentrations) in raw material, final products and during the fermentation in industrial bioprocess, such as wine, hydrated alcohol, penicillin production is very important [10].

1.5 Invert Sugar in Wine

Wine has been made for several thousand years by the fermentation of the juice of grape. The quality of the wine product is largely related to grape, soil, sun, climatic conditions, use of pesticides, wine making procedures, storage and technical factors, resulting in variation in flavor, bouquet, and aroma [11]. Sugar is an essential component in the production of wine. During alcoholic fermentation, yeast feeds on

the sugar found in grape juice and converts it to ethanol and carbon dioxide. The amount of sugar fermented determines the wine's alcohol level and the amount of invert sugar left in the wine. Glucose and fructose are the main fermentable sugars in grape juice. Although each type of sugar exists in approximately equal concentrations in wine, fructose is roughly twice as sweet as glucose. [12].

Invert sugar determination is important for routine quality control to follow the fermentation process and also to check the conformity to the requirements for certain types of wine [13]

1.6 Catecholamines

Dopamine, norepinephrine and epinephrine are all biogenic amines based on a benzene ring with hydroxyl groups at 3- and 4- positions, and with an amino group in a side chain (Figure 1.3). Due to the fact that the benzene ring with the two vicinal groups is called a catechol ring, the name catechloamines were given to these substances. Free catecholamines have a strong physiological potential as a neurotransmitter and as a hormone of the adrenal medulla [14]. They take part in the regulation of the response to stress, physicomotor acitivity, emotional processes, learning, sleep, and memory and have important regulatory function in the immune system. In addition, catecholamines play an important role in higher animals in the metabolism of sugar, lipid and so on [15].

They are now widely used in the treatment of bronchial asthma, hypertension, heart failure associated with organic heart disease, and

in cardiac surgery [16]. For this reason, determination of catecholamines and their metabolites in body fluids such as urine, plasma and serum is one of the most important ways to evaluate the activity of the sympathetic nervous system and support the diagnosis of many diseases [17, 18].

Quantification is also needed in the fields of pharmacological and biological sciences [19]. The catecholamines are present in relatively large amounts in drugs and many efforts have been made to develop rapid, simple and accurate analytical procedures for their determination. [20]

1.7 Importance of Dopamine

Dopamine is derived from tyrosine and is the precursor to norepinephrine and epinephrine [21].

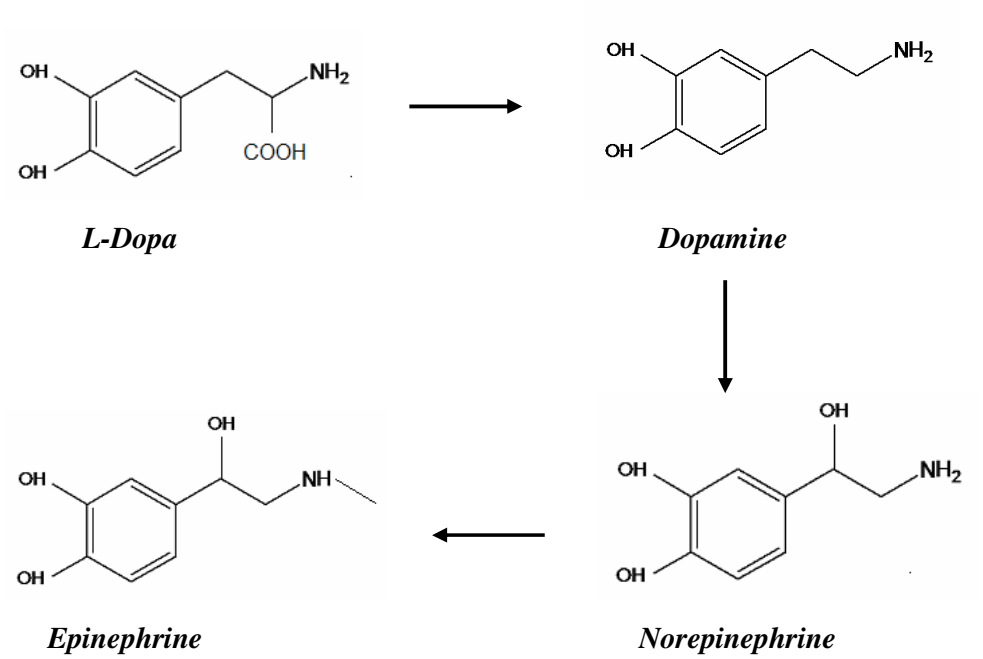

Figure 1.3 The Metabolic Pathway for the Major Catecholamine Synthesis.

Dopamine affects brain processes (control movement, emotional response, the ability to experience pleasure and pain, mental and physical health) and a number of psychiatric disorders are attributed to imbalances in dopamine levels, particularly schizophrenia and Parkinson disease [22].

Dopamine hydrochloride injection (Dopamine hydrochloride salt) is used in Parkinson disease [22], in the treatment of shock, which may

be caused by trauma, hearth attack, open heart surgery, heart failure, kidney failure and severe bacterial infections of the blood [23-26]. Hence simple, rapid and reliable determination of dopamine in pharmaceutical injections and in biological fluids with relatively low cost instruments is strongly required.

1.8 Luminescence

Luminescence is the emission of photons from electronically excited states; thermal excitations are excluded. Luminescence is divided into three types, fluorescence and phosphorescence and chemiluminescence. Fluorescence and phosphorescence are emissions which result due to singlet-singlet and triplet-singlet states transitions, respectively. Substances which display significant fluorescence generally have delocalized electrons formally present in conjugated double bounds. Typical fluorescence lifetime ranges are typically near 10^{-8} second. The third type of luminescence, chemiluminescence, is based upon the emmission spectrum of an excited species that is formed in the course of a chemical reaction [27]; in case that it takes place in a living organism, it is called bioluminescence.

A diversity of molecules display fluorescence, and several interactions and processes can alter the spectral properties of fluorophores. As a result, fluorescence methods are widely used in analytical chemistry [27].

Fluorescence quenching refers to any process which decreases the fluorescence intensity of a given substance. A variety of processes can result in quenching. These include excited state reactions, energy transfer, complex formation, and collisional quenching.

In the case of dynamic collisional quenching, the quencher must diffuse to the fluorophore during the lifetime of the excited state. Upon contact, the fluorophore returns to the ground state, without emission of photon.

Fluorescence quenching has been widely studied both as a fundamental phenomenon, and in the application of fluorescence to chemical and biological problems [27].

Quenching of fluorescence is described by the Stern-Volmer equation:

$$\boldsymbol{Io/I}=1+k_q\tau_o[Q]=1+K_D[Q]$$

In this equation Io and I are the fluorescence intensities in the absence and presence of quencher, respectively, k_q is the bimolecular quenching constant, τ_o is the life time of the fluorophore in the absence of quencher, [Q] is the concentration of quencher, and K_D is the Stern-Volmer quenching constant. Quenching data are frequently presented as a plot of Io/I versus [Q] because Io/I is expected to be linearly dependent upon the concentration of quencher [27].

1.9 Photo Induced Electron Transfer (PET) Sytems

Generally, PET model compounds are composed of three parts: Fluorophore-Spacer-Receptor.

- A fluorophore module is usually based on a polycyclic aromatic system (e.g. anthracene, naphthalene, pyrene) and is the site of both photonic transactions of excitation and emission.

- A spacer module (at least one methylene group) holds the fluorophore and receptor close to, but separate from each other.

- A receptor module (e.g. a crown ether, an amine or a boronic acid) with the role of signaling is specific for a particular species and responsible for guest complexation [1,28-30].

Dansylaminophenyl boronic acid, DAPB, used in our fluorescence measurements, is a PET molecule (Figure 1.4);

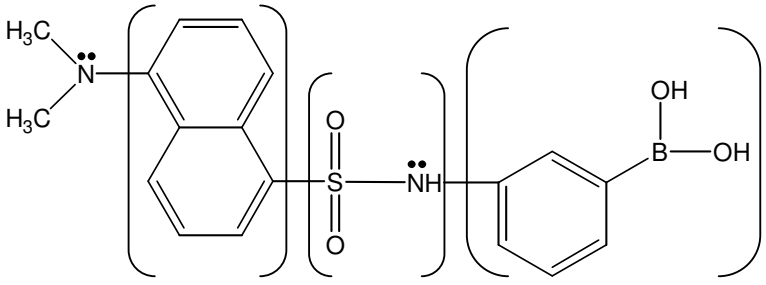

Figure 1.4 Chemical Structure of m-Dansylaminophenyl Boronic Acid, a PET molecule.

Boronic acids [R-B$(OH)_2$] have the unique properties of forming reversible complexes with diol-containing compounds to give boronate esters [31-33]. All saccharides and polysaccharides have a number of cis- and trans-diols and therefore rapidly form diol-boronic acid complexes when dissolved in basic aqueous media [28]. Such tight binding allows boronic acids to be used as the recognition moeity in the construction of PET molecules specific for saccharides [34].

The response which signals an interaction between carbohydrate and boronic acid receptor is usually communicated by changes in fluorescence intensity either through chelation enhanced quenching (CHEQ), or chelation enhanced fluorescence (CHEF) [35].

Charge-separating processes, especially those separating a full electronic charge, are highly sensitive to environmental stimuli. PET is ideal process fitting this description, even though charge shift (or translocation) can be seen in intrinsically charged systems. This environmental sensitivity shows up in fluorescence (quantum yield and lifetime but not wavelength) since it competes with electron transfer to deactivate the photoproduced excited (normal PET) (Figure 1.5 and 1.6) state or to activate the photoproduced excited state (reverse PET) (Figure 1.7) [29].

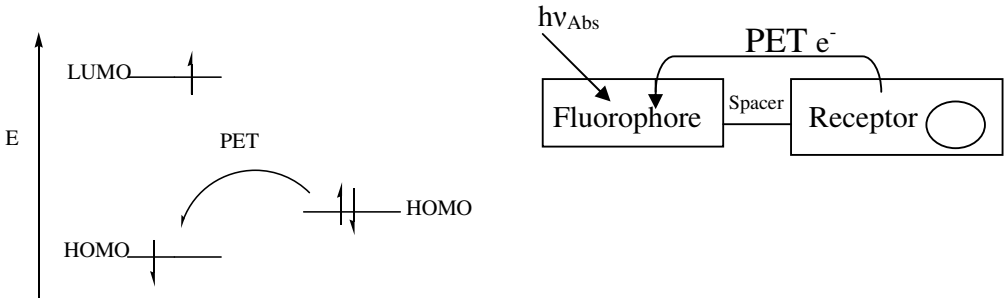

Figure 1.5 Frontier Orbital Energy Diagram and Spaced Fluorophore-Receptor System for "off" (Non-fluorescence) State [29].

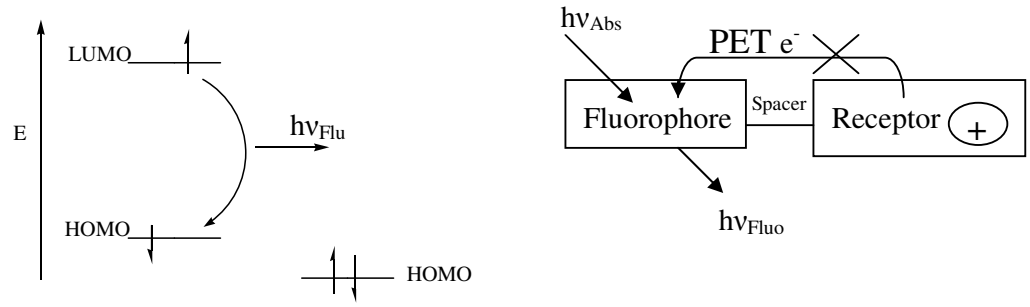

Figure 1.6 Frontier Orbital Energy Diagram and Spaced Fluorophore-Receptor System for "on" (Fluorescence Emission) State [29].

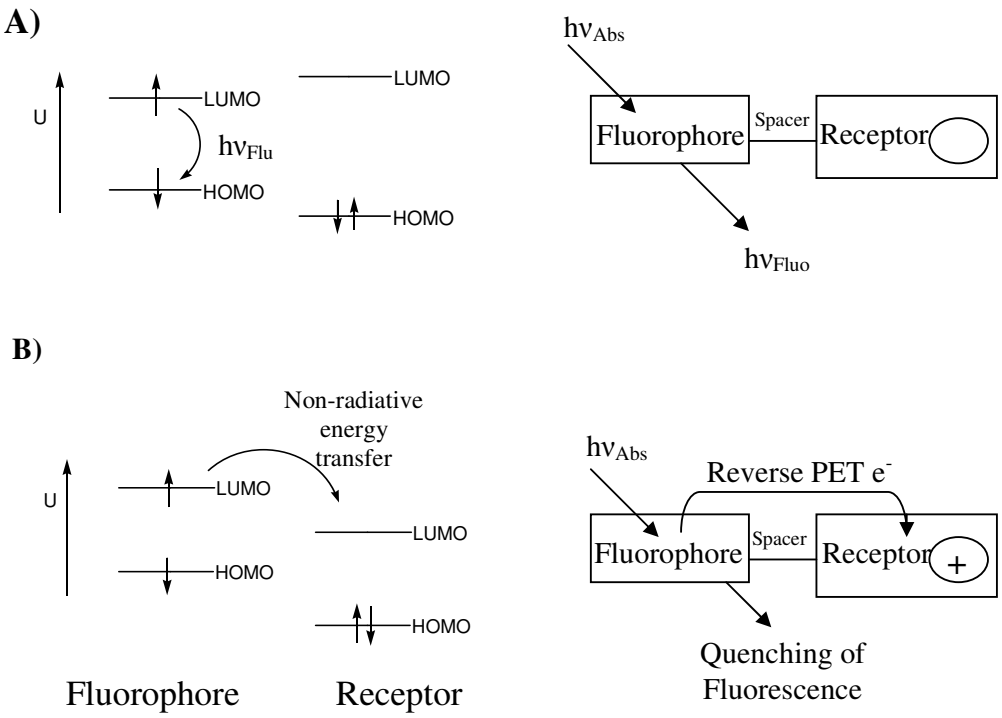

Figure 1.7 Schematic Representation of Reverse PET Mechanism [29].

1.10 Flow Injection Analysis

Ruzicka and Hansen proposed the term "flow injection analysis" (FIA) to describe a procedure in which the on-line formation of a derivative for a spectrophotometric monitoring was made by injecting a discrete volume of sample into a continuously flowing carrier stream containing the reagent [36].

The use of FIA system replaces manual operations like pippetting, dispensing, mixing, and separating; this results in a drastic reduction in the volume of sample and reagent solutions that are required, with

no loss of reproducibility. Thus material and time will be saved, and less energy will be needed [37].

Flow injection technique became a versatile instrumental tool that contributed substantially to the development of automation in clinical, agricultural, pharmaceutical, industrial analysis. [38,39].

1.11 Methods Used for the Determination of Saccharides

The most common analytical methods are based on GC [40] or HPLC [41,42] determinations with either UV–VIS spectrophotometric [43,44] or refractive index [41] detection but often involve tedious sample preparation and derivation. Alternatively, enzyme biosensors are commonly used for the determination of glucose or fructose, based on the reaction of glucose or fructose with enzymes [45]. Though this method has the advantage of the specificity inherent in enzymes, the long-term stability at room temperature can sometimes be poor [5].

In the sugar industry, Lane-Eynon and Luff-Schoorl methods are used for invert sugar determination. These medhods are based on reducing Cu^{+2} to Cu_2O which forms a red precipitate by invert sugar [6]. However, these procedures need a time consuming heating step, before the reducing sugar content determination.

R. P. Sartini et al. reported a flow injection procedure for a gravimetric determination of reducing sugars based on Lane-Eynon method [46]. A. Oliveira described an unsegmented liquid vapor phase FIA system for the determination of reducing sugars by

oxidation with potassium hexacynoferrate in a hot reaction medium and then the excess of oxidant reagent was measured by molecular spectrophotometry [44]. H. L. Thanh developed a method for the rapid determination of sugars in soft drinks by sequential injection FTIR spectrometry [47]. A. Cáceres et al. proposed a continuous spectrophotometric method for the determination of monosaccharides in foods by using methylamine as derivatising reagent [48].

H. Kubo et al. proposed a chemiluminescence flow injection analysis of reducing agents (such as fructose and glucose) based on the luminol reaction [49]. Nicholaos P. Evmiridis et al. described a kinetic method for the determination of glucose and fructose in sample solutions combining flow injection and chemiluminescence (CL) detection based on CL signal generated during oxidation of pyrogallol by periodate [50].

N. D. Danielson et al. reported the fluorimetric FIA method for the determination of fructose and glucose using zirconyl chloride [51].

1.12 Methods Used for the Determination of Dopamine

The most common methodologies used for the determination of dopamine are based on chromatography [16, 17, 19], electrochemistry [52] and amperometry [53].

A chemiluminescence flow injection determination of cathecholamines with electrogenerated hypochlorite in pharmaceutical injections was proposed by Chengxiao Zhang et al. [16]. Lihe Zhang

et al. [54] and Feng Li et al. [55] reported flow injection methods based on the inhibition effect of dopamine on the iron(II)-induced chemiluminescence of lucigenin and on the electrochemiluminescence of ruthenium complexes respectively in pharmaceutical injections.

Wang et al. [23,25] reported fluorimetric determinations of dopamine using methanol as sensitizing reagent and ethylene diamine as the fluorigenic reagent in pharmaceutical products and urine respectively.

1.13 Aim of the Work

Luminescence methods find applications in many analytical studies due to their inherent sensitivity and simplicity. However, the use of fluorescence methods for the determination of monosaccharides and neurotransmitters is rather rare. This fact probably due to the lack of suitable fluorophores for these compounds. Even though considerable effort has been given for the preparation of boronic acid-appended fluorophores that react selectively with carbohydrates and catecholamines within the last decade only few of them are available commercially.

In this study, a fully automated fluorimetric FIA method has been developed utilizing m-dansylaminophenyl boronic acid (DAPB), a PET molecule, for the determination of monosaccarides (fructose, glucose) and neurotransmitters (dopamine, epinephrine and noradrenaline).

The application of the method was investigated for determination of invert sugar in commercial sugar syrups and wine samples.

The proposed method was also applied successfully to the determination of dopamine and epinephrine in pharmaceutical injections.

CHAPTER 2

EXPERIMENTAL

2.1 Chemicals and Reagents

i) m-Dansylaminophenyl boronic acid (DAPB) solution ($2.00x10^{-4}$ M): Prepared by dissolving 3.7 mg DAPB (Fluka) in 1 mL dimethylsulfoxide (DMSO, Labscan) and diluting with deionized water to 50 mL.

ii) Fructose stock solution (0.5 M): Prepared by dissolving 2.25 g fructose (Fluka) in 25 mL phosphate buffer (pH 9.00).

iii) Glucose standart solution (0.5 M): Prepared by dissolving 2.25 g glucose in 25 mL pH 9.00 buffer.

iv) Ascorbic acid solution (0.1 mM): Prepared by dissolving 0.0018 g. ascorbic acid (Merck) in 100 mL deionized water.

v) Dopamine hydrochloride salt stock solution (0.1 M): Prepared by dissolving 0.1896 g of dopamine hydrochloride salt (Fluka) in ascorbic acid solution.

vi) Aspartic acid (Merck), L-cysteine (Merck), alanine (Merck), glycine (Merck), leucine (Merck), norepinephrine (Fluka), epinephrine (Fluka), tyrosine (Merck), tryptophan (Merck), galactose (Merck), sucrose (Merck) solids were used for interference studies.

vii) Dibasic sodium phosphate ($NaH_2PO_4.7H_2O$) (Fisher) and monobasic sodium phosphate ($Na_2HPO_4.H_2O$) (Fisher) solids were used for buffer preparations.

All chemicals were of analytical reagent grade unless stated otherwise. De-ionized water obtained from a Millipore water purification system was used for sample and standard preparations. All the glass and plasticware were soaked in 10% HNO_3 for at least 24 hours and then rinsed with de-ionized water (18 MΩ cm^{-1}).

2.2 Apparatus

Perkin-Elmer LS-50B spectrophotometer was used for all fluorescence measurements. Maximum excitation and emission wavelengths were 324 nm and 529 nm. The emission and excitation slits were positioned at 5 nm and 7.5 nm, respectively.

The spectrophotometer equipped with a Perkin-Elmer flow-through cell (Light Path: 10mmx6.5mm, Cell Volume: 750 µl) was connected to a home-made flow injection analysis (FIA) system for FIA measurements. The fully automated system consisted of two syringe pumps and three distribution valves (5-way). PTFE and FEP tubings of outer diameter (od) 1/32´´- 1/16´´ and inner diameter (id) 0.020´´-1/32´´ were used for all connections throughout the system.

2.3 Spectral Behaviours of m-Dansylaminophenyl Boronic Acid (DAPB)

2.3.1 Selection of the Excitation and Emission Wavelengths for DAPB

In order to select the optimum excitation and emission wavelengths of DAPB, first the fluorescence emission was measured at a fixed wavelength while the excitation wavelength was varied. The excitation wavelength was held constant while scanning the emission wavelength. Maximum excitation and emmission wavelengths of DAPB are found to be 324 nm and 529 nm, respectively.

2.3.2 Effect of pH on the Emission Profile of DAPB

The emission profile of DAPB (3.5×10^{-5}M) was examined in the pH range of 2.00-10.00 by preparing the DAPB solution in phosphate buffer. The buffer compositions used are given in Table 2.1.

2.3.3 Calculation of the First Derivative of Fluorescence Intensity for DAPB

The graphical approach used in the calculations involves a plot of change in the fluorescence intensity of DAPB per unit change in pH ($\Delta F/\Delta pH$) as a function of the average pH of DAPB solution [56]. First derivative of fluorescence intensity ($\Delta F/\Delta pH$) for DAPB was calculated and plotted versus the average of pH.

Table 2.1 Compositions of Buffers

Reagents	Concentration, M	pH
NaH_2PO_4 H_3PO_4	0.0414 0.0586	2.00
NaH_2PO_4 H_3PO_4	0.0876 0.0124	3.00
NaH_2PO_4 H_3PO_4	0.0986 0.0014	4.00
NaH_2PO_4 Na_2HPO_4	0.0993 0.0007	5.00
NaH_2PO_4 Na_2HPO_4	0.0939 0.0061	6.00
NaH_2PO_4 Na_2HPO_4	0.0608 0.0392	7.00
NaH_2PO_4 Na_2HPO_4	0.0134 0.0866	8.00
NaH_2PO_4 Na_2HPO_4	0.0018 0.0982	9.00
Na_2HPO_4 Na_3PO_4	0.0995 0.0005	10.00

2.3.4 Effect of Ionic Strength on the Fluorescence Signal of DAPB

The effect of ionic strength on the fluorescence signal of DAPB was examined by adding KCl in the range of 0.01 M-0.1 M to $3.5x10^{-5}$ M DAPB solutions prepared in pH 9.00 buffer. The fluorescence intensity was measured for each case.

2.3.5 Effect of Diol Containing Compounds (Fructose, Glucose and Dopamine) on the Emission Intensity of DAPB.

In order to examine the effect of diol containing compounds on the emission intensity of DAPB, emission signal of $3.5x10^{-5}$M DAPB solution was measured in the presence of fructose, glucose ($2.00x10^{-4}$ M) and dopamine ($5.00x10^{-5}$M) at the pH range of 4.00-9.00.

2.4 Calibration Studies for Fructose, Glucose and Dopamine

Calibration plots for fructose ($1.00x10^{-4}$M-$1.00x10^{-3}$M), glucose ($1.00x10^{-4}$M-$1.00x10^{-3}$M) and dopamine ($1.00x10^{-5}$M-$1.00x10^{-4}$M) were obtained by using $3.5x10^{-5}$M DAPB. Emission signal of DAPB was measured at 508 nm (λ_{exc}=324 nm, excitation slit=5.00 nm and emission slit=7.5 nm). For having further information about the selectivity of DAPB, sucrose ($1.00x10^{-4}$M-$1.00x10^{-3}$M) and galactose ($1.00x10^{-4}$M-$1.00x10^{-3}$M) were also examined under similar conditions.

2.5 Optimization of DAPB Concentration at Low Fructose Concentrations

DAPB-fructose complexation was examined by varying the concentrations of DAPB and fructose in the range of $1.00x10^{-6}$M-$7.00x10^{-5}$M and $1.00x10^{-5}$M-$1.00x10^{-4}$M, respectively. Emission signals were obtained at 508 nm (λ_{exc}: 324 nm) by changing the excitation and emission slit widths between 5.0 nm and 15.0 nm.

2.6 Interference Studies for Fructose, Glucose and Dopamine

For fructose, the expected interferences from amino acids ($6.00x10^{-3}$ M), maltose ($1.5x10^{-3}$M), sucrose (0.08 M), glucose (0.01 M), MgO (0.01 M),CaO ($9.00x10^{-3}$ M) and saponine ($3.00x10^{-3}$M) were studied.

Maltose($3.00x10^{-3}$M-$1.5x10^{-2}$M), glycerol ($3.00x10^{-3}$M) and ethanol ($6.00x10^{-3}$M) were studied as potential interferants for glucose.

The interference effects of glycine (0.385 M), alanine (0.05 M), leucine (0.015M), tyrosine (0.03 M), tryptophan (0.03 M), cysteine ($6.00x10^{-8}$ M-0.06 M), epinephrine ($9.00x10^{-6}$M), norepinephrine ($3.45x10^{-5}$M), ascorbic acid (0.02 M), citric acid ($9.00x10^{-7}$M) and glucose (0.07 M) were studied for dopamine.

The fluorescence measurements were performed both in the presence and absence of these potential interferants.

2.7 Preparation of Sample Solutions

2.7.1 Preparation of Sample Solutions for the Determination of Glucose and Fructose in their Commercial Sugar Syrups

Commercial fructose syrups, consisting of fructose and glucose are labelled as F42 or F55 according to their fructose content. Sample solutions of F42 and F55 (Cargill Products) syrups were diluted 500 and 1000-fold with pH 9.00 phosphate buffer. For glucose determination, a glucose syrup labelled as 01612 Nişkoz Reks (Pendik Nişasta Product) was utilized and the sample solution was diluted 10-fold with pH 9.00 phosphate buffer. Content of commercial syrups are given in Table 2.2

Table 2.2 Content of Commercial Syrups

Sugar Syrups	**Density ($g\ mL^{-1}$)**	**Dry Matter Substance (w/w) %**	**Fructose Content (w/w) %**	**Glucose Content (w/w) %**	**Maltose Content (w/w) %**
F42 Syrup	1.3474	71.37	42.5	54.0	2.1
F55 Syrup	1.4896	72.5	55.0	44.0	1.0
01612 Nişkoz reks	1.4264	81	—	32	68

2.7.2 Preparation of Sample Solutions for the Determination of Dopamine and Epinephrine in their Pharmaceutical Injections

Two pharmaceutical injections; Solvay Pharmaceuticals and Drogsan Products, were examined for their dopamine content by preparing the sample solutions in 0.1 mM ascorbic acid by 500-fold dilution.

For epinephrine determination, Drogsan and Biofarma Products injections were examined and the sample solutions were prepared in 0.1 mM ascorbic acid by 10-fold dilution.

2.8 Invert Sugar Determination in Wine by Lane-Eynon Method

2.8.1 Preparation of Solutions Used for Lane-Eynon Method

Methylene Blue Solution: 1 g of methylene blue was dissolved in deionized water and diluted to 100 mL.

Sodium Hydroxide Solution (1.00 M): 4 g of sodium hydroxide was dissolved in deionized water and made up to 100 mL.

Hydrochloric Acid Solution (0.5 M): 4.45 mL of concentrated HCl solution was diluted with deionized water to 100 mL.

Phenolphthalein Solution: 1 g of phenolphthalein was dissolved in 60 mL of ethanol (96%) and diluted to 100 mL with deionized water.

Standard Invert Sugar Solution (1% w/v): 0.95 g of sucrose was dissolved in 10 mL deionized water. 5 mL of concentrated HCl was added to this solution and this mixture was allowed to stand for 3 days at 20-25 °C for inversion of sucrose and again diluted with deionized water to 80 mL.

Benzoic Acid Solution: 0.2 g of benzoic acid was dissolved in 7.5 mL of hot water and added to the standard invert solution stated above. The volume of the mixture was made up to 100 mL with deionized water.

Diluted Invert Sugar Solution: 25 mL of standard invert solution was transferred into a 100 mL volumetric flask and two drops of phenolphthalein was added to invert solution. 1 M NaOH solution was added dropwise until a pink colour developed. By the addition of one or two drops of 0.5 M HCl, the pink colour of invert solution was discharged and finally volume of the this solution was made up to 100 mL with deionized water.

Copper Solution (Solution A): 6.928 g of cupric sulphate pentahydrate was dissolved in deionized water and the volume was made up to 100 mL.

Alkaline Tartrate Solution (Solution B): 34.6 g of potassium sodium tartrate and 10 g of sodium hydroxide were dissolved in sufficient volume of deionized water separately. These solutions were mixed and the mixture was diluted to 100 mL with deionized water.

Mixed Fehling's Solution: Equal volumes of Solution A and Solution B were mixed together and stored in a suitable container.

Preparation of Wine Sample: 25 mL of wine was transferred into a 50 mL volumetric flask and the rest was as described in the preparation of diluted invert solution. Finally, the sample volume was made up to 50 mL with deionized water.

2.8.2 Application of Lane-Eynon Method for the Determination of Invert Sugar in Wine

5 mL of Fehling solution, 15 mL of standard invert sugar or wine sample solution and 3.75 mL of deionized water were mixed in a boiling flask. This solution was boiled precisely for 2 minutes and two drops of methylene blue was added to the boiling mixture. Invert sugar solution or wine sample was added to the boiling solution in small increments. At the end point, blue colour of boiling solution turning into red indicates the occurence of cuprous oxide.

2.9 Flow Injection Analysis System Applications

For the determination of fructose, glucose or dopamine, a 5.00 mL syringe pump was chosen and the two five-way distribution valves were used for all connections. FIA (Figure 2.1) system was connected to the 750 µL flow through cell. Dispense volumes were selected as 775 µL for DAPB (5.00×10^{-5}M) and 80 µL for fructose and dopamine. When the injections were done the volume was adjusted to fill the

tubing (75 µL for DAPB and 30µL for sample) and the cell (700 µL for DAPB and 50 µL for sample)

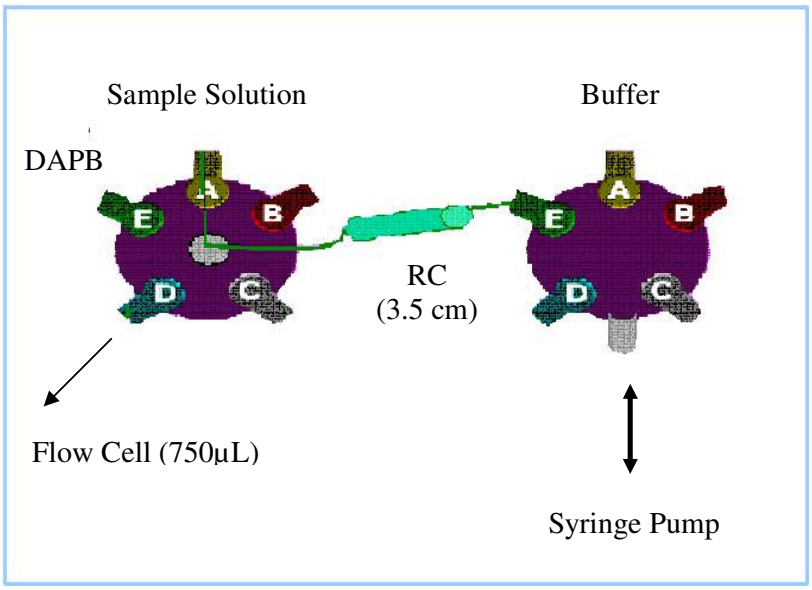

Figure 2.1 Schematic Representation of the Flow Injection System. RC: Reaction Coil (3.5 cm). Capital letters indicate the ports.

Before each measurement cycle, a pre-washing step was applied and 5.00 mL phosphate buffer (pH 9.00) was dispensed through the system.The experimental procedure used is summarized below:

- At the beginning of each measurement cycle, a pre-washing step was applied by aspirating 5.00 mL of phosphate buffer through the flow path.

- 775 µL of DAPB and 5.00 mL of phosphate buffer were aspirated sequentially through the flow cell to measure the fluorescence intensity of DAPB.

- 775 µL of DAPB and 80 µL of sample solutions were sequentially aspirated through the reaction coil (3.5 cm). Then, the flow was reversed and the mixture was driven through the flow cell by 5.00 mL of phosphate buffer and the alteration of fluorescence intensity of DAPB was measured.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Spectral Behaviours of DAPB

3.1.1 Emission and Excitation Spectra of DAPB

Maximum excitation and emission wavelengths of DAPB were found to be 324 nm and 529 nm, respectively. The excitation and emission spectra of DAPB are shown in Figure 3.1.

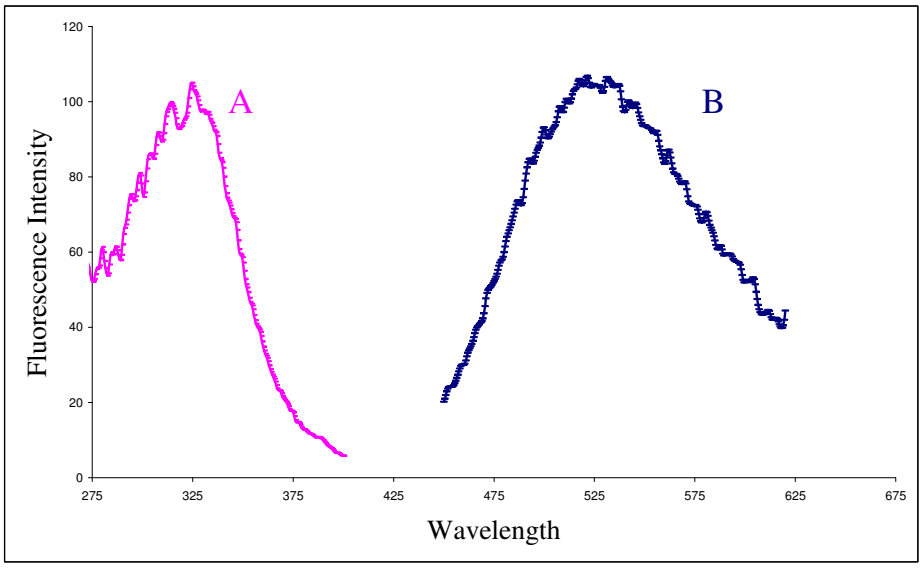

Figure 3.1 Excitation (A) and Emission (B) Profile of DAPB (3.5×10^{-5} M), using 529 nm emission and 324 nm excitation.

3.1.2 Effect of pH on the Emission Intensity of DAPB

The luminescence behaviour of $3.5x10^{-5}$ M DAPB was examined at the pH range of 2.00-10.00 by the use of phosphate buffers. Figure 3.2 corresponds to the fluorimetric titration of the acidic hydrogen's of boronic acid group. As can be seen from the figure, the fluorescence intensity of DAPB is very low at acidic pH values; starts to increase after pH 7.00, becomes maximum at pH 9.00 and starts to decrease afterwards. In other words, the fluorescence signal is enhanced in alkaline conditions. This is probably due to the loss of electron withdrawing properties of the boronic acid group, following the formation of boranate, which diminishes the tendency for the nitrogen lone pair electrons to be donated to the open shell of boron through naphthalene moiety. Hence quenching is prevented in basic medium.

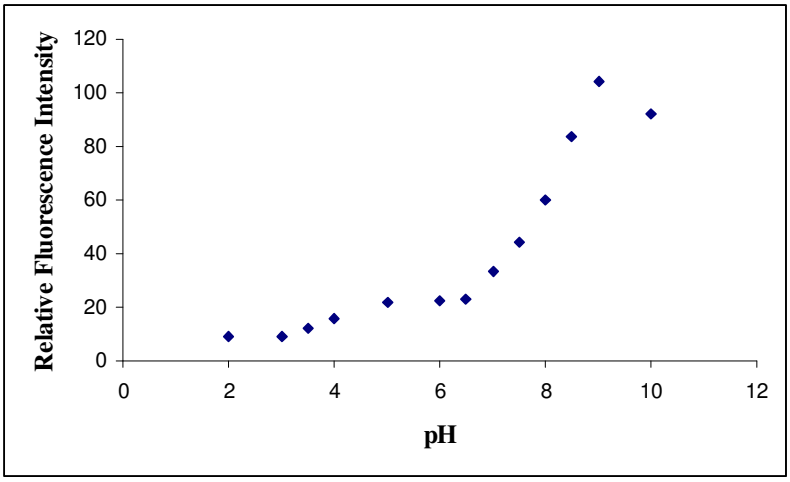

Figure 3.2 Influence of pH on the Emission Intensities of 3.5×10^{-5} M DAPB in 1% (v/v) Dimethylsulfoxide (DMSO). (λ_{exc}= 324 nm, λ_{em}= 529 nm exc. slit= 5.00 nm and em. slit= 7.5 nm)

Figure 3.3 displays the first derivative of the titration curve. As can be seen from the figure, end point pH values are located at 3.50 and 8.07.

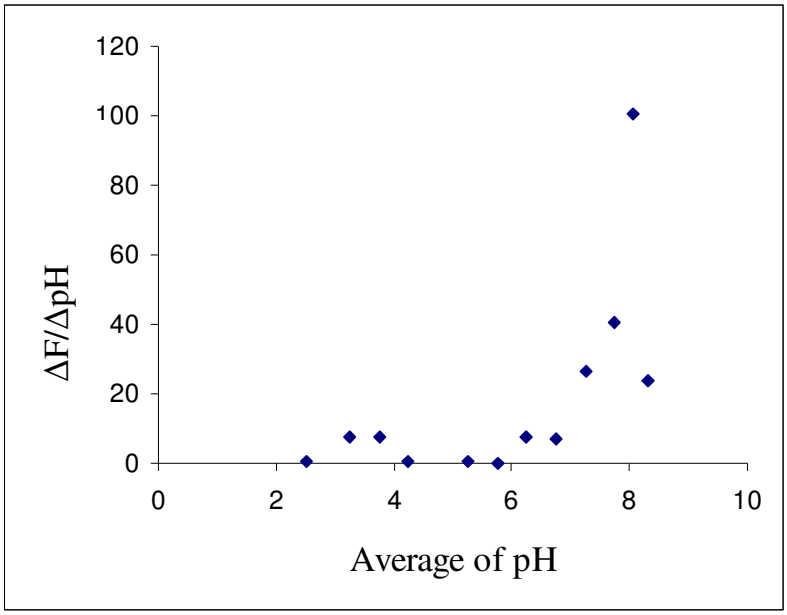

Figure 3.3 First Derivative of the Fluorescence Intensity of DAPB (ΔF/Δ pH) vs pH. (λ_{exc}= 324 nm, λ_{em}= 529 nm exc. slit= 5.00 nm and em. slit= 7.5 nm)

3.1.3 Effect of Ionic Strength on the Fluorescence Signal of DAPB

In order to check the effect of ionic strength on the emission profile of DAPB, pH 9.00 buffer solutions containing varying concentrations of KCl (0.01 M-0.1 M) were used in the fluorescence measurements. Results demonstrated that the ionic strength did not significantly affect the fluorescence intensity of DAPB at pH 9.00.

3.1.4 Effect of Diol Containing Compounds (Fructose, Glucose, Dopamine) on the Emission Intensity of DAPB

Fructose, glucose and dopamine are all diol containing compounds (diol). They give complexes with boronic acids. In order to examine the influence of DAPB-diol complexation on the emission intensity of DAPB, the fluorimetric titration of $3.5x10^{-5}$ M DAPB was repeated in the presence of $2.00x10^{-4}$ M fructose and glucose, and $5.00x10^{-5}$ M dopamine in the pH range 4.00-9.00.

As can be seen from Figure 3.4 and 3.5, the fluorescence intensities of DAPB-diol complexes and free DAPB are almost the same up to pH 8.00. At higher pH values, on the other hand, fluorescence intensities of DAPB decrease upon binding with diol containing compounds.

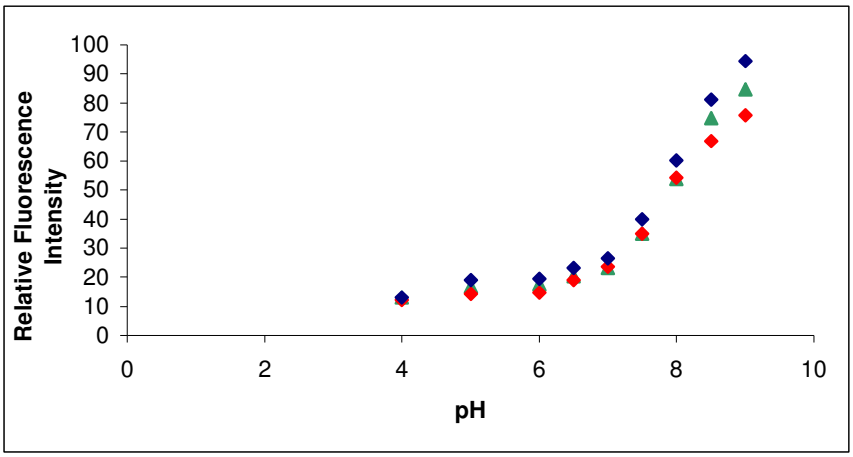

Figure 3.4 Fluorescence Intensity versus pH Profile of 3.5×10^{-5} M DAPB alone (♦), in the Presence of $2.00x10^{-4}$ M Fructose (♦), and in the Presence of $2.00x10^{-4}$ M Glucose (▲). ($\lambda_{exc.}$= 324 nm, λ_{em}= 529 nm exc. slit= 5.00 nm and em. slit= 7.5 nm)

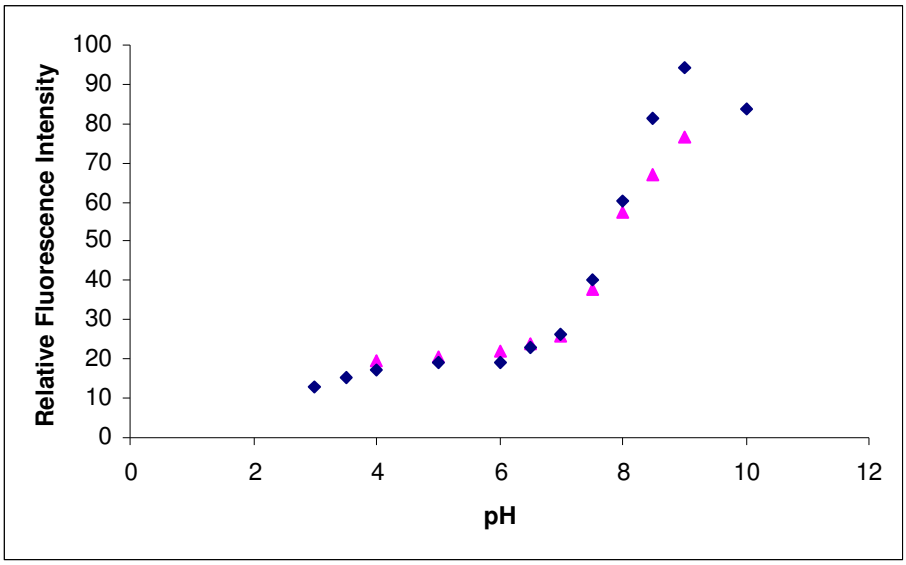

Figure 3.5 Fluorescence Intensity versus pH of 3.5×10^{-5} M DAPB alone (♦), in the Presence of $5.00\text{x}10^{-5}$ M Dopamine (▲). (λ_{exc}= 324 nm, λ_{em}= 529 nm exc. slit= 5.00 nm and em. slit= 7.5 nm)

Both the fluorescence intensity of pure DAPB and the degree of quenching of DAPB in the presence of diol containing compounds are reaching their maximum values at pH 9.00. Hence, in all fluorescence measurements the pH of the solutions was adjusted to 9.00, using a phosphate buffer.

The decrease in fluorescence intensity upon binding indicates an oxidative PET mechanism in DAPB-diol complex in which an inverse photo induced electron transfer occurs. Figure 3.6 provides a summary of this fact in terms of frontier orbital energies. Most probably, the fluorescence intensity of the naphthalene moiety is decreased with the formation of an ester with a cis diol of the molecule because of the increased acidity of the boron atom after the ester formation. This

increases the tendency for the lone-pair electrons of the nitrogens to be donated to the open shell of boron and through this reverse photoinduced electron transfer (PET) and fluorescence of the naphthalene moiety is quenched.

Thus, complex formation will activate the quenching mechanism and therefore, decreases the fluorescence intensity.

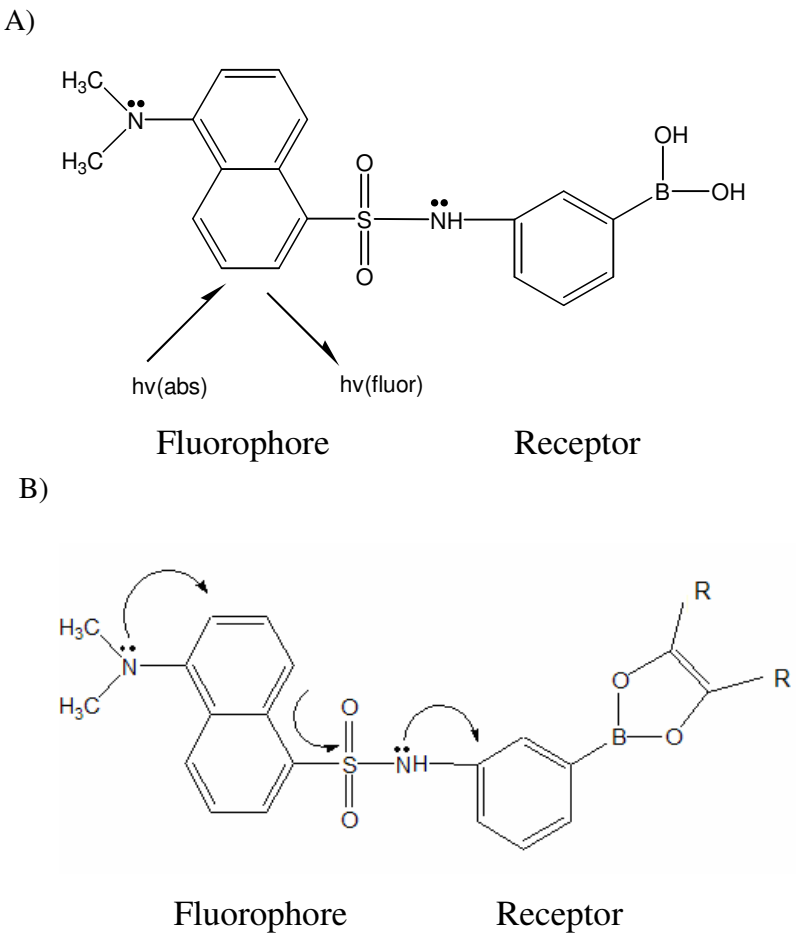

Figure 3.6 Schematic Representation of Reverse PET Mechanism.

3.2 Determination of Carbohydrates

3.2.1 Calibration Data for Fructose

Fructose determination is based on the quenching of the DAPB fluorescence signal due to the reaction of fructose with boronic acid group.

In order to see the change in the quenching of DAPB at different concentrations of fructose, DAPB concentration was kept constant at $3.5x10^{-5}$ M while fructose concentration was varied in the range of $1.00x10^{-4}$ M-$1.00x10^{-3}$ M and the fluorescence spectra of DAPB were recorded as shown in Figure 3.7.

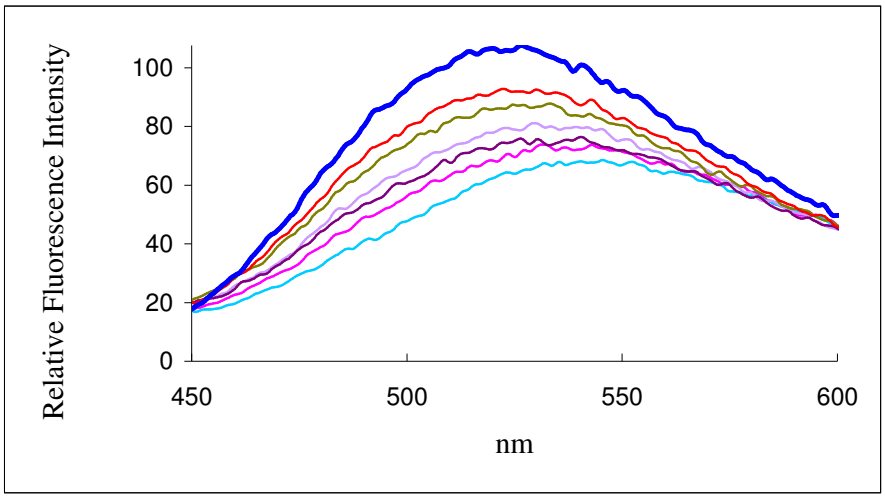

Figure 3.7 Fluorescence Spectra of $3.5x10^{-5}$ M [a] DAPB at Various Fructose Concentrations; [b], $1.00x10^{-4}$ M; [c], $2.00x10^{-4}$ M; [d], $4.00x10^{-4}$ M; [e], $6.00x10^{-4}$ M; [f], $8.00x10^{-4}$ M; [g], $1.00x10^{-3}$ M. ($\lambda_{exc.}$= 324 nm, exc. slit= 5.00 nm and em. slit= 7.5 nm)

In Figure 3.7, blue color indicates the fluorescence spectrum of DAPB alone. Fructose concentrations lower then $1.00x10^{-4}$M did not make any distinguishable quenching in the fluorescence of $3.5x10^{-5}$ M DAPB.

As can be seen from Figure 3.7, a red shift is observed at fructose concentrations higher than $4.00x10^{-4}$ M. Besides, above this concentration the spectra start to overlap with each other. Due to this fact, absolute quenching values (I_{DAPB}-$I_{Fructose}$) of DAPB corresponding to different concentrations of fructose ($1.00x10^{-4}$ M-$1.00x10^{-3}$ M) are higher at 508 nm as compared to at 529 nm which was the peak maximum. Hence, Stern-Volmer calibration plot was constructed by following the quenching of $3.5x10^{-5}$ M DAPB at 508 nm instead of the peak maximum (529 nm).

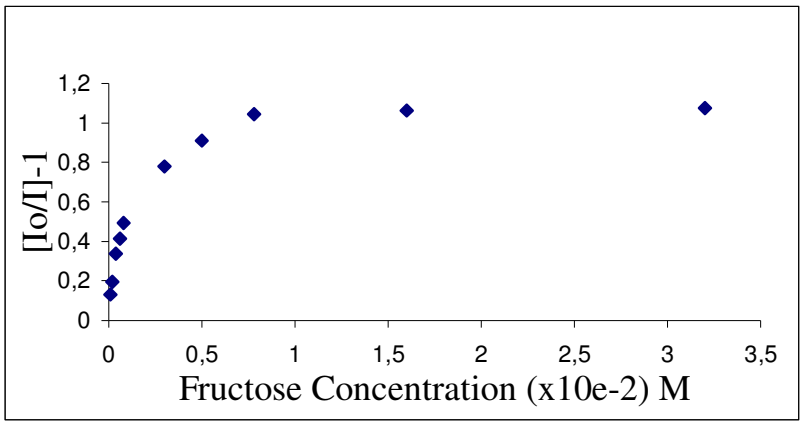

Figure 3.8 Stern-Volmer Plot for DAPB in the Presence of Fructose (1.00×10^{-4} M-3.2×10^{-2} M), Io=Fluorescence Signal of the DAPB, I=Fluorescence Signal of the DAPB in the Presence of Fructose as Quencher. ($\lambda_{exc.}$= 324 nm, $\lambda_{em.}$= 508 nm, exc. slit= 5.00 nm and em. slit= 7.5 nm)

As can be seen from Figure 3.8, the quenching is linear up to fructose concentration of $1.00x10^{-3}$ M. At higher fructose concentrations, a curvature and a plateau region are observed.

3.2.2 Optimization of DAPB Concentration at Low Fructose Concentrations

The studies given in section 2.5 have shown that the lowest fructose concentration that can be measured was $1.00x10^{-4}$ M when $3.5x10^{-5}$ M DAPB was used in the fluorescence measurements.

In order to be able to detect lower concentrations of fructose the concentration of DAPB was optimized thoroughly. DAPB

concentrations ($1.00x10^{-6}$ M-$7.00x10^{-5}$ M) and fructose concentrations examined for each DAPB concentration are given in Table 3.1. The third column of the Table 3.1 states whether the quenching corresponding to the given fructose concentration was observable or not. In order to enhance the signal, for DAPB concentrations lower than $3.5x10^{-5}$ M, excitation and emission slits were set at 15 nm and for concentrations higher than $3.5x10^{-5}$ M, fluorescence intensities of DAPB ($5.00x10^{-5}$ M and $7.00x10^{-5}$ M) were measured at excitation slit of 5.0 nm and emission slit of 7.5 nm.

Table 3.1 Optimization of DAPB Concentration for Low Fructose Concentrations

DAPB Concentration, M	Fructose Concentration, M	Quenching
$1.00x10^{-6}$	$1.00x10^{-5}$	Not Detectable
	$5.00x10^{-5}$	Not Detectable
	$1.00x10^{-4}$	Detectable
$5.00x10^{-6}$	$1.00x10^{-5}$	Not Detectable
	$5.00x10^{-5}$	Not Detectable
	$1.00x10^{-4}$	Detectable
$7.00x10^{-6}$	$1.00x10^{-5}$	Not Detectable
	$5.00x10^{-5}$	Not Detectable
	$1.00x10^{-4}$	Detectable
$1.00x\ 10^{-5}$	$1.00x10^{-5}$	Not Detectable
	$5.00x10^{-5}$	Not Detectable
	$1.00x10^{-4}$	Detectable
$3.5x10^{-5}$	$1.00x10^{-5}$	Not Detectable
	$5.00x10^{-5}$	Not Detectable
	$1.00x10^{-4}$	Detectable
$5.00x10^{-5}$	$1.00x10^{-5}$	Not Detectable
	$5.00x10^{-5}$	Not Detectable
	$1.00x10^{-4}$	Detectable
$7.00x10^{-5}$	$1.00x10^{-5}$	Not Detectable
	$5.00x10^{-5}$	Not Detectable
	$1.00x10^{-4}$	Detectable

As can be seen from the Table 3.1 quenching was not observed for fructose concentrations less than $1.00x10^{-4}$ M at the stated conditions. Therefore, $3.5x10^{-5}$M DAPB was decided to be used as fluorophore throughout this study.

The linear region of Stern-Volmer graph corresponding to 1.00×10^{-4} M-$1.00x10^{-3}$ M fructose concentrations is displayed in Figure 3.9.

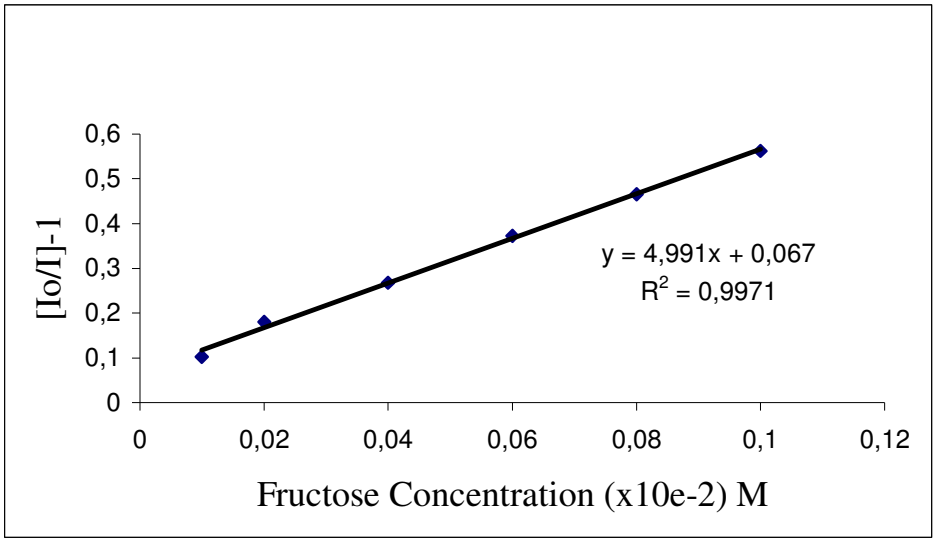

Figure 3.9 Calibration Line for Fructose ($1.0x10^{-4}$ M-$1.0x10^{-3}$ M), Io=Fluorescence Intensity of DAPB, Io=Fluorescence Signal of DAPB, I=Fluorescence Signal of DAPB in the Presence of Fructose as Quencher. ($\lambda_{exc.}$= 324 nm, $\lambda_{em.}$= 508 nm, exc. slit= 5.00 nm and em. slit= 7.5 nm)

The detection limit of the system, based on 3s, was $2.3x10^{-5}$ M. However, experimentally it was not possible to measure the fructose

concentration less than $1.00x10^{-4}$ M. Hence, $1.00x10^{-4}$ M fructose concentration was considered as limit of quantitation.

DAPB could also recognize other sugars such as glucose, galactose and sucrose in their aqueous solutions. Figure 3.10 shows the calibration lines obtained for glucose, galactose and sucrose.

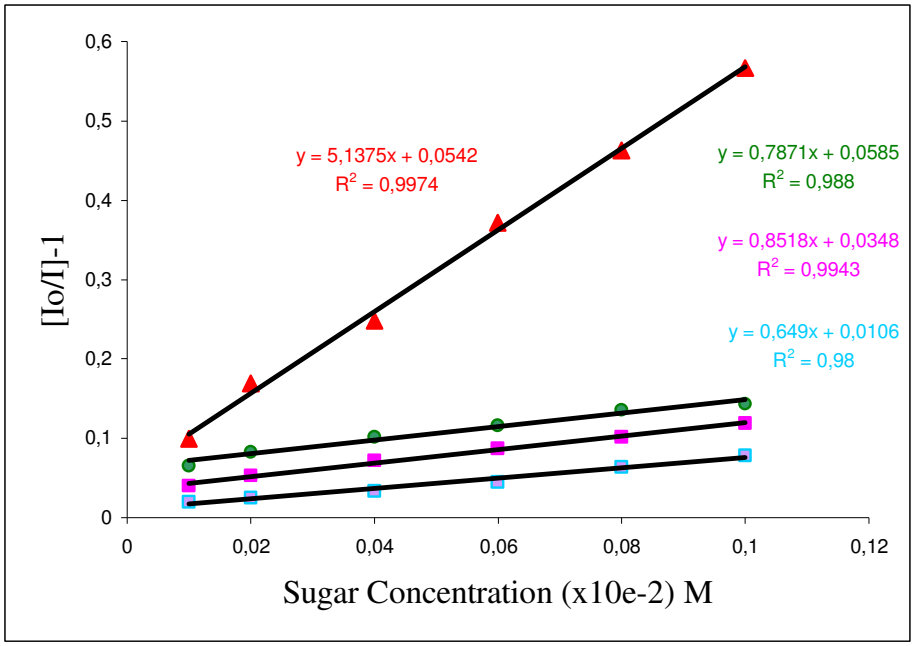

Figure 3.10 Calibration Lines for Fructose ($1.00x10^{-4}$ M-$1.00x10^{-3}$ M) (▲), Galactose ($1.00x10^{-4}$ M-$1.00x10^{-3}$ M) (●), Glucose ($1.00x10^{-4}$ M-$1.00x10^{-3}$ M) (■), Sucrose ($1.00x10^{-4}$ M-$1.00x10^{-3}$ M) (■), Io=Fluorescence signal of the DAPB, I=Fluorescence Signal of the DAPB in the Presence of Quencher (Fructose, Galactose, Glucose and Sucrose). ($\lambda_{exc.}$= 324 nm, $\lambda_{em.}$= 508 nm, exc. slit= 5.00 nm and em. slit= 7.5 nm)

It was seen that the highest sensitivity was obtained for fructose compared to that of glucose, galactose and sucrose. It has been reported [28] that the effect of spacer length on PET chemicals is an important factor influencing the specificity of the supramolecule to particular saccharide. As the spacer length is increased, receptor parts on PET molecules show tendency to bind higher-membered saccharides than that of lower-membered rings. Fructose is a five membered ring whereas glucose and galactose are six-membered rings. Sucrose is composed of glucose and fructose units. In m-dansylaminophenyl boronic acid, the spacer is the sulphonamide group. Most probably, due to the short length of the spacer, DAPB is more sensitive to fructose compared to glucose and galactose. Because of the two cyclic rings of sucrose, DAPB binds to sucrose hardly, thus the sensitivity of DAPB to sucrose was the lowest among them.

3.2.3 Interference Studies for Fructose Determination

In the sugar industry, amino acids, maltose, sucrose, MgO, CaO and saponine are expected as interfering species for the determination of fructose in the course of process control. Commercial fructose syrups (F42 and F55) include fructose, glucose and maltose.

The interfering effects of glucose, sucrose, maltose, amino acids (Glycine, Alanine, Leucine, Tyrosine, Tryptophan, Cysteine), MgO, CaO and saponine were examined for the determination of fructose. The results and the concentrations of the interfering species [I] relative to the fructose [F] concentration (3.00×10^{-3} M) are given in Table 3.2.

Table 3.2 Results of Interference Studies for Fructose Determination

Interfering Species [I]	**Concentrations of [I]**	**Interference Effect**
Glycine	2x[F]*	None
Alanine	2x[F]*	None
Leucine	2x[F]*	None
Tryptophan	2x[F]*	None
Tyrosine	2x[F]*	None
Cysteine	2x[F]*	None
Maltose	0.5x[F]	None
Sucrose	27x[F]	None
Glucose	1x[F] 4x[F]	None None
MgO	4x[F]	None
CaO	3x[F]	None
Saponine	1x[F]	None

*Fructose [F] concentration: 3.00×10^{-3} M.

Experiments demonstrated that, fluorescence signal of fructose was not affected by the presence of the interfering species. The stated concentrations were selected taking into account of their maximum levels in the course of fructose process control. Thus, it was concluded

that these species at the stated concentrations did not produce any interference effect on the determination of fructose.

3.2.4 Determination of Fructose in Commercial Fructose Syrups

There is a considerable demand for the determination of fructose and glucose for quality control, nutritional purposes and for monitoring the industrial process.

Table 3.3 Commercial Fructose Syrups Analysis

Sample	Fructose Found /g per 100 g		$\pm ts/N^{0.5}$ Values
	Reference Value	Proposed Method	
F42 Commercial Fructose Syrup	42	41.9±1.6 (n=3)	3.90
F55 Commercial Fructose Syrup	55	56.3±1.4 (n=3)	3.32
Standard Invert Sugar Solution	0.25	0.25±0.006 (n=3)	0.016

Student- t test. If $\pm ts/N^{0.5}$> absolute difference of the results compared, there is no significant difference at the given confidence level.

Student-t test results at 95 % confidence level have shown that there was no difference between the results of the proposed method and the reported results of the standard samples (Table 3.3). Hence our method can safely be applied for the determination of fructose in the commercial fructose syrups.

3.2.5 Calibration Data for Glucose

The linear region of Stern-Volmer graph corresponding to 1.00×10^{-4} M-$1x10^{-3}$ M glucose concentrations is given in Figure 3.11.

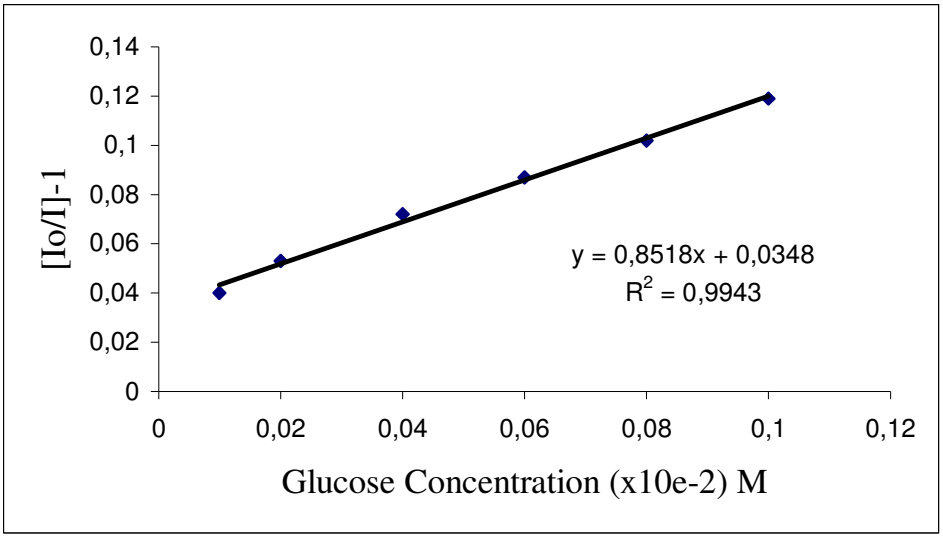

Figure 3.11 Calibration Line for Glucose ($1.0x10^{-4}$ M-$1.0x10^{-3}$ M), Io=Fluorescence Signal of the DAPB, I=Fluorescence Signal of the DAPB in the Presence of Glucose as Quencher. (λ_{exc}= 324 nm, $\lambda_{em.}$= 508 nm, exc. slit= 5.00 nm and em. slit= 7.5 nm)

3.2.6 Interference Studies for Glucose Determination

Commercial glucose syrups include glucose, maltose and maltose polymers. Glycerol and ethanol were also expected to be present in various glucose containing samples. So maltose, glycerol and ethanol were studied as potential interferants to glucose. The results and the concentrations of the interfering species [I] relative to the glucose [G] concentration of 3.00×10^{-3} M are given in Table 3.4.

Table 3.4 Results of Interference Studies for Glucose Determination

Interfering Species [I]	**Concentrations of [I]**	**Interference Effect**
Maltose	1x[G]*	None
	5x[G]	None
Glycerol	1x[G]	None
Ethanol	2x[G]	None

*Glucose [G] concentration: 3.00×10^{-3} M.

Experiments demonstrated that, fluorescence signal of glucose was not affected by the presence of the interfering species. Thus, it was concluded that these species at the stated concentrations (which were maximum levels in various glucose containing samples) did not produce any interference effects on the determination of glucose.

3.2.7 Determination of Glucose in Commercial Glucose Syrup

Table 3.5 shows the results of commercial glucose syrup analysis.

Table 3.5. Commercial Glucose Syrup Analysis

Sample	Glucose found/ g per 100 g		$\pm ts/N^{0.5}$ Values
	Reference Value (w/w)%	Proposed Method (w/w)%	
01612 Nişkoz Reks, Commercial Glucose Syrup	32	32.7±1.4 (n=3)	3.42
Standard Invert Sugar Solution	0.25	0.244±0.003 (n=3)	0.016

Student- t test. If $\pm ts/N^{0.5}$> absolute difference of the results compared, there is no significant difference at the given confidence level.

Student-t test results at 95 percent confidence level have shown that there were no difference between the results of the proposed method and the reported results of the standard samples (Table 3.5). Hence, our method can safely be applied for the determination of glucose in the commercial glucose syrups.

3.2.8 Invert Sugar Determination in Wine Samples

Invert sugar determination in wine is important for routine quality control to follow the fermentation process and also to check the conformity to the requirements for certain types of wine.

In sugar industry, Lane-Eynon and Luff-Schoorl methods are used for invert sugar determination. These methods are based on reducing Cu^{+2} to Cu_2O which forms a red precipitate by invert sugar [6].

Six wine samples were analyzed for their invert sugar content. They were not standard wine samples. Hence, to validate the accuracy of the proposed method for the invert sugar determination in wines, Lane-Eynon titration method was selected as an independent analysis method. Table 3.6 gives percent invert sugar values in wine samples obtained by these two methods.

Invert sugar contents of wines obtained by two methods were compared according to *t*-test at 95 % confidence level. Table 3.7 gives the outcomes of the statistical calculations.

As can be seen from Table 3.7, at the 95% confidence level, no significant differences have been established between the results of two independent methods.

These results demonstrated that our method is a very good candidate for the routine determination of invert sugars in wine samples.

Table 3.6 Percent Invert Sugar Values in Wine Samples Obtained by Lane-Eynon and Proposed Methods

Wine Sample	Lane-Eynon Method Invert Sugar %(w/v) (g per100 mL) (n=3)	Proposed Method Invert Sugar %(w/v) (g per 100 mL) (n=3)
Doluca White Dry Wine	0.222±0.005	0.265±0.047
Sevilen/Altıntepe White Dry Wine	0.240±0.021	0.230±0.021
Pamukkale/Senfoni White Dry Wine	0.206±0.006	0.226±0.013
Sevilen/Tellibağ White Dry Wine	0.232±0.005	0.254±0.015
Kavaklıdere/Çankaya White Dry Wine	0.198±0.02	0.20±0.03
Doluca/Safir White Dömisek Wine	2.77±0.04	2.74±0.01

Table 3.7 The Outcomes of the Statistical Calculations for the Results Given in Table 3.6

Wine Sample	S_{pooled}	X_p-X_{LE}	Calculated Values at 95% CL	Significant Difference
Doluca White Dry Wine	0.0336	0.043	0.076	No
Sevilen/Altıntepe White Dry Wine	0.0211	0.017	0.048	No
Pamukkale/Senfoni White Dry Wine	0.0478	0.020	0.023	No
Sevilen/Tellibağ White Dry Wine	0.0112	0.022	0.025	No
Kavaklıdere/Çankaya White Dry Wine	0.0254	0.002	0.047	No
Doluca/Safir White Dömisek Wine	0.030	0.030	0.068	No

X_p; Mean of the percent invert sugar values obtained with proposed method.

X_{LE}; Mean of the percent invert sugar values obtained with Lane-Eynon titrimetric method.

$N_P=N_{LE}=3$, no of replicates.

For the calculations following formulas were used [56];

$$S_{pooled}=\{[\Sigma(X_i-X_p)^2/(N_P-1)+\Sigma(X_j-X_{LE})^2/(N_{LE}-1)]/(N_P+N_{LE}-2)\}^{1/2}$$

Degree of freedom= $N_P+N_{LE+}-2$

If $X_P - X_{LE} < \pm ts_{pooled} [(N_P+N_{LE})/ N_P N_{LE}]^{1/2}$ there is no significant difference at the given confidence level.

3.3 Determination of Catecholamines

3.3.1 Calibration Data for Dopamine

In order to see the change in the quenching of DAPB at different concentrations of dopamine, DAPB concentration was kept constant at $3.5x10^{-5}$M while dopamine concentration was varied in the range of $1.00x10^{-5}$M-$1.00x10^{-4}$M and the fluorescence spectra of DAPB were recorded as shown in Figure 3.12.

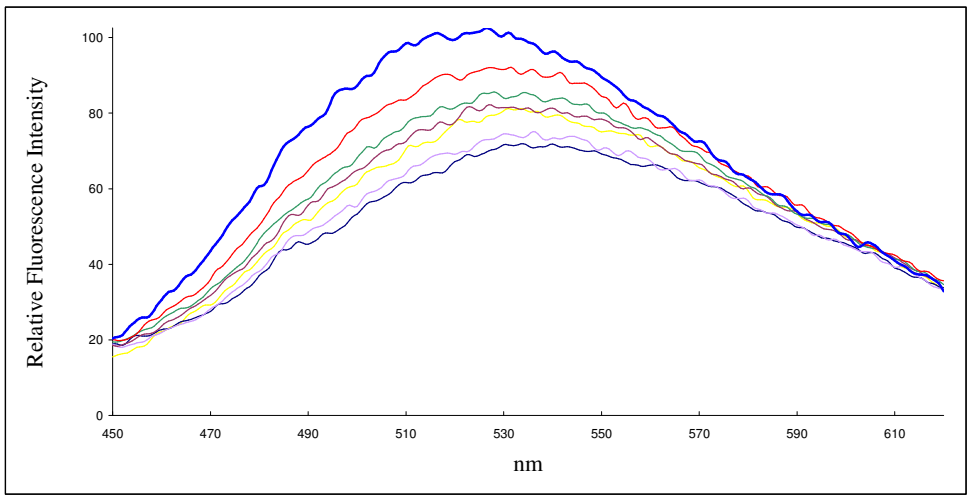

Figure 3.12 Fluorescence Spectra of $3.5x10^{-5}$ M DAPB [a] at Various Dopamine Concentrations; [b], $1.00x10^{-5}$ M; [c], $2.00x10^{-5}$ M; [d], $4.00x10^{-5}$ M; [e], $6.00x10^{-5}$ M; [f], $8.00x10^{-5}$ M; [g], $1.00x10^{-4}$ M. ($\lambda_{exc.}$=324 nm, exc. slit=5.00 nm and em. slit=7.5 nm)

The linear region of Stern-Volmer graph corresponding to 1.00×10^{-5} M-$1.00\text{x}10^{-4}$ M dopamine concentrations is shown in Figure 3.13.

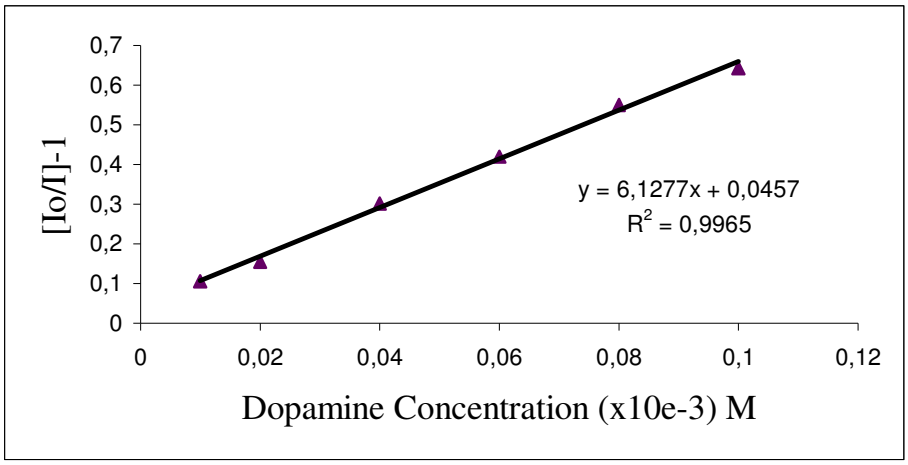

Figure 3.13 Calibration Line for Dopamine ($1.00\text{x}10^{-5}$ M-$1.00\text{x}10^{-4}$ M), Io=Fluorescence Signal of the DAPB, I=Fluorescence Signal of the DAPB in the Presence of Dopamine as Quencher. ($\lambda_{exc.}$=324 nm, $\lambda_{em.}$=508 nm, exc. slit=5.00 nm and em. slit=7.5 nm)

Detection limit based on 3s was $3.7\text{x}10^{-6}$ M. The percent relative standard deviation was 1.2 for 10 replicates of $1.00\text{x}10^{-5}$ M dopamine standard solution.

3.3.2 Interference Studies for Dopamine Determination

Pharmaceutical dopamine hydrochloride injections include cystine and citric acid as the stabilizing agents. Interference effects of these additives and several other substances such as cathecholamines (noradrenaline and adrenaline), glucose, amino acids, which are

expected to be present in urine, were thoroughly examined. For that purpose the quenching effect of dopamine in the presence (ΔI_i) and absence (ΔI_d) of interferant were measured and percent interference effect was calculated by taking the ratio of (ΔI_i) to (ΔI_d) ($\Delta I_i/\Delta I_d$ x100). The interferant to analyte ratios used in Table 3.8 are similar to the ratios of these substances to dopamine present in the pharmaceutical injections and normal urine samples.

Table 3.8 Interference Effects of Various Species for Dopamine Determination

Interferant [I]	**Concentrations of [I]**	**Interference Effect**
Glycine	1280x[D]*	8.6 %
Alanine	165x[D]	None
Leucine	47x[D]	None
Tyrosine	105x[D]	None
Tryptophan	94x[D]	None
Cysteine	0.002x[D]** 192x[D]	None None
Epinephrine	0.03x[D]	4.6 %
Noradrenaline	0.115x[D]	7.6 %
Ascorbic Acid	65x[D]	12.4 %
Citric acid	0.03x[D]***	None
Glucose	220x[D]	None

*Dopamine [D] concentration: 3.00×10^{-4} M

**Cysteine concentration in dopamine pharmaceutical injection.

***Citric acid concentration in dopamine pharmaceutical injection.

Stated concentrations for interfering species were choosen at their maximum levels which expected to be present in the pharmaceutical injections and normal urine samples related to dopamine levels.

As can be seen from Table 3.8, other catecholamines (epinephrine, noradrenaline), that have the same catechol group on dopamine give interference together with glycine and ascorbic acid. However, usually as only one cathecholamine is present in pharmaceutical preparations, interfering effects of epinephrine and noradrenaline are not important for dopamine determination in dopamine pharmaceutical injections.

It should be pointed out that the ascorbic acid concentration (0.1 mM) used in the preparation of dopamine standards does not cause any interference effect.

Very high interference effect observed for catecholamines indicated that epinephrine and noradrenaline can also be determined using the proposed method.

3.3.3 Dopamine and Epinephrine Determination in Their Pharmaceutical Injections

The proposed method was applied to the determination of dopamine and epinephrine in pharmaceutical injection. Results are presented in Table 3.9.

Table 3.9 Determination of Catecholamine Levels in Their Pharmaceutical Injections

Sample	Reference Value (mg/mL)	Proposed Method (mg/mL) (n=3)	± $ts/N^{0.5}$ values
Dopamine Injection (a), I	20	19.7±0.42	1.04
II	40	40.4±0.50	1.24
Epinephrine Injection (b), I	0.5	0.50±0.02	0.05
II	1	1.00±0.01	0.03

Student- t test. If ± $ts/N^{0.5}$> absolute difference of the results compared, there is no significant difference at the given confidence level.

a) Dopamine Hydrochloride Injection; Solvay Pharmaceuticals, 20 mg/mL of Dopamine Hydrochloride injection (**I**), Drogsan Pharmaceuticals, 40 mg/mL of Dopamine Hydrochloride Injection (**II**).

b) Epineprine Injection; Drogsan Pharmaceuticals, 0.5 mg/mL of Adrenaline Injection (**I**), Biofarma Pharmaceuticals, 1 mg/mL of Adrenaline Injection (**II**).

Student-t test results at 95 percent confidence level have shown that there were no difference between the results of the proposed method and the results of the standard samples (Table 3.9). Hence, the method can safely be applied for the determination of dopamine and epinephrine in their pharmaceutical injections.

3.4 Flow Injection Analysis System Applications

Buffer (pH 9.00), DAPB ($5.00x10^{-5}$ M) and standard or sample solutions at various concentrations and volumes were aspirated through different ports of the distribution valves using the injection pump. First DAPB (775 µL) solution was propelled to the flow-cell and fluorescence signal was measured (Io). Then, DAPB (775 µL) was mixed with either standard (80 µL) or sample (80 µL) solution in reaction coil and fluorescence of the DAPB-diol complex was measured (I).

3.4.1 Optimization of the Flow Injection System for Fructose and Dopamine Determination

The effects of reaction variables such as flow rate, concentration of DAPB were studied. It was observed that the highest signal intensities (in terms of peak height) for both DAPB and DAPB-diol complex and

the best peak shapes were obtained when flow rate of the reagents was adjusted as 300 μL s^{-1}.

Batch type experiments have shown that the rate of DAPB-diol complex formation was very high (30 s). Consequently, the length of the reaction coil (3.5 cm.) was not very critical. The injection volumes of DAPB and diol were adjusted according to the flow cell dimensions.

The concentration of DAPB was optimized for a wide range of dopamine hydrochloride ($1.00x10^{-5}$ M-$1.00x10^{-4}$ M) and fructose ($1.00x10^{-4}$ M-$1.0x10^{-3}$ M) concentration. It was found that $5.00x10^{-5}$ M concentration of DAPB gave the best signal in flow, when the volume ratio of DAPB to diol molecule was 700 μL/50 μL.

Table 3.10 Parameters Used in FIA-Fluorescence System

Reagents	**Flow Rate**	**Injection Volume**
DAPB ($5.00x10^{-5}$M)	300 μL s^{-1}	775 μL
Dopamine ($1.00x10^{-5}$M-$1.00x10^{-4}$M)	300 μL s^{-1}	80 μL
Fructose ($1.00x10^{-4}$M-$1.00x10^{-3}$M)	300 μL s^{-1}	80 μL
Buffer (0.1 M, at pH 9.00)	300 μL s^{-1}	5.00 mL

The flow parameters are summarized in Table 3.10. The reaction cell and tubings were cleaned thoroughly by flushing the system with 5.0 mL phosphate buffer (at pH 9.00) before each measurement. The

reproducibility of FIA fluorescence signals for DAPB is presented in Figure 3.14.

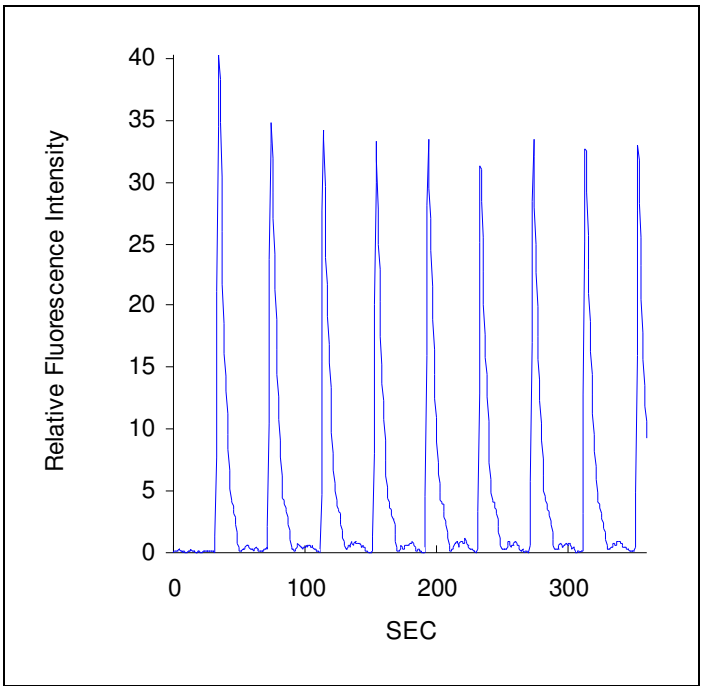

Figure 3.14 Reproducibility of the Fluorescence Intensities of DAPB (5.00×10^{-5} M) in Flow Injection System (FIA) with 300 µL s^{-1} of Flow Rate. ($\lambda_{exc.}$=324 nm, $\lambda_{em.}$=508 nm, exc. slit=5.00 nm and em. slit=7.5 nm)

Figure 3.15 and Figure 3.16 depict the inhibition of the DAPB signal after complexing with $4.00\text{x}10^{-5}$ M dopamine and $8.00\text{x}10^{-4}$ M fructose.

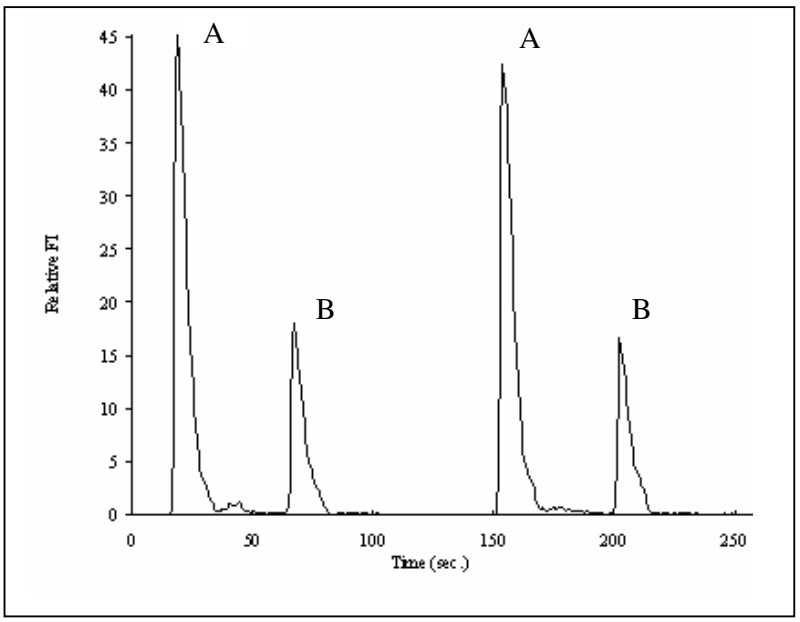

Figure 3.15 Reproducibility of Fluorescence Intensities of $5.00x10^{-5}$ M DAPB (A) alone and in the Presence of $4.00x10^{-5}$ M Dopamine (B) in FIA system with 300 μL s^{-1} of Flow Rate. ($\lambda_{exc.}$=324 nm, $\lambda_{em.}$=508 nm, exc. slit=5.00 nm and em. slit=7.5 nm)

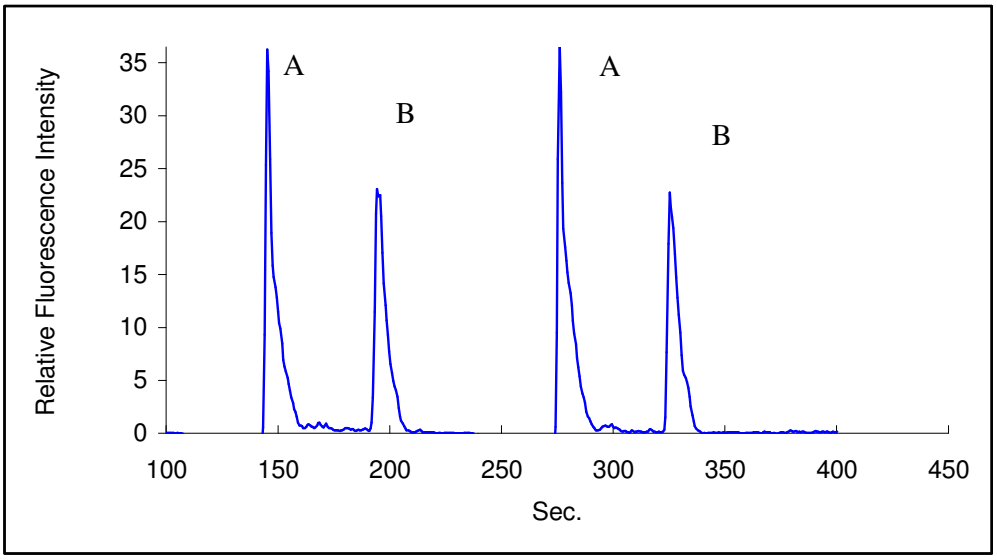

Figure 3.16 Reproducibility of Fluorescence Intensities of $5.00x10^{-5}$ M DAPB (A) alone and in the Presence of $8.00x10^{-4}$ M Fructose (B) in FIA system with 300 µL s^{-1} of Flow Rate. ($\lambda_{exc.}$=324 nm, $\lambda_{em.}$=508 nm, ex. slit=5.00 nm and em. slit=7.5 nm)

At the specified conditions, one measurement (including washing step) lasts 150 s, which allows the measurement of 24 samples per hour.

CHAPTER 4

CONCLUSION

In this study, a simple, selective and reliable method is developed for the rapid determination of fructose, glucose, dopamine, and epinephrine based on quenching of the fluorescence of m-dansylaminophenyl boronic acid (DAPB). Quenching occurs due to the reaction of boronic acid 1,2 or 1,3-diol groups of Saccharides or catecholamines which activates the reverse photoinduced electron transfer (PET).

Quenching of fluorescence was linearly related with fructose and glucose concentrations over the range of $1.00x10^{-4}$ M-$1.00x10^{-3}$ M. DAPB was more selective for catecholamines compared to monosaccharides, relatedly the linearity for dopamine and epinephrine was in between $1.00x10^{-5}$ M and $1.00x10^{-4}$ M, ten fold lower than that of monosaccharides. Detection limit of fructose, glucose and dopamine were $2.3x10^{-5}$ M, $6.7x10^{-5}$ M and $3.7x10^{-6}$ M, respectively.

The validation of the proposed method was done using standard samples. Potential interferants were examined thoroughly.

The flow injection system is composed of two five-way distribution valves, one syringe pump and a reaction coil. System parameters were optimized. The percent relative standard deviation of the

measurements performed by FIA system was 1.2 for ten replicates of $1.00x10^{-5}$ M dopamine standard solution. At the optimized conditions, one measurement (including washing step) lasts 150 s. Hence the proposed fluorescence FIA method is completely suitable for the fast analysis of the samples at an analytical frequency of 24 sample per hour. Both the repeatibility and the speed of the system are suitable for routine analysis.

The proposed method was applied succesfully for the determination of fructose and glucose in their commercial syrups and invert sugar in wine samples.

The proposed method was also applied succesfully for the determination of dopamine and epinephrine in their pharmaceutical injection products.

REFERENCES

1. T. D. James, K. R. A. Samankumara, Sandanayake, R. Iguchi, S. Shinkai, *Journal of American Chemical Soceity*, 117, 1995, 8982-8987.

2. J. L. Groff, S. S. Grapper, *Advanced Nutrition and Human Metabolism*, 3rd Edition, Wadsworth Thomson Learning, Belmont, CA, 2000, 70.

3. http://dl.clackarmas.cc.or.us./ch 106-07/reducing.htm

4. http://www.elmhurst.edu/543fructose.htm

5. M. F. Kavas, J. Leblebici, *Kalite ve İşletme Kontrol Laboratuarları El Kitabı,* 1st Edition, Mars Yayınevi, Ankara, 2004, 130.

6. W. Surareungchai, W. Deepunya, P. Tasakorn, *Analytica Chimica Acta*, 448, 2001, 215–220.

7. http://www.britishsugar.co.uk/bsweb/sfi/pages/invert.htm

8. http://www.lurgilifescience.com/lurgi_ls/ fructose.pdf

9. A. Caballo-Lo´pez, M.D. Luque de Castro, *Talanta*, 59, 2003, 837-843.

10. A. F. Oliveira, O. Fatibello-Filho, *Talanta*, 50, 1999, 899–904.

11. R. N. Shreve, *Chemical Process Industries*, 3th edition, McGraw-Hill Kogakusha Book Company, New York, 1967, 591.

12. http//www.winemakermag.com

13. B. Roig, O. Thomas, *Carbohydrate Research*, 338, 2003, 79

14. O. Nozaki, H. Kawamoto, H. Moriyama, *Luminescence*, 14, 1999, 369-374.

15. J. Bergquist, A. Sciubisz, A. Kaczor, J. Silberring, *Journal of Neuroscience Methods*, 113, 2002, 1-13.

16. C. Zhang, J. Huang, Z. Zhang, M. Aizawa, *Analytica Chimica Acta*, 374, 1998, 105-110.

17. M. A. Fotopoulou, P. C. Ioannou, *Analytica Chimica Acta*, 462, 2002, 179-185.

18. A. T. Wood, M. R. Hall, *Journal of Chromatography*, 744, 2000, 221-225.

19. S. Higashidate, K. Imai, *Analyst*, 117, 1992, 1863-1868.

20. L. Rover Ju´nior, J. C. B. Fernandes, G. de Oliveira Neto, L. T. Kubota, *Journal of Electroanalytical Chemistry*, 481, 2000, 34–41.

21. E. Nalewajko, R. B. Ramýrez, A. Kojło, *Journal of Pharmaceutical and Biomedical Analysis*, 36, 2004, 219–223.

22. http://tcw2.ppsw.rug.nl/vdbosch/pd.html

23. H. Y. Wang, Q. S. Hui, L. X. Xiao, J. G. Jiang, Y. Sun, *Analytica Chimica Acta*, 497, 2003, 93-99.

24. X. Lin, J. Gong, *Analytica Chimica Acta*, 507, 2004, 255–261.

25. H. Y. Wang, Y. Sun, *Talanta*, 57, 2002, 899-907.

26. http://www.netdoctor.co.uk/medicines/100000840.html

27. J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, 3rd edition, Plenum Corporation, New York, 1986, 257.

28. B. Appleton, T. D. Gibson, *Sensors and Actuators B*, 2000, 65, 302- 304.

29. A. P. Silva, H. Q. N. Gunaratne, T. Gunnlaugsson, A. J. M. Huxley, C. P. McCoy, J. T. Rademacher, T. E. Rice, *Chemical Reviews*, 97, 1997, 1515-1566.

30. Masashi Yamamoto, M. Takeuchi, S. Shinkai, *Tetrahedron*, 54, 1998, 3, 125-140.

31. C. W. Gray, B. T. Walker, R. A. Foley, T. A. Houston, *Tetrahedron Letters*, 44, 2003, 3309-3312.

32. K. R. A. Samankumara Sandanayeke, S. Shinkai , *Nature*, 374, 1995, 345-347.

33. H. Suenega, K. R. A. Samankumara Sandanayeke, S. Shinkai, *Pergamon*, 36, 1995, 4825-4828.

34. G. Springsteen, B. Wang, *Tetrahedron*, 58, 2002, 5291-5300.

35. D. P. Adhikiri, M. D. Heagy, *Tetrahedron Letters*, 40, 1999, 7893-7896.

36. E. H. Hansen**,** *Analytica Chimica Acta*, 308, 1995, 3-13.

37. J. Ruzicka, *Flow Injection Analysis*, 2nd edition, Wiley-Interscience Publication, 12-13.

38. J. Ruzicka, G. D. Marshall, *Analytica Chimica Acta*, 237, 1990, 329.

39. E. M. Garrido, J. Lima, C. Delerue-Matos, *Journal of Pharmaceutical anf Biomedical Analysis*, 15, 1997, 845.

40. F. O. Silva , V. Ferraz, *Food Chemistry*, 88, 2004, 609-612.

41. J. L. Chávez-Servýn, A. I. Castellote, M. C. López-Sabater, *Journal of Chromatography A*, 1043, 2004, 211-215.

42. R. Vonach, B. Lendl, R. Kellner, *Journal of Chromatography A*, 824, 1998, 159-167.

43. B. Roig, O. Thomas, *Analytica Chimica Acta*, 477, 2003, 325-329.

44. A. F. Oliveira, O. F. Filho , J. A. Nobrega, *Talanta*, 55, 2001 677-684.

45. S. Campuzano, O. A. Loaiza, M. Pedrero, F. J. Manuel de Villena, J. M. Pingarro, *Bioelectrochemistry*, 63, 2004, 199-206.

46. R. P. Sartini, C. C. Oliveira, E. A. G. Zagatto, H. B.Filho, *Analytica Chimica Acta*, 366, 1998, 119-125.

47. H. L. Thanh, B. Lend, *Analytica Chimica Acta*, 422, 2000, 63–69.

48. A. Cáceres, S. Cárdenas, M. Gallego, M. Valcárce, *Analytica Chimica Acta*, 404, 2000, 121–129.

49. H. Kubo, A. Toriba, *Analytica Chimica Acta*, 353, 1997, 345-349.

50. N. P. Evmiridis, N. K. Thanasoulias, A. G. Vlessidis, *Analytica Chimica Acta*, 398, 1999, 191–203.

51. N. D. Danielson, C. A. Heenan, F. Haddadian, A. Numan, *Microchemical Journal*, 63, 1999, 405-414.

52. A. Dalmia, C. C. Liu, R. F. Savinell, *Journal of Electroanalytical Chemistry*, 430, 1997, 205.

53. V. S. Bezerra , J. L. de Lima Filho, M. Conceic¸ B. S. M. Montenegro, A. N. Arau,V. Lins da Silva, *Journal of Pharmaceutical and Biomedical Analysis*, 33, 2003, 1025-1031.

54. L. Zhang, N. Teshima, T. Hasebe, M. Kurihara, T. Kawashima, *Talanta*, 50 (1999) 677.

55. F. Li, Y. Q. Pang, X. Q. Lin, H. Cui, *Talanta*, 59, 2003, 627.

56. D. A. Skoog, D. M. West, F. J. Holler, *Fundemantals of Analytical Chemistry* , 7th Edition, Saunders College Publishing, Philadelphia, 1996, 53.