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Optimization of a Flow Injection Indicator
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presented by

Pavlos Aspris

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OPTIMIZATION OF A FLOW INJECTION INDICATOR REACTION WITH APPLICATIONS IN DETERMINING SUGARS IN FRUITS

By

Pavlos Aspris

A THESIS

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ABSTRACT

OPTIMIZATION OF A FLOW INJECTION INDICATOR REACTION WITH APPLICATIONS IN DETERMINING SUGARS IN FRUITS

By

Pavlos Aspris

In this research a new dye, leucomalachite green (LMG), has been introduced for the determination of glucose by flow injection analysis (FIA) using immobilized glucose oxidase.

By means of general univariate and computerized simplex optimization procedures, nine analytical parameters affecting the indicator reaction and the FIA system were optimized. Through a specific optimization procedure, four crucial analytical parameters were further optimized. The optimum conditions obtained were: flow rate, 1.47 ml/min; 0.1 M phosphate buffer, pH 6.0, as a carrier; peroxidase activity, 143 units per 10 ml of reagent; concentration of LMG reagent solution, 9.09×10^{-2} mM; concentration of LMG stock solution, 1.5 mM; 0.1 M phosphate buffer, pH 6.0, 2.4 ml per 10 ml reagent solution; 0.1 M acetate buffer, pH 4.0, 7 ml per 10 ml reagent solution; length of enzyme single bead string reactor (SBSR), 11.6 cm; length of unmodified SBSR reactor, 35 cm; and temperature, 40 ^{o}C.

The optimized FIA procedure was applied to the analysis of glucose directly, and fructose and sucrose after proper enzyme conversions to

glucose. The results of the FIA analysis of 26 fruit samples for these sugars were compared with the results obtained by conventional sugar enzyme analysis and found to be in good agreement.

To my parents,

and brother, George.

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I am extremely indebted to Professors Pericles Markakis and Stanley Crouch for their guidance, enthusiasm, encouragement, and understanding throughout the course of my research effort. Their willingness to give me the freedom to do what I want, but offering help when needed, built my confidence and independence in doing research.

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I. INTRODUCTION

The significance of chemical analysis in Food Science and Human Nutrition is steadily increasing. New techniques are sought which are rapid, do not require highly skilled personnel and are not very costly.

Dramatic advances in electronics, computer technology and biotechnology over the past decades have led to the emergence of a number of techniques to fulfill the needs of the modern food analysis laboratory. The availability of fast, low-cost computers allows automation in instrument control and data handling so that the classical methods themselves can be automated. In addition, the data handling capability allows the development of sophisticated techniques based on measurement of physical properties of the sample; these include spectroscopy, chromatography and electrochemistry.

Flow Injection Analysis (FIA) is a fairly recent technique (1) and features major methodological innovations such as simplicity, relatively inexpensive equipment, handy operation and great capacity for achieving results that are excellent in rapidity, accuracy and precision. The extreme versatility of this methodology makes it stand out from most new analytical techniques. For example, FIA can be adapted to meet many types of requirement without major technical changes. FIA differs from traditional analytical techniques in that it is not necessary for measurements to be made at a state of equilibrium with respect either to the course of the chemical reaction or flow dynamics. FIA is a microchemical technique in which beakers, pipettes and volumetric flasks are replaced by small (0.5 mm i.d.) open-ended tubes through which the solutions are pumped. Since FIA has proven to be very effective by

making the handling of liquid samples an easy task, it has great potential in all areas that require chemical analysis.

This research contributes to the development of a rapid, parallel continuous flow analyzer, for the simultaneous enzymatic determination of six nutritionally important sugars present in food samples with complex matrices, without prior separation. Work on this project has been going on for several years already under the supervision of Dr. Crouch, with various researchers focusing on different aspects, such as enzyme immobilization procedures, sample preparation, and construction of the FIA manifold. Specifically this research deals with the optimization of a new flow-injection indicator reaction using Leucomalachite Green (LMG) and the application of this novel technique to the direct and indirect determination of three major free sugars (glucose, fructose and sucrose) in fruits using immobilized glucose oxidase.

II. BACKGROUND INFORMATION

A. Methods of Sugars Analysis in Fruits

Fresh fruits and certain vegetables are major sources of unprocessed sugars in the human diet. In addition, fruits contain a higher proportion of free sugars than vegetables and a lower proportion of unavailable carbohydrates than most vegetables (2). Recently, high fiber natural health foods, consumption of unprocessed vs. processed foods, and metabolic differences in fructose, glucose and sucrose have all received wide spread attention.

The free sugars in fruits are usually mixtures of glucose, fructose and sucrose. Occasionally maltose and other oligosaccarides are present. The proportions of the different sugars are characteristic of the fruit, although different varieties of the same fruit show some variations.

1. Non-Specific and Specific Methods

High performance liquid chromatography (HPLC) and methods of enzymatic analysis have rapidly become the techniques of choice for the quantitative analysis of sugars and other carbohydrates in most fruits. Prior to the development of such analytical techniques, sugars were determined quantitatively in many fruits as total reducing sugars and total non-reducing sugars. These non-specific methods had serious limitations (3). In those measurements fructose and glucose were usually assumed to be the reducing sugars and sucrose the only nonreducing sugar. These assumptions are generally true for most fruits. Some fruits, however, contain significant amounts of sorbitol,

which is not accounted for by those earlier measurements, or maltose, which would be included in the total reducing sugar value.

As the role of individual sugars, such as fructose, in health and nutrition became more well defined (4), the need for rapid and simple quantitative methods for determining individual sugars (specific methods) in foods became more important. Individual sugars have been determined by GC and by enzymatic methods (using soluble enzymes). HPLC was shown to be generally faster than either of those two methods. A relatively new analytical technique, FIA, based on continuous flow and immobilized enzymes has been applied for the determination of individual sugars in fruits with greater advantages than the HPLC technique.

2. Comparison of HPLC with FIA

Figure 1 shows two flow diagrams corresponding to FIA and liquid chromatography.

Table 1 lists the common and differential features of FIA and HPLC. The following similarities should be emphasized: miniaturization capability, injection, unsegmented flow, small sample volume, and signal profile. On the other hand, there are substantial differences between them, the most important of which is probably their principle, since in HPLC there is always an interface which affects the separation of a mixture of substances passing through the column, and this is not so common in FIA. The similarity between both techniques becomes more apparent when a column (packed, open or single bead sting reactor) with an ion exchange resin or an immobilized enzyme is used in the manifold behind the injection point in a FIA system. The working pressure is a

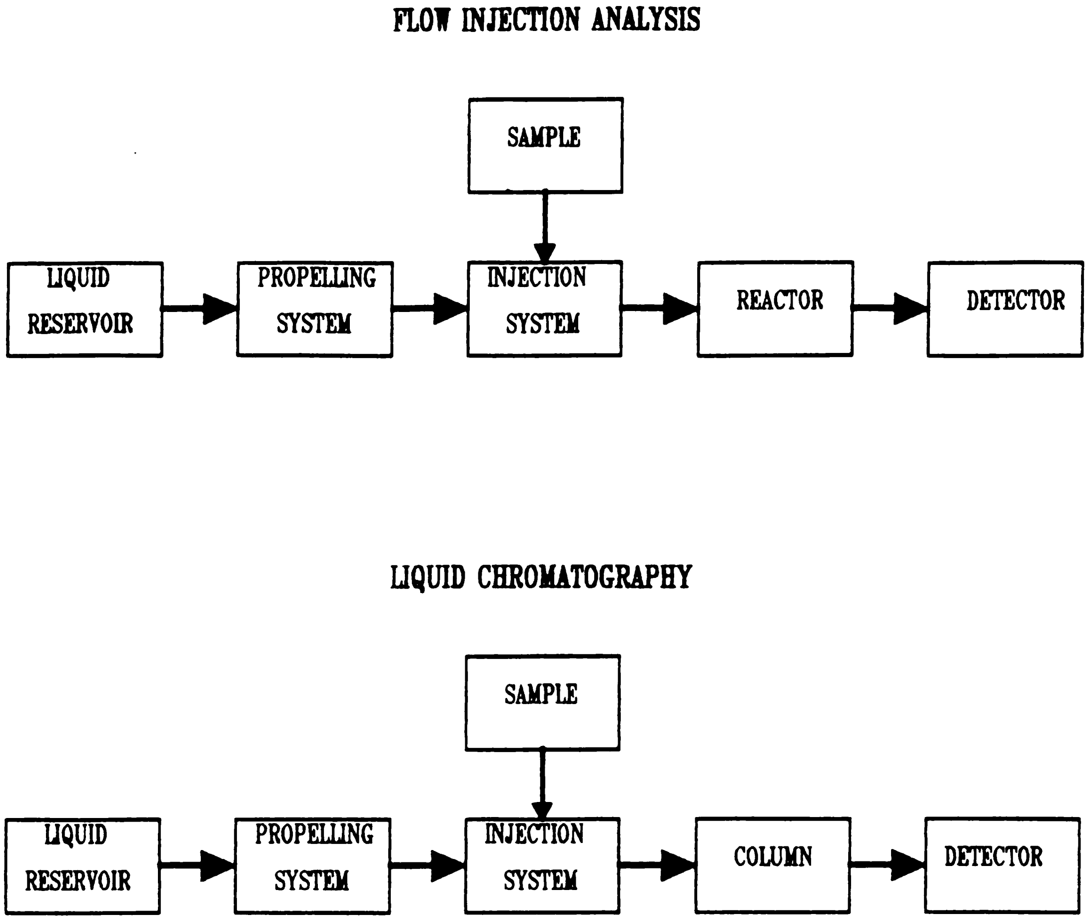


Figure 1. Flow diagrams of Flow Injection Analysis and Liquid Chromatography.

Table 1. Comparison of attributes between FIA and HPLC.

Characteristics	HPLC	FIA
Pressure	high	low
Column	essential	possible
Interface	always	occasionally
Data produced	peak height/area	peak height/area/ width/peak-to-peak distance
Cost	high	low
Versatility	limited	great
Main analytical purpose	several components in a single sample	a single component in many samples
Tubing diameter	small	small
Flow rate	variable	variable
Sample volume	small	small
Sample introduction	injection	injection
Unsegmented flow	yes	yes

major factor responsible for significant differences between the two techniques. FIA uses low pressures, whereas in HPLC the pump (usually dual-piston) must exert a high pressure to overcome the hydrodynamic resistance of columns packed with material that is finely divided to improve the efficiency of the separation process. Despite the fact that some FIA methods have been developed with the aid of HPLC components, typical FIA systems are much simpler, since they are designed to at low pressures. Therefore, HPLC instruments are much more expensive.

The scope of application of the two techniques is very different. The basic aim of an HPLC instrument is to separate and analyze a complex mixture of substances, whereas FIA is mainly devoted to the rapid determination of a single species in a large number of samples.

B. Introduction to Continuous Flow Methods

The analytical procedures in which the analyte concentration is measured without stopping the flow of a gas or liquid are referred to as continuous flow methods (CFA) (5). There are two general types of continuous flow methods: <u>segmented</u> and <u>unsegmented</u>.

In the segmented flow methods, the samples are introduced onto the manifold which made of interconnected tubing, by aspiration for a defined period of time, and air bubbles separate (segment) the flow. In each segment complete mixing takes place so that the signal obtained at the output has a rectangular shape similar to what would be expected in the ideal case of a plug-shape sample. The air bubbles are usually removed before they reach the detector cell.

Unsegmented flow methods, are the Flow Injection Analysis (FIA) methods and differ from segmented flow methods in that the flow is not

segmented by air bubbles, the sample is injected instead of aspirated and neither flow homogenization nor chemical equilibrium has been accomplished by the time the signal is recorded (5). In addition, FIA methods require less sophisticated and expensive equipment.

C. Principles and Function of Flow Injection Analysis

The simplest flow injection analyzer (Figure 2a) consists of a pump, which is used to propel the carrier stream through a narrow tube; an injection port, for injection of a well defined volume of a sample solution into the carrier stream in a reproducible manner; and a microreactor in which the sample zone disperses and reacts with the components of the carrier stream, forming a species that is sensed by a flow through detector and recorded.

A typical recorder output has the form of a peak (Figure 2b), the height H and width W, or area A, of which is related to the concentration of the analyte. The time span between the sample injection and the peak maximum, is the residence time T during which the chemical reaction takes place. A well designed FIA system has an extremely rapid response, because T is in the range of 5-20 sec. Therefore, a sampling cycle is less than 30 sec ($T+t_b$), and thus, typically, two samples can be analyzed per minute. The injected sample volumes may be between 1 and 200 μL (typically 25 μL). This makes FIA a simple, automated microchemical technique, capable of having a high sampling rate and a minimum sample and reagent consumption.

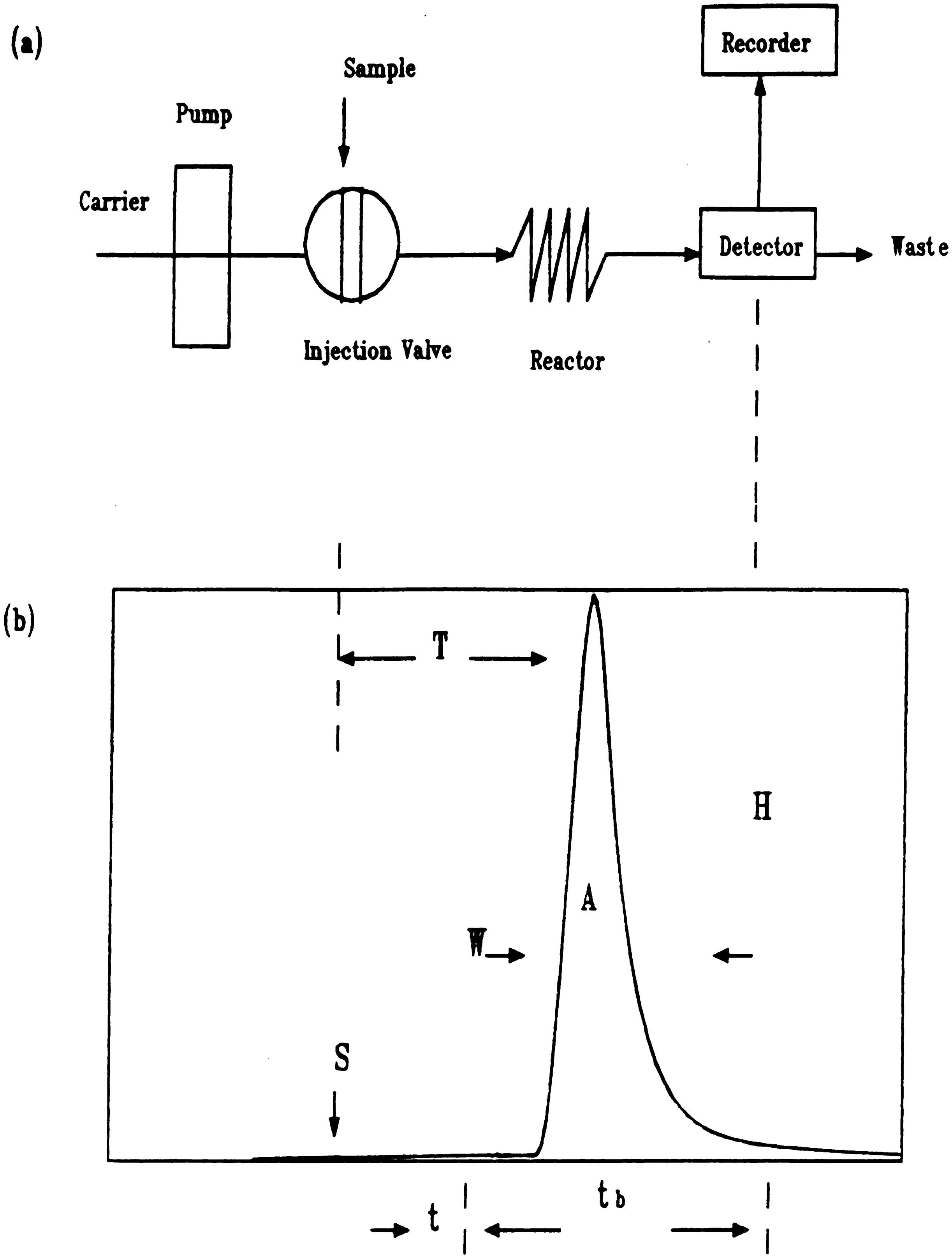


Figure 2. (a) The main parts of the simplest flow injection analyzer. (b) Typical output corresponding to analyte.

Flow Injection Analysis is based on a combination of three principles: sample injection, controlled dispersion of the injection sample zone, and reproducible timing of its movement from the injection point toward and into the detector. Thus, in contrast to all other methods of instrumental analysis, the chemical reactions are taking place while the sample material is dispersing within the reagent prior to the detection point. This is why the concept of dispersion, controlled within space and time, is the central issue of FIA.

1. Definition

Flow Injection Analysis (FIA) is 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 toward a detector that continuously records a physical parameter, such as absorbance or electrode potential, as it continuously changes due to the passage of the sample material through the flow cell (1,5).

2. Essential Features

In principle, Flow Injection Analysis is an automatic method of analysis. The essential features of the FIA used here are the following:

(a) The flow is not segmented by air bubbles, which is the fundamental difference from classical CFA methods.

(b) The sample is injected or inserted directly into the flow stream instead of being aspirated into it.

(c) The injected plug is carried along the system. A physicochemical process (chemical reaction, dialysis, liquid-liquid extraction, etc.) may occur in addition to transport.

(d) The partial dispersion or dilution of the analyte throughout this transport operation can be manipulated by controlling the geometric and hydrodynamic characteristics of the system(tubing i.d., length, flow rate ect).

(e) A continuous sensing system yields a transient signal which is recorded.

(f) Neither physical equilibrium (which would involve the homogenization of a portion of the flow) nor chemical equilibrium (completeness of reaction) has been attained when the signal is detected.

(g) The operational timing must be highly reproducible because measurements are made under non-equilibrium conditions and small variations may result in serious alterations in the results.

3. Dispersion

The flow injection technique involves the injection of a sample into a nonsegmented carrier stream. Since the conditions are usually such that laminar flow is predominant, the development of a parabolic velocity profile is responsible for the dispersion of the sample along the axis of the tube. This dispersion, although much greater than that found in CFA, can be controlled by appropriate choices of tubing length and inner diameter, flow rate, sample size, and other components such as valves and flow cells which determine the overall volume of the reactor in the FIA system.

Ruzicka and Hansen have proposed an empirical method by which dispersion can be measured (5). The sample has an initial concentration C_0 as it enters the carrier stream. As the plug travels through the manifold, axial and radial mixing take place. This result is a predominantly Gaussian-shaped signal profile. The maximum concentration

sensed by the detector, C_{max}, is only a fraction of C_0. The formula for the dispersion is:

$$D = C_0/C_{max} = H_0/H \times Const'/Const''$$

The height of the peak obtained with the undiluted sample is H0. After the sample has traversed the manifold a lower peak height, H, will be obtained due to dispersion. If the two constants are equal, as in the case of photometric detection for a system that obeys Beer's law, the peak heights of the signals can be used to determine the dispersion of the FIA system. The amount of dispersion that can be tolerated in an FIA system depends on the application for which it is applied. For mere transportation of a sample, limited dispersion is ideal. On the other hand, for a chemical reaction requiring reagent additional dispersion must take place.

In conclusion, an FIA peak is a result of two kinetic processes that occur simultaneously: <u>the physical process of zone dispersion and the chemical processes resulting from reactions between sample and reagent species</u>.

4. Types of FIA Manifolds

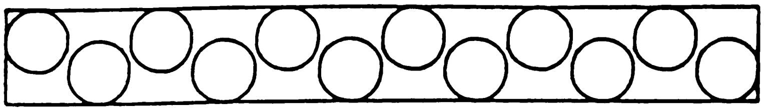
The physical foundations of FIA are related to dispersion, which is caused by injecting a sample volume into the flowing stream. The dispersion is characterized by the concentration profile adopted by a zone or plug inserted at a given point in the system without stopping the flow. The recorder output from the detector (the analytical signal) is representative of the dispersion at such a point, and can be used to access the extent of dispersion.

One way of manipulating dispersion is by selection of the appropriate type of manifold. A comparison between various types of FIA manifolds was carried out by Ruzicka and Hansen (6). Three general types of reactors have been used in FIA: the open tubular (OTR), packed bed (PBR), and single bead string reactors (SBSR) (Figure 3).

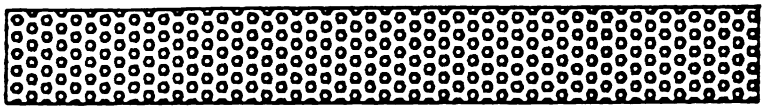
Since straight open tubes yield relatively large amounts of dispersion (7), little attention has been paid to the preparation of reactors with enzymes immobilized on the inner wall of the tubes (8-10). However, such reactors have advantages over packed beds in certain applications because they permit an unobstructed flow of the substrate solution. Coiled tubes show less dispersion due to the presence of a secondary flow (11). This type of flow is a result of the centrifugal forces which affect the flow perpendicular to the axis of the tube.

A relatively small amount of dispersion has been found in the case of packed tubes (12-13). This is due to the fact that the parabolic profile is broken up as the sample passes through the packed material. The high pressure drop associated with tubes that have been packed with very small diameter particles makes them difficult to use with the peristaltic low pressure pumps normally present in an FIA system.

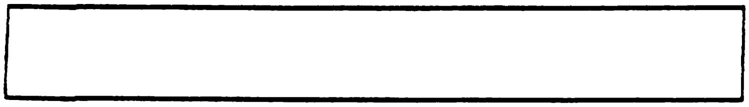
The single bead string reactor (14-15) has gained acceptance as a viable alternative to open tubes and those packed with small particles. The SBSR consists of ordinary Teflon tubes packed with glass beads having diameters that are 60-80 % of that of the tube. This type of reactor in FIA provides the following advantages:



Single Bead String Reactor



Packed Bed Reactor



Open Tubular Reactor

Figure 3. Types of enzymatic reactors that can be used in the Flow Injection Analysis method.

(a) Decreased dispersion due to the break up of the velocity profile.

(b) The sampling rate is significantly high, with up to 500 samples assayed per hour.

(c) It allows easy merging of streams, which is especially desirable when there is a chemical reaction between species dissolved in the streams.

(d) The pressure drop is small. Therefore, the SBSR can be used to provide longer residence time without an increase in dispersion.

(e) For use with immobilized enzymes an added advantage of the SBSR is the additional surface area available compared to that of an open tube.

D. Use of Immobilized Enzymes in FIA

Enzymes have been insolubilized by irreversible covalent attachment to various organic polymers (16-22), and cellulose derivatives (23-25). Immobilization has also been accomplished by entrapment in starch (26) and acrylamide gel (27-28). These types of derivatives have been studied in detail by Silman and Katchatski (29). Also, enzymes can be irreversibly covalently coupled to inorganic carriers (30-33). In general, inorganic carriers are not subject to microbial attack. The carrier does not change configuration over an extensive pH range or under various solvent conditions, and is therefore, easier to use in continuous flow systems. In addition, the inorganic carriers have greater rigidity and they immobilize enzymes to a greater degree than do organic polymers.

In general, immobilization by covalent attachment has proved to be the most suitable for continuous flow analysis and can be applied with

the three different types of immobilized enzyme reactors, such as packed columns, open tubular wall reactors, and single bead string reactors.

1. Reversible Immunological Immobilization

The use of immobilized enzymes packed into reactors and coupled to flow systems in analytical applications has been well demonstrated (34-35). However, the immobilized enzymes in these configurations suffer from several limitations. Some of these are a) limited lifetime, and b) susceptibility to inhibitory and steric problems created by immobilization, which limit the transfer of substrate to the enzyme layer and block access to the active site. By using immobilized antibodies which are specific to the enzyme or using indirect immunochemical reactions, enzymes can be immobilized with high efficiency while retaining maximum enzymic activity. The use of antibodies in the immobilization of enzymes allows the operator to replace the bound enzyme reproducibly in a few minutes in the event of a loss of enzyme activity without removing or replacing the packing material (Figure 4). The flow injection analysis method has the advantage of rapid sample throughput and minimal sample handling.

Another coupling technique is based on the fact that avidin binds to biotin with a binding constant of 10^{15} (36) and that the resulting binding is therefore irreversible under conditions where the antibody-antigen interactions can be reversed (37-40). This situation provides a method for immobilizing the primary antibody with high efficiency (41-46). Furthermore, the use of avidin-biotin interactions for the reversible or irreversible immobilization of enzymes is very critical (Figure 4). These methods of immobilization of enzymes in reactors use

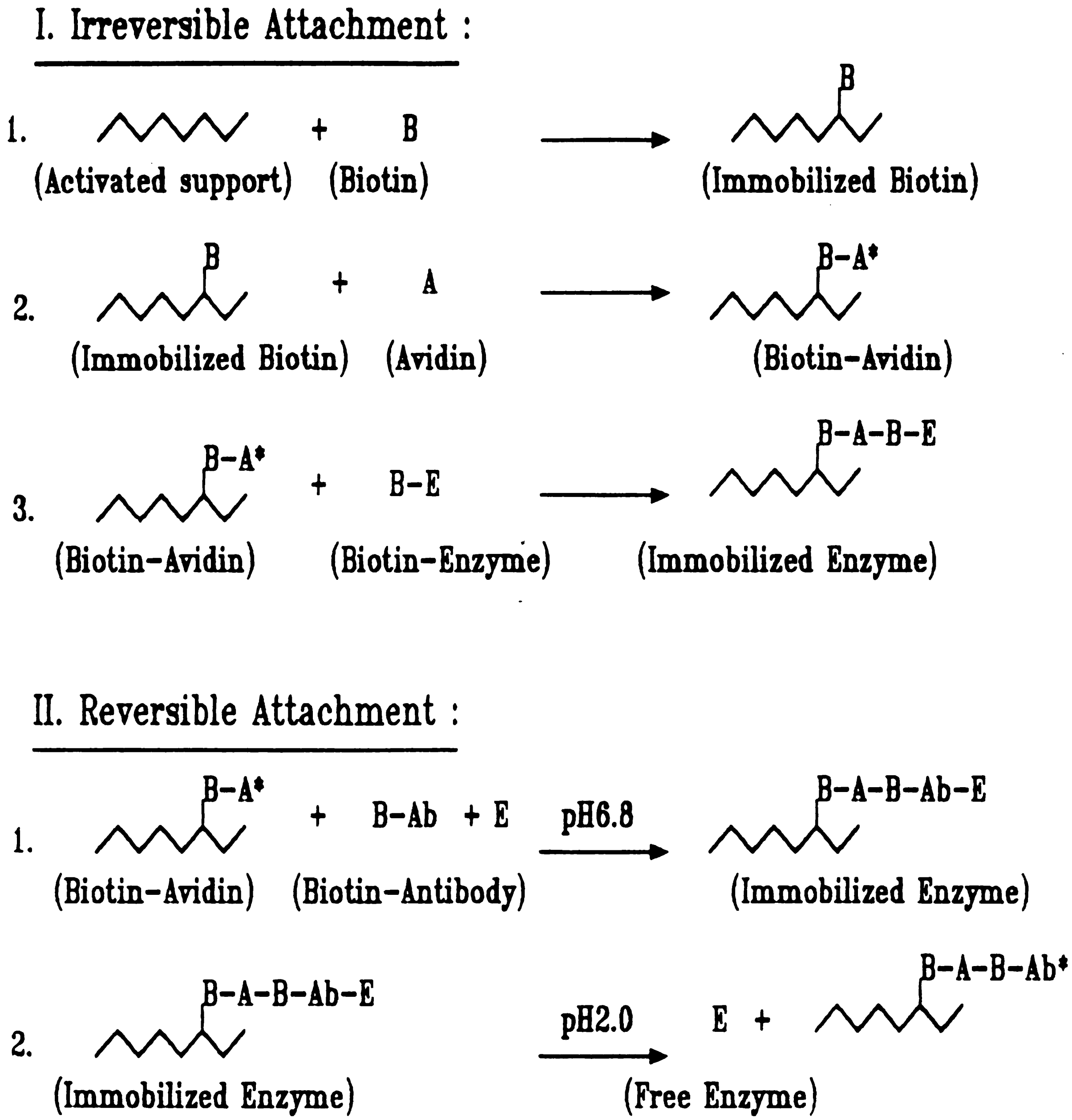


Figure 4. Attachment of the enzyme in two ways by using avidin-biotin interaction.

two biospecific reactions where one of the reactions is irreversible and the other reversible (Figure 4).

2. Advantages of Immobilized Enzymes

In recent years there has been an increasing use of immobilized enzyme preparations in industrial, analytical and medical procedures (47). The most obvious advantages are products free from enzymes, continuous run, greater efficiency of substrate conversion, higher yields and good product uniformity. However, these advantages must be balanced against the additional costs of enzyme immobilization, and the relatively poor stability of purified soluble enzymes. Additionally, some enzyme solutions cannot be used in organic solvents, and all are very sensitive to elevated temperatures. These particular drawbacks have slowed the advancement of enzyme applications, and much research effort has been expended to overcome these problems. Large scale procedures of enzyme immobilization have helped to reduce enzyme immobilization costs. New procedures of enzyme immobilization like pre-treatment (for maximizing the surface area on support) or new immobilization methods such as affinity chromatography and immuno-techniques have provided useful preparations, with greater stability, that are also suitable for reuse (48).

3. Applications of Immobilized Enzymes

The benefits of an increased understanding of enzymes, and especially immobilized enzymes, should allow many novel solutions to analytical problems involving substrates, activators or inhibitors of these enzymes. In addition, the potential for using immobilized enzymes as catalysts in areas such as food and clinical analysis, medicine, chemical synthesis and conversions, has been widely promoted. The scope for using enzymes as industrial catalysts is indicated by the wide range of reaction types that can be catalyzed by enzymes. These include oxidation/reduction, inter and intramolecular transfer of a variety of chemical groups, hydrolysis, cleavage of covalent bonds, isomerization and addition of chemical groups across double bonds; so some organic and many inorganic reactions can be catalyzed by one or more enzymes.

The best known and widely used enzymic assay is for glucose. Glucose Oxidase, oxidizes β-D-glucose to gluconic acid and hydrogen peroxide. The latter is used to oxidise an appropriate dye using horseradish peroxidase, giving a useful colorimetric assay (20,49 and 50). Some mutarotase activity should be present to convert α-D-glucose to β-D-glucose, as the latter is oxidized 150 times faster at 20 ^{0}C by the glucose oxidase used. Furthermore, many other substrates can be assayed using immobilized enzymes and FIA, including: Vitamin C by Ascorbate Oxidase (51), Ethanol by Alcohol Dehydrogenase (52), Amino Acids by L-Amino Acid Decarboxylases (53), L-Lactic acid by Lactate Oxidase (54), Oxalate by Oxalate Oxidase (55), Penicillin by Penicillinase (56), Urea by Urease (57), Malate by Malate Dehydrogenase (58), Cholesterol by Cholesterol Oxidase (59-61) etc.

III. METHODS AND MATERIALS

Unlike many other theses, in this work the development and optimization of an analytical method was the major objective. For this reason in this section only the general interactions on which the method is based are described, and the details of the proposed method are given in the Results and Discussion section. The conventional method for the determination of sugars is described here.

A. Methodology of FIA for Sugars

1. Apparatus

For the determination of the six nutritionally important sugars, the proposed novel parallel continuous flow analyzer and the appropriate enzyme reaction schemes are shown in Figures, 5 and 6.

In this work, direct determinations of glucose and indirect determination of fructose and sucrose were done with the flow injection apparatus shown in Figure 7 and the enzymatic reaction schemes shown in Figure 8. The flow injection analysis apparatus consisted of a 12-channel peristaltic pump (Ismatec, Glattbrugg, Switzerland) with flow-rated pump tubing (Technicon Instruments, Tarrytown, NY), a pneumatically activated injection valve with a 30 μL sample loop (Rheodyne Inc., Cotati, CA), and a miniaturized flow through filter colorimeter designed and constructed by Patton and Crouch (62). A light source of variable intensity was connected to the channel of the detector via a fiber optic. The wavelength of the operation was 620 nm and was accomplished by a filter. An IBM PC compatible microcomputer, equipped with an RTI-815 (Analog Devices, Norwood, MA) interface board,

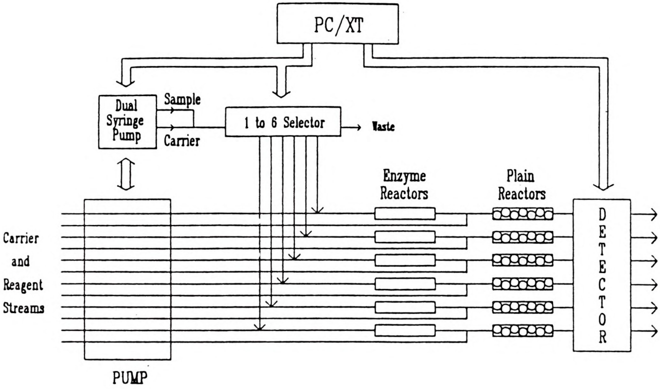


Figure 5. Parallel multichannel flow injection analyzer for the enzymatic determination of sugars.

$$\text{D-glucose} + O_2 + H_2O \xrightarrow{\text{Glucose Oxidase}} \text{D-glucono-}\delta\text{-lactone} + H_2O_2$$

$$\text{D-galactose} + O_2 + H_2O \xrightarrow{\text{Galactose Oxidase}} \text{D-galacto-hexodialdose} + H_2O_2$$

$$\text{Sucrose} + H_2O \xrightarrow{\text{Invertase}} \xrightarrow{\text{Mutarotase}} \text{D-glucose} + \text{D-fructose}$$

$$\text{Lactose} + H_2O \xrightarrow{\beta\text{ - galactosidase}} \text{D-glucose} + \beta\text{-D-galactose}$$

$$\text{Maltose} + H_2O \xrightarrow{\alpha\text{ - glucosidase}} \text{D-glucose} + \alpha\text{-D-glucose}$$

$$\text{D-fructose} \xrightarrow{\text{Glucose Isomerase}} \text{D-glucose} + \text{D-fructose}$$

Figure 6. Enzymatic reactions for each sugar.

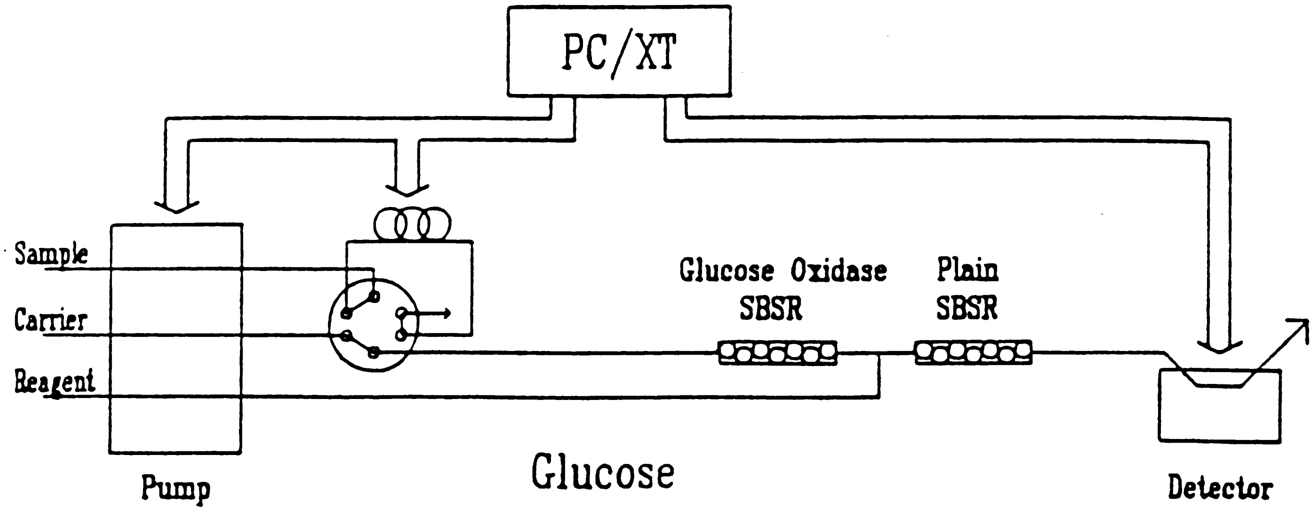


Figure 7. Diagram of the apparatus and the reaction manifold used for the analysis of glucose.

$$\text{D-glucose} + O_2 + H_2O \xrightarrow{\text{Glucose Oxidase}} \text{D-glucono-}\delta\text{-lactone} + H_2O_2$$

$$\text{D-fructose} \xrightarrow{\text{Glucose Isomerase}} \text{D-glucose} + \text{D-fructose}$$

$$\text{Sucrose} + H_2O \xrightarrow{\text{Invertase}} \xrightarrow{\text{Mutarotase}} \text{D-glucose} + \text{D-fructose}$$

Figure 8. Enzymatic reaction schemes for glucose, fructose and sucrose.

controlled the pump speed, sample injection and data acquisition. Software was written in Quick BASIC (Microsoft Corp., Rendmond, WA). More details about this apparatus are given by Stults (63).

This system was based on immobilized enzyme SBSRs for high selectivity and sensitivity. The sample containing glucose passed first through a SBSR which had glucose oxidase covalently bonded to 0.6 mm diameter non-porous glass beads (Propper MFG. CO., L. I. City, NY) (64). The beads were packed into a reactor of 0.86 mm i.d. poly(tetrafluoroethylene) tubing (Benton-Dickinson, Parsipanny, NJ). The sample was then mixed with a reagent stream that contained horseradish peroxidase, and Leucomalachite Green (LMG) (Figure 9). A colored product (MG^+) was formed in a plain SBSR and was detected photometrically.

The pump tubing for the carrier, the sample and the reagent stream were, 0.42 cc/min, 0.32 cc/min, and 0.06 cc/min respectively. Different pieces of tubing were connected to one another as well as to the different components of the system by means of connectors. Particular care was taken, in making connections, to avoid dead volumes, leakage or the introduction of air bubbles.

The parameters and their ranges of the values studied for the optimization effort were the following: concentration of LMG stock solution, 0.303-15.151 mM; concentration of LMG reagent solution, 6×10^{-2} - 12×10^{-2} mM; activity of peroxidase, 72-178 units; pH of LMG stock solution, 1.65-3.0; flow rate, 0.2-1.8 ml/min; length of enzymatic SBSR, 8-16 cm; length of unmodified SBSR, 14-40 cm; pH of carrier, 5.0-6.5; and effect of temperature, 20-50 ^{0}C.

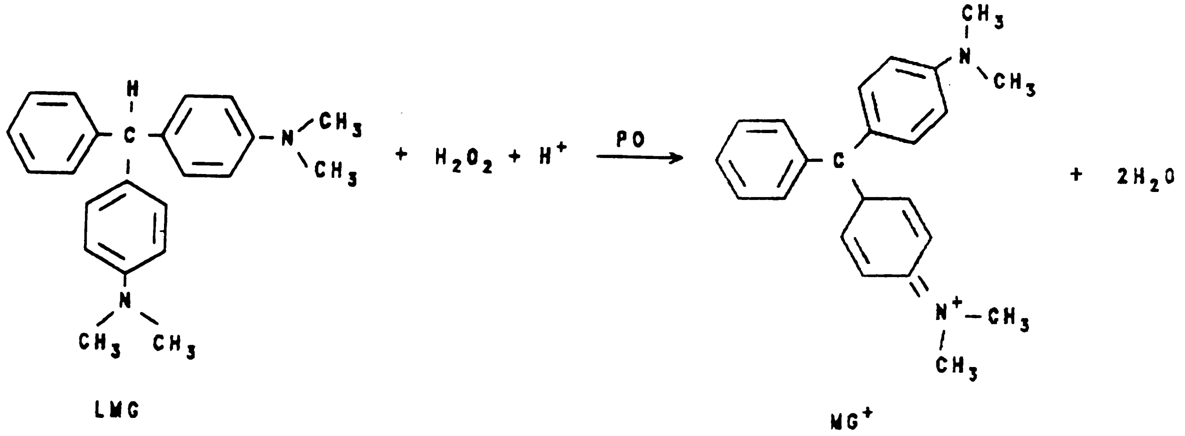


Figure 9. Oxidation of Leucomalachite Green to Malachite Green.

For the optimization of the FIA method and for the applications in determining fruit sugars, six replications were performed for each sample.

2. Reagents

All stock solutions were prepared with distilled water and filtered before use. All stock solutions were diluted with 0.05 M phosphate buffer, pH 6.85. All chemicals (reagent grade) were used without further purification.

Anhydrous β-D(+)-glucose grade III, sucrose grade II and β-D(-)-fructose crystalline (all from Sigma Chemical Co., St.Louis, MI USA) were used to prepare the standard solutions (all 0.01 M) that contained 0.5 g L^{-1} benzoic acid as preservative. The reagent for the Leucomalachite Green (p,p'-Benzylidene-bis-N,N-dimethylaniline) (LMG) indicator reaction was prepared immediately before use and contained peroxidase, dissolved in 0.1 M phosphate buffer, pH 6.0, LMG, and 0.1 M acetate buffer, pH 4.0. For comparison purposes the Trinder indicator reaction (66) was also employed. The reagent for the Trinder indicator reaction was prepared immediately before use and contained 143 units peroxidase, 1 mM 4-aminoantipyrine (from Sigma), 1 mM 3,5-dichloro-2-hydroxyphenyl sulfonic acid mixed together and then diluted with 0.05 M phosphate buffer, pH 6.85, to 10 ml in a volumetric flask (63). A stock solution of 0.06 M of H_20_2 (30 % W/W from Sigma Chemical Company) was used for the preparation of the standard solutions. Stock magnesium chloride ($M_gCl_2.6H_2O$) solution 0.1 M and cobalt chloride ($C_OCl_2.6H_2O$) solution 0.01 M were prepared for use as activators for the glucose isomerase conversion reaction in 0.05 M phosphate buffer and pH 7.5 environment.

The enzymes used were horseradish peroxidase (Sigma, Type II, from Aspergillus niger, activity approximately 17800 units g^{-1}), invertase (Sigma, grade VII, from Baker's Yeast, activity approximately 400 units mg^{-1}), glucose isomerase (Spezyme GI-M600) (Finnsugar Biochemicals, Inc., activity approximately 3290 units g^{-1}), and ascorbate oxidase (Boehringer Mannheim, from Cucurbita species, activity approximately 170 units mg^{-1}).

Glucose oxidase was immobilized on non-porous glass beads by the procedure described by Stults (64).

3. Preparation of the Samples

All solutions of H_2O_2 and standard sugars, for the optimization studies , were prepared with 0.05 M phosphate buffer pH 6.85.

The fruit samples tested were olives from California (Manzanillo and Ascolano), from Greece (Coroneiki and Amphisis) and from Cyprus (Cypriot, Manzanillo and Ascolano); cherries from Michigan (Wolynska, Montmorency and I 20(36)); and citrus fruits (oranges "NAVEL" from California, lemons from California, and grapefruit from Florida). The olive and cherry samples were in different maturity stage, but the citrus fruit samples were in the ripe stage. Fifty milliliters of the citrus juice samples, which weighed 52.5 g, 52.3 g and 52.4 g for the oranges, lemons and grapefruit juice, respectively, were diluted 1:10 prior to use for preparation of the FIA working solution . Ten grams of olive fruit without seeds were blended for 5 min with 30 ml water. The slurry was centrifuged at 2100 rpm for 10-15 min and then filtered under vacuum twice. The filtrate was diluted to 50 ml total volume with water and used for the preparation of the FIA working solution without any further dilution. Cherry selections were harvested at the

Clarksville Horticultural Experiment Station and frozen at -20 °C under nitrogen, a few hours after harvesting. Fifty grams of frozen cherries without seeds were blended at high speed with 50 ml water for 10-15 min. The slurry was centrifuge at 2100 rpm for 10-15 min and then filtered under vacuum, twice. The solution was diluted to 100 ml by using distilled water. A 1:10 dilution was done prior to use for preparing the FIA working solution. All working solutions were prepared with 0.05 M phosphate buffer, pH 6.85, and used 0.2 ml of the sample solution.

4. Procedure

Six replicate measurements were done for each sample. The conditions for all measurements were the optimum for the new indicator reaction of LMG (see below).

a. Glucose Analysis

For the determination of glucose, each sample was transferred into a separate 10 ml volumetric flask; the amounts taken were 0.2 ml of the citrus juice (after dilution), 0.2 ml of cherry juice, and 0.2 ml of olive juice. 1 ml stock ascorbate oxidase solution was prepared using 0.1 M phosphate buffer pH 5.5 and 10 mg ascorbate oxidase (1700 units mg^{-1}). 0.02 ml of this stock solution was transferred into all juice samples to destroy the undesirable ascorbic acid. The volumetric flasks were filled to volume with 0.05 M phosphate buffer, pH 6.85, to make the working solutions. The FIA determinations begun after a delay of 15 min to allow the α and β forms of D-glucose to reach equilibrium in the phosphate buffer (65). After injection, approximately 85 sec passed under the conditions used before the FIA signal was obtained with the

computer data acquisition system. Peak absorbance values were used for the calculations.

b. Fructose Analysis

For the determination of fructose, the same procedure was used as for glucose except that the samples were treated with glucose isomerase (GI) prior to the glucose determination. The conversion reaction of fructose to glucose proceeded in the presence of the enzyme activators, M_gCl_2, C_oCl_2, at pH 7.5 and at 60 °C. A stock activator mixture was prepared, which contained 2 ml of 0.1 M M_gCl_2, 2 ml of 0.01 M C_oCl_2, 2 ml of 1M phosphate buffer, pH 7.5 and 6 ml of H_2O. A 0.3 ml volume of the activator stock solution and 0.1 ml glucose isomerase enzyme were transferred to all 10 ml volumetric flasks. The reaction was run at pH 7.5 at 60 °C for 25 min. Then the volumetric flasks containing the treated samples for fructose analysis were filled to volume with the buffer solution. The FIA determinations of glucose were again begun 15 min after the dilution. A blank sample without glucose isomerase was run and the appropriate corrections to the samples with enzyme were done. A relatively high concentration of glucose isomerase was used in order to speed up the conversion reaction.

c. Sucrose Analysis

For the determination of sucrose, the same procedure was used except that the samples were treated with invertase prior to the glucose determination. Ten milliliters of stock solution of invertase was prepared using 0.1 M acetate buffer pH 4.5 and 5 mg invertase. A 0.1 ml volume of this stock solution was transferred to all volumetric flasks to convert the sucrose to the invert sugar. The conversion reaction proceeded under optimum conditions: one unit of invertase hydrolyzed

1.0 μmole of sucrose to invert sugar per min at pH 4.5 and 55 °C for 15 min.

B. Conventional Enzyme Methods for Sugar Analysis

1. Apparatus

The apparatus used for the conventional enzyme method of sugar analysis consisted of a Perkin-Elmer Lambda 4B, UV/VIS spectrophotometer with a Perkin-Elmer Lambda Accessory Interface and an Epson printer. The wavelength of the determination was 340 nm and Absorbance was measured.

2. Reagents

All stock solutions were prepared with distilled water. For these experiments a kit for sucrose, D-glucose and D-fructose was used (Methods of Food Analysis using Test-Combinations, Boehringer Mannheim). This kit consisted of five bottles. Bottle 1 with approximately 0.5 g lyophilisate, contained: citrate buffer, pH 4.6; b-fructosidase, 720 units; stabilizers. Bottle 2 with approximately 7.2 g powder mixture contained: triethanolamine buffer, pH 7.6; NADP, 110 mg ; ATP, 260 mg; magnesium sulfate; stabilizers. Bottle 3 with 1.1 ml enzyme suspension, consisting of: hexokinase, 320 units; glucose-6-phosphate dehydrogenase, 160 units. Bottle 4 contained approximately 0.6 ml phosphoglucose isomerase suspension, 420 units. And bottle 5 contained standard sucrose.

Anhydrous β-D-glucose grade III and β-D-fructose crystalline (all from Sigma Chemical Co.) and sucrose (Boehringer Mannheim) were used to prepare the standard solutions (2 g/L) for the evaluation of this method.

3. Preparation of the Samples

The samples tested were olives (from California, Greece and Cyprus), cherries (from Michigan) of different maturity, and citrus fruits (oranges from California, lemons from California, and grapefruit from Florida) in the ripe stage. Fifty milliliters of the citrus juice samples weighing 52.5 g for orange, 52.3 g for lemon and 52.4 g for grapefruit juice were diluted 1:25 prior to use for the soluble enzyme analysis. Ten grams of olive samples without seeds were used for the sugar extraction. Fifty milliliters distilled water were used as extraction solvent and the sample was diluted to 1:10 prior to analysis. Fifty grams of cherry without seeds were blended with water for 10 min and the slurry was centrifuged and filtered. The final volume of the filtrate was made to 100 ml and diluted 1:50 prior to use for the sugar analysis.

4. Procedure

The D-glucose concentration was determined before and after the enzymatic hydrolysis of sucrose; D-fructose was determined after the determination of D-glucose. In all determinations the volume of the fruit sample solution used was 0.1 ml. For each measurement of D-glucose or D-fructose the whole procedure took 50-60 min plus 50-60 min for the D-glucose\D-fructose blank sample. For the sucrose measurement the procedure took 35-40 min plus 35-40 min for the sucrose blank sample. Three replicate measurements were done for each standard sample of glucose, fructose and sucrose, but only one measurement was made for the fruit samples. All the appropriate corrections in the calculations were applied.

a. Determination of D-glucose before inversion

The enzyme hexokinase (HK) catalyzes the phosphorylation of D-glucose by adenosine-5-triphosphate (ATP) with the simultaneous formation of adenosine-5-diphosphate (ADP).

$$\text{D-Glucose} + \text{ATP} \text{ -------->} \text{ G-6-P} + \text{ADP}$$

In the presence of glucose-6-phosphate dehydrogenase (G6P-DH) the glucose-6-phosphate (G-6-P) formed is specifically oxidized by nicotinamide-adenine dinucleotide phosphate (NADP) to gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH).

$$\text{G-6-P} + \text{NADP}^+ \text{ -------->} \text{ gluconate-6-phosphate} + \text{NADPH} + \text{H}^+$$

The NADPH formed in this reaction is stoichiometric with the amount of D-glucose and is measured by means of its absorbance at 340 nm.

b. Determination of D-fructose

Hexokinase also catalyzes the phosphorylation of D-fructose to fructose-6-phosphate (F-6-P) with the aid of ATP.

$$\text{D-fructose} + \text{ATP} \text{ -------->} \text{ F-6-P} + \text{ADP}$$

On completion of this reaction F-6-P is converted by phosphoglucose isomerase (PGI) to G-6-P.

$$\text{F-6-P} \text{ -------->} \text{ G-6-P}$$

G-6-P reacts again with NADP^+ with formation of gluconate-6-phosphate and NADPH. The amount of NADPH formed now is stoichiometric with the amount of D-fructose.

c. Determination of sucrose

Sucrose is hydrolyzed by the enzyme invertase to D-glucose and D-fructose.

$$\text{Sucrose} + H_2O \text{ --------> D-glucose} + \text{D-fructose}$$

The determination of D-glucose after inversion (total D-glucose) is carried out according to the reaction above. The sucrose content is calculated from the difference of the D-glucose concentrations before and after enzymatic inversion.

IV. RESULTS AND DISCUSSION

The main objective of this work was to optimize the conditions for the FIA determination of glucose, using a new indicator reaction. Two more sugars, fructose and sucrose, were subjected to this analysis, after their conversion to glucose, and a comparison was made with a conventional method of sugar analysis.

A. Optimization of the FIA System

1. Initial Optimization of the Indicator Reaction

One of the most commonly used method, for the colorimetric determination of glucose is the Glucose Oxidase / Trinder reaction (66-68). In this reaction, glucose and molecular oxygen in the presence of glucose oxidase (GO) produce hydrogen peroxide (Figure 10a). The H_2O_2 then reacts with 4-aminoantipyrine (AAP) and 3,5 dichloro-2-hydroxyphenyl sulfonic acid (DCPS) in the presence of peroxidase (PO) to produce a colored compound with an absorbance maximum at 510 nm (69). The Trinder reaction has been used by many researchers for glucose determinations in clinical applications using immobilized glucose oxidase and flow injection analysis (FIA) systems (70-72). Also Stults (64) optimized the Trinder reaction for the enzymatic determination of glucose with a flow injection analysis system. However, the Trinder reaction has certain shortcomings such as limited sensitivity and a small linear dynamic range. Also, for applications with food samples with high concentration of sugars, the Trinder reaction appeared to be not the reaction of choice, because the samples had to be diluted several times.

(a) **Trinder Reaction:**

$$\text{DCPS} + \text{AAP} + H_2O_2 \xrightarrow{\text{Peroxidase}} \text{Quinonimine Dye} + H_2O$$

DCPS = 3,5-dichloro-2-hydroxyphenyl sulfonic acid
AAP = 4-aminoantipyrine

(b) **Malachite Green Reaction:**

$$\text{LMG} + H_2O_2 \xrightarrow{\text{Peroxidase}} \text{Malachite Green} + H_2O$$

LMG = leucomalachite green

Figure 10. (a) Detection of hydrogen peroxide with the Trinder Reaction. (b) Detection of hydrogen peroxide with the Malachite Green Reaction.

Several other dyes have been used by many workers for the coloremetric assays of H_2O_2 in the presence of peroxidase (PO) such as benzidine (73), leucomalachite green (74-82), and o-dianisidine (73).

In this work the LMG reaction has been optimized and used as the new indicator reaction in order to overcome the limitations of the Trinder reaction for practical applications in the food science area (Figure 10b). There are several advantages of using LMG in the indicator reaction. First, LMG is more sensitive in its response over the desirable absorbance range than the Trinder reaction. Figures 11, and 12 show a comparison of the LMG and Trinder reactions, using H_2O_2 and glucose standard solutions in 620 nm and 510 nm, respectively. The experimental conditions for this comparison were as follows: 1M acetate buffer, pH 2.25, for LMG stock solution; 3 mM of stock LMG solution; 143 units of peroxidase per 10 ml of reagent; 12×10^{-2} mM LMG of reagent solution; 5.0 ml, 0.1 M phosphate buffer, pH 6.0, per 10 ml of reagent; 4.2 ml, 0.1 M acetate buffer, pH 4.0, per 10 ml of reagent; 10 cm of enzymatic SBSR; 30 cm of unmodified SBSR; pH 6.5, 0.1 M phosphate buffer as a carrier; and pump setting 80. The pump setting of 80 was not the optimum but gave higher absorbance values than the pump setting of 45, used with the Trinder reaction (63). For the LMG and Trinder reaction, the slopes in Figure 11 were 0.1147 and 0.0406 A/mM, and the standard errors of the estimate (relative to the mean of absorbance), were 1.55 % and 1.78 %, respectively. In addition, absorption measurements at 620 nm, as used for LMG, are often an obvious advantage, because fewer potentially interfering materials absorb significantly at

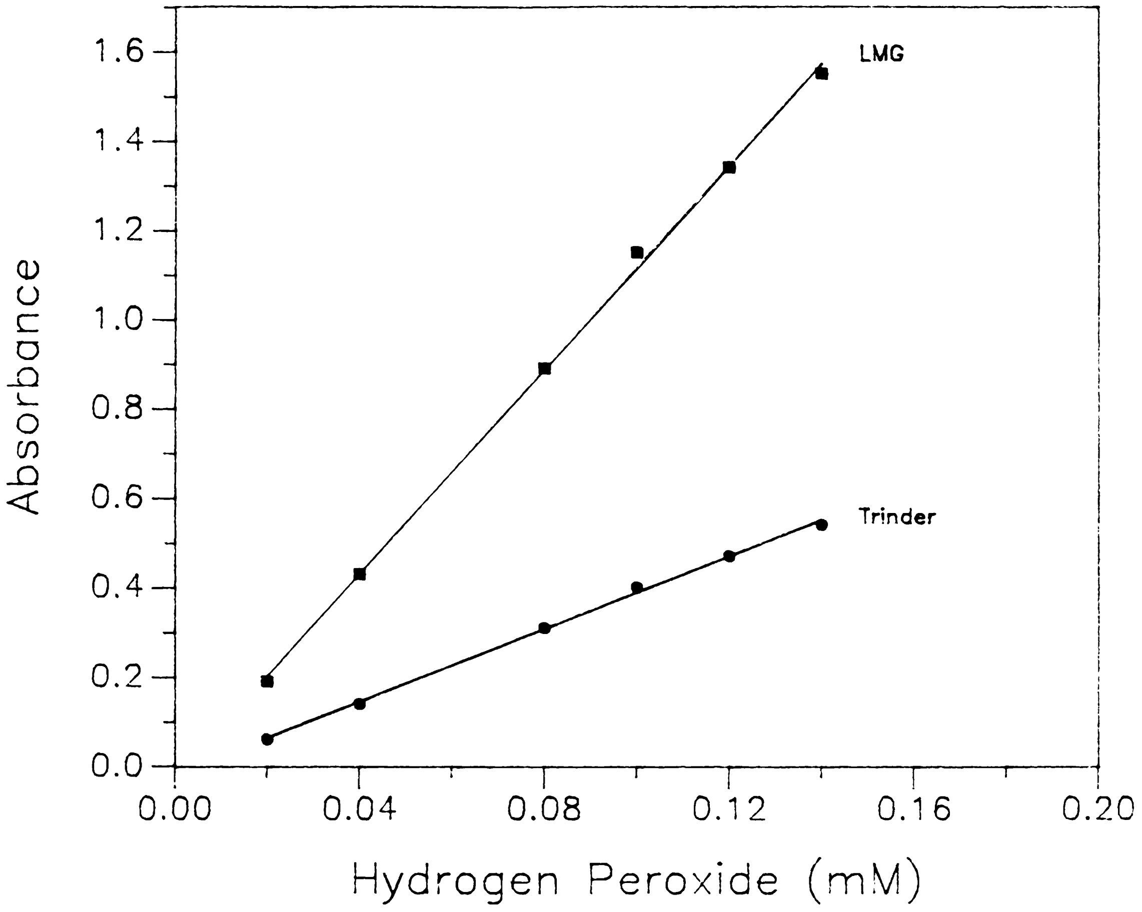


Figure 11. Comparison of LMG and Trinder reaction using hydrogen peroxide. The lengths of the enzymatic and plain SBSRs were 10 cm and 30 cm, respectively.

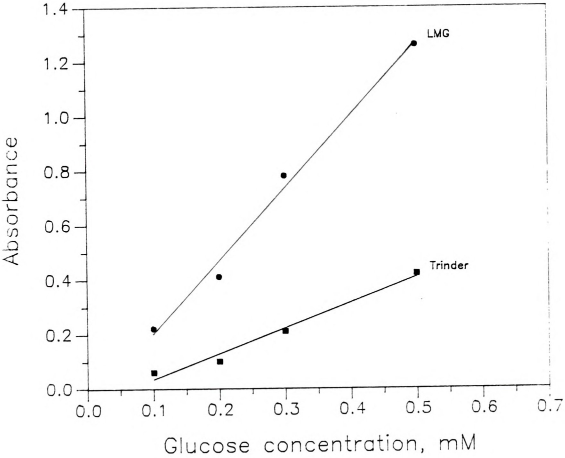


Figure 12. Comparison of LMG and Trinder reaction using a glucose oxidase reactor. The lengths of the enzymatic and plain SBSRs were 10 cm and 30 cm, respectively.

this wavelength than at 510, 520 or 395-460 nm, the wavelength used in analyses with the Trinder, benzidine or o-dianisidine, reactions, respectively (73).

For the initial optimization of the LMG indicator reaction univariate methods were carried out in order to optimize primarily the conditions of the reaction. The simplex optimization methods were used in order to optimize the indicator reaction adapted by the FIA system. For the univariate experiments all the FIA parameters such as flow rate, tubing size, injected sample size, and pH of the carrier stream were kept constant. The variables that optimized were the following: concentration of the LMG stock solution; concentration of the LMG reagent solution; activity of peroxidase; and pH of the LMG stock solution. For the general simplex optimization, nine variables were employed in the procedure. Those nine variables were: the pump setting, the carrier pH, the carrier concentration, the length of enzyme SBSR, and the length of plain SBSR as the instrumental variables; and the peroxidase activity, the volume of the LMG stock solution, the volume of 0.1 M phosphate buffer, pH 6.0, and the volume of 0.1 M acetate buffer, pH 4.0, used for the preparation of 10 ml of reagent.

a. Concentration of LMG stock solution

According to Ahlquist (73), on a molar basis, the optimum concentration of LMG was 50 % less than all the other dyes tested (benzidine, o-dianisidine), while the optimum concentration of H_2O_2 for LMG was 25 % less than all the other dyes tested. This difference in H_2O_2 requirement seems reasonable, because twice as many hydrogen atoms are lost from LMG in the oxidation reaction than for any of the other dyes tested by the same author (Figure 9). This fact makes LMG very

sensitive even at very low concentrations. Also, according to the same author (73), the sensitivity is dependent on the ratio of the LMG to hydrogen peroxide. In excessive amounts of either LMG or hydrogen peroxide, the peroxidase activity is inhibited. This could have been a disadvantage of the LMG, but working with low concentrations of LMG stock solutions the absorbance values were in a desirable range (up to 1.6) and the hydrogen peroxide concentration low. For concentrations up to 0.3 mM H_2O_2 the peroxidase enzyme was not inhibited (73). The optimum concentration for the LMG stock solution was selected to be approximately 1.515 mM for 0.06 mM H_2O_2 solution (Figure 13). The experimental conditions applied here were as follows: 1M acetate buffer, pH 2.25, for the LMG stock solution; 143 units of peroxidase per 10 ml of reagent; 12 x 10^{-2} mM LMG in the reagent solution; 5.0 ml, 0.1 M phosphate buffer, pH 6.0, per 10 ml of reagent; 4.2 ml, 0.1 M acetate buffer, pH 4.0, per 10 ml of reagent; 10 cm of enzymatic SBSR; 30 cm of unmodified SBSR; pH 6.5, 0.1 M phosphate buffer as a carrier; and pump setting 80. For the preparation of 10 ml of reagent solution, 143 units of peroxidase were dissolved in 5.0 ml of 0.1 M phosphate buffer, pH 6.0. To this 0.8 ml of 1.515 mM LMG stock solution and 4.2 ml of 0.1 M acetate buffer, pH 4.0, were added. Always, the same sequence was remained for the preparation of the reagent solution. For such low H_2O_2 concentrations (0.06 mM) the absorbance values were in the range of 0.3-0.64 for LMG concentrations in the range of 0.303-15.151 mM (Figure 13).

In order to ascertain the stability of the LMG/peroxidase reagent, the activity of this solution was examined at room temperature every 30 min. Comparing 1.515 mM and 3.030 mM LMG, at higher concentrations of

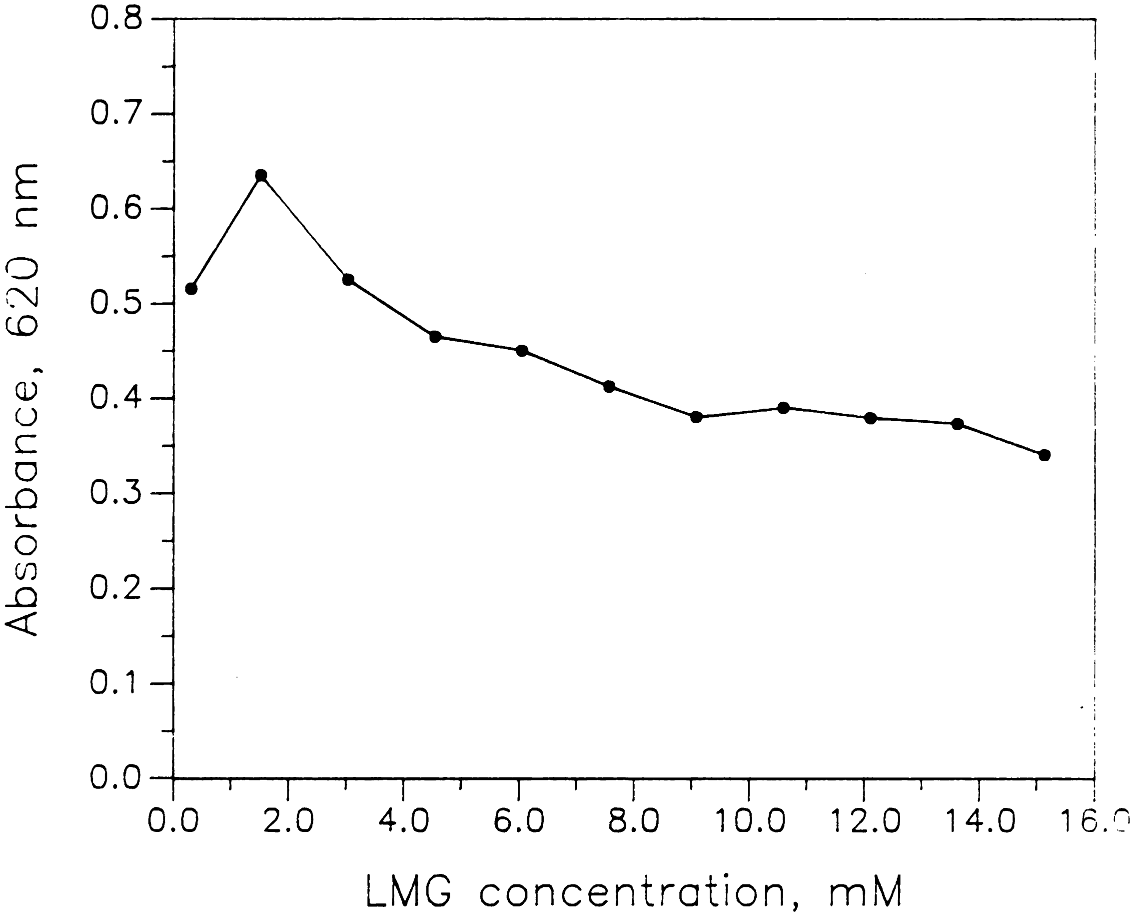


Figure 13. Relationship between LMG concentration and absorbance of the H_2O_2 reaction product. The lengths of enzyme and plain SBSRs were 10 cm and 30 cm, respectively.

H_2O_2, such as 0.12 mM, the two mixed solutions showed no considerable differences (Figures 14, and 15). A small loss of activity can be seen over the 270 min periods for both LMG concentrations. The experimental conditions for this comparison were as follows: 1M acetate buffer, pH 2.25, for the LMG stock solution; 143 units of peroxidase per 10 ml of reagent; 12 x 10^{-2} mM LMG of reagent solution; 5.0 ml, 0.1 M phosphate buffer, pH 6.0, per 10 ml of reagent; 4.2 ml, 0.1 M acetate buffer, pH 4.0, per 10 ml of reagent; 10 cm of enzymatic SBSR; 30 cm of unmodified SBSR; pH 6.5, 0.1 M phosphate buffer as a carrier; and pump setting 80. As can be seen in Figure 14 and 15, the solutions prepared from 1.515 mM and 3.030 mM had a similar profile of losing activity over time (up to 270 min), and the same maximum absorbance of 1.27 at time zero, which is sufficient and accurate for making calibration curves and measuring real food samples.

For low concentrations of H_2O_2, the mixed solution which was prepared with the 1.515 mM stock LMG solution was slightly more sensitive than that prepared with 3.030 mM (Figure 16). When the concentration of H_2O_2 was increased, the mixed solution, which was prepared with 3.030 mM LMG stock solution, had the disadvantage of losing its sensitivity. For the LMG stock solutions of 0.303, 1.515 and 3.030 mM, the slopes of the Figure 16 were 0.1225, 0,1147, 0.1048 A/mM, and the standard errors of the estimate (relative to the mean of absorbance), were 1.16 %, 1.32 %, 1.45 %,, respectively. Also, disadvantages such as precipitation in the plain reactor and flow cell occurred with the 3.030 mM stock LMG solution. As a result, the instrument was noisy and sluggish in returning to the base line for a new injection. In this case more reagent and more solution were consumed

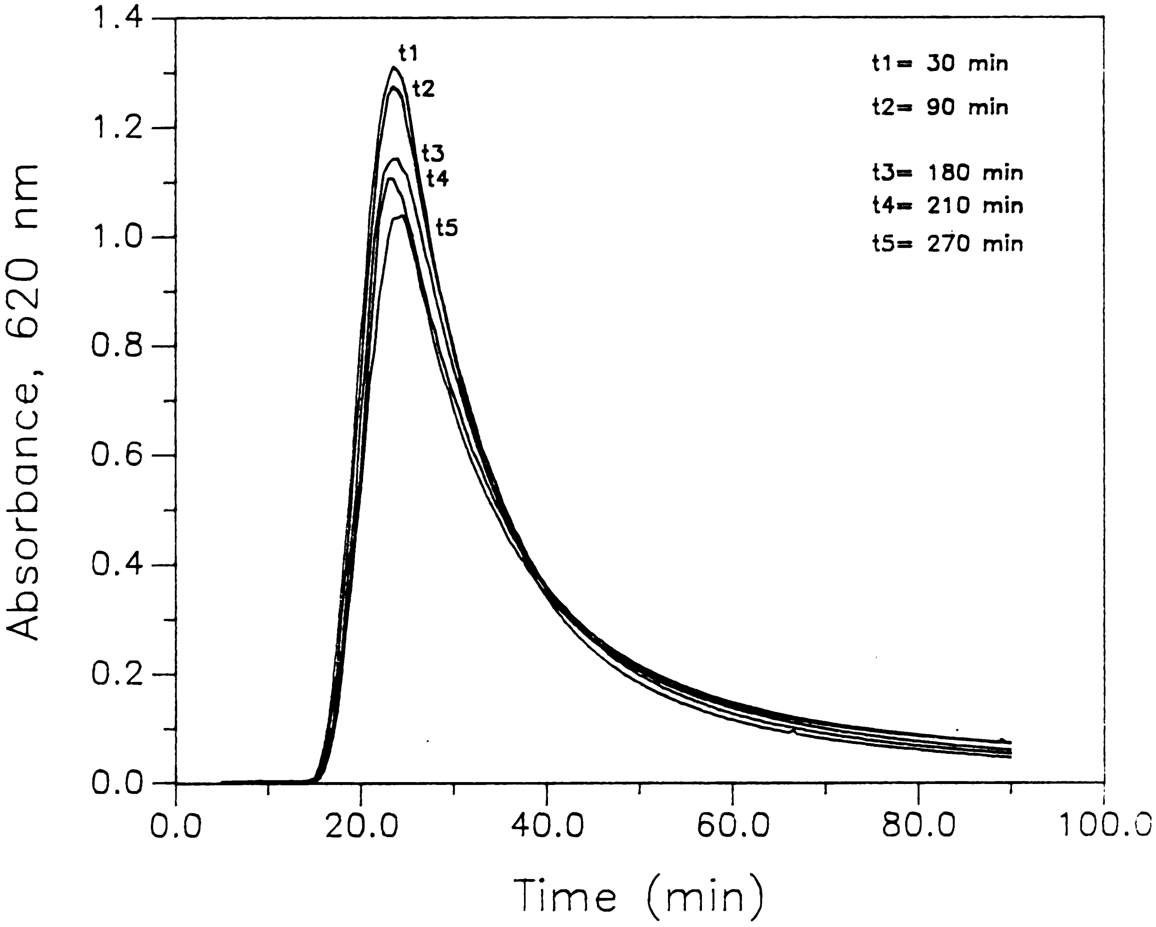


Figure 14. Loss of LMG reagent activity during storage up to 270 min. The concentration of the stock LMG solution was 1.5 mM. The lengths of enzyme and plain SBSRs were 10 cm and 30 cm, respectively. X-Axis is time of signal detection.

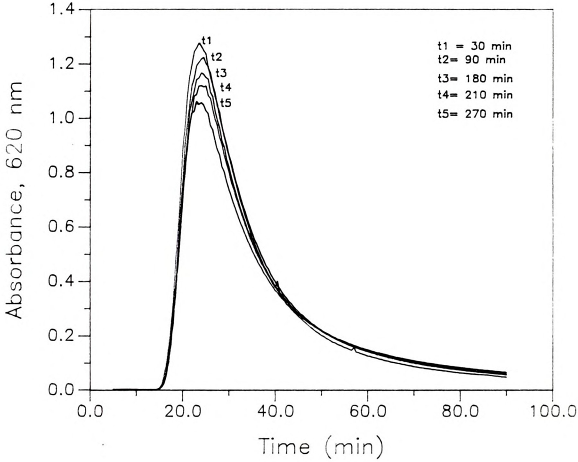


Figure 15. Loss of LMG reagent activity during storage up to 270 min. The concentration of the stock LMG solution was 3.0 mM. The lengths ofenzyme and plain SBSR were 10 cm and 30 cm,respectively X-Axis is time of signal detection.

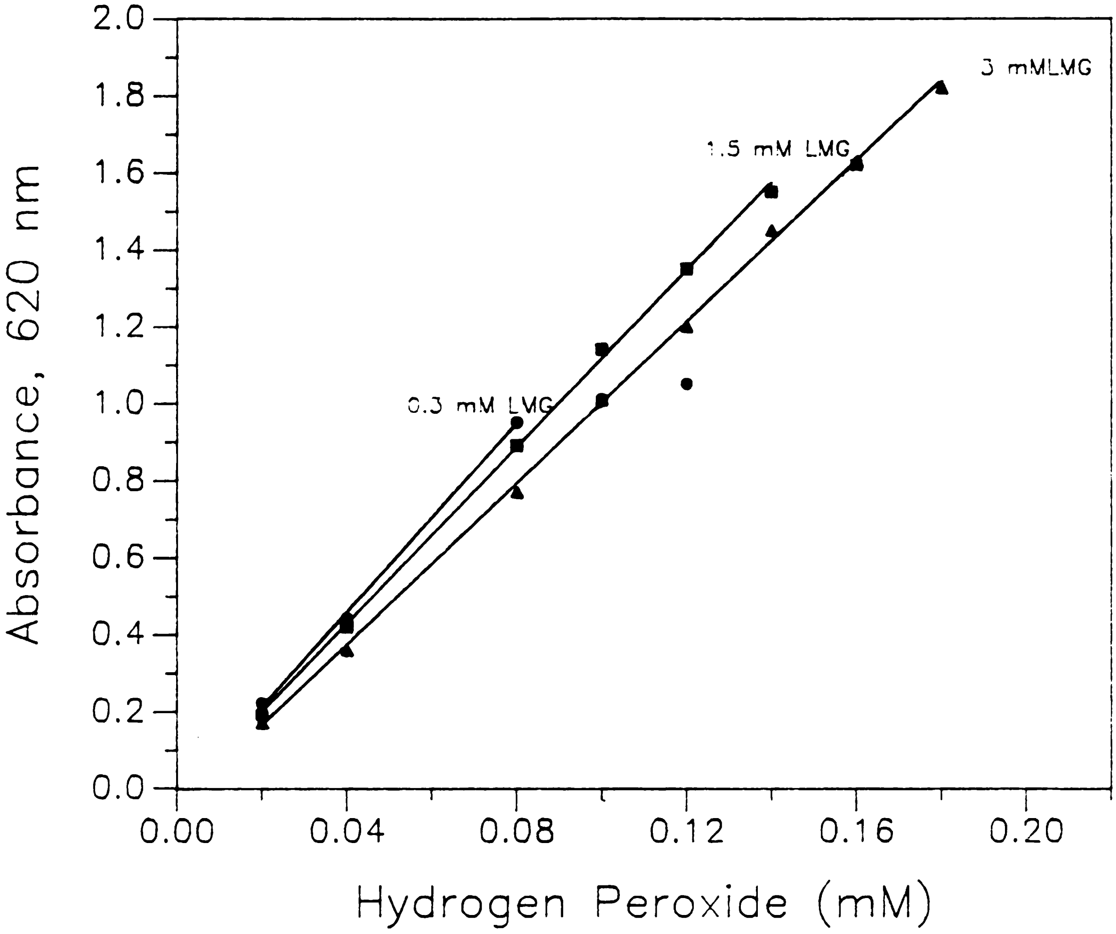


Figure 16. Comparison of two concentrations of LMG stock solution for reaction sensitivity. The lengths of enzyme and plain SBSRs were 10 cm and 30 cm, respectively.

The biggest disadvantage was the insolubility of the LMG at higher concentrations.

b. Concentration of LMG Reagent Solution

Since the concentration of stock solution of LMG of 1.515 mM was sufficient for linearity and sensitivity in food applications, the concentration of the LMG reagent solution was optimized. This experiment was performed only to find an optimum volume (ml) of the stock LMG solution needed to prepare 10 ml of reagent. The experimental conditions applied here were as follows: 1M acetate buffer, pH 2.25, for the LMG stock solution; 143 units of peroxidase per 10 ml of reagent; 5.0 ml, 0.1 M phosphate buffer, pH 6.0, per 10 ml of reagent; 4.2 ml, 0.1 M acetate buffer, pH 4.0, per 10 ml of reagent; 10 cm of enzymatic SBSR; 30 cm of unmodified SBSR; pH 6.5, 0.1 M phosphate buffer as a carrier; and pump setting 80. Figure 17 shows the optimum concentration of LMG reagent solution to be 9.09×10^{-2} mM (equivalent to 0.6 ml of 1.515 mM LMG stock solution), when the peroxidase activity was 143 units per 10 ml of reagent. The standard solution of H_2O_2 was 0.1 mM and the absorbance found under the optimum conditions was 1.42.

c. Activity of Peroxidase

Once the concentrations of stock and reagent solutions of LMG were optimized, the testing of the activity of peroxidase was carried out using 72, 143, and 178 units of peroxidase per 10 ml reagent. This experiment was performed only to find an estimate of the optimum activity of the peroxidase enzyme. The experimental conditions applied here were the following: 1M acetate buffer, pH 2.25, for LMG stock

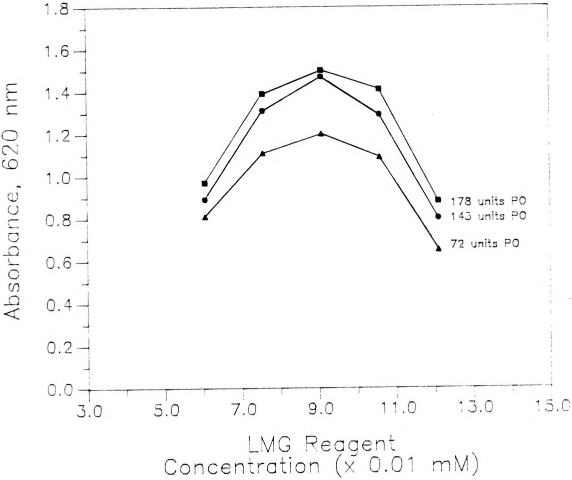


Figure 17. Relationship between LMG reagent concentration and absorbance of reaction product at three concentrations of peroxidase (PO). The lengths of enzyme and plain SBSRs were 10 cm and 30 cm respectively. The standard hydrogen peroxide solution was 0.1 mM.

solution; 5.0 ml, 0.1 M phosphate buffer, pH 6.0, per 10 ml of reagent; 0.6 ml of 1.515 mM LMG stock solution; 4.4 ml, 0.1 M acetate buffer, pH 4.0, per 10 ml of reagent; 10 cm of enzymatic SBSR; 30 cm of unmodified SBSR; pH 6.5, 0.1 M phosphate buffer as a carrier; and pump setting 80. Figure 17 shows the optimum LMG reagent concentration to be 9.09×10^{-2} mM at all these different activities of peroxidase. The concentration of the standard H_2O_2 used was 0.1 mM. Since the results for 143, and 178 peroxidase (PO) units at the optimum LMG reagent concentration are very close, the value of 143 units peroxidase per 10 ml of reagent solution was selected as optimum for technical and economic reasons.

d. pH of LMG Stock Solution

According to Ahlquist (73) LMG is sparingly soluble in water, but is quite soluble in low concentrations, in organic liquids, such as acetic acid. It is nearly ideal in producing a intense and stabile color with low reagent blank.

LMG was tested for solubility and stability in solution with four preparations of acetate buffer with different molarities and acidities. In phosphate buffer LMG was oxidized to MG^+, as noted by the stock solution turning dark green upon preparation. Also, the absorbance values obtained with the phosphate buffer preparation of LMG were low. The optimum conditions of LMG stock reagent concentrations and peroxidase activity were used for the preparation of the reagent solution. The concentration of the standard H_2O_2 was 0.1 mM. Table 2 shows that the absorbance values were higher at a carrier pH of 6.0 with all LMG stock buffers. Also, the absorbance values increased when the

Table 2. Comparison of Buffer Conditions for LMG Stock Solution

	Absorbance (620 nm)	
	Carrier pH	
LMG Stock Buffer	6.85	6.00
Acetate 1.0 M, pH 3.00	0.45	0.66
Acetate 1.0 M, pH 2.25	1.40	1.60
Phosphate 1.0, M pH 1.65	0.20	0.30
Acetate 2.0 M, pH 1.65	2.00*	2.20*
30% Acetic Acid, pH 1.65	2.20*	2.40*

* Values at upper limit of detection

pH values of the LMG stock buffers were lower. LMG gave the best results in 30 % acetic acid adjusted to pH 1.65. In general the solubility of LMG increased in higher acidity.

e. General Simplex Optimization of FIA

The major assumption during the univariate experiments was that there was no interaction between variables. The nine variables mentioned previously were studied in order to find the optimum indicator reaction activity and the optimum FIA system. Under the conditions of optimization of the nine different variables, which may interact with each other, the univariate method of optimization has the disadvantage of requiring several thousand experiments. Therefore, a better alternative is the simplex optimization (83), which allows simultaneous variation of all parameters.

The simplex method is widely applied and accepted in many research areas. A simple two dimensional surface, as pictured in Figure 18, can be used to illustrate the principles employed. The x and y axes represent the two parameters to be varied and each concentric circle represents combinations of those two which have the same response. This surface can be thought of as a topographical map; as the circles get smaller the response increases in magnitude. The simplex is generated initially by choosing a set of experimental conditions which are known to be suboptimal. After the response from that experiment is obtained another set of conditions is specified. This process is repeated until the geometric shape made of n+1 vertices is obtained, where n is the number of parameters. In the two parameter case, three experiments are required and the simplex takes the shape of a triangle.

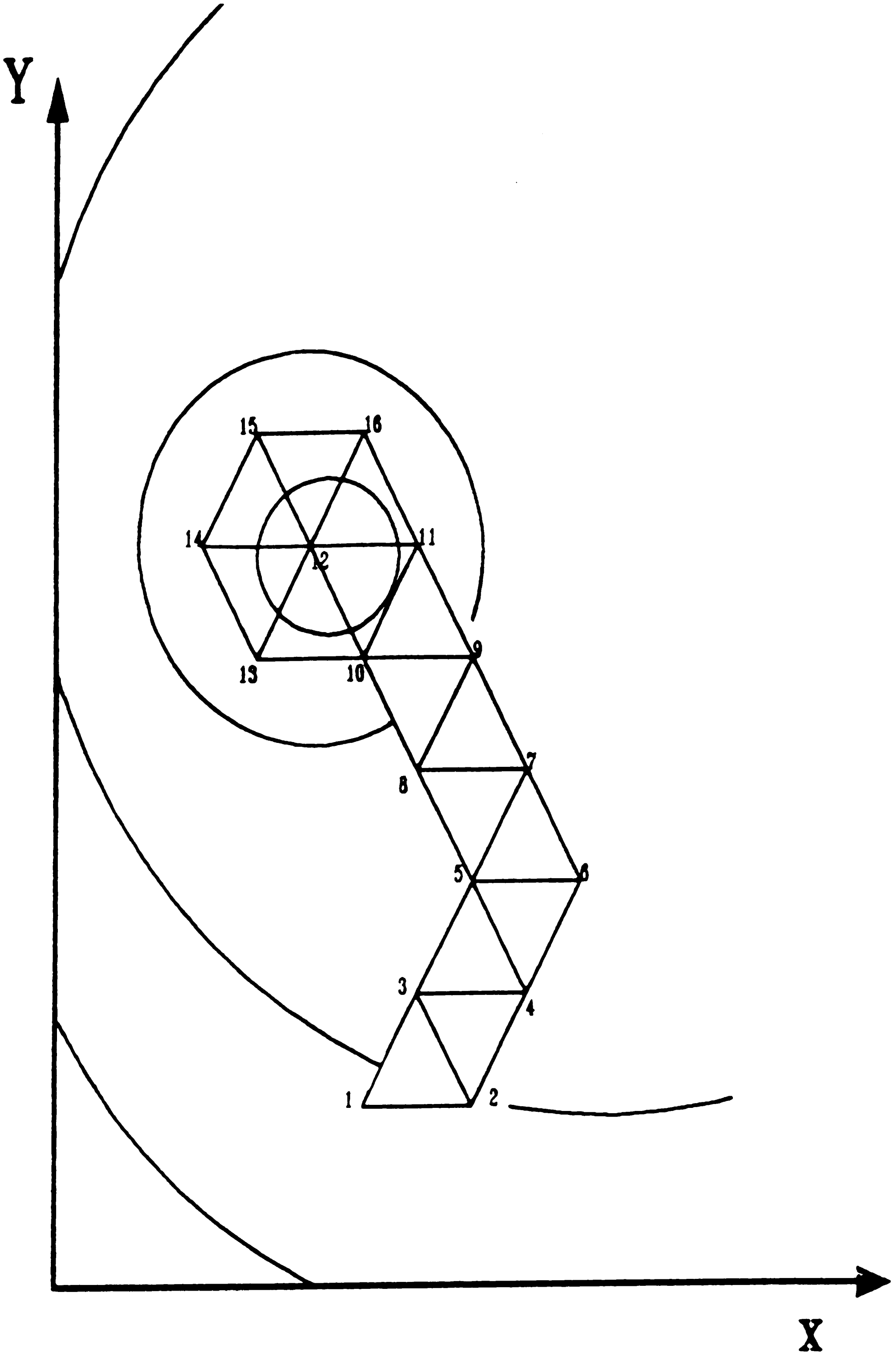


Figure 18. Movements of standard step size simplex procedure on response surface. The initial simplex is 1, 2, 3 and the optimum region lies close to point 12.

The response at each vertex is ranked based on its magnitude: the largest is taken as best, the next largest as next best, etc. In the triangular case, the point that gives the worst response (point 1 in the Figure 18) is reflected an equal distance through the line between the other two points. A new set of conditions is specified, the response obtained, and the responses are again ranked. In this example, the response surface is shown so that the movement of the simplex can be understood. Normally, the response surface is unknown and becomes defined by the movement of the simplex. As the optimum set of conditions is reached the simplex may begin to oscillate. Such behavior can be thwarted by reflecting the next best point rather than the worst.

The response function chosen was based only on the maximum absorbance obtained. It is also possible (64) to optimize a more complex function that includes response time and peak width as well as peak absorbance. The whole simplex optimization was carried out only with standard hydrogen peroxide solutions, in order to avoid the problems arising from loss of activity of the immobilized enzymes with time and the consequent enprecision. Basically the reason for doing a preliminary optimization of all nine variables was to find an estimate of the optimum conditions of the FIA system using the optimum experimental conditions of the univariate methods for the indicator reaction and adapting that into the FIA system.

The initial simplex was obtained by entering the information listed in Table 3 into the modified simplex program which was run on an IBM PC compatible microcomputer. From the univariate experiments that were done prior to this optimization, the acceptable range for each of the parameters were identified. For the parameters that were not tested

Table 3. Range and Precision of Variables for Simplex Optimization

Experimental Variables	Forward Boundary	Reverse Boundary	Precision
Pump Setting	99	10	5
Carrier pH	8	5	0.5
Carrier Concentration.	0.3	0.05	0.05
Peroxidase Activity	322	72	36
LMG Stock Vol.(ml/10ml)	1	0.1	0.1
Phosphate Buffer(ml/10ml)	5	0	0.1
Acetate Buffer(ml/10ml)	4	0	0.1
Length Enzyme SBSR(cm)	14	8	2
Length Plain SBSR(cm)	40	20	5

Response to optimize: Absorbance
Precision of Absorbance: 0.05

in the univariate experiments, we used reasonable values to start with. The settings listed under "Reverse" are those which gave low absorbance values and those under "Forward" gave high absorbance values (Table 3).

Since there were nine variables, ten experiments were needed to form the initial simplex. These were performed and the results entered. The first point of the simplex was the response obtain from the baseline experiment with the initial conditions. Therefore, each time the simplex program specified a set of experimental conditions, this experiment was performed and the absorbance value was entered. Sixty-two times the program specified a set of experimental conditions and sixty-two absorbance values entered. Table 4 shows the current optimum values which are the current top 5 values after sixty-two experiments. The concentration of standard hydrogen peroxide was 0.012 mM. Six replications were done for each set of experimental conditions, and the

Table 4. Initial and Optimal Experimental Conditions for the Flow Injection Analysis System.

	Exp. No	Pump Sett.	Carrier pH	Carrier Con.	PO Con.	LMG(ml)	Phosh. Buffer	Acetate Buffer	SBSR Enzyme	Plain SBSR	Absorbance 620 nm
Start	1	70	6.85	0.10	10	0.6	5.0	4.4	10	30	0.236
Top 5	17	80	6.0	0.20	6	0.6	1.9	7.5	12	30	0.294
	31	80	6.5	0.15	10	0.7	2.7	6.6	10	30	0.304
	37	75	5.5	0.10	8	0.6	2.5	6.9	12	35	0.310
	52	80	5.5	0.10	8	0.6	2.4	7.0	12	35	0.326
	60	75	6.0	0.10	6	0.5	2.5	6.9	10	30	0.341
	Mean:	78	5.9	0.13	7.6	0.6	2.4	7.0	11.2	32	0.315
	SD:	2.74	0.52	0.07	1.67	0.07	0.3	0.33	1.10	2.74	0.02

SD= Standard Deviation

2. Specific Optimization of Rate-Dependent Variables

In a FIA system where many variables can interact with each other, optimization is not an easy task. The overall dispersion within the FIA system can be considered as the sum of the dispersion originating in the three main parts of the system (injector, detector and tubing-pump) (6). The dispersion due to the injector ($D_{injection}$) depends on the sample volume and the geometric aspects of the system. The dispersion due to the detector ($D_{detector}$) expresses the contribution of the flow cell geometry (shape and dimensions) to the dilution. The most significant contribution to the overall dispersion is the dispersion due to the reactor geometry and the flow rate, $D_{transport}$. In our FIA system this was the only dispersion that we tried to minimize.

During the initial optimization of the indicator reaction (above), the optimum conditions for the preparation and adaptation of the reagent into the FIA system were established. The general simplex optimization of FIA gave a new set of current optimum variables which improved the performance of the system. These values were 33 % higher than the original ones. In this part of the work, a more specific optimization was done for variables such as <u>flow rate</u>, <u>pH of the carrier</u>, and <u>length of the enzymatic</u> and <u>unmodified reactors</u>, using both univariate and simplex optimization methods. The effect of temperature in FIA using an immobilized enzyme in the enzymatic reactor was studied separately. The variables for the preparation of the reagent were fixed. This reagent was prepared immediately prior to every experiment and consisted of 7 ml 0.1 M acetate buffer, pH 4.0, 2.4 ml 0.1 M phosphate buffer, pH 6.0, 0.6 ml LMG stock solution 1.515 mM and 143 units peroxidase per 10 ml of reagent. The flow rated tubing (with special diameter) used for all the experiments consisted of a 0.32 cc/min tube for the sample channel, a 0.42 cc/min tube for the carrier stream and a 0.06 cc/min tube for the reagent stream. The flow rate of the carrier stream was selected to be seven times higher than that of the reagent stream in order to minimize both the consumption of peroxidase and dilution of the sample.

a. Univariate Methods

For the optimization of rate dependant variables using univariate methods, one variable was changed at a time while all others were held constant.

i. Flow Rate

By having the tubing in the FIA manifold and all the other variables constant, except the pump setting, an optimum for the pump setting was reached. The experimental conditions applied here were: 30 % acetic acid, pH 1.65, for the LMG stock solution (1.5 mM); 0.1 M phosphate buffer, pH 6.0, as a carrier; 143 units of peroxidase per 10 ml of reagent; concentration of LMG reagent solution, 9.09×10^{-2} mM, equivalent to 0.6 ml of 1.5 mM LMG stock solution per 10 ml of reagent; 2.4 ml of 0.1 M phosphate buffer, pH 6.0, per 10 ml of reagent; 7.0 ml of 0.1 M acetate buffer, pH 4.0, per 10 ml of reagent; a enzyme SBSR of 12 cm length; and a plain SBSR of 35 cm length.

The flow rate (FR) was related linearly to the pump speed (PS). This relationship was obtained by measuring the time required to fill a fixed volumetric flask (2 ml) with water at different pump settings. The results were expressed as ml/min and the relationship between pump setting and flow rate was a straight line. The calculated equation for this relationship was: $(FR) = (0.01828)(PS) + (0.01014)$ and was a characteristic of the pump. As can be seen in Figures 19 and 20, there is a marked increase in absorbance as the flow rate is increased by using H_2O_2 and glucose standard solutions. The concentration of the standard solutions of H_2O_2 and glucose, were 0.04 mM and 0.4 mM, respectively. In Figure 20, an optimum flow rate of 1.47 ml/min was obtained with the enzymatic reactor containing glucose oxidase. Further, increases the flow rate resulted in decreasing absorbance due to the fact that the glucose did not reside long enough in the immobilized glucose oxidase reactor. A rate of 1.47 ml/min was chosen for subsequent experiments. This gave an absorbance that was large enough to give very reproducible results yet small enough that values obtained with more concentrated samples would not be beyond the range of detection. This also allowed an acceptable sampling rate.

ii. Length of Enzymatic SBSR Reactor

A basic issue of enzymatic reactors is dispersion, which occurs when the sample travels through the reactor. One way of manipulating dispersion is by selection of the appropriate type of manifold.

The experimental conditions applied here were: 30 % acetic acid, pH 1.65, for the 1.5 mM LMG stock solution; 0.1 M phosphate buffer, pH 6.0 and 5.0, as carriers; 143 units of peroxidase per 10 ml of reagent; concentration of LMG reagent solution, 9.09×10^{-2} mM, equivalent to 0.6 ml of 1.5 mM LMG stock solution per 10 ml of reagent; 2.4 ml of 0.1 M phosphate buffer, pH 6.0, per 10 ml of reagent; 7.0 ml of 0.1 M acetate buffer, pH 4.0, per 10 ml of reagent; flow rate, 1.47 ml/min; and length of plain SBSR, 35 cm.

Figure 21 shows that the maximum absorbance (minimum dispersion)

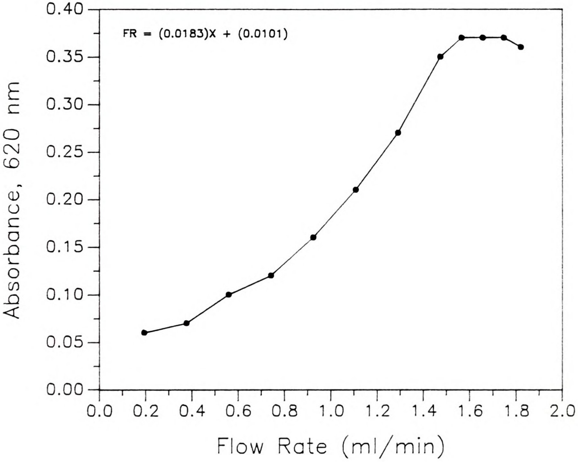


Figure 19. Relationship of absorbance and flow rate without glucose oxidase. The standard hydrogen peroxide solution was 0.04 mM and the carrier was a 0.1 M phosphate buffer pH 6.0. The lengths of enzyme and plain reactors were 12 cm and 35 cm, respectively.

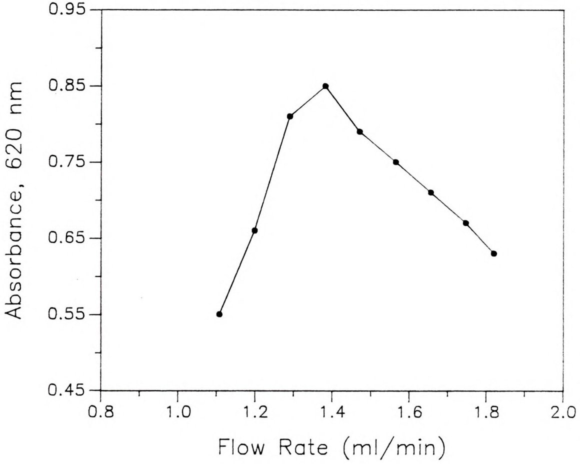


Figure 20. Relationship of absorbance and flow rate in the presence of glucose oxidase. The standard glucose solution was 0.4 mM and the carrier pH was 6.0. The lengths of enzyme and plain reactors were 12 cm and 35 cm, respectively.

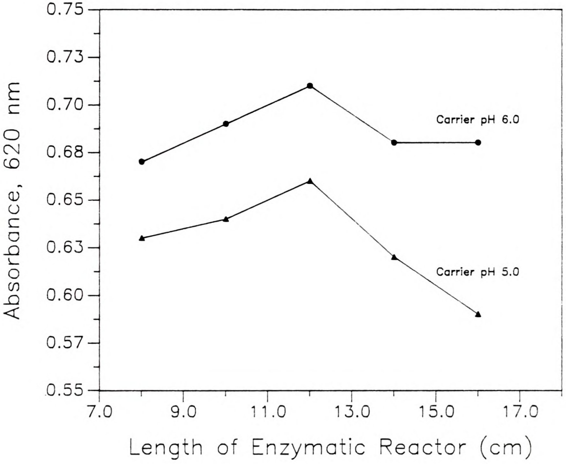


Figure 21. Relationship of length of the enzyme reactor and absorbance (MG^+ concentration). The standard hydrogen peroxide solution was 0.04 mM. The length of the plain reactor was 35 cm.

was obtained with a 12 cm enzymatic reactor. When the length was increased, the dispersion increased, and the absorbance decreased. The absorbance values using a carrier pH of 5.0 were lower than those obtained with a carrier pH of 6.0.

iii. Length of Unmodified SBSR Reactor

The length of unmodified reactor was tested with different concentrations of hydrogen peroxide (0.04-0.10 mM). The experimental conditions applied here were: 30 % acetic acid, pH 1.65, for the 1.5 mM LMG stock solution; 0.1 M phosphate buffer, pH 6.0, as carrier; 143 units of peroxidase per 10 ml of reagent; concentration of LMG reagent solution, 9.09×10^{-2} mM, equivalent to 0.6 ml of 1.5 mM LMG stock solution per 10 ml of reagent; 2.4 ml of 0.1 M phosphate buffer, pH 6.0, per 10 ml of reagent; 7.0 ml of 0.1 M acetate buffer, pH 4.0, per 10 ml of reagent; flow rate, 1.47 ml/min; and length of enzyme SBSR, 12 cm.

At higher concentrations of H_2O_2 (0.1 mM, 0.08 mM) or eventually high glucose concentrations in a sample, higher absorbance values were obtained with a SBSR of 35 cm length with all the other reactors tested as can be seen in Figure 22. With small lengths, such as 14 cm, the mixing of the sample and reagent was not enough when the sample concentration was high. On the other hand, samples at low concentration had sufficient mixing in the 14 cm reactor. When the length increased, the dispersion increased, and the absorbance decreased (Figure 22).

iv. Flow Rate and Length of Unmodified Reactor.

The optimum length of the plain reactor can be affected by the pH of the carrier and perhaps by the flow rate. The experimental conditions of the parameters applied here were: 30 % acetic acid, pH 1.65, for the 1.5 mM LMG stock solution; 0.1 M phosphate buffer, pH 6.0,

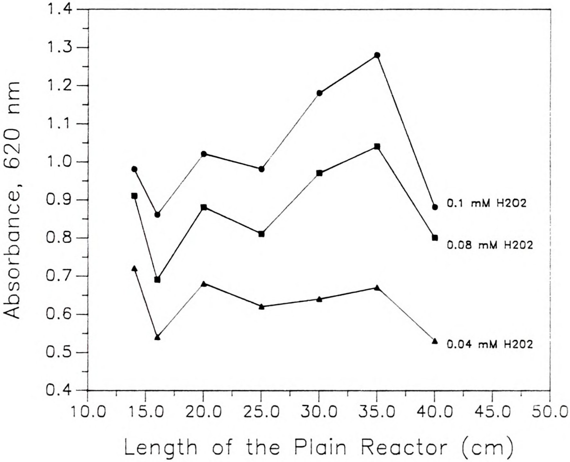


Figure 22. Relationship of length of the plain reactor and absorbance (MG^+ concentration) at three concentrations of H_2O_2. The length of the enzyme reactor was 12 cm.

as carrier; 143 units of peroxidase per 10 ml of reagent; concentration of LMG reagent solution, 9.09 x 10^{-2} mM, equivalent to 0.6 ml of 1.5 mM LMG stock solution per 10 ml of reagent; 2.4 ml of 0.1 M phosphate buffer, pH 6.0, per 10 ml of reagent; 7.0 ml of 0.1 M acetate buffer, pH 4.0, per 10 ml of reagent; and length of enzyme SBSR, 12 cm.

Figures 22 shows that reactors of 14 cm and 35 cm, length gave higher absorbance values than the other reactors for 0.04 mM of H202. The 35 cm reactor gave the highest absorbance values for higher concentrations of H_2O_2, such 0.08 and 0.1 mM.

Figure 23 shows the behavior of three plain reactors (14 cm, 30 cm, and 35 cm) at different flow rates. With different reactor lengths and constant tube diameter and flow rate, the dispersion coefficient, travel time and peak width increase with increasing reactor length. A 14 cm length of plain SBSR was sufficient for a good mixing and low dispersion due to the low sample concentration (0.04 mM H_2O_2). Increasing the length, increases the dispersion while the absorbance values decrease. In addition, when the mixing of sample and reagent is sufficient in a 14 cm reactor, increasing the flow rate results in decreased absorbance because the high flow prevents good mixing.

The 30 cm and 35 cm long plain SBSRs were too long for that concentration of H_2O_2. The dispersion was increased, and lower absorbance values were obtained. Also, upon increasing the flow rate, the dispersion decreases and the absorbance values increase. That was why the absorbance was still increasing even with a flow rate of 1.7 ml/min. Also, with increasing flow rate, the mixing in these reactors

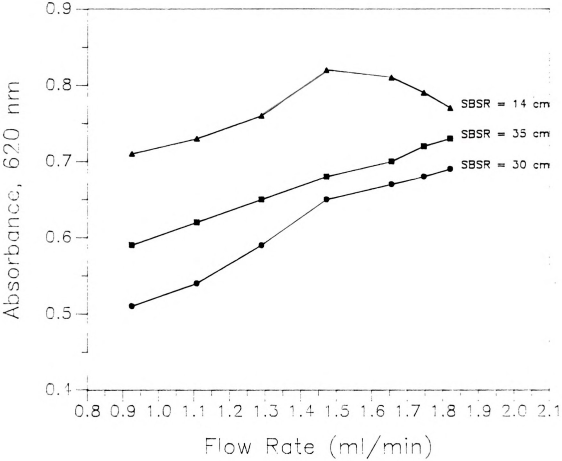


Figure 23. Relationship of absorbance (MG$^+$ concentration) and flow rate with three different lengths of plain reactors. The carrier was pH 6.0 and the length of the enzymatic reactor was 12 cm. The standard solution of hydrogen peroxide was 0.04 mM.

gets better, and the absorbance increases. Figure 24 shows the effect of flow rate on dispersion: increasing the flow rate, decreases the dispersion .

v. pH of Carrier

The pH of the carrier must be proper both for the LMG reaction and for the immobilized enzyme reactors. The pH of the carrier was optimized within the range of 5.0 to 6.5. The experimental conditions of the parameters applied here were the following: 30 % acetic acid, pH 1.65, for 1.5 mM LMG stock solution; 143 units of peroxidase per 10 ml of reagent; concentration of LMG reagent solution, 9.09 x 10^{-2} mM, equivalent to 0.6 ml of 1.5 mM LMG stock solution per 10 ml of reagent; 2.4 ml of 0.1 M phosphate buffer, pH 6.0, per 10 ml of reagent; 7.0 ml of 0.1 M acetate buffer, pH 4.0, per 10 ml of reagent; flow rate, 1.47 ml/min; length of plain SBSR, 35 and 14 cm; and length of enzyme SBSR, 12 cm. The concentration of the standard H_2O_2 was 0.04 mM. Figure 25 shows the optimum pH for the carrier to be approximately 6.0.

vi. Comparison of Different Lengths of Plain SBSR for Sensitivity.

The sensitivity of the method for different lengths of unmodified SBSR was tested by using the current optimum values. The experimental conditions used in this study were: 30 % acetic acid, pH 1.65, for the 1.5 mM LMG stock solution; 143 units of peroxidase per 10 ml of reagent; concentration of LMG reagent solution, 9.09 x 10^{-2} mM, equivalent to 0.6 ml of 1.5 mM LMG stock solution per 10 ml of reagent; 2.4 ml of 0.1 M phosphate buffer, pH 6.0, per 10 ml of reagent; 7.0 ml of 0.1 M acetate buffer, pH 4.0, per 10 ml of reagent; carrier, pH 6.0;

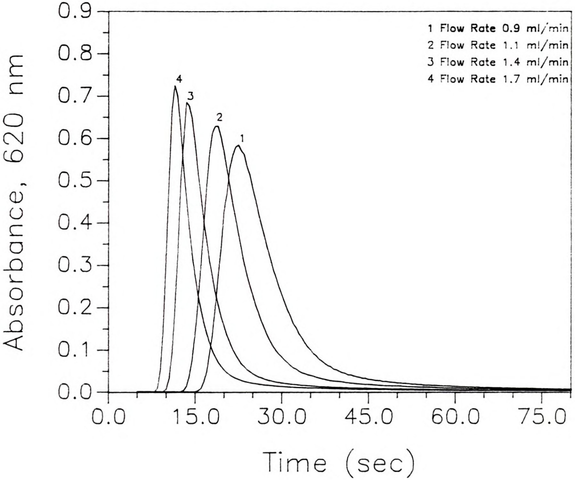


Figure 24. The effect of flow rate on dispersion. The lengths of enzyme and plain reactors were 12 cm and 35 cm, respectively. The carrier pH was 6.0 and the standard solution of hydrogen peroxide was 0.04 mM.

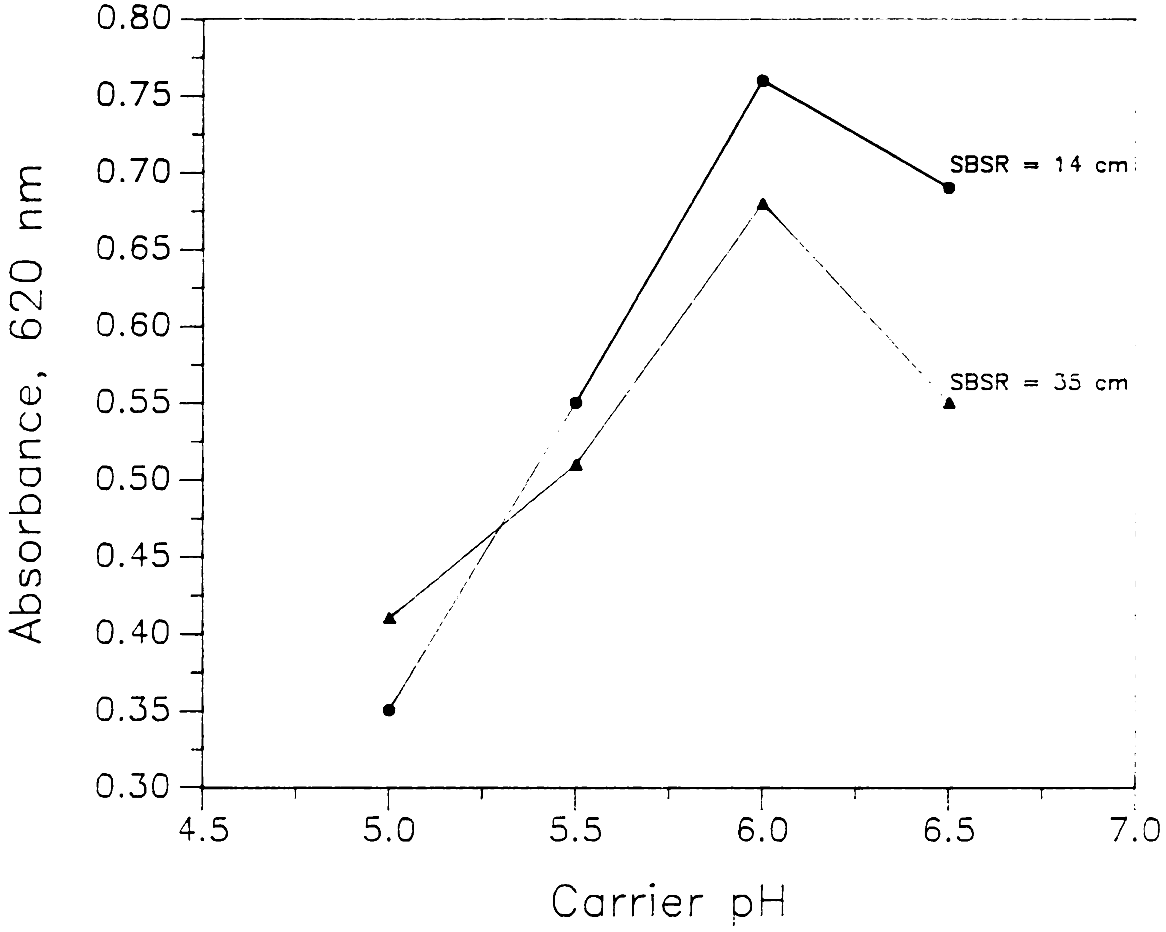


Figure 25. Relationship between the carrier pH and absorbance (MG^+ concentration) with different lengths of the plain reactor. The length of the enzyme reactor was 12 cm and the standard solution of hydrogen peroxide was 0.04 mM.

flow rate, 1.47 ml/min; and length of enzyme SBSR, 12 cm. Also, glucose oxidase was already immobilized onto glass beads.

The sensitivity of the method increased when the length of the plain SBSR increased in the range tested due to better mixing. The 35 cm plain reactor had the highest sensitivity with a slope of 2.520 A/mM glucose (Figure 26). For plain SBSR lengths of 14, 30, and 35 cm, the standard errors of the estimate (relative to the mean of absorbance), were 6.68 %, 1.08 %, and 1.10 %, respectively.

vii. Sensitivity for Different Lengths of Enzymatic SBSR.

For comparing the sensitivities with different lengths of enzymatic reactor, the current above optimum values were used. The lengths of the enzymatic reactor SBSR tested were 8 cm, 10 cm, 12 cm, 14 cm, and 15 cm (Figure 27). The corresponding linear relationships had standard errors of the estimate (relative to the mean of absorbance), 6.47 %, 1.89 %, 2.21 %, 2.16 %, and 0.39 %, respectively. The 12 cm reactor showed the highest sensitivity.

viii. Effect of Temperature

Most chemical reactions proceed at higher velocity as the temperature, T, is raised. An increase in T imparts more kinetic energy to the reactant molecules resulting in more productive collisions per unit time (81). Enzyme-catalyzed reactions behave similarly, up to a point. Enzymes are complex protein molecules. Their catalytic activity results from a precise, highly ordered tertiary structure which juxtaposes specific amino acid R groups in such a way as to form the stereospecific substrate binding sites and the catalytic center (82). The tertiary structure of an enzyme is maintained primarily by a large number of weak noncovalent bonds. If the molecule absorbs too much

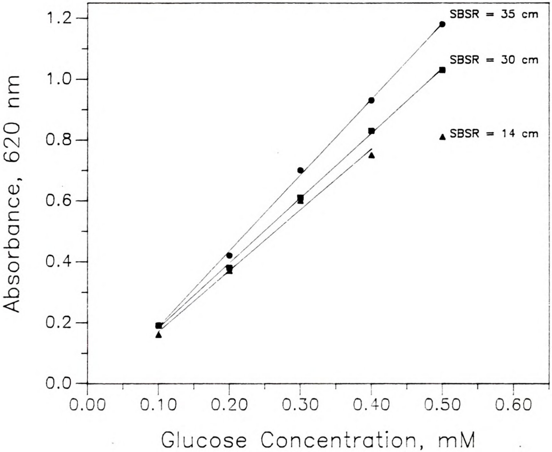


Figure 26. Comparison of the sensitivity of LMG reaction with different lengths of plain SBSR. The enzyme reactor was 12 cm and the pH of the carrier was 6.0.

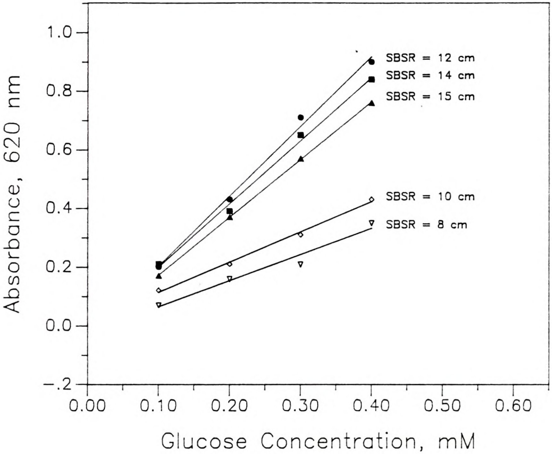


Figure 27. Sensitivity of the LMG reaction for different lengths of enzymatic SBSR. The plain SBSR was 35 cm and the pH of the carrier was 6.0.

energy, the tertiary structure will disrupt and the enzyme will be denatured and lose catalytic activity. Therefore, as the temperature increases, the expected increase in velocity (v) resulting from increased (Enzyme + Substrate) collisions is offset by the increased rate of denaturation. Immobilized enzymes behave similarly.

In this work the enzyme SBSR (12 cm), the unmodified SBSR (35 cm) and the tee, in which the reagent was introduced to the carrier stream, were placed under controlled temperature as shown in Figure 28. A transparent nylon tube (0.5 cm i.d.) was used as a jacket for the enzymatic and unmodified SBSR; controlled temperature water flowed very slowly through this jacket. A specially constructed glass tee shown in Figure 29 was used in order to have the appropriate flow of the warm water around the tee. The manifold used for this experiment was exactly the same as the one used for the optimization of the method, and only the two SBSRs were in the nylon tube (0.5 cm i.d.). The temperature of the ingoing and outgoing water was measured. For each temperature point there were six replications; an interval of a few minutes was used between experiments in order to ensure that the enzyme was at the measured temperature. The enzyme reactions show increased activity when the temperature is raised up to 40 ^{o}C. After that the enzyme activities become lower. In Figure 30 the absorbance values show this optimum temperature. During that experiment all the variables used were at their optimum and kept constant.

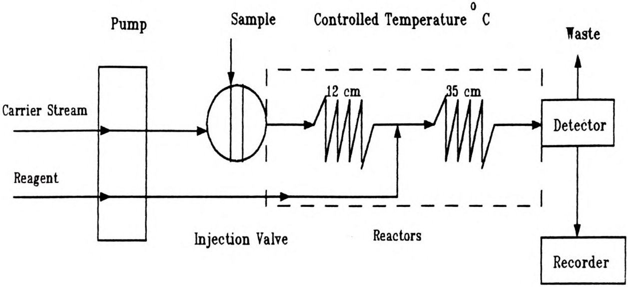


Figure 28. Apparatus for the controlled temperature determination of glucose using immobilized glucose oxidase.

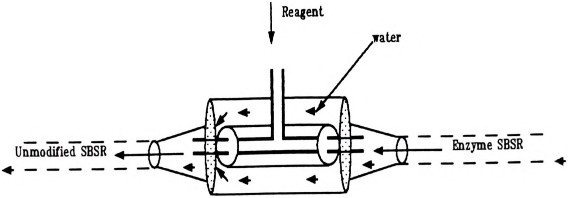


Figure 29. Diagram of the glass part used for the temperature experiments with glucose oxidase. Water with specific temperature was passing around the glass tee.

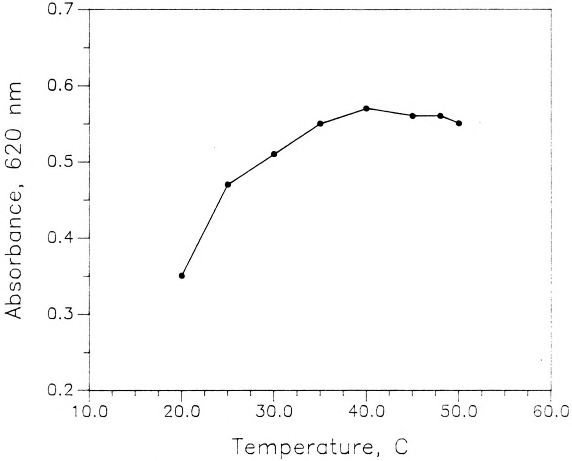


Figure 30. Effect of temperature on the activity of the glucose oxidase reactor. The enzyme and plain SBSRs were 12 cm and 35 cm, respectively; the pH of the carrier was 6.0; the flow rate was 1.47 ml/min and the standard glucose solution was 0.2 mM.

b. Specific Simplex Optimization of FIA

The basic rate dependent variables which were already optimized were the following: flow rate, pH of the carrier and lengths of the enzymatic and unmodified (plain) reactors. The specific simplex optimization of FIA dealt with those four variables. The initial specific simplex optimization was obtained by entering the information listed in Table 5 into the modified simplex program. From the univariate experiments that were done prior to this optimization, an acceptable range for each of the parameters was identified. Since there were four variables, five experiments were needed to form the initial specific simplex. These were performed and the results entered. The first point of the simplex was the response obtained from the baseline experiment with the initial conditions. Therefore, each time the simplex program specified a set of experimental conditions, this experiment was performed and the absorbance value was entered. The summary of progress in cycle 45 of the program where the optimum was reached is illustrated in Table 6. The optimum for the set of variables was obtained by measuring the absorbance at 620 nm with a precision of 0.05. In Table 7 the optimum values of the variables after fifty-eight experiments are shown with a standard deviation for the current top 5 values. The concentration of standard hydrogen peroxide was 0.04 mM. Six replications were done for each set of experimental conditions, and the entered value of absorbance was the average.

Table 5. Range and Precision of Variables for the Specific Simplex Optimization.

Experimental Variables	Forward Boundary	Reverse Boundary	Precision
Pump Setting	99	40	1
Length Enzyme SBSR(cm)	16	8	1
Length Plain SBSR(cm)	40	10	5
Carrier pH	6.5	5	0.5

Response to optimize: Absorbance
Precision of Absorbance: 0.05

Table 6. Optimum information given by Simplex Optimization Program.

```
Summary of Progress so far - Cycle: 45


Values of Absorbance:

point 13 = 0.688

point 56 = 0.687

point 54 = 0.686

point 58 = 0.686

point 51 = 0.684


Best Response = 0.688 (13)

Mean Response = 0.6862

Std. Dev. = 1.483239E-03

Rel.Improvement: 1.075


Carrying out a SUBOPTIMAL REDIRECTION.
```

Table 7. Initial and Optimal Experimental Conditions for the Specific Simplex Optimization of FIA system.

	Exp. No	Pump Sett.	SBSR Enzyme cm	Plain SBSR cm	Carrier pH	Absorbance 620 nm
Start:	1	75	12	30	6.0	0.640
Top 5:	13	78	12	35	6.0	0.688
	56	79	12	35	6.0	0.687
	54	79	13	35	6.0	0.686
	58	77	10	35	6.0	0.686
	51	77	11	35	6.0	0.684
Mean:		78	11.6	35	6.0	0.686
(SD):		1.0	1.14	NV	NV	0.0015

NV - The Simplex program did not call for variations of the length of the plain SBSR or the carrier pH in the top 5 experiments.

c. Evaluation of the optimization methods

A comparison of the specific simplex and univariate methods of optimization was done by comparing the slopes of the two calibration curves that were obtained by applying the optimum conditions for each method. The glucose oxidase immobilized enzyme used for this comparison was three weeks old. Therefore, the activity of such a reactor was lower than that of a fresh one. Figure 31 shows that the optimum values obtained from the Simplex method gives a slightly higher sensitivity than those obtained by the univariate method. The improvement of the simplex method was only 7 %, because we already used the improved points of the general simplex optimization as the initial points for the new optimization. For the simplex and univariate optimizations, Figure 31, the calculated slopes were 2.160, 1.793 A/mM and the standard errors of the estimate (relative to the mean of absorbance), were 2.34 %, 2.98 %, respectively.

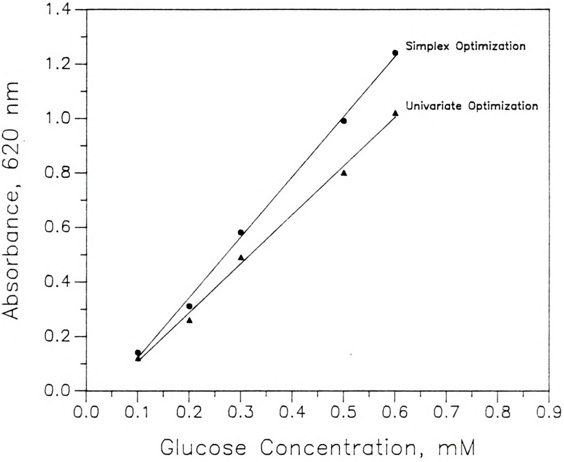


Figure 31. Comparison of specific Simplex and Univariate methods of optimization for sensitivity.

B. Application of FIA in Determining Fruit Sugars

Flow injection analysis can be an excellent way of determining fruit sugars, rapidly and simply. Eventually, the portability of the FIA analyzer can facilitate its applications in agriculture and the food industry.

In this work, the determination of sugars (glucose, fructose and sucrose) in fruits was applied for ripe fruits (oranges, lemons, and grapefruit), and fruits of different maturity (olives, cherries). The procedure was described in the chapter III. The determination of fruit sugars is of interest to horticulturists, food technologists, nutritionists, chemists and others. Calibration curves for glucose, fructose and sucrose, using immobilized glucose oxidase were prepared. The sugar analysis in fruits was performed in three individual experiments. For each type of fruit (olives, cherries and citrus fruits), new calibration curve was prepared, because the enzyme activity of glucose oxidase reactor was decreasing over time.

1. Determination of Sugars in Fruits of Different Maturity

The fruit used for this work were olives and cherries of different cultivars and varying maturity. Coroneiki and Cypriot were known to be olive cultivars used for oil production; Ascolano and Manzanillo were known to be olive cultivars preferred for table olive production. All the cherry cultivars used were sour cherries.

a. Olives

In olives, as the degree of maturation advances, the sugar concentration decreases (Table 8). Similar results have been found by other workers (83-88). This is significant because olives destined for oil extraction must be harvested when the sugar content is minimal and

Table 8. Determination of Sugars in Fruits using FIA Method.

Sample	Harvesting Time	Glucose % w/w	Fructose % w/w	Sucrose % w/w	Total Sugars % w/w
<u>Olives:</u>					
Cypriot (Cyprus)	Sept.	1.29	0.39	0.14	1.82
Coroneiki (Greece)	Sept.	2.19	0.14	0.14	2.47
Amphisis (Greece)	Sept.	1.29	0.21	0.13	1.63
Manzanillo (Cyprus)	August	2.55	0.77	0.11	3.43
Manzanillo (Cyprus)	Sept.	1.69	0.40	0.07	2.16
Ascolano (Cyprus)	August	2.87	0.19	0.29	3.35
Ascolano (Cyprus)	Sept.	1.46	0.14	0.09	1.69
Manzanillo (Calif.)	August	2.92	1.23	0.20	4.35
Manzanillo (Calif.)	Sept.	2.23	1.11	0.18	3.52
Manzanillo (Calif.)	Nov.	1.62	0.91	0.11	2.64
Ascolano (Calif.)	August	2.94	1.38	0.60	4.92
Ascolano (Calif.)	Sept.	2.90	1.29	0.48	4.67
Ascolano (Calif.)	Nov.	2.69	0.99	0.40	4.08
<u>Cherry from Michigan</u>:					
Wolynska	July 17	1.71	1.45	0.46	3.62
Wolynska	July 27	2.51	2.39	0.69	5.59
Wolynska	August 1	2.61	2.49	0.79	5.89
I 20(36)	July 14	4.23	3.21	0.17	7.61
I 20(36)	July 17	4.36	4.47	0.47	9.30
I 20(36)	July 24	4.45	4.52	0.78	9.75

(Table Continued)

(Continued)

Table 8. Determination of Sugars in Fruits using FIA Method.

Sample	Harvesting Time	Glucose % w/w	Fructose % w/w	Sucrose % w/w	Total Sugars % w/w
Montmorency	July 7	3.12	2.52	0.52	6.16
Montmorency	July 14	3.12	2.72	0.65	6.49
Montmorency	July 21	3.30	2.73	0.81	6.84
Montmorency	August 3	3.42	3.70	0.99	8.11
Citrus:					
Orange		1.69	1.71	3.70	7.10
Lemon		1.13	1.58	0.89	3.60
Grapefruit		2.24	2.19	3.40	7.83

the oil content maximal. Olives to be pickled (table olives) must contain sugars for lactic acid fermentation. The rate of olive fruit maturation is affected by several factors including cultivars, age of the tree, health of the tree, moisture of the soil, fertilization and light. Therefore, a fast, simple and accurate method of sugar determination is of great interest to the olive industry because the sugar content can be used to tell precisely when to harvest. Such analytical information is more reliable than conventional methods which involve observing the color and feeling the fruit. The calibration curves used for the analysis of glucose, fructose, and sucrose in olives by FIA had slopes of 2.901, 1.151, 2.504 A/mM and standard errors of the estimate, (relative to the absorbance), 2.62 %, 2.68 %, 1.22 %, respectively.

b. Cherries

The degree of maturation in cherry fruit is related to the sweetness of the cherry. Sugar determination is important both as a criterion of fruit maturity and for comparisons among cherry cultivars. The calibration lines used for the analysis of glucose, fructose, and sucrose in cherries had slopes of 2.320, 1.185, 1.834 A/mM and standard errors of the estimate, (relative to the absorbance), 3.20 %, 5.53 %, 3.24 %, respectively.

2. Determination of Sugars in Ripe Fruits

The fruits used for this work were citrus fruits (oranges, lemons and grapefruit). Table 8 shows that grapefruit juice (fruit from Florida) has the highest concentration of sugars of all the citrus fruits. For the direct determination of glucose and the indirect determination of fructose and sucrose with flow injection analysis and

immobilized glucose oxidase, the slopes of the calibration lines were 1.837, 0.549, 1.289 A/mM and the standard errors of the estimate (relative to the absorbance), 2.49 %, 5.08 %, 3.46 %, respectively.

C. Determination of Fruit Sugars using Conventional Enzyme Methods.

The conventional enzyme methods used for the determination of fruit sugars were described in chapter III. Only one determination was performed with the conventional method for each of the 26 samples because this method is time consuming and the reagents are expensive. Table 9 summarizes the results of the determination of sugars in olives, cherries and citrus fruits. There is a reverse relationship between the sugar concentration and degree of maturity in olives. The Manzanillo and Ascolano olive cultivars show this relationship for all three sugars (glucose, fructose and sucrose).

The cherry values of the Wolynska cultivar show that the sugar concentration of the fruit increases with maturity up to a certain date (Table 9).

D. Comparison of the two Methods

Since FIA is a new method, a comparison with an accepted method is desirable. For the comparison of the FIA and the conventional method we used the procedure of Fisher's Least Significant Difference (LSD_a) (89) at significant level of 1 %. The samples that were compared were three samples of citrus fruits (oranges, lemons, and grapefruit), thirteen samples of olives and ten samples of cherries. Six replications were done for each sample with the FIA method, with x_i the mean of each

Table 9. Determination of Fruit Sugars using Conventional Enzyme Methods

Sample	Harvesting Date	Glucose % w/w	Fructose % w/w	Sucrose % w/w	Total Sugars % w/w
Olives:					
Cypriot (Cyprus)	Sept.	1.37	0.31	0.13	1.82
Coroneiki (Greece)	Sept.	2.20	0.07	0.12	2.39
Amphisis (Greece)	Sept.	1.35	0.15	0.15	1.65
Manzanillo (Cyprus)	August	2.58	0.78	0.11	3.47
Manzanillo (Cyprus)	Sept.	1.72	0.45	0.01	2.18
Ascolano (Cyprus)	August	2.91	0.14	0.30	3.35
Ascolano (Cyprus)	Sept.	1.44	0.10	0.03	1.57
Manzanillo (Calif.)	August	2.95	1.25	0.20	4.40
Manzanillo (Calif.)	Sept.	2.23	1.12	0.15	3.50
Manzanillo (Calif.)	Nov.	1.67	0.93	0.08	2.68
Ascolano (Calif.)	August	3.02	1.41	0.60	5.03
Ascolano (Calif.)	Sept.	2.93	1.29	0.48	4.70
Ascolano (Calif.)	Nov.	2.71	1.02	0.40	4.13
Cherries from Michigan:					
Wolynska	July 17	1.74	1.51	0.48	3.75
Wolynska	July 27	2.50	2.33	0.66	5.49
Wolynska	August 1	2.58	2.44	0.77	5.79
I 20(36)	July 14	4.31	3.23	0.14	7.68
I 20(36)	July 17	3.70	4.51	0.52	8.73
I 20(36)	July 24	4.04	3.80	0.81	8.65

(Table Continued)

(Continued)

Table 9. Determination of Fruit Sugars using Conventional Enzyme Methods

Sample	Harvesting Date	Glucose % w/w	Fructose % w/w	Sucrose % w/w	Total Sugars % w/w
Montmorency	July 7	3.15	2.56	0.53	6.24
Montmorency	July 14	2.34	2.80	0.69	5.83
Montmorency	July 21	2.57	2.81	0.82	6.20
Montmorency	August 3	3.48	3.21	0.97	7.66
<u>Citrus:</u>					
Orange (Calif.)		1.71	1.70	3.97	7.38
Lemon (Calif.)		1.14	1.10	0.89	3.13
Grapefruit (Florida)		2.32	2.41	3.63	8.36

sample, and one with the conventional method (x_j). Analyses of variance (ANOVA) were performed to test the null hypothesis that the means were equal (H_o: $\mu_i = \mu_j$), where μ_i and μ_j are the true means of the FIA and conventional methods for each sugar. For the calculations of the analyses of variance for each sugar (glucose, fructose and sucrose), only the results of the FIA method were used. Table 10 shows the analyses of variances for glucose, fructose and sucrose. In all sugars the calculated F is a large number; therefore, the F test for treatments was shown to be significant. Fisher's LSD must therefore be applied. The calculated Pool Variance (S_p^2) was used for the calculation of the Variance of the Difference ($S_d^2 = S_p^2(1/n_i+1/n_j) = S_p^2(1/n_i+1)$). For a specified significance level a (e.g. a=0.01), the least significant difference for comparing μ_i to μ_j is: $LSD_a = t_{a/2}\ S_d = t_{a/2}\ S_p\ (1/n_i + 1)^{-1/2}$, where n_i and n_j (equal to 1) are the respective sample sizes for each fruit sample treatment and t is the critical t value for a=a/2 and the number of degrees of freedom (df) for S_p^2 from the analysis of variance (Error degree of freedom). Three LSD_as were calculated from the three analyses of variance corresponding to the three sugars (glucose, fructose and sucrose); they were 0.082, 0.080 and 0.061, respectively. For all three ANOVAs we had the same number of treatments (t), which was equal to the number of different fruit samples (t=26), and for each treatment we had the same replication number (r=6) for each individual sugar.

If the difference between the means of the FIA and the experimental value of the Conventional method of glucose for each treatment is in the range of $+LSD_a$ to $-LSD_a$ for glucose, then the two methods are not significantly different and the null hypothesis is

Table 10. Analyses of Variance for sugars with treatment and replication numbers equal to 26 and 6, respectively (FIA method).

(a) ANOVA for glucose:

source	df	SS	MS	F
Total	(rt-1) = 155	131.89		
Treatment	(t-1) = 25	131.78	5.271	6358.27
Error	t(r-1) = 130	0.1078	0.000829*	

* Pool Variance (S_p^2)

(b) ANOVA for fructose:

source	df	SS	MS	F
Total	(rt-1) = 155	250.25		
Treatment	(t-1) = 25	250.14	10.0056	12713.59
Error	t(r-1) = 130	0.10234	0.000787*	

* Pool Variance (S_p^2)

(c) ANOVA for sucrose:

source	df	SS	MS	F
Total	(rt-1) = 155	120.84		
Treatment	(t-1) = 25	120.78	4.8312	10323.077
Error	t(r-1) = 130	0.06079	0.000468*	

* Pool Variance (S_p^2)

accepted. Tables 11, 12 and 13 show the statistical comparison of glucose, fructose and sucrose of both methods respectively, at significance level of a = 0.01 for each treatment. For glucose measured by both methods (Table 11) the differences of twenty-two out of twenty-six fruit samples were found to be not significant at a = 0.01. Therefore, the null hypothesis is accepted for those samples. For fructose (Table 12) the differences of twenty-three out of twenty-six fruit samples were found to be not significant at a = 0.01. Finally, for sucrose (Table 13) the differences of twenty-four out of twenty-six fruit samples were found to be not significant at a = 0.01. For nine out of the seventy-eight total runs the null hypothesis was rejected indicating that the two methods did not give the same values. This discrepancy may not have appeared if we had more than one value obtained by the conventional method. In addition, in six out of those nine cases the FIA method gave slightly higher values than the conventional one.

E. Test of the Conventional Method against Sugar Standards.

In this test, three standard pure sugar solutions (glucose, fructose and sucrose) with known concentration (2 g/L) were used as unknowns and determined using the conventional method. Each solution was used to prepare and run three replications by the conventional method. All nine experimental values, were slightly lower than 2.0.

The analysis of variance was performed to test the null hypothesis (H_o: $\mu_i = 2.0$) against the alternative hypothesis that the true mean was not equal to 2.0 (H_1: μ_i # 2.0) for the level of a=0.01. The calculated pool variance (S_p^2) for the analysis of variance was 0.0000321, and the calculated standard deviation of the sample mean (S_x) was 0.0032711.

Table 11. Statistical Comparison of Glucose Measurements for FIA and Conventional Methods by Using The Least Significant Difference (LSD) Procedure.

Sample	FIA % w/w	Conventional % w/w	Difference	LSDa	Significance at a=0.01
<u>Citrus</u>:					
Orange	1.69	1.71	-0.02	0.082	-
Lemon	1.13	1.14	-0.01	0.082	-
Grapefruit	2.24	2.32	-0.08	0.082	-
<u>Olives</u>:					
Cypriot Olive	1.29	1.37	-0.08	0.082	-
Coroneiki Olive	2.19	2.20	-0.01	0.082	-
Amphisis Olive	1.29	1.35	-0.06	0.082	-
Manzanillo Cyprus#1	2.55	2.58	-0.03	0.082	-
Manzanillo Cyprus#2	1.70	1.72	-0.02	0.082	-
Ascolano Cyprus#1	2.87	2.91	-0.04	0.082	-
Ascolano Cyprus#2	1.46	1.44	+0.02	0.082	-
Manzanillo Calif.#1	2.92	2.95	-0.03	0.082	-
Manzanillo Calif.#2	2.23	2.23	0.00	0.082	-
Manzanillo Calif.#3	1.62	1.67	-0.05	0.082	-
Ascolano Calif.#1	2.94	3.02	-0.08	0.082	-
Ascolano Calif.#2	2.90	2.93	-0.03	0.082	-
Ascolano Calif.#3	2.69	2.71	-0.02	0.082	-

(Table Continued)

(Continued)

Table 11. Statistical Comparison of GLucose Measurements for FIA and Conventional Methods by Using The Least Significant Difference (LSD) Procedure.

Sample	FIA % w/w	Conventional % w/w	Difference	LSDa	Significance at a=0.01
<u>Cherries</u>:					
Wolynska Cherry#1	1.71	1.74	-0.03	0.082	-
Wolynska Cherry#2	2.51	2.50	+0.01	0.082	-
Wolynska Cherry#3	2.61	2.58	+0.03	0.082	-
I 20(36) Cherry#1	4.23	4.31	-0.08	0.082	-
I 20(36) Cherry#2	4.36	3.70	+0.66	0.082	+
I 20(36) Cherry#3	4.45	4.04	+0.41	0.082	+
Montmorency #1	3.12	3.15	-0.03	0.082	-
Montmorency #2	3.12	2.34	+0.78	0.082	+
Montmorency #3	3.30	2.57	+0.73	0.082	+
Montmorency #4	3.42	3.48	+0.06	0.082	-

(-) = Not Significant, then the null hypothesis is accepted (H_o: FIA - Conventional = 0)

(+) = Signifigant, then the null hypothesis is rejected and the way of evedence says that the alternative hypothesis is reasonable (H_1: FIA - Conventional # 0)

LSD_a = Least Signifigant Difference ($LSD_a = t_{a/2} S_d$)

Table 12. Statistical Comparison of Fructose Measurments for FIA and Conventional Methods by Using the Least Significance Difference (LSD) Procedure.

Sample	FIA % w/w	Conventional % w/w	Difference	LSDa	Significance at a=0.01
Citrus:					
Orange	1.71	1.70	+0.01	0.080	-
Lemon	1.58	1.10	+0.48	0.080	+
Grapefruit	2.19	2.41	-0.22	0.080	+
Olives:					
Cypriot Olive	0.39	0.31	+0.08	0.080	-
Coroneiki Olive	0.14	0.07	+0.07	0.080	-
Amphisis Olive	0.21	0.15	+0.06	0.080	-
Manzanillo Cyprus #1	0.77	0.78	-0.01	0.080	-
Manzanillo Cyprus #2	0.41	0.45	-0.04	0.080	-
Ascolano Cyprus #1	0.19	0.14	+0.05	0.080	-
Ascolano Cyprus #2	0.14	0.10	+0.04	0.080	-
Manzanillo California #1	1.23	1.25	-0.03	0.080	-
Manzanillo California #2	1.11	1.12	-0.01	0.080	-
Manzanillo California #3	0.90	0.93	-0.03	0.080	-
Ascolano California #1	1.39	1.41	-0.02	0.080	-
Ascolano California #2	1.29	1.29	0.00	0.080	-
Ascolano California #3	0.99	1.02	-0.03	0.080	-

(Table Continued)

(Continued)

Table 12. Statistical Comparison of Fructose Measurments for FIA and Conventional Methods by Using the Least Significance Difference (LSD) Procedure.

Sample	FIA % w/w	Conventional % w/w	Difference	LSDa	Significance at a=0.01
<u>Cherries</u>:					
Wolynska Cherry #1	1.46	1.51	-0.05	0.080	-
Wolynska Cherry #2	2.39	2.33	+0.06	0.080	-
Wolynska Cherry #3	2.50	2.44	+0.06	0.080	-
I 20(36) Cherry #1	3.21	3.23	-0.02	0.080	-
I 20(36) Cherry #2	4.47	4.51	-0.04	0.080	-
I 20(36) Cherry #3	4.52	3.80	+0.72	0.080	-
Montmorency #1	2.52	2.56	-0.04	0.080	-
Montmorency #2	2.72	2.80	-0.08	0.080	-
Montmorency #3	2.74	2.81	-0.07	0.080	-
Montmorency #4	3.70	3.21	+0.49	0.080	+

(-) = Not Significant, then the null hypothesis is accepted (H_o: FIA - Conventional = 0)

(+) = Significant, then the null hypothesis is rejected and the way of evidence says that the alternative hypoithesis is reasonable (H_1: FIA - Conventional # 0)

LSD_a = Least Significant Difference ($LSD_a = t_{a/2}\ S_d$)

Table 13. Statistical Comparison of Sucrose Measurements for FIA and Conventional Methods by Using Least Significant Difference (LSDa).

Sample	FIA % w/w	Conventional % w/w	Difference	LSDa	Significance at a=0.01
Citrus:					
Orange	3.70	3.97	-0.27	0.061	+
Lemon	0.89	0.89	0.00	0.061	-
Grapefruit	3.40	3.63	-0.23	0.061	+
Olives:					
Cypriot Olive	0.14	0.13	+0.01	0.061	-
Coroneiki Olive	0.14	0.12	+0.02	0.061	-
Amphisis Olive	0.13	0.15	-0.02	0.061	-
Manzanillo Cyprus #1	0.11	0.11	0.00	0.061	-
Manzanillo Cyprus #2	0.07	0.01	+0.06	0.061	-
Ascolano Cyprus #1	0.29	0.30	-0.01	0.061	-
Ascolano Cyprus #2	0.09	0.03	+0.06	0.061	-
Manzanillo California #1	0.20	0.20	0.00	0.061	-
Manzanillo California #2	0.18	0.15	+0.03	0.061	-
Manzanillo California #3	0.11	0.08	+0.03	0.061	-
Ascolano California #1	0.60	0.60	0.00	0.061	-
Ascolano California #2	0.48	0.48	0.00	0.061	-
Ascolano California #3	0.40	0.40	0.00	0.061	-

(Table Continued)

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Table 13. Statistical Comparison of Sucrose Measurements for FIA and Conventional Methods by Using Least Significant Difference (LSDa).

Sample	FIA % w/w	Conventional % w/w	Difference	LSDa	Significance at a=0.01
Cherries:					
Wolynska Cherry #1	0.46	0.48	-0.02	0.061	-
Wolynska Cherry #2	0.69	0.66	+0.03	0.061	-
Wolynska Cherry #3	0.79	0.77	+0.02	0.061	-
I 20(36) Cherry #1	0.17	0.14	+0.03	0.061	-
I 20(36) Cherry #2	0.46	0.52	-0.06	0.061	-
I 20(36) Cherry #3	0.78	0.81	-0.03	0.061	-
Montmorency #1	0.53	0.53	0.00	0.061	-
Montmorency #2	0.65	0.69	-0.04	0.061	-
Montmorency #3	0.81	0.82	-0.01	0.061	-
Montmorency #4	0.99	0.97	+0.02	0.061	-

(-) = Not Significant, then the null hypothesis is accepted (H_o: FIA - Conventional = 0)

(+) = Significant, then the null hypothesis is rejected and the way of evidence says that the alternative hypothesis is reasonable (H_1: FIA - Conventional # 0).

LSD_a = Least Significant Difference ($LSD_a = t_{a/2} S_d$)

$\frac{16 - [illegible]}{3 - 2}$ ①

$s'(t) = $ [illegible]

$=$ [illegible] ④

[illegible]

$f'(t) = $ [illegible]

6. a) [illegible]

b) [illegible]

c) [illegible]

9. $s(t) = t^3 - $ [illegible]

a) v [illegible]

b) [illegible]

c) [illegible]

The t test for glucose samples gave a calculated t value (2.139) smaller than that of the table for a two tailed test with a=0.01 ($t_{a/2,6}$=3.707). Therefore, for the level of a=0.01 the null hypothesis was accepted as reasonable (μ_i = 2.0). On the other hand, the t test for fructose and sucrose was significant (14.97, 15.591 respectively), and the null hypothesis was rejected for these two sugars.

Hence the conventional method appears to give slightly low values for fructose and sucrose. If we take into account the fact that six out of nine values obtained by the FIA method for the food sugars were higher than those obtained by the conventional method, it may be concluded that the FIA method may be more accurate for those nine food samples showing statistical differences by the two methods.

In addition, statistical comparison by the least significant difference procedure between standard pure sugar solutions with the FIA method (with three replications) and the Conventional method (with three replications) shows that the two methods are not significantly different at the 99 % confidence level.

Hence we may conclude that the FIA method is at least as accurate as the conventional method and may, in fact, be more reliable. Further comparisons need to be done to prove or disprove this last hypothesis.

SUMMARY

In this work a new dye, leucomalachite green (LMG), was introduced for the indicator reaction of the Flow Injection Analysis (FIA) determination of glucose, based on immobilized glucose oxidase.

First, an initial optimization of the indicator reaction was carried out using univariate methods and optimizing the concentration of the LMG stock solution, the concentration of the LMG reagent solution, the activity of peroxidase, and the pH of the LMG stock solution. Then, a general simplex optimization of the FIA system was performed for the following nine variables: pump setting, pH of the carrier, concentration of the carrier, activity of peroxidase, volume of LMG stock solution (ml/10ml), volume of 0.1 M phosphate buffer, pH 6.0 (ml/10ml), volume of 0.05 M acetate buffer, pH 4.0 (ml/10ml), length of enzyme Single Bead String Reactor (SBSR) (cm) and length of plain SBSR (cm). The experimental conditions used as the initial points for the general simplex optimization were the optimal of the initial optimization. The general simplex optimization after sixty-two runs gave 33 % improvement in absorbance values.

A more specific optimization of rate dependent variables, such as flow rate, length of enzymatic and unmodified SBSRs and pH of the carrier were carried out using univariate and simplex optimization methods. A new set of optimum values was obtain by those two methods. An additional improvement of 7 % in absorbance values was achieved. A comparison of the specific simplex and univariate methods of optimization was done by comparing the slopes of two calibration curves which were obtained by applying the optimum conditions for each method. The calibration curve obtained, using the optimum values given by the

Simplex method gave a slightly higher sensitivity than that of the univariate method. The optimum values which were obtained after the optimization of the FIA system for all variables were the following: flow rate, 1.47 ml/min; 0.1 M phosphate buffer, pH 6.0, as a carrier; peroxidase activity 143 units per 10 ml of reagent; concentration of LMG reagent solution, 9.09×10^{-2} mM; concentration of LMG stock solution, 1.5 mM; 0.1 M phosphate buffer, pH 6.0, 2.4 ml per 10 ml reagent solution; 0.1 M acetate buffer, pH 4.0, 7 ml per 10 ml reagent solution; length of enzyme SBSR, 11.6 cm; length of unmodified SBSR reactor, 35 cm; and temperature 40 ^{o}C. The injected volume of the sample was 30 μL.

The above optimum conditions were applied to the determination of glucose, fructose and sucrose in citrus fruits (oranges, lemons, and grapefruit), thirteen olive samples and ten cherry samples.

Conventional enzyme methods for the analysis of these sugars were also used. When pure sugar solutions were analyzed by the FIA and conventional methods, the agreement was satisfactory. When the sugars present in the fruit samples were analyzed by the two methods, the results in nine out seventy-eight samples were different at the 0.01 probability level. This discrepancy may be due to the fact that only one value for each food samples was available by the conventional method, compared to six such values by the FIA method. The FIA method appears to be as accurate as the conventional method.

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